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Research Article

Production of rare cycloartane saponins from *Astragalus thracicus* (Griseb) compared to *Astragalus membranaceus* (Fisch.) Bunge – native and biotechnological sources

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Abstract

The aim of this study is a comparative metabolomic analysis between the endangered species *Astragalus membranaceus* and endemic species *Astragalus thracicus* concerning cycloartane saponins. In addition, *in vitro* shoots, callus, and suspension cultures of *A. thracicus* were successfully established to conserve the biodiversity of those endemic species and to increase the amount of produced saponins. The comparison was made according to the quantity of cycloartane saponins astragaloside I (**1**), astragaloside II (**2**), and astragaloside IV (**4**) to the reference standards for the same compounds by UHPLC-HRESI-MS analysis. The *in vitro* root cultures of *A. thracicus* reached two folds higher amounts of saponins (1.50 mg/g DW (**1**), 1.01 mg/g DW (**2**), and 0.91 mg/g DW (**3**)) than the native root of *A. thracicus* (1.14 mg/g DW (**1**) 0.47 mg/g DW (**2**), 0.40 mg/g DW (**3**)), and up to six times higher when compared with roots *A. membranaceus* (0.23 mg/g DW (**1**), 0.18 mg/g DW (**2**) and 0.05 mg/g DW (**3**)).

Keywords

LC/MS analysis, cycloartane saponins, *Astragalus thracicus*, *Astragalus membranaceus*, *in vitro* cultures

Introduction

The proven immunomodulatory, antiviral, and antitumor effects of representatives of the *Astragalus* genus are mainly due to the triterpene saponins of the cycloartane type contained in them. Due to the complexity of their structure, they are still most efficiently obtained from native plants. Variations in the quantity and quality of the plant material, a long period of development before starting saponin production (*Astragalus* roots), and the excessive collection of endangered species for pharmaceutical purposes (*A. membranaceus*) are just some of the problems associated with obtaining these natural products. Therefore, the discovery of new plant species (*A. thracicus*) that contain them is essential for the pharmaceutical industry. Thus, comparative metabolomic analysis between endangered Asia species *A. membranaceus* and Bulgaria endemic species *A. thracicus* could provide important information about possible alternative sources. In addition, biotechnological methods could serve as an opportunity to protect those endemic species and to increase the amount of produced saponins.

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PENSOFT.

Astragalus L. Fabaceae (Leguminosae) is the largest genus of herbaceous plants in the pea and bean family it includes approximately 3000 species, distributed across Asia, Africa, Europe, South, and North America, although the center of origin and biodiversity of plants of the genus *Astragalus* is Eurasia, and in particular, the mountainous parts of Southwest Asia (Podlech 2008).

The most used species of the genus is *A. membranaceus* Bunge var. *mongholicus* (Bunge), (syn. *A. mongholicus* var. *mongholicus*), the drug used to be the dried whole or thinly sliced roots. The plant substance, due to its wide use, has been included in the editions of the European Pharmacopoeia after 2012 (Ph. Eur. monograph, as well as in the Chinese Materia medica.

Phytochemical studies on *Astragalus* species have been conducted due to their effects as immunostimulants or as anticancer agents (Ionkova et al. 2014; Krasteva et al. 2016). In particular, the chemical composition of the dried roots of *Astragalus* spp. protects the heart, brain, kidneys, intestines, liver, and lungs from various diseases associated with oxidative stress (Hong et al. 1992; Shahzad et al. 2016). Various isolated components of *Astragalus* spp. show significant antiproliferative activity. The highest activity against T-cell leukemia (SKW-3) cells was registered for rhamnocitrin 4'-D-galactopyranoside isolated from *A. hamosus* (Krasteva et al. 2008).

Biotechnological techniques and approaches are an extremely attractive alternative to over-exploited wild species. Some of the advantages of the *in vitro* techniques are the propagation of the plants in aseptic controlled conditions and their large-scale production in a year-round system without seasonal constraints (Isah et al. 2018). The plant cell techniques provide some high-efficiency methods for isolation and extraction of the secondary metabolites within a short time compared to the wild plant populations and the simplicity of the methods from *in vitro*-produced tissues makes it suitable for commercial application (Kolewe et al. 2008). Apart from these advantages, some metabolites can be produced by *in vitro* cultures but are generally not found in intact plants (Pavlov et al. 2005).

Materials and methods

Plant material

The *in vitro* cultures of *A. thracicus* were successfully established and maintained in our lab. The native *A. thracicus* roots were carefully collected from their natural environment, while native *A. membranaceus* roots were delivered by HerbaSinica Hilsdorf GmbH (Ch.-B. 160601H004).

General experimental procedures

All solvents were at least of analytical grade, whereas solvents used for semi-preparative HPLC analysis, i.e., EtOAc and MeOH were HPLC grade and were purchased from Fischer Scientific (Loughborough, UK). The following reference substances of cycloartane saponins were used: Astragaloside I (≥ 95.0%) delivered by Cayman chemical company; Astragaloside II (≥ 99.8%) obtained from Sigma-Aldrich and Astragaloside IV (≥ 98.0%) purchased from Tokyo chemical industry Co., LTD.

All analyses were performed with a Dionex Ultimate 3000 RSLC UHPLC-HRESI-MS system from Thermo Scientific (Germering, Germany) consisting of 6-channel degasser SRD-3600, high-pressure gradient pump HPG-3400RS, autosampler WPS-3000TRS, and column compartment TCC-3000RS coupled to Thermo Scientific Q Exactive Plus (Bremen, Germany) mass spectrometer. The full scan MS was set at 16 minutes duration with runtime from 1.06 to 13.96 minutes, resolution 70000; AGC target $3e^6$, max IT 100 ms, scan range 150 to 2000 *m/z*. The MS scan was set at 17500 resolution and AGC target $1e^5$, maximum IT, scan range 200 to 2000 *m/z*, isolation window 2.0 *m/z*, and (N) CE 20, 40, 60. The ionization source (HRESI) was set at: +3.5 to -2.5 kV spray voltage and 320 °C capillary and probe temperature, 38 arbitrary units (a.u., as set by the Extractive 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 C18 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 performed as follows: 25% B for 0.5', gradient to 35% B for 1.5', gradient to 40% B for 5', increase to 45% in 4', increase to 95% in 3', and isocratic 95% for another 2'.

Analysis and quantification of cycloartane saponins

Astragaloside I (**1**), Astragaloside II (**2**), and Astragaloside IV (**3**) were used as external standards for the quantitative analysis of saponins. Each of the standards was dissolved in 50% MeOH and then subsequently diluted to reach 5 concentration levels used to build the calibration curves for quantitative assay covering the concentration range of 1.35–21.60 μg/mL for Astragaloside I; 1.20–19.00 μg/mL for Astragaloside II and 2.00–27 μg/mL for Astragaloside IV, respectively. All solutions were stored in the refrigerator at 4 °C.

Extraction and purification of crude extracts

The dried plant material from *in vitro* cultures of *A. thracicus*, native *A. thracicus* roots, and *A. membranaceus* roots were exhaustively extracted with 80% MeOH. The extracts were filtered and concentrated under reduced pressure and after that fractionated by solid phase extraction (SPE) using C18 cartridges (500 mg) to obtain H_2O, EtOAc, and MeOH fractions. Further separation of the fractions was achieved by blotting the samples onto Diaion HP 20, and subsequently eluted with 100 mL 40% and 90% MeOH for each

of the EtOAc fractions and 30% and 90% MeOH respectively for each of the MeOH fractions, which resulted in 4 fractions for each initial extract (Scheme 1). Each of the fractions was subjected to UHPLC-HRESI-MS analysis.

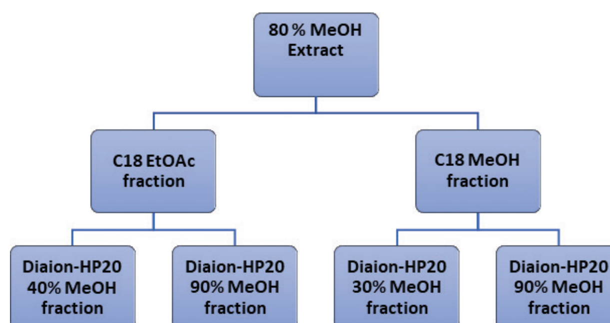
Results and discussion

In vitro cultivation

Shoot culture from *A. thracicus* was derived from MS medium, while the roots were obtained by cultivation in the dark regimen of cultivation using modified MS medium supplemented with 2 mg/L NAA (Ms-Li). The suspension cultures were initiated when cultivated on MS medium supplemented with 2 mg/L kinetin, 0.2 mg/L IAA, 0.1 mg/L 2,4-D, and 1 g/L casein (G48) and cultivated in a dark and light regimen of cultivation (Ionkova et al. 2010).

Identification of cycloartane saponins

Within UHPLC-HRESI-MS analysis astragaloside I, II, and IV were determined at negative ion mode as adducts with HCOOH. For astragaloside I (**1**) HRESIM spectrum showed a protonated molecular ion at *m/z* 913.4805 $[M+FA-H]^-$ corresponding to molecular formula $C_{46}H_{73}O_{18}$ (calc. *m/z* 913.4791) and t_R 9.12' (Suppl. material 1: fig. S1). Astragaloside II (**2**) was observed as a protonated molecular ion at *m/z* 871.4699 $[M+FA-H]^-$ corresponding to molecular formula $C_{44}H_{71}O_{17}$ (calc. *m/z* 871.4686) and t_R 5.61' (Suppl. material 1: fig. S2), while astragaloside IV (**3**) produce a protonated molecular ion at *m/z* 829.45941 $[M+FA-H]^-$ corresponding to molecular formula $C_{42}H_{69}O_{16}$ (calc. *m/z* 829.4580) and t_R 4.31' (Fig. 1). The compounds in samples were identified according to the described above retention times, *m/z* ratios and spectrum fragmentations. The three important cycloartane metabolites (**1**–**3**) were found in all analyzed samples from *A. thracicus* and *A. membranaceus*.



Scheme 1. Extraction and purification of crude extracts from *in vitro* cultures of *A. thracicus*, native *A. thracicus* roots, and native *A. membranaceus* roots.

Calibration model

A visual evaluation of the linear regression line plot showed that the method was linear for all of the standards (Fig. 2). The determination coefficient for compound **1** was r^2 = 0.9858, for compound **2** r^2 = 0.9932 and compound **3** r^2 = 0.9960, respectively. After the linearity was investigated the regression analysis was performed. The determination coefficients and regression equations are presented in Table 1.

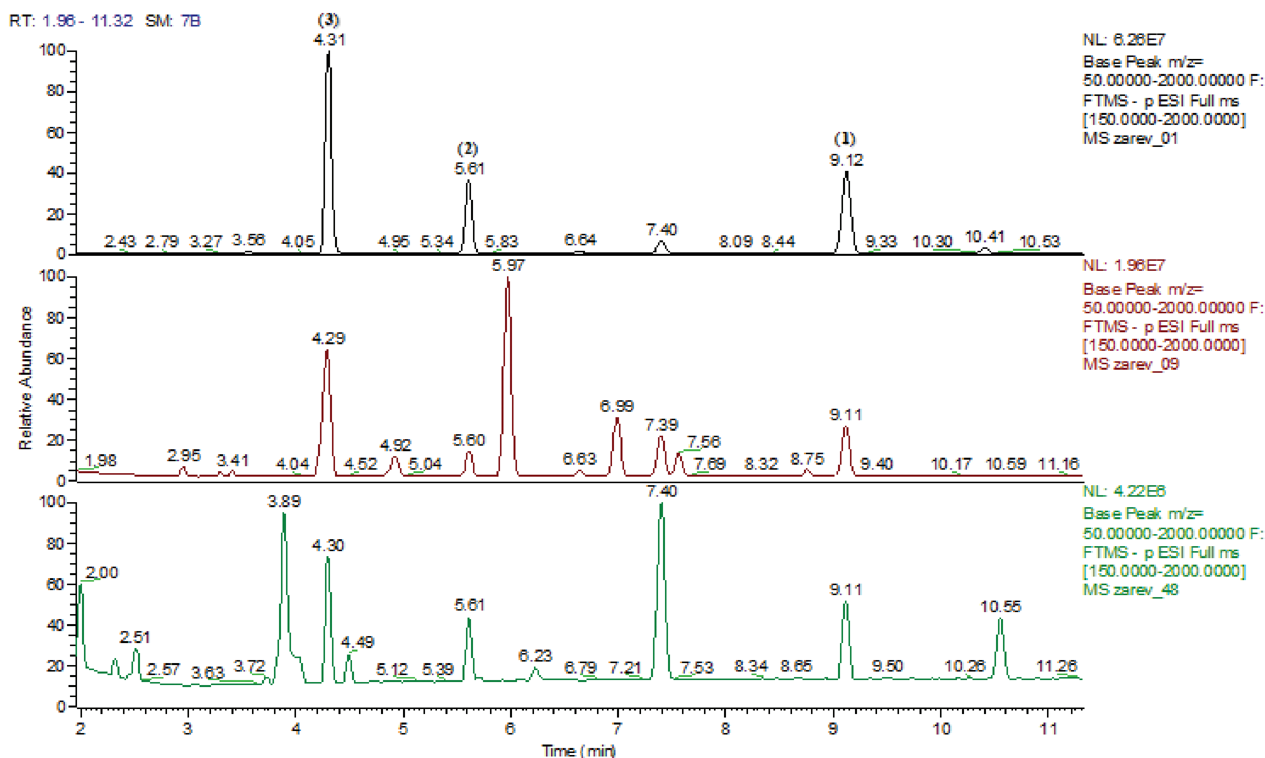


Figure 1. A chromatogram of the standard mixture of saponins.

Table 1. Determination coefficient and regression equations of compound 1–3.

	Astragaloside I (1)	Astragaloside II (2)	Astragaloside IV (3)
Determination coefficient	0.9858	0.9932	0.9960
Linear range (µg/mL)	1.35–21.60	1.20–19.20	1.66–26.60
Regression equations	Y = 7E+06X–1E+07	Y = 6E+06X–7E+06	Y = 1E+07X–1E+07
Number of standards	5	5	5
Rt (min)	9.12	5.61	4.31

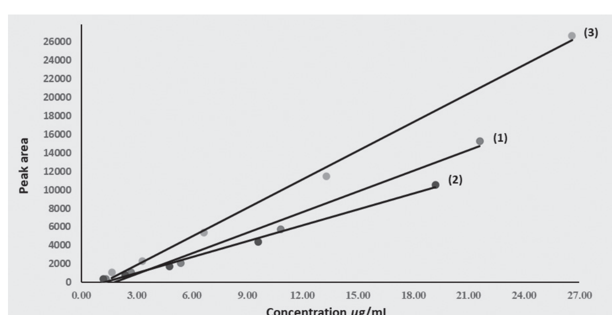
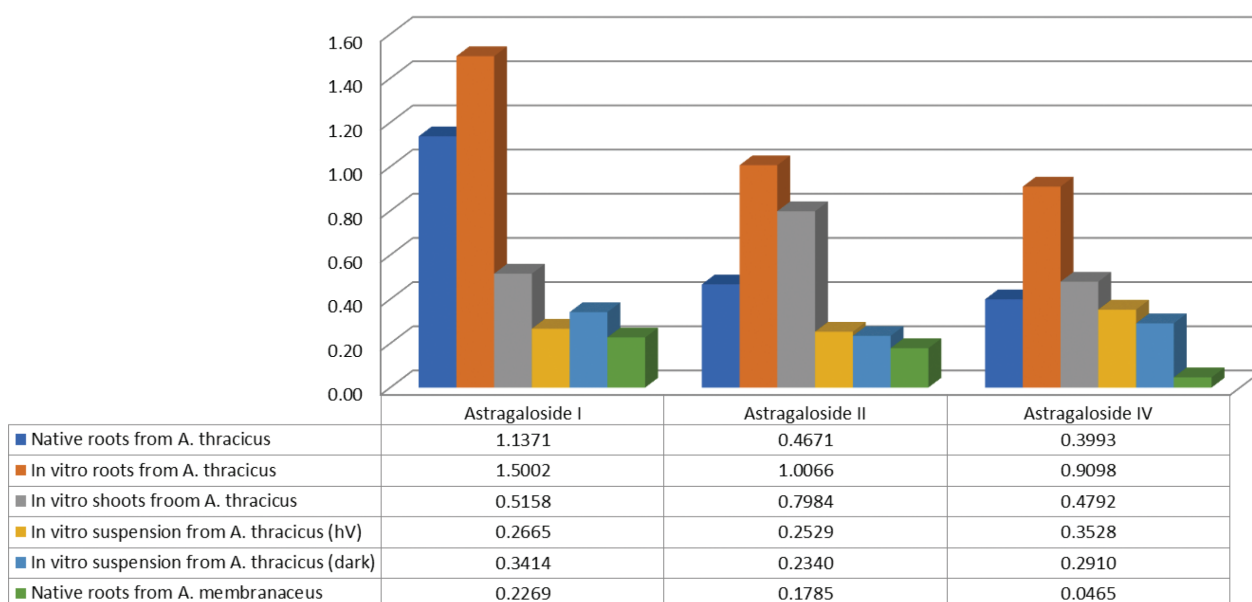


Figure 2. Calibration curve for Astragaloside I (**1**), Astragaloside II (**2**), and Astragaloside IV (**3**).

Quantification of cycloartane saponins

The amount of the individual saponins was calculated due to the calibration equation formula for Astragaloside I y = 7E+06x–1E+07; Astragaloside II y = 6E+06x–7E+06 and Astragaloside IV y = 1E+07x–1E+07 within each of the derived fractions from *in vitro* cultures of *A. thracicus*, native roots of *A. thracicus* and native roots of *A. membranaceus*. In general, the highest amount of compounds **1**–**3** was found in EtOAc 90% fractions, except compound **3**, which eluted in large amounts also in MeOH 90% (Suppl. material 1: figs S4–S6). This unexpected chromatographic behavior may be related to the absence of an acetylene residue in the sugar moiety of the molecule, which probably makes it more polar. This can also be confirmed by the retention times from the LC-HRESI-MS analysis of a mixture including the three saponins (Fig. 1).

Cycloartane saponins were proved in the highest amount (1.50 mg/g DW (**1**), 1.01 mg/g DW (**2**), and 0.91 mg/g DW (**3**)) in *in vitro* root cultures from *A. thracicus*. Even for astragaloside II and IV, the amount was two folds higher than the amount in native roots (0.47 mg/g DW (**2**), 0.40 mg/g DW (**3**)). *In vitro* shoot cultures of *A. thracicus* also produce a higher amount of astragaloside II and IV (0.80 mg/g DW (**2**), 0.48 mg/g DW (**3**)) than the native source provides. An expected low amount of cycloartane saponins was observed in suspension culture cultivated on G48 medium such as those grown in the dark could produce a higher amount of astragaloside I (0.34 mg/g DW), while astragaloside II and IV are observed in higher amounts (0.25 mg/g DW and 0.35 mg/g DW) when suspension cultures are cultivated at light regimen. In all, *in vitro* cultures of *A. thracicus* the observed metabolites were in higher abundance than in native roots of *A. membranaceus* (0.23 mg/g DW (**1**), 0.18 mg/g DW (**2**) and 0.05 mg/g DW (**3**)) (Fig. 3).



	Astragaloside I	Astragaloside II	Astragaloside IV
Native roots from A. thracicus	1.1371	0.4671	0.3993
In vitro roots from A. thracicus	1.5002	1.0066	0.9098
In vitro shoots froom A. thracicus	0.5158	0.7984	0.4792
In vitro suspension from A. thracicus (hV)	0.2665	0.2529	0.3528
In vitro suspension from A. thracicus (dark)	0.3414	0.2340	0.2910
Native roots from A. membranaceus	0.2269	0.1785	0.0465

Figure 3. Cycloartane saponins mg/g DW in native roots of *A. thracicus* and *A. membranaceus*, compared to *in vitro* cultures of *A. thracicus*.

Conclusion

The comparative LC-HRESI-MS analysis showed for the first time that the endemic species *A. thracicus* biosynthesized identical rare cycloartane saponins as *A. membranaceus*. In the present study, a new biotechnological platform was also created that provides a higher production of cycloartane saponins–astragaloside I, II, and IV compared to the wild species. The *in vitro* root cultures of *A. thracicus* reached two folds higher amounts of saponins than native root of *A. thracicus*, and up to six times higher when compared with *A. membranaceus*. In addition, our results provide a fast LC-HRESI-MS protocol for the identification of cycloartane saponins.

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Supplementary material 1

HR-ESI-MS of Astragaloside I, Astragaloside II and Astragaloside IV

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Data type: .zip file

Explanation note: The amount of Astragaloside I (1), Astragaloside II (2) and Astragaloside IV (3) determined in each of the fraction obtained from in vitro cultures of *A. thracicus*, native roots of *A. thracicus* and native roots of *A. membranaceus*.

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