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Research Article

Flavonoids in *in vitro* cultures of *Astragalus hamosus*

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Abstract

Astragalus hamosus contains valuable biologically active compounds, incl. flavonoids. The possibility for *in vitro* cultivation of the species as a source of important flavonoids was studied. Shoot and callus cultures were established and successfully cultivated on different nutrition media, complemented or not with growth regulators. An ultra-high performance liquid chromatography – high-resolution electrospray ionisation mass spectrometry (UHPLC-HRESIMS) qualitative and quantitative analysis of non-purified methanol extracts of these cultures was performed. It was found that the cultures produced rutin in comparable quantity. Interestingly, both shoots and callus cultures accumulated the rare triglycosides alcesefoliside and mauritianin. The quantity of mauritianin, biosynthesized in shoots, was significantly higher to that in callus cultures. Alcesefoliside, was in lower quantity, compared to mauritianin. In addition, callus cultures produced alcesefoliside trice as the shoots, besides their lower level of differentiation. These findings could serve as initial research to establish the value of *in vitro* cultures from *A. hamosus* as an alternative mean of production of pharmaceutically important flavonol glycosides.

Keywords

Astragalus, quantitative analysis, flavonoids, UHPLC-MS, *in vitro* cultures

Introduction

Astragalus hamosus L. (Fabaceae) is an annual or biennial ascending plant, distributed in the Mediterranean, Southern Europe, Caucasus, Central and Southwest Asia. The species is spread in Bulgaria as well. Due to its early flowering, it is difficult to obtain in the wild. Moreover, the species is quite small and thus the naturally growing biomass is expensive to collect in quantities, suitable for the practice (Valev 1976; Asyov et al. 2012). From the overground parts of the wild grown species 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. Recently, *N*-(8-methylkaempferol-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidine-2-one, quercetin-3-O-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside], kaempferol-3-O-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside], alcesefoliside and mauritianin were proved (Shkondrov and Krasteva 2021b). Hyperoside, astragalin and isoquercitrin were identified in introduced plants (Krasteva et al. 2007). Rutin, astragalin and isoquercitrin were found in callus and suspension cultures of the species (Ionkova and Alfermann 1990; Ionkova 1995). Extracts from the fruits of the plant exhibited *in vivo* anti-inflammatory and analgesic activity (Shojaii et al. 2015). Rhamnocitrin-4'-β-D-ga-

lactopyranoside protected human kidney cells HEK-293T against the cytotoxic effects of nephrotoxic drugs (Krasteva et al. 2008) and exhibited hepatoprotective activity against liver cancer in Wistar rats (Saleem et al. 2013). Alcesefoliside exhibited excellent neuroprotective activity *in vivo* (Simeonova et al. 2019). Mauritianin, on the other hand has an antiviral potential, incl. against Covid-19 (Owis et al. 2020). Wild grown plants have their major drawbacks considering the low amount of these compounds. Biotechnological approach has been proved as reliable and modern alternative. Many studies proved that flavonoid production is possible in *Astragalus* plants *in vitro* (Wink et al. 2005; Krasteva et al. 2016)

The aim of the study was to establish *in vitro* cultures of *A. hamosus*, and to preform phytochemical analysis by ultrahigh performance liquid chromatography – electrospray ionisation mass spectrometry (UHPLC-HRESIMS).

Materials and methods

In vitro cultivation

Seeds from *Astragalus hamosus* were obtained from a wild plant in May 2020, growing in Sofia, Bulgaria. One of us (I. K.) confirmed the identity of the species. Seeds were surface-sterilized with 95% EtOH for 60 s, then in a 20% solution of commercial bleach (20 min), followed by three times rinsing with sterile water. After sterilization, the seeds were germinated aseptically in DoH culture media (Zdraveva et al. 2017). The germinated seeds were transferred into flasks containing solid Murashige and Skoog (MS) plant growth media (Murashige and Skoog 1962), and grown in an illuminated chamber (3000 Lx, 20 °C) to produce shoots. After four weeks, shoots were sub-cultured. Callus culture was initiated when shoot explants were cultivated on G48 medium in light regimen (Ionkova et al. 2010). Every three weeks the calli were transferred in fresh medium.

Extraction

The samples (shoots and callus) were dried at room temperature and 200 mg of each were extracted in reflux twice with 2.5 mL 80% MeOH on a boiling water bath for 30 min. The obtained extracts were filtered and combined in a volumetric flask. The volume was adjusted to 10.0 mL with 80% MeOH. An aliquot of 2 µL was injected to the UHPLC system after filtration through a membrane PVDF syringe filter (0.22 µm).

UHPLC-HRESIMS analysis

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 $3e^6$, max IT 100 ms, scan range 250 to 1700 *m/z*. The MS^2 conditions were: resolution 17500 (at *m/z* 200), AGC target $1e^5$, 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. 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

N-(8-methylquercetin-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidine-2-one (**QueFA**), *N*-(8-methylkaempferol-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidine-2-one (**KaeFA**), quercetin-3-O-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside] (**QueHMG**), kaempferol-3-O-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside] (**KaeHMG**), quercetin-3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranoside (**alcesefoliside**) and kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranoside (**mauritianin**) were obtained from *A. monspesulanus* subsp. *monspesulanus* (purity > 95%). The compounds were identified by extensive MS and NMR analyses and comparison to literature (Krasteva et al. 2015). Rutin was purchased from Sigma Aldrich (Germany). Standard solutions of each reference were prepared in MeOH (1000 ng/mL). Subsequent serial dilutions were made from these stock solutions. Two µL of each solution were injected in the UHPLC-HRESIMS system three times to obtain the mean retention time. The mean AUC of each concentration of the flavonoids was used to construct the calibration curves.

Results and discussion

In vitro cultivation

Seeds form the species were successfully germinated in *in vitro* conditions on casein-containing DoH medium. The seedlings were aseptically transferred on MS medium solidified with agar-agar. The shoots obtained were growing

well (Fig. 1) and after four sub-cultivation cycles enough biomass was obtained for phytochemical analysis.

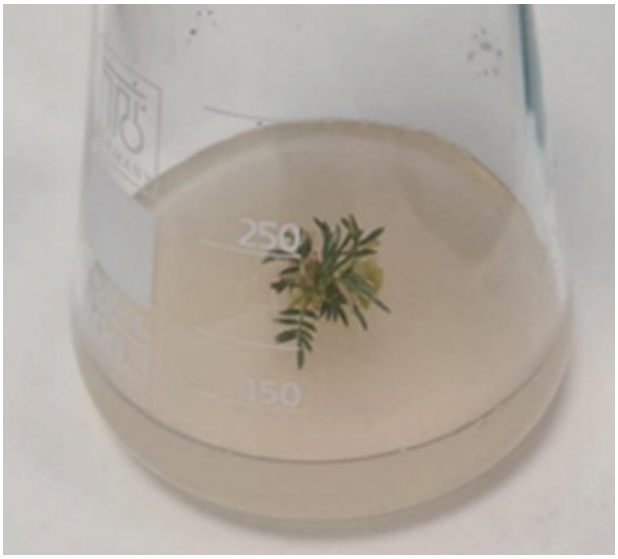

Figure 1. Shoot culture of *A. hamosus*.

Unlike the wild grown plant, the shoots have predictable time of harvest and it is possible to calculate the expected quantity of plant material. This is crucial to further investigations, because as it is well-known, phytochemical analysis and isolation of perspective compounds often requires large quantities of plant material (Ionkova 2009). In the case of *A. hamosus*, a stable and predicted growth is a preferred feature, compared to the wild-growing and often difficult to collect plant (Krasteva et al. 2008).

Callus was initiated after transferring sterile explants from large intact shoots of the plant on G48 medium. The most successful explants are often young tissues of one or a few cell types. Pith cells of young stem are usually a good source of explant material. Initially, callus cells proliferate without differentiating, but eventually differentiation occurs within the tissue mass. Actively dividing cells are those uppermost and peripheral in the callus. When cultivated in light regimen, the growth was substantial (Fig. 2).

The extent of overall differentiation usually depends on the hormone balance of the support medium and the physiological state of the tissue. Actively growing callus can be initiated on culture media with an even physiological balance of cytokinins and auxins. After callus biomass increases two to four times (after two to four weeks of growth), callus can be divided and placed on fresh medium for multiplication. This procedure can be repeated several times before gross chromosome instability (or contamination) occurs (Ionkova 2007).

Identification of compounds by LC – HRESIMS

Identification of analytes in all samples was done based on retention time, and the mass spectral fragmentation of the compound, compared to the corresponding standard, injected at the same conditions. The retention times and

Figure 2. Callus culture of *A. hamosus*.

the spectral fragmentation pattern of the reference substances was previously described (Shkondrov and Krasteva 2021a). Results from the identification are presented in Table 1.

Table 1. Compounds identified in the samples.

Compound	Callus, G48	Shoots, MS
Rutin	present	present
Mauritianin	present	present
Alcesefoliside	present	present
QueFA*	not present	not present
KaeFA*	not present	not present
QueHMG*	not present	not present
KaeHMG*	not present	not present

*see Reference substances

Rutin, alcesefoliside and mauritianin were identified in callus grown on G48 medium and in shoots grown on MS. These findings coincide with data on wild grown plant (Shkondrov and Krasteva 2021b). Moreover, rutin was previously reported only in suspension cultures of the species (Ionkova and Alfermann 1990). Compounds QueFA, KaeFA, QueHMG and KaeHMG were not identified in the samples. In contrast to the wild plant, both *in vitro* cultures did not produce the kaempferol flavoalkaloid and the hydroxymethylglutaric acylated flavonoids (QueHMG and KaeHMG) (Shkondrov and Krasteva 2021b). This could be explained by two aspects – the culturing media is not optimized to meet the specific needs of this particular species; secondly, there is a possibility of metabolic interference of the arbuscular mycorrhiza in the wild on the secondary metabolism of *Astragalus* plants (Smith and Read 2010), incl. *A. hamosus*.

LC-MS quantitation

Based on the qualitative analysis, only the identified compounds were used to construct the calibration curves and

Table 2. Limits of detection and quantitation.

Parameter	Rutin, ng.mL^{-1}	Mauritianin, ng.mL^{-1}	Alcesefoliside, ng.mL^{-1}
LOD	0.001	0.002	0.001
LOQ	0.010	0.02	0.014

to perform quantitative assays. Validation of LC-MS method in respect of rutin, alcesefoliside and mauritianin was performed according to ICH guideline (Guideline 2005). Calibration curves of the flavonoids are presented in Suppl. material 1. The equations and the correlation coefficients were: $y = -4.06921e^{+006} + 5324.8 \times$ ($r^2 = 0.9999$) for rutin; $y = -338029 + 13361 \times$ ($r^2 = 0.9937$) for mauritianin; $y = 89288.7 + 8839.1 \times$ ($r^2 = 0.9942$) for alcesefoliside. Specificity in respect of solvents was examined on blank solution except rutin, alcesefoliside and mauritianin. There were no peaks in the chromatogram of this solution with t_R corresponding to that of the references. For repeatability eight solutions containing rutin, alcesefoliside and mauritianin were analysed. Standard deviation (SD) was found to be ± 1.0% for these solutions. The limit of detection (LOD) was calculated by the equation $y_{LOD} = y_b + 3S_b$. The limit of quantitation (LOQ) was determined by $y_{LOQ} = y_b + 10S_b$. In both equations, y_b is the analytical signal of the blank solution and S_b is the standard deviation of that signal.

Linearity was studied in concentration range 0.002–1520 ng for the three flavonoids; the correspondence between the area of the peaks and concentrations in ng.mL^{-1} was proportional in the intervals with $r^2 > 0.99$. The mean values from three injections were calculated and the standard deviation was determined. The results are presented in Table 3.

Table 3. Content of flavonoids.

Culture	Rutin ng/mg dw ± SD	Mauritianin ng/mg dw ± SD	Alcesefoliside ng/mg dw ± SD
Callus, G48, hv	79.4 ± 0.01	43.4 ± 0.03	18.9 ± 0.01
Shoots, MS, hv	80.6 ± 0.01	77.3 ± 0.03	6.1 ± 0.02

Rutin content was not significantly different between the two cultures. Rutin was previously found only in suspension cultures of *A. hamosus* (Ionkova and Alfermann 1990). Surprisingly, mauritianin quantity was nearly double in shoot culture than in callus. This could be explained with the level of differentiation of the shoots, compared to callus. On the contrary, alcesefoliside, although metabolically connected to mauritianin, was the other way round; its quantity in the callus culture was higher than alcesefoliside found in shoots. Besides their lower level of cell differentiation (compared to shoots) callus culture accumulated alcesefoliside. Nevertheless, biosynthetic pathways of these two flavonoids have not been studied yet to allow such conclusions. LC-MS is considered to be one of the most accurate methods to identify and quantify multiple compounds in complex mixtures, including plant extracts (Tolonen and Uusitalo 2004). It is known that culturing media composition has significant influence on the production of secondary metabolites, incl. flavonoids (Zdraveva et al. 2017). Moreover, mauritianin and alcesefoliside, as relatively rare compounds, are interesting markers to perform such analysis. This study could serve as the basis for further optimization of the media composition in order to fine-tune the biosynthetic potential of *in vitro* cultures from *A. hamosus* in order to produce alcesefoliside and mauritianin.

Conclusion

In vitro cultures of *Astragalus hamosus* were successfully developed. Rutin, alcesefoliside and mauritianin were proved and quantified in the cultures by UHPLC-HRESIMS method. Significant differences in the quantity of both the flavonol triglycosides were found. Callus cultures could be used to produce quercetin derivatives, and shoot cultures - to biosynthesize kaempferol glycosides. A further investigation of the growth media composition is needed in order to investigate the influence of the components on the production of these valuable flavonoids in the established *in vitro* cultures.

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Supplementary material 1

Figures S1–S3

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Data type: Docx file.

Explanation note: **Fig. S1.** Calibration curve of rutin. **Fig. S2.** Calibration curve of mauritianin. **Fig. S3.** Calibration curve of alcesefoliside.

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