

Bulgarian species of genus *Astragalus* as potential sources of mauritianin

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Abstract

Mauritianin is a relatively rare flavonoid, but several studies revealed its pharmacological potential. In this study overground parts of ten *Astragalus* species were investigated for mauritianin content to find a reliable source of the compound. The quantity of the flavonoid in each extract was determined by a novel high performance liquid chromatography-high resolution mass spectrometry method. For the first time mauritianin is reported in *A. cicer*, *A. onobrychis*, *A. glycyphyllos*, *A. glycyphylloides*, *A. corniculatus* and *A. ponticus*. Only in *A. depressus* the compound was not found. Significant differences in mauritianin content (from 4 to 1642 ng/mg dry weight) of the samples were observed. Noteworthy, *A. onobrychis* var. *chlorocarpus* and *A. cicer* could be considered as a potential source of the compound, substituting the rare *A. monspessulanus* subsp. *monspessulanus*.

Keywords

Astragalus, mauritianin, quantitative analysis, flavonoids, UHPLC-MS

Introduction

Mauritianin (kaempferol-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 2)-[α -L-rhamnopyranosyl-(1 \rightarrow 6)]- β -D-galactopyranoside) was isolated for the first time from *Lysimachia mauritiana* (Yasukava and Takido 1987). Due to the relatively rare occurrence of mauritianin in the plant kingdom, only few studies of its pharmacological action have been conducted. A methanol extract of *Swartzia apetala* var. *glabra*, containing the compound, showed antifungal activity against nine strains of *Candida* spp. (de Araujo et al. 2013). Mauritianin, obtained from leaves of *Maytenus ilicifolia* exhibited significant gastro protective effect (Leite et al. 2013). The flavonoid isolated from leaves of *Catharanthus roseus* enhanced the 12-*O*-tetradecanoylphorbol-13-acetate-suppressed delayed-type hypersensitivity in mice, indicating that the

compound may augment the immune resistance to cancer (Nishibe 2013). Mauritianin was found in *Acalypha indica* leaves as well (Nahrstedt et al. 2006). Lately, a DNA-topoisomerase I inhibition activity was proved for the compound, similar to that of camptothecin in higher concentration levels, making it a potential candidate for investigation of its antiproliferative effects (Ma et al. 2005).

Our previous phytochemical study of *Astragalus monspessulanus* subsp. *monspessulanus* afforded mauritianin and its cytoprotective effect was evaluated in a model of *tert*-butylhydroperoxide-induced oxidative stress on isolated rat hepatocytes (Krasteva et al. 2015). Due to the rare occurrence of *A. monspessulanus* in Bulgaria (Asyov et al. 2012), ten different species from genus *Astragalus* L. (Fabaceae) were examined in order to reveal the quantity of mauritianin and to select a reliable source of this flavonoid.

Materials and methods

Plant material

The overground parts of ten *Astragalus* species (Table 1) were collected in two phenological phases (flowering and fructification), different years and localities in Bulgaria. The species were identified by Dr. D. Pavlova from Faculty of Biology, Sofia University, Bulgaria and two of us (E. K. and S. S.). Voucher specimens were deposited in the Herbarium of the Sofia University (SO) or at the Herbarium of the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences (SOM).

Extraction

Each plant sample (200 mg) was refluxed twice with 3 mL 80% MeOH on a water bath for 30 min each. The obtained extracts were filtered, combined in a volumetric flask and the volume adjusted to 10.0 mL with the same solvent. An aliquot of 2 μ L was injected to the ultra-high-performance liquid chromatography (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 ionization (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 MS² 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: -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 C₁₈ column (1.9 μ m, 2.1 \times 50 mm, Akzo Nobel, Sweden) maintained at 40 °C. The mobile phase was H₂O + 0.1% HCOOH (A) and MeCN + 0.1% HCOOH (B) with a flow rate of 0.3 mL/min. Gradient elution was performed as follows: 10% B for 0.5 min, then 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, then return to 10% B for 0.1 min.

Reference substance and calibration curve

Mauritianin (Fig. 1) was used as a reference. It was isolated from the overground parts *A. monspessulanus* subsp. *monspessulanus* (99.8%) and its structure was confirmed by MS and NMR analyses and comparison to the literature (Krasteva et al. 2015). The HRESIMS spectrum of mauritianin showed an ion [M-H]⁻ at $m/z = 739.2104$ (calcd. 740.2164, C₃₃H₄₀O₁₉). The deprotonated molecule is suitable as a marker to perform quantitation. Standard solutions of mauritianin were prepared in MeOH as follows: 1; 10; 50; 100; 600; 1000; 1500 and 2000 ng/mL. To obtain the calibration curve points 2 μ L of each solution were injected in the UHPLC-HRESIMS system three times.

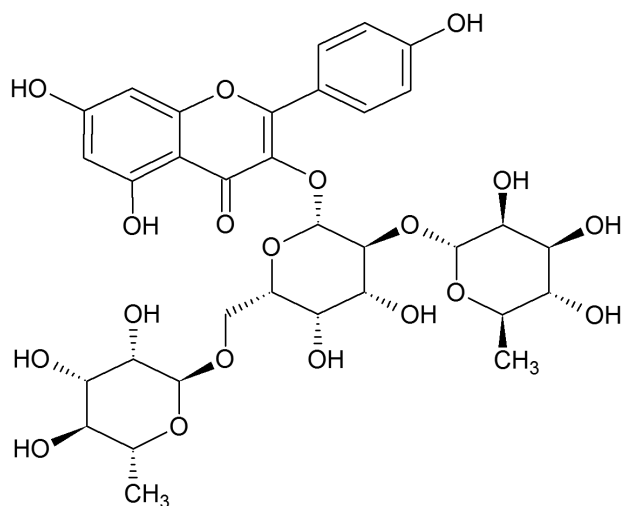


Figure 1. Structure of mauritianin.

Table 1. Content of mauritianin in several *Astragalus* species in Bulgarian flora.

Species (aerial parts)	Year	Locality	Voucher specimen	Phenological stage	Mauritianin, ng/mg dry weight \pm SD
<i>A. cicer</i>	2013	Sofia	SO 102681	flowering	1472.43 \pm 0.03
	2016	Sofia	SOM 1394	fructification	238.67 \pm 0.02
<i>A. corniculatus</i>	2018	Gorna Studena	SOM 1399	flowering	4.53 \pm 0.02
<i>A. depressus</i>	2015	Erma	SOM 1402	flowering	not detected
<i>A. glycyphylloides</i>	2008	Vitosha	SO 093817	flowering	381.81 \pm 0.03
<i>A. glycyphyllos</i>	2009	Rila	SO 107612	flowering	4.34 \pm 0.02
	2012	Vitosha	SO 107613	flowering	11.48 \pm 0.02
<i>A. hamosus</i>	2013	Sofia	SOM 1398	flowering	150.02 \pm 0.02
<i>A. monspessulanus</i> subsp. <i>monspessulanus</i>	2016	Devin	SOM 1391	fructification	706.84 \pm 0.02
	2016	Slavianka	SOM 1392	flowering	1642.33 \pm 0.02
<i>A. monspessulanus</i> subsp. <i>ilyricus</i>	2015	Erma	SO 107532	flowering	6.33 \pm 0.02
<i>A. onobrychis</i> var. <i>chlorocarpus</i>	2013	Stara Zagora	SO 107538	flowering	1008.68 \pm 0.02
	2016	Golo Bardo	SOM 1393	flowering	927.52 \pm 0.03
	2016	Vitosha	SOM 1390	flowering	1121.86 \pm 0.02
<i>A. ponticus</i>	2013	Pleven	SO 107539	flowering	13.98 \pm 0.03

Detection

Detection of mauritianin in plant samples was performed by a set range of m/z 739.19 to 739.22 with an additional time filter, corresponding to the retention time of the standard (5.37 ± 0.02 min). Identification of the flavonoid was supported by a MS² 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 mauritianin.

Statistical analysis and calculation

The software Xcalibur, Version 4.2 (Thermo Scientific) was used to collect raw data, to obtain the calibration curve and to calculate the results.

Results and discussion

For determination of mauritianin in plant extracts a novel UHPLC-HRESIMS method was developed. Identification of the compound in the samples was achieved by comparison with the standard (retention time and MS² fragmentation pattern). Mauritianin was detected as a deprotonated molecule $[M-H]^-$ at m/z 739.21 (for $C_{33}H_{39}O_{19}$), for which MS² fragmentation resulted in product ions as follows: $[M-rha-rha-H_2O-H]^-$ at m/z 465.0275 (Cuyckens et al. 2000); the subsequent loss of 162 Da (galactose moiety, Hvattum and Ekeberg 2003) yielded product ion $[M-rha-rha-gal-H_2O-H]^-$ at m/z 285.0396 (deprotonated kaempferol, Kae); at m/z 284.0328 for $[Kae-2H]^{2-}$; and a product ion at m/z 151.0023, indicative of flavonols' cleavage ^{1,3}A, as adopted from the fragmentation pattern, given by Fabre et al. (2001). The reference mauritianin maintained a retention time of 5.37 ± 0.02 min, which corresponded well to that of the flavonoid in the samples (5.35 ± 0.02 min) (Fig. 2).

The equation $y = 608416 + 24831.2 x$ ($r^2 = 0.9976$) was obtained from the calibration curve. The method was validated following the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) requirements (2005). Specificity was examined on blank solutions. There were no peaks in the chromatogram of the blank solution with t_R simi-

lar to that of mauritianin. The limit of detection, based on three times the signal-to-noise ratio, was calculated as 0.018 ng/mL by injecting 2 μ L of standard solution. The linearity was studied in the interval 0.018 to 1800 ng/mL ($r^2 > 0.99$). The precision and accuracy were evaluated by spiking extract from *A. monspessulanus* subsp. *monspessulanus* with mauritianin to obtain concentration of 5 and 1800 ng/mL. Method precision was 5% (as RSD%, by six replicates of each concentration level). The accuracy was 0.9%. The repeatability (SD%) on six solutions containing mauritianin was $\pm 1.2\%$.

Liquid chromatography, coupled with mass spectrometry is considered to be one of the most accurate methods to identify multiple compounds in complex mixtures, including plant extracts (Tolonen and Uusitalo 2004).

The method was applied to examine mauritianin content of ten *Astragalus* species. The compound was detected in all species except *A. depressus*. MS² analysis was used to confirm its identity, taken together with the retention time and the m/z of the deprotonated molecule. Noteworthy, in the samples the cleavage of the sugar moiety attached to the third position of kaempferol was a two-step process, involving loss of the two rhamnose units (274 Da) (Cuyckens et al. 2000) and the galactose part (162 Da, Hvattum and Ekeberg 2003). The kaempferol aglycone was registered (285 Da) as well (Fabre et al. 2001). The MS² fragmentation pattern was identical to that of the reference substance mauritianin as described above.

Significant differences in mauritianin content of the samples were found. The highest amount of the compound was determined in *A. monspessulanus* subsp. *monspessulanus* (1642 ng/mg), *A. cicer* (1472 ng/mg) and *A. onobrychis* (1009 ng/mg). The lowest quantity was found in *A. glycyphyllos* (4 ng/mg) (Table 1).

Although closely related, the subspecies of *A. monspessulanus* (*monspessulanus* and *illyricus*) were established to have different flavonoid composition (Krasteva et al. 2015). Moreover, the quantitative analysis of mauritianin is another factor to differ the subspecies. Results showed that subsp. *monspessulanus* had the highest mauritianin content, whereas subsp. *illyricus* accumulated only 6 ng/mg.

There were no significant differences in mauritianin quantity in samples from *A. glycyphyllos* and *A. onobrychis* var. *chlorocarpus*, collected in different years and localities (Table 1).

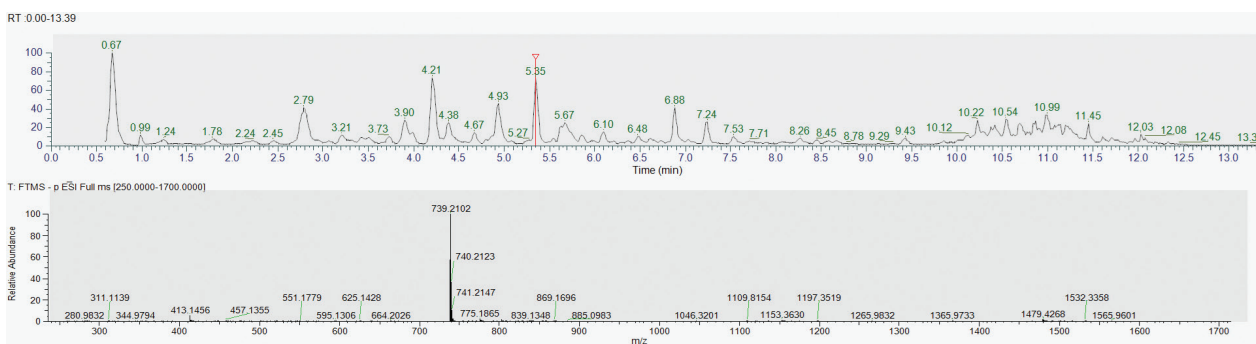


Figure 2. A base peak full scan chromatogram of an extract of *A. cicer* ($t_R = 5.35$ min, $[M-H]^-$ m/z 739.2102, mauritianin).

Fructification stage reduced significantly the amount of the flavonoid, compared to the flowering stage, as seen from the results for *A. cicer* and *A. monspessulanus* subsp. *monspessulanus*. Nearly five times fold reduction in mauritianin content during fructification is in direct correlation with the general rule of collection of flavonoid-rich plant substances – only in the flowering stage (Koes et al. 1994).

Conclusion

Using a novel UHPLC-HRESIMS method mauritianin was determined in *A. cicer*, *A. onobrychis*, *A. glycyphyllos*, *A. glycyphylloides*, *A. corniculatus* and *A. ponticus* for the first time. The highest amount of the compound was found in *A. onobrychis* var. *chlorocarpus* and *A. cicer*. These species could be considered as a reliable source of this rare flavonol mauritianin.

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