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Original Article

Identification of phenolic compounds in *Myricaria bracteata* leaves by high-performance liquid chromatography with a diode array detector and liquid chromatography with tandem mass spectrometry

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Myricaria bracteata Royle, Tamaricaceae, is a species with a wide geographic range that encompasses Eastern Europe, Western and Central Siberia, Central Asia, and the Himalayas. This plant is used in traditional folk medicine in Russia (Siberia) and in China typically as an analgesic and for the treatment of some infections and certain types of intoxication. The aim of this study was to identify phenolic constituents of the leaves of *M. bracteata* from two considerably distant populations. Chromatographic profiles of the leaves of *M. bracteata* were analyzed for the first time. Seventeen compounds, mainly methyl ethers of quercetin (isorhamnetin, rhamnazin), kaempferol (kaempferide, rhamnocitrin), and ellagic acid as well as quercetin, quercetin 3-glucoside, kaempferol, luteolin, chrysoeriol, citric acid, gallic acid, methyl gallate, ethyl gallate, and ferulic acid were identified in hydrolyzed aqueous ethanol extracts of the leaves. Flavonols and ellagic acid were the major compounds in both samples. Isorhamnetin was the main flavonoid constituent. Kaempferide and rhamnazin were also abundant in the flavonoid complex of the leaves of *M. bracteata* from the Altai. This study shows that *M. bracteata* leaves are a source of flavonoids with possible biological activities.

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Introduction

Myricaria Desv., Tamaricaceae, is a genus of herbs with a potential for applications to conventional medicine. *Myricaria* species are used in folk medicine in Russia (Siberian regions) and in many Asian countries (more commonly in China, Mongolia, and India) for the treatment of some infections, certain types of intoxication, liver diseases, scalds, and arthritis (Semenova, 1993; Gewali, 2008; Kletter et al., 2008; Kirbag et al., 2009; Singh, 2012). Some species were reported to have high antimicrobial and acetylcholinesterase inhibitory activities and thus can be considered natural sources of antibiotics and drugs for the treatment of neurological disorders such as Alzheimer's disease (Mukherjee et al., 2007).

High antimicrobial activity of the volatiles of intact *Myricaria bracteata* plants against *Staphylococcus epidermidis* as well as a pronounced antifungal activity against *Candida albicans* were also revealed (Lyakh and Tsybulya, 2009). Crude ethanol extract from the above-ground part of *M. bracteata* and its fractions have been shown to have a strong antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Micrococcus luteus*, and *Pseudomonas aeruginosa* (Gonchig et al., 2008).

The literature lacks detailed information on regular constituents of *M. bracteata*, their geographic variability, and the key active ingredients. The aim of this study was to identify the phenolic constituents of the leaves of *M. bracteata* from two considerably distant populations.

Materials and methods

All chemicals were mass spectrometry (MS) and analytical grade. Chemical reference standards of gallic and ferulic acids were purchased from Serva (Heidelberg, Germany); quercetin, kaempferol, rhamnetin, isorhamnetin, luteolin, citric and ellagic acids were purchased from Sigma (St. Louis, MO, USA).

The samples of *Myricaria bracteata* Royle, Tamaricaceae, leaves were collected in the fruiting period of 2011. Sample A was collected at 1800 m above the sea level (a.s.l.) on a pebble floodplain of the Bolshoi Yaloman River, 1 km from Bolshoi Yaloman village, Ongudaysky Region, the Altai Republic. Sample B was collected at 3100 m a.s.l. on a pebble and sandy floodplain of the Tokuz-Bulak

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River (the left tributary of the Gunt River), 1.5 km from the Kuygantukoy village, Shugnon Region, the Republic of Tajikistan. The collected *Myricaria* samples were dried and ground into powder using a household mill.

A precisely weighed sample of air-dried plant material (0.3 g) was exhaustively extracted with an ethanol:water mixture (70:30, v/v) in a water bath at 60–70 °C. The aqueous ethanol extract was hydrolyzed with 2 N HCl for 2 h in a boiling water bath. The hydrolysate was purified by means of a C16 Diapack cartridge and redissolved in ethanol.

Mass spectrometric analysis was carried out at the Core Facility of Mass Spectrometric Analysis (ICBFM SB RAS). An Agilent 1200 liquid chromatography system, with an Agilent 6410 QQQ mass spectrometer (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source, served as an LC-MS/MS system. The chromatographic separation was carried out at 25 °C on a Zorbax Eclipse XBD-C18 Column (4.6 mm × 150 mm, 5 μm i.d.) with an Eclipse XBD-C18 guard column (4.6 mm × 12.5 mm, 5 μm i.d.).

For the gradient elution, 10 mM NH_4COOH in water (pH 4.0, adjusted with formic acid), *i.e.*, solvent A, and 10 mM NH_4COOH (pH 4.0 adjusted with formic acid) in acetonitrile–water 95:5 (v/v), *i.e.*, solvent B, were used. The run was started with 0.6 ml min^{-1} flow rate at a solvent A:solvent B ratio of 100:0 (v/v) followed by a linear gradient to 50:50 (v/v) for the first 5 min, then to 48:52 (v/v) from min 5 to min 10, then to 0:100 (v/v) from min 10 to min 20; and returned to 35:65 (v/v) from min 20 to min 35, then to 100:0 (v/v) from min 35 to min 40. The sample injection volume was 30 μl. All data acquisition and peak integration tasks were performed in the MassHunter software (version 1.3) from Agilent Technologies.

At the first step of the analysis, the samples were analyzed in full-scan negative mode from 100 to 1500 Da. The multiple-reaction monitoring (MRM) transitions for possible molecular structures that have been proposed for a detected ion (Supplementary data A) were based on the RIKEN MSn spectral database for phytochemicals (http://spectra.psc.riken.jp), MassBank (http://www.massbank.jp) and the Human Metabolome Database (HMDB) (http://www.hmdb.ca).

The HPLC system for absolute quantification of phenolic consisted of an Agilent 1200 with a diode array detector (DAD) and the ChemStation (Agilent Technologies, USA) software for data processing. The chromatographic separation was carried out at 25 °C on a Zorbax SB-C18 Column (4.6 mm × 150 mm, 5 μm i.d.) with an Agilent Guard Column Hardware Kit (p.n. 820888-901).

The mobile phase consisted of MeOH (solvent A) and 0.1% orthophosphoric acid in water (solvent B). The run was started at 1 ml min^{-1} rate with a solvent A–solvent B mixture at 50:50 (v/v) followed by a linear gradient to 52:48 (v/v) for the first 15 min, then to 100:0 (v/v) from min 15 to min 17. It was returned to 50:50 (v/v) from min 17 to min 20. The sample injection volume was 10 μl.

The quantification of phenolic compounds was conducted as previously reported (Karpova and Khramova, 2014) (see Supplementary data B). All the data were reported as mean ± standard deviation (SD) of three replicates. The results were compared by Student's *t* test. The data analysis was performed using the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA), and differences between the means were considered statistically significant at the 5% level ($p < 0.05$).

Results and discussion

Seventeen constituents, mainly flavonoids, were identified in the hydrolyzed extracts of each geographic sample of the leaves of *M. bracteata*. The composition of the leaves was identical (Fig. 1).

At the beginning of the analysis, three phenolic acids were identified in *M. bracteata* leaf extract (Table 1). Citric (**1**), gallic (**2**), ellagic

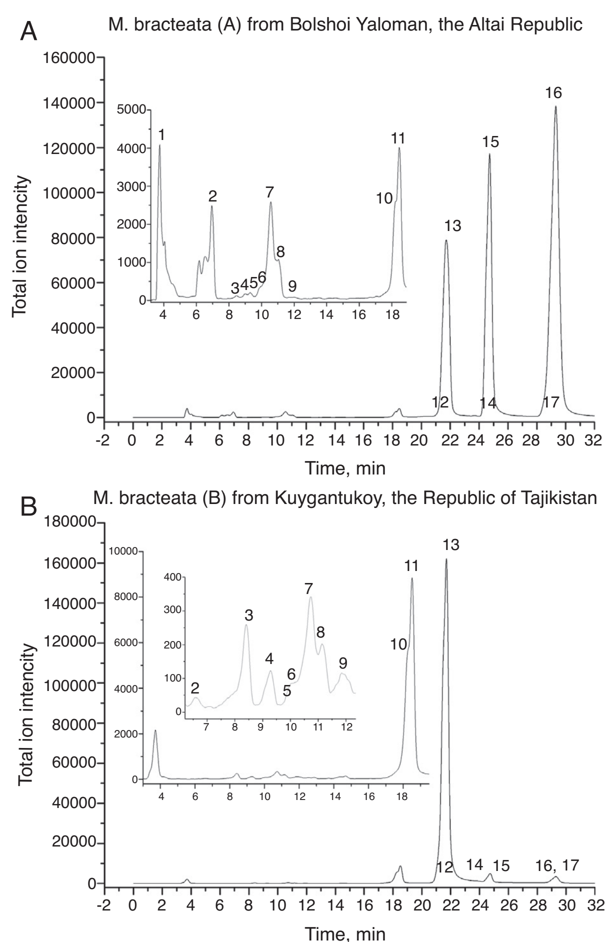

Fig. 1. LC-MS/MS base peak chromatograms of MS in negative ion mode for the hydrolyzed aqueous ethanol extracts of the leaves of *Myricaria bracteata* from the Altai (A) and from the Tajikistan (B).

(**7**), and ferulic (**9**) acids were directly identified by comparison of their retention time with that of standards and were confirmed in the MS/MS spectrum. Citric acid is being suggested as a constituent of *M. bracteata* for the first time and characterizes this plant as alkali-tolerant (Nawwar et al., 2013).

Ethyl gallate (**8**) was tentatively identified according to $[M-H]^-$ at *m/z* 197 and MS/MS fragment ions at *m/z* 124 and 169, where the latter corresponds to gallic acid. Compounds **3** and **4** with $[M-H]^-$ at *m/z* 183 and an MS/MS fragment ion at *m/z* 124 (Rendekova et al., 2015) were tentatively identified as methyl gallate isomers. An additional fragment ion at *m/z* 140 was detected for **3** (Rendekova et al., 2015); however, this fragment ion was not revealed for **4**, probably because of low quantity. Methyl gallate and other derivatives of hydroxybenzoic acids have been reported to be frequent constituents of *M. bracteata* (Zhang et al., 2011; Zhao et al., 2005).

A total of ten flavonoid derivatives were identified in *M. bracteata* leaves. Quercetin (**11**), luteolin (**10**), kaempferol (**12**) and isorhamnetin (**13**) were identified by comparison of retention time and the MS/MS spectra with those of authentic standards.

Compound **14** was tentatively identified as rhamnocitrin by its molecular ion $[M-H]^-$ at *m/z* 299.2 and fragment ions at *m/z* 271, 256, and 227, which reflect decarbonylation, *i.e.*, $[M\text{-}H\text{-}CO]^-$, the loss of a methyl radical with subsequent double decarbonylation, *i.e.*, $[M\text{-}H\text{-}CH_3\text{-}CO\text{-}H]^-$ and $[M\text{-}H\text{-}CH_3\text{-}CO\text{-}CO\text{-}H]^-$, respectively. Compound **15** with deprotonated ion $[M-H]^-$ at *m/z* 329 and the fragment ions *m/z* 314 $[M\text{-}H\text{-}CH_3]^-$, *m/z* 299 $[M\text{-}H\text{-}2CH_3]^-$, and *m/z* 271 $[M\text{-}H\text{-}CO\text{-}2CH_3]^-$ was tentatively identified as rhamnazin.

Table 1
Phytochemical constituents identified in *Myricaria bracteata* leaves of plants from the Altai (A) and Tajikistan (B) and some phenolic compounds concentrations.

No.	T_R (min)	$[M-H]^-$, m/z	Fragment ions, m/z	Identified compounds	Concentration in leaves, mg/g of dry weight	
					A	B
1	3.7	191	111, 87, 67, 57	Citric acid[a]		
2	6.8	169	125, 97, 79, 69	Gallic acid[a]	1.69 ± 0.19[b]	1.71 ± 0.06
3	8.4	183	140, 124	Methyl gallate I		
4	9.3	183	124	Methyl gallate II		
5	9.7	317	179, 151, 137, 107	Myricetin[a]		
6	10	463	301	Quercetin hexoside/Ellagic acid hexoside		
7	10.6	301	284, 257, 229, 185	Ellagic acid[a]	5.72 ± 0.22	4.29 ± 0.15
8	11.1	197	169, 124	Ethyl gallate		
9	11.9	193	178, 149, 134	Ferulic acid[a]	0.15 ± 0.02[b]	0.11 ± 0.03
10	18.2	285	151,133, 121, 107	Luteolin[a]	0.00 ± 0.00	0.03 ± 0.01
11	18.3	301	245, 229, 179, 151, 121, 107	Quercetin[a]	0.25 ± 0.02	1.64 ± 0.08
12	21.2	285	255, 227, 211, 187, 159, 143, 117, 108, 93	Kaempferol[a]	0.42 ± 0.06[b]	0.37 ± 0.06
13	21.8	315	300, 283, 271, 255, 243, 227, 164, 151, 148, 136, 107, 83, 63	Isorhamnetin[a]	2.25 ± 0.06	11.73 ± 0.19
14	24.6	299.2	271, 256, 255, 227, 211, 199, 151	Rhamnocitrin		
15	24.7	329.2	314, 299, 271, 227	Rhamnazin		
16	29.3	299	284, 255, 227, 164, 163, 132, 107, 83, 63	Kaempferide		
17	29.3	299	226, 211, 200, 199, 183, 158, 151, 133, 107	Chrysoeriol		

[a] Identified by comparison with a standard.
[b] Insignificant differences between samples ($p > 0.05$).

Kaempferide (**16**) and chrysoeriol (**17**) were tentatively identified by their molecular ion $[M-H]^-$ at m/z 299 and fragmentation patterns containing specific ions at m/z 63, 83, 163, and 164 or m/z 151, 158, and 183, respectively.

Compound **5** was tentatively identified as myricetin with a molecular ion $[M-H]^-$ at m/z 317 and typical MS/MS fragments at m/z 179 and 151 that corresponded to retrocyclization on the A–C ring ($^{1,2}A^-$) and the consecutive loss of CO ($^{1,2}A^-$-CO), respectively. Myricetin has not been detected in Tamaricaceae before, and the lack of that aglycon is regarded as a characteristic of the family (Harborne, 1975). It was detected in the samples by MS in small amount. Its absence in other previously studied samples of *Myricaria* was most likely due to the peculiarities of the methods or sample preparation.

Compound **6**, with molecular ion $[M-H]^-$ at m/z 463 and a fragment ion at m/z 301, could be proposed as one of ellagic-acid or quercetin hexoside structure. Indirect confirmation of the quercetin derivative could be the fact that quercetin 3-glucoside was found in the majority of *Myricaria* species earlier (Iwashina, 2013).

Concentrations of some phenolic compounds in leaves of *M. bracteata* are shown in Table 1. Significant differences between the geographic locations ($p < 0.05$) were revealed in ellagic acid, quercetin, luteolin, and isorhamnetin. Most significantly, the samples differed in concentrations of quercetin and isorhamnetin.

Isorhamnetin was dominant in the leaves of plants from the Tajikistan. Kaempferide and rhamnazin also made a significant contribution to the total phenolic content in the leaves of plants from the Altai (Fig. 1). Ellagic acid was found to be the second major phenolic constituent of leaves of *M. bracteata*. Results of both HPLC and LC-MS/MS analysis showed higher concentration of ellagic acid in sample A compared to sample B. Citric acid, phenolic acids, and their derivatives, luteolin, kaempferol, and rhamnocitrin were the minor constituents in both samples.

These results are in agreement with the findings of Chumbalov et al. (1975) who studied samples of *M. bracteata* from Kazakhstan, and in contrast to Wang et al. (2008), reported flavones as the major active ingredients of *M. bracteata*. In other studies, there are no data on the abundance of such compounds.

A broad geographic distribution of the species is suggestive of quantitative or qualitative differences in the chemical composition of the plants owing to different environmental circumstances (Keinänen et al., 1999). The results obtained in this study indicate that the samples differ mainly in flavonol accumulation. The chief difference between the samples is the lower concentration of isorhamnetin and the higher levels of rhamnazin and kaempferide, other methylated metabolites of quercetin and kaempferol, in sample A.

There are no reports in the literature about the quantification and variation of phenolic compounds in species *M. bracteata*. Our results suggest that it is important to determine the chromatographic profile of this species to reveal possible chemotypes.

The progress in the research on chemical constituents of *M. bracteata* from different geographical areas forms the basis for identification of pharmacological leads in medicinal plant materials and for setting the standards of quality control.

The diversity of phenolic compounds of this plant (flavonoids in complex with phenolic acids and citric acid) indicates the possibility to isolate drugs with different pharmacological properties. This study shows potential biological activities of this plant, some of them have been previously revealed in other *Myricaria* species (Zeng et al., 2005; Mukherjee et al., 2007; Kletter et al., 2008; Kirbag et al., 2009; La et al., 2010).

Authors' contributions

AC conducted the HPLC-MS/MS analysis and wrote the manuscript. EK contributed to the HPLC experiments and wrote the manuscript. EL collected and identified the samples. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2017.07.001.

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