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Preliminary Communication

Chemical Differentiation of *Berberis croatica* and *B. vulgaris* Using HPLC Fingerprinting

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Abstract. In Croatia, two indigenous species of the genus *Berberis* L. occur: common barberry (*B. vulgaris* L.) and Croatian barberry (*B. croatica* Horvat), endemic to the *Balkano-Illyric* region. As a contribution to the chemotaxonomy of the two species, differences of their RP-HPLC chromatograms were investigated. A HPLC method combined with Unweighted Pair Group Method with Arithmetic means (UPGMA) was developed for species differentiation and compared to several previously described procedures for the differentiation of other species. The results indicate that common and Croatian barberry are closely related species, but can be differentiated according to their HPLC fingerprints under appropriate chromatographic conditions and selection of clustering variables. Generally, the amount of berberine was higher in samples obtained from *B. vulgaris* than from *B. croatica*.

Keywords: Berberis croatica, *Berberis vulgaris*, HPLC, chemotaxonomy

INTRODUCTION

The genus barberry (*Berberis* L.) contains about 450 species distributed in Europe, North Africa, America, East and Central Asia.[1] Many are used in traditional medicine for lumbago, rheumatism, fever, and pyrexia and to treat liver and gallbladder diseases.[2–6]

There are two *Berberis* species present in Croatia: common barberry (*B. vulgaris* L.) and Croatian barberry (*B. croatica* Horvat). While common barberry is widely distributed in Croatia, Croatian barberry is an endemic Balkano-Illyric species, growing on rocky slopes at the upper limit of the wooded zone in Croatia, Bosnia and Herzegovina, Montenegro and Macedonia.[7] The taxonomic status of *B. croatica* is not completely resolved and the following synonyms are used in the literature: *B. aetnensis* var. *brachyacantha*,[8] *B. vulgaris* var. *aetnensis*[9] and *B. aetnensis*.[10] In references describing the European flora, Croatian barberry is not listed as a separate species.[11]

HPLC is a commonly used method for chemical authentication and profiling of plants and medicinal products. If the peaks in chromatograms are well separated, a plant sample can be authenticated by chemical fingerprinting based on the presence or absence of a limited number of peaks.[12,13] On the other hand, a larger number of variables (peaks) is required to efficiently discriminate a large number of similar samples by multivariate statistical analysis.[14] There are a variety of clustering methods that can be used for analysis of chromatograms. Unweighted Pair Group Method with Arithmetic means (UPGMA) is widely used for classification purposes due to its accuracy.[15–17]

According to their morphological features, *B. vulgaris* and *B. croatica* are similar species. *B. vulgaris* is widely used as medicinal plant. The medicinal parts of *B. vulgaris* consist of the dried stem-bark (Berberidis cortex), root-bark (Berberidis radicis cortex), roots (Berberidis radix) as well as fruits (Berberidis fructus). The fruits are used for ailments and discomforts of the

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kidneys and urinary tract, gastrointestinal tract, liver diseases, bronchial discomfort, spleen ailments, spasms, and as a stimulant for the circulatory system. The root and stem of plant are mainly used for ailments and complaints of the gastrointestinal tract, liver, gallbladder, kidney and urinary tract, respiratory tract, heart and circulatory system and as an antipyretic.[18] Some of these effects may be attributed to the presence of berberine, an isoquinoline alkaloid found to possess many pharmacological effects, such as hypotensive, antiarrhythmic, cholesterol lowering, choleretic, hepatoprotective and many other activities.[2,4,5]

On the other hand, knowledge of the chemical characteristics of *B. croatica* is very limited. Since results from an earlier study by Karlović *et. al.* (2009) regarding morphological and anatomical differentiation between *B. croatica* and *B. vulgaris* were inconclusive,[19] the possibility of chemical differentiation between *B. vulgaris* and *B. croatica* was investigated using the UPGMA method for analysis of HPLC chromatogram fingerprints.

EXPERIMENTAL

Plant Material

Roots, twigs, leaves and fruits of *Berberis vulgaris* and *B. croatica* were collected at three locations per species (height above sea-level; geographical latitude and longitude are given in brackets) in Croatia in September 2007. *B. croatica* was collected in Balinovac (Mt Velebit; 1575 m a.s.l.; 44°59' N; 14°54' E), Kiza (Mt Velebit; 1210 m a.s.l.; 44°32' N; 15°09' E) and Mt Biokovo (1410 m a.s.l.; 43°17' N; 17°01' E) while *B. vulgaris* was collec-ted in Rakov Potok (150 m a.s.l.; 45°48' N; 15°39' E), Samobor (210 m a.s.l.; 45°48' N; 15°42' E) and Pharmaceutical Botanical garden "Fran Kušan" in Zagreb (195 m a.s.l.; 45°47' N; 15°59' E). Voucher specimens are deposited in the Herbarium Collection of the Department of Ornamental Plants, Landscape Architecture and History of Garden art, Faculty of Agriculture University of Zagreb, Zagreb, Croatia.

Sample Preparation

Roots, twigs, leaves and fruits of *B. vulgaris* and *B. croatica* were dried for three weeks in one layer, protected from direct solar light in a well-ventilated room. Air-dried material was placed in double paper bags, closed in a dark container and stored in a dry place protected from light and saved three months until the analysis. For preparation of extracts, 1.0 g of powdered plant material and 5.0 mL of methanol were shaken overnight at room temperature. The extract was filtered, and the residue on filter paper re-extracted. Extracts were combined and evaporated to dryness under reduced pressure. Extraction yields were calculated as the weight of extract obtained from 100 g of plant material. For chromatographic analysis, dry extracts were diluted with methanol to a volume of 25.0 mL. An aliquot of 20 μL of each sample solution was injected into the HPLC system for analysis.

Apparatus and Reagents

The RP-HPLC-DAD system was employed for obtaining the chromatograms of *B. vulgaris* and *B. croatica*. The experiments were carried out with an Agilent 1100 high performance liquid chromatographic system (Santa Clara, US) equipped with degasser, autosampler (Agilent 1100) and quaternary pump (Agilent 1100). Compounds were separated on a prepacked analytical reversed-phase column Merck LiChroCART 250 × 4 mm, filled with LiChroSpher 100, RP-18 (5 μm). For chromatography, HPLC-grade acetonitrile, phosphoric and formic acid were used (Fluka, USA).

Chromatographic Conditions

Two types of chromatographic systems were used: isocratic and gradient elution. For isocratic elution, TEA-adjusted 0.02 mol L^{-1} H_3PO_4 (pH = 4.82)/acetonitrile (φ = 75 %) was used with signal detection at 254 nm (mobile phase 1, MP1).[20] The mobile phase for gradient elution consisted of water containing HCOOH (φ = 0.1 %) (A) and acetonitrile containing HCOOH (φ = 0.1 %) (B) which were applied according to the following profile: φ(B) = 10–50 % (20 min), then φ(B) = 90 % (10 min) (mobile phase 2, MP2). The flow rate was kept constant at 1.0 mL min at room temperature (20 °C). Signal detection at 205, 254, and 330 nm was employed.[21]

Determination of the Berberine Content

Berberine content in extracts was determined in MP1 according to the procedure previously described in the literature.[20] The quantity of berberine was calculated from a berberine (Sigma, USA) calibration curve.

Data Analysis

Peaks in each chromatogram were integrated. The area of each peak was expressed as a percentage of the total peak area (PTPA). For comparison purposes of chromatograms, the retention times of all peaks were calculated relative to the highest peak identified in all chromatograms. The peaks (with the exception of berberine) were not identified. Four approaches were used for the comparison of chromatograms. First, as previously described in the literature,[13] the main peaks were determined in all chromatograms and samples analyzed with regard to the PTPAs of those peaks (in further text:

"main peaks approach"). Then, the chromatograms were divided into ten sections containing 10 % of the peaks and PTPAs of the peaks in the sections were compared ("section approach"). Furthermore, samples were also analyzed according to Reference 22, with regard to the following two parameters: sum of PTPAs and average PTPA ("parameters approach"). Each peak was considered as an independent variable. Finally, complete chromatograms were compared according to PTPAs of all peaks ("complete approach"). Cluster analysis was done by means of the UPGMA using Euclidean distance (D_E).

RESULTS AND DISCUSSION

The quantity of berberine in extracts of *B. vulgaris* and *B. croatica* was determined. In addition, the possibility of chemical differentiation between *B. vulgaris* and *B. croatica* was investigated using the UPGMA method for analysis of HPLC chromatogram fingerprints. The overall metabolite profile was based upon both known and unknown metabolites in HPLC chromatograms obtained by two mobile phases and at different wavelengths. Similar approaches have recently been used for the classification of plants[17,23] and fungi.[24]

Table 1 shows that, the yield of crude methanolic extract varied among organs. It was highest in fruits, followed by leaves, while the quantity of dry matter isolated from roots and twigs was lower. Extraction yield was somewhat higher in samples obtained from *B. vulgaris* than from *B. croatica*.

Under the selected conditions, the retention time for berberine was 19.2 min, varying less than 2 %. For the determination of the standard calibration curve, 2.0 mg berberine chloride (1.8 mg of berberine) was dissolved in 25.0 mL of 0.02 mol L^{-1} NaH_2PO_4/Na_2HPO_4 buffer (pH = 4.82) to obtain 7.24×10^{-2} g L^{-1} berberine stock solution. Then, stock solution of 0.05, 0.1, 0.2, 0.4, 0.8 and 1 mL were each diluted into a 10 mL volumetric flask using the same buffer. A series of different concentrations of sample solutions (x) was prepared, which was injected into the column one by one to obtain y (peak area in mAU s). A calibration graph was plotted (peak area *vs.* concentration). The regression equation of berberine was $y = 27.202x - 7.5787$ ($r^2 = 0.9995$) which was linear within the range of 0.89×10^{-6} to 17.82×10^{-6} g L^{-1}.

While the content of berberine in leaves and fruits was negligible, significant quantities of berberine were found in twigs and roots of all investigated plants (Table 1). The highest amount of berberine (12.19 %) was found in the root extract of *B. vulgaris* from the Pharmaceutical Botanical garden "Fran Kušan" in Zagreb. In *B. croatica*, the highest amount of berberine (7.29 %) was found in the root extract of plants from Balinovac population. Generally, the amount of berberine was somewhat higher in samples obtained from *B. vulgaris* than from *B. croatica*. As expected, the content of berberine in twigs was lower than its content in roots of specimens.

Table 1. Yield and quantity of berberine in extracts

Origin	Organ	Yield / %	Berberine / %
Berberis croatica			
Balinovac	root	11.2	7.29
	twigs	10.9	0.63
	leaves	27.6	(–)[b]
	fruits	37.5	(–)[b]
Kiza	root	7.0	1.31
	twigs	12.6	0.48
	leaves	27.7	(–)[b]
	fruits	(–)[a]	(–)[a]
Biokovo	root	11.6	1.22
	twigs	4.9	0.11
	leaves	30.2	(–)[b]
	fruits	44.6	(–)[b]
Berberis vulgaris			
Rakov potok	root	12.6	9.29
	twigs	14.5	1.21
	leaves	34.1	(–)[b]
	fruits	51.4	(–)[b]
Samobor	root	9.1	7.85
	twigs	15.2	1.53
	leaves	31.4	(–)[b]
	fruits	43.2	(–)[b]
Pharmaceutical Botanical Garden "Fran Kušan", Zagreb	root	11.3	12.19
	twigs	9.7	0.82
	leaves	27.1	(–)[b]
	fruits	53	(–)[b]

[a] The organ was not available.
[b] Berberin was not detected in the extract.

Some examples of the chromatograms of *B. vulgaris* and *B. croatica* in MP1 are presented in Figure 1. Although the chromatograms are quite similar, differences are evident. Depending on the organ, most of the differences in chromatograms are visible in the regions with retention times of 2–4 min and 8–9 min.

The success of the UPGMA separation of specimens of one species per cluster in an approach was dependent on the organ, the mobile phase and the wavelength at which the chromatograms were recorded, and on the applied comparison approach. The results are

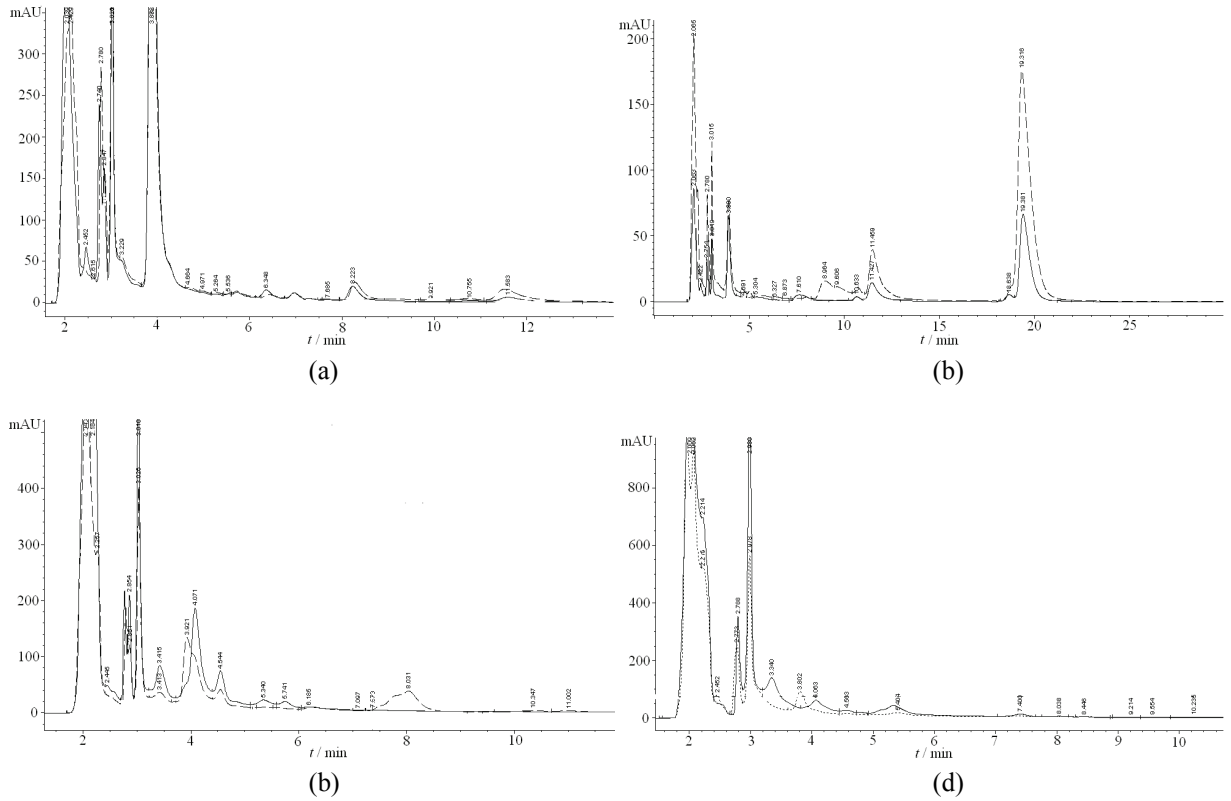

Figure 1. HPLC chromatograms of twigs (a), roots (b), leaves (c), and fruits (d) of *B. vulgaris* (straight line) and *B. croatica* (dotted line) (berberine peak is at retention time of 19.3 min).

summarised in Table 2. When the most common method of fingerprint analysis was used, where the highest 15–25 peaks per organ were selected for analysis ("main peak approach"), good separation of at most two organs (MP1 and MP2 at 205 nm) was obtained.

If chromatograms were divided into sections containing approximately 10 % of peaks ("sections approach"), the success of UPGMA separation of organs into clusters was not dramatically increased. Of the ten sections, good separation was rarely achieved in more than three. Only twigs and fruits were separated in four sections in MP2 at 254 nm. Among the sections obtained in MP2 at 254 nm, the best separation was achieved with retention times of 27.25–31.01 min. The UPGMA of that section separated twigs, leaves and fruits in clusters according to species.

Obradovič *et al.*[22] reported that some parameters obtained from the mathematical description could provide more objective data for reliable authentication systems and allow better classification of species than a conventional approach with identification of chromatographic peaks. In this paper, two parameters obtained from the mathematical description of chromatograms were used as variables for UPGMA separation: the sum of PTPA and average PTPA in the section ("parameters approach"). This approach indeed provided a better

Table 2. Separation using different HPLC conditions and UPGMA separation approaches (MP1 = TEA-adjusted 0.02 mol L^{-1} H_3PO_4 (pH = 4.82)/acetonitrile (φ = 0.75); MP2 = HCOOH/H_2O, φ = 0.1 % (A), acetonitrile (B): 10–50 % B (20 min), 90 % B (10 min); + = successful separation; – = unsatisfying separation)

Mobile phase ($\lambda_{det.}$ / nm)	Main peaks				Sections[(a)]				Parameters				Complete			
	MP1 (254)	MP2 (205)	MP2 (254)	MP2 (303)	MP1 (254)	MP2 (205)	MP2 (254)	MP2 (303)	MP1 (254)	MP2 (205)	MP2 (254)	MP2 (303)	MP1 (254)	MP2 (205)	MP2 (254)	MP2 (303)
Roots	–	–	–	–	2	1	1	1	+	–	–	+	–	–	+	+
Twigs	+	+	–	–	2	0	4	2	–	–	–	+	–	–	+	+
Leaves	–	+	–	+	3	2	2	3	+	–	+	+	+	–	+	+
Fruits	+	–	–	–	2	3	4	2	–	–	–	–	+	–	+	–

[(a)] Number of sections in which samples were well separated.

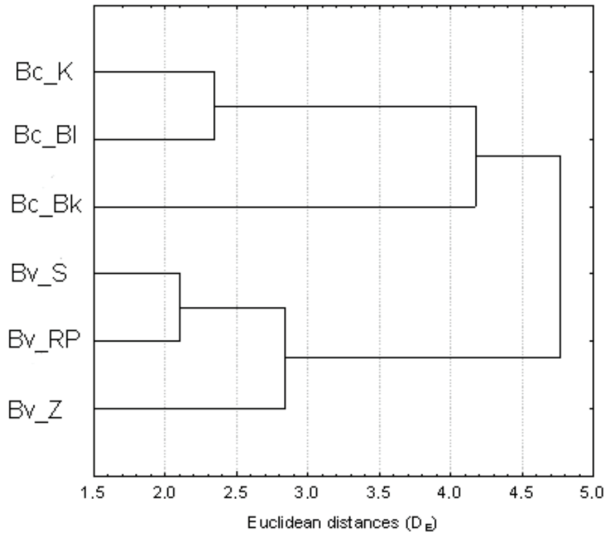

Figure 2. UPGMA dendrogram in "complete approach": *B. croatica* (Bc) collected in: Kiza (K), Balinovac (Bl), Biokovo (Bk); *B. vulgaris* (Bv) collected in: Samobor (S), Rakov Potok (RP), Zagreb (Z).

separation of the two species than the conventional approach, especially if chromatograms obtained in MP2 with detection at 303 nm were evaluated. Under these conditions, the UPGMA analysis grouped specimens of one species together in the case of three organs (roots, twigs and leaves).

Finally, the UPGMA separation resulted in the most successful separation of the two species when the "complete approach" was used. For example, use of this approach in analysis of chromatograms obtained by MP2 with detection at 254 nm allowed for separation of all the organs of *B. vulgaris* and *B. croatica* in clusters according to the respective species. Furthermore, UPGMA analysis of chromatograms obtained in MP2 with detection at 303 nm grouped three (roots, twigs and leaves) of four organs according to the species. Generally, this was the most successful of all four investigated approaches. An example of a dendrogram obtained by this method is given in Figure 2.

CONCLUSION

HPLC was used for quantification of berberine in extracts of *B. vulgaris* and *B. croatica*. Generally, the amount of berberine was higher in samples obtained from *B. vulgaris* than from *B. croatica*. Some approaches for the analysis of chromatograms previously described in the literature did not prove satisfying for separation of the two *Berberis* species into clusters by the UPGMA method indicating that common and Croatian barberry are closely related species. However, they can be differentiated according to their HPLC fingerprints under appropriate conditions and selection of variables for UPGMA clustering. Gradient elution with water and acetonitrile containing HCOOH (φ = 0.1 %) with detection at 254 nm, combined with UPGMA where all peaks were used as variables, was successful in separating all the investigated organs of *B. vulgaris* and *B. croatica*.

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SAŽETAK

Kemijska diferencijacija biljnih vrsta *Berberis croatica* i *B. vulgaris* pomoću tekućinske kromatografije visoke djelotvornosti

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U Hrvatskoj su rasprostranjene dvije vrste roda žutika (*Berberis* L.): obična žutika (*B. vulgaris* L.) i hrvatska žutika (*B. croatica* Horvat). Dok je obična žutika široko rasprostranjena vrsta, hrvatska žutika je endemična vrsta balkansko-ilirske regije. Kao prilog kemotaksonomiji ovih dviju vrsta, istraživane su razlike u njihovim RP-HPLC kromatogramima. Za razlikovanje ovih dviju vrsta razvijena je kombinacija HPLC metode s UPGMA (Unweighted Pair Group Method with Arithmetic mean) metodom klaster analize te uspoređena s prethodno opisanim metodama za diferencijaciju drugih vrsta. Rezultati su pokazali da su obična i hrvatska žutika bliske vrste, ali i to da se mogu razlikovati prema područjima otiska prsta u njihovim RP-HPLC kromatogramima dobivenima pri odgovarajućim kromatografskim uvjetima uz prikladan izbor UPGMA varijabli. Utvrđeno je i da je količina berberina viša u obične negoli u hrvatske žutike.