

Short communication

Compounds of the methanolic leaf extract as chemotaxonomic markers for the *Campanula pyramidalis* complex (Campanulaceae)

IVANA B. JANKOVIĆ¹, MILICA M. DROBAC², DMITAR V. LAKUŠIĆ^{1*}

¹ Institute of Botany and Botanical Garden, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia

² Department of Pharmacognosy, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11000 Belgrade, Serbia

Abstract – During the past few years, the isophylloid *Campanula pyramidalis* complex has been the subject of studies aimed at an improved understanding of the relationships within it. The center of distribution of the *C. pyramidalis* complex is in the Balkan Peninsula with some smaller parts of the area located in the south Apennines. Although 21 taxa of the *C. pyramidalis* complex were described, only four species are accepted: *C. pyramidalis*, *C. versicolor*, *C. secundiflora* and *C. austroadriatica*. In the present study, we propose compounds of the methanolic leaf extract as possible chemotaxonomic markers for the *C. pyramidalis* complex. Eleven flavonoids and two phenolic acids were detected in leaf extract using high-performance liquid chromatography with diode-array detection analysis. The investigated taxa of the *C. pyramidalis* complex differ in terms of the composition of the methanolic leaf extract. Clustering of investigated taxa is not completely consistent with the previously reported molecular and morphometric data.

Keywords: *Campanula pyramidalis*, chemotaxonomy, flavonoids, methanolic leaf extract, phenolic acids

Introduction

Many studies have been performed to elucidate some of the complicated relationships that exist within the Campanulaceae family. However, due to the large number of taxa it is hard to make a comprehensive study and a single classification system. Endemic or rare *Campanula*, as well as smaller groups, aggregates and complexes are at the focus of interests, since usually they are molecularly, morphologically, karyologically, and biogeographi-

* Corresponding author, e-mail: dlakusic@bio.bg.ac.rs

cally distinctive (KOVANDA and ANČEV 1989, EDDIE and INGROUILLE 1999, EDDIE et al. 2003, SHULKINA et al. 2003, COSNER et al. 2004, NIKOLOV 2005, KOVAČIĆ and NIKOLIĆ 2006, CUPIDO et al. 2011, MANSION et al. 2012, STAMENKOVIĆ et al. 2012).

Such a group is constituted by the Balkan »isophylloid« bellflowers (KOVAČIĆ 2004, TKALEC et al. 2004) that belong to the *Campanula pyramidalis* complex also informally referred to as the »pyramidalis« aggregate (GESLOT 1984) or subsection *Pyramidalis* (KOLAKOVSKY 1992). The Balkan Peninsula is the center of the distribution of the *C. pyramidalis* complex with some small disjunct parts of the range that lay in the south Apennines (Fig 1.) (LAKUŠIĆ et al. 2013).

The *Campanula pyramidalis* complex is traditionally represented with three generally accepted species *Campanula pyramidalis* Linnaeus (1753: 164), *Campanula versicolor* Andrews (1804: 396) and *Campanula secundiflora* Visiani et Pančić (1862: 20) (FEDOROV and KOVANDA 1976, GREUTER et al. 1984). According to the results of molecular phylogenetic study, the south Adriatic populations represent separate species, recently described as new – *Campanula austroadriatica* D. Lakušić et Kovačić (2013: 519) (LAKUŠIĆ et al. 2013).

Apart from the above mentioned species, there are 13 taxa at specific and at intraspecific level and four hybrids described within the *C. pyramidalis* complex (LAKUŠIĆ et al. 2013). However, they are not geographically and taxonomically well-defined (LAKUŠIĆ et al. 2013).

In this paper we rely on groups of populations that are presented with five clades (P. 1, P. 2, P. 3, S, V), well-supported on the phylogenetic networks and trees (LAKUŠIĆ et al. 2013):

- P. 1 clade – *C. pyramidalis* s. str. – northern and central Adriatic coast (Fig. 1),
- P. 2 clade – *C. austroadriatica* sp. nova – southern Adriatic coast from the Neretva River canyon to northern Albania (Fig. 1),
- P. 3 clade – *C. »montenegrina«* prov. – populations from the continental part of Montenegro traditionally considered as part of *C. pyramidalis* s. lato (Fig. 1). Molecularly this clade is much closer to *C. secundiflora*. Therefore, we apply the concept of R. Lakušić, who

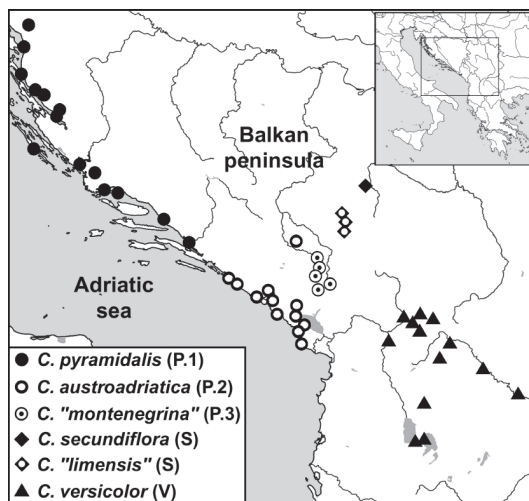


Fig. 1. Distribution of the *Campanula pyramidalis* complex in the investigated area. Symbols P.1–3, S and V correspond to molecular clades (LAKUŠIĆ et al. 2013).

originally recognized these groups of populations as *C. secundiflora* subsp. *montenegrina* R. Lakušić (LAKUŠIĆ et al. 2013),

S clade – *C. secundiflora* s. lato – which includes *C. secundiflora* subsp. *secundiflora* from the gorge of the Panjica River in south-west Serbia, and *C. secundiflora* subsp. *limensis* R. Lakušić (LAKUŠIĆ et al. 2013) from the canyons of the Lim and the Mileševka in south-west Serbia and northern Montenegro (Fig. 1), and

V clade – *C. versicolor* – southern parts of the Balkan Peninsula (Macedonia, Albania, Greece, Bulgaria) (Fig. 1).

Previous phytochemical studies on the plants of the genus *Campanula* revealed the presence of different secondary metabolites such as flavonoids, phenolic acids, anthocyanins and triterpenoids (CUENDET et al. 2001, YAYLI et al. 2003, YAYLI et al. 2005, TOUAFEK et al. 2011). The presence of volatile oils in some *Campanula* species was described as well (TOSUN et al. 2011, KADRIYE et al. 2012, POLITEO et al. 2013). Isoenzyme variability among nine *Campanula* taxa from Croatia and Bosnia and Herzegovina, including *Campanula pyramidalis*, has been studied (TKALEC et al. 2004).

The main goal of our present phytochemical analysis is to investigate the possibility of the use compounds of the methanolic leaf extract as possible chemotaxonomic markers for the *C. pyramidalis* complex. Furthermore, our goal is to determine if phytochemical data provide any correspondence with and support for the establishment of currently recognized or previously described taxa within this complex, especially for the unrecognized taxa *C. »montenegrina«* and *C. »limensis«*.

Materials and methods

Plant material

The aerial parts of *Campanula* species were collected from different locations in Croatia, Serbia, Montenegro and Macedonia (Tab. 1, Fig. 1). Voucher specimens of each sample have been deposited in the Herbarium of the Institute of Botany and Botanical Garden (BEOU), Faculty of Biology, University of Belgrade (Tab. 1).

In addition to the formal names *C. pyramidalis*, *C. versicolor*, *C. secundiflora* and *C. austroadriatica* that are registered in the International Plant Names Index (IPNI), in this paper we have used informal names *C. »montenegrina«* and *C. »limensis«*. These informal names are used in order to emphasize morphological (JANKOVIĆ et al. 2013) and molecular specificities of the population from the canyon of the Morača River and the mountains in its surroundings (*C. »montenegrina«*), as well as the populations from the canyons of the rivers Lim and Mileševka (*C. »limensis«*) (LAKUŠIĆ et al. 2013).

Phytochemical analysis

The air-dried and finely ground leaves were extracted twice with methanol (1:15 w/v) using an ultrasonic bath for 30 min, left to stand for 24 h and filtered. The extract was combined, concentrated in a rotary evaporator under reduced pressure for total solvent removal and dissolved in methanol prior to analysis in the concentration of 10 mg mL⁻¹.

High-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1100 Series system consisting of a G 1312A binary pump, a G1328B injector (20 µL

Tab. 1. Sites, voucher numbers, dates of collecting and clades (according to Lakušić et al. 2013) of examined populations of the *Campanula pyramidalis* complex. Compounds present in the methanolic leaf extracts: 1) luteolin-7-O-heteroside, 2) chlorogenic acid, 3) luteolin-7-O-rutinoside, 4) luteolin-7-O-glucoside, 5) flavonoid 1 (luteolin derivative), 6) flavonoid 2 (7-O- heteroside of luteolin or its derivative), 7) apigenin-7-O-glucoside, 8) flavonoid 3 (apigenin derivative), 9) apigenin-7-O-diheteroside, 10) flavonoid 4 (heteroside of apigenin or its derivative), 11) flavonoid 5 (apigenin derivative), 12) luteolin, 13) caffeic acid. Compounds are marked as present (+) or absent (-).

Clades	Taxon	Site	Voucher	Date	Compounds																					
					1	2	3	4	5	6	7	8	9	10	11	12	13	Σ								
P.1	<i>C. pyramidalis</i>	Velebit	30297	18.11.2009.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	2	
P.2	<i>C. austroadriatica</i>	Orijen	30298	18.11.2009.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
P.2	<i>C. austroadriatica</i>	Risan	30291	28.11.2009.	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
P.2	<i>C. austroadriatica</i>	Orahovac	30289	28.11.2009.	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
P.2	<i>C. austroadriatica</i>	Budva	30288	28.11.2009.	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
P.2	<i>C. austroadriatica</i>	Stari Bar	30287	28.11.2009.	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
P.2	<i>C. austroadriatica</i>	Rumija	30299	30.12.2009.	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
P.2	<i>C. austroadriatica</i>	Virpazar	30286	28.11.2009.	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
P.3	<i>C. »montenegrina«</i>	Morača	30285	28.11.2009.	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
S	<i>C. »limensis«</i>	Dobrun	30284	28.11.2009.	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	11
S	<i>C. secundiflora</i>	Panjica	30300	06.12.2009.	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7
V	<i>C. versicolor</i>	Veles	27623	06.08.2008.	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	6
				Σ	11	7	5	4	3	3	3	3	2	1	1	1	1	1	1	1	1	1	1	1	2	1

sample loop) and G1315B DAD detector, equipped with ZORBAX Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm). A gradient elution was performed with solvent A (0.03% (v/v) H₃PO₄, pH = 2.8) and solvent B (solvent A:acetonitrile = 10:90) as follows: in 0 min, 15% B; in 25 min 25% B; in 30 min 35% B; in 35 min 50% B; in 40 min 70% B and in 45 min 15% B. The column temperature was 25 °C, flow rate 1.0 mL min⁻¹ and the injection volume was 30 μL. The spectra were acquired from 190 to 400 nm. Detection was performed at 250, 320, 340, 350 and 370 nm. All analyses were carried out in triplicate.

Identification of compounds was carried out by comparing their spectra and their retention times with those of standards (luteolin, luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside, chlorogenic acid and caffeic acid) as well as with literature spectral data (MABRY et al. 1970, MARKHAM 1982).

Statistical analysis

FLORA software (KARADŽIĆ et al. 1998) was used for cluster analysis (Fig. 2) in order to determine the structure and separation of the populations based on presence / absence of compounds of methanolic leaf extract. Populations were classified using the optimal cluster method based on Jaccard's distance as a heterogeneity measure. The relation between populations and components of methanolic leaf extract originated by principal component analysis (PCA) was also obtained by using FLORA software and it is presented in form of bi-plot (Fig. 3).

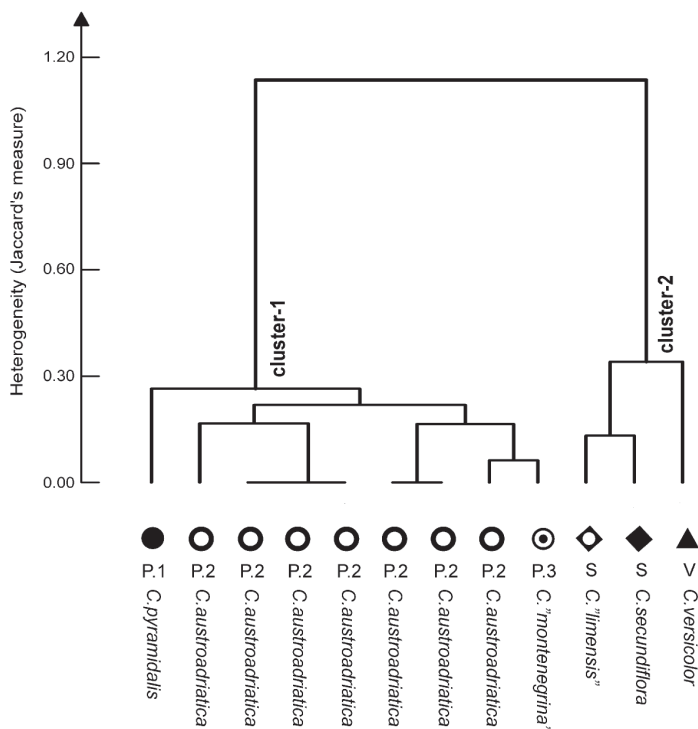


Fig. 2. Cluster analysis for whole data set. Symbols P.1–3, S and V correspond to molecular clades (LAKUŠIĆ et al. 2013).

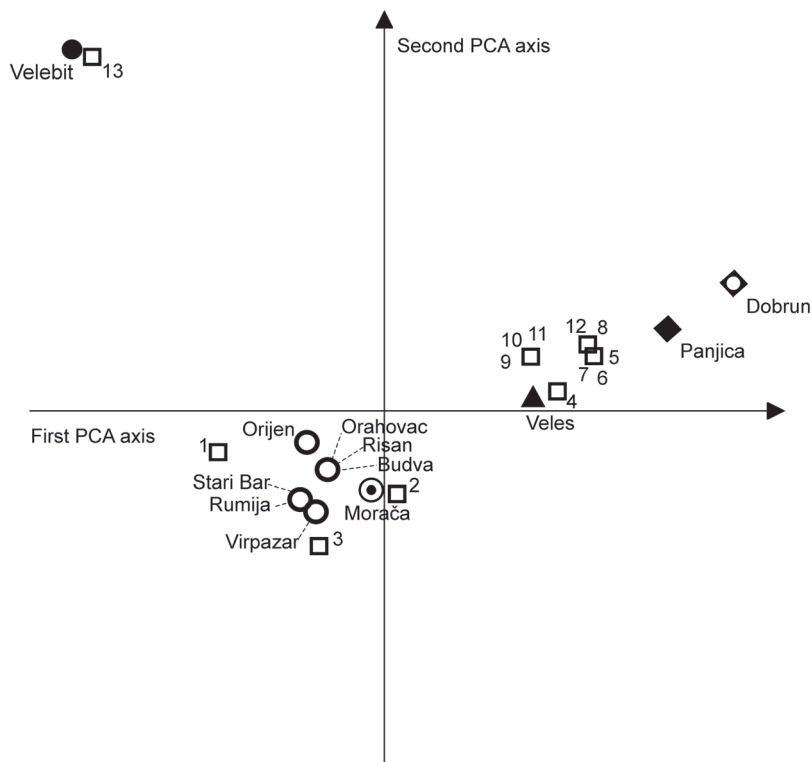


Fig. 3. Bi-plot principal component analysis (PCA) for populations and compounds detected in the methanolic leaf extract of the *Campanula pyramidalis* complex. Numbers from 1 to 13, next to the white rectangles, correspond to number of compounds from table 1. Other symbols correspond to clades as explained in figure 1.

Results and discussion

This study represents one of the first insights into the chemical compounds of the *Campanula pyramidalis* complex. A total of 11 flavonoids and 2 phenolic acids were detected in the methanolic leaf extract of 12 populations of 6 taxa (Tab. 1). The flavonoids present are flavones, either free aglycon (luteolin) or heterosides of apigenin, luteolin and its derivatives. Luteolin and its glycosides were also identified previously in the genus *Campanula* (TESLOV 1990, YAYLI et al. 2003, TOUAFEK et al. 2011). Concerning the phenolic acids, chlorogenic acid was identified in the methanolic leaf extract of *C. austroadriatica*, *C. »montenegrina«*, *C. »limensis«* and *C. secundiflora* whereas caffeic acid was present only in *C. pyramidalis*. The mentioned acids were previously reported in some species of the genus *Campanula* (*C. cephalotes*, *C. maleevii*, *C. rotundifolia*, *C. persicifolia*) (TESLOV and BLINOVA 1973, TESLOV et al. 1983, TESLOV and PODUSHKIN 1988).

The result of the cluster analysis has shown that two main clusters stand out (Fig. 2): (i) cluster 1 – *C. pyramidalis*, *C. austroadriatica* and *C. »montenegrina«*; (ii) cluster 2 *C. secundiflora*, *C. »limensis«* and *C. versicolor*. Luteolin 7-O-heteroside is present in almost all populations but absent only in *C. secundiflora*. Generally, we can conclude that popula-

tions are characterized by the regular presence of luteolin 7-*O*-heteroside and the occasional presence of chlorogenic acid and/or luteolin 7-*O*-rutinoside (Tab. 1). Therefore, we can conclude that luteolin 7-*O*-heteroside, chlorogenic acid and luteolin 7-*O*-rutinoside are less responsible for separation of taxa than other compounds (Fig. 3). A luteolin derivative (labeled as flavonoid 1 in Tab. 1), 7-*O*-heteroside of luteolin or its derivative (flavonoid 2 in Tab. 1) and apigenin 7-*O*-glucoside are the compounds that separate cluster 1 from cluster 2. *Campanula pyramidalis* s.s. is unique in producing caffeic acid besides luteolin 7-*O*-heteroside. Caffeic acid is a common phenolic constituent of Campanulaceae (LAMMERS 2007). Luteolin 7-*O*-glucoside is present in taxa that belong to cluster 2. However, luteolin 7-*O*-glucoside has also been detected in *C. »montenegrina«* from cluster 1. The presence of luteolin 7-*O*-glucoside is another evidence that *C. »montenegrina«* is associated with *C. secundiflora* and *C. versicolor* as molecular data has showed. *Campanula secundiflora* and *C. »limensis«* differ from other taxa by the presence of two flavonoids, luteolin and an apigenin derivative (flavonoid 3 in Tab. 1). *Campanula »limensis«* is distinguished by the presence of three compounds: apigenin 7-*O*-diheteroside, heteroside of apigenin or its derivative (flavonoid 4 in Tab. 1) and an apigenin derivative (flavonoid 5 in Tab. 1), which are not found in other taxa examined.

The chemical data indicate that within the *C. pyramidalis* complex the Mediterranean and sub-Mediterranean populations *C. pyramidalis* s. s. (P.1), *C. austroadriatica* (P.2) and *C. »montenegrina«* (P.3) form one group, while other group is represented by the continental populations of *C. secundiflora* (S), *C. »limensis«* (S) and *C. versicolor* (V). However, the results of methanolic leaf extract analysis are inconsistent with molecular phylogenetic data. In fact, molecular data has showed that *C. pyramidalis* s. s. (P.1) is the most distant taxon at genetic level, forming one main cluster, while *C. austroadriatica* (P.2) forms a second main cluster. The third main cluster is represented by *C. »montenegrina«* (P.3) and *C. secundiflora* (S) (LAKUŠIĆ et al. 2013).

Especially interesting is the position of *C. »montenegrina«* (P.3), due to its chemical characteristics nested within cluster 1 with *C. pyramidalis* s. str. (P.1) and *C. austroadriatica* (P.2). This relation of *C. »montenegrina«* (P.3) with other investigated taxa is inconsistent with respect not only to molecular but also to morphometric data, which suggest that *C. »montenegrina«* (P.3) is part of *C. secundiflora* s. l. (JANKOVIĆ and LAKUŠIĆ 2011, JANKOVIĆ et al. 2013, LAKUŠIĆ et al. 2013). *Campanula »montenegrina«* occupies a transition zone between the Adriatic and the continental bellflowers belonging to the *C. pyramidalis* complex (JANKOVIĆ et al. 2013). Also, it inhabits a wider range of altitudes from canyons to high mountains. The chemotaxonomic, molecular phylogenetic and morphometric results emphasize the necessity to investigate the populations that lie in the continental part of Montenegro more fully. Those populations are morphologically and geographically very specific and therefore they require a new taxonomic treatment (LAKUŠIĆ et al. 2013).

The second cluster, to which belong *C. secundiflora* s. s. (S), *C. »limensis«* (S) and *C. versicolor* (V), is differentiated in the same way chemically and molecularly (LAKUŠIĆ et al. 2013). From all taxa investigated in this study, *C. »limensis«* is highly differentiated in the composition of the methanolic leaf extract by the number of different flavonoids. Eleven different compounds were detected in the methanolic leaf extract of *C. »limensis«*. It differs even from its closest taxon *C. secundiflora* s. s. Morphometric data (JANKOVIĆ and LAKUŠIĆ 2011, JANKOVIĆ et al. 2013) also suggest that *C. »limensis«* is separate entity and its taxonomic status should be seriously reconsidered in future studies.

Chemotaxonomic results based on the analysis of methanolic leaf extract demonstrated that investigated taxa of the *C. pyramidalis* complex are different. Therefore, our results suggest that the presence or absence of certain compounds in the methanolic leaf extract can be used as chemotaxonomic markers for further studies of the *C. pyramidalis* complex.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the Serbian Ministry of Science and Technological Development (Project No. 173021 – Examination of potential medicinal plants: morphological, chemical and pharmacological characterization and 173030 – Biodiversity of the plant life of Serbia and Balkan Peninsula – Assessment, sustainable use and conservation). Also, we would like to offer our special thanks to Dijana Dobrota (Magdeburg, Germany) for improving the language of the manuscript.

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