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Original Scientific Paper

Fatty acid composition of the cypselae of two endemic *Centaurea* species (Asteraceae)

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ABSTRACT: The fatty acid composition of cypselae of two endemic species from Macedonia, *Centaurea galicicae* and *C. tomorosii*, is analysed for the first time, using GC/MS (gas chromatography/mass spectrometry). In the cypselae of *C. galicicae*, 11 fatty acids were identified, palmitic (hexadecanoic) acid (32.5%) being the most dominant. Other fatty acids were elaidic [(*E*)-octadec-9-enoic] acid (13.9%), stearic (octadecanoic) acid (12.8%) and linoleic [(9*Z*,12*Z*)-9,12-octadecadienoic] acid (10.6%). Of the 11 identified fatty acids, seven were saturated fatty acids, which represented 41.5% of total fatty acids, while unsaturated fatty acids altogether constituted 58.5%. In the cypselae of *C. tomorosii*, five fatty acids were identified. The major fatty acid was linolelaidic [(9*E*,12*E*)-octadeca-9,12-dienoic] acid (48.8%). The second most dominant fatty acid was oleic [(9*Z*)-octadec-9-enoic] acid (34.2%). Thus, unsaturated fatty acids were present with 83%. The other three fatty acids identified were saturated fatty acids, which represented 17% of total fatty acids. As a minor fatty acid, levulinic (4-oxopentanoic) acid was determined in both *C. galicicae* and *C. tomorosii* (0.3% and 3.2%, respectively). The obtained results differ from published data on dominant fatty acids in the cypselae of other species belonging to the same section as the species investigated in the present paper (section *Arenariae*, subgenus *Acrolophus*, genus *Centaurea*). They also, differ from published data referable to other genera belonging to the same tribe (Cardueae). The general chemotaxonomic significance of fatty acids is discussed.

Keywords: fatty acids, *Centaurea*, *Arenariae*, Compositae, chemotaxonomy

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INTRODUCTION

The genus *Centaurea* L., one of the largest genera of the Asteraceae (Compositae) family, comprises between 400 and 700 species (Dittrich 1977; Bremer 1994; Wagenitz & Hellwig 1996; Garcia-Jacas *et al.* 2001). It belongs to the tribe Cardueae Cass. and subtribe Centaureinae (Cass.) Dumort. The genus is distributed in the Mediterranean region and in South-East Asia. On the Balkan Peninsula, there are about 146 species (Lovrić 1990). Taxonomy of the genus is somewhat vague: sections are still being revised, and interrelationships are not well resolved (Garcia-Jacas *et al.* 2006).

Fatty acids are distributed in all plant organs and occur as free fatty acids or in the form of esters. From the chemotaxonomic point of view, the most important are fatty acids from seeds and fruits (cypselae in Asteraceae) because of their conservative nature. The cypsela can be defined as a dry, indehiscent, unilocular fruit with a single seed not adnate to the pericarp (linked only

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by the funicle), originating from an inferior ovary and with the pericarp formed by the real pericarp (cells from the ovarian wall) and extracarpelar tissues from the receptacle (Marzinek *et al.* 2008). Seed fatty acids can be good taxonomic markers at the intra- and interspecies levels and can indicate hybridisation between related taxa or even reflect the phylogeny of certain plant groups (Aitzetmüller 1996; Janaćković *et al.* 1996; Özcan 2008; Coutinho *et al.* 2015). In many plants, unusual fatty acids have been recorded that are also considered important as taxonomic characters (Aitzetmüller 1993). Unusual fatty acids can sometimes be used as a fingerprint in delimitation of taxa (Aitzetmüller 1996; Aitzetmüller 1997; Tsevegsüren *et al.* 1997). The composition of seed fatty acids is considered to be a good taxonomic character at the subfamily level of the Lamiaceae family (Marin *et al.* 1991). As a rule, the oils from seeds of the family Asteraceae are rich in linoleic acid and contain smaller amounts of palmitic and oleic acid, stearic and linolenic acids being minor fatty acids (Shorland 1963). Some Asteraceae taxa possess unusual C_{18} unsaturated acids as dominant, i.e., > 10% of total seed oil fatty acids (Shorland 1963; Hegnauer 1964; Hilditch & Williams 1964). Seed oils of some Cardueae taxa contain hydroxy- and epoxy-fatty acids with conjugated double bonds. Such fatty acids are found in *Calendula, Artemisia*, *Helianthus*, *Cosmos*, *Vernonia*, *Tragopogon*, *Osteospermum* and *Dimorphoteca* species (Wagner 1977). An unusual fatty acid, γ-linolenic, was found in oil of the seeds of some *Saussurea* species (Tsevegsüren *et al.* 1997). Oil of the seeds of some Mongolian Compositae contains unusual *trans*-fatty acids (Tsevegsüren *et al.* 2000).

There are a number of phytochemical investigations of lactones and flavonoids in members of the genus *Centaurea* (Youssef & Frahm 1995; Fortuna *et al.* 2001; Janaćković *et al.* 2004a; López-Rodríguez *et al.* 2009). The genus has also been the subject of some phytochemical research on the fatty acid composition of the aerial part of the plant (Tekeli *et al.* 2010; Zengin *et al.* 2010; Aktumsek *et al.* 2011; Zengin *et al.* 2011a; Zengin *et al.* 2011b; Erdogan *et al.* 2014).

The endemic species *C. galiciae* Micevski is a perennial herbaceous plant with purple flowers and a cypsela 2.5 mm long with a pappus twice as long as the cypsela. It grows in the southwestern part of the Former Yugoslav Republic (FYR) of Macedonia, in the foothills of Mt. Galičica in the Galičica National Park (Micevski 1985) (Fig. 1a, b, c). A new record for the flora of Albania was confirmed in the Prespa National Park (Shuka & Tan 2013). The endemic species *C. tomorosii* Micevski is a perennial herbaceous plant with pale yellow flowers and a cypsela 4 mm long with a short pappus 3 mm long. It grows among calcareous rocks, on Mt. Galičica: [Tomoros, 1450 m.a.s.l. (Micevski 1985) (Fig. 1d, e, f)]. Both species, *C. galicicae* and *C. tomorosii*, belong to the section *Arenariae* (Hayek) Dostál of the subgenus *Acrolophus* (Cass.) Dobrocz of the genus *Centaurea* L. (Micevski 1985).

Phytochemical study of these two species is scarce. There is only one study dealing with secondary metabolites of three endemic species, including *C. galiciace* and *C. tomorosii*, where the lactone cnicin and seven flavonoids were isolated and identified (Tešević *et al.* 2014). No previous phytochemical study dealing with the fatty acids of these species has been reported. The present study therefore aims to investigate fatty acid composition of the cypselae of *C. galicicae* and *C. tomorosii* in order to further elucidate phytochemistry of the genus *Centaurea*. In addition to this, we here discuss the general usefulness of fatty acid composition as a taxonomic marker.

MATERIAL AND METHODS

Plant Material. Mature cypselae of *C. galicicae* were collected during the flowering period on July 7, 2012 at Konjsko, close to Lake Prespa, FYR Macedonia (N 40.93504, E 20.93702201). Mature cypselae of *C. tomorosii* were collected during the flowering period on July 7, 2012 at a locality on Mt. Galičica, FYR Macedonia (N 40.98119000, E 20.86370006). Voucher specimens (accession numbers BEOU 38477 and BEOU 38474) were deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU)—Faculty of Biology, Serbia.

Isolation of Fatty Acids. Mature cypselae (100 mg) of the investigated species were separately boiled for 10 min at 80°C in 1 mL of 2-propanol (iPrOH) (Kurata *et al.* 2005; Sokovic *et al.* 2009). The samples were then homogenised with 8 mL of undecanoic acid (conc. 0.2 mg/mL CH_2Cl_2) used as an internal standard. The homogenised samples were stored at 4°C for 24 h (lipid extraction), after which 2 mL 0.7% NaCl in H_2O was added. The lower phase was taken from the funnel, transferred into a test tube and dried with anhydrous Na_2SO_4. The samples were then evaporated, after which 6 mL of a mixture of 1% H_2SO_4 in MeOH was added and the material heated for 2 h. A saturated solution of $NaHCO_3$ was then added to the samples. After neutralisation, the samples were extracted with dichlormethane (CH_2Cl_2), and the lower phase from the funnel was taken for analysis.

Gas Chromatography/Mass Spectrometry (GC/MS) aAnalysis. An Agilent 7890A GC instrument equipped with two detectors, a flame ionisation detector (FID) and an Agilent 5975C mass selective detector (MSD), was used for GC and GC/MS measurements. The analyses were performed with a DB-23 fused silica capillary column (60 m × 250 μm × 0.25 μm). The carrier gas was helium at an initial rate of 4.1 mL/min (in constant pressure mode). The injection volume was 1 μL, the mode was splitless and the injector temperature was 220°C.

Figure 1. *Centaurea galicicae* and *C. tomorosii*: **a** - aerial part of *C. tomorosii*; **b** - capitulum of *C. tomorosii*; **c** - cypselae of *C. tomorosii*; **d** - aerial part of *C. galicicae*; **e** - capitulum of *C. galicicae*; **f** - cypselae of *C. galicicae*.

The oven temperature was programmed as follows: 50°C for 1 min; 50–175°C, 25°C/min; 175–235°C, 4°C/min; and 235°C for 5 min. The FID temperature was 300°C and total run time was 26 min. The MS transfer line was heated at 250°C. Mass spectra were collected in the electron ionisation mode (EI energy 70eV). The ion source was heated at 230°C and the quadrupole at 150°C. The mass range was 33-550 m/z.

Identification of methyl esters of fatty acids was done by comparison of mass spectra and retention times with standard Supelco® 37 Component FAME Mix and with the NIST11 and Wiley 7 commercial mass spectral libraries.

Quantification methyl esters of fatty acids was done using the internal standard mass and relative percentage of GC in relation to each component. Fatty acids are expressed as the percentage of total fatty acids and as the fatty acid mass (mg) in 100 mg of mature cypselae.

RESULTS AND DISCUSSION

In this work the fatty acid composition of cypselae of two endemic species, *C. galicicae* and *C. tomorosii*, was analysed using GC/MS (Table 1). In the cypselae of *C. galicicae*, 11 fatty acids were identified. The major fatty acid was palmitic (hexadecanoic) acid (32.5%). Other fatty acids with a high percentage were elaidic [(*E*)-octadec-9-enoic]) (13.9%), stearic (octadecanoic) (12.8%) and linoleic [(9*Z*,12*Z*)-9,12-octadecadienoic] (10.6%) acids. Levulinic (4-oxopentanoic) acid was a minor fatty acid (0.3%). Out of the 11 identified fatty acids, seven were saturated, and they constituted 41.5% of total fatty acids. On the other hand, monounsaturated fatty acids were present with 33.9% and polyunsaturated fatty acids with 24.6% of total fatty acids. We identified five isomers of C18:2 fatty acids with an undetermined position of

Table 1. Fatty acid composition of investigated *Centaurea* species.

Fatty acids			*Centaurea galicicae*		*Centaurea tomorosii*	
Systematic name	Trivial name	Rt, min	%	fatty acids, mg / 100 mg of cypselae	%	fatty acids, mg / 100 mg of cypselae
4-oxopentanoic acid (C5:0)	levulinic acid	7.298	0.3	0.038	3.2	0.175
tetradecanoic acid (C14:0)	myristic acid	9.766	1.4	0.190	-	-
pentadecanoic acid (C15:0)	pentadecylic acid	10.637	0.5	0.076	-	
hexadecanoic acid (C16:0)	palmitic acid	11.639	32.5	4.438	10.0	0.538
heptadecanoic acid (C17:0)	margaric acid	12.740	0.8	0.114	-	-
octadecanoic acid (C18:0)	stearic acid	13.967	12.8	1.752	3.8	0.203
(*E*)-octadec-9-enoic acid (C18:1n9t)	elaidic acid	14.173	13.9	1.905	-	-
(9*Z*)-octadec-9-enoic acid (C18:1n9c)	oleic acid	14.341	7.2	0.990	34.2	1.845
C 18:2 *	-	14.596	1.5	0.209	-	-
(9*E*,12*E*)-octadeca-9,12-dienoic acid (C18:2n6t)	linolelaidic acid	14.647	2.1	0.286	48.8	2.634
C 18:2 *	-	14.720	2.5	0.343	-	-
C 18:2 *	-	14.833	3.5	0.476	-	-
(9*Z*,12*Z*)-9,12-octadecadienoic acid (C18:2n6c)	linoleic acid	15.034	10.6	1.448	-	-
C 18:2 *	-	15.341	1.5	0.209	-	-
C 18:2 *	-	15.421	2.9	0.381	-	-
icosanoic acid (C20:0)	arachidic acid	16.634	6.0	0.819	-	-

* isomers of c18:2 fatty acid with an undetermined position of the double bond; Rt, min - retention time, minutes.

the double bond. In total, unsaturated fatty acids were present with 58.5% of total fatty acids. In the cypselae of *C. tomorosii*, five fatty acids were identified. The major fatty acid was linolelaidic [(9*E*,12*E*)-octadeca-9,12-dienoic] acid (48.8%). The second most dominant fatty acid was oleic [(9*Z*)-octadec-9-enoic] acid (34.2%). Thus, unsaturated fatty acids were present with 83%. Out of the five identified fatty acids, three were saturated, and they constituted 17% of total fatty acids. Levulinic (4-oxopentanoic) acid was present as a minor fatty acid (3.2%), the same as in *C. galicicae*.

In the present work, moreover, two free amino acids were identified in the cypselae of *C. galicicae* (L-aspartic and L-glutamic acid) that were not detected in the cypselae of *C. tomorosii*. This finding could be of importance because it has been shown that seed free amino acid composition can be used as a taxonomic marker (Bhunia & Mondal 2014).

Eleven fatty acids were identified in the cypselae of *C. galicicae*, as compared with five in *C. tomorosii*. The results showed that in both investigated species unsaturated fatty acids were present in greater amounts than saturated fatty acids. However, the two species differed with respect to the most dominant fatty acid, which in *C. galicicae* was palmitic (32.5%), a saturated fatty acid, while in *C. tomorosii* it was linolelaidic (48.8%), a polyunsaturated fatty acid. Palmitic acid was also identified in the cypselae of *C. tomorosii*, but in a smaller percentage (10%), while the percentage of linolelaidic acid in *C. galicicae* was only 2.1%. With respect to the dominant fatty acids in their cypselae, the investigated species differed from other species of the section to which they belong (section

Arenariae, subgenus *Acrolophus*, genus *Centaurea* L.) according to available published data. Linolenic, linoleic and stearic acids were found in the cypselae of *C. arenaria*; linolenic, linoleic, stearic and palmitic acids were detected in the cypselae of *C. derventana*; and linolenic, stearic, palmitic and oleic acids were found in the cypselae of *C. incompta* (Janaćković *et al.* 2004b). According to Flora Europaea, all three of those species belong to the same section as *C. galicicae* and *C. tomorosii* (section *Arenariae*, subgenus *Acrolophus*, genus *Centaurea*). By way of contrast, linolenic acid was not identified in the cypselae of our investigated species.

The dominant fatty acids in cypselae of *C. galicicae* and *C. tomorosii* are also different in comparison with major fatty acids found in the seed oil of other Cardueae species. The major fatty acids of *Carlina acaulis* L. and *C. corymbosa* L. are oleic (21-24%) and linoleic (50-52%) acids (Spencer *et al.* 1969). *Arctium minus* L. has an oil rich in linoleic acid (74%) as well as *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid (10%) (Morris *et al.* 1968). Also, *Xeranthemum annuum* L. produces seed oil rich in linoleic acid, which is accompanied by measurable amounts (25%) of a mixture of 5-*cis*-,9-*cis*,12-*cis*-octadecatrienoic acid, coronaric acid, epoxystearic acid, vernolic acid and two unsaturated hydroxy acids (Powell *et al.* 1967). It has been shown that seed fatty acids can be a good taxonomic marker at the generic level in analysis of *Ptilostemon strictus*, *P. afer*, *Cirsium candelabrum* and *C. eriophorum* (Janaćković *et al.* 1996). To be specific, the saturated lauric, myristic, palmitic and stearic acids were found, as well as the unsaturated oleic, linoleic and linolenic acids. However, linolenic acid was detected only in cypselae of the *Cirsium* species, which supports separation of *Ptilostemon* from the genus *Cirsium*. Ozcan *et al.* (2016) also concluded that seed fatty acid composition can be used as an additional chemotaxonomic marker of *Cirsium*.

CONCLUSION

In this paper, fatty acid composition of the seeds of *Centaurea galicicae* and *C. tomorosii* is analysed for the first time. The dominant fatty acid in cypselae of *C. galicicae* was palmitic acid, while in *C. tomorosii* it was linolelaidic acid. Regarding dominant fatty acids, our results differ from previously published data on all studied species belonging to the same section (section *Arenariae*, subgenus *Acrolophus*, genus *Centaurea*). Further phytochemical analysis of all *Arenarie* taxa, together with morphological and molecular analyses, would be of interest for better resolution of the infrageneric classification of *Centaurea*, one of the most complex genera of the Asteraceae.

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REZIME

Sastav masnih kiselina cipsela dve endemične vrste *Centaurea* (Asteraceae)

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U ovom radu je po prvi put analiziran sastav masnih kiselina cipsela dve endemične vrste *Centaurea galicicae* i *C. tomorosii* iz Makedonije, upotrebom GH/MS (gasne hromatografije i masene spektrometrije). U cipselama vrste *C. galicicae* identifikovano je 11 masnih kiselina, među kojima je dominantna palmitinska kiselina (32,5%). Ostale visoko zastupljene masne kisleine su elaidinska (13,9%), stearinska (12,8%) i linolna (10.6%). Od prisutnih, 7 su zasićene masne kiseline i predstavljaju 41,5% od ukupnih masnih kiselina, dok su nezasićene masne kiseline zastupljene sa 58,5%. U cipselama vrste *C. tomorosii* identifikovano je 5 masnih kiselina. Među njima su dominantne linolelaidinska (48,8%) i oleinska masna kiselina (34,2%). Nezasićene masne kiseline predstavljaju 83%, dok zasićene predstavljaju 17% od ukupnih masnih kiselina. Najmanje zastupljena masna kiselina je levulinska kod *C. galicicae* (0,3%) kao i kod *C. tomorosii* (3,2%). Naši rezultati pokazali su razliku u poređenju sa literaturnim podacima u odnosu na prisustvo dominantnih masnih kiselina u cipselama drugih ranije istraživanih vrsta koje, takođe, pripadaju sekciji *Arenariae* podrodu *Acrolophus* rodu *Centaurea*. Takođe, uočene su razlike u prisustvu dominantnih masnih kiselina cipsela u odnosu na druge rodove iz istog tribusa Cardueae. Taksonomski značaj masnih kiselina je diskutovan.

Ključne reči: masne kiseline, *Centaurea*, *Arenariae*, Compositae, hemotaksonomija