Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *79* (11) 1355–1362 (2014)
JSCS–4670
UDC **Centaurea*:547.972:543.422.25
Original scientific paper

Secondary metabolites of three endemic *Centaurea* L. species

VELE TEŠEVIĆ[1]*, IVANA ALJANČIĆ[2], SLOBODAN MILOSAVLJEVIĆ[1#], VLATKA VAJS[1,2], IRIS ĐORĐEVIĆ[3], MILKA JADRANIN[2], NEBOJŠA MENKOVIĆ[4] and VLADO MATEVSKI[5]

[1]*Faculty of Chemistry, University of Belgrade, Studentski trg 12–16, 11158 Belgrade, Serbia,* [2]*Institute of Chemistry, Technology and Metallurgy, Center for Chemistry, University of Belgrade, Njegoševa 12, 11001 Belgrade, Serbia,* [3]*Faculty for Veterinary Medicine, University of Belgrade, Bulevar oslobodenja 18, 11000 Belgrade, Serbia,* [4]*Institute for Medicinal Plant Research, "Dr. Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia and* [5]*Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University in Skopje, Blvd. Goce Delcev 9, 1000 Skopje, FYROM*

(Received 18 March, revised 25 April 2014, accepted 6 May 2014)

Abstract: The aerial parts of three endemic *Centaurea* L. species, namely *C. tomorosii* Micevski, *C. soskae* Hayek and *C. galicicae* Micevski, afforded the sesquiterpene lactone cnicin (**1**) and seven flavonoids: apigenin (**2**), isokaempferide (**3**), hispidulin (**4**), eupatorin (**5**), cirsimaritin (**6**), santoflavone (**7**) and salvigenin (**8**). The structures of the isolated compounds were determined by UV, ^{1}H-NMR and ^{13}C-NMR spectroscopy and HR-ESI-MS spectrometry. ^{1}H-NMR spectroscopy was used as a method for the quantitative analysis of cnicin.

Keywords: *Centaurea*; cnicin; flavonoids.

INTRODUCTION

The genus *Centaurea* L. is one of the largest genera of the Asteraceae family and also one of the most representative genera within the tribe Cardueae Cass. and the subtribe Centaureinae (Cass.) Dumort. with around 250 species.[1] It is mainly distributed in the Mediterranean region and South–East Asia and is characterized by a great degree of endemism,[2] which is usually associated to restricted geographical areas.[3]

The species of the genus *Centaurea* have been used in folk medicine for a long time. Various *Centaurea* species have certain biological activities, such as antifungal,[4] antimicrobial,[5] anti-ulcerogenic,[6] anti-inflammatory,[7] antioxidant,[8] antiviral,[9] anti-*Helicobacter pylori*,[10] anticancer and cytotoxic.[11] The genus was

* Corresponding author. E-mail: vtesevic@chem.bg.ac.rs
# Serbian Chemical Society member.
doi: 10.2298/JSC140318048T

also the subject of interest of several phytochemical investigations for their potentially active constituents, particularly sesquiterpene lactones[12–16] and flavonoids.[17,18]

As a part of ongoing and systematic investigations on the composition of the plants of this genus,[19–24] the chemical constituents of the extracts of the aerial parts of three species: *C. tomorosii* Micevski, *C. soskae* Hayek and *C. galicicae* Micevski, all are endemic to Balkan Peninsula, were investigated. No previous phytochemical study on these species has been reported.

EXPERIMENTAL

General

The UV spectra were recorded on a GBC Cintra 40 UV–Vis spectrometer. The ^{1}H- and ^{13}C-NMR data were acquired on Varian Gemini 2000 NMR spectrometer (200 MHz for ^{1}H- and 50 MHz for ^{13}C-NMR, in $CDCl_3$, with TMS as an internal reference). Dry column flash--chromatography (DCFC) and column chromatography (CC) were performed on silica gel (ICN Silica 12–26 60 Å and 70–230 mesh, ASTM, Merck, respectively). Silica gel 60 F_{254} precoated aluminum sheets (layer thickness 0.25 mm, Merck) for TLC control and preparative TLC plates (2 mm Merck) for preparative purification were used. The high-resolution liquid chromatography/photo-diode array/electrospray ionization/time of flight mass spectra (HRLC/PDA/ESI/TOF MS) were measured on a HPLC instrument (Agilent 1200 Series) equipped with an autosampler, using a Zorbax Eclipse Plus C_{18} analytical column (1.8 μm particle size, 4.6 mm×150 mm i.d., Agilent Technologies), and a PDA detector (DAD) coupled with a 6210 TOF LC/MS system (Agilent Technologies).

Plant materials

The aerial parts of the investigated species were collected during the flowering period (17 July 2010) at the following localities: *C. tomorosii* Micevski at Tomoros (*ca*. 1700 altitude), on Mount Galičica, *C. soskae* Hayek near Lake Ohrid, and *C. galicicae* Micevski close to Lake Prespa. The plants were identified by Vlado Matevski, Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University of Skopje, where voucher specimens are deposited at the Macedonian National Herbarium (MKNH) under accession numbers: MKNH135338 (*C. tomorosii*) MKNH135336 (*C. soskae*) and MKNH135337 (*C. galicicae*).

Extraction and isolation

The dried, ground aerial parts of *C. tomorosii* (100 g) were extracted twice with petroleum ether–Et_2O–methanol (1:1:1) at room temperature and re-extracted with Et_2O–MeOH.[25] The crude extract (5 g) was fractionated by dry-column flash chromatography on silica gel using petroleum ether–Et_2O–MeOH with increasing polarity to yield 25 fractions. The fractions eluted with Et_2O–MeOH 9:1 (Fr 19), 8:2 (Fr 20) and 7:3 (Fr 23) contained flavonoids and the sesquiterpene lactone cnicin (according to the ^{1}H-NMR and IR spectra), and were further purified by CC on silica gel. Elution with CH_2Cl_2–MeOH (1:1) afforded cnicin (**1**, 123 mg) from Fr 23, while the combined fractions Fr 19 and Fr 20 yielded apigenin (**2**, 5 mg), isokaempferide (**3**, 3 mg), hispidulin (**4**, 6 mg), cirsimaritin (**6**, 10 mg) and santoflavone (**7**, 8 mg).

HRLC/PDA/ESI/TOF MS analyses

Crude extract of each endemic *Centaurea* species was dissolved in methanol to an approximate concentration of 5 mg mL^{-1}. The HRLC/PDA/ESI/TOF MS analyses were realized under the following conditions: the mobile phase consisted of water containing 0.2 % formic acid (A) and acetonitrile (B). A gradient program was used as follows: 0–1.5 min 5 % B, 1.5––26 min, 5 %–95 % B, 26–35 min, 95 % B. The flow rate of the mobile phase was 1.4 mL min^{-1}, the column temperature was kept at 40 °C and the injection volume was 5 μL. UV spectral data from all peaks were accumulated in the range of 190–450 nm and chromatograms were recorded at 240 nm. MS data were collected by applying the following parameters: ionization, negative ESI capillary voltage 4000 V, gas temperature 350 °C, drying gas 12 L min^{-1}, nebulizer pressure 45 psi*, fragmentor voltage 140 V, mass range 100–2000 *m*/*z*. A personal computer system running MassHunter Workstation software was used for data acquisition and processing.

Quantification of cnicin content

The quantitative analysis of cnicin was performed according to the previously described procedure,[26] using BHT (2,6-bis(1,1-dimethylethyl)-4-methylphenol) as the internal standard. The ^{1}H-NMR spectrum of known amounts of crude plant extract and internal standard was recorded and quantification was performed by calculating the ratio of the peak areas of selected proton signals of the target compound and the internal reference standard.

RESULTS AND DISCUSSION

According to HPLC analysis, all the three studied taxa of *Centaurea* exhibited very similar flavonoid patterns, differing only in the relative amounts of the constituents (Fig. 1).

Exact mass measurements of pseudomolecular ions of the analytes was realized with a time-of-flight (TOF) mass spectrometer operating in the negative polarity mode, which enabled the determination of the molecular formula of most of the constituents. All the identified compounds exhibited a quasi-molecular ion $[M–H]^-$ signal in the negative mode, confirming their molecular masses (Table I).

Peak identification was performed by comparison of their retention time, mass, and UV spectra with those of previously isolated compounds from *C. tomorosii*. From the crude extract of *C. tomorosii*, sesquiterpene lactone cnicin (**1**) and flavonoids apigenin (**2**), isokaempferide (**3**), hispidulin (**4**), cirsimaritin (**6**) and santoflavone (**7**) were isolated and indentified by means of UV, MS and NMR spectral data.[27] Flavonoids eupatorin (**5**) and salvigenin (**8**) were tentatively identified based on their UV and MS spectra, as well as by comparison with the literature data.[21] Formulae of these compounds are represented in Fig. 2.

All compounds represent mainly the characteristic secondary metabolites of the genus that were previously reported from different *Centaurea* species.[18] The observed differences between the taxa is the absence of cirsimaritin (**6**) and predominance of santaflavone (**7**) in *C. soskae* and *C. galicicae*, compared to *C.*

* 1 psi = 6894.757 Pa

tomorosii. Similarly, the germacranolide cnicin (**1**) was detected, as was the case for a large number of *Centaurea* species.[28]

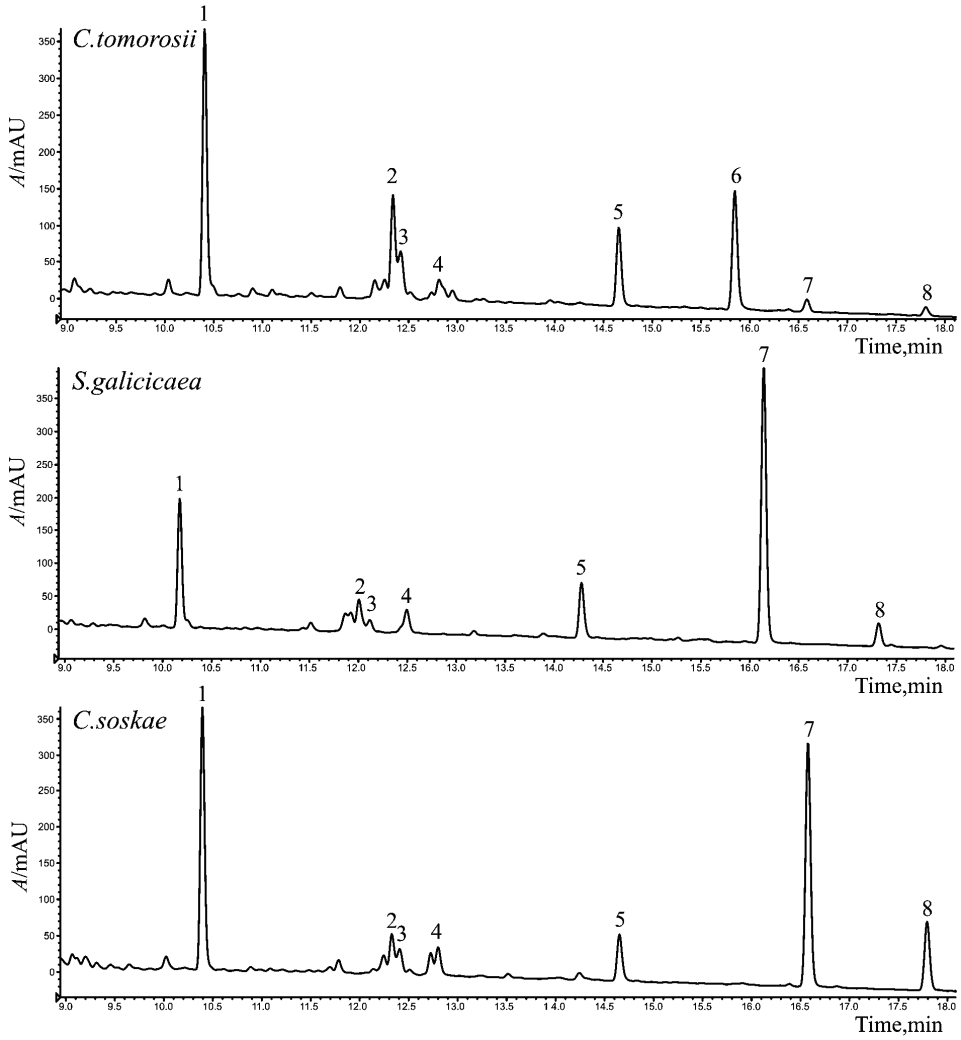


Fig. 1. HPLC profiles of the crude extracts of *C. galicicae*, *C. soskae* and *C. tomorosii*.

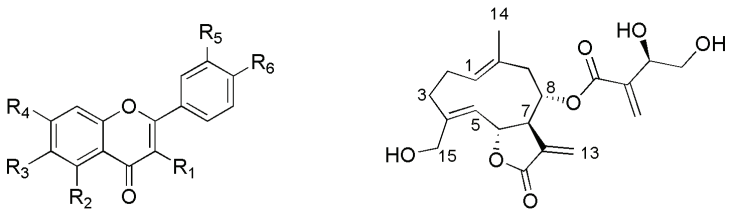
The HPLC chromatograms and ^{1}H-NMR spectra of the crude extracts of the aerial parts of all samples revealed the sesquiterpene lactone cnicin (**1**) as the major constituent (Figs. 1 and 3).

The sesquiterpene lactone cnicin was first isolated from *Cnicus benedictus* (blessed thistle) and found in 83 *Centaurea* species.[28] Since mediaeval times, preparations of blessed thistle (*e.g.*, bitter tonics) have been used to treat various

disorders, such as lack of appetite, dyspeptic troubles, liver diseases, and biliousness, while in the form of an external local remedy, blessed thistle was effective against ulcers and chilblains.[29] Nowadays, liver and bile tea compositions contain blessed thistle herb, which is also known to stimulate salivary secretion and gastric juice formation. Cnicin was established as the main active principle in blessed thistle, exhibiting antibacterial, antifungal, and strong anti-inflammatory effects. Known to be cytotoxic as some other sesquiterpene lactones,[30] cnicin exhibited cytotoxic activity against some tumor cell lines including leukemia (HL-60), hepatomas, sarcomas, lymphoid leukemia and multiple myeloma.[31–34]

TABLE I. Composition of flavonoid complex of *C. tomorosii*, *C. soskae* and *C. galicicae*

Compound	$[M–H]^-$ *m/z*	Acc. mass	Molecular formulae	Spectral methods used in identification	*C. tomorosii*	*C. soskae*	*C. galicicae*
Apigenin (**2**)	269.0438	270.0528	$C_{15}H_{10}O_5$	^{1}H-, ^{13}C-NMR, HRMS, UV	+	+	+
Isokaempferide (**3**)	299.0545	300.0633	$C_{16}H_{12}O_6$	^{1}H-, ^{13}C-NMR, HRMS, UV	+	+	+
Hispidulin (**4**)	299.0541	300.0633	$C_{16}H_{12}O_6$	^{1}H-, ^{13}C-NMR, HRMS, UV	+	+	+
Eupatorin (**5**)	343.0811	344.0896	$C_{18}H_{16}O_7$	HRMS, UV	+	+	+
Cirsimaritin (**6**)	313.0702	314.0793	$C_{17}H_{14}O_6$	^{1}H-, ^{13}C-NMR, HRMS, UV	+	–	–
Santoflavone (**7**)	359.1474	360.1209	$C_{19}H_{19}O_7$	^{1}H-, ^{13}C-NMR, HRMS, UV	+	+	+
Salvigenin (**8**)	327.2148	328.0974	$C_{18}H_{16}O_6$	HRMS, UV	+	+	+



1

Cmpd.	R_1	R_2	R_3	R_4	R_5	R_6
Apigenin (**2**)	H	OH	H	OH	H	OH
Isokaempferide (**3**)	OH	OH	H	OMe	H	OH
Hispidulin (**4**)	H	OH	OMe	OH	H	OH
Eupatorin (**5**)	H	OH	OMe	OMe	OH	OMe
Cirsimaritin (**6**)	H	OH	OMe	OMe	H	OH
Santoflavone (**7**)	H	OH	OMe	OMe	OMe	OMe
Salvigenin (**8**)	H	OH	OMe	OMe	H	OMe

Fig. 2. Chemical structures of the compounds detected in the studied *Centaurea* species.

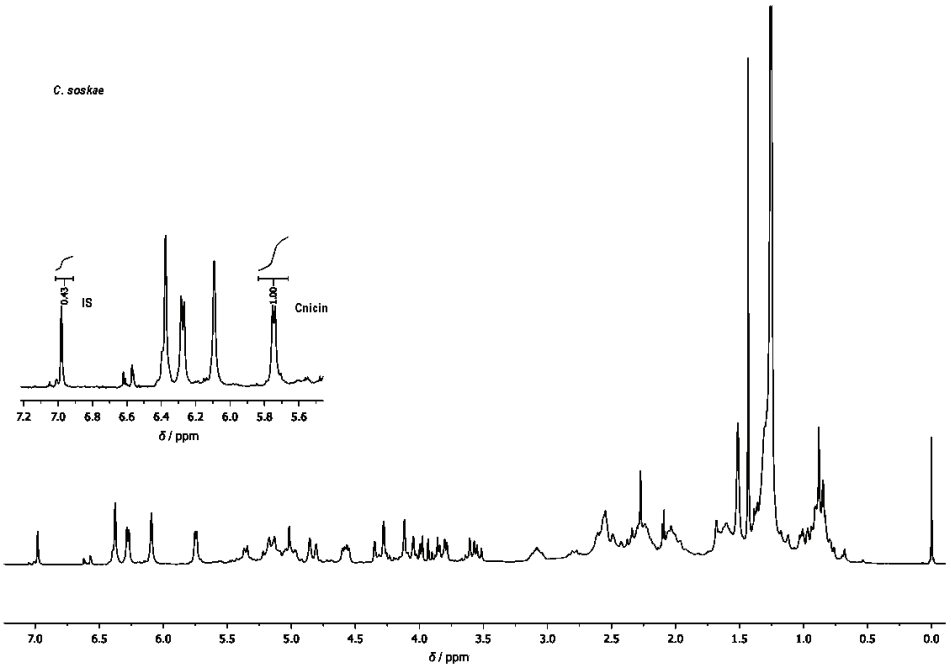


Fig. 3. Representative ^{1}H-NMR spectrum of the crude extract of *C. soskae* with internal standard (IS).

In previous phytochemical studies, the content of cnicin in six *Centaurea species* was analyzed by ^{1}H-NMR spectroscopy. The examined extracts were made from freshly collected plants. The highest content of cnicin was found in *C. affinis* (0.61 %). The content of cnicin in *C. arenaria*, *C. cuneifolia*, *C. glaberrima*, *C. splendens* and *C. stoebe* was lower (0.11–0.55 %).[26] According to quantitative ^{1}H-NMR measurements, based on the integrals of the one-proton doublet of cnicin (centered at δ 5.74 ppm, H-13) and the two-proton singlet (at δ 6.98 ppm) of a known amount of BHT, used as an internal standard, the content of cnicin was 2.9, 9.6 and 3.6 % in the dry plant extracts of *C. tomorosii*, *C. soskae* and *C. galicicae*, respectively (Table II). This amount of cnicin indicates potentially alternative sources of cnicin in these three *Centaurea* species, compared to one of the main natural sources, *i.e.*, *C. benedictus*, in which this metabolite is found at a concentration of 0.5 % of the dry mass.[29]

TABLE II. Content of cnicin in the aerial parts of *C. tomorosii*, *C. soskae* and *C. galicicae*

Specimen	Content±*SD*, % dry plant
C. tomorosii	2.9±0.08
C. soskae	9.6±0.15
C. galicicae	3.6±0.11

CONCLUSIONS

No previous phytochemical study has been performed on these three *Centaurea* species.

Among the three *Centaurea* species examined in this work, *C. soskae* showed the highest content of cnicin 9.6 %, while *C. tomorosii* and *C. galicicae* were found to contain 2.9 and 3.6 % of cnicin, respectively. All isolated compounds represent mainly the characteristic secondary metabolites of the genus. Methoxylated flavones, such as hispidulin (**4**), eupatorin (**5**), cirsimaritin (**6**), santoflavone (**7**) and salvigenin (**8**), as well as apigenin (**2**) and flavonol isokaempferide (**3**) were described previously as constituents of different *Centaurea* species. Similarly, the germacranolide cnicin (**1**) was detected in a large number of *Centaurea* species.

Acknowledgement. This work was financially supported by Ministry of Education, Science and Technological Development of the Republic of Serbia, (Project No. 172053). We thank Andon Bojadži and Oliver Avramoski, National Park Galičica, Ohrid, FYROM for supplying the plant material.

И З В О Д

СЕКУНДАРНИ МЕТАБОЛИТИ ТРИ ЕНДЕМСКЕ ВРСТЕ РОДА *Centaurea* L.

ВЕЛЕ ТЕШЕВИЋ[1], ИВАНА АЉАНЧИЋ[2], СЛОБОДАН МИЛОСАВЉЕВИЋ[1], ВЛАТКА ВАЈС[1,2], ИРИС ЂОРЂЕВИЋ[3], МИЛКА ЈАДРАНИН[2], НЕБОЈША МЕНКОВИЋ[4] и ВЛАДО МАТЕВСКИ[5]

[1]Хемијски факултет, Универзитет у Београду, Студентски трг 16, 11158 Београд, [2]Институт за хемију, технологију и металургију, Центар за хемију, Универзитет у Београду, Његошева 12, 11001 Београд, [3]Факултет ветеринарске медицине, Универзитет у Београду, Булевар ослобођења 18, 11000 Београд, [4]Институт за лековито биље, „Др Јосиф Панчић“ Тадеуша Кошћушка 1, 11000 Београд и [5]Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University in Skopje, Blvd. Goce Delcev 9, 1000 Skopje, FYROM

Из надземног дела три ендемске врсте рода *Centaurea* L., *C. tomorosii* Micevski, *Centaurea soskae* Hayek и *Centaurea galicicae* Micevski, изоловани су сесквитерпенски лактон кницин (**1**) и седам флавоноида: апигенин (**2**), изокемферид (**3**), хиспидулин (**4**), еупаторин (**5**), цирсимаритин (**6**), сантофлавон (**7**) и салвигенин (**8**). Структуре изолата су одређене UV и ^{1}H-NMR спектроскопијим и HR-ESI-MS спектрометријом. ^{1}H-NMR спектроскопија је коришћена као метода за квантитативну анализу сесквитерпенског лактона кницина.

(Примљено 18. марта, ревидирано 25. априла, прихваћено 6. маја 2014)

REFERENCES

1. A. Susanna, N. Garcia-Jacas, in *The families and genera of vascular plants*, J. W. Kadereit, C. Jeffrey, Eds., Springer, Berlin, 2007, p. 123
2. B. Colas, I. Olivieri, M. Riba, *Proc. Natl. Acad. Sci. USA* **94** (1997) 3471
3. V. N. Suárez-Santiago, M. J. Salinas, N. Garcia-Jacas, P. S. Soltis, D. E. Soltis, G. Blanca, *Mol. Phylogenet. Evol.* **43** (2007) 156
4. C. Koukoulitsa, G. D. Geromichalos, H. Skaltsa, *J. Comput.-Aided Mol. Des.* **19** (2005) 617
5. C. Karamenderes, S. Khan, B. L. Tekwani, M. R. Jacob, I. A. Khan, *Pharm. Biol.* **44** (2006) 534

6. E. Yeşilada, E. Sezik, G. Honda, Y. Takaishi, Y. Takeda, T. Tanaka, *J. Ethnopharmacol*. **64** (1999) 195
7. N. Garbacki, V. Gloaguen, J. Damas, P. Bodart, M. Tits, L. Angenat, *J. Ethnopharmacol*. **68** (1999) 235
8. J. F. Severino, K. Stich, G. Soja, *Environ. Pollut*. **146** (2007) 707
9. G. Rusak, M. Krajacic, N. Plese, *Antiviral Res*. **36** (1997) 125–129
10. G. Stamatis, P. Kyriazopoulos, S. Golegou, A. Basayiannis, S. Skaltsas, H. Skaltsa, *J. Ethnopharmacol*. **88** (2003) 175
11. M. Shoeb, S. M. MacManus, M. Jaspars, J. Trevidu, L. Nahar, P. Kong-Thoo-Lin, S. D. Sarker, *Tetrahedron* **62** (2006) 11172
12. A. G. Gonzalez, J. B. Barrera, T. Z. Garcia, F. E. Rosas, *Phytochemistry* **23** (1984) 2071
13. D. Youssef, A. W. Frahm, *Planta Med.* **60** (1994) 572
14. A. M. Fortuna, E. C. de Riscala, C. A. Catalan, T. E. Gedris, W. Herz, *Biochem. Syst. Ecol.* **29** (2001) 967
15. K. Medjroubi, F. Benayache, J. Bermejo, *Fitoterapia* **76** (2005) **744**
16. M. López-Rodriguez, V. P. Garcia, H. Zater, S. Benayache, F. Benayache, *Acta Crystallogr., E* **65** (2009) 1867
17. Z. F. Ahmed, H. Rimpler, A. M. Rizk, F. M. Hammouda, S. I. Ismail, *Phytochemistry* **9** (1970) 1595
18. M. Kaij-A-Kamb, M. Amoros, L. Girre, *Pharm. Acta Helv*. **67** (1992) 178
19. V. Tešević, V. Vajs, P. Janaćković, N. Todorovic, D. Đoković, P. Marin, S. Milosavljević, *Planta Med.* **64** (1998) 488
20. V. Vajs, N. Todorović, M. Ristić, V. Tešević, B. Todorović, P. Janaćković, P. Marin, S. Milosavljević, *Phytochemistry* **52** (1999) 383
21. P. Janaćković, V. Tešević, S. Milosavljević, V. Vajs, P. D. Marin, *Biochem. Syst. Ecol.* **32** (2004) 355
22. V. Tešević, V. Vajs, N. Todorović, D. Đoković, P. Marin, S. Milosavljević, *J. Serb. Chem. Soc.* **63** (1998) 131
23. V. Tešević, D. Đoković, V. Vajs, P. Marin, S. Milosavljević, *J. Serb. Chem. Soc.* **59** (1994) 979
24. V. Tešević, S. Milosavljević, V. Vajs, P. Janaćković, M. Popsavin, *Biochem. System. Ecol.* **31** (2003) 89
25. F. Bohlmann, C. Zdero, H. R. King, E. H. Robinson, *Phytochemistry* **23** (1984) 1979
26. V. Tešević, S. Milosavljević, V. Vajs, P. Janaćković, I. Djordjević, M. Jadranin, I. Vučković, *J. Serb. Chem. Soc.***72** (2007) 1275, and references therein
27. T. J. Mabry, K. R. Markham, M. B. Thomas, *The Systematic Identification of Flavonoids*, Springer, Berlin, 1970
28. M. Bruno, S. Bancheva, S. Rosselli, A. Maggio, *Phytochemistry* **95** (2013) 19
29. S. Berger, D. Sicker, *Classics in Spectroscopy, Isolation and Structure Elucidation of Natural Products*, Wiley–VCH, Weinheim, 2009, p. 443, and references therein
30. M. Bruno, S. Rosselli, A. Maggio, R. A. Raccuglia, K. F. Bastow, C. C. Wu, K. H. Lee, *Planta Med.* **71** (2005) 1176.
31. R. Vanhaelen-Fastré, *Pharm Belg*. **27** (1972) 683
32. R. Vanhaelen-Fastré, M. Vanhaelen, *Planta Med*. **29** (1976) 179
33. K. Johrer, M. Obkircher, D. Neureiter, J. Parteli, C. Zelle-Rieser, E. Maizner, J. Kern, M. Hermann, F. Hamacher, O. Merkel, N. Wacht, C. Zidorn, M. Scheideler, R. Greil, *J. Mol. Med*. **90** (2012) 681
34. M. Bach, M. A. Fortuna, R. Attarian, J. T. de Trimarco, C. A. Catalán, Y. Av-Gay, H. Bach. *Nat. Prod. Commun*. **6** (2011) 163.