

SHORT COMMUNICATION

**Quantitative analysis of sesquiterpene lactone cnicin in seven
Centaurea species wild-growing in Serbia and Montenegro
using ¹H-NMR spectroscopy**

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Abstract: ¹H-NMR spectroscopy was applied for the quantitative analysis of cnicin, a bioactive germacranolide type sesquiterpene lactone, in the aerial parts of seven wild-growing *Centaurea* species collected in Serbia and Montenegro. The analysis was performed by comparison of the integral of the one-proton signal of cnicin (H-13, δ 5.75) with that of the two-proton singlet (δ 6.98) of 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT), used as the internal standard. Cnicin, within the concentration range 1.06–6.12 mg/g, calculated per weight of the fresh plant material was detected in six species, the exception being *C. salonitana*. This method allows the rapid and simple quantification of cnicin without any pre-purification step.

Keywords: ¹H-NMR, *Centaurea*, cnicin, BHT.

INTRODUCTION

Centaurea, the largest genus of the family Compositae (Asteraceae), is represented with about 40 species in Serbia and Montenegro and has been the subject of many chemical investigations, which have led to the isolation of various types of compounds, such as sesquiterpene lactones,^{1–5} acetylenes,^{5–7} flavonoids^{5,8} and lignans.^{3–5} Many of the *Centaurea* species are known for their anti-diabetic, anti-diarrhetic, anti-rheumatic, anti-inflammatory, cholagog, cholaretic, digestive, stomachic, diuretic, emenagogue, astringent, hypotensive, anti-pyretic, cytotoxic and antibacterial effects, and are used single or mixed.^{9–11}

One of the first biologically active compounds to be isolated from the genus *Centaurea* was cnicin (C, Fig. 1), a sesquiterpene lactone of the germacranolide

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type, known for a long time (since 1837) as the main “bitter” ingredient in blessed thistle, *Cnicus benedictus*.^{12,13} Cnicin has been reported as a constituent of several species of the *Centaurea* genus,¹⁴ such as *C. diffusa* (0.81 %),¹⁵ *C. squarrosa* (0.8 %),¹⁶ *C. affinis* (0.46 %),³ *C. stoebe* (0.45 %),¹⁷ *C. derventana* (0.2 %),¹ *C. nicaensis* (0.19 %),¹⁸ *C. bruguierana* (0.16 %)¹⁹ and *C. calcitrapa* (0.06 %).²⁰ Cnicin could be found in the glandular epithelial trichomes of the leaves and, to a lesser extent, the shoots of knapweeds and it finds its way into the soil *via* leaching or litter.²¹ This compound has broad effects on other taxa (allelopathic effect). Various bioassays of cnicin revealed antibiotic, antifungal, phytotoxic, and anti-herbivore properties.^{14,22,23} Additionally, it has been shown to be auto-toxic to knapweeds themselves.²¹ Spotted (*C. maculata*), diffuse (*C. diffusa*) and Russian knapweeds (*C. repens*) all produce cnicin at fairly high levels.²⁴ Cnicin is used as a bitter tonic and the bitterness value is approximately 1,500.²⁵ The bitterness of these compounds stimulates digestive activity, including the flow of saliva and secretion of gastric juice, which leads to improved appetite and digestion.

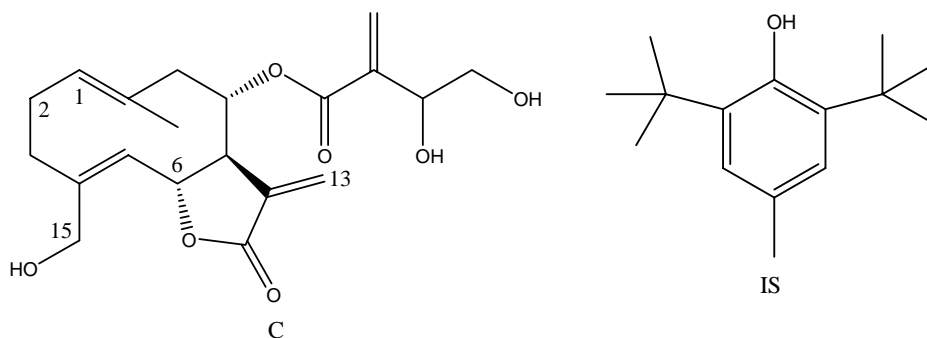


Fig. 1. Structure of cnicin (C) and BHT (IS).

Hitherto, various methods for the analysis of sesquiterpene lactones (SLs) in complex mixtures have been reported.²⁶ Most of them involve chromatographic techniques, such as GC and HPLC, or a combination of these with spectrometric methods, such as GC–MS, HPLC–MS and/or NMR. Since SLs are not volatile compounds and many of them are thermolabile, HPLC seems to be more suitable than GC. However, the lack of a specific and strong absorbing UV chromophore in SLs makes sensitivity a problem when conventional HPLC–UV detection is used. The chromatographic analysis usually requires tedious clean-up procedures, evaluation of suitable elution conditions, preparation of calibration curves for quantifications, and in some cases derivatization in order to enhance sensitivity and to remove compounds which interfere with the detection of the analyte.

Representing the method of choice in structure determination of natural products, NMR spectroscopy is mainly used qualitatively, although is generally accepted as a quantitative tool. The decisive advantage of NMR spectroscopy for

quantitative analysis over chromatographic methods is due to the fact that the preparation of the sample is much simpler. Nowadays, due to the enhanced sensitivity and introduction of the new pulse sequences, NMR spectroscopy (especially $^1\text{H-NMR}$) is becoming a routine quantitative method in natural products laboratories. There are numerous examples of the application of quantitative $^1\text{H-NMR}$ in the analysis of different natural products, such as cannabinoids from *Cannabis sativa*,²⁷ terpene trilactones in *Ginkgo biloba*,^{28,29} ephedrine alkaloids, the composition of *Ephedra* species,^{30,31} camptothecin derivatives in *Nothapodytes foetida*³² and parthenolide in feverfew products³³ and in the aerial parts of *Tanacetum larvatum*.³⁴

In this paper, a survey of the cnicin content in seven wild-growing *Centaurea* species from Serbia and Montenegro measured using $^1\text{H-NMR}$ spectroscopy is reported.

EXPERIMENTAL

Plant material

The studied species and collection data are listed in Table I.

TABLE I. The studied *Centaurea* species, collection data and the average content of cnicin in the aerial parts (estimated recovery of cnicin: 95.51±2.2 %)

Species	Collection data ^a	Content, mg/g fresh plant ±S.D.
<i>C. affinis</i> Friv.	Vlasina lake, SE Serbia, July 2001 (CAf06072001)	6.12±0.15
<i>C. arenaria</i> Bieb. ex Willd.	New Belgrade, Serbia, June 2001 (CAr18062001)	1.06±0.06
<i>C. cuneifolia</i> S. S.	Progorelica, C. Serbia, June 2001 (Ccu11062001)	2.08±0.15
<i>C. glaberima</i> Tausch.	Kotor, Montenegro, June 2003 (Cgl29062003)	5.48±0.21
<i>C. splendens</i> L.	Mt. Orjen, Montenegro, June 2001 (CSpl3062001)	4.12±0.03
<i>C. stoebe</i> L.	Sićevo, SE Serbia, September 1998 (CSt391998)	2.61±0.06
<i>C. salonitana</i> Vis.	Niševac, SE Serbia, July 2001 (CS18072001)	Not detected

^aVoucher specimens deposited at the Herbarium, Faculty of Biology, University of Belgrade – Herbarium Code BEOU

Sample preparation

An aliquot (1 g) of fresh aerial parts of the studied *Centaurea* species (cut into small pieces) was ultrasonicated, after addition of 1 ml (2.4 mg/ml) of BHT (IS, Fig. 1), with 25 ml of Et₂O at room temperature for 30 min. The extraction was repeated three times. The combined extracts were filtered and evaporated to dryness *in vacuo*. The residues were dissolved in 1 ml of CDCl₃ (99.9 %) and used for $^1\text{H-NMR}$ measurements. All results are based on triplicate measurements.

Recovery test of cnicin

A standard solution containing 1.87 mg of cnicin in Et₂O (1 ml) was spiked onto 1 g of filter paper disks (5891 white, Schleicher & Schuell GmbH, Kassel, Germany) cut into circles of *ca.* 1.0 cm

diameter, placed in an extraction vessel and dried at room temperature for 24 h. The NMR samples were prepared using the same procedures as above. The sample preparation and NMR measurements were carried out in triplicate.

¹H-NMR analysis

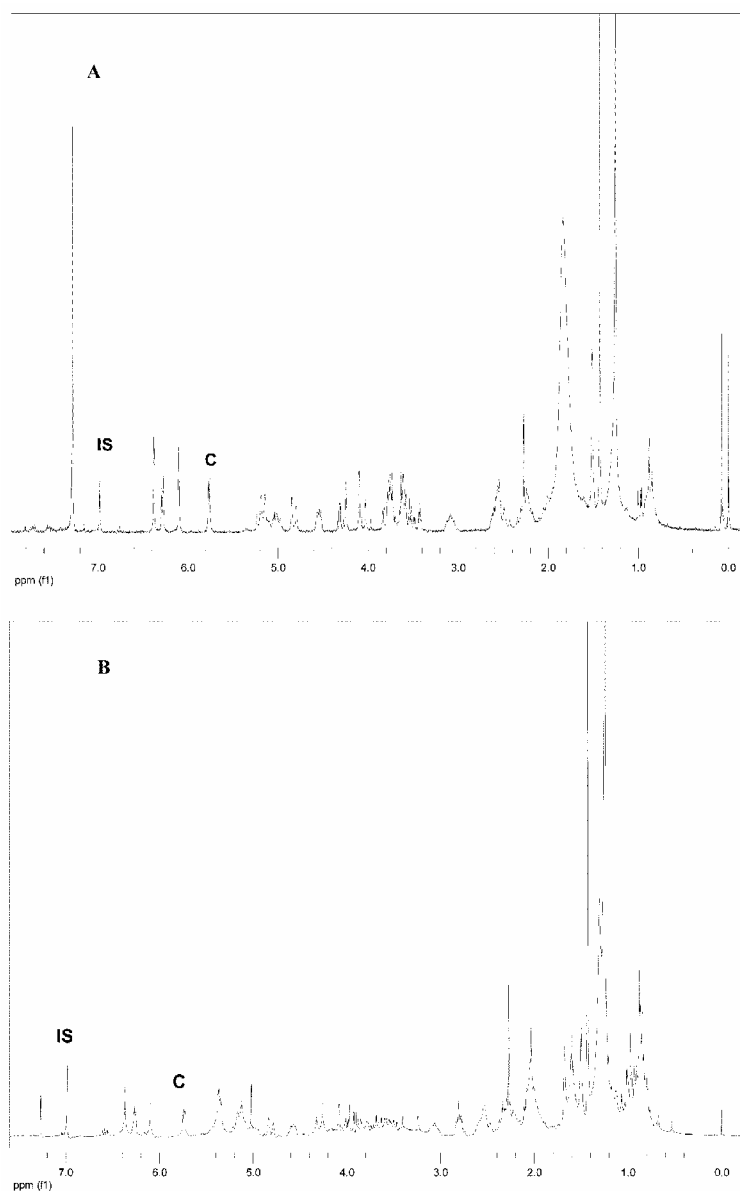


Fig. 2. ¹H-NMR spectra (200 MHz) in CDCl₃ of: cnicin + internal standard from the recovery experiment (A) and extract from *C. affinis* + internal standard (B). IS: the signal of H-3/5 from internal standard (BHT) and C: the signal of H-13 from cnicin.

The $^1\text{H-NMR}$ (200 MHz) spectra (in CDCl_3) were recorded using a Varian GEMINI 2000 spectrometer. For each sample, 100 scans were recorded with the following parameters: 0.187 Hz/point; spectral width, 3005.5 Hz; pulse width, 20 μs ; relaxation delay, 4 s. For quantitative analysis, peak area was used and the start and end points of the integration of each peak were selected manually. The quantitative results were based on the integrals of the one-proton signal of cnicin (H-13, δ 5.75), not overlapped with other signals (see Fig. 2), and the two-proton singlet (δ 6.98) of 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT), used as the internal standard (I_C and I_S , respectively), using the following equation:

$$\text{Cnicin (mg/g)} = (I_C \times M_C \times G_S) / [(I_S/2) \times M_S \times G]$$

where M_C and M_S are the molar masses of cnicin and the standard, respectively, while G and G_S are the weighed masses of the sample (g) and standard (mg), respectively.

RESULTS AND DISCUSSION

The results of the quantification of cnicin in the studied *Centaurea* species are given in Table I. The content of cnicin in six of them varied within the range 1.06–6.12 mg/g of fresh plant material, whereas cnicin was not detected in *C. salonitana*. It was demonstrated that using an appropriate choice of experimental conditions, $^1\text{H-NMR}$ spectroscopy is a suitable tool for the quantitative determination of cnicin in crude extracts. The results obtained by the $^1\text{H-NMR}$ method were found to be sufficiently accurate, with a standard deviation of between 0.03 and 0.21. The application of the $^1\text{H-NMR}$ method enabled the quantification of cnicin in a much shorter time than that required by the conventional chromatographic methods. *C. affinis*, *C. glaberima* and *C. splendens*, with a content of cnicin in the range of about 4–6 mg/g could be regarded as new sources of this valuable compound.

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ИЗВОД

КВАНТИТАТИВНА АНАЛИЗА СЕСКВИТЕРПЕНСКОГ ЛАКТОНА КНИЦИНА У СЕДАМ САМОНИКЛИХ БИЉНИХ ВРСТА РОДА *Centaurea*, КОЈЕ РАСТУ У СРБИЈИ И ЦРНОЈ ГОРИ, КОРИШЋЕЊЕМ $^1\text{H-NMR}$

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$^1\text{H-NMR}$ спектрометрија је примењена за квантитативну анализу кницина, биоактивног сесквитерпенског лактона гермакранолидног типа, у надземном делу седам самониклих биљних врста рода *Centaurea* сакупљених у Србији и Црној Гори. Анализа је извршена поређењем интеграла сигнала једног протона кницина (H-13, δ 5.75) са синглетом који потиче од два протона (δ 6.98) интерног стандарда, 2,6-бис(1,1-диметилетил)-4-метилфенола (БХТ). Кницин је детектован у свим врстама, осим у *C. salonitana*, у концентрацијама у опсегу од 1,06 до 6,12 mg/g, рачунато на масу свежег биљног материјала. Овај метод омогућава брзу и једноставну квантификацију кницина без претходног пречишћавања.

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