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NATURAL DRUGS

TERPENOIDS AND STEROLS FROM *NEPETA CATARIA* L. *VAR. CITRIODORA* (LAMIACEAE)

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Abstract: Isolation and GC/MS quantitative determination of ursolic acid in the herb of *Nepeta cataria* var. *citriodora* have been performed. The content of this compound was in the range 0.95 – 1.30%. Daucosterol (β-sitosterol 3-O-β-D-glucoside) was also isolated from the plant, in addition to small amounts of β-sitosterol, campesterol, α-amyrin and β-amyrin. The content and composition of essential oil in samples of the *Nepeta cataria* var. *citriodora* herb have been analysed as well.

Keywords: *Nepeta cataria* var. *citriodora;* essential oil, ursolic acid, daucosterol; GC quantitative estimation

Nepeta cataria L. (catnip) and *Nepeta cataria* L. var. *citriodora* (Becker) Balb. are perennial herbs native to Asia Minor and south-eastern Europe. In other parts of Europe and also in northern Africa and North America the plants are often naturalized (1). *Nepeta cataria* var. *citriodora* is a plant of interest in fragrances industry due to the essential oil containing citral (2,3). In Poland it was investigated in the fifties of the last century as a potential *corrigens* of pharamceutical preparations (4,5). In traditional medicine *Nepeta cataria* var. *citriodora* was used as a sedative, spasmolytic and tonic remedy similar to the lemon balm (*Melissa officinalis*) (1) and in the textbooks of pharmacognosy it is mentioned as a possible adulteration or substitute of *Melissae folium* (6, 7).

Previous papers on *Nepeta cataria* var. *citriodora* reported mainly on essential oil (4,8,9). Other constituents have not been examined so far. In our laboratory, the investigations on *Melissae* folium, obtained from domestic cultivation have been conducted in recent years (10-14) and also preliminary investigations of volatile components of *Nepeta cataria* var. *citriodora* have been carried out (11). The current study deals with the isolation and identification of non-volatile terpenoids from *Nepeta cataria* var. *citriodora*, apart from the detailed analysis of essential oil.

EXPERIMENTAL

Plant material

Plant material was obtained from the plants *N. cataria* L. var. *citriodora* cultivated in the Garden of Medicinal Plants, the Chair of Pharmacognosy, Medical University of Łódź. The seeds for cultivation were obtained from the „Kawon" (Gostyń). Identification of plant material was performed on the basis of morphological features described in the literature (1,15). A herbal specimen is deposited in the Department of Pharmacognosy, Medical University of Łódź.

The samples of aerial parts of the plant were harvested in the period 2000 – 2004 at different stages of vegetation: sample 1 (herb with fruits) – 07.09.2000; sample 2 (herb in beginning of flowering) – 14.07.2001; sample 3 (herb with fruits) – 05.09.2002; sample 4 (young, non-blossoming herb) – 13.06.2003; sample 5 (herb with buds) – 09.07.2003; sample 6 (herb with flowers and fruits) – 19.08.2003; sample 7 (herb with partly brown leaves) – 29.09.2003; sample 8 (herb with fruits) – 18.08.2004.

Methods and equipment

A rotating evaporator (Rotavapor R-200, Büchi) was used to the concentration of extracts. Chromatograms were visualized under a quartz lamp Camag. Melting points were determined on the microscopic table Boetius; not corrected. IR spectra were recorded in KBr on ATI Mattson Infinity FTIR, NMR spectra on a Bruker DRX 500. Gas chromatography was carried out using Agilent – 6890N Network GC System, 7683 Injector, conjugated with a mass spectrometer 5973 Mass Selective Detector (Agilent). The analysis of the components of the essential oil was performed with the use of a DB-PE-

TRO column, 50m long, 0.2mm in diameter, film layer – 0.5 μm. To the determination of ursolic acid an HP-5MS column was used, 30m long, 0.25mm in diameter at film layer = 0.25 μm.

Chromatography

Thin-layer chromatography was performed with the use of glass plates, 10 x 20 cm, covered with silica gel Kieselgel 60 (Merck). Mixtures: S1: $CHCl_3$ /cyclohexane/MeOH (3: 2: 1) and S2: *n*-hexane/EtOAc (9:1) were used as mobile phases. Chromatograms were visualized by sprying with a Liebermann-Burchard reagent (5cm^3 of $(MeCO)_2O$ and 5cm^3 of H_2SO_4 conc. were added to 50cm^3 of anhydrous EtOH during continuous cooling and mixing) followed by UV light at 365 nm.

In the column chromatography Diaion HP-20 (Supelco), silica gel (Kieselgel- Merck 60) 0.063-0.2mm, silica gel MN-Kieselgel 60 0.125-0.25mm) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used. Solvents: S1, S2 and S3: cyclohexane/$CHCl_3$ gradient 3: 2 – 1: 4 were used as eluents in column chromatography on silica gel. The column with Sephadex LH-20 was eluted with MeOH.

Quantitative determination of ursolic acid by means GC/MS

Preparation of a calibration curve

In a test-tube with a teflon stopper, 2.5mg of ursolic acid (Roth) was placed. Silylation was performed by the addition of 0.5 mL of BSTFA (N, O--*bis*-(trimethylsilyl)-trifluoroacetamide, Fluka) and heating at a temperature of 90°C in a thermostatic block for 45min. After the reaction was complete the mixture was cooled and 0.5 mL of cyclohexane (HPLC purity) was added. Several dilutions from the basic solution of the concentration 2.5 mg/mL were prepared and autocalibration using an internal standard was done.

A relationship between peak area *(y)* and the compound concentration in the analyzed sample *(x)* is depicted by the equation:

$y = 4E + 06x^2 - 264924x + 83223 \quad R = 0{,}9987$

Preparation of sample for analysis

A sample, 0.15 g of raw material, weighed accurately to 0.0001 g, was extracted with 10 mL of $CHCl_3$ (HPLC purity) for 30 min at boiling. Extraction was repeated twice. The extracts were combined, filtered through a filter paper, concentrated in a rotating evaporator to the volume of 1 mL, quantitatively transferred into a test-tube and the solvent was evaporated by gentle heating. The dried residue was dissolved in 1mL of $CHCl_3$. 0.5 mL of this extract was quantitatively transferred into another test-tube, the solvent was evaporated and silylation was performed as described above.

Based on peak area, the content of ursolic acid in the sample was calculated and subsequently the percentage of this compound was determined. The results are presented in Table 1.

Determination of the content and composition of essential oil

The content of essential oil was calculated by hydrodistillation in a Deryng apparatus according to the Polish Ph.VI (16), method 1. The estimations were performed in 4 – 5 weeks after harvesting. The results are presented in Table 2. Components of the oil obtained from sample 6 were identified on the basis of GC/MS analysis using the above-mentioned equipment. Identification of components was done on the basis of retention time and mass spectrum, database Wiley7n. 1 was employed to the identification. The results of GC analysis of sample 6 are presented in Table 3.

Table 1. Ursolic acid content in the sample of *Nepeta cataria* L. var. *citriodora* herb

Sample No.	Weighed amount	Ursolic acid in the sample [mg]	Ursolic acid [%]	Ursolic acid mean content [%]
3	0.1503	2.12	1.41	1.22
	0.1431	1.48	1.03	
4	0.1508	1.46	0.97	0.95
	0.1516	1.41	0.93	
5	0.1571	1.72	1.10	1.30
	0.1555	2.32	1.49	
6	0.1564	1.80	1.15	1.18
	0.1557	1.88	1.21	

Table 2. Essential oil content in different samples of *Nepeta cataria* var. *citriodora* herb

Sample No.	Content of essential oil [mL/100g]
1	0.70
2	0.72
3	0.74
4	0.80
5	0.65
6	0.80
7	0.45
8	0.80

Table 3. Composition of essential oil of *Nepeta cataria L.* var. *citriodora*

Components	Retention time	Content % of total oil
Monoterpene alcohols		
geraniol	16.44	23.49
nerol	16.13	24.36
citronellol	16.03	13.96
p-cymene-3-ol	16.86	1.14
linalool	14.05	0.96
α-terpineol	15.70	0.43
Sum of alcohols		64.34
Monoterpene aldehydes		
geranial (citral a)	16.63	8.22
neral (citral b)	16.26	6.63
citronellal	14.85	1.62
Sum of aldehydes		16.47
Sesquiterpenes		
β-caryophyllene	19.49	1.83
β-caryophyllene oxide	21.62	2.48
Other components		
α-terpinolene	14.05	0.11
rose oxide	14.33	0.86
nerol oxide	14.99	1.21
3,7-dimethyl-6-octen-1-ol formate	16.72	0.37

Extraction of raw material and isolation of non--volatile terpenoids

From the raw material (435 g of the sample 6) volatile constituents were removed by steam distillation in the Deryng apparatus. Raw material was filtered off, dried at a temperature of 40-50 °C and extracted four times with MeOH, each time using 500 mL of the solvent and heating in a boiling water bath for 4 hours. MeOH extracts were combined, a solvent was distilled off and dry residue was dissolved in hot H_2O. A dark green resinous substance was removed by filtration and the solution was extracted with *n*-hexane (8x100 cm^3). After evaporation of *n*-hexane, 21 g of dry extract was obtained which was preliminary separated on a Diaion HP-20 column using methanol as an eluent. Fractions of similar composition were combined and then chromatographed on silica gel columns using the mixtures of cyclohexane, chloroform and methanol in various proportions to the elution. From the fraction 18-32 (Diaion), after further separation on silica gel (eluent: S1), compound **1** (45 mg) and compound **2** (85 mg) were obtained. From fractions 93-122 (Diaion) – a mixture of compounds **3** and **4** (2 mg) and from fractions 123-134- a mixture of compounds **5** and **6** (4 mg) were obtained.

Identification

Compound 1 (β-sitosterol 3-O- β-D-glucopyranoside)

white, crystalline powder, m. p. 286-290 °C;

C – 69.34%, H – 9.53%, O – 20.01% ($C_{35}H_{60}O_6+H_2O$)

IR $_{(KBr)}$ ν cm^{-1}: 3404 (OH), 2959, 2931, 2867 (C-H), 1638 (C=C)

^{1}H NMR [$CDCl_3$] δ (ppm): 5.19 d J=5Hz (H-6), 4.22 d J=7.8Hz (H-1'), 3.67 dd J=3Hz and 12Hz (H-6'), 3.57 dd J=4.8Hz and 12Hz (H-6'), 3.42 m (H-3), 3.24 dd J=9Hz and 3.5 Hz (H-3'), 3.21 dd J=9.5 Hz and 3 Hz (H-4'), 3.11 m (H-5'), 3.05 dd J=4.5 Hz and 8Hz (H-2'), 2.18 dd 2.5Hz and 5.2Hz (2H-4), 2.08 t J=13Hz (2H-2), 1.83 dd (H-7), 1.35 m (2H-1), 0.51s (Me-18), 0.84 s (Me-19), 0.75 d 6.4Hz (Me--21), 0.64 d 6.8Hz (Me-26), 0.65 d 6.8 Hz (Me-27), 0.67 t 7.4Hz (Me-29)

^{13}C NMR [$CDCl_3$] δ (ppm): 38.30 (C-2), 78.60 (C--3), 38.70 (C-4), 121.60 (C-6), 11.40 (C-18), 18.90 (C-19), 18.30 (C-21), 18.70 (C-26), 11.90 (C-29), 100.70 (C-1'), 73.20 (C-2'), 76.14 (C-3'), 69.80 (C--4'), 76.50 (C-5'), 61.40 (C-6').

The assignments were proved by HMQC experiment.

Compound 2 (ursolic acid)

fine, colorless needles, m. p. 278-282°C (283 – 284 °C (17))

IR $_{(KBr)}$ ν cm^{-1}: 3358 – 3440 (OH), 2965, 2930, 2869 (C-H), 1675 (COOH), 1456, 1375, 1187

^{1}H NMR [$(CD_3)_2CO$] δ (ppm): 0.77, 0.89, 0.94, 0.98, 1.12 (singlets 5 x Me); 0.83 (d, J=6.1Hz) Me--29; 0.93 (d, J=5Hz) Me-30, 2.25 (d, J=12Hz) H-18; 5.21 (t, J=3,7Hz) H-12

^{13}C NMR [$(CD_3)_2CO$] δ (ppm): 38.45 (C-1); 79.28 (C-3); 39.00 (C-4); 56.85 (C-5); 19.76 (C-6); 34.61 (C-7); 40.53 (C-8); 48.93 (C-9); 38.25 (C-10); 18.18 (C-11); 126.88 (C-12); 138.01 (C-13); 43.21 (C-14);

29.33 (C-15); 28.74 (C-16); 49.12 (C-17); 54.52 (C--18); 40.16 (C-19); 40.53 (C-20); 31.99 (C-21); 37.01 (C-22); 30.48 (C-23); 17.00 (C-24); 16.57 (C--25); 18.18 (C-26); 24.60 (C-27); 179.32 (C-28); 25.64 (C-29); 22.13 (C-30)

Compounds 3 and 4 (α-amyrin, β-amyrin)
TLC R_f: 0.26 (silica gel, S2)
GC: t_{R1} = 17.47 min (α-amyrin), t_{R2} = 17.1 min (β--amyrin) 10: 1
GC/EIMS *(m/e)*: 426 [M^+], 411 [M^+-CH_3], 218 [M^+-$C_{15}H_{21}$], 203 [218–CH_3].

Compounds 5 and 6 (β-sitosterol and campesterol)
TLC R_f: 0.15 (silica gel, S2)
GC: t_{R1} = 17.13 min (β-sitosterol), t_{R2} = 16.62 min (campesterol) 4: 1
GC MS: 414 [M^+], 396 [M^+-H_2O], 381 M^+-[CH_3--H_2O], 329 M^+-[H_2O+67], 303 M^+-[H_2O+67], 273 [M^+-side chain] (SC), 255 M^+-[SC+H_2O], 231 M^+-[SC+42 (C_{15}+C_{17})].

RESULTS AND DISCUSSION

The results obtained in the study of essential oil in *N. cataria* var. *citriodora* herb were consistent with the results previously reported (4, 8, 9). In the majority of samples, the essential oil content amounted to 0.7-0.8 mL/100g. It was significantly higher than in Melissae folium which contained from 0.08 to 0.25 mL essential oil in 100 g of dry raw material derived from plants cultivated in Poland (13). Monoterpene alcohols: geraniol, nerol, citrenolol and the corresponding aldehydes: geranial, neral, citrenolal are the major constituents of the essential oil from *N. cataria* var. *citriodora*. It also contains β-caryophyllene and its oxide. The same compounds were found in Melissa oil, but in that oil monoterpene aldehydes dominated (above 60% of the whole oil) and the sum of monoterpe alcohols amounted to a few per cent (13). The content of the essential oil in Melissae folium and its composition may vary significantly (10,11,13). Our investigations and the data from the literature on oils from plants growing in France (8), Finland and Scotland (7) point out that the content and composition of the essential oil in *N. cataria* var. *citriodora* are rather stable.
Due to the oil composition, *N. cataria* var. *citriodora* can show a mild sedative action which depends on monoterpene aldehydes and to a lesser extent on alcohols and β-caryophyllene (15). According to the data from the literature, geraniol, nerol, citral and β-caryophyllene can be essential for antiinflammatory and also bacteriostatic activities (18-20).

The isolation of terpenoids other than volatile compounds was the next step of our study. The methanolic extract prepared from the plant material remained after essential oil distillation was further extracted with *n*-hexane. From the *n*-hexane extract, compounds **1** and **2** were isolated by means of column chromatography.
Compound **1** was analyzed by NMR including ^{1}H-H COSY and HMQC experiments. A doublet at 5.2 ppm with coupling constant J=5Hz suggested that compound **1** was a sterol with a double bond between C5 and C6 and the chemical shift of six triproton singlets derived from -CH_3 groups indicated the structure of β -sitosterol. A signal of anomeric sugar proton at δ=4.22 ppm was a doublet with J=7.8Hz, which pointed out the β-configuration of glycosidic bond. The remaining signals located between 4.22 and 3.05 ppm were identified by means of ^{1}H-^{1}H COSY as H-2, H-3, H-4, H-5 and H-6 of glucose, respectively. On the basis of ^{1}H-^{1}H COSY and HMQC data, compound **1** was identified as β-sitosterol-3-O-β-D-glycopyranoside (daucosterol). This compound was detected in the examined taxon for the first time. Previously, it was isolated from *Nepeta caesarea* (21). Apart from daucosterol free β- sitosterol in a mixture with campesterol was identified by means GC/MS. A quantitative ratio of the compounds was 4: 1. Free sterols occur in small amounts in *N. cataria* var. *citriodora*. They are probably substrats for the synthesis of β-sitosterol-β-D-glucoside.

Compound **2** was identified as ursolic acid on the basis of melting point, Rf values and ^{1}H and ^{13}C NMR spectral analysis. Ursolic acid is present in different amounts in various plants and especially often it occurs in Lamiaceae plants. In *N. cataria* var. *citriodora* was found to be one of the major constituents. Ursolic acid as a compound having lipophilic triterpenoid sceleton and hydrophilic -COOH group is soluble in water, alcohols as well as in lipophilic solvents such as *n*-hexane and chloroform; therefore the isolation efficacy from *n*-hexane extract was not high. A compound which often accompanies ursolic acid is oleanolic acid (an isomer with β-amyrine sceleton). These two isomers are hardly separated by column or thin-layer chromatography. Using GC/MS analysis, traces of oleanolic acid were detected in the purified fraction of hexane extract form *N. cataria* var. *citriodora*.

Quantitative determination of ursolic acid in *N. cataria* var. *citriodora* was performed using gas chromatography after silylation of the investigated product. The results obtained confirmed that ursolic acid was one of the predominant constituents of *N. cataria* var. *citriodora* herb. Among four samples investigated only one revealed the content of ursolic acid be-

low 1% of the dry mass. In the remaining three samples more than 1.2% of ursolic acid was determined.

Apart from ursolic acid, two triterpenoid alcohols were detected in *N. cataria* var. *citriodora* and identified as β-amyrine and α-amyrine by means of GC/MS.

A fraction of non-volatile terpenoids can play a significant role in the biologicaly activity of medicinal herbs from Lamiaceae family. According to the recent biological and pharmacological studies triterpenoid acids revealed, among other, antiinflammatory (22-24) and cytotoxic (25-26) action. Recently, their inhibitory effect on cyclooxygenase (27-28) and leucocyte elastase was proved (29). Beta-sitosterol and its glycoside (daucosterol) are regarded as the plant constituents reducing cholesterol level in man (30-31). β-sitosterol glucoside, according to the recent reports, shows some immunomodulative properties (increases proliferation of T-lymphocytes and increases secretion of IL-2 and gamma-interferon) (32). It can be also used as an adjuvant because it facilitates absorption of some active substances (verapamil) administered to the nasal cavity (33).

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