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The Essential Oil and Glycosidically Bound Volatile Compounds of *Calamintha nepeta* (L.) Savi*

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Results of the investigation of the essential oil and glycosidically bound volatile compounds of *Calamintha nepeta* (L.) Savi are presented in the paper. The essential oil was isolated by hydrodistillation and glycosides were extracted with ethyl acetate. The yield of essential oil was $W = 0.91\%$. The essential oil was fractionated on a microcolumn with solvents of different polarity. One fraction of terpene hydrocarbons and four fractions of oxygenated terpene compounds were obtained. All fractions were analyzed by gas chromatography-mass spectrometry (GC-MS) on two columns with different polarity of the stationary phases. The purpose of the fractionation was to obtain a more completely analysis. In this way, sixty-five compounds were identified. In contrast, only twenty-four compounds were identified in the essential oil without fractionation. The main components were piperitone oxide, piperitenone oxide, limonene, caryophyllene, thymol, linalool, 3-octanol and other compounds in smaller amounts. After isolation, final purification of the glycosides by »flash« chromatography on silicagel column and enzymatic hydrolysis, the liberated aglycones were analyzed in the same way as the essential oil fractions. The content of volatile aglycones was $W = 0.00081\%$ or 8.1 mg kg^{-1} of the plant material. Eighteen compounds were identified. The main aglycones were eugenol, carveol, 3-octanol, 3-hexene-1-ol, 2-butanol, 2-phenyletha-

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nol, methyl salicylate, benzyl alcohol, acetophenone derivates, and others. Some of the aglycones were the same or structurally similar to the essential oil components. Glycosidically bound volatiles in this plant have not been investigated so far.

INTRODUCTION

Calamintha nepeta (L) Savi (*Lamiaceae*) is a perennial green shrub, widely spread over dry parts of the Mediterranean region.This plant grows wild along the Adriatic coast and the submediterranean part of Croatia. This plant also grows in Montenegro, Macedonia and Greece. *Calamintha nepeta* (L.) Savi grows up to a height of 40 cm and has small leaves which are alternately arranged. The plant flowers in July and August. It is not known whether this plant is used in traditional herbal medicine in Croatia.

The essential oils of aromatic plants have been extensively investigated. On the other hand, the glycosidically bound volatiles of these plants are unsufficiently investigated. They have been identified in aromatic[1–5] and non-aromatic plants.[6–9] Glycosides with β-glycosidic linkage are more common than those with α-glycosidic linkage.[10] β-Glucosidase from almonds is commonly used for hydrolysis of most β-glycosides. The essential oil of *Calamintha species* has been studied but glycosidically bound volatiles have not been investigated to date.[11–14]

EXPERIMENTAL

Plant Material

The plant material was collected in the Mediterranean region of Dalmatia, near Split. The plant material sample was collected during flowering, in August 1995. Young stems with leaves and flowers (15–20 cm) were harvested and dried at room temperature. Voucher specimens are deposited in the Department of Pharmaceutical Botany and Pharmacognosy, University of Zagreb.

Isolation of Essential Oil and Glycosides

Essential oil was isolated by hydrodistillation in a modified Clevenger type apparatus for three hours. Essential oil was dried over anhydrous sodium sulphate and stored under nitrogen in a sealed vial, at –20 °C, until required. The yield of essential oil was $W = 0.91\%$.

The glycosides of volatile compounds were isolated by extraction at room temperature. Extraction was effected by percolation with ethyl acetate from 100 g of plant material. As internal standard, 500 μg of octyl-β-D-glucoside was added to ethyl acetate. After percolation and evaporation of the solvent, the residue was dissolved in 50 mL ethanol. Ballast components were removed from the ethanol extract with 50 mL of water in the form of precipitate. The remaining ethanol-water extract was then concentrated to 2 mL in a rotating evaporator under reduced

pressure. To the obtained syrup, 30 mL of absolute ethanol and a few drops of concentrated ammonia were added. The precipitated acid ballast compounds were filtered off. The ethanol extract was mixed with 3 g of silica gel 60 (0.040–0.063 mm) and the mixture was dried in a rotating evaporator under reduced pressure . This sample was applied to the top of a glass column (2.5 cm i.d.) packed with 15 g of silica gel 60. Elution was performed with 400 mL ethyl acetate – ethanol – concetrated ammonia 6:3:1 *v*/*v*/*v*. Air pressure in the chromatographic column was about 80 kPa. The obtained fractions were tested by thin layer chromatography (TLC) on silicagel with the same solvent and vanillin-sulphuric acid as reagent. All fractions with R_F-values of 0.25–0.75 were combined.

Fractionation of Essential Oil

20 μL of essential oil was fractionated on a silica gel 60 microcolumn (0.040––0.063 mm) and five fractions were obtained. 10 mL pentane was used for fractionation (fraction I) and a mixture of pentan-ether: 5 mL 5% ether (fraction II), 5 mL 10% (fraction III), 5 mL 50% (fraction IV) and 5 mL pure ether (fraction V). All fractions were concentrated to 0.5 mL and tested by thin layer chromatography (TLC). The first fraction contained only nonpolar monoterpene and sesquiterpene hydrocarbons. Other fractions contained polar, oxygenated compounds as per to the increasing polarity from fraction II to fraction V. These results were also confirmed by GC-MS analysis.

Enzymatic Hydrolysis of Glycosides

Pooled glycosidic fractions were concentrated to dryness and the residue dissolved in a citrate buffer (pH 5.5; 5 mL) The aqueous solution was washed with 5 × 5 mL of pentane-dichloromethane 2:1 *v*/*v* and with 5 × 5 mL of pure pentane to remove free terpenes and other hydrophobic compounds that might be present. After being concentrated to a few drops, the last pentane extract (5 mL) was tested by TLC and GC and found to have no traces of free terpene and other hydrophobic compounds. Then, 20 mg β-glucosidase from almonds (»Fluka«) was added to the glycosidic solution, along with 3 mL pentane for trapping liberated aglycones. Hydrolysis was carried out at 30 °C for 72 hours, the mixture being shaken occasionally. After hydrolysis, the pentane layer was separated. The remaining aglycones from the aqueous layer were extracted with 4 × 5 mL of pentane. The combined pentane extracts were dried over anhydrous sodium sulphate and concentrated to a final volume of 0.5 mL.

Analysis of Essential Oil and Aglycones

All fractions of the essential oil and volatile aglycones were analyzed by gas chromatography-mass spectrometry (Hewlett-Packard, model 5890, with a mass selective detector, model 5971A). Two columns with different polarity of stationary phases, HP-20M and HP-101, were used.

GC operating conditions: column HP-20M (Carbowax 20M), 50 m × 0.2 mm i.d., film thickness 0.2 μm; column temperature programmed from 70 °C isothermal for 4 minutes, then increased to 180 °C at a rate of 4 °C min^{-1}; column HP-101 (Methyl-silicone), 25 m × 0.2 mm i.d., film thickness 0.2 μm; column temperature programmed from 70 °C isothermal for 2 minutes, then increased to 200 °C at a rate of

3 °C min^{-1}. Carrier gas: helium, flow rate: 1 mL min^{-1}, injector temperature: 250 °C; volume injected: 1 μL; split ratio: 1:50.

MS conditions: ionization voltage: 70 eV; ion source temperature: 280 °C; mass range: 30–300 mass units. Individual peaks were identified by comparing their retention indices to those of authentic samples, as well as by comparing their mass spectra with those stored in the data base (Wiley library). The percentage composition of the samples was computed from the GC peak areas without correction factors.

RESULTS AND DISCUSSION

Essential Oil

The yield of the essential oil obtained by hydrodistillation of dry plant material was 0.91%. The chemical composition and content of the essential oil of *Calamintha nepeta* are given in Table I. Monoterpene and sesquiterpene hydrocarbons and oxygen containing compounds were identified. Sixty--five compounds were identified to represent 91.6% of total oil. Several compounds (8.4% of essential oil) remained unidentified. The major components of the essential oil were monoterpene oxides such as piperitone oxide (46.0%), piperitenone oxide (12.7%) and limonene (10.9%). This essential oil also contained smaller quantities of α-pinene, caryophyllene, thymol, β-myrcene, β-cube-

TABLE I

Identified constituents of the *Calamintha nepeta* (L.) Savi essential oil

No. Compound	% (area)	Mode of identification
Hydrocarbons		
1. α-pinene	1.6	I_1, I_2, MS
2. sabinene + β-pinene	1.6	I_1, I_2, MS
3. β-myrcene	1.2	I_1, I_2, MS
4. limonene	10.9	I_1, I_2, MS
5. α-terpinene	0.2	– I_2, MS
6. *cis*-β-ocimene	0.1	I_1, I_2, MS
7. γ-terpinene	0.4	I_1, I_2, MS
8. *p* + *m*-cymene	t	I_1, –, MS
9. α-terpinolene	0.2	I_1, I_2, MS
10. alloocimene	0.1	I_1, I_2, MS
11. α-copaene	0.1	I_1, I_2, MS
12. α-cubebene	t	–, I_2, MS
13. β-bourbonene	0.3	I_1, I_2, MS
14. β-elemene	0.2	–, I_2, MS
15. *trans*-caryophyllene	1.5	I_1, I_2, MS
16. α-humulene	0.2	I_1, I_2, MS
17. β-cubebene	1.1	I_1, I_2, MS
18. α-muurolene	t	–, I_2, MS
19. γ-cadinene	0.1	–, I_2, MS
20. δ-cadinene	0.1	I_1, I_2, MS

TABLE I, continued

No. Compound	% (area)	Mode of identification
Oxygen containing compounds		
21. methyl-2-methyl-butanoate	0.2	I_1, I_2, MS
22. 2,5-diethyltetrahydrofurane	0.2	I_1, I_2, MS
23. acetic acid	0.1	I_1, –, MS
24. 2-pentanol	0.1	I_1, –, MS
25. 2-butanol	0.1	I_1, –, MS
26. 1,8-cineol	0.5	I_1, I_2, MS
27. *Z*-2-hexenal	t	I_1, –, MS
28. 3-heptanol	t	I_1, –, MS
29. 2-methylbutil-2-methylbutanoate	0.3	I_1, I_2, MS
30. 3-octanol	0.8	I_1, I_2, MS
31. 1-octen-3-ol	t	I_1, –, MS
32. menthone	1.1	I_1, I_2, MS
33. *cis*-sabinenhydrate	0.5	I_1, –, MS
34. linalool oxide	t	I_1, –, MS
35. 3-hexenyl-2-methylbutanoate	0.5	–, –, MS
36. 2-ethyl-1-heksanol	t	I_1, –, MS
37. 3-nonanol	0.1	I_1, –, MS
38. *trans*-sabinenhydrate	t	I_1, –, MS
39. linalool	0.6	I_1, I_2, MS
40. bornyl acetate	0.2	I_1, –, MS
41. terpinen-4-ol	0.2	I_1, –, MS
42. pulegone	1.0	I_1, I_2, MS
43. *Z*-citral	t	I_1, –, MS
44. borneol	0.4	I_1, I_2, MS
45. α-terpineol	0.3	–, I_2, MS
46. piperitone	0.3	I_1, I_2, MS
47. piperitone oxide	46.0	I_1, –, MS
48. β-citronellol	t	I_1, –, MS
49. methyl salicylate	0.2	I_1, –, MS
50. myrthenol	t	I_1, –, MS
51. benzyl isobutanoate	0.1	I_1, I_2, MS
52. nerol	t	I_1, –, MS
53. 2-hydroxy-3-methyl-6-isopropyl-2-cyclo-hexene-1-on	1.0	I_1, I_2, MS
54. isopiperitenone	0.4	I_1, I_2, MS
55. carveol	t	I_1, –, MS
56. benzyl-2-methylbutanoate	0.3	I_1, I_2, MS
57. 6-hydroxy-6-isopropyl-3-methyl-2-cyclohexene-1-on	0.8	I_1, I_2, MS
58. 2,3-dimethyl-5-isopropenyl-2-cyclo-penten-1-on	0.1	I_1, I_2, MS
59. piperitenone	0.3	I_1, I_2, MS
60. 3-phenyl-2-butanone	0.3	I_1, –, MS
61. piperitenone oxide	12.7	I_1, –, MS
62. diphenylamine	0.3	I_1, I_2, MS
63. eugenol	0.1	I_1, I_2, MS
64. thymol	1.5	I_1, I_2, MS
65. α-terpenyl isobutanoate	0.1	I_1, –, MS
Hydrocarbons	19.9	
Oxygen containing compounds	71.7	
Identified	91.6	
Unidentified	8.4	

I_1 – retention indices on HP-20M, I_2 – retention indices on HP-101
MS – mass spectra, t – trace < 0.1%

bene, menthone and other compounds. In contrast, only twenty-four compounds were identified in the essential oil without fractionation.

Some components are very common in many essential oils, *viz.* α- and β-pinene, limonene, β-caryophyllene and humulene, geraniol, borneol, camphor. Piperitone oxide and piperitenone oxide have been identified only in a small number of plants. These components were found only in some *Mentha, Calamintha* and *Satureja* ssp. Piperitone oxide, the main component of this oil, was also found in the essential oil of *Calamintha nepeta* of Greek origin.[11] According to an earlier observation,[15] piperitone oxide and piperitenone oxide ratio in the essential oil of *Calamintha* depends on the maturity of the plant, and probably on the climate and soil.[14] From the toxicological point of view, the presence of a significant quantity of oxides in this oil may represent a possible hazard to the tissues of mammals.

Glycosidically Bound Volatiles

Glycosidically bound volatile compounds in dried green parts of this plant amounted to 0.00081%, or 8.1 mg/kg. The GC-MS analysis of the aglycones on two columns revealed eighteen compounds. The results are shown

TABLE II

Identified aglycones of *Calamintha nepeta* (L.) Savi

No. Compound	% (area)	Mode of identification
1. 2-butanol	4.9	I_1, –, MS
2. 3-hexene-1-ol	5.7	I_1, –, MS
3. 3-octanol	6.2	I_1, I_2, MS
4. benzaldehyde	1.1	I_1, –, MS
5. terpinen-4-ol	1.0	I_1, –, MS
6. isopulegol	0.5	I_1, –, MS
7. α-terpineol	0.7	I_1, –, MS
8. 2-methyl-5-isopropenylcyclohexanol	0.8	I_1, –, MS
9. *cis*-carveol	9.5	I_1, –, MS
10. methyl salicylate	1.5	I_1, –, MS
11. 2-hydroxy-3-methyl-6-isopropyl-2-cyclohexene-1-on	0.8	I_1, –, MS
12. *trans*-carveol	0.8	I_1, –, MS
13. benzyl alcohol	1.3	I_1, –, MS
14. 2-phenylethanol	3.2	I_1, I_2, MS
15. phenylpropanol	0.5	I_1, –, MS
16. eugenol	19.2	I_1, –, MS
17. 3,4-dimethoxyacetophenone	2.8	I_1, I_2, MS
18. 4-hydroxy-3-methoxyacetophenone	1.2	–, I_2, MS
Identified	61.7	
Unidentified	38.3	

I_1 – retention indices on HP-20M, I_2 – retention indices on HP-101, MS – mass spectra

in Table II. These compounds represented about 61.7% of total aglycones. About ten compounds (37.3% of volatile aglycones) remained unidentified. Among the aglycones, aliphatic alcohols, terpene compounds, derivates of acetophenone and phenylpropanes were identified.

Major aglycones included eugenol (19.2%), *cis*-carveol (9.5%), 3-octanol (6.2%), 3-hexene-1-ol (5.7%), 2-butanol (4.9%). Aglycones also contained 2-phenylethanol, 3,4-dimethoxyacetophenone, methyl salicylate, benzyl alcohol, 4-hydroxy-3-methoxyacetophenone, benzaldehyde, terpinen-4-ol, α-terpineol, *trans*-carveol and other compounds. Some of the aglycones are the same or structurally similar to the essential oil components, such as eugenol, carveol, 3-octanol, 2-butanol, terpinen-4-ol, α-terpineol and methyl salicylate.

Like in essential oils, some of the components are very common in aglycones of different plants. Aglycones such as aliphatic acohols, 2-phenylethanol, benzyl alcohol, eugenol, linalool, geraniol, nerol, α-terpineol and terpinen-4-ol might be considered as ubiquitous in aglycone fractions.[16,17] Some of them were not identified among the aglycone compounds from *Calamintha nepeta*.

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S A Ž E T A K

Eterično ulje i glikozidno vezani hlapljivi spojevi biljke *Calamintha nepeta* (L.) Savi

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Prikazani su rezultati istraživanja eteričnog ulja i glikozidno vezanih hlapljivih spojeva biljke *Calamintha nepeta* (L.) Savi. Eterično ulje izolirano je vodenom parom, a glikozidi su ekstrahirani etil-acetatom. Prinos ulja bio je $W = 0.91\%$. Eterično ulje frakcionirano je na mikrokoloni otapalima različite polarnosti. Dobivena je jedna frakcija terpenskih ugljikovodika i četiri frakcije oksidiranih terpenskih spojeva. Sve su frakcije bile analizirane vezanim sustavom plinska kromatografija-masena spektrometrija (GC-MS) na dvije kolone različite polarnosti stacionarnih faza. Svrha frakcioniranja bila je dobiti potpuniju analizu ulja. Identificirano je 65 spojeva, a u ulju bez frakconiranja samo 24 spoja. Glavne komponente bile su piperiton-oksid i piperitenon-oksid, limonen, kariofilen, timol, linalol, 3-oktanol i drugi spojevi u manjim količinama. Poslije izolacije i konačnog pročišćavanja glikozida »flash« kromatografijom na stupcu silikagela i enzimske hidrolize, oslobođeni aglikoni analizirani su na isti način kao i frakcije eteričnog ulja. Maseni udjel hlapljivih aglikona bio je $W = 0{,}00081\%$ ili 8,1 mg kg^{-1}. Glavni su aglikoni eugenol, karveol, 3-oktanol, 3-heksen-1-ol, 2-butanol, 2-feniletanol, metil-salicilat, benzil-alkohol, derivati acetofenona i drugi. Neki od aglikona jednaki su ili strukturno slični komponentama eteričnog ulja. Glikozidno vezani hlapljivi spojevi ove biljke nisu do sada bili istraživani.