Chemical Composition and Biological Activity of *Nepeta parnassica* Oils and Isolated Nepetalactones

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Essential oils of *Nepeta parnassica*, collected at different developmental stages, were analyzed by means of GC/MS. From the fifty-five identified constituents in samples A and B, representing 94.8 % and 98.7 % of the oils respectively, 4aα,7α,7aβ-nepetalactone (22.0 %), 1,8-cineole (21.1 %), α-pinene (9.5 %) and 4aα,7β,7aβ-nepetalactone (7.9 %) were the major components of sample A (vegetative stage), whereas in sample B (flowering stage) the main contributors were 1,8-cineole (34.6 %), 4aα,7α,7aα-nepetalactone (17.3 %), α-pinene (11.4 %) and 4aα,7α,7aβ-nepetalactone (8.9 %). The oils were tested on human health important insects such as the *Pogonomyrmex* sp. ants and the *Culex pipiens molestus* mosquitoes with promising results on insect repellency/toxicity.

Key words: Nepeta parnassica, Essential Oil, Nepetalactones, Insect Repellency

Introduction

Nepeta L. is a genus of annual or perennial herbs found in temperate Europe, Asia, North Africa, in mountains of tropical Africa and comprises of approximately 250 species (Mabberly, 1997). Many *Nepeta* species have been investigated for their oil constituents, but only a limited number have been studied thoroughly.

Nepeta parnassica Heldr. & Sart. is an aromatic perennial herb, endemic of Greece and South Albania, growing on Mt Parnassos (Central Greece) and Mt Helmos (Peloponnisos, Greece). It is usually found in dry stony places, rocky habitats and scree (Turner, 1972; Baden, 1987).

In literature, the iridoid monoterpenes nepetalactones frequently appear as the main constituents of *Nepeta* essential oils. In Table I is attempted to show the complication in the published data of the nepetalactone content in this genus covering the literature up to 2000.

Previous reports on the biological activity of nepetalactones include the repellant activity against different types of insects (Eisner, 1965; Regnier *et al.*, 1967).

The aim of this study was the chemical analysis of *N. parnassica* volatile constituents and the evaluation of the essential oils and isolated nepetalactones on *Pogonomyrmex* sp. ants and *Culex pipiens molestus* mosquitoes.

Results and Discussion

The essential oils of *N. parnassica* (samples A and B) were analyzed by means of GC/MS. Fifty-five compounds were detected and identified, representing 94.8 % and 98.7 % of the total oils, respectively (Table II). Oxygenated monoterpenoids were the dominant contributors in the oils accounting for 73.6 % of sample A and 75.6 % of sample B. The monoterpene hydrocarbons accounted for 13.0 % and 19.2 % of samples A and B, respectively.

The chemical composition of the two samples was qualitative similar. However, significant differences in the quantitative composition of the two samples were observed. The main metabolites of sample A were 4aα,7α,7aβ-nepetalactone (22.0 %), 1,8-cineole (21.1 %), α-pinene (9.5 %) and 4aα,7β,7aβ-nepetalactone (7.9 %). In sample B the major constituents were 1,8-cineole (34.6 %), 4aα,7α,7aα-nepetalactone (17.3 %) and α-pinene (11.4 %), while 4aα,7α,7aβ-nepetalactone and 4aα,7β,7aβ-nepetalactone were found in 8.9 % and 2.0 %, respectively.

Chemical investigation of essential oils of the genus *Nepeta* have shown increased lactone con-

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Table I. Nepetalactone content of *Nepeta* species.

	Nepetalactones												
Species	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**l**	**m**
N. argolica subsp. *argolica*[1]	3.2–10.5	64.5–91.3											
N. argolica subsp. *argolica*[2]	26.5	12.9	14.5, 0.4	29.4, 1.9			4.7						
N. asterotrichus[3]	2.7		14.8										
N. beltranii[4]	0.6–0.2	0.4–0.4	0.4–0.5										
N. binaludensis[5]	25.2			0.7									
N. caesarea[6]	91.2–95.3	0.1–0.2	0.1–0.2										
N. cataria[4]	90.5	0.5											
N. cataria[7]									77.6		15.0		0.3
N. cataria[8]	1.3–2.8	11.4–56.9				2.0–1.7							
N. cataria[9]		24.0–78.0		11.0–6.0	15.0	10.0							
N. cephalotes[10]	35.1												
N. citriodora[7]									9.4		1.6		1.2
N. coerulea[4]	11.9	21.5	3.7	19.3									
N. crassifolia[11]	16.3	7.7	9.6	27.2				1.5					0.5
N. elliptica[12]												80.0	
N. grandiflora[9]		2.4		41.0									
N. mussini[7]									16.7		70.0		
N. nepetella[13]									76.5	0.4	0.6		
N. nepetella subsp. *aragonensis*[4]	3.5	57.7											
N. nuda[9]				62.0				1.3					
N. nuda[14]	0.9–7.1	6.7–76.6	1.2–54.8	0.1–18.4									0.1–0.2
N. nuda subsp. *albiflora*[15]*	1.0	37.5	37.6										
N. parnassica[16]									1.9–7.6	3.2–0.4	1.6		0.4–0.7
N. racemosa[17]		91.6–31.5	0.7–1.3										
N. racemosa[18]	64.9	7.4	1.7										
N. rtanjensis[19]		86.4	0.9										
N. sulfuriflora[20]**	0.5												
N. teydea[21]	89.5–1.4	0.9–0.4	0.5–1.5	tr–0.1				0.3–0.5					
N. troodi[22]									0.1–2.2	1.1–3.8			
N. tuberosa subsp. *tuberosa*[23]								69.4					

a: 4aα,7α,7aα-nepetalactone; **b**: 4aα,7α,7aβ-nepetalactone; **c**: 4aβ,7α,7aβ-nepetalactone; **d**: 4aβ,7α,7aα-nepetalactone; **e**: 3,4α-dihydro-4aα,7α,7aβ-nepetalactone; **f**: 3,4β-dihydro-4aα,7α,7aα-nepetalactone; **g**: 3-hydroxy-4α,4aα,7α,7aα-dehydronepetalactone; **h**: 5,9-dehydronepetalactone; **i**: nepetalactone; **j**: neonepetalactone; **k**: epinepetalactone; **l**: (7*R*)-*trans*,*trans*-nepetalactone; **m**: dihydronepetalactone.

[1] Tzakou *et al.* (2000); [2] Skaltsa *et al.* (2000); [3] Rustaiyan *et al.* (1999); [4] Velasco-Negueruela *et al.* (1998); [5] Rustaiyan and Nadji (1999); [6] Baser *et al.* (1994); [7] Regnier *et al.* (1967); [8] Bourrel *et al.* (1993); [9] Handjieva *et al.* (1996); [10] Rustaiyan *et al.* (2000a); [11] Moghaddam *et al.* (1996); [12] Bottini *et al.* (1987); [13] Bicchi *et al.* (1984); [14] De Pooter *et al.* (1987); [15] Kökdil *et al.* (1996); [16] Arnold *et al.* (1993a); [17] Baser *et al.* (1993); [18] Rustaiyan *et al.* (2000b); [19] Chalchat *et al.* (2000); [20] Kökdil *et al.* (1997); [21] Velasco-Negueruela *et al.* (1989); [22] Arnold *et al.* (1993b); [23] Cotrim *et al.* (1994).

* Sarer and Konuklugil (1996) no nepetalactones in *N. nuda* subsp. *albiflora* oil.

** Baser *et al.* (1998) no nepetalactones in *N. sulfuriflora* oil.

tent during the flowering stage. In the present study, nepetalactone contribution was rather similar (31.3 % for sample A and 28.2 % for sample B) in the two samples.

The oil of *Nepeta parnassica* was found to contain the array of monoterpenes usually found in the *Nepeta* oils. Nepetalactones, 1,8-cineole, α-pinene, α-terpineol and caryophyllene oxide, were the main metabolites that have been found in almost all the studied species of genus *Nepeta*.

Nepeta parnassica oils from different plant parts have been previously studied by Arnold *et al.* (1993a). Leaf and flower oils were dominated by 1,8-cineole (46.4 %). The reported nepetalactones were neonepetalactone (3.2 %), nepetalactone (1.9 %) and epinepetalactone (1.6 %). Noteworthy is the excessive contribution of citronellol (63.8 %) in the oil of the stems. In the present study nepetalactones were present in significantly higher amounts.

Table II. Chemical composition of *Nepeta parnassica* oils.

Component[a]	KI[b]	Sample A[c] (%)	Sample B[c] (%)
α-Pinene	928	9.5	11.4
Camphene	930	tr	
Verbenene	935	tr	tr
Sabinene	944	tr	tr
β-Pinene	952	3.5	6.4
Myrcene	962	tr	
1,8-Cineole	1015	21.1	34.6
cis-Sabinene hydrate	1045	0.6	
(*Z*)-β-Ocimene	1051		0.5
γ-Terpinene	1056		0.4
cis-Linalool oxide	1065		tr
6-Camphenone	1066	tr	
p-Cimenene	1078		0.5
Linalool	1090		tr
α-Campholenal	1116	tr	1.3
trans-Limonene oxide	1125		0.6
trans-Pinocarveol	1131	1.9	1.2
cis-Verbenol	1133	tr	tr
trans-Verbenol	1141	6.4	1.7
Pinocarvone	1142	1.5	tr
p-Mentha-1,5-dien-8-ol	1166		2.6
Terpin-4-ol	1170	tr	0.5
α-Terpineol	1173	5.0	4.3
Myrtenal	1176	tr	
Myrtenol	1182	tr	tr
Verbenone	1195	4.5	0.6
trans-Carveol	1201	1.1	tr
Citronellol	1217		tr
cis-Carveol	1220		tr
Cuminal	1226		tr
Carvone	1231		tr
Bornyl acetate	1261	tr	
Ethyl 3-phenylpropanoate	1324	tr	
4aα,7β,7aβ-Nepetalactone	1337	7.9	2.0
4aα,7α,7aα-Nepetalactone	1344	1.5	17.3
α-Copaene	1351	1.3	
4aα,7α,7aβ-Nepetalactone	1366	22.0	8.9
α-Humulene	1436		tr
(*E*)-β-Farnesene	1439		tr
cis-Muurola-4(14),5-diene	1445		0.5
Germacrene-D	1464		0.6
β-(*E*)-Ionone	1469		tr
β-Bisabolene	1489	tr	tr
γ-Cadinene	1491	tr	tr
cis-Calamenene	1496	1.1	
δ-Cadinene	1508		2.8
trans-Calamenene	1509		tr
Cadina-1,4-diene	1513		tr
α-Calacorene	1524	tr	tr
Spathulenol	1557	tr	tr
Caryophyllene oxide	1561	4.8	tr
β-Copaen-4-α-ol	1565	1.1	tr
α-Cadinol	1632	tr	tr
Cadalene	1652		tr
cis-14-Muurol-5-en-4-one	1666	tr	tr
Total of identified		94.8	98.7

[a] Components listed in order of elution from an HP 5MS column.
[b] Kováts indices calculated against C_9-C_{24} *n*-alkanes.
[c] A and B represent the essential oils of *Nepeta parnassica* collected in vegetative and in full flowering stage, respectively.
tr = mass fraction less than 0.05 %.

Evaluation of the *N. parnassica* essential oils showed significant ant toxicity and mosquito repellency. Considering the available literature on the biological activity of nepetalactones and the fact that these metabolites are the main constituents of the oils, it was presumed that there might responsible for these activities. For this purpose three nepetalactones (Fig. 1) were isolated by means of HPLC separations and identified as 4aα,7α,7aα-nepetalactone (**1**), 4aα,7α,7aβ-nepetalactone (**2**) and 4aα,7β,7aβ-nepetalactone (**3**), by comparison of their ^{1}H and ^{13}C NMR spectra with literature values (Bottini *et al.*, 1987; Boros and Stermitz, 1991; Kökdil *et al.*, 1999).

The insecticidal activity of the essential oils and nepetalactones was studied on *Pogonomyrmex* sp. ants (Tsoukatou *et al.*, 2001). Both oils and tested nepetalactones showed significant levels of toxicity (Table III). Oils from samples A and B showed high toxicity in bioassay I (Table III), where 100 % mortality was observed within 12h, while nepetalactone **2** showed worth noting toxicity in the feeding bioassay II (Table III).

In the course of the present study, the repellency of *N. parnassica* oil was evaluated against *C. pipiens molestus* mosquitoes. This experiment showed that the oil from the vegetative stage was very active. Quantities of 1 and 10 mg repelled significantly female mosquitoes from approaching the applied with oilskin surface (Table III).

The promising results of this preliminary insect repellency/toxicity investigation of the *Nepeta* oils and nepetalactones need to be supplemented with more sophisticated experiments that are already in progress.

Experimental

General experimental procedures

Optical rotations were measured using a Perkin-Elmer model 341 polarimeter and a 10 cm cell. NMR spectra were recorded using a Bruker AC 200 and a Bruker DRX 400 spectrometer. High resolution mass spectra data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, Indiana. EIMS data were recorded on a Hewlett Packard 5973 mass selective detector. CC separation was performed with Kieselgel 9385 (Merck), TLC were performed with Kieselgel 60 F_{254} (Merck alu-

Table III. Bioassays.

I	Hours	Sample A		Sample B		Control
		0.1 mg	1.0 mg	0.1 mg	1.0 mg	
	6 h	0/16*(0.00)[#]	4/16(0.05)	8/16(0.05)	0/16(0.03)	0/16(0.00)
	12 h	16/16(0.03)	16/16(0.00)	16/16(0.03)	16/16(0.03)	8/16(0.00)

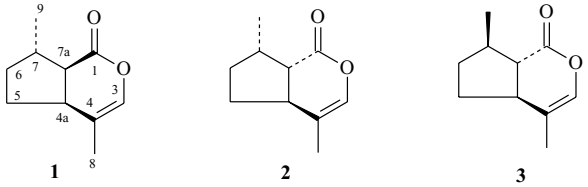
II	Hours	Metabolite 1	Metabolite 2	Metabolite 3	Control
		0.1 mg	0.1 mg	0.1 mg	
	12 h	0/12*(0.00)	0/16(0.00)	0/14(0.00)	0/12(0.00)
	24 h	0/12(0.04)	8/16(0.05)	2/14(0.06)	2/12(0.00)
	48 h	2/12(0.07)	16/16(0.03)	4/14(0.06)	4/12(0.07)
	72 h	8/12(0.16)	16/16(0.00)	8/14(0.08)	4/12(0.07)

III	Hours	Metabolite 1	Metabolite 2	Metabolite 3	Control
		0.5 μg	0.5 μg	0.5 μg	2/14(0.06)
	12 h	4/12*(0.07)	2/14(0.00)	0/12(0.04)	2/14(0.06)
	48 h	4/12(0.07)	2/14(0.00)	0/12(0.04)	2/14(0.06)
	96 h	4/12(0.07)	4/14(0.06)	4/12(0.07)	2/14(0.06)
	144 h	4/12(0.07)	4/14(0.06)	4/12(0.07)	2/14(0.06)

IV	Repellency (%)		
	mg	Sample A	Deet[**]
	0.1	6.3	72.2
	1.0	54.0	81.1
	10.0	88.5	95.5

I: Ant body contact bioassay.
II: Ant feeding toxicity bioassay.
III: Ant body diffusion bioassay.
IV: Mosquito repellency.
[#] Numbers in parentheses represent the standard deviation.
[*] Dead/total.
[**] Registered insect repellant (*N*, *N*-diethyl-*m*-toluamide).

minum support plates) and spots were detected with 15 % H_2SO_4 in MeOH reagent. HPLC separation was conducted using a Pharmacia LKB 2248 model and a GBC LC-1240 refractive index detector, with a Supercosil SPLC-Si column (5 μm; column size, 10 × 250 mm).



1 **2** **3**

Fig. 1. Isolated nepetalactones. **1**, 4aα,7α,7aα-nepetalactone; **2**, 4aα,7α,7aβ-nepetalactone; **3**, 4aα,7β,7aβ-nepetalactone.

Plant material

The aerial parts of a wild population of *N. parnassica* were collected during vegetative stage in June 2000 (sample A) and during flowering stage in September 2000 (sample B) from the same population, on Mt Parnassos at an altitude of 1600 m. The plant was identified by Dr. Th. Constandinidis (Institute of Systematic Botany, Agricultural University of Athens) and a voucher specimen of the collection (OT-14) has been deposited in the Herbarium of the University of Athens (ATHU).

Isolation of oils

Air-dried aerial parts of the plant material were subjected to hydrodistillation for 3 h using a modified Clevenger-type apparatus with a water-cooled receiver, to reduce hydrodistillation-overheating

artifacts. The essential oils were dried over anhydrous sodium sulfate and were stored under N_2 atmosphere in amber vials at 4 °C until they were analyzed. The essential oils were yellow in color, with a strong pleasant odor. The oil yields, estimated on the basis of plant dry weight, were 1.8 % and 0.8 % for samples A and B, respectively. The physicochemical characteristics of the oil were: $[\alpha]_D^{20} = +1.1°$ and $[\alpha]_D^{20} = -1.4°$ for samples A and B, respectively.

GC-MS analysis

GC-MS analyses were carried out using a Hewlett-Packard 5973–6890 GC-MS system operating in the EI mode at 70 eV, equipped with an HP-5 MS capillary silica column (30 m × 0.25 mm; 0.25 μm film thickness). The initial temperature of the column was 60 °C and was raised to 280 °C at a 3 °C/min rate. Carrier gas was He, flow rate = 1 ml/min. Split ratio was 1:10. The injection volume of each sample was 1 μl. *n*-Alkanes were used as reference points to calculate the Kováts' indices (KI). Identification of the chemical constituents was based on comparison of their relative retention times and mass spectra with those obtained from authentic samples and/or the NIST/NBS and Wiley libraries spectra as well as literature data (Adams, 1995). Quantitative analyses were performed of individually prepared samples A and B on two sets. The contribution shown in Table II is the mean of the two measurements.

Isolation of nepetalactones

Plant material (271 g) in the flowering stage was subjected to hydrodistillation for 3 h using a modified Clevenger-type apparatus to yield 2.26 g of oil. Separation of compounds was obtained by column chromatography (60 × 2.5 cm) on silica gel, eluting with *n*-pentane-diethyl ether (100:0 → 0:100 v/v) to give twelve fractions. Fraction 7 (515.6 mg) was subjected to normal phase HPLC chromatography with cyclohexane-ethyl acetate (98:2 v/v) as eluent, to afford compounds **1** (1.7 mg), **2** (7.6 mg) and **3** (6.1 mg) in pure form.

Biological evaluation

Ant toxicity

Bioassay I (ant body contact bioassay)

Quantities (0.1 and 1 mg) of the essential oils obtained from samples A and B, were applied on the bottom of glass petri dishes, which were aerated for 2 min, to allow evaporation of the solvent. Subsequently four *Pogonomyrmex* sp. ants were placed in every dish. Assays were run in quadruplicates. Control dishes were prepared in a similar way. Mortality of the ants was recorded after 6 and 12 h.

Bioassay II (ant feeding toxicity bioassay)

Quantities of the three nepetalactones (0.1 mg) were applied on 0.5 cm × 0.5 cm pieces of corn flakes. The food substrates were aerated for 2 min, to allow evaporation of the solvent. After evaporation, the corn flakes were placed in 12 petri dishes hosting 54 *Pogonomyrmex* sp. ants, in total. Control dishes were prepared in a similar way. Mortality was recorded after 12, 24, 48 and 72 h.

Bioassay III (ant body diffusion bioassay)

Quantities of the three nepetalactones (0.5 μg) dissolved in dichloromethane, were applied on the bodies of 38 *Pogonomyrmex* sp. ants. The ants were placed in petri dishes, while control assays were run with the solvent. Mortality was recorded after 12, 48, 96 and 144 h.

Mosquito repellency

Quantities of the sample A (0.1, 1 and 10 mg) were examined for their repellant activity on *Culex pipiens molestus* mosquitoes. The assay was performed according to the protocol described by Grannett (1940). The cages in all replications were hosting at least 800 female mosquitoes.

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