

# Composition and antimicrobial activity of the essential oil of *Clinopodium ascendens* (Jordan) Sampaio from Madeira

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Received 15 December 2005; Revised 26 March 2006; Accepted 6 April 2006

**ABSTRACT:** *Clinopodium ascendens* (Jordan) Sampaio [synonyms = *Calamintha ascendens* Jordan = *C. officinalis* Moench ssp. *ascendens* (Jord.) Mateo = *C. sylvatica* Bromf. ssp. *ascendens* (Jord.) P.W. Ball] is a small herbaceous plant with a very strong and distinctive mint aroma. The plant is known for its medical uses in folk medicine and as a spice in Italian kitchens. In Madeira Island, *Clinopodium ascendens*, known locally as ‘neveda’, grows wild along the shady paths of the Laurissilva forest. The local population uses the leaves of calamint as a mouth freshener and to alleviate headache and toothache. The essential oil obtained by hydrodistillation of the aerial parts of *C. ascendens* growing wild in Madeira was analysed by a combination of CC, GC, GC–MS, <sup>1</sup>H- and <sup>13</sup>C-NMR. The oil was dominated by C<sub>3</sub> oxygenated *p*-menthane derivatives: *cis*-isopulegone (75.2%), pulegone (6.9%), *neiso*-isopulegol (6.0%) and *trans*-isopulegone (4.5%). The whole essential oil was tested against a variety of bacteria, both Gram-positive and Gram-negative, and two fungi; it exhibited remarkable activity against *Escherichia coli* and was active against *Agrobacterium tumefaciens* and *Staphylococcus aureus* and the phytopathogenic fungus *Botrytis cinerea*. It was ineffective against *Streptococcus faecium*, *Streptococcus mutans* and *Candida albicans*. Copyright © 2006 John Wiley & Sons, Ltd.

**KEY WORDS:** *Clinopodium ascendens* (Jordan) Sampaio; essential oil; *cis*-isopulegone; *neiso*-isopulegol; antimicrobial activity; <sup>13</sup>C-NMR

## Introduction

The botanical name *Calamintha officinalis* Moench evidently refers to a plant for which a herbal medical use has been recognized. However, the actual identity of the plant in use is not certain, it being possible that early herbal practitioners did not distinguish between closely related taxa, as evidenced by the cross-application of common names between the three taxa: (a) *Calamintha nepeta* Savi, lesser calamint, mountain calamint, mountain balm; (b) *Calamintha sylvatica* Bromf. (syn. *Calamintha intermedia* Braun), wood calamint, woodland calamint; (c) *Calamintha sylvatica* Bromf. ssp. *ascendens* P.W. Ball, common calamint [syn. *Calamintha ascendens* Jordan, *Satureja ascendens* Maly, *Calamintha menthifolia* Host, *Clinopodium ascendens* (Jordan) Sampaio, and *Calamintha clinopodium*, *Melissa calamintha*]. Marin *et al.*<sup>1</sup> recall that the boundaries between the five closely

related genera *Calamintha* Miller, *Micromeria* Benth, *Satureja* L., *Clinopodium* L. and *Acinos* Miller are poorly defined and suggest the use of chemotaxonomic markers to differentiate them.

Stuart,<sup>2</sup> referring to *C. ascendens* Jordan as the common calamint, mountain calamint or mountain balm, notes that the leaves may be used as a poultice for bruises. However, Wren<sup>3</sup> refers to this use for a plant identified as *Calamintha officinalis* Moench for which the synonym *Calamintha menthifolia* Host and the names common calamint, mountain mint, wild basil and basil thyme were provided.

Common calamint was used as a poultice to ease the pains of sciatica. It was a great favourite with the old herbalists. Calamint is said to have psychic effects as well. It has been used to calm hysteria, cure melancholy, and bring gladness to the heart. The tea was used to strengthen the stomach and to help with gas and colic.<sup>4</sup> It is useful in jaundice, being a liver and spleen cleanser. Externally, it is used in poultices for bruises and its use has been recommended as an addition to warm baths, especially for children, as a strengthner and nerve soother. It is used in syrups for coughs and colds as an expectorant. The essential oil is used as a rubefacient, applied to the skin in sciatica and neuralgia. One drop of

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the oil on cotton wool, put into a decayed tooth, is said to alleviate the pain.

Only a few papers report on the chemical composition of the essential oil from aerial parts of *Calamintha sylvatica* ssp. *ascendens*, all of Spanish origin. Pulegone, isomenthone, cineole and menthol were the major components of the oil, which possesses antimicrobial activity<sup>5</sup> and exerts significant sedating and antipyretic activities in the rat.<sup>6</sup> Conversely, the second oil, which is fungistatic against most of the common moulds, was dominated by isomenthone, cineole and pulegone.<sup>7</sup>

Our attention was called to this plant precisely by the offer of a leaf to deal with a toothache during a field collection of other plants. In the present study, we used plants collected in the heart of Madeira natural park and identified by two independent taxonomists as *Clinopodium ascendens* (Jordan) Sampaio. The composition of this essential oil was determined and its antimicrobial properties established.

## Materials and Methods

### Plant Material, Extraction of the Essential Oil, Optical Rotation

Aerial parts of *Clinopodium ascendens* were collected in the full flowering period (July 2003) and during the vegetative phase (April 2004) in several locations of Madeira Island at the heart of the Natural Park: in Fajã da Nogueira in the shade of the Laurissilva forest, and in Santo da Serra on a sunny cliff of the south coast (flowering, only). Three voucher specimens, one for each collection, were deposited in the herbarium of Madeira Botanical Garden. The plants were dried at room temperature, away from direct sunlight, spread over plastic grids to allow ventilation.

The dried leaves and thin stalks were ground to a powder and submitted to hydrodistillation on a Clevenger-type apparatus for 3 h. The essential oils were collected by decantation, dried over sodium sulphate and weighed.

Optical rotation of the pure oil was measured using a Perkin-Elmer Model 241 polarimeter, at  $\lambda$  589 nm and 21.2 °C (room temperature) according to the international norm ISO 592 (1981).

### Chromatographic Fractionation of the Essential Oil

A sample of the oil (531 mg) was chromatographed on a silica gel column (200–500  $\mu$ m, 25 g) and seven fractions (F1–F7 = 8, 132, 184, 95, 79, 12 and 18 mg, respectively) were eluted with a gradient of solvents of increasing polarity (pentane:diethyl oxide 100:0 to 0:100). All fractions were submitted to GC–RI and <sup>13</sup>C-NMR analysis.

## Analytical GC

GC analysis was carried out using a Perkin-Elmer Auto-system apparatus equipped with FID and two fused-silica capillary columns (50 m  $\times$  0.22 mm i.d., film thickness 0.25  $\mu$ m), BP-1 (polydimethyl siloxane) and BP-20 (polyethylene glycol). The oven temperature was programmed from 60 °C to 220 °C at 2 °C/min and then held isothermal at 220 °C for 20 min; injector temperature, 250 °C; detector temperature, 250 °C; carrier gas, helium (1 ml/min); split, 1/60. The relative proportions of the essential oil constituents were expressed as percentages, obtained by peak area normalization. Retention indices (RI) were determined relative to the retention times of a series of *n*-alkanes with linear interpolation, using the Target Compounds software from Perkin-Elmer.

## GC–MS Analysis

Samples were analysed with a Perkin-Elmer TurboMass detector, directly coupled to a Perkin-Elmer Autosystem XL equipped with fused-silica capillary columns (60 m  $\times$  0.22 mm i.d., film thickness 0.25  $\mu$ m), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethyleneglycol). Ion source temperature, 150 °C; energy ionization, 70 eV; electron ionization mass spectra were acquired over the mass range 35–350 Da. Other GC conditions were the same as described for GC, except split, 1/80.

## <sup>13</sup>C-NMR Analysis

NMR spectra were recorded on a Bruker AVANCE 400 Fourier Transform spectrometer operating at 100.13 MHz for <sup>13</sup>C-NMR, equipped with a 5 mm probe, in deuteriochloroform, with all shifts referred to internal tetramethylsilane (TMS). <sup>13</sup>C-NMR spectra were recorded with the following parameters: pulse width, 4  $\mu$ s (flip angle 45°); acquisition time, 2.7 s for 128 K data table with a spectral width of 25 000 Hz (250 p.p.m.); CPD mode decoupling; digital resolution = 0.183 Hz/pt. The number of accumulated scans was 5000 for each sample (around 40 mg of the oil in 0.5 ml of CDCl<sub>3</sub>).

## Microorganisms

The micro-organisms used were: Gram-positive bacteria, *Staphylococcus aureus* CCM1 335, *Streptococcus faecium* CCM1 338 and *Streptococcus mutans* CCM1 1022; Gram-negative bacteria, *Agrobacterium tumefaciens* and *Escherichia coli* CCM1 270; fungi, *Botrytis cinerea* CCM1 899 and *Candida albicans* CCM1 209; culture media, Brain Heart Infusion Agar (BHIA) (Merck) for *S. mutans*, Nutrient Agar (Merck) for the other bacteria and

Malt Extract Agar (Merck) for *B. cinerea* and *C. albicans*.

The procedure was described by Hong-Xi and Song.<sup>8</sup> Solutions of the microorganisms under study at  $10^8$  cfu/ml were incorporated in molten suitable culture medium in Petri dishes. Filter paper discs were impregnated with 0.02 ml of the test compound and set on the agar. After 24–48 h incubation, the plates were observed for antimicrobial activity, indicated by the formation of a clear zone around the discs. The inhibition zone was measured in mm. Positive controls were used: rifampicin (Sigma) for bacteria, 5-fluorocytosine (Sigma) for *C. albicans* and Carbendazim<sup>®</sup> for *B. cinerea*. The *C. ascendens* was tested pure, 20 µl/disc. Rifampicin, 5-fluorocytosine and carbendazim were tested at 1 mg/ml.

### Identification of Components

Identification of the individual components was based on: (a) comparison of their GC retention indices (RI) on apolar and polar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation (Target Compounds software of Perkin-Elmer) with those of authentic compounds or literature data; (b) computer matching with a laboratory-made mass spectral library and commercial libraries,<sup>9,10</sup> and comparison of spectra with literature data;<sup>11–13</sup> (c) comparison of the signals in the <sup>13</sup>C-NMR spectra of all the fractions of chromatography with those of reference spectra compiled in the laboratory spectral library, with the help of a laboratory-made software.<sup>14–16</sup> The essential oil and the fractions of chromatography were analysed by <sup>13</sup>C-NMR spectroscopy, based on the pioneering work of Formacek and Kubeczka<sup>17</sup> and following a methodology developed and computerized in our laboratories.<sup>14–16</sup> This technique allows the direct and unequivocal identification of the main constituents of a mixture (up to 38 compounds) to a content as low as 0.5–0.3%. The computer program compared the chemical shift of each carbon of the compounds in the experimental spectrum with the spectra of pure components compiled in our spectral data library. The identification of all the compounds was carried out, taking into account: (a) the number of observed carbons with respect to the number of expected signals; (b) the number of overlapped signals of carbons which fortuitously possess the same chemical shift; (c) the difference of the chemical shift of the signals in the mixture spectrum from those of reference spectra compiled in the laboratory spectral library. <sup>13</sup>C-NMR is particularly suited for the identification of diastereoisomers. Indeed, the slightest structural modification of the skeleton of a given compound induces measurable chemical shift variation on the signal of most, if not all, carbons of that molecule.

### *cis*-Isopulegone 13

Computer matching against commercial MS libraries suggested the structure of isopulegone (*cis* or *trans* isomer) for compound 13. The structure of the molecule and the *cis* stereochemistry of the substituents were confirmed by NMR analysis of fraction F2, where compound 13 accounted for 87% of the fraction. Assignment of proton and carbon chemical shifts was ensured by recording 1D and 2D NMR spectra (<sup>1</sup>H, <sup>13</sup>C, DEPT, HSQC). <sup>1</sup>H-NMR, δ, p.p.m. = 4.96 (s), 4.79 (s), (H9, H9'); 2.98 (t, *J* = 6.4 Hz, H4), 2.39 (d, *J* = 9.3 Hz, H2), 2.15 (m, H1, H2, H5), 1.84 (m, H5, H6), 1.73 (s, H10), 1.61 (m, H6), 0.98 (d, *J* = 6.6 Hz, H7). <sup>13</sup>C-NMR, δ, p.p.m. = 211.79 (C3), 142.77 (C8), 112.58 (C9), 56.91 (C4), 48.20 (C2), 33.34 (C1), 30.12 (C6), 27.61 (C5), 21.65 (C10), 20.56 (C7).

### *trans*-Isopulegone 14

This was identified by comparison of its mass spectrum and <sup>13</sup>C-NMR data with those reported in the literature.<sup>18</sup>

### Component 16

MS suggested the structure of isopulegol. It was obviously a misidentification. Indeed, the <sup>13</sup>C-NMR data of that compound, which accounted for 71% in fraction F7, did not fit with either those of an authentic sample or with those of neo-isopulegol or iso-isopulegol, whose data are present in our <sup>13</sup>C-NMR library. The <sup>1</sup>H- and <sup>13</sup>C-NMR of fraction F7 allowed us to suspect the structure of the fourth isomer, *neoiso*-isopulegol. Thus, *neoiso*-isopulegol was prepared by LiAlH<sub>4</sub> reduction of *cis*-isopulegone (75% in the whole essential oil) and its structure was established using 1D and 2D NMR.

### Reduction of *cis*-isopulegone

A solution of *C. ascendens* essential oil (104 mg, *cis*-isopulegone 75%, *neoiso*-isopulegol, 6% by GC) in Et<sub>2</sub>O was added dropwise, at room temperature, to a suspension of LiAlH<sub>4</sub> (28 mg) in Et<sub>2</sub>O. The mixture was refluxed for 2 h. Then a solution of NaOH (15%, 2 ml) was added, the organic layer was separated, washed to neutrality and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure to yield 80 mg of an alcohol (79% pure by GC). This alcohol was submitted to NMR analysis without further purification and was identified as *neoiso*-isopulegol. <sup>1</sup>H-NMR, δ, p.p.m. = 4.97 (s), 4.86 (s) (H9, H9'), 3.93 (q, *J* = 4.0 Hz, H3), 2.17 (dt, *J* = 9.8 and 3.7 Hz, H4), 1.86 (td, H5 axial), 1.83 (m, H1), 1.79 (s, H10), 1.71 (m, H2 + H2'), 1.57 and 1.53 (2 m, H6, H6'), 1.36 (m, H5 equatorial), 1.11 (d, *J* = 7.1 Hz, H7). <sup>13</sup>C-NMR, δ, p.p.m. = 146.84 (s, C8), 112.16 (C9), 68.32 (C3), 48.04 (C4), 37.80 (C2), 31.12 (C6), 27.88 (C1), 23.48, (C10), 21.29 (C5 + C7).

## Results and Discussion

Aerial parts of *Clinopodium ascendens* produced, by hydrodistillation, an almost colourless essential oil with refractive index  $1475 \pm 0.002$  and density of  $0.903 \pm 0.06$ . The yields were 1.78% and 2.26% for dried plants harvested during the vegetative phase and the flowering period, respectively. Each determination is the average of three independent distillations. Optical rotation was  $-53.1 \pm 0.1^\circ$ . The three essential oils were analysed by GC-RI with identical results, so all the further work was performed using only the oil from one location (Fajã da Nogueira, flowering period) of which an amount of ca. 3 g was available. The essential oil was also submitted to GC-MS analysis on two columns of different polarities and to  $^{13}\text{C}$ -NMR analysis. The oil was chromatographed on  $\text{SiO}_2$  and the fractions of chromatography were analysed by GC (RI) and  $^{13}\text{C}$ -NMR. The composition of the essential oil is presented on Table 1. Twenty-three components were identified and they represented 97.4% of the oil. The bulk of this oil, 93.2%, is constituted by eight C-3 oxygenated *p*-menthane compounds. By far the main component of the essential oil is *cis*-isopulegone, accounting for more than 75% of the oil. The *trans* isomer of isopulegone and pulegone were detected in 4.5% and 6.9%, respectively. Three of the four isopulegol isomers (Figure 1) were identified in the oil: *neoiso*-isopulegol (6.0%), *neo*-isopulegol (0.2%) and *iso*-isopulegol (0.2%), while isopulegol itself was not detected.

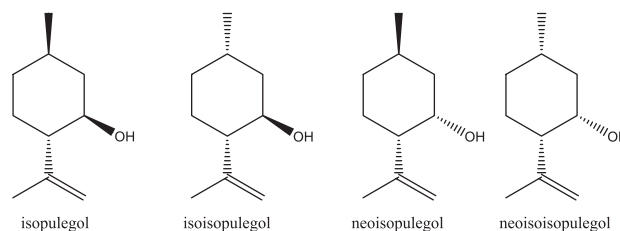


Figure 1. The four geometrical isomers of isopulegol

The four identified sesquiterpenes accounted for less than 1%. No significant variations were found for these oils with either location or stage of plant development.

The two isopulegone isomers have similar retention times on apolar or slightly polar columns. Indeed, both BP1 and DB5 GC columns proved to be inefficient for the separation of these two compounds, while they are well separated on a polar column (retention indices 1587 and 1592, respectively). As expected, they exhibit very similar mass spectra. Conversely, they are easily differentiated by  $^{13}\text{C}$ -NMR, taking into account the  $\gamma$  steric effect of the axial (or pseudo-axial) methyl C7 in *cis*-isopulegone. Up to now, the presence of the two *cis*- and *trans*-isopulegone isomers have been reported only in *Agathosma crenulata* (buchu)<sup>19</sup> essential oils in very small amounts and in relevant amounts in *Cyclotrichium oranifolium*.<sup>20</sup>

The *cis* isomer of isopulegone resulting from the biosynthetic pathway of (-)-limonene, found in several

Table 1. Composition of the essential oil of *Clinopodium ascendens* from Madeira

	Components	RI Apolar	RI Polar	(%)	Identification
1	$\alpha$ -Pinene	930	1026	0.2	RI, MS, $^{13}\text{C}$ -NMR
2	Sabinene	964	1120	tr	RI, MS
3	$\beta$ -Pinene	970	1110	0.2	RI, MS, $^{13}\text{C}$ -NMR
4	Myrcene	980	1164	0.2	RI, MS, $^{13}\text{C}$ -NMR
5	<i>p</i> -Cymene	1011	1274	0.1	RI, MS
6	Limonene	1022	1206	2.5	RI, MS, $^{13}\text{C}$ -NMR
7	( <i>Z</i> )- $\beta$ -Ocimene	1024	1235	0.1	RI, MS
8	Terpinolene	1080	—	tr	RI, MS
9	Linalool	1083	1554	0.1	RI, MS, $^{13}\text{C}$ -NMR
10	Menthone	1133	1467	0.1	RI, MS
11	<i>neo</i> -Isopulegol	1142	1568	0.2	RI, MS, $^{13}\text{C}$ -NMR
12	Borneol	1148	1707	tr	RI, MS, $^{13}\text{C}$ -NMR
13	<i>cis</i> -Isopulegone*	1159	1587	75.2	RI, MS, $^1\text{H}$ -NMR, $^{13}\text{C}$ -NMR
14	<i>trans</i> -Isopulegone*	1159	1592	4.5	RI, MS, $^{13}\text{C}$ -NMR
15	<i>iso</i> -Isopulegol*	1159	1632	0.2	RI, MS, $^{13}\text{C}$ -NMR
16	<i>neoiso</i> -Isopulegol	1166	1614	6.0	RI, MS, $^1\text{H}$ -NMR, $^{13}\text{C}$ -NMR
17	$\alpha$ -Terpineol	1173	1703	tr	RI, MS, $^{13}\text{C}$ -NMR
18	Pulegone	1216	1654	6.9	RI, MS, $^{13}\text{C}$ -NMR
19	Piperitone	1228	1736	tr	RI, MS, $^{13}\text{C}$ -NMR
20	( <i>E</i> )- $\beta$ -Caryophyllene	1420	1600	0.3	RI, MS, $^{13}\text{C}$ -NMR
21	Bicyclogermacrene	1493	1708	0.1	RI, MS, $^{13}\text{C}$ -NMR
22	Spathulenol	1564	2133	0.2	RI, MS, $^{13}\text{C}$ -NMR
23	Caryophyllene oxide	1571	1990	0.2	RI, MS
	Total (%)			97.4	

Order of elution and percentages are given on apolar column (BP-1), except for compounds with an asterisk (\*), percentage on BP-20. tr, traces.

**Table 2.** Antimicrobial activity of *C. ascendens* essential oil against bacteria and fungi

Microorganisms	Products			
	<i>Clinopodium</i>	Rifampicin	Carbendazim <sup>®</sup>	5-Fluorocytosine
<i>A. tumefasciens</i>	20	40	n.t.	n.t.
<i>E. coli</i>	22	15	n.t.	n.t.
<i>S. aureus</i>	15	35	n.t.	n.t.
<i>S. faecium</i>	—	20	n.t.	n.t.
<i>S. mutans</i>	—	45	n.t.	n.t.
<i>B. cinerea</i>	20	n.t.	30	n.t.
<i>C. albicans</i>	—	n.t.	n.t.	15

—, absence of activity; n.t., not tested.

species of mentha, is the dextrorotatory form 2*R*,5*R*.<sup>21,22</sup> The essential oil of *Clinopodium ascendens* analysed in the present work is laevorotatory, thus dominated by 2*S*,5*S*-(-)-*cis*-isopulegone.

The biological activity of the essential oil was tested over a variety of bacteria in order to validate the traditional use of this plant. The results obtained are presented in Table 2. *A. tumefasciens* is a phytopathogenic bacteria, *E. coli*, *S. aureus* and *S. faecium* are pathogenic in humans, whereas *S. mutans* is a human pathogen of the oral cavity. *C. albicans* is a human pathogenic yeast and *B. cinerea* is a widely distributed phytopathogenic. *C. ascendens* has inhibited the growth of the phytopathogenic *A. tumefasciens*, and the human pathogenics *E. coli* and *S. aureus*. The growth of the phytopathogenic *B. cinerea* was equally inhibited. It was inactive against *S. faecium*, *S. mutans* and *C. albicans*. The results obtained may be of interest for further investigation in the search for other types of bioactivity, such as the validation and quantification of the sedative action, using *in vivo* models. In fact, it is surprising that this plant, used traditionally against toothache, is ineffective against *S. mutans*, which can be explained by an analgesic rather than antimicrobial effect.

The composition of the essential oil of *Clinopodium ascendens* (syn = *Calamintha sylvatica* ssp. *ascendens*) of Madeira differs drastically from those of the samples of Spanish origin<sup>5,6</sup> which exhibited pulegone (54.5%), isomenthone (15.2%), cineole (12.6%) and menthol (10.2%), or isomenthone (36.8%), cineole (18.4%) and pulegone (16.6%) as main components.<sup>7</sup> It also differs from those of other species of calamint.

Indeed, two studies which have been carried out on the volatile constituents of *Calamintha sylvatica* Bromf. subsp. *sylvatica*<sup>23,24</sup> of southern European species, show major differences: the first concludes that pulegone is the major product (54.2%), whereas the second states that the major product is *cis*-piperitone oxide (66.6%, 69.2% and 74.6%, depending on the stage of plant evolution, pre-full blossom and post-blossom).

The essential oil of three populations of *Calamintha sylvatica* Bromf. (subspecies not mentioned), native to

the mountain region of southwestern Serbia<sup>25</sup> exhibited *cis*-piperitone oxide as major compound (48.9–59.2%).

A literature survey shows that *Calamintha* taxa produce almost exclusively monoterpenes bearing an oxygen function at C3. The only exception found was one study<sup>26</sup> on *Calamintha officinalis*, in which carvone, a C6 oxidized compound, was predominant (60%).

The essential oils from *Calamintha nepeta* ssp. *nepeta* or ssp. *glandulosa* (lesser calamint) are much better studied. According to Baldovini *et al.*,<sup>27</sup> it seems that the pattern of volatiles in *C. nepeta* varies to a large extent. In Corsica, the existence of three chemotypes was demonstrated. Some samples have pulegone (associated with a wide range of substances; menthone, isomenthone, menthol, piperitenone) or menthone, or carvone as the main components of their essential oils, others have piperitone oxide and piperitenone oxide. Flamini *et al.*<sup>28</sup> observed a wide antimicrobial spectrum of action for the essential oil of a *Calamintha nepeta* (*sensu lato*) from Italy, which composition reveals pulegone as the major component. Previously, Särer *et al.*<sup>29</sup> have found pulegone as major component in *Calamintha nepeta* ssp. *glandulosa*.

The compositions of the essential oil of other *Calamintha* species were studied. For instance, the essential oils of one population of *Calamintha vardarensis* from southern former Yugoslav Republic of Macedonia<sup>25</sup> contained *cis*-piperitone oxide as a major compound (65.6%).

Baser<sup>30</sup> studied five *Calamintha* taxa from Turkey, finding isopinocampone (49–56%) as the major component of the oil of *C. grandifolia* and piperitenone oxide (67%) dominating the composition of *C. incana*. *C. pamphylica* ssp. *pamphylica* presented pulegone (36%) associated with menthyl acetate (28%) and menthol (9%), whereas in *C. pamphylica* ssp. *davisii*, pulegone (38%) is associated with menthone (10%), menthyl acetate (9%) and menthol (9%). In that study, *C. nepeta* ssp. *glandulosa* had *trans*-piperitone oxide as the main component (25–58%).

In our survey, isopulegone was never found as a significant component of *Calamintha* essential oils. On the other hand, Baser *et al.*<sup>20,30</sup> found *cis*-isopulegone (4–52%) in the essential oil of *Cyclotrichium organifolium*,

an endemic taxon from Turkey. These are the largest amounts of *cis*-isopulegone reported in the literature. Relevant amounts of isopulegone (13%, no isomer referred) were found in *Tanacetum khorassanicum* (Krasch.) Parsa<sup>31</sup> and *Micromeria libanotica* Boiss.<sup>32</sup> (6.5%).

From the present findings, it seems that the calamint under study is an adapted species with an essential oil profile quite different from previously analysed counterparts, with isopulegone fulfilling the antibiotic role in the same way as pulegone does. If so, this could be an interesting replacement for pulegone-rich oils. Pulegone shows hepatotoxicity<sup>33</sup> that is higher for the *R*(+) than for the *S*(-) isomer, due to the extent of their metabolism and different metabolic profiles. When *cis*, *S,S*(-)-isopulegone was used as the substrate, only a very small amount of a single metabolite (menthofuran) was detected, the substance remaining essentially unmetabolized. WHO<sup>34</sup>, FEMA<sup>35</sup> and AFC<sup>36</sup> are unanimous in stating that isopulegone is significantly less hepatotoxic than pulegone.

*Acknowledgements*—We are indebted to Mr J. M. Desjobert for technical assistance and to Mrs F. Rocha for help with plant collection and taxonomic identification.

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