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

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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 Laurisilva 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, 1H- and 13C-NMR. The oil was dominated by C3 oxygenated p-menthane derivatives: cis-isopulegone (75.2%), pulegone (6.9%), neoiso-isopulegone (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; neoiso-isopulegol; antimicrobial activity; 13C-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.1 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,2 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, Wren3 refers to this use for a plant identified as *Calaminta officinalis* Moench for which the synonym *Calamintha officinalis* (Jord.) Sampaio is the common name. 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.4 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 strengthener 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
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 and exerts significant sedating and antipyretic activities in the rat. Conversely, the second oil, which is fungistatic against most of the common moulds, was dominated by isomenthone, cineole and pulegone.

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 λ 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 µm, 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 13C-NMR analysis.

**Analytical GC**

GC analysis was carried out using a Perkin-Elmer Autosystem apparatus equipped with FID and two fused-silica capillary columns (50 m × 0.22 mm i.d., film thickness 0.25 µm), BP-1 (polydimethylsiloxane) 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 × 0.22 mm i.d., film thickness 0.25 µm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). 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.

**13C-NMR Analysis**

NMR spectra were recorded on a Bruker AVANCE 400 Fourier Transform spectrometer operating at 100.13 MHz for 13C-NMR, equipped with a 5 mm probe, in deuterochloroform, with all shifts referred to internal tetramethylsilane (TMS). 13C-NMR spectra were recorded with the following parameters: pulse width, 4 µs (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 CDCl3).

**Microorganisms**

The micro-organisms used were: Gram-positive bacteria, *Staphylococcus aureus* CCMI 335, *Streptococcus faecium* CCMI 338 and *Streptococcus mutans* CCMI 1022; Gram-negative bacteria, *Agrobacterium tumefasciens* and *Escherichia coli* CCMI 270; fungi, *Botrytis cinerea* CCMI 899 and *Candida albicans* CCMI 209; culture media, Brain Heart Infusion Agar (BHLA) (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. Solutions of the microorganisms under study at 10⁵ 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® for *B. cinerea*. The *C. ascendentis* 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, and comparison of spectra with literature data; (c) comparison of the signals in the ¹³C-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. The essential oil and the fractions of chromatography were analysed by ¹³C-NMR spectroscopy, based on the pioneering work of Formacek and Kubeczka and following a methodology developed and computerized in our laboratories. 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. ¹³C-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 (*H*, ¹³C, DEPT, HSQC). ¹H-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). ¹³C-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 ¹³C-NMR data with those reported in the literature.

**Component 16**

MS suggested the structure of isopulegol. It was obviously a misidentification. Indeed, the ¹³C-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 ¹³C-NMR library. The *H*- and ¹³C-NMR of fraction F7 allowed us to suspect the structure of the fourth isomer, *neoiso*-isopulegol. Thus, *neoiso*-isopulegol was prepared by LiAlH₄ 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. ascendentis* essential oil (104 mg, *cis*-isopulegone 75%, *neoiso*-isopulegol, 6% by GC) in Et₂O was added dropwise, at room temperature, to a suspension of LiAlH₄ (28 mg) in Et₂O. 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 Na₂SO₄. 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. ¹H-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). ¹³C-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 ± 0.002 and density of 0.903 ± 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 ± 0.1°. 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 13C-NMR analysis. The oil was chromatographed on SiO 2 and the fractions of chromatography were analysed by GC (RI) and 13C-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 isopulegone 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](image-url)

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 13C-NMR, taking into account the γ 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) 19 essential oils in very small amounts and in relevant amounts in Cyclotrichium origanifolium. 20

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

| Components | RI Apolar | RI Polar | (%) | Identification |
|------------|-----------|----------|-----|----------------|
| 1 α-Pinene | 930       | 1026     | 0.2 | RI, MS, 13C-NMR |
| 2 Sabinehe | 964       | 1120     | tr  | RI, MS          |
| 3 β-Pinene | 970       | 1110     | 0.2 | RI, MS, 13C-NMR |
| 4 Myrcene  | 980       | 1164     | 0.2 | RI, MS, 13C-NMR |
| 5 p-Cymene | 1011      | 1274     | 0.1 | RI, MS          |
| 6 Limonene | 1022      | 1206     | 2.5 | RI, MS, 13C-NMR |
| 7 (Z)-β-Ocimene | 1024 | 1235     | 0.1 | RI, MS |
| 8 Terpinolene | 1080 | —        | tr  | RI, MS          |
| 9 Linalool  | 1083      | 1554     | 0.1 | RI, MS, 13C-NMR |
| 10 Menthone | 1133      | 1467     | 0.1 | RI, MS          |
| 11 neo-isopulegol | 1142 | 1568     | 0.2 | RI, MS, 13C-NMR |
| 12 Borneol  | 1148      | 1307     | tr  | RI, MS, 13C-NMR |
| 13 cis-isopulegone* | 1159 | 1587     | 75.2 | RI, MS, 1H-NMR, 13C-NMR |
| 14 trans-isopulegone* | 1159 | 1592     | 4.5 | RI, MS, 13C-NMR |
| 15 iso-isopulegol* | 1159 | 1632     | 0.2 | RI, MS, 13C-NMR |
| 16 neo-trans-isopulegol | 1166 | 1614     | 6.0 | RI, MS, 1H-NMR, 13C-NMR |
| 17 α-Terpinol | 1173  | 1703     | tr  | RI, MS, 13C-NMR |
| 18 Pulegone | 1216      | 1654     | 6.9 | RI, MS, 13C-NMR |
| 19 Piperitone | 1228 | 1736     | tr  | RI, MS, 13C-NMR |
| 20 (E)-β-Caryophyllene | 1420 | 1600     | 0.3 | RI, MS, 13C-NMR |
| 21 Bicyclomacrene | 1493 | 1708 | 0.1 | RI, MS, 13C-NMR |
| 22 Spathulenol | 1564 | 2133 | 0.2 | RI, MS, 13C-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 | Clinopodium | Rifampicin | Carbendazim\(^{a}\) | 5-Fluorocytosine |
|----------------|-------------|------------|---------------------|-----------------|
| A. tumefaciens  | 20          | 40         | n.t.                | n.t.            |
| E. coli        | 22          | 15         | n.t.                | n.t.            |
| S. aureus      | 15          | 35         | n.t.                | n.t.            |
| S. faecium     | —           | 20         | n.t.                | n.t.            |
| S. mutans      | —           | 45         | n.t.                | n.t.            |
| B. cinerea     | 20          | n.t.       | 30                  | n.t.            |
| C. albicans    | —           | n.t.       | 15                  |                 |

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

The essential oil of Clinopodium ascendens analysed in the present work is laevorotatory, thus dominated by 2S,5R\(^{21,22}\). The essential oil of Clinopodium ascendens 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\(^{26}\) 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.,\(^{27}\) 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; menthol, isomenthone, menthone, piperitenone) or menthone, or carvone as the main components of their essentials oils, others have piperitone oxide and piperitenone oxide. Flaminia et al.\(^{28}\) 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, Sarer et al.\(^{29}\) 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\(^{25}\) contained cis-piperitone oxide as a major compound (65.6%).

Baser\(^{30}\) studied five Calamintha taxa from Turkey, finding isopinocamphone (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.\(^{20,30}\) found cis-isopulegone (4–52%) in the essential oil of Cyclotrichium origanifolium,
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 and Micromeria libanotica Boiss. (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 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, FEMA and AFC are unanimous in stating that isopulegone is significantly less hepatotoxic than pulegone.

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