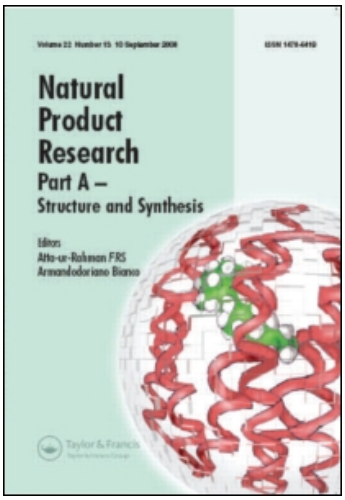


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# Composition and antifungal activity of the essential oil of Mentha cervina from Portugal

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# Composition and antifungal activity of the essential oil of *Mentha cervina* from Portugal

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The chemical composition of the essential oils obtained by hydrodistillation from the aerial parts of *Mentha cervina* collected during the flowering and vegetative phases of the plants were investigated by GC and GC-MS. Quantitative differences were observed in the compositions, particularly in the amounts of pulegone (12.9–79.6%) and isomenthone (8.7–77.0%). Antifungal activity of the oils was evaluated by minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) against *Candida*, *Apergillus* and dermatophyte strains. Antifungal activity of the sample containing lower amounts of pulegone was the highest for dermatophytes, particularly for *Epidermophyton floccosum* with MIC and MLC values of $0.63\,\mu L\,mL^{-1}$. *Mentha cervina* oils with low content of pulegone, may be an alternative as antifungal agents in dermatophytosis.

*Keywords:* Essential oils; *Mentha cervina*; Mint; Antifungal activity

## 1. Introduction

Mints (*Mentha* spp.) are aromatic plants with high industrial and commercial value, used in food industries, flavouring, perfumery and pharmaceutical preparations. Peppermint (*Mentha* × *piperita*) is one of the most popular herbs for use in teas and flavourings [1]. Some mints are used in folk medicine as an antispasmodic, choleretic, carminative and their essential oils are used as secretolytic/mucolytic and have been known to have antibacterial and antifungal proprieties since antiquity [1–4].

In Portugal there are about eight species of *Mentha* L. (Lamiaceae) with some hybrids. *Mentha cervina* L. [*Preslia cervina* (L.) Fresn.] is a subglabrous perennial herb, with scent of penny-royal, with stems procumbent and erect above; leaves sessile, linear-oblanceolate, entire or obscurely toothed, glabrous; flowers in verticillasters many-flowered; bracts leaflike and bracteoles digitately lobed; calyx 4-teethed, with the throat hairy within and corolla-tube straight, lilac or white [5].

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This species grows wild in stony places, on the banks of rivers, in septentrional regions of the Iberian Peninsula. In some localities it is regarded as a flavouring for fish dishes and it is usually used in the treatment of cutaneous infections.

The aim of the present work was to characterise the volatile oils isolated by hydrodistillation of aerial parts of *M. cervina* growing in the north of Portugal, as well as their antifungal activity. To our knowledge this is the first report on the composition of the oil of *M. cervina* by gas chromotography (GC) and gas chromotography-mass spectrometry (GC/MS) and on its antifungal activity against *Candida, Aspergillus* and dermatophyte strains.

## 2. Experimental

### 2.1. *Material and methods*

**2.1.1. Materials.** Wild plants were harvested in Monção, near the Minho river (North Portugal), maintained in plastic plots and propagated by herbaceous stems, in a greenhouse. In Spring, rooted cutting stems were planted in the field, located at Monte Redondo, Arcos de Valdevez (Northern region of Portugal), using black plastic to control infesting plants.

Aerial parts of *M. cervina* were collected during the flowering phase (August, sample 1) and during the vegetative phases of the plants (October-sample 2, December-sample 3 and February-sample 4). After harvesting the aerial parts were air-dried in the shade. *Mentha cervina* plants were authenticated by Dr Jorge Paiva, University. of Coimbra, voucher specimens were deposited at the Herbarium of the Instituto Botânico of the University of Coimbra (COI) with the numbers LS 320-323.

**2.1.2. Hydrodistillation.** The plants were submitted to a water distillation for 3 h in a Clevenger-type apparatus, in accordance with the European Pharmacopoeia method [6].

**2.1.3. GC-MS analysis.** Analysis of oils was carried out by GC and by GC/MS. Analytical GC was carried out using a Hewlett Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent Technologies, Part Number 5021-7148) was used for simultaneous sampling in two Supelco (Supelco Inc., Bellefont, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm, film thickness 0.20 μm), and SupelcoWax 10 (polyethyleneglycol 30 m × 0.20 mm, film thickness 0.20 μm). Oven temperature program: 70–220°C ($3°C\,min^{-1}$), 220°C (15 min); injector temperature: 250°C; carrier gas: helium, adjusted to a linear velocity of $30\,cm\,s^{-1}$; splitting ratio 1 : 40; detectors temperature: 250°C.

Gas chromatography-mass spectrometry analyses were carried out using a Hewlett Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm, film thickness 0.25 μm), interfaced with an Hewlett Packard

mass selective detector 5973 (Agilent Technologies, Palo Alto, CA, USA) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as above; interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 m; scans $sec^{-1}$: 4.51.

The identity of the components was assigned by comparison of their retention indices, relative to $C_8$–$C_{17}$ *n*-alkanes, and GC/MS spectra with corresponding data of components of reference oils and commercial available standards from a home-made library [7,8]. Relative amount of individual components was calculated based on GC peak areas without FID response factor correction.



**2.1.4. Antifungal activity.** Antifungal activity of sample 1 (75.1% pulegone and 8.7% isomenthone) and sample 4 (12.9% pulegone and 77.0% isomenthone) was evaluated against yeasts, *Aspergillus* and dermatophyte strains: two clinical *Candida* strains isolated from recurrent cases of vulvovaginal candidosis (*C. krusei* H9, *C. guillermondii* MAT23), three type strains from the American Type Culture Collection (*Candida albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. parapsilosis* ATCC 90018) and one type strain from the Colección Española de Cultivos Tipo (*Cryptococcus neoformans* CECT 1078); three dermatophyte clinical strains isolated from nails and skin (*Epidermophyton floccosum* FF9, *Trichophyton mentagrophytes* FF7, *Microsporum canis* FF1) and two type strains from the Colección Española de Cultivos Tipo (*Trichophyton rubrum* CECT 2794, *M. gypseum* CECT 2908); and one *Aspergillus* clinical strain isolated from bronchial secretions (*A. flavus* F44) and two type strains from the American Type Culture Collection (*Aspergillus niger* ATCC 16404, *A. fumigatus* ATCC 46645).

The fungal isolates were identified by standard microbiology methods and stored on Sabouraud broth with glycerol at −70°C. Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud agar to ensure optimal growth characteristics and purity.

A macrodilution broth method was used to determine the minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC), according to NCCLS references M27A [9] and M38P [10] for yeasts and filamentous fungi, respectively.

The serial doubling dilution of each oil were prepared in dimethyl sulfoxide (DMSO), with concentrations ranging from 0.16 to 20 μL $mL^{-1}$. Final concentration of DMSO never exceeded 2%. Recent cultures of each strain were used to prepare the cell suspension adjusted to $1–2 \times 10^3$ cells $mL^{-1}$ for yeasts and $1–2 \times 10^4$ cells $mL^{-1}$ for filamentous fungi. The concentration of cells was confirmed by viable count on Sabouraud agar. The test tubes were incubated aerobically at 35°C for 48 h (yeasts) and at 30°C for 3/7 days (filamentous fungi/dermatophytes) and MICs were determined. To evaluate MLC, aliquots (20 μL) of broth were taken from each negative tube after MIC reading, and cultured in Sabouraud dextrose agar plates. Plates were then incubated for 48 h at 37°C (yeasts) and 3/7 days at 30°C (filamentous fungi/dermatophytes). In addition, two reference antifungal compounds, amphotericin B and fluconazole (Pfizer), were used to control the sensitivity of tested microorganisms. All tests were performed in RPMI medium. For each strain tested, the growth conditions and the sterility of the medium were checked in two control tubes.

The innocuity of the DMSO was also checked at the highest tested concentration. All experiments were performed in triplicate and repeated if the results differed.

## 3. Results and discussion

The results concerning the qualitative and quantitative analysis of the volatile oils are presented in table 1, where the compounds are listed in order of their elution from SPB-1 column. In total 28 compounds were identified, accounting for 94.9–99.7% of the oils. The oxygenated monoterpenes were shown to be the main group of constituents in all samples. Nevertheless, some important quantitative differences were found, particularly in the amounts of pulegone (12.9–79.6%) and isomenthone (8.7–77.0%). Within the hydrocarbons, limonene was found to be the main constituent.

Evaluation of MIC and MLC of the two samples showed antifungal activity against all the fungi investigated (table 2). Antifungal activity of sample 4 (containing 77.0% of isomenthone and 8.7% of pulegone) was the highest for dermatophytes, particularly for *E. floccosum* FF9 with MIC and MLC values of $0.6\,\mu l\,mL^{-1}$. Although the MIC and MLC results varied among tested organisms, in most cases MIC was equivalent to the MLC, particularly for dermatophyte strains, indicating fungicidal activity of this oil.

Table 1. Constituents of four *M. cervina* essential oils: flowering phase (August, sample 1) and vegetative phases of the plants (October-sample 2, December-sample 3 and February-sample 4).

| RI (SPB-1) | RI (SuperW-10) | Compound | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| 923 | 1030 | α-Thujene | 0.1 | | | 0.1 |
| 930 | 1030 | α-Pinene | 0.5 | 0.5 | 0.4 | 0.3 |
| 962 | | Octan-3-one | 0.1 | 0.1 | 0.1 | 0.1 |
| 969 | 1127 | Sabinene | 0.1 | 0.1 | 0.1 | 0.1 |
| 970 | 1118 | β-Pinene | 0.4 | 0.5 | 0.4 | 0.3 |
| 981 | 1387 | 3-Octanol | 1.2 | 1.6 | 1.5 | 1.3 |
| 981 | 1164 | Myrcene | 0.5 | 0.5 | 0.4 | 0.2 |
| 998 | 1171 | α-Phellandrene | t | t | | t |
| 1020 | 1215 | 1.8-Cineole | 0.2 | 0.2 | 0.1 | 0.1 |
| 1020 | 1207 | Limonene | 4.3 | 3.2 | 1.2 | 0.8 |
| 1025 | 1235 | *Z*-β-Ocimene | 0.1 | 0.1 | | |
| 1035 | 1253 | *E*-β-Ocimene | 0.5 | 0.4 | 0.3 | 0.1 |
| 1077 | 1464 | *t*-Sabinene hydrate | | t | | |
| 1077 | 1288 | Terpinolene | t | | | |
| 1106 | 1555 | *cis*-*p*-Menth-2-en-1-ol | t | | | t |
| 1130 | 1461 | Menthone | 1.0 | 0.8 | 1.7 | 4.4 |
| 1139 | 1491 | Isomenthone | 8.7 | 9.5 | 33.3 | 77.0 |
| 1144 | 1483 | Menthofurane | | 0.1 | t | |
| 1147 | 1591 | *neo*-Menthol | | | | 0.3 |
| 1147 | 1580 | *cis*-Isopulegone | 0.7 | 1.3 | 0.8 | 0.4 |
| 1147 | 1570 | *trans*-Isopulegone | 0.5 | 0.6 | 0.2 | 0.1 |
| 1154 | 1635 | Menthol | | 0.1 | | |
| 1159 | 1599 | Terpinene-4-ol | t | t | t | |
| 1212 | 1640 | Pulegone | 75.1 | 79.6 | 58.3 | 12.9 |
| 1226 | 1731 | Piperitone | | t | 0.2 | 0.3 |
| 1303 | 1906 | Piperitenone | 0.3 | 0.1 | 0.2 | |
| 1410 | 1594 | *E*-Caryophyllene | 0.4 | 0.3 | 0.5 | 0.3 |
| 1558 | 1968 | Caryophyllene oxide | | | | 0.1 |

Note: Compounds listed in order to their elution on the SPB-1 column.
t = traces (<0.05%).

Table 2. Antifungal activity (MIC and MLC) of *M. cervina* oils: sample 1 (75.1% pulegone and 8.7% isomenthone) and sample 4 (12.9% pulegone and 77.0% isomenthone).

| | 1 | | 4 | | Fluconazole | | Amphotericin B | |
|---|---|---|---|---|---|---|---|---|
| Strains | MIC[a] | MLC[a] | MIC[a] | MLC[a] | MIC[b] | MLC[b] | MIC[b] | MLC[b] |
| *C. albicans* ATCC 10231 | 1.25 | 1.25–2.5 | 2.5 | 2.5 | 1 | >128 | N.T[(c)] | N.T |
| *C. tropicalis* ATCC 13803 | 1.25 | 1.25–2.5 | 2.5 | 2.5 | 4 | >128 | N.T | N.T |
| *C. krusei* H9 | 1.25 | 1.25–2.5 | 2.5 | 2.5 | 64 | 64–128 | N.T | N.T |
| *C. guillermondii* MAT23 | 1.25 | 1.25–2.5 | 2.5 | 2.5 | 8 | 8 | N.T | N.T |
| *C. parapsilosis* ATCC 90018 | 1.25–2.5 | 2.5 | 2.5 | 5 | <1 | <1 | N.T | N.T |
| *C. neoformans* CECT 1078 | 1.25 | 1.25 | 1.25 | 1.25 | 16 | 128 | N.T | N.T |
| *E. floccosum* FF9 | 1.25 | 1.25 | 0.64 | 0.64 | 16 | 16 | N.T | N.T |
| *T. rubrum* CECT 2794 | 1.25 | 1.25 | 1.25 | 1.25 | 16 | 64 | N.T | N.T |
| *T. mentagrophytes* FF7 | 1.25–2.5 | 1.25–2.5 | 2.5 | 2.5 | 16–32 | 32–64 | N.T | N.T |
| *M. canis* FF1 | 1.25 | 1.25 | 1.25 | 1.25 | 128 | 128 | N.T | N.T |
| *M. gypseum* CECT 2905 | 2.5 | 2.5 | 1.25 | 1.25 | 128 | >128 | N.T | N.T |
| *A. niger* ATCC16404 | 1.25 | 5 | 2.5 | >20 | N.T | N.T | 1-2 | 4 |
| *A. fumigatus*ATCC 46645 | 1.25 | 2.5 | 2.5 | 5 | N.T | N.T | 2 | 4 |
| *A. flavus* F44 | 2.5 | 2.5 | 5 | 5 | N.T | N.T | 2 | 8 |

Note: [a]MIC and MLC was determined by a macrodilution method and expressed in $\mu L\,mL^{-1}$ (V/V).
[b]MIC and MLC was determined by a macrodilution method and expressed in $\mu g\,mL^{-1}$(W/V).
[c]Not tested.
Results were obtained from three independent experiments performed in duplicate.



Pulegone is a terpenoid ketone, which is toxic to the liver because it is metabolised into epoxides. Essential oils rich in pulegone, like *M. pulegium* and *Calamintha nepeta* oils, should not be used in aromatherapy [11]. *Mentha cervina* oil with low level of pulegone, obtained in the vegetative phase of the plants, may be useful for therapeutic purposes without toxic risk, particularly in the dermatophytosis generated by *E. floccosum*.

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