COMPOSITION, ANTIFUNGAL ACTIVITY AND MAIN FUNGITOXIC COMPONENTS OF THE ESSENTIAL OIL OF MENTHA PIPERITA L.

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ABSTRACT

Twenty-one components (93.9% of the total chromatographic peak area) were tentatively identified in the essential oil (EO) of Mentha piperita L., based on Kováts retention indices (RIs), a mass spectral database (gas chromatography-mass spectrometry, GC-MS) and visual comparison of the mass spectra of the sample peaks with those of the database. The presence of 15 compounds (corresponding to 90.7% of the total chromatographic peak area) was confirmed by authentic standards. The EO presented a good activity against the following important postharvest deteriorating fungi: Aspergillus flavus, Aspergillus glaucus, Aspergillus niger, Aspergillus ochraceous, Colletotrichum gloeosporioides, Colletotrichum musae, Fusarium oxysporum and Fusarium semitectum. At a concentration of 0.2% of the EO, all the fungi were completely inhibited, except for A. glaucus and C. musae which were inhibited 90 and 98%, respectively. TLC-bioautography yielded three subfractions that prevented fungal growth, suggesting the presence of antifungals. Bioassay data of the crude EO were compared with those of the three subfractions. Based on these tests, it was concluded that several fungitoxics were responsible for the antifungal activity of M. piperita, with the principal ones being menthone, neomenthol, menthol and carvone. However, participation of other compounds cannot be ruled out. This is the first study in the literature that presents data on the activity of the crude EO against eight important postharvest deteriorating fungi, characterizing the amounts and types of compounds. In addition, also for the first time, the active fractions of the crude EO were isolated, identified and the components quantified. More detailed fungal tests are being conducted to confirm the tentative preliminary antifungal data.

PRACTICAL APPLICATIONS

Deterioration of agricultural products provoked by fungi accounts for considerable loss of crops of economic importance. Presently, these fungi are controlled by toxic synthetic chemicals. The food industry has significantly reduced the use of chemical preservatives. EOs have been known for their biological activities for many decades and they should, in principle, not be toxic to man and could replace toxic synthetic fungicides. This study demonstrates the potential of M. piperita EO as antifungal against C. gloeosporioides, C. musae, F. oxysporum, F. semitectum, A. niger, A. flavus and A. glaucus. Further studies are underway to evaluate M. piperita EO as a feed preservative. In addition, its principal active compounds were isolated and characterized. Identification of such components also helps to understand the mode of action of the extract, which can lead to the discovery of new antifungal compounds.
INTRODUCTION

Deterioration of agricultural products provoked by fungi accounts for considerable loss of crops of economic importance (Ehlers et al. 2006). Besides causing damage, some fungi produce mycotoxins, which are considered dangerous to human and animal health (Soković et al. 2009). This problem is aggravated in warm and humid regions, ideal for fungal growth and mycotoxin production (Ehlers et al. 2006).

The food industry has significantly reduced the use of chemical preservatives (Tassou et al. 2000) because of their adverse effects (Soković et al. 2009). Consumers and legal authorities have pressured the food industry to either completely remove these toxic compounds or use natural food preservatives (Tassou et al. 2000).

Biological activities of essential oils (EOs) have been known for many decades and are widely used as bactericidal, virucidal, fungicidal, antiparasitics, insecticidal, cosmetic, and pharmaceuticals (Dhingra et al. 2002; McKay and Blumberg 2006). Mentha piperita EOs are used in flavors, fragrances and pharmaceuticals (Işcan et al. 2002; McKay and Blumberg 2006). M. piperita EOs have several important properties, such as antifungal (Schuhmacher et al. 2003; Matan et al. 2009; Soković et al. 2009), antiviral (Schuhmacher et al. 2003), antibacterial (Işcan et al. 2002; Schuhmacher et al. 2003; McKay and Blumberg 2006; Bakkali et al. 2008; Rasooli et al. 2008; Vuuren et al. 2009), insecticidal (Samarasekera et al. 2008), larvicidal and mosquito repellent, and are considered nontoxic for humans (Ansari et al. 2000). M. piperita EO presents low mice toxicity, evidenced by high lethal dose (LD₅₀) value (2,000 mg/kg) (Debbab et al. 2007).

Identification of M. piperita EO components has been mostly based on Kováts retention indices (RIs) and a mass spectral database. However, such identification is tentative and must be confirmed by authentic standards. A very wide variation in the chemical composition (terpenes) of M. piperita has been reported (Ansari et al. 2000; Işcan et al. 2002; Dwivedi et al. 2004; Duarte et al. 2005; Debbab et al. 2007; Bakkali et al. 2008; Golebiowski et al. 2008; Rasooli et al. 2008; Matan et al. 2009; Soković et al. 2009).

Use of EO to protect agricultural products requires consistent performance, which can only be obtained if a preparation is standardized against an active fungitoxic component. Identification of active components also helps in understanding the mode of action of the extract, which can lead to the discovery of new antifungal compounds. However, major antifungals in M. piperita have not been isolated, characterized and quantified.

In this study we have: (1) tentatively identified the chemical composition of the crude M. piperita EO by RIs, a mass spectral database and by visual comparison of mass spectra of the samples peaks with that of the database. Tentative identifications were unequivocally confirmed by standards; (2) determined the activity of the crude M. piperita EO against the following eight major postharvest deteriorating fungi: Aspergillus flavus, Aspergillus glaucus, Aspergillus niger, Aspergillus ochraceus, Colletotrichum gloeosporioides, Colletotrichum musae, Fusarium oxysporum and Fusarium semitectum; and (3) located the fungitoxic fraction by TLC-bioautography. In addition, the chemical compositions of the three sub fractions were determined along with their antifungal activities.

MATERIALS AND METHODS

Plant Materials and Chemicals

M. piperita leaves (16 kg), lot MENI09/02 of Polish origin were purchased from Santos Flora Comércio de Ervas Ltda (São Paulo, SP, Brazil). All solvents were of analytical grade (Vetec Quimica Fina, Rio de Janeiro, RJ, Brazil) and were distilled before use. Analytical thin layer chromatography (TLC) analyses were conducted on precoated silica gel plates (5 × 10 cm, 0.1-mm thick, 60 GF254) while preparative TLC on precoated silica gel plates (20 × 20 cm, 1-mm thick, 60 GF254). TLC plates were purchased from Merck, Rio de Janeiro, RJ, Brazil. Disposable Petri dishes (60 × 15 mm and 90 × 15 mm) and potato dextrose agar (PDA) were obtained from Prolab, Rio de Janeiro, RJ, Brazil. Standard 1,8-Cineole, menthone, neomenthol, menthol, terpin-4-ol, isomenthol, methyl chavicol, pulegone, carvone, eugenol (E)-caryophyllene, α-terpineol and neomenthol acetate were purchased from Sigma (St. Louis, MO, USA), and piperitone from Pflatz & Bauer (Waterbury, CT, USA).

EO Extraction

EO was obtained by hydrodistillation of shade-dried M. piperita leaves (200 g) with 1.4 L of distilled water (1:7 w/v). The distillate (1 L) was extracted twice with dichloromethane (5:1 v/v). The combined organic phases were dried with anhydrous sodium sulfate and filtered. Dichloromethane was evaporated in a rotary evaporator at 30°C, under reduced pressure. The EO was weighed, stored at 4°C in sealed ampoules and used within a few days.

Identification of the Compounds in the Crude EO

Compounds in the crude EO were tentatively identified by RIs, a mass spectral database (Wiley/NBS Mass Spectral
Registry) using similarity indices (SIs) and by visual comparison of the mass spectra of the peaks in the crude EO with those of the database. Only compounds with SIs of 90% or higher, provided by the gas chromatograph–mass spectrometer were considered as positive identifications. Tentative identifications were based on the best SI and RI fits (Adams 2007). The presence of 15 compounds was confirmed by standards. Further confirmation was based on spiking the crude EO with individual standards.

RIs data were obtained on a gas chromatograph with a flame ionization detector (Shimadzu, Kyoto, Japan; model GC 17 A), an auto sampler and workstation. A mixture of linear saturated hydrocarbons and the crude EO were co-injected. The RIs were calculated from the following formula: 

$$RI = 100y + 100(z - y) \times R(\alpha) - R(\beta) - R(y)/R(z)$$

where $y$ and $z$ are the carbon numbers in the hydrocarbons eluting before and after a gas chromatography (GC) peak of interest, respectively; $R(\alpha)$ and $R(\beta)$ are the retention times of the hydrocarbons, eluting before and after a GC peak of interest. Fused silica capillary column (30 m × 0.25 mm; film thickness of 0.25 μm) coated with DB-5 stationary phase was purchased from Supelco (Bellefonte, PA, USA). The following conditions were used: oven temperature was programmed from 60°C (1 min hold) to 240°C (9 min hold) at 3°C/min; the injector and detector were maintained at 240 and 250°C, respectively. Nitrogen was the carrier gas at a flow rate of 1.3 mL/min.

Mass spectral data were obtained on a gas chromatograph–mass spectrometer (Shimadzu; model QP 5000 and software program-Class-5000, Version 1.2), fitted with an auto sampler, workstation and a database with around 350,000 entries. Conditions described for GC analysis were also used for GC-mass spectrometry (GC-MS) with the transfer line at entries. Conditions described for GC analysis were also used sampler, workstation and a database with around 350,000 ware program-Classs-5000, V ersion 1.2), fitted with an auto mass spectrometer (Shimadzu; model QP 5000 and soft-

Antifungal Activity

EO activity was tested on PDA, using the poison food assay (Dhingra and Sinclair 1995). The antifungal potential of the crude EO was evaluated at concentrations of 0.1 and 0.2% (v/v). The EO was dissolved in Tween 20 (0.1% v/v) and added to molten PDA (35°C) together with streptomycin sulphate. Ten milliliters of the medium was poured into 9-cm culture plates. The medium in each plate was spot-seeded with each of the test fungus conidia. The colony diameter was measured after 7 days of incubation at 25°C. All tests were carried out in triplicate. Inhibition percentage (IP) was calculated according to the equation: 

$$IP = 100 - \left[ \frac{(100 \times B/A)}{A} \right]$$

where $A$ and $B$ are the radial growth inhibitions of the control and the sample, respectively. Blanks were run in exactly the same manner without the EO.

Identification of the Fungitoxic Fraction of the EO, Determination of its Antifungal Activity and Chemical Composition

To identify the antifungal fraction of *M. pipertia*, the crude EO was fractionated by TLC-bioautography (Rahalison et al. 1991; Wedge and Nagle 2000). The crude EO (75 mg dissolved in 0.5 mL of dichloromethane) was coated horizontally as a narrow streak about 2 cm above the base of a preparative silica gel TLC plate (20 × 20 cm) using a disposable Pasteur pipette. The TLC plate was placed in a transparent glass chamber and eluted with dichloromethane: hexane (2:1 v/v) as the solvent. After elution, the plate was removed from the development chamber, placed on a clean metal tray and allowed to dry for several minutes. After evaporation of the solvent, molten PDA containing streptomycin sulfate and conidia (10⁴/mL) of *A. flavus* was sprayed over the plate and incubated for 7 days at 25°C in a moisture-saturated plastic box. The bands on the TLC were visualized under ultraviolet (UV) light or by spraying with a solution (about 5 mL) of vanillin and concentrated sulfuric acid (prepared by dissolving 15 g of vanillin in 250 mL of ethanol, followed by the addition of 2.5 mL of concentrated sulfuric acid). Visualized bands were arbitrarily designated as SF₁, SF₂ and SF₃ (lowest to highest retention factor [Rₙ]). The area on the TLC plate with no fungus development, considered to contain antifungals was delineated and the Rₙ along with the longitudinal width (Lw) were measured. A total of 63 plates were coated with the crude EO and worked up in exactly the same manner.
Appropriate bands from all the plates containing the fungitoxic fractions were scraped, pooled, extracted with two portions of dichloromethane (250 mL each), filtered, dehydrated with anhydrous sodium sulfate and filtered. The solvent was evaporated in a rotary evaporator at room temperature; the residues were weighed and stored at 4°C until use. For identification, the residues were redissolved in dichloromethane and analyzed by GC and GC-MS. The antifungal activity and the chemical composition of the fungitoxic fraction were determined as described for the crude EO. Blanks were run in exactly the same manner without the EO.

**RESULTS**

**EO Yield and Chemical Composition**

Hydrodistillation of the *M. piperita* dry leaves yielded 0.6% (w/w) of the EO. Twenty-one components (93.8% of the total chromatographic peak area) were tentatively identified in the crude EO, based on RIs, mass spectral database and by visual comparison of the mass spectra of the sample peaks with those of the database (Fig. 1, Table 1). Although 21 compounds were tentatively identified by RIs and a mass spectral database, the

FIG. 1. GAS CHROMATOGRAM OBTAINED ON THE ANALYSIS OF CRUDE ESSENTIAL OIL (A) AND PURIFIED SUBFRACTIONS SF1 (B), SF2 (C) AND SF3 (D) OF *MENTHA PIPERITA* L.

Chromatography conditions: oven temperature programmed from 60°C (1 min hold) to 240°C (9 min hold) at 3°C/min; N2 carrier gas (1.3 mL/min), temperatures of injector and a flame ionization detector 220 and 240°C, respectively, fused silica capillary column coated with a DB-5 stationary phase. Peaks identified in the chromatograms refer to the compounds listed and numbered in Table 1.
The presence of the following 15 compounds (corresponding to 90.7% of the total chromatographic peak area) was confirmed by authentic standards (elution order): 1,8-cineole, menthone, neomenthol, menthol, terpin-4-ol, isomenthol, \( \alpha \)-terpineol, methyl chavicol, pulegone, carvone, piperitone, neomenthyl acetate, menthyl acetate, eugenol and \((E\)-caryophyllene. Menthol, menthone, neomenthol, carvone and menthyl acetate corresponded to 76.9% of the peak area, while the remaining 19% of the peak area was made up by 16 constituents with relative concentrations less than 3.0%.

The crude EO inhibited the growth of all the eight test fungi at concentrations of 0.1 and 0.2% (Fig. 2A). At 0.1%, all fungi were moderately inhibited, ranging from 44.1% (\(A. \) niger) to 85.4% (\(F. \) oxysporum), except for \(C. \) gloeosporioides, where the inhibition was only 17.4%. The crude EO completely inhibited the radial growth of all fungi at a concentration of 0.2%, except \(A. \) glaucus and \(C. \) musae (90 and 98% inhibition, respectively).

Fractionation of the crude EO by TLC-biautobiography yielded three bands without fungal growth. \(R_f\) of the subfractions SF\(_1\), SF\(_2\) and SF\(_3\) was 0.47, 0.60 and 0.68, respectively. Lw of the subfractions SF\(_1\), SF\(_2\) and SF\(_3\) was 8.0, 5.0 and 1.0 cm, respectively. Bands SF\(_1\) and SF\(_2\) could be visualized under a UV lamp while the band SF\(_3\) only after spraying with a solution of sulphuric acid and vanillin. On GC analysis, the subfractions SF\(_1\), SF\(_2\) and SF\(_3\) presented one, two and three major components, respectively (Fig. 1, Table 1). In the subfractions SF\(_1\), SF\(_2\) and SF\(_3\), the principal components (%) were menthol (88.3), neomenthol (16.4) and menthone (15.0), neomenthol (24.4) and carvone (35.5), respectively.

All the eight fungi were sensitive to the active subfractions at the concentration of 0.1% (Fig. 2B). Subfraction SF\(_1\) was more active against \(A. \) flavus, \(A. \) niger, \(F. \) oxysporum, and \(F. \) semitectum, while SF\(_2\) and SF\(_3\) were more fungitoxic to \(A. \) glaucus, \(C. \) musae, \(F. \) oxysporum and \(F. \) semitectum.

**DISCUSSION**

**EO Yield**

Yield of \(M. \) piperita (0.60%) EO in this study was similar to that reported by other authors. Yields of 0.40, 0.50 and 0.70% of the peppermint EO were reported from India (Ansari et al. 2000; Dwivedi et al. 2004). Brazilian cultivars from Paraná yielded 0.15–0.67% in summer and 0.05–0.45% in winter (Deschamps et al. 2008), while the cultivars from São Paulo (Brazil) yielded 0.42 (Sartoratto et al. 2004) and 0.8% (Duarte et al. 2005). Plants grown in Morocco yielded 1.72% of EO (Debbab et al. 2007). Variation in EO

<table>
<thead>
<tr>
<th>Peak no (Fig. 1)</th>
<th>Retention index (RI)</th>
<th>Compound</th>
<th>Crude EO</th>
<th>SF(_1)</th>
<th>SF(_2)</th>
<th>SF(_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>978</td>
<td>Octen-3-ol</td>
<td>0.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>1,033</td>
<td>1,8-Cineole</td>
<td>2.6*</td>
<td>–</td>
<td>1.4</td>
<td>6.8</td>
</tr>
<tr>
<td>3</td>
<td>1,154</td>
<td>Menthone</td>
<td>7.3*</td>
<td>–</td>
<td>–</td>
<td>15.0</td>
</tr>
<tr>
<td>4</td>
<td>1,165</td>
<td>Neomenthol</td>
<td>6.3*</td>
<td>–</td>
<td>16.4</td>
<td>24.4</td>
</tr>
<tr>
<td>5</td>
<td>1,173</td>
<td>Menthol</td>
<td>54.2*</td>
<td>88.3</td>
<td>47.5</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>1,177</td>
<td>Terpin-4-ol</td>
<td>1.7*</td>
<td>–</td>
<td>5.7</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>1,182</td>
<td>Isomenthol</td>
<td>0.9*</td>
<td>–</td>
<td>1.9</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>1,188</td>
<td>(\alpha)-Terpineol</td>
<td>1.3*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>1,195</td>
<td>Methyl chavicol</td>
<td>0.7*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>Oct-7-en-1-yl acetate</td>
<td>1.1</td>
<td>–</td>
<td>2.5</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>1,237</td>
<td>Pulegone</td>
<td>1.8*</td>
<td>–</td>
<td>2.8</td>
<td>5.3</td>
</tr>
<tr>
<td>12</td>
<td>1,242</td>
<td>Carvone</td>
<td>5.0*</td>
<td>–</td>
<td>2.0</td>
<td>35.5</td>
</tr>
<tr>
<td>13</td>
<td>1,252</td>
<td>Piperitone</td>
<td>1.5*</td>
<td>–</td>
<td>5.5</td>
<td>1.8</td>
</tr>
<tr>
<td>14</td>
<td>1,275</td>
<td>Neomenthyl acetate</td>
<td>0.5*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>1,294</td>
<td>Menthyl acetate</td>
<td>4.0*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>1,306</td>
<td>Iso menthyl acetate</td>
<td>0.2</td>
<td>–</td>
<td>–</td>
<td>2.9</td>
</tr>
<tr>
<td>17</td>
<td>1,348</td>
<td>4-Methoxyacetophenone</td>
<td>0.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>1,356</td>
<td>Eugenol</td>
<td>0.4*</td>
<td>4.3</td>
<td>–</td>
<td>1.6</td>
</tr>
<tr>
<td>19</td>
<td>1,418</td>
<td>((E)-Caryophyllene</td>
<td>2.4*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>1,458</td>
<td>((E)-(\beta)-Farnesene</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>21</td>
<td>1,507</td>
<td>?</td>
<td>2.0</td>
<td>4.0</td>
<td>8.1</td>
<td>3.4</td>
</tr>
<tr>
<td>22</td>
<td>1,524</td>
<td>(\delta)-Cadinene</td>
<td>0.5</td>
<td>3.4</td>
<td>–</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Total identified</strong></td>
<td>93.9 (90.7*)</td>
<td>100</td>
<td>93.8</td>
<td>97.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Confirmed by authentic standards.
yield and its chemical composition was expected as they are affected by several factors, such as genotype, agroclimatic conditions, fertilization, plant organ, age and vegetative cycle stage (Gobbo-Neto and Lopes 2007; Bakkali et al. 2008).

**Chemical Composition of the EO**

Since the crude EO presented a complex chromatogram, identification of components based on RIs, mass spectral database and by visual comparison of the mass spectra of the sample peaks with those of the database would not be reliable. Hence, this identification was followed by confirmation by standards. The presence of 15 compounds (corresponding to 90.7% of the total chromatographic area) was confirmed by standards (Table 1, Fig. 1). It is interesting to note that all the compounds identified tentatively based on both the RI and the database were confirmed by standards. Despite this agreement, it must be pointed out that MS database identifications are considered tentative since they depend on several factors, such as chromatographic conditions, database used and the type of the mass spectrometer. In addition, RIs are known to vary slightly with chromatographic conditions, particularly the age of chromatographic column.

Most of the compounds identified in the *M. piperita* EO were monoterpens, the main components (in %) being menthol (54.2), followed by menthone (7.3), neomenthol (6.3), carvone (5.0) and menthyl acetate (4.0). The following components (%) have been identified in the *M. piperita* EO: menthol (33–60), menthone (15–32), isomenthone (2–8), 1,8-cineole (5–13), menthyl acetate (2–11), menthofuran (1–10), limonene (1–7), β-myrcene (0.1–1.7), β-caryophyllene (2–4), pulegone (0.5–1.6) and carvone (1) (McKay and Blumberg 2006). Accession of *M. piperita* from India presented menthol (29.8–37.3), p-menthone (27.1–33.0), isomenthone (4.4–8.5), pulegone (0.2–7.5), menthyl acetate (1.6–4.3), cineole (0.5–6.5) (Dwivedi et al. 2004). Menthol and menthone were major components in the *M. piperita* EO from Turkey, U.S.A., India, Germany, Sri Lanka, Argentina and Poland, presenting the following percentage composition: 27.5 and 27.9, 42.3 and 21.1, 34.6 and 18.4, 42.8 and 14.6, 41.2 and 24.3, 41.5 and 20.9, and 49.4 and 23.5, respectively (İşcan et al. 2002; Schuhmacher et al. 2003; Golebiowski et al. 2008; Samarakera et al. 2008; Palacios et al. 2009). Menthol was the major component of the *M. piperita* EO from Thailand (61%) and India (81.1%) (Ansari et al. 2000; Matan et al. 2009); while in EO from Serbia, the main components (%) were menthol (37.4), menthyl acetate (17.4) and menthone (12.7), respectively (Soković et al. 2009). However, in the Brazilian EO the percentage of linalool, 3-octanol and carvone was 51, 10.1 and 23.4%, respectively, while menthone, isomenthone, menthyl acetate, neomenthol and menthol were not detected (Duarte et al. 2005). In the peppermint EO from Portugal, menthol was not detected, with linalyl acetate (72%) and linalool (12.3%) being the main compounds (Martins et al. 2004), EO from Iran contained α-terpine (19.7%), isomenthone (10.3%), trans carveol (14.5%), pipertitinitone oxide (19.3%) and β-caryophyllene (7.6%) (Rasooli et al. 2008). The major compounds (%) characterized in *M. piperita* EO grown in Morocco were linalool (60.7), linalyl acetate (20.7), geraniol (3.2), 1,8-cineole (2.3) and limonene (1.5) (Debbab et al. 2005).
2007). However, the chemical composition reported in many literature studies have used only RI and a mass spectral database for identification of EO components (Işcan et al. 2002; Sridhar et al. 2003; Dwivedi et al. 2004; Sartoratto et al. 2004; Duarte et al. 2005; Soković et al. 2009). While these techniques are widely used and very convenient, they are not reliable, and the final identification must be confirmed by authentic standards.

Results on fractionation of *M. piperita* EO have not been reported and, hence, it will not be possible to compare our results with those of the literature. The chemical composition of the three subfractions was based on RIs, mass spectral database and by visual comparison of the mass spectra of the sample peaks with those of the database. In addition, identification was based on comparison of *t*<sub>s</sub> and *t*<sub>0</sub> of peaks in the three subfractions with those of the crude EO, where several peaks were confirmed with authentic standards.

**Antifungal Activity**

Crude *M. piperita* EO inhibited the growth of all the eight test fungi. The sensitivity of the test fungi to the *M. piperita* EO at a concentration of 0.1% differed significantly. The most susceptible fungus was *F. oxysporum* with 85% inhibition, and the least sensitive was *C. gloeosporioides*, with 17% inhibition. Percentage inhibition increased with the concentration. At a concentration of 0.1%, all the fungi were moderately inhibited, while at a concentration of 0.2%, the crude EO completely inhibited the radial growth of all fungi, except for *A. glaucus* and *C. musae,* which were, respectively, 90 and 98% inhibited.

The effectiveness of *M. piperita* EO as an antifungal agent against 17 micromycetial food poisoning, plant, animal and human pathogens was reported by Soković et al. (2009). *Mentha* sp. showed strong antifungal activity, higher than bifonazole (commercial fungicide used as a control) but lower than that of pure menthol. Carvone presented the best antifungal properties. At a concentration of 0.1%, the EO totally inhibited *A. flavus* in corn (Belmont and Carvajal 1998). *M. piperita* drastically reduced viable cells of *Staphylococcus aureus,* a foodborne bacteria, suggesting that the EO oil can be a promising preservative for some foods (Tassou et al. 2000). However, the chemical composition of this EO was not determined.

At 0.1%, antifungal activity of the subfractions depended on the fungus. For example, for the fungi *A. flavus,* *A. niger* and *C. gloeosporioides,* the activities increased as follows: SF<sub>1</sub> = SF<sub>3</sub> = SF<sub>2</sub>; for *A. glaucus,* the antifungal activities were essentially the same (Fig. 2B). The EO activity probably could be attributed to menthol, considering that, generally, their biological properties are determined by the major components (Bakkali et al. 2008). In addition, menthol was the main inhibitory component of *M. piperita* EO against the fungus *Trametes versicolor* (Matan et al. 2009). This observation is coherent with the fact *M. piperita* EO from Brazil, containing high amount of linalool (51%), but no menthol, had only a moderate activity (minimal inhibition concentration [MIC] 0.6 mg/mL) against *Candida albicans* (Duarte et al. 2005). Bioautography assay against *C. albicans* suggested that menthol was responsible for the antimicrobial activity of the EO obtained from commercial sources (Işcan et al. 2002). However, further tests must be conducted before attributing the activity of *M. piperita* EO to menthol.

The subfraction SF<sub>3</sub> was constituted mainly of menthol (47.5%) and neomenthol (16.4%), both antifungal compounds (Niridy 1998). However, menthol was not detected in the subfraction SF<sub>2</sub>, which had carvone, neomenthol and menthene as main constituents (35.5, 24.4 and 15.0%, respectively), compounds with an antifungal activity (Niridy 1998; Sridhar et al. 2003; Soković et al. 2009). According to Niridy (1998), menthol, neomenthol, menthyl acetate, menthone and linalool inhibited the mycelial growth of *C. gloeosporioides*.

Although the chemical identifications of the crude EO and the active fraction are unequivocal, the antifungal activity tests in this study are still preliminary. Other antifungal tests (broth, diffusion and disc), including EO effects on more fungal strains of the same genera/species and maximal effective concentration (EC50) with pure standards will be conducted. Such studies would be beyond the scope of this study but will be reported in other communications. Despite these limitations, this is the first study in the literature where the chemical composition of the Brazilian *M. piperita* EO was unequivocally determined simultaneously with antifungal activity and isolation/characterization of the active fraction. The information generated in this study will be useful for further studies in our laboratory, as well as to scientists worldwide.

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