Morphological alterations and time-kill studies of the essential oil from the leaves of *Coriandrum sativum* L. on *Candida albicans*

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Abstract: The objective of this study was to evaluate the morphological alterations and time-kill of the essential oil of the leaves of *C. sativum* L. on strains of *C. albicans*. The essential oil was submitted to gas chromatography-mass spectrometry analysis. The predominant component identified was linalool (39.78%). Minimal inhibitory concentration and minimal fungicidal concentration of the essential oil were respectively 512 and 1024 μg.mL⁻¹ for 90% of the strains tested. In the time-kill curves, the essential oil showed a concentration-dependent fungicidal effect. In the micromorphology assay it caused a significant reduction in pseudohyphae, an important pathogenic factor of *C. albicans*.

Keywords: Apiaceae, *Coriandrum sativum* L., linalool, time-kill, pseudohyphae.

Resumen: El objetivo de este estudio fue evaluar las alteraciones morfológicas y de letalidad del aceite esencial de las hojas de *C. sativum* L. en cepas de *C. albicans*. El aceite esencial se presentó a gas análisis de espectrometría de cromatografía-masa. El componente predominante identificado fue linalol (39.78%). Concentración inhibitoria mínima y concentración mínima fungicida del aceite esencial fueron, respectivamente, 512 y 1024 μg.mL⁻¹ para 90% de las cepas probadas. En las curvas el tiempo-matar, el aceite esencial mostró un efecto fungicida dependiente de la concentración. En el ensayo de micromorfología causó una reducción significativa en pseudohifas, un importante factor patógeno de *C. albicans*.

Palabras clave: Apiaceae, *Coriandrum sativum* L., linalol, el tiempo-matar, pseudohifas.
INTRODUCTION

The opportunistic pathogenic fungus *Candida albicans* is a common component of the human intestinal microbiota, but in immunocompromised individuals, it is responsible for a large variety of infections (White et al., 2010). Gastrointestinal candidiasis causes diarrhea, vomiting, irritation, anal itching and ulcerative lesions of the intestinal mucosa. It can also lead to episodes of vulvovaginal candidiasis through vaginal infection caused by contiguity with the digestive tract. Besides, after translocation to the bloodstream, it is responsible for serious cases of systemic candidiasis (Murzyn et al., 2010).

With the increase of vulnerable individuals (recipients of organ transplantation, patients treated with immunosuppressive agents, and patients with acquired immunodeficiency syndrome virus or other conditions of immunodeficiency), the incidence of opportunistic fungal infections has also increased (Pappas, 2010). Therapeutic limitations, the development of resistance to antifungal drugs, drug-related toxicity, significant drug interactions, or insufficient bioavailability of current antifungals (Silva et al., 2009) have prompted the continuous search for new more potent antifungal drugs, but mainly safer than existing ones (Fenner et al., 2006).

Since ancient times, aromatic spices and herbs have been utilized in the preparation of foods to improve flavor and their organoleptic properties. Currently, they have great potential in the food industry, because many phytochemical preparations derived from plants prevent the deterioration of foods and are efficacious against a wide gamut of microorganisms (Samojlik et al., 2010).

*Coriandrum sativum* Linn (Umbelliferae/Apiceae), popularly known as coriander, has been consumed by people for centuries without demonstrating any signs of toxicity (Matasyoh et al., 2009). In the food industry, coriander oil is used as a condiment, with approval for food use by the FDA (U. S. Food and Drug Administration), FEMA (Flavor and Extract Manufacturers’ Association) and European Council (Burdock & Carabin, 2009). Besides, the essential oils and various extracts of coriander have been demonstrated to possess various therapeutic properties, including antioxidant, hypolipidemic, hypoglycemic, anti-inflammatory, analgesic, sedative, anxiolytic, antimutagenic, anti-hypertensive, antimicrobial, diuretic and antispasmodic activities (Begnami et al., 2010; Duarte et al., 2016).

However, studies of antimicrobial activity of leaves of *C. sativum*, which is the plant part most utilized, against human clinical isolates are limited, mainly in relation to their effect on fungal micromorphology and the time-kill kinetics. Therefore, the objective of this study was to evaluate the morphological alterations and time-kill of the essential oil of leaves of *C. sativum* against strains of *C. albicans* isolated from human feces.

MATERIALS AND METHODS

**Essential oil**

The essential oil of *Coriandrum sativum* L., extracted from the leaves by steam distillation, was acquired from Ferquima Industria e Comercio Ltda. (Sao Paulo, Brazil). Emulsions of essential oil at different concentrations were prepared at the time of the assays. The essential oil was solubilized in 5% dimethylsulfoxide (DMSO) and 2% Tween 80. Next, sterile distilled water was added and the tubes mixed for 5 min using a Vortex (Fanem), to obtain the desired concentration.

**Standard antifungal**

Nystatin (Pharma Nostra, Brazil) was used as the standard antifungal.

**Microorganisms**

The microorganisms used in the tests for antifungal activity included two standard strains (ICB 12 and ATCC 76.485) and 10 strains of *C. albicans* isolated from human feces (LM 018, LM 497, LM 336, LM 420, LM 601, LM 138, LM 109, LM 188, LM 168 and LM 104). The microorganisms were isolated, identified and stored in the Laboratory of Mycology, Department of Pharmaceutical Sciences, Center of Health Sciences, Federal University of Paraiba, Brazil.

**Essential oil analysis**

For the analysis of the essential oil constituents of *C. sativum* was used a gas chromatography mass spectrometer coupled to GC17-A (GC-MS) Shimadzu operated by electron impact. The mobile phase consisted of helium and was pumped at a flow rate of 1.6 mL min⁻¹ at split 1:5. Chromatographic separation was performed using a DB-5 capillary column (30 m x 0.25 mm, 0.25 μm). The column oven temperature was programmed to move from an
initial temperature from 60°C to 105°C at 5°C min⁻¹, 105°C to 190°C at 10°C min⁻¹ and 280°C to 20°C min⁻¹. The temperature of injector and detector were 260 and 280°C, respectively. The total time was 22 minutes and the injection volume was 1.0 μL (Adams, 1995). The identification of the essential oil constituents was performed by the computer system and data processing (workstation) connected to the GC-MS. The system is equipped with a database of Wiley library, 6th edition of the class 5000-1999, with 229,119 spectra.

**Determination of minimal inhibitory concentration (MIC)**

MIC of the essential oil was determined by the microdilution technique in broth medium. Cultures of *C. albicans* were seeded in Sabouraud dextrose agar (Difco Lab., USA) and incubated at 35°C for 24 - 48 h. Colonies of this culture were suspended in sterile 0.85% NaCl and the inoculum was standardized at 0.5 tube of McFarland scale (1-5 x 10⁶ CFU mL⁻¹). Sabouraud dextrose broth (Difco Lab., USA) was added to all wells of 96-well plates. Next, serial dilutions were made to obtain concentrations varying between 4 and 1024 μg mL⁻¹. The same procedure was carried out with nystatin. DMSO (5%) and Tween 80 (2%), without drugs, serving as the positive control. Finally, 10 μL of yeast inoculum were added to all wells, and the plates were incubated at 35°C for 24 - 48 h. MIC was defined as the lowest concentration capable of visually inhibiting fungal growth seen in the wells (Souza et al., 2007).

**Determination of minimal fungicidal concentration (MFC)**

Aliquots of 20 μL of supernatant from each well of the microtiter plate with no visible fungal growth were transferred to wells of a new microtiter plate containing 100 μL of Sabouraud dextrose broth, devoid of any antifungal. The plates were incubated at 35°C for 24 - 48 h. MFC was defined as the lowest concentration of essential oil that caused total inhibition of visible growth (Ernst et al., 2002). Based on the MIC and MFC results, two representative strains were selected, a clinical strain (*C. albicans* LM 336) and a standard strain (*C. albicans* ATCC 76485), for the subsequent assays.

**Effect of essential oil on micromorphology of *C. albicans***

Possible alterations in the micromorphology of *C. albicans*, caused by the action of *C. sativum* essential oil was studied by microculture on glass slide in Petri dish (moist chamber) (Alves et al., 2013). Melted cornmeal agar-Tween 80 culture medium (HiMedia Lab., India) was added to sterile tubes: without oil or antifungal (control), containing essential oil at concentrations corresponding to the MIC and 2x MIC, and nystatin at a concentration corresponding to the MIC. After mixing, the culture medium was spread over the slide. Yeasts were seeded on the slides, and the plates were incubated at 35 °C for 24 - 48 h. The slides were examined with a light microscope at 400 x magnification to determine the formation or not of characteristic structures of *C. albicans* such as blastoconidia, pseudohyphae and chlamydoconidia.

**Time-kill**

The kinetic assays of microbial killing of *C. albicans* in presence of the essential oil were performed according to Klepser et al. (1998). One milliliter of fungal suspension (1-5 x 10⁶ CFU mL⁻¹) was added to 9 mL of Sabouraud broth with or without the essential oil at various appropriate concentrations (0.5, 1, 2 and 4 times the MIC). The standard antifungal (nystatin) was tested at MIC. These cultures were incubated at 35°C and at various time periods (0, 2, 4, 8, 12 and 24 h); an aliquot of 100 μL was removed from each solution and serially diluted in sterile distilled water. An aliquot of 10 μL of each dilution was removed and plated on Sabouraud dextrose agar. When a count of less than 1000 CFU.mL⁻¹ was expected, 10 μL samples were plated directly onto Sabouraud dextrose agar without dilution. The plates were incubated at 35°C for 24-48 h and CFU were counted. The experiment was performed in duplicate. The minimum detection limit of this method is 100 CFU.mL⁻¹. Plots of log₁₀ CFU.mL⁻¹ versus time were used to compare the rate and extent of antifungal activity at various concentrations of essential oil. Fungicidal activity was defined as a ≥3 log₁₀ (99.9%) decrease in CFU.mL⁻¹ from the initial inoculum. A lower activity was considered fungistatic (Ernst et al., 2002).

**Statistical analysis**

The results obtained in the experiments had their values expressed as mean ± standard of the mean (SEM) error and analyzed employing the Student’s t-test for analysis of two columns. Results were considered significant when p < 0.05. For data analysis, we used the statistical program GraphPad Prism® version 5.0.
RESULTS

The essential oil of C. sativum, obtained by steam distillation, was analyzed by gas chromatography-mass spectrometry (GC-MS). The constituents in the essential oil are summarized in Table 1. The predominant component identified was linalool (39.78%).

The MIC values of C. sativum essential oil in strains of C. albicans obtained in this work varied from 128 μg.mL⁻¹ (LM 018) to 1024 μg.mL⁻¹ (LM 104) and the MFC values of C. sativum essential oil varied between 128 and 1024 μg.mL⁻¹ (Table 2).

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time (min)</th>
<th>Compounds</th>
<th>Kovats retention index</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.547</td>
<td>α-pinene</td>
<td>933</td>
<td>4.95</td>
</tr>
<tr>
<td>2</td>
<td>3.756</td>
<td>camphene</td>
<td>953</td>
<td>1.49</td>
</tr>
<tr>
<td>3</td>
<td>4.233</td>
<td>β-pinene</td>
<td>980</td>
<td>1.38</td>
</tr>
<tr>
<td>4</td>
<td>5.204</td>
<td>p-cymene</td>
<td>1026</td>
<td>17.62</td>
</tr>
<tr>
<td>5</td>
<td>6.192</td>
<td>linalool oxide</td>
<td>1074</td>
<td>27.33</td>
</tr>
<tr>
<td>6</td>
<td>7.042</td>
<td>linalool</td>
<td>1098</td>
<td>39.78</td>
</tr>
<tr>
<td>7</td>
<td>7.546</td>
<td>camphor</td>
<td>1143</td>
<td>7.45</td>
</tr>
</tbody>
</table>

Table 2

MIC and MFC of essential oil of Coriandrum sativum on 10 strains of C. albicans isolated from feces and 2 standard strains.

<table>
<thead>
<tr>
<th>C. albicans</th>
<th>Essential oil (μg.mL⁻¹)</th>
<th>Nystatin (μg.mL⁻¹)</th>
<th>Control strains⁹</th>
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<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
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<tr>
<td>LM 018</td>
<td>128</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>LM 497</td>
<td>512</td>
<td>512</td>
<td>16</td>
</tr>
<tr>
<td>LM 336</td>
<td>512</td>
<td>512</td>
<td>16</td>
</tr>
<tr>
<td>LM 420</td>
<td>512</td>
<td>512</td>
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<tr>
<td>LM 601</td>
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<td>512</td>
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<td>LM 138</td>
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<td>LM 109</td>
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<td>LM 188</td>
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<td>512</td>
<td>16</td>
</tr>
<tr>
<td>LM 168</td>
<td>512</td>
<td>512</td>
<td>16</td>
</tr>
<tr>
<td>LM 104</td>
<td>1024</td>
<td>1024</td>
<td>16</td>
</tr>
<tr>
<td>ICB 12</td>
<td>256</td>
<td>256</td>
<td>16</td>
</tr>
<tr>
<td>ATCC 76485</td>
<td>512</td>
<td>1024</td>
<td>16</td>
</tr>
</tbody>
</table>

⁹ Growth of the microorganism in Sabouraud dextrose broth, 5% DMSO and 2% Tween 80, without addition of essential oil or antifungal.
The results of the effect of *C. sativum* essential oil on the micromorphology of *C. albicans* LM 336 and *C. albicans* ATCC 76485 are shown in figures 1 and 2, respectively. As can be seen in figures 1A and 2A, the assays of the yeast control (without drugs) in the two strains tested showed normal fungal growth, with the formation of all the morphological structures of *C. albicans*: blastoconidia, pseudohyphae and chlamydoconidia. These data confirmed the cellular viability of the samples and their normal capacity of morphogenesis.

**Figure 1**

*Effect of essential oil of Coriandrum sativum L. on the micromorphology of Candida albicans LM 336: A) yeast control; B) in the presence of *C. sativum* essential oil at MIC; C) in the presence of *C. sativum* essential oil at 2x MIC; D) in the presence of nystatin at MIC. Bar: 100 μm (400x)*

**Figure 2**

*Effect of essential oil of Coriandrum sativum L. on the micromorphology of Candida albicans ATCC 76485: A) yeast control; B) in the presence of *C. sativum* essential oil at MIC; C) in the presence of *C. sativum* essential oil at 2x MIC; D) in the presence of nystatin at MIC. Bar: 100 μm (400x)*
The results of time-kill were expressed as curves of $\log_{10}$ CFU mL$^{-1}$ of *C. albicans* LM 336 (Figure 3A) and *C. albicans* ATCC 76485 (Figure 3B) versus time.

**Figure 3**

Time-kill curves of *Candida albicans* LM 336 (A) and *Candida albicans* ATCC 76485 (B), exposed to *Coriandrum sativum* L. essential oil at different concentrations. Growth control (●), 0.5 x MIC (▲), 1 x MIC (▼)*, 2 x MIC (■)* and nystatin control (♦)*. *p<0.05 test versus growth control.

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**DISCUSSION**

The essential oil of *C. sativum*, obtained by steam distillation, was analyzed by gas chromatography-mass spectrometry (GC-MS). The predominant component identified was linalool (39.78%), followed by linalool oxide (27.33%) and p-cimene (17.62%).

These results differ from those found by other authors. Bhuiyan et al. (2009) found the major constituents of essential oil from leaves of *C. sativum* to be aromatic acids, such as 2-decenoic acid (30.8%), E-11-tetradecenoic acid (13.4%) and capric acid (12.7%). In a study carried out by Begnami et al. (2010), the principal constituents identified were the alcohols 1-decanol (24.17%), (E)-2-decenol (18.05%), 2(Z)-dodecenol (17.55%) and 3-hexenol (10.34%). Other authors found the major components to be the aldehydes decanal (Fan & Sokorai, 2002), (E)-2-decenal (7), and (E)-2-dodecenal (Msaada et al., 2007). This difference can be explained by the change in place to get the plant and the subsequent extraction of the oil, which can be altered climate and altitude of vegetable cultivation location.

Delaquis et al. (2002) also identified linalool as the major component of essential oil from the leaves of *C. sativum*, while other authors identified this component as the major one in the essential oil of fruits (Grosso et al., 2008; Zoubiri & Baaliouamer, 2010; Soares et al., 2012).

Bandoni et al. (1998) when analyzing the essential oils obtained from fruits of coriander growing in six different zones of Argentina observe that twenty components were identified which accounted for 96.6 - 99.7% of the total oils composition. The main constituents were linalool (68.9 – 83.7%), γ-terpinene (2.2 - 5.1%), camphor (3.2 - 4.8%), α-pinene (1.0 - 6.5%), geraniol (1.4 - 3.2%) and geranyl acetate (0.8 - 3.8%). The contents of cis- and trans-linalool oxide (0.1 - 0.4%) were low. In this study, the high percentage of linalool oxide may have arisen by a degradation of the majority of the oil compound, linalool.

An analysis of the MIC values of *C. sativum* essential oil in strains of *C. albicans* shows that 11/12 (91.6%) of the strains submitted to biological assays
had their growth inhibition up to a concentration of 512 μg.mL⁻¹ of *C. sativum* essential oil. The MIC₅₀ (MIC for 50% of the strains tested), as well as the MIC₉₀ (MIC for 90% of strains tested) was 512 μg.mL⁻¹.

The results found in our study corroborate those obtained by Begnami et al. (2010), who found a MIC of 500 μg.mL⁻¹ for *C. albicans*. However, our findings showed greater antimicrobial potential, when compared to those of Matasyoh et al. (2009), who reported a MIC of 163 mg mL⁻¹ for *C. albicans*.

When comparing the activity of natural products with that of standard antimicrobials, there is still no consensus on the level of acceptable inhibition. Aligiannis et al. (2001) proposed a classification of antimicrobial potential for plant products based on MIC results. In accordance with this classification, *C. sativum* essential oil, with a MIC₉₀ of 512 μg.mL⁻¹, showed strong antimicrobial activity against the strains of *C. albicans* tested.

The yeast grew in Sabouraud dextrose broth, 5% DMSO and 2% Tween 80, without drugs, demonstrating the viability of the fungal strains utilized and confirming that the impedance of their growth was truly the consequence of the presence of the essential oil or antifungal.

Analysis of the MFC values of *C. sativum* essential oil in strains of *C. albicans* showed that 10/12 (83.3%) of the strains submitted to the assays had MFC values up to 512 μg.mL⁻¹ and that 100% of strains tested had MFC values up to 1024 μg.mL⁻¹. MFC₅₀ (MFC for 50% of the strains tested) was 512 μg.mL⁻¹, while the MFC₉₀ (MFC for 90% of the strains tested) was 1024 μg.mL⁻¹.

In the presence of *C. sativum* essential oil at MIC (Figure 1B and 2B) and 2x MIC (Figure 1C and 2C), there was a significant reduction in pseudohyphae, an important pathogenic factor of *C. albicans*. The morphological alterations induced by the essential oil were similar to those induced by the standard antifungal, nystatin (Figures 1D and 2D). Blastocandidiasis were observed in all tests, yeast control, presence of essential oil and standard antifungal (Figures 1 and 2), which are the commensal form of the yeast, and thus, it is not of interest whether these are affected since *C. albicans* makes up part of the normal microbiota of humans.

Pseudohyphae are an important pathogenic factor of *C. albicans*. Studies have been demonstrated that mutants of *C. albicans* that do not produce hyphae are incapable of causing invasive candidiasis in mice. Strains of *Candida albicans* in the form of yeast are less virulent and more sensitive to the phagocytic activity of macrophages (Murzyn et al., 2010).

For strain *C. albicans* LM 336, coriander essential oil had a fungicidal effect (≥ 3 log₁₀ decrease in CFU.mL⁻¹ relative to the initial inoculum) from the MIC at 8 h, and as the concentration was increased, this fungicidal effect was seen earlier at 2x MIC starting at 4 h and at 4x MIC as of 2 h. MIC of nystatin, the standard antifungal used as control, showed fungicidal effects as of 2 h. In the strain *C. albicans* ATCC 76485 (Figure 3B), *C. sativum* essential oil showed a fungistatic effect at MIC, and a fungicidal effect at 2x MIC as of 4 h, and at 4x MIC as of 2 h.

In the strain *C. albicans* ATCC 76485 (Figure 3B), nystatin at MIC did not show a fungicidal effect, but only a fungistatic effect. In a study by Gunderson et al. (2000), it was demonstrated that the fungicidal activity of nystatin is concentration-dependent. In their study, fungistatic activity was generally observed between 0.5 and 2 times MIC, and rapid fungicidal activity was observed with concentrations equal to or greater than 2x MIC. Therefore, it is possible that for this ATCC strain used in this study, nystatin presents a fungicidal effect only starting at 2x MIC.

The time-kill curves (Figure 3) show that at concentrations lower than MIC, *C. sativum* essential oil had fungistatic activity and at concentrations equal to or greater than 2x MIC fungicidal activity. Therefore it suggests that the essential oil has fungicidal activity that is concentration-dependent, like nystatin. The essential oil differs of fluconazole, for example, whose effect is not concentration-dependent, since an increase in concentration does not cause a significant increase in activity (Klepser et al., 1997). We also observed that the greater the concentration of *C. sativum* essential oil, the less time was necessary for fungicidal activity.

Clinically, the differences in the dynamics of the antifungal can influence the selection of the ideal dose regimes for these agents. Agents whose degree and range of antifungal activity increase with increase in concentration (for example, amphoterocin B) can be optimized by the administration of relatively high doses. In contrast, the antifungal activity of agents such as fluconazole is not significantly reinforced by a higher concentration than the MIC (Ernst et al., 1996).
For the two strains, the time-kill results were compatible with those for MFC of C. sativum essential oil. The clinical strain C. albicans LM 336, in the assay for determination of MFC, showed an MFC equal to the MIC (Table 2), as in the microbial kill assay, which showed a fungicidal effect at the MIC (Figure 3A). The standard strain ATCC 76485, in the assay for determination of MFC, showed a two times greater MFC compared to MIC (Table 2), as in the microbial kill assay, which showed a fungicidal effect starting at 2x MIC (Figure 3B).

Fungicidal effect of C. sativum essential oil was noted in the kinetic assays of microbial kill of C. albicans strains. Fungicidal activity is clinically more important than fungistatic activity, particularly in HIV patients, because the prophylactic use of fungistatic drugs has been associated with an increase in the frequency of innate or acquired resistance in clinical isolates (Monk & Goffeau, 2008). Flow cytometric evaluation indicates that the fungicidal effect is a result of cytoplasmic membrane damage and subsequent leakage of intracellular components such as DNA (Silva et al., 2009).

CONCLUSION

Thus, the essential oil of C. sativum represents a natural product with potential antifungal activity against C. albicans. In the time-kill curves, the essential oil showed a concentration-dependent fungicidal effect. In the micromorphology assay it caused a significant reduction in pseudohyphae, an important pathogenic factor of C. albicans.

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