A novel nerolidol-rich essential oil from *Piper clausseenianum* modulates *Candida albicans* biofilm


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Candidiasis is a major opportunistic fungal infection in humans, and its incidence has increased steadily over the last two decades. *Candida albicans*, the main species of the genus, has a large arsenal of virulence attributes that contribute to successful infections, such as dimorphism and biofilm formation. The adverse effects of eukaryotic antimicrobial therapies associated with an increase in resistance to the compounds presently available have boosted efforts to improve the therapeutic arsenal against candidiasis with a newer and cheaper range of drugs. In this study, a novel nerolidol-rich essential oil (EO) derived from *Piper clausseenianum* (Miq.) C. DC., Piperaceae, was tested on the growth, transition (yeast to hyphae), formation and stability of biofilms produced by *C. albicans*. Both inflorescence and leaf EOs were evaluated and revealed MIC values ranging from 0.04 to 0.1 % and 0.2 to 1.26 %, respectively. Furthermore, leaf EO managed to downregulate the yeast-to-hyphae transition by 81 %, as well as reducing biofilm formation by about 30 and 50 % after incubation for 24 and 48 h, respectively. The EO was also able to reduce the viability of pre-formed biofilm by 63.9 %. Finally, the association between the leaf EO and fluconazole was evaluated and revealed an interesting synergistic effect. Taken together, these results demonstrate that this novel compound could be a promising agent and could reinforce the arsenal of therapeutic alternatives for the treatment of candidiasis. Furthermore, it may represent a novel and natural source of nerolidol, which could be of interest pharmaceutically.

INTRODUCTION

Candidiasis represents the major opportunistic fungal infection in humans and is often associated with the patient’s clinical condition. This infection has a wide range of severe implications that may be explained by virulence factors intrinsic to the micro-organism. These factors are responsible for a successful colonization or fungal infection, and in particular, *Candida albicans* has developed an extraordinary set of strategies for such purposes, including biofilm formation and morphological conversion or dimorphism (Odds, 1994). The ability of the cell to differentiate from the yeast form to hyphal morphology is considered essential for pathogenicity and mandatory for establishment of the disease (Kumamoto & Vinces, 2005). This process occurs in an autoregulatory mechanism called quorum sensing, commanded by molecular signalling between yeast and hyphae. This mechanism is notoriously mediated by autoregulatory molecules, such as the aromatic alcohols tryptophol, tyrosol and phenylethyl alcohol, dodecanol, farnesoic acid, HTDE [3(R)-hydroxy-tetradecanoic acid], MARS (morphogenic autoregulatory substance), *trans*,*trans*-farnesol and *trans*-nerolidol (Martins et al., 2007; Han et al., 2013), and these two latter compounds are known to downregulate biofilm formation by *C. albicans*, which has been determined as a pivotal virulence factor (Baillie & Douglas, 2000). Biofilm is clinically very relevant, as this structure is able to endure a variety of environmental assaults, including resistance to conventional antifungal drugs (Baillie & Douglas, 2000; Uppuluri *et al.*, 2010).

The well-known side effects of conventional antifungal therapy and the spread of antimicrobial resistance to compounds that have been on the market for a long time have encouraged the search for unconventional drugs able to overcome or control fungal infections. Essential oils (EOs) and natural extracts have been studied intensely and have been proven to have significant antimicrobial
properties (Alviano et al., 2005). Recently, the EOs extracted from the leaves and inflorescences of *Piper claussenianum* have revealed a remarkably high concentration of terpenes such as trans-nerolidol and linalool (Marques et al., 2010).

As sesquiterpenes have been associated with quorum sensing processes in fungi, and the monoterpen linalool has been reported as having intense antifungal activity (Alviano et al., 2005; Martins et al., 2007; Marques et al., 2010), we hypothesized that the *P. claussenianum* EOs might have antifungal potential against *C. albicans*. Therefore, the aim of the present study was to measure the influence of these EOs on *C. albicans* growth and resistance to fluconazole, its yeast-to-hyphae transition and its biofilm development.

**METHODS**

EOs extracted from the leaves and inflorescences of *P. claussenianum* were obtained at the Natural Products Research Centre of the University of Rio de Janeiro, Brazil, through hydrodistillation. The oils were chemically analysed and the identification of their constituents was performed by gas chromatography/flame ionization detection, gas chromatography/mass spectrometry and headspace/solid phase microextraction. The major component in the EO from the leaves was the sesquiterpene trans-nerolidol (81.4 %), and its structure was confirmed by the 1H and 13C nuclear magnetic resonance spectra of the crude EO from the leaves. The main constituent of the EO from inflorescence was identified as the monoterpen linalool (50.2 %) (Marques et al., 2010).

The viability assays were assessed on four *C. albicans* strains: two clinical strains isolated from the oral mucosa of human immunodeficiency virus-positive paediatric patients, one resistant and one susceptible to fluconazole, namely PRI and 88, respectively (Portela et al., 2010; Garcia-Gomes et al., 2012), and two reference strains, one resistant and the other susceptible to fluconazole, ATCC 60193 and ATCC 10231, respectively. Biofilm and dimorphism experiments were assessed on the clinical isolate resistant to fluconazole (PRI). All strains were stored on Sabouraud dextrose agar slants at 4 °C at the Institute of Microbiology Paulo de Góes/University of Rio de Janeiro.

The MICs of the EOs were determined according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2002). Serial dilutions of the EOs were prepared in the range 1.0–0.0004 %, equivalent to 100 %, 50 %, 25 %, 12.5 %, 6.25 %, 3.1 %, 1.5 %, 0.75 % and 0.375 %, respectively. The compound cis-nerolidol and a mixture of cis,trans-nerolidol (Sigma Aldrich) were used as controls. The minimum concentrations that inhibited yeast growth completely and by 50 % were defined as MIC and MIC50, respectively.

The effect of the EO extracted from the leaves of *P. claussenianum* was evaluated on the yeast-to-hyphae transition. For this, 100 cells were incubated in 1.0 ml FBS in the presence or absence of the EO (ranging from 4.0 to 0.062 %) for 3 h at 37 °C with shaking. Cell viability was tested by the trypan blue exclusion method. The cells were suspended in 1 M NaOH containing 10 mM EDTA and 1 % (v/v) 2-mercaptoethanol and then subjected to vigorous vortexing for 1 min to eliminate the clusters that may hamper counting accuracy (Casanova et al., 1997). A haemocytometer Neubauer chamber was used for differential counting, and the percentage of germ tubes was determined. This procedure was performed four times, and a minimum of 500 cells were used for each experiment.

The effect of the EO from the leaves of *Piper claussenianum* in the formation and stability of *C. albicans* biofilm was evaluated by the methodology recommended by Thein and collaborators (2007) with slight modifications. Briefly, 105 cells were added to a 96-well flat-bottomed microtitre plate and incubated at 37 °C for 90 min with gentle agitation (75 r.p.m.). The supernatant was removed and the biofilms formed were washed twice gently with PBS (pH 7.2) to remove non-adherent cells. Brain–heart infusion medium (200 μl) containing *P. claussenianum* EO from leaves (range 1.0–0.02 %) was placed over the adhered cells, and the plates were incubated at 37 °C for 24 and 48 h. The influence of the leaf EO on mature biofilms was also assessed as described previously (Vila et al., 2013). Briefly, biofilm was allowed to form for 48 h, and then serial concentrations of the oil (range 0.001–1.0 %) were washed over the mature biofilms and incubated at 37 °C for another 48 h. All biofilm quantification was performed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay with slight modifications (Thein et al., 2007). Briefly, an MTT solution (3.0 mg ml−1) was prepared in PBS (pH 7.2) and sterilized using a 0.22 μm pore-size filter. A 0.4 mM menadione solution was prepared, filtered and added to the MTT solution at a volume ratio of 20:1. The MTT/menadione solution was added to each well, followed by incubation at 37 °C for 3 h in the dark, and the result was read spectrophotometrically by determining A590.

To evaluate the possible toxicity of EOs to mammal cells, a cytotoxicity assay was performed using L929 mouse fibroblast cells. Cells were grown in Eagle’s minimum essential medium supplemented with 10 % (v/v) FBS, 0.03 mg glutatione ml−1, 50 μg garamycin ml−1, 2.5 mg fungzone (amphotericin B) ml−1, 0.25 % NaHCO3 and 10 mM HEPEs (pH 7.2). Cell cultures were prepared in 96-well microtitre plates and incubated at 37 °C in a 5 % CO2 atmosphere. Several concentrations of compounds (range 5–0.07 %) were placed in contact with the confluent L929 cell monolayer and incubated at 37 °C in a 5 % CO2 atmosphere for 48 h. Cytotoxicity was evaluated by neutral red dye uptake method described elsewhere (Borefreund & Puerner, 1985). Briefly, cells were incubated in the presence of 0.01 % neutral red solution for 3 h at 37 °C in a 5 % CO2 atmosphere and then fixed with 4 % formalin in PBS (pH 7.2). Dye incorporated by the viable cells was eluted using methanol:acetate acid:water (50:1:49), and dye uptake was determined by measuring the absorbance at 490 nm (A490). The concentration that caused a 50 % reduction in the number of viable cells was defined as the 50 % cytotoxic concentration.

The combined effect of the EO from leaves of *P. claussenianum* with fluconazole was also evaluated. For this purpose, checkerboard experiments were conducted with both resistant strains (PRI and ATCC 60193) as recommended by Mukherjee et al. (2005). Briefly, after culturing, yeast cells were washed and 5 × 104 cells ml−1 were incubated in 96-well plates containing RPMI 1640 in the absence and presence of different concentrations of the EO and fluconazole, alone or in combination. Fluconazole was used in a range 256–2 μg ml−1 and EO in the range 1.0–0.01 %, within horizontal and vertical line wells, respectively. Interaction between compounds was classified based on the fractional inhibitory concentration (FIC) index (FICI), defined by the following equation: FICI = FIC of EO + FIC of fluconazole, where the first FIC is defined as the MIC of EO in combination with fluconazole divided by the MIC of EO alone, and the second as the MIC of fluconazole combined with the EO divided by the MIC of fluconazole alone. Combined MICs represent the concentration of both compounds at the isoeffective combination (Odds, 2003). The values calculated by the FICI equation defined the following interactions: ≤0.5, synergistic interaction; >0.5 to ≤4, no interaction; >4, antagonistic effect.

All experiments were repeated at least three times, all systems were performed in triplicate sets, and the results were expressed as means ± SD. Data were analysed by Student’s t-test at a significance level of P<0.05.
Table 1. Inhibitory concentration values of inflorescent and leaf P. clausenianum EOs

Inhibitory concentrations were determined according to CLSI (2002).

<table>
<thead>
<tr>
<th>C. albicans strain</th>
<th>Leaf EO (%)</th>
<th>Inflorescence EO (%)</th>
<th>cis,trans-Nerolidol (%)</th>
<th>cis-Nerolidol (%)</th>
<th>Fluconazole (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC₅₀</td>
<td>MIC</td>
<td>MIC₅₀</td>
<td>MIC</td>
<td>MIC</td>
</tr>
<tr>
<td>PRI</td>
<td>0.43 ± 0.02</td>
<td>0.91 ± 0.11</td>
<td>0.05 ± 0.02</td>
<td>0.10 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>0.69 ± 0.091</td>
<td>1.26 ± 0.03</td>
<td>0.01 ± 0.00</td>
<td>0.04 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>0.96 ± 0.04</td>
<td>0.24 ± 0.03</td>
<td>0.02 ± 0.00</td>
<td>0.04 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>ATCC 60193</td>
<td>0.41 ± 0.07</td>
<td>0.96 ± 0.09</td>
<td>0.05 ± 0.02</td>
<td>0.09 ± 0.01</td>
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</tr>
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</table>

RESULTS AND DISCUSSION

The MIC data obtained by broth microdilution against the C. albicans strains are summarized in Table 1, and revealed that both inflorescent and leaf P. clausenianum EOs were able to inhibit yeast growth in all strains tested. In fact, the EO extracted from the inflorescences was more active against yeast growth, reaching a lower MIC compared with the EO extracted from the leaves. This result could be attributed to a higher presence of linalool in its composition (50.2%). This monoterpenic has already been reported to have a strong antifungal activity against C. albicans (Alviano et al., 2005; Khan et al., 2010; Hsu et al., 2013), but, interestingly, it seemed to be more effective against susceptible strains compared with the resistant ones, as the calculated MICs were significantly lower. Moreover, the cis,trans-nerolidol mixture acquired commercially and used as a control was able to inhibit fungal growth at a concentration greater than the EO extracted from leaves; however, the cis-nerolidol isomer showed the lowest activity in all strains tested, reaching an MIC about threefold higher compared with the cis,trans-nerolidol mixture. This finding suggests that trans-nerolidol, which is the major component of the EO extracted from leaves, is probably the compound responsible for modulating fungal growth among the nerolidol isomers. The effect of the EO extracted from the leaves of P. clausenianum on the morphological differentiation of the resistant clinical C. albicans isolate (PRI) is shown in Fig. 1. This particular EO was chosen due to its high concentration (81.4%) of trans-nerolidol, strongly associated with the quorum sensing phenomena and its ability to downregulate germ-tube formation (Martins et al., 2007). After a 3 h incubation in FBS, the percentage of germ tubes reached 74.2%. However, with the presence of 0.062% EO, this percentage was significantly reduced to 17.2% (inhibition of 76.8%). Inhibition of germ-tube differentiation could be seen in all concentrations tested. When higher concentrations of EO (0.12–0.25 and 0.5%) were evaluated, morphological transition rates were 10.2–4.2 and 2.0%, demonstrating an inhibition in germ-tube formation of 86.2–94.3 and 97.3%, respectively. Similar results could be seen with the cis,trans-nerolidol mixture, used as control. Using this compound at 0.062, 0.12, 0.25 and 0.5% concentrations, the inhibition rates were 72.7, 80.9, 91.9 and 98.9%, respectively. Interestingly, when the cis-nerolidol isomer was evaluated using the same concentration rates, the inhibition levels were significantly lower reaching 60.7, 60.9, 67.5 and 88.1%, respectively. These results suggested that trans-nerolidol has a stronger activity in inhibiting C. albicans germ-tube formation than cis-nerolidol. This suggestion was reinforced by the similar results achieved with the EO extracted from the leaves, which contains 81.4% of trans-nerolidol (Marques et al., 2010).

The filamentous morphology can exert mechanical force, favoring epithelial penetration and endothelial damage, which can aid the escape of the micro-organism into deeper tissues. Therefore, C. albicans morphogenesis is an integral part of an overall fungal strategy, and is considered essential for fungal virulence (Kumamoto & Vinces, 2005). Thus, any compound able to inhibit this transition, or at least reduce it, would be acting directly against the disease installation process. The EO from the leaves of P. clausenianum showed an important effect in inhibiting the differentiation from yeast to hyphae in C. albicans and could be considered a promising agent targeting mechanisms that are considered essential for the beginning of the infection.

Fig. 1. Effect of the EO of P. clausenianum derived from leaves on germ-tube formation by C. albicans. Cells (10⁵) were incubated in FBS containing the EO or not at the concentrations indicated for 3 h at 37 °C. The percentage of germ tubes was calculated by differential counting by optical microscopy.
The influence of the EO from the leaves of *P. claussenianum* on biofilm formation by *C. albicans* resistant clinical strain (PRI) is shown in Fig. 2. When the biofilm formation was measured after 24 h (Fig. 2a), the results showed that the EO had reduced the biofilm formation process by about 30% for all concentrations tested. A reduction of 28.9, 37.4, 31.0, 31.6 and 32.5% in biofilm formation was observed for concentrations of 0.06, 0.12, 0.25, 0.5 and 1% EO, respectively. However, in this test, the results obtained from the mixture of the cis,trans-nerolidol used as a control were not significant, suggesting that, despite its strong inhibitory activity on germ-tube transformation, this mixture of nerolidol isomers is not efficient against the biofilm formation process by itself. This indicated that other important signalling routes and processes may be active in the formation and architecture of biofilms developed by *C. albicans*, besides morphological transition (Ramage et al., 2005). After 48 h of incubation (Fig. 2b), the results showed an even greater activity. EO concentrations ranging from 1.0 to 0.25% decreased the biofilm formation process by approximately 50%, demonstrating a significant activity, which was independent of the dosage, similar to the results seen for the 24 h incubation. The reductions in biofilm formation were 56.1, 49.2 and 50.8% for 0.25, 0.5 and 1% concentrations, respectively. With regard to mature biofilms, the EO from the leaves of *P. claussenianum* was able to exert a significant effect on its stability, even at the lowest concentration used in these experiments, pointing to a promising ability to disrupt these pre-formed structures at the beginning of incubation. The results are summarized in Fig. 3 and revealed that the viability was significantly reduced by 47.2% when mature biofilms were incubated with 0.06% EO; this reduction maintained its significance at all concentrations tested. This decrease was 52.2, 56.5, 62.0 and 63.9% when the mature biofilms were incubated with 0.12, 0.025, 0.5 and 1.0% EO respectively. The commercial mixture of cis,trans-nerolidol showed an even stronger activity, achieving 76.1% inhibition when the mature biofilms were incubated with 1.0% of this sesquiterpene. In fact, mature biofilms were reduced significantly by 46.1, 59.0, 62.2 and 65.2% when incubated with 0.06, 0.12, 0.25 and 0.5% cis,trans-nerolidol, respectively. Interestingly, the commercial cis-nerolidol standard showed a significant inhibition activity similar to the EO. This inhibition was about 64.6, 64.3, 65.0, 65.8 and 67.0% when the mature biofilms were incubated with 0.06, 0.12, 0.25, 0.5 and 1.0% of this sesquiterpene, respectively. Although different, the inhibition values among EO and the cis- and cis,trans-nerolidol isomers were not significant. The similarities in the inhibition values among the isomer controls and the EO suggested that trans-nerolidol is the main oil component responsible for the reduction in pre-formed biofilms.

*C. albicans* biofilm can be defined as a mixture of cells in yeast, hyphae and pseudo-hyphae forms with a basal yeast layer anchoring the rest to an inert surface (Baille & Douglas, 2000). Genetic analyses have indicated that these morphologies are crucial for biofilm formation, which suggests that each cell type has a unique role in the development of biofilm. Formation begins with adherence of yeast cells to a substrate, and these cells proliferate across

![Fig. 2. Effect of the EO extracted from the leaves of *P. claussenianum* on the *C. albicans* biofilm formation process. Different concentrations of the EO as indicated were placed on formed biofilm and incubated at 37 °C for 24 h (a) and 48 h (b). Biofilm quantification was performed by measuring A630 in an MTT reduction assay.](image)

![Fig. 3. Effect of the EO extracted from the leaves of *P. claussenianum* on mature biofilm formed by *C. albicans*. Serial concentrations of the EO were placed on biofilms that had been allowed to form for 48 h and incubated at 37 °C for 48 h. Biofilm quantification was performed by measuring A630 in an MTT reduction assay.](image)
the surface and produce elongated projections that grow into filamentous forms. Extracellular matrix accumulates causing a high level of drug resistance. Finally, non-adherent cells are released into the surrounding medium. Several noteworthy classes of gene products govern this process, including the known cell-wall proteins. In fact, studies indicate that putative cell-wall adhesins, such as enhanced adherence to polystyrene 1 (Eap1), hyphal wall protein 1 (Hwp1), agglutinin-like sequence 1 (Als1) and Als3 have a strong role in these developmental events (Nobile et al., 2008). Many of the *C. albicans* genes involved in biofilm development encode predicted transcription factors or protein kinases, for example, the transcription factor biofilm and cell wall regulator 1 (Bcr1) is required for biofilm formation, and its expression is upregulated in hyphae (Nobile & Mitchell, 2005).

 Similarly, the zinc-responsive transcription factor (Zap1) is a regulator of extracellular matrix accumulation, indicating that alterations in zinc levels might alter matrix formation. Several alcohol dehydrogenases have an impact on biofilm development, and therefore the role of these proteins indicates that substrate specificity is crucial for biofilm development (Mukherjee et al., 2006). Quorum sensing mechanisms rule microbial behaviour by cell density. This phenomenon is usually determined by secreted signalling molecules and has a critical role in all kinds of biofilms. Farnesol and tyrosol are signalling molecules produced by *C. albicans*: the former suppresses filamentation and the latter stimulates the yeast-to-hyphae conversion. Application of exogenous farnesol inhibits biofilm formation, but its effects are overcome by the addition of exogenous tyrosol on the same biofilm. Both molecules are observed in the supernatant of mature biofilms, as well as other small molecules taken as regulators, such as nerolidol. Each of these compounds can inhibit hyphae formation, and together they may aid in biofilm dispersal by promoting yeast morphology (Finkel & Mitchell, 2011; Han et al., 2011). Despite many studies showing the effect of small molecules on fungal morphogenesis and biofilm formation, the signalling pathways responsible for its dynamics are not fully understood. Due to the structural similarity between farnesol and nerolidol, the latter compound should be able to exert a similar inhibitory effect on yeast-to-hyphae transition as the former. Our results point to nerolidol as a strong downregulator, not only in the formation process of *C. albicans* biofilm but also in the stability of pre-formed structures, probably by promoting dispersion events, whilst maintaining yeast morphology. Nevertheless, the activity of individual molecules can provide a better understanding of the biological functions of biofilm development. A crucial feature associated with *C. albicans* biofilm, which has a severe impact in clinical settings, is the high resistance to antifungal drugs. Therefore, biofilm formation on medical devices can impact negatively on patient survival, especially in patients admitted to intensive care units, by causing device failure as well as serving as a reservoir of the micro-organism, perpetuating the infection (Kojic & Darouiche, 2004; Ramage et al., 2005).

Therefore, the discovery of any new methodology able to modulate the formation or the stability of biofilms would have great therapeutic relevance.

Mice fibroblasts were challenged with the EO extracted from the leaves of *P. clausenianum* demonstrating a 50 % cytotoxic concentration of 0.5 %. This finding agrees with the literature, which has already reported that no toxic effect was observed *in vivo* either after an intraperitoneal 100 mg nerolidol kg⁻¹ injection or with a 5 % topical administration, using the murine model (Arruda et al., 2005). *In vivo* studies have demonstrated that animals treated with nerolidol at concentrations of up to 2000 mg kg⁻¹ showed no clinical indications of morbidity, and, furthermore, peripheral blood cells and liver taken from these animals showed no DNA damage (Piculo et al., 2011). In fact, this sesquiterpene has already been approved by the US Food and Drug Administration as a food flavouring agent, which favours its reliability and the possibility of applying it in clinical practice in the near future (Arruda et al., 2005).

Interaction between the EO from leaves of *P. clausenianum* and fluconazole was determined according to the FICI, and the results are shown in Table 2. The FICI values indicated a clear synergistic profile among the compounds for both fluconazole-resistant strains, reinforcing its supposed therapeutic usefulness. The combined use of antimicrobial drugs seems to be advantageous, as it achieves a satisfactory clinical response using lower concentrations of each medication and therefore reduced toxicity, which is a serious and recurrent issue among current conventional antifungal drugs (Mukherjee et al., 2005). Furthermore, previous studies have suggested that the combined use of drugs may reduce the emergence of resistant strains (Lupetti et al., 2003).

Taken together, the results presented in this work suggest that *P. clausenianum* EOs may represent an important alternative for the treatment and prophylaxis of candidal infections due to their ability to control fungal growth as well as modulate factors strongly associated with the virulence of this organism, such as yeast-to-hyphae transition and biofilm formation. These effects are due to the presence of linalool in the EO extracted from the inflorescences, as well as the remarkable content of *trans*-nerolidol, a well-known

<table>
<thead>
<tr>
<th><em>C. albicans</em> strain</th>
<th>FICI</th>
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<tr>
<td>PRI</td>
<td>0.38 ± 0.1</td>
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<tr>
<td>ATCC 60193</td>
<td>0.27 ± 0.2</td>
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FICI values <0.5 are indicative of synergism. Data represent the means ± SD of three independent experiments.
molecule associated with the quorum sensing phenomena, in the EO extracted from the leaves of this plant. Furthermore, the high concentration of this sesquiterpene in the leaves of this plant indicates a novel and natural source to obtain this bioactive principle for pharmaceutical preparations, instead of conventional synthesis, which could be industrially advantageous.

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