








Article

Antibiotic Potential and Chemical Composition of the Essential Oil of *Piper caldense* C. DC. (Piperaceae)

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Abstract: Infections by multiresistant microorganisms have led to a continuous investigation of substances acting as modifiers of this resistance. By following this approach, the chemical composition of the essential oil from *Piper caldense* leaf and its antimicrobial potential were investigated. The antimicrobial activity was determined by broth microdilution method providing values for minimum inhibitory concentration (MIC), IC₅₀, and minimum fungicidal concentration (MFC). The essential oil was tested as a modulator for several antibiotics, and its effect on the morphology of *Candida albicans* (CA) strains was also investigated. The chemical characterization revealed an oil composed mainly of sesquiterpenes. Among them are caryophyllene oxide (13.9%), spathulenol (9.1%), δ -cadinene (7.6%) and bicyclogermacrene (6.7%) with the highest concentrations. The essential oil showed very low activity against the strains of CA with the lowest values for IC₅₀ and MFC of 1790 μ g/mL and 8192 μ g/mL, respectively. The essential oil modulated the activity of fluconazole against CA URM 4387 strain, which was demonstrated by the lower IC₅₀ obtained, 2.7 μ g/mL, whereas fluconazole itself presented an IC₅₀ of 7.76 μ g/mL. No modulating effect was observed in the MFC bioassays. The effect on fungal morphology was observed for both CA INCQS 40006 and URM 4387 strains. The hyphae projection was completely inhibited at 4096 μ g/mL and 2048 μ g/mL, respectively. Thus, the oil has potential as an adjuvant in antimicrobial formulations.

Keywords: pimenta d'água; *Candida*; fungistatic effect; inhibition of dimorphism; GC/MS

1. Introduction

Infections caused by fungi, are the major problem of hospital infections, mainly due to the emergence of new infections and the generalized resistance to antibiotics [1]. Due to the rapid resistance acquired by microorganisms the search for natural products with antimicrobial activity has

been constant in recent years and drugs derived from plants have contributed largely to human health, historically representing a source for the creation of new drugs [2].

One way of combating microbial resistance is the investigation of substances that can act as resistance modifiers by combining common antimicrobial drugs with some phytochemicals, and in some cases observed synergism [3,4].

Yeasts of the genus *Candida* are found as commensals in the human organism, however, because of factors that disturb the dynamics of the host can favor the growth of these fungi, from superficial infections to systemic infections [5]. Among the yeasts of this genus, *Candida albicans* stands out as the main cause of infections, being a species with great ability to change its morphology, a necessary factor for its virulence and pathogenicity [6,7].

For the treatment of infectious diseases, populations without access to medicines, especially those from underdeveloped and developing countries, use medicinal plants to combat disease. Such use of these vegetables is due to a number of advantages, such as their availability, low purchasing power and being in popular knowledge for several generations [2,8]. Among the species of the Brazilian flora, the genus *Piper* L. is one of the largest in the Piperaceae family, with 290 species and 45 varieties occurring in Brazil [9]. Some species of the genus are used as a flavoring of food and treatment of diseases [10]. A scientific study evidenced the antimicrobial potential of four species of the genus against *Staphylococcus aureus* and three strains of *Candida*, including *C. albicans* [11]. Among the species of the genus, *Piper caldense* C. DC., is popularly known in Brazil as “pimenta d’água” or “pimenta d’arda”, being commonly used for the treatment of snake bites, sedative and stomach problems [10]. Research using *P. caldense* essential oil has revealed promising antimicrobial potential [12,13].

In view of the above problem, this study aimed to evaluate the antibiotic and modulator potential of volatile terpenes of *P. caldense* against *Candida albicans*, as well as to determine if the natural product is capable of reducing pleomorphism. Finally, it was evaluated by gas chromatography which terpenes were present in the essential oil.

2. Results

2.1. Chemical Composition

The chromatographic analysis of the essential oil of *P. caldense* identified 25 phytochemical constituents, corresponding to 94.3% of the total composition. Sesquiterpenes are the main class observed, reaching a percentage of 89.7% of the sample. Monoterpenes were found in very low concentration (4.6%). There were no major constituents (>20%) and no constituent in trace concentration (<1%). All the identified phytochemicals are secondary metabolites, with caryophyllene oxide (13.9%), spathulenol (9.1%), δ -cadinene (7.6%), and bicyclogermacrene (6.7%) as constituents with the highest concentrations (Table 1).

2.2. Antifungal Activity

2.2.1. Cell Viability Curve and IC₅₀

Regarding the antifungal activity of the essential oil of *P. caldense*, it was found that it has a low antifungal effect against strains of *C. albicans* because the IC₅₀ values were high, 2256.24 and 1790.24 μ g/mL for the CA INCQS 40006 and CA URM 4387, respectively (Table 2) (Figures 1 and 2). However, it was observed that for the *C. albicans* URM 4387 strains, the oil potentiated the effect of the drug used, fluconazole, since it had an IC₅₀ of 7.73 μ g/mL alone, and when associated with the essential oil, the value of evaluated parameter dropped to 2.7 μ g/mL, i.e., a significant reduction. It is noteworthy that for *C. albicans* 40006 strains, no potentiating effect was found for fluconazole but an antagonistic effect, since there was an increase in the IC₅₀ of the oil associated with fluconazole. Thus, based on the behavior of the curve through non-linear regression, the IC₅₀ values of *P. caldense* oil were clinically irrelevant when evaluated alone.

Table 1. Chemical composition of the essential oil of leaves from *Piper caldense*.

Compounds	%	AI _{lit}	MF
α-Pinene	2.5	935	C ₁₀ H ₁₆
Camphene	2.1	952	C ₁₀ H ₁₆
α-copaene	2	1381	C ₁₅ H ₂₄
(E)-Caryophyllene	2.6	1426	C ₁₅ H ₂₄
Aromadendrene	2.7	1445	C ₁₅ H ₂₄
γ-muurolene	4	1478	C ₁₅ H ₂₄
β-selinene	3.2	1490	C ₁₅ H ₂₄
Bicyclogermacrene	6.7	1492	C ₁₅ H ₂₄
Germacrene D	5.3	1493	C ₁₅ H ₂₄
α-muurolene	4.2	1498	C ₁₅ H ₂₄
δ-Cadinene	7.6	1520	C ₁₅ H ₂₄
γ-Cadinene	3.3	1521	C ₁₅ H ₂₄
α-Calacorene	2.2	1551	C ₁₅ H ₂₀
β-Calacorene	1.9	1572	C ₁₅ H ₂₀
Spathulenol	9.1	1576	C ₁₅ H ₂₄ O
Caryophyllene oxide	13.9	1580	C ₁₅ H ₂₄ O
Globulol	2.3	1594	C ₁₅ H ₂₆ O
Rosifoliol	1.3	1597	C ₁₅ H ₂₆ O
Humulene epoxide II	1	1619	C ₁₅ H ₂₄ O
1.10-di-epi-Cubenol	1.6	1624	C ₁₅ H ₂₆ O
1-epi-Cubenol	3.4	1638	C ₁₅ H ₂₆ O
epi-alfa-muurolol	3.1	1640	C ₁₅ H ₂₆ O
α-cadinol	4.2	1650	C ₁₅ H ₂₆ O
α-Muurolol	2.1	1651	C ₁₅ H ₂₆ O
Cadalene	2	1667	C ₁₅ H ₁₈
Total sesquiterpenes	89.7		
Total monoterpenes	4.6		
Total identified (%)	94.3		

AI_{lit}—Arithmetic Retention Indices from literature; MF: Molecular Formula.

Table 2. IC₅₀ of the essential oil of *Piper caldense* (EOPc) against *Candida albicans*.

Products Tested	Yeast	
	<i>Candida albicans</i> INCQS 40006	<i>Candida albicans</i> URM 4387
Fluconazole (FCZ)	7.76 µg/mL	7.73 µg/mL
EOPc	2256.24 µg/mL	1790.24 µg/mL
EOPc + FCZ	12.37 µg/mL	2.7 µg/mL

INCQS: National Institute for Health Quality Control. URM: University Recife Mycology.

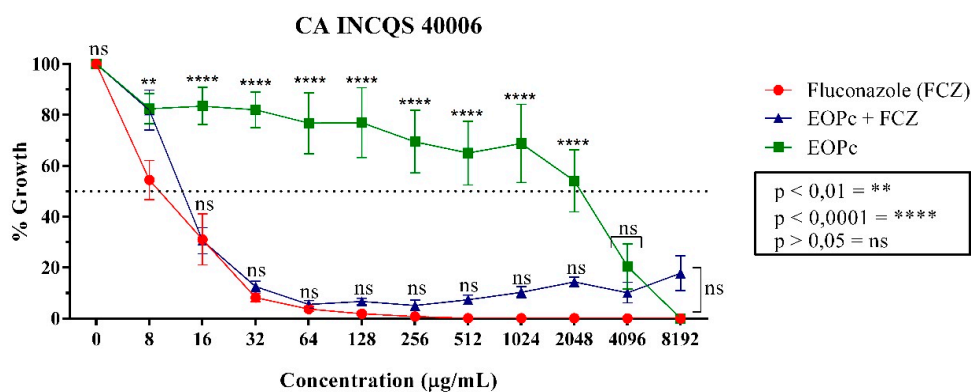


Figure 1. Anti-*Candida* potential of essential oil of *Piper caldense* (EOPc) against strains of *Candida albicans* 40006 INCQS.

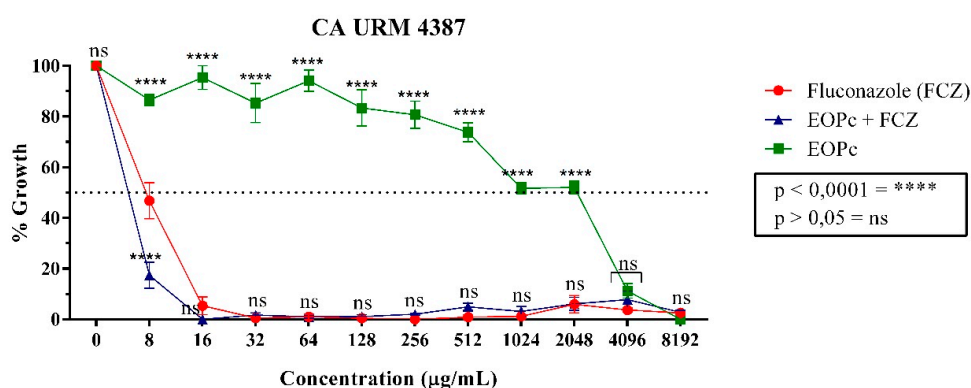


Figure 2. Anti-*Candida* potential of essential oil of *Piper caldense* (EOPc) against strains of *Candida albicans* URM 4387.

2.2.2. Cell Viability Curve and Minimum Fungicidal Concentration (MFC)

In determining MFC it was considered those samples capable of inhibiting the growth of the fungal colonies. Thus, for the CA INCQS 40006 line, none of the products alone or in combination were able to totally inhibit colony growth, since MFC was $\geq 16,384 \mu\text{g/mL}$ (Table 3). However, there was an MFC for the oil against CA URM 4387, in which fluconazole alone and combined with *P. caldense* oil showed a much lower MFC, $16 \mu\text{g/mL}$, so that the oil did not modulate the fungicidal effect of fluconazole for this lineage (Table 3).

Table 3. Minimum fungicidal concentration (MFC) of essential oil of *Piper caldense* (EOPc) and fluconazole associated and isolated against strains of *Candida albicans*.

Products Tested	Yeast	
	<i>Candida albicans</i> INCQS 40006	<i>Candida albicans</i> INCQS 40006
Fluconazole (FCZ)	$\geq 16,384 \mu\text{g/mL}$	$16 \mu\text{g/mL}$
EOPc	$\geq 16,384 \mu\text{g/mL}$	$8192 \mu\text{g/mL}$
EOPc + FCZ	$\geq 16,384 \mu\text{g/mL}$	$16 \mu\text{g/mL}$

INCQS: National Institute for Health Quality Control. URM: University Recife Mycology.

2.2.3. Activity of the *Piper caldense* in the Control of Virulence of *Candida albicans*

In the evaluation of the activity of the oil in the morphology of the yeasts of *C. albicans*, the effect was caused by impoverishment of the culture medium, so that the yeasts project the hyphae and pseudohyphae in search of nutrients. For the growth control of CA INCQS 40006 (Figure 3, Slides 1–7), it is possible to observe the formation of several hyphae (S1), whereas in the treatments with fluconazole, there is a significant decrease in the hyphae projection (S2–4), resulting in a complete inhibition of hyphae at concentration of $4096 \mu\text{g/mL}$ (S2). The EOPc showed a similar result, being as effective as fluconazole.

For the CA URM 4387 strain, fluconazole at a concentration as low as $8 \mu\text{g/mL}$ could diminish the virulence (Figure 4, S2). The oil at higher concentrations, such as $2048 \mu\text{g/mL}$ (S3), was able to inhibit the hyphae projection; on the other hand, at concentration of $512 \mu\text{g/mL}$ (S5), there was a significant decrease in hyphae projections.

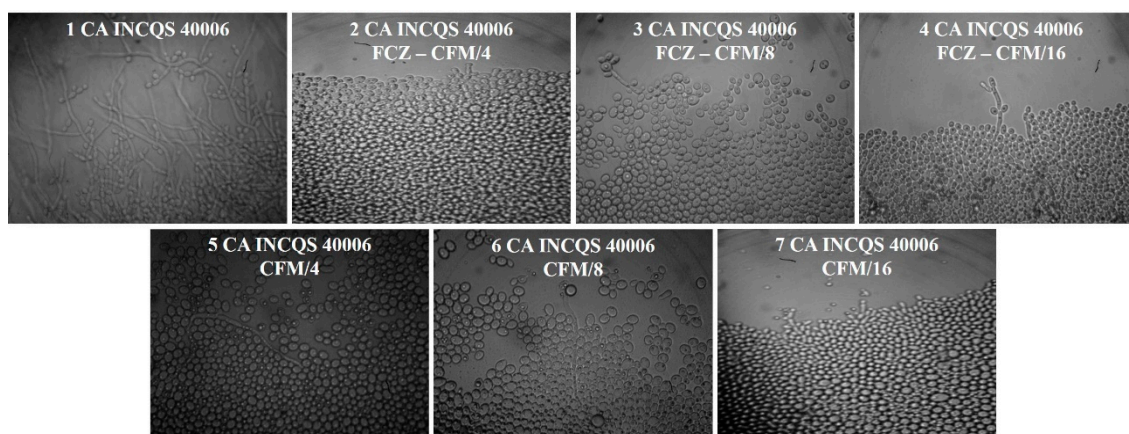


Figure 3. Effects of essential oil of *Piper caldense* on the dimorphism of *Candida albicans* INCQS 40006. Slide (S1): Growth control; S2–4: Effect of fluconazole at concentrations 4096 µg/mL (S2), 2048 µg/mL (S3), 1024 µg/mL (S4). S5–7: Effect of essential oil at concentrations of 4096 µg/mL (S5), 2048 µg/mL (S6), 1024 µg/mL (S7). Display 400× increased.

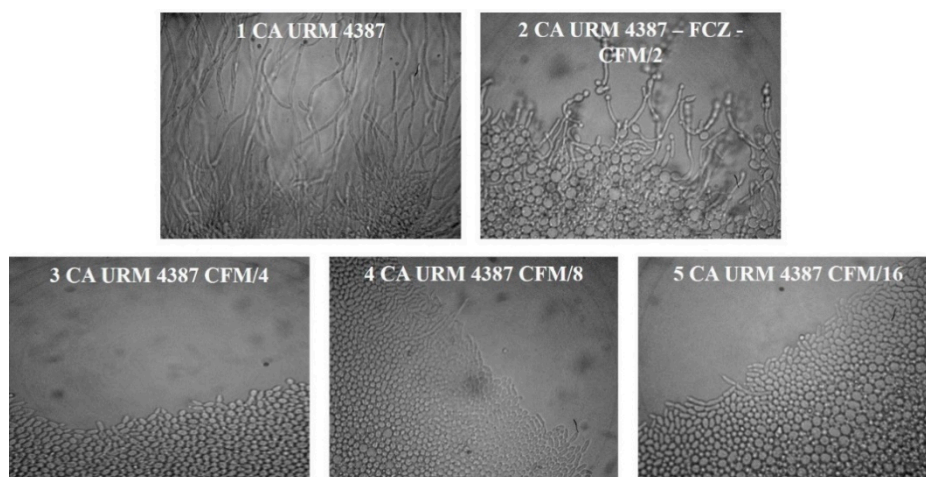


Figure 4. Effects of essential oil of *Piper caldense* on the dimorphism of *Candida albicans* URM 4387. Slide (S1): Growth control; S2: Effect of fluconazole at the concentration of 8 µg/mL. S3–5: Effect of the essential oil at 2048 µg/mL (S3), 1024 µg/mL (S4), 512 µg/mL (S5).

3. Discussion

The genus *Piper* L. presents a high number of species with medicinal, insecticidal and condiment applications, since the representatives are sources of volatile oils produced by the secondary metabolism [14]. Some species of this genus present antifungal activities, such as *Piper amalago* L. [15], *Piper aduncum* L. and *Peperomia pelúcida* (L.) Kunth [16], and antibacterial activity, among them *Piper betle* L. [17]. Thus, the selection strategy of *P. caldense* for the investigation of the antimicrobial activities of this study was based on chemotaxonomy, since there is a phylogenetic relationship between *P. caldense* and other species of the genus and possibly in the evolutionary historical branch the biosynthetic routes are similar [18].

The phytochemicals (monoterpenes and sesquiterpenes) of the essential oil of the species under study were elucidated by Rocha et al. [19], however, there were marked differences in the constitution, the first is that in our study, the caryophyllene oxide was the major constituent (13.9%), whereas in the results of the aforementioned study this sesquiterpene is not present in the oil of leaves, but in the stem essential oil (6.2%). In addition, Rocha et al. [19] states that the constituent in higher percentage is the α -cadinol, reaching compose 19% of the total composition, and in our study this sesquiterpeno is in

low percentages (4.2%). This variation is justified by several factors, both intrinsic and genetic, as well as extrinsic factors such as geographic origin of the plant, cultivation, collection form, and especially the period of the year that was collected [20–22].

Although the essential oil of *P. caldense* did not present antifungal activity in low concentrations (≤ 500 $\mu\text{g/mL}$), it presented a modulating effect for fluconazole against strains of *C. albicans* URM 4387. So that this finding is relevant, since the introduction of azole class antibiotics (miconazole, econazole, ketoconazole, fluconazole and triazonazole) for the treatment of infections caused by *Candida* species, a growing emergence of resistant *Candida* species has been observed [23,24].

The activity of caryophyllene oxide, an oxygenated terpenoid, was tested in the laboratory against dermatophyte fungi, showing significant results, and their activity has been compared with antifungals such as cyclopiroxolamine and sulconazole [12].

Silva [25], evaluating the antifungal activity of 2-geranyl-3,4-dihydroxybenzoic acid and 3-geranyl-4-hydroxybenzoic acid, both substances isolated from the fruits of *P. caldense*, demonstrated, respectively, a moderate and high activity against *C. albicans* strains (LM-86 and LM-111), so that the first substance showed a MIC of 512 $\mu\text{g/mL}$ for both strains and the second one 32 $\mu\text{g/mL}$, also for the two strains. It is important to highlight that in our study the essential oil was used as the product, and this is a mixture of mono and sesquiterpenes, whereas in the study mentioned above the substances were derived from the benzoic acid prenylate, so that the chemical structures are quite different.

Inhibition of virulence of *C. albicans* strains by natural products was also shown by other scientists, among them Santos et al. [26], who evaluated the essential oil of *Eugenia uniflora* L. (Myrtaceae) and demonstrated that at concentrations of 8192 $\mu\text{g/mL}$ there is inhibition of hyphal projection. Two other species also from the same family that have the ability to inhibit the virulence of *C. albicans* are *Psidium brownianum* Mart. ex DC. And *Psidium guajava* L., the former having medicinal properties and is used to combat infections caused by fungi of the genus *Candida* [6,27].

The activities of the natural products, concerning the antimicrobial agents act by diverse mechanisms of action such as the disintegration of the cytoplasmic membranes, destabilization of the motor proton force (MPF), altering the polarization of the membrane, and the coagulation of the cellular content [28–30].

4. Materials and Methods

4.1. Botanical Material

The collection of leaves of *Piper caldense* was performed in the municipality of Piraquara in the state of Paraná, Brazil, under the coordinates 25°29.693' S and 49°00.844' W at 528 m elevation (Figure 5). An exsiccata was identified and deposited in the Herbarium of the Faculdades Integradas Espírita under voucher 9.103.

4.2. Extraction of Volatile Terpenes and Determination of the Chemical Composition

Healthy *P. caldense* leaves were selected and dried in a incubator at 40 °C. After dehydration, the leaves were crushed to increase their contact surface and maximize the extraction of their volatile components. For such extraction, the hydrodistillation system was used, in which 50 g of the plant material was placed in a volumetric flask with 1000 mL of distilled water, being constantly heated for 4.5 h, until the oil extraction [21].

For the chemical characterization of *P. caldense* essential oil, it was made by gas chromatography-mass spectrometry (GC/MS). Initially, the essential oil was diluted in dichloromethane to 1% concentration, then 1 μL of this solution was injected (1:20) into Agilent 6890 chromatograph, coupled to Agilent 5973N mass selective detector, wherein the injector temperature was 250 °C. For the separation of the constituents, helium gas was used as a carrier (1 mL/min) and an HP-5MS capillary column with the following specifications: 5% phenyl-95%-dimethylpolysiloxane, 30 m \times 0.25 mm \times 0.25 μm . For the heating ramp, the temperature started at 60 °C with a heating rate of 3 °C/min to

240 °C, totaling 60 min. The mass detector was operated in the electronic ionization mode (70 eV), at a rate of 3.15 s^{-1} sweeps and a mass range of 40 to 450 u. The transfer line was maintained at 260 °C, the ion source at 230 °C and the analyzer (quadrupole) at 150 °C.

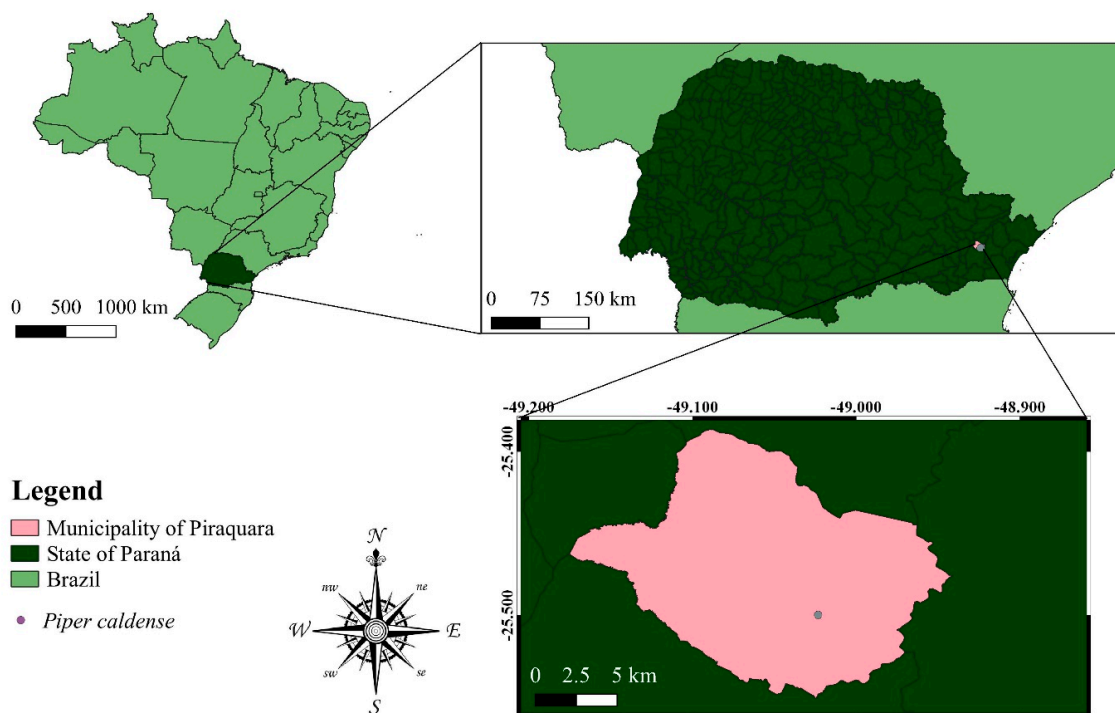


Figure 5. Location of the species *Piper caldense* in the municipality of Piraquara in the state of Paraná, Brazil.

For quantification, the diluted samples were injected into an Agilent 7890A chromatograph equipped with a flame ionization detector (FID), operated at 280 °C. We employed the same column and analytical conditions described above except for the carrier gas used, which was hydrogen at a flow rate of 1.5 mL/min. The percentage composition was obtained by the electronic integration of the FID signal by dividing the area of each component by the total area (area%).

For the determination of chemical constituents, the mass of the constituents was compared with the library (NIST and Wiley) e and also by their linear retention indexes, calculated from the injection of a homologous series of hydrocarbons (C₇-C₂₆) and compared with data from the literature [31].

4.3. Drugs, Reagents, Solution Preparation and Fungal Strains

For the antifungal test, the essential oil stock solution was prepared from 0.15 g and diluted in 1 mL of dimethyl sulfoxide (DMSO). To obtain the initial concentration of 16,384 µg/mL, the stock solution was diluted in sterile distilled water so that the DMSO concentration in the natural product had no activity in the cells tested. The reference antifungal was fluconazole (Capsule-FLUCOMED), diluted in sterile water at the same oil concentration [27]. For microbiological assays, two *Candida albicans* strains were used: CA INCQS 40006 (standard strain), obtained from the Oswaldo Cruz Culture Collection of the National Institute for Quality Control in Health (INCQS) and CA URM 4387 (clinical isolate), provided by mycology collection of the Federal University of Recife (URM - University Recife Mycology). For the antifungal activity test the culture media were used: Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB). For the fungal micromorphology evaluation test the nutrient-poor Potato Dextrose Agar (PDA) culture medium prepared with solidification Agar was used. The media were prepared according to the supplier's guidelines (Difco®) and autoclaved at 121 °C within 15 min.

4.4. Determination of Minimum Inhibitory Concentration (MIC)

To perform this experiment the broth microdilution method was used according to Javadpour. First the yeasts were inoculated in ASD and kept incubated at 37 °C for 24 h. Subsequently, the inoculum were prepared by transferring small aliquots of the strains to tubes containing sterile saline, the inoculum were compared to McFarland scale resulting in a concentration of 1×10^5 cells/mL. 96-well plates were filled containing in each well 100 μ L of SDB and 10% fungal inoculum. The plates were then microdiluted with 100 μ L of the essential oil of *P. caldense*, where the well concentrations were from 8192 μ g/mL to 64 μ g/mL. The last well was not diluted as a growth control. Media sterility controls and substance dilution controls were also used, using only saline without fungal inoculum. The plates were incubated at 37 °C for 24 h and subsequently read on a 630 nm wavelength ELISA spectrophotometer (Thermoplate®). All assays were performed in triplicate and the results obtained were used to construct the cell viability curve and the IC₅₀ of essential oil and fluconazole [6,32]. MIC was defined as the lowest concentration able to reduce the fungal growth curve.

4.5. Evaluation of Modulating Activity of Natural Product

The essential oil was tested at sub-inhibitory concentration (MIC/16) according to the method proposed by Coutinho et al., 2008 [33]. The plates were filled with a solution containing SDB, fungal inoculum and the essential oil, then 100 μ L of fluconazole was mixed into the first well and serially microdiluted at a ratio of 1:1 to the penultimate well, at 1 μ g/mL. Control of culture media sterility and antifungal dilution control were performed, and the MIC of fluconazole was also determined. The tests were performed in triplicate and the plates were incubated at 37 °C for 24 h. The reading was performed in an ELISA spectrophotometer (Thermoplate®). The modulatory activity is defined when used in combination. The natural product enhances the action of the antifungal, showing synergism. If the opposite occurs and the natural product interferes with the action of the drug, the effect is considered antagonistic.

4.6. Determination of Minimum Fungicidal Concentration (MFC)

After the MIC test, a sterile stem was placed in each well of the plates, first the stem was used to mix the solutions contained in the wells, then small aliquots with medium, inoculum and essential oil were transferred to Petri dishes containing solid medium SDA, for yeast subculture and verification of cell viability. After 24 h of incubation, the plates were analyzed and the concentration at which no fungal colony growth was observed is considered the Minimum Fungicidal Concentration of the essential oil [34].

4.7. Effect of Natural Product on Fungal Morphology

To verify whether *P. caldense* volatile terpenes cause any change in fungal morphology by inhibiting hyphae emission, micromorphological sterile chambers were mounted for yeast observation. On the blade chamber (sterile) were poured 3 mL of medium PDA, poor nutrient for dilution, containing the natural product in CFM/4 concentrations CFM/8 and CFM/16. Aliquots of fungal inoculum were taken from SDA-containing Petri dishes to make two parallel strips in the solidified solid medium (PDA) and then covered by a sterile coverslip. The chamber were taken to the incubator and after 24 h (37 °C) the culture was visualized under optical microscopy using a 400 X objective. A camera was attached to the microscope for image capture. A control for yeast growth (with hyphal emission stimulated by depletion of the medium) was performed, as well as a control with the reference antifungal fluconazole was also used for comparative purposes [35].

4.8. Statistical Analysis

The IC₅₀ was calculated by means of linear regression. Subsequently, data from *P. caldense* antifungal assays were investigated by one-way analysis of variance (ANOVA), using the Bonferroni

test and considered significant when $p < 0.05$. All analyzes were performed on the GraphPad Prism 6.0 software.

5. Conclusions

The essential oil of *P. caldense* presented mono- and sesquiterpene components, though not presented a major constituent. In addition, it showed low antifungal activity for *C. albicans* strains but was able to modulate the effect of standard drug (fluconazole) and decreased the virulence of these strains. In this way, the oil has in its composition constituents that are promising in the formulation of drugs used in the treatment of infectious diseases.

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