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# Bioprospecting of the antifungal activity of Patchouli essential oil (*Pogostemon cablin* Benth) against strains of the genus *Candida*

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**The incidence of infections caused by *Candida* has increased worldwide and these strains are becoming resistant to their respective drugs, requiring the search for new drugs with greater efficacy and safety. Natural products are an alternative means of treatment. The essential oil (EO) stands out among these products, due to some biological activities relevant to human health, including antibiotic and antifungal activities, among others. The objective of this study was to evaluate the antifungal activity of the EO of *Pogostemon cablin Benth* using laboratory and experimental model. In evaluating antifungal activity, different strains of *Candida* genus was used, the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) were determined by microdilution technique. The interference of the EO on the cell wall and its potential for rupture was done by MIC in the presence of sorbitol, and the interference on the cell membrane was done by MIC with ergosterol. The interference of the EO on the resistance to the standard antifungal drug, in the absence and in the presence of the EO in sub-inhibitory concentrations, was also evaluated. The analysis performed revealed that the EO showed excellent antifungal activity against clinical strains of C. *parapsilosis* and *C. albicans* with a MIC and CFM between 4 and 16 µg/mL. Mechanism of action involved no effect on the cell wall as well as on the plasma membrane. It promoted a synergistic effect when associated with amphotericin B. It is hoped that these results can contribute to the study of biological activities of natural products.**

**Key words:** *Pogostemon cablin* Benth essential oil, anti-fungal activity, *Candida* genus.

## INTRODUCTION

Invasive fungal infections represent an increasingly common threat to human health (Firacative, 2020), with *Candida* species serving as the leading cause of fungal infections and the fourth most prominent source of bloodstream infections globally, resulting in over 750,000 infections and a 40% mortality rate globally each year (McCarty and Pappas, 2016; Tsay et al., 2020). The incidence of infections caused by *Candida* has increased

worldwide, with mortality rates exceeding 70% in certain populations. *C. albicans*, *C. glabrata, C. tropicalis, C. parapsilosis* and *C. krusei* account for more than 90% of *Candida* infections (Singh et al., 2020).

The emergence of antifungal resistance remains an ever present threat to the limited antifungal arsenal. Treatment of systemic *Candida* infections is currently limited to only three major drug classes; azoles, polyenes and echinocandins (Whaley et al., 2017; Mohammad et al., 2018). The scarcity of antifungal drug classes, together with the intrinsic plasticity of the fungal genome, promotes fungal adaptation and survival under antifungal drug stress. An increasing number of Candida species resistant to first-line antifungal treatments (azoles or echinocandins) have been identified alongside high antifungal usage environments, eliminating almost all current treatment options (Healey et al., 2016). This trend is accompanied by an increase in the clinical prevalence of multidrug-resistant isolates (Murphy and Bicanic, 2021).

Given this premise, arises a great need for development of new drugs with greater efficacy and safety, and in this logic, the use and search for plant-derived drugs have accelerated in recent years. Natural products possess several biological activities relevant to human health, including antibiotic, antifungal, anticancer, immunosuppressive, anti-inflammatory and biofilm inhibitory activities (Pham et al., 2019).

The essential oils (EOs) have been prominent among natural products used all over the world. They are derived from aromatic plants, are volatile oily liquids composed mainly of terpenoids and phenolic acids (Da silva et al., 2021). Some of the most reported properties of EOs are antibacterial (Cho et al., 2020), antifungal (D'agostinho et al., 2019), antiviral (Ma and Yao, 2020) and antioxidant (Leyva-lópez et al., 2017), mainly due to the disruption of bacterial and fungal membranes and viral envelopes (Böhme et al., 2014).

Among these oils, the one that deserves research interest is the essential oil from the Patchouli ("Guanghuoxiang") plant or scientifically known as *Pogostemon cablin* Benth, belonging to the Lamiaceae family. Several bioactive compounds have been identified in Patchouli, including terpenoids, phytosterols, flavonoids, lignins, glycosides, alcohols, pyrones, and aldehydes. Among the numerous compounds, Patchouli alcohol, β-patchoulene, patchoulene epoxide, pogostone and pachypodol are of great importance (Junren et al., 2021). Modern studies have revealed several biologica activities such as antioxidant, analgesic, anti-inflammatory, antiplatelet, antithrombotic, aphrodisiac, antidepressant, antimutagenic, antiemetic, fibrinolytic and cytotoxic activities (Hussain et al., 2011; Junren et al., 2021; Swamy and Sinniah, 2015). The antifungal activity of some compounds from the essential oil of *P. cablin* Benth against *C. albicans* has been reported in the literature (Zhou et al., 2018).

Based on the information about the therapeutic potential of medicinal plant essential oils and the importance of combating fungal infections, this work aims to evaluate the possible antifungal activity of *P. cablin* Benth essential oil against some strains of the genus *Candida*.

## MATERIALS AND METHODS

### Test substance

The product submitted for biological tests was coded as OE-*Pogostemon cablin* Benth from the Quinarí® located in Ponta Grossa-Paraná. This Quinari oil comes from Indonesia and is obtained by steam-dragging the leaves of the *Pogostemon cablin* Benth plant. It is a brown, woody-smelling liquid. It was solubilized in 0.02% between 80 and dimethyl sulfoxide (DMSO) up to 0.5% and completed with deionized water.

### Culture media

The culture media to be used in the biological activity evaluation were Sabouraud Dextrose Agar (ASD), Sabouraud Dextrose Broth (CSD) (DIFCO Laboratories/USA/France) and RPMI 1640-L-glutamine without bicarbonate (LGC Biotechnology/Brazil) prepared according to the manufacturers' descriptions.

### Fungical cepas

For the EO biological activity assays, the following yeasts of the genus *Candida* were used: (1). *Candida albicans* (C a ATCC 76485, C a LM - 62, C a LM - 106, C a LM - 108 and C a LM - 111): (2) *Candida parapsilosis* (C. p. ATCC 22019, C. p. LM - 02, C. p. LM - 04, C. p. LM - 09 and C. p. LM - 14).

These belong to the Mycology Laboratory, Research Laboratory: Antibacterial and Antifungal Activity of Natural and/or Synthetic Bioactive Products Department of Pharmaceutical Sciences (DCF), Center of Health Sciences (CCS), Federal University of Paraiba. All strains were kept in ASD at 4°C. For the assays, replicates of 24 to 48 h in ASD, incubated at 35 ± 2°C were used.

### *Inoculum*

For preparation of the inoculum, colonies obtained from cultures of *Candida* spp. strains maintained in DSA were suspended in sterile 0.85% sodium chloride solution and adjusted according to McFarland's 0.5 standard to first obtain an inoculum of $10^6$ CFU/mL and then diluted in saline solution in a 1:9 ratio to finally result in a fungal suspension containing $10^5$ CFU/mL that was used in the assays (Koneman et al., 2008; Ostrosky et al., 2008).

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**Minimum inhibitory concentration (MIC)**

The antifungal activity assays were performed according to the protocols of Cleeland and Squires (1991), Hadacek and Greger (2000) and NCCLS/CLSI (2002). The determination of the MIC of the essential oil on *Candida* strains was performed using the broth microdilution technique in a cell culture plate (TPP/ SWITZERLAND/EUROPA) containing 96 wells. Initially, 100 µL of double concentrated RPMI 1640 was dispensed into the holes of the microdilution plates.

Then, 100 µL of the double concentrated essential oil test emulsion was dispensed into the wells of the first row of the plate. And by serial dilution at a ratio of two, concentrations from 1024 to 4 µg/mL were obtained. Finally, 10 µL of the *Candida* spp. inocula were added to the cavities, where each column of the plate refers to a fungal strain, specifically. In parallel, it was inoculum control (RPMI 1640 + yeast); and standard drug control (RPMI 1640 + inoculum + amphotericin B 100 µg). The prepared and aseptically closed plates were subjected to incubation at a temperature of 35 ± 2°C for 24 to 48 h. The MIC was defined as the lowest concentration of the EO, capable of producing visible inhibition on fungal growth seen in the holes, when compared to their controls. The result was expressed as the arithmetic mean of the MICs obtained in the assay performed in duplicate.

The antifungal activity of the essential oil was interpreted and considered as active or inactive, according to the following criteria, 50-500 µg/mL= strong/optimal activity, 600-1500 µg/mL= moderate activity; > above 1500 µg/mL=weak activity or inactive EO (Holetz et al., 2002; Sartoratto et al., 2004; Houghton et al., 2007).

**Minimum fungicidal concentration (MFC)**

After reading the MIC, aliquots of 10 µL of the supernatant from the wells where complete inhibition of fungal growth was observed (MIC, MIC x 2 and MIC x 4) in the microdilution plates, were subcultured in 100 µL of RPMI 1640 contained in new cell culture plates. Subsequently, these properly prepared plates were incubated at 35± 2°C for 24 to 48 h. The CFM was considered as the lowest concentration at which there was no yeast growth in the culture medium. The assays were performed in duplicate and the result expressed by the arithmetic mean of the MFC's obtained in the two assays (Ncube et al., 2008).

**Essential oil action on the fungal cell wall**

To investigate the action of EO on the fungal cell wall, an assay was performed with sorbitol, an osmotic protector used to stabilize fungal protoplasts. If OE acts in any way on the fungal cell wall, it will cause lysis of its cells when in the absence of an osmotic stabilizer, but will allow its growth in the presence of this osmotic support. Thus, this assay compares the MICs of the EO in the absence and presence of 0.8 M sorbitol.

The determination of the EO MIC, in the presence of sorbitol (0.8M), was performed by the microdilution method, using microdilution plates containing 96 wells, similar to the this assay was performed with *C. albicans* ATCC 76485 and *C. albicans* LM-62 strains. In a column of two separate plates, 100 µL of the liquid medium Sabouraud dextrose broth - CSD previously added to 0.8 M sorbitol was added to all 12 wells. In the next column, 100 µL of CSD without sorbitol was added to all 12 wells. Subsequently, 100 µL of the solution of *P. cablin* Benth EO, at a concentration of 1024 µg/mL, were dispensed in the wells of the first row of the column of the plate to which it was determined. By serial dilution at a ratio of two, concentrations from 512 up to 1 µg/mL of *P. cablin* Benth EO were obtained.

Finally, 10 µL of the species inoculum was added to the cavities of both plates, where each column of the plate refers to a fungal strain (Frost et al., 1995).

A microorganism control was performed by placing in the cavities 100 µL of the same CSD and sorbitol (0.8 M) also doubly concentrated and 10 µL of the inoculum of each species. A sterility control was also performed, where 200 µL of the CSD was placed in a hole without the fungal suspension. The same procedure was performed with one wall-acting and one non-wall-acting antifungal to control the results. The plates were aseptically closed and incubated at 35°C for 48 h and then read (Frost et al., 1995).

**Interaction with ergosterol**

Many drugs available for clinical use interact directly with ergosterol, causing the rupture of the fungal cell membrane and loss of intracellular content. To determine whether the essential oil or its constituents bind to fungal membrane sterols, the MIC of these products for *C. albicans* was determined with and without the addition of ergosterol. If the activity of the essential oil is caused by binding to ergosterol, the exogenous ergosterol will prevent the binding with the fungal membrane ergosterol and as a consequence, the MIC of this EO tends to increase in the presence of the exogenous ergosterol compared to the control assay. If the MIC of the EO remains unchanged in the presence of different exogenous ergosterol concentrations, it is suggested that this EO does not act by binding to membrane ergosterol. Similarly, it can be observed if this behavior is specific for ergosterol or if it happens similarly with cholesterol.

The determination of the MIC of the products (OE and amphotericin B) against strains of *C. albicans* ATCC 76485 and *C. albicans* LM- 62 was performed by microdilution, using microdilution plates containing 96 wells, similar to the protocol described in item 6. The culture medium (CSD) was used in the absence and in the presence of 400 µg/mL of ergosterol. A micro-organism control was performed by placing in the wells 100 µL of the same CSD and ergosterol at the same concentrations and 10 µL of the inoculum of each species. A sterility control was also performed, where 200 µL of the CSD was placed in a hole without the fungal suspension. Finally, the same procedure was performed with amphotericin B, whose interaction with ergosterol is already known, to serve as a positive control of the results. The plates were sealed and incubated at 35°C for 24 to 48 h and readings were taken (Escalante et al., 2008).

**Interference of patchouli essential oil (*P. cablin* Benth) against Amphotericin B**

To evaluate if the EO modulates the action of amphotericin B against *C. albicans* ATCC 76485 and *C. albicans* 62 strains, the MIC of this antifungal was determined by microdilution technique in the absence and in the presence of EO in subinhibitory concentrations (MIC/8). The MIC values of the antifungal were then compared in the absence and presence of the OE MIC/8. The plates were incubated for 24 h at 35 to 37°C. The tests were performed in triplicate and the result expressed by the geometric mean of the results (Coutinho et al., 2008; Eliopoulos and Moellering, 1991).

## RESULTS AND DISCUSSION

The minimum inhibitory concentration (MIC) in liquid medium was tested and determined for the essential oil of *P. cablin* Benth at the different concentrations suggested in the methodology and determined by the

**Table 1.** The evaluation of the MIC (µg/mL) of *P. cablin* essential oil on *C. parapsilosis* strains.

| Fungal strain | *Pogostemon cablin* EO | Anfotericina B (100 µg) | Yeast control |
|---|---|---|---|
| *C. p.* ATCC 22019 | NI | - | + |
| *C. p.* LM - 02 | 8 | - | + |
| *C. p.* LM - 04 | 8 | - | + |
| *C. p.* LM - 09 | 8 | - | + |
| *C. p.* LM - 14 | 8 | - | + |

(-) no yeast growth; (+) yeast growth; (NI) not identified
Source: Authors 2022

**Table 2.** Evaluation of the CFM minimum fungicidal concentration (µg/mL) of *P.cablin* essential oil on *C. parapsilosis* strains.

| Fungal strain | *Pogostemon cablin* EO | Anfotericina B (100 µg) | Yeast control |
|---|---|---|---|
| *C. p.* ATCC 22019 | NI | - | + |
| *C. p.* LM - 02 | 8 | - | + |
| *C. p.* LM - 04 | 8 | - | + |
| *C. p.* LM - 09 | 8 | - | + |
| *C. p.* LM - 14 | 8 | - | + |

(-) absence of yeast growth; (+) yeast growth; (NI) Not identified.
Source: Authors 2022

**Table 3.** The evaluation of the MIC (µg/mL) of *P. cablin* essential oil on *C. albicans* strains.

| Fungal strain | *Pogostemon cablin* EO | Anfotericina B (100 µg) | Yeast control |
|---|---|---|---|
| *C a* ATCC 76485 | 4 | - | + |
| *C a* LM - 62 | 4 | - | + |
| *C a* LM - 106 | 4 | - | + |
| *C a* LM - 108 | 4 | - | + |
| *C a* LM - 111 | 16 | - | + |

(-) absence of yeast growth; (+) yeast growth; (NI) Not identified.
Source: Authors 2022

lowest concentration capable of inhibiting visually. The results of the assays evaluating the antifungal activity of Patchouli (*P. cablin* Benth) essential oil against strains of *C*. *parapsilosis* and *C. albicans* are shown below.

It was observed that for *C*. *parapsilosis* all clinical strains tested were sensitive to *P. cablin* Benth essential oil showing an MIC of 8 µg/mL for most strains, as shown in Table 1.

Regarding the minimum fungicidal concentration (MFC) of *P. cablin* Benth essential oil on *C. parapsilosis* strains, it was observed that the values were 8 µg/mL for most strains, shown in Table 2. Similar to C. *parapsilosis*, all clinical strains of *C. albicans* tested were sensitive to *P. cablin* Benth essential oil, with MICs ranging from 4 to 16 µg/mL for all strains, shown in Table 3. It can be seen that Patchouli essential oil showed superior activity against *C. albicans* strains when compared to the activity against C. *Parapsilosis* strains.

Regarding the Minimum Fungicidal Concentration (MFC) of *P. cablin* Benth essential oil on *C. albicans* strains it was observed that the values ranged from 4 to 16 µg/mL for all strains, shown in Table 4

According to Sartorato et al. (2004) the antimicrobial activity of essential oils is classified as strong when they have MICs up to 500 µg/mL, moderate for MICs from 600 to 1500 µg/mL and weak for MICs above 1500 µg/mL.

The essential oil of *P. cablin* Benth showed a strong fungicidal effect against all strains, as the MIC for the *Candida* genus was less than 500 µg/mL.

According to Hafidh et al. (2011) for a compound to be considered fungicidal or fungistatic according to the minimum fungicidal concentration (MFC) should be equal to or twice the MIC or the MFC should be greater than twice the MIC. Analyzing the result of CFM it can be observed that *P. cablin* has fungicidal activity against all strains since the MIC was equal for CFM. This result

**Table 4.** Evaluation of the CFM minimum fungicidal concentration (µg/mL) of *P. cablin* essential oil on *C. albicans* strains.

| Fungal strain | *P. cablin* EO | Anfotericina B (100 µg) | Yeast control |
|---|---|---|---|
| *C a* ATCC 76485 | 4 | - | + |
| *C a* LM - 62 | 4 | - | + |
| *C a* LM - 106 | 4 | - | + |
| *C a* LM - 108 | 4 | - | + |
| *C a* LM - 111 | 16 | - | + |

(-) no yeast growth; (+) yeast growth; (NI) not identified
Source: Authors 2022

**Table 5.** Effect of *P. cablin* EOs on *C. albicans* strains ATCC 76485 and *C. albicans* LM- 62 in the absence and presence of ergosterol 400 µg/mL and 0.8 M sorbitol.

| Microorganism | MIC EO of *P. cablin* (µg/mL) | | | |
|---|---|---|---|---|
| | Absence of sorbitol | Presence of sorbitol | Absence of ergosterol | Presence of ergosterol |
| *C. albicans* ATCC 76485 | 512 | 512 | 512 | 512 |
| *C. albicans* LM-62 | 256 | 256 | 64 | 64 |

Source: Authors 2022

corroborates with the literature, where antifungal activity against *C. albicans* was demonstrated with MIC values of 300 to 500 µM of *P. cablin* Benth oil (Zhou et al., 2018).

Other studies that corroborate the importance of this EO and the data from this research include Pimenta et al. (2019) when evaluating the antifungal activity of *P. cablin* Benth essential oil, observed antifungal activity on *C. glabrata* strain. As well, Cavalcante et al. (2018) tested the antifungal ability of patchouli oil against *Candida tropicalis* strains. The author in question concluded that *P. cablin* oil can be considered a strong inhibitor against *C. tropicalis* strains, as it showed a MIC of 128 µg/ml (capable of inhibiting the growth of 50% of the strains).

Moreover, Alves et al. (2019) demonstrated that *P. cablin* oil has antifungal ability against *Candida krusei* strains, showing MIC and CFM of 32 µg/mL. Whereas, Adhavan et al. (2017), demonstrated that patchouli oil has antimicrobial activity against Gram-positive and Gram-negative microorganisms, with MIC of 25 mg/mL against *Streptococcus mutans* isolates and 12.5 mg/mL against *Shigella flexneri* and *S. aureus* in *in vitro* studies.

Antifungal activity was also demonstrated recently by Aisyah et al. (2021), who reported the combination with citronella oil and Patchouli oil resulted in more effective inhibition of the fungus *Aspergillus niger* compared to nystatin, which is a commercial antifungal.

The fungal cell wall acts as a barrier, conferring rigidity and strength. It is composed of several macromolecules essential for fungal survival such as β-glucan, chitin and other proteins and is an important target for antifungal agents (Lee and Kim, 2016). To evaluate the action on the cell wall, the sorbitol technique was used and it was observed that even in the presence and absence of sorbitol, *P. cablin* essential oil showed the same MIC value in both situations, the same profile was observed when analyzed in the presence and absence of ergosterol.

As shown in Table 5, the EO of *P. cablin* Benth had no alteration in the MIC both in the presence and absence of sorbitol and ergosterol, demonstrating that the mechanism of action does not involve the cell wall or plasma membrane, and may involve another mechanism of action, thus suggesting that further studies should be carried out to understand the mechanism of action.

Other mechanisms can be suggested by which essential oils can act, promoting their antifungal action, such as: Inhibition of the efflux pump, the action on the membrane of fungal mitochondria, acting in the inhibition of biofilm development and inhibiting the synthesis/ production of mycotoxins, among others (Nazzaro et. al., 2017).

Another hypothesis to be looked at is strain resistance that results in reduced sensitivity to certain compounds and is caused by a hereditary adjustment of the fungus to that compound. It is usually due to single or multiple genetic mutations. Intrinsic resistance can be explained by the combined effect of an expansion of multi-drug transporters in the genome and nine amino acid changes in Erg11 sequence, which are known to confer significant resistance in *C. albicans* (Nishikawa et al., 2016; Revie et al., 2018). Fungicide resistance may be due to (a) reduced fungicide binding due to altered target site, (b) overexpression of target protein, (c) reduced fungicide uptake due to efflux pump removing toxic compounds,

**Table 6.** Modulation of the antifungal action in its MIC by *P. cablin* in MIC/8 against *C. albicans* strains.

| Microorganism | Amphotericin B MIC (µg/mL) | |
|---|---|---|
| | Only Amphotericin B | Associated with EO of *P. cablin* (MIC/8) |
| C. albicans ATCC 76485 | 0.5 | 0.5 |
| C. albicans LM-62 | 2 | 0.5 |

Source: Authors 2022

and (d) metabolic degradation of fungicide through detoxification (Sánchez-Torrez, 2021).

Some works reported the combination of different antifungal agents or natural products and standard drugs in reducing their MIC values (D'agostino et al., 2019). With the performance of this work, it can be observed that the two types of strains of the genus *Candida* exposed to the combination of the EO and the standard drug obtained as a value of MIC 0.5, giving emphasis to the clinical strain *C. albicans* LM-62, shown in Table 6.

The synergistic effect was evident when amphotericin B was linked with Patchouli essential oil (*P. cablin* Benth) by reducing the MIC, according to Lewis et al. (2002). Therefore, a synergistic interaction is observed when the killing ability of the first agent is increased by a sub-inhibitory concentration of the second agent. An antagonistic interaction is present if the antimicrobial effect of the first component is inhibited by the second. Because one agent is used at sub inhibitory concentrations, this assay cannot distinguish additive interactions (combined activity equals the sum of individual activities) from indifferent interactions (Singh et al., 2000).

The importance of synergism in clinical research is referenced in some studies. Fu et al. (2007) observed increased antifungal effects caused by combinations (1:5, 1:7 and 1:9) of essential oils of *S. aromaticum* (clove) and *R. officinalis* against *C. albicans*. Therefore, this result is very significant, since the use of antifungal therapy combined with an EO to obtain a synergistic effect may be a therapeutic option. From the study there need for new antifungal drugs due to the increasing resistance of fungal strains against the limited repertoire of commercialized antifungal drugs (Lee et al., 2021).

## Conclusion

This study had shown that the essential oil of *P. cablin* Benth showed a strong fungicidal effect against *C. albicans* and *C. parapsilosis* strains. In relation to *C. albicans*, it was observed that the effect is independent of the action on the cell wall as well as on the plasma membrane and the EO shows synergistic effect when associated with the standard drug, amphotecin B. However, more studies should be conducted to better unveil the antifungal effect observed by this natural product. Therefore, Patchouli essential oil has becomes a promising candidate for use, because of its bioactive properties as phyto-therapeutics and antifungal drugs. This class of drugs is highly needed in clinics.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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