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Effects of associating linalool with nystatin and fluconazole against *Candida albicans* strains from vulvovaginal secretions

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ABSTRACT. Vulvovaginal candidiasis is one of the most common fungal infections in women, at some point in their lives affecting approximately 75% of this population. It is primarily caused by the fungus *Candida albicans* and can be triggered by many factors, such as antibiotic use, diabetes, pregnancy, and use of hormonal contraceptives. The current large increase in mycotic infections caused by *C. albicans* and frequent therapeutic failure of conventional antifungals, when added to the development of microorganism resistance has led to the need for antifungal studies on the capacity of monoterpenes. Monoterpenes present excellent microbicidal potential in addition to their capacity in combination therapies. Our work aimed to investigate the effects of associating the monoterpene linalool with the antifungal drugs nystatin and fluconazole against *C. albicans* strains from vulvovaginal secretions. *In vitro* microdilution assays were conducted in RPMI-1640 broth, and the Checkerboard method applied, using amphotericin B as a control. The study results indicate linalool's strong anti-*C. albicans* activity and synergism with fluconazole. Linalool presented a fractional inhibitory concentration index (FICI = 0.25) against *C. albicans* ATCC 76485. However, linalool in combination with nystatin resulted in an indifferent effect. Yet linalool presented antifungal potential, as its association with fluconazole reversed *C. albicans* resistance in *in vitro* assays.

Keywords: Linalool; *Candida albicans*; Synergism; Fluconazole; Fungal resistance.

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Introduction

Candidiasis is a fungal disease that affects many individuals. It is characterized by exacerbated yeast growth with the potential to affect multiple anatomical sites. The skin and mucous membranes are the most common sites of infection, and especially the mouth and vagina (Ben-Ami, 2018). Vulvovaginal candidiasis (VVC) is a fungal infection caused by many *Candida* species, yet in most cases, *Candida albicans* is involved. A total of 75% of women of fertile age develop VVC at least once in their lives (Carvalho et al., 2021), and the disease brings various discomforts such as: pruritus, pain, irritation, dyspareunia, and abnormal vaginal discharge among others causing great distress and impacting the overall health of the female population (Yano et al., 2019).

Standard treatment for VVC consists of therapy with a topical antifungal agent or oral fluconazole (Pappas et al., 2016; Sarsi et al., 2021). Fluconazole is a fungistatic agent and selectively inhibits enzyme-dependent fungal cytochrome P-450 lanosterol 14 α-demethylase, which is encoded by the *ERG11* gene. It converts lanosterol into ergosterol, the principal lipid of the fungal cell membrane (Whaley et al., 2017). Since fluconazole is fungistatic rather than fungicidal, the possibility of fungal cells developing resistance is greater (Berkow & Lockhart, 2017).

In this context, discovery and development of antifungal molecules with fungicidal properties and few adverse effects is important (d'Enfert et al., 2021). Linalool, a lipophilic monoterpene from aromatic plants such as *Lavandula angustifolia* (Lavender) (24.30%), of the *Lamiaceae* Martinov family and native to the Mediterranean coast, presents known antifungal activity and appears to be promising (Chen et al., 2020). Combined pharmacological therapy is important in fungal infections involving resistant pathogens. Concomitant administration of two or more drugs enables a better spectrum of action, use of smaller doses, and minimization of adverse effects (Nidhi et al., 2020; Rosata et al., 2021).

Our study aimed to investigate the effects of combining linalool with the antifungal drugs nystatin and fluconazole against *C. albicans* strains from vulvovaginal secretions.

Material and methods

Chemicals and microorganisms

The monoterpene linalool [3,7-dimethyl octa-1,6-dienol-3] (purity: 98.7%) was purchased commercially from Quinarí®, Ponta Grossa, PR, Brazil. The working solution was prepared at the time of testing and was duly solubilized in 150 µL (3%) of dimethyl sulfoxide (DMSO) with 100 µL (2%) of Tween 80. This was completed with sterile distilled water (q.s.p. 5.0 mL^{-1}) to obtain an emulsion at an initial concentration of 1024 µg mL^{-1} (Nascimento et al., 2007). The medications used in this study were fluconazole (FLU), nystatin (NYS), and amphotericin B (AMB), which were purchased commercially from Sigma-Aldrich® (São Paulo, Brazil) and prepared according to the manufacturers' specifications and in the desired concentrations.

The fluconazole-resistant clinical strains of *C. albicans* LM 129 originated from vulvovaginal secretions. The *C. albicans* strain ATCC® 76485 was used as the standard. Both strains belong to the collection of the Antibacterial and Antifungal Activity Research Laboratory for Natural and/or Synthetic Bioactive Products at the Federal University of Paraíba, Paraíba, Brazil (UFPB). The fungal strains were maintained in test tubes containing Sabouraud dextrose agar (SDA) culture medium (DIFCO Laboratories Ltd), at a temperature of 4°C. For the assays, 48-hour replicates incubated at 35 ± 2°C were used.

Checkerboard synergy assays

Combinations of linalool and the commercial antifungals (nystatin and fluconazole) were tested in triplicate against *C. albicans* strains (LM 129 and ATCC 76485) using a 96-well plate microdilution method. Initially, 100 µL of RPMI-1640 broth was added to the plate wells and linalool (50 µL) was then added in the vertical direction and the antifungal agent (nystatin or fluconazole) (50 µL) was added in the horizontal direction. Linalool was thus tested at different concentrations (MIC × 8, MIC × 4, MIC × 2, MIC, MIC/2, MIC/4, and MIC/8). Finally, 20 µL of the fungal suspensions were supplemented with an inoculum of approximately 1-5 × 10^6 colony-forming units per milliliter (CFU mL^{-1}) standardized according to the 0.5 tube on the McFarland scale. The plates were then aseptically sealed and incubated at 35 ± 2ºC for 48 hours (Bassolé & Juliani, 2012; Clinical And Laboratory Standards Institute [CLSI]., 2017).

The fractional inhibitory concentration index (FICI) was calculated by summing the FIC(A) + FIC(B), where A stands for antifungal and B for linalool. The FIC(A) was calculated by the relation: minimum inhibitory concentration (combined MIC(A) / MIC(A) alone), and FIC(B) analogously (Equation1).

$$\text{FIC index} = \text{FIC}_\text{A} + \text{FIC}_\text{B} = \frac{\text{MIC}_\text{A} \text{ in combination}}{\text{MIC}_\text{A} \text{ tested alone}} + \frac{\text{MIC}_\text{B} \text{ in combination}}{\text{MIC}_\text{B} \text{ tested alone}} \quad (1).$$

The index was interpreted as follows: synergism (⩽ 0.5), additivity (> 0.5 and < 1), indifference (⩾ 1 and < 4), and antagonism (⩾ 4.0) (Lewis et al., 2002).

Results

Effects of combining linalool and nystatin against *C. albicans*

The MIC of linalool alone against the strains analyzed was 64 µg mL^{-1} (Table 1). Further, it was observed that the *C. albicans* strains studied were sensitive to nystatin, and that association with linalool resulted in an indifferent interaction, as can be verified through the FIC index. Linalool has significant antifungal activity, however, from the point of view of multidrug therapy, its combination with nystatin was not interesting since it resulted in an indifferent interaction, not improving nor impairing the sensitivity profile of the microorganism under study.

Table 1. MIC of linalool and the effects of the combination with the antifungal nystatin (NYS) against *C. albicans* LM 129 and *C. albicans* ATCC 76485.

Substance/	*C. albicans* LM 129		*C. albicans* ATCC 76485	
Drug	MIC (µg mL^{-1})	FIC index (Interaction type)	MIC (µg mL^{-1})	FIC index (Interaction type)
Linalool	64	-	64	-
NYS	4	-	4	-
Linalool/NYS	64/4	3.0 (indifferent)	64/4	2.25 (indifferent)

Cut off points: MIC of Nystatin ⩽ 4 (S); 8 - 32 (S-DD); ⩾ 64 (R) μg mL^{-1}. S: susceptible; S-DD: susceptible dose-dependent; R: resistant (Pádua et al., 2003).

Effects of combining linalool and fluconazole against *C. albicans*

For the drug fluconazole, both strains tested were resistant (Table 2). In addition linalool (given its lower MIC) was shown to have a better activity profile compared to fluconazole. For the strain LM 129 we verified an indifferent effect for the linalool - fluconazole association, and synergism for the strain ATCC 76485. Thus, it seems that the linalool and fluconazole associating may be promising, since it was able to improve the sensitivity profile of the microorganism.

Table 2. MIC of linalool and the effects of association with fluconazole (FLU) against *C. albicans* LM 129 and *C. albicans* ATCC 76485.

Substance/	*C. albicans* LM 129		*C. albicans* ATCC 76485	
Drug	MIC (μg mL^{-1})	FIC index (Interaction type)	MIC (μg mL^{-1})	FIC index (Interaction type)
Linalool	64	-	64	-
FLU	> 1024	-	32	-
Linalool/FLU	64/ >1024	3.0 (indifferent)	64/32	0.25 (synergistic)

Cut off points: MIC of fluconazole ⩽ 2 (S); 4 (S-DD); ⩾ 8 (R) μg mL^{-1}, - Document M60. S: susceptible; S-DD: susceptible dose-dependent; R: resistant ([CLSI], 2017).

Discussion

Combination drug therapy is recommended and used to treat various mycoses (Hsieh et al., 2018). However, combination therapy with natural products is less explored. The advantages over monotherapy include increases in fungal elimination, reductions in the emergence of resistant strains, and minimization of antifungal drug dose-related toxicity (Maurya et al., 2020).

Recent studies relate that antifungal agent associations or combinations with natural products can reduce individual MIC values against fungal strains (Herman & Herman, 2021).

In this study, the association of nystatin with the monoterpene linalool was used against strains of *C. albicans* (Table 1) which were found sensitive to nystatin according to the interpretation criteria reference values (Pádua et al., 2003). Further, it is observed that the tested strains were sensitive to linalool, which inhibited fungal growth at 64 μg mL^{-1}. This demonstrates a pharmacological effect on these strains which Morales et al., 2008 classify as strong antifungal activity (MIC < 100 μg mL^{-1}). However, linalool in association with nystatin demonstrated an indifferent effect, and consequently no advantages in their concomitant administration.

The need to search for new alternatives and/or different antifungal therapeutic approaches has been emphasized by the growing incidence of fungal resistance to classical drugs, their toxicity, and the costs of the available treatments. Many studies report a growing incidence of *C. albicans* strains resistant to commonly used antifungals. For fluconazole, this phenomenon is quite common (Sobel & Sobel, 2018; Awad et al., 2021). Combining antifungals with phyto-medicines is often an effective method for improving their activity (Bhattacharya et al., 2021).

Houshmandzad et al., (2022) observed that linalool presents antifungal activity against both *Candida* sp. and azole-resistant strains, resulting in further research involving clinical applications. (Table 2) highlights the antifungal activity of linalool (alone) (MIC = 64 μgmL^{-1}), as well as the resistance of fungal strains tested against fluconazole (alone) (MIC > 1024 μg mL^{-1}). However, from another perspective, the synergistic effects of linalool when in association with fluconazole (FICI of 0.25) were observed for *C. albicans* strain ATCC 76485.

For Park et al. (2007), the synergistic interactions reported for phyto-pharmaceuticals with fluconazole may be related to simultaneous inhibition of differing fungal targets by that of the test molecule and of fluconazole. Fluconazole acts by inhibiting the activity of lanosterol 14α-demethylase which results in the interruption of ergosterol biosynthesis. The integrity of the cell wall and cell membrane, together with other membranous structures are the target sites for linalool. This effect can be attributed to the lipophilic properties of the molecule and indicates its ability to penetrate the plasma membrane (Tariq et al., 2019). In addition, the fungicidal action of many monoterpenes (including linalool) has been observed to reduce the usual required dose of fluconazole. Since fluconazole is a hydrophilic azole, it is less effectively absorbed than

lipophilic azoles such as ketoconazole, clotrimazole, and itraconazole by lipidic components of fungal cells (Scheven & Schwegler, 1995).

Cell wall lysis by lipophilic compounds can result in higher absorption, more availability, and greater lethality to these microorganisms. Cell wall lysis also facilitates the conversion of fluconazole from a fungistatic mode to a fungicidal mode of action. The linalool - fluconazole association is comparable to the synergistic interaction of azoles with inhibitors of cell wall synthesis (i.e., echinocandins) (Spitzer et al., 2016). The exact mechanism of cell wall and membrane synthesis disruption by monoterpenes is not yet fully understood, however in combination therapy, multiple active target sites are an advantage.

Importantly, linalool exhibits no photo-toxicity or genotoxicity. Several *in vitro* models, such as bacteria and fibroblast cells, as well as *in vivo* models with (Sprague-Dawley rats) have been used to assess the genotoxicity of linalool, and all have confirmed that it causes no changes in genetic material (Aprotosoaie et al., 2014; Marnett et al., 2014; Api et al., 2015). *In vivo* rabbit and rat studies have indicated that after rapid intestinal absorption, linalool functions as an enzymatic inducer of the microsomal cytochrome P-450 system, which metabolizes the monoterpene into 8-hydroxy linalool and 8-carbox linalool, which are mainly excreted by the urinary tract (Chadha & Madyastha, 1984).

Despite their therapeutic properties, the use of phytochemicals extracted from essential oils, such as linalool, may be limited due to their low solubility and bioavailability. To overcome these limitations, drug delivery systems can be used as alternative and versatile platforms to enhance their bioactive effects (Koziol et al., 2014). Although solubilizing agents, such as dimethyl sulfoxide (DMSO), might improve linalool bioavailability, they may also increase cellular toxicity and undesirable side effects. Further, due to its volatile nature, linalool is unstable and has a short shelf life, limiting its clinical application (Rodríguez-López et al., 2020). Linalool is lipophilic, this results in low water solubility. To overcome this limitation, recent studies have explored the complexation of linalool with cyclodextrins (Paiva-Santos et al., 2022).

Cyclodextrins are supramolecular structures characterized as forming rings, with β-cyclodextrins as the most common form used in drug delivery. The β-cyclodextrins have a truncated cone shape and are composed of seven glucopyranoside units. In cyclodextrin complexes, the hydrophilic outer surface confers water solubility while the hydrophobic inner cavity allows the inclusion of lipophilic compounds such as linalool. Nanoscale delivery systems can also be used to naturally encapsulate linalool (Carneiro et al., 2019; Paiva-Santos et al., 2022).

The use of lipid nanoparticles is widely described in the scientific literature as a solution to overcome challenges associated with the delivery of natural compounds such as flavonoids, monoterpenes, polyphenols and carotenoids (Al-Nasiri et al., 2018). Such nanoparticles exhibit a wide range of important characteristics, including adequate particle size (40-1000nm), large surface areas, high loading capacity, controlled release of the active compound, and ease of large-scale production, as well as both biocompatibility and biodegradability (Carbone et al., 2018; Rodríguez-López et al., 2020). The physicochemical difficulties associated with linalool are largely avoided by its loading in lipidic nanoparticles (Pereira et al., 2018).

Conclusion

The combination therapies with synergistic activity are being explored as an alternative to the singular antimicrobials currently in clinical use which have become ineffective due to microbial resistance.

In the present study, it was possible to observe both the antifungal activity of linalool and the synergistic effect of this monoterpene when in association with fluconazole against *C. albicans* strains, making these once-resistant strains susceptible. Additionally, toxicological analysis of linalool in the scientific literature is vast, demonstrating its pharmacokinetic parameters - Administration, Distribution, Metabolism, Excretion and possible Toxicity (ADMET) which are similar to licensed drugs. However, pharmacological and pharmacodynamic evaluation including toxicity studies of its synergistic phytochemical/antifungal associations need to be explored before studies in humans.

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