Interactions between Terpinen-4-ol and Nystatin on biofilm of *Candida albicans* and *Candida tropicalis*

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The aim of this study was to evaluate the antifungal activity of Terpinen-4-ol associated with nystatin, on single and mixed species biofilms formed by *Candida albicans* and *Candida tropicalis*, as well as the effect of terpinen-4-ol on adhesion in oral cells and the enzymatic activity. The minimum inhibitory concentrations and minimum fungicide concentrations of terpinen-4-ol and nystatin on *Candida albicans* and *Candida tropicalis* were determined using the microdilution broth method, along with their synergistic activity ("checkerboard" method). Single and mixed species biofilms were prepared using the static microtiter plate model and quantified by colony forming units (CFU/mL). The effect of Terpinen-4-ol in adhesion of *Candida albicans* and *Candida tropicalis* in coculture with oral keratinocytes (NOK Si) was evaluated, as well as the enzymatic activity by measuring the size of the precipitation zone, after the growth agar to phospholipase, protease and hemolysin. Terpinen-4-ol (4.53 mg mL-1) and nystatin (0.008 mg mL-1) were able to inhibit biofilms growth, and a synergistic antifungal effect was showed with the drug association, reducing the inhibitory concentration of nystatin up to 8 times in single biofilm of *Candida albicans*, and 2 times in mixed species biofilm. A small decrease in the adhesion of *Candida tropicalis* in NOK Si cells was showed after treatment with terpinen-4-ol, and nystatin had a greater effect for both species. For enzymatic activity, the drugs showed no action. The effect potentiated by the combination of terpinen-4-ol and nystatin and the reduction of adhesion provide evidence of its potential as an anti-fungal agent.

Key Words: terpinen-4-ol, nystatin, *Candida albicans*, *Candida tropicalis*, synergism.

Introduction

In the oral cavity, Candida species adhere to many sites, such as mucosal cells, the tongue, surfaces of teeth and dentures, and other microorganisms. To induce disease, they must adhere to epithelial cells of the oral mucosa and subsequently invade and destroy these cells. To improve its ability to colonize and establish infections, the fungus produces exoenzymes, such as aspartyl protease and phospholipase, which degrade the extracellular matrix and inhibit phagocytosis by neutrophils to induce inflammatory reactions, and hemolysin. Then, they acquire iron, which facilitates the invasion and hyphal development of disseminated candidiasis (1,2).

Most cases of candidiasis have been attributed to *Candida albicans* and *Candida tropicalis*, identified as the most causative agents of candidemia. Recently, an increasing number of infections caused by non-albicans Candida species has been reported, and have been identified as the infecting pathogens isolated from various clinical specimens, which also demonstrated the production of virulence factors once attributed to *Candida albicans* (2,3).

Several antifungal agents are used in the prevention and treatment of Candida genus, as nystatin, the most commonly used in the treatment of oral candidiasis due to its fungicidal and fungistatic characteristics (4). However, the widespread use of topical and systemic antifungal agents has resulted in the development of resistance in Candida species.

Several mechanisms of azole resistant species have been reported, including changes in the cell wall or plasma membrane, which impairs azole uptake; overexpression or mutations in the target enzyme of azoles and the efflux of drugs mediated by membrane transport proteins (5). Resistance appears to increase proportionally with the extent of previous exposure to antifungal drugs. In addition, studies have shown some medications may cause injuries in the kidney and liver (6), which highlights the need to develop new therapeutic strategies, including, for example, a search for agents with novel mechanisms of action that may be used independently or in combination with conventional medicines.

Plant extracts and phytochemical compounds are derived from constituents of plants and/or semi-synthetic derivatives and have shown encouraging results on oral pathogens and control of the inflammatory response, serving as a vehicle for the prevention and control of infectious oral disease (7).

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Terpinen-4-ol is a monoterpene that is found in plants such as Melaleuca alternifolia, Hajeb Layoun arboreta (Tunisia) and Alpinia zerumbet; it has broad-spectrum antimicrobial activity and anti-inflammatory properties (8). In vitro antifungal activity was demonstrated in planktonic cultures of Candida albicans in different types of assays, such as minimum inhibitory concentration and minimum fungicidal concentration assays (9,10).

The combination of drugs is an approach that has been used to enhance the effect of antimicrobial therapy for resistant microorganisms. Studies have shown synergistic effects when antifungal agents are combined (11). Thus, the nature and severity of drug interactions should be studied to verify that small concentrations of drug combinations have better effects on isolates, resulting in better efficacy and low toxicity (12,13).

Thus, the aim of this study was to evaluate the synergistic/additive antifungal effect of terpinen-4-ol and nystatin on planktonic cultures and biofilms of Candida albicans and Candida tropicalis in vitro. In addition to analyze the inhibition of the adhesion of yeast in oral epithelial cells (NOK) and the inhibition of the hydrolytic enzymes protease, phospholipase and hemolysin.

Material and Methods

Fungal Strains and Growth Conditions

Candida albicans (ATCC 90028) and Candida tropicalis (ATCC 4563) were used in this study. The Candida species were grown at 37 °C for 18 h in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA), pH 7.0 buffered with 0.165 M MOPS (3- (N-morpholino) propanesulfonic acid) supplemented with 2% glucose. The obtained cultures were adjusted to OD at 600 nm (OD 600), until the absorbance of 1.0 to 0.75 for Candida albicans and Candida tropicalis, corresponding to 1.0×10^7 colony forming units per mL (CFU mL^{-1}).

Preparation Solutions

Dilutions of terpinen-4-ol (Sigma-Aldrich, St. Louis, MO, USA; CAS number: 20126-76-5) were prepared at concentrations ranging from 9.16 to 1.06 mg mL^{-1} in RPMI-1640 medium, pH 7.0 (0.165 M MOPS), 2% glucose (Life Technologies, Carlsbad, CA, USA) together with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 0.4% to increase the solubility of the oil (14).

Nystatin solution (Sigma-Aldrich, St. Louis, MO, USA; CAS Number: 1400-61-9) was prepared (DMSO) as a stock solution at a concentration of 0.064 mg mL^{-1} and subsequently diluted from 0.064 to 0.00012 mg mL^{-1} (RPMI-1640 medium, pH 7.0, 0.165 M MOPS, 2% glucose), according to the M27-A2 standard CLSI (15).

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC was determined using the broth microdilution technique (CLSI - M27-A2) (15). The compounds in serial dilutions were added to 96-well microtiter plates. Terpinen-4-ol and nystatin were added to rows and columns, respectively. The fungal suspensions prepared in RPMI with a final concentration of 1.0×10^3 CFU mL^{-1} were added to each well. The microorganism in the absence of terpinen-4-ol and nystatin was used as a growth control, and the absence of microorganisms and drugs was used to verify no medium contamination. A group for DMSO was performed to prove that it does not interfere with the results. After 24 h at 37 °C in a shaker at 75 rpm (incubator, 430, Vargem Grande Sao Paulo, SP, Brazil), the wells were assessed visually and by absorbance at OD 590 nm in an ELISA reader (Multiskan, Ascent 354 EC Labsystems, Les Ulis, France), and the MIC was defined as the lowest concentration of each drug that was able to inhibit the growth completely according to spectrophotometry (590 nm).

The wells that showed no visible growth, that is, 600 nm≤0.05, 10 µL samples were withdrawn and subsequently plated on SDA culture medium (Saboraude Dextrose Agar, Acumedia, Lansing, MI, USA) and maintained at 37 °C for 48 h. MFC was defined as the lowest concentration able to reduce the initial inoculum ≥99.9%. All experiments were conducted in triplicate and in three different moments.

Single and Mixed Species Biofilms

According to Rossoni et al. (16), for the formation of either single or mixed species biofilms, Candida albicans suspensions and/or Candida tropicalis were transferred to microtiter plates (96 wells) and maintained at 37 °C for 1 h and 30 min with stirring at 75 rpm. The wells were washed twice with sterile PBS, RPMI 1640 was added. After 48 h, the culture medium was removed, and the tested solutions (terpinen-4-ol and nystatin) were added as performed in the tests with planktonic microorganisms. After 24 h at 37 °C, the resulting biofilm was washed with PBS.

The biofilm was shaved, carefully aspirated and transferred to Falcon tubes. The suspension was diluted and plated in culture medium (for single specie biofilms, SDA (Sabouraud Dextrose Agar Acumedia, Lansing, MI, USA) and mixed species biofilms, CHROMagar Candida). The plates were maintained at 37 °C for 48 h and quantified results in CFU mL^{-1}.

Fractional Inhibitory Concentration Index (FICI)

The results of both tests with planktonic microorganisms and biofilms were evaluated by the lowest concentrations of each drug able to inhibit the growth completely. For

this, the MICs defined for terpinen-4-ol and nystatin from the Candida strains were converted to fractional inhibitory concentrations (FICs) with the "checkerboard" method. These are equivalent to the ratio of the MIC of drug A and B combined with the MIC of each drug, as described below. Thus, the fractional inhibitory concentrations index (FICI) is calculated by the sum of the fractional inhibitory concentrations of each drug used (17).

FICA = MIC of drug A with B / MIC of drug A alone
FICB = MIC of drug B with A / MIC of drug B alone
FICI = FICA + FICB

In this evaluation, the FICI was interpreted as indicative of synergism when ≤0.5, additive when >0.5 and≤1, indifferent when >1.0 and and ≤4.0, and antagonism when >4.0 (17). All experiments were conducted in triplicate and in three different moments.

CLSM Analysis

In order to verify the biofilms formation, *Candida albicans* and *Candida tropicalis* biofilms were developed on glass cover slip discs for 48 h and, after treatment with terpinen-4-ol and nystatin, the discs were transferred to a new 12-well plate and washed 3 times with 1 mL of sterile phosphate-buffered saline (PBS) to remove unattached microorganisms. The microorganisms were then labeled with 0.01 mM of Syto-9 and 0.06 mM of propidium iodide (PI), LIVE/DEAD stain BacLight Bacterial Viability Kit (Invitrogen Corporation, Grand Island, NY), for 15 min, according to the manufacturer's instructions. Then, the discs were visualized with a 40x oil-immersion objective using an confocal laser scanning microscopy (CLSM) (Leica, TCS SPE, Leica Microsystems, Mannheim, Germany). The excitation/emission used for these stains were approximately 480/500 nm for SYTO-9 stain and 490/635 nm for PI. A series of optical cross-sectional images was acquired at 0.63μm deep intervals from the surface through the vertical axis of the specimen. The confocal images were then exported to LAS AF – TCS SP5.

Adhesion Assay in Co-culture

The normal cell line NOK Si (normal immortalized oral cells) was used. The cells were maintained and cultured as monolayers in cell culture flasks in DMEM (Dulbecco's Modified Eagle's medium - Gibco Laboratories) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 2% TBA antibiotic (complex streptomycin, penicillin, gentamicin) and 2.20 g L^{-1} sodium bicarbonate, and maintained in an atmosphere of 5% CO_2 at 37 °C. After confluency, the medium was removed, and the cells were washed with PBS (phosphate buffered saline) and trypsinized. Using this cell suspension, 24-well plates were prepared containing 1 x 10^5 cells per well (5). After the

formation of plaques in the cell monolayer, the inoculum of *Candida albicans* or *Candida tropicalis* (1x10^3 CFU mL^{-1}) together with terpinen-4-ol or nystatin at a concentration established in previous tests (1,06 mg mL^{-1} terpinen-4-ol for both species and 0,002 and 0,001 mg mL^{-1} nystatin for *Candida albicans* and *Candida tropicalis,* respectively) were added to the wells, and the plates were incubated at 36.5 °C and 5% CO_2 for 2 h (the incubation time for *Candida albicans* and *Candida tropicalis* with NOK Si cells was determined with a compliance curve from previously performed tests). Then, cells were washed three times with sterile PBS, trypsinized and plated onto Sabouraud agar. After 48 h, the colonies that grew on the plates that corresponded to the number of cells that adhered to yeast were counted, and the data were transformed into colony forming units (CFU mL^{-1}). As a control, adhesion tests were performed without treatment and were considered 100% adhesion. The results were analyzed as percentage graphs.

Proteinase, Phospholipase and Hemolysin Assay

Candida species were tested for phospholipase, protease and hemolysin activity according Sardi et al. (2), in which the test medium was agar proteinase basic bovine serum albumin and phospholipase medium containing egg emulsion. The hemolysin activity was evaluated using blood agar plates. Candida species were grown on SDA for 24 h, subjected to contact with the tested solutions (terpinen-4-ol and nystatin at the concentrations established in previous tests) for the time previously determined in the adhesion test (2 h); after this time, a single drop (10 μL) of each species containing 10^8 CFU mL^{-1} (absorbance 0.5 at 600 nm) was inoculated into the assay medium. The plates were incubated at 37 °C for 48 h. The assay was performed on three separate occasions for each isolate. As a negative control, the inoculum of each species was incubated with culture medium (RPMI 1640).

The enzyme activity was determined by measuring the halo formed around the yeast colony and the ratio between the diameter of the colony and the overall diameter of the colony plus the precipitation zone (PZ) according to the method described by Sardi et al. (2). According to this system, Pz=1.0 indicates that the test strain was negative for proteinase/phospholipase/hemolysin, while a value of Pz=0.63 means that this species releases large amounts of enzymes (strongly positive). Pz values between 0.64 and 0.99 represent smaller quantities of enzymes (positive).

Statistical Analysis

The data on the activity of terpinen-4-ol in the adhesion of *Candida albicans* and *Candida tropicalis* in NOK Si cells were analyzed for the presence of outliers and for the assumptions of normality (Shapiro-Wilk test) and

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homogeneity of variances (Levene's test). Then, ANOVA was performed, followed by Tukey's HSD test to identify the existence of significant differences between the groups (control, terpinen-4-ol and nystatin) for each studied strain and the dependent variable was analyzed was colony-forming units (CFU mL^{-1}) after being log transformed (CFU mL^{-1}). Inferential analyses were performed with SPSS software (v.17, SPSS Inc, Chicago, IL) considering statistically significant effects whose p-value was less than or equal to 0.05.

Results

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

For both species, *Candida albicans* (ATCC 90028) and *Candida tropicalis* (ATCC 4563), the MIC of terpinen-4-ol was 2.31 mg mL^{-1} and the CFM was 4.53 mg mL^{-1}. The MIC for nystatin in Ca was 0.004 mg mL^{-1} and Ct was 0,002 mg mL^{-1}, whereas the MFC was 0.008 and 0.004 mg mL^{-1} for Ca and Ct, respectively.

Table 1 shows the values of the Fractional Inhibitory Concentration Index and activity presented by the combination of terpinen-4-ol and nystatin as tested by the "checkerboard" method on planktonic cultures of *Candida albicans* and *Candida tropicalis*. For *Candida albicans*, an additive effect was found at some concentrations and synergism with concentrations of terpinen-4-ol of 1.06 mg mL^{-1} and nystatin 0.00012 mg mL^{-1}. For *Candida tropicalis*, an additive effect was observed at terpinen-4-ol concentration of 1.06 mg mL^{-1} and nystatin concentrations of 0.001-0.0003 mg mL^{-1}, and terpinen-4-ol was able to reduce the MIC of nystatin up to 128 times in Ca and up to 64 times in Ct. This demonstrates that terpinen-4-ol and nystatin possess an antifungal effect on the two species when used singly and that when used in combination, the effect is potentiated.

Single and Mixed Species Biofilms

The concentration of terpinen-4-ol able to inhibit biofilm growth was 4.53 mg mL^{-1} and 2.31 mg mL^{-1} for *Candida albicans* and *Candida tropicalis*, respectively. Nystatin inhibited the growth of both Candida species at a concentration of 0.008 mg mL^{-1}.

In single biofilms, *Candida albicans* exhibited an additive relationship with the combination of terpinen-4-ol (2.31 mg mL^{-1}) and nystatin (0.002 to 0.001 mg mL^{-1}), in which terpinen-4-ol was able to reduce the inhibitory concentration of nystatin up to 8 times. However, *Candida tropicalis* showed no synergism/additive effect (Table 2).

In a mixed species biofilm of Ca and Ct, the concentration of terpinen-4-ol able to inhibit biofilm growth for both species was 9.16 mg mL^{-1}, and nystatin was 0.008 mg mL^{-1}. The combination of both drugs resulted in a indifferent effect, except for *Candida tropicalis* at concentrations of terpinen-4ol 2.31 mg mL^{-1} and nystatin 0.004 mg mL^{-1} and terpinen-4-ol reduced the inhibitory concentration of nystatin 2 times (Table 2).

Table 2 shows the concentrations produced by synergism/additive effect. Therefore, the results for *Candida tropicalis* in a single biofilm and *Candida albicans* mixed species biofilms are not shown because they did not exhibit any type of activity.

CLSM Analysis

The representative image of viability and spatial arrangement of the biofilms formed by *Candida albicans* and *Candida tropicalis* was obtained from CLSM (Fig. 1). The images (z-slices) (Fig. 1D, E) showed that after applications

Table 1. Synergistic / modulating activity of the terpinen-4-ol and nystatin on planktonic culture of *Candida albicans* and *Candida tropicalis*

| Planktonic suspension | Terpinen-4-ol + Nystatin | | | Effect |
	Terpinen-4-ol (CIM)	Nystatin (CIM)	FICI	
Candida albicans	1.06 mg mL^{-1}	0.002 mg mL^{-1}	0.96	Aditism 2x
	1.06 mg mL^{-1}	0.001 mg mL^{-1}	0.71	Aditism 4x
	1.06 mg mL^{-1}	0.0005 mg mL^{-1}	0.58	Aditism 8x
	1.06 mg mL^{-1}	0.00025 mg mL^{-1}	0.52	Aditism 16x
	1.06 mg mL^{-1}	0.00012 mg mL^{-1}	0.49	Sinergism 32x
	1.06 mg mL^{-1}	0.00006 mg mL^{-1}	0.61	Aditism 64x
	1.06 mg mL^{-1}	0.00003 mg mL^{-1}	0.53	Aditism 128x
Candida tropicalis	1.06 mg ml^{-1}	0.001 mg mL^{-1}	0.96	Aditism 2x
	1.06 mg ml^{-1}	0.0005 mg mL^{-1}	0.71	Aditism 4x
	1.06 mg ml^{-1}	0.00025 mg mL^{-1}	0.58	Aditism 8x
	1.06 mg ml^{-1}	0.00012 mg mL^{-1}	0.52	Aditism 16x
	1.06 mg ml^{-1}	0.00006mg mL^{-1}	0.49	Aditism 32x
	1.06 mg ml^{-1}	0.00003mg mL^{-1}	0.61	Aditism 64x

Table 2. Synergistic / modulating activity of the terpinen-4-ol and nystatin on single and mixed species biofilm of *Candida albicans* and *Candida tropicalis*

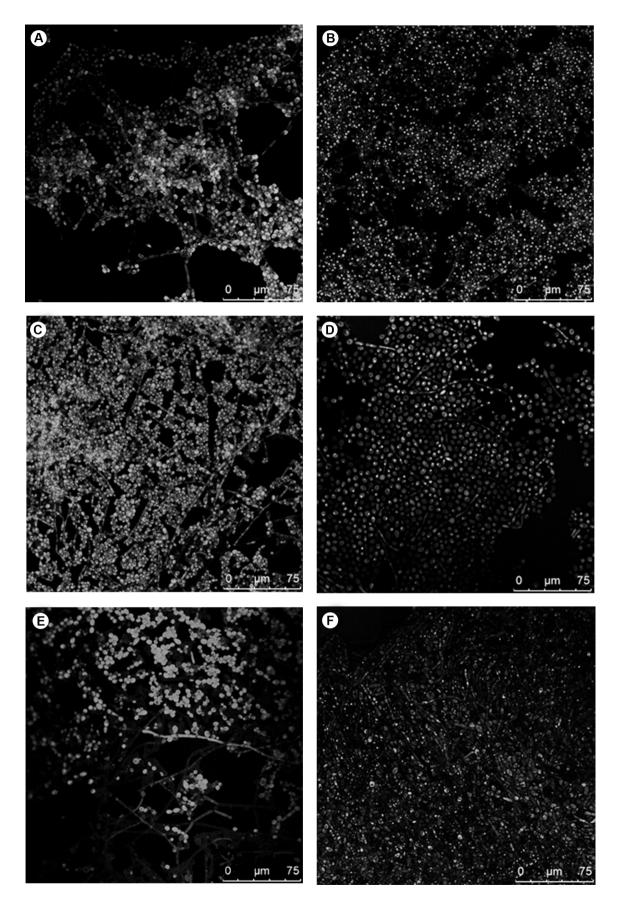| Biofilm | Terpinen-4-ol + Nystatin | | | | |
	Specie	Terpinen-4-ol (CIM)	Nystatin (CIM)	FICI	Effect
Simple biofilm	*Candida albicans*	2.31 mg mL^{-1}	0.002 mg mL^{-1}	0.76	Aditism 4x
		2.31 mg mL^{-1}	0.001 mg mL^{-1}	0.63	Aditism 8x
Dual biofilm	*Candida tropicalis*	2.31 mg mL^{-1}	0.004 mg mL^{-1}	0.76	Aditism 2x

Figure 1. CLSM images (z-slices) of biofilms grown on cover slip discs stained with fluorochromes SYTO-9 and PI. A, B Images of single biofilm of *Candida albicans* and *Candida tropicalis*, respectively, from the control group. C Image of mixed species biofilm, from the control group. D, E Images of single biofilms of *Candida albicans* and *Candida tropicalis*, respectively, after application of Terpinen-4-ol 2.31 mg mL^{-1} and nystatin 0.001 mg mL^{-1}. F Image of mixed species biofilms after application of Terpinen-4-ol 2.31mg mL^{-1} and nystatin 0.004 mg mL^{-1}.

of Terpinen-4-ol 2.31mg mL^{-1} and nystatin 0.001 mg mL^{-1} on single biofilms of Ca and Ct, respectively, and the image (Fig. 1F) showed mixed species biofilm after application of Terpinen-4-ol 2.31mg mL^{-1} and nystatin 0.004 mg mL^{-1}, there were a visual increase in cell death (dark grey cells, which corresponds to red fluorescence), great amount of black spaces between cells when compared with control groups, respectively (Fig. 1A, B, C).

Adhesion Assay in Co-culture

The standardization curve for the adhesion of *Candida albicans* and *Candida tropicalis* cells in NOK Si (time of the first occurrence of yeast adhesion in cell culture and the time with a higher compliance rate) was determined, and after two hours, there was a greater association.

When the adhesion of *Candida albicans* in NOK Si cells was assessed after contact with terpinen-4-ol (1.06 mg mL^{-1}) and nystatin (0.004 mg mL^{-1}), there was no statistically significant difference between the group tested with terpinen-4-ol and the control group (no treatment). Regarding the group tested with nystatin, there was a significant reduction in the adhesion of *Candida albicans*, as shown in Figure 2. The evaluation performed with *Candida tropicalis*, there was a small reduction in adhesion when terpinen-4-ol was used (1.06 mg mL^{-1}) but this reduction was greater for nystatin (0,002 mg mL^{-1}) compared to the control group ($p < 0.05$).

Proteinase, Phospholipase and Hemolysin Assay

Candida species were tested for phospholipase and proteinase activity according to Sardi et al. (2012). The activity of hydrolytic enzymes was detected by measuring the size of the precipitation zone, after the growth agar to phospholipase, protease and hemolysin.

As shown in Table 3, terpinen-4-ol and nystatin had no action on these virulence factors (production of proteinase, phospholipase and hemolysin). The values of the ratio of the measures of the colonies and their halos did not decrease compared to the control group, which did not receive any treatment. For *Candida tropicalis*, no phospholipase was produced in any of the groups studied.

Discussion

Low concentrations of terpinen-4-ol were able to inhibit the growth of planktonic Candida

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species in vitro, presenting a fungicidal effect that was also shown in the literature (18,19). In single and mixed species biofilms, terpinen-4-ol also showed antifungal activity for *Candida albicans* and *Candida tropicalis*, corroborating the findings of Ramage et al. (19), which showed similar results as the present study and suggested that biofilms composed of more than one species are more resistant.

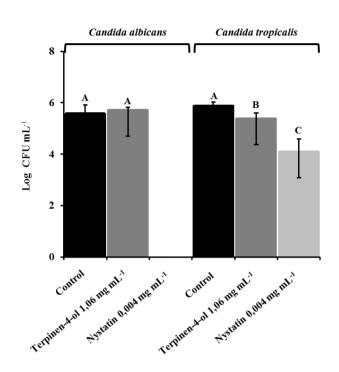Scientific evidence shows that the use of nystatin in the

Figure 2. Effect of terpinen-4-ol and nystatin on adhesion of *Candida albicans* and *Candida tropicalis* co-cultured with normal immortalized oral cells (NOK Si). Statistics differences are represented by different letters for comparison between treatments (ANOVA One-way and Tukey test, $p < 0.05$).

Table 3. Average values of the ratios between the measures of the colonies and their halos (Pz) and enzymatic activity (proteinase, phospholipase and hemolysin) of *Candida albicans* (Ca) and *Candida tropicalis* (Ct) after 2 h of contact of terpinen-4-ol and nystatin

Pz		Control		Terpinen-4-ol		Nystatin	
		Enzimatic Activity	Pz	Enzimatic Activity	Pz	Enzimatic Activity	
Proteinase	Ca	0.474446	Moderate	0.626366	Moderate	0.583228	Moderate
	Ct	0.366024	High	0.378112	High	0.464813	Moderate
Phosfolipase	Ca	0.676846	Moderate	0.647597	Moderate	0.508426	Moderate
	Ct	-	-	-	-	-	-
Hemolisyn	Ca	0.491761	Moderate	0.495198	Moderate	0.398225	High
	Ct	0.417553	Moderate	0.41421	Moderate	0.349847	High

treatment of oral fungal infections is recommended because it can be applied topically and presents reduced side effects (20). However, fungal resistance has been reported, and the association between synthetic and natural antifungals presents itself as an alternative to reducing the dosage and unwanted side effects and prevent the development of resistance to antifungal drugs (21).

One of the simplest and best known protocols to investigate the antimicrobial activity of synthetic products or natural agents is the "checkerboard" test, which provides a two-dimensional arrangement of different concentrations of substances and allows the required FICI to be calculated to evaluate the combination of synergism, additive effect, indifference or antagonism (17). The simultaneous use of antimicrobial agents can maximize the chances of success in the therapeutic process, thereby improving the spectrum of action, the time optimization of treatment intervals and doses, and even strength reduction.

The results shown here using the "checkerboard" technique were effective, and when used together, terpinen-4-ol and nystatin have synergistic and additive effects in some combined concentrations. To illustrate the effect of this combination on biofilm, analysis by CLSM was performed. The images show a great amount of black spaces between cells when the applications of terpinen-4-ol and nystatin were performed, in comparison with the controls groups. Thus, the imagens obtained by CLSM confirm the data obtained in this study.

Furthermore, as previously described, nystatin may produce side effects in the liver and kidneys and cause microbial resistance. Thus, the results presented herein show that terpinen-4-ol was able to lower the inhibitory concentrations of nystatin when used together, reducing planktonic cultures up to 128 times in *Candida albicans* and up to 64 times in *Candida tropicalis*. In sinlge-specie biofilms terpinen-4-ol was able to reduce the inhibitory concentrationof nystatin up to 8 times with Ca, and 2 times in mixed biofilms, with Ct. Thus, this combination (terpinen-4-ol and nystatin) may reduce the quantity of nystatin used in antifungal therapies, thus reducing undesirable side effects.

Synergistic effects were also observed among other essential oils and medicines. Monoterpenes (thymol and carvacrol) and fluconazole were also synergistic against 38 isolates of Candida (12). In addition, other studies have been conducted involving antimicrobials that are already available on the market, such as fluconazole, amphotericin, and caspofungin (13).

The elucidation of the mechanisms of action of agents with pharmacological potential, regardless of whether they are of natural or synthetic origin, contributes to the rational development of therapeutic approaches, especially in terms of infections caused by resistant microbes, which often require combinations of drugs or the use of new drugs when the first-choice agent is not effective.

Monoterpenes such as terpinen-4-ol are hydrophobic molecules that can attach to the lipophilic structures of microorganisms, such as the plasma membrane, which leads to increased permeability and loss of essential electrolytes for cell survival (22). According to Carson et al. (22), treatment with Melaleuca alternifolia oil results in changes in mitochondrial membranes for 95% of *Candida albicans*, Candida glabrata and Saccharomyces cerevisiae by glucose-induced acidification. The acidification occurs to expel the plasma membrane proton ATPase, which is powered by ATP derived from mitochondria. The inhibition of this function suggests that plasma and/or mitochondrial membranes were negatively affected.

In the study by Ramage et al. (19), terpinen-4-ol (MIC_{50}=0.25%) was cytotoxic to both fibroblasts and epithelial cells, but at $0.5 \times MIC_{50}$, terpinen-4-ol showed no toxicity. In the study by Nogueira et al. (8), terpinen-4-ol was cytotoxic to macrophages between 0.238 and 0.119%. Over time, evidence has suggested that topical use is safe and that adverse events are minor, self-limited and infrequent.

Microbial adhesion to biological materials is an essential element for the initial step in the infection process. In the presence of microbial adhesins and receptors on host cells, the hydrophobicity of the microbial surface has been described as an important factor that influences the adhesion of microorganisms. To understand the pathogenesis of candidiasis in vivo, in vitro studies have been developed to characterize and quantify the adhesion of Candida to cell surfaces (2). The data from this study indicate that terpinen-4-ol can affect the adherence of *Candida tropicalis* at the MIC_{50} concentration, but only nystatin was effective in inhibitin g the adhesion of both species, especially in *Candida albicans*.

Sudjana et al. (23) have studied the effect of Melaleuca alternifolia oil on the adhesion of *Candida albicans* isolates in mammalian cells (buccal epithelial cells, HeLa, and A549) and showed no significant results, which suggested that changes in the surface hydrophobicity play only a small role in the reduction of adhesion and that additional factors, such as interactions with receptor binding, as mentioned above, and the inhibition of the formation of germ tubes, may be involved.

A statistically significant decrease in the adherence of *Candida albicans* (ATCC 10231) to fibroblasts compared to controls was demonstrated in a study where the organisms were exposed to MIC of essential oils (lemon balm, citronella, geranium clove). However, when the same test was carried out in the presence of half of the CIM, as in this study,

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their adhesion ability was not significantly affected, which corroborates our results (24). According to Sun et al. (11), the resistance of *Candida albicans* biofilms to the penetration of liquid antimicrobials, including terpinen-4-ol, is limited due to its low wettability. This could explain why terpinen-4-ol did not interfere with the adherence of *Candida albicans* in oral cells, as it did not penetrate fungal cells at an MIC_{50}.

The production of virulence factors, such as exoenzymes, by fungi improve the adhesion ability and penetration and proliferation in tissues and thus facilitates the establishment of Candida infections (2). Thus, the study of mechanisms that reduce virulence becomes extremely important to combat oral candidiasis. In the present study, the inhibition of proteolytic activity by terpinen-4-ol and nystatin showed no satisfactory results on *Candida albicans* and *Candida tropicalis*. The production of these virulence factors is in line with reports from other researchers (1). Secreted aspartyl proteases (SAPs) degrades many proteins, such as albumin, hemoglobin, keratin, and secretory immunoglobulin, in lesion sites. The proteolytic activities of these SAPs are important for the virulence of *Candida* spp. Therefore, the inhibition of extracellular proteinase production weakens the colonization of Candida cells and can be easily circumvented by the cells of the immune system.

In the 2009 study by Patel et al. (25), the plant extract used (Dodonaea viscous var. Angustifolia) also had no effect on the production of proteinase and phospholipase. Thus, it was suggested that the low concentrations used in this study altered the surface characteristics but did not penetrate the yeast cells, thus causing changes in the production of hydrolytic enzymes. In addition, the drugs tested were also not effective in inhibiting the production of hemolysin at the concentration used (MIC_{50}). A study of nystatin, fluconazole and amphotericin B showed that isolates of *Candida albicans* and *Candida tropicalis* exhibited reduced hemolysin activity (26). In the study by Khan et al. (27), the oils tested (Carum copticum and Thymus vulgaris) were effective in inhibiting the production of hemolysin using (CIM_{25}); however, the authors claim to have no knowledge of cases of the inhibition of hemolysin production in *Candida albicans* by essential oils. Studies by Costa et al. (28) demonstrated that high concentrations of azoles increased SAP activity on resistant strains. Tests with fluconazole and voriconazole (0,1,1/2 and 1/4 MIC) showed that resistant isolates of *Candida albicans* exhibited high levels of SAP.

The combination of terpinen-4-ol and nystatin presented a potentiated antifungal effect on the two *Candida* species evaluated, showing synergistic and additive effects on planktonic and biofilm cultures. Terpinen-4-ol

reduces the adhesion of *Candida tropicalis* and nystatin reduces the adhesion of both species in immortalized oral cells (NOK Si). The sub-inhibitory concentrations (MIC_{50}) of terpinen-4-ol and nystatin that were used did not show activity in the production of proteinase, phospholipase and hemolysin, except for nystatin that increased the hemolysin activity in both species.

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Resumo

O objetivo desse estudo foi avaliar a atividade antifúngica do Terpinen4-ol associado à nistatina em biofilmes simples e misto, formados por *Candida albicans* e *Candida tropicalis*, bem como o efeito do terpinen-4-ol na adesão em células orais e atividade enzimática. As concentrações inibitórias mínimas e as concentrações fungicidas mínimas do terpinen-4-ol e da nistatina em *Candida albicans* e *Candida tropicalis* foram determinadas pelo método de microdiluição em caldo, juntamente com a atividade sinérgica (método do tabuleiro de "xadrez"). Biofilmes simples e misto foram preparados usando o modelo de placa de microtitulação estática e quantificados por unidades formadoras de colônias (CFU/mL). O efeito do Terpinen-4-ol na adesão de *Candida albicans* e *Candida tropicalis* em co-cultura com queratinócitos orais (NOK Si) foi avaliado, bem como a atividade enzimática, medindo o tamanho da zona de precipitação, após o crescimento em ágar fosfolipase, protease e hemolisina. O terpinen-4-ol (4.53 mg mL-1) e a nistatina (0,008 mg mL-1) conseguiram inibir o crescimento de biofilmes e um efeito antifúngico sinérgico foi demonstrado com a associação de fármaco, reduzindo a concentração inibidora de nistatina até 8 vezes em biofilme simpes de *Candida albicans* e 2 vezes em biofilme misto. Uma pequena diminuição na adesão de *Candida tropicalis* em células NOK Si foi mostrada após o tratamento com terpinen-4-ol e a nistatina teve um efeito maior para ambas as espécies. Para a atividade enzimática, as drogas não apresentaram ação. O efeito potencializado pela combinação de terpinen-4-ol e nistatina e a redução de adesão evidenciam seu potencial como agente anti-fúngico.

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