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Combined effect of Cinnamomum zeylanicum blume essential oil and nystatin on Candida albicans growth and micromorphology

Efeito da combinação entre o óleo essencial de Cinnamomum zeylanicum blume e nistatina sobre crescimento e micromorfologia de Candida albicans

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Abstract

Introduction: *The combination of a natural product and a synthetic antifungal may lead to a lower dose of each individual agent and consequent reduction of adverse effects and greater pharmacological synergism.* ***Objective:*** *This study investigated the antifungal activity of the essential oil (EO) from Cinnamomum zeylanicum Blume alone and combined with nystatin on Candida albicans growth and micromorphology.* ***Methodology:*** *We determined the Minimum Inhibitory Concentration (MIC), Fractional Inhibitory Concentration Index (FIC) and the effect of the EO alone and combined with nystatin on the growth kinetics and production of virulence-related structures by the yeasts, such as pseudohyphae and chlamydoconidia.* ***Results:*** *When tested alone, the EO from C. zeylanicum and nystatin showed MIC of 312.5 µg/ml and 64 µg/ml, respectively. When combined, MIC values decreased to 39 µg/ml and 32 µg/ml for the EO and nystatin, respectively. The value of the Fractional Concentration Index (FIC) was 0.6024, indicating additivity. It could be observed that at all concentrations the products tested alone and in combination were able to reduce the number of CFU/mL, when compared to the control group (p<0.0001) from 30 min. In addition, both the products alone and combined inhibited production of pseudohyphae and chlamydoconidia compared to the control.* ***Conclusion:*** *The combination between the essential oil from C. zeylanicum and nystatin potentiated the inhibitory effect on C. albicans growth. Furthermore, it reduced the production of pathogenicity-related morphological structures such as pseudohyphae and chlamydoconidia.*

Key-words: *Cinnamomum zeylanicum. Nystatin. Essential Oil. Natural Product. Drug synergism. Candidiasis.*

Resumo

Introdução: A combinação entre produtos naturais e antifúngicos sintéticos pode acarretar em uma menor dose individual de cada agente e consequente diminuição dos efeitos adversos e maior sinergismo farmacológico. **Objetivo:** O presente estudo investigou a atividade antifúngica do óleo essencial (OE) de Cinnamomum zeylanicum Blume sozinho e combinado com nistatina sobre o crescimento e micromorfologia de Candida albicans. **Metodologia:** Determinou-se a Concentração Inibitória Mínima (CIM), índice de Concentração Inibitória Fracional (FIC) e o efeito do OE sozinho e combinado com nistatina sobre a cinética de crescimento e produção de estruturas de virulência relacionadas tais como pseudohifas e clamidoconídios. **Resultados:** Quando testados isoladamente, o OE de C. zeylanicum e nistatina apresentaram CIM de 312,5 µg/mL e 64 µg/mL, respectivamente. Quando combinados, os valores de CIM referente ao OE e nistatina diminuíram, respectivamente, para 39 µg/mL e 32 µg/mL. O valor do índice de concentração fracional (FIC) foi de 0,6024, indicando aditividade. Pôde-se observar que, em todas as concentrações, os produtos testados isoladamente e em combinação reduziram o número de UFC/mL a partir de 30 minutos quando comparados ao grupo controle (p<0,0001). Além disso, ambos os produtos sozinhos e combinados inibiram a formação de pseudohifas e clamidoconídios em comparação ao grupo controle. **Conclusão:** A combinação entre o óleo essencial de C. zeylanicum e nistatina (antifúngico padrão) potencializou o efeito inibitório sobre o crescimento de *C. albicans*, além disso, reduziu a formação de estruturas morfológicas relacionadas à patogenicidade, tais como pseudohifas e clamidoconídios.

Palavras-chave: Cinnamomum zeylanicum. Nistatina. Óleo Essencial. Produto Natural. Sinergismo farmacológico. Candidíase.

INTRODUCTION

The incidence and prevalence of candidiasis have increased in the last decades, particularly in the large population of HIV-immunocompromised and/or hospitalized patients (1). It is estimated that Candida albicans accounts for over 40 % of all fungal infections worldwide, followed by non-albicans species such as C. tropicalis, C. parapsilosis, C. glabrata and C. krusei (2,3).

Although C. albicans is an opportunistic microorganism that commensally inhabits the human body in up to 75% of healthy individuals (1), its clinical relevance as the major human fungal pathogen has been

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extensively addressed (3-5). In addition to changes in the host's immune response, a mechanism by which C. albicans causes disease lies in its morphological plasticity. This microorganism converts from unicellular yeast cells to either pseudohyphal or hyphal filaments. As such, the growth of hyphae constitutes a pathogenicity mechanism and plays a critical role in enhanced biofilm formation (6), tissue invasion and resistance to phagocytosis (7).

Currently, the relative small number of antifungal classes therapeutically available favors mortality, which is attributed to systemic infections. Allied to this, fungal resistance to antifungal agents becomes a problem for some groups of patients, including the immunocompromised ones (8). Hence, given the evident growth in the number of pathogens resistant to antibiotics used in the medical and dental clinics, there has been an emerging need to introduce new antimicrobial agents in the therapeutic arsenal (9).

In this respect, naturally-occurring products are used as a great source of effective therapeutic agents, offering a wide range of biological active molecules (10). Evidence-based studies have proposed the combination of antifungal compounds conventionally used in the clinics with natural products, in an attempt to promote greater effectiveness of each drug, thus allowing the use of lower doses of both products (11-16). This combination may represent an alternative to eliminate multi-resistant microorganisms by preventing contact with synthetic agents and reducing the risk for selecting new or improved mechanisms of resistance.

Amongst the species with promising antimicrobial activity is Cinnamomum zeylanicum, popularly known as cinnamon (9,17-21). In addition to the antimicrobial property, many beneficial health effects of cinnamon have been reported, such as anti-inflammatory activity, blood glucose control, reducing cardiovascular disease, boosting cognitive function and reducing risk of colonic cancer (22).

In the perspective of drug discovery and improvement, this study investigated the antifungal effect of the combination between the essential oil from Cinnamomum zeylanicum Blume and nystatin on Candida albicans growth and micromorphology.

MATERIALS AND METHODS

Microorganisms

Microbiological tests were performed in the Mycology Laboratory of the Center for Health Sciences, Federal University of Paraiba, which provided strains of C. albicans ATCC 40277.

Essential Oil

The EO of C. zeylanicum was obtained from Ferquima Ind. and Comp. Ltd (Vargem Grande Paulista, São Paulo, Brazil). Its physical and chemical parameters were described by the supplier, which produces and markets essential oils on an industrial scale.

Considering the lipid-solubility of the essential oil, an emulsion was prepared by adding Tween 80 and sterile distilled water, and this mixture was stirred for five minutes in Vortex apparatus. The essential oil concentration used in the study was determined based on the product's density (d = 1.040 g/ml).

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the essential oil and nystatin was determined by the microdilution technique, using 96-well U-bottom microtiter plates (ALAMAR®). Initially, 100 µL of Sabouraud Dextrose Broth (HIMEDIA®, São Paulo, Brazil) doubly concentrated were placed in each well on the plate. Then 100 µL of the emulsion of C. zeylanicum EO and nystatin were added at an initial concentration of 5,000 µg/ml and 128 µg/ml, respectively. Serial dilutions were prepared from these concentrations by taking 100 µL from the most concentrated well and placing it in the following well. Finally, 10 µL aliquots of inoculum were placed into the wells of each column. At the same time, yeast viability was checked. Tests were performed in triplicate, and the plates were incubated at 35°C for 48 hours (23).

The MIC of the EO and control on the yeast strain was determined visually. The formation or non-formation of cell clusters ("buttons") at the bottoms of the wells was considered. MIC was taken as the lowest concentration of the study product capable of producing visible inhibition of the growth of the yeast strain used in the microbiological assay (23).

In order to confirm the presence of viable microorganisms at non-inhibitory concentrations, 10 µL of TTC dye (2, 3, 5 triphenyl tetrazolium chloride) were placed in the wells after 24 hours of incubation. The detection of microorganism viability reflects the activity of dehydrogenase enzymes, which are involved in the fungal respiration process. It makes possible to distinguish the live samples, red-colored, from the dead samples that keep their color (24).

Synergism assay by the Checkerboard method

The combined effect between C. zeylanicum EO and nystatin was determined by the microdilution technique – checkerboard method – for derivation of the Fractional Inhibitory Concentration index (FIC index).

The turbidity of the fungal suspension of C. albicans ATCC 40277 was compared and adjusted to that presented by the barium sulphate suspension (tube '0.5' of McFarland scale), corresponding to an inoculum of approximately 5,0x106 Colony-Forming

Units/ml (CFU/ml). Solutions of the products tested were used at concentrations determined from their respective MIC. Initially, 100 µL of Sabouraud Dextrose culture medium were added into the wells of a 96-well U-bottom microtiter plate (ALAMAR®). Then, 50 µL of each product tested in the concentrations MIC÷4, MIC÷2, MIC, MICx2 and MIC×4 were added in the horizontal (nystatin) and vertical (EO) directions of the plate. Finally, the culture medium was inoculated with 10 µL of fungal suspension. Fungal growth was evidenced through the use of TTC dye. The test was performed in triplicate, and the microplates were incubated at 35ºC for 48 hours (25).

The FIC index was calculated as FICA + FICB, in which A represents the EO and B is nystatin. FICA is calculated through the ratio MICA combined/MICA alone, while FICB is the ratio MICB combined/MICB alone. This index was interpreted as follows: synergism (<0.5), additivity (0.5-1.0), indifference (> 1 and <4) or antagonism (> 4.0) (25,26).

Effect of the essential oil alone and combined with nystatin on yeast growth kinetics

The study of interference of the test product (C. zeylanicum EO) alone or combined with nystatin on the viability of fungal strains was performed using the method of counting viable cells. In this assay, it was observed the behavior of the yeasts strains compared to EO MIC values as well as to those values obtained from the association with nystatin. Initially, 0.5 ml of fungal suspension was inoculated in 4.5 ml of sabouraud broth containing different concentrations of the EO, nystatin and combination of these products (MIC÷2; MIC, MIC×2, MIC×4 and MIC×8). In the intervals 0, 30 min, 60 min, 120 min, 180 min and 24 h after incubation, a 10 µL aliquot of inoculum was uniformly inoculated on Petri plates containing sabouraud dextrose agar (HIMEDIA®, São Paulo, Brazil). An antifungal-free growth control was also checked. The inoculated plates were incubated at 35°C for 48 hours. Thereafter, the counting of viable cells was carried out, and values were expressed as CFU/ml and presented in a graphical form of microbial death curve (27).

Effect of the essential oil alone and combined with nystatin on yeast micromorphology

Morphological changes in the C. albicans yeasts (ATCC 40277) were identified by the technique of microculture for yeasts, using agar-rice in moist chamber (28,29).

Different amounts of the EO emulsion and nystatin, alone and in combination, were added to the agar-rice culture medium, in order to obtain various final concentrations of the products (MIC, MICx2, MICx4 and MICx8). Then 3 ml of agar-rice associated with the products were placed in a Petri plate (90x15 mm) containing a sterile glass slide on a supporter for the microcultive (another slide).

After culture medium solidification, the yeasts were seeded using a needle in "L" and 02 parallel striations were made. The striations were covered with sterile coverslips. To avoid desiccation of the medium, a moist chamber was made during the incubation period by adding 2 ml of distilled water on a piece of sterile filter paper (3x3 cm), which was placed on the plate. The plate was closed and after specific intervals (24h, 48h and 72h) slides underwent analysis by optical microscopy. In each slide, it was observed the presence of typical structures such as pseudohyphae, blastoconidia and chlamydoconidia.

Statistical analysis

Data were statistically analyzed by analysis of variance for parametric distributions, considering a type I error (α) of 0.05 ($p<0.05$). The statistical software package Graphpad Prism version 5.0 (GraphPad, San Diego, CA, USA) was used.

RESULTS AND DISCUSSION

The essential oil of C. zeylanicum and nystatin showed MIC of 312.5 µg/ml and 64 µg/ml on C. albicans, respectively, when evaluated alone (table 1). These findings confirm the data presented by other studies (9,30,31).

The phytochemical profile of the essential oil from C. zeylanicum leaves was previously determined by gas chromatography coupled to a mass spectrometer (GC-MS). A total of 17 components were identified. Among the phytochemicals, eugenol was presented as the major analyte, accounting for over 73 %, followed by trans-β-caryophyllene and benzyl benzoate (data of our research group not published yet).

The antimicrobial activity of the EO obtained from C. zeylanicum has been attributed to the action of trans-cinnamaldehyde, a compound found in large quantity in its composition32. Moreover, eugenol plays an important role against microorganisms of clinical interest (9). This information is reinforced by Schmidt et al. (31), who found that the chemical compounds present in this EO, particularly eugenol, are able to inhibit growth of Candida strains.

With regard to antimicrobial drugs, the use of double or triple combinations starts with in vitro studies, which indicate that positive interactions can promote the inhibition of microbial growth. There are several experimental models measuring the effects of drug combinations. One of the simplest and effective protocols is the "checkerboard" test, which provides a two-dimensional array of different concentrations of the substances evaluated and allows the calculation of the Fractional Inhibitory Concentration index (FIC) (33).

Table 1. *Minimum Inhibitory Concentration (MIC) (µg/ml) of the essential oil from C. zeylanicum and nystatin alone and combined and Fractional Inhibitory Concentration Index (FIC) against C. albicans ATCC 40277.*

Product	**MIC (alone)**
C. zeylanicum EO	312.5 µg/ml
Nystatin	64.0 µg/ml
Product	**MIC (combined)**
C. zeylanicum EO	39 µg/ml
Nystatin	32 µg/ml
	FIC = 0.6024 (Additivity)

The combination of the EO from C. zeylanicum and nystatin potentiated the inhibitory effect on the growth of C. albicans, which is involved with mucocutaneous infections of the oropharynx. After combining the EO and nystatin, it was verified a reduction in MIC values for both substances to 39 µg/ml and 32 µg/ml, respectively (table 1). This represents a decrease of 87.52 % and 50 %, respectively, from the concentrations initially found. As shown in table 1, the value of the Fractional Inhibitory Concentration Index (FIC) was 0.6024, indicating an additive effect of the combination for the growth inhibition of C. albicans.

According to Estrella-cuenca (34), the combined antifungal compounds might promote greater effectiveness of each drug, thus allowing the use of lower doses of each drug. The checkerboard method and the microbial death curve are often used in the in vitro evaluation of antimicrobials' combined activity.

In the literature, there have been no studies evaluating the antifungal effect of the combination between the EO from C. zeylanicum and nystatin on Candida albicans. Nevertheless, the association of other naturally-occurring products with conventional antibiotics has been reported by some authors (12,13,15).

In order to understand the mechanisms involved in the additivity of the effect produced by this combination, the completion of further studies is required. Johnson et al. (35) pointed out that the synergistic activity of antifungal combination may occur through inhibition of different stages in the yeast intracellular biochemical pathways, which are essential for: cellular survival; increasing penetration of the antifungal agent provided by the action of other antifungal in the fungal cell membrane; and for inhibiting carrier proteins, which would have the task to promote the extrusion of drugs and inhibition of different cellular targets simultaneously.

The results of this study suggest the conduction of antimicrobial tests simulating biofilm formation, as these mimic clinical situations characterized by increased resistance to available antifungal agents and also suggest the use of clinical isolates resistant to conventional antifungals. Hence, our findings represent the possibility of a new pharmaceutical formulation for the treatment of superficial fungal infections, particularly those affecting the oropharyngeal mucosa.

As an additive effect of the combination of the EO from C. zeylanicum and nystatin in inhibiting C. albicans cell growth was verified, we also studied the effect of that combination on the yeasts growth kinetics. It was observed that at all concentrations (MIC÷2, MIC, MICx2, MICx4 and MIC×8) the products tested alone or in combination were able to reduce the number of CFU/ml when compared to the control group (p<0.0001). These data are shown in figures 1, 2, 3, 4 and 5, respectively.

Figure 1. *Growth kinetics of C. albicans yeasts (ATCC 40277) under activity of the test products at MIC÷2 (p<0.0001).*

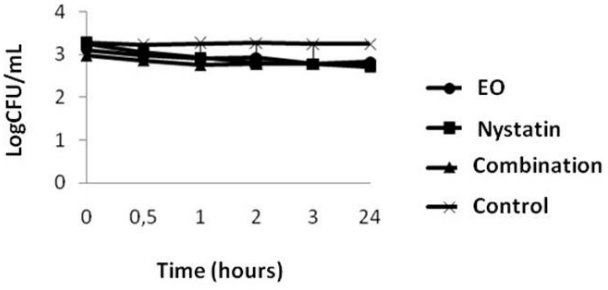

Figure 2. *Growth kinetics of C. albicans yeasts (ATCC 40277) under activity of the test products at MIC (p<0.0001).€*

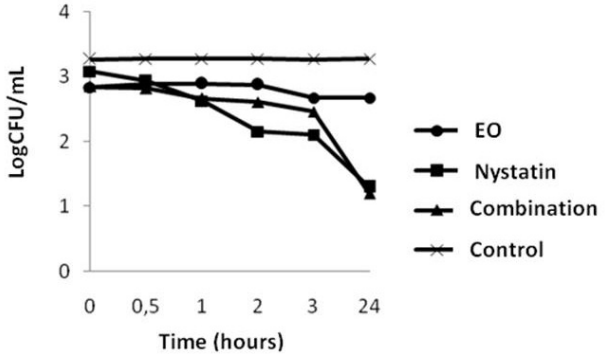

Figure 3. *Growth kinetics of C. albicans yeasts (ATCC 40277) under activity of the test products at MICx2 (p<0.0001).*

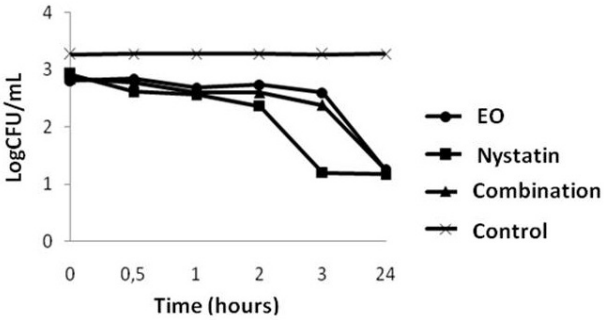

Figure 4. *Growth kinetics of C. albicans yeasts (ATCC 40277) under activity of the test products at MICx4 (p<0.0001).*

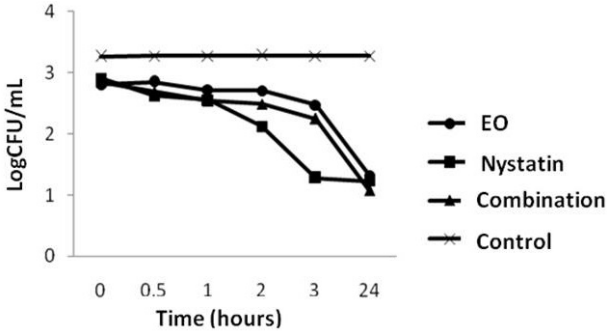

Figure 5. *Growth kinetics of C. albicans yeasts (ATCC 40277) under activity of the test products at MICx8 (p<0.0001).*

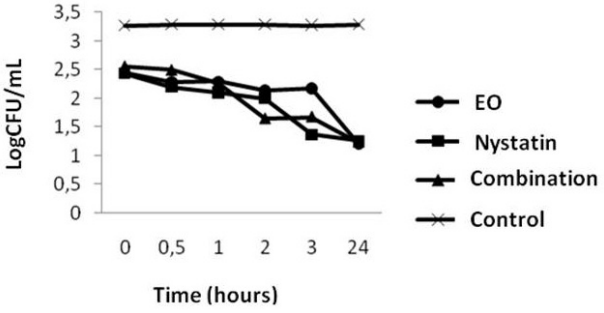

Figure 6. *Micromorphology of C. albicans yeasts (ATCC 40277). A: growth control; B: activity of the EO from C. zeylanicum (39 µg/ml) combined with nystatin (32 µg/ml); C: activity of nystatin alone (64 µg/ml); D: activity of the EO from C. zeylanicum alone (312.5 µg/ml). (1): Pseudo-hyphae; (2): Chlamydoconidia; (3): Blastoconidia.*

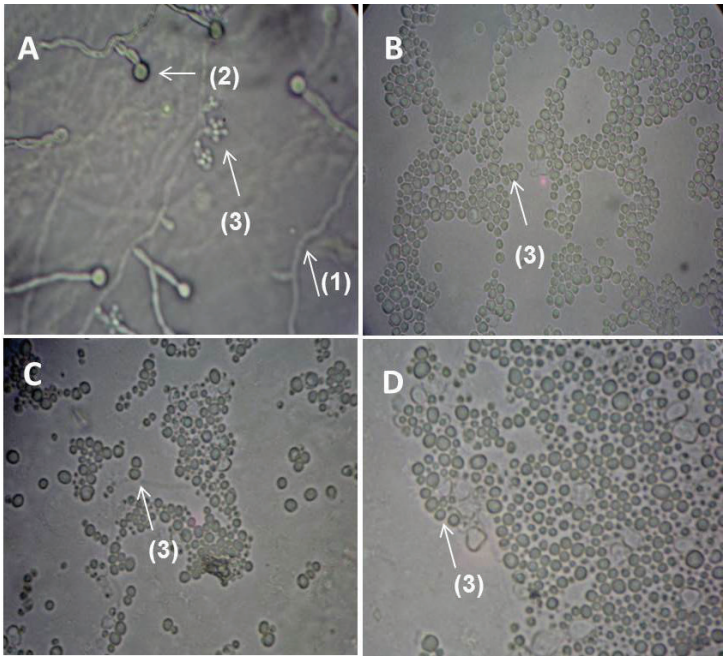

Table 2. *Micromorphological changes produced by the essential oil from C. zeylanicum and nystatin alone and in combination against C. albicans yeasts (ATCC 40277).*

Test prod uct	Concentration	Pseudohyphae	Blastoconidia	Chlamydoconidia
C. zeylanicum Alone	MIC	-	+	-
	MIC x 2	-	+	-
	MIC x 4	-	+	-
	MIC x 8	-	+	-
Nystatin alone	MIC	-	+	-
	MIC x 2	-	+	-
	MIC x 4	-	+	-
	MIC x 8	-	+	-
C. zeylanicum + nystatin	MIC	-	+	-
	MIC x 2	-	+	-
	MIC x 4	-	+	-
	MIC x 8	-	+	-
H_2O	-	+	+	+

(+): Presence / (-): Absence.

The results obtained in the kinetics study indicated that the combination of products (EO from C. zeylanicum and nystatin), even at lower concentrations, significantly reduced the number of viable cells and that the contact time of the substances with the cells positively favored such reduction.

When test products were combined, even at initial concentrations of 39 µg/ml (EO) and 32 µg/ml (nystatin), similar results were observed for the inhibition of fungal cell development. On the other hand, as shown in figure 6A, the cells grown in antifungal-free culture medium were able to develop, and therefore presented inherent characteristics of the specie, which reflects viability and normal capability of morphogenesis.

The formation of hyphae and pseudohyphae is related to pathogenicity factors expressed by C. albicans, as these structures represent a barrier to phagocytosis and allow the settlement of yeasts on the epithelial tissue. Morphological changes are associated with microorganism pathogenicity, and it is believed that environmental factors influence the physiological state of the commensal yeast (36). This is the first study demonstrating the activity of the essential oil from C. zeylanicum and nystatin alone and in combination on fungal micromorphology, which makes it difficult to compare our findings with the literature.

The results indicated that, when tested in combination, or alone at the lowest concentrations (312.5 µg/ml for the EO and 64 µg/ml for nystatin), C. zeylanicum and nystatin were able to inhibit the formation of pseudohyphae and chlamydoconidia, as seen in table 2 and figures 6B, 6C and 6D. These data are consistent with information reported in the literature about the recognized activity of these products against C. albicans strains (9,17,30,37).

There are few studies designed to evaluate the activity of essential oils on fungal micromorphology. In the study by Martins (38), it was found that in presence of the EO from Citrus limon Linn essential oil at 78 µg/ml, clinical isolates of C. albicans were able to grow incipiently, and microscopic analysis showed blastoconidia and rare rudimentary hyaline pseudohyphae.

This study brings a considerable advance in the knowledge on the antifungal activity of the EO from C. zeylanicum alone and combined with nystatin against C. albicans. However, further researches are needed to assess this activity on resistant clinical isolates. In addition, the use of experimental models reproducing the formation of biofilms should be considered.

CONCLUSION

In conclusion, the combination between the essential oil from C. zeylanicum and nystatin potentiated the inhibitory effect on C. albicans growth. Furthermore, it reduced the production of pathogenicity-related morphological structures such as pseudohyphae and chlamydoconidia.

The association of this naturally-occurring agent (C. zeylanicum) and synthetic antifungal (nystatin) may represent a new therapeutic option for the treatment of oral and pharyngeal candidiasis.

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