ARTÍCULO ORIGINAL

Combined effect of *Cinnamomum zeylanicum* blume essential oil and nystatin on strains of non-albicans *Candida*

Efecto combinado del aceite esencial de *Cinnamomum zeylanicum* blume y nistatina sobre cepas de *Candida* no-albicans

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ABSTRACT

Introduction: considering the emergence of resistant species of *albicans* and non-*albicans Candida* to agents therapeutically available as a result of the increased number of immunocompromised population and of the increasingly frequent use of prophylaxis and empirical treatment with antifungals, it's verified that there is a clear and emerging need to introduce new antimicrobials agents in the therapeutic arsenal. The purpose of this study was to evaluate the antifungal activity of essential oil of *Cinnamomum zeylanicum* Blume alone and combined with Nystatin on strains of *C. tropicalis* and *C. krusei*.
Methods: this was an experimental research in laboratory. It was determined the Minimum Inhibitory Concentration, using the microdilution method, as well as the Fractional Inhibitory Concentration to determine the possible synergistic effects of the association. Strains of *C. tropicalis* ATCC 40147 and *C. krusei* ATCC 40042 were used in the tests. When assessed separately, *C. zeylanicum* essential oil and Nystatin presented Minimum Inhibitory Concentration of 312,5 µg/mL and 64 µg/mL, respectively, on both tested strains.

Results: When combined, were found Minimum Inhibitory Concentration of 39 µg/mL and 32 µg/mL for the essential oil and for Nystatin, respectively. The Fractional Inhibitory Concentration value was 0,6024 for both tested strains, indicating additivity of the inhibitory effect on fungal growth.
Conclusions: the results indicate that *C. zeylanicum* essential oil has antifungal activity against the strains of non-albicans *Candida* evaluated and that its association with Nystatin potentiates this effect.

Key words: *Cinnamomum zeylanicum*; Drug Synergism; *Candida*; *Candida tropicalis.*

RESUMEN

Introducción: es necesaria la introducción de nuevos agentes antimicrobianos por el surgimiento de especies de *Candida albicans* y no *albicans* resistentes a los agentes terapéuticos disponibles .El objetivo del estudio fue evaluar la actividad antifúngica del aceite esencial de *Cinnamomum zeylanicum* Blume aislado y asociado con nistatina sobre cepas *Candida tropicalis* y *Candida krusei*.
Métodos: se realizó una investigación experimental de laboratorio. La concentración mínima inhibitoria fue determinada utilizando el método de microdilución, y la concentración inhibitoria fraccionada se usó para determinar los posibles efectos sinérgicos de la asociación. Para las pruebas fueron utilizadas las cepas de *C. tropicalis* ATCC 40147 y *C. krusei* ATCC 40042. Se usaron el aceite esencial de *C. zeylanicum* y nistatina. Cuando fueron evaluados por separado presentaron la concentración mínima inhibitoria de 312,5 µg/mL y de 64 µg/mL, respectivamente, sobre ambas cepas ensayadas.
Resultados: una vez asociados, la concentración mínima inhibitoria fue de 39 µg/mL para el aceite esencial y de 32 µg/mL para la nistatina. El valor de la concentración inhibitoria fraccionada para ambas cepas probadas fue de 0,6024, lo que indica adicción del efecto inhibidor sobre el crecimiento de hongos.
Conclusiones: los resultados indican que el aceite esencial de *C. zeylanicum* tiene actividad antifúngica frente a las cepas de *Candida* no albicans y que la asociación del mismo con la nistatina promueve la potenciación de este efecto.

Palabras clave: *Cinnamomum zeylanicum*; sinergia farmacológica; *Candida*; *Candida tropicalis*.

INTRODUCTION

Candidiasis is a fungal infection caused by the presence of yeasts of *Candida* genus, which is a member of the family Cryptococcaceae. In total, about 81 species are recognized, especially *C. albicans* for its virulence and potential to promote disease. Besides that, other species also contribute to the development of disease, such as *C. tropicalis* and *C. krusei.*[1-3]

In immunocompromised individuals, especially those affected by HIV / AISD, about 74 % have lesions in the oral mucosa resulting from infections caused by *Candida*

spp.[4] It is noteworthy that oral candidiasis in these subjects can act as a marker of disease progression and as a predictive for increasing immunosuppression.

Clinically, the disease may arise as mucosal until systemic manifestations, characterized by the invasion of various organs. The oral, vaginal and esophageal mucosas are the most affected sites in cases of candidiasis. Systemic infections, occurring as a result of hematological dissemination, may cause microabscesses throughout the body. For spreading *C. albicans* cells, the vascular endothelium actively participates in the process, through interaction between receptors present on endothelial cells and adhesins expressed by yeasts.[5,6]

Candidiasis has been considered the most frequent infection of the oral cavity. In most cases, it is clinically characterized into fours patterns: erythematous, pseudomembranous, hyperplastic and angular cheilitis.[7] Erythematous candidiasis associated to the use of prosthesis, popularly known as *denture sore mouth*, stands out owing to its high prevalence and clinical manifestations (hyperemia, edema and moderate inflammation which are likely to be associated with pain, itching and burning). [8] In this sort of infection, one can observe reddish spots that appear at sites of contact between the prosthesis and oral mucosa, in addition to texture changes on this surface.

Histopathologically, in the oral mucosa infected by *Candida* species it is possible to observe tissue invasion of these microorganisms as a result of phospholipases and proteinases production, which favors hyphae and pseudo-hyphae adherence and formation.[9]

The higher prevalence of oral colonization by *Candida* may be pointed out as a predisposing factor for subsequent onset of clinical candidiasis. Its diagnosis is usually performed based on clinical manifestations and species of *C. albicans, C. krusei* and *C. tropicalis* are likely to be found in large numbers.[10]

The medication approach to treat candidiasis includes topical and systemic antifungal agents. The three major classes of antifungal agents currently used are polyenes (e.g. Nystatin and Amphotericin B), imidazoles (such as Clotrimazole and Miconazole) and triazoles (e.g. Fluconazole and Itraconazole).[11]

Whereas oral candidiasis is a superficial infection, usually the initial treatment is done with a topical agent. Nystatin and Miconazole are the drugs of initial choice. If topical therapy fails to submit results, systemic treatment is initiated, and Fluconazole is the most prescribed drug.[11]

However, considering the emergence of resistant species of *albicans* and non-*albicans Candida* to agents therapeutically available as a result of the increased number of immunocompromised population and of the increasingly frequent use of prophylaxis and empirical treatment with antifungals, [12] it's verified that there is a clear and emerging need to introduce new antimicrobials agents in the therapeutic arsenal.[13,14]

In this perspective, comes up the possibility to investigate the interactive effects of conventional antifungal compounds and natural products.[15] This interaction can promote greater effectiveness of each drug, thus allowing the use of lower doses of both the substances.[16]

Thus, considering the known antimicrobial activity of *Cinnamomum zeylanicum* Blume essential oil. [13-14,17-18] The purpose of this study was to evaluate the

antifungal activity of essential oil of *Cinnamomum zeylanicum* Blume alone and combined with Nystatin on strains of *C. tropicalis* and *C. krusei*.

METHODS

This was an experimental research in laboratory**.**

Microbiological tests were performed in the Mycology Laboratory of the Center for Health Sciences, Federal University of Paraiba, which provided strains of *C. tropicalis* ATCC 40042 and *C. krusei* ATCC 40147.

The essential Oil (EO) whose antifungal activity is under study was obtained from Ferquima Ind. and Comp. Ltd (Vargem Grande Paulista, Sao 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).

The Minimum Inhibitory Concentration (MIC) determination for the essential oil and for Nystatin was performed by microdilution technique, using 96-well U-bottom microtiter plates (ALAMAR®). Initially, 100 µL of Sabouraud Dextrose Broth doubly concentrated were distributed into the plate's wells. Then, 100 µL of the emulsion of *C. zeylanicum* EO and Nystatin were distributed at an initial concentration of 5.000 µg/mL and 128 µg/mL, respectively. From these concentrations were conducted serial dilutions by withdrawing an aliquot of 100 µL from the most concentrated well and inserting it into the following well. Finally, aliquots of 10 µL of inoculum correspondent to the strains under test were dispensed into the wells of each column. In parallel, it was made a yeast viability control. Tests were performed in triplicate, and the plates were incubated at 35°C for 24-48 hours.[19]

The reading to determine the essential oil MIC on the yeast strains was made through the visual method. It was taken into consideration the formation or non-formation of cellular clusters («button») at the bottoms of the wells. Thus, MIC was considered as the lowest concentration of the product under test capable of producing visible inhibition on the growth of yeast strains.[19]

In order to confirm the presence of viable microrganisms at non-inhibitory concentrations, 10 µL of TTC dye (2,3,5 triphenyl tetrazolium chloride) were inserted into the wells after 24 hours of incubation. The detection of microrganisms 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.[20]

Combined effect between Nystatin and *C. zeylanicum* EO was determined by the microdilution technique - *checkerboard* - for derivation of the Fractional Inhibitory Concentration index (FIC index).

The turbidity of the fungal suspensions of *C. tropicalis* ATCC 40042 and *C. krusei* ATCC 40147 was compared and adjusted to that presented by the barium sulphate suspension referent to the tube 0.5 of McFarland scale, which corresponds to an

inoculum of approximately 10^6 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 holes of a 96-well U-bottom microtiter plate (ALAMAR®). Then, 50 µL of each product tested whose concentrations ranged among MIC÷4, MIC÷2, MIC, MICx2 and MIC×4 were added in the horizontal (Nystatin) and vertical (essential oil) 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 37ºC for 48 hours.[21,22]OuvirLer foneticamente Dicionário - Ver dicionário detalhado

The FIC index was calculated as $FIC^A + FIC^B$, in which A represents the EO and B is Nystatin. FIC^A is calculated through the ratio MIC^A combined / MIC^A alone, while $FIC^B = MIC^B$ combined / MIC^B alone. This index was interpreted as follows: synergism (<0.5), additivity (0.5-1.0), indifference (> 1 and <4) or antagonism (> 4.0[21-23].

RESULTS

The essential oil of *C. zeylanicum* and Nystatin, when assessed separately, presented MIC of 312.5 µg/mL and 64 µg/mL, respectively, on both tested strains, *C. tropicalis* ATCC 40042 and *C. krusei* ATCC 40147, as seen in table 1.

Table 1. Minimum Inhibitory Concentration of *C. zeylanicum* essential oil and Nystatin on strains of *C. tropicalis* ATCC 40042 and *C. krusei* ATCC 40147.

	Minimum Inhibitory Concentration (µg/mL)	
Strains	*C. zeylanicum*	Nystatin
C. tropicalis ATCC 40042	312.5	64
C. krusei ATCC 40147	312.5	64

As seen in tables 2 and 3, there was a decrease in MIC values for both substances. The values found were 39 µg/mL and 32 µg/mL for the EO and Nystatin, respectively, representing a reduction of 87.52% and 50% from the concentrations initially used. After obtaining these findings, FIC was calculated and its value was 0.6024 for both strains tested, indicating additivity of the inhibitory effect on fungal growth.

Table 2. Fractional Inhibitory Concentration Index after combination between *C. zeylanicum* essential oil and Nystatin on *C. tropicalis* strain ATCC 40042.

Products	Concentration
C. zeylanicum EO MIC	39 µg/mL
Nystatin MIC	32 µg/mL
Fractional Inhibitory Concentration Index	0.6024 (Additivity)

Table 3. Fractional Inhibitory Concentration Index after combination between *C. zeylanicum* essential oil and Nystatin on *C. krusei* strain ATCC 40147.

Products	Concentration
C. zeylanicum EO MIC	39 µg/mL
Nystatin MIC	32 µg/mL
Fractional Inhibitory Concentration Index	0.6024 (Additivity)

DISCUSSION

The antifungal activity evidenced of *C. zeylanicum* essential oil found in this research confirm the data presented by other studies.[15, 24-27]

The antifungal activity of *C. zeylanicum* EO has been attributed to its major constituents.[24] As regards the chemical composition, studies indicate that eugenol is the main component of this essential oil.[15] *Meades et al.*[28] suggest that this activity may be due to the action of trans-cinnamaldehyde.

Once identified the antifungal activity of *C. zeylanicum* essential oil on the species of non-albicans *Candida* under test, it was sought to evaluate whether this activity would suffer influence when the EO were combined with Nystatin, a conventional antifungal used for the treatment of mucocutaneous candidiasis.

This is the first study evaluating the antifungal effect of the combination between *C. zeylanicum* EO and Nystatin against non-albicans *Candida* species. However, the association of other natural products to conventional antibiotics has been reported by some contemporary authors,[29-32] what reflects an increasing interest for this type of theoretical and methodological approach.

There are several experimental models that measure the effects of drug combinations. One of the simplest and well known protocols is the «checkerboard» test, which provides a two-dimensional array of different concentrations of the substances evaluated and allows the calculation of Fractional Inhibitory Concentration index (FIC).[15,16]

Johnson et al.[33] point out some probable mechanisms responsible for synergistic activity presented by the combination of antifungal agents, as follows: a) inhibition of different stages in the yeast intracellular biochemical pathways, essential for cellular survival; b) increased penetration of the antifungal agent provided by the action of other antifungal in the fungal cell membrane. This interaction can be observed, for instance, through the interaction between Amphotericin B or Fluconazole and Rifamycin; c) inhibition of carrier proteins. For example, Amphotericin B inhibits the action of plasma membrane proteins that would promote the extrusion of flucytosine, which remains inside the cell and exerts its effect; d) inhibition of different cellular targets simultaneously. This effect can be observed in drugs that exert their effects on the cell wall and another that acts on the plasma membrane.

Given the above, it is recognized as promising the possibility of using natural products combined with traditional antimicrobials in order to increase the antimicrobial potential of drugs.[34] These combinations may represent a new option for elimination of multiresistant fungi and for reducing the exposure of conventional antifungal agents to these microrganisms, thus reducing the risk of selecting new or improved mechanisms of resistance.[32]

The results of this research had allowed to conclude that the essential oil of *C. zeylanicum* alone and combined with Nystatin is able to promote reduction in the non-albicans *Candida* cells development capacity.

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