Combined effect of *Cinnamomum zeylanicum* Blume essential oil and miconazole against *Candida* spp

Efeito combinado do óleo essencial de Cinnamomum zeylanicum *Blume e miconazol sobre* Candida *spp*

Ricardo Dias de Castro[1], Edeltrudes de Oliveira Lima[2], Irlan de Almeida Freires[3], Alessandro Leite Cavalcanti[4]

[1]Post-Graduate Program in Dentistry, Federal University of Paraíba, João Pessoa-PB, Brazil; [2]Department of Pharmaceutical Sciences, Federal University of Paraiba, Joao Pessoa-PB, Brazil; [3]Department of Physiological Sciences, Piracicaba Dental School, University of Campinas, Piracicaba-SP, Brazil; [4]Department of Dentistry, School of Dentistry, State University of Paraiba, João Pessoa-PB, Brazil.

Abstract

Objective – To evaluate the combined effect of the essential oil (EO) of *Cinnamomum zeylanicum* Blume and miconazole against *Candida* spp. **Methods** – It was determined the minimum Inhibitory Concentration (MIC) of both products and the Fractional Inhibitory Concentration index (FIC) by means of the Checkerboard Test. Data were analyzed descriptively. **Results** – When assessed alone, *C. zeylanicum* and miconazole showed MIC of 312.5µg/mL and 32µg/mL, respectively, against all strains tested. After combination, it was observed that the EO inhibited the yeast growth at a concentration of 39µg/mL. On the other hand, miconazole combined showed MIC of 128, 32 and 32µg/mL against strains of *C. albicans*, *C. tropicalis* and *C. krusei*, respectively. These results indicate FIC values of 4.1248 (antagonism), 1.1248 (indifference) and 1.1248 (indifference), respectively, for *C. albicans*, *C. tropicalis* and *C. krusei*. **Conclusions** – The combination between *C. zeylanicum* essential oil and miconazole was not found to be an advantageous possibility for growth inhibition of *Candida* spp. The combination of this EO with other standard antifungals should be considered in further trials.

Descriptors: *Cinnamomum zeylanicum*; Miconazol; Drug synergism; *Candida albicans*

Resumo

Objetivo – Avaliar o efeito combinado do óleo essencial (OE) de *Cinnamomum zeylanicum* Blume e miconazol sobre cepas de *Candida*. **Métodos** – Foi determinada a concentração inibitória mínima (CIM) de ambos os produtos e o índice de concentração inibitória mínima fracionada (FIC) – checkerboard Test. Os dados foram avaliados descritivamente. **Resultados** – Quando avaliados isolados, *C. zeylanicum* e miconazol apresentaram CIM de, respectivamente, 312,5µg/mL e 32µg/mL sobre todas as cepas ensaias. Após a associação dos produtos, foi observado que o OE de *C. zeylanicum* inibiu o crescimento das leveduras na concentração de 39µg/mL. Por outro lado, o miconazol, quando associado, apresentou CIM de, respectivamente, 128,32 e 32µg/mL, sobre cepas de *C. albicans*, *C. tropicalisi e C. crusei.* **Conclusão** – A associação do óleo essencial de *C. zeylanicum* ao miconazol não constitui em uma possibilidade vantajosa para inibição de crescimento de *Candida* spp. A combinação desse OE com outros antifúngicos padrão deve ser considerada em outros estudos.

Descritores: *Cinnamomum zeylanicuym*; Miconazol; Sinergismo farmacológico; *Candida albicans*

Introdução

C. albicans is an opportunistic pathogen that inhabits the human body as a commensal microorganism, and it is considered to be the major cause of fungal infections in humans[1]. Usually, these infections have arisen due to the virulence of *C. albicans*, which presents considerable morphological plasticity as a result of changes in immune response[2].

The molecular mechanisms of virulence are mostly related to the activation of MAP (mitogen-activated protein) Kinase signal transduction via. In this sense, cellular responses involved in invasive growth, cell wall formation, osmotic stress adaptation and reproduction occur through intracellular signaling pathways as MKc1, Cek1/2 and HOG1 MAP Kinase[2].

The activation of MAPK pathway also provides activation of the transcription of Cph1 factor, which is responsible for the filamentous form, considered a virulence factor for the occurrence of systemic infections, and CLA4, responsible for the formation of the germ tube and hyphae. The PKA pathway activation provides the formation of cyclic AMP, which regulates the Efg1 factor, also responsible for the hyphal formation[3].

In regards to superficial infections, especially those affecting the oropharynx, object of interest in this study, it is known that the mucosa of this region is the most frequent site affected by superficial *candidiasis*, and colonization by *C. albicans* occurs in 10-50% of healthy individuals. The drug approach to treat this type of candidiasis includes topical and systemic antifungal agents. Miconazole and nystatin have been the drugs of initial choice. If topical therapy fails to submit results, systemic treatment is initiated, and fluconazole is therefore the most prescribed drug in such cases[4].

Nevertheless, the number of *Candida* species resistant to the antifungal agents available has been increasing considerably in the last years[5]. Prolonged use of these agents may act as a risk factor for the development of fungal resistance by adaptive mutagenesis[6]. Moreover, there has been a growing population of immunocompromised individuals and an increasingly frequent use of prophylaxis and empirical treatment with antifungals[7].

Given the above, natural products have been proposed in an attempt to obtain new drugs, since they differ from synthetic products as regards molecular diversity, which is much higher in natural products than in those derived from synthesis processes, that despite of considerable advances, have been still limited[8]. This provides the development of numerous new drugs with diverse therapeutic functions. Within this context, it is highlighted the recognized antifungal activity of the essential oil from *C. zeylanicum* Blume[9-10].

Some studieshave proposed the combination of natural products and conventional antimicrobial agents as a way to introduce new formulations in the therapeutic arsenal, capable of tackling multi-resistant microorganisms and preventing or minimizing contact of these microorganisms with synthetic products, thus reducing the risk of selecting new or improved mechanisms of resistance[11-12].

In this perspective, this study aimed to evaluate the combined effect between *C. zeylanicum* essential oil and miconazole against *Candida* strains.

Methods

Strains

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, *C. tropicalis* ATCC 40042 and *C. krusei* ATCC 40147.

Essential Oil

The EO whose antifungal activity has been 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 produced and marketed 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 that 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=1.040g/mL).

Minimum Inhibitory Concentration (MIC)

The MIC determination for the essential oil and for miconazole was performed by the microdilution technique, using 96-well U-bottom microtiter plates (ALAMAR®). Initially, 100µL of Sabouraud Dextrose Broth doubly concentrated were dispensed in the wells. Then, 100µL of the emulsion of *C. zeylanicum* EO and miconazole were distributed at an initial concentration of 5,000µg/mL and 128µg/mL, respectively. From these concentrations, serial dilutions were conducted by withdrawing an aliquot of 100µL from the most concentrated well and inserting it into the following well. Finally, aliquots of 10µL of inoculum corresponding to the strains under test were dispensed into the wells of each column. In parallel, a yeast viability control was made. Tests were performed in triplicate, and plates were incubated at 35°C for 24-48 hours[13].

The reading to determine the essential oil MIC on the yeast strains was made through 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[13].

In order to confirm the presence of viable microrganisms at non-inhibitory concentrations, 10µL 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[14-15].

Synergism assay – Checkerboard method

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

The turbidity of the fungal suspensions 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 1.5x106 Colony Forming Units/mL (CFU/mL). Solutions of the products tested were used at concentrations determined from their respective MIC. Initially, 100µL of Sabouraud Dextrose culture medium were added into the holes of a 96-well U-bottom microtiter plate (ALAMAR®). Then, 50µL of each product tested whose concentrations ranged among MIC÷4, MIC÷2, MIC, MICx2 and MIC×4 were added in the horizontal (miconazole) and vertical (essential oil) directions of the plate. Finally, the culture medium was inoculated with 10µL of fungal suspension. Fungal growth was evidenced by means of the TTC dye. The test was performed in triplicate, and microplates were incubated at 37ºC for 48 hours[16-17].

The FIC index was calculated as FICA + FICB, in which A represents the EO and B is miconazole. FICA is calculated through the ratio MICA combined / MICA alone, while FICB = 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)[16,18].

Results and Discussion

As seen in Table 1, *C. zeylanicum* EO and miconazole when assessed alone presented, respectively, MIC of 312.5µg/mL and 32µg/mL on strains of *C. albicans*, C. tropicalis and *C. krusei*. These findings confirm the data presented by other studies[19-22].The antimicrobial activity of the EO from *C. zeylanicum* may be related to the action of trans-cinnamaldehyde, an important compound found in large amounts in this EO composition[23].

Table 1. Minimum Inhibitory Concentration (MIC) of *C. zeylanicum* essential oil and miconazole on *Candida* strains

Strains	MIC (µg/mL) *C. zeylanicum*	Miconazole
C. albicans ATCC 40277	312.5	32
C. tropicalis ATCC 40042	312.5	32
C. krusei ATCC 40147	312.5	32

After products were combined, a change in MIC values was observed for both substances. Values of 39 µg/mL and 128µg/mL were found, respectively, for EO and miconazole on *C. albicans* 40277 strains (Table 2). These data represent a decrease of 87.52% in the essential oil MIC. Nevertheless, it was necessary to increase the concentration of miconazole in 400% in order to promote growth inhibition of the strains assessed. The FIC value was 4.1248, indicating antagonist effect. It is worth noting that, even considering a reduction in the concentration of the EO studied herein, the increase in miconazole concentration may represent a greater possibility for *Candida* strains to develop resistance mechanisms.

Table 2. Fractional Inhibitory Concentration Index (FIC) and MIC (µg/mL) after combination between *C. zeylanicum* essential oil and miconazole against strains of *C. albicans* ATCC 40277, *C. tropicalis* ATCC 40042 and *C. krusei* ATCC 40147

	C. albicans ATCC40277	*C. tropicalis* ATCC40042	*C. krusei* ATCC40146
C. zeylanicum EO MIC	39 µg/mL	39 µg/mL	39 µg/mL
Miconazole MIC	128 µg/mL	32 µg/mL	32 µg/mL
FIC	4.1248	1.1248	1.1248
Effect	Antagonism	Indifference	Indifference

The combined use of medicinal plants and/or their products and byproducts with the concomitant use of conventional drugs may act by inhibiting, enhancing the therapeutic effects of drugs or otherwise by interfering with the expected response[24]. Oliveira *et al.*[25] emphasize that such combined use on special occasions may put the patient at risk, since it might trigger acquisition of resistance by microorganisms or might initiate mechanisms of irritation or other adverse effects.On the other hand, with respect to strains of *C. tropicalis* and *C. krusei*, there was a decrease in the EO MIC (39 µg/mL) and unaltered miconazole MIC (32µg/mL) values. Then, it was observed a FIC value of 1.1248 for both strains, thereby indicating indifference of the effect produced by the association, when compared with the products tested alone.

In the literature, no study has been found evaluating the antifungal effect of the combination between *C. zeylanicum* EO and miconazole against *Candida* spp. The findings of the present investigation warrant the completion of other studies to further investigate the association of *C. zeylanicum* with other conventional agents used in the medical and dental fields.

According to Cuenca-Estrella[26], the combined antifungal compounds can promote greater effectiveness of each drug, thus allowing the use of lower doses of each product. The checkerboard method and the microbial death curve have been often used in the in vitro evaluation of combined antimicrobials activity[19]. This information was pointed out by Odds[27], who reaffirmed the viability of the checkerboard test in the study of interactive effects between molecules.

Conclusions

The findings of this study indicated that the combination between *C. zeylanicum* essential oil and miconazole was not found to be an advantageous possibility for in vitro growth inhibition of *Candida* spp., since antagonist or indifferent effects were verified when compared with the potential of these products alone.Nevertheless, the combination of this essential oil with other standard antifungals should be considered in further trials in order to diminish the use/dose of synthetic agents due to their adverse effects.

References

1. Pfaller M, Neofytos D, Diekema D, Azie N, Meier-Kriesche HU, Quan SP, Horn D. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance®) registry, 2004-2008. Diagn Microbiol Infect Dis. 2012;74(4):323-31.

2. Monge RA, Román E, Nombela C, Pla J. The MAP Kinase signal transduction network in *Candida albicans*. Microbiology 2006; 152(4):905-12.

3. Phan QT, Belanger PH, Filler SG. Role of hyphal formation in interactions of *Candida albicans* with endothelial cells. Infect Immun. 2000;68(6):3485-90.

4. Paiva LCA, Ribeiro RA, Pereira JV, Oliveira NMC. Clinical and laboratorial evaluation of Uncaria tomentosa (Cat's Claw) gel on oral candidiasis. Rev Bras J Pharmacogn. 2009;19(2).

5. Andes D, Forrest A, Lepak A, Nett K, Marchillo K, Lincoln L. Impact of antimicrobial dosing regimen on evolution of drug resistance *In Vivo*: fluconazole and Candida albicans. Antimicrob Agents Chemother. 2006;50(7):2374-83.

6. Quinto-Alemany D, Canerina-Amaro A, Hernández-Abad LG, Machín F, Romesberg FE, Gil-Lamaignere C. Yeasts Acquire Resistance secondary to antifungal drug treatment by adaptive mutagenesis. PLoS One. 2012;7(7):e42279.

7. Rautemaa R, Richardson M, Pfaller M, Koukila-käkhölä P, Perheentupa J, Saxén H. Decreased susceptibility of *Candida albicans* to azole antifungals: a complication of long-term treatment in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients. J Antimicrob Chemother. 2007;60(4):889-92.

8. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 Years from 1981 to 2010. J Nat Prod. 2012; 75(3):311-35.

9. Pozzatti P, Scheid LA, Spader TB, Atayde ML, Santurio JM, Alves SH. *In vitro* activity of essential oils extracted from plants used as spices against fluconazole-resistant and fluconazole-susceptible *Candida* spp. Can J Microbiol. 2008;54(11):950-60.

10. Moreira ACP, Lima EO, Souza EL, Van Dingenen MA, Trajano VN. Inhibitory effect of *Cinnamomum zeylanicum* Blume (Lauraceae) essential Oil and β-pinene on the growth of dematiaceous moulds. Braz J Microbiol. 2007;38(1):33-8.

11. Calabrese EC, Castellano S, Santoriello M, Sgherri C, Quartacci MF, Calucci L *et al*. Antifungal activity of azole compounds CPA18 and CPA109 against azole-susceptible and resistant strains of *Candida* albicans. J Antimicrob Chemother. 2013;68(5):1111-9.

12. Coutinho HD, Costa JG, Falcão-Silva VS, Siqueira-Júnior JP, Lima EO. In vitro additive effect of Hyptis martiusii in the resistance to aminoglycosides of methicillin-resistant *Staphylococcus aureus*. Pharm Biol. 2010;48(9):1002-6.

13. Ellof JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med. 1998;64(8):711-3.

14. Deswal DP, Chand U. Standartization of the tetrazolium test for viability estimation in ricebean (*Vigna umbellate* T.) seeds. Seed Sci Technol. 1997;25(1):409-17.

15. Gabre DF. Tetrazolium manual test. Brasília: AGIPLAN; 1976.

16. Dutta NK, Dastidar SG, Kumar A, Mazumdar K, Ray R, Chakrabarty NA. Antimycobacterial activity of the anti-inflammatory agent sodium diclofenac, and its synergism with streptomycin. Braz J Microbiol. 2004;35(4):316-23.

17. Eliopoulos GM, Moellering RC. Antimicrobial combinations. *In*: Lorian V, editor. Antibiotics in Laboratory Medicine. Baltimore: Williams & Wilkins; 1991. p. 434-41.

18. Nightingale CH, Ambrose PG, Drusano GL, Murakawa T. Antimicrobial pharmacodynamics. *In:* Theory and clinical practice. 2ª ed. New York: Medical; 2002.

19. Cardozo EI, Pardi G, Perrone M, Salazar E. Estudio de la Eficacia del miconazol tópico (Daktarin jalea oral) en pacientes con estomatitis subprotesica inducida por *Cándida*/study of the effectiveners of Topical miconazole (Daktarin oral) on patients with Candida-induced denture stomatitis. Acta Odontol Venez. 2001; 39(3):45-53.

20. Lima IO, Oliveira RAG, Lima EO, Farias NMP, Souza EL. Antifungal activity of essential oils on *Candida* species. Braz J Pharmacogn. 2006;16:197-201.

21. Schmidt E, Jirovetz L, Wlcek K, Buchbauer G, Gochev V, Girova T, Stoyanova A, Geissler M *et al*. Antifungal activity of eugenol and various eugenol-containing essential oils against 38 clinical isolates of *Candida albicans*. J Essent Oil-Bearing Plants. 2007;10(5):421-9.

22. Van Roey J, Haxaire M, Kamya M, Iwanga I, Katabira E. Comparative efficacy of topical therapy with a slow-release mucoadhesive buccal tablet containing miconazole nitrate versus systemic therapy with ketoconazole in HIV-positive patients with oropharyngeal *candidiasis*. J Acquir Immune Defic Syndr. 2004; 35(2):144-50.

23. Meades GJR, Henken RL, Waldrop GL, Rahman MM, Gilman SD, Kamatou GP, Viljoen AM, Gibbons S. Constituents of Cinnamon inhibit bacterial acetyl CoA carboxylase. Planta Med. 2010;76(14):1570-5.

24. Nascimento AMA, Brandão MGL, Oliveira GB, Fortes ICP, Chartone-Souza E. Synergistic bactericidal activity of Eremanthus erythropappus oil or β-bisabolene with ampicillin against *Staphylococcus aureus*. Antonie Van Leiwenhock. 2007;92(1):95-100.

25. Oliveira RAG, Lima EO, Vieira WL, Freire KRL, Trajano VN, Lima IO, Souza EL, Toledo MS *et al*. Study of the interference of essential oils on the activity of some antibiotic used clinically. Braz J Pharmacogn. 2006;16(1):77-82.

26. Cuenca-Estrella M. Combinations of antifungal agents in therapy – what value are they? J Antimicrob Chemother. 2004; 54(5):854-69.

27. Odds FC. Synergy, antagonism, and what the checkerboard puts between them. J Antimicrob Chemother. 2003;52(1):1.

Corresponding author:

Dr. Ricardo Dias de Castro
Universidade Federal da Paraíba – Campus I – Cid. Universitária
Centro de Ciências da Saúde
Dep. Odontologia Clínica e Social
João Pessoa - PB, CEP 58059-900
Brazil

E-mail: irlan.almeida@gmail.com,
ricardodiasdecastro@yahoo.com.br

Received August 14, 2013
Accepted November 13, 2013