



## Antifungal activity of carvacrol and antifungal agent combinations against non-*albicans* *Candida* species

Atividade antifúngica do carvacrol e associação entre agentes antifúngicos contra espécies de *Candida* não-*albicans*

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The aim of this study was to determine the minimum inhibitory concentration of carvacrol and to evaluate the antifungal activity of carvacrol when combined with fluconazole, ketoconazole or amphotericin B against non-*albicans* *Candida* species. MICs were determined using microdilution method, and the combination study was conducted using the checkerboard method. The carvacrol MIC ranged between 128 and 512 µg/mL. The greatest antifungal activity was displayed with ketoconazole. Conversely, fluconazole displayed the lowest activity. Amphotericin B showed MICs ranging from 0.5 to 2 µg/mL. The best combinations were observed against *Candida guilliermondii* and *Candida parapsilosis*, which presented synergistic effects. Additive effect was observed in three combinations, whereas, indifferent interactions were seen in six combinations. No antagonistic effects were observed. Based on these results, we conclude that carvacrol retains high activity against non-*albicans* *Candida* strains, and the combinations between carvacrol and the antifungals tested yielded significant effects.

Keywords: antifungal, association, terpene.

O objetivo deste estudo foi determinar a concentração inibitória mínima de carvacrol e avaliar a atividade antifúngica desse fitoconstituente quando combinado com fluconazol, cetoconazol ou anfotericina B contra espécies de *Candida* não-*albicans*. As CIMs foram determinadas usando o método de microdiluição, e o estudo da combinação foi conduzido usando o método de *checkerboard*. A CIM carvacrol variou entre 128 e 512 µg / mL. A maior atividade antifúngica foi demonstrada com cetoconazol. Por outro lado, o fluconazol exibiu a menor atividade. A anfotericina B mostrou CIM variando de 0,5 a 2 µg / mL. As melhores combinações foram observadas contra *Candida guilliermondii* e *Candida parapsilosis*, que apresentaram efeitos sinérgicos. Efeito aditivo foi observado em três combinações, enquanto, interações indiferentes foram vistas em seis combinações. Nenhum efeito antagonístico foi observado. Com base nesses resultados, concluímos que o carvacrol retém alta atividade contra cepas de *Candida* não-*albicans*, e as combinações entre o carvacrol e os antifúngicos testados produziram efeitos significativos.

Palavras-chave: antifúngicos, associação, terpeno.

### 1. INTRODUCTION

The incidence and prevalence of fungal infections caused by species of *Candida* are increasing rapidly, especially in large populations of immunocompromised patients and/or those hospitalized with underlying grave diseases [1]. *Candida* spp. are considered opportunistic microorganisms present in the normal microbiota of the oral cavity, gastrointestinal tract and urogenital tract in humans. They can become pathogenic species when there is weakening of the host defense system through multiple risk factors [2]. Although *C. albicans* is the most prevalent species involved in invasive fungal infections, the incidence of infections caused by non-*albicans* species has increased significantly in recent years [3]. The high prevalence of non-*albicans* *Candida* species

involvements may be a reflection of their inherently higher resistance levels to antifungals when compared to *C. albicans* [4].

These increases in micro-organism resistance and antifungal agent toxicity, as well as the lack of drugs available for the treatment of fungal infections have brought attention to the search for compounds with antimicrobial activities that arise mainly from natural products, which are more effective in treating these types of infections [5]. In this context, essential oils, which are complex mixtures of volatile compounds extracted from aromatic plants, are rich in terpenes; which, with their special structures are possibly the best of sources for obtaining a great variety of drugs [6]. Carvacrol (2-methyl-5-isopropylphenol) is a phenolic monoterpene present in the essential oils of many plants of the Lamiaceae, Euphorbiaceae, Verbenaceae and Poaceae families [7]. In relation to its biological activity: antinociceptive action [8], anti-inflammatory [9], antioxidant [10] and gastro-protective [11] activities have already been evidenced.

Rational combinations of antimicrobial substances may be an effective strategy for fighting infections caused by organisms insensitive to conventional therapies. Combination antimicrobial therapy looks to promote greater effectiveness in treatment, as well as permitting the use of lower concentrations of each drug. It can also increase the spectra of action, and thus prevent resistant organism development [12].

Considering the clinical importance of the infections caused by non-albicans species, and the necessity for the development of new antifungal drugs, the objective of this study was to investigate the antifungal activity of carvacrol both alone and in combination with fluconazole, ketoconazole, or amphotericin B against non-albicans *Candida* species strains.

## 2. MATERIALS AND METHODS

### 2.1 Strains

The following strains were used in this study: *C. tropicalis* (LM-10, ATCC 13803); *C. guilliermondii* (LM-103, LM-703); *C. krusei* (LM-120, LM-13) and *C. parapsilosis* (ATCC 20019, ATCC 22019). These strains were kindly provided by the collection of fungi cultures at the Mycology Laboratory of Paraíba Federal University, Brazil.

### 2.2 Test substances

The substances carvacrol, fluconazole, ketoconazole and amphotericin B were purchased from Sigma-Aldrich® (Steinheim, Germany). Culture media utilized were Sabouraud dextrose agar (SDA) from Difco Laboratories Ltd (Le Pont de Claix, France) and RPMI-1640-L-glutamine (without sodium bicarbonate) from Sigma-Aldrich (São Paulo, Brazil). Carvacrol and antifungals solutions were prepared at the time of the testing, these substances were first solubilized in 0,02% Tween 80 and 2% dimethyl sulfoxide [13]. They were then solubilized in sterile distilled water (q.s. 2 mL) to give an initial concentration of 1,024 µg/mL.

### 2.3 Inoculum

For inoculum preparations, cultures of *Candida* spp. were first grown in culture medium SDA (for 24-48 h, at 37 °C). Subsequently, colonies from this culture were suspended in tubes containing sterile saline (0.85%). These suspensions were then agitated and turbidities compared and adjusted to the 0.5 McFarland scale, corresponding to an inoculum of approximately 10<sup>6</sup> CFU/mL [14].

### 2.4 Minimum inhibitory concentration (MIC)

The MICs of carvacrol, fluconazole, ketoconazole and amphotericin B were determined using the broth microdilution method. MIC determinations for each substance were performed in 96-well microplates and using 100 µL of RPMI-1640-L-glutamine (without sodium bicarbonate) as the culture. Each substance was serially diluted in proportions of two. Carvacrol concentrations were diluted from 1,024 to 2 µg/mL, the antifungals were varied in concentrations of from 1,024 to

0.0625 µg/mL. Then 10 µL of the fungal suspension was added to each well. The plates were incubated at 37 °C and the reading was taken after 24h, noting the presence or absence of visible fungal growth [14]. The MIC was defined as the lowest concentration of carvacrol or antifungal agents capable of inhibiting visible fungal growth, as indicated by the color change in the colorless to pink cavities, indicating growth of the microorganism. Both negative controls (RPMI-1640-L-glutamine) and positive controls (RPMI-1640-L-glutamine and the micro-organism) were tested, to prove the sterility of the medium and the viability of the strains, respectively. Sensitivity controls (for DMSO and Tween 80) were also included in the studies. All tests were performed in triplicate.

## 2.5 Checkerboard assay

The study of combinations between carvacrol and the antifungal agents was conducted using checkerboard technique. Initially, 100 µL of RPMI-1640-L-glutamine (without sodium bicarbonate) was distributed into the 96 wells of the micro-titer plate. Subsequently, 50 µL of antifungal agent in various concentrations (MICx8, MICx4 MICx2, MIC, MIC/2 MIC/4 and MIC/8) were added in the vertical direction, and 50 µL of carvacrol also in different concentrations (MICx8, MICx4 MICx2, MIC, MIC/2 MIC/4 and MIC/8) were added in the horizontal direction of the micro-titer plate. In this way, the various concentrations of carvacrol were tested in the presence of various concentrations of antifungal agent. Finally, we added 20 µL of inoculum strains *C. guilliermondii* LM-103, *C. tropicalis* ATCC 13803, *C. krusei* LM-13 or *C. parapsilosis* ATCC 22019 each previously adjusted in accordance with the 0.5 McFarland scale. The plates were incubated at 37 °C and readings taken after 24h to observe the presence or absence of fungal visible growth, as indicated by the color change in the colorless to pink cavities, indicating growth of the microorganism [15]. All assays were performed in triplicate and controls tested.

The fractional inhibitory concentration (FIC) indexes were calculated, where  $FIC_A = MIC$  of substance A (in combinations) /  $MIC$  of the substance A alone, and  $FIC_B = MIC$  of the substance B (in combinations) /  $MIC$  of the substance B alone. Subsequently, the fractional inhibitory concentration index (FICI) for each antifungal agent combined with carvacrol was determined by summing  $FIC_A + FIC_B$  [16]. The FICI was interpreted in the following way: synergism  $\leq 0.5$ ; additivity  $>0.5$  to  $<1.0$ ; indifference  $\geq 1$  and  $<4$ ; or antagonism  $\geq 4.0$  [17].

## 3. RESULTS AND DISCUSSION

The carvacrol MIC values against *Candida* species are shown in Table 1. The concentration of 512 µg/mL inhibited the growth of all strains, while 128 µg/mL was capable of inhibiting 50% of strains tested.

Table 1: Minimum inhibitory concentrations (µg/mL) of carvacrol against non-albicans *Candida*.

Strains	Carvacrol
<i>C. tropicalis</i> LM-10	128
<i>C. tropicalis</i> ATCC 13803	128
<i>C. guilliermondii</i> LM-103	512
<i>C. guilliermondii</i> LM-703	256
<i>C. krusei</i> LM-120	512
<i>C. krusei</i> LM-13	256
<i>C. parapsilosis</i> ATCC 20019	128
<i>C. parapsilosis</i> ATCC 22019	128

In accordance with the values proposed by Sartoratto et al. (2004) [18], values of MIC between 50 - 500 µg/mL are to be considered as high activity; values from 600 - 1,500 µg/mL as medium activity; and those above 1,500 µg/mL as low activity. In this study, the phytoconstituent showed

high antifungal activity and it may be said that carvacrol was prominently revealed as a non-*albicans Candida* species antifungal agent. The literature reports that the carvacrol has antibacterial activity against *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Streptococcus mutans* (MIC 0.25% v/v) [19]. Activity was also demonstrated against fungi, among them: *C. albicans* (MIC 128 µg/mL) [20], and also on 33 clinical isolates resistant to fluconazole, carvacrol yielded results (of between MIC 75 to 100 µg/mL) for *Candida* spp. (*C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei*) [21]. Yet another study demonstrated carvacrol's activity against *Aspergillus fumigatus*, *A. flavus* (MIC 100 µg/mL), *Penicillium chrysogenum* (MIC 125 µg/mL) and against *Rhizopus oryzae* (MIC 200 µg/mL) [22].

The antifungal MIC values (fluconazole, ketoconazole, and amphotericin B) against non-*albicans Candida* species are shown in Table 2. The antifungals showed varied activity patterns; the highest activity presented was that of ketoconazole, in contrast, fluconazole showed the lowest activity against the strains tested. Amphotericin B presented inhibition against 100% of the strains at the concentration of 2 µg/mL. As for ketoconazole, the concentration of 0.5 µg/mL inhibited the growth of 50% of all isolates. To carry out individual evaluations of the antifungal agents and their activity against non-*albicans Candida* species, as well as for their achievements through combination with carvacrol, it was crucial to first determine their MICs.

Table 2: Minimum inhibitory concentrations (µg/mL) of antifungals against non-*albicans Candida*.

Strains	Fluconazole	Ketoconazole	Amphotericin B
<i>C. tropicalis</i> LM-10	32	2	2
<i>C. tropicalis</i> ATCC 13803	32	0.5	2
<i>C. guilliermondii</i> LM-103	2	32	1
<i>C. guilliermondii</i> LM-703	8	0.125	0.5
<i>C. krusei</i> LM-120	*> 1,024	64	2
<i>C. krusei</i> LM-13	256	0.5	2
<i>C. parapsilosis</i> ATCC 20019	16	0.5	2
<i>C. parapsilosis</i> ATCC 22019	16	0.5	2

\*>: MIC was larger than the tested concentrations.

Confirming the results obtained in this study, Lima et al. (2013) [20] obtained MICs for amphotericin B against 16 strains of *C. albicans* of between 0.5 and 4 µg/mL. Another study evaluated the activity of amphotericin B and fluconazole against non-*albicans Candida* isolates (*C. tropicalis*, *C. krusei* and *C. parapsilosis*), where the MICs ranged between 0.125 to 32 µg/mL [23].

The results from combinations against non-*albicans Candida* specie strains are shown in Table 3. Various forms of interaction between the terpene and the antifungals were observed, among them: synergism in 25% of the combinations, additivity in 25% and, finally indifferent for 50% of the combinations. However, no results were found to be antagonistic. When compared to the use of isolated antifungal medications, combinations between substances with promising antifungal properties can bring new mechanisms of action or intensify them, and possibly bring other benefits such as: decreasing adverse reactions, increasing the spectrum of action, capacity to treat resistant microorganisms, applications for differing infection types, providing treatment alternatives, or decreased toxicities [24].

Table 3. Minimum inhibitory concentration (combinations); fractional inhibitory concentration (FIC); and fraction inhibitory concentration index (FICI) of combination between carvacrol and antifungals against non-*albicans* *Candida* species.

	FIC of compounds		FICI	Outcome
	Antifungals	Carvacrol		
<i>C. guilliermondii</i> LM-103				
Fluconazole	0.06	1	1.06	Indiference
Ketoconazole	0.125	0.125	0.25	Synergism
Amphotericin B	0.125	0.125	0.25	Synergism
<i>C. tropicalis</i> ATCC 13803				
Fluconazole	1	0.25	1.25	Indiference
Ketoconazole	0.5	0.125	0.625	Additivity
Amphotericin B	0.125	1	1.125	Indiference
<i>C. krusei</i> LM-13				
Fluconazole	0.5	0.125	0.625	Additivity
Ketoconazole	0.125	1	1.125	Indiference
Amphotericin B	0.125	1	1.125	Indiference
<i>C. parapsilosis</i> ATCC 22019				
Fluconazole	0.125	0.125	0.25	Synergism
Ketoconazole	0.125	1	1.125	Indiference
Amphotericin B	0.5	0.125	0.625	Additivity

In a study conducted by Ahmad et al. (2013) [25] against both fluconazole -susceptible and -resistant *Candida* spp., it was noted that *C. tropicalis* strains sensitive to the carvacrol/drug combination resulted in synergistic and additive effects. However, for the strains of *C. tropicalis* resistant to fluconazole, results for the combinations with the monoterpene were synergistic. For the fluconazole resistant species *C. krusei*, the results demonstrated were both additive and indifferent to the combinations presented. For *C. parapsilosis*, the combination between fluconazole and carvacrol achieved synergistic results.

#### 4. CONCLUSION

This study has demonstrated the antifungal activity of carvacrol against non-*albicans* *Candida* species, a fact that is important because such information provides clearer expectations for future pharmacological studies; as to its toxicity, possible mechanisms of action, and its effectiveness for possible in vivo use. Testing of these combinations between the carvacrol and the chosen antifungal agents has led to the conclusion that some of these combinations promote synergistic effects. An important point to be considered is that antagonistic results were not found. However, applied studies in animal models, toxicological evaluations, and clinical studies are needed to generate solid evidence based data to support the use of such therapies.

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