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INVESTIGATION OF THE ACTIVITY AND MECHANISM OF ACTION OF CARVACROL AGAINST RHODOTORULA RUBRA

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Abstract:

The genus Rhodotorula is considered saprophytes. However, in recent decades they have been emerging with the ability to colonize and infect susceptible patients. Most cases of Rhodotorula infections are fungemia associated to catheters, endocarditis, cancer, acquired immunodeficiency syndrome, meningitis, and pleural tuberculosis. With increasing fungal resistance and the reduced spectrum of antifungal drugs available for clinical use, it is important that new alternatives be sought. In the context of this search for new drugs, carvacrol emerges as an option of natural origin, which has a wide range of biological activities. Thus, this study aimed to evaluate the antifungal activity of carvacrol against Rhodotorula rubra and to investigate the mode of action of this phytoconstituent. The Minimum Inhibitory Concentration (MIC) was determined by the broth microdilution method and the mechanism of action on the membrane and on the cell wall of the microorganism was evaluated by the addition of ergosterol and sorbitol to the culture medium, respectively, with subsequent verification of carvacrol MIC in the presence of these two substances. Carvacrol showed significant antifungal activity, and the concentration of 128 µg/mL inhibited the growth of all strains, while 64 µg/mL inhibited half of them. The results suggest that the mechanism of action of carvacrol occurs through binding to ergosterol present in the cell membrane and not by modifying the cell wall of the microorganism. Further studies are needed for further clarification and, thus, allow possible use of carvacrol against R. rubra.

Key-words: *Rhodotorula rubra; carvacrol; ergosterol, sorbitol, monoterpene.*

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INTRODUCTION:

Rhodotorula yeasts belong to the family Sporidiobolaceae, of the order *Sporidiales*. This genus has a mucoid appearance and its colonies can be found in the color spectrum ranging from yellow to red [1]. Such species are considered saprophytes; however, in recent decades they have been emerging with the ability to colonize and infect susceptible patients. Most cases of *Rhodotorula* infections are fungemia associated to catheters, endocarditis, cancer, acquired immunodeficiency syndrome, meningitis, and pleural tuberculosis [2,3]. In addition, there are species in this genus with large capacity to form biofilms [4].

The pharmacotherapy to combat *Rhodotorula* infections is preferably with amphotericin B, 5-flucytosine and fluconazole. Considerable rates of strains belonging to this genus that are resistant to fluconazole, caspofungin and micafungin are reported [5]. The increased resistance of microorganisms to antifungal drugs, toxicity of these drugs, reduced number and high cost of drug options available for the treatment of fungal infections have also drawn attention to the search for compounds with antimicrobial activity [6,7]. One of the main sources of discovery of new drugs is products of natural origin, which are recognized as important in pharmacological research and in the development of new therapeutic tools [8].

Essential oils are natural products that constitute a complex mixture of phytochemicals, such as terpenes, which can be extracted from various aromatic plants and are produced as a consequence of secondary metabolismo [8]. Carvacrol (2-methyl-5-isopropylphenol), belonging to the monoterpenes class, present in the essential oil extracted from plants of various families such as Lamiaceae, Euphorbiaceae, Verbenaceae and Poaceae, has a wide range of biological activities with potential use in practice. clinic, such as: antimicrobial, antioxidant and antitumor [9].

Considering the clinical importance of the infections caused by *Rhodotorula* species, and the need for the development of new antifungal drugs, the objective of this study was to investigate the antifungal activity of carvacrol and the possible mechanisms of action of the phytoconstituent on the plasma membrane and fungal cell wall against strains of *R. rubra*, in order to contribute to the elucidation of the mechanism of action of this terpene.

MATERIALS AND METHODS:

Fungal strains and culture mediums:

The strains used in the study were *R. rubra* LM-680, LM-840, LM-940, LM-139, LM-702 e LM-1. These strains were kindly provided by the collection of fungi cultures at the Mycology Laboratory of "Universidade Federal da Paraíba", Brazil. 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).

Substances:

The substances carvacrol and amphotericin B were purchased from Sigma-Aldrich® (Steinheim, Germany). The solutions were prepared at the time of the testing, these substances were first solubilized in 2% dimethyl sulfoxide (DMSO) and 0,02% Tween 80. They were then solubilized in sterile distilled water to give an initial concentration of 1024 µg/mL.

Inoculum:

For inoculum preparations, cultures of *Rhodotorula* 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 106 CFU/mL.

Minimum inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) of carvacrol and amphotericin B was made in accordance with the CLSI M27-A2 standard (2002) [10] with modifications, using the broth microdilution method. To this end, 100µl of RPMI-1640-L-glutamine (without sodium bicarbonate) concentrate broth was distributed in 96-well microdilution plates. Each substance was serially diluted in proportions of two. Carvacrol concentrations were diluted from 1024 to 2 µg/mL. Then 10µL of the fungal suspension was added to each well. The plates were incubated at 37°C and the reading was taken after 48h, noting the presence or absence of visible fungal growth. The MIC was defined as the lowest concentration of carvacrol or amphotericin B capable of inhibiting visible fungal growth. 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.

Ergosterol binding assay

To determine whether carvacrol interacts with fungal plasma membrane sterols, the carvacrol MIC against *R. rubra* LM-702 was determined by the broth microdilution method as described above, in the absence and presence of different concentrations (100, 200 and 400 µg/ml) of exogenous ergosterol (Sigma-Aldrich®, Steinheim, Germany). Amphotericin B was used as a positive control for the test [11, 12]. This assay was conducted in triplicate and the controls described above were tested.

Sorbitol assay

To assess whether carvacrol acts on the yeast cell wall, the carvacrol MIC was determined for the *R. rubra* LM-702 strain by broth microdilution method as described above, in the presence and absence of 0.8M sorbitol. The microplate was then incubated at 37°C, and the MIC was determined after 2 and 7 days [11, 12]. All experiments were submitted to controls (medium sterility, microorganism viability and solvent interference) and performed in triplicate.

RESULTS & DISCUSSION:

The values of carvacrol MICs against *R. rubra* strains are shown in Table 1. The carvacrol MICs ranged from 8 to 128 µg/mL against the tested strains. The concentration of 128 µg/mL inhibited the growth of all isolates, while 64 µg/mL was able to inhibit 50% of the tested strains.

Table 1: MIC (µg/mL) of carvacrol against *R. rubra* strains.

***R. rubra* strains**	**MIC (µg/mL)**
LM-680	32
LM-840	64
LM-940	64
LM-139	8
LM-702	64
LM-1	128

Studies have shown that carvacrol has antibacterial activity against *Streptococcus mutans* and *Fusobacterium nucleatum* (MIC 0.25% v/v) [13], as well as action on erythromycin-resistant group A *Streptococcus* (MIC 64 to 256 µg/mL) [14]. Moreover, antifungal activity against *Candida albicans* (MIC 128 µg/mL) [15], as well as against *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis* and *Candida krusei* strains resistant to fluconazole was also evidenced. (MIC 75 to 100 µg/mL) [16]. Another study showed carvacrol action against *Aspergillus niger* (MIC 50 µg/mL), *Aspergillus fumigatus*, *Aspergillus flavus*, *Cladosporium* spp. (MIC 100 µg/mL), *Penicillium chrysogenum*, *Fusarium oxysporum* (MIC 125 µg/mL), *Penicillium citrinum* (MIC 150 µg/mL) and against *Rhizopus oryzae* (MIC 200 µg/mL) [17]. The MIC values found in this study are close to those obtained in other studies, and it is important to highlight that no reports on the activity of carvacrol against *Rhodotorula* strains were found in the literature.

Considering the various possibilities of carvacrol interactions with the fungal cell membrane, it was tested whether the antifungal activity observed was due to the ability of the phytoconstituent to interact with ergosterol. The carvacrol MIC value increased as the exogenous ergosterol concentration increased. Similar behavior was observed for MIC (16 µg/mL) of amphotericin B when tested in the presence of ergosterol (Figure 1). The results shown suggest that carvacrol interacts with ergosterol present in the cell membrane of the microorganism. This fact is relevant since this sterol is characteristic of fungal cells.

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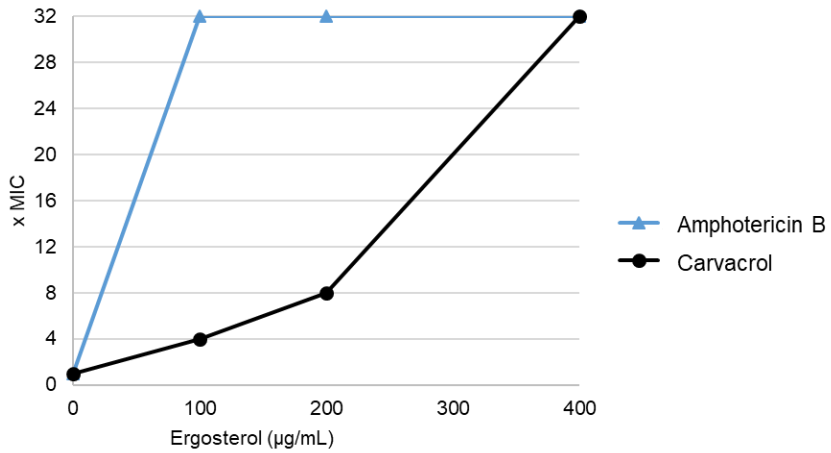
Fig. 1 Effect of different concentrations of exogenous ergosterol on MIC of carvacrol and amphotericin B against *R. rubra* LM-702



The method for assessing the ability of the phytoconstituent to form complexes with ergosterol is based on the exposure of a test compound to an exogenous sterol, where an affinity for sterol will lead to rapid complex formation, thus preventing substance complexation test with membrane ergosterol, resulting in increased MIC [12]. The hydrophobicity shown by the essential oils and their phytoconstituents is probably what allows them to interact with the fungal cell membrane, making them more permeable and liable to damage the integrity of the cell membrane, consequently causing the death of the microorganism [18].

In the assay that consisted of evaluating the carvacrol activity on the cell wall, carvacrol MIC values against *R. rubra* LM-702 in the presence and absence of 0.8 M sorbitol were 64µg/mL in both cases. Sorbitol is an osmotic protector used to stabilize the fungus protoplast when these microorganisms are exposed to substances that act on the fungal cell wall [19]. Based on sorbitol's ability to act as an osmotic protective agent for the fungal cell wall, when higher MIC values were observed in the sorbitol-added medium compared to the absent protector medium, it is understood that the cell wall is one of the possible cellular targets for the compound tested [11]. In the sorbitol assay no increase in MIC was observed, suggesting that this phytoconstituent does not act on the cell wall of the microorganism. As observed in this study, Lima et al. (2013) [15] also report that carvacrol has no cell wall activity against *C. albicans* strains.

CONCLUSIONS:

The present study corroborated with the discovery of the *in vitro* antimicrobial activity of carvacrol against *R. rubra*, which is a fungal species in evidence. Evidence suggests that the possible mechanism of action of carvacrol involves interaction with ergosterol of the fungal plasma membrane. In addition, carvacrol has no activity on the cell wall. It is important to know the mechanism of action of a compound, because such information provides clearer expectations for future pharmacological studies aiming at a therapeutic application of carvacrol against infections caused by *R. rubra*.

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