Artículo original

Determinación de la actividad antifúngica de geraniol frente aislados de *Candida tropicalis* de origen pulmonar

Determination of the Antifungal Activity of Geraniol against Candida Tropicalis Isolates of pulmonary Origin

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RESUMEN

Introducción: La prevalencia de infecciones nosocomiales causadas por levaduras del género *Candida* ha aumentado en especial por especies no albicans. Las formas de candidiasis superficiales pueden evolucionar a formas sistémicas afectando diferentes órganos alcanzando el tracto respiratorio inferior y dando lugar a una candidiasis pulmonar diseminada de diagnóstico complejo, tratamiento difícil y elevada mortalidad, debido al limitado arsenal de antifúngicos que obliga a buscar nuevas alternativas, en las que los monoterpenos derivados de las plantas han mostrado potencial antifúngico.

Objetivo: Evaluar la actividad antifúngica del monoterpeno geraniol frente a aislados de *Candida tropicalis* de origen pulmonar.

Métodos: Se utilizó la técnica de microdilución para determinar la concentración inhibitoria mínima y la concentración fungicida mínima del geraniol. Además, se evaluó el impacto del geraniol y la anfotericina B sobre la micromorfología fúngica y tiempo de muerte frente a cepas de *Candida tropicalis*.

Resultados: El geraniol mostró concentración inhibitoria de 32-64 µg/mL y una concentración fungicida mínima de 128-256 µg/mL. El geraniol (64 µg/mL) inhibió la aparición de estructuras de virulencia como pseudohifas y redujo la formación de blastoconidias en el ensayo de micromorfología. Los resultados mostraron que el geraniol es fungicida concentración-dependiente.

Conclusiones: El monoterpeno geraniol mostró una actividad fungicida significativa frente a aislados pulmonares de *Candida tropicalis* e inhibió la aparición de estructuras de virulencia fúngica, con eficacia dependiente de la concentración.

Palabras-clave: fungicida; monoterpenos; drogas naturales; micosis pulmonar; geraniol; candidiasis.

ABSTRACT

Introduction: The prevalence of nosocomial infections caused by yeasts of the genus Candida has increased especially by non-albicans species. The superficial

forms of candidiasis can evolve to systemic forms affecting different organs reaching the lower respiratory tract and giving rise to disseminated pulmonary candidiasis of complex diagnosis, difficult treatment and high mortality, due to the limited arsenal of antifungals that forces to look for new alternatives, in which monoterpenes derived from plants have shown antifungal potential.

Objective: To evaluate the antifungal activity of the monoterpene geraniol against Candida tropicalis isolates of pulmonary origin.

Methods: The microdilution technique was used to determine the minimum inhibitory concentration and the minimum fungicidal concentration of geraniol. In addition, the impact of geraniol and amphotericin B on fungal micromorphology and time to death against Candida tropicalis strains was evaluated.

Results: Geraniol showed inhibitory concentration of 32-64 µg/mL and a minimum fungicidal concentration of 128-256 µg/mL. Geraniol (64 µg/mL) inhibited the appearance of virulence structures such as pseudo hyphae and reduced ballistoconidia formation in the micromorphology assay. The results showed that geraniol is concentration-dependent fungicidal.

Conclusions: The monoterpene geraniol showed significant fungicidal activity against pulmonary isolates of Candida tropicalis and inhibited the appearance of fungal virulence structures, with concentration-dependent efficacy.

Keywords: fungicide; monoterpenes; natural drugs; pulmonary mycosis; geraniol; candidiasis.

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Introduction

Candida is a common fungus found in the human microbiome. It is an opportunistic pathogen and the leading cause of fungal infections worldwide, ranging from superficial manifestations to systemic forms with high mortality, especially in immunocompromised patients.[1,2]

Recently, there has been an increase in the number of infections caused by non-albicans species. *Candida tropicalis* is one of these emerging non-albicans species with concerning resistance to standard antifungal agents such as azoles and amphotericin B.[3,4]

Pulmonary candidiasis is a rare fungal infection that can seriously compromise respiratory functions, leading to more serious pathologies and increasing the cost and length of hospitalization, as well as the risk of secondary bacterial infection.[5,6]

The diagnosis of pulmonary candidiasis is difficult and often inaccurate, as it is assumed that *Candida* yeasts in respiratory samples are colonization rather than an invasive infection. More accurate diagnostic methods are still being tested to prove their effectiveness.[7,8]

In addition to the vulnerability of immunocompromised patients, the irrational use of antibiotics and azole fungicides in agriculture has led to the emergence of antifungal resistance. The growth of multi-resistant strains complicates the treatment of patients.[9,10]

Natural plant products, such as monoterpenes, are an important source of research for new antifungal metabolites. Geraniol is a monoterpene with high pharmacological value that can be found in a variety of plants.[11,12]

Geraniol (3,7-Dimethyl-2,6-octadien-1-ol) is a monoterpene of a high pharmacological value that can be found in a variety of plants such as *Cymbopogon martini, Pelargonium graveolens, Rosa damascene, Rosa centifolia,* and *Cymbopogon nardus*.[13]

Geraniol is commercially important for the cosmetics industry due to its desirable aroma, but this compound also displays an interesting bioactive profile with

relevant pharmacological functions such as anti-inflammatory, antidepressant and antitumoral activity.[13,14,15]

However, to the best of our knowledge, there has been no report of studies about the effect of geraniol against pulmonary *Candida tropicalis*. In this work we tested the antifungal potential effect of geraniol against yeasts of pulmonary origin by determining its minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against *Candida tropicalis*. We also tested the effects of this compound on the fungal micromorphology and its influence on yeast growth through a time-kill kinetic assay.

Methods

Drugs

Geraniol 95% and Amphotericin B were purchased from Sigma-Aldrich®. Both substances were solubilized in Tween80 2% and dimethyl sulfoxide (DMSO) in a 0.5% proportion. Sabouraud Dextrose Agar (SDA) (Difco Laboratories, Detroit, MI, USA) was used for the cultivation of *Candida* strains. Liquid culture medium Roswell Park Memorial Institute (RPMI)-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich®, São Paulo, SP, Brazil) was used for the antifungal assay. Both culture media were prepared according to the manufacturer's instructions.[16,17]

Isolates

The following strains were used in the experiments: *Candida tropicalis* ATCC-13803, *Candida tropicalis* LM-07, *Candida tropicalis* LM-26, *Candida tropicalis* LM-56, *Candida tropicalis* LM-165, *Candida tropicalis* LM-268, *Candida tropicalis* LM-271. All strains were provided by the Mycology Laboratory of Microorganisms at the Federal University of Paraiba.

All the strains were kept on SDA at 4°C. Fungal inoculum were prepared from *Candida tropicalis* colonies on SDA at 35°C for 24-48 h. They were suspended in sterile 0.85% NaCl solution and adjusted according to the 0.5 McFarland turbidity scale obtaining a final concentration of about 106 colony-forming units per milliliter (CFU/mL). The inoculum was diluted with saline solution in a ratio of 1:1, and a fungal suspension containing 105 CFU/mL was obtained.[16,17]

Antifungal susceptibility testing

Minimum Inhibitory concentration

The minimum Inhibitory concentration (MIC) is defined as the lowest concentration of a drug that is able to produce visible inhibition of fungal growth compared to the control.

The MIC values of geraniol and Amphotericin B was obtained by microdilution technique using RPMI medium in a 96-well microplate. Geraniol and Amphotericin B concentrations range were from 1024 to 0.5 µg/mL and 0.25 to 4 µg/mL, respectively.

The microplates were sealed and incubated at 35 ± 2°C for 24 to 48 h. A negative control (RPMI 1640 + inoculum) and a control with DMSO 0.5% (DMSO + inoculum + RPMI 1640) were included. MIC values were expressed as mode.

The tests were performed in triplicate.[17]

Minimum fungicidal concentration

The minimum fungicidal concentration (MFC) is the lowest concentration of a drug that results in less than 3 colony growths or 99.5% death.

Briefly, 10 µL aliquots of supernatant from the wells that presented complete inhibition of fungal growth (MIC, MIC × 2, and MIC × 4) in the MIC test were placed on ASD plates (Difco®) and incubated for 24 - 48 h at 35 ± 2°C.

Assays were performed in triplicate and the results were expressed as mode. A drug demonstrates fungicidal action when the MFC/MIC ratio does not exceed the value of 4 and it is considered fungistatic when MFC/MIC is greater than 4.[18]

Effect on fungal micromorphology

To observe the fungus morphological changes caused by the drugs, it was used the microculture technique for yeasts in a moist chamber. *Candida tropicalis* ATCC-13803 and *Candida tropicalis* LM-165 strains were chosen in accordance with MIC results.

First, a liquefied corn-meal agar containing the test compound in concentration equal to its MIC was poured onto a slide until solidification. Then, inoculum suspensions were prepared from fresh cultures of each strain and seeded to form two parallel striations on the cornmeal agar and then covered with a sterile coverslip. Finally, filter paper soaked into sterile distilled water was used to keep the system humid. Each plate was sealed and incubated at 35°C for 24-72 h.

After the incubation period, each sample was examined under the optical microscopy with a magnification of 40x to observe the formation (or not) of yeast-typical structures like *ballistoconidia, pseudo hyphae*, and *chlamydoconidia*.

The experiment was performed in duplicate.[19,20]

Time-kill assay

The analysis of the interference of the test compounds on the viability of *Candida tropicalis* ATCC-13803 and *Candida tropicalis* LM-165 was performed.[21]

Three tubes with 9 mL of SDB and Geraniol at MIC, MIC x 2 and MIC x 3 (64 µg/mL, 128 µg/mL and 256 µg/mL, respectively) concentration were prepared.

To each tube, 1 mL of fungal was added. Amphotericin B in MIC, MIC x 2 and MIC x 4, corresponding to 1 µg/mL, 2 µg/mL and 4 µg/mL, respectively was used as control.

A negative control (SDB + DMSO + inoculum) and sterility control (SDB + DMSO, without inoculum) were also included.

An aliquot of 10 µL of the samples was collected in different time exposure intervals (0 h, 2 h, 4 h, 8 h and 24 h) and they were uniformly seeded on SDA plates.

The plates were incubated at 35 ± 2°C for 24 to 48 h.

After incubation, the number of viable cells was counted and expressed as number of colony-forming units per milliliter (CFU/mL).

Mean CFU counts were plotted as a function of time (log10 CFU/mL vs hours of incubation) for each isolate at each concentration of antifungal tested.

All tests were performed in triplicate.

Fungicidal activity was defined as a ≥ 3 log10 (99.9%) decrease in CFU.mL-1 from the initial inoculum. A lower activity was considered fungistatic.[21]

A one-way analysis of variance (ANOVA) was used to determine significant differences ($p < 0.05$) between treatments at each incubation time (2 h, 4h, 8h and 24 h).

When significant, the results were submitted to Tukey's test, using the GraphPad Prism 8.0.0. software for Windows, San Diego, CA, USA, http://www.graphpad.com.

Results

Determination of minimum Inhibitory Concentration (MIC) and minimum fungicidal concentration (MFC).

MIC and MFC values of Geraniol and Amphotericin B are shown in table 1. Geraniol showed MIC values varying from 32 to 64 µg/mL against *Candida tropicalis*. It presented fungicide action against 71% of the tested strains.

Amphotericin B inhibited the growth of *Candida tropicalis* strains at 1 µg/mL and showed fungicide action against 100% of the tested strains.

Table 1- MIC and MFC values (µg/mL) of Geraniol and Amphotericin B against *Candida tropicalis* strains

Candida tropicalis	Strains	Geraniol				Amphotericin B			
		MIC	MFC	MFC/MIC	Action mode	MIC	MFC	MFC/MIC	Action mode
	ATCC-13803	64	128	2	Fungicide	1	2	2	Fungicide
	LM-07	32	256	8	Fungistatic	1	1	1	Fungicide
	LM-26	32	256	8	Fungistatic	1	1	1	Fungicide
	LM-56	64	256	4	Fungicide	1	1	1	Fungicide
	LM-165	64	256	4	Fungicide	1	1	1	Fungicide
	LM-268	64	64	1	Fungicide	1	1	1	Fungicide
	LM-271	64	256	4	Fungicide	1	1	1	Fungicide

MIC, minimal inhibitory concentration; MFC, minimal fungicide concentration.

Effect on fungal micromorphology

The results in table 2 shows that geraniol at MIC of 64 µg/mL inhibited pseudo hyphae and chlamydoconidium formation in the *Candida tropicalis* strains assayed.

Table 2 - Micromorphological changes promoted by 64 µg/mL Geraniol essential oil against *Candida tropicalis* strains after 48 h and 72 h (chlamydoconidium) of incubation

-	Pseudo hyphae	Ballistoconidia	Chlamydoconidium
Control (distilled water)	+	+	-
Candida tropicalis ATCC-13803	-	+/-	-
Candida tropicalis LM-165	-	+/-	-

Presence (+), few (+/-) and absence (-) of the structure.

Time-kill assay

To evaluate the antifungal potential of Geraniol, we observed the microbial growth of yeasts exposed to Geraniol and Amphotericin B at different time intervals. Figures 1 and 2 present the log10 of CFU/mL of *Candida tropicalis* ATCC 13803 and

LM-165 versus the time of exposure in the presence of Geraniol and Amphotericin B (MIC, MIC x 2 and MIC x 4), respectively.

We observed that Geraniol (MIC, MIC x 2, MIC x 4) significantly reduced growth of *Candida tropicalis* ATCC-13803 ($P < 0.05$) and *Candida tropicalis* LM-165 ($P < 0.05$) starting from 2 hours of exposure (fig. 1 and 2). Similar performance was observed with the standard treatment amphotericin ($p < 0.05$).

Geraniol, at MIC x 4, exhibited fungicidal activity against *Candida tropicalis* ATCC-13803 after 8 hours of exposure, resulting in a reduction of 3 log10 CFU/mL (99,9%) of the initial inoculum (fig. 1). Similar results were observed with amphotericin MIC x 4 after 4 h of exposure.

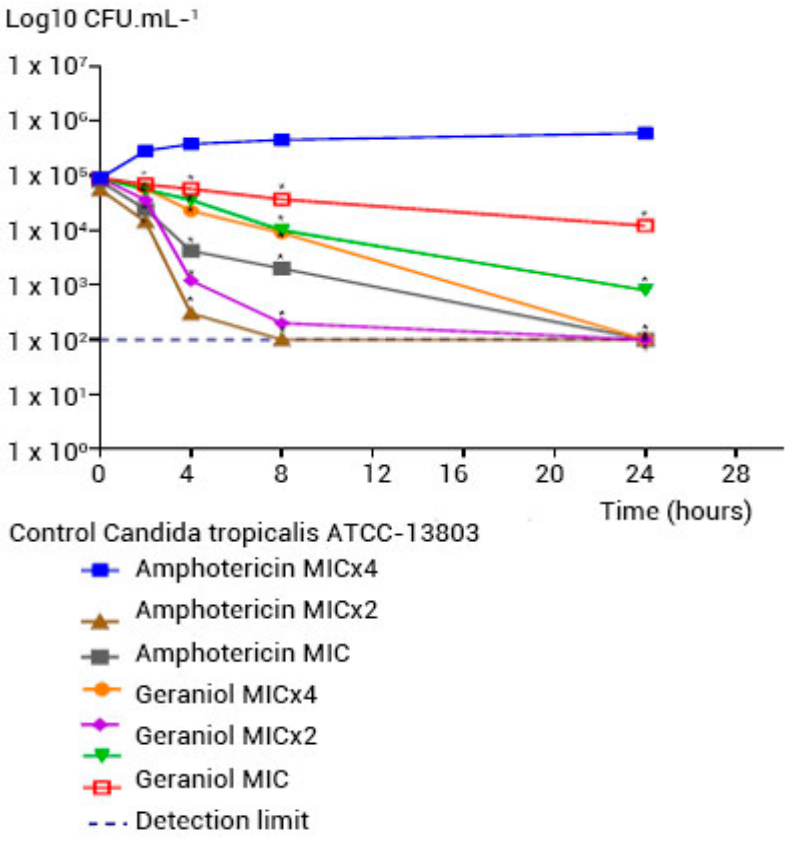

Fig. 1- Time-kill curve of *Candida tropicalis* ATCC-13803 when exposed to various concentrations of geraniol and Amphotericin B at different time intervals (0, 4, 8, 24h). (*) Significant difference ($p < 0.05$) when compared to control.

In figure 2 Geraniol MIC x 4 produced fungicidal activity against the clinical isolate *Candida tropicalis* LM-165 after 8 h. Geraniol MIC x 2 (128 µg/mL) induced the elimination of 99.9% (reduction of 3 log10 CFU/mL) of the *Candida tropicalis* population at 24 hours when compared to the starting inoculum.

The Geraniol and Amphotericin B showed fungicidal activity against *Candida tropicalis* isolates in a concentration-dependent relationship.

All strains demonstrated satisfactory growth under positive control conditions, indicating yeast viability. No microbial growth was observed on sterility control plates.

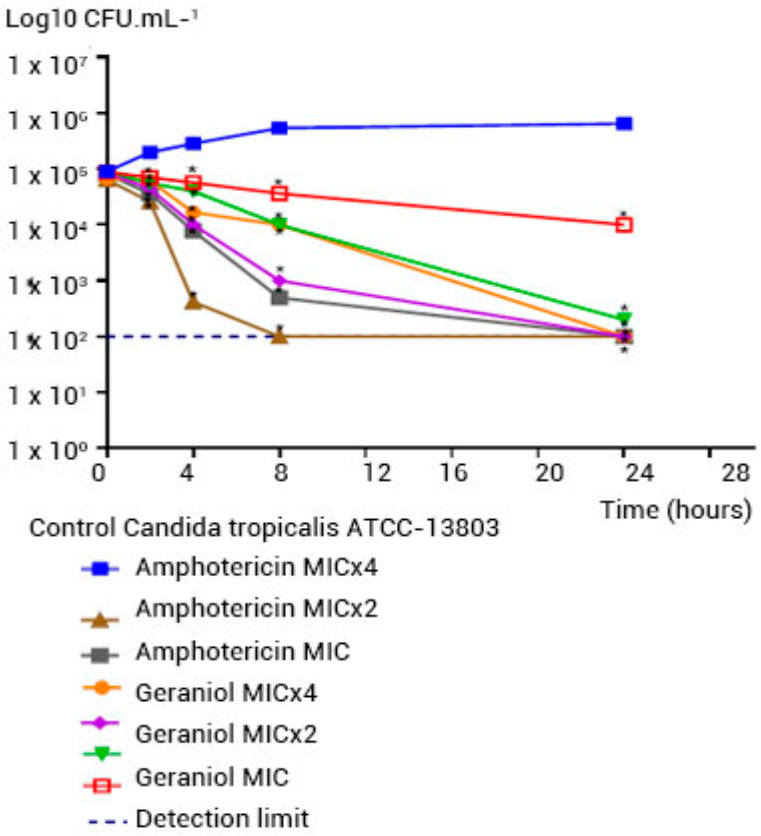

Fig. 2 - Time-kill curve of *Candida tropicalis* LM-165 when exposed to various concentrations of Geraniol and Amphotericin B at different time intervals (0, 4, 8, 24 h). (*) Significant difference ($p < 0.05$) when compared to control.

Discussion

Candida tropicalis has become a prevalent isolate in invasive candidiasis, dividing the attention to emerging species like *Candida auris*, which is now recognized for its resistance to conventional antifungal drugs.[4,21] Consequently, there is a demanding need for natural antifungal alternatives that can effectively address resistance issues while minimizing adverse reactions.[22]

Over the decades, the use of azole fungicides in agriculture and livestock has led to the emergence of resistance, creating a challenging situation.[21] Hence, there is a need for natural antifungal alternatives that can overcome resistance concerns and unwanted side-effects.[22]

Geraniol (C10H18O) a monoterpene commonly found in various volatile oils and distinguished by its characteristic odor and flavor, emerges as a promising alternative.[20]

In this work, results of MIC and MFC of Geraniol in cultures of *Candida tropicalis* demonstrate effectiveness against the clinical and standard strains. The concentrations found revealed more effective than in previous studies.[22] Our MICs (32 and 64 µg/mL) were lower than 100 µg/mL and therefore considered as good/significant.[23,24]

Despite similar methodologies employed in both studies, disparities in outcomes may be attributed to variations in isolate origins. Specifically, we utilized clinical pulmonary isolates, which usually exhibit higher virulence, supporting the antifungal potential of Geraniol.[25,26,27]

The nature of the antifungal effect produced by a drug against a specific microorganism can be classified according to the ratio between its MFC and MIC, where a MFC/MIC ratio ≤ 4:1 indicates a fungicidal characteristic of the product, and a MFC/MIC ratio > 4:1 suggests this product is fungistatic.[28]

The fungicidal effect is ideal for treating invasive *Candida* infection because it is associated with a greater chance of therapeutic success and a lower chance of

recurrent infection.[25] Remarkably, in the present study, Geraniol demonstrated fungicidal activity against the majority of clinical isolates *Candida tropicalis*.

Our study observed the inhibition of pseudo hyphae formation in strains treated with geraniol. These structures play a crucial role in *Candida* virulence contributing to biofilm formation, acting as a barrier against phagocytosis, leading to increased resistance to antifungal agents and immune system evasion.[23,26] In addition, due to their morphology, can easily penetrate epithelial and endothelial tissues, facilitating tissue invasion.[4,26]

This finding corroborates with the results reported by *Souza* et al.,[23] which likewise demonstrated the inhibitory effects of linalool and geraniol on *Candida tropicalis* biofilms obtained from dental prostheses, achieving 50% biofilm inhibition at concentrations ranging from 500 to 8000 µg/mL.

However, geraniol exhibits characteristics such as hydrophobicity, high volatility, and potential toxicity, which may hinder its utility. Nevertheless, strategies such as encapsulation with lipid-based carriers can mitigate these limitations by enhancing shelf life, reducing toxicity, and improving efficacy.[22] Moreover, due to their volatility, terpenes like geraniol can readily access the respiratory tract via inhalation, offering a viable option for pulmonary infections.[28,29,30]

The antifungal potential of geraniol is undeniable and has been demonstrated against various fungi.[21] This is primarily attributed to its ability to regulate ergosterol biosynthesis, a critical biological pathway in fungal organisms. Disruption of ergosterol equilibrium in fungal cell membranes enhances permeability, compromising structural integrity, and ultimately leads to cellular demise.[20,21,22]

It is necessary to underline that this study presents the first data on the antifungal activity of Geraniol against pulmonary clinical isolates of *Candida tropicalis*. Our findings suggest that geraniol could serve as an effective alternative antifungal agent in natural medicine, possibly complementing standard antifungal therapies, particularly in severe respiratory mycoses.

In conclusion, geraniol exhibits promising in vitro antifungal potential against C. tropicalis at concentrations as low as 32 and 64 µg/mL. It also induced significant morphological changes in fungal cells and exhibited a concentration-dependent fungicidal effect.

Furthermore, our outcomes contribute treatment to the field for invasive candidiasis, the most common human fungal disease among hospitalized patients, particularly in the context of emerging resistant non-albicans pathogens.[21]

However, further research is necessary to investigate the effect of geraniol in combination with other antifungals and its efficacy against resistant strains. Additionally, in vivo toxicological studies are required to confirm the effectiveness and safety of geraniol for clinical application.

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Conflict of Interest

The authors declare no conflict of interest.

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