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Evaluation of the antifungal activity and mode of action of citral against *Cladophialophora carrionii.*

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Summary

Introduction: *Cladophialophora carrionii* is one of the most frequent etiologic agents of human chromoblastomycosis, a chronic cutaneous disease. Such fungal infections are difficult to treat and underlines the need for new antifungal agents. Citral is a monoterpene with known pharmacological properties, including antimicrobial activity. **Aims:** To investigate the antifungal activity of citral against strains of *C. carrionii*. **Methodology:** The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined by broth microdilution techniques. Citral was tested to evaluate its effects on *C. carrionii* mycelia growth and germination of fungal conidia. Next, it was investigated the possible citral action on cell walls (sorbitol assay) and cell membranes (ergosterol binding assay). **Results:** The MIC_{50} and MFC_{50} of citral were respectively 128 μg/mL and 512 μg/mL. The study shows that citral displayed *in vitro* antifungal potential against strains of *C. carrionii*, where it was capable of inhibiting both its mycelial growth and germination of conidia for *this fungus*, whilst affecting the structure of fungal

cell membranes. Citral's mechanism of action involves binding to ergosterol. Further study is needed to completely describe its effects before clinical use as a therapeutic antifungal agent.

Keywords: Antifungal, Chromoblastomycosis, Citral, *Cladophialophora carrionii*, Monoterpene.

Resumen

Evaluación de la actividad antifúngica y forma de acción de citral frente a *Cladophialophora carrionii.*

Introducción: *Cladophialophora carrionii* es uno de los agentes etiológicos más frecuentes de la cromoblastomicosis humana, una enfermedad cutánea crónica. Tales infecciones fúngicas son difíciles de tratar y subrayan la necesidad de nuevos agentes antifúngicos. Citral es un monoterpeno con propiedades farmacológicas conocidas, incluida la actividad antimicrobiana. **Objetivo:** investigar la actividad antifúngica de citral contra cepas de *C. carrionii*. **Metodología:** la concentración inhibitoria mínima (CIM) y la concentración fungicida mínima (CFM) se determinaron mediante técnicas de microdilución de caldo. Citral se probó para evaluar sus efectos sobre *C. carrionii* crecimiento de micelios y germinación de conidios fúngicos. A continuación, se investigó la posible acción citral sobre las paredes celulares (ensayo de sorbitol) y las membranas celulares (ensayo de unión al ergosterol). **Resultados:** La CIM_{50} y CFM_{50} de citral fueron respectivamente 128 µg/mL y 512 µg/mL. El estudio muestra que citral muestra potencial antifúngico *in vitro* contra cepas de *C. carrionii*, donde fue capaz de inhibir tanto su crecimiento micelial como la germinación de conidios para este hongo, a la vez que afectaba a la estructura de las membranas celulares fúngicas. El mecanismo de acción de Citral implica la unión al ergosterol. Se necesitan estudios adicionales para describir completamente sus efectos antes del uso clínico como agente antifúngico terapéutico.

Palabras clave: Antifúngico, chromoblastomicosis, citral, *Cladophialophora carrionii*, monoterpeno.

Resumo

Avaliação da atividade antifúngica e modo de ação do citral contra *Cladophialophora carrionii.*

Introducão: *Cladophialophora carrionii* é um dos agentes etiológicos mais frequentes da cromoblastomia humana, uma doença cutânea crônica. Tais infecções fúngicas são difíceis de tratar e sublinham a necessidade de novos agentes antifúngicos. Citral é um monoterpeno com propriedades farmacológicas conhecidas, incluindo atividade antimicrobiana. **Objetivos:** investigar a atividade antifúngica do citral contra cepas de *C. carrionii*. **Metodologia:** a concentração inibitória mínima (CIM) e a concentração fungicida mínima (CFM) foram determinadas por técnicas de microdiluição. Citral foi testado para avaliar seus efeitos em crescimento micelar e germinação conidialde *C. carrionii.* Em seguida, foi investigada a possível ação do citral na parede celular (ensaio de sorbitol) e membrana celular (ensaio de ligação ergosterol) fúngicas. **Resultados:** a CIM_{50} e CFM_{50} do citral foram respectivamente 128 μg/mL e 512 μg/mL. O estudo mostra que o citral exibiu potencial antifúngico *in vitro* contra *C. carrionii*, onde era capaz de inibir tanto seu crescimento micelar quanto a germinação conidial, ao mesmo tempo em que afetava a estrutura da membrana celular fúngica. O mecanismo de ação do citral envolve a ligação ao ergosterol. Mais estudos são necessários para descrever completamente seus efeitos antes do uso clínico como um agente antifúngico terapêutico.

Palavras-chave: Antifúngico, cromoblastomicose, citral, *Cladophialophora carrionii*, monoterpeno.

Introduction

Cladophialophora carrionii are saprobic dematiaceous fungi, associated with opportunistic human and animal infections. This fungus is one of the relatively most common causative agents of chromoblastomycosis, a chronic, progressive, polymorphic implantation mycosis of skin and subcutaneous tissue, causing hyperproliferation leading to verrucous nodular clinical features, and is histologically characterized by muriform cells. Infection occurs after the etiologic agent gains entrance through a traumatic lesion [1].

The disease is seen worldwide, but most reports are from tropical and subtropical areas, with greater prevalences in Africa and Latin America [2]. The lesions associated with the disease are polymorphic or hyperkeratotic and in therapy must be differentiated

from those associated with other infectious or autoimmune disorders [3]. This infection is very difficult to treat. Various therapies are commonly applied, such as amphotericin, which binds to the fungal ergosterol and creates pores in the fungal membrane leading to the leakage of intracellular contents, and voriconazole an ergosterol biosynthesis inhibitor. However, there is no standard treatment [1].

The increased incidence of these fungal infections, especially dangerous hospital-acquired infections and infections in immunocompromised patients, has underlined the need for new antifungal treatments. Since there are limited antifungals options and some strain are able to exhibit antifungal resistance, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants [4].

Plants and their derivatives are known to be important in pharmacological research; this is due to their great potential as a source for a variety of biologically active components used in drug development. Among these products are the terpenes, a class of phytoconstituents formed by the 5-carbon alkene isoprene (C_5H_8) [5].

Citral (3,7-dimethyl-2-6-octadienal) is a natural mixture of two geometric isomers: geranial (*trans*-citral) and neral (*cis*-citral), which are acyclic α,β-unsaturated monoterpene aldehydes that occur naturally in many essential citrus fruit oils and in other herbs or spices [6]. Citral has become a flavoring substance of great importance and a heavily used raw material for the pharmaceutical, food, perfume, and cosmetics industries [7].

Various biological activities such as anti-inflammatory, anti-tumor, antiprotozoal and spasmolytic have been reported for citral [8-10]. Besides its low toxicity (LD_{50}>1000mg/kg), antimicrobial activity was exhibited as demonstrated through *Trichophyton mentagrophytes* hyphae growth inhibition, where it was observed that the cell membrane and organelles were irreversibly damaged by citral, as well as a potent *in vitro* activity against *Candida* spp. [11-13].

Given the above, the aim of this study was to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of citral, and to also investigate its mechanism of action against *C. carrionii* regarding its mycelial growth, conidial germination, cell wall formation, and interactions involving ergosterol.

Materials and methods

Micro-organisms

The strain *C. carrionii* URM-2871 used in the antifungal assay was obtained from the fungal collection of the Mycology Department (URM), Biological Sciences Center, Federal University of Pernambuco, Brazil and randomly selected *C. carrionii* strains (LM-0212 and CQ-02) were obtained from the Micro-organisms Collection of the Mycology Laboratory, at the Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil. The samples were maintained on Sabouraud dextrose agar (SDA) (DIFCO®) under refrigeration (4 °C).

Stock inoculations (suspensions) of *C. carrionii* were prepared from 7-14 days old SDA (Difco Lab., USA); the cultures were grown at room temperature (28 °C to 30 °C). Fungal colonies were covered with 5 mL of sterile saline solution (0.9%), and then, gently agitated with vortexes. Next, the fungal elements in saline solution were transferred to sterile tubes. The inoculum was standardized according to the 0.5 McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the micro-organisms in a Neubauer chamber [14-16].

Chemicals

The product tested was the monoterpene Citral, obtained from (Sigma Aldrich, Brazil) as well as Amphotericin B and Voriconazole. The monoterpene was dissolved in 2% tween 80 and DMSO (dimethylsulfoxide). The antifungal standards were dissolved in DMSO, and sterile distilled water to obtain solutions of 2048 μg/mL for each substance. The concentration of DMSO did not exceed 0.5% in the assays.

Culture media

The culture media employed to test the biological activity of the products were SDA, purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil). Both were prepared and used according to the manufacturers' instructions.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

Broth microdilution assays were used to determine the MICs of monoterpene citral, amphotericin B and voriconazole against *C. carrionii* (URM-2871, LM-0212 and CQ-02). RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the agents were prepared to obtain concentrations varying between 4 μg/mL

and 1024 μg/mL. Finally, 10 μL aliquots of the inoculum suspension were added to the wells, and the plates were incubated at 28 °C for 5 days. Negative controls (without drugs) were used to confirm conidia viability and sensitivity controls (for DMSO and Tween 80) were also included in the studies. At 5 days, there were visual observations of fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 99.9%. The results were expressed as the arithmetic mean of the three experiments [15, 16].

The MFC was determined by microdilution method to verify if the inhibition was reversible or permanent [17]. Aliquots of 20 μL (from the wells without microbial growth detected in the MIC procedure) were transferred to 96-well plates previously prepared with 100 μL of RPMI-1640. The plates were aseptically sealed followed by mixing on a plate shaker (300 rpm) for 30 seconds, incubated at 28 °C and read at 5 days of incubation. Tests were performed in duplicate and the geometric mean values were calculated. MFC was defined as the lowest citral concentration in which no visible growth occurred when subcultured on the 96-well plates containing broth without antifungal products.

After determination of the MIC and MFC, one strain (*C. carrionii* URM 2871) was selected to continue the citral antifungal activity study.

Effects on mycelia growth

Analyses of the interferences of citral, voriconazole and amphotericin on *C. carrionii* URM-2871 mycelia growth were determined using poisoned substrate technique (dilution in solid medium) by daily measuring of radial mycelial growth on SDA by adding products in an amount adjusted to provide final concentrations similar to the MIC, MIC × 2, and MIC × 4 previously found in order to assess if its effect occurs in a dose-dependent manner. For this, 2 mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28 °C were placed at the center of the sterile SDA Petri dishes containing the test drugs. At different intervals (0, 2, 4, 6, 8, 10, 12, and 14 days) of incubation at 28 °C, the mold's radial mycelial growth was measured (mm) with calipers. The controls in this assay revealed the mold's radial growth on SDA without adding drugs. Each assay was performed twice and the results were expressed as the average of the two repetitions [18, 19].

Conidial germination assay

Citral, voriconazole and amphotericin B were tested to evaluate the effects on the germination of *C. carrionii* URM-2871 fungal conidia. Flasks containing MIC, MIC × 2 and MIC × 4 of citral, voriconazole, amphotericin and a control with distilled water

were used. In sterile test tubes, 500 μL of RPMI-1640 plus citral were evenly mixed with 500 μL of fungal conidia suspension and immediately incubated at 28 ºC. Samples of the mixture were taken after 48h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the spore germination inhibition percentage at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment. The analysis was conducted under an optical microscope (Zeiss Primo Star) [20].

Sorbitol assay effects

The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *C. carrionii* URM-2871 cell wall at the starting concentration of 1024 μg/mL. The sorbitol was added to the culture medium in a final concentration of 0,8M. The assay was performed by microdilution method in 96-well plates in a "U" (Alamar, Diadema, SP, Brazil) [15, 16]. The plates were sealed aseptically, incubated at 28 ºC and readings were taken at 5 days. Based on the ability of sorbitol to act as an osmotic protective agent of the fungal cell wall, the higher MIC values observed in the medium with sorbitol added (as compared to the standard medium) suggests that the cell wall is a possible target for the product tested [12, 21, 22]. The assay was performed in duplicate and expressed as the geometric mean of the results.

Ergosterol binding assay: MIC value determination in the presence of ergosterol.

To assess if the product binds to fungal membrane sterols, an experiment was performed according to the method described by Escalante *et al.* (2008) [23], with some modifications. Ergosterol was prepared as described by Leite *et al.* (2014) [12]. The MIC of citral, against *C. carrionii* URM 2871 was determined by using broth microdilution techniques at the starting concentration of 1024 μg/mL [15, 16], in the presence and absence of exogenous ergosterol added to the assay medium, in different lines on the same microplate. Briefly, a solution of citral was two-fold diluted with RPMI-1640 (100 μL) containing ergosterol added at a concentration of 400 μg/mL. 10 μL of the yeast suspension (0.5 McFarland) was added to each well. The same procedure was realized for amphotericin B, whose interaction with membrane ergosterol is already known, serving as a control drug. The plates were sealed and incubated at 28 ºC. The plates were read after 5 days of incubation and the MIC was determined as the lowest concentration of test agent inhibiting visible growth. The assay was carried out in duplicate and the geometric mean of the values was calculated. The binding assay reflected the ability of the compound to bind with ergosterol.

Statistical analysis

The results are expressed as mean ± S.E. Differences between the means were statistically compared using the Student's t-test. The values were considered significant with $p < 0.05$.

Results and discussion

The results for citral's antifungal activity against *C. carrionii* were determined using the MIC and MFC in broth microdilutions. The MIC of citral varied between 128 and 256 μg/mL. The MIC_{50} (minimum fungicidal concentration for 50% of strains tested, calculated via arithmetic mean), was 128 μg/mL. Amphotericin B and voriconazole retained a lesser MIC_{50} than the phytoconstituent at 16 μg/mL MIC (Table 1).

Table 1. MIC and MFC of citral, amphotericin B and voriconazole against *C. carrionii.*

Micro-organisms	Citral (μg/mL)		AMB (μg/mL)		VCZ (μg/mL)		Control strains*
	MIC	MFC	MIC	MFC	MIC	MFC	
C. carrionii URM-2871	128	512	16	64	16	16	+
C. carrionii LM-0212	256	1024	>1024	ND	>1024	ND	+
C. carrionii CQ-02	128	256	16	64	8	16	+

Legend: AMB, Amphotericin B; VCZ, Voriconazole; MIC, Minimum Inhibitory Concentration; MFC, Minimum Fungicidal Concentration; ND, Not determined; *, Micro-organism growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal agents or monoterpenes.

The MFC of citral varied between 256 and 1024 μg/mL. The MFC_{50} (minimum fungicidal concentration for 50% of strains tested) was 512 μg/mL. The MFC_{50} for amphotericin B and voriconazole were 64 μg/mL and 16 μg/mL, respectively (Table 1).

The results for the control (Tween 80 and DMSO) showed no fungal growth inhibition; fungal growth in the medium without drug added was detected (viability control). In accordance with the results aboce, the *C. carrionii* URM-2871 strain was selected for further testing.

In previous studies, it was observed that *Melissa officinalis* essential oil (at 256 μg/mL) has antifungal activity, inhibiting the mycelial growth and conidial germination of *C. carrionii* [24]. *M. officinalis* essential oil is characterized by monoterpene compounds and citral is the principal component present [25].

The antimicrobial activity of citral has been confirmed. More recently, studies have shown that citral exhibits antimicrobial effect against *C. sakazakii* strains, with MICs ranging from 0.27 to 0.54 mg/mL [26]. Khan and Ahmad (2013) [27] showed that citral has effective antifungal activity against azole-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. In recent studies, citral showed *in vitro* antifungal potential against strains of *C. albicans* [12] and *C. tropicalis* [13].

In the present study, citral showed activity against *C. carrionii* URM-2871. The product is therefore considered actively antifungal in accordance with the parameters defined by Sartoratto *et al.* (2004) [28]: strong activity (MIC<0.50 mg/mL), moderate activity (0.6<MIC<1.50 mg/mL) and weak activity above 1.50 mg/mL.

This study also verified citral's action against *C. carrionii* mycelial growth and spore germination. The effect of differing concentrations of the test drug (MIC, MIC × 2 and MIC × 4) on mycelia growth was determined by measuring radial mycelial growth and the results are shown in Figure 1. Concerning *C. carrionii*, it can be seen that citral at its MIC concentration (128 μg/mL) was not capable of inhibiting mycelial growth, but at MIC × 2 (256 μg/mL) and MIC × 4 (512 μg/mL) concentrations, normal mycelia growth was inhibited when compared to the control (mycelia diameter being 99.9%). The control strains showed a constant rate of mycelial growth over the time evaluated, indicating good antifungal effect for citral (Fig.1A).

The voriconazole test against *C. carrionii* URM-2871 showed significant inhibition of mycelial growth at all concentrations tested (Fig. 1B). With amphotericin B, inhibition of mycelial growth was only observed at a higher concentration, MIC × 4 (64 μg/mL) (Fig. 1C).

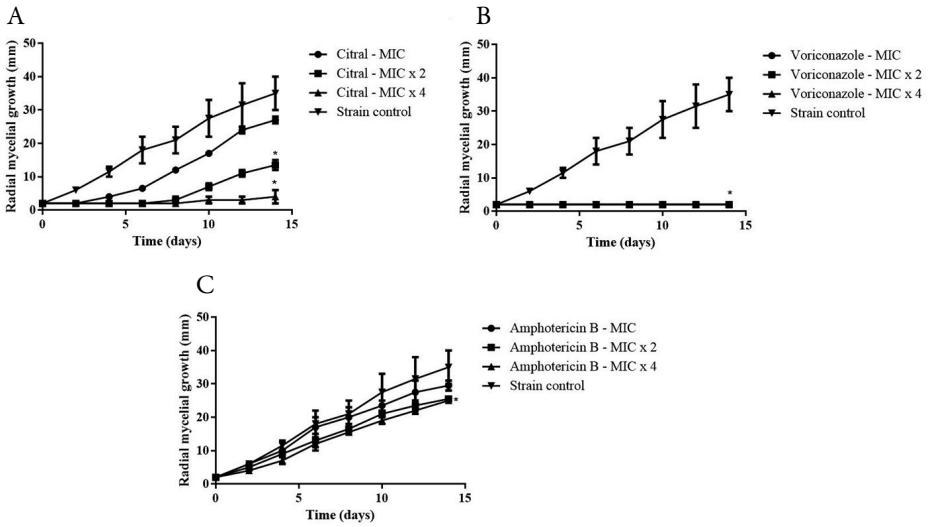

Figure 1. Radial mycelial growth produced by *C. carrionii* URM-2871 in the absence (control), and presence of citral (A), voriconazole (B) and amphotericin B (C). $^{*}p < 0.05$ compared to control.

The results show that citral at its MIC × 2 and MIC × 4 concentrations, was more potent than amphotericin B at its respective MIC × 2 and MIC × 4 concentrations ($p < 0.05$). However, the action was shown to be less potent ($p < 0.05$) when compared with respective MIC and MIC × 2 concentrations of voriconazole.

The production of hyphae and consequent mycelium formation are important virulence factors for fungal filaments as well as serving as relevant protective factors which demands higher doses of antifungal agents to exert inhibitory effect. In an infection, longitudinal growth of the hyphae facilitates penetration into the inner layers of the skin, while lateral growth exacerbates the damage [29]. Hyphae are more difficult to phagocytize and can induce apoptosis in macrophages, since they often form inside the macrophage after phagocytosis [30].

These results corroborate with the data obtained in previous studies of investigation of the antifungal potential of citral in inhibiting the mycelial growth of pathogenic and non-pathogenic fungi [31, 32]. A previous study reported that citral at concentrations of 50 and 100 μg/mL can inhibit *Trichophyton mentagrophytes* growth in PDA by 8.5% and 99.9%, respectively [11].

More recently, OuYang *et al.* (2016) [33] observed that citral inhibited the mycelial growth of *Penicillium digitatum*, with the MIC of 1.78 mg/mL in a dose-dependent

manner. Given the importance of mycelial growth to the development of mycoses, the inhibition of *C. carrionii* mycelial growth caused by citral (as observed in the present study), proving superior to amphotericin B in its respective concentrations, is a significant contribution to the search for new natural products with antifungal activity.

The study of the conidia germination has great implications for clinical practice, because it is possible to develop new therapeutic approaches that block the infection at its onset [34]. From this perspective, the effect of citral on the germination of *C. carrionii* URM-2871 conidia was investigated. The effects of different concentrations (MIC, MIC × 2 and MIC × 4) of citral, voriconazole and amphotericin B on the germination of conidia are shown in Fig. 2.

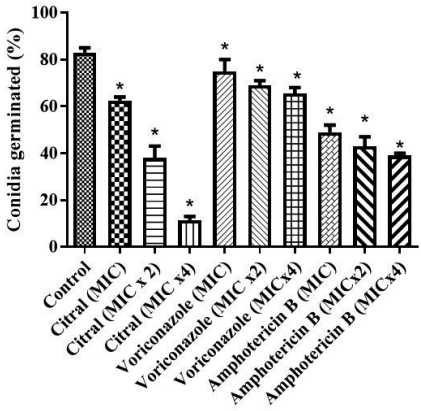

Figure 2. *C. carrionii* URM-2871 conidial germination percentage in the absence (control) and presence of citral, voriconazole and amphotericin B. $^{*}p < 0.05$ compared to control.

In the three test concentrations (MIC, MIC × 2 and MIC × 4), citral displayed significant inhibitory action against *C. carrionii* as compared to the control. The tested antifungals standards (voriconazole and amphotericin B), also showed significant inhibition on conidia germination. However, the results shows that citral at its MIC × 4 concentration was more potent than amphotericin B and voriconazole at their respective MIC × 4 concentrations ($p < 0.05$).

These results are in accordance with previously published work, as the one conducted by Li *et al.* (2014) [35], who described the effect of citral on *Magnaporthe grisea*. In this study, it was found that germination in a concentration-dependent manner was significantly inhibited by citral and a similar trend was observed for mycelial growth, demonstrating the efficacy of citral for inhibition of pathogenic fungal growth.

Conidia represent the most common mode of asexual reproduction; they play an important role in natural fungal propagation and are structurally resistant. It is important to note that the conidia are not merely quiescent cells; a basal level of RNA and protein synthesis is required for survival of the spores [21]. Conidia are distributed in large quantities in the atmosphere and some have the ability to cause disease in humans, animals and plants [36]. Thus, it is important to quantitatively evaluate the power of a product to interfere with fungal spore germination [30].

The great challenge when developing new antifungal drugs is in the similarity between fungal cells and human cells. Thus, the targets for a new antifungal's action must be unique or at least sufficiently different from the host [37]. Based on this, two important fungal structures become targets for detecting antifungal agents; the fungal cell wall and the ergosterol present in the plasma membrane.

Ergosterol is one of the principal sterol components in the fungal membrane and plays the same role in fungal membranes that cholesterol plays in the mammalian cell. Generally, a decrease in ergosterol content results in osmotic disturbances with disruption of cell growth and proliferation [32, 38]. Many drugs available for clinical use interact directly with ergosterol, causing damage to the fungal cell membrane.

Considering possible fungal cell membrane interference of citral, the compound was tested to investigate its ability to form complexes with ergosterol (Table 2). Whether the effects of citral on the fungal cell are due to ergosterol binding in the membrane can be verified if they interact directly. In the presence of exogenous ergosterol in the culture medium, decreased binding of the product to the ergosterol of the membrane occurs. Thus, the product's MIC tends to increase in the presence of exogenous ergosterol, needing a much higher concentration to interact with ergosterol in the fungal membrane [12].

The MIC of citral against *C. carrionii* increased sixteen times in the presence of ergosterol at 400 μg/mL. Amphotericin B, the positive control that has a known interaction with ergosterol showed a 341-fold higher MIC in the presence of ergosterol. These results suggest that the mechanism of citral's antifungal action involves direct interaction with ergosterol, which leads to the disruption of the fungal membrane and loss of intracellular contents.

To investigate the action of the product on the fungal cell wall, was performed an assay with sorbitol (Table 2). Sorbitol is an osmotic protective used to stabilize fungi protoplasts. Specific fungal cell wall inhibitors share a distinctive characteristic where their antifungal effects are reversed in mediums containing sorbitol [22]. Cells protected with sorbitol can grow in the presence of fungal cell wall inhibitors, whereas growth

is inhibited in the absence of sorbitol. This effect is detected by increases in the MIC value as observed in medium with sorbitol as compared to the MIC value in medium without sorbitol (standard medium).

Table 2. MIC values (μg/mL) of drugs in the absence and presence of sorbitol (0.8 M) and ergosterol (400 μg/mL) against *C. carrionii* URM-2871.

Drugs	Sorbitol		Ergosterol	
	Absence	Presence	Absence	Presence
Citral	128	128	128	2048
Amphotericin B[a]	-	-	6	2048

Legend: [a], Positive control; -, Not tested.

In this work, the MIC values of citral in both experiments (in mediums with and without sorbitol) were identical, suggesting that citral does not act by inhibiting fungal cell wall synthesis, but probably by affecting another target.

These results are in agreement with those reported by Miron *et al.* (2014) [39], who evaluated the antifungal activity of citral against seven opportunistic pathogenic yeasts and four dermatophyte species; no changes were observed in the MICs of this monoterpene in the sorbitol protection assay.

The lipophilic nature of terpenoids enables them to preferentially enter the lipid membrane, which results in an increased membrane fluidity and eventually to an increase in membrane permeability [38].

According to Harris (2002) [40], citral appears to act predominantly on the fungal cell membrane, affecting its structure, blocking its synthesis and causing cell death; inhibiting spore germination, proliferation and cellular respiration.

The action of citral on the cell membrane has been widely studied. In a recent study Tao *et al.* (2014) [32] showed that citral considerably impaired ergosterol biosynthesis in *Penicillium italicum* cells, significantly decreasing lipid levels; suggesting that the plasma membrane may well be an important citral antifungal target.

Zhou *et al.* (2014) [31] evaluated the antifungal activity of three volatile compounds: citral, octanal and α-terpineol against *Geotrichum citri-aurantii*. It was found in the study that citral was able to significantly inhibit mycelial growth. Antifungal activity was attributed to cell membrane disruption and to the consequent loss of cellular components.

Another study also showed that citral at a concentration of 200 μg/mL irreversibly damaged cell organelles and the cell membrane of *Trichophyton mentagrophytes* [11].

More recently OuYang *et al.* (2016) [33] suggested that citral might exhibit its antifungal activity against *P. digitatum* by down-regulation of ergosterol biosynthesis. The positive results of citral in the "Ergosterol Affinity Assay" and other reports on the subject strongly suggest that the mechanism of action of this monoterpene is related to ergosterol-binding and a subsequent destabilization of fungal cell membranes.

The test product presents as a relevant and promising antifungal agent which may be considered as an alternative prototype for production of a new and future antifungal drug, thus contributing to the existing arsenal of products which have proven antifungal activity against *C. carrionii*. Investigations of this nature are important since they provide clearer expectations for future pharmacological studies, with a view to a better understanding of citral's mode of action, its toxicity and possible therapeutic applications.

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Conflict of interest

All authors report that they do not have any conflicts of interest.

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