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ARTICLE

Investigation on mechanism of antifungal activity of citral against *Cladosporium sphaerospermum* Penz.

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Resumen

Investigación del mecanismo de la actividad antifúngica del citral contra Cladosporium sphaerospermum *Penz.*

El objetivo de este estudio fue investigar la actividad antifúngica del citral contra *Cladosporium sphaerospermum*, mediante la determinación de la concentración mínima inhibitoria (CMI) y la concentración mínima de fungicida (CMF), los efectos sobre el crecimiento micelial y la germinación de conidios, y también investigar la posible acción de citral en paredes celulares y membranas celulares. La CMI del citral varió de 128 a 256 µg/mL y la CMF varió de 256 a 1024 µg/mL. La CMI_{50} y CMF_{50} fueron, respectivamente, 128 µg/mL y 256 µg/mL. El citral inhibió el desarrollo micelial y la germinación de conidios y mostró interacción con ergosterol. Estos datos indican que el citral tiene una fuerte actividad antifúngica, que puede estar relacionada con su interacción con el ergosterol.

Palabras clave: Citral; Actividad antifúngica; *Cladosporium; C. sphaerospermum.*

Abstract

The aim of this study was to investigate the antifungal activity of citral against *Cladosporium sphaerospermum,* by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), effects on mycelial growth and conidia germination, and also investigated the possible action of citral in cell walls and cell membranes. The MIC of citral ranged from 128 to 256 µg / ml and the MFC ranged from 256 to 1024 µg/mL. The MIC_{50} and MFC_{50} were, respectively, 128 µg/mL and 256 µg/mL. Citral inhibited mycelial development and conidia germination and showed interaction with ergosterol. These data indicate that citral has strong antifungal activity, which may be related to its interaction with ergosterol.

Key words: Citral; antifungal activity; *Cladosporium; C. sphaerospermum.*

Introduction

Nosocomial infections caused by fungi dematiaceous have increased greatly in recent years, becoming a risk for many patients, especially the immunocompromised patients (Revankar & Sutton 2010, Calumby *et al.* 2019).

Cladosporium spp. Penz. are dematiaceous fungi usually identified as common airborne contaminants occupying a wide variety of habitats (Zalar *et al*. 2007, Cordeiro *et al*. 2021).

C. sphaerospermum is an important pathogen, being very harmful to crops (Ahmed 2015). For humans and animals, not all pathogenic strains are, however, some strains can occasionally cause skin and brain phaeohyphomycosis regardless of the immune status of the host (Tasic & Miladinović-Tasić 2007, Maduri *et al.* 2015, Soumagne *et al*. 2015).

This species is one of the most widely distributed allergens causing serious problems in patients with respiratory tract disease (Ng *et al*. 2012, Segers *et al*. 2015). May cause allergic airways diseases, pulmonary emphysema, and intrabronchial lesions (Yano *et al*. 2003, Yew *et al*. 2016).

The resistance of microbes to antimicrobial agents has potentially serious implications for the management of infections (Sanglard & Odds 2002). However, the available antifungal therapeutic arsenal is limited, and the development of new drugs has been slow. Therefore, the search for alternative drugs with low resistance rates and fewer side effects remains a major challenge. Plants produce a variety of medicinal components that can inhibit pathogen growth. A considerable number of studies of medicinal plants and alternative compounds, such as secondary metabolites, phenolic compounds, essential oils and extracts, have been performed (Negri *et al*. 2014).

Terpenes are compounds found in essential oils from several aromatic plant and form structurally and functionally different classes. Terpenes can be classified according to their number of isoprene units: monoterpenes (C10), the most representative molecules, and sesquiterpenes (C15), but there are also hemiterpenes (C5), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) (Bakkali *et al*. 2008).

Citral (3,7-dimethyl-2,6-octadienal) is a natural mixture of two acyclic monoterpene aldehyde geometric isomers, geranial (trans-citral or citral A), and neral (cis-citral or citral B). It is present in the essential oil of many plants including lemon and orange species (Fisher & Phillips 2008, Saddiq & Khayyat 2010, Saraiva-Filho *et al.* 2021). Citral presents different pharmacological properties, such as: anti-inflammatory (Ponce *et al*. 2010), anti-tumor (Chaouki *et al*. 2009, Xia *et al*. 2013), bronchodilator (Mangprayool *et al*. 2013) antiprotozoal (Armas *et al*. 2015) and antimicrobial (Belga-galbis *et al*. 2013, Shi *et al*. 2016, Oliveira *et al.* 2021) effects. The antifungal activity exerted by citral against molds and yeasts has already been demonstrated in varied conditions (Leite *et al*. 2014, Zhou *et al*. 2014, Sousa *et al*. 2016).

Although there are many reports on the antimicrobial properties of citral, there are few studies on its antifungal modes of action against strains of *C. sphaerospermum*. Given the above, the aim of this study was to determine the minimum inhibitory concentration (MICu) and the minimum fungicidal concentration (MFC) and to investigate the action mechanism of citral against *C. sphaerospermum* in its mycelial growth, conidial germination, cell wall formation, and ergosterol interactions.

Materials and methods

Microorganisms

Cladosporium sphaerospermum (URM 5962, URM 5455, URM 5350, URM 6120) strains used in the antifungal assay were obtained from the Mycology Department fungal collection (URM), Biological Sciences Center, Federal University of Pernambuco, Brazil. The samples were maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28 ºC) and under refrigeration (4 ºC).

Stock inoculations (suspensions) of *C. sphaerospermum* were prepared from 7-14 day old sabouraud dextrose agar (Difco Lab., USA); the cultures were grown at room temperature. Fungal colonies were covered with 5 mL of sterile saline solution (0.9%), the surface was gently agitated with vortexes, and fungal elements in saline solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (106 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber (Hadacek & Greger 2000, Cleeland & Squires 1991, Sahin *et al*. 2004).

Chemicals

The product tested was the monoterpene Citral, obtained from Sigma Aldrich, Brazil. Amphotericin B and voriconazole were obtained from Sigma Aldrich, Brazil. The monoterpene was dissolved in Tween 80

(2%) and DMSO (dimethylsulfoxide). The antifungal standards were dissolved in DMSO, and sterile distilled water to obtain solutions of 2048 µg/mL for each antifungal. The concentration of DMSO did not exceed 0.5% in the assays.

Culture media

To test the biological activity of the products, Sabouraud dextrose agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media were used. 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 voriconazol against *C. sphaerospermum* (URM 5962, URM 5455, URM 5350, URM 6120). 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 inoculate suspension were added to the wells, and the plates were incubated at 28 ºC 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 100%. The results were expressed as the arithmetic mean of three experiments (Cleeland & Squires 1991, Hadacek & Greger 2000).

The MFC was determined by microdilution method to verify if the inhibition was reversible or permanent (Denning *et al*. 1992, Rassoli & Abyaneh 2004). Aliquots of 20 µL (from the wells that did not show growth 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 ºC 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 MIC and MFC we selected 1 strain (*C. sphaerospermum* URM 6120), to continue the study of antifungal activity of citral.

Effects on mycelia growth

Analyses of the interferences of citral, voriconazole, and amphotericin on *C. sphaerospermum* URM 6120 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 x 2, and MIC x 4 previously found. For this, 2 mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28 ºC 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 ºC, 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 (Thyágara & Hosono 1996, Adam *et al*. 1998, Dafera *et al*. 2003).

Conidial germination assay

Citral, voriconazole, and amphotericin B were tested to evaluate effects on the germination of *C. sphaerospermum* URM 6120 fungal conidia. Flasks containing MIC, MIC x 2 and MIC x 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 48 h 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) (Rana *et al*. 1997, Pereira *et al*. 2013).

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. sphaerospermum* URM 6120 cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8 M. The assay

was performed by microdilution method in 96-well plates in a "U" (Alamar, Diadema, SP, Brazil) (Cleeland & Squires 1991, Hadacek & Greger, 2000). 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 a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with sorbitol added (as compared to the standard medium), suggest the cell wall as a possible target for the product tested (Frost *et al*. 1995, Liu *et al*. 2007, Leite *et al*. 2014). 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) with some modifications. Ergosterol was prepared as described by Leite *et al*. (2014) The MIC of citral, against *C. sphaerospermum* URM 6120 was determined by using broth microdilution techniques (Cleeland & Squires 1991, Hadacek & Greger 2000), in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines on the same microplate. Briefly, a solution of citral was diluted serially twice with RPMI-1640 (volume = 100 µL) containing ergosterol added at a concentration of 400 µg/mL. A volume yeast suspension 10 µL (0,5 McFarland) was added to each well. The same procedure was realized for amphotericin B, whose interaction with membrane ergosterol is already known, which served 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 obtained reinforce the importance and necessity of research on the potential use of products naturals as a new therapeutic alternative in the treatment of fungal infections, due to emerging drug resistance, mainly related to dermatiaceous fungi.

The results for citral's antifungal activity against *C. sphaerospermum* were determined using the MIC and MFC in broth microdilutions. The MIC of citral varied between 128 and 256 µg/mL. The MIC50 (minimum fungicidal concentration for 50 % of strains tested) was 128 µg/mL, inhibiting the growth of tested fungal strain. Amphotericin B and voriconazol retained a lesser MIC50 than the constituent at 16 µg/mL MIC. However, the strain *C. sphaerospermum* URM 6120, presented the MIC >1024 µg/mL for amphotericin B (Table 1).

According to the criteria proposed by Sartoratto *et al*. (2004), since products naturals with a MIC between 50 and 500 µg/mL are considered to have strong antimicrobial activity, while MICs between 500 and 1,500 µg/mL and over 1,500 µg/mL indicate moderate and weak activity, respectively. These results indicate that citral showed strong antifungal activity against strains of *C. spahaerospermum*.

After determination of the MIC, the fungicidal effect of the products was investigated. The MFC of citral varied between 256 and 1024 µg/mL. The MFC50 (Minimum Fungicidal Concentration for 50 % of strains tested) was 256 µg/mL. The MFC50 for amphotericin B and voriconazole was 32 µg/mL (Table 1).

According Siddiqui *et al*. (2013), the MFC/MIC ratio is used to specify the nature of the antimicrobial effect against a particular pathogen. The ratio MFC/MIC was calculated in order to determine if the compound had a fungistatic (MFC/MIC ≤ 4) or fungicidal (MFC/MIC > 4) activity.

In the present study, the MFC of the citral was found to be two or four folds higher than the corresponding MIC results. The MFC/MIC ratios of citral were ≤ 4; this suggests that citral has a fungicidal effect against the strains tested.

The results for the control (Tween 80 and DMSO) showed no fungal growth inhibition; fungal growth in the medium without added drug was detected. In accordance with the above results, the strain *C. sphaerospermum* URM 6120 was selected for further testing.

Earlier studies demonstrated that the citral display a wide spectrum of antifungic activity. Garcia *et al*. (2008) demonstrated the activity of citral against the fungi *Colletotrichum musae* (Berk. & M.A. Curtis), *Colletotrichum gloeosporioides* (Penz.) Penz. e Sacc. and *Fusarium subglutinans* (Wollenw. & Reinking). Citral is active against of

Microorganisms	Citral (µg/mL		Amphotericin B (µg/mL)		Voriconazole (µg/mL)		Control strains*
	MIC	MFC	MIC	MFC	MIC	MFC	
C. sphaerospermum URM 5962	128	256	8	16	32	64	+
C. sphaerospermum URM 5455	256	512	16	32	16	32	+
C. sphaerospermum URM 5350	128	256	64	128	16	64	+
C. sphaerospermum URM 6120	256	1024	>1024	ND	16	32	+

* Microorganism growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal or monoterpenes. ND: Notdetermined.

Tabla 1. MIC y MFC de citral, anfotericina B y voriconazol contra *C. sphaerospermum*.

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

methicillin-resistant, *Penicillium italicum* (Wehmer), *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. *and Staphylococcus aureus* (Rosenbach) (Saddig & Khayyat 2010)

Citral showed strong inhibition on *Geotrichum citri-aurantii* (Ferraris) E.E. Butler. with MIC and MFC of 0.50 µL/mL and 1.00 µL/mL (Zhou *et al*. 2014). Zheng *et al.* (2015) demonstrated the antimicrobial activity of citral front of fungal strains of *Penicillium digitatum* (Pers.) Sacc.

Recently, our research group showed that the citral is in vitro antifungal potential against strains of *Candida albicans* (Robin) Berkh. (Leite *et al*. 2014) and *Candida tropicalis* Berkh. (Sousa *et al*. 2016).

Wei *et al.* (2021) evaluated the inhibitory effects of citral against various fungal pathogens viz: *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., *Botrytis cinerea* Pers. and *Phomopsis macrospore* Tak.Kobay. & Chiba.

After determination of MIC and MFC, we selected a strain *C. sphaerospermum* URM 6120 which was resistant to amphotericin B, to continue the study of antifungal activity of citral.

Macromolecules whose functionality is related to growth, survival, virulence or cellular morphogenesis are pointed out as promising targets for new antifungal agents (Odds *et al*. 2003). Thus, taking into consideration the promising antifungal activity of citral, the effect of different concentrations of that substance on mycelial growth and the germination of conidia of *C. sphaerospermum* URM 6120 was investigated.

The effect of differing concentrations of the test drug (MIC, MIC x 2 and MIC x 4) on mycelia growth was determined measuring of the radial mycelial growth, and the results are shown in figure 1. With to effects on *C. sphaerospermum* URM 6120, it can be seen that citral in MIC concentrations of (256 µg/mL), MIC x 2 (512 µg/mL) and MIC x 4 (1024 µg/mL) inhibited normal mycelia growth ($p < 0.05$) when compared to the control (mycelia diameter being 100 %). Amphotericin B did not showed activity in all concentrations tested (Fig. 1C). However, the voriconazole test on *C. sphaerospermum* URM 6120 showed significant inhibition of mycelial growth at all concentrations tested (Fig. 1B). Strain controls showed a constant rate of mycelial growth over the time evaluated, indicating good antifungal effect for the citral.

These results suggested that the substance evaluated inhibited normal mycelial development of *C. sphaerospermum* at all concentrations tested. And that citral at its MIC, MIC x 2 and MIC x 4 concentrations was more potent when compared to amphotericin B at its respective MIC, MIC x 2 and MIC x 4 concentrations ($p < 0.05$).

These results corroborate the data obtained by some researchers who have investigated the antifungal potential of citral in inhibiting the mycelial growth of pathogenic and non-pathogenic fungi (Li *et al*. 2014, Zhou *et al*. 2014).

The results reported to date can be considered of great relevance, due to the importance of mycelial growth in the development of infection fungal. Good fungal growth of *Cladosporium* species, similarly to other filamentous fungi, produce hyphae which can penetrate the innermost skin layer and aggravate the damage in the host (Gupta *et al.* 2003, Zuritta & Hay 1987). Therefore, some researchers are investigating the products naturals potential in inhibiting mycelial growth of pathogenic fungi due to their importance in the mycosis development (Pereira *et al*. 2011, Guerra *et al*. 2015).

The results obtained in this study agree with those of Zhou *et al*. (2014) that evaluated the antifungal activity of three volatile compounds: citral, octanal, and α- terpineol against *Geotrichum citri-aurantii.* It was found that citral in the study was able to significantly inhibit mycelial growth.

More recently, Ouyang *et al*. (2016) observed that citral dose-dependently inhibited the mycelial growth of *Penicillium digitatum*, with the minimum inhibitory concentration (MIC) of 1.78 mg/mL.

Thus, the study of the germination of conidia has

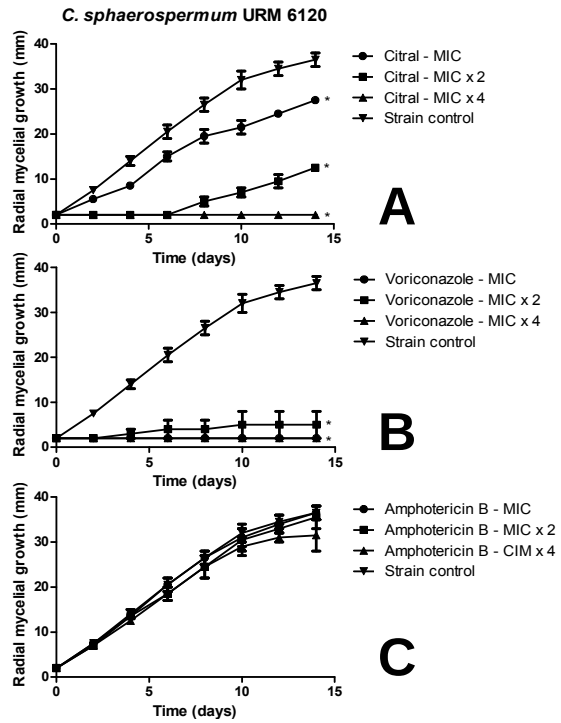

Figura 1. Comparación del crecimiento radial del micelio de por *C. sphaerospermum* URM 6120 entre el control (ausencia) y las sustancias activas. **A:** Citral; **B:** Voriconazol; **C:** Anfotericina B. *p<0,05 comparado con el control.

Figure 1. Comparison of radial mycelial growth produced by *C. sphaerospermum* URM 6120 with the control (absence) and active susbtances. **A:** Citral; **B:** Voriconazole; **C:** Amphotericin B. *p<0.05 compared to control.

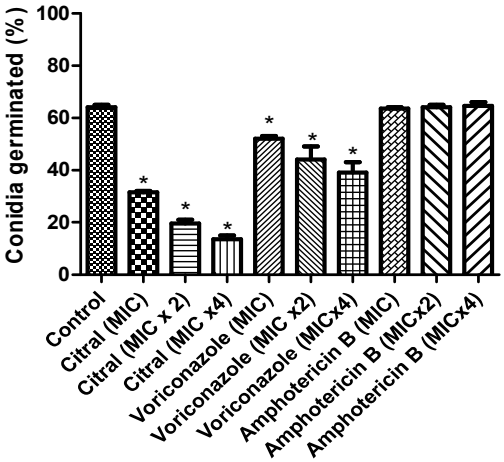

Figura 2. Porcentaje de germinacion de conidios de *C. sphaerospermum* URM 6120 en ausencia (control) y presencia de citral, vorticonazol y anfotericina B. *p< 0,05 comparado con el control.

Figure 2. Percentage of conidial germination of *C. sphaerospermum* URM 6120 in the absence (control) and presence of citral, voriconazole and amphotericin B. *p<0.05 compared to control.

great implications in clinical practice, because it is possible to develop new therapeutic approaches that block the infection at its onset (Osherov & May 2001). In this perspective, the effect of the citral on the germination of the conidia of *C. sphaerospermum* URM 6120 was investigated. The effects of different concentrations (MIC, MIC x 2 and MIC x 4) of citral, voriconazole and amphotericin B on the germination of conidia are shown in figure 2.

Conidia represent the most common mode of asexual reproduction, they play an important role in natural fungal propagation and are structurally resistant (Thabulsi & Alterthum 2004). Fungal spores are distributed in large amounts in the outdoor air, and some of them may cause diseases in human beings, animals, and plants. Among several taxa, *Cladosporium spp* is one of the most ubiquitous and most widely distributed, being found in high concentrations in the air. *C. sphaerospermum* spores have been classified as important allergens, and are, therefore, important to the study of allergies (Zoppas *et al*. 2011). Thus, it is deemed important to quantitatively evaluate the power of a product to interfere with fungal spore germination (Chotirmall *et al*. 2014).

The antifungal potential of essential oils in inhibiting the germination of conidia has been extensively studied. It has been reported that the essential oil of *Cymbopogon winterianus* L. has a strong inhibitory effect on the germination of conidia of *Trichophyton mentagrophytes* (CP Robin) Sabour. (Pereira *et al*. 2011). The essential oil of *Cinnamomum verum* J.Presl (as *C. zeylanicum*), was shown to inhibit the germination of the conidia of *A. fumigatus* Fresenius*, A. flavus* Link. and *A. niger* Van Tiegh. (Carmo *et al.* 2008). Recently, our research group showed that the essential oil of *Melissa officinalis* L. inhibited the germination of the conidia of *Cladophialophora carrionii* (Trejos) de Hoog, Kwon-Chung & McGinnis (Menezes *et al*. 2015).

Antifungal activities of 15 different plant essential oils or its components were evaluated during conidial germination and mycelial growth of *C. gloeosporioides*. It was found that citral in the study was able to significantly inhibit conidial germination (Hong *et al*. 2015).

The results obtained in this study corroborate those observed in previous studies, thus revealing the antifungal potential of the citral in blocking the infection induced by *C. sphaerospermum* soon after onset, since they significantly inhibit the germination of conidia.

The antifungal mechanism of volatile compounds has been attributed to its capacity to disturb the cellular membrane, interfere with the cellular metabolism, react with active sites of enzymes, or act as H^+ carriers (Ultee *et al*. 2002, Bajpai *et al*. 2013).

Several targets including cell wall, cell membrane, mitochondrion, and genetic material, have been proposed to account for the antifungal activity of essential oils or their volatile compositions (Parveen *et al*. 2004, Rao *et al*. 2010, Shao *et al*. 2013, Zheng *et al*. 2015). The cell wall is an extracellular layer outside the cell membrane which protects the cell against mechanical damage, osmotic strength and determines the cell shape.

To investigate the action of the product on the fungal cell wall, we performed an assay with sorbitol (Table 2), which has an osmoprotectant function. Sorbitol, an osmotic protective is used to stabilize the yeast protoplasts. Specific fungal cell wall inhibitors share a distinctive characteristic where their antifungal effects are reversed in mediums containing sorbitol (Frost *et al*. 1995). Cells protected with sorbitol can grow in the presence of fungal cell wall inhibitors, whereas growth would be 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) (Frost *et al*. 1995, Svetaz *et al*. 2007). Osmotic destabilizing agents and disrupting the cell wall lead to rearrangements of the cell wall and allow the fungal cells to survive (CLSI 2008).

In this paper, 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 in *C. sphaerospermum* URM 6120 (Table 2).

This is the first study to demonstrate the action of citral on the cell wall of *C. sphaerospermum* using sorbitol tests. The results are in agreement with those reported by Miron *et al.* (2014) who no changes were observed in the MIC of citral in the sorbitol protection assay against *T. asahii* Behrend. and with those reported by Leite *et al*. (2014) and Sousa *et al*. (2016) who have shown that citral does not act on the cell wall of *C. albicans* and *C. tropicalis*.

Since it appears that citral does not act at the level of the fungal cell wall, another possibility investigated was that it might act at the level of the cell membrane.

Considering the lipophilic nature of terpenes, as well as the interaction of these products with biological membranes, it was decided to investigate the participation of membrane sterols in the antifungal effect exerted by citral. Ergosterol is the principal sterol present in yeasts and filamentous fungi, where it is necessary for the growth and normal function of the fungal cell membrane. Besides controlling the fluidity, asymmetry and integrity of the membrane, ergosterol contributes to the proper functioning of enzymes bound to the membrane (Lupetti *et al*. 2002). The majority of existing drugs for the treatment of fungal infections target the cell wall or plasma membrane directly or indirectly, particularly ergosterol and its biosynthesis (Lupetti *et al*. 2002, Odds *et al*. 2003).

Considering this possible fungal cell membrane interference of citral, the compound was tested to investigate its ability to form complexes with ergosterol. This method is based on the exposure of a test compound to exogenous ergosterol, where an affinity for sterol will lead to the rapid formation of a complex, thereby impeding complexation with sterols of the membrane and resulting in an increase in MIC (Escalante *et al*. 2008). The MICs of citral against *C. sphaerospermum* URM 6120 increased eight times in the presence of 400 µg/mL ergosterol. Amphotericin B, the positive control that has a known interaction with ergosterol, showed a higher MIC in the presence of this sterol (Table 2).

The mechanism of action of monoterpenes has not been completely clarified. Some studies showed the breakdown of cytoplasmic and organelle membranes exposed to certain volatile oils. The loss of membrane integrity can cause changes in membrane function leading to the antifungal activity (Sikkema *et al*. 1995, Pinto *et al*. 2006, Park *et al*. 2009). Despite these findings, it is not known how volatile oils damage the membranes. The discovery of the mechanism of action can help maximize the effect of natural products, either by concentration of active ingredients or formulation optimization.

In this study, citral showed an affinity for ergosterol relating their mechanism of action to cell membrane destabilization. Recent studies have shown that citral inhibits ergosterol biosynthesis in *C. albicans* (Rajput & Karuppayil 2013) and *Penicillium italicum* (Tao *et al*. 2014). Ergosterol biosynthesis inhibition has also been observed for citral at 200 µg/mL in *Aspergillus ochraceus* K.Wilh. (Hua *et al*. 2014).

	Sorbitol		Ergosterol	
Drugs	Absence	Presence	Absence	Presence
Citral	256	256	256	2048
Amphotericin B[a]	-	-	>1024	>2048

[a]Positive control. —: not tested.

Tabla 2. Valores de CMI de los fármacos (μg/mL) en ausencia y presencia de sorbitol (0,8 M) y ergosterol (400 μg/mL) contra *C. sphaerospermum* URM 6120.

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. sphaerospermum* URM 6120.

Miron *et al*. (2014) evaluated the antifungal activity of geraniol, nerol, citral, neral (citral B) and geranial (citral A) against seven opportunistic pathogenic yeasts and four dermatophyte species. It was found that citral in the study showed an affinity for ergosterol relating their mechanism of action to cell membrane destabilization.

Wei *et al*. (2021) suggested that the antifungal activity of citral could be attributed to the increase in membrane permeability and the damage in the cell membrane.

Conclusions

On the basis of the data presented, the monoterpene citral has promising fungicidal activity, whereby is capable of inhibiting an infection at its onset. Such activities can be related to an interaction with ergosterol, a sterol present in the cell membrane of *C. sphaerospermum*, which plays an important role in the mycelial growth and germination of conidia of these fungi. Therefore, this monoterpene, may represent new alternative therapeutic agents in the treatment of mycosis by dematiaceous fungi. However, there is a need for more studies aimed at correlating their potent antifungal activity *in vitro* and *in vivo* and proving their safety for clinical application.

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