Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(11):279-282

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Evaluation of Antifungal Activity of citral associated with antifungals Amphotericin B and Voriconazole, against *Cladophialophora carrionii* and *Cladosporium spp*

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ABSTRACT

Combining antifungal drugs may improve therapeutic response. The potential benefits of using therapeutic combinations include a broader spectrum of efficacy, improved cure rates, safety, and tolerability, reduction of resistance to antifungal drugs, dose reduction, and thus reduced toxicity. The research aimed to study the antifungal properties of citral associated with synthetic antifungal (Amphotericin B and Voriconazole) against strains of Cladophialophora carrioni and Cladosporium spp. The parameters used for this purpose were based on the determination of Fractional Inhibitory Concentration (FIC) Index (Method of association – Checkerboard). The study shows that citral in combination with voriconazole an indifferent effect, and resulting in a FIC index varied between 1.5-3.0. However, the combination of the citral and amphotericin B showed FIC index varied between 6.0-8.0, for four species tested, obtained antagonistic effects. This study contributes to understanding the citral's antifungal activity associated with antifungals agents against dematiaceous fungi. However, more studies are needed to investigate whether citral interations with drugs.

Keywords: Monoterpene; Citral; Antifungal agents; *Cladophialophora carrionii*; *Cladosporium* spp checkerboard

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INTRODUCTION

Fungal participation in the aetiology of infections has increased considerably [1, 2]. However, as medical technology has improved, the survival of patients with severe and life-threatening illnesses has led to a rapid increase in the immunosuppressed population [3]. These changes are correlated with a substantial increase in the rate of invasive fungal infections. Moreover, drug-resistant strains are emerging, and the number of infections by intrinsically drug-resistant species has increased rapidly [4].

Synergistic drug combination has been proved a valid and pragmatic strategy to seek drugs with novel mode of actions. It can potentially reduce the dose of single drug usage with increased drug-efficacy, and subsequently lower the drug toxicity. The practice of targeting 2 or more drug targets simultaneously is consistent with the philosophy that a disease is a systematic and complicated outcome caused by multi-effects. Furthermore, the development of drug resistance can be slowed down by the multi-target strategy [5, 6].

Despite the constant introduction of new and effective synthetic drugs to the market, medicinal plants, which are the historical basis of therapeutic health care, represent an alternative that is economical, accessible, and applicable to various pathologies, particularly in developing countries [7]. Therefore, parallel to the development of synthetic drugs, substantial attention has focused on natural products with antifungal properties, which has stimulated the search for therapeutic alternatives [8, 9].

In addition to their inherent antimicrobial properties, natural products and their derivatives may alter the effects of standard antifungal agents (those used in clinical practice). The use of two or more antifungal combinations can lead to a reduction in the required drug dosages and decrease the normally produced adverse event profile [10, 11]. Amongst these products, we find the terpenes a class of natural substances of vegetable origin formed by combining five carbons called isoprene (C_5H_8). 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) [12].

Citral is a lemon scented acyclic monoterpene aldehyde consists of a racemic mixture of two isomers geranial (*trans*-citral or citral A) and neral (*cis*-citral or citral B). Citral possesses many significant bioactivities such as, antimicrobial, anti-inflammatory, antiparasitic, allelopathic and mosquito repellent. Citral is most valuable monoterpene in flavors, fragrances, cosmetics, perfumery and pharmaceuticals [13].

Given the above, the aim of this study was to determine the antifungal properties of citral associated with synthetic antifungal (Amphotericin B and Voriconazole) against strains of *Cladophialophora carrioni* and *Cladosporium spp.*

EXPERIMENTAL SECTION

Microorganisms

Cladophialophora carrionii URM 2871, *Cladosporium oxysporum* URM 5234, *Cladosporium spherospermum* URM 6120 and *Cladosporium cladosporioides* (INCQS 40188) strains used in the antifungal assay were obtained from the Biological Sciences Center, Mycology Department fungal collection (URM), Federal University, Pernambuco (Brazil) and the collection of Sanitary Surveillance Reference Microorganisms, National Institute of Health Quality Control (INCQS), Oswaldo Cruz Institute (OCI). The sample was maintained on Sabouraud Dextrose Agar (SDA) (DIFCO®) at room temperature (28°C) and under refrigeration (4°C). Stock inoculators (suspensions) of strains tested were prepared from 7-14 day old potato dextrose agar (Difco Lab., USA), the cultures grown at room temperature. Fungal colonies were covered with 5mL of sterile saline solution (0.9%), the surface was gently agitated with vortexes, and fungal elements with saline solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber [14-16].

Chemicals

The product tested was the monoterpeno 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 standard were dissolved in DMSO and sterile distilled water was used to obtain solutions of 2048µg/mL for each antifungals. The concentration of DMSO did not exceed 0.5% in the assays.

Culture media

To test the biological activity of the products, RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media was used. They were prepared and used according to the manufacturer's instructions.

Checkerboard assay

A checkerboard microtiter test was performed to evaluate the interaction of citral with the antifungal drugs (voriconazole and Amphotericin B) against *C. carrionii* URM 2871, *C. oxysporum* URM 5234, *C. sphaerospermum* URM 6120 and *C. cladosporioides* (INCQS 40188). A series of 2 fold dilutions, in eight for citral and each antifungal drug, were made in RPMI-1640 to obtain four times the final concentration being achieved in the microtiter well. Furthermore, 50µL of each dilution of citral was added to the 96-well microtiter plates in the vertical direction, while 50µL of each dilution of antifungal drugs was added in the horizontal direction, so that various combinations of citral and antifungal drugs could be achieved. In addition, 10µL of inoculum from the spore suspension (1.5×10^5 CFU/mL) was added to each well, and the plates were incubated at 28ºC for 5 days. In order to evaluate the activity of the combinations of drugs, fractional inhibitory concentration (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the minimum concentrations inhibiting the fungal growth for drugs A and B, respectively: $FIC^A = MIC^A$ combination/MIC^A alone and $FIC^B = MIC^B$ combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = $FIC^A + FIC^B$. In addition, the interpretation was made as follows: synergistic (<0.5), additivity (0.5–1.0), indifferent (>1), or antagonistic (>4) [17, 18].

RESULTS AND DISCUSSION

Several reports have been made concerning different antifungal combinations assayed *in vitro* and applied in the clinic [10, 19, 20], and with other plant derivatives [21].

This study evaluated the effect of citral in association with the antifungals amphotericin B and voriconazole against *C. carrionii and Cladosporium spp.* strains, using the checkerboard technique. The results are shown in Tables 1 and 2.

Table 1: MIC of Antifungal drugs and effect of combination with citral, against *C. carrionii* URM 2871 and *C. oxysporum* URM 5234

Citral + Antifungal	*C. carrionii* URM 2817		*C. oxysporum* URM 5234	
	MIC* (µg/mL)	FIC* index (type of interaction)	MIC* (µg/mL)	FIC* index (type of interaction)
Citral	128		256	
Amphotericin B	16		16	
Voriconazole	16		16	
Citral/ Amphotericin B	256/64	6.0 (antagonistic)	256/64	6,0 (antagonistic)
Citral/Voriconazole	128/8	1.5 (indifferent)	256/16	2.0 (indifferent)

*MIC, minimal inhibitory concentration; *FIC, fractional inhibitory concentration

Table 2: MIC of Antifungal drugs and effect of combination with citral, against *C. sphaerospermum* URM 6120 e *C. cladosporidioides* INCQS 40188

Citral + Antifungal	*C. sphaerospermum URM 6120*		*C. cladosporidioides INCQS 40188*	
	MIC* (µg/mL)	FIC* index (type of interaction)	MIC* (µg/mL)	FIC* index (type of interaction)
Citral	256		64	
Amphotericin B	>1024		16	
Voriconazole	16		16	
Citral/Amphotericin B	-	-	256/64	8.0 (antagonistic)
Citral/Voriconazole	256/16	2.0 (indifferent)	128/16	3.0 (indifferent)

*MIC, minimal inhibitory concentration; *FIC, fractional inhibitory concentration

As can be seen, antagonistic effects were observed for the combinations of citral with Amphotericin B, resulting in a fractional inhibitory concentration (FIC) index varied between 6.0-8.0 against respective species tested. However, the combination of the citral and voriconazole showed FIC index varied between 1.5 -3.0, for four species tested, obtained indifferent effects. The strain of *C. sphaerospermum* URM 6120 was not evaluated with the citral-Amphotericin B combination because the MIC of Amphotericin B was greater than 1024µg/mL.

Sousa et al. [22] evaluated the effect of citral in association with the antifungal fluconazole, and amphotericin B against *C. tropicalis* strains, using the checkerboard technique. It was found that the citral-amphotericin B combination was for indifferent (FICI = 1.0) for the *C. tropicalis* ATCC strain. In *C. albicans* strains, previous studies have shown for citral-amphotericin B; effects ranging from indifferent to synergistic [23]. 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 [24-26]. The action of citral on the cell membrane has been widely studied. In a recent study, Tao et al. [27] showed that citral considerably impaired ergosterol biosynthesis in cells of *Penicillium italicum*, significantly decreasing lipid levls, suggesting that the plasma membrane may well be an important citral antifungal target. More recently, OuYang et al. [28] suggests that citral could exhibit its antifungal activity against *P. digitatum* by the down-regulation of ergosterol biosynthesis. These studies suggest that the mechanism of the antifungal action of the citral involves a direct interaction with ergosterol, which leads to the disruption of the fungal membrane and loss of intracellular contents [29]. Amphotericin B is one of the most potent antifungals, demonstrating activity against an array of yeast and filamentous fungal pathogens. Amphotericin B exerts its activity through hydrophobic interactions with cell membrane ergosterol, subsequently disrupting membrane function. Pores formation allows the efflux of potassium, leading to cell death [30]. The voriconazole is antifungal azole drug class. These agents impair ergosterol synthesis by inhibiting C14-a sterol demethylase. Cell membrane integrity is disrupted by the accumulation of sterol precursors and the reduction of ergosterol [31,32]. The antagonistic and indifferent effects observed in this study when the citral was associated with amphotericin B and voriconazole, respectively, can be explained by the monoterpene, although action also interacting with the ergosterol of fungal membrane, as well as the antifungals possibly acts in different pharmacological conditions, blocking or otherwise interfering with the final effect of fungal growth inhibition. According to published reports, the effect of combining amphotericin B and flucytosine, for example, has varied between synergism and antagonism, and also changes according to the species, and even which strain is tested [33,34].

The focus of this evaluation is of the efficacy of combination antifungal drugs with respect to the extent or rate of death of the fungal pathogen, although other potential interactions (such as pharmacokinetic drug interactions), may well impact efficiency when agents are used together, increased penetration of the antifungal

agent provided by the action of the another cell membrane antifungal; inhibition of protein carriers; simultaneous inhibition of different cellular targets [34].

CONCLUSION

This study represents an advance in our understanding of citral's antifungal activity associated with antifungals agents against dematiaceous fungi. However, more studies are needed to investigate whether citral interations with others drugs.

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