



Antifungal Activity and Association Study of (R)-(+)-citronellal Enantiomer with Amphotericin B, Fluconazole, Itraconazole and Miconazole against *C. tropicalis* Isolated from Vulvovaginal Secretions

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ABSTRACT

Vulvovaginal candidiasis (VVC) is among the most common diagnosis in women who seek gynecological services. The *C. albicans* is the main etiological agent. However, nowadays can be observed an increase of the prevalence of non-*albicans* species, such as *C. tropicalis*. Assess the antifungal potential of the (R)-(+)-citronellal [(R)-(+)-CT] isolated and associated to amphotericin B, fluconazole, itraconazole and miconazole, against *C. tropicalis* from vulvovaginal secretions. The enantiomer was solubilized in tween 80 and DMSO, posteriorly diluted in sterile distilled water up to the concentration of 2048µg/mL. The minimum inhibitory concentration (MIC) of the product was determined by microdilution in RPMI-1640, obtaining dilutions of 1024-4µg/mL. The minimum fungicidal concentration (MFC) was determined by the sabouraud dextrose agar (SDA) depletion technique from aliquots of 10µL of the MIC, MIC × 2 and MIC × 4. The antifungal susceptibility testing and the interfering effects of the association of the enantiomer with the standard drugs, were determined by disk-diffusion in SDA. The MIC of (R)-(+)-CT was 16µg/mL and the MFC 32µg/mL. A high resistance of the strands *C. tropicalis* to amphotericin B, itraconazole and miconazole was observed. The combination test of the enantiomer with the amphotericin B, as well as with the itraconazole, resulted in synergism 2 (66.6%) of the yeasts, and in association with the fluconazole 1 (33.3%) and miconazole 3 (100%) of synergic effect. The (R)-(+)-CT alone is fungicide for the 3 fungal strains and in association with the four antifungals increased the inhibition zones, increasing the sensitivity.

Keywords: Enantiomer; Antifungal agents; Combination studies; Vulvovaginal candidiasis

INTRODUCTION

Both *Candida albicans* and *C. non-albicans* species are known to colonize the skin, gastrointestinal tract and reproductive tract in humans [1]. Among the infections of the genital tract in women in fertile age, vaginitis is the most common infection which compromises the quality of life of many women and who need to be seen by their gynecologists [2]. Although bacteria are the most prevalent agents, which cause this infection, 20-25% of cases are due to *Candida* species [3, 4]. It is estimated that about three quarters of all health women will experience at least one episode of VVC during their reproductive lives and that 6-9% of them suffer from recurrent, chronic or refractory episodes of the infection [5, 6].

There are many reports, which indicate that 85-95% of the VVC cases are caused by *C. albicans*. However, other species of *Candida* are now emerging as identifiable causes of VVC and differ considerably regarding the epidemiology, virulence and antifungal susceptibility [7, 8].

Although the clinical experience shows that the isolates have a smaller virulence in the lower genital tract infections, the presence of potential risk factors in the host such as pregnancy, uncontrolled diabetes mellitus, use of antibiotics, immune-suppression and hormone replacement therapy, predisposes to the development of VVC [9]. Among the most commonly identified non-*Candida albicans* species in women with VVC are *C. glabrata* and *C. tropicalis* followed by

C. parapsilosis, *C. krusei*, *C. kefyr*, *C. guilliermondii* and others, which have been reported in different countries [1, 10, 11].

The emergence of drug-resistant strains reinforces the need for studies of these pathogens, and the vigilance of the antimicrobial susceptibility is commonly used in the therapy and monitoring of the rapid changes in the resistance patterns [12, 13].

The prolonged therapy and the increase the use of antifungal drugs in the treatment of the recurrent cases of VVC are the most common risk factors in the development of azole resistance in the isolates of vaginal *Candida*. However, the azoles have the advantage of being administered orally, which increases their power [14]. However, due to the dynamics antimicrobial resistance process, and in particular in the practice of monotherapy, the azoles, commonly used antifungal drugs in the treatment of the VVC have been presenting a unfavorable clinical picture [15, 16].

The anti-*Candida* activity of several terpenoids has been broadly studied. The monoterpene phytoconstituent citronellal is one of the major substances of the essential oils of aromatic plants, such as those of the *Cymbopogon* and *Eucalyptus* genus, which present this property [17, 18].

Furthermore, there is a growing interest in the use of combination therapy, which includes the use of combinations of synthetic substances, as well as natural products, together with the conventional medicines against several infectious diseases, such as candidiasis. Some essential oils and phytoconstituents are reported to synergistically improve the activities of antibiotics such as amphotericin B, ketoconazole e fluconazole [19, 20].

In this context, it was aimed to assess the antifungal potential of the enantiomer (*R*)-(+)-citronellal [(*R*)-(+)-CT] isolated and associated to amphotericin B, fluconazole, itraconazole and miconazole against strands of *C. tropicalis* originated from vulvovaginal secretions.

EXPERIMENTAL SECTION

Phytoconstituent, antifungal standards and substances

The following substances used in this work were obtained commercially: enantiomer (*R*)-(+)-CT [(3*R*)-3,7-dimethyloct-6-enal], (Purity > 90%), dimethylsulfoxide (DMSO) and Twee 80 (0.02%) (all from Sigma-Aldrich, São Paulo, SP, Brazil). The twee 80 and the DMSO were solubilized in a proportion that did not exceed 0.5% in the test, and was posteriorly diluted in sterile distilled water in order to reach the initial concentration of 2048µg/mL [21, 22]. Furthermore, fluconazole, itraconazole and miconazole were respectively, purchased from Control Center and Products for Diagnosis (CECON) Ltd. (São Paulo, SP, Brazil).

Culture media

To test the biological activity of the products, Sabouraud dextrose broth (SDB) and Sabouraud dextrose agar (SDA) were purchased from Difco Laboratories (Detroit, MI, USA). Furthermore, RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, São Paulo, SP, Brazil) culture media were used. They were prepared and used according to the manufacturers' instructions.

Fungal strains

The assays were performed with two strains of *C. tropicalis*: LM 665, LM 255 (isolated from vaginal), and one standard strains: *C. tropicalis* ATCC 13803. All strains belong to the collection of the Mycology Laboratory, Department of Pharmaceutical Sciences, Federal University of Paraíba (LM, DCF, UFPB). These strains were maintained in SDA at 35±2°C and 4°C until used in tests.

Inoculum

The suspensions were prepared from recent *C. tropicalis* cultures, plated on SDA, and incubated at 35±2°C for 24-48h. After incubation, was transferred roughly 4-5 yeast colonies (with a sterile loop) to test tubes containing 5.0mL of sterile saline (NaCl 0.85%). The resulting suspensions were stirred for 15 seconds with the aid of a Vortex apparatus (Fanem Ltd., Guarulhos, SP, Brazil). The turbidity of the final inoculum was standardized using a barium sulfate suspension (tube 0.5 on the McFarland scale). The final concentration obtained was about 1-5 × 10⁵ colony forming units per milliliter (CFU/mL) [23, 24].

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

The determination of the products' MIC on the ten strains used in the biological assays was determined by the broth microdilution method [25-27]. One hundred microliters (100µL) of liquid medium RPMI-1640 was transferred into the wells of a 96-well microdilution plate with a "U" shaped bottom (Alamar, Diadema, SP, Brazil). Then, 100µL of (*R*)-(+)-CT emulsion was inoculated in the first horizontal row of the plate wells. Doubled serial dilutions, where a 100µL aliquot removed from the most concentrated well went to the next well, and yielded concentrations of 1024-4µg/mL. Finally, 10µL of *C. tropicalis* inoculum suspension was added to each well of the plate, where each column represented a yeast strain. In parallel, controls were made for yeast viability and for susceptibility with the standard antifungal nystatin (100 IU/mL). The plates were incubated at 35±2°C for 24-48 h. After the appropriate incubation time, the presence (or absence) of growth was observed visually. The formation of cell clusters or "buttons" in the plate wells was considered. The MIC was defined as the lowest (*R*)-(+)-CT concentration that produced visible inhibition of yeast

growth. The antimicrobial activity of the products was interpreted (considered active or not), according to the criteria proposed by (Morales *et al.*, 2008) [28]: strong/good activity (MIC: <100µg/mL); moderate activity (MIC: 100-500µg/mL); weak activity (MIC: 500-1000µg/mL); and inactive product/no antimicrobial effect (MIC: >1000µg/mL). To determine the MFC, we subcultured 10µL aliquots of MIC, MIC × 2, and MIC × 4 of the product, nystatin (100IU/mL), and the control yeast growth onto Petri dishes containing SDA. After 24-48 hours of incubation at 35±2°C, a reading was made to evaluate the MFC as based on the growth of the controls. The MFC was defined as the lowest product concentration that inhibited growth of the yeast or permitted less than three CFUs to occur, resulting thus in 99.9% fungicidal activity [29, 30]. Biological activity assays were performed in duplicate, and the results were expressed as the arithmetic mean of the MIC and MFC.

Susceptibility assays

The fungal susceptibility test was carried out based on the disk-diffusion method in solid mean [26, 31]. In this test the following antifungal medications were used: amphotericin B (100µg), fluconazole (25µg), itraconazole (10µg) and miconazole (50µg). The interpretation of the results was carried out using the sensitive or resistant criteria recommended by the (CECON) Ltd. (São Paulo, SP, Brazil) and the [32].

Combination studies in vitro

The susceptibility tests of the combination of (R)-(+)-CT with the antifungal agents were also carried out based on the disk-diffusion method in solid media [24, 33].

In this test, the antifungal disks in their respective concentrations were soaked with 10µL of the MIC of (R)-(+)-CT, and posteriorly dispensed in Petri dishes containing SDA inoculated with 1mL of the fungal suspensions. Then, the dishes were incubated at 35±2°C for 24-48h. The interactions of the (R)-(+)-CT with the antifungal agents were considered as being positive (synergism), when the inhibition zone of the combined application was (≥ 2mm) in relation to the antifungal medication alone, and as being negative (antagonism), when the inhibition zone of the association was (≤ 2mm) to the presented by the isolated antifungal medication and “0 interaction” (indifferent), when the inhibition zone of the combination was the same as the antifungal medication alone [25, 34].

The tests were carried out in duplicate and the results were expressed by the arithmetic mean of the diameters formed in the two tests in parallel.

RESULTS AND DISCUSSION

The results of the antifungal activity of the enantiomer (R)-(+)-CT against the *C. tropicalis* strains were determined using the MIC and MFC by micro-dilution in broth. The MIC value of the enantiomer was 16µg/mL, corresponding to the inhibition of fungal growth on the 3 tested strains (Table 1).

Table 1: MIC values (µg/mL) of (R)-(+)-CT against *C. tropicalis* strains by broth microdilution

Specie fungi/substance	<i>C. tropicalis</i> LM 665	<i>C. tropicalis</i> LM 255	<i>C. tropicalis</i> ATCC 13803
(R)-(+)-CT (1024µg/mL)	+	+	+
(R)-(+)-CT (512µg/mL)	+	+	+
(R)-(+)-CT (256µg/mL)	+	+	+
(R)-(+)-CT (128µg/mL)	+	+	+
(R)-(+)-CT (64µg/mL)	+	+	+
(R)-(+)-CT (32µg/mL)	+	+	+
(R)-(+)-CT (16µg/mL)	+	+	+
Negative control	-	-	-
Positive Control	+	+	+

(+) inhibition (-) no inhibition

The terpenoids such as the enantiomer (R)-(+)-CT, major phytoconstituent of the essential oils of plants of the *Cymbopogon* and *Eucalyptus* genus present an excellent antifungal activity [17, 18]. In this study, was observed that this molecule presented an excellent antifungal efficiency of *C. tropicalis* strains. According to Morales *et al.*, 2008 [28], this phytoconstituent showed a strong anti-*C. tropicalis* activity, as a value of the MIC was lower than 100µg/mL (MIC <100µg/mL). In literature, (R)-(+)-CT also showed a good fungicide, bactericide, tripanocidal and leishmanicidal activity [36, 37].

The MFC was of 32µg/mL, corresponding to the MIC × 2 for the 3 *C. tropicalis* strains as can be observed in (Table 2).

Table 2: MFC values (µg/mL) of (R)-(+)-CT against *C. tropicalis* strains

Specie fungi/substance	<i>C. tropicalis</i> LM 665	<i>C. tropicalis</i> LM 255	<i>C. tropicalis</i> ATCC 13803
(R)-(+)-CT (1024µg/mL)	+	+	+
(R)-(+)-CT (512µg/mL)	+	+	+
(R)-(+)-CT (256µg/mL)	+	+	+
(R)-(+)-CT (128µg/mL)	+	+	+
(R)-(+)-CT (64µg/mL)	+	+	+

(R)-(+)-CT (32µg/mL)	+	+	+
Negative control	-	-	-
Positive control	+	+	+

(+) inhibition (-) no inhibition

In this work, was also found the fungicide effect of the (R)-(+)-CT in 3 strains of *C. tropicalis* (MFC 32µg/mL) corresponding to a MIC × 2. According to Hafidh *et al.*, 2011 [38], the fungicide effect of a natural product such as citronellal, is observed when the coefficient between the MFC/MIC is between 1 and 2.

The results of the fungal susceptibility tests for *C. tropicalis* for the standard antifungal agents were determined by the disk-diffusion test in solid mean. The resistance profile was observed for the 3 fungal strains to the itraconazole, miconazole and to the amphotericin B. However, for fluconazole the resistance was of 2 (66.6%) of the fungal strains (Table 3).

Table 3: Susceptibility testing of *C. tropicalis* strains to standard antifungal. Average diameters of halos expressed in (mm)

Antifungals	Fungal strains			Classification
	<i>C. tropicalis</i> LM 665	<i>C. tropicalis</i> LM 255	<i>C. tropicalis</i> ATCC 13803	
Amphotericin B 100µg	14**	12**	11**	>15(S) ≤15(R)
Fluconazole 25µg	0**	0**	28*	≥20(S) <20(R)
Itraconazole 10µg	16**	0**	18**	≥20(S) <20(R)
Miconazole 50µg	18**	15**	18**	>20(S) ≤20(R)
Control yeast	+	+	+	--

*Sensible (S); **Resistant (R)

The results for the combination tests are shown in the (Table 4), where can be observed that the effects of the (R)-(+)-CT interference on the antifungal medications varied according to the type of the therapeutic agent and the fungal strain tested. However, synergism was predominant on the four tested antifungal medications. The association of the (R)-(+)-CT with amphotericin B, as well as to itraconazole, resulted in synergetic effect in 2 (66.6%) of the fungal strains. The enantiomer in combination with fluconazole and miconazole showed synergism in 1 (33.3%) and 3 (100%) of the yeast respectively.

Furthermore, it was also observed that for some of the strains previously resistant to isolated antifungal medications, became sensitive when faced with the combination of the phytoconstituent with the antifungal agents.

Table 4: Average diameters (in mm) of the test (R)-(+)-CT combination of patterns and antifungal against *C. tropicalis* in solid medium

Fungal strains	(R)-(+)-CT + Antifungals			
	Amphotericin B 100µg	Fluconazole 25µg	Itraconazole 10µg	Miconazole 50µg
<i>C. tropicalis</i> LM 665	17 ↑	0 I	14 ↓	25 ↑
<i>C. tropicalis</i> LM 255	12 I	0 I	24 ↑	25 ↑
<i>C. tropicalis</i> ATCC 13803	16 ↑	40 ↑	30 ↑	45 ↑
Control yeast	+	+	+	+

↑ Synergism; ↓ Antagonism; I Indifferent

The high incidence of fungal infections of the feminine genital tract by emerging strains species such as *C. tropicalis* as a consequence of the development of new resistance mechanisms to antifungal drugs, accentuates the need for studying new molecular prototypes aspirant to drugs, such as natural products and their phytoconstituents, as well as molecules originated from the laboratorial chemical synthesis, with a possible modulation activity of the microbial resistance [35]. For over a decade, cases of reduced sensitivity to fluconazole and itraconazole have been observed [39, 40], with the observation of crossed resistance to isolates of *C. albicans* and non-*albicans*, by the previous and prolonged exposure to fluconazole [41]. Therefore, a smaller susceptibility to these antifungal drugs reported for vulvovaginal clinical samples (Table 3) [42, 43]. This way, *C. tropicalis* have shown to be predominantly resistant resembling this work's profile [44]. In the light of this context, the reality for the current clinical situation of the emerging cases of antimicrobial resistance, makes the treatment of infections by *C. albicans*, *C. tropicalis* and several other pathogenic microorganisms even harder, reflecting a higher frequency of therapeutic failure to monotherapy [45].

In these cases the researches of the interactions of natural and synthetic products on the effectiveness of the conventional antifungal agents seems very promising to us if the combinations results in a better spectrum of activity and reduced toxicity in comparison with the complementary schemes of a single agent [45, 46]. This way, it seems that the modification of the antimicrobial activity resulting from the associations, with the expansion of the sensitivity profile of resistant fungal strains is a new clinical strategy, with the potential of being a modifier of the resistance profile [33, 47].

The mechanisms of anti-*Candida* activity of the terpenoids are not very clear, but are reported to be from modular to mevalonate pathway (MP), altering the cellular levels of the intermediary molecules and associated functions in eukaryotic cells [48]. In addition to the modulation of the MP, terpenoids are reported to destabilize the membrane and

modulate the functions associated to the membrane, such as the permeability, the cell signaling etc., leading to the cellular death [49, 50].

It is also probable that due to the lipophilicity level, the (*R*)-(+)-CT may have interacted with the components of the phospholipid bilayer of the fungal membrane affecting the degree of fluidity, besides interfering in signaling routes involved in the synthesis of polysaccharides such as β -glucan, mannan and chitin, important for the maintenance of the cellular wall of *C. tropicalis*. Therefore, these interactions may cause a greater influx of the antifungal agents, resulting in the increase of the inhibition zones and this way reducing these yeasts' resistance (Table 4) [36, 51, and 52].

CONCLUSIONS

Based on these results, the present study showed that the citronellal has significant antifungal activity against *C. tropicalis*, acting as a fungicide for the majority of the tested strains. Furthermore, this monoterpene also proved to act synergically with the four antifungal medications tested, important for the monotherapy and the combination therapy in the treatment of the VVC. This way this product shows itself as being relevant and promising as a potential antifungal drug and can be considered as an alternative prototype for the production of a new and future antifungal agent, thus contributing to the existing arsenal of products with confirmed antifungal activity against *C. tropicalis*. Investigations of this nature are important, once that they provide clear expectations for future pharmacological studies, aiming, with a view to reaching a common understanding of the action mechanism of the citronellal, its toxicity, and its possible therapeutic application.

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DECLARATION OF INTEREST

The authors report no declarations of interest.

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