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Original Article

ACTIVITY ANTI-*C. TROPICALIS* AND EFFECTS OF THE COMBINATION OF (*S*)-(-)-CITRONELLAL WITH FOUR ANTIFUNGAL APPLIED IN VULVOVAGINAL CANDIDIASIS

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ABSTRACT

Objective: Assess the antifungal potential of the enantiomer (*S*)-(-)-citronellal [(*S*)-(-)-CT] isolated and associated to amphotericin B, fluconazole, itraconazole and miconazole against *C. tropicalis* from vulvovaginal secretions.

Methods: The enantiomer was solubilized in Tween 80 and DMSO. Posteriorly diluted in sterile distilled water up to the concentration of 2048 µg/ml. The MIC of the product was determined by microdilution in RPMI-1640 obtaining dilutions of 1024-4 µg/ml. The MFC was determined by the SDA depletion technique from aliquots of 1 µl of the MIC, MIC × 2 and MIC × 4.

Results: 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 (*S*)-(-)-CT was 64 µg/ml and the MFC 128 µg/ml. A high resistance of the strands *C. tropicalis* to amphotericin B, itraconazole and miconazole were 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.

Conclusion: The (*S*)-(-)-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 that 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 vulvovaginal candidiasis (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. kefir*, *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 an unfavorable clinical picture [15, 16].

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

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

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

MATERIALS AND METHODS

Phytoconstituent, antifungal standards and substances

The following substances used in this work were obtained commercially: enantiomer (*S*)-(-)-CT [(3*S*)-3,7-dimethyl-6-octenal] (Purity>96%), 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,

amphotericin B, 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 5.0 ml 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 × 10^5colony 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 three strains used in the biological assays was determined by the broth microdilution method [25-27]. One hundred microliters (100 µl) 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, 100 µl of (*S*)-(-)-CT emulsion was inoculated in the first horizontal row of the plate wells. Doubled serial dilutions, where a 100 µl aliquot removed from the most concentrated well went to the next well, yielded concentrations of 1024-4 µg/ml. Finally, 10 µl 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 (*S*)-(-)-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: <100 µg/ml); moderate activity (MIC: 100-500 µg/ml); weak activity (MIC: 500-1000 µg/ml); and inactive product/no antimicrobial effect (MIC: >1000 µg/ml).

To determine the MFC was subcultured 1 µl aliquots of MIC, MIC × 2 and MIC × 4 of the test product. Nystatin (100 IU/ml) was the control yeast growth onto Petri dishes containing SDA. After 24-48 h 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 (100 µg), fluconazole (25 µg), antibiotics itraconazole (10 µg) and miconazole (50 µg). 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 (*S*)-(-)-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 10 µl of the MIC of the (*S*)-(-)-CT and posteriorly dispensed in Petri dishes containing SDA inoculated with 1 ml of the fungal suspensions. Then, the dishes were incubated at 35±2 °C for 24-48h. The interactions of the (*S*)-(-)-CT with the antifungal agents were considered as being positive (synergism) when the inhibition zone of the combined application was (≥ 2 mm) in relation to the antifungal medication alone and as being negative (antagonism) when the inhibition zone of the association was≤(2 mm) 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

The results of the antifungal activity of the enantiomer (*S*)-(-)-CT against the *C. tropicalis* strains were determined using the MIC and MFC by micro-dilution in broth. The MIC values of the enantiomer was 64µg/ml corresponding to the inhibition of fungal growth on the three tested strains (table 1).

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

Specie fungi/substance	*C. tropicalis* LM 665	*C. tropicalis* LM 255	*C. tropicalis* ATCC 13803
(*S*)-(-)-CT (1024 µg/ml)	+	+	+
(*S*)-(-)-CT (512 µg/ml)	+	+	+
(*S*)-(-)-CT (256 µg/ml)	+	+	+
(*S*)-(-)-CT (128 µg/ml)	+	+	+
(*S*)-(-)-CT (64 µg/ml)	+	+	+
Negative control	-	-	-
Positive control	+	+	+

(+) inhibition (-) no inhibition, The MFC was of 128 µg/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 (*S*)-(-)-CT against *C. tropicalis* strains

Specie fungi/substance	*C. tropicalis* LM 665	*C. tropicalis* LM 255	*C. tropicalis* ATCC 13803
(*S*)-(-)-CT (1024 µg/ml)	+	+	+
(*S*)-(-)-CT (512 µg/ml)	+	+	+
(*S*)-(-)-CT (256 µg/ml)	+	+	+
(*S*)-(-)-CT (128 µg/ml)	+	+	+
Negative control	-	-	-
Positive control	+	+	+

(+) inhibition (-) no inhibition

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
	C. tropicalis LM 665	*C. tropicalis* LM 255	*C. tropicalis* ATCC 13803	
Amphotericin B 100 µg	14**	12**	11**	>15(S) ≤15(R)
Fluconazole 25 µg	0**	0**	28*	≥20(S) <20(R)
Itraconazole 10 µg	16**	0**	18**	≥20(S) <20(R)
Miconazole 50 µg	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 (*S*)-(-)-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 (*S*)-(-)-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 (*S*)-(-)-CT combination of patterns and antifungal against *C. tropicalis* in solid medium

Fungal strains	(*S*)-(-)-CT+Antifungals			
	Amphotericin B 100 µg/ml	Fluconazole 25 µg/ml	Itraconazole 10 µg/ml	Miconazole 50 µg/ml
C. tropicalis LM 665	16 ↑	15 ↑	21 ↑	30 ↑
C. tropicalis LM 255	10 ↓	0 I	0 I	28 ↑
C. tropicalis ATCC 13803	17 ↑	22 ↑	24 ↑	42 ↑
Control yeast	+	+	+	+

↑ Synergism; ↓ Antagonism; I Indifferen

DISCUSSION

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].

The terpenoids such as the enantiomer (*S*)-(-)-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 against *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 100 µg/ml (MIC<100 µg/ml). In literature (*S*)-(-)-CT also showed a good fungicide, bactericide, tripanocidal and leishmanicidal activity [36, 37].

In this work, the fungicide effect of the (*S*)-(-)-CT in three strains of *C. tropicalis* (MFC 128 µg/ml) corresponding to a MIC × 2 was found. 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.

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 of 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 (*S*)-(-)-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].

CONCLUSION

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 and RVVC. 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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CONFLICT OF INTERESTS

The authors report no declarations of interest

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