

Antifungal Activity of Geraniol on *Candida albicans* Isolates of Pediatric Clinical Importance

Lima A L A^{1*}, Pérez A L A L², Sousa J P³, Pinheiro L S¹, Oliveira-Filho A A⁴, Siqueira-Júnior J P¹, Lima E O¹

¹Program in Natural Products and Synthetic Bioactive, Federal University of Paraiba, Paraiba, Brazil.

²Posgraduate Program in Dentistry School of Dentistry, Federal University of Paraiba, Paraiba, Brazil.

³Mycology Laboratory, Department of Pharmaceutical Sciences, Federal University of Paraiba, Brazil.

⁴Academic Unit Biological Sciences, Federal University of Campina Grande, Paraiba, Brazil.

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ABSTRACT

Geraniol is a plant-derived monoterpene alcohol that has antifungal effect. The aim of this study was to evaluate the geraniol for antifungal activity against *Candida albicans* isolates of pediatric clinical importance. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined by the broth microdilution techniques. We also investigated possible geraniol action on cell walls (0.8M sorbitol) and cell membranes (Geraniol to ergosterol binding). For 90% of isolates, the MIC and MFC of the phytochemical was 64 µg/ml. Involvement with the cell wall and ergosterol binding were excluded as possible mechanisms of action. Thus, geraniol showed *in vitro* antifungal potential against strains of *C. albicans*, but did not involve action on the cell wall or ergosterol and further study is needed to completely describe its mechanism of action.

Keywords: Antifungal activity, geraniol, *Candida albicans*, pediatric.

INTRODUCTION

Candida spp., particularly *C. albicans*, are an opportunistic fungus residing in the human body due to its commensal nature¹. This fungus can also cause systemic infection and induce damage to many organs especially in immunocompromised patients². Candidemia is an important concern in the pediatric clinical medicine, mainly because *Candida* species represents the third most common cause of healthcare-acquired blood stream infections (BSI) and the mortality can exceeds 30% in hospitalized and immunocompromised children³.

The constrained armory of conventional antifungal treatments for systemic pediatric candidiasis depends profoundly on polyenes, azoles and echinocandins that have high costs and toxicity or stern side effects^{4,5}. Increased resistance to antifungal agents represents the major deterrent against effective therapies⁶. Therefore, it highlights the importance of research of new antifungals compounds which could constitute alternatives to the existing drugs^{7,8}.

Monoterpenes have been proposed to play beneficial roles in diverse physiological systems; geraniol is a plant-derived monoterpene with a rose scent and a slightly sweet flavor, widely found in the volatile oil of various plants: citronela, geranium, vanilla and rose oils. It is widely used as a spice ingredient and in cosmetics, fragrances, shampoos, soaps and other non-cosmetic products, including household and other detergents⁹. The pharmacological activities of geraniol include:

cardioprotection¹⁰, antioxidant¹¹, anti-inflammatory¹², antinociceptive¹³, antitumor¹⁴, antibacterial¹⁵, insecticide¹⁶ and antimycotic activities, including against *Candida* spp.¹⁷.

Due the antifungal properties of this monoterpene, the aim of our study was to evaluate the geraniol for antifungal activity against hospital strains of *Candida albicans* from BSI of pediatric patient.

MATERIAL AND METHODS

Chemicals

Geraniol, amphotericinB, fluconazole, ergosterol and sorbitol were obtained from Sigma-Aldrich (São Paulo, SP, Brazil), whereas dimethylsulfoxide (DMSO) and Tween 80 were purchased from Labsynth products for Laboratories Ltd. (Diadema, SP, Brazil). The emulsion used in the antifungal assays was prepared at the time of the execution of the tests. The drugs were dissolved in DMSO, Tween 80 and sterile distilled water was used to obtain an initial concentration of 1024 µg/ml. The mixture was kept under stirring for 3 minutes, in a Vortex apparatus (Fanem® Ltd., Guarulhos, SP, Brazil).

Growth media

To test the biological activity of the products, Sabouraud glucose agar (SGA) were purchased from Difco Laboratories (Detroit, MI, USA), agar-cornmeal from HiMédia Laboratories (Mumbai, MH, India), and RPMI-1640, with L-glutamine, without sodium bicarbonate (Sigma-Aldrich, São Paulo, SP, Brazil) culture media were

used. They were prepared and used according to the manufacturers' instruction. The media were solubilized in distilled water and sterilized by autoclaving at 121°C, 1.0 atm. for 15 min.

Fungal strains

The assays were performed with nine hospital strains, AM-02, AM-04, AM-06, AM-07, AM-08, AM-09, AM-10, AM-13 and AM-15 of *Candida albicans* isolated from BSI of pediatric patients and one strain used as standard, *C. albicans* ATCC 60193. These strains belong to the collection of the Mycological Laboratory of the UFPB and were maintained in Sabouraud dextrose agar (SDA) at 4°C until used in tests.

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

The MIC was determined by the microdilution method¹⁸. Cultures of *Candida* spp. were placed on Sabouraud Dextrose Agar (SDA) and incubated for 24-48 hours at temperature 37°C. Colonies of this culture were suspended in sterile 0.85% NaCl and the inoculum was standardized according to the scale of 0.5 McFarland (1-5 x 10⁶ CFU/ml). In a 96-well plate was added liquid medium RPMI-1640 and geraniol concentrations of 1024 to 0.5 µg/ml. The MIC determination was conducted with approximately 1-5 x 10⁵ CFU/ml of the microorganism in each well. The plates were incubated at 37°C for 24-48 hours. In 24-48 hours there was a visual observation of fungal growth. The MIC was defined as the lowest oil concentration that inhibited visible growth of the yeast^{19,20}. The antimicrobial activity of the products was interpreted (considered active or not), according to the criteria proposed by Morales *et al.* (2008): 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)²¹.

To determine the MFC, 10µl of each of the wells without fungal growth was seeded on a plate containing SDA, the SDA plating were incubated at 37°C for 24-48 hours. The MFC was considered as the lowest concentration cultivated in plate with SDA in which growth was less than 3 CFU²². A negative control (without drugs) was performed to confirm cell viability¹⁸. A sensitivity control to Tween 80 and DMSO was performed at the same concentrations used to dissolve the products. There were three independent experiments in duplicate on different occasions and the results were expressed as the arithmetic mean of the MIC and MFC.

Sorbitol assay

MIC of geraniol was determined with *C. albicans* (ATCC 60193 and AM-09) using the broth microdilution method in 96-well plates (Alamar, Diadema, SP, Brazil) as previously described²⁰. 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 yeast cell wall. The sorbitol (Sigma-Aldrich, São Paulo, SP, Brazil) was added to the culture medium to give a final concentration of 0.8 M. Following incubation at 37°C, the plates were read at 48 hours and after 7 days^{23,24}. This assay was carried out in two

independent assays, in duplicate and the geometric means were calculated.

Effect of ergosterol on MIC of geraniol

The MIC of geraniol against *C. albicans* (ATCC 60193 and AM-09) was determined by the microdilution method using microplates of 96 wells in the absence and in the presence of 400 µg/ml of ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil). Amphotericin B was used as a control drug. The MIC was determined after 48 h of incubation. This assay was carried out in two independent assays, in duplicate and the geometric means were calculated^{24,25}.

RESULTS AND DISCUSSION

Candida albicans is a major fungal pathogen of the pediatric patients causing a variety of infections including blood stream infections (BSI) with high mortality rate despite antifungal therapy^{26,27}. The resistance of microbes to antimicrobial agents has potentially serious implications for the control and treatment of invasive candidiasis²⁸. Thus, there is a need for the development of novel antifungal agents, which may meet the above challenges. The natural products, particularly their phytochemicals, persist as an important source of new therapeutic agents against diseases²⁹. Phytoconstituents are therefore important due to their various pharmacological activities including antifungal and antibacterial effects^{30,31}. Geraniol is a monoterpene alcohol constituting about 20 % as a main component of *C. winterianus* essential oil^{32,17} and anti-*Candida albicans* potential of the geraniol was tested in this study against isolates of pediatric clinical importance. The MIC of geraniol tested ranged between 32 and 128 µg/ml. The concentration of 128 µg/ml inhibited the growth of all strains, while 32 and 64 µg/ml was able to inhibit 80% and 90% of the strains tested, respectively. MFC of the oil ranged between 32 and 256 µg/ml; being 64 µg/ml able to inhibit 90% of the fungal strains. Amphotericin B and fluconazole were used as positive controls because they are the most commonly used antifungal drugs for the treatment of candidemia in pediatrics⁵. The MIC of the amphotericin B and fluconazole ranged between 0.5-1 and 0.5-8 µg/ml, respectively. The results for the control showed no fungal growth inhibition (Table 1).

According to the above results and to the criteria proposed by Morales *et al.* (2008), the geraniol, amphotericin B and fluconazole exhibited a strong antifungal activity against *Candida albicans* because their MIC values were lower than 100 µg/ml. In the literature, geraniol proved to be active against bacteria, including against *Streptococcus mutans*^{15,33} fungi: against *Trichophyton* species³⁴, *Aspergillus* species and against *Candida* spp.³⁵, including fluconazole-resistant and susceptible-dose dependent *Candida* isolates³⁶.

In accordance with the geraniol MIC value (MIC₉₀ = 64 µg/ml) in the present study, similar study results were found by Leite *et al.* (2015) (MIC₉₀ = 16 µg/ml)¹⁷, by Tampieri *et al.* (2005) (MIC = 100 µg/ml)³⁷, by Sharma (2016) (MIC of 30-130µg/ml)³⁸ and by Shweta *et al.* (2016) (MIC = 256 µg/ml)³⁹, who considered geraniol to be a good anti-*Candida* agent. It was even more effective than that

Table 1: MIC, MFC, MFC:MIC and effect of the geraniol, MIC of amphotericin B and fluconazole in *Candida albicans* strains.

Yeasts	Geraniol (µg/ml)		MFC:MIC	Effect	AmB (µg/ml)	Fluc (µg/ml)	Control strains ^a
	MIC	MFC			MIC	MIC	
<i>C. albicans</i>							
ATCC 60193	32	64	2:1	cide	0.5	0.5	+
AM-02	32	32	1:1	cide	1	1	+
AM-04	32	64	2:1	cide	1	1	+
AM-06	32	32	1:1	cide	1	1	+
AM-07	64	64	1:1	cide	0.5	0.5	+
AM-08	32	64	2:1	cide	0.5	0.5	+
AM-09	32	32	1:1	cide	0.5	0.5	+
AM-10	32	32	1:1	cide	1	8	+
AM-13	32	64	2:1	cide	1	0.5	+
AM-15	128	256	2:1	cide	0.5	0.5	+

Legend: MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; AmB, amphotericin B; Fluc, fluconazole; cide, fungicide; tatic, fungistatic; ^ayeast growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal or oil essential.

Table 2: Effect of geraniol against *Candida albicans* ATCC 60193 and AM-09 in the absence and presence of 0.8M sorbitol.

Drug	MIC (µg/ml)			
	ATCC 60193		AM-09	
	Without sorbitol	With sorbitol	Whitout sorbitol	With sorbitol
Geraniol	32	32	32	32

previously reported where it exhibited antifungal activity against *C. albicans* at or above 300 µg/ml^{40,41,36}.

In addition, according Hafidh *et al.* (2011) the MFC/MIC ratio is used to specify the nature of the antimicrobial effect against a particular pathogen. When the MFC/MIC ratio is between 1:1 and 2:1, the chemical is considered fungicidal. On the other hand, if the ratio is > 2:1, it is more likely to be fungistatic⁴². In the present study, the MFC/MIC ratios of geraniol were 1 or 2, this suggests that pytochemical has a fungicidal effect against the strains tested. This result is in accordance with previous studies that also reported the fungicidal effect of geraniol, using the kill time method in strains of *C. albicans*¹⁷ (Leite *et al.* 2015) and using agar disc diffusion assay in strains of *C. albicans*, *C. tropicalis* and *C. glabrata*³⁸. Fungicidal activity is clinically more important than fungistatic activity. The prophylactic use of fungistatic drugs has been associated with an increased frequency of innate or acquired resistance in clinical isolates⁴³. Our results are encouraging as they indicate that geraniol is fungicidal and not fungistatic and also suggest that it may help in resolving the issue of drug resistance due the use of fungistatic drugs in fungal strains.

Due to its pronounced anti-*C. albicans* activity, geraniol has been studied in more detail using two strains of *Candida albicans* (ATCC 60193 and AM-09). To investigate the action of the product on the fungal cell wall we performed the sorbitol assay. Sorbitol is an osmotic protector used to stabilize fungi protoplasts, protecting the fungal cell wall from environmental stresses, particularly osmotic changes. Products that act on the cell wall cause lysis of fungal cells in the absence of sorbitol, but fungi can grow in the presence 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)^{7,23}. In the present work, the MIC of the geraniol against *C. albicans* ATCC 60193 and AM-09 in the absence and presence of sorbitol was the same: 32 µg/ml (Table 2). The results suggest that this phytochemical does not act by modifying the fungal cell wall, but probably by affecting another target. Similar results were observed by Leite *et al.* (2015) where the MIC values were unchanged for geraniol in presence and absence of sorbitol against *Candida albicans* strains¹⁷.

The next step of this work was to determine if geraniol acts by affecting ergosterol in the fungal cell. Ergosterol is the main sterol of yeasts and thus is necessary for growth and normal membrane function of cells. Besides serving as a bioregulator of membrane fluidity, asymmetry and membrane integrity, ergosterol contributes to the proper function of membrane-bound enzymes⁴⁴. If the activity of compound was caused by binding ergosterol, it would increase the MIC of compound when the assay was conducted with the presence of ergosterol because the exogenous ergosterol would prevent the binding to ergosterol in the fungal membranes²⁴. Thus, the MIC of geraniol and amphotericin B was determined with and without the addition of ergosterol. As can be seen in table 3, geraniol displayed no changes in MIC values. This indicates that the primary mechanism of action of geraniol does not involve complexation with ergosterol. Amphotericin B, a positive control having a known interaction with ergosterol²⁴, showed a MIC value about 100 times greater in the presence of sterol (Table 3). Leite *et al.* (2015) observed similar results in *Candida albicans*,

Table 3: Effect of geraniol and amphotericin B against *Candida albicans* ATCC 60193 and AM-09 in the absence and presence of ergosterol 400 µg/ml.

Drugs	MIC (µg/ml)			
	ATCC 60193		AM-09	
	Without ergosterol	With ergosterol	Whitout ergosterol	With ergosterol
Geraniol	32	32	32	32
Amphoterecin B	0.5	64	0.5	64

when described that the MIC value of geraniol was not altered in the presence of exogenous ergosterol¹⁷.

Several studies have reported the mechanism of anti-*Candida* activity of geraniol appears to be associated with damage in the membrane integrity. According to Sharma *et al.* (2016) the geraniol disrupts cell membrane integrity and function by interfering with ergosterol biosynthesis and inhibiting the PM-ATPase that plays a crucial role in fungal cell physiology and hence is a promising new antifungal target for drugs³⁸. Zore *et al.* (2011) have shown that geraniol increased the rate of potassium leakage out of whole cells, increasing the membrane permeability (by decreasing phase transition temperature of dipalmitoyl phosphatidyl choline vesicles), and inhibited growth of *C. albicans* and *S. cerevisiae*⁴¹.

In addition, Shweta *et al.* (2016) reveals the mechanisms of action of geraniol on clinical *Candida albicans* isolates from diabetic patients suffering from oral candidiasis affects the fungal membrane and cell wall. The membrane tampering was observed by depleting ergosterol levels and altering plasma membrane ATPase activity leading disruption not only membrane but cell wall integrity as well. The data also reveal that geraniol causes mitochondrial dysfunction, impaired iron homeostasis and the function calcineurin signaling pathway is indispensable for *C.albicans* cells to sustains geraniol stress³⁶. The difference between ours and the results presented above could be explained by the different methodologies and different microorganisms used in the works.

Furthermore, it has been reported in the literature that geraniol inhibits both virulence attributes of hyphal morphogenesis and biofilm formation^{17,36,38}. The findings of this work are interesting, but mechanisms of action for geraniol against *Candida albicans* isolates from pediatric clinical importance need to be better investigated in order to justify and validate the later clinical application.

CONCLUSION

The present study demonstrated that geraniol has excellent antifungal activity against *Candida albicans* isolates from pediatric clinical importance. The likely primary mechanism of the geraniol's action appears not to involve cell walls, or binding to membrane ergosterol. Therefore, the test product is presented as a relevant and thus contributing to the existing arsenal of products with proven antifungal activity against *Candida albicans*. In addition, this information is important for future pharmacological applications of geraniol with the prospect of developing a new, safe and effective antifungal for the treatment of systemic mycoses. However, preclinical and clinical studies are needed to investigate whether geraniol acts on other targets in the fungal cell and to correlate the potent

in vitro - *in vivo* antifungal activity, thus confirming the efficacy and safety of the compound for later clinical application.

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