Investigating the antifungal activity and mechanism(s) of geraniol against *Candida albicans* strains

Maria Clerya Alvino Leite<sup>1,2,*</sup>, André Parente de Brito Bezerra<sup>2</sup>, Janiere Pereira de Sousa<sup>2</sup> and Edeltrudes de Oliveira Lima<sup>2</sup>

<sup>1</sup>Federal Institute of Education, Science, and Technology of Paraíba (IFPB), Itaporanga, Paraíba, Brazil and <sup>2</sup>Mycology Laboratory, Department of Pharmaceutical Sciences, Federal University of Paraíba, João Pessoa, 58051-970, Brazil

*To whom correspondence should be addressed: Maria Clerya Alvino Leite, Federal Institute of Education, Science, and Technology of Paraíba (IFPB), Rodovia BR-361, 58780-000, Itaporanga, Paraíba, Brazil. Tel.: +55-83-8845-6030; +55-83-9967-4508; E-mail: cleryaalvino@hotmail.com

Received 13 August 2014; Revised 29 September 2014; Accepted 11 October 2014

**Abstract**

*Candida albicans* can be a yeast that is a commensal on the human body but can cause opportunistic or pathogenic infections. *Candida* infections may create serious health problems and as a result has initiated a search for new drugs with an antifungal action. Geraniol is an acyclic monoterpene alcohol with known pharmacological properties, including antimicrobial activity. The aim of this work was to evaluate the antifungal activity and mechanism(s) of geraniol against *C. albicans* strains. The minimum inhibitory concentration (MIC) was determined through broth microdilution techniques. We investigated possible geraniol activity on the fungal cell wall (sorbitol protect effect), cell membrane (geraniol to ergosterol binding), the time-kill curve, and its biological activity on the yeast’s morphology. Amphotericin B was used as control, and all tests were performed in duplicate. The MIC of geraniol was 16 µg/ml (for 90% of isolates) but its probable mechanism of action did not involve the cell wall and ergosterol binding. In the morphological interference assay, we observed that the product inhibited pseudo- hyphae and chlamydoconidia formation. Time-dependent kill curve assay demonstrated that the fungicidal activity for MIC × 2 started at 2 h for the ATCC 76485 strain, and at 4 h for the LM-70 strain. Geraniol showed *in vitro* antifungal potential against strains of *C. albicans* but did not involve action on the cell wall or ergosterol. This study contributes to the development of new antifungal drugs, especially against *Candida* spp.

**Key words:** antifungal activity, biological products, *Candida albicans*, geraniol, terpenes.

**Introduction**

In recent years, opportunistic mycoses have been characterized by a considerable increase in both their prevalence and diversity of the suspected etiologic agent, as well as by the increased severity of such infections, often due to new predisposing factors [1].
*Candida albicans* is a common opportunistic human fungal pathogen that inhabits the human body as a commensal and can cause superficial mucosal infection, such as oral thrush and vaginitis, as well as potentially life threatening systemic disorders in immunocompromised individuals, such as organ transplant recipients, cancer patients receiving chemotherapy, and people with HIV/AIDS. In the last few years, *C. albicans* infections occur more frequently with high mortality rates and are recognized as a major cause of hospital-acquired infections [2]. *C. albicans* is the most important opportunistic pathogenic yeast, as it is the most frequently isolated fungus isolates from cases of human infections [3]. The therapeutic approach for fungal infections is a significant challenge due to the limited arsenal of antifungal drugs that are commercially available, their relatively high toxicity, and the well-recognized increase in the number of resistant yeasts during therapy [3,4].

Fungal infections present a unique problem because both the mammalian host and the invading fungi are eukaryotic, making it difficult to develop a specific antifungal aimed only at the pathogen [5]. From this perspective, there is a growing demand for new antifungal agents that are more effective and less toxic than those already in use. This is the major factor that initiate an intensive search among various sources, including natural products [4,5]. Antifungal compounds of natural origin, such as terpenes, have received much attention in recent times. They are a promising therapeutic tool for treating fungal infections and are known for their antimicrobial properties.

Terpenes are a class of natural substances of vegetable origin formed by the condensation of isoprene units (C₅H₈) and are classified as monoterpenes (C₁₀), the most representative molecules, and sesquiterpenes (C₁₅). A terpene containing oxygen is called a terpenoid [6]. Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is an acyclic monoterpene alcohol with the chemical formula C₁₀H₁₈O. Geraniol is the name given to the natural mixture of the two isomers properly named geraniol (trans) and nerol (cis). Geraniol is widely used in the flavor and fragrance industries as one of the most important molecules and is a common ingredient in consumer products produced by these industries [7]. Geraniol is a major component of geranium oil, and its content is about 20%. Geranium oil is one of the most popular essential oils in aromatherapy and has traditionally been used for vaginal candidiasis [8].

The geraniol exhibits various biochemical and pharmacological properties, including insecticide and repellent effects, besides anthelmintic, antibacterial, antioxidant, anticancer, and anti-inflammatory activities. Scientific literature also supports the antifungal properties of these compounds. It has been reported that geraniol shows antifungal activity against *C. albicans* and *Saccharomyces cerevisiae* [9]. Zhang et al. [10] investigated the antifungal activity of seven volatile constituents toward the mycelial growth of *Colletorichum camelliae*, and of the seven, geraniol had the most potential as an antifungal agent. The antifungal activity of palmarosa oil (*Cymbopogon martinii*) against *S. cerevisiae* is mainly attributed to its geraniol content [11]. Geraniol exhibited strong antifungal activity against *C. albicans*, with an MIC value of 19.5 mM [12].

Previous studies have reported that geraniol shows antifungal activity against *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium digitatum* [13]. However, knowledge about the antifungal activity of this monoterpene against the classic yeasts which cause opportunistic infections is very limited. This study seeks to contribute to the literature by providing data for the development of new antifungal drugs. The study therefore aims to investigate the antifungal activity of geraniol against strains of *C. albicans*. For this purpose, the minimum inhibitory concentration (MIC) of geraniol was determined, along with its effects on cell viability, morphogenesis, the fungal cell wall, and the fungal cell membrane.

### Materials and methods

#### Chemicals

Geraniol, amphoterin B (positive control), 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), and Vetec Fine Chemicals Ltd. (Duque de Caxias, RJ, Brazil), respectively. The emulsion used in the antifungal assays was prepared at the time of the execution of the tests. The drugs were dissolved in DMSO (dimethylsulfoxide), 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 dextrose broth (SDB) and Sabouraud glucose agar (SGA) were purchased from DiCo 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 strains of *C. albicans* tested were obtained from the collection of the Mycology Laboratory, Department of Pharmaceutical Sciences, Federal University of Paraíba (LM, DCF, UFPB) and included LM-14, LM-17, LM-70, and LM-520 (isolated from blood cultures), LM-11, LM-15, LM-94, and LM-410 (lung secretion), and two standard *C. albicans* strains from international collections, in other words, ATCC 76485 and ICB-12. All cultures were maintained on nutrient agar slants at 4°C. Overnight cultures on SGA slants at 35°C were used to prepare the fungal inoculum to be used in the antimicrobial assays.

Inoculum preparation
The inoculum was prepared from recent *C. albicans* cultures, plated on SGA and incubated for 24–48 h at temperature 35°C. After incubation, we transferred roughly 4–5 yeast colonies (with a sterile loop) to culture tubes containing 5 ml of sterile saline solution 0.9% (Farmax-Distributor Ltd. Amaral, Divinópolis, MG, Brazil). The resulting mixture was strongly agitated using a Vortex apparatus (Fanem Ltd., Guarulhos, SP, Brazil) for 15 s. The inoculum was standardized according to the 0.5 tube of the McFarland turbidity scale; the final concentration obtained was about 1–5 × 10^6 colony-forming units per milliliter (cfu/ml). The final concentration was confirmed by counting the microorganisms in a Newbauer chamber [14–16].

Determination of minimum inhibitory concentration (MIC)
The MIC was determined by the broth microdilution techniques [14–16]. In brief, 100 µl of liquid medium RPMI-1640 was dispensed aseptically to the wells of a 96-well microdilution plate with a “U” shaped bottom (Alamar, Diadema, SP, Brazil). Afterwards, 100 µl of geraniol emulsion was added in the first horizontal row of the wells. Next, doubling dilutions of test compound was aseptically incorporated in to these wells to obtain concentrations ranging from 1024 to 1 µg/ml. Finally, 10 µl of fungal inoculum suspension was added to all well of the plate. To verify the absence of interference in results by the emulsifying agent (DMSO and Tween 80) used in preparing the emulsion, a sensitivity control was made in which RPMI 1640, DMSO (5%), Tween 80 (2.5%) and the fungal suspension were added to the wells. A control microorganism (strain viability) was performed by placing 100 µl RPMI 1640, 100 µl of sterile distilled water, and 10 µl of fungal suspension. Sterility control of medium was also performed in which 200 µl RPMI-1640 was added in wells in the absence of fungal inoculum. The plates were incubated at 35°C for 24–48 h to allow for a visual observation of the growth inhibition in each well compared with that of the control (without drugs) well. The formation of cell clusters or “buttons” in the plate wells was considered. The MIC was defined as the lowest drug concentration that showed absence of visible growth or complete fungal growth inhibition (100% inhibition). Grading of the antimicrobial activity of test product was done according to literature [17,18], which consisted of a product with MICs less than 100 µg/ml was considered as good/significant, from 100 µg/ml to 500 µg/ml was classified as moderate; from 500 µg/ml to 1000 µg/ml the antimicrobial activity was defined as weak and finally, above 1000 µg/ml the product was considered inactive/no antimicrobial effect.

Determination of the kill time
The analysis of the interference of test product and amphotericin B on the viability of fungal strains was conducted according to Klepser et al. [19], with some modifications involving the use of viable cells count procedures. To determine the kinetics of fungal death, two strains were selected, in other words, a standard strain ATCC 76485, and a clinical strain LM-70, in accordance with the MIC results. In this test, the behavior of selected yeast strains was observed for 24 h. A series of sterile culture tubes was prepared, and we added 4.5 ml of RPMI 1640 medium containing various three different concentrations of the antifungals, in other words, MIC/2, MIC, and MIC × 2. Subsequently, 0.5 ml of the yeast suspension was added to the system and followed by shaking using a Vortex for 30 s. The assay was incubated at 35°C for 24 h, which permitted collecting aliquots at different time intervals (0 h, 2 h, 4 h, 6 h, and 24 h) of exposure, an aliquot of 10 µl from the tubes was removed with a sterile calibrated loop, and uniformly streaked on the surface of SGA culture medium. The Petri dishes were incubated at 35°C for 24–48 hours, and at the elapsed incubation time, the count was done (cfu/ml). Controls for yeast growth and the antifungal standard were also performed. All tests were performed in duplicate. The minimum detection limit of this method is 100 cfu/ml.

After the incubation period, the number of viable cells was counted and expressed in log of cfu/ml. The results were analyzed and represented graphically, that is a microbial death curve as a function of time. Data analysis for the test product was considered as showing fungicidal activity when there was a decrease greater than or equal to 3 log_{10} cfu/ml of the initial inoculum, resulting in reduction.
of 99.9% or more cfu/ml in 24 h compared with the initial inoculum. Fungistatic activity was considered as reduction in growth lower than 99.9% or < 3 log10 in cfu/ml from the initial inoculum [19].

Interference on fungal micromorphology

In view of observing morphological changes caused by geraniol and amphotericin B against *C. albicans* ATCC 76485 and LM-70, this study employed Dalmau slide culture technique involving the use of cornmeal agar in a moist chamber/Petri dish [20]. Initially, 1 ml of liquefied cornmeal agar containing the test product was poured onto a slide and after it solidified an inoculum suspension was prepared from recent cultures of the strains and seeded to form two parallel striations on the cornmeal agar and covered with a sterile coverslip. Then, the filter paper was soaked in sterile distilled water to keep system humidity. Each plate was closed and incubated at 35°C for 24–48 h. After the incubation period, each preparation was examined under optical microscopy at a magnification of 40× to observe the formation (or not) of yeast-typical structures like blastoconidia, pseudohyphae, and chlamydoconidia, and images were registered. Microculture controls (including the antifungal standard) were tested in the same manner [21–23]. The experiment was performed in duplicate.

Determination of effects of geraniol on fungal cell wall

**Sorbitol assay**

The MIC of geraniol was determined with *C. albicans* ATCC 76485 and LM-70 using the broth microdilution method in 96-well plates in a “U” as previously described. The sorbitol was added to the culture medium as osmotic support (final concentration 0.8 M). The plates were sealed aseptically and incubated at 35°C for 24 and 7 days. Based on the ability of sorbitol to act as an fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with added sorbitol compared to the standard medium implicated the cell wall as one of the possible cell targets for the product tested [24]. The assay was performed in duplicate, and the geometric mean of the results was calculated.

**Ergosterol binding assay**

**MIC value determination in the presence of ergosterol**

To assess if the test product binds to the fungal membrane sterols, this experiment as described by Escalante et al. [25] was adapted. The preparation of ergosterol followed the protocol described by Leite et al. [26]. The product was prepared at the time of test execution, where it was first pulverized and dissolved in DMSO and Tween 80, in accordance with the desired concentration and volume. The formed emulsion was then homogenized, heated, and diluted with the liquid culture medium.

The MIC of geraniol against *C. albicans* was determined by the microdilution method, in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines of the same microplate. Briefly, a solution of geraniol was doubly diluted serially with RPMI-1640 (volume = 100 µL) containing plus ergosterol at a concentration of 400 µg/ml. A volume of 10 µL of yeast suspension (0.5 Mc Farland) was added to each well. Amphotericin B was used as positive control in this experiment since it is known to bind ergosterol in the fungal membrane. The vehicle alone served as a negative control, and yeast viability in the presence of ergosterol was also checked. The plates were sealed and incubated at 35°C for 24 h and read afterward. MIC was defined as the lowest concentration of test agent capable of visually inhibiting 100% the fungal growth. This assay were carried out in duplicate, and the geometric mean values were calculated. Thus, this binding assays reflected the ability of compound to bind with the ergosterol.

Results

Table 1 shows the test results from studies of the antifungal activity of geraniol against *C. albicans* strains. The MIC of geraniol by the broth microdilution was 16 µg/ml for 90% of the tested fungal strains as compared to 2 µg/ml for the positive control (amphotericin B). There was no inhibition of the growth of controls by either DMSO or Tween 80, confirming that inhibition was due to antifungal agents. Yeast growth in the absence of antifungals demonstrated the viability of the fungal inoculum. Since there was no bacterial growth in the sterility controls the RPMI-1640 medium used in the tests was uncontaminated by microorganisms.

In accordance with the MIC results, ATCC 76485 and LM-70 were chosen for further studies of micromorphology and time-kill. The MIC of geraniol and amphotericin B for both strains were 16 and 2 µg/ml, respectively.

Based on the MIC, time-kill assay was conducted for *C. albicans*, with the viability of fungal structures analyzed at time intervals selected on the time-kill assay. In this test, we made viable cell counts by checking whether the drug used had fungistatic or fungicidal action, in addition to analyzing the interaction between the microorganism and the antifungal, in order to characterize a dynamic relationship between
Table 1. Results for MIC of amphotericin B and geraniol in µg/ml against Candida albicans- broth microdilution technique.

<table>
<thead>
<tr>
<th>C. albicans strains</th>
<th>Control</th>
<th>AmB (MIC) (µg/ml)</th>
<th>Geraniol (MIC) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) ATCC-76485</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>2) ICB-12</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>3) LM-11</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>4) LM-14</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>5) LM-15</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>6) LM-17</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>7) LM-70</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>8) LM-94</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>9) LM-410</td>
<td>+</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>10) LM-520</td>
<td>+</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

Legend: (+), growth of the microorganism. AmB, amphotericin B; MIC, minimum inhibitory concentration.

The sorbitol assay consisted of determining the MIC in the presence and absence of 0.8 M sorbitol, an osmotic protector. We found that the geraniol MICs against ATCC 76485 and LM-70 strains remained the same at 16 µg/ml in either the absence or presence of sorbitol. There was no change in the geraniol MIC values in media with and without addition of ergosterol, but the amphotericin B MIC value increased 64 times in the presence of ergosterol. The control with sorbitol ensured the viability of the strain since it was able to grow in the presence of sorbitol and the absence of either drug (Table 2).

Discussion

The high incidence of C. albicans infections and emergence of resistance to antifungal drugs underlines the need to study new sources of drugs, such as natural products and their phytochemicals

Plant substances are used in the treatment of various diseases, but their potential application as a source of new drugs is still largely unexplored. Of the estimated 250,000 to 500,000 species of plants, only a small percentage have had their pharmacological properties studied. Thus, compounds derived from plants are potential valuable sources of new medicinal agents, justifying the need to find new drugs from plants that have therapeutic potential. Phyto-constituents are therefore important due to their various pharmacological activities including antifungal, antibacterial, and anti-parasitic effects.

The present work studied the activity mode of action of geraniol against C. albicans, in other words, MIC$_{90}$ was 16 µg/ml or 90% of the test strains were found to have this MIC (Table 1).

Mesa-Arango et al. [35] showed geraniol antifungal activity against strains of Candida parapsilosis, Candida krusei, Aspergillus flavus, and Aspergillus fumigatus.
A previous study had shown that low doses of geranium oil, and its main component, geraniol, in combination with the vaginal washings showed both anti-inflammatory and anti-Candida activity for the treatment of vaginal candidiasis in similar models [8]. Marcos-Arias et al. [36] demonstrated the antifungal activity of geraniol against C. albicans, C. glabrata, C. tropicalis, C. guilliermondii, C. parapsilosis, C. dubliniensis, and C. krusei isolates from denture wearers. The authors showed that geraniol had significant in vitro activity against the fluconazole-resistant and susceptible-dose dependente Candida isolates. In addition, another study reported that geraniol showed an MIC of 100 µg/ml against Streptococcus mutans, the causal organism of dental caries. Thus, this compound might well be used for preparing different formulations for dental caries [37].

Dalleau et al. [38] demonstrated that geraniol is able to significantly reduce biofilm development of C. albicans, showing one of the strongest of anti-biofilm activities. According to the authors, geraniol inhibited biofilm formation by > 80% when used at concentrations of 0.06%.

In the present study, geraniol showed potential antifungal activity against C. albicans confirming the results obtained in previous studies [37–39]. Similar study results were found by Tampieri et al. [40] (MIC = 100 µg/ml), who considered geraniol to be a good anti-Candida agent. It was even more effective than that previously reported where it exhibited antifungal activity against C. albicans at or above 300 µg/ml [9,31,36].

Due to its pronounced anti-C. albicans activity, geraniol has been studied in more detail using the time-kill...
Figure 2. Micromorphology of *Candida albicans* strains in the absence (control) and presence of geraniol and amphotericin B. Slides A and B: *C. albicans* ATCC 76485 and *C. albicans* LM-70, respectively, in the product’s absence (control) showing the presence of blastoconidia, chlamydoconidia, and pseudohyphae. Slide C: under the action of amphotericin B - MIC. Slide D: under the action of geraniol - MIC. Legend: (1) Pseudo-hyphae; (2) chlamydoconidia; (3) Blastocinidia.

Table 2. MIC values (µg/ml) of drugs in the absence and presence of sorbitol (0.8 M) and ergosterol (400 µg/ml) against *Candida albicans* ATCC 76485 and LM-70.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Sorbitol Absence</th>
<th>Sorbitol Presence</th>
<th>Ergosterol Absence</th>
<th>Ergosterol Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geraniol</td>
<td>16</td>
<td>16</td>
<td>+</td>
<td>16</td>
</tr>
<tr>
<td>Amphotericin B³</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>2</td>
</tr>
</tbody>
</table>

Legend: (+), presence de fungal growth in liquid medium plus sorbitol and absence of drugs; MIC, minimum inhibitory concentration.
³Positive control.

method. The time of death curves showed that the higher concentrations of geraniol increased the antifungal activity from fungistatic to fungicidal. Thus, geraniol has a concentration dependent antifungal activity. The time kill characterization is very important because it has valuable therapeutic implications, such as adjusting the dose for a more effective treatment [41]. Thus, antifungal agents whose activity increases with increasing concentration can be optimized by the administration of larger doses (Klepser ME, Wolfe EJ, Jones RN, Nightingale CH, Pfaller MA. Characterization of the fungicidal dynamics of fluconazole (FLU) and amphotericin B (AB) against *C. albicans* via kill-curve methodologies. In *Program and Abstracts of the 36th Iiztierscience Conference on Antimicrobial Agents*
The analysis of the log$_{10}$ cfu/ml versus time graph shows that the fungicidal activity against Candida albicans ATCC 76485 was achieved after 2 h at MIC × 2, while for C. albicans LM 70, after 4 h. Similarly, Lima et al. [42] found antimicrobial effectiveness of phytochemicals (citral, eugenol, α-pinene and β-pinene) against pathogen yeasts (C. albians, C. krusei and C. tropicalis) providing a intense fungicide effect in 2 h of exposure. A previous study examined the kill-curve of three isoprenoid constituents of geranium oil reported geraniol to be the most effective with an MFC = 640 µg/ml, and MIC = 561 µg/ml killing 99.9% of the inoculum just within 15 min, while to obtain the MFC with geranium oil, and geranyl acetate required 30 min of exposure [31].

Fungal micromorphology assay indicated that the geraniol was able to inhibit formation of chlamydoconidia and pseudo-hyphae. The formation of pseudo-hyphae in response to various environmental signals is considered to be essential to Candida virulence [43], since these structures represent a barrier for phagocytosis (owing to their morphology and/or size) and can also easily penetrate in the epithelial and endothelial tissues [44]. There have been reports in the literature that mutant strains of C. albicans unable to form filaments are avirulents in models of disseminated or mucosal candidiasis [45–47].

Thus, change in morphology from the yeast form to the filamentous form plays a vital role in the pathogenesis of fungal infections and suggests that associated factors for this conversion process represent promising therapeutic targets [48,49]. Few studies have focused on the effect of test products on the micromorphology of opportunistic yeasts. A recent study reported that plant terpenoids inhibit morphogenesis, adhesion, and biofilm formation by C. albicans [50]. Results obtained by Zore et al. [31], demonstrate that geraniol was highly effective inhibiting serum-induced morphogenesis, in other words, dimorphism (yeast to hyphal) and also caused 50% inhibition of germ tube induction at very low (non-toxic) concentrations to mammalian. The germaniol results in this work may be of great value in developing newer drugs.

To investigate the action of the geraniol on the fungal cell wall we performed an assay with sorbitol, an common osmotic protector used to stabilize fungal protoplasts. The sorbitol protection assay is compatible with a range of natural products, as well as pure chemicals. Compounds acting on the fungal cell wall share a distinctive feature where their antifungal effects are reversed in a medium containing an osmotic stabilizer such as sorbitol. Cells protected with sorbitol can grow in the presence of inhibitors of fungal cell wall synthesis, 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). This assay is known as a broad spectrum screen that can find not only agents that directly interfere in cell wall synthesis and assembly but also regulatory mechanisms involved in this process, including signal transduction pathways [24,51].

Based on the results of the present study, the MIC values of geraniol in media with and without sorbitol, were identical, suggesting that geraniol does not act by inhibiting the fungal cell wall synthesis, but probably by affecting another target. This is the first study to demonstrate the action of geraniol on the cell wall of C. albicans under sorbitol testing, which complicates comparison with other investigations. However, the results confirm earlier studies for other terpenoids such as citral [27] and carvacrol [52], which described their activities was not related to modifying the C. albicans cell wall.

To explore the possible mechanism of interaction of geraniol with fungal cell membrane, we studied the ability of the compound to form complexes with ergosterol. Ergosterol is the main sterol component in the plasma membrane of fungi and plays the same role in the fungal membranes that cholesterol does in mammalian cell membranes [53]. Therefore, these two sterols seem to exhibit qualitatively similar properties. If the activity of geraniol is caused by binding to ergosterol, the exogenous ergosterol would prevent the binding to the fungal membrane’s ergosterol. Consequently, it would cause an increase in MIC of geraniol in the presence of exogenous ergosterol with respect to the control experiment [25,54]. Thus, the effect of exogenous ergosterol on the MIC of geraniol and amphotericin B was determined.

The ergosterol binding assay revealed that geraniol MICs were identical in medium with and without additional ergosterol. Thus, our findings indicates that the mechanism of action of geraniol does not involve complexation with ergosterol. However, amphotericin B does complex with ergosterol, in that its MIC increased 64 times. Several studies have reported the action of geraniol on fungal cell membranes. According to Zore et al. [31], the mechanism of anti-Candida activity of geraniol appears to be associated with damage in the membrane integrity. It has been reported that the geraniol interferes with membrane functions in fungal strains. This compound was shown to increase the rate of potassium leakage out of whole cells, to increase membrane permeability (by decreasing phase transition temperature of dipalmitoyl phosphatidyl choline vesicles), and to inhibit growth of C. albicans and S. cerevisiae [9]. Confirming our results, the authors also stated that the geraniol does not appear to
inhibit ergosterol biosynthesis (as its primary mode of action) but instead appears to partition membranes where it alters bilayer properties. Geraniol has been reported to show an enhancing effect on percutaneous absorption of highly lipophilic drug like midazolam in mice [55]. Carnevecchi et al. [56] reported that geraniol causes perturbation of cell membrane permeability (induced membrane depolarization) and signal transduction pathways (reduction of protein kinase C (PKC) activity) in animal cells. Therefore, according to these authors, geraniol has antiproliferative effects on human colon cancer cells.

From the results obtained, it was concluded that geraniol showed excellent antifungal activity against C. albicans. 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 opportunistic mycoses. However, we suggest further tests and clinical studies to correlate the potent in vitro - in vivo antifungal activity, thus confirming the efficacy and safety of the compound for later clinical application.

Acknowledgment
The authors are grateful to the Federal University of Paraíba (UFPB) and to David Harding who edited the English manuscript.

Declarations of Interest
The authors report no conflicts of interest. The authors alone are responsible for the contente and the writing of the paper.

References
5. Khan MSA, Ahmad I, Cameotra SS. Phenyl aldehyde and propanoids exert multiple sites of action towards cell membrane and cell wall targeting ergosterol in Candida albicans. AMB Express 2013; 3.