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In vitro synergistic efficacy of nystatin combined with geraniol against *Candida albicans* and *Candida glabrata* biofilm on acrylic resin

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ABSTRACT

Candida albicans and *Candida glabrata* predominate in the oral cavities of elderly people with denture stomatitis. Prolonged nystatin treatment can alter the surface properties of acrylic resin, while geraniol can inhibit *Candida* biofilm without affecting the surface roughness or color of the acrylic resin. This study investigated the efficacy of nystatin/geraniol combinations against *C. albicans* and *C. glabrata* biofilms on acrylic resin. Checkerboard assay revealed synergistic activity of nystatin/geraniol combinations. The minimum biofilm eradication concentration (MBEC) and efficacy against *Candida* biofilm were evaluated by crystal violet assay. The $MBEC_{90}$ of nystatin against *C. albicans* and *C. glabrata* biofilms was 55 and 131 µg/mL, respectively. The $MBEC_{90}$ of geraniol against *C. albicans* and *C. glabrata* biofilms was 330 and 45,367 µg/mL, respectively. A significantly higher percentage of *C. albicans* biofilm reduction was observed when combining nystatin at 0.25 MBEC with geraniol at 1 MBEC (70.89%) ($p<0.05$), compared to geraniol monotherapy (42.35%). However, the percentage of *C. glabrata* biofilm reduction at sub-inhibitory concentrations did not differ from single-agent treatment. In conclusion, nystatin/geraniol combinations had a synergistic effect against *C. albicans* and *C. glabrata*, and combinations at sub-inhibitory concentrations can diminish the biomass of *Candida* biofilms on acrylic resin.

Keywords: *C. albicans*; *C. glabrata*; biofilm; geraniol; nystatin; acrylic resin

1. INTRODUCTION

Older patients with edentulism may have difficulty with food intake, negatively affecting their nutritional status and further compromising their general health. Replacing edentulous areas with acrylic dentures is common due to their affordability and acceptable mechanical characteristics in most clinical conditions. However, acrylic resin is easily colonized by microorganisms, especially *Candida spp.*, which can induce a biofilm-mediated chronic inflammatory response in the oral mucosa known as denture stomatitis (DS) (McReynolds et al., 2023). The main oral yeasts in older patients with *Candida*-associated DS are *C. albicans* and *C. glabrata*. An association between mixed colonization by these two *Candida* species and higher grades of inflammation has been previously demonstrated (Coco et al., 2008). Several methods have been suggested for the prevention and

treatment of DS, such as topical antifungal therapy, denture disinfection, denture base modification, and the integration of antimicrobial agents into denture base material (Gad & Fouda, 2020). However, clinical relapse and post-treatment recurrence remain the greatest challenges for DS treatment (McReynolds et al., 2023).

Nystatin oral suspension is a common antifungal drug used for oral candidiasis treatment (Pappas et al., 2016). The advantages of nystatin over other antifungals are highlighted by the rare presence of nystatin-resistant fungi and its affordable price. However, its slight solubility in water, short contact time in the oral cavity, and unpleasant taste are notable disadvantages of nystatin oral suspension. Common adverse effects of nystatin also include mouth irritation, nausea/vomiting, skin rash(es), and diarrhea (Lyn et al., 2016). In the case of *Candida*-associated DS, the eradication of *Candida* on the oral mucosa and denture is essential for effective treatment. Therefore, nystatin oral suspension is prescribed for mouth rinsing and immersion of the prosthesis. However, continued exposure to nystatin could adversely affect surface roughness, contact angle, and *Candida* adhesion on the acrylic resin (Al-Dwairi et al., 2012). Surface roughness has been shown to be a major contributing factor to *Candida* adhesion (Le et al., 2020). Irreversibly attached microorganisms on the micropores of rough surfaces lead to complex biofilm development on acrylic surfaces, inducing a recurrence of DS. Additionally, wearing a fungal-infected denture can cause serious life-threatening diseases such as aspiration-related pneumonia or gastrointestinal fungal infection (van der Maarel-Wierink et al., 2013).

Geraniol is classified as a terpene commonly found within various essential oils such as geranium oil, rose oil, lemongrass, and other aromatic plants (Sharma et al., 2018). Geraniol is a natural monoterpenic alcohol with numerous biological properties, including antioxidant, antimicrobial, anti-inflammatory, and anti-cancer effects (Lei et al., 2019). Geraniol significantly decreases *C. albicans* biofilm biomass, impairs *C. albicans* adherence to epithelial cells (Singh et al., 2018), and inhibits the biofilm formation of *C. albicans*, *C. glabrata*, and *C. tropicalis* on self-polymerizable acrylic resin (Kaypetch et al., 2022). However, surface roughness and the color of the denture acrylic have been shown to not alter after immersion with geraniol at various concentrations for 28 days (Kaypetch et al., 2022). In combination with antifungals, geraniol has exhibited synergistic interactions with both fluconazole and amphotericin B (Khan et al., 2012).

To overcome the effects of nystatin on the properties of an acrylic resin denture base, combination therapy with a natural phytocompound has potential benefits over monotherapy in terms of decreasing side effects. Geraniol exhibits antifungal inhibitory activity without affecting the acrylic resin properties and is considered safe. Its combination with nystatin could improve efficacy, decrease inhibitory dosages, and reduce side effects, resulting in more cost-effective and safer formulations. Therefore, our objective is to determine the antifungal effect of the geraniol and nystatin combination on *Candida* biofilm formed on an acrylic resin denture base, focusing on the potential synergistic effects.

2. MATERIALS AND METHODS

2.1 Materials

The nystatin solution (100,000 U/mL) was purchased from T.O. Pharma Co., Ltd. (Bangkok, Thailand). Geraniol (98%) was acquired from Ji'An Zhong Xing Natural Plants Co., Ltd. (Jiangxi, China). Sabouraud dextrose broth was obtained from HiMedia (Mumbai, India). Morpholinepropanesulfonic acid (MOPS), glucose, and RPMI 1640 without sodium bicarbonate supplemented with L-glutamine, were procured from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Massachusetts, USA). The crystal violet (CV) solution was acquired from Panreac Quimica SLU (Barcelona, Spain). Heat-polymerized acrylic resin (ACRY POL R. POLVERE V7) was obtained from Ruthinium® Dental Manufacturing S.p.A. (Rovigo, Italy). The polystyrene, flat bottom, 96-well microtiter plates, and 24-well tissue culture plate were procured from Corning Incorporated (New York, USA).

C. albicans (ATCC10231) and *C. glabrata* (DMST46683) were kindly provided by the Department of Medical Sciences, Ministry of Public Health, Thailand. Stock cultures were divided into small portions and stored at -80°C in a 20% glycerol tryptone soil broth.

2.2 Preparation of *Candida* cells

Thawed *C. albicans* and *C. glabrata* were grown in Sabouraud dextrose broth and incubated on an orbital shaker at 37°C for 24 h. The cells were harvested, enumerated using a hematocytometer, resuspended, and adjusted to 10^6 or 10^8 cells/mL with RPMI1640.

2.3 Acrylic resin strip fabrication

Acrylic resin specimens were fabricated using heat-polymerized techniques. The main composition of the powder was methyl methacrylate, with benzoyl peroxide as an initiator, while the liquid consisted of methyl methacrylate and ethylene dimethacrylate. A total of 216 square-shaped acrylic resin strips (W:10 mm, L:10 mm, H:2 mm) were fabricated following a conventional flasking and pressure-pack procedure for acrylic resin denture bases.

Briefly, wax denture baseplates (W:50 mm, L:50 mm, H:2 mm) were embedded in flasks with dental stone and compressed with a hydraulic dental flask press at a pressure of 300 kPa for 1 h. After the dental stone setting, the flasks were immersed in boiling water for 5 min for dewaxing. The mold spaces were cleaned with boiling water, and a thin film of separating medium was applied. The acrylic resins, with a 3:1 ratio of powder to liquid by volume, were thoroughly mixed for 30 s. and rested for 15 min in glass containers, according to the manufacturer's instructions. The plastic dough of acrylic resin was packed in the flasks under a final pressure of 300 kPa. The flasks were then immersed in water at 70°C for 30 min and 100°C for an additional 30 min.

After polymerization, the curing flasks were bench-cooled overnight at room temperature, and the specimens were deflasked. Both surfaces of the specimens were polished with 100-, 400-, 800-, 1000-, and 2000-grit wet sandpaper. The specimens were then sectioned into square-shaped acrylic resin strips (W:10 mm, L:10 mm, H:2 mm) by microcutting. All strips were immersed in distilled water for 48 h. to leach the excess monomer,

disinfected by dipping in 1% sodium hypochlorite for 15 min (Orsi et al., 2011), washed with sterile distilled water, and dried before use.

2.4 *Candida* biofilm formation

2.4.1 *Candida* biofilm formation for MBEC

Biofilms were attached to polystyrene, flat bottom, 96-well microtiter plates, as previously described (Pesee et al., 2016). The culture medium was RPMI 1640 without sodium bicarbonate supplemented with L-glutamine, 2% glucose, and buffered with morpholinepropanesulfonic acid (MOPS). Briefly, aliquots of 100 µL of the standardized *Candida* cell suspensions (10^6 cells/mL) and 100 µL of culture medium were transferred into each well of the microtiter plate. After incubating the plate for 24 h at 37°C, the non-adherent cells were carefully removed by washing with 200 µL of sterile phosphate-buffered saline (PBS) three times.

2.4.2 *Candida* biofilm on resin acrylic strips

Aliquots of 100 µL of the standardized *Candida* cell suspension (10^8 cells/mL) were applied to the surfaces of acrylic resin strips placed in a 24-well tissue culture plate. The strips were then submerged in 2 mL of culture medium. Strips to which no cells were added served as negative controls. All strips were incubated at 37°C for 24 h After biofilm formation, the non-adherent cells were removed from the strips by gently washing with 1 mL PBS. They were then transferred to a new 24-well tissue culture plate for experimentation.

2.5 Antifungal test

2.5.1 Minimum inhibitory concentration (MIC) determination

The antifungal activity of nystatin and geraniol against planktonic *Candida* was evaluated by determining the MIC using a broth microdilution assay, as described in the Clinical and Laboratory Standards Institute document M27-A3. The nystatin solution was prepared with 1% DMSO and sterile PBS to create an initial concentration of 2 mg/mL, and it was then serially two-fold diluted to the designed concentrations. The initial concentration of geraniol was 871.612 mg/mL. The geraniol was then serially two-fold diluted in culture medium and 2% DMSO. The final concentrations tested for nystatin were 0.5 to 16 µg/mL for both *C. albicans* and *C. glabrata*. The final concentrations tested for geraniol ranged from 4 to 256 µg/mL and 7.5 to 7680 µg/mL for *C. albicans* and *C. glabrata*, respectively.

One hundred microliters of standardized *C. albicans* or *C. glabrata* (10^6 cells/mL) was exposed to 100 µL of nystatin or geraniol in flat-bottomed, polystyrene, 96-well microtiter plates and incubated for 48 h at 37°C. *Candida*, without drug treatment, served as the positive control, with culture media only serving as the negative control. The *Candida* growth was measured as turbidity using a microtiter plate reader (Opsys MR, Dynex, USA) at 590 nm. MIC_{90} values were expressed as the lowest concentrations inhibiting *Candida* growth by 90%, compared to the agent-free growth control. All assays were performed in triplicate and on three separate occasions.

2.5.2 Determination of MBEC

The MBEC was determined by inoculating *Candida* biofilm in a 96-well microtiter plate with 100 µL of various concentrations of nystatin or geraniol for 48 h at 37°C (Ramage et al., 2001). The serial two-fold dilution of nystatin prepared for the final concentration ranged from 2 to 256 ug/mL for *C. albicans* and 4 to 512 ug/mL for *Candida glabrata*. The final concentration of geraniol ranged from 8 to 1,024 ug/mL and 512 to 65,536 ug/mL for *C. albicans* and *C. glabrata*, respectively. *Candida* biofilm without any agents was included as the positive control. The negative control was culture media without biofilm and agents. The biofilm biomass was then determined using CV assay. The $MBEC_{90}$ was the lowest drug concentration at which the *Candida* biofilm biomass decreased by 90%. All assays were performed in triplicated wells and on three separate occasions.

2.6 The Effect of the nystatin/geraniol combinations on *C. albicans* and *C. glabrata*

2.6.1 The potency of the nystatin/geraniol combination on *Candida* planktonic cells by checkerboard assay

One hundred microliters of the standardized *Candida* (10^6 cells/mL) were placed in each well of a 96-well microtiter plate. Fifty microliters of nystatin and geraniol, at various concentrations, were allocated to each well along the columns and rows following the pattern shown in Figure 1. The concentrations of agents for the checkerboard assay were designed based on the MIC of each agent. The nystatin was tested at concentrations of 0.25 to 32 µg/mL for *C. albicans* and 0.45 to 28.4 µg/mL for *C. glabrata*. The concentrations of geraniol ranged from 4 to 256 µg/mL and 191 to 12230 µg/mL for *C. albicans* and *C. glabrata*, respectively. Wells with *Candida* absent of nystatin and geraniol served as the positive controls. Culture media without *Candida* and drugs served as the negative control. After incubation for 48 h at 37°C, the plate was examined using a microtiter plate reader at 590 nm. The checkerboard endpoint was defined as the lowest concentrations of the combination observed at a growth inhibition of ≥90% compared to those of the growth control. The fractional inhibitory concentration index (FICI) was calculated as per Equation 1.

$$FICI = FIC_N + FIC_G = (C_N/MIC_N) + (C_G/MIC_G) \quad (1)$$

where MIC_N and MIC_G are the MIC_{90} of nystatin and geraniol alone, and C_N and C_G are the concentrations of the nystatin and geraniol in combination in all wells corresponding to an MIC_{90}. The interaction was considered synergistic when the FICI was ≤0.5, indifferent at >0.5 to ≤4.0, and antagonistic at >4 (Parker et al., 2022). All were performed in triplicate.

2.6.2 Effect of the nystatin/geraniol combination on preformed *Candida* biofilms on acrylic resin

In a 24-well plate, mature *Candida* biofilms on acrylic resin were co-incubated for 24 h at 37°C with 1,000 µL of nystatin or geraniol at MBEC and with the combination of nystatin (500 µL) and geraniol (500 µL) at various concentrations. The synergism of the combinations was revealed from the checkerboard assay, leading us to

speculate whether the combinations at sub-inhibitory concentrations (0.5 and 0.25 MBEC) can reduce the biofilm mass of *Candida*. Thus, the efficacy of nystatin and geraniol combinations at MBEC, and the sub-inhibitory concentrations on *Candida* biofilm were evaluated. Untreated *Candida* biofilms on acrylic resin were included to serve as positive controls. The biofilm biomass remaining on the acrylic resin after drug treatment was examined by CV staining and calculated for the percentage of biofilm reduction. The biofilms of each group were triplicated, and the experiments were performed on three separate occasions.

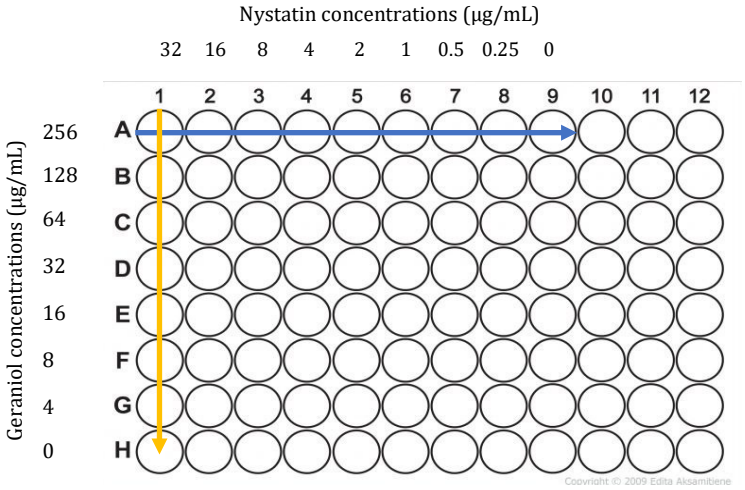


Figure 1. Layout of the nystatin and geraniol combination treatment in a 96-well microtiter plate for the checkerboard assay

Note: The blue arrow indicates the same concentration of geraniol being placed along the row, while the yellow arrow indicates the same concentration of nystatin being placed along the column. Wells H1 to H8 contain nystatin without geraniol, while wells A9 to G9 contain geraniol without nystatin. Columns 11 and 12 were preserved for positive and negative controls.

2.7 Quantification of the total biofilm biomass

2.7.1 CV assay for the biofilm on a 96-well microtiter plate

The quantitation of biofilm biomass on the 96-well microtiter plate was measured after 48 hours of incubation at 37°C with antifungal agents using the CV assay, as described by Pesee et al. (2016). The CV assay was performed at room temperature. Briefly, 100 ml of 99% methanol was added after washing the adherent cells twice with PBS. The methanol was discarded after 15 min of fixation, and 100 µL of 0.5% (w/v) CV solution was added into each well. The excess CV was then removed after 20 min of incubation, while 150 mL of 33% acetic acid was added and slowly pipetted for 1 min to release the CV bound to the biofilm. The density of the solubilized CV was read using a microtiter plate reader at 590 nm. The anti-biofilm effect was expressed as the percentage of *Candida* biofilm reduction using Equation 2.

$$\text{Percentage of } \textit{Candida} \text{ biofilm reduction} = ((OD_{control} - OD_{agent})/OD_{control}) \text{ X } 100 \quad (2)$$

2.7.2 CV assay for the *Candida* biofilm on acrylic resin

The quantitation of biofilm biomass on acrylic resin strips was determined using a CV assay (Monteiro et al., 2011). After 24 h of incubation at 37°C with antifungal agents, the medium was aspirated from each well, and the non-adherent cells were washed twice with PBS. The acrylic resin was then placed into a polypropylene tube containing 1 mL of sterilized PBS and vortexed for 20 min to disrupt the biofilm cell aggregates. Visual inspection with an inverted microscope revealed that no biofilm cells remained on the acrylic resin strips. The resultant suspension containing the detached biofilm cells was centrifuged at 3000 x g for 5 min, the supernatant was discarded, and 1 mL of sterilized PBS was added (Marra et al., 2012). Two hundred microliters (200 µL) of the suspension culture was added to each well of the 96-well microtiter plate. The cells were fixed with 1 mL of 99% methanol for 15 min before evaluating with a CV assay, and the obtained absorbance was read in a microtiter plate reader at 590 nm.

2.8 Statistical analysis

The analyses were performed by GraphPad Prism version 8.0.2 (GraphPad Software, Inc.). Differences between the mean MIC or MBEC values for each agent were assessed using Student's t-test. Differences between the mean percentage of biofilm mass reduction in response to nystatin-geraniol combinations were assessed by one-way analysis of variance (ANOVA) with Tukey's post hoc tests. A value of $p<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Antifungal susceptibility of nystatin and geraniol against planktonic and *Candida* biofilm

The antifungal activities (MIC_{90} and $MBEC_{90}$) of nystatin and geraniol, when used alone against *Candida* planktonic and biofilm, are summarized in Table 1. For the planktonic cells, the MIC_{90} of nystatin and geraniol against *C. glabrata*

was significantly higher than those of *C. albicans* (p<0.0001). Additionally, the $MBEC_{90}$ of nystatin for *C. glabrata* biofilms was significantly higher than that for *C. albicans* (p<0.01). The $MEBC_{90}$ of geraniol for *C. glabrata* biofilms was also significantly greater than that for *C. albicans* (p<0.0001).

Table 1. Antifungal susceptibilities of planktonic and biofilm of *C. albicans* and *C. glabrata* determined by a crystal violet assay

Antifungal agents	*Candida* species	MIC_{90} (µg/mL) (means±SD)	$MBEC_{90}$ (µg/mL) (means±SD)
Nystatin	*C. albicans*	9.65±0.27	55.04±2.39
	C. glabrata	14.2±0.26****	131.34±31.83**
Geraniol	*C. albicans*	127.0±4.08	330.68±27.94
	C. glabrata	6115.05±166.52****	45367.62±5826.52****

Note: ** *p<0.01*, **** *p<0.0001* compared between the MIC or MBEC of each agent

The MIC_{90} of geraniol for *C. albicans* reported here was relatively consistent with the results of Sharma et al. (2018) and Singh et al. (2018) but greater than that reported by Leite et al. (2015). Our results indicate a weaker inhibitory effect for geraniol against planktonic *C. glabrata* than *C. albicans*, as the MIC_{90} of geraniol for *C. albicans* and *C. glabrata* was 127 and 6,115 µg/mL, respectively. This MIC_{90} of geraniol for *C. glabrata* was notably higher than that found in previous reports (Gupta et al., 2021; Sharma et al., 2018). In comparing the MEBC of geraniol against *Candida* biofilms, *C. glabrata* was revealed to be more resistant to geraniol than *C. albicans*. This contrasts with a previous study using an XTT assay, which demonstrated that 69% of *C. albicans* and 93% of *C. glabrata* biofilm formation were inhibited after treatment with geraniol at 640 µg/ mL (Kaypetch et al., 2022). Different methodologies, *Candida* strains, source of geraniol, and the way the results were analyzed and interpreted would provide a possible explanation for these discrepancies.

Several virulence factors contribute to *Candida* pathogenesis, including the adherence to host tissues, transition from blastospores to hyphae, hydrophobicity, biofilm development ability, and extracellular hydrolytic enzyme secretion. *Candida* possesses several gene families encoding adhesion proteins, and the regulation of biofilm formation involves complex and varied gene networks that are strain-specific and allele-specific (Malinovská et al., 2023). Additionally, hydrophobicity plays a crucial role in the adhesion process, and clinical isolates of *Candida* exhibit significant variability in cellular surface hydrophobicity (CSH), which correlates with biofilm production (Dabiri et al., 2018). Variations in CSH may also influence the response of *Candida* strains to antifungal treatments (Danchik & Casadevall, 2021). Differences in the production of hydrolytic enzymes, such as esterases and hemolysins, were observed in *C. glabrata* strains with different azole susceptibility profiles (Figueiredo-Carvalho et al., 2017). Therefore, the variant antifungal susceptibility profiles between *Candida* strains may be due to gene variability, differences in CSH, or the heterogenous production of extracellular hydrolytic enzymes.

3.2 *In vitro* antifungal effect of the nystatin and geraniol combination against planktonic *Candida* cells

The antifungal activity of nystatin and geraniol combinations has primarily been studied against *Candida* planktonic cells using a checkerboard assay. As shown in Table 2, the combination of nystatin/geraniol displayed synergistic activity against both *C. albicans* and *C. glabrata*. The MIC_{90} of nystatin in combination was 1 and 1.78 µg/mL against *C. albicans* and *C. glabrata*, respectively. The MIC_{90} of geraniol in combination with *C. albicans* ranged from 1 to 8 µg/mL, whereas the MIC_{90} of geraniol in combination with *C. glabrata* was 764 µg/mL. The FICI values for this combination tested on *C. albicans* and *C. glabrata* were 0.130 and 0.25, respectively.

The observed synergistic interactions between nystatin and geraniol on *C. albicans* align with previous studies investigating other antifungal drugs (Singh et al., 2018; Khan et al., 2012). When combined with fluconazole, geraniol exhibited a synergism by reducing the MIC of fluconazole against clinical *C. albicans* strains (Khan et al., 2012; Singh et al., 2018). Additionally, the combination of amphotericin B and geraniol showed greater antifungal efficacy against multi-drug resistant *C. albicans* isolates than the sum of their individual effects (Khan et al., 2012).

Nystatin exerts its antifungal effects by disrupting the fungal cell membrane. It binds to sterols in the plasma membranes of *Candida*, resulting in a change to membrane permeability, allowing leakage of the cellular component and eventually leading to fungal cell death. Geraniol, when used alone, targeted numerous cellular mechanisms in the cell wall, plasma membrane, and other membrane structures of *C. albicans* and *C. glabrata* cells (Gupta et al., 2021; Leite et al., 2015; Singh et al., 2016, 2018). Geraniol affects cell wall function and alters mitochondrial function, reducing plasma membrane activity (ATPase) and ergosterol levels in the membrane of *C. albicans* (Singh et al., 2016, 2018). Similarly, geraniol disrupts *C. glabrata* by disturbing β-glucan and chitin in the cell wall and decreasing ergosterol content in the membrane. It also reduces hydrolytic enzyme activity in the extracellular matrix, alters mitochondria activity, disrupts membrane permeability, and inhibits ATP-binding cassette (ABC) drug efflux pumps (Gupta et al., 2021). The cell wall rupture, induced by geraniol, may facilitate the entry of nystatin into the cell membrane of *Candida*. Additionally, the cell membrane dysfunction caused by geraniol may compromise membrane integrity and the fluidity of yeasts. The blocking of ABC drug efflux pumps by geraniol diminishes the ability of *Candida* to escape from the action of nystatin. These explanations may support the synergistic antifungal activity of nystatin against *Candida* in the presence of geraniol. Therefore, disruption of cell membrane ergosterol and permeability, alteration of the cell wall structure, as well as the inhibition of ABC drug efflux pumps of *Candida* need to be further investigated to confirm the antifungal mechanisms involved in the synergistic effect of the nystatin and geraniol combination.

Table 2. Fractional inhibitory concentration index (FICI) of nystatin/geraniol combinations using the checkerboard assay

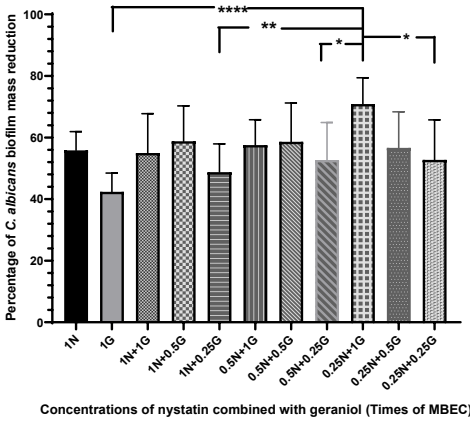
Species	Nystatin			Geraniol			FICI	outcome
	MIC (μg/mL)	MIC in Combination (μg/mL)	FIC	MIC (μg/mL)	MIC in combination (μg/mL)	FIC		
C. albicans	9.65	1	0.10	127	1–8	0.008–0.063	0.130	Synergy
C. glabrata	14.2	1.78	0.125	6115	764	0.125	0.25	Synergy

3.3 *In vitro* antifungal effect of nystatin combined with geraniol against *Candida* biofilm performed on acrylic resin

The synergistic efficacy of nystatin/geraniol combinations against *C. albicans* biofilms on acrylic resin is depicted in Figure 2(A). A significant increase in antifungal activity against *C. albicans* biofilm on the acrylic resin was demonstrated when geraniol at the concentration of 1 MBEC (330 μg/mL) was combined with nystatin at 0.25 MBEC (13.75 μg/mL), compared to geraniol alone at 1 MBEC (p<0.0001). The percentage reduction in *C. albicans* biofilm mass when combining geraniol at 1 MBEC with nystatin at 0.25 MBEC (70.89±8.51; mean±SD) was significantly higher than that of geraniol alone (42.35±6.11). However, the efficacy of geraniol at sub-inhibitory concentrations (0.5 MBEC, 0.25 MBEC), combined with either 1 MBEC or sub-inhibitory concentrations of nystatin, was similar to geraniol or nystatin alone at 1 MBEC. Surprisingly, combinations of geraniol at 1 MBEC with nystatin at 1 MBEC could exhibit an indifferent reduction in the biomass of *C. albicans* biofilm on acrylic resin compared to geraniol or nystatin alone at 1 MBEC (Figure 2A).

Figure 2(B) illustrates the efficacy of nystatin/geraniol combinations against *C. glabrata* biofilms on acrylic resin. The addition of geraniol increased the efficacy of nystatin at a sub-inhibitory concentration to levels comparable with nystatin at 1 MBEC. The efficacy of geraniol at 1 MBEC (45,368 μg/mL), 0.5 MBEC (22,684 μg/mL), or 0.25 MBEC (11,342 μg/mL) combined with nystatin at 0.5 MBEC (65.5 μg/mL), 0.25 MBEC (32.75 μg/mL) against *C. glabrata* biofilm on acrylic resin was similar to geraniol or nystatin alone at 1 MBEC. Likewise, the percentage reduction in *C. glabrata* biofilm mass when combining geraniol at 1 MBEC with nystatin at 1 MBEC (81.30±8.33) was comparable to nystatin alone at 1 MBEC (83.19±1.84) or geraniol alone at 1 MBEC (83.17±7.04).

(A)



(B)

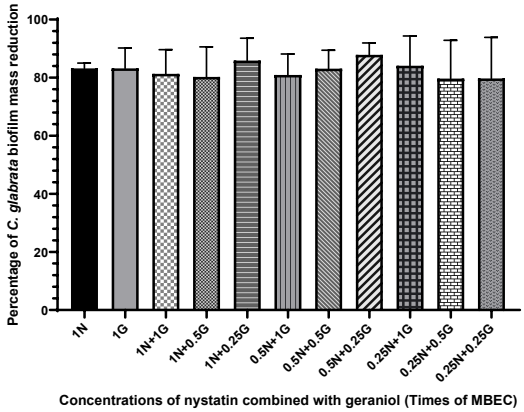


Figure 2. Assessment of (A) *C. albicans* and (B) *C. glabrata* biofilms on acrylic resin following nystatin combined with geraniol treatment at sub-inhibitory concentrations (times of MBEC)
Note: Bars represent means ± SD from three experiments. * p<0.05, ** p<0.001, **** p<0.0001 among experiment groups using one-way ANOVA. 1N abbreviated from 1 MBEC of nystatin, 1G abbreviated from 1 MBEC of geraniol.

Our observations indicate that the minimum efficient dose of nystatin against *Candida* biofilm can be depleted in the presence of geraniol. Geraniol exhibits fungicidal action against strains of *C. albicans* and *C. glabrata* through multiple cellular mechanisms. Geraniol alters the morphology of cell walls and membrane structure, eradicates pseudohyphae and chlamydoconidia formations, diminishes ergosterol biosynthesis, and impairs biofilm development of *C. albicans* (Leite et al., 2015; Singh et al., 2016). A reduction in ergosterol biosynthesis by geraniol may reduce the efficacy levels of nystatin required for antifungal actions. Thus, nystatin at 1 MBEC may be excessive for anti-biofilm activity when combined with geraniol, resulting in equivalent efficacy of the nystatin/geraniol combination at 1 MEBC compared to monotherapy. However, the indifferent efficacy of nystatin/geraniol combinations at sub-inhibitory concentrations against *Candida* biofilm confirms that the required concentration of nystatin for *Candida* biofilm inhibition could be reduced in the presence of geraniol.

Fabrication techniques affect the physical properties and *Candida* adhesion on the denture base. A recent study revealed greater numbers of attached *C. albicans* on a-3D printed denture base compared with heat-polymerized resin

acrylic, whereas a computerized milling technique significantly reduced the adhesion of *C. albicans* on the denture base (Meirowitz et al., 2021). Therefore, additional studies using diverse types of denture materials, such as 3D printing or CAD/CAM milling, are needed to better understand the effects of the nystatin/geraniol combination. Moreover, their effects on acrylic resin properties, such as surface roughness, hardness, and color change, need to be further investigated.

Although the evaluation biofilm quantification can be explored using several techniques, a simple CV assay was performed in this study because the dye density is directly correlated with the biofilm size and is stated to be the appropriate method for biofilm quantification (Jogalekar et al., 2014). However, since both alive and dead cells, as well as the extracellular matrix, are dyed by CV, this technique provides no information on the actual number of living cells. Therefore, the cell viability and microstructure of *Candida* biofilm in response to the nystatin/geraniol combination should be further evaluated. The anti-biofilm activity of the nystatin-geraniol combination against other clinical *Candida* isolates should also be further evaluated.

4. CONCLUSION

This research demonstrates that geraniol exhibits synergistic behavior with nystatin against *C. albicans* and *C. glabrata* planktonic cells. The efficacy of nystatin combined with geraniol at sub-inhibitory concentrations in reducing Candida biofilm mass on heat-polymerized acrylic resin is comparable to that of nystatin monotherapy at the MBEC. These findings suggest that the minimum efficient concentration of nystatin could be decreased when geraniol is combined. The synergism activity of combinations against *Candida* biofilm on acrylic resin suggests that the nystatin/geraniol combination could be a possible therapeutic strategy for the treatment of *Candida*-associated DS. A limitation of this study was the assessment of *C. albicans* and *C. glabrata* biofilm biomass on heat-polymerized acrylic resin. Therefore, further investigations involving a larger number of *Candida* species and the isolation of diverse denture material types should be performed to confirm our findings.

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