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Cell Viability of *Candida albicans* Against the Antifungal Activity of Thymol

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Candida albicans is a commensal fungus, but circumstantially it may cause superficial infections of the mucous membranes, such as denture stomatitis, when a biofilm is formed on the surface of dental prostheses. This study evaluated the cell viability of *C. albicans* biofilms against the antifungal activity of thymol when compared with miconazole, by the fluorescence imaging using SYTO 9 and propidium iodide dyes, and counting of colony forming units. *C. albicans* standard strains (ATCC 11006) were used. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of drugs were determined by broth microdilution tests and the inoculum was standardized to match 0.5 on the McFarland scale (10^6 cfu/mL). Biofilms were grown on the surface of acrylic resin disks in parallel flow chambers from Sabouraud broth supplemented with 10% dextrose. For counting of colony forming units, the fungal solution was sequentially diluted and plated in Sabouraud dextrose agar. Data were analyzed using two-way ANOVA and Tukey's test (α=5%). Biofilms treated with thymol and miconazole presented low numbers of viable cells at the evaluated exposure times. There was statistically significant difference ($p<0.05$) when compared with control, and the mean value of the exposure times between miconazole and thymol did not differ significantly ($p>0.05$). In conclusion, both drugs have similar efficiency as antifungal agents against biofilms of *C. albicans* formed on acrylic surfaces.

Key Words: *Candida albicans*, thymol, microbial viability.

Introduction

Candida is a fungus that harmlessly inhabits niches of various parts of the human body, including the oral cavity, but may occasionally cause superficial infections or contribute to the worsening of systemic diseases (1). The formation of *Candida albicans* biofilms has been described as a process that begins with the adhesion to a substrate, followed by proliferation of yeast cells on the entire surface of this substrate, early development of hyphae and maturation (2).

Superficial infections of *Candida* associated with certain prosthetic devices are frequently found, being most common the denture stomatitis occurring in the oral cavity. In this condition, a biofilm of *Candida* containing a large number of bacteria, particularly streptococci and fungal yeasts, may be formed on the surface of the dental prostheses (3). One of the major consequences of fungal growth in biofilms is the increasing resistance to antimicrobial therapy, which is the reason why infections associated with biofilm formation are often refractory to conventional antibiotic therapy (4,5).

Thymol is a phenol monoterpene, being a major component in several plant species as *Thymus*, particularly *T. vulgaris,* and it is acknowledged for having a number of pharmacological properties, including antimicrobial activity against oral bacteria and also demonstrating some antifungal activity, which may involve effects on the cell membrane (6,7).

The anti-*Candida* activity of various constituents of essential oils, such as thymol, eugenol and carvacrol is well recognized and a number of pharmacological properties are credited to thymol including antibacterial and antifungal effects. The main therapeutic application of thymol is in oral preparations to suppress bacterial and fungal activity, and it is also employed as a preservative and an antioxidant (8). For Omran and Esmailzadeh (9), the essential oil obtained from *Thymus vulgaris* L. can be used in the control and treatment of candidosis. The authors evaluated the anti-*Candida* activity of thyme (*Thymus vulgaris* L.), pennyroyal (*Mentha pulegium* L.) and lemon (*Citrus aurantifolia* Christm.) on different species of *Candida*, including *C. albicans, C. glabrata* and *C. krusei*, and found that thyme essential oil had the highest inhibitory effect against various *Candida* species.

Thymol has also been successfully used for *in vitro* studies against pathogenic fungi, including *Aspergillus* and *C. albicans* (10), and may also be tested in fungal suppression on acrylic resin surfaces that mimic the inner surface of dentures, to be assessed as a substance for cleaning prosthetic devices.

Considering the potential antifungal effect of thymol, this study aimed at evaluating the cell viability of *C. albicans* biofilms grown on the surface of acrylic resin discs against this substance compared with miconazole, by

the fluorescence imaging and counting of colony forming units (CFU).

Material and Methods

Miconazole and thymol antifungal agents (Sigma-Aldrich®, São Paulo, SP, Brazil) were prepared in 10% dimethyl sulfoxide (DMSO) for the tests. To evaluate the antifungal activity, Sabouraud dextrose agar and Sabouraud dextrose broth media were solubilized with deionized water and autoclaved. Standard strains from the American Type Culture Collection of *C. albicans* (ATCC 11006) were used.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC and MFC of the antifungal agents (miconazole and thymol) were determined by microdilution assays in microplates. The inoculum was standardized by absorbance reading to match the 0.5 McFarland scale (10^6 cfu/mL). The antifungal agents were prepared so that different concentrations ranging from 15 µg/mL to 400 µg/mL were obtained. After filling all wells, the microplates were sealed with plastic paraffin film and incubated at 37 °C for 48 h. After the incubation period, 35 µL resazurin (0.01%, 10 mg diluted in 80 mL) were added to each well and mixed with the well contents. The plates were sealed with plastic paraffin film and re-incubated for further 1 h before visual reading. Holes with pink-violet or pink color indicated fungal activity with chemical reaction of oxidation-reduction of resazurin into resofurin, therefore the last blue well (left to right) was the one with the MIC. This step was performed in triplicate. Subsequently, 100 µL were removed from the concentration considered as inhibitory and those immediately more concentrated for subculture on Sabouraud dextrose agar plates. After 48 h of incubation at 37 °C, the lowest concentration capable of preventing visible growth of subculture was regarded as the MFC.

Preparation of Acrylic Resin Discs

Chemically activated acrylic resin discs (VIPI Flash®; VIPI, São Paulo, SP, Brazil) were fabricated to reproduce the material commonly used in the manufacturing of the palatal baseplate of a dental prosthesis. For this purpose, condensation silicone molds (Optosil/Xantopren®, Heraeus, São Paulo, SP, Brazil) measuring 12.7 mm diameter and 3.81 mm thickness were used and were then filled with acrylic resin. Monomer and polymer were incorporated in the 3:1 ratio forming a liquid, but consistent mixture that was inserted into the silicone mold with a dental spatula. All surfaces of the samples were polished with abrasive sandpaper and rubber polishers. Twenty samples were fabricated and divided into two groups according to the tested drug. Cultivation was performed in duplicate.

Fungal Biofilm Formation

Pure cultures of *C. albicans* biofilms were grown in Sabouraud dextrose broth supplemented with 10% dextrose in parallel flow chambers (FC-274 PC; BioSurface Technologies Corporation, Bozeman, MT, USA) on the surface of the acrylic resin discs. Before the fungal biofilm formation, acrylic resin disks were immersed in 1.5 mL of sterile and centrifuged human saliva for film formation on the disc surface, which were incubated in this saliva for 4 h at room temperature in a shaker.

The biofilm formation system is composed by parallel chambers where the discs are inserted. These chambers are connected to a peristaltic pump by silicone tubes that reach the containers. As the system is in operation, a continuous flow of medium and fungi passes in contact with the discs and, at the end, the material reaches a discard container.

The standard fungal solution was added to the medium at a rate of 3 mL fungal solution to 500 mL of medium. The system was kept at 37 °C and the experiment lasted 12 h under constant agitation.

After this period, the flow was discontinued and resin discs were removed from the cell and placed in cryogenic tubes containing the minimum fungicidal concentration of each antifungal agent. The MIC, characterized by the absence of cellular activity, corresponded to 350.0 and 75.15 µg/mL for thymol and miconazole, respectively. After subculture, the MFC was established for both drugs, with values of 400.0 and 150.0 µg/mL for thymol and miconazole, respectively.

Three exposure times (5, 15 and 30 min) for thymol and miconazole were evaluated. After exposure, the discs were removed and placed into Falcon tubes with 3 mL of saline. The biofilm formed on the discs was then dispersed in a sonicator (Ultrasonic Cleaner USC 750; Unique Group, São Paulo, SP, Brazil) and homogenized by vortexing (Vortex Mixer; Vision Scientific Co. Ltd, Seoul, South Korea). The fungal solution was transferred to pre-weighed Eppendorf tubes, which were centrifuged in a microcentrifuge and the supernatant was carefully discarded with pipette. The Eppendorf tubes were weighed to obtain the biofilm wet weight by adding saline at a rate of 1 mL per 35 mg weight.

Cell Quantification

For cell quantification by fluorescence imaging, a calibration curve was constructed initially, and was used as a reference for the readings of the examined wells. For this purpose, a combination of living (maintained in saline) and dead fungi (maintained in 70% isopropyl alcohol) was made and the curve had the following concentrations: 0%, 20%, 50%, 80% and 100% viable fungi and the

complement reverse (100%, 80%, 50%, 20% and 0%) of dead fungi. Mixtures of 30 µL of living and dead fungus were transferred to a 96-well black plate suitable for fluorescence. Likewise, 30 µL of each fungal solution to be evaluated (after exposure periods of 5, 15 and 30 min) were placed into the fluorescence plate wells.

Cell viability was quantified by fluorescence imaging using a mixture of dyes (SYTO 9 and propidium iodide) from the cell viability kit LIVE/DEAD® *Bac*Light™ (Molecular Probes; Invitrogen, Carlsbad, CA, USA). After exposure of each well to the reagent mixture, was made the feasibility analysis on the fluorescence microplate reader (FluoStar OPTIMA; BMG LabTech, Germany).

For CFU/mL counting, serial dilutions were made and then an aliquot of 50 µL of each dilution was seeded in sterile Petri dishes, containing Sabouraud dextrose agar supplemented with 10% dextrose, incubated for 48 h.

Data Analysis

Data were analyzed statistically by two-way ANOVA and Tukey's test at 5% significance level. 'Antifungal agent' and 'exposure time' were considered as factors.

Results

The results concerning the cell quantification by fluorescence are shown in Table 1. The numbers of CFU per milliliter of fungal solution are shown in Table 2. Saline was used as the control group.

The average value of the three exposure times comparing miconazole and thymol did not differ statistically by the t-test at 5% probability ($p>0.05$) demonstrating that both drugs have similar efficiency.

Discussion

It is increasingly clear that infections caused by *C. albicans* biofilms are becoming a serious clinical problem. Since these infections are facing a shortage of active antifungal molecules, new treatments are needed and must be continuously investigated. For Baillie and Douglas (11) studying microbial biofilms, particularly fungal biofilms, should be of great interest because, unlike the typical development of a bacterial biofilm, *C. albicans* may change from a yeast form into a filamentous form, which provides this fungus unique growth characteristics.

According to Ramage et al. (12), most information on *C. albicans* biofilm development and architecture comes from *in vitro* experiments in which different biofilm models are implemented and, although some of these models utilize static incubation conditions, the use of flow-through conditions, as the one used on this research, are important for trying to mimic the environment encountered within the host. A limitation of the current study was the inability to test the action of drugs on multispecies biofilms, which would reflect more accurately the conditions found in the oral cavity. Likewise, adding artificial saliva to the culture medium would contribute to simulate events that occur in the oral environment, such as increasing initial adhesion and colonization.

It is appropriate to emphasize that this research used the MFC of drugs to evaluate the inhibition of fungal biofilms, whose architecture and cellular interactions are responsible for the increasing resistance of these microorganisms to antifungal agents. Silva et al. (13) claim that it is expected that concentrations higher than the MIC could interfere on biofilms at metabolic levels, but it is not fully understood whether antifungal agents at concentrations higher than MIC could actually affect the biofilm architecture.

According to Jin et al. (14), many coloring substances may be used to allow quantification of microbial biofilms, such as the FUN-1 dye, widely used in the investigation of antifungal resistance and cell viability of *C.* biofilms. However, these authors pointed out that the FUN-1 could lead to overestimation of living cells, especially when cell density is high. The combination of SYTO-9 and propidium iodide dyes used in he present study uniformly stains tissues in green (live) or red (dead) regardless of cell morphology, since they are based on membrane integrity, being therefore better suited

Table 1. Percentage of mean values of cell viability and standard deviation of controls and drugs tested depending on the incubation time

Antifungal agent	Control (saline)	Exposure time (min)		
		5	15	30
Thymol	90.91 ± 4.77 aA	28.01 ± 3.72 bB	17.96 ± 1.33 bC	31.24 ± 8.91 bB
Miconazole	96.72 ± 3.77 aA	24.42 ± 2.74 bB	25.22 ± 1.95 bB	29.31 ± 3.89 bB

Means followed by the same lowercase letter in the rows and the same capital letter in the columns do not differ significantly by the Tukey's test and t-test at 5% significance level.

Table 2. Colony forming units (CFU) counts per milliliter of fungal solution (CFU/mL x 10^{-1})

Antifungal agent	Control (saline)	Exposure time (min)		
		5	15	30
Thymol	8250 x 10^{-1}	70 x 10^{-1}	45 x 10^{-1}	80 x 10^{-1}
Miconazole	11250 x 10^{-1}	70.9 x 10^{-1}	62.5 x 10^{-1}	70 x 10^{-1}

for viability tests in fungal biofilms.

Investigating the capacity of thymol to interfere with the hyphal formation of *C. albicans* and its viability, a study (15) demonstrated that in the absence of thymol, about 93% of fungal cells were found viable, while after 6 h of incubation with 1x MIC, ½ x MIC and ¼ x MIC there were 54%, 29% and 23% damaged cells, respectively. The results of the present study corroborate the aforementioned study when, in absence of antifungal agent, approximately 90% of *C. albicans* cells were viable; however, the biofilms had their viable mass reduced in the presence of CFM thymol by an even greater proportion, exhibiting 28%, 18% and 31% living cells after 5, 15 and 30 min exposures, respectively (Table 1). In this research, the average obtained after 15 min of activity showed that thymol exhibited a greater reduction in the number of viable cells compared to control, and statistically similar reduction with 5 and 30 min exposures. These data indicate that *C. albicans* may have a resistance mechanism to the drug after periods of time over 15 min. In addition, it cannot be ruled out that a rapid degradation of the agent might occur, resulting in decreased efficacy of the antifungal drug after 15 min. It is not clear if both factors are operating simultaneously and have any cumulative effect. Nevertheless, both situations are a matter of concern for any antifungal drug since good substantivity is a key feature when delivering a drug in the oral cavity.

Supporting the antifungal effect of thymol on mature biofilms of *C. albicans*, Braga et al. (16) observed that after 6 h of incubation with thymol, biofilms of *C. albicans* showed reduction of 45.1% in their metabolic activity; and the same occurred after 12 h, but with 68% inhibition, and after 24 h of incubation, the percentage inhibition was 88.3%. These results are consistent with the aforementioned authors; however, in the current research the antifungal activity of thymol was demonstrated in much shorter exposures (5, 15 and 30 min) (Table 1).

In agreement with the present study, Ahmad et al. (17) investigated the efficacy of thymol in sensitive and resistant clinical isolates of *C. albicans* and stated that the antifungal activity occurred rapidly and that propidium iodide penetrated more than 95% of sensitive fungal cells, indicating structural rupture of cell membrane. In the present study, the permeation of propidium iodide occurred especially after a short exposure period, indicating, by analogy, that the drug's mechanism of action involves a primary lesion in the cell membrane resulting from its solubilization. Another study (18) tested the *in vitro* antibiofilm activity of 10 terpenes against three *Candida* species and showed that thymol, carvacrol and geraniol were the most effective in reducing the development of *C. albicans* in both planktonic and biofilm forms, since these compounds induced the inhibition of about 80% biofilm fungal mass. After 15 min of exposure, thymol was also able to inhibit 82% of fungal mass of biofilms of *C. albicans* in the current research, supporting the potential interest in the use of terpenes as antibiofilm agents.

Sánchez et al. (6) and Pina-Vaz et al. (19) argue that thymol interferes with the production of viable forms of *C. albicans*, i.e., shows antimicrobial effect. Those authors support the idea that this antifungal agent is able to interfere negatively with the ergosterol biosynthesis and can affect the structure and electrostatic surface of cell membrane, increasing its fluidity and changing its permeability.

Lamfon et al. (20) conducted a study to estimate the *in vitro* susceptibility of biofilms of *C. albicans* against miconazole on acrylic resin discs, as used in this study. According to those authors, exposure to miconazole for 24 h resulted in a 99.2% reduction of viability. In the present study, biofilms of *C. albicans* exposed to the CFM miconazole also showed large reduction in the number of viable cells (approximately 75%); however, it was found that the onset of drug action has occurred within the first 5 min of exposure, with no difference among the tested times (15 and 30 min). This result demonstrates that periods longer than that do not represent any increase in its role as a fungicidal agent (Table 1).

Investigating the antifungal activity of miconazole alone and in combination with berberine (an alkaloid found in several plant species) on biofilms of *C. albicans* formed *in vitro* in parallel flow chambers, Wei Xu and Wu (21) found that when the drugs were tested alone no significant inhibition was observed in the biofilm formation compared with the control. These results are consistent with those of another study in which miconazole was able to promote rapid inhibition of *C. albicans,* with approximately 25% cell viability after exposure, with significant difference compared to the control. Currently, it is known that the fungicidal effect of miconazole occurs by the action in the biosynthesis of ergosterol, which is important in cell membrane integrity (17) and induction of oxygen reactive species (22).

In conclusion, this study demonstrated by fluorescence imaging that thymol and miconazole are effective to reduce cell viability of *C. albicans* biofilms grown on acrylic surfaces. Thus, prospects of additional studies include the possibility of micro- or nanoencapsulation of these antifungal agents, which could prevent their oxidation and reduce their volatility. Moreover, further research to investigate the incorporation of these drugs in denture cleansing products must be considered.

Resumo

Candida albicans é um fungo comensal que circunstancialmente pode

causar infecções superficiais das mucosas, como a estomatite protética, na qual o biofilme se forma sobre a superfície das próteses dentárias. Este estudo avaliou a viabilidade celular de biofilmes de *C. albicans* frente à ação antifúngica do timol em comparação com o miconazol por meio da técnica de fluorescência, empregando os corantes SYTO 9 e iodeto de propídio, e contagem das unidades formadoras de colônia. Utilizaram-se cepas de *C. albicans* (ATCC 11006). As concentrações inibitórias mínimas (CIM) e fungicidas mínimas (CFM) das drogas foram determinadas com testes de microdiluição em caldo, sendo o inóculo padronizado para corresponder a 0,5 da escala de McFarland (106 UFC/mL). Os biofilmes foram cultivados sobre a superfície de discos de resina acrílica, em células paralelas de fluxo, a partir de caldo Sabouraud suplementado com dextrose 10%. Para a contagem das unidades formadoras de colônia, as soluções fúngicas foram sequencialmente diluídas e semeadas em agar Sabouraud dextrose Os dados foram analisados por meio de estatística ANOVA A dois fatores e teste de Tukey ($\alpha = 5\%$). Biofilmes tratados com o timol e miconazol apresentaram baixos números percentuais de células viáveis nos tempos de exposição avaliados. Houve diferença estatística significante ($p<0.05$) em comparação com o controle, e o valor médio dos tempos de exposição entre miconazol e timol não diferiu estatisticamente ($p>0.05$). Concluindo, ambas as drogas possuem similar eficiência como agentes antifúngicos contra a viabilidade de biofilmes de *C. albicans* formados em superfícies de resinas acrílicas.

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