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Original Article

Antifungal Activity of Eugenol and its Association with Nystatin on *Candida albicans*

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Abstract

Objective: To evaluate the anti-*Candida* effect of eugenol and its antimicrobial interaction with nystatin. **Material and Methods:** The antimicrobial potential was assessed by microdilution technique (M27A3 reference method), by determining the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) against *C. albicans* (ATCC 90028). The possible action of eugenol on the fungal cell wall was evaluated with the assistance of the osmotic protector sorbitol (0.8 M). The antimicrobial interaction with nystatin was assessed through the checkerboard method. All tests were performed in triplicate. **Results:** All groups showed reductions in PI and GBI values and improvements in oral health knowledge, but IG1 and IG2 showed statistically significant differences in these variables compared to CG. **Conclusion:** The eugenol has antifungal activity against *C. albicans* and its mechanism of action is probably not related to damage to the fungal cell wall. Association between eugenol and nystatin was not found to be an advantageous possibility for growth inhibition of *C. albican.*

Keywords: Eugenol; Nystatin; Drug Synergism; *Candida albicans.*

Introduction

Candidal species are opportunistic microorganisms involved in the most prevalent fungal infection in humans, known as candidiasis [1,2]. Among the various *Candida* species, *C. albicans* is considered the most frequent and pathogenic strain, being able to colonize and invade many mucosal surfaces [1,3,4]. The virulence of *C. albicans* is linked to the ability of such microorganism in forming biofilms, performing morphogenesis to a filamentous form, invading soft tissues, and secreting phospholipases and proteinases [1,3].

Many strategies are known to fight candidal infections, including the use of antifungals with systemic or topic effect, showing a fungistatic or fungicidal action [5]. Antimycotic agents such as azoles, polyenes and antimetabolites agents (i.e. nystatin, amphotericin b, fluconazole, miconazole, itraconazole, and 5-Fluorocytosine) are recognized as the main choice-drugs for candidiasis treatment [5,6]. However, with regards to oral candidiasis, a local antimicrobial agent is preferred, in order to produce better efficacy due to higher drug penetration and retention; also avoiding systemic side effects [7].

Resistance to some of widely-used antifungals has been recently reported in the literature [8,9]. Considering this, new strategies for fungal infection control are necessary to improve the efficacy of such treatments [10]. Based on that, recent studies had given evidence to antimicrobial compounds extracted from plants, in a form of essential oils, crude extracts, and molecules, such as flavonoids and terpenes [8,11,12]. Recent studies have also focused on the association between natural products and conventional pharmacological agents in order to achieve better efficacy of treatments [12-15].

Regarding the clinical use of natural compounds extracted from plants, eugenol is recognized as a therapeutic agent widely handled in Dentistry, also presenting antimicrobial, anti-septic, analgesic, and anti-inflammatory properties [16]. Given the biological properties of such compound, it is hypothesized that association with a synthetic antifungal would improve efficacy of both. Therefore, the aim of this study is to evaluate the anti-*Candida* effect of eugenol and its antimicrobial interaction with nystatin.

Material and Methods

Experimental Design

An *in vitro* study was performed to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of eugenol and nystatin against *C. albicans*. In addition, the influence of eugenol on the integrity of cell wall was determined by the sorbitol test. Finally, a checkerboard method was used to determine the interaction between eugenol and nystatin.

Inoculum standardization

C. albicans (ATCC 90028) reference strain was used in all experiments. Lyophilized stocks were acquired from the National Institute of Quality in Health (INCQS, Fundação Oswaldo Cruz,

Rio de Janeiro, RJ, Brazil). Reactivation procedures involved suspension of microorganisms in Sabouraud Dextrose Broth (SDB, HiMedia Laboratories, Mumbai, India) and cultivation in Sabouraud Dextrose Agar plates (SDA, HiMedia Laboratories, Mumbai, India). Inoculum was standardized by suspension of three to five *C. albicans* colonies in 5 mL of saline. Resulted suspension was then compared to the point 0.5 from the McFarland scale, also showing an absorbance of 0.1 at 600 nm, which corresponds to approximately 1×10^6 colony forming units per milliliter (CFU/mL). For all assays, the standardized suspension of *C. albicans* was diluted 1000× Sabouraud Dextrose Broth (HiMedia Laboratories, Mumbai, India), in order to generate a working suspension with 1×10^3 CFU/mL, following the Clinical and Laboratory Standards Institute (CLSI) recommendation [17].

Testing Substances and Preparation

Eugenol was obtained in its commercial formula used in Dentistry (Eugenol USP, 99.9% purity, Maquira Dental Products, Maringa, PR, Brazil). Eugenol working solutions were prepared at 10,000 µg/mL by diluting 100 µL of eugenol in 9.9 mL of SDB with 2% emulsifier agent (v/v) (Tween 80, Sigma-Aldrich, St. Louis, MO, USA). Nystatin (Sigma-Aldrich, St. Louis, MO, USA) working solutions were prepared at 800 µg/mL by dilution in SDB.

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

MIC was determined by the micro-dilution method, following the M27A3 guideline of the CLSI [17]. Initially, 100 µL of SDB culture medium was distributed along all wells from a 96-well microtiter plate. After that, 100 µL of eugenol (10,000 µg/mL) and nystatin (800 µg/mL) working solutions were added to the first line of the microtiter plate, followed by a two-fold serial dilution along all subsequent wells. Concentrations of eugenol ranged from 2,500 µg/mL to 19.5 µg/mL; whilst nystatin concentrations ranged from 200 µg/mL to 1.56 µg/mL. Finally, 100 µl of *C. albicans* (1×10^3 CFU/mL) inoculum was added to each test well [17]. Positive and negative controls consisted of wells without antimicrobials and without microorganisms, respectively. Plates were then incubated at 37°C, for 24 h.

The MIC was defined as the lowest concentration that inhibited at least 80% of microbial growth, initially identified by visual method. Microbial growth was perceptible by culture medium turbidity and/or by cellular precipitation. In order to confirm the presence of microbial viability in non-inhibitory concentrations, an aliquot of 50 µL of 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) was added to each test well. After incubation (37°C, 24 h), viable microorganisms resulted in a color change for non-inhibitory wells [18].

After that, MFC was assessed by sub-cultivation of 20 µL aliquots from tested wells on SDA plates. MFC was defined as the lowest concentration that yielded no cultured microorganisms.

Eugenol Effect on Fungal Cell Wall

The possible mechanism by which eugenol would affect *C. albicans* viability was assessed by sorbitol test [19]. In this evaluation, an osmotic protector (Sorbitol 0.8 M) is used during antimicrobial assays in order to check differences on MIC values, in the presence or absence of sorbitol. Therefore, MIC evaluation was replicated using culture medium supplemented with Sorbitol 0.8 M. Briefly, if the MIC of tested substance does not change, it is said that the mechanism of action is not involved with osmotic pressure and cell wall degradation; however, if the MIC value increases in the presence of sorbitol, it is said that tested substance interferes with osmotic pressure and cell wall degradation. Caspofungin was used as a positive control in this assay at an initial concentration of 5 μg/mL (caspofungin diacetate – Sigma-Aldrich, São Paulo, SP, Brazil).

Antimicrobial Interactions: Checkerboard Method

The checkerboard method was used to evaluate the interaction between eugenol and nystatin in producing an antimicrobial effect. By means of checkerboard, a fractionated inhibitory concentration index (FICI) was determined and the interaction between substances was interpreted as synergistic, additive, indifferent or antagonist.

Briefly, 100 μL of culture medium containing 1×10^3 CFU/mL of *C. albicans* was added in each well of a 96-well microtiter plate. Each of the tested solutions was evaluated at seven different concentrations, according to previously determined MIC. Therefore, working solutions at concentrations MIC÷8, MIC÷4, MIC÷2, MIC, MIC×2, MIC×4 and MIC×8 were previously prepared. After that, 50 μL of eugenol solutions were added horizontally, whilst 50 μL of nystatin solutions were added vertically in seven subsequent wells. According to that, each working concentration of eugenol was combined with each concentration of nystatin, and the opposite is also true. After incubation at 37°C, for 24 h, the inhibitory concentrations of each substance were assessed. The absence of cellular growth and viability was determined as previously described [20,21].

Within each substance, the lowest inhibitory concentration was considered as minimum inhibitory concentration in combination (MICC). Interpretation of data was based on FICI calculation, which considered both the MIC and MICC of each substance, using the following formula:

$$FICI = \left(\frac{MICC}{MIC}eugenol\right) + \left(\frac{MICC}{MIC}nystatin\right)$$

Resulting values were interpreted as the following: synergistic (FICI < 0.5), additive (0.5 < FICI < 1.0), indifferent (1.0 < FICI < 4.0), or antagonist (FICI > 4.0) [21,22].

Results

Both eugenol and nystatin, at evaluated concentrations, inhibited the cellular growth of *C. albicans* (ATCC 90028) in the present study. MIC and MFC values of both substances under the conditions studied are shown in Table 1. The presence of an osmotic protector did not interfere with the antimicrobial effect of eugenol, as also shown in Table 1.

Table 1. MIC and MFC, in µg/mL, of eugenol and nystatin against *C. albicans* (ATCC 900280). MIC and MFC were evaluated in the presence (+) or absence (−) of an osmotic protector (sorbitol).

Substance	MIC		MFC	
	+ sorbitol	− sorbitol	+ sorbitol	− sorbitol
Eugenol	625	625	625	625
Nystatin	25	*	25	*
Caspofungin	< 0.0003	> 0.0045	*	*

* Not evaluated.

Antimicrobial interaction of eugenol and nystatin was assessed by checkerboard method, by which a MICC was determined for each substance. The lowest inhibitory concentration of eugenol persisted at 625 µg/mL ($MICC_{eugenol}$), whilst lowest inhibitory concentration of nystatin in the presence of eugenol was detected at 3.125 µg/mL ($MICC_{nystatin}$), as shown in Table 2. Antimicrobial interactions between eugenol and nystatin were considered "indifferent".

Table 2. MICC, in µg/mL, of eugenol and nystatin against *C. albicans* (ATCC 900280), assessed by the checkerboard method. Antimicrobial interaction was determined by FICI calculation and interpretation.

Substance	MICC	$\frac{MICC}{MIC}$	FICI	Interaction's Interpretation
Eugenol	625	1.0	1.125	Indifferent
Nystatin	3.125	0.125		

Discussion

Natural products consist a very rich source of new bioactive compounds, which deserve investigation to find out or confirm its popular use [10, 23,24]. The eugenol has been widely used in Dentistry for many years, due to its antimicrobial, analgesic, anti-oxidant, and anti-inflammatory properties [25]. Eugenol is more frequently used in the composition of some dental materials; usually in association with zinc oxide, in order to improve the biological effects. Considering that the microorganism *C. albicans* can habit many sites of the human body, causing significant health problems, authors were interested in detecting the antimicrobial effect of eugenol and its association with nystatin. In the present study, authors demonstrated that eugenol has a significant inhibitory effect on *C. albicans*. In addition, it is shown that association with nystatin is not antagonistic, although it is not synergistic either.

It is well known that *Candida* species can colonize both hard and soft tissues [4]. Therefore, with regards to oral environment, *Candida* can participate not only in the pathogenesis of oral candidiasis [1], but also in endodontic, periodontal and peri-implant infections [26]. Clinically, it is

suggested that eugenol could be used as an adjuvant substance to the control of *C. albicans* infection, especially in endodontic infection, as a component of zinc oxide pastes [27].

Although several studies have investigated the antifungal effect of eugenol [14,28-30], the mechanisms by which eugenol induces *C. albicans* cell death are not completely understood yet. In this study, the osmotic protective effect of sorbitol was not present, since there was no variation in the values of MIC, similarly to a previous investigation [30]. Based on that, it can be stated that the mechanism of action of eugenol against *C. albicans* is probably not related to the degradation of fungal cell wall. Possibly, other mechanisms such as inactivation of ergosterol synthesis and production of free radicals, can be responsible for the antimicrobial effect of eugenol [28,29].

In vitro studies have shown satisfactory results regarding the association between natural products and conventional antimicrobials directed for antifungal therapy [13-15]. In a study conducted in India [15], the authors showed that the addition of eugenol and carvacrol to fluconazole have significantly reduced the concentration of the latter, in inhibition of biofilm growth by *C. albicans*. According to the aforementioned study, these terpenes cause instability in the fungal membrane and intervention in specific signals, facilitating greater fluconazole influx to the intracellular environment and culminating in inhibition of biofilm formation [15].

In the present study, the antimicrobial interaction test resulted in "indifference", regarding the association between eugenol and nystatin against *C. albicans*. This means eugenol does not constitute an advantageous possibility to nystatin in inhibiting fungal growth. Similarly, Indian researchers [14] evaluated the antifungal activity of the association between terpenes (thymol, eugenol, and menthol) and fluconazole through antimicrobial interaction test. The authors found better results by associating the drug with thymol; whilst eugenol showed higher fungicidal activity when used alone [14].

Overall, the absence of additive or synergistic interaction between nystatin and eugenol does not invalidate the development of further pharmacological and microbiological studies involving such substances. Considering an endodontic infection with the presence of *C. albicans*, eugenol and nystatin could be an additive substance for additional antimicrobial effect. In addition, eugenol combination with other conventional anti-fungal and antimicrobials should be considered in future investigations.

Conclusion

The results of present study support that eugenol has antimicrobial potential against *C. albicans*, being its mechanism of action not related with damage to the fungal cell wall. The association between eugenol and nystatin does not constitute a significant pharmacological advantage against *C. albicans*; although those substances did not influence each other.

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