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In Vitro Antifungal Effect of Isoeugenol Against *Penicillium citrinum* Strains

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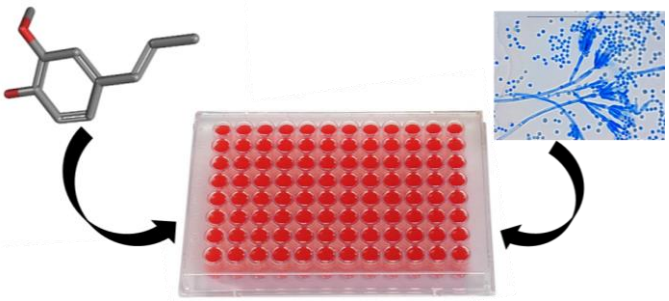
Graphical Abstract



Abstract.

Human mycoses have controversial treatment, since the available antifungal drugs besides favoring the appearance of resistant isolates, can present great toxicity to the human organism. This fact has driven the search for more efficient, safe and natural therapeutic alternatives with the objective of reversing this scenario of resistance presented by pathogenic fungi, for this, new natural and effective alternatives, such as the use of plant extracts, natural compounds and semi-synthetic, have been extensively investigated for the development of new drugs. In view of this scenario, this study aims to evaluate the antifungal activity of isoeugenol in vitro against strains of *Penicillium citrinum*. For this, the broth microdilution technique was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC) of isoeugenol and voriconazole. The concentrations by which the strains were submitted, 1024 µg/mL to 0.5 µg/mL, were obtained by means of serial dilution at a ratio of two, so that in the first row of the plate is the highest concentration and in the last , the lowest concentration. Finally, 10 µL of the fungal inoculum of each isolate was added to the wells, where each strain was placed on a plate

column. MIC was defined as the lowest concentration capable of inhibiting fungal growth visually verified by maintaining the original coloration of the medium. After reading the MIC, aliquots of 20 μL of the supernatant from the wells where complete inhibition of fungal growth in the microdilution plates were observed were plated on Sabouraud dextrose agar plates. Plates were incubated, and MFC was considered the lowest concentration at which growth was less than 3 colonies. The assays were performed in triplicate and the geometric mean was calculated. The CIM of isoeugenol varied between 256 and 32 μg/mL, being the highest MIC value for the LM-21 strain and the lowest value for the LM-02, LM-03, LM-08, LM-155, LM- 157 and LM-161. The MIC range of voriconazole was 0.5 to 256 μg/mL. The MFC of isoeugenol varied between 64-512 μg/mL and for voriconazole it was 2 μg/mL and above 1024 μg/mL. In this way, we can conclude that isoeugenol presented an antifungal effect, which enables it as a potential antifungal drug, requiring complementary tests that clarify the mechanism of action involved in its antimicrobial activity.

Introduction

The indiscriminate use of antifungal compounds led to the emergence of multiresistant microorganisms for the drugs used in the medical routine. Antifungals considered first choice, especially for dermatophyte and nondermatophytic fungi and new opportunistic pathogenic fungi isolated from immunocompromised patients, have become intrinsically resistant, with resistance developed in response to drug exposure during pharmacological treatment [1-4].

This problem is further accentuated, since treatment with antifungal drugs available in addition to favoring the emergence of resistant isolates can present great toxicity to the human organism. Furthermore, the increase in the number of immunocompromised patients, associated with a greater resistance of fungal strains, poses a challenge to the pharmaceutical industry in the search for new therapeutic agents that present greater safety, efficacy and low toxicity [5-6].

Given this scenario, there was a greater search for more efficient, safe and natural therapeutic alternatives. As alternatives, plants are extremely rich sources of molecules with medicinal potential, however, only about 25% of the medicines produced in Brazil are of vegetable origin. However, the academic interest in this topic has been growing, especially due to indications of empirical origin [7-9]. Among the secondary metabolites produced by plants, we have the essential oils. They are volatile compounds extracted from plants, often as part of the natural defense of plants against microorganisms.

There is still a lot to learn about the mode of action of essential oils, but a common and important feature is their hydrophobicity, which allows interaction with the cell membrane, leading to leakage of important ions and other compounds [10].

Isoeugenol is an essential oil of the subgroup of phenylpropanoids, being chemically designated as 2-methoxy-4- (1-propenyl) phenol, it is naturally present in a naturally occurring phenolic constituent of clove oil, monkey orange, basil, the petunia flower [11-12]. It's used in perfumes, soaps, detergents, air purifiers and as a flavoring agent in cosmetics and food products. In addition, isoeugenol has a propenyl moiety and beneficial properties such as antioxidants and anti-inflammatory [13-16].

The aim of the present study was to verify the in vitro antifungal effect of isoeugenol against strains of *Penicillium citrinum*. The objective of the present study was to verify the in vitro antifungal effect of isoeugenol in the literature on the antimicrobial activities of essential oils and of the various biological properties of phytoconstituents.

Materials and Methods

Isoeugenol and voriconazole were purchased from Sigma-Aldrich® (São Paulo, SP, Brazil). The solutions were prepared at the time of the tests, dissolving them first in 5% dimethylsulfoxide (DMSO) and 2% Tween 80 (Sigma-Aldrich®, São Paulo, Brazil), and using sterile distilled water to perform the dilutions employed in the tests.

The fungal strains used were of the species Penicillium citrinum of clinical origin (LM-02, LM-03, LM-04, LM-08, LM-30, LM-145, LM-155, LM-157, LM-161, LM -171 and LM-278) and standard INCQS 40011 (National Institute for Quality Assurance in Health) and performed an inoculum a fungal suspension and adjusted 0.5 scale Mc Farland, which corresponds to approximately 1-5 x 106 CFU / mL [17-19].

For the determination of Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of isoeugenol and voriconazole were carried out using the broth microdilution technique [17-20]. Sterile, capped 96-well plates were used. At each well in the plate, 100 µl of doubly concentrated RPMI-1640 was added. Then 100 µl of the solution of the doubly concentrated products were dispensed into the wells of the first row of the plate. The concentrations by which the strains were submitted, 1024 µg / mL to 0.5 µg / mL, were obtained by means of serial dilution at a ratio of two, so that in the first row of the plate is the highest concentration and in the last , the lowest concentration. Finally, 10 µL of the fungal inoculum of each isolate was added to the wells, where each strain was placed on a plate column.

The plates were then aseptically closed and incubated at the temperature and time suitable for the reading. Subsequently, the results were observed observing the change of the RPMI medium. MIC was defined as the lowest concentration capable of inhibiting fungal growth visually verified by maintaining the original (pink) coloration of the medium. The assays were performed in triplicate.

After reading the CIM, aliquots of 20 µL of the supernatant from the wells where complete inhibition of fungal growth in the microdilution plates were observed were seeded on Sabouraud dextrose agar plates and incubated. CFM was considered the lowest concentration in which growth was less than 3 colonies (approximately 99 to 99.5% of death activity). The assays were performed in triplicate and the geometric mean was calculated [21].

Results and Discussion

According to results obtained from the Minimum Inhibitory Concentration test described in Table 1, we can observe that the MIC of isoeugenol ranged between 256 and 32 μg/mL among the strains, being the highest MIC value for the LM-21 strain and the lowest value for LM-02, LM-03, LM-08, LM-155, LM-157 and LM-161 strains.

Table 1 - Minimum Inhibitory Concentration and Minimum Fungicide Concentration values for isoeugenol and voriconazole against *Penicillium citrinum* strains.

Strains of *Penicillium citrinum*	Isoeugenol (μg/mL)		Voriconazol (μg/mL)		*C1	**C2
	MIC	MFC	MIC	MFC		
INCQS 40011	64	64	256	+	+	-
LM-02	32	64	64	128	+	-
LM-03	32	64	0,5	2	+	-
LM-04	256	512	+	+	+	-
LM-08	32	64	0,5	+	+	-
LM-30	128	256	8	16	+	-
LM-145	64	128	32	32	+	-
LM-155	32	64	64	64	+	-
LM-157	32	64	1	4	+	-
LM-161	32	64	2	8	+	-
LM-171	64	64	2	8	+	-
LM-278	64	128	256	+	+	-

* C1 - Control of microbial growth: wells containing RPMI-1640 broth, DMSO (5%), Tween 80 (2%) and the inoculum of each strain, in the absence of the phytoconstituent or antifungal. ** C2 - Culture medium sterility control: wells containing RPMI-1640 broth, DMSO (5%), Tween 80 (2%), in the absence of the phytoconstituent or antifungal. (+): fungal growth. (-): absence of fungal growth.

After the MIC of isoeugenol was found for the strains of *P. citrinum*, the Minimum Fungicide Concentration (CFM) was determined. As described in Tables 2 and 4, we can observe that isoeugenol presented a CFM at the concentration of 2 μg/mL for 8.33% of the strains, from 4 μg/mL to 16.7% of the strains, 8 μg/mL for 33.33% of the strains, from 16 μg/mL to 41.67% of the strains, from 32 μg / mL to 50% of the strains, from 64 μg/mL to 58.33% of the strains, from 128 μg/mL to 66.67% of the strains and 512 μg/mL to 100% of the samples.

Pizzolitto et al. [22] also screened strains of the filamentous fungus *Aspergillus parasiticus* using the phytochemicals: thymol, carvacrol, eugenol, isoeugenol, creosol, m-Creosol, p-Creosol, o-Cresol and phenol. In this study, they found that isoeugenol had a MIC of 206 μg/mL, which was lower than those found in the other compounds tested, showing its potency against the microorganism, including carvacrol. This result corroborates the MIC values obtained in this study, since the highest MIC value for Penicillium citrinum was 256 μg/mL.

For the control of the experiments, voriconazole was used as standard antifungal. Although there are studies in the literature using voriconazole in several species of fungi, this study unprecedentedly carried out the determination and characterization of the antifungal activity of the substance against standard and clinical strains of *Penicillium citrinum*.

In the experiments performed in this study, the MIC of voriconazole had a considerable variation, showing a great difference of sensitivity between the strains used. The MIC range of antifungal was 0.5 to 256 μg/mL, and for LM-21 strain it was not possible to define the minimum inhibitory concentration because it was higher than 1024 μg/mL and the lowest MIC value was for strain LM-03 (Table 1).

Regarding CFM, we can observe that voriconazole presented a variation of 2 μg/mL at values higher than 1,024 μg/mL among the strains used, establishing a CFM at the concentration of 2 μg/mL for 8.33% of the strains. 4 μg/mL for 16.67% of the strains, from 8 μg/mL to 33.33% of the strains, from 16 μg/mL to 41.67% of the strains, from 32 μg / mL to 50% of the strains. 64 μg/mL for 58.33% of the strains and from 128 μg/mL to 66.67% of the strains.

It is important to note that for 33.33% of the samples it was not possible to detect CFM because there was growth above 1024 μg/mL (Table 1). Voriconazole was first introduced in clinical use in 1995 and approved by the Food and Drug Administration (FDA) in the United States in May 2002 and in Brazil in July 2012 [23-25].

It is a new anti-fungal agent of the triazole class that shows promise for the treatment of a broad spectrum of fungal pathogens, including species of *Aspergillus*, *Candida, Cryptococcus neoformans*, *Penicillium marneffei, Scedosporium apiospermum* among others [26-35].

Conclusions

The demonstration of the antifungal effect of the phenylpropanoic acid isoeugenol on strains of *P. citrinum* in vitro makes the molecule a candidate for a possible drug to be used as an agent in the fight against agricultural pests and in infections against other microorganisms.

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