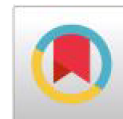




Science

## **DRUG ENHANCEMENT, SYNERGISM AND ANTIFUNGAL ACTIVITY OF MICONAZOLE ASSOCIATED POLYGODIAL AGAINST *CANDIDA ALBICANS***



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### **Abstract**

Miconazole has low toxicity, however, there is a high incidence resistance of *Candida* sp. In the search for new drugs or improve existing ones, the originating products of medicinal plants has been the target of constant studies. The aim of this work was to verify *in vitro* synergism and antifungal activity of miconazole associated with polygodial. Miconazole (MCZ) and Polygodial (P) were dissolved in dimethylsulfoxide (DMSO) 1% done successive dilutions of each product ranging from 25mg to 0,19mg/mL (Polygodial)( $10^{-1}$  to  $10^{-9}$ mg / ml); 14mg to 0,10mg/mL (miconazole). Antifungal test were based by CLSI diffusion agar against *C. albicans* (ATCC 18804), *C. albicans* (LMB01) and *C. albicans* (LMB02), *C. tropicalis* (ATCC 18807), *C. lusitaniae* (ATCC 42720), *C. krusei* (ATCC 20405). The results had shown all compounds that were effective in inhibiting *Candida* species. However, the zones of inhibition in agar diffusion test were higher for Miconazole (16,83mm) and Polygodial (15,16mm) while the MIX showed lower inhibition zones (14,43mm) when compared with controls. MIX was more effective in MIC test, and lowest concentration were at  $10^{-6}$  dilution (0,60mg / ml) compared to MCZ and P controls.

**Keywords:** *Candida* sp; Miconazole; Polygodial; Antifungal Activity; MIC.

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## 1. Introduction

An increase incidence of fungal infections is associated with some predisposing factors as the use of dentures, xerostomia, prolonged therapy with antibiotics, local trauma, malnutrition, endocrine disorders, increased longevity of people, among other states that diminish the quality of individual defense [1-4]. Oral candidiasis is one of the most common clinical features of those patients infected with the human immunodeficiency virus - HIV [5, 6]. Oral candidiasis treatment is based on four fundamentals: making an early and accurate diagnosis of the infection; correcting the predisposing factors or underlying diseases; evaluating the type of *Candida* infection; appropriate use of antifungal drugs [7-10]. Miconazole (MCZ) is a time-honored antifungal of the imidazole class. MCZ exerts a multipronged effect on fungi. It inhibits the cytochrome P450 complex. Disturbances in oxidative and peroxidative enzyme activities lead to an intracellular toxic concentration of hydrogen peroxide. As a result, intracellular organelle destruction then leads to cell necrosis [11-15].

Natural products derived from plants, have over the years greatly contributed to the development of therapeutic drugs. Polygodial is a sesquiterpene isolated from the bark of the plant *Drymiswinteri* (Winteraceae), *Polygonum punctatum*, and other plants, that exhibit antinociceptive properties [16-18]. Polygodial exhibits several pharmacologically interesting activities. Among them, its antifungal properties have been more thoroughly studied.

Polygodial on the *Saccharomyces cerevisiae* mitochondrial energy metabolism were described and has been suggested as one of the possible targets for polygodial action inhibited the mitochondrial ATP synthesis coupled to succinate oxidation [15-22]. Polygodial showed in vitro strong antifungal activity, comparable to amphotericin B, against *Candida albicans*, *C. utilis*, *C. krusei*, *Cryptococcus neoformans*, *S. cerevisiae* and also filamentous fungi including *Trichophyton mentagrophytes*, *T. rubrum*, and *Penicillium marneffei*. Other strains such as *Aspergillus fumigatus*, *A. flavus*, *P. chrysogenum*, *C. lipolytica*, and *C. tropicalis* showed moderate to low susceptibility to polygodial [23-24]. So, this paper aims to evaluate in vitro the antifungal property of a product compound miconazole and polygodial against *Candida* species.

## 2. Material and Methods

### 2.1. Chemicals

Miconazole (Sigma Miconazole Nitrate Aldrich- salt- USA), Polygodial (SC 201489<sup>a</sup>; Santa Cruz Biotechnology, USA) were used in the experiment.

### 2.2. Suspensions Compounds Preparation

Each chemical was dissolved in dimethylsulfoxide at 0.1%: 14,0mg miconazole (MCZ), and 25,0mg Polygodial(P). For isolated tests Polygodial(P) was diluted successively ( $10^{-1}$  to  $10^{-7}$ ) corresponding concentrations of 25 mg to 0.19 mg; while Miconazole was also diluted successively ( $10^{-1}$  to  $10^{-7}$ ) corresponding concentrations of 14mg to , 0.10 mg. For tests with the MIX (MCZ + P) was associated with 3 ml of each original suspension and became the successive dilutions to obtain the various concentrations for procedure Minimum Inhibitory Concentration (MIC).

### 2.3. Microorganisms and Antifungal Testing

*Candida albicans* (ATCC 18804), *Candida tropicalis* (ATCC 18807), *Candida lusitanae* (ATCC 42720), *Candida krusei* (ATCC 20405) was acquired by donation from the National Institute of Quality Control in Health (INCQS), Oswaldo Cruz Institut Foundation, Rio de Janeiro. Two *C. albicans* species (LMB01 and LMB02) were collected from the mouth of patients with candidiasis associated mucositis in Dentistry Clinical Assistance for Cancer Patient Irradiated in Head and Neck region, School of Dentistry, Federal University of Minas Gerais (FOUFMG). The two *Candida* samples were identified in the Laboratory of Microbiology of the School of Dentistry using the CANDIFAST® kit (International Microbio, France) and through the chromogenic medium CHOMagar®(Difco, USA). The ATCC lyophilized microorganisms were kept in freezer -20° C until the time of inoculum preparation. The *Candida albicans* inoculum was made using Sabouraud dextrose broth medium (Himedia, India). An aliquot of 0.1mL was left suspended overnight for 18 hours. Then 0.5mL suspension containing  $1,0 \times 10^6$  UFC/ml corresponding to the 0.5 McFarland standard were plated on Sabouraud dextrose agar. For antimicrobial diffusion tests were used in accordance with Kirby-Bauer test and Clinical Laboratory Standard Institute (CLSI) [25, 26]. Discs blanc antibiogram (Laborclin, Pinhais, PR, Brazil) were soaked with 20µL of each concentration of each compound (MCZ and P) and also the associated compounds (MCZ + P) and dispensed on agar containing *Candida* sp. After 24 hours at 37° C in bacteriological and greenhouse, readings and measurements were made the zones of inhibition observed and the minimum inhibitory concentration (MIC). The results were given considering the mean and the standard deviation in mm of the inhibition zones, three experiments. MIC was considered the lowest concentration that inhibited growth of the microorganism.

### 2.4. Ethical Aspects

The project study was approved by the Etic Committee of UFMG (Number CAAE-0561.0.203.438-11).

### 3. Statistical Analysis

Statistical analysis was performed using the nonparametric Kruskal-Wallis test and was considered significant difference when  $p < 0.5$ .

### 4. Results and Discussions

All the samples of *Candida* spp tested were sensitive to antifungal. However, differences in behavior were observed. Figure 1 shows the MIC in diffusion agar test. It can be observed that the MIC for MIX compound occurred on the disk 7, is the dilution  $10^{-6}$  (0,65mg/mL). This value is smaller and better than was observed for miconazole and polygodial separately. In the Table 1 the antimicrobial susceptibility tests showed inhibition zones were lower for Miconazole compared with Polygodial. Moreover, the combination of the two drugs (MIX = MCZ + P) showed greater sensitivity of the microorganism when compared with both antifungal alone and this showed that there was synergism between the drugs. However, it is noted that containing around the disks Miconazole there are two zones of inhibition being one as evident and

considered as a result, and a brighter region (arrow) which can mean colonies of resistant microorganisms and also observed by Martins et al (2002). This proves the high resistance of *C. albicans* to Miconazole, which leads us to look their best performance in the control of these microorganisms. Wild *Candida albicans* samples (LMB01 and LMB02) were more sensitive to the three antifungals tested. However, they demonstrated greater resistance to Miconazole compared to Polygodial and MIX. *C. tropicalis*, *C. lusitaniae* e *C. krusei* were more sensitive to MIX and Polygodial. Dimethyl sulfoxide 0.1% used as a control did not show any inhibition signal to any of the microorganisms.

Table 1: Antifungal activity of miconazole (MCZ), poligodial (P), and MCZ+P (MIX) association against *Candida* sp. Minimum Inhibitory Concentration (MIC = mg/mL) and Means and Standard Deviations (M ± SD = mm). Results of three experiments. \*Significant difference.

Microorganisms	Antifungals					
	Inhibition zones (mm/ M±DP)			MIC(mg/mL)		
	MCZ	P	MIX	MCZ	P	MIX
<i>C.albicans</i> (ATCC18804)	14,83±0,55	15,16±0,50	16,43±0,25*	0,43*	0,78*	0,65**
<i>C. albicans</i> LMB01	13,00±0,00*	14,50±0,50	17,00±0,00*	0,87*	0,78	0,32**
<i>C. albicans</i> LMB02	12,75±0,25*	14,00±0,00	16,85±0,75*	0,87*	0,39*	0,65**
<i>C. tropicalis</i> (ATCC18807)	13,00±0,00	14,36±0,76*	17,00±0,00*	0,21*	0,39	0,32**
<i>C. lusitaniae</i> (ATCC42720)	15,00±0,00	14,60±0,50*	18,50±0,34*	0,87	0,78	0,65**
<i>C. krusei</i> (ATCC20405)	11,00±0,00*	15,75±0,45	18,00±0,00*	1,75*	0,39**	0,65

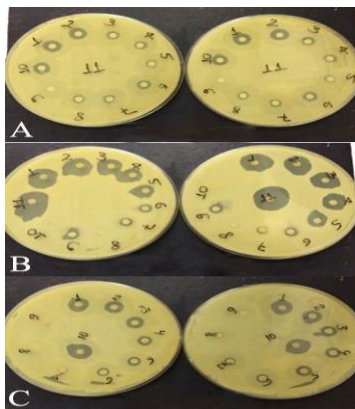


Figure 1: Antimicrobial diffusion agar test (MIC) against *Candida albicans*. Inhibition zones (A=Miconazole; B= Polygodial; C=MIX).

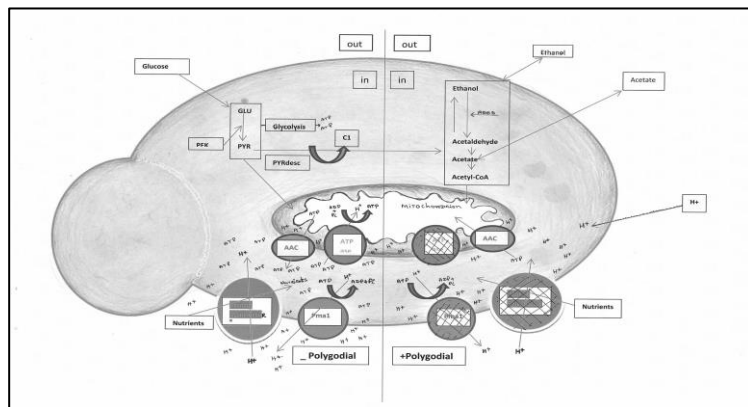


Figure 2: Polygodial activity mechanism of action in OUT and IN mitochondria yeast cell. (Adapted from Linde and Kubo, 2001; Kubo and Himejima,1992)<sup>20-23</sup>

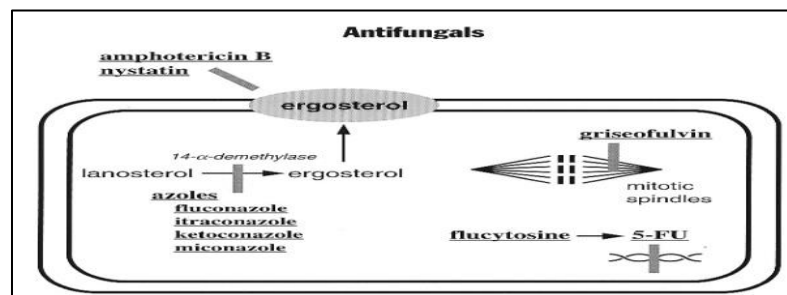


Figure 3: Mechanism of action involving the inhibition of miconazole synthesis of ergosterol (MCZ) of class Imidazole. MCZ inhibits the cytochrome P450 complex, including the enzyme 14α-demethylase required for ergosterol biosynthesis, in fungal cell membranes(39)

## 5. Discussion

The choice of two wild *C. albicans* species, collected from the mouth of patients with candidiasis and irradiated in the head and neck has allowed us to observe the behavior of the standard ATCC species comparing it to generally resistant samples by exposure to chemotherapy and radiation and, also, because the vast majority of irradiated patients generally had contact with some antifungal during treatment. Wild specimens of *Candida* spp showed increased resistance to Miconazole. This was probably due to prior contact of patients with some of the antifungal azoles group due to treatment of candidiasis. This resistance can also be caused by radiation and chemotherapy, despite not having a concrete base on this fact.

This study suggests that the mixture compound MIX has a very good profile of antifungal activity in terms of effectiveness and spectrum of action while being devoid of any significant toxicity. The antifungal activity of two drimanesquiterpenedialdehydes, polygodial and warburganal, alone and in combination with several other substances, was examined against three fungi, *Candida albicans*, *Saccharomyces cerevisiae* and *Pityrosporum ovale* employing a broth dilution method. Anethole significantly synergized the activity of the two sesquiterpenoids against *C. albicans* and *S. cerevisiae*; however, it had only an additive effect against *P. ovale* [27].

Miconazole (MCZ) inhibits the cytochrome P450 complex, including the 14 $\alpha$ -demethylase enzyme required for ergosterol biosynthesis, in fungal cell membranes. Disturbances in oxidative and peroxidative enzyme activities lead to an intracellular toxic concentration of hydrogen peroxide. As a result, intracellular organelle destruction then leads to cell necrosis [12].

These biological activities add up and act synergistically enhancing the antimicrobial activity of MIX (MCZ + P). Polygodial acts as a surfactant and this feature makes it easy to miconazole activity. The polygodial mechanism of action against *S. cerevisiae* were proposed by Lunde and Kubo [28].

The fungicidal action of the primary Polygodial comes in part from its ability to function as a nonionic surfactant, disrupting the protein-lipid interface and integral proteins denaturing its conformation. As a result, the antifungal dialdehydesesquiterpene this mechanism is associated with the functions of the membrane or membrane disarray. For example, the process of acidification of the medium induced by glucose *Saccharomyces cerevisiae* was inhibited by Polygodial presumably caused by inhibition of H<sup>+</sup> in the plasma membrane ATPase by. Thus, the potent antifungal activity of Polygodial holds in its multiple functions [23].

As resistance to azoles (the preferred treatment) is occurring, we aimed at identifying compounds that increase the activity of miconazole against *Candida albicans* biofilms [34]. Enhancement of the antibiofilm activity of miconazole by artesunate, pointing to potential combination therapy consisting of miconazole and artesunate to treat *C. albicans* biofilm-related infections [29]. Combinations of MUC7 12-mer and Hsn5 12-mer, and of MUC7 12-mer and miconazole have a synergic antifungal effect on *C. neoformans* and *C. albicans* [30-33].

There was a significant difference between the zones of inhibition when compared miconazole, Polygodial and MIX. These differences may be related to the types of molecules of each compound, whereas polygodial displays a heavier molecule that miconazole this contributes to the diffusion in agar is smaller [34]. This difference was also observed in the MIX and probably the polygodial interfere with the spread of miconazole. Moreover, the association of the two compounds improved antifungal activity as shown in Table 1, for the MIC was 0,65mg / ml, the observed difference was significant when compared separately. The polygodial surfactant activity improves the action of miconazole and *Candida* is attacked on two fronts: in the cell wall by the absence of ergosterol and fall of the energy produced by mitochondria and disruption of intracytoplasmic membrane system [20, 35]. The search for novel antifungal agents and the attempt to modify and improve the features of the currently available chemotherapeutic agents are in high demand as a consequence of the rapid spread of multidrug-resistant fungal pathogens. In this regard, naturally occurring antimicrobial peptides are an attractive option, what is discussed by others [36-38]. In conclusion, these results suggest that the combination of miconazole with polygodial may be a promising avenue in the search for new antifungal formulations for treatment of oral candidiasis.

**Conflict of Interest:** The authors declare no conflicts of interest related to this research.

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