

In vitro anti-*Candida* activity and mechanism of action of the flavonoid isolated from *Praxelis clematidea* against *Candida albicans* species

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ARTICLE INFO

Article history:

Received on: 03/10/2015

Revised on: 15/11/2015

Accepted on: 30/11/2015

Available online: 26/01/2016

Key words:

Flavonoid, antifungal, *Candida*, *Praxelis clematidea*.

ABSTRACT

The prevalence of candidiasis in the world is high. *Candida* species are able to create superficial and systemic infections. *Candida albicans* is an opportunistic pathogen, causing mycoses in immunocompromised patients as well as long-term antibiotic users. The present study objective was to evaluate *in vitro* anti-*Candida* effect of this compost isolated from *Praxelis clematidea*. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined by the broth microdilution techniques. We also investigated possible flavonoid 5,7,4'-trimethoxyflavone (TMF) action on cell walls (0.8 M sorbitol) and cell membranes (TMF to ergosterol binding). The MIC₅₀ of flavonoid were 64 µg/mL and the MFC₅₀ was 64 µg/mL. Involvement with the cell wall and ergosterol binding were improved as possible mechanisms of action. In conclusion the flavonoid showed *in vitro* antifungal potential against strains of *C. albicans*.

INTRODUCTION

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide. Human infections, particularly those involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries (Portillo *et al.*, 2001). In humans, fungal infections range from superficial to deeply invasive or disseminated, and have increased dramatically in recent years. Although new drugs have been introduced to combat this problem, the development of resistance to antifungal drugs has become increasingly apparent, especially in patients who require long-term treatment or who are receiving antifungal prophylaxis, and there is growing awareness of shifts of flora to more-resistant species (Pradeepa *et al.*, 2014). Fungal infections are usually associated with *Candida*, *Aspergillus* and *Cryptococcus* species but those due to *Candida* species represent the main opportunistic fungal infections

worldwide, leading to high morbidity and mortality in the population (Low and Rotstein, 2011). These changes are linked to the growing population of immuno-compromised patients. During the last three decades, *Candida albicans* has been the most prevalent pathogen in systemic fungal infections (Pfaller and Diekema, 2004). Although the antifungal active principles are diverse and numerous, only few classes of antifungal agents are currently available to treat yeast infections due to the high toxicity of many of them (Spampinato and Leonardi, 2013). The high morbidity and mortality rates associated with opportunistic yeast infections indicate that current anti-fungal therapy to combat candidiasis is still ineffective (Dzoyem *et al.*, 2014).

Therefore, it has become essential to develop new drugs and alternative therapies (including natural products) for treatment of *Candida albicans* infections. Plants and their derivatives are known to be important in pharmacological research due to their great potential as a source for a variety of biologically active ingredients used in drug development. Amongst these products we find the flavonoids that are considered as constitutive antimicrobial ingredients, especially those belonging to prenylated flavonoids, flavones and isoflavones (Filho *et al.*, 2012; Leite *et al.*, 2014).

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Considering the few studies on the antifungal effects of the flavonoid 5, 7, 4'-trimethoxyflavone (TMF), the aim of the present study was to evaluate *in vitro* anti-*Candida* effect of this compost isolated from *Praxelis clematidea*.

MATERIALS AND METHODS

Isolated of the flavonoid

Maia *et al.* (2011) describe the method of obtaining the flavonoid.

Fungal strains

For antifungal activity assays, were selected 8 strains of fungi (*Candida albicans* – LM 86, *Candida albicans* – LM 111, *Candida albicans* – LM 122, *Candida albicans* – LM 108, *Candida albicans* – LM 20, *Candida albicans* – LM 189, *Candida albicans* – ATCC 90028, *Candida albicans* – ATCC 76645). All the microorganism strains were obtained from the Laboratory of Mycology collection. Fungi was kept on Nutrient Agar (NA) slants at 4 °C. Inocula were obtained from overnight cultures grown on NA slants at 37 °C and diluted in sterile saline solution (NaCl 0.85% w/v) to provide a final concentration of approximately 10⁶ count forming unit per mL (CFU.mL⁻¹) adjusted according to the turbidity of 0.5 McFarland scale tube.

Determination of the minimum inhibitory concentration (MIC) and minimum fungicide concentration (MFC)

The microplate bioassay was used to determine the minimum inhibitory concentration (MIC) of flavonoid (Viljoen *et al.*, 2003; Sahin *et al.*, 2004). One hundred milliliters (100 µL) of liquid medium RPMI-1640 was transferred into the wells of a 96-well microdilution plate with a “U” shaped bottom (Alamar, Diadema, SP, Brazil). Then, 100 µL of flavonoid emulsion was inoculated in the first horizontal row of the plate wells. Doubled serial dilutions, where a 100 µL aliquot removed from the most concentrated well went to the next well, and yielded concentrations of 1024-16 µg/mL. Finally, 10 µL of *C. albicans* inoculum suspension was added to each well of the plate, where each column represented a yeast strain. In parallel, controls were made for yeast viability and for susceptibility with the standard antifungal Nystatin (100 UI/mL). To verify the absence of interference in the results for the solvent used in the preparation of the substance in the event the cremophor, in which a control was placed in the cavities 100 µL of the double-concentrated broth, 100 µL of cremophor and 10 µL of the suspension was made. The plates were incubated at 35° C for 24–48 h.

After the appropriate incubation time, the presence (or absence) of growth was observed visually. The formation of cell clusters or “buttons” in the plate wells was considered. The MIC was defined as the lowest TMF concentration that produced visible inhibition of yeast growth.

To determine the MFC, we subcultured 20 µL aliquots of MIC, MIC × 2, and MIC × 4 of the TMF, and the control yeast growth onto Petri dishes containing SDA. After 24–48 hours of

incubation at 35° C, a reading was made to evaluate the MFC, as based on the growth of the controls. The MFC was defined as the lowest product concentration that inhibited growth of the yeast or permitted less than three CFUs to occur, resulting thus in 99.9% fungicidal activity (Ernst *et al.*, 1996, Espinel-Ingroff *et al.*, 2002). Biological activity assays were performed in duplicate, and the results were expressed as the arithmetic mean of the MIC and MFC.

Sorbitol assay-effect of TMF on the cell wall of *C. albicans*

The assay was performed using medium with and without sorbitol (control) to evaluate possible mechanisms involved in the antifungal activity of the test product on the yeast cell wall.

The sorbitol was added to the culture medium in a final concentration of 0.8 M. The assay was performed by microdilution method in 96-well plates in a “U” (Alamar, Diadema, SP, Brazil). The plates were sealed aseptically, incubated at 35° C, and readings were taken at 2 and 7 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with added sorbitol compared to the standard medium implicated the cell wall as one of the possible cell targets for the product tested (Frost *et al.*, 1995). The assay was performed in duplicate and expressed as the geometric mean of the results.

Ergosterol binding assay-MIC value determination in presence of ergosterol

To assess if the product binds to the fungal membrane sterols, this experiment was performed according to the method described by Escalante *et al.* (2008), with some modifications. The ergosterol was prepared at the time of test execution, where it was first pulverized (with the help of a pre-sterilized porcelain mortar and pestle) and dissolved in DMSO (no more than 10% of final volume), and Tween 80 at 1%, in accordance with the desired concentration and volume. The formed emulsion was then homogenized, heated to augment the solubility, and diluted with the liquid culture medium.

The MIC of TMF against *C. albicans* was determined by using broth microdilution techniques according to the guidelines of the CLSI for yeasts (M27-A3) (CSLI, 2008), in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines of the same microplate. Briefly, a solution of flavonoid was doubly diluted serially with RPMI-1640 (volume = 100 µL) containing plus ergosterol at concentration of 400 µg/mL.

A volume of 10 µL of yeast suspension (0.5 McFarland) was added to each well. The plates were sealed and incubated at 35 °C. The plates were read after 24 h of incubation and MIC was determined as the lowest concentration of test agent inhibiting the visible growth. This assay was carried out in duplicate and the geometric mean values were calculated. Thus, this binding assay reflected the ability of compound to bind with the ergosterol.

RESULTS AND DISCUSSION

Nowadays, fungal diseases have emerged and are being increasingly recognized as important public health problems owing to an ever-expanding population of immunocompromised patients (Miceli *et al.*, 2011). Linked to this the prevalence of candidiasis has been increased. *Candida* species are able to create superficial and systemic infections. *Candida albicans* is an opportunistic pathogen, causing mycoses in immunocompromised patients as well as long-term antibiotic users (Zhang *et al.*, 2002).

Furthermore Pierce and Lopez-Ribolt *et al.* (2013) reported that, the current arsenal of antifungal drugs is exceedingly short and no new antifungal drugs are expected to reach the market any time soon. Therefore, the discovery of new antimicrobial agents is still relevant. Among the potential sources of new agents, plants have long been investigated because they contain many bioactive compounds that can be of interest in therapy. Because of their low toxicity, there is growing interest in using spices as a source of bio- active phytochemicals for their antimicrobial properties in preventing pathogenic diseases, in addition to their flavor and fragrance qualities (Arora and Kaur, 1999). The results for TMF's antifungal activity against *C. albicans* strains were determined using the MIC and MFC in broth microdilutions (Table 1). The MIC₅₀ (minimum inhibitory concentration able to inhibit 50% of the fungal strains) of TMF was 64 µg/mL, inhibiting the growth of all tested fungal strains. The results for the negative control (cremophor) showed no fungal growth inhibition, the results for the positive control (nystatin) showe fungal growth inhibition and fungal growth in the medium without added drug was detected (sterile control). The MFC₅₀ (minimum fungicid concentration able to inhibit 50% of the fungal strains) of TMF was 64 µg/mL.

Table 1: Antifungal activity for determination of the MIC and MFC of the TMF.

Substance / Fungal strains	TMF (MIC µg/mL)	TMF (MFC µg/mL)	Negative control	Positive control
<i>C. albicans</i> (ATCC 90028)	32	64	-	+
<i>C. albicans</i> (ATCC 76645)	32	64	-	+
<i>C. albicans</i> (LM 20)	64	-	-	+
<i>C. albicans</i> (LM 86)	512	1024	-	+
<i>C. albicans</i> (LM 108)	64	-	-	+
<i>C. albicans</i> (LM 111)	64	64	-	+
<i>C. albicans</i> (LM 122)	64	64	-	+
<i>C. albicans</i> (LM 189)	64	64	-	+

(-) No inhibition (+) inhibition

According with literature results strong activity is for MIC values between 0.05 – 0.50 mg/mL, moderate activity MIC values between 0.6 – 1.50 mg/mL and weak activity above 1.50 mg/mL (Sartoratto *et al.*, 2004). The results showed that TMF present de strong effect against *C. albicans* strains with MIC₅₀ for TMF is 64 µg/mL. These results are in agreement with the data obtained by Filho *et al.* (2012) in their study using the flavonoid TMF against various strains of *Candida*. Analyzing the results of the MFC can be seen that the flavonoid does have fungicide

activity against *C. albicans* species, because when the ratios of MFC/MIC were 1 or 2, indicating that the effect of the compound was fungicide in nature (and not fungistatic) (Hafidh *et al.*, 2011).

In accordance with the above results, the strains ATCC 76645 and LM 122 were selected for further testing. The MIC for TMF both strains was 64 µg/mL. To investigate the action of the product on the fungal cell wall we performed an assay with sorbitol, which has an osmoprotectant function. Sorbitol is an osmotic protector used to stabilize fungi protoplasts. Specific fungal cell wall inhibitors share a distinctive characteristic where their antifungal effects are reversed in mediums containing sorbitol (Frost *et al.*, 1995). Cells protected with sorbitol can grow in the presence of fungal cell wall inhibitors, whereas growth would be inhibited in the absence of sorbitol. This effect is detected by increases in the MIC value as observed in medium with sorbitol as compared to the MIC value in medium without sorbitol (standard medium) (Frost *et al.*, 1995; Svetaz *et al.*, 2007). Osmotic destabilizing agents and disrupting the cell wall lead to rearrangements of the cell wall and allow the fungal cells to survive (CSLI, 2002). In this paper, the MIC values of TMF in experiments, in mediums with sorbitol (Table 2), were increased, suggesting that flavonoid does act by inhibiting fungal cell wall synthesis.

Table 2: Antifungal activity of the TMF in presence and absence of sorbitol (0.8M).

Substance / Fungal strains	TMF (MIC µg/mL)	TMF (MIC µg/mL) + sorbitol (0.8 M)	Negative control
<i>C. albicans</i> (ATCC 76645)	32	256	-
<i>C. albicans</i> (LM 122)	64	1024	-

(-) No inhibition.

Considering this possible fungal cell membrane interference of TMF, the compound was tested to investigate its ability to form complexes with ergosterol. Ergosterol is the principal sterol present in yeasts and filamentous fungi, where it is necessary for the growth and normal function of the fungal cell membrane. Besides controlling the fluidity, asymmetry and integrity of the membrane, ergosterol contributes to the proper functioning of enzymes bound to the membrane (Alves *et al.*, 2013). Ergosterol plays the same role in fungal membranes that cholesterol plays in mammalian cell membranes (Bowman and Free, 2006). Thus, these two sterols seem to exhibit qualitatively similar properties. The majority of existing drugs for the treatment of fungal infections target the cell wall or plasma membrane directly or indirectly, particularly ergosterol and its biosynthesis (Odds *et al.*, 2003; Lupetti *et al.*, 2002).

If the activity of flavonoid is caused by binding to ergosterol, the exogenous ergosterol would prevent the binding to ergosterol in the fungal membranes. Consequently, MIC increase for TMF (in the presence of exogenous ergosterol in relation to the control assay) would occur because only increased product concentration in the growth medium might assure interaction with ergosterol in the fungal membranes (Escalante *et al.*, 2008; Lunde

et al., 2000). Thus, the effect of exogenous ergosterol on TMF's MIC was determined. As can be seen, flavonoid displayed changes in MIC values; the values were increased in medium with additional ergosterol (Table 3). This indicates that the mechanism of action of TMF does involve too complexation with ergosterol.

Table 3: Antifungal activity of the TMF in presence and absence of ergosterol (400 µg/mL).

Substance / Fungal strains	TMF (MIC µg/mL)	TMF (MIC µg/mL) + ergosterol (400 µg/mL)	Negative control
<i>C. albicans</i> (ATCC 76645)	32	128	-
<i>C. albicans</i> (LM 122)	64	512	-

(-) No inhibition

CONCLUSION

Based on these results, the present study demonstrated that flavonoid TMF has significant antifungal activity against *C. albicans*. Another important aspect was that the probable mechanism of action does involve interactions either the cell wall or ergosterol. Therefore, the test product is presented as a relevant and promising antifungal which can be considered as an alternative prototype for production of a new and future antifungal, and thus contributing to the existing arsenal of products with proven antifungal activity against *C. albicans*.

COMPETING INTERESTS

The authors declared no potential conflicts of interest.

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How to cite this article:

deOliveira Filho AA, de Oliveira HMBF, Sousa JP, Meireles D, de Azevedo Maia GL, Filho JMB, Lima EO. In vitro anti-Candida activity and mechanism of action of the flavonoid isolated from *Praxelis clematidea* against *Candida albicans* species. *J App Pharm Sci*, 2016; 6 (01): 066-069.