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# *Mitracarpus frigidus*: A promising antifungal in the treatment of vulvovaginal candidiasis

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ABSTRACT

*Candida species* are opportunistic pathogens associated with some important clinical infections such as vulvovaginal candidiasis (VVC). Nowadays, candidiasis is a public health problem that is aggravated by inefficiency of the traditionally used antifungal agents. The aim of the work was to evaluate the *in vitro* and *in vivo* antifungal activity of *M. frigidus* methanolic extract (MFM) against *C. albicans* ATCC® 10231 drug resistant strain. The *in vitro* assays (Minimal Inhibitory Concentration (MIC) = 500 μg $ml^{-1}$; fungal cell density; nucleotide leakage; permeability with crystal violet; sorbitol protection; ergosterol effect and time-kill kinetics) demonstrated that MFM has a growth inhibition effect on *C. albicans* acting on the cellular envelope by increasing cell permeability and interfering in the fungus growth. The *in vivo* assays (therapeutic treatment of the experimental VVC) showed that MFM is able to decrease the fungal infection. Altogether, the *in vitro* and *in vivo* results of this study proved that MFM is active for the VVC caused by *C. albicans* ATCC® 10231 strain. Considering the non-toxicity of the extract reported in previous studies from our group and the present results, MFM may be considered a promising antifungal agent.

## 1. Introduction

*Candida* species are fungi commonly found in the human microbiota. In healthy individuals, the fungus is in equilibrium with the host. However, a microbial dysbiosis leads *Candida* to act as an opportunistic pathogen. In this case, it is responsible for important clinical infections in different anatomical sites of the human body (Dühring et al., 2017; Mayer et al., 2013; Panpetch et al., 2017).

One of the most important sites of candida infection is the vulvovaginal mucosa leading to the so called vulvovaginal candidiasis (VVC). This is a worldwide condition that affects immunocompetent and immunocompromised women. Several factors as hormonal conditions, contraceptive and antibiotic use, among others can lead to microbial dysbiosis contributing in the VVC establishment. Most episodes of VVC is caused by *C. albicans*, which is able to adhere to the vaginal epithelial cells thus causing cell damage and inflammatory response, with vaginal discharge and severe itching being the main symptoms (Miró et al., 2017a; Verma et al., 2017).

Epidemiological data estimate that 70–75% of women will experience at least an episode of VVC in their life; 50% of initially infected women will suffer a second VVC event and 5–10% will develop recurrent VVC (Brandolt et al., 2017; Gonçalves et al., 2016). Currently, candidiasis is a public health problem with high morbidity and mortality due to the ability to become invasive in immunocompromised patients (Ou et al., 2017). In addition, the Candida Drug Resistance (CDR) and Multidrug Resistance (MDR) genes (Khosravi et al., 2016) associated with issues from current candidiasis treatment (side effects, toxicity, and inefficiency), have become challenging to its therapeutic (Bhattacharjee, 2016; Perlin et al., 2017).

The species *Mitracarpus frigidus* (Willd. Ex Roem. & Schult.) K.

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Shum, belonging to Rubiaceae family, is found in South America, mainly in Brazil (Pereira et al., 2006). Biological studies using oral doses of this plant to rodents showed antimicrobial, leishmanicidal, cytotoxic and laxative activities, with no toxicity in acute and subchronic assays (Fabri et al., 2012a, 2014a). Recently, kaempferol, kaempferol-*O*-rutenoside, rutin, ursolic acid, methyl ursolate, scopoletin and psychorubrin were identified in *M. frigidus* extract (Fabri et al., 2012b, 2014b, 2014c). Previous studies with *M. frigidus* reported by our group showed a potential antifungal activity (Fabri et al., 2009). In the present work, we used different approaches to investigate the *in vitro* and *in vivo* antifungal activity of *M. frigidus* extract against a drug-resistant *C. albicans* strain.

## 2. Material and methods

### 2.1. Chemicals

Sabouraud Dextrose agar (SDA) medium, sabouraud Dextrose broth (SDB) and brain heart infusion (BHI) broth Acumedia ® were obtained from Neogen ® (São Paulo, SP, Brazil). Nystatin and fluconazole were obtained from Teuto ® (Anápolis, GO, Brazil) and itraconazole from Geolab ® (Anápolis, GO, Brazil). 4′,6-diamidino-2-phenylindole (DAPI), ergosterol and sorbitol were purchased from Sigma Aldrich ®, (São Paulo, SP, Brazil). The solvents methanol, dimethyl sulfoxide and formalin were obtained from Labsynth ® (Diadema, SP, Brazil). Cyclophosphamide (Genuxal ®) was obtained from Baxter ® (São Paulo, SP, Brazil); estradiol cypionate (E.C.P ®) from Pfizer ® (São Paulo, SP, Brazil) and clotrimazole cream from Medley ® (São Paulo, SP, Brazil).

### 2.2. Plant material

*Mitracarpus frigidus* aerial parts were collected in May, 2011 in Juiz de Fora, state of Minas Gerais, Brazil. Geographic coordinates: –43.38223, –21.78073 (WGS84). A voucher specimen (CESJ 46076) was deposited at the Leopoldo Krieger Herbarium at the Universidade Federal de Juiz de Fora (UFJF).

### 2.3. Preparation of the extract

The aerial parts (1 kg) of *M. frigidus* were powdered and macerated with methanol (5 × 2000 ml) for five days at room temperature. After evaporation of the solvent under reduced pressure at 45 °C, the methanolic extract was obtained (MFM), and kept in tightly stoppered bottles under refrigeration for subsequent biological tests.

### 2.4. Fungal strain

*Mitracarpus frigidus* methanolic extract (MFM) was evaluated against *C. albicans* ATCC ® 10231, a strain resistant to Anidulafungin, Voriconazole, Itraconazole, and Fluconazole (ATCC, 2018).

### 2.5. Serial dilution assay for determination of the Minimal Inhibitory Concentration (MIC)

The antifungal activity of MFM was performed according CLSI (2017) in order to determine the Minimal Inhibitory Concentration (MIC). The fungus was cultured at 35 °C for 24 h in SDA. Sample stock solution of MFM was two-fold diluted from 1000 to 7.8 μg ml$^{-1}$ (final volume = 80 μl) and a final DMSO concentration ≤ 1%. Then, 100 μl of SDB was added onto microplates. Finally, 20 μl of $10^6$ CFU ml$^{-1}$ (according to 0.5 McFarland turbidity standards) of standardized fungal suspension was inoculated onto microplates and the test was performed in a volume of 200 μl. Plates were incubated at 35 °C for 24 h. The same tests were performed simultaneously for growth control (SDB + fungus + MFM vehicle) and sterility control (SDB + MFM vehicle). Nystatin (100 to 0.78 μg ml$^{-1}$), fluconazole (10,000 to 78.1 μg ml$^{-1}$) and itraconazole (400 to 3.12 μg ml$^{-1}$) was used as positive control. The MIC values were calculated as the highest dilution showing complete inhibition of tested strain. The analyses were performed in duplicate.

### 2.6. Minimum Fungicidal Concentration (MFC)

To determine the MFC, a sample from each well that showed no visible fungus growth in the MIC assay was plated on freshly prepared SDA plates and later incubated at 35 °C for 24 h (Spencer and Spencer, 2004). The MFC was expressed as the concentration of the MFM that did not show any growth on a new set of agar plates.

### 2.7. Fungal cell density

To determine the total number of fungal cells, samples were cytocentrifuged as previously described (Silva et al., 2014). Fungal strain of *C. albicans* was inoculated in tubes of SB containing MFM (MIC value) incubated at 35 °C for 24 h. Fungal strain inoculated in SB with vehicle served as negative control and the positive control was incubated with Nystatin (MIC value). Samples were diluted 10 times (1 ml) in saline, fixed with free-particle formaldehyde (final concentration 4%) and stained with DAPI at 0.1 μg ml$^{-1}$ final concentration for 10 min. Next, samples were placed in mega funnels (Shandon Mega Funnel, Thermo, UK) for immediate centrifugation in a cytocentrifuge (Shandon Cytospin 4, Thermo, United Kingdom), at 254 *g* at high acceleration for 10 min. Analyses were performed on a fluorescence microscope (BX-60, Olympus, Melville, NY, USA) and U-MWU2 filter (330–385 nm excitation wavelengths). The number of fungal organisms was determined by counting 20 random fields at 1000 x magnification using an ocular grid. The final count was calculated by multiplying by the dilution factor (10 ×).

### 2.8. Nucleotide leakage

The experiment was performed according to Tang et al. (2008) with some modifications. *C. albicans* was incubated in SB at 35 °C for 24 h. The culture then was washed and resuspended in 10 mM PBS (pH 7.4), reaching the final density of about $10^6$ cells ml$^{-1}$. Strains were incubated with MFM and Nystatin (MIC value) for different time intervals (0, 1, 2, 3, 4 and 5 h); strains incubated with 10 mM PBS (pH 7.4) were used as control. Following incubation, the cell suspensions were centrifuged at 10,000*g* for 10 min and the supernatants were then diluted appropriately and the optical density (OD) at 260 nm was recorded in a spectrophotometer (Multiskan Go, Thermo Scientific, Waltham, MA, United States) at room temperature (25 °C). The experiment was carried out in triplicate.

### 2.9. Measurement of permeability with crystal violet

Membrane permeability changes were also evaluated by crystal violet (CV) assay as described by Devi et al. (2010) with some modifications. Freshly grown fungal strain ($10^6$ cells ml$^{-1}$) (according to McFarland turbidity standards) in SDB was inoculated posteriorly in tubes supplemented with different concentrations of MFM (MIC, 0.5MIC and 0.25MIC values) at 35 °C and nystatin (MIC), followed by incubation for 4 h. Fungal cell suspensions were centrifuged at 1000*g* for 10 min and the pellet was resuspended in 10 μg ml$^{-1}$ of solution crystal violet prepared in sterile water and incubated 10 min at 35 °C. After incubation, the tubes were centrifuged at 1000*g* for 15 min and the OD of the supernatant was measured at a wavelength of 570 nm in a spectrophotometer (Multiskan Go, Thermo Scientific, Waltham, MA, United States). The supernatant OD of the normal untreated cell group was used as growth control. The OD value of crystal violet solution was considered as 100%. The percentage of crystal violet uptake was expressed as follows: (OD value of sample/OD value of CV solution) ×