In vitro Evaluation of the Curcumin against Fluconazole-Resistant Strains of Candida spp. and Cryptococcus neoformans

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A B S T R A C T

The isolates of Candida spp. and Cryptococcus neoformans were inhibited by curcumin, with different degrees of inhibition among species, with MICs ranging from 8-64 µg/mL. After exposure of the C. albicans strains to curcumin, it was observed a decrease in the number of viable cells and damage to cell membranes, the mitochondrial function of C. albicans cells appears to be affected after exposure to curcumin. Our data suggest that after exposure to curcumin, C. albicans cells showed total strand breaks in the DNA, where the nuclear DNA fragmentation was evident in the comet assay. Oxidative damage to DNA was verified by the alkaline comet assay in the presence of Fpg, where it was observed an increase in DNA migration of cells that were treated with curcumin. Based on the characteristics of cell death observed, it is raised the hypothesis that curcumin seems to exert its antifungal activity at specific sites near the DNA, resulting in cell death by apoptosis.

Keywords
Curcumin, Resistance, Fluconazole, Candida, Cryptococcus neoformans

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Introduction

Fungal infections caused by the genus *Candida* represent a concern in clinical medicine related to public health, because these infections show high prevalence in different medical centers, and also due to their complications, difficult diagnosis, in addition to being associated with mortality in the order of 30-50% in children and adults (Nucci et al., 2013; Colombo et al., 2007).

The candidemias are considered the most common invasive mycoses, accounting for 80% of hospital fungal infections. *Candida* spp. is currently considered the fourth agent of bloodstream infections [representing 8-10% of cases], due to a 500% increase in its incidence from the decade of 80 (Colombo et al., 2007; Bergamasco et al., 2013).

Other fungal infections of opportunistic character, which also represent a public health problem worldwide, affecting not only immunocompromised, but also immunocompetent individuals, are infections caused by the genus *Cryptococcus* (Del Poeta and Casadevall, 2012).

The *Cryptococcus* genus contains about 48 species, two being clinically relevant, *Cryptococcus neoformans* and *Cryptococcus gattii*. The scope of cryptococcosis by *C. neoformans* prevails when associated with low cellular immunity conditions. This characteristic is due to its tendency to infect immunocompromised individuals, particularly those infected with the Human Immunodeficiency Virus [HIV] (Del Poeta and Casadevall, 2012; Kronstad et al., 2011).

The antifungal resistance, especially to azole-based drugs, has emerged as a major clinical problem for immunosuppressed patients and hospitalized at high risk for fungal infections (Pfaller et al., 2012). Given the small pharmacological arsenal to antifungal treatments when compared to the arsenal of antibiotics, often studies look for new antifungals that have greater spectrum of action, low cost and lower evidence of antifungal resistance, considering that this is a current problem (Zhang et al., 2009).

In this context, herbal antifungal drugs have gained importance due to their natural origin. Curcumin has demonstrated its therapeutic potential with anti-inflammatory, antioxidant, antibacterial, antiparasitic action (Maheshwari et al., 2006), being also well documented in the treatment of breathing problems (Shaikh et al., 2009), in various chronic diseases presenting inflammation (Aggarwal and Harikumar, 2009), and the antifungal activity is another major biological effect of curcumin, in particular against *Candida* spp. (Khan et al., 2012; Martins, 2009).

The aim of the study was to evaluate the antifungal potential of curcumin against fluconazole-resistant strains of *Candida* spp. and *Cryptococcus neoformans*, elucidating the possible mechanisms involved in the cytotoxic action of curcumin through procedures such as flow cytometry and comet assay.

Materials and Methods

Isolates

We used nine fluconazole-resistant strains of *Candida* spp. (Ramos et al., 2016). Fluconazole-resistant *C. albicans* (three strains), *C. tropicalis* (three strains), *C. parapsilosis* (three strains) and *Cryptococcus neoformans* (four strains) clinical strains were used. These strains are from the yeast collection of the Laboratory of Bioprospection in Antimicrobial Molecules (LABIMAN/FF/UFC). The strains were inoculated in Sabouraud dextrose agar (Himedia, Mumbai, India) and incubated at 37°C for 24 h. Then,
they were grown in CHROMagAar Candida (Himedia) to evaluate their purity.

**In vitro antifungal activity**

The drug was tested according to the document M27-A3 using RPMI broth (pH 7.0) buffered with 0.165 M MOPS (Sigma Chemical, St. Louis, MO). Fluconazole (Merck Sharp & Dohme, São Paulo, Brazil) was dissolved in distilled water, and solution of curcumin chloride form (Sigma Chemical) was prepared in dimethylsulfoxide (DMSO; Sigma Chemical). Fluconazole (FLC) and curcumin were tested at concentrations ranging from 0.125 to 64 mg/L. Yeasts and compounds were incubated in 96-well culture plates at 35°C for 24 h and the results were examined visually, as recommended by CLSI (2008). The following cut-off points were determined according to the document M27-S4 (CLSI, 2012). The strains C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were used as control.

**Cell treatments**

To assess cell density, membrane integrity, mitochondrial transmembrane potential and DNA damage (comet assay), the fluconazole-resistant strain C. albicans was exposed for 24 h to increasing curcumin concentrations (MIC, 2x MIC, and 4x MIC). All tests were conducted in triplicate, in three independent experiments (Da Silva et al., 2013; Andrade Neto et al., 2014).

**Preparation of yeast suspensions**

Cell suspensions were prepared from cultures in the exponential growth phase. The cells were collected by centrifugation (1600 g for 10 min at 4°C), washed twice with a 0.85% saline solution (1200 g for 5 min at 4°C) and then resuspended (~10^6 cells/mL) in HEPES buffer (pH 7.2) supplemented with 2% glucose. Amphotericin B (Ampho B; Sigma Chemical) was used as cell death control (Da Silva et al., 2013; Andrade Neto et al., 2014).

**Determination of cell density and membrane integrity**

Cell density and membrane integrity of fungal strains were evaluated by the exclusion of 2 mg/L PI (propidium iodide). Aliquots from yeasts incubated for 24 h with the drugs (curcumin, FLC, Ampho B) were analysed using flow cytometry. 10,000 events were evaluated per experiment (n=2) and the cellular debris was omitted from analysis. Cellular fluorescence was then determined by flow cytometry using a Guava EasyCyte™ Mini System cytometer (Guava Technologies Inc., Hayward, CA, USA) and analysed using CytoSoft 4.1 software (Da Silva et al., 2013; Andrade Neto et al., 2014).

**Measurement of mitochondrial transmembrane potential [Δψm]**

The mitochondrial transmembrane potential was determined by measuring the retention of rhodamine 123 dye by the mitochondria of yeast cells after 24 h exposure. The cells were washed with PBS, incubated with 5 mg/L rhodamine 123 at 37°C for 30 min, in the dark, and then washed twice with PBS. Their fluorescence was measured by flow cytometry (Guava EasyCyte™ Mini System). A total of 10,000 events was evaluated per experiment [n=2] and cellular debris was omitted from analysis (Da Silva et al., 2013; Andrade Neto et al., 2014).

**Yeast comet assay**

This assay was performed essentially as described by Da Silva et al., (2016). For each experimental group, it was under analysis images obtained by fluorescence microscopy
of 100 randomly selected cells (50 cells from each of 2 replicate slides). The cells were scored visually and depending on their tail size they were assigned to one of five classes (from undamaged [class 0] to maximally damaged [class 4]), and a damage index value was calculated for each sample of cells. The damage index values, thus, ranged from 0 (completely undamaged: 100 cells x 0) to 400 (maximum damage: 100 cells x 4). The tailed cells were taken as an indicator of DNA damage and its frequency was calculated based on the numbers of cells with tails (DNA strand breaks) and without them (Da Silva et al., 2016).

Analysis of oxidized purine and pyrimidine bases in yeast DNA

The alkaline comet assay was performed as described above. The slides were removed from the lysis solution and were washed three times in an enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, and 0.2 mg/mL BSA, pH 8.0), drained and incubated with 70 µL of formamidopyrimidine DNA-glycosylase (FPG) for 30 min at 37ºC. Images of 100 randomly selected cells per group [50 cells from each of two replicate slides] were visually analysed. The number of oxidized purines (FPG-sensitive sites) was then determined by subtracting the amount of strand breaks observed in the control (samples incubated only with buffer) from the total amount of breaks obtained after incubation with FPG (Da Silva et al., 2013; Andrade Neto et al., 2014).

Annexin V staining

Treated and untreated C. albicans cells were collected by centrifugation and digested with 2 mg/mL zymolyase 20T (Seikagaku Corp., Japan) in potassium phosphate buffer (PPB, 1 M sorbitol, pH 6.0) for 2 h at 30ºC. Protoplasts of C. albicans were stained with FITC-labelled Annexin V and PI using a FITC-Annexin V apoptosis detection kit (Guava Nexin Kit, Guava Technologies, Inc., Hayward, CA, USA). Subsequently, the cells were washed with PPB and incubated in an Annexin binding buffer containing 5 µl/ml of FITC-Annexin V and 5 µl of PI for 20 min. The cells were then analyzed by flow cytometry (Guava EasyCyte™ Mini System). For each experiment (n=2), 10,000 events were evaluated, and cell debris was omitted from analysis (Andrade Neto et al., 2014).

Statistical analysis

In vitro susceptibility experiments were repeated at least three times on different days. Geometric means were used to compare MIC results. The data obtained from flow cytometry and alkaline comet assays were compared using a one-way analysis of variance [ANOVA] followed by Newman-Keuls test (p<0.05).

Results and Discussion

The sensitivity of each strain to fluconazole was assessed using the broth microdilution method (CLSI, 2008). According to table 1, after 24 hours of exposure to fluconazole, all tested strains showed MICs between 16 and 64 µg/mL, thus confirming their resistance. When the strains in studies were treated with curcumin, varying degrees of inhibition between species were observed. Table 1 shows an antimicrobial activity against clinical isolates of yeasts, with MIC to Candida albicans (8 µg/mL), Candida parapsilosis (8 µg/mL), Candida tropicalis (8-32 µg/mL) and Cryptococcus neoformans (8-64 µg/mL).

Neelofar et al., (2011) show the antifungal properties of curcumin against 14 strains of Candida spp., with MICs ranging from 250 to 2000 µg/ml. Not withstanding, our results
demonstrated much lower concentrations than the described above. In a study by Ludwing et al., (2013), curcumin was able to inhibit the growth of *C. neoformans* ATCC strains with MICs between 31.25-62.5 μg/ml. These data corroborate those of the present study, considering that the band was found between 8-64 mg/ml.

Treatment of strains with fluconazole showed no DNA damage after 24 hours of exposition. As shown in Figure 5, curcumin promoted DNA damage in the cells of fluconazole-resistant *C. albicans*. Cells treated with curcumin exhibited percentage damage index of 22.50 ± 5.04 (MIC), 37.50 ± 5.09 (2x MIC) and 74.50 ± 4.59 (4x MIC), when compared to the control group (10.50 ± 4.08) (p < 0.05). It was also observed that *C. albicans* cells resistant to FLC, when incubated with the Fpg enzyme, showed a significant increase in the values of the damage index compared to the control group (p<0.05) after treatment with curcumin (Figure 6).

Lu et al., (2009) found that after 24 and 48 h of incubation at a concentration of 10, 25 and 30 μM, the compound curcumin led to increased DNA migration. According to Ogiwara et al., (2013), curcumin promotes the breakage of double-stranded DNA through inhibition of histone acetylation in DNA. According to Cao et al., (2007), curcumin at high doses (≥10μg/ml) promotes damage to the nuclear DNA and to the mitochondrial DNA, leading the cell to trigger a process of cell death by apoptosis. In this study, curcumin showed breaks in DNA strands at doses greater than 8 μg/ml, which corroborates the findings of the above authors.

Also according to Cao et al., (2007), the damage to nucleic acids caused by curcumin could be due to oxidative stress conditions. It is important to stand out that structural modification in the nucleotide bases may occur as a result of oxidative stress. Several studies point to the role of reactive oxygen species (ROS's) in the mechanism of action of curcumin (Kumar et al., 2014; Sharma et al., 2010).

The strain treatment with fluconazole (24 h exposure) showed no externalization of phosphatidylserine. Cells treated with curcumin (24 h exposure) exhibited annexin-positive cell percentage of 8.89 ± 2.21% (MIC) 21.61 ± 5.42% (2x MIC) and 46.74 ± 5.46 % (4x MIC) compared with the control group (3.97 ± 0.67%) (p < 0.05), according to Figure 4. Cell death by apoptosis is a peculiar suicide program characterized by the externalization of phosphatidylserine on the plasma membrane, the condensation of chromatin and fragmentation of DNA, mitochondrial damage and cytochrome c release from mitochondria to the cytosol (Sukhanova et al., 2012).

Curcumin demonstrated a significant reduction in the number of viable cells after different concentrations (MIC, 2x MIC and 4x MIC) (Figure 1). Moreover, curcumin has caused damage to the cell membrane of fluconazole-resistant *C. albicans*, as shown in Figure 2. According to the experiments of Azad et al., (2013), in mutant strains of *Saccharomyces cerevisiae*, the results of the viability test have suggested that the cells started to become metabolically inactive after treatment with curcumin.

Recently, Kumar et al., (2014) reported that after treatment with curcumin alone or associated with antifungal in *C. albicans* cells, there was an increase in the membrane permeabilization to the PI dye, which agrees with our results, where the fungal growth and the development and the ability to survive under environmental stress conditions are dependent on the integrity of the membrane and the cell wall.
Table 1: Antifungal effect of curcumin against strains of yeast resistant to fluconazole and isolated in Ceará

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC b</th>
<th>Standard MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLC</td>
<td>Curcumine</td>
</tr>
<tr>
<td>C. albicans 1 (289)</td>
<td>16 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. albicans 2 (442)*</td>
<td>32 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. albicans 3 (103A)</td>
<td>32 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. parapsilosis 1 (71A)</td>
<td>32 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. parapsilosis 2 (356)</td>
<td>32 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. parapsilosis 3 (271A)</td>
<td>32 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. parapsilosis 4 (43)</td>
<td>32 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. parapsilosis 5 (72A)</td>
<td>32 µg/mL</td>
<td>32 µg/mL</td>
</tr>
<tr>
<td>C. parapsilosis 6 (848)</td>
<td>16 µg/mL</td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>C. parapsilosis 7 (423)</td>
<td>16 µg/mL</td>
<td>16 µg/mL</td>
</tr>
<tr>
<td>C. tropicalis 1 (574A)</td>
<td>16 µg/mL</td>
<td>16 µg/mL</td>
</tr>
</tbody>
</table>

a FLC-resistant strains of yeast isolated from biological samples.
b FLC – Fluconazole. The MIC was defined as the lowest concentration that produced a 50% reduction in growth of fungal cells after 24h of incubation. The procedure was performed according to CLSI protocol M27-A3. Values are expressed in µg/mL for FLC and Curcumine. MICs represent geometric means of at least three MICs determined on different day’s c.

*The strains were used for determination of action mechanism.

Fig. 1: The cell number viable

The effect of the curcumin on the cell number viable of representative FLC-resistant strain of C. albicans
Fig. 2 The cell membrane stability

The effect of the curcumin on the cell membrane stability of FLC-resistant strains of *C. albicans* curcumin was tested at MIC, 2x MIC and 4x MIC values.

Fig. 3 The mitochondrial membrane potential

An assessment of the mitochondrial membrane potential ($\Delta \psi_m$) of fluconazole-resistant *C. albicans* strains. The cells were labeled with Rh123 (50 nM). The graph shows strains incubated for 24 hours with RPMI-1640 (control), with FLC (64 µg/mL) and Ampho (4 µg/mL) and curcumin at concentrations of MIC, 2x MIC and 4x MIC. The percentage of cells with mitochondrial dysfunction in the representative strains FLC-resistant of *Candida* spp. was evaluated for 24 hours.
Phosphatidylserine externalization, which is observed at an early stage of apoptosis, was shown by annexin V staining. This probe enabled us to detect alterations in phosphatidylserine localization from the inner membrane to the outer membrane. The intensity of fluorescence indicates the quantity of exposed phosphatidylserine on cells treated with curcumin at concentrations of MIC, 2x MIC and 4x MIC. The percentage of Annexin V+ cells in the in the representative strains FLC-resistant of *C. albicans* was evaluated for 24 hours. *p <0.05 compared to control by ANOVA followed by the Newman-Keuls test.

The effects of 24 h incubations with RPMI-1640 (control), with FLC (64 µg/mL), Ampho (4 µg/mL) and curcumin at concentrations of MIC, 2x MIC, 4x MIC on the DNA damage index in *C. albicans*. *p < 0.05 compared with the control using an ANOVA followed by the Newman-Keuls test.
Effects of different treatments on the distribution of damage DNA classes using modified alkaline versions (FPG) of the comet assay were used in FLC-resistant strains of *C. albicans*. The yeasts were exposed to RPMI-1640 (control), with FLC (64 µg/mL), Ampho (4 µg/mL), and curcumin at concentrations of MIC, 2x MIC, 4x MIC. * p< 0.05 compared with the control using an ANOVA followed by the Newman-Keuls test.

When the strains of *C. albicans* were treated with fluconazole, in the 24 hour period, there were no changes observed in Δψm. However, after 24 h of exposure to curcumin, at different concentrations, significant changes were observed in the Δψm of *C. albicans* resistant to fluconazole compared to the control group (p <0.05), shown in Figure 3.

Several studies on the mitochondrial functions and dynamics have been highlighting the crucial role of this organelle in biological processes, such as aging and programmed cell death (Mazzoni *et al.*, 2013). According to Morin *et al.*, (2011), curcumin induces an increase in mitochondrial membrane permeability, resulting in the loss of membrane potential and the inhibition of ATP synthesis, through the opening of the permeability transition pore.

Studies recently published by our group demonstrate that damage to the mitochondria is shown to be precursors of the cell death process, acting irreversibly (Andrade Neto *et al.*, 2014; Silva *et al.*, 2013). According to the experiments of Mahmoud *et al.*, (2011) and Neelofar *et al.*, (2011), curcumin seems to act, also, inhibiting various eukaryotic ATPases, which can come to contribute to changes in the potential of mitochondrial membrane of fungi.

Our results suggest that curcumin induces apoptotic cell death in fluconazole-resistant *C. albicans* strains. In a study conducted by Sharma *et al.*, (2010), curcumin induced apoptosis in strains of *C. albicans*, a fact that corroborates our findings. The apoptotic process induced by the compound curcumin is due to the activation of a transduction pathway with multiple signals (Liu *et al.*, 2013).

In conclusion, the compound curcumin has *in vitro* antifungal activity against fluconazole-resistant strains of *Candida* spp. and *Cryptococcus neoformans*. Based on the
characteristics of cell death observed, it is raised the hypothesis that curcumin seems to exert its antifungal activity at specific sites near the DNA, resulting in cell death by apoptosis. This study points to an effective biological activity of this compound, suggesting that it might be used as an adjunct to the treatment of candidemia.

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