INTRODUCTION

Candidiasis is one of the most common fungal infections in humans caused by Candida species, most notably by Candida albicans. Currently, other species of this genus have been found to cause an increasing number of cases of candidiasis (Mokaddas et al., 2007; Pereira et al., 2010; Das et al., 2011).

The therapy of invasive candidiasis remains a difficult medical problem. Despite the availability of extended-spectrum triazoles, the incidence of invasive infections and resistance to antifungal therapy continue to increase (Pfaller & Diekema, 2010). Widespread use of antifungal agents could be an explanation for the emergence of the more resistant non-albicans species of Candida, such as Candida glabrata (reviewed by Kothavade et al., 2010; Silva et al., 2012).

The azole fluconazole is effective against most Candida species and is used currently as a first-line drug, although different degrees of susceptibility among species have been described. For instance, C. glabrata is inherently less susceptible to fluconazole. In addition, the emergence of fluconazole resistance has been reported in species typically susceptible to this agent, such as C. albicans, Candida tropicalis and Candida parapsilosis (Hajjeh et al., 2004; Yang et al., 2004; Lyon et al., 2010; Oxman et al., 2010; Arendrup et al., 2011), including that observed in Brazilian tertiary-care hospitals (Bruder-Nascimento et al., 2010; Favalessa et al., 2010; Pereira et al., 2010; Furlaneto et al., 2011).

These factors present an urgent need to search for novel compounds with antifungal activity. To this end, efforts have been made in the search for novel antifungal agents from various natural sources. In this regard, baicalein (5,6,7-trihydroxyflavone; Fig. 1) is a flavonoid originally isolated from the roots of the Chinese medicinal plant Scutellaria baicalensis Georgi that has been shown to present a variety of biochemical and pharmacological activities, including antifungal activity. With regard to C. albicans, baicalein was found to inhibit growth (Wong & Tsang, 2009), decrease biofilm production (Cao et al., 2008) and exhibit in vitro synergism with fluconazole and amphotericin B (Huang et al., 2008; Fu et al., 2011). For non-albicans Candida species, baicalein also exhibited potent activity against Candida krusei (Kang et al., 2010).

The aims of this study were: (i) to evaluate the in vitro antifungal activity of baicalein against C. albicans, C. tropicalis and C. parapsilosis; (ii) to evaluate the interaction between baicalein and fluconazole against Candida strains;
and (iii) to monitor yeast growth in the presence of baicalein alone or baicalein plus fluconazole at an ultrastructural level.

**METHODS**

**Fungal strains and growth medium.** Four strains were obtained from the American Type Culture Collection (ATCC): *Candida albicans* ATCC 65458 and ATCC 64530, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 200956. Three clinical isolates, *C. tropicalis* 186.06 and *C. tropicalis* 170.06 recovered from tracheal secretions and *C. parapsilosis* 153.07 recovered from urine, were obtained as stock cultures from the Fungal Genetics Laboratory, University of Londrina, Brazil. Stock cultures were stored at −70 °C, subcultured on Sabouraud dextrose agar and stored at 4 °C.

**Antifungal agents.** Baicalein was purchased from Sigma-Aldrich (purity 98 %), dissolved to a concentration of 5200 μg ml⁻¹ in DMSO and stored at −20 °C. Fluconazole was purchased from Sigma-Aldrich; stock solution was prepared by dissolving in sterile distilled water to a concentration of 6400 μg ml⁻¹. Stock solutions were stored frozen at −70 °C until the day of the test. The final concentration of DMSO was 1 % in all assays.

**Antifungal activity.** MICs were determined using the microdilution method described in the guidelines of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (CLSI, 2002). Serial twofold dilutions were prepared exactly as described in the CLSI document. The spectrophotometric method of inoculum preparation, an inoculum concentration of 1.5 × 10⁸ ± 1.0 × 10⁸ cells ml⁻¹ and RPMI 1640 (Sigma-Aldrich) buffered to pH 7.0 with MOPS (Sigma-Aldrich) at 0.165 mol l⁻¹ were used. To each well of the microdilution plates, 0.1 ml yeast inoculum was added. Drug-free and yeast-free controls were included. Plates were incubated at 37 °C and MIC end points were read after 24 h. The MIC₅₀ was assigned as the lowest concentration of the drugs at which there was 50 % inhibition of growth. Experiments were performed three times with three replicate wells for each experiment.

After MICs had been read, 100 μl samples from the wells corresponding to either MIC₅₀ or 260 μg ml⁻¹ (highest concentration tested) of the baicalein MICs were withdrawn and plated onto Sabouraud dextrose agar plates. Inoculated plates were incubated at 35 °C and the percentage of viable cells was calculated at 24 h.

**Evaluation of drug interactions.** The interaction between baicalein and fluconazole against *C. albicans* ATCC 64550, *C. tropicalis* 170.06 and *C. parapsilosis* 153.07 strains was determined using the checkerboard microdilution method (CLSI, 2002) and included the MIC determinations of each drug alone. Drug concentrations with a range of 0.031–16 μg ml⁻¹ (fluconazole) or 0.13–260 μg ml⁻¹ (baicalein) were used for MIC determinations. The MIC₅₀ value was determined as the lowest concentration of the drugs (alone or in combination) that inhibited growth by 50 % compared with that of drug-free wells. The interaction was classified on the basis of the fractional inhibitory concentration index (FICI). The FICI was calculated using the formula \( \text{FICI} = \left( \frac{\text{MIC}_{A}}{\text{MIC}_{B}} \right) + \left( \frac{\text{MIC}_{B}}{\text{MIC}_{A}} \right) \), where \( \text{MIC}_{A} \) and \( \text{MIC}_{B} \) are the MICs of drugs A and B in combination and Aa and Ba are the MICs of drugs A and B alone. The drug interaction was defined as: synergistic (FICI ≤ 0.5), partial synergistic (0.5 < FICI < 1.0), additive (FICI = 1.0), indifferent (1.0 < FICI < 4.0) or antagonistic (FICI ≥ 4.0) (Eliopoulos & Moellering, 1996).

**Scanning electron microscopy (SEM).** For SEM, *Candida* strains were incubated in RPMI 1640 for 24 h at 37 °C with fluconazole alone (MIC₅₀ value), baicalein alone (MIC₅₀ value) and fluconazole combined with baicalein at MIC values of each compound in combination (see Table 2). A negative control (untreated cells) was carried out simultaneously. After the incubation period, samples were fixed in 2.5 % glutaraldehyde (Electron Microscopy Sciences) in 0.1 M phosphate buffer. Samples were then carefully washed with 0.1 M phosphate buffer. Post-fixation was carried out for 1 h at 25 °C with 1 % osmium tetroxide in 0.1 M phosphate buffer. Samples were gently dehydrated in graded ethanol, critical-point dried in CO₂ (BALTEC DCP 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and viewed in a FEI Quanta 200 scanning electron microscope.

**RESULTS AND DISCUSSION**

**Antifungal activity**

Although antifungal activity of baicalein has been shown previously, the data are largely limited to *C. albicans*, which is a commonly chosen model organism. To the best of our knowledge, there have been no studies of baicalein activity against *C. tropicalis* and *C. parapsilosis*.

The antifungal activity of baicalein against the *Candida* strains tested is shown in Table 1. Among the *Candida* strains, there was a variation in sensitivities. The MIC₅₀ of baicalein alone against the six *Candida* strains ranged from 13 to 104 μg ml⁻¹. *C. albicans* ATCC 64548 and both *C. parapsilosis* strains were the most susceptible to baicalein, whereas *C. tropicalis* 186.06 was less sensitive than the other strains.

In this study, for all three species tested, exposure to baicalein at MIC₅₀ values obtained for each strain and at 260 μg ml⁻¹ resulted in a high loss of viability, revealed by a reduction in fungal colony counts compared with the untreated control (Table 1), suggesting that baicalein is capable of antifungal activity against *C. albicans* and non-*albicans Candida* species. These data represent what we believe to be the first report of the antifungal capability of baicalein against *C. tropicalis* and *C. parapsilosis*. According to Huang et al. (2008), the mechanism of baicalein against fluconazole-resistant *C. albicans* may be in part due to a reduction in the extrusion of drug out of the yeast cells by inhibition of efflux pumps. Baicalein also appears to inhibit *C. albicans* cells by inducing programmed cell death (apoptosis) (Dai et al., 2009).

**Drug interaction assay**

Further studies of fluconazole plus baicalein combinations were conducted against the three *Candida* strains that were

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Fig. 1. Chemical structure of baicalein.
Table 1. Inhibitory concentrations of baicalein (BE) against Candida strains and effects on the yeast viability

The data are mean values of the percentage of viable cells at 24 h with respect to the controls.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; of BE (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Viable cells (% of c.f.u.)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>BE at MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>BE at 260 µg ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. albicans ATCC 64548</td>
<td>13</td>
<td>4.5</td>
</tr>
<tr>
<td>C. albicans ATCC 64550</td>
<td>26</td>
<td>9.78</td>
</tr>
<tr>
<td>C. tropicalis 186.06</td>
<td>104</td>
<td>3.23</td>
</tr>
<tr>
<td>C. tropicalis ATCC 200956</td>
<td>52</td>
<td>8.8</td>
</tr>
<tr>
<td>C. parapsilosis ATCC 22019</td>
<td>13</td>
<td>10.0</td>
</tr>
<tr>
<td>C. parapsilosis 153.07</td>
<td>13</td>
<td>6.0</td>
</tr>
</tbody>
</table>

less susceptible to fluconazole: fluconazole-susceptible dose-dependent C. albicans ATCC 64550 and C. parapsilosis 153.07 and fluconazole-sensitive C. tropicalis 170.06 exhibiting a high MIC value. As shown in Table 2, the antifungal activity of fluconazole plus baicalein was greater than the individual contribution of each agent for the strains analysed. According to FICI values, the combination demonstrated partial synergistic properties in vitro against C. albicans and C. tropicalis. The combination of baicalein and fluconazole produced a synergistic action against C. parapsilosis. In this case, the fluconazole MIC changed from susceptible dose-dependent (MIC<sub>50</sub>=16 µg ml<sup>-1</sup>) to susceptible (MIC<sub>50</sub>=0.125 µg ml<sup>-1</sup>). For C. parapsilosis, baicalein significantly potentiated the antifungal effect of fluconazole. Thus, the combination of baicalein with fluconazole may represent an attractive prospect for the development of new management strategies for candidiasis caused by C. parapsilosis. The incidence of C. parapsilosis has increased dramatically over the past decade. This yeast can cause candidiasis that can vary from relatively mild skin mycoses to life-threatening systemic or disseminated disease (reviewed by van Asbeck et al., 2009). It is also worth noting the emergence of fluconazole resistance in C. parapsilosis worldwide, including in Brazilian tertiary-care hospitals (Bruder-Nascimento et al., 2010; Furlaneto et al., 2011). Recently, Huang et al. (2008) demonstrated that baicalein in combination with subinhibitory concentrations of fluconazole was able to strongly inhibit the proliferation of C. albicans. Fu et al. (2011) showed that a combination of baicalein and the polyene macrolide amphotericin B enhanced C. albicans apoptosis accompanied by an increase in reactive oxygen species. According to these authors, these characteristics suggest a promising application of baicalein for clinical drug-resistant yeasts.

**SEM analysis**

In this study, we used SEM to monitor yeast growth in the absence or presence of baicalein (Fig. 2). Untreated cells of C. albicans ATCC 64550 consisted of blastoconidia presenting a smooth surface with a high capacity for producing filamentous forms, mostly pseudohyphae (Fig. 2a). Cells exposed to fluconazole alone (MIC<sub>50</sub>=16 µg ml<sup>-1</sup>) had a smooth surface and oval shape, and showed a tendency to aggregate (Fig. 2b). For cells exposed to baicalein alone (MIC<sub>50</sub>=26 µg ml<sup>-1</sup>), we observed the presence of blastoconidia, as well as filamentous forms (Fig. 2c), similarly to what was seen after the treatment with baicalein in combination with fluconazole (Fig. 2d). In addition, under these growth conditions, we observed the presence of flocculent extracellular material associated with filamentous cells.

C. tropicalis 170.06 untreated cells exhibited a normal budding profile with a lower proportion of filamentous forms (Fig. 2e). Extracellular material was seen surrounding the filamentous cells. Cells exposed to fluconazole alone (MIC<sub>50</sub>=8 µg ml<sup>-1</sup>) had an oval shape and aggregated (Fig. 2f), similarly to what was found for C. albicans. After exposure to baicalein alone (MIC<sub>50</sub>=2.6 µg ml<sup>-1</sup>), we

Table 2. MICs of baicalein (BE) and fluconazole (FLC), alone and in combination, and FICI results against Candida strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>FICI</th>
<th>Interaction</th>
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<tbody>
<tr>
<td></td>
<td>BE</td>
<td>FLC</td>
<td>BE + FLC&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. albicans ATCC 64550</td>
<td>26</td>
<td>16</td>
<td>13/0.5</td>
</tr>
<tr>
<td>C. tropicalis 170.06</td>
<td>2.6</td>
<td>8</td>
<td>0.13/4</td>
</tr>
<tr>
<td>C. parapsilosis 153.07</td>
<td>13</td>
<td>16</td>
<td>2.6/0.125</td>
</tr>
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<sup>*</sup>MICs in combination are expressed as [BE]/[FLC].
observed the presence of blastoconidia, as well as a great capacity for producing filamentous forms (Fig. 2g). Following treatment with baicalein in combination with fluconazole, cells did not produce pseudohyphae, and a profusely flocculent extracellular material connecting yeast cells was seen, resembling a biofilm-like structure (Fig. 2h).

For C. parapsilosis 153.07, SEM analyses showed that untreated cells consisted of blastoconidia with a normal budding profile, and no extracellular material was seen (Fig. 2i). A similar morphology was observed for cells exposed to fluconazole alone (MIC$_{50}$=16 µg ml$^{-1}$), except that they were aggregated (Fig. 2j). However, cells exposed to baicalein (MIC$_{50}$=13 µg ml$^{-1}$) and baicalein in combination with fluconazole showed a profusely flocculent extracellular material connecting yeast cells (Fig. 2k, l), similarly to that found for C. tropicalis. In general, for C. parapsilosis, pseudohyphal cells did not occur. These data represent a preliminary investigation at an ultrastructural level of the effect of baicalein on Candida growth. It is therefore essential that more studies be conducted to investigate the effect of baicalein alone and in combination with fluconazole on yeast-cell morphology.

This study represents the first report, to our knowledge, of the presence of profusely flocculent extracellular material following yeast growth in the presence of baicalein alone or in combination with fluconazole. The flocculent extracellular material was seen either associated with filamentous cells or connecting yeast cells, suggesting that the flocculent material did not represent the baicalein and/or fluconazole drugs. According to the literature, extracellular material is often associated with Candida biofilm structure. In biofilms, the role of extracellular material includes adhesion (McCourtie & Douglas, 1985) and resistance to antifungal agents (Baillie & Douglas, 2000; Al-Fattani & Douglas, 2006). Although it has been shown that baicalein affects biofilm production by C. albicans (Cao et al., 2008), analysis of the effect of baicalein on the biofilm matrix was not carried out. According to these authors, biofilm inhibition occurred as a consequence of the effect of baicalein on the cell-surface hydrophobicity of the biofilm.

Taken together, the results obtained in this study demonstrated that baicalein reduces growth and cell viability. A synergistic effect of baicalein and fluconazole against C. parapsilosis was observed. More studies need to be conducted in order to establish the mechanism of growth inhibition by baicalein and the mechanism of synergy between baicalein and fluconazole.

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