Anti-candida and anti-enzyme activity and cytotoxicity of 2-phenyl-4H-chromen-4-one

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This study determined the fungicidal concentration (MFC), anti-enzymatic (production of proteinases and phospholipase), and cytotoxic activities of 2-phenyl-4H-chromen-4-one against different strains of Candida sp. The results were: MIC> 62.5 µg/ml and MFC>250 µg/ml for C. albicans; MIC/MFC = 31.25 µg/ml for C. parapsilosis; MIC/MFC= 62.5 µg/ml for C. famata; MIC/MFC=125 µg/ml for C. glabrata and MIC = 15.62 µg/ml for C. lipolytica. Values for phospholipase and proteinase before and after exposure of yeast to the compound had no statistical differences. For the cytotoxicity test, there were no statistical differences between the tested and control groups. Data from this study provide evidence that 2-phenyl-4H-chromen-4-one could be an alternative source for the treatment of fungal infections caused by Candida.

Key words: Candida albicans, antifungal activity, antifungal agents, anti-enzyme activity, flavone.

INTRODUCTION

Species of Candida spp. are often cited as opportunistic microorganisms and / or commensal found in the normal flora of the digestive and genitourinary tracts of humans and animals (Kam and Xu, 2002). Lately, these species have become clinically important, because they are responsible for superficial and deep infections (Saghrouni et al., 2013) and due to the increasing number of immunocompromised patients and the advances in intensive medical care (Pfaller and Diekema, 2007).

Some species have become important pathogens of nosocomial infections, including C. albicans, which is associated with high mortality rates of above 30% despite the availability of already established (azoles) or new antifungal treatment options (Pfaller and Diekema, 2007; Horn et al., 2009). Some authors emphasize the need to implement appropriate control measures, which requires a high knowledge of the biology, epidemiology, and antifungal treatment of Candida species, including C.
albicans, which are recognized as being particularly complex (Saghrouni et al., 2013).

However, the interaction between antifungal drugs and fungi, molds, and hosts as well as between the antifungal drug and the host are very complex. The establishment of the antifungal activity of a new drug offers only an in vitro of the information needed to predict the outcome of treating infection with a new drug. The improvement of in vitro assays and susceptibility testing against the constant emergence of new drugs makes for indispensable in vitro tools and meaningful contributions to the selection of the most appropriate antifungal agent (Johnson, 2008).

Medicinal plants have been used to fight diseases since the early days of mankind, and today these plants serve as a guide in supporting drug discovery. One of the main groups of phenolic compounds found in plants is the flavonoids. It has been known for many years that this class of compounds of plant origin has potential bioactivity. Flavonoids are subdivided into flavones according to their chemical structure. Flavones are heterocycles of six members which contain one oxygen and ketone group. The general methods to obtain flavones are the Allan–Robinson reaction, Auwers synthesis and Baker–Venkataraman rearrangement (Eicher and Hauptmann, 2003). The flavones are a class of naturally occurring compounds that are widely distributed in vascular plants, which are well known for having a wide range of pharmacological activities such as antiallergic, antibacterial, antidiabetic, antiinflammatory, antiviral, antiproliferative, antimutagenic, antithrombotic, anticarcinogenic, hepatoprotective, oestrogenic, insecticidal, and antioxidant activities (Fesen et al., 1994; Duthie and Crozier, 2000; Midleton et al., 2000; Fukai et al., 2002; Sohn et al., 2004; Cushnie and Lamb, 2005; Isobe et al., 2006). However, the antifungal properties of the flavone, 2-phenyl-4H-chromen-4-one, remain unknown.

The aims of this study were to evaluate the antifungal activity and the anti-enzymatic activity of 2-phenyl-4H-chromen-4-one against different strains of Candida and to evaluate their cytotoxicity. The hypothesis to be tested is that 2-phenyl-4H-chromen-4-one will show antifungal activity against Candida and low cytotoxicity.

MATERIALS AND METHODS

Mass spectrometry

The mass spectra were obtained on a Shimadzu GCMS-QP2010 SE with a split–splitless injector and equipped with a Rtx-5MS capillary column (30 m x 0.25 mmID); helium was used as the carrier gas (56 Kpa).

Infrared analysis

The IR spectra were taken on a Agilent Technologies Cary 640 FTIR spectrometer in KBr pellets.

Nuclear magnetic resonance

NMR spectra were recorded on a Bruker DPX 300 spectrometer (300.13 MHz for 1H and 75.48 MHz for 13C) at 300 K.

Strains

Candida albicans (33), C. parapsilosis (2), C. famata (2), C. glabrata (2) and C. lipolytica (2) strains were used for determining the in vitro anti-Candida activity of 2-phenyl-4H-chromen-4-one. These strains of C. albicans were collected from patients with Chronic Atrophic Candidiasis (CAC) who use dentures and who were enrolled in the Center for Diagnosis of Diseases of the Mouth (CDDB), School of Dentistry, Federal University of Pelotas (FOP-UFPel). The diagnosis of CAC was clinical, and microbiological as previously described (Lund et al., 2009).

In vitro anti-candida activity

Inoculum

The strains of C. albicans and non-albicans Candida were subcultured onto Sabouraud Dextrose Agar with 0.2 mg/mL of chloramphenicol at 36°C for 24 h. After incubation, these strains were individually inoculated into tubes containing 5 mL of a sterile 0.85% saline solution and the yeast suspension was adjusted to a 0.5 McFarland standard (which is approximately 10^6 CFU/mL). After that, the inoculum was re-suspended to obtain a final concentration of 0.5 × 10^−2.5 × 10^7 in RPMI 1640 medium (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with 165 mmol L\(^{-1}\) of morpholinepropanesulfonic acid (MOPS; Vetec).

Determination of MIC

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 2-phenyl-4H-chromen-4-one was determined by using broth microdilution techniques as described by the Clinical and Laboratory Standards Institute for yeasts (M27-A2). The stock solution of 2-phenyl-4H-chromen-4-one was prepared in a 70% ethanol solution at the concentration of 500 μg mL\(^{-1}\). A powder consisting of 2-phenyl-4H-chromen-4-one was previously weighed and dissolved in ethanol at the concentration of 500 μg mL\(^{-1}\). The solutions were diluted in RPMI medium, and the final drug concentrations ranged from 0.49 to 250 μg mL\(^{-1}\). Two replicates were made for each concentration of the tested compounds.

After 48 h of incubation at 35°C, the MIC was determined visually by comparison with the drug-free growth control well. The MIC was defined as the lowest concentration of the antifungal agent that prevented visible fungal growth.

Determination of MFC

Each inoculum from the previous test that did not show growth was subcultured on agar plates. After 24 h of incubation, the reading was determined by the visible growth of strains. The CFM was considered to be the lowest concentration that prevented visible growth.

In vitro anti-enzyme activity (Carvalho et al., 2015)

Phospholipase

Reduced egg yolk agar (Reya) was used and the plates were
inoculated (4 per isolate) with a 5 µL suspension of each isolate (108 cells/mL) and then cooled until they were dry. After the plates were incubated at 37°C aerobically for 48 h, the diameters of zones of precipitation around the colonies were measured. The enzyme activity was determined by the radius hyaline zone/diameter of the colony (Pz). Pz values equal to 1 (Pz=1) indicated the absence of enzyme activity, while values greater than 1 (Pz>1) indicated positive levels of phospholipase. Each experiment was repeated twice.

**Proteinase**

Clinical isolates were inoculated in tubes containing 5 mL of YPD medium and incubated at 37°C for 18 h. After the incubation, aliquots of 1.5 mL of culture were transferred to Eppendorf tubes and centrifuged at 3000 rpm for 5 min at 4°C. The cell pellets were re-suspended in saline (NaCl 0.9 wt%) and centrifuged using double speed that was used for removal of debris through cultivation. The concentrations of the suspensions of the strains were standardized, using the index range of 0.5 MacFarland (Approximately 1x106 CFU/mL), and 1 mL volumes were inoculated at equidistant points in the middle Proteinase Agar. The plates with different inocula were inoculated at 37°C for four days. The tests were performed in duplicate. The values of the Precipitation Zone (PZ) were obtained by dividing the diameter of the colony by the diameter of the colony plus the precipitation zone (PZ = 1, Negative; PZ ≥ 0.64 <1, Middle-Positive, Positive-PZ ≤ 0.63 High).

**Cytotoxicity assay (Carvalho et al., 2015)**

The cell culture medium was Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL). Mouse fibroblasts of the 3T3/NIH immortalized cell line were maintained as a stock culture in DMEM and incubated at 37°C in a humidified atmosphere of 5% CO2 in air until subconfluency was reached. Cells were incubated with the same concentrations of 2-phenyl-4H-chromen-4-one were used in the antifungal assay. These compounds were dissolved in DMSO and added to the DMEM supplemented with 10% FBS to the desired concentrations.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell metabolic function according to mitochondrial dehydrogenase activity. Mouse fibroblasts (3T3/NIH; 2 x 104/well) were maintained in DMEM in 96 well plates for 24 h. DMEM was removed and replaced with 200 µL of extract from different groups with 10% FBS. Cytotoxicity produced by the different extracts was assessed at 24 h after cell exposure time. After removing the extracts, the cells were washed with phosphate-buffered saline (PBS), and then 200 µL of medium in 20 µL of MTT solution (5 mg of MTT/ml DMEM) were added to each well. After 5 h of incubation at 37°C, the blue formazan precipitate was extracted from the mitochondria using 200 µL/well of dimethyl sulfoxide (DMSO) on a shaker for 5 min at 150 rpm. The absorption at 540 nm was determined spectrophotometrically.

The cell viability was analyzed using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA) statistical analysis software. The statistical analysis was performed by one-way ANOVA with the level of significance set at p<0.05.

### RESULTS AND DISCUSSION

The results obtained for the minimum inhibitory concentration and minimum fungicide are shown in Table 1. The results show respectively, MIC and MFC 62.5 µg/ml for *C. albicans*; MIC 0.48µg/ml and fungistatic behavior for *C. parapsilosis*; MIC and MFC 62.5 µg/ml for *C. glabrata* and *C. famata*; MIC 15.62 µg /ml and fungistatic behavior for *C. lipolytica*. The syntheses of flavones usually start from o-hydroxyacetophenones. The 2-phenyl-4H-chromen-4-one was obtained conform literature (Munawar and Groundwater, 1999). The Flavone was crystallized in colourless needles of mp 97°C; yield of isolated product was70%. The structure of flavone was confirmed by infrared analysis, mass spectrometry and nuclear magnetic resonance of 1H and 13C; MW: 222.06. CG-MS m/z (%): 120 (M+, 75), 194 (M+, 60), 222 (M+, 100). NMR 1H: 7.3-7.8 (Ph, 5H); 7.13 (H, 1H); 7.89-8.17 (Ar, 4H). NMR 13C: 131.7; 129.0; 126.2; 131.1; 123.2; 155.7; 118.4; 134.2; 125.7; 177.0; 106.9; 162.5. IR: ν (cm⁻¹) 3100-3200; 1600-1700, 1300-1400, 700-800.

The research hypothesis was confirmed since data from this study provide evidence that 2-phenyl-4H-chromen-4-one could be an alternative source for the treatment of fungal infections caused by *C. albicans* and *non-albican Candida*. The fungal species chosen for this study were *C. albicans*, *non-albicans Candida* (*C. parapsilosis*, *C. famata*, *C. glabrata*, *C. lipolytica*). The Candida species were specially chosen because they are present in 75% of the commensal microflora of normal healthy individuals (Mulu et al., 2013) and infections caused by the same have increased dramatically in the last three decades, setting up a current clinical challenge (Samaranayake et al., 2002).

Several factors determine the severity and type of

<table>
<thead>
<tr>
<th>Structural formula</th>
<th>C. albicans</th>
<th>C. parapsilosis</th>
<th>C. Famata</th>
<th>C. glabrata</th>
<th>C. lipolytica</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC(µg/ml)</td>
<td>&gt;62.5</td>
<td>31.25</td>
<td>62.5</td>
<td>125</td>
<td>15.6</td>
</tr>
<tr>
<td>MFC(µg/ml)</td>
<td>&gt;250</td>
<td>31.25</td>
<td>62.5</td>
<td>125</td>
<td>15.6</td>
</tr>
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*Table 1. In vitro antifungal activity of 2-phenyl-4H-chromen-4-one dissolved in ethanol 70% against Candida albicans and non-albicans Candida.*

2-phenyl-4H-chromen-4-one
infection that may be caused by this type of yeast, but the mechanism by which the transformation occurs from a harmless commensal to an aggressive pathogen is not completely understood (Santos and Braga, 2013). However, it is known that C. albicans virulence has attributes that promote adaptation, interaction with host structures, and the circumvention of host immune responses (Santos and Braga, 2013). One attribute of virulence is the secretion of hydrolytic enzymes such as phospholipase and proteinase. Thus, compounds that inhibit or decrease the release of these enzymes could prevent the establishment and proliferation of yeast, preventing the development of the disease. Therefore, it was necessary to test the activity of phospholipase anti-enzyme and proteinase with the administration of flavone.

Moreover, the diagnosis and the inappropriate and indiscriminate use of antifungals cause a predisposition to recurrent infections as a consequence of the acquisition of antifungal resistance by common pathogenic microorganisms (Akpan and Morgan, 2002). Two types of resistance are detectable in fungi: innate resistance, as in C. krusei against fluconazole, or acquired, as in after an antifungal treatment, as in the case of C. albicans being responsible for oral infections in HIV-positive patients treated with azole compounds (Yang and Lo, 2001). Because these episodes of resistance to conventional antifungals it is essential to search for new treatment alternatives in order to ensure the death or inhibition of the growth of pathogenic microorganisms or the inhibition of the enzymes that are responsible for their adhesion and subsequent colonization.

Flavonoids are polyphenolic compounds that are biosynthesized through the phenylpropanoid pathway and the acetate (Mann, 1987). The main sources of Flavonoids are fruits like grapes, cherries, apples, currants, citrus fruits and vegetables like peppers, tomato, spinach, onions, broccoli (Barnes et al., 2001). Medicinal and spice plants that contain these flavonoids have been used for thousands of years in Oriental medicine, but have still not spread to the West (Midleton et al., 2000). Flavones are a class of flavonoids that are divided in two groups. Flavones constructed by two-phenylchromone are characterized by a natural product of low molecular weight that is a participant in the photosynthetic reaction. Some important biological characteristics are already linked to the flavones, including antioxidant, anti-inflammatory, antiviral, antitumor, and antiatherosclerotic properties (Midleton et al., 2000). However, since the antifungal and anti-enzymatic activities of this compound have not yet been reported in the scientific literature, it is necessary to fill this gap. Based on the methodology applied in this study, the results show significant antifungal activity. Moreover, in this study, most of the compounds have similar results for MIC and MFC, as drugs behaving as fungicide. Immune depression in patients with only the inhibition of fungal growth may not be sufficient to prevent the spread of Candida (Elewiki and Ohio, 1993).

The choice of a broth dilution test to determine the sensitivity of yeasts to antifungal therapy, as recommended by The National Committee for Clinical Laboratory Standards (M27-A2 protocol), was chosen for the advantages of easy reproducibility (Arthington-Skaggs et al., 2002), low cost, and sensitivity. In addition, this method requires only a small amount of each sample, which is used in various tests (Ostrosky et al., 2008). The methodology of the Standard M27-A2 recommends the reading of the results for Candida after 48 h (NCCLS). In our study, we chose to read at two times: 24 and 48 h. This was due to a lack of knowledge about the action and behavior of this novel compound. The two required readings and their comparison resulted in the observance of the occurrence of trailing and the partial inhibition of growth over a wide range of concentrations of the antifungal.

In addition, the antifungal activity of the compound 2-phenyl-4H-chromen-4-one was tested for its possible ability to inhibit the secretion of the hydrolytic enzymes, proteinase and phospholipase. Protein and enzyme secretions are considered an essential process in fungi survival, and the characteristics of these secreted proteins defined many of the functional capabilities of these microorganisms (Carvalho et al., 2015). These enzymes appear as important pathogenic factors of C. albicans. Values for phospholipase and proteinase (Pz) before and after exposure of yeast to the compound had no statistical differences. Figures 1 and 2 illustrate the dose-response curves of the action of flavone tested on the inhibition of proteinase and phospholipase.

The results of the test for cytotoxicity (Figure 3) indicate that these products showed a low toxicity against fibroblasts 3T3/NH concentrations of the flavones tested. For a better interpretation of the results, these data were analyzed statistically and the wells containing the product were compared to the control. One way analysis of variance (ANOVA) was not statistically significant. In order to compare the action of the compounds on cells and control cells respectively we used the Duncan's statistical test. The results show that there was a significant difference between the wells in which compound was added and those in which it was not added.

Regarding cytotoxicity, according to the present findings, the compounds tested showed no significantly different results where there was the same inclusion. They showed low cytotoxicity, reinforcing the possible use of these antifungal agents. Among antifungal agents, therapeutic agents that are used in most topically in most cases of oral candidiasis, are polienios (Nystatin and amphotericin B) and azoles (itraconazole, miconazole and clotrimazole) (Akpan and Morgan, 2002; Yang and Lo, 2001). Even the most widespread treatments have certain limitations due to side effects such as toxicity and the emergence of resistant strains (Yagiela et al., 2004),
Figure 1. Dose response curve in the production of extracellular proteinase.

Figure 2. Dose response curve in the production of extracellular phospholipase.

Figure 3. Test for cytotoxicity against fibroblasts 3T3/NIH concentrations of the flavones tested.
which confirms the need for new studies investigating other agents with potential for treating this mycosis and low cytotoxicity (Nobre et al., 2002).

**Conclusion**

Based on the methods employed, it was found that 2-phenyl-4H-chromen-4-one is a promising antifungal agent that has low cytotoxicity. However, there is a need for a specific study on the safety and efficacy of this in vivo use, and clinical trials are still needed to evaluate the practical relevance of in vitro results.

**Authors’ contributions**

Simone Oliveira, Rafael Lund, Rodrigo V. Carvalho and Evandro Piva were responsible for the biological assays and writing and revision of the final scientific paper. Claudio de Pereira was responsible for the synthesis, purification and identification of flavone.

**Conflict of interest**

The authors have not declared any conflict of interest.

**Abbreviations**

CAC, Chronic atrophic candidiasis; DMEM, Dulbecco’s modified eagle’s medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; IR, infrared analysis; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; MOPS, morpholinepropanesulfonic acid; NMR, nuclear magnetic resonance; RPMI, roswell park memorial institute; PZ, precipitation zone; YPD, yeast peptone dextrose.

**REFERENCES**


