Original article

Synergistic antifungal activity of the lipophilic fraction of Hypericum carinatum and fluconazole

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ABSTRACT

Hypericum species, Hypericaceae, are recognized as a source of therapeutical agents. Purified fractions and isolated compounds have been shown antimicrobial activity. As the indiscriminate use of antifungals and the increase of infections caused by emerging species are leading to the search of new alternative treatments, the aim of this study was to continue the study with Hypericum carinatum Griseb. lipophilic fraction, rich in phloroglucinol derivatives, investigating the effect of its association with fluconazole against emerging yeasts (Candida krusei, C. famata, C. parapsilosis and Cryptococcus neoformans). The synergistic activity between H. carinatum lipophilic fraction and fluconazole was assessed by two methodologies for multiple dose–response analysis: checkerboard and isobologram. Regarding synergistic experiments, the effect of the association was higher than the effect of fluconazole alone against Candida krusei and C. famata isolates (MIC fluconazole decreased about eight and four folds, respectively), suggesting that somehow, H. carinatum lipophilic fraction compounds are facilitating the action of this drug. On the other hand, when tested against Cryptococcus neoformans and C. parapsilosis, fluconazole showed better results than the association. Thus, against Candida krusei and C. famata, the lipophilic fraction of H. carinatum was able to reduce the MIC values of fluconazole and could be considered as a potential alternative to be used against emerging yeast species.

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Introduction

Fungal infections are associated with high morbidity and mortality rates. In the last decades, emerging fungal infections, also called opportunistic infections, have drawn attention due to the high number of immunocompromised patients affected (Silva et al., 2012). Some species of Candida and Cryptococcus, previously considered nonpathogenic, are now recognized as opportunistic pathogens responsible for deep-seated mycoses (Vandeputte et al., 2012; Alcazar-Fuoli and Mellado, 2014).

The high incidence of infection by Candida species is due to many factors such as immuno suppressive therapies, invasive surgical procedures and use of broad-spectrum antibiotics (Pfaller et al., 2012). Candida albicans is still the most prevalent species but infections caused by non-Candida albicans (NCA) have significantly increased, bringing even more worrying scenario due to high resistance to antifungal exhibited by these microorganisms (Pfaller et al., 2010, 2012). Since the epidemiology of these fungal infections is currently changing, new alternatives are needed in case of antifungal therapy failure (Alcazar-Fuoli and Mellado, 2014).

Because of yeasts inconstant susceptibility profiles and lack of different molecular targets, drug combinations appear as a strategy for therapy due to the multiplicity of targets (Musiol et al., 2014). The main advantage of these combinations is the synergistic interaction, in which the antifungal activity is better than the individual effects of each compound.

Plants from genus Hypericum, Hypericaceae, are an important source of therapeutic agents. Purified fractions and isolated compounds have shown antibacterial and antifungal activities (Barros et al., 2013; Dulger and Dulger, 2014). Barros et al. (2013) have reported the antifungal activity of lipophilic extracts of five Hypericum species (H. carinatum, H. caprifoliatum, H. linoides, H. myriathum and H. polyanthemum) against several emerging fungal strains, with better results for H. carinatum. According to these authors, dimeric phloroglucinol derivatives (uliginosin B, hyperbrasilol B and japonicin A), present in lipophilic fractions could

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be responsible for the antifungal activity showed by *Hypericu-
mum* species. Other compounds with phloroglucinol pattern such as benzo-
pyrans and benzophenones also showed antifungal activity.

Due to the indiscriminate use of antifungals and the increase of
infections caused by emerging species new alternative treat-
ments are necessary. Thus, the aim of this work was to continue
the study with *Hypericum carinatum* Griseb. lipophilic fraction
(LF), investigating the effect of its association with fluconazole
against the emerging yeasts *Candida krusei*, *C. famata*, *C. parapsilosis*
and *Cryptococcus neoformans*. The synergistic activity between LF
and fluconazole was assessed by two methodologies for multiple
dose–response analysis: checkerboard and isobologram.

**Materials and methods**

**Plant material**

Aerial parts of *Hypericum carinatum* Griseb., Hypericaceae, were
collected in Rio Grande do Sul, Brazil, in December of 2009. Voucher
specimens are deposited in the herbarium of Federal University
Rio Grande do Sul (ICN). Plants collection was authorized by
IBAMA (Brazilian Institute of Ambient Media and Renovable
Natural Resources) (n° 003/2008, protocol: 02000.001717/1008-60).

**Lipophilic fraction preparation**

The dried and powdered plant material (ca. 500 g) was extracted
with hexane at room temperature. The extract was pooled, evap-
orated to dryness under reduced pressure, and the epicuticular
waxes were removed by acetone treatment. The lipophilic fraction
(LF) was stored at −20 °C until biological and chemical evaluation.

LF was analyzed by HPLC using a Shimadzu 600 pump (LC-
6AD) and a Shimadzu SPD-10A dual absorbance detector.

The separations were carried out with an isotropic solvent system
(60% acetonitrile:40% water) to benzophenones determination and
(95% acetonitrile, 5% water, 0.01% trifluoroacetic acid) to
phloroglucinol derivatives using a Waters Nova-Pack C18 column
(4 μm, 3.9 mm × 150 mm) adapted to a Waters Nova-Pack C18 60 A
(3.9 mm × 20 mm) guard column. The flow rate was 1 ml/min, the
detection sensitivity was 1.0 AUs, and the detection was performed
at 270/220 nm at room temperature.

Constituents were identified by comparison with the retention
times of the authentic samples and co-injection of isolated com-
ponents. The yields were expressed in % (weight compound per
weight dry extract) as mean of two injections.

**LF toxicity**

The experimental protocol was approved by Local Ethical
Committee (Protocol 23081, UNIPAMPA). The toxicity of LF was
evaluated by cell viability test and comet assay, according to Güez
et al. (2012), analyzing three different fraction concentrations: 500,
250 and 100 μg/ml.

**Fungal strains**

Four resistant strains to fluconazole were used in this study.
Interpretative criteria of resistance were used according to break-
points from M27-S4 document (CLSI, 2012) to *Candida* and
according to Espinel-Ingroff et al. (2012) to *Cryptococcus neofo-
rmans*. All strains are deposited in the Mycology Collection of Federal
University of Rio Grande do Sul, Brazil: *Candida famata* (RL23) ori-
ginates from hemoculture, *C. krusei* (CK03) from National Program
of Quality Control, *C. parapsilosis* (RL11) from urine and *Crypto-
coccus neoformans* (HCCR 01) from environment (environmental
pathogenic). *C. krusei* ATCC 6258 was included as control in the
susceptibility testing.

**Antifungal activity**

The screening for antifungal activity was carried out with a
concentration of 500 μg/ml. In order to achieve the test con-
centration, samples were solubilized with dimethyl sulfoxide 2%
(DMSO) and sabouraud dextrose broth (SDB). Further, the minimal
inhibitory concentration (MIC) was determined by the broth
microdilution method according to M27-A3 protocol (CLSI, 2008).
The MIC was defined as the lowest concentration of LF in which
the microorganism tested did not demonstrate visible growth. In
microdilution experiments, samples were solubilized with DMSO
2% and RPMI-MOPS medium (RPMI 1640 medium) containing l-
glutamine, without sodium bicarbonate buffered to pH 7.0 with
0.165 mol/l of MOPS buffer. The concentrations of LF ranged from
1.9 to 500 μg/ml and all experiments were carried out in duplicate.
Control with DMSO 2% was previously performed.

**Association studies**

**Checkerboard assay**

The effect of fluconazole combined with LF was evaluated in
quadruplicate using the checkerboard method (Johnson et al., 2004)
with slightly modifications. The fluconazole final concentrations
ranged from 0.5 to 32 μg/ml for *C. famata* and *C. neoformans*,
and 4 to 64 μg/ml for *C. krusei* and *C. parapsilosis*. On the other
hand, the concentration of LF ranged from 31.25 to 250 μg/ml
for *C. famata* and *C. neoformans* and 4 to 250 μg/ml for *C. kru-
sei* and *C. parapsilosis*. Plates were incubated at 37 °C for 48 h
and then, the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) was used to assess the fungal
cell viability. Interaction was evaluated algebraically by deter-
mining the fractional inhibitory concentration index (FICI) defined as
the sum of the MIC of each drug in combination, divided by the
MIC of the drug used alone. An FICI < 0.5 is considered synergic;
>0.5 and ≤1 additive; >1 and ≤4 indifferent, and >4 antagonistic
(Kontoyiannis and Lewis, 2003).

**Isobologram**

The isobologram was performed with the association of LF and
fluconazole against *C. krusei* (CK03) and *C. parapsilosis* (RL11).

A curve concentration-effect of LF or fluconazole was deter-
mined with logarithmic concentrations, in order to obtain the IC$_{50}$
(inhibitory concentration 50%) by non-linear regression. Then, with
these results, curves concentration-effect of association were also
performed by non-linear regression (Tallarida, 2006, 2007). The
proportion of combinations is demonstrated in Table 1.

Theoretical additive curves (IC$_{50}$ add) were calculated to each
combination according the equation:

\[
\text{Conc.add} = f \times \text{Conc. fluconazole} + (1 - f) \times \text{Conc. Fraction}
\]

where, Conc.fluconazole and Conc.Fraction represent the equi-
effective concentration of each treatment alone and f is the fraction
of each sample that composes the active concentration of associ-
ation (in this study two f values 0.5 (50:50) and 0.7 (70:30) were
used): Conc.add is the total concentration and its variance was cal-
bulated by this equation:

\[
\text{Var IC}_{50}\text{add} = f^2 \times \text{Var IC}_{50}\text{ fluconazole} + (1 - f)^2 \times \text{Var IC}_{50}\text{ fraction}
\]

From these variances, confidence intervals were calculated
according to the proportion of each sample in the association.
Besides, the interaction magnitude was calculated through interaction index (γ), following the formula:

\[ γ = \text{dose fluconazole IC}_{50} \text{mixture} + \text{dose fraction IC}_{50} \text{mixture} \]

The interaction index is an indicator of the potency of the association. Values next to 1 indicate additive interaction; values higher than 1, antagonistic interaction, and values lower than 1, synergistic interaction (Grabovsky and Tallarida, 2004).

### Statistical analysis

Checkerboard and toxicity data were evaluated using one-way analysis of variance (ANOVA) followed by Tukey’s test (Sigma Stat 3.2 software, Jandel Scientific Corporation®). In checkerboard, the difference between antifungal activity of fluconazole alone and in combination with LF was evaluated. Differences were considered statistically significant at p < 0.05. The isobologram data were performed with Student t test, where IC50 mixture is significantly shorter than IC50 calculated (IC50 add) to a determined combination, there is a synergic interaction (Codd et al., 2008).

### LF toxicity

The investigated fraction (LF) did not show toxic effects at the concentration used (250 µg/ml) in association studies as demonstrated in Fig. 1. According to these results, the concentration of 500 µg/ml showed DNA damage (Fig. 1A) as well as reduced cellular viability (Fig. 1B). Therefore, the higher LF concentration used at this study (250 µg/ml) is considered safe by these two toxicity methodologies.

---

**Table 1**

<table>
<thead>
<tr>
<th>Yeasts strains</th>
<th>Fluconazole concentration (%IC50)</th>
<th>LF concentration (%IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida krusei</em> CK03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>25</td>
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<tr>
<td>70</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>17.5</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>8.75</td>
<td>3.75</td>
<td></td>
</tr>
<tr>
<td>4.38</td>
<td>1.875</td>
<td></td>
</tr>
<tr>
<td>2.19</td>
<td>0.938</td>
<td></td>
</tr>
<tr>
<td>1.095</td>
<td>0.496</td>
<td></td>
</tr>
</tbody>
</table>

| *Candida parapsilosis* RL11 |                                   |                          |
| 50                     | 50                                |                          |
| 25                     | 25                                |                          |
| 12.5                   | 12.5                              |                          |
| 6.25                   | 6.25                              |                          |
| 3.125                  | 3.125                             |                          |
| 70                     | 30                                |                          |
| 35                     | 15                                |                          |
| 17.5                   | 7.5                               |                          |
| 8.75                   | 3.75                              |                          |
| 4.38                   | 1.875                             |                          |
| 2.19                   | 0.938                             |                          |
| 1.095                  | 0.496                             |                          |

*Candida parapsilosis* RL11: IC50 fluconazole: 26.55 µg/ml and IC50 LF: 174.7 µg/ml; *Candida krusei* CK03: IC50 fluconazole: 35.58 µg/ml and IC50 LF: 35.76 µg/ml.

The non-linear regression analysis was performed using GraphPad Prism® version 4.02.

### Results and discussion

#### Chemical analysis

HPLC analysis were carried out to quantify the major constituents of LF. As demonstrated by Barros et al. (2013), the main constituents of *H. carinatum* lipophilic fraction are the phloroglucinol derivative uliginosin B (1) (1.65 ± 0.08%) and the benzophenones cariphenone A (2) (0.08 ± 0.001%) and cariphenone B (3) (0.58 ± 0.009%), confirming the previous results.
Table 2
Minimal inhibitory concentration (MIC) of Hypericum carinatum lipophilic fraction (LF) against emerging yeasts strains.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>MIC LF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida famata</td>
<td>RL23</td>
<td>250</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>CR03</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>RL11</td>
<td>250</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>HCCRF01</td>
<td>125</td>
</tr>
</tbody>
</table>

* MIC (μg/ml): minimal inhibitory concentration.

Antifungal activity
Concerning the antifungal capacity, LF was capable of inhibit the fungal growth in a moderate way (Table 2). This capacity may be attributed to the presence of dimeric chlorogigocilin derivatives as uliginosin B (1) and the benzophenones cariphenone A (2) and cariphenone B (3). These results are in accordance with those described by Barros et al. (2013).

Association studies
The results obtained in the checkerboard analysis (Fig. 2) are interesting, since LF was capable of reducing the fluconazole MIC values for all species tested. For C. neoformans, C. krusei and C. parapsilosis the fluconazole MIC decreased about eight fold (% Cell damage = 75.6%, ICIF = 0.375; % Cell damage = 91.2%, ICIF = 0.25 and % Cell damage = 71.3%, ICIF = 0.5, respectively), while for C. famata this value was about four fold (% Cell damage = 94.4%, ICIF = 0.5). Nevertheless, for C. neoformans and C. parapsilosis, the fluconazole MIC was capable of achieving a higher cell damage in comparison with association. Therefore, the use of the combinations is only justified when decrease of drug dose is needed, especially in cases where the microorganisms are resistant to this azole.

Concerning the isobologram analysis the curves concentration effect of each compound tested (fluconazole and LF) showed IC₅₀ values of 35.58 μg/ml and 35.70 μg/ml for C. krusei and 26.55 μg/ml and 174.7 μg/ml for C. parapsilosis, respectively. It is important to note that this methodology was not applied to C. famata and Cryptococcus neoformans due to the impossibility of to construct dose response curves with fluconazole alone.

The results obtained in the isobologram (Fig. 3), are in agreement with those obtained by the checkerboard analysis, where synergistic effect was found to both species tested (C. krusei CK 03 and C. parapsilosis RL11). The interaction index (γ) = 1 for all proportions tested for C. krusei (γ₋₅₀₋₅₀ = 0.36; γ₋₇₀₋₃₀ = 0.57) and for C. parapsilosis (γ₋₅₀₋₅₀ = 0.79; γ₋₇₀₋₃₀ = 0.75). However, this index against C. parapsilosis was closer to 1, indicating a probable presence of additive effect instead of synergistic, corroborating with the ICIF (0.5) found for this association in the checkerboard analysis.

The increased incidence of systemic infections caused by NCA species and the high mortality rates due to acquired resistance against drugs current utilized is worrisome, as well as the high incidence of polymicrobial fungal infections (Ruhnke, 2014; Trifilio et al., 2015). Therefore, the association between different compounds could be an excellent strategy to reduce the drug doses, and thus, achieve the resistance reversion.

There are two hypotheses to lipophilic fraction of H. carinatum decreases the MIC of fluconazole. The first could be related to the general action mechanism of phenolic compounds, change the fungal dimorphism (Zhang et al., 2011) and/or opening of membrane channels (Rao et al., 2010) both found for C. albicans. The second hypothesis lies in the fact that some benzophenones are able to block the cytochrome P-450 (Podust et al., 2007). Nevertheless, since it is a fraction, the synergistic effects of the bioactive compounds mixture could be responsible by increasing the effectiveness.

Fig. 2. Interaction between the lipophilic fraction of Hypericum carinatum (LF) and fluconazole against Cryptococcus neoformans HCCRF01 (MICᵢ₉₅ᵢ₉₅ = 32 μg/ml, MICᵢ₉₅ᵢ₉₅ = 250 μg/ml, Association = MICᵢ₉₅ᵢ₉₅:MICᵢ₉₅ᵢ₉₅) (A), Candida famata RL 23 (MICᵢ₉₅ᵢ₉₅ = 8 μg/ml, MICᵢ₉₅ᵢ₉₅ = 250 μg/ml, Association = MICᵢ₉₅ᵢ₉₅:MICᵢ₉₅ᵢ₉₅) (C) and Candida parapsilosis RL 11 (MICᵢ₉₅ᵢ₉₅ = 32 μg/ml, MICᵢ₉₅ᵢ₉₅ = 250 μg/ml, Association = MICᵢ₉₅ᵢ₉₅:MICᵢ₉₅ᵢ₉₅) (D) by checkerboard method, stained with tetrazolium salt MTT. Vertical bars are mean ± SD of four different replicates. Different letters represents significant differences at p < 0.05 (Tukey test).
of itself, and then, the antifungal effect is achieved by a sum of mechanisms (Wagner, 2011).

Some studies report association between extracts and antifungal drugs such as essential oils in association with ketaconazole against several fungal species (Giordani et al., 2004) and benzophenone enriched fraction from Brazilian red propolis with fluconazole and anidulafungin against C. parapsilosis and C. glabrata (Pippi et al., 2015). On the other hand, many studies have demonstrated the association between plant metabolites and antifungal drugs against Candida species. For example, the association of the tannin punicalagin and fluconazole against C. albicans and C. parapsilosis (Endo et al., 2010) and flavonoids (catechin, quercetin and epigallocatechin gallate) associate with fluconazole against C. tropicalis (Da Silva et al., 2014).

There are no doubts that combined therapy between LF and fluconazole is benefic, but further studies must be performed in order to determine the nature of this interaction. The analysis of isolated compounds of this fractions alone and/or combined with fluconazole is needed aiming to standardize this association in cases where the monotherapy with fluconazole is ineffective.

Conclusion

The results of this study reinforce the use of Hypericum species as source of products with biological importance. Association studies are very significant, especially in emerging fungi, which are worldwide distributed and frequent causes of infections in immunocompromised patients. The lipophilic fraction of H. carinatum was able to reduce the MIC of fluconazole, probably by facilitating the access of the drug within the fungal cell. These results are important due to the increasing resistance of emerging yeast species to available drugs used for a variety of fungal infections and the exploration of potential alternative therapeutic sources for multidrug therapy.

Authors’ contributions

GCM (PhD student) contributed in fraction preparation, chemical characterization, biological studies (antifungal activity and association studies), analysis of data and drafted the paper. BP contributed to biological studies (antifungal activity and association studies – checkerboard) and critical reading of manuscript, CH contributed to biological studies (antifungal activity), FMCB contributed to chemical characterization, LFSO contributed to toxicity studies, GLVP and AMF supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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