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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.

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 imunossupressive 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 *Hypericum* species. Other compounds with phloroglucinol pattern such as benzopyrans and benzophenones also showed antifungal activity.

Due to the indiscriminate use of antifungals and the increase of infections caused by emerging species new alternative treatments 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 Renewable 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, evaporated 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 isocratic 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 C_{18} column (4 μm, 3.9 mm × 150 mm) adapted to a Waters Nova-Pack C_{18} 60 Å (3.9 mm × 20 mm) guard column. The flow rate was 1 ml/min, the detector sensitivity was 1.0 Aufs, 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 compounds. 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 breakpoints from M27-S4 document (CLSI, 2012) to *Candida* and according to Espinel-Ingroff et al. (2012) to *Cryptococcus neoformans*. All strains are deposited in the Mycology Collection of Federal University of Rio Grande do Sul, Brazil: *Candida famata* (RL23) originates from hemoculture, *C. krusei* (CK03) from National Program of Quality Control, *C. parapsilosis* (RL11) from urine and *Cryptococcus neoformans* (HCCRY 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 concentration, 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. krusei* 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 determining 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 synergistic; >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 determined 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 equieffective concentration of each treatment alone and f is the fraction of each sample that composes the active concentration of association (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 calculated 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.

Table 1
Proportion of combinations used in isobologram studies.

Yeasts strains	Fluconazole concentration (%IC_{50})	LF concentration (%IC_{50})
Candida krusei CK03	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	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: IC_{50} fluconazole: 26.55 μg/ml and IC_{50} LF: 174.7 μg/ml; *Candida krusei* CK03: IC_{50} fluconazole: 35.58 μg/ml and IC_{50} LF: 35.76 μg/ml.

Besides, the interaction magnitude was calculated through interaction index (γ), following the formula:

$$\gamma b = \text{dose fluconazole } IC_{50\,\text{mixture}} + \text{dose fraction } IC_{50\,\text{mixture}}$$

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).

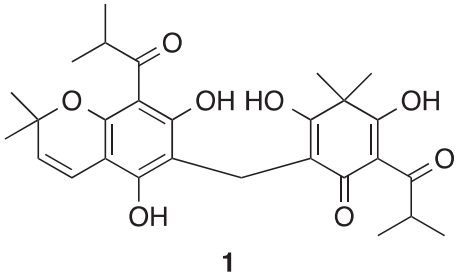
Statistical analysis

Checkerboard and toxicity data were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test (Sigma Stat 3.2 software, Jandel ScientificCorporation®). 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 IC_{50} mixture is significantly shorter than IC_{50} calculated (IC_{50} add) to a determined combination, there is a synergistic interaction (Codd et al., 2008). 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.



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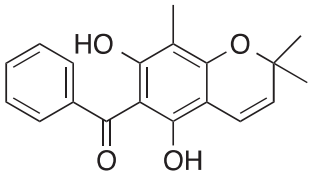

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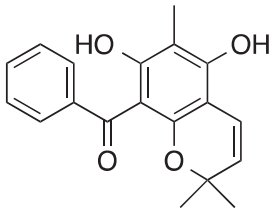

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LF toxicity

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.

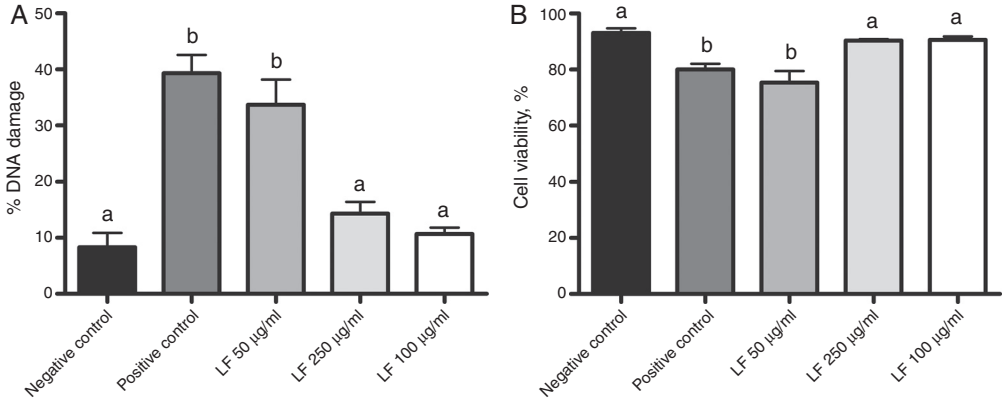

Fig. 1. (A) DNA damage index determined by comet assay and (B) Cell viability in leucocytes for *Hypericum carinatum* lipophilic fraction (LF) in three different concentrations. Phosphate buffered saline (PBS) was used as negative control and hydrogen peroxide (10 μM) (H_2O_2) as positive control in both experiments. DMSO 2% was used as diluent control in these assays. Vertical bars are mean ± SD of three different replicates. Different letters represents significant differences at $p < 0.05$ (Tukey test).

Table 2
Minimal inhibitory concentration (MIC) of *Hypericum carinatum* lipophilic fraction (LF) against emerging yeasts strains.

Species	Strains	MIC[a] LF
Candida famata	*RL23*	250
Candida krusei	*CK03*	>1000
Candida parapsilosis	*RL11*	250
Cryptococcus neoformans	*HCCRY01*	125

[a] 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 phloroglucinol 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 comparision 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 IC_{50} values of 35.58 μg/ml and 35.76 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) was less than 1 for all proportions tested for *C. krusei* ($\gamma_{50:50} = 0.36$; $\gamma_{70:30} = 0.57$) and for *C. parapsilosis* ($\gamma_{50:50} = 0.79$; $\gamma_{70:30} = 0.75$). 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 ionic 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 effectivity

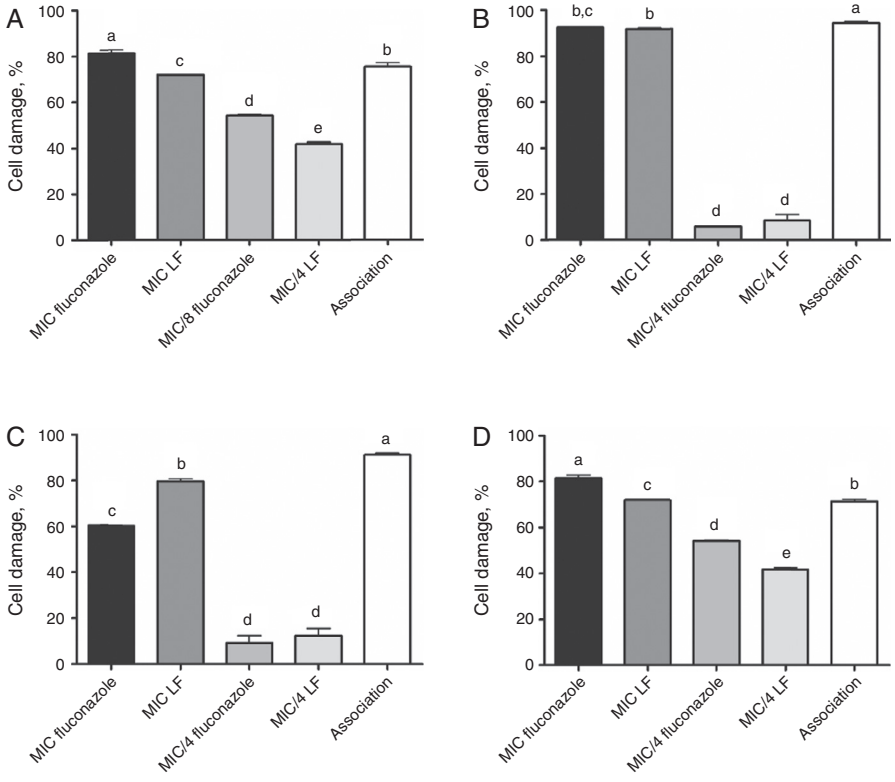

Fig. 2. Interaction between the lipophilic fraction of *Hypericum carinatum* (LF) and fluconazole against *Cryptococcus neoformans* HCCRY01 (MIC_{Fluco} = 32 μg/ml, MIC_{LF} = 250 μg/ml, Association = $MIC/8_{Fluco}$:$MIC/4_{LF}$) (A), *Candida famata* RL 23 (MIC_{Fluco} = 8 μg/ml, MIC_{LF} = 250 μg/ml, Association = $MIC/4_{Fluco}$:$MIC/4_{LF}$) (B), *Candida krusei* CK03 (MIC_{Fluco} = 32 μg/ml, MIC_{LF} > 250 μg/ml, Association = $MIC/4_{Fluco}$:$MIC/4_{LF}$) (C) and *Candida parapsilosis* RL 11 (MIC_{Fluco} = 32 μg/ml, MIC_{LF} = 250 μg/ml, Association = $MIC/8_{Fluco}$:$MIC/4_{LF}$) (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).

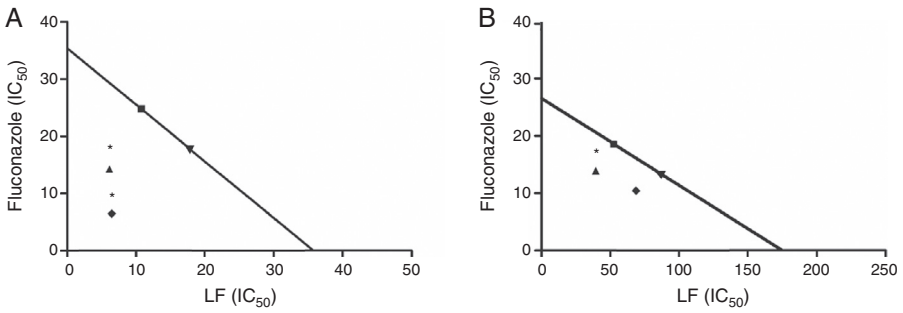

Fig. 3. Interaction analysis of fluconazole with the lipophilic fraction of *Hypericum carinatum* (LF) (IC_{50}) against *Candida krusei* (CK03) (A) and *Candida parapsilosis* (RL11) (B). The continuous line represents the additivity line and the points the experimental combinations at different levels. * represents significant differences between DeqADD (calculated) and Deqmix (experimental) with $p < 0.05$. (■) Additive equieffective concentration (70:30), (▲) Concentration equi-effective of the association (70:30), (▼) Additive equi-effective Concentration (50:50) and (♦) concentration equi-effective of the association.

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 ketoconazole 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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