Revista Brasileira de Farmacognosia xxx (2016) xxx–xxx

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original article

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

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ARTICLE INFO

Article history:
Received 9 June 2016
Accepted 29 August 2016
Available online xxx

Keywords:
Candida
Checkerboard
Fluconazole
Hypericum carinatum
Isobologram
Synergistic activity

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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http://dx.doi.org/10.1016/j.bjp.2016.08.001

Please cite this article in press as: Meirelles, G.C., et al. Synergistic antifungal activity of the lipophilic fraction of *Hypericum carinatum* and fluconazole. Revista Brasileira de Farmacognosia (2016), http://dx.doi.org/10.1016/j.bjp.2016.08.001

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.

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