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Essential oils and major compounds of *Hedychium coronarium* Koenig (Zingiberaceae) against pathogenic yeast of *Candida* and *Cryptococcus* genus

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ABSTRACT

Among the major causative agents of invasive fungal infections stands out the opportunistic yeasts of *Candida* and *Cryptococcus*. Regarding the problem of the high incidence of infections by these agents and the difficulty of treating the low stockpile of antifungal drugs and the high toxicity of most therapies, the search for new antifungal compounds has been highlighted in recent decades. *Hedychium coronarium*, popularly known as "lírio-do-brejo" or "gengibre-branco" features several previously reported biological activities, including antimicrobial activity. Compound 1.8-cineole is the major compound in essential oil extracted from roots of *H. coronarium*, while caryophyllene oxide presents itself as the major in essential oil extracted from leaves of this plant. Our data show strong antifungal activity of compounds, against species of *Candida albicans, Candida parapsilosis, Candida krusei, Cryptococcus neoformans and Cryptococcus gattii,* with minimal inhibitory concentration and minimal fungicidal concentration equal to 0.2 % (v/v) for essential oil extracted from roots, while the essential oil extracted from leaves showed no activity against yeasts. The caryophyllene oxide showed higher antifungal activity for *Cryptococcus* spp. Thus, our results showed that the essential oil of rhizome is a promising antifungal agent against pathogenic yeasts.

Keywords: Candida spp. Cryptococcus spp. Hedychium coronarium, 1.8-cineole, caryophyllene oxide.

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INTRODUCTION

Invasive fungal infections are a major cause mortality and morbidity among critically ill patients in hospitals. Most fungal infections are caused by yeasts of the genus *Cryptococcus* spp. and *Candida* spp. (Enoch *et al*., 2006; Prado *et al*., 2009).

Cryptococcus species are yeast widespread in nature, an ubiquitous and opportunistic yeast. This yeast is commonly associated with pigeon droppings, decaying leavesand wood debris (Nasser *et al*., 2011). *Cryptococcus* can be inhaled by humans or animals and are able to survive within the alveolar macrophages of the host. In the case the yeast if multiply locally in the lung, producing a focal inflammatory reaction. The cryptococcosis disesase is caused, mainly, by two species of fungi *Cryptococcus neoformans* and C. *gattii* (Severo *et al*., 1999).

Candida species are yeast, that inhabit various ecosystems, including the oral cavity, vagina, other dermal and mucosal sites and participate as normal commensal microbiota without harming the host. This yeast are most commonly associated with superficial and disseminated *Candida* infections in humans. *Candida* spp. are considered a major cause of blood stream infections and are associated with a large number nosocomial fungal infections. *Candida albicans* is the most common species in skin and oropharyngeal candidiasis. Therefore, the species such as *Candida parapsilosis* and *Candida krusei*, have increased in number and importance in vaginal and systemic candidiasis (Rex *et al*., 2000).

However, systemic diseases like diabetes and AIDS, physiological conditions such as pregnancy, infancy or old age, nutritional factors, treatment with broad-spectrum antibiotics, immunosuppressive corticosteroids, in addition to local factors such use of prosthetic devices are conditions that predispose to the development of infections by *Candida* spp.

Fungal infections in humans have increased alarmingly in recent years, particularly in immunocompromised individuals. The fungal infections

caused by yeasts *Cryptococcus* spp. and *Candida* spp. are among the main causes of death in immunocompromised patients.

The incidence of cryptococcosis and candidiasis in immunocompromised patients varies according to the population, the region and the period studied, and are among the main causes of death in immunocompromised patients (Prado *et al*., 2009).

The major causative agents are *Candida albicans* with percentage of mortality 20 to 40%, *Aspergillus fumigatus* with 50 to 90% and *Cryptococcus neoformans* with 20 to 70% (Park *et al*., 2009; Nucci *et al*., 2010; Butts and Kysan, 2012).

The problem of high mortality of invasive fungal infection (IFI) is mainly due to three problems. The first problem is the weakened immune system of patients, since patients with HIV, transplant, and who use steroids are the groups with the highest prevalence. The second problem is the limited antifungal arsenal. And the third problem is the indiscriminate use of antifungal drugs (Butts and Kysan, 2012). Amphotericin B, fluconazole and itraconazole are the current drugs used in treatment of invasive fungal infections (Enoch *et al*., 2006). But, one of the recurring difficulties in clinical practice is the emergence of fungal strains resistant to medication. In addition to the current treatment of fungal infections showed high toxicity for human cells.

Therefore, natural products has been the subject of many studies, in search for new antifungal, that are more active, safer and less toxic (Santoro *et al*., 2007; Nibret *et al*., 2010; Gullo *et al*., 2012; Butts and Kysan, 2012; Gullo *et al*., 2013).

In recent decades antimicrobials of plant origin have received special attention due to resistance to traditional antibiotics developed microorganisms. Many plants can serve as an alternative therapy for antimicrobial activity and, among them, aromatic plants due their essential oils revealed particularly effective.

Hedychium coronarium J. Koenig (Zingiberaceae) is monocotyledon macrophyte, popularly known as "lírio-do-brejo", "gengibre-branco" or "lírio-borboleta" (Pio Correa, 1969). It is an invasive tropical plant, commonly found in regions of swamp, originally from Asia, however well adapted in South-America, especially in Brazil. It is popularly used for healing bruise injuries, infections, sore throats, rheumatism, diabetes, headaches, severe pain (Couto *et al*., 2005; Valadeau *et al*., 2009; Facundo *et al*., 2005). The chemical composition of the essential oils from the rhizome and leaves from *H. coronarium* was previously described by Rodrigues *et al* (2013). 1.8-cineole (31.7 %), α-terpineol (12 %), β-pinene (11 %) and terpinene-4-ol (6.8 %) are the major components of the essential oil from rhizomes. In essential oil from leaves the major constituents were caryophyllene oxide (43.9%), E-caryophyllene (12.1%), caryophylladienol I (7.7%), caryophyllenol II (5.6%), caryophyllenol I (2.0%), and caryophylladienol II (1.8%) (Rodrigues *et al*., 2013).

Several studies reported the antimicrobial activity of essential oil from *H. coronarium*. The essential oil of rhizomes proved to be active against Gram-negative and Gram-positive bacteria, namely, Staphylococcus *aureus*, *Shigella flexneri*, *Pasteurella multocida*, *Escherichia coli*, *Salmonella enterica*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Joy *et al*., 2007; Joshi *et al*., 2008; Suksathan *et al*., 2013). Recently, Reuk-Ngam *et al* (2014) showed the antimicrobail activity of coronarin D, isolated from the corms of *H. coronarium*, against Staphylococcus *aureus*, S. epidermidis, *Pseudomonas* aeruginosa, Enterococcus faecalis, *Escherichia coli*, *Salmonella* typhimurium, *Bacillus* cereus and Acremonium sp. The Coronarin D also showed antifungical activity against C. *albicans*, C. albidus, A. niger, A. flavus, *Aspergillus* sp., Acremonium sp. and Penicillium sp.

The essential oil from rhizomes of *H. coronarium* was also effective against fungi (*Candida albicans*, Trichoderma sp) using the diffusion technique (Joy *et al*., 2007). No references were found concerning the activity of the essential oil from leaves.

The present study aimed to evaluate the action of essential oils of the *H. coronarium* (leaves and rhizomes) and the major components, against others pathogenic yeast of *Candida* and *Cryptococcus* genus.

MATERIALS AND METHODS

Plant material

Leaves and rhizomes of *H. coronarium* were collected in the Ecological Station Juréia-Itatins by Prof. Dr. Maria Bernadette G. Martins on 15/03/2011 and identified by Dr. Vinícius Castro Souza. Voucher specimens were deposited in the Herbarium of the College of Agriculture - ESA/USP being registered with number 93272 ESA.

Essential oils

The essential oils were obtained by hydrodistillation for 4 h in Clevenger apparatus according to the procedure described in the European Pharmacopoeia (Council of Europe, 2014). The 1.8-cineole and Caryophyllene oxide were acquired from Sigma-Aldrich, St. Louis, MO, USA.

Microorganisms

American Type Culture Collection (ATCC) strains and isolates belong to the mycology collection of the Laboratory of Clinical Mycology, Department of Clinical Analysis, Faculty of Pharmaceutical Sciences, UNESP, Araraquara, São Paulo state, Brazil, were used in the current study. The strains used were *Candida albicans* ATTC 64548, *Candida parapsilosis* ATTC 22019, *Candida krusei* ATTC 6258, *Cryptococcus neoformans* ATTC 90112, environmental isolates of *Cryptococcus neoformans* (Cn16.3 and Cn21.2) and *Cryptococcus gattii* (Cg118).

Table 1: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of essential oil from the rhizomes and leaves from *Hedychium coronarium*, as well as the major compounds.

Yeast/ Compounds	Essential oil rhizomes		Essential oil leaves		1.8-cineole		Caryophyllene oxide		Amphotericin B	
	MIC (SD)	MFC (SD)	MIC (SD)	MFC (SD)	MIC (SD)	MFC (SD)	MIC (SD)	MFC (SD)	MIC (SD)	MFC (SD)
C. *albicans* ATCC 64548	0.2 % (0.11)	0.2 % (0.11)	>100% (0.0)	>100 % (0.0)	1.56 % (1.20)	1.56 % (1.20)	100 % (0.00)	100 % (0.00)	8.0 µg/mL (2.30)	8.0 µg/mL (2.30)
C. *parapsilosis* ATCC 22019	0.2 % (0.00)	0.2 % (0.00)	>100% (0.0)	>100 % (0.0)	6.25 % (1.79)	6.25 % (1.79)	100 % (0.00)	100 % (0.00)	2.0 µg/mL (0.57)	2.0 µg/mL (0.57)
C. *krusei* ATCC 6258	0.2 % (0.11)	0.2 % (0.11)	>100% (0.0)	>100 % (0.0)	1.56 % (1.20)	1.56 % (1.20)	25 % (7.21)	25 % (7.21)	1.0 µg/mL (0.28)	1.0 µg/mL (0.28)
C. *neoformans* ATCC 90112	0.2 % (0.00)	0.2 % (0.00)	>100% (0.0)	>100 % (0.0)	0.78 % (0.21)	0.78 % (0.21)	1.56 % (0.45)	1.56 % (0.45)	2.0 µg/mL (0.57)	2.0 µg/mL (0.57)
C. *neoformans* isolate Cn16.3	0,2 % (0.11)	0.2 % (0.11)	>100% (0.0)	>100 % (0.0)	3.12 % (0.90)	3.12 % (0.90)	0.78 % (0.21)	0.78 % (0.21)	1.0 µg/mL (0.28)	1.0 µg/mL (0.28)
C. *neoformans* isolate Cn21.2	0.2 % (0.00)	0.2 % (0.00)	>100% (0.0)	>100 % (0.0)	0,78 % (0.21)	0,78 % (0.21)	1.56 % (0.45)	1.56 % (0.45)	2.0 µg/mL (0.57)	2.0 µg/mL (0.57)
C. *gattii* Isolate Cg118	0.2 % (0.11)	0.2 % (0.11)	>100% (0.0)	>100 % (0.0)	3.12 % (0.90)	3.12 % (0.90)	6.25 % (1.80)	6.25 % (1.80)	2.0 µg/mL (0.57)	2.0 µg/mL (0.57)

Standard Deviation: SD

Determination of Minimal Inhibitory Concentration (MIC)

The test for yeast was carried out in accordance with the broth microdilution method described according to the M27-A2-S3 of the CLSI (Clinical and Laboratory Standards Institute) (2008), with modifications, which were based on document Ef7.1 of EUCAST (European Subcommittee on Antifungal Susceptibility Testing). The inoculates were prepared in culture medium RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) with L-glutamine, without sodium bicarbonate, supplemented with 2% glucose, and buffered to a pH of 7.0 using 0.165M MOPS (Sigma-Aldrich, St. Louis, MO, USA). Yeast suspension was adjusted to a final concentration in the plate of 2.5 x 104 to 5.0×103 cells/mL. In the 96-well plates, initially was added 200 µL of the essential oils (rhizomes and leaves) and 1.8-cineole1.8-cineole. Serial dilutions were performed from the concentration of 100% (v / v) to 0.2% (v / v) with a final volume of 100 µL. In this way the essential oils, as well as their major components, were evaluated in the % v / v concentration; which ranged from 100 to 0.2% v / v. As antifungal control was used amphotericin B, according to CLSI. The negative control used was 200 µL of RPMI culture media (without yeast inoculum), and how positive control was used 100µl RPMI culture media more 100 µL. of inoculum of yeast. The plates were incubated in a shaker at 37 ºC / 150 rpm to 24 h for *Candida* species and 48 h for isolates of *Cryptococcus* spp. Then, were performed the visual reading of fungal growth by turbidity of the culture medium.

Determination of Minimal Fungicidal Concentration (MFC)

After reading the MIC results, the content of each well was transferred to a Sabouraud-Dextrose Agar for to determine the lowest concentration that kills the yeast. The plates were incubated at room temperature for 24 h for *Candida* and 48 h for *Cryptococcus* isolates. Results were checked by visual observation of the growth of colonies.

Statistical Analysis

The statistical analysis was performed by two-way ANOVA test with Bonferroni post test through PrismaGraph Pad v.5. Assays were performed in triplicate in three independent experiments.

RESULTS AND DISCUSSION

Aromatic plants have been widely used in folk medicine, and their volatile oils, are known to possess antifungal activity (Kalemba and Kunicka, 2003). However, limited information exists about activity of the essential oils on pathogenic yeasts to humans. Therefore, the discovery of new essential oils for the treatment of these infections, mainly for diseases with mucosal, cutaneous and respiratory tract involvement, is very important.

The results of the essential oils from *H. coronarium* and major compounds, against pathogenic yeast, are showed in Table 1. The essential oil of the leaves of *H. coronarium* was not active against the tested yeast (MIC and MFC > 100 %). Surprisingly, caryophyllene oxide, the major constituent of this oil, revealed to be active against the *Cryptococcus* strains. Due the content of this component (43.9%), it was expected that the oil of the leaves of *H. coronarium* was active against *Cryptococcus*. This was not the case, probably due to antagonistic effects of other components.

Differently the essential oil of the rhizomes of *H. coronarium* was active against all the tested strains of *Candida* and *Cryptococcus* (MIC and MFC values, equal to 0.2 %) (Table 1). The activity can be related with the content of 1.8-cineole (31.7%). All tested strains were susceptible to 1.8-cineole with MIC and MFC values ranging from 0.78% to 6.25% (Table 1). Salgueiro *et al* (2006) showed antifungical activity of the essential oil of Thymus capitellatus oils and the major component, 1.8-cineole on Candida, *Aspergillus* and dermatophyte strains. The oils exhibited antifungal activity for the dermatophyte strains, with MIC values of 0.32–1.25 µL/mL. 1.8-cineole proved

to be against Epidermophyton floccosum, Trichophyton rubrum, T. mentagrophytes, Microsporum canis, M. gypseum with values among 25 and 100 µg/mL.

Some authors indicate that one possible mechanism of action may be associated with a disruption of the membrane of the yeast cell, having ergosterol as the major sterol component (Rodriguez *et al*., 1985; Kaomongkolgit *et al*., 2012; Rodrigues *et al*., 2013; Reuk ngam *et al*., 2014). But, the difficulties for exploring the mechanism of antifungal action of the natural bioactive can be influenced by a wide variety of factors, such as the different quantitative and qualitative ratios of the compounds in essential oils. Therefore, there is need for studies to determine the mechanism of action. The different components of an essential oil can have antagonistic, synergistic, or additive effects on microbial cells, which can influence their overall antifungal activity. In addition, the synergic effects between the major or minor compounds also may be responsible for antifungal activity of the essential oil of the rhizomes of *H. coronarium*.

Thus, our results showed that the essential oil of rhizomes of *H. coronarium* is a promising antifungal agent against pathogenic yeasts, mainly against isolates of *Candida* spp. and *Cryptococcus* spp. However, further study is necessary to elucidate the mechanisms of action and synergic effect of essential oil of the rhizome of *H. coronarium*, against pathogenic yeasts and others pathogenic fungi.

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RESUMO

Óleos essenciais e compostos majoritários de Hedychium coronarium Koenig (Zingiberaceae) contra leveduras patogênicas dos gêneros Candida e Cryptococcus

Candida **spp e** *Cryptococcus* **spp estão classificadas entre os maiores causadores de infecções fúngicas invasivas em pacientes imunocomprometidos. Diante a alta incidência destas infecções por estes agentes e a dificuldade do sucesso no tratamento, decorrente do baixo arsenal de fármacos antifúngicos e da alta toxicidade presente na maioria dos esquemas terapêuticos, a busca por novos compostos antifúngicos tem sido alvo de diversos estudos nas últimas décadas.** *Hedychium coronarium***, popularmente conhecido como "lírio-do-brejo" ou "gengibre-branco", apresenta diversas atividades biológicas já descritas, entre elas a atividade antimicrobiana. O composto 1.8-Cineol é o composto majoritário presente no óleo essencial extraído de raízes de** *H. coronarium* **e o composto óxido de cariofileno é o composto majoritário extraído das folhas desta planta. Nossos resultados mostram que os compostos extraídos de** *H. coronarium* **apresentam forte atividade contra** *Candida albicans***,** *Candida parapsilosis***,** *Candida krusei***,** *Cryptococcus neoformans* **e** *Cryptococcus gattii***, com valores de concentração inibitória minima e concentração fungicida minima igual a 0,2 % (v/v) para o óleo essencial extraído das raízes, enquanto que o óleo essencial extraído das folhas, não mostrou atividade contras as leveduras. O composto óxido de cariofileno mostrou maior atividade antifúngica para Crytopcoccus spp. Assim, nossos dados mostraram que o óleo essencial extraído das raízes de** *H. coronarium***, é um agente antifúngico promissor contra leveduras patogênicas.**

Palavras-chave: *Candida* spp., *Cryptococcus* spp., *Hedychium coronarium*, 1.8-cineole, caryophyllene oxide.

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