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THE "*in vitro*" ANTIFUNGAL ACTIVITY EVALUATION OF PROPOLIS G12 ETHANOL EXTRACT ON *Cryptococcus neoformans*

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SUMMARY

Cryptococcosis is a worldwide disease caused by the etiological agent *Cryptococcus neoformans*. It affects mainly immunocompromised humans. It is relatively rare in animals only affecting those that have received prolonged antibiotic therapy. The propolis is a resin that can present several biological properties, including antibacterial, antifungal and antiviral activities. The standard strain *C. neoformans* ATTC 90112 was used to the antifungal evaluation. The tests were realized with propolis ethanol extract (PEE) G12 in concentrations from 0.1 to 1.6 mg mL^{-1}. The evaluation of MIC and MFC were done according to DUARTE (2002)[5]. The inhibitory effect of PEE G12 on the fungal growing was seen at the concentration of 0.2 mg mL^{-1} and 1.6 mg mL^{-1} was considered a fungicidal one.

KEYWORDS: Propolis; Antifungal activity; *Cryptococcus neoformans*.

INTRODUCTION

With an enlarging immunocompromised population through the panepidemic of HIV infection and the aggressive use of immunosuppressant agents such as corticosteroids for cancer, organ transplantation and other serious medical conditions, cryptococcosis has become a relatively common infection worldwide as we begin the new millennium. It is caused by the encapsulated pathogenic yeast *Cryptococcus neoformans*[8].

According to BERNARDO *et al.*[2], the disease is relatively rare in animals except for those that were submitted to large antibiotic and anti-inflammatory therapies. There are some reports of bovine mastitis and pulmonary infections caused by *C. neoformans*. COSTA *et al.*[4] found 12% of the lactating cow mastitis caused, mainly, by this yeast.

At the last decades, it has been observed a growth on the interests in alternative medicines and natural therapies, especially, those involving substances with antimicrobial properties like propolis among others[14].

The propolis is a complex resinous bee product with a physical appearance that varies widely, depending on many factors. It is collected by bees - *Apis mellifera* - from the buds or other parts of the trees. It is known for its antibacterial, antifungal and healing properties[12]. As the most important chemical weapon of bees against pathogenic microorganisms, propolis has been used as a medicine by human being since ages ago for treatment of wounds, burns, sore throats and stomach ulcers. For this reason, propolis has become the subject of intense pharmacological and chemical studies for the last 30 years[1].

The propolis composition is extremely complex. Some factors as the vegetal ecology from the region where propolis was collected and even the genetic variability in queen bee can influence the chemical composition of this resinous material[14].

Due to the wide variability of its chemical compositions depending on its origin, the chemical standardization is extremely difficult[3]. Several biological and therapeutic activities have been associated with the presence of flavonoids, aromatic acids and esters. In the Brazilian samples other classes of bioactive components, instead of flavonoids, have been described, such as prenylated phenolic acids and specific terpenoids. It is also known that the biological activities of a sample depend on the extraction methodology employed.

The Brazilian propolis has been classified into 12 groups based on physicochemical characteristics: five in the Southern Brazil group (group 3), one in the Southeastern Brazil group (group 12) and six in the Northeastern Brazil group (group 6)[15].

Numerous studies carried out with combined efforts of phytochemists and pharmacologists, led on recent years to the idea that different propolis samples could be completely different in their chemistry and biological activity[1].

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The propolis is used traditionally in folk medicine mainly because its antibacterial effect. Other properties, as antifungal, antiviral, anti-inflammatory and immuno-stimulating have also been described to this resin[3,10].

By this way, with the intention to prevent some undesirable effects arisen from the use of antibiotic and antifungal drugs and also to obtain an alternative and efficient substance, efficient against pathogenic yeast growth. The goal of this work was to evaluate the propolis from group 12 (G12) that according to FERNANDES *et al*.[6] has showed the best antifungal activity in susceptibility evaluations. It was evaluated the "in vitro" antifungal activity of propolis G12 ethanol extract (PEE) on *Cryptococcus neoformans.*

MATERIAL AND METHODS

The antifungal activity of the propolis G12, so named "green propolis", was evaluated in the concentration of 0.1; 0.2; 0.4; 0.8; 1.2 and 1.6 mg mL^{-1} of propolis in a 80% ethanol solution according to IKEGAKI[9].

The susceptibility test employed the *C. neoformans* standard strain ATCC 90112. The strain was subcultured on Sabouraud dextrose agar at 25 °C during 48 h. The inoculum was diluted in 0.9% NaCl solution to the concentration of 2 $.10^8$ UFC mL^{-1}.

The determination of Minimum Inhibitory Concentration (MIC) was realized according to the methodology proposed by DUARTE[5]. A volume of 0.5 mL from the standardized inoculum was added to 49.5 mL Sabouraud broth and the resulting colonies were counted.

The control was eight tubes with Sabouraud broth inoculated with *C. neoformans* ATCC90112, ATCC90112 and ethanol 80%, ATCC90112 in each one of the 6 PEE concentrations respectively. The medium was agitated and incubated during 48 h at 28 °C. The growth evaluation was done by absorbance reading.

The Minimum Fungicidal Concentration (CFM) and MIC were determined according to DUARTE[5] across the colony counting on Sabouraud agar dishes. The inoculum was all the suspension that presented an absorbance result minor or equal than 0.05 at 660 nm. The volume of 50 µL was added on to the agar and spread with a swab. The incubation was at 28 °C during 48 h. The MFC was defined as the lowest PEE concentration in which there wasn't cellular growing on to the agar surface.

RESULTS AND DISCUSSION

According to the methodology, the MIC was observed in the concentration of 0.2 mg mL^{-1} to the standard strain *C. neoformans* ATCC 90112 (Table 1). Other researches have shown MIC and MFC at higher concentrations of the PEE G12 than ours[6,13]. FERNANDES Jr. *et al*.[7] verified fungistatic effect at the concentration of 3.8 mg.mL^{-1} to *Candida albicans* and 2.1 mg.mL^{-1} to *C. tropicalis*.

The MFC was observed at the concentration of 1.6 mg.mL^{-1} (Table 1). To *Malassezia pachydermatis,* a yeast that is frequently isolated from otitis externa in dogs, LILENBAUM & BARBOSA[11] used propolis extract from apiary Itamel in Itaboraí, Rio de Janeiro State and observed fungicidal effect only into the concentration of 2.4 mg mL^{-1}. OTA *et al.*[13] found propolis fungicidal activity inside a range from 9 to 10 mg mL^{-1} in 65% of *Candida* sp. strains.

Table 1
The results of MIC and MFC of PEE G12 to the Standard Strain *Cryptococcus neoformans* ATCC 90112

PEE G12 concentrations (mg.mL^{-1})	MIC	MFC
0.1	-	-
0.2	+	-
0.4	+	-
0.8	+	-
1.2	+	-
1.6	+	+

The signs + and – indicate positive and negative results respectively.

According to SALATINO *et al.*[17] many compounds may be involved in the biological activity of G12 propolis. Prenylated cinnamic acid-derived compounds, such as 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) has shown to possess antimicrobial activity. Mono and sesquiterpenes are frequently detected in propolis G12, probably contributing to the antimicrobial activity.

Beyond that compounds, the propolis G12 has flavonoids in its composition. These phenolic compounds are considered as one of the mainly responsible for its antimicrobial activity[1,15]. QUIROGA *et al.*[16] demonstrated that pinocembrin and galangin were partially responsible to the toxic activity against several strains of phytopathogenic fungi. The minimal inhibitory concentration values for pinocembrin and galangin were between 14-40 µg mL^{-1}.

The beneficial effects of the propolis have been mentioned since ages ago. According to our results, the analyzed standard strain was shown to be sensitive to the PEE G12 in almost all the evaluated concentrations. Cryptococcosis is until now a worldwide mycose that has been characterized by the development of resistance among many strains after antifungal therapies. So, alternative therapies can be an option since more "in vitro" studies may be done and related to some established parameters of the "in vivo" efficacy.

RESUMO

Avaliação da atividade antifúngica do extrato etanólico de própolis G12 sobre *Cryptococcus neoformans*

Criptococose, doença cosmopolita, causada pelo agente etiológico *Cryptococcus neoformans,* está associada, principalmente, a indivíduos imunocomprometidos. O acometimento de animais é relativamente raro, exceto, nos casos associados à prolongada antibioticoterapia. A própolis é uma resina que pode apresentar diversas propriedades biológicas, incluindo atividades antibacterianas, antifúngicas e antivirais. Amostra padrão de *C. neoformans* foi utilizada no teste de atividade antifúngica do extrato etanólico de própolis (EEP) G12 nas

concentrações de 0,1 a 1,6 mg.mL^{-1}. As avaliações da Concentração Inibitória Mínima (CIM) e Concentração Fungicida Mínima (CFM) foram realizadas conforme DUARTE[5]. O efeito inibitório do EEP G12 sobre o crescimento fúngico foi observado na concentração de 0,2 mg.mL^{-1}. A concentração de 1,6 mg.mL^{-1} foi considerada fungicida.

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