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Antifungal activity of the essential oil isolated from *Laurus nobilis* L. against *Cryptococcus neoformans* strains

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ABSTRACT

Cryptococcosis is an opportunistic fungal infection that evolved to an important cause of morbidity and mortality in recent years. Even with advances in medicine, the treatment of ringworm still presents a therapeutic system with limited availability of antifungal agents, and so, research is needed to identify new agents fungistatic or fungicides effectives. The natural products are known to represent an arsenal of compounds, some with antifungal properties. Thus, this study aimed investigates the antifungal activity *in vitro* of the essential oil of *Laurus nobilis* L. (leaves) against *Cryptococcus neoformans* strains. The chemical composition of the oil was analyzed by gas chromatography coupled to mass spectrometry (GC / MS) and minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined by the broth micro dilution techniques. The MIC100 of essential oil were 256 μg/mL and the MFC_{50} was 1024μg/mL. In conclusion the essential oil showed *in vitro* antifungal potential against strains of *C. neoformans*.

INTRODUCTION

Cryptococcosis is an opportunistic fungal infection that has featured between mycoses due to its high incidence in patients with Acquired Immunodeficiency Syndrome (AIDS), and the difficulties of treatment, high mortality in humans and animals with nervous system involvement central.*Cryptococcus neoformans* is a major etiological agents of cryptococcosis, and has the ability to infect healthy individuals and with impaired cellular immunity (Chayakulkeeree and Perfect 2006; Dromer *et al.*, 2004; Mirza *et al.*, 2003; Pukkila-Worley and Mylonakis, 2008; Singh *et al.*, 2008). Since the treatment this mycosis, involves a therapeutic system with limited antifungal agents, some of these, with high toxicity and microbial resistance (Girois *et al.*, 2006). Among the antifungal agents are used amphotericin B, flucytosine and fluconazole or itraconazole administered in combination or individually. At the beginning of treatment can be used Amphotericin B associated with flucytosine, where for more prolonged treatment is recommended the use of fluconazole (Saag *et al.*, 2000). The medicinal and aromatic plants have been the subject of various studies in order to find biologically effective compounds with lower toxicity and which can possibly be used to treat various types of infectious diseases (Nakamura *et al.*, 2004; Oliveira *et al.* 2006; Prabuseenivasan; Jayakumar; Ignacimuthu, 2006; Saad, 2010). Essential oils are produced from the secondary metabolism of plants and considered important in various functions necessary for the survival of the plant, as a defense mechanism against microorganisms (Gonçalves *et al.*, 2003; Siqui *et al.*, 2000). Since ancient times, they are known through its antibacterial and antifungal properties (Cavaleiro *et al.*, 2006; Cowan, 1999; Tenpone *et al.*, 2008). *Laurus nobilis* L. is a small aromatic plant belonging to the family *Lauraceae*, popularly known as laurel. It is widely used by the food industry, pharmaceuticals and cosmetics.

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The dried leaves are considered important ingredients in food preparation, and the essential oil is a valuable complement in the aroma of various types of food, especially meat, sausages, canned soups and bakery products (Patrakar; Mansuriy; Patil, 2012).

The antimicrobial properties and insecticides have made the use of this common plant for preserving food by the food industry. In folk medicine, it is used in rheumatic processes, dermatitis, gastrointestinal disorders such as epigastric distention, and flatulence impaired digestion. The aqueous extract is commonly used as anti-haemorrhoidal, antirheumatic, diuretic, antidote for snake bites and for the treatment of stomach pains (Baytop, 1984; Gulcin, 2006).

Because the clinical and epidemiological importance of cryptococcosis, further investigations are necessary to contribute with safer treatment options, low cost for the population, assessing the chances of successful treatment.Therefore, the search for less toxic antifungal agents and products derived from medicinal plants is vital and considered an excellent alternative for this purpose. The objective of this study was to investigate the antifungal activity in vitro of the essential oil of *Laurus nobilis L.* (leaves) against strains of *C. neoformans*.

MATERIALS AND METHODS

Essential Oil

The essential oil of *L. nobilis* L. was purchased from Company Quinarí® House of Essences (Ponta Grossa, Parana, Brazil), being extracted from the leaves by steam distillation. This product has met the purity requirements specified by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) on food additives and their quality parameters were described in a technical bulletin sent by the company.

The essential oil was solubilized in 5% dimethylsulfoxide (DMSO) and 2% Tween 80. Next, sterile distilled water was added and the tubes mixed for 5 min using a Vortex (Fanem), to obtain the desired concentration.

Fungal strains

For antifungal activity assays, were selected strains of *C. neoformans:* FCF10, LM 0109, LM 310, LM 2601, LM 2301, LM 1909and LM 0310. All the microorganisms were obtained of collection of Laboratory of Mycology, Federal University of Paraiba – Brazil.Fungi were maintained on Sabourad Dextrose Agar (SDA) (Difco Lab., USA)inclined at 4 °C. Inoculums were obtained from fresh cultures of each of the selected strains of *C. neoformans*, previously grown in sterile tubes containing tilted Sabouraud dextrose agar (SDA; Difco Lab., USA), incubated at 37 °C for 72 hours.

Colonies of this culture were suspended in sterile 0.85% NaCl to provide a final concentration of approximately 10^6 count forming unit per mL ($CFU.mL^{-1}$) adjusted according to the turbidity of 0.5 McFarland scale tube.

Standard antifungal

Flucytosine (Sigma-Aldrich®, São Paulo-SP) was used as the standard antifungal.

Essential oil analysis

The sample was analyzed with a SHIMADZU GC/MS QP2010 Ultra gas chromatographer coupled to a mass spectrometer with highly purified helium as the carrier gas. An aliquot of 1 µL of the prepared sample solution was injected in split mode in ratio 1:20 with a purge time of 3 min. The injection ports, the GC to MS transfer line and ion source temperatures were held at 250 °C. A 30m long RTX-5 MS column (25.0 µm i.d.; and 0.25 µm film thickness; Restek Corporation) was used to separate all the target compounds. The GC oven temperature program was as follows: 60 °C, 3 °C/min to 240 °C.

Determination of minimal inhibitory concentration (MIC)

MIC of the essential oil was determined by the microdilution technique in broth medium (Cleeland; Squires, 1991; Eloff, 1998; Hadacek and Greger, 2000). Cultures of *C. neoformans* were seeded in SDA and incubated at 35 ºC for 24-72 h. Colonies of this culture were suspended in sterile 0.85% NaCl and the inoculum was standardized at 0.5 tube of McFarland scale (10^6 CFU mL^{-1}). Sabouraud dextrose broth (Difco Lab., USA) was added to all wells of 96-well plates. Next, serial dilutions were made to obtain concentrations varying between 1 and 1024 $\mu g.mL^{-1}$. The same procedure was carried out with flucytosine. DMSO (5%) and Tween 80 (2%), without drugs, serving as the negative control to verify the absence of interference on fungal growth. Finally, 10 µL of yeast inoculum were added to all wells, and the plates were incubated at 35°C for 24 - 72 h. The test was performed in duplicate. MIC was defined as the lowest concentration capable of visually inhibiting fungal growth seen in the wells (Souza *et al.*, 2007).

Determination of minimal fungicidal concentration (MFC)

Aliquots of 10 µL of supernatant from each well of the microtiter plate with no visible fungal growth were subcultured on dextrose saubourad agar plates, devoid of any antifungal. The plates were incubated at 35°C for 24-72 h. MFC was defined as the lowest concentration of essential oil that caused total inhibition of visible growth (Espinel-Ingroff *et al.*, 2002;Ernst *et al.*, 2002; Pereira *et al.*, 2011a; Trajano *et al.*, 2010). The test was performed in duplicate.

RESULTS AND DISCUSSION

The essential oil of *L. nobilis* L., obtained by steam distillation, was analyzed by gas chromatography-mass spectrometry (GC-MS). The predominant component identified was isoeugenol (57%), followed by myrcene (15.9%), chavicol

(9.3%) and methyl eugenol (2.43%) (Table 1). The main constituents of the essential oil of *L. nobilis* L. leaves are referred to in the literature as 1.8-cineole, terpineol, β-pinene, α-pinene, p-cymene, β-caryophyllene. The acyclic monoterpenes (linalool and myrcenol) may also be present, as well as, dimethyl styrene, eugenol, methyl eugenol and carvacrol (Riaz *et al.*, 1989; Rizi, 2008, 2009; Rosa and Assunta, 2008; Yalcin *et al.*, 2007).

Table 1: Essential oil components from the leaves of *L. nobilis* L.

Peak	Time to retention (min)	Constituent	% the sample	Basis m/z
16	23,137	Isoeugenol	57,0	164,15
4	7,556	Myrcene	15,9	93,10
15	18,401	Chavicol	9,3	134,15
18	25.029	Methyl eugenol	2,43	178,15
11	11,568	Linalool	2,14	71,10
19	25,688	Caryophyllene	2,10	93,10
8	8,883	Limonene	2,02	68,10
Others	-	-	9,11	-

Analyzes of the MIC values of *L. nobilis* L.essential oil on strains of *C. neoformans* showed that all strains submitted to biological assays had their growth inhibition in a concentration of 256 μg.mL^{-1} of *L. nobilis* L.essential oil.The MIC_{100} (MIC for 100% of the strains tested) was 256 μg.mL^{-1} (Table 2).

Table 2: MIC of the essential oil components from the leaves of *L. nobilis* L *againstC. neoformans* strains.

Substance/ Fungal strains	Essential oil MIC (μg/mL)	Flucytosine MIC (μg/mL)	Negative control
FCF 10	256	32	-
LM 0109	256	32	-
LM 310	256	64	-
LM 0310	256	32	-
LM 2601	256	32	-
LM 2301	256	64	-
LM 1909	256	128	-

(-) No inhibition.

The flucytosine (positive control) exerted inhibitory effects against all strains tested. The MIC values of antifungal ranged from 32-128 μg.mL^{-1}, with 50% of the strains tested inhibiting 32 μg.mL^{-1}. The MIC_{50} value for the antifungal used in the study was lower than those found by Pfaller *et al.* (1990) against strains of *C. neoformans* that exhibited MIC values greater than 64 μg.mL^{-1}.

According to the literature results is considered strong antifungal activity MIC values between 0.05-0.50 mg/mL, MIC values between 0.6 to 1.50 mg/mL are considered moderately active and values MIC greater than 1.50 mg/mL are related to a weak antifungal activity (Sartoratto *et al.*, 2004). The results showed that the essential oil used in this study showed strong activity against *C. neoformans* strains with MIC_{100} 256 μg.mL-1.These results are in agreement with the data obtained by Ozcan *et al.* (2010) in their study using the *L. nobilis* L. essential oil against various strains of *Candida*. Analyzing the results of the MFC_{50} can be seen that the essential oil (Table 3) does have fungistatic activity against *C. neoformans* species, because when when the ratios MFC / MIC was greater than 1 or 2, indicating that the effect of the compound was fungistatic in nature (and not fungicide) (Hafidh *et al.*, 2011).

Table 3: MIC and MFC of the essential oil components from the leaves of *L. nobilis* L *againstC. neoformans* strains.

Substance/ Fungal strains	MIC (μg/mL)	MFC (μg/mL)
FCF 10	256	512
LM 0109	256	1024
LM 310	256	1024
LM 0310	256	512
LM 2601	256	1024
LM 2301	256	1024
LM 1909	256	512

CONCLUSION

Thus, the essential oil of *L. nobilis* L represents a natural product with potential antifungal activity against *C. neoformans*. In the study, the essential oil showed a fungistatic effect. Therefore, the test product is presented as a relevant antifungal and may be contributing to the existing arsenal of products with proven antifungal activity against *C. neoformans*.

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