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Short Communication

In vitro antifungal activities of leaf extracts of *Lippia alba* (Verbenaceae) against clinically important yeast species

***Graziela Teixeira de Oliveira*[1], *Jaqueline Maria Siqueira Ferreira*[1], *Luiz Henrique Rosa*[2], *Ezequias Pessoa de Siqueira*[3], *Susana Johann*[2] *and Luciana Alves Rodrigues dos Santos Lima*[1]**

[1]. Campus Centro-Oeste Dona Lindu, Universidade Federal de São João Del-Rei, Divinópolis, MG. [2]. Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG. [3]. Laboratório de Química de Produtos Naturais, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG.

ABSTRACT

Introduction: There are few studies reporting the antifungal activities of *Lippia alba* extracts. **Methods:** A broth microdilution assay was used to evaluate the antifungal effects of *Lippia alba* extracts against seven yeast species of *Candida* and *Cryptococcus*. The butanol fraction was investigated by gas chromatography-mass spectrometry. **Results:** The butanol fraction showed the highest activity against *Candida glabrata.* The fraction also acted synergistically with itraconazole and fluconazole against *C. glabrata*. The dominant compounds in the butanol fraction were 2,2,5-trimethyl-3,4-hexanedione, 3,5-dimethyl-4-octanone and hexadecane. **Conclusions:** The butanol fraction may be a good candidate in the search for new drugs from natural products with antifungal activity.

Keywords: *Lippia alba*. Antifungal activity. Yeast.

Plants exhibit antifungal properties, and several compounds, such as coumarins, terpenes and flavonoids[1], could be responsible for these activities. The incidence of invasive opportunistic mycoses has increased in immunosuppressed patients, including those undergoing organ transplantation or hematopoietic stem cell therapy and those suffering from cancer or acquired immunodeficiency syndrome (AIDS)[2]. Clinical *Candida* species remain the most important cause of opportunistic mycoses worldwide. The majority of invasive infections due to *Candida* spp. are attributed to several species, including *Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis* and *Candida krusei*[2]. *Cryptococcus neoformans* is the etiological agent of cryptococcosis and is responsible for up to 30% of deaths in AIDS patients[3]. *Cryptococcus gattii* causes cryptococcal infections mostly in immunocompetent individuals[4]. Despite some effective treatment options, such mycoses are associated with high morbidity[2]. To overcome this problem, identifying new compounds, especially natural products, that exert antifungal activities, is very important.

Lippia alba (Mill.) N.E. Brown (Verbenaceae) is a shrub widely distributed throughout South America and is popularly known as *cidreira* or *false melissa*. In traditional popular medicine, the tea from its leaves is largely utilized as a tranquilizer but is also used to treat gastrointestinal and respiratory infections[5]. Several studies have reported on the antimicrobial activity of the essential oil of *L. alba*, but there are few reports in the literature on the extracts from this species. In the present work, the extract and fractions obtained from the leaves of *L. alba* were evaluated in respect to their antifungal activities against seven clinically important yeast species. We also tested the synergistic interaction of the butanol fraction with commercial antifungal drugs.

Leaves of *L. alba* were collected in the City of Carmopólis de Minas, State of Minas Gerais (MG), Brazil, in March 2011. A voucher specimen (BHCB 147243) was deposited at the *Instituto de Ciências Biológicas* Herbarium, *Universidade Federal de Minas Gerais, Belo Horizonte, MG*, Brazil. The fresh leaf material (216.73g) was extracted by cold maceration in 95% ethanol (Vetec, Brazil) over a period of 10 days at room temperature. The extract was then filtered and concentrated in a rotary evaporator at 40°C under reduced pressure to yield the ethanol extract (Et, 5.83g). Part of this extract (2.05g) was dissolved in EtOH/H_2O (7:3) and then partitioned successively with hexane, dichloromethane, ethyl acetate and butanol (Vetec, Brazil; 15mL, 3 times with each solvent), resulting in 1.13, 0.15, 0.08, 0.26 and 0.35g of hexane (Hex), dichloromethane (DCM), ethyl acetate (Ac), butanol (But) and hydroalcoholic (HE) fractions, respectively.

Seven yeast species, *Candida albicans* ATCC 18804*, C. glabrata* ATCC 2001*, C. krusei* ATCC 200298, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 22019, *Cryptococcus gattii* ATCC 32608 and *Cryptococcus neoformans* ATCC 2467, were used in the biological assays. All the strains were stored at -80ºC.

For screening of antifungal activities, the ethanol extract and fractions of *L. alba* (dissolved in dimethylsulfoxide) were diluted to a final concentration of 2,000μg/mL for use in the antifungal assay. The yeast species were grown at 37°C in Sabouraud (Himedia, India) media. After 24h under these

Address to: Drª Luciana Alves Rodrigues dos Santos Lima. Campus Centro-Oeste Dona Lindu/UFSJ. Rua Sebastião Gonçalves Coelho 400, Chanadour, 35501-296 Divinópolis, MG, Brasil.
Phone: 55 37 3221-1393; **Fax:** 55 37 3221-1614
e-mail: luarsantos@ufsj.edu.br
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conditions, a yeast inoculum was prepared by diluting the cell suspensions appropriately in RPMI (Roswell Park Memorial Institute) 1640 (supplemented with 2% glucose). A total of 50µl of yeast inoculum was added to each well of a 96-well plate and adjusted to 1.0×10^6 cells/mL. After adding 25µL of the extract or control solution and 25µL of each medium to attain the desired concentrations, the plates were incubated at 37°C for 24h for *Candida* or 48h for *Cryptococcus*. As an indicator of microorganism growth, 10µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (Sigma, USA) (dissolved in sterile water at 5mg/mL) was added to each well and incubated at 37°C for 4h. The results are expressed as the percent inhibition with respect to the controls without drugs[6]. Amphotericin B (2µg/mL) (Sigma, USA) was used as a positive control. All assays were performed in triplicate.

The minimal inhibitory concentration (MIC) was obtained from the broth microdilution tests performed in accordance with the guidelines in the Clinical and Laboratory Standards Institute (CLSI) document M27-A3[7]. Suspensions from the cultures of the *Candida* and *Cryptococcus* species were prepared according to the CLSI document M27-A3[7] and the modifications suggested by Johann et al.[1] to obtain a final inoculum of 2.5 x 10^3 cells/mL. Amphotericin B (Sigma, USA), fluconazole (Pfizer Pharmaceutical, USA) and itraconazole (Jansen Pharmaceutical, Belgium) were included as positive controls; the stock solutions of amphotericin B and itraconazole were prepared in dimethylsulfoxide, and fluconazole was prepared in water. Two-fold serial dilutions were prepared exactly as outlined in CLSI document M27-A3[7]. All assays were performed in triplicate.

Eight serial dilutions of the butanol fraction (1.9 to 250µg/mL) and amphotericin B (0.008 to 1µg/mL) were prepared with the same solvents used in the MIC test. Fifty-microliter aliquots of each dilution of the butanol fraction were added to ninety-six-well plates in a vertical orientation, and fifty microliters of each amphotericin B dilution was added in a horizontal orientation so that each well on the plate contained various concentrations of each sample (amphotericin B and butanol fraction). After the addition of 100µl of *C. glabrata* inoculum (the same as the one used in the MIC test) to each well, the plate was cultured for 48h at 37ºC. The fractional inhibitory concentration (FIC) of amphotericin B was calculated as the MIC of amphotericin B in the presence of the butanol fraction divided by the MIC of amphotericin B alone. The FIC of the butanol fraction was calculated in the same fashion. The fractional inhibitory concentration index (FICI) was calculated by adding both FICs. With this method, FICI ≤ 0.5 indicated synergistic activities, $0.5 < \text{FICI} < 2.0$ defined additive or indifferent effects, and FICI > 2.0 described antagonistic effects[8]. This analysis was also performed for the butanol fraction with fluconazole (0.062 to 8µg/mL) and itraconazole (0.015 to 2µg/mL). All assays were performed in triplicate.

Gas chromatography-mass spectrometry (GC-MS) of the butanol fraction was performed on a Shimadzu model QP5050A instrument equipped with a DB-5 column (30m x 0.25mm, 0.25µm). The initial temperature of the column was 80°C for 1min, which was then increased at a rate of 7°C/min to 300°C and held for 5min; the injector temperature was kept at 250°C (split 1:20), and the detector temperature was kept at 260 °C. Helium (He) was used as the carrier gas, with a linear flow-rate of 39.3mL/min (115.4 Kpa). For each analysis, 1µL of the sample was injected into the GC. The scan range was from 50 to 500*m/z* at a scan rate of 0.50 scan/s. The solvent delay was 2.5min. The compounds were identified by a mass spectral database search (NIST) (National Institute of Standards and Technology), followed by matching of MS data and expressed as relative percentages of each compound, calculated by internal normalization of the chromatographic peak area. All volatile compounds showing mass spectra with match factors ≥ 90% were put on a *positive list* of tentatively identified metabolites.

The antifungal effect of the ethanol extract and fractions of *L. alba* was screened against seven yeast species of clinical interest at a concentration of 2,000µg/mL **(Figure 1)**. *C. krusei* was the species most sensitive to the ethanol extract

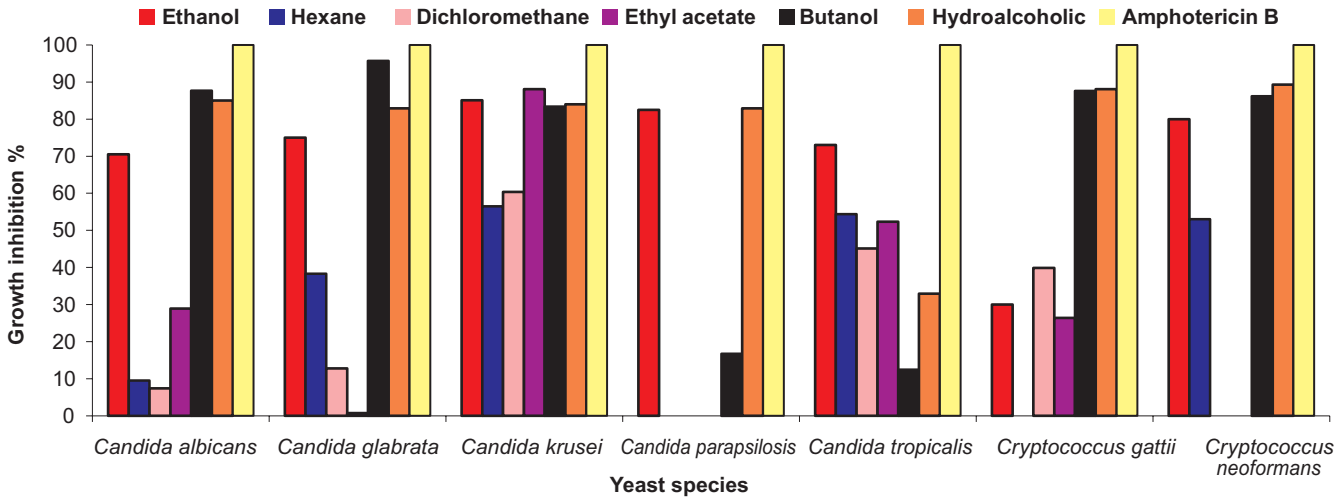

FIGURE 1 - Growth inhibition of seven yeast species of clinical interest by ethanol extract and fractions of *Lippia alba* at concentration of 2,000µg/mL.

and fractions, with inhibition rates ranging from 57% to 88%. The ethanol extract and hydroalcoholic fraction were also active against the yeast species. The butanol fraction showed the highest growth inhibition against *C. glabrata* (95%) compared with the other fractions and with the ethanol extract of *L. alba*.

After the screen, all fraction activities were evaluated in the MIC tests. The butanol fraction showed the highest activity against *C. glabrata,* with a MIC value of 62.5µg/mL, and was also active against the other yeast species tested, *C. albicans*, *C. krusei*, *C. gattii* and *C. neoformans*. However, for the other species, the MIC value was 2,000µg/mL **(Table 1)**. The ethanol extract and ethyl acetate fraction showed activity against *C. krusei,* with a MIC value of 1,000µg/mL. The ethanol extract also presented activity against *C. parapsilosis* and *C. neoformans* with a MIC of 1,000µg/mL, and against *C. albicans*, *C. glabrata* and *C. tropicalis*, with a MIC of 2,000µg/mL.

The hydroalcoholic fraction was active against *C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. gattii* and *C. neoformans*, with a MIC of 2,000µg/mL. The dichloromethane fraction displayed activity only against *C. krusei*, with a MIC of 2,000µg/mL. In contrast, the hexane fraction was inactive against all the yeast species studied. Notably, the ethanol extract and fractions of *L. alba* were less potent than amphotericin B, fluconazole and itraconazole.

The activity of the butanol fraction against *C. glabrata*, but not for other *Candida* species or *Cryptococcus*, could be explained because these yeast species are phylogenetically different. *C. glabrata* is a species more phylogenetically related to *Saccharomyces cerevisiae* than other pathogenic *Candida* species[9]. The specific activity of the butanol fraction against *C. glabrata* also suggests a selectivity of the natural products present in this fraction.

The antifungal activities observed in the present work have been confirmed by other studies on *L. alba* extracts, being used against *C. albicans*, *C. krusei*, and *C. tropicalis*. To the best of our knowledge, this study is the first to show the antifungal activity of the ethanol extract and fractions of leaves of *L. alba* against *C. glabrata* and *C. gattii*.

The hexane and chloroform extracts from the leaves and flowers of *L. alba* showed activity against *C. albicans*[10]. The hydroalcoholic and methanol extracts obtained from the leaves of *L. alba* exerted effects against *C. krusei*, with MIC values of 125 and 165.2µg/mL, respectively[11,12]. The hydroalcoholic extract also showed activity against *C. tropicalis*, with a MIC of 1,000µg/mL[11].

The effect against *Candida* species is interesting, mainly because of the innate resistance of *C. krusei* to fluconazole. *Candida glabrata* shows less susceptibility than other *Candida* species, and the development of resistance to fluconazole among the clinical strains of *C. albicans, C. parapsilosis* and *Candida tropicalis*, hampers the treatment of these infections[13]. The activity against *C. neoformans* is of particular importance, as this yeast is the major cause of meningitis in AIDS patients and has been identified as the fourth most common cause of life-threatening infection in AIDS patients[14].

To explore the possibility of developing more effective combination therapies, the butanol fraction from the leaves of *L. alba* was combined with amphotericin B, itraconazole and fluconazole and tested against *C. glabrata*. **Table 2** shows the additive or indifferent effect of combining the butanol fraction with amphotericin B, and the synergistic effect of this fraction with itraconazole and fluconazole. These results are very interesting because *C. glabrata* exhibits intrinsic resistance against azole antifungal drugs[15].

In this study, we showed that the butanol fraction from the leaves of *L. alba,* combined with fluconazole and itraconazole, had a synergistic effect against *C. glabrata* and, therefore, could be an alternative in the treatment of candidemia caused by this yeast species. However, additional studies are needed to evaluate the toxicity of this fraction and the *in vivo* action of fluconazole and itraconazole combined with the butanol fraction against this opportunistic yeast.

TABLE 1 - Minimal inhibitory concentration activity of ethanol extract and fractions of *Lippia alba* against seven clinically important yeast species.

	MIC (µg/mL)								
Fungi	Et	Hex	DCM	Ac	But	HE	amphotericin B	itraconazole	fluconazole
Candida albicans	2,000	≥ 2,000	≥ 2,000	≥ 2,000	2,000	2,000	0.25	0.031	2
Candida glabrata	2,000	≥ 2,000	≥ 2,000	≥ 2,000	62.5	2,000	0.125	0.25	4
Candida krusei	1,000	≥ 2,000	2,000	1,000	2,000	2,000	0.5	0.125	32
Candida parapsilosis	1,000	≥ 2,000	≥ 2,000	≥ 2,000	≥ 2,000	2,000	0.5	0.062	1
Candida tropicalis	2,000	≥ 2,000	≥ 2,000	≥ 2,000	≥ 2,000	≥ 2,000	1.0	0.062	2
Cryptococcus gattii	≥ 2,000	≥ 2,000	≥ 2,000	≥ 2,000	2,000	2,000	1.0	0.031	nt
Cryptococcus neoformans	1,000	≥ 2,000	≥ 2,000	≥ 2,000	2,000	2,000	1.0	0.031	nt

MIC: minimal inhibitory concentration; Et: ethanol extract; Hex: hexane fraction; DCM: dichloromethane fraction; Ac: ethyl acetate fraction; But butanol fraction; HE: hydroalcoholic fraction; nt: not tested.

TABLE 2 - FIC and FICI of the butanol fraction from *Lippia alba* leaves against *Candida glabrata* ATCC 2001.

Samples	MIC in combination (μg/mL)	MIC alone (μg/mL)	FIC	FICI
1. Butanol fraction	0.975	62.5	0.015	1.015
2. Amphotericin B	0.125	0.125	1	
1. Butanol fraction	0.975	62.5	0.015	0.075
2. Itraconazole	0.015	0.25	0.06	
1. Butanol fraction	0.975	62.5	0.0156	0.031
2. Fluconazole	0.062	4	0.0155	

FIC: fractional inhibitory concentration; FICI: fractional inhibitory concentration index; MIC: minimal inhibitory concentration.

The butanol fraction showed a higher antifungal activity than the other fractions or the ethanol extract against *C. glabrata*. Therefore, GC/MS was used to identify the main compounds. The dominant compounds were 2,2,5-trimethyl-3,4-hexanedione, 3,5-dimethyl-4-octanone and hexadecane. There are currently no reports in the literature that describe the antifungal activity of these compounds, suggesting that the antifungal activity of plant extracts or fractions could be linked to the synergistic interactions between their components.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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