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Atividade Antifúngica e Efeito Sinérgico contra *Trichophyton rubrum* e *Candida* spp. do Óleo Essencial das Partes Aéreas de *Lippia alba*

Abstract: Pesquisas relacionadas à prospecção de substâncias isoladas de plantas com potencial antimicrobiano, especialmente contra cepas do gênero *Candida*, tem se mostrado uma estratégia importante em tratamentos alternativos contra infecções causadas por fungos, uma vez que novas cepas desse microorganismo são relacionadas com infecções hospitalares graves. Estudos sobre a capacidade antifúngica de óleos essenciais têm recebido considerável atenção do setor farmacêutico devido à grande variedade de compostos presentes em amostras com propriedades farmacológicas e ao alarmante aumento de infecções micóticas em indivíduos imunocomprometidos por espécies de *Candida* spp. Assim, este estudo teve como objetivo descrever a composição química do óleo essencial de *Lippia alba*, avaliar atividade antifúngica e efeito modulador, investigar a toxicidade *in vivo* e *in vitro* utilizando *Artemia salina* e células sanguíneas. O óleo essencial foi obtido por hidrodestilação e a composição química determinada por CG-EM e CG-DIC. A atividade antifúngica foi analisada pelo método de microdiluição em caldo usando linhagens de dermatófitos e leveduras. A atividade sinérgica foi determinada pela técnica *Checkerboard*, utilizando cetoconazol como padrão para dermatófitos e anfotericina B para leveduras. Quanto à composição química, o óleo essencial mostrou que os principais constituintes foram sabineno (19,34 %), *E*-cariofileno (18,21 %), limoneno (16,47 %) e γ-elemeno (9,09 %). O óleo essencial apresentou baixa toxicidade e atividade intrinseca contra dermatófitos, bem como apresentou efeito sinergico contra todas as cepas de *Tricophyton rubrum* quando combinado com cetoconazol e efeito de sinergismo com anfotericina B contra duas cepas de *C. tropicalis* e uma de *C. parapsilosis*.

Keywords: *Lippia*; composição volátil; atividade antimicrobiana.

Resumo

Research related to the prospecting of substances isolated from plants with antimicrobial potential, especially against *Candida* strains, is an important strategy for developing alternative treatments against infections caused by fungi, since new strains of such microorganisms are associated with serious hospital infections. Studies on the antifungal capacity of essential oils have received considerable attention from the pharmaceutical sector, owing to the wide variety of compounds present in samples with pharmacological properties and the alarming increase in mycotic infections in individuals immunocompromised by *Candida* spp. Therefore, this study was aimed at determining the chemical composition of the essential oil of *Lippia alba*, evaluating its antifungal activity and modulatory effect, and investigating its toxicity *in vivo* and *in vitro* using *Artemia salina* and blood cells, respectively. The essential oil was obtained by hydrodistillation, and its chemical composition was determined using CG-EM and CG-DIC. Antifungal activity was analyzed *via* the broth microdilution method, using dermatophyte and yeast strains. Synergistic activity was determined using the *checkerboard* technique, with ketoconazole and amphotericin B as the standards for dermatophytes and yeasts, respectively. The main constituents of the essential oil were sabinene (19.34 %), (*E*)-caryophyllene (18.21 %), limonene (16.47 %), and γ-elemene (9.09 %). The essential oil showed low toxicity and intrinsic activity against dermatophytes, a synergistic effect against all strains of *Trichophyton rubrum* when combined with ketoconazole, and a synergistic effect with amphotericin B against two strains of *Candida tropicalis* and one of *Candida parapsilosis*.

Palavras-chave: *Lippia*; volatile composition; antimicrobial activity.

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Antifungal Activity and Synergistic Effect of Essential oil from *Lippia alba* Against *Trichophyton rubrum* and *Candida* spp.

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1. Introduction

The emergence of new microbial species has alerted scholars around the world, as resistance to antimicrobials has become prevalent. Currently, new strains of the genus *Candida* are being found and have been associated with serious hospital infections[1]. The problems caused by resistant or mutant microorganisms are serious, and hence, mortality rates have increased dramatically. Such microbes have become great threats to global health, affecting people of any age anywhere in the world. Among the most recurrent hospital infections, fungal infections of *Candida* spp. (such as *Candida albicans, Candida parapsilosis*, *and Candida tropicalis*) have significantly increased in number in recent decades. In Brazil, several pathogens exhibit high levels of resistance to conventional antimicrobials. However, it is known that *Candida* spp. are the most prevalent causes of nosocomial fungal infections[2-4].

Trichophyton rubrum is a dermatophytic fungus. Dermatophytes can cause infections of the skin, hair, and nails because of their ability to use keratin. Superficial mycoses are probably the most prevalent infectious diseases found worldwide[5]. The fungus colonizes keratin tissues and causes inflammation as a result of the host's response to its metabolic byproducts. Dermatophytes such as *T. rubrum* are distributed worldwide. They are seldom isolated from animals and are rarely found in soil. The infections caused by dermatophytes may manifest differently depending on the site of infection. The inflammatory reaction caused by *T. rubrum* can cause severe damage to the skin and, in particular, the nails[6].

The current increase in resistance to antimicrobials has encouraged the search for new agents capable of preventing the growth of resistant microorganisms. In view of this, several research groups have shown increased interest in essential oils obtained from plants with antimicrobial potential; the use of such oils is an important strategy in alternative treatments against fungal infections[7,8].

Studies on the antifungal capacity of essential oils have received considerable attention from the pharmaceutical sector, owing to the wide variety of compounds present in samples with pharmacological properties and the alarming increase in mycotic infections in individuals immunocompromised by *Candida* spp. *Candida albicans* is one of the most prevalent fungal species both in hospital environments and healthy people in cities, peripheries, or urban centers, owing to its resistance to the small arsenal of conventional drugs, which are used ineffectively[9].

The genus *Lippia* L. has approximately 200 species of herbs, bushes, and small trees. Their major centers of dispersion are concentrated in countries of the Southern Hemisphere and some tropical regions of North America and Australia. Essential oils from *Lippia* spp. contain a wide variety of volatile constituents with a range of pharmacological properties, particularly antimicrobial activity[10]. *Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson (Figure 1), popularly known as erva-cidreira, is used in folk medicine in the form of infusions made from the flowers, leaves, stems, and roots to treat dermatological diseases as well as gastrointestinal and respiratory disorders; pharmacological studies have demonstrated the promising biological activities of this plant, such as its antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, sedative, cytotoxic, analgesic, antipyretic, anti-hypertensive, antispasmodic, and anti-Leishmania effects[11-18].

Figure 1. General aspect of the floral branch of *Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson

In this context, the search for promising sources of natural products with antimicrobial activity has become increasingly important, since microorganisms gradually show resistance to conventional antimicrobials, even at high concentrations. In this study, we aimed to determine the chemical composition of the essential oil from aerial parts of *L. alba* collected from the Meruoca mountain region of Sobral, Ceará, and evaluate the synergistic effect of the *L. alba* essential oil and antifungal drugs against *T. rubrum* and *Candida* spp.

2. Material and Methods

2.1. Plant material

Aerial parts from *L. alba* were collected in the flowering period in the Meruoca mountain region of Sobral, Ceará state, Brazil, in a semideciduous forest environment located around 800 m above sea level. This region is located in the middle reaches of the Acaraú River, about 250 km from the Fortaleza, the state capital. A voucher specimen (Nº 18716) was deposited in the Prof. Francisco José de Abreu Matos Herbarium (HUVA) and authenticated by Dr. Elnatan Bezerra de Souza of the Center for Agricultural Sciences and Biological Sciences of the State University Vale do Acaraú.

2.2. Extraction of the essential oils

For the extraction of the essential oil, the hydrodistillation method was used, where 3.54 Kg of aerial parts from *L. alba* were introduced in a volumetric flask and 1500 ml of distilled water were added. The flask was attached to the Clevenger apparatus under the heating mantle for 2 hours extraction. At the end of the process, the essential oil was collected, dried with anhydrous sodium sulfate (~1.0 g), filtered and stored in a sealed and labeled glass vial, stored in a thermal box under a low temperature for future analysis and biological assays. Calculation of the essential oil yield was achieved by dividing the total value of oil obtained by the total value of the foliar mass.

2.3. Gas Chromatography-Flame Ionization Detection (GC-FID)

GC-FID for the quantitative analysis was carried out on a Shimadzu GC-17A gas chromatography using a dimethylpolysiloxane DB-5 fused silica capillary column (30 mm x 0.25 mm, film thickness 0.25 m). H_2 was used as the carrier gas at a flow rate of 1 mL/min and 30 psi inlet pressure; split, 1:30; temperature program: 35-180°C at 4°C/min, then heated at a rate of 17°C/min to 280°C and held isothermal for 10 min; injector temperature, 250°C; detector used FID, detector temperature, 250°C.

2.4. Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS for the analysis of the volatile constituents was carried out on a Hewlett-Packard Model 5971 GC/MS using a non-polar DB-5 fused silica capillary column (30 mm x 0.25 mm i.d., 0.25 m film thickness); carrier gas helium, flow rate 1 mL/min and with split ratio 1:1. The injector temperature and detector temperature were 250º C and 200º C, respectively. The column temperature was programmed from 35 C to 180º C at 4º C/ min and then 180 C to 250º C at 10º C/min. Mass spectra were recorded from 30 - 450 *m/z*. Individual components were identified by matching their 70 eV mass spectra with those of the spectrometer database using the Wiley L-built library MS searches using retention indices as a preselection routine, as well as by visual comparison of the fragmentation pattern with those reported in the literature[19,20].

2.5. Brine shrimp lethality bioassay

The toxicity test for *A. salina* was performed following the methodology proposed by Meyer and collaborators[21], which consists of the hatching of *A. salina* eggs in artificial saline water, then the larvae were collected for the bioassays. The dissolution of the samples and of the blank test was performed with 3.9 mL of saline water, 1mL of saline water with *A. salina,* and 0.1 mL of concentrated DMSO. The procedure was performed in triplicate at concentrations of 1000, 100, 10 and 1 µL, and added 10 *Artemia* in each vial. The survivors count being after 24 hours. The negative control was 100 µL to 2% of DMSO and 4.9 mL of distilled water. The experiment was performed in triplicate. After analyzing the results, the procedure is repeated at intermediate concentrations to find the lethal concentration, capable of killing 50% of microcracks. For the calculation of $LD_{50,}$ the Microsoft Excel Program was used by calculating linear regression with the following formula: 5 - b / a**,** at where, a

and b are values of the first-order equation y = ax + b, being considered active when DL50 <1000 µg / mL degree y = ax + b, being considered active when CL_{50} < 1000 µg/ mL.

2.6. Hemolysis assay

This test was performed in 96-well plates using 1% fresh blood erythrocytes suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the methods described in Jimenez *et al*. (2003). The essential oil was assayed at concentrations ranging from 0.039 to 2.5 mg/mL. After 1h incubation, the plate was centrifuged, and the supernatant containing hemoglobin was measured spectrophotometrically for the absorbance 540 nm (Multimode Detector DTX 880, Beckman Counter)[22].

2.7. Fungal strains

The strains were obtained from the fungal collection of the Specialized Medical Mycology Center (CEMM), Federal University of Ceará, the URM Culture Collection of the Department of Mycology, Federal University of Pernambuco, and Holy House of Mercy of Sobral Hospital. *Candida* species were isolated from clinical cases, and its use was approved by the Ethics Committee on the advice number 644.365. In all these collections, the strains were maintained in saline (0.9% NaCl) at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated onto potato dextrose agar (Difco, Detroit, MI, USA) and then incubated at 28°C for 2–10 days. A total of four strains of *T. rubrum*, two strains of *C. albicans*, three strains of *C. tropicalis*, one *C. parapsilosis,* and one strain of *C. famata* were included in this study.

2.8. Preparation of inocula

For the broth microdilution method, standardized inocula ($2.5–5\times10^3$ CFU mL^{-1} for *Candida* spp. and 5.0×10^4 CFU mL^{-1} for *T. rubrum*) were prepared by turbidimetry. Stock inocula were prepared on day 2 and day 5 for *Candida* spp. and *T. rubrum*, respectively, grown on potato dextrose agar at 28°C. Sterile saline solution (0.9%) was added to the agar slant, and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *T. rubrum* and *Candida* spp., respectively. The suspensions were diluted to 1:2000 for *Candida* spp. and 1:500 for *T. rubrum*, both with RPMI 1640 (Roswell Park Memorial Institute-1640) with l-glutamine without sodium bicarbonate (Sigma Chemical Co., St. Louis, MO, USA), and then buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co.), to obtain inocula of $2.5–5\times10^3$ CFU mL^{-1} and 5.0×10^4 CFU mL^{-1}, respectively[23].

2.9. Broth microdilution method

The minimum inhibitory concentration (MIC) for *Candida* spp. was determined by the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute[24]. The broth microdilution assay for *T. rubrum* was performed based on the M38-A document[25]. The minimum fungicidal concentrations (MFC) for both *Candida* spp. and *T. rubrum* were determined according to Fontenelle and collaborators[26,27]. The EOLA was prepared in 100% mineral oil. Amphotericin B (AMB) and ketoconazole (Sigma, Chemical Co., USA) were prepared in distilled water. For the susceptibility analysis, the essential oil samples were tested in concentrations ranging from 0.078 to 5.0 mg/mL. The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for the EOLA. The microplates were incubated at 37°C and read visually after two days for *Candida* spp. and five days for the *T. rubrum*. The assay for the essential oil was run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 100% inhibition of visible fungal growth. The results were read visually, as recommended by CLSI. The MFC was determined by subculturing 100 µL of solution from wells without turbidity on potato dextrose, at 28°C. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after two days for *Cândida* spp. and five days for *T. rubrum*[26].

2.10. Microdilution checkerboard assay

The tests performed with *T. rubrum and Candida* spp. were followed by the Checkerboard technique to determine the combined effect between essential oil and a standard antifungal. To obtain these necessary results, first, consider the values found for the Minimum Inhibitory Concentration (MIC) corresponding to essential oil, isolated and combined with antifungal, and antifungal, isolated and combined with essential oil. Then, the Fungicide

Inhibitory Concentration (FIC) is measured through the quotient between isolated MIC and combined MIC of essential oil and antifungal.

FIC (A) = MIC of agent (A) in combination/MIC of agent (A) alone
FIC (B) = MIC of agent (B) in combination/MIC of agent (B) alone

where A = Essential oil, and B = AMB.

The IFIC, in turn, is defined according to the addition of the values corresponding to the Fungicide Inhibitory Concentration (FIC) of each of the compounds considered in the assay.

IFIC = FIC (A) + FIC (B)

The turbidity of the fungal suspensions was adjusted to McFarland standard (10^5 CFU/mL). In the solutions, the samples tested were used in the concentrations of their respective MICs. Initially, 50 µl RPMI 1640 medium was added to all 96 wells of the microdilution plate. Then 50 µL chalcone was added to the first column, where serial dilutions were performed on the plate to the eighth column, with sample concentrations varying from 0.002 to 2.5 mg/mL. In the vertical lines, 50 µL of the standard AMB was placed in concentrations ranging from 0.5 to 16 µg/mL, and finally, 100 µL of inoculum was added to all wells. RPMI 1640 medium with inoculum was used as the positive control, while wells containing only RPMI 1640 were considered as the negative control. The microplates were incubated at 37 ºC and read visually after 48h.[24]

3. Results and Discussion

The essential oil of the aerial parts of *L. alba* was obtained with a yield of 0.04 (w/w) dry weight, half the 0.08% yield obtained by Shukla *et al*.[28] on the other hand, Castro *et al*.[16] obtained yields from 0.15% to 0.61% for the same species. Chemical analysis of the essential oil revealed the presence of 24 components, mainly mono- and sesquiterpenes, representing 94.80% of the essential oil chemical composition. The chemical composition of the essential oil and the respective percentages are reported in Table 1. The major constituents were sabinene (19.34%), (*E*)-caryophyllene (18.21%), and limonene (16.47%), followed by the sesquiterpene, γ-elemene (9.09%). The chromatogram of the essential oil from the aerial parts of *L. alba* is depicted in Figure 2.

The antifungal activity of sabinene[29] and limonene[30] against *Saccharomyces cerevisiae, Candida* spp., *Cryptococcus neoformans*, and *Malassezia furfur* has been reported. Deena and Thoppil[31] investigated the antibacterial activity of the essential oil from *L. camara* collected in Calicut, India. They found that the major compound in this oil was (*E*)-caryophyllene. The sesquiterpene, γ-elemene, one of the major constituents found in the present study, has also been identified as one of the factors that determines *the antifungal activity of some essential oils of Piper* spp., as reported by Silva *et al*.[32]. These results indicate that the antimicrobial activity identified in our study may represent a combined effect of these components of the *L. alba* essential oil.

Although the chemical composition of a plant's essential oil is genetically determined and is generally specific to a determined origin, as necessary for its development stage, environmental variables can cause significant alterations, giving rise to what are called breeds or chemotypes. These are frequently found in plants rich in essential oils[33].

According to Oliveira *et al*.[11], 13 chemotypes of *L. alba* have been identified to date, *viz.*, myrcene-citral, limonene-citral, limonene-carvone, limonene-1,8-cineol, citral-germacrene D, caryophyllene-citral, linalool-1,8-cineol, limonene, piperitone, camphor-1,8-cineol, citral-terpinene, linalool, and carvone. Blank *et al*.[34], in a study of the chemical diversity of the essential oils of this species obtained from different Brazilian regions, identified many constituents, some common to those found in the present study, such as α-thujene sabinene, myrcene, *p*-cymene, limonene, 1,8-cineol, γ-terpinene, linalool, (*E*)-caryophyllene, γ-elemene, germacrene D, (*E*)-nerolidol, and spathulenol.

The various results observed in each study are related to differences in the chemical composition of the different oils tested, which can be influenced by several factors, such as seasonality, soil type, collection period, extraction method, extract drying temperature, and genotype[35-37]. Taking into account the studies on *L. alba*, it is possible to identify diversity in the literature on cases of antifungal activity, most of which does not indicate activity against strains of *Candida* spp. According to our study, the *in vitro* antifungal activity summarized in Table 2 reveals a MIC of 2,500 µg/mL against dermatophytes.

Table 1. Chemical composition of essential oil of aerial parts from *L. alba*

Constituents	IK_{calc} [1]	IK_{lit} [2]	%
α-thujene (1)	931	930	1.36
α-Pinene (2)	939	939	1.89
Camphene (3)	958	954	0.50
Sabinene (4)	977	975	19.34
β-Pinene (5)	984	979	0.53
Myrcene (6)	992	990	1.06
α-Phellandrene (7)	1015	1002	1.29
p-Cymene (8)	1028	1026	1.14
Limonene (9)	1032	1029	16.47
1,8-Cineole (10)	1037	1031	2.95
Z-β-Ocimene (11)	1041	1044	1.44
γ- Terpinene (12)	1062	1059	3.93
Linalool (13)	1096	1096	3.12
Thujone (14)	1108	1102	0.77
4-Terpineol (15)	1183	1176	1.47
α-Terpineol (16)	1200	1188	0,61
E-Caryophyllene (17)	1341	1338	18.21
α-Humulene (18)	1458	1454	1.29
Germacrene D (19)	1435	1436	2.93
γ-Elemene (20)	1484	1484	9.09
Germacrene B (21)	1563	1559	0.51
E-Nerolidol (22)	1567	1563	2.73
Spathulenol (23)	1581	1578	0.88
Total			94.51

[1]IK_{calc}: Calculated Kovats Index, [2]IK_{lit} : Literature Kovats Index.

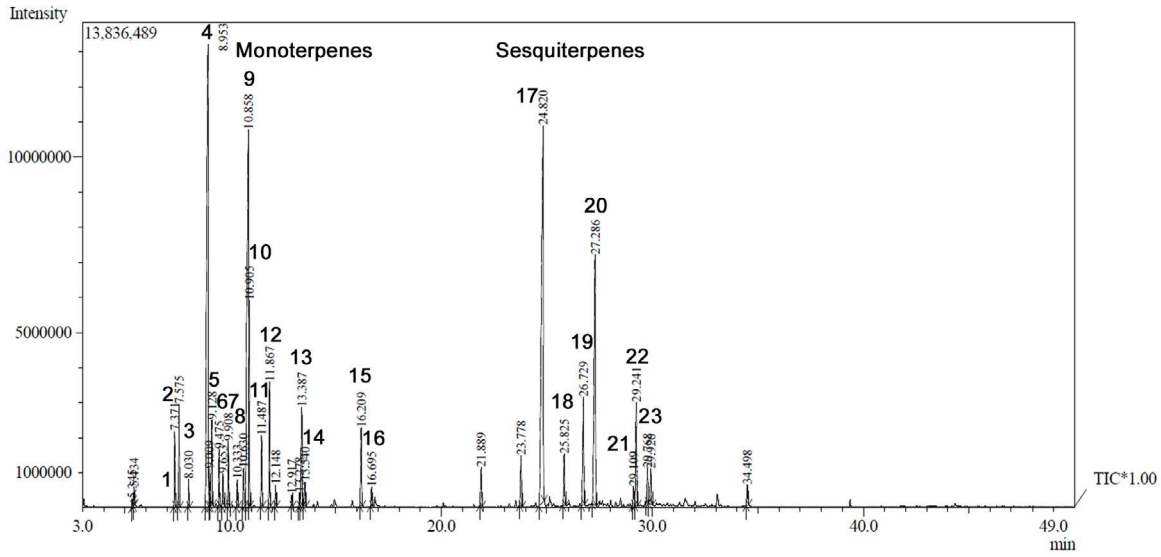

Figure 2. Chromatogram of the essential oil of aerial parts from *L. alba*

Table 2. Minimum Inhibitory Concentration and Minimum Fungicide Concentration of the essential oil of aerial parts from *L. alba*

Strains	MIC	MFC	Amphotericin B	Ketoconazole
C. albicans LABMIC 0101	NI	NI	1.0	-
C. albicans LABMIC 00102	NI	NI	2.0	-
C. tropicalis LABMIC 0109	NI	NI	2.0	-
C. tropicalis LABMIC 0111	NI	NI	4.0	-
C. tropicalis LABMIC 0112	NI	NI	2.0	-
C. parapsilosis LABMIC 0301	NI	NI	8.0	-
C. famata LABMIC 0120	NI	NI	2.0	-
T. rubrum LABMIC 0203	2.5	5.0	-	0.5
T. rubrum LABMIC 0204	2.5	5.0	-	0.5
T. rubrum CSMM 05-1-08	2.5	5.0	-	0.5
T. rubrum CSMM 05-1-034	2.5	5.0	-	0.5

LABMIC: Laboratory of Microbiology; CSMM: Center of Specialties in Medical Mycology; NI: Not Inhibited.

To identify the possible mechanism of action of essential oils, researchers have used crystal violet to observe changes in membrane permeability caused by the oils, as detected in the test described by Devi *et al*.[38]. In the assay, ethylenediaminetetraacetic acid (EDTA) and *Candida* spp. cultures in Sabouraud dextrose broth, untreated and treated with nystatin, were used. The DO_{570nm} value of the crystal violet solution (10 µg/mL in PBS) was considered 100% absorption. The following formula was used to calculate the crystal uptake: OD of the sample / OD of the crystal violet solution × 100. The experiments were carried out in triplicates, and the results were expressed as% CV uptake.

Fungi have intracellular signaling pathways, including the cAMP-PKA and MAPK pathways[39]. Hence, tissue penetration can occur, allowing the yeasts to spread through the bloodstream, invading endothelial and epithelial cells, and releasing hydrophilic enzymes that are capable of causing tissue damage[40].

However, the *L. alba* essential oil did not inhibit the growth of the strains *C. albicans* LABMIC 0101 and LABMIC 0102, *C. parapsilosis* LABMIC 0116, *C. famata* LABMIC 0120, and *C. tropicalis* LABMIC 0109, LABMIC 0111, and LABMIC 1112. The essential oil achieved the best results against the strains *T. rubrum* CEMM 05-1-034 and 05-01-08, with an MIC of 2.5 mg/mL and MFC of 5 mg/mL, and LABMIC 0203 and LABMIC 0204, with an MIC of 2.5 mg/mL and MFC of 5.0 mg/mL. In previous studies with essential oils of some *Lippia* species against *Candida* spp. strains, the essential oil of *L. sidoides* was active against *C. albicans* and *C. tropicalis*[27,41]. Research into the bioactivity of the essential oils of *L. alba* and its chemotypes is diversified[17,42-44]; however, in the case of antifungal activity, much of the available literature indicates no activity against strains of *Candida* spp.

To determine the modulatory activity, four strains of *T. rubrum* (LABMIC 0203, LABMIC 0204, CSMM 05.01.034, and CSMM 05.01.08) and seven strains of *Candida*, *C. albicans* LABMIC 0101 and LABMIC 0102, *C. tropicalis* LABMIC 0109, LABMIC 0111, and LABMIC 0112, *C. parapsilosis* LABMIC 0116, and *C. famata* LABMIC 0120, were used. The essential oil of *L. alba* reduced the MIC of all the *T. rubrum* strains (Table 3).

The combination of antifungals with non-antifungal agents, such as antibacterials, especially for deep fungal infections, is an important therapeutic alternative. Other non-antifungal agents can act as calcineurin and heat shock protein 90 inhibitors, and calcium homeostasis regulators have been combined with fluconazole, producing synergistic results.

The minimum fractional inhibitory concentration index (MFICI) was 0.17 against the strains LABMIC 0203 and LABMIC 0204, and 0.18 for CEMM 05.01.034 and CEMM 05.01.08. This reveals a synergistic modulatory effect with the drug ketoconazole. The yeast strains for which the MFICI was best were *C. tropicalis* LABMIC 0109, *C. tropicalis* LABMIC 0111, and *C. parapsilosis* LABMIC 0116, with values of 0.5, 0.25, and 0.25, respectively (Table 4). They enhanced the antifungal effect of amphotericin

Table 3. MIC of the Ketoconazole in the presence and absence of essential oil from aerial parts from *L. alba* against *T. rubrum*

Strains	Essential oil (mg/mL)		Ketoconazole (µg/mL)		
	MIC isolated	MIC combined	MIC isolated	MIC combined	FICI
T. rubrum LABMIC 0203	2.5	0.015	0.5	0.156	0.171
T. rubrum LABMIC 0204	2.5	0.015	0.5	0.156	0.171
T. rubrum CSMM 05-1-08	2.5	0.031	0.5	0.156	0.187
T. rubrum CSMM 05-1-34	2.5	0.031	0.5	0.156	0.187

*FICI: Fractional Inhibitory Concentration Index; LABMIC: Laboratory of Microbiology; CSMM: Center of Specialties in Medical Mycology

Table 4. MIC of amphotericin B in the presence and absence of essential oil of aerial parts from *L. alba* against *Candida* spp.

Strains	Essential oil (mg/mL)		Amphotericin B (µg/mL)		
	MIC isolated	MIC combined	MIC isolated	MIC combined	FICI
C. albicans LABMIC 0101	NI	NA	2.0	2.0	1.0
C. albicans LABMIC 0102	NI	NA	1.0	1.0	1.0
C. tropicalis LABMIC 0109	NI	0.312	2.0	1.0	0.5
C. tropicalis LABMIC 0111	NI	0.625	4.0	1.0	0.25
C. tropicalis LABMIC 0112	NI	NA	3.0	3.0	1.00
C. parapsilosis LABMIC 0116	NI	0.078	8.0	2.0	0.25
C. famata LABMIC 0120	NI	NA	2.0	2.0	1.00

*FICI: Fractional Inhibitory Concentration Index; NA – No Activity; NI- Not Inhibited; LABMIC: Laboratory of Microbiology.

B because the oil alone did not inhibit the growth of the strains tested. These results corroborate the observation that the oil possibly causes alterations of the integrity of the membrane that favor the entrance, and therefore, the action of antifungals[38].

The first azole drug was reported in 1944; however, it was not until 1958 that chlormidazol began to be marketed. Previously, treatment for fungi was carried out with nonspecific medications, such as keratolytics, antiseptics, and antibiotics. In 1951, Brown and Hazen discovered polyenes, nystatin, and amphotericin B. However, their use, mainly that of amphotericin B, caused several adverse effects, especially in patients who had nephrological problems. The constant problems with amphotericin B led researchers to find analogs of the compound that had much less adverse effects and did not affect patients (e.g., *via* nephropathogenesis). For a period, the search for new antifungals was neglected compared to the development of antibiotics, as the rates of fungal infections were low and a highly selective fungal target was lacking[45].

The assay of activity against *A. salina* is used for screening to isolate and monitor the phytochemical properties of plant extracts, in order to assess the bioactive capacity of these substances[21]. The toxicity of the *L. alba* essential oil was determined by testing against larvae of this microcrustacean, and the concentration that could kill 50% of the larvae (LC_{50}) was 53.01 µg/mL, indicating that this oil had significant activity, because the LC_{50} was lower than 1,000 µg/mL, the limit proposed by Meyer *et al*. (1982). This LC_{50} is similar to that found in other studies of biological potential, such as antifungal[23,45], bactericidal[46], and antioxidant activity[47].

The test to determine the hemolytic activity of the *L. alba* essential oil *in vitro* indicated concentrations varying from 0.039 to 2.5 mg/mL, whereas the hemolysis percentages ranged from 0.46% to 1.52%. Based on these results, it was possible to extrapolate the hemolysis values and estimate an LC_{50} of 11.94 mg/mL for the essential oil. The hemolytic effect of drugs or natural products can occur through different mechanisms, including solubilization and alteration of the membrane of red blood cells, causing cytolysis in both situations[48]. According to Miyazaki *et al*.[49], the hemolytic action *in vitro* is

ascertained according to the presence of damage to the red blood cell membrane caused by the substances contained in essential oils. These cells, when submitted to lysis, release hemoglobin.

The MIC values of the *L. alba* essential oil indicated a hemolysis percentage of 1.52%. A comparison of the LC_{50} value for hemolytic activity with the MIC indicated that the concentration of the oil necessary for fungicidal activity was lower than that needed to damage red blood cells *via* membrane rupture. However, the *L. alba* essential oil presented a weak cytotoxic effect according to the hemolytic activity test. A similar study was carried out by Sobrinho *et al*.[23], with the essential oil of *Vernonia chalybaea* Mart. ex DC., and 4.89% hemolysis was observed at the MIC values of the essential oil. In the present study, we found a lower percentage of hemolytic effect. However, the low toxicity and cytolysis at the MIC values indicated herein are similar to the results obtained by Sobrinho *et al*.[23].

4. Conclusion

The essential oil extracted from the aerial parts of *L. alba* contained monoterpenes (58.87%) and sesquiterpenes (35.64%). The main constituents of the oil were sabinene (19.34%), (*E*)-caryophyllene (18.21%), and limonene (16.47%). Its antifungal potential was evaluated against dermatophyte and yeast strains; the essential oil could not inhibit the growth of *Candida* spp., but showed *in vitro* activity against *T. rubrum*. Modulatory activity assays with combinations of standard antifungals and the essential oil showed synergistic effects against all the tested dermatophytes as well as two strains of *C. tropicalis* and one of *C. parapsilosis*. Toxicity tests showed that the essential oil exerts bioactivity against *A. salina*, indicating a correlation with the antifungal activity observed in this study.

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