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Antifungal and Modulatory Activity of Lemon Balm (*Lippia alba* (MILL.) N. E. BROWN) Essential Oil

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Abstract: Fungal diseases and the progressive development of resistance are a challenge. In this context, *Lippia alba* (lemon balm) is a species used in folk medicine, being described with antimicrobial potential. The aim of this study was to determine the antifungal activity and modulating effect of the essential oil of *Lippia alba* (Mill.) N.E. Brown leaves (LaEO). The antifungal activity of LaEO on eight Candida strains was determined by minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC), minimum biofilm inhibition concentration (MBIC), minimum biofilm eradication concentration (MBEC) and time-kill. The checkerboard technique was used to determine the modulating effect of LaEO on antifungal activity. The results indicate the presence of 11 constituents, with a predominance of carvone (58.15%) and limonene (25.37%). LaEO was able to inhibit the growth of all tested microorganisms, with MIC and MLC ranging from 0.078 to 1.25 mg/mL and MBIC and MBEC ranging from 0.156 to 5 mg/mL. The time-kill assay showed that LaEO was able to eliminate the strains after two hours of exposure and the best association was observed for the combination of LaEO and ketoconazole. The results of the study indicate that LaEO has excellent antifungal activity with potential biotechnological application.

Keywords: *Lippia alba*; essential oil; *Candida*; antifungal activity; modulatory effect

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1. Introduction

Antimicrobial resistance has increased exponentially in recent decades and is considered one of the greatest health challenges of the current century. It is estimated that 700,000 individuals die worldwide every year due to infections caused by resistant strains, and this number could increase to approximately 10 million worldwide by 2050 if there is no intervention [1].

Fungal infections are consequences of the health problems often associated with immunosuppression. Clinical success depends on the response to the antifungal therapy, presenting high mortality. Unfortunately, treatment options are restricted due to the limited availability of antifungals [2].

Candida sp. are opportunistic agents responsible for several diseases, ranging from skin and mucosal infections to systemic infections. *Candida albicans* accounts for more than 70% of all *Candida* infections; however, there has been an increase in clinical cases related to species such as *Candida parapsilosis* and *Candida krusei* [3].

Several mechanisms of resistance to antifungals have been described; among them is the formation of biofilm, which can lead to limitations in the use of available drugs. Furthermore, the indiscriminate use of these drugs increases the resistance, which has stimulated the use of gradually higher doses and broad-spectrum antifungals, which has increased the reporting of adverse reactions [4].

Considering the increased microbial resistance to multiple drugs, such as antifungals, synergistic combinations between natural molecules and commercial drugs may be good

alternatives for the control of these microorganisms, reducing the doses and consequently the adverse effects associated with the treatment [5].

Lippia alba (*Verbenaceae*), popularly known as lemon balm or lemon grass, is widely used by the population in the form of tea, macerated leaves, in compresses, baths or as an alcoholic extract, due to its calming effects, as well as for the treatment of skin diseases and gastrointestinal problems [6,7]. Several studies on the biological activities of *L. alba* essential oil (LaEO) have demonstrated anxiolytic, antioxidant, anesthetic, antigenotoxic, antiviral, antibacterial and antiparasitic activities, thus supporting its use in the treatment of several diseases [8–11]. In northeastern Brazil, different chemotypes of *L. alba* can be found, named according to the major constituents: chemotype I (citral, b-myrcene and limonene), chemotype II (citral and limonene) and chemotype III (carvone and limonene) [12,13]. Thus, the present study aimed to investigate the antifungal activity of LaEO against planctonic cell and *Candida* sp. biofilm and its modulatory effect on the activity of clinically used antifungal agents.

2. Materials and Methods

2.1. Obtaining and Characterization of Lippia Alba Essential Oil

The leaves of *Lippia alba* (Mill.) N. E. Brown were collected at the Medicinal Plants Garden Prof. Francisco José Abreu Matos of the Federal University of Ceará (−3.74578127 S, −38.57743103 W). The identification of the plant was carried out through exsiccate registered (no. 59675) at the Prisco Bezerra Herbarium of the Federal University of Ceará. The access to the Genetic Patrimony was registered in the National Genetic Patrimony and Associated Traditional Knowledge Management System (SisGen) under registration number A0F234C.

The *Lippia alba* essential oil (LaEO) was obtained by the hydrodistillation of fresh leaves, and the study of its composition was performed at the Brazilian Agricultural Research Corporation (Embrapa) by gas chromatography coupled to mass spectrometry (GC/MS), using the CG/EM-QP 2010 equipment (SHIMADZU, Kyoto, Japan), under the following conditions: OV-5 capillary column (5% phenyl, 95% dimethylpolysiloxane) measuring 30 m long × 0.25 mm of internal diameter × 0.25 µm film thickness, with a total flow of 1 mL/min of helium as carrier gas, and temperature gradient of 4 °C/min (70–180 °C) and 10 °C/min (180–300 °C), with an injector temperature of 230 °C. The mass spectra were produced by electron impact (70 eV). The identification of the compounds was performed by analyzing the fragmentation patterns displayed in the mass spectra with those present in the database provided by the equipment (NIST) and from literature data [14]. The Kovats retention index (KI) was determined under the same chromatographic conditions of the samples, using a series of n-alkanes (6 to 22 carbons), using the Van den Dool and Kratz equation [15].

2.2. Microbial Strains and Media

Eight microbial strains obtained from the American Type Culture Collection (ATCC) were used: *C. albicans* ATCC 64124; *C. albicans* ATCC 90028; *C. albicans* ATCC 90029; *C. albicans* ATCC 44858; *C. albicans* ATCC 10231; *C. tropicalis* ATCC 750; *C. tropicalis* ATCC 13803; and *C. parapsilosis* ATCC 90018. Microbial cultures were maintained in Sabouraud dextrose agar (SDA) under refrigeration. Before use, they were added to Sabouraud dextrose broth (SDB) and incubated at 35 °C until reaching the exponential growth phase. The antimicrobials and reagents used in the study were obtained from Sigma Chemical Co. (St Louis, MO, USA) with 99% analytical purity; culture media were obtained from Himedia® (Mumbai, Maharashtra, India).

2.3. Evaluation of Effect of LaEO on Planktonic Fungus

2.3.1. Determination of the Minimum Inhibitory Concentration—MIC

MIC of LaEO and antifungal (ANF) ketoconazole, nystatin, miconazole, ciclopirox olamine and amphotericin B for *Candida* sp. strains were determined by the culture broth

microdilution method adapted [16]. Initially, 100 µL of SDB was distributed in 96-well plates. Then, 20 µL of LaEO (0.002 to 5.000 mg/mL) or ANF (0.078 to 160.000 µg/mL) was added to the respective wells. Standardized aliquots of the appropriate inoculum (80 µL) for each strain were dispensed into the wells at a final concentration of 2.5×10^3 CFU/mL; diluents (1% Tween 80 and 1% DMSO) were used as vehicle controls.

Microplates were incubated at 35 °C for 24 h and submitted to microbial growth visual inspection and absorbance reading at 620 nm in a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The MIC was considered the lowest concentration of LaEO or ANF capable of completely inhibiting microbial growth, as evidenced by the absence of visible turbidity.

2.3.2. Determination of the Minimum Lethal Concentration—MLC

After determining the MIC, 5 µL of the wells treated with subsequent higher concentrations that did not show any visible fungal growth (no turbidity) was plated on the surface of plate-count agar (PCA) by spread-plate method to determine the minimum lethal concentration (MLC) [17]. Plates were incubated at 35 °C for 24 h, and the colonies that grew on the agar surface were counted. The concentration of LaEO that determined a microbial growth $\leq$ 0.1% of the initial inoculum was considered the MLC.

2.3.3. Effect of LaEO Exposure Time on Microbial Viability (Time-Kill)

In order to determine the dead time, 20 µL of LaEO at concentrations equal to $^1/_2 \times$ MIC, MIC and 2 × MIC was added to wells containing microbial suspension (1×10^3 CFU/mL) in SDB and incubated at 35 °C. 1% Tween 80 and 20 µg/mL miconazole were used as vehicle and positive control, respectively [18]. After 0, 2, 4, 6, 8, 10, 12 and 24 h, 5 µL aliquots were seeded on PCA using the microdrop technique [17] and incubated for 24 h at 35 °C, and the colonies were counted. Results were expressed as logarithm (log) of CFU/mL.

2.4. Evaluation of Effect of LaEO on Fungal Biofilm

2.4.1. Evaluation of *Candida* Strains Regarding Biofilm Adhesion and Formation

Aiming to evaluate biofilm adhesion, 100 µL of SDB was added to 96-well microplate, followed by the addition of 100 µL of the inoculum (2.5×10^5 CFU/mL) prepared with SDB supplemented with 2% sucrose. SDB and microbial culture were used as experimental controls. Microplates were incubated under agitation (125 rpm) at 35 °C for 48 h, the supernatant was removed, and the wells were washed with sterile distilled water to remove non-adherent cells.

After, microplates were dried at room temperature (10 min), and cells were fixed with 200 µL of absolute methanol (10 min) and dried (10 min). Then, 200 µL of an aqueous 0.4% crystal violet solution was added to each well and incubated for 10 min, and the wells were washed with sterile distilled water to remove excess crystal violet. After drying (10 min), crystal violet accumulated in biofilm cells was decolorized with 200 µL of 33% acetic acid (10 min). Finally, supernatant solution was transferred to another microplate and the absorbance measured in a microplate reader at 570 nm.

The biofilm formation cutoff was established according to optical density (ODc), defined as the mean absorbance at 570 nm ($OD_{570\ nm}$) of wells containing only SDB, the control group [19]. Tested strains were classified as non-biofilm-producers (OD $\leq$ ODc), weak producers (ODc < OD $\leq$ 2 × ODc), moderate producers (2 × ODc < OD $\leq$ 4 × ODc) or strong producers (4 × OD > ODc) [20].

2.4.2. Determination of the Minimum Biofilm Inhibition Concentration (MBIC)

Minimum biofilm inhibition concentration (MBIC) is the lowest concentration capable of inhibiting biofilm formation. One strain of each species that presented better capacity for biofilm production was selected. For this, the protocol of evaluation of biofilm formation

was followed including the addition of 100 µL of LaEO at concentrations of 0.002 to 5.000 mg/mL to each microplate well.

Controls used in this experiment were: culture medium, 20 µg/mL miconazole and 1% Tween 80. The MBIC was considered as the lowest concentration capable of inhibiting biofilm formation [19].

2.4.3. Determination of the Minimum Biofilm Eradication Concentration (MBEC)

This assay was performed to determine the lowest concentration of LaEO capable of eradicating the already formed biofilm [21]. *Candida* strains were grown at 37 °C in SDB supplemented with 100 mM glucose and incubated overnight. Cultures were centrifuged (5000× *g*/5 min), washed with sterile phosphate-buffered saline (PBS) and resuspended in SDB supplemented with 100 mM glucose at a standardized density of 1×10^7 CFU/mL.

For biofilm formation, 100 µL of microbial suspension was added to the microplate wells and incubated under agitation (75 rpm) at 35 °C for 90 min for the initial phase of cell adhesion. The supernatant was aspirated, each well washed with sterile PBS, followed by the addition of 200 µL of SDB with 100 mM glucose and incubation at 37 °C for 48 h. Then, the supernatant from each well was aspirated and the biofilm washed with PBS to remove non-adhered cells. A total of 20 µL of LaEO at different concentrations (40, 20, 10, 5 and 2.5 mg/mL) and 180 µL of SDB supplemented with 100 mM glucose were added to the microplate wells and incubated for 24 h at 35° C under orbital shaking (120 rpm). Sterile PBS and 20 µg/mL miconazole were used as controls.

After incubation, the supernatant was discarded, and the biofilm was scraped off and resuspended in sterile PBS. Subsequently, the contents of each well were aspirated, homogenized and serially diluted in sterile PBS. A total of 5 µL aliquots of each dilution were plated on SDA plates. After incubation at 37 °C for 48 h, colonies were counted, and the values obtained were expressed in logarithm (log) of CFU/mL.

2.5. Determination of the Modulatory Effect of LaEO on the Activity of Clinically Used Antifungals

The modulatory effect of LaEO on the activity of antifungals was evaluated using the checkerboard method [22]. For this assay, 5 µL of microbial cultures containing approximately 2.5×10^3 CFU/mL was added to 80 µL of Sabouraud broth and 10 µL of 1/2, 1/4, 1/8 and 1/16 × MIC of LaEO or ANF in the microplate wells. Microplates were incubated for 24 h at 35 °C. After the incubation period, visual inspection of the microbial growth was performed. Aiming to analyze the effect of LaEO–ANF associations, the fractional inhibitory concentration indices (FICIs) were calculated according to [23]:

$$FICI = FIC_{LaEO} + FIC_{ANF} = [LaEO]/MIC_{LaEO} + [ANF]/MIC_{ANF} \quad (1)$$

where FICI is the sum of the fractional inhibitory concentration of LaEO (FIC_{LaEO}) and ANF (FIC_{ANF}), represented by the fraction of the lowest concentration of LaEO or ANF with antifungal activity in the association [LaEO] or [ANF] derived by the MIC of LaEO or ANF, respectively. The synergistic effect was considered when FICI $\leq$ 0.5, additive or indifferent effect for 0.5 < FICI < 1.0 and antagonistic effect for FICI > 1.0 [24].

2.6. Statistical Analysis

All the experiments were performed in triplicate, with three replicates. The statistical analyses were performed using the GraphPad Prism 6.0 software, and the results were expressed as mean ± standard error of the mean (SEM). To verify the statistical differences between the groups, the analysis of variance (ANOVA) and Tukey post-test were performed. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Essential oil Composition

Gas chromatography coupled with mass spectrometry (GC-MS) of the *Lippia alba* essential oil (LaEO) led to the identification of 11 different components, representing 99.24% of the essential oil constituents (Table 1). The main components were limonene (25.37%) and carvone (58.15%). The identified compounds belong to the class of monoterpenes (34.4%), oxygenated terpenes (60.63%) and sesquiterpenes (4.21%). LaEO showed to be a yellowish-green oil with a very citric odor, with an extraction yield of 0.38%.

Table 1. Chemical constituents of LaEO.

No.	T. R. [1]	Constituent	I.K [2]	$I.K_E$ [3]	% Total
1	4.638	Sabinene	976	980	5.23
2	4.892	Myrcene	991	993	0.59
3	5.643	*P*-cymene	1026	1030	0.9
4	5.745	Limonene	1031	1035	25.37
5	6.419	γ-Terpinene	1062	1064	2.31
6	6.617	*cis*-Sabinene hydrate	1068	1071	0.9
7	7.386	*trans*-Sabinene hydrate	1097	1100	0.99
8	9.594	4-terpineol	1177	1181	0.59
9	11.617	Carvone	1252	1250	58.15
10	15.918	β-bourbonene	1384	1388	0.71
11	18.813	γ-murolene	1477	1484	3.5
		Total identified			99.24%
		Grouped components (%)			
		Monoterpene hydrocarbons			34.4
		Monoterpene containing oxygen			60.63
		Sesquiterpene hydrocarbons			4.21

LaEO: *Lippia alba* essential oil; [1] T.R.: time of retention (min); [2] I.K: index of Kovats in the literature; [3] $I.K_E$: index of Kovats experimental.

3.2. Minimum Inhibitory (MIC) and Lethal (MLC) Concentrations

MIC and MLC values of LaEO were the same for each *Candida* strain used in the present study and varied from 0.078 mg/mL to 1.250 mg/mL (Table 2). *C. albicans* ATCC 64124 strain presented the lowest value of MIC and MLC, being the most susceptible studied yeast to the LaEO effect. On the other hand, *C. tropicalis* ATCC 13803 presented the highest values of MIC, equal to 1.250 mg/mL.

The tested antifungal MIC varied among the assessed strains: ketoconazole (0.078 to 40.000 μg/mL), nystatin (5 to 20.000 μg/mL), ciclopirox olamine (0.078 to 5.000 μg/mL), miconazole (0.078 to 10.000 μg/mL) and amphotericin B (0.156 to 10.000 μg/mL). The *C. parapsilosis* ATCC 90018 strain presented lower MIC values when compared with other ANFs, except in the AMPHO B group, which presented *C. albicans* ATCC 10231 as a more susceptible strain in the experimental conditions, with an MIC equal to 0.156 μg/mL. Additionally, the highest value of MIC was presented by the *C. albicans* ATC90029 strain (40.000 μg/mL) when exposed to ketoconazole. Nystatin presented higher MIC values when compared with other ANFs, ranging from 5.000 to 20.000 μg/mL (Table 3).

Table 2. MIC and MLC of LaEO for *Candida* strains, determined by microdilution techniques in culture broth and viable cell counts, respectively.

Strains	[1] LaEO (mg/mL)	
	MIC [2]	MLC [3]
C. albicans ATCC 90028	0.625	0.625
C. albicans ATCC 44858	0.625	0.625
C. albicans ATCC 10231	0.625	0.625
C. albicans ATCC 90029	0.312	0.312
C. albicans ATCC 64124	0.078	0.078
C. tropicalis ATCC 13803	1.250	1.250
C. tropicalis ATCC 750	0.625	0.625
C. parapsilosis ATCC 90018	0.156	0.156

Controls: Tween 80 at 1%, miconazole 20 μg/mL, culture medium. [1] LaEO: *Lippia alba* essential oil; [2] MIC: minimum inhibitory concentration; [3] MLC: minimum lethal concentration.

Table 3. MIC and MLC of the antifungals for reference strains of *Candida*, determined by microdilution techniques in culture broth and viable cell counts, respectively.

	ANTIFUNGAL AGENTS (μg/mL)									
	[1] KET		[2] NYS		[3] CIC		[4] AMPHO B		[5] MICO	
Strains	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
1	0.312	0.312	10.000	20.000	0.078	0.156	1.25	2.500	10.000	10.000
2	5.000	5.000	10.000	20.000	0.312	0.625	0.625	2.500	0.156	0.156
3	40.000	40.000	10.000	10.000	0.078	0.078	0.625	1.250	0.078	0.078
4	10.000	10.000	5.000	10.000	0.156	0.625	0.625	1.250	0.078	0.078
5	20.000	20.000	5.000	5.000	0.078	0.312	0.156	0.312	0.625	0.625
6	1.250	1.250	10.000	10.000	0.312	0.625	5.000	20.000	1.250	1.250
7	1.250	1.250	20.000	20.000	5.000	10.000	10.000	20.000	0.156	0.156
8	0.078	0.078	5.000	10.000	0.078	0.312	1.250	2.500	0.078	0.078

1—*C. albicans* ATCC 64124, 2—*C. albicans* ATCC 90028, 3—*C. albicans* ATCC 90029, 4—*C. albicans* ATCC 44858, 5—*C. albicans* ATCC 10231, 6—*C. tropicalis* ATCC 750, 7—*C. tropicalis* ATCC 13803, 8—*C. parapsilosis* ATCC 90018. [1] ketoconazole (KET), [2] nystatin (NYS), [3] ciclopirox olamine (CIC), [4] amphotericin B (AMPHO B) and [5] miconazole (MICO). Control: 1% DMSO.

As in the MIC, the antifungal MLC tested varied among the strains evaluated: ketoconazole (0.078 to 40.000 μg/mL), nystatin (5.000 to 20.000 μg/mL), ciclopirox olamine (0.078 to 5.000 μg/mL), miconazole (0.078 to 10.000 μg/mL). However, amphotericin B presented a different value range (0.312 to 20.000 μg/mL). The MLC values for CIC and MICO demonstrate that most strains have a high sensitivity to these ANFs, and two of the ANFs tested showed values that coincided with the MICs (ketoconazole, miconazole), while the others varied among the tested strains.

3.3. Effect of LaEO Exposure Time on Microbial Viability (Time-Kill)

LaEO MIC and 2 × MIC were able to eliminate all *Candida* strains after 2 h of contact. For 1/2 × MIC, a reduction in microbial growth was observed in the first two hours of exposure for strains *C. albicans* ATCC 64124, *C. albicans* ATCC 90028, *C. albicans* ATCC 90029, *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 90018, with different inhibition profiles. The time-kill assay demonstrated the rapid antifungal action of LaEO on the tested strains (Figure 1).

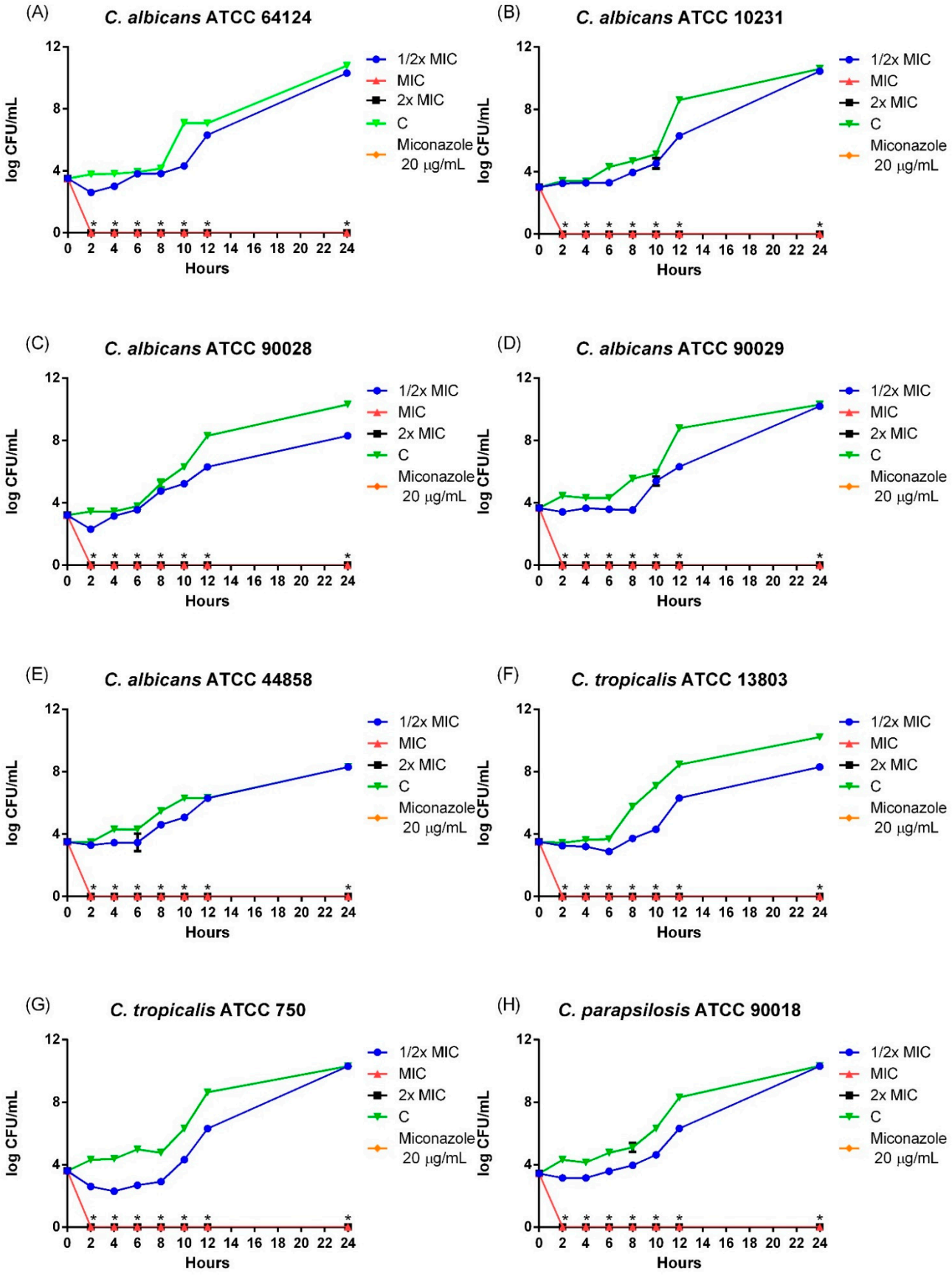

Figure 1. Effect of the time of exposure on the minimum inhibitory concentration of LaEO on the viability of *Candida* strains, determined by the microdilution technique in culture broth and viable

cell count. (**A**) MIC: 0.078 mg/mL. (**B**) MIC: 0.625 mg/mL. (**C**) MIC: 0.625 mg/mL. (**D**) MIC: 0.312 mg/mL. (**E**) MIC: 0.625 mg/mL. (**F**) MIC: 1.250 mg/mL. (**G**) MIC: 0.625 mg/mL. (**H**) MIC: 0.156 mg/mL. Control for all groups (**C**): culture medium + microorganism + 1% Tween 80. The values are expressed as mean ± SEM of three experiments, with three replicates. Data analysis was performed by ANOVA, with Tukey post-test and $p < 0.05$ (*).

3.4. Study of LaEO Effect on the Candida Biofilm

3.4.1. Classification of *Candida* Strains Regarding Biofilm Adhesion and Production

The tested strains were evaluated regarding biofilm adhesion and formation, and the results are shown in Table 4. *C. parapsilosis* ATCC 90018 showed the greatest biofilm formation capacity, being classified as a strong biofilm producer. *C. albicans* strain ATCC 10231 was shown to be a moderate biofilm producer, while *C. albicans* ATCC 44858, 90028, 64124 and *C. tropicalis* ATCC 13803 were classified as poor producers. *C. albicans* ATCC 90029 and *C. tropicalis* ATCC 750 strains did not produce biofilm. The selection of *C. parapsilosis* ATCC 90018, *C. tropicalis* ATCC 13803 and *C. albicans* ATCC 10231 to continue the biofilm study was performed according to the strongest biofilm production capacity in each species.

Table 4. Classification of *Candida* strains regarding biofilm adherence and production.

Strains	Mean $OD_{570\ nm}$	[1] Classification
C. parapsilosis ATCC 90018	1.725	Strong producer
C. tropicalis ATCC 13803	0.595	Weak producer
C. tropicalis ATCC 750	0.297	Non-producer
C. albicans ATCC 10231	0.821	Moderate producer
C. albicans ATCC 90028	0.589	Weak producer
C. albicans ATCC 64124	0.454	Weak producer
C. albicans ATCC 44858	0.387	Weak producer
C. albicans ATCC 90029	0.349	Non-producer
Control	0.361	

[1] The strains were classified as non-biofilm-producers (OD ≤ ODc), weak producers (ODc < OD ≤ 2 × ODc), moderate producers (2 × ODc < OD ≤ 4 × ODc) and strong producers (4 × OD > ODc).

3.4.2. Minimum Biofilm Inhibition (MBIC) and Minimum Biofilm Eradication (MBEC) Concentrations

In order to evaluate the potential of LaEO to inhibit biofilm formation, MBIC and the MBEC were determined. The minimum concentrations of LaEO capable of inhibiting biofilm formation (MBIC) were the same as the previously determined MIC, as shown in Table 5.

Table 5. MBIC and MBEC of LaEO for *Candida* strains.

Strains	[1] LaEO (mg/mL)	
	[2] MBIC	[3] MBEC
C. albicans ATCC 10231	0.625	5.000
C. tropicalis ATCC 13803	1.250	5.000
C. parapsilosis ATCC 90018	0.156	5.000

Controls: Tween 80 at 1% and miconazole 20 µg/mL. [1] LaEO: *Lippia alba* essential oil; [2] MBIC: minimum biofilm inhibitory concentration; [3] MBEC: minimum biofilm eradication concentration.

When the minimum concentrations capable of eradicating the already formed biofilm (MBECs) were evaluated, LaEO was found at 5 mg/mL for all tested strains. In comparison to the MBICs, MBECs were 4 to 32 times higher in the studied strains.

3.5. Modulatory Effect of LaEO on the Activity of Clinically Used Antifungal Agents

The modulatory effect of LaEO on ANF activity was evaluated using the checkerboard method. The association of LaEO–ANF was performed for all strains; however, only associations capable of inhibiting the visual growth of the tested strains were listed in the tables. Thus, the index of fractional inhibition concentration (FICI) was calculated using the lowest concentrations of LaEO and ANF.

The LaEO–NYS associations (Table 6) inhibited *C. albicans* strain ATCC 64124, synergistically modulating NYS action. Even presenting high MIC values for nystatin against this strain (10.000 µg/mL), the association with LaEO positively modulated its action, reducing its concentration by up to 1/16 × MIC. For other strains, no LaEO–NYS associations were observed capable of inhibiting the visual growth of the tested strains.

Table 6. Modulatory effect of LaEO on the antifungal activity of nystatin (NYS) on *Candida* strains.

Strain	[1] FIC_{LaEO}	[2] FIC_{NYS}	[3] FICI	[4] Effect
C. albicans ATCC 64124	0.250	0.125	0.375	S
	0.125	0.062	0.187	S

Results of associations in which there was at least one modulatory effect. Fractional inhibitory concentration (FIC); fractional inhibitory concentration index (FICI). [1] FIC_{LaEO} = MIC of LaEO in the association/MIC of isolated LaEO. [2] FIC_{NYS} = MIC of NYS in the association/MIC of isolated NYS. [3] FICI = FIC_{LaEO} + FIC_{NYS}. [4] FICI $\leq$ 0.500: synergism (S); FICI > 0.500 and <1.000: additive (I); FICI $\geq$ 1.000: antagonism (A).

In LaEO–KET associations (Table 7), the inhibition of all tested strains was observed, with 68.42% (13) of synergistic, 10.52% (2) of indifferent and 21.05% (4) of antagonistic effects. Comparatively, our study showed that ketoconazole had the best results when associated with LaEO, reducing by up to 16-fold the antifungal MIC in the tested associations, with synergism in five of the eight studied strains.

Table 7. Modulatory effect of LaEO on the antifungal activity of ketoconazole (KETO) on *Candida* strains.

Strains	[1] FIC_{LaEO}	[2] FIC_{KETO}	[3] FICI	[4] Effect
C. albicans ATCC 64124	0.500	0.500	1.000	A
	0.500	0.250	0.750	I
C. albicans ATCC 90028	0.062	0.250	0.312	S
	0.062	0.125	0.187	S
	0.125	0.125	0.25	S
C. albicans ATCC 90029	0.125	0.125	0.250	S
	0.062	0.125	0.187	S
	0.062	0.062	0.124	S
C. albicans ATCC 44858	0.250	0.250	0.500	S
	0.125	0.125	0.250	S
	0.062	0.062	0.124	S
C. albicans ATCC 10231	0.062	0.062	0.124	S
	0.062	0.125	0.187	S
	0.125	0.125	0.250	S
C. tropicalis ATCC 13803	0.500	0.500	1.000	A
	0.250	0.250	0.500	S
C. tropicalis ATCC 750	0.500	0.500	1.000	A
C. parapsilosis ATCC 90018	0.500	0.500	1.000	A
	0.500	0.250	0.750	I

Results of associations in which there was at least one modulatory effect. Fractional inhibitory concentration (FIC); fractional inhibitory concentration index (FICI). [1] FIC_{LaEO} = MIC of LaEO in the association/MIC of isolated LaEO. [2] FIC_{KETO} = MIC of KETO in the association/MIC of isolated KETO. [3] FICI = FIC_{LaEO} + FIC_{KETO}. [4] FICI $\leq$ 0.500: synergism (S); FICI > 0.500 and <1.000: additive (I); FICI $\geq$ 1.000: antagonism (A).

The LaEO–CIC associations (Table 8) inhibited the growth of five of the eight tested strains, with a predominance of additive effects (12) (63.15%). None of the antifungal agents studied showed a synergistic effect on the strain of *C. parapsilosis*, and only ketoconazole and ciclopirox olamine presented an effect, either additive or antagonistic, on this yeast.

Table 8. Modulatory effect of LaEO on the antifungal activity of ciclopirox olamine (CIC) on *Candida* strains.

Strains	[1] FIC_{LaEO}	[2] FIC_{CIC}	[3] FICI	[4] Effect
C. albicans ATCC 64124	0.115	0.500	0.615	I
	0.051	0.500	0.551	I
	0.115	0.250	0.365	S
	0.051	0.250	0.301	S
C. albicans ATCC 44858	0.500	0.500	1.000	A
	0.250	0.500	0.750	I
	0.125	0.500	0.625	I
	0.062	0.500	0.562	I
C. tropicalis ATCC 13803	0.125	0.500	0.625	I
	0.062	0.500	0.562	I
	0.125	0.062	0.187	S
	0.062	0.062	0.124	S
C. tropicalis ATCC 750	0.500	0.500	1.000	A
	0.250	0.250	0.500	I
	0.250	0.500	0.750	I
C. parapsilosis ATCC 90018	0.500	0.500	1.000	A
	0.250	0.500	0.750	I
	0.125	0.500	0.625	I
	0.062	0.500	0.562	I

Results of associations in which there was at least one modulatory effect. Fractional inhibitory concentration (FIC); fractional inhibitory concentration index (FICI). [1] FIC_{LaEO} = MIC of LaEO in the association/MIC of isolated LaEO. [2] FIC_{CIC} = MIC of CIC in the association/MIC of isolated CIC. [3] FICI = FIC_{LaEO} + FIC_{CIC}. [4] FICI $\leq$ 0.500: synergism (S); FICI > 0.500 and <1.000: additive (I); FICI $\geq$ 1.000: antagonism (A).

For the LaEO–MICO association (Table 9), only the presence of additive (5) (62.5%) and antagonistic effects (3) (37.5%) was observed. This association was not promising as it presented no synergism, furthermore, acting only against *C. albicans* strains.

Table 9. Modulatory effect of LaEO on the antifungal activity of miconazole (MICO) on *Candida* strains.

Strains	[1] FIC_{LaEO}	[2] FIC_{MICO}	[3] FICI	[4] Effect
C. albicans ATCC 64124	0.500	0.500	1.000	A
	0.500	0.250	0.750	I
	0.500	0.125	0.625	I
	0.500	0.062	0.562	I
C. albicans ATCC 90029	0.500	0.500	1.000	A
C. albicans ATCC 10231	0.500	0.500	1.000	A
	0.500	0.250	0.750	I
	0.250	0.500	0.750	I

Results of associations in which there was at least one modulatory effect. Fractional inhibitory concentration (FIC); fractional inhibitory concentration index (FICI). [1] FIC_{LaEO} = MIC of LaEO in the association/MIC of isolated LaEO. [2] FIC_{MICO} = MIC of MICO in the association/MIC of isolated MICO. [3] FICI = FIC_{LaEO} + FIC_{MICO}. [4] FICI $\leq$ 0.500: synergism (S); FICI > 0.500 and <1.000: additive (I); FICI $\geq$ 1.000: antagonism (A).

The LaEO–AMPHO B associations (Table 10) inhibited only two strains, showing 37.5% (3) of synergistic effects, 50% (4) of indifferent effects and 12.5% (1) of antagonistic effects.

These results show a synergic potential in *C. tropicalis* strains, reducing by up to 8 to 16-fold the antifungal MIC, which is important due to toxicity associated with amphotericin use.

Table 10. Modulatory effect of LaEO on the antifungal activity of amphotericin B (AMPHO B) on *Candida* strains.

Strains	[1] FIC_{LaEO}	[2] $FIC_{AMPHO\ B}$	[3] FICI	[4] Effect
C. tropicalis ATCC 13803	0.500	0.250	0.750	I
	0.500	0.125	0.625	I
	0.250	0.250	0.500	S
	0.250	0.125	0.375	S
C. tropicalis ATCC 750	0.500	0.500	1.000	A
	0.500	0.250	0.750	I
	0.250	0.500	0.750	I
	0.250	0.250	0.500	S

Results of associations in which there was at least one modulatory effect. Fractional inhibitory concentration (FIC); fractional inhibitory concentration index (FICI). [1] FIC_{LaEO} = MIC of LaEO in the association/MIC of isolated LaEO. [2] $FIC_{AMPHO\ B}$ = MIC of AMPHO B in the association/MIC of isolated AMPHO B. [3] FICI = FIC_{LaEO} + $FIC_{AMPHO\ B}$. [4] FICI $\leq$ 0.500: synergism (S); FICI > 0.500 and <1.000: additive (I); FICI $\geq$ 1.000: antagonism (A).

4. Discussion

The present study showed that different concentrations of LaEO were able to inhibit the growth of *Candida* sp. strains associated with the inhibition of the biofilm formation capacity in the early hours of incubation. Furthermore, LaEO eradicated the already formed biofilm, although at higher concentrations. In this sense, LaEO presented a modulatory effect on the activity of clinically used antifungal agents, leading to a possible reduction in the concentration of these substances with better effects.

Several studies have reported the antimicrobial activity of the species *L. alba* and that it has antifungal properties due to the presence of compounds such as coumarins, terpenes and flavonoids [25]. The main presence of limonene and carvone in this oil allows its classification as chemotype III [26].

Of the compounds most found in essential oils, carvone has shown antibacterial and antifungal activity [27]. The mechanism of action of carvone involves structural destabilization of the phospholipid and interaction with membrane proteins, in addition to acting as a proton exchanger, altering the pH gradient across the membrane [9].

Recent work has shown that monoterpenes, such as carvone, have been described as having activity against *Candida*. Among the tested monoterpenes, (±)-citronellol was the most potent compound followed by (+)-α-pinene and menthol [28]. It has already been shown that carvone interferes with the serum-induced formation of filamentous structures *Candida albicans* at substantially lower concentrations than those causing significant inhibition of growth [29].

Limonene, another major component of LaEO, inhibited the growth of *C. albicans* by causing oxidative stress in the cell envelope, also leading to DNA damage, resulting in cell-cycle modulation and apoptosis induction through nucleolar stress [28]. Indeed, it has already been described that limonene, a component generally recognized as safe (GRAS), inhibits *Candida albicans* growth by inducing apoptosis through downregulation of Tps3 and activation of caspase (CaMca1) [30].

For natural products, MIC values between 50 and 500, 600 and 1500 or above 1600.0 µg/mL are considered to have strong, moderate and weak activity, respectively [31]. In the present study, LaEO showed an antifungal effect against eight *Candida* strains, with MIC ranging from 0.078 mg/mL to 1.250 mg/mL, with values similar to those of MLC. This demonstrates that LaEO has antifungal effects rated from strong to weak, depending on the strain tested. Previous studies showed that LaEO showed fungicidal and fungistatic effects against saprophytic fungus at concentrations around 1.0 mg/mL, even with higher antifungal activity due to a synergistic effect with some active components [32].

The evaluation of microbial growth kinetics is an important factor, as it provides valuable information for conducting microbiological experiments and determining the mechanism of action. Among the mechanisms evaluated, the inhibitory and lethal activity of substances can be determined by temporal killing assays, which allow the identification of the speed of antimicrobial action [33,34].

Importantly, the effects observed for LaEO occurred within the first hours of incubation, reducing microbial growth even at half MIC, indicating potent antifungal activity. Essential oil extracted from *Lippia* sp. species has been shown to reduce growth by preventing fungal recovery [35].

Eradicating the formation of these fungal biofilms is a challenge. Biofilm formation is an important strategy for survival, resistance and pathogenicity. Terpenes and flavonoid-rich extracts have been shown to reduce the expression of adhesion and aggregation molecules, reducing biofilm viability [36].

Biofilms are usually composed of one or several species, forming so-called mono-species or multi-species biofilms, respectively, which also show increased resistance to antimicrobial and antifungal agents and may involve spoilage and pathogenic microorganisms and also involve sessile microbial cells that are different from their planktonic form as they have an altered phenotype and different metabolic characteristics [37,38].

Clinically used antifungals are generally ineffective against certain *C. albicans* biofilms or require high doses to eradicate them, which can result in serious adverse effects. In general, antifungals eliminate free planktonic cells but are often ineffective against biofilms, mainly due to their incomplete removal, which can result in resistant reinfections [39].

The prevention of *Candida* biofilms is an important assay since they are associated with severe infections and the fact that microorganisms with high resistance to antifungal drugs and immune defenses occur in these structures [40], which justifies the search for essential oils and other bioactive compounds with antibiotic activity.

The use of phytochemicals to treat biofilm-forming pathogens is a breakthrough, bringing with it new prospects for product development to prevent or eradicate the biofilm-forming microbial community [41]. Several studies have demonstrated the inhibition of bacterial and fungal biofilm formation by phytoconstituents such as essential oils and flavonoids [16,42–45] as in the case of LaEO, which was able to inhibit all strains tested.

In fact, EOs can act on the mechanisms involved in biofilm formation, thus making it possible to eradicate and control this process. The effect exerted depends both on the chemical characteristics associated with its chemical composition and on the target microorganism. It is noteworthy that both hydrophilic and hydrophobic compounds in essential oils can contribute to antibiofilm activity [46].

In order to reduce the dosage of antifungal agents, preventing toxicity, adverse effects, collateral effects and drug interactions, several studies have developed strategies to combine bioactive molecules, which can interact and determine synergistic, additive or antagonistic effects. Synergism can be defined as the occurrence of a greater effect when combined than the sum of the individual effects of the assessed substances [47]. Thus, synergistic interactions can increase efficacy and lead to a reduction in effective doses for the treatment of infectious diseases [48].

Combination therapies that incorporate substances such as natural products can enhance the activity of an antimicrobial agent and are suggested as strategies to combat microbial resistance [49]. LaEO showed different modulation profiles, with a predominance of synergistic and additive effects for the tested strains when associated with standard commercial antifungal agents.

Previously, a synergistic potential of limonene-rich *Citrus aurantium* L. essential oil with antibiotics against *Candida albicans* was demonstrated, indicating a potential interaction of this compound with the enzymes N-myristoyl transferase (NMT) and cytochrome P450 14-α sterol demethylase (CYP51), essential for the redox metabolism of the fungus [50].

In in vitro experiments using LaEO (chemotype—geranium, neral and myrcene), a positive modulation of the action of ciprofloxacin, cefepime and ceftriaxone on Salmonella and

Shigella strains was observed, also reducing the biofilm mass of both strains when exposed to the combination LaEO–ciprofloxacin [45]. In another study using LaEO (chemotype—sabinene, (E)-caryophyllene and limonene), a positive modulatory activity (synergism) was observed in combinations with standard antifungal drugs, resulting in the inhibition of all *Trichophyton rubrum* strains tested and of two strains of *C. tropicalis* and one of *C. parapsilosis* [51].

Synergism assays have played a leading role in the search for new antimicrobial and antifungal compounds to improve the use of already known substances. One study showed antibacterial and antibiofilm activities and synergism with florfenicol from the essential oils of *Lippia sidoides* and *Cymbopogon citratus* against *Aeromonas hydrophila*, a Gram-negative bacterium that has a high potential for resistance [52].

Extracts from species of the genus Lippia have already been studied as modulators of antibiotic activity on *S. aureus*. A study performed with *Lippia origanoides* showed a modulating effect on antibacterial activity at subinhibitory concentrations of neomycin and amikacin. A similar modulation was found when the natural products were switched to chlorpromazine, an inhibitor of bacterial efflux pumps, suggesting the involvement of resistance mediated by the efflux system in MRSA [53].

Altogether, our results demonstrate the good antifungal potential of LaEO. Interestingly, the oil is promising against planktonic fungus and in biofilms. It is a valuable finding, opening a field of study aiming at the development of a pharmacological and biotechnological tool, being a candidate for the development of a new antifungal agent for clinical use.

5. Conclusions

LaEO showed excellent antifungal activity on the tested *Candida* strains, with fungicidal and fungistatic action on planktonic cells, as well as an antibiofilm agent. It positively modulated the anti-*Candida* action when combined with antifungals, especially when used together with ketoconazole. However, additional studies are required, such as in vivo studies, to further demonstrate its antifungal-action mechanism.

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