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Original Article

Lavandula dentata L. essential oil: a promising antifungal and antibiofilm agent against oral *Candida albicans*

Óleo essencial de *Lavandula dentata* L.: um agente antifúngico e antibiofilme promissor contra *Candida albicans* oral

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Abstract

Candida albicans is the main fungal species involved in oral candidiasis, and its increasing resistance to pharmacological treatment encourages the search for improved antifungal agents. *Lavandula dentata* L. essential oil (LD-EO) has been recognized for its antimicrobial activity, but little is known about its role against oral *C. albicans*. This study evaluated the antifungal and antibiofilm activities, mechanisms of action, and toxicity of LD-EO from Brazil against oral strains of *C. albicans*. Antifungal activity was assessed based on Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC), association study with miconazole (Checkerboard method), and sorbitol and ergosterol assays. Inhibition of biofilm formation and disruption of preformed biofilm were considered when studying the effects of the product. Additionally, the toxicity of LD-EO was evaluated by a hemolysis assay on human erythrocytes. Phytochemical analysis by gas chromatography-mass spectrometry identified eucalyptol (33.1%), camphor (18.3%), and fenchone (15.6%) as major constituents. The test substance showed mainly fungicidal activity (MIC100 = 8 µg/mL; MFC = 16 µg/mL), including against two miconazole-resistant isolates of *C. albicans*. The effects of LD-EO were synergistic with those of miconazole and appeared not to involve damage to the fungal cell wall or plasma membrane. Its effectiveness in inhibiting biofilm formation was higher than the effect of disrupting preformed biofilm. Finally, the product exhibited low hemolytic activity at MIC. Based on the favorable and novel results described here, LD-EO could constitute a promising therapeutic alternative for oral candidiasis, including miconazole-resistant cases.

Keywords: oral candidiasis, natural products, antimicrobial activity, antifungal agents, essential oil.

Resumo

Candida albicans é a principal espécie fúngica envolvida na candidíase bucal, e sua crescente resistência ao tratamento farmacológico encoraja a busca por melhores agentes antifúngicos. O óleo essencial da *Lavandula dentata* L. (LD-EO) tem sido reconhecido por sua atividade antimicrobiana, porém pouco se conhece o seu papel contra *C. albicans* bucal. Este trabalho avaliou as atividades antifúngica e antibiofilme, mecanismos de ação e toxicidade do LD-EO do Brasil contra cepas de *C. albicans* bucal. A atividade antifúngica foi avaliada baseando-se em: Concentração Inibitória Mínima (CIM), Concentração Fungicida Mínima (CFM), estudo de associação com o miconazol (método *Checkerboard*) e ensaios com sorbitol e ergosterol. A inibição da formação do biofilme e a ruptura do biofilme pré-formado foram considerados no estudo dos efeitos do produto. Adicionalmente, a toxicidade do LD-EO foi avaliada pelo ensaio de hemólise em eritrócitos humanos. A análise fitoquímica por cromatografia gasosa-espectrometria de massa identificou eucaliptol (33.1%), cânfora (18.3%) e fenchona (15.6%) como constituintes majoritários. A substância teste revelou atividade principalmente fungicida (CIM100 = 8 µg/mL; CFM = 16 µg/mL), inclusive contra dois isolados de *C. albicans* resistentes ao miconazol. Os efeitos do LD-EO foram sinérgicos aos do miconazol e parecem não envolver danos à parede celular ou à membrana plasmática fúngica. Sua efetividade em inibir a formação do biofilme foi maior que o efeito de eliminação do biofilme pré-formado. Finalmente, o produto exerceu baixa atividade hemolítica na CIM. Considerando os resultados favoráveis e inéditos aqui descritos, o LD-EO poderia constituir uma alternativa terapêutica promissora para a candidíase bucal, incluindo casos resistentes ao miconazol.

Palavras-chave: candidíase bucal, produtos naturais, atividade antimicrobiana, agentes antifúngicos, óleo essencial.

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1. Introduction

Candidiasis is the most common fungal disease affecting the oral cavity. It generally manifests as a mild condition limited to the mucous membranes. However, in some cases, it can spread to other body regions and even lead to fungemia. Severe cases of the disease, capable of causing the patient's death, often occur in people with some form of immunodeficiency, such as acquired immunodeficiency syndrome (AIDS), diabetes, or those undergoing immunosuppressive therapy (Bhattacharya et al., 2020; Fang et al., 2021; Karpiński et al., 2021). Although there have been changes in the profile of *Candida* species identified in oral candidiasis lesions due to the widespread use of azoles (Moghadam et al., 2020; Muhaj et al., 2022), *Candida albicans* remains the primary species responsible for this disease, identified in over 80% of lesions (Millsop and Fazel, 2016; Miranda-Cadena et al., 2021).

Generally, its treatment involves control of underlying systemic diseases, oral hygiene guidance, and topical antifungal agents (Miranda-Cadena et al., 2021; Shui et al., 2021). Systemic administration is reserved for cases of therapeutic failure or in the face of disseminated or recurrent infections (Lyu et al., 2016; Quindós et al., 2019; Xiao et al., 2022). Unfortunately, commercially available antifungals not only often cause side effects, but they also face the issue of increasing microbial resistance, a potential threat to public health worldwide (Shui et al., 2021; Iyer et al., 2022; El Hachlafi et al., 2023).

The need for new and effective antifungal agents with lower toxicity and a broader or different spectrum of action than conventional drugs has become urgent. In this regard, natural products obtained from plants have been of great interest, given the biological and structural diversity of plants' chemical constituents (Sakkas and Papadopoulou, 2017; Silva et al., 2021). Essential oils (EOs) extracted from plants of the Lamiaceae family, especially those of the *Lavandula* genus, have been highlighted for their antimicrobial effects, including against *Candida* spp. (Waller et al., 2017). Among these, the EO from *Lavandula dentata* Linnaeus (*Lavandula dentata* L.), known as French lavender, is one of the most promising as it contains substances with proven antifungal activity, such as eucalyptol, camphor, and fenchone (Zuzarte et al., 2009; Pessoa et al., 2020; Ivanov et al., 2021).

Scientific literature has shown promising results regarding the antimicrobial activity of *Lavandula dentata* L. essential oil (LD-EO) against standard strains of *C. albicans* (Justus et al., 2018; Müller-Sepúlveda et al., 2020). However, there is a lack of research on its efficacy against oral isolates of *Candida* spp. Furthermore, there has been no investigation into the potential of combining LD-EO with miconazole, one of the leading antifungals for treating oral candidiasis, which is facing increasing and concerning drug resistance.

This study evaluated the antifungal and antibiofilm activities and mechanisms of action of LD-EO cultivated in Brazil. The effects of this natural product were specifically evaluated against strains of *C. albicans* isolated from patients with oral candidiasis, including a miconazole-resistant strain. Additionally, its toxicity was evaluated using hemolysis assay. By assessing the promising but underexplored biological activity of LD-EO, this research may contribute to developing strategies for the treatment of oral candidiasis and also for overcoming the issue of miconazole resistance.

2. Material and Methods

2.1. Essential oil and reagents

The test substance was pure LD-EO, extracted through steam distillation, and commercially obtained from Laszlo® (Belo Horizonte, MG, Brazil). Miconazole, dimethyl-sulfoxide (DMSO), Tween 80, sorbitol, and ergosterol were purchased from Sigma-Aldrich® (São Paulo, SP, Brazil).

2.2. Chemical analysis of the essential oil

The chemical composition of LD-EO was analyzed by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu QP2010 system equipped with a Rtx-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) operating at 70 eV. Helium was the carrier gas at a flow rate of 3.0 mL/min (split ratio = 1:100). The injector and detector temperatures were set at 220 °C and 280 °C, respectively. The column temperature was programmed from 40 °C (for 1 min) to 220 °C at a rate of 10 °C/min, then from 220 °C (for 2 min) to 280 °C at 20 °C/min. Subsequently, the column remained at 280 °C for 5 min. Ion scanning was performed (*m*/*z* range = 50–500). The test substance was diluted with hexane (999:1, v/v), and then 1 µL of the solution was injected into the chromatograph (Ferreira et al., 2023).

2.3. Fungal strains and growth conditions

This study focused on clinical strains of *C. albicans* isolated from patients with oral candidiasis, registered in the Culture Collection of the Mycology Laboratory at the Federal University of Paraíba. The selected strains included: LM-4 (isolated from palate); LM-70, LM-38, LM-86, LM-80, LM-128, and LM-13B (isolated from buccal mucosa); LM-42, LM-115, LM-106, LM-125, LM-12B, and LM-19P (isolated from prostheses). A standard strain of *C. albicans* (ATCC-76485) from the American Type Culture Collection (ATCC, Rockville, MD, USA) was also evaluated. All fungal strains analyzed in this research are registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under code A2DA181.

Sabouraud Dextrose Agar (SDA) (Difco Laboratories, Detroit, MI, USA) and RPMI-1640 cell culture medium with L-glutamine, without sodium bicarbonate (Sigma-Aldrich®, São Paulo, SP, Brazil) were used for maintaining the strains and executing antifungal assays, respectively. Both culture media were prepared according to the manufacturers' instructions. Strains of *C. albicans* were grown in SDA at 35–37 °C for 24-48h before conducting microbiological assays. Subsequently, microbial colonies were suspended in a sterile 0.85% NaCl solution and adjusted according to the 0.5 McFarland standard to obtain an inoculum of 1–5

× 10^6 colony-forming units/mL (CFU/mL) (Cleeland and Squires, 1991; Hadacek and Greger, 2000; CLSI, 2008).

2.4. Minimum Inhibitory Concentration (MIC)

To evaluate the antifungal activity of LD-EO and miconazole, the Minimum Inhibitory Concentration (MIC) was determined through a microdilution technique based on established protocols (Cleeland and Squires, 1991; Hadacek and Greger, 2000; CLSI, 2008). A sterile 96-well microplate for cell culture with a "U"-shaped bottom (Inlab, São Paulo, SP, Brazil) was used for this assay. MIC was defined as the lowest concentration at which the product visibly inhibited fungal growth in the wells compared to controls. Initially, 100 µL of doubly concentrated RPMI medium was dispensed into the microdilution plates. Then, 100 µL of LD-EO and control (miconazole) were inoculated into the wells of the first row of each plate. A serial dilution was performed at a two-fold ratio, resulting in different concentrations (1,024–2.0 µg/mL). Aliquots (10 µL) of fungal strain suspensions were added to the wells, each plate column corresponding to a specific strain. The plates were sealed and incubated at 35 ± 2 °C for 24–48h for subsequent data reading. Controls of the culture medium (RPMI) sterility and strain viability (RPMI + 3% DMSO + 2% Tween 80 + yeasts) were prepared concomitantly. This assay was conducted in triplicate, and the result was expressed as the modal value of the MICs obtained.

The products (LD-EO and miconazole) were considered active if they inhibited at least 50% of the microorganisms used in the experiment (Cleeland and Squires, 1991; Hafidh et al., 2011). Antifungal activity was categorized as strong (<600 µg/mL), moderate (600–1500 µg/mL), or weak (>1500 µg/mL), adapting the classification system adopted by Silva et al. (2020).

2.5. Minimum Fungicidal Concentration (MFC)

An assay to determine the Minimum Fungicidal Concentration (MFC) was conducted after reading the MIC. Aliquots (10 µL) of supernatant from the wells showing complete inhibition of fungal growth (MIC, MIC×2, and MIC×4) were transferred to the wells of a new microplate containing 100 µL of RPMI medium, and this plate was incubated at 35 ± 2 °C for 24–48h (Silva et al., 2020). Controls of the culture medium (RPMI) sterility and strain viability (RPMI + 3% DMSO + 2% Tween 80 + yeasts) were prepared concomitantly. MFC was defined as the lowest concentration of the product capable of inhibiting fungal growth (Ncube et al., 2008; Balouiri et al., 2016). This assay was performed in triplicate, and results were expressed as the modal value obtained with the three experiments.

To specify the nature of the antifungal effect of LD-EO and miconazole, the MFC/MIC ratio was calculated. These products were classified as fungicidal (ratio between 1:1 and 2:1) or fungistatic (ratio > 2:1), as proposed by Hafidh et al. (2011).

2.6. Time-kill curves assay

The effect of LD-EO on the cell death curves of yeasts was analyzed using an adaptation of the methodology by Silva et al. (2020). Two strains of *C. albicans* (ATCC-76485 and LM-4) were selected based on MIC results and evaluated over 24h.

A microdilution of RPMI medium was performed similarly to that previously described to obtain three concentrations (MIC, MIC×2, and MIC×4) of the test substance. Next, 10 µL of fungal strain suspensions were added to each well. Then, aliquots (1 µL) of each concentration were collected using disposable bacteriological loops (K30-0101, Kasvi Olen, Belo Horizonte, MG, Brazil) and streaked on the surface of 90 × 15 mm Petri dishes (Inlab, São Paulo, SP, Brazil) containing SDA at 0h, 1h, 2h, 4h, 8h, and 24h. The plates were kept incubated at 35 ± 2 °C between these intervals. Viability controls for the fungal strains were prepared at each interval. All Petri dishes were incubated at 35 ± 2 °C for 48h after inoculation.

The experiment was performed in triplicate, and curves were constructed by plotting the mean colony count (CFU/mL) at different time intervals. If the substance caused a reduction in microbial growth of 3 log units (≥99.9%) or higher from the initial inoculum, it was considered fungicidal; otherwise, if it caused a reduction of less than 3 log units (<99.9%), it was fungistatic (Silva et al., 2020).

2.7. Effect on fungal cell wall (Sorbitol assay)

To assess whether LD-EO causes damage to the fungal cell wall, its MICs were compared in the presence and absence of an osmotic stabilizer (sorbitol), which penetrates cells and makes them less sensitive to osmotic changes. If the test substance alters the fungal cell wall, it will cause cell lysis when sorbitol is absent. Otherwise, if LD-EO does not affect the fungal cell wall, its presence should result in cell growth and an increase in the MIC value. Therefore, if the MIC values remain unchanged, it suggests that the mechanism of action of LD-EO does not involve damage to the fungal cell wall. In this assay, MIC was determined using the microdilution method, similar to the one previously described. Yeasts of *C. albicans* (ATCC-76485 and LM-4) were exposed to different concentrations of LD-EO in a medium containing 0.8M sorbitol. Simultaneously, controls of the culture medium (RPMI) sterility and strain viability (RPMI + 3% DMSO + 2% Tween 80 + yeasts) were prepared (Frost et al., 1995).

2.8. Effect on the cell membrane (Ergosterol assay)

Several antifungal agents available for clinical use interact directly with ergosterol, causing fungal cell membrane disruption and loss of intracellular content. MICs were determined for the selected strains (ATCC-76485 and LM-4) in the absence and presence of ergosterol to clarify whether LD-EO acted through binding to cell membrane sterols. An unchanged MIC value (compared to the control group) in the presence of exogenous ergosterol demonstrates that the action of the substance does not result from binding to fungal cell membrane ergosterol. Otherwise, an increase in MIC in the presence of exogenous ergosterol indicates that this molecule prevented binding to membrane ergosterol. Therefore, it is suggested that the test substance acts by binding to ergosterol (Valgus, 2003).

Values of MIC were determined through the microdilution method in triplicate, similar to the protocol described

previously, except that culture medium was used with and without the addition of ergosterol (400 µg/mL) (Sigma-Aldrich®, São Paulo, SP, Brazil). Simultaneously, sterility controls of the culture medium (RPMI) and strain viability (RPMI + 3% DMSO + 2% Tween 80 + yeasts) were prepared (Escalante et al., 2008).

2.9. Association study (Checkerboard method)

The combined effect of LD-EO and miconazole, both at different concentrations (below and above their MICs), was investigated through the checkerboard method. For this assay, 100 µL of RPMI culture medium was added to a 96-well microplate (Inlab, São Paulo, SP, Brazil). Next, 50 µL of substance A (LD-EO) at different concentrations (MIC×8, MIC×4, MIC×2, MIC, MIC/2, MIC/4, and MIC/8) and 50 µL of substance B (miconazole) at the same concentrations were added to the plates (A dispensed vertically, and B dispensed horizontally). Subsequently, 20 µL of strains of C. albicans (ATCC-76485 and LM-4) were added, and plates were incubated at 35 ± 2 °C for 24-48h. After incubation time, readings were performed to detect the presence or absence of visible fungal growth (White et al., 1996). Sterility controls of the culture medium (RPMI) and strain viability (RPMI + 3% DMSO + 2% Tween 80 + yeasts) were prepared concomitantly. This assay was conducted in triplicate, and results were expressed in percentages, representing the modal value of the three measurements.

A Fractional Inhibitory Concentration Index (FICI) was determined as follows: FIC_A = MIC of substance A in the combination ÷ MIC of substance A individually; FIC_B = MIC of substance B in the combination ÷ MIC of substance B individually. Afterward, the FICI was calculated using the equation: $FICI = FIC_A + FIC_B$. The obtained values were interpreted as synergism (FICI ≤ 0.5), additivity (0.5 < FICI < 1), indifference (1 ≤ FICI < 4), or antagonism (FICI ≥ 4.0) (Lewis et al., 2002).

2.10. Inhibition of biofilm formation

This experiment aimed to evaluate the inhibitory effect of LD-EO on biofilm formation by oral strains of *C. albicans*. Initially, 10 µL of the inoculum of strains ATCC-76485 and LM-4 were incubated in 100 µL of RPMI medium containing the test substance at different concentrations (MIC/2, MIC, and MIC×2) at 35 ± 2 °C for 48h. The wells were emptied, washed with running water to remove non-adherent cells, and air-dried at room temperature. The well contents were then stained with 125 µL of 1% crystal violet solution (Newprov, Pinhais, PR, Brazil) for 20 min. After washing off the excess dye and drying, 125 µL of absolute ethanol was added for 30 min (Onsare and Arora, 2015).

The counting of fixed and dyed cells in the well walls was performed using a microplate spectrophotometer (Multiskan GO, Thermo Scientific) at 540 nm. Simultaneously, a negative control was prepared using only RPMI medium and the inoculum of the fungal strains. The percentage of inhibition of biofilm formation was assessed using the following formula: % biofilm formation = [(ABS_{540} test/ABS_{540} control) x 100] (Onsare and Arora, 2015; Rajasekharan et al., 2017). All analyses were performed in triplicate, and results were expressed as the arithmetic mean (± standard error) of the absorption values obtained, plotted on graphs using GraphPad Prism software (version 8.0 for Windows, San Diego, CA, USA). The ability to inhibit biofilm formation was interpreted as low (≤40% inhibition), moderate (40%< inhibition <80%), or strong (≥80% inhibition) based on adaptations from a previous study (Kwasny and Opperman, 2010).

2.11. Disruption of preformed biofilm

To assess the activity of LD-EO on elimination of preformed biofilm by *C. albicans*, 10 µL of the inoculum of fungal strains (ATCC-76485 and LM-4) were incubated in 100 µL RPMI medium at 35 ± 2 °C for 48h. After removing the well contents, 100 µL of RPMI containing the test substance at MIC×5 was added and incubated at 35 ± 2 °C for an additional 48-h period (Onsare and Arora, 2015; Rajasekharan et al., 2017).

As described in the previous section, after staining cells fixed in the wells, the optical density of the crystal violet-ethanol solution was measured using a microplate spectrophotometer (Multiskan GO, Thermo Scientific) at 540 nm. Simultaneously, a negative control was prepared by adding RPMI medium without LD-EO to the wells with formed biofilm. Disruption of preformed biofilm was evaluated using the following formula: % biofilm formation = [(ABS_{540} test/ABS_{540} control) x 100] (Onsare and Arora, 2015; Rajasekharan et al., 2017). All analyses were conducted in triplicate, and results were expressed as the arithmetic mean (± standard error) of the obtained absorption values, plotted on graphs using GraphPad Prism software (version 8.0 for Windows, San Diego, CA, USA). The ability of LD-EO to eliminate preformed biofilm compared to the control group was interpreted as low (≤40% elimination), moderate (40%< elimination <80%), or strong (≥80% elimination), adapting the classification from a study by Kwasny and Opperman (2010).

2.12. Hemolysis assay

The hemolytic activity of LD-EO was tested using human erythrocytes from healthy young adults of both sexes, aged between 18 and 40 years. Participants were Biological Sciences and Dentistry students at the Federal University of Campina Grande (UFCG, Paraíba, Brazil). The local Ethics Committee approved the study protocol (approval number: 6.076.256), and experiments followed the Ethics Code of the World Medical Association.

To obtain the erythrocytes, fresh blood aliquots (types A, B, and O) were mixed with 0.9% NaCl (at a ratio of 1:30) and centrifuged at 2500 rpm for 5 min. This process was repeated twice, and the pellet was resuspended in 0.9% NaCl to obtain a 0.5% suspension free of leukocytes and platelets. Samples of LD-EO (0.5 mL) at different concentrations (5, 10, 50, and 100 µg/mL) were added to 2 mL of the erythrocyte suspension. A positive control with 1% Triton X-100 was used to test for full hemolysis, and a negative control (without LD-EO), for no hemolysis. Samples were incubated at 22 ± 2 °C under slow and constant agitation (100 rpm) for 1h and then centrifuged at 2500 rpm for 5 min. Hemolysis was quantified by spectrophotometry at a wavelength of 540 nm (Rangel et al., 1997). All assays

were performed in triplicate. Results were expressed as percentage values, representing the arithmetic mean (± standard error) of three measurements, and compared to the positive control. Hemolytic activity was classified into low (<40%), moderate (40–80%), or high (>80%) categories based on the obtained values for the percentage of hemolysis (Figueiredo-Júnior et al., 2021).

2.13. Statistical analysis

Differences between groups were analyzed using a One-way Analysis of Variance (ANOVA), followed by a Tukey or Dunnet post hoc test. GraphPad Prism software (version 8.0 for Windows, San Diego, CA, USA) was used for the analyses. Results were considered statistically significant for p-values<0.05.

3. Results

3.1. Essential oil composition

According to Table 1, LD-EO from Brazil consisted mainly of monoterpenes. The three most abundant phytoconstituents were eucalyptol (33.13%), camphor (18.34%), and fenchone (15.67%). Some sesquiterpenes, including *cis*-α-Bisabolene, β-Bisabolene, and β-Selinene, were found in smaller quantities.

Table 1. Chemical composition of *L. dentata* L. essential oil (LD-EO) from Brazil obtained by gas chromatography-mass spectrometry (GC-MS).

Retention time (min)	Compound	Area (%)
6.520	α-Pinene	2.71
6.797	Camphene	0.92
7.216	Sabinene	0.60
7.297	β-Pinene	3.81
8.115	p-Cymene	0.63
8.273	Eucalyptol	33.13
8.927	*cis*-Linalool oxide	0.42
9.267	Fenchone	15.67
9.347	Linalol	1.50
9.690	Fenchol	5.01
10.148	*trans*-Pinocarveol	1.67
10.256	Camphor	18.34
10.554	δ-Terpineol	1.96
10.723	Terpinen-4-ol	0.63
10.927	L-α-Terpineol	1.73
11.067	Myrtenal	1.23
14.558	*cis*-α-Bergamotene	0.42
15.386	β-Selinene	0.96
15.513	β-Bisabolene	0.55
15.942	*cis*-α-Bisabolene	1.16

3.2. Antifungal activity

Following phytochemical characterization of the test substance, assays were conducted to determine the MIC and MFC for LD-EO and miconazole against oral strains of *C. albicans*.

As shown in Table 2, MIC values for LD-EO ranged from 2 to 8 µg/mL, and MIC_{100} was established at 8 µg/mL, effectively inhibiting all strains evaluated. Hence, the test substance demonstrated strong antifungal activity. In turn, miconazole had MIC values ranging from 4 to 512 µg/mL and inhibited most strains (n=14; 88%) at MIC_{80} (8 µg/mL). In this study, strains LM-4 and LM-12B showed MICs of 512 µg/mL and 16 µg/mL, respectively, and were classified as resistant according to a classification system by Nawrot et al. (2005). Subsequently, MFC values were established at 16 µg/mL for both LD-EO and miconazole. At this concentration, LD-EO and miconazole exhibited inhibitory activity in all analyzed strains (n=16; 100%) and 14 (88%), respectively.

After establishing MIC and MFC values, the antifungal effects of LD-EO and miconazole were assessed according to the MFC/MIC ratio (Table 3). As proposed by Hafidh et al. (2011), ratios between 1:1 and 2:1 were classified as fungicidal, and MFC/MIC values > 2:1 were considered fungistatic (Hafidh et al., 2011). Based on this criterion, LD-EO showed predominantly fungicidal activity (n=9 strains; 56.2%). In turn, miconazole played a fungicidal role in 12 (75%) strains. From the results of these experiments, a standard strain (ATCC-76485) and a miconazole-resistant strain (LM-4) were selected for subsequent tests.

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *L. dentata* L. essential oil (LD-EO) and miconazole (Mic) against oral strains of *C. albicans*.

Strains	LD-EO (µg/mL)*		Mic (µg/mL)*		C1	C2
	MIC	MFC	MIC	MFC		
ATCC-76485	8	8	8	8	+	–
LM-02	4	8	8	32	+	–
LM-03	2	8	8	16	+	–
LM-4	8	8	512	>1024	+	–
LM-12B	4	8	16	16	+	–
LM-13B	8	8	8	16	+	–
LM-19P	8	16	8	16	+	–
LM-38	8	8	8	16	+	–
LM-42	4	16	8	16	+	–
LM-70	4	16	8	16	+	–
LM-80	4	16	8	16	+	–
LM-86	4	16	8	16	+	–
LM-106	8	16	8	16	+	–
LM-115	4	16	4	16	+	–
LM-125	4	16	4	16	+	–
LM-128	8	16	8	16	+	–

*Modal values of three experiments; C1, Control of fungal growth; C2, Control of the culture medium sterility; (+), Presence of antifungal growth; (-), Absence of fungal growth.

Table 3. Determination of MFC/MIC ratio of *L. dentata* L. (LD-EO) and miconazole (Mic) against oral strains of *C. albicans*.

Strains	LD-EO (μg/mL)		Mic (μg/mL)	
	MFC/MIC	Effect	MFC/MIC	Effect
ATCC-76485	1	Fungicidal	1	Fungicidal
LM-02	2	Fungicidal	4	Fungistatic
LM-03	4	Fungistatic	2	Fungicidal
LM-4	1	Fungicidal	>2	Fungistatic
LM-12B	2	Fungicidal	1	Fungicidal
LM-13B	1	Fungicidal	2	Fungicidal
LM-19P	2	Fungicidal	2	Fungicidal
LM-38	1	Fungicidal	2	Fungicidal
LM-42	4	Fungistatic	2	Fungicidal
LM-70	4	Fungistatic	2	Fungicidal
LM-80	4	Fungistatic	2	Fungicidal
LM-86	4	Fungistatic	2	Fungicidal
LM-106	2	Fungicidal	2	Fungicidal
LM-115	4	Fungistatic	4	Fungistatic
LM-125	4	Fungistatic	4	Fungistatic
LM-128	2	Fungicidal	2	Fungicidal

MFC, Minimum Fungicidal Concentration; MIC, Minimum Inhibitory Concentration.

3.3. Time-kill curves

The time-kill curves of strains ATCC-76485 and LM-4 illustrate the mean colony count (CFU/mL) over time in the presence of LD-EO at different concentrations and a control (miconazole). According to Figure 1, LD-EO had fungicidal properties against strain ATCC-76485 at the highest concentration (MIC×4) up to 8h (decrease greater than 3 log units). At lower concentrations (MIC and MIC×2), it only showed inhibitory effects, irrespective of the time interval. For strain LM-4, LD-EO demonstrated fungicidal activity throughout the analyzed intervals, except at MIC, where a lethal effect remained for up to 8h. Miconazole exhibited a predominantly fungicidal effect on ATCC-76485, particularly at higher concentrations (MIC×2 and MIC×4). However, it did not show any fungicidal action against LM-4 at any of the evaluated concentrations, and instead only demonstrated inhibitory effects. This result was expected due to the resistance of LM-4 to the studied antifungal.

3.4. Effects on fungal cell wall and cell membrane

Table 4 indicates MIC values of LD-EO determined for oral strains of *C. albicans* (ATCC-76485 and LM-4) in the absence and presence of sorbitol (0.8M) or exogenous ergosterol (400 μg/mL). Sorbitol, an osmotic stabilizer, did not modify the referred MICs. Similarly, the presence of ergosterol in the culture medium did not alter the MIC values of LD-EO for both strains. Such findings suggest that the antifungal mechanisms of action of LD-EO may not involve damaging the fungal cell wall or binding of the mentioned sterol to the cell membrane.

3.5. Association study

The results of the association between LD-EO and the antifungal miconazole through the checkerboard assay are shown in Table 5. As observed, the mentioned combination resulted in synergy (FICI ≤ 0.5) between strains ATCC-76485 (FICI = 0.25) and LM-4 (FICI = 0.375).

Table 4. Effect of *L. dentata* L. essential oil (LD-EO) against oral strains of *C. albicans* in the absence (-) and presence (+) of sorbitol (0.8M) and ergosterol (400 μg/mL).

Strains	MIC (μg/mL)*			
	Sorbitol (-)	Sorbitol (+)	Ergosterol (-)	Ergosterol (+)
ATCC-76485	8	8	8	8
LM-4	8	8	8	8

MIC, Minimum Inhibitory Concentration; *Modal values of three experiments.

Table 5. Effect of the association between *L. dentata* L. essential oil (LD-EO) and antifungal miconazole (Mic) against oral strains of *C. albicans*.

Strains	FIC	FIC	FICI	Interaction type
	LD-EO	Mic		
ATCC-76485	0.125	0.125	0.25	Synergism
LM-4	0.25	0.125	0.375	Synergism

FIC, Fractional Inhibitory Concentration; FICI, Fractional Inhibitory Concentration Index.

3.6. Antibiofilm activity

The percentages of biofilm formation by oral strains of *C. albicans* (ATCC-76485 and LM-4) in the presence and absence of the test substance (LD-EO) and miconazole are represented in Figure 2. A strong inhibitory effect was evidenced for LD-EO, which reduced biofilm growth by over 80% in both strains compared to negative control ($p<0.0001$). In the presence of miconazole, strains ATCC-76485 and LM-4 exhibited inhibition of biofilm formation at low concentrations (MIC/2 and MIC), respectively. After exposure to miconazole at MIC/2, strain LM-4 showed higher biofilm growth than negative control. Nevertheless, this difference was not statistically significant ($p>0.05$).

Figure 3 shows the results of the preformed biofilm assay. As evidenced, LD-EO had weak activity in disrupting preformed biofilm at MIC×5. In the groups treated with the test substance, the percentage of preformed biofilm remained above 80% of that observed in the negative control. There were no statistically significant differences between the analyzed groups ($p>0.05$).

3.7. Hemolytic activity

Cytotoxicity of LD-EO was analyzed by conducting a hemolysis assay with human erythrocytes (blood groups A, B, and O). Results of the percentage of hemolysis caused by the test substance, compared to a positive control (1% Triton X-100), are presented in Figure 4. The findings revealed that LD-EO caused hemolysis on erythrocytes from all three blood types in a concentration-dependent manner, and susceptibility to hemolysis was significantly lower than in the positive control ($p<0.001$). Hemolytic activity of the product was considered as low (<40%) up to a concentration of 50 μg/mL and moderate (40–80%) at 100 μg/mL in all blood types.

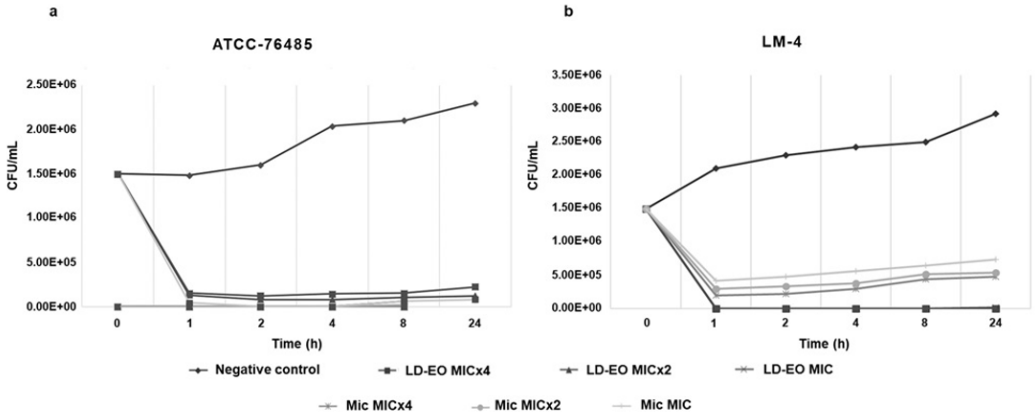

Figure 1. Time-kill curves for *C. albicans* strains ATCC-76485 (a) and LM-4 (b) after treatment with *Lavandula dentata* L. essential oil (LD-EO) and miconazole at different concentrations (MIC, MICx2, and MICx4) during a 24h period. CFU, Colony-Forming Units; MIC, Minimum Inhibitory Concentration.

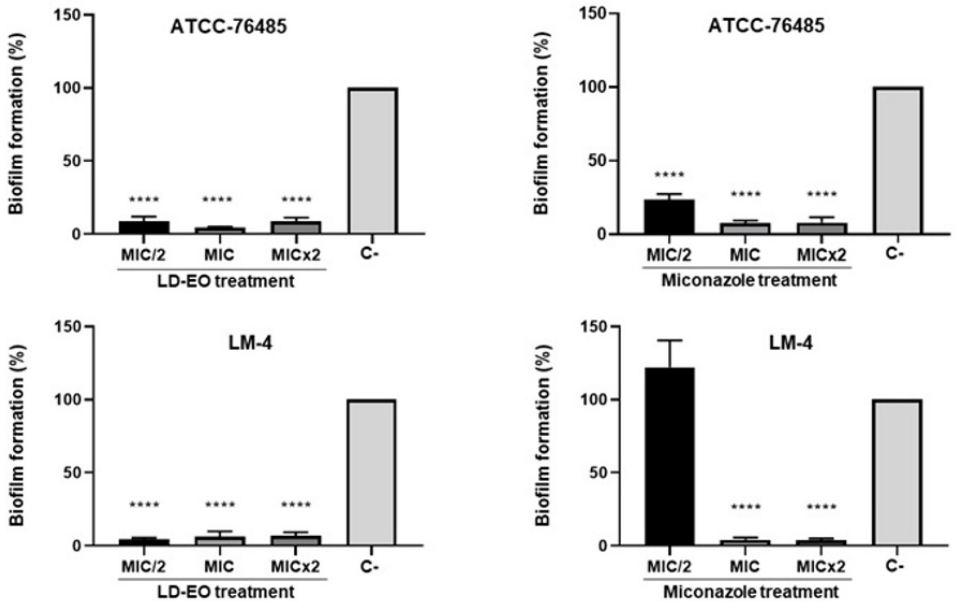

Figure 2. Impaired biofilm formation by *C. albicans* after treatment with *Lavandula dentata* L. essential oil (LD-EO) and miconazole. Bars represent the mean (± standard error) of the percentage of adhered cells relative to the negative control (C-). Fungal cells were exposed to LD-EO or miconazole at different concentrations (MIC/2, MIC, and MICx2). MIC, Minimum Inhibitory Concentration. ****p<0.0001 compared to the C- (One-way ANOVA, Tukey post hoc test, n=3).

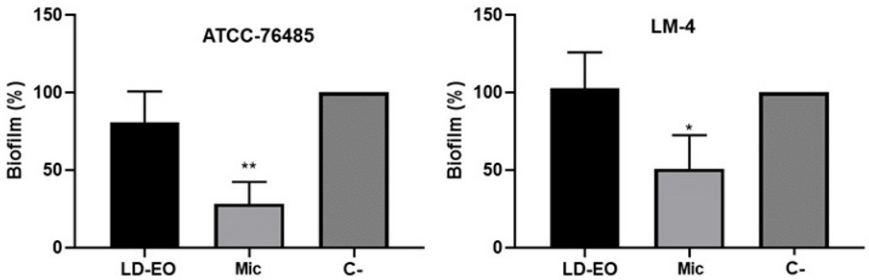

Figure 3. Disruption of preformed biofilm by *C. albicans* after exposure to *Lavandula dentata* L. essential oil (LD-EO) and miconazole (Mic). Bars represent the mean (± standard error) of the percentage of adhered cells relative to the negative control (C-). Fungal cells were exposed to LD-EO or miconazole at MICx5. MIC, Minimum Inhibitory Concentration. *p<0.05 compared to the C-; **p<0.01 compared to the C- (One-way ANOVA, Tukey post hoc test, n=3).

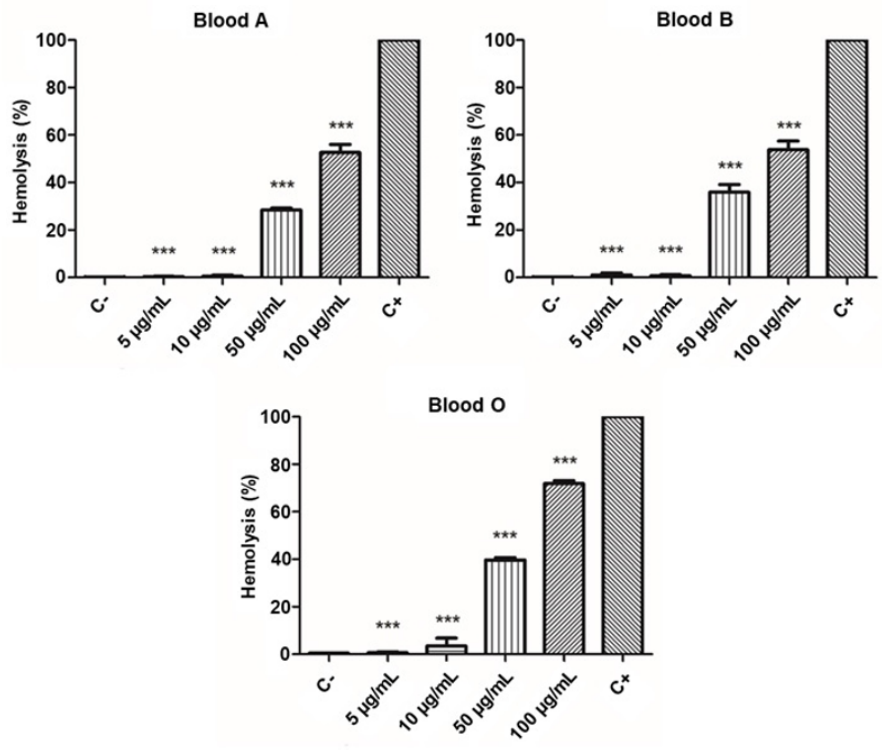

Figure 4. Hemolytic activity of *Lavandula dentata* L. essential oil (LD-EO) on human erythrocytes (blood types A, B, and O) compared to the positive control (C+, 1% Triton X-100). C-, negative control. Results are expressed as the mean (± standard error). ***p<0.001.

4. Discussion

The phytochemical characterization of an EO is crucial for comparing its biological activities across different studies. Gas chromatography coupled with mass spectrometry (GC-MS) was used to analyze the composition of LD-EO. Eucalyptol, camphor, and fenchone, the major phytoconstituents of LD-EO explored in our study, are often identified as the main compounds in *Lavandula* spp. EOs (Vairinhos and Miguel, 2020). *L. pedunculata* represents a classic example of this, as it generally has these three phytoconstituents as its main components (Zuzarte et al., 2009, 2022; Nafis et al., 2021; Marques et al., 2023).

Regarding *L. dentata*, other studies focusing on specimens cultivated in Brazil have found a composition very similar to that described in our study. Vicenço et al. (2021) also identified eucalyptol, camphor, and fenchone as major compounds. Moreover, Martins et al. (2019) compared the EOs from the inflorescence and the aerial parts of *L. dentata* in Brazil. They found similar major components for both oils, with no statistically significant differences between the two types of oil.

However, most studies highlight differences in the chemical composition of EOs depending on the plant part used for their extraction. For example, Justus et al. (2018) have found a significant predominance of eucalyptol (63.25%) over all other phytoconstituents when evaluating the EO obtained exclusively from the leaves and stems of *L. dentata* in Brazil. This composition differs from that reported by most studies, which more frequently use the EO from the flowers of *L. dentata*, as in our study. Corroborating these findings, Angioni et al. (2006) found higher concentrations of fenchone in the EO from the flowers of *L. stoechas* compared to that obtained from stems and leaves.

We highlight that the chemical composition of a given plant species can vary considerably due to various factors other than the part used for extraction, including climate, altitude, soil, seasonal conditions, harvesting methods, and timing (Justus et al., 2018; Müller-Sepúlveda et al., 2020; El-Abdali et al., 2022). However, the major components found in most *L. dentata* oils from Brazil were also identified in oils from other countries, with minor variations in some cases. Eucalyptol and camphor were identified as predominant phytoconstituents in oils from Tunisia (Moumni et al., 2021), Chile (Müller-Sepúlveda et al., 2020), Morocco (Imelouane et al., 2009; El-Abdali et al., 2022), and Italy (Giuliani et al., 2020).

This research demonstrated a strong inhibitory activity of LD-EO against all evaluated oral strains of *C. albicans*, consistent with the well-established antifungal effects of EOs. These properties are attributed to the isolated or synergistic action of their main phytoconstituents, especially monoterpenes, sesquiterpenes, and diterpenes (Nazzaro et al., 2017; El-Abdali et al., 2022). Monoterpene-rich EOs have been shown to exhibit relatively low inhibitory concentrations for *C. albicans*, such as *Tetradenia riparia* (MIC ≥ 31.2 µg/mL) (Gazim et al., 2010) and *Coriandrum sativum* L. oils (15.6 µg/mL ≤ MIC ≤ 31.2 µg/mL)

(Freires et al., 2014; Barbosa et al., 2023), corroborating our results. Other EOs with a chemical composition similar to LD-EO have also demonstrated activity against *C. albicans*, such as *Artemisia annua* (Trinh et al., 2011), *Foeniculum vulgare* (Cabral et al., 2017; Bassyouni et al., 2019), *Ocimum forskolei* (Ali et al., 2017), and *Thuja occidentalis* (Bai et al., 2020), in addition to other *Lavandula* species including *L. stoechas* (Benali et al., 2023; El Hachlafi et al., 2023), *L. luisieri* (Zuzarte et al., 2012), *L. pedunculata* (Zuzarte et al., 2009), and *L. angustifolia* (Mijatovic et al., 2022).

Although LD-EO has shown promising results regarding its antifungal activity against standard strains of *C. albicans*, no studies evaluating its activity against clinical oral isolates of this species have been published. For instance, research by Justus et al. (2018) demonstrated a strong inhibitory effect of LD-EO from Brazil against the strain ATCC-10231 (MIC = MFC = 54.7 µg/mL). In another study, LD-EO from Chile was found to inhibit the strains ATCC-90029 and ATCC-10231 at 156 µg/mL and 130 µg/mL, respectively (Müller-Sepúlveda et al., 2020). The MICs of LD-EO have been reported as generally low, which is consistent with the findings of this research. However, it is worth highlighting that these values can be influenced by various factors, including the culture medium used, pH, incubation time and temperature, purity of substances, density of fungal suspensions, and sensitivity of strains to chemical agents (Karpiński et al., 2021).

The finding that the two miconazole-resistant strains (LM-4 and LM-12B) were sensitive to low concentrations of LD-EO is significant, as it suggests that the test substance could be an effective treatment for miconazole-resistant oral candidiasis cases. In a study by Müller-Sepúlveda et al. (2020), LD-EO inhibited a fluconazole-resistant strain of *C. albicans* (ATCC-10231), corroborating our results. It is worth emphasizing that miconazole is the topical agent that enables the most comfortable treatment of oral candidiasis, and, therefore, it is widely used in the current therapeutic approach to this disease (Quindós et al., 2019). However, few studies have sought to improve its effectiveness and performance against the growing fungal resistance, which justifies choosing this antifungal as a control for this research.

Concerning the fungicidal activity of LD-EO, scientific literature reports on other terpene-rich EOs that have demonstrated a lethal effect on *Candida* spp. at relatively low concentrations. For example, *Coriandrum sativum* L. oil is active against strains isolated from the oral cavity (MIC = 15.6 µg/mL and MFC = 31.2 µg/mL) (Freires et al., 2014). Similarly, Zuzarte et al. (2009) analyzed the *L. pedunculata* EO with the same major components used in the present study and found fungicidal activity against the strain ATCC-10231 of *C. albicans* (MIC = 2.5 µL/mL and MFC = 2.5–5 µL/mL). Likewise, Mijatovic et al. (2022) suggested that the effect of *L. angustifolia* EO against strains of *C. albicans* was more fungicidal than fungistatic during a 24-h exposure, given that the MFC values were at most twice those of the MIC. Finally, El Hachlafi et al. (2023) analyzed *L. stoechas* EO and found MIC and MFC of 0.125% v/v. In light of these findings, the mentioned authors concluded that this oil also exerts a fungicidal effect on *C. albicans*. It is worth highlighting that the fungicidal nature of LD-EO observed in this study is significant, given that fungistatic agents have shown a higher risk of causing resistance than fungicidal substances. A possible explanation is that fungistatic agents, by merely inhibiting microbial growth, allow the occurrence and perpetuation of mutations and, consequently, the development of resistance phenomena (Kumar et al., 2018; Bhattacharya et al., 2020).

Regarding the time-kill curves assay, this technique enables determining whether the analyzed substance has fungicidal or fungistatic activity and also allows for establishing a dynamic relationship between concentration and substance activity over the evaluated time intervals. Through this test, we observed that the fungicidal effect of LD-EO was concentration-dependent, being found mainly in the first 8h of the experiment. This finding may have relevant clinical applications, including defining drug administration time intervals and developing strategies to improve its release and bioavailability.

Another interesting finding in this experiment was the tendency for microbial regrowth after 24h, especially at lower concentrations of LD-EO. Eradication (absence of subsequent regrowth) of the inoculum of strain LM-4 was achieved only with the highest concentrations (MIC×2 and MIC×4). A possible explanation for this phenomenon is that, between 8h and 16h of the experiment, there may have been growth through cell duplication among those cells that survived the lower concentrations of the test substance. It is worth noting that in a population of microorganisms, there is often a heterogeneous susceptibility to chemical agents, influenced by physiology, growth rate, and duration of the cell cycle. Such factors may lead to the survival of few cells (Ranieri et al., 2018). In this context, López-Rojas et al. (2017) reported the emergence of new microbial growth after 24h of applying a biocidal substance (polyhexanide). They observed a complete elimination of inoculum at variable concentrations for different bacterial strains and species, consistent with our findings. The hypothesis raised by the authors was that the emergence of new microbial growth was due to the loss of substance activity, not the development of tolerance or resistance to the product.

The phenomenon of new microbial growth in cultures exposed to biocidal agents has been long-reported, and one of the explanations, in addition to resistance-related mechanisms, is the consumption or degradation of the drug (Hamano et al., 1984). The degradation of the drug may have occurred through oxidation or volatilization, as EOs are highly volatile and susceptible to degradation and oxidation. Technological strategies such as microencapsulation (Zhao et al., 2023) can overcome these limitations. Therefore, the emergence of a new microbial population in a quantity close to the negative control (<99% difference) during the last analyzed period may be explained by the likely absence of the test substance in the medium due to its degradation over an extended period (16h).

This study performed sorbitol and ergosterol assays to evaluate if the mechanisms of action of LD-EO on fungal cells involves the cell wall or cell membrane. The cell wall is a crucial structure for fungal cells' survival against osmotic stress and is absent in mammalian cells, making it an attractive antifungal target. Ergosterol is the principal type of sterol in fungal cell membranes,

including the plasma and mitochondrial membranes, and is essential for maintaining their structures and function (Bhattacharya et al., 2020). However, sorbitol and ergosterol assays did not show changes in the MICs, suggesting that LD-EO does not affect the integrity of the fungal cell wall or cell membranes through binding to ergosterol. Further tests, such as flow cytometry, are necessary to determine the product's targets and mechanisms of action.

Scientific literature has established that EOs damage bacterial and fungal cells through similar mechanisms. Cell membranes are among the main targets and are also the most studied. EOs' lipophilicity enables them to interact with the lipid bilayer membrane, causing damage to this cellular structure. This leads to increased cell permeability or rupture of the plasma membrane, resulting in the leakage of cytosolic content and subsequent cell death (Ngo-Mback et al., 2019; El-Abdali et al., 2022; Angane et al., 2022; El Hachlafi et al., 2023). These products can also damage mitochondrial membranes, alter electron transport, and affect ATPases' functioning, leading to cell death (El-Abdali et al., 2022; Mijatovic et al., 2022; El Hachlafi et al., 2023). Thus, LD-EO may exert antifungal action through other mechanisms related to cell membranes, even if it does not directly bind to ergosterol (Bhattacharya et al., 2020).

In addition to exerting biocidal mechanisms, EOs and monoterpenes can inhibit microbial growth (fungistatic action), including the biosynthesis of ergosterol, through gene inactivation. The biosynthesis of ergosterol is an ideal drug target since it is found in fungi and plants but not humans, ensuring greater selectivity. Other important fungistatic mechanisms are related to the reduction of virulence factors, such as the decrease in hyphae formation (Bhattacharya et al., 2020; Ivanov et al., 2021), inhibition of the phospholipase enzyme (El-Abdali et al., 2022), and efflux pumps disruption (Mijatovic et al., 2022). Given that LD-EO is a terpene-rich product, its antifungal mechanisms of action may involve different cellular targets, collectively responsible for its predominantly biocidal activity.

This study conducted a combination assay of LD-EO with the antifungal miconazole using the checkerboard technique to reduce the concentrations necessary to achieve the inhibitory effect against *C. albicans*. This method of associating substances at lower concentrations can reduce their toxicities and provide other advantages, including an increased spectrum of activity and potency of drugs, faster effects, and a decreased risk of microbial resistance. The observed synergy between LD-EO and miconazole can be explained by EOs altering the permeability of microbial cell walls and membranes, thereby facilitating the penetration of azole agents into the fungal cell (Bassyouni et al., 2019). This reduces the drug concentrations required to achieve activity and transforms the fungistatic mechanism of action into a fungicidal one. EOs may also disrupt the effect of efflux pumps, which is a recognized mechanism of microbial resistance. The synergy against the miconazole-resistant LM-4 strain results from the inhibition of efflux pumps that would actively transport this drug to the extracellular environment (Bhattacharya et al., 2020; Mijatovic et al., 2022).

Scientific literature has described synergism between EOs and standard antimicrobials. For instance, oils from different lavender species demonstrated synergism with ciprofloxacin against three foodborne pathogenic bacteria, especially *Salmonella* spp. (Nafis et al., 2021). Similarly, *Salvia officinalis* EO containing bicyclic monoterpenes combined with the antifungal terbinafine against *C. albicans* significantly reduced the MIC of the standard drug (Kodadová et al., 2017). These studies demonstrated the efficacy of combining substances to achieve a synergistic effect. The present study was the first to report the synergy between LD-EO and miconazole against a strain of *C. albicans* resistant to this antifungal agent. Therefore, this result is of great significance, as it may contribute to reducing the toxicity of the drug and overcoming the increasing resistance to miconazole. Considering that the treatment of oral candidiasis primarily involves the topical application of antifungal agents to the lesions and that the effective concentrations obtained in the *in vitro* tests were relatively low, our results suggest that LD-EO is a promising antifungal agent for the therapeutic management of this disease.

The formation of biofilms by *Candida* spp. is a major factor in controlling these microorganisms, as it allows them to spread on surfaces and makes them more resistant to environmental factors and antifungal drugs. Therefore, a product that can prevent or reduce the formation of biofilms is an important strategy to prevent fungal infections (Ranieri et al., 2018; Karpiński et al., 2021). The present study aimed to determine the inhibitory activity of LD-EO on the biofilm formation of *C. albicans*. Our results showed that LD-EO strongly inhibited the biofilm formation of both analyzed fungal strains, even at a subinhibitory concentration (MIC/2).

Other studies have also reported a reduction in the biofilm formation of *Candida* spp. at subinhibitory concentrations of the analyzed EOs. For example, Ngo-Mback et al. (2019) reported that the administration of EOs from the Lamiaceae family at subinhibitory concentrations led to a reduction in biofilm formation due to their high terpene content. Similarly, Freires et al. (2014) demonstrated that low concentrations of *Coriandrum sativum* L. EO, which is rich in terpenes, led to the inhibition of biofilm formation by different *Candida* species isolated from the oral cavity. For *Candida tropicalis*, for instance, there was inhibition at 15.6 µg/mL, corresponding to approximately half the inhibitory concentration. The standard antifungal used by these authors (nystatin) also inhibited the same species at MIC/2.

Antibiofilm activity of *L. dentata* against *Candida* spp. has been studied to a limited extent, but the few researches conducted have yielded promising results. *L. dentata* was identified as one of the species, along with others from the Lamiaceae family, that had antibiofilm effects against *Candida* spp. among 29 EOs and 16 plant extracts, according to a review by Karpiński et al. (2021). LD-EO from Chile reduced the biofilm formation by two standard strains of *C. albicans* (ATCC-90029 and ATCC-10231), exhibiting inhibitory activity at low concentrations (Müller-Sepúlveda et al., 2020). The strong inhibitory effect of LD-EO at low concentrations on the biofilm formation of these oral strains is a novel finding of this study. The results suggest that LD-EO could be an effective inhibitor of

biofilm formation by *Candida* spp., indicating its potential for preventing or treating oral candidiasis. It is worth mentioning that the major components of LD-EO, such as camphor and eucalyptol, have demonstrated effectiveness in reducing biofilm formation by *C. albicans* at MIC and MIC/2 (Ivanov et al., 2021). Similarly, Manoharan et al. (2017) reported that camphor and fenchone inhibited over 80% of the biofilm formation by this same *Candida* species.

The biofilms produced by *Candida* spp. are composed of various cell forms, including yeasts, hyphae, and pseudohyphae, surrounded by an extracellular polymeric matrix. The transformation of yeast cells into filamentous forms is a crucial step in the early phase of biofilm formation and in the development of fungal infections and drug resistance. Many antibiofilm substances act during this stage (Manoharan et al., 2017; Müller-Sepúlveda et al., 2020). Studies have examined the inhibitory activity of terpene-rich EOs on the hyphae formation of *Candida* spp. One study found that LD-EO from Chile effectively inhibited the filamentation process and reduced the adhesion of two standard strains of *C. albicans* (ATCC-90029 and ATCC-10231) at MIC and MIC×2 (Müller-Sepúlveda et al., 2020). Another research found that cedar EO and some monoterpenes inhibited hyphae formation, which may explain the antibiofilm effect of the studied substances. The antibiofilm mechanism of action of camphor involves the negative regulation of hyphae-specific genes related to biofilm formation. The concentrations with an antibiofilm effect (~0.01%) were 50 times lower than MIC (Manoharan et al., 2017), supporting the results of biofilm formation inhibition at subMIC observed in the present study. As suggested by Manoharan et al. (2017), such findings confirm that the biofilm formation by *C. albicans* was effectively reduced by the antibiofilm activities of the studied compounds and not by their antimicrobial effect, which could also justify our results.

In addition to inhibiting biofilm formation, the ability of a product to penetrate and damage the preexisting structure is highly relevant, as a preformed biofilm increases the resistance of microorganisms to external aggressors, including antimicrobial agents. The effect of LD-EO on the disruption of the preformed biofilm of *C. albicans* was evaluated at a concentration five times higher than MIC but did not show a statistically significant effect. As established in scientific literature, biofilms of *C. albicans* consist of a dense network of multiple layers of cells, in which fungal cells become more resistant to environmental adversities and antimicrobial substances (Raut et al., 2013; Ranieri et al., 2018; Karpiński et al., 2021).

Some reports in the scientific literature suggest that EOs can eliminate preformed biofilms of *Candida* spp. at concentrations similar to the MIC, which contradicts the results of the present study. Müller-Sepúlveda et al. (2020) found that LD-EO from Chile effectively decreased the levels of preformed biofilm in standard strains of *C. albicans* in values similar to MIC. This result differed from the findings of our study and could be because these authors analyzed two standard strains that may form biofilms less resistant to environmental adversities, including antimicrobial substances, compared to the strains selected for the present research.

We investigated the hemolytic effect of LD-EO on human erythrocytes of blood types A, B, and O. Our results showed that the product had low cytotoxicity at MIC values for *C. albicans*. Toxicological assays are necessary to explore the potential use of a biological product in the pharmaceutical industry, and the hemolytic activity of a substance has been used as an indicator of its cytotoxic effect (Figueiredo-Júnior et al., 2021). The toxicity of terpene-rich EOs has been extensively explored in scientific literature, with most studies corroborating the findings of this research. For example, *Coriandrum sativum* L. EO showed low cytotoxicity in human HeLa cells (Freires et al., 2014), while *Foeniculum vulgare* EO was considered safe up to specific concentrations for various cell types, including keratinocytes, hepatocytes, fibroblasts, and macrophages (Cabral et al., 2017). The cytotoxicity of *L. luisieri* EO was assessed in mouse macrophages using the MTT assay, and it was considered safe for mammalian cells at < 0.32 µL/mL. However, this concentration was below the MIC for *C. albicans* (0.64–2.5 µL/mL), indicating toxicity at inhibitory concentrations (Zuzarte et al., 2012). Nevertheless, Arantes et al. (2016), when analyzing *L. stoechas* subsp. *luisieri* EO in mice, including histological and morphometric studies of the liver and kidney, concluded that this oil had a low acute oral toxicity.

Regarding LD-EO, El-Abdali et al. (2022) reported that high concentrations and durations of exposure to this product resulted in higher mortality in *C. maculatus*. We emphasize that assays with mammalian cells and *in vivo* studies are more suitable for accurately estimating the toxicity of a biological product in humans. Within this context, Cossetin et al. (2018) investigated the cytotoxicity, mutagenicity, and genotoxicity of LD-EO for human leukocytes. The test substance at 1 and 10 µg/µL was considered safe, as it did not induce significant cell death or lysis. Safety regarding potential genotoxic or mutagenic effects was also evidenced. According to the authors, their results could be justified by the predominance of monoterpenes in the chemical composition of EOs, given that these compounds do not cause toxic effects on DNA. These findings, together with the low toxicity of LD-EO for human erythrocytes found in our research, suggest that the studied product is probably safe at concentrations close to MIC for treating oral candidiasis. Despite this, additional types of toxicity assays should be conducted to clarify the safety of LD-EO for future clinical trials in humans.

5. Conclusion

This study is the first to report the excellent antifungal and antibiofilm activities of *Lavandula dentata* L. essential oil (LD-EO) from Brazil. The potential of this natural product as an antifungal agent with greater efficacy and safety for treating oral candidiasis, including its miconazole-resistant forms, is evident. Therefore, we encourage further research to elucidate the mechanisms of action, toxicity, and *in vivo* efficacy and future clinical trials focusing on LD-EO.

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