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Original Article

Bioactive potential of *Eugenia luschnathiana* essential oil and extract: antifungal activity against *Candida* species isolated from oncological patients

Potencial bioativo do óleo essencial e extrato de Eugenia luschnathiana: atividade antifúngica contra espécies de Candida isoladas de pacientes oncológicos

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Abstract

Immunosuppressed individuals, including those undergoing cancer treatment, are more vulnerable to fungal infections, such as oral candidiasis, impacting their quality of life. Given the limitations of current therapies, the discovery of new antifungal agents, including those of natural origin, is crucial for the proper managing of these infections. We investigated the phytochemical profile and antifungal activity of both the essential oil and crude ethanolic extract (CEE) obtained from *Eugenia luschnathiana* against reference strains and clinical isolates of *Candida* from oncology patients. Toxicological characterization was also conducted. Gas chromatography coupled to mass spectrometry (GC-MS) and ^{1}H Nuclear Magnetic Resonance (NMR) were used for phytochemical analysis. Antifungal evaluation was conducted to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC); evaluation of potential mechanisms of action; activity on a fungal biofilm; evaluation of the cytotoxic effect on human keratinocytes of the HaCat lineage by the MTT method; determination of lethality for *Artemia salina* larvae. GC-MS identified a predominance of sesquiterpenes in the essential oil, notably *(E)*-Caryophyllene. The ^{1}H NMR spectrum identified aliphatic, osidic, and aromatic compounds in the crude ethanolic extract. The essential oil showed no antifungal activity. However, the CEE exhibited fungicidal activity, with MIC and MFC ranging from 1.95 µg/mL to 3.90 µg/mL. The antifungal effect was affected by sorbitol, indicating a possible mechanism targeting fungal cell wall structures. At low concentration (19.5 µg/mL), the CEE inhibited 62,78% of *C. albicans* biofilm. The CEE demonstrated a promising toxicity profile, with an LC_{50} of 142.4 µg/mL against *Artemia salina*. In conclusion, the CEE from *Eugenia luschnathiana* exhibited potent antifungal activity, likely through cell wall disruption, biofilm inhibition, and a favorable toxicity profile for further exploration.

Keywords: candidiasis, antifungals agents, oils, volatile, plant extracts, phytochemicals.

Resumo

Indivíduos imunossuprimidos, a exemplo dos que fazem tratamento para o câncer, são mais suscetíveis a infecções fúngicas, como a candidíase oral, que podem afetar diretamente sua qualidade de vida e, consequentemente, seu processo de recuperação. Diante das limitações relacionadas às atuais opções terapêuticas, a descoberta de novos agentes antifúngicos, incluindo os de origem natural, é fundamental para o correto manejo dessas infecções. Este estudo investigou o perfil fitoquímico, a atividade antifúngica do óleo essencial e extrato etanólico bruto (EEB) obtidos de *Eugenia luschnathiana* sobre cepas referência e isoladas clínicas de *Candida* de pacientes sob tratamento oncológico e realizou a caracterização toxicológica. Para análise fitoquímica foram usados cromatografia gasosa acoplada ao espectrofotômetro de massas (CG-EM) e Ressonância Magnética Nuclear (RMN) de 1H. A avaliação antifúngica foi conduzida para determinação da Concentração Inibitória Mínima (CIM) e da Concentração Fungicida Mínima (CFM); avaliação dos potenciais mecanismos de ação; avaliação da atividade sobre biofilme fúngico; determinação da letalidade para larvas de *Artemia salina*. A CG-EM identificou predominância de sesquiterpenos no óleo essencial, sendo (E)-Caryophyllene o composto majoritário. O espectro de RMN de 1H identificou compostos alifáticos, osídos e aromáticos no extrato etanólico bruto. O OE não apresentou atividade antifúngica. O EEB mostrou atividade fungicida, com CIM e CFM variando de 1,95 µg/mL a 3,90 µg/mL para cepas testadas, estes valores permaneceram inalterados na presença de ergosterol exógeno, com possível mecanismos de ação sobre estruturas que envolvem parede celular fúngica. Em baixa concentração (19.5 µg/mL), o EEB inibiu 62.78% do

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biofilme de *Candida albicans*. A CL_{50} do EEB para *A. salina* foi de 142,4 µg/mL. Observa-se, então, que o EEB possui atividade antifúngica muito forte, com ação provável sobre parede celular, efeito sobre biofilme e perfil de toxicidade compatível para realização de outras investigações.

Palavras-chave: candidíase, antifúngicos, óleo essencial, extrato vegetal, compostos fitoquímicos.

1. Introduction

Organisms of the Fungi kingdom are responsible for infecting and causing the death of approximately 1.5 million people annually. The increase in this number in recent decades is due to the growing number of elderly and immunocompromised patients, such as cancer patients, transplant recipients, and HIV^+ individuals. Among the main etiological agents of fungal infections in immunocompromised patients are fungi of the genus *Candida*, such as *C. albicans*, the predominant species (Lee et al., 2020). Other species, including *C. tropicalis, C. parapsilosis, C. glabrata, C. krusei, and C. guilliermondii*, are frequently associated. Mortality associated with *Candida* species varies from 46 to 75%, characterized by the Centers for Disease Control and Prevention of the United States as a serious threat to human health (Chowdhary et al., 2023; Rai et al., 2022).

The species *C. albicans* is responsible for symbiotically colonizing the human microbiota and is present in the gastrointestinal and reproductive tracts of a large proportion of healthy individuals. This usually commensal microorganism has its growth favored by local (dentures and poor oral hygiene) and systemic factors (anemia, uncontrolled diabetes, prolonged systemic therapies, and immunodeficiencies) that can lead to an imbalance in the microbiota (Contaldo et al., 2023; Vila et al., 2020). In this context, oral candidiasis, an opportunistic fungal infection, manifests itself superficially and usually mildly on the mucosa. However, due to the virulence factors of *C. albicans* and the increasing association of infections with other *Candida* species, this infection can become resistant to existing treatments and present high rates of recurrence (Quindós et al., 2019).

Oral candidiasis can lead to discomfort, oral burning sensation, and taste alteration, impacting on the quality of life and nutrition of the affected population, as well as on the recovery of hospitalized patients (Monsen et al., 2023; Vila et al., 2020). Proper nutrition is crucial for the recovery of immunocompromised patients, especially those with cancer, who are constantly undergoing radio and chemotherapy. Moreover, due to the risk of dissemination to adjacent mucosa and systemic infection, these patients are in a highly vulnerable position. Hence, effective management of oral candidiasis is essential for maintaining the quality of life of the population, especially immunodeficient patients (Rai et al., 2022; Ramírez-Carmona et al., 2023).

Treatment with conventional antifungals, however, has become ineffective due to the indiscriminate and prolonged use of the limited available arsenal (polyenes, azoles, and echinocandins). Therapies for oral candidiasis primarily involve topical (nystatin and miconazole) and systemic (fluconazole and itraconazole) options, depending on the severity of the infection (Pristov and Ghannoum, 2019; Rai et al., 2022). Besides the drug therapy, the success of the treatment is related to the removal of local or systemic causes. If this is not possible, oral candidiasis can relapse and lead to a cycle of chronicity and drug resistance, resulting in a more aggressive condition with systemic dissemination (Contaldo et al., 2023).

Therefore, it is emphasized the need to discover new antifungals with greater efficacy and different mechanisms of action to properly manage the patient (Alves et al., 2021). In this sense, the use of natural products has been considered promising for the development of new therapeutic agents, especially for the treatment of oral candidiasis (Silva-Rodrigues et al., 2024). This alternative appears viable considering the tendency for fewer adverse effects and low cost compared to allopathic drugs (Souza-Melo et al., 2021; Ferreira et al., 2015).

The Myrtaceae family, mostly found in tropical and subtropical regions, has its use as a functional food and biological activity well-documented, being widely studied in natural product research (Araújo, 2018; Henriques et al., 2021). The genus *Eugenia*, one of the main representatives of the Myrtaceae family, is described in the literature for possessing plants with pharmacological activity such as antioxidant, antibacterial, antidiarrheal, antifungal, anti-inflammatory, and antipyretic, with the antifungal potential well-documented. Among the plants of this family is *Eugenia luschnathiana* (O. Berg) Klotzsch ex BD. Jacks, popularly known as "pitomba-da-bahia" and found in several states of northeastern Brazil (Sardi et al., 2017; Henriques et al., 2021). A previous study indicates that an extract/essential oil of *E. luschnathiana* has activity against gram-positive and gram-negative bacteria (Araújo, 2018). This result supports the hypothesis that the essential oil and crude ethanolic extract obtained from *E. luschnathiana* showed antifungal activity against standard strains and clinical isolates of *Candida* obtained from the oral cavity of individuals undergoing oncologic treatment.

Although the antimicrobial activity of several species of the genus *Eugenia* is described in the literature, studies on the biological activities of *E. luschnathiana* are limited. Thus, the present article aims to determine the phytochemical characteristics of the EO and CEE from the leaves of *E. luschnathiana*, investigate their antifungal activity, including possible mechanisms of action and inhibitory effect on biofilm, against reference strains and clinical isolates of *Candida* spp. from patients undergoing oncologic treatment, as well as determine toxicity parameters.

2. Material and Methods

2.1. Plant material

Before collecting the plant product, the research was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), under number A4B2595. The leaves of

E. luschnathiana were collected in the municipality of Tibau do Sul, RN, Brazil (S 6º 13' 39", W 35º 02' 51").

The Herbarium of the Center for Exact and Natural Sciences of the Federal University of Paraíba, João Pessoa, holds the voucher specimen of *Eugenia luschnathiana* (O. Berg) Klotzssch ex B.D. Jacks, under the number JPB 63845.

2.2. Extraction of the essential oil and crude ethanolic extract

The essential oil was extracted using the steam distillation method and the Linax mini distiller, as described by Rostagno and Prado (2013), with modifications. Fresh leaves were placed inside the extractor with water, which was heated to produce steam. At the outlet of the distiller, there is a condenser and a separating funnel. At this point, after being cooled by water, the steam is condensed and stored in the separating funnel, where the separation between water and oil occurs due to differences in density.

For the crude ethanolic extract, the method recommended by de Souza-Melo et al. (2021) was adopted, with modifications. Fresh leaves of *E. luschnathiana* were placed in the percolator with 96% ethanol for 72 hours to obtain the extractive solution. This process was repeated 3 times for the effective extraction of all components. This solution was concentrated in a rotary evaporator under reduced pressure at an average temperature of 45°C to obtain the crude ethanolic extract (CEE).

2.3. Microorganisms and reagents

Strains of *C. albicans* ATCC 76645 and *C. albicans* ATCC 90028 from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used. Clinical strains, belonging to the microorganism collection of the Laboratory of Experimental Pharmacology and Cell Culture of the Federal University of Paraíba, João Pessoa, Paraíba, Brazil, were isolated from the oral cavity of patients undergoing oncologic treatment: *C. albicans* (A1), *C. albicans* (A5), *C. glabrata* (A14), *C. krusei* (A18), *C. glabrata* (A19), *C. tropicalis* (A20).

The culture medium RPMI-1640, Nystatin, DMSO (dimethyl sulfoxide), and ergosterol 400µg/mL, used during the assays, were obtained from Sigma-Aldrich® Chemical Co. (St. Louis, MO, USA). Sorbitol 0.8M (D-sorbitol anhydrous) was obtained from INLAB® (São Paulo, Brazil), and Sabouraud Dextrose Agar from KASVI® (Kasv Imp and Dist e Prod) Laboratórios LTDA (Curitiba, Brazil).

2.4. Phytochemical evaluation

To characterize and identify the components of the EO, gas chromatography coupled with mass spectrometry (GC-MS) was performed. A gas chromatograph coupled to a mass spectrometer was used, with a capillary column and stationary phase of 5% phenyl and 95% dimethylpolysiloxane, 30m in length, 0.25mm internal diameter, and 0.25µm film thickness. Initially, the temperature was programmed from 60 to 240ºC (3ºC/min). The run time was set to 60 min, and the injector temperature was 250ºC. Helium was used as the carrier gas (mobile phase) at a flow rate of 1.0 mL/min, with a split ratio of 1:20 and an injection volume of 1µL. The components were ionized by electron impact at 70eV, using a 1.25-kV detector. The spectrometer operated in SCAN mode, scanning a mass range of 40 to 500 atomic mass units (u.m.a). The ion source temperature was 250ºC. The oil sample was injected at a concentration of 2 ppm, using hexane as the solvent. Chromatogram and mass spectra analyses were performed by comparing their mass spectra with those in the equipment's database. Integration parameters used were width, 3, and slope, 2000 (Trindade et al., 2015). Under the same conditions, a series of hydrocarbons (C10 to C40) (Sigma-Aldrich®) was injected for the calculation of the compounds' retention indices. According to the libraries (Nist 08 and Wiley 9) used for compound identification, each retention index was determined based on a similarity greater than 89%. Retention indices were determined using the chromatogram obtained based on the Van Den Dool and Kratz equation (Van Den Dool and Kratz, 1963).

For the CEE, the assays were conducted according to the methodology proposed by Matos (1997), with modifications. The extracts were subjected to tests for alkaloids, steroids, tannins, flavonoids, and saponins.

2.4.1. Alkaloids test

The CEE of *E. luschnathiana* was evaporated to dryness and alkalized with 0.8 mL of 1% NaOH. Then, 6 mL of distilled water and 6 mL of $CHCl_3$ were added to filter and separate the extract from the chloroform layer. To this layer, 6 mL of 1% HCl was added. After decantation, 1 mL of the upper HCl layer was used for the tests with the Bouchardat, Mayer, Dragendorff, and Silicotungstic Acid reagents. The presence of alkaloids was identified by the formation of a precipitate.

2.4.2. Steroids test

For the identification of steroids, 2.5 mL of $CHCl_3$ was added to the extract and dissolved for subsequent distribution into test tubes (0.12; 0.25; and 0.5 mL). To each tube, 2 mL of $CHCl_3$ and 1 mL of acetic anhydride were added, and after shaking, 2 mL of H_2SO_4 was added. The results were read according to the preparation containing cholesterol, the standard steroid.

2.4.3. Tannins test

For this assay, 10 mL of distilled water was added to the extract for subsequent filtration. The obtained solution was distributed into 6 test tubes, three with 0.5% gelatin and three with 2% FeCl3. The presence of tannins was observed by the color change of the solutions.

2.4.4. Flavonoids test

15 mL of distilled water was added to the extract of *E. luschnathiana* and, after shaking, allowed to settle. Then, 15 mL of chloroform was added, and the solution was allowed to settle until the layers separated, followed by the discard of the chloroform layer. This process was repeated until no more chlorophyll was present. The obtained solution was subjected to rotary evaporation, and then 3 mL of methanol was added. This preparation was added to 2 test tubes, the first containing 0.5 mL of 10% HCl and

1 strip of magnesium, the second containing 0.05 mg of oxalic acid and 0.05 mg of boric acid in acetone, with the subsequent addition of 10 mL of ethyl ether. The presence of flavonoids was determined by a pink color in the first tube and fluorescence under UV light in the second tube.

2.4.5. Saponins test

The presence of saponins was determined by shaking the extract with 10 mL of water. After resting for 10 minutes, the formation of foam indicates the presence of saponins.

2.4.6. Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy of the Extract of E. luschnathiana

A 20 mg aliquot of the *E. luschnathiana* extract was dissolved in 600 µL of deuterated methanol, and the suspension was subjected to an ultrasonic bath for 30 minutes and filtered. The resulting solution (550 µL) was placed in a 5 mm diameter tube for analysis by Nuclear Magnetic Resonance (NMR). The assays were performed on the Bruker Ascend equipment operating at 400 MHz for ^{1}H-NMR and 100 MHz for 13C-NMR (Bruker, Billerica, MA, USA). The following parameters were used to obtain the sequence of spectra: zg30; temperature: 26°C; number of scans: 16; dummy scan: 4; receiver gain: 64; acquisition time: 4.0894 s. The spectra were processed using the Bruker TopSpin 4.1.1 software.

2.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

Based on Clinical Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2008), the MIC, the lowest concentration capable of visually inhibiting fungal growth, was determined by the microdilution technique in RPMI-1640. The strains used were suspended in 0.9% NaCl solution, with a transmittance of 0.5 on the McFarland scale, at a wavelength of 530 nm, to obtain a suspension of $5x10^2$ to $2.5x10^3$ CFU/mL. For the serial dilutions, sterile 96-well flat-bottom plates containing RPMI-1640 were used. These were incubated for 24 hours at 35°C, and the results were read from the observation of cell aggregates at the bottom of the wells. The essential oil was tested at concentrations ranging from 1000 µg/mL to 7.81 µg/mL, and the CEE was tested at concentrations ranging from 62.5 µg/mL to 0.48 µg/mL. DMSO (dimethyl sulfoxide) (Sigma-Aldrich, São Paulo, Brazil) and Tween 80 (Sigma-Aldrich) were used to prepare the solutions. Nystatin (Sigma-Aldrich, São Paulo, Brazil) was used as a positive control. Controls were performed to ensure the sterility of the culture medium and the absence of the antifungal effect of DMSO (5%) and Tween 80 (2%).

The MFC, defined as the lowest concentration of the substance capable of inhibiting visible growth on solid medium, was determined from subcultures on Petri dishes containing Sabouraud Dextrose Agar of 20µL aliquots corresponding to the MIC and the two immediately more concentrated concentrations (MICx2 and MICx4). The plates were incubated for 24 hours at 35°C, and the results were read from the observation of fungal growth in the culture medium. The MFC/MIC ratio was calculated to determine if the substance has fungicidal activity (MFC/MIC<4) or fungistatic activity (MFC/MIC>4) (Popiołek et al., 2016). All experiments were performed in triplicate and three independent experiments.

2.6. Extract's effect on fungal cell wall and membrane

2.6.1. Effect of the extract on fungal cell wall

This assay aims to evaluate the potential mechanism of action of CEE on structures that affect the function of the fungal cell wall. Sorbitol is an osmotic protector, and an increase in MIC values with its addition will imply determining the cell wall as one of the possible cellular targets of the tested compound (Lima et al., 2012). For this test, the microdilution technique was performed. Initially, 100 µL of RPMI-1640 was added to each well, followed by the addition of 100 µL of the test substance to the first wells. A serial microdilution was performed at a ratio of 2 to obtain concentrations from 62.5 µg/mL to 0.48 µg/mL (Freires et al., 2014). The strains *C. albicans* ATCC 90028 and *C. albicans* (A5) were used at a concentration of $5x10^2$ to $2.5x10^3$ CFU/mL. To the plates, 100 µL of these inoculums, prepared with RPMI-1640 supplemented with sorbitol (0.8 M), were added and subsequently added to the wells. The microplates were incubated for 24 hours at 35°C, and the reading was performed from the observation of cell aggregates at the bottom of the wells. Caspofungin was used as a positive control at an initial concentration of 4 µg/mL, due to its activity on the fungal cell wall (Perlin, 2011). Growth and sterility controls were performed simultaneously with the assay and were performed in triplicate and three repetitions.

2.6.2. Effect of the extract on fungal cell membrane

To determine if the extract will interact with ergosterol, the MIC was determined against the strains *C. albicans* ATCC 90028 and *C. albicans* (A5) in the absence and presence of exogenous ergosterol, one of the main sterols present in the plasma membrane, at a concentration of 400 µg/mL. The microdilution technique, previously described, was used for this purpose, with extract concentrations ranging from 62.5 µg/mL to 0.48 µg/mL (Freires et al., 2014). Nystatin was used as a positive control at an initial concentration of 48 µg/mL, due to its activity on the fungal cell membrane (Peixoto et al., 2017). An increase in MIC values in media with the addition of ergosterol will imply determining the cell membrane as one of the possible cellular targets of the extract (Lima et al., 2012). Growth and sterility controls were performed simultaneously with the assay, wich was performed in triplicate and three repetitions.

2.7. Extract's antimicrobial activity on fungal biofilm

To form the biofilm, 1 mL samples of the inoculum of *C. albicans* ATCC 9002, with approximately 10^6 CFU/mL, were transferred to 24-well microdilution plates, which were then incubated for 48 hours at 35°C. After incubation, the wells were washed with phosphate-buffered saline (PBS) to remove loosely adhered cells. Subsequently, sterile culture medium was added, followed by the addition of the CEE of *E. luschnathiana* at predetermined concentrations: MIC (1.95 µg/mL); MICx2 (3.90 µg/mL);

MICx4 (7.8 µg/mL); MICx10 (19.5 µg/mL) and nystatin (positive control) at concentrations of MIC (1.5 µg/mL); MICx2 (3 µg/mL); MICx4 (6 µg/mL); MICx10 (15 µg/mL). The plates were incubated again for 48 hours at 35°C. For biofilm quantification, the wells were washed twice with PBS, followed by air drying for 45 min. The biofilm was stained with a 0.4% crystal violet solution. The reading was performed using a microplate reader with a wavelength of 595 nm (DJORDJEVIC, WIEDMANN, MCLANDSBOROUGH, 2002).

2.8. Cytotoxicity assay on non-tumor human keratinocytes

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was employed to evaluate the cytotoxicity of the ethanolic extract of E. luschnathiana against human keratinocytes cell line HaCaT. This method involves assessing cell viability and proliferation through the reducing activity of mitochondrial and cytoplasmic enzymes. MTT is a water-soluble yellow dye converted into insoluble blue-purple formazan crystals in the viable cellular cytosol by the activity of dehydrogenases, mainly succinate dehydrogenase. Then, the amount of formazan produced is directly proportional to the number of viable cells (Mosmann, 1983; Kumar et al., 2018).

The HaCaT cell line was obtained from the Rio de Janeiro Cell Bank (BCRJ – Brazil), and cultured in DMEM medium (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA), and 1% penicillin-streptomycin (Sigma Aldrich), maintained at 37 ºC with 5% CO2.The cells were seeded in 96-well plates (3 x 105 cells/mL). After 24 hours, the cells were treated with the extract at different concentrations (0.487, 0.975, 1.95, 3.9, 7.8, 19.5, 39 and 78 µg/mL) for 72 hours. After treatment, 110 µL of supernatant was removed, and 10 µL of MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added for an additional 4 hours. After that, 100 µL of a 10% sodium dodecyl sulfate hydrochloric acid solution (SDS-HCl) was added to dissolve the formazan crystals produced. Absorbance was measured using a spectrophotometer (BioTek Instruments microplate reader, Sinergy HT, Winooski, VT, USA) at a wavelength of 570 nm.

Three independent experiments were conducted in quadruplicate. The results are expressed as the mean of the percentage of cell viability ± SEM (standard error of the mean) and were compared by one-way analysis of variance (ANOVA), followed by Tukey's test ($p<0.05$).

2.9. Toxicity on Artemia salina

The protocol established by Meyer et al. (1982), with modifications (Oliveira et al., 2021), was adopted to establish a preliminary toxicity profile. To do so, *Artemia salina* cysts were incubated at 28ºC for 48 hours in an aerated system to reach the metanauplius maturation stage. After the incubation period, the extract was prepared at concentrations of 1.95, 3.9, 7.8, 19.5, 39, 97.5, and 195 µg/mL and added to falcon tubes containing ten *A. salina* larvae. Artificial seawater (3.5% NaCl) and DMSO were used as solvents at a ratio of 95:5. After 24 hours of exposure, the dead larvae were counted, and the median lethal concentration (LC_{50}) value was established using the concentration-response regression model, which is suitable for binary or count data, such as survival or mortality results at different doses. Sodium lauryl sulfate was used as a positive control, and the assay was performed in triplicate.

3. Results

3.1. Phytochemical characterization of the essential oil

The major components present in the EO of *E. luschnathiana* were tabulated according to name, retention time (RT), area (%), experimental Kovats index, and literature Kovats index, according to the NIST library (Table 1). A total of 20 components were identified, with a predominance of sesquiterpene compounds: (E)-Caryophyllene (26.34%), β-Spathulenol (11.88%), Bicyclogermacrene (11.44%), and (+)-Caryophyllene oxide (9.39%).

3.2. Phytochemical characterization of the CEE

Qualitative assays for the detection of secondary metabolites allowed the identification of tannins and flavonoids in the extract, both moderately positive. The results for alkaloids, steroids, and saponins were negative (Table 2).

In the ^{1}H NMR spectrum, signals were observed in the δH 2.5-0.6 ppm region, consistent with the presence of compounds with aliphatic carbon chains. In the δH 5.3-3.0 ppm region, the signals present are consistent with hydrogens belonging to osidic units, while the signals in the δH 8.8-6.0 ppm region indicate the presence of aromatic compounds or compounds with olefinic hydrogens. Thus, a fingerprint of the crude ethanolic extract (CEE) of *E. luschnathiana* was obtained by ^{1}H NMR, expanding the chemical characterization of its composition, in addition to assisting in its authenticity (Figure 1).

3.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC and MFC values of the CEE, the EO, and the positive control, nystatin, are expressed in Table 3. The MIC and MFC values of the CEE ranged from 1.5 µg/mL to 3.90 µg/mL, while the EO showed a MIC >1000 µg/mL for all tested strains. The concentration of nystatin showed no variation, being equal to 1.5 µg/mL for all strains tested. The MFC/MIC ratio indicates a fungicidal action for both tested substances (Popiołek et al., 2016).

3.4. Extract's effect on fungal cell wall and membrane

The MIC of the extract increased in the presence of sorbitol from 1.95 µg/mL to 3.90 µg/mL for both tested strains, like the positive control, caspofungin, an agent known for its activity on the fungal cell wall (Table 4). In the presence of exogenous ergosterol, however, the MIC remained unchanged for the extract, unlike nystatin, a substance used as a positive control (from 1.5 µg/mL to 6 µg/mL) (Table 5).

3.5. Extract's antimicrobial activity on fungal biofilm

Figure 2 illustrates the effect of the extract on fungal biofilm inhibition. A 13% reduction in fungal biofilm was

Table 1. Phytochemical composition of the essential oil of *E. luschnathiana* by GC-MS.

Component	RT (min)	Area (%)	KI (exp.)	KI (lit)
(E)-Caryophyllene	14,48	26,34	1445	1416
β-Spathulenol	16,60	11,88	1606	1572
Bicyclogermacrene	15,51	11,44	1523	1505
(+) Caryophyllene oxide	16,70	9,39	1614	1581
Germacrene D	15,30	6,35	1507	1519
α-Humulene (CAS)	14,94	6,23	1480	1488
β-Bourbonene	13,95	4,75	1405	1412
Spathulenol	17,33	3,66	1661	1622
α-Copaene	13,79	3,06	1393	1376
δ-Cadinene	15,79	2,88	1544	1524
Salvial-4(14)-en-1-one	16,81	2	1622	1599
Thymol	12,42	1,99	1290	1292
γ-Muurolene	15,19	1,88	1499	1481
Humulene epoxide II	17,02	1,44	1638	1620
1,10-di-epi-Cubenol	17,19	1,44	1650	1619
Caryophyllene <9-epi-(E)->	15,05	1,30	1449	1455
Aromadendrene	14,74	1,06	1465	1484
α-Cadinol	17,51	1,01	1675	1673
β-CubCEEne	14,58	0,96	1452	1388
β-Selinene	15,39	0,94	1514	1489

TR = Retention time; KI (exp.) = Experimental Kovats index; KI (lit.) = Literature Kovats index. Source: Data collected by the authors.

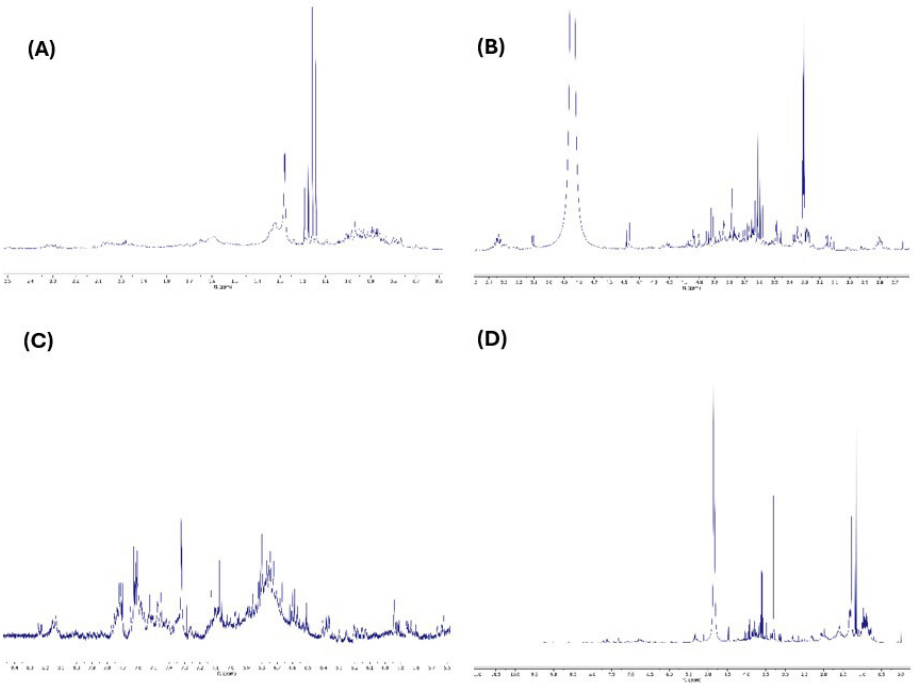

Figure 1. Assignment of ^{1}H NMR spectra of the extract of E. *luschnathiana*. (A) ^{1}H NMR spectrum expansion in the region of 2.5 – 0.6 ppm. (B) 1H NMR spectrum expansion in the region of 5.5 – 2.6 ppm. (C) 1H NMR spectrum expansion in the region of 8.5 – 5.6 ppm. (D) ^{1}H NMR spectrum of extract *Eugenia luschnathiana*.

observed at the concentration equivalent to the MIC, with significant reductions ($P < 0.05$) starting from 3.9 µg/mL. The extract also showed a 56.67% reduction in biofilm at MICx4 and a 62.78% reduction at MICx10. Nystatin, the positive control, showed a significant reduction in fungal biofilm starting from the MIC (43.32%).

Table 2. Results obtained in the screening of functional groups of the extract of *E. luschnathiana*.

Tests performed	Extract of *E. luschnathiana*
Alkaloids	-
Steroids	-
Tannins	++
Flavonoids	++
Saponins	-

Strongly positive (+++); Moderately positive (++); Positive (+); Negative (-).

3.6. Cytotoxicity assay on non-tumor human keratinocytes

The cytotoxic potential of the ethanolic extract from *E. luschnathiana* was assessed on the human keratinocyte cell line HaCaT after 72 hours of treatment. It was noted that the extract only impacted cell viability at the three highest concentrations tested (MICx10, MICx20 and MICx40), resulting in reductions of 19.93%, 24.36%, and 34.26% in this parameter, respectively (Figure 3). Moreover, half-maximal inhibitory concentration (IC_{50}) cannot be calculated due to the weak cytotoxic effect of the extract at the tested concentrations. Therefore, it can be considered that the IC_{50} is greater than 78 µg/mL.

Table 3. MIC and MFC of the extract and essential oil of *E. luschnathiana* and nystatin on reference strains of *Candida* spp. and clinical isolates from the oral cavity of oncological patients (A). Concentration values are expressed in µg/mL.

Cepa	Extract of *E. luschnathiana*			EO of *E. luschnathiana*			Nystatin		
	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC
C. albicans ATCC 76645	1.95	1.95	1	>1000	>1000	>1	1.5	1.5	1
C. albicans ATCC 90028	1.95	1.95	1	>1000	>1000	>1	1.5	1.5	1
C. albicans (A1)	3.90	3.90	1	>1000	>1000	>1	1.5	1.5	1
C. albicans (A5)	1.95	1.95	1	>1000	>1000	>1	1.5	1.5	1
C. glabrata (A14)	1.95	1.95	1	>1000	>1000	>1	1.5	1.5	1
C. krusei (A18)	1.95	1.95	1	>1000	>1000	>1	1.5	1.5	1
C. glabrata (A19)	1.95	1.95	1	>1000	>1000	>1	1.5	1.5	1
C. tropicalis (A20)	3.90	3.90	1	>1000	>1000	>1	1.5	1.5	1

Source: Data collected by the authors.

Table 4. MIC values of the extract of *E. luschnathiana* and Caspofungin in the absence and presence of 0.8M sorbitol against strains of *C. albicans* ATCC 90028 and *C. albicans* (A5). Values expressed in µg/mL.

Concentration (µg/mL)	*E. luschnathiana*				Concentration (µg/mL)	Caspofungin			
	C. albicans ATCC 90028		*C. albicans* A5			*C. albicans* ATCC 90028		*C. albicans* A5	
	Without sorbitol	With sorbitol	Without sorbitol	With sorbitol		Without sorbitol	With sorbitol	Without sorbitol	With sorbitol
62.5	-	-	-	-	**4**	-	-	-	-
31.25	-	-	-	-	**2**	-	-	-	-
15.62	-	-	-	-	**1**	-	-	-	-
7.81	-	-	-	-	**0.5**	-	-	-	-
3.90	-	-	-	-	**0.25**	-	+	-	-
1.95	-	+	-	+	**0.125**	-	+	-	-
0.97	+	+	+	+	**0.062**	+	+	-	+
0.48	+	+	+	+	**0.031**	+	+	+	+

Note: +, fungal growth; -, no fungal growth.
Source: Data collected by the authors.

3.7. Toxicity on *Artemia salina*

From the equation obtained by linear regression analysis, an LC_{50} of 142.4 µg/mL was obtained for the extract of *E. luschnathiana* (Figure 4). The positive control showed 100% mortality, and there were no deaths in the negative control, indicating that the solvent used did not exhibit toxicity on *A. salina*.

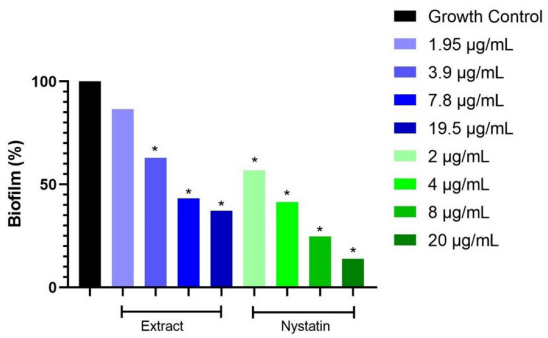

Figure 2. Inhibitory effect of the extract of *E. luschnathiana* and nystatin on biofilm of *C. albicans* ATCC 90028. Results presented as mean ± SD of three independent experiments performed in triplicate. (One-way ANOVA analysis followed by Tukey's post-hoc test, $p < 0.05$, extract and nystatin vs. growth control). Source: Data collected by the authors.

4. Discussion

Plant-derived products are constantly associated with antimicrobial activities due to their complex chemical composition, which may include alkaloids, tannins, terpenoids, and flavonoids, among other organic components. This action is essentially due to phytochemicals, which can lead to synergy among the components and potentiation of their individual effects (Rahman et al., 2021). Thus, the analysis of the chemical constituents of these products allows the delineation of their main characteristics. With this intention, the essential oil was characterized by GC-MS, as well as a phytochemical screening and analysis by ^{1}H Nuclear Magnetic Resonance of the crude ethanolic extract of *E. luschnathiana*. In line with other species of *Eugenia* spp. and a study by Monteiro et al. (2016) to determine the

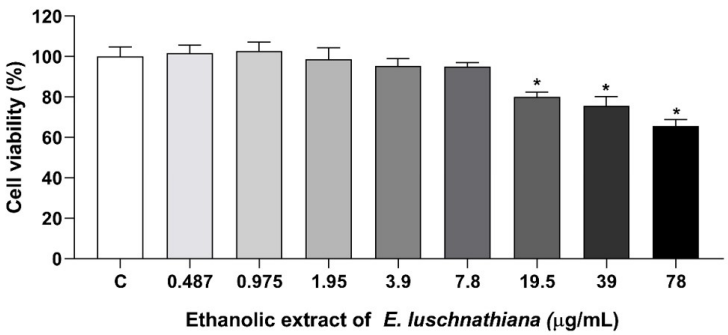

Figure 3. Cytotoxic activity of the ethanolic extract of *E. luschnathiana* on the human keratinocyte cell line HaCaT after 72 h of treatment. Source: Data obtained from three independent experiments performed in quadruplicate and presented as the mean of the percentage of cell viability ± SEM. One-way analysis of variance (ANOVA), followed by Tukey's test. *p<0.05 compared to the control group.

Table 5. MIC values of the extract of *E. luschnathiana* and Nystatin in the absence and presence of exogenous ergosterol (400 µg/mL) against strains of *C. albicans* ATCC 90028 and *C. albicans* (A5). Values expressed in µg/mL.

Concentration (µg/mL)	*E. luschnathiana*				Concentration (µg/mL)	Nystatin			
	C. albicans ATCC 90028		*C. albicans* (A5)			*C. albicans* ATCC 90028		*C. albicans* (A5)	
	Without ergosterol	With ergosterol	Without ergosterol	With ergosterol		Without ergosterol	With ergosterol	Without ergosterol	With ergosterol
62.5	-	-	-	-	48	-	-	-	-
31.25	-	-	-	-	24	-	-	-	-
15.62	-	-	-	-	12	-	-	-	-
7.81	-	-	-	-	6	-	-	-	-
3.90	-	-	-	-	3	-	+	-	+
1.95	-	-	-	-	1.5	+	+	+	+
0.97	+	+	+	+	0.75	+	+	+	+
0.48	+	+	+	+	0.37	+	+	+	+

Note: +, fungal growth; -, no fungal growth.
Source: Data collected by the authors

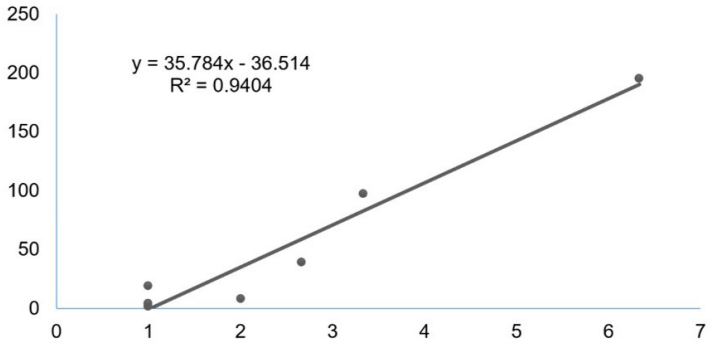

Figure 4. Linear regression curve for determination of the LC50 of the Extract of *E. luschnathiana* on *Artemia salina*. Source: Data collected by the authors.

phytochemical profile of the essential oil of *E. luschnathiana*, the GC-MS analysis demonstrated the prevalence of sesquiterpene compounds. However, Monteiro et al. (2016) identified 2-β-Hexenal, α-Thujen, and α-Pinene as the main compounds, while the present analysis showed β-Caryophyllene, β-Spathulenol, and Bicyclogermacrene as predominant components. This divergence may be due to environmental and climatic factors, as well as the period of collection, which directly affects the composition and properties of essential oils (Costa et al., 2020).

The main secondary metabolites found in the extract were tannins and flavonoids. Tannins have well-described anti-inflammatory, antiviral, antibacterial, and antifungal activity in the literature, as do flavonoids. Aliphatic and aromatic compounds, present in the extract of *E. luschnathiana*, are functional groups commonly associated with constituents with fungicidal activity (Seleem et al., 2017; Morey et al., 2016; Wei et al., 2017).

In the literature, the antimicrobial potential of species of the genus *Eugenia* is well-documented. However, for the species *E. luschnathiana*, there is little information about the biological activity of extracts obtained from this plant. Araújo (2018) investigated the activity of the EO of *E. luschnathiana* against Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermis*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and identified weak activity. In this study, we did not observe the susceptibility of *Candida* strains to the EO of *E. luschnathiana*. A previous study demonstrated that β-Caryophyllene, the major component present in the EO, showed weak bioactivity against strains of *S. aureus, E. coli, Salmonella typhimurium, Enterococcus faecallis, Aspergillus niger, A. fumigates, A. parasiticum, and Fusarium solani* (Selestino Neta et al., 2017)

According to the classification proposed by Ferreira et al. (2021), the CEE showed very strong fungicidal activity against the reference strains and clinical isolates (1.95 µg/mL and 3.90 µg/mL). Previous studies have demonstrated that extracts of *E. leitonii* and *E. brasiliensis* showed antifungal activity against strains of *C. albicans* ATCC 90028 (Sardi et al., 2017). Several *Candida* species can be found in the oral cavity of advanced cancer patients, where there is a prevalence of 36%-86% of mixed infections. The increased presence of species beyond *C. albicans* promotes a more complex clinical picture with direct consequences on the treatment plan (Monsen et al., 2023). The use of strains isolated from cancer patients is relevant for *in vitro* studies, given the predominance of oral candidiasis in this population. The fungicidal activity of the CEE against these strains is therefore promising for the development of new therapeutic strategies.

Terpenes and flavonoids may have their mechanism of action associated with the fungal cell wall of *Candida* spp. strains (Martínez et al. 2014; Nguyen et al., 2021). The MIC of the CEE of *E. luschnathiana*, in the presence of sorbitol, increased from 1.95 µg/mL to 3.90 µg/mL, while in the presence of exogenous ergosterol, these values remained unchanged. Hence, it is suggested a possible effect on the cell wall and not on the plasma membrane. Products that exert their effects on cell wall structures, absent in human cells, have an additional advantage since there is an estimate of lower adverse effects (Liu et al., 2020)

Candida spp. can form highly drug-tolerant biofilms in the human body, leading to unviable treatments. These biofilms are an important virulence factor and consist of a dynamic and structured community of various cell types (Barros et al., 2020). The anti-biofilm activity of the CEE of *E. luschnathiana* at low concentrations (3.90 µg/mL; 7.81 µg/mL; 19.5 µg/mL) demonstrates its potential as a possible alternative in the treatment of oral candidiasis.

The toxicity of natural products can originate from various factors, including their chemical constituents. Evaluating this potential is an essential step in advancing future clinical phases. Several tests can be used to determine the preliminary toxicological profile of natural extracts. Among the methods used are the use of human keratinocytes, which play a fundamental role in the oral epithelium, and alternative biological assays, such as those performed with *A. salina* (Alves et al., 2020, 2021; Hamidi et al., 2014). In the cytotoxicity assay conducted on non-tumorigenic human keratinocytes, the EEB (ethyl acetate extract) of *E. luschnathiana* demonstrated inhibition of less than 25% at concentrations below MICx20 (39 µg/mL) when compared to the control group (100% cell viability). At the highest concentration tested, MICx40 (78 µg/mL), the EEB showed slight cytotoxicity. Other studies have investigated the cytotoxicity on HaCat keratinocytes of extracts from the genus *Eugenia*, such as *E. dysenterica* DC.,

maintaining 80% cell viability at concentrations of up to 160 µg/mL (Moreira et al., 2017). These findings indicate a promising toxicological profile.

Toxicity tests on *A. salina* with the extract of *E. luschnathiana* showed promising results, with LC_{50} values above 2590 µg/mL, indicating a toxicity profile compatible with studies involving more complex animals, such as rodents (Araújo, 2018). In this study, we identified that the LC_{50} of the EEB from *E. luschnathiana* is 142.4 µg/mL. This concentration is approximately 70 times the concentration capable of inhibiting the growth of *Candida* spp. Employing alternative models enhances toxicity screening by providing robust scientific evidence, thereby substantially reducing costs and minimizing vertebrate animal sacrifice (Freires et al., 2017).

The EEB of *E. luschnathiana* represents a promising option for developing new therapeutic approaches in treating infections caused by *Candida* spp. Our results suggest that further investigations should be conducted to elucidate the mechanism of action, expand the toxicological characterization, including experimental models with rodents, as well as clinical trials to evaluate the safety and effectiveness of the product for the treatment of superficial fungal infections, including oral candidiasis.

5. Conclusion

The essential oil (EO) of *E. luschnathiana* is predominantly composed of sesquiterpenes, with (E)-Caryophyllene as the major constituent, and exhibited no effect on *Candida* spp. The crude ethanolic extract (CEE) of this plant is rich in flavonoids and terpenes, showing potent fungicidal activity against *Candida* species, possibly by targeting fungal cell wall. Additionally, the CEE promoted the inhibition of Candida biofilm. at low concentrations and a toxicity profile that is favorable for the survival of *A. salina*.

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