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Research Article

Eucalyptus spp: *Candida albicans* Antibiofilm Activity

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Abstract

The genus *Eucalyptus* comprises more than 900 species and subspecies, belonging to the family Myrtaceae. The essential oil obtained from the leaves is known to have antioxidant activity, anticancer, insecticide, antibacterial, antiviral and antifungal. The formation of biofilms by *Candida* spp. is a virulence factor providing an environment of persistence and survival for the cells. Faced with difficulties in the treatment of biofilms of *Candida* spp., Medicinal plants have been seen as an alternative agent for the development of new therapeutic drugs. Therefore, the present study evaluated the activity of the essential oils of *Eucalyptus citriodora* and *Eucalyptus globulus* against the formation and mature biofilm of *Candida albicans* (SC 5314), and their respective cell viabilities were obtained by XTT dye and spectrophotometer reading. Images of the biofilms treated with the oils were captured by Scanning Electron Microscopy (SEM). The oils presented antibiofilm action against the biofilm in the formation of *C. albicans*, which presented low metabolic activity up to the concentration 1 mg/mL with 7.2% biofilm cell viability for *E. citriodora* and 4 mg/mL with 10.7% viability for *E. globulus*; the mature biofilm showed reduced metabolic activity to 0.5 mg/mL for *E. citriodora* with 14.1% cell viability and 8 mg/mL for *E. globulus* with 9.9%. SEM analyzes of *C. albicans* biofilms showed decreased hyphae and deformities as roughness and wilting in the cells and hyphae present. The EO of the leaves of *Eucalyptus citriodora* and *Eucalyptus globulus* are biologically active in the formation and mature biofilms of *C. albicans*, decreasing the metabolic activity of both biofilms and SEM images have shown that OEs act on the morphology and structure of the biofilm of *C. albicans*, presenting roughness and decreased cell volume in yeast cells and hyphae, promoting hyphae decrease.

Keywords: *Biofilm; Eucalyptus spp; Essential Oil; Antifungal; Candida spp*

Abbreviations

CG: Gas Chromatography; DMSO: Dimethyl Sulfoxide; h: Hours; SEM: Scanning Electron Microscopy; mg: Milligrams; mL: Milliliters; nm: Nanometer; OEs: Essential Oil; PBS: Phosphate Buffered Saline; PS: Polystyrene; XTT: 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; YNB: Yeast Nitrogen Base; YPD: Yeast Peptone Dextrose

Introduction

The formation of biofilms by *Candida* spp. is a virulence factor providing an environment of persistence and survival for the cells; these arranged in biofilm exhibit distinct phenotypic properties of cells in the planktonic state, demonstrating greater resistance to antimicrobial agents. Biofilm formation is an important contributor to mortality rates associated with *Candida* spp. Infections. In this

sense, the biofilms of *Candida* spp. are clinically problematic because they are intrinsically resistant to antimicrobial agents [1-4]. The biofilms of *Candida albicans* have been shown to be highly resistant to antifungal class Azole, including the new drugs of this class, voriconazole and posaconazole. The cells in biofilm are up to 1000 times more resistant to fluconazole compared to planktonic cells; triazoles are 50% less effective in biofilm cells. Studies have shown that biofilms of *C. albicans* also showed resistance to amphotericin B [5].

Medicinal plants have now been considered a promising source for new therapeutic agents, due to the presence of phytochemicals that can lead to the development of new drugs. Most of the phytochemicals present in medicinal plants have had a positive impact on health and cancer prevention [6].

Members of the genus *Eucalyptus* belonging to the Myrtaceae family originate in Australia but have been naturalized on most continents. There are approximately 900 species and subspecies of *Eucalyptus* [7]. *Eucalyptus citriodora* is a species of the genus *Eucalyptus* belonging to the family Myrtaceae, is a vegetable cultivated for several purposes, being used as an insect repellent, controlling the microbiological activity of fungi and bacteria and activating the latent defense mechanisms through its essential oil [8]. The essential oil of the leaves of *Eucalyptus globulus* consists of several volatile compounds in great concentrations, accumulate abundantly throughout the parenchyma of leaves and bark of the plant. It has been used worldwide as an antiseptic, and also for treating coughs, colds, sore throats and other infections; antimicrobial and antiviral activity; and is commonly used in the cosmetic industry [9-11].

In the face of increased cases of resistance to commercial antifungal drugs, studies that seek new drugs, having medicinal plants as a promising source or coadjuvants for the treatment of *Candida* spp biofilms have been a research alternative. The objective of this study was to evaluate the antifungal activity of *Eucalyptus* spp essential oils against formation and mature biofilms of *Candida albicans* (SC 5314), through cell viability obtained by XTT dye by reading in Spectrophotometer and biofilm images treated with the captured oils by Scanning Electron Microscopy (SEM).

Material and Methods

Essential oils

The essential oils of *Eucalyptus citriodora* and *Eucalyptus globulus* were purchased commercially from TERRA FLOR (Street 09, block 10, Lot 01 - Sector Planalto, Alto Paraíso de Goiás - GO, 73770-000).

- *Eucalyptus citriodora* (Lot: C064/16)
- *Eucalyptus globulus* (Lot: L173)

Dilution of essential oils and antifungals

The essential oils of *Eucalyptus citriodora* and *Eucalyptus globulus* were diluted in Tween 80 solution (0.025%) and DMSO solution (0.5%), YNB (Yeast Nitrogen Base) culture medium.

Effect of essential oils on Candida albicans biofilm

The essential oils of *E. citriodora* and *E. globulus* were tested against *C. albicans* biofilms (MYA-2876).

Inoculum adjustment

The culture was incubated overnight in YPD at 30°C under 180 rpm shaking. A 7 mL aliquot of the inoculum was centrifuged at 3,000 rpm for 5 minutes and washed 2x with PBS for removal of the culture medium. After the last centrifugation at 3,000 rpm for 5 min the supernatant was discarded and the pellet was resuspended in 7 mL of YNB. From the resulting cell suspension a 1:100 dilution was prepared for cell counting in Neubauer chamber by light microscopy (400x magnification). After counting and performing the calculation the inoculum was adjusted to 1.0 x 10^6 cells/mL in YNB [12].

For the biofilm in formation, 100 µl of inoculum was deposited in the 96-well sterile microplate type PS (background U), which was incubated for 90 minutes under agitation (75 rpm at 37°C) in a microplate incubator. The plate was then washed 3x with PBS and 100 µl

of diluted essential oil was added at each concentration tested (16 mg/mL to 0.03125 mg/mL for *E. citriodora* and 64 mg/mL to 0.125 mg/mL for *E. globulus*). The plate containing the inoculum and oil was incubated for 24h at 37°C in an aerobiose oven [13].

Biofilm cell viability analysis

For biofilm cell viability analysis, the plates were previously washed 3x with PBS and then stained with 80 µl of XTT [2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl) -2H- tetrazolium-5-carboxanilide] for 2 hours. The biofilm was then measured (A490 nm) on an ELISA microplate reader (Versa MAX, molecular Devices, USA). Absorbance values were subtracted from the absorbance values of the control in order to evaluate the amount of viable cells in the biofilm [12].

Biofilm analysis by scanning electron microscope (SEM)

For the analysis of the effect of the essential oils on the biofilm of *Candida albicans* (MYA-2876) by MEV, the inoculum was also adjusted to that previously described in the biofilm assay, resulting in a concentration of 1 x 10^6 UFC/mL. From the final cell suspension, *C. albicans* biofilm was produced on culture slides (BD Falcon). For the biofilm assay in formation, the cells were incubated with shaking 75 rpm on a microplate shaker at 37°C for 90 minutes and then added the essential oils at concentrations of 0.5 mg/mL, 1 mg/mL and 2 mg/mL for the essential oil of *E. citriodora* and at concentrations of 4 mg/mL, 8 mg/mL and 16 mg/mL for *E. globulus* essential oil and incubated in an aerobic oven at 37°C for 24 hours. For the mature biofilm, the cell suspension was incubated at 37°C for 24 hours and after the incubation period, the essential oils were added at the concentrations of 0.5 mg/mL, 1 mg/mL and 2 mg/mL for *E. citriodora* essential oil and at concentrations of 4 mg/mL, 8 mg/mL and 16 mg/mL for *E. globulus* essential oil and incubated again for another 24 hours. After the incubation period, the culture medium and glutaraldehyde fixed biofilm (2.0%) were removed for 30 minutes, followed by drying at room temperature. Specimens were dehydrated in baths with increasing concentrations of ethanol (50%, 70%, 90% and 100%) for 10 minutes. Specimens were dried, metallized and stored in desiccator for subsequent observation in MEV (JEOL, JSM 5600LV, Japan). Only yeast samples were added to the control.

Statistical analysis

For the comparative evaluations we used the statistical analysis ANOVA, *Dunnett* variation, bilateral ($p < 0.05$), (Biostat 5.0 program).

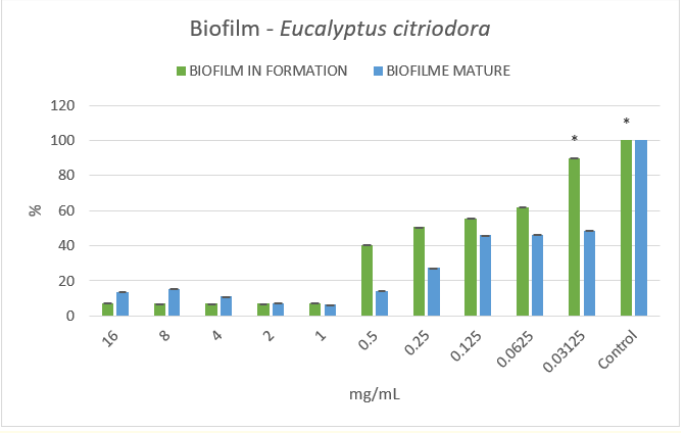
Results

Analysis of cell viability in biofilms of *Candida albicans*

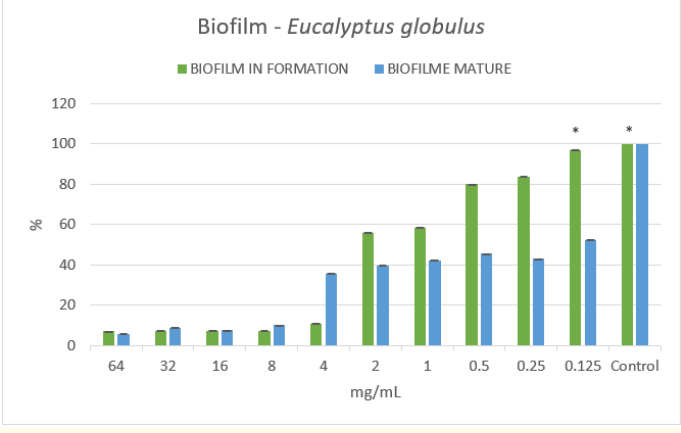
The essential oils of *E. citriodora* and *E. globulus* were tested in different concentrations against the formation and mature biofilm of *Candida albicans* (MYA-2876), in order to evaluate the effect of the oils by the biofilm cell viability.

Compared to the control group (untreated), the *C. albicans* biofilm in *E. citriodora* treated formation had low metabolic activity up to 1 mg/mL concentration with 7.2% of biofilm cell viability and treatment with *E. globulus* at 4 mg/mL concentration presented 10.7% viability. The increase of the metabolic activity of the biofilm in formation is observed from the 0.5 mg/mL concentration for *E. citriodora* and 2 mg/mL for *E. globulus* ($p < 0.05$); where there was increase of viable cells in the biofilm 40.5% and 56% respectively. The concentration of 0.0312 mg/mL of *E. citriodora* and 0.125 mg/mL of *E. globulus* did not demonstrate statistical differences compared to control (Graph 1 and 2).

Compared to the control group (untreated), the biofilm of mature *C. albicans* showed reduced metabolic activity up to the concentration of 0.5 mg/mL for *E. citriodora* essential oil with 14.1% cell viability and 8 mg/mL of *E. globulus* with 9.9% cell viability. The mature biofilm had increased cell viability from 0.25 mg/mL for *E. citriodora* and 4 mg/mL for *E. globulus*, 27.3% and 35.4%, respectively. All tested concentrations of *E. citriodora* and *E. globulus* had statistical difference when compared to control ($p < 0.05$) (Graph 1 and 2).



Graph 1: *Cell viability (%) of the biofilm in formation and mature treated with E. citriodora.*
**: Statistical significance p < 0.05 ANOVA test 1 criterion, Dunnett variation.*



Graph 2: *Cell viability (%) of the biofilm in formation and mature treated with E. globulus.*
**: Statistical significance p < 0.05 ANOVA test 1 criterion, Dunnett variation.*

Scanning electron microscopy (SEM)

Biofilm in formation treated with *Eucalyptus citriodora* and *Eucalyptus globulus*

The scanning electron microscopy analyzes showed control cells with regular surface and pseudohifas in considerable amounts and with homogeneous surface; the control shows the *C. albicans* cells with their intact morphology (Figure 1 and 2). When treated with *E. citriodora* at a concentration of 0.5 mg/mL, a significant decrease in hyphae is observed and some cells with deformities can be detected losing their original form (Figure 3 and 4), at a concentration of 1 mg/mL, the decrease in hyphae and deformities as well as roughness in the cells and hyphae present (Figure 5 and 6) and in the highest concentration tested (2 mg/mL), a large variation in cell morphology when compared to the control, observing a decrease in the cellular volume (Figure 7 and 8). When treated with the 4 mg/mL *E. globulus* essential oil, only a small decrease in hyphae was observed when compared to the control (Figure 9 and 10), and at 8 mg/mL, it was also observed (Figure 11 and 12), in the highest concentration (16 mg/mL) the phenotype was repeated (Figure 13 and 14) and there were no significant morphological changes between the tested concentrations.

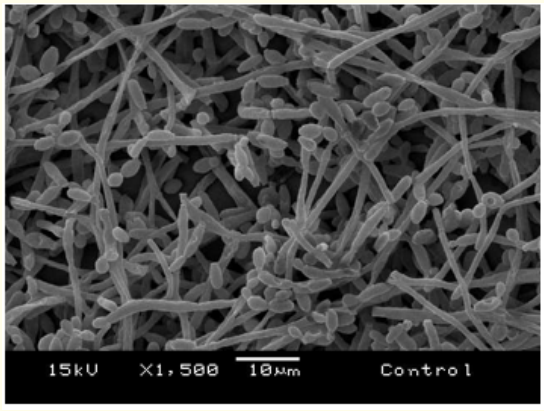


Figure 1: *Control of biofilm formation of C. albicans. 1.500x magnification.*

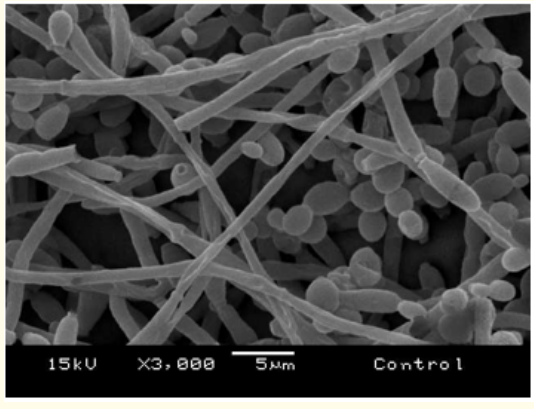


Figure 2: *Control of biofilm formation of C. albicans. 3.000x magnification.*

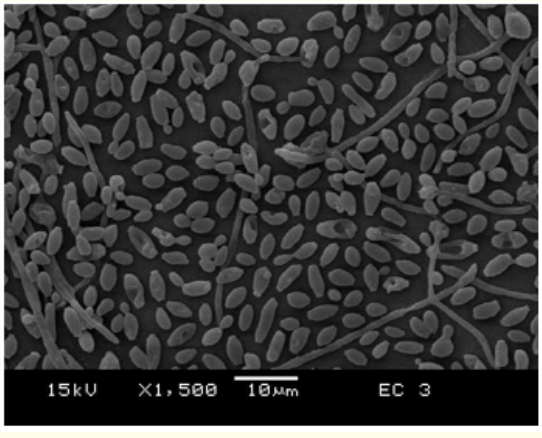


Figure 3: *Biofilm in formation of C. albicans, exposed to E. citriodora oil (0.5 mg/mL). 1,500x magnification.*

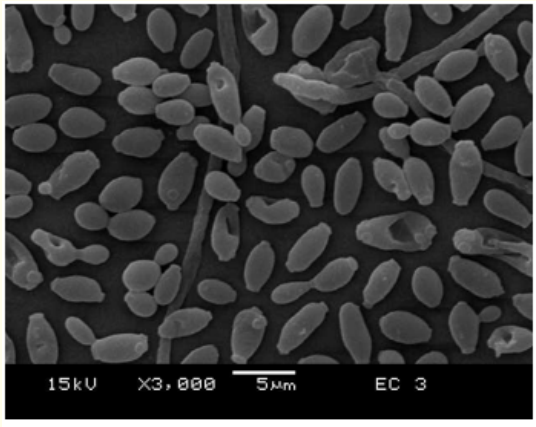


Figure 4: *Biofilm in formation of C. albicans, exposed to E. citriodora oil (0.5 mg/mL). 3,000x magnification.*

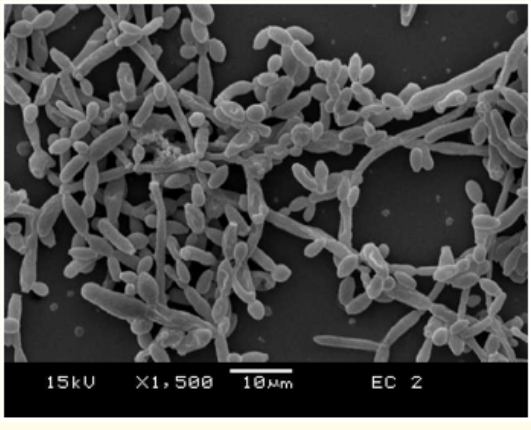


Figure 5: *Biofilm in formation of C. albicans, exposed to E. citriodora oil (1 mg/mL). 1,500x magnification.*

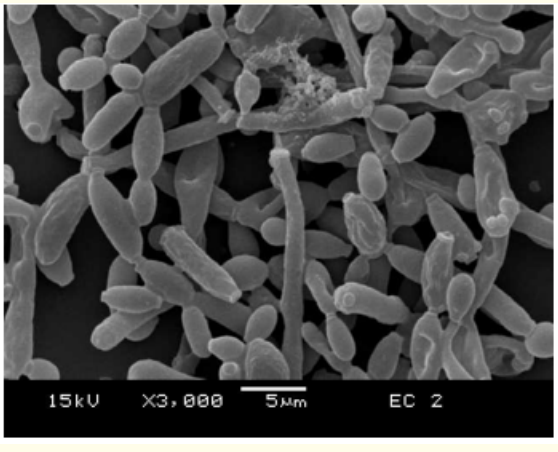


Figure 6: *Biofilm in formation of C. albicans, exposed to E. citriodora oil (1 mg/mL). 3,000x magnification.*

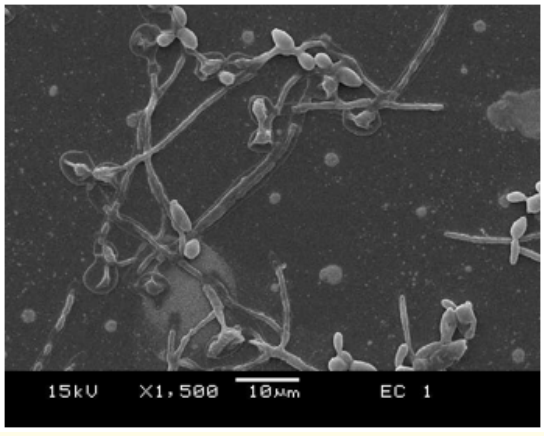


Figure 7: *Biofilm in formation of C. albicans, exposed to E. citriodora oil (2 mg/mL). 1,500x magnification.*

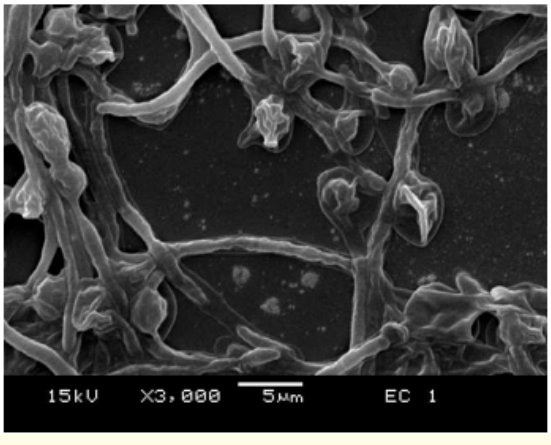


Figure 8: *Biofilm in formation of C. albicans, exposed to E. citriodora oil (2 mg/mL). 3,000x magnification.*

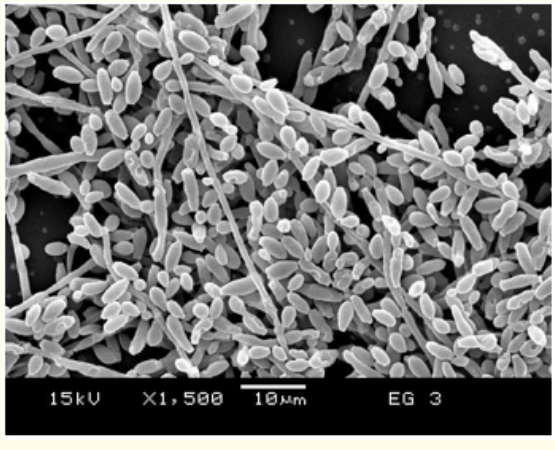


Figure 9: *Biofilm in formation of C. albicans, exposed to E. globulus oil (4 mg/mL). 1,500x magnification.*

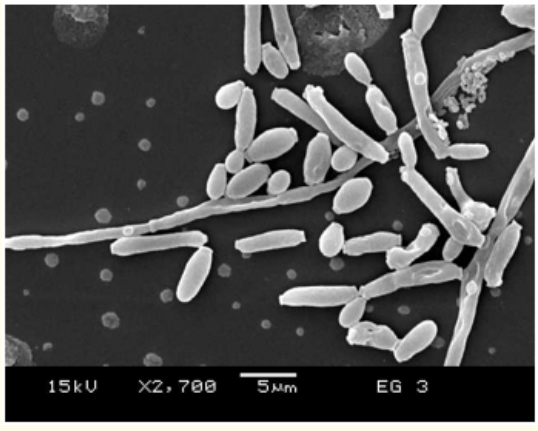


Figure 10: *Biofilm in formation of C. albicans, exposed to E. globulus oil (4 mg/mL). 3,000x magnification.*

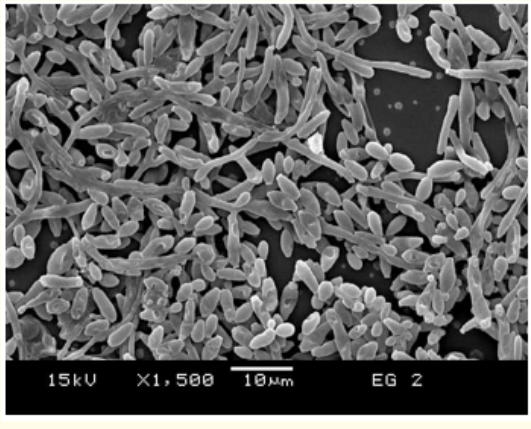


Figure 11: *Biofilm in formation of C. albicans, exposed to E. globulus oil (8 mg/mL). 1,500x magnification.*

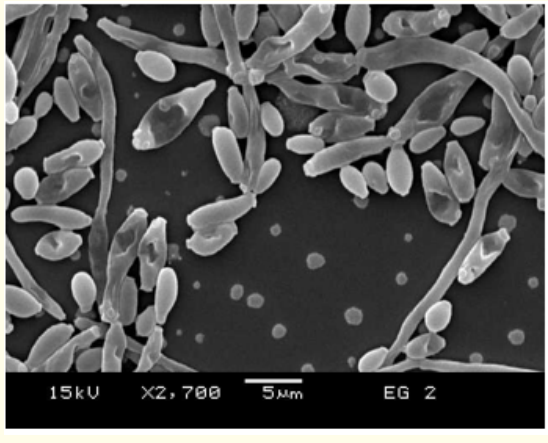


Figure 12: *Biofilm in formation of C. albicans, exposed to E. globulus oil (8 mg/mL). 3,000x magnification.*

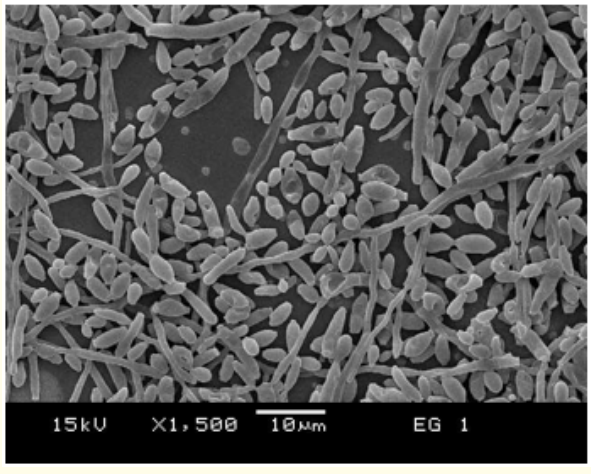


Figure 13: *Biofilm in formation of C. albicans, exposed to E. globulus oil (16 mg/mL). 1,500x magnification.*

Figure 14: *Biofilm in formation of C. albicans, exposed to E. globulus oil (16 mg/mL). 3,000x magnification.*

Mature biofilm treated with *Eucalyptus citriodora* and *Eucalyptus globulus*

In the scanning electron microscopy analyzes, in the control of the mature biofilm, cells with a regular surface and hyphae with a homogeneous surface were observed, as shown in figure 15 and 16. In the images of treatment with *E. citriodora* at the concentration of 0.5 mg/mL, deformities were observed in the hyphae and cells, such as decrease in cell volume and roughness (Figure 17 and 18), at a concentration of 1 mg/mL, the deformities presented in the previous concentration are more pronounced (Figure 19 and 20), and at the highest concentration of 2 mg/mL, a great variation in the cell morphology can be observed when compared to the control, detecting the same cell phenomenon and roughness, and the production of viscous material involving the cells can also be observed (Figure 21 and 22). When treated with the essential oil of *E. globulus* at the concentration of 4 mg/mL, only roughness was observed in the hyphae (Figure 23 and 24), already at the concentration of 8 mg/mL, more pronounced roughnesses were observed in the hyphae and cells and also the rupture of the hypha (Figure 25 and 26), at the highest concentration 16 mg/mL, the phenomenon was repeated (Figure 27 and 28).

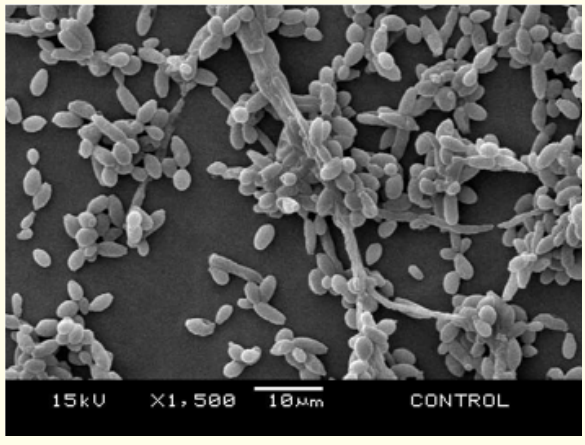


Figure 15: *Control of the mature biofilm of C. albicans. 1,500x magnification.*

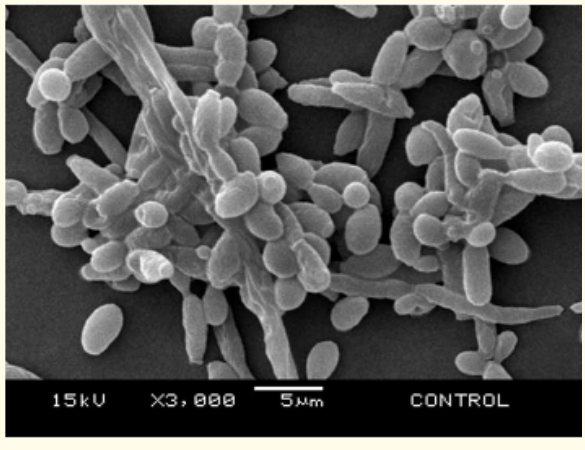


Figure 16: *Control of the mature biofilm of C. albicans. 3,000x magnification.*

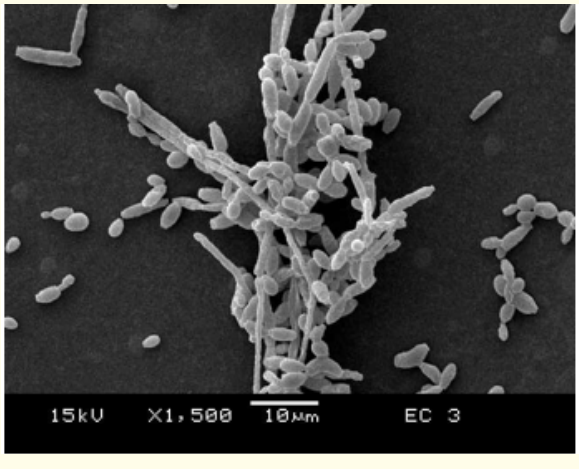


Figure 17: *Mature biofilm of C. albicans, exposed to E. citriodora oil (0.5 mg/mL). 1,500x magnification.*

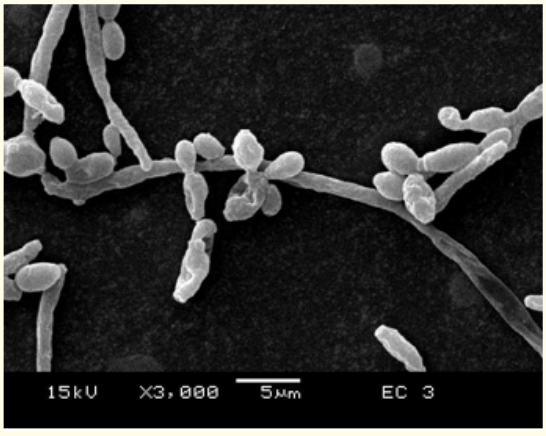


Figure 18: *Mature biofilm of C. albicans, exposed to E. citriodora oil (0.5 mg/mL). 3,000x magnification.*

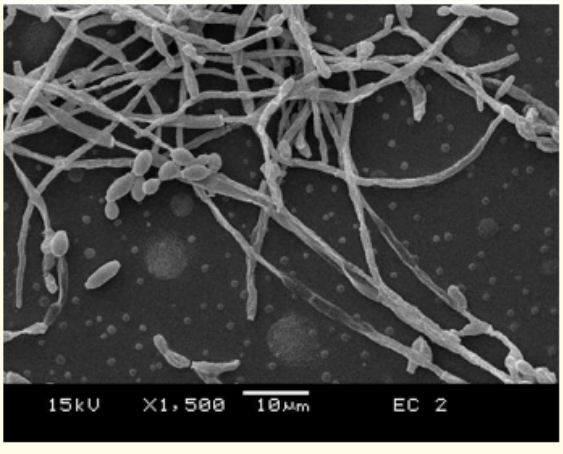


Figure 19: *Mature biofilm of C. albicans, exposed to E. citriodora oil (1 mg/mL). 1,500x magnification.*

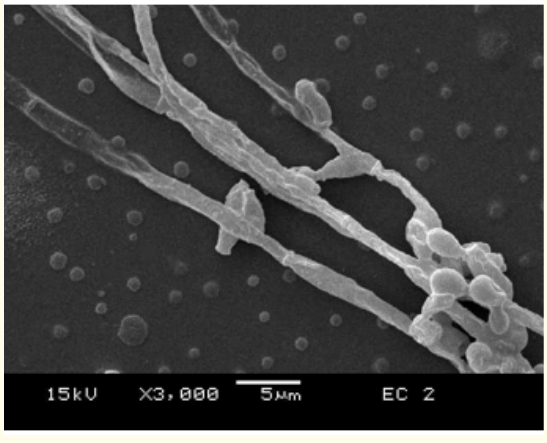


Figure 20: *Mature biofilm of C. albicans, exposed to E. citriodora oil (1 mg/mL). 3,000x magnification.*

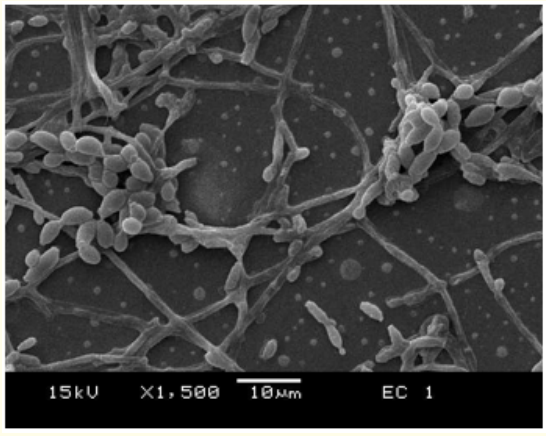


Figure 21: *Mature biofilm of C. albicans, exposed to E. citriodora oil (2 mg/mL). 1,500x magnification.*

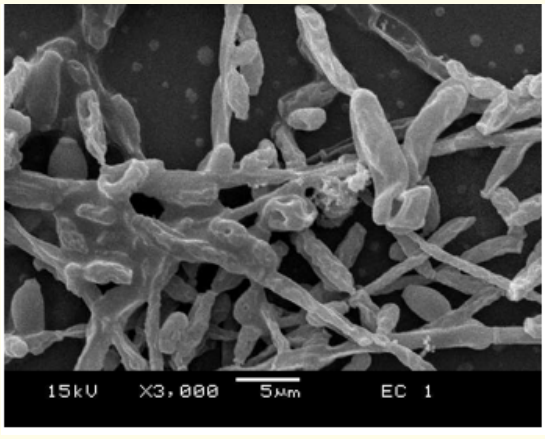


Figure 22: *Mature biofilm of C. albicans, exposed to E. citriodora oil (2 mg/mL). 3,000x magnification.*

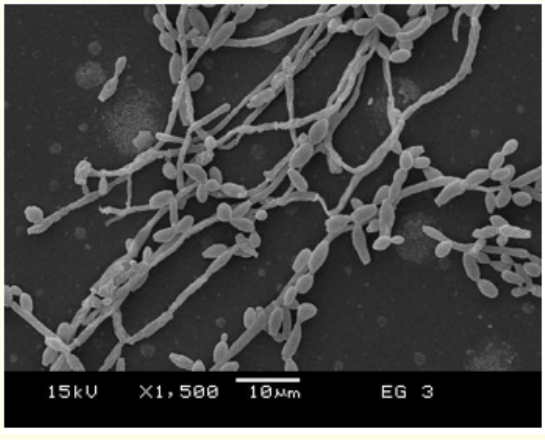


Figure 23: *Mature biofilm of C. albicans, exposed to E. globulus oil (4 mg/mL). 1,500x magnification.*

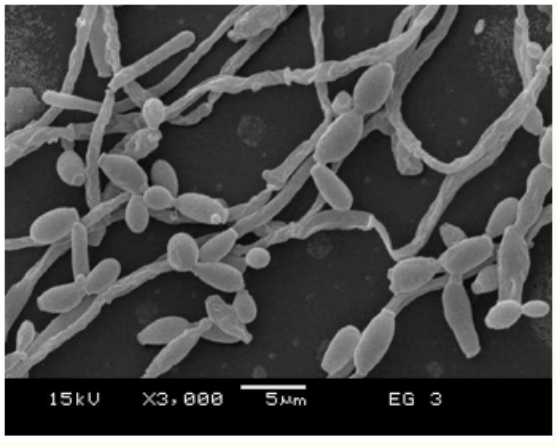


Figure 24: *Mature biofilm of C. albicans, exposed to E. globulus oil (4 mg/mL). 3,000x magnification.*

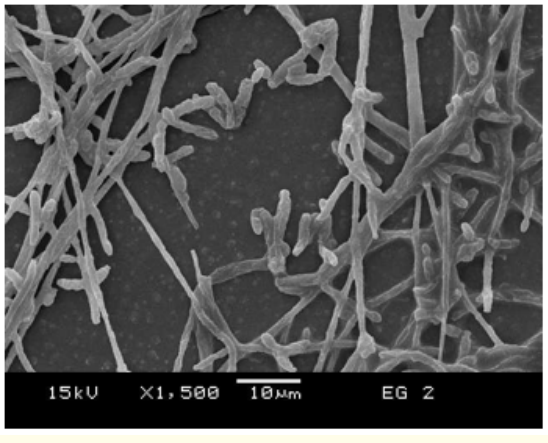


Figure 25: *Mature biofilm of C. albicans, exposed to E. globulus oil (8 mg/ mL). 1,500x magnification.*

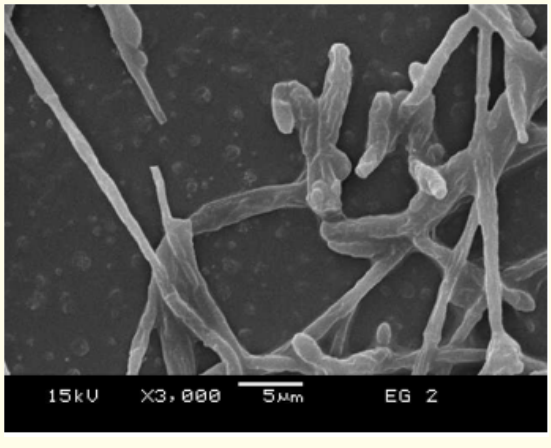


Figure 26: *Mature biofilm of C. albicans, exposed to E. globulus oil (8 mg/mL). 3,000x magnification.*

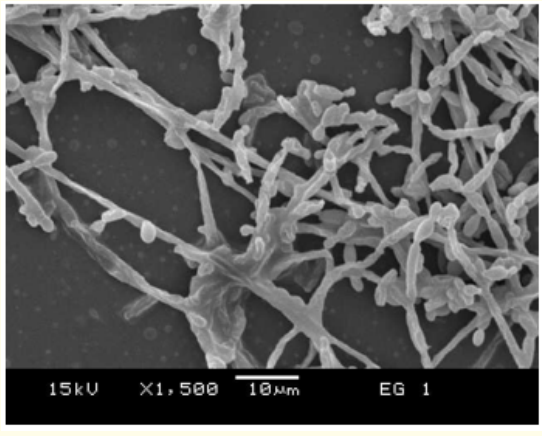


Figure 27: *Mature biofilm of C. albicans, exposed to E. globulus oil (16 mg/mL). 1,500x magnification.*

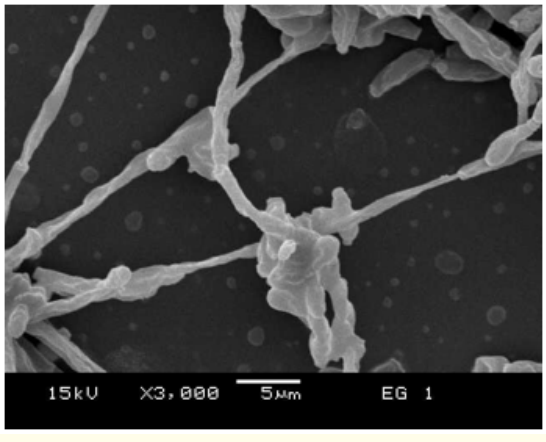


Figure 28: *Mature biofilm of C. albicans, exposed to E. globulus oil (16 mg/mL). 3,000x magnification.*

Discussion

In recent years, treatment against candidiasis has become a concern due to toxicity and the development of resistance to commercial antifungal agents [14]. In this sense, the search for alternatives of substances that have the property to act against microorganisms without changing the conditions of the host, has led researchers to look for these substances in plants, since they have demonstrated a healing power, even if empirically years. In the last decades, however, it has been possible to validate these properties scientifically, through numerous researches.

Eucalyptus spp. It has long been used in folk medicine due to its antiseptic action and applied in the treatment of infections of the upper respiratory tract. The essential oils of the species *E. citriodora* and *E. globulus* demonstrate antimicrobial, antioxidant and anti-inflammatory action [15].

Biofilms are defined with a microbial community attached to any surface, where the cells of this community secrete and are surrounded by a matrix of extracellular polymeric substances, exhibiting different phenotypic characteristics of planktonic cells [16]. The formation of this structure is one of the most important virulence factors of microorganisms, especially *Candida*, due to its resistance to the treatment

of several antifungal agents and affecting the immunological responses of the host. It is believed that the order of events of *Candida* biofilm development "*in vitro*" is initiated by the adhesion of yeast cells to a substrate, cell proliferation and the formation of filamentous projections, such as hyphae and pseudohifas, interspersed with matrix accumulation extracellular [17]. *Candida* biofilms are colonizers of various medical devices, such as central venous catheters, heart valves, and also oral prostheses. Infections caused by biofilm-forming *Candida*'s are often associated with higher mortality rates [18].

The evaluation of the action of the essential oils of the genus *Eucalyptus* on the structure of the forming biofilm and the mature biofilm of *Candida albicans* (MYA-2876) were carried out in this work. These essential oils proved to be efficient against the progression of the biofilm in formation, inhibiting by approximately 50% the growth of the biofilm in the concentration 0.25 mg/mL for *E. citriodora* and 2 mg/mL for *E. globulus.* In the initial concentrations tested for *E. citriodora* (16 mg/mL) and *E. globulus* (64 mg/mL) the inhibition of biofilm growth was approximately 92% for both compared to the control group. These data suggest the potential action of these substances, preventing the normal process of formation of *Candida albicans* biofilm when tested with the essential oils of these species.

The essential oils were also efficient in the disorganization of the mature biofilm, compromising about 50% of the biofilm in lower concentrations when compared to the biofilm in formation, 0.125 mg/mL for *E. citriodora* and 0.5 mg/mL for *E. globulus*. At the initial concentrations tested for *E. citriodora* (16 mg/mL) and *E. globulus* (64 mg/mL) the disorganization of the mature biofilm was approximately 86% for *E. citriodora* and 94% for *E. globulus* when compared to the control group. The results obtained with the tested oils showed satisfactory results regarding the deconstruction of the mature biofilm, suggesting its use as an antimicrobial alternative in the action against this structure, either as main agent or as adjuvants.

The antibiofilm activity of the genus *Eucalyptus* was also observed by Mathur., *et al.* [19] against the urinary tract pathogen *Proteus mirabilis*, presenting approximately 90% inhibition of biofilm formation. It is suggested that 1,8-cineol (44.2%) and α-pinene (13.6%) present in the essential oils of *Eucalyptus* are the main components that are affecting the normal process of biofilm formation of *P. mirabilis*. A more accurate knowledge about the profile of the bioactive substances present in these species could contribute greatly to the selection of these species in relation to their action against microorganisms.

In order to investigate possible alterations in the biofilm architecture after treatment with the essential oils, the samples were visually analyzed by Scanning Electron Microscopy (SEM). For *E. citriodora* oil, the concentrations were 0.5 mg/mL, 1 mg/mL and 2 mg/mL; for *E. globulus* oil the concentrations 4 mg/mL, 8 mg/mL and 16 mg/mL were used. At the highest concentrations of the essential oils deformities were observed in the hyphae and in the cells as roughness and decrease of the cellular volume, compromising the structure of the biofilm due to the reduction of the hyphae, which suggests an effective action in the destructuring and in the mechanisms of development of this structure.

The mechanisms of *Candida* antibiofilm activity are not very clear, but the main agents are the action of terpenoids by destabilizing the cell membrane and modulating membrane-associated functions, such as permeability, cell signaling (*quorum sensing*) [20]. Anibal., *et al.* [21] investigating other plant species such as *Mentha* spp., Suggest that the presence of flavonoids and terpenes present in the essential oils of these medicinal plants are responsible for the toxicity to fungal cells due to a certain degree of lipophilicity and the interactions with the membrane constituents and their arrangement.

Since the main constituents of the essential oils of *Eucalyptus* species contain terpenes, 1-8-Cineol (75.8%), α-Pinene (7.4%), limonene (6.4%) in *E. globulus*; and citronellal (69.77%), citronellol (10.63%) in *E. citriodora* this may be indicative that the essential oils of *Eucalyptus* may have antibiofilm activity, promoting morphological and structural alterations in the biofilm of *Candida albicans* as demonstrated in this research [22,23].

In general, the results presented in this research corroborate with other studies in this line of research, demonstrating the antimicrobial effects of the essential oils of the genus *Eucalyptus.* Future studies involving more species in terms of knowledge of the profiles of these bioactive substances, other host cell cytotoxicity assays and *in vivo* model in *Galleria mellonella* larvae, as well as the action on a wide range of microorganisms, especially fungi, will be able to amplify and more appropriately base its use as an effective alternative of antimicrobial action, at least as coadjuvants, according to data available in the literature.

Conclusion

- EO of leaves of *Eucalyptus citriodora* and *Eucalyptus globulus* are biologically active in the formation and mature biofilms of *C. albicans*, reducing the metabolic activity of both biofilms.
- The EO of the leaves of *Eucalyptus citriodora* and *Eucalyptus globulus* demonstrated by the MEV, act on the morphology and structure of the *C. albicans* biofilm, presenting roughness and decrease of the cellular volume in yeast cells and hyphae, promoting hyphae decrease.

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