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# Susceptibility Profile of the Essential Oil from *E. muticus* against *C. albicans* Biofilm

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## Abstract

The increase in fungal infections associated with the selection of resistant fungi has led researchers to look for new bioactive substances or components as therapeutic alternatives. Infections caused by *Candida* species may involve the formation of biofilms resulting in superficial and systemic infections. Due to the resistance of *Candida* species to conventional drugs added to the increase of infections, research with medicinal plants has been an alternative in the discovery of new biocomponents for several applications in popular medicine. The use of essential oils extracted from different types of plants, has been showed antiseptic, antifungal and antibacterial actions. Essential oils with diversified potential have been an alternative to clinical studies in the area of Health. *Elionurus muticus* is popularly used in the treatment of various diseases, including those caused by fungi. Known as Brazilian lemongrass, it presents antioxidant activity related to the content of its phenolic compounds, besides containing antiseptic properties. The essential oil of *Elionurus muticus* may contain limonene, linanol, citronellal, geraniol, among others. Studies aiming to increase pharmacological knowledge about potentially medicinal plants are important in order to improve the understanding of the substances and biocomponents present in extracts and essential oils with emphasis on their use as phytotherapics.

***Keywords:*** *Elionurus muticus; Candida spp; Biofilms; Essential Oil; Antifungal Action*

## Introduction

Globally, every year, 300 million people of all ages suffer from severe fungal infections and more than 1.5 million of these people die as result of such diseases. According to the latest data estimates by the World Health Organization, this mortality rate now exceeds up on other serious infectious diseases such as malaria (445,000 deaths in 2016), hepatitis B (887,000 deaths in 2015) and AIDS (1 million deaths in 2016). Invasive candidiasis affects more than 700,000 people worldwide each year and, with a mortality rate unacceptably greater than 40%, is the cause of more than 280,000 deaths [1]. The incidence of these infections has increased in recent years due to the widespread use of broad-spectrum antimicrobials and the growing number of HIV-infected and immunocompromised individuals [2].

*Candida* species are considered commensal microorganisms in most healthy individuals, but may become pathogenic when host defenses are breached or under microbial dysbiosis [3]. Although the most frequently isolated species are *C. albicans*, in the last decade the number of infections caused by other *Candida* species have increased significantly, such as candidiases caused by *C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei* [4,5]. Candidiasis is a broad term that refers to mucosal and organ infections caused by fungi of the genus *Candida* [5].

The severity of these infections are related to the pathogenicity of the species involved. The pathogenicity of *Candida* species depends on several factors of virulence, among them, ability to adhere to host cells, secretion of degradative enzymes, pleomorphism and the ability to form biofilm [6,7].

One of the virulence factors that contribute to infections of *Candida* species is the formation of biofilms. In this structure, the fungal cells are usually incorporated into an autoproduced extracellular matrix containing proteins, carbohydrates, phosphorus and hexosamines. The advantages of forming a biofilm are: protection against *Candida* cells against drugs and other microorganisms; removal of harmful metabolic products; favoring the acquisition and processing of nutrients; facilitating communication through the secretion of signaling molecules in a population (quorum sensing); production of secreted proteases for nutrient acquisition and degradation of host proteins [6,8] and can occur on biotic and abiotic surfaces such as medical devices, prostheses, defibrillators, urinary catheters, vascular and cardiac devices [9].

The biofilm matrix of *C. albicans* is complex and has polysaccharide constituents: α-mannan, β-1,6 glucan and β-1,3-glucan. At least five species of *Candida (C. albicans, C. glabrata, Candida guilliermondii, C. parapsilosis and C. tropicalis)* are capable to forming biofilms and have also been investigated *in vitro*. Among these, *C. albicans* and *C. parapsilosis* showed the greatest capacity to form biofilms [6].

The therapeutic options for the treatment of these infections caused by *Candida* are mainly the antifungals of the type azoles, polienes and echinocandins. The clinical use of these agents has been limited, due to the increased resistance of *Candida* species to these drugs, contributing to the increase of morbidity and mortality rates [10].

Faced with these facts, research with medicinal plants has been an alternative in the discovery of new biocomponents for various applications in folk medicine. The use of essential oils extracted from several types of plants, presented several indications, among them antiseptic, antifungal and antibacterial actions [11].

In this context, the genus *Elionurus* is popularly used in the treatment of various pathogens. This genus is found in Africa, Asia, North America and South America. It has complex taxonomy with great variability, but still little known, another taxonomic difficulty is related to synonymies. The synonyms of *E. muticus* include the species *E. rostratus, E. latiflorus* and *E. adustrus* [12].

*Elionurus muticus* is a grass of the Brazilian pampas known as Brazilian lemongrass. This has aroused considerable interest in the phytochemical industry. It presents antioxidant activity related to the content of its phenolic compounds. It is also notable for its antiseptic properties. It produces essential oil in all its extension: in the aerial part, leaves and inflorescences, as in the roots. However, both the composition and the yield are variable according to the region of cultivation and the seasonality. The essential oil of *Elionurus muticus* may contain alpha-pinene, myrcene, limonene, eucalyptol, cis-beta-ocimene, linanol, cis-photocythral, trans-photocylthral, citronellal, neral, geraniol, geranial, bornyl acetate, alpha-ilangene, geranyl acetate, beta-caryophyllene, ar-curcumene, zingiberene, elemol, trans-nerolidol, caryophyllene oxide, 10-epi-gamma-eudesmol and isospathulenol [13].

The antimicrobial activity of essential oils, in general, may be related to the set of substances in their composition, and not only to each of the major compounds. Therefore, both the chemical composition and the antimicrobial action may vary in the same plant due to factors related to biology (genetics, nutrition and development phase), besides other factors (local, climatic conditions and soil type) [14].

In this line of research, the antimicrobial activity of the essential oil of *Elionurus muticus* has been demonstrated in several articles against *Candida* species [15,16], *Escherichia coli, Staphylococcus aureus* [17], *Penicillium expansum, Botrytis cinera, Botrytis allii* [13], coagulase-negative *Staphylococcus, Staphylococcus pseudintermedius, Streptococcus uberis* and *Pseudomonas aeruginosa* [18,19].

Medicinal plants may be considered toxic causing various reactions, as skin and mucosal allergies, cardiovascular, respiratory, metabolic, gastrointestinal and neurological disorders, and in some cases even death. The factors that influence toxicity are concerned to the chemical composition, the used dose and the form of use [20]. There are several *in vivo* and *in vitro* experimental models used to assess the

toxicity of the substances. Generally toxicity studies are initiated with *in vitro* experimental models and then with experimental models *in vivo. In vitro* cell sorting provides a cost-effective method. Cell culture experiments are suitable for developing mechanistic models for reporting toxicity of the compounds. Epithelial cells are in direct contact with substances from the external environment. Thus, the result of cytotoxic effects on a human derived cell line (Hacat) may be useful for a better understanding of toxic mechanisms and potential risks to human health [21].

In addition to *in vitro* assays using cell culture, there has been a growing interest in using insects as alternative models replacing conventional animal models. *Galleria mellonella* is a lepidopteran (*Pyralidae)* that provides important advantages as a study model. *G. mellonella* larvae have a low acquisition cost and results can be obtained within 48 hours and a large number of insects can be inoculated in a short period of time [22].

Studies aiming to increase pharmacological knowledge about potentially medicinal plants are important in order to improve the understanding of the substances and biocomponents present in extracts and essential oils with emphasis on their use as phytotherapics. Therefore, the present work aimed to evaluate the antifungal and cytotoxic properties of *Elionurus muticus*, determining its phytochemical composition by Gas Chromatography in apparatus with Mass Spectrometry (GC/MS). Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (CFM) were assessed visually according to CLSI [23]. Biofilm of *Candida albicans* was quantified by XTT and evaluated by Scanning Electron Microscopy (SEM). Cytotoxic activity was quantified by sulforhodamine B in Hacat cells and by the survival rate of *Galleria mellonella* larvae.

## Material and Methods

The essential oil of *Elionurus muticus* (Lot: 002) was purchased commercially from the company Harmonia Natural, Canelinha - SC, Brazil.

### Gas chromatographic (GC) and mass spectrometry (GC-MS) analyses

The identification of volatile constituents from *Elionurus muticus* essential oil was performed using a Hewlett-Packard 5890 Series II gas chromatograph, equipped with a HP-5971 mass selective detector and capillary column HP-5 (25 m × 0.2 mm × 0.33m diameter). GC and GC-MS were performed using split/splitless injection, with injector set at 220°C, detector set at 280°C, column set at 60°C, with heating ramp of 3°C $min^{-1}$ and final temperature of 240°C for 7 minutes and the FID detector set up at 250°C. Helium was used as the carrier gas at 1 ml $min^{-1.}$

This assay was performed at Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas, Paulínia, São Paulo, Brazil.

### Microorganisms

Reference strains of *Candida* spp., were: *C. albicans* (ATCC 90028, ATCC MYA-2876 and CBS 562), *C. guilliermondii* (CBS 566), *C. krusei* (CBS 573), *C. utilis* (CBS 5609), *C. rugosa* (IZ 12), *C. lusitaniae* (IZ 06), *C. glabrata* (IZ 07), *C. dubliniensis* (CBS 7987), *C. parapsilosis* (CBS 604) and *C. tropicalis* (CBS 94) (CBS - Centraalbureau voor Schimmelcultures, Dutch collection; IZ: Instituto Zimotécnico, Bank of Superior School of Agriculture "Luiz de Queiroz", ESALQ/USP, Piracicaba, São Paulo, Brazil).

### Planktonic anti-*candida* assay

The essential oil of *Elionurus muticus* was diluted in Tween 80 solution (0.05%) added of culture medium and tested for antimicrobial activity against planktonic anti-*Candida* spp. The yeast was grown overnight at 37°C in Sabouraud Dextrose Agar (Merck®) plates, and the inoculum for the assays was prepared by diluting scraped cell mass in 0.9% NaCl solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometric reading at 530 nm. Cell suspensions were finally diluted to $10^{-4}$ UFC/ml for use in assays. Minimum inhibitory concentration (MIC) tests were carried out according to M27-A3 protocol [23], using a test plate (96 wells). The stock solutions of the oil

were diluted and transferred into the first well, and serial dilutions were performed in sequence where concentrations in the range of 2.000 to 7.8 µg/mL were obtained. Fluconazole and amphotericin B (Sigma) were used as reference antimycotic control in the range of 64 to 0,5 µm/L, and 16 to 0,12 µm/L, respectively. The yeast inoculum was added to all wells and the plates were incubated at 37°C for 24h. MIC was defined as the lowest concentration of oil that inhibited visible growth.

After determining the MIC, Minimal Fungicide Concentration (CFM) was determined by transferring 10 µL of each microtiter well from the MIC to an SDA (HIMEDIA®) plate which was incubated at 37°C for 24 hours, according to the protocol with modifications of Gullo., *et al*. [24]. CFM was determined as the lowest concentration of essential oil that did not show colony growth.

The interpretation of the essential oil susceptibility profile data was determined according to Sardi., *et al*. [25]. Therefore, it was established through the CFM/MIC ratio, where values less than 4 exhibit a fungicidal profile and values greater or equal to 4 exhibit a fungistatic profile.

### Test of essential oil in *Candida albicans* biofilms

The essential oil of *Elionurus muticus* was tested in *C. albicans* biofilm (ATCC 90028 and MYA-2876) in formation (2 hours) as described by Silva., *et al*. [26] with adaptations and mature biofilm (24 hours) as described by Pierce., *et al* [27].

### Preparation of inoculum

The yeast strains were incubated overnight at 37°C in SDA plate, a loopful from these cells were inoculated in 50 ml of YPD (Accumedia®) liquid medium and grown overnight in an orbital shaker (150 to 180 rpm) at 30°C. The cells were washed twice with sterile PBS, centrifuged at 3000 g, and were resuspended in RPMI. After that, the cells were counted using a haemocytometer on a bright field microscope (Dilutions 1:100 in PBS). The concentration of inoculum was in $1.0 \times 10^{-6}$ cells/ml.

### Biofilm formation

In a microtiter plate pre-sterilized, polystyrene, flat-bottomed, 96-well (Kasvi®), were inoculated 100 µl to RPMI with essential oil at the preestablished concentration and 100 µl to inoculum adjusted. Cells were incubated in microplate incubator for 2 hours under shaking at 100 rpm at 37°C. After the incubation period, the plate was washed 3 times with PBS and 100 µl of the essential oil was added in decreasing concentrations from 1000 µg/ml to 125 µg/ml. The plates were incubated at 37°C for 24h.

### Mature biofilm

In a microtiter plate with same specifications were inoculated 100 µl to inoculum adjusted and were incubate at 37°C for 24 h. Then, the plates were washed 3x with sterile PBS and added 100 µl to RPMI with essential oil in the pre-established concentration. The plates were incubated at 37°C for 24h.

### Biofilm quantification

After incubation, the plates were washed 3x with sterile PBS for remove planktonic cells. The semi-quantification of the fungal cell viability was calculate using a colorimetric XTT (Sigma-Aldrich) reduction assay, in which were add in the plates 80 µl solution XTT and the absorbance measurement above in reader microtiter spectrophotometer at 490 nm. The assays have been done in three independent experiments. The absorbance values of the treated biofilm have been subtracted from the absorbance values of the control to obtain the percentage of metabolic activity of the biofilm [27].

### Biofilm statistical analysis

For the comparative evaluations we used the statistical analysis ANOVA a criterion, Dunnett variation, bilateral ($p < 0.01$), (Biostat Program 5.0), where the means of concentrations and control were compared to each other.

### Scanning electron microscopy (SEM)

For the analysis of the effect of the essential oils on the biofilm of *Candida albicans* ATCC 90028 and MYA-2876 by MEV, the inoculum was adjusted in a concentration of 1 x $10^{-6}$ CFU/mL. From the final suspension of cells, *C. albicans* biofilm was produced on culture slides (BD Falcon). For the biofilm assay in formation, the slide was incubated at 37°C with shaking at 75 rpm on a microplate shaker for 90 minutes. The concentrations of 125 µg/mL, 312.5 µg/mL, 1250 µg/mL of the essential oil of *E. muticus* were then added and incubated 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 PBS was rinsed, and then at a concentration of 125 µg/mL, 312.5 µg/mL, 1250 µg/mL of the essential oil of *E. muticus* was incubated again for another 24 hours.

After incubation, the culture medium was removed and biofilm fixed with glutaraldehyde (2%) for 30 min. Samples were then dehydrated using sequential rinses of ethanol at concentrations of 50, 70, 90% and absolute ethanol. Each rinse concentration was used twice. The samples were sputtered with gold for 120s (BAL-TEC SCD 050, Balzers Liechtenstein). The analysis of samples was performed using an scanning electron microscope operating at 15 KV acceleration (JSM-5600 Lv; JEOL, Tokyo, Japan).

### In vitro toxicity: Hacat cells

The epithelial cells used for this assay are from the untransformed immortalized human normal keratinocyte cell line called Hacat. These cells were kindly donated by Prof. Dr. Ricardo Della Coletta, from the Pathology Area (FOP/UNICAMP), which were cultivated in the presence of the essential oil to determine a toxicity effect on it. The assay of cytotoxicity in Hacat cells was done according to Vichai and Kirtikara [28] with modifications. The inoculum adjustment was done by counting cells in the Neubauer chamber to obtain a value of 1.9 x $10^{-4}$ cells/mL. Cells were cultured with RPMI + 10% fetal bovine serum (FBS) in flat bottom 96 well plates, suitable for cell culture (KASVI®) and incubated at 37°C in $CO_2$ (5%) for 24h. After 24h, 100 µl of *E. muticus* essential oil was added at concentrations of 125 µg/ml to 16000 µg/ml and incubated for 24h at 37°C in $CO_2$ (5%). The determination of essential oil concentrations was done through MIC and concentrations greater than ten times MIC. After this time, the cells were fixed with 50 µl of 50% Trichloroacetic Acid (TCA) (SYNTH®) and incubated for 1 h at 4°C. Thereafter, the plates were washed with distilled water (4x) and dried at room temperature. After drying, they were stained with 100 µl of 0.4% Sulforhodamine B (SRB) solution (SIGMA-ALDRICH®) for 1 hour at 4°C.

After incubation the plates were washed with 1% acetic acid (SYNTH®) to remove nonspecific bindings from the dye and unbound cells and were dried again at room temperature. After drying, 100 µl of Trizma Base (SIGMA-ALDRICH®) was added for resuspension of the dye previously attached to cell surface proteins. The plates were read in Microplate Spectrophotometer (510 nm) to measure the optical density. The absorbance was expressed in numerical values of percentage in relation to the control group. The assays were done in three independent experiments.

### Toxicity in vivo: Galleria mellonella

The whole life cycle of *Galleria mellonella* was carried out in the area of Pharmacology (FOP/UNICAMP) under the supervision of Prof. Dr. Pedro Luiz Rosalen, to whom he graciously ceded the larvae in their last stage to carry out the evaluation of the *E. muticus* essential oil *in vivo* toxicity

*Galleria mellonella* larval toxicity assay was described by Scorzoni., *et al*. [29] with modifications. For each tested essential oil concentration and control, 10 larvae were used. The larvae were weighed (0.3 - 0.5g), placed in Petri dishes and incubated at 37°C in the dark by night before the experiment. Larvae with color changes were excluded, (dark spots) or with apparent melanization. The larvae's death was monitored by visual inspection of color (brown - dark brown) and lack of movement after touch.

Prior to any procedure, the pro-paw was previously washed with 70% ethanol and 10 µl of the essential oil suspension in various concentrations (125 - 1200 µg/ml) was applied to the hemocele through the last left larval prophila using the 1 ml Hamilton syringe. The determination of these concentrations was followed by the MIC up to ten times the MIC value. After inoculation, the larvae were incubated at 37°C and death monitored for 72 hours.

## Results

### Gas chromatography

Chromatographic analysis of the essential oil detected a ratio of miscellaneous compounds and their percentages in a 100 µl sample of oil. The most abundant compound was citronellal, with 87.27%, follow by citronellol 7.73%, isopulegol 2.91%, cineole < 1,8 > 0.64%, dihydrocitronellol acetate 0.44%, citronellyl acetate 0.40%, caryophyllene < (z-) > 0.39%, and others not identified 0.23%.

### Susceptibility test of Candida species

The essential oil was tested against standard strains of *Candida* spp. in order to determine the minimum inhibitory concentration by broth microdilution technique. The data demonstrated inhibitory activity at concentrations of 31.2 µg/mL to 125 µg/mL for the essential oil of *E. muticus*, as shown in table 1.

| Microrganisms | MIC (µg/mL) | MFC (µg/mL) | MFC/MIC | Oil profile | MIC – Fluconazole (µg/mL) | MIC –Amphotericin B (µg/mL) |
|---|---|---|---|---|---|---|
| *C. albicans* ATCC 90028 | 125 | 250 | 2 | Fungicidal | 0.25 | 1.0 |
| *C. albicans* CBS 562 | 125 | 250 | 2 | Fungicidal | 1.0 | 0.5 |
| *C. albicans* MYA 2876 | 125 | 250 | 2 | Fungicidal | 0.125 | 0.25 |
| *C. guilliermondii* CBS 566 | 62,5 | 125 | 2 | Fungicidal | 4.0 | 0.5 |
| *C. krusei* CBS 573 | 31,2 | 125 | 4 | Fungistatic | 8.0 | 0.5 |
| *C. utilis* CBS 5609 | 31,2 | 125 | 4 | Fungistatic | 2.0 | 0.5 |
| *C. rugosa* IZ 12 | 31,2 | 62,5 | 2 | Fungicidal | 8.0 | 0.5 |
| *C. lusitaniae* IZ 06 | 250 | 250 | 1 | Fungicidal | 0.125 | 0.5 |
| *C. glabrata* IZ 07 | 125 | 250 | 2 | Fungicidal | 8.0 | 2.0 |
| *C. dubliniensis* CBS 7987 | 125 | 125 | 1 | Fungicidal | 0.25 | 0.5 |
| *C. parapsilosis* CBS 604 | 62,5 | 500 | 8 | Fungistatic | 8.0 | 2.0 |
| *C. tropicalis* CBS 94 | 125 | 250 | 2 | Fungicidal | 0.5 | 0,5 |

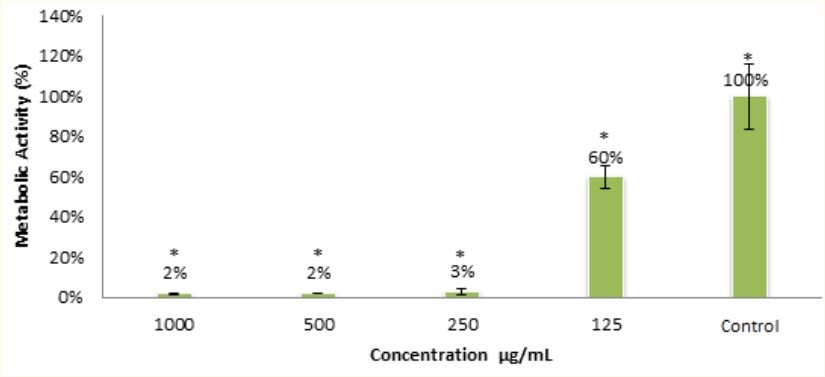
***Table 1:*** *Susceptibility profile of essential oil from Elionurus muticus.*

After the determination of MIC, an aliquot of the susceptibility assay was used to determine the minimum fungicidal concentration (MFC) against standard strains of *Candida* spp. The essential oil of *E. muticus* presented antifungal action at concentrations ranging from 62.5 µg/mL to 500 µg/mL, as shown in table 1.

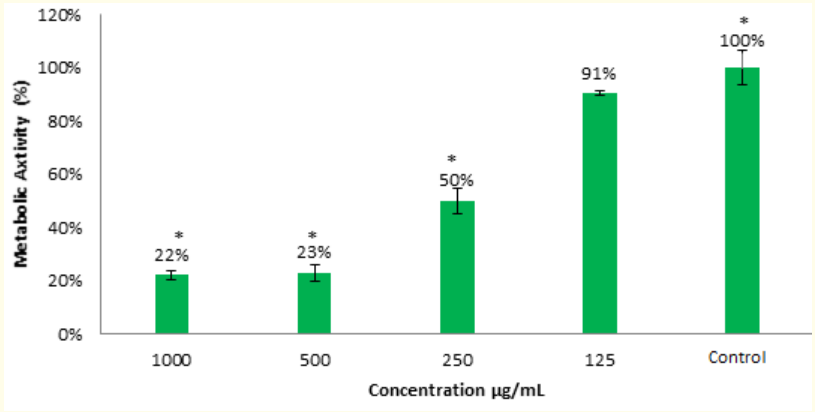
The data interpretation of the essential oil susceptibility profile was established according to the CFM/MIC ratio, where values less than 4 exhibit a fungicidal profile and values greater than or equal to 4 exhibit a fungistatic profile. The essential oil of *Elionurus muticus* showed a fungistatic profile for *C. parapsilosis* CBS 604, *C. krusei* CBS 573 and *C. utilis* CBS 5609 at MIC and CFM concentrations: 31.2 and 125 µg/mL; 31.2 and 125 µg/ml; 62.5 and 500 µg/mL, respectively. The fungicidal profile was reported for *C. albicans* ATCC 90028, *C. albicans* ATCC MYA-2876, *C. guilliermondii* CBS 566, *C. rugosa* IZ 12, *C. lusitaniae* IZ 06, *C. glabrata* IZ 07, *C. dubliniensis* CBS 7987, *C. albicans* CBS 562, *C. tropicalis* CBS 94 at concentrations of MIC and CFM: 125 and 250 µg/ml; 125 and 250 µg/ml; 62.5 and 125 µg/ml; 31.2 and 62.5 µg/mL; 250 and 250 µg/ml; 125 and 250 µg/ml; 125 and 125 µg/ml; 125 and 250 µg/ml; 125 and 250 µg/mL, respectively.

### Biofilm tests of *Candida albicans* ATCC MYA-2876 XTT assay

The essential oil was tested with the purpose of evaluating the metabolic activity of *Candida albicans* biofilm ATCC MYA-2876 by colorimetric quantification with XTT. The results of this assay were analyzed using the statistical analysis ANOVA, Dunnet variation, bilateral ($P < 0.01$) of the Biostat 5.0 Program and they are expressed in graph 1 and 2.




***Graph 1:*** *Metabolic activity of the biofilm in formation from C. albicans ATCC MYA-2876 exposed to the essential oil of Elionurus muticus. *Statistically significant data according to Anova a criterion followed by Dunnett with p < 0.01.*




***Graph 2:*** *Metabolic activity of the mature biofilm from C. albicans ATCC MYA 2876 exposed to the essential oil of Elionurus muticus. *Statistically significant data according to Anova a criterion followed by Dunnett with p < 0.01.*

In graph 1, was observed a decrease in the metabolic activity of the biofilm in a dose dependent formation of increasing concentrations of the essential oil. At the concentrations of 1000, 500 and 250 µg/mL, the biofilm in formation showed metabolic activity of 2%, 2% and 3%, respectively. At the concentration of 125 µg/mL, corresponding to MIC of this strain, the biofilm in formation expressed 60% of metabolic activity. All concentrations tested showed statistical differences compared to control.

In graph 2, we also observed a decrease in the metabolic activity of the mature biofilm dose dependent on increasing concentrations of the essential oil. The treatment of the essential oil at concentrations of 1000, 500 and 250 µg/mL showed metabolic activity of 22%, 23% and 50%, respectively. At the concentration of 125 µg/mL, corresponding to MIC of this strain, the mature biofilm revealed 91% of metabolic activity, not showing statistical difference compared to the control.

## Scanning electron microscopy (SEM)

The evaluation of the essential oil effect on the biofilm cellular morphology of *C. albicans* ATCC MYA 2876 and *C. krusei* CBS 573 was done through visualization of this structure in the Scanning Electron Microscope (SEM). Various concentrations of the essential oil were used in the analysis.

The morphological changes in the cells of the biofilm in formation were observed after the treatment with the essential oil of *Elionurus muticus* and are expressed in figure 1A to 1H. The control of the biofilm in formation is showed in A and B, showing the cells in the form of hyphae or yeasts with uniform cell surface. Scanning electron microscopy images of the treated biofilm (in formation) showed morphological changes according to the increase of the *E. muticus* essential oil concentration. Subitems C and D concern to the biofilm in formation when treated with 125 µg/mL, showing that the cells are mostly in the form of hyphae with smooth surface, revealing similar morphological aspects to the control. The subitems from E to H represent the biofilm in formation treated with 312.5 µg/mL and 1250 µg/mL, demonstrating that the cells in the form of hyphae or yeasts show a low cytosolic volume and irregularities on the cell surface.

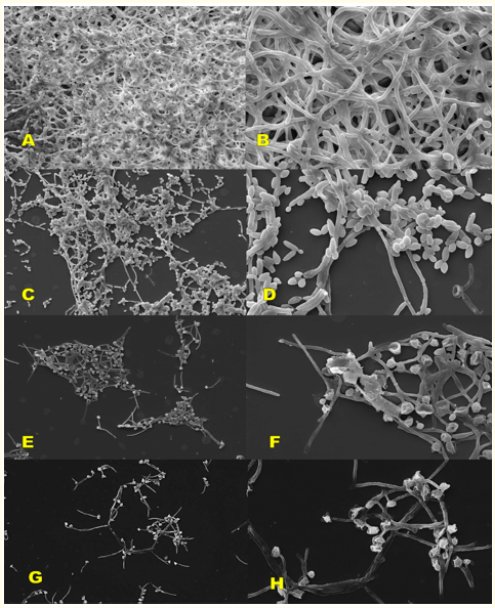



***Figure 1:*** *SEM Images of the C. albicans ATCC MYA 2876 biofilm in formation treated and untreated with the essential oil of Elionurus muticus.*

*A) Untreated mature biofilm, control (500x); B) Mature biofilm without treatment, control (1500x); C) Biofime mature with treatment of 125 µg/mL (500x); D) Biofilm mature with treatment of 125 µg/mL (1500x); E) Biofilm mature with treatment of 312.5 µg/mL (500x); F) Biofilm mature with treatment of 312.5 µg/mL (1500x); G) Biofilm mature with treatment of 1250 µg/mL (500x); H) Biofilm mature with treatment of 1250 µg/mL (1500x). The increase of 500x corresponds to 50 µm and the increase of 1500x corresponds to 10 µm in scale.*

The morphological changes of the mature biofilm after treatment with the essential oil of *Elionurus muticus* are shown in figure 2, in subitems A to H. The control of mature biofilm is represented in subitems A and B, showing cells in the form of hyphae or yeasts with uniform cell surface in considerable amounts. Subitems C and D represent the mature biofilm when treated with 125 µg/mL, showing that the cells are mostly in the form of hyphae with smooth surface, demonstrating similar morphological aspects to the control, but there was a decrease in the amount of hyphae. The subitems E and F represent the mature biofilm when treated with 312.5 µg/mL, observing some hyphae with thickness decrease regions and low cytosolic volume, in relation to the control. Subitems G and H represent the mature biofilm when treated with 1250 µg/mL, showing hyphae and yeasts with low cytosolic volume and irregular cell surface, with a significant decrease in the amount of hyphae. It was observed in the mature biofilm that according to the increase of the concentration tested there was a gradual decrease in the amount of hyphae.

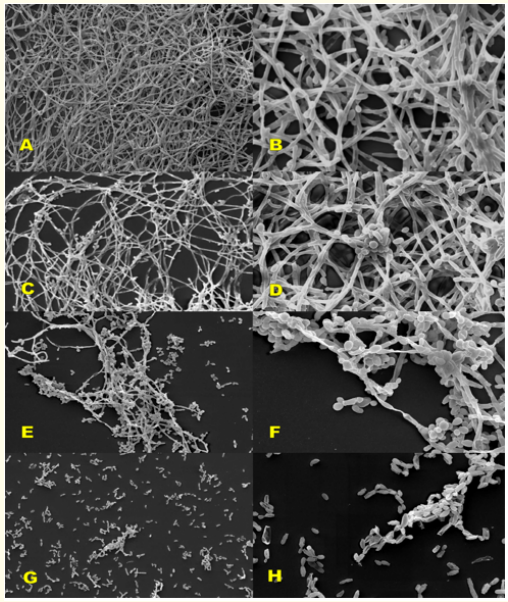
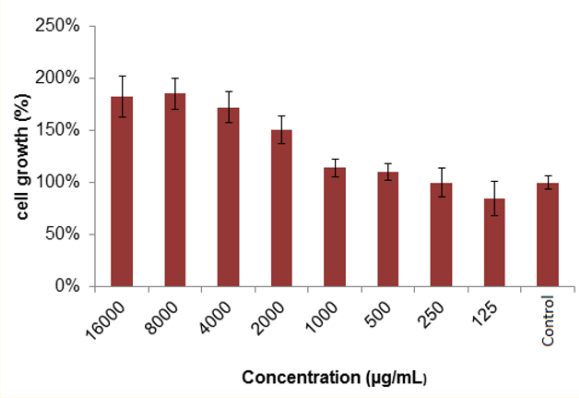



***Figure 2:*** *SEM images of the mature biofilm from C. albicans ATCC MYA 2876 treated and untreated with the essential oil of Elionurus muticus.*

*A) Untreated mature biofilm, control (500x); B) Mature biofilm without treatment, control (1500x); C) Biofime mature with treatment of 125 µg/mL (500x); D) Biofilm mature with treatment of 125 µg/mL (1500x); E) Biofilm mature with treatment of 312.5 µg/mL (500x); F) Biofilm mature with treatment of 312.5 µg/mL (1500x); G) Biofilm mature with treatment of 1250 µg/mL (500x); H) Biofilm mature with treatment of 1250 µg/mL (1500x). The increase of 500x corresponds to 50 µm and the increase of 1500x corresponds to 10 µm in scale.*

## In vitro toxicity of E. muticus essential oil in Hacat cells

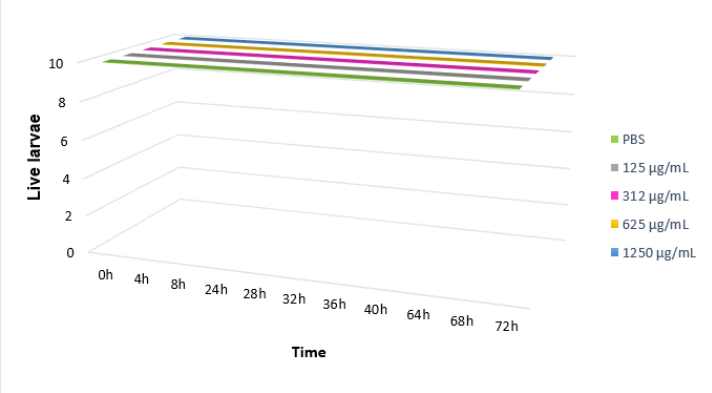
The *in vitro* toxicity test performed on immortalized, unprocessed normal human keratinocytes - Hacat (Graph 3) incubated at different concentrations of the essential oil of *Elionurus muticus* (125 to 16000 µg/mL) showed cell growth above 50% when compared to the control at all concentrations tested, indicating low cytotoxicity in this assay.




***Graph 3:*** *In vitro toxicity of Elionurus muticus essential oil in Hacat cells.*

### *In vivo* toxicity of the essential oil from *E. muticus* in *G. mellonela* larvae

*In vivo* toxicity was assessed by the amount of live larvae after treatment with different concentrations of essential oil. It was found after 72 hours of exposure to the essential oil that the survival rate of the larvae was 100% at all concentrations tested (Graph 4), indicating no toxicity in this assay.




***Graph 4:*** *In vivo toxicity of Elionurus muticus essential oil in Galleria mellonella larvae.*

## Discussion

In the last decades, research with medicinal plants has been a viable resource in the discovery of new drugs for various applications in medicine. The use of essential oils extracted from several genera of plants presented several therapeutic indications, among them the antiseptic, antifungal and antimicrobial action [11]. *Elionurus muticus* is a Brazilian pampas grass that has aroused interest in containing antiseptic properties [30].

The gas chromatography of *E. muticus* essential oil revealed the presence of important monoterpenoids, mainly citronellal (87.27%) and others, such as isopulegol and 1,8-cineol. In general, the antifungal mechanism of monoterpenoids involves cytoplasmic membrane rupture, inactivation and inhibition of intracellular and extracellular enzyme synthesis [31,32].

Citronellal is a monoterpenoid that has anti-*Candida* action showed in several scientific articles. Its anti-*Candida* mechanism of action is mainly due to its harm on the cell membrane, mitochondria and DNA. In the cell membrane, according to Sigh et al. [33], citronellal reduced levels of ergosterol. Citronellal also, according to Saibabu et al., [34], resulted in dysfunctional mitochondria reducing their metabolic activity (61.5%) and inducing DNA damage showing significant differences in gene expression.

The MIC data of the present study showed values of 31.2 to 125 µg/mL and MFC values of 625 to 500 µg/mL. These results showed the profile of fungicidal activity of the *E. muticus* essential oil against most of the strains *Candida* spp. tested (Table 1).

These data, in principle, demonstrate antifungal action of this oil corroborating with the literature. Chagonda and Fungirayi [15] also describe the antifungal activity against *C. albicans, C. krusei* and *Cryptococcus neoformans* with MIC values ranging from 0.5 µg/mL to 5 µg/mL and MFC ranging from 2.5 to 10 µg/mL, using the diffusion disc technique, a methodology different from that used in this study.

The antimicrobial activity is also presented in some articles, using the microdilution technique in the broth and disc diffusion, obtaining MIC values ranging from 1000 µg/mL to 150000 µg/mL against *Escherichia coli, Staphylococcus aureus, Staphylococcus coagulase-negative, Staphylococcus pseudintermedius, Streptococcus uberis, Pseudomonas aeruginosa* and *Bacillus cereus* [17-19].

These data, when analyzed together, demonstrate that the essential oil of *E. muticus* exhibits antibacterial and antifungal action against the microorganisms tested in different ways, indicating an antimicrobial potential.

Biofilms are well organized microbial clusters that produce a self-produced matrix from a series of compounds that include extracellular DNA (eDNA), proteins and polysaccharides. These compounds are bound to a surface (when they originate in medical devices or teeth) or are suspended (in mucus or in chronic wounds) [35]. Its form confers advantages to the microorganisms involved in this matrix when compared to planktonic cells, including antimicrobial tolerance, immune defense evasion of the host and persistence [36,37].

The essential oil was efficient in reducing the metabolic activity of the biofilm in formation, exhibiting approximately 60% of metabolic activity of the biofilm in the concentration of 125 μg/mL. At concentrations of 1000 μg/mL, 500 μg/mL and 250 μg/mL, there was a significant decrease in the metabolic activity of the biofilm in formation compared to the control, exhibiting metabolic activity of 2%, 2% and 3%, respectively. In the mature biofilm, the essential oil of *E. muticus* also showed a decrease in the metabolic activity of the mature biofilm up to the concentration of 250 μg/mL at a concentration of 125 μg/mL.

These observations demonstrate a potential action of the essential oil of *E. muticus* in the decrease of the metabolic activity of the biofilm in formation and mature. These results, in relation to the decrease in the metabolic activity of both biofilms, suggest its use as an antimicrobial alternative, as main agent or as adjuvants. These data, however, should be complemented with other studies aimed at determining the characteristics of its bioactive components added to its pharmacological properties, so that they can effectively make it a phytotherapic, as is usually the case.

Several articles have demonstrated a decrease in metabolic activity with the administration of citronellal or essential oils containing a significant amount of this active component in *Candida* biofilms [38-41], confirming the antimicrobial properties of this species. Further data on the essential oils from the extraction of the various parts of plants should also be investigated for the purpose of comparing their properties with respect to their bioactive components, ability to inhibit microorganisms and morphological changes in their cells.

In order to investigate possible morphological alterations of the biofilm after treatment with the essential oil, the biofilm samples were analyzed visually by SEM at the concentrations of 1250, 312.5 and 125 μg/mL. At the highest concentrations of the essential oil tested morphological alterations were observed, such as reduction in hyphae thickness, low cytosolic volume in yeasts and hyphae and irregularities on the cell surface.

Several studies have shown significant morphological changes in the *Candida* biofilm after treatment with citronellal or with oils containing a significant amount of this component [38,39,42], confirming this structural action property.

Since the main constituent of the essential oil of *Elionurus muticus* is the citronellal monoterpenoid (87.27%), it is indicative that the essential oil of *E. muticus* has antibiofilm activity, promoting morphological changes in the biofilm of *C. albicans* as demonstrated in this study. Other substances present in these oils should be investigated in order to broaden the basic knowledge about this plant.

The toxicity assays of the compounds under study are being steered steadily in an *in vitro* approach based on human cells and *in vivo* approach by unconventional animals for reasons including scientific relevance, efficiency, cost and ethical legitimacy [43].

In this study, *in vitro* and *in vivo* toxicity were tested with Hacat cells and *Galleria mellonella* larvae, respectively. In the *in vitro* toxicity test with Hacat cells, cell growth was above 50% at all concentrations tested as compared to control, indicating low cytotoxicity. In the *in vivo* assay, after 72 hours of exposure to essential oil, all larvae of *Galleria mellonella* remained alive at all concentrations tested demonstrating low toxicity, relevance and efficiency as a bioactive substance.

Some publications have shown low toxicity of citronellal and essential oils with a significant amount of this component in several studies, involving *in vitro* tests with human cells and *in vivo* tests [34,44,45]. These data indicate low toxicity, suggesting little or no toxic effect of this component, revealing possible therapeutic applications.

In general, the results presented in this research corroborate with other studies in this line of research, showing the antimicrobial effects of *Elionurus muticus* essential oil and its antimicrobial potential.

## Conclusion

The essential oil of *Elionurus muticus* presents citronelal as the main active ingredient; demonstrates antifungal action in a dose-dependent manner against standard *Candida* strains; it reveals antifungal action against the formation and mature biofilm of *C. albicans,* observing a decrease in the metabolic activity of these structures; morphologically alter the cells of *C. albicans* biofilm showing irregularities in the cell surface, low cytosolic volume and decrease in the amount of hyphae; no shows significant toxicity in human Hacat cells and *Galleria mellonella* larvae.

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