EVALUATION OF THE ANTIFUNGAL ACTIVITY OF THE BIOCOMPOUND BASED ON MELALEUCA (Melaleuca alternifolia) AND CLOVE (Eugenia caryophyllata) ESSENTIAL OILS ON DIFFERENT Candida spp.

AVALIAÇÃO DA ATIVIDADE ANTIFÚNGICA DO BIOCOMPOSTO À BASE DOS ÓLEOS ESSENCIAIS DE MELALEUCA (Melaleuca alternifolia) E DE CRAVO (Eugenia caryophyllata) SOBRE Candida spp.

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GENUS: Candida primarily responsible for infections associated with fungemia. Toxicity of antifungals is observed in the treatment of candidiasis. Interest in herbal medicine has grown. Plant essential oils are less harmful offering better results than synthetic medicines. Essential oils Melaleuca alternifolia (tea tree oil) and Eugenia caryophyllata (clove) have antimicrobial and antifungal activities. Biocompound a product with essential oils, has as its main constituents melaleuca and clove. Registered at ANVISA as antiseptic and healing. Objective: to evaluate the antifungal activity and cytotoxicity of the Biocompound against the genus Candida. Disk diffusion tests showed no results. Minimum Inhibitory Concentration: Candida albicans ATCC 24433 (256 μg/ml) ketoconazole (0.80 μg/ml), Candida albicans ATCC 14053 (154.60 μg/ml) ketoconazole (0.31 μg/ml), Candida krusei (30.80 μg/ml) ml) ketoconazole (3.16 μg/ml) and Candida glabrata (26.30 μg/ml) ketoconazole (3.18 μg/ml). Cytotoxicity: Biocompound showed low toxicity, being 8.30 times lower than ketoconazole. Selectivity Index: best results against Candida glabrata the Biocompound presented a value 14.70 times greater than ketoconazole and Candida krusei Biocompound presented a selectivity index 12.44 times greater than ketoconazole. Conclusion: Biocompound was effective against the genus Candida, mainly against Candida glabrata and Candida krusei, strains resistant to current drugs. Biocompound is a promising treatment against Candida.

KEYWORDS: Treatment, Essential Oils, Candida Genus.

RESUMO: Gênero Candida o principal responsável por infecções associadas à fungemia. A toxicidade dos antifúngicos é observada no tratamento da candidíase. O interesse pela fitoterapia tem crescido. Os óleos essenciais vegetais são menos nocivos, oferecendo melhores resultados do que os medicamentos sintéticos. Os óleos essenciais Melaleuca alternifolia (óleo de melaleuca) e Eugenia caryophyllata (cravo) têm atividades antimicrobiana e antifúngica. Biocomposto um produto com óleos essenciais, tem como seus principais constituintes melaleuca e cravo-da-índia. Registrado na ANVISA como antisséptico e cicatrizante. Objetivo: avaliar a atividade antifúngica e citotoxicidade do Biocomposto contra o gênero Candida. Os testes de difusão em disco não mostraram resultados. Concentração inibitória mínima: Candida albicans ATCC 24433 (256 μg/ml) cetoconazol (0.80 μg/ml), Candida albicans ATCC 14053 (154.60 μg/ml) cetoconazol (0.31 μg/ml), Candida krusei (30.80 μg/ml) ml) cetoconazol (3.16 μg/ml) e Candida glabrata (26.30 μg/ml) cetoconazol (3.18 μg/ml). Citotoxicidade: O biocomposto apresentou baixa toxicidade, sendo 8.30 vezes menor que o cetoconazol. Índice de seletividade: melhores resultados contra Candida glabrata o Biocomposto apresentou valor 14.70 vezes maior que o cetoconazol e Candida krusei Biocomposto apresentou índice de seletividade 12.44 vezes maior que o cetoconazol. Conclusão: O biocomposto foi efetivo.
contra o gênero Candida, principalmente contra Candida glabrata e Candida krusei, cepas resistentes às drogas atuais. Biocomposto é um tratamento promissor contra Candida.

PALAVRAS-CHAVE: Tratamento, Óleos Essenciais, Gênero Candida.

1. Introduction

Fungal diseases represent a critical public health problem due to the increase in the population at risk. In the last two decades, the incidence of fungal infections has grown dramatically due to the increase in individuals with compromised immune systems (Peixoto et al. 2014). Among the fungal species related to clinical manifestations, the Candida genus is the main responsible for infections associated with hospital fungemia, especially in critical sectors, such as intensive care units (Mimica et al. 2009). From 15 to 20 microorganisms of the Candida genus are described as causing infection. However, four species of this genus are responsible for causing 96% of infections: Candida albicans is the main etiologic agent, responsible for 42.5% of infections, followed by Candida tropicalis (27.3%), Candida parapsilosis (21.9 %) and Candida glabrata (4.4%) (Felipe et al. 2018).

Conventionally, the treatment of candidiasis consists of the use of topical or systemic antifungal agents, with azole and polyene derivatives being the most common. The toxic effects caused by conventional antifungals have been observed when used for the treatment of candidiasis (Abrantes et al. 2013, Rodrigues et al. 2018). There is much evidence that natural products such as essential oils and plant extracts may have fewer harmful health side effects than synthetic drugs. Plant derivatives such as essential oils can be successfully used to reduce the local fungal load (Rohilla et al. 2018).
Phytotherapy can be historically defined as the science that deals with health problems using plants (phytocomplexes), being contemporary with the beginning of civilization. Essential oils and their components have multiple biological activities, such as antimicrobial, antifungal, and antioxidant properties (Wang et al. 2010, Mertas et al. 2013). These oils can be used as important active principles in products intended for the treatment of humans. They are products of complex composition extracted from plants by various processes, the most used being steam distillation. They are known to have a variety of pharmacological effects, including antifungal, antiviral, antimicrobial, anti-inflammatory, healing, and cell regenerating activity (Silva et al. 2015, Abrantes et al. 2013). Several studies have been carried out “in vitro” and “in vivo”, confirming the effectiveness of essential oils such as tea tree and clove as antifungals (Vieira et al. 2018).

2. Description of the Product to be Tested

Biocompound is a product made with essential oils and vegetable oils, its main constituents are tea tree (*Melaleuca alternifolia*) and clove (*Eugenia caryophyllata*) essential oils. Biocompound was registered with ANVISA as a Grade II product, with antiseptic and healing properties, strengthening and revitalizing the skin, produced by the company Magia da Mata Cosméticos Ltda. The product has already been submitted to a clinical study of topical skin compatibility (Primary Dermal Irritability, Accumulated Dermal Irritability, Dermal Sensitivity, Dermal Photosensitivity, Dermal Phototoxicity) and none of the volunteers (n= 85) had a significant skin reaction (erythema, edema, papule or gallbladder), and this product was considered safe for topical use, as it did not induce irritative phenomena or skin sensitization and did not cause any type of photoallergy or detectable phototoxicity during the study period, according to a report sent to ANVISA. The product was registered (Grade II) and released to be marketed, with no
known side effects during the study phase. The objective of this work is to evaluate the antifungal activity of the Biocompound against *Candida* spp fungi and its cytotoxicity on mammalian cells.

3. Materials and Methods

The yeasts of *Candida albicans* (*C. albicans*) ATCC 24433, *Candida albicans* (*C. albicans*) ATCC 14053, *Candida krusei* (*C. krusei*) ATCC 6258, and *Candida glabrata* (*C. glabrata*) were seeded on Sabouraud dextrose agar (Fluka analytical) by the depletion technique in Petri dishes (90 mm) to evaluate the viability, purity and obtaining an isolated colony. All plates were incubated at 35°C for 24 hours.

3.1 Inoculum Preparation

The inoculum was prepared by choosing about five colonies of approximately 1 mm in diameter from a 24-hour culture. Colonies were suspended in 5mL of sterile saline solution (8.5g/L NaCl). After the colonies were suspended, the solution was vortexed for 15 seconds and the cell density was adjusted according to the standard solution on the 0.5 McFarland scale. The incubation temperature was 35°C, with a variation of +/- 2°C.

3.2 Antifungal Sensitivity Test

The antifungal sensitivity test was performed using the methodology of Kirk-Bauer CLSI (2015), in which filter paper discs soaked with the tested Biocompound (dilution of 5 µg/ml) were placed on a petri dish culture. The culture used was obtained from a 24-hour growth of *Candida* spp growth at 35°C, then a suspension of yeasts in saline adjusted to a 0.5 McFarland scale. From this dilution, an aliquot of 0.1 ml was added to a petri dish of Sabouraud
agar and swabbed. The plates were incubated at 35°C for 30 minutes and after this time, the discs were placed on the culture and impregnated with 20µL of Biocompound, and then the plates were incubated for 24 hours at 35°C. Sensitivity to Biocompound was determined by measuring the diameter, in millimeters, of the microbial growth inhibition halo (Marconi et al. 2008). Discs soaked in dimethylsulfoxide – DMSO and ketoconazole were used as negative and positive controls, respectively. After determining the sensitivity, the ImageJ image analysis software was used, and the analysis of the data obtained by an image in the statistical software GraphPadPrism V.7 was used.

3.3 Determination of the Minimum Inhibitory Concentration (MIC)

To determine the minimum inhibitory concentration (MIC), an inoculum will be prepared from a 24-hour repeat of the strain to be tested, and a Neubauer chamber count will be performed to adjust the cell/mL concentration required in the protocol to be performed. After counting, we will transfer an aliquot of the culture in 5 mL with saline solution, and from this suspension, 900 µL aliquots will be added in two tubes with 9 mL of Sabouraud dextrose medium. The determination of the minimum inhibitory concentration will be performed in triplicates, by the half dilution method as described by CLSI (2015). In an 8 x 12-well polystyrene microplate (96 wells), 180 µL of sterile Sabouraud medium will be added to the first row of the plate and 100 µL to the following wells (rows 2 to 12). Afterward, 20 µL of the chemical compound will be inoculated and serial dilutions will be made in decreasing concentrations from 500 µg/mL to 3.9 µg/mL, discarding the last 100 µL of row 12. After the dilution, an aliquot of 100 µL will be placed with approximately 5.0 x 106 CFU/ml. The total volume of each well will be a total of 200 µL. Three controls will be used in triplicates for each experiment, including three wells containing 100 µL of the pure medium,
three wells containing 100 µL of the inoculum without the derivative, and three tubes with 100 µL of the culture with dimethylsulfoxide, the diluent used with the Biocompound. Decreasing concentrations of ketoconazole will also be used as a positive control. The plates will be incubated at 35 + - 2°C for 24 hours on a turntable. The MIC will be defined as the lowest concentration of the derivative capable of completely inhibiting the visible growth of the culture (Mulu et al. 2013, Pfaller et al. 2012). Aliquots of 20 µL of resazurin (125 µg/ml) were added to all wells, the plate was incubated for 4 hours and read on the SpectraMax M4 spectrophotometer (Molecular Devices) at wavelengths 530 (excitation) – 585 nm (emission).

3.4 Assay to Determine the Concentration of Cytotoxicity in Mammalian Cells (CC50)

The “in vitro” cytotoxic test is used to identify (median lethal dose) the dose capable of killing 50% of the cells in a culture, to assess the toxicity of new substances. To analyze the cytotoxic effect, we used mouse bone marrow cells. For the culture assays, this procedure was carried out under sterile conditions and the cells were obtained in RPMI-1640 medium then the cell count was performed under an optical microscope in a Neubauer hemocytometer and the suspensions were adjusted in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 2-mercaptoethanol, L-glutamine, 100 µg/mL penicillin G potassium and 100 µg/mL streptomycin, maintained at 37 °C with 5% CO2. To perform the cytotoxicity assays, the cells will be cultured in 96-well microplates at a concentration of 5x105 cells/mL, in a final volume of 100 µL/well. After an incubation period of 24 hours, necessary for the cells to adhere to the plate, different concentrations of Biocompound (pure Biocompound, 5ug/mL, and 10ug/mL) will be added, in a volume of 100 µL. As a control, only half will be used. Then the culture will be kept in an oven at 37º C at 5% CO2 for 24 and 48 hours. Cell viability...
will be evaluated by colorimetry. The study was performed in 96-well polystyrene microplates. After 24 hours of incubation, an aliquot of 20 μL of resazurin (125μg/mL) was placed. The plates were then sealed and incubated at 37°C and, after 4h, the plates were analyzed in a SpectraMax M4 spectrophotometer (Molecular Devices) at wavelengths of 545 nm, to establish the value of the cytotoxic concentration (CC50) of the Biocompound.

3.5 Determination of the Selectivity Index (SI)

The selectivity index will be calculated by dividing the 24h average growth concentration limit values (MIC) by the CC50/24h values. The value obtained from this calculation will demonstrate the proportion of toxicity of the Biocompound on Candida spp yeasts and mammalian cells.

3.6 Statistical Analysis

The results of the experiments with fluorescence reading and MIC with resazurin were tabulated in Excel and analyzed by analysis of variance (ANOVA), followed by the Tukey t-test, using the GraphPad Prism 6 program. Values of p<0.05 were considered significant.

4. Results

4.1 Antifungal Sensitivity Test by the Diffusion Disk Method

For the antifungal susceptibility test, we tested the following strains Candida albicans ATCC 24433, Candida albicans ATCC 14053, Candida krusei ATCC 6258, and Candida glabrata. We performed the Kirk-Bauer methodology (CLSI), using the disk-diffusion method in which filter paper
disks were soaked with the product. We used the positive control ketoconazole and the negative control DMSO. We always perform this test in triplicate. As a result, the Biocompound did not present antifungal inhibition halos in the tested strains, only halos were formed in our positive control, ketoconazole, which showed the following inhibition area measurements: *Candida albicans* ATCC 24433 (30 mm), *Candida albicans* ATCC 14053 (14 mm), *Candida krusei* (20 mm) and *Candida glabrata* (18 mm) (Table I).

### Table 1 – Biocompound activity in disk diffusion with *Candida* spp. Yeasts.

<table>
<thead>
<tr>
<th>DERIVATIVES</th>
<th><em>C. albicans</em> ATCC 24433</th>
<th><em>C. albicans</em> ATCC 14053</th>
<th><em>C. krusei</em></th>
<th><em>C. glabrata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompound</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>30 mm</td>
<td>14 mm</td>
<td>20 mm</td>
<td>18 mm</td>
</tr>
</tbody>
</table>

Measurement of inhibition halos in (mm).
Source: The authors.

### 4.2 Minimum Inhibitory Concentration (MIC)

The determination of the minimum inhibitory concentration (MIC) was performed in triplicates, by the half dilution method as described by CLSI (2015). Serial dilutions were made in decreasing concentrations from 500 µg/mL to 3.80 µg/mL. For this test, we used pure Biocompound, ketoconazole as a positive control, and DMSO as a negative control. First, we will analyze the plates visually, and we did not observe any inhibition in the growth of the strains in the wells containing the Biocompound. The test was then repeated, and after 24 hours of the strains exposed to the drugs, we added resazurin to read the results on the spectrophotometer. After 4 hours of reaction, we observed activity of the Biocompound with the following concentrations on the tested strains, always comparing with our reference drug. For *C. albicans* ATCC 24433 (256 µg/mL) ketoconazole (0,80 µg/mL), for *C. albicans* ATCC 14053 (154,60 µg/mL) ketoconazole (0,31 µg/mL), for *C. krusei* (30,80 µg/ml) ketoconazole (3,16 µg/ml) and for *C. glabrata* (26,30 µg/ml) ketoconazole (3,18 µg/ml) (Table II).
### Table 2 – Biocompound and ketoconazole MIC values in Candida spp.

<table>
<thead>
<tr>
<th>Candida spp.</th>
<th>Biocompound *MIC (µg/mL)</th>
<th>Ketoconazole MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> 24433</td>
<td>256</td>
<td>0,80</td>
</tr>
<tr>
<td><em>C. albicans</em> 14053</td>
<td>154,60</td>
<td>0,31</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>30,80</td>
<td>3,16</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>26,30</td>
<td>3,18</td>
</tr>
</tbody>
</table>

*MIC (minimum inhibitory concentration).
Source: The authors.

### 4.3 Assay to Determine the Concentration of Cytotoxicity in Mammalian Cells (CC50/24h)

The pure Biocompound was submitted to an in vitro cytotoxic test on mouse bone marrow cells to determine the cytotoxic activity, which corresponds to the dose of the derivative capable of killing 50%/24h of cells in culture. In table III we can see that the Biocomposite showed low toxicity, reaching 8,3 times lower when compared to our ketoconazole control.

Table 3 – Cytotoxicity assessment (CC50%/24h) on bone marrow cell cultures after 24-hours of treatment.

<table>
<thead>
<tr>
<th>DERIVATIVES</th>
<th>CC50%/24h*MO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompound</td>
<td>244,20 µg/mL</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>2,02 µg/mL</td>
</tr>
</tbody>
</table>

*M (Mouse bone marrow cells).
Source: The authors.

### 4.4 Determination of the Selectivity Index (SI)

The selectivity index was calculated by dividing the 24h mean growth concentration limit values (MIC) by the values of (CC50%/24h). The value obtained from this calculation will demonstrate the proportion of toxicity of the Biocompound on Candida spp yeasts and on mammalian cells. Table IV shows the comparison between the selectivity indexes of the Biocompound on the strain of *C. albicans* ATCC 24433 with the reference drug. We verified that ketoconazole presented a selectivity index (SI) 2,6 times greater than the Biocompound.
Table 4 – Evaluation of the selectivity index of the Biocompound and ketoconazole on the C. albicans ATCC 24433 strain and mouse bone marrow cells.

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>C. albicans 24433</th>
<th>CC50%/24h *MO</th>
<th>*SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompound</td>
<td>256</td>
<td>244,20</td>
<td>0,95</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0,80</td>
<td>2,02</td>
<td>2,52</td>
</tr>
</tbody>
</table>

*MO (Mouse bone marrow cells). *SI (selectivity index).
Source: The authors.

Table 5 shows the comparison between the selectivity indexes (SI) of the Biocompound on the strain of C. albicans ATCC 14053 with the reference drug. In this result, we found that ketoconazole also showed better activity than the Biocompound, with (SI) values 4 times higher.

Table 5 – Evaluation of the selectivity index (SI) of the Biocompound and ketoconazole on the strain of C. albicans 14053.

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>C. albicans 14053</th>
<th>CC50%/24h *MO</th>
<th>*SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompound</td>
<td>154,60</td>
<td>244,20</td>
<td>1,57</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0,31</td>
<td>2,02</td>
<td>6,52</td>
</tr>
</tbody>
</table>

*MO (Mouse bone marrow cells). *SI (selectivity index).
Source: The authors.

Table VI shows the comparison between the selectivity index (SI) of the Biocompound against the C. krusei strain with the reference drug. In this case, it appears that the Biocompound presented (SI) 12,4 times greater than ketoconazole.

Table 6 – Evaluation of the selectivity index of the Biocompound and ketoconazole on a strain of C. krusei.

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>C. krusei</th>
<th>CC50%/24h *MO</th>
<th>*SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompound</td>
<td>30,80</td>
<td>244,20</td>
<td>7,94</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>3,16</td>
<td>2,02</td>
<td>0,63</td>
</tr>
</tbody>
</table>

*MO (Mouse bone marrow cells). *SI (selectivity index).
Source: The authors.

Table VII shows the comparison between the selectivity index (SI) of the Biocompound against the C. glabrata strain with the reference drug.
Table 7 – Evaluation of the selectivity index of the Biocompound and ketoconazole on a strain of *C. glabrata*.

<table>
<thead>
<tr>
<th></th>
<th>MIC (µg/mL) C. glabrata</th>
<th>CC$_{50%}/24$h</th>
<th><em>MO</em></th>
<th><em>SI</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompound</td>
<td>26,30</td>
<td>244,20</td>
<td>9,30</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>3,18</td>
<td>2,02</td>
<td>0,63</td>
<td></td>
</tr>
</tbody>
</table>

*MO (Mouse bone marrow cells). *SI (selectivity index).

Source: The authors.

5. Discussion

Treating diseases caused by fungi is an increasingly difficult task. Fungi are eukaryotic organisms that parasitize other eukaryotic organisms. In addition, there are few drugs currently available for this purpose, and many of them have low efficacy.

Another problem is that current medications such as Fluconazole and Itraconazole have many side effects, especially for people who have congenital or acquired immunodeficiencies. These effects range from nausea, abdominal pain, vomiting, diarrhea, headache, exfoliative dermatitis, anaphylaxis, thrombocytopenia, leukopenia to hepatotoxicity. Although these drugs have a broad spectrum of action against some *Candida* species como *Candida albicans*, other strains like *Candida krusei* and *Candida glabrata* they are very resistant (Santos Jr. et al. 2005).

*C. albicans* is still the most common fungal pathogen among hospitalized patients, but non-albicans Candida species are becoming increasingly common. Unfortunately, many of these fungal species are resistant to currently available therapeutic options, requiring further investigations to find alternative and effective drugs.

So, due to so many side effects and the high degree of resistance on the part of some pathogens, there is a growing interest not only in the activity of natural substances against resistant fungus but also in the synergistic interactions between these substances and conventional drugs, taking into account that these substances may intensify the action of these drugs (Mertas et al. 2015).
Research involving medicinal plants and essential oils has grown significantly in number and importance, mainly regarding their biological activity. However, these activities still lack much investigation. The possibility of obtaining less aggressive, lower cost, and therefore more accessible drugs has encouraged the evaluation of chemically pure compounds, essential oils, and crude extracts concerning their therapeutic actions, and toxic effects, among others, supporting the validity of drug principles to meet basic health needs (Abrantes et al. 2013). In this context, natural products represent an important and interesting area of development and their complete appreciation allows proving the legitimacy of their numerous benefits.

Biocompound is a product made from two essential oils melaleuca (Melaleuca alternifólia) and clove (Eugenia coryophyllata) that are proven to be effective as antifungals. Work carried out “in vitro” has shown that clove oil has antimicrobial and antifungal properties, including against yeasts of the genus Candida (Misner 2007, Affonso et al. 2012).

Previous studies carried out tests that proved the effectiveness of clove essential oil as a potent antifungal, whether against yeasts of C. albicans or non-albicans. Abrantes et al. (2013) carried out a study similar to ours, where they tested the antifungal potential of several essential oils, including Clove oil (Eugenia coryophyllata) “in nature”, against non-albicans candida yeasts, including C. glabrata, also used in our trials. This test revealed intense activity of clove oil against these strains, as there was the formation of halos that ranged between 17 mm and 49 mm in diameter. The authors highlighted that this result was superior to those of the control drug nystatin, which formed a 16-mm halo.

However, our results do not corroborate those of the authors, since the Biocompound, when tested on both C. albicans and non-albicans Candida yeasts such as C. glabrata and C. Krusei, using the same diffusion test, did not show formation halo against these strains. Our ketoconazole control
showed halos in all tested strains *C. albicans* 24433 (30 mm), *C. albicans* 14053 (14 mm), *C. krusei* (20 mm) and *C. glabrata* (18 mm).

Studies like that of Kouidhi et al. (2010) used the same test and reported the potent antibacterial and antifungal activity of *Eugenia caryophyllata* (clove) oil against 114 strains of cariogenic bacteria and 46 strains of yeast, including *Candida albicans*, *Candida guilliermondii*, *Candida glabrata*, and *Candida tropicalis*.

The search of Fu et al. (2007) corroborates with the previously mentioned studies, where they tested the essential oil of clove (*Eugenia coryophyllata*), and described the antifungal activity of this oil against strains of *C. albicans*, with inhibition halos of 32 mm.

Shetty et al. (2018) tested an autosomal clove oil gel for the treatment of cutaneous candidiasis. To evaluate the antifungal sensitivity “in vitro”, they used the diffusion test and compared it with nystadine. As a result, it was observed that the ethosomal gel incorporated into clove oil presented a halo of 21 ± 4,89 mm of inhibition when compared to 28,91 ± 5,23 mm of pure nystatin. According to the authors, this oil can be a promising new treatment against *C. albicans*.

In our work, we also used the Kirby-Bauer disk diffusion test against *Candida albicans* 24433 and *Candida albicans* 14053, and unlike the authors mentioned above, we did not observe the formation of inhibition halos for any of the tested strains.

Sharma & Hegde (2014) created a product containing (mistletoe gel + 30% of *Melaleuca alternifolia* essential oil), and tested its antifungal sensitivity in plaque, comparing the activity of this product with a control (mistletoe gel + 5% fluconazole) against strains of *C. albicans*. They observed that the gel containing *Melaleuca alternifolia* oil was effective, showing a halo of 21,89 mm in 24 hours and 20,56 mm after 7 days of incubation. In the control gel, despite having a larger halo (34,56 mm in 24h), on the seventh day, there was a decline in the size of the formed halo.
The authors highlighted in this study that although both products showed comparable antifungal activity at 24h against the tested yeast, fluconazole completely lost its activity after the seventh day, while the visco-gel containing tea tree oil substantially maintained its antifungal action (Sharma & Hegde 2014). In this work, the authors do not report what this gel was made of but described that they tested different concentrations of tea tree oil (1%, 5%, 10%, 20%, 25%, 27.5%, 30%, and 35%) mixing with this gel and in the concentration (gel + 30% tea tree essential oil) obtained better results.

The opposite results to our previous ones observed in diffusion tests, perhaps, can be explained, because the mentioned works have used only oils “in nature”. The Biocompound is a product, and its formulation features a mixture of oils, including vegetable oils and not just clove and tea tree essential oils, which may have interfered with its final result.

In contrast, the work of Hammer et al. (1998) analyzed the “in vitro” action of tea tree essential oil and three other formulations that contained this oil in their composition against C. albicans and non-albicans, as a result, they reported that the minimum concentration of oil that killed 90% of the isolates was 0.25% for C. albicans and 0.5% for non-albicans species. The three tested products containing tea tree oil were shown to have MIC and fungicidal properties comparable to the pure oil, indicating that tea tree oil contained in these products maintained its anticandidal activity. This work shows us that both the oil “in nature” and the products based on tea tree have antifungal action, however, this work did not reveal all the other compounds of the tested products nor the percentage of oil in each product.

Another reason for failure in disk diffusion tests may be due to the Biocompound being an oil, and when placed on disks, it does not diffuse well into the medium. Absence of an inhibition zone does not necessarily mean that the extract is inactive against the tested microorganism, but rather that the diffusion was not complete, especially for less polar compounds that
diffuse more slowly in the culture medium (Scorzoni et al. 2007, Bona et al. 2014).

Therefore, we performed other tests to prove the antifungal efficacy of the product. We carried out the MIC test to verify the minimum concentration of the Biocompound capable of killing 50% of the tested yeasts. In the literature, works carried out before ours, such as that of Pinto et al. (2009) showed that clove oil (Eugenia coryophyllata) exhibited broad-spectrum antifungal activity. MIC values ranged from 0,32 to 0,64 μg/ml, showing that this oil was active against all strains tested, including C. albicans, C. Krusei, and C. glabrata. According to the authors, the activity of this oil may be related to the presence of a high concentration of eugenol (85,3%). This component is considered the most active in clove oil, having antimicrobial, fungistatic, and fungicidal action.

Work carried out with the essential oil of Melaleuca alternifolia with the objective of evaluating its "in vitro" and "in vivo" effects on Candida albicans, reported that the minimum inhibitory concentration obtained was 0,195% (1,95 mg/mL) (De Campos Rasteiro et al. 2014). Tobolli et al. (2016) also carried out a test using tea tree essential oil against strains of C. albicans, as results showed that the oil of Melaleuca alternifolia inhibited the growth of C. albicans, presenting a MIC value of 0,375% (3,4 mg/ml).

These results corroborate our findings, as the Biocompound proved effective against all strains tested, always in comparison with our reference drug, at the following concentrations: for C. albicans ATCC 24433 (256 μg/mL) ketoconazole (0,80 μg/ mL), for C. albicans ATCC 14053 (154,60 μg/mL) ketoconazole (0,31 μg/mL), for C. krusei (30,80 μg/mL) ketoconazole (3,16 μg/mL) and C. glabrata ( 26,30 μg/ml) ketoconazole (3,18 μg/ml). According to Scorzoni et al. 2007, concentrations of MICs from 250 μg/mL are considered relevant for researching natural substances for therapeutic purposes, in most tests the Biocompound showed MICs lower than this. Our results only reinforce what the literature describes that tea
Tree and clove essential oils are excellent “in vitro” antifungals, being considered promising treatments in the fight against *C. albicans* and non-*albicans*. It is worth noting that *C. glabrata* and *C. krusei*, on which the Biocompound revealed a better selectivity index, are currently being considered highly resistant.

Corroborating our findings, Mertas et al. (2015) in their work, they tested tea tree essential oil in fluconazole-resistant *C. albicans*, performed the Kirby-Bauer disk diffusion test, and also did not observe growth inhibition halos in the tested strains. However, when performing the MIC, according to the authors, the tea tree essential oil was highly active against the tested strain, the MIC values ranged from 0,25% to 0,5%. The authors attributed the antimicrobial activity of tea tree essential oil mainly to terpinen-4-ol, the main bioactive component present in this oil. They also highlighted that tea tree essential oil can be used as a topical antiseptic to effectively treat superficial mycoses caused by *Candida* spp. resistant to fluconazole and other drugs.

Our next path was to analyze the cytotoxicity of the Biocompound in bone marrow cells and mice. We observed that the Biocompound showed low cytotoxicity values for these cells (244,20 µg/mL), reaching 8,3 times lower when compared to the reference drug ketoconazole (2,02 µg/mL).

Hammer et al. (2006) reported in their review on the toxicity of oil of *Melaleuca alternifoila*, that evidence from nearly 80 years of use suggests that topical use of this oil is relatively safe and adverse effects are minor and occasional. Research indicates that TTO is toxic if ingested in very high doses and can cause skin irritation only at very high concentrations. Allergic reactions to tea tree essential oil can occur in predisposed individuals and may be due to the various oxidation products that are formed by exposing the oil to light and/or air. Adverse reactions can be minimized by avoiding ingestion, applying only topically diluted oil, and using oil that has been
stored correctly. Although tea tree essential oil has the potential to be toxic if ingested in higher doses, the components are not genotoxic.

In the work of Prashar et al. (2006) the cytotoxicity of clove essential oil (*Syzygium aromaticum*) and its components against several mammalian cells was evaluated, and as a result this oil proved to be highly toxic at a concentration of 0.03%. The authors further reported that the cytotoxic activity of clove oil was attributed to a phenolic terpene, eugenol, which constitutes 78% of the oil and has been shown to be highly cytotoxic to skin cells when tested at concentrations as low as 0.06%. The second major component, β-caryophyllene (which constitutes 13% of the oil), did not contribute to cytotoxicity.

Assmann et al. (2018) investigated some possible mechanisms underlying the “in vitro” antitumor activity of tea tree essential oil (*Melaleuca alternifoila*) in human and mouse cells (MCF-7 and 4T1) and its cytotoxicity in fibroblasts (HFF-1) and peripheral blood mononuclear cells (PBMCs). As a result, it was reported that high concentrations of tea tree essential oil (≥ 600 μg/mL) showed remarkable antitumor activity, decreasing cell viability and cell proliferation of MCF-7 and 4T1 cells. Tea tree essential oil at 300 μg/mL increased the number of MCF-7 cells in the early stages of apoptosis. TTO, primarily at 300 μg/mL, slowed cell growth and stopped MCF-7 cells in the S phase of the cell cycle. Lower antitumor concentrations (≤300μg/mL) evaluated in MCF-7 and 4T1 cells were not cytotoxic to PBMCs and HFF-1. Furthermore, TTO (300 μg/mL) was not able to induce cell proliferation in fibroblasts after 72h, indicating a non-cytotoxic effect on these cells. Tea tree essential oil exhibited an “in vitro” antitumor effect on MCF-7 and 4T1 cells, decreasing cell viability and modulating apoptotic pathways, and blocking the cell cycle of MCF-7 cells.

Nikolić et al. (2017) investigated the chemical composition, antimicrobial, synergistic effect, and cytotoxic activity of essential oils *Citrus limon* (lemon), *Piper nigrum* (green pepper) and *Melaleuca alternifoila* (tea
Cytotoxic activity of essential oils was obtained by assay with MTT. This assay was performed on five tumor cell lines (HeLa, K562, A549, LS-174, and FemX) and one normal cell line (MRC5). The antitumor activity of this oil was verified, but it did not show toxic effects against the normal cell lineage (MRC5).

We know that the ideal drug should have high therapeutic effects and low levels of cytotoxicity, so we decided to analyze the selectivity index (SI) of this compound about our control. We observed that on the strain of *C. albicans* ATCC 24433 ketoconazole showed an index 2.6 times higher than the Biocompound. In *C. albicans* ATCC 14053, the reference drug also showed better activity than the Biocompound, with (SI) 4 times greater. On the other hand, in *C. krusei*, we verified the opposite, the Biocompound presented (SI) 12.4 times greater than ketoconazole. In *C. glabrata* we also observed that the Biocompound presented (SI) 14.7 times greater than ketoconazole. *C. glabrata* is the second most common causative agent of candidiasis in recent years, it acquires resistance quickly after exposure to azoles (Abrantes et al. 2013).

About the tests carried out here, we would like to point out that although the agar tests are the most used and have the advantage of simply evaluating the results by viewing the halo, they have the disadvantage of being very imprecise methods, with regard to the preparation of the plates and of the yeast inoculum. Also, depending on the chemical nature of the material added to the test disk, it may not diffuse to the area adjacent to the disk. The microdilution test, on the other hand, saves space, culture media and reagents, enabling the quantitative determination of the MIC, making it possible to perform several repetitions and several uniform dilutions of the extracts in only one plate per microorganism, increasing reliability of the tests. When comparing the results of the antifungal activity with the Biocompound using the agar diffusion method, it was observed that there was a significant difference between the analyzed agar diffusion methodology.
and the method of minimum inhibitory concentration in liquid medium. The broth microdilution method was the best option to determine the antifungal activity, as it provides quantitative data, in addition to being more reliable and economical to determine the antifungal activity of the Biocompound.

6. Conclusion

Our results suggest that the Biocompound is effective against fungi of the Candida genus in vitro, especially against C. glabrata and C. krusei, where it showed high rates of selectivity and low toxicity when compared to the reference drug Ketoconazole. These Candida strains have shown great resistance against current drugs, requiring new approaches in their combat, so the Biocompound can be a promising solution against fungi of the Candida genus.
References


