Antifungal activity of clove basil (Ocimum gratissimum L.) beverage fermented with kombucha culture

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ABSTRACT

Fungi are associated with several diseases in humans, however these diseases have been neglected over the years. Fungal infections are estimated to cause more than 1.5 million deaths annually. In addition, recent studies have revealed the presence of pathogens resistant to antifungal drugs available for treatment. In view of this scenario, it is necessary to invest in research aimed at the discovery and development of new antifungal therapies. An important aspect to be considered is the antimicrobial activity attributed to fermented beverages, such as kombucha, which is obtained from the fermentation of an infusion of plant parts by a symbiotic consortium of bacteria and yeast known as SCOBY (Symbiotic Culture of Bacteria and Yeast). In this context, the aim of this work was to elaborate a fermented beverage based on clove basil (*Ocimum gratissimum* L.) (CBK) and to evaluate its antimicrobial activity against fungi *Candida albicans*, *Candida auris*, *Candida glabrata*, *Candida tropicalis* and *Cryptococcus neoformans*. CBK has presented pH values within the required legislation for kombucha, demonstrating quality and safety standards in the fermentation process. As for antimicrobial analysis, CBK fermented for 15 days showed antifungal effect on all tested strains, however, the fungicidal effect was only verified against *C. albicans*, *C. auris* and *C. neoformans*. Thus, this study contributes to the description of a new source of antifungal substances and opens perspectives for future research on the biotransformation processes of chemical compounds during fermentation procedures.

Keywords: SCOBY, antifungal, fermentation.

RESUMO

Os fungos estão associados a diversas doenças no ser humano, porém essas doenças têm sido negligenciadas ao longo dos anos. Estima-se que as infecções fúngicas causem mais de 1,5 milhão de mortes anualmente. Além disso, estudos recentes revelaram a presença de patógenos resistentes aos antifúngicos disponíveis para tratamento. Diante deste cenário, faz-se necessário investir em pesquisas voltadas à descoberta e desenvolvimento de novas terapias antifúngicas. Um aspecto importante a ser considerado é a atividade antimicrobiana atribuída às bebidas fermentadas, como o kombucha, que é obtido a partir da fermentação de uma infusão de partes de plantas por um
consórcio simbiótico de bactérias e leveduras conhecido como SCOBY (Symbiotic Culture of Bacteria and Yeast). Neste contexto, o objetivo deste trabalho foi elaborar uma bebida fermentada à base de alfavaca-cravo (Ocimum gratissimum L.) (CBK) e avaliar sua atividade antimicrobiana contra os fungos Candida albicans, Candida auris, Candida glabrata, Candida tropicalis e Cryptococcus neoformans. A CBK apresentou valores de pH dentro da legislação exigida para kombucha, demonstrando padrões de qualidade e segurança no processo de fermentação. Quanto à análise antimicrobiana, a CBK fermentada por 15 dias apresentou efeito antifúngico em todas as cepas testadas, porém o efeito fungicida só foi verificado contra C. albicans, C. auris e C. neoformans. Assim, este estudo contribui para a descrição de uma nova fonte de substâncias antifúngicas e abre perspectivas para futuras pesquisas sobre os processos de biotransformação de compostos químicos durante procedimentos de fermentação.

Palavras-chave: SCOBY, antifúngico, fermentação.

1 INTRODUCTION

Fungi are associated with a wide spectrum of diseases in humans, ranging from acute pulmonary manifestations and skin lesions in immunocompetent individuals to severe, life-threatening infections, especially of immunocompromised patients (Romani, 2011). However, despite their alarming impact on human health, fungal infections have been continuously neglected over the years (RODRIGUES; ALBUQUERQUE, 2018).

Rodrigues and Albuquerque (2018), in a recent review, describe data from GRAFFI, Global Action For Fungal Infection, estimating that fungal infections kill more than 1.5 million people every year. Other estimates suggest an annual global occurrence of approximately 200,000 cases of cryptococcal meningitis, caused by fungi of the genus Cryptococcus, and 700,000 cases of invasive candidiasis, caused by fungi of the genus Candida. Another relevant fact is the occurrence of pathogens with resistance to antifungal drugs available for therapy, such as the multidrug-resistant pathogen Candida auris, which has emerged as a serious threat to human health, with some infections resistant to all major antifungal drug classes (LAMOTH; KONTOYIANNIS, 2018). It is worth to mention that there are no licensed antifungal vaccines and that the last antifungals approved for clinical use were echinocandins in 2002 (RODRIGUES;
ALBUQUERQUE, 2018).

Given the importance of human fungal infections, there is a clear need for further studies to develop more effective antifungal drugs that can overcome resistance to existing treatments (BROWN et al., 2012). In this sense, natural products can be sources of new antimicrobial molecules.

Kombucha is a fermented drink traditionally made from tea, sugar and a symbiotic colony of bacteria and yeast known as SCOBY (Symbiotic Culture of Bacteria and Yeast) (JAYABALAN et al., 2014). In vitro and in vivo studies point to potential biological effects of kombucha, including antimicrobial activity (VILLARREAL-SOTO et al., 2018; LEAL et al., 2018; KAPP et al., 2019; BARBOSA et al., 2022). Studies show that the benefits attributed to kombucha are due to compounds with bioactive properties, especially phenolic compounds and organic acids (CARDOSO et al., 2020).

Most of the metabolites present in kombucha come from the plant material used in its production, such as polyphenols. Nonetheless, many of these compounds have their structure modified and are transformed into new compounds during fermentation (DUTTA et al., 2019).

Although traditional kombucha is produced from the infusion of green or black tea (Camellia sinensis), the use of other products for the preparation of kombucha is described in the literature. Examples include herbal infusions, mallow flowers, coffee, oak leaves, eucalyptus, laurel, fruit juices, milk and soy products, which have been shown to be good alternative substrates (EMILJANOWICZ et al., 2019; SILVA et al., 2021; DE MIRANDA et al., 2022).

All these substrates stand out for their chemical composition rich in bioactive compounds, which favor several physiological effects, in addition to potential biological effects (ROCHA-GUZMÁN et al., 2019). Therefore, studies to investigate kombucha SCOBY-fermented beverages prepared from different substrates have been stimulated, mainly to develop new drinks with more attractive properties (EMILJANOWICZ et al., 2019).

Within this context, clove basil (Ocimum gratissimum L.) presents a high potential for the production of fermented beverage, since studies carried out with
its extracts have demonstrated many biological properties, among them antifungal (OLIVEIRA et al., 2016; MOHR et al., 2017) and antibacterial activity (FRANCO et al., 2007).

Reiterating the above data, the importance of fungal infections and the emergence of resistant strains justify the development of works that seek new molecules that exert antifungal effect and act in the elimination of fungi and in the resolution of these diseases. Since kombucha is a product with high antimicrobial potential, research using alternative substrates in the production of these beverages, exploring its antifungal effect is extremely relevant. Therefore, the aim of this work was to evaluate the antifungal activity of a fermented beverage based on clove basil (*Ocimum gratissimum* L.) against pathogenic and opportunistic fungal species, as well as to verify the toxicity absence or presence in this beverage.

2 MATERIAL AND METHODS

2.1 RAW MATERIAL

The clove basil (*Ocimum gratissimum* L.) sample was collected in the medicinal garden of Federal Institute of Mato Grosso, Juína campus. Crystal sugar, vinegar and mineral water were purchased from local shops in Juína. SCOBY used for the production of the fermented beverage was acquired through donation by Vida Probióticos company.

2.2 SCOBY ENVIRONMENT

The donated SCOBY was set in a clove basil infusion for the production of samples to be tested. The basil was dehydrated by incubation in an oven at 50°C for 24 hours to obtain a dry substrate. Afterwards, the substrate was transferred to a grail and crushed with the aid of a pestle to prepare the infusion. 15 g of SCOBY were added to 500 mL of infusion prepared with dehydrated clove basil at a concentration of 3%, containing 25 g of sugar (5%) and 1 mL of apple cider vinegar. The beverage was then incubated at room temperature (25±3°C) for 21 days (JAYABALAN et al., 2014).
2.3 PREPARATION OF FERMENTED BEVERAGE

The amount of 60 g of the dehydrated substrate (3%) was transferred to a glass jar, and then 2000 mL of natural mineral water at 90°C was added to obtain the base infusion. The substrate was infused for 10 minutes. An aliquot of 500 mL was separated for control experiments. 75 g of sugar (5%) was added to the remaining 1500 mL of infusion. Subsequently, this remaining infusion was aliquoted into 500 mL volumes in three glass jars for experimental triplicates. The inoculum composed of SCOBY (3%) and 50 mL of fermented product (10%) used to set the symbiotic culture were added to each beaker. The jars were covered with paper towels to protect against contaminants and still allow air entrance. The beverages were incubated without stirring at room temperature (25±3°C) for 15 days. Aliquots of 10 mL were taken from each jar on 0, 6 and 15 fermentation days, processed on sterile filter with 0.22 µm pore membrane (Millipore) and stored at -20°C.

2.4 PH MONITORING

The beverage pH was measured on days 0, 6 and 15 of the fermentation process, by direct reading, in a potentiometer (Bel Engineering PHS3BW) calibrated periodically with buffer solutions of pH 4.0 and 7.0 at 25°C (AOAC, 1992).

2.5 ANTIFUNGAL ACTIVITY

The antifungal activity was evaluated by the microdilution method in 96-well microplates according to the M27-A3 document proposed by the Clinical and Laboratory Standards Institute (CLSI) with determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) (CLSI; 2017).

2.5.1 Fungi and cultivation

For antifungal activity assays, Candida albicans, Candida auris, Candida glabrata, Candida tropicalis and Cryptococcus neoformans species were grown
in Sabouraud dextrose broth (Kasvi) at 30°C under 150 rpm stirring for 48 hours or in Sabouraud dextrose agar at 30°C for 48 hours.

2.5.2 Determination of Minimum Inhibitory Concentration (MIC)

For MIC tests, the number and viability of fungal cells were first determined. Yeasts were counted after addition of Trypan blue in a Neubauer hemocytometer in order to obtain a suspension of 10⁸ cells/mL. A 1:10 dilution was then made in sterile PBS and consecutively a 1:10 dilution in Sabouraud broth was carried out to obtain the desired concentration of 1 x 10⁶ cells/mL. 100 µL of this prepared inoculum was added to each well from the 96-well plate. Samples of the fermented beverage made from clove basil were tested at fermentation times of 0, 6 and 15 days, as well as the non-fermented drink. Serial dilutions were prepared by adding 100 µL of a sample into 100 µL of Sabouraud broth. Afterwards, 100 µL of the standardized fungal culture was inoculated into all wells, except for the negative control wells (Sabouraud broth only). The concentrations of the tested samples ranged from 500 to 1 µL/mL. The microplates were incubated at 30°C for 48 h. The concentrations of Amphotericin B, the antifungal agent used as control in the experiment, were 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625 (µg/mL). Results were obtained visually and by optical density reading. The MIC was obtained from three independent experiments.

2.5.3 Determination of the Minimum Fungicidal Concentration (MFC)

Qualitative analysis of fungal viability was performed to determine MFC. For this purpose, a 5 µL aliquot of the cultures from the inhibited growth wells was transferred to a plate containing Sabouraud agar and incubated at 30°C for 48 hours. The MFC was determined as the lowest sample concentration which prevented yeast growth in the solid medium after the incubation period. Results were obtained by visual reading of three independent experiments.
3 RESULTS AND DISCUSSION

Regarding the pH results of the beverages produced, a reduction of these values was observed at the end of 15 days of fermentation, the pH ranged from 4.21 to 3.13. The graph below (Figure 1) shows the variations of the pH parameters. The pH drop demonstrates the occurrence of the fermentative process, as acetic bacteria mainly participate in this process by producing acetic acid from the ethanol produced by yeasts (NEFFE-SKOCINSKA et al., 2017). Monitoring pH is essential to ensure the safety of these beverages, determine the end of fermentation and ensure that the values are between 2.5 and 4.2, parameters established in the Standard of Identity and Quality (SIQ) of kombucha (WANG et al., 2022; BRASIL, 2019). The results show that the beverage fermented from an infusion based on clove basil was within the pH parameters established for kombucha.

Figure 1. pH variation over days 0, 6 and 15 of the fermentation process. The values shown in the graph are the average of three pH values obtained from three independent samples.

Regarding the results of the antifungal activity evaluation of the analyzed beverages, only the highest concentration (500 µL/mL) of the drink fermented for 15 days showed an inhibitory effect on the growth of all fungal species tested. Moreover, the concentrations of 250 and 125 µL/mL also inhibited the growth of the fungus C. neoformans. Table 1 presents the MIC and MFC data of the beverages produced.
The determination of the minimum fungicidal concentration (MFC) of kombucha samples on the fungal strains *C. albicans*, *C. auris*, *C. glabrata*, *C. tropicalis* and *C. neoformans* was evaluated from the inoculum of aliquots taken from the wells of the microdilution assay that showed growth inhibition. This assay verifies whether or not the fungus resumes growth after removal of the antifungal test substance, which in this study were the CBK samples, and thus determining whether the activity was fungistatic or fungicidal. According to table 1 and figure 2, there was fungicidal effect on *C. albicans* and *C. auris* strains at the highest tested concentration of the CBK sample fermented for 15 days, determining the same MIC and MFC value. The 250 µL/mL concentration was the MFC, while the MIC was 125 µL/mL for *C. neoformans*. None of the concentrations tested had a fungicidal effect against the fungi *C. glabrata* and *C. tropicalis*.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Samples</th>
<th><em>C. albicans</em></th>
<th><em>C. auris</em></th>
<th><em>C. glabrata</em></th>
<th><em>C. tropicalis</em></th>
<th><em>C. neoformans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC (µL/mL)</td>
<td>CBI</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td>CBK₀</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>CBK₆</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td>CBK₁₅</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>125</td>
</tr>
<tr>
<td>MFC (µL/mL)</td>
<td>CBI</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>CBK₀</td>
<td>ND</td>
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<td></td>
<td>CBK₆</td>
<td>ND</td>
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<tr>
<td></td>
<td>CBK₁₅</td>
<td>500</td>
<td>500</td>
<td>ND</td>
<td>ND</td>
<td>250</td>
</tr>
</tbody>
</table>

ND – No detected
Source: authors.

Samples with fermentation time of 0 and 6 days and with pH of 4.2 and 3.8, respectively, showed no inhibitory effect on any species tested. However, the CBK fermented for 15 days, with a pH of 3.1, did presented. Thus, the antifungal effect can be attributed to both the lower pH and the substances produced and/or modified during the fermentation process, or even to the accumulation of these substances over time, reaching an amount necessary to inhibit the development of yeasts.

The infusion of unfermented basil (CBI) was tested at the same concentration used to produce the fermented beverages (3%) in order to
investigate whether the inhibitory effect observed on the fungi was related to the substances present in the plant. But no antifungal activity was observed. Therefore, products originating in the fermentation process present in the beverage fermented for 15 days are responsible for the antifungal activity.

Although not all fungal species were sensitive to the tested concentrations, these results are promising, since recent work carried out with *C. albicans* and *C. neoformans* strains showed no antifungal effect of mallow kombucha and little effect of green tea kombucha. Nevertheless, the authors argue that this antifungal effect was due to substances already present in unfermented green tea, as it showed similar antifungal activity to kombucha based on this tea (SILVA et al., 2021).

De Miranda et al. (2023) published results that corroborate the data presented in this study. The authors analyzed the antifungal activity of kombucha based on Arabica coffee infusion against fungal strains of *C. albicans*, *C. gattii* and *C. neoformans*. All fungi were sensitive to the beverage at the highest concentration tested (500 µL/mL) and at the longest fermentation time (21 days), except for *C. neoformans*, which also showed sensitivity to beverages with shorter fermentation time (12 and 15 days).

Another relevant fact to be pointed out was that the CBK fermented for 15 days showed fungicidal activity against the *C. auris* strain, an emerging pathogen resistant to all major classes of antifungal drugs (LAMOTH; KONTOYIANNIS, 2018).
Figure 2. Determination of the minimum fungicidal concentration (MFC). Image of Sabouraud agar plates inoculated with aliquot of cultures from the wells with inhibited growth in the minimum inhibitory concentration (MIC) determination test. The concentrations shown in the image correspond to the concentrations of the wells with no growth. C+ corresponds to positive control of fungal growth.

Source: authors.

4 CONCLUSION

Reiterating the exposed data, it was possible to prove the metabolic activity of the symbiotic culture of kombucha in clove basil infusion by reducing pH within the established range for kombucha; and that this drink showed antifungal activity against all fungal species tested on the 15th day of fermentation. However, the compounds or mechanisms involved in antimicrobial activity are still unknown and further studies are needed. Therefore, this study contributes with a description of a new source of antifungal substances and opens perspectives for more detailed studies on microbial biotransformation processes of chemical compounds during the fermentation process.

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REFERENCES


5. CARDOSO, RODRIGO REZENDE.; et al. Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. Food Res Internat, v.128, p.108782, 2020. doi:10.1016/j.foodres.2019.108782


11. FRANCO, ANA LUIZA PEREIRA; et al. Avaliação da composição química e atividade antibacteriana dos óleos essenciais de Aloysia gratíssima (Gillies &


20. RODRIGUES, Márcio L.; ALBUQUERQUE, Priscila C. Searching for a change: The need for increased support for public health and research on fungal diseases. *PLoS Neglected Tropical Disease*, v.12, n.6, p.e0006479, 2018. doi:10.1371/journal.pntd.0006479

