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COMPARISON OF THE ANTIFUNGAL ACTIVITY OF BLACK, WHITE, AND GREEN TEA KOMBUCHA EXTRACTS AGAINST DERMATOPHYTE FUNGI

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ABSTRACT

Kombucha is obtained by fermentation of tea and sugar by bacteria and yeast. This beverage has several beneficial effects, including antimicrobial activity. The objective of this study was to evaluate the antifungal activity of Kombucha extracts against dermatomycosis-causing fungi. Different types of Kombucha (black, white, green tea) and times of fermentation (10, 20, 30 days) were evaluated. Kombuchas were filtered, and extracts obtained by liquid-liquid extraction with ethyl acetate. Minimum inhibitory concentrations (MIC) were determined against clinical isolates of dermatophytes *Trichophyton rubrum* and *T. mentagrophytes* by broth microdilution. Toxicity tests and high-performance liquid chromatography (HPLC) were also performed. MIC ranged from 62.5–2000 µg/mL. Green tea Kombucha and 10-days fermentation extracts showed the best results. Toxicity was observed only for white tea Kombucha. Despite the chromatographic profiles, further studies are needed to characterize the active molecules. However, Kombucha extracts may constitute a viable alternative for new treatment strategies against dermatomycosis.

Keywords: Kombucha; Antifungal susceptibility testing; *Trichophyton mentagrophytes*; *Trichophyton rubrum*; Dermatophytosis; Liquid-liquid extraction

INTRODUCTION

Kombucha is a beverage originally obtained by the fermentation of *Camellia sinensis* leaves and sugar. It is believed that it has its origin in China, around 220 B.C. Recently, it has gained popularity for its detoxifying and energizing properties (**Jayabalan et al., 2014**). With a slightly acidic, sweetened, and carbonated taste, Kombucha is consumed worldwide as a homemade beverage, or it can be found on stores and supermarkets ready to drink (**Baschali et al., 2017; Marsh et al., 2014**). The fermentation process is carried out by a starter culture composed of acetic acid bacteria (for example, *Komagataeibacter*, *Gluconobacter*, and *Acetobacter* species) and yeast (*Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis*, among others) named SCOBY (symbiotic culture of bacteria and yeast) (**Coton et al. 2017; Primiani et al., 2018; De Roos and Vuyst, 2018**). Its composition is variable, depending on the geographic location, climate, local composition of bacterial and yeast species, and the origin of the inoculum (**De Filippis et al., 2018; Jayabalan et al., 2014**). The organisms on the SCOBY can ferment the sugary tea, producing a variety of metabolites, for example, carbohydrates; polyphenols, such as catechins; amino acids, such as lysine; essential elements, such as sodium, potassium, calcium, copper, iron, and zinc; water-soluble vitamins, such as C, B, and B2; hydrolytic enzymes, among others (**Jayabalan et al., 2014**).

Although Kombucha is traditionally prepared by fermenting black tea for 7 to 10 days, other types of plants can be used, including *Camellia sinensis* in distinct stages of processing (green and white teas), rosemary and hibiscus infusions, fennel, lemon balm, mint, among many others. Each type of infusion may alter the Kombucha's chemical composition and, consequently, its properties (**Battikh et al., 2012; Chen and Liu, 2000**).

Kombucha have many beneficial effects reported in the literature: antimicrobial, antioxidant and anticarcinogenic activities (**Jayabalan et al., 2011**); diabetes treatment and prevention (**Aloulou et al., 2012**); treatment of gastric ulcers (**Banerjee et al., 2010**); lowering cholesterol (**Yang et al., 2009**); and immune effects (**Ram et al., 2000**). Although toxicity related to the ingestion of Kombucha has already been reported, it was mainly attributed to inadequate conditions of hygiene and sterility (**Leal et al., 2018**).

Kombucha's antimicrobial activity is a result of biologically active components formed in fermentation. Consequently, the positive effects of different teas and infusions can be optimized by association with Kombucha (**Battikh et al., 2012**). In the current scenario, with increasing numbers of individuals susceptible to infections, to study variations of Kombucha can lead to the discovery of new molecules and beneficial effects.

Antifungal activity of Kombucha beverages has already been demonstrated against *Candida* spp., such as *C. albicans*, *C. glabrata*, and *C. tropicalis* (**Battikh et al., 2012**). Strong inhibitory activity was also observed against *Microsporum* spp. and *Malassezia* spp., two agents of superficial mycoses in humans and domestic animals (**Mahmoudi et al., 2016; Santos Jr et al., 2009; Yuniarto et al., 2016**). Kombucha's activity has also been reported against *Aspergillus flavus*, a fungus that can cause opportunistic infections, especially in immunocompromised patients (**Yuniarto et al., 2016**).

Fungal infections are often defined by the causing agent or affected area. Among the most prevalent, dermatophytosis are superficial infections caused by dermatophyte fungi (i.e., genera *Trichophyton*, *Microsporum*, and *Epidermophyton*). When they affect the nail, the infection is named onychomycosis, which is characterized by thickening, roughness, and fragmentation of the nail, and affects 10 to 30% of the world population. Its pathology evolves from discomfort and pain to physical and occupational limitations, reducing the patient's quality of life. Although onychomycoses can be caused by many agents, *Trichophyton rubrum* and *Trichophyton mentagrophytes* are the most frequent agents in most of the countries. Oral treatment is often necessary, and terbinafine is the primary drug of choice, followed by itraconazole, fluconazole, ketoconazole, and griseofulvin. However, prolonged treatment may cause systemic side effects, including liver and cardiac damage (**Angelo et al., 2017**).

Within the purpose of finding new antifungal strategies and improving the quality of life of patients with onychomycosis and dermatophytosis, the main objective of this study was to evaluate the antifungal activity of extracts obtained from Kombucha made from different types of tea against *Trichophyton rubrum* and

Trichophyton mentagrophytes. In addition, to assess the toxicity of these extracts in an in vivo model.

MATERIAL AND METHODS

Preparation of SCOBY, teas, and Kombucha

The SCOBY (Casa Amarela®, São José do Rio Preto, Brazil) was added to one litre of sterile distilled water with 20 g of sucrose (Synth®, Diadema, Brazil) and incubated at 25ºC, for 7 days. This procedure was performed twice to remove traces of the original medium where it was produced. The SCOBY was then fragmented into parts of 20g each and added to the different teas: black – *Camellia sinensis* (L.) Kuntze (Amaya Chás®, Registro, Brazil); white – *Camellia sinensis* (L.) Kuntze (Kampo de Ervas®, Ribeirão Preto, Brazil); and green – *Camellia sinensis* (L.) Kuntze (Kampo de Ervas®, Ribeirão Preto, Brazil). These mixtures (teas and SCOBY) are called *starters*. They were incubated at 25 ºC for 20 days for initial fermentation.
Teas and Kombucha were prepared as previously described (**Chen and Liu, 2000**), with modifications. Four grams (4 g) of black tea (BT), white tea (WT), and green tea (GT) were boiled separately in 450 mL of sterile mineral water for 5 minutes and filtered through sterile filter paper. In a beaker, the volume was adjusted to 800 mL with sterile mineral water at room temperature. Then, 80 g of sucrose, 20 g of SCOBY, and 160 mL of starter from the respective tea were added to each beaker. These solutions were incubated at 25ºC. To verify the influence of fermentation time, different beakers were incubated for 10, 20, and 30 days.

Preparation of the extracts

After each incubation period, the SCOBY was weighed to calculate the difference between the initial and final mass, followed by filtration on a Millipore® system, with a 0.45µm filter membrane.
The pH of the fermentations was measured with a pH indicator tape (MColorpHast®, Merck, Dermstadt, Germany) and the extracts were obtained as previously proposed (**Mahmoudi et al., 2016**), with modifications. The filtrates of each tea were subjected to liquid-liquid extraction by adding 96 mL of ethyl acetate (Synth®, Diadema, Brazil) as a counter-phase. This procedure was repeated twice for each Kombucha, allowing total extraction of chemical compounds. The ethyl acetate phase was subjected to a rotary evaporator, for concentration of the purified extract. Dried extracts were diluted in 10% DMSO.
Pure white, black, and green teas extracts were also prepared to determine the influence of the SCOBY fermentation on the antifungal activity.

Kombucha extracts antifungal susceptibility testing

Two clinical dermatophytes isolates from onychomycosis were included in this study, one *Trichophyton rubrum* and one *Trichophyton mentagrophytes*. They were from the Microbiology Laboratory of the São José do Rio Preto Medical School (FAMERP).
The antifungal activity of WT, BT, and GT of Kombucha and pure extracts were determined by the microdilution method, according to the Clinical Laboratory Standards Institute (CLSI), document M38-A2, with modifications (**CLSI, 2008**). The inoculums were adjusted to a concentration of 0.4 to 5 x 10^4 cells/mL. Each extract was prepared in sterile distilled water at a concentration of 2000 µg/mL and serially diluted in a 96-well plate until the concentration of 15.62 µg/mL. Controls included the extract solvent (10% DMSO), to prove that the solvent alone does not have antifungal activity, and RPMI-1640 (Roswell Park Memorial Institute) medium, with and without inoculum.
One hundred microlitres of inoculum was added to each well and the microplates were incubated at 35ºC, for 7 days. After incubation, readings were performed visually. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract that resulted in 100% of inhibition of visible growth. All controls showed the expected growth.
An aliquot from each well that showed inhibition was plated on Sabouraud Dextrose agar (SDA) (Difco®, Detroit, USA) to determine the minimum fungicidal concentration (MFC), which was defined as the lowest concentration of extracts that did not allow visible growth on the solid medium (**De Toledo et al., 2016**). Tests were performed in duplicate.
The fungal isolates were also submitted to susceptibility testing against fluconazole, according to the document M38-A2 of CLSI (**CLSI, 2008**), for comparison.

Toxicity Test on *Galleria mellonella* model

The toxicities of the Kombucha extracts were analysed in an in vivo model of sixth-instar *G. mellonella* larvae (weighing 250 to 350 mg each), according to the methodology previously described (**Gottardo et al., 2019**). The analysis included larvae bathed in the extracts and artificial inoculation into the larvae's haemolymph. For the first method, to assess surface toxicity, the larvae were bathed for 2s in each extract (BT, WT, and GT) at a concentration of (8 mg/mL). In the inoculation method, 5 µL of each Kombucha extract (8 mg/mL) was injected with a Hamilton 10 µL 7000.5 KH syringe into the larval hemocoel through the last right proleg. Quaternary ammonium and PBS solutions were used as positive and negative controls of toxicity, respectively. The larvae were incubated at 28°C, deprived of food and direct light. Every 12 hours, larvae were removed from the prepupae stage to delay their metamorphosis. Survival rate was analysed every 24 hours, and statistical analyses and graphs were generated by the log-rank method (Mantel-Cox) in Prism 5 software (GraphPad®).

Chromatographic analysis

The qualitative chemical composition of the Kombucha extracts were analysed by high performance liquid chromatography (HPLC). The chromatoplates were inspected using sulfuric anisaldehyde reagent, under ultraviolet light (wavelengths of 256 nm and 365 nm). The analyses were performed in a system equipped with a binary pump, autosampler column (Agilent® Technologies, Santa Clara, USA, model 1220 Infinity) and Zorbax Eclipse Plus C-18 column (4.6×250 mm, 5µm, Agilent® Technologies, Santa Clara, USA), using isocratic flow in methanol:water (3:1) at 1.0 mL/min. An aliquot of 20 µL of each sample was injected and analysed by a diode array detector at wavelengths from 250 nm to 800 nm.

RESULTS AND DISCUSSION

Dermatomycosis, such as onychomycosis and superficial skin infections, are immensely common worldwide. These infections have a negative psychosocial impact and may cause inflammation and physical discomfort (**Urban et al., 2021**). Treatment is often prolonged and ineffective. Besides, the number of classes of antifungals is limited, and oral drugs may present side effects, including hepatotoxicity (**Hay, 2018**). This study shows preliminary results demonstrating the antifungal activity of Kombucha tea extracts against dermatophytes commonly found in clinic. Although the relevance of dermatomycosis and the fact that Kombucha has been gaining attention in the last years, data regarding this topic is scarce. The results showed here should promote the search for new natural molecules that could result in better treatment options, with low toxicity, which could improve patient's quality of life.

Antifungal activity of Kombucha extracts

The pure tea extracts exhibited antifungal activity, with equal MIC values for *T. rubrum* and *T. mentagrophytes*, 250 µg/mL for white tea extract and 125 µg/mL for black and green teas extracts (Table 1). Antifungal activity of *Camellia* tea has been reported previously, including green tea extract against *T. mentagrophytes* (**Cheruiyot et al., 2015**). However, great MIC range can be observed due to differences in methodology, including amount of tea used, type of leaves, extract preparation, etc.
Kombucha tea is also known to have antifungal activity against a variety of fungi, including *Candida*, *Malassezia*, and *Microsporum* (**Mahmoudi et al., 2016; Yuniarto et al., 2016**). However, it was not found in the literature tests against *Trichophyton* spp. In this study, MIC values of Kombucha tea extracts against *Trichophyton* isolates ranged from 62.5 to 2000 µg/mL. Considering the type of tea, green tea exhibited the lowest mean MIC value (geometric mean – GM – of 125 µg/mL), while black tea had lower activity (GM 222.7 µg/mL). Concerning time of fermentation, a 10-days period exhibited better activity (GM 111.3 µg/mL), in relation to 20-days (GM 250 µg/mL) and 30-days (GM 176.8 µg/mL).
The 10-days fermentation extracts showed the same MIC values of pure extracts for *T. mentagrophytes* (250 µg/mL for WT and 125 µg/mL for BT and GT). For *T. rubrum*, antifungal activity was higher for the 10-days fermentation Kombucha extracts, with MIC values of 125 µg/mL for WT, and 62.5 µg/mL for BT and GT. For 20-days fermentation extracts, results varied according to the tea of origin. The best activity was observed for white tea Kombucha, followed by green and black teas (Table 1). MIC values for 30-days fermentation extracts were equal or higher than the pure tea extracts, except for the black tea against *T. rubrum*, which was 125 µg/mL for pure tea, and 62.5 µg/mL for Kombucha tea extract.
Regarding the analyses of MFC, tests revealed values equal or similar to the MIC values for most of the extracts (Table 1). These results indicate that the extracts tested exert a fungicidal activity, rather than fungistatic.

Table 1 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values (µg/mL) of each Kombucha tea extract, with different times of fermentation, against *Trichophyton mentagrophytes* and *T. rubrum*

Tea	Fermentation Days	*T. mentagrophytes*		*T. rubrum*	
		MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
White	0 (pure)	250	250	250	250
	10	250	250	125	125
	20	125	500	62,5	500
	30	500	500	250	1000
Black	0 (pure)	125	125	125	125
	10	125	125	62,5	62,5
	20	2000	2000	1000	1000
	30	125	250	62,5	62,5
Green	0 (pure)	125	125	125	125
	10	125	125	62,5	62,5
	20	250	500	62,5	62,5
	30	250	250	125	125

Kombucha fermentation time is often reported to be between 8 and 14 days (**Chen and Liu, 2000; Coton et al., 2017; De Filippis et al., 2018; Mahmoudi et al., 2016**). The literature reports, and it is corroborated here, the fermented Kombucha extract has the potential to show a higher activity than the pure extract (**Al-Mohammadi et al., 2021**). However, it has also been postulated that the antifungal activity of Kombucha tea decreases with longer periods of fermentation (**Yuniarto et al., 2016**). We could also observe it, since 10-days extracts showed the best antifungal activities. Metabolites and cell concentration vary during fermentation (**Chen and Liu, 2000**), consequently, time of fermentation plays a significant role in Kombucha antimicrobial activity.

The antifungal activity of the extracts was better on *T. rubrum* than on *T. mentagrophytes*, with geometric mean MIC values of 222.7 µg/mL for *T. mentagrophytes*, and 125 µg/mL for *T. rubrum*. The high proteolytic activity of *T. mentagrophytes* is a factor that helps in the fast mycelial growth of this fungus (**Pakshir et al., 2016**). This fact may help justify the difference in MIC values for *T. mentagrophytes* and *T. rubrum*.

Considering the biological divergence of the two fungal species, the different MIC values to fluconazole occurred as expected *T. rubrum* and *T. mentagrophytes* (1 µg/mL and 32 µg/mL, respectively). Moreover, the origin of the isolates (patient status, anatomical site of the infection, prior exposure of antifungal drugs or steroids), can also influence the difference of susceptibility of the isolates against the Kombucha tea extracts.

Galleria mellonella Toxicity Test

None of the superficial toxicity tests (bathed *G. mellonella* larvae) showed toxicity, i.e., all larvae remained alive after five days. Considering the inoculation tests, black or green tea Kombucha extracts did not show toxicity, results similar to the findings by **Silva et al. (2021)**. However, with extracts from white tea Kombucha, toxicity occurred, killing more than 50% of the larvae within five days (Figure 1).

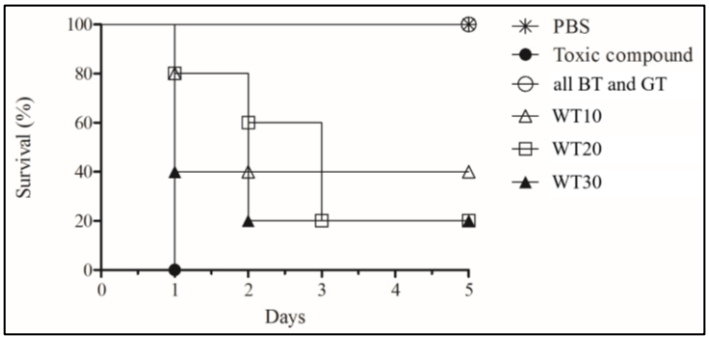

Figure 1 In vivo toxicity evaluation of Kombucha tea extracts. *Galleria mellonella* larvae survival rate after inoculation of the extracts. PBS: negative control (non-toxic compound), Toxic compound: positive control, BT: black tea Kombucha extract, GT: green tea Kombucha extract, WT: white tea Kombucha extract. Numbers correspond to days of fermentation

Due to its similarity to the innate immune system of insects and mammals, *G. mellonella* larvae have been used to assess virulence of microorganisms, as well as the toxicity of new antimicrobial compounds, as an alternative to mammalian models (**Binder et al., 2016**). The non-toxicity of black and green tea Kombucha extracts on *G. mellonella* is promising for their use in the control of onychomycosis and other dermatomycosis. Regarding white tea Kombucha extracts, further testing is necessary to verify its toxicity to humans with other methodologies.

Analysis of the chromatographic profile by High Performance Liquid Chromatography (HPLC) of Kombucha extracts

The extracts, including pure tea and Kombucha tea extracts, were submitted to HPLC analysis in lengths between 200 and 800 nm. The wavelength at 254 nm was selected to evaluate the chromatographic profile of the extracts as there was greater absorption in this spectral region. The chromatographic composition of the extracts can be seen on Figure 2.

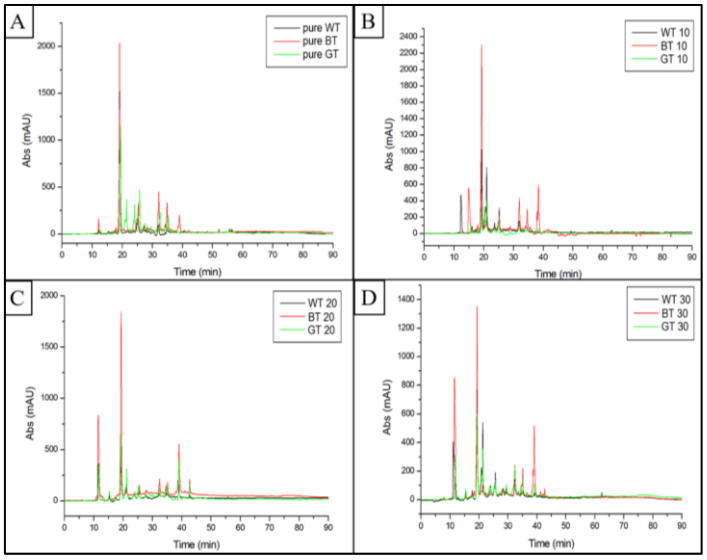

Figure 2 Chromatographic profiles obtained at 254 nm by high performance liquid chromatography of pure tea extracts (no fermentation), and white (WT), black (BT), and green (GT) tea Kombucha extracts. (a) pure tea extracts, (b) 10-days fermentation extracts, (c) 20-days fermentation extracts, and (d) 30-days fermentation extracts

HPLC analysis is an efficient and robust separation technique, it is simple to perform, and it has strong separation ability. Characterization of these profiles is a powerful tool to discriminate among metabolites and target possible active agents (**Wróblewski et al. 2019**). For pure white tea (pure WT), the analysis showed major chromatographic peaks in retention times between 15 and 20 min, with maximum absorptions between 1750 and 2000 mAU; and between 25 and 30 min with absorptions peaks between 250 and 500 mAU. In 10-days fermentation extracts (WT 10), chromatographic analysis showed chromatographic peaks at retention times of 10 to 15 min with maximum absorption of 400 to 500 mAU, indicating more polar metabolites than those observed in pure WT. In addition, there was a chromatographic peak in the retention time interval between 20 and 25 min, with maximum absorptions between 700 and 800 mAU; and a peak in the interval of 30 to 35 min with maximum absorption of approximately 50 mAU. Chromatographic peaks seen on pure WT were also seen on WT 10 (retention times between 15 and 20 min, and 25 and 30 min), however, on the latter, with lower absorption peaks (Figure 2B).

In 20-days and 30-days fermentation white tea Kombucha (WT 20 and WT 30), chromatographic profiles were similar to WT 10. However, some chromatographic peaks appeared at longer retention times (35 to 65 min), characterizing the formation of more hydrophobic metabolites (Figures 2C and 2D).

Chromatographic profiles from the extracts from the other teas were comparable to the ones from white tea Kombucha (Figure 2). These teas showed a similar composition, especially demonstrated by the peaks in retention times between 15 and 20 min. Moreover, these peaks were consistent in all black and green tea extracts after incubation periods of 10 to 30 days. In black and green teas extracts, similarly to white tea extracts, longer incubation periods resulted in the formation of new peaks, which is expected due to the fermentation process and the appearance of minor metabolites. This is supported by the literature. The number of volatile compounds in non-fermented tea can increase from 21 to 56, in fermented Kombucha (**Al-Mohammadi et al., 2021**). New peaks with longer

retention times have more hydrophobic characteristics, while shorter retention times reveal less hydrophobic characteristics.

The BT 10 extract showed a peak in retention times between 10 and 20 min that does not appear in the other chromatographs, which may be related to the higher antifungal activity of this extract, showed by the MIC results. Although the antimicrobial activity of the extracts seems to be attributed to the synergism among the components of the beverage, different substrates can lead to higher antimicrobial efficacy (**Nyiew et al. 2022**). To confirm these hypotheses, it is necessary the identification of the metabolites and further susceptibility testing using the isolated metabolites.

Future perspectives related to these results are to select the best extracts, considering antimicrobial activity and toxicity. In addition, to isolate and identify the active compounds, test a larger set of species and isolates, assess the activity of the extracts against fungal biofilms, and to perform drug interaction assays to determine if there are synergistic effects with available antifungal drugs.

CONCLUSION

Different types of Kombucha tea extracts exhibit antifungal activity against the isolates of *T. rubrum* and *T. mentagrophytes* included in this study. The best antifungal activities are observed for green tea Kombucha and 10-days fermentation extracts. Green and black tea Kombucha extracts do not present toxicity, according to the methodology used. Further testing is necessary to reveal the active compounds of the extracts and how to include them in a treatment alternative. However, this preliminary study shows promising results for the obtention of natural molecules with antifungal activity against common agents of dermatophytosis.

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