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Melaleuca alternifolia Cheel (Myrtaceae), *Citrus limon* (L.) Burm.f. (Rutaceae), *Caryocar brasiliense* Cambess (Caryocaraceae), *Pelargonium graveolens* L´Hér (Geraniaceae), and Propolis extract as *in vitro Sporothrix schenckii* inhibitors

Melaleuca alternifolia Cheel (Myrtaceae), *Citrus limon* (L.) Burm.f. (Rutaceae), *Caryocar brasiliense* Cambess (Caryocaraceae), *Pelargonium graveolens* L´Hér (Geraniaceae) e extrato de Própolis como inibidores *in vitro* de *Sporothrix schenckii*

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ABSTRACT

Sporotrichosis, a mycosis caused by fungus of the genus *Sporothrix*, has the itraconazole therapy of first choice. Resistance reports have been observed to both Itraconazol and amphotericin B, culminating in treatment failures and clinical cases like pulmonary or systemic infections. The aim of this study was to evaluate *in vitro* the effect of essential oils from *Melaleuca alternifolia* Cheel (Myrtaceae), *Citrus limon* (L.) Burm.f. (Rutaceae), *Caryocar brasiliense* Cambess (Caryocaraceae), *Pelargonium graveolens* L´Hér (Geraniaceae) and pure propolis extract or associated in inhibiting of *S. schenckii* growth. The methods used were Kirb-Bauer, disk diffusion on agar, Minimum Inhibitory Concentration and Minimum Fungicide Concentration. The essential oils and the propolis extract were effective in inhibiting fungal growth, overcoming the effects of itraconazole. Itraconazole was able to inhibit the growth of *S. schenckii* up to a dilution of 4mg.mL^{-1} (10^{-3} dilution). The essential oils of melaleuca, geranium, lemon, all at a concentration of 10mg.mL^{-1} and propolis extract at a concentration of 20mg.mL^{-1}, were able to inhibit, respectively, the development of this fungus at concentrations lower than 0.325mg.mL^{-1} (10^{-6} dilution), 0.15625mg.mL^{-1} (10^{-7} dilution), 0.325mg.mL^{-1} (10^{-4} dilution) and 0.625mg.mL^{-1} (10^{-6} dilution). Geranium and melaleuca essential oils showed the best inhibition and fungicidal potential against *S. schenckii*. These results suggest the importance of *in vivo* tests to evaluate the use of these herbal medicines as an alternative treatment against sporotrichosis.

Keywords: Essential oils. Phytotherapy. Sporotrichosis.

RESUMO

A esporotricose, uma micose subcutânea causada por fungos do gênero *Sporothrix*, tem o itraconazol como terapia de primeira escolha. Relatos de resistência têm sido observados tanto ao itraconazol quanto à anfotericina B, culminando em falhas nos tratamentos e surgimento de casos clínicos com infecções pulmonar ou sistêmica. O objetivo deste estudo foi avaliar *in vitro* o efeito dos óleos essenciais (OEs) de *Melaleuca alternifolia* Cheel (Myrtaceae), *Citrus limon* (L.) Burm.f. (Rutaceae), *Caryocar brasiliense* Cambess (Caryocaraceae), *Pelargonium graveolens* L´Hér (Geraniaceae) e extrato de própolis isolado ou em associação, na inibição do crescimento de *S. schenckii*. Os métodos utilizados foram Kirb-Bauer, difusão de disco em ágar, Concentração Inibitória Mínima e Concentração Fungicida Mínima. Os óleos essenciais e o extrato de própolis inibiram o crescimento fúngico, superando os efeitos do itraconazol. Esse último foi capaz de inibir o crescimento de *S. schenckii* até a diluição de 4mg.mL^{-1} (diluição 10^{-3}). Os OEs de melaleuca, gerânio, limão, todos na concentração de 10mg.mL^{-1} e extrato de própolis na concentração de 20mg.mL^{-1}, foram capazes de inibir, respectivamente, o desenvolvimento desse fungo em concentrações inferiores a 0,325mg.mL^{-1} (diluição 10^{-6}), 0,15625mg.mL^{-1} (diluição 10^{-7}), 0,325mg.mL^{-1} (diluição 10^{-4}) e a 0,625mg.mL^{-1} (diluição 10^{-6}). Os óleos essenciais de gerânio e melaleuca apresentaram os melhores efeitos inibitórios e potencial fungicida contra *S. schenckii*. Os resultados obtidos neste estudo sugerem que futuros testes *in vivo* sejam feitos para avaliar a eficácia desses fitoterápicos como tratamento alternativo contra a esporotricose.

Palavras-chave: Esporotricose. Fitoterapia. Óleo essencial.

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INTRODUCTION

The sporotrichosis is a subcutaneous mycosis caused by the fungi of the genus *Sporothrix* in which affects animals and humans, being domestic cat an important source of infection for humans. In felines, the most common symptoms are skin lesions in the cephalic region, limbs, and tail extremities. In humans, skin infection is frequent, however, it can evolve and affect various tissues and internal organs in immunocompromised individuals or with pre-existing chronic diseases (Gondim & Leite, 2020). The fungi contamination occurs by inoculating *Sporothrix* sp. into the dermis, mainly through scratches, injuries by wood barbs and plant thorns. It can also be transmitted by bite or scratch of infected animals, and even in rarer cases (Lima, 2017).

Fungi of the genus *Sporothrix* are ascomycetes, saprophytes, thermotolerant and dimorphic, growing as yeast at 37 °C and as mycelium in room temperature 25 ºC. Its main species are *S. schenckii, S. brasiliensis, S. globosa, S. mexican, S. albicans, S. luriei*, which can be found in saprophytic life in vegetables, soil, contaminated water, and parasitizing animals (Lima, 2017).

The spread of the disease is worldwide, occurring particularly in tropical and subtropical regions, such as Brazil, in which the incidence of sporotrichosis contracted by contact with infected cats is increasing. In a study with 161 strains of *Sporothrix* sp., from clinical and environmental samples from various regions of Brazil, it was proven that the species *S. brasiliensis*, *S. globosa*, *S. mexicana* and *S. schenckii* had the highest geographical distribution in the country (Macedo-Sales et al., 2019).

Itraconazole, terfinafine, potassium iodide and amphotericin B are drugs to treat sporotrichosis (Kauffman, Bustamante, Chapman & Pappas, 2007), but itraconazole is the first-choice drug prescribed. However, studies have reported the existence of resistant isolates to this drug, that has led to therapeutic failures and mycosis dissemination, apart from long treatment duration, high cost and toxicity (Queiroz-Fernandes & Magalhães, 2020). These reports reflect the necessity for alternative methods in sporotrichosis treatment, and medicinal plants is a possibility, since they have a high chemical compounds diversity and antimicrobial potential (Lima, 2017).

Herbal medicines are prepared by plant parts and have great treatment efficacy or prevention of various diseases worldwide, and can be used in the form of wax, extracts, and essential oils (EOs) (Cardoso et al., 2013). The use of EOs has been adopted since the beginning of primitive life and its use has grown among the current population. Studies report that the EOs have extensive septic and drug activity, being widely used in the treatment and prevention of diseases (Bakkali, Averbeck, Averbeck & Idaomar, 2008). This is because EOs are natural, liquid, complex, bioactive, volatile compounds with a characteristic color and odor, formed by secondary metabolites of aromatic plants (Almeida, Almeida & Gherardi, 2020).

In the present study, four EOs were chosen that presented relevant data as antimicrobials in the literature, being the EOs of *Melaleuca alternifolia* Cheel (Myrtaceae), *Citrus limon* (L.) Burm.f. (Rutaceae), *Caryocar brasiliense* Cambess (Caryocaraceae), *Pelargonium graveolens* L´Hér (Geraniaceae), and, in addition to these EOs, the propolis extract.

M. alternifolia belongs to the Myrtaceae family, commonly known as the "tea tree" or melaleuca, and has great medicinal importance, due to the presence in its composition active compounds that act with bactericidal, antifungal and virucidal action (Sampaio, 2022). It can be applied on skin cuts, wounds, scratches, fungal and bacterial skin infections, in addition to acting as a healing agent and obtaining antiseptic and expectorant action, which has highlighted this plant in phytotherapy studies worldwide (Silva, 2019).

A very common plant in Brazil that has shown potential as an antimicrobial is the *Caryocar brasiliense* Cambess (Caryocaceae), knowed as pequizeiro, which is one of the Brazilian's trees with the highest degree of utilization and is widely used in traditional medicine (Nascimento, 2018). Phenolic compounds, which are free radical blocking, were found in the pequi leaf and therefore it has been used as an antioxidant (Denger et al., 2020), besides presenting antifungal and molluscicides potential (Passos et al., 2002). This found may suggest the efficacy of pequi oil against fungi of *Sporothrix* sp.

Another extract very used by the Brazilian population against viral infections is the Propolis, which, in addition to acting as a plant hormone in the development of plants, protects it against attacks of fungi and bacteria. Due to its antimicrobial action, it has been widely used in studies that indicate its efficacy against human pathogens and other animals (Barbosa, Zuffi, Maruxo & Jorge, 2009). Many people, both in traditional and popular medicine, used to drink Propolis extract every day to avoid infections and to reduce flu symptoms (Ozarowski & Karpinski, 2023).

Also, the genus *Citrus* has several species, among them the *C. limon* (L.) Burm.f (Rutaceae), popularly known as lemon. The EO of *C. limon* has anticancer and antimicrobial activity, due to the phenolic and antioxidant compounds already described (Everton et al., 2018). In many countries, the lemon is popularly used for its effectiveness in treatment of infections, fevers, acidity in the stomach and for presenting antimicrobial, anti-inflammatory, antiparasitic and antiseptic properties. The EO of *Citrus limon* is composed mainly by limonene and citral, which have antimicrobial activity proven in studies on the bacteria *Escherichia coli* and *Staphylococcus aureus* (Santos, Carvalho, Barros & Guimarães, 2011).

Another common oil used by the population is the *Pelargonium graveolens* L´Hér (Geraniaceae), known as geranium, it has antioxidant, antibacterial, antifungal and acaricide properties. Its therapeutic effects are attributed to terpenoids and flavonoids in its composition (Lima, 2022). This oil has been used in aromatherapy against flu infections and has been suggested by companies that produce essentials oils for use during the menopause period to reduce the symptoms of undesirable estrogen deficit (Cheverria, Cheverria, Barreto, Echeverría & Mendoza, 2021).

By means of the presented, the present work verified the sensibility or resistance of *S. schenckii to* growth when essential oils and propolis extract were used like natural inhibitors.

MATERIALS AND METHODS

Culture of the microorganism

The microorganism used in this study was *Sporothrix schenckii*, which is part of the collection of microorganisms used in the Laboratory of Applied Microbiology (LAMAP) of the Minas Gerais State University (UEMG). It was isolated and identified from feline injury in the city of Ibirité-MG, having its identification followed the Manual of Detection and Identification of Fungi of Medical Importance of Brazilian Health Regulatory Agency (Anvisa) of 2004. A case of feline lesion was isolated and identified in the city of Ibirité - MG, and its identification was made according to the "Manual of Detection and Identification of Fungi of Medical Importance" of Brazilian Health Regulatory Agency (Anvisa) of 2004.

This process was carried out with subcutaneous tissue collected after antisepsis and placed in a sterile and sealed container, as directed for researching the agent, in sufficient quantity (> 2mL or 0.5cm^3 or twice). The culture was used for cultivation on Sabouraud Dextrose Agar according to the manufacturer's recommendations for observation of fine hyaline hyphae, septate with spores in arrangements, like a daisy flower. The visualization was possible after 10 days of cultivation at 30 ºC in a greenhouse.

The plate sample was also cultured at 25 ºC, which after 24 hours allowed the preparation of slides for the observation of small yeasts in 20% KOH, and by gram staining, for the visualization of cigar-type yeasts characteristic of *Sporothrix schenckii*. Genomic analysis by PCR was performed by a veterinary laboratory when the sample was collected while still in the clinic, confirming that it was *S. schenckii*.

Essential oils

The melaleuca, geranium and lemon EOs were obtained commercially from Laszlo® at the concentration of 10mg.mL^{-1}. The propolis extract was obtained commercially from the Santa Barbara® at the concentration of 20mg.mL^{-1}, and the vegetable oil of Pequi was obtained from *Mundo dos Óleos*® at the concentration of 10mg.mL^{-1}.

Kirb-Bauer test

For the Kirb-Bauer tests, 10µL of *S. schenckii* (1x10^8 CFU mL^{-1}) was inoculated by triplicate test using Petri dishes containing Mueller-Hinton Agar (MHA) prepared according to the manufacturer's recommendations. The inoculum was spread on a plate using Drigalsky's handle.

To make the disks in the laboratory, the filter paper was cut into circles using a paper punch with 0.4mm diameter. Inside the laminar flow chapel, the disks were soaked with 10µL of the extracts: M – melaleuca EO (10mg.mL^{-1}), L - lemon EO (10mg.mL^{-1}), G - geranium EO (10mg.mL^{-1}), Pr - propolis extract (20mg.mL^{-1}), Pe – pequi oil (10mg.mL^{-1}), I – itraconazole (16mg.mL^{-1}) was used as positive control, S1 – equal volumes of each oil were mixed in a sterile bottle, and S2 – equal volumes of each oil plus itraconazole were mixed in sterile vial.

To verify if pequi oil could inhibit the effect of other oils together to itraconazole, another treatment was named S3. The S3 had equal volumes of each essential oil and itraconazole except pequi oil. The disks were distributed on the plate containing the fungal inoculum and incubated at 37 °C for 48 hours. The zone of inhibition was measured with the aid of a caliper for further statistical analysis.

Minimum Inhibitory Concentration (MIC)

This test was based on the microdilution method in 96-well microplates suggested by the Clinical and Laboratory Standards Institute CSLI (2018). Then, 100µL of RPMI was distributed in wells of a micro-titration plate and 100µL of each phytotherapeutic products was added to the first well of its respective column. After homogenization, 100µL was transferred to the second well and so on.

The preparation of the inoculum consisted of resuspend from colonies developed in Muller-Hinton agar, a microbial suspension (*S. schenckii*) in saline, with turbidity equivalent to the 0.5 tube of the Mac Farland Scale (1x10^8 CFU mL^{-1} or 80% transmittance at 530nm). This suspension was diluted 1:100 with RPMI, obtaining as inoculum 1x10^6 CFU mL^{-1} in each well already containing the essential oils. The microplates were incubated at 37 °C for 24 hours. All tests were performed in triplicates.

Minimum Fungicide Concentration (MFC)

This assay allowed the determination of the lowest concentration of extracts capable of eliminating *S. schenckii*, demonstrating fungicide effect against the microorganism. This assay was performed in triplicates. From the wells that did not obtain visible fungal growth in MIC tests, a 10µL suspension was cultivated in plate with

YPD medium by Santurio et al. (2007) methodology.

Statistics

The statistical analyzes were performed by the GraphPad Prism 7.0 program by One-Way Anova, through the Tukey test, to obtain comparations on the inhibitory potential of the extracts studied.

RESULTS AND DISCUSSION

Antifungal activity assay

The data obtained after the agar diffusion test showed that all the extract studied, except for pequi oil, presented antifungal activity against *S. schenckii in vitro*. The results obtained by the Kirb Bauer test can be observed in Table 1 and Figure 1. In the Table 1, the average of the zone of inhibition was expressed in millimeters (mm), and the Figure 1 contains the statistical analyses of the value of *S. schenckii* growth according to zone of inhibition *in vitro*.

Table 1

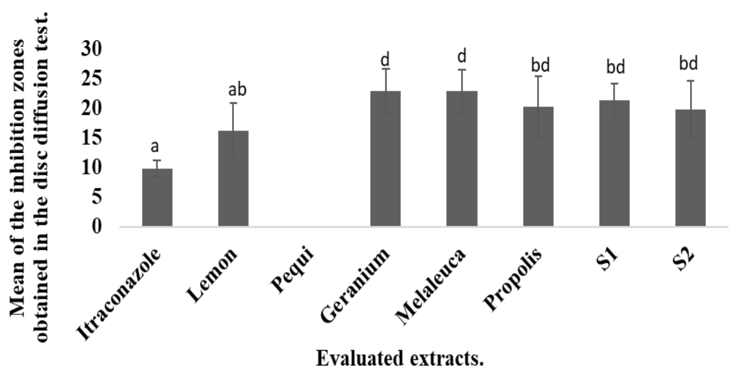
Medium Diameter of the zone of inhibition of *S.schenckii* growth *in vitro*, according to the studied tests in comparation with itraconazole, the drug of clinical choice.

	Lemon	Pequi	Geranium	Melaleuca	Propolis	S1	S2	Itraconazole
Average.	16.00	00.00	23.00	23.00	20.00	21.00	20.00	00.90
Standard deviation.	04.68	00.00	03.73	03.55	05.25	02.83	04.70	01.56

Source: The authors.
Note. The value is expressed in millimeters.

Figure 1

Comparison of the average of the value of growth inhibition *in vitro* of *S. schenckii* expressed in millimeters.



Source: The authors.
Note. The average was compared using ANOVA, followed by the Tukey test, with a significance level of 5%. Error bars indicate the standard deviation, while the letters on the bars indicate statistical equivalence between groups. Different letters show statistically significant difference by Turkey's test (p<0,05).

The geranium, melaleuca, lemon EOs and Propolis extract were able to inhibit the growth of *S. schenckii* in all disk inhibition tests. The geranium EO (23mm), melaleuca (23mm) and propolis extract (20mm) presented the highest average of inhibition zone of inhibition test among the tested phytotherapeutics compared *in vitro* to Itraconazole, which, despite being the antifungal of choice for the treatment of sporotrichosis, presented on average of zone inhibition test of 9mm.

Santos and Casteluber (2020) also demonstrated *in vitro* the effect of itraconazole against *S. schenckii* with zone of inhibition test of only 10mm, as found in this study. The data showed that geranium and melaleuca EOs were more effective against *S. schenckii*, while lemon EO did not show significantly higher performance when compared to Itraconazole. In addition, there was no statistically significant difference between the halos obtained in S1 (all phytotherapeutic drugs without Itraconazole) and in S2 (all herbal medicines with the addition of Itraconazole), allowing to infer that the tested phytotherapeutics have no antagonistic effect when added to Itraconazole.

These *in vitro* inhibition results permit to infer that, except for pequi, which did not demonstrate inhibition effect in any of the tests, the studied herbal medicines help positively in the treatment of sporotrichosis, given the antifungal activity observed. This is of great importance, mainly in view of the fungal resistance that the species of the *Sporothrix* complex have demonstrated (Pimentel, Santos & Cabral, 2017).

In addition, studies indicate that high doses and long treatments with Itraconazole in felines can generate toxic effects for fetuses and cause decreased appetite, weight loss, vomiting and diarrhea, added to liver problems. In humans, liver problems are also been reported as a result of its prolonged use (Meinerz et al., 2007; Pimentel et al., 2017).

Considering that essential oils and extracts are used in low concentrations, the chances of the appearance of side effects are reduced (Santos & Casteluber, 2020), however, such cytotoxic effects still need to be confirmed by *in vivo* tests. Although the cytotoxicity tests of the EOs alone or associated, have not yet been performed *in vivo*, Fogaça de Andrade, Batista, Lasakosvitsch, Souza Antunes and Oliveira (2018) performed the cytotoxicity of the *M. alternifolia* Chell (Myrtaceae) EOs from the method of incorporation of the blue dye of tripan and reduction of TM in fibroblasts L-929, and observed the low level of cytotoxicity of the oil, since, after the tests, there was no significant reduction of L-929 cells.

Peter et al. (2019) also described the antifungal activity of three Brazilian propolis extracts: brown, green and jataí bees (*Tetragonisca angustula*) against *Sporothrix brasiliensis*, and cell toxicity was evaluated in MDBK (Madin-Darby Bovine Kidney) cells, by microscopic observation and quantified by the MTT assay. The results showed that cell toxicity was not observed at concentrations of 0.097 to 0.39μg.mL^{-1}. The researchers also emphasize the importance of further research on the cytotoxicity of herbal medicines because they can present promising solutions for the control of drug-resistant microorganisms. Pequi oil showed no inhibition effects of this fungus when compared to other treatments, as shown in Figure 1.

The results obtained are promising, even for lemon, which was considered statistically equivalent to Itraconazole. The identification of herbal medicines capable of inhibiting growth in a similar way to that observed for itraconazole is of great importance, since it has not been described in the literature on resistance induced using herbal medicines, in addition to fungal resistance to Itraconazole that has been increasingly highlighted in the literature for *S. schenckii* (Meinerz, 2007; Queiroz-Fernandes & Magalhães, 2020).

According to Felipe et al. (2018), the use of the melaleuca EO is an alternative for the treatment of infection by fungi of the genus *Candida* that exhibit a high rate of resistance to antifungal drugs, mainly because of terpinen-4-ol, which is associated with hydrophobicity in which terpenes interact with lipids of the pathogen's cell membrane, changing the permeability of the membrane. These authors suggest that the use of EOs together with conventional antifungal therapy may be a very promising strategy to overcome microbial resistance, demonstrating that phytotherapy is effective in fungal species besides *S. schenckii*.

Chagas (2019) demonstrated *in vivo* the antifungal activity of phytotherapeutic *C. limon* (L.) Burm.f. (Rutaceae) together with *Mentha piperita* and *Pimpinella anisum* in the treatment against oral candidiasis, by creating a mouthwash containing the association of extracts. The rinse was tested for 15 days in selected volunteers with active picture of oral candidiasis, and the results were monitored on the 7th and 15th days of treatment, measuring the evolution of the lesions, and performing photographic records. It was possible to verify a decrease in white lesions and in reddish tone of the lesions, proving the antifungal activity of the extracts used.

Studies by Pereira et al. (2021) also analyzed the antifungal efficiency of crude extracts from pequi and the propolis extract against *C. albicans* yeast. Both pequi extract and propolis extract showed antimicrobial effect on yeast, with an average zone of inhibition test of 18mm and 26mm, respectively, while in the present study the mean halos were 0mm and 20mm, respectively. This data sampled by them for the effect of pequi extract confronts the present work, since pequi oil was not able to inhibit the development *in vitro* of *S. schenkii* in any of the tests and it may suggest that the extract contains more interesting bioactive compounds when the objective is to observe antifungal effect of this phytotherapeutic effect.

This statement is also based on the observation of studies by Martins et al. (2020), who verified *in vivo* the efficacy of an ointment based on the extract of the pequi leaf in the healing of skin wounds experimentally caused in rabbits. The control group was treated with the ointment only as a glycerin-based vehicle.

Macroscopic analyses of wounds were performed on the 7th and 14th days after the surgical procedure, and a lower frequency of hyperemia was observed in rabbits treated with ointment based on the pequi leaf. However, it was found that there was no significant reduction in the area when compared to wounds treated with glycerin-based ointment. This observation suggests that the presence of oilseed compound may have interfered in the results observed by it, as was seen in the present study when it was observed that pequi oil could not inhibit the growth of *S. schenckii*.

It is worth emphasizing the importance of further studies around the characteristics of pequi extracts, seeking to evaluate more accurately their antimicrobial activity against fungi, such as *S. schenckii*, and identify the chemical nature of antimicrobial compounds, which may be present in different fractions of the plant.

Studies by Ferrão et al. (2020) analyzed the *in vitro* antifungal activity of *P. graveolens* EO against *Candida* spp., using the broth microdilution method. The geranium EO showed strong antifungal activity for ten isolated species studied, indicating promising results, corroborating the present study, in which geranium EO proved effective in inhibiting the growth of *S. schenckii*, also at the minimum inhibitory concentration, as shown in Table 2.

Minimum inhibitory concentration results

Itraconazole was able to inhibit the growth of *S. schenckii* until dilution of $4mg.mL^{-1}$ (dilution 10^{-3}). The EOs of melaleuca, geranium, lemon – all in concentration of $10mg.mL^{-1}$ and propolis extract in a concentration of $20mg.mL^{-1}$ – were able to inhibit respectively the development of this fungus at concentrations lower than $0.325mg.mL^{-1}$ (dilution 10^{-6}), $0.15625mg.mL^{-1}$ (dilution 10^{-7}), $0.325mg.mL^{-1}$ (dilution 10^{-4}), and $0.625mg.mL^{-1}$ (dilution 10^{-6}), as shown in Table 2. These data indicate that essential oils and propolis extract, even diluted, still have satisfactory inhibition effect against *S. schenckii in vitro* and in smaller dilutions when compared to Itraconazole.

Meinerz et al. (2007) studied the *in vitro* activity of terbinafine and itraconazole through the broth microdilution technique (NCCLSM27-A2) against 12 clinical isolates of *S. schenckii,* six cases of feline sporotrichosis, five of human sporotrichosis and one from dog mycosis from the Oswaldo Cruz Institute (IOC). The MIC for terbinafine ranged from $0.055\mu g.mL^{-1}$ to $0.109\mu g.mL^{-1}$; for itraconazole, from $0.219\mu g.mL^{-1}$ to $1.75\mu g.mL^{-1}$; and for both drugs, the MICs among the IOC isolates were $0.875\mu g.mL^{-1}$. Resistance to itraconazole was observed in the dog isolate and in the other isolates from feline sporotrichosis.

In the present study, Itraconazole presented the MIC between $16mg.mL^{-1}$ and $4mg.mL^{-1}$, demonstrating a greater potential for resistance compared to the previous research, emphasizing the importance of alternative treatments against sporotrichosis.

Table 2
Minimum Inhibitory Concentration (MIC) observed in treatments with extracts medicines and Itraconazole.

	Itraconazole	Melaleuca	Geranium	Lemon	Propolis	Pequi	S1	S2	S3
10-1	-	-	-	-	-	+	-	-	-
10-2	-	-	-	-	-	+	-	-	-
10-3	-	-	-	-	-	+	-	-	-
10-4	+	-	-	-	-	+	-	-	-
10-5	+	-	-	+	-	+	-	-	-
10-6	+	-	-	+	-	+	+	+	-
10-7	+	+	-	+	+	+	+	+	-
10-8	+	+	+	+	+	+	+	+	-
10-9	+	+	+	+	+	+	+	+	-

Source: The authors.
Note. (-) Concentration in which there was no growth of *S. schenckii*; (+) Concentration in which there was growth of *S. schenckii;* S1- equal volumes of each oil were mixed in a sterile bottle; S2 – equal volumes of each oil and itraconazole were mixed in sterile vial; S3- equal volumes of each oil and itraconazole were mixed in sterile vial without pequi oil.

Junio et al. (2016) proved in their studies the inhibitory effect of melaleuca oil on the growth of *Staphylococcus aureus* strains isolated from animals with mastitis. Inhibitions of microorganisms were observed mainly when used at the concentration of 1.5% (v/v), in such concentration all isolates of *S. aureus* tested had their growth inhibited.

In another study, Cox et al. (2000) reaffirmed the broad-spectrum antimicrobial activity of melaleuca against *S. aureus* and proved effective action against the growth of *Escherichia coli* and *C. albicans*, data that corroborate the present study, given the great antimicrobial potential of melaleuca EO, with inhibition level of up to 0.325mg.mL^{-1} against *S. schenckii*.

The study by Gucwa, Milewski, Dymerski and Szweda (2018) investigated the antifungal activity of geranium essential oil and other herbal medicines against isolates of *C. albicans* and *C. glabrataisola*. In the MIC tests, the phytotherapy inhibited the growth of all strains tested, up to the concentration of 1.25%.

In addition to presenting fungicide effect against the isolates, this research data presents that the geranium EO obtained fungicide and fungistatic action against *S. schenckii*, as presented in Table 3. However, there are few studies on the antifungal activity of geranium EO. No data were found on the action of geranium essential oil or plant extract against *S. schenckii*, and this work is pioneering in the subject.

Essential oils of geranium and lemon were able to block ACE2 and TMPRSS2 receptors, with which the Spike protein of SARS-CoV-2 interacts for cell invasion, thus, pointed out by Kumar et al. (2020). In the present study, lemon EO was effective in inhibiting the growth of *S. schenckii* even at dilution 10^{-4} (0.325mg.mL^{-1}), presenting satisfactory data regarding the inhibition potential of this microorganism, as shown in Table 2.

In studies of Menezes (2020), the antifungal activity of essential oils produced from the green and ripe fruits of the *Citrus limonia* species was verified. The data obtained by him showed antifungal activity from 1.08 to 88.17% against *Sclerotinia sclerotiorum*; from 96.18%, and 6.51 to 93.51% against *Colletotrichum acutatum*, from 71.94 to 100%, and 25.18 to 90.03% against *Aspergillus flavus*, respectively, for green and ripe fruits of *C. limonia*. Thus, presenting important results of the antifungal activity of other species within the genus *Citrus*.

From the analysis of the data obtained by the MIC test with all oils acting together, it is noted that even with a good degree of inhibition, with dilution up to 10^{-5}, as shown in Table 2, geranium, melaleuca and propolis EOs performed better when tested alone, presenting degree of inhibition in dilutions 10^{-7}, 10^{-6} and 10^{-6}, respectively. As pequi oil was not able to inhibit the fungus *in vitro*, it may have acted antagonistically preventing the action of chemical compounds present in essential oils and propolis extract.

This was previously suggested in the work of Martins et al. (2020), in which, when testing the effect of the ointment compared to lotion, both elaborated with pequi leaf extract, it was shown that the ointment had no effect on inhibiting the growth of the fungus, acting only on local hyperemia, while the lotion was efficient, corroborating the statement. It emphasizes the importance of further studies on the molecular cytotoxic effects of essential oils, to combine the best herbal medicines against *S. schenckii*.

To verify whether pequi oil could have interfered with the antimicrobial action of the extracts, the effect of all added oils (being mixed by adding the same volume of each of them in a same vial) was also verified, which was called the S3 test (observed in Table 2). From this result, it is possible to infer that the antifungal activity of geranium and melaleuca oils (inhibition until dilution 10^{-7} and 10^{-6}, respectively) was impaired when combining them with the other oils, and it is possible to observe an antagonistic effect when mixing all the extracts tested.

From the analysis of the MIC test with all oils and Itraconazole acting together, it is inferable that Itraconazole does not exert antagonism or synergism with the essential oils and extract studied, maintaining the same level of inhibition in both cases (10^{-5}), as shown in Table 2. The same pattern of degree of inhibition is again noted when compared to the performance of essential oils alone. This data is important because it may indicate that oils and extracts do not interfere with the mechanism of action of the drug indicated in the medical clinic and allows to suggest that essential oils and propolis extract can be used in joint therapy to Itraconazole for the treatment of sporotrichosis.

The antifungal activity of the mixture of all oils without pequi oil was tested in MIC, data available in Table 2, to verify whether the phytotherapy acted antagonistically when added to the other extracts impairing the expression of antifungal activity.

From the analysis of the MIC test data of the synergy of geranium, melaleuca, lemon EOs and propolis, it is inferable that pequi oil acted with antagonistic effect to the tested phytotherapeutics, since the inhibition level increased from dilution 10^{-5} to dilution 10^{-9}, confirming the hypothesis placed from the results of the MIC test of the mixture of all oils. This behavior is consistent with the results of the agar diffusion test and its individuals MIC, presenting no action against *S. schenckii*.

Despite the description of its effect as antifungal and antibacterial in the literature, as in the study by Pereira et al. (2021), who verified antifungal action of the extract produced from the leafs of the pequi against the yeast *C. albicans in vitro*, pequi oil was not able to inhibit the growth of *S. schenckii* , and this finding confronts the use of pequi oil as antifungal and proves the statement that the oilseed extract may not have all the compounds necessary to inhibit the development of *S. schenckii* or other fungi, such as *C. albicans*, as observed by Martins et al. (2020), who, when testing the ointment against *C. albicans*, found no inhibitory effect.

There are still few studies verifying the antifungal activity against *S. schenckii*, and the data found reveal the potential of using essential oils and propolis extract to contain sporotrichosis, since many studies have been done evaluating herbal medicines against other fungal species.

Minimum fungicide concentration assay

In the data obtained by testing the Minimum Fungicide Concentration (MFC) of the tested herbal medicines observed in Table 3, it was possible to observe fungicide behavior by geranium and melaleuca EOs and antifungal Itraconazole at dilution 10^{-1}. Lemon EO and propolis extract showed fungistatic behavior against *S. schenckii.* While Itraconazole had fungicide effect at the concentration of 16mg.mL-^{1}, geranium and melaleuca EOs showed fungicide effect at the concentration of 10mg.mL^{-1}.

Table 3
Minimum Fungicide Concentration (MFC) of each herbal and Itraconazole against *S. schenckii.*

	Itraconazole	Lemon	Pequi	Geranium	Melaleuca	Propolis	S1	S2	S3
10^{-1}	-	+	+	-	-	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+	+
10^{-4}	+	+	+	+	+	+	+	+	+
10^{-5}	+	+	+	+	+	+	+	+	+
10^{-6}	+	+	+	+	+	+	+	+	+
10^{-7}	+	+	+	+	+	+	+	+	+
10^{-8}	+	+	+	+	+	+	+	+	+
10^{-9}	+	+	+	+	+	+	+	+	+
10^{-10}	+	+	+	+	+	+	+	+	+

Source: The authors.
Note: (-) Concentration in which there was no growth of *S. schenckii*; (+) Concentration in which there was growth of *S. schenckii*; S1- equal volumes of each oil were mixed in a sterile bottle; S2 - equal volumes of each oil and itraconazole were mixed in sterile vial; S3 - equal volumes of each oil and itraconazole were mixed in sterile vial without pequi oil.

The findings of this study suggest that geranium and melaleuca EOs can be considered as an alternative treatment for sporotrichosis, either alone or added to the antifungal of clinical choice to potentiate the inhibition capacity of the fungus. These oils could be a future alternative for the treatment of sporotrichosis caused by *S. schenckii* resistant to itraconazole and allow the cure of the disease in cats and humans affected.

CONCLUSION

The EOs of geranium, melaleuca, lemon and the propolis extract showed inhibitory effects on the growth *in vitro* of *S. schenckii*, while pequi oil has not demonstrated such effect. This study highlights the melaleuca and geranium EO antifungal activity, because they presented the best levels of inhibition against *S. schenckii in vitro*, in addition to the fungicide effects, such as that demonstrated by Itraconazole. Further studies are necessary to suggest alternative treatments alone or in combination with Itraconazole. In this context, it is suggested to perform *in vivo* tests to demonstrate the possibility of cytotoxic effects of the use of the essential oils studied.

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COMPETING INTERESTS

The authors declare that there are no conflicts of interest.

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Conceptualization: M. C. F. C. *Data curation*: S. R. M. G., M. C. F. C. *Formal analysis*: S. R. M. G., E. I. R., M. C. F. C., F. P. B. *Funding acquisition*: M. C. F. C., S. R. M. G. *Investigation*: S. R. M. G., E. I. R., L. T. F. R. *Methodology*: M. C. F. C., F. P. B. *Project administration*: M. C. F. C. *Resources*: M. C. F. C., S. S. L. *Software*: M. C. F. C., F. P. B. *Supervision*: M. C. F. C. *Validation*: S. R. M. G., E. I. R., M. C. F. C. *Visualization*: M. C. F. C., F. P. B., S. S. L. *Writing the initial draft*: M. C. F. C., S. R. M. G., E. I. R. *Revision and editing of writing*: M. C. F. C., F. P. B., S. S. L.

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