ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 11, Issue, 04, pp. 46059-46063, April, 2021
https://doi.org/10.37118/ijdr.21499.04.2021

RESEARCH ARTICLE OPEN ACCESS

POTENTIAL FUNGISTATIC AND FUNGICIDAL ACTIVITY OF ESSENTIAL OILS IN DERMATOMYCOSIS

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ARTICLE INFO

Article History:
Received 04th January, 2021
Received in revised form
11th February, 2021
Accepted 18th March, 2021
Published online 22th April, 2021

Key Words:
Antifungal, Cinnamon, Dermatophytes, Yeast, Onychomycosis.

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ABSTRACT

Objectives: The aimed to evaluate the fungistatic and fungicidal activity ofessential oils (EOs), against fungi that cause dermatomycosis. **Design:** The study design was experimental, with in vitro tests, with a control group and treatments. **Setting:** Fungal species *Trichophyton rubrum* ATCC MYA 4438, *T. mentagrophytes* var. *interdigitale* ATCC MYA 4439, *Candida albicans* ATCC 14053, and *C. parapsilosis* ATCC 22019, in addition to clinical samples of *Fusarium* spp., *Scytalidium* spp., and *T. mentagrophytes* were used in this study. **Interventions:** EOS of the Rosemary (Rosmarinus officinalis), Lavender (Lavandula angustifolia), Cinnamon (Cinnamomum cassia), and Tea tree (Melaleuca alternifolia) were used separately in the experimental group, and fluconazole and terbinafine were used as controls. **Main outcome measures:** The antifungal activity of EOs was evaluated by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). **Results:** Cinnamon performed best, with an MIC and MFC of 62.5–125 µg/mL against the *T. mentagrophytes* ATCC and clinical samples, and a MIC and MFC of 15.62–31.25 µg/mL against *T. rubrum* ATCC.The most promising result was against *Fusarium* spp., which are resistant to most antifungal agents, with an MIC and MFC of 62.5–125 µg/mL. Lavender and Tea tree showed fungicidal effects against yeasts, with an MIC and MFC of 2,000–4,000 µg/mL, whereas they showed only fungistatic effects against *T. rubrum* ATCC. **Conclusions:** Cinnamon showed a fungicidal effect against all evaluated microorganisms, and was more efficient than the other EOs tested.

Citation*: *Weder da Silva Mateus, Wellington Francisco Rodrigues, Camila Botelho Miguel, Larissa Beatriz Silva, Kamila Botelho Fernandes de Souza, Javier Emilio Lazo-Chica, Mônica Hitomi Okura and Tony de Paiva Paulino. 2021. "Potential fungistatic and fungicidal activity of essential oils in dermatomycosis*", International Journal of Development Research,* 11 (4), 46059-46063.

INTRODUCTION

Dermatomycoses are superficial fungal infections of the skin, hair and nails that affect between 20–25% of the population worldwide, particularly in tropical and subtropical regions, making them one of the most common dermatological diseases. These diseases, although not fatal, are considered a public health problem because they affect the quality of life of individuals. Fungi responsible for dermatomycosis include dermatophytes, yeasts, and filamentous fungi non-dermatophytes (FFND) (Silva, 2014 and Ghannoum, 2018). The treatment of onychomycosis depends on the clinical type, number of nails involved, and severity of the infection. The disadvantages of therapies are that oral treatments are often limited by drug interactions and can cause a high level of hepatotoxicity, while topical antifungal agents have limited effectiveness if used without debridement of the nail plate. A combination of both topical and systemic treatments is often the best choice (Jayatilake, 2009 and Bonhert, 2019). Currently, there is great variability in the range of antifungal options, both topical and systemic, but the therapeutic arsenal is still quite restricted.The need for new, more effective, and less toxic antifungal agents is apparent. Many have the same mechanism of action, and show the same pharmacological actions; however, the responses of fungi are significantly different according to the varying susceptibility to these drugs (Almeida, 2009 and Stultz, 2018). Considering the intrinsic and acquired resistance of some species of fungi to certain drugs, it has become evident that reference methods, duly standardized and validated, are necessary in order that susceptibility

tests can be widely used in clinical practice. Prolonged therapy together with the antifungal resistance of some fungi are important factors that explain the increasing use of these tests in an attempt to establish more adequate therapies for the treatment of fungal infections (Geddes-Mcalister, 2019). The high resistance of microorganisms to current medications and the search for new treatment alternatives were two of the main motivators of this study. Likewise, the various side effects of systemic drugs, such as hepatotoxicity, have been an incentive to identify topical natural products as treatment options. Therefore, this study aimed to evaluate, in vitro, the potential antifungal activity of essential oils (EOs) against agents related to onychomycosis.

MATERIALS AND METHODS

Experimental design: To examine the fungistatic and fungicidal effects of EOs, American Type Culture Collection (ATCC) strains of fungi of the species*Trichophyton rubrum*, *T. mentagrophytes var. interdigitale*, *Candida albicans,* and*C. parapsilosis*, and strains of clinical samples of *Fusarium spp., Scytalidium spp.*, and *T. mentagrophytes*were used. These strains were challenged against the oils of Lavender, Rosemary, Cinnamon, and Tea tree. The minimum inhibitory concentration (MIC) and minimum fungicide concentration (MFC)were determined individually for each microorganism and vegetable oil. As a reference parameter, control groups and two antifungal agents (fluconazole and terbinafine) were used. All procedures were performed in independent duplicates, with five repetitions for each stage. The tests were conducted in a randomized and blinded manner.

Eos: Essential oils were purchased commercially (Ferquima Industry and Trade of Essential Oils LTDA, Vargem Grande Paulista-SP, Brazil). The oils were solubilized in Tween 80. The critical micelle concentration (CMC) of Tween 80 was 0.06 mM (≅0.015 g/mL) and the density was 1.07 g/mL. Taking these values into account, 14 µL Tween was used to solubilize 1 mL of EO.

Filamentous fungi (dermatophytes and FFND): We used ATCC strains of dermatophyte fungi of the species *T. rubrum* ATCC® MYA 4438and *T. mentagrophytes var. interdigitale* ATCC® MYA 4439, and clinical samples of *Fusarium* spp., *Scytalidium* spp., and *T. mentagrophytes*, in addition to clinical samples of non-dermatophyte filamentous fungi of the species *Scytalidium spp.* and *Fusarium spp.* Fungi from clinical specimens were obtained from another study (SILVA et al., 2014), after approval by the UFTM Ethics Committee (protocol 1361/2010).

Maintenance, cultivation, and preparation of the inoculums: Strains were stored in a physiological solution at 2–7 °C, after being sub cultured on Sabouraud agar plates and incubated for 4 d at 28 °C. They were then sub cultured again in a 50 mL Falcon tube with potato dextrose agar (BDA) and incubated for 4 d at 28 °C for the production of conidia. Fungal colonies were covered with 5 mL of sterile saline (0.9%) and subsequently scraped using a Pasteur pipette. The resulting mixture of microconidia and hyphae fragments was filtered through gauze, and the density of the suspension was adjusted to a transmittance of 70–72% using a spectrophotometer at a wavelength of 520nm. This procedure generated an inoculum ranging from 2×10^6–4×10^6 CFU/mL, and was confirmed by plating 0.01 mL of BDA suspension and counting the fungal colonies after incubation of the plates for 7–10 d at 28 °C. The inoculum suspension was diluted (1:50) in RPMI medium to give a number of cells ranging from 4×10^4–8×10^4 CFU/mL.

MFC: The MFCs of the EOs were determined by sub culturing in 90 × 15 mm smooth petri dishes with 25 µL potato dextrose agar.Potato dextrose agarwas added to the contents of the wells where there was no fungal growth (MIC), and to the two wells with the next highest concentration and the growth control wells. After sowing with the aid of a Drigalski loop, the plates were incubated for 4 d at 28 °C, when MFC was determined as the lowest concentration of oil that inhibited any fungal growth in the subcultures.

Yeasts: ATCC strains of the yeasts C. albicans (ATCC® 14053) and C. parapsilosis (ATCC® 22019) were used.

Maintenance, cultivation, and preparation of the inoculums: The strains were stored at -20 °C and prepared according to aClinical & Laboratory Standards Institute (CLSI) reference method (M27-A3, 2008) with modifications. They were spiked onto a plate containing Sabouraud dextrose agar and incubated for 24 h at 35 °C. Afterwards, they were re-spiked onto a plate containing Sabouraud dextrose agar and incubated for 24 h at 35 °C. Using a platinum loop, some colonies were removed and placed in a test tube with 5 mL of sterile saline (0.9%) and shaken for 15 s. The density of the resulting suspension was adjusted to a transmittance of 85% using a spectrophotometer at a wavelength of 530 nm. This procedure generated an inoculum ranging from 1×10^6–5×10^6 CFU/mL, which was confirmed by counting in a Neubauer chamber. The inoculum suspension was diluted (1:100) in sterile saline (0.9%) and diluted (1:20) in RPMI medium to obtain a number of cells ranging from 5×10^2–2.5×10^3 UFC/mL.

MIC: To determine MIC, a second stock solution ('stock solution 2') was prepared from the stock solutionthat was used in section 2.3.2.This was a 1:5 dilution using standard RPMI 1640 (pH 7.0) medium, buffered with 0.165 M acid (MOPS) (34.54g/L),and Lavender, Rosemary, and Melaleuca EO (concentration 16,000 µg/mL),and a 1:20 dilution of Cinnamon EO (concentration 4,000 µg/mL). Flat-bottomed micro dilution plates (96 wells) were prepared according to a CLSI reference method (M27-A3, 2008), with modifications. Briefly, 100 µL standard RPMI 1640 medium was added to each well, except for plate 1, in which 200 µL stock solution 2 (EO + RPMI) was added. Afterwards, 100 µL of the contents of the wells of column 1, at concentrations of 4,000 µg/mL for Cinnamon and 16,000 µg/mL for the remaining oils, wasremoved and added to the wells inthe following column,resulting in a1:2 dilution.By repeating this process, serial dilutions were made. This procedure was performedfor each column up to column 10, then 100 µL of the solution was discarded. Finally, 100 µL diluted inoculum suspension (1:100 and 1:20) was added to the wells containing EOs. For each test plate, three controls were added: one containing 200µL medium (sterility control) in column 12; another containing 100 µL medium and 100 µL inoculum suspension (growth control) in column 11; and anothercontaining 100 µL medium plus Tween 80, at the same concentrations as those present in the EOs, and 100 µL inoculum suspension (solvent control). After the addition of the inoculum, all concentrations were reduced by half, thus obtaining a concentration ranging from 2,000–3.90 µg/mL for Cinnamon and 8,000–15.62 µg/mL for the other EOs. The micro dilution plates were incubated at 35 °C and visually read after 24 h of incubation.

MIC of fluconazole and terbinafine: Fluconazole and terbinafine were used as reference drugs in the fungal tests. The overall procedure was the same as that used for EOs, differing only in the variations of final concentrations tested. Fluconazole concentrations ranged from 64–0.125 µg/mL and those of terbinafine ranged from 16–0.031 µg/mL.Procedures were performed according to CLSI reference methods (M38-A2, 2008; M27-A3, 2008), with modifications. The MIC for fluconazole was defined as the lowest concentration that caused a prominent decrease in fungal growth, corresponding to approximately 50% of the growth control. The MIC for terbinafine was defined as the lowest concentration that inhibited 100% of fungal growth after 4 d at 28 °C for dermatophytes and FGFND, and after 24 h at 35 °C for yeasts.

Statistical analysis

Statistical analyses were performed using the Instat and Prisma programs of Graphpad (http://www.graphpad.com). For all variables, normal distribution (Kolmogorov-Smirnov with Dallal-Wilkinson-Liliefor P value) and homogeneous variance (Bartlett's test or F-test) were tested. We used the non-parametric Mann-Whitney test for

comparing two groups, or the Kruskal-Wallis with Dunn's multiple comparisontest for comparing three groups or more. Differences were considered significant when $p < 0.05$.[9]

RESULTS

MIC and MFC of EOs against dermatophytes: The data shown in Table 1 demonstrate that CinnamonEO was the most efficient of the oils ($p < 0.05$) and highlights, that against*T. rubrum,*it was effective at a lower concentration than those of the other EOs. However, Lavender and Tea tree obtained worse results in this evaluation than with yeasts, except for *T. rubrum*, which reached slightly lower concentrations. The results for Rosemary were equal to those of the previous test, being the least effective among all the oils. For *T. mentagrophytes ATCC*, the mean MIC was 62.5 µg/mL and the mean MFC was 125 µg/mLforCinnamon. For Lavender and Tea tree, the mean MICswere 8,000 µg/mL and the mean MFCs were > 8,000 µg/mL.For Rosemary, the mean MIC was > 8,000 µg/mL and MFC could not be determined.

Using the *T. mentagrophytes* clinical sample,Cinnamon had a mean MIC of 62.5 µg/mL and a mean MFC of 125 µg/mL. The other oils obtained the same values using the ATCC strains. For *T. rubrum*,the mean MIC value was 15.62 µg/mL and the mean MFCwas 31.25 µg/mL for Cinnamon.The mean MIC was 500 µg/mL and the mean MFCwas 2,000 µg/mL for Lavender and Tea tree.ForRosemary, the valueof MIC was > 8,000 µg/mL. After analyzing the results, CinnamonEO performed best against the dermatophyte fungi in vitro,showing a fungicidal effect against all fungi.Lavender and Tea tree showed only a fungistatic effect. Among all the dermatophytes evaluated, *T. rubrum* was the most sensitive to the effects of Cinnamon.

MIC and MFC of EOs against FFND: The data presented in Table 2 show that, against these species of fungi, Cinnamonobtained the best results ($p < 0.05$). However, in this analysis, none of the other oils achieved any effectiveness at the maximum concentration tested. ForCinnamon, a mean MIC value of 62.5 µg/mL and a mean MFC of 125 µg/mL was achieved against *Fusarium spp*. Lavender, Tea tree, and Rosemary obtained an MIC> 8,000 µg/mL.

Table 1.MIC and MFC of EOs against dermatophytes

Filamentous Dermatophytes						
Essential oil	*T. mentagrophytes* Clinical Sample		*T. mentagrophytes* ATCC MYA 4439		*T. rubrum* ATCC MYA 4438	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
Cinnamon	62.5	125	62.5	125	15.62	31.25
Lavender	8,000	>8,000	8,000	>8,000	500	2,000
Tea tree	8,000	>8,000	8,000	>8,000	500	2,000
Rosemary	>8,000	*	>8,000	*	>8,000	*

MIC and MFC values expressed in µg/mL
*MFC was not calculated because the MIC was greater than the maximum concentration tested.

Table 2. MIC and MFC of EOs against FFNDs

Filamentous Non-Dermatophytes				
Essential oil	*Fusarium spp.* Clinical Sample		*Scytalidium spp.* Clinical Sample	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
Cinnamon	62.5	125	62.5	62.5
Lavender	>8,000	*	>8,000	*
Tea tree	>8,000	*	>8,000	*
Rosemary	>8,000	*	>8,000	*

MIC and MFC values expressed in µg/mL
*MFC was not calculated because the MIC was greater than the maximum concentration tested.

Table 3. MIC and MFC of EOs against yeasts

Yeast				
Essential oil	*C. albicans* ATCC 14053		*C. parapsilosis* ATCC 22019	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
Cinnamon	62.5	62.5	31.5	31.5
Lavender	2,000	2,000	2,000	4,000
Tea tree	2,000	2,000	4,000	4,000
Rosemary	>8,000	*	>8,000	*

MIC and MFC values expressed in µg/mL
*MFC was not calculated because the MIC was greater than the maximum concentration tested.

Table 4. MIC and MFC of reference drugs against fungi

Reference drug				
Fungi	Fluconazole		Terbinafine	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
Yeast				
C. albicans ATCC 14053	2.0	2.0	NT	NT
C. parapsilosis ATCC 22019	4.0	4.0	NT	NT
Dermatophytes				
T. rubrum ATCC MYA 4438	NT	NT	≤0.031	0.031
T. mentagrophytes ATCC MYA 4439	NT	NT	≤0.031	0.031
T. mentagrophytes Clinical Sample	NT	NT	≤0.031	0.031
FFND				
Fusarium spp. Clinical Sample	≥64.0	*	>16.0	*
Scytalidium spp. Clinical Sample	≥64.0	*	2	*

MIC and MFC expressed in µg/mL; NT = not tested; *MFC was not calculated

For*Scytalidium spp*., Cinnamon obtained an MIC and MFC of 62.5 µg/mL, whereas the other oils had an MIC> 8,000 µg/mL. In the evaluation performed with NDFF, Cinnamon was the only EO that showed a fungicidal effect.

MIC and MFC of EOs against yeasts: The data described in Table 3 show that Cinnamon was the most effective among the evaluated oils ($p < 0.05$), being effective against the two species tested, with better results against*C. parapsilosis*. Lavender and Tea treeshowed similar inhibitory results, reaching medium efficiency. Rosemaryshowedthe worst results and did not inhibit any of the species at the maximum concentrations tested. Of note isCinnamon, which showed a mean MIC and MFC of 62.5 µg/mL against*C. albicans*, whereas Lavender and Tea tree had an MIC and MFCof 2,000 µg/mL, and the MIC for Rosemary was > 8,000 µg/mL. *C. parapsilosis* showed the highest sensitivity to the effects of Cinnamon, with an MIC and MFC of 31.5 µg/mL. The results show that all but Rosemaryshowed fungicidal effects.

MIC and MFC of reference drugs: The results shown in Table 4 demonstrate the viability of the strains used in the experiment, as they showed the expected responses to the drugs according to the CLSI. The sensitivity of yeast strains to fluconazole, which according to CLSI (2008) show MIC values of ≤ 8.0 µg/mL, demonstrated that strains of the genus *Candida spp*. are sensitive to this drug, because *C. albicans*showed an MIC and MFC of 2.0 µg/mL and for *C. parapsilosis,* an MIC and MFC of 4.0 µg/mL. These results also show that both the ATCC and clinical samples of dermatophytes were sensitive to terbinafine, with an MIC and MFC ≤ 0.031 µg/mL. Among the FFNDs, *Fusariumspp*. showed resistance to fluconazole, with an MIC ≥64 µg/mL, and terbinafine, with MIC > 16 µg/mL. *Scytalidiumspp*. was resistant to fluconazole with an MIC of ≥64 µg/mL, and sensitive to terbinafine with an MIC of 2 µg/mL.

DISCUSSION

Among the dermatological diseases, dermatomycoses have shown an increase in worldwide incidence in recent decades. They are an important cause of morbidity, especially in tropical countries, (Kayman, Sariguzel and Koc, 2012), and many studies around the world, including those in Brazil, have shown that onychomycosis is the most prevalent dermatomycosis (Silva et al., 2014). Treatment of these diseases is not always effective, given the possibility of recurrence of infection, resistance of microorganisms, and possible toxicity. This issue has led to the search for new drugs that are more effective and safer than those currently available. Although most of the antifungal agents on the market are of synthetic origin, the identification of natural products deserves attention (Fenner et al., 2006). The choice of Lavender, Rosemary, Tea tree, and Cinnamon EOs for this study was based mainly on the antifungal activities already ascribed to them. The species of fungi were chosen because they are among the main agents causing dermatomycosis. Cassella, Cassella, and Smith (2002) evaluated Lavender and Tea tree Eos (Cassella, Cassela and Smith, 2002). At high concentrations, both oils appeared to demonstrate antifungal effects against *T. rubrum* and *T. mentagrophytes*. These EOs achieved 100% inhibition of fungal growth at concentrations of 250,000 µg/mL, reflecting the results of the present study. In another study, the MIC of Cinnamon and Tea tree was 560 µg/mL against *C. albicans* ATCC 289065 (Cavalcanti et al., 2011), showing higher inhibitory concentrations for Cinnamon and lower concentrations for Tea tree than those found in the present study (62.5 and 2,000 µg/mL, respectively). In the study by Ouraini et al. (2005), for Rosemary EO tested against *T. rubrum*, the results were inconsistent with ours, with an MIC of 0.4 µg/mL and an MFC of 2 µg/mL (Ouraini et al., 2005). This study was not performed as recommended by the CLSI, as it used microdilution in Sabouraud liquid medium plate, and the recommended method uses RPMI. The two main constituents of the EO tested in the Ouraini study were 1.8 cineol 50.2% and camphor 9.1%, which are similar to those reported in the EO technical report as used in the present study, at 45% and 15%, respectively (Ouraini et al., 2005). In another study, Cleff et al. (2012) used Rosemary EO against *C. albicans* (ATCC 44858 and clinical sample) and *C. parapsilosis* (ATCC 22019), and obtained results with lower concentrations than those obtained here, with an MIC and MFC for *C. albicans* ATCC and C. parapsilosis of 1.25 µL/mL and 5.0 µL/mL, respectively (Cleff et al., 2012). For the *Candida* clinical sample, the MIC and MFC were ≥ 10 µL/mL, although the EO chromatography used showed that the concentrations of the two main constituents, 1.8 cineol and camphor, were 16.02% and 56.04%, respectively. Thus, Rosemary showed a large difference in its main constituents compared to that used in the present study, which had 45% 1.8 cineol and 15% camphor. This fact could justify the difference in results between the two studies, since the methodology was referenced by CLSI (CLSI, 2008).

In a study by Nasir, Tafess, and Abate (2015), Cinnamon EO against *C. zeylanicum*showed an MIC of 0.31 µL/mL against *Trichophytonspp*. isolated from *Tinea unguium* (Nasir, Tafess and Abate, 2015). These results demonstrate the effective antifungal activity of Cinnamon against dermatophyte fungi, with *T. rubrum* being the most sensitive, and *T. mentagrophytes* ATCC presenting the mean MIC of the clinical sample strains. Regarding the greater sensitivity of *T. rubrum* compared with *T. mentagrophytes*, Cassella and Smith (2002) also reported that there is a clear difference in susceptibility between the two *Trichophyton* species, in that *T. mentagrophytes* is less susceptible to antifungal drugs than *T. rubrum* (Cassella, Cassela and Smith, 2002). Almeida et al. (2012) reported results that support the antifungal effect of Cinnamon against strains of *C. albicans* isolated from HIV-positive patients and the standard ATCC 76845 strain, with an MIC ranging from 64–128 µg/mL, similar to the values found in our study (Almeida et al., 2012). Active constituent of Cinnamon acts alone to potentiate the antifungal effect, this can be explained by the fact that, on average, 81% of Cinnamon EO consists of cinnamaldehyde, but 19% are other constituents; thus, several hypotheses can be drawn from this. However, a comparative study between cinnamaldehyde and the other components of Cinnamon against the same fungal strains should be carried out to exclude, for example, intrinsic differences in the susceptibility of microorganisms to antifungal agents, since different strains were used in the two studies. It would then be possible to affirm the effectiveness of cinnamaldehyde and all the components of Cinnamon. The results obtained using Cinnamon against *Scytalidium* and *Fusarium* are promising because of the difficulty in treating dermatomycoses caused by these fungi. Microorganisms of the genus *Fusarium* are generally resistant to available treatments. Although they are sensitive to amphotericin B and present varying degrees of susceptibility to voriconazole and posaconazole, clinical therapy requires the combination of drugs for treatment (Spader et al., 2013). Furthermore was verified the synergistic effect when voriconazole was combined with terbinafine, with 84% of the species of the genus *Fusarium* being inhibited by this combination (Spader et al., 2013). The FFNDs used in this study were from clinical samples from another study, wherein the antifungal agents showed little activity against FFNDs, and the MICs for all the agents against *Fusariumspp*. were higher than those obtained using other FFNDs. This suggests a greater resistance to treatment, with MICs for ketoconazole, griseofulvin, itronazole, voriconazole, terbinafine, and fluconazole ranging from > 16 µg/mL to ≥ 64 µg/mL. Carmo et al. (2008) demonstrated the antifungal effects of Cinnamon on *Aspergillus genus* FFNDs with MIC values of 80 µL/mL. At concentrations of 80, 40, and 20 µL/mL, the oil demonstrated a potent fungicidal effect, inhibiting the radial mycelial growth of *A. niger*, *A. flavus*, and *A. fumigatus* over 14 d of exposure. At 80 µL/mL and 40 µL/mL, Cinnamon EO promoted 100% inhibition of spore germination of these three *Aspergillus* species (Carmo et al., 2008).

CONCLUSION

Our study demonstrated the fungicidal potential of Cinnamon *(C. cassia)* EOagainst all tested microorganisms, being more effective than Lavender and Tea tree EOs. RosemaryEO showed no antifungal effects at the studied concentrations. Compared with the reference

drugs fluconazole and terbinafine, Cinnamon was effective against *Fusariumspp*., which showed low sensitivity to these drugs. Further studies are needed to elucidate the mechanism of action of Cinnamon and its constituents, to evaluate its action in vivo, and to subsequently develop a new product for topical use in the treatment of dermatomycoses.

Author disclosures: All authors consent to this manuscript's publication

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

TPP and MHO: conceptualized and designed the study, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published. WSM, CBM, LBS, KBFS and JELC: collection and analysis of data, drafted the manuscript, and gave final approval of the version to be published.

Ethics statement: This study involved *in vitro* evaluations only, and had no use of humans or other animals; it did not require ethics approval or consent to participate.

Declaration of Competing Interest: The authors declare that they have no conflicts of interest

Acknowledgements: WFR, thanks for the postdoctoral fellowship for the PNPD-CAPES program.

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