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In vitro inhibitory activity of terpenic derivatives against clinical and environmental strains of the *Sporothrix schenkii* complex

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Abstract

Sporotrichosis is a subacute or chronic subcutaneous infection, caused by the fungus *Sporothrix schenkii* complex, occurring in human and animal tissues. Potassium iodide and itraconazole have been used as effective therapy for first-choice treatment, while amphotericin B may be indicated for disseminated infection. However, the adverse effects of potassium iodide and amphotericin B or the long duration of therapy with itraconazole often weigh against their use, leading to the search for alternatives for the treatment of severe infections. Terpinen-4-ol and farnesol are components of essential oils present in many plant species and have been described to have antifungal activity against microorganisms. In this study, 40 strains of *Sporothrix* spp. were tested for the susceptibility to terpinen-4-ol and farnesol. Changes in cytoplasmic membrane permeability were also investigated. Terpenes inhibited all *Sporothrix* strains with MIC values ranging from 87.9 to 1,429.8 μg/ml for terpinen-4-ol and from 0.003 to 0.222 μg/ml for farnesol.

The MFC values ranged from 177.8 to 5,722.6 μg/ml and from 0.027 to 0.88 μg/ml, respectively, for terpinen-4-ol and farnesol. Farnesol was the most active compound for the *Sporothrix* strains. Significant loss of 260 and 280 nm-absorbing material did not occur after treatment with concentrations equivalent to the MIC and sub-MIC of the tested terpenes, when compared to corresponding untreated samples. The failure of terpenes to lyse *Sporothrix* cells suggests that their primary mechanism of action is not by causing irreversible cell membrane damage. Thus, new studies are needed to better understand the mechanisms involved in the antifungal activity.

Introduction

Sporotrichosis is a subacute or chronic subcutaneous infection, caused by the dimorphic fungi of the *Sporothrix schenkii* complex, occurring in human and animal tissues [1,2]. Infection generally occurs by traumatic inoculation produced by plant fragments and animal biting or scratching or rarely by inhalation of conidia [3]. The *S. schenckii* complex is currently formed by five pathogenic species: *S. schenckii sensu stricto*, *S. brasiliensis*, *S. mexicana*, *S. globosa* and *S. luriei* [2]. Sporotrichosis is distributed throughout the world, especially in tropical and subtropical zones [2]. In Brazil, epidemics have occurred due to zoonotic transmission and the cats are seen as the main source of infection [4].

Solution of potassium iodide has been traditionally used as effective therapy for localized sporothrichosis. Itraconazole (ITC) is currently the first-choice treatment for the cutaneous and lymphocutaneous forms of the disease, while amphotericin B (AMB) may be indicated for disseminated disease or in cases of ITC therapeutic failure. However, the adverse effects of potassium iodide and AMB or the long duration of therapy with ITC often weigh against their use. Although these drugs are effective, the search for new alternatives for the treatment of severe infections is needed [1,5].

The use of products of plant origin with potential antimicrobial activity has acquired significance in therapeutic treatments. Thus, several studies on medicinal plants have been conducted to test the activity of their extracts, essential oils, and fractions with effect against several fungal species [6,7]. Essential oils are complex mixtures of natural products obtained from plants [8], known for their *in vitro* and *in vivo* antimicrobial properties [9,10]. These substances are capable to attack different microorganisms through several modes of action, depending on the compound [11]. Essential oils usually occur as monoterpenes (C_{10}) and sesquiterpenes (C_{15}), formed, respectively, from the coupling of two and three isoprene units (five carbon-base) [7]. Terpinen-4-ol and farnesol are important components of essential oils present in many plant species, belonging to the class of monoterpenes [9] and sesquiterpenes [12], respectively.

The antifungal activity of some major components of essential oils, such as terpinen-4-ol and farnesol, against several pathogenic fungal species has been described, including *Paracoccidioides brasiliensis* [13], *Candida* spp. [14–16], *Cryptococcus* spp. [17], and *Coccidioides posadasii* [18]. Thus, this study aimed at investigating the *in vitro* antifungal activity of terpenic derivatives, terpinen-4-ol and farnesol, against *Sporothrix* spp. isolates, as well as to evaluate the loss of intracellular material after exposure to inhibitory concentrations of terpene compounds.

Materials and methods

Microorganisms

In this study a total of 40 strains of *Sporothrix* spp. in the filamentous phase was utilized, including *Sporothrix brasiliensis* ($n = 24$); *S. schenckii* ($n = 6$); *S. mexicana* ($n = 7$), and *S. globosa* ($n = 3$). The isolates were obtained from the collection of organisms of the Laboratory of Molecular Medical Mycology, Federal University of São Paulo, from humans ($n = 15$), animals ($n = 22$), and the environment ($n = 3$). The strains are stored in the fungal collection of the Specialized Medical Mycology Center, Federal University of Ceará. All these isolates were identified as *Sporothrix* spp. by typical colony morphology on potato dextrose agar (Himedia, Índia) and microscopical appearance with septate hyaline hyphae, conidiophores, and typical conidia. For the quality control of the susceptibility experiments, the strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included.

Antimicrobial agents

A stock solution of terpenes terpinen-4-ol ((S)-p-Menth-1-en-4-ol; Sigma-Aldrich, USA) and farnesol (3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol; Sigma-Aldrich, USA) was prepared in DMSO (dimethyl sulfoxide) at

concentration 100% [19] and 30% [18], respectively. For control, a parallel series of dilutions containing DMSO alone was made to measure the effect of the solvent on the fungus. DMSO did not affect fungal growth at concentrations present in the wells of the microdilution plates. The antifungal drugs amphotericin B (AMB, Sigma-Aldrich, USA) and itraconazole (ITC, Janssen Pharmaceutica, Belgium) were used as control drugs. The concentration ranges tested were from 11.7 to 5,722.67 μg/ml (0.07246 to 37.1 mM) for terpinen-4-ol; from 0.0017 to 0.889 μg/ml (0.00781 to 4 μM) for farnesol; and from 0.03125 to 16 μg/ml for AMB and ITC [20].

Preparation of inoculum

The inocula of *Sporothrix* species in the filamentous phase were prepared from cultures maintained on brain heart infusion agar (BHI; Himedia, India) for 7 days, at 28°C. Each fungal culture was added to 0.9% sterile saline solution, and with the aid of microbiological loop, the surface of the colonies were softly scraped to form a suspension of conidia and hyphal fragments. Then, each fungal suspension was adjusted to 2 on the McFarland scale of turbidity. Finally, the suspensions were diluted 1:10 in RPMI medium to obtain the final inoculum at a concentration of 1.0 to 5.0 $\times 10^5$ CFU. ml^1 [19].

In vitro antifungal activity testing

Susceptibility testing of *Sporothrix* spp. to terpinen-4-ol; farnesol; AMB and ITC was performed according to the *Clinical and Laboratory Standards Institute* (CLSI) broth microdilution method - document M38-A2, with some modifications [20]. Minimum inhibitory concentration (MIC) of each compound terpinen-4-ol and farnesol was defined as the lowest concentration that produced an 80% growth reduction, when compared to growth at the drug-free control [18]. For AMB and ITC, MIC was considered the lowest concentration capable of inhibiting 100% and 50% of the fungal growth, respectively [21]. All samples were tested in duplicate, and the results were visually read after three days of incubation at 35°C [19]. The minimum fungicidal concentration (MFC) was defined as the lowest drug concentration resulting in the death of 99% of the initial inoculum. To determine MFC, 0.1 ml of each sample without visible fungal growth were inoculated onto potato dextrose agar and incubated at 35°C for 7 days [22].

Evaluation of the influence of terpenes on loss of 260 and 280-nm-absorbing material

In vitro antifungal activity against *Sporothrix* spp. was previously determined by broth microdilution assay [20]. In this test, the microorganisms were submitted to the fol-lowing treatments: RPMI without drug (growth control); AMB and ITC (antifungal control) and 0.2 g/ml thimerosal ($C_9H_9HgO_2SNa$, Sigma-Aldrich, USA) as the positive control for membrane irreversible damage. The plates were incubated as described above for the susceptibility assays. After this period, the wells corresponding to MIC and sub-MIC of test-compounds and controls were used for this experiment. Approximately 1.0 ml of each fungal suspension, adjusted to 0.5 on McFarland scale was transferred to sterile microtubes and centrifuged for 15 minutes at 13,416 $\times$ g. Then, the supernatant was diluted 1:10 with sterile distilled water and the content was submitted to a spectrophotometric reading at wavelength of 260 to 280 nm for analysis of the presence of nucleic acids and proteins, respectively [23]. In these analyses, 16 strains of *Sporothrix* spp. were used, including *S. brasiliensis* ($n = 9$); *S. mexicana* ($n = 3$); *S. schenckii* ($n = 2$); and *S. globosa* ($n = 2$).

Statistical analyses

In order to evaluate the occurrence of differences in MICs of terpinen-4-ol and farnesol among the analyzed *Sporothrix* strains, a *post hoc* analysis of variance was applied, through Fisher's least significant difference test, given the low asymmetry of the data. P-values lower than 0.05 indicate statistically significant conclusions. Additionally, in order to verify differences in absorbance values at a wavelength of 260 nm and 280 nm, to evaluate the loss of 260 and 280-nm-absorbing material, after exposure to the tested terpenic derivatives, Student's t test for paired samples was used. P-values lower than 0.05 indicate statistically significant conclusions.

Results

Terpinen-4-ol and farnesol inhibited all isolates of *Sporothrix* spp. tested with different MIC values. The MIC values ranged from 87.9 to 1,429.8 μg/ml (0.57 to 9.27 mM; geometric mean of 2.35) for terpinen-4-ol and from 0.003 to 0.222 μg/ml (0.015 to 1.0 μM; geometric mean of 0.09) for farnesol (Table 1). No statistically significant differences were observed among the terpinen-4-ol MICs against the four tested *Sporothrix* species. However, farnesol MICs against *S. brasiliensis* and *S. globosa* were statistically lower than those obtained against *S. mexicana* and *S. schenckii* ($P < 0.05$). The MFC values ranged from 177.8 to 5,722.6 μg/ml (1.15 to 37.1 mM) and from 0.027 to 0.88 μg/ml (0.125 to 4.0 μM), for terpinen-4-ol and farnesol, respectively. Farnesol was the most active compound *in vitro* against all *Sporothrix* strains tested, including less susceptible strains to AMB (MIC = 4 μg/ml) and ITC (MIC = 2 μg/ml) (data not shown). The MIC for the

Table 1. Minimum inhibitory and fungicidal concentrations of terpenen-4-ol and farnesol against strains of the *Sporothrix schenkii* complex in the filamentous form ($n = 40$).

| Species (*n*) | Origin (*n*) | Drugs | MIC | | | MFC | |
			Values (n)	GM	Range	GM	Range
S. brasiliensis (26)	Human (6)	T-OH	0.57 (1) 1.1 (4) 2.3 (14) 4.6 (6) 9.2 (1)	2.44	0.57–9.27	23.58	1.15–37.1
	Animal (20)	F-OH	0.01 (2) 0.03 (6) 0.06 (10) 0.12 (5) 0.25 (3)	0.06[a]	0.015–0.25	0.76	0.125–4.0
	Environmental (0)						
S. schenckii (6)	Human (4)	T-OH	1.1 (2) 2.3 (3) 9.2 (1)	2.31	1.15–9.27	29.44	18.55–37.1
	Animal (1)	F-OH	0.03 (1) 0.12 (2) 0.5 (1) 1.0 (2)	0.25[b]	0.03–1.0	1.0	0.125–4.0
	Environmental (1)						
S. mexicana (5)	Human (3)	T-OH	1.1 (2) 4.6 (2) 9.2 (1)	3.05	1.15 – 9.27	18.55	4.63–37.1
	Animal (0)	F-OH	0.01 (1) 0.06 (1) 0.5 (2) 1.0 (1)	0.18[b]	0.015–1.0	1.31	0.5–4.0
	Environmental (2)						
S. globosa (3)	Human (3)	T-OH	0.57 (1) 1.1 (1) 2.3 (1)	1.15	0.57 – 2.31	18.55	9.27–37.1
	Animal (0)	F-OH	0,03 (1) 0.06 (1) 0.25 (1)	0.078[a]	0.03–0.25	1.25	1.0–2.0
	Environmental (0)						

Note. MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; GM: geometric mean; T-OH: terpinen-4-ol (mM); F-OH: farnesol (μM). The MIC for the control strains *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were: 1.0 μg/ml for AMB; 0.25 μg/ml for ITC; 37.0 mM for terpinen-4-ol, and 2.0 μM for farnesol. Different letters indicate statistically significant differences among the farnesol MICs obtained against the *Sporothrix* species.

control strains agreed with the CLSI guidelines (CLSI 2008). The results are shown in Table 1.

After contact with inhibitory concentration (MIC) and subinhibitory concentration (MIC/2) of terpinen-4-ol and farnesol, no significant changes were observed in cell membrane permeability in *Sporothrix* species, when compared to the drug-free control (Figure 1). Thimerosal, used as positive control test, was capable of causing cell lysis, so there was a significant increase in the 260 and 280-nm-absorbing material, when compared to the drug-free control, due to the presence of nucleic acid and protein molecules in the extracellular medium, after contact of *Sporothrix* strains with this salt. AMB and ITC (antifungal controls) were also not capable of causing damage to the fungal cellular membrane, hence, not promoting the escape of these macromolecules (Figure 1).

Discussion

Despite the introduction of new antifungal drugs in recent years, the response to therapy particularly in immunocompromised patients remains unsatisfactory, since the frequency of fungal infections has rapidly increased [24]. In addition to the limited drug arsenal, the obstacle to antifungal therapeutic success is mainly related to the adverse effects, low-spectrum of antifungal activity and emergence of intrinsic antifungal resistance among pathogenic species [25]. Many compounds isolated from plant extracts and essential oils have shown biological activity *in vivo* and *in vitro* [10,16]. Such compounds are known for their an-

tibacterial [26], antifungal [16], and anti-inflammatory [27] properties, among others. Chemically, these compounds are mostly terpenes, such as terpinen-4-ol and farnesol [7].

Brilhante *et al.* [18] investigated the *in vitro* antifungal activity of farnesol against strains of *Coccidioides posadasii*, with MIC ranging from 0.0017 to 0.0136 μg/ml (0.0078–0.0616 μM), which are quite low compared to the MIC values found in this study from 0.003 to 0.222 μg/ml (0.015–1.0 μM). However, in a work described by Derengowski *et al.* [13] farnesol presented an average MIC of 5.55 μg/ml (25 μM) against the dimorphic fungus *Paracoccidioides brasiliensis*, while against *Sporothrix* species the average was 0.04 μg/ml (0.18 μM).

Against other fungal species, such as *Cryptococcus neoformans* and *C. gattii* MIC values ranged from 0.064 to 16.67 μg/ml (0.29–75 μM) [17]. Cordeiro *et al.* [16] have also shown the activity of farnesol against several *Candida* species with MIC values ranging from 2.08 to 33.35 μg/ml (9.37–150 μM), which are well above the values found in this study. In another study, Brilhante *et al.* [26] observed the *in vitro* activity of farnesol against the bacterium *Burkholderia pseudomallei*, showing MICs between 16.67 and 33.35 μg/ml (75–150 μM). In the present work, farnesol has shown a good inhibitory activity against all the tested *Sporothrix* species. However, the *in vivo* antifungal potential is unknown.

The data presented here also showed that terpinen-4-ol can inhibit the *in vitro* growth of strains of the *Sporothrix schenkii* complex. Marcos-Arias *et al.* [15] analyzed the *in vitro* antifungal effects of terpenic derivatives
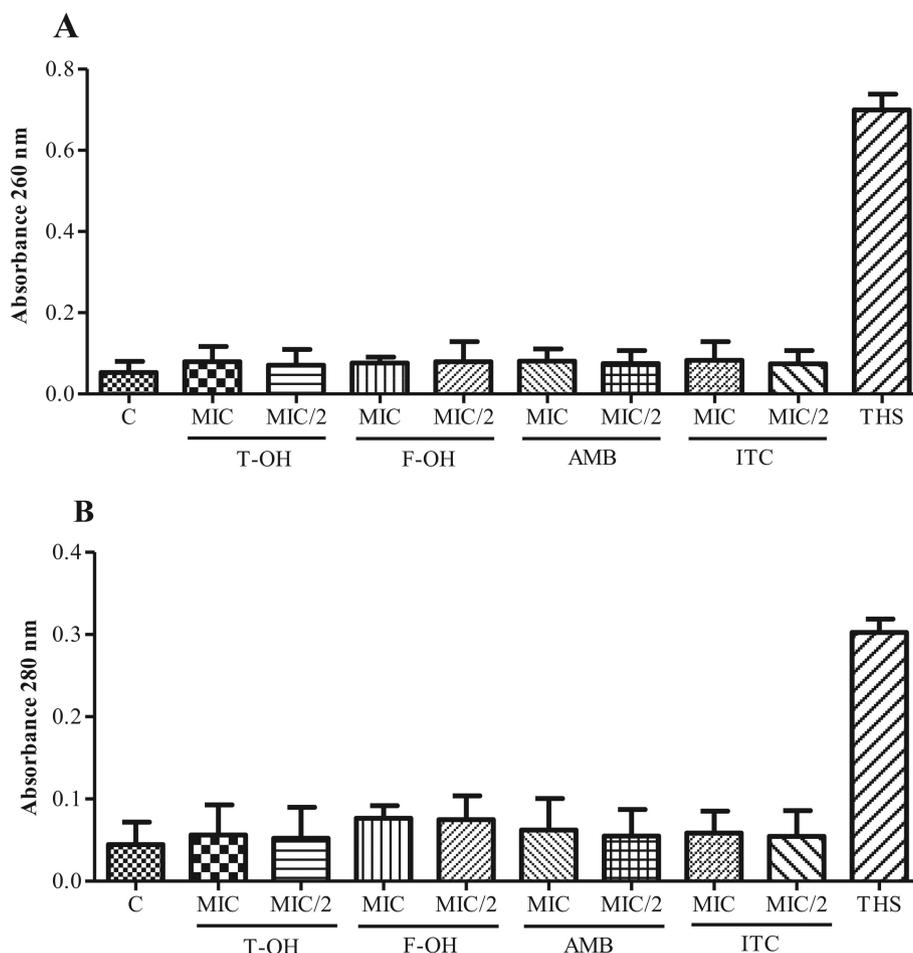
Figure 1. *Sporothrix* spp. strains ($n = 16$) were incubated in RPMI 1640 medium, without antimicrobials as growth controls (C) or with terpinen-4-ol (T-OH); farnesol (F-OH); amphotericin B (AMB), and itraconazole (ITC) at concentrations equivalent to MIC and MIC/2 for each strain, and 0.2 g/ml thimerosal (THS). All experiments were performed in duplicate. (A) Absorbance of extracellular contents at a wavelength of 260 nm. (B) Absorbance of extracellular contents at a wavelength of 280 nm.

against *Candida* isolates and terpinen-4-ol was one of the most active compounds against *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. guillermondii*. Inhibition of *C. albicans* biofilm formation was also assessed and terpinen-4-ol was able to effectively inhibit biofilm growth [28]. In other work, the effects of terpinen-4-ol on the *in vitro* growth of fluconazole-susceptible and -resistant *C. albicans* strains were also examined and showed MIC values ranging from 4,997.7 to 20,021.6 μg/ml (32.4–129.8 mM) [29]. The results of our study show an increased susceptibility of *Sporothrix* species to terpinen-4-ol, with MIC values ranging from 87.9 to 1,429.8 μg/ml (0.57–9.27 mM). Few data on *in vivo* trials with terpinen-4-ol are available; however, evidences of an anti-inflammatory effect of terpinen-4-ol have also been demonstrated in human skin [27].

The mechanisms of action of plant compounds are not well known [13]; however, the presence of terpenes in these compounds can indicate the occurrence of a toxic interaction with membrane constituents [9] and/or inhibition of ergosterol synthesis [18]. The OD_{260} and OD_{280} of filtrates treated with terpinen-4-ol and farnesol, when compared with the drug-free control suspensions, did not show statistically significant differences. The failure of terpenes to lyse *Sporothrix* cells suggests that these derivatives do not primarily cause gross and irreversible cell membrane damage, thus, not inducing the loss of 260 and 280 nm absorbing material, such as nucleic acids and proteins [18,30]. Studies with *C. albicans* using Methylene Blue staining demonstrated that terpinen-4-ol-treated cells did not present increased membrane fluidity [9].

In summary, the results of this work show that terpinen-4-ol and farnesol have *in vitro* antifungal activity against *Sporothrix* species. Thus, *in vivo* tests and new studies are needed to confirm the antifungal activity of terpenic derivatives and better understand the involved mechanisms of action.

Acknowledgments

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