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Original article

Antifungal activity and mechanism of action of monoterpenes against dermatophytes and yeasts

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ABSTRACT

Dermatomycosis causes highly frequent dermal lesions, and volatile oils have been proven to be promising as antifungal agents. The antifungal activity of geraniol, nerol, citral, neral and geranial (monoterpenes), and terbinafine and anidulafungin (control drugs) against seven opportunistic pathogenic yeasts and four dermatophyte species was evaluated by the Clinical and Laboratory Standards Institute microdilution tests. Monoterpenes were more active against dermatophytes than yeasts (geometric mean of minimal inhibitory concentration (GMIC) of 34.5 and 100.4 μg.ml^{-1}, respectively). *Trichophyton rubrum* was the fungal species most sensitive to monoterpenes (GMIC of 22.9 μg.ml^{-1}). The *trans* isomers showed higher antifungal activity than the *cis*. The mechanism of action was investigated evaluating damage in the fungal cell wall (Sorbitol Protection Assay) and in the cell membrane (Ergosterol Affinity Assay). No changes were observed in the MIC of monoterpenes in the sorbitol protection assay. The MIC of citral and geraniol was increased from 32 to 160 μg.ml^{-1} when the exogenous ergosterol concentrations was zero and 250 μg.ml^{-1}, respectively. The monoterpenes showed an affinity for ergosterol relating their mechanism of action to cell membrane destabilization.

Introduction

Human mycoses are caused by primary pathogenic fungi that invade the tissues of a normal host, or by opportunistic fungi that invade the tissues of individuals with severe alterations in the immune system. Dermatomycosis is one of the most frequent dermal lesions that affect humans and animals, and is often caused by dermatophytes of the genera *Epidermophyton*, *Microsporum* and *Trichophyton*. These fungi infect keratinized tissue such as skin, nails and scalp,

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and their lesions are characterized by irritation, scaling, local redness, swelling and inflammation (Kyle and Dahl, 2004; Gupta and Cooper, 2008; Patel and Schwartz, 2011). Meanwhile, some dermatomycoses may be associated with the presence of opportunistic yeast pathogens, including genus *Candida*, that can quickly colonize damaged nails or skin, especially the mucous membranes (Eggimann et al., 2003).

Citral (CIT), geraniol (GOL) and nerol (NOL) are monoterpenes constituents of volatile oils. CIT is a mixture of two geometric isomers, neral (NAL, *cis* isomer) and geranial (GAL, *trans* isomer). CIT obtained by organic synthesis has similar percentages of its isomers, whereas the volatile oils obtained by extraction from plant sources have a higher content of geranial (GAL:NAL (4:3)) (Weisheimer et al., 2010).

The diversity of medicinal plants contributes to several studies that aim to demonstrate the action and effectiveness of some compounds against fungi. It is worth noting that volatile oils containing CIT, GOL and NOL, have proved promising, and may be incorporated into pharmaceutical preparations, alone or in combination (Prasad et al., 2010; Saddiq and Khayyat, 2010; Zore et al., 2011a; Miron et al., 2014a).

The mechanism of action of monoterpenes has not been completely clarified. Some studies showed the breakdown of cytoplasmic and organelle membranes exposed to certain volatile oils. The loss of membrane integrity can cause changes in membrane function leading to the antifungal activity (Sikkema et al., 1995; Pinto et al., 2006; Park et al., 2009). Despite these findings, it is not known how volatile oils damage the membranes. The discovery of the mechanism of action can help maximize the effect of natural products, either by concentration of active ingredients or formulation optimization.

Considering the significance of dermatomycosis and candidiasis as prevalent diseases (Ghannoum et al., 2000; Caputo et al., 2001), the present study was undertaken to establish the antifungal activity of some monoterpenes found in volatile oils (GOL, NOL, GAL, NAL and CIT) and to investigate possible mechanisms of action for these substances.

Material and methods

Reagents

CIT 98.7% (containing 47.6% and 49.1% of NAL and GAL, respectively), GOL 99%, NOL 99% and ergosterol were purchased from Sigma-Aldrich® (Brazil). Lemongrass oil from *Cymbopogon citratus* was obtained from Ferquima (São Paulo, Brasil). Ecalta® (anidulafungin - AFG) was purchased from Pfizer® (USA) and each 1 g of injectable product contains 100 mg of AFG. Terbinafine hydrochloride (TBF) 100% and amphotericin B (AMP) 972 $\mu g.mg^{-1}$ were kindly supplied by Cristália® (Brazil). Methanol (Tedia®, HPLC grade) and water filtered through a Milli-Q purification system (Millipore®) were used for HPLC mobile phase preparation. RPMI 1640 medium containing L-glutamine, without sodium bicarbonate, Sigma-Aldrich® (USA), and buffered to pH 7.0 with MOPS buffer (Sigma®).

Isolates

The set of isolates included yeasts: *Candida albicans, Candida krusei, Candida glabrata, Candida tropicalis, Candida parapsilosis, Cryptococcus neoformans, Trichosporon asahii*; and dermatophytes, *Trichophyton rubrum, Trichophyton mentagrophytes, Microsporum canis* and *Microsporum gypseum,* from the culture collections of the Laboratory of Applied Mycological Research - Federal University of Rio Grande do Sul (LAMR-UFRGS).

Inocula preparation

Inocula were prepared according to M27-A3 and M38-A2 documents determined by the National Committee for Clinical Laboratory Standards (NCCLS, 2008a,b). Briefly, stock filamentous fungi inocula suspensions were prepared from 7-day cultures grown on potato dextrose agar (Oxoid) and adjusted in sterile saline 0.9% spectrophotometrically to optical densities that ranged from 0.085 to 0.100 (approximately 80 to 82% transmittance) and diluted (1:50) in RPMI 1640 with MOPS buffer (RPMI-MOPS). Yeasts were grown on Sabouraud Dextrose Agar (Oxoid) for 48 h at 35°C and standardized to the turbidity of a 0.5 McFarland standard, and diluted 1:50 in saline followed by dilution 1:20 in RPMI-MOPS.

Microdilution assay

Minimal inhibitory concentration (MIC) values were determined by broth microdilution using the twofold dilution method according to the NCCLS guidelines with RPMI-MOPS. Monoterpenes (CIT, GOL and NOL), isomers of CIT (GAL and NAL) and antifungal drugs (TBF and AFG) were tested in the range of 0.25 to 128 $\mu g.ml^{-1}$. Afterwards, 100 µl were inoculated into all but the negative control wells of a flat-bottom 96-well microtiter plate with each drug concentration range. The plates were incubated for 48 h at 35°C for the yeast; fungi and dermatophytes were incubated for seven days at the same temperature. All assays were tested in triplicate. The MIC was defined as the lowest concentration of compound at which the microorganism tested did not demonstrate visible growth.

Mechanism of action

Sorbitol Protection Assay

MIC values were determined using *T. asahii* TAH10, by the standard broth microdilution procedure described above. Duplicate plates were prepared: one of them containing two-fold dilutions of AFG (positive control), CIT, GOL or NOL from 1 to 256 $\mu g.ml^{-1}$ and the other contained the same concentrations plus 0.8 $mol.l^{-1}$ sorbitol. MIC was determined after 2 and 7 days.

Ergosterol Effect Assay

MIC of AMP (positive control), CIT, GOL or NOL against T. assahii TAH10 was determined following the guidelines of NCCLS as explained above, in the absence and in the presence of different concentrations (0, 50, 100, 150, 200 and 250 $\mu g.ml^{-1}$) of ergosterol added to the assay medium, in different lines of the same microplate. The antifungals were added to reach the following final concentrations: 0.125, 0.25, 0.5 or 1.0 $\mu g.ml^{-1}$ for AMP and

32, 64, 96, 128 or 160 µg.ml^{-1} for monoterpenes. The MIC was determined after 24 h according to the control fungal growth and further confirmed after 48 h.

Purification of citral isomers

Samples of standard CIT and lemongrass oil were subjected to analysis by Gas Chromatography – Flame Ionization Detection (GC-FID), as described in the Lemongrass monograph present in the Brazilian Pharmacopoeia (Farmacopeia Brasileira, 2010). The analyses were performed on a Perkin Elmer gas chromatograph model Autosystem XL (Shelton, USA).

Citral isomers (GAL and NAL) were isolated/purified from CIT solution (12 mg.ml^{-1}) and identified by a high-performance liquid chromatography (HPLC) method adapted from Miron et al. (2012). Purification was performed in an Agilent instrument (series 1200) using a reversed phase ACE® RP18 column (250 × 4 mm, 5 µm particle size). The system was set with a mobile phase consisting of methanol and water (67:33, v/v), injection volume was 50 µl, and flow rate 1.4 ml.min^{-1}. The CIT isomers were detected at 270 nm and the apex peak of each isomer was collected separately in glass tubes. The purified isomers were subsequently quantified with the same HPLC method (detection at 240 nm and injection volume 20 µl). Peak purity analysis was performed using the wavelength range of 200-300 nm, standard deviation of noise determined at 0.2 min with 50 spectra, reference spectra set in automatic mode and purity level calculated with apex spectrum.

Statistical analysis

Statistical analysis was carried out using Prism 5 statistical analysis software for Windows (GraphPad software®). MIC data were used to compare the activity of the antifungal agents using a non-parametric statistical test (Mann-Whitney Test). The geometric mean MIC (GMIC) was employed to summarize results. The values of MIC > 128 µg.ml^{-1} were replaced by 256 µg.ml^{-1} to enable the calculation and thus avoid the underestimation of GMIC by deletion of data.

Results

Purification of citral isomers

The analysis by GC-FID was performed to identify and quantify NAL and GAL in the CIT standard and the lemongrass oil. The identification of CIT isomers was achieved by comparing the experimental results of Kovats Index to the values established by the Brazilian Pharmacopoeia. NAL showed lower retention time (10.1 min) than GAL (10.7 min). The relative percentage area of the chromatogram peaks of NAL and GAL was calculated by electronic integration. The CIT standard showed 47.6 and 49.1% of NAL and GAL, respectively, whereas lemongrass oil had 33.5 and 44.6%. For CIT standard, NAL and GAL had similar peak intensities. Nevertheless, for the lemongrass oil, GAL showed a more intense peak than NAL.

The lemongrass oil analysis by HPLC at 240 nm showed the highest peak with retention time at 9.5 min, which was identified as GAL. A second peak, less intense, at 8.5 min was assigned to NAL.

NAL and GAL were purified by HPLC from a CIT standard solution containing 12 mg.ml^{-1} (Fig. 1a). The injection of the highly concentrated CIT solution was monitored at 270 nm to minimize the greater absorptivity of NAL and GAL at 240 nm. Four drops from the peak apex were collected in different tubes for each one of the isomers. This small volume collected allowed having purified isomers in concentrations greater than 1.3 mg.ml^{-1}. NAL and GAL obtained by purification were diluted with water to a concentration of 256 µg.ml^{-1}. Thus, the initial concentrations of GAL and NAL in the microdilution test were 128 µg.ml^{-1} with less than 7% methanol.

Purified isomers quantification was performed at 240 nm of the diluted samples (Fig. 1c) comparing its results to CIT standard solution (~ 0.00952 and 0.00982 mg.ml^{-1} of NAL and GAL, respectively). The purification of NAL results in solutions with higher concentrations (1.35-1.51 mg.ml^{-1}) than GAL (1.30-1.39 mg.ml^{-1}). The purity of the solutions containing the isolated isomers was estimated from the relative percentage area of the peaks in the chromatograms at 240 nm. Calculated purity percentages of NAL and GAL were 98.4% and 97.9%, respectively.

Purified and undiluted NAL and GAL were stable at 4°C for at least 72 h. The peak purity analysis did not detect impurities for the samples of NAL and GAL stored for 72 h at 4°C (Fig. 1d). The analysis of the same samples after five days showed impurities co-eluting with NAL (the similarity curve intersected the threshold curve) though the reduction in the concentration of the drugs was not significant (Fig. 1e). Keeping the purified isomers at room temperature during 48 h causes over 5% reduction of content.

In vitro *activity*

The isolates of yeasts tested in this study exhibited different sensitivities against monoterpenes (Table 1). CIT was more active against *C. neoformans* and *T. asahii*; however, CIT showed lower MIC against *Candida* species than its isolated isomers. The results of GOL and NOL against yeasts were not impressive, especially for *Candida* species. It should be mentioned that all yeast strains were susceptible to CIT in the concentration range evaluated (0.25 – 128 µg.ml^{-1}).

For dermatophyte isolates, the greatest effect of monoterpenes was observed against *T. rubrum* (GMIC of 22.9 µg.ml^{-1}). GOL and CIT were the most active monoterpenes against *Microsporum* strains (GMIC of 19.5 and 23.8 µg.ml^{-1}) and highly effective against dermatophytes (GMIC of 25.4 and 16.0 µg.ml^{-1}, respectively).

In general, the monoterpenes were more active against dermatophytes than yeasts (Fig. 2). The GMIC of monoterpenes were 34.5 and 100.4 µg.ml^{-1} for dermatophytes and yeasts, respectively.

CIT was active against all strains of yeasts and dermatophytes. GOL and GAL had intermediate activity, similar to CIT in many strains. Furthermore, *trans* isomers (GOL and GAL) showed greater activity than *cis* isomers (NOL and GOL). The Mann-Whitney test showed a statistical difference between MIC of *trans* and *cis* isomers (p = 0.007) with GMIC of 56.3 and 99.0 µg.ml^{-1}, respectively.

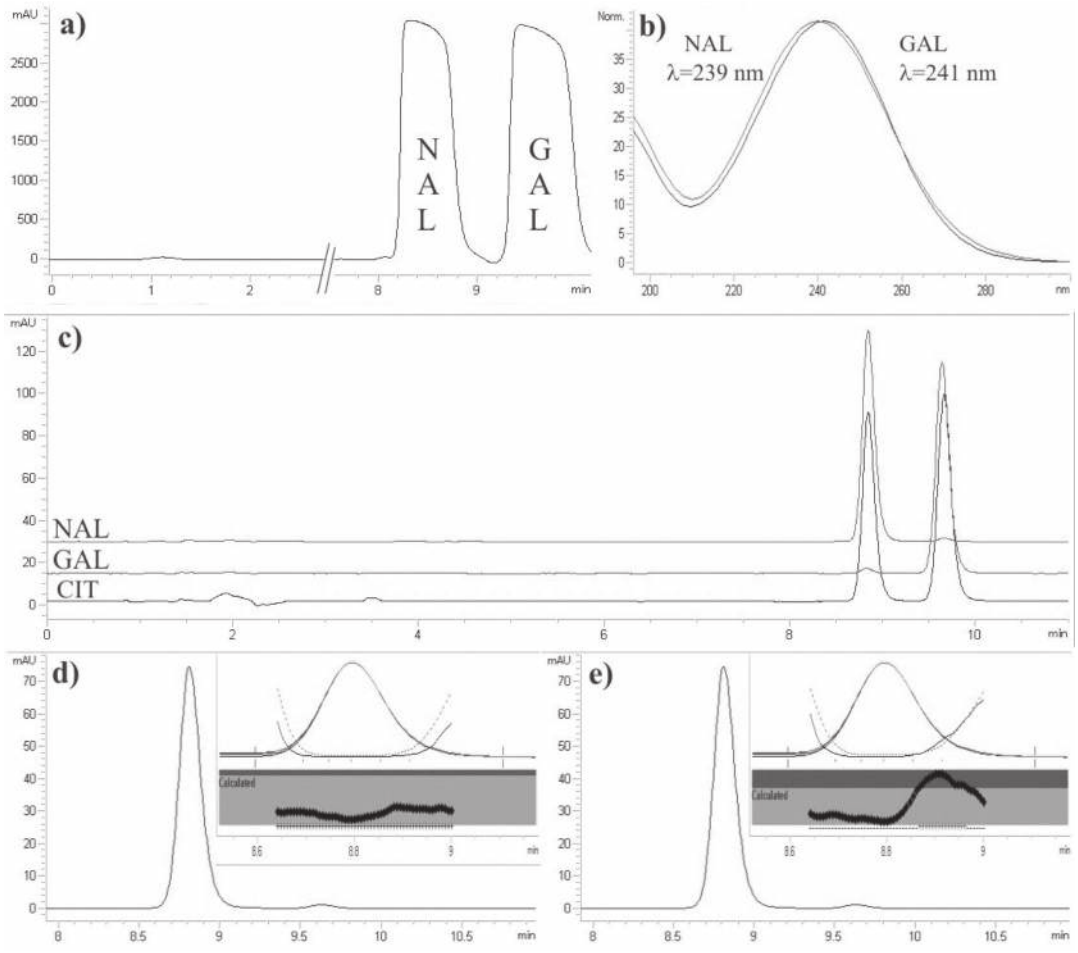

Figure 1 – Chromatograms, UV spectra and peak purity analysis of citral isomers. A. Chromatogram at 270 nm of high concentrated citral solution used to neral and geranial purification; B. UV spectra of neral and geranial; C. Chromatogram at 240 nm of citral standard and neral and geranial purified; D. Peak purity analysis of neral after 72 h of storage at 4°C – impurity not detected; E. Peak purity analysis of neral after five days of storage at 4°C – impurity detected.

Table 1
Susceptibility of yeast and dermatophytes against monoterpenes (NAL, GAL, CIT, NOL and GOL) and control drugs (AFG and TBF). Median MIC in μg.ml^{-1} (the results in brackets represent the maximum and minimum MIC for each species).

		MIC (μg.ml^{-1})					
	Microorganism	**NAL**	**GAL**	**CIT**	**NOL**	**GOL**	**TBF**
Dermatophytes	*T. rubrum* (n = 5)	32 (128,16)	8 (128,2)	4 (64,2)	64 (64,32)	64 (64,2)	0.25 (0.25,0.25)
	T. menthagrophytes (n = 3)	128 (128,16)	32 (64,8)	32 (128,2)	128 (128,64)	32 (64,32)	2 (4,0.25)
	M. canis (n = 4)	80 (>128,32)	48 (128,16)	48 (64,16)	128 (128,128)	40 (64,1)	0.25 (4,0.25)
	M. gypseum (n = 3)	>128 (>128,32)	32 (>128,16)	16 (128,1)	128 (128,1)	128 (128,1)	0.25 (0.5,0.25)
		NAL	**GAL**	**CIT**	**NOL**	**GOL**	**AFG**
Yeasts	*C. albicans* (n = 3)	>128 (>128, >128)	>128 (>128, >128)	64 (128,64)	>128 (>128,128)	>128 (>128,128)	8 (8,0.25)
	C. Krusei (n = 3)	>128 (>128, >128)	>128 (>128,64)	64 (64,64)	128 (128,128)	128 (128,64)	0.5 (32,0.25)
	C. glabrata (n = 3)	64 (>128,32)	64 (64,64)	64 (128,64)	>128 (>128,128)	>128 (128,32)	0.5 (4,0.25)
	C. tropicalis (n = 3)	>128 (>128, >128)	>128 (>128,128)	64 (128,64)	>128 (>128,128)	128 (128,128)	4 (32,2)
	C. parapsilosis (n = 3)	>128 (>128,128)	64 (>128,32)	64 (128,64)	128 (128,64)	128 (128,64)	2 (2,0.25)
	T. asahii (n = 3)	128 (>128,64)	128 (128,64)	32 (64,32)	64 (128,32)	128 (128,32)	0.25 (4,0.25)
	C. neoformans (n = 2)	17 (32,2)	8.5 (16,1)	12 (16,8)	128 (128,128)	72 (128,16)	1.1 (2,0.25)

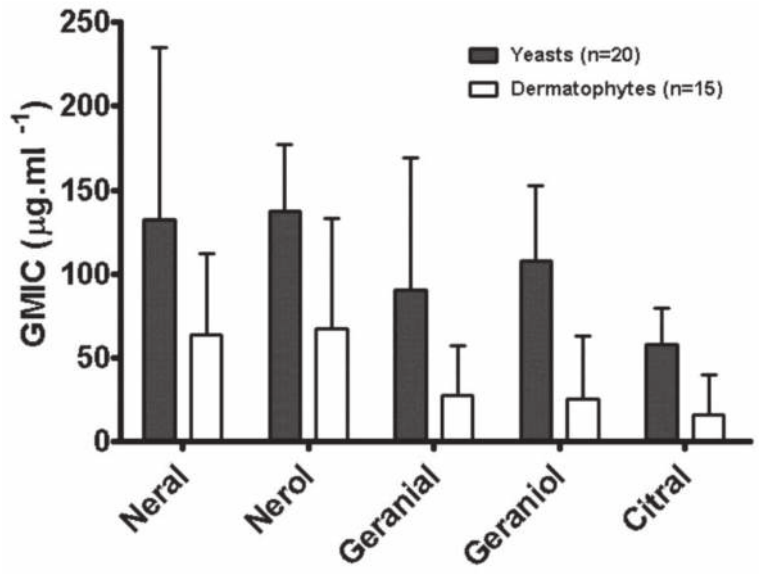

Figure 2 – Geometric mean of minimum inhibitory concentration (GMIC) of monoterpenes against yeasts and dermatophytes, and 95% confidence interval.

Mechanism of action

The MIC of the positive control (AFG), in the sorbitol protection assay, was increased by the presence of sorbitol (Table 2). However, after seven days of incubation, the MIC of monoterpenes did not increase. Morphological analysis of the microorganisms not treated with antifungals showed perfect

Table 2
MIC values (µg.ml^{-1}) of monoterpenes (CIT, GOL and NOL) and anidulafungin (AFG) against *T. asahii* TAH10 in the Sorbitol Protection Assay.

Sorbitol in the media (mol.l^{-1})		MIC (µg.ml^{-1})			
		AFG (control)	CIT	GOL	NOL
0.0	Day 2	1	32	32	32
	Day 7	1	32	32	32
0.8	Day 2	1	32	32	32
	Day 7	128	32	32	32

yeast cells in reproduction (Fig. 3). Cells treated with AFG and sorbitol, after seven days, are viable and recover their growth, but dysmorphic cells still occur due to damage to the cell wall. Sorbitol has no effect on monoterpene activity and, even after seven days, there was no microorganism growth.

In the "Affinity Ergosterol Assay", AMP and monoterpenes had their MIC increased since larger amounts of exogenous ergosterol were added to medium (Fig. 4). When ergosterol ranged from 0 to 250 µg.ml^{-1} in the growth medium, MIC for monoterpenes increased from 32 to 160 µg.ml^{-1}. Concentrations

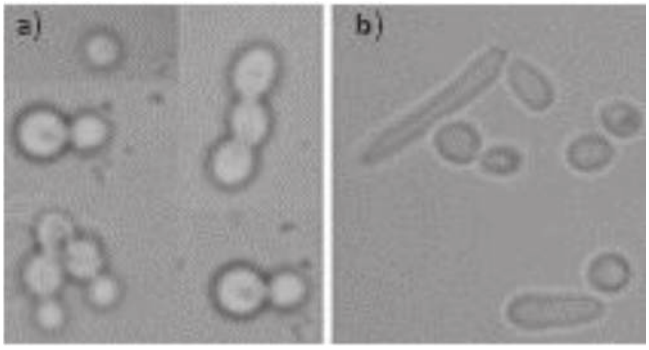

Figure 3 – Optical microscopy of *T. asahii* after seven days of incubation in culture medium (a) no antifungal, (b) medium with AFG and sorbitol.

of exogenous ergosterol, equal or greater than 100 µg.ml^{-1}, increased the MIC of AMP; and at concentrations of 150 µg.ml^{-1} of ergosterol in the medium, the MIC of AMP has increased eight-fold.

Discussion

Purification of citral isomers

Isomers were identified from information obtained from analysis by GC-FID of the CIT standard and sample of lemongrass oil which showed greater amount of GAL than NAL (relation of 4:3 was found). The peak with higher intensity in the analysis of the lemongrass oil by HPLC-UV was identified as GAL. The comparison with CIT standard solution allowed the identification of NAL peak. Furthermore, Miron et al (2012) showed that GAL and NAL can be identified by UV analysis since the *trans* isomer GAL has greater maximum in the UV spectrum (Fig. 1b).

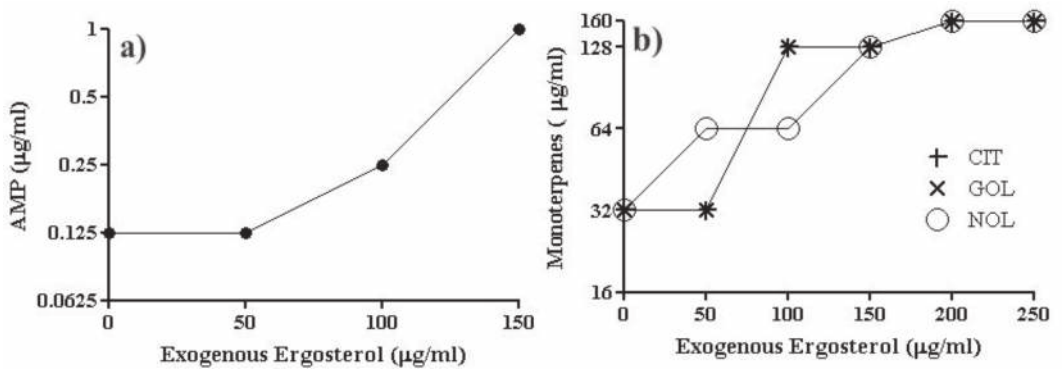

Figure 4 – Effect of exogenous ergosterol (50-250 µg.ml^{-1}) on the MIC of (a) amphotericin B and (b) monoterpenes against *T. asahii* (strain TAH10).

Essential oils, including CIT (Log $P^{o/w}$ 2.8-3.0), have pronounced lipophilic properties (OECD, 2001). Therefore, a large amount of the organic phase (67% methanol) was used for elution of the isomers (NAL and GAL). In this context, it was essential to collect small volumes of the apex of the chromatographic peak yielding high concentrations of the isolated isomers. This allowed a large dilution (at least 1:10) of the isolated isomers such that the final concentration of methanol in the tests of microbiological activity was less than 7% (there was no growth inhibition at this concentration of methanol for any of the strains tested).

CIT and its isomers are aldehydes that can be unstable in aqueous solution (Choi et al., 2009; Miron et al., 2014b). The CIT isomers were stored at 4°C because of its great instability at room temperature (more than 5% is degraded in 48 h). Peak purity analysis is a very sensitive tool and detected a co-eluting compound along NAL peak after 120 h of storage at 4°C. The purification/isolation process did not last more than 8 h. The purified isomers were kept in a refrigerator in a tight glass flask and used within 48 h after extraction in the microbiological assays. This procedure was used to minimize the effect of the instability of the CIT isomers and to ensure reproducibility of the results. For the same reason, CIT solutions were prepared at the same time of the purification process of its isomers to control any time effect on the microbiological assays. NOL and GOL are alcohols and previous studies did not show any stability problems in aqueous solution and in temperatures around 32°C (Miron et al., 2014a).

In vitro *activity*

CIT was the monoterpene with greater antifungal activity against yeasts. These results are consistent with results from Sajjad et al. (2012) and Zore et al. (2011a,b) that showed lower MIC values to CIT than to GOL.

CIT and its isomers were very effective against *T. rubrum*, which causes most cases of onychomycosis (Thomas et al., 2010; Nazar et al., 2012). *Microsporum* species infect animals more frequently than humans, but reports have shown that dogs and cats can transmit these microorganisms to man (Segundo et al., 2004; Grills et al., 2007). GOL and CIT lead the antifungal activity against this species as well as against dermatophytes in general.

Many studies on volatile oils evaluate the activity of monoterpenes against *Candida* species, probably due to its clinical relevance (Sajjad et al., 2012; Zore et al., 2011a,b). Despite this, our study demonstrated that monoterpenes were more active against dermatophytes than yeasts. These results are promising, demonstrating the potential of monoterpenes against dermatophyte species.

Mechanism of action

The Sorbitol Protection Assay was performed to test the effect of monoterpenes on the integrity of the fungal cell wall (Frost et al., 1995). In this assay, MIC determinations were conducted with and without 0.8 mol.l^{-1} sorbitol. Specific *T. asahii* strain TAH10 (Internal laboratory identification) was used because of its known sensitivity to AFG and other terpenes. Note that this specific strain was not tested in the antifungal activity assay and its MIC results are not presented in the Table 1.

Sorbitol is an osmotic protectant used for stabilizing fungal protoplasts and it is expected that the MIC of a compound that damages the cell wall will shift to a much higher value in the presence of the osmotic support. No variations in MIC were observed for monoterpenes in this assay suggesting that CIT, GOL and NOL would not act by inhibiting control of cell wall synthesis or assembly mechanisms.

To determine if monoterpenes bind to ergosterol, the assay based on the effect of exogenous ergosterol was performed. The affinity of a molecule with ergosterol can be established by determining the MIC in the presence of exogenous ergosterol in the extracellular media. If the compound binds to ergosterol, it will rapidly form a complex, preventing the cellular membrane from interacting with ergosterol. Consequently the observed MIC increases.

The MIC for monoterpenes increased five-fold in the presence of exogenous ergosterol revealing that CIT, GOL and NOL bind to the main sterol of fungal membranes. AMP also interacts with ergosterol, and at concentrations of 200 µg.ml^{-1} of sterol there was no more inhibition caused by AMP. This is probably due the large relative concentration of ergosterol

that result in very low concentrations of free AMP and, consequently, no inhibition of fungal growth.

The mechanism of action of AMP has recently been associated to sterol binding and channel formation (classical known mechanism of action) seems to be only one of multiple sterol-binding-dependent mechanism of action (Palacios et al., 2011; Wilcock et al., 2013). Park et al. (2009) reported the effect of citral at the cellular level in a strain of *T. menthagrophytes* employing the Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) techniques. The authors found less thickness and the presence of discontinuities in the cellular membrane, concluding that the membrane was degraded. The results at high concentrations of terpenes (citral, eugenol, nerolidol and α-pinene) also show the breakdown of the cell membrane, along with abnormalities in the structure of mitochondria.

The positive results of monoterpenes on the "Affinity Ergosterol Assay" and the other reports on the subject strongly suggest that the mechanism of action of this class of drugs is related to ergosterol-binding and a subsequent destabilization of fungal cell membranes.

Authors' contributions

DM was involved in all experimental studies and drafted the manuscript; FB contributed to purification and HPLC analysis; AF, FKS, ADL and BP designed and performed the antifungal activity assays; BC and SG designed and performed the mechanism of action assays; PM and ESSS supplied the materials and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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