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Farnesol inhibits *in vitro* growth of the *Cryptococcus neoformans* species complex with no significant changes in virulence-related exoenzymes

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ABSTRACT

Farnesol is a sesquiterpene alcohol that modulates cell-to-cell communication in *Candida albicans*. In recent years, several studies have shown that this molecule presents inhibitory effects against non-*albicans Candida* species, *Paracoccidioides brasiliensis* and bacteria. The present study aimed at determining the effect of farnesol on the growth of strains of the *Cryptococcus neoformans* species complex, through microdilution assays. In addition, the effect of farnesol on the synthesis of phospholipase and protease – important virulence-associated enzymes – by *C. neoformans* and *Cryptococcus gattii* was also investigated. A total of 36 strains were studied, out of which 20 were from veterinary sources, 8 were from human cases and 8 were from a reference collection. The minimum inhibitory concentrations (MICs) were determined in accordance with the M27-A3 protocol as described by the CLSI and farnesol was tested at a concentration range of 0.29–150 μM. Phospholipase and protease activities were evaluated through growth on egg yolk agar and spectrophotometry, respectively, after pre-incubating the strains at different farnesol concentrations (MIC/4, MIC/2 and MIC). It was observed that farnesol presents an inhibitory activity against *C. neoformans* and *C. gattii* (MIC range: 0.29–75.0 μM). Although farnesol did not significantly alter phospholipase activity, a tendency to decrease this activity was observed. Concerning protease, no statistically significant differences were observed when comparing the production before and after pre-incubation at different farnesol concentrations. Based on these findings, it can be concluded that farnesol has *in vitro* inhibitory activity against *C. neoformans* and *C. gattii*, but has little impact on the production of the analyzed virulence factors.

1. Introduction

The *Cryptococcus neoformans* species complex is formed by two distinct microorganisms, *C. neoformans* and *C. gattii*, which are sub-classified into at least two varieties, five serotypes and eight molecular types (Kwon-Chung and Varma, 2006; Bovers et al., 2008; Lester et al., 2011). Despite sharing several phenotypical and molecular

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characteristics, these species differ from each other concerning ecological and epidemiological aspects (Lin and Heitman, 2006; Costa et al., 2010). They are the major causative agents of cryptococcal meningoencephalitis, the most frequent clinical manifestation of cryptococcosis, which has a high mortality rate in developing countries (Kronstad et al., 2011).

To succeed in the host environment, *Cryptococcus* spp. produce a large amount of virulence factors, which directly influence the infectivity of an individual strain (Ma and May, 2009). Among these main virulence factors, hydrolytic enzymes able to degrade lipids and proteins have gained attention in recent years (Bien et al., 2009; Chayakulkeeree et al., 2011). Phospholipases are directly related to the destabilization of host cell membranes (Ma and May, 2009) and proteases have been shown to degrade several host molecules (Chen et al., 1996).

Even though new antifungal drugs have been developed in recent years, the availability of antifungal agents with anticryptococcal activity is still limited. This scenario has motivated the pursuit of new compounds that present antifungal properties against *Cryptococcus* spp. Based on this perspective, farnesol, a sesquiterpene alcohol, has been investigated for its antimicrobial potential (Jabra-Rizk et al., 2006; Semighini et al., 2008; Derengowski et al., 2009; Weber et al., 2010; Pammi et al., 2011).

Farnesol is found in plant extracts and also produced by *Candida albicans* as a quorum sensing molecule (Hornby et al., 2001, Derengowski et al., 2009). This autoregulatory compound alters *C. albicans* morphology and also has a dramatic impact on fungal growth and survival (Shirtliff et al., 2009; Weber et al., 2010). Several studies have shown that this molecule also has inhibitory effects against other pathogens, including non-*albicans Candida* species (Weber et al., 2010), *Paracoccidioides brasiliensis* (Derengowski et al., 2009), *Fusarium graminearum* (Semighini et al., 2008) and bacteria (Pammi et al., 2011; Brilhante et al., 2012).

The present study aimed at evaluating the antifungal potential of farnesol against *C. neoformans* and *C. gattii*. In addition, the effect of farnesol on phospholipase and protease activities was also investigated.

2. Material and methods

2.1. Fungal strains

A total of 36 strains from the culture collection of the Specialized Medical Mycology Center of Federal University of Ceará were included in this study, out of which 20 were from veterinary sources (2 from animal clinical cases and 18 from pigeon excreta) and 8 from human clinical cases. Eight international reference strains of *Cryptococcus* sp. obtained from the Evandro Chagas Clinical Research Institute, Brazil (IPEC/FIOCRUZ) were used as controls: *C. neoformans* var. *grubii* WM 148 (serotype A, VNI/AFLP1); *C. neoformans* var. *grubii* WM 626 (serotype A, VNII/AFLP1A); *C. neoformans* WM 628 (serotype AD, VNIII/AFLP2); *C. neoformans* var. *neoformans* WM 629 (serotype D, VNIV/AFLP3); *C. gattii* WM 179 (serotype B, VGI/AFLP4); *C. gattii* WM 178 (serotype B, VGII/AFLP6); *C. gattii* WM 175 (serotype B, VGIII/AFLP5); and *C. gattii* WM 779 (serotype C, VGIV/AFLP7), as described by Meyer et al. (2003). In addition, *Candida parapsilosis* ATCC 22019 was included as internal quality control for the susceptibility tests (CLSI, 2008). All isolates were stored on 2% potato dextrose agar supplemented with glycerol, at −20 °C, and were recovered through growth on 2% potato dextrose agar at 35 °C, for 2 days (Cordeiro et al., 2011).

2.2. Dilution of farnesol, amphotericin B and fluconazole

The strains were tested against farnesol diluted with 30% dimethyl sulfoxide (DMSO) at the moment of use, in order to obtain a stock solution at a concentration of 1892 μM. Afterwards, the stock solution was diluted with RPMI 1640 to a concentration of 600 μM, in which DMSO was at a concentration of approximately 10%. The tested concentration range was from 0.29 to 150 μM.

Amphotericin B (Sigma Chemical Corporation, Germany) and fluconazole (Pfizer Pharmaceuticals, USA) were tested against *C. parapsilosis* ATCC 22019 as internal quality control, and also against the eight international reference *Cryptococcus* strains. These drugs were diluted with sterile distilled water, as described by the document M27-A3 of the Clinical Laboratory Standards Institute (CLSI, 2008). The tested concentration ranged from 0.03125 to 16 μg/mL and from 0.125 to 64 μg/mL, for amphotericin B and fluconazole, respectively. Additionally, the effect of DMSO on the viability of *Cryptococcus* spp. was evaluated, at concentrations that ranged from 0.05 to 30%.

2.3. Testing in vitro susceptibility to farnesol

Fungal inocula were prepared from cultures grown on potato dextrose agar at 28 °C, for 48 h. Sterile 0.9% saline (5 mL) was added to sterile glass slants and a sample of the colony was added to the saline solution, adjusting its turbidity to 0.5 on the McFarland scale (CLSI, 2008). Afterwards, inocula were diluted to 1:100 and 1:20 with RPMI 1640 medium supplemented with L-glutamine (HiMedia, Mumbai, India), and buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS). The final inoculum concentration was $0.5–2.5 \times 10^3$ cells/mL (CLSI, 2008; Costa et al., 2010).

The minimum inhibitory concentrations (MICs) of farnesol and the tested drugs against *C. neformans* and *C. gattii* strains were determined through the broth microdilution method, in 96-well microdilution trays, with a capacity of 200 μL/well. These trays were properly prepared and incubated at 35 °C for 72 h, when they were visually read. The MIC for farnesol was defined as the lowest drug concentration able to inhibit 80% of fungal growth, when compared to the farnesol-free control wells (Jabra-Rizk et al., 2006). Concerning amphotericin B and fluconazole, the MICs were defined as the lowest concentrations capable of inhibiting 100% and 50% of fungal growth, respectively (CLSI, 2008).

2.4. Effects on phospholipase and protease activities after exposure to farnesol

In this step, all strains were grown on 2% Sabouraud dextrose agar and incubated at 37 °C, for 48 h. Afterwards,

the strains were cultured in RPMI 1640 medium, containing three decreasing concentrations of farnesol (MIC, MIC/2 and MIC/4), at 37 °C, for 48 h. A farnesol-free control for each strain was also included.

Phospholipase activity was evaluated according to Sidrim et al. (2010). Initially, the fungal suspensions in RPMI medium containing different farnesol concentrations were centrifuged at 805 × *g*. Then, from the fungal pellets, yeast inocula were prepared in 0.9% saline, reaching turbidity equivalent to 4.0 on the McFarland scale. The medium used was 2% Sabouraud dextrose agar, supplemented with 1 mol/L of sodium chloride, 0.05 mol/L of calcium chloride and 8% sterile egg yolk emulsion at 30%. The emulsion was heated to 40 °C and incorporated into the sterile Sabouraud medium after it reached a temperature of 40 °C. A volume of 5 μL of each inoculum was applied onto a 5-mm sterilized filter paper disk, which was placed on the agar surface. The plates were then incubated at 35 °C for seven days (Sidrim et al., 2010).

The phospholipase activity (Pz) was determined by calculating the ratio between the diameter of the fungal colony and the total diameter, including the colony and the precipitation zone. When Pz = 1, the isolate is phospholipase negative; when $1 > Pz \geq 0.64$ the isolate is positive for phospholipase activity; and when $Pz < 0.64$, the isolate is strongly positive for the enzymatic activity (Price et al., 1982; Sidrim et al., 2010).

For protease production, the analyses were performed according to Cenci et al. (2008), with modifications (Brilhante et al., 2011). Briefly, the strains were incubated in RPMI containing different decreasing farnesol concentrations, under slow agitation of 150 × *g*, for 48 h, at 28 °C. Afterwards, the material was centrifuged for 15 min at 805 × *g* to separate the enzymatic extract from the cells. Then, 2 mL of the enzymatic extract was incubated at 37 °C with 2 mL of 0.3% azoalbumine for 48 h. After this procedure, the reaction was stopped by the addition of 10% trichloroacetic acid. To the reaction mixture, 2 mL of 0.5 M KOH was added for posterior spectrophotometric analysis at 530 nm. The enzymatic unit (EU) was defined as the amount of enzyme that causes an increase in absorbance of 0.001 per mL at 530 nm.

2.5. Statistical analysis

To analyze the effects of farnesol on the growth of the strains and the activity of virulence factors, the data were analyzed through univariate analysis of variance. Farnesol MICs were evaluated considering species and source of each strain, whereas phospholipase and protease values were analyzed before and after exposure to farnesol. In this analysis, species and source of strains were also considered. *P*-values lower than 0.05 indicated statistically significant differences.

3. Results

Farnesol presented *in vitro* inhibitory effects against the tested strains, with MICs varying from 0.29 to 75 μM for both *C. neoformans* and *C. gattii*. MIC50 and MIC90 of farnesol against *C. neoformans* were 9.37 μM and 75 μM, respectively; while for *C. gattii* these values were 4.68 μM and 75 μM, respectively. Regarding antifungals, the MICs against *C. parapsilosis* ATCC 22019 were 0.5 μg/mL for AMB and 2 μg/mL for FLC. As for the reference strains of *Cryptococcus* sp. the MICs ranged from 0.0625 to 1.0 μg/mL for AMB and from 4.0 to 16.0 μg/mL for FLC. Information regarding susceptibility to farnesol of each tested strain and antifungal susceptibility of control strains is shown in Table 1. Susceptibility tests were performed with DMSO as solvent at 1.25%, which presented no inhibitory effect against *Cryptococcus* strains.

Furthermore, 18 (50%) out of the 36 tested strains secreted phospholipases without being exposed to farnesol (control). When the 36 strains were pre-incubated at three different concentrations of this compound, 8 (22.22%), 11 (30.55%) and 11 (30.55%) strains did not exhibit any alteration in phospholipase secretion after exposure to farnesol concentrations of MIC/4, MIC/2 and MIC, respectively (Table 2). Under experimental conditions, a reduction in enzymatic activity was observed in 17 (47.22%), 14 (38.88%) and 15 (41.66%) strains after pre-incubation at MIC/4, MIC/2 and MIC of farnesol, respectively. Increased phospholipase activity was observed in 11 (30.55%), 11 (30.55%) and 10 (27.77%) strains after pre-incubation at MIC/4, MIC/2 and MIC of farnesol, respectively.

Concerning protease activity, 18 strains (50%) secreted these enzymes without being exposed to farnesol (control). After pre-incubation at different concentrations of this sesquiterpene, no statistically significant differences were observed between the control and the tested farnesol concentrations. The mean ± SD values of protease activity (EU) of *C. neoformans* were as follows: 0.008 ± 0.013 for control; 0.017 ± 0.033 for MIC/4; 0.016 ± 0.031 for MIC/2 and 0.026 ± 0.033 for MIC. As for *C. gattii*, the mean values were 0.007 ± 0.009 for control; 0.015 ± 0.024 for MIC/4; 0.008 ± 0.008 for MIC/2 and 0.004 ± 0.008 for MIC.

4. Discussion

The mechanisms through which farnesol acts on fungal cells is still unknown (Derengowski et al., 2009; Langford et al., 2010), but it is believed that it damages the fungal cell membrane and impairs ergosterol synthesis, considering that farnesol and ergosterol share many precursors in the sterol biosynthetic pathway (Hornby and Nickeerson, 2004; Navarathna et al., 2005; Jabra-Rizk et al., 2006). In addition, as an efflux pump modulator, farnesol may affect membrane permeability through the disruption of cytoplasmic membranes (Jin et al., 2010; Sharma and Prasad, 2011).

The present article showed that farnesol can also inhibit growth of *C. neoformans* and *C. gattii* obtained from veterinary sources. The MIC values for both species were lower than those observed for other fungal pathogens, such as *P. brasiliensis*, for which inhibition was achieved at 25 μM (Derengowski et al., 2009), and *C. parapsilosis*, which was inhibited by 50 μM of farnesol (Rossignol et al., 2007).

Even though *C. gattii* has been described as being less susceptible to classical antifungal drugs (Trilles et al.,

Table 1
Minimum inhibitory concentration (MIC) of farnesol against strains of the *Cryptococcus neoformans* species complex.

Collection number	Specie	Serotype	Source	FNS	FLC	AMB
CEMM 03-2-067	*C. neoformans*	A	Veterinary	0.29	–	–
CEMM-05-1-044	*C. neoformans*	A	Human	0.58	–	–
CEMM-05-1-045	*C. neoformans*	A	Human	0.58	–	–
CEMM-05-1-048	*C. neoformans*	A	Human	2.34	–	–
CEMM-05-3-002	*C. neoformans*	A	Veterinary	2.34	–	–
CEMM 05-1-090	*C. neoformans*	A	Veterinary	2.34	–	–
CEMM-05-1-043	*C. neoformans*	A	Human	4.68	–	–
CEMM-05-1-050	*C. neoformans*	A	Veterinary	4.68	–	–
CEMM-05-1-047	*C. neoformans*	A	Human	9.37	–	–
CEMM-05-1-046	*C. neoformans*	A	Human	18.75	–	–
CEMM-05-1-042	*C. neoformans*	A	Human	18.75	–	–
CEMM-05-3-003	*C. neoformans*	A	Veterinary	37.5	–	–
CEMM 03-2-075	*C. neoformans*	A	Veterinary	37.5	–	–
CEMM 03-2-078	*C. neoformans*	A	Veterinary	37.5	–	–
CEMM-05-1-049	*C. neoformans*	A	Human	75.0	–	–
CEMM-05-3-001	*C. neoformans*	A	Veterinary	75.0	–	–
CEMM 03-2-061	*C. neoformans*	A	Veterinary	75.0	–	–
CEMM 05-3-033	*C. gattii*	B	Veterinary	0.29	–	–
CEMM 05-2-093	*C. gattii*	B	Veterinary	0.58	–	–
CEMM 05-3-032	*C. gattii*	B	Veterinary	1.17	–	–
CEMM 05-1-099	*C. gattii*	B	Veterinary	1.17	–	–
CEMM-03-2-074	*C. gattii*	B	Veterinary	4.68	–	–
CEMM 03-2-066	*C. gattii*	B	Veterinary	4.68	–	–
CEMM 05-2-087	*C. gattii*	B	Veterinary	4.68	–	–
CEMM 05-3-030	*C. gattii*	B	Veterinary	18.75	–	–
CEMM 05-2-091	*C. gattii*	B	Veterinary	37.5	–	–
CEMM 03-2-079	*C. gattii*	B	Veterinary	75.0	–	–
CEMM 05-3-031	*C. gattii*	B	Veterinary	75.0	–	–
WM626	*C. neoformans*	A	Reference	0.58	4.0	1.0
WM148	*C. neoformans*	A	Reference	1.17	16.0	0.0625
WM629	*C. neoformans*	D	Reference	2.34	8.0	0.5
WM628	*C. neoformans*	AD	Reference	18.75	4.0	0.5
WM178	*C. gattii*	B	Reference	0.58	4.0	1.0
WM779	*C. gattii*	C	Reference	1.17	16.0	1.0
WM179	*C. gattii*	B	Reference	4.68	16.0	0.0625
WM161	*C. gattii*	B	Reference	37.5	16.0	1.0

FNS, farnesol (μM); FLC, fluconazole (μg/mL); AMB, amphotericin B (μg/mL); (–), not tested.

2004), the farnesol MICs for *C. neoformans* serotypes A, D and AD (GM = 6.29 μM), were higher than those for *C. gattii* serotypes B and C (GM = 4.67 μM). Additionally, the veterinary strains of *C. neoformans* and *C. gattii* presented higher MIC values (GM = 10.91 and 5.30 μM, respectively) compared to those of human *C. neoformans* (GM = 5.56 μM) and *C. neoformans* and *C. gattii* reference strains (GM = 2.33 and 3.30 μM, respectively). In the present study, antifungal susceptibility profiles of *C. parapsilosis* ATCC 22019 (CLSI, 2008) and *Cryptococcus* spp. control strains were similar to previously described results (Chong et al., 2010; Hagen et al., 2010; Iqbal et al., 2010; Trilles et al., 2011), hence, validating the accuracy of the methodology employed for susceptibility assays.

In recent years, several studies have shown the antifungal potential of farnesol *in vitro* against many pathogens (Derengowski et al., 2009; Liu et al., 2010; Weber et al., 2010; Pammi et al., 2011; Brilhante et al., 2012). Nevertheless, the *in vivo* effect of this compound – and therefore its potential as a new antifungal drug – is still a matter of debate. As illustration, one study has shown that *C. albicans* cells pretreated with fluconazole were able

Table 2
Phospholipase activity of *Cryptococcus* strains following exposure to farnesol.

Farnesol concentration	*C. neoformans*				*C. gattii*			
	PZ Mean ± SD	No alteration[a] (*n*)	Decreased activity[b] (*n*)	Increased activity[c] (*n*)	PZ Mean ± SD	No alteration[a] (*n*)	Decreased activity[b] (*n*)	Increased activity[c] (*n*)
MIC/4	0.94 ± 0.13	6	12	3	0.75 ± 0.23	2	5	8
MIC/2	0.89 ± 0.20	5	9	7	0.92 ± 0.14	6	5	4
MIC	0.85 ± 0.22	4	9	8	0.90 ± 0.19	7	6	2

PZ, phospholipase activity; SD, standard deviation.
[a] No variation in enzymatic activity throughout the experiment, regardless the intensity of the response.
[b] Enzymatic activity variation from positive or strongly positive to negative.
[c] Enzymatic activity variation from negative to positive or strongly positive.

to secrete higher concentrations of farnesol and were more lethal in a mouse model (Navarathna et al., 2005). However, according to Hisajima et al. (2008), farnesol protects against candidiasis in mice. Although we could not predict the *in vivo* effect of farnesol during cryptococcosis, the present study expands the list of fungal pathogens that are inhibited *in vitro* by this compound. The great susceptibility of *Cryptococcus* to farnesol points to the antifungal potential of this compound *in vivo*.

It has been shown that phospholipase and protease production by *Cryptococcus* spp. is a strain-dependent phenomenon, presenting great frequency and intensity variation (Ma and May, 2009). In the present study, incubation with farnesol was able to change the enzymatic secretion in a strain-dependent manner: some isolates were not affected by the compound, whereas others showed an increase or decrease in enzymatic activities. Statistical differences among groups were not detected and the net effect of farnesol on the test strains was insignificant.

5. Conclusion

This study showed, for the first time, that farnesol has an inhibitory effect on strains of the *C. neoformans* species complex, with no relevant change in the secretion of virulence-related exoenzymes. These findings create the perspective for further investigation of the mechanisms through which this molecule inhibits *Cryptococcus* spp. and the potential use of this compound as an alternative for the treatment of cryptococcosis in the future.

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