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Antifungal activity of farnesol incorporated in liposomes and associated with fluconazole

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ABSTRACT

Candida infections represent a threat to human health. *Candida albicans* is the main causative agent of invasive candidiasis, especially in immunosuppressed patients. The emergence of resistant strains has required the development of new therapeutic strategies. In this context, the use of liposomes as drug carrier systems is a promising alternative in drug development. Thus, considering the evidence demonstrating that sesquiterpene farnesol is a bioactive compound with antifungal properties, this study evaluated the activity farnesol-containing liposomes against different *Candida* strains. The IC_{50} of farnesol and its liposomal formulation was assessed *in vitro* using cultures of *Candida albicans, Candida tropicalis*, and *Candida krusei*. The Minimum Fungicidal Concentration (MFC) was established by subculture in solid medium. The occurrence of fungal dimorphism was analyzed using optical microscopy. The effects on antifungal resistance to fluconazole were assessed by evaluating the impact of combined therapy on the growth of *Candida* strains. The characterization of liposomes was carried out considering their vesicular size, polydispersion index, and zeta medium potential, in addition to electron microscopy analysis. Farnesol exerted an antifungal activity that might be associated with the inhibition of fungal dimorphism, especially in *Candida albicans*. The incorporation of farnesol into liposomes significantly increased its antifungal activity against *C. albicans, C. tropicalis*, and *C. krusei*. In addition, liposomal farnesol potentiated the action of fluconazole against *C. albicans* and *C. tropicalis*. On the other hand, the association of unconjugated farnesol with fluconazole resulted in antagonistic effects. In conclusion, farnesol-containing liposomes have the potential to be used in antifungal drug development. However, further research is required to investigate how the antifungal properties of farnesol are affected by the interaction with liposomes, contributing to the modulation of antifungal resistance to conventional drugs.

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1. Introduction

While *Candida* species live as commensal organisms in healthy individuals, they do not cause disease. However, as opportunistic pathogens, these microorganisms can cause invasive fungal infections which represent a serious threat to human health. Especially in susceptible individuals, *Candida* spp. cause chronic diseases, increasing both mortality rates and costs with hospitalization (Lamoth et al., 2018; Barac et al., 2020).

Candida albicans, one of the main causative agents of fungal infections in humans, is commonly associated with severe disease (Kauffman, 2006; Zida et al., 2017), deep mycosis, and infiltrating candidiasis (Sobel, 2007; Cassone, 2015; Pfaller et al., 2019; Hendrickson et al., 2019). As dimorphic microorganisms, *Candida* species can change their morphology from yeast to hyphal, which significantly contributes to their ability to produce biofilms (Jacobsen et al., 2012; Mayer and Hube, 2013; Tsui et al., 2016). Also, the development of resistance to fluconazole (and many other antifungals), the mainstay of antifungal therapy (Fekkar et al., 2014), has impaired the treatment of several infectious diseases (Zomorodian et al., 2016), indicating the urgent need for new antifungal compounds (Fenner et al., 2006).

In this context, studies have identified farnesol as a bioactive compound present in the essential oils of some plant species (Weber et al., 2008). This sesquiterpene alcohol is also found as a product of the metabolism of yeasts belonging to the genus *Candida* (Hornby et al., 2001; Ramage et al., 2002a,b; Langford et al., 2009). Consistent evidence has demonstrated that farnesol acts as a virulence-repressing factor in *Candida* species (Enjalbert and Whiteway, 2005; Bandara et al., 2016) by preventing the morphological transition from yeast to hyphae (Yu et al., 2012).

Previous studies demonstrated that this compound has remarkable antimicrobial activity, with significant inhibitory effects on the pathogenicity of resistant yeasts (Dižová and Bujdáková, 2017). In this context, farnesol (300 μM) caused potent inhibition of biofilm formation by *C. albicans*, both directly and associated with fluconazole or 5-flucytosine (Xia et al., 2017).

The development of nanoparticles as drug carrier systems has represented an important milestone in drug development, especially in the context of chemotherapy. Studies have shown that the incorporation of drugs into carrier nanoparticles can increase bioavailability, reduce the therapeutic dose and increase the safety and efficiency of these molecules (De Jong and Borm, 2008; Shidhaye et al., 2008; Haider et al., 2020).

In this context, liposomes are highly efficient systems with a wide spectrum of clinical applications (Allen and Cullis, 2013; Johnsen and Moos, 2016). Structurally, liposomes consist of spherical vesicles formed by an internal aqueous nucleus surrounded concentrically by one or more phospholipid bilayers. These nanoparticles are notable for being biodegradable, biocompatible, and non-toxic. Besides, they are versatile, stable and compatible with the incorporation of either hydrophilic, lipophilic and amphiphilic compounds (Allen and Cullis, 2013; Johnsen and Moos, 2016).

Therefore, this study aimed to evaluate *in vitro* the antifungal activity of farnesol-containing liposomes associated with fluconazole against *Candida* strains. The study also reports the effects of this *in vitro* treatment on fungal dimorphism, one of the main virulence factors in the strains under investigation.

2. Materials and methods

2.1. Analysis of antifungal activity

2.1.1. Strains and culture media

Standard strains of *Candida albicans* (CA INCQS 40006), *Candida tropicalis* CT INCQS 40042, and *Candida krusei* (CK INCQS 40095) were obtained from the Oswaldo Cruz Culture Collection of the Brazilian Institute for Quality Control in Health (INCQS, FIOCRUZ, RJ). These strains were incubated in the Sabouraud Dextrose Agar medium (SDA, KASVI) at 37 °C for 24 h. Following incubation, a sample of each colony was transferred to test tubes containing 3 mL of sterile saline, and turbidity was assessed using a value of 0.5 on the McFarland scale (NCCLS, 2002). Double-concentrated Sabouraud Dextrose Broth (SDB, HIMEDIA) was used in the microdilution tests while depleted Potato Dextrose Agar (PDA) medium added with bacteriological agar was used in morphological analysis.

2.1.2. Drugs and reagents

Farnesol (95 % purity) was obtained from Sigma Aldrich (St. Louis, Missouri, United States) and dissolved in Dimethyl Sulfoxide (DMSO, Merck, Darmstadt, Germany). Fluconazole (Capsule, Prati Donaduzzi) was dissolved in distilled water. Liposomal farnesol was obtained as detailed in Section 2.2. After dissolution, the compounds were diluted in sterile distilled water to a final concentration of 16.384 μg/mL. The treatment solutions were then serially diluted as described below. Of note, the final concentration of DMSO does not exert any toxic or pharmacological activity (STOPPA et al., 2009).

2.1.3. Analysis of cell viability and determination of IC_{50}

The cultures and treatments were performed as described by Javadpour et al. (1996). Briefly, each inoculum was prepared in test tubes by adding 900 μL of culture medium (Broth Sabouraud Dextrose- BSD) and 100 μL a fungal suspension with a turbidity corresponding to 10^6 CFU according to the MacFarland scale. Each well on a 96-well plate was filled with 100 μL of this solution, followed by the addition of 100 μL of the treatment solution containing the compounds at concentrations ranging from 8192 to 8 μg/mL. Wells containing only the inoculum in the BSD medium were used as growth controls. Diluent controls, in which the inoculum was replaced with 0.9 % sodium chloride and sterile medium, were also used. All tests were performed in quadruplicate. The plates were incubated for 24 h at 37 °C, and then, the readings were performed at 630 nm using a spectrophotometer (Thermoplate®). The data were used to determine the cell viability and calculate the IC_{50} of each treatment, as previously described (Morais-Braga et al., 2016).

2.1.4. Determination of minimum fungicidal concentration (MFC)

The tip of a sterile stick was inserted into each well containing the cultures treated as described above. After homogenization, a subculture was made by taken the stick to a Petri dish containing SDA with the aid of a guide plate attached to the bottom of the dish and the growth of *Candida* colonies in the plates was analyzed 24 h later (Ernst et al., 1999). The MFC was defined as the lowest concentration capable of inhibiting the growth of fungal colonies.

2.1.5. Evaluation of antifungal enhancing activity in association with fluconazole

After evaluating the isolated action of farnesol and fluconazole, this study analyzed the ability of farnesol to enhance the antifungal activity of fluconazole. To this end, the MFC of fluconazole was determined in the presence or absence of farnesol at a concentration equivalent to its MFC/16 (Coutinho et al., 2008). The culture, controls, and readings were performed as described above.

2.1.6. Analysis of morphological changes

To assess the effects of the treatments on fungal morphology, the development of hyphae was evaluated in chambers containing slides for microscopic analysis, as described by Sidrin and Rocha (2010) and Mendes (2011), with adaptations. To this end, the chambers were filled with 3 mL of PDA medium depleted by dilution, in the presence of nerolidol at concentrations equivalent to its MFC/8 or MFC/16. Aliquots of the subcultures were removed from the Petri dishes to make two parallel streaks in the solid medium (PDA), which were later covered with a sterile coverslip. The chambers were then placed in the oven at 37

°C for 24 h, and the images were recorded under optical microscopy (AXIO IMAGER M2-3525001980- ZEISS- Germany). In Fig. 1, we observe the demonstration of the fungal micromorphology assay, with a wet chamber model that provides an ideal environment for fungal proliferation. Each slide was photographed and the length of the filament extensions (hyphae and pseudohyphae) was determined using Zen software version 2.0 (Cordeiro et al., 2019).

2.2. Preparation of liposomes

Each liposome batch was prepared by weighing 5 mg of dipalmitoylphosphatidylcholine (DPPC), 3 mg of dipalmitoylphosphatidylserine (DPPS), 1 mg of cholesterol (CHOL) and 1 mg (1 µL) of farnesol. These substances were mixed and dissolved with 1 mL of chloroform/methanol (purity > 99 %, Dynamic Reagents-Diadema, SP, Brazil) solution (1:1). Nitrogen (White Martins, Rio de Janeiro, RJ, Brazil) was used to evaporate the solvents, after which a thin layer was formed on the tube wall. The tube was kept in a desiccator overnight (18 h) and then, 1 mL of PBS buffer (pH 7.2) was added. The tube was then subjected to the shaker and water bath (at 57 °C) to resuspend the liposomes, forming an emulsion, which was then submitted to an extruder (LiposoFastTM, Avestin) with polycarbonate membranes with pores of 200 nm in diameter. This step was repeated 40 times producing a population of uniformly sized liposomes (Barros et al., 2013). Liposomes without the addition of farnesol were used as controls. The lipids used in the preparation of liposomes were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA)

2.2.1. Physicochemical characterization of liposomes

The physical-chemical characterization was performed through the analysis of the following parameters: vesicular size, morphology, polydispersity index, and average zeta potential. The determination of the particle size was performed through dynamic light scattering (DLS) measurements, using serial dilutions with Milli Q water in the proportions of 1:10, 1:20, 1:50, and 1: 100 at 25 °C. The particle size distribution was obtained by the polydispersity index (PDI/PI). The average zeta potential of the particles was calculated at 25 °C, using the microelectrophoresis technique associated with Laser Doppler Anemometry, by submitting the sample to an electric field. The analyzes were performed using the Zetasizer Nano ZS equipment (Malvern, version 6.20). The shape and morphology of the liposomes were analyzed by scanning electron microscope (FEG Quanta 450 EDS/EBSD).

2.3. Statistical analysis

The half-maximal inhibitory concentration (IC_{50}) was calculated by non-linear regression and expressed as arithmetic means ± standard error of the mean. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test. Fungal growth was expressed as the arithmetic means ± standard deviations and statistical significance was calculated using two-way ANOVA with Bonferroni's post hoc test. All experiments were performed in quadruplicate and analyzed using Graphpad Prism version 5.0.

3. Results

3.1. In vitro antifungal activity of farnesol alone and incorporated in liposomes

An analysis of the fungal growth curve in the presence of different concentrations of farnesol demonstrated that this compound exerted significantly less potent antifungal activity than fluconazole (pharmacological control) against all strains of *Candida* evaluated by this study (Fig. 2). However, the incorporation of farnesol into liposomes resulted in significantly increased antifungal activity. This finding is corroborated by the data shown in Table 1, which demonstates a reduction in the IC_{50} of farnesol when associated with liposomes.

3.2. Antifungal enhancing activity of liposomal farnesol in association with fluconazole

Following the characterization of the inhibitory effects of different farnesol formulations on *Candida* growth, this work evaluated their ability to modulate the antifungal resistance to fluconazole. To this end, we analyzed the growth curves of three different *Candida* strains treated with the standard antifungal drug, both alone or in association with free or liposomal farnesol at concentrations equivalent to their MFC/16 (subinhibitory concentrations). Of note, the MFC analysis found values above 16,384 µg/mL for all treatments and controls used in the present study.

As shown in Fig. 3, the association with subinhibitory concentrations of liposomal farnesol significantly increased the antifungal activity of fluconazole against *C. albicans* (Fig. 3A) and *C. tropicalis* (Fig. 3B), demonstrating a clinically useful potentiating effect, as the drug was found to cause 100 % of colony formation inhibition at 8 µg/mL when associated with the natural product. This finding is corroborated by the data shown in Table 2, which demonstrates a reduction in the IC_{50} of

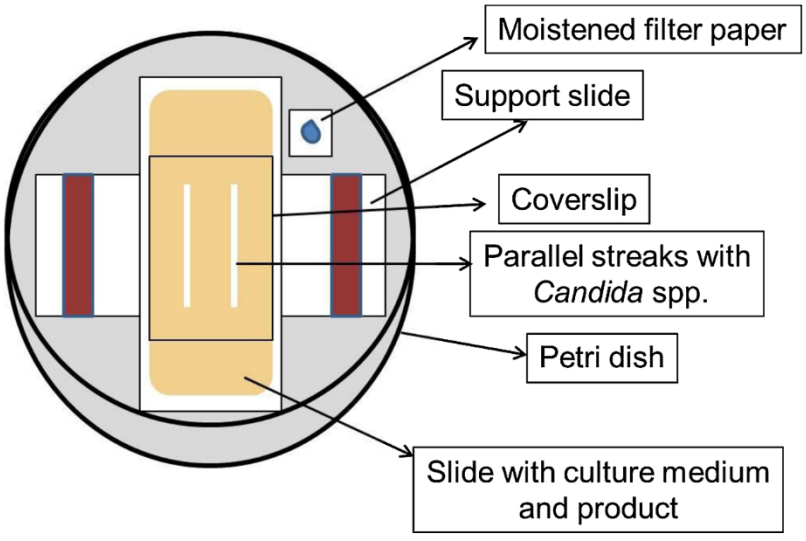

Fig. 1. Wet chamber model for fungal micromorphology assay.

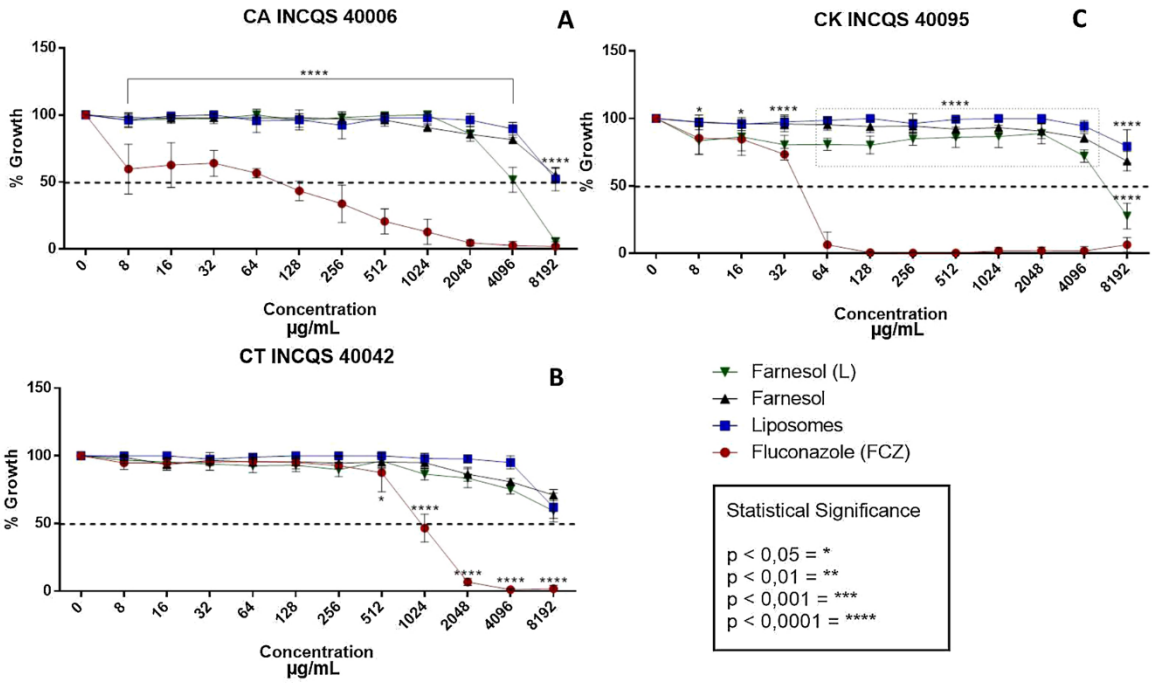

Fig. 2. Growth curves of different *Candida* strains in the presence of fluconazole (FCZ), farnesol, and liposomal farnesol (L). Liposomes without farnesol were used as controls. CA: *Candida albicans*; CT: *Candida tropicalis*; CK: *Candida krusei;* INCQS: National Institute for Quality Control in Health.

Table 1
IC_{50} values (µg/mL) of farnesol against *Candida* strains.

SUBSTANCE	CA INCQS 40006 IC_{50} (µg/mL)	CT INCQS 40042 IC_{50} (µg/mL)	CK INCQS 40095 IC_{50} (µg/mL)
Farnesol	12572 ± 923	27457 ± 2,667	31318 ± 5,134
arnesol (L)	4057 ± 125	15286 ± 1,419	6296 ± 722
Fluconazole	55,98 ± 12	1168 ± 118	35,68 ± 1.74
Liposome	13512 ± 1,369	≥ 16384	≥ 16384

Legend: CA: *Candida albicans*; CT: *Candida tropicalis*; CK: *Candida krusei.* L: Liposomal farnesol.

fluconazole when associated with liposomal farnesol. On the other hand, the association between non-liposomal farnesol and fluconazole presented antagonistic effects against *C. albicans* and *C. tropicalis*, suggesting that the controlled release of farnesol could contribute to its inhibitory effects on antifungal resistance to fluconazole. Interestingly, no significant change in the activity of fluconazole against *Candida krusei* was observed. Finally, the association between fluconazole and liposomes (control) presented antagonistic effects. However, further research is required to investigate how the antifungal properties of fluconazole are affected by the interaction with both control liposomes and drug-containing liposomes.

3.3. Effects of the treatments on fungal morphology

To investigate the effects of different *in vitro* treatments on fungal dimorphism, one of the main virulence factors in *Candida* species, strains of *C. albicans* and *C. tropicalis* were cultured in chambers containing test compounds at concentrations equivalent to their MFC/8 (2048 µg/mL) and MFC/16 (1024 µg/mL). The *in vitro* treatment with fluconazole at both concentrations prevented the formation of filamentous structures by all strains, demonstrating a potent inhibitory effect of fungal dimorphism. Unconjugated or liposomal farnesol also inhibited fungal dimorphism by 100 % in *C. albicans* (Fig. 4A), which is corroborated by evidence showing that farnesol acts as a regulatory factor for hyphal growth in this species. The morphological analysis of *C. tropicalis* cultures (4B) showed that farnesol significantly inhibited dimorphism, especially at the concentration equivalent to its MFC/8, which caused 100 % inhibition. Interestingly, liposomal farnesol demonstrated little inhibitory effects on fungal dimorphism. The analysis of *C. krusei* (4C) cultures found moderate activity for unconjugated farnesol, while the liposomal formulation exerted significant active only at the highest concentration. Interestingly, the control (liposome) exerted some intrinsic activity in this model, which points to a possible action of the lipids used in the formulation of this carrier nanoparticle.

3.4. Physicochemical characterization of liposomes

The morphological analysis using scanning electron microscopy (SEM) (Fig. 5A) revealed the presence of homogeneous populations of spherical vesicles with similar dimensions. However, the group of liposomes containing farnesol presented as larger structures (Fig. 5B), which may be related to the influence of the physicochemical characteristics of farnesol, as well as the interference of environmental factors, such as the temperature and method of conservation, causing agglutination or swelling of the liposomes.

The values of vesicular size, polydispersity index, and zeta potential of liposomes are shown in Table 3. The data demonstrate significant differences in the size of the vesicles between the formulations. The zeta potential values indicated that the surface of the nanoparticles is negatively charged, which together with the values of polydispersity indices and SEM images ensure that these formulations were obtained with satisfactory distribution, size, and reproducibility.

4. Discussion

The present study characterized the antifungal properties of farnesol against clinically important *Candida* species. Our results demonstrated that farnesol exerted weak antifungal activity in comparison with the standard antifungal drug fluconazole. Nevertheless, the incorporation of farnesol into liposomes significantly potentiated its inhibitory effects on the growth of *Candida* strains, indicating improved antifungal activity.

Farnesol is a sesquiterpene commonly found in the essential oils of plant species with notable antimicrobial properties, such as *Tetradenia*

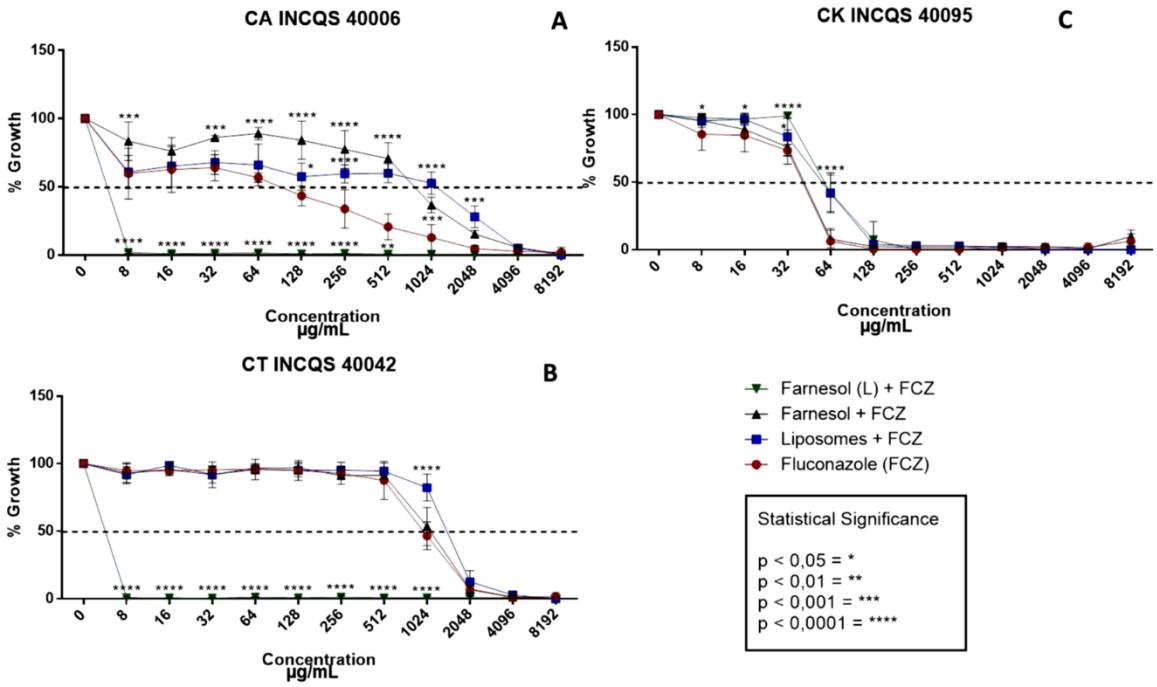

Fig. 3. Growth curves of different *Candida* strains in the presence of fluconazole (FCZ) alone or in association with farnesol, liposomal farnesol (L) or liposomes (L). CA: *Candida albicans*; CT: *Candida tropicalis*; CK: *Candida krusei;* INCQS: National Institute for Quality Control in Health.

Table 2
IC_{50} values (μg/mL) of fluconazole associated with farnesol against *Candida* strains.

TREATMENT	CA INCQS 40006 IC_{50} (μg/mL)	CT INCQS 40042 IC_{50} (μg/mL)	CK INCQS 40095 IC_{50} (μg/mL)
Farnesol + FCZ	905 ± 158	1297 ± 113	42,60 ± 4,11
Farnesol (L) + FCZ	2,52 ± 0,02	2,48 ± 0,01	67,68 ± 1,17
Fluconazole	55,98 ± 12,11	1168 ± 118	35,68 ± 1,74
Liposome + FCZ	788 ± 142,15	1737 ± 164	61,33 ± 6,75

Legend: CA: *Candida albicans*; CT: *Candida tropicalis*; CK: *Candida krusei.* L: Liposome; FCZ: Fluconazole.

riparia (De Melo et al., 2015). Accordingly, the effectiveness of this sesquiterpene against human pathogens, including bacteria of the genus *Staphylococcus* (Bonikowski et al., 2015) and filamentous fungi of the genera *Aspergillus, Paracoccidioides* and *Cryptococcus* (Semighini et al., 2006; Derengowski et al., 2009; Cordeiro et al., 2012) have been demonstrated by several studies. Additionally, consistent evidence has suggested that farnesol has the potential to be used in the combat of infections caused by diverse types of infectious agents (Derengowski et al., 2009; Dižová and Bujdáková, 2017).

A study by Hisajima et al. (2008) identified farnesol as a promising compound for the treatment of oral candidiasis caused by *C. albicans* in mice. Through histological analysis, the authors showed that this sesquiterpene reduced the formation of colonies and mycelia in the tongue, kidneys, liver, and feces of these animals. In fact, in the present study, the most potent effect of farnesol was demonstrated against *C. albicans*. Here, we demonstrated that incorporation into liposomes increased the effectiveness of farnesol as an antifungal compound.

To date, the effectiveness of nanoparticles in potentiating activity of antifungal compounds remains poorly investigated. A recent work by Fernandes Costa et al. (2019) demonstrated that farnesol, as well as miconazole-chitosan nanoparticles associated with farnesol, presented clinically useful results in a murine model of vulvovaginal candidiasis. This finding is corroborated by a study by Shirtliff et al. (2009) who analyzed the global expression of proteins in *Candida spp.* after the treatment with farnesol. These authors suggested that this compound could induce mitochondrial damage due to the accumulation of reactive oxygen species (ROS) with consequent activation of caspases and cell death by apoptosis. On the other hand, Navarathna et al. (2007) reported that farnesol could enhance the virulence of other *Candida* species, resulting in increased mortality of the mice in the same model. Therefore, the antifungal effects of farnesol against different *Candida* strains should be better investigated using other experimental models, both *in vitro* and *in vivo*.

The present research demonstrated that the association between liposomal farnesol and fluconazole resulted in potentiating effect. This finding is corroborated by previous studies showing that the association of farnesol with other drugs resulted in potentiated antifungal action (Yu et al., 2012; Cordeiro et al., 2013; De Cremer et al., 2015. Accordingly, it was demonstrated that farnesol potentiated the inhibitory effect of other antifungal drugs on biofilm formation by the strain of *C. albicans* SC5314 (Katragkou et al., 2015). Furthermore, Bozó et al. (2016) reported that the combined treatment using farnesol and fluconazole had potentiating effect against biofilms of susceptible and resistant strains of *Candida*, corroborating the data of the present study.

The morphological analysis revealed significant inhibitory effects of the *in vitro* treatment with farnesol, both free and liposomal. Our results indicate that these treatments inhibit dimorphism in *Candida* species, especially in *C. albicans*. Accordingly, previous studies have suggested that this compound is capable of preventing dimorphism in several species of fungi (Jabra-Rizk et al., 2006; Henriques et al., 2007; Rossignol et al., 2007; Weber et al., 2010; Monteiro et al., 2017; Polke et Al., 2018).

Dimorphism is characterized as the morphological change from yeast to a filamentous structure. Since this phenomenon is significantly related to the virulence of *C. albicans*, the effect demonstrated by farnesol highlights the potential of this compound for the development of antifungal drugs (Hornby et al., 2001; Cordeiro et al., 2019). A study by Agustín et al. (2019) demonstrated that farnesol alone or in association with natamycin inhibited biofilm formation in *C. tropicalis*, while Fleischmann et al. (2017) reported an inhibitory effect on biofilm formation by *C. Krusei*. The data of the present research demonstrated that farnesol has a less potent action against these strains, in comparison to

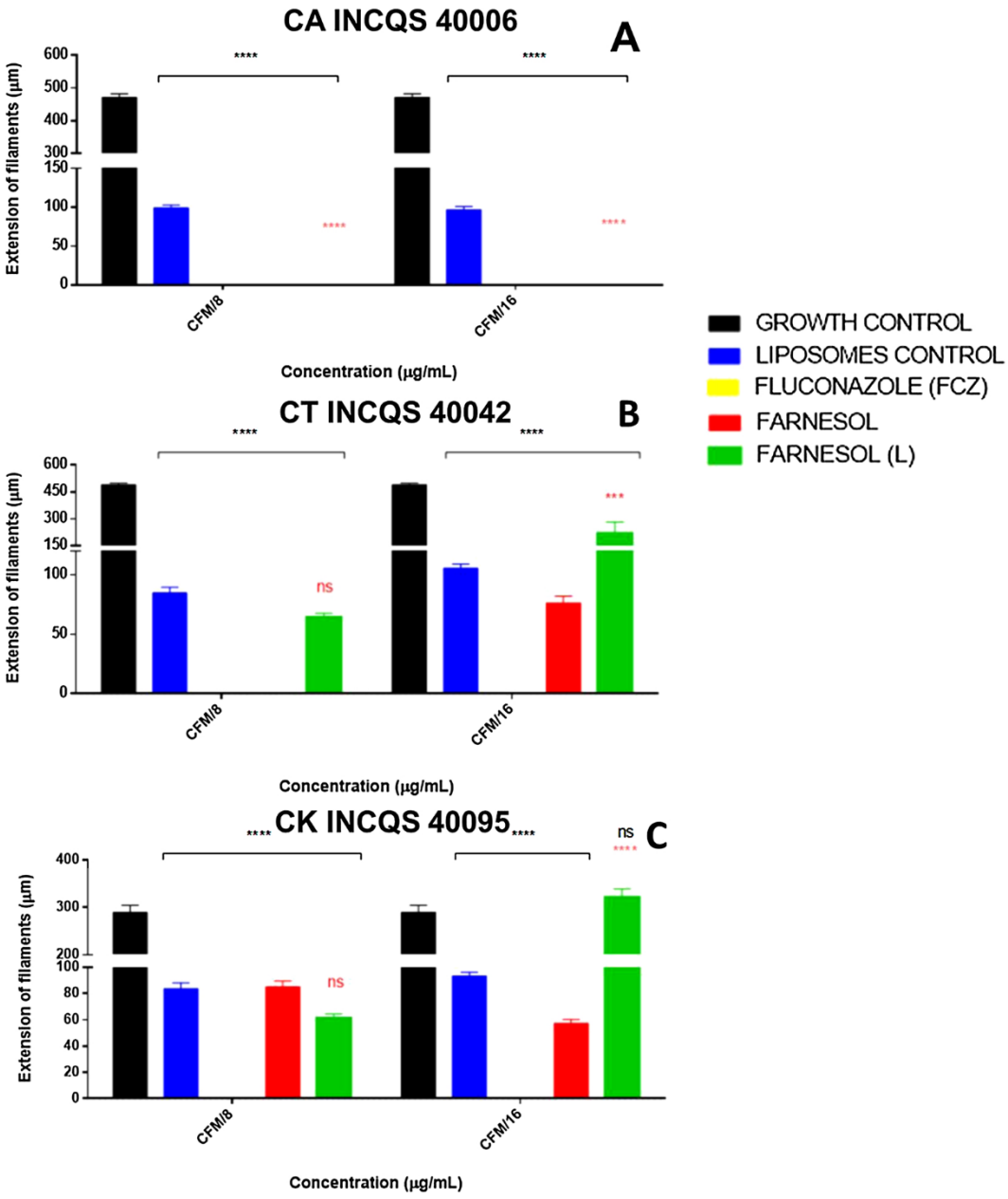

Fig. 4. Effects of the *in vitro* of fluconazole (FCZ), farnesol and liposomal farnesol (L) on fungal morphology. Changes in fungal morphology were expressed as a measure of the extension of filaments. Liposomes without farnesol were used as controls. CA: *Candida albicans*; CT: *Candida tropicalis*; CK: *Candida krusei;* INCQS: National Institute for Quality Control in Health.

the effect observed in *C. albicans*. However, it is worth mentioning that the experimental models and conditions can significantly influence the results, which could justify the differences observed between the studies.

The physicochemical characterization revealed the presence of homogeneous populations of vesicles with regular spherical pattern and similar dimensions. The analysis of the zeta potential, polydispersity indices and SEM images confirmed that the formulations were obtained with satisfactory distribution, size, and reproducibility. The zeta potential represents the measure of the magnitude of the repulsion or attraction between the particles and, therefore, is a fundamental parameter to prove the stability of the vesicles. A repulsion force between liposomes with similar electrical charges facilitates the dispersibility, impairing aggregation. Therefore, zeta potentials with positive or negative values are expected to ensure electrostatic stability, contributing to the aesthetics of a nanosuspension (Honary and Zahir, 2013; (Patil et al., 2007; Puttipipatkhachorn et al., 2001).

5. Conclusion

Farnesol exerted antifungal effects that might be associated with inhibition of fungal dimorphism, especially in *Candida albicans*. The incorporation of farnesol into liposomes significantly increased its antifungal activity against *C. albicans, C. tropicalis*, and *C. krusei*. In addition, the association with liposomal farnesol potentiated the action of fluconazole against *C. albicans* and *C. tropicalis*. On the other hand, the association of unconjugated farnesol with fluconazole presented antagonistic effects against these strains.

In conclusion, farnesol-containing liposomes have the potential to be used in antifungal drug development. However, further research is required to investigate how the antifungal properties of farnesol are affected by the interaction with liposomes, contributing to the

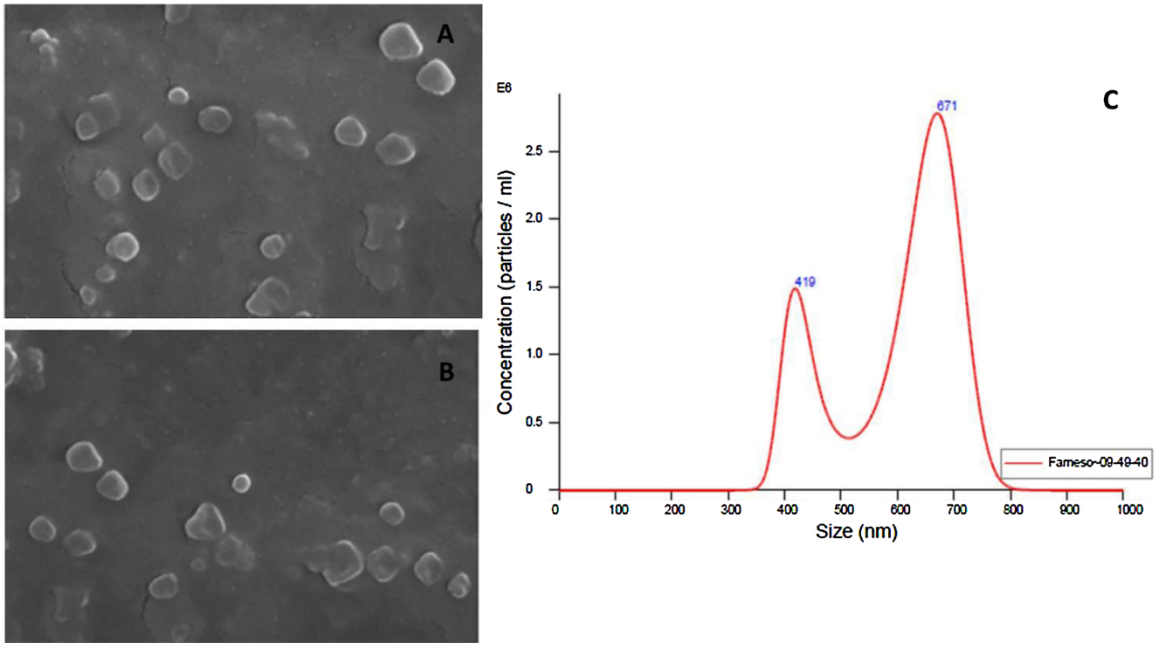

Fig. 5. Scanning electron microscopy images (SEM) (A and B) and diagram of concentration (particles/ mL) by vesicular size (nm) of liposomal farnesol (C).

Table 3
Vesicular sizes (*vs*), polydispersity indices (PI), and zeta potentials (ZP) of liposomes.

Formulation	Size (nm)	PI	ZP (mV)
Control liposome	185,46 ± 3,76	0,48 ± 0,01	− 40,9 ± 0,96
Liposomal farnesol	418,9 ± 108,7	0,320 ± 0,02	−35,1 ± 0,1

These results are expressed as the means ± standard deviations.

modulation of antifungal resistance to conventional drugs.

Declaration of Competing Interest

The authors deny the existence of any conflict of interest regarding this publication.

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