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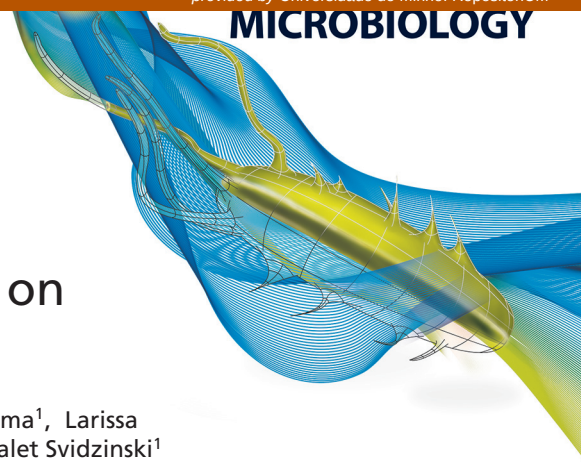

Antibiofilm activity of propolis extract on *Fusarium* species from onychomycosis

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Aim: The present study evaluated the capacity of three species of *Fusarium* isolated from onychomycosis to form biofilms and the antibiofilm effect of propolis extract on these biofilms. **Materials & methods:** The biofilms and antibiofilm effects were evaluated by quantifying the colony-forming units, mitochondrial metabolic activity assays, total biomass by crystal violet staining and scanning electron microscopy. **Results:** Propolis extract demonstrated significant antibiofilm efficiency on *Fusarium* spp. isolates and reduced *F. solani*, *F. oxysporum* and *F. subglutinans* mature biofilms. **Conclusion:** Propolis extract can be an alternative topical treatment of onychomycosis caused by *Fusarium* spp.

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Keywords: antifungal • fusariosis • paronychia • treatment • ungueal mycosis

Fusarium species are geophilic fungi, frequently isolated from opportunistic infections in immunocompromised patients, causing disseminated infections with high rates of mortality [1]. Onychomycosis, a fungal infection caused by *Fusarium* spp., can also affect immunocompetent patients [2]. It has previously been reported that paronychia, a characteristic of onychomycosis caused by this fungus, can be an entry portal to disseminated infections [3]. Moreover, fusariosis has the highest incidence of infection among invasive fungal diseases in Brazil, with high mortality in hematological patients [4].

While the pathogenesis of fusariosis is not completely understood, the virulence factors of these fungi appear to be determinants for infection. Of these, biofilms are related to antifungal resistance and greater difficulty in treatment [5]. Additionally, some studies have described the capacity of *F. oxysporum* to form biofilm *in vitro* [6] or as an onychomycosis agent [7].

Studies focusing on finding new antifungal agents are urgently required, as there is a limited therapeutic arsenal available for *Fusarium* spp. infections. Alternatives include natural products, especially as they are inexpensive and have low toxicity. Propolis is a natural product collected from the hives of *Apis mellifera* L. Several studies have described the medicinal properties of propolis, such as its anti-inflammatory and antitumor effects and antifungal and antibacterial activity [8,9]. In the present study, biofilms formed by three species of *Fusarium* were characterized and the activity of propolis extract (PE) against these biofilms was evaluated.

Materials & methods

Fusarium strains

Fungi were isolated from the nail samples of patients with onychomycosis. Nail scrapings were cultivated in three tubes containing Sabouraud dextrose agar (SDA; Himedia Laboratories, Mumbai, India) and three tubes that contained Mycosel medium (Himedia Laboratories, Mumbai, India). The identification of fungi was performed using classic methods, including the examination of colonies and microscopic morphology [10,11]. The fungi isolated and identified were maintained in a freeze-dried state in the Mycological Collection of the Laboratory of Medical Mycology of the State University of Maringá (UEM), Brazil. One clinical isolate was randomly chosen from each of

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the three most frequent species of *Fusarium* in our region: *F. oxysporum* (FO42), *F. solani* (FS04) and *F. subglutinans* (FSub39). We also used a reference strain of *F. solani* (ATCC 36031), kindly provided by Fiocruz (Oswaldo Cruz Foundation, Brazil).

Biofilm formation

This assay was based on the methodology described by Silva *et al.* [12] with modifications. The strains of *Fusarium* spp. were grown on potato dextrose agar (PDA; Himedia Laboratories, Mumbai, India) for 7 days at 25°C. The colonies were gently scraped, harvested in 0.85% sterile saline and the conidia were counted in a Neubauer chamber. The inoculum was adjusted to a final concentration of 1×10^6 conidia ml^{-1} [13,14] on Sabouraud dextrose broth medium (SDB; Himedia Laboratories), and 200 µl of this suspension was placed into 96-well plates. The plates were then incubated without agitation at 37°C for 2 h in order to settle and to adhere the conidia to the bottom of the plate, and were then incubated at 37°C in a shaker at 110 rev min^{-1}. Every 24 h, 100 µl of SDB was removed and an equal volume of fresh SDB without *Fusarium* was added to each well for renewal of the culture medium. The plates were then incubated for 72 h. All experiments were repeated on three occasions with individual samples evaluated in triplicate.

Biofilm biomass quantification by crystal violet staining

Biofilms were allowed to grow for 24, 48 and 72 h. They were then washed three-times with sterile saline and after drying, 200 µl of methanol was added to each well for 15 min to affix the biofilms. Later, 200 µl of crystal violet (1% v/v) was added for 5 min. The wells were washed with sterile distilled water and 200 µl of acetic acid (33% v/v) was then added to dissolve the stain. The obtained solution was read in a microtiter plate reader at 570 nm and the absorbance values were standardized per unit area of well (absorbance cm^{-2}).

Biofilm metabolic activity assay by XTT reduction

The reduction assay of the tetrazolium salt 2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-([phenylamino]carbonyl)-2H tetrazolium hydroxide (XTT; Sigma-Aldrich, MO, USA) was used to determine *in situ* biofilm mitochondrial activity. After biofilm formation, each well was washed three-times with sterile saline. A total of 100 µl of a solution containing XTT, phenazine methosulphate (PMS) and 0.85% sterile saline was added to each well: 60 µl of sterile saline, then 20 µl of XTT stock solution (500 µg ml^{-1}) and 20 µl of PMS stock solution (50 µg ml^{-1}) were added. The final concentrations of XTT and PMS in the wells were 100 and 10 µg ml^{-1}, respectively. The plates were then incubated at 35°C for 3 h, protected from the light. After this period, the absorbance of the obtained solution was read in a microtiter plate reader at 492 nm and the absorbance values were standardized per unit area of well (absorbance cm^{-2}).

Scanning electron microscopy

This assay was based on the methodology described by Silva *et al.* [12]. To examine the structure of biofilms by scanning electron microscopy (SEM), biofilms were allowed to form in 24-well polystyrene microtiter plates as described above for 72 h. The wells were washed with sterile saline and the biofilms were dehydrated with alcohol (using 70% for 10 min, 80% for 10 min, 95% for 10 min and 100% ethanol for 20 min) and air dried. Prior to observation, the bases of the wells were cut out and mounted onto aluminum stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, MA, USA).

Biofilm viability assay

Biofilms were formed for 24, 48 and 72 h and were washed three-times with sterile saline. Then, 200 µl of saline was added to each well and the biofilms were vigorously scraped with a pipette, transferred to a conical tube and vortexed for 1 min. Aliquots of 15 µl were placed in a plate containing Dichloran rose bengal chloramphenicol agar (Merck, Darmstadt, Germany) and were incubated at 35°C. Dichloran rose bengal chloramphenicol agar is a selective medium for the isolation and enumeration of viable molds and restricts colony size, improving the enumeration and detection of the molds. The number of colony-forming units was determined after 5 days of incubation.

PE & phenol content

The propolis source was as described by Capoci *et al*. [15], with green propolis collected from hives of *A. mellifera* in Cianorte (Paraná, Brazil). The samples were triturated and stored at -20°C until further analysis. PE was prepared with a propolis/ethanol ratio of 30/70 (w/w) by turbo extraction, at 3500 rpm, three-times at 15 min with two intervals of 5 min. PE was filtered through filter paper and adjusted to the initial weight with ethanol. The total phenol content (TPC) of PE was evaluated by the Folin–Ciocalteau method [16]. PE was added to 6 ml of the Folin–Ciocalteau and 6 ml of 20% Na_2CO_3 (Sigma-Aldrich). After 2 h, absorbance was determined by Shimadzu UV-1650PC spectrophotometer (Tokyo, Japan) at a wavelength of 760 nm. A calibration curve with solutions of gallic acid was used as a reference. TPC was expressed as a percentage of total phenolic substances in PE and corresponded to a mean of six replicates.

Antifungal assay

In vitro antifungal susceptibility testing of planktonic cells was performed using a microdilution method adapted from Capoci *et al*. [15] and the Clinical and Laboratory Standards Institute M38-A2 protocol [17] with serial dilution of PE 34.17, 68.35, 136.71, 273.43, 546.87, 1093.75, 2187.5, 4375, 8750 and 17,500 μg ml^{-1} of TPC expressed in gallic acid in RPMI 1640 (Roswell Park Memorial Institute; Gibco, NY, USA) with l-glutamine (with sodium bicarbonate) and 0.165 M 3-(N-morpholino) propanesulfonic acid (pH 7.2) as a buffer (Sigma-Aldrich). The strains of *Fusarium* spp. were grown on potato dextrose agar for 7 days at 25°C. The colonies were gently scraped and harvested in 0.85% sterile saline solution. The number of conidia were counted in a Neubauer chamber and the inoculum was adjusted to a final concentration of 5×10^4 conidia ml^{-1} in RPMI 1640. An aliquot of PE (100 μl) was dispensed into 96-well plates and further incubated with aliquots (100 μl) of the *Fusarium* inoculum. The 96-well plates were incubated at 35°C for 72 h. The minimal inhibitory concentration (MIC) values were determined by visualization, corresponding to the lowest concentration of antifungal agent in which no visible growth was observed, in comparison with the control (cells grown without PE) [15,17]. The minimal fungicidal concentration (MFC) was determined after 72 h of incubation at 35°C and 5 μl of each well was transferred to a plate containing sabouraud dextrose agar and incubated at 25°C for 48 h.

Antibiofilm assay

Once stable and mature biofilms were formed (for 24 h as described above) they were washed three-times with sterile saline. Biofilms were formed as described above for 24 h. Then, PE was added at the MFC concentration found in the latter assay. The 96-well plates were incubated at 37°C for a further 24 h. The activity of PE against biofilms was determined by biomass quantification using crystal violet staining and by biofilm viability assay described above.

Epifluorescence microscopy

Antibiofilm assay was also evaluated by epifluorescence microscopy. Biofilms were formed in 24-well polystyrene microtiter plates as described above for 24 h, then PE was added at the MFC concentration and the plates were incubated at 37°C for a further 24 h. The matrix of biofilms was then stained with calcofluor white (Fluka Analytical, MO, USA) diluted in sterile saline (1:4) for 5 min and washed twice with sterile saline. Stained biofilms were observed with a microscope (Evos FL, Live Technologies, CA, USA) with filters capable of detecting the fungi cell wall (BP 365–370, FT 400, LP 421).

Statistical analysis

Data were analyzed using Prism 6.0 software (GraphPad, CA, USA). The *t*-test and one-way analysis of variance with Bonferroni were used. All of the tests were performed with a confidence level of 95% and values of $p \leq 0.05$ were considered statistically significant.

Results

The three species of *Fusarium* tested in this study formed biofilms. Nevertheless, the *F. solani* strains were more efficient than the other species in relation to total biomass and the number of viable cells. Furthermore, the biomass produced by both the clinical isolates and reference strain of this species was directly proportional to incubation time (Figure 1). While the total biomass of biofilms formed by *F. solani* increased from 24 to 48 h and from 48 to 72 h of incubation, the total biomass of *F. oxysporum* and *F. subglutinans* biofilms increased only until 48 h

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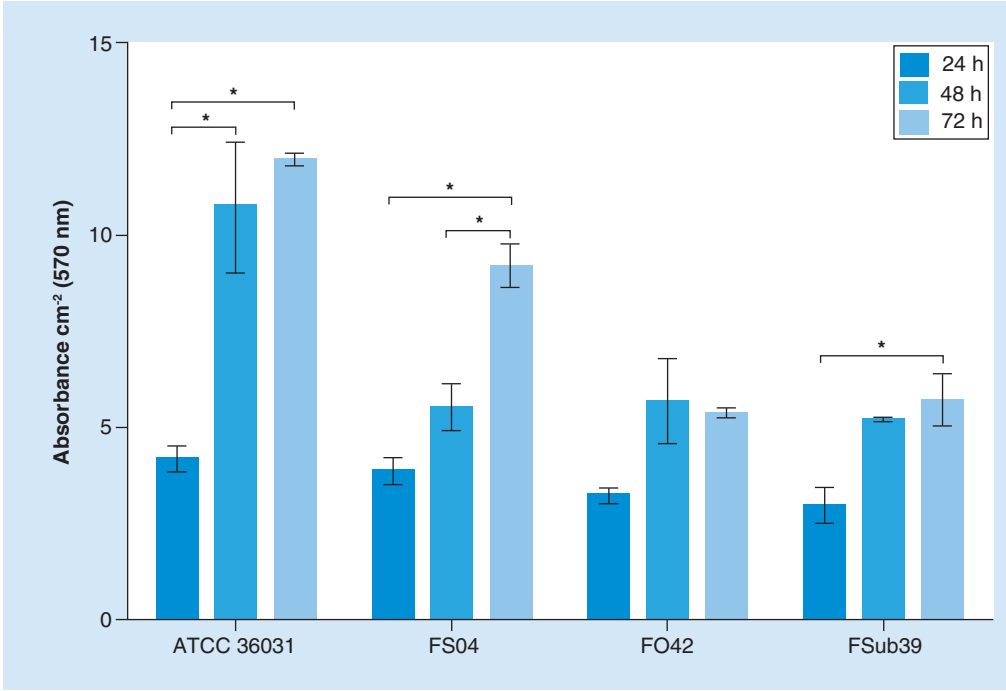

Figure 1. Crystal violet staining of biofilm biomass of *Fusarium solani* (ATCC 36031 and FS04), *Fusarium oxysporum* (FO42) and *Fusarium subglutinans* (FSub39) at different times of incubation. ANOVA with Bonferroni, *$p < 0.05$.

and apparently became stable after this period (Figure 1). However, these increases in biomass production were significant only for strains of *F. solani* and *F. subglutinans*, and mainly from 24 to 72 h (Figure 1).

In contrast, the absorbance values from the XTT reduction assay, related to biofilm metabolic activity, decreased with incubation time. The decrease in absorbance values was significant from 24 to 72 h for all the isolates (Figure 2).

The clinical isolate of *F. solani* also presented significantly more viable cells on the biofilm than the other species at 48 and 72 h of incubation. Most isolates, except for *F. oxysporum*, exhibited a significant increase in viable cells from 24 to 48 h and from 24 to 72 h (Figure 3). The number of viable cells and the quantity of biomass were quite similar among species with 24 h of incubation, but after this period, they began to show differences among themselves.

SEM analysis revealed that the morphologies of the biofilm structure formed by the three different *Fusarium* species differed. It was observed that biofilms of *F. solani* were formed by narrow, smooth hyphae; and conidiophores with monophiliades, as well as macroconidia, were found in the clinical isolate (FS04). The biofilm of *F. oxysporum* was formed by smooth hyphae with larger lumen, while *F. subglutinans* exhibited crystal-encrusted hyphae. The structure of biofilms examined by SEM is shown in Figure 4.

Regarding *in vitro* antifungal susceptibility testing, PE exhibited strong fungicidal activity against *Fusarium* spp. isolates in planktonic cells. The MIC50 (i.e., the MIC that inhibited 50% of the isolates tested) and MIC90 (i.e., the MIC that inhibited 90% of the isolates tested) corresponded to 1093.75 μg ml^{-1} of TPC expressed in gallic acid and was used to test the fungicide activity of PE against biofilms formed by *Fusarium* species. Furthermore, MIC and MFC were coincident (1093.75 μg ml^{-1} of TPC expressed in gallic acid) for all species.

In the exposure of the biofilms to PE, it was observed that the biomass of those treated with PE was significantly lower than control (Figure 5). Similarly, the number of viable cells also decreased significantly after exposure to PE (Figure 6). These results were confirmed by epifluorescence microscopy, in which it was observed that the number of cells of biofilms treated with PE decreased and were more disorganized and damaged than control, without PE (Figure 7). These data demonstrated the antifungal activity of PE not only on planktonic cells but also on *Fusarium* biofilms.

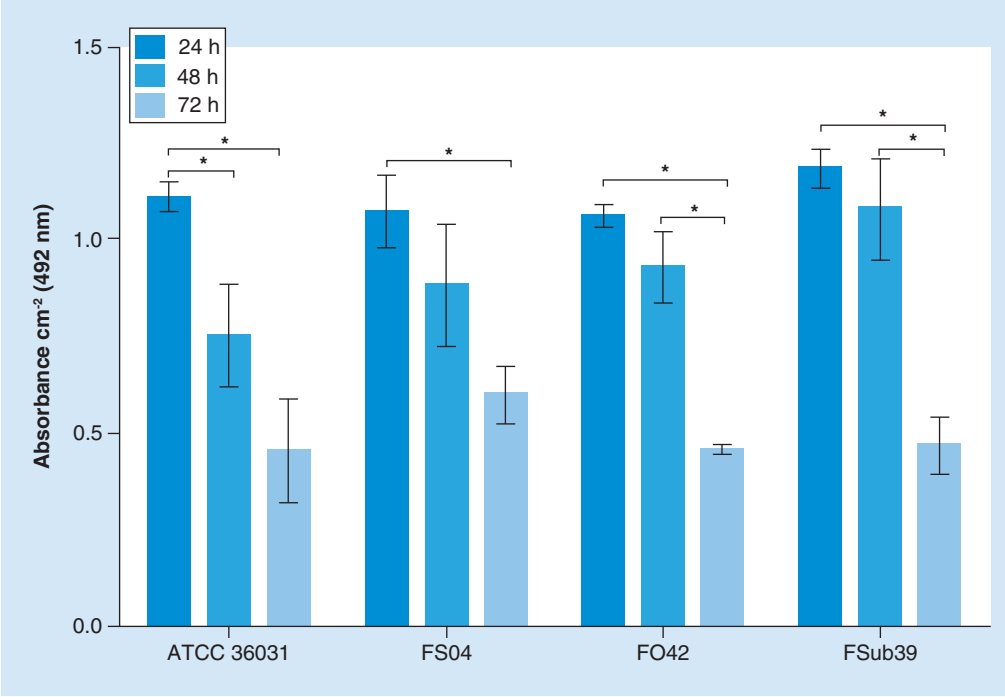

Figure 2. Absorbance values of XTT reduction assay of the three clinical isolates from onychomycosis and a reference strain (*Fusarium solani* ATCC 36031) biofilms at different times of incubation. ANOVA with Bonferroni, *$p < 0.05$.

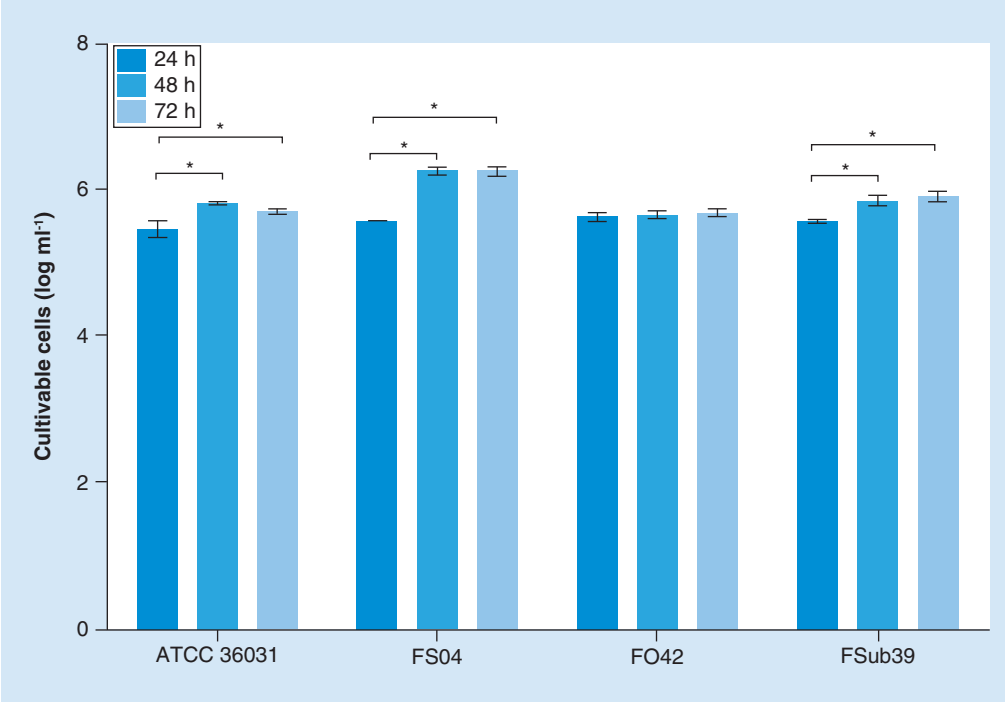

Figure 3. Colony forming units from *Fusarium solani* (ATCC 36031 and FS04), *Fusarium oxysporum* (FO42) and *Fusarium subglutinans* (FSub39) biofilms at different times of incubation. ANOVA with Bonferroni, *$p < 0.05$.

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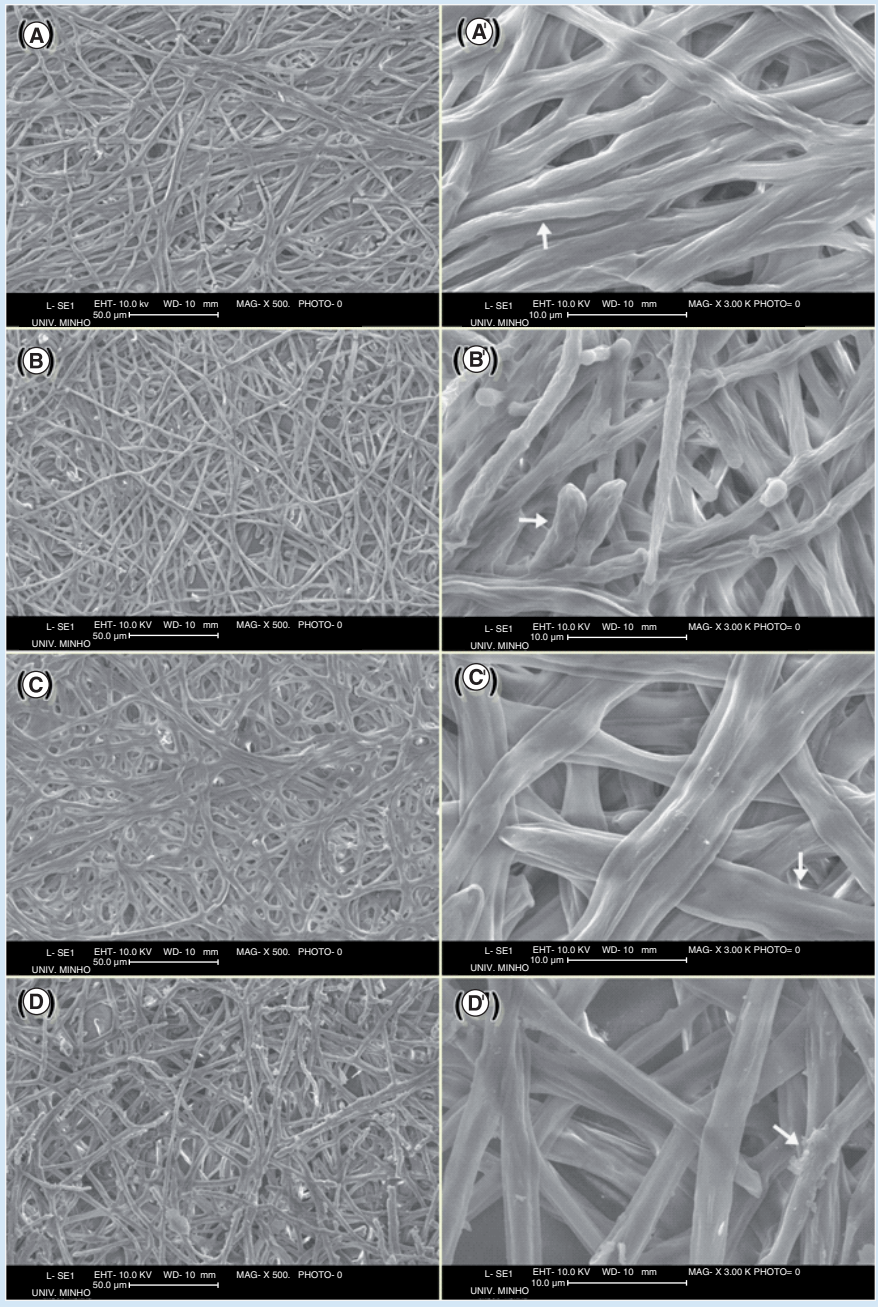

Figure 4. Scanning electron microscopy of *Fusarium solani* ATCC 36031 (A, A'), *Fusarium solani* FS04 (B, B'), *Fusarium oxysporum* FO42 (C, C') and *Fusarium subglutinans* FSub39 (D, D') biofilms with 72 h of incubation. First column 500× magnification (A, B, C, D), second column 3000× magnification (A', B', C', D').

Discussion

Onychomycosis caused by *Fusarium* spp. is frequent in Brazil and has been increasing in recent years [2,10]. Its pathogenesis and treatment, however, have not yet been completely elucidated.

According to our previous study [2], onychomycosis caused by *Fusarium* spp. is characterized by painful lesions, paronychia and inflammatory reaction. In addition, the primary skin lesions caused by *Fusarium* spp. have been considered a portal of entry for disseminated infections caused by this fungus, especially in neutropenic and

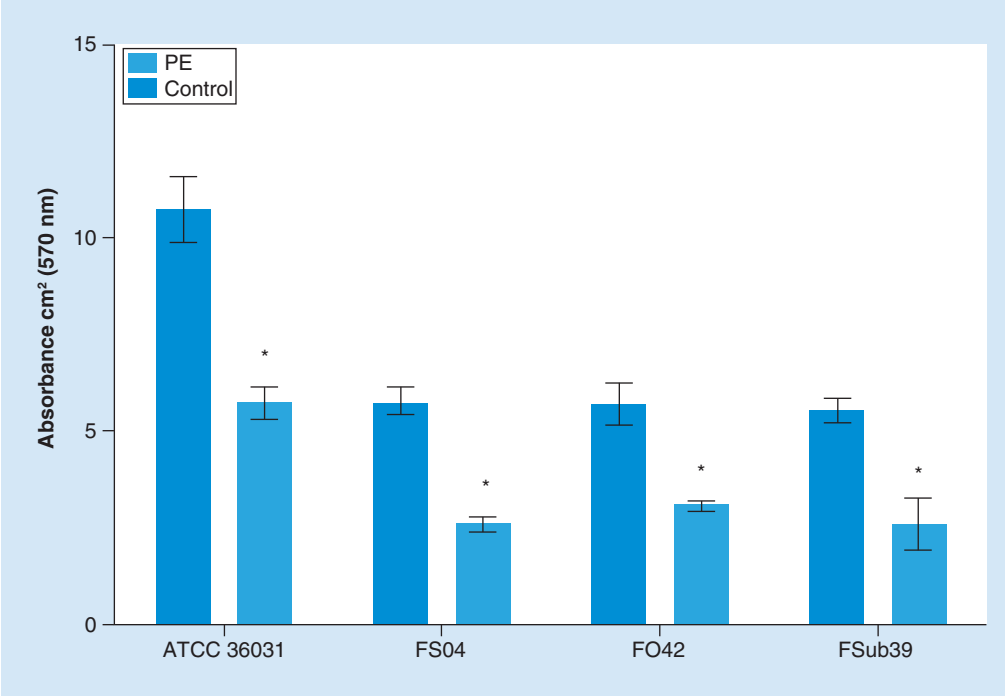

Figure 5. Crystal violet staining of biofilm biomass of *Fusarium solani* (ATCC 36031 and FS04), *Fusarium oxysporum* (FO42) and *Fusarium subglutinans* (FSub39) exposed to propolis extract and control, without propolis extract.
t-test, *p $<$ 0.05.
PE: Propolis extract.

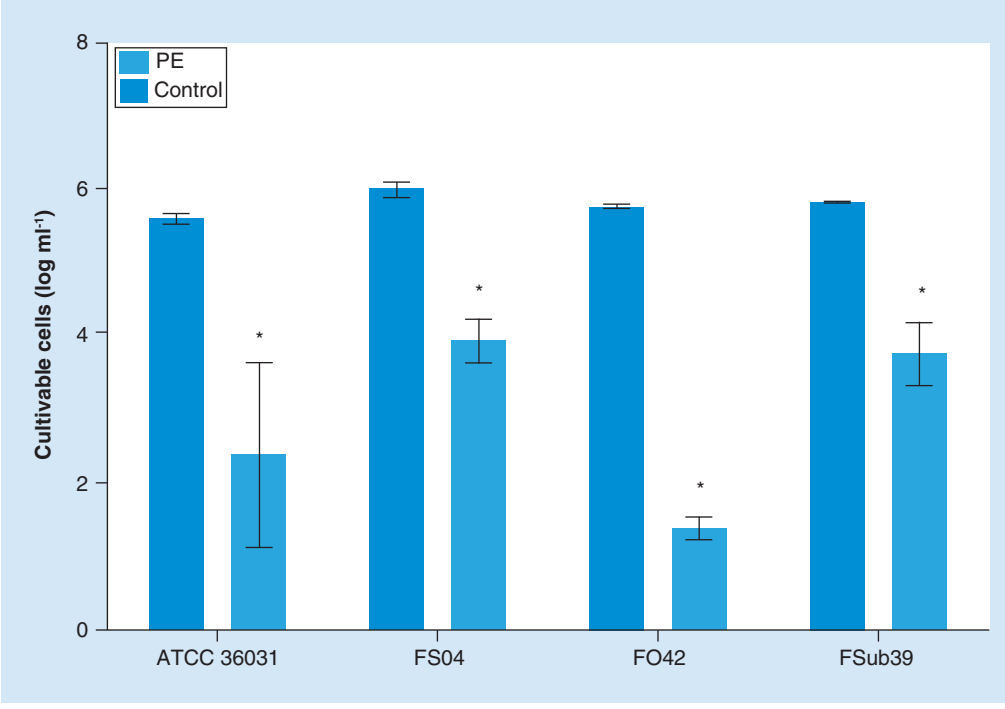

Figure 6. Colony forming units from Fusarium *solani* (ATCC 36031 and FS04), *Fusarium oxysporum* (FO42) and *Fusarium subglutinans* (FSub39) biofilms exposed to propolis extract and control.
t-test, *p $<$ 0.05.
PE: Propolis extract.

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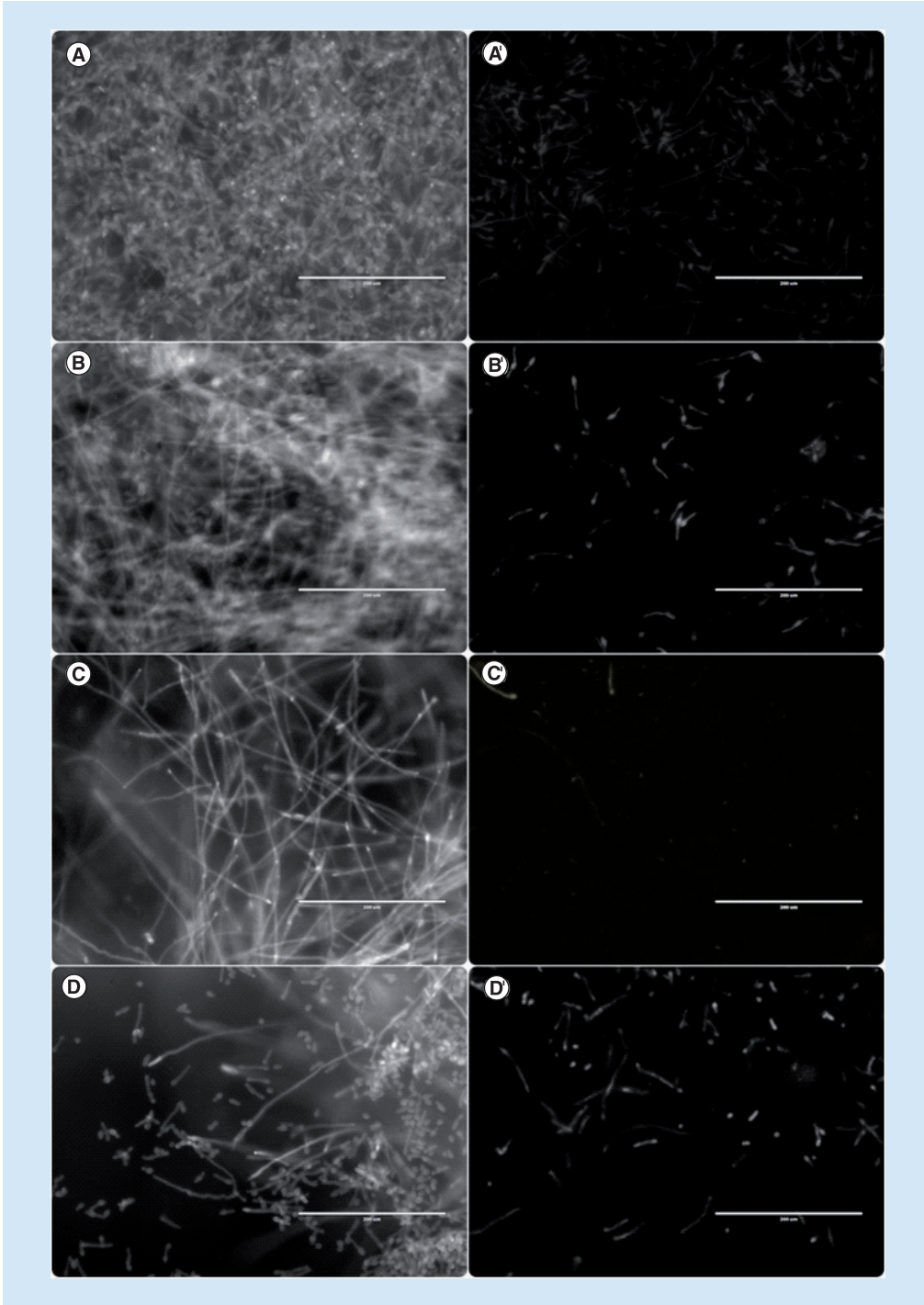

Figure 7. Epifluorescence of *Fusarium solani* ATCC 36031 (A, A'), *Fusarium solani* FS04 (B, B'), Fusarium *oxysporum* FO42 (C, C') and *Fusarium subglutinans* FSub39 (D, D'). First column shows control without PE, 2000× magnification (A, B, C, D), second column shows biofilms treated with PE, 2000× magnification (A', B', C', D').
PE: Propolis extract.

transplanted patients [3,18]. Although *Fusarium* spp. is considered a non-keratinolytic fungus, this genus is able to cause onychomycosis with the ability to form biofilms. According to Gupta *et al.* [7], there is evidence that in onychomycosis, the fungi may be arranged in biofilms in complex, sessile microbial communities attached

to epithelial surfaces. The capacity of *F. oxysporum* to form biofilms on fragments of human nails has also been documented [6].

It has recently been shown for the first time that *Fusarium* spp. can grow *in vitro* using human nails as a single source of nutrient; and also that members from this genus have the ability to invade healthy human nails [10]. In the current study, the capacity of clinical isolates of *F. solani*, *F. oxysporum* and *F. subglutinans* obtained from onychomycosis to form biofilms was demonstrated, a finding similar to those described by Costa-Orlandi *et al.* (2014) [14] for the dermatophytes *T. rubrum* and *T. mentagrophytes*, which are the most common agents of onychomycosis.

While all the species of *Fusarium* tested in this study formed biofilms, the *F. solani* strains were more efficient than the others for all the studied parameters. The results of total biomass quantification and viable cell characterization revealed the greater capacity of *F. solani* to form biofilms (Figures 1 & 2), suggesting that this species is more virulent than the others. This finding is in accordance with the fact that this species is most frequently isolated from invasive fusariosis [19]. Other studies have also described *F. solani* as the most virulent species, such as when *F. solani* was injected intravenously and caused the death of all the animals tested, which other *Fusarium* species did not [20].

Curiously, the values of XTT reduction assay in the present study decreased over time (Figure 2), suggesting a reduction in the metabolic activity of the biofilms. It is important to point out that according to biofilm structure, the fungal cells in sessile populations may display a poor physiological state, as confirmed by XTT-based assay [21]. Furthermore, *Fusarium* biofilms exhibit opposing kinetics (Figures 1 & 2), as metabolic activity was lowest at the moment of peak biomass production and the inverse effect was also observed, corroborating the findings of other studies [22,23]. This low metabolic activity could be due to the increased adsorption of compounds on the biofilm matrix, meaning there is less contact between the tetrazolium salt and the cells of the mature biofilm [21–23]. It is also important to emphasize that the association between these parameters (low activity and high biomass) is well established in literature and is considered responsible for biofilm antifungal resistance, as fungi arranged in biofilms are considered to be up to 1000-fold more resistant to antifungal agents than planktonic cells [21].

Regarding fungal biofilm formation, although most attention has been paid to the yeast pathogen *Candida albicans*, it is known that many fungal species, including filamentous fungi, can form such structures [24,25]. Biofilm morphology can vary according to the species involved and it has been found that even species from the same genus can form biofilms with distinct arrangements [14]. The SEM results of the present study (Figure 4) have shown that the *Fusarium* species used here can form biofilms with different morphologies and all the isolates were able to organize dense growth, with a well-structured community constituted by large hyphae, sometimes with conidiophores and conidia, suggesting a mature ecosystem.

When considering its ability to form biofilms, Peiqian *et al.* [13] reported that *F. oxysporum* when found in biofilms was less susceptible to cold, heat, UV light and some fungicides than those grown in planktonic (free-living) cells. It is well known that the capacity to form biofilms is related to higher resistance and difficulty in clinical treatment [5]. Considering that onychomycosis can be a portal of entry for invasive fusariosis, especially in immunocompromised patients, the search for effective new topical treatments for onychomycosis caused by *Fusarium* spp. is highly relevant. In addition, few antifungal agents are efficacious against fungal biofilms (echinocandins and liposomal amphotericin B) and these drugs are not used for onychomycoses [21].

In this way, it is important to evaluate if a new antifungal option for planktonic cells exists, as PE also acts against fungi organized in biofilms. Propolis is a resin that presents a number of medicinal properties [8], including anti-inflammatory activity, which could potentiate a possible antifungal topical action. Furthermore, the capacity of propolis to inhibit the biofilm formation of *C. albicans* has already been reported, along with the low cytotoxicity in human cells with the same PE used in the present study [15,26]. However, the antifungal property of PE has not yet been proved in biofilms formed by *Fusarium* species.

While microbiologists have historically tested the effect of drugs on planktonic cells, the present study evaluated the action of PE on both planktonic cells and biofilm formation by *Fusarium* species. Initially, good results were obtained for the MIC and MFC of PE against *Fusarium* spp. planktonic cells, which were 1093.75 μg ml^{-1}, similar to those described by Dota *et al.* [27] for *Candida* spp. The majority of isolates tested had MIC values between 550.3 and 1100.63 μg ml^{-1}. Capoci *et al.* [15] also found similar values of MFC and MIC for *Candida*, proving that propolis possesses a fungicide mechanism.

Next, the performance of this PE on biofilms formed by the *Fusarium* species was evaluated. The results were exciting, as PE appears to be efficient, significantly reducing both the biomass and the number of viable

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cell parameters ($p < 0.05$) of the biofilms produced by all the evaluated isolates (Figures 5 & 6). In addition, epifluorescence assay showed clearly that the number of cells of the biofilms formed by all the strains treated with PE were reduced and the structure of the biofilm was lost, leaving only some damaged and isolated cells (Figure 7).

A possible limitation of this study was the absence of a comparison between the antibiofilm effects of propolis and conventional antifungals. However, it should be pointed out that other works carried out by our group [15,26] have shown propolis to be as or more efficient than other antifungal drugs. Moreover, *Fusarium* spp. are highly resistant to azole antifungals which are not usually active against this fungus and terbinafine response varies according to species [10]. We have not therefore included a conventional antifungal as a control.

In summary, it is known that biofilm-forming capacity is related to greater resistance and difficulty in treatment [21]. PE is therefore promising as propolis can be found in its extract form in pharmacies, ready for topical use in patients with onychomycosis, justifying the importance of the development of new treatment approaches with the capacity to control fungal biofilms present in nails affected by *Fusarium* spp.

Conclusion

The results of the present study described the biofilm-forming capacity of three species of *Fusarium* isolated from onychomycosis, with *F. solani* exhibiting the greatest such capacity, reinforcing that it is more virulent than the other *Fusarium* species tested. We also demonstrated the antifungal and antibiofilm capacity of PE against clinical isolates of *F. solani, F. oxysporum* and *F. subglutinans*. This reinforces the previous results and suggests that PE could be an alternative option for the topical treatment of onychomycosis and avoid the possible dissemination of this fungi of onychomycosis, since the most frequent species that cause human invasive fusariosis, *F. solani* and *F. oxysporum*, are also the most frequent species that cause onychomycosis.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Summary points

- *Fusarium* species isolated from onychomycosis form biofilm efficiently.
- Propolis presents potential antifungal activity.
- Propolis is a strong inhibitor of *Fusarium* spp. from onychomycosis.
- Propolis is able to reduce and destroy biofilms formed by *Fusarium* species.
- Propolis is a promising alternative to traditional antifungal therapy.

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Papers of special note have been highlighted as: • of interest; •• of considerable interest

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•• **Excellent article for understanding *Fusarium* species infections.**

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