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Use of the amphotericin B, miconazole, and sodium hypochlorite to control the growth of the robust *Aspergillus flavus* and *Aspergillus fumigatus* biofilms on polyethylene support

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ABSTRACT

Aspergillus flavus and *Aspergillus fumigatus* are able to grow on surfaces, including medical equipment, forming robust and resistant biofilms, and protecting the fungal cells against antifungal agents. Based on that, *A. flavus* and *A. fumigatus* biofilms were treated for 48 h with amphotericin B, miconazole, and sodium hypochlorite. Amphotericin B and miconazole acted as fungistatic for both fungal strains. The minimal inhibitory concentration for the amphotericin B was 400 µg mL^{-1} and >833 µg mL^{-1} for *A. flavus* and *A. fumigatus*, respectively, for the miconazole was 600 µg mL^{-1} and 1200 µg mL^{-1}. Sodium hypochlorite presented fungicide activity at 8.3 µg mL^{-1} on *A. flavus* biofilms, while for *A. fumigatus* biofilm only fungistatic activity was observed. *A. flavus* biofilms were most susceptive to the treatment with the antifungal agents than were *A. fumigatus*. Our observation suggests that these robust structures as an interesting model to study fungal resistance.

1. INTRODUCTION

Fungi are one of the most diverse groups of organisms in our planet with an estimate of around 12 million of species. These microorganisms are found in several environments with different roles in the ecosystems as decomposers, pathogens, and mutualists [1]. In this context, the genus *Aspergillus* deserves attention, comprising a diverse and complex group with importance for such different areas as food microbiology, biotechnology, crop management, and animal health, including humans [2,3]. Some species as *Aspergillus fumigatus* and *Aspergillus flavus* can impact negatively on the human health and several economical activities as well.

The fungus *A. fumigatus* is responsible for the contamination of different products as maize, coffee, bean, soybean, fruits, and meat [4]. In addition, this fungus is responsible for most of the cases of aspergillosis, an infectious disease [5]. *A. flavus* is a saprophytic fungus that grows on seeds of important agro products as maize and rice, promoting their degradation. In addition, this fungus produces the mycotoxin known as aflatoxin, a secondary metabolite with potent toxic effect on the liver and other organs, causing serious health problems [6]. It can also act as carcinogenic and mutagenic agent [7].

Both *A. fumigatus* and *A. flavus* are able to grow on surfaces forming biofilms, complex structures composed of hyphae adhered to biotic or abiotic surfaces. The extracellular matrix of the fungal biofilms is constituted by proteins, lipids, and polysaccharides, presenting many channels that enable the nutrition of the cells, mass transfer, and cell communication [8]. The microbial development as biofilm gives advantages to the microorganism, such as the resistance to biotic and abiotic stresses. This resistance is dependent on the different factors as the structural complexity of the biofilm and the presence of extracellular matrix, impairing the penetration of antifungal agents [9].

Amphotericin B and miconazole are antifungal agents that have been used to control fungal development. The former is an agent included in the class of polienic used to treat systemic infections, acting on different species of yeasts and filamentous fungi, while miconazole is an antifungal agent included in the class of azoles that present high toxicity if used as systemic agent, but high efficiency if used topically [10]. Amphotericin B binds to ergosterol in fungal cell membrane, leading to the formation of aqueous porous affecting the permeability of the plasma membrane. The plasma membrane is also ruptured [11]. Two mechanisms are involved in the miconazole action: (i) Inhibition of the enzyme 14α-lanosterol dismutase associated to the

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biosynthesis of ergosterol, promoting the rupture of plasma membrane, and (ii) oxidative damage through the increase in the concentration of the reactive oxygen species (ROS) on account of the inhibition of the peroxidase and catalase activities [12]. Differing from amphotericin B and miconazole, sodium hypochlorite is used as disinfectant and sanitizer of residential, industrial, and medical devices. According to Han *et al*. (2016) [13], sodium hypochlorite modifies the cell membrane permeability promoting osmotic disequilibrium and intracellular damage, and also inhibits enzymes as the ones found in important metabolic ways.

The increase in fungal resistance to the conventional antifungal agents can cause a significant increase in the prevalence of infections and contamination of food and medical devices [14]. Based on that, the robust filamentous fungal biofilm is an interesting model to study the action of antifungal agents on a robust structure aiming, in the future, the understanding of mechanisms of resistance. In spite of the diversity of studies evaluating the action of different antifungal agents on fungal biofilms, this is the first study with the evaluation of the action of amphotericin B, miconazole, and sodium hypochlorite on dense and robust *A. flavus* and *A. fumigatus* biofilms developed on inert supports.

2. MATERIALS AND METHODS

2.1. Microorganisms

The filamentous fungi *A. flavus* (LH0106) and *A. fumigatus* (LH0206) were isolated from soil samples and deposited in the culture collection of the Laboratory of Microbiology and Cellular Biology, Department of Biology of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, São Paulo, Brazil. The strains were maintained on Potato Dextrose Agar (PDA) slants at 4°C and new cultures were obtained from 30-day-old cultures.

2.2. Preparation of the Spore Suspension and Fungal Biofilm Development

The suspension of spores of each fungal strain was prepared through the addition of sterile distilled water on the PDA slants with fungal growth and scraping of the surface. The suspension was filtered through gauze and adjusted to 10^6 spores/mL using a TC20 automatic cell counter (Bio-Rad).

The fungal biofilms were developed on polyethylene supports with dimensions of 1 cm × 1cm, previously washed with detergent and distilled water, dried, and sterilized under ultraviolet radiation for 20 min. The supports were added to a 24-well microplate containing spore suspensions, separately, and maintained under agitation (50 rpm) at 30°C for 90 min for spore adhesion. Thereafter, the spore suspension was removed of each well and the supports containing the spores were washed twice (45 min each one) with distilled water to remove the unadhered spores. The supports with adhered spores were transferred to a new microplate well containing 1.5 mL of Khanna medium [15] with 2% (w/v) glucose as carbon source and cultured under 50 rpm at 30°C for 36 h and 48 h for the development of the biofilms of *A. flavus* and *A. fumigatus*, respectively.

2.3. Antifungal Treatment

A. flavus and *A. fumigatus* biofilms were treated with different concentrations of the conventional antifungal amphotericin B (400–3200 μg mL^{-1} for the former and 0.83–833 μg mL^{-1} for the latter), miconazole (600–4800 μg mL^{-1}), and sodium hypochlorite (0.31–8.3 μg mL^{-1}) as determined through the previous experiments. The microplate wells containing the fungal biofilms of each strain, separately, and Khanna medium were added with the antifungal agents and maintained at 30°C for 48 h. After this period, metabolic activity and the potential of growth on PDA medium of the treated biofilms were analyzed. As control, untreated biofilms obtained as described above were used.

2.4. Analysis of the Metabolism and Potential of Growth

The metabolic activity of *A. flavus* and *A. fumigatus* biofilms after and before treatment with the antifungal agents for 48 h was evaluated using resazurin methodology [16] with modification. Resazurin (0.002 μg mL^{-1}) was added to each microplate well and the absorbance was monitored using a spectrophotometer with wavelength adjusted to 570 nm.

For the analysis of the growth potential, treated biofilms were washed with sterile distilled water and deposited on the surface of the PDA culture medium in Petri dishes and incubated at 37°C for 12, 24, and 36 h. The sizes of the biofilms for each period of incubation were measured using a pachymeter [17]. Fungal biofilms without treatment were used as controls.

The concentration of the antifungal agent that inhibited at least 30% of the development of the fungal biofilms on PDA slants for 72 h of incubation at 30°C was considered as the minimal inhibitory concentration (MIC) and the concentration that inhibited the growth completely (95–100%), under the same conditions, as the minimal fungicide concentration (MFC).

2.5. Statistics

All experiments were performed as biological triplicates and the values were expressed as mean ± standard error. The results were analyzed with one-way analysis of variance, followed by Tukey test ($P = 0.05$) using the software GraphPad Prism 9.

3. RESULTS

3.1. Effect of Amphotericin B

The effect of amphotericin B on the growth and metabolism of *A. flavus* and *A. fumigatus* biofilms is observed in Figure 1. *A. flavus* growth on PDA slants, for different periods of incubation (12, 24, and 36 h), after treatment with amphotericin B, was reduced when compared with the untreated biofilm, and this reduction was dependent of the concentration of amphotericin B [Figure 1a]. The growth reduction after 24 and 36 h of incubation was statistically significant for all concentrations of amphotericin B used when compared to the control. Fungal metabolism was also reduced in a concentration-dependent manner [Figure 1b], with significant differences among the treatments with exception between 1600 and 3200 μg mL^{-1}. When 3200 μg mL^{-1} of amphotericin B was used, *A. flavus* biofilm metabolized only 44.6% of the resazurin in the medium. The MIC estimated was 800 μg mL^{-1}, while MFC was >3200 μg mL^{-1} [Table 1], since under this concentration, only 57% of reduction in the growth was observed.

Considering the effect of amphotericin B on *A. fumigatus* growth evaluated 48 h after the treatment, it is possible to observe that the growth was reduced in a concentration-dependent manner for all periods of incubation analyzed [Figure 1c]. The highest significant reduction of the growth was achieved using 833 μg mL^{-1} of amphotericin B. It is also noted that when the treated biofilm was maintained at 30°C

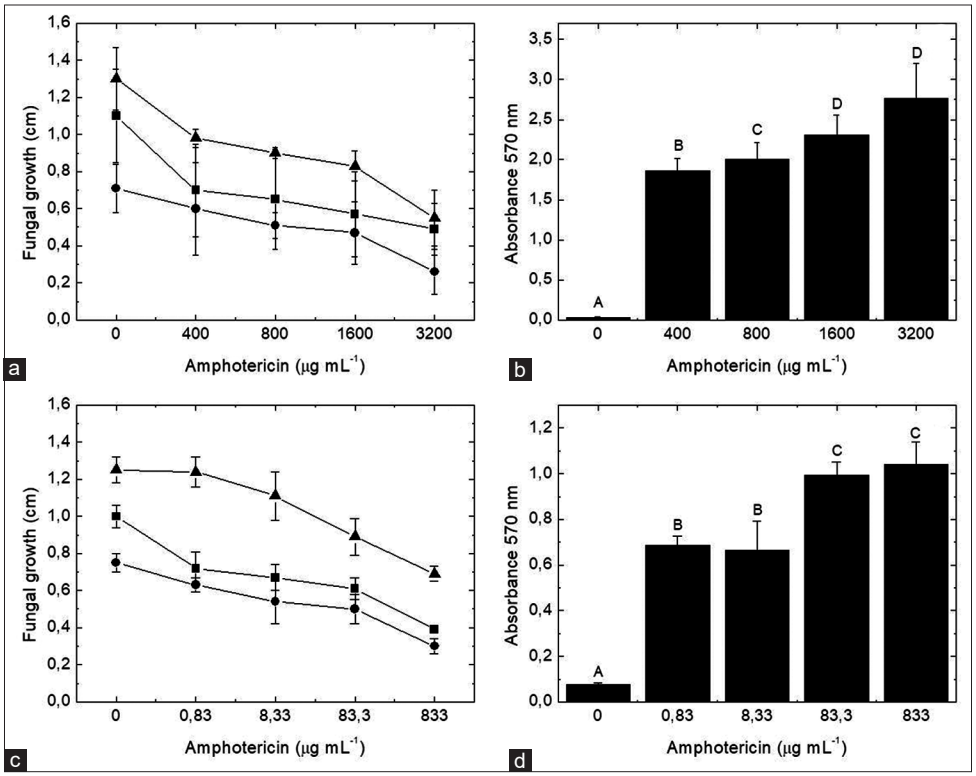

Figure 1: Effect of amphotericin B on the fungal development in Potato Dextrose Agar slants (a and c) for 12 h (●), 24 h (■), and 36 h (▲), and on the fungal metabolism (b and d) of *Aspergillus flavus* (a and b) and *Aspergillus fumigatus* (c and d) biofilms after the treatment for 48 h. The absorbance of the control (medium + resazurin) at 570 nm was 5 ± 0.1. Different capital letters indicate statistical difference considering the same period of growth after treatment with different antifungal concentrations. *P*-value was fixed at 0.05.

Table 1: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) for amphotericin B, miconazole, and sodium hypochlorite (free chlorine) used for the treatment of *A. flavus* and *A. fumigatus* biofilms developed on inert supports.

Antifungal agent	*A. flavus*		*A. fumigatus*	
	MIC (µg mL^{-1})	MFC (µg mL^{-1})	MIC (µg mL^{-1})	MFC (µg mL^{-1})
Amphotericin B	800	> 3200	83	> 833
Miconazole	600	4800	2400	> 4800
Free chlorine	<0.31	0.92	0.31	>8.3

A. flavus: Aspergillus flavus, A. fumigatus: Aspergillus fumigatus The MIC values were estimated considering the minor concentration of the antifungal agent that inhibited at least 30% of biofilm growth on a PDA slant for 72 h at 30°C, and the MFC that inhibited completely (100 ± 5%) the biofilm growth under the same conditions.

for 72 h, the growth was higher for all concentrations of amphotericin used, indicating a fungistatic action of this antifungal agent. This was confirmed by the analysis of the metabolism of the treated biofilm [Figure 1d]. There is no significant difference between the treatments with 0.83 and 8.33 µg mL^{-1} and 83.3 and 833 µg mL^{-1}. The increase in the absorbance indicates a reduction in the metabolism, but it was not fully stopped. At 833 µg mL^{-1}, *A. fumigatus* biofilm metabolized 79.2% of the resazurin in the medium and only a reduction of 40% in the growth was registered. The MIC and MFC values of amphotericin B were estimated as 83.3 µg mL^{-1} and >833 µg mL^{-1}, respectively, considering 72 h of incubation at 30°C [Table 1]. In Figures S1 and S2, it is possible to observe the macroscopic aspect of both *A. flavus* and *A. fumigatus* biofilms 48 h after treatment with different concentrations of amphotericin B. In Figure S1, it is possible to observe the conidiation of the untreated *A. flavus* biofilm, while in the presence of amphotericin, this event was impaired and uncolored mycelia was noted. For *A. fumigatus*, the conidiation occurred for both untreated and treated biofilms [Figure S2].

3.2. Effect of the Miconazole

Considering the use of miconazole in the treatment of *A. flavus* biofilm [Figure 2a], the profile of growth was similar to that obtained in the presence of amphotericin B, with the highest reduction achieved using both 2400 µg mL^{-1} and 4800 µg mL^{-1}. The growth reduction at these concentrations was statistically significant when compared to the control and also to 600 µg mL^{-1} and 1200 µg mL^{-1}. However, after treatment with 2400 µg mL^{-1} and 4800 µg mL^{-1} of miconazole, any significant difference was observed for the growth on PDA slants considering different periods of incubation at 37°C. In addition, the growth reduction of *A. flavus* biofilms treated with these concentrations was similar. No significant reduction in resazurin metabolization was observed since the biofilm treated with these concentrations of miconazole metabolized 93.6–95.8% of the resazurin [Figure 2b]. Figure S1 illustrates the macroscopic aspect of *A. flavus* biofilm 48 h after treatment with different concentrations of miconazole. Conidiation can be observed for the untreated biofilm, but using 4800 µg mL^{-1}, 2400 µg mL^{-1}, and 1200 µg mL^{-1} of miconazole, a translucent mycelium was noted. At 600 µg mL^{-1}, the pigmentation was affected when compared to the control biofilm.

The growth of *A. fumigatus* biofilm was not affected when 600 µg mL^{-1} of the miconazole was used, but at 1200 µg mL^{-1}, growth inhibition was observed, with most pronounced effect achieved at 2400 µg mL^{-1} and

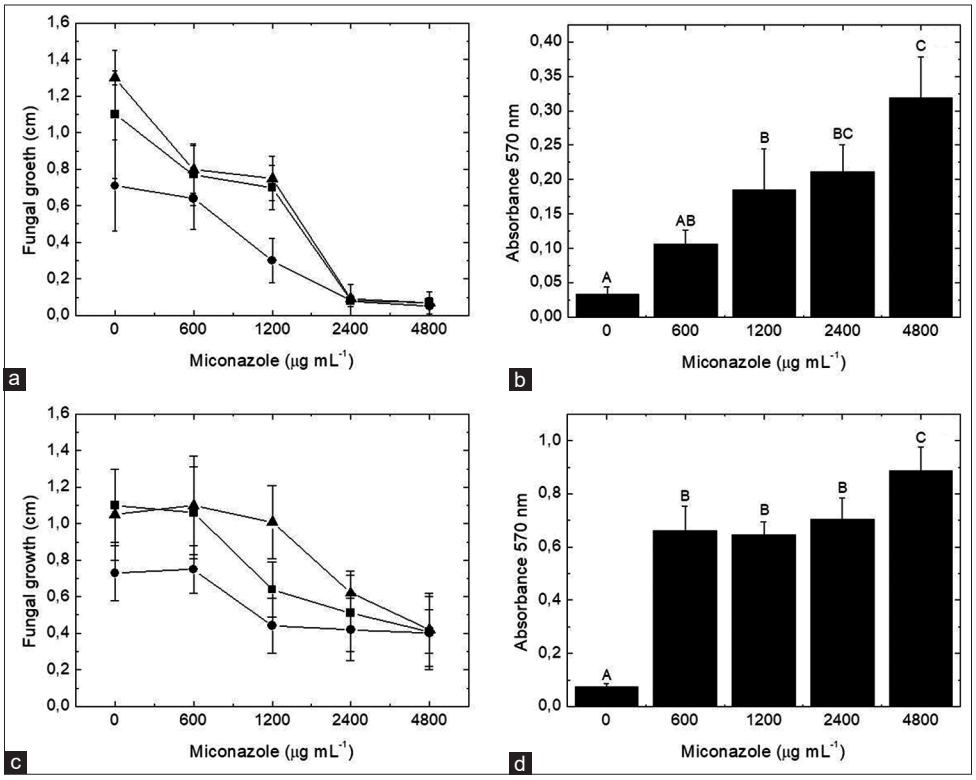

Figure 2: Effect of miconazole on the fungal development in Potato Dextrose Agar slants (A and C) for 12 h (●), 24 h (■), and 36 h (▲), and on the fungal metabolism (B and D) of *Aspergillus flavus* (A and B) and *Aspergillus fumigatus* (C and D) biofilms after the treatment for 48 h. The absorbance of the control (medium + resazurin) at 570 nm was 5 ± 0.1. Different capital letters indicate statistical difference considering the same period of growth after treatment with different antifungal concentrations. *P*-value was fixed at 0.05.

4800 μg mL^{-1} of miconazole [Figure 2c]. The fungal growth on PDA slants for 24, 48, and 72 h at 30°C was similar when the biofilms were treated with 2400 μg mL^{-1} and 4800 μg mL^{-1} of miconazole. The macroscopic aspect of *A. fumigatus* biofilm 24 h after treatment with miconazole is shown in Supplementary Figure S2. The untreated biofilm as well as the treated biofilms with 600 μg mL^{-1}and 1200 μg mL^{-1} presented gray pigmentation while yellow pigmentation was observed for the treated biofilms with 2400 μg mL^{-1} and 4800 μg mL^{-1} of miconazole. The effect of miconazole on the metabolism of *A. fumigatus* biofilm was more pronounced than on *A. flavus* biofilm. The metabolism of biofilms treated from 600 μg mL^{-1} to 2400 μg mL^{-1} of miconazole was similar (85.9–90.8%), and at 4800 μg mL^{-1}, 82.3% of the resazurin was metabolized [Figure 2d]. The MIC values of miconazole were estimated as 600 μg mL^{-1} and 2400 μg mL^{-1} for *A. flavus* and *A. fumigatus* biofilms, respectively. The MFC was 4800 μg mL^{-1} for *A. flavus*, with a reduction of 96% in the biofilm growth, while the reduction was only 41% for *A. fumigatus* biofilm [Table 1].

3.3. Effect of the Sodium Hypochlorite

The best results of inhibition of both fungal growth and metabolism of *A. flavus* biofilms were achieved using sodium hypochlorite. No growth was observed for 24 h after the treatment of the biofilms with 8.3 μg mL^{-1} of free chlorine [Figure 3a and Figure S1]; at 2.77 μg mL^{-1}, a minimal growth was observed. Considering the same concentration of the free chlorine used, no significant difference was found for the growth during different periods. When compared the results obtained at 2.77 μg mL^{-1} and 8.3 μg mL^{-1}, any significant difference was observed. These results were confirmed by the resazurin metabolization as only 26% of the resazurin was metabolized when the biofilm was treated with 8.3 μg mL^{-1} of free chlorine [Figure 3b]. The reduction of the resazurin metabolization was significant for the treatment using 8.3 μg mL^{-1} free chlorine when compared to the control and also to the other concentrations. The MIC value was estimated as <0.31 μg mL^{-1}, and the MFC was 0.92 μg mL^{-1} with 95% of reduction in biofilm growth observed 72 h after treatment [Table 1].

The use of sodium hypochlorite also caused the highest reduction in the growth of the *A. fumigatus* biofilm [Figure 3c and Figure S2]. A significant growth reduction was observed using 0.31 μg mL^{-1} compared to the control for all periods of incubation at 30°C after treatments. The most pronounced effect of free chlorine, without significant differences, was achieved in a range of 0.92–8.3 μg mL^{-1}. In these concentrations, the fungal growth was similar for all periods of incubation [Figure 3c]. According to Figure S2, the concentrations of free chlorine were not sufficient to impair the conidiation of *A. fumigatus* biofilms. The ability of *A. fumigatus* biofilm to metabolize resazurin after treatment with sodium hypochlorite was negatively affected, mainly in the presence of 8.3 μg mL^{-1} of free chlorine, in spite of the statistical similarity of the results obtained, but differing from the control. At this concentration, 59.5% of the resazurin present in the medium was metabolized by *A. fumigatus* biofilm [Figure 3d]. The MIC value of the free chlorine was estimated as 0.31 μg mL^{-1} for *A. fumigatus* biofilm, but the MFC was >8.3 μg mL^{-1} [Table 1] considering 36 h of incubation in PDA medium after treatment.

4. DISCUSSION

In general, studies on the action of antifungal agents are performed following the standards from the Clinical and Laboratory Standards

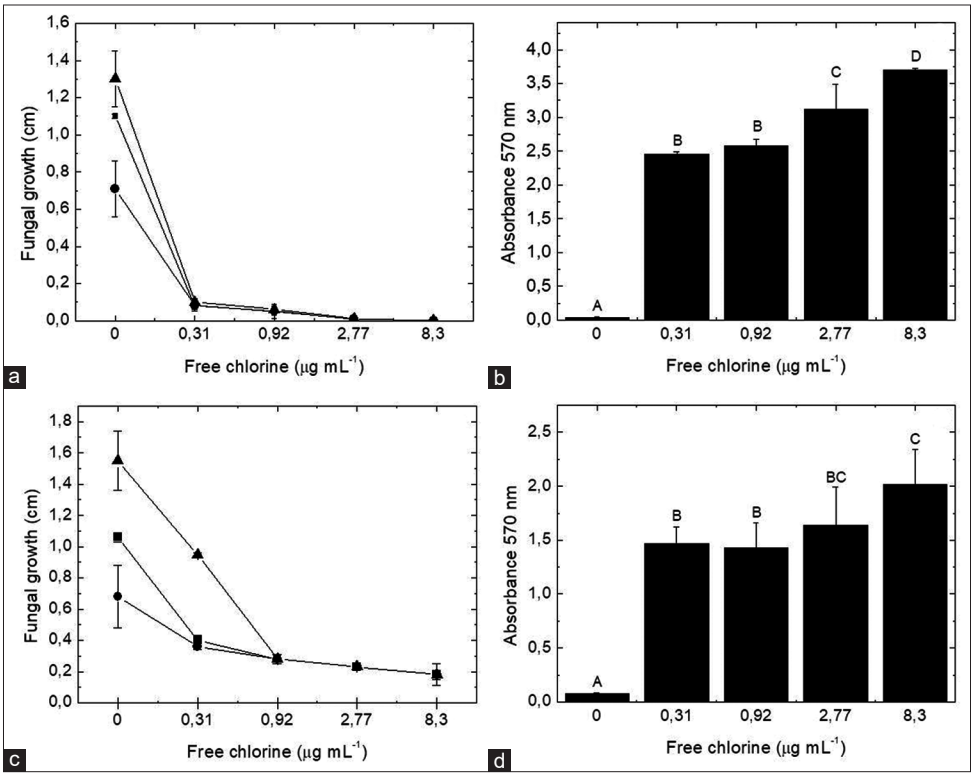

Figure 3: Effect of the sodium hypochlorite (free chlorine) on the fungal development in Potato Dextrose Agar slants (a and c) for 12 h (●), 24 h (■), and 36 h (▲), and on the fungal metabolism (b and d) of *Aspergillus flavus* (a and b) and *Aspergillus fumigatus* (c and d) biofilms after the treatment for 48 h. The absorbance of the control (medium + resazurin) at 570 nm was 5 ± 0.1. Different capital letters indicate statistical difference considering the same period of growth after treatment with different antifungal concentrations. *P*-value was fixed at 0.05.

Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST), which was not possible for this study. The CLSI and EUCAST recommendation is directed for studies using conidia and not for mature and robust structure as the biofilms used in the present study.

The fungal biofilm complexity is the result of the mycelia immersed in an extracellular matrix that protects the cells from the action of the antifungal agents and improves its resistance [18,19]. *A. flavus* biofilm was resistant to the treatment with amphotericin B and a high value of MIC was observed. According to Ellis *et al*. (2002) [20], the concentration of amphotericin B that inhibited 90% of the free mycelia of *A. flavus* was 2 µg mL^{-1}, significantly lower than that obtained for the biofilm. On the other hand, some resistant fungal strains have been isolated, as described by Reichert-Lima *et al*. (2017) [21]. According to these authors, the MIC value for amphotericin B found for *A. flavus* isolates was ≥2 mg L^{-1}.

No effect of amphotericin B was observed on the growth of *A. fumigatus* biofilm using the different concentrations of this antifungal agent, in spite of the citation of the susceptibility of this filamentous fungus to this antifungal [22]. Amphotericin B presents a broad spectrum of action and resistance to this antifungal is rare [23], although some resistant *A. fumigatus* isolates [21]. When resistance is observed, different mechanisms can explain it as the lack of ergosterol in the fungal cell membrane and increase in the antioxidant activity [22], but in the case of the low effectiveness observed for both *A. flavus* and *A. fumigatus* biofilms, the main reason is probably the structure of the biofilms and the presence of the extracellular matrix.

The extracellular matrix hinders the linkage of amphotericin B to ergosterol in the plasma membrane and, consequently, the modification in the permeability is not sufficient to compromise the cell viability. Amphotericin B also induces the accumulation of ROS, damaging the cells [22], but in the case of fungal biofilms, this way can be also compromised. To overcome this barrier, the use of high antifungal concentration is necessary for an effective control of the fungal growth, more than that required for the planktonic cells [18]. The use of high concentrations of amphotericin required for fungal biofilm treatment is a problem for the systemic application, because of its high toxicity [23]. However, its application in the cleaning process of different pieces of equipment is an alternative. Since amphotericin B does not impair the fungal biofilm development completely, it can be considered fungistatic for these biofilms.

Similar results, as those found for the use of amphotericin B, were observed using miconazole for the treatment of *A. flavus* biofilm. The development of the biofilm was mainly affected when high concentrations of miconazole were used in spite of the high level of metabolization of the resazurin, indicating its fungistatic activity for all concentrations used. Miconazole, an antifungal of the azole class, inhibits the synthesis of the ergosterol through its action on the enzyme lanosterol 14-alpha-demethylase, essential for this biosynthetic pathway [24]. Different mechanisms of resistance to the azoles have been reported as a reduction in the accumulation of the antifungal inside the cell, reduction in the affinity of the antifungal to its target, increase in the copy number of the target, and alteration of the fungal metabolism [24]. The development of *A. fumigatus* biofilm was mostly impacted when a high concentration of miconazole was applied, while the metabolism was not significantly affected, characterizing

its fungistatic activity. The resistance of *A. fumigatus* biofilm to the miconazole was reported by Shishodia *et al*. (2019) [19] and, according to these authors, the extracellular matrix is important to guarantee the junction of the hyphae to form the biofilm structure, improving the resistance. We can hypothesize, for both *A. flavus* and *A. fumigatus* biofilms, that the accumulation of miconazole inside the fungal cell is reduced because the extracellular matrix impairs the penetration of the antifungal agent, especially considering the most internal fungal cells. Consequently, its action on the lanosterol 14-alpha-demethylase is reduced.

Differing from the use of both amphotericin B and miconazole, the application of sodium hypochlorite, through the free chlorine, allowed the best control of the development of *A. flavus* biofilm, presenting both fungistatic and fungicidal activities. According to Estrela *et al*. (2002) [25], sodium hypochlorite can act on extracellular matrix through free chlorine and also by pH modification, facilitating the access of the agent to the fungal cells. The effect of free chlorine on the growth of *A. fumigatus* biofilm on PDA slants was not the same observed for the development of the *A. flavus* biofilm. The concentration that inhibited totally the development of *A. flavus* biofilm was not sufficient to produce the same result on *A. fumigatus* biofilm, indicating that the former is most susceptible to the action of this antifungal. The level of the metabolism observed for each one reinforces this observation. Some studies have indicated that *Aspergillus* genus is the prevalent fungi in hospital and veterinary settings [26], and the elimination of the contamination is essential. In this context, sodium hypochlorite has been mentioned as an effective disinfectant, as also demonstrated in this study for the robust filamentous fungus biofilms developed on inert support. In addition, sodium hypochlorite can be used in seeds, soils, and water treatment systems [27]. This is an important fact to be considered since *A. flavus* can contaminate different agro-byproducts. The inhibitory effect of free chlorine on the growth of fungal phytopathogens as *Phytophthora cactorum*, *Helicobasidium mompa,* and *Rosellinia necatrix* has also been reported [27]. Sisti *et al*. (2012) [28] reported a study on the use of sodium hypochlorite to control *Aspergillus* spp. and *Candida albicans* in drinking water.

5. CONCLUSIONS

A. flavus and *A. fumigatus* biofilms developed on polyethylene as inert supports are robust structures resistant to the treatment with conventional antifungal agents. The susceptibility of the fungal biofilms to each antifungal was different. However, sodium hypochlorite was the best antifungal agent to control the development of the biofilms of both fungal strains, what was expected since sodium hypochlorite is a sanitizer agent extensively used to clean the unanimated surfaces, acting on the components of extracellular matrix. The action of miconazole was more pronounced on *A. flavus* biofilm than the action on *A. fumigatus* biofilm while the opposite is true for amphotericin B, in spite of the high level of metabolization of the resazurin by both fungal biofilm species. Differences in the permeability of the extracellular matrix to these antifungals can explain the different responses. This study confirmed the importance of the extracellular matrix to protect the fungal cells and highlights the use of filamentous fungus biofilms as models of study on fungal resistance.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

12. PUBLISHER'S NOTE

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SUPPLEMENTARY FIGURE

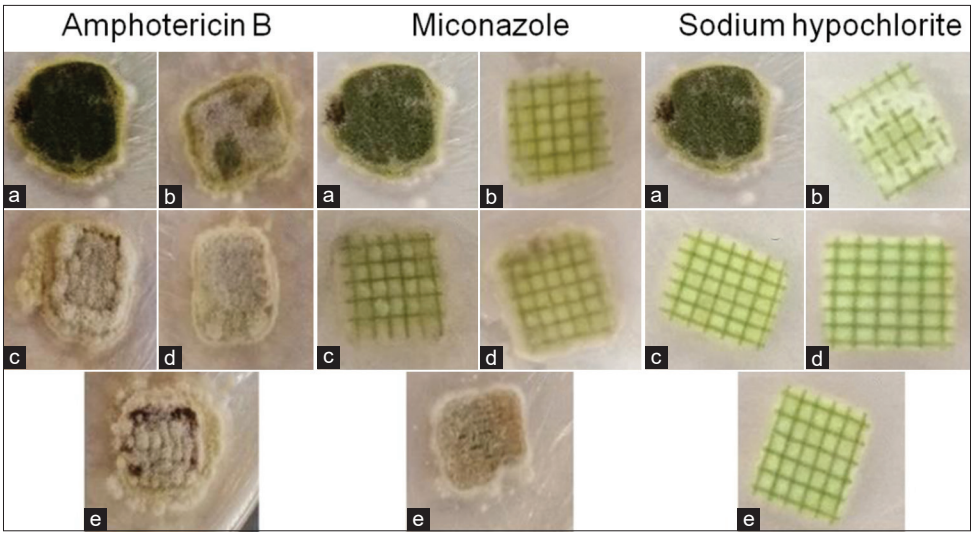

Figure S1: Development of *Aspergillus flavus* biofilm in Potato Dextrose Agar medium after 48 h of treatment with amphotericin B (a: Control, b: 3200 µg mL^{-1}, c: 1600 µg mL^{-1}, d: 800 µg mL^{-1}, and e: 400 µg mL^{-1}), miconazole (a: Control, b: 4800 µg mL^{-1}, c: 2400 µg mL^{-1}, d: 1200 µg mL^{-1}, and e: 600 µg mL^{-1}) and free chlorine (a: Control, b: 8.3 µg mL^{-1}, c: 2.77 µg mL^{-1}, d: 0.92 µg mL^{-1}, and e: 0.31 µg mL^{-1}) for 24 h of incubation at 30°C.

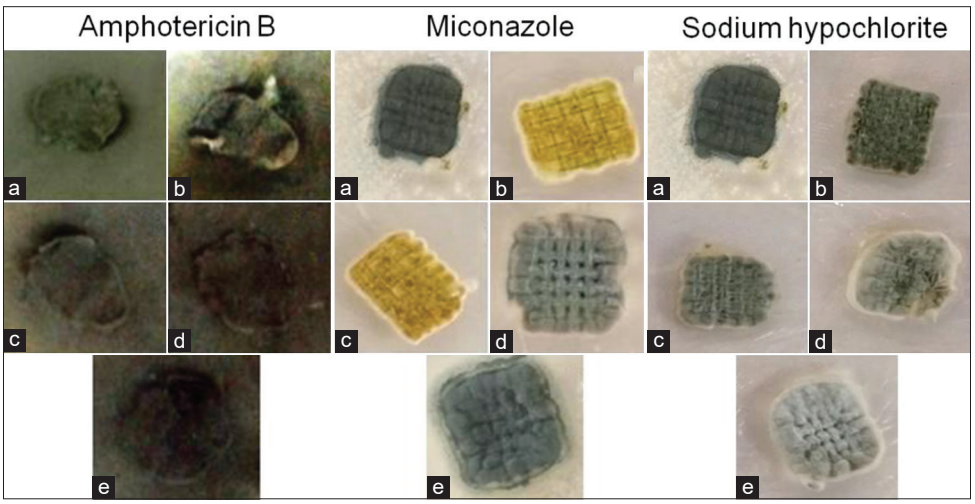

Figure S2: Development of *Aspergillus fumigatus* biofilm in Potato Dextrose Agar medium after 48 h of treatment with amphotericin B (a: Control, b: 833 µg mL^{-1}, c: 83 µg mL^{-1}, d: 8.3 µg mL^{-1}, and 0.83 µg mL^{-1}), miconazole (a: Control, b: 4800 µg mL^{-1}, c: 2400 µg mL^{-1}, d: 1200 µg mL^{-1}, and e: 600 µg mL^{-1}) and free chlorine (a: Control, b: 8.3 µg mL^{-1}, c: 2.77 µg mL^{-1}, d: 0.92 µg mL^{-1}, and e: 0.31 µg mL^{-1}) for 24 h of incubation at 30°C.