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Antifungal activity of silver nanoparticles and simvastatin against toxigenic species of *Aspergillus*

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ABSTRACT

Aspergillus spp. are ubiquitous fungi that grow on stored grains. Some species produce toxins that can harm human and animal health, leading to hepato- and nephrotoxicity, immunosuppression and carcinogenicity. Major fungicides used to prevent fungal growth may be toxic to humans and their repeated use over time increases levels of resistance by microorganisms. Nanotechnology is an emerging field that allows use of antimicrobial compounds in a more efficient manner. In this study, was evaluated the antifungal activity of biogenic silver nanoparticles (AgNPs, synthesized by fungi) and simvastatin (SIM, a semi-synthetic drug), alone and in combination against three toxigenic species belonging to the genera *Aspergillus* section *Flavi* (*Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus. parasiticus*) and two of section *Circumdati* (*Aspergillus ochraceus* and *Aspergillus melleus*). SIM exhibited a MIC_{50} of 78 μg/mL against species of Section *Flavi* and a MIC_{50} of 19.5 μg/mL against species of Section *Circumdati*. The MIC_{50} of AgNPs against *Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus parasiticus* was 8 μg/mL, while the MIC_{50} was 4 μg/mL against *Aspergillus melleus* and *Aspergillus ochraceus*. Checkerboard assay showed that these compounds, used alone and in combination, have synergistic and additive effects against toxicogenic species of *Aspergillus*. Analysis by SEM gives an idea of the effect of SIM and AgNPs alone and in combination on spore germination and vegetative growth. Ultrastructural analysis revealed that spore germination was prevented, or aberrant hyphae were formed with multilateral branches upon treatment with SIM and AgNPs. These results reveal potential benefits of using combination of AgNPs and SIM to control fungal growth.

1. Introduction

Food commodities are of great importance in the developing countries, where bulk cereal grains used for food and feed are main exports. Inadequate conditions pre-crop, during storage and post-harvest may predispose cereal grains to contamination with filamentous fungi that produce mycotoxins (Rocha et al., 2014). Mycotoxins are secondary metabolites, and the most prominent mycotoxins are mainly produced by toxigenic species of *Aspergillus*, *Penicillium* and *Fusarium* (Anfossi et al., 2016; Njobeh et al., 2010; Terzi et al., 2014). Among mycotoxins, aflatoxins and ochratoxins are of major concern, contaminating cereal grains stored in inappropriate condition (Strosnider et al., 2006; Yehia, 2014). The commodities susceptible to contamination by aflatoxins (AFs) are peanut, figs, maize, oil seeds, sorghum, cocoa beans, spices, rice, fruit and vegetables (Makun et al., 2012). In this way, fungi spoilage directly impacts the local diet. AFs are produced by fungi of the genus *Aspergillus*, predominantly by two species: *Aspergillus flavus* and *Aspergillus parasiticus.* There are four main AFs: B1, B2, G1 and G2. AFB1 is the most toxic. According to the (IARC-International Agency for Research on Cancer, 2010), AFs are classified as group 1 carcinogens.

Ochratoxin A (OTA), which can be produced by *Aspergillus ochraceus*, *Aspergillus melleus*, *Aspergillus westerdijkiae* and some species of *Penicillium* (Heussner and Bingle, 2015), is also of great importance. OTA is classified, according to the IARC, as a 2B carcinogen, and it poses a risk to human health as a nephrotoxic agent. OTA is often

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detected in many types of grains and cereal products such as coffee, cocoa, grapes, wine, soybeans, nuts, legumes and beer (Bellver-Soto et al., 2014; Haighton et al., 2012; Streit et al., 2012). In Brazil, one of the largest producers of coffee, contamination by OTA is of major concern (Pardo et al., 2004).

Development of new antifungal agents has always been in demand due to the surge of resistant fungal isolates and the need to produce antifungals with reduced toxicity and lower cost (Nyilasi et al., 2010). Cases of antifungal resistance have occurred worldwide, by repeated use of agrochemicals and/or shift of fungal population, favoring selection of resistant mutants (Deising et al., 2008; Guilger et al., 2017).

Simvastatin (SIM) is a semi-synthetic compound, isolated initially from *A. terreus*. This drug disrupts the synthesis of cholesterol and has medical applications, being one of the most used drugs globally. SIM acts on the enzyme HMG CoA reductase (3-hidroxy 3-methy-glutaril-CoA reductase), which also acts in the formation of ergosterol (Macreadie et al., 2006; Menezes et al., 2012). In this manner, the use of SIM as an antifungal drug has been studied (Nyilasi et al., 2010; Nyilasi et al., 2014).

Nanotechnology provides a means to develop new antifungal compounds to diminish the impact of emerging resistant fungi (Hahn, 2014; Markoglou et al., 2008). Nanoparticles, such as silver nanoparticles (AgNPs), have been used for a long time as antimicrobial agent and are highly effective against fungal pathogens (Auyeung et al., 2017; Hernández-Sierra et al., 2008; Kim et al., 2008). AgNPs have many mechanisms of action including linkage to phosphate groups in the DNA (Gupta et al., 1998; Matsumura et al., 2003), interaction with the plasma membrane leading dissipation of protons and cell death (Lok et al., 2006; Sondi and Salopek-Sondi, 2004), reacting with sulfhydryl groups of proteins and enzymes, interfering with the electron transport chain, disrupting membrane permeability to protons and phosphate groups (Feng et al., 2000). Myconanotechnology has the potential to produce nanoparticles easily without generating hazardous waste. Cell-free filtrate of *Fusarium oxysporum* has been used to produce stable AgNPs without generating toxic compounds which would cause damage to the environment (Durán et al., 2005). AgNPs synthesized by a strain of *Aspergillus flavus* showed antibacterial action and antifungal properties against *Trichoderma* spp. (Fatima et al., 2016).

AgNPs have been used extensively over the last years as antimicrobial agents in food storage, medical devices, clothes, paints, health industry and cosmetics (Backx et al., 2018; Kokura et al., 2010; Kumar et al., 2008). However, evidence of toxicity of silver nanoparticles to human health is still not clear. The use of protective coatings with silver AgNPs for packaging presents a great potential food industry, increasing the shelf life of food products (Echegoyen and Nerín, 2013; Tavakoli et al., 2017). However, there is a need to better understand if ingested AgNPs poses a risk to human health (McClements and Xiao, 2017). It is not possible to make a single general generalization about the safety of all nanoparticles, so it remains unclear whether nanoparticles used in food packaging may migrate into the food as it depends on the nature and size of nanoparticles (Gaillet and Rouanet, 2015; Sharma et al., 2014).

The aim of this study was to evaluate the antifungal activity of biogenic AgNPs produced by using cell-free filtrate from *F. oxysporum* and SIM alone and in combination against toxigenic *Aspergillus* species. Additive or synergistic effects for *A. flavus* and *A. parasiticus* (respectively) were observed. These compounds had low minimum inhibitory concentrations and prevented biofilm formation. Finally, analysis by scanning electronic microscopy revealed that both SIM and biogenic AgNPs compromised the germination of spores from *A. flavus* and *A. ochraceus*.

2. Material and methods

2.1. . Microorganism cultivation and storage

The microorganisms used in this study were toxicogenic species of filamentous fungi: *Aspergillus flavus* MCT 00335, *Aspergillus nomius* MCT 00328, *Aspergillus parasiticus* MCT 00336, which produce aflatoxins. *Aspergillus melleus* MCT 00144 and *Aspergillus ochraceus* MCT 00435, producers of ochratoxins, were kindly provided by Fiocruz from the Mycological Collection of *Trichocomaceae* in Manguinhos, Rio de Janeiro, Brazil. The species were grown on Sabouraud Dextrose Agar (Himedia®, Mumbai, India) and incubated for seven days at 30 °C. After cultivation, all species were stored in 40% glycerol (a cryoprotectant) and stored at −20 °C.

2.2. Antifungal agents

2.2.1. Simvastatin

SIM was obtained commercially (Henan Topfond Pharmaceutical Co, China) in the form of a pro-drug and was activated by hydrolysis in NaOH to 0.25 M at 37 °C for 1 h. The pH was adjusted to 7.4 with HCl 0.25 M and then stored at −20 °C at the concentration of 5000 μg/mL (adapted from Lorenz and Parks, 1990).

2.2.2. Silver nanoparticles (AgNPs)

The AgNPs were obtained biologically as described previously, with adaptations (Durán et al., 2005). The fungus *Fusarium oxysporum* was grown on Malt agar (Difco®, Sparks, USA) containing 5% yeast extract for seven days at 30 °C. Then, the fungal biomass was scrapped from the plate using a sterile wire loop. Ten grams of fungal biomass were added to 100 mL of sterile distilled water. Then, the suspension was incubated with agitation (150 rpm) for 72 h at 30 °C. The biomass was then filtered using a vacuum (paper filter Qualy 9.0 cm in diameter, 80 m^{-2} weight, 205 μm thick, 14 μm pores), and silver nitrate (Sigma Aldrich®, St Louis, MO, USA) was added to the supernatant to a final concentration of 10^{-3} M. The suspension was kept in the dark for 14 days at 30 °C for formation of AgNPs. The production of AgNP's is performed by an enzymatic reductive activity of nitrate reductase, according to Durán et al., 2005.

2.3. Characterization of biological AgNPs

At regular intervals, aliquots of the suspension were removed, and the absorbance was measured in a spectrophotometer UV–Vis at 440 nm (Fig. 1A) and X-ray diffraction (XRD) pattern was measured in XDR 7000 model (Shimadzu, Japan) (Fig. 1B). The biological nanoparticles were characterized by transmission electron microscopy (Fei Tecnai 12) (Fig. 2). The average size of nanoparticles was 90 nm. The zeta potential (measured using the equipment Potencial Zeta Matec/Zeta APS, USA) gave values higher than +30 mV or less than −30 mV, showing stable nanoparticles with a reduced tendency to aggregate.

2.4. Agar diffusion tests

2.4.1. Agar disk diffusion method

The tests of diffusion in agar were performed in accordance with the document M51-A2 of the *Clinical Laboratory Standard Institute* (CLSI, 2010), with adaptations. The species were initially grown in Sabouraud Dextrose Agar (Himedia®, Mumbai, India) and incubated at 30 °C for seven days. The fungal suspension was prepared in sterilized PBS (phosphate buffered saline). The inoculum was adjusted to 10^5 spores/mL after counting in a Neubauer chamber. 10^4 spores were uniformly distributed on agar plates containing Agar Sabouraud Dextrose (Himedia®, Mumbai, India).

Paper disks of 6-mm diameter (Laborclin, Pinhais, Brazil) were impregnated with 10 μL SIM at concentrations ranging from

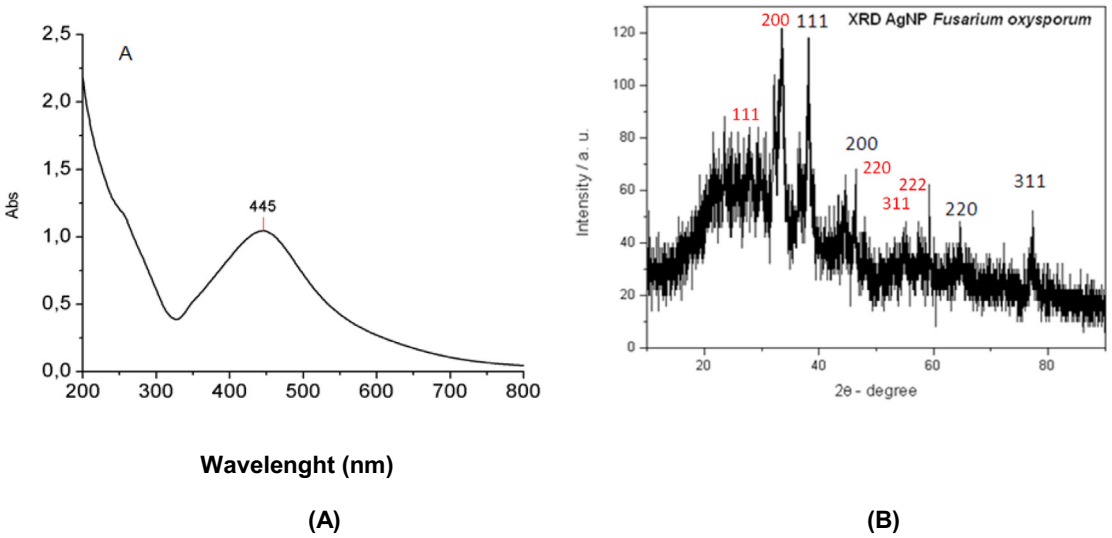

Fig. 1. Absorption spectrum of UV–Vis (A) and (B) XRD pattern of AgNPs.
(Note: In (B) the black numbers corresponding to AgNPs and in red AgCl in low concentration). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

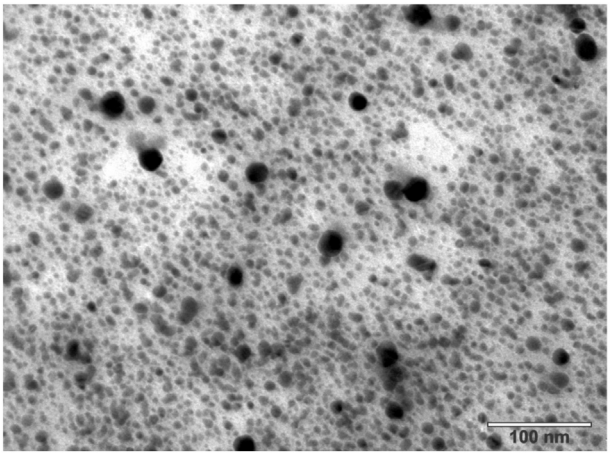

Fig. 2. Characterization by Transmission Electron Microscopy of AgNPs (viewed as black spheres) synthesized by the fungus *Fusarium* oxysporum.

Table 1
Evaluation of SIM antifungal activity at concentrations of 6.25–50 μg/disk in agar disk diffusion against five species of *Aspergillus*.

Inhibition zone in diameter (mm)				
Species	SIM concentration (μg/disk)			
	6.25	12.5	25	50
A. flavus	10 ± 1.89	14.6 ± 0.95	> 30	> 30
A. melleus	ni	9,7 ± 1.12	14.9 ± 0.04	20.1 ± 0.6
A. nomius	ni	ni	16.1 ± 0.8	22.5 ± 3.1
A. ochraceus	ni	14.5 ± 3	15.1 ± 1.05	21.3 ± 1.8
A. parasiticus	ni	ni	5.7 ± 1.2	10.1 ± 0,007

Agar disk-diffusion method. Agar plates were incubated at 30°C for 48 h.
ni: no inhibition.

6.25–50 μg/mL and placed on the surface of the culture plates, which were then incubated at 30 °C. After 48 h of incubation, the inhibition diameter of halos was measured.

2.4.2. Agar well diffusion method

Sabouraud Dextrose Agar plates were streaked by a cotton swab with 10^4 fungal spores and allowed to dry for 1 min. Agar plates were punched with a sterile 66 mm mm cork borer and 50 μL of SIM was added, in concentrations ranging between 0,04–250 μg/mL. The plates were incubated at 30 °C, and the inhibition halos were measured after 48 h of incubation.

2.5. Broth microdilution assays

2.5.1. Minimum inhibitory concentration (MIC) in 96-well microtiter plate

The minimum inhibitory concentration (MIC) was determined in triplicate from broth microdilutions in a 96-well plate according to the document M38-A2 of the Clinical Laboratory Standard Institute, with adaptations (CLSI, 2008). Different concentrations of SIM (2.44 μg/mL to 1250 μg/mL) and AgNPs (0.26 μg/mL to 135 μg/mL) were added. The species were initially grown in Sabouraud Dextrose Agar and incubated at 30 °C for seven days. The fungal suspension was prepared in PBS. After counting using the Neubauer chamber cell counting, the inoculum was adjusted to 10^4 spores/mL in medium RPMI 1640 (with glutamine (0.3 g/L) and phenol red (0.0053 g/L), without sodium bicarbonate – Sigma Aldrich®, St Louis, MO, USA) and buffer MOPS (3 – (N-Morpholino) propanesulfonic acid) (Inlab, São Paulo, Brazil), pH 7.2 to 7.4.100 μL aliquots of fungal suspension were added to each well. The plate was incubated for 48 h at 30 °C before a visual reading was performed. To estimate the interaction between the SIM and AgNPs, the fractional inhibitory concentration index (FICI) was determined according with Chin et al. (1997), following the equation:

$$FICI = \frac{MIC\ (SIM\ combination)}{MIC\ (SIM\ alone)} + \frac{MIC\ (AgNPs\ combination)}{MIC\ (\ AgNPs\ alone)}$$

FICI < 0.5 means that there is synergism; between 0.5 and 1.0 additive effect; between 1.0 and 2.0 means no interaction; and > 4 means antagonism.

2.5.2. Interaction between the compounds (checkerboard) in a 96-well microtiter plate

After determination of the MICs, SIM and AgNPs were also analyzed in combination against fungal growth. In 96-well microplates, SIM was serially diluted in the horizontal direction in concentrations varying from 0.305 to 625 μg/mL, while AgNPs were added in a vertical direction at concentrations ranging from 1 to 135 μg/mL. Both SIM and AgNPs were diluted in RPMI 1640. The species were grown in Sabouraud Dextrose Agar and incubated at 30 °C for seven days. The

Table 2
Evaluation of SIM antifungal activity at concentrations of 3.9–250 μg/well in agar well diffusion against five species of *Aspergillus*.

Inhibition Zone in Diameter (mm)							
Species	SIM Concentration (μg/well)						
	3.9	7.8	15.6	31.25	62.5	125	250
A. flavus	11 ± 1.6	15 ± 1.9	21.3 ± 3.5	26 ± 0.04	> 30	> 30	> 30
A. melleus	ni	ni	6.7 ± 0.77	15.9 ± 0.5	21.7 ± 1.9	> 30	> 30
A. nomius	ni	ni	14 ± 0.74	18 ± 1.2	25.8 ± 0.7	> 30	> 30
A. ochraceus	ni	ni	14 ± 0.97	15.6 ± 2.3	23 ± 0.67	> 30	> 30
A. parasiticus	ni	ni	13 ± 0.8	14.6 ± 2	18.6 ± 0.9	> 30	> 30

ni: no inhibition.

Table 3
Minimum inhibitory concentration and fractional inhibitory concentration index of simvastatin (SIM) and silver nanoparticles (AgNPs) isolated and in interaction against species of *Aspergillus*.

MIC (μg/mL) and compounds interaction				
Species	Antifungals	Compounds Alone	Compounds Combination	FICI
A. flavus	SIM	78	19.5	0.5
	AgNPs	8	2	
A. melleus	SIM	19.5	0.305	1
	AgNPs	4	4	
A. nomius	SIM	78	19.5	0.75
	AgNPs	8	4	
A. ochraceus	SIM	19.5	9.75	0.75
	AgNPs	4	1	
A. parasiticus	SIM	78	19.5	0.5
	AgNPs	8	2	

FICI ≤ 0.5 indicates synergy; FICI ≤ 1.0 indicates additive effect; FICI < 2.0–4.0 indicates no interaction; FICI ≥ 4.0 represents an antagonistic effect.

Table 4
Minimum inhibitory concentration and minimum fungicidal concentration of simvastatin (SIM) and silver nanoparticles(AgNPs) against five species of *Aspergillus*.

Minimum inhibitory concentration/minimum fungicidal concentration (μg/mL)		
Species	MIC/MFC SIM	MIC/MFC AgNPs
A. flavus	78/312	8/64
A. melleus	19.5/78	4/16
A. nomius	78/312	8/32
A. ochraceus	19.5/78	4/16
A. parasiticus	78/312	8/32

MFC: Minimum Fungicidal Concentration, MIC: Minimum inhibitory concentration,
SIM: Simvastatin, AgNPs: Silver nanoparticles.

fungal suspension was resuspended in PBS pH 7.4. The concentration of spores was determined by counting in a Neubauer chamber. The fungal suspensions containing 10^4 spores were then diluted in RPMI 1640 (RPMI 1640 (with glutamine (0.3 g/L) and phenol red (0.0053 g/L), without sodium bicarbonate – Sigma Aldrich®, St Louis, MO, USA) and added to buffer MOPS pH 7.4 (Inlab, São Paulo, Brazil). Aliquots of 100 μL were distributed across the board, except for the negative control. The plate was incubated for 48 h at 30 °C before a visual reading was performed.

2.6. Determination of minimum fungicidal concentration (MFC)

The minimum fungicidal concentration was determined according to the protocol described by Espinel-Ingroff et al. (2002). 20 μL of each well with complete inhibition of fungal growth was withdrawn and cultured in plates with Sabouraud Dextrose Agar for 72 h at 30 °C. The MFC was defined as the lowest drug dilution that yielded fewer than three colonies or complete absence of growth.

2.7. Inhibition of biofilm formation by AgNPs and SIM

The effect of the compounds in the formation of biofilms was analyzed in accordance with the protocol of Jin et al. (2003), with adaptations. The fungi were cultured in Sabouraud Dextrose Agar for seven days at 30 °C. A suspension containing 10^4 spores/mL was prepared. Concentrations of the compounds based on MIC were prepared, ranging from 9,75–625 μg/mL for SIM and 1–8 μg/mL for AgNPs. Aliquots of 100 μL of each compound, alone and in combination and 100 μL of cell suspension (containing 10^4 spores) were distributed in 96-well plate and incubated at 30 °C for 24 h.

After 24 h the contents of the wells were aspirated, and the wells were washed twice with PBS for the removal of cells that was not adhered. After cell adhesion to the plate, the drugs were added along with the medium, at the concentrations mentioned above. The plate was incubated for 72 h at 30 °C, it was removed all the contents from the wells, washed twice with PBS and left it drying at room temperature for 45 min. After this step, the biofilm was stained by adding in each well 110 μL of crystal violet 0.4%. Finally, the biofilm was washed four times with 300 μL/well of ultrapure sterile water and discolored with 200 μl/well of ethanol 95% for 45 min. Then, 100 μL of bleach solution were transferred to a new 96-well plate. The reading was performed at 470 nm (Bio Stack Ready). It was subtracted the value obtained for negative control in each sample for standardization of results.

2.8. Scanning electron microscopy (SEM)

For SEM, suspensions of *A. flavus* and *A. ochraceus* (10^4 spores) with and without the antifungals compounds (values found in the CIM) were placed in sterile slides, in a 24-well plate and incubated for 24 h. After incubation, the culture medium was removed, and each plate was fixed with glutaraldehyde 3% (Sigma Aldrich®, St Louis, MO, USA), paraformaldehyde 2% (Sigma Aldrich®, St Louis, MO, USA) in buffer solution of sodium cacodylate buffer 0.1 M at pH 7.2, for 16–18 h. After this period, all plates were rinsed in sodium cacodylate buffer solution 0.1 M at pH 7.2 (Sigma Aldrich®, St Louis, MO, USA) for 10 min, repeating three times. The buffer was withdrawn, and the samples were dehydrated in an ascending ethanol series (70, 80, 90, 100% ethanol) for 10 min, for three times in each concentration. Then, plates were taken to a critical point in CO_2 (BALTEC CPD 030 Critical Point Dryer), mounted on “stubs” and coated with gold (BALTEC SDC 050 Sputter Coater). Finally, the plates were examined under Scanning Electron Microscope (FEI Quanta 200, Netherlands).

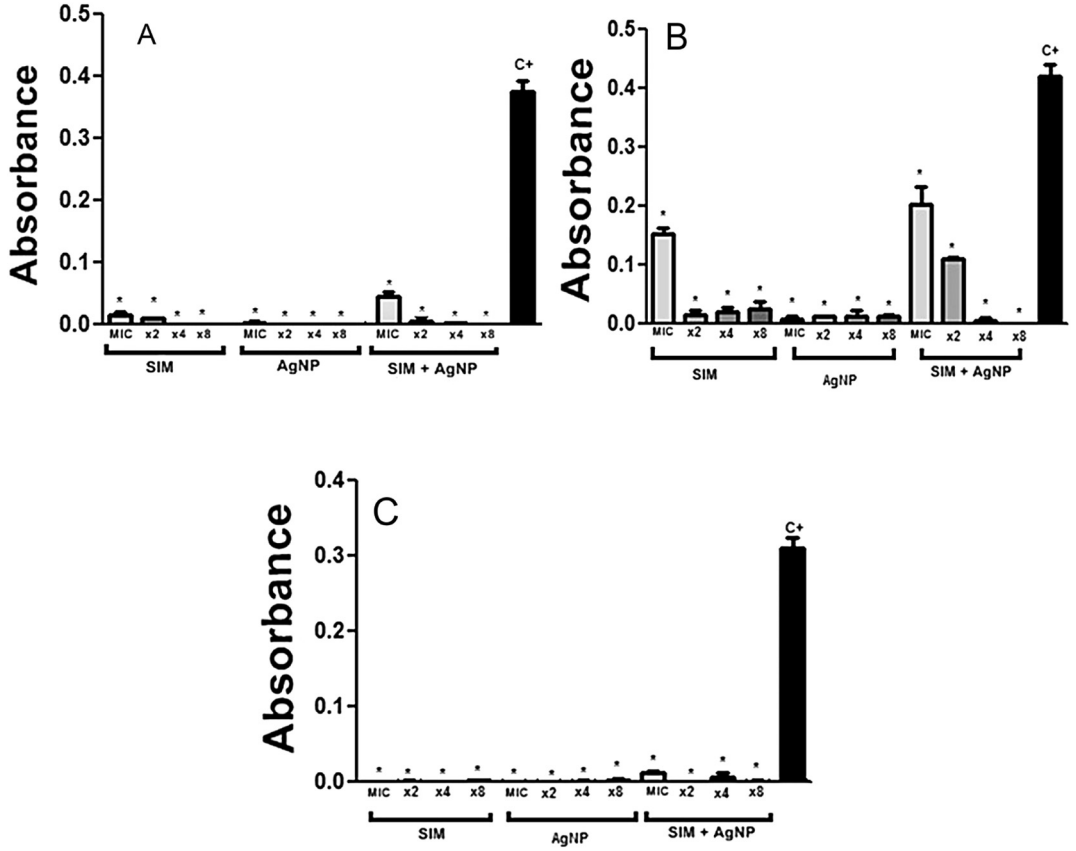

Fig. 3. *In vitro* effect of SIM, AgNPs isolated and in interaction using different concentrations (MIC, 2×, 4× and 8×) against biofilm formation by *Aspergillus flavus* (A); *Aspergillus nomius* (B); *Aspergillus parasiticus* (C); *B*lack bar represents the positive control. Wavelength 470 nm. *: represents a statistically significant difference ($p < 0.05$) compared with the positive control.

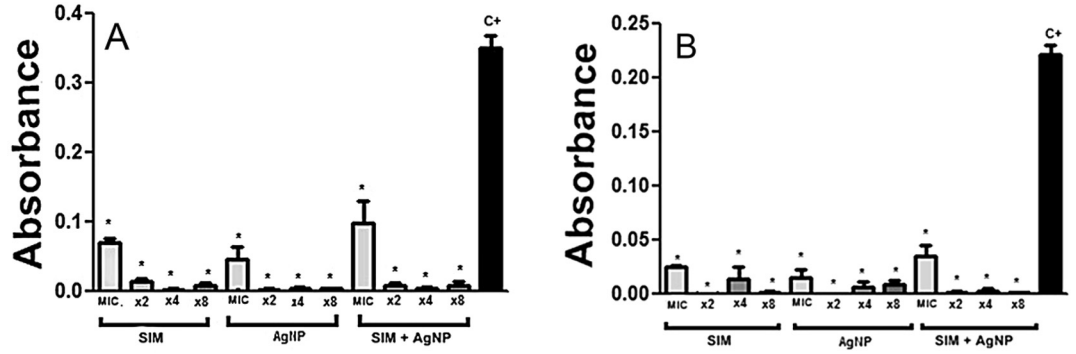

Fig. 4. *In vitro* effect of SIM, AgNPs isolated and in interaction using different concentrations (MIC, 2×, 4× and 8xMIC) against biofilm formation by *Aspergillus melleus* (A); *Aspergillus ochraceus* (B). *B*lack bar represents the positive control. Wavelenght 470 nm. *: represents a statistically significant difference (p < 0.05) compared with the positive control.

2.9. Statistical analysis

The results obtained are expressed as mean ± SD. The assays were performed in triplicate. Analysis of variance (ANOVA) was followed by Tukey's test using the software GraphPad Prism 5. Significance level was set for $p < 0.05$.

3. Results and discussion

3.1. Characterization of AgNPs

The UV–Vis spectrum showed the plasma absorption at 440 ηm (Fig. 1A) and X-ray diffraction (XRD) pattern (Fig. 1b) showed the typical Bragg reflections at 2Θ = 39.18, 44.81, 65.40 and 77.59, which correspond to the XRD pattern of indexed [111], [200], [220], [311], and [222] facets of AgNPs (Durán et al., 2016).

In this case also small contribution in the XRD pattern in 2θ peak appeared values on 27.9°, 32.3°, 46.3°, 55.0°, 57.6°, corresponding to the indexed (111), (200), (220), (311), (222) as mainly diffraction corresponding to facets of AgCl nanoparticles. Then, the AgNPs is the main product in the biogenic synthesis and a small contribution of AgCl nanoparticles. The biological nanoparticles were also characterized by Transmission Electron Microscopy (Fig. 2) showing spherical nanoparticles. The average size of nanoparticles was 93 ± 11 nm and zeta potential of −37,1 ± 2,6 mV were found (not shown).

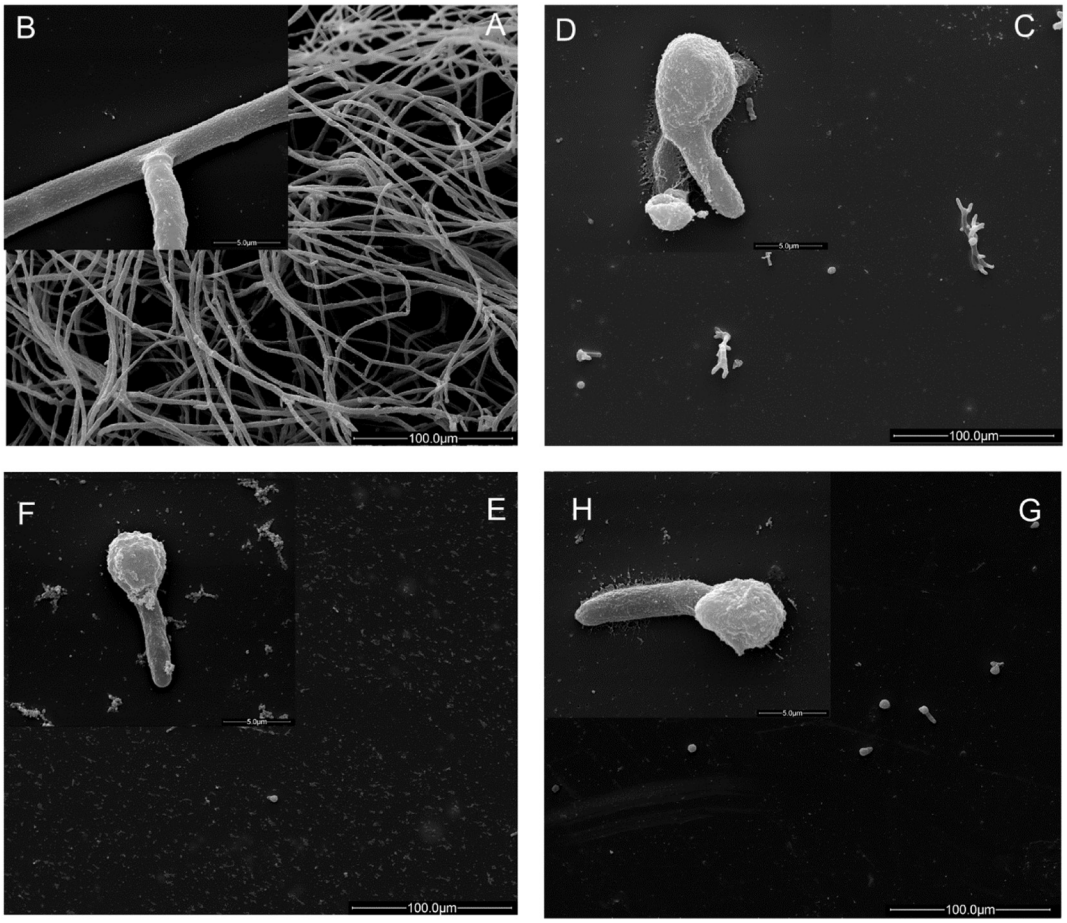

Fig. 5. Scanning electron microscopy of antifungal effect of SIM, AgNPs and interaction of compounds against *Aspergillus flavus* after 24 h of treatment. A (increased by 800x) and B (increased by 12kx): control group, without treatment; C (800x) and D (12kx): treatment with 78 µg/mL (MIC) of SIM; E (800×) and F (12kx): treatment with 8 µg/mL (MIC) of AgNPs; G (800x) and H (12kx): associated treatment with 19.5 µg/mL (MIC) of SIM and 2 µg/mL (MIC) of AgNPs.

3.2. Antifungal activity in agar diffusion

As shown in Tables 1 and 2, SIM was able to inhibit fungal growth. Using the disk diffusion method, it was possible to observe growth inhibition of *A. melleus* and *A. ochraceus* at concentrations of 50 µg/mL and 12.5 µg/mL, respectively. For A. *nomius* and *A. parasiticus*, there halo inhibition occurred at concentrations of 50–25 µg/disk, respectively. *A flavus* showed the greatest sensitivity to SIM, which inhibited growth at concentrations of 12.5–6.25 µg/disk. Qiao et al. (2007) showed that a higher concentration (200 µg/disk) was needed to inhibit *A. flavus* growth. Inhibition of growth of *A. flavus* was achieved at a concentration of 3.9 µg/well, while for the other fungal species (*A. ochraceus*, *A. parasiticus*, *A. nomius* and *A. melleus*), inhibition of growth was observed at 15.6 µg/well.

3.3. Determination of minimum inhibitory concentration of SIM and AgNPs and interaction of compounds

Using broth microdilution, all species demonstrated sensitivity to the SIM and AgNPs, as shown in Table 3. The MIC for SIM was 78 µg/mL for *A. flavus*, *A. nomius* and *A. parasiticus* and 19.5 µg/mL for *A. ochraceus* and *A. melleus*. The MIC for SIM was 8 µg/mL for *A. flavus*, *A. nomius* and *A. parasiticus* and 4 µg/mL for *A. ochraceus* and *A. melleus*.

The MIC results for AgNPs revealed a high susceptibility of the toxigenic species of the genus *Aspergillus* to this compound. Mousavi and Pourtalebi (2015) found much higher MIC levels for AgNPs against *A. parasiticus*. Kim et al. (2008) obtained an MIC of 1–7 µg/mL for AgNPs against *Candida* species and *Trichophyton mentagrophytes*. In our study, the values were between 4 and 8 µg/mL for *Aspergillus* species. Interaction of the compounds promoted a synergistic activity for the species of *A. flavus* and *A.parasiticus* and additive activity for the other species, located in Table 3. Synergistic activity, when the compounds are interacted, reduced the initial inhibitory concentration 4 times for *A. flavus* and *A. parasiticus*. The other species of toxigenic fungi did not occur an interaction equal or greater to 4 times, promoting an additive effect. It is known that mycotoxin production increases if fungi are under sub-inhibitory concentrations of antifungal compounds, but it was not addressed in this work if concentrations of AgNPs or SIM below the MIC have impact on mycotoxin production.

3.4. Minimum fungicidal concentration (MFC)

The minimum fungicidal concentrations of compounds were compared to the previously obtained MICs. The MFC of SIM for *A. flavus*, *A. nomius* and *A. parasiticus* was 312 µg/mL (Table 4). Compared to the MIC (78 µg/mL), fungicidal effects required a 4-fold greater quantity of SIM. For *A. melleus* and *A. ochraceus*, MFC obtained was 78 µg/mL of SIM, a value also 4-fold higher than that found in the MIC. Regarding the AgNPs, the MFC was 8-fold greater than the MIC for *A. flavus*. For *A. nomius*, *A. parasiticus*, *A. melleus* and *A. ochraceus*, concentrations that were four times higher were required to reach the MFC.

3.5. Effect of AgNPs and SIM on biofilm formation

SIM inhibited biofilm formation of *A. flavus* at concentrations of 4–8× MIC and significantly decreased biofilm formation at

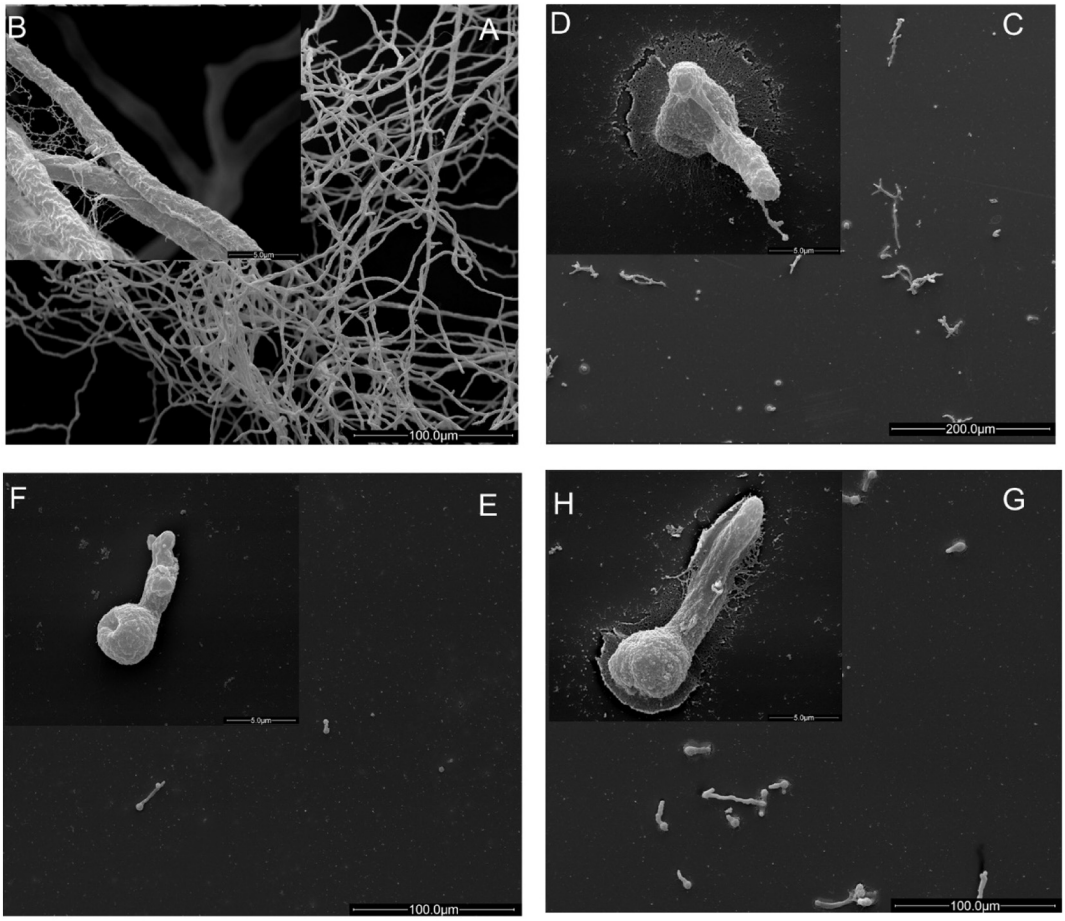

Fig. 6. Scanning electron microscopy of antifungal effect of SIM and AgNPs and interaction of compounds against *Aspergillus ochraceus* after 24 h of treatment. A (increased by 800x) and B (increased by 12kx): control group, without treatment; C (400x) and D (12kx): treatment with 19.5 µg/mL (MIC) of SIM; E (800x) and F (12kx): treatment with 4 µg/mL (MIC) of AgNPs; G (800x) and H (12kx): associated treatment with 9.75 µg/mL of SIM and 1 µg/mL of AgNPs (MIC).

concentrations of 2× MIC. Lara et al. (2015) and Liu et al. (2009) showed similar effects on *C. albicans*. The combination of SIM and AgNPs also prevented biofilm formation (Fig. 3A).

When SIM and AgNPs were combined, there was a significant reduction in the formation of biofilm, where the concentration of 8× MIC from the interaction of the compounds was able to inhibit biofilm formation (Fig. 3B). All tested concentrations of either SIM or AgNPs alone or in combination were able to inhibit biofilm formation by *A. parasiticus* (Fig. 3C).

Regarding the action of SIM against *A. melleus* biofilms, was found that the drug inhibits the formation of biofilms at the MIC and significantly reduced biofilm formation at concentrations of MIC, 2× and 8× MIC. AgNPS at concentrations 2×–8× MIC were able to inhibit biofilm formation, and the concentration equal to the MIC decreased biofilm formation. Using both compounds, the concentrations 2× and 8× MIC significantly decreased the formation of biofilm (Fig. 4A).

For *A. ochraceus* (Fig. 4B), all concentrations used were able to reduce progress of biofilm and some concentrations (2× MIC SIM; 2× MIC AgNPs) inhibited the formation of biofilm.

3.6. Scanning electron microscopy (SEM) of *A. flavus* and *A. ochraceus*

SEM analysis revealed that SIM and AgNPs caused severe damage to hyphae of *A. flavus* and *A. ochraceus* (Figs. 5 and 6). At the concentration of 78 µg/mL of SIM, was observed abnormal hypha formation by *A. flavus*, compared to control. In addition, the few spores that germinated made deformed and short hyphae with bizarre configurations and multiple short lateral branches (Fig. 5C). Conidial germination gives rise to germinative tubes with a rough surface. When exposed to AgNPs at a concentration of 8 µg/mL (Fig. 5E), was observed a conidial germination with short hyphae with little branching. An effect of AgNPs on spores (Fig. 5F) was observed, including roughness on the surface. When the compounds were combined (19.5 µg/mL of SIM + 2 µg/mL of AgNPs), was observed a more pronounced effect on spore germination with the presence of germinative tubes and without the formation of hyphae. Spore germination was severely compromised and hyphae formation was completely inhibited by treatment with a combination of SIM and AgNPs (Fig. 5H).

The effects of SIM and AgNPs on *A. ochraceus* were similar to those found on *A. flavus*. SIM (19 µg/mL) caused a marked decrease in the number of hyphae and abnormal filamentation, with hyphae showing irregular growth patterns and short and distorted hyphae (Fig. 6C). On the germinative tube was observed a very rough and fractured surface (Fig. 6D). Treatment with AgNPs at 4 µg/mL resulted in a significant decrease in spore germination and caused formation of unbranched and short hyphae (Fig. 6E). Silver nanoparticles also damaged hyphae of *Trichosporon asahii* in a similar way (Xia et al., 2016). However, in the present study AgNPs imposed a greater damage on fungal cells, preventing hyphae elongation and having a better effect on inhibition of germination than SIM. When the treatment was combined (9.75 µg/mL of SIM + 1 µg/mL of AgNPs), there was a decrease in spore germination compared to tests with AgNPs alone, despite the lower concentration of AgNPs. This fact can be explained by the small amount of silver in combination, which is additive but non-synergistic (contrary to what was observed in *A. flavus*, when there is synergism). The compounds (Fig. 6H) caused deformations in spores and loss of extracellular matrix.

AgNPs exhibited greater effects than SIM, presenting greater inhibition on spore germination. When SIM and AgNPs are combined, the effect of AgNPs is optimized, maybe because as an ergosterol synthesis inhibitor, SIM disrupts fungal cell membranes, allowing the entry of AgNPs.

4. Final remarks and conclusion

The present study encompasses the activity of biogenic AgNPs and SIM against five *Aspergillus* species. Alone or in combination, these compounds exhibited antifungal action, having additive or synergistic effect, depending on the fungal species. There is a promising use of AgNPs and SIM as potential substitutes for agrochemicals and food preservatives. The observed AgNPs-induced alterations in multiple cellular targets are crucial to avoid emergence of antifungal resistance. However, the use of AgNPs and SIM as safe preservatives must be more investigated, due to the controversy over toxicity by intake of metallic nanoparticles.

Conflict of interest

The authors declare that they have no conflict of interests.

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