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Research Article

Effect of a Silver Nanoparticles Solution on *Staphylococcus aureus* and *Candida* spp.

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An AgNPs solution was synthesized by chemical reduction, characterized, and tested against *Candida glabrata*, *Candida tropicalis*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Minimum inhibitory (MICs) and minimum fungicidal/bactericidal concentrations (MFC/MBC) were determined on planktonic cells. Also, total biofilm mass was determined by crystal violet (CV) staining and morphological changes by scanning electron microscope (SEM). MICs for *C. glabrata*, *C. tropicalis*, *S. aureus*, and MRSA were 15.63, 3.91, 1.95, and 1.95 μg/mL, respectively. MFC for *C. glabrata* was 62.5 μg/mL and for *C. tropicalis* 15.63 μg/mL The same MBC (3.91 μg/mL) was observed for *S. aureus* and MRSA. CV assay showed that the AgNPs (1000 μg/mL) promoted reductions in biofilm mass of ~60% for *C. glabrata* and ~35% for *C. tropicalis*. A reduction of ~20% in *C. tropicalis* biomass was also observed at the concentration of 3.91 μg/mL. No significant effect on total biomass was found for *S. aureus* and MRSA. SEM images revealed that *C. glabrata* and *C. tropicalis* biofilm cells, exposed to the AgNPs (1000 μg/mL), had an irregular and shriveled appearance. AgNPs solution exhibited considerable antimicrobial activity against important fungal and bacterial pathogens, associated with several oral and systemic diseases, and has potential as an antimicrobial agent.

1. Introduction

The oral cavity contains numerous types of microorganisms [1] that have the ability to attach to both biotic and abiotic surfaces, such as the denture base materials. Glass et al. [2] evaluated the spectra of microorganisms residing on the surfaces and depths of complete maxillary and mandibular dentures. A total of 2292 isolates were obtained from the dentures, among them *Candida albicans, Candida glabrata*, *Candida tropicalis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. Denture surfaces and the oral cavity have also been reported as important reservoirs of methicillin-resistant *Staphylococcus aureus* (MRSA), a strain of *S. aureus* that is highly resistant to many antibiotics [3, 4]. This resistance is associated with the gene *mecA*, which encodes an extra penicillin-binding protein (PBP2A). This protein, due to its extremely low affinity to all β-lactam antibiotics, provides the transpeptidase activity, which is essential to the cell wall peptidoglycan synthesis [4].

Oral microorganisms can cause local infections such as *Candida*-associated denture stomatitis that may affect 65% to 80% of denture patients [2]. Despite the fact that one of the main etiological factors of this disease is *C. albicans*, other species, such as *C. glabrata*, *C lusitaniae*, *C parapsilosis*, and *C. tropicalis* [2, 5], may also be involved. *C. glabrata* represents the second most prevalent fungal pathogen and exhibits innate resistance to azole-based drugs, and, for *C. tropicalis*, acquired resistance to these drugs has also been reported [6]. In addition to the local disease, *Candida* may penetrate the epithelium and invade the host tissues, resulting in systemic

infections. Invasive candidiasis (the invasion of *Candida* spp. in a human organ) and candidemia (invasion via the bloodstream) have shown an increasing incidence in the nosocomial setting [7]. Although *C. albicans* ranks first in isolation frequency of candidemia [8], the non-*albicans* species *C. tropicalis* and *C. glabrata* have been isolated in blood cultures [9]. Bacterial species (*Staphylococcus epidermidis*, *Enterococcus* spp., and *S. aureus*) were also coisolated from 372 patients with candidemia [10]. In addition, *C. albicans* may serve as a scaffold for the attachment of *S. aureus*, which is considered one of the most pathogenic human bacteria, and also contribute to its vancomycin resistance [11].

Systemic infections are difficult to treat and the mortality for invasive candidiasis and candidemia ranges between 36% and 63% depending on patient population [7]. Bacteremia due to MRSA has also been associated with increased mortality compared with methicillin-susceptible *S. aureus* bacteremia [12]. Gasch et al. [13] evaluated 579 episodes of MRSA bloodstream infection and reported that mortality was observed in 179 (32%) episodes. Thus, the occurrence of these systemic infections has become an important and growing public health problem worldwide and the development of new antimicrobial agents is needed. In this context, nanotechnology has contributed significantly to advances in health research and nanoparticles have attracted great scientific interest, particularly silver-containing nanoparticles (AgNPs). Because AgNPs use multiple mechanisms to kill and/or inhibit growth of microorganisms, they represent one of the most promising strategies for overcoming microbial resistance [14]. It has been reported that AgNPs are efficient antimicrobial agents against the growth of a broad spectrum of bacterial and fungal species [15–22]. In our previous study [23], an AgNPs solution was synthesized by chemical reduction method and characterized, and its activity against *C. albicans* was demonstrated. In this investigation, the biocidal action of the AgNPs solution against other important fungal (*C. glabrata and C. tropicalis*) and bacterial (*S. aureus* and MRSA) pathogens was further evaluated. The antibacterial properties of the AgNPs solution were investigated against both planktonic (free-floating) cells and biofilms.

2. Materials and Methods

2.1. Preparation and Characterization of AgNPs Solution. The AgNPs solution was synthesized by chemical reduction method and characterized as described in our previous publication [23]. Briefly, silver nitrate ($AgNO_3$, 90 mg mL^{-1}) was reduced by sodium borohydride ($NaBH_4$, 540 mg mL^{-1}) in the presence of PVA (230 mg mL^{-1}), and the molar ratio of $AgNO_3$ to PVA was maintained at 1 : 4. An absorption band at around 400 nm was observed in the UV-vis spectra of the solution, indicating the presence of spherical AgNPs [23]. The presence of PVA, which was used as the stabilizing agent to prevent agglomeration of nanoparticles, was confirmed in the FTIR spectra [23]. Broadening in the band from OH (3200–3400 cm^{-1}) and a significant decrease in the band at 840 cm^{-1} (out-of-plane CH vibration in PVA) suggested interactions between Ag and the OH groups from PVA. Absence of the band at about 1596 cm^{-1} indicated that the AgNPs influenced the vibration of the atoms of the polymer chains. The nanoparticles size was *c*. 9 nm, as determined by the DLS analysis [23].

2.2. Microbial Strains and Growth Conditions. Candida glabrata (ATCC 2001), *Candida tropicalis* (ATCC 4563), *Staphylococcus aureus* (ATCC 25923), and MRSA (ATCC 33591) were used in this study. The bacterial and fungal stock cultures were maintained at −70°C. During the experimental period, the microorganisms were maintained at 4–6°C, in appropriate medium (Sabouraud dextrose agar, SDA, for *C. glabrata* and *C. tropicalis*, and mannitol salt agar were for *S. aureus* and MRSA). Prior to MIC, MFC, and MBC testing, a loopful of each stock culture was streaked onto SDA (*C. glabrata* and *C. tropicalis*) or mannitol salt agar (*S. aureus* and MRSA) and incubated at 37°C for 48 h. From these young cultures, one loopful was transferred to 20 mL of yeast nitrogen base (YNB) medium with 50 mmol^{-1} glucose for the *Candida* cells or to tryptic soybean broth (TSB) medium for the *S. aureus* and MRSA cells and incubated at 37°C for 24 h, respectively. Yeast and bacteria cells of the resultant cultures were harvested and washed twice with phosphate buffered saline (PBS) (pH 7) at 3220 g for 5 min. Then, yeast and bacteria cells were resuspended in RPMI-1640 and TSB culture medium, respectively.

2.3. The In Vitro Antimicrobial Activity of the Synthesized AgNPs Solution. The antimicrobial activity of the AgNPs was evaluated against planktonic microbial strains by determining the minimum inhibitory concentrations (MICs) and minimum fungicidal/bactericidal concentrations (MFC/MBC). MIC and MBC/MFC were determined using 96-well microtiter plates, according to the microdilution method of the Clinical and Laboratory Standards Institute (CLSI) (document M27-A3, 2008, and document M7-A7, 2006) [24] with some modifications. For *Candida* spp., each well contained the microorganism at a final concentration of 2.5×10^3 cells/mL, RPMI-1640 culture medium, and the AgNPs solution. For bacteria, each well contained the cells at a final concentration of 2.5×10^5 cells/mL, TSB culture medium, and the AgNP solution. Inoculated RPMI-1640 or TSB culture medium (without the AgNPs solution) was included as positive control and uninoculated RPMI-1640 or TSB culture medium as negative control. The MIC values were determined by incubating for 24 h (*S. aureus* and MRSA) or 48 h (*Candida* spp.) at 37°C, exposed to serial 2-fold dilution in RPMI-1640 or in TSB culture medium of the AgNPs solution (from 1000 to 1.95 μg mL^{-1}). The MICs were defined as the lowest concentrations showing no growth by visual inspection. To establish MFC/MBC, aliquots from each well were removed and diluted serially 10-fold, and each dilution was plated, in duplicate, on SDA (for yeast) and mannitol salt agar (for bacteria). After 48 h of incubation at 37°C, the colony-forming units per millilitre (CFU mL^{-1}) were then determined. The MFC/MBC values were defined as the lowest concentration of the AgNPs solution resulting in no growth. These assays were performed in triplicate on three different occasions.

The influence of the AgNPs solution on the microbial biofilms was also evaluated by using the absorbance of the crystal violet staining to determine the total biomass of the biofilms. For this purpose, mature 48 h biofilms of *Candida* spp. and bacteria were formed in 96-well microtiter plates with suspensions of each microorganism spectrophotometrically standardized to 1×10^7 cells mL^{-1} in their respective culture media. In this study, tryptic soy broth medium (TSB, Acumedia Manufacturers, Inc., Baltimore, Maryland, EUA) was used to grow *Staphylococcus aureus* biofilms, while RPMI-1640 medium was used to grow *Candida* biofilms. The cell suspensions were placed into each well and incubated at 37°C in an orbital shaker at 75 rpm. After 90 min (adhesion phase), the loosely adhered cells were removed by washing twice with 200 μL of PBS to remove nonadherent cells. Wells were then filled with 200 μL of culture medium according to each microorganism, and the plates were incubated (37°C; 75 rpm) for 48 h. One hundred microliters of culture media were renewed after 24 h. Following biofilm formation (48 h), the medium was aspirated and nonadherent cells were removed by washing with 200 μL of PBS. Culture media according to each microorganism (200 μL) containing different concentrations (from 1000 to 1.95 μg mL^{-1}) of the AgNPs solution were then added to the preformed biofilms and maintained for 24 h. Biofilms incubated only with respective culture media were positive controls, while negative controls were wells containing only medium (without biofilms). To determine the total biomass, the biofilms were fixed with methanol and stained with 1% violet crystal solution for 5 min. The stain bonded to biofilms was dissolved in acetic acid at 33% and the optical density was measured (570 nm) using a spectrophotometer (Thermo Plate—TP Reader). These assays were performed in quadruplicate on two different occasions. Data obtained from total biomass were statistically analyzed by ANOVA and Tukey *post hoc* tests at 5% of significance.

2.4. Scanning Electron Microscopy. A scanning electron microscope (SEM) (JEOL JSM 6610LV) was used to observe the morphological changes of the microorganisms caused by AgNPs. For such purpose, the bottom of each well of the 12-well polystyrene plates, where the biofilms were formed, was extracted from the plate. The nonadherent cells were removed by washing in PBS and the cells were fixed in 2.5% glutaraldehyde for 24 hours at room temperature. Thereafter, they were dehydrated through a graded ethanol series (70, 85, 90, and 100%). Then, the sample from each group was sputter-coated with carbon.

3. Results

Planktonic MIC values for *C. glabrata*, *C. tropicalis*, *S. aureus*, and MRSA were 15.63, 3.91, 1.95, and 1.95 μg/mL, respectively. The MFC of the AgNPs solution for planktonic *C. glabrata* was 62.5 μg/mL and for *C. tropicalis* 15.63 μg/mL. The AgNPs solution also exhibited bactericidal effect against *S. aureus* and MRSA, with the same MBC value (3.91 μg/mL).

Results from the crystal violet assay (Figures 1 and 2) showed that the total biomass of *C. glabrata* was significantly

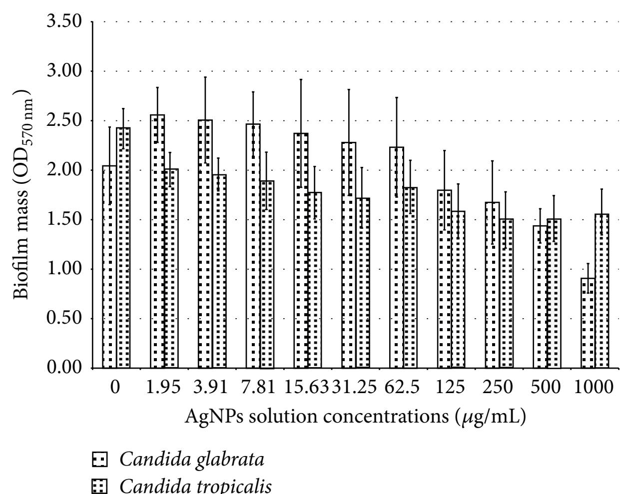


Figure 1: Absorbance values (570 nm) obtained with crystal violet staining assay for *Candida glabrata* and *Candida tropicalis* biofilms treated with different AgNps concentrations. Error bars are standard deviations.

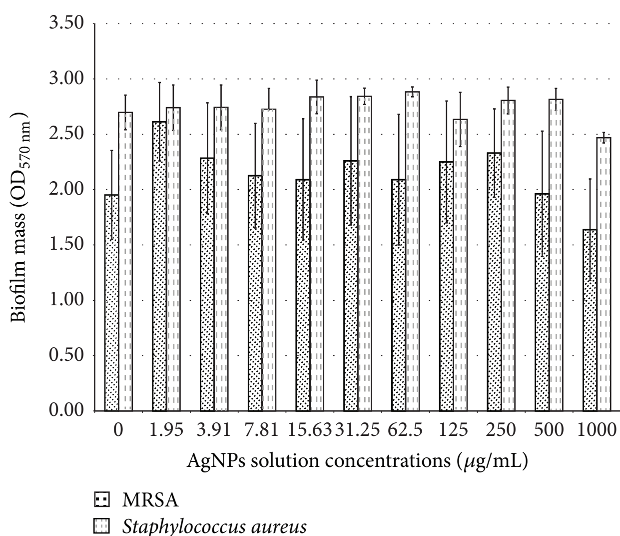
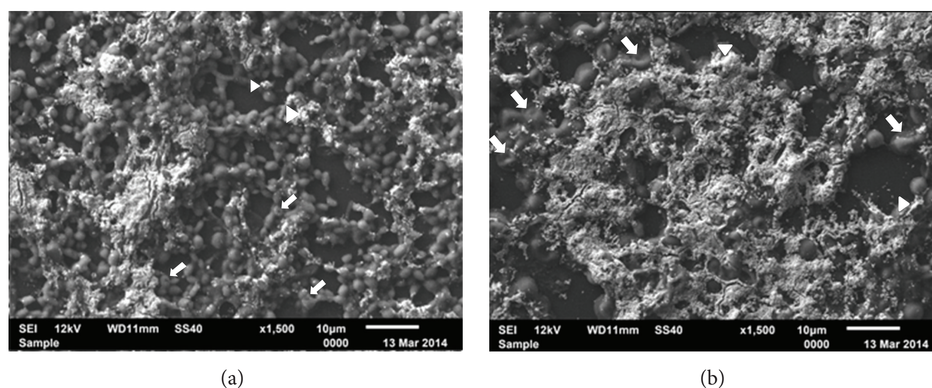


Figure 2: Absorbance values (570 nm) obtained with crystal violet staining assay for MRSA and *Staphylococcus aureus* biofilms treated with different AgNps concentrations. Error bars are standard deviations.

decreased by the AgNPs solution at a concentration of 1000 μg/mL compared to control (biofilm without AgNPs). For *C. tropicalis*, the AgNPs solution promoted a significant biomass reduction at concentrations greater than 3.91 μg/mL. The synthesized AgNPs solution had no significant effect on the total biomass for the bacterial strains evaluated (MRSA and *S. aureus*).

SEM analysis showed that, after exposure to the AgNPs (white arrowheads), the *C. glabrata* and *C. tropicalis* biofilm cells had an irregular and shriveled appearance (white arrows). Figures 3(a) and 3(b) are representative images of typical fields of view for *C. glabrata* and *C. tropicalis* exposed to the highest AgNPs concentration evaluated (1000 μg/mL).



(a) (b)

Figure 3: SEM analysis. (a) and (b) are representative images of typical fields of view for *C. glabrata* and *C. tropicalis* exposed to the highest AgNPs concentration evaluated (1000 μg/mL).

4. Discussion

Several new antimicrobial agents have been developed and investigated for their efficacy in inactivating clinically important pathogenic bacteria and fungi. In this context, silver, which is known for its antimicrobial property, has been evaluated recently in the form of nanoparticles. Compared to bulk materials, silver nanoparticles have a higher surface area to mass ratio, which provides notable physical, chemical, and biological properties [25]. The smaller the particle size, the higher the active or specific surface area to come in contact with the microbial cells, increasing the interaction and penetration into the cell membrane and, consequently, their antimicrobial activity [16, 25–27]. Furthermore, the type of molecule that stabilizes the nanoparticles may influence the activity, with polyvinyl alcohol (PVA) stabilized silver nanoparticles showing higher antibacterial properties against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* than polyvinylpyrrolidone (PVP) stabilized ones [28]. In addition, PVA has some advantages, such as easy processability, high transmittance, and water solubility [29]. Among the methods to synthesize silver nanoparticles, the chemical reduction of a silver salt solution by a reducing agent such as borohydrate ($NaBH_4$) is commonly used [17], due to simple operation, easily controlled reaction conditions, high reaction rate, and low cost. Hence, in this study, an AgNPs solution was synthesized by chemical reduction method described in our previous report [23], using borohydrate ($NaBH_4$) as the reducing agent and PVA to prevent agglomeration of the nanoparticles, whose size was *c*. 9 nm. This solution was further used to determine its antimicrobial activity against representative microbial species such as Gram-positive bacteria and fungi.

The results from the microbiological tests demonstrated that the synthesized AgNPs solution was not only fungistatic but also fungicidal for *C. glabrata* and *C. tropicalis*. Similarly, the synthesized AgNPs were capable of inhibiting the growth and also of killing the *S. aureus* and MRSA planktonic cells, regardless of the antibiotic susceptibility profile of these bacteria. The antimicrobial activity of silver nanoparticles has been attributed to different mechanisms [30], such as direct physical contact between the nanoparticles and the microorganism causing structural damage [15, 18], the generation of reactive oxygen species (ROS) damaging the membrane [18], and the slow release of silver ions which interfere with the DNA replication and inhibit enzymes by reacting with thiol groups [16]. These findings suggest that the activity of the AgNPs solution against the bacteria and fungi observed in this study was probably due to the destruction of membrane integrity. The small size of the synthesized AgNPs may also have accounted for their antimicrobial efficiency [16, 25–27].

In a previous study [23], MIC and MFC values for *C. albicans* were the same as those observed here for *C. tropicalis* (3.98 μg/mL and 15.63 μg/mL, resp.), indicating that both species were more sensitive to the synthesized AgNPs than *C. glabrata*, whose MIC/MFC values were 15.63 μg/mL and 62.5 μg/mL, respectively. Hence, the antimicrobial activity of AgNPs was affected by the characteristics of the microbial species, such as the cell wall, which may have played a role. Electron microscopy of *C. glabrata* cells showed that they were surrounded by a 100- to 200-nm-thick double wall, which contains the same basic components as those in *C. albicans* (1,3-β-glucan, 1,6-β-glucan, chitin, and mannoproteins) [31]. However, the contribution of individual components and the extent of cross-links between the components are different. The mannose/glucose ratio is higher in *C. glabrata* than in *C. albicans*. Moreover, the amount of alkali-insoluble glucans, relative to both total wall mass and total glucan, is lower than those in *C. albicans*, suggesting that there are fewer cross-links between glucan and chitin in the cell wall of *C. glabrata* [31]. Although there is less information available on *C. tropicalis*, electron micrographs also have shown yeast cells presenting a compact, double cell wall (average thickness of 0.13 μm) [32], with an outer layer with high electron density and an inner layer with less electron density [33]. Therefore, since the cell walls of pathogenic fungi form the first point of contact with the environment, these differences in architecture and components among *Candida*

species probably may have contributed to the higher MIC and MFC values observed for planktonic *C. glabrata* cells compared to *C. tropicalis* and *C. albicans* cells.

The composition and structural differences of cell wall may also have caused the bacteria *S. aureus* and MRSA to be more sensitive (MIC 1.95 μg/mL and MBC 3.91 μg/mL, for both strains) to the synthesized AgNPs than the fungi. The cell wall in Gram-positive bacteria, such as *S. aureus* and MRSA, is of a single layer, mainly consisting of a peptidoglycan chain of polysaccharide, teichoic acid, and phosphated sugar [34]. In addition, the outer membrane is absent. Because the outer membrane indirectly helps stabilize the inner membrane, the peptidoglycan mesh surrounding Gram-negative cells is relatively thin with only a few nanometers thick, while Gram-positive microorganisms are surrounded by many layers of peptidoglycan with 30–100 nm thickness [35]. Despite this thicker and more compact peptidoglycan layer of Gram-positive bacteria, with a three-dimensional rigid structure, it is likely that the bilayered structure of the cell wall of *Candida* species played an important role in its resistance to the AgNps. Another characteristic that may have contributed to these results is the size of the microorganisms. *Candida* species are eukaryotic cells, while *S. aureus* and MRSA are prokaryotic cells, and therefore the fungal cell is significantly larger than the bacterial cells [36] and thus required a higher AgNPs solution concentration to be inactivated. However, others have observed a different rank order of antifungal activity of AgNPs against *C. glabrata*, *C. tropicalis*, and *C. albicans* [20, 28, 37]. The dissimilar findings among studies suggest that the antimicrobial effect of Ag nanoparticles may be not solely dependent on the component and structure of the microbial cell wall but also dependent on the nanoparticles characteristics, such as size and shape, which are known to be affected by the synthesis method used.

The influence of the AgNPs solution on biofilms of each of the four microorganisms was also evaluated. In biofilms, the cells are fixed at a surface and embedded in a self-produced extracellular polymeric substance (EPS) matrix. In this study, the assay used was the crystal violet, which stains the bacteria as well as the biofilm EPS components, thus quantifying the total biofilm mass. The results confirmed that, when organized in biofilms, the microorganisms are more tolerant to antimicrobial agents. For *C. glabrata*, a ~60% decrease in biofilm mass was observed when the biofilm was treated with 1000 μg/mL of the synthesized AgNPs. In the case of *C. tropicalis*, a reduction of ~20% was observed at the concentration of 3.91 μg/mL, and ~35% decrease in biofilm mass was seen at the higher concentration (1000 μg/mL). Corroborating with these findings are SEM images of biofilms treated with the AgNPs solution: *C. glabrata* and *C. tropicalis* cells displayed morphological changes with shriveled cell walls and were collapsed to some extent. These alterations may have impaired the matrix production. Greater effect of silver nanoparticles on reducing *C. glabrata* biofilm biomass compared with other *Candida* species, as *C. albicans*, has also been found [21]. The AgNPs solution did not promote a significant biomass reduction for both MRSA and *S. aureus* biofilms. Similar findings were reported by Martinez-Gutierrez [22], who observed high resistance of established biofilm of MRSA to the action of the AgNPs.

Differences in the matrix components between the bacterial and the fungal biofilms evaluated in this study may have accounted for the less effective activity of the AgNPs solution against MRSA and *S. aureus*. The differences may also have accounted for a lower percentage of decrease in biofilm mass observed here for *C. tropicalis* when compared to *C. glabrata* biofilms. Costa et al. [38] reported that *C. glabrata* forms a thin, compact biofilm consisting of yeast cells embedded in an extracellular matrix, while *Candida tropicalis* develops a complex biofilm structure that contains coaggregated microcolonies of blastoconidia and a thick extracellular polymeric layer. With regard to the matrix composition, *C. glabrata* biofilms have relatively higher quantities of both protein and carbohydrate compared with *C. tropicalis*; however, the total biomass is lower in *C. glabrata* biofilms [39]. In addition, *C. tropicalis* biofilm matrix consists mainly of hexosamine in the form of a polysaccharide like that found in *Staphylococcal* biofilms. This polysaccharide is sometimes referred to as the intercellular polysaccharide adhesin (PIA) and has been associated with the lower susceptibility of *C. tropicalis* biofilms to the antifungal agents [40]. A review by Boles and Horswill [41] reported that, besides PIA, the second major biofilm matrix components of *Staphylococcal* biofilms are proteins, such as the fibronectin binding proteins, protein A, SasG, and beta toxin. eDNA is another recently described component, which is thought to serve a structural role in the *S. aureus* biofilm matrix [41].

5. Conclusion

In this study, an AgNPs solution, synthesized using a chemical reduction method, was tested against fungi (*C. glabrata and C. tropicalis*) and bacteria (*S. aureus* and MRSA). Different degrees of sensitivity were shown among the microorganisms, probably due to the differences in the structure and/or composition of their cell walls and extracellular matrix. Nevertheless, the synthesized AgNPs showed considerable activity against planktonic *S. aureus*, MRSA, *C. glabrata*, *C. tropicalis*, and also *C. albicans*, as reported previously [23]. Reductions in total biomass of mature biofilms of *C. glabrata* and *C. tropicalis* were also achieved. These microorganisms are important human pathogens and have been associated with several oral and systemic diseases. The fact that preparation method of the AgNPs used here is easy and cost-effective is also of importance. Thus, the synthesized AgNPs solution has potential as an antifungal agent, warranting further investigations on methods that allow the nanoparticles to more efficiently penetrate the biofilm and interact with the targeted microorganisms. From the SEM analysis, it could be observed that when this interaction occurred, the AgNPs were able to cause membrane damage.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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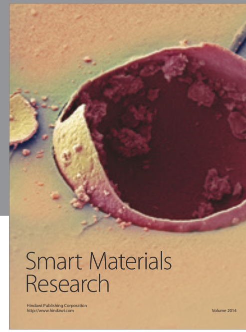
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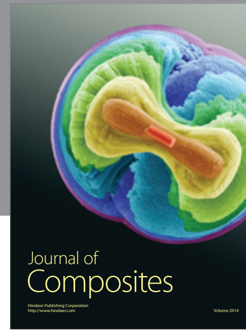
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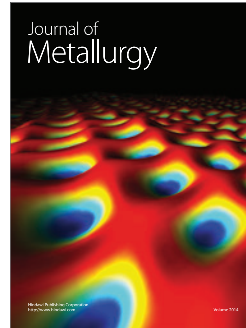
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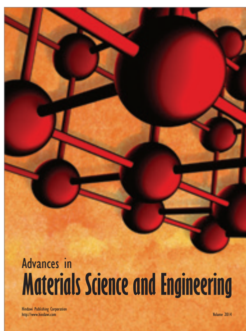
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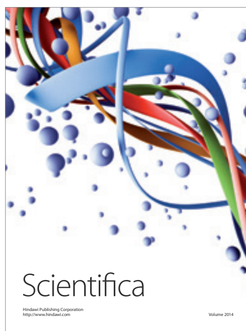
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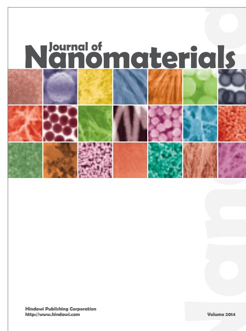
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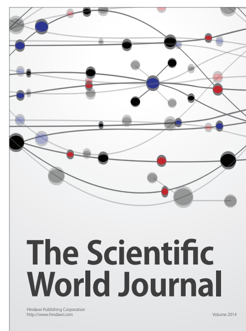
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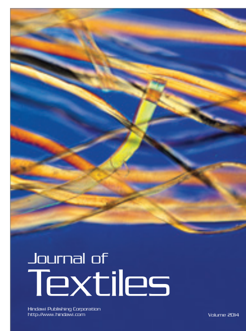
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