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Evaluation of Silver Nanoparticles for Antifungal Activity Against the Human Fungal Pathogen - *Candida albicans*

M. Kamal[1] and Vandana Ghormade[1*]

[1]*Nanobioscience Group, Agharkar Research Institute, Pune - 411 004, India.*
**Corresponding author Email:vandanaghormade@aripune.org*

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ABSTRACT

***Candida albicans* is an opportunistic human fungal pathogen causing candidiasis in immune-compromised patients. *C. albicans* is resistant to the existing drugs and exists on abiotic and biotic clinical surfaces, making it difficult to control. The biofilm formed by these fungi leads to multiple hospital-acquired fungal infections. Silver nanoparticles are reported for their antimicrobial activity and absence of resistance. Hence, the study aims at the application of silver nanoparticles against *C. albicans* and its biofilm formation. The synthesized AgNPs were ~ 37 nm, having a -37 mV charge. This process gives a yield of 880 µg/mL of AgNPs. These nanoparticles displayed antifungal activity against *C. albicans* with 2.97 µg MIC. Coating the coverslip with silver nanoparticles showed efficient inhibition of the biofilm formation by *C. albicans* with 97.51% inhibition by fluorescence microscopy. Impregnation of catheter surfaces with 1, 2, and 3 layers of silver nanoparticles showed 7.51, 15.23, and 43.02% reduced viability of *Candida* by MTT assay, respectively. The luminol assay could attribute the efficiency of AgNPs to their ROS generation. Moreover, the nanoparticles were non-cytotoxic. Hence, the silver nanoparticles exert antimicrobial activity, and their coating on catheter surfaces can show the antifungal effect on *C. albicans* biofilm formation.**

Keywords: *Candida albicans*, Silver nanoparticles, Biofilm, Antifungal

INTRODUCTION

Globally, over 150 million cases of fungal infections are reported annually, with ~1.7 million deaths (Kainz *et al.,* 2020). *Candida* are the most prevalent infectious fungi (Sheevani *et al.,* 2013). *Candida albicans* is an opportunistic fungal pathogen, accounting for 80% of candidiasis cases worldwide with high mortality (Ghrenassia *et al.,* 2019). In India, ~ 4% of the population is affected by fungal disease, among which the prevalence of candidiasis is 24.3 million (Ray *et al.,* 2022). Commensalic *C. albicans* is asymptomatically associated with normal host microbiota but becomes invasive due to host immunocompromised status (Atriwal *et al.,* 2021). Immuno-compromized hosts ailing from HIV/AIDS, diabetes, chronic pulmonary disease, cystic fibrosis, tuberculosis, or cancer are susceptible to fungal infections. Most nosocomial infections are caused by resistant *Candida* commonly present on biotic or abiotic surfaces like clinical surroundings or medical devices. In addition, *Candida* develops drug-resistant biofilms due to their encapsulation in an extracellular matrix. Most hospitalized patients are supported with catheters that act as a good substratum for fungal growth and biofilm formation. Catheters possess polymeric abiotic surface that is constantly in contact with body fluids, promoting microbial growth. *Candida* is a leading cause of 70 - 80% of catheter-associated urinary tract infections (Venkataraman and Yadav, 2022). Treating candidiasis is of major concern due to its resistance to antimicrobial drugs. Therefore, alternate strategies for coating devices with antimicrobial agents or nanoparticles for localized drug releases can control the growth of fungi (Lo *et al.,* 2014). Nanoparticles (NPs) have a larger surface area to volume ratio and unique physical and chemical properties. Antimicrobial properties of metal and metal oxide nanoparticles are reported to interfere with the cell membrane proteins and alter the membrane permeability (Yassin *et al.,* 2019). Thus, metal NP can potentially prevent fungal growth and tackle drug-resistant species (Yassin *et al.,* 2019). *Candida* growth inside and on the surfaces of in-dwelling catheters is being fought with various methods of coating medical devices with metal NPs. (Mussin *et al.,* 2019; Cruz-Luna *et al.,* 2021).

Here, we have synthesized and characterized silver nanoparticles and tested their antifungal efficacy. The silver NPs had a low MIC *in vitro* against *C. albicans* and effectively controlled the biofilm formation on the catheter surface. The NPs generated ROS activity that was responsible for its antifungal effect. The NPs had low toxicity against mammalian cell lines. Silver NP-coated catheters could help in controlling *Candida* infections.

MATERIALS AND METHODS

Synthesis of silver nanoparticles: Silver nanoparticles were synthesized by the Turkevich method (Turkevich *et al.,* 1951). Fifty mL of freshly prepared 5 mM $AgNO_3$ solution was boiled in a flask on a heating plate magnetic stirrer. Under continuous

stirring, 0.03 M tri-sodium citrate was added to the reaction dropwise until the solution turned light yellow. Stirring was continued for 4 minutes till the color changed to amber-yellow, indicating the formation of silver nanoparticles. The reaction flask was allowed to cool down to room temperature, after which 2 mL of liquid ammonia was added while stirring. Prepared nanoparticles were stored at 4°C in the dark.

Yield and characterization of silver nanoparticle: Spectroscopic analysis of nanoparticles was performed using Nanodrop 1000 (Thermofisher Scientific) to check the absorbance between 200-800 nm range. Zeta potential and nanoparticle size were confirmed by Zeta sizer nano ZS by Malvern. The yield of the nanoparticles was measured by taking the dry weight after lyophilization.

Antifungal activity of silver nanoparticles on *Candida albicans*: The antifungal activity was performed according to the CLSI guidelines (CLSI M602020). *Candida albicans* were grown on YPG broth containing 0.3% yeast, 0.5% peptone, and 0.1% glucose. 100 µL of *C. albicans*(1×10^5 cells/mL) in YPG broth was added to each well. The AgNPs were two-fold diluted, and 100 µL of different concentrations ranging from 88 to 0.75 µg was added into wells. The plate was incubated for 24 h at 30 °C. Amphotericin B (1 µg/mL and 10 µg/mL) was used as a standard against the growth of *Candida* species. The positive controls for the experiment were only media and media with *C. albicans.*

Biofilm inhibition test

1) fluorescence microscopy: Small rectangular pieces of sterile coverslips were submerged overnight in 0.5 % chitosan solution in 0.1 M glacial acetic acid for coating. After drying, the coverslip pieces were placed in a 6 well plate, and 50 µL of AgNPs were coated on a coverslip and allowed to dry. Likewise, three coatings were done in duplicates. 100 µL of *C. albicans* (1×10^5 cells/mL) was seeded onto the coverslip. The plate was incubated at 30 °C for 48 h. Later, coverslips were washed with 1X PBS, stained with SytoX green for 30 minutes, and observed under a fluorescence microscope. ImageJ software was used to analyse the percentage area coverage by *Candia albicans* and was correlated to its biofilm forming ability. The significance was analyzed with Graph Pad Prism version 8 software.

2) Cell viability test: Catheter samples were cut into thin slices and sterilized by autoclaving at 121 °C for 20 mins. The sterile catheter pieces were inoculated with *C. albicans* and incubated at 30 °C for at least 48 h. The formation of biofilm was confirmed microscopically.

The catheter pieces were transferred into 96 well plates containing 200 µL of YPG media. The 10 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added (Hjertstedt *et al.*, 2008). The plate was incubated at 30°C for 6 h. After incubation, the solution was removed, and 100 µL of DMSO was added to the wells. The absorbance was taken at 570 nm using an ELISA plate reader. The significance was analyzed with Graph Pad Prism version 8 software.

Cytotoxicity testing of AgNPs: Cytotoxicity of AgNPs was observed with A549 (adenocarcinomic human alveolar basal epithelial) cells cultured in Ham's F-12 Nutrient Mixture (Himedia) and Foetal Bovine Serum (Himedia) at a ratio of 1:0.1. Once the cells reached 80 to 100% confluency, they were trypsinized and 10^4 cells/well were inoculated in a 96-well plate for 24 h for cell adherence. The media was replaced with a serum-free Ham's F-12 Nutrient Mixture. Subsequently, the 10 µL of AgNPs were added at concentrations ranging from 100 ug/mL to 1 ng/mL, with the untreated cells serving as the control. After 24-48 h incubation, the media was replaced, and 10 µL MTT was added per well (5 mg/mL, Sigma Aldrich, USA). After 4 h, the media was substituted with 200 µl of DMSO (Spectrochem, India), and the cell viability was assessed by measuring the absorbance at A520 nm.

ROS measurement by Luminol assay: 5-Amino-2,3-dihydro-1,4-phthalazinedione solution (Luminol, 4 mM) stock solution was prepared by dissolving 7.1 mg of luminol in 10 mL of 30 mM aqueous sodium hydroxide and stored at -20 °C. To a microwell plate, 100 µL of AgNPs concentrations from 44 to 5.5 µg were added, followed by 85µl of phosphate buffer (10 mM pH 7.4,). Before taking luminescence, luminol (15 µL, 4mM) was added. The resulting mixture was measured for luminescence using the Bio-Tek microwell plate reader at 489 nm (Mamgain *et al.*, 2023)

RESULTS

Synthesis and characterization of silver nanoparticles: The chemical synthesis of silver nanoparticles by reduction technique resulted in the production of silver nanoparticles which is indicated by the amber-coloured solution upon the addition of trisodium citrate **(Figure 1i)**. The presence of silver nanoparticles in the solution was confirmed using UV spectroscopy, where the particles observed the optical density at 422 nm, an ideal range at which the AgNPs absorb the visible light **(Figure 1ii)**.

The dynamic light scattering studies of silver nanoparticles gave the size of the silver nanoparticles to be around 37.84 nm **(Figure 1iii)**. The zeta potential of the silver nanoparticles was -34 mV, suggesting the negatively charged silver nanoparticles **(Figure 1iv)**. The 50 mL reaction mixture was able to synthesize 44 mg of AgNPs. Therefore, the total yield of AgNPs is 880 mg/L.

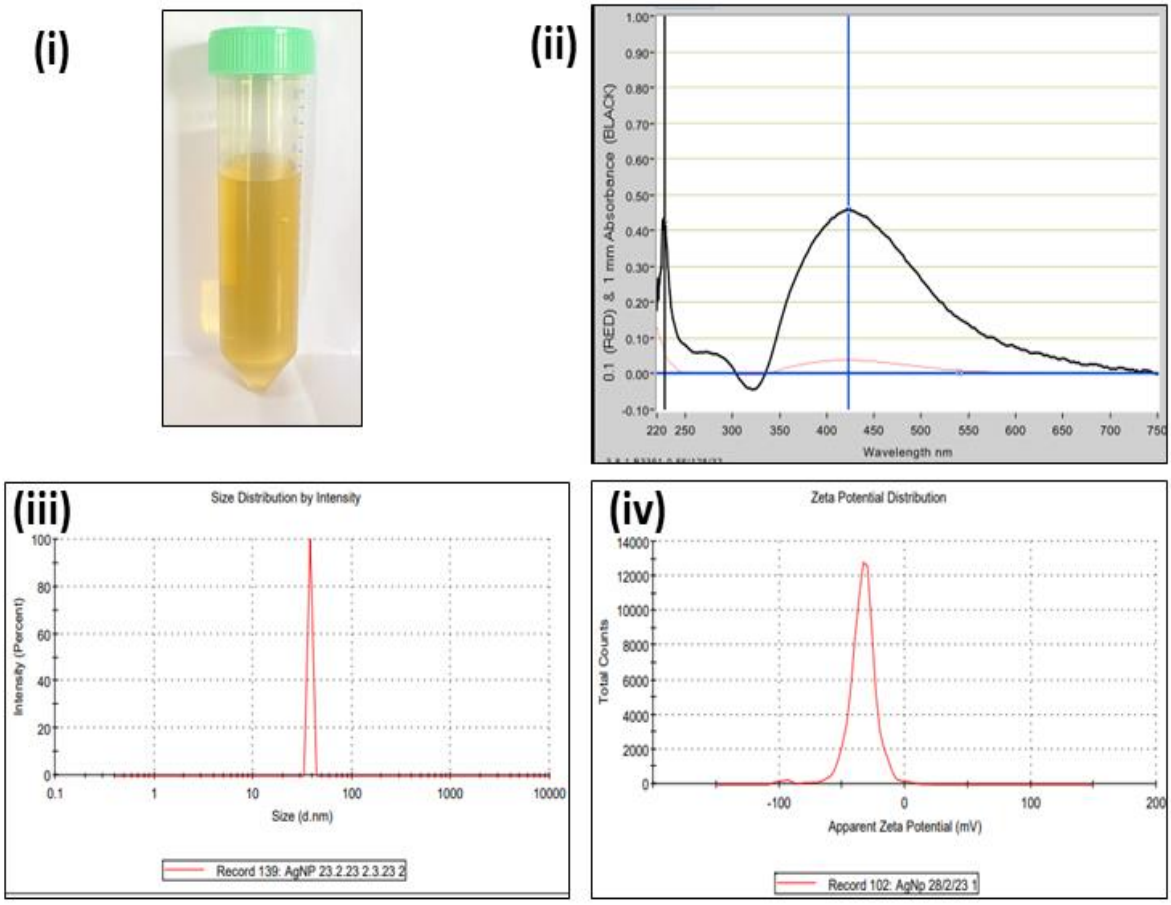

Figure 1: Synthesis and characterization of silver nanoparticles. i, Amber yellow solution indicates the formation of AgNPs; ii, characteristic absorbance peak of AgNPs at 422 nm; iii, AgNPs size 37.84 nm by dynamic light scattering; iv, Zeta potential.

Antifungal activity of silver nanoparticles on *Candida albicans*: The MIC_{70} and MIC_{50} of the silver nanoparticles using the broth dilution method on *C. albicans* were about 5.5 µg and 2.97 µg, suggesting that this concentration range of silver nanoparticle is capable of inhibiting around 70 to 50% of the *C. albicans* population **(Figure 2)**.

Biofilm inhibition test: The chitosan functionalized coverslip and catheter surfaces were used to coat AgNPs and form biofilm.

***Fluorescence microscopy*:** The coverslips coated with AgNPs were analyzed for the *Candida* biofilm. The live cells were stained with SytoX green **(Figure 3i)**. The biofilm growth was compared with the control having no AgNP coating. The coverslip with three coats showed a 97.5% significant reduction in cells compared to the single and double coats, which were 63.4 and 69%, respectively **(Figure 3ii)**. This shows that the AgNPs are efficient enough to reduce the biofilm forming ability of *Candida*.

Cell viability test by MTT: The *Candida* was grown on sterile catheters for 2 days to obtain a firm biofilm, and the same was visualized under a microscope **(Figure 4i)**. The MTT assay for *C. albicans* showed a significant decrease in the biofilm forming ability of the *C. albicans* with an increase in AgNP coating. The study suggests that the three coats of AgNPs could suppress 43.02% of the *Candida* species **(Figure 4 ii)**.

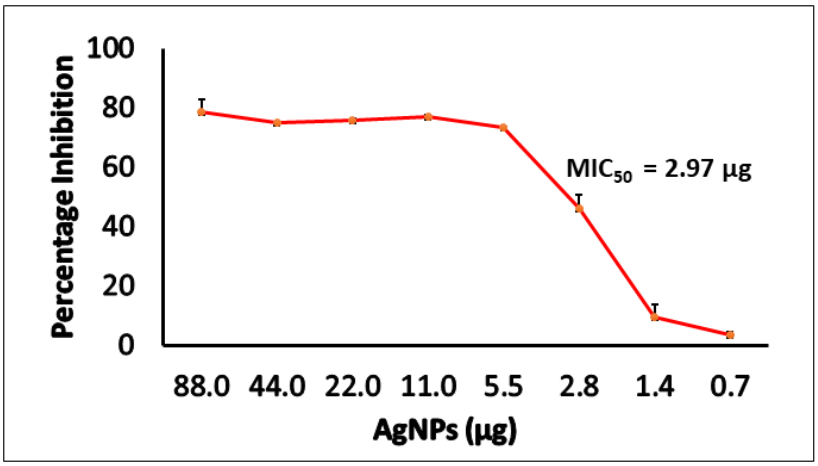

Figure 2: Determination of MIC of AgNPs against *C. albicans* using broth microdilution method.

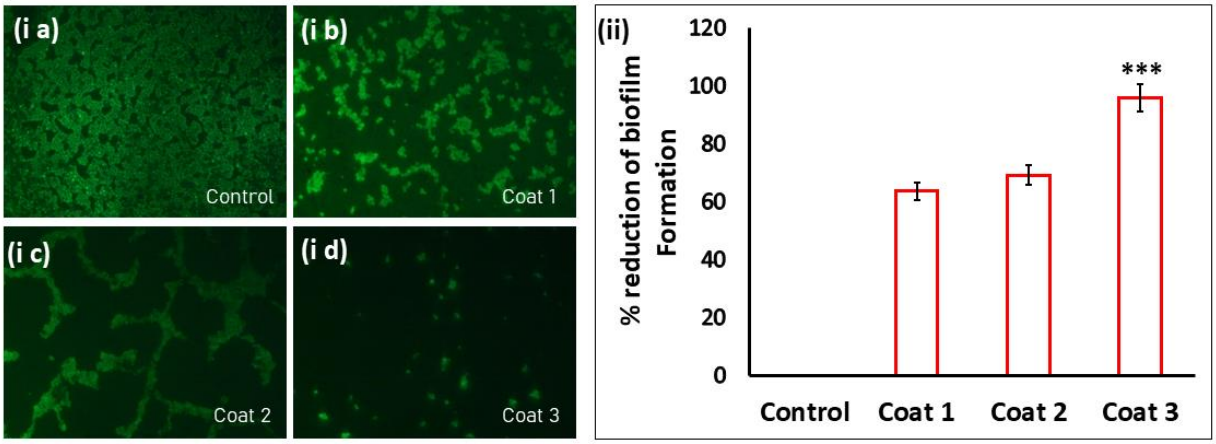

Figure 3: Antifungal effect of AgNPs coating on coverslip. i, The AgNPs coated coverslips were analyzed for the *Candida* biofilm formation under a fluorescence microscope at 40X magnification; ii, Images analyses by ImageJ software for area coverage to evaluate the percentage of living cells. Significance p = 0.001(***).

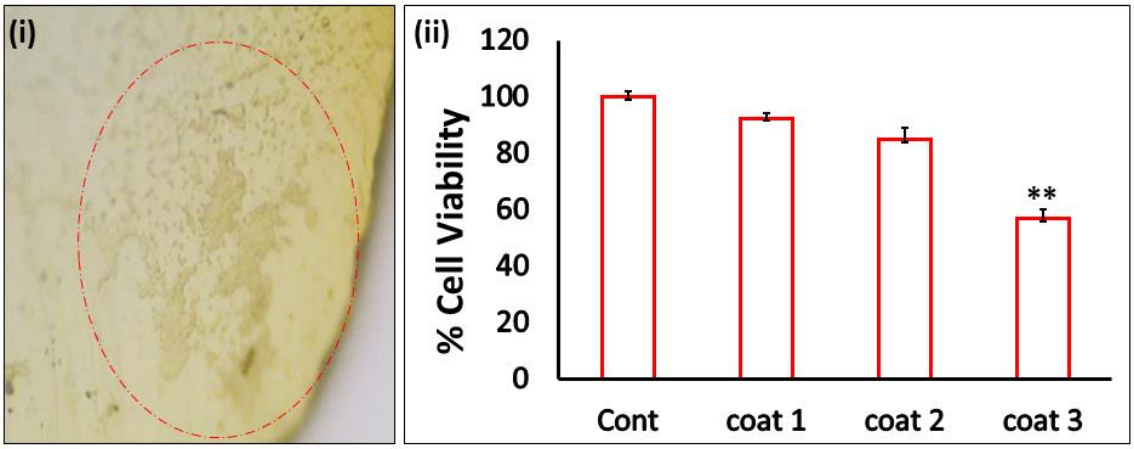

Figure 4: Antifungal effect of AgNPs coating on catheter surface. i, The catheter was incubated with *Candida albicans* for biofilm formation and observed at 40X magnification under brightfield microscopy; ii, MTT assay was conducted for the cells on the catheter surface and the percentage of cell viability was evaluated. Significance p = 0.01(**).

Cytotoxicity testing of AgNPs: AgNPs were not toxic to A549 adenocarcinomic human alveolar cells at the exposed concentrations (100 to 0.001 µg/mL) for 24 and 48 hours **(Figure 5)**. Therefore, silver nanoparticles are highly biocompatible.

ROS measurement by Luminol assay: The chemiluminescence-based assay for superoxide radical (O_2*) generation was conducted in a phosphate buffer having pH 7.4 using luminol. The relative luminescence intensity (RLI) of 1240 to 31 for 44 to 5.5 µg of AgNPs was observed, respectively, indicating a decreasing trend of chemiluminescence **(Figure 6)**. It confirms the ability of AgNPs to generate O_2* under aerobic conditions. From the literature, a mixture of hypoxanthine and xanthine oxidase (X+XO) was compared with the same reaction mixture with 5 units catalase as a scavenger for H_2O_2 as a positive control for ROS generation, which resulted in RLI of 1625.67 (Mamgain *et al.*, 2023).

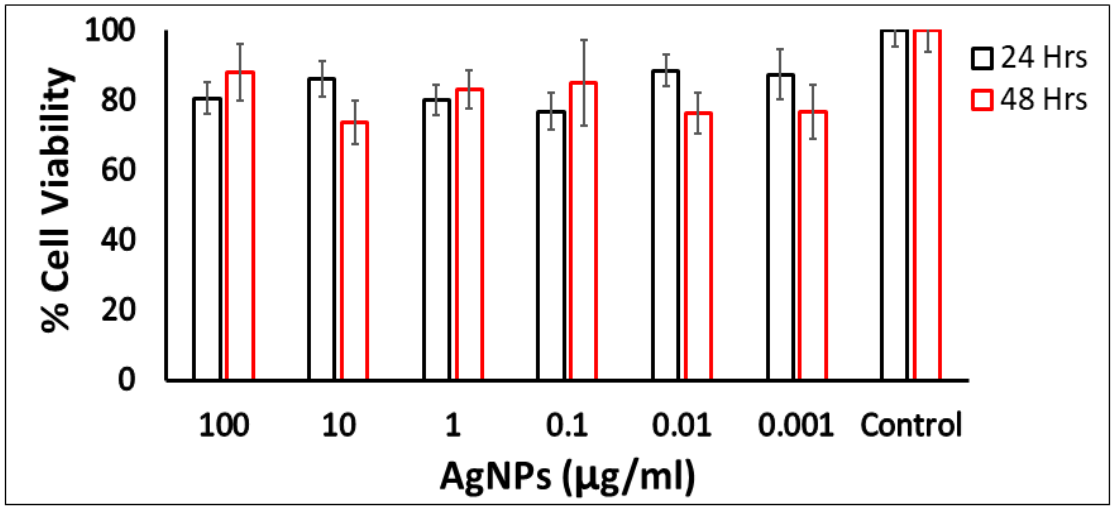

Figure 5: Cytotoxicity of silver nanoparticles with human A549 lung cell line.

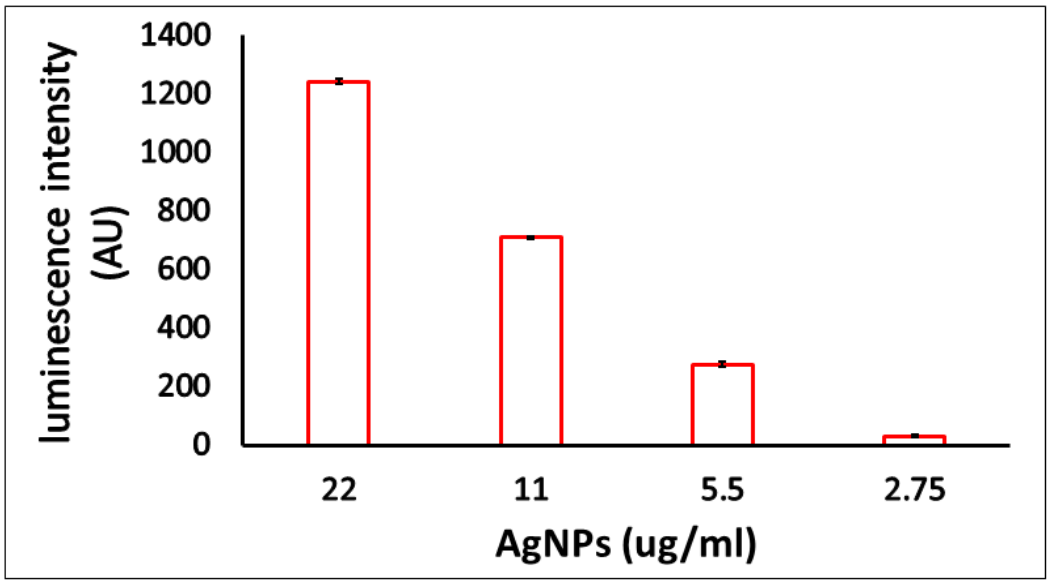

Figure 6: Generation of ROS by AgNPs was determined by luminol assay.

DISCUSSION

Candida infection and its growth on medical devices is difficult to control due to its omnipresence. The resistance developed by *Candida* towards several antifungals makes it difficult to manage disease condition and their biofilm (Cavalheiro *et al.,* 2018). Nanoparticles are an innovative strategy to control fungal growth and tackle resistance. Metal nanoparticles such as silver are known to have antimicrobial activity (Skłodowski *et al.,* 2023). Silver nanoparticles ranging from 1-100 nm can be synthesized with easy techniques, including biological, chemical, and physical methods. AgNPs damage the cell membrane due to structural and functional alterations such as membrane destabilization, cytoplasm leakage, and gap formation. The release of free Ag+ ions and the generation of ROS leads to sub-cellular structural damage. AgNPs can be synthesized in various shapes and sizes ranging from 1-100 nm. Some shapes include spherical, colloidal, rod-like, octahedron, tetrahedron, nanowire, etc (González *et al.,* 2014; Helmlinger *et al.,* 2016).

The present study uses a chemical synthesis method to synthesize colloidal AgNPs. These colloidal particles showed the minimum inhibitory concentration on the growth of *C. albicans* as 2.97 ug/mL, which is lower than reported earlier, proving that low doses of these AgNPs are capable of killing or suppressing the growth of species (Ahamad *et al.,* 2022). Due to their antimicrobial ability, they can be used to coat materials employed for bio-medicinal purposes, helping reduce infections and contaminations, hence improving patient safety.

Chitosan, a fibrous, linear cationic polysaccharide substance obtained from shells of crustaceans, is made of random β-(1,4)-D-glucosamine. Chitosan has exposed amine and hydroxyl functional groups, which act as a cross-linking agent between surface and nanoparticles. It also helps prevent nanoparticle agglomeration (Mirda *et al.,* 2021). It is evident from some studies that some cross-linking agent is required between the catheter surface and nanoparticles to achieve a strong link with low release (pharmaceuticals). Hence, chitosan solution was used to functionalize the catheter surface and nanoparticles. The chitosan coating efficiently held three coats of nanoparticles onto the surface and reduced the biofilm formation by *Candida*.

Candida showed a reduction in biofilm by 43% on the AgNPs coated catheter surfaces. The reduction in the case of the coverslips was higher, which was attributed to the uniform coating on the smooth coverslip surfaces. Previously, Roe *et al.* (2008) showed that silver nanoparticle-coated plastic catheters prevented biofilm formation by *C. albicans*. A previously reported study on filamentation showed that AgNPs of size 18-45 nm and 50-75 nm could inhibit the filamentation by 40 and 85.9%, respectively (Gómez-Garzón *et al.,* 2021). Hence, the synthesized nanoparticles of 37.84 nm affect the viability of the cells by 43%.

Metal nanoparticles act as antimicrobial agents due to their capacity for generating ROS that affects cell viability. In this study, the ROS generated by AgNPs was significant at MIC compared to the RLI of the reference controls. A study of AgNPs against *Candida albicans* showed that when compared to control, the ROS levels were significantly increased in *C. albicans* in the presence of 2, 20, and 50 µg/mL AgNPs (Lee *et al.,* 2019), which is similar to the ROS levels at 2.75, 5.5, 11 and 22 µg AgNPs in this study. The potent dose-dependent inhibitory effect of silver nanoparticles on biofilm formation on catheters showed >90% inhibition at highest dose (Lara *et al.,* 2015).

The above studies demonstrate that the prepared AgNPs are efficient in suppressing the growth and biofilm forming ability of the *C. albicans.* Hence, the silver nanoparticles exert an antimicrobial effect, and their coating on catheter surfaces can show an antifungal effect on *C. albicans* biofilm formation.

CONCLUSION

The silver nanoparticles were synthesized using a chemical reduction method to produce the AgNPs of size 37.84 nm having a particle charge of -34 mV. The particles showed absorbance at 422 nm. The presented procedure gave a yield of 880 µg/mL. Upon exposing the *C. albicans* with varying concentrations of AgNPs, the species showed no growth till 2.97 µg, suggesting that this concentration is the minimum inhibitory concentration required to inhibit the visible growth of the *C. albicans.* To their ability to inhibit the biofilm forming ability, the coverslips and catheters were coated with the AgNPs of a first, second, and third coat. The biofilm inhibition studies were concluded by the fluorescence assay and MTT assay. The fluorescence studies revealed that the three coats of AgNPs could show minimum growth and progression of biofilm as compared to the control and single coat.

Similarly, on catheter surfaces, the MTT assay confirmed that the biofilm-forming cells on the catheter surfaces are more disturbed by the three coats of the AgNPs. The antifungal activity of AgNPs was rendered by ROS generation without any risk of toxicity to human cells. Medical devices coated with

silver nanoparticles shall be a promising way to avoid the formation of infectious biofilms, which may lead to severe health consequences such as candidiasis. Using chitosan solution for the coated AgNPs shall help maintain and enhance the antifungal activity. This can reduce the risk of opportunistic fungal nosocomial infections in immune-compromised patients. Further, we plan to conduct *in vivo* systemic toxicity studies with mice to understand the effect of indwelling catheters.

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