

Anticandidal activity of biosynthesized silver nanoparticles: effect on growth, cell morphology, and key virulence attributes of *Candida* species

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Purpose: The pathogenicity in *Candida* spp was attributed by several virulence factors such as production of tissue damaging extracellular enzymes, germ tube formation, hyphal morphogenesis and establishment of drug resistant biofilm. The objective of present study was to investigate the effects of silver nanoparticles (AgNPs) on growth, cell morphology and key virulence attributes of *Candida* species.

Methods: AgNPs were synthesized by the using seed extract of *Syzygium cumini* (Sc), and were characterized by UV-Vis spectrophotometer, Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), energy-dispersive X-ray (EDX), and transmission electron microscopy (TEM). ScAgNPs were used to evaluate their antifungal and antibacterial activity as well as their potent inhibitory effects on germ tube and biofilm formation and extracellular enzymes viz. phospholipases, proteinases, lipases and hemolysin secreted by *Candida* spp.

Results: The MICs values of ScAgNPs were ranged from 0.125-0.250 mg/ml, whereas the MBCs and MFCs were 0.250 and 0.500 mg/ml, respectively. ScAgNPs significantly inhibit the production of phospholipases by 82.2, 75.7, 78.7, 62.5, and 65.8%; proteinases by 82.0, 72.0, 77.5, 67.0, and 83.7%; lipase by 69.4, 58.8, 60.0, 42.9, and 65.0%; and hemolysin by 62.8, 69.7, 67.2, 73.1, and 70.2% in *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis* and *C. krusei*, respectively, at 500 µg/ml. ScAgNPs inhibit germ tube formation in *C. albicans* up to 97.1% at 0.25 mg/ml. LIVE/DEAD staining results showed that ScAgNPs almost completely inhibit biofilm formation in *C. albicans*. TEM analysis shows that ScAgNPs not only anchored onto the cell surface but also penetrated and accumulated in the cytoplasm that causes severe damage to the cell wall and cytoplasmic membrane.

Conclusion: To summarize, the biosynthesized ScAgNPs strongly suppressed the multiplication, germ tube and biofilm formation and most importantly secretion of hydrolytic enzymes (viz. phospholipases, proteinases, lipases and hemolysin) by *Candida* spp. The present research work open several avenues of further study, such as to explore the molecular mechanism of inhibition of germ tubes and biofilm formation and suppression of production of various hydrolytic enzymes by *Candida* spp.

Keywords: ScAgNPs, virulence factors, hydrolytic enzymes, germ tubes, biofilm, LIVE/DEAD staining.

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Introduction

Candida spp. is one of the most common opportunistic human fungal pathogens, which are responsible for 90–100% of mucosal infections and the fourth-leading cause of nosocomial infections (candidemia and other forms of invasive candidiasis) that attribute a 35–50% mortality rate in immunocompromised and critically ill

individuals.^{1,2} Approximately 70% of women experienced vaginal infections by *Candida* spp, 70% of AIDS patients had oropharyngeal candidiasis, and 20% of them suffered from recurrence.³ Extracellular hydrolytic enzymes, most importantly proteinases, phospholipases, hemolysin, and lipases, are believed to play an important role in *Candida* overgrowth because these facilitate adherence, tissue penetration, and the subsequent invasion to the host.⁴ The formation of biofilms, communities of adhered cells that are embedded and encased in an extracellular polymeric matrix on both kind of surfaces, either abiotic or biotic (such as implanted medical devices and tissues), also play a major role in pathogenicity.⁵ It is believed that cells in a biofilm exhibit high resistance to antifungal drug and the host immune system, primarily due to inadequate penetration of antifungal agents.⁶ Due to the production of various extracellular hydrolytic tissue-damaging enzymes, hyphae transition, and formation of highly drug-resistant biofilm in isolates of *Candida* spp, innovative strategies are needed to develop newer and safer antifungal agents with broad-spectrum activity that suppress and eradicate the virulence factors involved in the pathogenicity of life threatening *Candida* spp. In most cases candidiasis is treated with the existing conventional antifungal drug that includes triazoles and polyene. Over the past decade the improper and misuse of existing antifungal drugs have led to the development of drug-resistant clinical isolates of *Candida* spp. Recently, nanomedicines have gained great attention to formulate nanoparticles based on antimicrobial agents which have the potential to combat microbial drug resistance either alone or in combination with existing drugs. The antimicrobial activity of biogenic AgNPs was well documented in the literature.⁷⁻²¹ However, there are very few studies available in the literature on the inhibition of production of various extracellular hydrolytic enzymes and germ tubes in *Candida* spp by nanoparticles.²²⁻²⁴ Due to the scarcity in the literature on the inhibition of production of various virulence factors by *Candida* spp, herein for the first time we have reported the potent inhibitory effects of green synthesized AgNPs on the production of different virulence attributes of *Candida* species isolated from oropharyngeal mucosa of HIV patients. The objective of the present study was i) the green synthesis of silver nanoparticles (AgNPs) using aqueous seed extract of *Syzygium cumini* (Sc). ii) Characterization of as-prepared ScAgNPs by a UV-Vis spectrophotometer, fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), energy-dispersive X-ray (EDX), and transmission electron microscopy (TEM). iii) Evaluation of antifungal

and antibacterial activity of ScAgNPs against *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. neoformans*, *S. aureus*, and *E. coli* using microbroth dilution, well diffusion, and time-dependent growth assay methods. iv) Investigation of potent inhibitory effects of green synthesized ScAgNPs on key virulence factors such as germ tube formation and extracellular enzymes viz. phospholipases, proteinases, lipases, and hemolysin secreted by *Candida* spp. v) Visualization of effects of ScAgNPs on biofilm formation by Congo red agar and Confocal laser scanning microscopy (CLSM), and vi) Examination of ultrastructural alteration caused by ScAgNPs in *C. albicans* by TEM.

Materials and methods

Preparation of the seed extract and biosynthesis of ScAgNPs

The fresh seeds of *Syzygium cumini* were washed with sterile water, and air-dried. About 10 g of seeds were ground to fine powder and dissolved in 100 mL sterile water, vigorously vortexed, and then boiled for 20 minutes. After cooling, the solutions were filtered by Whatman No. 1 paper (Maidstone, UK) and then the filtrate was collected and stored at 4°C.²⁵ For the synthesis of AgNPs, 25 mL of aqueous seed extract was transferred into 75 mL of 1 mM silver nitrate (AgNO₃, Sigma Aldrich, St. Louis, MO, USA) solution. The mixture was kept at room temperature overnight. The change in color of the solution from pale yellow to dark brown was an indication of the formation of AgNPs. The solution was washed with double distilled water three times at 14,000 rpm for 10 minutes to separate the AgNPs.²⁵

Characterization of ScAgNPs

The biosynthesized SCAgNPs were characterized by UV-Vis spectroscopy (LAMBDA 25, Perkin Elmer, USA), FT-IR spectrometer (SHIMADZU-8400, Japan), energy dispersive X-ray spectroscopy (JED-2300, Japan), scanning electron microscopy (SEM, JSM-6510LV, Jeol Ltd., Tokyo, Japan) and transmission electron microscopy (TEM, 2100, Jeol) by following the protocol of our previous study.²⁶

Strains

The clinical isolates of *Candida* (*C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. krusei*) and bacterial (*S. aureus* and *E. coli*) strains used in this study were collected from the department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India.

Antimicrobial activity of ScAgNPs

The zone of inhibition (in mm) test for *Candida* spp and bacterial stains were carried out on Sabouraud Dextrose Agar (SDA, Hi-Media, Mumbai, India) and Mueller Hinton Agar (MHA, Hi-Media) plates, respectively, by well diffusion methods, as described in a previous study.²⁶ The MICs, MBCs, and MFCs values of ScAgNPs against tested strains were examined by microbroth dilution method, as previously described with slight modification.²⁶ Further, the effects of ScAgNPs on the growth curve of *Candida* spp at different time interval and different concentration were determine as described in our previous study.²⁶

Effect of ScAgNPs on virulence factors of *Candida* spp

Phospholipase activity of untreated and ScAgNPs treated *Candida* isolates (*C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. krusei*) was assessed as described previously.^{24,27} The anti-proteinase activity of different concentration of green synthesized ScAgNPs against proteinase positive *Candida* spp were carried out by following the protocol of our previous study.²⁴ Lipase activity of control and ScAgNPs treated experiments was carried out using the previously reported protocols.^{28,29} The lysis efficacy of human red blood cells by *C. albicans* after treatment with varying concentration of ScAgNPs was analyzed as previously described methods with some modification.^{7,30} Further, the effect of ScAgNPs on the germ tube formation by *C. albicans* was examined as a method previously reported with some modification.²⁴

Effect of ScAgNPs on *C. albicans* biofilm

The effects of different concentration of ScAgNPs on *C. albicans* biofilm were assessed by Congo Red Agar method (qualitatively), as described in our previous study with slight modification.³¹ Furthermore, the inhibitory effect of ScAgNPs on biofilm formation of *C. albicans* was quantitatively analyzed and visualized by CLSM (FV1000, Olympus Latin America, Miami, FL, USA) using LIVE/DEAD staining, ie, Con-A-FITC (Sigma Aldrich) and propidium iodide (Sigma Aldrich).²⁴

Effects of ScAgNPs on *C. albicans* morphology and ultrastructure: TEM analysis

The morphological and ultrastructural alteration in *C. albicans* cells after treatment with ScAgNPs were examined by TEM (2100, Jeol). The sample preparation and analysis methods were similar to those described previously.²⁴

Results and discussion

Structural characterization of biosynthesized ScAgNPs

In the present study, aqueous seed extract of *Syzygium cumini*, a traditional medicinal plant, has been used as a reducing and stabilizing agent for the green synthesis of AgNPs. The bioreduction of Ag^+ ions to Ag^0 by seed extract of *Syzygium cumini* and formation of AgNPs was confirmed by UV-Vis absorbance spectroscopy, which showed an intense peak at 474.45 nm due to surface plasmon resonance (Figure 1) and is supported by the results of Banerjee and

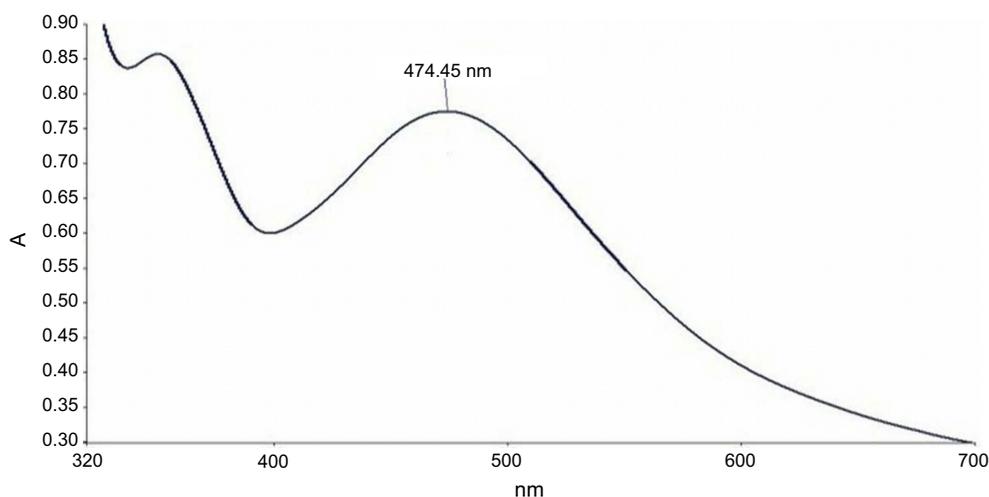


Figure 1 UV-Vis spectrum of AgNPs synthesized by aqueous seed extract of *Syzygium cumini*.

Abbreviation: AgNPs, silver nanoparticles.

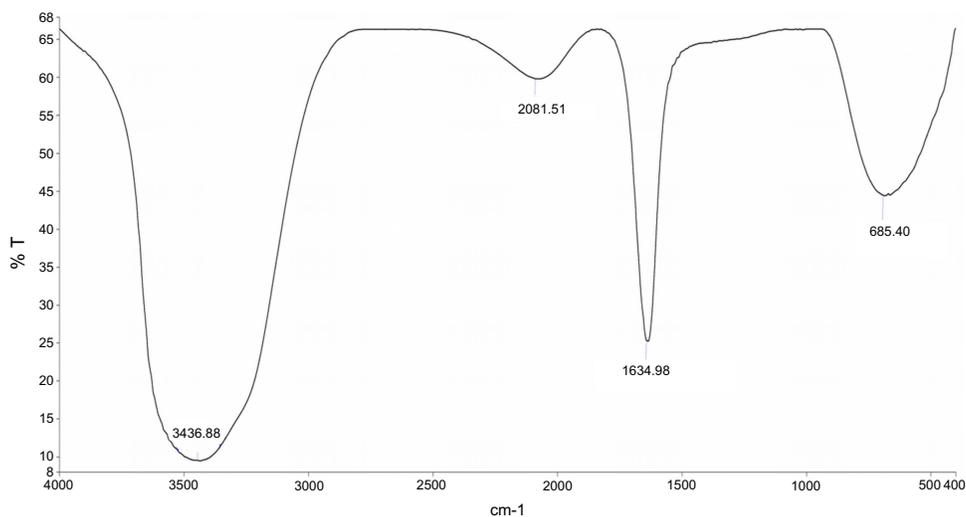


Figure 2 FTIR Spectrum of AgNPs synthesized by aqueous seed extract of *Syzygium cumini*.
Abbreviations: AgNPs, silver nanoparticles; FTIR, Fourier-transform infrared spectroscopy.

Narendhirakannan.³² The FTIR spectra (Figure 2) showed a broad peak at $3,436.88\text{ cm}^{-1}$, indicating the presence of hydroxyl group of polyphenolic component in seed extract.³² It has been reported that the seed extract of *S. cumini* contains a large amount of polyphenols, flavonoids, and gallic acid.³³ The peak at $1,634.98\text{ cm}^{-1}$ was due to the presence of carbonyl groups (C=O) of the polyphenolic compounds such

as epicatechin gallate, epigallocatechin gallate, catechin gallate, epi-gallocatechin, gallic acid, theaflavin present in the seed extract.^{32–34} The presence of a large amount of polyphenolic compounds in the seed extract of *S. cumini* might be responsible for the reduction of Ag^+ to Ag^0 and stabilization of AgNPs.^{33,34} The SEM micrograph revealed that the particles were hexagonal (Figure 3A). EDX analysis

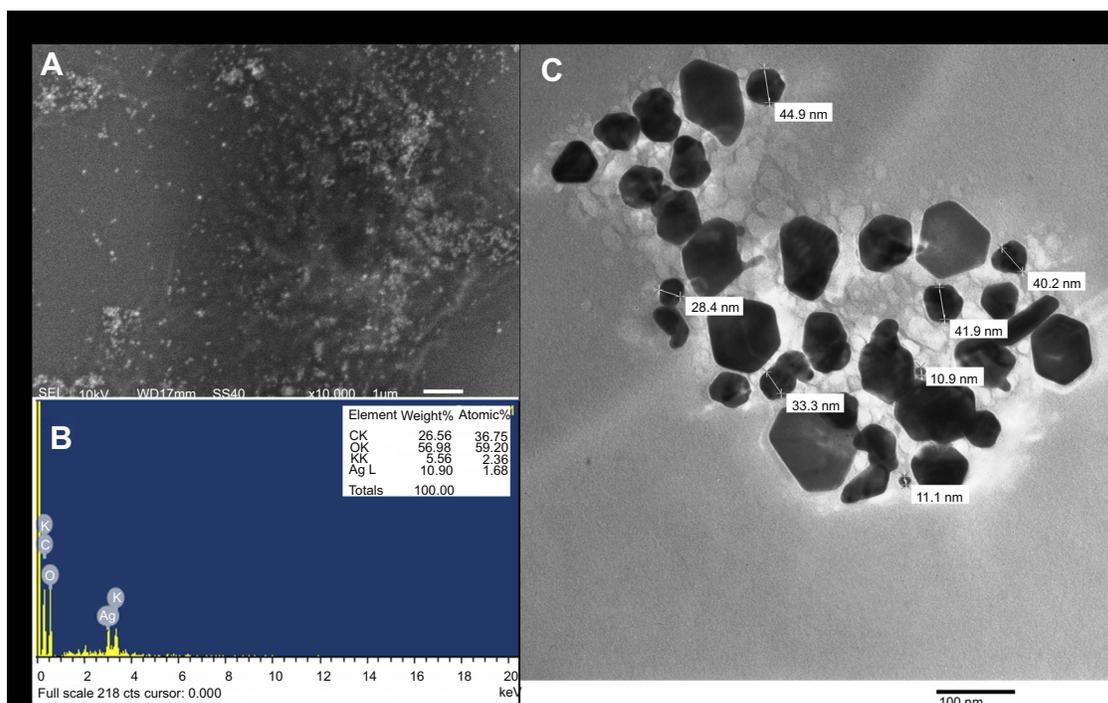


Figure 3 SEM (A) EDX (B); and TEM (C) analysis of AgNPs synthesized by aqueous seed extract of *Syzygium cumini*.
Abbreviations: AgNPs, silver nanoparticles; EDX, energy-dispersive X-ray; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

of the synthesized ScAgNPs showed a typical optical absorption peak at 3 keV that confirmed the presence of elemental silver in the form of AgNPs³³ (Figure 3B). The TEM analysis confirms that the shape of ScAgNPs was primarily hexagonal and was in the range of 10–100 nm (Figure 3C).

Antimicrobial activity of ScAgNPs

The antimicrobial activity of ScAgNPs was assessed by measuring the clear zone of inhibition around the wells supplemented with 0.031–0.5 mg/mL concentration of ScAgNPs. A significant reduction in the growth of *Candida* and bacterial species was observed at 0.5 mg/mL of ScAgNPs (Figure 4). The highest zone of inhibition (22 mm) was recorded for *C. albicans*, followed by *C. neoformans* (21 mm), *S. aureus* (20 mm), *C. parapsilosis* (19 mm), *E. coli* (18 mm), and *C. tropicalis* (17 mm). The zone of inhibition values obtained here is similar to those reported by Yasir et al.¹⁰ The MICs values of ScAgNPs against all tested *Candida* and bacteria species ranged from

0.125–0.250 mg/mL, whereas the MBCs and MFCs were 0.250 and 0.500 mg/mL, respectively. In a recent study,¹⁷ AgNPs synthesized by *Caesalpinia ferrea* seed extract showed MIC values in the range of 156.25–1,250 µg/mL, and MFC values in the range of 312.5–5,000 µg/mL against *C. albicans*, *C. glabrata*, *C. kruzei*, and *C. guilliermondii*.¹⁷ The MIC and MFC results of the present study demonstrate that the ScAgNPs exhibited high anticandidal activity. Further, the effects of a different concentration of ScAgNPs (ie, 62.5–1000 µg/mL) on the growth of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* examined by time-dependent growth inhibition assay shows that ScAgNPs inhibit the growth of tested *Candida* species at all doses (Figure 5). The growth of *Candida* species reached exponential phase rapidly in the absence of ScAgNPs. However, it was found that the growth of the *Candida* cells were significantly reduced when exposed at a higher concentration of ScAgNPs (500 and 1000 µg/mL). Figure 5 clearly shows that, as the concentration of ScAgNPs

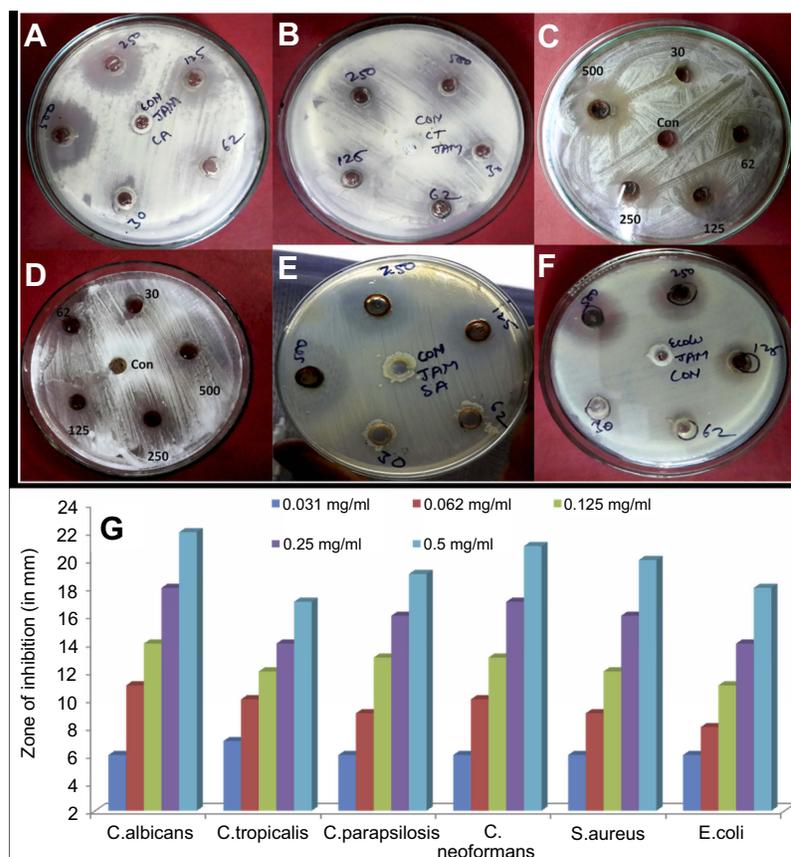


Figure 4 Antimicrobial activity of AgNPs synthesized by aqueous seed extract of *Syzygium cumini* against (A) *C. albicans*; (B) *C. tropicalis*; (C); *C. parapsilosis*, (D) *C. neoformans*; (E) *S. aureus*, and (F) *E. coli*. Panel (G) shows zone of inhibition in millimetres.

Abbreviation: AgNPs, silver nanoparticles.

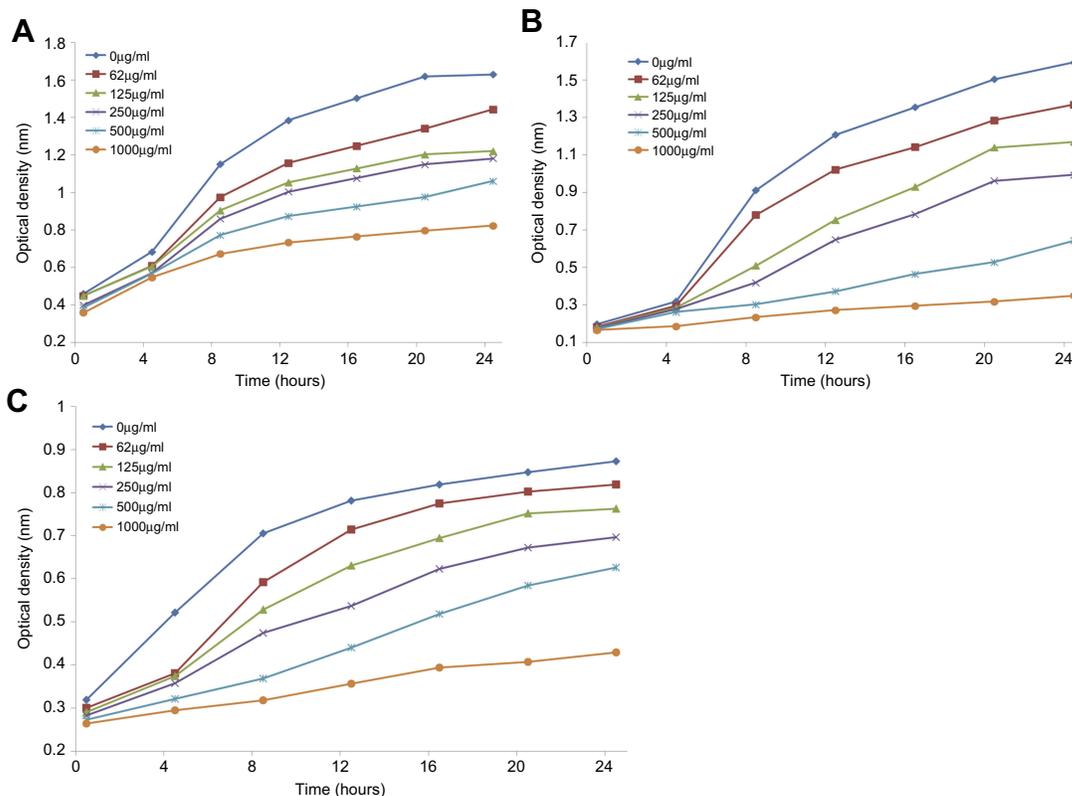


Figure 5 Growth curves of *C. albicans* (A); *C. tropicalis* (B); and *C. parapsilosis* (C) treated with different concentration of ScAgNPs.

increases, the growth inhibition of *Candida* cells was also increased. Dose-dependent anticandidal activity of AgNPs has been previously reported.^{26,35}

Effects of ScAgNPs on extracellular hydrolytic enzymes of *Candida* spp

The virulence in *Candida* species is attributed by a number of extracellular enzymes such as proteinase, phospholipase, lipase, hemolysin, chondroitinase, and hyaluronidase, which plays an important role in its pathogenicity.^{4,36} Thus, interference in these virulence factors has emerged as a novel target for developing new anti-infective agents. However, a huge number of studies on anticandidal activity of AgNPs synthesized by different approaches were reported in the literature.^{10,15,37–39} The effects and mechanism of AgNPs on the production of extracellular enzymes by *Candida* spp are still not reported. Very little is known about the inhibition of production of virulence factors by nanoparticles.^{22–24} In the present study, for the first time, the inhibitory effects of green synthesized ScAgNPs on the production of phospholipases, SAPs, lipases, and hemolysin by five *Candida* spp, ie, *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. krusei* were investigated. Phospholipases are associated to adherence, induction of germ tubes, transition from yeast to hyphal

forms, penetration, and tissue injury.⁴ In the present study, it was found that ScAgNPs at 500 µg/mL significantly inhibits production of phospholipases activity by 82.2, 75.7, 78.7, 62.5, and 65.8% in *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. krusei*, respectively, in a dose-dependent manner (Figure 6). Similar results were previously reported by Jalal et al,²⁴ who found that ZnO NPs synthesized by leaf extract of *Crinum Latifolium* inhibit the secretion of phospholipases in albicans and non-albicans isolates of *Candida*. It was observed that the suppression of phospholipases activity in albicans is more pronounced than that of non-albicans isolates (Figure 6).

The production of SAPs by *Candida* spp has been identified as one of most important virulence factors as it has the ability to degrade a number of human proteins on the lesion site, hemoglobin, albumin, secretory immunoglobulin A, and skin proteins. The proteolytic action of this enzyme also attributed the tissue invasion and penetration.⁴⁰ In the present study, it was found that ScAgNPs at 500 µg/mL inhibits production of SAPs by 82.0, 72.0, 77.5, 67.0, and 83.7% in *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. krusei*, respectively (Figure 7). Similar results were previously reported by Hamid et al,²³ who found that AgNPs synthesized by fungi *Aspergillus* spp inhibit the secretion of

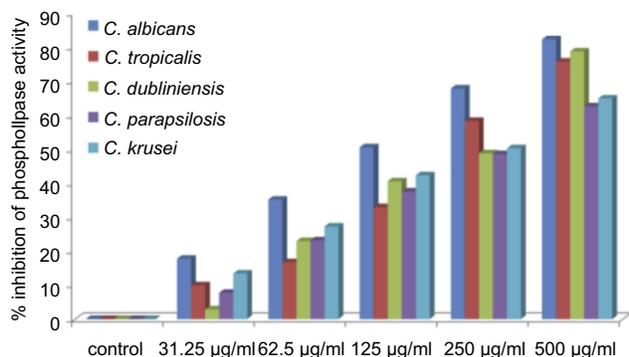


Figure 6 Effects of different concentration of ScAgNPs on production of extracellular phospholipases.

Abbreviation: ScAgNPs, *Syzygium cumini* silver nanoparticles.

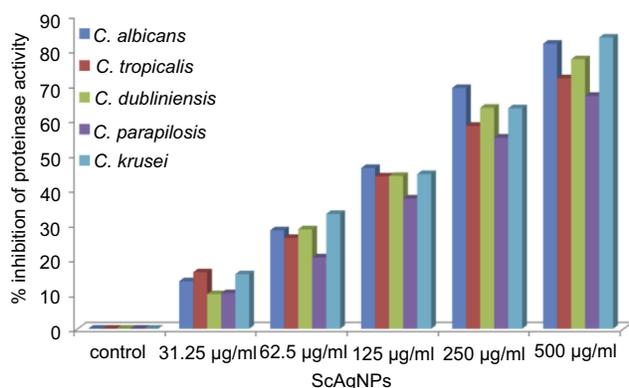


Figure 7 Effects of different concentration of ScAgNPs on production of extracellular secreted aspartyl proteinases.

Abbreviation: ScAgNPs, *Syzygium cumini* silver nanoparticles.

SAPs in albicans and non-albicans isolates of *Candida*. In another study, Hajjar et al²² reported the inhibition of *C. albicans* secreted aspartyl proteinase by exploring triangular gold nanoparticles.

Stehr et al⁴¹ reported that the secretion of extracellular lipases increases the pathogenicity of *Candida* by degrading lipids, and may also support the microorganism to stick to host tissue and/or neighboring cells.⁴¹ In the present study, it was found that ScAgNPs at 500 µg/mL suppress the production of lipases by 69.4, 58.8, 60.0, 42.9, and 65.0% in *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis* and *C. krusei*, respectively (Figure 8). Another putative virulence factors responsible for *Candida* pathogenesis in humans is secretion of hemolysins by *Candida* spp. Yeast cells destroy erythrocytes to acquire iron from the host by secreting hemolysins⁴² and the secretion of hemolysins followed by iron obtained facilitates invasion of hyphae and the development of disseminated candidiasis.⁴³ In the present study, we found that ScAgNPs at 500 µg/mL

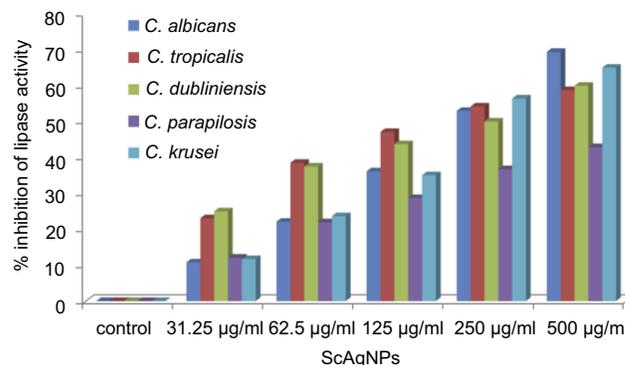


Figure 8 Effects of different concentration of ScAgNPs on lipase activity.

Abbreviation: ScAgNPs, *Syzygium cumini* silver nanoparticles.

suppress the production of hemolysins by 62.8, 69.7, 67.2, 73.1, and 70.2% in *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. krusei*, respectively (Figure 9).

Effect of ScAgNPs on germ tube formation

The morphological transitions between yeast and filamentous forms are probably to be one of the most important virulent factors in *C. albicans*.⁴⁴ The developments of hyphae or germ tube is an intriguing characteristic of *C. albicans* that plays a crucial role in adherence and biofilm formation, which indeed is essential for colonization and initiation for pathogenesis.^{45,46} Impeding or blocking of transformation from yeast to hyphal form would mean stopping the infection. In this study, it was found that ScAgNPs almost completely impede the germ tube formation in a dose-dependent manner (Figure 10). ScAgNPs inhibits germ tube formation by 97.1, 94.3, 57.1, and

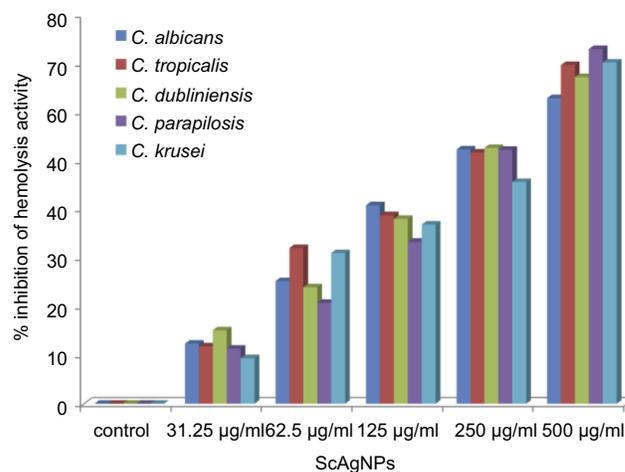


Figure 9 Effects of different concentration of ScAgNPs on hemolysin activity.

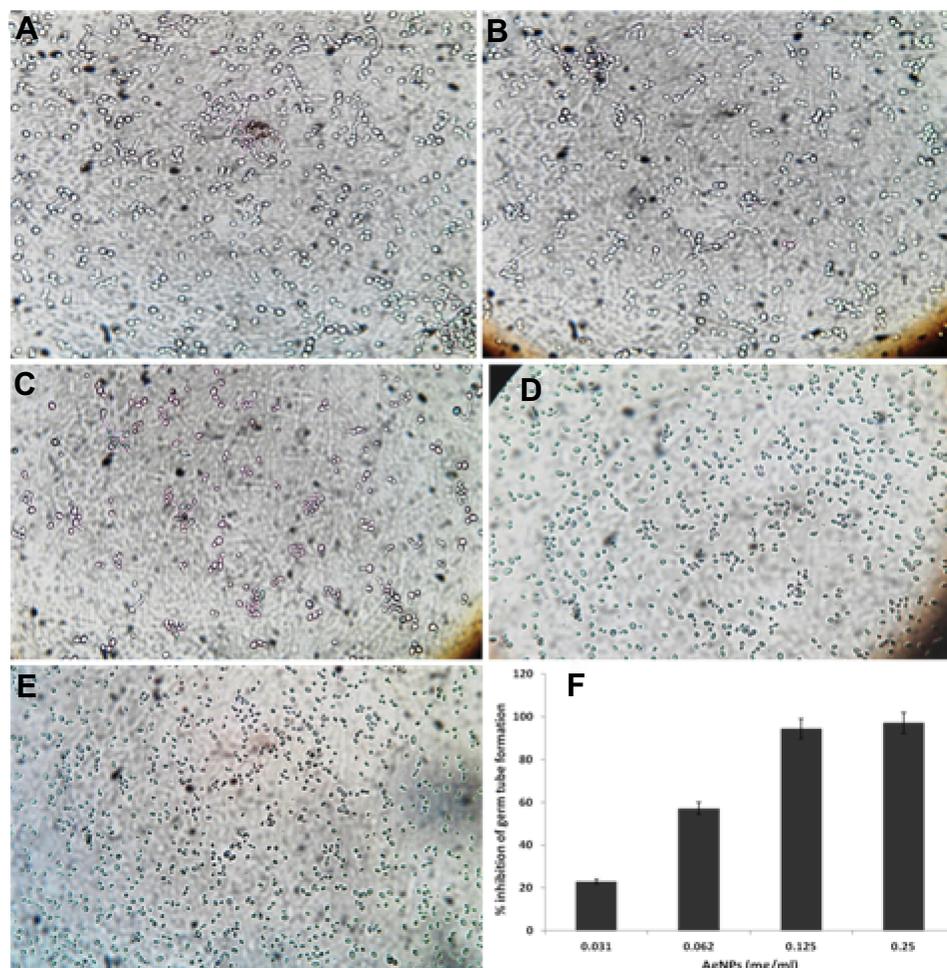


Figure 10 Effects of different concentration of ScAgNPs on germ tube formation of *C. albicans*. (A) control; (B) 0.031; (C) 0.062; (D) 0.125; and (E) 0.250 mg/mL of ScAgNPs. (F) Depicts the percentage inhibition of germ tube formation at various concentrations of ScAgNPs.

Abbreviation: ScAgNPs, *Syzygium cumini* silver nanoparticles.

22.9% at concentrations of 0.25, 0.125, 0.062, and 0.031 mg/mL, respectively (Figure 10F). Similar results were previously reported by Jalal et al,²⁴ who reported that ZnO NPs synthesized by leaf extract of *Crinum Latifolium* inhibit germ tube induction by 86.4% but at a high concentration, ie, 1.0 mg/mL²⁴. However, in the present study ScAgNPs inhibit germ tube formation by 97.1% at 0.25 mg/mL, which is 4-times lesser than that of ZnO NPs. Recently,⁴⁷ it was reported that CuO NPs at 300 mg/L completely inhibit the germ tube formation in *Candida* spp. However, the exact mechanism of germ tube inhibition by nanoparticles is not clear. Halbandge et al⁴⁸ reported that biosynthesized AgNPs affect Ras-mediated signal transduction pathways in *C. albicans* by downregulating the expression of cell elongation gene (ECE1), hyphal inducer gene (TEC), and yeast to hyphal transition genes (TUP1 and

RFG1) which are important for yeast to hyphal (Y-H) form transition.⁴⁸

Visualization of *C. albicans* biofilm by congo red agar and CLSM

Another most important virulence factor that plays a major role in pathogenesis in host is biofilm formation by *C. albicans*. *C. albicans* has the ability to build biofilms more or less on all kinds of medical device, eg, cardiac valves, indwelling prosthetic devices and catheters, joint prostheses, vascular and urinary catheters, ventricular assist devices, artificial vascular bypass devices, and pacemakers.⁴⁹ The extracellular polymeric substances act as a barrier to prevent the diffusion of drugs.⁵⁰ Further, it has been reported that sessile cells within biofilms are more difficult to eradicate, and they have the

ability to resist drug concentrations even 1,000-fold higher than the IC₅₀ reported for the planktonic yeasts.⁵¹ Therefore, there is an urgent need to design and develop novel anti-andidal and antibiofilm agents against these unmanageable infections. In the present study, qualitative inhibition of biofilm formation in *C. albicans* by ScAgNPs examined on BHIA supplemented with Congo red shows that the colonies of untreated *C. albicans* were black, which indicates the production of exopolysaccharide (EPS). However, cells treated with ScAgNPs not only inhibit the production of exopolysaccharides, but also inhibit the growth of cells (Figure S1). Muthamil et al⁵² reported that AgNPs strongly inhibit the EPS production in *C. albicans*, *C. glabrata*, and *C. tropicalis*. The visualization and quantification of biofilm and distribution of live and dead cells after treatment of ScAgNPs were evaluated by CLSM using LIVE/DEAD biofilm viability fluorescent stains, ie, ConA-FITC and PI. ConA (carbohydrate-binding lectin protein) conjugated with FITC is a green fluorescent stain which was used to study their binding to biofilms exopolysaccharide and live cells within the matrix. In contrast, PI is a red-fluorescent nucleic acid stain which can penetrate the cells with damaged membranes. Thus, *Candida* cells with intact cell membranes (ie, live) are fluorescent green, whereas cells with damaged membranes (ie, dead) are fluorescent red. In the present study, CLSM analysis showed that ScAgNPs not only act on the biofilm cells, but also penetrate and damage the exopolysaccharides matrix. It was observed that ScAgNPs at a concentration of 50 µg/mL resulted in almost complete inhibition of biofilm formation in *C. albicans* (Figure 11). Recently, AgNPs synthesized by *Dodonaea viscosa* and *Hyptis suaveolens* leaf extract have inhibited biofilm formation of *Candida*

spp. from 79 to 88% at 10 µg/mL.⁵² Monteiro et al⁵³ reported that colloidal suspensions of AgNPs at 54 µg/mL inhibit the biofilm formation approximately 54 and 90% in *C. albicans* and *C. glabrata*, respectively. The exact mechanism of inhibition of biofilm formation by AgNPs is not known. Rózsalska et al⁵⁴ suggested that antibiofilm activity was due to the extremely easy binding and enhanced penetration of the AgNPs into the biofilm structure which disturb the lipidome of cell membranes.⁵⁴ Another possible mechanism of antibiofilm activity of AgNPs could be due to inhibition of yeast morphogenesis. Inhibition of blastospores and hyphae forms by AgNPs also lead to the suppression of biofilm formation in *Candida*.⁵⁴ Lara et al⁵⁵ reported that the antibiofilm effect of AgNPs was mainly due to the disruption of the cell wall and survival of both the yeast and the filamentous forms of the *Candida* spp.

Morphological and ultrastructural alteration caused by ScAgNPs

Finally, the morphological and ultrastructural alteration caused by ScAgNPs on *C. albicans* was analyzed by TEM. It was observed that *C. albicans* cells treated with 50 and 100 µg/mL of ScAgNPs exhibited significant alterations in the cell wall and membrane (Figure 12). TEM analysis clearly shows that AgNPs not only attached and accumulate to the cell wall and membranes but also penetrates inside the cells and accumulated in the cytoplasm (Figure 12, red arrows) that may lead to the rupturing of the cell wall and disintegration of the cytoplasmic membrane (Figure 12, black arrows). The

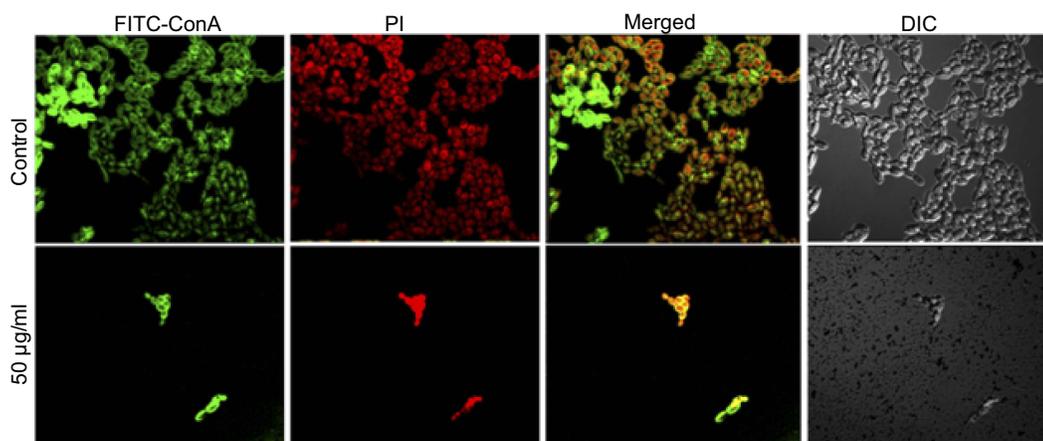


Figure 11 CLSM image of *C. albicans* biofilm. (A) Control. (B) *C. albicans* biofilm treated with 50 µg/mL of ScAgNPs. Biofilms were stained with ConA-FITC and PI. ConA-FITC stained *C. albicans* cells as well as exopolysaccharide matrix green. PI stained nucleic acid and fluorescent red.

Abbreviation: ScAgNPs, *Syzygium cumini* silver nanoparticles.

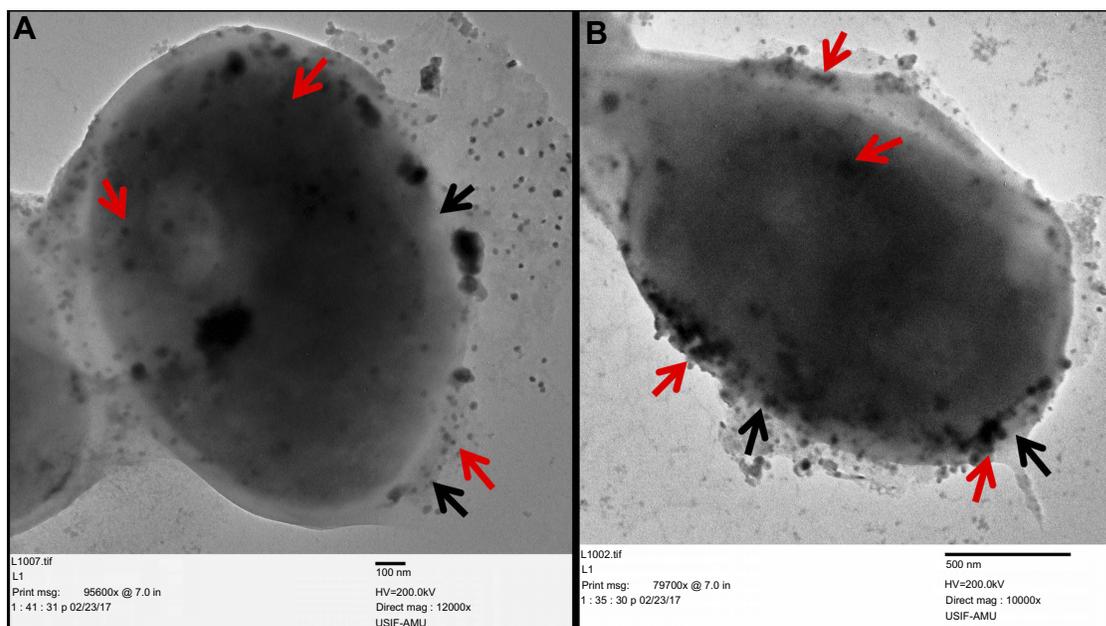


Figure 12 Ultrastructure alteration in *C. albicans* after treatment with 25 and 50 $\mu\text{g}/\text{mL}$ of ScAgNPs. The red arrows indicate attachment and internalization of AgNPs, whereas black arrows represent damage to the integrity of the cell wall and cytoplasmic membrane after treatment with AgNPs.

Abbreviation: ScAgNPs, *Syzygium cumini* silver nanoparticles.

present TEM result is in good agreement with the previous reports on ultrastructural analysis of the effects of AgNPs on *C. albicans*.³⁸ Anticandidal mechanisms of nanomaterials are not fully understood. Kim et al⁵⁶ reported that AgNPs damage the cell wall and membrane of *C. albicans* due to the formation of “pits and holes” on the cells surface that inhibits the budding process and finally leads to cell death. Gutierrez et al.³⁵ reported that the fungistatic effect of AgNPs was due to the inhibition of β -glucan synthase, and the fungicidal effect was due to changes in the cell wall integrity, and loss of its mechanic resistance that leads to the cell destruction by osmotic pressure variations. However, in another study, it has been reported that AgNPs promote mitochondrial dysfunctional apoptosis, phosphatidylserine externalization, DNA, and nuclear fragmentation, and the activation of metacaspases in *C. albicans* due to programmed cell death through generation and accumulation of intercellular ROS.⁵⁷ Further, Radhakrishnan et al.⁵⁸ reported that generation of intracellular ROS is not the only major cause of *C. albicans* toxicity, and they found that AgNPs altered surface morphology, membrane fluidity, cellular microenvironment and ultrastructure, cellular ergosterol content, and fatty acid composition, especially oleic acid, which is vital for hyphal morphogenesis.⁵⁸

Conclusion

In the present study, the biosynthesized ScAgNPs strongly suppressed the multiplication, germ tube and biofilm formation, and most importantly secretion of hydrolytic enzymes (viz. phospholipases, proteinases, lipases, and hemolysin) by *Candida* spp. The finding of the present study suggested that ScAgNPs could be employed as promising antifungal drugs to prevent the progress of pathogenesis in *Candida* spp by inhibiting the key virulence factors and development of biofilms on medical devices by applying ScAgNPs coating on medical devices and catheters. The present research work opens several avenues of further study, such as to explore the molecular mechanism of inhibition of germ tubes and biofilm formation and suppression of production of various hydrolytic enzymes by *Candida* spp. In vivo studies however, need to also be carried out to determine the biocompatibility, cytotoxicity, safety, and mode of action of AgNPs before being used for biomedical applications.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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Supplementary material

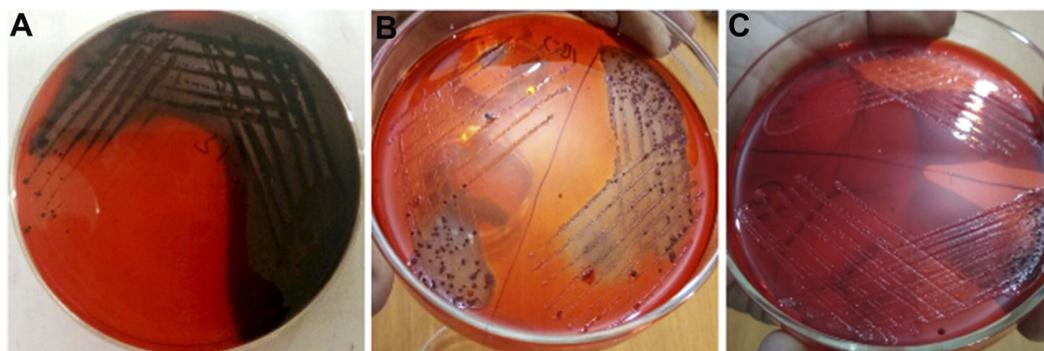


Figure S1 Ability of biofilm formation of *C. albicans* on BHI agar supplemented with Congo red and ScAgNPs. (A) Control (without ScAgNPs) showing black crystalline colonies indicate the exopolysaccharides production. Plates (B) and (C) treated with 0.025 and 0.05 mg/mL of ScAgNPs showing inhibition of exopolysaccharide synthesis. **Abbreviation:** ScAgNPs, *Syzygium cumini* silver nanoparticles.

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