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Fabricated silver nanoparticles by a combination of cell-free supernatant of *Fusarium solani* and *Comamonas aquatica* and its antibacterial activity

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Abstract

The current study involves silver nanoparticles (AgNPs) synthesis, characterization, and antimicrobial activity of nanoparticles produced by a combination of cell-free supernatant (C-FS) of the intimate organisms, *Fusarium solani* and *Comamonas aquatica* as synthesis catalysts against Gram-negative and positive human pathogens. The detailed characterization of the Ag NPs was carried out using UV-visible spectroscopy, field emission Scanning Electron Microscopy (FE-SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM). From the UV-visible spectroscopy, the absorption peak was found at 442 nm, and FE-SEM images confirmed the formation of AgNPs. Further, TEM and AFM analysis demonstrated that fabricated AgNPs were relatively monodispersed, approximately spherical, and of the size between 2.0 - 7.5 nm. Furthermore, the antibacterial activity of AgNPs was determined by the agar well diffusion method, and results showed that AgNPs exhibited excellent antimicrobial activity against Gram-negative *(E. coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*) and Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*). Finally, The MIC test was performed to test the inhibitory concentration of $AgNO_3$ against the bacteria under investigation. This is the first study proposing alternative sources to form AgNPs via synergistic metabolites of *F. solani* and *C. aquatica*. The results here offer a foundation for developing an effective therapy using AgNPs against various microorganisms which can endanger human beings.

Introduction

In the last decades, the rate of antibiotic resistance has considerably elevated as pathogens evolve a variety of mechanisms for the resistance to antibiotics ***[1]***. As a phenomenon, antibiotic resistance is often associated with infection and is also related to virulence ***[2]***. Most of the currently used antibiotics are becoming inefficient against multidrug-resistant microorganisms. Therefore, it is necessary to search for alternative solutions to mitigate resistance associated with pathogenic bacteria ***[3]***. Recently, nanomaterials such as nanoparticles (NPs) have received enormous attention because of their unique size-dependent electrical, optical, physical, and chemical properties ***[4]***. In particular, silver nanoparticles (AgNPs) possess a broad spectrum of highly efficient antimicrobial, anticancer, anticoagulant, anti-inflammatory, and antibiofilm activities ***[5, 6, 7]***. Other biological activities of AgNPs have also been explored, include the promotion of bone healing ***[8]*** and wound repair ***[9,***

10], which make them an ideal candidate in medical applications. In addition, silver represents less toxicity towards humans at lower concentrations. Thus, it has been widely incorporated with drugs in various forms such as salts, immobilized ions, or nanoparticles ***[11]***. The exact mechanism behind the antibacterial efficacy of AgNPs is still not precise. However, there are various proposed mechanisms of action, including disturbance of the cell membrane, alteration of cellular DNA and proteins, respiratory chain blockage by binding with enzymes, or the generation of reactive oxygen species (ROS), which lead to cell death ***[5, 12]***.

The standard methods used to produce AgNPs are chemical and physical. However, physical processes have low yields, whereas the chemical ones have harmful effects on the environment due to the use of toxic solvents and the regeneration of hazardous byproducts, limiting the use of AgNPs for clinical application ***[13, 14]***. Lately, researchers have turned to green methods (biogenic) for the synthesis of nanoparticles as an alternative ***[15,16]***.

The biogenic methods are mainly carried out by utilizing microorganisms, like bacteria, yeasts, fungi, and plants extracts ***[17]***. The main principle of all mechanisms of green synthesis nanoparticles is the reduction reaction ***[18]***. Microorganisms are essential Nano factories that can accumulate and detoxify heavy metals due to various reductase enzymes that can reduce metal salts to metallic nanoparticles ***[5]***. Biosynthesis processes are relatively simple, clean, sustainable, economical, and enable NPs to be obtained with lower toxicity, better physicochemical characteristics, and higher stability ***[19, 20]***. Biogenic synthesis of nanoparticles can be performed using microorganisms or the byproducts of their metabolism, which act as reducing and stabilizing agents ***[21]***. It is well known that many microbes, both unicellular and multicellular, can fabricate NPs either intra or extracellularly ***[22]***. The extracellular biogenic synthesis of the NPs is economical compared to the intracellular method because of the simplicity of the production process. Intracellular biogenic synthesis requires some additional steps, such as the application of suitable detergents or ultrasonic treatment for release of the synthesized NPs ***[23,24]***. Among microorganisms, filamentous fungi have advantages over other microorganisms for biogenic synthesis of NPs for their high tolerance to metals, high secretion of proteins, enzymes, and metabolites that contribute to the stability of the NPs, easy handling in large-scale production, and low-cost requirements for production procedures ***[25, 26]***. In addition, bacteria have received much attention in the biogenic synthesis of NPs due to their fast-growing capabilities, ease of handling, and ability to perform genetic modification ***[27]***.

In the present study, the tested hypothesis is that the intimate connection and synergistic associations between Gram-negative *Comamonas aquatica* and filamentous fungi *Fusarium solani* actively synthesize stable and robust silver nanoparticles (AgNPs) extracellularly. Therefore, the objective of this study is to use cell-free supernatant (C-FS) of *C. aquatica* and *F. solani* cooperatively for the biosynthesis of stable AgNPs. In addition, the synthesized AgNPs will be further characterized by UV-Visible absorption spectra and confirmed by FE-SEM, TEM, and AFM. Furthermore, AgNPs will be evaluated for their antimicrobial activity against clinical isolates Gram-negative and Gram-positive pathogens.

Materials and methods

A. Source of microorganisms

C. aquatica and *F. solani* were previously isolated and identified ***[28]***. For the antimicrobial experiment, Gram-negative and Gram-positive bacteria were used, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica, Enterococcus faecalis*, and *Staphylococcus aureus,* which were collected from Sulaymaniyah Teaching Hospitals and identified using VITEK2 ID card (BioMerieux, USA).

B. Synthesis of AgNPs using cell-free supernatant (C-FS)

The local isolates *C. aquatica* and *F. solani* were used for the synthesis of AgNPs. Fresh cultures of *C. aquatica* and *F. solani* were grown separately in a 250 mL Erlenmeyer flask that contained 100 mL of nutrient broth. Both organisms were incubated at 30°C for 48 and 72 hours for both the bacteria and the fungus. The supernatants of the cultures were collected by centrifugation (Biofuge Stratose, Germany) at 5,000 rpm for 20

minutes at 4°C in sterile tubes. Three Erlenmeyer flasks were prepared; the first containing $AgNO_3$ solutions (Sigma, USA, purity 99.9%) without the supernatant (used for comparison), the second containing only the supernatant (also used for comparison), and the third containing equal amounts of the bacteria and fungi supernatants. The latter was mixed with 1.0 mM of filter-sterilized $AgNO_3$ solution as a final concentration. Reaction mixtures were incubated for 72 hours at room temperature (~25°C) under static conditions. The extracellular synthesis of AgNPs was monitored by visual inspection of the flasks for a color change. The AgNPs were collected by high-speed centrifugation at 14,000 rpm for 20 minutes at 4°C using a cold centrifuge (Mikro 200R, UK). Supernatants were discarded, and pellets of AgNPs were washed three times with autoclaved distilled water to remove the unconverted metal ions or any other constituents. The obtained precipitation was kept in Petri dishes and left in the oven to dry at about 40°C for 24 hours (WTC Binder, Germany). The dried AgNPs were scraped out and obtained in powder form for further study ***[29, 30]***.

C. Characterization of AgNPs

C.1. UV–visible spectral analysis

The synthesized NPs were characterized based on specific surface plasmon resonance peaks, shapes, and sizes. In addition, the bio-reduction of Ag+ ions was monitored with UV-visible spectrum and was recorded with UV-vis spectrophotometer (Cary 60 Agilent, USA) with a resolution of 2 nm within a range of A_{300} to A_{800} nm ***[31]***.

C.2. Field emission scanning electron microscopy (FE-SEM)

Morphological characteristics of AgNPs were further characterized via field emission scanning electron microscopy (Quanta 450, Netherlands). The sample for FE-SEM was prepared by placing a drop of AgNPs solution on a sample holder and dried at room temperature, then coated by 300 Å gold and transferred for analysis to see a better resolution image ***[29]***.

C.3. Transmission Electron Microscopy (TEM)

The shape and size of the AgNPs were determined by TEM (PHILIPS model CM120, 137 Netherlands) operated at an accelerating voltage of 100 kV. The samples for TEM analysis were prepared by dispersing the samples in distilled water through sonication for 15 minutes. Then, AgNPs were poured on the carbon-coated copper grids and left to dry at room temperature, and scanned. At least three images of each sample were taken to have a clear representation of its morphology ***[32]***.

C.4. Atomic force microscopy (AFM)

The surface topography and morphology of AgNPs were studied by atomic force microscopy. A thin layer of the sample was prepared by dropping 100 μL of the sample on a glass slide and drying for 5 minutes. The slides were then scanned with AFM (BRUKER, ICON Instruments, USA). The AFM images were then taken with silicon cantilevers in contact mode. The AFM images were processed using ICON data processing software ***[33]***.

D. Antimicrobial activity of AgNPs

D.1. Agar well diffusion assay

Agar well diffusion assay method was conducted to analyze the antimicrobial activity of AgNPs according to a procedure obtained from previous work ***[34]***. Then, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Staphylococcus aureus* were grown overnight in nutrient broth (NB), and *Enterococcus faecalis* was grown in Luria-Bertani broth (LB), and all of the cultures were incubated at 37°C. The number of cells per milliliter was adjusted to be 10^8 CFU/mL, equivalent to 0.5 McFarland. Then, 100 µL of each culture broth isolate was spread evenly on Müeller-Hinton agar plates. Wells were made using gel puncture, and 100 µL of different concentrations of AgNPs (12.5, 25, 50, 75, and 100 %) were loaded into the designated wells. In addition, 100 µL broth was used for comparison as a control. The plates were incubated at 37°C for 24 hours, and the zone of inhibition was measured (mm). The assay was done in triplets.

D.2. Minimum inhibitory concentration assay (MIC)

Fresh overnight cultures of the bacterial isolates were adjusted to be 10^8 cells/mL, as mentioned previously. A bacterial culture of 120 µL was dispensed in 96-wells of sterile polystyrene microtiter plates, and then 80 µL of an appropriate concentration of AgNPs (3, 6, 9, 12.5, 25, 50, 75, and 100 %) was added. From the broth, 200

µL was used as a negative control, and 120 µL of bacterial culture mixed with 80 µL broth was applied as a positive control. The microdilution trays were incubated at 37°C overnight under a gentle shaking in the microplate incubator-shaker PST-60 HL Plus (BOECO, Germany). The absorbance of each well was measured at 600 nm using a microtiter ELISA reader (Biotech µQuant, USA). In addition, 5 µL was taken from each well and spotted onto Nutrient Agar (NA) plates, and then the plates were incubated at 37°C for 18 hours. Growth percentage was calculated based on the average and standard deviation of triplicate results. MIC was carried out according to the procedure of the previous study [35].

Results and discussions

A. AgNPs synthesis and characterization

In this study, we found that the combination of *F. solani* and *C. aquatica* supernatants could substantially reduce silver salt into silver nanoparticles. Initially, the extracellular biogenic synthesis of AgNPs was confirmed throughout the visual color change in the reaction mixture and via UV–vis spectroscopy (Fig.1). The color change started after about 25 minutes of mixing the C-FS with $AgNO_3$ solution. Gradually, the intensity of the reaction mixture changed from pale yellow to dark brown after about 18 hours of incubation due to the reduction of Ag^+ and the formation of the silver nanoparticles. A previous study reported that AgNPs in aqueous solutions show a dark brown color and displayed a broad peak at 408-411 nm ***[34]***. Such changes in the color of the solution are due to the excitation of the surface Plasmon resonance (SPR) with the nanoparticles ***[36]***.

Additionally, the formation of the AgNPs was confirmed by UV-visible spectrophotometric analysis in the range of 300 nm to 800 nm. The solution showed the characteristic SPR at 442 nm. This finding is in agreement with several other studies conducted by Singh *et al*. (2016) and Gopinath *et al*. (2015) ***[5, 37]***. According to previous studies, several proteins, free amino acids, and NADH-dependent reductases ***[23, 38]*** secreted by the fungus and bacteria were responsible for reducing Ag ions and biogenic synthesis nanoparticles.

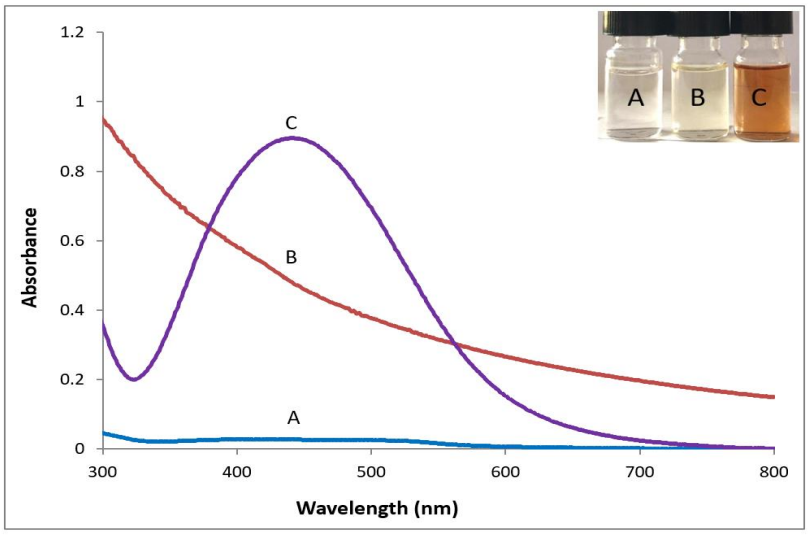

Figure1. The UV-Visible absorption spectrum of AgNPs using C-FS of *F. solani* and *C. aquatica* under light conditions at 25°C. (A) $AgNO_3$ solution (1 mM) control. (B) cell-free supernatant of *F. solani* and *C. aquatica* (control). (C) cell-free supernatant of *F. solani* and *C. aquatica* with $AgNO_3$ solution (1 mM). **Inset** shows biogenic silver nanoparticles–visual observation. (A) $AgNO_3$ solution without supernatant (no color change). (B) cell-free supernatant, (no color change) and (C) cell-free supernatant and $AgNO_3$ solution, (brown).

B. FE-SEM, TEM, and AFM

FE-SEM was applied to determine the formation of (AgNPs). After drying, the thin layer was analyzed, and the NPs were photographed. The FE-SEM confirmed the presence of high-density of AgNPs with aggregations (Fig. 2A). Accumulation of the particles may have occurred during the drying process that could have affected their sizes and shapes. Similar observations have been reported before ***[37, 39]***. The data obtained from TEM

images show that the synthesized AgNPs mostly have spherical shape and range in size from 3-5 nm and relatively monodispersed with a few agglomerated particles (Fig. 2B). The particle size distribution histogram extracted from TEM images confirmed that the size of AgNPs range between 2.0 - 7.5 nm with an average diameter of 4.5 nm (Fig. 2C). AbdelRahim *et al*. (2017) ***[15]*** synthesized spherical, monodispersed AgNPs from *Rhizopus stolonifer* with a diameter around 9.47 nm. Rudakiya & Pawar (2017) [30] obtained AgNPs from *C. acidovorance* with 6-53 nm having spherical, oval, and irregular shapes with a smooth surface. Also, previous findings have reported that mycosynthesized AgNPs have spherical shapes that range from 21.3-37.3 nm in size and were uniformly distributed without significant agglomeration ***[34]***. The surface topography and morphology of AgNPs were confirmed by AFM imaging (Fig. 2D). The three-dimensional configuration of the AgNPs indicates that the surface topography of the synthesized AgNPs was almost spherical. Gopinath *et al.* (2015) ***[37]*** synthesized AgNPs using the fungus *F. oxysporum* which showed a spherical arrangement of silver nanoparticles with a diameter in the range of 6.3-12.67 nm using AFM. Another study conducted by Hamzah *et al*. (2018) ***[35]*** indicated that the surface topography of AgNPs synthesized by fungus *F. mangiferae* was almost spherical in shape.

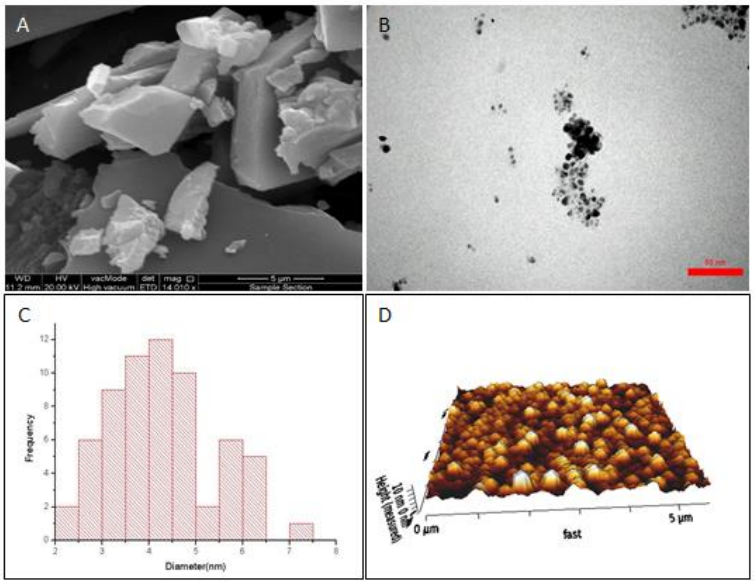

Figure 2. FE-SEM, TEM, and AFM of the synthesized silver nanoparticles. A) FE-SEM image. B) TEM image of relatively spherical-shaped AgNPs (scale bar is 50 nm) C) Histogram analysis of the particle size distribution. D) 3D AFM image.

C. Antimicrobial activity of AgNPs

C.1. Agar well diffusion assay

To investigate the ability of prepared AgNPs, we tested the biogenically synthesized AgNPs for their antimicrobial activity against clinical isolates Gram-negative (*E. coli, P. aeruginosa, S. enterica)* and Gram-positive (*E. faecalis and S. aureus*) bacteria. Results have shown that nanoparticles prepared by C-FS of *F. solani* and *C. aquatica* cooperatively proved effective against all of the bacterial species under investigation. In the case of the Gram-negative bacteria, at a concentration of 100% (v/v), the highest antimicrobial activity observed against *S. enterica* was 17 mm, followed by *E. coli* at 16 mm and the lowest inhibition zone marked against *P. aeruginosa*, which was 13 mm in diameter (Fig. 3).

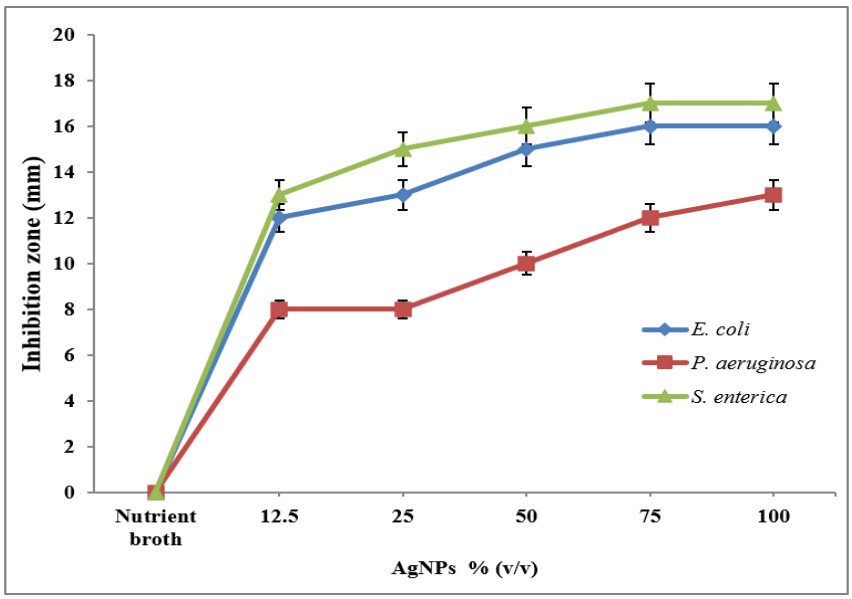

Figure 3. Inhibition zone assay of Gram-negative bacteria by synthesized AgNPs.

Furthermore, the inhibition zone against *E. faecalis* was studied and shown to be 16 mm and 15 mm against *S. aureus* (Fig. 4). At the same time, the well-containing broth culture (negative control) did not show any inhibition zones around the well. The bactericidal action of AgNPs may be due to the small size of the nanoparticles, as it has been shown in previous studies where smaller nanoparticles had more excellent antimicrobial effects ***[40, 41]***. A possible reason is that a greater surface area in contact with the cells facilitates membrane rupture and internalization *[42]*. Singh *et al*. (2018) ***[31]*** have proved that the antibacterial activity of AgNPs synthesized by *Pseudomonas* sp. have significant antimicrobial activity against various pathogens at a concentration of 30 µL. Another study conducted by Gopinath *et al*. (2015) and Saxena *et al*. (2016) ***[37, 43]*** showed an excellent inhibitory zone against all tested bacteria at a concentration of 40µg/ml AgNPs.

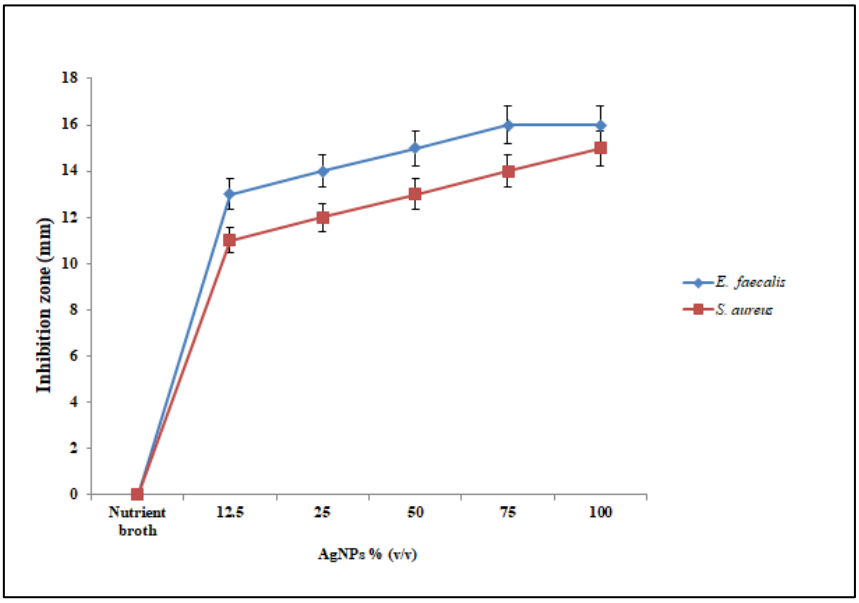

Figure 4. Inhibition zone assay of Gram-positive bacteria by synthesized AgNPs. X-axes represent different concentrations of AgNPs and Y-axes represent inhibition zone in (mm).

C.2. Minimum inhibitory concentration assay (MIC)

Antimicrobial activities of biogenic AgNPs in the current study have been investigated against both Gram-negative and Gram-positive bacteria. The effectiveness of AgNPs on clinical isolates was assessed by

comparing growth rates under control and test conditions. The lowest concentration, which exhibited no visible growth than the control wells, was considered the MIC value. Interestingly, our biogenic AgNPs showed potent antibacterial activity against all pathogens at the lowest concentrations. In the case of Gram-negative bacteria, 9% concentration (v/v) of NPs inhibited the growth of *E. coli* and *P. aeruginosa*, by about 63% and 90%, respectively, and complete bacterial growth was inhibited at a concentration of 12.5% (Fig. 5). In addition, the concentration of NPs at 12.5% showed a significant effect on *S. enterica*, which has resulted in about 65% growth inhibition and total inhibition at a concentration of 25% (v/v).

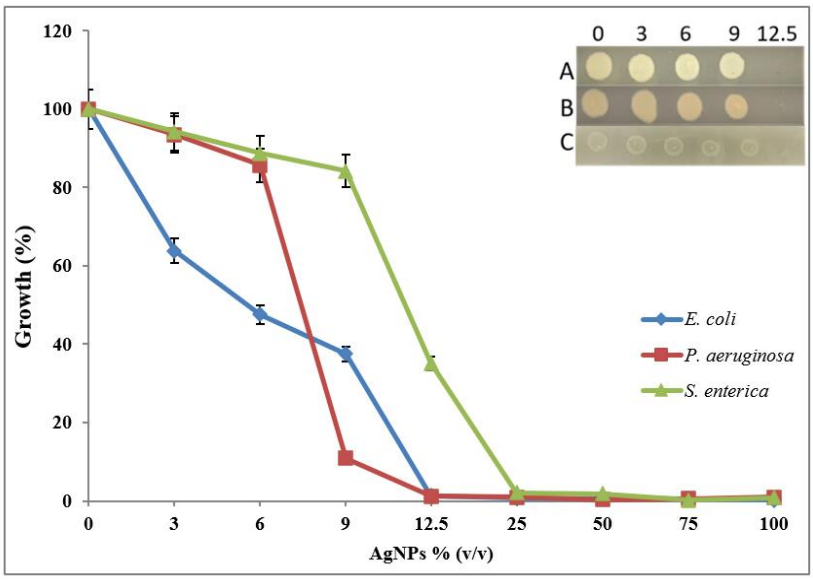

Figure 5. Effect of different concentrations of biosynthesized silver nanoparticles (AgNPs) on the growth of Gram-negative pathogenic bacteria using microtiter plate technique. **Inset:** Five µL was taken from each well and spotted onto the (NA) agar plates.

In the case of Gram-positive bacteria, a concentration of 9% NPs completely inhibited the growth of *S. aureus*, but only 28% growth inhibition for *E. faecalis* at same concentration was observed (Figure 6). However, the growth of *E. faecalis* was inhibited completely at a concentration of 12.5%.

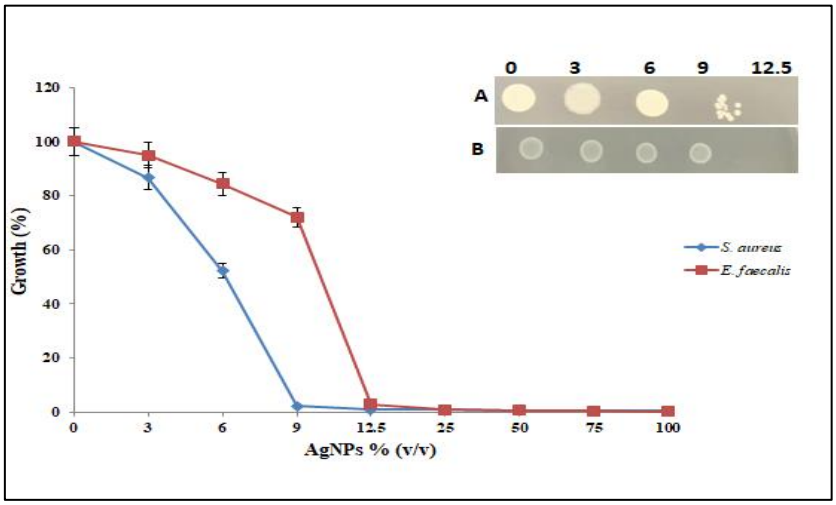

Figure 6. Effect of different concentrations of biosynthesized silver nanoparticles (AgNPs) on the growth of Gram-positive pathogenic bacteria using microtiter plate technique. **Inset:** Five µL was taken from each well of *S. aureus* and *E. faecalis* and spotted onto the NA and LB agar plates, respectively.

The growth of both Gram-negative and Gram-positive bacteria used in this study was shown to be inhibited significantly. Several mechanisms have been proposed for the bactericidal activity of AgNPs. However, the exact mechanism of bactericidal action of AgNPs remains unclear. One of the most accepted mechanisms is that the direct contact of AgNPs with large surface areas on a bacterial cell wall could produce pits resulting in the leakage of cellular contents and, eventually, cell death [44]. In certain cases, small nanoparticles of size less than 10 nm particularly, can penetrate the cytoplasm and damage the respiratory chain enzyme, generating reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl (OH–) and superoxide (O_2 −) radicals that induce oxidative stress and cause damage to proteins, reduce transcriptome and cause cell death [45]. Also, Agnihotri *et al*. (2014) ***[46]*** have investigated various sizes of AgNPs against *E. coli* and showed that AgNPs with sizes below 10 nm exhibited the best antibacterial activity. Besides, cell membranes carry a negative charge due to the presence of lipopolysaccharide, peptidoglycan, and multiple groups, including carboxyl, amino, and phosphate groups. Thus, positively charged silver ions can facilitate the adherence of AgNPs on bacterial membranes through electrostatic interaction between the bacteria and the treated surface, causing a structural change of bacterial cell wall and disruption of metabolic process which results in the degradation of cell wall and, finally, cell death ***[45]***.

Conclusion

In this study, we found that the C-FS combination of *F. solani* and *C. aquatica* showed synergistic effects for AgNPs synthesis. The promising activity of our fabricated AgNPs against both Gram-positive and Gram-negative pathogens suggests its potential to be used as an alternative therapeutic agent for certain medical conditions. Our results revealed that the C-FS of *F. solani* and *C. aquatica* has potential metabolites that reduce the silver into nanoparticles, shedding light on the interaction between both organisms. Moreover, small-sized and spherical-shaped AgNPs are supposedly stable particles. The promising antibacterial activity enables these nanoparticles as potential bactericidal material for various environmental and biomedical applications such as water treatment, food packaging films, healthcare products, antimicrobial textiles, and wound dressings. Although the obtained AgNPs show is promising antibacterial agents, further research is strongly recommended to investigate the safe usage of AgNPs.

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