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Original Research Article

Biosynthesis of Silver Nanoparticles from *Aspergillus flavus* and Aqueous Extract of *Gnetum africanum*, *Anacardium occidentale*, and *Landolphia owariensis*: Characterization and the Antimicrobial Evaluation

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ABSTRACT

The rapid increase in microbial infections and the failure of conventional therapeutic agents (antibiotics) are currently a global public health threat. However, nanoparticles can be used as a potential treatment alternative. This study investigated the antimicrobial potential of silver nanoparticles (AgNps) biosynthesized from culture cell-free supernatant of *Aspergillus flavus* (Af) and aqueous extract of plants: *Gnetum africanum* (Ga), *Anacardium occidentale* (Ao), and *Landolphia owariensis* (Lo). The AgNps were synthesized and characterized respectively, using standard procedures. Their antimicrobial efficacy were determined against *Staphylococcus spp*, *Klebsiella spp*., and *Salmonella spp*. using the agar well diffusion method with ciprofloxacin (CPX) as a positive control. The AgNps were confirmed by visible colour change in the reaction mixtures of the $AgNO_3$ solution and the samples. The characterization of the AgNps through UV-Visible spectroscopy showed that Ao-AgNps, Lo-AgNps, Ga-AgNps, and Af-AgNps had maximum absorbance at a wavelength of 439, 437, 428, and 453 nm respectively which correspond to the excitation of surface plasmon resonance (SPR). SEM and TEM studies revealed irregular spherical nanoparticles with sizes around 100 nm. The aqueous leaf extract synthesized AgNps displayed low antibacterial activity ($P < 0.05$) (possibly due to environmental conditions) against the test organisms compared to CPX except for Ga-AgNps with a substantially high inhibitory effect against *Klebsiella spp*. only. More so, the fungal biomass (Af-AgNPs) synthesized AgNps displayed substantial inhibitory efficacy against all the tested pathogens. This study reveals the potential antimicrobial abilities of the AgNps of the fungus and plant extracts.

Keywords: Silver nanoparticles, Biosynthesis, Antibacterial activity, *Gnetum africanum*, *Anacardium occidentale*, *Landolphia owariensis*.

Introduction

Despite the vast advances in public health, microbial infections are still on the increase. According to the Center for Disease Control (CDC),[1] resistance to antimicrobial agents is one of the most prominent public health burdens globally. The rapid increase in antimicrobial resistance is a global threat to current medicine, often leaving clinicians with no reliable option to manage infected patients.[2] Additionally, the failure of conventional antibiotics shows the need to develop an alternative treatment option, and nanoparticles promise to be a viable alternative.[3]

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The increasing interest given to nanoparticles is not surprising as they are particles with novel and intriguing properties with sizes ranging between 1-100 nm.[4] These particles are key components of nanotechnology and have been used in various sectors of human affairs to improve the standard of living.[5] They have received considerable attention due to their wide application. Generally, in molecular biology, nanotechnology have found extensive applications ranging from their use in targeted drug therapy and diagnostics to tissue regeneration and cell culture.[6] They have also been applied as antimicrobial agents in related fields such as agriculture,[7] pharmaceuticals,[8] cosmetics[9] and other consumer products, etc.[10]
More so, the application of nanoparticles has received accolades in the last decades due to their defined chemical, optical and mechanical properties as well as their large surface area to volume ratio.[11] This has instigated their interest in many multidisciplinary fields as mentioned above. More importantly, metallic-based nanoparticles are the most promising antimicrobial agents because they have a broad spectrum of antibacterial activity facilitated by their non-specific bacterial toxicity mechanisms which makes it difficult for bacteria pathogens to develop resistance, since they do not have a specific receptor binding site in the bacterial cell.[12] Different types of metallic and metal oxide nanoparticles such as silver nanoparticles (AgNps),[13] gold nanoparticles (AuNps),[14] zinc nanoparticles (ZnNps),[15] copper nanoparticles (CuNps),[16] titanium nanoparticles (TiNps),[17] and iron

nanoparticles (FeNps)[18] among others have been synthesized by different methods with evidence of good antimicrobial efficacy. However, the green synthesis of AgNps has attracted tremendous focus over the years by numerous researchers.[19]

Silver (Ag) has long been known to exhibit a broad-spectrum antimicrobial property. AgNps specifically has superior biological, chemical, and physiological attributes than other nanoparticles. Additionally, their well-structured shape, size, composition, and crystalline nature, low cytotoxicity, high thermal stability, and low volatility make them highly relevant.[20] These features have led to their extensive application in the biomedical sector. Therefore, AgNps represent a reasonable alternative for the treatment of drug-resistant infections, in addition to several other existing biomedical applications.[21] Several physical and chemical methods are available for the synthesis of metal nanoparticles. However, most of these methods are laden with several drawbacks such as the use of toxic chemicals, poor particle shape and size distribution, low yield of product, and the production of associated hazardous waste products, etc.[22] There is a growing need to develop an environmentally-friendly process for the synthesis of nanoparticles. This has given rise to the concept of green bio nanotechnology which involves the use of biological materials like plant extracts and microorganisms.[6] During the biosynthesis of nanoparticles, the extracts from plants and the metabolites produced by microbes serve as the reducing and capping agents; while appropriate reaction conditions are maintained to ensure high product yield, uniform shape, and particle size distribution of the synthesized nanoparticles.[23] This current research was aimed at assaying the antimicrobial activities of silver nanoparticles (AgNps) obtained from the culture cell-free supernatant of *Aspergillus flavus* isolated from the soil and the aqueous extract of selected plants; *Gnetum africanum* (Ukazi), *Anacardium occidentale* (Cashew), and *Landolphia owariensis* (Utu) against pathogenic clinical isolates.

Materials and Methods

Bacterial Identification and Characterization

All the bacterial isolates used in the study were cultivated on nutrient agar, blood agar (Oxoid, Basingstoke, UK), MacConkey agar (Biozyme Laboratories Ltd, San Diego, USA), chocolate agar (Oxoid, Hampshire, UK) and incubated at 37^{o}C for 24 h. The potato dextrose agar (PDA) (HiMedia, Mumbai, India) supplemented with chloramphenicol (0.05 mg/mL) was used for the cultivation of the fungi. The morphology (colour, elevation, edge, and density) of the colonies were thoroughly evaluated. The bacterial isolates were specifically identified through gram staining and standard biochemical characterization (citrate utilization test, catalase test, urease test, and sugar fermentation test) as described by Ezeonu *et al*.[24]

Collection and preparation of aqueous extract of the different plants

The leaf of the plants (*A. Occidental* (Cashew), *G. africanum* (Ukazi), and *L. owariensis* (Utuu)), grown locally were purchased in 2018 from a local market in Nsukka, Nigeria, and the aqueous extracts were prepared as described by Ahmed *et al*.[25] with slight modifications. They were properly identified by a certified Taxonomist, Mr. Ozioko, in the Department of Botany, University of Nigeria, Nsukka, Enugu State, Nigeria. The deposit voucher numbers are PGC/UNN/0031, PGC/UNN/0341, and PGC/UNN/0402 for *A. Occidental*, *G. africanum*, and *L. owariensis* respectively. Briefly, the leaves of different plants were carefully washed separately in water three times to remove dirt and then re-washed using distilled water. Further, 10 g of finely chopped leaves were suspended inside a conical flask containing 100 mL of distilled water and heated in a pot at 100^{o}C for 1 h. The solution was then filtered using Whatman filter paper no. 1 to obtain the aqueous extracts.

Preparation of fungal biomass

Similar to Birla *et al*.[26] the fungal biomass (*A. flavus*) obtained from the soil was prepared by inoculating the spores of a 7-day old culture on a PDA plate into a conical flask containing 120 mL of Potato dextrose broth and incubated at room temperature while shaking at intervals for 5 d. The fungal biomass was then obtained by filtration and washed with sterile distilled water. Thereafter, 13 g of the fungal biomass was suspended inside a conical flask containing 100 mL of sterile distilled water and incubated at room temperature for 48 h while shaking at intervals. The cell-free filtrate was used for the biosynthesis of AgNPs.

Biosynthesis of AgNps using the aqueous plant extracts

The aqueous extracts served as the reducing and capping agent while silver nitrate ($AgNO_3$) solution served as the metal precursor.[27] The $AgNO_3$ solution was prepared by dissolving 0.051 g of $AgNO_3$ salt in 100 mL of distilled water. Each plant extract (20 mL) was mixed with 20 mL of filter-sterilized (0.45 um) 3 mM $AgNO_3$ solution into a sterile 250 mL conical flask to make a ratio of 1:1. The mixture was further heated in a water bath at 100^{o}C for 15 min. The reduction of Ag+ to Ag0 was verified by a visual change of colour. The $AgNO_3$ solution without the plant extracts was used as the control.

Biosynthesis of AgNps using A. flavus cell-free filtrate

The cell-free supernatant was used as the reducing and capping agent.[28] In the brief procedure, the cell-free supernatant was mixed with filter-sterilized 3 mM $AgNO_3$ solution in the same volume as the plant-mediated synthesis to make a ratio of 1:1. The mixture was incubated under a dark condition at room temperature for 48 h while shaking at intervals. $AgNO_3$ solution without the cell-free supernatant (control) was incubated under the same conditions. The reduction of Ag^{+} was observed by a visible colour change.

Characterization of the biosynthesized AgNps

Similar to Saikhaldein *et al*.[29] fourier transform infrared spectroscopy (FTIR), UV spectroscopy, powdered X-ray diffractometer (PXRD), scanning electron microscopy (SEM), and transmission electron microscopy were used to characterize the biosynthesized AgNps. The FTIR spectra of the AgNps were recorded as a KBr pellet at a resolution of 4 cm^{-1} in the wavenumber region of 500–4000 cm^{-1} using FT-IR TENSOR27 PMA 50 (Brook, Germany). The Ag+ reduction was evaluated by measuring the UV Spectrum of the biosynthesized AgNps. UV spectra of AgNps were recorded as a function of wavelength using UV-Visible spectrophotometer (Shimadzu, Tokyo, Japan) with wavelength scan from 200-800 nm. The crystalline nature of the biosynthesized AgNps was studied with PXRD, and the diffraction patterns were recorded in the scanning mode on an Ultima IV (Kabuskiki Kaisha) X-ray diffractometer operated at 40 kV and with a current of 40 mA, with Cu/kα radiation (λ = 1.5418 Å) in the range of 10˚–60˚ in 2θ angles. The surface morphology of the biosynthesized AgNps was examined scanning electron microscopy (SEM), FEI-110730002486 instrument. In addition, JEOL (JEM-2100) transmission electron microscope was used to study the size and shape of the AgNps after dispersing in 100% ethanol before placing it on a carbon-coated copper TEM grid. The images were obtained by operating at an accelerating voltage of 200 Kv and a resolution power of 0.24 nm.

Determination of the antimicrobial activity of AgNps

The antimicrobial activities of the biosynthesized AgNps against three clinical isolates (*Staphylococcus spp*, *Klebsiella spp*, and *Salmonella spp*) were determined by the agar well diffusion method as described by Sanchooli *et al*.[30] Briefly, the cells were emulsified in sterile normal saline and standardized to 0.5 MacFarland (1×10^{6} CFU/mL). The standardized suspension of each tested isolate was swabbed uniformly onto sterile Muller-Hinton Agar (MHA) (Oxoid, Basingstoke, UK) plates using sterile cotton swabs. Wells of 6 mm diameter were bored into the agar medium using a 6 mm cork-borer. Using a micropipette, 5 drops of different AgNps solution were added into each well. After incubation at 37 ^{o}C for 24 h, the zones of inhibition were measured. Ciprofloxacin (CPX) (10µg) disc was used as a positive control.

Statistical Analysis

The obtained data were subjected to statistical analysis with SPSS software (version 23.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Tukey's multiple comparisons

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post-hoc test was used to assess and compare the differences in the antimicrobial potentials of the biosynthesized AgNps. Results with $P < 0.05$ were considered significant.

Results and Discussion

Biosynthesis of AgNps

The synthesis of AgNps was primarily confirmed by a visible colour change in the reaction mixtures. When the $AgNO_3$ solution reacted with the aqueous extract of *A. occidentale*, there was a rapid change in colour from the colourless cloudy mixture to a red cloudy mixture. The colour further deepened to reddish-brown indicating a rapid reduction of the silver ions to nanoscale silver. The application of heat to the mixture resulted in no further colour change. Upon the application of heat, the reaction mixture containing the aqueous extracts of *G. africanum* showed a colour change from pale green to reddish-brown while the aqueous extract of *L. owariensis* showed a rapid colour change from colourless to dark ash. In addition, after 48 h during the biosynthesis of AgNps using the cell-free filtrate of *A. flavus*, there was a visible colour change in the reaction mixture from its initial colourless to a dark brown indicating the formation of AgNps while the control showed no colour change.

The colour change observed during the biosynthesis of the AgNps is an indication of the synthesis of nanoparticles.[31] Bio-reduction of silver ions to zerovalent silver is mediated by enzymes such as nitrate reductase (NADPH-dependent reductases) and other proteins.[32] Thus, the production of this enzyme may be responsible for the reduction of Ag^+ and the subsequent production of AgNps.[33]

FT-IR Studies

The biological functional groups present in the synthesized AgNps from the aqueous plant extracts and fungal biomass were compared in Figure 1. The FT-IR spectra corresponding to the aqueous plant extract synthesized AgNps revealed the presence of flavonoids, phenols, tannins, alkaloids, and terpenes. Broad bands were obtained at 3450 cm -1 for Ao-AgNps and a series of weak bands at 1634-1599 and 1105 cm^{-1}. For, Lo-AgNps, broad bands were observed at 3443 cm^{-1} and weak bands at 2923, 1637-1400 and 1107 cm^{-1}. Go-AgNps displayed a broad band at 3471 cm^{-1} and weak bands at 2912, 1634-1397 and 1104 cm^{-1}, while Af-AgNps gave broad FT-IR bands at 3533-2926 cm^{-1} and other bands at 2354, 1671, 1146-1006 and 489 cm^{-1}. The phyto-constituents seen are most likely responsible for the capping and stabilization of the AgNps and prevention of agglomeration.[34] Specifically, broad bands obtained at 3450, 3443, 3471 cm^{-1} are associated with -OH stretching for Ao-AgNps, Lo-AgNps, and Go-AgNps respectively. Also, the presence of alkanic -CH stretching was revealed by the very weak bands at 2923 and 2912 cm^{-1} for Lo-AgNps and Go-AgNps respectively. However, not very prominent in Ao-AgNps. More so, series of weak bands appearing at 1634-1599, 1637-1400, and 1634-1397 cm^{-1} are corresponding to the phenolic C=C as well as other bands at 1105, 1107, and 1104 cm^{-1} tentatively assigned to C-O stretching were assigned to Ao-AgNps, Lo-AgNps, and Ga-AgNps respectively.[35]

Furthermore, the characteristic FT-IR broad band at 3533-2926 cm^{-1} for Af-AgNps could be attributed to the overlapping -OH and -NH groups due to glucose and proteins respectively present in the fungus cell. Also, the weak band at 2354 cm^{-1} is tentatively assigned to -CH stretching; while the band appearing at 1671 cm^{-1} could be associated with the amide band of *N*-acetyl glucosamine polymer or the protein-peptide bond. Additionally, the strong bands at 1146-1006 and 489 cm^{-1} may be associated with the C-O stretching of sugar alcohol and the scissoring of alkyl halide respectively of the biosynthesized AgNps from different aqueous plant extracts and fungal biomass.[36]

UV-Vis Spectroscopy Studies

The UV-Vis absorption spectra (Figure 2) showed the biosynthesized AgNps display characteristic absorption peaks ranging from 428 to 543. The results confirm the stability of the samples. The aqueous extracts of Ga-AgNps and Lo-AgNps showed broad peaks at 428 and 437 nm respectively, while Ao-AgNps displayed a series of weak absorption peaks at 345, 393, and 439 nm. Furthermore, the fungal biomass, Af-AgNps revealed a very broad peak at 453 nm.

The peaks correspond to the excitation of surface plasmon resonance (SPR) band for AgNps, i.e., an indication of Ag^+ reduction.[37] The broad peaks obtained in the study suggest that the synthesized AgNps are polydisperse, while the SPR bands appearing > 420 nm could be due to the large particle size distribution of AgNps.[38]

PXRD Studies

The degree of crystallinity of the biosynthesized AgNps was determined from PXRD studies and the obtained results were shown in Figure 3. Both Ao-AgNps and Lo-AgNps displayed a conspicuous diffraction peak at $2\theta = 25.29^o$ (0 2 1) as well as another peak at $2\theta = 32.48^o$ (1 2 0) for Ao-AgNps alone. Also, Ga-AgNps revealed three prominent diffraction peaks at $2\theta = 28.25^o$ (2 1 0), 32.37^o (1 2 2), and 46.17^o (2 3 1). Af-AgNps showed several diffraction peaks at $2\theta = 10.80^o$ (0 0 1), 12.51^o (2 0 0), 17.58^o (2 0 1), 18.58^o (1 1 0), 20.63^o (0 1 1), 23.64^o (2 0 -2), 24.92^o (2 1 1), 25.13^o (4 0 0), 26.50^o (2 0 2), 28.68^o (4 0 1), 31.87^o (2 1 2), 37.44^o (0 2 1), and 37.73^o (1 2 -1).

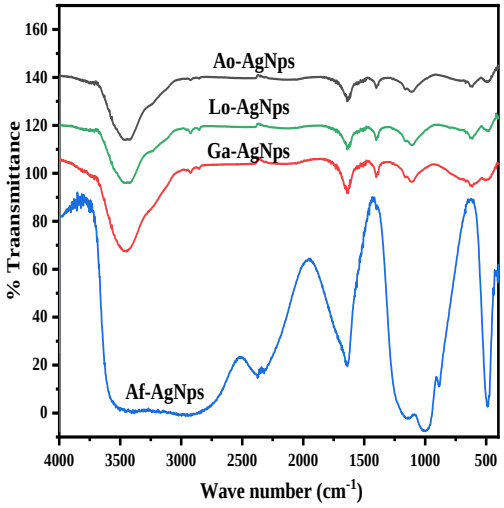

Figure 1: FT-IR spectra of the AgNps from different aqueous plant extracts and fungal biomass.

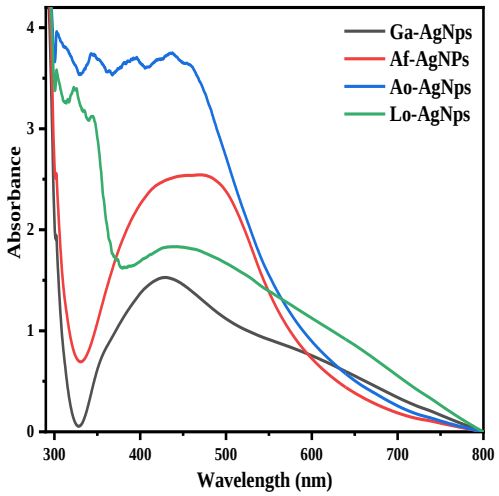

Figure 2: UV-visible absorption spectra of the AgNps.

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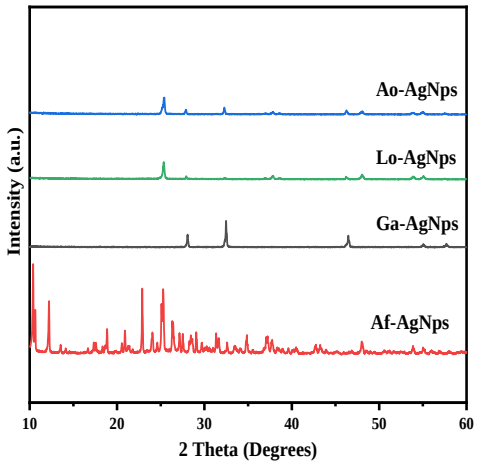

Figure 3: PXRD pattern of the AgNps from different aqueous plant extracts and fungal biomass.

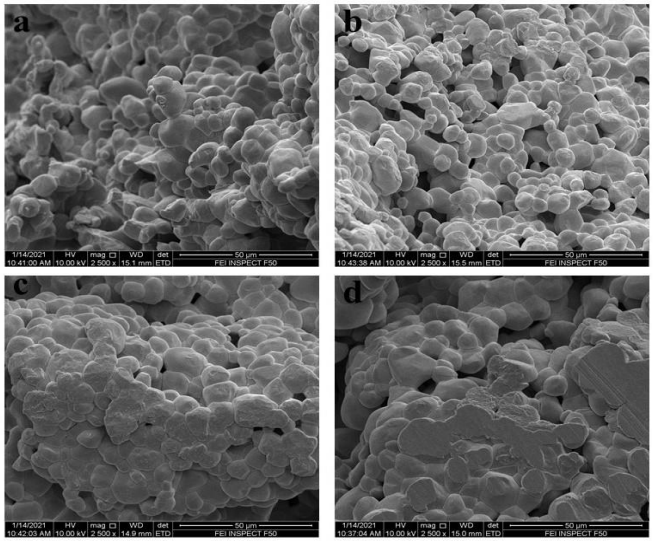

Figure 4: SEM images of (a) Ao-AgNps (b) Lo-AgNps (c) Ga-Nps (d) Af-AgNps

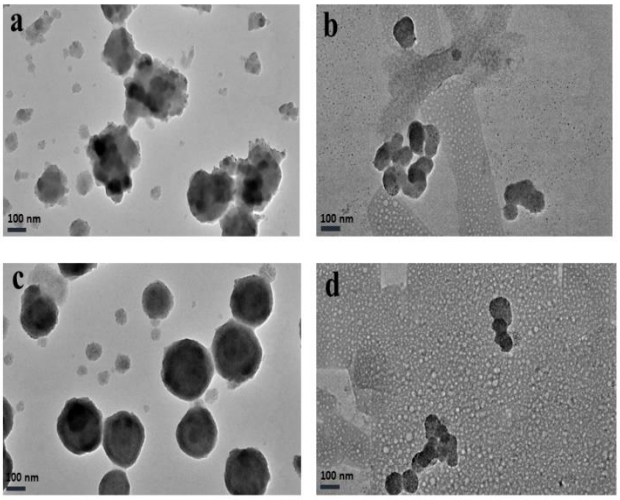

Figure 5: TEM images of (a) Ao-AgNps (b) Lo-AgNps (c) Ga-Nps (d) Af-AgNps

Besides, the calculated average crystallite sizes were 93.36, 92.31, 101.43, 80.36 nm for Ao-AgNps, Lo-AgNps, Ga-AgNps, and Af-AgNps respectively.

According to the PXRD patterns, all the biosynthesized AgNps were semi-crystalline in nature and having a series of weak diffraction peaks associated with the leaf extracts. The peaks observed for Ao-AgNps and Lo-AgNps corresponded well with the monoclinic crystal facet of silver oxide according to ICCD/JCPDS PDF No. 40-1054. Also, that of Ga-AgNps corresponded to orthorhombic crystal facet attributed to silver nitrate with ICCD/JCPDS PDF No. 43-0649. Furthermore, Af-AgNps peaks attributed well with the monoclinic crystal facet of ammonium silver nitrate due to ICCD/JCPDS PDF No. 36-0803.

SEM/TEM Studies

The characterization of the surface morphology of the AgNps as collected on a SEM showed irregular arrangements (Figure 4), while TEM images captured for the AgNps biosynthesized with the leaf extracts and fungal biomass showed monodispersed roughly spherical shapes with different size variations within the 100 nm range, as presented in Figure 5. The SEM micrograph shows that the nanoparticles were irregularly spherical with slight agglomeration.[39] Also, the layer of the organic components surrounding the biosynthesized AgNps could be responsible for the dispersion and agglomeration of the nanoparticles. The slight agglomeration observed in Af-AgNps may be attributed to the accumulation of proteins and enzymes secreted during the biosynthesis process.[40]

Antimicrobial activity of AgNps

The antimicrobial activities of the synthesized AgNps against three pathogenic clinical isolates were assessed by measuring the inhibition zone diameter (IZD) (Figure 6). An antibiotic disc containing CPX (10 µg) was used as positive control. Relatively, Af-AgNps had the best activity among the AgNps with the highest activity recorded against *Klebsiella spp.*, followed by Ga-AgNps, then Lo-AgNps and Ao-AgNps. The control, CPX (10 µg), had a significantly higher ($P < 0.05$) inhibition activity than all the AgNps.

Generally, the AgNps synthesized with all the aqueous leaf extract exhibited low antibacterial activity against the test organisms compared to ciprofloxacin (control) except for Ga-AgNps which displayed a substantially high inhibitory effect against *Klebsiella spp*. alone. The low inhibitory effect could be due to environmental and soil conditions of the plant such as water stress caused by different rainfalls and seasonal changes as suspected by Netshiluvhi and Eloff.[41]

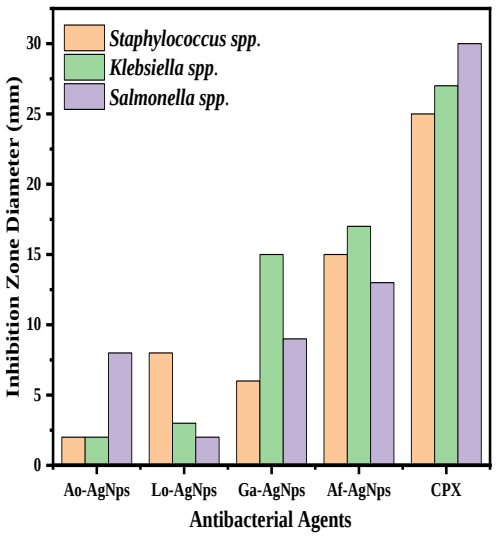

Figure 6: Inhibition zone diameter of the AgNps against the clinical isolates.

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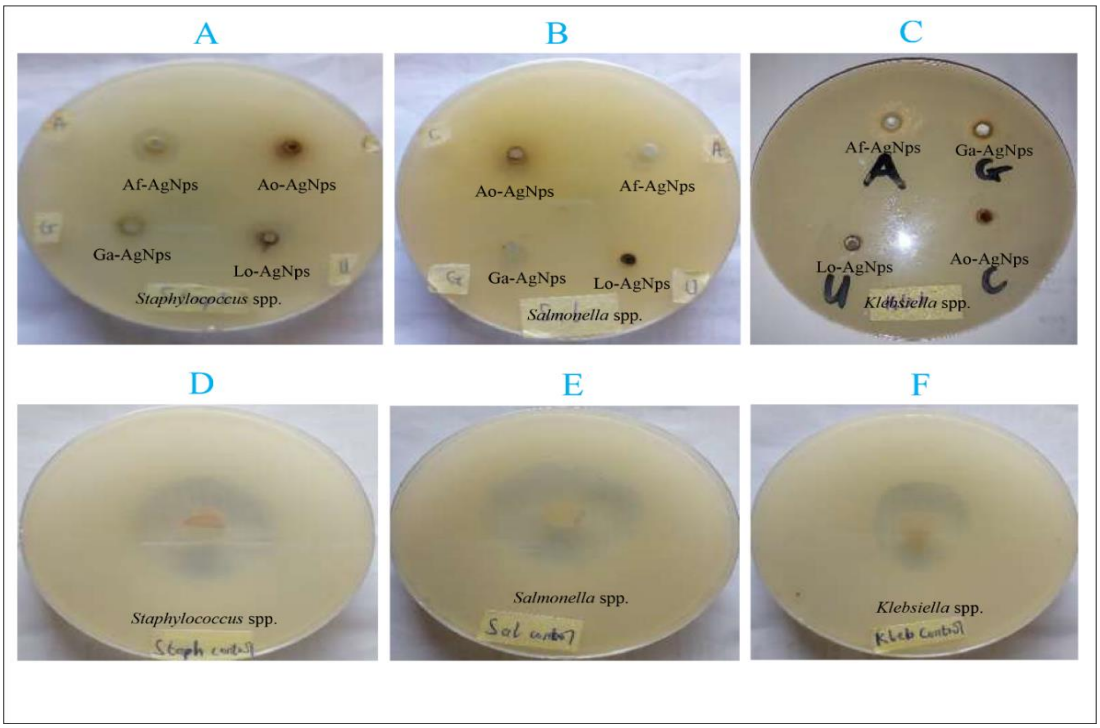

Figure 7: Inhibition efficacy of the AgNps against the clinical isolates. (A) *Staphylococcus* spp. (B) *Salmonella* spp. (C) *Klebsiella* spp. (D-F) Controls for A, B, and C respectively

The obtained efficacy of the biosynthesized AgNps could be due to the large sizes of nanoparticles.[42] Nevertheless, the AgNps synthesized from the fungal biomass (Af-AgNps) exhibited a substantial inhibitory efficacy towards all the tested organisms which is similar to some other studies. Additionally, the mechanism of antimicrobial activity of AgNps has been severally reported.[14] The first step is the attachment to cells, leading to changes in the conformation, architecture, and integrity of the cell membrane. Most of the damages caused by AgNps are due to the release of the Ag^+. The Ag^+ provoke the production of reactive oxygen species (ROS) and free radicals. The production of ROS leads to oxidative stress. It also generates pores on the cell, damage intracellular micro-organelles, and cause cell death.[43] Both the AgNps itself and its ion can interact with several proteins and DNA leading to inactivation of several important enzymes.

Conclusion

The study showed that aqueous extract of *A. occidentale*, *L. owariensis*, *G. africanum*, and cell filtrate of *A. flavus* could be utilized in the biosynthesis of AgNps. Currently, most research focused on AgNps is due to their superior features such as well-structured shape, size distribution, and non-toxicity. Based on the available studies, AgNps are regarded as the next generation antibiotics. Nevertheless, the approach in this study provides better alternatives in comparison to physical and chemical methods of synthesis. The AgNps synthesized from fungal biomass displayed a more promising antibacterial activity against all the tested pathogens.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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