WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 11, 765-776. Research Article ISSN 2277– 7105

GREEN SYNTHESIS OF SILVER NANOPARTICLES AND THEIR ANTIMICROBIAL ACTIVITY

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Article Received on
08 Sept. 2016,
Revised on 29 Sept. 2016,
Accepted on 19 Oct. 2016
DOI: 10.20959/wjpr201611-7169

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ABSTRACT

We reporting the synthesis of stable silver nanoparticles by the bioreduction method a low-cost, convenient, green synthesis approach to obtain large quantities of silver nanoparticles by reduction of silver ions with using *Caesalpinia bonduc* leaf extract. UV-Vis absorption spectroscopy was used to monitor the quantitative formation of silver nanoparticles. The characteristics of the obtained silver nanoparticles were studied using FT-IR, energy, scanning electron microscopy (SEM) and Transmission electron microscopy(TEM). The average diameter of the prepared nanoparticles in solution was about 29-68 nm. The synthesized silver nanoparticles were screened for biological activity.

KEYWORDS: *Green synthesis, Caesalpinia bonduc, FT-IR, Transmission electron microscopy (TEM), and Scanning electron microscopy (SEM), Biological activity.*

INTRODUCTION

Nanotechnology concerns with the development of experimental processes for the synthesis of nanoparticles of different sizes, shapes and controlled dispersity.[1] This provides an efficient control over many of the physical and chemical properties[2] and their potential application in photo-electronics.[3],[4], recording media[5],[6], sensing devices[7],[8], catalysis[9] and medicine.[10-12] To date, metallic nanoparticles are mostly prepared from noble metals(ie,Ag,Pt,Au, and Pd).[13] Among the noble metals,silver(Ag) is the metal of choice in the field of the biological system, living organisms and medicine[14] Green synthesis of nanoparticles is an emerging branch of nanaotechnology.[15] The use of environmentally

benign materials like plant leaf extract, bacteria, and fungi for the synthesis of silver nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and biomedical applications as they do not use toxic chemicals in the synthesis protocols.[16] Bio-inspired synthesis of nanoparticles provides an advancement over chemical and physical methods as it is a cost-effective and environment-friendly and in this method, there is no need to use high pressure, energy,temperature and toxic chemicals.[17] Disease-causing microbes that have become resistant to drug therapy are an increasing public health problem. Therefore there is an urgent need to develop new bactericides. Silver nanoparticles take advantages of the oligodynamic effect that silver has on microbes.[18] In the present study, reducing silver ions present in the aqueous solution of silver nitrate by the help of aqueous extract of some medicinal plants of Medaram forest and their antibacterial assessment will performed to produce novel drugs to overcome drug resistance and adverse reaction.

MATERIALS AND METHODS

Silver nitrate was purchased from Merck and used as received. Distilled deionized water was used throughout the reactions. The plant material was collected from the Medaram forest, Warangal, Telangana and washed with sterile distilled water. The leaf extract was prepared by taking 20 g of thoroughly washed plant material in a 250 ml Erlenmeyer flask with 100 ml of deionized water, and then boiling the mixture for 10 min in a water bath. The mixture was then filtered and centrifuged at 8000 rpm for 20 min. To obtain the extract of *Caesalpinia bonduc leaf,* 5 g of thoroughly washed leaf material of *Caesalpinia bonduc* was added to 100 ml of deionized water and incubated with shaking in dark conditions at 25 ◦C for 15 min. The obtained mixture was filtered and purified by centrifugation at 6000 rpm for 20 min. These solutions were stored at 4 ^{0}C and used within 1 week. For the preparation of silver nanoparticles, 15 ml of the aqueous leaf extract of *Caesalpinia bonduc* was added to 100 ml of 3 mM aqueous silver nitrate solution and incubated on a rotary shaker for 2 h,the mixture for reduction of Ag+ ions. The effect of temperature on the synthesis rate of the silver nanoparticles was studied by carrying out the reaction in a water bath at 25-95 ^{0}C. The silver nanoparticle solution thus obtained was purified by repeated centrifugation at 12,000 rpm for 20 min. The spectroscopic studies were carried out using a Shimadzu UV-2550 UV-Vis spectrophotometer equipped with matched quartz cells. After freeze-drying of the purified silver nanoparticles, the structure, composition, and average size of the synthesized silver

nanoparticles were analyzed by scanning electron microscopy (SEM; Philips XL-30), Transmission electron microscopy (TEM; Philips PW-180) and FT-IR.

Antibacterial assays

Materials and bacterial strains

The bactericidal experiments were carried out with gram-negative bacteria *E. coli* and gram-positive bacteria *B. Subtilis* and *S. aureus* in nutrient media, composed of peptone (Loba Chemie Ltd., Mumbai) and NaCl (Merck Ltd., Mumbai) 5 g l^{-1} each, and yeast extract (Central Drug House, New Delhi) and beef extract (S.D. Fine Chem Ltd., Mumbai) 1.5 g l^{-1} each. Throughout this study, the same nutrient media was used for all strains, unless otherwise specified. For preparing solid media, the nutrient media was supplemented with 2% bacteriological agar (Himedia Laboratories Ltd., Mumbai) as a solidifying agent. The silver nanoparticles were prepared by wet chemical synthesis involving a stoichiometric reaction between sodium borohydride and silver ions.[6]

E. coli strains MTCC 443 (ATCC 25922), MTCC 739 (ATCC 10536), MTCC 1302 (wild type), MTCC 1687 (ATCC 8739), and *B. subtilis* strain MTCC 441 (ATCC 6633) were procured from the Institute of Microbial Technology (Chandigarh, India). *S. aureus* strains NCIM 2079 (ATCC 6538P), NCIM 5021 (ATCC 25923) and NCIM 5022 (ATCC 29213) were procured from the Department of Microbiology, Kakatiya University, Warangal – 506009(T.S.), India

Disk diffusion test

Bacterial sensitivity to antibiotics is commonly tested using a disk diffusion test, employing antibiotic impregnated disks.[7] A similar test with nanoparticle-laden disks was used in this study. A 5 ml suspension of nanoparticles (5 mg ml^{-1}) was sonicated and subsequently filtered through a membrane filter (0.2 µm, 47 mm diameter Pall Gelman Laboratory). The nanoparticle-laden filter paper was dried in an oven for 1 h and small disks of uniform size (6 mm diameter) containing 100 ± 15 µg nanoparticles were punched out and stored in a desiccator at room temperature. The bacterial suspension (100 µl of $10^4 – 10^5$ CFU ml^{-1}) was applied uniformly on the surface of a nutrient agar plate before placing the disks on the plate (4 per plate). The plates were incubated at 35 ^{0}C for 24 h, after which the average diameter of the inhibition zone surrounding the disk was measured with a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for each type of nanoparticle and with each microbial strain were based on three replicates.

Determination of minimum inhibitory concentration

(MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC), defined as the lowest concentration of material that inhibits the growth of an organism[8], was determined based on batch cultures containing varying concentration of silver nanoparticles in suspension (20–300 mg l^{-1}). Sterile Erlenmeyer flasks (500 ml), each containing 100 ml nutrient broth was sonicated for 10 min after adding the nanoparticles to prevent aggregation of the nanoparticles. Subsequently, the flasks were inoculated with 1 ml of the freshly prepared bacterial suspension in order to maintain initial bacterial concentration 103–104 CFU ml^{-1}, and then incubated in an orbital shaker at 200 rpm and 30 ^{0}C. The high rotary shaking speed was selected to minimize aggregation and settlement of the nanoparticles over the incubation period. Lower rpm setting during incubation may cause underestimation of the antimicrobial activity of the nanoparticles. Bacterial growth was measured as an increase in absorbance at 600 nm determined using a spectrophotometer (Thermo Spectronic, Helios Epsilon, USA). The experiments also included a positive control (flask containing nanoparticles and nutrient media, devoid of inoculum) and a negative control (flask containing inoculum and nutrient media, devoid of nanoparticles). The negative controls indicated the microbial growth profile in the absence of nanoparticles. The absorbance values for positive controls were subtracted from the experimental values (flasks containing nutrient media, inoculum, and nanoparticles).[9] All the experiments were carried out in triplicate. Silver nanoparticles were tested for bactericidal effect using all the microbial cultures selected for the study.

The minimum bactericidal concentration (MBC), i.e., the lowest concentration of nanoparticles that kills 99.9% of the bacteria was also determined from the batch culture studies. For growth inhibitory concentration (PMIC) the presence of viable microorganisms was tested and the lowest concentration causing bactericidal effect was reported as MBC as suggested by Abadi et al.[10] To test for bactericidal effect, a loopful from each flask was inoculated on nutrient agar and incubated at 35 ^{0}C for 24 h. The nanoparticle concentration causing bactericidal effect was selected based on the absence of colonies on the agar plate.

RESULTS AND DISCUSSION

Silver reduction

It is well known that silver nanoparticles exhibit a yellowish-brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles.[11] Reduction of silver

ions to silver nanoparticles could be followed by a color change and UV-Vis spectroscopy. The technique outlined above has proven to be very useful for the analysis of nanoparticles.[12-14] Therefore, the progress in conversion reaction of silver ions to silver nanoparticles was followed by a color change and spectroscopic techniques. Figure 1 shows the photographs of sample solutions containing silver nitrate (left beaker) and silver nitrate in the presence of optimized amounts of *Caesalpinia bonduc* leaf extract. The appearance of a yellowish-brown color confirms the existence of silver nanoparticles in the solution (right beaker) **Fig 1**.

UV-Vis spectroscopy

The silver nanoparticles were characterized by UV-Vis spectroscopy, one of the most widely used techniques for structural characterization of silver nanoparticles.[15] The absorption spectrum (**Figure 2**) of the yellowish-brown silver nanoparticle solution prepared with the proposed method showed a surface plasmon absorption band with a maximum of 425 nm, indicating the presence of spherical Ag nanoparticles. This structure was confirmed by SEM images.

Particle size and chemical composition

The silver nanoparticle solution thus obtained was centrifuged at 12,000 rpm for 15 min, after which the pellet was redispersed in deionized water to get rid of any uncoordinated biological molecules. The purified pellets were then freeze-dried, powdered, and used for FT-IR, SEM, and TEM analyses.

SEM ANALYSIS

To gain further insight into the features of the silver nanoparticles, analysis of the sample was performed using SEM technique. The element analysis of the silver nanoparticles was performed using the SEM. The freeze-dried silver nanoparticles were mounted on specimen stubs with double-sided taps, coated with gold in a sputter coater (BAL-TEC SCD-005), and examined under a Philips XL-30 SEM at 12-16 kV with a tilt angle of 45◦.

Scanning electron microscopy provided further insight into the morphology and size details of the silver nanoparticles. Comparison of experimental results showed that the diameter of prepared nanoparticles in the solution was about 29-68 nm. **Figure 3A** shows the scanning electron micrograph of the plant extract as a positive control (incubated with deionized water

for 48 h), and **Figures 3B-3D** show the scanning electron micrographs of silver nanoparticles obtained from the proposed bioreduction method at various magnifications.

The diameter of inhibition zone (DIZ) for silver nanoparticle impregnated disks was almost 40–50% greater than that observed with the copper nanoparticle impregnated disks for all the E. coli strains selected for this study. Similarly, for S. aureus the silver nanoparticle impregnated disks were found to be more effective compared to copper nanoparticle impregnated disks, however, the difference in the DIZ was merely 10– 15%. In contrast, for B. subtilis, the disks impregnated with copper nanoparticles showed a significantly larger DIZ, almost 90% greater compared to that observed with silver nanoparticles. Since DIZ was measured on agar plates using a ruler with 1 mm resolution, the possibility of measurement errors exist; however, the method illustrates the potential biocidal effect of nanoparticles to different microbial strains.

The MIC and MBC representing the antimicrobial activity of nanoparticles dispersed in batch cultures is summarized in **Table 1.**

The MIC observed in this study for silver nanoparticles are 40 mg ml^{-1} for MTCC 443, 120 mg ml^{-1} for MTCC 1302, 140 mg ml^{-1} for MTCC 1687 and 180 mg ml^{-1} for MTCC 739. Our results are in contrast with some studies reporting the negligible inhibitory effect of silver nanoparticles on *E. coli* up to 100 mg ml^{-1}.[16,17] However, these studies employed silver nanoparticles of larger size (12–40 nm) and higher initial concentration of bacteria in the batch cultures (105–108 CFU ml^{-1}). For *E. coli* at an initial concentration of 106 CFU ml^{-1} suspended in distilled water, Li et al.[31] reported the MIC of silver nanoparticles (_~20 nm) as 40 mg ml^{-1}. The relatively low MIC is possibly due to the suspension of the cells in distilled water compared to suspension in nutrient media as employed in our study. For studies conducted on agar plates, the MIC of silver nanoparticles for E. coli was reported as 75 mg ml^{-1}.[18] In batch studies with *E. coli* and colloidal silver nanoparticle (size range 2–25 nm), MIC was reported to be in the range of 3–25 mg ml^{-1} for initial bacterial concentration 10^5– 10^8 CFU ml^{-1}.[19-21] Due to variation in the *E. coli* strain employed, variation in the size of silver nanoparticles and initial bacterial concentration, a direct comparison between the studies is not feasible. Several studies on the antimicrobial activity of silver nanoparticles were carried out with colloidal nanoparticles[20,22,21], while dry nanoparticles in powder form were re-suspended in the nutrient media in our studies. In aqueous suspension, the mean hydrodynamic diameter of the nanoparticles is expected to be in the same range as that based

on TEM.[22] However, in this study, the DLS results depicted that a maximum number of silver and copper nanoparticles were in the size range of 12–21 nm and 41–82 nm, respectively both in DI water and in nutrient media. No systematic variation in size was observed with increase in time up to 24 h. The measured particle size using DLS is higher compared to the size measured by TEM possibly due to agglomeration of nanoparticles in water. However, the agglomeration effect was not enhanced by the presence of salts in the nutrient media and increasing agglomeration over time was not observed. The agglomeration effect may have affected the bactericidal efficiency and MIC/MBC values as also suggested by Gan et al.[23]

TEM ANALYSIS

The TEM images of silver nanoparticles synthesized with different concentration of curry leaf extract for 2h. TEM images show that the synthesized silver nanoparticles are polydisperse and ranges approximately from 10-25nm. The shape of the nanoparticles is spherical with few exceptional as ellipsoidal. From **Fig. 4** it is found that decreasing leaf extract concentration in reaction mixture reduces the particle size and also their agglomeration tendency. Also, from **Fig. 4** it can be seen that the particles cluster together without much physical contact. This confirms the possibility of secondary material that binds the silver nanoparticles in clusters, which was further confirmed by FT-IR analysis. SEM analysis of the synthesized silver nanoparticles was made and typical SEM image is shown in **Fig. 3**, which confirms the spherical shape of the particles and again the tendency for particles to aggregate.

FTIR ANALYSIS

FTIR measurements were carried out to identify the biomolecules for capping and efficient stabilization of the metal nanoparticles synthesized. The FTIR spectrum of silver nanoparticles by *Caesalpinia bonduc* **(Figure:5)** was the band between 3490-3500 cm-1 corresponds to O-H stretching H-bonded alcohols and phenols. The peak found around 1500-1550 cm^{-1} showed a stretch for C-H bond, peak around 1450-1500 cm^{-1} showed the bond stretch for N-H. Whereas the stretch for Ag-NPs was found around 500-550 cm^{-1} in *Caesalpinia bonduc?* Therefore the synthesized nanoparticles were surrounded by proteins and metabolites such as terpenoids having functional groups.

Figure 1: A solution of silver nitrate (3 mM) before (left) and after (right) addition of plant extract solutions.

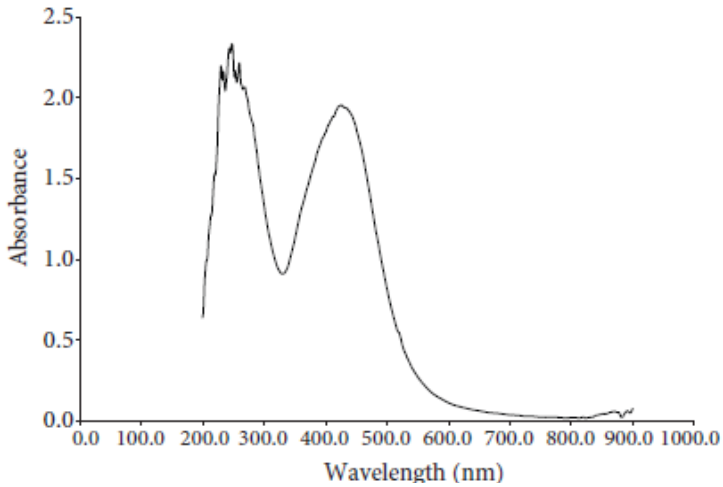
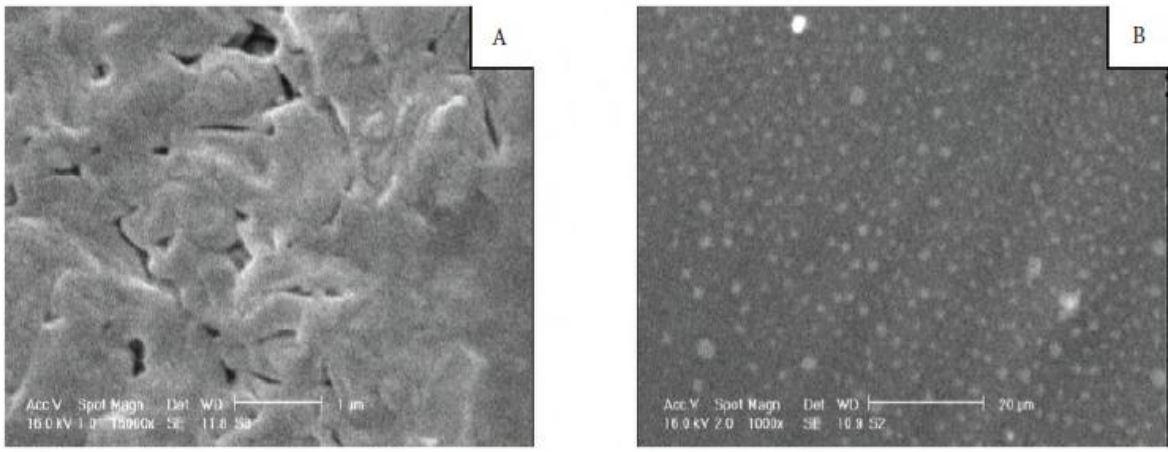


Figure 2: U.V spectroscopy of silver nanoparticles by Caesalpinia bonduc.



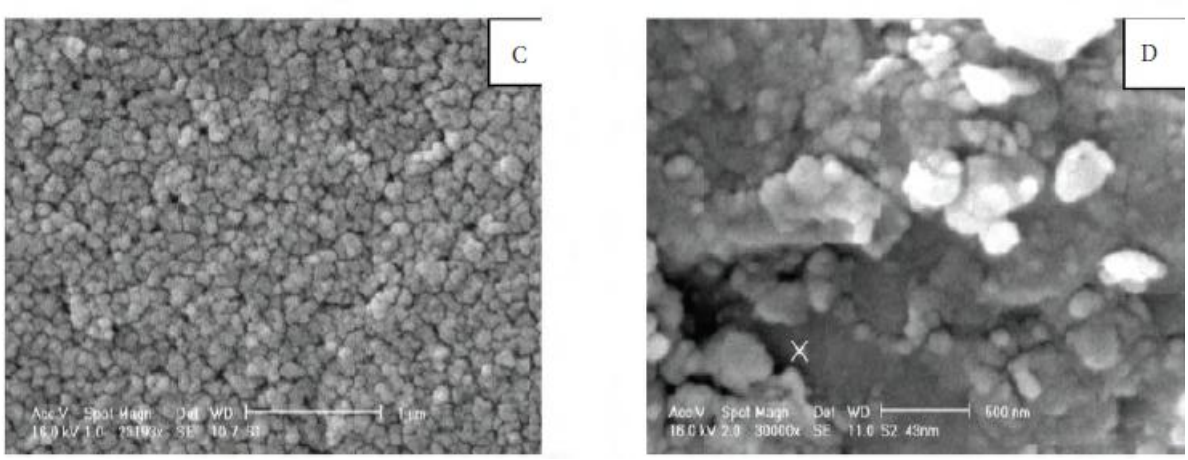


Figure 3. Scanning electron micrograph of the plant material incubated with deionized water (3A) and of the silver nanoparticles obtained with plant extract incubated with 0.003 M silver nitrate solution at 86 ^{0}C for 13 min (3B-3D).

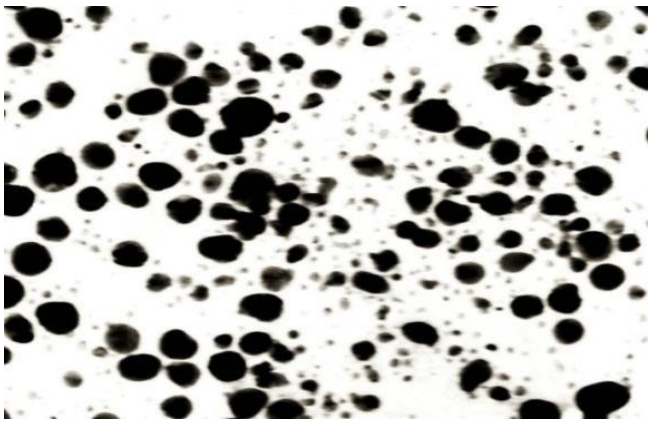


Figure 4: Transmission electron micrograph image of silver NPs by Caesalpinia bonduc.

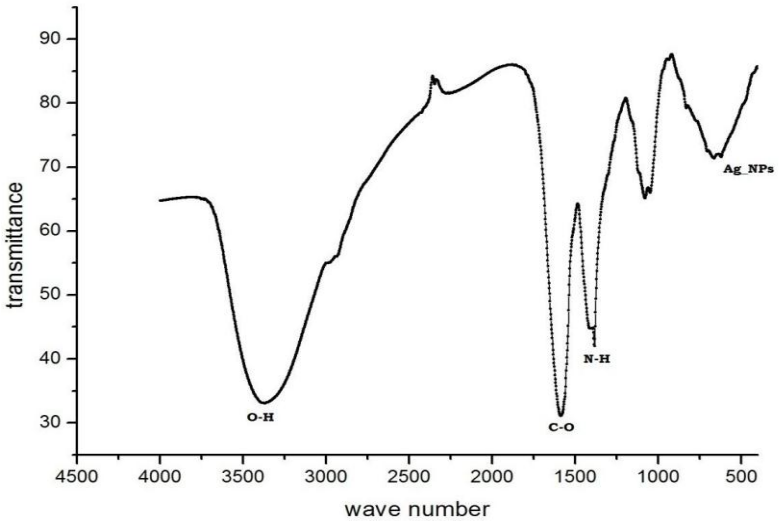


Fig 5: FTIR spectra of silver NPs by Caesalpinia bonduc.

Table 1: MIC (mg ml^{-1}) and MBC (mg ml^{-1}) of silver and copper nanoparticles for various microorganisms.

Culture	Strain no.	MIC	MBC
Escherichia coli	MTCC 443	40	60
Escherichia coli	MTCC 739	180	220
Escherichia coli	MTCC 1302	120	160
Escherichia coli	MTCC 1687	140	180
Bacillus subtilis	MTCC 441	40	60
Staphylococcus aureus	NCIM 2079	120	160
Staphylococcus aureus	NCIM 5021	120	160
Staphylococcus aureus	NCIM 5022	120	160

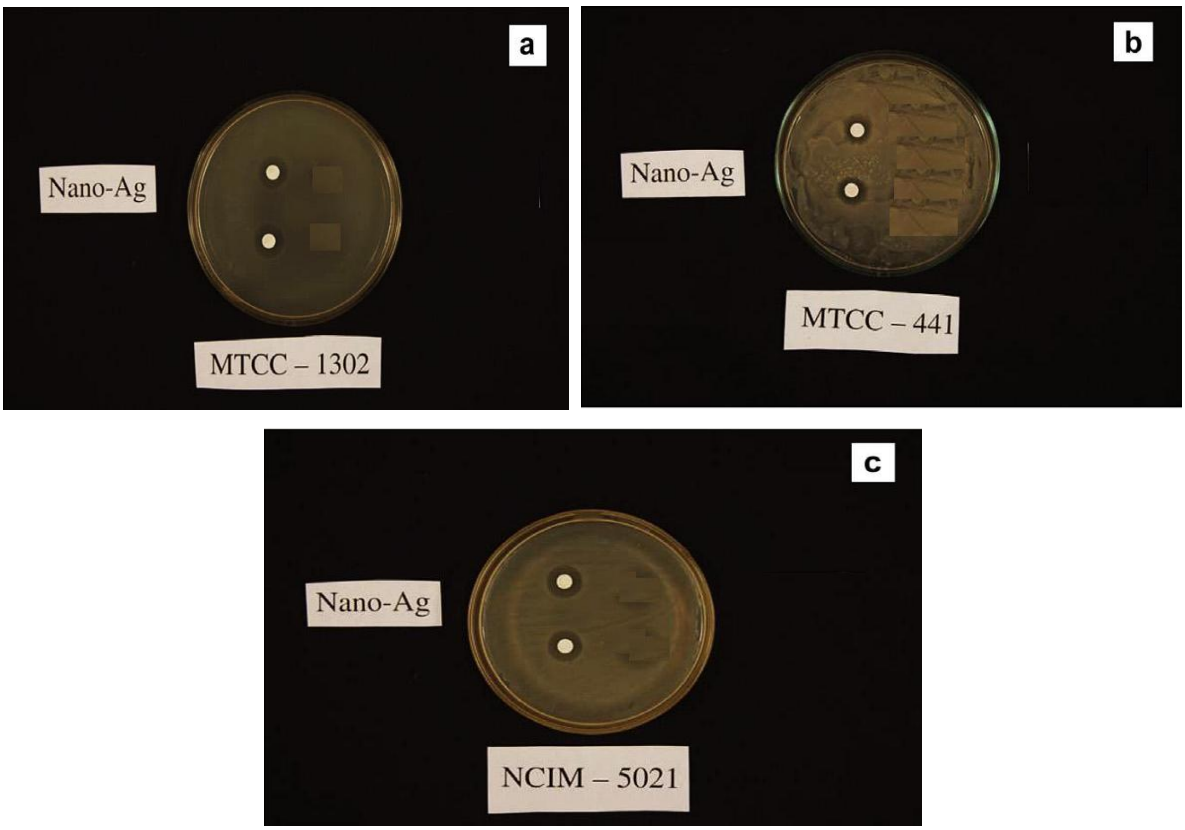


Fig. 6. Representative images of agar plates containing silver nanoparticle impregnated disks and DIZ for (a) *E. coli* (MTCC 1302), (b) *B. subtilis* (MTCC 441) and (c) *S. aureus* (NCIM 5021).

CONCLUSIONS

A critical need in the field of nanotechnology is the development of reliable and eco-friendly processes for the synthesis of metallic nanoparticles. Here, we have reported a simple biological and low-cost approach for the preparation of stable silver nanoparticles by reduction of silver nitrate solution with a bioreduction method using aqueous leaf extract of *Caesalpinia bonduc* as the reducing agent. The characteristics of the obtained silver

nanoparticles were studied using UV-Vis, FT-IR, TEM, and SEM techniques. The results confirmed the reduction of silver nitrate to silver nanoparticles with high stability and without any impurity. Comparison of experimental results showed that the average size of synthesized silver nanoparticles was about 40 nm. Growth studies of different microbial cultures were performed in the presence of nanoparticles to observe their effect on the growth profile. This study shows that silver nanoparticles have great promise as an antimicrobial agent against *E. coli*, *B. subtilis,* and *S. aureus.* MIC, MBC, and disk diffusion test suggest that for all cultures of *E. coli* and *S. aureus*, the antimicrobial action of the silver nanoparticles were superior.

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