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IN VITRO ANTIBACTERIAL POTENTIAL OF SYNTHESISED SILVER NANOPARTICLES FROM LEAVES OF AVERRHOA BILIMBI L.

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ABSTRACT

Nanotechnology is a rapidly growing field and plays an important role in most of the advanced science, medicine, and technology areas. Plants are the major sources of different types of phytochemicals with numerous biomedical applications. This study focused on the phyto-synthesis of silver nanoparticles (AgNPs) using the leaf extract of *Averrhoa bilimbi* L and their antibacterial potential. Phytochemical analysis revealed that the rich amount of biochemical present in leaf extract and these bio-surfactant molecules in the extract play a significant role both as stabilizing and reducing agents for converting silver nitrate into AgNPs. The silver nanoparticles synthesized were confirmed by their change of colour to dark brown due to the phenomenon of surface Plasmon resonance (SPR) and characterization was studied by UV-visible spectroscopy, Fourier-Transform Infrared Spectroscopy (FTIR) and X-Ray Diffraction (XRD) analysis. The morphology and size of synthesized silver nanoparticles were confirmed by Transmission Electron Microscopy (TEM) analysis with size range from 10 to 20 nm. The biosynthesized AgNPs were further studied for its anti-bacterial activity by using agar well diffusion and Minimum Inhibitory Concentration (MIC) methods at different concentrations against human pathogenic bacteria (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa*). AgNPs were showed potent antibacterial against all the pathogenic enteric

bacteria tested. The AgNPs showed maximum efficacy against *P.aeruginosa and E.coli*. The AgNPs were found to be more susceptible to all the organisms than the aqueous extract.

KEYWORDS: Bio-Surfactant Molecules, AgNPs, Averrhoa bilimbi, SPR.

INTRODUCTION

Nano-biotechnology is presently one of the most dynamic disciplines of research in contemporary material science whereby plants and its products are finding an imperative use in the synthesis of Nanoparticles (NPs). Entirely novel and enhanced characteristics such as size, distribution and morphology have been revealed by these particles in comparison to the larger particles of the mass material that they have been prepared from. In recent years, researchers in the field of nanotechnology are finding that there is an expanding research in the synthesis of metal nanoparticles due to the potential applications for the development of novel technologies. Noble metal nanoparticles are extensively studied because of their wide applications.[1,2,3]

Numerous methodologies are invented to synthesize noble metal nanoparticles of particular shape and size depending on specific requirements, because properties of metallic nanoparticles dependent on size and shape are of interest for applications ranging from catalysts and sensing to optics, antibacterial activity and data storage. The surface to volume ratio of nanoparticles is inversely proportional to their size. The biological effectiveness of nanoparticles can increase proportionately with an increase in the specific surface area due to the increase in their surface energy and catalytic reactivity.[4]

Silver has been known for many centuries worldwide for its antimicrobial activity. By reducing the bulk silver into nanoparticles, we can increase its efficacy against microbes.[5,6]

A.bilimbi is a small tree. It is easily available and cost effective. It holds a great value in complementary medicine as evidenced by the substantial amount of research on it. It is used in the treatment of fever, syphilis, stomach ache, ulcer, hypertension, obesity and diabetes. There are many reports on various aspects of fruits of *bilimbi*, however no work have been carried out on its leaves except the phytochemical profile. So here is an attempt in synthesizing the silver nanoparticles using the leaves of *Averrhoa bilimbi* and study on its antibacterial potential due to their chemical composition.[7]

MATERIALS AND METHODS

Collection and preparation of plant material

Fresh leaves of *Averrhoa bilimbi* were collected from Chennai. The collected leaves were washed thoroughly with tap water and rinsed twice with distilled water until no foreign material remained.

Preparation of aqueous leaf extract

10 gms of fresh leaves were boiled with 100ml distilled water for 10 min. The mixture was then filtered through Whatmann no.1 filter paper. The filtrate was concentrated at 30^{o}C and stored in sterile sample containers at 4^{o}C until further use.

Preparation of 1mM silver nitrate aqueous solution

An accurately weighed 0.017g of silver nitrate was dissolved in 100ml distilled water and stored in amber color bottle until further use.

Synthesis of silver nanoparticles

5ml of *Averrhoa bilimbi* leaf extract was added drop wise into 95ml of 1mM aqueous solution of AgNO3 with constant stirring at 50– 60^{o} C. *A. bilimbi* extract was mixed in aqueous solution of silver ion complex and stirred for 10 minutes; it starts to change color from whitish to reddish brown due to Surface Plasmon Resonance which indicates the formation of silver nanoparticles. It was found to be stable for more than 2 months without showing any precipitation or color change. The reaction was carried out in darkness, to avoid photo activation of $AgNO_3$. Both the aqueous and AgNPs solution were concentrated at 50^{o}C in hot air oven and kept this crude at 4 ^{o}C for further use.

UV-Visible spectrophotometer

The bio-reduction of silver ion in the solution was monitored by UV–Vis spectra of the reaction mixture using UV–Visible Spectrophotometer (Shimadzu UV-1650 pc) from 300 to 600nm at a resolution of 1nm.

Transmission Electron Microscopy

The size of silver nanoparticles was measured by TEM (Tecnai G 2 F 20), which operated at an acceleration voltage of 200 kV.

X-ray powder diffractometer

The crystallinity and phase composition of the silver nanoparticles were characterized by an X-ray powder Diffractometer, which carried out at 40 kV and 40 mA.

FTIR Analysis

FTIR measurement was carried out for functional group characterization through potassium bromide (KBr) pellet method and the spectrum was recorded using Fourier Transform Infrared Spectrometer.

Antibacterial activity

Test pathogenic microorganisms

Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa were procured from Lifeteck Research centre, Chennai. Bacterial strains were grown and maintained on Muller-Hinton Agar medium.

Preparation of inoculum

The bacterial strains were maintained in nutrient agar slants at 4°C. Loop full of organism from the stock cultures were inoculated in the test tubes containing nutrient broth and incubated for 24hrs at 37^{o}C. The turbidity was adjusted to 0.5 Mac Farland standards. Antibacterial screening was carried out using the standard well diffusion test, Minimum inhibitory concentration(MIC), Minimum bactericidal concentration (MBC).

Agar well diffusion method

The sterilized Muller Hinton agar medium was poured into petriplates and allowed to solidify. Wells were cut from the agar medium using sterile 6mm cork borer. Then, the inoculum was spread on the medium with the sterile swabs moistened with the bacterial suspension. The extracts of both aqueous and AgNPs was prepared in the different concentrations 125 µg/ml (C_1), 62.5 µg/ml (C_2), 31.25 µg/ml (C_3), 15.62 µg/ml (C_4) and 7.81 µg/ml (C_5) from the crude by diluting in DMSO. 100µl of extract of each concentration of aqueous plant extract and AgNPs was loaded in to each well. Ampicillin is used as standard antibiotic. After overnight incubation at 37°C, the agar plates were observed for zones of inhibition. The complete antibacterial analysis was carried out under strict aseptic conditions.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of the aqueous extract and synthesized silver nanoparticles of *Averrhoa bilimbi* were determined using tube dilution technique. A volume of 50 µl respective sterile MHB and aqueous and AgNPs extract solution from 62.5 µg/ml to 0.2 µg/ml to which only 100 µl of test product was added to wells of the microtitre plate separately. And to all the wells loaded with test sample, 100 µl bacterial suspension of approximately 10^5 CFU/ml was added. A growth control (bacterial cell suspension + 50 µl broth medium) and broth control (only broth medium 50 µl) was kept in the 96 wells microtitre plate. A positive control that consists of the Ampicillin was also placed on the microtitre plate. The plates were incubated at 37°C for 24 hours. After incubation, 10 µl of working solution of resazurin dye was added to all wells. The colour change was then assessed visually. Any colour change from purple to pink or colourless was recorded as positive growth. The lowest concentration at which there is no colour change occurred was taken as the MIC value. Experiments were performed in triplicate under aseptic condition.

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of the aqueous extracts and synthesized silver nanoparticles were determined by sub culturing 0.1 ml from the MIC test dilutions that showed visible growth in the sterile petri dishes having agar medium and incubated for 24 hrs. The highest dilution that shows no growth of culture was considered as minimum bactericidal concentration (MBC).

RESULTS

Characterization of Silver Nanoparticles

UV-Visible Spectrophotometer analysis

Reduction of silver nitrate to silver nanoparticles during exposure to plant extracts is followed by a gradual increase in color development from whitish to reddish brown after 30 minutes, of the reaction. The formation and stability of the reduced AgNP in the colloidal solution was firstly confirmed by the UV–Vis spectra. The UV-Vis Spectra showed maximum absorbance at 433.60 nm.

(a)$AgNO_3$ Solution **(b) Aqueous leaf extract** **(c) AgNPs Solution**

Fig. 1. Color change observed during bio reduction.

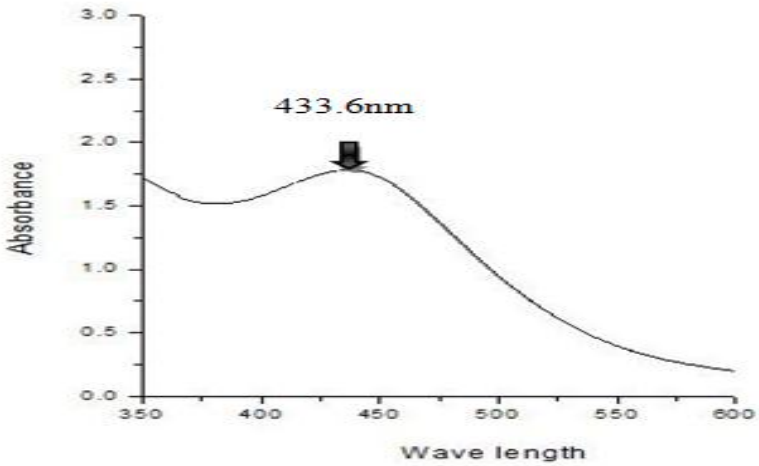


Fig. 2. UV-Vis Spectra of Silver nanoparticles.

Fourier Transform infra-red spectrum

FTIR analysis carried out to characterize the AgNPs obtained from the plant extract is shown in graph 2. In AgNP solution, prominent bands of absorbance were observed at around 500.55, 538.16, 567.1, 620.14, 645.22, 670.29, 1635.71, 2085.14, 2333.97, 2359.04, 2406.3, 2831.62, 2898.17 and 3453.69 cm−1. The observed peaks denote Carbon halide group, Carbon halide group, Carbon halide group, Carbon halide group, Carbon chloride group, Carbon chloride group, N-H bending medium bond, C=C- alkyne bonds, C=C- alkyne bonds, C=C- alkyne bonds, O-H stretch, acid, strong bond, O-H stretch, acid, strong bond, O-H stretch, acid, strong bond and O-H Strong intensity, very broad band respectively. These bands denote stretching vibrational bands responsible for compounds like flavonoids and terpenoids and so may be held responsible for efficient capping and stabilization of obtained AgNPs.

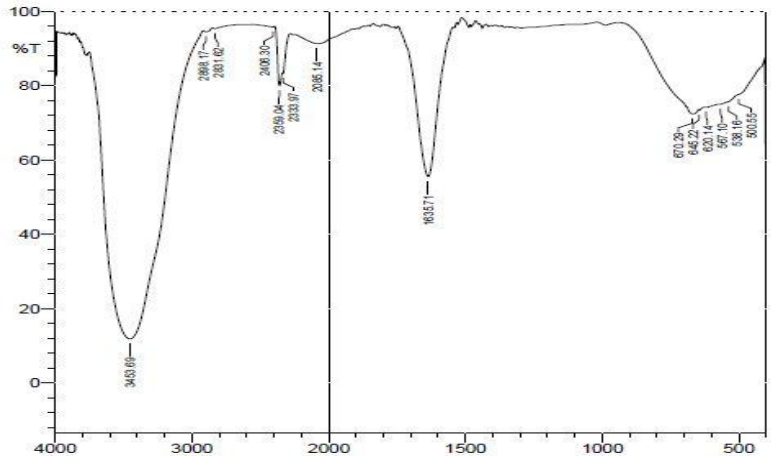


Fig. 3. FTIR spectra of Silver Nanoparticles.

XRD Analysis

The crystalline nature of the as-prepared silver nanoparticles was confirmed by XRD analysis. The acquired typical XRD pattern is depicted in Graph-3, suggesting the crystalline nature of prepared silver nanoparticles. The XRD peak positions are in agreement with metallic silver. Five diffraction peaks were observed at 31.92, 37.96, 45.88, 57.32, and 64.28 which correspond to crystal facets of (111), (200), (220), (311) and (222) reflections respectively, and represent the Face-Centered Cubic (FCC) structure of metallic silver. The mean crystallite diameter of silver nanoparticles obtained from reduction process, is determined by using Debye-Scherrer method, and is estimated to be 15 nm.

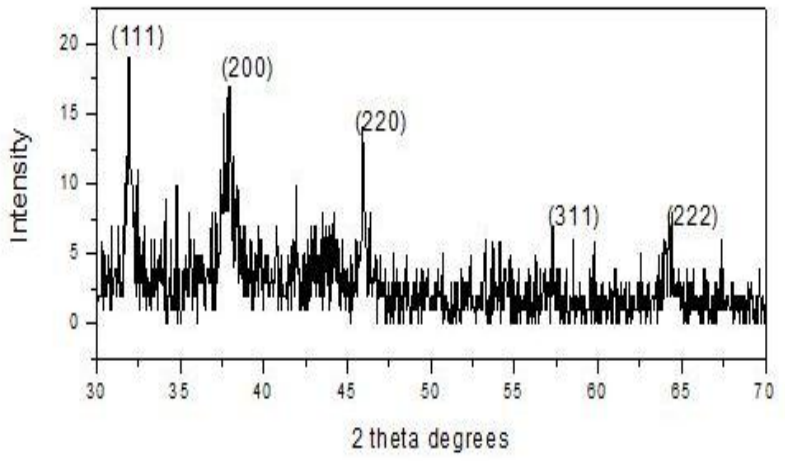
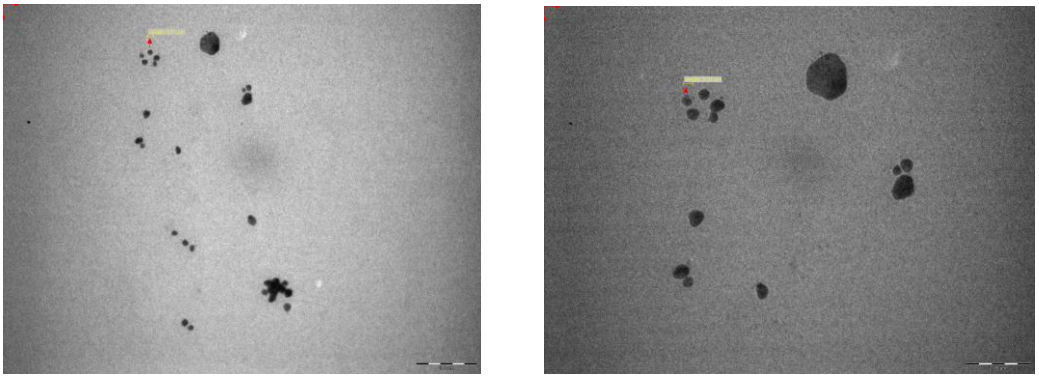


Fig. 4. XRD Pattern of Silver Nanoparticles.

Transmission Electron Microscopy

TEM was conducted in order to investigate the morphology and size distribution of silver nanoparticles. The typical TEM images of silver nanoparticles are shown in Fig. 3(a) and

3(b). As can be seen, the morphology of the prepared silver nanoparticles is mostly spherical, in accordance with the shape of the SPR band in the UV–Vis spectra. The particles size measured from the TEM images is from 10 nm to 30 nm. However, besides small amount of relatively bigger particles, most of the particles are below 15 nm, with a narrowly distribution from 10 to 30 nm. The average particles size of silver nanoparticles is about 15 nm.



a) Magnification – 65000X. **b) Magnification – 135000X.**

Fig. 5. Transmission Electron Microscope.

Antibacterial activity

The results of the antibacterial activity of the synthesized nanoparticles and the aqueous plant extracts were shown in the table 1 & 2 and in graph 1 & 2. In case of all bacterial species tested, the zone of inhibition of synthesized nanoparticle was found to be more than that of the aqueous plant extract. The inhibition zone showed decline while the concentration of extract decreases. AgNPs were showed potent antibacterial activity against all the pathogenic enteric bacteria tested. The maximum zone of inhibition was observed against *P. aeruginosa and E. coli* (22.6 mm, 21mm zone of inhibition) at 125 µg/ml of AgNPs extract than *S. aureus and K. pneumoniae* which showed 18mm and 16.5mm of inhibition zone respectively at 125 µg/ml AgNPs extract. The AgNPs showed maximum efficacy against *P.aeruginosa and E.coli*. The AgNPs were found to be more susceptible to all the organisms than the aqueous extract.

Table. 1. Table showing the Antibacterial activity aqueous plant extract.

No.	Micro organism	Aqueous plant extract Zone of inhibition (mm)				
		125 µg/ml	62.5 µg/ml	31.25 µg/ml	15.63 µg/ml	7.81 µg/ml
1	*Staphylococcus aureus*	13±1.15	11.2±1.15	10.3±1.53	9±1.15	0±0

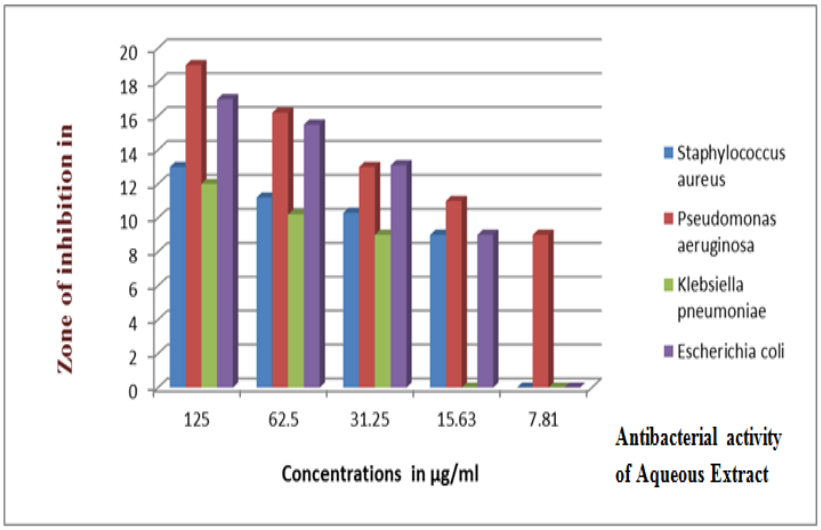
2	*Pseudomonas aeruginosa*	19±1.00	16.2±1.15	13±1.15	11±1.53	9±1.00
3	*Klebsiella pneumoniae*	12±1.00	10.2±0.58	9±1.00	0±0	0±0
4	*Escherichia coli*	17±0.58	15.5±1.00	13.1±1.15	9±1.00	0±0

**values are mean and standard deviation of three (3) replicates, 0 ±0.00 = No activity*

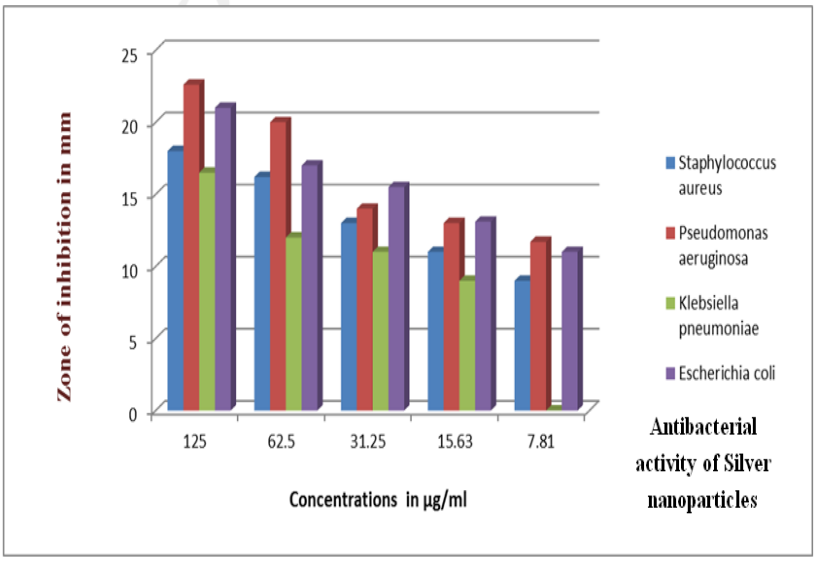
Table. 2. Table showing the Antibacterial activity of synthesised silver nanoparticles.

No.	Micro organism	Silver Nanoparticles Zone of inhibition (mm)				
		125 µg/ml	**62.5** µg/ml	**31.25** µg/ml	**15.63** µg/ml	**7.81** µg/ml
1	*Staphylococcus aureus*	18±1.00	16.2±1.00	13±1.00	11±1.15	9±1.00
2	*Pseudomonas aeruginosa*	22.6±1.15	20±1.53	14±1.15	13±1.53	11.7±1.15
3	*Klebsiella pneumoniae*	16.5±1.00	12±1.00	11±1.00	9±1.00	0±0
4	*Escherichia coli*	21±1.53	17±1.15	15.5±1.00	13.1±1.00	11±1.15

**values are mean and standard deviation of three (3) replicates, 0 ±0.00 = No activity*



Graph. 1. Graph showing the Antibacterial activity aqueous plant extract.



Graph. 2. Graph showing the Antibacterial activity of synthesised silver nanoparticles.

Determination of MIC and MBC

The MIC of *Staphylococcus aureus* was found to be 3.91µg/ml in aqueous plant extract, while it was 0.49µg/ml in synthesized nanoparticle. For *Pseudomonas aeruginosa* and *Escherichia coli*, it was found to be 7.81µg/ml in aqueous plant extract, while in synthesized nanoparticle it was 1.95µg/ml. And, for *Klebsiella pneumoniae*, it was 15.63µg/ml in aqueous plant extract, while it was 3.91µg/ml in synthesized nanoparticle. The MIC and MBC value is tabulated in table 3a & 3b.

Antibacterial activity of Silver nanoparticles

Table. 3a. Table showing the Minimum Inhibitory Concentra f extract and synthesized Silver Nanoparticles.

No.	Micro organism	Aqueous plant extract µg/ml	Silver Nanoparticles µg/ml
1	*Staphylococcus aureus*	3.91	0.49
2	*Pseudomonas aeruginosa*	7.81	1.95
3	*Klebsiella pneumoniae*	15.63	3.91
4	*Escherichia coli*	7.81	1.95

Table. 3b. Table showing the Minimum Bactericidal Concentration of Aqueous leaf extract and synthesized Silver Nanoparticles

No.	Micro organism	Aqueous plant extract µg/ml	Silver Nanoparticles µg/ml
1	*Staphylococcus aureus*	1.95	0.24
2	*Pseudomonas aeruginosa*	3.91	0.49
3	*Klebsiella pneumoniae*	7.81	-
4	*Escherichia coli*	3.91	0.49

- *No activity*

DISCUSSION

There are only few studies were reported in the antibacterial activity of leaves of *Averrhoa bilimbi*. Hence this study is focused in the synthesis Silver nanoparticle from aqueous leaf extracts of *Averrhoa bilimbi* and it was tested for its antibacterial activity, because of its wide usage in traditional system of medicine. The synthesis of Nanoparticles using the plant extracts with the 1mM $AgNO_3$ solution and the characteristic colour change noted, which is due to the excitation of the surface Plasmon resonance in the metal nanoparticles.[8] The UV-Visible spectrophotometer showed the maximum absorbance at 433 nm using the *A.bilimbi* leaf extract. This result is in conformity with Dinesh S et al 2012. The FTIR spectra of the extract showed the strong bands at 3453.69, 2898.17, 2831.62, 2406.3 and 1635.71 cm^{-1} assigned to O–H stretching vibration of alcohols and phenols[10], C=O stretching vibration of tertiary amines[11], and C–O stretching of aromatic ethers.[12] The amines and alcohols present in the sample along with ascorbic acid may be responsible for the reduction and capping of silver nanoparticles.[13] XRD is commonly used for determining the chemical composition and crystal structure of a material; therefore, detecting the presence of silver nanoparticles in plants extracts can be achieved by using XRD to examine the diffraction peaks of the plant.[14] XRD shows the crystalline nature of the synthesized nanoparticles. From the TEM images, it was observed that most of the silver nanoparticles are spherical in shape and also there is variation in particles size and distribution. It is shown that average particle estimated was 15nm. The results of the present work clearly showed that antibacterial activity was more in AgNPs than aqueous leaf extracts.

CONCLUSION

It has been demonstrated that the extract of *Averrhoa bilimbi* leaves are capable of producing silver nanoparticles extracellularly and the Ag nanoparticles are quite stable in solution. The biosynthesized silver nanoparticles showed excellent antimicrobial activity. The data represented in our study contribute to a novel and unexplored area of Nano-materials as alternative medicine. Therefore, further studies are needed to fully characterize the toxicity and the mechanisms involved with the antimicrobial activity of these particles.

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