

Biosynthesis and Characterisation of Silver Nanoparticles using Leaf Extract of Terminalia Belerica for its Antimicrobial Activity

Akila Elias

Department of Pharmacognosy,
RR College of Pharmacy, Bangalore, Karnataka, India

Dr. V. B. Narayana Swamy

Department of Pharmacognosy,
R.R. College of Pharmacy,
Bangalore, Karnataka, India

Hoonesh N. H.

Department of Pharmacognosy,
R.R. College of Pharmacy,
Bangalore, Karnataka, India

ABSTRACT

*In the current study, silver nanoparticles were synthesized using the ethanolic leaf extract of Terminalia belerica. Characterization was carried out using UV-Visible, FTIR, XRD, SEM and TEM. The formation of the AgNPs was confirmed through UV-Visible spectroscopy by the colour change. Based on the XRD pattern, the crystalline property of the AgNPs was established. The functional group present in the ethanolic leaf extract of Terminalia belerica is responsible for the reduction of the Ag⁺ ion which was studied through FTIR. From the SEM and TEM analysis, it was found that the formed nanoparticles are spherical in shape and nano in size. The biosynthesized AgNPs was evaluated for its antimicrobial activity against gram positive bacteria (*S. aureus* and *L. bacillus*) and gram negative bacteria (*E. coli* and *P. aeruginosa*) using disc diffusion and dilution method and it was found to exhibit potential antimicrobial activity.*

Keywords: Nanotechnology, Green synthesis, Silver nanoparticles, Antimicrobial activity.

INTRODUCTION:

Nanotechnology may be outlined as the manipulation of matter through certain processes to make materials with specific properties, which may be employed in specific applications. A nanoparticle is a microscopic particle that has at least one dimension less than a hundred nanometers in size. Materials within the nano dimensions (1-100 nm) have outstanding distinction in the properties compared to a similar material in the bulk. These distinction be the physical and structural properties of atoms, molecules and bulk materials of the component because of distinction in physico chemical properties and surface to volume quantitative relation.² Owing to the interest and importance of nanoparticles several researchers have centered on the synthesis of nanoparticles using chemical and physicals ways, however unfortunately they are quite costly and probably dangerous to the surroundings that involve use of harmful and threatening chemicals that are liable for numerous biological risk.³ A wide selection of biological resources like micro organisms (bacteria, yeast, fungi, algae, and viruses) and plants are often used for nanoparticle synthesis. The major drawback of microbe-mediated nanoparticle synthesis is that the obligatory constraint of antiseptic conditions, which requires trained workers and raises the scaling value. Of these reasons, at the side of the straight forward handiness of plants in nature, make them more preferred biological resources than microbes.¹ The synthesis of nanoparticles using plant extract provides advancement over different ways because it is easy, one step, price effective, surroundings friendly and comparatively reproducible results.³ There are differing types of nanomaterials. Depending on dimensions they are being classified into 4 types: one dimensional, two dimensional, three dimensional nanostructures. Nanomaterials are a product of inorganic or carbon (fullerenes, nanotubes, etc.) based materials. The latter, embrace metal oxides (iron oxide, cerium oxide,

zinc oxide, titanium oxide, etc.) metals (iron, silver, gold, etc.) and quantum dots (cadmium selenide and cadmium sulfide).⁴ The nanoparticles used for all same procedures, the metallic nanoparticles thought-about because the most promising as they contain exceptional antibacterial properties that is of interest for researchers because of growing microbial resistance against metal ions, antibiotics and also the development of resistant strains. Among all the metallic nanoparticles, silver nanoparticles are an arch product as a result of their distinctive properties like chemical stability, sensible conduction, catalytic and most vital antibacterial, antiviral, antifungal additionally to anti inflammatory properties.

MATERIALS AND METHODS:

Plant material collection and identification:

The plant *Terminalia belerica* leaf was collected from the surrounding areas of Bangalore. It is dried under shade and made into coarse powder. The plant was then identified and authenticated.

Pharmacognostical studies:

Different parameters viz; macroscopy and proximate values were investigated as per standard protocols which includes colour, taste, size, moisture content, foreign organic matter, foaming index, ash and extractives values.

Phytochemical studies:

Phytochemical screening was carried out by the methods referred from text book authored Pulok Mukherjee and Kokate.

Determination of total phenolic content (TPC):

Total phenolic compounds were determined by using Folin-Ciocalteu method.

Synthesis of nanoparticles:

Selection of leaf extract:

Terminalia belerica ethanolic leaf extract was selected for the synthesis of silver nanoparticles because of its high phenolic content.

Biosynthesis of Silver Nanoparticles:

For biosynthesis of silver nanoparticles, 5 ml of the prepared ethanolic leaf extract of *Terminalia belerica* was added to 95 ml of 1 mM aqueous silver nitrate solution and stirred properly. The visual colour change in the reaction mixture from colorless to dark brown should be observed and the reaction mixture was then incubated at room temperature for a period of 24 hrs for the complete reduction of silver ions to silver nanoparticles. The formation of silver nanoparticles was confirmed by UV-Visible spectroscopy. After the synthesis of AgNPs, the solution containing nanoparticles was centrifuged at 8000 rpm for 10 mins to separate silver nanoparticles from other composition of solution and the deposit was prepared for relevant analysis. The dried AgNPs were kept in microtube for further studies.

Characterisation of silver nanoparticles:

The formation of silver nanoparticles using plant extract was monitored by various analytical techniques like UV-Vis, FTIR, XRD, SEM and TEM.

Antimicrobial activity of the biosynthesized silver nanoparticles:

Preliminary test for antibacterial activity:

The antimicrobial activity of AgNPs was evaluated against gram negative microorganism (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram positive microorganisms (*Staphylococcus aureus* and *Lacto bacillus*) by cup diffusion method. Approximately 10^6 colony-forming units (CFU) of the microorganisms were inoculated on Luria Broth (LB) agar plate and then 50 µg/ml concentrations of AgNPs were loaded in the wells present in the LB agar plate. A reaction mixture lacking AgNPs and *Terminalia belerica* leaf extract were loaded in the wells in the LB plate and cultured under the same condition as the control test. All the LB plates were incubated in the incubator at 37°C overnight. After incubation period, the plates were observed for the zone of inhibition.

Evaluation of antibacterial effectiveness using Minimum Inhibitory Concentration method:

The antimicrobial effectiveness of AgNPs were evaluated by Minimum Inhibitory Concentration (MIC) which was determined by broth dilution method against microorganism including gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram positive (*Staphylococcus aureus* and *Lacto bacillus*). In 5 ml LB broth, 100

µL of AgNPs from different concentrations (50, 25, 10, 5, 1, 0.5, 0.25, 0 µg/ml) and 100 µL of each test microorganisms were added. The cultures were then incubated at 37°C for 24 hours with shaking. AgNPs with inoculum in Luria broth media were used as positive control. The results were then monitored by measuring the mean optical density OD at 600 nm.

Effect of AgNPs on bacterial growth:

To determine the growth curve in the presence of silver nanoparticles, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Lacto bacillus* were grown in the liquid LB medium. These microorganisms were activated by culturing 100 µL of each in 10 ml LB medium, and then incubated it at 37°C for 24 hours with shaking at 150 rpm. Then, in 10 ml LB broth, 100 µL of AgNPs from different concentrations (50, 25, 10, 5, 1, 0.5, 0.25, 0 µg/ml) and 100 µL of each microorganism were added. The microbial growth was then monitored by measuring the optical density (OD) at 600 nm.

RESULTS AND DISCUSSIONS:

Pharmacognostical studies:

Macroscopically, the leaf of *Terminalia belerica* was found to be highly ornamental, glossy, alternate, spirally clustered at the tips of the forked twigs.

- Colour: Dorsal view- Dark green, Ventral view- Pale green
- Odour: Characteristic
- Apex and Base: Acute
- Shape: Ovate elliptic and pointed at both ends
- Size: 7.5 - 11.5 cm long and 2.5 - 4 cm width
- Petiole: 4.5 cm long
- Margin: Entire or emarginated
- Midrib: Prominent

The proximate values are given in the following table (Table 1).

Table 1: Proximate values

Parameters	Yield % (W/W)
Loss on drying	6.9
Foreign organic matter	<2%
Foaming index	<100
Total ash	5.9
Acid insoluble ash	1
Water soluble ash	8.2
Petroleum ether soluble extractive	4.71
Chloroform soluble extractive	5.32
Ethanol soluble extractive	9.04
Water soluble extractive	8.28

Phytochemical studies (Table 2):

Table 2: Phytochemical preliminary screening

Chemical constituents	Test	Pet ether	Chloroform	Ethanol	Water
Alkaloids	- Dragendorff's	-	-	+	+

	reagent - Hager's reagent	-	-	+	+
Protein	Copper sulphate test	-	-	+	-
Phenol	Ferric chloride test	-	+	+	+
Glycoside	Borntrager's test	-	+	+	+
Carbohydrates	Molisch's test	-	-	+	+
Saponins	Frothing test	-	-	-	-
Steroids	- Liebermann - Burchard's test	+	+	-	-
Tannins	Lead acetate test	-	-	-	+
Flavonoids	Lead acetate test	-	-	+	+

(+ present, - absent)

1.1. Total phenolic content (Table 3)

Table 3: Total phenolic content

Solvents	Total Phenols (mg GAE/g DW)
Chloroform	1.29 ± 0.07
Ethanol	22.40 ± 0.05
Water	10.32 ± 0.08

1.2. Synthesis of silver nanoparticles

The formation of AgNPs by the reduction of silver nitrate was indicated by the change in colour of the reaction mixture from light yellow to brown colour (Figure 1) within 24 hours. The appearance of dark brown color within 24 hours of incubation confirms the reduction of AgNO₃ into AgNPs. After 24 hours, no change in the colour is observed which indicates that the AgNPs synthesis process was completed. On the other hand, no change in colour was observed in the control solution that contained only 1 mM AgNO₃ solution and had been kept under the same conditions as that in the other flask (Figure 1).

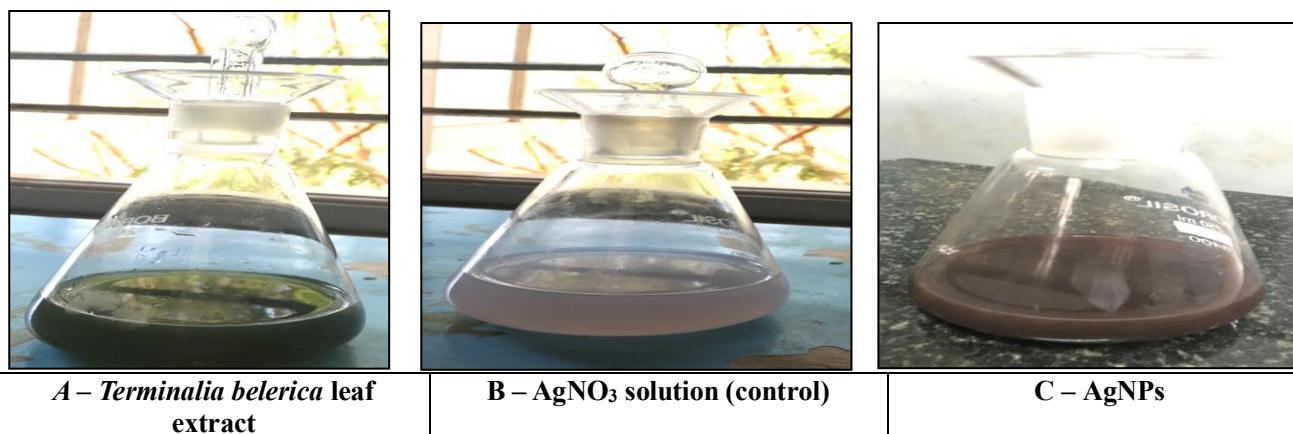


Figure 1: Silver nanoparticles formation

Characterisation of biosynthesized silver nanoparticles:

UV-Visible spectral analysis

The Surface Plasmon Resonance (SPR) band of silver nanoparticles occurs at 400-450 nm. In this study the synthesized AgNPs using ethanolic leaf extract of *Terminalia belerica* showed SPR band between 400-450 nm which confirms the formation silver nanoparticles (Figure 2). An early report suggests that single SPR band in the absorption spectra indicates spherical metal nanoparticles and two or more SPR bands indicate anisotropic particles.¹⁸ With reference to that AgNPs prepared by using ethanolic leaf extract of *Terminalia belerica* showed single and sharp SPR band which indicate that the nanoparticle is spherical in shape.

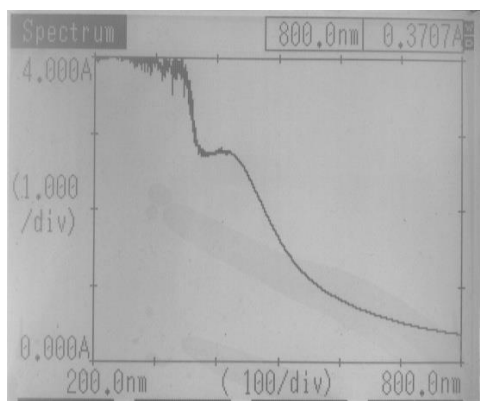


Figure 2: UV-Vis spectra for AgNPs

Fourier Transform Infrared:

The representative ATR FTIR spectra of the ethanolic leaf extract of *Terminalia bellerica* and the stabilized silver nanoparticles were shown in Figure 3 and 4. It can be seen that, in contrast to the ethanolic extract of *Terminalia bellerica*, the stabilized silver nanoparticles show significant changes in their respective vibrational spectra. The ethanolic leaf extract of *Terminalia bellerica* showed intense peaks at 3270.30 cm⁻¹ and 1633.01 cm⁻¹ (Figure 3). In stabilized silver nanoparticles the strong bands were observed at 3308.81 cm⁻¹, 1637.29 cm⁻¹, 1437.62 cm⁻¹, 1312.11 cm⁻¹, 1140.97 cm⁻¹, 986.94 cm⁻¹, 901.37 cm⁻¹ (Figure 4). In the Plant extract the peak was broad and blends, but after encapsulation of nanoparticles the peak was narrow and sharper. The absorption peak at 3270.30 cm⁻¹ observed in control extract, is due to OH stretching vibration, 1633.01 cm⁻¹ is due to C=O stretching, which indicates that the control extract may have the phenolic substances. These structural changes indicated that the reduction and stabilization of silver nanoparticles proceed via the coordination between the phenolic substances of the plant extracts with the silver ions. The FTIR studies have confirmed the fact that the Phenolic group has the stronger ability to bind metal indicating that the phenolic constituents could possibly form a layer covering the metal nanoparticles (i.e., capping of Silver nanoparticles) to prevent agglomeration and thereby stabilize the medium.

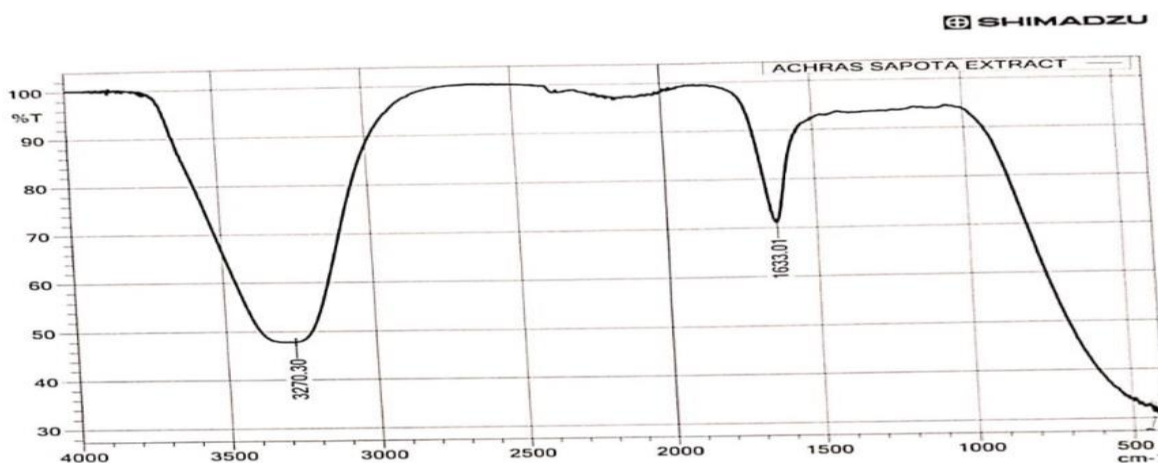


Figure 3: FTIR Spectra of ethanolic leaf extract of *Terminalia bellerica*

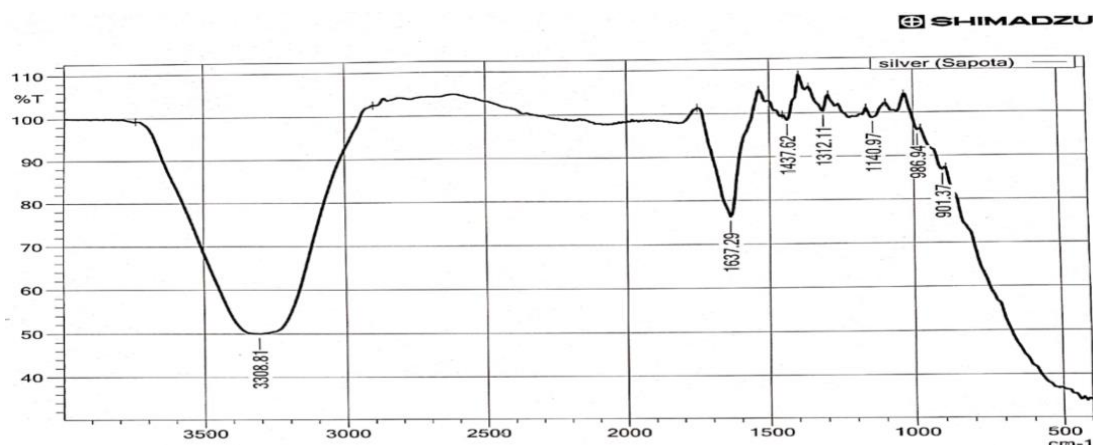
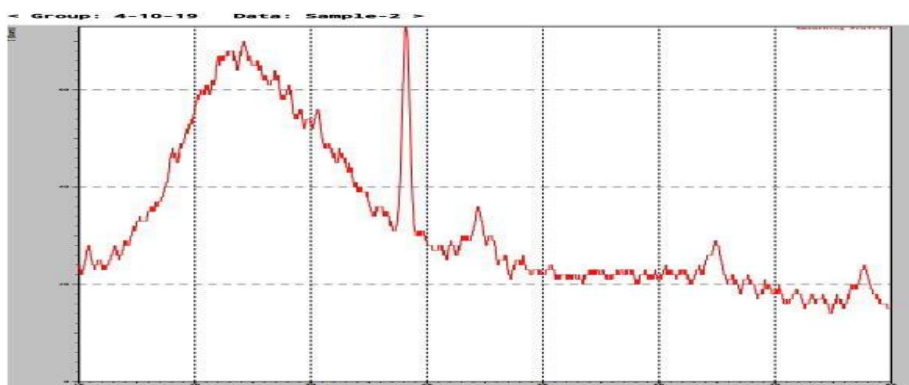


Figure 4: FTIR spectra of AgNPs synthesized by *Terminalia belerica*

X-ray Diffractometry:

The crystalline nature of Silver nanoparticles was confirmed from X-ray diffraction. XRD analysis shows that XRD pattern of the dried nanoparticles obtained from colloid samples. Three peaks were observed at 38.16°, 44.43° and 24.26° in the 2θ range 10-80° for silver nanoparticles (Figures 5 a and 5 b) (Table 4). These Bragg reflections clearly indicated that presence of (2.35646), (2.03738) and (3.66582) sets of lattice planes and further on the basis that they can be indexed as face centered-cubic (FCC) structure of silver. The observed peak broadening and noise were probably macromolecules present in the plant extract which may be responsible for the reduction of silver ions. Hence XRD pattern thus clearly illustrated that the Silver nanoparticles formed in this present synthesis are crystalline in nature. In addition to the Bragg peaks representative of FCC silver nanocrystals, additional as yet unassigned peaks are also observed suggesting that the crystallization of bio-organic phase occurs on the surface of the Silver nanoparticles.¹⁹



(a)

Figure 5 (a): XRD pattern of AgNPs synthesized by *Terminalia belerica*

Table 4: Details of Strongest 3 peaks of AgNPs XRD analysis

No.	2 Theta (deg)	D (A)	I/I1	Intensity (Counts)
1	38.1600	2.35646	100	28
2	44.4300	2.03738	29	8
3	24.2600	3.66585	21	6

Scanning Electron Microscopy:

SEM results suggest that synthesized AgNPs are present in the nanometer range and the particles are monodispersed (Figure 6). White particles were observed for AgNPs at different magnifications. The overall

morphology of most of AgNPs may be described as irregular, some possess spherical shape.

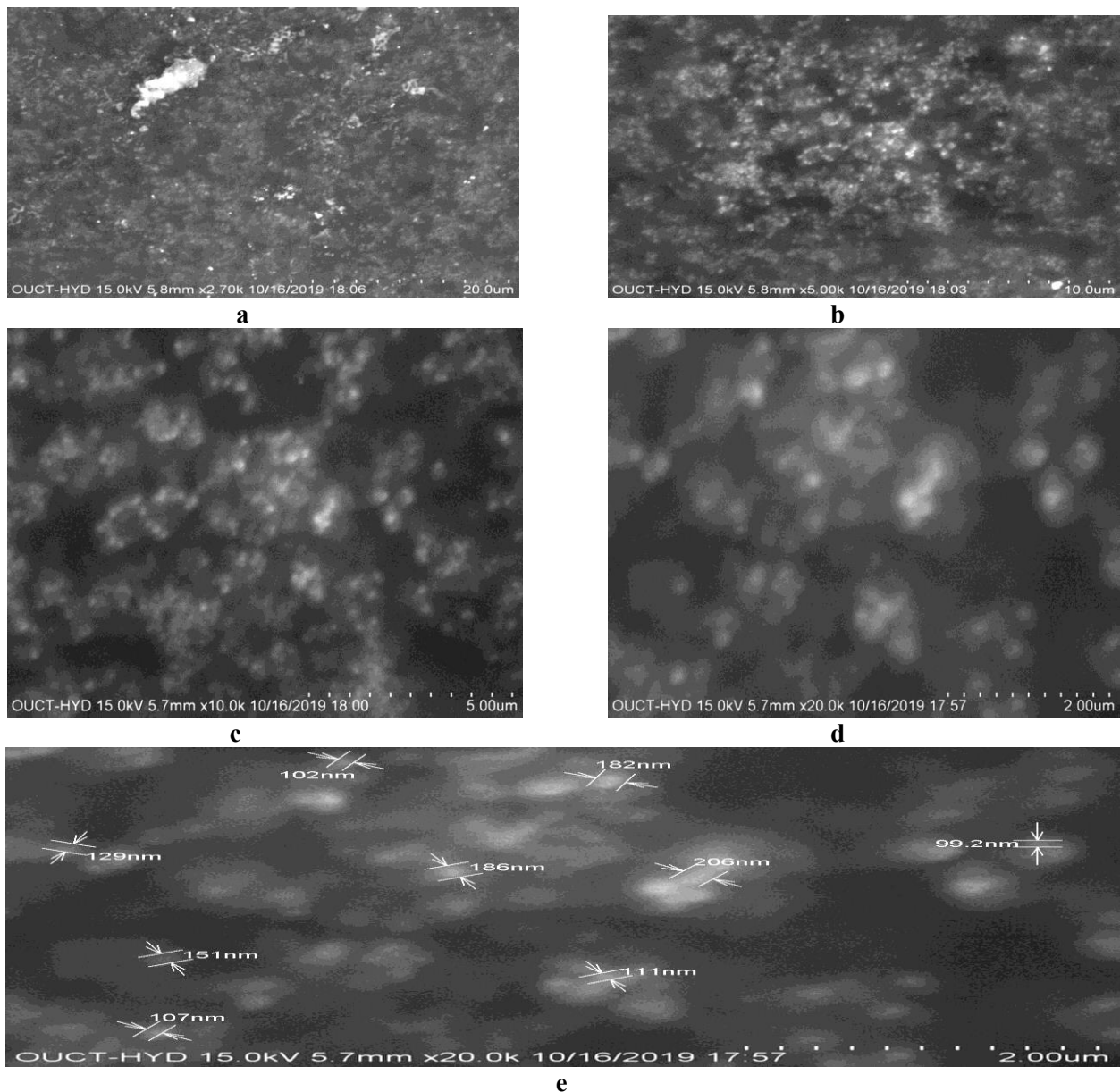


Figure 6 (a-e): SEM images of the biosynthesized AgNPs at different magnifications

Transmission Electron Microscopy:

Morphological structure and distribution of biosynthesized silver nanoparticles were characterized at high magnifications done by Transmission Electron Microscopy (TEM). Figure 7 shows the well dispersed predominantly spherical shape of silver nanoparticles and it also confirms that the biosynthesized AgNPs were in nanosize i.e., it ranges from 5-200 nm. It is also quite obvious from the images that the AgNPs were capped with phytoconstituents of *Terminalia belerica* ethanolic leaf extract.

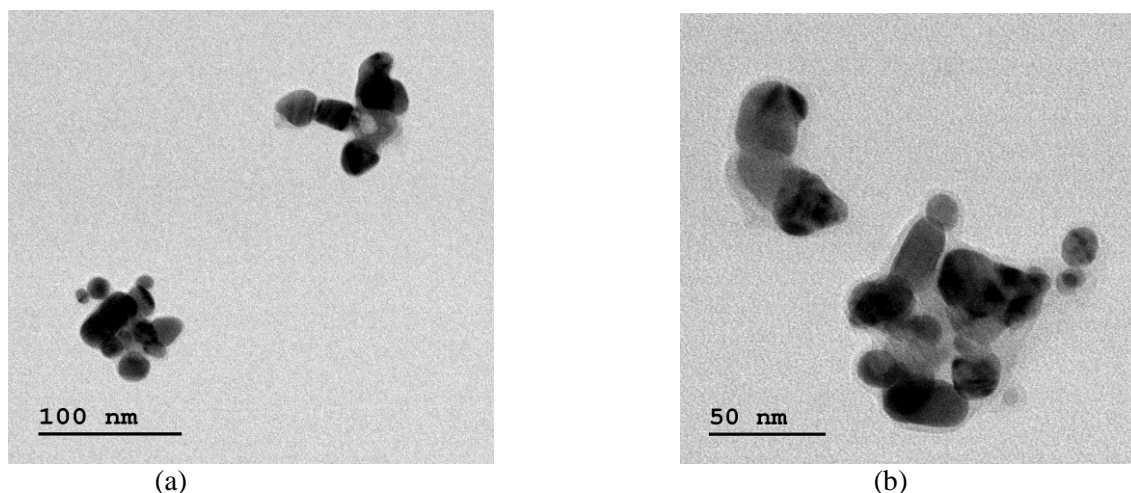


FIG 7 (a-b): TEM images of biosynthesized AgNPs prepared from the ethanolic leaves extract of *Terminalia belerica* at different magnification.

ANTIMICROBIAL ACTIVITY OF THE BIOSYNTHESED SILVER NANOPARTICLES:

Preliminary test for antibacterial activity:

The antibacterial activity of different concentrations of AgNPs was proved from the zone of inhibition (Figure 8) against gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and gram positive microorganisms (*Staphylococcus aureus* and *Lacto bacillus*). At concentration 50 µg/ml of both silver and gold nanoparticles a clear zone of inhibition is seen in all the 4 plates. No zone of inhibition is observed in the vehicle control well which is in the middle of the plate which suggested that the antimicrobial activity was specifically due to silver and gold nanoparticles.

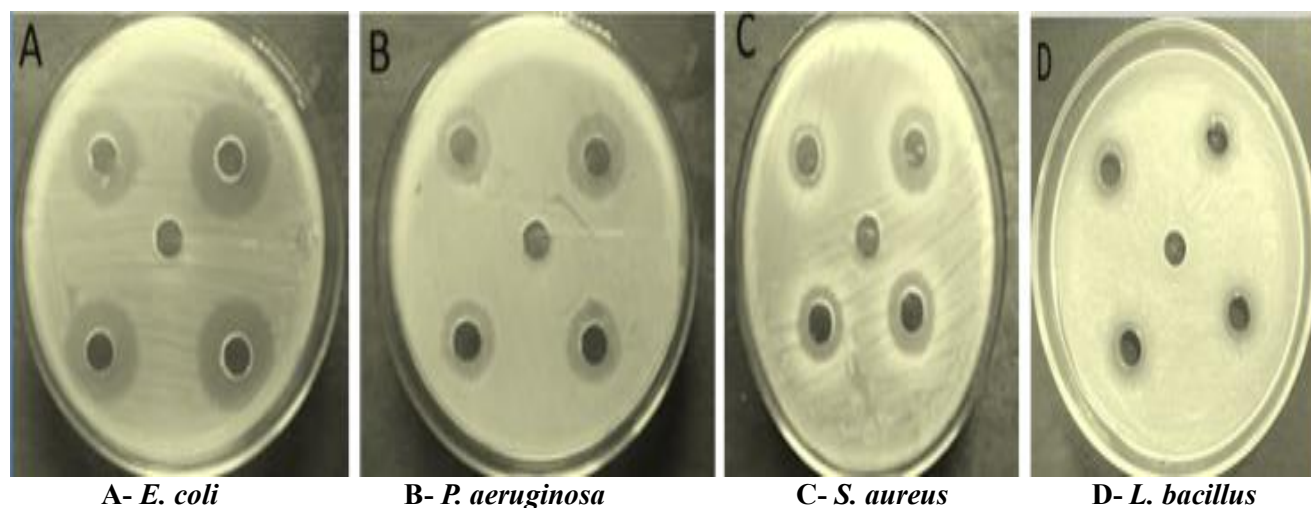


Figure 8 (A-D): Zone of inhibition of AgNPs

Evaluation of antibacterial effectiveness using Minimum Inhibitory Concentration method:

To study the antimicrobial effectiveness of silver nanoparticles, a bacterial concentration of high CFU (10^6 /ml) were treated with varying concentration of silver nanoparticles from 0.25 to 50 µg/ml. When the concentration of AgNPs was increased the bacterial concentration was found to decrease. At a concentration of 50 µg/ml of AgNPs, the growth of the microorganisms of gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and gram positive microorganisms (*Staphylococcus aureus* and *Lacto bacillus*) was completely inhibited, which indicated that the Minimum Inhibitory Concentration of AgNPs was 50 µg/ml (Figure 9-13).

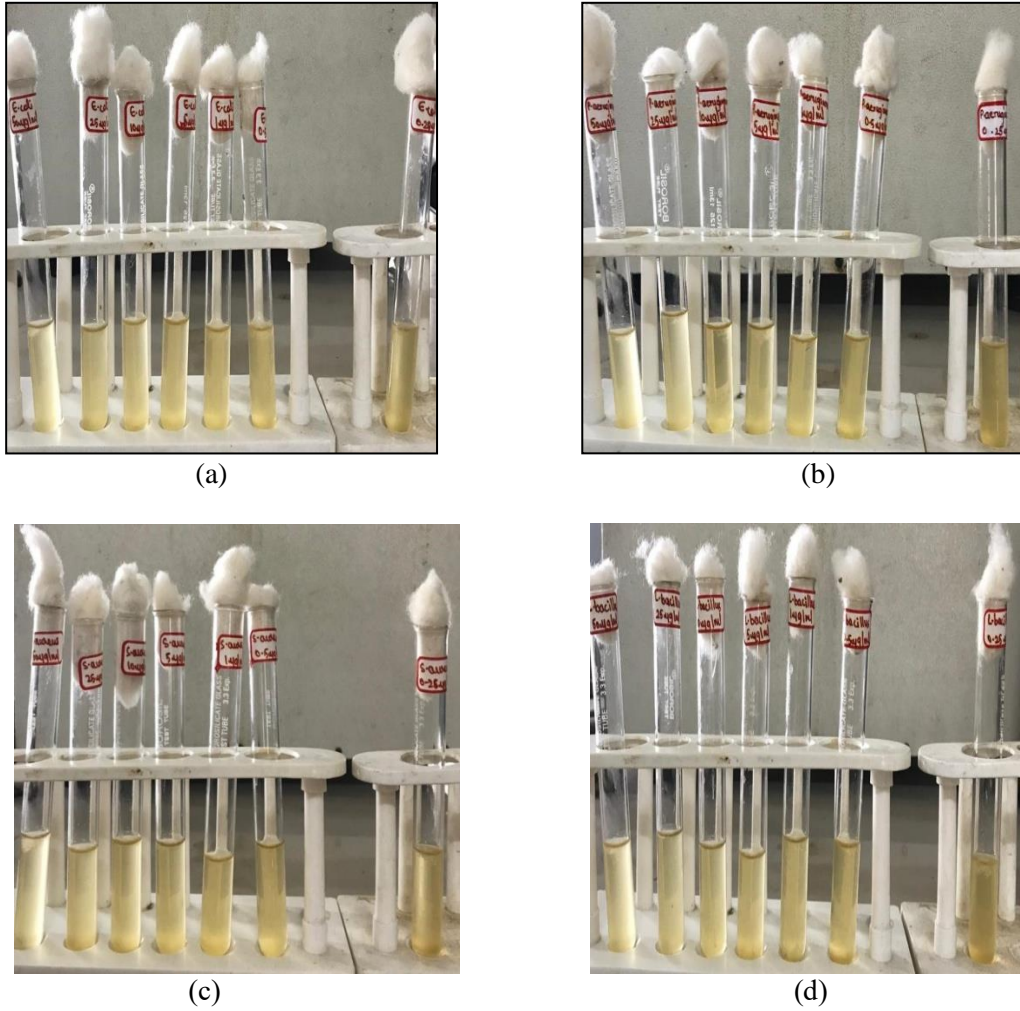


Figure 9 (a-d): Determination of MIC of AgNPs

where a, b, c and d indicates different concentrations of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. bacillus* respectively

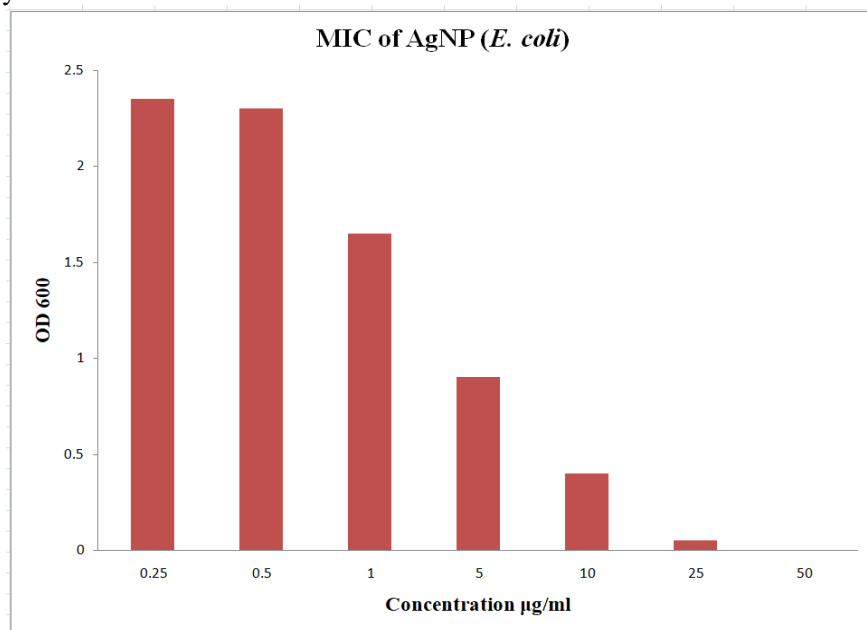


Figure 10: MIC of AgNPs on *E. coli*

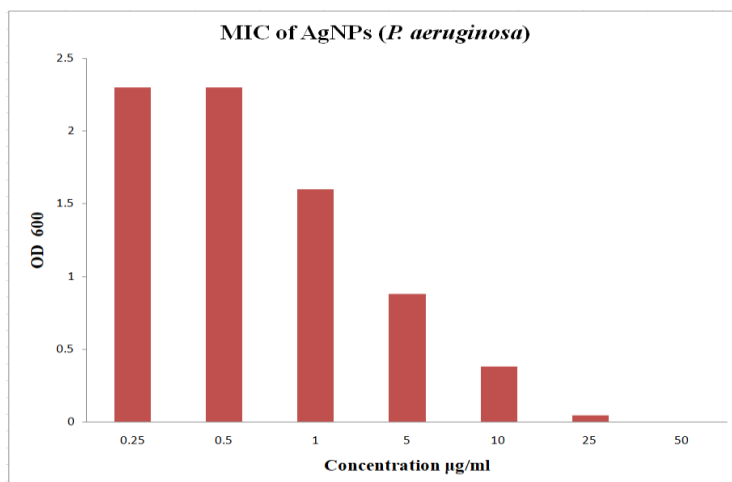


Figure 11: MIC of AgNPs on *P. aeruginosa*

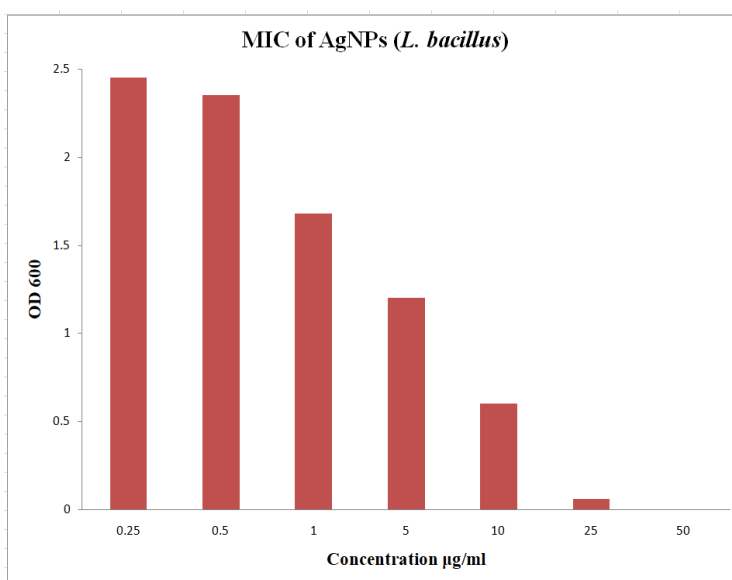


Figure 12: MIC of AgNPs on *L. bacillus*

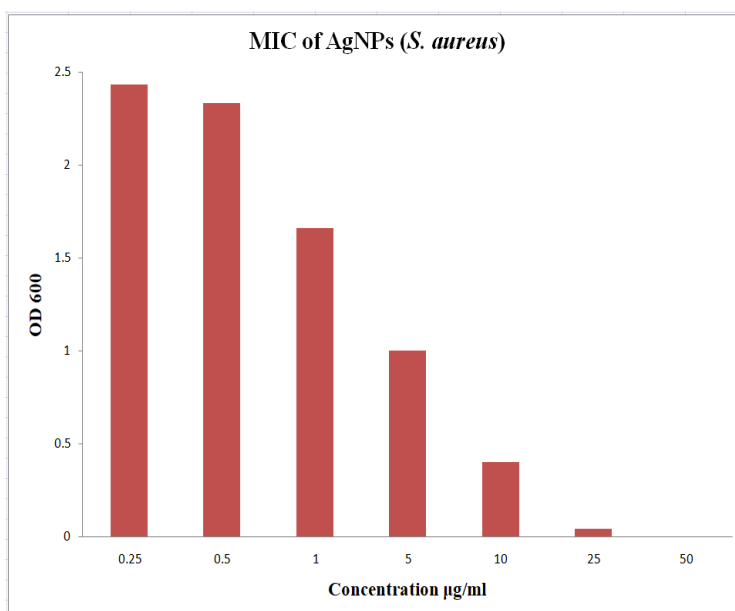


Figure 13: MIC of AgNPs on *S. aureus*

Effect of AgNPs on bacterial growth:

Growth rates and bacterial concentrations were determined by measuring OD at 600 nm at different time points i.e., from 1 to 6 hours (Figure 9). The slope of the bacterial growth curve continuously decreased with increasing silver nanoparticles concentration. This means that at low concentration of the nanoparticles, the growth of all the four i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Lacto bacillus* bacteria were delayed and at higher concentration, growth was completely inhibited. So it can be concluded that silver nanoparticles are bacteriostatic at low concentration and bactericidal at high concentration (Figure 14-17).

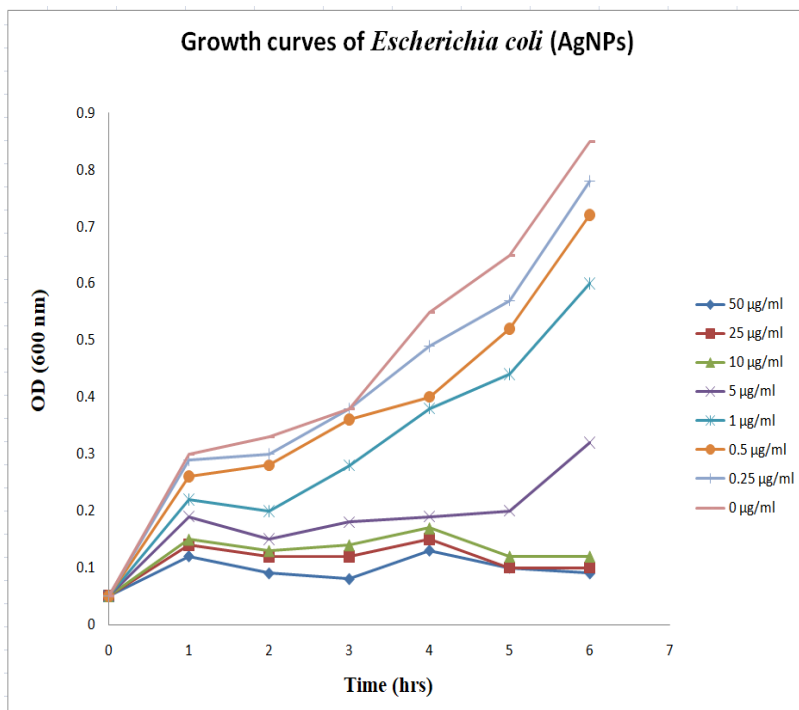


Figure 14: Growth curve of *E. coli* using biosynthesized AgNPs as an antibacterial agent

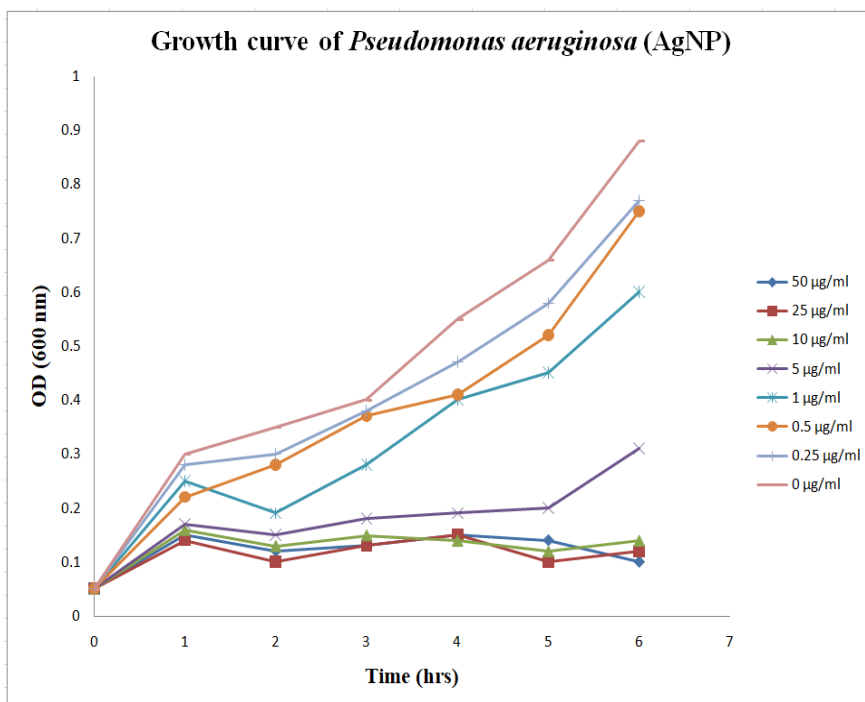


Figure 15: Growth curve of *P. aeruginosa* using biosynthesized AgNPs as an antibacterial agent

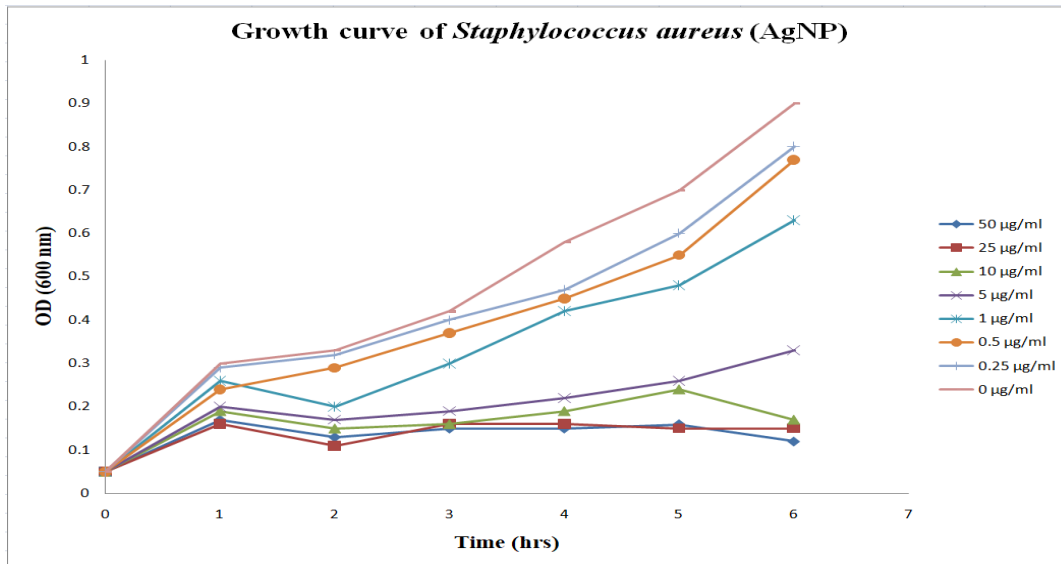


Figure 16: Growth curve of *S. aureus* using biosynthesized AgNPs as an antibacterial agent

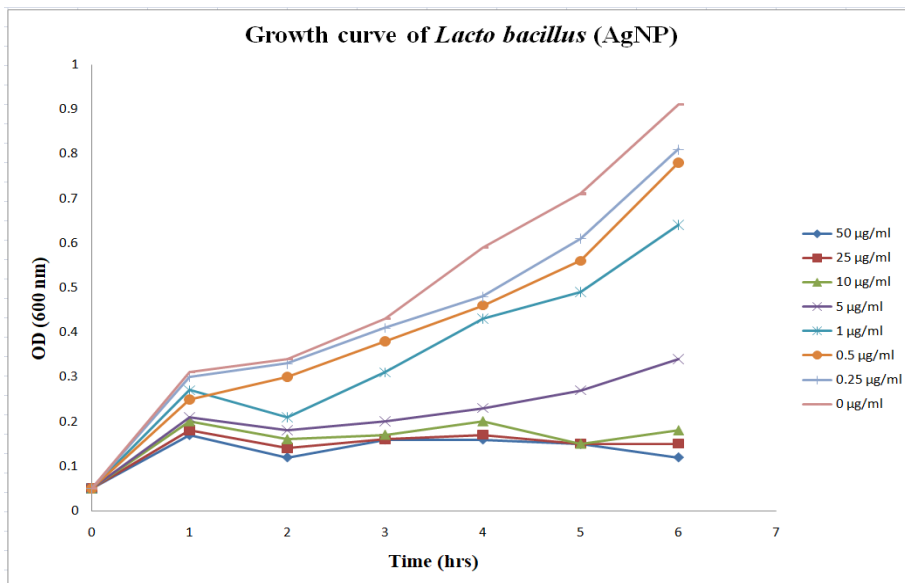


Figure 17: Growth curve of *L. bacillus* using biosynthesized AgNPs as an antibacterial agent

CONCLUSION:

A simple one-pot green synthesis of stable silver nanoparticles using the ethanolic leaf extract of *Terminalia belerica* L. at room temperature was reported in this study. Synthesis was found to be efficient in terms of reaction time as well as stability of the synthesized nanoparticles which exclude external stabilizers/reducing agents. It proves to be an eco-friendly, rapid green approach for the synthesis providing a cost effective and an efficient way for the synthesis of silver nanoparticles and it was also found out that the biosynthesized silver nanoparticles possess potent antimicrobial activity.

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