



Characterization Of Phyto-Synthesized Silver Nanoparticles And Their Antimicrobial Activity Against Diabetic Foot Ulcer Bacterial Isolates.

Chhajed Shweta ^{1*} and Arora Asha²

¹Researcher, Department of Biotechnology, M L S University, Udaipur, 313001, India.

²Head, Department of Biotechnology, BNPG college, Udaipur, Rajasthan 313001, India.

Abstract: Diabetic Foot Ulcer (DFU) is one of the major complications of Diabetes. Patients with Diabetic Foot ulcers have a high susceptibility to microbial infections and are the leading cause of hospitalization and amputation of lower limbs. In the era of increased prevalence of bacterial resistance and outbreak of resistant infectious diseases, it is very essential to develop effective therapeutic strategies towards multi-drug resistant pathogens. The antimicrobial properties of silver nanoparticles have been well studied, therefore their use in biomedicine and pharmacology is a trend. Herein we present the use of Phyto-mediated synthesized AgNPs for the treatment of diabetic foot ulcers by topical administration. The nanoparticles were synthesized by reducing silver nitrate using *Terminalia chebula* fruit extract. The nanoparticles were analyzed and characterized using UV-Visible Spectrophotometer, FTIR, XRD, SEM with EDAX, TEM, and DLS. The synthesized silver nanoparticles were assayed for antimicrobial activity against five Diabetic Foot Ulcer bacterial isolates i.e. *Escherichia coli*, *Klebsiella Pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus aureus*, and *Bacillus subtilis*. The bactericidal property of synthesized nanoparticles was analyzed by the Agar well diffusion method, which revealed the remarkable antimicrobial effects against all the selective pathogenic bacterial isolates of Diabetic foot ulcers in the present study. These results constituted the basis for further studies on the use of plant-based silver nanoparticles for the treatment of Diabetic Foot ulcers from different origins.

Keywords: Silver Nanoparticles, Amputation, *Terminalia chebula*, Bactericidal, Diabetic Foot Ulcer, *Escherichia coli*, *Klebsiella Pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus aureus*, and *Bacillus subtilis*.

*Corresponding Author

CHHAJED SHWETA I*, Researcher, Department of Biotechnology, M L S University, Udaipur, 313001, India.



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1. INTRODUCTION

Diabetes, an endocrine disease characterized by high blood glucose levels, is among the most frequent and fastest-growing diseases in the world, with around 693 million adults affected by this disorder, with a rise of more than 50% from 2017. Diabetes is linked to a slew of serious complications,^{1,2} retinopathy, neuropathy, atherosclerosis, nephropathy, and foot ulcers are among them. The untreated wound in diabetes, ruffed by infectious pathogens.³ In persons with Type 1 or Type 2 diabetes, the annual population-based rate of foot ulcers ranges from 1.9 percent to 2.2 percent.⁴ Diabetic foot ulcer patients are more likely to develop various other complications, hence careful management in a multidisciplinary approach is essential.⁵ Pathogen prevalence varies in diabetic foot ulcers.⁶ Foot infection is caused by several factors of wound microbiology including microbial burden, diversity of microorganisms, presence of infectious organisms, and synergistic connection among microbial species. Polymicrobial isolates have been found in severe cases of diabetic foot ulcers.^{7,8} *Staphylococcus*, *Streptococcus*, *Proteobacteria*, *Pseudomonas aeruginosa*, and coliform bacteria are thought to be the DFU's causal organisms. Silver-containing dressings are utilized exclusively to prevent microbial infections throughout the wound healing and tissue engineering processes.^{9,10} The presence of the low amount of growth factor in wounds has been linked to the delay in DFU.¹¹ To combat this, Silver nanoparticles have been shown to improve wound healing by promoting the proliferation and migration of keratinocytes. As a result, AgNPs may promote wound healing by preventing infections from Gram-negative or Gram-positive bacteria. Nanotechnology is the most dynamic subject of material science, and the production of nanoparticles (NPs) is rapidly increasing around the world.¹² It is gaining traction in a variety of disciplines, including health care, food, cosmetics, environmental health, biomedical science, chemical industries, medication and gene therapy, and biological sciences.¹³ Conventional physical and chemical methods have limited use in preparing metal nanoparticles due to toxic chemicals.¹⁴ To solve these issues, scientists have discovered the exact green routes, or naturally produced resources and their byproducts, that can be utilized to synthesize nanoparticles. Green synthesis can be divided into three categories: (a) microorganisms such as fungi, yeasts (eukaryotes), bacteria, and actinomycetes (prokaryotes); (b) plants and plant extracts; and (c) templates such as membranes, viruses DNA, and diatoms. In comparison to microorganism-based green synthesis, plant-mediated synthesis of noble metal nanoparticles is gaining a lot of attention these days, because of its distinctive features like the wide range of applications, faster synthesis rate, and flexible morphological properties. Allegedly, biological agents act as reducers, stabilizers, or both in the process of forming nanoparticles.^{15,16} The green synthesis approach is used to create silver nanoparticles of various shapes and sizes utilizing diverse plant parts such as root, stem, seed, leaves, peel, fruit, callus, and flower. The present study comprises the synthesis and characterization of silver nanoparticles using selected medicinal plants and evaluates their antibacterial activity against Diabetic Foot Ulcer bacterial isolates for improved treatment procedures.

2. Materials and Methods

2.1. Collection and Identification of Plant Sample

Fruits of *Terminalia chebula* were purchased from the local

market of Udaipur, Rajasthan, India.¹⁷ For the authentication and validation of plants, a herbarium sheet was prepared with taxonomical affiliation, and the plant material was authenticated by Dr. Sunita Jain, Department of botany, BN University, Udaipur, Rajasthan, India. Silver nitrate (AgNO₃) was purchased from Sigma-Aldrich, India. The media, chemicals, and antibiotics were purchased from Hi-media laboratory Pvt. Ltd. Mumbai.

2.2. Preparation of Plant Extract

The plant extract was prepared by adding 10 gm *Terminalia chebula* fruit powder in 100 ml sterile distilled water, boiled in the water bath at 80° C for 10 min, and after that cooled at room temperature.¹⁸ These extracts were filtered through Whatman No.1 filter paper and then with vacuum filtration unit using filter paper of 0.2 µm size in diameter. The filtrate is stored at 4° C for further analysis.

2.3. Synthesis Of Silver Nanoparticles

Synthesis of Silver nanoparticles was done by adding 5ml of plant extract to 100 ml 1mM aqueous solution of silver nitrate solution for the bio-reduction process at room temperature.¹⁹ The reaction was carried out in a dark room to minimize the photoactivation of silver nitrate in light. The formation of silver nanoparticles was observed after 24 hours and the solutions were turned from yellow to dark brown indicating the formation of Ag NPs. The silver nanoparticles formed in the solution were purified by centrifugation at 15,000 rpm for 30 min. The centrifugation process was repeated three times. This process was followed by the dispersion of pellets in deionized water.²⁰

2.4 Characterization of Silver Nanoparticles

The silver nanoparticles formed were monitored by a UV-vis spectrophotometer. The structure and composition of the purified silver nanoparticles were characterized by X-Ray diffraction, Scanning Electron microscopy, Transmission Electron microscopy, FTIR, and EDAX techniques.

2.4.1. UV-Vis Spectroscopy

It refers to absorption spectroscopy in the UV Visible spectral region. The absorbance spectrum of the colloidal sample was obtained in the range of 200–800 nm.²¹ On the addition of plant extract, the intensity of color from yellow to brown was increased with time at room temperature.

2.4.2. XRD Analysis

The particle size and nature of AgNPs were determined using X-Ray diffraction (XRD). X-ray diffraction (XRD) analysis was conducted by X-Ray Diffractometer using monochromatic Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) operated at 40 kV and 30 mA at a 2 θ angle pattern. The scanning was done in the region of 20°–80° with a step size of 0.02.²² The images obtained were compared with the Joint Committee on Powder Diffraction Standards (JCPDS) library to account for the crystalline structure. The coherent diffraction Crystallography domain size of the Silver nanoparticle was calculated from the width of the XRD peaks using the Scherer formula.

2.4.3. TEM

Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through.²³ An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera.

2.4.4. SEM & EDAX

The scanning electron microscope works on the same principle as an optical microscope, but it measures the electrons scattered from the sample rather than a photon.²⁴ Scanning electron microscopy and EDAX analysis were done using Scanning electron microscope XL 30 ESEM and EDAX. The powder sample was placed on an aluminum stub and coated with a carbon tap and then kept in an instrument for further analysis.²⁵

2.4.5. FTIR

This technique was used to determine the interaction between the compounds present in plant extract and silver nitrate. FTIR analysis of dried silver Nanoparticles was carried out through the Potassium bromide (KBr) pellet method in a 2:200 ratio and the spectrum was recorded using FTIR-8400s.

2.4.6. DLS Analysis

The principle of size measurements by DLS is based on measuring light scattering intensity fluctuations. This method was used for the measurement of narrow particle size distributions, especially in the range of 2–500 nm. Lasers of known wavelength passed through the diluted sample in solution and the intensity of scattered light was collected by a detector and deconvoluted by algorithms to determine the particle size distribution of the sample.²⁶ The measurements were performed in disposable polycarbonate folded capillary cells with gold-plated beryllium-copper electrodes. This analysis was performed at RCA College, MPUAT, Udaipur, Rajasthan.

2.5 Antimicrobial Assay

A stock solution of 1000 ppm was prepared by dissolving 100 mg of synthesized silver nanoparticles in 100 ml of deionized water. From the stock solution of 1000 ppm, 10 ml of 200 ppm, 400 ppm, 600 ppm, and 800 ppm solutions were prepared. The bacterial strain was prepared by inoculating pure culture of bacterial strain into 5 ml of nutrient agar

solution and then incubated at 37°C for 24 hours in the incubator. Preparation of test bacteria was carried out by inserting one inoculation loop of cultured bacteria into 5 ml of 0.19% NaCl solution.

The Agar well method was used to evaluate the antimicrobial activity of plants or microbial extracts.²⁷ 20ml of molten Muller Hinton Agar (MHA) was poured into sterile Petri plates (9 cm in diameter) and allowed to set at room temperature. 100 µl of standardized inoculum were swabbed uniformly to solidified MHA plates using sterile cotton swabs and allowed to dry for 5 min. Wells with a diameter of 6 mm have been punched aseptically with a cork borer on freshly seeded agar plates. Streptomycin is used as a positive control. Thereafter, 20µl of plant extracts, AgNO₃, and AgNPs with different ppm concentrations were transferred in wells. The Plates were labeled and incubated upside down at 37°C for 24 hours thereafter the zones of inhibitions were measured.

3. STATISTICAL ANALYSIS

The inhibitory effects of the plant extract and synthesized silver nanoparticles against the test pathogens were expressed as mean ± standard error of the mean inhibition zones diameter (mm). The significance of all the statistical tests was predetermined at P<0.05 using SPSS (Statistical Package for Social Science) version 17 software.

4. RESULTS AND DISCUSSION

4.1 Characterization of Silver-Nanoparticles (AgNP's)

4.1.1 UV-visible Spectroscopy

Plant-mediated silver nanoparticles were synthesized through the mentioned procedure and were initially confirmed by visual analysis (i.e. changing in color). The formation of AgNPs has been confirmed as the color of AgNO₃ solution turned from yellow to dark brown on the addition of plant extract solution.²⁸ The color changes that occurred in the solution after the addition of leaf extract are due to the excitation of NPs Surface Plasmon Resonance (SPR), which strongly indicates the formation of Ag NP. The biosynthesized silver nanoparticles were characterized in the wavelength range of 300-700 nm.²⁹ Sharpest peak was observed at 430 nm after 80 min. According to the present study, 80-160 minutes was the ideal time for plant-mediated synthesis of silver nanoparticles. The shape and position of the surface plasmon resonance (SPR) band are incumbent for the quality of synthesized AgNPs. And the maximum adsorption peak reveals the comparative size of nanoparticles, as absorption peak at a lower wavelength signifies a smaller size of nanoparticles.³⁰⁻³¹

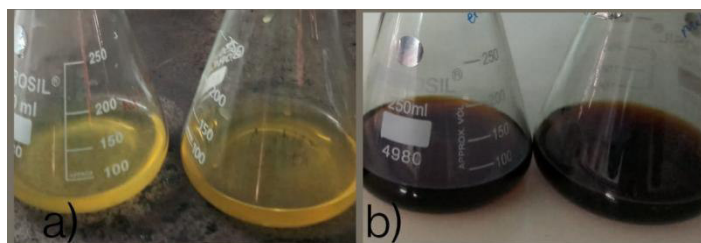


Fig 1: a) *Terminalia chebula* aqueous extract and b) Silver Nanoparticles synthesized using *Terminalia chebula* Fruit extract.

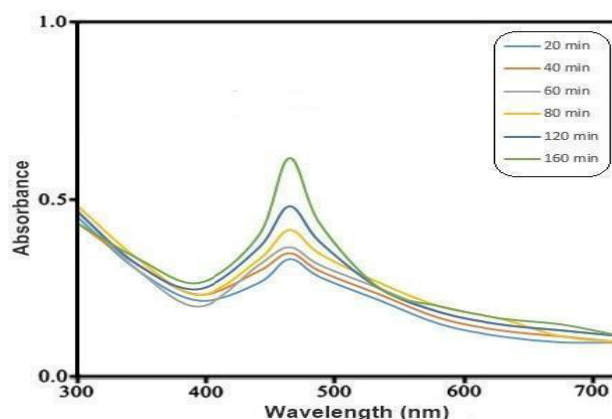


Fig 2: UV-visible spectrum of synthesized AgNP of *Terminalia chebula* at different time duration.

4.1.2. FTIR (Fourier Transform Infra-Red) Spectroscopy

Compounds responsible for the synthesis of AgNPs were identified through FTIR spectroscopy with the spectral range from 500 to 4000 cm^{-1} . FTIR spectrum of AgNPs synthesized from *T. chebula* (Figure 3) showed the most dominant peaks

at 1028.09, 1367.56, 1636.78, 2842.32, 2954.09, 3430.76 cm^{-1} which corresponds to CO stretching of spectroscopy confirmed the presence of several functional groups such as amines, phenol, nitrogen, aromatic compounds with a binding affinity to Ag^+ and responsible for the formation process of AgNPs.³²

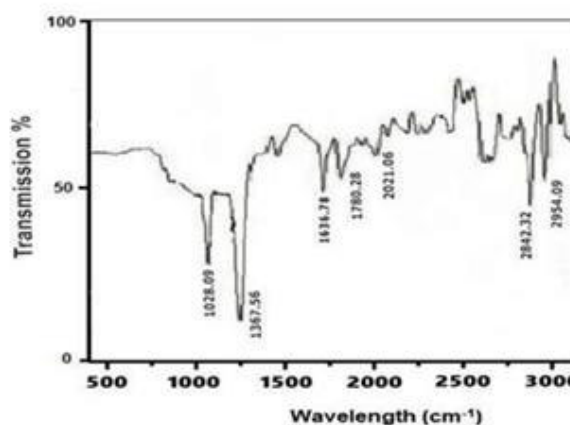


Fig 3: FTIR spectrum of synthesized AgNPs from *Terminalia chebula*

4.1.3. X-RAY DIFFRACTION (XRD)

The XRD patterns were analyzed to investigate the physical structure & properties of the synthesized AgNPs in the 20-90° range of 2θ .³³ The higher peaks obtained may be due to the capping agent that stabilizes the AgNPs and also represents the smaller crystal size of AgNPs.³⁴ XRD pattern

of *T. chebula*-AgNPs (Figure 4) showed the diffraction peaks at 36.79°, 45.38°, 64.85°, 79.15° related to (111), (200), (220), (311) planes respectively, which can be indexed to the face-centered cubic crystal structure of silver. In the present study, the XRD pattern of AgNP confirmed the crystalline structure of synthesized silver nanop

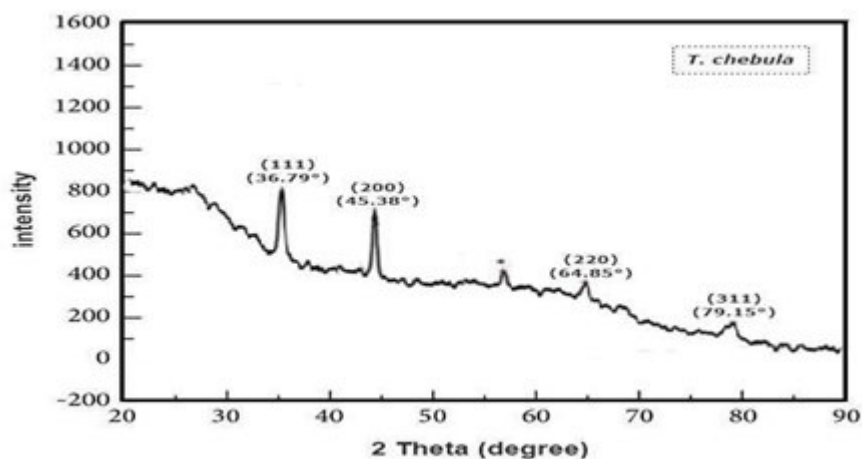


Fig 4: XRD pattern of synthesized AgNPs of *Terminalia chebula*

4.1.4. EDX (energy-dispersive X-ray) Analysis

The chemical composition of synthesized silver nanoparticles was analyzed through the EDX method, which provides the complete elemental profile of synthesized silver nanoparticles. The EDX spectra of silver nanoparticles from

fruit extract of *T. chebula* showed high intensity and significant absorption peak for Ag^+ at 3 keV, which is a well-known absorption signal for Ag^+ .³⁶ Some other eminent peaks were also shown for carbon, oxygen, and other elements because of plant residue as a capping agent.

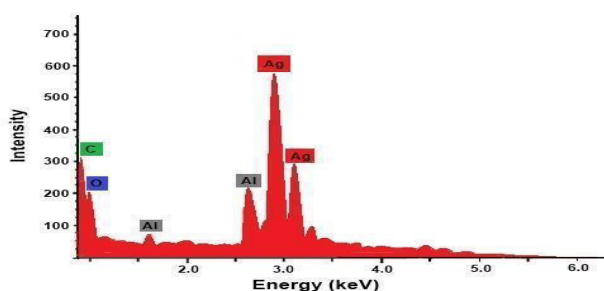


Fig 5: EDX spectra of synthesized AgNP from fruit extract of *T. chebula*.

4.1.5 SEM & TEM analysis

For *Terminalia chebula* AgNPs, the obtained micrographs showed that the synthesized nanoparticles with the size

ranging from 10- 80 nm (Figure 6). The synthesized nanoparticles were dispersed, spherical, and had a rough surface due to the smeared organic layer. A few earlier studies were also reported similar to the current.³⁷

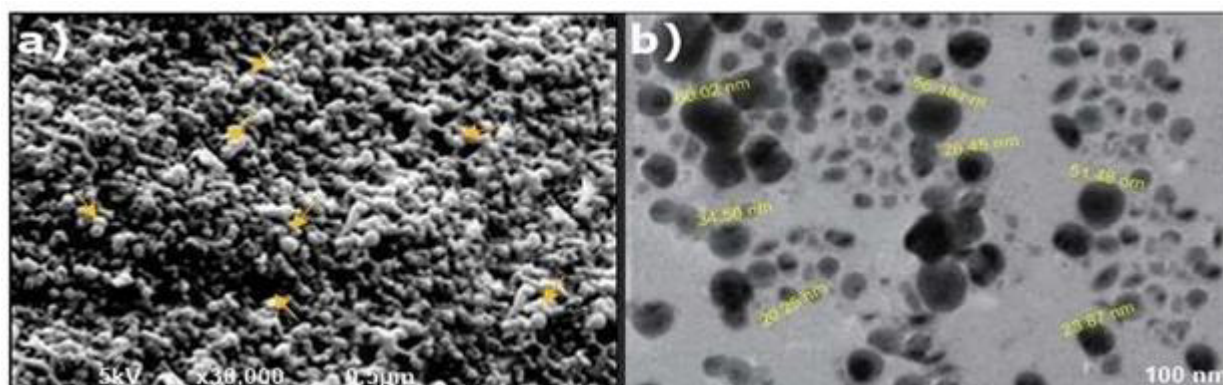


Fig 6: a) SEM & b) TEM micrograph of synthesized AgNPs using fruit extracts of *Terminalia chebula*

4.1.6 DLS Analysis

The mean of AgNPs at optimum condition was recorded at 236 nm and the range of nanoparticles was 200 to 350 nm.³⁸ The DLS measured size is larger than both XRD and TEM measurements because TEM only measures a number based size of the physical size and doesn't include any capping agent,

while DLS hydrodynamic diameter of a particle, plus ions or molecules that are attached to the surface of AgNPs in the solution. So these ions or molecules make the particle size larger in comparison to TEM. Many studies proposed the importance of hydrodynamic diameter for understanding and optimizing the size of nanoparticles.

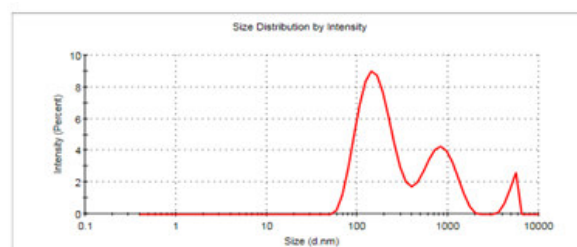


Fig 7: DLS Spectra of Fruit extract of *Terminalia chebula*.

4.2. Antimicrobial Assay

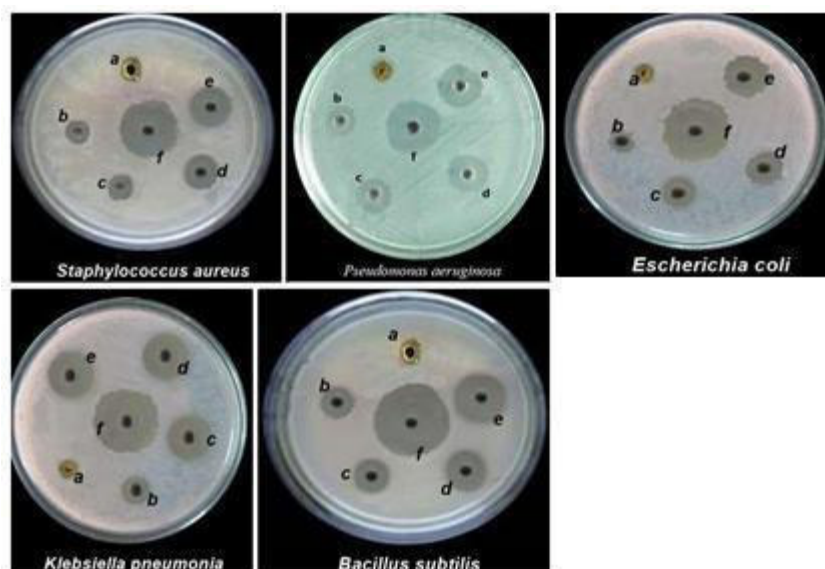
Silver nanoparticles have been noted as the most effective antimicrobial agent because of their sturdy biocidal effect against DFU bacterial strains.³⁹ Bacterial growth was reduced with an increase in AgNP concentration.⁴⁰ The antimicrobial activity of the synthesized silver nanoparticles of Fruit extract of *Terminalia chebula* showed effective results against all the selected pathogenic bacteria at higher concentrations (Table I, Figure 8). The activity of fruit extract was not so effective against the bacteria species, as compared to the synthesized silver nanoparticles, as for fruit extract (20µl), the highest zone of inhibition was found against for *P. aeruginosa* (10.67±0.50) and the least zone of inhibition was found against *S. aureus* (6.67±0.78), whereas zone of inhibition was found in between against *B. subtilis* (9.67±0.67), *E. coli* (7.33±0.22) and *K. pneumonia* (7±0.00). At the 200ppm concentration of silver nanoparticles, the highest zone of inhibition was found against *P. aeruginosa* (16±0.33), followed by *B. subtilis* (13.80±0.12), *K. pneumoniae* (12.4±0.25), *E. coli* (12.23±1.20), and the minimum zone of inhibition was found

against *S. aureus* (11.67±0.35). At the 400ppm concentration of silver nanoparticles, the highest zone of inhibition was found against *P. aeruginosa* (18.34±0.33), followed by *B. subtilis* (16.04±0.34), *K. pneumoniae* (14.78±0.12), *E. coli* (14.33±0.60), and the least zone of inhibition was found against *S. aureus* (14±0.57). At the 600ppm concentration of silver nanoparticles, the highest zone of inhibition was found against *P. aeruginosa* (21.60±0.4), followed by *B. subtilis* (19.17±0.63), *S. aureus* (16.55±0.23), *K. pneumoniae* (16.67±0.66), and the minimum zone of inhibition was found against *E. coli* (16.09±0.23). At the 800 ppm concentration of silver nanoparticles, the highest zone of inhibition was found against *P. aeruginosa* (23.23±0.40), followed by *B. subtilis* (22.67±0.56), *K. pneumoniae* (19.67±0.62), *E. coli* (19.13±0.78), and the least zone of inhibition was found against *S. aureus* (19±0.00). The synthesized silver nanoparticles from the fruit extract of *Terminalia chebula* showed significant antimicrobial activity, as bacterial species of DFU were susceptible to the higher concentration of AgNPs.

Table I: Zone of Inhibition

	Plant extract (20 µl)	AgNps (200ppm)	AgNps (400ppm)	AgNps (600ppm)	AgNps (800ppm)	Streptomycin
<i>Staphylococcus aureus</i>	6.67±0.78	11.67±0.35	14±0.57	16.55±0.23	19±0.00	23±0.1.2
<i>Pseudomonas aeruginosa</i>	10.67±0.50	16±0.33	18.34±0.33	21.60±0.4	23.23±0.40	29.67±1.0
<i>Klebsiella pneumonia</i>	7±0.00	12.4±0.25	14.78±0.12	16.57±0.66	19.67±0.62	27.67±0.78
<i>Bacillus subtilis</i>	9.67±0.67	13.80±0.12	16.04±0.34	19.17±0.63	22.67±0.56	31.33±0.22
<i>Escherichia coli</i>	7.33±0.22	12.23±1.20	14.33±0.60	16.09±0.23	19.13±0.78	27.67±0.12

*Values are means ± standard errors, n = 3



(*a: Plant extract 20 μ l, b: AgNP's 200 μ g/ml, c : AgNP's 400 μ g/ml, d: AgNP's 600 μ g/ml, e: AgNP's 800 μ g/ml, f: Streptomycin 20 μ l)

Fig 8: Antimicrobial activity of aqueous fruit extract and Silver nanoparticles synthesized from *Terminalia chebula* fruit extract.

5. CONCLUSION

The biosynthesis of silver nanoparticles using *Terminalia chebula* fruit extract was found to be an easy and safe method. The addition of silver nitrate solution to the extract begins to form nanoparticles and results were observed with the change in color and increase in absorbance peak. The XRD pattern revealed the physical structure and property of synthesized nanoparticles. Morphology and size were identified by SEM and TEM. The chemical composition of silver nanoparticles was revealed by the EDAX technique. FTIR spectroscopy confirmed the presence of various functional groups and revealed the interaction among the compounds present in plant extract and silver nitrate. The synthesized silver nanoparticles of selected plant extract showed significant antibacterial activity against the selected Gram-negative and Gram-positive pathogens. Thus, plant-based Silver nanoparticles might be a good alternative to develop as an antibacterial agent against multidrug-resistant strains and biofilm forming bacteria. The applications of AgNPs may lead to valuable findings in various fields such as medical devices and antimicrobial systems. The green synthesis of silver nanoparticles, especially for antibacterial purposes against human pathogens, opens a new path in

antibacterial drug discovery. The study reveals an encouraging in vitro efficacy of *Terminalia chebula* based AgNPs that can be used for topical application against MDR bacteria after careful in vivo investigation.

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7. AUTHORS CONTRIBUTION STATEMENT

Dr. Asha Arora Conceptualized and designed the work process, Shweta Chhajed collected, analyzed, and interpreted the data. Both authors contributed to editing and revising the manuscript. All the authors read and approved the final version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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