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DRUMSTICK TREE (*Moringa oleifera*) Lam. LEAF EXTRACT MEDIATED SYNTHESIS OF SILVER NANOPARTICLES AND THEIR ANTIBACTERIAL ACTIVITY AGAINST NOSOCOMIAL BACTERIAL PATHOGENS

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ABSTRACT

Nanoparticles are unique and show different physical and chemical properties from their bulk material. Silver nanoparticles have a variety of antimicrobial applications. In the present study Silver nanoparticles (Ag-NPs) were synthesized by green methodology using the plant *Moringa oliefera* Lam. leaf extract. The main objective of this study was to synthesize silver nanoparticles without using any hazardous chemicals for future biomedical uses. *M. oleifera* Lam. is a well-known medicinal plant and is used in the preparation of different ayurvedic medicines. This plant is a rich source of polyphenols, folic acid, and beta-carotene. The effect of temperature on the synthesis of nanoparticles was also determined. The synthesized silver nanoparticles showed an absorbance peak at 430 nm with a UV-Vis spectrophotometer. X-ray diffraction study and TEM analysis exhibited the crystalline nature of silver nanoparticles and revealed that their size is 20-30 nm. An antimicrobial activity study was also performed against the most common nosocomial pathogens and the results revealed that the synthesized Ag-NPs have significant antimicrobial properties against *MRSA*, *Escherichia coli*, *Klebsiella* (ESBL), and *Salmonella enterica*. The antibacterial efficacy of silver nanoparticles was also determined using MIC and MBC. This study concluded that *M. oliefera* Lam. exhibited strong potential for the synthesis of silver nanoparticles. These synthesized silver nanoparticles are stable and eco-friendly and could be used to control bacterial growth of common pathogenic microorganisms including some multi-drug resistant (MDR) bacterial strains such as *MRSA* and *Klebsiella* (ESBL).

Keywords: *Moringa oliefera* Lam., Silver nanoparticles, Multi-drug resistant, Nosocomial pathogens, TEM

INTRODUCTION

Silver nanoparticles (Ag-NPs) are incorporated into more than 200 consumer products utilized in the field of agriculture, pharmaceutical, biomedical, cosmetic industry, etc. **(Agrawal *et al.*, 2019; Ahmed *et al.*, 2016)**. Ag-NPs are widely used in photonics, sensing devices, and Surface Enhanced Raman Spectroscopy **(Gould *et al.*, 2000; Frederix *et al.*, 2003; Tian *et al.*, 2004)**. Silver is used in the medical field since ancient times **(Galdiero *et al.*, 2011)**. Silver nitrate is commonly used in wound dressing and as an antibacterial coating on medical devices **(Bhusnure *et al.*, 2017)**. Moreover 1% silver nitrate is also used as an eye drop for newborns to prevent *Neisseria gonorrhea*-infected mothers **(Burdușel *et al.*, 2018)**. In such applications, silver nanoparticles could work better than silver nitrate **(Xu *et al.*, 2020)**. It has been reported that silver produces free radicals and damages the bacterial cell and it is effective against a wide range of bacteria **(Rai *et al.*, 2012; Danilczuk *et al.*, 2016)**. Additionally, silver nanoparticles can create pores in the bacterial cell wall and also damage their genetic material as well as their respiratory enzymatic pathway **(Lara *et al.*, 2010)**. The overuse and misuse of antibiotics have led to the development of drug-resistant bacteria but silver nanoparticles do not induce the development of resistance in bacteria **(Alexandra *et al.*, 2016)**. Hence, silver nanoparticles can serve as good alternatives to antibiotics or can be used in combinatorial therapies.

Nanoparticle synthesis is usually mediated by all the three: physical, chemical, and biological approaches **(Pal *et al.*, 2020)**. However, the use of chemicals for nanoparticle synthesis is a toxic and hazardous process for the environment as well as for mankind. Additionally, many physical methods are available for nanoparticle synthesis but owing to their high cost, high energy requirement, and less stability they are not suitable for large-scale and stable production of nanoparticle **(Bloch *et al.*, 2021)**. The synthesis of silver nanoparticles using biological methods is an eco-friendly as well as a green method **(Elghanian *et al.*, 1997)**. Plant parts like leaves, stems, roots, seeds, and flowers have been used under the green preparation of nanoparticles **(Zahir *et al.*, 2015; Shende *et al.*, 2015; Velmurugan *et al.*, 2015)**. Medicinal plants contain many bio-reduction compounds and stabilizers that boost the nanoparticle synthesis in an aqueous solution **(Pal *et al.*, 2021)**. Some bioactive compounds present in medicinal plants reduce silver ions (Ag^{+}) and are also capped with nanoparticles. Therefore, it enhances the stability and antimicrobial property of silver nanoparticles in comparison to the Ag-NPs synthesized chemically **(Subasri *et al.*, 2019)**. Nanoparticles synthesized with the extract of medicinal plants can be used in pharmaceuticals, cosmetics, and personal care products.

Moringa oleifera Lam. is commonly known as the "drumstick tree" in India and it belongs to the *Moringaceae* family **(Anwar *et al.*, 2019)**. It is an ingredient of the Indian diet and is known as a medicinal plant. The plant is a rich source of polyphenols, folic acid, and beta carotene **(Garima *et al.*, 2011; Mónica *et al.*, 2015).** The plant leaves have antioxidant, antitumor, wound healing, and eye healing properties **(Devendra *et al.*, 2011; Fernandes *et al.*, 2016)**. In the preparation of nanoparticles aqueous and organic solvents both can be used but for the biomedical purposes use of water instead of any other chemical solvent is much safer **(Singh *et al.*, 2010).** Water as a solvent is also good for liquid packaging and helps in avoiding unwanted or harmful by-products **(Solomon *et al.*, 2007)**. *Escherichia coli*, *Salmonella enterica*, *Methicillin-Resistant Staphylococcus aureus (MRSA), and Klebsiella (ESBL)* are the common nosocomial infection-causing organisms **(Donkor *et al.*, 2019)**. *Staphylococcus aureus* causes serious infections in the hospital community **(Bouche *et al.*, 2010)**. *MRSA* is a strain of *S. aureus* resistant to methicillin antibiotics. It is difficult to treat *MRSA* with commonly used antibiotics **(Harbottle *et al.*, 2006)**. It can cause serious infections in the bloodstream, on surgical wounds, in the lungs, and, in the urinary tract **(Green *et al.*, 2012)**. Sometimes *MRSA* is called a "superbug" because they are hard to treat **(Turner *et al.*, 2019)**. *E. coli* mainly causes urinary infection, pneumonia, and neonatal septicemia **(Lee *et al.*, 2018)**. *S. enterica is* associated with typhoid fever and infect the intestinal tract of humans and animals **(Veeraraghavan *et al.*, 2018)**. Many healthy carriers of *S. enterica* can infect other people. *Klebsiella pneumonia (ESBL)* is resistant to beta-lactam drugs because it produces beta-lactamase enzymes. *K. pneumonia* is an opportunistic pathogen and mostly infects hospitalized patients **(Parveen *et al.*, 2011)**.

The present study aims to develop a simple novel and one-pot green methodology for the synthesis of silver nanoparticles using the medicinal plant *M. oleifera* Lam. leaf extract and study the effectiveness of the synthesized silver nanoparticles against common nosocomial infection causing bacterial species.

MATERIAL AND METHODS

All the chemicals used in the present study were of analytical grade and were used without any further purification. Silver nitrate was procured from Merck Mumbai, India.

Preparation of Plant Extract

Fresh leaves of *Moringa oleifera* Lam. were collected from Indore M.P. (22.7196° N, 75.8577° E) India, and were washed twice with distilled water. Then, 20 g of washed leaves were grounded using pestle mortar. Crushed leaves were then mixed in 100 ml distilled water and the mixture was then stirred on a magnetic stirrer at 70 ±2 ° C, 100 rpm for 10 -15 min. The mixture was then allowed to cool down at room temperature and it was then filtered through Whatman filter paper no. 1 **(Sathyavathi *et al*., 2011)**. This prepared extract can be stored for approximately 10 days in a refrigerator in a glass container with a lid for future use.

Synthesis of Silver Nanoparticles

Ten ml of leaf extract was added to 90 ml of 1mM silver nitrate solution followed by continuous stirring on a magnetic stirrer at 70 ±2 °C at 150 rpm for 20 min (**Girish, 2011**). The percentage yield was calculated according to the formula given below **(Sood, R & Chopra, D., 2017)**

% Yield= (Weight of lyophilized Silver Nanoparticles) / (Weight of Silver Nitrate used) ×100 (Equation 1)

Effect of Temperature on Silver Nanoparticles Synthesis

The reaction temperature is very crucial for nanoparticle synthesis. The reaction flask of leaf extract and silver nitrate were both kept at different temperatures (40, 50, 60, 70, 80 ° C) and under shaking condition (150 rpm). Optimum temperature facilitates the maximum synthesis of silver nanoparticles **(Ndikau *et al*., 2017)**.

Characterization of Silver Nanoparticles

The preliminary detection of synthesized silver nanoparticles was observed by the change in colour of the reaction mixture of silver nitrate and leaf extract. The absorption spectrum of the synthesized silver nanoparticles was determined using a UV-Vis spectrophotometer (Labtronics Model LT 2201).

The Transmission Electron Microscope (Talos, model FEI) was operated at 200 kV and an accelerating voltage was used to identify the size and shape of the silver nanoparticles. The solution of silver nanoparticles was loaded on a copper grid and a thin film was prepared and dried at room temperature. This thin film was placed in the sample chamber to observe the shape and size of the nanoparticles and the results were recorded.

An X-ray diffraction study was conducted using a 0.154 nm Cu-Kα radiation between the ranges of 30º to 90º. The size of the nanoparticles was estimated using the Debye-Scherrer equation **(Al-Shmgani *et al*., 2017)**.

$$D = \frac{K\lambda}{\beta \cos\theta}$$

(Equation 2)

Where, *K*=crystal shape factor usually, λ= wavelength of the X-ray radiation, β=FWHM i.e. peak width in half the maximum height, Θ = is the diffraction angle in degree.

All the data were analyzed by variance (ANOVA) using Tukey–Kramer multiple comparisons tests significant reading all the statistical test was considered when P was ≤ 0.05 using SPSS software (16.0).

Antibacterial Activity of Synthesized Silver Nanoparticles

Agar well diffusion method was used to evaluate the antibacterial property of synthesized silver nanoparticles **(Aritonang *et al*., 2019)**. Four common nosocomial pathogens *E. coli* (ATCC 25922), *Salmonella enterica* (ATCC 6539), *Klebsiella pneumonia* (ATCC 700603), and *MRSA* (ATCC 43300) were used for the antibacterial activity. These bacteria were sub-cultured in nutrient broth at 37 ± 2 °C for 24 h and log-phase bacterial cultures (10^6 cells/mL) were standardized using McFarland's standard and used as inoculum. The cultures were uniformly spread onto separate sterile nutrient agar plates. Four wells with a diameter of 8 mm were punctured aseptically with a sterile cork borer onto each plate and the wells were filled with 0.08 ml each of 1mM synthesized silver nanoparticles, 1 mM silver nitrate solution, plant extract, and distilled water respectively. All the plates were prepared in triplicates and were incubated at 35 ± 2 °C for 24 h. The different inhibition zones were measured at the end of the incubation period.

Assessment of Increase in the Fold Area

Each inhibition zone of silver nitrate and silver nanoparticles was measured and calculated using mean surface area to determine the increase in fold area. Fold increase in area was calculated by using the given equation **(Gajbhiye *et al*., 2019)**.

$$\frac{(B^2 - A^2)}{A^2}$$

(Equation 3)

Where, A=Zone of silver nitrate, B= Zone of silver nanoparticles

RESULTS AND DISCUSSIONS

Synthesis of Silver Nanoparticles

The aqueous extract of *M. oleifera* Lam. leaf was green, when this extract was mixed with the silver nitrate solution it became yellow and when heated to 70 ° C, the mixture started changing its colour from light yellow to dark brown. This colour modification was observed visually. The brown colour indicates the excitation of surface plasmon resonance of silver nanoparticles **(Mock *et al*., 2002)**. In the presence of bioactive compounds present in the leaf extract and due to the effect of temperature Ag^+ ions undergo reduction and form Ag-NPs. The colour change indicates the formation of silver nanoparticles **(Marslin *et al*., 2018)**. The percentage yield of synthesized silver nanoparticles is calculated with equation 1 and it was around 70.68 %.

UV-Vis Spectrophotometric Analysis

Silver nanoparticles showed strong absorption in the visible range of the electromagnetic spectrum. The synthesized silver nanoparticles showed absorption maxima at 430 nm [Figure 1] and the absorption remains unchanged indicating the formation of stable nanoparticles. According to the Mie's theory, when the number of peaks increases anisotropy also increases **(Philip *et al*., 2011)**. The free electrons are present in the metal nanoparticles which endow the Surface Plasmon Resonance (SPR) absorption band **(Narayanan *et al*., 2012)**. The specific colour alteration was observed due to the excitation of SPR in the metal nanoparticles **(Hanady *et al*., 2017)**. The colour change was observed when the leaf extract was incubated with the $AgNO_3$ solution. In this study, the SPR band exhibited the formation of spherical-shaped silver nanoparticles, which is further confirmed by TEM. In the plant extract-mediated synthesis of nanoparticles, less time is required to complete the reaction. The bioactive compound present in the plant extracts such as alkaloids of *M. oleifera* Lam. plays an important role as a reducing and capping agent in metal nanoparticle synthesis **(Sathyavathi *et al*., 2011)**.

Effect of Temperature on Synthesis of Ag-NPs

According to the study by **Iravani *et. al,* (2014)** the temperature affects the Ag-NPs synthesis. The absorbance of the reaction mixture gives information on the production of Ag-NPs. When the reaction temperature increases kinetic energy elevation begins and it facilitates Ag-NPs synthesis. A broad peak of nanoparticles was observed at 430 nm after heating at 70 ±2 °C. An increase in absorbance, indicating an increase in the yield of synthesized silver nanoparticles, is observed with an increase in temperature from 40-70 °C [Figure 2]. However, the temperature beyond 70 °C does not have the same effect, probably due to the denaturation or inactivation of some polyphenols, alkaloids, etc **(Bawazeer *et al*., 2021)**. The reaction maxima are noted at 70 ±2 ° C and at this temperature, the yield of Ag-NPs was maximum (equation 1).

XRD Study

The XRD pattern of dried silver nanoparticles showed peaks at 38 °, 42 °, 58 °, 65 °, and 68 ° of 2θ [Figure 3]. The peak feature indicates the crystalline nature of Ag-NPs. The average particle size of synthesized Ag-NPs was calculated by measuring the full width at half maximum (FWHM) using the Debye-Scherrer equation (Equation 2). The synthesized silver nanoparticles were crystalline with an average size of 28.2 nm **(Shameli *et al*., 2011)**.

TEM Study

The shape and size of the synthesized Ag-NPs were determined using transmission electron microscopy. The Ag-NPs sample was loaded onto a copper grid. Ag-NPs were synthesized using aqueous extract so they were well dispersed but some agglomerates were also observed. Most of the particles were separated from each other. TEM images of these synthesized nanoparticles revealed that the Ag-NPs were coated with an organic layer. Many polyphenolic components of *M. oleifera* Lam. reduced the Ag ion and also stabilized them and prevented their agglomeration. The TEM studies revealed that the synthesized Ag-NPs had an

average size of about 20 nm and their shape was spherical [Figures 4 and 5]. These results coincide with the results obtained through the XRD study.

Studies on Antibacterial Activity

In the present study, the synthesized silver nanoparticles exhibited antimicrobial activity against three Gram-negative bacteria - *Escherichia coli, S. enterica, and Klebsiella* (*ESBL)* and one Gram-Positive bacteria *MRSA*. Amongst these four bacteria, 2 were drug-resistant bacterial strains; *Klebsiella* (*ESBL*) and *MRSA*. A clear zone of inhibition appeared at the periphery of the well-containing sample. Along with the synthesized nanoparticles, $AgNO_3$, *Moringa oleifera* Lam. leaf extract, and distilled water was also analyzed as control [Table 1]. The synthesized Ag-NPs showed a significant difference in the size of the zone of inhibition as compared to the zone of inhibitions obtained with silver nitrate. However, distilled water and plant extract showed no inhibitory effect, and the zone of inhibition was not observed with all the four nosocomial pathogens taken under study. The zones of inhibition of silver nitrate were smaller in comparison to the zones of inhibition of Ag-NPs. The enhancement in diameter of zones of inhibition obtained with synthesized silver nanoparticles can also be understood by an increase in fold area as shown in table 1. Zone of inhibition with diameters 15.0 ± 1.0 mm, 24.5 ± 0.5 mm, 30.16 ± 1.25 mm, 21.66 ± 0.57 mm were observed for *E. coli, S. enterica, MRSA, and Klebsiella* (ESBL) respectively, with Ag-NPs [Figures 6, 7, 8 and 9]. The synergistic effects of plant extract and silver nanoparticles are more effective and beneficial to minimize the dosage of silver in drugs. *M. oleifera* Lam. (medicinal plant) and silver both are having antibacterial properties known for many years **(Shende *et al.*, 2015)**. Their combination could be a better choice for medical applications mainly against pathogenic bacteria **(Bhakya *et al.*, 2016)**. The exact mode of action of silver nanoparticles as an antimicrobial agent is not known but probably silver nanoparticles produce free radicals and they create pores in the cell membranes and damage the bacterial cell **(Pal *et al.*, 2021)**.

Minimum Inhibitory Concentration (MIC)

The antibacterial efficacy of silver nanoparticles was determined using the broth dilution method. The MIC was performed using nutrient broth with two-fold serial dilutions of synthesized silver nanoparticles **(Krishnan *et al.*, 2015)**. Standard bacterial cultures (1×10^8 CFU/ml) of all the four *E. coli, S. enterica, MRSA, and Klebsiella* (*ESBL*) bacteria were used as inoculum for MIC. The selected range of synthesized Ag-NPs for MIC was 0.0425 mg/ml to 0.51 mg/ml [Table 2]. Different dilutions of the synthesized Ag-NPs were inoculated in separate sterile nutrient broth test tubes inoculated with the test organisms. In this experiment, three nutrient broth tubes inoculated with standard bacterial culture were used as a positive control, and three un-inoculated nutrient broth tubes were used as a negative control. All the test tubes were incubated at 35 ± 2 °C for 24 h. After the incubation, all the tubes showing no turbidity were selected and a loopful of culture from the selected test tube was spread on nutrient agar plates to observe minimum bactericidal concentration (MBC). The presence and absence of bacterial growth were observed after incubation. Based on bacterial growth with the varying concentrations of Ag-NPs minimum inhibitory concentration was determined. According to the MIC and MBC results, 0.17 mg/ml concentration of synthesized Ag-NPs was observed as minimum inhibitory concentration [Table 3].

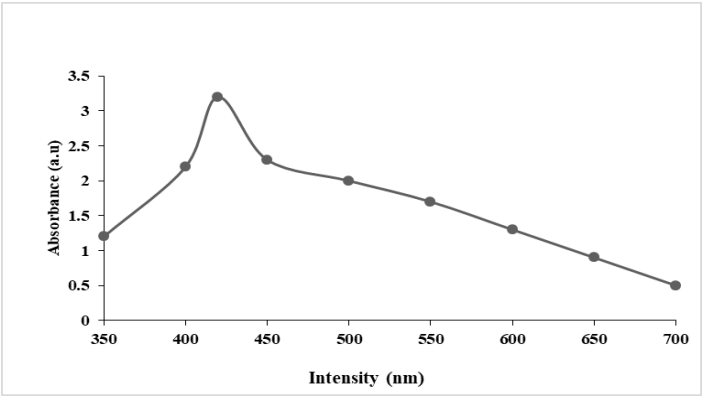


Figure 1 UV-Vis Spectra of Silver Nanoparticles Synthesized from *Moringa oleifera* Lam. Leaf extract

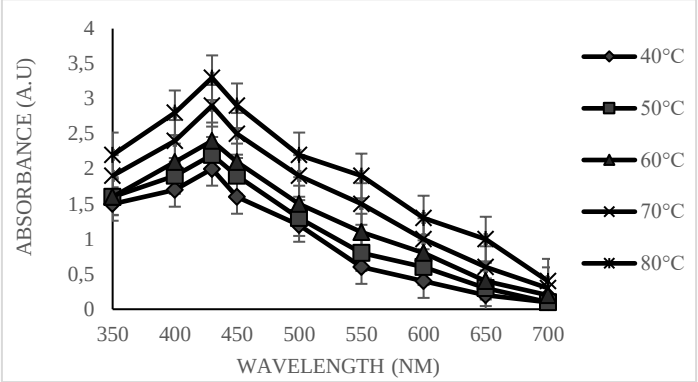


Figure 2 UV–Vis spectra of silver nanoparticles synthesized from *Moringa oleifera* Lam. leaf extract at different reaction temperatures.

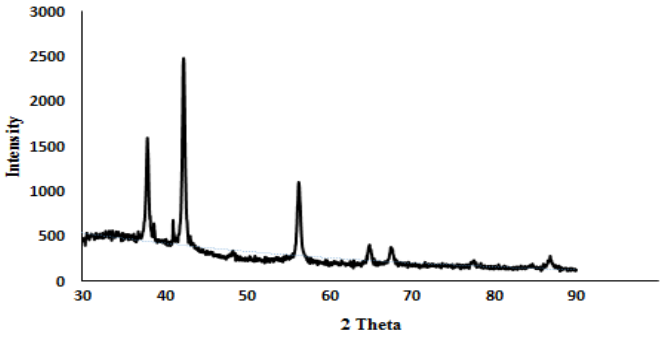
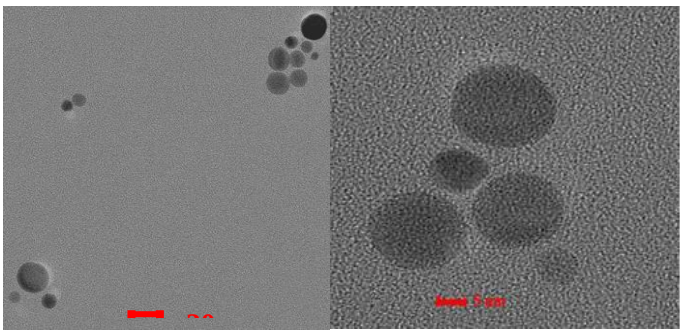


Figure 3 XRD pattern of silver nanoparticles synthesized from *Moringa oleifera* Lam. leaf extract.



Figures 4 and 5 TEM images of silver nanoparticles synthesized from *Moringa oleifera* Lam. leaf extract

Table 1 Antibacterial activity of synthesized silver nanoparticles against *MRSA, Escherichia coli, Salmonella enterica, and Klebsiella* (*ESBL*)

Test Organisms	Zone of Inhibition (mm, mean ± SD)*				Increase in the Fold Area
	Ag-NPs	1 mM of $AgNO_3$	Plant Extract	Distilled water	
MRSA	30.16 ± 1.25	19 ± 1.0	8.0	8.0	3.37
Escherichia coli	15.0 ± 1.0	13 ± 1.0	8.0	8.0	0.28
Salmonella enterica	24.5 ± 0.5	16.83 ± 0.28	8.0	8.0	3.08
Klebsiella (ESBL)	21.66 ± 0.57	14.5 ± 0.86	8.0	8.0	0.24

**Mean surface area of the inhibition zone was calculated for each from the mean diameter.*

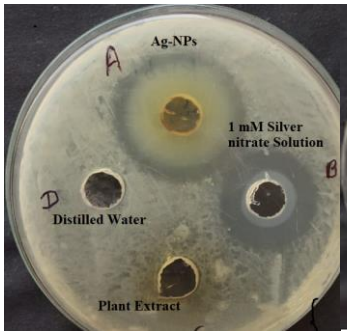


Figure 3 MRSA

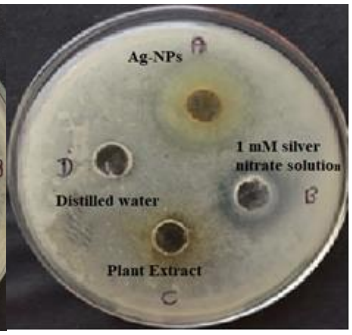


Figure 7 *Escherichia coli*

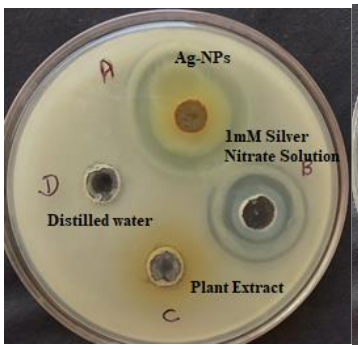


Figure 2 *Salmonella enterica*

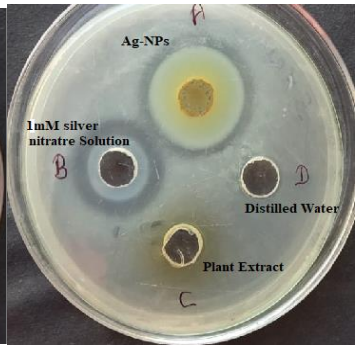


Figure 9 *Klebsiella* (ESBL)

Figures 6, 7, 8 & 9 Antibacterial activity of synthesized silver nanoparticles against *MRSA, Escherichia coli, Salmonella enterica, and Klebsiella* (*ESBL*) respectively.

Table 2 Minimum Inhibitory Concentration of synthesized silver nanoparticles against *MRSA, Escherichia coli, Salmonella enterica, and Klebsiella* (ESBL)

		Concentration (dilution) of synthesized silver nanoparticles in mg/ml				
S. No.	**Pathogens**	**0.0425**	**0.085**	**0.17**	**0.34**	**0.51**
i.	*MRSA*	+	+	-	-	-
ii.	*Escherichia coli*	+	+	-	-	-
iii.	*Salmonella enterica*	+	+	-	-	-
iv.	*Klebsiella (ESBL)*	+	+	-	-	-

Positive **(+)** = Turbidity indicating the growth of bacteria,
Negative **(-)** = No turbidity indicating the absence of bacterial growth

Table 3 Minimum Bactericidal Concentrations of Silver Nanoparticles against *MRSA, Escherichia coli, Salmonella enterica, and Klebsiella* (*ESBL*)

			Concentration (dilution) of synthesized Silver nanoparticles in mg/ml		
S. No.		**Pathogens**	**0.17**	**0.34**	**0.51**
i.	1	*MRSA*	-	-	-
ii.	2	*Escherichia coli*	-	-	-
iii.	3	*Salmonella enterica*	-	-	-
iv.	4	*Klebsiella (ESBL)*	-	-	-

Positive **(+)** = Turbidity indicating the growth of bacteria
Negative **(-)** = No turbidity indicating the absence of bacterial growth

CONCLUSION

The present study deals with an ecofriendly and green methodology for preparing silver nanoparticles using *Moringa oleifera* Lam. leaf extract. Characterization studies on the synthesized Ag-NPs revealed that the nanoparticles were distributed within a 20-30 nm size range with a spherical shape. In this preparation, silver nitrate was converted into silver nanoparticles. Significant antibacterial activity was observed against the four selected nosocomial pathogens including multidrug-resistant pathogens. This green approach is dedicated to serving mankind to fight against pathogens. It could be a new drug to cure drug-resistant bacterial diseases. These synthesized stable and eco-friendly Ag-NPs could be used in the food, pharmaceutical, and cosmetic industry for controlling bacterial growth. However further pharmacological study is still required.

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