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ORIGINAL ARTICLE

Optimization, Characterization and Antibacterial activity of Green Synthesized silver Nanoparticles using *Moringa oleifera* leaf extract

***Shilpashree Mayachar K, Nandini N, and, Alaknanda J Adur**
Department of Environmental Science, J B Campus, Bangalore University,
Bengaluru- 560056, India
***Corresponding Author 's** E-mail address: * shilpashreeshreya@gmail.com

ABSTRACT

Present study successfully demonstrates the extracellular synthesis of silver Nanoparticles using the Moringa oleifera leaf extract (MLE). Agroecological grown Moringa oleifera leaves was collected from Amrita Bhoomi Chamarajanagar- BR Hills Road, Hondarabalu in Karnataka, India. Moringa oleifera leaves have proven to contain antioxidizing phytochemicals which have reduced silver ions to spherical silver Nanoparticles (AgNPs). Characterization of green synthesized AgNPs was carried out using UV-Visible spectrophotometer and transmission electron microscopy (TEM). Optimization of the synthesis process is carried out to understand the reaction kinetics for optimum production of silver nanoparticles. X-ray diffractograms (XRD) of green synthesized silver nanoparticles have shown crystalline structure having with distinct peaks at Bragg's (2θ) values of around 38.62^0, 44.76°, 64.8°, 77.2° corresponding to Miller indices (hkl) of (111) (200) (220) and (311) corresponding to each of diffraction peaks. Selected area electron diffraction (SAED) pattern analysis of single spherical practical confirms the crystalline structure of green synthesized AgNP's within the face-centered cubic (FCC) nanocrystals similar to XRD. Using Fourier Transform Infra-Red Spectrophotometric (FTIR) analysis, possible phenol group in leaf extract which is responsible for the synthesis of stabilized AgNPs is characterized. The particle size of the nanoparticles formed was in the range of 10-40nm and an average diameter of spherical silver nanoparticles was 20nm as depicted by HR-TEM analysis. The antibacterial activity of green synthesized silver Nanoparticles was investigated against ATCC strains as Pseudomonas sp, Klebsiella pneumonia and Staphylococcus aureus at different concentrations, which has shown clear inhibition zones 1cm, 5mm, 2mm respectively thereby indicating its inhibitory activity.

Keywords: *green synthesis, Moringa oleifera, Nanoparticles, phytochemicals, bioreduction, antibacterial. TEM, FTIR, X-ray diffraction*
Abbreviation: MLE- Moringa oleifera leaf extract, AgNPs-silver nanoparticles

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INTRODUCTION

The increasing interest in the synthesis of nanoparticles (NPs) and its application in the field of therapeutics, environmental science, and material science, has opened a new vista in the scientific community to find out safest means of synthesis, utilization, and disposal of nanomaterials, and purge their possible harmful effects on nature. The historical evidence of eco-friendly nanomaterials is Ayurvedic Bhasma [1] Ayurvedic Bhasma is considered very safe and economical in comparison to contemporary metal based nanomedicines [2-5]. From past few decades, physical and chemical methods of synthesis of nanoparticles were used to obtain the uniform, monodispersed, circular metal nanoparticles. But In recent years, the process of conversion of bulk metals into nanomaterials using ~~of~~ microorganism and plant material has become a viable alternative to conventional physical and chemical methods, since it is facile, rapid, eco-friendly and economical benign [6]. Nanoparticles have a high specific surface area to volume ratio and small fraction of surface atoms which has gained importance in the field of research and application since it demonstrates high catalytic, electrical, magnetic and antimicrobial activity [7] and are potentially used in the field of sensors, catalyst, surface enhanced Raman spectroscopy and biomedicine [8]. Biosynthesis process involves the use of microorganisms such as bacteria, fungi, yeast etc. for the synthesis of nanoparticles. The extracellular synthesis is found to be more advantageous than intracellular synthesis, as the separation of nanoparticles is much easier than that in later.

Green synthesis of metal nanoparticles is the method in which different parts of plants like leaves, fruit, flower, bud, pod extracts etc. are used to convert metal ions to nanoparticles by the process of reduction. The phytochemicals present in the plant play an important role in nanoparticles synthesis. The Moringa oleifera Lam (family- Moringaceae) is commonly known as drumstick tree or horseradish tree. These are commonly found in South Asia and are good sources of ß-carotene, Vitamin C, protein, iron, and potassium. The leaves of *Moringa oleifera* are proven to have a higher amount of polyphenols, which are known to scavenge free radicals and high antioxidant potential [9-10]. Sathyavathi *et al.* reported a rapid synthesis of silver nanoparticles (AgNPs) using Moringa leaf extract, in which the average particle size is 46nm and dispersed nanoparticles were in the range of 5nm to 80nm.[11]. Moodley *et al.* able to synthesize silver nanoparticles using moringa leaf extract having a diameter in the range of 9nm to 11nm [12]. Prasad and Elumalai have reported the green synthesis of silver nanoparticles using 10ml of moringa leaf extract with 1mM silver nitrate heated to 60-80°C for 20 min and were able to synthesize silver nanoparticles having a p[article size of 57nm [13]. Shivashankar and Sisodia have reported the synthesis of silver nanoparticles using moringa leaf extract [14]. Das *et al*. have successfully synthesized silver nanoparticles of average 40nm size using Moringa oleifera leaf extract [15]. In the present paper, the synthesis of silver nanoparticles using organically grown Moringa oleifera leaf extract as reducing medium through the biochemical process is reported. The synthesis of the AgNPs has been optimized. The structural analysis by XRD and SAED, morphological studies using HR-TEM analysis and testing of antimicrobial properties of the green AgNPs have been done and reported.

MATERIAL AND METHODS

Materials

Fresh leaves of *M.oleifera* were collected from the model agroecology farm "Amrita Bhoomi" situated in Chamarajanagar- Biligiri Rangana Hills Road, Hondarabalu in Karnataka state, India. It is the International center for sustainable development which spread across 66 acres of land along with medicinal garden conserving indigenous medicinal plants which are facing near extinction. Double distilled water was prepared in the laboratory using quartz distillation assembly and sterilized before Moringa leaf extract (MLE) preparation and other reagents. Silver nitrate of AR grade, 99.9% purity was procured from M/s Sisco research laboratories Pvt. Ltd. Glass wear apparatus were thoroughly washed and dried in hot air oven at 60°C for 3hr. Magnetic stirrer with heat control and microcentrifuge (Remi) were used for synthesis and separation of silver nanoparticles.

Synthesis of Silver Nanoparticles (AgNPs) and their characterization

Fresh leaves of *M.oleifera* were cleaned and washed thrice with double distilled water and 10% fresh leaf extract was prepared using distilled water by heating it to 80^0 C for 20 min. Then, the moringa leaf extract (MLE) was filtered through Whatman's filter paper no 69 and stored it at 4^0C in the incubator. The green synthesis of silver nanoparticles was done by adding 10mL of leaf extract to 90 ml of 1mM of aqueous $AgNO_3$ aseptically and heated at 80^0 C for 20 min. A change in color was observed from pale yellow to reddish brown which confirmed the reduction of silver ions into nanoparticles. Optimization of green synthesis was carried out to study the effect of physical and chemical factors which are involved in the formation of silver nanoparticles. Subsequently, bio-reduction was monitored using UV-visible spectrophotometer of Systronics make. The samples of silver nanoparticles in the solution were scanned through the wavelength of 200 to 600nm using the spectrophotometer. The process optimization was carried out to determine the factors influencing on a green synthesis of nanoparticles The effect of pH, temperature, the volume of extract, the concentration of ionic solution and time were studied during optimization of the process. The green synthesized silver Nanoparticles synthesized by the optimized process were subjected to centrifugation using micro-centrifuge Remi at a speed of 10,000rpm for 20 min. Pellet was washed twice with sterile distilled water to remove the organic substance. The thin film of separated silver nanoparticles was prepared for XRD analysis. Prepared AgNP's were characterized using X-ray diffractometer, *RIGAKU, ULTIMA IV*, using Cu Kα radiations at the wavelength of 1.542 Å. The functional groups involved in the reduction during synthesis of silver nanoparticles were characterized by recording ATR-FTIR spectra of the leaf extract and its remnants in the range 4000 cm^{-1} to 500 cm^{-1} using FTIR (spectrophotometer, Bruker-alpha make). The particle size distribution was investigated using high-resolution Transmission Electron Microscope (HR-TEM) make Jeol/JEM2100, a drop of green synthesized nanoparticles was taken on carbon-coated copper TEM grid and left for few minutes, excess sample is removed by blotting paper and dried before measurement [16] The green synthesized silver Nanoparticles were analyzed for their antibacterial activity by diffused well plate method

RESULT AND DISCUSSION

Preparation of silver nanoparticles by green synthesis

Green synthesis of silver Nanoparticles using *M.oleifera leaf extract* (MLE) is an eco-friendly and economical approach. The formation of silver Nanoparticles from silver ions is due to the biochemical reaction of MLE as a reducing agent. Moringa leaves are reported to have the high amount of polyphenols which are known to have scavenging activity and metal chelating properties [9]. Synthesis of silver Nanoparticles is confirmed by the color change from colorless to yellow after heating to 80°C for 20 min. The bioreduction of Ag^{+} to Ag^{0} with the action of moringa leaf extract is observed with the characteristic change in color from pale yellow to reddish brown which may be attributed due to surface Plasmon resonance (SPR) of silver nanoparticles. The thematic representation of green synthesis method is depicted in Fig.1

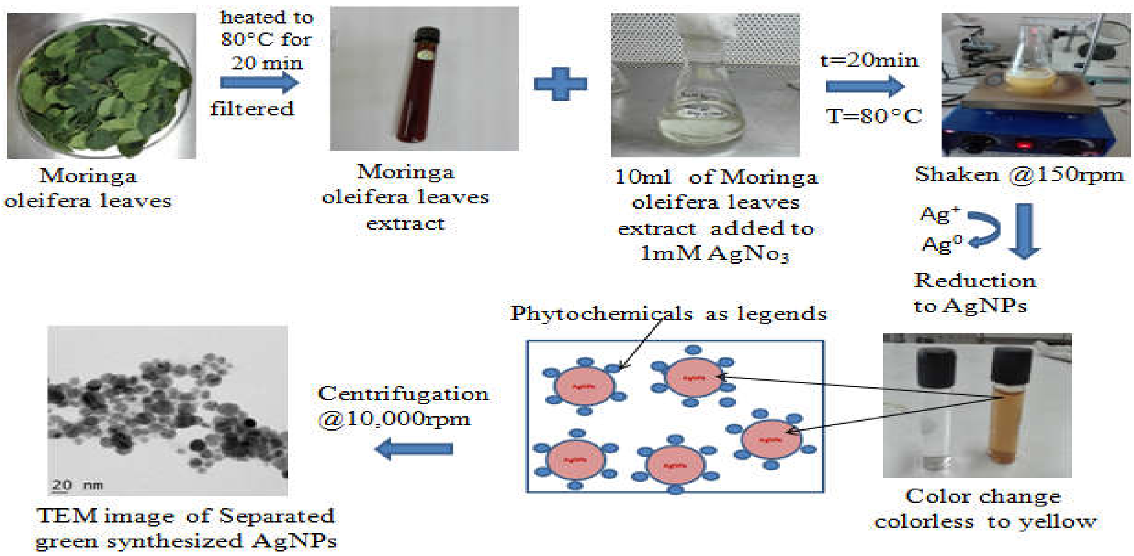


Fig.1: Green synthesis of silver nanoparticles using *Moringa oleifera* leaf extract.

Characterization of Silver nanoparticles

Silver nanoparticles (AgNPs) thus synthesized were characterized for the morphological feature, structural characteristics, estimation of functional groups responsible for biochemical reduction and antimicrobial activity.

- **Optimization of physical and chemical parameters effect of temperature on silver ion reduction and nanoparticle formation using UV-Visible Spectrophotometric Analysis**

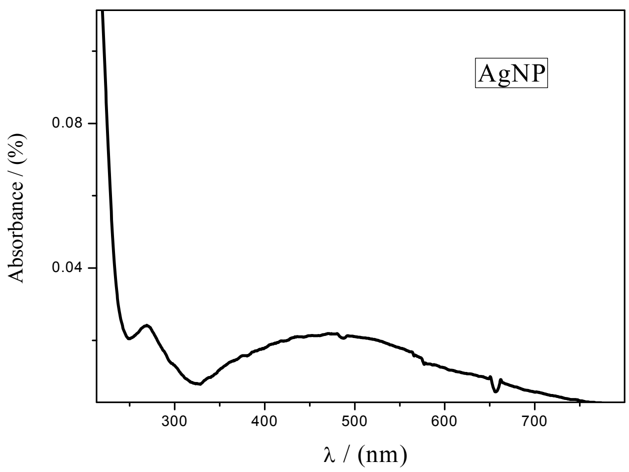


Fig.2: UV- Visible spectra of green synthesized AgNPs

The quantitative assessment of green synthesized silver nanoparticles was carried out using a UV-visible spectrophotometer. After visualization of color change from colorless to reddish brown to confirm the formation of silver nanoparticles, the solution of green synthesized AgNPs was scanned in the range 200 to 800nm wavelength using UV- Visible spectrophotometer. The characteristic rise in peak height at 410nm is attributed to excitation of localized surface Plasmon oscillation of conduction electrons of silver nanoparticles [16] The absorption spectra are shown in the Fig.2 which indicates band edge at 217 nm corresponding to the band gap energy of 3.02eV formed due to formation of smaller size of (10nm) AgNPs, the band gap energy is calculated using Eg=hc/λ where h is Plank's constant c is wavelength of light and λ is the absorption wavelength from absorption spectra of green synthesized AgNPs [17] .

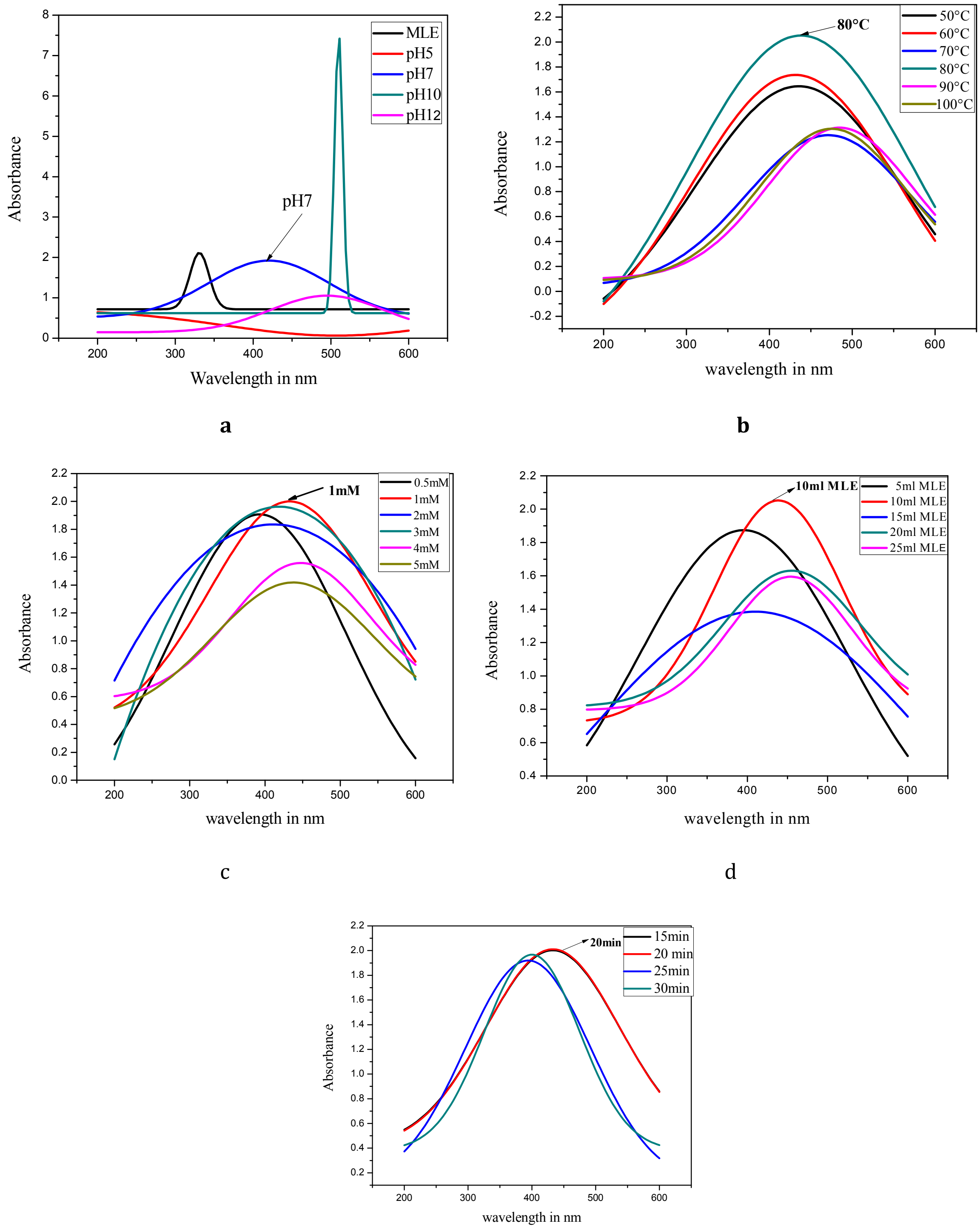


Fig.3 (a), (b), (c), (d) and (e): UV- Visible spectra recorded as a function of pH, temperature, pH, , ionic concentration of $AgNO_3$ volume of MLE and time on green synthesized AgNPs by MLE

After the synthesis of silver nanoparticles using MLE, the optimum conditions for synthesis of AgNPs were determined. Fig.3 shows the kinetic studies conducted during optimization. The factors considered for optimization process included pH, temperature, MLE volume, ionic concentration of silver nitrate and time. The factor which was under consideration was changed keeping the other factors as invariants [19-22].

- ✓ **Effect of pH on silver nanoparticles formation**

The 10ml of MLE is added to 90ml of 1mM silver nitrate solution then the pH of the synthesis mixture was adjusted to 5, 7, 10 and 12 using Sodium hydroxide and Hydrochloric acid and incubated to 80°C for 20min. the colloidal Solutions was scanned using a UV-Visible spectrophotometer between 200 to 600nm. It may be seen from the Fig.3(a) that the solution of extractant at pH 7 has an absorbance peak at 440nm, Solution of extract at pH 5 has not given shown any absorbance, the solution of extract at pH 10 has absorbance peak at 510 nm and the solution of extract at pH 12 has an absorbance peak at 490nm.

- ✓ **Effect of temperature on silver nanoparticles formation**

Effect of temperature on silver nanoparticles formation was studied by following the same procedure mentioned earlier. It may be seen from the Fig.3 (b) that the solution of extract at 80^{o}C has a maximum absorbance peak at 440nm, while Solutions of extract at a temperature of 50^{o}C, 60^{o}C, 70^{o}C, 90^{o}C and 100^{o}C gave the peak at same wavelength with low absorption

- ✓ **Effect of concentration of Silver nitrate on silver nanoparticles formation**

Silver nitrate concentration of 0.5 mM, 1.0 mM, 2.0 mM, 3.0 mM, 4.0 mM and 5mM silver nitrate was studied for optimization process. From the Fig.3(c), it is observed that 1.0 mM of Silver nitrate concentration showed maximum absorption at 440 nm results.

- ✓ **Effect of concentration of MLE on silver nanoparticles formation**

The effect of MLE on the synthesis of silver nanoparticles formation was studied by adding 5ml, 10ml, 15ml, 20ml and 25ml of MLE to each 1mM silver nitrate solution and heated to 80°C for 20 min. From the Fig.3 (d), it is inferred that 10ml of MLE is optimum volume required for maximum silver nanoparticles formation.

- ✓ **Effect of duration of treatment on silver nanoparticles formation**

The effect of duration of the treatment was done at a time intervals of 15min, 20min, 25min, and 30min for the formation and stability of green synthesized AgNPs. It may be seen from Fig.3 (e) that 20 min heating is required for optimum nanoparticles formation after which agglomerationssssss of AgNPs was observed.

Based on the above kinetic studies, it is inferred that the optimum parameters for the synthesis of AgNPs using MLE are 10ml MLE added to 1mM silver Nitrate solution heated at 80°C for 20min. at pH 7 to yield an optimum number of silver Nanoparticles and avoid agglomeration of AgNPs

- ✓ **X-ray Diffraction Analysis**

The concentrated silver nanoparticles were used for the preparation of thin film on a glass slide for XRD analysis. The glass slides were annealed at 240°C for 4 hours in the muffle furnace to remove organic matter and other impurities. The thin film of dispersed silver nanoparticles was used to record the X-ray diffractogram under reflection mode to estimate the crystalline structure and crystallinity and spotting of various peaks of reflection due to diffraction of the X rays corresponding to silver nanoparticles. Fig.2 shows distinct diffraction peaks at different Braggs' (2θ) values of around 38.62^0, 44.76°, 64.8°and 77.2° which correspond to planes (111) (200) (220) and (311) of virgin silver crystalline substance respectively. The observed peaks corresponding to various Braggs' angle were compared with the standard powder diffraction card of JCPDS, silver file No. 04-0783 (Fig.4). Other than silver nanoparticles peaks, additional peaks are present indicating the presence of organic matter associated with AgNPs. The crystallite size of silver Nanoparticles is determined by using Debye Scherrer's formula [23] and the crystallite size is found to be 11nm and 14nm corresponding to the planes (111) and (200) respectively

$$D = \frac{k\lambda}{\beta Cos\theta}$$

Where D is crystallite size, k is Debye Scherer's constant taken as 0.9, λ is the x-ray wavelength 0.1542 nm used Cu Kα, β is the FWHM calculated at peak corresponding (111) and (200) and θ is a Bragg's angle. Based on these, it is pointed out that the nanoparticles formed in the synthesis are silver nanoparticles.

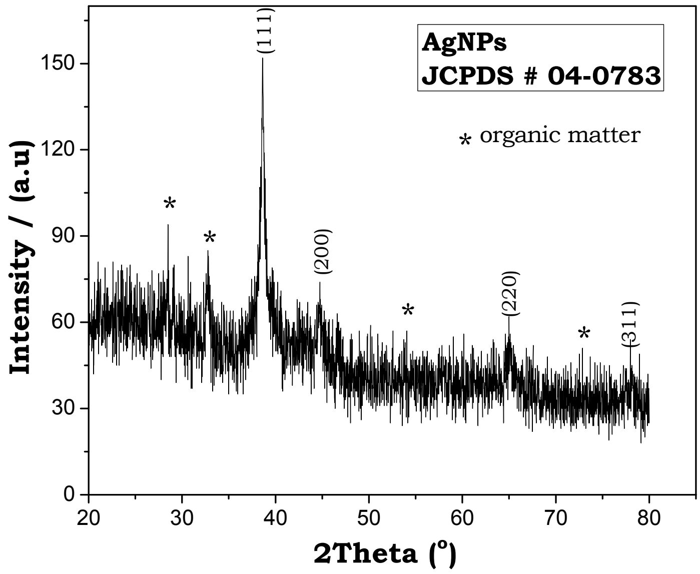
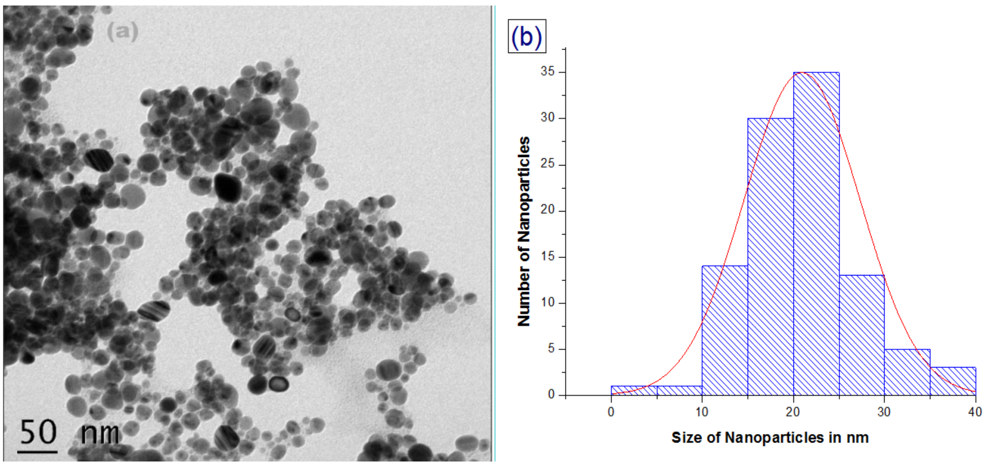


Fig.4: PXRD patterns of green synthesized AgNPs

➢ **Morphological and fine structural studies using High-Resolution Transmission Electron Microscopy (HR-TEM)**

The morphological study of a colloidal suspension of green synthesized silver nanoparticles is carried out using High-Resolution Transmission Electron Microscopy (HR-TEM); images obtained were analyzed and interpreted using ImageJ software. The TEM photograph [Fig.5 (a)] clearly exhibits the formation of mono-dispersed circular silver nanoparticles with uniform distribution. The twine defect was visualized in the TEM image of silver nanoparticles which are very common in face-centered cubic crystals, this confirms the formation of silver nanoparticles [24]. The histogram Fig.5 (b) depicts that the average particle diameter is 20nm and AgNPs size range is distributed between 10-40nm. Fig.5(c) represents the inter-planer spacing in nanoparticles, the calculated d spacing value is found to be 0.2244nm [25], corresponding to (h k l) value of (111) plane respectively. The selected area electron diffraction (SAED) pattern of single spherical practical is shown in Fig.5 (d) confirms the crystalline structure of green synthesized AgNP's within the face-centered cubic (FCC) nanocrystals. The diffraction pattern of silver nanoparticles seen as circular rings in SAED pattern correspond to (111), (200), (220) and (311) FCC crystal lattice plane thereby establish the formation of the synthesized silver Nanoparticles.



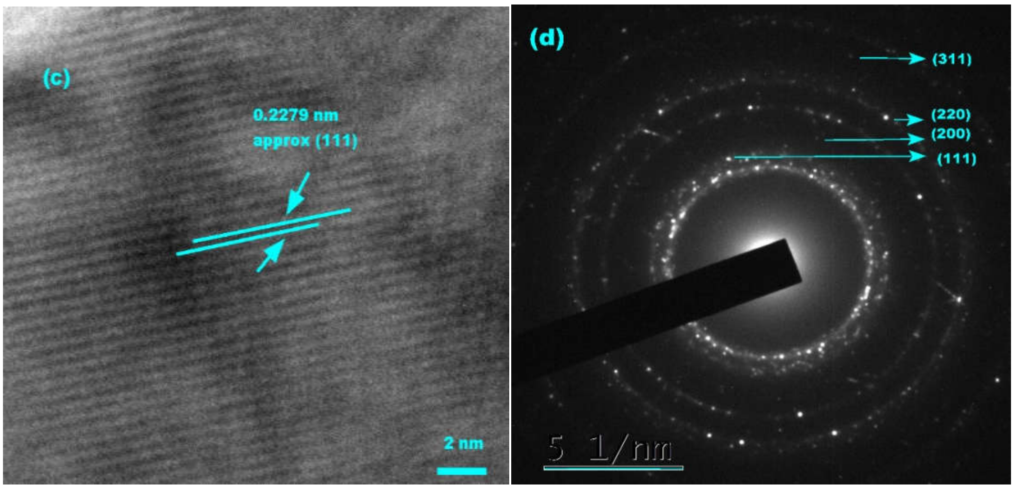


Fig.5: HR-TEM micrographs showing (a) size distribution of Ag nanoparticles; (b) corresponding histogram; (c) HR-TEM image showing *d* spacing and (d) SAED pattern of Ag nanoparticles.

➢ Fourier Transform Infra-Red Spectrophotometric (FTIR) analysis

The FTIR analysis is carried out to determine the possible phytochemicals in the MLE which is acting as the reducing agent during nanoparticles formation. The MLE and green synthesized AgNPs were scanned in the spectral range of 4000cm^{-1} to 500 cm^{-1}. The result obtained from the FTIR spectra of MLE and green synthesized AgNPs is shown in Fig.6. The MLE has six FTIR spectral transmittance troughs at 1217cm^{-1}, 1373cm^{-1},1443 cm^{-1} 1633 cm^{-1},1735 cm^{-1},2125 cm^{-1} and 3310 cm^{-1} respectively, whereas FTIR spectra of biosynthesized AgNPs showed three strong transmittance troughs at 1633 cm^{-1},2125 cm^{-1} and 3310 cm^{-1} region respectively.

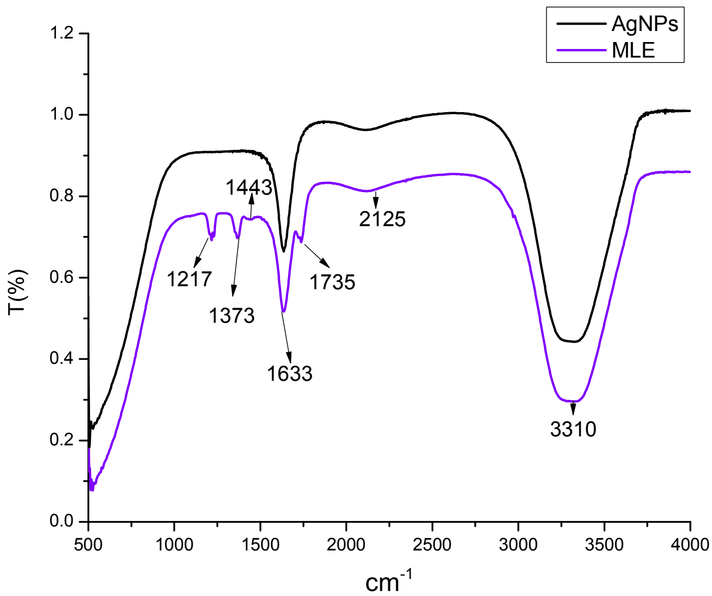


Fig: 6 FTIR spectra of moringa leaf extract and colloidal suspension of green synthesized silver nanoparticles

The medium transmittance trough corresponding to1217cm^{-1} is formed in the fingerprint region between 1200-1360cm^{-1} for MLE, is probably due to stretching and vibration corresponds to C-O bond related to aromatic hydrocarbons. The weak stretching of C-H wagging in −C≡CH alkynes has formed transmittance at 1373cm^{-1}. The medium transmittance trough at 1633cm^{-1} has arisen due to C=O carbonyl stretching vibration confirms the presence of amide I and is a characteristic of β- sheet protein, which has stabilized nanoparticles [21]. Medium stretching and vibration at 1735 cm^{-1} are due to the presence of C=O fragment of esters associated with chlorophyll [26-27]. Very strong band in 3310 cm^{-1} is stretching and vibration of the O-H bond confirms the presence of alcoholic and phenol compounds [28, 11]. The notable change in the transmittance trough is observed before and after biochemical reduction [29] at 1217 cm^{-1}, 1373 cm^{-1} and 1735 cm^{-1} indicating the action of alkynes, esters and carboxylic groups in the formation of silver nanoparticles and Amide group in the stabilization of nanoparticles.

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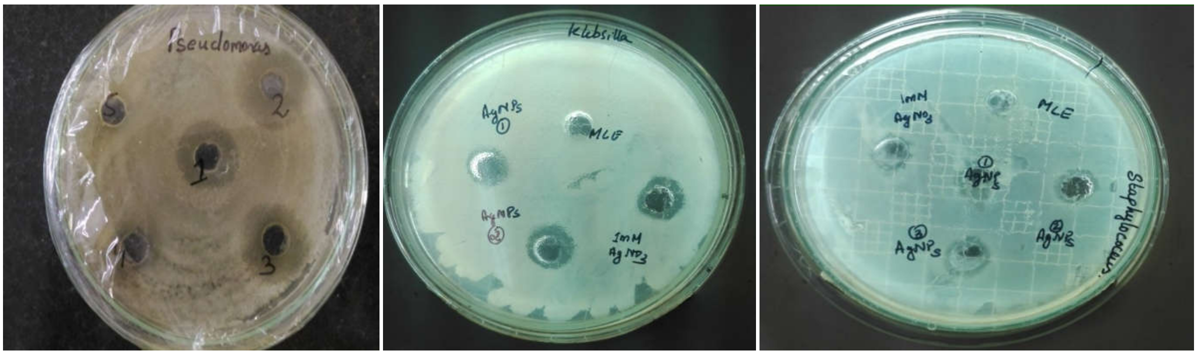

Pseudomonas aeruginosa	***Klebsiella pneumonia***	***Staphylococcus aureus***

Fig.7: Antibacterial Evaluation of Green synthesized silver Nanoparticle using MLE

✓ **Antibacterial evaluation through the 'Zone of Inhibition'**

The antibacterial sensitivity test was carried out against Pseudomonas (MTCC 9027), Klebsiella Pneumonia and Staphylococcus aureus to check the efficacy of green synthesized AgNPs, by agar well-method, the petri dish containing 20 ml of nutrient agar was evenly spread and incubated for 24 hrs at 37^0C with pure culture broth with the help of sterile buds and each well was filled with 100µl of 1mM $AgNO_3$, MLE, and AgNPs . Two different concentrations of AgNPs were experimented and demarketed as AgNPs.1 is 100µl and AgNPs.2 is 50µl as shown in Fig.6. After 24 hours of incubation at 37^0C, the distinct zone of bacterial growth inhibition was observed around all the experimental wells whereas no such zone was formed around the wells containing MLE. The zone of inhibition formed by Pseudomonas is 1cm, Klebsiella Pneumonia-4mm and Staphylococcus aureus is 2mm respectively The observed results revealed that silver nanoparticles thus formed possess antibacterial activity similar to $AgNO_3$.From the above evidence, it is clear that green synthesized silver nanoparticles have good inhibitory property against Klebsiella and Pseudomonas and less inhibitory capacity against E.Coli and Staphylococcus aureus plate, whereas MLE does not show any zone of inhibition [28]. Further, the green synthesized silver nanoparticles show higher antibacterial activity at the concentration of 100µl and 50 µl respectively.

CONCLUSION

Based on the studies, it is inferred that silver nanoparticles are successfully synthesized from the leaves of *M.oleifera*. Green synthesis is an environmentally friendly method as compared to other physical and chemical methods. The leaf extract was found to be highly effective as reducing and capping agent for the synthesis of AgNPs. The Surface Plasmon Resonance (SPR) property and band energy gap of synthesized nanoparticles were studied by UV-Vis spectroscopy and the peak of the spectra was found to be at 440nm confirming the formation of small size nanoparticles. The TEM characterization of AgNP's visualizes the formation of spherical silver Nanoparticles with the size distribution from 10-50nm with the mean size of 20nm. The FTIR spectral analysis of MLE and AgNPs confirms that alkynes, amide and ester group present in phytochemicals of MLE played important role in bioreduction of Ag^+ to Ag^0. The X-ray Diffraction analysis of AgNPs indicated the formation of Face Centered Cubic crystalline structure of the silver nanoparticles with characteristic peaks at Braggs' angle (2θ) value of 38.62°, 44.72°,64.95° and 77.44° corresponding to Miller indices of (111),(200),(220) and (311) lattice plane. Similar inferences are also drawn from SAED investigation. The antibacterial activity of silver nanoparticles showed significant antibacterial activity against MTCC strain *Pseudomonas (9027*) *Klebsiella pneumonia, Staphylococcus aureus and Escherichia coli.* Thus, the present study is an attempt of a greener approach for the production of silver nanoparticles using MLE. The MLE mediated synthesized silver nanoparticles were found to be stable for one about a month without aggregation. Hence, MLE can be used as reducing and capping agent for synthesis of the silver nanoparticles.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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