GREEN SYNTHESIZED SILVER NANOPARTICLES (AGNPs) USING AQUEOUS EXTRACT OF CALOTROPSIS PROCERA AND ITS ANTIMICROBIAL ACTIVITY ON CLINICAL BACTERIA ISOLATES

Mamman Abakeyah James*, Bako Myek, Zakari Ladan

Department of Pure and Applied Chemistry, Faculty of Physical Sciences, Kaduna State University (KASU), Tafawa Balewa Way, PMB 2339, Kaduna, Nigeria

*Corresponding Author Email Address: mammanabakeyah16@gmail.com

ABSTRACT

The green synthesis of nanoparticles, utilizing aqueous plant extract as a capping and stabilizing agent, has attracted significant attention in various domains, particularly in pharmaceuticals and drug delivery. In this investigation, silver nitrate (AgNO₃) salts were employed as precursors to fabricate silver nanoparticles using *Calotropsis procera* (leaves/flower) extract, and the resulting nanoparticles were characterized. Fourier Transform Infrared (FTIR) spectroscopy revealed three primary functional groups at peaks of 2851.4 cm⁻¹, 1543.1 cm⁻¹, and 1323.2 cm⁻¹, responsible for capping and stabilizing the synthesized C.p-AgNPs. Scanning Electron Microscopy (SEM) demonstrated that the synthesized C.p-AgNPs exhibited spherical shapes with an average particle size ranging from 20 nm to 30 nm. Energy-dispersive X-ray (EDX) analysis of the synthesized C.p-AgNPs indicated the presence of pure silver (Ag) at 54.32% in the region of 2.7 to 3.1 keV. Furthermore, the antimicrobial activity of C.p-AgNPs was examined, with the best inhibition observed at 0.5 mg/ml on Gram-negative bacteria *S. aureus* (12.0 mm) and *Streptococcus* spp (13.0 mm), and on Gram-positive bacteria *E. coli* (16.0 mm) and *Salmonella* spp (14.0 mm). The antimicrobial efficacy was dose-dependent, suggesting the potential for eradicating resistant human pathogenic bacteria. The antibacterial potential of C.p-AgNPs could be enhanced by increasing their concentration, depending on the specific application. Based on the study’s findings, C.p-AgNPs derived from *Calotropsis procera* can be employed for various biomedical purposes, such as textile coating by incorporating C.p-AgNPs in fibers and food storage by nanocapsulation of food items to extend their shelf life.

Keywords: silver nanoparticles, *Calotropsis procera*, *Salmonella* spp, *E. coli*, *S. aureus*, *Streptococcus* spp.

1.0 INTRODUCTION

Nanoparticles have attracted significant attention across various domains due to their substantial impact on drug delivery and targeting, reducing toxicity, enhancing efficacy, and creating new avenues for pharmaceutical and drug delivery enterprises (Lutfi et al., 2011; Bianco et al., 2015; Jokerst et al., 2017). Capping agents play a vital role in functionalizing and stabilizing synthesized nanoparticles. These biologically acceptable reducing, stabilizing, or capping agents are carefully chosen to ensure compatibility with living systems (Ocsoy et al., 2018; Javed et al., 2020). They shield nanoparticles from agglomeration and enhance reduction kinetics by forming complex structures with metallic ions in precursor salts (Campisi et al., 2016; Sharma et al., 2021). Silver nanoparticles (AgNPs) were synthesized using *Artemisia capillaris* extract, and their antimicrobial activity was tested on methicillin-resistant *Staphylococcus aureus* (MRSA) (Jang et al., 2015). AgNPs were also synthesized using aloe vera extract and tested against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Tippayawat et al., 2016). Additionally, AgNPs from Coffea arabica seed extract were tested on *Lactobacillus acidophilus* (Dhand et al., 2016). AgNPs from Rosmarinus officinalis leaf extracts exhibited activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Ghaedi et al., 2015), and AgNPs from *Calotropsis gigantea* flower extract showed efficacy against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* (Kemala et al., 2022). Furthermore, silver (Ag) and zinc (Zn) NPs extracted from *Calotropsis procera* fruits or leaves demonstrated activity against *Vibrio cholerae* and *Escherichia coli* (Saleem et al., 2015). AgNPs from *Helicetes isora* fruit extract exhibited activity against extensively drug-resistant (XDR) *Pseudomonas aeruginosa* isolates (Mapara et al., 2015). AgNPs from *Caesalpinia sappan* fruit extract demonstrated efficacy against *Streptococcus faecalis*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Aspergillus niger*, and *Candida albicans* (Jun et al., 2015).

Antimicrobial resistance is a persistent natural occurrence, with factors such as excessive and illicit antimicrobial use, inappropriate usage, sub-therapeutic dosing, and noncompliance with prescribed treatment courses accelerating the emergence of drug resistance (CDC, 2019).

Plants have been long recognized for their therapeutic attributes, with aboriginal cultures worldwide employing traditional herbal medicine for centuries to address various ailments (Li et al., 2017). The recent surge in demand for herbal medicines is attributed to their efficacy in treating diseases with minimal side effects (Priyanka and Brahmeshwar, 2017). Concerns have arisen regarding the use of modern medicines, such as chemotherapy, hormone-blocking therapy, monoclonal antibodies, and their equivalents, for diseases like cancer, given their adverse effects, therapeutic complexities, and cost implications. Ethnopharmacological remedies present a viable alternative to address these issues (Tannoy et al., 2017). Ethnopharmacology serves as an approach to identify potential medicinal compounds by assessing plants with therapeutic potential (Tita et al., 2020). Throughout history, plants and their secondary metabolites have consistently served as an exemplary source of medicine, renowned for their antibacterial and anticancer effects (Maya et al., 2016). *Calotropsis procera* (Plate 1), commonly referred to as the Apple of...
Sodom or Sodom Apple, belongs to the Apocynaceae family and the Asclepiadaceae subfamily (milkweed family) (Al-Rowaily et al., 2020). It is a perennial shrub or small tree native to North Africa, tropical Africa, and parts of Asia, reaching a height of up to 2.5 m. It holds significance in traditional medicinal practices across North Africa, the Middle East, South Asia, and Southeast Asia (Al Sulaihi et al., 2020). Additionally, it has been employed for fiber, fuel, fodder, and timber purposes since ancient times (Batool et al., 2020). Numerous studies explore its antimicrobial, anti-inflammatory, analgesic, anti-diabetic, anti-hypertensive, and anticancer properties. Its therapeutic potential stems from bioactive compounds like alkaloids, glycosides, flavonoids, lannins, glucosides, saponins, and terpenoids (Rahman et al., 2016; Kalu et al., 2022).

Plate 1. Calotropis Procera plant showing leaves, flowers, and fruit/pods.

Several studies have explored the antimicrobial properties of biosynthesized silver nanoparticles (AgNPs). However, only a limited number of these studies have utilized Calotropis procera as the stabilizing and capping agent. Notably, Salem et al. (2015) synthesized AgNPs using Calotropis procera and observed antimicrobial activity specifically against V. cholerae and E. coli. Meanwhile, Kemala et al. (2022) employed Calotropis gigantea flower extract, demonstrating activity against E. coli, S. aureus, and Candida albicans.

2.0 MATERIALS AND METHODS

2.1 Collection and Identification of Plant Extract and Bacterial Isolates

Calotropis procera leaves and flowers were obtained from Chikun LGA, Kaduna, Nigeria, and authenticated by a taxonomist at the Department of Biological Science Herbarium, Ahmadu Bello University with voucher number V/N-ABU900086. Clinical isolates of Staphylococcus Aureus (sourced from Urine), Streptococcus spp (sourced from Sputum), Salmonella spp (sourced from Stool), Escherichia Coli (sourced from Urine), and Staphylococcus Aureus (sourced from High Vaginal Swab) were collected at Oxford Hospital Chemical Pathology, Hematology, and Microbiology diagnostic laboratory in Kaduna State, Nigeria.

2.2 Preparation of Aqueous Silver Nitrate (AgNO₃)

A 1 mM AgNO₃ (Silver trionxide (V)) solution was prepared by weighing 0.0169 g of AgNO₃ using an analytical balance and dissolving it in distilled water to reach the 100 mL mark of a 100 mL volumetric flask.

2.3 Preparation of Aqueous Calotropis procera Extract and AgNPs Synthesis

Following the method of Chhangte et al. (2021) with slight modifications, Calotropis procera leaves (8.6 g) and flowers (7.4 g) were sliced into small pieces, pounded, and mixed with 100 mL of distilled water. The resulting extract was filtered, and 30 mL of the filtrate was added to 70 mL of 1 mM AgNO₃ solution in a 100 mL conical flask. The mixture was heated to 70 °C, stirred mechanically, and the pH adjusted to 9 using 0.1M NaOH (sodium hydroxide). The color change from light green to dark brown confirmed the synthesis of AgNPs. The synthesized C.p-AgNPs were obtained through centrifugation (KA-1000 model), eliminating the supernatant, and then oven-dried at 40 °C.

2.4 Characterization of Green Synthesized Nanoparticles

The synthesized silver nanoparticles (AgNPs) were characterized using Agilent Technology UV-Vis spectroscopy (Agilent Cary 5000 UV-Vis-NIR), Agilent Technology Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) model (Agilent Cary 630 FTIR), Scanning Electron Microscopy (SEM), and Energy Dispersive X-ray (EDX) SEM-EDX, NLNG model at the Multibuser Laboratory of Chemistry Department, Faculty of Physical Science, Ahmadu Bello University.

UV-Vis spectroscopy observed the synthesis of AgNPs, with successive dilutions of the initially prepared nanoparticles solution. For FTIR analysis, the Agilent ATR-FTIR instrument was used to collect the infrared spectrum reflecting the absorption of infrared light by the functional groups within Calotropis procera extracts and the synthesized silver nanoparticles. SEM was employed to capture images depicting the morphology and surface characteristics of the nanoparticles, and EDX analysis identified and quantified elements on the nanoparticle surface.

2.5 Antimicrobial Screening

The antimicrobial screening was carried out at Oxford Hospital Chemical Pathology, Hematology, and Microbiology diagnostic laboratory in Kaduna State, Nigeria. Streptococcus spp (High Vaginal Swab) Salmonella spp (Stool), Escherichia Coli (Urine), and Staphylococcus Aureus (High Vaginal Isolates)
Swab) were isolated, characterized, and identified using biochemical method. High profile positive/negative 10 tipped multiple susceptibility antibiotic discs containing Amoxicillin, Perfl oxacin, Erythromycin, Septrin, Streptomycin, Ciprofl oxacin, Rocephin, Zinncaf, Ampicloxi, Gentamycin, Sparfl oxacin, Chloramphenicol were used for screening of resistant bacteria, while the prepared sample mixtures ant imicrobial disk were used for minimum inhibitory concentration determination against the screened resistant bacteria using Kirby-Bauer disk diffusion test, as outlined by Ol gica et al., (2018), utilizing Mueller-Hinton Agar (MHA).

- Sgu for 5 mg of C. procera extract dissolved in 1 mL distilled water and was applied on 5mm diameter autoclaved filter paper to prepared the antimicrobial disk
- Tg1 for 0.1 mg AgNPs dissolved in 1 mL distilled water and was applied on 5mm diameter autoclaved filter paper to prepared the antimicrobial disk
- Tc2 for 0.25 mg AgNPs dissolved in 1 mL distilled water and was applied on 5mm diameter autoclaved filter paper to prepared the antimicrobial disk
- Sg3 for 0.5 mg AgNPs dissolved in 1 mL distilled water and was applied on 5mm diameter autoclaved filter paper to prepared the antimicrobial disk

To ensure sterility, area sterilization involved the use of a disinfectant and an open burner. A sterile loop was then employed to select a well-isolated bacterial colony from a pure culture plate, and the colony was subsequently transferred to the broth medium under aseptic conditions, as described by Sudhir et al., (2015). Inoculation of the entire surface of a Mueller-Hinton agar plate included streaking in three directions (north-south, east-west, and diagonally) to achieve uniform distribution. Following inoculation, the plate was allowed to briefly dry, facilitating bacterial adhesion to the agar surface, as suggested by Sudhir et al., (2015).

The application of prepared antimicrobial disks onto the agar surface was performed using sterile forceps with gentle pressure for proper contact. Plates were then inverted and incubated for 24 hours at 37 °C. Post-incubation, the plates were examined, and the diameter of the zone of inhibition around each disk was measured and recorded, indicating susceptibility or resistance, as detailed by Sudhir et al., (2015).

For confirmed clinical isolates of Staphylococcus Aureus from High vaginal swab (HVS), Escherichia Coli from stool, Streptococcus Spp from sputum, and Salmonella Spp from stool, antimicrobial susceptibility tests were conducted using the prepared samples:

3.0 RESULTS AND DISCUSSION

3.1 Synthesis of AgNPs

When C. procera leaf/flower extract was mixed with AgNO₃ solution, heated at 60 °C for 10 minutes, and alkaliized, a visible colour change from light green to dark brown or reddish-brown occurred. This colour change is attributed to the Surface Plasmon Resonance phenomenon in silver nanoparticles, resulting from the excitation of free electrons in nanoparticles, as explained by Kemala et al., (2022). Phytometabolites have been reported to act as electron donors, mediating the reduction of silver ions and initiating surface plasmon resonance (SPR), as noted by Al-otbi et al., (2021) and Kemala et al., (2022). SPR is a complex process defined as the excitation and coherent oscillation of electrons in an incoming electromagnetic field, as described by Pryshchepa et al., (2020) and Kemala et al., (2022). No further color change was observed after 24 hours, indicating the completion of the reduction process, consistent with literature reports by Kero et al., (2017), which indicated the synthesis of silver nanoparticles at 24 hours using Lippia citriodora leaf extract.

![UV spectrum of C.p-AgNPs](image.png)

The UV-VIS spectrum, depicted in figure 1, reveals absorbance peaks at a λmax of 368 nm, which is consistent with the characteristic absorption pattern of AgNPs. This observation aligns with the findings of Hao and Qinghua (2017), who conducted a study on the green synthesis of silver nanoparticles and their antimicrobial activities. In their research, they reported a distinct surface plasmon adsorption peak at approximately λmax 400 nm, indicative of silver nanoparticles.

Similarly, Nadia et al. (2014), in their investigation on the antimicrobial activity of latex silver nanoparticles using Calotropis procera, identified a surface plasmon resonance band with a peak centered around λmax 290 nm. Moreover, Chhangte et al. (2021), in their review of recent literature on the green synthesis of silver nanoparticles using plant extracts and their antimicrobial activities, highlighted that the absence of peaks in the 335-600 nm region in UV-Vis spectra is sometimes employed as an indication of the absence of aggregation in nanoparticles.
3.2 ATR-FTIR spectra of C. procera extract and the synthesized C.p-AgNPs

The ATR-FTIR spectroscopy results for both C. procera extract and the synthesized C.p-AgNPs are illustrated in figures 2a and 2b. The key characteristic peaks observed at 3280.1 cm\(^{-1}\), 2922.2 cm\(^{-1}\), and 2851.4 cm\(^{-1}\) were attributed to Amine N-H stretching, alcohol and carboxylic acid O-H stretching, and C-H stretching of terminal alkyne, vinyl, alkane, and aldehydes, as reported by Félix et al. (2021). Félix et al. (2021) also noted multiple bands between 3600 and 3200 cm\(^{-1}\), indicative of O-H stretching from phenol and N-H stretching from amide (acetaminophen), along with C-H stretching at 3000 – 2640 cm\(^{-1}\). Furthermore, peaks at 2117.1 cm\(^{-1}\), 1640.0 cm\(^{-1}\), 1543.1 cm\(^{-1}\), 1420.1 cm\(^{-1}\), and 1323.2 cm\(^{-1}\) were assigned to C=C, C=N, C≡C, C≡N stretching vibrations of alkyne, alkene, benzene, and aline, with support from Khtokoi (2019), Monowar et al. (2021), and Félix et al. (2021). The literature also

Figure 2a: ATR–FTIR of aqueous C.procera extract

Figure 2b: ATR–FTIR of C.procera-AgNPs
includes references to peaks at 2110 cm\(^{-1}\) and 1523 cm\(^{-1}\) corresponding to C\(\equiv\)C and C\(\equiv\)N stretching vibrations (Monowar et al., 2021), and a peak at 1329 cm\(^{-1}\) attributed to \(\approx\)C-C\(\equiv\) stretching for the benzene ring (Kihotoki, 2019). Félix et al. (2021) reported C=C double bond stretching at 1690 – 1635 cm\(^{-1}\). Peaks at 1241.2 cm\(^{-1}\), 1148.0 cm\(^{-1}\), and 1025.0 cm\(^{-1}\) were assigned to C-O stretching and \(-\)CH\(\equiv\) bending vibrations, aligning with the findings of Ibrahim et al. (2016), who reported C-O stretching at 1200 cm\(^{-1}\) and \(-\)CH\(\equiv\) bending vibrations at 1100 – 1000 cm\(^{-1}\). Distinctive peaks at 2851.4 cm\(^{-1}\), 1543.1 cm\(^{-1}\), and 1323.2 cm\(^{-1}\) observed in \(\text{C. procera}\) silver nanoparticles (\(\text{C.p-AgNPs}\)) disappeared, possibly due to conjugation resulting from synthesis temperature and/or sodium hydroxide addition. Two peaks at 3280.1 cm\(^{-1}\) and 2922.2 cm\(^{-1}\) remained unchanged, while the remaining six peaks exhibited variations in wave numbers. These changes in functional groups, coupled with others showing reduced or increased wave numbers, contribute to the reduction, capping, and stabilization of synthesized \(\text{C.p-AgNPs}\), in accordance with Javed et al. (2020). This observation aligns with Shakeela et al. (2019), who reported similarities between plant extracts and AgNPs, suggesting that residual plant material serves as capping agents for AgNPs, preventing agglomeration and stabilizing the medium. The shifts within the peaks (3000 cm\(^{-1}\) to 1600 cm\(^{-1}\)) and some unchanged peaks indicate the potential binding of proteins from the plant extract with Ag, potentially contributing to the capping of metal NPs and preventing agglomeration, as discussed by Shakeela et al. (2019).

3.3 Scanning Electron Microscope (SEM) Micrographs and Energy Dispersive X-ray (EDX) of Synthesized \(\text{C.p-AgNPs}\)

The SEM micrographs of synthesized \(\text{C.p-AgNPs}\), as presented in figure 3, reveal predominantly spherical nanoparticles with an average particle size of 20 – 30 nm. The non-uniform distribution and agglomeration of nanoparticles may be attributed to the precursor concentration, biomolecules in \(\text{C. procera}\) leaf extracts, and the presence of secondary metabolites in leaves and flower extracts. Similar findings were reported by Chhangte et al. (2021) in their study on the green synthesis of silver nanoparticles using plant extracts, indicating spherical particle sizes ranging from 10 – 20 nm. Alkammash (2017) also observed predominantly spherical particles with sizes ranging from 8 – 20 nm, with other non-spherical shapes present.

The analysis presented in figure 4, through Energy Dispersive X-ray (EDX) revealed the presence of pure Silver (Ag) at a range of 2.7 to 3.1 keV, constituting 54.32% of the material. This robust signal within the metallic silver range serves as confirmation of the formation of silver nanoparticles utilizing \(\text{C. Procera}\). Additional peaks were identified for Carbon (C) at 24.69%, Copper (Cu) at 14.81%, and Oxygen (O) at 6.17%. Notably, a significant percentage of Copper atoms were observed on the surface of \(\text{C.p-AgNPs}\) nanoparticles, potentially stemming from impurities in the precursor or agrochemicals used on the \(\text{C. Procera}\) plant sourced from a farm. These findings align with results reported by Dada et al. (2017), who observed a similar elemental composition in AgNPs synthesized using the leaf extract of \(\text{Calotropis procera}\).

3.4 Antimicrobial Assay

The antimicrobial assay of \(\text{C. procera}\) extracts and \(\text{C.p-AgNPs}\) is detailed in table 1 and figure 4. The susceptibility test revealed that \(\text{C. procera}\) extracts at 5 mg/mL exhibited no inhibition against the four tested bacterial strains. This lack of inhibition is attributed to the relatively low concentration of the extract used, as aqueous plant extracts typically require higher concentrations to demonstrate positive effects on bacterial growth, especially for resistant strains (Gideon, 2023).

**Table 1: MIC Zone of inhibition of \(\text{C.procera}\) synthesized silver nanoparticles \(\text{C.p-AgNPs}\)**

<table>
<thead>
<tr>
<th>Bacteria Isolates</th>
<th>(\text{C.procera}) extract</th>
<th>(\text{C.procera}) Silver Nanoparticles, AgNPs extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mg/mL</td>
<td>0.1 mg/mL</td>
</tr>
<tr>
<td>salmonella spp</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>streptococcus spp</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Furthermore, \(\text{C.p-AgNPs}\) at 0.1 mg/mL displayed negative inhibition against \(\text{Salmonella}\) spp. and \(\text{S. aureus}\). This result is attributed to the inherent nature of these bacterial strains, which necessitate a higher concentration of synthesized silver nanoparticles for inhibition. Comparable findings were reported by Mathew et al. (2023) and Siriporn et al. (2016), where lower concentrations of plant extracts and biologically synthesized silver nanoparticles were insufficient to inhibit the growth of various gram-positive and gram-negative bacteria at concentrations below 1 mg/mL.

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4.0 Conclusion

The study demonstrates the bio-reduction of aqueous silver ions using the leaf/flower extract of the *Calotropis procera* plant. Characterization of the resulting product was carried out through UV-Vis, ATR-FTIR, SEM, and EDX analyses. The metal ions were reduced, leading to the formation of silver nanoparticles with an average particle size of 20 – 30 nm, as indicated by SEM analysis. The UV-Vis analysis of AgNps revealed a surface plasmon resonance (SPR) characteristic peak at 368 nm. EDX confirmed the presence of pure silver (Ag) at a region of 2.7 to 3.1 keV. ATR-FTIR showed the disappearance of three distinctive peaks at 2851.4 cm⁻¹, 1543.1 cm⁻¹, and 1323.2 cm⁻¹, with others exhibiting reduced and increased wavenumbers. These changes are responsible for the reduction, capping, and stabilization of the synthesized C.p-AgNPs. The leaves/flowers of *Calotropis procera* emerge as a promising source for green synthesis of silver nanoparticles.

Furthermore, the antimicrobial activity of C.p-AgNPs was investigated, revealing dose-dependent inhibition. The C.p-AgNPs exhibited the best inhibition at 0.5 mg/mL against gram-negative bacteria *S. aureus* (12.0 mm) and *streptococcus* spp (13.0 mm), as well as gram-positive bacteria *E. coli* (16.0 mm) and *salmonella* spp (14.0 mm). The efficacy of the antimicrobial activity suggests the potential to eradicate resistant human pathogenic bacteria, and adjusting the concentration of C.p-AgNPs could further enhance their antibacterial potential.

Based on these findings, C.p-AgNPs derived from *Calotropis procera* can be utilized for various biomedical purposes. Additionally, incorporating C.p-AgNPs in fiber for textile coating and nanocapsulation of food items to increase shelf life are suggested applications.

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None.

Conflict of interests

The authors declare no competing conflict of interest.

Thank you for your adequate outstanding review of my manuscript. All corrections highlighted were effectively addressed, also in regards to the preparation of the disk, it is also addressed which can be seen in page 6. The antimicrobial activity was completely carried out in the oxford hospital laboratory, it was isolated and characterized by the laboratory technologist before we did the resistance test and our prepared disk Minimum inhibitory concentration using disk diffusion method was carried out on the resistant bacteria.

As for MBC minimum bactericidal concentration was not determine based on the scope of our work. Also the topic has been annul to emphasize the major aim of the work as directed by my supervisor.

I hope you will understand and consider all the corrections made noteworthy.

Yours faithfully

Mamman A. James

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Mapara, N., Sharma, M., Kumar, V. (2015). Antimicrobial potentials of Helicteres isora silver nanoparticles against extensively
Green Synthesized Silver Nanoparticles (AgNps) Using Aqueous Extract of Calotropis Procera and Its Antimicrobial Activity on Clinical Bacteria Isolates