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Green synthesis of silver nanoparticles utilizing *Gardenia latifolia* extract, characterization and *in vitro* antibacterial activity

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Abstract: Biogenic silver nanoparticle (AgNPs) formulations were synthesized using alcoholic extract from the bark of *Gardenia latifolia* Ait. as a reducing agent and a capping ligand for the silver ions present in an aqueous silver nitrate solution. The synthesis of AgNPs was noted by color, the presence of notable peaks in Fourier-transformed infrared spectra (FT-IR), and Raman spectra. High-resolution transmission electron microscopy (HRTEM) analyses of all the AgNP formulations confirmed the crystalline and spherical characteristics along with smooth surface that was further supported by low particle size (*Zavg*, <50 nm), low polydispersity index (*PDI*), and negative zeta potential. The crude extract and its various fractions did not show significant activity against *Staphylococcus aureus* MTCC-96 and the Methicillin-resistant *S. aureus* (MRSA) clinical isolate, yet in a combinatorial study, a concentration of 10 µg/mL, they reduced the minimum inhibitory concentration (*MIC*) of ethidium bromide (EtBr) to half against the MRSA clinical isolate, supporting the drug resistance reversal property of *G. latifolia* extracts and fractions. The *in vitro* evaluations of AgNP formulations against MRSA, *Staphylococcus epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae* revealed *MIC* ranging from 30.00 to 2.81 µg/mL. Among all, AgNP–**F3** may find its use in the development of cost-effective broad-spectrum antibacterial medications.

Keywords: biofabrication; AgNPs; HR-TEM; MRSA; drug resistance.

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INTRODUCTION

Plants and natural substances have traditionally played a vital role in historical medicinal practices, including Ayurveda, traditional Chinese medicine and native American healing, for the treatment of numerous health issues.[1,2] Traditional medicines use a single herb or a combination of two or more herbs as herbal formulations for the treatment of various diseases and debilities.[3] Besides being directly used in remedies, plants have significantly contributed to the advancement of modern medicine, acting as a valuable source of bioactive substances with therapeutic capabilities.[4–6] Throughout history, plants have been essential to traditional medical practices and continue to be a vital source of inspiration and materials for current drug discovery efforts.[7,8] Plants produce a variety of secondary metabolites, including alkaloids, terpenoids, flavonoids and polyphenols, which display a range of biological effects.[9,10] These compounds can function directly as medications or serve as lead compounds for the development of synthetic pharmaceuticals.[11] Although herbal formulations offer advantages, they encounter notable drawbacks, including fluctuations in the concentration of active ingredients that can vary based on the origin of the plant, the time of year, and the methods used for extraction.[12] Additionally, their low solubility and stability lead to poor bioavailability, which restricts their effectiveness in clinical settings.[13,14]

Nanoparticles (NPs) have transformed the fields of drug discovery and development by providing novel approaches to address issues related to drug delivery, effectiveness and safety.[15] These nanoparticles, generally measuring between 1 and 100 nm, display distinctive physicochemical characteristics, such as tiny size, elevated surface area-to-volume ratio and tunable surface chemistry, which renders them highly efficient in improving therapeutic results.[16] Silver nanoparticles (AgNPs) have become a significant area of interest in antimicrobial research owing to their remarkable attributes, such as a wide-ranging antimicrobial action and the capability to fight against antibiotic-resistant microorganisms.[14] Conventional techniques for synthesizing nanoparticles typically involve environmental and health hazards. On the other hand, green synthesis offers a sustainable and economical alternative.[17] The production of AgNPs through bio-fabrication utilizing plant extracts presents a sustainable, economical and environment-friendly option compared to traditional chemical and physical methods.[18] This eco-friendly synthesis method utilizes the inherent reducing, capping, and stabilizing properties found in plant extracts, including flavonoids, alkaloids, phenols and proteins, to generate AgNPs.[13,15]

Gardenia latifolia Ait., also referred to as “Indian boxwood”, is a deciduous tree that belongs to the Rubiaceae family.[19] This plant is indigenous to the tropical and subtropical areas of India, Bangladesh and Southeast Asia.[20] It is well--known in traditional medicine practices for its ethnomedicinal uses and is

known with many vernacular names, *viz*. Papra and Ban pindalu (Hindi), Parpataki (Sanskrit), Varkura (Bengali), Ghogari (Marathi), and Pedda bikki and Peddakaringuva (Telagu).[21] The various plant parts of *G. latifolia* are used to cure a variety of diseases as folklore remedies by villagers and tribes, as well as in well--developed Ayurveda and Siddha systems.[21–24] The leaves of *G. latifolia* are used to cure snake bites.[21] The stem bark extract with pepper and garlic is given in the treatment of fever.[24] The bark of *G. latifolia* is also used to cure skin diseases,[22] stomach troubles, constipation and caries.[25] The resinous sap extracted from the stem tips is applied to the sores of the hands and feet in the rainy season.[25] Fruit paste is given to cure amoebiasis, and the gum is used to cure cutaneous diseases.[25] The root is useful in the treatment of heavy bleeding during the menstrual cycle while the seed along with the leaf of the piper is taken for regular menstruation in women.[26] In the Ballari district of Karnataka (India), fruit pulp is used to cure fever.[27] The alcoholic extracts of the leaf, bark, and fruit of *G. latifolia* showed potent antioxidant and anti-inflammatory properties, which were later supported by the presence of glycosides, flavonoids, phenols, saponins, tannin and anthraquinones in phytochemical investigations.[21] In Bangladesh, different parts of *G. latifolia* are reported to be used traditionally in the treatment of a wide range of human diseases such as stomach pain, fevers, skin diseases, snake bites, inflammatory pain, caries, hemorrhage and ephemeral fever.[19] The leaf extract of *G. latifolia* showed analgesic, antipyretic, *α*-amylase enzyme inhibition, membrane stabilizing, antioxidant effect and antimicrobial activities.[28] There are several reports on the biological activity of fruit extracts of *G. latifolia*.[20,29] Despite a few reports on the fruits and leaf extract of *G. latifolia*, there is no detailed report on the phytochemical investigations and biological activity of the bark extract. In given frame of context, the present work deals with the primary phytochemical screening of *G. latifolia* bark extract, a sustainable synthesis of AgNPs using *G. latifolia* bark extract, characterization and antibacterial activity assessment of extracts and AgNPs for their possible use in the development of a broad-spectrum, cost-effective antibacterial formulation for treating multidrug-resistant bacterial infections.

EXPERIMENTAL

Chemicals and reagents

The solvents used in the extraction and fractionation were purchased from Merck/ /Thermo Fisher, India, Pvt. Ltd. Silver nitrate (analytical grade) and water (HPLC grade) used for the synthesis of AgNPs were purchased from Loba Chemie Pvt. Ltd., India. All the other chemicals and reagents were of analytical/synthetic grade, procured from Sigma–Aldrich Pvt. Ltd., India. All the solvents and reagents were used without further purification.

Collection of plant material

The authenticated samples of *G. latifolia* stem bark were collected in October 2023 from the Hathi Nala forest area of Sonbhadra (24°18'8.43"N and 83°5'31.8"E), Uttar Pradesh, India,

under the supervision of Dr. Deepak Gond, Department of Botany, C.M.P. College, University of Allahabad, India.

Extraction and fractionation

The bark of *G. latifolia*, which was shade-dried and ground into a powder, underwent extraction and fractionation following the previously outlined procedure (Fig. S-1 of the Supplementary material to this paper).[14,30] In summary, 1.2 kg of plant material was extracted three times using 2 L of methanol, and the solvent was removed under a vacuum with a rotary evaporator-chiller (Buchi, India) to produce methanolic extract. Next, the dried extract was dissolved in warm-distilled water and successively partitioned with 500 mL of *n*-hexane, chloroform and *n*-butanol three times each, with the collected fractions being dried separately under a vacuum, resulting in *n*-hexane, chloroform and *n*-butanol fractions (Fig. S-1).

Phytochemical investigation of the methanolic extract

The thin-layer chromatography (TLC) profile of the crude extract and the fractions of hexane, chloroform and *n*-butanol were individually analyzed on ready-made TLC plates (silica gel $60F_{254}$, Merck, India) in different solvent systems to assess the presence of phytoconstituents preliminary. The developed TLC plates were initially observed under UV light at 254 and 365 nm, then treated with a vanillin–sulphuric acid (1:5) solution in ethanol and subsequently heated at 95 °C for 5 min for visualization. Further, the analysis of the alcoholic extract was conducted to assess the presence of secondary metabolites utilizing standard methods.[9,31]

Synthesis of AgNPs

A 1 % *G. latifolia* bark extract was made by dissolving 1 g of the dried methanolic extract in 100 ml of ethanol, followed by sonication for 20 min using an ultrasonic cleaner (Science Tech, India) and subsequent filtration. A green synthetic method using a bottom-up approach was utilized, employing different concentrations (2 and 3 mM) of aqueous silver nitrate ($AgNO_3$) solution along with various volumes (25 and 50 μL) of 1 % plant extract solution to determine the optimal combination with suitable colloidal characteristics. To begin with, 3.5 mL of 2 mM and 3 mM $AgNO_3$ solutions were separately combined drop by drop with 25 μL/50 μL of the plant extract, and the total volume was brought up to 4 mL using millipore water, resulting in the final extract concentrations of 62.5/125 μg/mL. All four reaction mixtures were placed in screw-capped glass vials and maintained on an orbital shaker (Remi, India) for 24 h, during which they were continuously monitored for any color changes, indicating the formation of AgNPs. Following the incubation period, color alterations were noted and verified using spectroscopy. Afterward, each formulation was centrifuged and rinsed three times with Millipore water and then redeveloped.

Characterization of AgNPs

Organoleptic properties. Initially, the samples were observed visually to detect any color changes, the presence of aggregates, and levels of turbidity.

Raman spectroscopy. The Raman spectra were recorded using an alpha-300 Access confocal Raman spectrometer (WITec, Germany) that featured a UHTS 300S_VIS spectrometer with a 600 g/mm BLZ 500 nm grating. Excitation lasers of 532 nm at 3–5 mW power were employed. Subsequently, the data were processed for noise removal and baseline correction using polynomial functions.

Fourier-transformed infrared (FT-IR) spectroscopy. Fourier-transformed infrared spectra (FT-IR) spectroscopy was performed using a Nicolet iS5 spectrometer (Thermo Fisher Sci-

entific, USA) within the range of 400–4000 cm^{-1}, where samples were directly added to the diamond surface and the spectra were recorded considering water as the solvent for blank.

High-resolution transmission electron microscopy (HRTEM) and selected area electron diffraction (SAED). The surface morphology and SAED pattern of samples were investigated by HRTEM (FEI, TECNAI G2, USA) equipped with a HAADF detector at 200 kV. In HRTEM analysis, the sample was diluted five times with ultrapure water, and one drop of the samples was placed on a carbon-coated copper grid. The excess water was soaked with clean filter paper and dried overnight to remove the residual water.

Dynamic light scattering (DLS) analysis. Particle size (*Zavg*), polydispersity Index (*PDI*) and zeta potential (*ZP*) of AgNPs were measured by DLS method on Zetasizer Ultra (ZSU5700, Malvern Panalytical Ltd., UK) at 25°C.

In vitro *antibacterial activity*

Procurement of bacterial strains. The pathogenic *Staphylococcus aureus* MTCC-96, clinical isolates of *S. aureus (*which was resistant to methicillin and named MRSA), *Staphylococcus epidermidis*, *Klebsiella pneumoniae* and *Escherichia coli* were obtained from the ICMR-RMRC, Gorakhpur repository. Mueller–Hinton agar and broth (MHA and MHB, Hi-Media, Mumbai, India) were employed as media for bacterial culturing. Colony counts were assessed utilizing MHA plates.

Antibacterial activity assessment. The minimum inhibitory concentrations (*MIC*s) were established using a twofold serial dilution broth method in Mueller–Hinton broth using 96--well microtiter plates following the guidelines set by the Clinical & Laboratory Standards Institute (CLSI) for broth micro-dilution.[32,33] The extracts and fractions were diluted to achieve final concentrations ranging from 1000 to 1.95 μg/mL, and they were evaluated against *S. aureus* MTCC-96 and MRSA strains. In the study involving AgNPs, the strains of MRSA, *S. epidermidis*, *K. pneumoniae* and *Escherichia coli* were tested using a 30 μL aliquot from each (2/3 mole, aqueous) sample as the stock solution, which ensured concentrations of 30 μg/mL of AgNPs for samples **F1** and **F2** and 45 μg/mL of AgNPs for samples **F3** and **F4** as their initial concentrations. The starting inoculum concentration was 5×10^5 cfu/mL. The inoculated plates were incubated at 37 °C for 24 h, after which visual assessments were made, and the *MIC* was noted as the final dilution that showed no turbidity in accordance with CLSI guidelines. Ciprofloxacin was used as a positive control.

Combinatorial study. The combination experiments at a fixed 10 μg/mL concentration of extract and fractions were conducted using ethidium bromide (EtBr) concentrations between 0.48 and 250 μg/mL.[2,34] The microtitre plates were incubated with 10 μL of a diluted overnight culture of the test organism MRSA, adjusted to a titer equivalent to the 0.5 McFarland standard. Following inoculation, the microtitre plates were incubated at 37 °C for 24 h.[4]

RESULTS AND DISCUSSION

Phytochemical investigations

A methanolic extraction from 1.2 kg of shade-dried and ground bark of *G. latifolia* resulted in 84 g of a dried extract. Furthermore, the fractionation of 60 g dried methanolic extract yielded *n*-hexane (2.5 g), chloroform (0.6 g), and *n*-butanol (13.2 g) fractions (Fig. S-1). The TLC of the extract and its fractions in several combinations of hexane, chloroform, and methanol revealed the presence of plant secondary metabolites (Fig. S-2 of the Supplementary material). The

phytochemical investigation of the crude methanolic extract of *G. latifolia* bark revealed the existence of saponins, tannins, glycosides, terpenoids, phenolic compounds and flavonoids; nonetheless, alkaloids were not found in the crude extract (Table S-I of the Supplementary material). The results are more or less consistent with previous research reporting several bioactive groups of compounds in the extracts obtained from various parts of *G. Latifolia*.[21,35,36]

Synthesis of AgNPs

The eco-friendly and sustainable approach of synthesizing AgNPs with plant extracts serves as a cost-efficient alternative to traditional chemical methods.[14,16,18] This method provides greater biocompatibility, minimizes toxicity, decreases energy usage, and improves biological characteristics, making it suitable for use in medicine, agriculture and environmental science.[13,15] Using aqueous silver nitrate as a source for silver cations, the initial step includes reducing Ag^+ to Ag by utilizing plant secondary metabolites such as flavonoids, phenols, alkaloids, terpenoids and proteins to provide electrons.[37] After the metallic Ag is generated, it begins to aggregate into tiny clusters of 15–2000 atoms, which are subsequently enclosed by capping agents that are, in turn, the secondary metabolites of the plant.[38]

In our initial phytochemical studies, the bark extract of *G. latifolia* showed the presence of various types of bioactive secondary metabolites with hydroxyl, carboxylic and carbonyl groups, which could effectively reduce and cap silver ions.[20,35] To determine the optimal concentration of extract and silver ions, four different formulations were synthesized using 3.5 mL of 2 or 3 mM silver nitrate solution along with 25/50 μL of 1 % alcoholic extract from the bark of *G. latifolia* (Table I).

TABLE I. The concentrations of $AgNO_3$ and *G. latifolia* bark extract in various AgNP-formulations

AgNP-formulation	3.5 mL of $AgNO_3$, mM	1 % alcoholic extract, μL
F1	2	25
F2	2	50
F3	3	25
F4	3	50

Characterization of AgNPs

The initial sign in the formation of metal nanoparticles is a color change, which occurs because of the vibration of the plasmon base, a unique optical characteristic of noble metals.[16] In this study, the synthesis of AgNPs involved the gradual addition of the extract to the $AgNO_3$ solution accompanied by continuous shaking. This process resulted in a color change of the reaction mixture from colorless to brown, indicating the formation of AgNPs (Fig. 1).

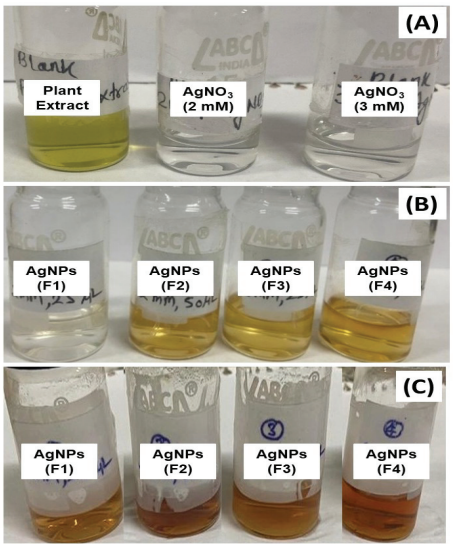

Fig. 1. Organoleptic changes observed in the reaction mixture during green synthesis of AgNPs before incubation (A) AgNP formulations after 1 h (B), and AgNP formulations after 24 h (C).

FT-IR spectroscopy. FT-IR, a technique that detects significant functional groups in plant extracts, serves as a crucial method for characterizing biofabricated AgNPs. Nanoscale silver particles synthesized through biofabrication are generally stabilized by phytochemicals that interact with the surfaces of the metal at a molecular level.[39] FT-IR analysis serves as a tool to investigate the characteristics of these molecular interactions and the associated functionalities, as noted in previous studies referencing specific functional group peaks.[14] In the present study, this examination might also reveal how silver nitrate engages with the secondary metabolites present in the bark extract of *G. latifolia*, which function as reducing and capping agents. A change in peak absorption or intensity indicates that a reaction has occurred between silver ions and the plant secondary metabolites containing functional groups, leading to the synthesis of nanoparticles. The FT-IR absorbance bands of the extract and the AgNP formulations in the range of 500 to 4000 cm^{-1} are presented in Fig. 2. The analysis revealed absorbance peaks at 3407, 2927, 2854, 1685, 1629, 1520, 1456, 1281, 1075 and 1048 cm^{-1}, primarily indicating the presence of carbonyl, alcoholic, and phenolic functional groups. A thorough examination of the FT-IR spectrum for the produced AgNPs revealed a strong and broad peak at 3452 (AgNP-F1), 3431 (AgNP-F2 and AgNP-F3) and 3442 (AgNP-F4), which appears to have shifted from 3407, the prominent peak of the extract. The significant peak observed at 1384 cm^{-1} could be attributed to the C–N stretching of secondary amines, amino acids, peptides, and proteins present in the plant extract, which might form a layer around the synthesized silver to serve as a capping agent.[40] The spectra of synthesized AgNPs revealed a few additional prominent and broad surface plas-

mon peaks ranging from 1400 to 1700 cm^{-1}, representing a significant band peak for silver ions.[16,18]

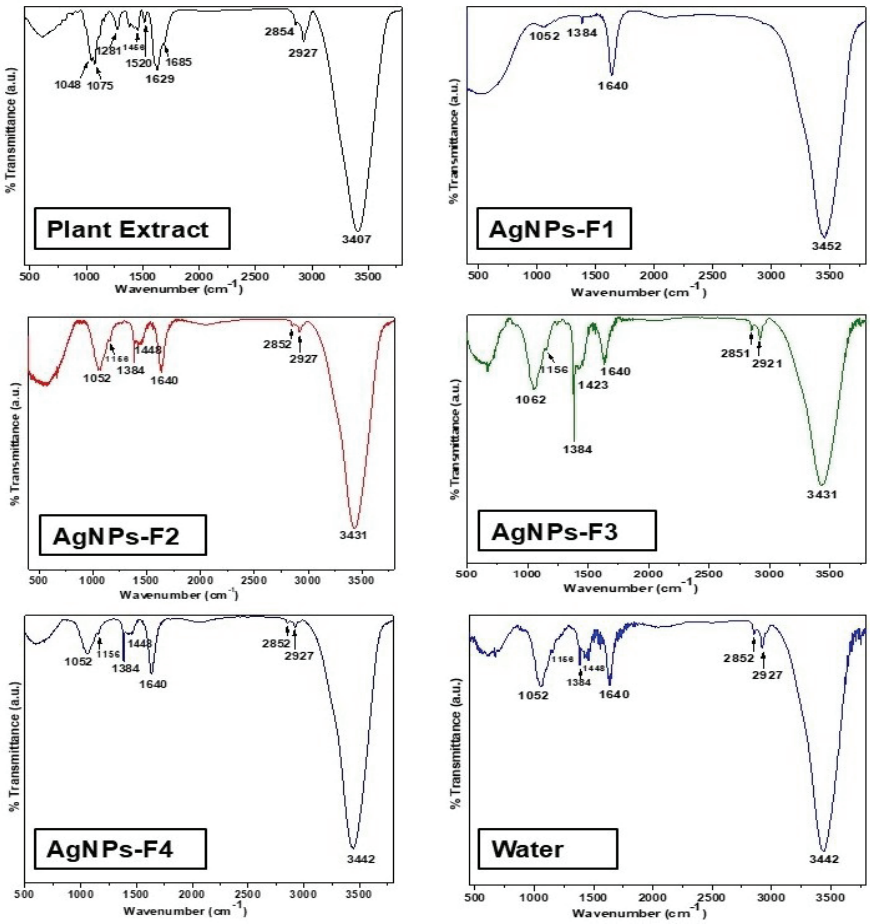

Fig. 2. Fourier transform infrared spectrum of plant extract and AgNP formulations.

Raman spectroscopy. Raman spectroscopy serves as an effective tool for analyzing AgNPs because of their distinctive surface-enhanced Raman scattering (SERS) characteristics. This enhancement takes place when molecules attach to the surface of silver nanoparticles, rendering it particularly beneficial for detecting chemical species and functional groups that are attached to the surface and play a role in the synthesis or stabilization process.[41] Raman spectroscopy was utilized over a spectrum from 200 to 4000 cm^{-1} to detect any possible changes in the reaction spectra leading to the synthesis of AgNPs (Fig. 3). In the case of the aqueous silver nitrate solution, no distinct peak was found in the range of 200–

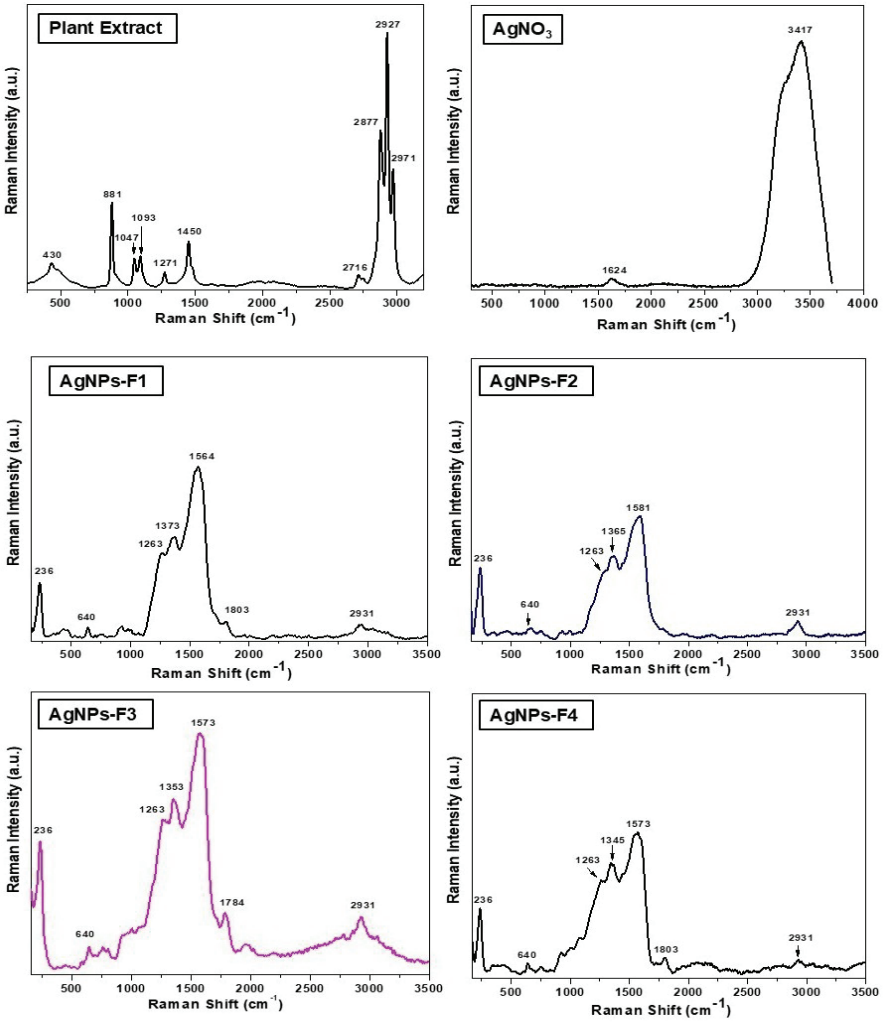

Fig. 3. Raman spectrum of plant extract and AgNP formulations.

–1500 cm^{-1}, suggesting that there were no organic components present. In contrast, the alcoholic extract exhibited distinct peaks for carbohydrates at 430, 881, 1047 and 1093 cm^{-1}, along with a pronounced peak probably for the phosphate group in DNA at 1271 cm^{-1}. Additionally, there were higher-frequency bond vibrations related to CH, NH and OH stretching in lipids at 2716, 2877, 2927 and 2971 cm^{-1}, which is supported by the literature.[42] The AgNP formulations revealed through Raman spectroscopy a fingerprint region ranging from 400 to 1500 cm^{-1}, along with the emergence of a pronounced peak at 236 cm^{-1}, attributed to the O=Ag_2/Ag–N stretching vibrations, which further confirms the conversion of Ag^+ to Ag.[43,44] The Raman spectrum exhibited prominent peaks at 1373 cm^{-1}

and 1564 cm^{-1} for the biosynthesized AgNP-F1, at 1365 and 1581 cm^{-1} for the biosynthesized AgNP-F2, at 1353 and 1573 cm^{-1} for the biosynthesized AgNP–**F3**, and at 1345 and 1573 cm^{-1} for the biosynthesized AgNP–**F4**, corresponding to the D and G bands. This is indicative of organic materials derived from the plant extract that function as capping agents.[41]

HR-TEM and SAED analysis. HR-TEM and SAED offer essential information regarding the nanoscale sizes, shapes, crystallinity and structural characteristics of the synthesized nanoformulations. The arrangement of spots in the chosen area diffraction pattern creates the TEM image, where each spot signifies a fulfilled diffraction state of the crystalline structure of the nanoparticle.[45] The various diffraction spots corresponding to different diffraction states reflect the crystallinity of the specimen. The HR-TEM photomicrographs of the produced AgNP formulations distinctly exhibited the presence of uniformly shaped spherical AgNPs with smooth surfaces, maintaining a consistent distance from one another, except for a few agglomerated AgNPs observed in certain areas of the AgNP formulation **F2** and occasionally in **F4** (see Figs. S3 and S4 of the Supplementary material). It was noted that the AgNP formulations created with a smaller quantity of plant extract (**F1** and **F3**) demonstrate consistent size and distribution of AgNPs with smooth surfaces (Fig. 4).

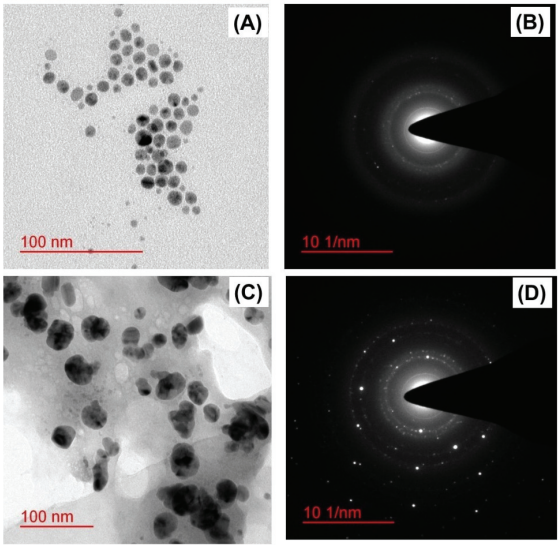

Fig. 4. HRTEM photomicrograph of: AgNP–**F1** (A) and AgNP–**F3** (C), SAED spot pattern of AgNP–**F1** (B) and AgNP–**F3** (D).

DLS analysis. DLS analysis is a reliable and economical method for determining the diffusion coefficient of suspended nanoparticles in a solution undergoing Brownian motion by analyzing the fluctuations in light intensity scattered

by the nanoparticles in the medium.[45] The intensity of the scattered light is inversely proportional to the diffusion coefficient of the nanoparticles and provides information on the hydrodynamic size of the nanoparticles. The average particle size (*Zavg*), polydispersity index (*PDI*) and zeta potential (*ZP*) of silver nanoparticles (AgNPs) were assessed through DLS techniques in bio-fabricated AgNP solutions, and all the relevant data is presented in Figs. S-5–S-8 of the Supplementary material. The *Zavg* indicates the mean diameter of nanoparticles in a sample, which was measured at 32.3±0.109 nm for formulation **F3** and 47.2±0.243 nm for formulation **F4** of the synthesized AgNPs. The *Zavg* values observed in this research are considered optimal for extract-mediated AgNPs.[14] The *PDI* is a dimensionless metric that indicates the uniformity of the particle size distribution in a sample. It is calculated based on the width of the size distribution curve. A lower *PDI* value approaching zero indicates that the nanoparticle sample is monodispersed, suggesting that the particles have comparable sizes, which is essential for the biological activity of extract-mediated AgNPs.[14] The *PDI* of the synthesized AgNP formulations **F3** and **F4** was measured at 0.102±0.0003, and 0.182±0.0002 respectively, suggesting a uniform distribution of the small-sized AgNPs. ZP refers to the electric potential at the particle surface, which is an indicator of the stability of nanoparticle dispersions. A high zeta potential indicates strong electrostatic repulsion between nanoparticles, which prevents aggregation and leads to better stability in suspension. The AgNP formulations **F3** and **F4** showed *ZP* at –20.65±0.897 and –5.657±1.023 mV (Fig. 5), hence may be considered to have appreciable stability in colloidal systems. Based on its smaller average particle size, lower *PDI*, and higher negative zeta potential, the AgNP formulation **F3** is deemed the optimal nanoformulation produced through a green synthesis method utilizing *G. latifolia* bark extract.

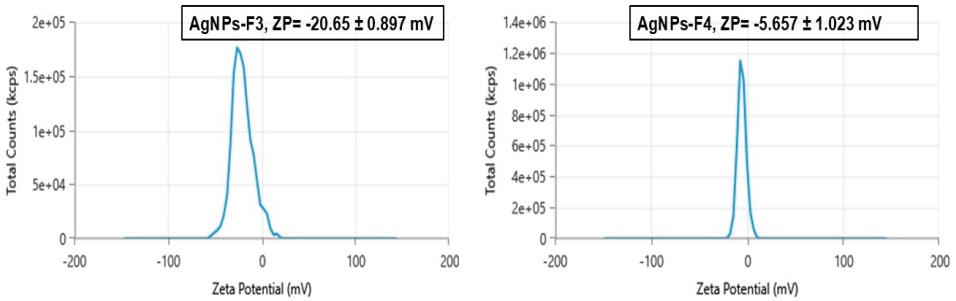

Fig. 5. Zeta potential of AgNP formulations **F3** and **F4**.

Antibacterial activity of extract, fractions and AgNPs

The crude methanolic extract of the bark of *G. latifolia* did not show any inhibitory effect on the *S. aureus* MTCC-96, and the MRSA clinical isolate (*MIC*

> 1000 μg/mL). Further, the medium polar chloroform fraction showed nominal activity (*MIC* = 1000 μg/mL) against *S. aureus* MTCC-96 while the non-polar hexane fraction and the polar n-butanol fraction were found inactive against both the *S. aureus strains* (*MIC* > 1000 μg/mL). In a combinatorial investigation, a concentration of 10 μg/mL of extracts and fractions was applied to evaluate a reduction in the *MIC* of EtBr against the MRSA clinical isolate. The crude methanolic extract and hexane and chloroform fractions reduced the *MIC* of EtBr to half (Table II), showing a synergistic effect with EtBr. The results suggest that the extract of the bark of *G. latifolia* may find its use in overcoming the drug resistance caused by *S. aureus* by inhibiting bacterial efflux pumps.

TABLE II. *In vitro* antibacterial activity of *G. latifolia* extract and fractions; Extr.: crude alcoholic extract, Fract.: fractions of crude extract, GL: crude alcoholic extract of *G. latifolia* bark, GLH: hexane fraction, GLC: chloroform fraction, GLB: *n*-butanol fraction, EtBr: ethidium bromide, Cipro.: ciprofloxacin

Extr./Fract.	*MIC* / μg mL^{-1} against *S. aureus* strains		
	MTCC-96	MRSA	EtBr against MRSA in combination with 10 μg/mL Extr./Fract.
GL	>1000	>1000	3.90
GLH	>1000	>1000	3.90
GLC	1000	>1000	3.90
GLB	>1000	>1000	15.62
EtBr	–	7.81	–
Cipro.	0.19	7.80	–

To evaluate the antibacterial effectiveness of the synthesized AgNP formulations, two strains of each gram-positive MRSA clinical isolate and *S. epidermidis*, as well as gram-negative *E. coli* and *K. pneumoniae*, were utilized under *in vitro* conditions. The measured *MIC* values varied from 30.00 to 2.81 μg/mL (Table III). The AgNP formulation **F3** exhibited demonstrated *MIC* values of 11.25, 2.81, 11.25 and 11.25 μg/mL, while the formulation **F1** demonstrated a *MIC* of 15.00, 3.75, 15.00 and 15.00 μg/mL against the MRSA, *S. epidermidis*, *E. coli* and *K. pneumoniae*, respectively. Among all formulations, AgNP formulation **F3** exhibited the strongest antibacterial activity against all the tested bacterial strains.

Concerning the positive control ciprofloxacin (*MIC* = 7.80 μg/mL), the *MIC* of AgNP–**F3** was measured at 11.25 μg/mL, which was the highest among all the AgNP formulations. The antibacterial effectiveness of AgNP–**F3** was observed to be over 11 times greater against the gram-positive *S. epidermidis* (*MIC* = 2.81 μg/mL) and more than 5 times greater against the gram-negative *E. coli* (*MIC* = 11.25 μg/mL) in comparison to ciprofloxacin, which exhibited *MIC* values of 31.25 and 62.5 μg/mL, respectively, for both bacterial strains. The antibacterial efficacy of AgNP–**F3** against the gram-negative bacterium *K. pneumoniae* (*MIC*

= 11.25 μg/mL) was significantly lower than that of ciprofloxacin (*MIC* = 0.97 μg/mL), yet it was the most potent among all AgNP formulations. The remarkable antibacterial effectiveness of the AgNP formulation **F3** (synthesized by reacting 3.5 mL of 3 mM aqueous $AgNO_3$ with 25 μL of 1 % plant extract) can be linked to the higher concentration of homogenous extract-stabilized AgNPs without aggregation in atom clusters. Despite the silver ion concentration being consistent, increasing the amount of extract (as in the case of **F2** and **F4**), plausibly due to a higher concentration of secondary metabolites, aggregation of silver atoms in the cluster has taken place, which is evident in HR-TEM analysis. It is evident that with an increase in size, the nano characteristic diminishes, leading to a decrease in antibacterial activity.

TABLE III. *In vitro* antibacterial activity of *G. latifolia* bio-fabricated AgNPs

AgNP Formulation	*MIC* / μg mL^{-1} against:			
	MRSA	*S. epidermidis*	*E. coli*	*K. pneumoniae*
F1	15.00	3.75	15.00	15.00
F2	30.00	7.50	15.00	30.00
F3	11.25	2.81	11.25	11.25
F4	22.50	5.63	22.50	22.50
$AgNO_3$	15.00	7.50	30.00	30.00
Ciprofloxacin	7.80	31.25	62.50	0.97

The antibacterial effect of AgNPs can occasionally surpass those of traditional antibiotics like penicillin, especially for those dealing with the resistance shown by various microbial strains to these antibiotics.[46,47] AgNPs demonstrate promising antibacterial properties through a distinctive mechanism that simultaneously affects multiple targets, leading to the death of various bacterial strains.[45,48] Due to their composition of 20–15,000 silver atoms and typically having a diameter of less than 100 nm, AgNPs possess a significant surface area that enables them to interact with proteins on bacterial surfaces and compromise the integrity of the cell wall.[49] Although the mechanism of action of AgNPs still needs to be explored, numerous studies suggest that the exceptional antibacterial properties of AgNPs can be linked to their extremely small size and increased surface area, allowing them to effectively disrupt membranes, enter microbial cells, and subsequently transform into silver ions within the cytoplasm, leading to damage to the internal structures as a secondary effect.[49] The production of free radicals by AgNPs could be regarded as an additional method for the elimination of bacterial cells. These free radicals harm the bacterial cell membrane, causing it to become porous, which ultimately results in cell death.[50,51] It has been noted that the engagement of AgNPs with the sulfur and phosphorus present in bacterial DNA can cause problems in DNA replication, ultimately leading to the termination of the microbes.[50,51] Moreover, the phytochemicals of the plant extract

as the capping ligand in the biofabricated AgNPs might be greatly enhancing the biological activity by interacting synergistically with AgNPs.

CONCLUSION

A rapid and environment-friendly protocol for the bio-fabrication of silver nanoparticles has been developed using *G. latifolia* bark extract, providing a superior alternative to traditional chemical and physical methods. The formation of AgNPs was monitored using FT-IR and Raman spectroscopy. The final characterization of the AgNPs was performed through HR-TEM, SAED, and DLS analysis, which confirmed that the extract-stabilized AgNPs were optimally sized, with a smooth surface and uniform distribution. However, the plant bark extract and fractions exhibited only moderate drug-resistance reversal potential against MRSA, the synthesized AgNPs displayed noteworthy antibacterial properties when tested *in vitro* against both gram-positive and gram-negative bacterial strains. Among all the formulations tested, AgNP–**F3** demonstrated the most favorable characteristics for extract-encapsulated AgNPs and displayed the highest antibacterial efficacy against both the gram-positive methicillin-resistant *S. aureus* clinical isolate and *S. epidermidis*, as well as the gram-negative strains *E. coli* and *K. pneumoniae*. The antibacterial potency of AgNP–**F3** was found to be more than 11 times superior against the gram-positive *S. epidermidis* and over 5 times more effective against the gram-negative *E. coli* when compared to the antibiotic ciprofloxacin. We recommend conducting further *in vivo* investigations of AgNP formulation **F3** for its development as a broad-spectrum antibacterial agent.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/13282, or from the corresponding author on request.

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ИЗВОД

ЗЕЛЕНА СИНТЕЗА НАНОЧЕСТИЦА СРЕБРА ПРИМЕНОМ ЕКСТРАКТА *Gardenia latifolia*, ЊИХОВА КАРАКТЕРИЗАЦИЈА И *IN VITRO* АНТИБАКТЕРИЈСКА АКТИВНОСТ

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Биогене форме наночестица сребра (AgNP) су синтетисане применом алкохолног екстракта коре *Gardenia latifolia* као редукујућег агенса и лиганда за јоне сребра из воденог раствора сребро нитрата. Синтеза честица AgNP праћена је променом боје и појавом специфичних максимума у FT-IR и Raman спектрима. Методом HRTEM утврђено је да су све AgNP формулације имале кристалну, сферну структуру са глатком површином, то је даље потврђено честицама мале величине (< 50 nm), ниским индексом полидисперзије (*PDI*) и негативним цета потенцијалом. Биљни сирови екстракт и његове фракције нису испољавали значајну активност спрам *Staphylococcus aureus* MTCC-96 и метицилин резистентном клиничком изолату *S. aureus* (MRSA), али у комбинованој студији, концентрација од 10 μg/mL је преполовила *MIC* етидијум-бромида спрам MRSA, указујући на способност *G. latifolia* екстраката и фракција да резистенцију на лек промене у активност. У *in vitro* одређивању активности AgNP формулација спрам MRSA, *Staphylococcus epidermidis*, *Escherichia coli* и *Klebsiella pneumoniae*, добијене су *MIC* вредности у опсегу од 30,00 до 2,81 μg/mL. Формулација AgNP–**F3** исказује потенцијал да се користи у развоју ефикасних антибактеријских лекова широког спектра дејства.

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