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Clerodendrum japonicum (*Thunb.*) Sweet leaf extract supported nanosilver particles: characterization, antioxidant and antibacterial activity

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Abstract. Silver nanoparticles have been synthesized in numerous ways, due to their diverse applications, including green procedures that use an extract of plants for the reduction of metal ions. This study delineates the green synthesis and characterization of silver nanoparticles (AgNPs) from the leaf extract of *Clerodendrum japonicum (Thunb.) Sweet* (CJ) and assesses the antioxidant as well as antibacterial characteristics. The biosynthesis of AgNPs was accomplished by reacting the aqueous leaf extract of the plant with a solution of silver nitrate ($AgNO_3$). The creation of AgNPs was indicated by the visual color change of the reaction concoction from golden to deep brown and the absorption peak at 442 nm in ultraviolet-visible spectroscopy. The Field Emission Scanning Electron Microscope (FESEM) and the High-Resolution Transmission Electron Microscope (HRTEM) images have revealed the generated AgNPs as spherical and oblate in shape which is 20 - 40 nm in size, whereas X-ray diffraction evaluation revealed the crystalline feature. The synthesized AgNPs showed free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with IC_{50} value, 7.02 ± 1 µg/mL. The antibacterial assay showed an effective activity towards *E. coli* and *S. aureus* by developing a well-defined zone of inhibition. The results of this study accentuate the biomedical potential of the above-mentioned plant, though further research is needed to implementing it in clinical practice.

Keywords: green synthesis, silver nanoparticles, nanotherapeutics

Classification numbers: 1.5.3, 2.7.1, 2.7.2

1. INTRODUCTION

Nanoparticles (NPs) can be synthesized by different procedures, namely, chemical, physical as well as biological [1, 2]. The synthesis of nanoparticles using a biological approach from herbal extracts and/or microorganisms has evolved as an alternative method, with significant advantages over the chemical and physical synthesis processes [3]. It is advantageous to utilize medicinally important plants because, during the biosynthetic process, the medicinal properties of the plants also get added to the nanoparticles. Plant phytocompounds like flavonoids, terpenoids, proteins, alkaloids, phenols, etc. play an important function in capping as well as reduction of biologically synthesized nanoparticles and thus providing extra antioxidant and antibacterial capabilities [4]. Toxic substances bind to the surface of chemically produced nanoparticles, making them unsuitable for medicinal use.

The promising characteristics (physicochemical and biological attributes) of nanomaterials have impacted various fields of health sciences such as regenerative medicine; gene, protein, and drug delivery; diagnosis; bio-imaging, etc.[5, 6]. Nanoparticles (NPs), particularly metallic nanoparticles (MNPs), have recently been the subject of research into their design and development through green synthesis as a potential treatment approach for various pathological conditions, including diabetic wounds [7]. Furthermore, green technology-based NPs outperform physical and chemical methods in various ways, including limited use of expensive chemicals, less energy, and the production of eco-friendly products and byproducts [8]. Green synthesis is a bottom-up approach in which costly and toxic chemical agents or solvents are replaced by naturally accessible materials such as whole plants, plant tissue, fruits, plant extract, bacteria, marine algae, and fungi to produce MNPs or metal oxide NPs [7, 9].

Among various MNPs or metal oxide NPs, silver has been widely used as a healing and antibacterial agent for many years because silver NPs (AgNPs), compounds based on silver, are significantly less expensive compared with gold-based compounds [10,11]. The emergence of antibiotic resistance in bacteria, particularly multidrug resistance, prompted scientists to look for new ways to combat multidrug-resistant bacteria. Synthesized AgNPs have bactericidal activity without affecting human cells, making them a good antibiotic substitute [12]. AgNPs' unique features have prompted researchers to investigate antimicrobial, targeted drug delivery, and various other applications in nanomedicine [13]. The redox interaction of bioactive phytomolecules (such as flavonoids, polyphenols, terpenoids, alkaloids, and sugars) with silver cations (Ag^{+}) in the plant extract is the basis for the production of AgNPs utilizing a reducing agent from plant extract. The Ag^{+} can be changed into silver atoms by functional groups in phytochemicals, which have a lower redox potential than Ag^{+}. These atoms then come together to form AgNPs. Newly formed AgNPs have surfaces on which biological phytochemicals are adsorbed, encapsulating them to prevent agglomeration and stabilize the colloidal solution. Thus, the synthesis of AgNPs using plant extract does not utilize any toxic chemicals, and the application of AgNPs colloidal solutions is becoming more promising in herbal drug delivery [14].

Clerodendrum japonicum (Thunb.) Sweet (CJ) is an ornamental plant belonging to the family Verbenaceae, which is widely distributed over South and East Asia, and the entire plant can be used for medicinal purposes [15, 16]. The phytochemical studies reveal the presence of triterpenoids (ursolic acid), flavonoids (tricin), phenylethanoids (martynosides, mono acetyl martinoside, clerodenoside A), and steroids (22,23 dihydrostigmasterol) in CJ leaf extracts [17]. Although there is no study that reports the discovery of fabricating AgNPs using CJ. Therefore, we have investigated the role of CJ aqueous extract in reducing Ag^{+} to Ag^{0} using spectroscopic and microscopic analysis, with an emphasis on antioxidant and antibacterial activity. The schematic representation of AgNPs extracted from plants is displayed in Fig. 1.

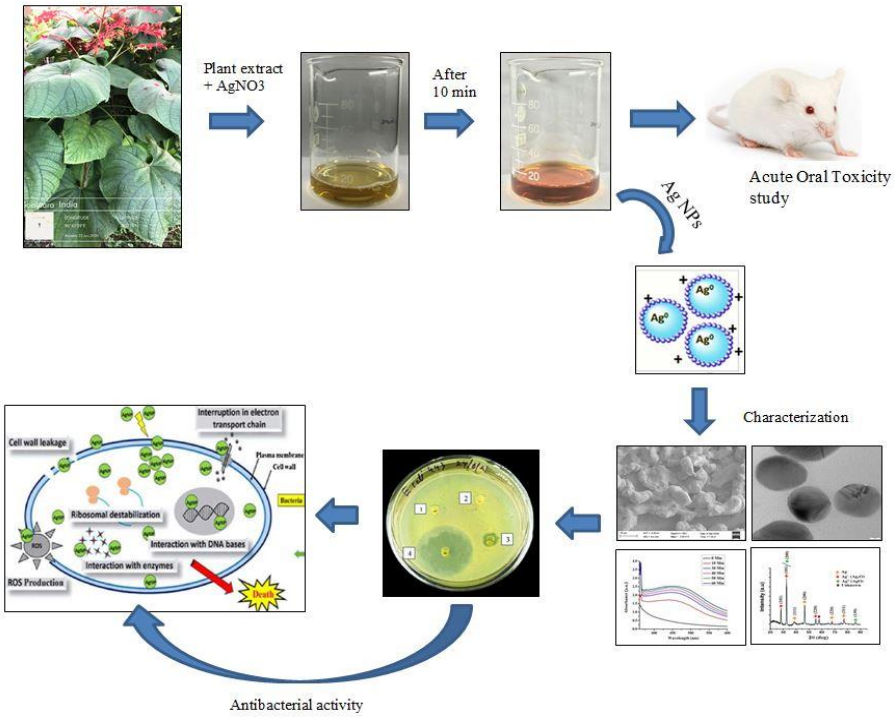

Figure 1. Schematic representation of Ag NPs extracted from plants.

2. MATERIALS AND METHODS

2.1. Materials

The chemicals used in the present study, such as $AgNO_3$ ($AgNO_3$, 99.9 % pure), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and ascorbic acid (AA) were procured from Sigma-Aldrich Chemicals (St Louis, MO, USA) and Merck Limited (Mumbai India). *E. coli* (MTCC 443) and *S. aureus* (MTCC 7443) strains were procured from the Institute of Microbial Technology, Chandigarh, India. The solvents and chemicals used throughout this research were of analytical grade.

2.2. Preparation of leaf extract

For the preparation of the aqueous leaf extract, fresh CJ leaves were collected from different areas of the Goalpara district of Assam (Lat N 26^o 10’ 24.7872 and long E 90^o 37’ 26.2848) and dried in the shade. The shade-dried leaves were ground to powder using mortar and pestle. A total of 0.1 g of powdered sample was mixed with 10 mL of distilled water. It was followed by boiling for 5 minutes and then subsequently cooled down to room temperature. It is then filtered with Whatman's No.1 filter paper.

2.3. Biosynthesis of AgNPs

The green synthesis of AgNPs was accomplished by reacting 10 mL of CJ aqueous leaf extracts with $AgNO_3$ according to the procedure described by Sahoo *et al.* [11]. Briefly, a freshly

prepared 1 mM aqueous solution of $AgNO_3$ was mixed with aqueous leaves extract of CJ at the ratio of 9:1 at room temperature (29 ± 3 ºC). While the reaction mixture was stirred continuously at room temperature, the color change was observed by ultraviolet-visible spectrophotometry (UV-Vis Spectroscopy). The color of the reaction mixture was noticed to change gradually from colorless to reddish brown. After a 10-minute incubation period, a persistent reddish-brown tint was observed (Fig. 2). The color shift indicated that the silver (Ag^+) had been reduced to silver nanoparticles (Ag^0).

2.4. Characterization of the AgNPs

The biosynthesized AgNPs were analyzed by using a UV-Vis spectrophotometer (Shimadzu UV-1800). Structural as well as crystallographic information of the synthesized silver nanostructured system was revealed using an Equinox 3000 diffractometer employing Cu-Kα radiation in the range of diffraction angle (2θ) between 20^0 to 90^0. The morphological details of the synthesized AgNPs were revealed using field-emission scanning electron microscopy or FESEM [SEM-SIGMA-VP (ZEISS)] as well as high resonance transmission electron microscope or HRTEM (TEM-2100 PLUS Electron Microscope). To find out the functional groups that are responsible for the reduction of silver ions, FTIR spectrum analyses were performed on pellets of synthesized AgNPs with potassium bromide (1:100) using a PerkinElmer FT-IR Spectrometer [7].

2.5. Antibacterial activities of green synthesized AgNPs

The antibacterial activities of biosynthesized AgNPs were studied against one gram-positive bacteria and one gram-negative bacteria performed according to a slight modification of Hazarikaa and co-workers' methods [18]. The bacterial strains were spread on 19 mm of Petri plates containing autoclaved Muller Hinton agar with the help of sterile cotton. Then biosynthesized AgNPs were placed on media along with aqueous extract and $AgNO_3$. Petri plates were incubated at 37 ºC and recorded the zone of inhibition after 24 hours. After 24 hours of incubation, antimycobacterial activity was recorded by measuring the diameter of the zone of inhibition using a transparent ruler under the colony counter. The test was performed in triplicate to minimize the errors and the average zones of inhibition (mm) were recorded.

2.6. DPPH free radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a well-known procedure for the determination of the antioxidant capacities of plant products. In this assay, five varied concentrations (20, 40, 60, 80, and 100 µg/mL) of the synthesized nanoparticles were reacted with 100 µL of 0.2 mM DPPH solution (3.9 mg DPPH powder dissolved in 50 mL of methanol). The reaction mixture was incubated at room temperature in a dark place for 30 minutes. Finally, absorbance was recorded at 517 nm keeping ascorbic acid as a standard. To estimate the percent inhibition of aqueous leaf extract using the following formula [15]

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control- Absorbance of plant extract}}{\text{Absorbance of control}} \times 100$$

3. RESULTS AND DISCUSSION

3.1. Color change of solution

The obtained leaf specimens from the wild condition for this investigation, and leaves were used to assess their capacity for reducing the silver ions. Initially, the aqueous extract of CJ

leaves was a golden tint; however, with the addition of $AgNO_3$ solution and stirring at room temperature, the color gradually converted to deep brown, indicating that Ag^+ ions were reduced to Ag^0, as shown in Fig. 2

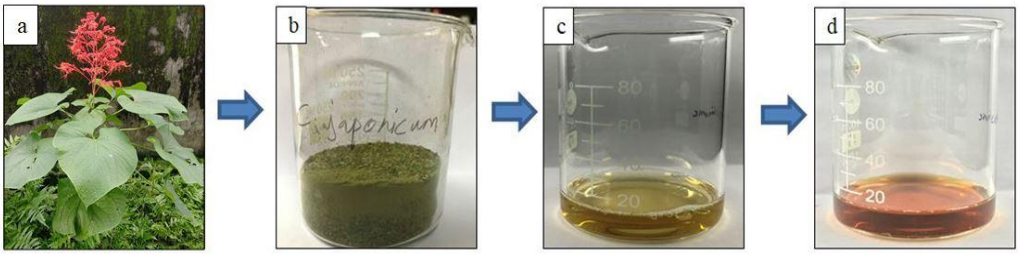

Figure 2. Color change in the Plant extract after adding $AgNO_3$ solution. a) *C. japonicum*, b) *C. japonicum* powder, c) mixture of plant extract with $AgNO_3$; d) 10 minutes after the reaction.

3.2. UV–Vis analysis

The UV-Vis spectrogram of the biogenic AgNPs is presented in Fig. 3. The UV–Visible absorption spectra of the biosynthesized AgNPs prepared at room temperature were recorded at regular intervals of time, viz., at 0, 10, 30, 40, 50, and 60 minutes. When the absorption study was done, no absorption peak was observed on the sample instantaneously after mixing.

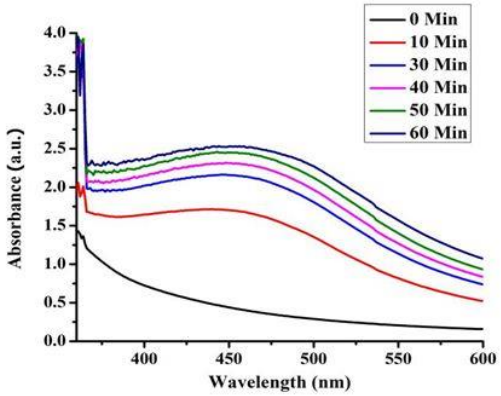

Figure 3. UV-Vis absorption spectra of synthesized AgNPs.

3.3. Structural and crystallographic studies

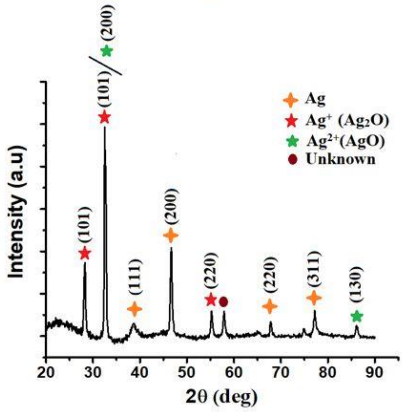

Figure 4. X-ray diffraction profile from synthesized AgNPs using *C. japonicum* extract.

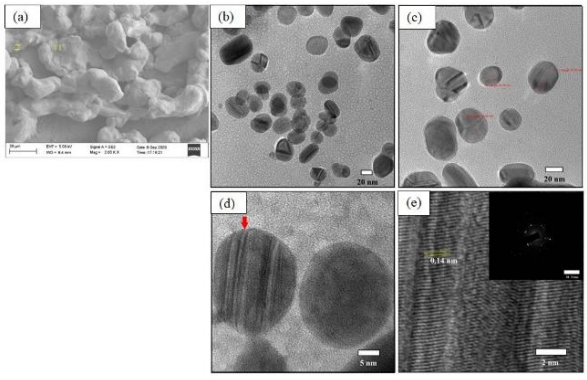

Figure 5. (a) FESEM, (b, c, d) HRTEM images of synthesized AgNPs of different magnifications, (e) SAED.

The X-ray diffraction (XRD) pattern of the synthesized silver nanostructure system is shown in Figure 4. There are eight distinct peaks that match well with those of metallic silver. However, the diffractogram showed one obscure peak.

3.4. Morphological study using FESEM and HRTEM

FESEM and HRTEM were used to examine the morphology of the biogenic AgNPs. As observed from FESEM Fig. 5 (a), it is evident that the AgNPs tend to agglomerate in clusters that are formed by large aggregates of nanoparticles of spherical shape with a diameter of hundreds of nanometers to a few micrometers. No nanoparticles of smaller size could be perceived by FESEM; hence we took the help of HRTEM for an in-depth study. As evident from the HRTEM images, most of the particles are spherical; however, a few particles of oblate shape were also detected, as portrayed in Fig. 5 (b). The nanoparticles were found to be ~20 - 40 nm in size (Fig. 5 (c)).

3.5. FTIR spectroscopy analysis

The FTIR spectrogram of biogenic AgNPs is shown in Fig. 6. Besides the interaction among the molecules, FTIR can be used to study the infrared (IR) radiation of the electromagnetic spectrum (IR = 4000 - 400 cm^{-1}). The FTIR spectrum reveals the possible functional groups of phytocompound responsible for the bio-reduction of silver ions.

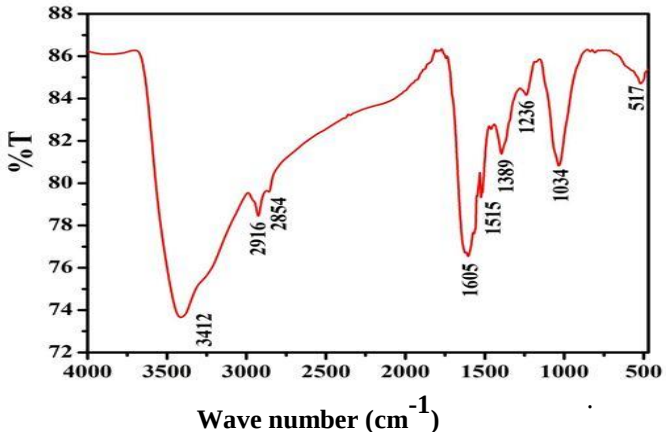

Figure 6. FTIR spectra of biosynthesized Ag NPs by CJ extract

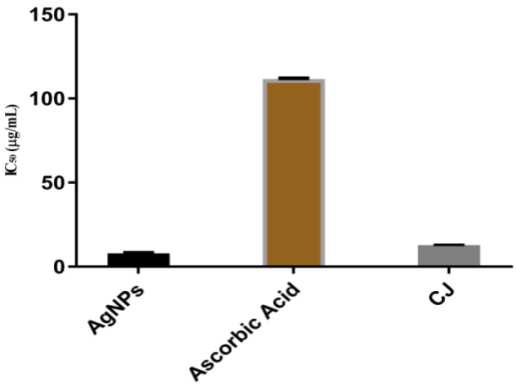

Figure 7. IC_{50} value of AgNPs for DPPH scavenging assay.

The DPPH free radical scavenging assay with an IC_{50} 7.02 ± 1 µg/mL revealed that the biologically fabricated AgNPs have remarkable antioxidant characteristics (Fig. 7). The ascorbic acid was used as a standard.

3.7. Antibacterial activities of the AgNPs

As Figure 8 depicts, the biogenic AgNPs have significant antibacterial properties. The plant extract, distilled water, and $AgNO_3$ were used as a control. After 48 hours of inoculation, the

zone of inhibition in *E. coli* and *S. aureus* was measured to be 36 mm and 25 mm in diameter, respectively.

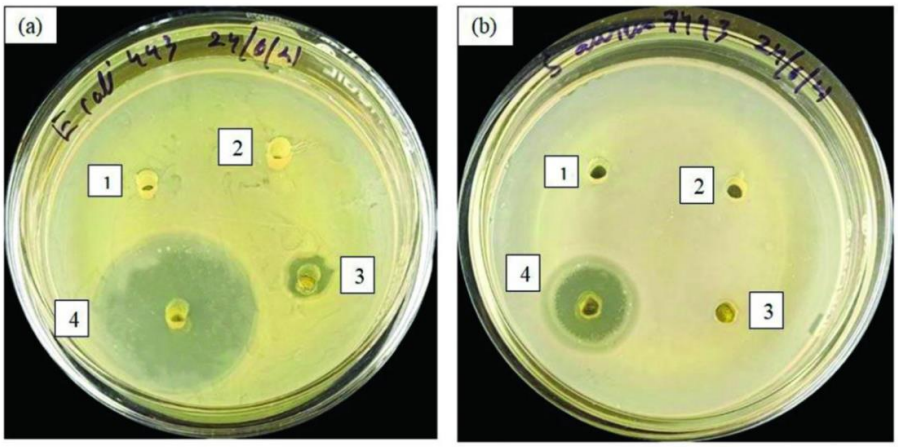

Figure 8. Antibacterial activity: (1) distilled water, (2) $AgNO_3$, and (3) CJ extract, (4) biosynthesized AgNPs from CJ extract, against (a) *S. aureus*, (b) *E. coli.*

Among the procedures used for the synthesis of nanoparticles, the biosynthetic mode is considered safe as well as eco-friendly. In the present study, the initial golden color of the aqueous extract of CJ leaves was gradually changed to deep brown after adding $AgNO_3$ solution with stirring at room temperature. This color change indicates that Ag^{2+} ions were reduced to Ag^0. The intensity of color increases with the increase in incubation time, indicating silver ions are being reduced and AgNPs are being formed. The primary and noble evidence for the creation of AgNPs is the color shift in the solution caused by $AgNO_3$ surface plasmon excitation.

In the UV-Vis analysis, instantly after mixing, there was no apparent peak on the sample, which suggests that a few times is required for the nucleation to start and for the formation of AgNPs. The appearance of a peak in the wavelength range of 400 - 500 nm after 10 minutes of incubation time (Fig. 3) suggests a rapid formation of AgNPs. It is noticeable that the intensity of the absorbance pattern increases with increasing time of incubation and is maximum at 60 minutes. The distinctive surface plasmon resonance absorption band of silver is accountable for this absorption profile and thus indicates the formation of AgNPs. As reported by Mie theory, the spherical shape nanoparticle shows only a single surface plasmon resonance band. The peak amplifies with the increasing heterogeneity of particle shapes. Thus, it can be inferred that the bio-fabricated AgNPs are spherical [19]. However, the plasmon band becomes wide when different metabolites of plant extract are added to the $AgNO_3$ solution since they can also be perceived in this spectrophotometric range [20].

The observed XRD pattern suggests the formation of AgNPs along with AgO and Ag_2O nanoparticle systems. The XRD peaks observed at 2θ values ~38.6, 46.6, 67.8, and 77.21 deg could be ascribed to (111), (200), (220) and (311) planes matching the face-centered cubic (fcc) crystal of AgNPs, respectively as shown in the results. The fcc lattice is responsible for peak formation [20]. The XRD peaks observed at 2θ values ~ 55.1 and 86.5 deg pertained to the planes of (220) and (130) of the AgO nanoparticles, whereas the XRD peak observed at 2θ value ~28.2 can be attributed to (110) crystalline planes of the Ag_2O nanoparticles [21]. Noticeably the most intense XRD peak observed at 2θ ~32.4 deg may correspond to (200) crystalline planes of AgO nanoparticle or (101) crystalline planes of Ag_2O nanoparticle system or maybe superimposed of both [22]. Another XRD peak at 2θ value ~ 57.89 deg could not be distinctly

identified and may be attributed to the remnants of the plant extract's organic content [23]. Peaks that are narrow and intense are the key sign of the crystalline nature of the observed substance [24]. The observed XRD pattern heralds the fabrication of AgNPs and their crystalline nature.

FESEM was used to characterize the surface morphology of the biosynthesized nanoparticles at multiple resolutions. The FESEM images revealed spherical shapes with diameters ranging from hundreds of nanometers to a few micrometers. Furthermore, the size and shape of the biologically synthesized AgNPs were evaluated using HRTEM imaging, which showed most of the particles to be spherical in shape with a few oblates. At a higher magnification of a single particle, the crystal lattice fringes can easily distinguish with inter-planar pacing of ~0.14 nm. The selected area electron diffraction (SAED) pattern of the extract, shown in the results, is characterized by diffused diffraction rings along with scattered bright spots. It indicates that the extract is primarily polycrystalline, along with the presence of a low amount of mono-crystalline phase.

The bio-fabrication of plant-mediated AgNPs can be determined by employing FTIR spectroscopy (Fig. 6). An IR spectrum can be separated into two approximate sections, viz, the functional group section (~4000 - 1000 cm^{-1} range) and the fingerprint section (<1000 cm^{-1} region). It was observed by researchers that numerous functional groups of organic molecules absorb IR radiation at around the same wavelength region, despite the structural organization of the remaining molecules [25]. In our sample, we got a number of stretching bands in the functional group regions. The stretching band at ~3412 cm^{-1} pertains to the existence of alcohols and phenols with a free OH group. This band is superimposed by the stretching peak of the NH group [33]. The bands at 2916 and 2854 cm^{-1} represent the C-H stretching bands. Other vibrations at ~ 1605 and 1515 cm^{-1} may be attributed to different functional groups such as C-O stretching and C=C aromatic ring. The peaks 1389, 1236, and 1034 cm^{-1} might be assigned to C-H stretching in alkanes or alkyl group, C-O stretching in phenol, ether, or ester, as well as C-N stretching of an aliphatic amine, respectively [26]. Furthermore, the weak peak at ~517 cm^{-1} in the fingerprint region can be assigned to C-H groups [27]. Thus, we can conclude that any of the biomolecules containing these bonds can function as a reducing or capping agent in the process of fabrication of AgNPs.

Albeit the actual mechanism of the biological process of synthesis of nanoparticles using plant extract is not clear, the involvement of plant secondary metabolites in the biosynthetic avenue is incontestable. The occurrence of flavonoids and carsonic acid containing $RCOO^{-}$ (carboxylate group) has been observed in the aqueous leaf extract of the CJ plant. The reduction of $AgNO_3$ is caused by the interaction between these metabolites and Ag ions which leads to the synthesis of AgNPs. The presence of negatively charged groups like $RCOO^{-}$ or polar groups like CO, OH, etc. in plant extract has a higher inclination for adherence to the plane of silver ions. Hence, such groups can assist in both stabilization as well as reduction of silver ions. It is conceivable that the presence of the groups' CO, OH, especially $RCOO^{-}$, like flavonoids, carsonic acid, and proteins in plant metabolites, has a crucial role in the mechanism of reduction of Ag ions.

Many experimental studies have reported that the green synthesized AgNPs can be used as a significantly free radical scavenging activity. For example, Priya and co-workers [28] reported that the *in-vitro* antioxidant property of biosynthesized AgNPs from *Pongamia pinnata* leaf extracts has a significant free radical scavenging function. Recently, Mahanta and co-workers [29] have also reported that AgNPs from *Erythrina suberosa (Roxb.)* leaf extract has potential antioxidant, antimicrobial, and cytotoxic activity. The results from our study suggest that AgNPs

may have antioxidant properties that might help to protect against various oxidative stresses related to degenerative diseases. Nonetheless, further evaluation of AgNPs as an antioxidant is required before being used in human as well as *in vivo* models. Most metallic nanoparticles possess considerable antimicrobial activity against bacteria, fungi, and viruses [30]. In the present study, both the gram-positive and gram-negative bacteria were assessed for antibacterial susceptibility. From our results, it was observed that gram-positive bacteria are more vulnerable to AgNPs than gram-negative ones. Eventually, several reports have proven that the synthesized AgNPs are bactericidal in nature. This potential has made a multifarious approach to synthesized AgNPs in the manifestation of bacteria. The precise mechanism behind the bactericidal activity is when the AgNPs are attached to the cell membranes generating free radicals. Over and above that, AgNPs have been found in the cell membranes of bacteria in the previous investigation too. By penetrating the cell membranes, AgNPs disrupt the permeability of the membrane, causing leakage of intracellular ATP and death of cells [31]. Ag ions are released from AgNPs and serve as reservoirs, leading AgNPs to have antibacterial action. The positively charged ions, for instance, Ag+ in this study, appear to have a great affinity for phosphorus and sulfur, which are abundant in macromolecules like DNA and RNA. As a result of such association, the functions of DNA and RNA are affected [32].

4. CONCLUSIONS

Because of the low cost, less harmful byproducts, congenial procedures, and the fabrication of metallic nanoparticles by biological method, employing plants has garnered more attention. The formation of AgNPs utilizing an aqueous extract of CJ leaf is described as environmentally safe, thrifty as well as non-hazardous in the current study. In addition to the bio-reduction, capping of $AgNO_3$ into AgNPs is caused by bioactive chemicals found in the leaves of the noted plant. The functional groups present on the plane of AgNPs are responsible for the antioxidant properties of those particles. Thus, biogenic AgNPs can be utilized in the production of potent antioxidants for biomedical applications. The AgNPs also possess a high antibacterial effect on the microorganisms investigated, owing to their nano dimension and the occurrence of capping agents. Thus, AgNPs may be used as a replacement for antibiotics in the future since they are affordable, ecologically friendly, and very efficient against bacteria.

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Credit authorship contribution statement. DR, HS & MB have contributed to the designing, literature search and execution of the experimental studies, data acquisition, compilation of the results and manuscript preparation. VG & DB have collected the samples, executed the work, analyzed and interpreted the results. RD, DCB & SR have provided scientific advice and discussions, analyzed the results, reviewed and revised the manuscript. RD & DCB have designed the research and guided the overall work.

Declaration of competing interest. The authors declare that they have no conflict of interest.

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