CHARACTERIZATION AND APPLICATION OF GREEN SYNTHESIZED SILVER NANOPARTICLES DERIVED FROM LEAF AND CALLUS OF *Viola canescens* WALL. ex ROXB.

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Abstract. Green production of nanoparticles using plant extracts is an intriguing field of study that has the potential to serve as an alternative to the hazardous chemical synthesis processes. In this study, silver nanoparticles (AgNPs) were prepared utilizing aqueous extracts of the mature leaves (PAgNPs) and seedling leaf derived callus (CAgNPs) of *Viola canescens* Wall. ex Roxb. Callus growth was obtained on Murashige and Skoog (MS) medium supplemented with 2,4-D (2.0 mg/L) and BAP (0.5 mg/L) was harvested and used for biogenic synthesis of AgNPs. The UV–visible spectra of leaf and callus mediated AgNPs revealed the SPR absorption band at 424.8 nm and 437 nm, respectively. The biochemical interaction and crystalline nature of the AgNPs were evaluated by Fourier transformation infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis. The surface morphology and composition of both the samples were confirmed by HRTEM and EDS analyses. The average particle size as calculated from HR-TEM histogram study of both CAgNPs and PAgNPs was found 9.15 nm and 13.9 nm, respectively. The synthesized AgNPs showed significant antibacterial activity against *Bacillus cereus, Bacillus subtilis* and *Escherichia coli*. The inhibition zone of both gram-negative and gram-positive bacteria reflected the broad spectrum antibacterial properties of AgNPs. Callus mediated AgNPs showed better antibacterial results with 30±0.90 mm inhibition zone. The protocol of antibacterial potential of silver nanoparticles generated from *V. canescens* plant and callus extracts is a very important aspect in technological point of view in having applications in the biomedical field and deserves to be recognized.

Keywords: antimicrobial activity; callus; FTIR; silver nanoparticles; TEM; *Viola canescens*; XRD.

INTRODUCTION

Viola canescens Wall. ex Roxb. (family: Violaceae) commonly known as Himalayan White Violet, is a prostrate, hairy perennial herb found mostly at an altitude of 2000 meters all the way through temperate Himalayan regions of Pakistan, India, Bhutan, and Nepal. It is used to medicate various conditions such as bronchitis, cold, cough, flu, cancer of the throat, respiratory tract problems, eczema, epilepsy, gastric acidity, pyrexia, dysentery, rheumatism etc [29, 31]. Qualitative testing of ethanolic and methanolic extract of this plant revealed the presence of different phytochemicals like alkaloids (violin), quercitrin, methyl salicylate, saponins, phenols, flavonoids, carbohydrates, tannins and triterpenes [9, 38]. Plant also exhibits antimicrobial and antispasmodic activity, antifungal activity, hepatoprotective activity and properties like antioxidant, analgesic, diaphoretic, carminative and aphrodisiac [25, 35, 36].

In recent past, interest in plant parts and plant based products of Ayurveda has led a sharp jam in bulk requirement and resulting in illegal and large scale extraction of medicinal and aromatic plants and this causes the reduction in plant diversity [44, 45]. Owing to its far-ranging use, this plant was over uprooted from wild threatening to its extinction. Various factors like extensive grazing, overexploitation, deforestation, effect of invasive plants, change in environmental conditions, and attack of pathogens are liable for making the conservation status of *V. canescens* as endangered in different regions [20, 29]. Thus, micropropagation is an efficient method for mass multiplication.

The modern biotechnological techniques have resulted in the establishment of economically beneficial green industries. Nanoscience deals with the structural and functional aspects of matter in atomic and molecular scale [24, 47]. In these dimensions, the characteristic physio-biochemical properties of material often change dramatically with high surface to volume ratio. It has dramatically revolutionized the interdisciplinary research by generating importance in various fields of material science, optics, mechanics, electronics, biotechnology, agriculture and the pharmaceuticals industry [1, 17].

Silver is a precious noble metal because of their usefulness in antimicrobial applications, biosensors, cosmetic fibres etc [10]. AgNPs preparation by green synthesis approaches have advantages over conventional methods involving physical and chemical synthesis by being environmental friendly, economical and need of fewer instruments [28]. The advantage of plant and plant derived extracts for green synthesis of AgNPs is that they can be easily available, easily cultured, safe and non-toxic. Moreover, plant extract contains a broad spectrum of metabolites such as enzymes, proteins, amino acids, polysaccharides, vitamins, organic compounds etc. resulting as natural reducing, capping and stabilizing agents to prepare nanoparticles without the use of any hazardous, toxic and expensive chemical substances [2]. Thus, AgNPs have emerged as a potential solution to fight against the diverse pathogenic microorganisms.

Various parts of the *Viola sp.* are used to develop antibacterial medications based on medicinal plant extracts. In nanomedicine, antimicrobial properties of AgNPs are among the most promising materials, now under investigation. Silver nanoparticles has the ability to interact with the cell wall of microorganisms, producing ROS (reactive oxygen species) that eventually kill the cell. As a result, we can hypothesize that combining both, i.e. *V. canescens* extract and AgNPs, can enhance their antibacterial properties.

To date, only a few investigations have been published on the antibacterial activity of callus mediated green synthesized AgNPs [22, 32, 43]. Khajuria et al. [23] also reported the callus mediated synthesis of ZnO nanoparticles (NPs) from *V. canescens* but the production of various other metallic NPs such as silver and, gold using various plant extracts of *V. canescens* are still required to develop an efficient and healthy green route for controlling the various endemic diseases with less adverse effect. So, the present study was undertaken with an objective to synthesize and characterize biogenic AgNPs from leaf and leaf derived callus of *V. canescens*, and to test them for antibacterial activity against *Escherichia coli*, *Bacillus cereus*, and *Bacillus subtilis*.

MATERIALS AND METHODS

Callus induction

Healthy seeds of *Viola canescens* were collected from Morni hills, Panchkula, Haryana, India. Selected seeds were surface sterilized using different concentration of mercuric cloride ($HgCl_2$) and germinated on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L GA_3 [39]. The MS media containing 2,4-D (2.0 mg/L) and BAP (0.5 mg/L) was used for callus initiation from seedling leaf explants of *Viola canescens* (Fig. 1). The MS basal medium consisted of 3% sucrose, 0.8% agar. The pH of the medium was adjusted to 5.8. The cultures were incubated at 25±2 °C with 55–65% relative humidity under a 16-h light/8-h dark cycle at 27 µM $m^{-2}s^{-1}$ PAR light intensity from 6500 K colour temperature white fluorescent tubes. The best *in vitro* regenerated mature callus considered to be rich in primary and secondary metabolites was excised aseptically and used for biogenic synthesis of AgNPs.

Preparation of aqueous extract

Fresh biomass of callus and leaves were collected and dried in hot air oven at 40° C for 48 hr. Five grams of air dried biological material was immersed in 100 mL of deionized water and kept on magnetic stirrer at 60°C for 6 hr in a dark room before being centrifuged at 5500 r/min for 10 min. This aqueous solution was then filtered with the help of Whatman's Filter paper no. 1. The filtrate was stored at 4° C for further experiment.

Biosynthesis of AgNPs

The reaction mixture was prepared by adding 10 mL of the aqueous callus/leaf extract and 90 mL of 1 mM silver nitrate ($AgNO_3$) in a 250 mL flask and incubated in a dark place at room temperature for about 48 h. Visual examination was carried out by observing change in color of the solution. When the plant and callus extracts were heated and kept in the dark, their colors turn rusty brown and dark brown, indicating the production of AgNPs (via reduction reaction i.e., Ag^+ reduced to Ag^0 nanoparticles). Both plant leaf mediated silver nanoparticles (PAgNPs) and callus mediated silver nanoparticles (CAgNPs) were subjected to spectrophotometry analysis.

Ultraviolet-visible (UV-vis) spectra analysis

The synthesis and characterization of AgNPs of leaf and callus extracts were confirmed by using a double beam UV- Visible spectrophotometer (Model- 2202, Systronics, India). The spectra were recorded between 200 and 800 nm after 48 hrs for studying the optical property of AgNPs. After the biosynthesis, the reduced solution containing the AgNPs was separated by centrifugation at 12,000 rpm for 10 min at room temperature. Supernants were discarded and AgNPs were redispersed in distilled water and purified by repeated centrifugation for five times. The pellet obtained was air-dried in hot air oven to evaporate excess liquid; and was used for further characterization.

X-ray Diffraction spectroscopy

The silver nanoparticles derived from different extracts were examined by XRD measurements. The crystalline structure of synthesized AgNPs was investigated. The silver nanoparticles were centrifuged at 12,000 rpm for 20 minutes and the pellets were re-dispersed in ethanol and centrifuged again for 10 minutes to get rid of any unwanted entities. The centrifugation and re-dispersion process in ethanol was carried out three times for better removal of impurities from silver nanoparticles. After purification, the pellets were dried at 60°C in an oven. Afterward, the crystalline metallic Ag was examined by PANalytical X'Pert Pro –PW 3040/60 X-ray Diffractometer (45 kV,

Figure 1. *In vitro* regenerated mature callus from leaf explants of *V. canescens* on MS media aliquoted with 2,4-D (1.5 mg/L) + BAP (0.5 mg/L)

40 mA) with Cu-Kα radiation in scattering range m(2θ) of 30–80°. The particle size of synthesized NPs was calculated using Scherrer's equation, which is as follows: $D = \frac{K\lambda}{\beta \cos\theta}$

where:

D = mean crystal size;
K= 0.94 (Scherrer's constant);
λ = 1.5406 Å (X-ray wavelength);
β = X-ray diffraction broadening, in radians;
θ = Bragg's peak angle (2θ).

High Resolution Transmission Electron Microscopy (HR-TEM) and Energy Dispersive X-ray Spectroscopy (EDX)

The morphology, structure, and composition of silver nanoparticles using high resolution-transmission electron microscopy using HR-TEM (JEM-2100 Plus electron microscope, JEOL Ltd.), with 200 kV accelerating voltage and EDX at Sophisticated Analytical Instrumentation Facility, CIL and UCIM, Punjab University, India.

Dynamic light scattering (DLS) and Zeta Potential analysis

The particle mean size and size distribution of AgNPs were measured at 25°C using Particle Size Analyzer by dynamic light scattering (DLS). The dried AgNPs were dissolved in double distilled water and loaded in quartz curvet for analysis using a particle size analyzer. The standard solutions were first run to known the size distribution, the detectors record the energy scattered, absorbed at particular angle and scattering patterns. Then, the values of samples were compared with these standard values. For this, Microtrac Nanotrac Wave Particle Size, Zeta Potential analyzer was used to evaluate the biologically produced solutions.

Fourier Transform Infrared Spectroscopy Analysis (FTIR)

AgNPs prepared with dried callus extract were subjected to FTIR spectroscopy by fourier transform infra-red spectrophotometer (Perkin Elmer, Model RZX) in the range of 4000-450 cm^{-1}. The solid powder sample of AgNPs was crushed, mixed with potassium bromide (KBr) in the ratio of 2:98 by weight and subjected to hydrolic pressure of about 1.5 bar for few seconds to make a disc. The spectrum was collected with eight scans co-added at 4 cm^{-1}. The abscissa range of the instrument is 4000 to 400 cm^{-1}. The detector was purged carefully using clean nitrogen gas to increase the signal level and reduce moisture. The sample discs were then introduced into the instrument and the spectrum was recorded.

Antibacterial Assay

The antibacterial activity of the synthesized AgNPs was determined by using the agar well diffusion method. Stock cultures of all the gram-positive (*Bacillus cereus* MTCC 430), *B. subtilis* MTCC 441) and gram-negative (*Escherichia coli* MTCC 1885) bacteria were maintained at 4°C and transferred to Mueller-Hinton broth and incubated for 24 h at 37°C for the preparation of fresh active cultures. The bacterial strains were obtained from the Department of Biotechnology, Kurukshetra University, Haryana (India). The inoculum density was maintained at 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL). Freshly prepared solidified Mueller-Hinton agar plates were inoculated by spreading 100 µl of bacterial inoculum and 6 mm diameter wells were made by using sterilized cork borer. A total of 100 µg of samples (leaf extract (PE) and silver nanoparticles) were sonicated in 100 µL of dimethyl sulfoxide (DMSO) and poured in the well. Plates were allowed to settle for 1 hr at room temperature for the diffusion of samples and then incubated for 24 hrs at 37°C [33]. DMSO and Ciprofloxacin were served as negative and positive control respectively.

RESULTS

Synthesis of AgNPs

The synthesis of AgNPs showed change in colour from light brown to dark brown colour in aqueous $AgNO_3$ solution due to excitation of surface plasmon vibrations phenomena in these nanoparticles thereby initially confirming the bioreduction of 1 mM $AgNO_3$ solution. When the combinations were heated and incubated at ambient temperature for 24 hr, the reduction process was complete; no additional color change was detected. Leaf extract had a stronger color intensity than callus extract. In control trails, no brown color was observed in the absence of extracts, thereby showing that change in color is associated with the presence of phyto-extracts.

UV-visible spectrum analysis

The silver metal has free electrons which produce surface plasmon resonance (SPR) absorption band because of the combined vibration of electrons of metal nanoparticles in resonance with light waves. The nanoparticles were ultrasonically dispersed in distilled water for absorbance measurements between 200 nm – 800 nm. The peak of nanoparticles produced by leaf and callus extracts, which were assigned to the SPR of AgNPs, was measured at wavelengths of 424.8 nm and 437 nm, respectively (Fig. 2a,b).

XRD analysis

The XRD pattern of the leaf and callus extracts derived AgNPs showed well defined diffraction peaks. The typical diffraction peaks of leaf extract mediated AgNPs lied at 2θ = 38.07, 43.96, 63.78 and 76.96, while callus mediated AgNPs peaks lied at 8.19, 44.28, 64.52 and 77.46. According to the results of the XRD spectrum, the produced silver nanoparticles were found to be crystalline in nature. The peaks were ascribed to silver face-centered cubic (fcc) and crystalline reflection planes (111), (200), (220) and (311) respectively (JCPDS no. 04-0783). XRD examination revealed the existence of high peaks, indicating the presence of active Ag content, as confirmed by indexing (Fig. 3a,b). The mean diameter of synthesized AgNPs using plant and callus was estimated as 13.67

nm and 10.39 nm, respectively, which corresponded to the nanoparticle size measured by HRTEM analysis (Table 1).

HR-TEM analysis of synthesized AgNPs

The HR-TEM provided additional information regarding the size and morphological characteristics of the silver nanoparticles *viz*, spherical in shape, well dispersed, and there was no evidence of aggregation (Fig. 4a-d). The mean particle size of CAgNPs and PAgNPs, as determined by HR-TEM image obtained, was found to be 9.15 nm and 13.9 nm, respectively. The particle size histograms of PAgNPs and CAgNPs indicated that the maximum NPs are present in the range between 4 nm to 18 nm and 6 nm to 9 nm, respectively (Fig. 4e-f). These results are consistent with the SPR vibrations geometry (Fig. 2).

Energy dispersive X-ray (EDX) analysis of synthesized AgNPs

The elemental composition of AgNPs was detected by EDX analysis [7]. The highest proportional of Ag signals revealed qualitative and quantitative determination of silver (Fig. 5). A few peaks of copper (Cu), carbon and other elements were also found, confirming the presence of *V. canescens* biomolecules on the surface of produced silver nanoparticles. Consequently, the copper peak was used to coat the sample.

DLS and Zeta Potential analysis

The particle size of AgNPs synthesized by leaf extract varied between 150-250 nm (Fig. 6). Plant leaf extract produced silver nanoparticles with mean size of 210 nm. The synthesized particles possess a charge of -39.86 mV. Polydispersity Index (PDI) for AgNPs was below 0.282. The size of AgNPs synthesized by using callus extract was found to vary between 130-200 nm. Mean size of CAgNPs was around 180 nm.

FTIR analysis

FTIR provides molecular fingerprint for the identification organic materials and helps us in the confirmation of numerous functional groups of biomolecules involved in the capping, production, and stability of AgNPs. FT-IR analysis of *V. canescens* leaf extract-AgNPs and callus extract-AgNPs, revealed various bands of absorption spanning from 3460 - 613 cm^{-1}, indicating the existence of functional groups. The absorption spectra of AgNPs produced from leaf extract (Fig. 7a) exhibits peaks at 3458 cm^{-1}, 2062 cm^{-1}, 1634 cm^{-1}, 1390 cm^{-1}, 1111cm^{-1} and 618 cm^{-1}. The peaks of CAgNPs (Fig. 7b) are visible at 3460 cm^{-1}, 2050 cm^{-1}, 1630 cm^{-1}, 1389 cm^{-1}, 1252 cm^{-1}, 1074 cm^{-1} and 613 cm^{-1}.

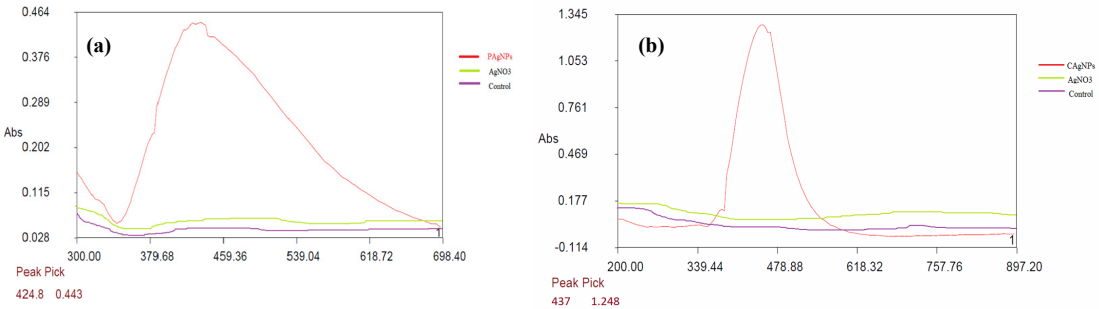

Figure 2. UV-Visible spectral analysis of AgNPs synthesized by using a) leaf extracts (PAgNPs), b) callus extracts (CAgNPs) of *V. canescens*.

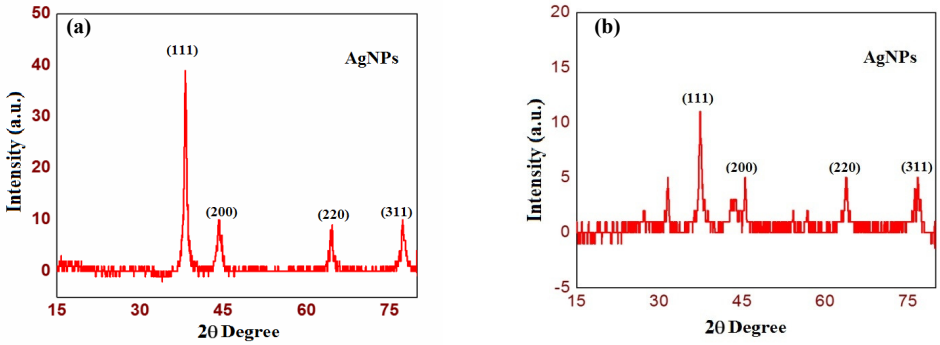

Figure 3. XRD analysis of green synthesized AgNPs using a) Leaf extract, and b) Callus extract.

Table 1. Size of biologically synthesized AgNPs calculated by Debye-Scherrer's equation

PAgNPs			CAgNPs		
Position (2θ)	**FWHM**	**Size (nm)**	**Position (2θ)**	**FWHM**	**Size (nm)**
38.07	0.576	14.56	38.19	0.665	12.64
43.96	1.152	7.42	44.28	1.052	8.15
63.78	0.48	19.49	64.52	0.800	11.74
76.96	0.768	13.21	77.46	1.127	9.03

*FWHM - Full Width at Half Maximum

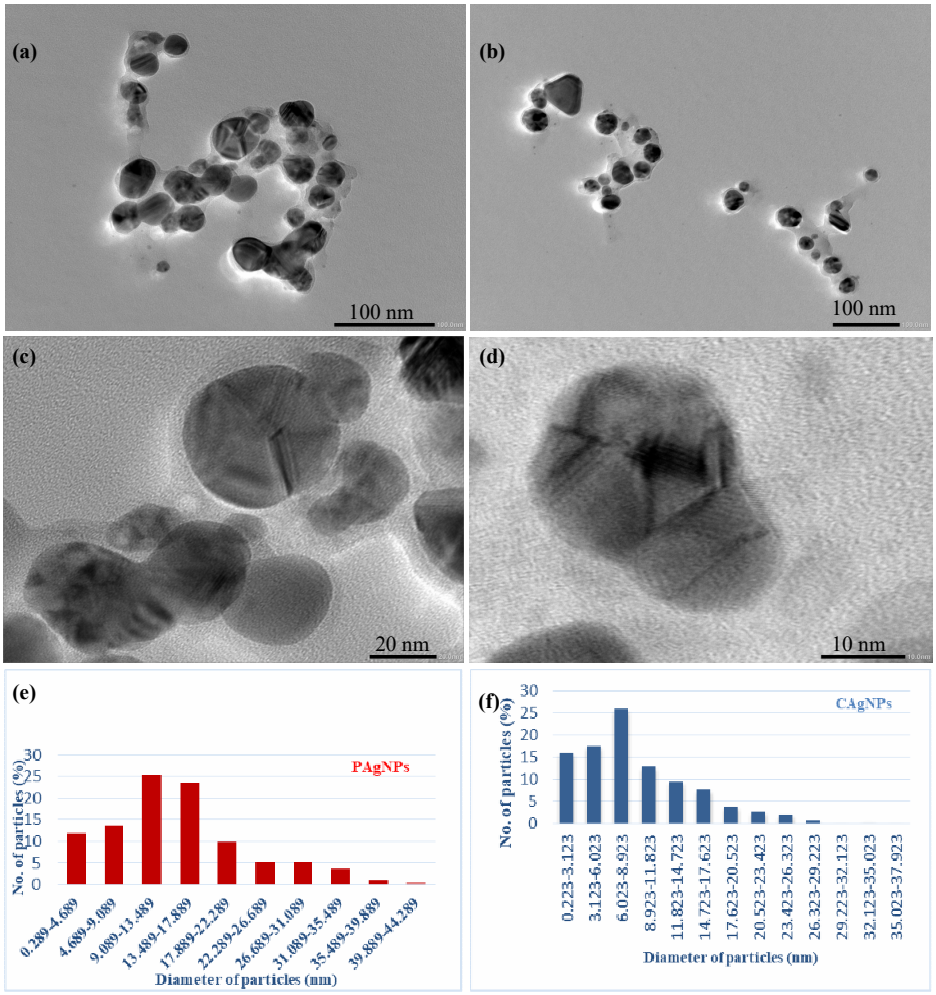

Figure 4. HR-TEM images showing different magnifications of AgNPs (a-d); and histogram of PAgNPs (e) and CAgNPs (f).

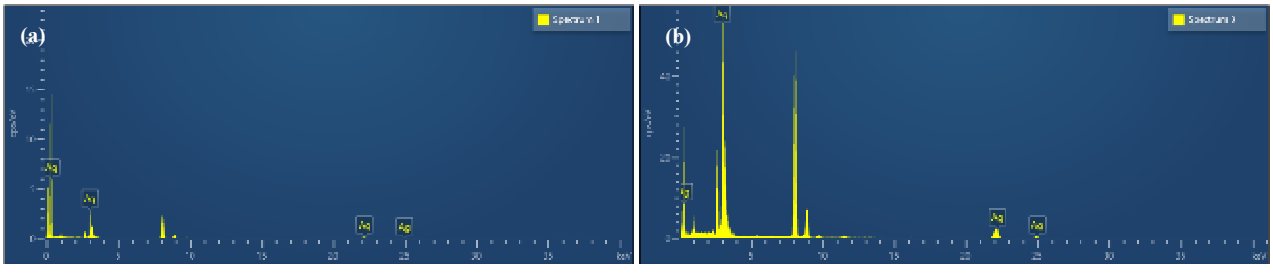

Figure 5. EDX spectroscopy of AgNPs synthesized using (a) leaf extract; (b) callus extract

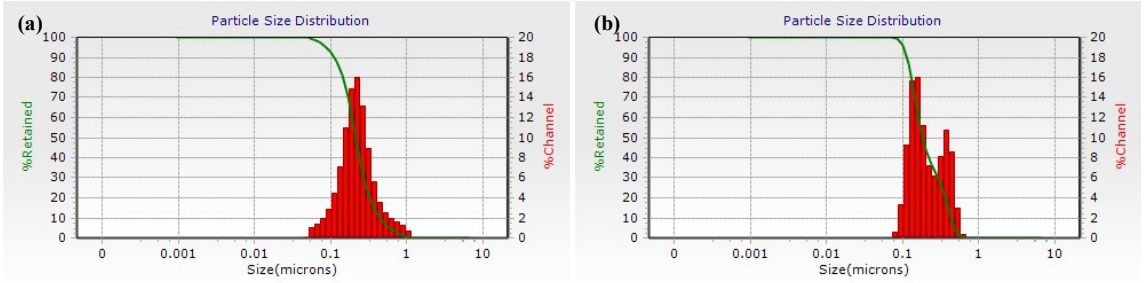

Figure 6. PSA analysis of biologically synthesized AgNPs by: (a) Leaf extracts (b) Callus extract

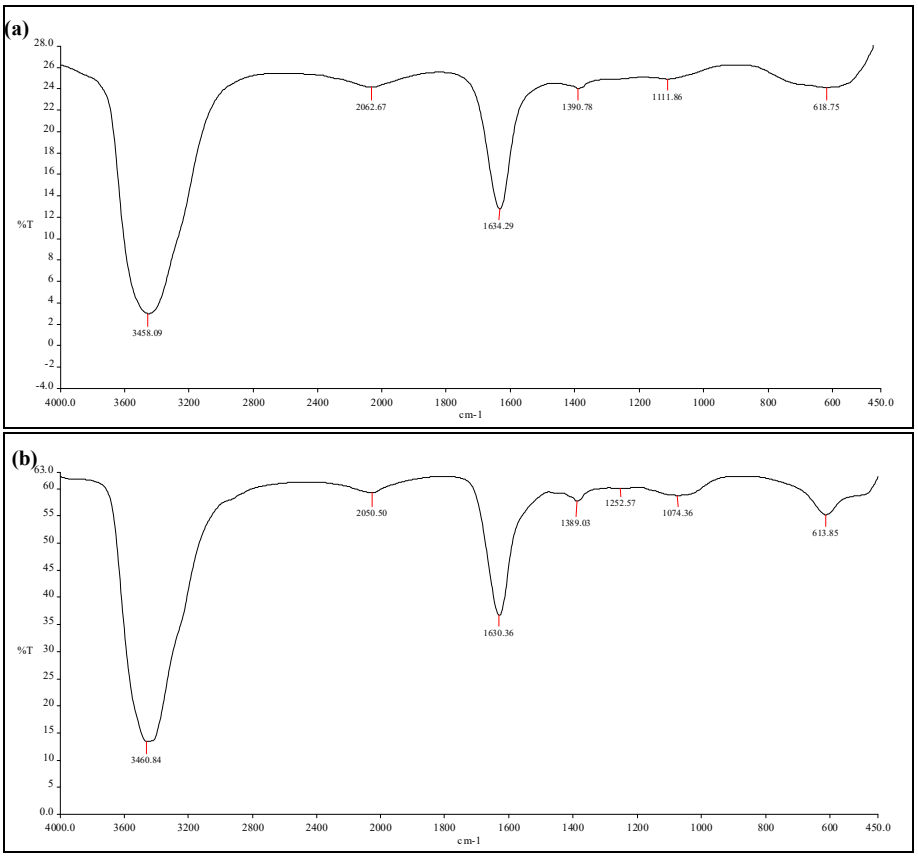

Figure 7. (a) FT-IR profile of Silver nanoparticles prepared from leaf extract (b) FT-IR profile of Silver nanoparticles prepared from callus extract.

Antimicrobial activity of AgNPs

After 24 hours of incubation, the leaf extracts, AgNPs and positive control (ciprofloxain) effectively inhibited bacterial growth over DMSO (Table 2). PAgNPs demonstrated strong antibacterial activity against gram-positive bacteria. Leaf extracts mediated AgNPs showed the highest ZOI of 27.7±1.00 mm towards *B. subtilis*, while a lower degree of ZOI was found gradually in *B. cereus* (27.3±0.45 mm) as gram-positive bacteria and *E. coli* (25±0.80 mm) as gram-negative bacteria (Table 2 and Fig. 8). In the case of callus generated silver nanoparticles, the greatest ZOI was reported in *B. cereus* (30±0.90 mm) followed by *B. subtilis* (27±0.76 mm) and *E. coli* (26.9±1.2 mm). In all the tested microorganisms, the ZOI of the callus mediated AgNPs found higher than that of leaf extract-mediated AgNPs.

Table 2. Antibacterial activity (zone of inhibition, mm) of AgNPs synthesized using leaf extract and callus extract of *V. canescens*

Microbial strains (zone of inhibition in mm)	Leaf extract (PE)	PAgNPs	CAgNPs	Control
E. coli	23±0.90	25±0.80	26.9±1.21	36.66±1.2
B. cereus	25.7±1.21	27.3±0.45	30±0.90	34±1.57
B. subtilis	24±0.57	27.7±1.00	27±0.76	37±0.82

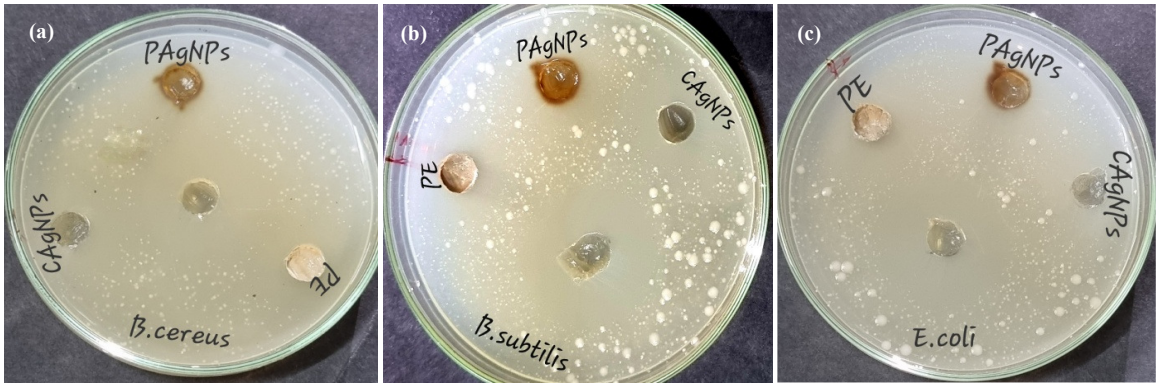

Figure 8. Antibacterial activity of PAgNPs and CAgNPs against: (a) *Bacillus cereus*, (b) *B. subtilis* and (c) *E. coli*.

DISCUSSION

Our findings substantiate the results reported by Cruz et al. [15]; Anjum & Abbasi, [4] and Groach et al. [19] indicating the completion of reduction reaction. The absorption band in the 400 to 450 nm region is typically for the silver nanoparticles [11, 19, 27]. According to XRD analysis, a sharp peak at 2θ of ~38.3° reveals the location (111) of silver, corresponding to face-centered cubic [6, 12]. The findings demonstrated that the AgNPs generated by plants and calli were made of high purity crystalline silver. The K value (constant) of 0.94 in the FCC and crystalline form of NPs, confirm the formation of silver NPs [16].

Similar observations regarding the size and morphological characteristics of the silver nanoparticles produced with leaf extracts of *Acacia nilotica* [40], flower extracts of Jasmine [5], and aqueous extracts of *Dracocephalum kotschyi* [13] respectively. The particles carry a charge of -33.75mV with a PDI below 0.276, demonstrating the stability of the green synthesized AgNPs [21]. The absorption bands at 3460 cm^{-1} & 3458 cm^{-1} in the FTIR spectra are due to OH stretch vibration of hydroxyl group and N–H stretch vibration of amines indicating the presence of the amine linkages of aniline. The band at 1634 cm^{-1} and 1630 cm^{-1} corresponds to C=O stretch bands of the carboxylic acid group [37]. The bands at 1252 cm^{-1} and 1074 cm^{-1} correspond to aryl -O stretch of aromatic ethers and C–N (amines) stretch vibration of proteins, respectively [22]. The bands at 618 cm^{-1} and 613 cm^{-1} correspond to S-S stretch vibration of disulphides bond [14].

The capping of several functional groups such as alkaloids, flavonoids, proteins, phenols, and glycosides was visible in the FT-IR spectra of plant and callus produced silver nanoparticles. Our results are in good agreement with other reports available on biosynthesis of AgNPs [19, 34, 46].

Silver nanoparticles may connect to the exterior of the plasma membrane interfering with components of the microbial electron transport system due to electrostatic attraction factors between the cell membranes of microbes of negative charge and positively charged NPs [3, 41]. The generation of silver metal free radicals also increases the oxidation forces responsible for the deterioration of membrane and nucleic acids causing cell death [26-42]. Similar reports on the inhibitory effects of green synthesized AgNPs on bacteria had been reported in *Azadirachta indica* [2]; *Crocus sativus* [8]; *Elaeagnus angustifolia* [30] and *Origanum majorana* [46]. Our study integrates nanotechnology and microbial biotechnology, leading to possible developments in the formulation of new types of bactericides [18].

In conclusion, silver nanoparticles were successfully produced from both plant leaves and *in vitro* developed callus of *V. canescens*. Green synthesis of AgNPs utilizing fresh leaves and callus from *V. canescens* has the potential to serve as an eco-friendly alternative to the hazardous chemical synthesis processes. The *in vitro* raised callus was proved to contain secondary metabolites resulting in antimicrobial activity. Thus, callus extract can be directly substituted in the extraction of certain useful drugs. Aside from that, *in vivo* studies are required to fully comprehend the function of *V. canecsens* mediated AgNPs and to assess their prospective uses in the biomedical area.

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Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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