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Biosynthesis of Silver Nanoparticles Using Callus Extract of *Catharanthus roseus* var. *alba* and Assessment of Its Antimicrobial Activity

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Authors' contributions

This work was carried out in collaboration between all authors. Author MAB carried out the plant tissue culture and analytical studies as well as protocol design. Author MM carried out the plant tissue culture study and supervisor of author MAB. Author MS carried out the silver nanoparticles synthesis and supervisor of author SV. Author SV carried out the silver nanoparticles synthesis. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Objective: To express a cost efficacious and environment friendly method for the green synthesis of silver nanoparticles (AgNPs) from *Catharanthus roseus* var. *alba* (*C. roseus* var. *alba*) callus extract.
Methodology: The aqueous solution of sliver nitrate containing Ag+ ions (1 mM) are integrating 100 µL of aqueous extract of callus of *C. roseus* var. *alba* and 10 mL of 1% w/v aqueous solution of polyethylene glycol 4000 to 90 mL. This was then alkalized with 0.1 NaOH (20 µL) and treated in a microwave oven (800 W) for 40 sec for the reduction of metal ion and the reaction takes place at room temperature (250C). The reduction of the Ag+ ions by aqueous callus extract of *C. roseus* var. *alba* in the solutions was monitored by UV–Visible spectrum and further characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM) and x-ray diffraction (XRD). Antibacterial activity was assessed on bacterial strain of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (*S. aureus* and *P. aeruginosa*) by using the disc-diffusion assay method.
Results: Characterization of AgNPs was done DLS, TEM and XRD methods. The Dynamic Light Scattering (DLS) showed the particle size distribution of the AgNPs

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synthesized from *C. roseus* var. *alba* callus extract was found 86.95 nm with polydispersity index (PDI) value of = 0.304. TEM images showed the formation of AgNPs with an average size of 10 nm to 20 nm. And the XRD analysis showed that the AgNPs were crystalline in nature with face-centered-cubic (FCC). For the assessment of antibacterial activity the concentration of AgNPs 25 µL, 50 µL, 100 µL and 150 µL were used against both the bacterial strain *S. aureus* and *P. aeruginosa*, the zone of inhibition found 4 mm, 7 mm, 16 mm and 23 mm as well as 5 mm, 9 mm, 19 mm and 26 mm respectively.

Conclusions: Aseptically engendered callus of *C. roseus* var. *alba* demonstrates vigorous potential for synthesis of silver nanoparticles by rapid reduction of Ag+ to Ag. The biologically synthesized AgNPs showed more preponderant antibacterial effect against *S. aureus* and *P. aeruginosa*.

Keywords: AgNPs; Catharanthus roseus var. alba; UV-Vis spectrophotometry; DLS; TEM; XRD.

1. INTRODUCTION

The development of green processes for the synthesis of silver nanoparticles is evolving into an important branch of nanotechnology [1,2]. Biologically synthesized AgNPs have drawn the attention of scientists [3], because of their extensive application in the development of new technologies in the areas of electronics, material sciences and medicine at the nanoscale [4,5,6]. AgNPs are highly toxic against human pathogen showing a strong biocidal effect against microbial species because these are highly reactive species with a large surface area. AgNPs exhibit potent antimicrobial activity that is produced by using microbes and plant extracts [7]. Nanoparticles play an important role in several aspects such as targeted drug delivery, diagnosis, tissue engineering and antimicrobial activities [8,9,10,11]. The use of environmentally benign materials like herbal extract, callus extract [12], bacteria, fungi and enzymes in green process for the synthesis of AgNPs [13], which offers many advantages over traditional, chemical and physical methods in terms of cost effective, eco-friendliness and compatibility for pharmaceutical and biomedical applications as they do not use toxic chemicals in the synthesis protocols, large scale production, easily affordable and there is no need to use high pressure, energy, temperature and toxic chemicals [10,11,5]. Extracts from natural sources are suitably scaled up for large scale biosynthesis of AgNPs in a controlled manner according to their shape, size and sensitivity [14,15,16]. Several plant species have been used for the synthesis of AgNPs including aseptically produced callus of *Caricapapya* [12]. Silver has been known to have a disinfecting effect and this property of silver has been exploited for the pharmaceutical applications. Several salts of silver and their derivatives are commercially manufactured as antimicrobial agents [4,17,18]. In addition, very low concentrations of silver are safe for human cells, but lethal for human pathogen [4,19]. It has also been reported that AgNPs are nontoxic to humans and most effective against human pathogenic bacteria, virus and other eukaryotic microorganisms at low concentrations without any side effects [4,17,18]. There has been an extensive research on the chemical synthesis of nanoparticles, but these particles have several potential hazards also, including carcinogenicity, genotoxicity, cytotoxicity and general toxicity [20,21]. A number of chemical and physical methods have been employed for the synthesis of AgNPs of different size and shape, such as UV irradiation [22,23], microware irradiation [24,25], chemical reduction [26,27], photochemical method [28,29], electron irradiation [30,31] and sonoelectrochemical method [32]. AgNPs also showed activity like- anti-fungal [33], anti-

inflammatory [34], anti-viral [35], anti-angiogenesis [36] and anti-platelet activity [37]. Recently, the development of silver nanoparticles is expanding. It is now being used as a part of clothing, food containers, wound dressings, ointments and implant coatings. US Food and Drug Administration approved some application of AgNPs [38]. Hence, the aim of present study is to synthesize silver nanoparticles by using callus extract of *C. roseus* var *alba*, characterization of these silver nanoparticles and to see the antimicrobial activity.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals used in this experiment were of analytical grade and obtained from sigma-aldrich (Bangalore, India) and Merck (Mumbai, India). Callus of *C. roseus* var *alba* were aseptically developed in plant tissue culture laboratory, Faculty of Pharmacy, Jamia Hamdard New Delhi 110062.

2.2 Preparation of the Callus Extract

Dried yellowish green callus (10 g) were used to make the aqueous extract of *C. roseus* var *alba* callus weighing were powdered and boiled in a 500 mL Erlenmeyer flask with 250 mL of sterile distilled water for 10 min and filtered through Whatman No.1 filter paper (pore size 25 µm) and the solution was used as stock solution for further experimental use.

2.3 Synthesis and Characterization of AgNPs

Aqueous solution containing Ag^+ ions (1 mM) purchased from Merck India Ltd were added 100 µL of aqueous extract of callus of *C. roseus* var *alba* and 10 mL of 1% w/v aqueous solution of polyethylene glycol 4000 (Merck India Ltd) to 90 mL of silver nitrate solution. This was then alkalized with 0.1 NaOH (20 µL) and treated in a microwave oven (800 W) for 40 sec for the reduction of metal ions. In a series of parallel experiments, the reaction takes place at room temperature. The reduction of the Ag^+ ions by aqueous callus extract of *C. roseus* var *alba* in the solutions was monitored by sampling the aqueous component (2 mL) and by quantifying the UV–visible spectrum of the solutions. UV– visible spectra of these aqueous colloid samples (1 mM) were quantified on a Labomed Model UVD-2950 UV-VIS Double Beam PC Scanning spectrophotometer, operated at a resolution of 2 nm. Furthermore, AgNPs were characterized by transmission electron microscopy (CM 200 FEG, Philips), Malvern Zetasizer Ver. 6.01, as well as by XRD analysis [39].

2.4 UV-Vis Spectra Analysis

The reduction of pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 3 hours after diluting a small aliquot of the sample into distilled water. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer.

2.5 Size Distribution of AgNPs by DLS

A laser diffraction method with a multiple scattering technique was used to determine the particle size distribution of the powdered AgNPs sample using a Zetasizer Nano Instrument (Malvern Instruments, Nano ZS, ZEN3600, UK) operating with a 532 nm laser. In order to find out the particle size distribution, the nanoparticles powder sample was dispersed in milli-

Q water by horn type ultrasonic processor (Vibronics, model: VPLP1) creating a total concentration of 1% at 37ºC.

2.6 X-ray Diffraction Analysis of AgNPs

The solution of AgNPs was purified by reiterated centrifugation at 5000 rpm for 20 min followed by redispersion of the pellet of AgNPs into 10 mL of deionized water. The structural composition were analyzed by XRD after freeze drying of the purified silver particles. The dried mixture of AgNPs was collected for the determination of the formation of AgNPs by an X'Pert Pro x-ray diffractometer (PAN analytical BV, The Netherlands) operated at a voltage of 45 kV and a current of 40 mA with Cu Kα radiation in a θ- 2 θ configuration. The crystalline domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer's formula:

$$D = 0.94 \lambda / \beta \text{ Cos } \theta$$

Where,

D is the average crystallite domain size perpendicular to the reflecting planes,

λ is the X-ray wavelength,

β is the full width at half maximum (FWHM), and

θ is the diffraction angle.

To eliminate additional instrumental broadening the FWHM was corrected, using the FWHM from a large grained Si sample.

$$\beta \text{ corrected} = (FWHM^2_{sample} - FWHM^2_{si})1/2$$

This modified formula is valid only when the crystalline size is smaller than 100 nm [40].

2.7 TEM Analysis of Silver Nanoparticles

Silver nanoparticles were characterized by transmission electron microscopy (CM 200 FEG, Philips). Transmission electron micrographs recorded from a small region of a drop-coated film of silver nitrate solution treated with the white Madagascar periwinkle callus extract (left picture) for 40 sec in a microwave oven (scale bars correspond to 50 nm).

2.8 Antibacterial Assays

Silver nanoparticles bactericidal effect was studied against one gram-positive (*S.aureus*ATCC-29213)and one gram-negative(*P. aeruginosa* ATCC-27853) bacterial pathogens by the use of filter paper discs by standard disc-diffusion assay method, provided from Hamdard Institute of Medical Science and Research, Hamdard University, New Delhi (INDIA) [41,42,43]. Briefly Luria Bertani (LB) agar medium was used to cultivate bacteria. Fresh overnight cultures of inoculum (100 µL) of each culture were spread on to LB agar plates. Filter paper discs (Whatman No 3 and 5 mm in diameter) were sterilized by autoclaving. In this method, different concentration of AgNPs prepared from callus extracts was mixed in 1 ml distilled water and then applied to sterile paper discs of 5mm diameter

(Whatman Filter papers) [4]. Sterile paper discs of 5 mm diameter containing AgNPs along with standard antibiotic or reference drug (Ampicillin 10 µg/disc and Gentamicin 10 µg/disc) containing discs were placed in each plate. The plates containing the bacterial and AgNPs were incubated at 37ºC. The plates were examined for evidence of zones of inhibition, which appeared as a clear area around the paper disk. The diameter of such zones of inhibition was measured using a meter ruler and the mean value for each organism was recorded and expressed in millimeter (mm).

3. RESULTS AND DISCUSSION

3.1 Synthesis of AgNPs

The chemical reduction of aqueous solution of silver nitrate is one of the most widely used methods for the synthesis of silver colloids. In this study, the formation of AgNPs by *C. roseus* var *alba* callus extract was investigated. The appearance of a yellowish brown colour in the reaction vessels suggested the formation of AgNPs [44]. Fig. 1 shows the bottles containing the silver nitrate (1 mM) before and after reaction with *C. roseus* var *alba* callus extract for 40 sec under heating in microwave oven. Also, no color change was observed when the procedure took place at room temperature or stayed for 24 hr in the same conditions (figure not shown).

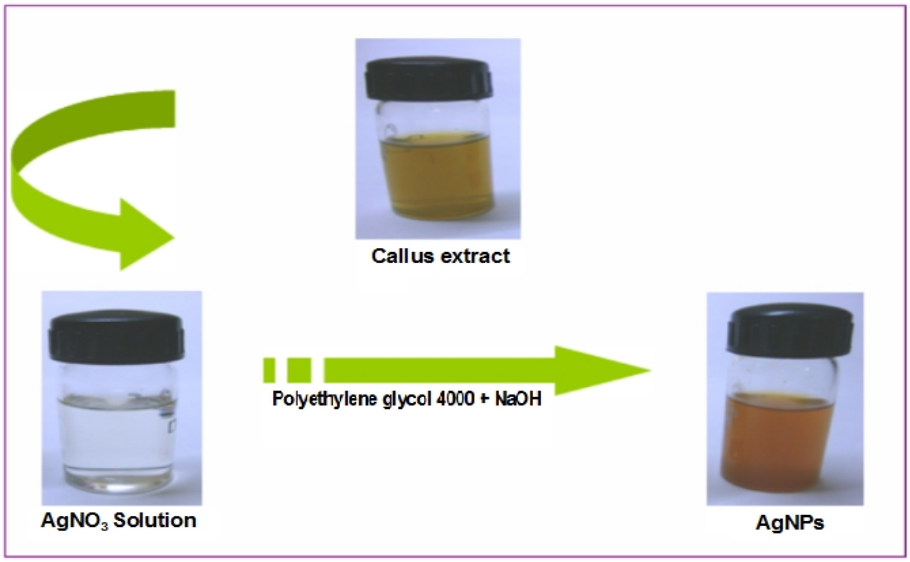


Fig. 1. Green synthesis of silver nanoparticles

3.2 UV-visible Spectra Analysis of AgNPs

The changes in the yellowish green color of callus extracts into yellowish brown indicate the presence and synthesis of AgNPs. The extract contents were then centrifuged at 10,000 rpm for 20 min. For the spectrometric UV analysis and for the evaluation of antibacterial activity the supernatants were used [45,4]. In the tested samples the spectrometric analyzed results showed the presence and reduction of silver ions. The reduction of silver ions was monitored by measuring the absorbance of the reaction mixture in a range of wavelength from 300 to

600 nm using spectrocolorimeter to find the absorbance peak [4]. As illustrated in Fig. 2, a well-defined plasmon band at 421 nm was observed due to formation of AgNPs produced due to the reducing properties of *C. roseus* var. *alba* callus extract. This peak is assigned to a surface plasmon, phenomenon that is well documented for various metal nanoparticles with sizes ranging from 2 nm to 100 nm [46,47]. UV-Vis spectrograph of the colloidal solution of AgNPs has been recorded as a function of time.

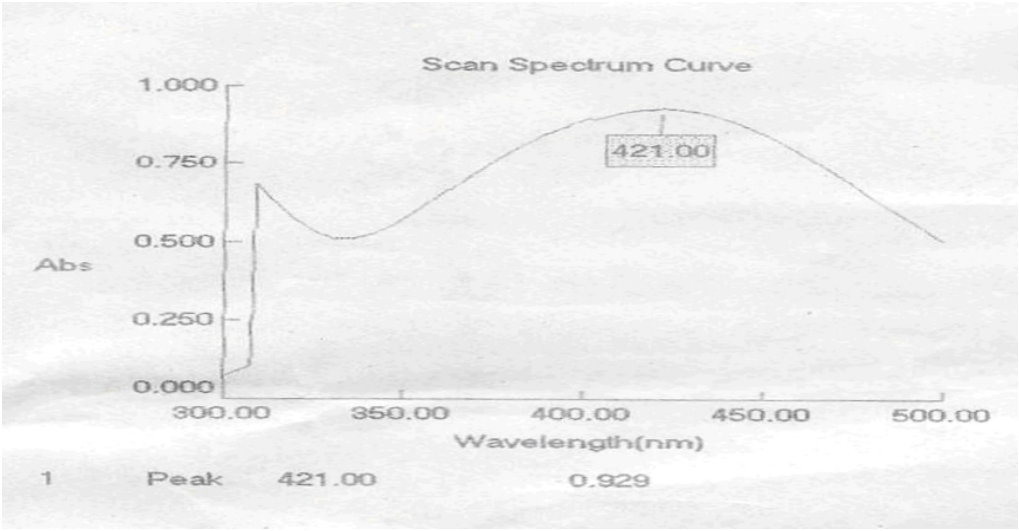


Fig. 2. UV-Vis absorption spectra of silver nanoparticles synthesized by *Catharanthus roseus* var. *alba* callus extract after 3 hrs

3.3 Size Distribution Analysis of Silver Nanoparticles by DLS

The AgNPs exhibited relatively narrow particle size distribution (z-average = 86.95 d.nm), as the relatively low polydispersity index (PDI = 0.304) values of DLS measurements, which is illustrated in Fig. 3.

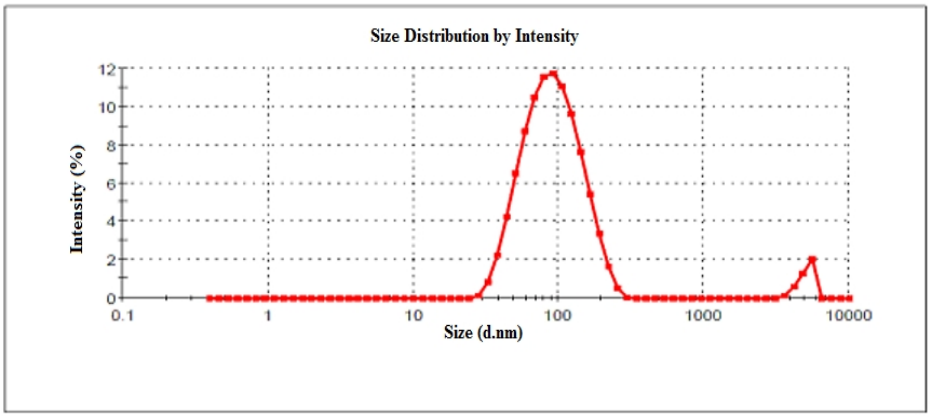


Fig. 3. Particle size distribution of Silver Nanoparticles synthesized by *C. roseus* var. *alba* callus extract

3.4 XRD Analysis of Silver Nanoparticles

The XRD patterns of AgNPs formed shown in the Fig. 4, which indicates the formation of the silver crystalline structure. The XRD peaks at 2θ degrees of 38.2, 44.4, 64.6 and 77.5 can be attributed to the (111), (200), (220) and (311) crystalline planes of face-centered-cubic (FCC) crystalline structure of metallic silver, respectively. It confirmed that the main composition of the nanoparticles was silver.

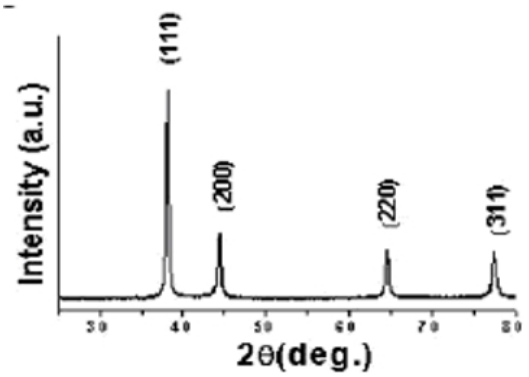


Fig. 4. XRD patterns of silver nanoparticles by treating *C. roseus* var. *alba* callus extract with $AgNO_3$ aqueous solutions

3.5 TEM Analysis of AgNPs

TEM techniques confirmed the reduction of silver ions to silver nanoparticles shown in the Fig. 5. The size of the silver nanoparticles based on the TEM micrographs was about 10–20 nm smaller than the size determined by DLS.

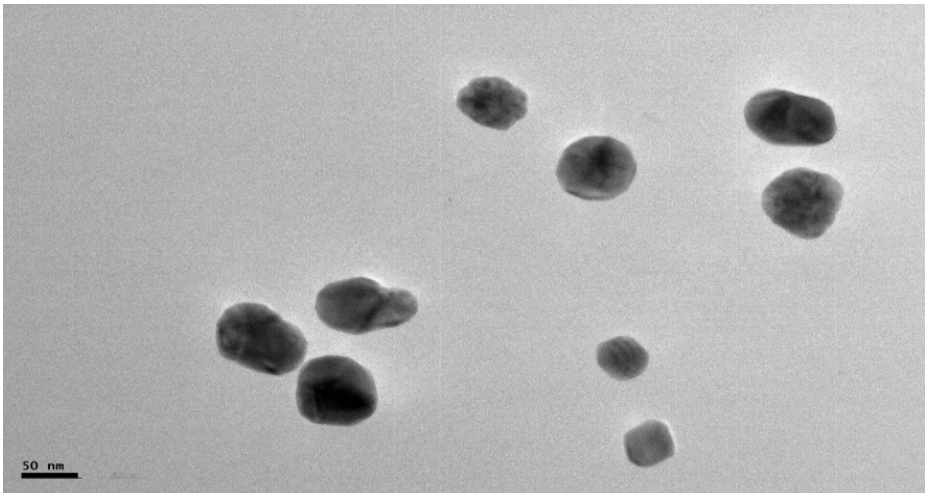
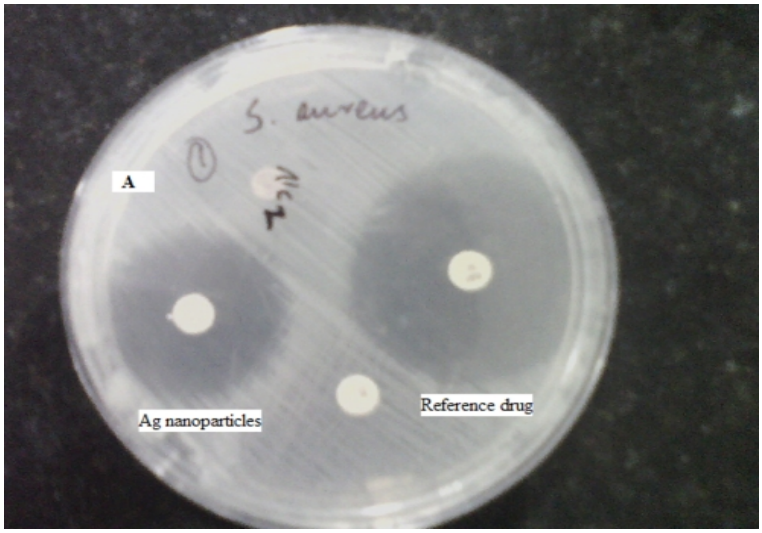


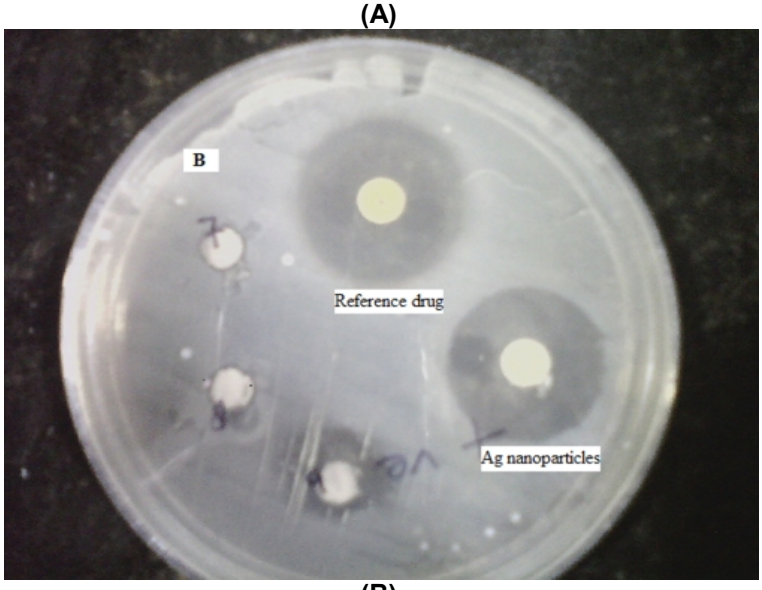
Fig. 5. Transmission electron micrographs recorded from a small region of a drop-coated film of silver nitrate solution (scale bars correspond to 50 nm)

3.6 Antibacterial Analysis of AgNPs

Silver nanoparticles exhibited antibacterial activity against multi-drug resistant (MDR) human pathogenic *S. aureus* and *P. aeruginosa* as it showed a clear zone of inhibition Table 1, Fig. 6 A and B. The inhibition rate at the concentration of 25 µL, 50 µL, 100 µL and 150 µL of AgNPs against *S. aureus* showed 4 mm, 7 mm, 16 mm and 23 mm. The inhibition rate against *P. aeruginosa* by AgNPs was 5 mm, 9 mm, 19 mm and 26 mm respectively. The action of silver nanoparticles is onto the surface of bacterial cell membrane that disturbing their permeability and respiration functions. Decrease in the size of silver nanoparticles showed the large surface area that is available for interaction would give more bactericidal effect than the larger silver nanoparticles [48].



(A)



(B)

Fig. 6. Antibacterial activity of silver nanoparticles against: (A) *Staphylococcus aureus* and (B) *Pseudomonas aeruginosa*

Table 1. Zone of inhibition showed by silver nanoparticle against bacterial pathogens

Name of the test bacteria	Concentration of silver nanoparticle (in µL)	Zone of inhibition (in mm)
Staphylococcus aureus	25	4
	50	7
	100	16
	150	23
Pseudomonas aeruginosa	25	5
	50	9
	100	19
	150	26

4. DISCUSSION

The research work included the synthesis and characterization of AgNPs from the extract of aseptically produced callus of *C. roseus* var. *alba* and their antimicrobial activity. The potential ability of *C. roseus* var. *alba* callus extract for the reduction of silver ions to AgNPs was investigated in different condition. From the study, it was accomplished that the aqueous silver ions exposed to the callus extract of *C. roseus* var. *alba* were reduced and the nanoparticles were synthesized. The presence of nanoparticles was confirmed by the yellowish brown color formation from yellowish green callus extract. The silver ions reduction and stabilization of the AgNPs was thought to occur through the participation of callus proteins and secondary metabolites. It is also a well known fact that proteins can bind to AgNPs through either free amino groups or cysteine residues in the proteins and the surface bound proteins stabilize the AgNPs during synthesis [12]. The polyphenols or flavonoids were main biomolecules which are responsible for nanoparticle synthesis [7]. The applications of such eco-amicable AgNPs in bactericidal, wound rejuvenating and other medical and electronic applications, makes this method potentially exhilarating for the sizably voluminous-scale synthesis of other inorganic materials (nanomaterials). Toxicity studies of AgNPs on bacterial pathogen opens a door for an incipient array of antibacterial agents [49]. The AgNPs showed antimicrobial activity when DNA molecules are in relaxed state and the replication of DNA can be effectively conducted, but when DNA is in condensed form it loses its replication ability hence, the DNA molecules turns into condensed form and loses its replication ability leading to cell death, when the silver ions penetrate inside the microbial cell [16]. AgNPs are larger in size than a silver ion, which makes them react with more molecules, leading to more antimicrobial activity [7,14]. According to another report, major mechanism of action of AgNPs were found, through which AgNPs manifest antibacterial properties was either by anchoring or penetrating the bacterial cell wall and modulating cellular signaling by dephosphorylating putative key peptide substrates on tyrosine residues [14]. For the killing of microbes present on the surface of the clothing material was done by incorporating silver of nanoscales into textiles, thus can be treated with AgNPs to help prevent spoilage rising from microbial growth in the damp areas [16]. Characterization of AgNPs done by UV-visible spectrophotometer, XRD, DLS and TEM analysis confirmed the reduction of silver ions to AgNPs. The UV-visible absorption spectra of AgNPs showed absobance peak at 421 nm and peak broadening showed that the particles are polydispersed. By the use of x-ray diffraction to confirm the crystalline nature of the particles and the XRD pattern showed numbers of Bragg's reflections that may be indexed on the basis of the face centered cubic structure of AgNPs. Therefore, AgNPs were formed having cubical with uniform shape. The obtained AgNPs

were analysed by the use of TEM, which confirm the size ranging from 10–20 nm. The TEM image of AgNPs showed that there is variation in particle sizes. Also, in this investigation, antibacterial analysis was used to assess the effect of AgNPs against humane pathogens. The AgNPs prepared by *C. roseus* var. *alba* callus extract showed significant antibacterial effect. The minimum concentration of AgNPs may be used to inhibit the bacterial pathogens is 25 µL and 150 µL is the maximum concentration.

5. CONCLUSION

Silver nanoparticles with the size ranging from 10–20 nm were synthesized using *C. roseus* var. *alba* callus extract. The biosynthesis of silver nanoparticles using the aqueous callus extract of *C. roseus* var. *alba* was explored under different conditions. The synthesized silver nanoparticles were characterized by UV-Vis spectroscopy, DLS, TEM and XRD measurements. This green synthesis method is alternative to chemical method, since it is cheap, pollutant free and eco-friendly. The results showed that *C. roseus* var. *alba* callus extract plays an important role in the reduction and stabilization of silver to silver nanoparticles. Further, these synthesized silver nanoparticles from *C. roseus* var. *alba* callus extract shows antibacterial activity on both gram-positive and gram-negative bacteria.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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