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Research article

GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *VINCA ROSEUS* LEAF EXTRACT AND EVALUATION OF THEIR ANTIMICROBIAL ACTIVITIES

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ABSTRACT: In the present study, silver nanoparticles were rapidly green synthesized from $AgNO_3$ solution using leaf extract of *Vinca Roseus* at room temperature. The green synthesized Silver Nanoparticles were characterized by UV–Visible absorption spectroscopy and further by analytical techniques such as, DLS, X-ray diffraction (XRD) and SEM. X-ray diffraction and Scanning Electron Microscope analysis studies confirmed the formation of well-dispersed silver nanoparticles with average particle size to be in the range of 20-80 nm as well as revealed their cubic structure. Further these green synthesized silver nanoparticles were evaluated for their antimicrobial activities against different pathogenic bacteria like *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa,* and *Staphylococcus aureus* by using Disc - diffusion methods and the bacterial growth kinetics. The activity was monitored by measurement of zone of inhibition and optical density (OD). The results evaluated that the antimicrobial activity of green synthesized silver nanoparticles was higher against gram - positive bacteria compared to gram-negative bacteria.

Keywords: Green synthesis, silver nanoparticles, gram –positive bacteria, gram negative bacteria

INTRODUCTION

Nanobiotechnology is at the cutting edge of this rapidly evolving area. In recent year's synthesis of silver Nanoparticles has attracted considerable aid from researchers mainly due to its advantage over other metal nanoparticles (e.g., Gold and copper). Because the surface plasmon resonance energy of silver is located far from the interband transition energy(Sathyavathi, Krishna, Rao, Saritha, & Rao, 2010) and also owing to their potential applications in biology, medicine(A. Shahverdi, Minaeian, Shahverdi, Jamalifar, & Nohi, 2007), optics(Shiraishi, 2000) and in modern electronic devices(Chang & Yen, 1995).

To synthesize the silver nanoparticles several approaches are available. For example, silver ions are reduced by radiation, chemical-reduction, electrochemical, photochemical methods, Langmuir–Blodgett, sol-gel, and biological techniques. Among those methods, biosynthesis of silver nanoparticles using microorganism(Nair & Pradeep, 2010), enzyme(I. Willner, Baron, & Willner, 2006) and plant(Bar et al., 2009; Dubey, Bhadauria, & Kushwah, 2009; Krishnaraj et al., 2010; Li et al., 2007; Nabikhan, Kandasamy, Raj, & Alikunhi, 2010; R. Sarkar, Kumbhakar, & Mitra, 2010; Sathishkumar et al., 2009; Shankar, Rai, Ahmad, & Sastry, 2004; Tripathy, Raichur, Chandrasekaran, Prathna, & Mukherjee, 2009) have been suggested as possible ecofriendly alternatives to chemical and physical methods. Using plant extract for nanoparticles synthesis can be advantageous over other biological processes by eliminating the elaborate process of maintaining cell cultures.

It is well known that metal nanoparticles have good antimicrobial properties(Shanmugam, Viswanathan, & Varadarajan, 2006). Among that silver nanoparticles are an obvious choice due to their antimicrobial effects effectively(Duncan, 2011; M. Rai, Yadav, & Gade, 2009; Sharma, Yngard, & Lin, 2009). It can be expected due to the high specific surface area and high fraction of surface atoms of Ag-NPs which lead to show high antimicrobial activity compared to the bulk silver metal(J. S. Kim et al., 2007).

The present study was aimed to synthesize the silver nanoparticles using aqueous plant leaves extract of Vinca roseus and evaluates its antimicrobial activities against pathogenic bacteria. Here the Vinca roseus plant leaves have the source of bio-redutant and capping agent for the silver nanoparticles synthesis due to its significance in medicinal field.

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MATERIALS AND METHODS

Preparation Vinca rosea of plant leaf extract

10g of Vinca roseus plant leaves were taken and cut down into small pieces, then they were surface cleaned by running tap water and followed by distilled water and boiled in 100ml of distilled water at 60°C for 20mins.After that the aqueous leaf extract was filtered through the whatsmann.No.1 filter paper, followed by Millipore filter (0.22µm) and the filtrate was stored at 4°c for further experiments.

Silver Nanoparticle synthesis

For preparation of a silver nanoparticle synthesis 2ml of aqueous leaf extract was added to the Erlenmeyer flask containing 98ml of AgNO3 (10-3M).The mixture was kept in incubation period for 20 minutes at room temperature.

Characterization

UV-VIS spectral analysis

The reduction in Ag+ ions monitored by measuring UV-VIS spectra of the silver nanoparticle solution and UV-VIS spectra was recorded by Shimadzu UV-VIS3600 Spectrophotometer from 200nm-800nm.The $AgNO_3$ solution used as blank.

Dynamic Light Scattering Analysis

For the green synthesized silver nanoparticles the nanostructure diameter measurements were performed using the Zetasizer Nano-S system (Malvern Instruments Ltd., Malvern, UK).A Sample was dispersed in water at a concentration of 50 µg/ml(Murdock, Braydich-Stolle, Schrand, Schlager, & Hussain, 2008).

XRD Analysis

The green synthesized silver nanoparticle solution thus obtained was purified by repeated centrifugation at 8000 rpm for 15 min followed by redispersion of the pellet of silver nanoparticles into 10 ml of deionized water. After freeze drying of the purified silver particles, the structure and composition were analyzed by XRD. The dried mixture of silver nanoparticles was collected for the determination of the formation of Ag nanoparticles by an X'Pert Pro x-ray diffractometer (PANalytical BV, The Netherlands) operated at a voltage of 40 kV and current of 30 mA with Cu Kα radiation in θ- 2θ configurations. The analysis was carried out 2θ with ranging from 10 to 80^0.with step size 0.05.

SEM Analysis

Scanning Electron Micrographs of the purified, dried silver nanoparticles was taken using Jeol JSM-6480 LV SEM machine. Thin films of the sample were prepared on a glass slide by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the slide was allowed to dry by putting it under a mercury lamp for 10 min. The sample was applied for SEM images.

Antimicrobial activity study

To evaluate the antimicrobial activity, green synthesized silver nanoparticles were applied against to the following pathogenic bacteria's like gram-negative bacteria *E.coli* and *Pseudomonas aeruginosa* among gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* were assayed and activity was monitored by the a Disc - diffusion method and Growth kinetics.

Disc- diffusion Assay

To evaluate the antimicrobial activity using the disc-diffusion method.18ml of autoclaved media (nutrient media) was poured in sterilized Petri dishes and allowed it to for 15minutes for the solidification of the agar media in the Petri dishes. To check any contamination to appear the plates were kept at room temperature for 24hrs. The bacterial test organisms were grown in nutrient broth for 24h.From that broth culture of each bacterial organism 0.1ml of inoculums were spreaded on the agar Petri plates with the help of spreader and the paper discs which were containing the silver nanoparticles with 40 µg/ml concentration were kept in agar plates at certain distances along with the antibiotic (erythromycin) discs in each plate and kept in incubator for 24hrs at 37 □c. After the incubation period of 24hrs the inhibition activity was monitored.

Growth kinetics

For this assay 1ml (10^4 cells/ml) of freshly grown *Bacillus subtilis* (gram-positive) and *Pseudomonas aeruginosa* (gram-negative) were inoculated to the each flask containing 50ml of prepared nutrient broth and the culture was incubated with silver nanoparticles with the concentration 40 µg/ml for 24hrs in orbital shaking incubator for 24hrs at 37^0c with 120rpm.To know the growth kinetics the OD values were taken at 600nm for each and every 2hours of interval time along the control.

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RESULTS AND DISCUSSION

As the *Vinca roseus* aqueous leaf extract was added to the silver nitrate solution and incubated the mixture color was changed rapidly from the transparent color to brown yellowish color due to the formation of AgNP's. (See fig.1) The appearance of yellowish brown color was due to the excitation of surface plasmon vibrations of silver nanoparticles(Ahmad et al., 2003). (Figure 1)

Figure-1 (a) Figure-1(b)

Figure 1 Digital photographs of (a) Pure 1 mM $AgNO_3$ solution (b) 1 mM $AgNO_3$ and leaf extract solution after 20mins of incubation at RT

UV-VIS Spectral analysis:

The formation of silver nanoparticles was confirmed using UV-Vis spectroscopy the broad plasma resonance peak around 448.5 nm corresponds to silver nanoparticles (see Fig.2).So it was reported that the nanoparticles which are showing maximum absorbance around the 450nm will have the spherical shape, and the silver nanoparticles that are formed may be Polydispersed in condition because the spectra exhibiting the broadening of the peak(Martínez-Castañón, Niño-Martínez, Martínez-Gutierrez, Martínez-Mendoza, & Ruiz, 2008).(Figure 2).

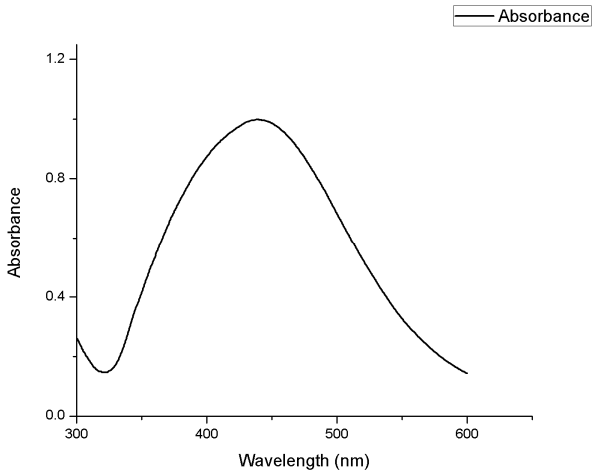

Figure 2 UV-Vis spectroscopy analysis of green synthesized silver nanoparticles by treating 1mM aqueous AgNO3 solution with 10% leaf extract after 20 mins

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XRD Analysis

The biosynthesized silver nanostructure was further demonstrated and confirmed by the characteristic peaks observed in the XRD pattern(Hall, 2000) (Figure.3). The synthesized silver nanostructure by employing leaf extract was confirmed by the characteristic peaks observed in the XRD image. The analysis was carried out 2θ value ranging from 10^0 to 80^0, with step size 0.05. All diffraction peaks correspond to the characteristic face centered cubic (FCC) silver lines. These diffraction lines observed at 2Θ angle 32.8^0, 38.2^0, 55.1^0 and 65.7^0 respectively, have been indexed as (111), (200), (220) and (311) respectively. The typical XRD pattern revealed that the sample contains a cubic structure of silver nanoparticles (Martínez-Castañón et al., 2008), (Thiel et al., 2007). (Figure 3)

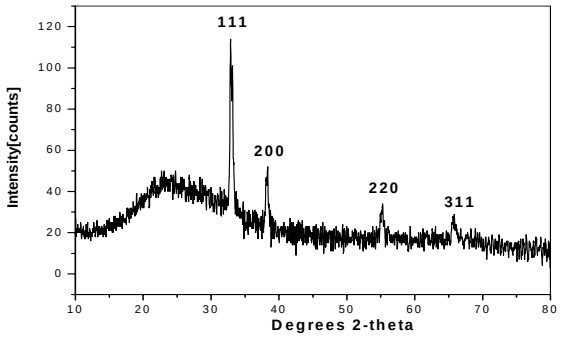

Figure 3 XRD pattern of silver nanoparticles synthesized by treating 1mM aqueous $AgNO_3$ solution with 10% leaf extract

Dynamic Light Scattering

Dynamic light scattering (DLS) of Ag nanoparticles represented the average particle size distribution of silver nanoparticles which is shown in figure 4. From the graph it has been concluded that the average particle size distribution of silver synthesized nanoparticles was 45nm. (Figure 4).

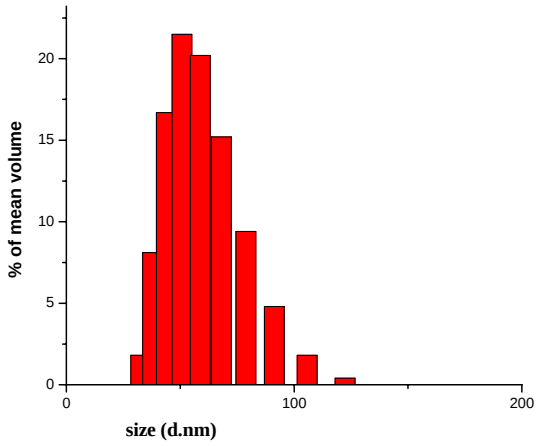

Figure 4 Particle size distributions of silver nanoparticles synthesized by leaf extract

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SEM Analysis

Scanning electron microscopy analysis was carried out to understand the topology and the size of the silver nanoparticles, which showed the synthesis of Polydisperse spherical silver nanoparticles in the range 10 to 80 nm with average size of 45 nm (Figure.5).

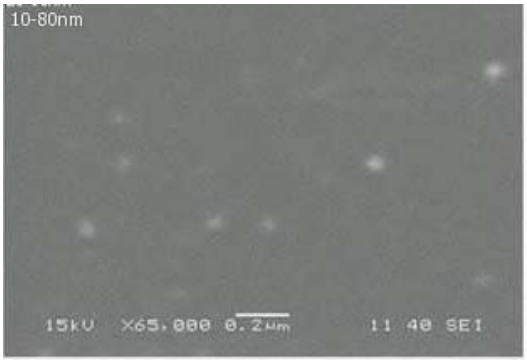

Figure 5 SEM micrograph of silver nanoparticles synthesized by leaf extract

Antimicrobial studies

Disc-diffusion Method

To evaluate the antimicrobial activity, green synthesized silver nanoparticles were treated against different pathogenic bacteria like *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *S. aureus using* disc-diffusion method with a paper discs containing 40µg/ml AgNP's concentration and erythromycin of 10mg/ml concentration was used as a control Antimicrobial agent. The green synthesized silver nanoparticles showed clear zone of inhibition against all studied pathogenic bacteria. The maximum zone of inhibition was found in gram positive bacteria and minimum zone of inhibition in gram-negative bacteria. (Table 1).

Table 1 Antimicrobial activity of green synthesized Ag Nanoparticles using *vinca roseus*

Name of the bacterial sps	Zone of inhibition(mm)	
	Ag Nanoparticle	Erythromycin
Staphylococcus aureus	11	20
Bacillus subtilis	14.4	21.7
Escherichia coli	8.3	16
Pseudomonas aeruginosa	6.6	11

Growth kinetics

Silver nanoparticles were added to the liquid growth medium Number of bacteria cells (10^5cells/ml) added in a flask containing 50 ml of solution. To know the growth kinetics of the *Bacillus subtilis* & *Pseudomonas aeruginosa* the OD values were taken at 600nm for each and every 2hours of interval time along the control. And the curve was plotted and shown in (Figure 6).

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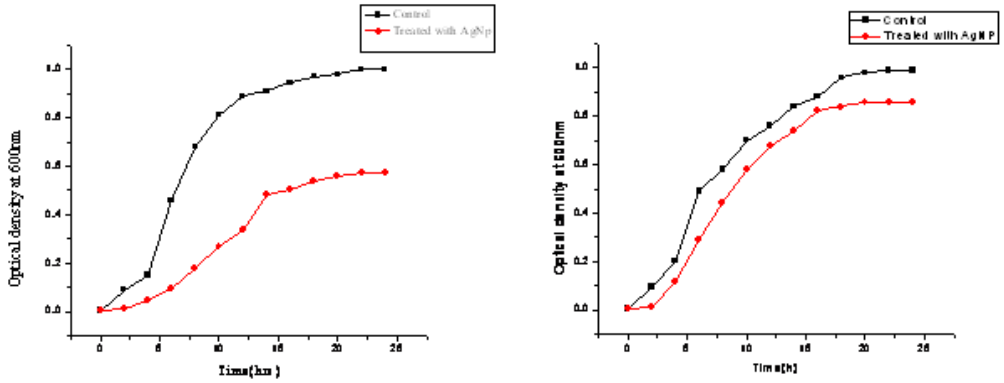

Figure 6 The effect of green synthesized silver nanoparticles on the growth of a) *B. subtilis* b) *P.aeruginosa*

It has been observed from the OD plot given in Figure 6 that the optical absorption in the growth medium decreased in comparison to the control

This was on the basis of the composition of the cell membrane structure the bacteria are classified into Gram negative or Gram positive. In the cell membrane structure mainly the structural difference was due to the organization of peptidoglycan. Gram-positive bacteria exhibit a thick layer of peptidoglycan between the cytoplasmic membrane and the outer cell wall. The outer membrane of *B. Subtilis* cells are constructed from tightly packed lipopolysaccharide (LPS) molecules, which provide an effective permeability barrier in the cell wall of the bacteria. The negative charge will be present on the surface of the bacterial cells will adsorb the opposite charge of nanoparticles via the electrostatic forces between the nanoparticle and the surface of the bacterial cell wall. Whenever the nanoparticles were absorbed in the bacterial cell they will penetrated into the cytoplasm by the breakdown of the cell wall of the bacteria through the change in the permeability of the cell membrane, The smaller nanoparticles which are present inside the cell will have more surface area to volume ratio leads to interactions in the cytoplasmic environment as well in the nucleus , results in the cytotoxic effect to the bacterial cell due to the conformational changes in the metabolic pathways of bacteria(J. S. Kim et al., 2007; Martínez-Castañón et al., 2008; Sharma et al., 2009; Thiel et al., 2007). Another reason is that Silver nanoparticles which were showing higher activity were suggested to use for monitoring the antimicrobial activity due to species differences as they dissolve to release Ag^0 (atomic) and Ag^+ (Ionic) clusters, whereas other silver sources such as silver nitrate and silver sulfadiazine release Ag^+ only(Words & Nanomaterials, 2008). It is believed that silver nanoparticles whenever penetrates inside the bacteria they inactivate microbial enzymes, and helps in the production of hydrogen peroxide which leads to the bacterial cell death(Allahverdiyev, 2011).

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