BIOSYNTHESIS OF SILVER NANOPARTICLES USING VITIS VINIFERA EXTRACT AND EVALUATION OF THEIR ANTIMICROBIAL ACTIVITY

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ABSTRACT

In the present investigation, synthesis of silver nanoparticles from Vitis vinifera extract and their bactericidal potential against four pathogenic bacteria was investigated. In this study, we have developed an environment friendly technique for the green synthesis of silver nanoparticles from AgNO₃ solution using Vitis vinifera extract. Silver Nanoparticles were characterized using UV–Visible spectroscopy whose absorbance measured at 440nm followed by Dynamic light scattering particle size analyser, X-ray Diffraction and Scanning Electron Microscopy showed the formation of nanoparticles in the range of 10-80nm. The biologically synthesized nanoparticles at concentration of 0, 20, 40, 60, 80 µg/ml were screened against two gram-positive (Bacillus subtilis ATCC-6633 and Streptococcus pneumonia ATCC 49619) and two gram-negative (Escherichia coli ATCC-25922 and Pseudomonas aeruginosa ATCC-27853) bacterial pathogens in both solid and liquid growth medium. The results confirmed that silver nanoparticles to be an effective bactericide at the concentration 60 µg/ml against pathogenic bacteria.

KEYWORDS: Nanoparticles, Silver; Green synthesis, Bacteria, Bactericide

INTRODUCTION

Nanotechnology is one of the most active research area in the modern material science. Based upon their specific characteristics such as size, distribution and morphology nanoparticles have distinct properties compared with the bulk form of the same material. New application of nanoparticles and nonmaterials are emerging rapidly [9, 11, 12]. Silver nanoparticles have tremendous applications in the field of high sensitivity bimolecular detection and diagnostics [20], antimicrobials and therapeutics [5, 13], catalysis [4] and micro-electronics [6]. The development of reliable green processes for the synthesis of silver nanoparticles is an important aspect of current nanotechnology research. Nanomaterials such as Ag, Au, Pt and Pd have been synthesized by different methods, including chemical reduction method [25] using bacteria [7] fungi [15] and plants [18]. Stable silver nanoparticles have been synthesized by using soluble starch as both the reducing and stabilizing agents [22]. Synthesis of gold nanotriangles and silver nanoparticles using Aloe vera plant extracts was reported [3].

Chemical synthesis methods lead to the presence of numerous toxic chemical species adsorbed on the surface of nanoparticles that may have adverse effects in medical applications. Synthesis of nanoparticles using microorganisms or plant parts can potentially eliminate this problem by making the nanoparticles more biocompatible. Biological synthesis of nanoparticles provides advancement over chemical and physical methods as it is a cost effective and environment friendly and in this method there is no need to use high pressure, energy, temperature and toxic chemicals. Using plant parts for nanoparticles synthesis can be advantageous over other biological processes by eliminating the elaborate process of maintaining cell cultures [16, 17]. It can also be suitably scaled up for large-scale synthesis of metal nanoparticles.
It is well known that inorganic nanomaterials have good antimicrobial properties. Silver nanoparticles are the metal of choice as they have the capability to kill microbes effectively [21]. The strong toxicity of silver against wide range of microorganisms is well known and silver nanoparticles have been recently shown to be an effective antimicrobial agent. The silver nanoparticles act on a broad range of target sites both extracellularly as well as intracellularly. The most widely used and known applications of silver and silver nanoparticles are in the medical industry [10]. These include topical ointments and creams containing silver to prevent infection of burns and open wounds [8]. In addition, silver-containing consumer products such as colloidal silver gel and silver-embedded fabrics are now used in sporting equipment. In fact, microbes generally have a harder time developing resistance to silver than they do to antibiotics. Silver nanoparticles takes advantages of the oligodynamic effect that silver has on microbes, whereby silver ions bind to reactive groups in bacterial cells, resulting in their precipitation and inactivation. Silver nanoparticles shows very strong bactericidal activity against gram-positive as well as gram-negative bacteria including multiresistant strains [19], can be considered as potential antifungal agent. The antifungal effect of silver nanoparticles has received only marginal attention and just a few studies on this topic have been published [24, 14].

Here in, we report for the synthesis of silver nanoparticles, reducing the silver ions present in the solution of silver nitrate by the aqueous extract of *Vitis vinifera*. Further these biosynthesized silver nanoparticles have high efficacy of antimicrobial activity against different multi drug pathogenic bacteria.

**MATERIALS AND METHODS**

**Microorganisms Tested**

Two gram-positive (*Bacillus subtilis* ATCC-6633 and *Streptococcus pneumonia* ATCC 49619) and two gram-negative (*Escherichia coli* ATCC-25922 and *Pseudomonas aeruginosa* ATCC-27853) were used in this study. All the bacterial strains were grown and maintained on nutrient agar slants at 4°C.

**Preparation of *Vitis vinifera* Extract**

Extract was prepared by weighing 25gm *Vitis vinifera* after thoroughly washed in distilled water. It was dried and cut into fine pieces and crushed into 100 ml sterile distilled water and filtered through Whatman No.1 filter paper (pore size 25 µm). The filtrate was further filtered through 0.45 µm filters. The extract was stored at 4ºC.

**Synthesis of Silver Nanoparticles**

100 ml aqueous solution of 1mM AgNO₃ was prepared and used for the synthesis of silver nanoparticles. 10 mL of fruit extract was added into 90 mL of aqueous solution of 1 mM AgNO₃ and kept at room temperature for 10 hours. To exclude bigger particles the solution was centrifuged at a rate of 2000 rpm up to 15 minutes.

**UV-Vis Spectroscopy Analysis**

The reduction of pure Ag⁺ ions to pure Ag was monitored by UV-Vis spectroscopy. UV-Vis spectral analysis was recorded by a UV-Vis spectrophotometer UV-2450 (Shimadzu) from 200 to 800 nm.

**XRD Measurement**

The 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 and SEM. The dried mixture of silver nanoparticles was collected for the determination of the formation of Ag nanoparticles by an X’Pert Pro x-ray
Biosynthesis of Silver Nanoparticles using Vitis Vinifera Extract and Evaluation of their Antimicrobial Activity

diffraction (PANalytical BV, The Netherlands) operated at a voltage of 35 kV and a current of 25 mA with Cu Kα radiation in θ-2θ configurations. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula.

\[ D = \frac{0.94 \lambda}{\beta \cos \theta} \]  

where \( D \) is the average crystallite domain size perpendicular to the reflecting planes, \( \lambda \) is the X-ray wavelength, \( \beta \) is the full width at half maximum (FWHM), and \( \theta \) is the diffraction angle. To eliminate additional instrumental broadening the FWHM was corrected, using the FWHM from a large grained Si sample.

\[ \beta \text{ corrected} = (\text{FWHM}^2_{\text{sample}} - \text{FWHM}^2_{\text{Si}})^{1/2} \]  

This formula is valid only when the crystallite size is smaller than 100 nm [2].

**SEM Analysis of Silver Nanoparticles**

Scanning Electron Microscopic (SEM) image was produced using Jeol JSM-6480 LV SEM machine. The freeze dried sample of silver nanoparticles was sonicated with distilled water; small drop of this sample was placed on glass slide allowed to dry. A thin layer of platinum was coated to make the sample conductive. SEM machine was operated at a vacuum of the order of 10\(^{-5}\) torr. The accelerating voltage of the microscope was kept in the range 10-20 kV.

**DLS Particle Size Analyzer**

A laser diffraction method with a multiple scattering technique was used to determine the particle size distribution of the powdered silver nanoparticle sample. 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). Then experiment was carried out in computer controlled particle size analyzer ([ZETA Sizers Nanoseries (Malvern Instrument Nano ZS)]) to find out the particles size distribution.

**Antibacterial Assay**

Silver nanoparticles bactericidal effect was studied against two gram-positive (Bacillus subtilis ATCC-6633 and Streptococcus pneumonia ATCC 49619) and two gram-negative (Escherichia coli ATCC-25922 and Pseudomonas aeruginosa ATCC-27853) bacterial pathogens. The nanoparticles were dispersed in autoclaved deionized water by ultrasonication. Aqueous dispersion of silver nanoparticles of desired concentration was made.

**Disc Diffusion Method**

The overnight culture inoculum (100 µl) of each bacteria was spread on to nutrient agar plates. Sterile paper discs (Whatman filter paper) of 5 mm diameter (containing 60 µg/ml silver nanoparticles) along with four standard antibiotic containing discs (10 mcg/disc) were placed in each plate. The cultured agar plates were incubated at 37°C for 24 hours. After 24 hours of incubation the zone of inhibition was measured.

**CFU Measurement**

Bacterial culture was collected for colony forming units (CFU) measurements on the solid medium. These samples were diluted at 10\(^9\) folds to get the better colonies on Petri plates. Samples were administered with different concentrations of silver nanoparticles (0, 20, 40, 60, and 80 µg/ml) and spreaded on nutrient agar plates. After incubation at 37°C for 24 hours, the numbers of CFU were counted.
Optical Density Measurement

The antibacterial efficacy of silver nanoparticles, in liquid nutrient growth medium was studied. Frozen bacterial cells were grown overnight in the nutrient medium to prepare cultures for the solution study. The bacteria cultures were grown in a shaker incubator at 37°C and 200 rpm. Shaking provided the bacteria aeration and homogeneity. The nanoparticles were dispersed in autoclaved deionized water by ultrasonication. Aqueous dispersion of silver nanoparticles of desired concentration was made. For this experiment, freshly grown inoculums (10⁴ cells/ml) of bacterial culture were incubated in the presence of a range of silver nanoparticles loading of 0, 20, 40, 60 and 80 µg/ml added in each flask to observe the bacterial cell growth pattern at 37°C and 150 rpm. Total solution volume used in each flask was 50 ml. Growth of pathogenic bacteria was indexed by measuring optical density (OD). OD measurements on the samples collected from the solution were carried out at λmax 600 nm against growth media control by UV-Vis spectroscopy after every 2 hrs and up to 24 hrs. Flasks containing 50 ml of all the initial reaction components except the silver nanoparticles was treated as control.

RESULTS AND DISCUSSIONS

Silver nanoparticles exhibit yellowish brown colour in aqueous solution due to excitation of surface plasmon resonance in silver nanoparticles [17]. As the fruit extract was mixed in the aqueous solution of the silver ion complex, it started to change the colour from watery to yellowish brown due to reduction of silver ions (Figure1) which indicates the formation of silver.

![Figure 1: Digital Photographs of (A) Vitis vinifera Extract (B) 1 mM AgNO3 without Vitis vinifera Extract (C) 1 mM AgNO₃ with Vitis vinifera Extract after 10 hrs of Incubation](image)

UV-Vis spectrograph of the colloidal solution of silver nanoparticles has been recorded as a function of time. Absorption spectra of silver nanoparticles formed in the reaction media after 10 hours has absorbance peak at 440 nm, broadening of peak indicated that the particles are polydispersed (Figure 2).

![Figure 2: UV-Vis Absorption Spectrum of Silver Nanoparticles Synthesized by Treating 1mM Aqueous AgNO3 Solution with 10% Vitis vinifera Extract after 10 hrs.](image)
The graphical representation of an average particle size distribution of silver nanoparticles shown in figure 2. From the graph it have been concluded that the average particle size of silver nanoparticles synthesized by fruit extract was 50 nm.

![Graph showing particle size distribution](image)

**Figure 3: Particle Size Distribution of Silver Nanoparticles Synthesized by Vitis vinifera Extract**

The biosynthesized silver nanostructure by employing fruit extract was further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Figure 3) and the structural view under the scanning electron microscope (Figure 4). The silver nanostructure synthesized by employing fruit extract was confirmed by the characteristic peaks observed in the XRD image. The analysis was carried out 2θ value ranging from 10° to 80°, 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°, 38.2°, 55.1° and 65.7° respectively, have been indexed as (111), (200), (220) and (311) respectively.

![XRD pattern](image)

**Table 1: XRD Data of Silver Nanoparticles Synthesized by Vitis vinifera Extract**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>2θ Value</th>
<th>Plane</th>
<th>Element</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32.844</td>
<td>111</td>
<td>Ag</td>
<td>Cubic</td>
</tr>
<tr>
<td>2</td>
<td>38.2349</td>
<td>200</td>
<td>Ag</td>
<td>Cubic</td>
</tr>
<tr>
<td>3</td>
<td>55.1855</td>
<td>220</td>
<td>Ag</td>
<td>Cubic</td>
</tr>
<tr>
<td>4</td>
<td>65.7352</td>
<td>311</td>
<td>Ag</td>
<td>Hexagonal</td>
</tr>
</tbody>
</table>

**Figure 4: XRD Pattern of Silver Nanoparticles Synthesized by Treating 10% Vitis vinifera Extract with 1 mM AgNO₃ Aqueous Solution**

XRD patterns were analyzed to determine peak intensity, position and width. Full-width at half-maximum (FWHM) data was used with the Scherrer formula. Average size of the particles synthesized was 50 nm with size range 10 to 80 nm with cubic and hexagonal shape. The typical XRD pattern reveled that the sample contains a mixed phase (cubic and hexagonal) structures of silver nanoparticles. The average estimated particle size of this sample was 50 nm derived from the FWHM of peak corresponding to 111 plane. The SEM image (Figure 5) showing the silver nanoparticles synthesized by the *vitis vinifera* extract further confirmed the development of silver nanostructures.
Further the silver nanoparticles synthesized by green route are found highly toxic against multi drug resistant human pathogenic bacteria at a concentration of 60 µg/ml (Figure 6). Silver nanoparticles exhibited antibacterial activity against *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Streptococcus pneumoniae* as it showed a clear inhibition zone whereas the standard antibiotics like Ampicillin, Tetracycline, Erythromycin and Vancomycin shows smaller zone of inhibition as compared to the nanoparticles treated discs (Table 1).

**Table1: Zone of Inhibition of Antibacterial Test of Silver Nanoparticles against Pathogenic Bacteria**

<table>
<thead>
<tr>
<th>Bioactive agent</th>
<th>Zone of inhibition (Diameter, mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>R. subtilis</em></td>
</tr>
<tr>
<td>Silver nanoparticles (60 µg/ml)</td>
<td>25 ± 0.1</td>
</tr>
<tr>
<td>Ampicillin (10 mcg/disc)</td>
<td>nil</td>
</tr>
<tr>
<td>Erythromycin (10 mcg/disc)</td>
<td>10 ± 0.1</td>
</tr>
<tr>
<td>Vancomycin (10 mcg/disc)</td>
<td>nil</td>
</tr>
<tr>
<td>Tetracycline (10 mcg/disc)</td>
<td>nil</td>
</tr>
</tbody>
</table>

Fig. 6: Images of Antibacterial Activities of Discs 60 µg/ML Silver Nanoparticles and Other Antibiotics on (A) *B. subtilis* (B) *E. coli* (C) *S. pneumonia* (D) *P. aeruginosa* (N=Nanoparticles, A= Ampicillin, E= Erythromycin, VA = Vancomycin, T = Tetracycline)
Optical densities as a function of time were measured periodically up to 24 h at 2 hr interval of the control and silver nanoparticles solutions of different concentration are plotted and shown in Figure 7. It has been observed that optical absorption in the growth medium decreased in comparison to the control with increasing concentration of silver nanoparticles. This has been attributed to the reduced growth of bacterial cells. Silver nanoparticles at concentration of 40 µg/ml and higher were found effective bactericides, but a complete bacterial growth inhibition has been witnessed at 60 µg/ml and above because there was virtually no bacterial growth, as a result optical absorption was insignificant.

Figure 8 shows the number of bacterial colonies grown on nutrient agar plates as a function of concentration of silver nanoparticles. The bacterial cell colonies on agar-plates were detected by viable cell counts. Viable cell counts are the counted number of colonies that are developed after a sample has been diluted and spread over the surface of a nutrient medium solidified with agar, contained in a petri dish.

![Figure 7: Optical density as a function of time in the solution studies (A) E.coli (B) Bacillus Subtilis (C) Pseudomonas aeruginosa (D) Streptococcus pneumoniae in LB medium inoculated with 10^7 CFU of bacteria in the presence of different concentrations of silver nanoparticles: (■) 0, (●) 20, (▲) 40, and (▼) 60 (◄) 80 µg/ml](image)

As the number of CFU have reduced significantly with increasing silver nanoparticles loading, therefore, virtually no CFU were observed in the samples containing silver nanoparticles loading of 60 µg/ml and higher. The bacterial growth inhibition trend found in CFU data has matched well with the results of OD.
The reduction of silver ions present in aqueous solution of silver complex during the reaction with the ingredients present in the *Vitis vinifera* extract observed by the UV-Vis spectroscopy revealed the presence of silver nanoparticles may be correlated with the UV-Vis spectra. UV-Vis spectroscopy is well known to investigate shape and size controlled of nanoparticles in aqueous suspension [23]. The XRD and SEM analysis showed the particle size between 10-80 nm as well the cubic structure of the nanoparticles.

Figure 8: Antibacterial Characterization by CFU as a Function of Silver Nanoparticles Concentration on Agar Plates Inoculated with (A) *E. coli* (B) *P. aeruginosa* (C) *B. subtilis* (D) *S. pneumoniae* after 24 H of Incubation

Bacteria have different membrane structure on the basis of which, they are classified as Gram negative or Gram positive. At the exterior, the gram-negative bacteria have a layer of lipopolysaccharide (LPS), followed underneath by a thin (about 7–8 nm) layer of peptidoglycan [11]. The overall charge of bacterial cells at biological pH values is negative because of excess number of carboxylic groups, which upon dissociation make the cell surface negative. Weak positive charges present on silver nanoparticles [23] are attracted towards negative charges on the lipopolysaccharides [16]. It is logical to state that binding of the nanoparticles to the bacteria depends on the surface area available for interaction. Nanoparticles have larger surface area available for interaction which enhances bactericidal effect than the large sized particles; hence they impart cytotoxicity to the microorganism [1].

Conversely, the cell wall in gram-positive bacteria is composed of a thick layer (about 20–80 nm) of peptidoglycan, consisting of linear polysaccharide chains cross-linked by short peptides to form a three dimensional rigid structure [27]. The rigidity and extended cross-linking not only provide the cell walls with fewer anchoring sites for the silver nanoparticles but also make them difficult to penetrate. The extent of inhibition of bacterial growth reported in this
study was dependent on the concentration of nanoparticles in the medium. Interaction between nanoparticles and the cell wall of bacteria would be facilitated by the relative abundance of negative charges on the gram-negative bacteria, which was congenial to the fact that growth of gram-negative bacteria was more profoundly affected by the silver nanoparticles than that of the gram-positive organisms. It is believed that silver nanoparticles after penetration into the bacteria inactivate their enzymes, generate hydrogen peroxide and cause bacterial cell death. The silver nanoparticles synthesized via green route are highly toxic to multidrug resistant bacteria hence has a great potential in medical applications.

CONCLUSIONS

The present study concluded that fruit Vitis vinifera can be used as an excellent source for biosynthesis for the silver nanoparticles in aqueous solution. The reduction of the metal ions through leaf extracts leading to the formation of silver nanoparticles of fairly well-defined dimensions. But the capabilities of the other plant part such as fruit as a capping and reducing agent is not tested and not well defined. In the present study we found that fruits can be also good source for synthesis of silver nanoparticles.

This green chemistry approach toward the synthesis of silver nanoparticles has many advantages such as, ease with which the process can be scaled up, economic viability, etc. Applications of such eco-friendly nanoparticles in bactericidal, wound healing and other medical and electronic applications, makes this method potentially exciting for the large-scale synthesis of other inorganic materials (nanomaterials). Toxicity studies of silver nanoparticles on human pathogen opens a door for a new range of antibacterial agents. We are currently pursuing further studies on proper identification and isolation of compounds responsible for the reduction and capping of the metal nanoparticles.

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