Green synthesis, characterization and antimicrobial activity of silver nanoparticles using methanolic root extracts of Diospyros sylvatica

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ABSTRACT

The current research study focuses to formulate the biosynthesized silver nanoparticles for the first time from silver acetate using methanolic root extracts of Diospyros sylvatica, a member of family Ebenaceae. TEM analysis revealed the average diameter of Ag NPs around 8 nm which is in good agreement with the average crystallite size (10 nm) calculated from X-ray Diffraction (XRD) analysis. Further the study has been extended to the antimicrobial activity against test pathogenic Gram (+) ve, Gram (-) ve bacterial and fungal strains. The bioinspired Ag-NP showed promising activity against all the tested bacterial strains and the activity was enhanced with increased dosage levels.

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Introduction

There is a vast scope in material research in interdisciplinary fields drawing new inspirations from biological systems. The environmental aspects have made the researchers to focus on methods to synthesize nanomaterials using green chemistry approaches i.e.; biological systems such as bacteria, fungi, algae, plants as well as biomolecules (Mandal et al., 2006; Xie et al., 2007; Kim et al., 2009; Siavash, 2011). One of the most considered methods is Green synthesis where plants and their crude extracts were employed for large-scale biosynthesis of nanoparticles, with controlled size and shape, thereby exploiting unique properties of metal tolerance by plants (Jha et al., 2009; Mukherjee et al., 2011). Further many biomolecules in plants such as alkaloids, alcholic compounds, aminoacids, enzymes, proteins, polysaccharides and vitamins could also be involved in bioreduction, formation and stabilization of metal nanoparticles (Iravani et al., 2009; Tolaymat et al., 2010; Makarov et al., 2014). The first report of the plant is from Medicago sativa (alfalfa) to synthesize gold and silver nanoparticles (Jae and Beom, 2009). From then onwards much attention has been paid to plants to synthesize nanoparticles.

Green synthesized silver nanoparticles are known to have diverse advantages in applications in medicine and biological
fields, and are known to be potent antimicrobial agents against various human and phytopathogenic microorganisms (Ratul and Satinder, 2013; Allaker and Ren, 2008; Rai et al., 2009; Shameli et al., 2012). Their extended application to medicine as anti-inflammatory, anti-angiogenesis, anti-platelet agents, dental materials, Cosmetics, nanodevice fabrication, as a dopant in photocatalysis field, biosensing, imaging and drug delivery have made them future model antimicrobial agents (Jia et al., 2007; Justin and Thomas, 2012).

The present investigation on Diospyros sylvatica (Gamble, 1997), a herbal tree used for the treatment of whooping cough, leprosy, dysentery, menstrual troubles, abdominal pains (Chopra et al., 1956; Watt and Breyer-Brandwijk, 1962). The genus has been reported to contain triterpenoids, polyphenols, steroids, naphthols and naphthoquinones (Mallavadhani et al., 1998). The antibacterial, antifungal and termite-resistant properties of Diospyros have all been attributed to the presence of naphthoquinones (Ganapathy et al., 2004). With the exception of gold and silver nanoparticles production using leaf broth of Diospyros kaki (Persimmon from china) (Jae and Beom, 2008), no other species of Diospyros has been worked out for the production of silver nanoparticles till date. We report for the first time, the synthesis and characterization of Ag NPs generated by the reduction of silver acetate using the methanolic root extracts of Diospyros sylvatica. The biologically synthesized nanoparticles were further analyzed and tested against several different pathogenic microorganisms.

1. Materials and methods

1.1. Preparation of Diospyros sylvatica root extract

The plant material Diospyros sylvatica (roots) was collected in and around Vizianagaram district of Andhra Pradesh, India and was authenticated by Prof. T. Pulliah, taxonomist, Department of Botany, Sri Krishnadevaraya University, Anantapur, India. The specimens were preserved in the Department of Botany, Andhra University, Visakhapatnam, India (Herbarium code = SKU). The collected plant materials were air-dried and coarsely powdered in Willey mill. Later soxhlet extracted with methanol and subsequently concentrated under reduced pressure to get the crude residue. The dried extract is dissolved in dimethyl sulfoxide (DMSO) and stored in refrigerator for further use. All chemicals and solvents used were of analytical grade and are obtained from Ranbaxy Fine Chemicals and Merck Ltd., Mumbai.

1.2. Synthesis of Ag NPs using Diospyros sylvatica root extract

In the present study, the metal ion concentration was standardized to 0.01 mol/L for studying the amount of root extract and reaction time. Initially, 10 mL of metal ion concentration of 0.01 mol/L was mixed with 2 mL of root extract to study the effect of reaction time. After optimization of the reaction time, the effect of amount of root extract (varying from 2 to 10 mL) was recorded, by fixing the reaction time and metal ion concentration. After optimization of parameters such as metal ion concentration, amount of root extract and reaction time, the Ag NPs were separated by centrifuge with 10,000 rpm for 8 min and washed with D.I water at least three times and then dried at 60°C for 3 hr for further characterization. For antimicrobial applications, the Ag NPs were redispersed in D.I water without drying.

1.3. Characterization of the synthesized silver nanoparticles

The biosynthesized Ag NPs were characterized by various instrumental analyses. The formation and optimization of Ag NPs synthesis were monitored by UV–Vis (Shimadzu UV–Vis 2450) spectroscopy. Crystalline metallic silver was examined by (PANalytical. XPERT–PRO) X-ray diffractometer, using Cu Kα radiation (λ = 0.1546 nm). The morphological analysis of Ag NPs was carried out using a Zeiss EVO 18 (Carl Zeiss SMT Ltd.) Scanning Electron Microscopy (SEM). Transmission Electron Microscopic (TEM) analysis is done by using a TEM, PHILIPS, CM200 with an accelerating voltage of 200 kV in order to find the exact morphology and size of the silver nanoparticles.

1.4. Anti-microbial activity of biosynthesized Ag NPs

The biologically synthesized silver nanoparticles of average size around 8 nm were tested against pathogenic Gram (+) and Gram (−) bacteria, fungi, and yeast. Stock solutions of the test residual extract of bioinspired silver nanoparticles were prepared in concentrations of 100, 50, 25, 12.5, 6.25, 3.125 μg/mL, respectively. Using sterile petriplates, 100 μL of each concentration were placed in cups of each petriplate. Two cups were allotted for control (DMSO) and standard respectively in every petriplate. Antibiotic chloramphenicol (100 μg/mL) was used as standard. The prepared petridishes were incubated for 16 hr at 30°C and later allowed to solidify in a refrigerator for about 30 min. In each plate, 4 cups were made at equal distances with 5 mm diameter. The prepared petridishes were then transferred into sterile 15 cm diameter petriplates. The medium in the plates was allowed to set at room temperature for about 10 min and later allowed to solidify in a refrigerator for about 30 min. In each plate, 4 cups were made at equal distances with 5 mm diameter. The medium in the plates was allowed to set at room temperature for about 10 min and later allowed to solidify in a refrigerator for about 30 min. In each plate, 4 cups were made at equal distances with 5 mm diameter. The medium in the plates was allowed to set at room temperature for about 10 min and later allowed to solidify in a refrigerator for about 30 min. In each plate, 4 cups were made at equal distances with 5 mm diameter.
1.4.2. Determination of MIC by macrodilution broth method

The Minimum Inhibitory Concentration (MIC) was determined by macrodilution broth method (Kavanagh, 1992; Edwin et al., 1985). Using Muller–Hinton broth (Hi-media), 5 mL of broth along with 0.5 mL of bacterial inoculum (Obeidat et al., 2012) and different concentrations of 1 mL test sample (100, 50, 25, 12.5, 6.25, 3.125 μg/mL) were added respectively and incubated for 8 hr at 30°C and then examined for viability by spread plate technique. Two tubes were incubated with standard (antibiotic chloramphenicol—100 μg/mL) and control (DMSO). The experiments were carried out in duplicate.

1.4.3. Antifungal activity

The nutrient PDA medium (Hi-media) was prepared and inoculated with 0.5 mL of aqueous suspension of the above mentioned fungal test organisms, which were prepared from 48 hour cultures and then transferred into sterile petridishes. The medium in the plates was allowed to set at room temperature for 10 min. In each plate, 4 cups of 5 mm diameter were made at equal distances. Stock solutions of the test residual extract were prepared in concentrations of 100 μmol/L, 50, 25, 12.5, 6.25; 3.125 μg/mL. One hundred microliters of each of the above stock concentrations was poured into cups using sterile pipettes. In each plate one cup was used for control (DMSO) and standard. Nystatin (100 μg/mL) is used as reference standard. The arranged petridishes were incubated for 48 hr at 37°C and then examined by measuring the zones of inhibition. The experiments were run in duplicate and the average diameter of the zones of inhibition was recorded and the results are shown in Table 2.

2. Results and discussion

The formation and optimization of Ag NPs was monitored using UV–Vis spectroscopy by measuring the absorbance in the range of 200–800 nm, by varying the reaction time and amount of Diospyros sylvatica root extract, the silver acetate ion concentration was fixed to 10 mmol/L at room temperature in order to complete the reaction. The change in color indicates the formation of Ag NPs which was further confirmed by the appearance of the SPR band between 400 to 800 nm. By fixing the amount of root extract as 2 mL and increasing the reaction time from 30 min to 4 hr, the intensity of SPR band has increased and got shaper indicating small size of Ag NPs (Fig. 1a). Further increasing in the intensity of SPR showed broad band implying agglomeration of Ag NPs or increase in particle size. We have investigated the effect of the amount of root extract from 2 to 10 mL by fixing metal ion concentration as 10 mmol/L and reaction time 4 hr. With increasing amount of root extract from 2 to 8 mL, there is slight variation in λmax values signifying the changes in particle size, owing to change in concentration of extract solution (Fig. 1b). On increasing the amount of root extract to 10 mL, displayed broadening of peak indicating the inhomogeneous shape and size of the particles. From the above optimized conditions, we have synthesized the Ag NPs and subjected for further characterization and evaluation of antimicrobial activities.

The present study has accomplished nanoparticle synthesis by using plant (methanolic) extracts of Diospyros sylvatica (roots) which has been thoroughly exploited for its secondary metabolites and their medicinal significance. Besides naphthoquinones and naphthalene derivatives that have been isolated previously from this genus, a survey of the literature revealed that triterpenes namely a-amyrin, lupeol and betulin were also reported from the bark of this genus (Ganapaty et al., 2004), which might have contributed to the formation of nanoparticles. It has been reported that, various secondary metabolites like terpenoids, polyphenols, sugars, alkaloids, phenolic acids, ...
and proteins play an important role in the bioreduction of metal ions, yielding nanoparticles (Makarov et al., 2014).

In the present study the mechanism for the formation of Ag NPs using root extract of *Diospyros slyvatica* might be attributed from naphthoquinones and naphthalene derivatives. Quinones are oxidized derivatives of aromatic compounds and are often readily made from reactive aromatic compounds with electron-donating substituents such as phenols and catechols, which increase the nucleophilicity of the ring and contributes to the large redox potential needed to break aromaticity. Depending on the quinone and the site of reduction, reduction can either rearomatise the compound or break the conjugation. Some serve as electron acceptors in electron transport chains such as those in photosynthesis (plastoquinone, phylloquinone), and aerobic respiration (ubiquinone). Naphthoquinones are also a class of natural phenolic compounds formed on a C6–C4 skeleton. Therefore the formation of Ag nanoparticles can be attributed to the presence of secondary metabolites like triterpenes or quinones which are reported to be present in the crude extracts.

The morphology and crystallite structure of Ag NPs using *Diospyros sylvatica* root extract has been characterized by SEM and TEM and XRD analysis. The nature of Ag NPs were identified to be crystalline using XRD analysis with Cu Kα target at 2θ = 20–80° C. The XRD pattern of the silver nanoparticles is shown in Fig. 2. There were four well-defined characteristic diffraction peaks at 38.3°, 44.6°, 64.8°, and 77.6° respectively corresponding to (111), (200), (220), and (311) planes of face-centered cubic crystal structure of metallic silver (JCPDS card file no.03-0921). The interplanar spacing (d_hkl) values (2.348, 2.030, 1.437, 1.229, and 1.175 Å) calculated from the XRD spectrum of silver nanoparticles was in agreement with the standard silver values. The crystallite size of Ag nanoparticles was estimated by applying the Scherrer’s equation,

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

where, \( D \) denotes the average crystallite size, \( K \) Scherer’s constant (\( K = 0.94 \)), \( \lambda \) is wave length of the Cu-Kα irradiation (0.1546 nm), \( \beta \) is full-width at half-maximum and \( \theta \) is the diffraction angle for the (111) peak of Ag NPs.

The resultant in the mean crystallite size of Ag NPs according to Scherrer equation is about 10 nm. The synthesized Ag NPs were spherical in shape as observed in the Scanning Electron Microscope image. The particle sizes observed from SEM images are in the range of 10 to 40 nm (Fig. 3a). SEM image of Ag NPs shows the existence of some large particles, which might be due to aggregation or overlapping of smaller particles. The morphology in terms of size and shape of Ag NPs visualized from TEM analysis (Fig. 3b) exhibited uneven shapes with almost similar size with rough edges. Most of Ag NPs observed as spherical with sizes ranging from 5 to 16 nm. The average particle size calculated from TEM image is about 8 nm which is in good agreement with XRD analysis.

### 2.1. Antibacterial

The bioinspired Ag-NPs showed excellent antibacterial activity against all the tested bacterial strains. They exhibited maximum activity against *Bacillus pumilis*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, moderate activity towards *Staphylococcus*...
aureus, Klebsiella pneumoniae, and Escherichia coli, and mild activity towards Streptococcus pyogenes and Proteus vulgaris. The values of Zone of Inhibition against various tested bacterial strains such as pathogenic Gram (+)ve and Gram (-)ve with different concentrations of Ag NPs were given in Table 1. The activity was enhanced with increased dosage levels.

2.2. MIC

Further the work has been extended to determine the MIC for the two bacterial strains which displayed maximum susceptibility. MIC of biosynthesized Ag NPs including standard antibiotic using macro dilution method against Bacillus pumilis (MTCC-441) as shown in Fig. 4a. The plates inoculated with Bacillus pumilis displayed MIC between 25–50 μg/mL and the plates above 25 μg/mL showed no growth (Fig. 4b), similarly for Pseudomonas aeruginosa, the MIC was found to be between 12.5–25 μg/mL. A concentration-dependent bacterial growth inhibition was observed with increase in silver nanoparticle concentration. A concentration of 25 μg/mL was able to inhibit both strains of the bacteria’s growth to some extent with fewer bacterial colonies formed on these plates, compared to the bacterial lawn present in the control plates. The AgNP, at concentrations of 50 μg/mL and 100 μg/mL, completely killed Bacillus pumilis and Pseudomonas aeruginosa, which is very clearly visualized from the plates that shows complete absence of colonies. Fig. 4b1–b4 shows the effect of Ag NP on the growth inhibition of B. pumilis.

Silver has a greater affinity to sulfur and phosphorus containing bio-molecules in the cell. The high bactericidal activity is certainly due to the silver cations released from Ag NPs that act as reservoirs for the Ag+ bactericidal agent, which changes membrane permeability and integrity. Thus the desired sites for silver nanoparticles are the sulfur containing proteins in the cell membrane and phosphorus containing elements like DNA (Mc Farland, 2012; Jung et al., 2008). The various mechanisms by which silver ions were thought to act, include structural changes in the cell wall of bacteria; interactions with thiol groups in proteins and...
enzymes, and interruption of DNA replication due to damage of the DNA (Fabrega et al., 2009; Fayaz et al., 2010). However, further studies are still required to know the exact mechanisms of antibacterial activity of the nanoparticles.

2.3. Antifungal

The values of Zone of Inhibition against various tested fungal strains with different concentrations of Ag NPs are given in Table 2. The green synthesized Ag-NP showed promising activity against all the tested fungal strains. They displayed maximum activity against *Aspergillus niger* and *Pencillium notatum*, moderate activity towards *Aspergillus flavus*, Saccharomyces cerevisiae and mild activity towards *Candida albicans*. The activity was enhanced with increased dosage levels. The antifungal activity can be attributed to silver cations causing membrane depolarization, pits in the cell wall and pores in the plasma membrane leading to the destruction of the fungal membrane integrity and inhibition of the fungal cell cycles (Kvitek et al., 2008; Kim et al., 2007). Fig. 5 shows the Zone of Inhibition of Ag NPs with different concentrations against *Klebsiella pneumoniae* and *Aspergillus niger* pathogens.

3. Conclusions

The present study demonstrates the use of unreported methanolic root extracts of *Diospyros sylvatica* for the consistent and quick synthesis of silver nanoparticles from silver acetate. Variation in reaction conditions affected nanoparticle synthesis where the reaction mixtures displayed typical colors and UV-visible spectra, characteristic of silver nanoparticles. The biosynthesized nanoparticles produced by this novel, cost-effective, non-toxic, environmentally safe protocol were characterized by a variety of standard analytical techniques like XRD, SEM, TEM and were further tested against bacterial and fungal strains. Nanoparticle synthesis is a novel research area to search for an eco-friendly manner and green materials for potential applications in the fields of medicine and drug delivery.

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