

Biosynthesis of Silver Nanoparticles Using the Fruit Extract of *Diospyros Malabarica* (Desr.) Kostel And Its Antibacterial Activity Against Human Pathogens

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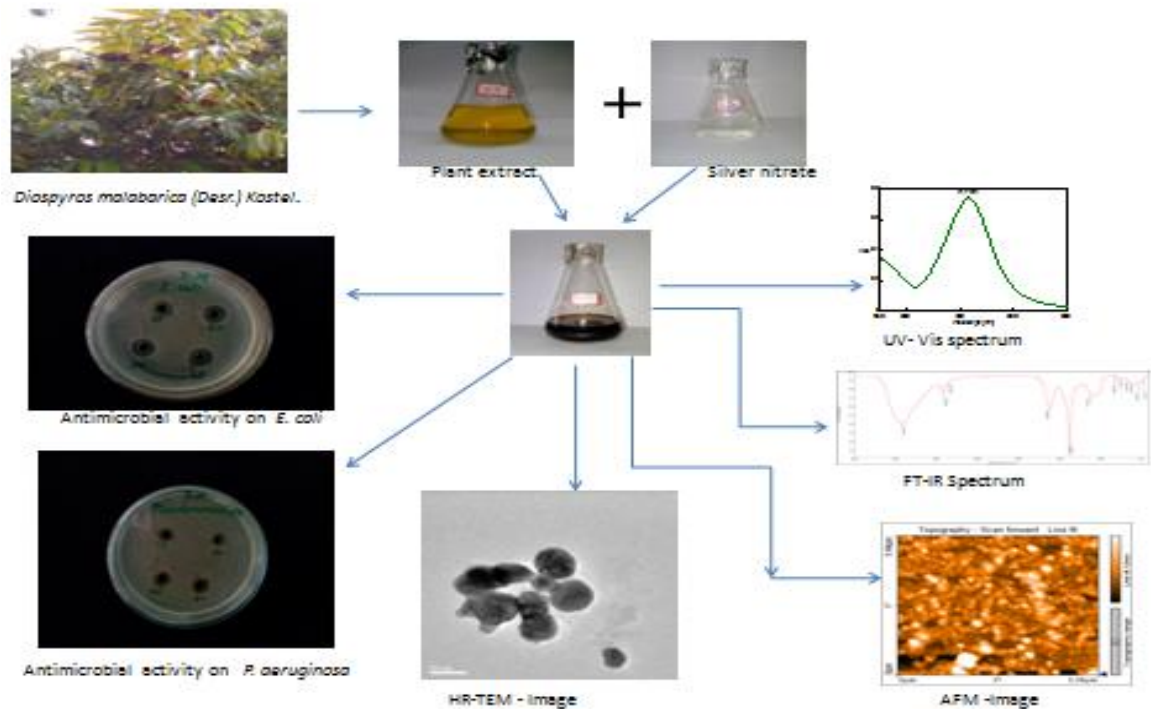
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ABSTRACT

Nanobiotechnology is a quickly rising scientific field of generating nanoparticles of about nanometer scale level (1 to 100 nm). The multidrug-resistant strains are a main problem in the control of infections in hospitals. Among the nanoparticles, silver nanoparticles are playing a major duty in the field of biomedical nanotechnology and nanomedicine. Silver nanoparticle has a natural antimicrobial effect against many pathogens such as bacteria and fungus. In this present study, we tried to set up eco-friendly, cost effective small sized silver nanoparticles (AgNPs) by using fruit extract of selected plant. These AgNPs are powerful tool against multidrug-resistant bacteria. The synthesized silver nanoparticles were characterized by Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM) and high resolution transmission electron microscopy. FTIR data reveals the possible functional group of biomolecules involved in the bioreduction and capping for efficient stabilization of Ag-NPs. Atomic force microscopy and High resolution transmission electron microscopy studies showed that the spherical shape silver nanoparticles with size ranges from 05nm to 50nm. Further antibacterial and antifungal activity of synthesized silver nanoparticles against multidrug resistant strains was determined by agar well diffusion assay and Minimum Inhibitory Concentration (MIC) was estimated. In summary the fruit mediated AgNPs showed acceptable size and possess very good antimicrobial activity which makes them a strong source of antimicrobial agent.

Keywords: Biosynthesis, *Diospyros malabarica* (Desr.) Kostel., HR-TEM, AFM, Antibacterial activity.



1. Introduction

Nanoparticles are viewed as the fundamental building blocks of nanotechnology. [1] Nano size particles are quite unique in nature because nano size increase surface to volume ratio and also its physical, chemical and biological properties are different from bulk material. So the main aim to study its minute size is to trigger chemical activity with distinct crystallography that increases the surface area [2 -3].

Silver nanoparticles (Ag-NPs) have become focus of intensive research owing to their wide range of application in areas such as catalyst, optics, antimicrobials and biomaterial production. Ag-NPs exhibit new or improved properties depending upon their size, morphology and distribution [4]. The processes used for nanoparticle synthesis are chemical, physical and recently developed biological method. Chemical methods have various drawbacks including the use of toxic solvents, generation of hazardous by-products and high energy consumption, which pose potential risks to human health and to the environment. Most of the physical methods deal with enormous consumption of energy to maintain the high pressure and temperature employed in the synthesis procedure. Therefore, the biological method has an advantage over chemical and physical method of nanoparticle synthesis, as it is cost effective and environmentally friendly [5]. The major biological systems involved in this are bacteria; fungi [6] and plant extract [7]. In recent years, the biosynthesis of nanoparticles using plant extracts has gained more importance. The synthesis and application of Ag-NPs from several plants have been studied by many researchers [8-11].

Diospyros malabarica (Desr.) Kostel belongs to the family Ebenaceae, is evergreen and it has high medicinal value used in treatment of dyspepsia, leprosia, diarrhoea, dysentery, haemorrhages, skin burning, diabetes, spermatorrhea, vaginal diseases, wounds, flatulence, proleptis, scabies and intermittent fever. In this study, we evaluated the synthesis of silver nanoparticles using the fruit extract of *D. malabarica* and their antibacterial activities against human pathogenic bacteria.

2. Materials and Methods

2.1 Preparation of fruit extract

Fruits of *D. malabarica* were collected from the Botanical garden at Karnatak University Dharwad, Karnatak, India. Fruits were washed 3-4 times with tap water followed by double distilled water to remove dust and impurities. Fruits were shade dried to remove the residual moisture and weighed about 30 g. The weighed fruits were cut into small pieces and boiled in a glass beaker containing 300 ml of sterile distilled water for 30 minutes [12]. The aqueous extract was separated by filtration with Whatman No. 1 filter paper and stored in refrigerator at 4°C for further use.



Fig. 1 Experimental plant

2.3 Preparation of Silver nitrate solution:

1mM AgNO_3 solution was prepared by dissolving 0.08493g AgNO_3 (AR grade) in 500ml double distilled water.

2.4 biosynthesis of Silver nanoparticles

For reduction of silver ions, 5 ml of plant extract was added to 250ml Erlenmeyer flask containing 95 ml of 1mM aqueous AgNO_3 solution. Simultaneously, the reaction mixture was adjusted to pH 9. Then the flask containing reaction mixture was incubated at 40-50°C, resulting in the formation of pale yellow to dark brown solution indicating the synthesis of silver nanoparticles [13].

2.5 Characterization

The bioreduction of silver ions in the solution was monitored by using UV- Vis spectroscopy (Jasco V-670 UV-Vis NIR spectrophotometer) operated at resolution of 1nm. The solution containing bioreduced silver ions was centrifuged at 3000 rpm for 45 min to remove the unwanted biomass residue; the resulting suspension was then dispersed in 10ml of double distilled water and centrifuged again at the same condition. Centrifugation process was repeated for 4-5 times to obtain the pellet of silver nanoparticles free from any biomass residue. A sample taken from pellet was dispersed on a slide and dried slide was observed on contact mode of AFM. The pellet thus obtained was redispersed in double distilled water and oven dried at 50°C to obtain the powder. The powder was used for FTIR and HRTEM (TECNAI 20 G2-electron microscope) analysis.

2.6 Antibacterial Activities

The silver nanoparticles synthesized using *D. malabarica* fruit extract was tested for antibacterial activity by agar well diffusion method against human pathogenic Gram negative *Escherichia coli* (NCIM 2931) and *Pseudomonas aeruginosa* (NCIM 5029). This method depends on the radial diffusion of an antibiotic

from the well through semisolid agar layer in Petri plate, which prevents the growth of bacteria in a circular area or the zone around the well.

The pure cultures of bacteria were sub-cultured on nutrient broth at 30⁰C. The hot sterile medium was poured into the sterile Petri plates to form 2-3mm thick uniform layer and allowed to solidify. Each strain was swabbed uniformly on the individual plates using sterile cotton swab. Wells of size 6 mm were made on nutrient agar plates using gel puncture. 15, 30, 45 and 60 µl of nanoparticle solution was poured onto each well on all plates using micropipette. After incubation at 37⁰C for 24h, the diameter of zone of inhibition was measured in millimeters and tabulated.

3. Results and Discussion

The present study was carried out to synthesize Ag-NPs using fruit extract of *D.malabarica* to study their biological properties. Nanoparticles are generally characterized by their size, shape, surface area and dispersity. Homogeneity of these properties is important in many applications [14]. when the fruit extract was mixed with AgNO₃, pH was adjusted to 9 and incubated at 40-50⁰C. The color of the reaction mixture changes from pale yellow to dark brown within few seconds as shown in fig-1, indicating the formation of AgNPs. It is an efficient and rapid method, which was very well explained by other researchers who worked with different plant system [15-18]. The change in color was due to the excitation of surface Plasmon resonance in the metal nanoparticles [11]. Our results are in conformity with Kiruba et al., 2012, who reported the formation of AgNPs with adjustment of pH 10. Acidic condition suppresses the formation of AgNPs (pH 2 and 4); whereas the slight basic condition enhances the formation of the nanoparticles (pH 6-8). Large sized nanoparticles were formed at lower pH which is indicated by the color change and aggregation in the solution, but at pH 8-10 small and highly dispersed nanoparticles were formed [12].

UV-Vis absorption spectroscopy is one of the main tools to analyze the formation of metal nanoparticles in aqueous solution [13]. The AgNPs formation was confirmed by UV-Vis spectrophotometric analysis .The analysis confirmed surface plasmon resonance peak at 417nm. (Figure 2). This SPR bands which was specific to the substrate or an organism involved in the biosynthesis of AgNPs. It clear that bioreduction of silver ions occurred in presence of fruit extract.

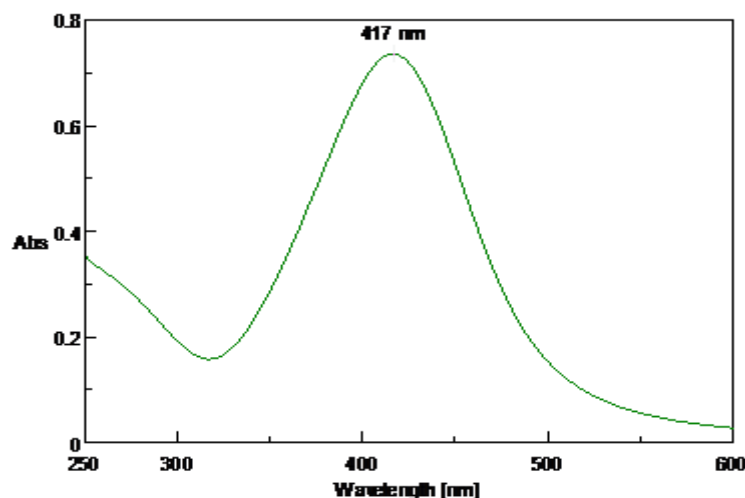


Figure 2: UV-Vis spectrum of AgNPs in an aqueous solution.

FTIR data reveals the possible functional group of biomolecules involved in the bioreduction and capping for efficient stabilization of AgNPs synthesized using *D. malabarica*. The FTIR spectrum (Figure 3) showed the peaks at 3426.10 cm^{-1} , 2925.22 cm^{-1} , 2857.89 cm^{-1} , 1622.56 cm^{-1} , 1384.51 cm^{-1} , 1115.02 cm^{-1} , 825.20 cm^{-1} and 673.75 cm^{-1} . The broad intense peak at 3426.10 cm^{-1} is associated with inductive O-H stretching bonded to alcohols and phenols arising from carbohydrates and proteins present in the sample [24]. The two small peaks at about 2925.22 cm^{-1} and 2857.89 cm^{-1} are found to be associated with the carboxylic acid and methylene antisymmetric and symmetric vibration of hydrocarbon [25]. The band 1622.56 cm^{-1} was characteristics of amide associated with the stretch of carbonyl group coupled to amide linkage [26]. The peak at 1384.51 cm^{-1} arises due to NO_3^- and associated with C-N stretching vibration of aliphatic and aromatic amines indicating the presence of protein in small concentration [27]. The absorption peak at 825.20 cm^{-1} was associated with C-C and C-H phenyl ring substitute and 673.75 cm^{-1} was with aromatic compounds. [28]. These functional groups associated with polyphenolic compounds as flavonoids and also to proteins indicate their involvement in capping of formed nanoparticles leading to stabilization.

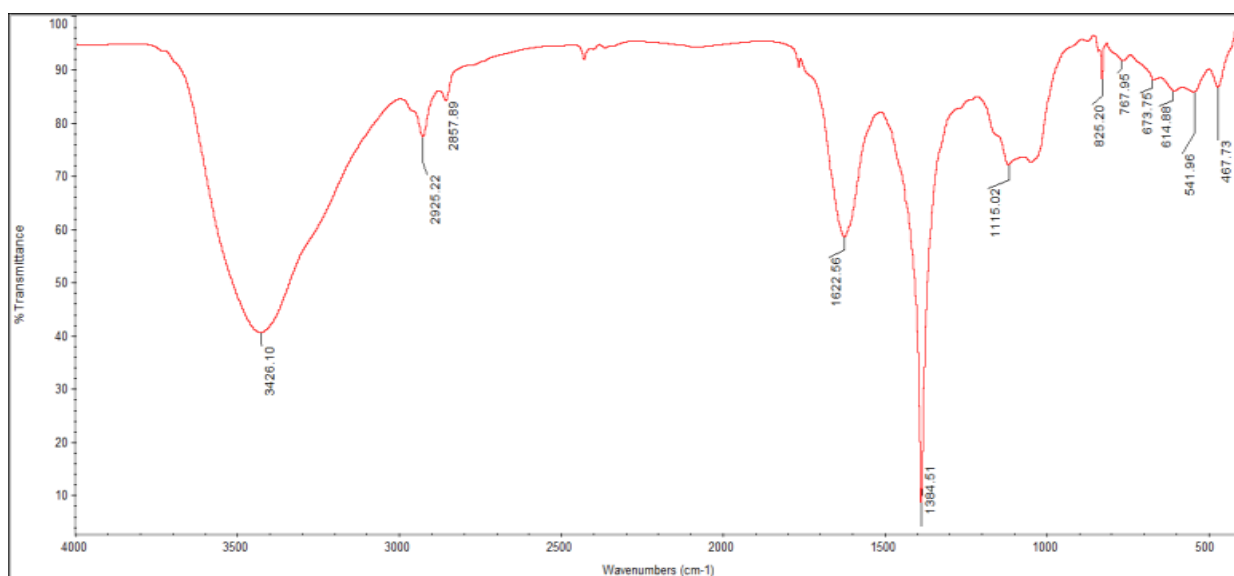


Figure 3: FTIR spectrum of silver nanoparticles.

Table 1: FTIR absorption peaks value and their functional groups

Sl. No.	Absorption peak (cm^{-1})	Functional groups
1	3426.10	O-H stretching H-bonded alcohols and phenols
2	2925.22	O-H stretch, carboxylic acid
3	2857.89	Asymmetric stretching of the C-H group
4	1622.56	Amide group from carbonyl stretch in proteins
5	1384.51	C-C , C-N stretching
6	1115.02	Aromatic amines
7	825.20	C-C and C-H phenyl ring substitute
8	673.75	Aromatic compounds

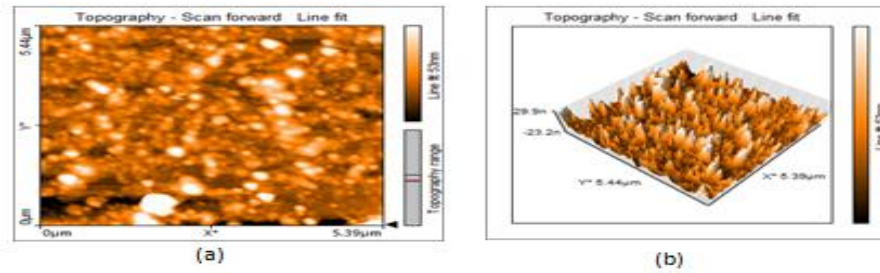


Figure 4: AFM images of silver nanoparticles synthesized from *D. malabarica* (Dear.) Kostel. Topography (a) and 3D image (b).

The synthesized silver nanoparticles were characterized by AFM. The topographic image of silver nanoparticles was shown in Figure 4(a). Where the formation of spherical silver nanoparticles and its agglomeration was clearly observed and Figure 4(b) represents three dimensional views of synthesized silver nanoparticles. The size of the silver nanoparticles ranges from 05-50 nm. AFM images were taken with silicon cantilevers with force constant and the particle size was measured using line profile. This could be attributed to the fact that the compounds present in the leaf extract were responsible for the particle morphology and were kinetically controlled [29].

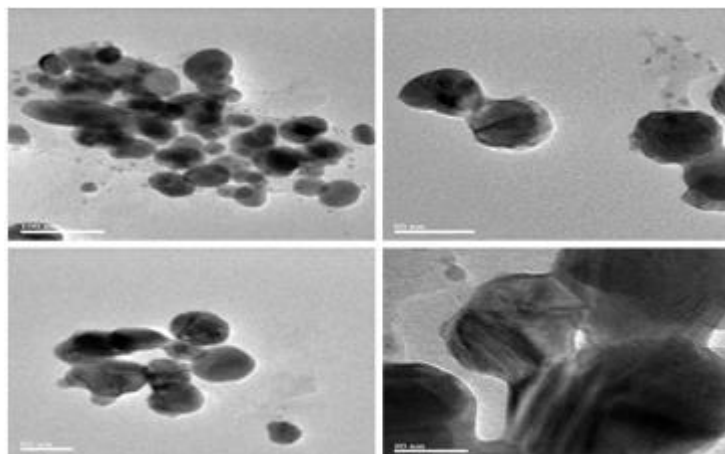


Figure 5: HR TEM images of biosynthesized silver nanoparticles

The silver nanoparticles were further characterized by HRTEM micrograph, these Silver nanoparticles showed spherical shape with the size range from 05 to 50nm. (Figure 5) also shows that the biomolecules of fruit extract bounded with the nanoparticles acts as capping agents to hinder further oxidation of nanoparticles. Hence from the HRTEM analysis, it was confirmed that all the particles (AgNPs) exist in the nanoscale range and possess spherical shape.

Antibacterial activity of silver nanoparticles

The present research reveals that the silver nanoparticles synthesized by fruit extract of *D. malabarica* exhibited antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*. The inhibitory zone of two replicates of diameter was measured and tabulated (Table 2). The antibacterial activity of silver nanoparticles tested against gram negative *Escherichia coli* showed an inhibition zone of 12, 13, 14 and 15.5 mm for concentration of 15, 30, 45 and 60 μ l respectively and 9, 10.3, 11.4 and 12mm for concentration of 15, 30, 45, and 60 μ l respectively against gram negative *Pseudomonas aeruginosa*

(Figure 6). It was notice that the zone of inhibition increased with increased concentration of Ag NPs [30-31]. Similarly, Dipankar and Murugan (2012) have reported dose- dependent inhibition by Ag NPs synthesized from Iresine herbstii leaf aqueous extract [32]. This might be due to the denaturation of bacterial cell wall, blocking bacterial respiration, destabilization of outer membrane, and depletion of intracellular ATP [33]. E. coli and P. aeruginosa are gram negative bacteria, thus cell wall possesses thinner peptidoglycan layer [34]. The high bactericidal activity is certainly due to the silver cat ions released from Ag nanoparticles that act as reservoirs for the Ag⁺ bactericidal agent. Changes in the bacterial membrane structure bacteria as a result of the interaction with silver cat ions leads to the increased membrane permeability [35-36] Lin et al. explained that in general, silver ions from silver nanoparticles are believed to become attached to the negatively charged bacterial cell wall and rupture it, which leads in to denaturation of protein and finally cell death [37]. The attachment of either silver ions or nanoparticles to the cell wall causes accumulation of an envelope protein precursor, which results in dissipation of the proton motive force. On the other hand, silver nanoparticles exhibited destabilization of the outer membrane and rupture of the plasma membrane, thereby causing depletion of intracellular ATP [38] Sacker et al. reported that for E. coli (ATCC 10536) and Staphylococcus aureus (ML 422), silver nanoparticles demonstrated greater bactericidal efficiency compared to penicillin [39]. Silver has a greater affinity to react with sulphur or phosphorus-containing biomolecules of the cell. Thus, sulphur-containing proteins the membrane or inside the cells and phosphorus-containing elements like DNA are likely to be the preferential sites for silver nanoparticle binding [40-41].

Table 2: Antibacterial activity of biosynthesized silver nanoparticles against E. coli and P. aeruginosa.

Silver nanoparticle sample	Zone of inhibition(mm)against pathogenic bacteria							
	E. coli				P. aeruginosa			
D. malabarica (Desr) Kostel.	15µl	30µl	45µl	60µl	15µl	30µl	45µl	60µl
	12 mm	13mm	14 mm	15.5mm	9mm	10.3mm	11.4mm	12mm

Silver nanoparticles are positively charged and it will attach to the negative charged bacteria by the electrostatic attraction in the cell wall and silver nanoparticles associated with thiol groups of cell wall results in the generation of reactive oxygen species and disrupting the cell [42]. Close association of silver nanoparticles with bacterial cell wall and forms the pits in the cell wall affecting the permeability and finally cause cell death. The smaller size of silver nanoparticles facilitates their easy entry into the bacterial cell and affects the intracellular processes such as DNA, RNA and protein synthesis. Silver nanoparticles were binding with bacteria depends on the surface area for the interaction. Smaller particles affect the larger surface area of the bacteria thus it has more antibacterial activity than the larger sized nanoparticles [43-45].

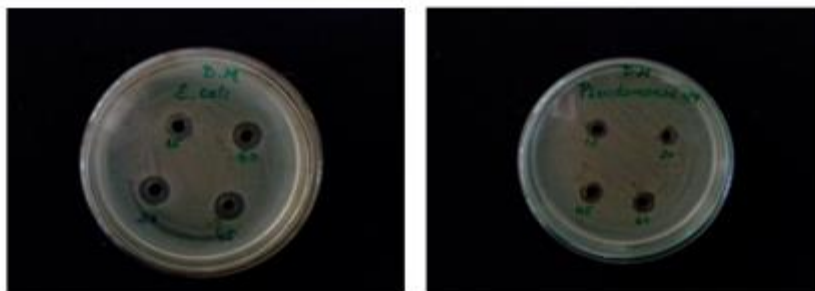


Figure 6: Antimicrobial activity of Biosynthesized nanoparticles

Conclusions:

Synthesis of ecofriendly, stable and spherical shaped nanoparticles using aqueous fruit extract of *Diospyros malabarica* (Desr.) Kostel has been described in the present research article. This protocol offers a viable and an ecofriendly way for fabrication of benign nanoparticles as it is a simple and carried out at room temperature without any huge inputs in terms of energy and waste. It is advantageous over the microbial synthesis as it is carried out using in aqueous solutions at ambient temperature, without any toxic chemicals in lesser time and could be exploited for developing cost effective biosynthesis of Ag nanoparticles. Role of phytochemicals such as flavonoids, tannins, saponins and triterpenoids may be significant in reduction and stabilization of the silver nanoparticles. The synthesized silver nanoparticles were evaluated for antibacterial activity against human pathogens viz. *Escherichia coli* and *Pseudomonas aeruginosa*. The biosynthesized silver nanoparticles showed antimicrobial activity against both the pathogens.

Acknowledgements

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