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ORIGINAL RESEARCH

Biosynthesis of silver nanoparticles using stem bark extracts of *Diospyros montana* and their antioxidant and antibacterial activities

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Abstract

The present study reports an eco-friendly, biosynthesis of silver nanoparticles (AgNPs) using stem bark extract of *Diospyros montana*. Initially, the synthesis of AgNPs was confirmed by visual observation as color change. Further, the morphology of the biosynthesized nanoparticles, average size and presence of elemental silver were characterized by UV–Visible spectroscopy, scanning electron microscopy, transmission electron microscopy, energy dispersive X-ray and dynamic light scattering spectrometer. Qualitative phytochemical screening and FTIR spectral peaks supported the role of phytochemicals in bark extract for the metal reduction, stabilization and capping of silver nanoparticles. XRD studies demonstrated that crystalline nature and their average size of nanoparticles was 28 nm as determined by Scherrer's formula. The antioxidant ability of AgNPs and plant extract was analyzed using DPPH and Hydrogen peroxide assay. The percentage of DPPH and H_2O_2 activity was increased with increasing concentration of AgNPs. In vitro antibacterial effect of various concentration of AgNPs was investigated against both Gram positive (*B.subtilis* and *S.aureus*) and Gram negative (*E.coli* and *K.aerogenes*) bacterial strains. The result shows that biosynthesized AgNPs have significant antibacterial activity.

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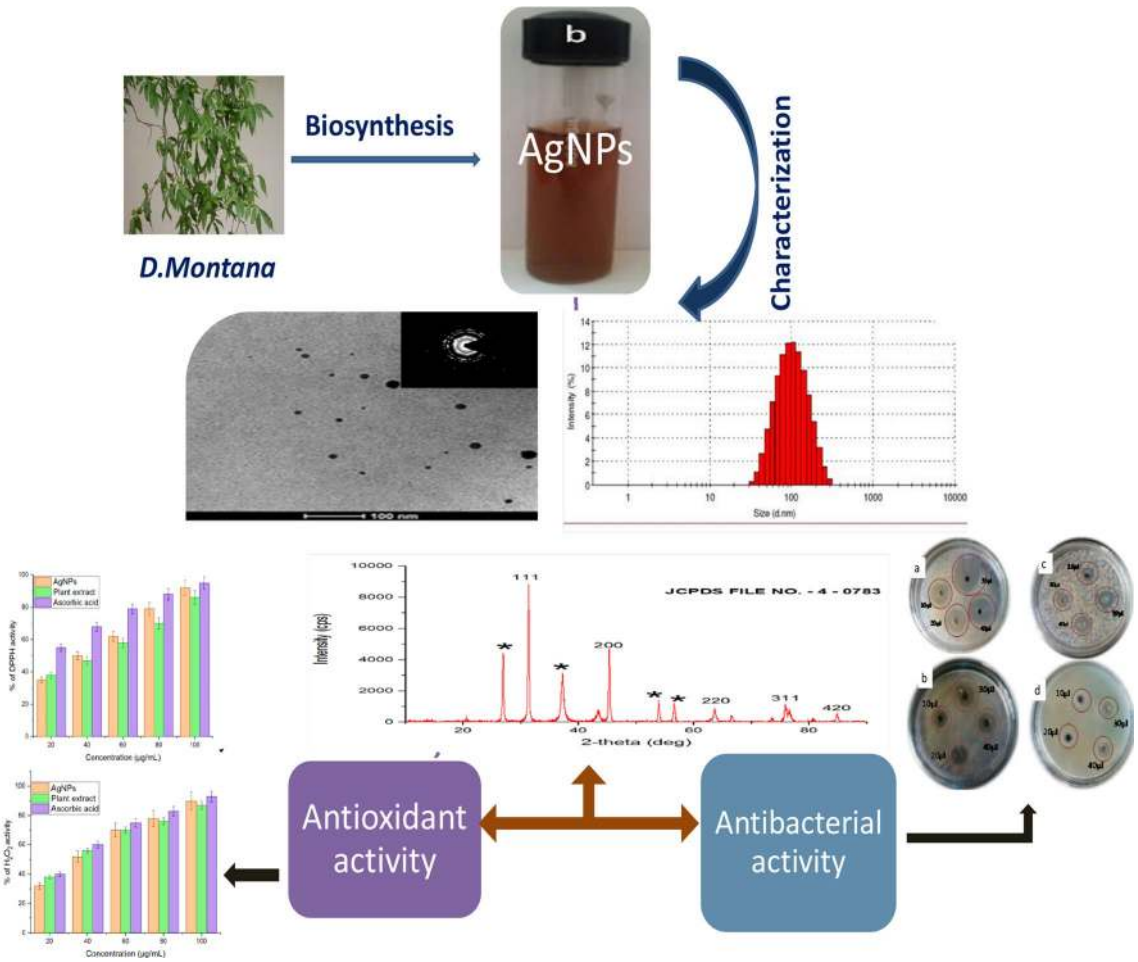
Graphical abstract



Keywords *Diospyros montana* · Silver nanoparticles · TEM · XRD · Antioxidant · Antibacterial activity

Introduction

Nanoscience is emerging as a fast growing area with tremendous application in biomedical science and technology. Nowadays, metal nanoparticles of silver, gold and platinum have been the subject of focused research area due to their unique optical, mechanical and chemical properties that are different from those large materials [1]. Among these metal nanoparticles, silver has become a focus of interest because they play a significant role in textile and pharmaceutical industries [2].

A number of approaches are available for the synthesis of silver nanoparticles (AgNPs) using electrochemical [3], microwave assists process [4], sono-chemical [5], radiation assist [6], reverse micelles process [7], phase transfer process [8] and photochemical synthesis [9]. Most of these methods are very expensive and also uses toxic chemicals which may lead to potential environmental and health risks [10]. In current scenario, metal nanoparticles protected by bio-organic ligands have attracted much interest due to their different applications [11].

In recent years, phytocompounds-facilitated synthesis of AgNPs is gaining significance due to their availability and eco-friendly [12]. Biosynthesis of silver nanoparticles using plants such as *Eucalyptus chapmaniana* [13], *Momordica charantia* [14], *Terminalia bellirica* [15], *Cochlospermum religiosum* [16], *Eucalyptus chapmaniana* [13] and many more plants has been reported. Silver nanoparticles have numerous applications such as bio-labeling, combating of microbes, detection of cancer, drug delivery and other diseases [17]. Antimicrobial activity of green synthesized nanoparticles allows them to use in water filtration process, textiles and food industries [18].

Diospyros montana also known as Bombay ebony and belong to the family of Ebanaceae. Diospyros genus is receiving increased attention as it is used in Indian traditional medicines like Ayurveda and Unani [19]. *Diospyros montana* is mainly distributed in Western Ghats of India, Sri Lanka and Australia. It was reported that genus Diosyros contain various phytochemicals such as phenols, flavonoids, saponins, terpinoids and reducing sugars [20]. *Diospyros montana* has significant pharmacological activities, used in the treatment of cough, ulcer, anti-hypersensitive and snake bites [21, 22]. Bark extract of *D.montana* contains diospyrin compound, which acts as a tumor inhibitory agent [23]. However, to date, less attention has been paid to synthesis nanoparticles from this plant. To the best of our knowledge, this is the first report on biosynthesis of AgNPs from stem bark extract of *D.montana*. We have demonstrated the phytochemical screening of methanolic stem bark extract, biosynthesis, characterization of silver nanoparticles and their free radical scavenging and antibacterial activities.

Materials and methods

Chemicals and reagents

Silver nitrate ($AgNO_3$), methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid was purchased from Sigma Aldrich. All other reagents and chemicals used in this research were of analytical grade.

Bacterial source

Bacterial strains *Escherichia coli* (MTCC 443), *Klebsiella aerogenes* (MTCC 98), *Bacillus subtilis* (MTCC 441) and *Staphylococcus aureus* (MTCC 3160) were used and maintained in nutrient agar (Himedia, Mumbai) slants at 4 °C.

Plant collection and authentication

The stem barks of *D.montana* were collected from Wayanad District (Western Ghats), Kerala, India. It was identified and authenticated (Ref. BSI/SRC/21/12/2015/Tech/1219) at Botanical Survey of India, Coimbatore, India.

Preparation of methanolic stem bark extract

The stem barks were washed thoroughly thrice with distilled water and dried for 7 days. The fine powder was obtained from stem bark using kitchen blender. About 25 g of stem bark dry powder was extracted with 250 ml of methanol solvent using soxhlet extractor. After completion of the extraction, the methanol solvents were evaporated using rotatory evaporator. Prepared extract was concentrated, dried and kept at 4 °C until further use.

Qualitative phytochemical screening

Phytochemical screening of stem bark extract of *D.montana* was performed for the qualitative detection of different phytocompounds such as alkaloids, flavonoids, saponins, glycosides, phenols, steroids, carbohydrates, oils and fats, proteins, amino acids, tannins, gums and reducing sugars. All phytochemical tests were performed according to the standard methods described by Al-Owaisi [24] and Sheel [25].

Biosynthesis of silver nanoparticles

Silver nitrate solution (1 mM) was prepared in amber bottle and stored at dark place. The prepared stem bark extract was mixed with 1 mM $AgNO_3$ solution in 1:9 proportions and kept at room temperature for 30 min. Purified AgNPs from the samples were obtained by centrifuging the solution at 10,000 rpm for 20 min. The precipitate was re-dispensed with 10 ml double distilled water and again centrifuged at 10,000 rpm for 10 min for removing excess biomass. The pellet of Ag nanoparticles was collected carefully and dried in desiccators for 12 h. The dried powder of AgNPs was used for further test.

Characterization of AgNPs

UV–Visible spectroscopy

The formation of nanoparticles was preliminarily confirmed by visual observation as color change from watery to brown color. Further, the reduction of Ag ions in the solution mixture was confirmed by UV–Visible spectroscopy (JASCO UV–Vis NIR V-670). The spectral data of synthesized AgNPs were recorded from 200 to 600 nm at a resolution of 2 nm.

SEM and EDX

The morphology of AgNPs was determined by SEM (Hitachi S -340 N). SEM samples were prepared on a carbon coated copper grid by placing a small amount of AgNPs on the SEM grid, excess samples was cleaned by blotting paper and then samples were allowed to dry under mercury lamp. This experiment was conducted at an accelerator voltage of 20 kV. Energy dispersive X-ray analysis was also taken on the same instrument.

TEM

Transmission electron microscope (TEM) (JEOL JEM-2100) analysis was conducted by drop coating of Ag nanoparticles on a copper grid and operated at an accelerated voltage at 80 kV. The grid was dried for 2 h and imaged.

DLS particle size analysis

Average size and polydispersity index of deionised water-diluted silver nanoparticles were measured by dynamic light scattering spectroscopy (DLS) (Malvern-zeta analyser) operated with a He–Ne laser.

XRD analysis

The structure of the AgNPs was determined using XRD technique. For X-ray diffraction studies (XRD), dried silver nanoparticles were coated on XRD grid (XPERT–PRO) and diffraction recorded in the 2θ range from 10° to 90°. The spectral data were operated at 40 kV and a current of 40 mA.

FT-IR

The biologically active components that are responsible for silver reduction, formation and capping were identified using Fourier Transform Infra-Red (FTIR) spectroscopy (Nicolet is 5, Thermo Scientific) by KBr pellet method. FTIR spectral reading was carried out in the range from 500 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Antioxidant activity

DPPH and H_2O_2 free radical scavenging activity

The DPPH free radical scavenging activity of synthesized nanoparticles and plant extract were carried out spectrophotometrically (517 nm) according to the method reported by Govindappa [26]. The Hydrogen peroxide assay, a method for measuring free radical scavenging activity, was assessed spectrophotometrically (230 nm) according to Yadav [27]. Antioxidant activity was noted in terms of stranded control ascorbic acid antioxidant equivalents. Percentage of the activity was calculated using the following equation; Percentage of antioxidant activity $= [(a - b)/a] \times 100$, where a was the absorbance of the control (blank) and b was the absorbance of samples.

Antibacterial activity

The antibacterial activity of AgNPs were carried out by agar well diffusion method described by Gudikandula [28] against Gram negative bacteria such as *E.coli* (MTCC 443) and *K.aerogenes* (MTCC 98), Gram positive bacteria such as *B.subtilis* (MTCC 441) and *S.aureus* (MTCC 3160). Muller-Hinton Agar (Himedia, Mumbai) plates were prepared, sterilized and solidified. Each bacterial strain (1×10^5 CFU/mL) was swabbed uniformly on the prepared individual petri plates using sterile cotton swabs. Four wells of size approximately 6 mm are made on prepared plates using gel puncture. Different concentration of AgNPs (10, 20, 30 and 40 µL) was loaded into the wells of all plates. After incubation for 24 h at 37 °C, the plates were examined for zone of incubation in mm.

Statistical analysis

All the experiments were carried out in triplicates and the results were expressed as mean ± standard deviation. Statistical analysis was done using origin software (Origin pro evaluation, 2018).

Results and discussion

Qualitative phytochemical screening

The results of qualitative phytochemical screening of methanolic stem bark extract of *D.montana* are shown in Table 1. Phytochemical screening showed the presence of saponins, alkaloids, glycosides, phenols, steroids, flavonoids and tannins. It was reported that the leaves of *D.montana* contain steroids, phenols, flavonoids, saponins, terpenoids [19]. The presence of these biologically active compounds may play a significant role in formation, capping and stabilization of AgNPs [29].

Table 1 Phytochemical screening of methanolic stem bark extract of *D.montana*

S. no.	Phytochemicals	Methanolic stem bark extract
1	Alkaloids	+
2	Saponins	+
3	Flavonoids	+
4	Glycosides	+
5	Phenols	+
6	Steroids	+
7	Oils and fates	–
8	Proteins and amino acids	+
9	Carbohydrates	+
10	Tannins	+
11	Gums	–
12	Reducing sugars	–

+ sign denotes positive and − sign denotes negative

Fig. 1 Biosynthesis of AgNPs using *D.montana*: **a** stem bark extract of *D.montana* and **b** AgNPs

Biosynthesis of AgNPs using *D.Montana*

For the biosynthesis, bark extract was mixed in the aqueous solution of $AgNO_3$ at room temperature. Initially, the color changed from watery to brown color (Fig. 1) within 30 min after which no color change was observed. The turn in color from watery to brown color indicates the formation of AgNPs and this change may be due to the SPR (Surface Plasmon Resonance) of AgNPs in the reaction samples [30, 31].

Characterization of AgNPs

The formation of silver nanoparticles was measured using UV–Visible Spectroscopy from 200 to 600 nm wavelength range and the highest spectral peak was observed at 432 nm (Fig. 2). It was reported earlier that the absorbance around 432 nm for Ag is a characteristic feature of metal nanoparticles [32].

SEM image (Fig. 3) showed the morphology of AgNPs is predominantly spherical shape with aggregation. TEM images showed that the size of individual AgNPs ranged from 5 to 40 nm. The morphology of Ag nanoparticles was also spherical (Fig. 4). Similar to our study, Krishnaraj [33] synthesized the silver nanoparticles from *Bacoba monnieri* extract and they obtained varying size and morphology of the silver nanoparticles ranged between 2 and 50 nm from TEM analysis. SAED (selected area electron diffraction) pattern of synthesized nanoparticles was also shown in the inset of Fig. 4. In this pattern, a circular ring was observed. This was due to the crystalline nature of silver nanoparticles.

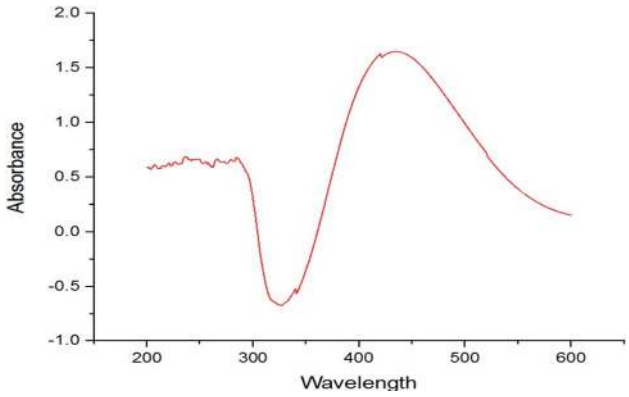


Fig. 2 UV–Visible spectrum of synthesized AgNPs

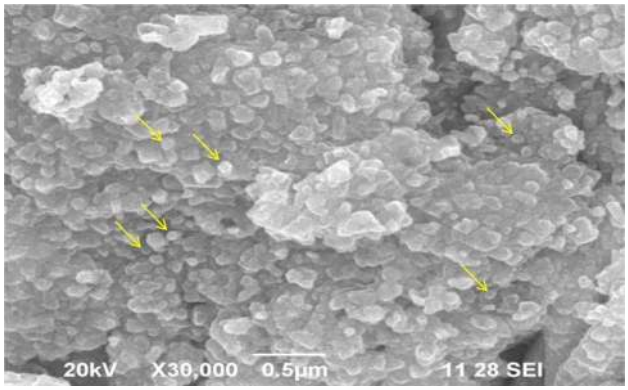


Fig. 3 SEM image of silver nanoparticles

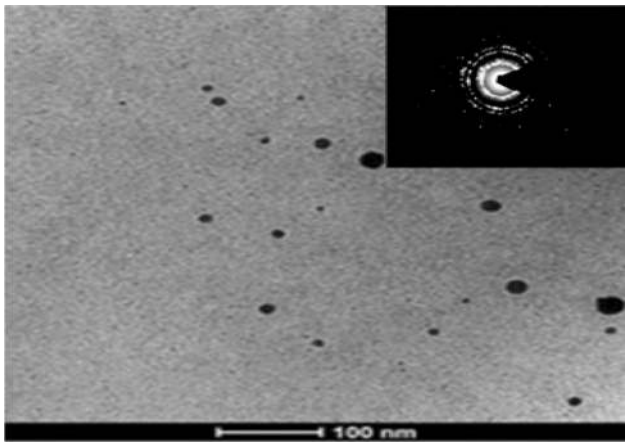


Fig. 4 TEM image of silver nanoparticles and insets show the SEAD pattern

EDX spectrum from Fig. 5 represents signal from Ag (0.3 and 3 keV) together with O (0.5 keV). The spectrum at 3 keV indicates that the Ag has been correctly identified. Generally, Ag nanoparticles show typical optical peak at

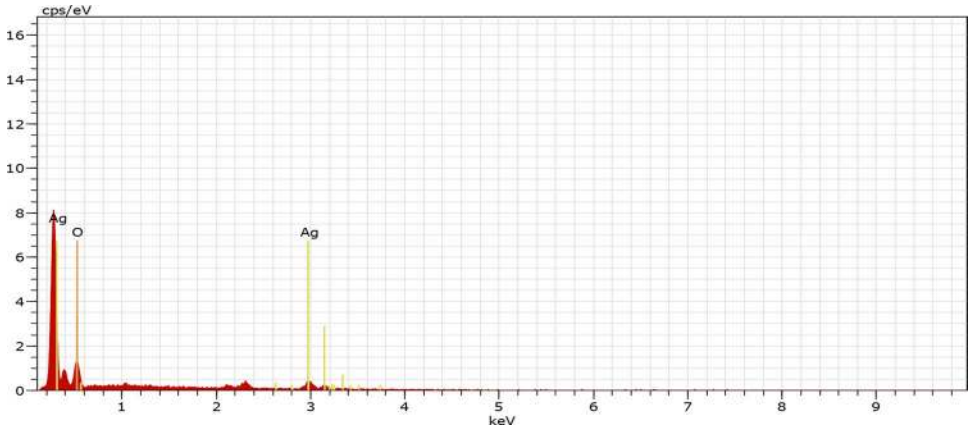


Fig. 5 EDX spectrum of silver nanoparticles

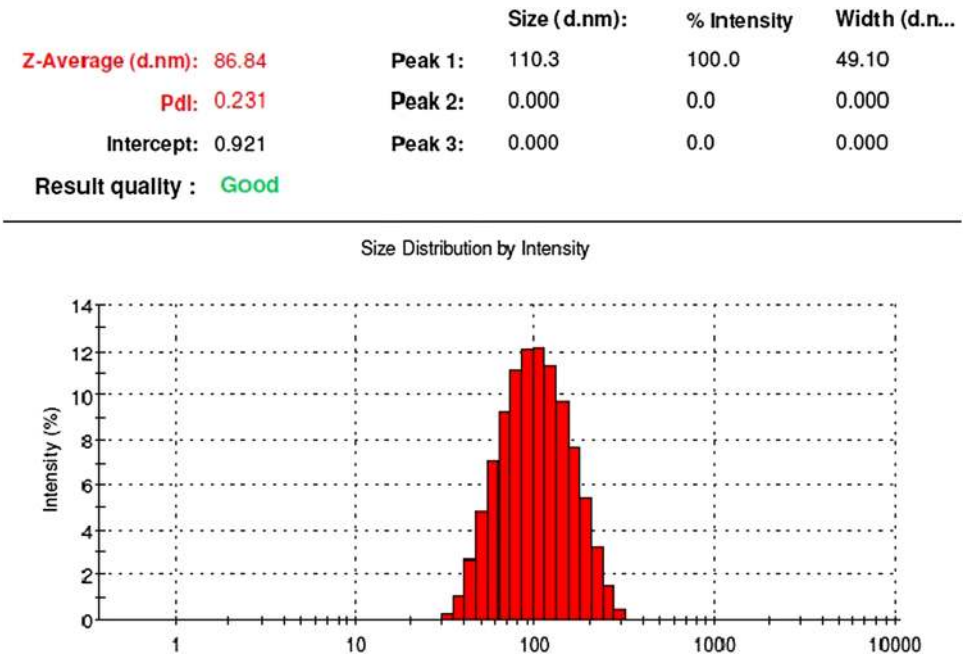


Fig. 6 DLS analysis of synthesized silver nanoparticles

3 keV [34]. The absorption peak of O in EDX spectrum might be due to the involvement of phytocompounds in capping and stabilizing of Ag nanoparticles through O related groups [14, 15].

The size distribution histogram of DLS revealed the *Z* average diameter of 86.84 nm with polydispersity (0.231) for synthesized AgNPs (Fig. 6).This is quite larger than the size reported by TEM and the variation mainly due to the process involved in sample preparation [35]. The polydispersity index values are less than 0.7 indicating the quality of nanoparticles to be good [36].

The XRD patterns shown in Fig. 7 demonstrate the crystalline nature of synthesized AgNPs. Highest 2θ diffraction

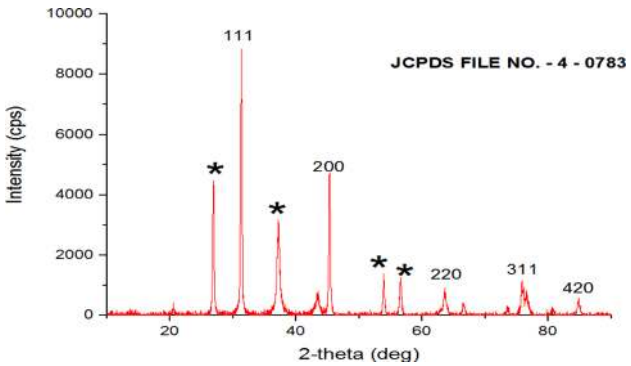


Fig. 7 XRD pattern of Ag nanoparticles

peaks observed at 37.4°, 46.1°, 64.2°, 78.1° and 85.2° which corresponding to (111), (200), (220), (311) and (420) planes, respectively [15, 33], which confirms the face-centered cubic structure of synthesized nanoparticles [32]. Corresponding planes were matched with JCPDS, File No. 4-0783 (Joint Committee on Powder Diffraction Standards) values for silver. The average mean size of biosynthesized nanoparticles was determined using Debye–Scherrer's formula, $D = 0.9\,\lambda/\beta\cos\theta$ and is estimated to be approximately 28 nm from the breadth of the reflection. Where λ is the wavelength of X-rays used, β is the broadening of diffraction line used measured at half maximum intensity and θ is Bragg's diffraction angle. The extra diffraction peaks (*) in the XRD pattern may be due to the crystallization of bio-organic phase in the plant extract [28].

FTIR spectra in Fig. 8 indicated the presence of phytochemicals which may be responsible for the synthesis of AgNPs from *D.montana*. The highest peak at 3364.92, 2978.32 and 1080.30 cm^{-1} attributed to N–H stretch, C–H stretch vibration of amides, alkanes and aliphatic amines. The medium bands at 3616.26, 2940.05, and 1099.58 cm^{-1} corresponded to O–H stretch and C–H stretch possible of alcohol, phenols and amines. The other peaks at 2396.10, 1581.48, 1462.08 and 853.15 cm^{-1} were assigned to the presence of aldehydes (H–C = O, C–H), nitrogen (N–O) and aromatic compounds (C–H), respectively [16, 35]. This spectroscopic study confirmed the presence of amides, phenol, nitrogen and aromatic compounds has a strong binding affinity with Ag and also plays a significant role in reducing and capping of Ag ions for the conversion of Ag^+ into AgNPs [37].

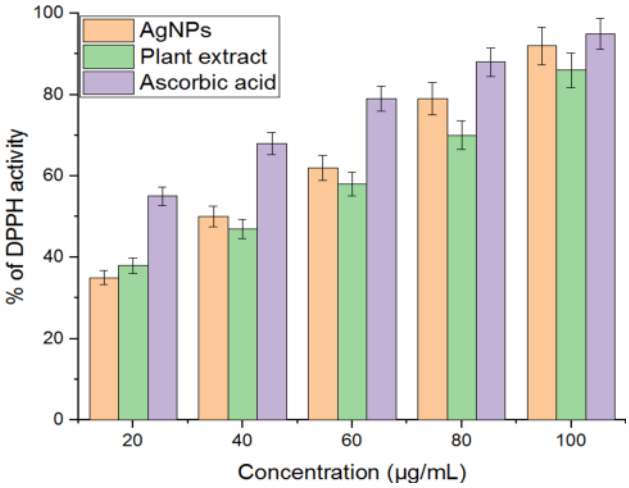


Fig. 9 DPPH antioxidant activity of AgNPs and stem bark extract of *D.montana*

Antioxidant activity

The free radical scavenging ability of biosynthesized AgNPs and plant extract was determined using DPPH and H_2O_2 assay. In DPPH assay, the antioxidant activity of AgNPs and plant extract improved as the concentration of samples used for assay was increased from 20 to 100 µg/mL (Fig. 9). It was observed that free radical scavenging activity of AgNPs and bark extract of *D.monanta* increased in a concentration-dependent manner. In addition, the antioxidant potential of biosynthesized Ag nanoparticles was significant to plant extract and similar to standard (ascorbic acid) control.

H_2O_2 assay is a photometric test for the determination of hydrogen peroxide in biological fluids. In H_2O_2 assay,

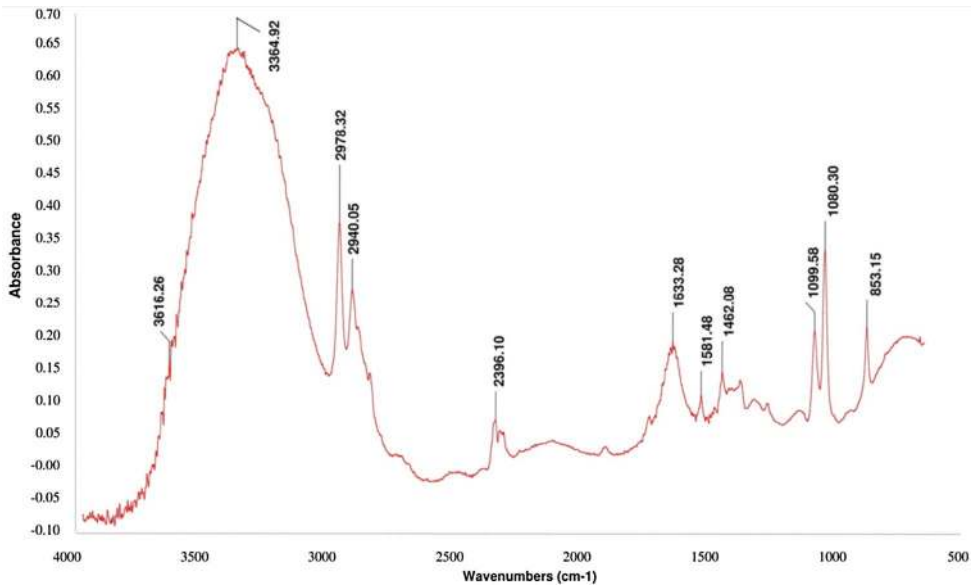


Fig. 8 FTIR analysis of biosynthesized AgNPs

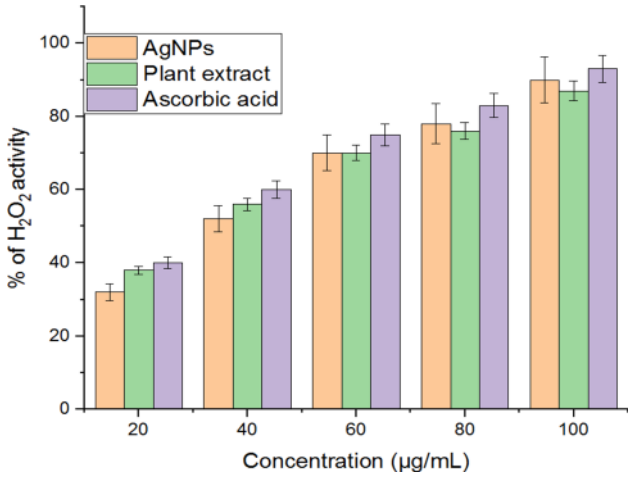


Fig. 10 H_2O_2 antioxidant activity of synthesized AgNPs and stem bark extract of *D.montana*

silver nanoparticles showed equal potency of activity compared to methanolic stem bark extract of plant (Fig. 10). The free radical scavenging activity might be due to the presence of phyto compounds, especially phenols that have the ability to donate the hydrogen atoms in their OH groups [38]. Antioxidant results indicated that there was increase in free radical scavenging activity of AgNPs in a concentration-dependent manner.

Antibacterial activity

The synthesized AgNPs showed significant antibacterial activity against both Gram positive and Gram negative bacteria. The antibacterial effect of different concentration of AgNPs (10, 20, 30 and 40 µL) was shown in Fig. 11. The highest anti-bacterial zone of inhibition was recorded in *K.aerogenes* followed by *E.coli*, *B.subtilis* and *S.aureus* (Fig. 12). From this study, AgNPs showed high zone of inhibition against Gram negative bacterial strains compared with Gram positive bacteria. This may be due to the presence of thin peptidoglycan layer in the Gram negative bacteria but in case of Gram positive bacteria, which are made up of thick peptidoglycan layer [39]. It was also reported that the charged AgNPs have the ability to bind negatively charged Gram negative bacterial cell wall better on contrary [19].

Conclusions

In this study, we have synthesized AgNPs by eco-friendly and cost effective method using *D.montana* stem bark extract. The phytochemicals present in the stem bark extract of plant act as a strong reducing and capping agents for the formation of AgNPs. The morphology and size of the silver nanoparticles were characterized by UV–Visible spectroscopy, SEM, TEM, EDX and DLS. XRD reports revealed that

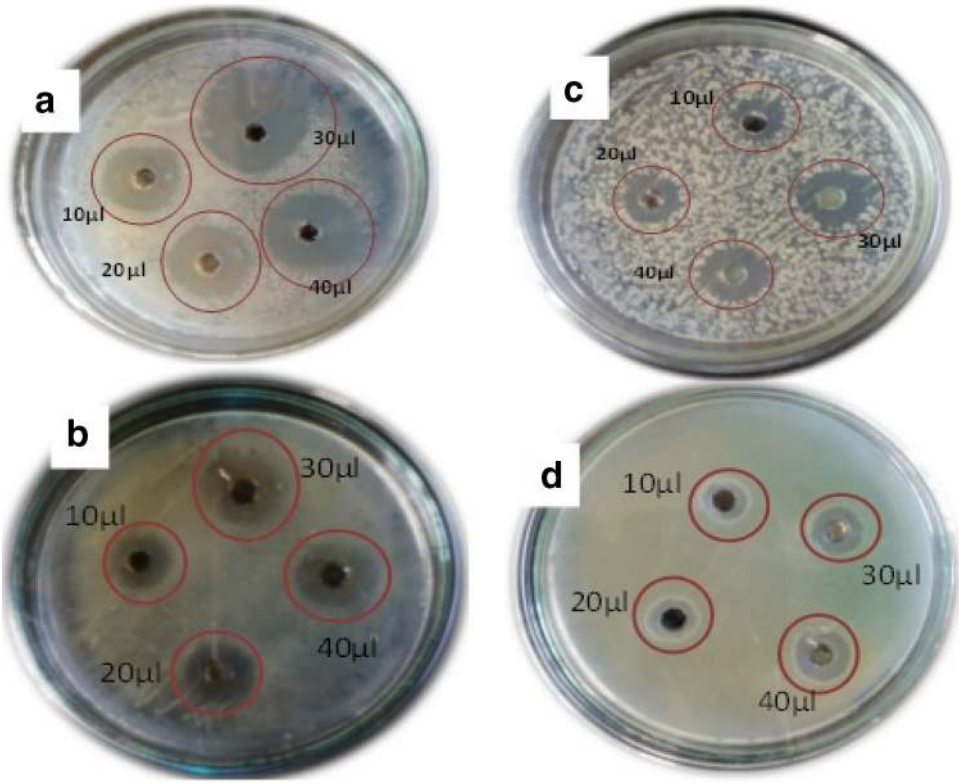


Fig. 11 Antibacterial activity of AgNPs against **a** *K.aerogenes* **b** *E.coli* **c** *B.subtilis* **d** *S.aureus*

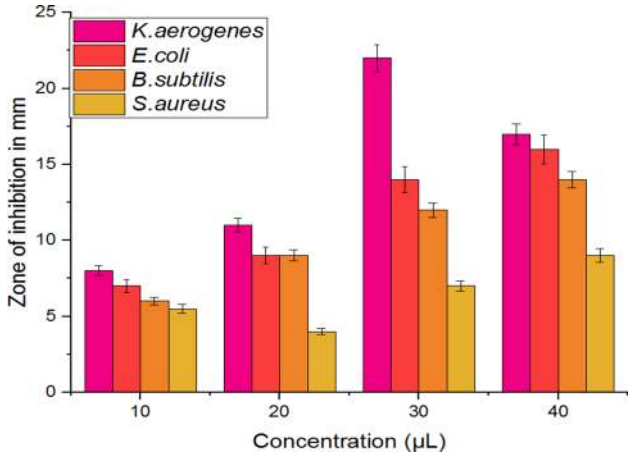


Fig. 12 Zone of inhibition of silver nanoparticles against Gram positive and Gram negative bacteria via agar well diffusion method

synthesized silver nanoparticles were crystalline in nature with average mean size as 28 nm and FTIR studies supported the confirmation of bioactive compounds for the Ag^+ reduction. Biosynthesized AgNPs showed significant antibacterial activity against Gram negative bacteria compared to Gram positive bacteria and also exhibit remarkable free radical scavenging activity. Further studies on AgNPs will be carried out for pharmacological applications.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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