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Research Article

Antibacterial and Antioxidant Studies of Green Synthesized Silver Nanoparticles using *Azadirachta indica* (Neem) Leaf Extract

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Abstract

Silver nanoparticles (AgNPs) have been synthesized by green synthesis using *Azadirachta indica* leaf extract as both reducing and stabilizing agent. Synthesis of colloidal AgNPs was monitored by UV- visible spectroscopy. The UV-visible spectrum showed a peak at 455 nm corresponding to the plasmon absorbance of the silver nanoparticles. Crystallite structure of silver nanoparticles was studied using X-ray diffraction (XRD) analysis which revealed the face-centered cubic structure (FCC) with average particle size of 8.9 nm, calculated using Debye-Scherrer's equation. Transmission electron microscopy (TEM) image revealed the agglomeration of small grain with particle size ranging from 2 to 14 nm. FCC crystalline nature was also evident from selected area electron diffraction (SAED) analysis. High purity of as-synthesized AgNPs was analyzed using energy dispersive X-ray (EDX) spectroscopy. Band gap energy was calculated to be 2.7 eV from UV- Visible spectra. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was stabilized by AgNPs which reveals its antioxidant efficacy. Well diffusion method showed 7 mm to 12 mm zone of inhibition (ZOI) against Gram-positive and Gram-negative bacteria, respectively confirming the antibacterial potential of AgNPs.

Keywords: AgNPs, Green synthesis, Antioxidant efficacy, TEM, XRD

Introduction

Nanoparticles possess very high surface to volume ratios. This property of nanoparticles can be utilized in various scientific fields, where high surface is needed (Brijesh et al., 2014; Harikrishna *et al.*, 2009). Nanoparticles have been successfully used in nanochemistry to enhance the immobilization and activity of catalysts, in medical and pharmaceutical nanoengineering for delivery of therapeutic agents, in chronic disease diagnostics, and in sensors. Besides, these nanoparticles have been used in clothing and in the food industry to limit bacterial growth (Harikrishna *et al.*, 2009; Fidel *et al.*, 2010). Because of wide applications in various fields, there are numerous methods for the fabrication of silver nanoparticles. They can be synthesized by several physical, chemical and biological methods (Regmi *et al.*, 2019). The synthetic methods used for the preparation of AgNPs often use toxic chemicals as a reducing agent such as $NaBH_4$, citrate, ascorbate etc. (Akl *et al.*, 2012; Florence *et al.*, 2013). Recently, there has been an increased emphasis on the topic of green synthesis of silver nanoparticles (Singh *et al.*, 2010). Green synthetic route for the synthesis of nanoparticles have received much attention to overcome these fallacies. Plants extract synthesis of nanoparticles is gaining importance due to its simplicity and eco-friendly.

AgNPs are synthesized using different plant extracts in literatures such as *Dalbergia spinose, Buddleja globose, Argemone Mexicana,* etc. In this study, silver nanoparticles are synthesized using plant leaves extract of *Azadirachta indica* (Neem). *A. indica* trees are attractive broad-leaved evergreen trees that can grow up to 30 meters in height and have attractive rounded crowns and thick furrowed bark. *A. indica* belongs to Meliaceae family and are native to Nepal, India and South Asian countries (Biu *et al*., 2009). It is typically grown in tropical and subtropical regions. *A. indica* leaves was found wide scale application in medicine, fertilizers and in industry such as making soaps, etc (Erico *et al.*, 2017).

The historical use of silver as an antibacterial agent made the progression to silver nanoparticles a logical and compelling step. As a naturally antibacterial metal, a silver nanoparticle likely has multiple mechanism of antibacterial activity (Erico *et al*., 2017); (Rongwei *et al*., 2009). Silver nanoparticles are effectively applied for the inhibition of Gram-negative and Gram-positive bacteria so it can also be used as killing agent. Besides this, it can also be used in antibiotic resistant strain (Fidel *et al.*, 2010). Similarly, silver nanoparticles have been known to show significant antioxidant efficacy. Antioxidant are those which are capable of inhibiting or delaying the effect of oxidation process due to the atmospheric oxygen and other reactive species (Hyatham *et al.*, 2015).

In this paper, we focus on the preparation of AgNPs by green synthesis method using *A. indica* leaf extract and their antibacterial activity and antioxidant efficacy were studied.

Materials and Methods

Synthesis of Silver Nanoparticles

Silver nanoparticles (AgNPs) has been synthesized by using various methods such as chemical synthesis, biosynthesis, and green synthesis (Hyatham *et al.*, 2015; Michael *et al.*, 2001). In this present work, we have chosen green synthesis as the appropriate method. Silver nitrate is used as a source compound for synthesis of silver nanoparticles and green leaves of *A. indica* as reducing agent. Double distilled water was used to prepare the silver nitrate solution and leaf extract.

The fresh leaves of *A. indica* were collected from Janakpur, Nepal. The collected leaves were washed thoroughly and allowed for air dry in room temperature**.** Exactly, 25 g of leaves were washed with distilled water thoroughly, dried in room temperature and crushed into fine powder. It was boiled in distilled water for 15 minutes in 80°C. The solution was filtered using Whatman's filter paper no. 42 and the filtrate was taken as leaves extract to proceed further experiment.

0.2 M $AgNO_3$ solution was prepared and stored in black bottle to avoid photodegradation. 20 mL of plant extract was added to the 80 mL of prepared $AgNO_3$ solution, shaken for 30 min and incubated in dark for 24 hr. Its maximum absorbance was measured using UV- Visible spectroscopy. The mixed solution was heated at 80°C, filtered and dried to obtain AgNPs. The reaction involved during the process is as follows.

$$AgNO_3 \rightarrow Ag^+ + NO_3^-$$

$$Ag^+ + e^- \xrightarrow{\text{(Neem extract)}} Ag^0$$

Characterization

Synthesized AgNPs was characterized using different surface characterization techniques. The crystallite size and structure were determined from XRD patterns obtained using Cu k_α radiations (λ = 1.514 Å) for 2θ value ranging from 10° to 75° in X-ray diffractometer (Rigaku Ultima IV model). The morphology of the samples was analyzed by transmission electron microscopy (FET Technai ST F20) images and selected area electro diffraction (SAED) pattern. The UV-Vis absorption spectra were obtained from UV-Vis spectrophotometer (ELICO SL 177) and their elemental detection and atomic weight percentage was identified via EDX (JEOL-JSM-6700F) analysis.

Antimicrobial Activity

The antibacterial activity was observed in two Gram-positive bacteria *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6051 and Gram-negative *Enterobacter aerogenes* ATCC 29007 in Polytechnic Research Institute of Nepal (PORIN), Kathmandu and stored at 4°C. The media plates were made of Himedia Nutrient Broth and bacterial lawn cultures were prepared by taking the respective bacteria for the different Petri plates labelled accordingly (Shetty *et al.*, 2017).

100 μL culture broths were spread into nutrient-agar plate (2.5 g/L nutrient media and 2.5 g/L agar) and incubated at 37°C for 15 min. The agar plate is levelled half with the help of marker then 0.1gm of silver nanoparticles synthesized using *A. Indica* was placed separately on central part of each half of agar plate carefully and incubated overnight at 37°C. The antibacterial activity of AgNPs was observed after 24 hr.

Antioxidant Activity

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The free radical scavenging activity of AgNPs was determined using the stable radical DPPH as shown in the Fig. 1.

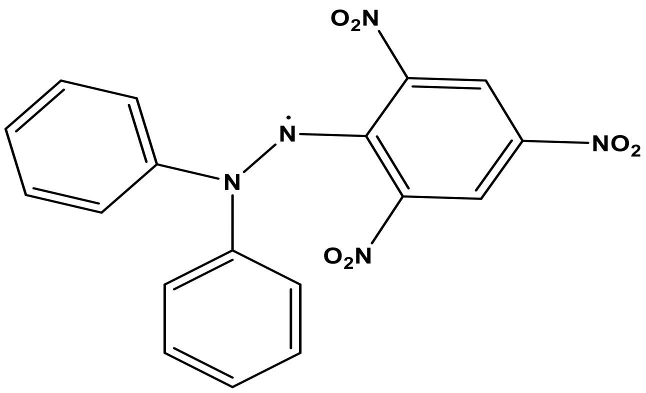

Fig. 1: 2,2-diphenyl-1-picrylhydrazyl (DPPH)

1 mL of different concentrations (10, 20, 30, 40, 50, 75, and 100) µg/mL of AgNPs was mixed with 1 mL freshly prepared DPPH (by dissolving 0.77 mg of DPPH in 100 mL of methanol). The solution was incubated at room temperature in the dark for 30 mins. The absorbance was recorded at 517 nm using UV-Vis spectrophotometer. DPPH was used as a control and methanol was used as a blank solution. The free radical scavenging activity was expressed as the percentage of inhibition which was determined using the following formula.

$$\% \text{ of Scavenging} = \frac{Pc-Ps}{Pc} \times 100$$

Where, Pc is the absorbance of control (methanol) and Ps is the absorption of AgNPs (Michael *et al.*, 2001).

Results and Discussion

i. X-ray diffraction (XRD) studies

XRD is a unique technique which provides a wide variety of information of crystalline property of nanoparticles. The average crystalline size can be calculated by Lorentzian profile fitting of most intense XRD diffraction peak using Debye-Scherrer's equation.

$$\text{Average crystalline size (D)} = \frac{0.94\lambda}{\beta\cos\theta}$$

Where, λ is the wave length of X-ray whose value is 0.15406 Å, β is full width at half maximum (FWHM) of most intense peak expressed in radians and θ is Bragg's diffraction angle (Dhungana *et al.*, 2016; Regmi *et al.*, 2019; Gautam *et al.*, 2020).

Figs. 2 (a) and 2 (b) depict the XRD pattern of the AgNPs and corresponding Lorentzian fitting, respectively. The average particle size was found to be 8.9 nm calculated from Debye-Scherrer's equation. The diffraction peaks observed at 32.4°, 46.3°, 64.7°, and 77.4° correspond to (111), (200), (220), and (311) planes of face centered cubic (FCC) crystal structure (JCPDS card 04-0783) (Mehta *et al.*, 2017).

ii. Transmission electron microscopy (TEM) studies

The typical transmission electron microscopic (TEM) image of well distributed silver nanoparticles is displayed in Fig. 3(a). The agglomeration of nanoparticles may be due to surface forces: van der Waal forces, capillary forces, and electrostatic forces (Muniyappan *et al.*, 2014; Jagtap and Bapat, 2013). The TEM images were processed with the ImageJ software to obtain diameter size distribution of particle. The histogram of particle sizes ranging between 2 nm to 12 nm are observed using ImageJ software on TEM image as shown in Fig. 3(b) which is in agreement with the size calculated from XRD data (Gautam *et al.*, 2008).

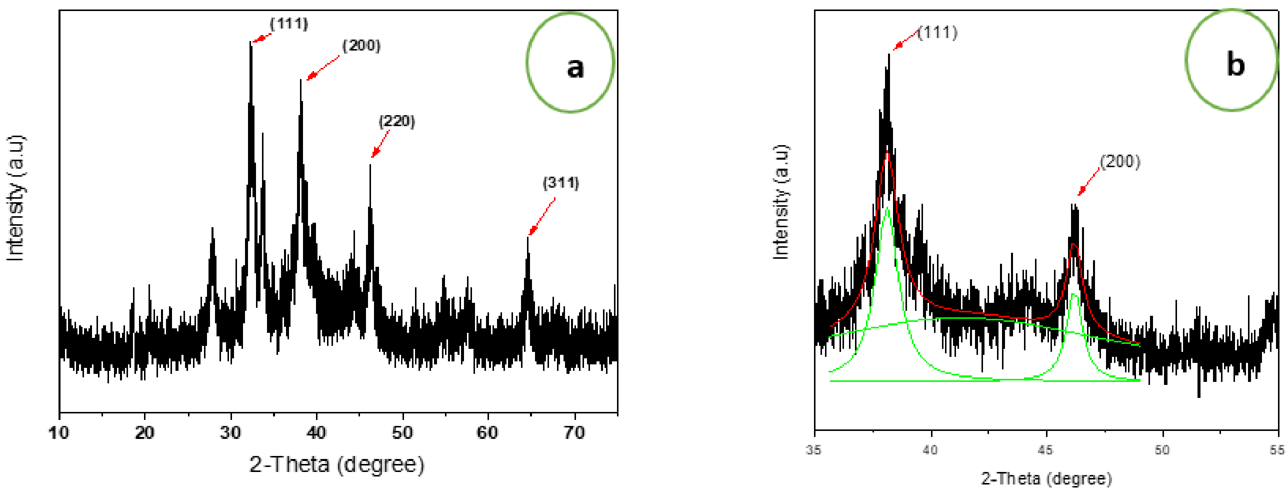

Fig. 2: (a) XRD pattern of AgNPs synthesized using. *A. indica* leaf extract and (b) corresponding Lorentzian fitting

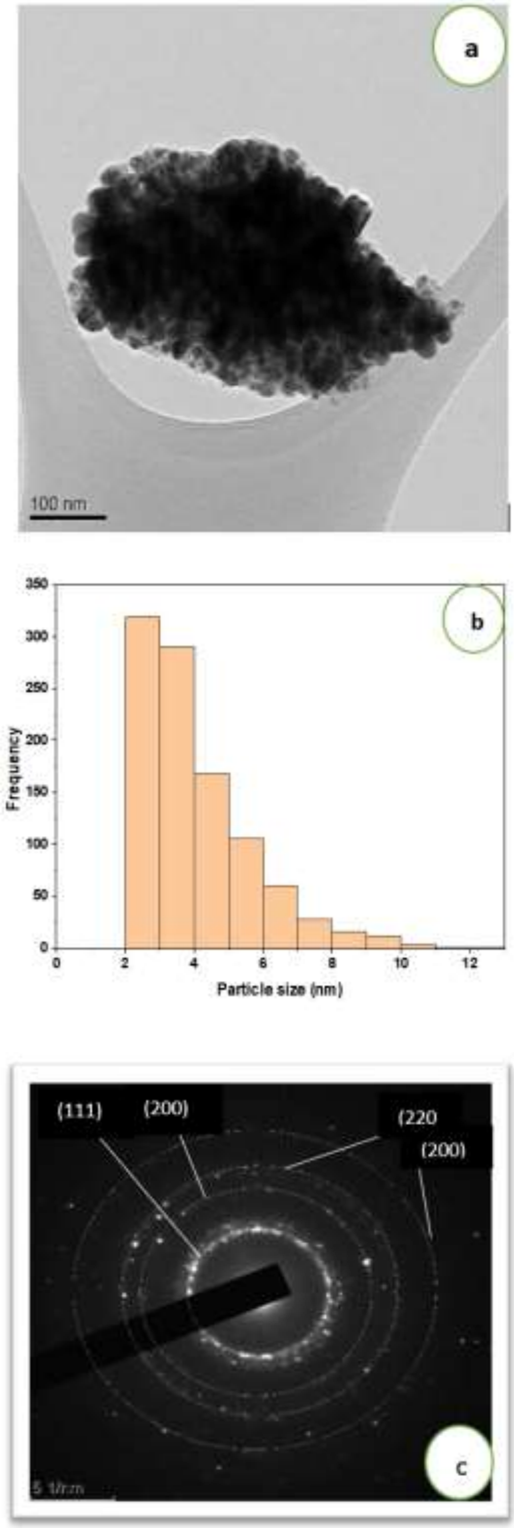

Fig. 3: (a) TEM micrograph of AgNPs, (b) histogram showing particle size of AgNPs and (c) SAED ring pattern of AgNPs synthesized using *Azadirachta indica* leaf extract

Selected area electron diffraction (SAED) is a crystallographic experimental technique associated with TEM and basically useful for the phase identification (Prakash *et al*., 2017). SAED image shown in Fig. 3(c) displays characteristics ring patterns which are consistent with the plane indices (111), (200), (220), and (311) of pure face centered cubic (FCC) silver structure (Harikrishna *et al*., 2009).

iii. EDX studies

The purity of Ag nanoparticles was analyzed by EDX pattern as revealed in Fig. 4. Sharp and distinct peak of silver is observed at 3 keV along with other small peaks at 0.1, 0.3, 0.6, 1.5, 2.1, 2.3, and 2.7 keV. The peaks appeared at 2.7 keV is of chlorine may be due to water present in the leaf extract. Silicon peaks is observed at 1.7 keV which may be contaminated from the borosilicate glass used to store synthesized powdered silver nanoparticles. Peak of oxygen is observed at 0.5 keV may be due to the oxidation of sample with atmosphere (Hyatham *et al*., 2015). Thus, presence of sharp and intense peaks of Ag shows the high purity of synthesized nanoparticles.

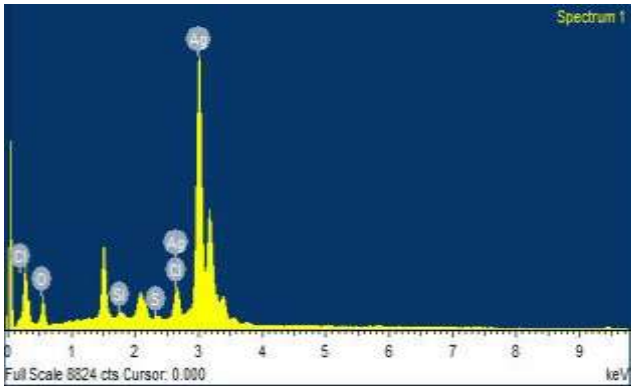

Fig. 4: EDX studies of Ag NPs synthesized using Azadirachta *indica* leaf extract

iv. UV-Visible Studies

UV-Visible absorption spectroscopy is considered as an efficient technique to monitor the optical property of quantum sized particles. The surface plasmon peak occurred at 355 nm is due to the presence of ingredients in the leaves for the formation of silver as shown in Figure 5(a). This peak shifted slowly towards the lower wavelength due to blue shift and depends on the particle size and shape. The band corresponds to the absorption by colloidal silver nanoparticles in the region 455 nm is due to the excitation of surface plasmon vibration (Aziz *et al*., 2018).

The UV-Vis absorption ability of nanoparticles is related with the band gap energy of that particle and provides a reliable estimate of band gap of any system (Bhujel *et al*., 2021). The band gap energy is calculated by extrapolating the curve between $(\alpha h\nu)^2$ versus hν to the x-axis as shown in Fig. 5(b) and is found to be 2.7 eV.

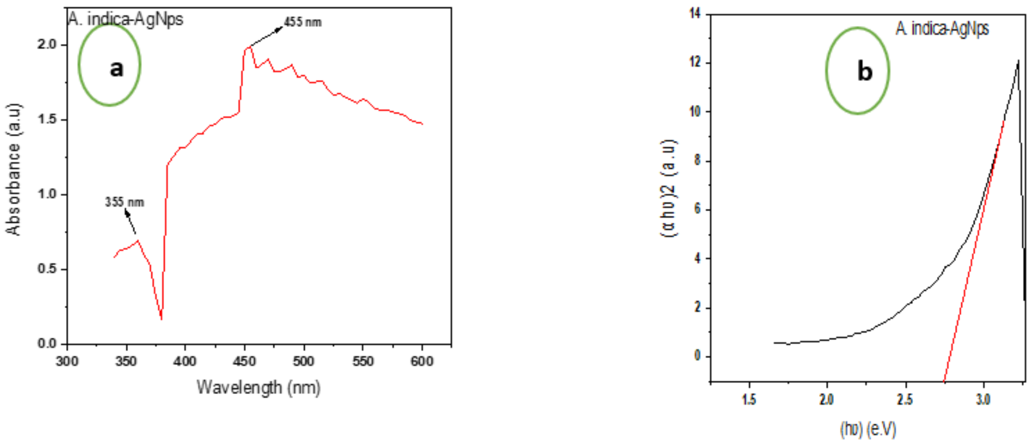

Fig. 5: (a) UV-Visible spectra and (b) Plot of $(\alpha h\upsilon)^2$ versus photon energy (hυ) of Ag NPs

Fig. 6: Antibacterial activity of Ag Nps against bacteria (a) *Enterococcus faecalis* (b) *Bacillus subtilis* and (c) *Enterococcus aerogenes*, respectively.

v. Antibacterial Activities

The antibacterial activity of green synthesized AgNPs was performed using well diffusion method. *Enterococcus faecalis* and *Bacillus subtilis* (Gram-positive bacteria) and *Enterococcus aerogenes* (Gram-negative) bacterium were cultured in HiMedia broth and incubated at 37ºC for 24 hours. Then the bacteria were swabbed onto Agar plate using sterile metal swab. 1 mg of AgNPs powder was loaded into the agar plate by making 1 mm well at the center of the plate. The plate is incubated for 24 hr and the zone of inhibition (ZOI) was measured. *Enterococcus faecalis* and *Bacillus subtilis* showed zone of inhibition (ZOI) of 12 mm and 7 mm, respectively while the Gram-negative bacteria *Enterococcus aerogenes* observed ZOI of 11 mm which approves the antibacterial activity of AgNPs (Fig. 6) (Gautam *et al.*, 2021).

Antibacterial effects of AgNPs obeyed a dual action mechanism of antibacterial activity; the bactericidal effects of Ag^+ and membrane disrupting effects of the polymer subunits. The AgNPs synthesized via green route are highly toxic to multidrug resistant bacterial hence has much potential in biomedical applications (Maribel *et al.*, 2009).

vi. Antioxidant Activities

Antioxidant activity of as-synthesized AgNPs is studied using DPPH assay method. The DPPH radical absorbs at 517 nm and antioxidant activity can be determined by monitoring the decrease in this absorbance. The percentage of free radical scavenging activity at different concentration ranging from 5 mM to 9 mM for AgNPs was evaluated. It was found that the efficacy of AgNPs as an antioxidant activity increases with increase in concentration which is displayed in Fig. 7.

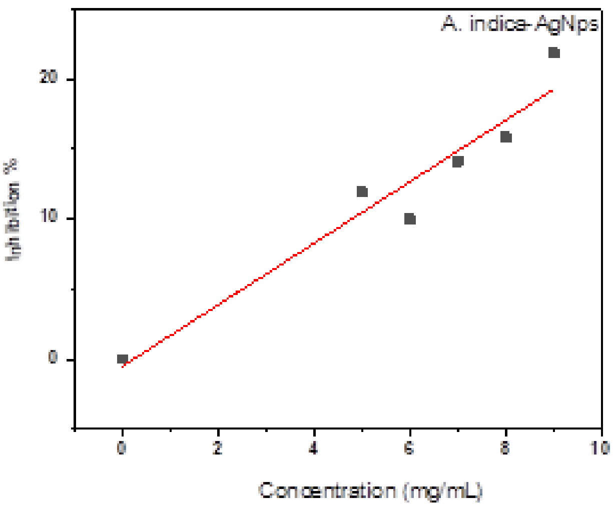

Fig. 7: Graphical representation of antioxidant efficacy of AgNPs assessed using DPPH assay method.

Conclusion

The result shows that the substantial amount of AgNPs was synthesized using *Azadirachta indica* leaf extract acting as both reducing and capping agents. UV-Visible spectra display surface plasmon resonance absorption peak at 455 nm and band gap calculated as 2.7 eV. XRD analysis reveals the face-centered cubic (FCC) structure with average particle size of 8.9 nm. TEM analysis shows the particles ranges from 2 to 12 nm and SAED verifies FCC crystalline structure of AgNPs. EDX analysis signifies the high purity of synthesized samples. AgNPs shows significant antibacterial activity against *Enterococcus faecalis*, *Bacillus subtilis* and *Enterococcus aerogenes* bacteria. AgNPs possesses substantial antioxidant efficacy and can be used in medical purposes.

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Conflicts of Interest

The authors declare no conflicts of interest with present publication.

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Ethical Approval

Not required given the nature of the article.

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