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Green Synthesis and Characterization of Silver Nanoparticles from *Azadirachta indica* Leaf Extract and its Antibacterial Study

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Abstract

Nanotechnology is the emerging branch in the field of science. The current study was conducted on plant-derived material for the green synthesis of silver nanoparticles (AgNPs) using neem leaf extract. The flavonoid and terpenoids found in the extract behaved as a reducing and capping agent. Here, the formation of NPs occurred by the bioreduction of Ag salts into AgNPs by the addition of leaf extract. The reduction was validated by the change in color that is from pale green to brown. The ion to NP reduction occurred due to the natural reducing agent in the neem leaf extract. The functional group presents in the extract and AgNPs are investigated using Fourier transform infrared. The synthesized NPs have undergone characterization by ultraviolet spectrometry between 300 and 700 nm. The peak was spotted at 308 nm. Antibacterial properties of AgNPs were studied against *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus.* From these results, it can be effectively reported that plant extract can be used in the synthesis of AgNPs and could be used for better results in future in the field of medicine, nano-related works, and so on.

Keywords: Antibacterial activity, *Azadirachta indica*, Characterization, Green synthesis

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Introduction

Nanoparticles (NPs) are known to be the fundamental blocks of nanotechnology. The main difference between NPs from other particles is that they are small in size and have different physical and chemical properties such as colloidal, optical, and electrical properties. They are mostly used as biological research tool for cell labeling and biomolecule tracking. The NPs show characteristic colors and properties with variation of size and shape, which could be effectively utilized for bioimaging applications.[1] Silver has a profound role in the field of high sensitivity biomolecule detection, catalysis, biosensor, and medicine. They also have a strong inhibitory and bactericidal effects along with the anti-fungal, anti-inflammatory, and anti-angiogenesis activity.[2] Silver is preferred as a NP due to its antibacterial, catalytic properties and their non-toxicity toward humans. The synthesis of silver NPs (AgNPs) using ecofriendly materials such as bacteria, fungi, and various plant extract is known as green synthesis. It also provides advancement over the other method as they are simple, one-step, cost-effective, environment-friendly, and relatively reproducible which result in more stable materials.[3] The green synthesis of NPs helps in reducing the generated waste and implementing sustainable process. The vast capability of a plant could be used for the synthesis of AgNPs through biological method. The stabilization of AgNPs was achieved by the plant extract which acts as the capping material.[4] The leaf extract of *Azadirachta indica (A. indica)* a member of Meliaceae family which is commonly known as neem is used for the green synthesis of NPs. The extract contains many phytochemicals such as terpenoids and flavonoids, which act as both capping and reducing agents.[5] The present work focusses on the synthesis of Ag NPs from neem leaf extract and its antibacterial effect.

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Materials and Methods

Sample Collection

Fresh leaves were collected from botanical garden of university campus in the month of February 2021 [Figure 1]. They were surface cleaned thoroughly with running water to remove any dirt or debris or any other contaminated organic contents. The wet leaves were kept for air dry at room temperature and fresh weight was determined.

Preparation of Neem Leaf Extract

Neem leaf extract was used to prepare AgNP on the basis of easy availability, cost-effectiveness, and also for its enriched medicinal properties, as for evaluating antibacterial, antimicrobial, antioxidative, and green synthesis of AgNPs.

Twenty grams of leaves were finely chopped using scissors and grinded using mortar and pestle. The chopped leaves were

boiled in 150 ml of double-distilled water for about 14 min in a heating mantle (Ragaa) [Figure 2a]. Then, the extract was left to cool down and after cooling it was filtered. From that, about 100 ml of the leaf extract was taken and the rest was stored in refrigerator for further use [Figure 2b]. This extract was used for reducing the silver ions (Ag+) in green synthesis to AgNPs.

Synthesis of AgNPs

Solution of 0.1 M $AgNO_3$ was prepared in 50 ml distilled water. To this solution, 100 ml of leaf extract was added and kept it in a magnetic stirrer (hot plate magnetic stirrer) for about 1 h. After cooling, it was taken for centrifugation in a cooling centrifuge at 4500 rpm at 37°C for 10 min [Figure 3]. Supernatant and pellet were separated. The pellet was dried and taken for further spectroscopic studies.

Characterization of AgNPs

Ultraviolet (UV)–Visible spectroscopy

The periodic scans of optical absorbance between 200 and 700 nm with UV–Visible spectrophotometer (Thermo Scientific, India) were performed to investigate the reduction rate of silver ions by neem leaf extract. The reaction mixture was diluted to ¾ ratio with water and used for UV–visible spectrophotometry. Distilled water was used to adjust the baseline.

Figure 1: Young neem leaves

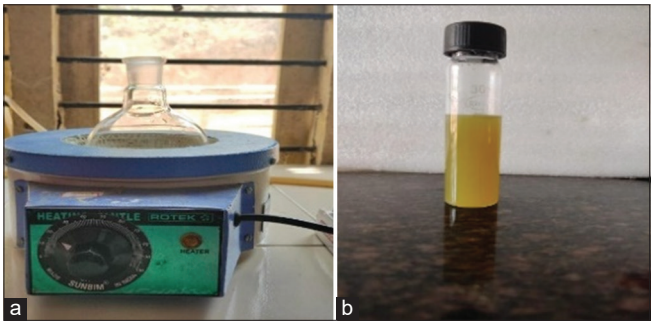

Figure 2: (a) Preparation of leaf extract by boiling. (b) Leaf extract

Fourier transform infrared (FTIR) analysis

The FTIR analysis of AgNPs helps in the result confirmation of the dual role of plant extract as a reducing and capping agent. The FTIR spectrophotometer was used to determine the functional group and chemical bond. The wavelength of the light absorbed was the characteristic of the chemical bond.

Antibacterial assay

The cultures were obtained from the standard culture collections maintained at Biovent, Department of Biotechnology, University of Kerala, Thiruvananthapuram. *Bacillus subtilis* (ATCC 25922), *Pseudomonas aeruginosa* (MTCC 424), and *Staphylococcus aureus* (ATCC 25923) were the bacterial cultures selected for the study.

Agar Well-diffusion Method

The antibacterial activity of AgNPs was done by agar well-diffusion assay. One hundred microliters of culture (0.5 McFarland Turbidly Standard, i.e. approximately $1–2 \times 10^6$ colony-forming units per ml) of test bacteria were loaded on to the sterility checked nutrient agar plates. The inoculum was swabbed uniformly over the entire agar surface using a sterile swab and allowed to dry for 5 min. Four wells of 8 mm were punched in the plate and added 50 µl of neem leaf extract, $AgNO_3$, separately. Streptomycin (0.125 mg/ml) was used as a positive control and $AgNO_3$ as negative control. Then, the Petri plates were kept for 24 h of incubation at 37°C and the result was observed by measuring the zone of inhibition (ZOI).

Results and Discussion

Synthesis of AgNPs

By the addition of neem leaf extract to silver nitrate solution, a visible color change from transparent green to dark brown was observed that indicates the formation of AgNPs. The brown color was due to the excitation of the surface plasmon resonance (SPR), which is a characteristic property of AgNPs.[5] SPR band depends on the particle size and refractive index of solution. After an incubation of 1 h, a complete color change was observed which indicates that the silver salt is completely reduced to AgNPs. The

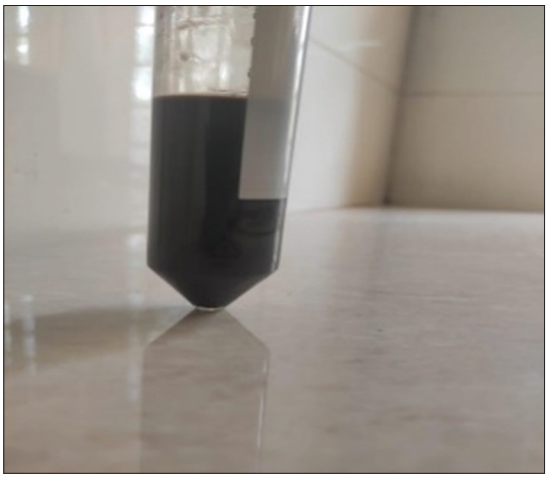

Figure 3: Silver nanoparticles

natural agents such as flavonoids and terpenoids present in the neem extract are responsible for the reduction of silver salt to its corresponding NPs.[6] The synthesis of novel metal NPs has been subjected for many applied researches due to its unique properties. Nano is used to indicate one billionth of a meter (or 10^{-9}). Particles with size less than 100 nm are referred to as NPs. *Pinus eldarica* was used[7] to obtain an aqueous extract that was further used to synthesize predominantly spherical AgNPs with diameter between 10 and 40 nm. A study from Ahmad and Sharma [8] reported that AgNPs synthesized by utilizing *Ananas comosus* (pineapple juice) as stabilizing as well as reducing agent. The AgNPs synthesized by utilizing *Argemone mexicana* leaf extract acted both as capping and reducing agent by adding to the aqueous solution of $AgNO_3$.[9] The properties of NPs are characterized by UV–Visible spectrometer, X-ray diffractometer, scanning electron microscopy, and FTIR spectrophotometer. The study[10] showed that the average size of NPs was 30 nm. The AgNPs synthesis was mostly spherical and oval in shape with average diameter up to 10–50 nm.[10] In another method, the AgNPs synthesis by Roy *et al*.[11] using the fruit extract of *Malus domestica* acted as capping agent with an average diameter of 20 nm. Report by Kaviya *et al*.[12] says that the synthesized AgNPs using *Polyalthia longifolia* leaf extract acted as reducing agent along with D-sorbitol enhance the stability of NPs. The plant parts such as roots, latex, stem, seeds, and leaves are used for NPs synthesis.[13] The active agents present in these parts make the stabilization and reduction possible and plant extracts incorporate biomolecule which influence the reduction and capping of NPs are terpenoids, polysaccharides, phenolics, alkaloids, flavonoids, amino acids, alcoholic compounds, enzyme, and protein. The chlorophyll pigments and quinal, methyl, chavicol, linabol, caffeine, eugenol, ascorbic acid, theophylline, and other vitamins have also been investigated.[14]

Characterization of AgNPs

The UV–Visible spectral analysis

The presence of shape and size controlled NPs in the aqueous suspension was recognized by UV–visible spectroscopy.[15] The samples were observed under UV–visible spectrophotometer for its maximum absorbance and wavelength to confirm the reduction of silver nitrate. The maximum peak was found to be 308 nm for AgNPs. From the study carried out by Lalitha *et al*.,[16] the peak value for AgNPs was 351 nm. The maximum peak value of 420 nm was found for AgNPs in the study done by Gavhane *et al*.[17] The UV–Visible spectrum of AgNPs using the fungi *Trichoderma viride* and *Trichoderma harzianum* showed a range of 420 nm, as reported by Fayaz *et al*.[18] and Singh and Raja[19] A range of wavelength from 200 to 800 nm was observed for Mulberry leaves extract.[20] The results obtained from bioreduction of AgNPs using *Spirulina platensis*, showed a SPR silver band at 400–800 nm.[21] In several literatures, the SPR peak of AgNPs was around 351 nm.[18]

FTIR Spectroscopy

FTIR analysis of neem leaf extract

According to Niraimathi *et al*.,[22] the FTIR spectra suggested that the proteins present in the plant extract act as capping agents. In the present study also, the neem leaf extract acted as reducing and capping agent. The broad band of 3491 cm^{-1} is due to the N-H stretching vibration of group NH_2 corresponding to amide and 3217cm^{-1} due to OH group corresponds to alcohols overlapping of stretching vibration for *A. indica* leaf extract molecules. The observed peak at 1018.41 cm^{-1} denoted C-O stretch corresponds to carboxylic acids. It can be inferred that terpenoids present in neem leaf extract act as stabilizing as well as capping agents. Besides terpenoids, the presence of flavonoids is also possible. The observed peak was attributed to the flavonoids and terpenoids present in plants extract.[23] The extract sample showed a wide and strong peak with maximum intensity at 1018.41 cm^{-1}. From FTIR result [Figure 4], it can be concluded that some of the bioorganic compounds from *A. indica* extract formed a strong capping on the NPs.

FTIR Analysis of AgNPs

By FTIR spectroscopy, biomolecules responsible for capping and stabilization of AgNPs can be identified. The peaks between regions 3727 and 3629 cm^{-1} correspond to OH stretching of alcohol and phenol compounds. The peak in the region 1982 cm^{-1} corresponds to the carbonyl group. A medium bond at 2323 cm^{-1} was attributed to the presence of C-N group. The 1701 cm^{-1} corresponds to C=O stretch of α,β-unsaturated aldehydes, ketones and 1509 cm^{-1} represents N-O asymmetric stretch which corresponds to nitro compounds alkenes. These bands denote stretching vibrational bands responsible for compounds such as flavonoids and terpenoids[24] and so may be held responsible for efficient capping and stabilization of obtained AgNPs. From FTIR analysis [Figure 5], it was found that the carbonyl group from amino acid reduces and proteins form a layer over the NPs to prevent agglomeration and stabilization.[25] Therefore, the FTIR analysis of synthesized Ag NPs using neem leaf extract showed that their functional group from the aqueous extract of neem was responsible for the reduction of Ag+ to Ag, the Ag NP synthesis, and stabilization, and is present on the surface of synthesized NPs. In aqueous medium, the biomolecules act as capping agents for stabilizing NPs.

Antibacterial Activity

In the present study for AgNPs, no ZOI was found for *B. subtilis*, 18 mm for *S. aureus*, and 24 mm for *P. aeruginosa* [Table 1 and Figure 6]. The silver ions and silver salts are used as antibacterial agents.[26] Even though the AgNPs are widely used as an antibacterial agent, their exact mechanism of inhibition is still unclear. The respiratory function and permeability of bacterial

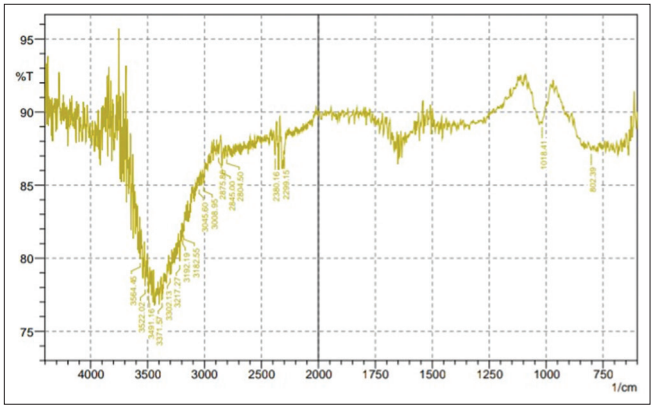

Figure 4: Fourier transform infrared analysis of neem leaf extract

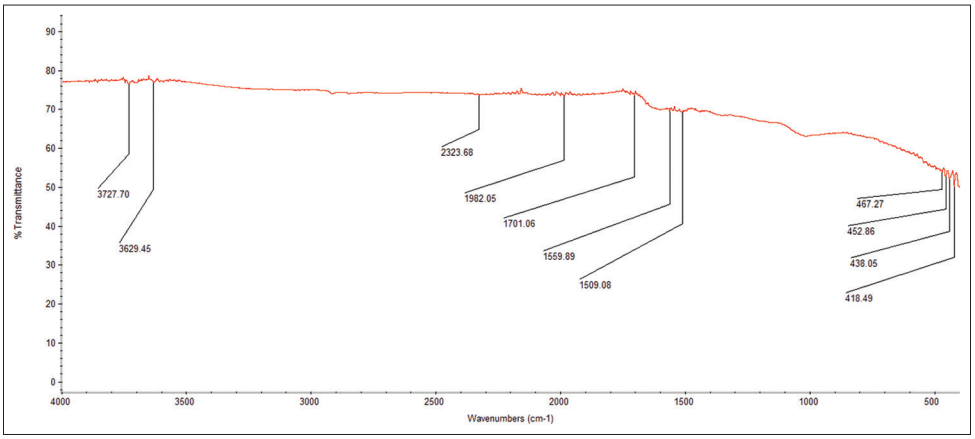

Figure 5: Fourier transform infrared analysis of silver nanoparticles

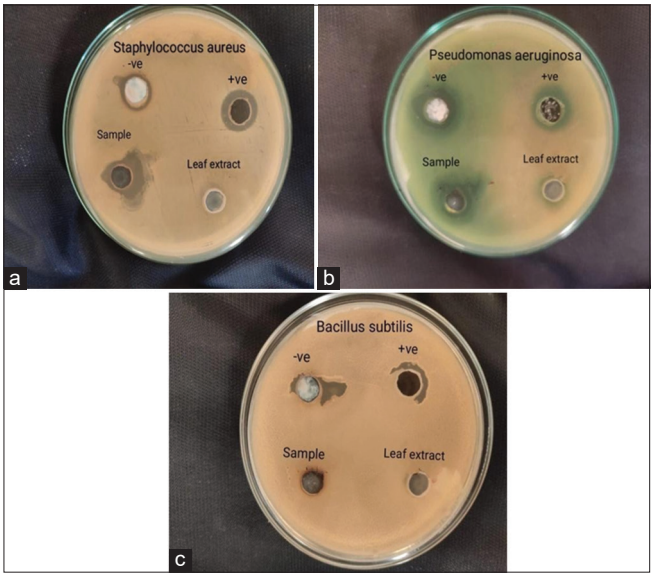

Figure 6: Antibacterial activity of –ve ($AgNO_3$), +ve (streptomycin), sample (silver nanoparticles), leaf extract against (a) *Staphylococcus aureus*, (b) *Pseudomonas aeruginosa*, (c) *Bacillus subtilis*

Table 1: Antibacterial activity of $AgNO_3$ (–ve control), streptomycin (+ve control), leaf extract, and AgNPs

Bacterial strain	*$AgNO_3$ (–ve)*	*Streptomycin (+ve)*	*Leaf extract*	*Sample*
Bacillus subtilis	22	16	0	0
Staphylococcus aureus	22	15	0	18
Pseudomonas aeruginosa	19	25	0	24

AgNPs: Silver nanoparticles

cells become unstable when the AgNPs attach to the surface of the cell membrane.[27] The AgNPs are effective at lower concentration than that of ions.[28] The silver ions and NPs are attached to the cell membrane and cause acclimatization of the envelope protein precursors which lead to the dissipation of proton motive force.[29]

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