Evaluation of antibiofilm and catalytic activity of biogenic silver nanoparticles synthesized from Acacia nilotica leaf extract

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Abstract
In the current research of bacteriology, the biofilm infections are becoming serious issues. The biofilm has potential resistant towards antibiotics, even at higher concentration, due to the protective covering that enable unavailability of these antibiotics towards bacteria residing inside. Thus to combat these problems, biogenic silver nanoparticles (AgNPs) of spherical shape were synthesized having potential against biofilm forming bacteria. Here, we focused on the green synthesis owing to its inherent features such as simplicity, eco-friendliness, cheap rate and rapidity. We have synthesized AgNPs using the aqueous extract of Acacia nilotica leaf. The bioactive compounds present in the extract are responsible for the reduction of Ag⁺ to Ag⁰. The synthesis conditions were optimized using critical parameters and the optimized one was then characterized via UV-Vis spectroscopy, dynamic light scattering, transmission electron microscopy, x-ray diffraction and Fourier transform infrared spectroscopy. Further, synthesized AgNPs were used to evaluate strong antibacterial activity and catalytic potential that was assessed against degradation of anthropogenic pollutant 4-NP and azo dyes: methylene blue and congo red.

Keywords: green synthesis, silver nanoparticles, antibacterial activity, catalytic potential

Classification numbers: 2.00, 2.04, 2.05

1. Introduction
In the modern era, indwelling devices have become the major part of our healthcare and normal lifestyle. But all these modernizations are somewhere associated with bacterial infection risk that is causing serious health issues, specially the biofilms. The biofilm acts as a protective covering for the bacteria in unfavourable environmental conditions as well as a barrier for antibiotics which inhibit their access to the bacteria that leads to continuous growth resulting in a mature biofilm. Further, these mature biofilm bacteria can invade the immune system by producing super antigen within the biofilm. Thus, these biofilms are a serious threat to biomedical and healthcare section [1, 2].

Further, increasing industrialization is creating major ecological problem as they are releasing hazardous chemical waste affecting the mankind with serious health issues. Among all, organic dyes that are non-biodegradable and various other
nitro aromatic compounds comprise the major portion of these wastes. The main sources of waste are various pharmaceutical, textile, food and packing industries. Although various conventional methods are employed to process these wastes, such as flocculation, UV-light degradation, activated carbon sorption, reduct treatment and adsorption, it is limited by its improper ability to remove the hazardous substances in an effective manner as these are known to change only the chemical phase. These azo dyes and nitro aromatic compounds are potentially stable and show carcinogenic effect [3, 4].

Nanomaterials have been gaining tremendous attention owing to their beneficial physicochemical properties and applications in the field of catalysis and water treatment. Among the available metal nanoparticles, silver nanoparticles have proved to be a potent agent for their antibacterial, antimicrobial activity and catalytic degradation of various pollutant compounds [5, 6]. In addition, AgNPs can be employed as anti-fungal, anti-bacterial, anti-inflammatory, anti-viral, anti-angiogenesis and antiplatelet agent. Also, silver is known to have a recorded history of its medical and therapeutic benefits more than its limitations which date back to the period before realization of microbes as the source of infections [7]. These particles also exhibit surface plasmon resonance (SPR) that results in a characteristic band due to collective oscillation of itinerant electrons in resonance with frequency of the light in visible and infrared region [8, 9].

Metal nanoparticles are synthesized by different methods such as chemical and physical methods. These methods are capable of producing small sized and well-defined particles. However, the chemicals used are toxic, energy consuming and expensive, making them unsuitable for biological purposes [10]. In comparison to this, green synthesis approaches have proven to be a promising method for synthesis as these are cost effective, eco-friendly and can be easily scaled up for large scale production of the particles [11]. Numerous studies have investigated plants extract for the biosynthesis of AgNPs such as Terminalia arjuna, Canarium ovatum, Prosopis juliflora bark, Cordia dichotoma, Cicer arietinum and Dichoma tomentosa [12–17]. Nanoparticles synthesized from microbial process and whole plant is an expensive process, whereas particles synthesized from plant extract can be used in various applications including drugs, targeted drug delivery and cosmetic applications [18, 19].

In the present work, we are introducing a rapid, eco-friendly and cost-effective method for catalytic degradation of hazardous chemicals including 4-nitrophenol and azo dye. Here, AgNPs were synthesized using cell free aqueous extract of Acacia nilotica (A. nilotica) leaves and silver nitrate solution. This biogenic synthesis of AgNPs was optimized using crucial parameters. The particles were then analyzed by UV-Vis spectroscopy, dynamic light scattering technique (DLS), transmission electron microscopy (TEM), x-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy. These AgNPs were then examined for their catalytic potential against methylene blue (MB), congo red (CR) and 4-nitrophenol (4-NP). Further, antibacterial activity of the particles was determined against pathogenic bacteria.

2. Materials and methods

2.1. Materials
Silver nitrate (AgNO₃, 99.99% MW = 169.87), Luria Bertani broth, Luria Bertani agar, 4-nitrophenol and kanamycin were purchased from Central Drug House (CDH) Bioscience (p) Ltd. New Delhi, India. The in-house prepared milli-Q water (EMD millipore) was used for the experiment. Whatman filter paper No. 1 was used for filtration and disk preparation.

2.2. Sample collection and extract preparation
The leaves of A. nilotica were collected from the campus of Central University of Rajasthan Bandarsindri, Kishangarh, Ajmer. The collected leaves were then separated and cleaned by washing under running tap water. Double distilled H₂O was used for rinsing. The cleaned leaves were left for 7–10 days to dry. 10 g of this powder was weighed and boiled in 100 ml of double-distilled water (ddH₂O) at 60 °C (Stuart UC152, Biocote) for 20 min. The obtained mixture was then cooled and filtered under reduced pressure condition (Lab. Companion VE-11, Korea) using Whatman filter paper no. 1.

2.3. Biosynthesis of silver nanoparticles
The biosynthesis reaction was performed by dropwise addition of this extract into aqueous AgNO₃ solution under continuous stirring at 500rpm. The reaction was then stirred continuously for a color change and after that the synthesized AgNPs were separated by centrifugation at 10000rpm (Heraeus, Fresco 17, Thermoscientific, table top refrigerated centrifuge) for 10 min. The obtained pellet was washed thrice and re-dispersed in milli-Q water.

2.4. UV-Vis spectrophotometric analysis
Due to the unique optical properties of AgNPs, UV–visible spectrophotometer scanning was done for the confirmation of our visual observation of AgNPs synthesis. For this, AgNPs were scanned between the range of 300 to 700 nm. The procured data gave a confirmation of synthesis along with a rough estimation about the size and morphology of nanoparticles that helped in the optimization studies of reaction parameters.

2.5. Optimization of different parameters
Further, various crucial parameters essential for the synthesis of Acacia nilotica leaf mediated AgNPs (ANL-AgNPs) were optimized including the time point of the reaction, the ratio of extract to AgNO₃, concentration of AgNO₃ and finally temperature of the reaction that control the size, morphology, yield and agglomeration state. The synthesized AgNPs were centrifuged, washed and sonicated prior to its analysis in UV-visible spectrophotometer at different time points (10, 20, 40, 80, 120, 160 and 200 min), by keeping the other three parameters constant. The UV-Vis spectrum was evaluated, and the optimized time was used for further reactions.
Further, different concentrations 0.1, 0.5, 1.0, 1.5 and 2mM of silver nitrate solution were prepared for the biosynthesis reaction including extract to AgNO₃ ratio taken as, taken as 1:40, 1:20, 1:10, 1:6.5 and 1:5 ratio. Further, an optimum temperature for maximal and homogenous synthesis of AgNPs was drawn by conducting reactions at different temperatures such as 4, 25, 40, 60, and 80 °C. All the reactions were subjected to UV-visible spectrophotometer analysis.

2.6. Physicochemical characterization of silver nanoparticles

After confirmation of synthesis, AgNPs were analysed through dynamic light scattering (DLS) technique for determining their average hydrodynamic diameter along with polydispersity index (Pdi) using zetasizer nano ZS (Malvern Instruments UK). Fully dispersed AgNPs in milli-Q water were employed with a nominal 5 mW HeNe laser run at 633 nm wavelength by a scattered light at the angle of 173°. The observation was taken in triplicates. Thereafter, AgNPs were subjected to zetasi- zurizer nano ZS with a laser doppler velocimetry for the measurement of zeta potential. Disposable zeta cell was used, and electrophoretic mobility was measured in triplicate and the average calculated in an automatic mode.

Morphological characterization was done using transmission electron microscopy (TEM). X-ray diffraction (XRD) was employed to determine the crystalline nature of the synthesized AgNPs.

Fourier transform infrared spectroscopy (FTIR) data of AgNPs was evaluated for the functional group analysis. AgNPs and the extract were dried for the preparation of pellet with KBr that were scanned against the blank KBr pellet in the range of 4000 to 450 cm⁻¹. The procured data gave the information analysis of interacting functional groups and capping agent involved in the synthesis and protection, respectively.

2.7. Evaluation of antibacterial potential via disc-diffusion assay

Evaluation of antibacterial potential of ANL-AgNPs was performed via disc-diffusion assay executed against E. coli and P. aeruginosa. For the preparation of primary culture, a single colony was picked from the Luria Bertani (LB) agar culture plate and inoculated in LB broth. The culture was kept at 37 °C for 16 h at 150rpm. From this primary culture, 1% inoculum was inoculated in fresh broth and incubated to grow till it reached 0.4 optical density at 600 nm. The secondary culture was evenly spread on separate LB agar plate and sterile six paper discs (5 mm) were placed. Four of them were impregnated with different concentrations of ANL-AgNPs (0.25 µg to 1 µg). Remaining discs were used as control impregnated with de-ionised water as negative and antibiotic (kanamycin) as positive control. The plates were then kept at 37 °C for 24 h and zone of inhibition was measured.

2.8. Evaluation of anti-biofilm activity using tissue culture method

Tissue culture plate (TCP) or microtiter plate (MTP) assay was performed to evaluate the antibiofilm activity of synthesized ANL-AgNPs [20, 21]. Firstly, primary cultures of Pseudomonas aeruginosa and Staphylococcus aureus were prepared by inoculating single colonies of both bacteria in 5 ml of tryptic soy broth (TSB) separately and incubated for 16 h at 37 °C. The primary culture was further diluted in 1:100 with fresh TSB and treatment of different concentration of ANL-AgNPs (0.25, 0.50, 0.75 and 1 µg) were given to bacteria in 96-well plate along with negative control (only TSB) and positive control (TSB with culture). The plates were then incubated at 37 °C for 24 h. On the next day, the content of 96-wells was discarded, and well were washed with phosphate buffer saline (PBS) thrice for the removal of free-floating bacteria. The wells were then treated with 100 µl of 0.1% crystal violet and incubated for 10–15 min. The wells were then rinsed with PBS to remove excess stain and air dried for 15 min. Finally, the biofilm bound crystal violet was dissolved by using 100 µl of 30% acetic acid for 15 min, the content was mixed by pipetting and monitored at 570 nm using microtiter plate reader [22, 23].

2.9. Microscopic study of anti-biofilm activity of AgNPs

Coverslip methods was performed to evaluate the microscopic study of anti-biofilm activity of ANL-AgNPs [24]. Firstly, primary cultures of P. aeruginosa and S. aureus were prepared by inoculating single colonies of both bacteria in 5 ml of tryptic soy broth (TSB) separately and incubated for 16 h at 37 °C. The primary culture was further diluted in 1:100 with fresh TSB and bacterial colonies were allowed to grow on coverslip of 1 x 1 mm diameter placed in 6-well plate. The treatment of 1 µg concentration of ANL-AgNPs was given to both bacteria along with negative control (only TSB) and positive control (TSB with culture). The plates were then incubated at 37 °C for 24 h. On the next day, the content of 6-wells was discarded, and wells were washed with 1 × PBS thrice for the removal of free-floating bacteria. The same procedure was followed for crystal violet staining as mentioned above. After washing of stains the coverslips were carefully put on the glass slides and observed under the bright field microscope [25].

2.10. Evaluation of catalytic activity of AgNPs

Catalytic degradation of 4-NP was carried out in three set of reaction using 2 mM of 4-NP and 0.03 M of NaBH₄. Set 1 (control) included the 200 µl of 4-NP in 2 ml water, set 2 involved the same as in control 1 along with 1 ml NaBH₄. The third set (test reaction) involves both ingredient of 1 and 2 along with 30 µg x ml⁻¹ AgNPs [26]. After a stipulated time points starting from 0, 5, 10, 20 and 40 min, the mixture was monitored for degradation of 4-NP using UV-Vis spectrophotometer after centrifugation or removal of AgNPs [27].

Further, the catalytic degradation of MB and CR involves two sets of reaction. Set 1 (control) reaction involved 10 mM of 1 ml of NaBH₄ that was mixed with 1 mM (1.5 ml) of dye separately and the volume of both the reaction were made up-to 10 ml by adding ddH₂O. The set 2 (test reaction) involved the same ingredient as in control along with AgNPs. The reaction was then monitored using UV-Vis spectrophotometer [28, 29].
3. Results and discussion

3.1. Biosynthesis of silver nanoparticles

A clear, light brown color aqueous extract of *A. nilotica* leaf was obtained by boiling the powdered leaf followed by filtration under reduced pressure condition. The filtrate was added to AgNO₃ solution in a drop wise manner under continuous stirring for biosynthesis reaction. The reaction resulted in the change of color from light yellow to a dark greyish solution that visually confirmed the synthesis of AgNPs due to the reduction of Ag⁺ into Ag⁰ (figure 1(A)). The resulting reduction of Ag⁺ can be attributed to the action of phytochemicals present in the extract. The synthesized AgNPs were then subjected to centrifugation followed by washing thrice with ddH₂O and resuspension in milli-Q water.

3.2. UV-visible spectrophotometric analysis and optimization study

The first qualitative confirmation of AgNPs synthesis was done by UV-Vis spectroscopy. The scanning data of AgNPs ranging from 300 to 700 nm showed a maximum absorbance peak at 420 nm. This peak arose due to the surface plasmon resonance (SPR) of AgNPs in 400–440 nm, where the free electron in the conduction band undergoes collective oscillation or vibration after excitation by an incident light of particular wavelength [30, 31]. The data procured after scanning gives a rough estimation about size and morphology of synthesized AgNPs.

Further, optimization of several critical parameters are very significant and essential due to sensitivity of synthesis reaction for AgNPs that control size, shape, yield and agglomeration.
The absorbance spectrum of AgNPs (figure 1(B)) was obtained at different time points and revealed that at reaction time 20 min, the absorbance maxima of SPR was obtained around 420 nm. Wherein at other time points such as at 40 and 80 min the absorbance maxima intensity decreased as the reaction saturated. The absorbance maxima usually increases with increase in the synthesis of smaller particles, that can ultimately lead to aggregation of the particles due to increased collision frequency of AgNPs [32, 33]. Along with this, at 200 min the SPR band showed bathochromic shift, indicating larger particles formation [33, 34]. So, synthesis for a duration of 20 min could be considered as the optimized time for reaction. Further, in case of 0.1 mM AgNO₃ concentration, synthesis of AgNPs does not occur as the reactants do not attain their threshold. The highest absorbance was observed for the reaction with 1.5 mM AgNO₃ concentration showing SPR band near 420 nm (figure 1(C)). Subsequently, different extract to AgNO₃ ratio were used for optimization. The procured data from UV-visible spectra (figure 1(D)) suggested that 1:10 (1 ml of extract) aided in the synthesis of nanoparticles with absorbance spectra near 420 nm generating higher yield. Similarly, in case of temperature optimization the
3.3. Physicochemical characterization of AgNPs

The optimized AgNPs were further characterized by DLS, TEM, XRD and FTIR to evaluate their physicochemical properties. The average hydrodynamic size of the particles was evaluated for the freshly prepared AgNPs using DLS technique. The data procured determined the size distribution profile to be in the range of 57.17 nm along with a polydispersity index (PdI) of 0.29, representing the homogeneous state of AgNPs nanoparticles in terms of size (figure 2(A)). Further, the evaluated zeta potential measurement of the suspension was found to be $-14.5$ mV (figure 2(B)). For average size and morphological analysis, the AgNPs were evaluated through TEM, where the image (figure 2(C)) revealed that the size of AgNPs was in the range of 10–20 nm along with spherical morphology. XRD data of particles revealed the main peaks of Bragg reflection at $2\theta$ near (1 1 1), (2 0 0), (2 2 0) and (3 1 1) (figure 2(D)). The high intensity on these set of lattice planes shows a typical pattern of face centered cubic (fcc) structure of AgNPs, which was matched with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783.

Further, FTIR spectroscopy was evaluated for the functional group and capping agent analysis that were involved in the synthesis and protection of AgNPs (figure 3). The leaf extract band (band (a)) indicates peak of –OH bend shift from 3487.8 to 3368.6 cm$^{-1}$. Similarly, the peak of phenolic compounds also shifted. These data indicated alcoholic and phenolic compounds to be involved in the synthesis of AgNPs. Along with this, the peaks near 1715, 1618 and 1390 cm$^{-1}$ depicts aromatic compounds and proteins. On the other hand, AgNPs showed (band (b)) indicated peak near 3487.8 and 2750.7 cm$^{-1}$ depicting the presence of –OH (alcoholic) and phenolic compounds with carboxylic group stretching. Peak near 1764, 1569 and 1390 indicated the presence of proteins and metabolites that play role for capping and stability of the AgNPs.

3.4. Evaluation of antibacterial potential via disc diffusion assay

The potent antibacterial efficacy of synthesized AgNPs was evaluated using disc diffusion assay performed against *E. coli* and multi-drug resistant *P. aeruginosa* on LB agar plates. In both the cases the efficient antibacterial activity was evaluated for stipulated concentrations (0.25, 0.5, 0.75 and 1 $\mu$g) of AgNPs on discs (C, D, E, F). Discs ‘A’ and ‘B’ were taken as negative and positive control with autoclaved deionized water and antibiotic (kanamycin), respectively. As evident from the image (figures 4(a) and (b)), the growth of bacteria was suppressed in an appreciable manner. Furthermore, the zone of inhibition for both the bacterium were measured and quantitatively presented by bar graph (figure 4(c)), revealing the concomitant decrease in the growth rate with increase in the concentration of ANL-AgNPs from 0.25 $\mu$g to 1 $\mu$g, indicating that the antibacterial potential was highly dose-dependent. The zone of inhibition diameter was presented in millimeter by taking as mean ± SD of duplicates. Thus, AgNPs could play an important role in surmounting the problem of multi-drug resistance.
3.5. Evaluation of anti-biofilm activity using TCP or MTP assay

Biofilm is described as the aggregated species on a solid surface of extracellular polymeric substance (EPS) which is secreted outside by these bacteria itself. These biofilms act as barrier, and are resistant towards antibiotics even at higher concentration due to the protective covering that enable unavailability of antibiotics towards bacteria residing inside [1, 2]. The tissue culture plate (TCP) or microtiter plate (MTP) assay was performed for quantitative evaluation of in vitro antibiofilm activity. This method studies the early stage biofilm formation in the static condition, and our synthesized ANL-AgNPs were examined for their antibiofilm potential against gram-negative \( P. \) aeruginosa and gram-positive \( S. \) aureus that was done by checking the extent of crystal violet (CV) absorbed by biofilm forming bacteria using microtiter plate reader [23]. CV, a basic dye, non-specifically binds to the negatively charged moieties. Here in this assay, CV binds to polysaccharides EPS secreted by bacteria for biofilm formation and extent of binding or absorption of CV indicates the strength or active biofilm formation [22]. Here, the result indicated (figure 5) the efficient antibiofilm potential of ANL-AgNPs against \( P. \) aeruginosa and \( S. \) aureus. The procured data revealed only 30% of biofilm formation (in case of \( P. \) aeruginosa) and 47% (in case of \( S. \) aureus) even at the smallest concentration of 0.25 \( \mu \)g as compared to the control one where no treatment was given. These data revealed 70% and 53% inhibition of biofilm in case of \( P. \) aeruginosa and \( S. \) aureus respectively at 0.25 \( \mu \)g concentration. Further the % of inhibition of biofilm was observed in a dose dependent manner where at 1 \( \mu \)g concentration, the % of biofilm was 22 and 19 that means the % of inhibition was 78 and 81 for \( P. \) aeruginosa and \( S. \) aureus, respectively.

3.6. Microscopic study of anti-biofilm activity using coverslip method

Here in the experiment, both the bacteria \( P. \) aeruginosa and \( S. \) aureus were allowed to grow on the coverslips that were kept in 6-well plate having TSB and diluted culture. After staining, the coverslips were dried well. The coverslips were then placed upside down on glass slide and observed under light microscope and it was evaluated that the non-treated culture of both \( P. \) aeruginosa and \( S. \) aureus absorbed higher crystal violet stain due to successful development of biofilm layer. The blank coverslip (TSB only) did not absorb the CV stain due to the absence of bacterial colonies. Further, the ANL-AgNPs treated coverslip also absorbed the CV stain, but the extent of absorption was reduced due to inhibition of formation of bacterial biofilm. In the results, figure 6 depicts the microscopic view of CV stained slide where (A) displays the blank slide that contain only TSB and CV not absorbed, (B) shows the PA culture slide that absorbed stain indicating the formation of bacterial biofilm and (C) with 1 \( \mu \)g ANL-AgNPs treated PA culture slide where the extent of CV absorption is reduced as compared to PA culture coverslip. This indicates the biofilm inhibition potential of our synthesised ANL-AgNPs against PA. Figure 6(D) shows the images of all three slide for PA. Further, figures 6(E)–(H) shows the same pattern for SA culture. These images clearly shows the effective biofilm formation and their inhibition in ANL-AgNPs treated cultures at low concentration.

3.7. Evaluation of catalytic activity of AgNPs

Catalytic activity of AgNPs was evaluated by testing its degradation potential on anthropogenic pollutant (4-NP) and azo dyes (MB and CR). In case of 4-NP, all the 3 set of reactions were scanned in the range of 200–500 nm through UV-Vis
spectrophotometer (figure 7(a)). The set 1 (control) exhibited the absorbance maxima near 317 nm indicating 4-NP where in set 2, due to the addition of NaBH₄, the peak was red shifted to 400 nm. This peak indicates the synthesis of sodium phenolate [35]. After addition of AgNPs in the test reaction, the mixture was monitored through UV-Vis spectrophotometer at different time points after centrifugation. The results here indicate that the degradation of 4-NP start immediately with addition of AgNPs and after 10 min, a significant decrease in the intensity of 4-NP was observed. Along with the reduction of 4-NP peak, there was emergence of a new peak at 296 nm which indicates the conversion of 4-NP to 4-AP [36, 37]. So, these data indicates the catalytic potential of synthesised ANL-AgNPs.

The degradation assay of MB was performed in comparison with NaBH₄ as control at different time points. Here two sets were involved where set 1, that is control, involved dye with aqueous NaBH₄ and set 2 involved the same along with AgNPs. The reaction mixture of both sets were monitored through UV-visible spectrophotometer. The data of the reaction 1 (control) indicates that even after 90 min (figure 7(b)) of incubation the degradation of MB was not very efficient. Whereas in set 2 (test reaction) the degradation immediately
started (figure 7(c)) with the addition of AgNPs and the dye was completely degraded within 20 min [29].

The degradation assay of CR was performed in comparison with NaBH₄ as control at different time points in a similar way as for MB mentioned above. Also in control reaction, the degradation was not efficient even after 80 min of incubation (figure 7(d)), wherein second test reaction data (figure 7(e)), the AgNPs acting as catalyst showed complete degradation of CR in 20 min [28].

4. Conclusion

The present study explains the successful green synthesis of silver nanoparticles using A. nilotica leaf extract that have potential to combat various harmful environmental pollutant by their catalytic action. In contrast to previous studies, our study showed smaller particle size (10–20 nm) with spherical morphology after thorough optimization of various parameters. In addition, the synthesized AgNPs also displayed antibacterial activity like that of commercial antibiotics. Further, the catalytic application of AgNPs evaluated against degradation of 4-NP, MB and CR depicted its immediate degradation on addition of AgNPs.

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