Green Synthesis of Silver Nanoparticles using *Azadirachta indica*(Neem) Leaf Extract and their Antibacterial Activity

Dr. T. Venugopala Swamy
Assistant Professor of Zoology
Girraj Government College (A), Nizamabad, Telangana

Abstract

In the present study, an aqueous extract of fresh leaves of Azadirachta indica (Neem) was used for the synthesis of silver nanoparticles. The formation of silver nanoparticles was confirmed using different methods of characterization such as UV-Visible spectroscopy (UV-Vis), transmission electron microscopy(TEM), Dynamic light scattering (DLS) and zeta potential. The antibacterial effect of biosynthesized AgNPs and aqueous leaf extract was performed against Gentamycin(Control) using different concentrations (10µg/ml, 20µg/ml and 40µg/ml) against Gram -ve (E.coli and P.aeruginosa) and Gram +ve (B.subtilis and S. aureus) bacterial strains that are sensitive to a large number of antibiotics. The biosynthesized AgNPs showed maximum zone of inhibition against all the four bacterial strains that were tested while the control and the aqueous leaf extract did not exhibit any zone of inhibition. However, maximum zone of inhibition was observed in Gram-ve bacteria when compared to Gram +ve bacteria. Further, the zone of inhibition increased with increased concentrations.

The results of the present study indicates that Neem leaf extract can be effectively used in the synthesis of silver nanoparticles and serve as potential antibacterial agent.

***Key words**: Silver nanoparticles. Azadirachta indica, Gram + ve bacteria, Gram – ve, antibacterial activity.*

Introduction:

Over the last decades, nanotechnology emerged as one of the active field of research in material science. The nanoparticles (NPs) exhibit unique properties owing to their specific characteristics such as size, distribution, and morphology that leads themselves to wide variety of applications (Tolaymat et al, 2010). AS a result, they possess enormous potential to serve all facets of life. Nanoparticles are basically clusters of atoms ranging between 1 and 100 nm in size. The word ‘nano’ is used to indicate one billionth of a meter (Brigger et al, 2002; Rai et al, 2009; Sudarenkov, 2013). Among the metal nanoparticles, the silver nanoparticles (AgNPs) possess wide area of interest varied applications in different fields such as clothing, dentistry, catalysis, mirrors, optics, photograph, electronics, food industry etc. They have immense significance in biological sciences and its role in different areas of biological research including medical and biotechnology has a great contribution towards the development of newer areas in the field of medicine. AgNPs show different physical and chemical properties to that of metallic silver due to their size(Nordberg et al; Marcone, GP, 2011).

It has been reported that silver is effective against more than 650 pathogens possessing broad spectrum of activity. The use of silver in the form of nanoparticles enhances this property thus allowing its use in wide range of applications (Yoon et al, 2007; Dastjerdi and Montazer, 2010). Hence nano-silver is considered as one of the most important and viable alternative to antibiotics because of its high potential to solve the problem of multidrug resistance which is generally observed in several bacterial strains(Rai et al, 2012; Franci et al, 2015; Salomoni et al, 2015).

Silver is known to possess inhibitory effect towards commonly present in medical and industrial processes(Jiang et al, 2004). Because of their antimicrobial properties, silver nanoparticles have been used in health, medicine, textile coatings, food storage, due reduction, dressing of wounds, antiseptic creams and varied number of environmental applications(Gao et al, 2014). They possess broad applications especially in skin ointments and creams to avoid infection of burns and open wounds . A number of studies have demonstrated the effective use of AgNPs as dressings for covering the burns to surgical devices and also for bone prostheses. AgNPs are always incorporated into clothing with the purpose of producing antibacterial effect (Lansdown, 2006; Chen et al, 2006; Cohen et al, 2007; Lee et al, 2007).

Elemental silver and its compounds were used since ancient times as microbial agents and were used in the form of silver coins/silver vessels to preserve water (Padalia et al, 2014, Devi and Joshi, 2015).

Various techniques are available for the synthesis of AgNPs that are capable to control the size of AgNPs. The AgNPs with small size and without bulking between the particles is highly favourable (Rai et al, 2009). Several physical and chemical routes and green synthesis are known for the preparation of metal nanoparticles. However, most of these methods are costly and toxic (Nanda, 2009; Sathish Kumar, 2016). On the other hand, the biological method was reported to be clean, non-toxic and environmentally acceptable route (Njagi, 2011; Muthukrishnan, 2015) as it utilizes non-toxic chemicals, ecofriendly solvent and renewable materials. The use of plants and its extracts as reducing agents for the synthesis of AgNPs is very feasible, cost- effective and eco-friendly (Mervat et al, 2012). The various biochemicals such as protein, phenols and flavonoids present in plants not only play an important role in reducing the ions to nano-size, but also help in capping of nanoparticles (Kuppuswamy et al, 2015). The present study was thus performed to evaluate the antibacterial activity of biosynthesized AgNPs and aqueous leaf extract using different concentrations (10 µg/ml, 20 µg/ml and 40 µg/ml) on both Gram-negative bacteria (E. coli and P. aeruginosa) and Gram-positive bacteria (*B. subtilis* and *S. aureus*) against Gentamycin (Control).

Materials and Methods:

Analytical grade silver nitrate was purchased from Merck (Mumbai, India).All the other chemicals were of analytical grade and were used without any purification. Whatman filter paper no-42 were purchased from Eppendorf, India. The microorganisms were collected from the National Chemical Laboratory, Pune, India. Double distilled(deionised) water was used for the experiment. Fresh leaves of *Azadirachta indica* (Neem) were collected from a greenhouse facility in Hyderabad, Telangana, India.

Preparation of Plant Extract:

Azadirachta indica (Neem) leaf extract was used to prepare the silver nanoparticles. Fresh neem leaves were collected and the surfaces of the leaves were cleaned with running tap water to remove the debris and other contaminated organic contents followed by washing with deionized water and drying at room temperature. About 15gm of finely cut leaves were taken in a 250ml Erlenmeyer flask containing 50ml deionized water and boiled for 25 minutes at 60°C in a water bath. Then the extract was cooled to room temperature, filtered using Whatman filter paper no-42 under vacuum and stored at 4°C for further use.

Synthesis of Silver Nanoparticles:

About 100mL of 1mM solution of silver nitrate ($AgNO_3$) was prepared in an Erlenmeyer flask. The silver nanoparticles (AgNPs) were synthesized by adding 10mL of the A. indica (Neem) leaf extract to 50mL of 1mM aqueous $AgNO_3$ solution by stirring continuously for 20 minutes at room temperature. The mixture thus obtained was incubated in a dark chamber at room temperature to prevent auto-oxidation of silver nitrate. The reduction of silver ions to silver nanoparticles was confirmed by the colour change from reddish to dark brown solution indicating the synthesis of AgNPs. The formation of AgNPs was also confirmed using UV-visible spectroscopy.

Characterization of Biosynthesized Silver nanoparticles (AgNPs):

The UV-Visible Spectrophotometric analysis of the biosynthesized Ag-NPs was carried out at room temperature using V-670, UV-visible spectrophotometer(JASCO) with a resolution of 0.5 nm. The absorbance of the sample was read at wavelengths ranging between 200 to 700nm. Subsequently one millilitre of the sample was pipetted into a test tube and analysed at room temperature. The chemical structure of the biosynthesized AgNPs was determined using Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS). The TEM measurements were done using a field emission JOEL-JEM-2100F TEM, operating at 200 KV (JEOL, Tokyo,Japan). DLS and zeta potential measurements were performed using a Zetasizer (Malvern, Worcestershire, UK).

Antimicrobial Activity:

The antimicrobial activities of the biosynthesized AgNPs and aqueous leaf extract was determined using Gram -ve(E. coli and P. aeruginosa) and Gram +ve(B. subtilis and S. aureus) following disc diffusion method (Kurby bauer,1966). The bacteria were cultured in Muller Hinton broth at 35^{o}C ± 2^{o}C on an orbital shaking incubator at 160rpm. Then a lawn of bacterial culture was prepared by spreading 100µL of broth culture having each test organism on a solid nutrient agar plate. The plates were left overnight at room temperature to allow the culture for absorption or to dry. About 8mm size wells were punched into the agar with sterile micropipette tips and then 100 µL of different concentrations ((10 µg/ml, 20 µg/ml and 40 µg/ml) of biosynthesized AgNPs and aqueous leaf extract were poured into each of the wells on all inoculated agar plates and incubated at 37^{o}C for 24 hours.A solvent blank was used as a negative control while the antibiotic i.e, Gentamycin (10 µg/ml) was used as a positive control. The antibacterial activity was measured based on the inhibition zone around the disc impregnated with the biosynthesized AgNPs and aqueous leaf extract.

Results and Discussion:

The formation of silver nanoparticles was indicated by a colour change from yellowish to reddish-brown upon addition of aqueous Azadirachta indica (neem) leaf extract to $AgNO_3$ solution. The biosynthesized AgNPs were characterized using UV-Visible Spectroscopy. (Figure-1) shows the UV-Vis Spectrum which confirms the formation of AgNPs. It was observed that the plasmon resonance band of biosynthesized AgNPs appeared at 412nm which corroborates with the findings as reported by several authors where the bands were mostly shown in the range of 400-450nm which was indicative of silver nanoparticle formation. Thus the visual observation and the UV-Vis spectra revealed the formation of silver nanoparticles.

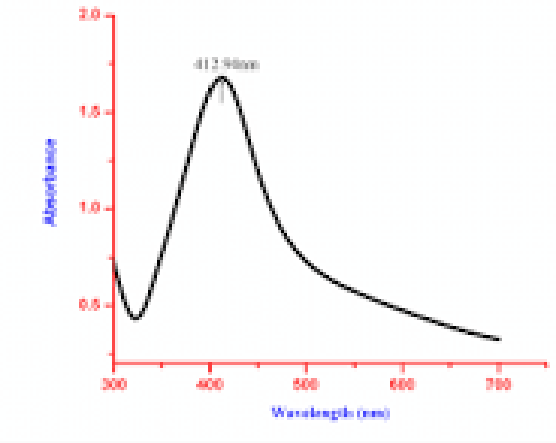

Figure-1: UV-Visible Spectra of Biosynthesized Silver nanoparticles.

Transmission electron microscopy was used to identify the size, shape and morphology of the nanoparticles. The results showed that the silver nanoparticles are well dispersed, homogenous and are predominantly spherical in shape as shown in (Figure-2). The results from DLS showed agglomeration of the biosynthesized AgNPs and the zeta potential value was shown to be -33.2 mV, revealing that the synthesized AgNPs are highly stable (Figure-3A&3B). The particle size agrees with that calculated from DLS with an average diameter of 38.5 nm(86.4%) and at least 6.5nm diameter(13.6%).

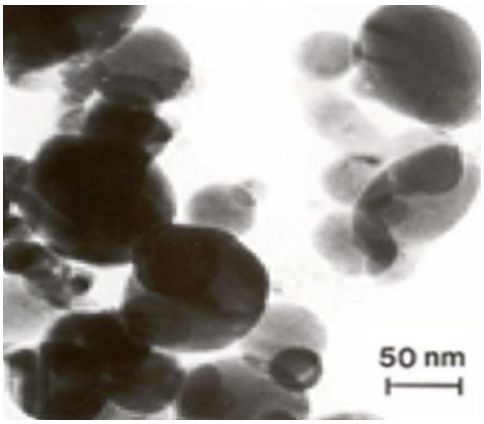

Figure-2: TEM Image of Biosynthesized Silver nanoparticles

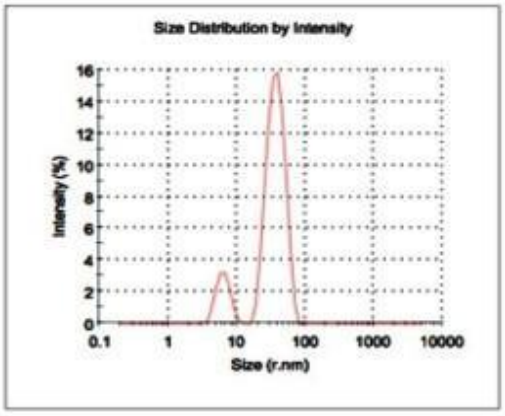

Figure-3A: DLS of Biosynthesized Silver nanoparticles

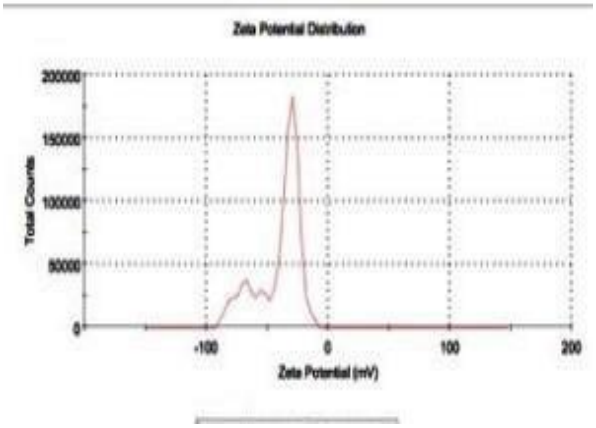

Figure-3B: Zeta potential of Biosynthesized Silver nanoparticles

The antibacterial effect of biosynthesized AgNPs using different concentrations(10 µg/ml, 20 µg/ml and 40 µg/ml) and the aqueous leaf extract was investigated against both Gram +ve (B.subtilis and S. aureus) and Gram -ve (E.coli and P.aeruginosa) bacterial strains. In the present study, AgNPs showed significant antibacterial activity on all the four bacterial strains that were tested, while the control and the aqueous leaf extract did not exhibit any bacterial activity. Though it is suggested that the leaf extract possess antibacterial activity and should reflect greater inhibition zone, but it alone shows very low activity due to its medium of extraction and lower concentration during experimentation. Moreover, the AgNPs exhibited maximum zone of inhibition against the Gram-ve (E.coli and P.aeruginosa) bacteria compared to the Gram+ve (B.subtilis and S. aureus) bacteria. Furthermore, maximum zone of inhibition was observed with increased concentrations as shown in Table-1.

Table:1

Antibacterial activity of Biosynthesized AgNPs against the bacterial strains.

S.No	Bacterial strain	Concentration(µg/ml)	Zone of Inhibition(mm)
1.	E.coli(Gram-ve)	10	13
		20	15
		40	17
2.	P.aeruginosa(Gram-ve)	10	12
		20	15
		40	18
3.	B.subtilis(Gram+ve)	10	7
		20	9
		40	10
4.	S.aureus(Gram+ve)	10	6
		20	8
		40	9

Bankar, et al (2010) reported similar results on the antibacterial activity of AgNPs using E.coli, E. aerogenes, Klebsiella sp and Shigella sp. Similar results are reported by Salari, et al and Anandalakshmi, et al (2016) using Prosopis farcta fruit extract and Pedalium murex leaf extract respectively. Further, Saravanakumar, et al (2015) also reported higher antibacterial activity by Cassia tora against Gram -ve bacteria compared to Gram +ve bacteria which corroborates with the findings of the present study. Several authors suggest that AgNPs attach to the surface of the cell membrane thereby disturbing the permeability and respiratory functions of the cell.It has also been indicated that AgNPs not only interact with the surface of the cell membrane but may also penetrate the bacteria. Thus higher antibacterial activity of AgNPs against Gram -ve bacteria may be due to its thinner peptidoglycan layer which can easily enter into the cell wall to denature or kill the bacteria as reported by Asmathunisha, et al (2010). Based on the zone of inhibition evaluated from the disc diffusion method, the biosynthesized AgNPs showed efficient antimicrobial property in the present study when compared to the control and aqueous leaf extract . This may be due to their large surface area which provide better contact with the bacterial cell wall as suggested by Ibrahim (2015).

Conclusion: The results of the present study indicate that neem leaf extract can be effectively used for synthesis of AgNPs as the green synthesis method is eco-friendly, of low cost, capable of producing at room temperature and serve as a potential antibacterial agent.

References:

1. Tolaymat, T.M.,Badawy, A.M.E., Genaidy, A., Scheckel, K.G.,Luxton, T.P & Suidan, M.(2010). An evidence based environmental perspective and manufactured silver nanoparticle in synthesis and applications: a systematic review and critical appraisal of peer-reviewed scientific papers. Total Environ.408,999-1006.
2. Brigger, I., Dubernet,C & Couvreur, P.(2002). Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Res. 54(5), 631-651.
3. Rai, M., Yadav, A & Gade, A.(2009). Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 27(1), 76-83.
4. Sudarenkov, V. (2013). Nanotechnology: balancing benefits and risks to public health and the environment. Strasbourg: Council of Europe, Committee on Social Affairs, Health and Sustainable Development.
5. Nordberg, G.F., Fowler, B.A., Nordberg, M & Friberg, L.T.(2007). Handbook on the Toxicology of Metals. 3rd. ed. San Diego: Elsevier.
6. Marcone, G.P. (2011). Avaliacao da ecotoxicidade de nanoparticulas de dioxide de titanio e prata [Doutorado] [Assessment of ecotoxicity of nanoparticles of titanium dioxide and silver]. Campinas. UNICAMP. Portuguese.
7. Yoon, K., Hoon Byeon, J., Park, J.H & Hwang, J. (2007). Susceptibility constants of Escherichia coli and Bacillus subtilis to silver and copper nanoparticles. Sci Total Environ. 373(2-3), 572-575.
8. Dastjerdi, R & Montazer, M. (2010). Areview on the application of inorganic nano-structured materials in the modification of textiles: focus on antimicrobial properties. Colloids Surf E Biointerfaces. 79(1), 5-18.
9. Rai, M.K., Deshmukh, S.K., Ingle, A.P& Gade, A.K.(2012). Silver nanoparticles: The powerful nanoweapon against multidrug-resistant bacteria. J Appl. Microbiol. 112(5), 841-852.
10. Franci, G., Falanga, A., Galdiero, S et al.(2015). Silver nanoparticles as potential antibacterial agents. Molecules. 20(5),8856-8874.
11. Salomoni, R., Leo, P & Rodrigues, M.F.A.(2015). Antibacterial activity of silver nanoparticles (AgNPs) in Staphylococcus aureus and cytotoxicity effect in mammalian cells. Formatex Microbiol. 5, 851-857.
12. Jiang, H., Manolache, S., Lee wong, A.C & Denes, F.S.(2004). Plasma enhanced deposition of silver nanoparticles onto polymer and metal surfaces for the generation of antimicrobial characteristics. J. Appl. Polym. Sci. 93, 1411-1422.
13. Gao, X., Yourick, J.J., Topping, V.D., Black, T., Olejnik, N., Keltner,Z et al.(2014). Toxicogenomic study in rat thymus of F_1 generation offspring following maternal exposure to silver ion. Toxicology Reports. https://dx.doi.org/10.1016/ j. toxrep. 12.008.
14. Lansdown, A.B.(2006). Silver in health care: antimicrobial effects and safety in use. Curr Probl Dermatol. 33, 17-34.
15. Chen, J., Han, C.M., Lin, X.W., Tang, Z.J & Su, S.J.(2006). [Effect of silver nanoparticle dressing on second degree burn wound] Zhonghua Wai Ke Za Zhi. 44(1), 50-52.

16. Cohen, M.S., Stern, J.M., Vanni, A.J et al (2007). In vitro analysis of a nano-crystalline silver- coated surgical mesh. Surg Infect (Larchmt). 8(3),397-403.
17. LEE, H.Y., Park, H.K., Lee, Y.M., Kim, K & Park, S.B.(2007). A practical procedure for producing silver nanocoated fabric and its antibacterial evaluation for biomedical applications. Chemic Communic (Comb). 28,2959-2961.
18. Padalia, H.,Moteriya, P & Chanda, S. (2014). Green synthesis of silver nanoparticles from marigold flower and its synergistic antimicrobial potential. Arabian Journal of Chemistry. http://dx.doi.org/10.1016/j.arabjc.2014.11.015.
19. Devi, L.S & Joshi, S.R.(2015). Ultrastructure of silver nanoparticles biosynthesized using endophytic fungi. Journal of Microscopy and Ultrstructure. 3, 29-37.
20. Nanda, B.A & Saravanan, M.(2009). Biosynthesis of silver nanoparticles from Staphylococcus aureus and its antimicrobial activity against MRSA and MRSE. Nanomedicine. 5, 452-456.
21. Sathish kumar, G., Jha, P.K., Vignesh,V., Rajkuberan, C., Jeyaraj, M., Selvakumar,M., Jha, R & Sivaramakrishnan, S.(2016). Cannonball fruit (Couroupita guianensis) extract mediated synthesis of gold nanoparticles and evaluation of its antioxidant activity. J.Mol. Liquids. 36, 229.
22. Njagi, E.C., Huang, H., Stafford, L., Genuino, H., Galindo, H.M., Collins, J.B.,Hoag, G.E & Suib. (2011). Biosynthesis of iron and silver nanoparticles at room temperature using aqueous sorghum bran extracts. Langmuir. 27,264- 271.
23. Muthukrishnan, S., Bhakya, S., Senthil Kumar, T & Rao, M.V. (2015). Biosynthesis, characterization and antibacterial effect of plant-mediated silver nanoparticles using Ceropegia thwaitesii- An endemic species. Ind. Crops. Prod.63, 119-124.
24. Mervat, F., Zayed, W.H., Eisa, W.H & Shabaka, A.A.(2012). Malva parviflora extract assisted green synthesis of silver nanoparticles. Spectrochim. Acta Mol. Biomol. Spectros. 98,423-428.
25. Kuppusamy, P., Yusoff, M.M & Govindan, N.(2015). Biosynthesis of metallic nanoparticles using plant derivatives and their new avenues in pharmacological applications. An updated report. Saudi Pharm J. doi: http://dx.doi.org/10.1016/j.jsps.
26. Bauer, A.W., Kurby, W.M., Sherris, J.C & Turck, M.(1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J.Clin.Pathol. 45(4), 493-496.
27. Bankar, A., Joshi, B., Kumar,A.M &Zinjarde, S.(2010). Banana peel extract mediated novel route for the synthesis of silver nanoparticles. Coll Surf A Physiochem Eng Asp. 368, 58-63.
28. Anandalakshmi, K., Venugopal, J & Ramasamy, V.(2016). Characterization of silver nanoparticles by green synthesis method using Pedalium murex leaf extract and teir antibacterial activity. Appl. Nanosci. 6, 399-408.
29. Saravanakumar, A., Ganesh, M., Jayaprakash, J & Jang, H.T. (2015). Biosynthesis of silver nanoparticles using Cassia tora leaf extract and its antioxidant and antibacterial activities. J.Ind. Eng. Chem. 28,277.
30. Asmathunisha, N., Kathiresan, K., Aburaj & Nabeel, M.A. (2010).Synthesis of antimicrobial silver nanoparticles by callus and leaf extracts from Saltmarsh plant, Sesuvium portulacastrum L. Coll. Surf. B: Biointer. 79, 488-493.