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ORIGINAL ARTICLE

Green synthesis of silver nanoparticles using *Azadirachta indica* leaf extract and its antimicrobial study

Pragyan Roy[1] · Bhagyalaxmi Das[1] · Abhipsa Mohanty[1] · Sujata Mohapatra[1]

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Abstract In this study, green synthesis of silver nanoparticles was done using leaf extracts of *Azadirachta indica.* The flavonoids and terpenoids present in the extract act as both reducing and capping agent. Microbes (*Escherichia coli* and Gram-positive bacteria) were isolated from borewell water using selective media. The silver nanoparticles showed antimicrobial activities against Gram-positive bacteria and *E. coli*. However the silver nanoparticles were more effective against *E. coli* as compared to Gram-positive bacteria. Various techniques were used to characterize synthesized silver nanoparticles such as DLS and UV–visible spectrophotometer. The absorbance peak was in the range of 420–450 nm, that varied depending upon the variation in the concentration of neem extract. This is a very rapid and cost-effective method for generation of silver nanoparticle at room temperature, however, its exact dose in water purification has to be determined.

Keywords Green synthesis · Silver nanoparticles · *Azadiracta indica* · *E. coli* · Antimicrobial

Introduction

About 5000 years ago, silver was used to store food by Romans, Persians, Egyptians and Greeks (Mody et al. 2010). The age-old application of silver in the making of utensils for drinking water and eating was probably due to its antibacterial nature. Materials in the nano-dimensions (1–100 nm) have very high surface to volume ratio that gives them certain unique properties that are different from the same material in bulk which are useful in different fields such as electronics, photonics, biomedical, catalysis, etc.(Saha et al. 2017). This property of nanoparticles is utilized in the areas of biomedicine, solar energy conversion, catalysis and water treatment. Among the various noble metals, silver is preferred as a nanoparticle because of its antibacterial catalytic properties and their nontoxicity towards human (Rai et al. 2009) in comparison to other metals.

Several methods have been used for the preparation of silver nano-particles which can be either physical, chemical or biological methods. Earlier methods used for the synthesis of silver nano-particles were toxic and hazardous chemicals were used for their synthesis. Thus the use of eco-friendly processes, for the synthesis of silver nano-particles is known as "Green synthesis". Green synthesis is preferred over conventional synthesis because it is eco-friendly, cost-effective, single-step method that can be easily scaled up for large scale synthesis and does not require high pressure, temperature, energy and toxic chemicals (Saha et al. 2017). Many researchers have reported the use of materials such as plant leaf extract, root, stem, bark, leaf, fruit, bud and latex (Mariselvam et al. 2014), fungi (Bhainsa 2006), bacteria (Saifuddin et al. 2009) and enzymes (Willner et al. 2007) for the synthesis of silver nano-particles. A lot of work has been done on green synthesis of silver nano-particles using microorganisms including bacteria, fungi and plants because of their antioxidant properties capable of reducing metal compounds in their respective nanoparticle. Plant extracts produce best capping material for the stabilization of silver nanoparticles (Ahmed et al. 2015).

✉ Pragyan Roy
roy.pragya@gmail.com

[1] Department of Biotechnology, Rama Devi Women's University, Bhubaneswar, India

The present work aims to use the leaf extract of *Azadirachta indica* (commonly known as neem) a member of the Meliaceae family used for the green synthesis of silver nanoparticles. This is a medicinal plant and is used for the treatment bacterial, fungal, viral and many types of skin ailments since ancient times. The aqueous neem extract is used in the synthesis of various nanoparticles such as gold, zinc oxide, silver, etc. Terpenoids and flavanones are the two important phytochemicals present in neem which play a vital role in stabilizing the nanoparticle and also act as capping and reducing agent (Banerjee et al. 2014). Aqueous neem leaf extract reduces silver salt to silver nitrate, this capped nanoparticle with neem extract exhibit antibacterial activity.

In the present study, the antibacterial effect of green synthesized silver nano-particle and its role in water purification was studied. Even the concentration of silver nano-particle was determined that was most effective in controlling the growth of Gram-positive and Gram-negative bacteria isolated from the water sample. Effect of silver nano-particle on the bacterial count was also studied.

Materials and methods

Preparation of leaf extracts from *Azadirachta indica* (Neem) leaves

Fresh neem leaves (Fig. 1) were collected from University Campus in the month of February. Leaves were thoroughly washed in running water to remove the dirt and dust on the surface of the leaves. Twenty g of finely chopped neem leaves were added to 100 ml of double-distilled water and boiled for 10 min. The extract was cooled and filtered and store for further use (Fig. 2). This solution was used for green synthesis of silver nanoparticle (AgNP) or reducing the silver ions.

Fig. 1 Neem leaves collected from campus

Synthesis of silver nanoparticles

Silver nitrate (Merck, India) GR was used to prepare 100 ml of 1 mM solution of silver nitrate. Then 1, 2, 3, 4 and 5 ml of neem extract was added separately to 5 ml of silver nitrate solution. This set up was incubated in dark chamber to minimize photo-activation of silver nitrate at room temperature. The colour change from colourless to brown in colour confirms the reduction of silver ions.

Collection of water sample

Water from borewell was collected and stored in sterilized glass bottle early in the morning. 10 ml of sample water was used for serial dilution and remaining water stored in refrigerator for further use. However, to repeat the experiment water was collected fresh from borewell and stored water was not used, for reproducibility of the result.

Preparation of Nutrient Agar, EMB (eosin methylene blue) Agar and MacConkey Agar

All the petridishes, conical flasks and beakers were cleaned with detergent and autoclaved then dried in hot air oven. Nutrient Agar (Hi-Media), EMB Agar (Hi-Media) and MacConkey Agar (Hi-Media), was prepared as per manufacture's instruction.

Isolation of pure culture

The water sample was diluted 10^4 times and plated on Nutrient Agar plates. Some isolated colonies were picked up and Gram staining was done. Gram-positive colonies were maintained as pure culture. Purplish black-centered colonies with greenish metallic sheen were picked from EMB plates. The same colonies were streaked on MacConkey plates and it gave pink to red colour colonies.

Assessment of antimicrobial assay

The antimicrobial activity of the AgNPs was determined on a Gram-positive bacteria and *Escherichia coli* (Gram-negative bacteria) by diffusion method. Wells of 2 mm diameter were bore on 4 mm-thick Nutrient Agar plates and EMB plates. A lawn culture of the Gram-positive bacteria and *Escherichia coli* (Gram Negative bacteria) was done on Nutrient Agar plates and EMB plates, respectively. Then 20, 50 and 100 µl of neem extract and

silver nitrate solution was added to the wells. The plates were incubated overnight at 37 °C. The diameter of clear zone was measured.

Particle size estimation of silver nanoparticles

UV–visible spectroscopy was used to monitor colour changes in the mixture using Shimadzu UV–visible spectrophotometer (UV-2450, Japan). The UV–Vis spectral analysis was done in the wavelength of 200–800 nm in the UV spectrophotometer. Dynamic light scattering (Malvern, UK) was used to determine the average particle size of the synthesized silver nanoparticles (Fig. 6a and b).

Results

Analysis of silver nanoparticle by UV–Vis spectroscopy

A distinct colour change was observed after addition of aqueous neem extract to silver nitrate solution. The colour of the solution changed from pale yellow to brown as it can be seen in Figs. 3 and 4. Sample S1 (1 ml plant extract + 10 mL silver nanoparticle) S2 (2 mL plant extract + 10 mL silver nano particle), S3 (3 mL plant extract + 10 mL silver nanoparticle), S4 (4 mL plant extract + 10 mL silver nanoparticle), S5 (5 mL plant extract + 10 mL silver nanoparticle).

As it can be seen from the graph for S1 (1 ml plant extract + 10 mL silver nanoparticle) at 350 nm wavelength absorbance was recorded 1.3, at 400 nm wavelength 0.6, at 450 nm 0.8, after 500, 550 and 600 nm wavelength absorbance was constant at 0.5. For S2 (2 mL plant extract + 10 mL silver nano particle) absorbance at 350 nm was 1.8, at 45 nm it was 1.4 and gradually decreased and at 600 nm it was 0.5. In case of S3 (3 mL plant extract + 10 mL silver nanoparticle) absorbance was maximum at 350 nm, at 450 nm it was 2 which gradually decreased to 0.5 at 600 nm. Similarly, for S4 (4 mL plant extract + 10 mL silver nanoparticle) and S5 (5 mL plant extract + 10 mL silver nanoparticle) absorbance at 350 nm is 3.5 that gradually decreases at 450 nm to 2.3 and 2.6, respectively. Absorbance at 500 nm for S4 and S5 is recorded to be 1.5, at 550 nm it is 1 and at 600 nm it is 0.5. This absorbance value is recorded after 24 h of incubation of plant extract.

In Sample S1, S2 and S3 peak was seen at 420, 425 and 430 nm, respectively. In sample S4 and S5 peak was observed at 440 and 445 nm, respectively (Fig. 5). Since S3 peak was nearest to the expected peak it was used for

Fig. 2 Neem leaves extract

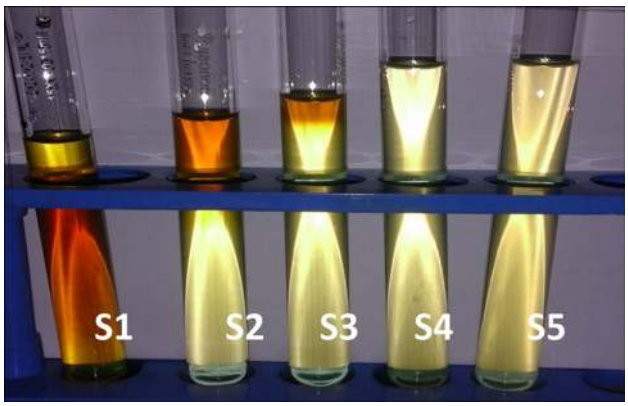

Fig. 3 Neem leaves extract and silver nitrate

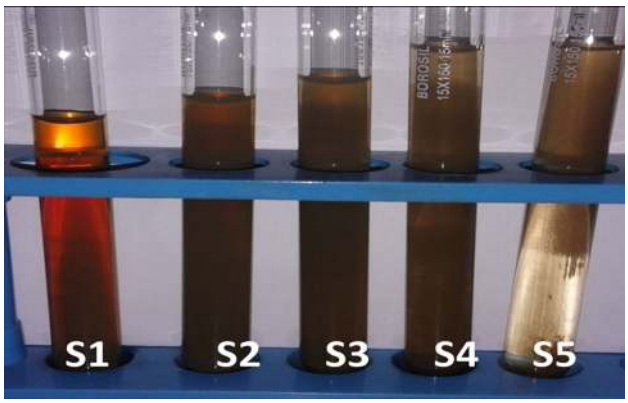

Fig. 4 Colour change in the extract and silver nitrate after 1 h incubation (S1 = 1 mL plant extract + 10 mL silver nitrate, S2 = 2 mL plant extract + 10 mL silver nitrate S3 = 3 mL plant extract + 10 mL silver nitrate, S4 = 4 mL plant extract + 10 mL silver nitratee S5 = 5 mL plant extract + 10 mL silver nitrate)

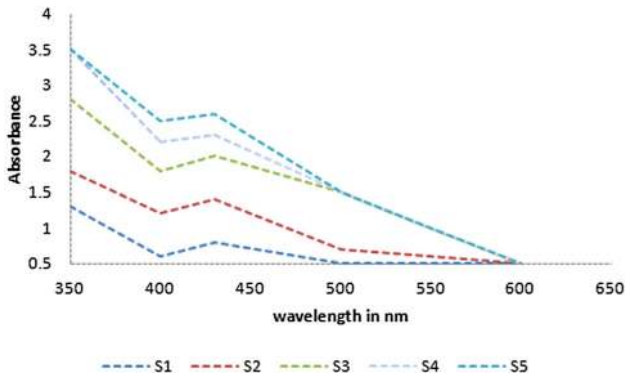

Fig. 5 UV vis spectra showing the absorbance of different concentration of plant extract in silver nitrate solution

further antimicrobial assay. Very high concentration of neem extract was not suitable for silver nanoparticle formation. The solution containing AgNP was stable for several weeks.

DLS analysis

The Z-average mean (d.nm) in case of S1 (Fig. 6b) was 65.67 and in case of S3 (Fig. 6a) it was 66.98. The Polydispersity Index was 0.299 in S1 and 0.280 in S3. Overall, the size of the nanoparticle was good in both S1 and S3.

Antimicrobial analysis

Isolation of E. coli and Gram-positive bacteria from water sample

The water sample (10^4 times diluted) was plated on Nutrient Agar (NA) plate and many different type of colonies was seen on the NA plate after overnight incubation at 37 °C. Some colonies were picked and Gram staining was done. Gram-positive culture was isolated and maintained as pure culture. The water sample was streaked on EMB (Fig. 7a) plate. Purplish black centered colonies with greenish metallic sheen was *Escherichia coli* ATCC 25922 (manufacture data sheet). These colonies were further streaked on MacConkey plates (Fig. 7c) and luxuriant pink colonies were seen on MacConkey plate.

Determining the exact concentration of AgNPs for antimicrobial assay

The lawn culture of Gram-positive bacteria was done on NA plate and wells were bore on it.

The silver nanoparticles with different concentrations of neem extract S1 (1 ml plant extract + 10 mL silver nanoparticle), S2 (2 mL plant extract + 10 mL silver nano particle), S3 (3 mL plant extract + 10 mL silver nanoparticle), S4 (4 mL plant extract + 10 mL silver nanoparticle), S5 (5 mL plant extract + 10 mL silver nanoparticle) was added 20, 50 and 100 µl on these culture plates. These plates were kept overnight in incubator at 37 °C. The next day, zone size was measured (Fig. 8). In sample S1, the zone size for 20, 50 and 100 µl was 12.5, 16.66 and 20.33 mm, respectively (Fig. 9a). For sample S2, no zone size was seen at 20 µl, and at 50 µl it was 13.83 mm and 100 µl it was 16.33 mm. In sample S3, for 20 µl it was 8.66 mm, 50 µl it was 18.66 mm and 100 µl it was 22 mm. While for S4 and S5, no zones were seen up to 100 µl silver nanoparticle concentration.

Lawn culture of *E. coli* was done on EMB plate and then 20, 50 and 100 µl of AgNPs were added to the wells bore on the plate (Fig. 10). No zone was seen when 20 µl of AgNp was added. For sample S1, a zone size of 13.6 mm

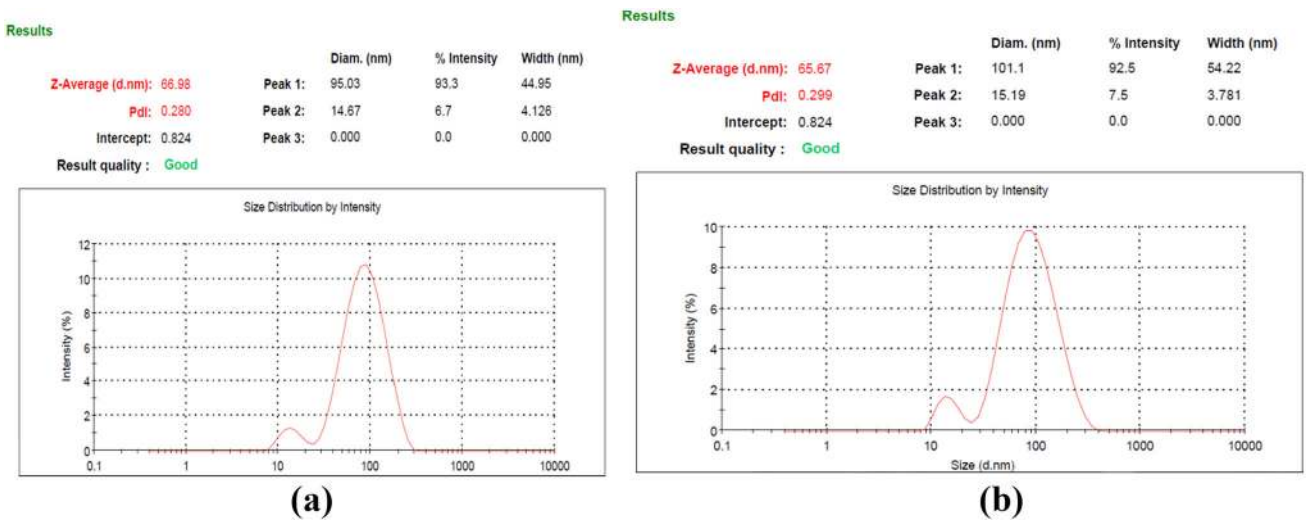

Fig. 6 **a** DLS result for S3. **b** DLS result for S1

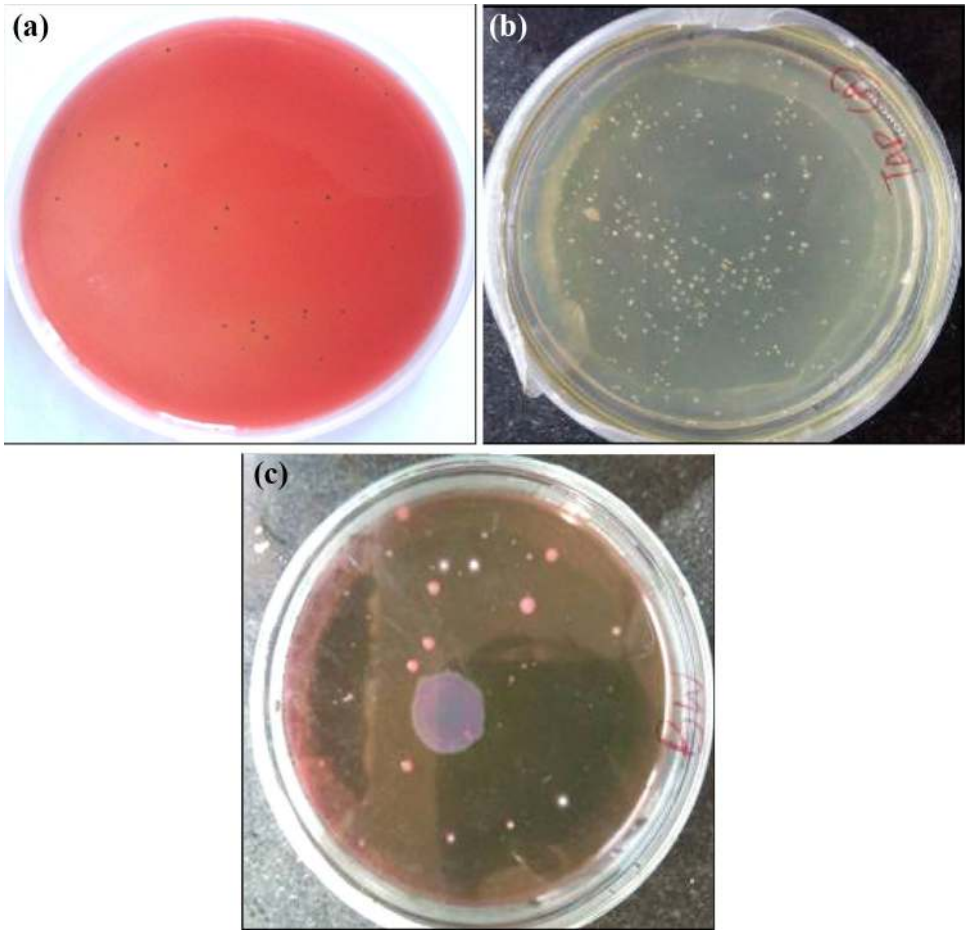

Fig. 7 **a** Purple with black-centered colonies with greenish metallic sheen, *E. coli* EMB plate. **b** Bacterial colonies on NA plate. **c** Pink colonies (*E. coli*) on MacConkey plate

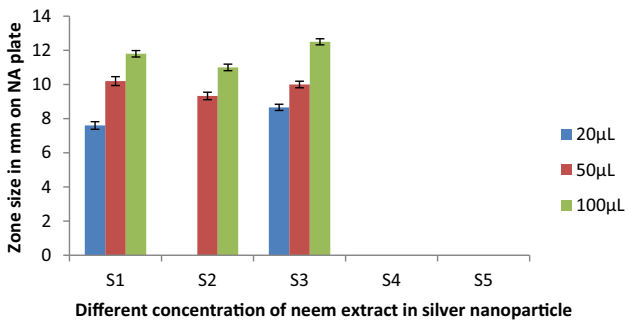

Fig. 8 The different zone size formed by adding (20, 50 and 100 µl) of neem extract and AgNP after streaking Gram-positive bacteria on NA plate

was formed at 50 µl and 16.67 mm at 100 µl (Fig. 11a). Sample S2 did not show any zone at 50 µl and at 100 µl, it was 14.16 mm (Fig. 11b). A zone size of 11.83 mm was measured at 50 µl AgNp and 12.5 mm at 100 µl AgNP (Fig. 11c). While sample S4 and S5 were not successful in inhibiting growth of *E. coli*, so no zone was seen.

Discussion

Bioreduction of silver ions into AgNP after addition of aqueos neem extract was confirmed with change in colour. Initially, after addition of aqueous neem extract, the colour was pale yellow with the increase in incubation time the colour changed from pale yellow to light brown and after 24 h incubation it was deep brown in colour. Slight variation in the peak absorbance was observed which might be due to variation in particle size which was further confirmed after DLS. The brown colour was due to the excitation of the surface plasmon resonance (SPR), very much a characteristic property of silver nanoparticle (Banerjee et al. 2014). According to Amendola (Amendola et al. 2010), SPR band is depended on the particle size and refractive index of the solution. The flavenoids and terpenoids present in neem extract act like natural reducing agent which are responsible for reducing silver salts to silver nanoparticles (Verma and Singh Mehata 2016). A complete colour change was seen within 1 h of incubation after which no colour change was seen which indicates that all the silver salts are completely reduced to AgNP. From several literatures, it was reported that the SPR peak of

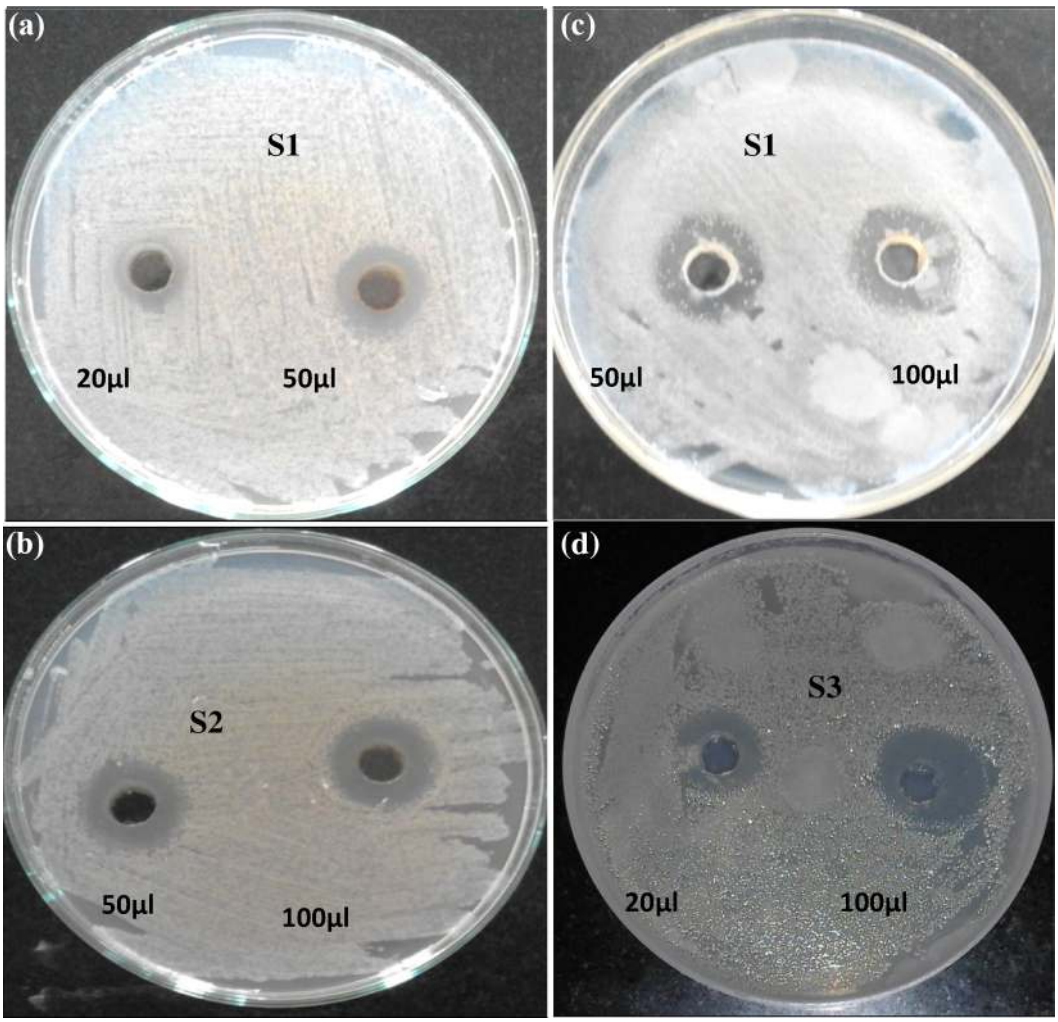

Fig. 9 **a**, **c** The different zones formed by adding 20, 50 and 100 µl of neem extract and AgNPs of sample S1 on Gram-positive bacteria. **b** The different zones formed by adding 50 and 100 µl of neem extract and AgNPs of sample S2 on Gram-positive bacteria. **d** The different zones formed by adding 20 and 100 µl of neem extract and AgNPs of sample S3 on Gram-positive bacteria

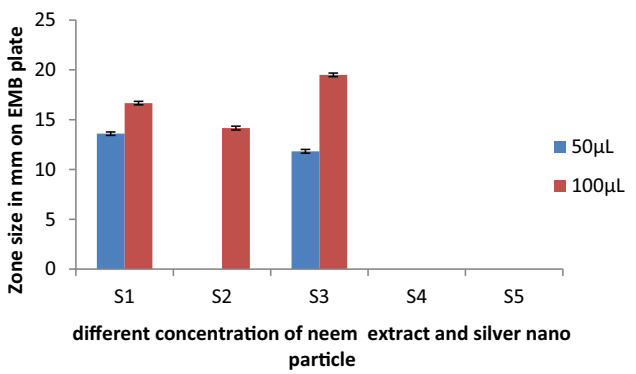

Fig. 10 The different zone size formed by adding (50 and 100 µl) of neem extract and AgNP after streaking *E. coli* on EMB plate

silver nanoparticles is around 420 nm and in the present study it was centered at 430 nm (Kumar et al. 2014). According to ISO 22412 (International Standard ISO 2008) *Z* average size or *Z* average diameter is a hydrodynamic parameter and predicts particle shape to be spherical or nearly spherical if we get a monomodal (i.e., only one peak), however, it has to be further confirmed with TEM analysis. The Polydispersity Index values less than 0.05 are rarely seen and values greater than 0.7 indicate that the sample has very broad size (Malvern, Instrument manual). For both the samples the Pdi was below 0.7 indicating the quality of nanoparticle to be good.

Though silver nanoparticles are extensively used as an antimicrobial agent, their exact mechanism of inhibition is still unclear. One of the probable mechanism is that silver nanoparticles attach to the surface of the cell membrane, the respiratory function and permeability of the bacterial cells become unstable (Kvitek et al. 2008). According to Gogoi (Gogoi et al. 2006), the negatively charged cell surface of *E. coli* is easily dislodged by Ag^{+} ions thus interrupting metabolic activity and subsequently leading to

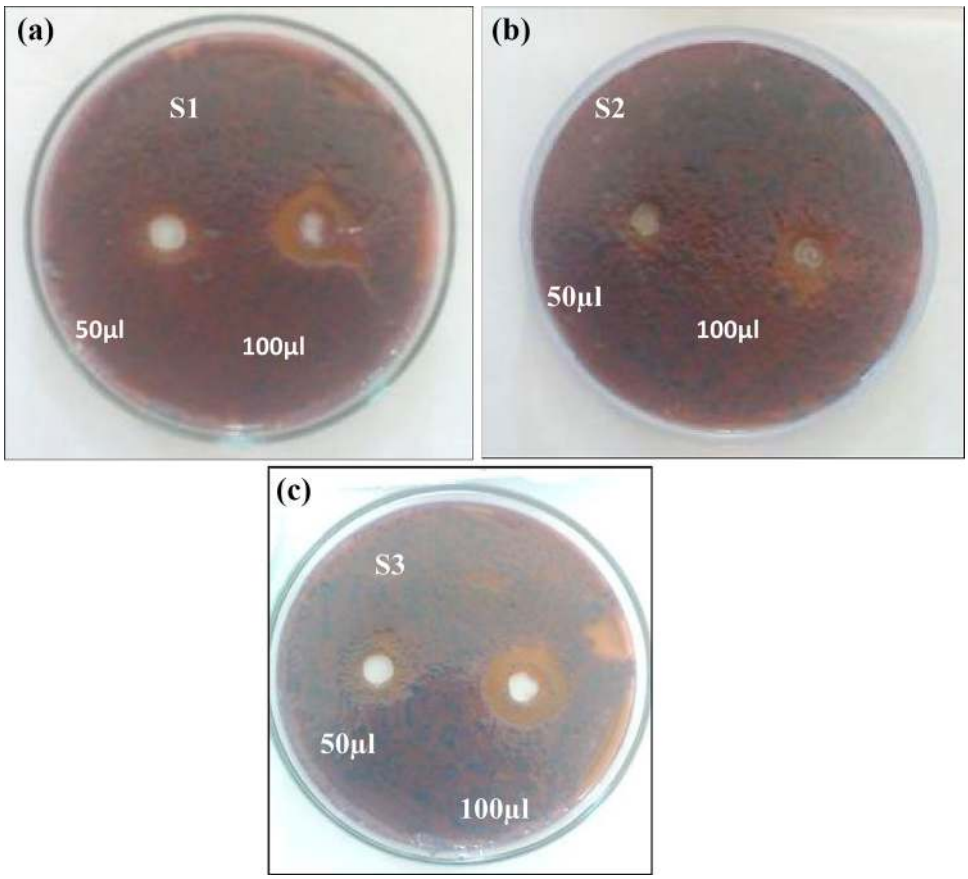

Fig. 11 **a** The different zones formed after adding 50 and 100 µl of neem extract AgNPs of sample S1 on *E. coli* on EMB plate. **b** The different zones formed after adding 50 and 100 µl of neem extract AgNPs of sample S2 on *E. coli* on EMB plate. **c** The different zones formed after adding 50 and 100 µl of neem extract and AgNPs of sample S3 on *E. coli* on EMB plate

denaturation of protein and cell death (Pal et al. 2007). Reactive Oxygen species (ROS) such as singlet oxygen $^1O_2$, hydroxyl radical OH^- and peroxide radical O_2^-, are produced by silvernano particle which are toxic to the bacteria (Carlson et al. 2008). In the present study, the antimicrobial activity of silver nanoparticle for Gram-positive bacteria was less compared to Gram-negative bacteria. Similar results have been reported earlier for neem as well as other plant extracts. This is attributed to the peptidoglycan layer which is negatively charged and prevents the free entry of Ag ions into the cell wall (Ankanna et al. 2010; Kim et al. 2011).

Conclusion

The present work highlights one of the most simple and economical methods for the green synthesis of silver nano-particles from *Azadirachta indica* leaves. Both silver ions and silver nano-particles can break the disulphide bonds and interfere with the metabolic activities of the microorganisms which determine its antimicrobial properties. Though the green synthesis of silver nano-particle is cost effective, environment friendly, yet large scale production is still at a very preliminary stage and the effective dose for its antimicrobial activity is yet to be decided. In this study, we have isolated pathogenic bacteria *E. coli* and some Gram-positive bacteria from the borewell water and studied the impact of silver nanoparticles in inhibiting the growth of microbes. We found that the effect of silver nanoparticle is dose sensitive and depends on the capping agent as reported by previous workers. Lower ratio of plant extract is optimum for the synthesis of silver nano-particle. Further work has to be done to determine the toxicity level of silver ions that can be suitable for human consumption so that water can be made microbe free before human consumption.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest with other authors.

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