

Original Research Article

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Study of Production of Green Conjugates of Silver Nanoparticles for Determination of Antimicrobial Potential against Biofilm Producing *Staphylococcus aureus*

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ABSTRACT

In recent era, Nanotechnology is a burning field for the researchers. Nanotechnology deals with the Nanoparticles having a size of 1-100 nm in one dimension used significantly concerning medical chemistry, atomic physics, and all other known fields. Nanoparticles are used immensely due to its small size, orientation, physical properties, which are reportedly shown to change the performance of any other material which is in contact with these tiny particles. These particles can be prepared easily by different chemical, physical, and biological approaches. But the biological approach is the most emerging approach of preparation, because, this method is easier than the other methods, eco-friendly and less time consuming. *Staphylococcus aureus* is a leading cause of nosocomial infections and the etiologic agent of a wide range of diseases associated with significant morbidity and mortality. Some of the diseases mediated by this species include endocarditis, osteomyelitis, toxic shock syndrome, food poisoning, and skin infections. Biofilms are well organized and complex aggregate of microorganisms, surrounded by a protective matrix of exo-polysaccharides and can adhere to each other on various surfaces. Because of increase in the drug resistance and the side effect of over use of antibiotic, it is necessary to find a suitable agent to reduce the growth of microorganism. Biofilm are known as a significant problem because biofilm formation protects pathogenic bacteria against antibiotics and is one of the main causes of development of chronic infections. Thus biofilm is the main cause of drug resistance in microbial strains. These strains cause pathogenicity to a maximum extent and thus cause resistance against the conventional antibiotics/drugs. The nanoparticles were synthesized by the concept of green synthesis by using the aqueous solution of *Azadirachta indica* leaf extract and silver nitrate. Silver was of a particular interest for this process due to its evocative physical and chemical properties. *Staphylococcus aureus* strains were isolated and screened for biofilm production. A fixed ratio of plant extract to metal ion was prepared and the color change was observed which proved the formation of nanoparticles. These nanoparticles were characterized by UV-Vis Spectrophotometer and SEM. The nanoparticles were found have the size ranges from 160-180 nm. These fused Ag-Azadirachta indica* (Ag-AI*) nanoparticles were screened against biofilm producing strains of *S. aureus*. The results showed promising antimicrobial activity against these biofilm producers.

Keywords

Green synthesis,
Silver fused
Azadirachta indica
nanoparticles,
Biofilm, *S. aureus*,
Antimicrobial
activity.

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Introduction

Nanotechnology is a latest field of modern research dealing with synthesis, design and manipulation of nanosized particles.

Remarkable development in nanotechnology has opened a novel and very wide frontier of application that includes the synthesis of

nanoscale material, exploration of their unique physical chemical and optoelectronic properties. Nanotechnology has gained tremendous importance in state of the art techniques for health care, food technology, cosmetics, environmental health, mechanics, optics, chemical industries, biomedical, electronics, space engineering, energy science, drug-gene delivery, optoelectronics, catalysis, light emitters, nonlinear optical devices, single electron transistors, and photo electrochemical applications etc (Colvin *et al.*, 1994; Wang and Herron, 1991; Schmid, 1992; Mansur, 1995). In the present scenario, there is a growing interest in biological reduction of metal ions into metal nanoparticles particularly in the field of biology and medicine because of their distinct particle size and shape dependent properties (Sun *et al.*, 2008; Ko, 2007). Bio-nanotechnology, a new approach, has emerged as the integration between biotechnology and nanotechnology for developing biosynthetic and environmental-friendly technology for the synthesis of nanomaterials. Nanoparticles are of different size and shapes (Sun and Xia, 2002; Manna *et al.*, 2000). These can be synthesized in different material compositions and surface modifications (Gref, 2003). The synthesized nanoparticles are found to exhibit size (Park, 2008) or shape dependent properties (Anker, 2008). The present study deals with the synthesis of silver nanoparticles using leaves extracts of *Azadirachta indica* (Neem). These nanoparticles were prepared were screened for antimicrobial activity against biofilm producing *S. aureus*.

Materials and Methods

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

Fresh leaves of *Azadirachta indica* (Neem) were collected from the local garden. Leaves were thoroughly washed to remove the dust

particles. Further, dried in the sun to remove moisture and then grinded to fine powder. 1% of Neem leaves were added to 250 ml conical flask containing deionized water followed by stirring for 15 minutes followed by incubation for 30 minutes at 25°C. Solution was centrifuge at 5000 rpm for 30 minutes at 25°C. Clear solution was collected followed by filtration (using 2.5 microns filter paper) with the help of vacuum pump. This solution was used for biosynthesis of silver nanoparticles or reducing the silver ions to metal silver forms.

Synthesis of silver nanoparticles

Concentration ratios of plant extract: AgNO₃ was prepared i.e. 3:2 by varying the plant extract concentration and keeping the concentration of silver nitrate constant. 1 mM silver nitrate was added to plant extract to prepare a final volume of 200 ml of a solution. Typically, 10 mL of plant extract was added to 190 ml of 1 mM aqueous AgNO₃. The change in the colour of solution was observed during the whole process while taking OD of the solution. The silver nanoparticles solution that was formed was centrifuged at 12,000 rpm for 15-20 minutes followed by dilution of pellet.

UV-Vis spectral analysis

This bioreduced aqueous component was taken to measure absorbance of the solution. U.V spectra of the ratio 3:2 showed the best results. The absorbance was determined from 200-700 nm.

FT-IR spectral analysis

The bioreduced silver nitrate solution (nanoparticles) was centrifuged at 12,000 rpm for 20 minutes and the pellet was collected and characterized using FT-IR (Bruker, Germany) in the range of 4000- 400cm⁻¹

SEM analysis of silver nanoparticles

Morphology and size of the silver nanoparticles were investigated by SEM images at an accelerating voltage of 5 KV.

Antimicrobial activity of silver nanoparticles against biofilm forming *Staphylococcus aureus*

Agar well diffusion method

The agar well diffusion method was modified (Perez and Anesini, 1993). Nutrient agar medium (SCDM) was used for bacterial cultures. The culture medium was inoculated with *Staphylococcus aureus* (isolated locally) suspended in nutrient broth. A total of 8 mm diameter wells were punched into the agar and filled with silver nanoparticles. Standard antibiotic (Erythromycin 1 mg/ml) were simultaneously used as the positive control. The plates were incubated at 37°C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The procedure for assaying antibacterial activity was performed in triplicates to confirm the readings of diameter of zone of inhibition observed for each of the test organism.

Measurement of minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Silver nanoparticles were added in LB medium, respectively. Each bacterium culture *Staphylococcus aureus* were controlled at 10^5 - 10^6 Cfu/mL and incubated at 37°C. To establish the antimicrobial activity of nanoparticles on the bacterial growth, the minimum inhibitory concentration of nanoparticles for *Staphylococcus aureus* were determined by optical density of the bacterial culture solution containing different concentration of nano particles after 24h. All

of the experiments (MIC) were triplicate, on three different days (Melaiye *et al.*, 2005). The minimum bactericidal concentration (MBC), i.e., the lowest concentration of nanoparticles that kills 99.9% of the bacteria were also determined from the batch culture studies. For growth inhibitory concentration (PMIC) the presence of viable microorganisms were tested and the lowest concentration causing bactericidal effect were reported as MBC as suggested (Avadi *et al.*, 2004). To test for bactericidal effect, a loopful from each flask were inoculated on nutrient agar and incubated at 35°C for 24 h. The nanoparticles concentration causing bactericidal effect was selected based on absence of colonies on the agar plate.

Results and Discussion

In the present study, the silver nanoparticles were prepared by the green synthesis which was characterized by the change in color from pale yellow to brown as shown in figure 1. Initially the solution colour was light yellowish than with time duration it turned from yellowish to light brown to dark brownish after 24 hours, after this no further colour change was observed. Colour change indicates the formation of silver nanoparticles. The sharp clear intense bands of silver nanoparticles were observed at 415 nm in case of 3:2 ratios (Figure 2). FT-IR spectrum was analyzed for identification of different biomolecules adsorbed on the surface of nanoparticles, and also to find out their role in reduction and stabilizing the nanoparticles. The FT-IR spectrum of synthesized silver nanoparticles shows strong bands at 3372.33, 2932.20, 2366.24, 2109.93, 1640.23, 869.22, 819.26 and 779.09 cm^{-1} . The peaks at 3372.33, 2932.20 and 1640.23 corresponds to O-H groups, H bonded alcohols and phenols respectively. The peaks at 2366.24 cm^{-1} and 2109.93 corresponds to secondary amine. The spectral bands

(1055.76-1413.76 cm^{-1}) show presence of proteins which are responsible for the reduction of metal ions or affinity for metal nanoparticles. The bands (779.09-869.22 cm^{-1}) show the presence of phytochemical

constituents in the form of flavanoids/terpenoids. This illustrates that that silver ions in aqueous medium have been converted to nanosized elemental silver (Fig. 3).

Table.1 Antimicrobial activities of silver nanoparticles (AgNps) against *S. aureus*

Biofilm forming pathogen	Doses (ppm)	Diameter of zone of inhibition (mm)
<i>S. aureus</i>	5	00
	10	18
	20	35

Table.2 MIC and MBC of silver nanoparticles (AgNps) against *S. aureus*

Biofilm forming pathogen	Doses (ppm)	MIC/MBC (ppm)	
		MIC	MBC
	10	6.0	8.5
	20	3.5	17.6

Fig.1 Preparation of silver nanoparticles via green synthesis

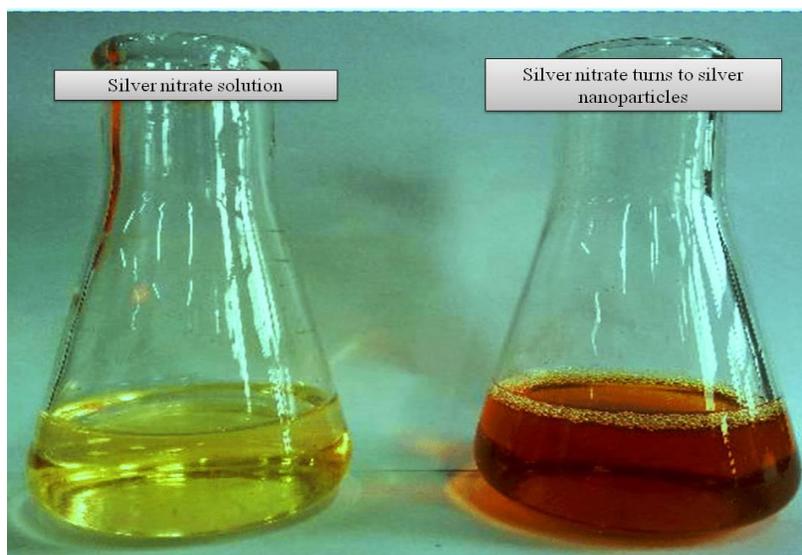


Fig.2 UV- absorption spectra of Ag nanoparticles

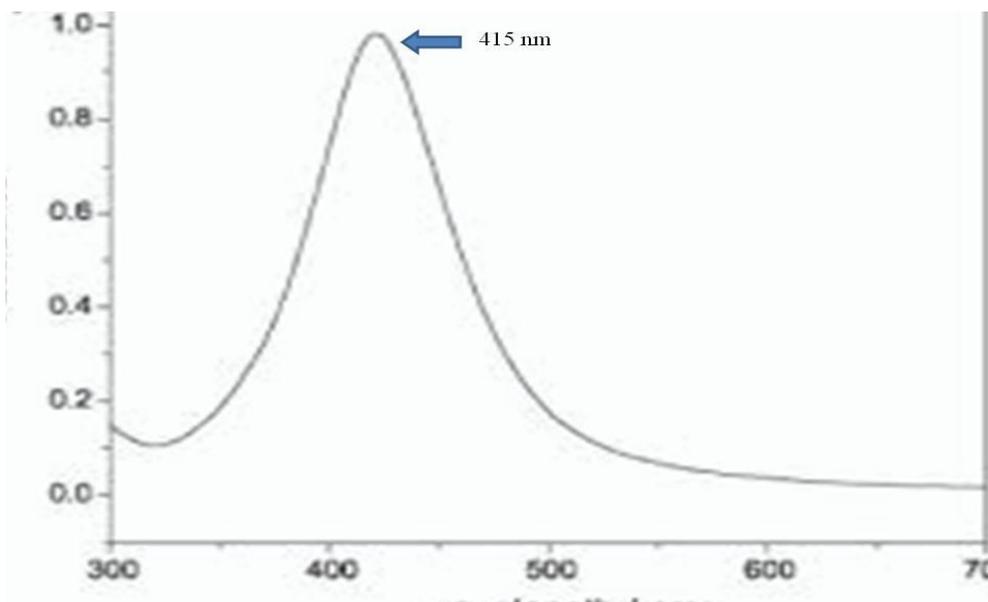


Fig.3 FT-IR spectra of silver nanoparticles prepared in 3:2 ratio (plant extract: AgNO₃)

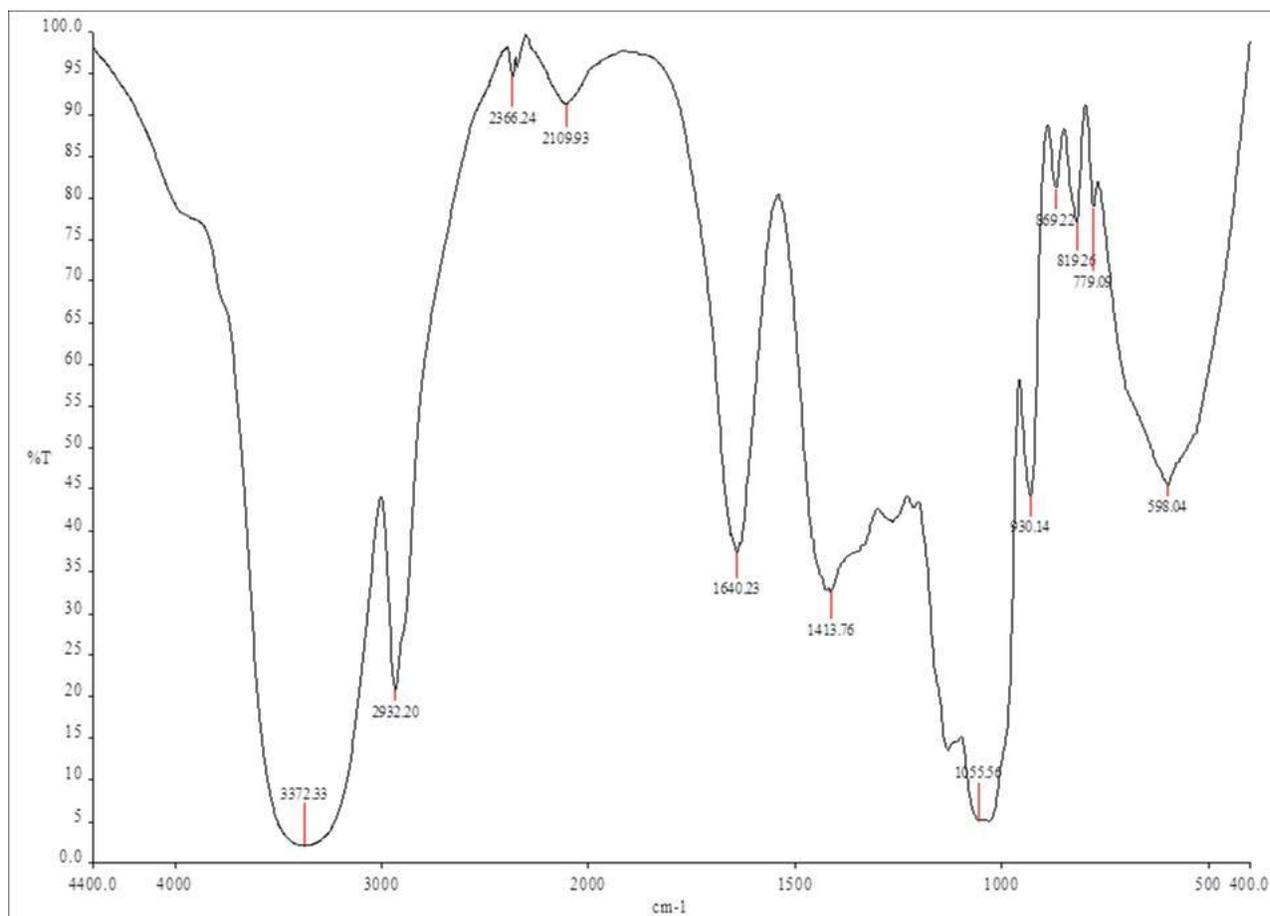


Fig.4 SEM image of silver nanoparticles prepared in 3:2 ratios (plant extract: AgNO₃)

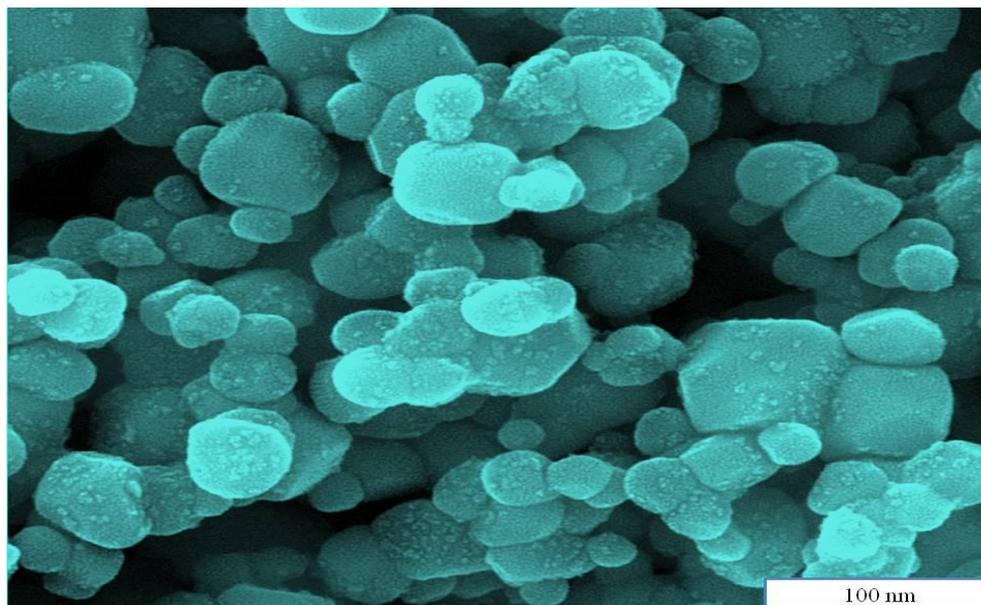
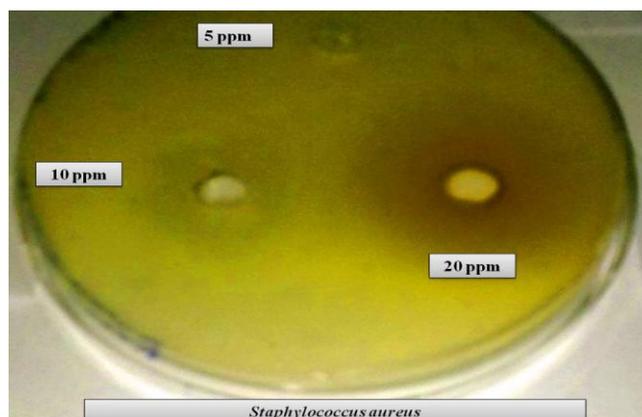


Fig.5 Antimicrobial activity of silver nanoparticles against biofilm producing *S. aureus*



The nanoparticles prepared were of 100 nm size as determined by SEM (Figure 4). The antimicrobial activity of the nanoparticles synthesized was determined against isolated biofilm producing strain of *S. aureus* at 10, 15 and 20 ppm. The results were found to be effective at 20 and 10 ppm while at 5 ppm there was no antimicrobial activity (Figure 5; Table 1). The results of MIC and MBC were also determined of the effective doses against the bacterium (Table 2). Silver nanoparticles were prepared by the use of different stabilizing agents viz. SDS and sodium citrate

and were evaluated against biofilm producing *S. aureus* (Bharti *et al.*, 2017). *Trichoderma* fused silver nanoparticles were evaluated for antifungal potential against phytopathogens (Gupta *et al.*, 2016).

In conclusion, the present study suggests the antimicrobial significance of silver nanoparticles prepared by green synthesis. The nanoparticles are found to be the effective suitable agents for different pharmacological properties. From the present studies, it is clear that, minimum

concentration of plant extract is suitable for preparation of green synthesis of silver nanoparticles. It is also concluded that, plant part(s) extracts containing secondary metabolites/active compounds are best capping agent for binding and providing a unique surface area to the nanoparticles. The results thus conclude that, silver nanoparticles synthesized by green chemistry are effective antimicrobial agents against *S. aureus* strains and thus can be utilized to formulate and effective antibacterial drug.

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