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Synthesis and Evaluation of Antimicrobial Property of Bionanoparticles

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Abstract: The nanoparticles are used for an efficient removal of pollutants and germs in the area of water purification. Among them, the use of silver nanoparticles have obtained massive attraction. Different types of physical and chemical methods are employed for the synthesis of silver nanoparticles. But, synthesis of silver nanoparticles using plant extract has been advantageous over other methods as they are cost effective, environmental friendly and easily scaled up for large scale synthesis. This study was mainly focused on synthesis of silver nanoparticles using *Azadirachta indica* (Neem) leaf extract. In this method, physiologically stable, bio-compatible silver nanoparticles were synthesized by reacting an aqueous solution of 10^{-3} M Silver Nitrate ($AgNO_3$) and 2.5 g/100 mL crude neem leaf extract at nine different ratios at room temperature without varying the other conditions. Initially, biosynthesized silver nanoparticles were observed by color change and then characterized by using ultraviolet-visible spectroscopy. The synthesized silver nanoparticles were then separated by centrifuging the silver nanoparticle solution at 5000 rpm for 20 min and separated nanoparticles were kept in oven for 16 h to collect silver nanoparticles. Antibacterial activity of silver nanoparticles made from 1:8 ratio was evaluated against selected highly abundant bacteria in wastewater such as *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* by using spread plate method. Both the color change from pale yellow to yellowish brown color and the absorption peak observed in ultraviolet-visible spectrum in the range of 325-477 nm confirmed the formation of silver nanoparticles in the solution. However, strong peak was observed in 1:8 with the maximum absorbance value of 3.733. These silver nanoparticles showed effectiveness in suppressing *E. coli* and *Staphylococcus aureus*. In conclusion, silver nanoparticles can possibly be used as effective growth inhibitors in microorganisms thereby applicable to water purifications and wastewater treatments.

Key words: Antibacterial activity, *Azadirachta indica*, Silver nanoparticles

INTRODUCTION

Nanotechnology deals with the materials at nano level which have profound application in various areas. The use of these nanoparticles is gaining significant attention at present as they possess important chemical and mechanical properties. Among them, metallic nanoparticles use in many researches as they contain remarkable antibacterial properties to act against various harmful microorganisms (Liau *et al*., 1997; Feng *et al*., 2000).

Different types of physical and chemical methods are employed for the synthesis of nanoparticles. Physical and chemical methods require toxic and flammable chemical reducing agents such as sodium borohydride (Solomon *et al*., 2007), formaldehyde and glycol ethylene (Hsu and Wu, 2010). The biological method provides a feasible alternative for the synthesis of nanoparticles. Among the biological methods, synthesis of nanoparticles using plant extracts is considered to be safe and ecologically feasible. Several plant extracts such as geranium leaves (Shankar *et al*., 2003), lemon grass (Shankar *et al*., 2004), aloe vera (Chandran *et al*., 2006) and several other leaves have been used to produce nanoparticles. In the present study, leaf extract of *Azadirachta indica* (Neem) was chosen to produce silver nanoparticles as it is a commonly available medicinal plant and the biosynthesized silver nanoparticle has efficient antibacterial activity as it is capped with the neem leaf extract (Shankar *et al*., 2004; Tripathy *et al*., 2010).

Synthesis of silver nanoparticles is much of interest because of their wider range of applications in catalysis,

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optical sensing, electronics, textile engineering and pharmaceuticals. In addition, it is suggested that silver nanoparticles may be used in future at large scale water purification. In the area of water purification, nanotechnology offers the possibility of an efficient removal of microorganisms and other contaminants. By focusing on removal of microorganisms, the present study mainly aimed to test the antibacterial properties of biosynthesized silver nanoparticles against selected bacterial strains such as *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* which are commonly found in contaminated water sources.

MATERIALS AND METHODS

Preparation of leaf extract: Total 2.5 g of fresh leaves of *Azadirachta indica* (Neem) and *Ocimum tenuiflorum* (Tulsi) were washed thoroughly with deionized water and cut into small pieces. These finely cut pieces were then mixed with 100 mL of deionized water and kept for boiling for a period of 2 min. After cooling, it was filtered through whatmann filter paper to obtain clear solution (De Silva *et al*., 2013; Kumari *et al*., 2014).

Synthesis of AgNPs: Silver nanoparticles were synthesized by the addition of 10 mL of aqueous extract of neem leaves to different volumes (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mL) of 10^{-3} M silver nitrate solutions without varying the other conditions. The solution was allowed to react at room temperature. Periodic sampling after 30 min was carried out to monitor the formation of AgNPs.

Analysis of bioreduced silver nanoparticles: The formation of silver nanoparticles was observed by the color change and also monitored by UV-visible spectroscopy. UV visible characterization of colloidal silver nanoparticle solutions prepared in different ratios was performed using a shimadzu double beam UV visible spectrophotometer (model U-1800). The bioreduction of $AgNO_3$ was monitored by sampling of 3 mL aliquot and absorbance was recorded in the range of 200-800 nm using 1 cm path length quartz cuvettes at different time intervals (30, 60, 90 and 120 min). The sterile distilled water was used as a reference. The measurements were carried out as a function of reaction time at room temperature.

Separation of AgNPs: Centrifugation was done to separate silver nanoparticles at 5000 rpm for 20 min after silver nanoparticle solution turned into reddish brown color and resulted the maximum absorbance in the UV-visible spectrum in the range of 400-500 nm. These separated nanoparticles were kept in oven for 16 h to obtain silver nanoparticles in the powder form.

Assessment of antibacterial activity: The antibacterial assays were done using silver nanoparticles capped with neem on gram negative bacteria *E. coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* and gram positive bacteria *Staphylococcus aureus* by spread plate method. Luria Bertani (LB) broth/agar medium was used to cultivate bacteria. Serial dilution of overnight culture was done and silver nanoparticles (0.1 mg) were added to the diluted samples. Each sample was agitated at 120 rpm for overnight. Fresh overnight cultures of inoculum (1 μL) were further diluted 10 times and were spreaded on to LB agar plates. The colony count was taken after 12 h of incubation at 37°C. In each plate without addition of nanoparticles was placed as control.

RESULTS

Biosynthesis and UV-visible spectral analysis of silver nanoparticles: Silver nanoparticles were synthesized according to the method described in De Silva *et al*. (2013). The silver nitrate solution turned pale yellow to reddish brown color in addition of neem leaf extract indicating the formation of AgNPs. The change in the color of the reaction mixture after 2 h is presented in Fig. 1. Further, formation of reddish brown color occured within 2 h indicating rapid biosynthesis of silver nanoparticles.

UV-visible spectroscopy is one of the most widely used techniques for characterization of silver nanoparticles. To confirm the formation of silver nanoparticles, UV visible spectroscopy was done by measuring the absorbance values of silver nanoparticle

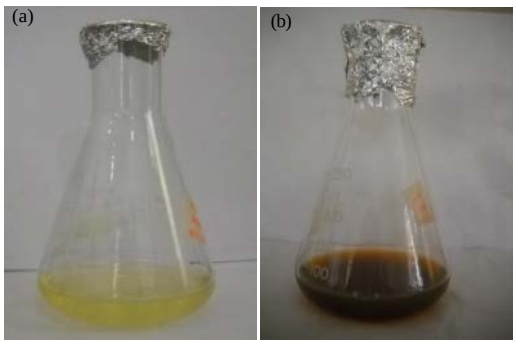

Fig. 1: The color change in reaction mixture (silver nitrate and neem leaf extract): a) 0 h; b) After 2 h

solution. Maximum absorbance for carboxylic-acid derivatized silver colloidal particles was reported in the visible range of 400-500 nm (Sastry *et al*., 1997). Strong absorption of electromagnetic waves of visible range (400-500 nm) was observed in the previous studies where silver nanoparticles were synthesized by using neem leaf extraction of 2.5 g mL^{-1} (De Silva *et al*., 2013). This was further confirmed by the present study by analyzing UV-visible spectra of Ag nanoparticles in aqueous solution at different reaction times. Absorbance values were varied with different reaction time. However, formation of silver nanoparticles by surface plasmon absorption maxima was detected at 2 h in the expected range (Fig. 2).

UV-visible spectral analysis was also done to detect the best reaction mixture. It showed a plasmon resonance band in the expected wavelength range for all the tested ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10) of neem leaf extract (data not shown). However, strong plasmon resonance band was obtained for both 1:8 and 1:10, peak at 412 nm with absorbance at 3.733 and 3.028, respectively (Fig. 3).

In addition, leaf extract of *Ocimum tenuiflorum* (Tulsi) as a control plant was tested to compare the effectiveness and efficiency of neem leaf extract as a bio-reductant to produce silver nanoparticles. Results revealed that the maximum absorbance (3.733) of neem leaf extract was greater than the *Ocimum tenuiflorum* (0.805) confirming the potential of neem leaf extract in synthesizing silver nanoparticles (Fig. 4).

Antibacterial activity of silver nanoparticles: Based on UV-visible spectroscopy results, silver nanoparticles

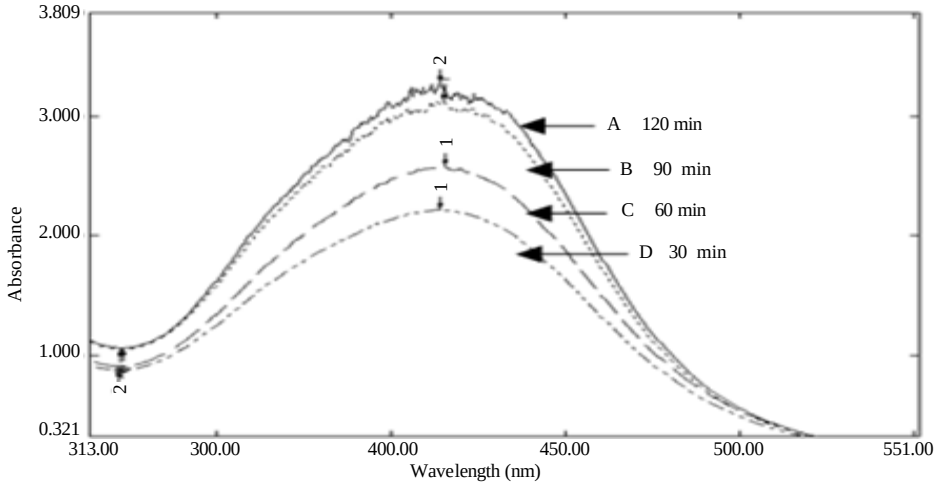

Fig. 2: UV-visible absorption spectrum of AgNPs recorded after regular intervals

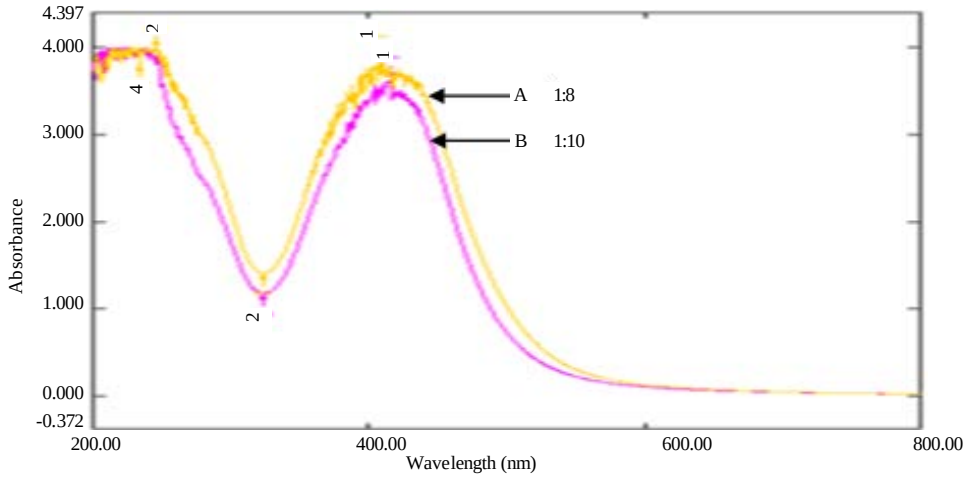

Fig. 3: UV-visible spectra of AgNPs recorded for different reaction mixture

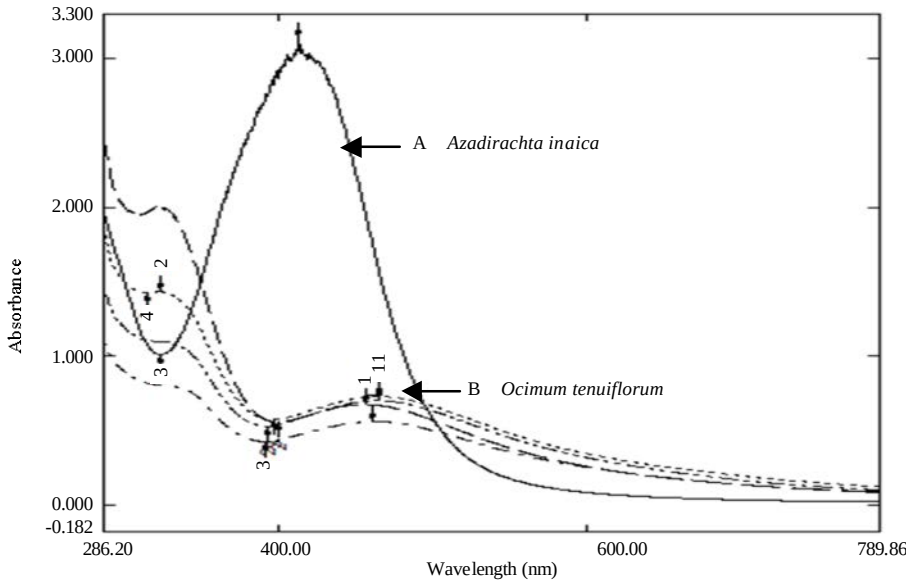

Fig. 4: UV-visible spectra of AgNPs recorded for both *Azadirachta indica* and *Ocimum tenuiflorum* leaf extract

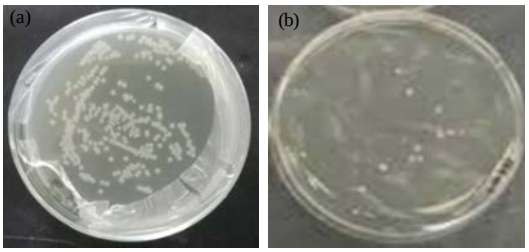

Fig. 5: Antimicrobial activities of Ag nanoparticles on *E. coli*; a) *E. coli* growth on LB medium without any treatment; b) *E. coli* growth treated with Ag nanoparticles

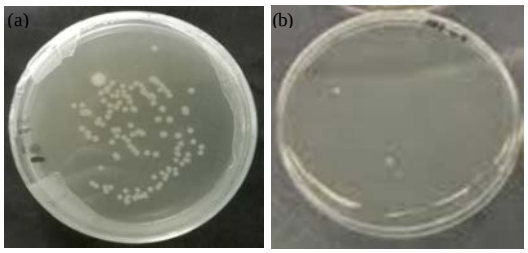

Fig. 6: Antimicrobial activities of Ag nanoparticles on *Staphylococcus aureus*; 1) *S. aureus* growth on LB medium without any treatment; 2) *S. aureus* growth treated with Ag nanoparticles

made from 1:8 ratio which gave the maximum absorbance at 3.733 was used to test the antimicrobial activity (Table 1). The experimental results indicated that silver nanoparticles were found to be effective against

Table 1: Colony counts of selected bacteria at 10^{-15} dilution

Tested organisms	Control (without silver nanoparticles)	After treatment with silver nanoparticles (0.1 mg)
S. aureus	210	51
E. coli	864	198
S. typhimurium	956	10065
P. aeruginosa	5487	10163

Escherichia coli (Fig. 5) and *Staphylococcus aureus* (Fig. 6). However, the highest antimicrobial activity was detected against *Staphylococcus aureus* (Fig. 6).

DISCUSSION

Formation and stability of Ag nanoparticles in aqueous colloidal solution are initially confirmed by using color change and then by UV-visible spectral analysis. It is well known that silver nanoparticles exhibit reddish brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles (Shankar *et al*., 2004; Ankamwar *et al*., 2005; Chandran *et al*., 2006). Initially, the reduction of Ag^+ ions leads to the formation of silver atoms (Ag) which may follow by agglomeration into oligomer clusters. These clusters eventually lead to the formation of colloidal Ag particles. On adding, aqueous extract of *Azadirachta indica* to silver nitrate solution, the yellowish color changed to reddish brown color after 2 h resulting reduction of the silver ion to silver nanoparticles (Fig. 1).

The formation of the silver nanoparticles was confirmed by UV-visible spectroscopy. It has been reported that wavelength for maximum absorbance value

(λ max) of silver nanoparticles lies in the visible range from 400-500 nm (Sastry *et al*., 1997) indicated the synthesis of silver nanoparticles in the solution. The neem leaf extract solution exposed to $AgNO_3$ solution showed a distinct absorption in the range of 400-500 nm which corresponds to surface plasmon resonance of silver nanoparticles established at 412 nm (Fig. 2). The absorption band in visible light region (400-500 nm, plasmon peak at 412 nm) is typical for silver nanoparticles.

UV-visible spectroscopy was performed further to detect the best reaction time needed for the synthesis of nanoparticles. Bioreduction at 2 h resulted with maximum absorption indicating the minimum time period required for the synthesis of silver nanoparticles (Fig. 2). In addition, different ratio of reaction mixture determined the effect of reaction mixture in converting silver ions into silver nanoparticles (Fig. 3). Further, the highest absorbance value of neem leaf extract compared with *Ocimum tenuiflorum* (Tulsi) exhibited competence of neem leaf extract in synthesizing nanoparticles (Fig. 4). It is well documented by this study that plant extract of neem leaves have the potential to synthesize AgNPs.

AgNPs have received increasing attention because they are able to control pathogenic bacteria of various sources. A number of theories for antimicrobial actions of colloidal silver solution have been proposed for example, alteration of permeability of cell membrane (Sondi and Salopek-Sondi, 2004), release of lipopolysaccharides and membrane proteins (Amro *et al*., 2000), generation of free radicals responsible for the damage of membrane (Kim *et al*., 2007), dissipation of the proton motive force resulting in the collapse of the membrane potential (Lok *et al*., 2006). However, the mechanism for the antimicrobial action of silver ions is not properly understood.

The growth of *Escherichia coli* (Fig. 5) and *Staphylococcus aureus* (Fig. 6) at 0.1 mg of AgNPs in the LB agar plate proved the effectiveness of silver nanoparticles by significantly reducing the (CFU) Colony Forming Units by >75% when compared to the control. In this study, *Salmonella typhimorium* and *Pseudomonas aeruginosa* were observed to be more resistance than *Escherichia coli* and *Staphylococcus aureus* (Table 1). The total reduction of these bacteria in the media may require >0.1 mg AgNPs in the growth media. The inhibitory effect of AgNPs may also depend on the CFU of bacteria used in this experiments.

Future research is required to evaluate number of parameters such as optimum concentration of AgNPs needed for the inhibitory effect on bacteria, CFU of the bacteria in the media and size and shape of the silver nanoparticles to understand the relationship between the size of nanoparticles and antibacterial activity. Better understanding of the mechanisms of silver biosynthesis using neem extract will be enable to achieve better control over size, shape and monodispersity which will lead to the development of high precision in the production level and the application of nanoparticles for controlling antibacterial activity.

CONCLUSIONS

AgNPs are synthesized using leaf extract of a well-known medicinal tree, Neem. The data from UV-visible spectroscopy supports the formation of biologically synthesized AgNPs. It confirms the efficiency of synthesized AgNPs by using Neem than the other medicinal plant (*Ocimum tenuiflorum*) used for the present study. The synthesis is found to be efficient in terms of silver Surface Plasmon Resonance (SPR) band in 400-500 nm range. Investigation of antibacterial activity of AgNPs on *E. coli* and *S. aures* reveals higher potential of silver nanoparticles capped with Neem extract to be used as antibacterial agent. In conclusion, AgNPs treatment in LB media with bacterial inoculum showed promising results on controlling the bacterium *S. aureus* than *E. coli*.

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