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SYNTHESIS OF SILVER NANO PARTICLES FROM NEEM LEAF (AZADIRACTA INDICA) EXTRACT AND ITS ANTIBACTERIAL ACTIVITY

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ABSTRACT

Eco friendly and cost effective methods of green mediated synthesis of nanoparticles are the present research in the limb of nanotechnology. The present work leads to the synthesis of silver nanoparticles from 1 mM AgNO₃ solution through various concentration of aqueous leaf extract of *Azadirachta indica* reducing as well as capping agent. The characteristics of silver nanoparticles were studied by using UV-VIS spectroscopy, SEM and EDX. Ultraviolet scanning spectroscopy was used to detect the distinct absorption spectrum of silver nanoparticles. The peak value observed at 435 nm. The EDX spectrum of the silver nanoparticles confirmed the presence of elemental silver signal. The size of synthesized silver nanoparticle was 146 nm. Green synthesized silver nanoparticle showed zone of inhibition against isolated Gram positive (*Micrococcus*, *Bacillus* and *Staphylococcus species*) and Gram negative (*Klebsiella species* and *E.coli*) bacteria. Based on the result obtained it can be said that the plant resources can efficiently use in the production of silver nanoparticle and it could be utilized in various fields in biomedical and nanotechnology.

Keywords: Nanoparticles; Neem Leaves; Antibacterial Activity; SEM, EDX

INTRODUCTION

Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process (Morones *et al.*, 2005; Lok *et al.*, 2007). The most important application of silver and silver nanoparticles is in medical industry such as topical ointments to prevent infection against burns and open wound. Silver ions (Ag⁺) and its compounds are highly toxic to microorganisms exhibiting strong biocidal effects on many species of bacteria but have a low toxicity towards animal cells (Prema, 2011). Studies indicated that the reducing phytochemicals in the neem (*Azadirachta indica*) leaf consisted mainly of terpenoids. It was found that these reducing components also served as capping and stabilizing agents in addition to reduction as revealed from FTIR studies. The major advantage of using the Neem leaves is that it is a commonly available medicinal plant and the antibacterial activity of the biosynthesized silver nanoparticle might have been enhanced as it was capped with the neem leaf extract. The major chemical constituents in the extract were identified as nimbin and quercetin (Shankar *et al.*, 2004; Tripathy *et al.*, 2009).

Feng *et al.*, (2000) conducted a study to observe the effects of silver ions on gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*). Under TEM they observed that cells exposed to the Ag⁺ ions seemed to have activated a stress response that led to the condensation of DNA in the center of the cell. They also observed cell membrane detachment from the cell wall, cell wall damage, and electron dense granules outside and, in some instances, inside the cell. It was proposed that condensation of DNA occurred as a protective measure in order to protect the genetic information of the cell (Feng *et al.*, 2000), however condensation of DNA could also prevent cell replication by preventing the DNA from being accessed by transcriptional enzymes such as DNA polymerase. The electron dense granules that formed inside and outside the cell were extracted and subjected to X-ray microanalysis to determine their composition. It was found that the granules were in part composed of silver and sulfur. This finding supports the idea that silver inactivates proteins by binding to sulfur-containing compounds (Klueh *et al.*, 2000). It was also observed that when treated with Ag⁺, *E. coli*, a gram-negative bacterium, sustained more structural damages than the gram-positive *Staphylococcus aureus* (Feng *et al.*, 2000). It

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was also reported that treating cells with silver leads to cell shrinkage and dehydration (Guggenbichler *et al.*, 1999).

Studies shows that silver nanoparticles anchor to and penetrate the cell wall of Gram-negative bacteria (Morones *et al.*, 2005), it is reasonable to suggest that the resultant structural change in the cell membrane could cause an increase in cell permeability, leading to an uncontrolled transport through the cytoplasmic membrane, and ultimately cell death. It has also been proposed that the antibacterial mechanism of silver nanoparticles is related to the formation of free radicals and subsequent free radical-induced membrane damage (Danilczuk *et al.*, 2006; Kim *et al.*, 2007).

Novel wound dressings have been developed that use silver to help prevent wound infections (Joshua *et al.*, 2008). Silver nanoparticles are incorporated into the wound dressing, and the silver-enhanced wound dressings were found in vitro to consistently kill *Pseudomonas aeruginosa* cultures entirely and kill *Staphylococcus aureus* cultures with >99.99% efficiency (Ong *et al.*, 2008). In mice, the silver-enhanced wound dressings were also found to reduce mortality from *Pseudomonas aeruginosa* wound infections from 90% to 14.3% (Ong *et al.*, 2008).

Among hospital patients that require ventilator-assisted breathing, ventilator-associated pneumonia is the most common illness (Olson *et al.*, 2002). Endotracheal tubes are used by patients needing ventilator-assisted breathing. Silver coatings on the inside of endotracheal tubes have been shown to delay the appearance of bacteria on the insides of these tubes, and subjects that used the silver-coated tubes also showed decreased lung colonization by *Pseudomonas aeruginosa* (Olson *et al.*, 2002). Kollef *et al.*, (2008) showed that silver-coated endotracheal tubes actually do reduce the incidence or increase the onset time of ventilator-associated pneumonia in patients using a ventilator.

Studies revealed the antibacterial properties of surgical masks coated with silver nanoparticles (Li *et al.*, 2006). Nanoparticle coated masks were capable of a 100% reduction in viable *Escherichia coli* and *Staphylococcus aureus* cells after incubation. Additionally, the study reported no signs of skin irritation in any of the persons wearing the masks (Li *et al.*, 2006).

Silver nanoparticles have been used to impart antimicrobial activity to cotton fibers. Cotton samples were immersed in silver nanoparticle solutions and then subjected to a curing process to allow the nanoparticles to adhere to the cotton (El-Rafie *et al.*, 2010). A chemical binder was then applied to the fabric to help maintain nanoparticle-cotton binding. Cotton samples prepared in this manner were able to reduce *Staphylococcus aureus* and *Escherichia coli* cell counts by 97% and 91% respectively. Even after subjecting the fabric to 20 laundry cycles, the cotton samples were still able to reduce *Staphylococcus aureus* and *Escherichia coli* cell counts by 94% and 85% respectively. Cotton prepared in this manner could be used by individuals working in the medical field or those who often work with microbes to prevent the spread of infectious bacteria (El-Rafie *et al.*, 2010).

Although chlorine has long been used as the primary drinking water disinfectant, it has been shown that the chlorination of water can lead to the formation of many hazardous compounds (Moudgal *et al.*, 2000). Based on its low known toxicity to humans, silver has been suggested as a possible disinfectant of drinking water (Silvestry-Rodriguez *et al.*, 2007). Water recycling systems on the Mir space station and NASA shuttles have used silver as an effective water disinfectant, and in the United States, faucet-mounted and pitcher home water purification units contain carbon filters that are supplemented with silver (Silvestry-Rodriguez *et al.*, 2007).

AGC Flat Glass Europe has developed a glass with antimicrobial properties (AGC: Antibacterial glass). Silver ions incorporated into the glass are responsible for the antimicrobial activity. The company reports that 99.9% of bacteria that come in contact with the surface of the glass are killed. The glass was produced to help prevent the spread of pathogens in a hospital setting. It could also be used to maintain the integrity of sterile workspaces.

Various types of food packaging have been supplemented with silver-containing compounds to determine microbial growth and extend product shelf life. Some of these packaging types include bulk food storage containers, paperboard cartons, plastic or paper food wraps, and milk containers (Appendini and Hotchkiss, 2002). Silver zeolite is the silver-containing compound used in food packaging (Appendini

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and Hotchkiss, 2002). Although few silver-containing compounds are approved by the FDA for direct food contact, silver-incorporated food packaging is quite widespread in Japan (Appendini and Hotchkiss, 2002).

In the past few decades, researchers are taking interest in the development of textile fabrics containing antibacterial agents. As, silver is non-toxic and possesses antimicrobial properties it has encouraged workers to use silver nanoparticles in different textile fabrics. In this direction, silver nanocomposite fibers were prepared containing silver nanoparticles incorporated inside the fabric but from the scanning electron microscopic study it was concluded that the silver nanoparticles incorporated in the sheath part of fabrics possessed significant antibacterial property compared to the fabrics incorporated with silver nanoparticles in the core part (Yeo and Jeong, 2003).

Toxicity from silver is observed in the form of argyria, only when there is a large open wound and large amount of silver ions are used for dressing. There are no regular reports of silver allergy (Leaper, 2006). Silver nanoparticles in most studies are suggested to be non-toxic. But due to their small size and variable properties they are suggested to be hazardous to the environment (Braydich-Stolle *et al.*, 2005). Hussain *et al.*, (2005) studied the toxicity of different sizes of silver nanoparticles on rat liver cell line (BRL 3A) (ATCC, CRL-1442 immortalized rat liver cells). The authors found that after an exposure of 24 hour the mitochondrial cells displayed abnormal size, cellular shrinkage and irregular shape. Cytotoxicity study of silver nanoparticle impregnated five commercially available dressings was undertaken by Burd *et al.*, (2007). In the study, it was found that three of the silver dressings depicted cytotoxicity effects in keratinocytes and fibroblast cultures. Braydich-Stolle *et al.*, (2005) reported the toxicity of silver nanoparticles on C18-4 cell, a cell line with spermatogonial stem cell characteristics. From the study, it was concluded that the cytotoxicity of silver nanoparticles to the mitochondrial activity increased with the increase in the concentration of silver nanoparticles.

From the above studies, it can be concluded that the use of nanoparticles in biomedical and therapeutic applications has opened up a wide area to nanotechnology in the fields like electronics, engineering, medicine, etc. but the possible side effects of nanoparticles have not been much studied hence, detailed studies should be carried out before the introduction of products related to nanomedicine in the market (Oberdorster *et al.*, 2005).

Silver has been known to possess strong antimicrobial properties both in its metallic and nanoparticle forms hence; it has found variety of application in different fields. The Fe_3O_4 attached Ag nanoparticles can be used for the treatment of water and easily removed using magnetic field to avoid contamination of the environment (Gong *et al.*, 2007). Silver sulfadiazine depicts better healing of burn wounds due to its slow and steady reaction with serum and other body fluids (Fox and Modak, 1974). The nanocrystalline silver dressings, creams and gels effectively reduce bacterial infections in chronic wounds (Richard *et al.*, 2002; Leaper, 2006; Ip *et al.*, 2006). The silver nanoparticle containing poly vinyl nano-fibers also show efficient antibacterial property as wound dressing (Jun *et al.*, 2007). The silver nanoparticles are reported to show better wound healing capacity, better cosmetic appearance and scar less healing when tested using an animal model (Tian *et al.*, 2006). Silver impregnated medical devices like surgical masks and implantable devices show significant antimicrobial efficacy (Furno *et al.*, 2004). Environmental-friendly antimicrobial nano paint can be developed (Kumar *et al.*, 2008). Inorganic composites are used as preservatives in various products (Gupta and Silver, 1998). Silica gel micro-spheres mixed with silica thio-sulfate are used for long lasting antibacterial activity (Gupta and Silver, 1998) and treatment of burns and various infections (Feng *et al.*, 2000). Silver zeolite is used in food preservation, disinfection and decontamination of products (Matsuura *et al.*, 1997; Nikawa *et al.*, 1997). Silver nanoparticles can be used for water filtration (Jain and Pradeep, 2005).

Antibacterial properties of silver are documented since 1000 B.C., when silver vessels were used to preserve water (Richard *et al.*, 2002; Castellano *et al.*, 2007). Use of plant sources offers several advantages such as cost-effectiveness, eco-friendliness and the elimination of high pressure, energy, temperature, and toxic chemicals necessary in the traditional synthesis methods (Sun and Xia, 2002). The main objectives of this study were to (1) Synthesize the silver nanoparticles using aqueous extract of

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neem leaves, *Azadirachta indica* (2) characterization of silver nanoparticles by using UV-Vis spectroscopy, SEM-EDX (3) analyze antimicrobial properties against gram-positive and gram-negative bacteria.

MATERIALS AND METHODS

Sample Collection

Neem leaves were collected from Karimkunnamp, Kottayam district of Kerala state, India. The fresh leaves were collected in poly ethylene zipper bags, later washed two times with distilled water. The plant materials were thoroughly washed with distilled water and fresh weight were determined. The samples are then oven dried (KOA4, KEMI lab equipments, Ernakulam, India) at 60°C for 24 h. The dried samples were powdered using a waring blender (Magic V2, Preethi Kitchen Appliances Pvt Ltd, Chennai, India) and stored in air-tight polyethylene bottles until further analysis.

Extraction Method

Neem leaf extract was prepared with 10 g of fresh curry leaves taken in 3 separate beakers each. It was thoroughly washed with tap water and then with distilled water for at least 2 times and cut into small pieces.

The chopped leaves were boiled in 75ml of distilled water for 3 minutes in 1st beaker, for 5 minutes in 2nd beaker and for 10 minutes in 3rd beaker separately. The leaf broth was then cooled and filtered. It was then stored at 4°C after covering the beaker with aluminum foil for further use. The obtained curry leaf extract which appeared light green in color was stored 4°C for further use.

Synthesis of Silver Nanoparticles

Stock solution was prepared by dissolving 1mM silver nitrate (AgNO_3 ; Merck, Mumbai, India) and volume made up to 250 ml with distilled water. 5ml of neem leaf extract of different concentration (3 min boiled, 5 min boiled and 10 min boiled separately) was added to 100 ml of 1mM AgNO_3 solution and allowed to react at room temperature.

Test Microorganisms

The organisms used comprise of two gram-negative organisms (*Klebsiella* and *E.coli*) and three gram-positive organisms (*Staphylococcus*, *Bacillus* and *Micrococcus*). The test organisms were obtained from microbial stock cultures, Department of Biotechnology, Mar Augusthinose College, Ramapuram.

Escherichia Coli

These are gram negative, facultative or anaerobic rods (commonly abbreviated *E.coli*) commonly found in the lower intestine of warm blooded organisms. The organisms are relatively heat sensitive and are readily destroyed at high temperature. The optimal temperature for growth is 37°C. *E. coli* is responsible for intestinal tract infection and diarrhea.

Staphylococcus Species

These are spherical in shape, non-motile, gram positive and facultative anaerobes which are positive in the catalase test. The coagulase test is used to broadly demarcate *Staphylococcus* species into coagulase positive and coagulase negative species. *Staphylococcus* species grow readily on ordinary media with a temperature range of 10 to 40°C, the optimum being 37°C and a pH of 7.4-7.6. *S. aureus* strains have emerged resistant to the penicillinase-stable penicillins (cloxacillin, dicloxacillin, methicillin, nafcillin, and oxacillin).

Klebsiella Species

The genus *Klebsiella* consists of non-motile, capsulated rods that grow well on ordinary media forming large, dome shaped, mucoid colonies of varying degrees of stickiness. *Klebsiella species* are widely distributed in nature, occurring both as commensals in the intestines and as saprophytes in soil and water. *Klebsiella species* can cause diseases like pneumonia, ozena, rhinoscleroma etc.

Micrococcus Species

These are positive cocci which occur mostly in pairs, tetrads or irregular clusters. They are catalase and oxidase positive. They are aerobic with a strictly respiratory metabolism. They are parasitic on mammalian skin and are ordinarily non-pathogenic.

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Bacillus Species

The genus *Bacillus* consists of anaerobic bacilli forming heat resistant spores. They are gram positive but tend to be decolourised easily so as to appear gram variable, or even frankly gram negative. They are generally motile with peritrichous flagella. *Bacillus anthracis*, the causative agent of anthrax, is the major pathogenic species. *B. cereus* can cause food borne gastroenteritis. Some species may be responsible for opportunistic infections.

Characterization of Silver Nanoparticles

UV–Vis Spectroscopy

The periodic scans of the optical absorbance between 385 and 500nm with a UV- Vis spectrophotometer (Model 118, Systronics, Mumbai, India) at a resolution of 1 nm were performed to investigate the reduction rate of silver ions by neem leaf extract. The reaction mixture was diluted 20 times and used for UV-Vis spectrophotometry. Deionised water was used to adjust the baseline.

SEM-EDX Analysis

SEM- EDX Analysis was carried out in instrument JSM 6390 with acceleration voltage 20 kV. SEM reveals information about the sample including external morphology, chemical composition and crystalline structure and orientation of materials making up the sample. SEM provides detailed high resolution images of the sample by rastering a focused electron beam across the surface and detecting secondary or back scattered electron signal. The EDX spectrum of the silver nanoparticles was performed to confirm the presence of elemental silver signal and provides quantitative compositional information.

Antibacterial Assay

Nutrient broth, nutrient agar and Muller Hinton agar plates were made according to standard microbiological protocol. Filter paper discs of approximately 6 mm diameter were soaked with 50 µl of the plant extract, AgNO₃ and silver nanoparticle separately and allowed to dry at room temperature for 15 minutes. Muller Hinton Agar plates were prepared and the test microorganisms were inoculated by the spread plate method. Prepared discs were placed in the previously prepared agar plates. Each plate of every test organisms contained discs impregnated with Ag nanoparticle, leaf extract, silver nitrate solution and an antibiotic disc. The discs were pressed down to ensure complete contact with the agar surface and distributed evenly so that they were not closer than 24 mm from each other, center to center.

The agar plates were then incubated at 37°C. After 16 to 18 hours of incubation, each plate was examined. The resulting zones of inhibition were uniformly circular with a confluent lawn of growth. The diameters of the zones of inhibition were measured, including the diameter of the disc where the antibiotic was used as control (NCCLS, 1997).

Statistical Analysis

The survey results were analyzed and descriptive statistics were done using SPSS 12.0 (SPSS Inc., an IBM Company, Chicago, USA) and graphs were generated using Sigma Plot 7 (Systat Software Inc., Chicago, USA).

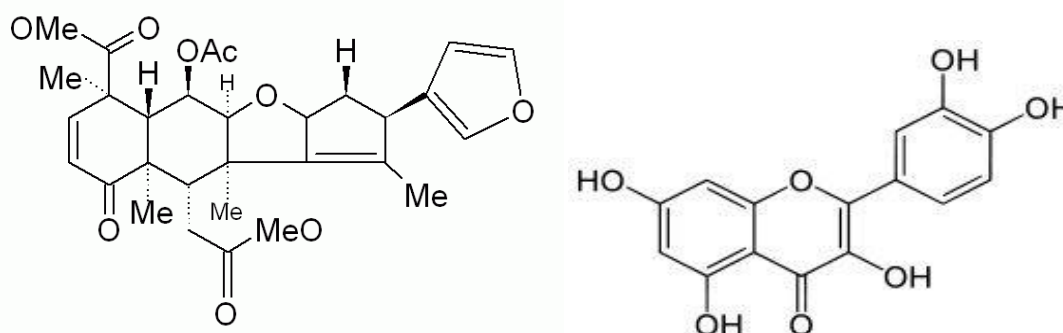


Figure 3: Structure of nimbin (left); Structure of quercetin (right)

(<http://mason.gmu.edu/~vandrade/projects/plant/plant.htm>;

<http://ayurvedicteas.files.wordpress.com/2011/06/quercetin.jpg>)

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Figure 3: *Azadirachta indica* plant with fruits in the field (top left); young leaves (top right); young leaves collected for making extract (bottom left); extracts of fresh leaves (bottom right). Photo credit: actaplantarum.org; davesgarden.com

RESULTS AND DISCUSSION

Synthesis of Silver Nanoparticles

After the addition of Neem leaf extract to AgNO_3 solution a visible color change from transparent to dark brown was observed which indicates the formation of silver nanoparticle. This occurred due to the reduction of silver ions present in the solution due to terpenoids present in neem leaf extract. After 90 minutes there was no change in the intensity of color developed, which indicates the completion of reduction reaction. The reduced silver particles are in the range of nano size.

Characterization of Silver Nanoparticles

UV Spectrometry

The UV absorption spectrum of silver nanoparticles from neem leaf extract of different concentrations was obtained as given in Figure 3.

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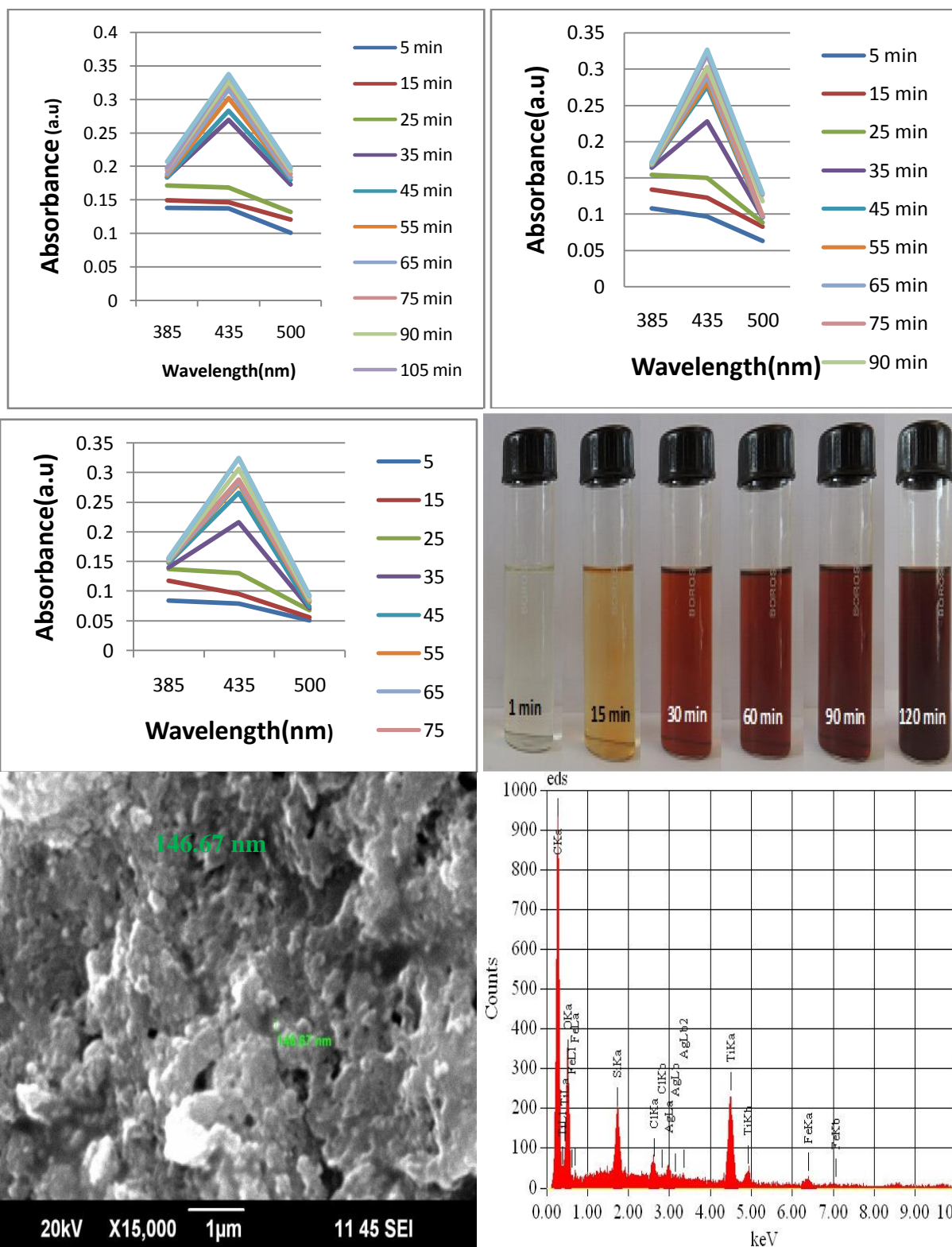


Figure 3: UV absorption spectrum of silver nanoparticles formed from neem leaves extracted at 10 minutes (top left); 5 minutes (top right); 3 minutes (middle left); mixture of neem leaves extract and silver nitrate over 2 hrs incubation (middle right); SEM micrograph of silver nanoparticles (bottom right); EDX spectra (bottom left)

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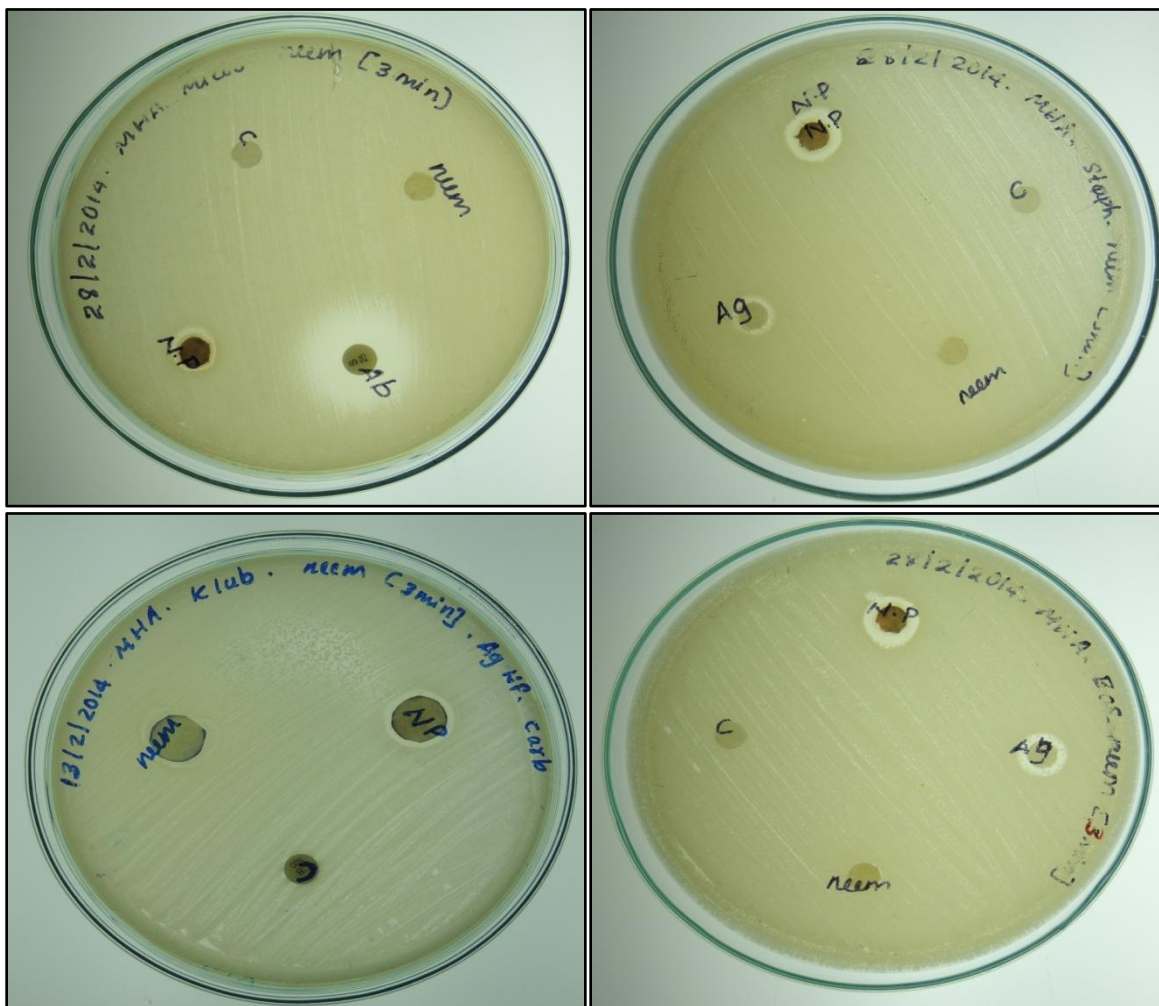


Figure 3: Antimicrobial activity of silver nanoparticles against *Micrococcus* species (top left); *Staphylococcus* species (top right); against *Klebsiella* species (bottom left); against *Bacillus* species (bottom right)

It is generally recognized that UV-VIS spectroscopy could be used to examine size and shape controlled nanoparticles in aqueous suspensions (Wiley *et al.*, 2006). Synthesized silver nanoparticles were characterized by UV-VIS spectrometry. The peak occurs at 435 nm (λ max) which corresponds to the absorbance of silver nanoparticles.

The intensity of the peak at 435 nm was increased with time until the reduction completes. The formation of silver nanoparticles slows after 120 min. The maximum peak was found to be 435nm for *Azadirachta indica*. From the studies carried out by Gavhane *et al.*, (2012) for the Neem plant, the maximum peak found at 420 nm.

SEM-EDX

The freeze-dried silver nanoparticles were mounted on specimen stubs with double-sided taps, coated with gold in a sputter coater, and examined under a Title PC SEM at 20 kV with a tilt angle of 45°. The silver nanoparticles formed were spherical in shape with diameter 146.67 nm as per the SEM analysis.

The EDX spectra of the synthesized silver nanoparticle from neem leaf extract confirmed the presence of elemental silver in the sample. The peaks around 3.40 keV correspond to the binding energies of AgL. Quantitative analysis proved 3.3% silver contents in the examined samples (the Mass% of silver in the sample is 3.3 and Atom% is 0.45).

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Anti Bacterial Assay

For *Azadirachta indica* the zone of inhibition was found to be 9-11 mm for *Klebsiella* species, 13-14 mm for *Bacillus* species, 10-11mm for *E.coli*, 13-15 mm for *Staphylococcus* species and 10-14 mm for *Micrococcus* species. The study done by Gavane *et al.*, (2012) the zone of inhibition found was 11-14 mm for *Klebsiella* species. Silver ions and silver salts are used as antimicrobial agents (Russel *et al.*, 1994). However, the high concentrations of silver salts restrict the use of them in present day medicine. Use of metal nanoparticles decreases the concentration of silver and other metal salts. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio which allows them to interact closely with microbial membranes and is not merely due to release of metal ions in solution or in culture plates (Morones *et al.*, 2005). The mode of action of both silver nanoparticles and silver ions were reported to be similar, although the nanoparticles were reported to be effective at significantly lower concentration than that of the ions (Morones *et al.*, 2005). According to Lok (2006), the attachment of both silver ions and nanoparticles to the cell membrane caused acclimatization of envelope protein precursors causing dissipation of the protein motive force.

Table 1: Zone of inhibition shown by microorganisms (*Bacillus*, *E.Coli*, *Staphylococcus*, *Klebsiella*, *Micrococcus*) against neem leaf extract, Ag (Sliver) nanoparticle and Ab (antibiotic)

Organism	Zone of inhibition (mm)				5 min		10 min	
	Ag [*]	Ab [#]	3 min Extract	NP [^]	Extract	NP	Extract	NP
<i>Bacillus</i>	11	14	11	14	-	13	-	13
<i>E.coli</i>	9	12	-	11	-	10	-	11
<i>Staphylococcus</i>	11	15	-	14	-	13	-	15
<i>Klebsiella</i>	8	-	-	10	-	11	-	9
<i>Micrococcus</i>	11	15	-	10	-	12	-	14

Key: *Ag (Silver), #Ab (Antibiotic), ^NP (Nanoparticle)

Conclusion

The neem leaf extract was prepared from fresh neem leaves by boiling it for 3 minutes, 5 minutes and 10 minutes separately. The obtained extract was of greenish color. Freshly prepared leaf extract was added to 1mM silver nitrate solution and the reaction takes place at room temperature which resulted in the synthesis of silver nanoparticles. The synthesized silver nanoparticles were characterized by UV-VIS spectrometry, SEM and EDX measurements. The UV-VIS spectra of silver nanoparticles formed in the reaction media has absorbance peak at 435 nm. Silver nanoparticles with an average size of 146 nm was synthesized. It has been demonstrated that Neem leaf extract is capable of producing silver nanoparticles that shows good stability in solution, under the UV-VIS wavelength of 435 nm nanoparticles shown quiet good surface Plasmon resonance behavior. The synthesized silver nanoparticles were characterized by UV-VIS spectrum, SEM and EDX measurements. This green synthesis method is alternative to chemical method, since it is cheap, pollutant free and eco-friendly. The results showed that neem plays an important role in the reduction and stabilization of silver to silver nanoparticles. Further, these synthesized silver nanoparticles from neem shows antibacterial activity on both Gram positive and Gram negative bacteria.

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