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Evaluation of the Antibacterial Activity of Silver Nanoparticles of *Carica papaya, Allium cepa and Azadirachta indica*: an Invitro Study

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A B S T R A C T

The integration of nanomaterials with biology is finding wide applicability in various areas of medical science. Silver has been long recognized as having inhibitory effect on various microorganisms present in medical and industrial processes. But silver nanoparticles (SNPs), when synthesized by wet chemical reduction method are found to be toxic, flammable and not at all environment friendly. Therefore in present study, an attempt was made to formulate a cost effective and environment friendly technique for green synthesis of SNPs, which were synthesized using extract of *Carica papaya, Allium cepa and Azadirachta indica* with $AgNO_3$ solutions and their antibacterial activity against clinical isolates viz., *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus and Steptococcus pyogenes* isolated from bed sheets of casualty ward from Prince Aly Khan Hospital was studied using Disk Diffusion Method. The SNPs were then impregnated onto cotton bedsheets and their antibacterial activity on the same isolates was studied. The effect of consecutive washing of the coated disks with distilled water on the antibacterial property was also investigated. The antibacterial activity of the cotton bed sheet coated with SNPs of all the three extracts was found to be retained up to three consecutive washings. This work demonstrates the possible use of biologically synthesized SNPs incorporated in cotton bed sheets to minimize nosocomial infections.

Introduction

The rates of nosocomial infections, especially by those caused by antibiotic resistant bacteria, are increasing alarmingly over the globe. Although more rigorous infection control measures are being implemented, it is clear that the current modalities to reduce nosocomial infections are not sufficient. In hospitals, textiles are an excellent substrate for bacterial growth under appropriate conditions of moisture and temperature.

Several studies have found that personnel in contact with contaminated textiles were the source of transmission of the micro-organisms to susceptible patients causing infection. Due to the growing demand for comfortable, clean, and hygienic textile goods, an urgent need for production of antimicrobial textile bed sheets has arisen. With the advent of new technologies, the growing needs of consumers in terms of health and hygiene can be fulfilled without

compromising issues related to safety, human health, and the environment (Wasif *et al.,* 2009).

Nosocomial Infection National Surveillance Service states that organisms like *Staphylococcus aureus*, *Escherichia coli*, Coagulase negative *Staphylococcus*, *Enterobacter species, Klebsiella species, Pseudomonas aeruginosa* and *Candida* are found to cause hospital acquired infections (HAI).These pathogens if not detected and dealt with in time, may turn out to be opportunistic and may cause a secondary infection in a patient already suffering from an infection or in an immunocompromised patient. (Gericke *et al.,* 2006). The use of antimicrobial textiles, especially in those textiles that are in close contact with the patients, may significantly reduce bioburden in clinical settings and consequently reduce the risk of nosocomial infections. These textiles should possess broad spectrum biocidal properties. They should be safe for use and highly effective against antibiotic resistant micro-organisms, including those that are commonly involved in hospital-acquired infections, and they should not permit the development of resistant micro-organisms to the active compound (Barkow and Gabbay, 2008).

Nanotechnology involves the tailoring of materials at the atomic level to attain unique properties, which can be suitably manipulated for the desired applications like biomedical and biotechnological, including drug delivery, enzyme immobilization, DNA transfection (Martin and Kohli, 2003), imaging, sensing, gene delivery system and artificial implants (Leela T and Shechinah Felice.Choragudi, 2014). Innovations in nanoscale processes includes, nanofiber enhanced clothing that protects the wearer or resists stains, nanoparticle based cosmetics and sunscreens that improve product efficacy (Wissing and Muller, 2003).

The *invitro* antimicrobial activity of silver containing polymers and bioglasses has been extensively studied the bactericide action. The bactericidal activity results show that this nanocomposite is strongly active against some of the most common Gram positive and Gram negative bacterial strains, so it can be considered as an antimicrobial biomaterial that can be be used in implant and reconstructive surgery applications (Diaz *et al.,* 2009).

Silver possesses antimicrobial activity against a broad spectrum of medically relevant microorganisms including bacteria, fungi, and yeasts. Antimicrobial silver nanoparticles are now used extensively to combat organisms in wounds and burns. Silver in its positively charged ionic form is highly toxic for microorganisms but has relatively low toxicity for human tissue cells. Silver nanoparticles simultaneously attacks multiple sites within the cell to inactivate critical physiological functions such as cell-wall synthesis, membrane transport, nucleic acid (such as RNA and DNA) synthesis and translation, protein folding and function, and electron transport, which is important in generating energy for the cell. Without these functions, the bacterium is either inhibited from growing or, more commonly, the microorganism is killed (Chen *et al.,* 2003).

Medicinal plants are used traditionally to prevent or cure diseases all over the world (Nair *et al.,* 2005). The medicinal values of these plants lie in bioactive phytochemical constituents that produce definite physiological actions on the human body. (Krishnaiah *et al.,* 2009). In recent years, these bioactive secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been

extensively investigated as a source of medicinal agents.

Therefore, with this in mind, the aim of the present investigation was to carry out green synthesis of silver nanoparticles using plant extracts, characterize them and study their application on hospital bed sheets so as to help in curbing nosocomial infections.

Materials and Methods

Clinical Isolates

The clinical isolates isolated from bed sheets of casualty ward from Prince Aly Khan Hospital were enriched in Nutrient Broth at 36 ± 1 ^{o}C for 2 hours after which they were aseptically inoculated on Nutrient Agar and incubated at 36 ± 1 ^{o}C for 24 hours. The organisms were identified to be *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pyogenes* on the basis of their colonial, morphological and biochemical characteristics using standard microbiological techniques (William and Wilkins, 1986; Kurtzman *et al.*, 1995). All the isolates were maintained at 4^{o}C.

Synthesis of Silver Nanoparticles (SNPs)

Using *Allium cepa*

Allium cepa extract was prepared by taking 25g of thoroughly washed and finely crushed onion mixed with 100ml deionized water in an Erlenmeyer flask and then boiling the mixture for 10 min. before finally decanting it. Then, this onion extract was mixed with 0.1mM aqueous solution of $AgNO_3$ in a 1:10 ratio drop wise with constant stirring at 50^{0}-60^{0}C.

Using *Carica papaya*

Fresh leaves of *C. papaya* (25 g) were diced into fine pieces and transferred to an Erlenmeyer flask. Deionized water (200 mL) was added to the flask and heated at 60 °C for 5-10 min. This extract was then added to 0.1mM aqueous solution of $AgNO_3$ in a 1:4 ratio dropwise and kept at room temperature for 5 h.

Using *Azadirachta indica*

Fresh Neem leaves (20g) were cut, washed and added to deionized water (100ml) and boiled for 15 min. in a water bath. The mixture was then filtered to obtain aqueous extract. The extract was immediately mixed with 0.01M aqueous solution of $AgNO_3$ at 1:4 ratio dropwise and incubated on a rotary shaker at 120rpm for 5 h. at 27±1^{0}C.

Characterization of Silver Nanoparticles by Visual Inspection of Colour

The mixture formed by mixing the various extracts with silver nitrate solution was observed for a change in colour characteristic of SNP formation.

Ultraviolet-visible (UV-Vis) Spectroscopy

The reduction of pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction mediums 5 h. after diluting a small aliquot of the sample with distilled water. UV-Vis spectral analysis was done by using Shimadzu 1800 UV-Vis spectrophotometer.

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopic (SEM) analysis was done using Hitachi S-4500 SEM machine at CIRCOT, Mumbai. Thin films of the sample were prepared on a carbon coated copper grid by just placing a very small amount of the sample on the grid, extra solution was removed using a blotting paper. Then the films on the SEM grid were

allowed to dry by successive ethanol dehydration and images were developed

Incorporation of Plant Extracts (*Allium cepa, Azadirachta indica* and *Carica papaya*) onto Cotton Disks

Cotton Disks (7mm) punched out and sterilized. Disks were added to the extracts of *Allium cepa, Azadirachta indica* and *Carica papaya*. The individual tube was centrifuged at 3000rpm / 30 min. The disks were removed and dried in the laminar flow.

Incorporation of Silver Nanoparticles of Plant Extracts onto Cotton Disks

The same procedure was carried out as above using silver nanoparticles of *Allium cepa, Azadirachta indica* and *Carica papaya* instead of the plant extracts.

Evaluation of the Antimicrobial Property of Respective Plant Extracts as well as their SNPs Coated onto Cotton Disk by Disc Diffusion Method (Bauer *et al.*, 1996)

Eighteen hour old cultures of *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus and Streptococcus pyogenes* (cell density 10^8 org /ml) were seeded onto Sterile Nutrient Agar plates. The coated discs (7mm discs individually coated with respective plant extracts as well as SNPs of the plant extracts used in the study were placed on plates containing the test isolates. The plates were then incubated at 36±1ºC for 24h in an upright position. The results were recorded and analyzed in terms of the zones of inhibition formed around each well.

Evaluation of Antibacterial Coated Textile (bed sheet) after Repeated Washings

The procedure used was exactly the same as above but, in this case discs (7mm) were cut out from the SNP coated cotton textile (bedsheet) after every step of washing with D/W and the antibacterial activity of the same was evaluated upto the stage where no antibacterial activity could be detected.

Results and Discussion

Characterization of Silver Nanoparticles by

Visual Inspection of Colour

As the *Carica papaya* fruit extract was mixed with the aqueous solution of the silver nitrate, it started to change its color from watery to yellowish brown due to reduction of silver ions which indicated formation of silver nanoparticles. During the biosynthesis using aqueous extract of *Allium cepa*, the color of the reaction medium changed rapidly from pale pink to amber on formation of the silver nanoparticles. During the biosynthesis using aqueous extract of *Azadirachta indica* leaves, the color of the reaction medium changed rapidly from colourless to brown indicating formation of the silver nanoparticles. The appearance of typical color of these SNPs in all the three cases was due to the excitation of surface plasmon vibrations which is characteristic of silver nanoparticles (Plate 1).

Ultraviolet-visible (UV-Vis) Spectroscopy

The formation of metal nanoparticles by reduction of the aqueous silver metal ions on exposure of various extracts to silver nitrate may be easily followed by UV–Vis spectroscopy (Shimadzu 1800 Spectrophotometer). UV-Vis absorption spectrum of silver nanoparticles in the presence of various extracts is depicted in Graph 1. It is generally recognized that UV–Vis spectroscopy could be used to examine size- and shape-controlled nanoparticles in aqueous suspensions. Absorption spectra of silver nanoparticles formed in the reaction

media (all three extracts) showed maximum absorbance at around 400 nm and a broadening of the peak indicated that the particles are poly dispersed.

Scanning Electron Microscopy (SEM)

The silver nanoparticles on scanning using SEM (Hitachi S-4500) showed a variable morphology and an average mean size of 2µm (*Allium cepa and Azadirachta indica*) and 1 µm (*Carica papaya*). The high density nanoparticles observed under the Scanning Electron Microscope confirmed the development of silver nanostructures of the various extracts used in the study (Plate 2, 3, 4).

Table.1 Comparitive Antibacterial Activity of Plant Extracts with their SNPs against Pathogenic Isolates

	Diameter of zone of inhibition (mm)						
	Allium cepa		***Carica papaya***		***Azadirachta indica***		**Control**
	Extract	**SNPs**	**Extract**	**SNPs**	**Extract**	**SNPs**	
Escherichia coli	44	46	62	71	60	63	NO ZONE
Pseudomonas aeruginosa	40	43	47	52	50	50	NO ZONE
Klebsiella pneumoniae	61	63	65	70	62	65	NO ZONE
Staphylococcus aureus	65	67	68	75	70	72	NO ZONE
Streptococcus pyogenes	61	67	68	73	68	72	NO ZONE

Table.2 Antibacterial Activity of the SNPs of Various Plant Extracts Coated on Textile Fabric after Repeated washings against *Staphylococcus aureus*

Number of Washings Given to the textile fabric	**Diameter of zone of inhibition (mm) FOR *Staphylococcus aureus***								
	***Allium cepa* SNPs**			***Carica papaya* SNPs**			***Azadirachta indica* SNPs**		
1ST	80	80	82	90	88	86	86	86	84
2ND	64	52	54	80	78	62	62	62	62
3RD	20	21	22	36	24	20	27	25	24
4TH	NO ZONE	NO ZONE	NO ZONE	NO ZONE	NO ZONE	NO ZONE	NO ZONE	NO ZONE	NO ZONE

Fig.1 Ultraviolet-visible (UV-Vis) Spectroscopy of:a) SNP made using *Allium cepa* b) SNP made using *Carica papaya* c) SNP made using *Azadirachta indica*

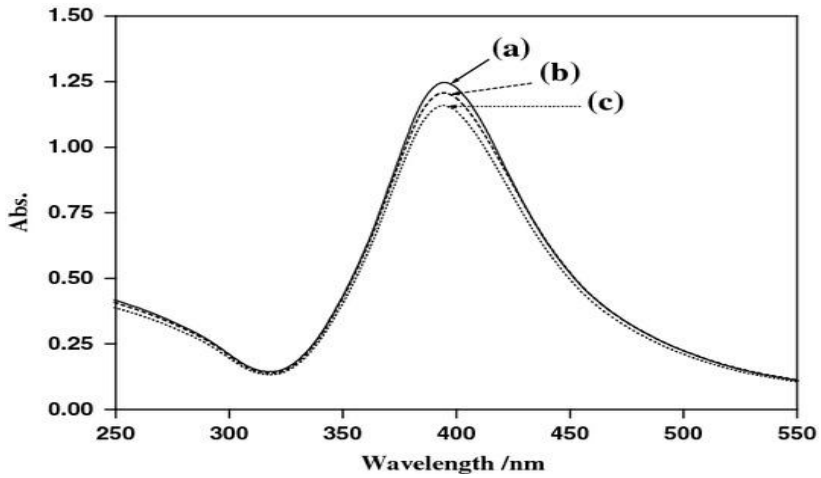


Plate.1 Visual Inspection of the SNPs Obtained: a) Using *Allium cepa* b) Using *Carica papaya* c) Using *Azadirachta indica*

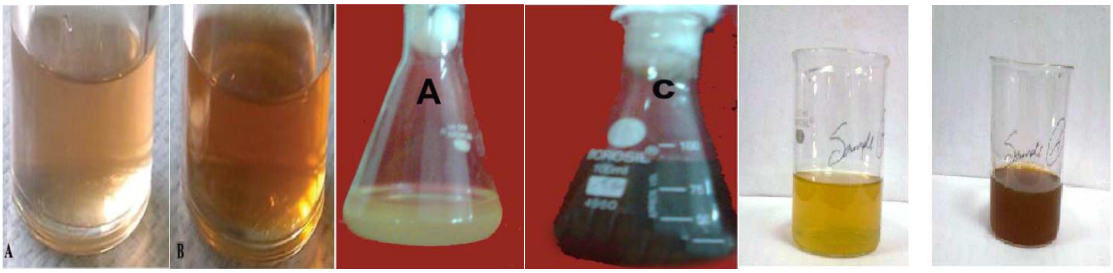


Plate.2 Scanning Electron Microscopy of SNPs made using *Allium cepa*

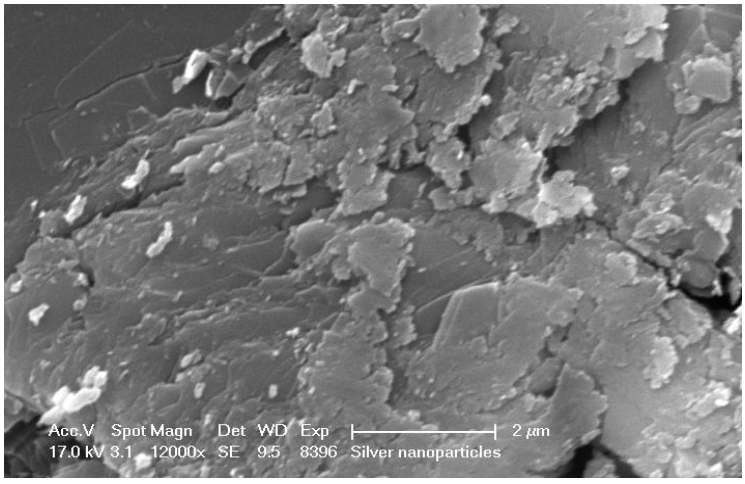


Plate.3 Scanning Electron Microscopy of SNPs made using *Carica papaya*

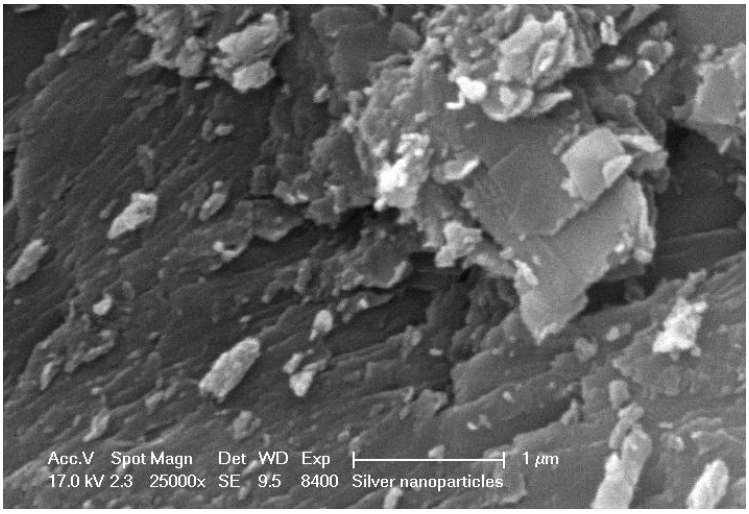
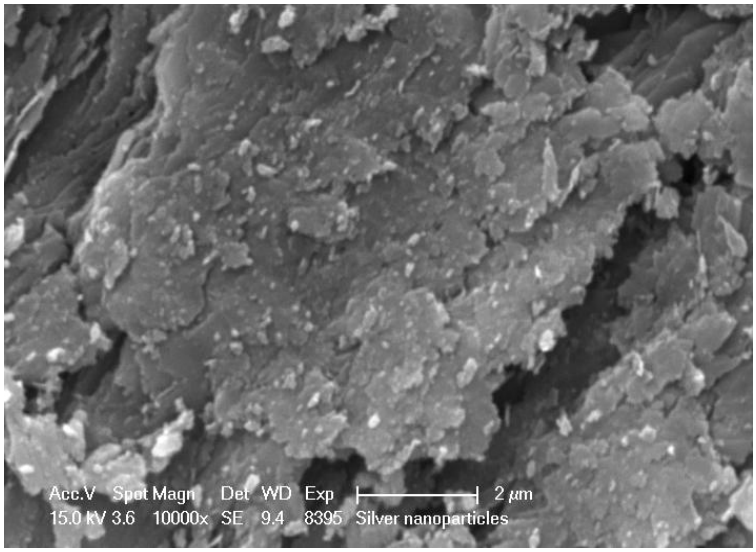


Plate.4 Scanning Electron Microscopy of SNPs made using *Azadirachta indica*



Evaluation of the Antimicrobial Property of Respective Plant Extracts as well as their SNPs Coated onto Cotton Disk by Agar Diffusion Method

The SNPs of the plant extracts were found to be more antimicrobial compared to the extracts. *Carica papaya* was found to be the most antimicrobial of all the three extracts used in the study. It showed bactericidal action against both Gram positive as well as Gram negative organisms. Gram positive organisms were found to be more susceptible in comparison with Gram negative organisms. The order of susceptibility was found to be *Staphylococcus aureus* > *Streptococcus pyogenes* > *Klebsiella pneumonia* > *Escherichia coli* > *Pseudomonas aeruginosa* (Table 1). Earlier reports have always maintained that plant extracts are more active against Gram positive bacteria

than Gram negative bacteria and the result observed in the study are found to be as per those reported earlier (Fyhrquist *et al*, 2004).

Evaluation of Antibacterial Coated Textile (bed sheet) after Repeated Washings

The washing effect on the coated cotton textiles is depicted in Table 2. A decrease in average size of inhibition zone around the cotton textiles was noted after each washing step with a complete loss in the antibacterial activity after the 4th wash. The washings obtained after each of the three washing steps were checked by UV Visible scan for presence of silver nanoparticles. No noticeable absorption maxima was observed in 400 - 500 nm range confirming that nanoparticles coated on the cotton disks were not washed away. Therefore it can be inferred that the decrease in inhibition zone was not due to washing away of the nanoparticles. Some part of the capping material might have been washed off, which could not be detected in the UV Visible scan, but yet resulting in a decrease in the antibacterial effect after the first washing. However, the antibacterial activity was found to be retained upto three consecutive washings.

This work thus highlights the importance of biologically synthesized silver nanoparticle impregnated cotton bed sheets to minimize nosocomial infections and alleviate the sufferings of the patients.

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