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Synthesis of Silver Nanoparticles (Ag-NPs) by *Ficus benghalensis* Plant Extract and their Applications against Methicillin Resistant *Staphylococcus aureus* (MRSA)

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A B S T R A C T

This study aims to investigate the silver nanoparticles (Ag-NPs) prepared by green synthesis method using *Ficus benghalensis* plant extract from which nanoparticles are in the range of 1 to 100 nm in size and unique properties. The Banyan plant leaves were selected and extracted by the addition of 100 ml deionized water to the leaves and boiled for 15 min in a water bath. The mixture was filtered to obtain extract for the green synthesis of nanoparticles. The biosynthesized nanoparticles were characterized by UV–Vis Spectrophotometer, FTIR, XRD and SEM. The results of various characterizations indicated that the sample of Ag have the optimum morphology and structure. The major applications of silver nanoparticles in the medical field include antimicrobial property that is being majorly explored. Silver nanoparticles are rampantly planned to use in many medical procedures and devices as well as in various biological fields. So the biosynthesized nanoparticles were tested against *Staphylococcus aureus*. The *Staphylococcus aureus* clinical isolates were challenged with two fold 100 nm nanosilver serial dilutions for 24h. Silver nanoparticles affected bacterial cellular viability in a dose-dependent manner inhibiting the biofilm formation of MRSA effectively.

Introduction

Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical, optical and electronic fields. Nanoparticles are of great scientific interest as they are effectively a bridge between bulk

materials and atomic or molecular structures. Generally, metal nanoparticles can be prepared and stabilized by physical and chemical methods; the chemical approach such as chemical reduction, electrochemical techniques, and

photochemical reduction is most widely used. Synthesis of nanoparticles through biological method is a good alternative over the chemical and the physical methods as they are both environment friendly and economic. The synthesis of nanomaterials by the use of green chemicals has vital importance as medicinal and technological aspects.

The green synthesis of silver nanoparticles using *Ficus benghalensis* leaf extract has been reported (1). The polyol components and the water-soluble heterocyclic components are mainly responsible for the reduction of silver ions and the stabilization of the nanoparticles, respectively (2).

The green synthesis techniques are generally synthetic routes that utilize relatively non-toxic chemicals to synthesize nanomaterials, and include the use of non-toxic solvents such as water, biological extracts, and biological systems synthesis.

The green synthesis of silver nanoparticles involves three main steps, which must be evaluated based on green chemistry perspectives, including selection of solvent medium, selection of environmentally benign reducing agent and selection of non-toxic substances for the silver nanoparticles stability (3).

As with all surgical procedures, implantation comes with the added risk of infection. The goal of this in vitro study was to explore the use of nanoparticles as a multifunctional platform to prevent biofilm formation. Infection has been reported on an array of implantable devices including joint prostheses, central venous catheters and needleless connectors, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, tympanostomy tubes, and voice

prostheses. A biofilm serves to promote bacteria persistence by resisting antibiotic treatment and host immune responses. Antibiotics are rendered ineffective when biofilms form due to their relative impermeability, the variable physiological status of microorganisms, sub-populations of persistent strains, and variations of phenotypes present.

Nanoparticles are small enough to penetrate the biofilm, large enough to have a long plasma half-life, and additionally offer a surface to volume ratio optimized for mass loading of targeting moieties, drugs and antibiotics (4).

Materials and Methods

Preparation of Leaf Extract

The plant Banyan (*Ficus benghalensis*) tree leaves were collected from Virudhunagar (5). Fresh leaf extract used for the synthesis of nanoparticle was prepared from 35 g of leaves comprehensively washed and cut into small pieces of leaves in a 500ml Erlenmeyer flask and boiled in 100 ml deionation water for 15min water bath for 80°C. And the extract was filtered with help of Whatman 40 filter paper (6).

Synthesis of Silver Nanoparticles by *Ficus benghalensis* Leaf Extract

The synthesis of silver nanoparticles by using aqueous solution (1mM) of silver nitrate (AgNO_3) was prepared. 4 ml of *Ficus benghalensis* leaf extract was added into 100 ml of aqueous solution of 1 mM silver nitrate for reduction into silver ions. The color change from colorless to yellowish brown color confirmed the synthesis of silver nanoparticles. The formation of green synthesis silver nanoparticles were confirmed by

spectrophotometric determination. An after 2 hours the reduced solution, was centrifuged at 8000 rpm for 30 min. The supernatant was discarded and the pellet was added to deionized water. The centrifugation process was repeated three times to wash off. Thereafter, the purified suspension was freeze dried to obtain dry powder (7).

Concentration of Silver Nitrate

The silver nitrate concentration above mentioned procedure was repeated for optimization, where the reaction was monitored using different silver nitrate (0.5, 1, 2 and 5 mM) concentration.

The resulting solution was measured spectrophotometrically absorbance (8).

Concentration Ratio of Silver Nitrate and Leaf Extract

The silver nitrate and leaf extract concentration above mentioned procedure was also repeated for optimization required for the maximum production of silver nanoparticles, where the different ratio of silver nitrate and leaf extract solution reaction was monitored (20:80, 15:85, 10:90, 5:95, 5:100, 10:100 and 15:100). The absorbance of the resulting solutions was spectrophotometrically measured (9).

Characterization of Silver Nanoparticles

UV–Vis Spectra Analysis

The synthesis of silver nanoparticles analyzed by UV–visible absorption spectrophotometer with a resolution of 1 nm between 300 to 800 nm possessing a scanning speed of 300 nm/ min was used. Similar amounts of the suspension 0.5 ml were diluted in a determined volume of

distilled water 4 ml and analyzed at room temperature. The reaction between silver metal ions and the plant leaf extracts were checked by UV–visible spectra of silver nanoparticles in aqueous solution with different reaction times, concentration ratio and pH. The distilled water was used as blank and nanoparticle solution showed maximum absorbance at 418 nm.

Fourier Transformed Infra-Red Analysis

Fourier transformed infra-red radiation (FTIR) spectroscopy measurements of the solid residue layer which contains silver nanoparticles were second time dispersed in sterile distilled water three times to remove the unattached biological impurities.

The pure residue was then dried perfectly in an oven overnight at 65°C. Thus obtained powder was subjected to FTIR measurements carried out at a resolution of 4cm⁻¹ in Potassium bromide pellets (10).

X-Ray Diffraction Analysis (XRD)

The synthesized silver nanoparticle characterized by XRD spectra. The size of particles was determined and XRD patterns were calculated using x per Rota flex diffraction meter using scherrer equation (11).

Isolation of Methicillin Resistant *Staphylococcus aureus* (MRSA)

The clinical sample was streaked on to selective media. The Methicillin Resistant Screening agar (MeReSA), (HIMEDIA M 1454) plates were prepared. A loopful of culture was streaked onto the selective plates. The plates were incubated at 37°C for 24 hrs. The sample culture was also streaked onto agar slant for further processing (12).

Antimicrobial Activity of Synthesized Silver Nanoparticles

Well diffusion assay

In this study, isolated MRSA were used as test organisms and grown in nutrient broth media for overnight and in Mueller-Hinton agar (MHA) medium.

Well diffusion assay was used to determine the antimicrobial activity of plant leaves synthesized using silver nanoparticles. 30 microlitre of silver nanoparticles were loaded separately into each well of the microtitre plates.

The antimicrobial activity of synthesized nanoparticles and the Plant leaves extract was compared against MRSA.

After inoculation, the plates were incubated at 37 °C for 24 hours and the zone of inhibition was measured in terms of millimeter. These assays were carried out in triplicates.

Determination of the Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentration was determined by a microdilution method, using LB broth (Sigma-Aldrich) and final inocula of 10^6 cfu/ml. Bacteria were incubated with silver nanoparticles, and the effect on cell viability was measured after a 24-h period of incubation. The MIC value corresponded to the doses that inhibited 99% and 90% of bacterial growth, to the silver nanoparticles doses where 100% of the bacterial growth was incubated compared with positive control (No treatment). The diluted sample was streaked on the Mannitol salt agar medium. The plates were incubated for 24 hour. After incubation, the inhibited colonies were observed(13).

Assessment of antibacterial activity of nanoparticles treated bandage material cloth

The antibacterial activity of the bandage material cloth coated with nanoparticles was assessed. Fabric cloth was washed, sterilized, and dried before use. The fabrics were submerged in a final filtrate (100 ml with 0.01 M of silver nanoparticles), shaken at 600 rpm for 24 hours and dried at 70°C. The fabric was cut into 38 mm diameter circles.

Before inoculation of the bacteria, the pieces of fabric were disinfected by ultraviolet irradiation for 1 min the circular pieces were divided into nine groups, and each group consisted of six pieces. One group (Flask 1) was seeded with 0.5 ml Nutrient broth (Hi-media); this group served as a sterility control. Eight groups (Flasks 2-9) were seeded with 0.5 ml fresh *Staphylococcus aureus* culture at a concentration of 10^5 colony-forming units per ml (cfu/ml).

Fifty milliliters saline was added immediately to four groups (Flasks 6-9) of bandage material cloth and vortexed, so as to neutralize the antimicrobial effect of the nanoparticles. All nine groups were incubated at 37°C for 24 hour. After incubation, 50 ml saline was added to each of the remaining four groups (Flasks 2-5) and to the control tube; all tubes were vortexed.

Fifty-microlitre sample was drawn from each of the nine groups, spread on to nutrient agar plate and incubated at 37°C for 48 hours for viable counts. The same procedure was performed on three groups of untreated bandage material cloth, one group served as the sterility control, and the other two groups represented the 0-h and 24-h contact intervals.

Results and Discussion

A simple plant extract reduction method has been developed for synthesizing silver nanoparticles. This methodology could be used for synthesizing a number of metallic nanoparticles involving other metals with good size and shape morphology (14).

The results related to the metallic silver nanoparticles indicates the reduction of silver ions by *Bacillus* sp. and *Streptomyces* sp. Therefore, it can be concluded that the resting cells of *Bacillus* sp. and *Streptomyces* sp. can reduce silver ions in their periplasmic space. Initially, the synthesis of silver nanoparticles was confirmed by observing the colour change of the reaction mixture.

The appearance of a yellowish brown in the reaction vessels after 2 hour of incubation at room temperature suggested the formation of silver nanoparticles. The confirmation of formation and stability of the silver nanoparticles in the colloidal solution was monitored by using UV-Vis spectral analysis, for which after completion of reaction (after 2-3 hrs) aliquots of the reaction sample were removed and subjected to UV-Vis spectroscopy measurements.(fig 1)

On adding the aqueous extract of banyan leaves to silver nitrate solution, the colour of the reaction medium changed rapidly from colorless to yellowish brown. The appearance of brown colour was due to the excitation of surface plasmon vibrations, typical of silver nanoparticles and yellowish colour in silver nanoparticle. In this study, the formation of silver nanoparticles was initially confirmed using SPR phenomenon. For silver nanoparticles, λ max values were reported in the visible range of 410-430nm. The results obtained with UV-Vis spectra of

the colloidal silver nanoparticles solutions synthesized from the plants leaves of *Ficus benghalensis*.

FTIR measurements were carried out to identify the possible biomolecules responsible for the reduction of the silver ions, capping of the bio-reduced silver nanoparticles synthesized by Banyan leaves extract filtrate. Representative spectra of obtained nanoparticles manifests absorption peaks located at about 969.16, 881.41, 800.01, and 883.34 in the region 1,000-400 cm^{-1} . The FTIR spectra reveal the presence of different functional groups like C-N, C-O-C, and amide linkages and -COO- between amino acid residues in protein and synthesized silver nanoparticles(15). We can presume that the flavonoids and terpenoids, which are abundant in Banyan leaves, show characteristic absorption peaks that appear to be responsible for accelerated reduction and capping process, which give rise to the well-known signatures in the infrared region of the electromagnetic spectrum (fig 2).

The XRD pattern shows four peaks at 38.41° , 44.23° , 64.65° , and 77.13° , which are assigned to the (111), (200), (220) and (311) planes of face-centered cubic (FCC) Ag. The absence of silver oxide peaks shows that the prepared nanoparticles are made of pure silver. The mean particle diameter of silver nanoparticle was calculated from the XRD pattern according to the line width of the (1 1 1) plane, reflection peak using the following Scherrer equation:

$$D = \frac{K\lambda}{\beta \cos \theta}$$

The equation uses the reference peak width at angle, where λ is the X-ray wavelength (1.5418 Å), β is the width of the XRD peak at half height and K is a shape factor. The calculated average particle size of the silver was found to be 20 nm, which was

also in line with the observation of the SEM results discussed (Fig 3). The surface morphology of the silver nanoparticles were studied by scanning electron microscopy method. The SEM micrograph showed nanoparticles aggregates. In the micrograph

observed in the size range between 20 - 100 nm. The nanoparticles were not in direct contact even with in the aggregates, which indicates the stabilization of the nanoparticles by a protein capping agent (Fig 4).

Fig 1: UV-vis spectral analysis of synthesized AgNPs from *Ficus benghalensis*

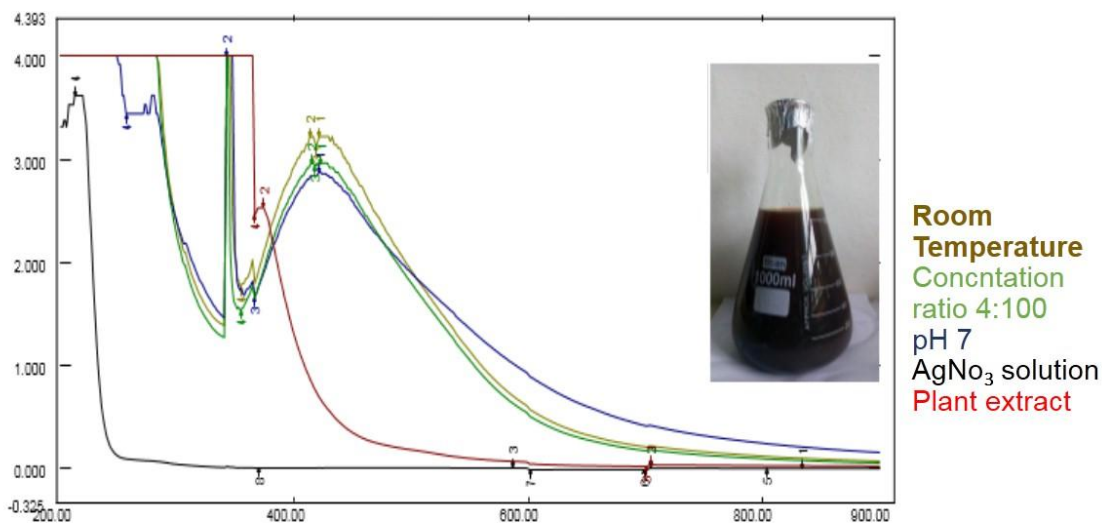


fig 2: FTIR spectra of dried, ultra centrifuge separated silver nanoparticles powder in potassium bromide pellets synthesized by Banyan leaves extract.

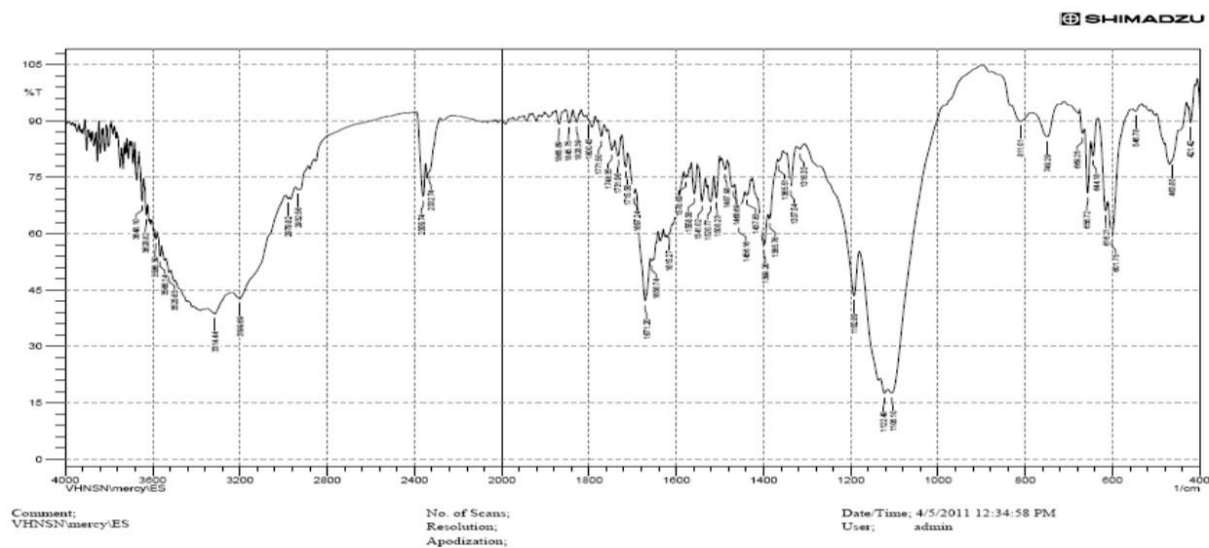


Fig 3: XRD diffractogram result of the nanoparticles synthesised from extracts of the plant

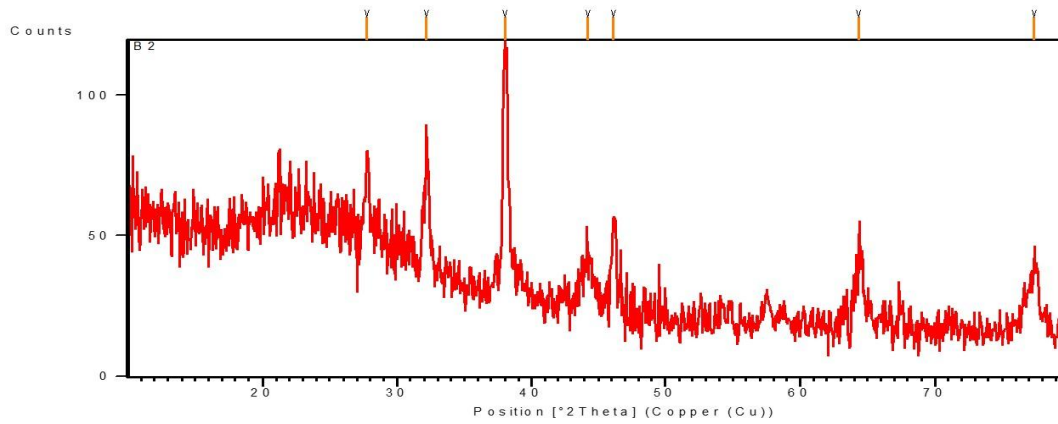


Fig4: SEM Micrograph of silver nanoparticles at 85kx magnification

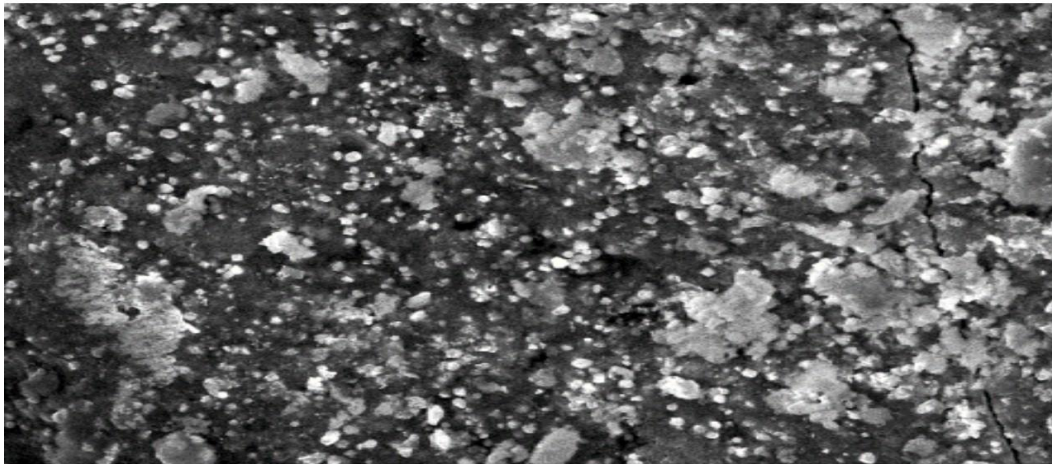
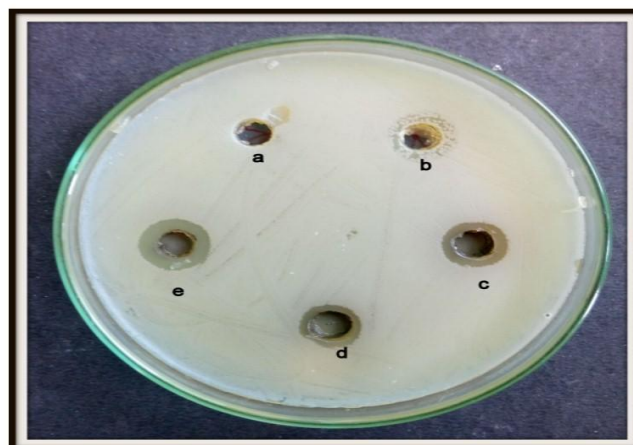


Fig 5: Antimicrobial Activity of Synthesized Silver Nanoparticles Well diffusion assay



a- plant extract, b- silver nitrate, c- 1 μ /ml concentration, d-2 μ /ml concentration and e- 3 μ /ml concentration

Table 1: Antibacterial effect of silver nanoparticles

Sample	MIC						MBC
	8 µg	6 µg	4 µg	2 µg	1 µg	Without nanoparticle	
Silver Nanoparticles	-	-	-	+	+	+	3.5 µg

Table 2: Bactericidal effect of silver nanoparticles against MRSA after 0 hr, 24 hrs and 48 hrs of incubation period.

S. No	Contact time	Silver Nanoparticles	
		Coated	Uncoated
1	0 hours	3.5×10^4	7×10^5
2	24 hours	0	3×10^6
3	48 hours	0	4×10^6

The antimicrobial activity of silver nanoparticle was evaluated against *S. aureus* by well diffusion method. This method displays the zone of bacterial colonies grown in the presence of different amount of AgNO₃ solution, plant extract and silver nanoparticle at different concentrations (µl) of sample was applied to each well. The results clearly indicate that at a given concentration of silver, inhibition of bacterial growth depends on the initial zone of cells. An amount of 3µg of nanoparticles almost completely prevented bacterial growth at silver amounts of 20µg and above, 100% inhibition of bacterial growth was also observed. This is markedly different from the results obtained when the initial number of bacterial cells was 10⁷ CFU (Fig. 5).

The bacterial species used in this study is a resistant strain of *S. aureus*, which is known

to be one of the common causes of nosocomial infections. This microorganism was isolated from the clinical samples collected from the Hospitals and the effects of the combinations on this microorganism were compared with that of a standard strain (16).

The MICs of the nanoparticles against *Staphylococcus aureus* were studied. The *Staphylococcus aureus* isolated were challenged with two fold 100 nm nanosilver serial dilutions for 24h. Silver nanoparticles affected bacterial cellular viability in a dose-dependent manner. The *Staphylococcus aureus* were inhibited at concentrations over 4 µg for 10⁵ CFU/ml inoculum for silver nanoparticles (Table 1). As expected, the antibacterial effect of 100 nm nanosilver was inversely related to the amount of bacteria, since the best

performance was achieved at 10^5 CFU/ml than 10^6 CFU/ml, even though the latter is 1,000 times higher than the standard for susceptibility tests. Performance was defined as the capacity to inhibit bacterial growth under these conditions (17).

The treated and untreated bandage material cloth were challenged with *Staphylococcus aureus*; no bacterial growth was obtained from the sterility control. The viable bacterial counts were recovered from the bandage material cloth before and after incubation. After 48 h of incubation, there was a 90% reduction in viable *Staphylococcus aureus* on the treated bandage material cloth. For the uncoated mask material, there was no reduction in viable counts; on the contrary, there was a 10% and 15% increase in the viable counts of *Staphylococcus aureus*, respectively (Table 2). The percentage reduction in bacterial count was calculated by based on the growth at 0 hrs and 48 hrs comparison.

The findings have shown that bandage material cloth coated with nanoparticles at low concentration are effective against *Staphylococcus aureus*, and that bacteria attached to the surface of nanoparticle-treated bandage material cloth were killed completely. Effective inhibition of microorganisms present on bandage material cloth is crucial for protection against infectious agents. The use of a combination of antimicrobial agents may be of value in preventing the emergence of resistant bacterial strains.

Despite the findings of this study, there are several aspects that require further investigation. Firstly, clinical evidence is required to demonstrate the impact of the bandage material cloth in the reduction of hospital-acquired infections. Secondly, as other microorganisms are involved in

hospital-acquired infections, it is necessary to test the nanoparticles against more microbial species, such as methicillin-resistant *Staphylococcus aureus*. Thirdly, the killing rate of the nanoparticles needs to be assessed to gain an accurate picture of the antimicrobial efficacy. Fourthly, this study showed that the nanoparticle coated bandage material cloth did not cause local skin allergy problems in volunteers. However, only a small number of subjects were involved in this study and more subjects need to participate in the skin allergy test.

In deduction, the bio-reduction of Ag⁺ ions by the *Ficus benghalensis* leaf extract has been established. The reduction of the metal ions through leaf extracts lead to the materialization of silver nanoparticles of equally definite dimensions. In the current study, we found that leaf can be also a respectable source for silver nanoparticles synthesis. This approach toward the silver nanoparticles synthesis has many advantage. Applications of such environmental friendly nanoparticles in bactericidal, wound healing and other medical applications, makes this method potentially for across-the-board synthesis of other inorganic silver nanomaterials.

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