Antibacterial Activity of Nanomaterials Synthesized from Plant Extracts against Methicillin Resistant *Staphylococcus aureus* (MRSA)

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Isolation of *Staphylococcus aureus*

For Bacterial sample collection, blood agar plates were exposed at different places such as surgical ward, Burn and wound section and orthopedic ward in Government run Hospital, Bangalore. After that all plates were incubated at 37°C for 24 hrs. Grown colonies were isolated, purified and identified by Gram's staining and coagulase test.

The bacterial strains that were confirmed as *Staphylococcus aureus* by Gram's staining, Catalase and coagulase test were further analyzed by several microbiological diagnostics tests including mannitol fermentation and growth on high salt concentration, gelatin hydrolysis, urea hydrolysis, protease activity on milk agar medium, lipase production on egg yolk agar medium and hydrolysis of esculin by standard methods (Blair et al., 1967; Chapman, 1945; Cruickshank et al., 1975). Hemolytic activity was determined on sheep blood agar. The confirmed *S. aureus* strains were further tested for methicillin resistance by following antibiotic sensitivity tests.

Oxacillin and Cefoxitin disc diffusion method

All strains were tested with 1 mg oxacillin discs (Hi-Media) and 30 mg cefoxitin discs on Mueller–Hinton agar plates. For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. The zone of inhibition was determined after 24 h incubation at 35°C. Zone size was interpreted according to CLSI (2008).

Oxacillin screen agar test

A bacterial inoculum of each strain was made and turbidity was adjusted to 0.5 McFarland. One drop of this suspension was inoculated on Mueller-Hinton agar containing 4% NaCl and 6 mg oxacillin ml^{-1} (Hi-Media). Plates were incubated at 35°C for 24 h. Any strains showing growth on the plate containing oxacillin were considered to be resistant to methicillin.

CHROMagar

CHROMagar (Hi-Media) is a new chromogenic medium for the identification of MRSA. For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. Subsequently, a swab was dipped in the suspension and streaked onto a CHROMagar plate. The growth of any green colony was considered to be positive, indicating MRSA.

Synthesis of silver nano particles from *Sesbania grandiflora* leaf extracts

Silver nanoparticles were prepared by mixing 10ml of the aqueous extract of *Sesbania grandiflora* with 90 ml of $AgNO_3$ (1 mM) solution. This setup was incubated in dark room at 37°C for 24hrs to avoid the photo activation of silver nitrate. A control setup was also maintained without leaf extract. The colloidal suspension thus obtained was centrifuged at 4000rpm for 30 min and the pellet after discarding the supernatant was re-dispersed in deionized water. The centrifugation process was repeated 2 to 3 times for the removal of any absorbed substances on the surface of silver nanoparticles (AgNPs). The synthesized nanoparticles were lyophilized and recovered in powdered form using a freeze dryer.

Synthesis of silver nano particles from *Solanum xanthocarpum*

Methanol extracts of *Solanum xanthocarpum* berries were centrifuged at 4000rpm for 30 min and the pellet after discarding the supernatant was re-dispersed in deionized water. The centrifugation process was repeated 2 to 3 times for the removal of any absorbed substances on the surface of silver nanoparticles (AgNPs). The synthesized nanoparticles were lyophilized and recovered in powdered form using a freeze dryer.

Characterization of silver nano particles using SEM analysis

Scanning electron microscopy (SEM) analysis was done using Hitachi s-4500 sem machine. thin films of the sample were prepared on a carbon coated grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film sem grid we allowed to dry by putting it under a mercury lamp for 5 min.

Minimal Inhibitory Concentration (MIC)

MRSA strains were grown overnight on MHA plates at 37°C before being used. The antimicrobial activity of Ag-NPs was examined using the standard broth dilution method (CLSI M07-A8). The MIC was determined in Luria Bertani (LB) broth Hi-Media (Mumbai,

India) using serial two-fold dilutions of Ag-NPs in concentrations ranging from 25 to 1.56 µg/mL, initial bacterial inoculums of 2×10^8 CFU/ml and the time and temperature of incubation being 24 h at 37°C, respectively.

Minimal Bactericidal Concentration (MBC)

After MIC determination of the Ag-NPs tested, aliquots of 50 µl from all tubes in which no visible bacterial growth was observed were seeded in MHA plates not supplemented with Ag-NPs and were incubated for 24 h at 37°C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population.

Bacterial Growth Curve

To examine the bacterial growth curve in liquid broth, inoculations were given from fresh colonies on MHA plates into 100 ml of LB culture medium. Growth was allowed until the optical density reached 0.1 at 600 nm. Subsequently, 2×10^8 CFU/ml from above were added to 100 ml of liquid LB media supplemented with 1.56, 3.125, 6.25, 12.5 and 25 µg/ml of Ag-NPs. All the flasks were put on rotatory shaker (150 rpm) and incubated at 37°C. Control broths were used without nano particles. The bacterial growth was determined by measuring optical density after every 2 hour (up to 20 h) at 600nm using spectrophotometer

Cytotoxicity Test

Cytotoxicity evaluation was assessed by monitoring the neutral red uptake (NRU) assay using tumoral HeLa (cervix) (100 µL; $1x10^5$ cells/ml) seeded into 96 well micro litre plates and left to adhere for 24 h. The next day, the medium was removed from the wells and the cells were exposed to 1.56 µg/mL, 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL and 25 µg/mL of nanosilver dispersed in complete medium with 5% FBS (100 µL/well). After 24 hours exposition, the medium was replaced with complete media 5% FBS containing neutral red dye (1 mg/mL) Plates were incubated for a further 3 h. Then the medium was removed and after dye extraction using ethanol/ acetic acid/water (50%/1%/49%) the absorbance was measured at 540 nm in spectrophotometer.

Results Achieved

In the present study, 106 stains were isolated collected from hospital environments. Among 106 samples, 55 stains (51.8%) were isolated from a selective mannitol salt agar media and then these isolates were identified for *Staphylococcus aureus* by different biochemical tests. Gram staining, catalase and coagulase were important phenotypic identifying markers of *Staphylococcus aureus*. In this study we found that 100% and 92% isolates were positive for catalase and coagulase activity. The enzyme gelatinase was secreted by *S. aureus* liquefy gelatin protein. Present study also showed that 61% of *Staphylococcus aureus* isolates were able to produce gelatinase. In general, 54.5%, 49% of isolated strains produced protease and lipase.

The study showed that 54.5% of *Staphylococcus aureus* isolates were able to produce clearing zone surrounding their growth on blood agar media demonstrating that they can produce hemolysin. A total of 55 isolates of *Staphylococcus aureus* were tested for the methicillin resistant *Staphylococcus aureus* (MRSA). Out of the 55 stains, 12 were MRSA and the prevalence of MRSA was 21.8%. The disc diffusion methods revealed that 96.2% sensitivity for cefoxitin disc and 90.2% sensitivity to oxacillin disc. This higher sensitivity to cefoxitin can be explained by the increased expression of the mecA-encoded protein PBP2a, cefoxitin being an inducer of the mecA gene. This study suggests the point that cefoxitin is superior to oxacillin as an indicator of MRSA for the detection of methicillin resistance. The oxacillin screen agar test showed 94.3% sensitivity and 100 % specificity for MRSA detection in the study.

Bactericidal activity of Ag-NPs

The aim of present study was to evaluate the antibacterial effects of Ag-NPs against the MRSA strains isolated from hospital environments. The MIC and MBC values of *S. grandiflora* Ag-NPs against MRSA strains were observed very low (i.e. in the range of 1-64 µg/ml), indicating very well bacteriostatic (represented by the MIC) and bactericidal activity (represented by MBC) of the antibacterial agents. However, *S. xanthocarpum* Ag-NPs exhibited poor effect in the case of MIC and MBC values.

Effects of Ag-NPs on bacterial growth

Bacterial cell growth enhances the turbidity of the liquid medium and as a result, the absorption increases. It is clear that at all these concentrations, the nanoparticles caused a growth delay of the bacterial cells; slope of the bacterial growth curve continuously decreased with increasing nanoparticles concentration. Nanoparticles with highest concentration showed almost no growth for upto 16 hrs representing a bactericidal effect at this concentration. The growth curve of standard strain of MRSA was plotted in the presence of 1.56, 3.125, 6.25, 12.5 and 25 µg/ml concentration of Ag-NPs. The results clearly indicate that as the concentration of Ag-NPs increases, reduction in bacterial growth was observed and this was even continued for 16 hrs.

The results indicate no cytotoxicity potential at the 70 nm AgNP on HeLa cells using the AgNP concentration of 1.56 µg/mL. When cells were exposed to 6.25 µg/mL and above, the cells showed little sensibility. These results contributed with the information that these bactericidal AgNP concentrations were non-cytotoxicity in normal cells by any concentration of AgNP used in this study.