

Antibacterial Activity of Nanomaterials Synthesized From Plant Extracts Against Methicillin Resistant *Staphylococcus aureus* (MRSA)



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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) are resistant to all β -lactam antibiotics and patients suffering from MRSA infections may lead to higher mortality rate and prolonged antibiotic therapy. Use of metallic nanoparticles treating infections caused by antibiotic resistant strains is a new paradigm. *Sesbania grandiflora* and *Solanum xanthocarpum* were used for the synthesis of silver nanoparticles (Ag-NPs). A total of 55 strains were isolated from hospital environments in which 12 were identified as MRSA. Antibacterial activity of the green synthesized Ag-NPs against MRSA was assessed by determining the minimal inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and by measuring the dynamic growth curve of the bacteria. Further, toxicity assay of synthesized Ag-NPs on HeLa cell lines were performed. The MIC and MBC values of *S. grandiflora* Ag-NPs against MRSA strains were observed very low (i.e. in the range of 1-25 $\mu\text{g/ml}$), indicating the potential antibacterial activity of Ag-NPs. Based on preliminary evaluation of cytotoxicity of green synthesized silver nano particles on HeLa cell lines, using neutral red uptake assay, healthy human cells were not adversely affected. This approach appears to be non-toxic, ecofriendly initiative over the conventional methods for treatment of MRSA infections.

Keywords: Silver nanoparticles; MRSA; *Sesbania grandiflora*; *Solanum xanthocarpum*

Abbreviations: Methicillin Resistant *Staphylococcus Aureus* (MRSA); Minimal Inhibitory Concentration (MIC); Minimum Bactericidal Concentration (MBC); Mueller Hinton Agar (MHA); Scanning Electron Microscopy (SEM)

Introduction

Staphylococcus aureus cause complications ranging from minor to life-threatening infections in wide range of host species. Methicillin resistant *Staphylococcus aureus* (MRSA) is resistant to methicillin, oxacillin, amoxicillin and penicillin [1]. MRSA are resistant to all β -lactam antibiotics and some strains additionally evolved resistance to multiple antibiotics. Methicillin resistant Staphylococcal infections lead to a higher mortality rate and prolonged antibiotic therapy as compared with methicillin sensitive Staphylococcal infection [2,3]. It is important to develop alternative and more effective therapeutic strategies to treat multidrug resistant pathogens. Nanoparticles are being used for combating microbial resistance and have advantages over traditional antibiotics [4]. The antimicrobial activity mechanism by antibiotics is quite different from the mechanisms by which nanoparticles inhibit microbial growth. Therefore, nanoparticles have the potential to serve as an alternative to antibiotics [5].

Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-100 nm [6]. Nanomaterials for the delivery of antimicrobials represent a new paradigm for treating antibiotic resistant strains [7]. Silver is more toxic element to microorganisms than many other metals and low toxicity to mammalian cells [8]. Microbial resistance to silver is lower than other antimicrobial agents [9,10]. Silver nanoparticles (Ag-NPs) have been demonstrated as effective antibacterial agents [11-18]. The size, shape, surface finish and surface charge of Ag-NPs affect the rate, location and time of release of ionic silver [19]. The binding of Ag⁺ ions with respiratory enzymes, DNA bases and internalization with thiol groups of cellular proteins results in antibacterial activity [18,20].

Eco-friendly initiatives are used to synthesis metal nanoparticles in which biological agents have replaced toxic chemicals to reduce metal ions to metal nanoparticles. Among the biological agents, plant extracts are widely used as reducing

agents for green synthesis of metal nanoparticles. The objective of this study is to determine the effects of green synthesized Ag-NPs using *Sesbania grandiflora* and *Solanum xanthocarpum* extracts against MRSA isolated from hospital environments. The antibacterial activity of the Ag-NPs against MRSA was assessed by determining the minimal inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), and by measuring the dynamic growth curve of the bacteria. Further, toxicity assay of synthesized Ag-NPs on HeLa cell lines were performed.

Materials and Methods

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 at Bengaluru. After exposure, the 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 [21-23]. Hemolytic activity was determined on sheep blood agar according to Rodgers et al. [24]. 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 µg oxacillin discs (Hi-Media) and 30 µg 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 for interpretation [25].

Oxacillin screen agar test

Bacterial suspension of each isolated strain adjusted to 0.5 McFarland was inoculated on Mueller-Hinton agar containing 4% NaCl and 6 µg oxacillin ml⁻¹. The plates were incubated at 35°C for 24 h and the presence of growth indicated that the strains were methicillin resistant [26].

CHROMagar

A bacterial suspension adjusted to 0.5 McFarland was swabbed onto a CHROMagar plate. The presence of green coloured colony after the incubation period was considered as MRSA positive [27].

Collection of plant samples

Leaves of *Sesbania grandiflora* and berries of *Solanum xanthocarpum* were collected from authorized local vendors of Bengaluru and identified by Taxonomist from Government Ayurveda Hospital, Bengaluru.

Preparation of leaf extracts of *Sesbania grandiflora*

Leaves of *Sesbania grandiflora* were used to make the aqueous extract. The leaves were surface cleaned with running tap water, followed by double sterilized distilled water and then shade dried for 5 days to completely remove the moisture. A fine powder was obtained from the dried leaf using a blender. The leaf powder (10g) was taken and mixed with 10ml of milli Q water and kept in a water bath at 60°C for 10min. The extracts were filtered through a nylon mesh followed by Whatman filter paper. The filtered extract was stored at 4°C for further studies.

Preparation of berry extracts of *Solanum xanthocarpum*

In the case of *Solanum xanthocarpum*, 200 g plant material was washed and air-dried in the shade for 15 days. The dried berry was ground to a fine powder by using a blender. A measured quantity of 20 g of dried *S. xanthocarpum* berry powder was soaked in 200 ml methanol in round bottom flask at room temperature for 24 hours. The extracts were filtered with Whatman No.1 filter paper. The filtrate was allowed to dry at room temperature until dry methanol extract was obtained. The extract was stored in airtight containers at 4°C for further use.

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

Silver nanoparticles were prepared by mixing 10 ml of the aqueous extract of *Sesbania grandiflora* with 90 ml of AgNO₃ (1 mM) solution. This setup was incubated in dark room at 37°C for 24 hrs 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 4000 rpm 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 (Ag-NPs). The synthesized nanoparticles were lyophilized and recovered in powdered form using a freeze dryer.

Synthesis of silver nanoparticles using *Solanum xanthocarpum*

Aqueous extracts of *Solanum xanthocarpum* berries were centrifuged at 4000 rpm for 30 min and the pellet 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 (Ag-NPs). 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.

Antibacterial activity of green synthesized Ag-NPs

Antibacterial activity of Ag-NPs was performed using a Mueller-Hinton agar (MHA) culture medium. The samples (10^8 CFU/mL) were inoculated in agar medium and then paper discs of 5 mm in diameter were laid on the inoculated test organism, which was instilled with Ag-NPs at different concentrations (1.56, 3.125, 6.25, 12.5, and 25 μ g/mL). The plates were incubated at 37°C over 24 hours and antimicrobial activity was determined by measuring the zone inhibition around the disc.

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. The MIC was determined in Luria Bertani (LB) broth using serial two-fold dilutions of Ag-NPs in concentrations ranging from 25 to 1.56 μ g/mL. Initial bacterial inoculums of 2×10^8 CFU/ml was used and the time and temperature of incubation were 24 h at 37°C, respectively. The MIC is the lowest concentration of antimicrobial agents that completely visually inhibits 99% growth of the microorganisms. The MIC measurement was done in triplicate to confirm the value of MIC for each tested bacterial strain.

Minimal Bactericidal Concentration (MBC)

After MIC determination of the Ag-NPs tested, aliquots of 50 μ l 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 (OD of 0.1 corresponds to 10^8 CFU/ml of medium). 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 μ g/ml of Ag-NPs. All the flasks were put on rotatory shaker (150 rpm) and incubated at 37°C. Control broths were used without nanoparticles. The bacterial growth was determined by measuring optical density after every 2 hours (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) (100μ L; 1×10^5 cells/ml) seeded into 96 well micro liter plates and left

to adhere for 24 h. The medium was removed from the wells and the cells were exposed to 1.56, 3.12, 6.25, 12.5 and 25 μ g/mL of Ag-NPs dispersed in complete medium with 5% FBS (100 μ l/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. Absorbance measurements of cells exposed only to medium were considered as 100% cell viability. Inhibition of growth of cells was calculated from the relative absorbance of untreated control cells at 540 nm.

Statistical Analysis

All the tests were performed in triplicate, and the results were expressed as the mean \pm standard error. Students 't' test was used to compare these results. P values lower than 0.05 were considered significant.

Results and Discussion

Staphylococcus aureus causes bacteremia, pneumonia, osteomyelitis, endocarditis, sepsis, and toxic shock syndrome [28]. It can cause skin infections and associated with nosocomial infections [29]. Methicillin resistant *S. aureus* (MRSA) are resistant to all β -lactam antibiotics due to mutation and horizontal gene transfer. MRSA has led to serious concern in human medicine [30] and patients suffering from MRSA infections may need antibiotics that are more potent and less toxic.

Table 1: Hemolysis pattern by the isolates in Blood agar.

Hemolysis pattern	No. of isolates
Alpha hemolysis	30.1%
Beta hemolysis	14.4%
Gamma hemolysis	10%

In the present study, 106 stains were isolated from hospital environments. Among the 106 strains, 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's 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 respectively. 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. As shown in Figure 1, 54.5% and 49% of isolated strains produced protease and lipase respectively (Figure 1). 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. Hemolysis patterns by the isolates are represented in (Table 1).

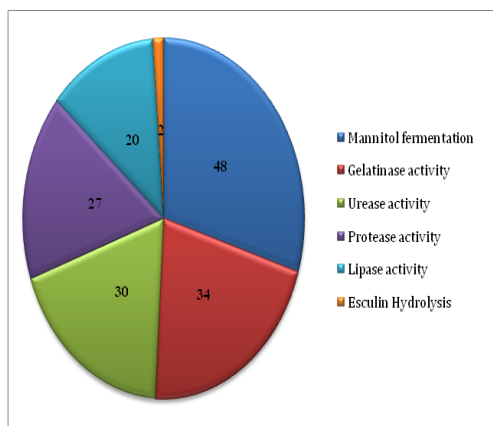


Figure 1: Biochemical characteristics of *Staphylococcus aureus* isolates.

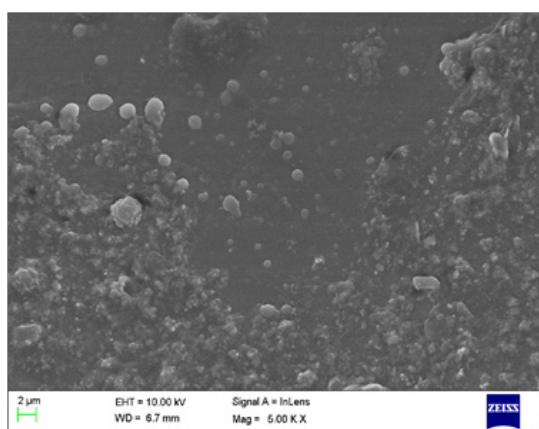


Figure 2: SEM image of *Sesbania grandiflora* synthesised Ag-NPs. Scale bar represents 2 µm.

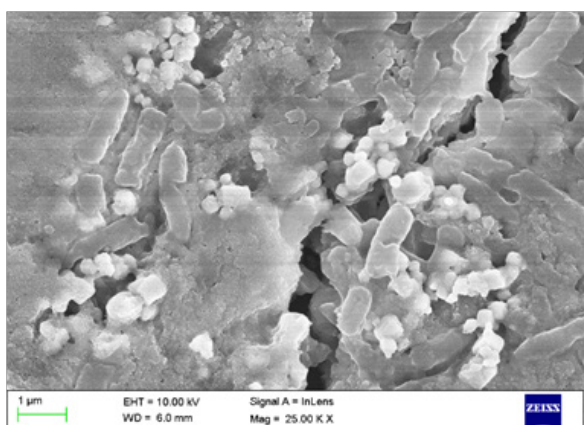


Figure 3: SEM image of *Solanum xanthocarpum* synthesised Ag-NPs. Scale bar represents 1 µm.

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. The oxacillin screen agar test showed 94.3% sensitivity and 100% specificity for MRSA detection in the study. The average diameters of the Ag-NPs synthesized from *S. grandiflora* and *S.*

xanthocarpum were mostly spherical and measured 45 nm and 80 nm respectively from SEM images (Figures 2 & 3).

Bactericidal activity of Ag-NPs

Silver nanoparticles are synthesized by chemical synthesis, radiation-assisted synthesis and electrochemical sonication [31] but these methods are laborious, expensive and have environmental defect [32]. Biological methods using plants for nanoparticle synthesis are more advantageous than other methods [33]. The use of plant extracts for the synthesis of Ag-NPs reduces the use of hazardous chemicals and contributes to the ecofriendly initiatives. Mechanisms involved in antibacterial properties of silver nanoparticles are attachment to cell surface and disturb important cell functions; interaction with DNA, proteins and other cell constituents; release of silver ions for biocidal effect [34-38].

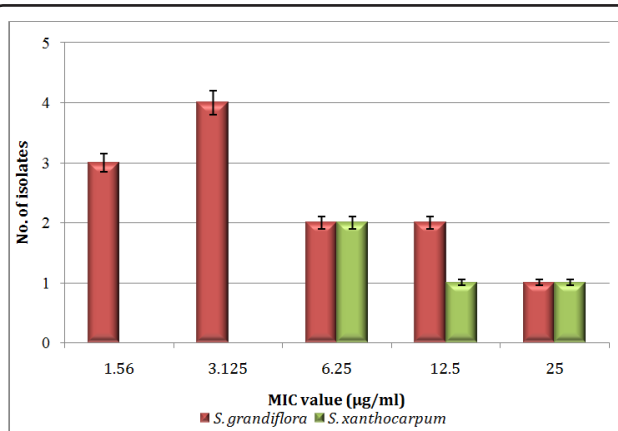


Figure 4: Clinical isolates of MRSA showing MIC treated with serial two fold dilutions of green synthesised Ag-NPs.

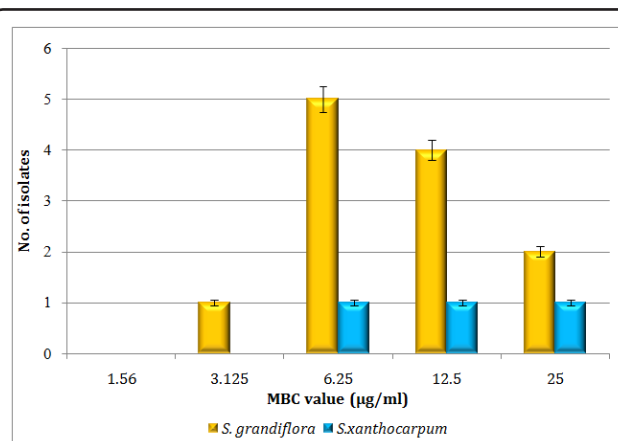


Figure 5: Clinical isolates of MRSA showing MBC treated with serial two fold dilutions of green synthesised Ag-NPs

The antimicrobial properties of silver nanoparticles were analyzed by means of MIC and MBC. MIC is defined as the lowest concentration of the antimicrobial agent that prevents visible growth of a microorganism under defined conditions. MBC is defined as the lowest concentration of antimicrobial agent that will prevent the growth of microorganism after subculture onto nanoparticles free media. The aim of this study was to evaluate

the antibacterial effects of green synthesized 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 1-25 $\mu\text{g/ml}$), indicating very well bacteriostatic (MIC) and bactericidal activity (MBC) of the nanoparticles (Figures 4 & 5). The MBCs of silver nanoparticles were found to be 6.25 $\mu\text{g/ml}$. However, *S. xanthocarpum* Ag-NPs exhibited poor effect in the case of MIC and MBC values hence *S. grandiflora* Ag-NPs were used for assays of dynamic growth curve and NRU (Figures 4 & 5).

Effects of Ag-NPs on bacterial growth

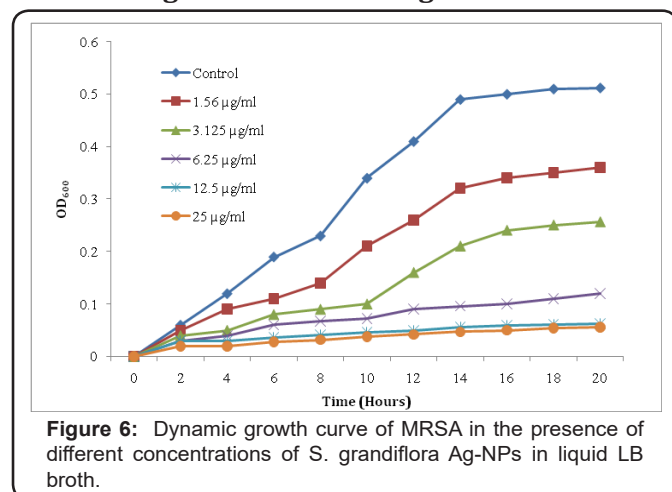


Figure 6: Dynamic growth curve of MRSA in the presence of different concentrations of *S. grandiflora* Ag-NPs in liquid LB broth.

The dynamics of bacterial growth curve was monitored in liquid LB broth. Time-dependent changes in the bacterial growth were monitored at a regular interval of 2 h (up to 20 h) by measuring the OD (at 600 nm) of the control and bacterial solutions supplemented with different concentration of *S. grandiflora* Ag-NPs are shown in (Figure 6). The growth curve of standard strain of MRSA was plotted in the presence of 1.56, 3.125, 6.25, 12.5 and 25 $\mu\text{g/ml}$ concentration of Ag-NPs. The results clearly indicate that as the concentration of Ag-NPs increases, reduction in bacterial growth was observed. Nanoparticles with highest concentration showed poor growth for up to 16 hrs representing a bactericidal effect at this concentration (Figure 6).

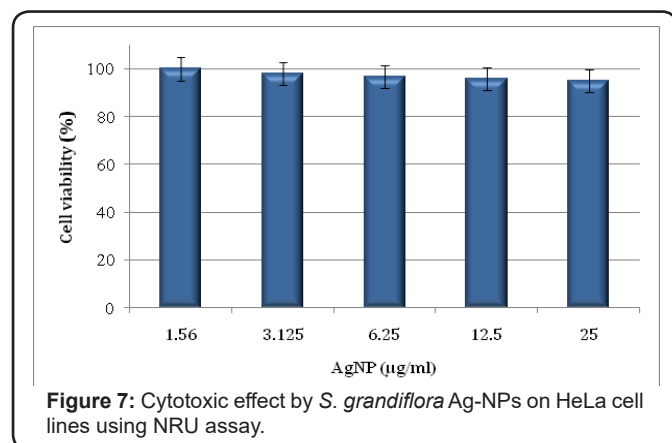


Figure 7: Cytotoxic effect by *S. grandiflora* Ag-NPs on HeLa cell lines using NRU assay.

Nanoparticles were investigated for their cytotoxicity aspect and silver nanoparticles showed different degrees of in vitro cytotoxicity in cell lines [39,40]. The neutral red uptake (NRU) assay provides a quantitative estimation of the number of viable cells in a culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. NRU assay results indicate no cytotoxicity potential of the Ag-NPs on HeLa cells at a concentration of 1.56 $\mu\text{g/ml}$. When the cells were exposed to 3.125 $\mu\text{g/ml}$ and above, the cells showed little sensitivity with a maximum of 5% cell toxicity at 25 $\mu\text{g/ml}$ concentration. These results contributed with the information that these bactericidal AgNP concentrations were non-Cytotoxic in HeLa cells (Figure 7).

Conclusion

Sesbania grandiflora and *Solanaum xanthocarpum* were used for the synthesis of silver nanoparticles. The green synthesized Ag-NPs were characterized by SEM. Antimicrobial assay revealed that significant antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) isolated from hospital environments. Based on preliminary evaluation of cytotoxicity of green synthesized silver nano particles on HeLa cell lines, healthy human cells were not adversely affected. This approach appears to be non-toxic, ecofriendly initiative over the conventional methods for treatment of MRSA infections.

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