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Synergistic antibacterial and antibiofilm activity of garlic mediated silver nanoparticles against methicillin-resistant *Staphylococcus aureus*

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Abstract
Methicillin-resistant *Staphylococcus aureus* (MRSA) is increasingly recognized in infections among persons in the community without established risk factors for MRSA. Besides the resistance to antibiotics, their capability to form biofilm also increased the pathogenicity of MRSA. Garlic exhibits broad pharmaceutical properties and inhibitory activities against MRSA. This work investigated the synergistic effect of garlic mediated silver nanoparticles against MRSA. The synthesized garlic mediated silver nanoparticles are characterised by UV-Visible spectrophotometer and SEM analysis. It shows a peak at 420nm in UV-Visible spectrum and on SEM analysis it is estimated that the synthesised nanoparticles are spherical in morphology, regular in shape with 100 nm particles. Both antibacterial and antibiofilm activity of AgNPs were determined by microtitre plate method. The garlic extract by itself shows antibacterial activity of approximately 22.8%, 30.6%, 40.9% and 50.8% for the application of 25µl, 50µl, 75µl and 100µl respectively. Whereas the antibacterial activity of garlic mediated silver nanoparticles for the identical concentration were 50.5%, 62.7%, 73.6% and 74.7% in approximate respectively. Likewise, antibiofilm activity of garlic extract was obtained as 19.5%, 29.5%, 31.7% and 40.1% correspondingly for the concentrations of 25µl, 50µl, 75µl and 100µl. While, 34.9%, 39.4% 49.4% and 66.9% of antibiofilm activity was shown by garlic mediated silver nanoparticles in that order of concentration. Antibacterial and antibiofilm activity of garlic mediated silver nanoparticles was owing to the synergistic properties of garlic and silver in nanosize. Thus the results pay way for new medical innovation.

Keywords: Antibacterial activity, antibiofilm activity, methicillin-resistant *Staphylococcus aureus*, silver nanoparticles

1. Introduction

Staphylococcus aureus is one among the leading causative pathogen of health-care-associated infections worldwide. About 50 to 70% of nosocomial *S. aureus* diseases emerge from methicillin-resistant *Staphylococcus aureus* (MRSA). It has been associated with various hard to treat infections, implying the antibiotic-resistant characteristics of these microorganisms. Therefore, MRSA infected persons might be at increased risk for delayed treatment, and morbidity [1]. Though most MRSA infections aren't serious, some can be life-threatening. Currently, there is a significant need to find alternative antimicrobials, especially those that might be for the treatment of MRSA infections [2] and also requires an antibiofilm agent against MRSA [3].

Nanotechnology has dragged considerable attention during the present years. The influence of the nanostructured elements can produce enhancement to the quality of life [4], moreover assuring further advancement in biomedical applications [5]. Silver is extensively studied and used antimicrobial agents from time immemorial [6]. Using garlic clove extract as a reducing and stabilizing agent AgNPs can be synthesized [7]. Garlic enhances immune functions and has antibacterial, antifungal and antivirus activities [8]. Thus this combination may direct to the expansion of an effective drug against MRSA infection. The fabrication of garlic-infused AgNPs may check MRSA infections. This synergestic effect can pave way for new techniques with minimal side effects and maximum antibacterial and antibiofilm activity, giving new hope to the medical world for better prevention and treatment methods. The present study aimed to analyze the synergistic antibacterial and antibiofilm activity of garlic mediated silver nanoparticles against methicillin resistant *Staphylococcus aureus*.

2. Materials and Methods

2.1 Garlic mediated silver nanoparticles synthesis

Commercially available fresh garlic was collected from a local market. 6 gm of freshly peeled garlic were crushed using mortar and pestle. To the well-crushed paste, 10 ml of purified water is supplemented and combined well. The mixture was transferred to Eppendorf tubes are set aside in a water bath at 55 °C for 30 minutes. After 10 min centrifugation of the mixture at 8000 rpm the supernatant collected was transferred to a disinfected beaker. The supernatant of the garlic extract was supplemented to 0.98mM $AgNO_3$ solution. The mixture was kept at 27 °C for 24 hrs to observing the possible colour change from pale white to brown.

2.2 Characterisation of silver nanoparticles

UV-VIS spectrophotometer: The Silver nanoparticles were characterized in a UV-VIS spectrophotometer to know the kinetic behaviour of nanoparticles at a scanning range of 200-800 nm. The spectrophotometer was equipped with software to record and analyze data. UV-visible spectrum of the prepared nanoparticle is analyzed. Scanning Electron Microscope (SEM) analysis: The SEM analysis of nanoparticles was done in JOEL JSM-6480 LV SEM machine at STIC under Cochin University of Science And Technology (CUSAT), Kerala, India.

2.3 Bacterial sample collection and Sub culture maintenance
Broth culture of methicillin-resistant *S. aureus* obtained from the stock culture maintained in Unibiosys Biotech Research Labs, Kalamassery, Kerala, India**.** A quadrate streak plate of the bacterial was prepared on Mannitol Salt agar plates. Plates were kept inverted and nurtured overnight at 37 ^{0}C.Grams staining: The bacterial smear was prepared on a sterile glass slide followed by heat fixing. The heat-fixed smear was then overwhelmed by crystal violet staining reagent for a minute. After washing off the stained crystal Violet, Grams iodine solution was infested for a minute followed by water and 95% ethanol wash. Subsequently, the bacterial smear was again washed with water and dipped in Safranin for 45 seconds. The smear was washed, air-dried, and observed under a microscope (10X/100X).Screening the antibiotic resistance of *Staphylococcus aureus*: MHA plates were prepared for screening the antibiotic resistance of *S. aureus*. A swab culture of the microorganism was prepared using sterile cotton buds followed by preparation of five wells, using sterile micropipette tips, which was approximately equidistance from each one. To each wells five different antibiotics such as Amoxicillin, Ampicillin, Erythromycin, Tetracycline and Vancomycin at a volume of 5 µl were added. The plates were incubated overnight for observing the antibiotic activity.

2.4 Molecular detection of the of β-lactamase gene in *S. aureus.*
PCR reactions for β-lactamase gene were carried out in Biorad Personal Thermal Cycler, USA employing the primers: forward primer 5' AGTCAGTATTTGGTAATTTACTA 3' and reverse primer 5' CATTGCGGGCGGGAACATTGCG3'. PCR amplifications were performed in 25µl reactions containing 1X assay buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) with 1.5mM MgCl2 (Genei, Bangalore, India), 5p moles of all primer, 200µM dNTPs (Genei, Bangalore, India), 20ng of template DNA and 1.5U Taq DNA polymerase (Genei, Bangalore, India). The PCR conditions normalized with primary denaturation temperature of 95 °C for five minutes; 30 cycles of denaturation (94 °C) for 30 seconds, annealing (55 °C) for 30 seconds, extension (72 °C) for 45 seconds followed by a final extension at 72 °C for ten minutes. The annealing temperature of the primers was also standardized by gradient PCR. All the primers manufactured by Sigma aldrich, Bangalore. To check the amplification, 4µl of each PCR product were loaded on 1.5% agarose gels and 1X TBE as running buffer. Commercially available 100 base-pair ladders (Banglore Genei) was served as a conventional molecular weight DNA marker to ascertain the load of the amplified output. The augmented products were sent for purifying and DNA sequenced at Scigenom Labs Pvt Ltd, Cochin. The edited sequences were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) programme in the NCBI GenBank (www.ncbi.nlm.nih.gov) DNA database for confirming the presence of β-lactamase gene in *S. aureus* sample.

2.5 Antimicrobial activity and antibiofilm activity
Bacterial culture preparation: Practicing aseptic conditions, a separate colony was shifted into 10 ml nutrient broth and placed in an incubator overnight at 37 °C. After 18 hrs of incubation, with the aid of a centrifuge and an aseptic condition, a clean sample of bacteria was prepared. The broth was spun down using a centrifuge set at 5000 rpm for 5 minutes with appropriate aseptic precautions. Antibacterial activity determination on microtitre plates: Microtitre plates were prepared under aseptic conditions. A sterile 96 well plate was labelled. This was followed by pipetting out 25 µL, 50 µl, 75 µl, and 100 µl of garlic mediated silver nanoparticles into the first row of the plate. Similarly, garlic extract of volume 25 µL, 50 µl, 75 µl and 100 µl was pipette into the second row of the microtitre plate and 100 µl of the bacterial suspension was added to each well. Each well is made up to 200 µl by adding the required quantity of nutrient broth. The plate was wrapped loosely with cling film to ensure prevention of dehydration of the bacteria. Each plate had a set of controls: a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 µl of nutrient broth instead. The plates were incubated at 37 °C for 24 hours and OD reading was taken (OD 600) after sufficient incubation. Antibiofilm activity determination on microtitre plate: 200 µl of 1% crystal violet stain was added to each well after removing the culture from the microtitre plate. Following 1minute of incubation the crystal violet stain is removed from the wells and gently washed with water. OD reading was taken (OD 600).

3. Result & Discussion
3.1 Characterisation of silver nanoparticles
UV visible spectrum of silver nanoparticles: In the UV spectra of prepared silver nanoparticles, a peak at 420 nm (fig.1) was observed which indicates the production of the silver nanoparticle and there is only a single peak at the spectrum which intimates that the prepared silver nanoparticle is pure. Silver nanoparticles morphology was studied by using Scanning electron microscopy (fig.2). It is observed that silver nanoparticles having spherical morphology and regular in shape with 100 nm particles.

3.2 Sub culturing of MRSA
S. aureus was sub-cultured from the provided stock culture on Mannitol salt agar (fig.3). Bacterial culture appeared as a yellow coloured colony.
Gram staining: The colony appeared as spherical in purple colour, indicating the purity of the *S. aureus.*
Antibiotic profiling
Antibiotic resistance of the *Staphylococcus aureus* is screened on MHA plate using Amoxicillin, Ampicillin, Erythromycin, Tetracycline and Vancomycin by well diffusion method. On incubation of the bacterial culture with these antibiotics no zone were observed (fig.4). This indicates the resistance of the strain to these antibiotics.

3.3 Molecular detection of the of β-lactamase gene in *S. aureus*
The bacterial DNA was isolated to detect the strain of the microorganism. The isolated DNA is then visualized by Agarose Gel Electrophoresis (0.8% Agarose). The separation of the DNA sample is succeeded by, the amplification of the β-lactamase gene from the isolate using Polymerase Chain Reaction (Figure 5). The antibiotic resistance of the *S. aureus* is ensured, by the incidence of β-lactamase gene. With NCBI-BLAST program the β-lactamase gene presence is confirmed in the *S. aureus.*

3.4 Antibacterial and antibiofilm activity
The antibacterial (fig.6) and antibiofilm (fig.7) susceptibility assay show promising proof for the antibacterial and antibiofilm

effect of garlic extract and garlic mediated silver nanoparticles against methicillin-resistant *Staphylococcus aureus*. The garlic extract by itself shows the antibacterial activity of approximately 22.8%, 30.6%, 40.9% and 50.8% for 25µl, 50µl, 75µl and 100µl respectively. Whereas the antibacterial activity of garlic mediated silver nanoparticles for the similar concentration were 50.5%, 62.7%, 73.6% and 74.7% in approximate respectively. Likewise, antibiofilm activity of garlic extract was obtained as 19.5%, 29.5%, 31.7% and 40.1% correspondingly for the concentrations of 25µl, 50µl, 75µl and 100µl. While, garlic mediated silver nanoparticles showed 34.9%, 39.4% 49.4% and 66.9% antibiofilm activity with increasing concentration. Antibacterial and antibiofilm activity of garlic mediated silver nanoparticles was owing to the synergistic effect of both garlic and silver and it is having an effect elevated than pure garlic extract. The present study indicated that Allicin and AgNPs when applied in a blend, exhibit synergistic activity. Thus it provides a new scope for preventing, newly emerging MRSA infections. The fabrication of AgNPs through green chemistry is an emerging field of medical nanotechnology. Wipes, tissues, or related cleaning agents can be infused with garlic infused AgNPs, which will have the antibacterial and antibiofilm activity at an enhanced level. These products find application in MRSA related infections transferred through contact as in the athletes. Thus it provides a primary protection to people who are having a chance of MRSA infections. The result of the present study thus can be considered as novel therapeutic agents for the prevention and treatment of MRSA infection

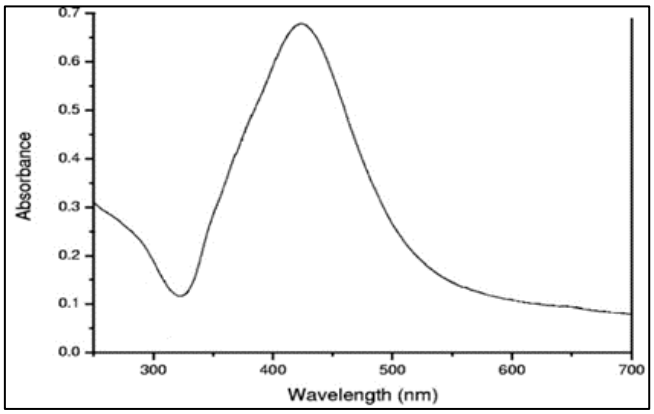

Fig 1: UV-Visible spectrum of synthesized Garlic mediated silver nanoparticles

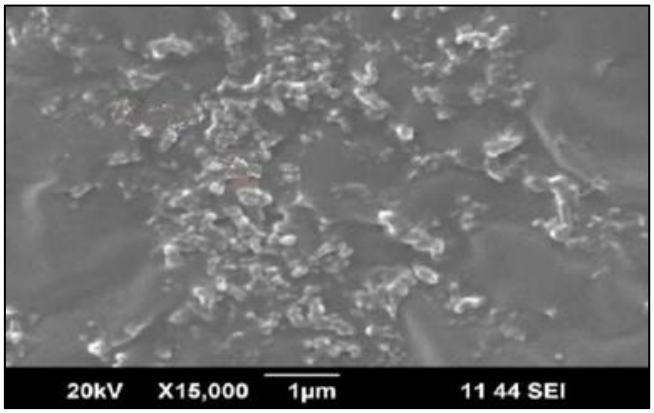

Fig 2: SEM analysis of Silver nanoparticles

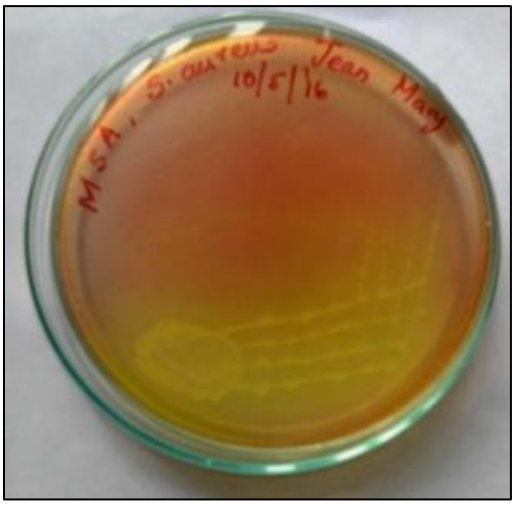

Fig 3: Streak plate of *S. aureus* on MSA.

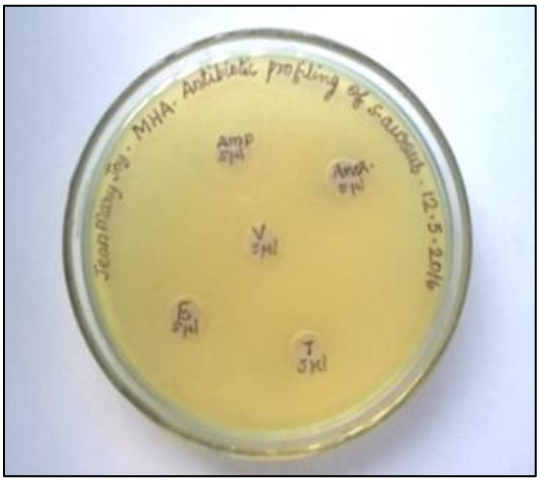

Fig 4: Antibiotic resistance of *S. aureus*

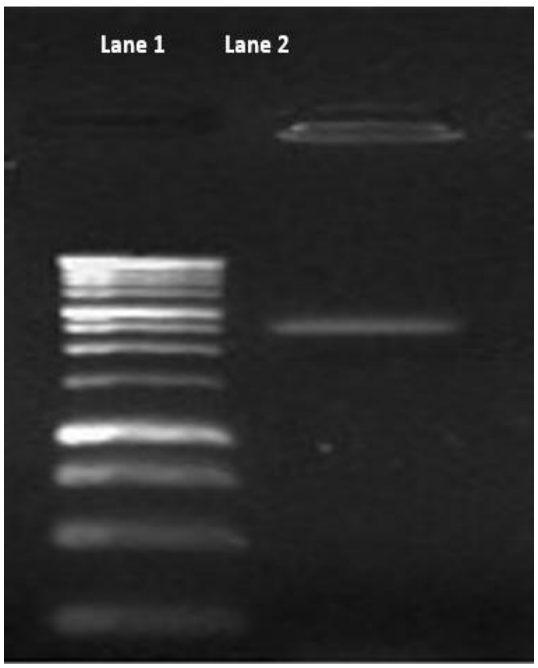

Fig 2: Agarose gel electrophoresis of amplified PCR product (Lane1: 1000bp marker, Lane2: Amplicon)

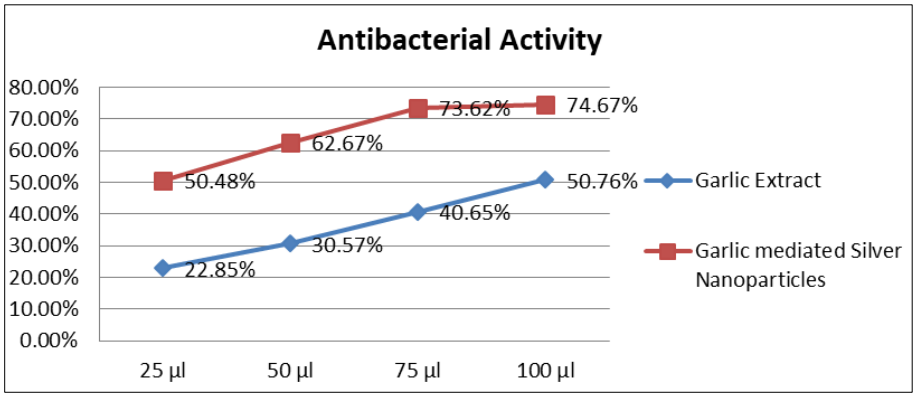

Fig 3: Antibacterial activity of Garlic extract and Garlic mediated Silver nanoparticles

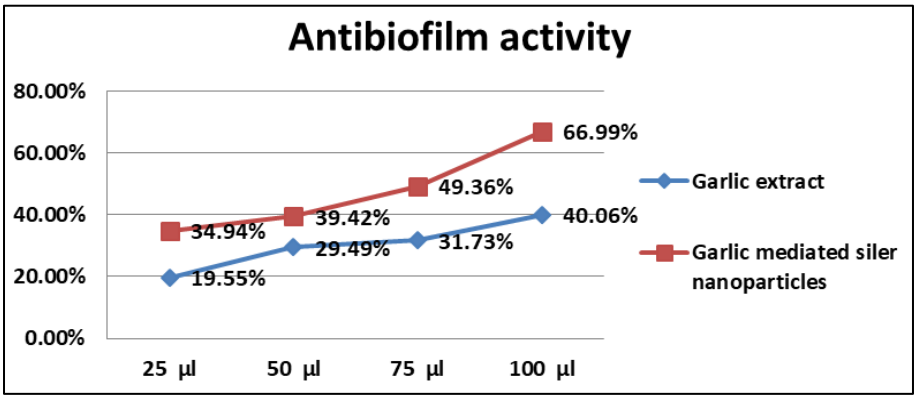

Fig 4: Antibiofilm activity of Garlic extract and Garlic mediated Silver nanoparticles.

4. Conclusion

Staphylococcus aureus is the leading causative pathogen of hospital acquired infections worldwide. Currently, there is a significant need to find alternative antimicrobials, especially those that could be employed for the treatment of MRSA infections and also requires an antibiofilm agent against MRSA. AgNPs constitute a very promising approach for the expansion of new antimicrobial systems. The approach of green synthesis method seems to be cost efficient, eco-friendly and easy alternative to conventional methods of AgNPs synthesis. Using garlic clove extract as a reducing and stabilizing agent AgNPs can be synthesized. Garlic and garlic extracts have been previously demonstrated as effective in hindering the growth of different bacterial pathogens, including *Staphylococci* and MRSA. This work investigated the synergistic effect of garlic mediated AgNPs against MRSA by microtitre plate method. Results shows an average of approximately 29% increased antibacterial activity by garlic mediated AgNPs against MRSA than garlic extract alone and 17% more antibiofilm activity by garlic mediated AgNPs against MRSA than garlic extract. This is overdue to the synergistic antibacterial and antibiofilm properties of garlic and silver in nanosize. Thus it provides a new scope to find solutions for emerging MRSA infections.

5. References

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