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INHIBITORY EFFECT OF SILVER NANOPARTICLES ON METHICLLIN RESISTANT *STAPYLOCOCCUS AUREUS*

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ABSTRACT

Staphylococcus aureus is a gram-positive coccus causing a wide variety of illnesses; most commonly pyogenic infections. It accounts for 30-50% of skin and soft tissue infections. Over the years there has been an increasing incidence of Methicillin Resistant Staphylococcus aureus. Antimicrobial resistance poses a threat to human health as it leads to increased morbidity and mortality. Due to this antibiotic resistance there is a need for new antibacterial agents that will be effective. Nanoparticles are gaining popularity as novel antimicrobial agents due to their high surface area to volume ratios as well as their physical and chemical properties. The present study was carried out to document the potential inhibitory effect of silver nanoparticles synthesized using leaf extract of Azadirachta indica (neem) on Methicillin-resistant Staphylococcus aureus (MRSA). A total of 100 MRSA isolates were collected from pus, blood, sputum, endotrachael secretions and drain samples. Antimicrobial assay was performed using the well diffusion method. The silver nanoparticles showed promising results of being able to inhibit the growth of the isolates.

KEY WORDS

antimicrobial resistance, Methicillin resistant Staphylococcus aureus (MRSA), neem leaves, silver nanoparticles

INTRODUCTION

Staphylococcus aureus is a gram-positive coccus that is 1µm in diameter occurring in clusters[1] and is a frequent colonizer of the skin and mucosal surface [2,3]. In addition, the carriage rates in hospital patients and staff reach up to 80%. The primary reservoir of organism is the anterior nares from where the bacteria spread to hands or with dust in the air[2]. However, the organism may be found colonizing axillae, vagina, pharynx and other skin surfaces. Infection occurs when colonizing strains gain access to sites on the body which are normally sterile as a result of trauma or abrasion to the skin or mucosal surfaces [3.] Once the organism is transmitted it may be established as part of the recipient's normal flora and then later is introduced to normally sterile body sites by either trauma or invasive procedures[3].

Since the 1990's, there has been an increasing incidence of Methicillin Resistant *Staphylococcus aureus* [4,5]. The colonization rate is higher in patients with insulin dependent diabetes and in HIV patients [4]. *S.aureus* has the ability to develop resistance to penicillin, methicillin, cephalosporin (all beta-lactam antibiotics) and other antimicrobial agents. However, they are susceptible to anti-MRSA 5th generation cephalosporins such as ceftobiprole and ceftarolin [6].

Methicillin resistant *Staphylococcus aureus* (MRSA) strains develop due to the acquisition of *mec*A gene encoding for altered penicillin binding protein PBP2a. This *mec*A gene is carried on staphylococcal cassette

chromosome *mec,* a mobile genetic element [6]. The mecA gene is 2.1Kb long and is found on a genomic island[5]. The altered penicillin binding protein –PBP2a has low affinity for all β-Lactam antibiotics and is hence not inactivated by β-Lactam antibiotics. Organisms proceed to replicate permitting the formation of a stable peptidoglycan structure which leads to the normal division and growth of the microorganism [7].

Antimicrobial resistance poses a threat to human health as it leads to increased morbidity and mortality [4]. In India, a study conducted by the Indian Network for Surveillance of Antimicrobial Resistance in 2008-2009 found that there was a prevalence of 41% of MRSA in Indian hospitals[8].

These MRSA strains are an important cause of nosocomial infections and they are classified as either Hospital-associated MRSA (HA-MRSA) or Community-associated MRSA (CA-MRSA) which is more virulent and infiltrates hospitals [8].

HA-MRSA commonly infect patients that are hospitalized while CA-MRSA infect younger patients[9]. Infections are similar to those caused by Methicillin-sensitive *S.aureus* (MSSA) however they are difficult to treat as MRSA strains do not respond to most antibiotics targeting the beta-lactam ring which result in passage of the specific gene encoding the resistance to strains that lack it, increasing the pool of organisms that demonstrate resistance[10].

Due to this antibiotic resistance there is a need for new antibacterial agents that will be effective[11]. Nanoparticles are becoming popular as novel antimicrobial agents due to their high surface area to volume ratios as well as their physical and chemical properties[12]. Silver is a metallic compound with noteworthy antimicrobial activity; it is a more toxic element than other metals, has low cytotoxicity and has a lesser tendency to induce microbial resistance than other antimicrobial materials[13]. Nanoparticles can be synthesized chemically, physically or biologically (green synthesis). When nanoparticles are synthesized chemically, toxic by-products are obtained which are not ideal for biochemical use. The use of green nanotechnology (plants or microorganisms) for the synthesis of nanoparticles is superior because there is no production of toxic by-products[14,15].

Plants contain natural compounds such as alkaloids, flavonoids, saponins, steroids, tannins and nutritional compounds[16]. Plant extracts act as pre-cursors for the synthesis of nanoparticles in a harmless way. Due to the fact that plant extract contains secondary metabolites, it acts as a reducing and stabilizing agent for the bioreduction reaction of silver ions metallic nanoparticles[16]. The biological synthesis of nanoparticles involves a single reduction step which requires less energy[16].

Azadirachta indica (Indian neem) is a plant which is a species of family Meliaceae[17]. In India, every part of the neem tree is used as a remedy against a variety of ailments and as treatment for viral, bacterial and fungal infections[17]. Furthermore, the reducing sugar present in neem extract is thought to be responsible for the reduction of metal ions and stabilization of the nanoparticles while flavonoids and terpenoids stabilize the nanoparticles [18].

MATERIALS AND METHODS

Bacterial Identification

The MRSA isolates were isolated from various clinical samples namely pus, blood, endotrachael secretions, sputum, pleural fluid and blood. Direct microscopy of most of these clinical samples revealed gram-positive cocci in clusters and pairs and these confirmed the presence of bacteria. These clinical samples were then inoculated onto blood agar. Once grown, culture smears were made and catalase was performed. The isolates suspected to be *Staphylococcus aureus* were subjected to susceptibility testing and identification with VITEK 2 system. Isolates confirmed by VITEK 2 system to be MRSA were used in the present study.

Green Synthesis of Silver Nanoparticles

Silver nanoparticles were synthesised by using 0.1M silver nitrate solution and leaf extract of *Azadirachta indica* (neem).]

Preparation of leaf extract:

The neem leaves were collected, weighed to 20g and washed with sterile distilled water. Thereafter, the neem leaves were cut into smaller pieces, kept in oven at 50ºC for 15 minutes and then added to 100 mL of distilled water. Neem leaves and distilled water were kept in a hot water bath at 60ºC for 15 minutes to obtain the leaf extract. Whatmann filter paper no.1 was used to filter the neem leaf extract. Preparation of silver nitrate solution: 0.679 grams of silver nitrate was mixed with 40 mL of distilled water.

Synthesis of silver nanoparticles was by addition of 20 mL of leaf extract to 40 mL of silver nitrate solution. This

was the 1:2 preparation of the silver nanoparticles. Preparation of 1:1 silver nanoparticle was by addition of 40 mL plant extract to 40 mL silver nitrate solution. The mixture was kept in the dark for 24 hours and observed for colour change. A change in colour to brown-black indicated the formation of silver nanoparticles.

Characterization of silver nanoparticles

The aim of characterization is to determine the surface area, porosity, particle size distribution, aggregation, and shape [19]. A range of techniques are available to achieve characterization of silver nanoparticles. These include ultraviolet (UV) visible spectroscopy, atomic force microscopy (AFM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS) and powder x-ray diffraction (XRD) and others[19]. In the present study, ultraviolet visible spectroscopy (Figure 1), dynamic light scattering (Figure 4 & 5) and powder x-ray diffraction (Figure 2 & 3) was used to characterize the silver nanoparticles. This was to determine the size and nature of the synthesized nanoparticles. The UV-vis (ultraviolet visible spectroscopy) results shown in figure showed an absorbance of 421 nm. This confirmed that it might be nanoparticles that were formed.

RESULTS

The present study was carried out in the Department of Microbiology, J.S.S. Hospital from January 2017 to December 2017. The initial stage of the study which involved the synthesis of the silver nanoparticles was carried out at the Faculty of Life Science, J.S.S Academy of Higher Education & Research. A total of 59% MRSA isolates were from males while 41% were from females. These isolates were then subjected to sensitivity testing with green synthesized silver nanoparticles by well diffusion method.

The average zone diameter for the 1:1 ratio preparation of the silver nanoparticles was found to be 13.65 mm. In contrast, the average zone diameter for the 1:2 ratio preparation of silver nanoparticles was found to be 13.36 mm. The two average zone diameters were almost equivalent to each other with a difference of 0.29 mm. Therefore, there was no major significant difference. However, it was noted that the 1:1 ratio preparation of the silver nanoparticles seemed to have slightly more action against MRSA than the 1:2 ratio. In comparison, the average zone diameter for the silver nitrate solution was 12.2 mm which was fairly less than the zone diameter obtained for either ratios of silver nanoparticles. The neem plant (*Azadirachta indica*) extract showed no zone of inhibition. This may have been due to its method of extraction. Similarly, the negative control showed no zone of inhibition as expected.

The sensitivity pattern of the MRSA isolates was obtained from VITEK 2 system and the antibiogram of these was isolates was determined (Figure 7). Out of the 100 isolates, 92% were resistant to ciprofloxacin. Furthermore, 47% and 46% of the isolates were resistant to clindamycin and erythromycin respectively. Only 6% of the isolates were intermediate to erythromycin while 53% and 48% were sensitive to clindamycin and erythromycin respectively. All the isolates were sensitive to daptomycin, linezolid and tigecycline. Furthermore, 98% of the isolates were sensitive to teicoplanin, 97% to vancomycin, 96% to cotrimoxazole, 94% to tetracycline, 92% to rifampicin and 83% to gentamicin. Levofloxacin was intermediate with 92% of the isolates, while only 3% were sensitive.

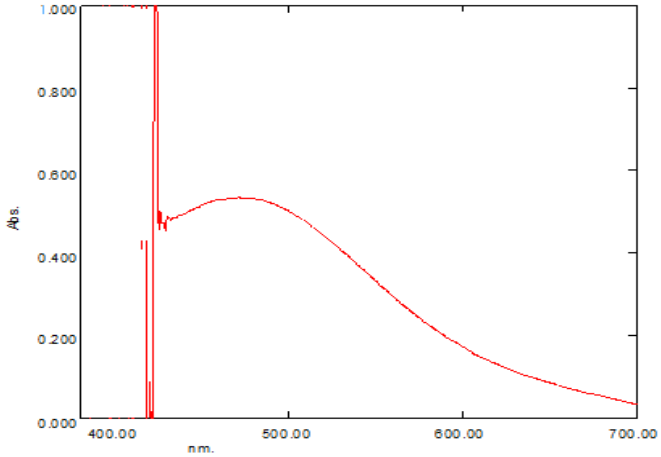

Figure 1: UV-vis graph showing the absorbance peak

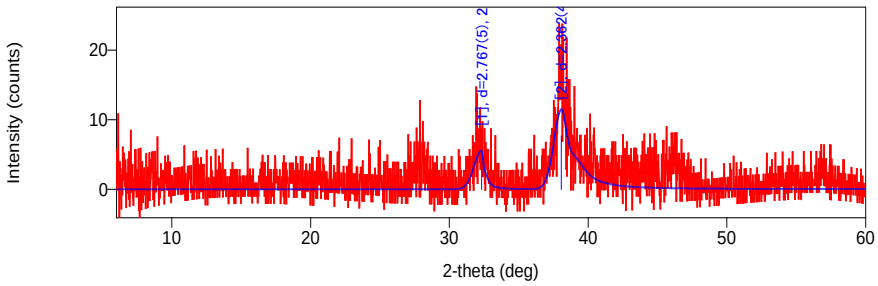

Figure 2: Graph displaying XRD results for 1:1 silver nanoparticle

It was observed that neem left extract/Ag showed strong reflections at 2θ of 32° and 38°respectively plus the shift of the position of the longer diffraction peak suggest the formation of silver nanoparticles.

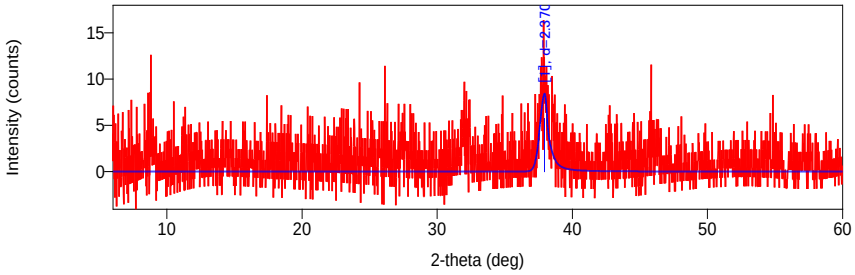

Figure 3: Graph displaying XRD results for 1:2 silver nanoparticles

One diffraction peak was obtained for the 1:2 preparations of silver nanoparticles. This peak was in the plane of (111) which is one of the characteristic peaks for silver cube crystals. This interesting feature indicates that silver nano-crystals are in the film are predominantly (111)-oriented. Therefore, this gives clear evidence for the presence of Ag NPs in the Ag/leaf extract.

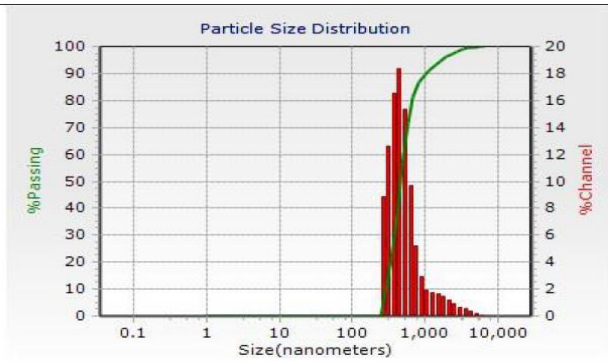

Figure 4: DLS histogram displaying results for 1:1 silver nanoparticle

The size distribution histogram of dynamic light scattering (DLS) revealed that the size of the nanoparticles was predominantly 500 nm. The particle size range began from approximately 480 nm.

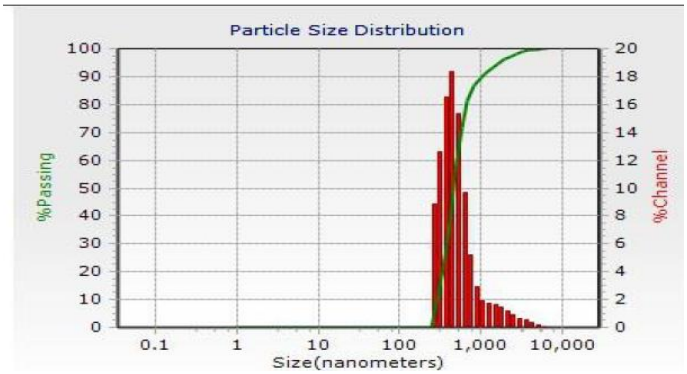

Figure 5: DLS histogram showing results for 1:2 silver nanoparticles

Similar results as obtained in were obtained with 1:1 ratio of silver nanoparticles whereby the particle size distribution was approximately 500 nm as well.

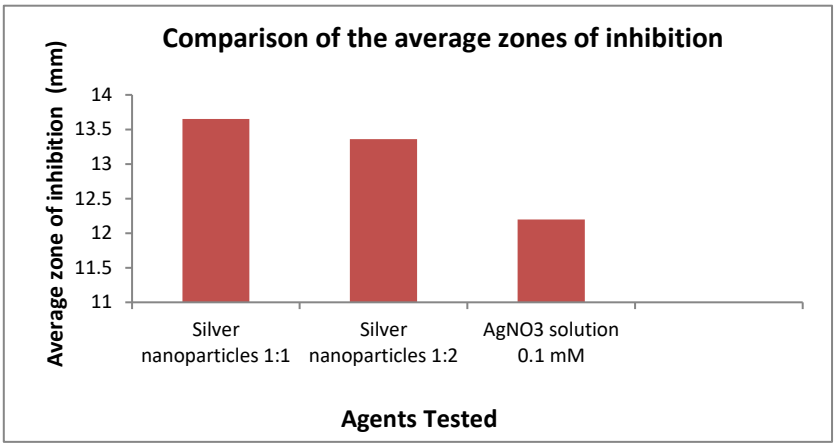

Figure 6: Showing the differences in the average zones of inhibition obtained

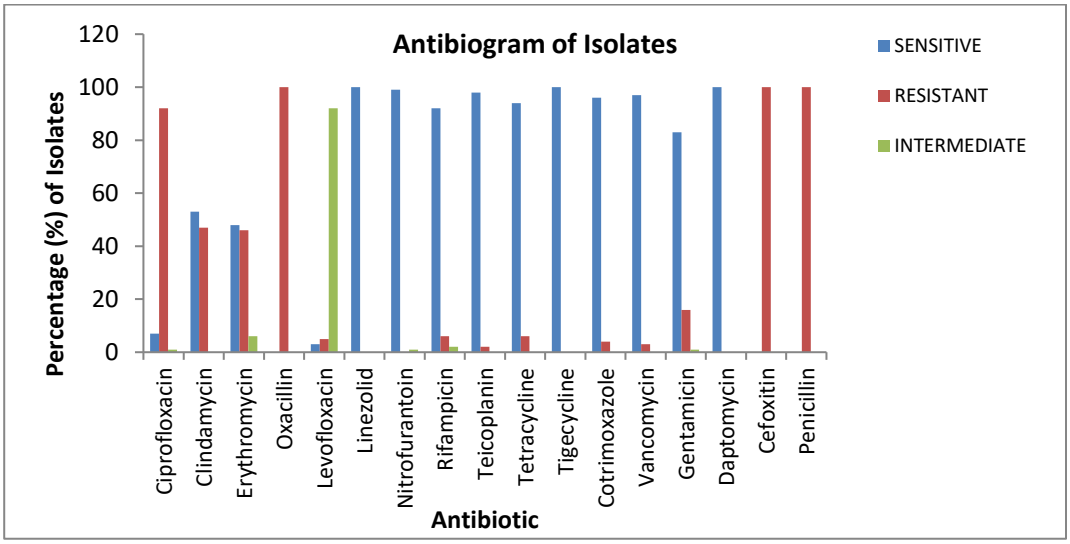

Figure 7: Graph summarizing the antibiotic Vitek 2 susceptibility results

DISCUSSION

Drug resistance usually requires multiple treatment with broad-spectrum antibiotics which are less effective, more toxic and expensive [20]. Therefore, there is a need for new antimicrobial agents. The present study investigated the potential of silver nanoparticles as antimicrobial agents against MRSA.

The silver nanoparticles were green synthesized according to the protocol followed by Sharma *et al.*, 2013. In addition, a second ratio of 1:1 was prepared by adding 20 mL of neem extract to 40 mL of silver nitrate solution. Combination of leaf extract with silver nitrate solution led to the formation of the silver nanoparticles. The plant extract was pale yellowish before the addition of silver ions and changed brown-black suggesting the formation of silver nanoparticles. A colour change confirms the formation of silver nanoparticles.

Further confirmation of the silver nanoparticles was performed using characterization techniques; namely UV-Visible spectroscopy (UV-vis), X-ray diffraction (XRD) and direct light scattering (DLS). In UV-vis, an absorbance of between 400 and 450 nm indicates that silver nanoparticles were formed [19]. The crystalline nature of the silver nanoparticles were analyzed using XRD whereby the diffracted powder specimen's angle of diffraction is measured as an X-ray beam is made incident to it [19].

The average zones of inhibition obtained for 1:1 and 1:2 ratios of silver nanoparticles were 13.65 mm and 13.36 mm respectively. Therefore, there was no significant difference between the activity of the two ratios except that of 0.29 mm. However, it was inferred that the silver nanoparticles of 1:1 had slightly more activity than 1:2. Ahmed *et al.*, 2015 obtained zones of inhibition of 9 mm

for *Staphylococcus aureus* which is less than the zones of inhibition of 10-13 mm obtained in present study. However, the present study focused on susceptibility testing with MRSA. Furthermore, in a study by Sharma *et al*., 2013 the zones of inhibition obtained with silver nanoparticles against Staphylococcus aureus ranged between 10-13 mm which were close to zone sizes obtained in this study. Another study conducted by Paredes *et al*., 2014 found that the zones of inhibition were 9-12 mm when silver nanoparticles were tested against MRSA.

Silver nitrate solution of a concentration of 0.1M was also subjected to susceptibility testing and gave an average zone diameter of 11.34 mm. This zone of inhibition was less than the zones of inhibition of either ratio. Based on these results it was concluded that there was enhancement of antimicrobial activity when silver was in nanoparticles form.

The neem leaf extract was presumed some inhibitory activity. However, no zone of inhibition was obtained for the extract during susceptibility testing. The lack of zone of inhibition may be attributed to the method of extraction. In a study conducted by Ahmed *et al*., 2015 the leaf extract of *Azadirachta indica* (neem) failed to yield any zone of inhibition. In the same study, the researchers suggested that lack of zone may have been due to using lower concentration of the leaf extract. In the present the concentration of the neem leaf extract was doubled, tested and still yielded no results.

There are mechanisms proposed to explain how nanoparticles act against microorganisms. These are as follows; direct interaction of silver nanoparticles with the cell membrane cause membrane damage and are complexed inside cells, production of reactive oxygen species and release block or inhibit respiratory enzymes [20, 21]. It is also believed that DNA stops replicating and cellular proteins are inactivated [22]. In addition, the DNA may become denatured once the silver ions enter and intercalates between the purine and pyrimidine bases [22].

Although more than 90% of MRSA (Figure 7) were determined by VITEK 2 system to be sensitive to linezolid, nitrofurantoin, rifampicin, teicoplanin, tetracycline, tigecycline, cotrimoxazole, vancomycin, daptomycin and gentamicin; each antibiotic has its own limitations. Linezolid needs to reserve for treatment of tuberculosis but presently it is overused and is available as an oral formulation while nitrofurantoin is used only for urinary isolates. Rifampicin has great potential in killing MRSA however it has to be reserved for the treatment of tuberculosis as well. Even though Cotrimoxazole can be used easily with availability of oral formulation, it cannot be used carelessly because of sulphonamide resistance in many patients. Gentamicin is an injectable aminoglycoside but for treating infections caused by gram-positive bacteria. It needs to be combined with beta-lactam antibiotics

CONCLUSION

Antimicrobial resistance in *Staphylococcus aureus* is on the increase and poses a challenge to health professionals including the pharmaceutical industry. Therefore, there is a dire need for the development of novel compounds active against microorganisms such MRSA that are not susceptible to the usual antimicrobial agents. Nanotechnology has a potential to alleviate this burden of antibiotic resistance through the use of nanoparticles.

Antimicrobial assay of synthesized silver nanoparticles against MRSA provided evidence that they are effective in inhibiting the growth. This is not only a cost-effective process but environmentally friendly as well. Furthermore, there is no evidence so far of microorganisms developing resistance to silver nanoparticles.

We conclude that the use of silver nanoparticles to combat drug resistance has significant potential. It is an avenue that should be explored and improved through optimisation as well as standardisation.

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