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SYNERGISTIC EFFECT OF SILVER NANOPARTICLES AND ANTIBIOTICS AGAINST METHICILLIN RESISTANCE *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Silver nanoparticles are the emerging Therapeutic molecules for life and medical field which have many characteristics in their nature act as drug carrier, have antibacterial, antifungal and antiviral activity. Green technology used for the synthesis of silver nanoparticles (AgNPs) gained significant importance in recent year within research community due to their simple, nontoxic, less time consumption, cost effective nature and also due to their effectiveness in large scale production. The present research study was focused on biosynthesis of AgNPs from the extract of *Azadirachta indica* and then different analytical techniques such as UV visible spectroscopy, Scanning Electron Microscopy (SEM), and Fourier Transform Infrared Spectroscopy (FTIR) were used for the characterization of AgNPs. In addition, the capability of AgNPs was also determined to enhance the activity of commercially available antibiotics *Oxacillin* 1µg, *ceftazidime* 30µg, *ceftriaxone* 30µg, *cefotaxime* 30µg, and *meropenem* 10µg, against bacterial isolates i.e. *Staphylococcus aureus* isolated from urine and wounds. In the current study, it was observed that the UV-visible spectroscopy gave absorption peak at 450 nm which was in the prescribed range and confirmed the synthesis of AgNPs. The SEM micrograph demonstrated the morphology of AgNPs. FTIR analysis gave confirmation about capping of AgNPs. Furthermore, it was suggested that AgNPs synthesized from the extract of *Azadirachta indica* could be of great importance in the pharmaceutical and medical science for their bio catalytic activities while, the combination of AgNPs and antibiotics have great effect against MDR Bacterial species.

Keywords: AgNPs, *Azadirachta indica*, *Staphylococcus aureus*, antibacterial, antifungal

INTRODUCTION

Methicillin resistance *staphylococcus aureus* is a bacterium which causes infection in different parts of the body. The MRSA commonly found in hospitals, and nursing home where patients with open wounds are present. The major reservoir of MRSA in institution are colonized in patients which transmit from person to person via hands of such a health worker which is major cause for a transmission [1, 2]. Methicillin resistance *staphylococcus aureus* have multi drug resistance to all classes of common antibiotics that makes it a serious nosocomial pathogen [3, 4]. MRSA infections are a global healthcare issue & the concerning factor is the bacteremia, which not only cause high morbidity & mortality but also cause metastatic infections like sepsis & endocarditis. As compare to MSSA the MRSA is responsible for more cases of bacteremia. Bacteremia caused by *S.aureus* can be managed by timely identification of source of infection and infectious strain, secondly treatment by antibiotics. [5, 6]. There are 4 types' penicillin binding proteins PBP1, PBP2, PBP3, and PBP4 as high molecular weight PBPs are two protein domains. 1 transpeptidation which proceed to cross linkage to other involved group of molecules. 2 trans glycosylation which elongates the glycan chain. The major mechanism of methicillin resistance in *S. aureus* through expression foreign PBP PBP2a that is not to confused with PBP2. This resistance to the actual mechanism of MRSA to takeover transpeptidation reaction the host PBPs. MRSA genetically distinguishes from the methicillin sensitive *staphylococcus aureus*. 40 to 60 (kb) foreign DNA stretches from the chromosome that present in MSA to isolate MRSA [7]. It is referred to the mec present the mec A gene which is encoded for the synthesis of penicillin binding proteins. The mec A gene has been originated from the staphylococcus sciurid [8]. Mec A gene highly conserved itself, PBPs and PBP2a are structural motifs that associated with penicillin binding interaction with beta -lactam antibiotics are highly reduced. mecR1, and mec I, are two gene which is co -transcribed from mec A, it is present on the staphylococcal chromosome. These two genes play a key role in the MRSA mechanism mecR1 encoded membrane bound signal transduction protein. While the mec I gene is responsible encoding for transcriptional regulators. Promoters are crucial for these genes and operator region that have 10 sequences for mec A and 35 sequences of mecR1[9]. Green synthesis is a field of biotechnology to make a compatible material such as bacteria, fungi, plants, synthesis of nanoparticles [10-12]. In the nanotechnology widely accepted of nanoparticles because it is very safe and eco- friendly. Which is development in Nano science [13-15]. From so many years to use traditional method but researchers are proved that the green method are more effective for the synthesis or generation of nanoparticles. In such some advantages of green method has been proved less cost, no chances of failure, and easy to characterization [16]. AgNPs has been widely used in medicine cause of its antibacterial activity. Its potential of antibacterial application is obvious by its action of destroying the MDR strains and also by its prevention of Bio-film formation. Contact killing & Ion-mediated killing are two anti-bacterial mechanisms of AgNPs that are wide accepted. AgNPs can anchor and eventually infiltrate the bacterial cell-wall; which leads to physical changes in the bacterial membrane, causes content leakage from the cell and death of bacteria. (Ag+) silver ions are thought to be essential in the antibacterial activity of AgNPs [17]. The thickness of gram-positive bacterial cell-wall is (30nm) as compare to gram negative (3-4nm) mainly composed of peptidoglycan the reason why antibacterial activity of AgNPs is weaker against gram positive as compare to the gram negative. The bactericidal activity of AgNPs makes them proper substitution for antibiotics and more importantly they show no toxicity to the human cell. The generation of free radicals after attachment to the cell-wall is thought to be mechanism of bactericidal activity of the AgNPs [18-20]. Penetrating the cell membrane and disturbing the permeability of the membrane that eventually leads to intracellular ATP leakage and cell death is the mechanism that AgNPs follow. The positively charged ions such as Ag+ in the experiment have tendency to act with sulfur & phosphorus which are present in bio-molecules such as the DNA & RNA. Disruption of the DNA & RNA function can be the consequence of this attachment[21]. The current research work was designed to study the antibiotic resistance of MRSA and also to observe the effect of AgNps alone against MRSA in vitro and observation of synergistic effect of AgNps and antibiotics *Oxacillin* 1µg, *ceftazidime* 30µg, *ceftriaxone* 30µg, *cefotaxime* 30µg, and *meropenem* 10µg, against MRSA invitro. Nanoparticles combine with antibiotics to enhance their

effect is called a synergistic effect. Commonly some antibiotic has not shown good result against bacteria such as MRSA, K. pneumonia, etc. but with silver- nanoparticles have shown a good result against MDR.

MATERIALS AND METHODS

Biochemical identification of MRSA

Total 200 bacterial samples are collected from wounds and urine by using sterile cotton swab and urine cups. Samples were streaked on Mannitol salt Agar which is selective media for *s. aurous*. The bacterial growth was observed on this media for bacterial morphology, for further identification and characterization the bacterial isolates were examined under microscope. for further confirmation gram staining and biochemical tests were performed [22].

Drug resistance pattern by disc diffusion method

For antibiotics sensitive assay disk diffusion method or Kirby Bauer method was used. For disk diffusion method the Mueller-Hinton agar media was used. These antibiotics are used for checking Antibiogram of MRSA. *Oxacillin* 1µg, *ceftazidime* 30µg, *ceftriaxone* 30µg, *cefotaxime* 30µg, and *meropenem* 10µg

Preparation of aqueous extract of Azadirachta indica plant

The AgNPs solution was prepared with concentration of 20µg/1µL by mixing of 20 mg AgNPs in 1 ml distilled water. Then the different amount of (40µg/2µL) and (80µg/4µL) AgNPs suspension was prepared. First take 100 ml of water and add 10 gram of *Azadirachta indica* leave powder and boil this solution at 80°C for 20 minutes in water bath at Microbiology Research Lab Abasyn University Peshawar .Now Cool it and filter out the extract and put into the burette this extract will be used as reducing and capping agent for Nano particles synthesis. Further we take 1 litter of distal water in conical flask and pour 0.17 mg of silver nitrate and max it to form solution also don't forget to covered the conical flask with aluminum coil to avoid the photodegradation of silver nitrate .In next step take 20ml of filter *Azadirachta indica* leave extract and max in already prepared 1 litter distal water+silver nitrate solution and cover it with aluminum coil and keep in dark place for 24 hours where light cannot reach. Now take this solution in beaker and take in microwave oven for 2 days at temperature 50 to 60°C to evaporate the water till totally dry the beaker.

Synthesis and characterization of silver nanoparticles A. indica

For Characterization of AgNPs was made through UV-visible spectroscopy (Model; UV 1902 PC, UV-Vis Spectrophotometer) in the Department of Pharmacy, Abasyn University Peshawar while, SEM (Model; JSM5910, JEOL, Japan) and FTIR (Model; IRAffinity-1S. Shimadzu) were performed in the Centralized Resources Laboratory (CRL), University of Peshawar, Pakistan. UV-Vis spectroscopy gave absorption peaks in visible and ultra-violet region of spectrum and hence gave quantitative data about sample nature. In SEM analysis the small amount of desired sample is put on grid by using copper coated grid, then SEM machine use electrons instead of light to make output image of samples. FTIR technique was used to confirm the presence of different functional groups in samples by producing different peak values.

Preparation of Antibiotic Disc with AgNPs

The AgNPs solution was prepared with concentration of 20µg/1µL by mixing of 20 mg AgNPs in 1 ml distilled water. Then the different amount of AgNPs 2 µL, and 4 µL, suspension was poured on antibiotic discs in petri dishes and placed these plates at 50 °C for 30 minutes to dry [23].

Antimicrobial Activity of AgNps against MRSA

For checking the Antimicrobial activity of AgNPs the agar well diffusion method was used, for this purpose the Muller-Hinton agar media was used in plates. After Preparation of media five wells were bored by sterilize crock borer have 6 mm diameter. One well is used as negative control and one well

is used as positive control, and in more two wells 2µL, and 4µL, AgNPs suspension was poured[24, 25].

Synergistic effect of silver nanoparticles and antibiotics against MRSA in vitro

To examine the synergistic effect of silver nanoparticles and antibiotics against MRSA, the AgNPs coated Antibiotics are used by Kirby Bauer disc diffusion method assay on base of CLSI, 2017 by measuring the zone of inhibition in millimeters.

RESULTS

Biochemical identification of MRSA

Staphylococcus aureus was isolated from these clinical samples through pure culture techniques including two types of media i.e. Nutrient agar (NA), and Mannitol salt agar (MSA). While characterization of these collected *S. aureus* isolates was screened out by performing microscopy and different biochemical tests. In Gram staining *S. aureus* was Gram positive. *S. aureus* displayed negative response to Indole and Oxidase. While *S. aureus* showed positive response in Urease test Coagulase, Citrate, catalase, and Urease.

For MRSA isolation from MSSA we use *oxacillin* antibiotic by disc diffusion method [26]. After these all confirmation tests 48 MRSA Bacterial species are isolated from 200 raw samples.

Table.1: Table show the results of different biochemical tests use *S. aureus*

Biochemical tests	Results
MSA test	+
Oxidase	-
Urease	+
Catalase	+
Coagulase	+
Hemolysis	-

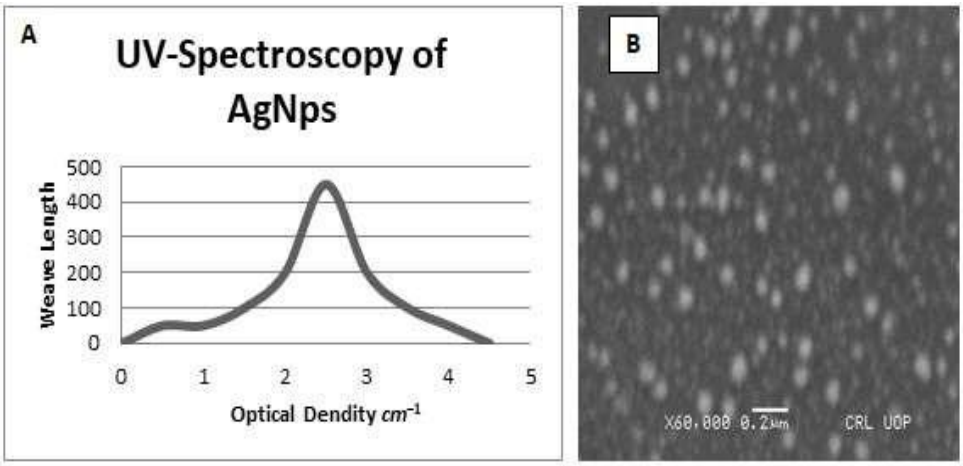
Drug resistance pattern by disc diffusion method

For Antibiotic sensitive assay we use Muller-Hinton agar medium and five different Antibiotics *Oxacillin* 1µg, *ceftazidime* 30µg, *ceftriaxone* 30µg, *cefotaxime* 30µg, and *meropenem* 10µg, are used, for these all five Antibiotics MRSA show resistance and no zone of inhibition was seen. It shows that the under-research specie of *staphylococcus aureus* (MRSA) is MDR multi drug resistance.

Characterization of silver nanoparticles A. indica

From UV-visible spectroscopy, it was observed that sample had absorbed energy at 450 nm which was a characteristic peak value of AgNPs. Beside this, absorption peak at 450 nm with other peaks displayed high purity of the nanoparticles. Beside this, SEM technique was used in the current study for overall appearance of the AgNPs.

The SEM micrograph of the synthesized AgNPS using aqueous extract of *Azadirachta indica* confirmed the mono dispersed and irregular morphology of the AgNPS showing particle size in the range of 1µm at magnification of 20,000X, 0.5 µm at magnification of 30,000Xand 0.2 µm at magnification of 60,000X. The SEM results indicated that aqueous extract of *Azadirachta indica* acts as a strong reducing agent during synthesis of irregular shaped AgNPs. For further characterization,the peaks in the FTIR spectrum of aqueous extract of *azadirachta indica*(control) at 3260.54 , 2932.89 , 1587.22 , 1390.88 , 1046.94 , 817.54 , 776.96 , 522.80 , 434.65 , 445.45 , 408.19 cm^{1} showed interrelation of different functional groups such as alcohol, alkanes, carboxylic acid or ester, amide, alkanes, anhydride , 1,2,3-Trisubtituted and amines respectively.



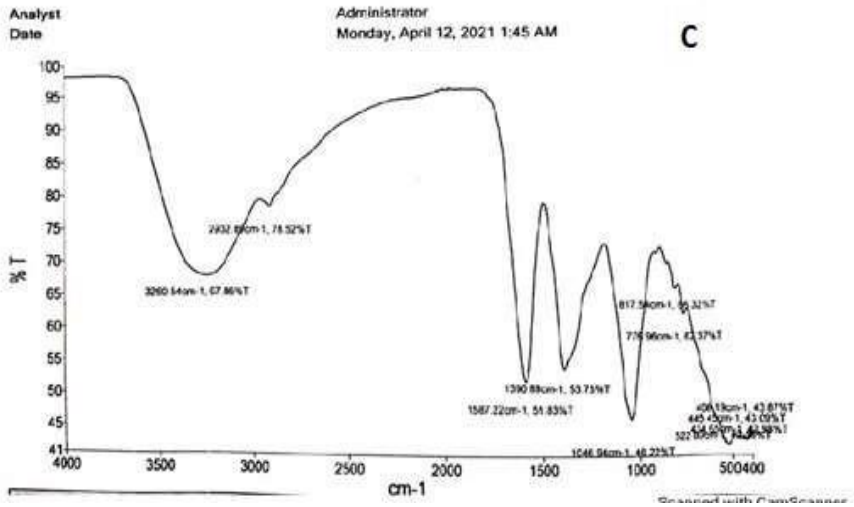


Figure. 1: (A) UV-visible spectroscopy of AgNPs (B) scanning electron microscope (SEM) of AgNPs and (C) Fourier transform infrared of AgNPs

Antimicrobial Activity of AgNps against MRSA

Disc diffusion assay method was used for observation of AgNPs activity against MRSA, in this procedure 2 well were form and fill it by 2 µl (40µg), and 4 µL (80µg) AgNPs was poured in petri dish. 11 mm zone of inhibition was caused by 40µg of AgNPs, and 15 mm zone of inhibition was caused by 80µg of AgNPs.

Synergistic effect of silver nanoparticles and antibiotics against MRSA in vitro

Oxacillin Antibiotics were coated with 80µg AgNPs which show 22 mm zone of inhibition, and 40µg AgNPs coated *Oxacillin* show 14 mm zone of inhibition. *Ceftriaxone* Antibiotics were coated with 80µg AgNPs which show 29 mm zone of inhibition, and 40µg AgNPs coated *Ceftriaxone* show 20 mm zone of inhibition. *Ceftazidime* Antibiotics were coated with 80µg AgNPs which show no zone of inhibition, and 40µg AgNPs coated *Ceftazidime* show no zone of inhibition. At these two potency powers MRSA show resistance to *Ceftazidime* if the amount of AgNPs increases the zone of inhibition may appear. *Cefotaxime* Antibiotics were coated with 80µg AgNPs which show 24 mm zone of inhibition, and 40µg AgNPs coated *Cefotaxime* show 12 mm zone of inhibition. *Meropenem* Antibiotics were coated with 80µg AgNPs which show 7 mm zone of inhibition, and 40µg AgNPs coated *Meropenem* show no zone of inhibition.

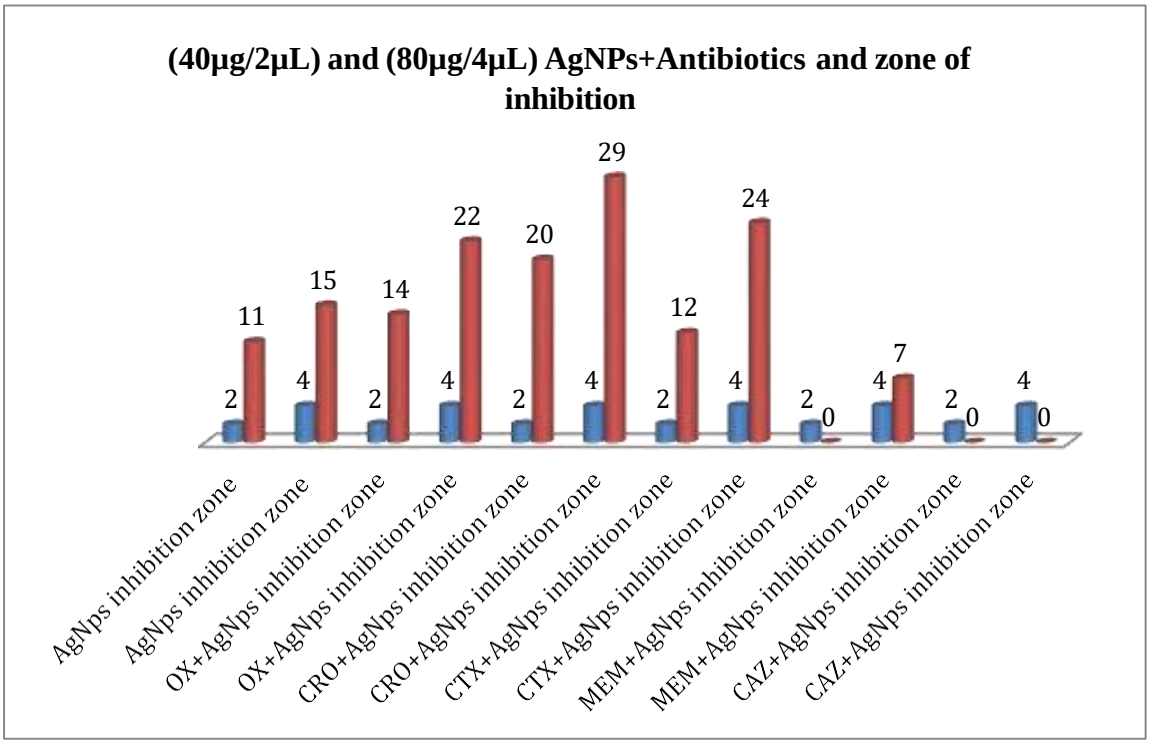


Figure.2: This graph shows the activity of AgNps and AgNps coated antibiotics against MRSA

DISCUSSION

The MRSA is emerging global issue for health, which cause life threating disease in humans and MRSA is one of the leading MDR bacterial species among bacterial species. In the current research study 200 samples of wounds and UTI were collected from different kpk hospitals *Staphylococcus aureus* was isolated from these clinical samples through pure culture techniques. collected *S. aureus* isolates were screened out by performing microscopy and different biochemical tests, In Gram staining *S. aureus* was Gram positive. *S. aureus* displayed negative response to Indole and Oxidase. While *S. aureus* showed positive response in Urease test Coagulase, Citrate, catalase, and Urease. For MRSA isolation from MSSA we use *oxacillin* antibiotic by disc diffusion method. the ratio of our collected samples (MRSA) was 19% from wound and UTI samples. for antibiogram we use MHA media and *Oxacillin* 1µg, *ceftazidime* 30µg, *ceftriaxone* 30µg, *cefotaxime* 30µg, and *meropenem* 10µg, are used for the MRSA which show resistance and no zone of inhibition was seen. AgNPs were prepared from the aqueous extract of *Azadirachta indica* by utilizing green synthesis approach and then different analytical techniques such as UV-visible spectroscopy, SEM, OD and FTIR were used for the characterization of AgNPs. UV Spectroscopy absorption peak at 450 nm with other peaks displayed high purity of the nanoparticles, SEM confirmed the mono dispersed and irregular morphology of the AgNPS showing particle size in the range of 1µm at magnification of 20,000X, 0.5 µm at magnification of 30,000Xand 0.2 µm at magnification of 60,000X, The FTIR spectrum of aqueous extract of *azadirachta indica*(control) at 3260.54 , 2932.89 , 1587.22 , 1390.88 , 1046.94 , 817.54 , 776.96 , 522.80 , 434.65 , 445.45 , 408.19 cm^{1} showed interrelation of different functional groups such as alcohol, alkanes, carboxylic acid or ester, amide, alkanes, anhydride , 1,2,3-Trisubtituted and amines respectively. 2 well were form by disc diffusion method and fill it by 2 µl (40µg), and 4 µL (80µg) AgNPs was poured in petri dish. 11 mm zone of inhibition was caused by 40µg of AgNPs, and 15 mm zone of inhibition was caused by 80µg of AgNPs. AgNps enhance the activity of antibiotics against MRSA in synergistic effect with antibiotics which was Multi drug resistance. In current research work for this MDR MRSA AgNps and synergistic effect of AgNps with antibiotics were used for observation of antimicrobial activity which showed that AgNps enhance the antimicrobial mechanism of antibiotic against MRSA. For characterization of AgNps Optical density, FTIR, and SEM, were performed. The different amount of AgNPs (40 µg/2 µl), and

(80 µg /4 µl) suspension was used against MRSA. The minimum inhibitory concentration of AgNps was 40 µg/2 µl.

CONCLUSION

The ratio of our collected samples (MRSA) was 19% from UTI and Wounds samples. The antibiotics used in this research *Oxacillin* 1µg, *ceftazidime* 30µg, *ceftriaxone* 30µg, *cefotaxime* 30µg, and *meropenem* 10µg, are resistance and no zone of inhibition was seen against MRSA. The AgNps were prepared from *azadirachta indica* plant by green Synthesis method and then different analytical techniques such as UV-visible spectroscopy, SEM, OD and FTIR were used for the characterization of AgNPs. This Research work clearly confirms that there is a good potential in AgNps against MRSA which is multi drug resistance bacterial species. AgNps alone show good result against MRSA, and form zone of inhibition, and in synergistic effect with antibiotics also form zone of inhibition against MRSA. The zone of inhibition was formed against MRSA at concentration of 40µg of AgNps. In synergistic effect AgNps enhance the activity of antibiotics which were resistive toward MRSA.

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