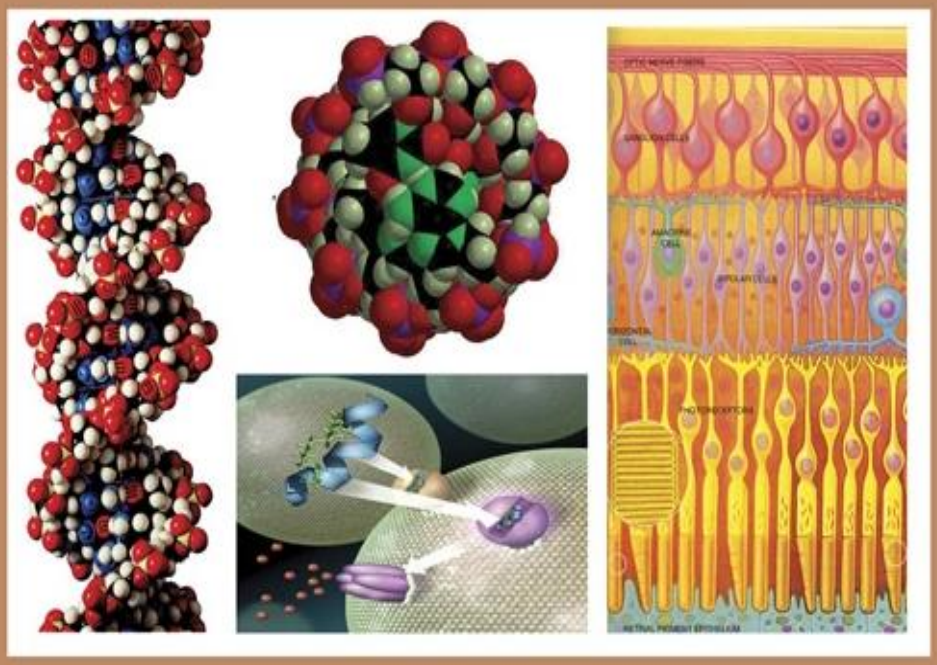
EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
C
PHYSIOLOGY & MOLECULARBIOLOGY



ISSN
2090-0767

WWW.EAJBS.EG.NET

Vol. 15 No. 1 (2023)

.

Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 15(1) pp319-337 (2023)*
DOI: 10.21608/EAJBSC.2023.303331

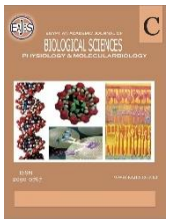
Egypt. Acad. J. Biolog. Sci., 15(1):319-337 (2023)

Egyptian Academic Journal of Biological Sciences

C. Physiology & Molecular Biology

ISSN 2090-0767

www.eajbsc.journals.ekb.eg



Synergistic Effect of Biogenic Silver Nanoparticles and Antibiotics Against Multidrug-Resistant *Pseudomonas aeruginosa*

Naif A. Jalal [1], Sumyya H. Hariri [1], Noha E. Abdel-razik [2], Abdulrahman Alzahrani [3], Suleman Khan [4] and Farkad Bantun [1]

[1]Department of Microbiology, Faculty of Medicine, Umm Al-Qura University, Makkah Saudi Arabia.

[2]Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Jazan University, Gizan, Saudi Arabia.

[3]Department of Applied Medical Sciences, Applied College, Al-Baha University, Al-Baha, Saudi Arabia.

[4]Department of Agricultural Sciences, Food, Natural Resources and Engineering, Università degli Studi di Foggia, Italy.

***E-mail:** sulemankhanazmat333@gmail.com

ARTICLE INFO

Article History

Received:24/1/2023

Accepted:25/3/2023

Available:1/4/2023

Keywords: Carbapenemase, *P. aeruginosa*, Modified carbapenem inactivation method, Sensitivity, Specificity, Kirby Bauer Disc Diffusion Method.

ABSTRACT

Infections caused by Pseudomonas aeruginosa may be either acquired in the community or contracted in a healthcare setting. Multidrug-resistant (MDR) *P. aeruginosa* is a growing problem; a new treatment approach is required to tackle this. Combination therapy of antibiotics and nanoparticle is thus applied to overcome this problem. Therefore, this study was planned to evaluate the synergistic effect of AgNPs along with different antibiotics against MDR P. aeruginosa. A total of 120 surgical or burn wound samples were collected from a tertiary care hospital. The plates containing the samples cultivated on cetrimide agar were then heated to 37°C. Isolates were identified based on colony shape, Gram staining, and several biochemical tests. A Kirby-Bauer disc diffusion technique antibiogram was conducted following CLSI 2022 recommendations. A minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined. The Agar well diffusion technique and the microdilution method were used to test the antibacterial activity of the AgNPs, respectively. The synergistic effect of antibiotics and AgNPs was estimated by the Checkerboard method. Out of 120 samples, 46 (38.8%) were confirmed positive for P. aeruginosa, and out of that, 33 were confirmed as MDR P. aeruginosa. Seven representative isolates proceeded for further procedures. Antibacterial activity of AgNPs revealed a maximum zone of inhibition of 12 mm at 4 mg/ml and a minimum of 2.5 mm at 1 mg/ml by agar well diffusion method. MIC and MBC of AgNPs showed that all the isolates were inhibited at 250 mg/ml. The FIC index of checkerboard results showed that colistin and gentamicin exhibited complete synergism with AgNPs, while ciprofloxacin showed partial synergism with AgNPs.

INTRODUCTION

Infections from bacteria are one of the major public health concerns nowadays because of the increase in resistance of bacteria because they can survive under the influence of antimicrobial agents. Bacteria do this by reducing the permeability of cells, inactivation of antimicrobial agents by enzymes, production of flow pumps, and altering binding sites, which offer safety and stability to microorganisms (de Lacerda Coriolano *et al.*, 2021).

These resistant bacterial strains are present all over the world. *P. aeruginosa* belongs to the class Gamma proteobacterium, is gram-negative, non-spore forming and aerobic. It is a rod-shaped organism responsible for causing infection in humans, plants, and animals (de Lacerda Coriolano et al., 2021). It is an opportunistic pathogen that produces indophenol oxidase enzyme, which means it is oxidase positive.

P. aeruginosa can also synthesize pyoverdine and pyocyanin. It can be found in soil, water, fresh vegetables, and fruits. It produces a sweet grape-like odor in the laboratory. *P. aeruginosa* gets energy by oxidizing sugar. It causes both community-acquired and nosocomial (like urinary tract infections, bacteremia, pneumonia, and ulcerative) infections. *P. aeruginosa* resists antiseptic solutions used for disinfecting surgical instruments (Salman *et al*., 2019). It causes hospital-acquired wound infections and bloodstream infections. It is also responsible for opportunistic infection in immunocompromised patients (Bayroodi & Jalal, 2016). The release of intracellular and extracellular virulence factors (Hydrogen cyanide, alkaline protease, elastase, phospholipases, and zinc metalloprotease) increase the pathogenesis of *P. aeruginosa* after colonization and results in high tissue damage due to toxA gene that encodes tissue damage enzyme and dissemination into the blood. Patients with persistent lung infections, pyocyanin may harm lung tissue (Haghi *et al*., 2018). *P. aeruginosa* now become able to overcome antibiotic treatment and has become resistant to various antibiotics. The misuse of antibiotics is a contributing factor, as is the organism's ability to thrive in new habitats and devise novel defensive mechanisms, such as biofilms (Hemmati *et al*., 2020; Hassani Sangani *et al*., 2015). In order to improve patient health, it is crucial to find an effective treatment for any barriers to treatment. *P. aeruginosa* has two primary antibiotic resistance types: intrinsic and acquired resistance (Fadwa *et al*., 2021). The pace of vaccine and antibiotic development has struggled to keep up with the emergence and spread of multidrug-resistant strains, highlighting the urgent need for more robust efforts in this area. That is why antibiotic resistance is a serious and major world problem. In the past few years, nanoparticles become popular and have been proven useful in various fields of diagnosis, medicine, and therapeutics. Nanoparticles have great antibacterial activity against all microorganisms, including bacteria and fungi. Nanotechnology in drug delivery systems consists of design, production, synthesis, characterization, device, and systems of nanoparticles with sizes ranging between 1-100 nm. Effective combinations of nanoparticles with antibiotics are among those techniques that are used nowadays to avoid bacterial growth (Abo-Shama *et al*., 2020; Sharma *et al*., 2016). Nanoparticles have garnered significant attention due to their unique properties and potential applications in various fields (Mba *et al*., 2021). Bose and Chatterjee (2016) conducted an environment-friendly, cheap, and simple method to synthesize silver nanoparticles from the leaf extract of guava (*Psidium guava*) as a capping and reducing agent. This plant was used because of its medicinal properties and is available in every season. TEM and UV analysis verified the synthesis of AgNPs. Typically, nanoparticles were 40 nm in size. Disc diffusion, serial dilution turbidity assay, and agar cup assay were used to calculate the relative activity of AgNPs against the test organism. The findings show that guava leaf extract used in the green synthesis of AgNPs may reduce bacterial growth. The antibacterial activity of biosynthesized AgNPs against *E. coli* and *P. aeruginosa* was estimated by Ramalingam *et al*. (2016). The final findings show that AgNPs exhibit antibacterial action that is concentration-dependent and that these nanoparticles result in the complete eradication of both bacteria. By employing Atomic Force Microscopy (AFM), researchers have successfully visualized and characterized changes occurring at the nanoscale level, shedding light on the intricate modifications

within cellular components and their mechanical behavior. Ramachandran and Sangeetha (2017) evaluate the activity of AgNPs against the biofilm production ability of MDR gram-negative bacteria. They studied the antibiofilm activity of AgNPs against five biofilms-producing MDR bacteria, *P. aeruginosa, K. pneumoniae, E. coli, Acinetobacter baumannii,* and *Proteus mirabilis*.

Jaiswal and Mishra (2018) studied formation of AgNPs from curcumin. These curcumin AgNPs were 25-35 nm in size, highly stable with low silver content, and had a bactericidal effect on gram-negative and gram-positive bacteria. In addition to their antibiofilm activity, silver nanoparticles (AgNPs) exhibited minimal cytotoxicity towards human cells, specifically keratinocytes. The assessment of bactericidal activity of AgNPs involved multiple methods, including determining the minimum inhibitory concentration (MIC), conducting post-agent effect studies, and performing time-kill assays. To further evaluate the antibacterial effect of curcumin-loaded AgNPs, scanning electron microscopy (SEM) and live-dead imaging techniques were employed. Cytotoxic results showed that AgNPs were cytotoxic at 156 mg/l, which is a very high concentration compared to its bacterial MIC value. The skin-keratinocytes test also revealed that AgNPs had anti-inflammatory activity on human macrophages.

MATERIALS AND METHODS

Study Design: The research study was carried out within the postgraduate research laboratories of the Department of Microbiology. Samples were obtained from patients having burns or surgical wounds from a tertiary care hospital.

Sample Collection:A total collection of 120 samples was obtained using sterile swabs from patients' burn or surgical wounds at a tertiary care hospital (Omer *et al*., 2020). Samples were then transported to the postgraduate research lab in the Department of Microbiology.

Isolation and Identification of Bacteria:Each sample was separately grown on selective media. Petri plates were incubated at 37^{o}C for 24 hours (Ijaz *et al*., 2019). After 24 hours, plates were taken, and Gram's staining was performed by picking isolated colonies from each plate. Bacteria were identified by observing morphological and cultural characteristics and biochemical tests (Omer *et al*., 2020).

Preparation of *Pseudomonas* Cetrimide Agar: Cetrimide agar is commercially available in powder form that is used to culture Pseudomonas bacteria. It is selective media for the growth of *P. aeruginosa*.

Composition:Pancreatic digest of gelatin (Peptone): 20.0g, Potassium sulfate:10.0g, Magnesium chloride: 1.4g, Cetyl-trimethyl-ammonium bromide: 0.3g, Glycerin: 10ml, Agar: 13.6g and Distilled water: 1000ml.Final pH: 7.2 ± 0.2 at 25 ^{o}C. Smear was made by adding one drop of water and then mixing with a colony of bacteria from cetrimide agar. Smear was also fixed by heat on the slide. Heat fixed slide was then allowed to cool before stains were applied.

Biochemical Tests: Biochemical tests (catalase , oxidase, urease, citrate utilization test, triple sugar iron test, methyl red, Voges Proskauer, and indole) were performed for all the 120 isolates to confirm bacterium (*P. aeruginosa*) (Quinn *et al*., 2002).

Molecular Identification:All the positive isolates were further confirmed through Polymerase Chain Reaction (PCR) by amplification of bacterial DNA using OprL Gene-specific primer (Table 1)..

DNA-Extraction: DNA is extracted from bacterial cells for use in molecular analysis. The lysis of bacterial cells is accomplished via chemical or physical means. Proteinase K and lysozyme are utilized for gram-negative bacterial lysis. In this study, DNA extraction was done by boiling method according to the protocol of (Ahmed & Dablool, 2017). Isolated DNA is then directly used for PCR. The broth culture of bacteria was transferred into Eppendorf tube and centrifuged for 10 minutes at 13000 rpm. The supernatant was

discarded. The pallet was resuspended with normal saline or PBS buffer and centrifuged again at 13000 rpm for 10 minutes. The supernatant was discarded. The pallet was resuspended again in normal saline or PBS and boiled for about 10 min at 95 C. Immediately, Eppendorf tubes were transferred into an ice box and centrifuged for 10 minutes at 13000 rpm. The supernatant was collected and transferred to a fresh Eppendorf tube. Eppendorf tubes then stored at -20 C.

Polymerase Chain Reaction:

Table1: List of Primer Sequences**.**

Gens Type	Primer sequences 5'-sequences-3'	Reference
OprL	F-ATG GAAATGCTGAAATTCGGC R-CTTCTTCAGCTCGACGCACG	(Abdulhaq *et al*.,2020)
OprL	F-ATGAACAACGTTCTGAAATTCTCTGCTR- CTTGCGGCTGGCTTTTTCCAG	(Abdulhaq *et al*.,2020)

Protocol:

The amplification was carried out in a total volume of 20 µl containing template DNA, forward/reverse primers, and Master Mix. The composition of the reaction reagent is described in Table 2. The conditions used in the thermo-cycler for PCR were as follows. In the first step, initial denaturation was carried out at 95^{o}C for 4-5 minutes. Then in the second step, denaturation was again accomplished at 95^{o}C for 45 seconds. In the third step, annealing was done at 57 C for 1 minute. In the fourth step, the extension of DNA was done at 72 C for 1 minute. Thirty cycles were repeated from the second to the fourth step and in the last step, the final polymerization of DNA was completed at 72 C for 5 minutes. After completing all steps, the reaction mixture was further analyzed by AgaroseGel (Table 3).

Table 2: PCR Mixture and Concentration of each Reagent.

Reagent	Volume (µl) for 1 sample	Final concentration
Master mix	12.5µl	IX
Total DNA	2.0 µl	100-500ng
Forward primer (10pm)	1.0 µl	1 µl
Reverse primer (10pm)	1.0 µl	1 µl
RNase-free water	8.5 µl	1 µl
Total volume	25 µl	1 µl

Table 3: Ingredients for preparation of Gel.

Ingredients	Amount
Agarose gel	0.8g
Ethidium bromide	6µl
0.5X TBE buffer	100ml

Agarose Gel Electrophoresis: Gel electrophoresis facilitates the separation of DNA molecules based on their size. Under the influence of an electric current, negatively charged DNA migrates towards the positive electrode, allowing for distinct banding patterns to emerge. Genomic DNA was visualized to check the quality and purity by gel electrophoresis. Agarose gel was prepared to undergo electrophoresis (Lee *et al*., 2012).

Preparation of Agarose Gel: Weigh 0.8 grams of agarose on the scale. Using a sterile measuring cylinder, 100ml of 0.5X TBE buffer was measured and deposited into the sterile beaker. After adding weighed agarose to 100ml of 0.5X TBE buffer, the beaker was sealed with aluminum foil. Microwave it for 1 to 2 minutes to dissolve agarose completely, but do not boil it. It was cooled to 50 degrees Celsius, and after 4-5 minutes, 6l of ethidium bromide was added. It was thoroughly combined to ensure that all ingredients were thoroughly mixed. The tray's rubber bands were affixed, and a 20-well comb was placed in its position. Agarose gel was steadily poured into a clean tray to prevent bubble formation. Allow the gel to solidify at an ambient temperature of 20 to 30 degrees Celsius. After solidification, rubber bands and a comb were removed with caution so as not to rupture the substance. The gel tray was deposited within the electrophoresis tank, and the gel was covered with 0.5X TBE buffer.

Protocol of Sample Loading: In the initial well, a 6µl molecular weight ladder of *P. aeruginosa* was introduced. For each well, 1 µl of stain/dye was combined with 5 µl of DNA sample, using a micropipette for precise measurements. The dye-infused DNA samples were then meticulously loaded into their respective wells. The electrophoresis tank was securely covered, and the electrodes

were appropriately connected, supplying a voltage of 120-135 for 45 minutes. To visualize the results, the spectrophotometer was utilized to observe the distinct bands obtained.

Antimicrobial Susceptibility Testing (AST): Kirby Bauer disc diffusion technique using several antibiotic discs (meropenem 10 g, imipenem 10 g, colistin 10 g, Amikacin 30 g, ciprofloxacin 5 g, and ceftazidime 30 g) was used to test for antimicrobial resistance. Clinical and Laboratory Standards Institute (CLSI) 2021 criteria were used to quantify and interpret the zone of inhibition.

McFarland Standard Preparation:The McFarland standard plays a crucial role in estimating bacterial density as it consists of a suspension containing either barium sulfate or latex particles. By comparing the turbidity of a test suspension with the standardized McFarland standards (Hudzicki, 2009), bacterial density can be accurately assessed. To establish a 0.5 McFarland standard, a solution was created by combining 0.05 ml of a 1.175% barium chloride solution with 9.95 ml of a 1% sulfuric acid solution. Following thorough mixing, the turbidity of the solution was carefully adjusted to an absorbance range of 0.08-0.1 OD by measuring the absorbance at 625nm (Table 4). This specific absorbance range ensured the accuracy of the McFarland standard for subsequent bacterial density estimations.

Table 4: Preparation of 0.5 McFarland standard**.**

McFarland Standard	$BaCl_2$. $2H_2O$	H_2S0_4	Number of bacterial cells
0.5	0.05 ml	9.95 ml	1.5×10^8

Bacterial Inoculum Preparation: The inoculum was prepared using an 18-24 hours old culture of *P. aeruginosa* grown on cetrimide agar. To achieve a uniform bacterial suspension, 4-5 isolated bacterial colonies were carefully selected and placed into small test tubes containing normal saline. The mixture was vortexed for 5 minutes to ensure thorough dispersion of the bacteria (Figure 1).

The turbidity of the bacterial suspension was then visually compared to the 0.5 McFarland standard. This assessment was done with the unaided eye against a black-lined background. If the bacterial suspension appeared more turbid than the standard, it was diluted by adding normal saline. Conversely, if the suspension appeared less turbid, 1-2 additional colonies of bacteria were added to enhance turbidity.

It's important to note that the prepared suspension was utilized within 30 minutes after its preparation to ensure accurate and reliable results. This time frame helped maintain the viability and consistency of the bacterial suspension for subsequent experimental procedures (Momenah *et al*., 2023).

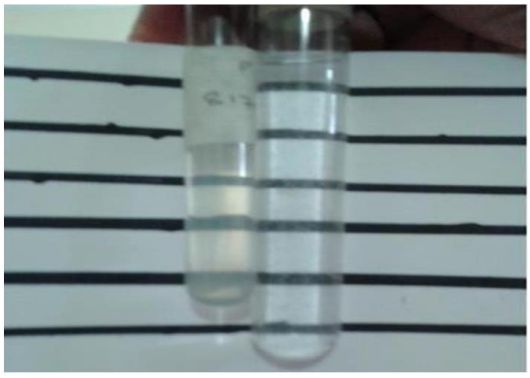


Fig. 1: Prepare bacterial inoculum according to McFarland standards.

Preparation of Muller-Hinton Agar (MHA)*:*For antibiogram testing, MHA was used. For disc diffusion assay, it has been the standard medium.

Composition: Beef (dehydrated infusion from): 150g, Casein hydrolysate: 8.75g, starch: 1.5g, Agar 8.5g, Deionized water: 500ml.Final pH: 7.3 ± 0.1 at 25 ^{o}C

Testing of Antibiotic Resistance: Taken first were Muller Hinton agar plates. Create a lawn of the bacterial suspension with the use of a sterile swab, ensuring that the turbidity meets 0.5 McFarland criteria before disc displacement. The inoculum was swabbed onto the agar plate in a three-dimensional pattern to ensure uniform distribution. Within

15 minutes after inoculating the plates, place antibiotic discs on top of them. The plates should be inverted and incubated at 35-37 degrees Celsius for 16-18 hours after the discs have been added. Antibiotic discs' diameters inside their inhibition zones are measured using CLSI interpretation criteria. Placing the plate against a dark background and reflecting light enabled the results to be measured. A ruler was utilized to measure the diameter of each antimicrobial agent's zone. The results were analyzed in accordance with CLSI guidelines 2022.

Determination of Minimum Inhibitory Concentration (MIC) of Antibiotics: The MIC value of the antibiotics (Colistin, Gentamicin, ciprofloxacin, Amikacin, ceftazidime, and cefepime) were determined using two folds broth microdilution method (Parvekar *et al*., 2020) in 96-well plates. A 1mg/ml stock of antibiotics (prepared in DMSO) was used to determine the MIC value against *P. aeruginosa*. Twenty-four hours old bacterial culture was used to prepare 0.5 McFarland. Next, on a sterile microtiter plate, 100 µl of MHB was placed into each well, and an equivalent volume of antibiotics stock solution was poured into well one and diluted by twofold serial dilution up to well 10. To ensure that each well had the same volume, 100 µl from well 10 was thrown away. Each well had 100 µl of bacterial inoculum put to it, except well 12, which served as a control. MHB and the positive-control bacterial inoculum are found in well 11. The plates were hermetically wrapped with parafilm to avoid evaporation, and then they were placed in a 37C incubator for 18 to 24 hours. After incubation, the optical density (OD) value was observed at 620 nm. Finally, 30 µl of Nitro-blue tetrazolium (NBT) redox dye was dispensed in each well and incubated at 37 ^{o}C for 4 hours. Color change from Yellow to blue was observed to detect bacterial growth. Wells with blue color indicate growth, while wells with yellow color indicate no growth.

Determination of Minimum Bacterial Concentration (MBC): The MBC was performed as described previously (Parvekar *et al*., 2020). Briefly, 50 l from each well exhibiting MIC and values above MIC were subcultured with the aid of a glass spreader on sterile petri plates containing MHA. Incubate these plates at 37 ^{o}C for 24 to 48 hours and observe for bacterial growth. Plates with the lowest antibiotic concentration that showed no visible growth were determined as MBC.

Evaluation of Antibacterial Properties of AgNPs:

Preparation of 0.5 McFarland Standard:A 0.5 McFarland standard containing 1.5 108 CFU was created by combining 0.05 ml of a 1.175% barium chloride solution and 9.95 ml of a 1% sulfuric acid solution. After combining, the solution's turbidity was set between 0.08 and 0.1 O.D. by measuring absorbance at 625 nm (Table 5).

Table 5: Preparation of 0.5 McFarland standard.

McFarland Standard	$BaCl_2.2H_2O$	H_2S0_4	Number of bacterial cells
0.5	0.05 ml	9.95 ml	1.5×10^8

Preparation of Bacterial Inoculum: The inoculum was prepared using an 18-24 hours old culture of *P. aeruginosa* grown on cetrimide agar. To ensure a uniform bacterial suspension, 4-5 isolated bacterial colonies were carefully selected and transferred into small test tubes containing normal saline. The mixture was then vortexed for 5 minutes to achieve thorough dispersion of the bacteria. The turbidity of the bacterial suspension was then compared to the 0.5 McFarland standard using the unaided eye against a black-lined background. If the bacterial suspension appeared more turbid than the standard, it was appropriately diluted by adding normal saline. Conversely, if the suspension showed less turbidity, 1-2 additional colonies of bacteria were added to increase the turbidity. It isimportant to note that the prepared suspension was used within 30 minutes after its preparation to maintain the viability and consistency of the bacterial suspension. This

time frame ensured reliable and accurate results in subsequent experimental procedures.

Preparation of AgNPs Concentrations: Three different concentrations of AgNPs, including 20 mg, 15 mg, 10 mg, and 5 mg, were weighted and mixed in 5 ml DMSO (dimethyl sulfoxide) to make 4 mg/ml, 3 mg/ml, 2 mg/ml, and 1 mg/ml solutions as shown in Figure 2. The solution of AgNPs was then sonicated for 2 hours to completely dissolve nanoparticles. These concentrations were then used for different antimicrobial tests.

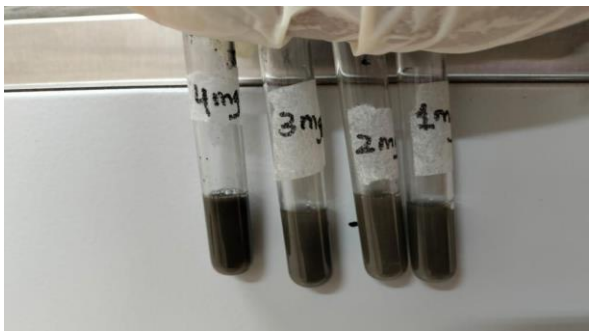


Fig. 2: Stock solution of 1mg/ml, 2mg/ml. 3mg/ml and 4mg/ml of AgNPs.

Agar Well Diffusion Method: The effect of different concentrations of AgNPs was tested against MDR strains of *P. aeruginosa*. The agar well diffusion method (Prasad *et al*., 2011) was used to check antibacterial potential. To perform this procedure, 24 hours old bacterial cultures were used to prepare an inoculum of 0.5 McFarland. A sterile cotton swab was used to spread 100 µl of bacterial culture on the surface of Muller Hinton agar, then left for 5 minutes to settle down. After swabbing, four wells of 5 mm diameter were punctured at appropriate positions with the help of a steel well borer. Each time a well was drilled, the good borer was heated to red-hot temperatures and then permitted to cool. Then 100 µl of prepared concentrations of AgNPs were added in all wells except the control well. The negative control well was punctured in the middle of the plate and filled with DMSO only. All the plates were left at 4 ^{o}C for 15-30 minutes to provide proper diffusion of AgNPs into media and then placed in the incubator for 24 hours at 37^{o}C. After incubation zone of inhibition was measured.

Preparation of Muller Hinton Broth (MHB):Muller Hinton broth was used as a general-purpose medium for the growth of microorganisms. The media was prepared by dissolving 10.5 grams of MHB in 500 milliliters of distilled water. The media was then autoclaved at 121°C for 30 minutes to sterilize it. After autoclaving, allow the substance to cool and preserve it at 4 ^{o}C for future use (Table 6).

Table 6: Ingredients for Preparation of MHB.

Ingredients	Quantity
Beef extract	2g/1000ml
Starch	1.5g/1000ml
Casein hydrolysate	17.5g/100ml
pH	7

Determination of Minimum Inhibitory Concentration (MIC) of AgNPs:The smallest amount of an antibiotic or other substance needed to stop the development of a particular strain of bacteria is known as its minimum inhibitory concentration (MIC). The antimicrobial activity of AgNPs was determined by measuring the MIC value using 2 folds broth microdilution method (Parvekar et al., 2020) in 96-well plates. A stock solution of 1mg/ml of AgNPs (prepared in DMSO) was used to determine the MIC value against P. aeruginosa. Twenty-four hours old bacterial culture was used to prepare 0.5 McFarland. Next, on a clean microtiter plate, 100 µl of MHB was put into each well, followed by 100 µl of AgNPs stock solution in well 1, serially diluted by 2 up to well 10. To ensure that each well had the same volume, 100 µl from well 10 was thrown away. Each well had 100 µl of bacterial inoculum put to it, with the exception of well 12, which served as a control. MHB and the positive-control bacterial inoculum are all that can be found in well 11. Parafilm was used to prevent evaporation while the plates were incubated at 37 C for 18 to 24 hours. An OD value of 620 nm was measured both before

and after incubation**.**

Determination of Minimum Bacterial Concentration (MBC): Minimum bacterial concentration (MBC) determines the lowest concentration of material that kills the bacteria. The MBC was performed by the method of (Parvekar *et al*., 2020). Pick up 50 µl from all wells that show MIC and above MIC value showing no visible growth and subculture on sterile petri plates containing MHA with the help of a glass spreader. Incubate these plates at 37 ^{o}C for 24 to 48 hours and observe for bacterial growth. Plates that contained no visible growth were determined as MBC.

Checkerboard Assay To Estimate The Synergistic Effect of AgNPs and Antibiotics: The combined effect of AgNPs and different antibiotics was determined by the calculation of fractional inhibitory concentration (FIC) in the two-dimensional checkerboard method (Fadwa *et al*., 2021). In the current research, we checked the activity of different antibiotics combined with *Moringa oleifera* synthesized silver nanoparticles against *P. aeruginosa*. The checkerboard method was used to perform FIC studies in a 96-well plate. A proper amount of Muller Hinton broth (MHB) was poured into all wells, then a two-fold serial dilution of antibiotics was poured into all the wells (in combination with AgNPs) from A to G (mentioned on 96 well plates). Similarly, a two-fold serial dilution of AgNPs, combined with antibiotics, was meticulously dispensed into all wells, numbered 1 to 11, on the 96-well plates. Column 1 contains a two-fold serial dilution of antibiotics alone to determine the MIC of antibiotics, while row A contains a two-fold serial dilution of AgNPs alone to determine the MIC of AgNPs. Initial OD600 was determined just before completing the experiment, and then incubate the plates at 37C for 18-24 hours. The plates were parafilm to prevent evaporation. After 18- 24 hours, the final OD600 was recorded again.

RESULTS

Isolation and Confirmation:

To isolate the bacterium, it was cultured on cetrimide agar. On cetrimide agar, *P. aeruginosa* showed large smooth, yellow-to-green colonies with green pigmentation of pyocyanin after incubation at 37^{o}C for 24- 48 hours (Fig.3). Out of 120 samples, 46 (38%) were *P. aeruginosa* positive (Fig.4).

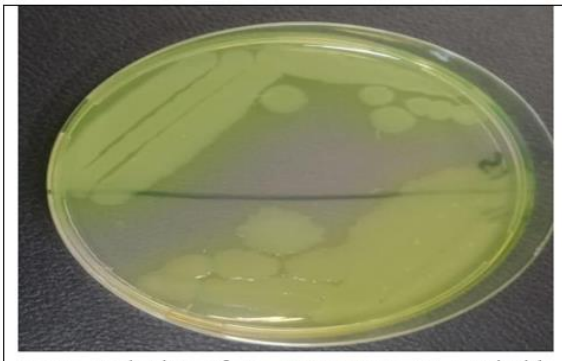


Fig:3. Colonies of *P. aeruginosa* on cetrimide agar.

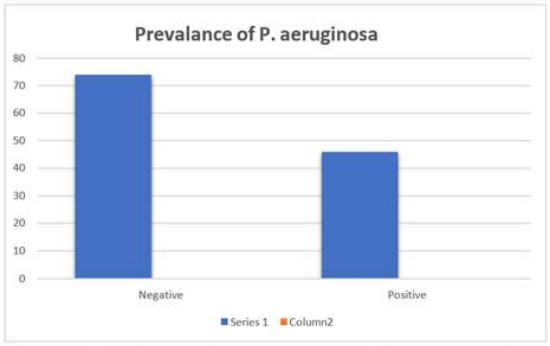


Fig. 4: Prevalence of *P. aeruginosa* from surgical or burn wounds.

Gram Stain:

Smear was prepared on a clean glass slide by picking characteristics growth from cetrimide agar plates, and staining was performed using standard Gram's staining procedure as explained by (Boyanova, 2018). All the samples that showed growth on cetrimide agar were found pink with a rod-shaped appearance at 100X under the microscope (Fig. 5).

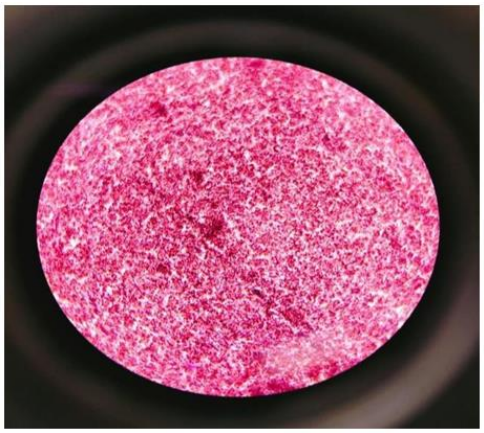


Fig. 5: Gram negative rods of *P. aeruginosa* (100X).

Biochemical Characterization:

All the isolates were subjected to biochemical testing. The following biochemical tests were performed i.e., catalase, oxidase (Figure 6), urease (Figure 7), citrate utilization test (Figure 8), triple sugar iron test, methyl red, Voges Proskauer, and indole. Results are demonstrated in Table 7.

Table 7: Biochemical tests for identification of *P. aeruginosa.*

Tests	Results
Catalase	Positive
Oxidase	Positive
Simon citrate	Positive
Voges Proskauer	Negative
Methyl Red	Negative
Triple sugar iron	Non-Fermenter
Urease	Negative
Indole	Negative

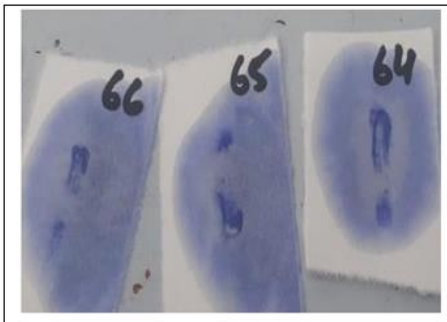


Fig. 6: Oxidase Positive

Fig. 7: Urease positive

Fig. 8: Citrate Positive.

Molecular Identification:

Microscopically and biochemically identified *P. aeruginosa* were confirmed at the genetic level as well. By using primers of gene OprL, *P. aeruginosa* was confirmed (Fig.9). OprL is peptidoglycan-associated lipoprotein (PAL) of about 20kDa. By using ethidium bromide-stained agarose gel, the OprL amplicon was visualized.

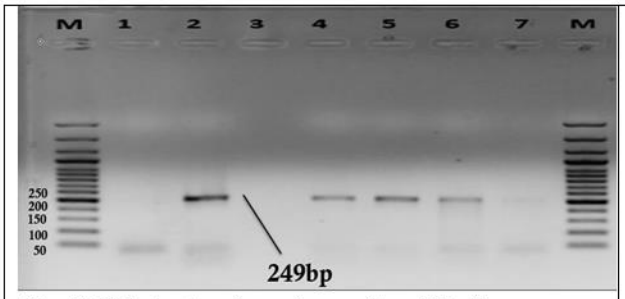


Fig. 9: Gel electrophoresis results of OprL gene.

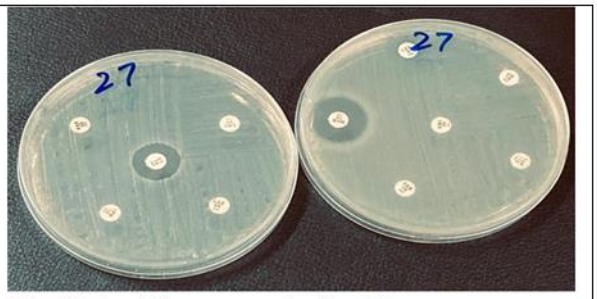


Fig. 10: Antibiogram analysis of *P. aeruginosa*.

Antimicrobial Susceptibility Testing (AST):

Antimicrobial susceptibility testing was performed to test whether *P. aeruginosa* is multi-drug resistant (MDR) (Fig. 10). To perform this, Muller Hinton agar plates were prepared and swabbed with bacterial inoculums. After swabbing antibiotic discs of imipenem (IMP), meropenem (MER), colistin (CT), gentamicin (GEN), cefepime (FEP) ciprofloxacin (CIP), ceftazidime (CAZ), ampicillin (AMP), and Amoxicillin/clavulanic acid were applied and incubated for 18-24 hours at 37°C (Fig. 11). Out of 46 isolates, 27 (58%) were MDR. Among these MDR isolates, 10 (37%) showed resistance to colistin (Fig. 12). The zone of inhibition was measured in mm (millimeter) and declared sensitive, intermediate, or resistant according to Clinical and Laboratory standard institute (CLSI) 2020 guidelines (Table 8).

Table 8: Interpretation of Antibiotic susceptibility testing.

Antibiotics	Sensitive	Intermediate	Resistant
Gentamicin	12	13	80
Ampicillin	20	13	67
Amikacin	24	15	61
Tobramycin	17	13	57
Imipenem	15	9	76
Meropenem	18	3	81
Cefepime	25	12	62
Cefoxitin	22	13	65
Ceftriaxone	11	12	75
Ceftazidime	13	9	78
Ciprofloxacin	22	13	65
Colistin	49	11	72
Piperacillin	54	15	35

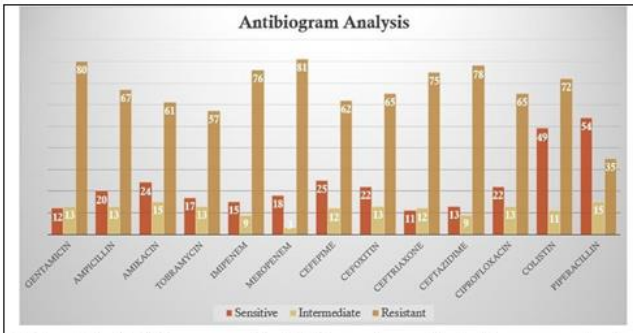


Fig.11: Efficacy of different antibiotics against *P. aeruginosa* isolates.

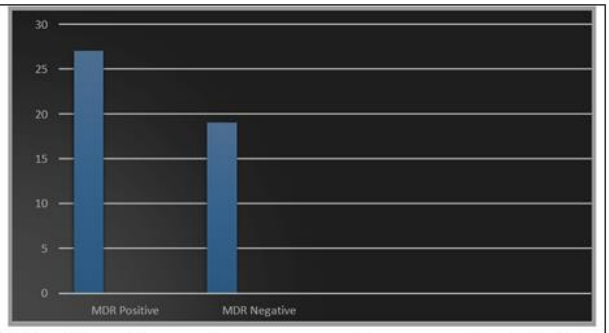


Fig:12. Percentage prevalence of MDR isolates of P. aeruginosa among (46) isolates.

Minimum Inhibitory Concentration (MIC) of antibiotics:

The minimum amount of antibiotic that inhibits the growth of microorganisms is called MIC. MIC of different antibiotics was evaluated in a 96-well plate; columns 11 and 12 act as a positive and negative control, and results were evaluated via optical density measured at 600nm (Fig. 13). The interpretation of each antibiotic's MIC was also performed (Table 9).

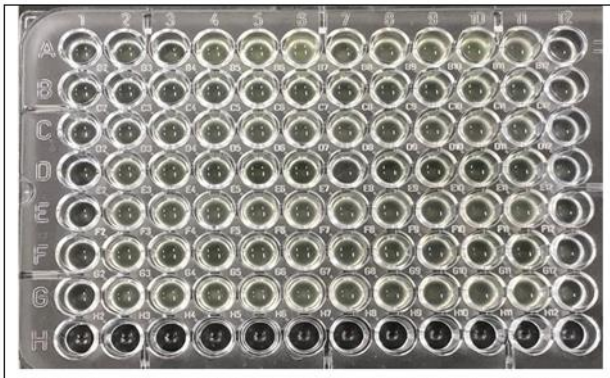


Fig:13. Microtiter plate showing MIC of antibiotics against *P. aeruginosa.*

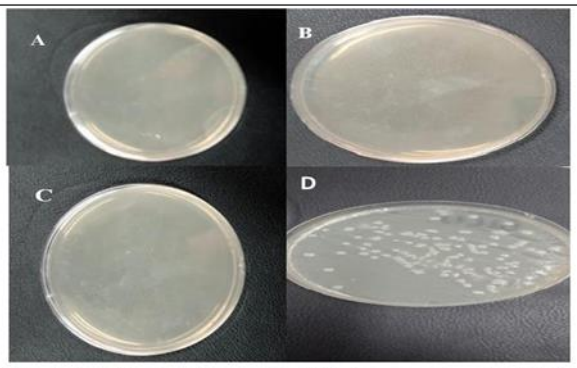


Fig.14: Evaluation of Minimum bactericidal concentration (MBC) on MHA plates.

Table 9 : Interpretation of MIC of Antibiotics.

Bacterial isolates no.	Colistinµg/ml	Gentamicin µg/ml	Amikacin µg/ml	Cefepime µg/ml	Ciprofloxacin µg/ml	Ceftazidime µg/ml
10	16	64	256	128	16	128
24	8	32	128	64	8	128
32	16	32	256	128	8	128
54	8	64	256	128	8	64
61	8	64	128	64	16	64
74	16	64	128	64	16	64
96	8	32	256	64	16	64

Minimum bactericidal concentration (MBC):

The minimum bactericidal concentration (MBC) represents the lowest concentration of antibiotics required to effectively eliminate bacteria. To determine the MBC, 50 µl of the content from each well of the microtitration plate was carefully spread onto autoclaved Muller Hinton Agar plates. These plates were then incubated for one day. Following the incubation period, the plates were thoroughly examined. Any plates showing no visible growth were identified as indicating the minimum bactericidal concentrations. This observation signifies the concentration at which the antibiotics successfully eradicated the bacteria, resulting in no detectable growth on the agar plates (Fig. 14).

The Minimum Bactericidal Concentration (MBC) values of antibiotics tested against Pseudomonas aeruginosa were also provided (Table 10). These values indicated the lowest concentration of each antibiotic that effectively kills the *P. aeruginosa* bacteria.

Table 10: Interpretation of MBC of Antibiotics.

Bacterialisolates	Colistin	Gentamicin	Amikacin	Cefepime	Ciprofloxacin	Ceftazidime
10	8	32	128	64	8	64
24	4	16	64	32	4	64
32	8	16	128	64	4	64
54	4	32	128	64	4	32
61	4	32	64	32	8	32
74	8	32	64	32	8	32
96	4	16	128	32	8	32

Antibacterial Activity of AgNPs:
Agar Well Diffusion Method:

The antibacterial activity of biologically produced silver nanoparticles against multidrug-resistant *P. aeruginosa* was shown using the agar well diffusion technique. To estimate antibacterial activity, the zone of inhibition was measured. Different concentration of AgNPs, i.e., 1 mg/ml, 2 mg/ml, 3 mg/ml, and 4 mg/ml (prepared in DMSO), was used against MDR *P. aeruginosa* (Fig. 15). The Maximum zone of inhibition was measured at 62.5 µg/ml, while the minimum zone of inhibition was measured at 7.8 µg/ml (Table 11). No zone of inhibition was observed against DMSO. This indicates that the antibacterial activity of AgNPs is concentration dependent. As concentration increases zone of inhibition also increases.

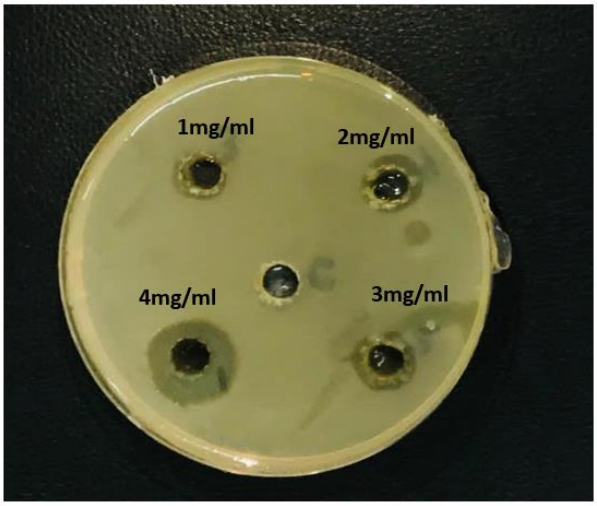


Fig.15: Zone of inhibition of AgNPs by agar well diffusion

Table 11: Zone of inhibition of AgNPs against *P. aeruginosa* by agar well diffusion method

Bacterial isolates	Zone of inhibition at different concentration				DMSO (Control)
	4mg/ml	3mg/ml	2mg/ml	1m/ml	
10	12mm	9mm	5mm	2.5mm	No zone
24	11.5	8mm	6mm	2mm	No zone
32	12.5mm	9mm	5.5mm	2.5mm	No zone
54	12mm	10mm	5mm	3mm	No zone
61	11mm	9.5mm	4mm	2mm	No zone
74	13mm	10mm	6mm	3.5mm	No zone
96	12.5mm	9mm	5.6mm	3mm	No zone

Minimum Inhibitory Concentration:

The minimum inhibitory concentration that inhibits the growth of microorganisms is called MIC. MIC of AgNPs was evaluated in a microtitration (96 well) plate, and results were evaluated by visible eye or via optical density at 600nm.

MIC of *P. aeruginosa* was found at 250 µg/ml of AgNPs. Results from the OD value are shown in Table 12. The results revealed a distinct pattern in the optical density readings of the wells. From well 4 onwards, an increase in optical density was observed, indicative of bacterial growth and turbidity. In contrast, wells 1 to 3 exhibited a decrease in optical density, suggesting that the presence of AgNPs effectively inhibited bacterial growth. Furthermore, a notable difference was observed within the first three wells. Well, 1 exhibited a higher optical density compared to wells 2 and 3. This discrepancy can be attributed to the higher concentration of AgNPs present in well 1, resulting in a stronger inhibitory effect on bacterial growth. These findings demonstrate the effectiveness of AgNPs in impeding bacterial growth, as evidenced by the decrease in optical density in wells 1 to 3. The subsequent increase in optical density from well 4 onwards indicates the absence of inhibitory effects, allowing for bacterial growth and turbidity to occur.

Table 12: Interpretation of MIC of AgNPs.

Isolate No.	Concentration of AgNPs											
	1000	500	250	125	62.5	31.2	15.6	7.8	3.9	1.95	P.C	N.C
10	0.451	0.324	0.198	1.307	1.458	1.656	1.695	1.706	1.721	1.749	1.847	0.043
24	0.323	0.311	0.211	1.369	1.388	1.403	1.406	1.473	1.567	1.598	1.788	0.032
32	0.435	0.367	0.276	1.105	1.154	1.209	1.274	1.319	1.432	1.545	1.877	0.045
54	0.476	0.318	0.295	1.264	1.288	1.292	1.328	1.481	1.51	1.61	1.687	0.024
61	0.387	0.267	0.214	1.357	1.362	1.435	1.44	1.518	1.534	1.548	1.662	0.033
74	0.312	0.297	0.206	1.318	1.368	1.456	1.497	1.668	1.677	1.734	1.767	0.029
96	0.412	0.378	0.165	1.254	1.394	1.471	1.488	1.569	1.673	1.698	1.752	0.054

Minimum Bactericidal Concentration:

The minimum bactericidal concentration was the amount of AgNPs that kill bacteria. MBC was found by spreading 50µl of solution from MIC and below MIC well on MH agar plates. Plates that exhibited no visible growth after 24 hours of incubation were identified as representing the minimum bactericidal concentration (MBC). In our study, the results indicated that the Muller Hinton Agar (MHA) plate containing 250 µg/ml of the test substance effectively inhibited the growth of P. aeruginosa, as no visible growth was observed on this particular plate (Fig. 16).

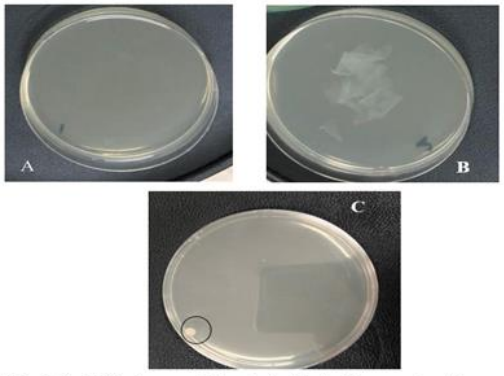


Fig.16: Minimum Bactericidal Concentration of AgNPs against *P. aeruginosa* showing (A) 1000 µg/ml, (B) 500 µg/ml, (C) 250 µg/ml

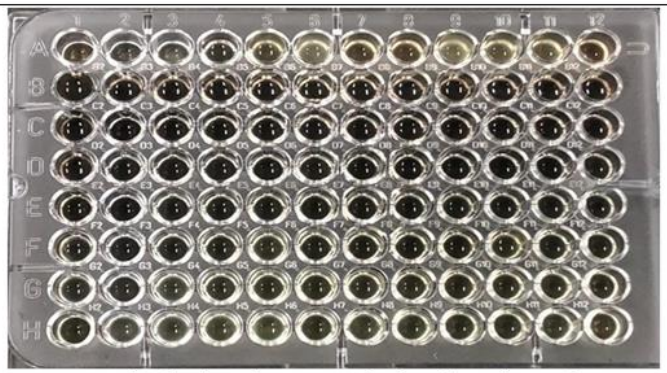


Fig. 17: Checkerboard assay of ciprofloxacin and AgNPs against *P. aeruginosa.*

Synergistic Effect of AgNPs with Drugs*:*

The checkerboard method was utilized to evaluate the combined effect of AgNPs and antibiotics. In this assay, only nanoparticles are serially diluted in column 1, while only antibiotics (ciprofloxacin, gentamicin, and colistin) are serially diluted in row 1 (Table 13-15). The rest of the wells have a serial dilution of both AgNPs and antibiotics in which antibiotic concentration decreases horizontally, and the dilution of nanoparticles decreases vertically. The MIC alone was used to calculate the FIC index to find the synergistic effect of synthesized AgNPs when combined with the drugs. The FIC index was calculated for P. aeruginosa, demonstrating a strong synergistic effect between antibiotics and AgNPs (Fig. 17). This synergy indicates that the combined application of these agents results in a highly effective inhibition of bacterial growth for P. aeruginosa.

Table 13: Interpretation of checkerboard assay of Sample 10.

Agents	MIC (µg/ml)		FIC of Antibiotic	FICI of AgNPs	FICI	Interpretation
	Alone	Combination				
Colistin	16	4	0.25	0.124	0.374	Synergism
NPs	250	31.2				
Gentamicin	64	16	0.25	0.25	0.5	Synergism
NPs	250	62.5				
Ciprofloxacin	16	8	0.5	0.124	0.624	Partial Synergism
NPs	250	31.2				

Table 14: Interpretation of checkerboard assay of Sample 32.

Agents	MIC (µg/ml)		FIC of Antibiotic	FICI of AgNPs	FICI	Interpretation
	Alone	Combination				
Colistin	16	8	0.5	0.06	0.56	Synergism
NPs	250	15.2				
Gentamicin	32	16	0.5	0.06	0.56	Synergism
NPs	250	15.2				
Ciprofloxacin	8	4	0.5	0.249	0.749	Partial Synergism
NPs	250	62.4				

Table 15: Interpretation of checkerboard assay of Sample 74.

Agents	MIC (µg/ml)		FIC of Antibiotic	FICI of AgNPs	FICI	Interpretation
	Alone	Combination				
Colistin	16	8	0.5	0.06	0.560	Synergism
NPs	250	15.2				
Gentamicin	64	16	0.25	0.124	0.374	Synergism
NPs	250	31.2				
Ciprofloxacin	16	8	0.5	0.219	0.749	Partial Synergism
NPs	250	62.4				

DISCUSSION

One of the leading pathogens that can grow on any surface with very low growth requirements is *P. aeruginosa*. As an opportunistic pathogen it can grow in different types of wounds, ICU, and immunocompromised patients (Ijaz *et al*., 2019). Harsh or misuse of antibiotics administration leads to the occurrence of MDR *P. aeruginosa*, which is regarded as a red alert (Mohamed, 2019). It is a prevalent pathogen, accounting for a substantial proportion (38%) of such infections (Ijaz *et al*., 2019). More than 7 million people are annually affected by *P. aeruginosa* (Haghi *et al*., 2018). Biofilm producing ability of *P. aeruginosa* make its association more strong with chronicity (Farva & Bhutta, 2021). Out of a total of 120 clinical samples, *P. aeruginosa* was found in 46 of them (33.33%). Our study's findings were similar to those of Farva and Bhutta (2021), who reported a prevalence of 30%, and Ijaz *et al*. (2019), who reported a prevalence of 38%, but they were lower than those of Nasser *et al*. (2018), who reported a prevalence of 46%. Several classes of antibiotics have been described as being utilized as anti-pseudomonas medications in various research (Nasser *et al*., 2018; Bazghandi *et al*., 2020; Abdulhaq *et al*., 2020). We used the following antibiotics in this study: imipenem (10 g), meropenem (10 g), cefepime (30 g), ciprofloxacin (5 g), amikacin (30 g), ceftriaxone (30 g), ceftazidime (30 g), and gentamycin (10 g). The most often prescribed antibiotics for *P. aeruginosa* therapy were amikacin (30 mg), colistin (10 mg), and piperacillin (30 mg). The disc diffusion technique was used to determine the antibiotic susceptibility pattern of the aforementioned medications. Gentamycin (83%), meropenem (85%), and ceftriaxone (87%) showed the highest levels of resistance among *P. aeruginosa* isolates in the antimicrobial susceptibility pattern, followed by ciprofloxacin (65%), cefepime (70%), imipenem (76%), cefoxitin (65%), ampicillin (67%), amikacin (61%), colistin (33%), and piperacillin (59%). Overuse of antimicrobials may contribute to the alarming rise of antibiotic-resistant infections. Furthermore, mobile genetic elements play a crucial role in the acquisition and loss of resistance genes, which is a key factor in the emergence of MDR isolates. Results are consistent with those found by Nasser *et al*. (2018), who found that 65.2% of isolates were resistant to ciprofloxacin, 85.0% to Gentamycin, 76% to ceftazidime, and 71.0% to cefepime. The results are lower than the observation of Abdulhaq *et al*. (2020) with imipenem (90.38%), meropenem (92.3%), and ceftriaxone (94.3%).In our study, 70% of isolates showed resistance to one or more antimicrobial agents, and all were multi-drug resistant. As compared to other studies, MDR isolates were (16.5%) by Mirzaei *et al*. (2020), (38.46%) by Abdulhaq *et al*. (2020), (65.2%) by Nasser *et al*. (2018),(52%) by Bazghandi *et al*. (2020) and (100%) by Akinloye *et al*. (2021).To overcome these complications and antibiotic resistance, scientists have focused on nanomaterials to solve this challenge for their use in the medical industry for the last 30 years. Nanoparticles have an important role in different fields of biology and medicine (D'Lima *et al*., 2020). According to this data, the present study shows better performance than the study conducted by Abdullah *et al*. (2021), where they reported a mean zone of 8 mm against *P. aeruginosa* by agar well diffusion method. Another study by Ulagesan *et al*. (2021) determined the antibacterial activity of AgNPs against *P. aeruginosa*. They recorded a maximum zone of 24 mm at 50 mg/ml and a minimum zone of 12 mm at 4 mg/ml. Their results were consistent with the present study findings as they also reported a mean zone of 12 mm at 4 mg/ml concentration. In addition, Lefta *et al*. (2016) investigated the activity of AgNPs against *P. aeruginosa* using different concentrations (1,2,4,8 and 16) mg/ml. They recorded (7, 9, 11, 7, and 22) mm, which indicates an increase in the zone of inhibition with the increase of AgNPs concentration. As agar well diffusion method is a qualitative assay and do not tell the exact value of inhibition of microorganism. Thus, MIC and MBC were conducted because they are quantitative techniques and give exact

values that inhibit and kill the microorganisms. Broth microdilution assay carried in 96 well plates was performed to calculate MIC and MBC. For MIC, 1 mg/ml concentration of AgNPs was used as starting concentration to make two-fold serial dilution, and MBC was determined by sub-culturing the solution from MIC well and below MIC wells on Muller Hinton agar plates. Results of MIC and MBC showed 250 µg and 500 µg for *P. aeruginosa.* MIC points were also seen visually, with bacterial growth showing turbidity, while wells with no visible growth showed no turbidity. Absorbance value (OD600) was also recorded, which indicates an increase in absorbance below 250 µg concentration. As compared to the study conducted by Ulagesan *et al*. (2021) recorded MIC and MBC of AgNPs 200 µg and 400 µg, respectively, for *P. aeruginosa.* The results of our study were less than the results recorded by Abdolhosseini *et al*. (2019). Danjuma and Abdullah (2021) recorded MIC of AgNPs 500 µg/ml against both gram-positive and gram-negative bacteria. The aim of the study was to check the synergistic effect of AgNPs with various antibiotics for different strains of *P. aeruginosa* isolated from surgical or burn wounds. *P. aeruginosa* strains that were used in our study showed resistance to multiple classes of drugs; however, these antibiotics may show synergistic effects when combined with nanoparticles. Results showed that drugs combined with silver nanoparticles have a good synergistic effect. The synergistic effect between AgNPs and various antibiotics was determined using the FIC (Fractional Inhibitory Concentration) index. The findings of the present study revealed notable synergism between AgNPs and colistin (mean FIC index: 0.49) as well as gentamicin (mean FIC index: 0.470). Additionally, a partial synergistic effect was observed with the combination of AgNPs and ciprofloxacin (mean FIC index: 0.70).

However, it is important to note that the results of the ciprofloxacin and AgNPs combination showed a slight antagonistic effect, indicating reduced effectiveness when used together, as opposed to the synergistic effects observed with the other antibiotic combinations.

These results provide valuable insights into the interactions between AgNPs and different antibiotics, highlighting the potential for enhanced efficacy and therapeutic benefits through synergistic combinations. The slight antagonistic effect observed in the ciprofloxacin and AgNPs combination warrants further investigation and consideration when developing treatment strategies (Mohamed, 2019). They recorded the FIC index of AgNPs with ciprofloxacin (0.5), which shows complete synergism. Likewise, another research conducted by Abdolhosseini *et al*. (2019) showed complete synergism of ciprofloxacin with AgNPs. The study conducted by Fadwa *et al*. (2021) showed that colistin showed a complete synergistic effect against *P. aeruginosa,* while ciprofloxacin showed partial synergism against clinical strains of *P. aeruginosa.* Esmaili and Hosseini Doust (2019) concluded in their research that AgNPs increased the antibacterial activity of gentamicin when both are used in combination. The best synergistic effect was observed when both were mixed equally at their MIC level. Colistin, as described (Dupuy *et al*., 2018), is a cationic polypeptide antibiotic that disrupts the bacterial outer membrane. The findings of the present research work showed that a combination of AgNPs and drugs (colistin, ciprofloxacin, and gentamicin) can be used as a strong antimicrobial agent against MDR *P. aeruginosa* (Tasleem *et al*., 2022). No resistance, even by a single isolate of *P. aeruginosa,* against AgNPs alone or in combinations of AgNPs with drugs was observed. Thus these nanoparticles could be used in combination with diverse antimicrobial agents for enhanced and significant antimicrobial effects against MDR bacteria. Moreover, to use these nanoparticles as a safe medicine, possibly for the treatment of human bacterial infection, there is a need to perform cytotoxic assays on numerous human cell lines (Jalal *et al*., 2023).

CONCLUSION

P. aeruginosa is among the most

critical pathogens declared by WHO. Due to the increase in antibiotic resistance, now the world is moving toward using nanotechnology to overcome all these issues. It is shown by this research that infections caused by MDR *P. aeruginosa* can be overcome by using nanoparticles in combination with drugs. In the current study, a total of 120 samples were collected from surgical or burn wounds of patients 46 (38.3%) were positive, and 74 (61.7%) were negative for *P. aeruginosa*. Antimicrobial susceptibility test results showed that out of 46 positive isolates, 33 isolates of *P. aeruginosa* were multi-drug resistant (MDR). These isolates were further used in this study to check the antibacterial action of AgNPs individually or along three drugs. Results indicate that activity was higher when a higher concentration was used, as the highest zone at 4 mg/ml concentration was 12 mm. As the concentration decreased, activity was also decreased, such as at 1 mg/ml concentration zone size was only 2.5 mm. In addition, MIC and MBC of AgNPs were recorded at 250 µg/ml and 500 µg/ml. All tested isolates had MIC at 250 µg/ml and MBC at 500 µg/ml. 3 Isolates out of all 27 MDR *P. aeruginosa* isolates were processed via checkerboard assay to check the combined effect of AgNPs with 3 drugs (colistin, ciprofloxacin, and gentamicin). According to current study results, all 3 isolates showed synergistic and additive.

REFERENCES

Abdolhosseini, M., Zamani, H., & Salehzadeh, A. (2019). Synergistic antimicrobial potential of ciprofloxacin with silver nanoparticles conjugated to thiosemicarbazide against ciprofloxacin resistant *Pseudomonas aeruginosa* by attenuation of MexA-B efflux pump genes. *Biologia, 74*(9), 1191-1196.

Abdulhaq, N., Nawaz, Z., Zahoor, M. A., & Siddique, A. B. (2020). Association of biofilm formation with multi drug resistance in clinical isolates of *Pseudomonas aeruginosa*. *EXCLI journal, 19*, 201.

Abdullah, S. R. A., Hasson, S. O., & Al-Yasari, A.-K. S. (2021). Antimicrobial effect of green synthesized silver nanoparticles (AgNPs) against multidrug resistant *Pseudomonas aeruginosa*. *Annals of the Romanian Society for Cell Biology*, 5033-5045.

Abo-Shama, U. H., El-Gendy, H., Mousa, W. S., Hamouda, R. A., Yousuf, W. E., Hetta, H. F., & Abdeen, E. E. (2020). Synergistic and antagonistic effects of metal nanoparticles in combination with antibiotics against some reference strains of pathogenic microorganisms. *Infection and drug resistance, 13*, 351.

Ahmed, O. B., & Dablool, A. (2017). Quality improvement of the DNA extracted by boiling method in gram negative bacteria. *International Journal of Bioassays, 6*(4), 53475349. Akinloye, A. O., Adefioye, J. O., Adekunle, C. O., Anomneze, B. U., Makanjuola, O. B., Onaolapo, O. J., . . . Olowe, O. A. (2021). Multidrug-Resistance Genes in *Pseudomonas aeruginosa* from Wound Infections in a Tertiary Health Institution in Osogbo, Nigeria. *Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders), 21*(1), 90-98.

Bayroodi, E., & Jalal, R. J. I. j. o. m. (2016). Modulation of antibiotic resistance in *Pseudomonas aeruginosa* by ZnO nanoparticles. *8*(2), 85.

Bazghandi, S. A., Safarirad, S., Arzanlou, M., Peeri-Dogaheh, H., Ali-Mohammadi, H., & Khademi, F. (2020). Prevalence of multidrug-resistant *Pseudomonas aeruginosa* strains in Ardabil. *Journal of Ardabil University of Medical Sciences, 20*(2), 280-286.

Bose, D., & Chatterjee, S. (2016). Biogenic synthesis of silver nanoparticles using guava (Psidium guajava) leaf extract and its antibacterial activity against *Pseudomonas aeruginosa*. *Applied Nanoscience, 6*(6), 895-901.

Boyanova, L. (2018). Direct Gram staining and its various benefits in the

diagnosis of bacterial infections. *Postgraduate medicine*, *130*(1), 105-110.

Danjuma, L., & Abdullahi, U. (2020). Antibacterial Efficacy Of Some Standard Antibiotics Discs Coated With Biologically Synthesized Silver Nanoparticles From Cassia Occidentalis Leaves. *Journal of Medicine and Health Research*, 47-53.

de Lacerda Coriolano, D., de Souza, J. B., Bueno, E. V., Medeiros, S. M. d. F. R. d. S., Cavalcanti, I. D. L., & Cavalcanti, I. M. F. (2021). Antibacterial and antibiofilm potential of silver nanoparticles against antibiotic-sensitive and multidrug-resistant *Pseudomonas aeruginosa* strains. *Brazilian Journal of Microbiology*, *52*(1), 267-278.

Dupuy, F. G., Pagano, I., Andenoro, K., Peralta, M. F., Elhady, Y., Heinrich, F., & Tristram- Nagle, S. (2018). Selective interaction of colistin with lipid model membranes. *Biophysical journal*, *114*(4), 919-928.

Esmaili, S., & Hosseini Doust, R. (2019). Synergistic effect of silver nanoparticles (AgNPs) and gentamicin against clinical isolates of Pseudomona aeruginosa. *Medical Science Journal of Islamic Azad Univesity-Tehran Medical Branch*, *29*(1), 64-70.

Fadwa, A. O., Alkoblan, D. K., Mateen, A., & Albarag, A. M. (2021). Synergistic effects of zinc oxide nanoparticles and various antibiotics combination against *Pseudomonas aeruginosa* clinically isolated bacterial strains. *Saudi Journal of Biological Sciences*, *28*(1), 928-935.

Farva, K., & Bhutta, G. A. (2021). Prevalence of gram-negative bacteria in infected burn wounds and antibiotic susceptibility pattern; A study of stable burn patients. *Professional Medical Journal*, *28*(10).

Haghi, F., Zeighami, H., Monazami, A., Toutouchi, F., Nazaralian, S., & Naderi, G. (2018). Diversity of virulence genes in multidrug resistant *Pseudomonas aeruginosa* isolated from burn wound infections. *Microbial pathogenesis*, *115*, 251-256.

Hassani Sangani, M., Nakhaei Moghaddam, M., & Forghanifard, M. M. J. N. J. (2015). Inhibitory effect of zinc oxide nanoparticles on *Pseudomonas aeruginosa* biofilm formation. *Nanomedicine Journal*, *2*(2), 121-128.

Hemmati, F., Salehi, R., Ghotaslou, R., Kafil, H. S., Hasani, A., Gholizadeh, P., & Rezaee, M. M. (2020). The assessment of antibiofilm activity of chitosan-zinc oxide- gentamicin nanocomposite on *Pseudomonas aeruginosa* and Staphylococcus aureus. *International Journal of Biological Macromolecules*, *163*, 2248-2258.

Hudzicki, J. (2009). Kirby-Bauer disk diffusion susceptibility test protocol. *American Society for Microbiology*, *15*, 55-63.

Ijaz, M., Siddique, A. B., Rasool, M. H., & Shafique, M. (2019). Frequency of multi drug resistant *Pseudomonas aeruginosa* in different wound types of hospitalized patients. *Pakistan journal of pharmaceutical sciences*, *32*(2), 865-870.

Jaiswal, S., & Mishra, P. (2018). Antimicrobial and antibiofilm activity of curcumin-silver nanoparticles with improved stability and selective toxicity to bacteria over mammalian cells. *Medical microbiology and immunology*, *207*(1), 39-53.

Jalal, N. A., Hariri, S. H., Momenah, A. M., Khan, S., & Bantun, F. (2023). Molecular detection of blaPER-1, blaVEB-1, and blaPSE-1 β-lactamase genes from *P. aeruginosa* Severe Urogenital UTI Infection. *Journal of Survey in Fisheries Sciences*, *10*(3S), 1628-1635.

Momenah, A. M., Hariri, S. H., Abdel-razik, N. E., Bantun, F., Khan, S., Alghamdi, S., & Tasleem, S. (2023). Aspergillus fumigatus-Mediated Biosynthesis of Silver Nanoparticles Efficiency, Characterization, and Antibacterial Activity Against Different Human Pathogens. *Egyptian Academic Journal of Biological Sciences. C, Physiology and Molecular Biology*, *15*(1), 353-364.

Tasleem, S., Moon, S., Alghamdi, S., Suliman, R. S., Ateeq, M., Salman, M., & Bantun, F. (2022). Presence of BlaPER-1 and BlaVEB-1 Beta-Lactamase Genes among Isolates of Pseudomonas Aeruginosa from Burn and Trauma Hospital Peshawar, Pakistan. *Journal of Bioresource Management*, *9*(2), 15.