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The Combating Impact of Biogenic Silver Nano Particles and Antibiotics on Combating Multidrug-Resistant Pseudomonas

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Abstract: ***This study aimed to assess the synergistic effect of silver nanoparticles (AgNPs) in combination with different antibiotics against multidrug-resistant Pseudomonas aeruginosa (MDR P. aeruginosa) infections. A total of 120 surgical or burn wound samples were collected from a tertiary care hospital. The samples were cultured on cetrimide agar plates and then heated to 370 degrees Celsius. The isolates were identified based on colony shape, Gram's staining, and various biochemical tests. The antibiotic susceptibility profile was determined using the Kirby-Bauer disc diffusion technique according to CLSI 2022 recommendations. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined. The antibacterial activity of AgNPs was evaluated using the agar well diffusion technique and the microdilution method. The synergistic effect of antibiotics and AgNPs was assessed using the Checkerboard method. Out of the 120 samples, 46 (38.8%) were confirmed as positive for P. aeruginosa, and among them, 33 were identified as MDR P. aeruginosa. Seven representative isolates were selected for further analysis. The antibacterial activity of AgNPs showed a maximum zone of inhibition of 12mm at 4mg/ml and a minimum of 2.5mm at 1mg/ml using the agar well diffusion method. The MIC and MBC of AgNPs indicated that all isolates were inhibited at a concentration of 250mg/ml. The Checkerboard results revealed that colistin and gentamicin exhibited complete synergism with AgNPs, while ciprofloxacin showed partial synergism with AgNPs.***

Keywords: Nano Particles, Multi Drug, Resistance, Bacterial Concentration.

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1. INTRODUCTION

Infections caused by antibiotic-resistant bacteria, such as Pseudomonas aeruginosa, have become a major public health concern worldwide. These bacteria have developed mechanisms to survive and persist despite the use of antimicrobial agents, leading to treatment failures and increased morbidity and mortality rates. Pseudomonas aeruginosa is a gram-negative bacterium that can cause infections in humans, animals, and plants. It is an opportunistic pathogen and exhibits resistance to various antibiotics commonly used in clinical settings.The bacterium possesses several virulence factors that contribute to its pathogenicity, including enzymes, toxins, and surface structures. These factors enable Pseudomonas aeruginosa to colonize host tissues, cause tissue damage, and evade the immune system. It can cause a range of infections, such as urinary tract infections, bacteremia, pneumonia, and wound infections, particularly in immunocompromised individuals.One of the challenges in treating Pseudomonas aeruginosa infections is its ability to develop resistance to antibiotics. The bacterium exhibits both intrinsic and acquired resistance mechanisms. Intrinsic resistance is due to factors such as reduced permeability of the bacterial cell membrane and efflux pumps. Acquired resistance can occur through horizontal gene transfer, allowing the bacterium to acquire resistance genes from other bacteria. Adaptive resistance, which occurs during persistent infections, is another obstacle to effective treatment. Moreover, nanoparticles have also demonstrated antibiofilm activity. Biofilms are structured communities of bacteria that are highly resistant to antibiotics. Nanoparticles can disrupt biofilm formation, reduce bacterial growth, and inhibit the production of extracellular polysaccharides, which are essential components of biofilms. This makes nanoparticles potentially useful for coating medical devices to prevent biofilm-associated infections.In summary, the rise of antibiotic-resistant bacteria, including Pseudomonas aeruginosa, poses a significant threat to public health. Nanoparticles have emerged as a promising tool in combating bacterial infections due to their antibacterial and antibiofilm properties. Further research is needed to optimize nanoparticle formulations, understand their mechanisms of action, and evaluate their effectiveness in clinical settings

2. MATERIALS AND METHODS

All the media and chemicals used in this study were highly pure and sterilized. All mediaand solution were prepared in distilled water before use. All glassware's were washed properlyfirst with tap water then distilled water and placed in hot air oven for sterilization before use.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. Identification of bacteria was done by observing morphological and cultural characteristics along with biochemical tests (Omer et al., 2020).Cetrimide agar is commercially available in powder from medium that is used to culture

Catalase test

Catalase test used to make sure that bacterium forms the catalase enzyme that hydrolyzeH2O2 into water and hydrogen gas or not.Microorganisms having catalase enzyme breaks the

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hydrogen peroxide into hydrogen and water with bubbling. Oxidation of flavoproteins is caused by hydrogen peroxide. Air bubble formation indicate positive result.

Oxidase test

Oxidase test is used to classify bacteria that can generate oxidase enzyme andcytochrome C. Cytochromes have a catalytic enzyme which is closely attached with cells of plasma membranes. Catalytic enzymes act a H2 shippers of electron and biological oxidation. Cytochrome bacteria produce oxidase enzymes. Oxidase enzyme catalyze cytochrome C. Bacteria that have cytochrome as a component of their respiratory chain change the reagent into blue color. This blue color indicates positive result which means oxidase positive. While those bacteria that do not have cytochrome c as component of their respiratory chain are colorless and indicate negative results, hence oxidase negative.

Citrate utilization test

Citrate utilization test was used to catalog the potential for the citrate usage of microorganisms.This test is used to make distinction among enteric bacteria on their potential to utilize their fundamental carbon sources. It is based on presence of citrate permease enzyme. This enzyme produced by the organism that support the cell to move the inner side.

Triple sugar iron Test (TSI)

The TSI test is used to observe either bacteria have ability to ferment glucose, lactose and sucrose, also its ability to contain hydrogen sulfide.This test depends on ability of a variety of microorganisms to modulate sulfur and to ferment carbohydrates. The fermentation of carbohydrates signals the formation of gas and color change of pH indicator from red to yellow.

Methyl Red Test

This test is used to distinguish between members of family Enterobacteriaceae andused to detect production of acid during fermentation of glucose (McDevitt, 2009). The MR test is used to ascertain whether or not a given organism is capable of producing and maintaining a stable acidic end product from glucose fermentation.

Urease Test

This test used to identify those organisms that are capable to hydrolyze urea into ammonia and carbon dioxide (Brink, 2010).Urease enzyme convert urea to ammonia and carbon dioxide. Urea is the most important waste produced through protein digestion by majority of vertebrates and is excreted in urine. Urease enzyme attack amide linkage stimulating ammonia.

Indole test

This test determines the ability of organism to extract amino acid tryptophan and createindole. It is part of IMViC procedures. That are used to distinguish between members of family Enterobacteriaceae (MacWilliams, 2012).Tryptophan is an important amino acid that undergo hydrolysis by different bacteriathat express tryptophanase enzyme.

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Molecular Identification

All the positive isolates were further confirmed through Polymerase Chain Reaction (PCR) by amplification of bacterial DNA using OprL Gene specific primer.Genomic DNA from bacterial cells are extracted which are used in molecular analysis. Lysis of bacterial cell is done by using chemical or physical method. Proteinase K or lysozyme is used for lysis of gram-negative bacteria. In this study DNA extraction was done by boiling method according to protocol of (Ahmed & Dablool, 2017). Isolated DNA then directly used for PCR.Broth culture of bacteria was transferred into Eppendorf tube and centrifuged for 10 minutes at 13000 rpm.Supernatant was discarded.Pallet was resuspended with Normal saline or PBS buffer and centrifuged again at 13000 rpm for 10 mins.Again, supernatant was discarded.Pallet was resuspended again in Normal saline or PBS and boiled for about 10 min at95 C for 15 mins.Immediately Eppendorf tubes were transferred into ice box for 30 in and centrifuged for 10 min at 13000 rpm.The supernatant was collected and transferred to fresh Eppendorf tube.Eppendorf tubes then stored at -20 C.

Polymerase Chain Reaction

Table: 1 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)

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

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RNase-free water	8.5 µl	1 µl
Total volume	25 µl	1 µl

Agarose Gel Electrophoresis

Gel electrophoresis is separation of DNA on basis of their size. Negatively charge DNAmove towards positive electrode in the presence of electric current. Genomic DNA was visualized to check the quality and purity by gel electrophoresis. Agarose gel was prepared to undergo electrophoresis (Lee et al., 2012).

Table: 3 Ingredients for preparation of Gel

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

Preparation of Agarose Gel

Gel tray including gel was placed into electrophoresis tank and gel was covered by adding 0.5X TBE buffer.

Antimicrobial susceptibility testing (AST)

Kirby Bauer disc diffusion technique using several antibiotic disc (meropenem 10g, imipenem 10g, colistin 10g, Amikacin 30g, ciprofloxacin 5g, and ceftazidime 30g) 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 is very important; it is suspension of either barium sulfate or latex particles which help to estimate bacterial density. The test suspension turbidity compared with McFarland standards (Hudzicki, 2009). A 0.5 McFarland standard containing 1.5×10^8 CFU was set by mixing 0.05 ml solution of 1.175% barium chloride and 9.95 ml solution of 1% sulfuric acid. After mixing, turbidity of solution

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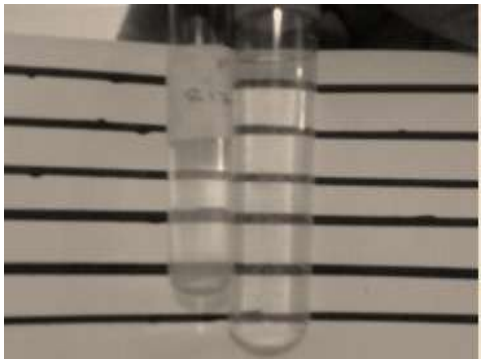

Fig: 1. Prepare bacterial inoculum according to McFarland standards

was adjusted between 0.08-0.1 OD by taking
the absorbance at 625nm.

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

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. The results readings were carried out by placing the plate in the dark background and reflected light. A ruler used to measure diameter of zone for each antimicrobial agent. Analysisof result were done according to CLSI guidelines 2022.

Determination of minimum inhibitory concentration (MIC) of antibiotics
When trying to stop the spread of germs, the best place to start is with the lowest effective dose of antibiotic possible, known as the minimum inhibitory concentration (MIC). drugs' antimicrobial effects (Colistin, Gentamicin, ciprofloxacin, Amikacin, ceftazidime and cefepime) was determined by measuring the MIC value using 2 folds broth microdilution method of (Parvekar et al., 2020) in 96 well plate.

Preparation of AgNPs concentrations
Three different concentrations of AgNPs including 20mg, 15mg, 10mg and 5mg 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. Solution of AgNPs was then sonicated for 2 hours to completely dissolve nanoparticles. These concentrations were then used for different antimicrobial tests.

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Agar well diffusion method

Effect of different concentrations of AgNPs was tested against MDR strains of Pseudomonas aeruginosa. Agar well diffusion method of (Prasad et al., 2011) was used to check antibacterial potential. To perform this procedure, 24 hours old bacterial cultures were used to prepare inoculum of 0.5 McFarland. Sterile cotton swab was used to spread 100 µl of bacterial culture on surface of Muller Hinton agar then left for 5 minutes to settle down. After swabbing 4 wells of 5mm diameter were punctured at appropriate positions with the help of steel well borer. Each time a well was bored, well borer was red hot and allowed to cool.

Preparation of Muller Hinton Broth (MHB)

Muller Hinton broth was used for growth of bacteria as general-purpose media. Media was prepared by taking 10.5 g MHB and dissolved in 500ml of distilled water in a flask. Media was then autoclaved for sterilization at 121 ^{o}C for 30 min. After autoclave let it cooled down and stored at 4 ^{o}C for further use.

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). Antimicrobial activity of AgNPs was determined by measuring the MIC value using 2 folds broth microdilution method of (Parvekar et al., 2020) in 96 well plate. A stock solution of 1mg/ml of AgNPs (prepared in DMSO) was used to determine MIC value against Pseudomonas aeruginosa. 24 hours old bacterial culture was used to prepare 0.5 McFarland. Next, on a clean microtiter plate, 100 l of MHB was put into each well, followed by 100 l 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 l from well 10 was thrown away. Each well had 100 l 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 degrees Celsius for 18 to 24 hours. An OD value of 620nm was measured both before and after incubation.

Determination of minimum bacterial concentration (MBC)

Minimum bacterial concentration (MBC) determined 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 glass spreader. Incubate these plates at 37 ^{o}C for 24 to 48 hours and observed for bacterial growth. Plates that contain no visible growth were determined as MBC.

Checkerboard assay to estimate Synergistic effect of AgNPs and antibiotics

3. RESULTS AND DISCUSSION

To isolate the bacterium, it was cultured on cetrimide agar. On cetrimide agar Pseudomonas aeruginosa showed large smooth, yellow to green colonies with green pigmentation of

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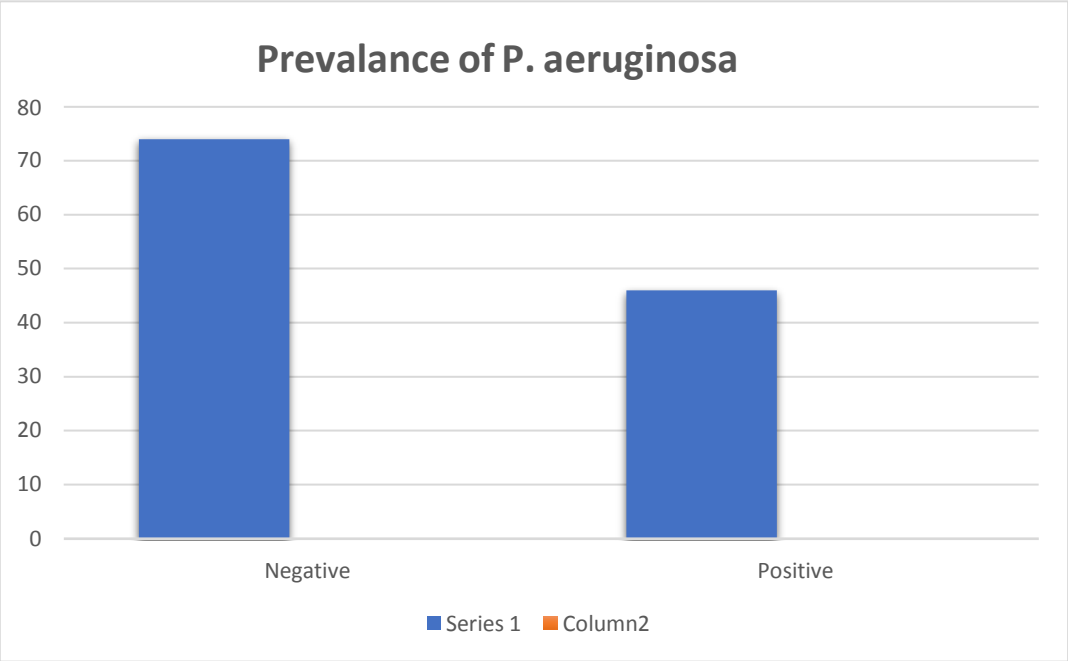

Fig: 4 .Prevalence of P. aeruginosa from surgical or burn wounds

pyocyanin after incubation at 37°C for 24- 48 hours. Out of 120 samples 46 (38%)were P. aeruginosa positive.

Gram's staining

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

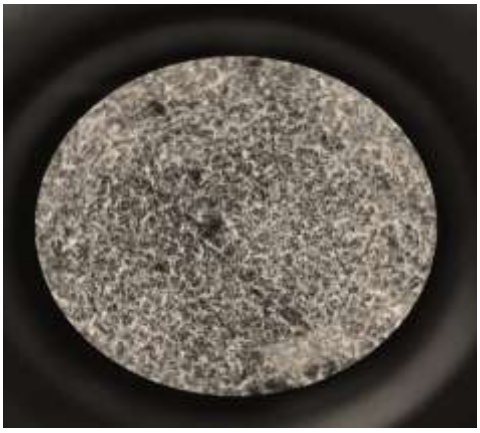

Fig: Gram negative rods of P. aeruginosa (100X)

Biochemical characterization

All the isolates were subjected to biochemical testing. Following biochemical tests were performed i.e. catalase, oxidase, urease, citrate utilization test, triple sugar iron test, methyl

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red, Voges Proskauer and Indole.

Table: Biochemical tests for identification of Pseudomonas 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. Oxidase Positive

Molecular Identification

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

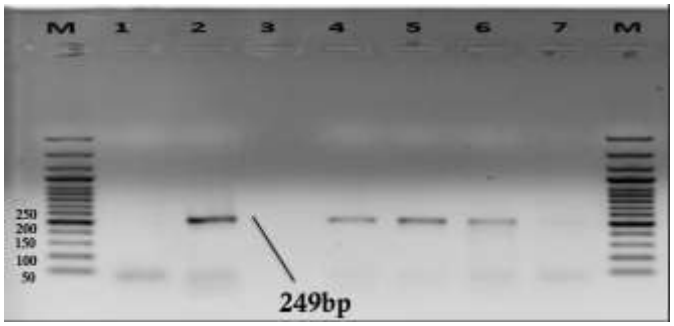

Fig: Gel electrophoresis results of OprL gene

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Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing was performed to test weather under study Pseudomonas aeruginosa is Multi drug resistant (MDR) or not. To perform this Muller Hinton agar plates were prepared and swabbed with bacterial inoculums. Antibacterial activity of biologically produced silver nanoparticles against multidrug-resistant P. aeruginosa was shown using the agar well diffusion technique. To estimate antibacterial activity, zone of inhibition was measured. Different concentration of AgNPs i.e. 1mg/ml, 2mg/ml, 3 mg/ml and 4 mg/ml (prepared in DMSO) was used against MDR P. aeruginosa. Maximum zone of inhibition was measured at 62.5 µg/ml while minimum zone ofinhibition was measured at 7.8 µg/ml. No zone of inhibition was observed against DMSO. This indicate that antibacterial activity of AgNPs are concentration dependent. As concentration increases zone of inhibition also increases.

Table: 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**	**1mg/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

Minimum inhibitory concentration that inhibits growth of microorganism is called MIC. MIC OF AgNPs was evaluated in 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 Ag NPs. Results from OD value shown in Table 12 below. Results showed that optical density was increasing in well 4 to onward which was due to turbidity of bacterial growth while wells at right from 1 to 3 showed a decrease in optical density that indicate growth of bacteria was inhibited by AgNPs. Well 1 showed more optical density than well 2 and 3 which is due to concentrated amount of AgNPs in well one.

Table 1: 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

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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

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 showed no visible growth after 24 hours were considered as MBC. Our result showed no growth on MHA plate that contained 250 µg/ml for P. aeruginosa.

Synergistic effect of AgNPs with drugs

The Combined effect of AgNPs along with antibiotics was estimated by checkerboard method. In this assay column 1 contain serial dilution of nanoparticles only, while row 1 contain serial dilution of antibiotics (Ciprofloxacin, Gentamicin and Colistin) only. Rest of the wells have serial dilution of both AgNPs and antibiotics in which antibiotic concentration decreasing horizontally and dilution of nanoparticles decreasing vertically. The MIC alone was used to calculate the FIC index to find the synergistic effect of or synthesized AgNPs when combined with the drugs. FIC index calculated for P. aeruginosa which shows combined effect of antibiotic and AgNPs are in good synergism for those bacteria.

Table: Interpretation of checkerboard assay of Sample 10

Agents	MIC (µg/ml)		FIC of Antibiotic	FICI of AgNPs	FICI	Interpretation
	Alone	Combination				
Colistin NPs	16 250	4 31.2	0.25	0.124	0.374	Synergism

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Gentamicin NPs	64 250	16 62.5	0.25	0.25	0.5	Synergism
Ciprofloxacin NPs	16 250	8 31.2	0.5	0.124	0.624	Partial Synergism

Table: Interpretation of checkerboard assay of Sample 32

Agents	MIC (µg/ml)		FIC of Antibiotic	FICI of AgNPs	FICI	Interpretation
	Alone	Combination				
ColistinNPs	16 250	8 15.2	0.5	0.06	0.56	Synergism
Gentamicin NPs	32 250	16 15.2	0.5	0.06	0.56	Synergism

4. DISCUSSION

Pseudomonas aeruginosa is a highly adaptable pathogen that can thrive on various surfaces and is particularly problematic in wounds, intensive care units, and immunocompromised patients. Misuse of antibiotics has led to the emergence of multidrug-resistant (MDR) P. aeruginosa, which poses a serious threat. MDR strains are difficult to treat with antibiotics and are associated with severe infections such as those acquired from medical devices or burn and surgical wounds. P. aeruginosa is a common pathogen in hospital settings and accounts for a significant percentage of skin and wound infections, which are major causes of hospital-associated deaths.To address the challenge of antibiotic resistance, researchers have explored the potential of nanomaterials in the medical field for the past three decades. Nanoparticles have shown promise in the diagnosis and treatment of various diseases, including infections that affect multiple organs in the body. In the case of P. aeruginosa, nanoparticles can be used as a potential treatment option. In this study, the antibacterial activity of silver nanoparticles (AgNPs) against MDR P. aeruginosa was investigated.

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5. CONCLUSION

Pseudomonas aeruginosa, recognized as one of the most critical pathogens by the World Health Organization, poses a significant threat to patients and healthcare workers in hospital settings. Traditional antibiotic usage against P. aeruginosa often leads to the emergence of multi-drug resistant (MDR) bacteria by eliminating beneficial normal flora. To address these challenges, researchers are now turning to nanotechnology as a potential solutionThe results indicated synergistic and additive interactions between AgNPs and colistin, AgNPs and gentamicin, while the combination of AgNPs and ciprofloxacin exhibited partial synergism. To minimize potential toxic effects in humans, it may be significant to reduce the concentrations of both AgNPs and drugs within the recommended dose interval for these combinations. In conclusion, this study demonstrated the ability of AgNPs to modulate the antibiotic susceptibility of P. aeruginosa. AgNPs exhibited excellent antibacterial activity and enhanced the effectiveness of drugs when used in combination. This combination therapy holds promise for the treatment of diseases caused by MDR P. aeruginosa. As a future perspective, this research could be extended to explore other pathogenic MDR, extensively drug-resistant (XDR), or pandrug-resistant (PDR) organisms. Furthermore, the effectiveness of nanoparticles, apart from AgNPs, should be tested for their antibiofilm and antioxidant properties. Cytotoxic activity assessments and in vivo studies on AgNPs may also be conducted to evaluate their therapeutic safety.

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