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Study for Possible Synergism of Bio Produced Silver Nanoparticles with Carbapenem Antibiotics against Carbapenem Resistant *Klebsiella pneumoniae* using *In Vitro* and *In Silico* Approaches

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Abstract: The emergence of Carbapenem-resistance among Enterobacteriaceae has been increasingly reported worldwide. The most common form of Carbapenem resistance in *Klebsiella pneumoniae* is carbapenemase production usually associated with prolonged use. This necessitates an alternate way to treat *K. pneumoniae* infections. Molecular docking is computational method in computer-aided drug designing helps in the right choice of antibiotics from the available arsenal. Silver nanoparticles attracted the attention in different fields due to their promising applications in the health care sector. In this study, we have produced and characterized AgNPs from *Bacillus subtilis* NCIM 2920 by fermentative method. *In silico* molecular docking was used to estimate the probable binding orientation of Carbapenem with the *K. pneumoniae* carbapenemase 2 (KPC-2) which confers resistance. The antimicrobial activity of the carbapenems and β-lactam antibiotics alone and in combination with synthesized AgNPs was evaluated against *K. pneumoniae* NCIM 2957, NCIM 5656 and clinical isolates by disc diffusion method. Minimum inhibitory concentration of the selected antibiotics was also determined using serial dilution method.

Keywords: *Klebsiella pneumoniae*, Silver nanoparticles, Carbapenem resistance, Molecular Docking

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Introduction

The global use and abuse of antibiotics have led to the development and spread of bacterial resistance to all regularly used antibiotics. To effectively tackle the spread of Antimicrobial Resistance (AMR) we should develop novel antimicrobial compounds/ treatment options. The composition, structure, and interactions within microbial populations affect the Horizontal Gene Transfer of antibiotic resistance, it is becoming evident that intra- and interspecies interactions

also influence how species respond and evolve under antibiotic exposure within microbial populations. As a result, susceptibility to antibiotics may not translate well to the effective treatment of polymicrobial infections (Prestinaci *et al*., 2015). β-Lactam antibiotics act on the cell wall of a bacteria. These antibiotics bind and inhibit the carboxypeptidases and transpepti-dases. The cell wall synthesizing enzymes also called the penicillin-binding proteins (PBPs) catalyze the D-alanine cross-linkages of the peptidoglycan wall surrounding the bacterium leads weakening of the cell wall structure (Zhu *et al*., 2020).

Silver Nanoparticles (*AgNPs*)

Silver Nanoparticles (AgNPs) have received particular interest, especially in biomedicine. AgNPs are famed for their broad-spectrum activity. Numerous studies focus on the synthesis of AgNPs with controlled size and shape, and a variety of specific synthetic methods have been developed, including physical, chemical, and biological methods. Biological synthesis can obtain AgNPs with uniform size distribution and high purity. Chemical synthesis is the most commonly used method to get AgNPs and it pollutes the environment (Vikram *et al*., 2021). Biological method proves an economical and eco-friendly approach for AgNPs production. Microorganisms include bacteria, fungi and algae are widely used in biological synthesis (Bakthavatchalam *et al*., 2020).

Although the exact mechanism of AgNPs antimicrobial effects has not been entirely clarified, various antimicrobial actions have been proposed. AgNPs can continually release silver ions, which may be considered the mechanism of killing microbes. Owing to the electrostatic attraction and affinity to sulfur proteins, silver ions adhere to the cell wall and cytoplasmic membrane (Kannan *et al*., 2011).The adhered ions can enhance the permeability of the cytoplasmic membrane and disrupt the bacterial envelope. Reactive oxygen species can be a principal agent in the provocation of cell membrane disruption and deoxyribonucleic acid (DNA) modification. As sulfur and phosphorus are essential components of DNA, the interaction of silver ions with the sulfur and phosphorus of DNA can cause DNA replication, cell reproduction, or even result in the termination of the microorganisms (Rossello and Gabriel, 2017)

The Protein Data Bank is a database for the 3D data of biological molecules, such as proteins and nucleic acids. Molecular docking is a practical tool used in the drug discovery field to investigate the binding compatibility of molecules ligands to target receptor (Lan *et al*., 2019).

In this study, we have produced and characterized AgNPs from *Bacillus subtilis* NCIM 2920 by fermentative method. *In silico* molecular docking was used to estimate the probable binding orientation of Carbapenem with the *K. pneumoniae* carbapenemase 2 (KPC-2) which confers resistance.

Materials and Methods

Procurement of strains

Reference strains of *K. pneumoniae* NCIM 2957 and 5656 were procured from the National Chemical Laboratory, Pune, and Clinical isolates of *K. pneumoniae* (n=60) were procured from a Multi-Specialty Corporate Ramakrishna Hospital, Coimbatore. Three to five well-isolated colonies of the same morphological type were selected from selective media and the broth culture was incubated at 37°C until it achieved the turbidity of the 0.5 McFarland standards (Balouiri *et al*., 2016; Dolinsky and Arthur, 2017).

In silico studies

Smiles of the molecular structures for a range of antibiotics were downloaded from PubChem and converted using an online smiles translator into their 3D PDB format. The preparation of the target proteins was downloaded from the PDB data bank with AutoDock 4.2. Software was used to add all hydrogen atoms to the macromolecule, which was necessary to calculate partial atomic charges correctly. In the Auto Grid procedure, the target

enzyme was embedded on a 3D grid point. AutoDock 4.2 would be run to get various docked conformations and analyse the predicted docking energy. The binding sites for these molecules would be selected based on the ligand-binding pocket of the templates. The selected Antibiotics would be docked with the target proteins. The docking orientations, binding interactions, binding energy and intermolecular energy were calculated based on the docking parameters (Meng *et al*., 2011)

Fermentative production of Silver nanoparticles

Culture containing flasks were placed in a shaking incubator at 37°C at 150 rpm for 24 and 48 h. After 24 h of bacterial growth, the liquid broth containing grown culture was centrifuged for 20 min at 8000 rpm to separate the bacterial culture and supernatant. The same procedure was adopted for 48 h flask (El-Bendary *et al*., 2020). For the synthesis of AgNPs, 1 mM sterile silver nitrate was treated with the bacterial supernatant solution (1:1). The reaction mixture and control tubes were placed under dark condition in an incubator at 150 rpm. After 24 and 48 h, the reduction of the silver ions to silver atoms was observed. Control samples without culture supernatant were placed under the same conditions as the supernatant reaction mixture. The extracellular synthesis of AgNPs was monitored by observing the colour change from pale yellow to deep brown (Kannan *et al*., 2011).

Characterization

UV–Visible investigation: The biosynthesized AgNPs were characterized by spectral absorption scan using double beam UV–Visible spectrophotometer (Jasco V 680) at a resolution of 1 nm in a wavelength range between 300 and 700 nm.

Sensitivity testing of the Reference and clinical isolates of K. pneumoniae: As per CLSI guidelines, the disc diffusion test was carried out for all the isolates. Plates were prepared and incubated at 37°C for 24 h and zone of inhibitions were measured using zone reader (Dolinsky and Arthur, 2017).

Minimum Inhibitory Concentration determination: Serial two-fold dilutions of the antimicrobial agent were made in MH broth. The overnight culture was diluted 10% (5x10 CFU/ml). All tubes, including a control tube without the drug (positive control), were inoculated with 50 µl of the diluted inoculum and incubated for 24 h at 37°C and the MIC was noted.

Results and Discussion

Biochemical test results

The standard biochemical tests were carried out to confirm the organisms procured and clinical isolates obtained from the hospital.

Determination of Binding energy and Inhibition constant of antibiotics

The AutoDock 4.2 program was used to investigate ligand binding to structurally refined KPC 2 enzymes in perfusion state protein model using a grid spacing of 2.0 Å and the grid points in X, Y, and Z axis were set to 90 X 90 Y 90 Z. For each ligand, a docking simulation was performed and the analysis was based on binding energies and inhibition constants. The data are given in Table 1 and Figure 1. From the results, antibiotics with binding energy between -6.46 kcal/mol to -1.82 kcal/mol were selected for further studies. The weaker binding energy (>1) indicates a lesser affinity towards the target enzyme as observed with 11 antibiotics among 18 studied.

Characterization of AgNPs using UV–Visible spectroscopy

UV–visible spectroscopy is the initial step for analysing the formation of AgNPs in aqueous solutions. Free electrons give rise to an absorption band (420-430 nm). This is due to combined vibrations of the electrons of AgNPs in resonance with the oscillating electric field of the incident light. The spectrum of the fermentation broth (Fig. 2) confirms the presence of AgNPs.

Table 1: Summary of the docking parameters of the antibiotics against KPC II

Tested antibiotics	Binding Energy (kcal/mol)	Inhibition constant (µmol)
Piperacillin	-6.46	18.4
Amoxicillin + Clavulanic acid	-6.33	23.01
Ticarcillin	-5.87	10.02
Meropenem	-5.12	177.69
Imipenem	-2.41	144.69
Cilastatin	-1.95	37
Amikacin	-1.82	46.33
Cefazolin	1.25	29.33
Levofloxacin	1.95	37
Nitrofurantoin	2.71	14.6
Tobramycin	2.82	4.33
Ceftriaxone	2.95	27
Ceftazidime	3.33	28.01
Ertapenem	4.12	17.69
Cefepime	4.41	44.69
Trimethoprim	5.12	17.9
Ciprofloxacin	6.33	13.1
Gentamicin	6.87	5.02

Visual identification of silver nanoparticles

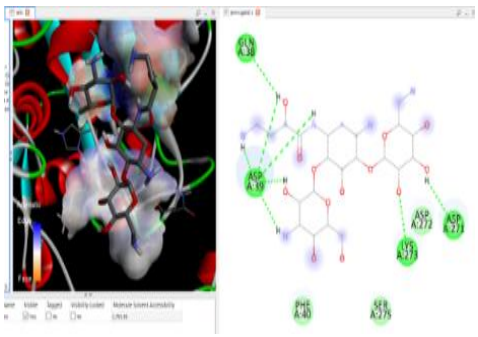
After centrifugation of fermentation medium, $AgNO_3$ was added to the collected supernatant and incubated at 24 h at 37°C, the colour of the solution changed from pale yellow to brown colour as shown in Figure 3 due to reduction of $AgNO_3$ and possible formation of AgNPs. The visibility of laser beam (due to the dispersion of light rays) passed through the solution confirms the presence of nanoparticles (Fig. 4) (Ramanathan and Gopinath, 2017).

MIC values of the selected antibiotics against NCIM strains using Vitek 2 compact

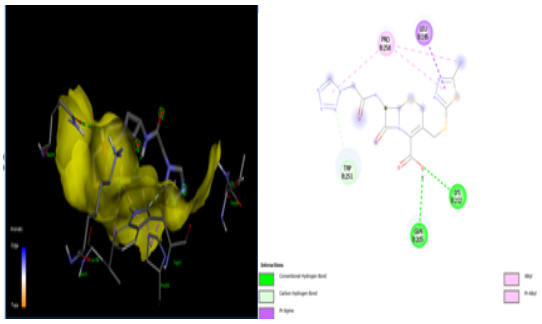
Vitek 2 Compact screening method was used for further selection of NCIM strain and clinical isolates to be used in this study. The results revealed that Levofloxacin has good antibacterial activity (<=0.12) among the 16 antibiotics against NCIM reference strains. Ciprofloxacin and Imipenem possess high inhibitory activity (<=0.25) against NCIM reference strains. Ertapenem showed slightly less activity (<=0.5) compared to the other antibiotics. Cefepime, Ceftazidime, Ceftriaxone, Gentamicin and Tobramycin inhibited Gram-negative NCIM strains with almost similar inhibitory activity (<=1) rates. Amoxicillin/clavulanic acid, Trimethoprim, Nitrofurantoin, Cefazoline, Piperacillin and Ampicillin (4-32) showed least activity compared to the other antibiotics. Clinical isolates (60 numbers) were procured and tested for sensitivity pattern. The results of MIC were used to classify the strains into Non-ESBL and ESBL strains (Table 2).

(i) *Sensitivity pattern of non ESBL Klebsiella pneumoniae*

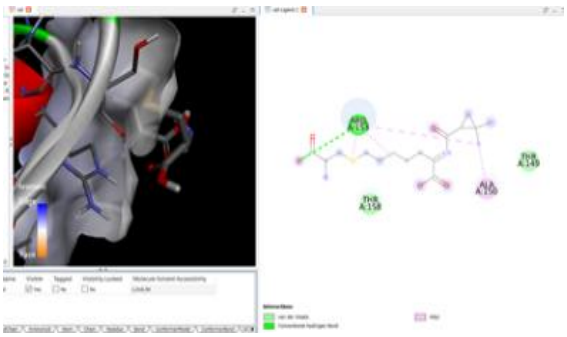
The clinical isolates that tested negative for ESBL were sensitive to most of the selected antibiotics on the GN card. Since the study focused on Carbapenems and β-lactams, the selected drug class was interpreted from the results. The results suggest that Imipenem has a high antibacterial action against non-ESBL strains. Among the 30 numbers of clinical isolates tested, Amoxicillin/ Clavulanic Acid and Gentamicin were less sensitive (21 numbers) when compared with Ertapenem and Ampicillin (25 numbers).



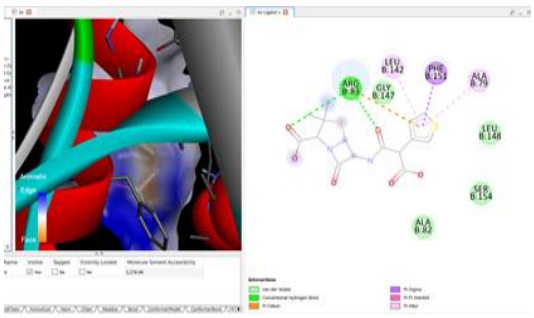
Amikacin



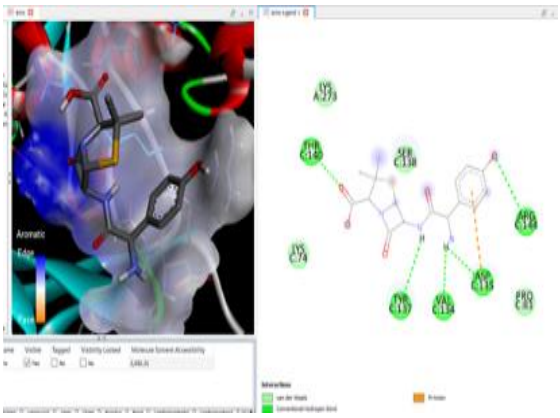
Imipenem



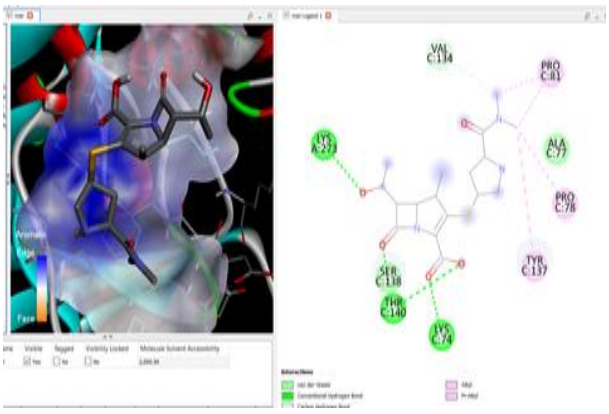
Cilastatin



Ticarcillin



Amoxycillin + Clavulanic acid



Meropenem3

Fig. 1: *In-silico* molecular docking images of antibiotics against KPC II enzyme of *K. pneumoniae* using AutoDock 4.2.

(ii) *Sensitivity pattern of ESBL Klebsiella pneumoniae*

ESBL positive clinical isolates was less sensitive to the majority of the antibiotics provided on the GN card. From the MIC value, Ertapenem and Imipenem showed good inhibitory activity against ESBL bacteria (23 numbers). Amoxicillin/ Clavulanic Acid and Ampicillin exhibit low activity (13 numbers) to ESBL strains than Ampicillin/ Sulbactam and Gentamicin (18 numbers). Among

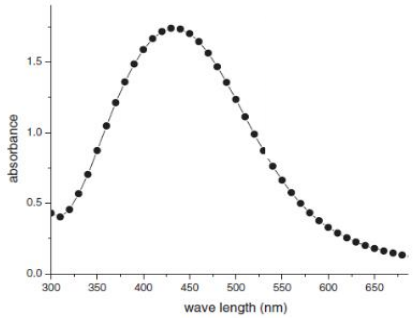


Fig. 2: UV–Visible spectrum of $AgNO_3$ treated *B. subtilis* supernatant.

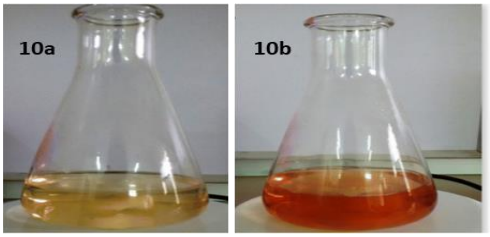


Fig. 3: Extract after centrifugation (10a) and after 24 h of incubation (10b).

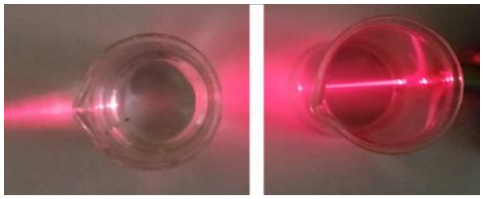


Fig. 4: Path of laser light not visible in water and path of laser visible in bacterial supernatant.

Table 2: Vitek 2 compact MIC values of the selected antibiotics against NCIM strains

Antimicrobial Agents	***Klebsiella pneumoniae*** NCIM5656		***Klebsiella pneumoniae*** NCIM2957	
	MIC value	**Interpretation**	**MIC value**	**Interpretation**
Amoxicillin / clavulanic acid	<=4	S	<=4	S
Ampicillin	<=2	S	<=2	S
Ampicillin / sulbactam	4	S	4	S
Piperacillin / tazobactam	<=4	S	<=4	S
Cefazolin	<=4	S	<=4	S
Ceftazidime	<=1	S	<=1	S
Ceftriaxone	<=1	S	<=1	S
Cefepime	<=1	S	<=1	S
Ertapenem	<=0.5	S	<=0.5	S
Imipenem	<=0.25	S	<=0.25	S
Gentamicin	<=1	S	<=1	S
Tobramycin	<=1	S	<=1	S
Ciprofloxacin	<=0.25	S	<=0.25	S
Levofloxacin	<=0.12	S	<=0.12	S
Nitrofurantoin	32	S	<=16	S
Trimethoprim	<=20	S	<=20	S

(S)- Sensitive; (R)-Resistant

Table 3: Classification of Clinical Isolates into Susceptible, intermediate and resistant based on the MIC values

Antimicrobial agents	Interpretation *K. pneumoniae* Non ESBL (n=30)			Interpretation *K. pneumoniae* ESBL (n=30)		
	Susceptible	Intermediate	Resistance	Susceptible	Intermediate	Resistance
	ESBL Negative			ESBL Positive		
Amoxicillin / Clavulanic Acid	18	8	4	7	4	19
Ampicillin	22	7	1	6	5	19
Ampicillin / Sulbactam	26	3	1	13	9	8
Piperacillin / Tazobactam	22	6	2	10	9	11
Cefazolin	13	8	9	5	8	17
Ceftazidime	25	3	2	16	7	7
Ceftriaxone	24	4	2	10	12	8
Cefepime	27	2	1	25	3	2
Ertapenem	25	3	2	23	3	4
Imipenem	28	2	-	26	2	2
Gentamicin	21	5	4	18	4	8
Tobramycin	14	8	8	11	9	10
Ciprofloxacin	18	5	7	21	4	5
Levofloxacin	23	4	3	20	4	6
Nitrofurantoin	17	7	6	13	7	10
Trimethoprim	20	5	5	18	3	9

the 30 numbers of clinical isolates tested Piperacillin/Tazobactam showed susceptible (10 numbers), intermediate (9 numbers), and resistance (11) patterns, respectively. Based on the results the best acting antibiotics which are available in the laboratory were further investigated.

Antibacterial activity of AgNPs and selected antibiotics using Kirby Bauer method

Antibiotic susceptibility testing was carried out for AgNP's and selected antibiotics using the Kirby Bauer disc diffusion method against NCIM, non-ESBL, and ESBL of *K. pneumoniae*. The test was carried out in triplicates. The zones of inhibition and CLSI guidelines are used to categorize the strains into Sensitive (S), Intermediate (I), and Resistant (R).

(i) *Antibiotics sensitivity pattern of K. pneumoniae NCIM 2957*

AgNP's demonstrate remarkable action in the case of the reference strain. Among the tested antibiotics the Carbapenem antibiotics exhibit high sensitivity with zone of inhibition (>31mm). The other tested antibiotics exhibit sensitivity with zones of inhibition from 14 to 29 mm.

(ii) *Antibiotics sensitivity pattern of K. Pneumonia non ESBL strain*

AgNP's exhibits exceptional activity in non-ESBL strain. The Carbapenem antibiotics display greater sensitivity against the non-ESBL strain of *K. pneumoniae* (>39 mm). The other tested antibiotics exhibit sensitivity with zones of inhibition from 14 to 24 mm.

(iii) *Antibiotics sensitivity pattern of K. pneumoniae ESBL strain*

ESBL strain of *K. pneumoniae* showed lesser sensitivity in comparison with reference strain and non ESBL strain against AgNP's. From the results (Table 4; Fig. 5), the ESBL strain was found to be resistant to Imipenem and Piperacillin (19 mm and 16 mm) and are intermediately sensitive to Imipenem + Cilastatin, Ticarcillin + Clavulanic acid, and Amoxicillin Clavulanic acid (23 mm, 16 mm and 17 mm). The test results of Meropenem, Colistin, and Amikacin barely met the set of criteria for sensitivity 30 mm, 12 mm, and 24 mm

Table 4: Sensitivity pattern of *K. pneumoniae* NCIM 2957 and non ESBL strain by Kirby Bauer method

Antibacterial agents	***K. pneumonia* NCIM 2957**				***K. pneumonia* Non ESBL**			
	Test (mm)			**S.D *Value* (n=3) *mm***	**Test (mm)**			**S.D Value (n=3) mm**
AgNPs	15	15	15	15	15	15	15	15
Imipenem (10mcg)	32	31	32	31.6 ±1.3	30	29	28	29±1.8
Imipenem+Cilastatin (10/10mcg)	35	37	36	36 ±0.47	30	28	28	28±1.4
Meropenem (10mcg)	34	34	33	33.6 ±1.3	32	31	32	31.6±1.3
Colistin(25mcg)	14	13	14	13.6 ±1.3	13	12	12	12.6±1.3
Amikacin (30mcg)	23	23	24	23.6±1.3	23	23	21	22.3±1.7
Piperacillin (100mcg)	24	24	23	24.6±1.3	25	24	23	24±1.8
Ticarcillin /Clavulanic acid(20/10mcg)	26	27	26	26.6±1.3	25	24	23	24±1.8
Amoxicillin /Clavulanic acid(20/10mcg)	29	30	29	29.6±1.3	24	25	24	24.3±1.3

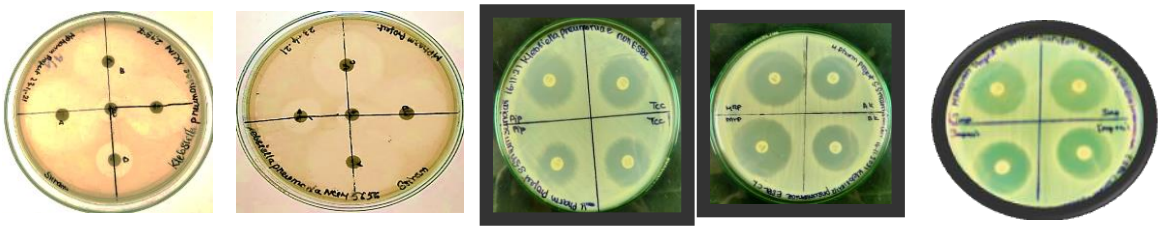


Fig. 5: Sensitivity pattern of *K. pneumoniae* NCIM, non ESBL and ESBL strain by Kirby Bauer method.

respectively.

Minimum Inhibitory Concentration determination

MIC determination was carried out for selected antibiotics and AgNP's using the broth microdilution method against NCIM, non ESBL, ESBL and lysozyme treated *K. pneumoniae.* The test was interpreted using CLSI guidelines and categorized as Sensitive (S), Intermediate (I), and Resistant (R).

(a) *MIC determination of K. pneumoniae NCIM 2957 (reference strain)*

AgNP's showed an inhibitory effect on the reference strain *K.pneumoniae* NCIM 2957 in the concentration of 32 µg/ml. The Carbapenem group of antibiotic MIC values for the NCIM strain was found to be in the range 1-0.5 µg/ml. The MIC values of other tested antibiotics having met the standard of sensitivity as per CLSI standards.

(b) *MIC determination of K. pneumoniae non ESBL strain*

AgNP's exhibit similar activity against non ESBL strain as that of NCIM strain (32 µg/ml). The Carbapenem group of antibiotics displays slightly low MIC values against the non ESBL, when compared to the NCIM reference strain with the values ranging from 8-2 µg/ml. Among the tested antibiotics, Amikacin possesses a high MIC value of 64 µg/ml. This may be due to increased resistance of C.I. since Amikacin is regular prescription drug and patient non-compliance.

(c) *MIC determination of K. pneumoniae ESBL strain*

AgNP's exhibit very low activity against ESBL strain compared to the reference strain (128 µg/ml). The Carbapenem group of antibiotics display slightly low MIC values against the ESBL, when compared to the NCIM reference strain with

Table 5: Minimum Inhibitory Concentration determination of AgNPs and selected Antibiotics

Antimicrobial agents	*K. pneumoniae* NCIM 2957 (µg/ml)	*K.pneumoniae* Non ESBL (µg/ml)	*K. pneumoniae* ESBL (µg/ml)
Amikacin	8	64	64
Meropenem	0.5	2	4
Imipenem+Cilastatin	1	8	8
Piperacillin	8	16	128
Colistin	1	4	2
Ticarcillin+Clavulanic acid	4	8	8
Amoxycillin+Clavulanic acid	0.25	2	8
Silver Nanoparticles	32	32	128

values range from 8-4 µg/ml. Piperacillin + Clavulanic acid has high MIC value of 128 µg/ml. to other tested antibiotics due to increased resistance of C.I. this may be due to KPC production (Table 5).

The mortality rate associated with Cr-KPN infection and the limited therapy options for treating Cr-KPN infection highlight the need for better detection of Cr-KPN infection, identification of effective preventive measures, and development of novel agents with reliable clinical efficacy against Cr-KPN (Falcone *et al*., 2022)

Molecular docking and dynamic study were carried out to analyze the resistance mechanism of KPC-2 at the structural level; this helps determine binding affinity and inhibition constant of the antibiotics (Reyes *et al*., 2017). In this study, the antimicrobial activity of AgNPs is very pronounced and it is particularly significant when used in combination with the selected Carbapenems (Imipenem and Meropenem). When combined with conventional antibiotics, they have a synergistic and additive effect on the strains of *K. pneumoniae.* The AgNPs were produced economically and in an eco-friendly manner using *Bacillus subtilis* strain using an earlier reported method (Bakthavatchalam *et al*., 2020). The susceptibility of the bacteria was tested by the Kirby-Bauer disk diffusion technique. The results demonstrated that AgNPs have efficient antibacterial activity against *K. pneumoniae*. This makes available an alternative nano-biotic antimicrobial therapy to overcome resistance in *K. pneumoniae*. Similar research carried out earlier, demonstrated considerable fluctuation in MIC values. The inherent variations in resistance pattern exhibited by clinical isolates studied in our work make the comparison of the research data with earlier work challenging for assessing the antibacterial activity of AgNPs.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, Tamil Nadu, India.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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