Synthesis of Imidazole conjugated silver nano-particles and evaluation of their antimicrobial potential

Thesis submitted in partial fulfilment of the requirement for the degree of

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In

MICROBIOLOGY

Submitted by

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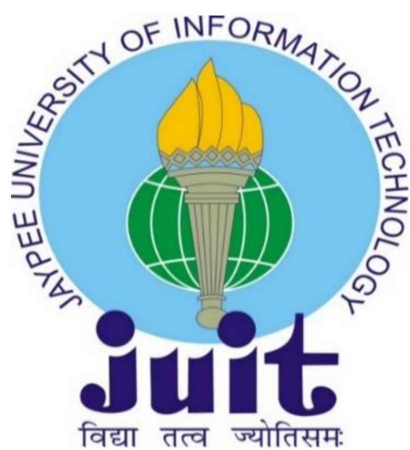

Department of Biotechnology & Bioinformatics

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

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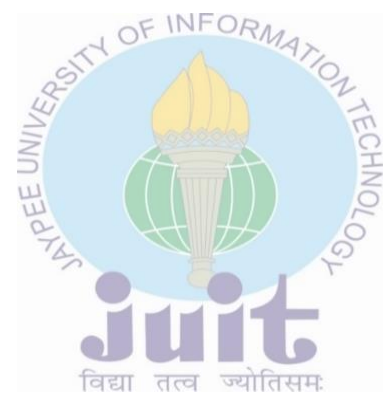

Certificate

This is to certify that thesis entitled **“Synthesis of Imidazole conjugated silver nano-particles and evaluation of their antimicrobial potential”**, submitted by **Purba Kundu** in partial fulfilment for the award of degree of **Master of Science** in **Microbiology** to Jaypee University of Information Technology, Waknaghat, Solan has been made under my supervision.

Dr. Jitendraa Vashistt

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Candidate's Declaration

I hereby declare that the work presented in this thesis entitled **"Synthesis of Imidazole conjugated silver nano-particles and evaluation of their antimicrobial potential"**in partial fulfilment of the requirements for the award of the degree of **Master in Science** in **Microbiology** submitted in the Department of Biotechnology & Bioinformatics**,** Jaypee University of Information Technology, Waknaghat is an authentic record of my work carried out over a period from August 2022 to May 2023 under the supervision of **Dr. Jitendraa Vashistt** (Associate Professor, BT&BI).

The matter embodied in the thesis has not been submitted for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is true to the best of my knowledge.

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LIST OF ABBREVIATION

AMR	Antimicrobial resistance
MDR	Multi drug resistance
XDR	Extreme drug resistant
VRE	Vancomycin resistant Enterococci
VISA	Vancomycin-intermediate Staphylococcus aureus
VRSA	Vancomycin-resistant Staphylococcus aures
MRSA	Methicillin-resistant Staphylococcus aureus
ESBL	Extended-spectrum beta-lactamase
CRAB	Carbapenem-resistant Acinetobacter baumannii
PRSP	Penicillin-resistant Streptococcus pneumoniae
AgNP	Silver nanoparticle
AgNP@Imidazole	Conjugated nano-formulation
ATCC	American type collection culture
CRHP	Clarithromycin-resistant Helicobacter pylori
Ab	Acinetobacter baumannii
UTI	Urinary tract infections
µM	Micromolar
µg	Microgram
ml	Milli-litre

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Abstract

Antimicrobial agents are a group of medications that include antibacterial, antifungal, antiviral, and antiparasitic compounds and are used to treat an array of medical ailments. Over administration of antimicrobial drugs has led to an issue regarding resistance against antimicrobial agents, which has now become a major challenge to treat microbial infections. Multidrug-resistant microorganism strains have doubled globally in recent years. Resistance against antimicrobial agents is a serious danger to patient care, resulting in a rise in morbidity and mortality. Several common genera of bacteria involving *Staphylococcus aureus*, *Enterococcus faecium*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter* spp. quickly acquire resistance to antibiotics and become prevalent in the hospital environment.

A relatively new field of nanomedicines has lately emerged as a promising possibility for addressing the long-standing problem of drug resistance. Nano-particles are unique entities having one of their dimensions in nano-meter range. This offers various advantageous physio-chemical properties which greatly differ from their bulkier counterparts. In this work we have tried to conjugate imidazole a prominent class of antibacterial agent with silver nano-particles and assessed its antimicrobial activity using broth microdilution technique. The effort has mostly concentrated on decreasing the imidazole dosage to the point where the conjugated system's effectiveness is proved to be efficient against bacterial isolates. We found that minimum inhibitory concentration got lower in conjugated system's as compared to unalloyed Imidazole. The strategy may be utilized further to synthesize the nano-particle conjugates with efficient activity.

Chapter 1

INTRODUCTION

The emergence of rapid peak of multidrug-resistant infections of bacterial isolates has becoming a threat to human health across the globe. The dynamicity of its genome composition provides an edge to accommodate several resistance mechanisms, leaving ineffective antibiotic doses.

World health organisation has already declared some bacterial strains and ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp*.) as group 1 MDR pathogens.[1].

There are more than 60% mortality rates of infections causing by MDR pathogens with respect to its specific strain. Its ability to survive on antimicrobial drugs, provides resistance to various antibiotics including colistin, carbapenem, chloramphenicol etc. the specific changes in their genome sequences imposes a challenging breakthrough in treatment [1], [2].

According to the data provided by WHO the MDR pathogens have been affecting approximately 2M people and 20,000 deaths in United States and European countries. The rate of infection rises rapidly with the inverse ratio of new antibiotic development. The microorganisms are commonly found in soil, water, skin of animals and humans as they have the ability to contaminate surfaces of hospitals and its equipment. It causes a diverse scale of infections especially in immunocompromised individuals that ultimately lead those to prone of infections. Some chronic infections of pneumonia which are ventilator related and also UTIs, and cerebrospinal fever which are causing by this nosocomial superbug. The pathogen can easily spread through various means such as sinks, doors, curtains, medical equipment etc. [3].

Considering the challenge of antimicrobial drug resistance, the present study has been carried to develop imidazole-silver conjugates (AgNP@Imidazole). Under optimized conditions, AgNP@Imidazole were developed by synthesizing silver nano-particles via a wet chemistry approach. In present approach, silver nanoparticle was synthesized and further consolidated by observing the absorption maxima using a UV spectrometer. Effective imidazole concentration was then added and incubated with silver nano-particles to synthesize silver-imidazole conjugate having an enhanced antimicrobial activity. Forthcoming studies were focusing on optimizing and enhancing these submicron-dimension antimicrobial conjugates via evaluating their minimal inhibitory concentrations. These

formulations were used to counteract the threat posed by the problematic MDR bacterial isolates.

AIMS AND OBJECTIVES

Since there is negative impact of bacterial pathogen on human mankind there are various strategies that have been implemented to reduce the resistant mechanism of the isolate. Subsequently, the amalgamation of antimicrobial compound has been on trend from a long time so an alternative strategy can be developed to formulate an antimicrobial enhanced complex compound that can exhibit potential activity against multi-drug resistant bacteria. Therefore, the following objectives were made to achieve the aim to utilize the nano-particles as antibacterials and to get rid of bacterial resistance.

- To synthesize silver nano-particles and its conjugated system.
- To assess the antimicrobial activity of silver nano-particles and its conjugated system against pathogenic bacterial isolates.

Chapter 2
REVIEW OF LITERATURE

2.1 Emergence of infections from MDR pathogens

Continuous use of antibiotics, medications, and regular exposure to hospital acquiring infections has increase the outbreak of multi drug resistant (MDR) bacteria. These nosocomial infections are caused by various types of microorganisms such as bacteria, viruses, and other pathogenic agents.

The transmission of infections may occur either from endogenous or exogenous sources and indirect and direct contact with infected patients, contaminated surfaces, or environmental sources. Hospital-acquired infections (HAI) has become the reason for emergence of multi drug resistant bacteria. In United states 722,000 infected cases with 75,000 deaths cases has been reported in 2011 that were associated with hospital acquired infections. Approximately 15.5% cases have been found responsible due to MDR bacteria [4].

These infections effects in high mortality rates, costs increment, uncertain diagnostics and beliefs in orthodox medicine. The increasing numbers of antibiotic-resistant pathogens causing significant pressure in healthcare systems and global economic systems. According to Infectious Diseases Society of America, the surveillance studies in hospitals have reported these pathogens as ESKAPE group nosocomial pathogens. Acronym ESKAPE is a group of MDR and virulent bacterial pathogens that encompasses both gram-negative and gram-positive species: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp* [5].

These nosocomial pathogens have become resistant to various antibiotics and escaping the bactericidal action of antimicrobial agents. Among immunocompromised and ventilator associated ICU patients these bacteria has shown their action as potential drug resistance nosocomial pathogens and causes life threatening diseases.

2.2 Bacterial pathogen coinfection in COVID-19

The unpredictable world health crisis has increased the concerning factors due to antimicrobial resistance (AMR) bacterial coinfections. The prevalence of antimicrobial resistance infections has increased its mechanical factors because of COVID-19.

COVID-19 affects widely through systematic infections in tissues and cells and this disease gets more fatal when it gets associated with bacterial coinfections of MDR or AMR species. This is the reason for 50% deaths in COVID-19 [6].

Various investigations have been found as an evidence of bacterial coinfection interference such as VRE, VISA/VRSA, MRSA, ESBL *Klebsiella pneumoniae*, CRAB, CRHP, PRSP etc. The ESKAPE MDR pathogens are broadly categorized into three groups:

- Antibiotic binding target sites may modify.
- Decrease in permeability or increase in efflux systems causes reduction in accumulation of drugs.
- Enzyme catalysed irreversibility leads to drug inactivation.

2.3 Evoking measures for evolving antimicrobial agents

Throughout these passing years the total number of antibiotics that are effective against ESKAPE pathogens has been declining which leads us to a conclusion that in near future the antibiotic ineffectiveness may induce major outbreaks.

Clinical & Laboratory Standards Institute has analysed and suggested certain guidelines that may effective against ESKAPE by including antibacterial combinations/complexes.

The combinations may be therapeutic, peptides, adjuvants, antibacterial compounds, phytochemicals, nano-particles etc [5].

The combinational approaches involve two or more monomeric antimicrobial agents co-integrally such as co-administrated, conjugated/functionalized to improve the efficacy of the antimicrobial agents [6].

2.4 Combinational antibiotics

Combinational antibiotics have been trial and tested as a potential approach for treatment due to this the chances of pathogen to induce resistance against combinational drugs is much less than that of against single drug. This approach is used to increase the synergistic effects of the drugs, also combinational drugs increase the coverage spectrum against severe infections that are caused by multiple pathogens. Some of the combinational drugs are tested against ESKAPE that were listed in table 2.1[4].

Table 2.1 List of combinational drugs against their respective pathogen

Combinational antibiotics	Tested against pathogen					
	E	S	K	A	P	E
Netilmicin, Oxacillin, Tobramycin	0	0	1	0	0	0
Cefazolin, Cefepime, Ceftazidime	0	0	1	0	0	1
Dalbavancin, Telavancin, Oritavancin, Tedizolid	1	1	0	0	0	0
Doripenem, Imipenem, Meropenem	0	0	1	1	1	1
Piperacillin-tazobactam, Ticarcillin-clavulanate	0	0	0	0	1	0

These combinational drugs may made with potential molecules and antibiotics that can make an ineffective drug to be effective by inhibiting the mechanism of resistance and increasing the uptake of drug through bacterial membrane, changing the physiology of resistant cells and blocking of efflux systems. The molecules are termed as adjuvants that may have very less amount of potential antimicrobial activity. They mainly induce to form planktonic cells by dispersing the biofilms. Commonly known adjuvants are β-lactamase inhibitors and are explained against MDR pathogen in table 2.2[4].

Table 2.2: List of β lactamase inhibitors against its respective pathogens.

β-lactamase inhibitors	Against MDR pathogens
Vaborbactam	*K. pneumoniae* carbapenemase
Avibactam	*S. aureus*
Nacubactam	*S. aureus*
Tazobactam	*S. aureus*
EDTA, deferasirox, deferoxamine	*P. aeruginosa*, and *E. coli*

The most promising agent that have been reported to shows antimicrobial activity against persisted cells of *P. aeruginosa* and also ESKAPE members is 1-[(2,4-Dichlorophenethyl) amino]-3- Phenoxypropan-2-ol. This agent with an antibiotic combination making it potential candidate as an adjuvant to kill the ESKAPE strains in both sessile and planktonic forms. Combinational drugs coverage is mainly for broad spectrum resistance. But certain combinations that are meant to treat infections can cause antagonistic effects.

Antagonistic effects case study:

In a clinical trial in Italy a combination of colistin and rifampicin were used against XDR *A. baumannii* has no improvement result rather it led to hepatic toxicity. A combination of colistin, tigecycline and carbapenems showed futile results against *A. baumannii* [6].

An alternative to combinations drugs and antibiotics is there that are basically experimentally synthesized constructs of two or more pharmacophore compounds to elicit antimicrobial activities.

The advantage of this therapy is overcoming the problems related to non-complementarity of pharmacodynamics in antibiotics and curbed the resistance chances.

Extensive research of alternative strategies was developing to utilise these combinational approaches against MDR pathogens.

2.5 Plant based antimicrobial compounds

There are numerous plants based antimicrobial compounds that have been screened against multi drug resistant bacteria. Out of which terpenoids, secondary metabolites, organic acids, polymers and alkaloids have found to be potential antimicrobial agents. Some alkaloids and polymers have antimicrobial activities at a very high concentration such as Imidazole is an alkaloid that has shown minimum inhibitory concentrations at higher concentrations. However, imidazole ring is present in various drug molecules such as miconazole, also in amino acids that are reported as a bioactive compound in medicinal chemistry. The imidazole and imidazolium skeleton bearing compounds can be used promisingly as potent pharmacological activity. Various mono- imidazolium salts have been used as good bacterial toxicity [7], [8].

The antibacterial activity of imidazolium salts of amino acids against Gram-negative *Escherichia coli* DH5-α and Gram-positive *Bacillus subtilis* 1904-E have already been

evaluated with their cytotoxic effect and clinically studied on human podocytes (HEK-293) [7].

Moreover, imidazole is present in biological system as histidine amino acid. The one reason for biofilm activity of MDR bacterial pathogens are extensively regulated by histidine metabolism. The upregulation of the proteins that are involved in histidine metabolism is carried out by urocanate hydratase (*Hut*U) and the downregulations are done by SA (*Hut*). Basically, L-histidine metabolism influences the biofilm formation which is reported by performing an experiment upon different type strain of cultivated with L-amino acids. However, D-histidine also found to be inhibited the biofilm formation in *Staphylococcus aureus* and *Pseudomonas aeruginosa*. urocanase has the vital functional role in histidine metabolism also D-His interferes with the degradation of L-His pathway [7].

Since, imidazole have been reported to be potential antimicrobial compound therefore to enhance its activity against MDR bacterial pathogens, many combinational drugs have been found out to be conjugated with nanoparticle formulations. Therefore, metal nanoparticle conjugation may lead to an outcome for evaluating the antimicrobial activities,

2.6 Therapeutic measures of silver nano-particles

Metal nano-particles are profoundly used in biomedical and nanomedicine branches as antimicrobial properties bearing agents because of their unique chemical and physical properties. Among various metal nano-particles, silver (AgNP) has shown as for approaching multi-targeted sites and reduces the resistance mechanism. Silver nano-particles acts by interfering the biofilm assembly of MDR pathogens. Silver nano-particles can be prepared by various processes plant extract, electrochemically, photodynamically [9].

The AgNP has been orally administered at 10-32 ppm and proved to be non-toxic. However, the conjugator effect of chitosan, nylon and collagen functionalised with AgNP has exhibited antibacterial activities in case of ESKAPE pathogens.

It has been reported that silver nano-particles entrapped allows a controlled dispersion of silver ions for prolonging antibacterial action. This formulation was been introducing nowadays for the enhancement of therapeutic actions just like co-incubation with antibiotics showed synergistic activities against ESKAPE.

2.7 Physiological targets for antibiotics against MDR bacterial pathogens.

Almost 2% of nosocomial infection is caused by MDR bacterial pathogens in various parts of Europe, United States, Middle East with double the rate. Due to overuse of antibiotics such resistance induced by pathogens has been reported.

The pathogen employs several mechanisms to counter the reactions of antibiotics by modifying their internal aminoglycoside enzymes, β-lactamase production, receptor conformation, ion-exchange pumps, membrane permeability defects etc.

MDR bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* have low rate of pathogenesis compared to *A. baumannii* which triggers by multiple of four. As per reports 90% of isolates were highly resistant-carbapenems and due to this the phenotype of MDR bacterial pathogens dominate the susceptibility of carbapenem antibiotic as per the survey of WHO 2016 [10].

The isolate resistance towards antibiotic have been evolved from MDR-PDR-XDR (multi-drug resistant-pan-drug resistant-extensively-drug resistant). Therefore, strict calls have to be implemented in order to control the rate of pathogenesis [10].

There are various molecular level-targets through which MDR bacterial pathogens are inhibited by using specific antibiotics in table 2.3[1].

Table 2.3: Antibiotics and their targets in MDR bacterial pathogens.

ANTIBIOTICS	TARGET
Polymyxin B and E (Colistin)	lipopolysaccharide and Outer membrane
Carbapenem, Sulbactam and Cephalosporins	cell wall
Tetracycline (minocycline), Aminoglycoisdes (amikacin) and Glycylcyclines (tigecycline)	Protein synthesis
Fluoroquinolones	DNA replication

Beside the accessibility of these antibiotics, still MDR bacterial pathogens builds resistance against antimicrobial drugs through various mechanisms, due to this there is a limited therapeutical advancement for getting active against bacterial pathogens.

2.8 Mechanism of resistance for MDR bacterial pathogens.

2.8.1 Enzymes-based resistance

i. **β-lactamases:** These are specific enzymes that inhibit cell wall synthesis. The property of acetylation of the serine site of the penicillin-binding protein in bacteria, basically leads to exert antimicrobial activity by hydrolysing the β-lactam ring. According to Ambler classification there are four classes of β-lactamases in which class A, C and D are depends on serine site whereas B class depends on metal derived enzymes. Below is a constructed table 2.4 for detail information [2].

Table 2.4: Classification of β lactamases and their resistant element.

CLASSIFICATION OF β-LACTAMASES	RESISTANT ELEMENT
Class A (TEM, SHV, GES, SCO, VEB)	Clavulanate
Class B (IMP and VIM)	All β-lactam ring antibiotic
Class C (Amp C)	Cephamycin, Penicillin, Cephalosporins combinations
Class D (OXA-23, OXA-24/40, OXA-58, OXA-143 and OXA-235)	Carbapenem

Thereby, enzyme β-lactamase can be a suitable target for developing a therapeutical reaction to increase the antimicrobial properties of drugs.

ii. **Alternate aminoglycoside enzymes:** these enzymes alter the morphological skeleton of aminoglycosides and lowers the potential activity of MDR bacterial pathogens**.** Mainly three types of aminoglycosides are present in MDR bacterial pathogens that are: AAC (acetyltransferases) that is it transfers the acetyl group of amino acid into the drug, which ultimately boost resistance against antibiotics due to inert drugs, APH (phosphotransferases) just like similar mechanisms it transfers its phosphate group to the drug and ANT (nucleotidyl transferases) which transfers the adenosine

monophosphate from the adenosine triphosphate, in the antibiotic which leads to inactive the drug functioning. Aminoglycosides the used in various treatment to eradicate MDR bacterial pathogens infections.

Likewise, there is an enzyme found in 16srRNA, they functioning in the methylation of guanine bases in the active site to ensure the unbinding of aminoglycoside. This resistance mechanism is due to the modification of RNA methyltransferases of MDR bacterial pathogens. The A side of methyltransferases (*Arm*A) provide resistance towards various antibiotics commonly amikacin, gentamicin, etc [2], [3]

2.8.2 Nonenzymic-based resistance

i. **Interruption in permeability:** The MDR bacterial pathogenesis a gram-negative pathogen whose outer membrane has lipopolysaccharide layer and porin proteins (outer membrane porins) that provides a key factor in resistance.

The porins forms a special structure to form a gateway through the outer membrane. It is a slender span of β- barrel skeletons with a collective 8 to 26 strands. There are size independent porin proteins such as *Omp*33-36, *Omp*37, *Omp* 43, *Omp* 44, *Omp* 47, *Opr*D and *Car*O. The outer membrane porin protein A provides resistance to carbapenem antibiotics by triggering the biofilm association which leads to reduction in density of porins from aztreonam, chloramphenicol, etc [2].

ii. **Efflux pumps:** The resistance mechanisms induced by MDR bacterial pathogens is due to these turning pumps 'on' in order to eject the antimicrobial drugs from their corresponding systems. The activation of these efflux pumps is due to the alteration in chromosomal genes to induce their expressions.

There are four families of efflux pumps through which MDR bacterial pathogens *induce* resistance against antibiotics. Figure 1 describe a brief idea about their resistance criteria [3], [11]

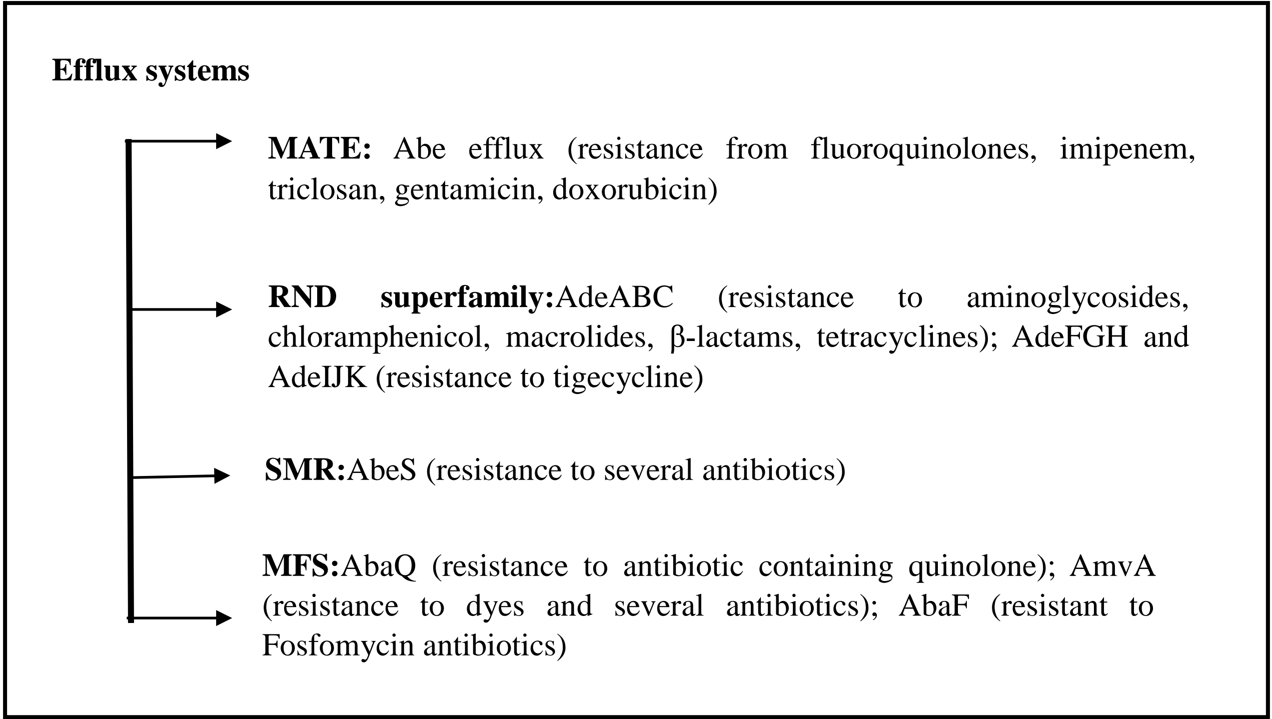


Figure 2.1: Resistance criteria of efflux systems.

iii. Modification of active site:

Due to random occurrence of mutation in prokaryotic cells, there is an induced modification in the active site of enzymes where antibiotic gets bind easily to degrade the pathogen. This develops a resistance against the antibiotics. Table 2.5 represents the list of antibiotics and their affecting target sites respectively [3], [11].

Table 2.5: Antibiotics and their target site.

ANTIBIOTIC	TARGET SITE
Rifampicin	ADP-ribosyl transferase
Fluoroquinolones	DNA Gyrase and topoisomerase IV enzymes
Colistin	Makes changes in the LPS leads to cell death due to: osmotic imbalance, mutation in *PmrAB* site and lack of production of lipid A.

2.9 Virulent nature of MDR bacterial pathogens and its pathogenicity

2.9.1 Proteins present in outer membrane

There are several outer membrane proteins present over the surface of MDR bacterial pathogenssuch as *BamA, OmpW, CarO*, etc. in which *OmpA* is considered as potential virulent and pathogenic porin protein. This porin is responsible for biofilm formation and strong hydrophobic interactions with the host cells. The infection can be formulated by various processes.

The *OmpA* porin binds through the fibronectin protein present over the surface of host cell and move inside the host mitochondria to activate the formation of Cytochrome C. This protein stimulates and release apoptosis inducing factors into the cytoplasm which ultimately leads it to the nucleus and damage the genetic material. However, *OmpA* which are present over the surface of epithelial cells, releases an enzyme nitric oxide synthase that induces over-regulation of TLR-2 causes death of epithelial cells.

Depending on the concentration of *OmpA*, the mitochondria produce ROS upon high concentrations of porins, in low concentration of porins it activates the immune and dendritic cells. The presence of these radicals prevents the stimulations of immune cells as well as causes death to dendritic cells.

There are several β-barrel shaped porins like *CarO* OMP and *OprD* OMP that are present over the surface of MDR bacterial pathogens which enables the entry of carbapenem based antibiotics. These proteins get defunctionalized due to conformational change, transcription and post-translational change, genetic alteration. Thereby, leads to development of resistance against carbapenem whereas in case of *OprD* the resistance is developed due to SNP in MDR bacterial pathogens.

There is another factor which is key of strong virulence under biotic or abiotic conditions is biofilm formation. The isolates micro colonizes by adhering with each other through epithelial cells. The biofilm formation is due to biofilm associated protein and MDR bacterial pathogens *OmpA* porins. This leads to inhibition of the antibiotics and provides a hostile condition [1], [2].

2.9.2 Glycolipids (LPS) and capsules

Lipid A is responsible for the stimulatory modulations of LPS and capsules. There are three genes which are mainly responsible for the synthesis of lipid A (*lpxA, lpxC and lpxD*). The capsules protect the isolate by blocking the entry of antimicrobial peptides into the bacterial cell for the survival in the host [2].

2.9.3 Mechanism of Quorum sensing

This is a sensing mechanism through which cells communicate with adjacent cells for their survival. There is a secretion N-acyl homoserine, when its concentration crosses a threshold value, the mechanism activates and induces binding of a regulator *AbaR* to *AHL* synthase prompter gene *AbaI*.

This results into the sensing ability in biofilm formation. the virulence is induced by the *AbaI* mutants through quorum sensing interactions [1], [3], [12].

2.9.4 Regulations of secretion systems

MDR bacterial pathogens various secretory systems. Type I, II, V, VI and others. The T6SS secretory system that is molecular syringe helps to import toxins into bacterial cell to eliminate other pathogenic cells. It secretes various proteins but most importantly valine-glycine repeat protein and haemolysin co-regulated protein.

The T2SS is known as general secretory pathway in which 12 genes are translated to form this type II secretory system.

T1SS comprises membrane bound fused proteins across the inner membrane and outer membrane and membrane transporter stack that binds to ATP. It binds two lipid membranes and induces secretions.

Type V also known as autotransporter system in which the C-terminal end of the peptide generates pore in outer membrane through which proteins can leak out [2], [11].

2.10 Therapeutic inhibitors against MDR bacterial pathogens.

2.10.1 Natural therapeutical products

There are some natures originated bioproducts that are used in numerous therapeutical causes. Approximately extracts of 60 herbal plants are found that are active against MDR that is resistant to AMK and 18 extracts against cefotaxime, IMI and piperacillin [13].

Some herbal extractions of *Scutellaria baicalensis, Rosa rugosa, Rabdosia rubescens* and *Magnoliaofficinalis* were found to be very potent with a MIC factor of 1.4-4.6 mg/ml. similarly following is the summarized list table 2.6 of potent herbal extracts that are active against MDR bacterial pathogens [2], [11].

Table 2.6: Minimum inhibitory concentrations of herbal extracts.

HERBAL EXTRACTS	MIC RANGE
Anigozanthos rufus, Anigozanthos pulcherrimus	52- 95 µM
Schinus terebinthifolia	CRAB- 256 µg/ml
Penta galloyl glucose	64 µg/ml
Chitosan	0.5- 1 mg/ml
Thymus daenesis	30-45 µg/ml
Root tuber of *Polygonum multiflorum* and flowers of *Rosa rugosa*	Inhibit >50% growth at 10µg/ml

2.10.2 Synthetic compounds

i. Imidazole containing compounds

There are numerous derivatives of naphthylamide nitroimidazole that were synthesized to enhance the bactericidal activity at minimum concentration levels. Following is a complete list of compounds that were proposed in table 2.7 [14]

Table 2.7: List of Imidazole containing compounds with their MIC values.[2], [14]–[16]

Serial No.	Compounds	MIC/IC values
1		0.013 µM/ml
2		0.03µM(IC90)
3		6.25µM
4		8±0.32µM, 13±0.70µM(IC50)
5		26.8±2.28µM(IC50)
6		2.8µM(IC50), 0.98µM(IC50)
7		6.25µM
8		32µg/ml
9		0.5-4µg/ml

10		0.25-0.5µg/ml
11		0.25-4µg/ml
12		0.25µg/ml
13		0.25µg/ml

ii. Thiazole skeleton compounds

A list of 2-aminothiazolyl berberine were synthesized in order to enhance antibacterial effect on MDR bacterial pathogens. Some enhancing agent with 2-aminothiazolyl berberine has been showed potential activity of MIC 2nmol/ml, 0.25µg/ml and a zone of inhibition of 7mm at 500µg/ml [17].

iii. Indole ring containing compounds

There is chain of compounds of pyrimidol [4,5-b] indole derivatives, that are synthesized against various Gram-negative bacteria including MDR bacterial pathogens with MIC 0.25-1µg/ml. below is the list of compounds that have been screened for antibacterial activity against MDR bacterial pathogens in table 2.8[1].

Table 2.8: List of Indole containing derivatives with their MIC values**.**

PYRIMIDOL[4,5-B] INDOLE DERIVATIVES	MIC VALUES
3-amino indoles	8µg/ml
4-hydroxy-2-pyridone	8 and 16µg/ml
N-(1H,1'H[2,2'-bipyrrol]-5-ylmethylene)-1H-indol-7-aminium	12.5µM
(E)-2-(2-(1H-indol-2-yl) vinyl)-1-methylquinolin-1-ium	16µg/ml

iv. β-lactam skeleton compound

The compounds are basically based upon biscatecholate-monohydroxamate mixed ligand that showed bactericidal activity against MDR bacterial pathogens with a MIC of 0.0078µM. There is a silver nanoparticle-based compound that shows synergistic effect with imidazole as IMI@AgNPs-PEG-NOTA which shows bactericidal activity against CRAB. They show reverse resistance as β-lactam ring is protected by AgNP[11].

2.10.3 Antimicrobial inhibitors for MDR bacterial pathogens.

Following is the list of inhibitors that can profoundly use against MDR bacterial pathogens in table 2.9[7].

Table 2.9: List of antimicrobial inhibitors with their MIC/IC values.

ANTIMICROBIAL INHIBITORS	MIC / IC VALUES
Curcumin	10µg/ml
Coriander oil	1-4µg/ml
Chitosan	1-5µg/ml
Pyrrole TAGE	400µM
Pyrroloindoline triazole amides	IC50 20±2.0µM

2.11 Assumption of conjugated compound for inhibition of bacterial isolates

As histidine is an essential amino acid though it contains imidazole ring in its skeleton, and it has already been discussed that if an urocanase inhibitor can be used for preventing the degradation of L-histidine we can reduce the biofilm assemblage ofMDR bacterial pathogens. For developing a urocanase inhibitor we will be going to remodel the pure imidazole compound by mimicking the structural characteristics of L-histidine, that can act as substrate in replacement of L-histidine for urocanase [7], [19], [20].

Also, pure imidazole shows minimum inhibition at higher concentrations so we concluded that if we can increase the efficiency of imidazole by conjugating it with metal nanoparticle then there would be a chance of getting minimum inhibition at low concentrations [20], [21].

2.12 Mechanism of formulated compounds

2.12.1 Silver nanoparticle

Through the bottom-up approach of nanoparticle synthesis, silver nitrate is used as a precursor for the synthesis of silver nanoparticle by using strong reducing agent trisodium citrate through citrate-reduction method. These approaches determined to produce spherical shape as well as low in cost. After the formation of silver nano-particles in one-pot synthesis by Turkevich method the particles dispersed in colloidal suspension is visualised by its pale-yellow appearance and optical density was checked by UV-Vis spectrophotometer at peak 420-435nm and Fourier-transform infrared spectroscopy[22]– [24].

The antibacterial action of silver nano-particles has been based on three mechanisms: The size of AgNP is in nano-range enables it to penetrate the outer membrane and adhere to the inner membrane where the nano-particles may cause damage, membrane leakage etc. [22]. Furthermore, the ability of the AgNP may interact with the sulphur and phosphorous groups of proteins and DNA that may lead to functional and structural interference. One the mechanism of AgNP is to interfere and altering the metabolic pathways, and genetic material [16], [25]

It has shown its effectivity against *E. coli* MTCC 443, *B. subtilis* MTCC441 with an inhibitory concentration at 10-20µg/ml[26].

The silver nano-particles get conjugated with the imidazole to enhance its chemical properties against MDR pathogen ATCC19606.

2.12.2 Conjugated formulation

The silver nano-particles were conjugated to the imidazole in a 9:1 ratio volume and incubation resulting the conjugation process. The mode of action is that this imidazole inhibits the biofilm assemblage by disrupting their antimicrobial resistance. The metal-Imidazole complex has also been reported to have antimicrobial activity [27]. These formulated compounds may bind to the EPS matrix or interfere with the His-metabolism of the bacterial strain just like miconazole conjugated with silver nanoparticle to induce antibiofilm activity and provides drug delivery system for antifungal activities[28], [29]. The activity is monitored by antibiotic susceptibility testing guided by CLSI [30].

2.13. Antibiotic susceptibility testing

The most commonly utilised method to determine the inhibitory concentrations of antimicrobial compounds that shows bactericidal and bacteriostatic activities. There are two types of methods that are grouped under antimicrobial susceptibility testing: agar dilution and broth dilution.

Agar dilution shows the number of bacterial cells spotted onto the agar plate and zone of inhibitions shows the susceptible, resistant and intermediate behaviour of the culture against antimicrobial compound. Whereas broth dilution shows the minimum concentration at which the compound inhibits the growth of bacterial cells, also known as broth microdilution as it is carried out in 96-well plates having final volume of 200µl.

In both the approaches, the antimicrobial compounds basically prevent the visible growth of bacterial culture. The standards are guided by Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing.

For the development of any drug MIC determination is a step to evaluate the potential antimicrobial compound. The dilution methods are very likely used for in vitro susceptibility testing to evaluate the activity of antimicrobial compound [30].

Chapter 3
MATERIAL &METHODOLOGY

3.1 Materials

3.1.1 Glassware and Instruments

The glass wares used such as conical flasks, measuring cylinders, burette etc. were acquired from Borosil (India). Tips, tarsons, eppendorf tubes and 96-well plates were acquired from Eppendorf (India). Antibiotic disks (amikacin) were obtained from Hi-Media (India). UV-Vis Spectrophotometer used in this experiment was from Thermo scientific (Evolution 220); JUIT (India) and Fourier transform infrared spectrophotometer from Agilent cary630; Shoolini University (India). For photosensitive chemicals, brown reagent bottles and aluminum foil were used.

3.1.2 Reagents

- Chemicals: Silver nitrate (AR/ACS LOBA Chemie), trisodium citrate (Fisher Scientific), imidazole (Hi-Media).
- Media: Mueller–Hinton broth (MHB) (Hi-Media- M1657-500G) and Mueller-Hinton agar (MHA) (Hi-Media- M173-500G)[India] sterilized by autoclaving.
- Stock: McFarland standard: $BaSO_4$(1.17%),H_2SO_4 (1%); 0.4mMAgNP; for first system 50.8mg/ml AgNP and 50.8mg/ml $AgNO_3$; for 9:1 ratio system 45.7mg/ml AgNP, 3mg/ml imidazole and conjugated stock having 45.7mg/ml│3mg/ml concentrations.
- MiliQ water

3.1.3 Microbial Culture

The bacterial strain used in current study was collected from ‘Proteomics Laboratory’ of ‘Department of Biotechnology and Bioinformatics’, JUIT, Sloan. The suspension of bacterial culture was kept in Mueller Hinton broth at 4ºC for extended period of time and all the microbiological processes, like inoculation, spreading were performed under aseptic conditions in laminar air flow (LAF) chamber. The protocols that we used for antimicrobial susceptibility test was standardized as per the CLSI guidelines.

3.1.4 Experimental design

The silver nano-particles were prepared and characterised by illustrated procedure in section 3.2.2. Furthermore, the activity of these formulations as antibacterial agent, antibiotic susceptibility test was performed by broth microdilution method section 3.2.5.

The resultant concentrations of the compounds against bacterial isolate were further optimized to check the minimum inhibitory concentrations of the compounds by broth micro-dilution method and compared with their positive and negative control respectively.

WORK PLAN

Bacterial strain was revived from glycerol stock and inoculated on MHB media to obtained bacterial suspension and further streaked on MHA media for isolated colonies

Silver nano-particles and its conjugated formulations were prepared by citrate reduction method and characterized by Spectroscopic analysis (200nm-800nm).

Evaluating the therapeutical properties of complex compounds (nano-particles and its conjugated formulation) by antibiotic susceptibility testing methods (broth micro-dilution assay)

Minimum inhibitory concentrations acquiring wells were used to assess the effectivity of the antimicrobial compounds.

3.2 Methodology

3.2.1 Revival of culture

- Glycerol stock of bacterial isolates was thawed, and 100µl of the inoculum was transferred to 10ml of sterilized MHB media.
- The inoculated sample was incubated at 37 ºC for overnight.

3.2.2 Silver nanoparticle synthesis

- 3.39mg of silver nitrate was weighed and added to 50ml of milli-Q water in 150 ml conical flask.
- The prepared solution of 0.4 mM silver nitrate was heated at 100 ºC.
- In the boiling condition, 5 ml of 1% trisodium citrate was added dropwise and vigorously stirred in the hot-plate stirrer.
- 30 mins of continuously stirring and dropwise addition of trisodium citrate led to a colour change of the solution from transparent solution to pale yellow colloid.
- The appearance of a pale-yellow colour indicates the formation of silver nano-particles in the colloidal solution.
- The solution was further characterized by spectrophotometric analysis using UV-Vis spectrophotometer.
- The UV-Vis spectroscopic analysis was done in the range of 200 nm-800 nm and the spectral data was monitored.

3.2.3 Antibiotic susceptibility test of prepared AgNP

- 20 µl of inoculum from the overnight culture was transferred to a sterile 10 ml MHB tube.
- Fresh culture of the microbial strain was adjusted to 0.5 McFarland.
- The preparations were diluted using sterile MHB, in microtiter well plates.
- Stock preparation was done according to the given table 3.1 below; two-fold dilutions of compounds were carried out in triplicates and further diluted by MHB.

Table 3.1: System having 1ml of compound for preparation of stock concentrations.

Compound	**Concentration (mg/ml)**
Silver nitrate ($AgNO_3$)	50.8 mg/ml
Silver nano-particles (AgNP)	50.8 mg/ml

- 100 µl of prepared fresh culture, having 2×10^5 CFU/ml was transferred into each well of the microtiter plate to bring the final volume up to 200 µl.
- The microtiter plate was incubated at 37 ℃ for 18 hrs.
- After the incubation period, the results were interpreted, and basic calculations were done to obtain MIC values by plotting the graph of concentrations vs absorbance.

3.2.4 Preparation of AgNP-Imidazole conjugates

- Conjugates were prepared by dissolving pre-synthesized silver nano-particles from its precursor salt $AgNO_3$ with imidazole in a ratio of 9:1 and mixing gently in 2 ml of Eppendorf.
- The concoction was incubated for 30 mins at room temperature.
- After preparing conjugates, their formation was confirmed by analysing the spectral data obtained from UV-Vis spectrophotometer.

3.2.5 Antibiotic susceptibility of conjugates using broth microdilution

- 20 µl of inoculum from the overnight culture was transferred to a sterile 10 ml MHB tube.
- Fresh culture of the microbial strain was adjusted to 0.5 McFarland.
- The preparations were diluted using MHB in microtiter wells as given in the table below
- Dilutions were carried out by adding sterile MHB.
- Stock preparation was done according to the given table 3.2 below; two-fold dilutions of compounds in 9:1 format was carried out in triplicates and further diluted by MHB.

Table 3.2: System having compounds in a volume ratio of 9:1.

Compounds	**Dilutions (9:1)**	**Final Concentrations (mg/ml)**
Imidazole	Milli-Q water (900 µl)**:** Imidazole (100 µl)	3 mg/ml
AgNP	AgNP (900 µl)**:** Milli-Q water (100 µl)	45.7 mg/ml
AgNP@Imidazole	AgNP (900 µl)**:** Imidazole (100 µl)	45.7mg/ml**:** 3mg/ml

- 100 µl of prepared fresh culture, having 2×10^5 CFU/ml was transferred into each well of the microtiter plate to bring the volume up to 200 µl.
- The microtiter plate was incubated at 37 ℃ for 18 h.
- After the incubation period, the results were interpreted, and basic calculations were done to obtain MIC values by plotting the graph of concentrations vs absorbance.

Chapter 4

RESULTS AND DISCUSSION

4.1 Formation of silver nano-particles

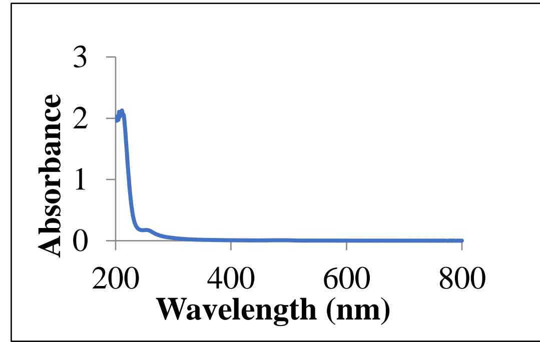
0.4mM silver nitrate was taken as a precursor for silver nanoparticle preparation. Trisodium citrate was used as reducing as well as stabilizing agent in the chemical reaction. The presence of transformed solution (pale-yellow color) from silver-nitrate solution (transparent) confirmed the presence of silver nano-particles.

4.2 Characterization of silver nano-particles

4.2.1 UV-Vis Spectroscopic analysis

Further the UV spectroscopy was utilized to confirm the presence of synthesized silver nano-particles. While using the UV-visible spectrum it was found that silver nitrate (0.4 mM) shown peak at 219 nm (figure 4.1), whereas the silver nano-particles prepared from its precursor has shown peak at 420-430 nm as shown in figure 4.2. The optical evaluations by UV-Vis spectrophotometer depicts spectral shift of silver nano-particles and its precursor that confirms the formation of silver nano-particles.

(4.1) (4.2)



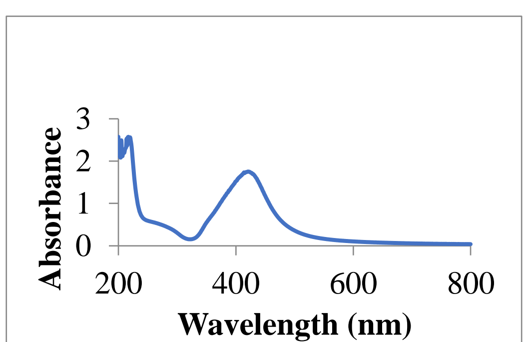
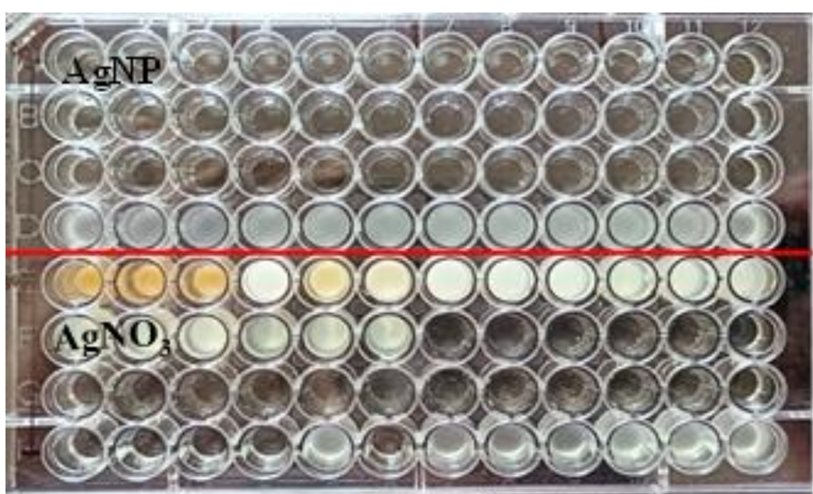


Figure 4.1 and 4.2: UV-Vis spectrum to depict the characterization of **(4.1)** 0.4mM silver nitrate solution and **(4.2)** Silver nano-particles synthesized from its precursor ($AgNO_3$).

4.3 Antimicrobial activity of silver nanoparticle against MDR microbial strain.

The broth micro-dilution assay was done to get the minimum inhibitory concentrations of the silver nanoparticle and its precursor as shown in figure 4.3 (a) and (b). The stock concentrations of both $AgNO_3$ and AgNP was at 50.8mg/ml and the comparison were done on the basis of activity induced by silver nano-particles and its precursor salt for the quantification of the toxicity.

(a)



(b)

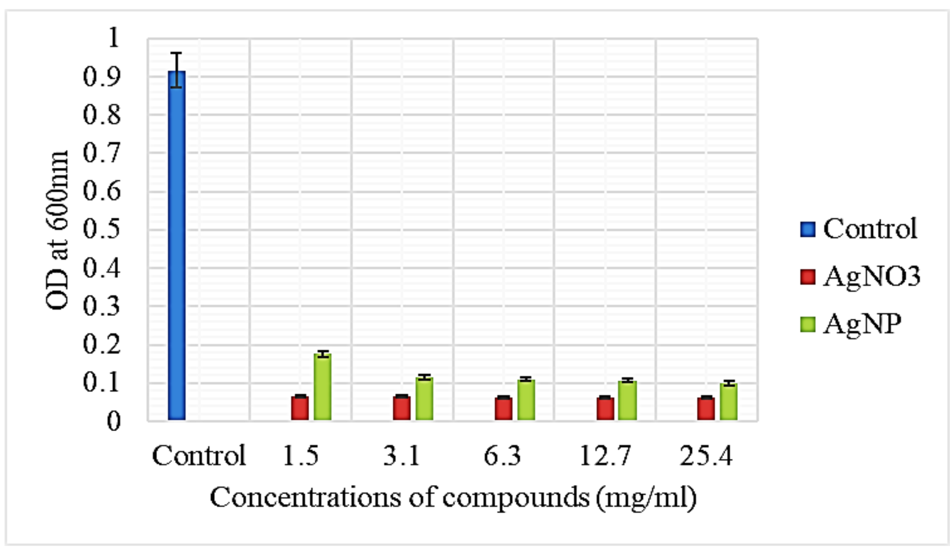


Figure 4.3(a) MIC plate assay of AgNP and $AgNO_3$ **(b)** MIC of silver nano-particles (AgNP) and its precursor $AgNO_3$. Data is representing the mean value of triplicates with ± SD value.

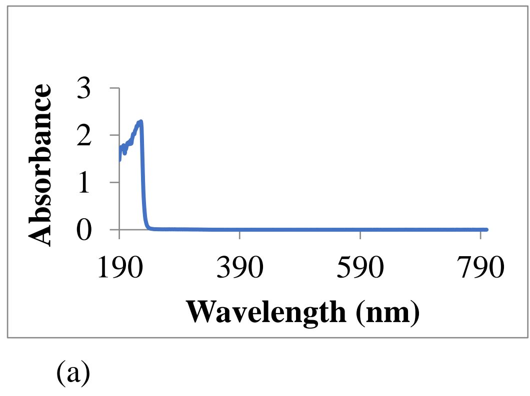
4.4 Assessment of imidazole conjugated silver nano-particles.

4.4.1 Formulation of conjugated system

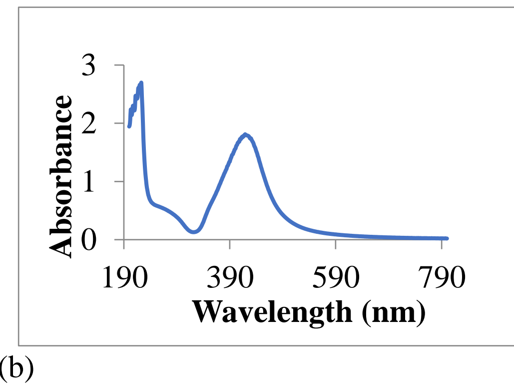
The optimized silver nano-particles were used for conjugation of silver nano-particles with imidazole so that the antimicrobial activity of the compounds can be enhanced at low concentrations. The functionalization was done by incubating the AgNP and Imidazole at 9:1 volume ratio for 30 minutes with their control systems and UV-Vis spectral analysis was used to characterize the conjugation step in the chemical reaction.

4.4.2 Characterization of conjugated system

The peak of conjugated compound at 9:1 volume ratio was determined by the spectral shifts of its precursor compounds, imidazole and silver nano-particles only. The observable peaks depict that the maximum absorbance of imidazole was at 200nm and silver nano-particles at 426nm. However, the maximum absorbance of imidazole conjugated silver nano-particles i.e., the conjugated system was at 210nm as shown in figure 4.4 This spectral shift ensures that the conjugation may occur during the reaction process and their antimicrobial property may get enhanced. The antimicrobial activity of the conjugated system was further tested against the bacterial isolate.



(a)



(b)

Figure 4.4: UV-Vis spectral analysis of compounds in 9:1 volume ratio of (a) Imidazole and **(b)** Imidazole conjugated silver nano-particles (AgNP@Imidazole).

4.5 Antimicrobial susceptibility testing for conjugated system against bacterial strain by micro-broth dilution method.

The antimicrobial activity of conjugated system was evaluated by micro-broth dilution method with their precursor compounds that has been taken as control. Therefore, 9:1 volume ratio was determined for alone silver nano-particles and imidazole with their conjugated system (AgNP@Imidazole) mentioned in section3.2.5.

In the conjugated system, the silver nano-particles may act as an elicitor to imidazole so that, the imidazole can be effective at low concentration or it may possess synergistic effects against bacterial isolates. The inhibitory effects of imidazole, silver nanoparticle and conjugated system as an antibacterial agent has been shown in figure 4.5 (a), (b) and (c) by observing no visible growth and MIC was determined as shown in figure 4.5 (d), (e) and (f).

(a)

Imidazole 1.5 mg/ml

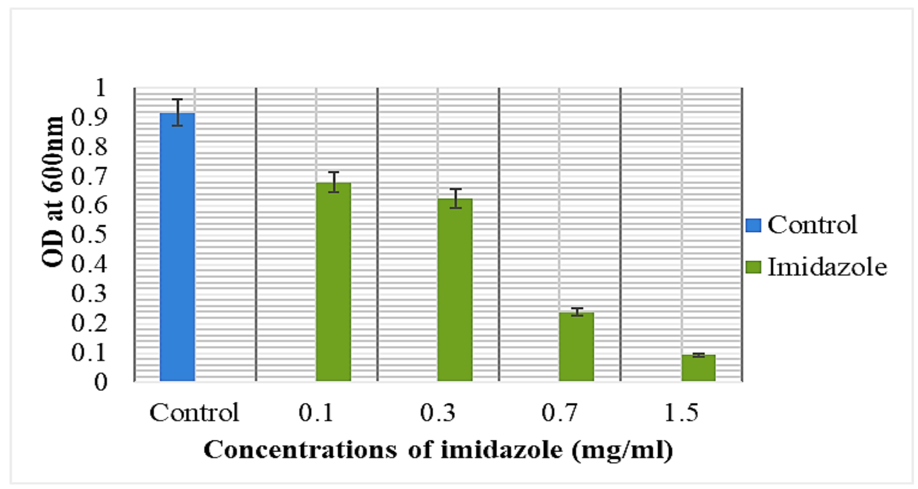
Silver nano-particles 22.8 mg/ml

(b)

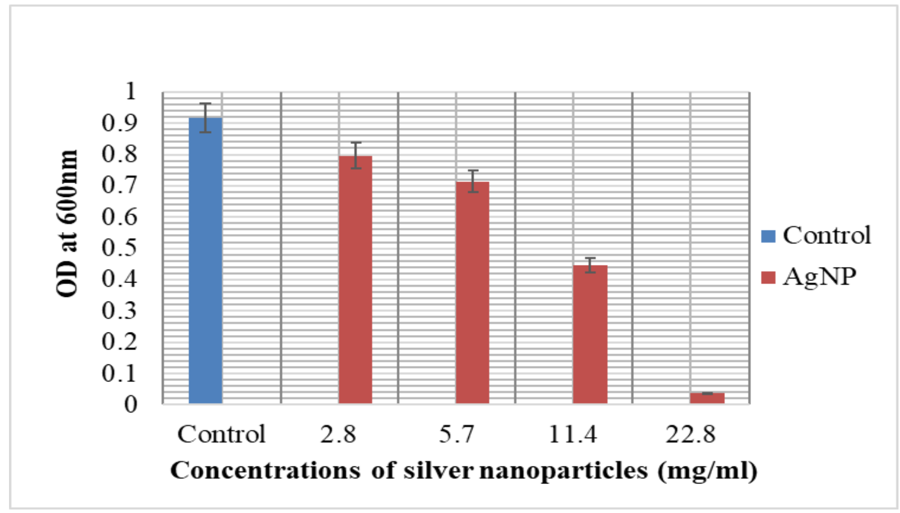
(c)

Imidazole conjugated silver nano-particles 11.4 mg/ml|0.7mg/ml

(d)



(e)



(f)

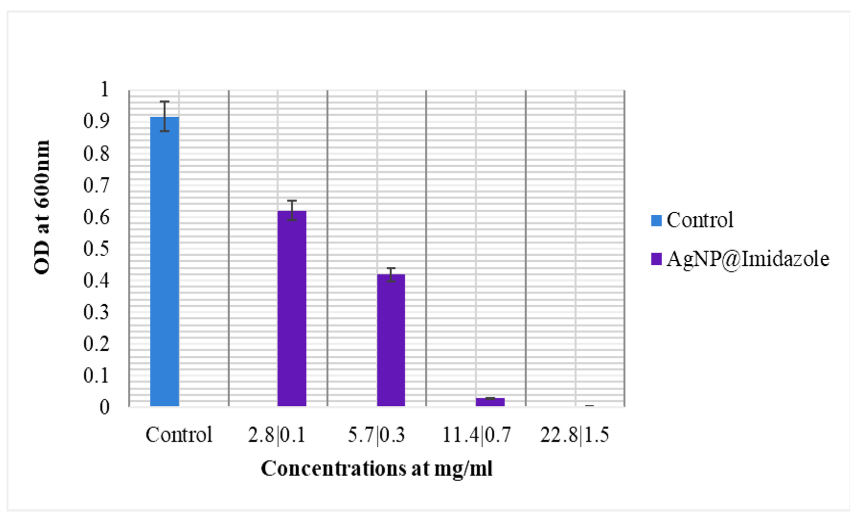


Figure 4.5: Micro-broth dilution assay of compounds at 9:1 volume ratio (a) Imidazole, **(b)** Silver nano-particles, **(c)** Imidazole conjugated silver nanoparticle **and MIC determination of compounds at 9:1 volume ratio (d)** Imidazole, **(e)** Silver nano-particles, **and (f)** Imidazole conjugated silver nano-particles. Data is representing the mean value of triplicates with ± SD value.

4.6 Comparative analysis of conjugated system with its precursors in 9:1 volume ratio.

The comparative analysis that has been shown in figure 4.6 illustrates that the combination of silver nano-particles and Imidazole possess efficient activity at low concentrations. Therefore, the synergistic effect of the compounds shows the bacteriostatic effect against bacterial isolate. However, the optimized activity of compounds for bactericidal effect is still in progress.

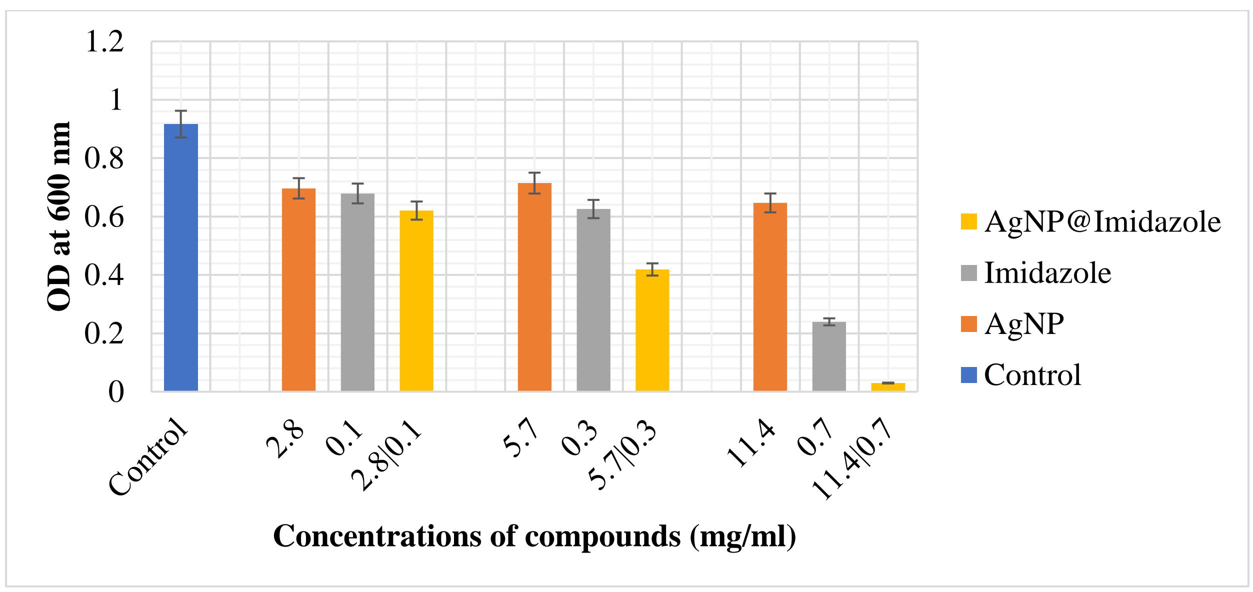


Figure 4.6: Comparative analysis of Imidazole, silver nano-particles (AgNP) and conjugated system (AgNP@Imidazole) at 9:1 volume ratio. Data is representing the mean value of triplicates with ± SD value.

Chapter 5

CONCLUSION

The drug-resistant potential of various bacterial strains challenges researchers in the exploration of new drugs system. Nanotechnology has gained attention for its application in drug discovery. In the present study, the silver nano-particles and their conjugated system with imidazole were utilized for their antimicrobial potential against MDR bacterial strain. The citrate reduction method was found to be an economical way to form AgNP where trisodium citrate act as a reducing agent as well as stabilizing agent that causes the change in colour of the precursor solution (i.e., $AgNO_3$).

The broth micro-dilution method assessed the antimicrobial potential of AgNP and its conjugate with imidazole (AgNP@Imidazole) which revealed that the imidazole conjugated silver nano-particles system has more efficiency than its alone form (i.e., AgNP and imidazole) against pathogenic bacterial isolate even at very low concentrations (i.e., 11.4mg/ml|0.7mg/ml of silver nano-particles and imidazole). Therefore, the nanotechnology approach can be explored more for its application in drug discovery.

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