

PREPARATION OF CARBAPENEM ENCAPSULATED NANO-MATERIAL FOR TREATING MULTI-DRUG RESISTANT MICROBIAL SKIN INFECTIONS

**Submitted in partial fulfilment of the requirements for the award of Bachelor
of Science degree in Biotechnology.**

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Harsha Varthan M K (41300055), V Sailaja (41300039)** who carried out the project entitled **"PREPARATION OF CARBAPENEM ENCAPSULATED NANO-MATERIAL FOR TREATING MULTI-DRUG RESISTANT MICROBIAL SKIN INFECTIONS"** under my supervision from October 2023 to April 2024.

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DECLARATION

We, **Harsha Varthan M K (41300055)** and **V Sailaja (41300039)** hereby declare that the Project Report entitled "**PREPARATION OF CARBAPENEM ENCAPSULATED NANO-MATERIAL FOR TREATING MULTI-DRUG RESISTANT MICROBIAL SKIN INFECTIONS**" done by us under the guidance of **Dr. DEVI, Assistant Professor at Sathyabama Institute of Science and Technology** is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in **Microbiology**.

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ABSTRACT

This study aimed at developing of a pioneering nanomaterial to combat carbapenem-resistant multidrug-resistant (MDR) bacterial skin infections. The formulation comprises silver nanoparticles (Ag NPs) encapsulated with the carbapenem antibiotic imipenem, integrated into a chitosan hydrogel matrix. Antimicrobial efficacy testing was centred on normal strains, with a specific focus on *Pseudomonas* and *Acinetobacter* species, offering insights into potential future applications against carbapenem-resistant MDR bacteria.

The methodology involved the green synthesis of Ag NPs utilizing optimized levels of turmeric extract. Subsequently, imipenem and Ag NPs were loaded into a chitosan hydrogel cross-linked with glutaraldehyde. Employing a Box-Behnken design, 15 samples with varying concentrations of drug, silver NPs, and chitosan were generated. Antimicrobial testing employed agar well diffusion, with observations of inhibition zones serving as indicators of sample efficacy. Furthermore, swelling tests conducted over 8 hours elucidated the hydrogel's water absorption capacity.

Results showcase the formulation's remarkable efficacy against microbial infections, particularly notable against *Pseudomonas* compared to *Acinetobacter*. Although not specifically targeted at MDR bacteria, the formulation's robust antimicrobial performance hints at its potential effectiveness against carbapenem-resistant MDR strains. This study underscores the significance of the novel formulation in antimicrobial research, with promising implications for future therapeutic interventions. Additionally, the hydrogel's exceptional water-holding capacity, as demonstrated in the swelling test, further bolsters its utility.

Keywords: MDR, carbapenem, silver nps and chitosan hydrogel

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ABBREVIATIONS

NPs – Nanoparticles

Ag- Silver

bla – Beta lactamases

Amp – Ampicillin

MDR- Multi Drug Resistant

ESBL- Extended-spectrum beta-lactamases

AETP -Aqueous Extract of Turmeric Powder

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CHAPTER 1 INTRODUCTION

1.1 MICROBES AND THEIR RAISING RESISTANCE:

A major threat to global public health has emerged from the rise in multidrug-resistant (MDR) strains of gram-negative bacteria over the past years, especially among Enterobacteriaceae family mainly *P. aeruginosa*, and *A. baumannii*. Hospitalized patients are experiencing an increasing number of problems from these organism-caused infections, especially since there haven't been many new drugs developed recently to combat these multi drug resistant infections. Because MDR gram-negative infections require more resources to manage, they are linked to greater death rates as well as an ever-increasing socioeconomic burden (1).

To choose the best treatment plans for MDR patients in a hospital context, good interdisciplinary antimicrobial stewardship is required. This involves regularly evaluating bacterial resistance patterns and the possibility of antibiotic medicines working synergistically. A prudent approach for the first empirical antimicrobial therapy in cases where a seriously ill hospitalized patient has a high risk of multidrug-resistant (MDR) gram-negative organisms is to choose a broad-spectrum regimen consisting of medications with distinct modes of action and mechanisms through which resistance may develop. This will increase the antimicrobial exposure and take advantage of potential synergistic effects. When susceptibility reports become available, the initial observed routine can be modified (1). This rise of multi-drug-resistant bacteria poses a serious threat to human health, leading to a decrease in the efficacy of antibiotics used for treatment. One such class of antibiotics that has lost its effectiveness against bacterial resistance is carbapenem, a broad-spectrum β -lactam antibiotic. Carbapenems serve as a last line of defence against gram-negative bacterial diseases, with organisms like *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *E. coli* and *Klebsiella pneumoniae* being the most common and highly infectious culprits that have developed resistance to this antibiotic class. The World Health Organization recently designated carbapenem resistant Enterobacterales as a global health priority pathogen, recognizing it as a

major cause of mortality and a public health concern (2). WHO has classified these carbapenem resistant *A. baumannii* & *P. aeruginosa* as critical category in the priority pathogen list which requires the topmost attention in creating new therapeutic approaches for their treatment. The mode of action of carbapenems involves disrupting the gram-negative cell wall by binding to cell wall synthesizing sites, preventing the formation of the cell wall (3)(4). However, these bacteria have developed resistance to specific members of the carbapenem class, such as imipenem and meropenem, through constant exposure over time. They employ resistive mechanisms like reduced permeability, overexpression of efflux pumps, degradative enzymes, and mutations (3)(5). For active infections such as wound infections caused by this carbapenem resistant microbes, the accurate diagnosis and early administration of suitable antimicrobial medication are critical for active infections, in order to improve patient survival and lower the high rates of morbidity and fatality caused by severe spreading of these wound aided infections (4).

1.2 Carbapenem and Silver Nanoparticles in Infection Treatment:

1.2.1 CARBAPENEM ANTIBIOTICS:

Carbapenems, belonging to the β -lactam antibiotics class like penicillin and cephalosporins, possess a unique chemical structure setting them apart. They are crucial in combating severe infections caused by ESBL-producing bacteria, standing as the last resort in treatment. However, there's a concerning rise in carbapenem-resistant bacterial strains, challenging this vital defence. Carbapenems boast the broadest spectrum among antibiotics and offer the most compelling evidence for efficacy against ESBL producers. The molecular structure of all β -lactam antibiotics shares a common feature: the presence of a beta-lactam ring. Carbapenems, while resembling penicillin with a five-membered ring (6,7), feature a unique alteration where the sulphur at C-1 is replaced by a carbon atom, and a double bond is introduced between C-2 and C-3. Additionally, carbapenems distinguish themselves by placing the side chain in the trans position instead of the more common cis position found in other β -lactams. This structural difference renders carbapenems insensitive to the effects of β -lactamases (8). Beta-lactamases are categorized into four primary groups based on their amino acid sequences: classes A, B, C, and D. Typically, the genes encoding all four classes (A, B, C, and D) are found on the

bacterial chromosome. Class A includes extended spectrum β -lactamases (ESBLs) and *Klebsiella pneumoniae* carbapenemase enzymes. Class B enzymes are known as metallo- β -lactamases (MBLs), which exhibit a wide substrate range, capable of degrading all beta-lactam antibiotics except monobactams. Class C enzymes, cephalosporinases, are widely distributed enzymes often resistant to cephamycins, penicillin, and cephalosporins. Class D enzymes are oxacillinases. Recently, it has been acknowledged that *A. baumannii*, though previously considered less virulent compared to *K. pneumoniae* and *P. aeruginosa*, significantly contributes to the dissemination of broad-spectrum resistance genes among other Gram-negative bacteria (9). Yet, the escalating resistance among Enterobacteriaceae strains to β -lactamase inhibitor combinations, often due to secondary, inhibitor-resistant "OXA" β -lactamases, poses a significant challenge. Thus, while carbapenems remain crucial, their effectiveness is increasingly under threat, emphasizing the pressing need for novel solutions in antibiotic development and management strategies (10). Phenotypic resistance to carbapenems typically stems from two primary mechanisms: β -lactamase activity combined with structural mutations and the production of carbapenemases—enzymes specifically designed to hydrolyse carbapenem antibiotics. The former mechanism involves extended-spectrum β -lactamases (ESBLs) and AmpC cephalosporinases, often encoded by plasmids, and their ability to confer carbapenem resistance when combined with mutations affecting porins. Porins, proteins in the outer membrane of Gram-negative bacteria, play a crucial role in facilitating antibiotic diffusion. Mutations in porins can retard this process, enabling ESBLs and AmpC enzymes to effectively degrade carbapenems. Other mechanisms contributing to carbapenem resistance such as drug efflux pumps and alterations in penicillin-binding proteins as stated earlier. The emergence and spread of carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains are of particular concern. These organisms, often associated with healthcare settings, are notorious for their ability to develop resistance to multiple antibiotics, including carbapenems (11). *A. baumannii* has become more associated with the dissemination of broad-spectrum resistance genes, whereas *P. aeruginosa* presents challenges due to its inherent resistance mechanisms and capacity to acquire additional resistance traits. Specifically, resistance to class D carbapenem oxacillinases (OXA types) is increasing in these

bacteria. These resistances, driven by bla genes, are transferred horizontally between individuals of the same species producing more resistant microbes (12).

1.2.2 ANTIMICROBIAL PROPERTY & GREEN SYNTHESIS OF SILVER NANOPARTICLES:

Since, the resistance obstructs the delivery of antibiotics into the bacteria, overcoming this challenge requires alternative approaches for better drug delivery methods. Nanotechnology emerges as a promising tool for solving this problem. Nanoparticles can serve as an excellent carrier of antibiotics, possessing their own antimicrobial properties. Some nanoparticles are inert and non-toxic, providing an ideal medium for antibiotic containment and efficient delivery. Due to their small size, nanoparticles exhibit a high surface-to-volume ratio, enabling them to absorb more antibiotic and enhance reactivity at the target site. When in contact with the bacterial surface, nanoparticles exhibit properties such as ROS generation, evading efflux pumps, protection against bacterial enzymes and toxins, and cell disruption. These align with the challenges faced when using carbapenem antibiotics against resistant gram-negative bacterial species. To address these challenges, nanoparticles can be used as carrier molecules facilitating efficient antibiotic transfer into the gram-negative cell wall.

Silver nanoparticles, with their inherent therapeutic properties and ability to act on multiple sites, possess broad-spectrum antibacterial capabilities against various microorganisms. This makes them promising candidates for addressing emerging challenges in microbial resistance across different applications, especially in improving healthcare. Silver nanoparticles have shown immense potential in diverse areas such as detection and diagnosis, drug delivery systems, coating biomaterials and medical devices, developing novel antimicrobial agents, and creating regenerative materials. The antimicrobial properties of silver nanoparticles have fuelled a growing demand for their medical applications, including wound dressings, artificial implants, and carriers for antitumor drugs (13)(17). While the antimicrobial activity of silver nanoparticles (AgNPs), either alone or in combination with standard antibiotic drugs, has been demonstrated against a wide range of microorganisms, including Gram-negative and Gram-positive bacteria, as well as fungi, the precise mechanism of their antimicrobial action remains incompletely understood. Nonetheless, extensive research has been conducted to elucidate their mode of action, and three well-defined mechanisms have been proposed thus far: (i) damage

to the cell wall and membrane, (ii) penetration into the cell and subsequent damage, and (iii) induction of oxidative stress (14).

In order to create silver nanoparticles (Ag-NPs) from plants or plant extracts, different plant parts such as roots, leaves, stems, and seeds are used as stabilising and reducing agents. These plants have active ingredients that help in the reduction and capping of the nanoparticles, including terpenoids, polysaccharides, phenolics, alkaloids, flavones, amino acids, enzymes, and proteins. Comparing the synthesis of Ag-NPs with plant extracts to other processes that involve microorganisms such as bacteria and fungi, has drawn interest because of its ease of use, speed, economy, and environmental friendliness. Plants are a great source of nanoparticle synthesis materials due to their wide spectrum of active agents, ease of accessibility, and safety during handling. Depending on the plant extract utilised and other variables including concentration, pH, temperature, metal salt, and contact duration, the synthesis process can proceed quickly at room temperature. Because the plant extracts contain biomolecules, the resulting nanoparticles are stable and have control over their structure, guaranteeing the generation of stable nanoparticles.

Promising uses for Ag-NPs have been demonstrated by their environmentally friendly synthesis from plants or plant extracts, which includes antifungal and antibacterial properties. Numerous plant species, including *Curcuma longa*, alfalfa, *ananas comosus*, *Argemone mexicana*, peanut shell, *Malus domestica*, neem leaf broth (*Azadirachta indica*), *Pelargonium graveolens*, geranium leaves, *Medicago sativa* (Alfalfa), Aloe vera, *Emblica officinalis* (Amla, Indian Gooseberry) and few microorganisms *Polyalthia longifolia*, papaya, and many more, were used in the synthesis of these nanoparticles. They are still being researched and studied because of their potential in many domains and their potency against a variety of bacteria (15)(16).

1.2.3.POTENTIAL SYNERGISTIC EFFECTS IN COMBATING MICROBIAL INFECTIONS:

Since bacterial resistance can be overcome through the empirical properties of silver nanoparticles, combining them with antibiotics can produce a synergistic effect to combat antibacterial resistance. Implementing silver nanoparticles into drug delivery systems has proven effective in recent studies against multidrug-resistant (MDR) pathogens. The preparation of nano-carriers for drug delivery is a complex

process requiring careful inspection to address various issues that may arise during their intracellular and extracellular activities inside the body. Consequently, the physical, chemical, and spectral properties of the synthesized nano-carriers are closely monitored and later checked for their cytological effects before being used as medicinal tools. Overcoming these challenges and producing an effective and efficient carbapenem-encapsulated nanocarrier helps in tackling life-threatening carbapenem-resistant bacterial infections.

Combining silver nanoparticles with imipenem (IMP-AgNPs conjugates) has shown promising results in treating bacterial infections caused by multidrug-resistant isolates. This combination provides a continuous and stable dose of imipenem over several days, enhancing the antibiotic's effectiveness against bacteria. Despite the presence of antibiotic-resistance genes in some isolates, IMP-AgNPs demonstrated potent antibacterial activity, surpassing the inhibitory effects of equivalent antibiotic concentrations alone. However, further studies are needed to elucidate the specific mechanisms of action and potential interactions of this combination for therapeutic applications. Additionally, while chemical methods are commonly used for producing silver nanoparticles, they can pose risks due to the use of toxic chemicals. In contrast, green synthesis (biosynthesis) offers a low-risk alternative, producing silver nanoparticles with various sizes and shapes without the need for toxic reductants. Although green synthesis may have a longer reaction time compared to the chemical method, its safety and environmental benefits make it a viable option for future studies and applications (18)(19).

In conclusion, imipenem and silver nanoparticles together provide a number of benefits for treating bacterial infections that are resistant to several drugs, such as increased antibacterial activity and less environmental hazards related to synthesis processes. More investigation into this combination's mechanisms of action may result in safer, more efficient treatment methods as well as better therapeutic outcomes.

1.2.4 CHITOSAN HYDROGEL NANOMATERIAL:

Chitosan hydrogels have garnered significant attention in biomedical applications, particularly in wound dressing due to their remarkable properties. As a biocompatible and biodegradable natural polymer, chitosan forms hydrogels that exhibit mucoadhesivity and moisture retention capabilities, creating an ideal environment for wound healing. These hydrogels can conform to irregular wound

shapes, providing a protective barrier against external pathogens while promoting tissue regeneration. Moreover, chitosan's antimicrobial properties make it particularly suitable for preventing infections, enhancing the efficacy of wound healing. Its ability to absorb exudates from wounds further contributes to maintaining a moist wound environment, facilitating faster healing and reducing scarring. Consequently, chitosan hydrogels have emerged as promising candidates for advanced wound dressing materials, offering improved patient outcomes and wound management strategies.

When combined with silver nanoparticles (AgNPs) and antibiotics, chitosan hydrogels exhibit enhanced capabilities as drug delivery systems. Silver nanoparticles possess inherent antimicrobial properties, effectively combating a broad spectrum of pathogens, including antibiotic-resistant strains. Integration of AgNPs into chitosan hydrogels not only augments their antimicrobial activity but also provides sustained release of silver ions, prolonging the therapeutic effect. Furthermore, incorporating antibiotics into the chitosan hydrogel matrix enables targeted delivery to the site of infection, maximizing drug efficacy while minimizing systemic side effects. This synergistic combination of chitosan hydrogel, silver nanoparticles, and antibiotics offers a multifaceted approach to combating microbial infections, with potential applications in wound care, topical treatments, and targeted drug delivery systems. By utilising the unique properties of each component, these innovative formulations hold promise for addressing the challenges of antimicrobial resistance and improving patient outcomes in clinical settings (20)(21).

1.3 Applications:

In response to this critical need, this study focuses on the preparation of a carbapenem-encapsulated nanomaterial tailored for treating MDR microbial infections, particularly wound infections. The approach involves the synthesis of silver nanoparticles using turmeric extract as a reducing agent, followed by the incorporation of these nanoparticles into a chitosan hydrogel matrix along with the antibiotic imipenem. The rationale behind this approach lies in the unique properties of both silver nanoparticles and imipenem. Silver nanoparticles exhibit potent antimicrobial activity, capable of disrupting bacterial cell membranes and inhibiting microbial growth. Imipenem, a broad-spectrum carbapenem antibiotic,

complements the antimicrobial action of silver nanoparticles by targeting bacterial cell wall synthesis, thereby exerting bactericidal effects. By encapsulating imipenem within a chitosan hydrogel matrix embedded with silver nanoparticles, the nanomaterial aims to achieve targeted and sustained release of the antibiotic at the site of infection. This localized delivery strategy enhances the efficacy of imipenem while minimizing systemic side effects, thereby overcoming challenges associated with carbapenem-resistant microbial infections.

Through this innovative approach, the study seeks to address the urgent need for effective treatments against MDR microbial infections, particularly those caused by carbapenem-resistant pathogens. By harnessing the synergistic effects of silver nanoparticles and imipenem within a nanocomposite framework, this research holds promise for advancing therapeutic options in the fight against antibiotic-resistant infections.

CHAPTER 2

LITERATURE SURVEY

2.1 MULTI DRUG RESISTANT BACTERIA:

Resistance among deadly Gram-negative pathogens has risen to epidemic proportions, particularly within hospitals and acute care settings. Infections with bacteria such as MDR and XDR *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* contribute to alarmingly high rates of mortality in our most vulnerable populations and add billions of dollars in healthcare costs through lengthening hospital stays, utilization of resources, lost productivity, and high acuity care needs. Addressing this problem requires both infection prevention and appropriate treatment (Morris S & Cerceo E 2020).

Antimicrobial resistance in these strains is genetically determined and most commonly mediated by the acquisition of extra-chromosomal genetic elements via horizontal gene transfer. Low permeability of the outer membrane in gram negative bacteria, efflux pumps, production of degrading enzymes, and modification of targets are examples of mechanisms used by bacteria to resist the toxicity of antibiotics. Globalization, excessive use of antibiotics in animal husbandry and aquaculture, use of multiple broad-spectrum agents, and lack of good antimicrobial stewardship can be listed as the factors most responsible for the spread of antibiotic resistance species (Vivas R et al. 2019).

Carbapenems are used as 'last resort' antibiotics. We found that CRAB – a critical global priority pathogens (GPP) has been reported globally, regionally, and in Bahrain, and has dramatically increased over the last decade. The heterogeneity of carbapenem-hydrolyzing class D B-Lactamases (CHDL)-encoding genes as the source of CRAB isolates was identified among eight patients in different units of SMC, leading to the death of four patients. Also, the first GCC regional study of the molecular epidemiology of CRAB found that the OXA-23-type was the major carbapenemase mechanism for the resistance phenotype; one-fourth represented healthcare-associated infections, while half associated with nosocomial infections indicating the need for optimizing infection control practices and antibiotic stewardship (Asokan GV et al 2019).

Among the clinically important extensively drug-resistant (XDR) Gram-negative bacteria (GNB) XDR-GNB species that harbor plasmidic genes encoding a wide

variety of carbapenemases, isolates of sequence type (ST) 258, ST11, and ST147 Enterobacterales (mainly producing *Klebsiella pneumoniae* carbapenemase [KPC], metallo- β -lactamase [MBL, especially New Delhi MBL {NDM}], and oxacillinase [OXA], etc.), ST111 and ST235 *Pseudomonas aeruginosa* (mainly producing NDM, imipenemase [IMP], Verona integron encoded MBL [VIM], and OXA, etc.), and ST2, ST32, ST92, and ST368 *Acinetobacter baumannii* complex (mainly producing MBL, NDM, and OXA, etc.), they usually co-harbor other resistant β -lactamases (various extended-spectrum β -lactamases [ESBLs] and/or AmpC β -lactamases), thus constitute a worrisome global threat because of their high potential for transmission (Jean et al. 2022, Tsakris et al., 2006; Vourli et al., 2006; Miriagou et al., 2007; Zappas et al., 2008; Giakkoupi et al., 2009; Da Silva et al., 2010; Kumarasamy et al., 2010; Papagiannitsis et al., 2010; Wang et al., 2010; Gupta et al., 2011; Chang et al., 2015; Huang et al., 2016; Roy Chowdhury et al., 2017; Yang et al., 2018; Nordmann and Poirel, 2019; Tavošchi et al., 2020; Yoon and Jeong, 2021)

Table 1 : Number of publications globally, and from the GCC and BAHRAIN indexed in PubMed for 2017 WHO global priority pathogens list stewardship (Asokan GV et al 2019).

Priority category	Pathogens	Antibiotic resistance	Gram's stain	Global	GCC	Bahrain
Critical	<i>Acinetobacter baumannii</i>	Carbapenem-resistant	Gram-negative	768	30	2
	<i>Pseudomonas aeruginosa</i>	Carbapenem-resistant	Gram-negative	467	9	NR
	<i>Enterobacteriaceae</i>	Carbapenem-resistant	Gram-negative	1169	16	NR
	<i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Enterobacter spp.</i> , <i>Serratia spp.</i> , <i>Proteus spp.</i> , <i>Providencia spp.</i> , <i>Morganella spp.</i>	Third generation cephalosporin-resistant		4001**	33	4

MDR *Enterobacteriaceae* showed increased resistance to aminoglycosides, quinolones, trimethoprim-sulfamethoxazole, but not to imipenem, ertapenem,

meropenem and aztreonam. Non-fermenting Gram negative bacteria showed the highest resistance to chemotherapy, some strains even to imipenem, meropenem, ertapenem, and aztreonam, but very few. This is why these four antibiotics, along with piperacillin-tazobactam remain reserve antibiotics for MDR Gram negative bacteria (fermenting and non-fermenting). CRE continue to evolve, posing an increasing threat to patients of all ages. Mechanisms of carbapenem resistance are variable, and the breadth of MGEs in Enterobacteriaceae—carbapenemase genes and other antibiotic resistance mechanisms and virulence determinants—continues to expand. (Pirvanescu H et al. 2014) (Logan LK et al. 2017).

2.2 SILVER NANOPARTICLES:

The metal nanoparticles have proven to be one of best choices to treat drug resistant bacterial infections. The high surface to volume ratio of NPs increases their affinity towards the cellular membrane. In addition, the positive charge on the NPs can further enhance the affinity for the negatively charged membrane due to the presence of either lipopolysaccharide or teichoic acid. It is not easy for bacteria to develop the resistance against NPs, since NPs simultaneously target several pathways to kill bacterial cell. The exact mechanism through which NPs antimicrobial activity occurs is only hypothesized and needs to be studied further. Although the multiple pathways that seem to be simultaneously activated by NPs make elucidation a difficult task, they are also the reason why NP exposure is so effective. The combination of ROS production, gene regulation changes, cell wall penetration, and metabolite binding are challenges for adaptation and survival, and the bacteria fail to establish a defense simultaneously against all of the interactions (Singh A et al. 2020) (Slavin YN et al. 2017).

silver has been most extensively studied and used since ancient times to fight infections and prevent spoilage. The antibacterial, antifungal and antiviral properties of silver ions, silver compounds and silver nanoparticles have been extensively studied. Silver is also found to be non-toxic to humans in minute concentrations. The microorganisms are unlikely to develop resistance against silver as compared to antibiotics as silver attacks a broad range of targets in the microbes. The silver nanoparticles with their unique chemical and physical properties are proving as an alternative for the development of new antibacterial agents. The silver nanoparticles

have also found diverse applications in the form of wound dressings, coatings for medical devices, silver nanoparticles impregnated textile fabrics, etc. the advantage of using silver nanoparticles for impregnation is that there is continuous release of silver ions and the devices can be coated by both the outer and inner side hence, enhancing its antimicrobial efficacy. The burn wounds treated with silver nanoparticles show better cosmetic appearance and scarless healing (Rai M et al. 2009).

In green synthesis, plants, bacteria and fungi are extensively used. These organisms are the major sources of metabolites that can work as stabilizing and reducing agent during NPs synthesis. NPs can be synthesized both extracellularly and intracellularly.

Extracellular method is more preferred over intracellular method, since it does not need any downstream process for harvest of NPs from the organisms. Plant assisted synthesis is cheaper and more proficient in getting higher yields of NPs than the microbes. Plants have numerous metabolites and biochemicals that can function as stabilizing and/or reducing agent in green synthesis of NPs (Singh A et al. 2020). Biosynthesis of Ag-NPs using green resources like *C. longa* is a better alternative to chemical synthesis, since this green synthesis is pollutant-free and eco-friendly. *C. longa* tuber powder can play an important role in the bio reduction and stabilization of silver ions to Ag-NPs (Shameli K et al. 2012). Turmeric powders contain some proteins and a high level of terpenoids. Moreover, 178

turmeric is rich in chemicals, such as zingiberene, α -phellandrene, sabinene, and sesquiterpenes. Most of these compounds are polyphenolic bioactive constituents called curcumin that contribute substantially to its unique colour and flavour. Terpenoids in geranium leaf possibly play a key role in the synthesis of AgNPs, and polyols in *Cinnamomum camphora* leaf were confirmed to be the primary agents for the bio reduction of silver and chloraurate ions. Researchers have reported that proteins in turmeric powder played a role in the stabilization of biosynthesized AgNPs through cysteine or free amino group residues that worked as capping agents for AgNPs to protect them from being aggregated in aqueous extract of turmeric powder (AETP) (Alsammarrhaie FK et al. 2018).

2.3 CHITOSAN HYDROGEL:

Chitosan is considered as an ideal material for hydrogels due to its biodegradable, biocompatible, non-toxic, antimicrobial, biologically adhesive, biological activity and haemostatic effect, as well as its amino and hydroxyl groups can be easily reacted and chemically modified, thus allowing a high chemical versatility. The conditions employed for amino group chemical modification may interfere with the final degree of deacetylation and therefore with the cationic nature of the obtained materials. The positively regulatory factors makes chitosan more susceptible to interact with negatively charged molecules such as proteins, anionic polysaccharides and nucleic acids in bacterial membrane, which is the key to antibacterial properties. In terms of wound healing, chitosan-based hydrogels could provide a moist wound environment, offer protection from secondary infections, remove wound exudate, be biocompatible, induce faster wound healing, and produce smoother scarring. Chitosan has shown high biocompatibility and biodegradability qualities. Amongst its features, chitosan is a matrix-forming material that can reach NP sizes. The chitosan mechanism is based on the interaction of its protonated glucosamine groups with cell membranes. As a result, chitosan-based hydrogels are considered advantageous in their application as a wound dressing material. Especially, when the chitosan is modified and/or combined with other polymers, chitosan-based hydrogel dressings will have some better properties to promote wound healing. In addition, these chitosan-based hydrogels as DDS, such as chitosan–PVA hydrogel, can deliver bioactive substances (drug, growth factors or stem cells and etc.) and controlled release at the wound. (Liu H et al. 2018)(Wunnoo S et al. 2023). silver nanoparticles into hydrogel materials has a certain impact on properties of the mentioned polymers. Such additives cause a slight decrease of porosity of hydrogels and affect their sorption capacity. Presented hydrogels based on chitosan and modified with nano silver exhibit biocompatibility in relations to the simulated body fluids such as Ringer's liquid (Tyliszczak B et al. 2017).

The antimicrobial properties of chitosan and its derivatives have attracted great attention from researchers (Kong et al., 2010). Antimicrobial activity of chitosan has been demonstrated against many bacteria, filamentous fungi, and yeasts. Chitosan has a wide spectrum of activity and high killing rates against gram-positive and gram-negative bacteria (Ueno et al., 1997), with low toxicity toward mammalian

cells. Antibacterial effects of chitosan are reported to be dependent on its molecular weight (Jeon et al., 2001). To prove this idea, No et al. (No, Park, Lee, and Meyers, (2002)) examined the antibacterial activity of six chitosan and six chitosan oligomers with widely different molecular weights against gram-negative and gram-positive bacteria (Hamed H et al. 2018).

CHAPTER 3

AIM AND OBJECTIVES

AIM:

To Prepare carbapenem encapsulated nanomaterials for treating multi-drug resistant microbial skin infections

OBJECTIVES:

- To synthesis and characterize the nanoparticles using scanning electron microscope (SEM), x-ray diffraction (XRD), FTIR etc.
- To encapsulate the carbapenem into the nanoparticles.
- To optimize the Carbapenem-silver NPs drug encapsulated Chitosan hydrogel.
- To study the effect of carbapenem-silver NPs drug encapsulated nanomaterial against the multidrug resistant microbial infections.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS:

Silver Nitrate (Purity: >99%, was Purchased from Thermo Fisher Scientific), Deionised water (Purchased from VITSZEE chemicals, pharmaceutical grade), Fresh Turmeric rhizome, Dried turmeric, Ethanol 99%, Petri plates, Cotton swabs, Magnetic stirrer, Imipenem drug (Lupin Ltd, Salt Composition: Imipenem (500mg) + Cilastatin (500mg), Chitosan (Purchased from SRL, 99.8%), Acetic acid (Purchased from OXFORD chemicals, 1%), Glutaraldehyde (SDFL chemicals, 25%), Ethanol 99% (Laboratory grade), Conical flasks, Micropipette, Para film, Nutrient broth, Agar powder, Muller Hinton agar.

4.2 METHODS:

4.2.1 PREPARATION OF TURMERIC EXTRACTS:

4.2.1.1 Preparation of Fresh turmeric extract:

Fresh turmeric rhizome was finely cut into pieces, and a sample was precisely weighed. The weighed sample underwent a slight crushing process, ensuring uniformity in particle size. Subsequently, the crushed turmeric was measured according to the required amount, with 25 grams selected for the extraction process. To facilitate extraction, 100 ml of ethanol was added to the turmeric sample in a ratio of 1:4, ensuring thorough mixing by stirring. The resulting mixture was then allowed to sit undisturbed in a dark room free from light for a period of one week. This duration was chosen to enable the efficient extraction of compounds present in turmeric. Following the one-week extraction period, the solution mixture underwent filtration using Whatman filter paper. This filtration step served to effectively separate and remove the residual crushed pieces of turmeric, ensuring the acquisition of a clean and clarified fresh turmeric extract. The use of Whatman filter paper provided an efficient means of achieving fine particulate filtration, thereby enhancing the purity of the extracted solution. This carefully performed filtration process yielded a clear turmeric extract devoid of any solid impurities.

4.2.1.2 Preparation of Dried turmeric extract:

Dried turmeric extract was carefully prepared by using 0.5 grams of finely ground dried powder derived from turmeric rhizome. This precise amount of dried turmeric powder was selected to ensure optimal extraction efficiency. Subsequently, 100 ml of water were carefully added to the powdered turmeric in a controlled manner while stirring, employing a heat magnetic stirrer set at 85 degrees Celsius. This elevated temperature and continuous stirring facilitated the extraction of bioactive compounds from the dried turmeric powder within a defined timeframe of 30 minutes. To minimize potential degradation of sensitive constituents, the extraction process was conducted in a darkened environment. Following the completion of the extraction period, the resulting mixture was subjected to filtration to isolate the dried turmeric extract. Utilizing a filtration apparatus, the solution was filtered to remove any solid residues, ensuring the attainment of a clarified and purified dried turmeric extract.

4.2.2.SYNTHESIS OF SILVER NANOPARTICLES FROM FRESH VS DRIED TURMERIC EXTRACT:

4.2.2.1 From Fresh turmeric extract:

Silver nanoparticles were synthesized using fresh *Curcuma longa* rhizome extract. A silver nitrate solution with a concentration of 1 mM was prepared by dissolving 0.0085 g of silver nitrate powder in a beaker containing 50 mL of water while being stirred magnetically. Subsequently, 5 mL of fresh turmeric extract was slowly added to the stirring solution. The reaction mixture was allowed to stir for 24 hours overnight in a dark room without exposure to light. Confirmation of silver nanoparticle formation was achieved through the observation of a colour change from the initial bright yellow to brown after the 24-hour incubation period.

4.2.2.2 From Dried turmeric extract:

Silver nanoparticles were synthesized using dry powdered fresh *Curcuma longa* rhizome extract by following a process similar to that carried out with dried turmeric extract. Confirmation of silver nanoparticle formation was achieved through the observation of a colour change from the initial bright yellow to brown after the 24-hour incubation period.

4.2.3.SYNTHESIS OF SILVER NANOPARTICLES FROM FRESH TURMERIC EXTRACT WITH DIFFERENT AMOUNTS (5ML VS 10ML):

4.2.3.1 From 5ml Fresh turmeric extract:

Silver nanoparticles were synthesized using fresh *Curcuma longa* rhizome extract. A silver nitrate solution with a concentration of 1 mM was prepared by dissolving 0.0085 g of silver nitrate powder in a beaker containing 50 mL of water while being stirred magnetically. Subsequently, 5 mL of fresh turmeric extract was slowly added to the stirring solution. The reaction mixture was allowed to stir for 24 hours overnight in a dark room without exposure to light. Confirmation of silver nanoparticle formation was achieved through the observation of a colour change from the initial bright yellow to brown after the 24-hour incubation period.

4.2.3.2 From 10ml Fresh turmeric extract:

Silver nanoparticles were synthesized using fresh *Curcuma longa* rhizome extract by following a similar process carried out with 5ml fresh turmeric extract but 10ml was used instead of 5ml. Confirmation of silver nanoparticle formation was achieved through the observation of a colour change from the initial bright yellow to brown after the 24-hour incubation period.

4.2.4 PURIFICATION OF SILVER NP FROM REACTION MIXTURE:

The prepared nanoparticle solution mixture was centrifuged at 8000 RPM for 20 minutes to facilitate the sedimentation of nanoparticles into a pellet. The resulting pellet was carefully collected and transferred to a watch glass where it was left to dry. Once dried, the sample was gently powdered to obtain a fine silver nanoparticle powder.

Centrifugation at 8000 RPM for 20 minutes effectively separated the nanoparticles from the solution, allowing for their isolation in the pellet form. This step ensures the concentration and purification of the nanoparticles, enhancing the efficiency and quality of the final product. Subsequent drying on the watch glass eliminates excess solvent, further refining the nanoparticle sample. Finally, the powdered form enables convenient storage and further applications using it.

4.2.5 PREPARATION OF CHITOSAN HYDROGEL USING CROSS LINKER VS AGAROSE

4.2.5.1 Preparation of chitosan hydrogel using cross linker glutaraldehyde:

A beaker containing 10 mL of 1% acetic acid solution was placed on a magnetic stirrer set at 300 rpm. Slow addition of 200 mg of chitosan powder ensued, ensuring gradual dissolution of the chitosan within the solution. Into this stirring mixture, a few drops of the cross-linker glutaraldehyde were carefully introduced. The solution was kept in stirrer for a duration of 30 minutes, facilitating the formation of chitosan hydrogel. This careful process enabled the chitosan powder to dissolve thoroughly, thereby homogenizing the solution and promoting effective cross-linking with glutaraldehyde. The resultant chitosan hydrogel exhibits desirable properties for various applications, owing to its controlled synthesis under optimized conditions.

4.2.5.2 Preparation of chitosan hydrogel using Agarose:

A beaker containing 10 mL of 1% acetic acid solution was placed on a magnetic stirrer set at 300 rpm. Slow addition of 200 mg of chitosan powder ensued, ensuring gradual dissolution of the chitosan within the solution. Into this stirring mixture, 200 mg of agarose powder was carefully added instead of the cross-linker glutaraldehyde. The solution was then kept on the stirrer for a duration of 30 minutes to facilitate the formation of chitosan hydrogel.

4.2.6 PREPARATION OF SILVER NP AND IMIPENEM LOADED CHITOSAN HYDROGEL:

Statistical design of Box-Behnken was used to maximize the responses with precision of factor which has the largest effect on variables equidistant from the centre points. Three levels with three variables such as chitosan concentration, silver nps concentration, and Imipenem concentration are taken and show in table 5.5.2, 15 samples were prepared as per Box-Behnken method with 3 variables.

In each run, a beaker containing 10 mL of a 1% acetic acid solution was positioned on a magnetic stirrer operating at 300 rpm. Gradual addition of x mg of chitosan powder concentration corresponding to the specific run was meticulously performed, ensuring the gradual dissolution of chitosan within the solution. Subsequently, the respective concentration of silver nanoparticle solution

corresponding to the run was added to the stirring mixture and stirred for 1-2 minutes. Following this, the respective concentration of the Imipenem drug was introduced into the solution, and after 5 minutes of stirring, a few drops of the cross-linker glutaraldehyde were carefully added.

The solution underwent continuous stirring for a duration of 30 minutes, promoting the formation of chitosan hydrogel through cross-linking. At the end of the experimentation, 15 distinct samples with varying concentrations were obtained. Each sample represented a unique combination of chitosan concentration, silver nps concentration, and Imipenem concentration as determined by the Box-Behnken statistical design. These samples were collected and numbered to respective runs. This methodical approach allowed for the exploration of various combinations of Chitosan, Silver NPs, and Imipenem concentrations, enabling the production of silver nanoparticles and Imipenem incorporated chitosan hydrogel formulations

4.2.7 ASSESMENT OF ANTIMICROBIAL ACTIVITY OF PREPARED HYDROGELS:

To conduct the antimicrobial susceptibility assay, 3 Muller Hinton agar plates were prepared for each bacteria, namely *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Each plate was carefully poured with an equal amount of Muller Hinton agar (MHA), allowing it to solidify for a brief period. Following solidification, bacterial inoculum cultures were incubated and evenly swabbed onto the agar surface, ensuring uniform coverage. Then, agar wells were created using the ends of 1 mL micro tips, resulting in a total of 15 wells across the three plates, with 5 wells on each plate. These wells were meticulously numbered to facilitate sample loading. Equal amounts of the 15 different concentrations obtained from the hydrogel samples were loaded into their corresponding numbered wells.

To ensure the integrity of the experimental setup and prevent any potential contamination, leakage or displacement of the hydrogel from the wells, the sealing process using parafilm was executed carefully. Special attention was paid to avoid shaking or disturbing the plates, thereby minimizing the risk of gel movement or leakage. This careful handling ensured that the hydrogel remained confined within the wells throughout the duration of the assay, maintaining the accuracy and

reliability of the results. The sealed plates were then placed in an incubator set at 37 degrees Celsius for a duration of 24 hours.

After the incubation period, the plates were carefully examined to assess the presence of zones of inhibition produced by the hydrogels. The diameter of the inhibition zones surrounding each well was measured and recorded. This comprehensive assay allowed for the evaluation of the antimicrobial efficacy of the chitosan hydrogel samples against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, providing valuable insights into their potential therapeutic applications.

4.2.7 SWELLING STUDIES:

Initially, a single prepared hydrogel was weighed to record its initial weight accurately. Subsequently, at regular intervals of 2 hours, 200µl of water was incrementally added to the hydrogel. The corresponding increase in weight of the hydrogel was meticulously noted, allowing for the inference of its water absorption and swelling capacity over time.

Utilizing the formula $SWELLING\ RATIO = \frac{W - W_0}{W_0}$ (W weight of swelled gel, W_0 initial weight) the swelling ratio of the sample was calculated. This swelling ratio represents the extent of swelling exhibited by the hydrogel in response to water absorption. The data obtained from these measurements were utilized to construct a graph illustrating the swelling capacity of the hydrogel over time.

This experimental procedure provides valuable insights into the dynamic behaviour of the hydrogel, particularly its ability to absorb water and undergo swelling over time.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 FE-SEM:

The synthesized silver nanoparticles using various types of turmeric extracts were characterized using FESEM.

5.1.1 FE-Sem of Fresh vs Dried Turmeric Silver NP solution:

The freshly prepared and dried turmeric extracts served as effective reducing agents in the green synthesis of silver nanoparticles from silver nitrate. After synthesis, the resulting nanoparticles were gathered as a pellet, subsequently dried, and then forwarded for FE-SEM (Field Emission Scanning Electron Microscopy) analysis. The FE-SEM analysis included various levels of magnification to examine the synthesized nanoparticles thoroughly.

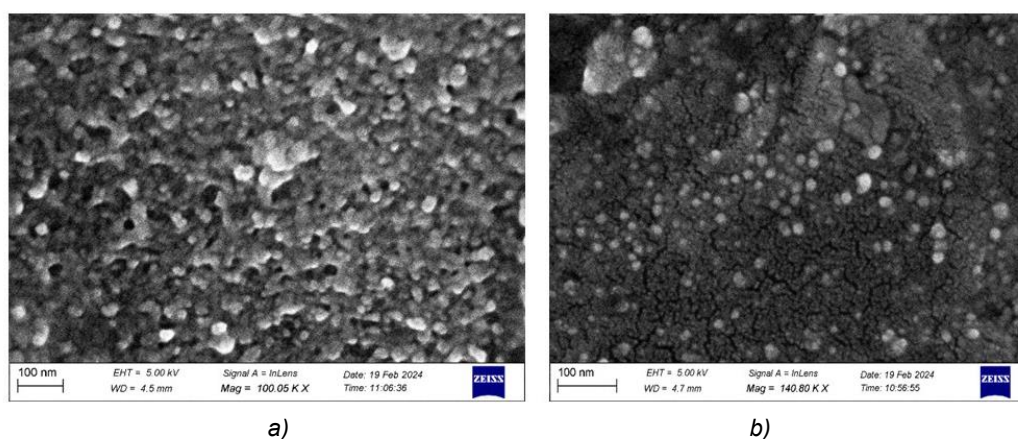


Figure 5.1 FE-SEM images silver nanoparticles synthesized using a)dried and b)fresh turmeric extract on magnification level of 100nm

FE-SEM images of silver nanoparticles derived from both dried and fresh turmeric extracts reveal intriguing insights into their respective reduction processes. In the case of dried and fresh turmeric extract, successful reduction of silver nitrate is evident, as indicated by the presence of nanoparticles at approximately 100KX magnification. However, at a closer inspection, at 100nm scale, the images unveil a notable difference: while the nanoparticles synthesized from fresh turmeric extract exhibit excellent dispersion, forming a uniform distribution, those from dried turmeric extract show significant agglomeration. Despite both types displaying spherical shapes at this scale, the fresh extract showcases superior dispersion and distribution without any agglomeration as seen in dried extract. This difference

highlights the effectiveness of fresh turmeric extract in producing nanoparticles with enhanced stability and uniformity, compared to its dried counterpart. According to the SEM image, the size of the silver nanoparticles appears to be approximately 50-60 nm based on observation. This observation suggests that the nanoparticles fall within a range of 50 to 60 nanometres in diameter.

While both dried and fresh turmeric extracts demonstrate proficiency as reducing agents, the stability and quality of the synthesized nanoparticles differ significantly. Fresh turmeric extract yields nanoparticles with superior dispersion and distribution, making it a more promising option. Therefore, fresh turmeric extract emerges as the preferred sample for applications requiring efficient reduction of silver nitrate and the production of high-quality nanomaterials.

5.1.2 FE-Sem Interpretation and Results of 5ml vs 10 ml Fresh Turmeric Silver NP solution:

Since the freshly prepared turmeric extract served as effective reducing agents in the green synthesis of silver nanoparticles from silver nitrate different amount of the extracts (5ml & 10ml) were used to produce nanoparticles. After synthesis, the resulting nanoparticles were gathered as a pellet, subsequently dried, and then forwarded for FE-SEM (Field Emission Scanning Electron Microscopy) analysis. The FE-SEM analysis included various levels of magnification to examine the synthesized nanoparticles thoroughly.

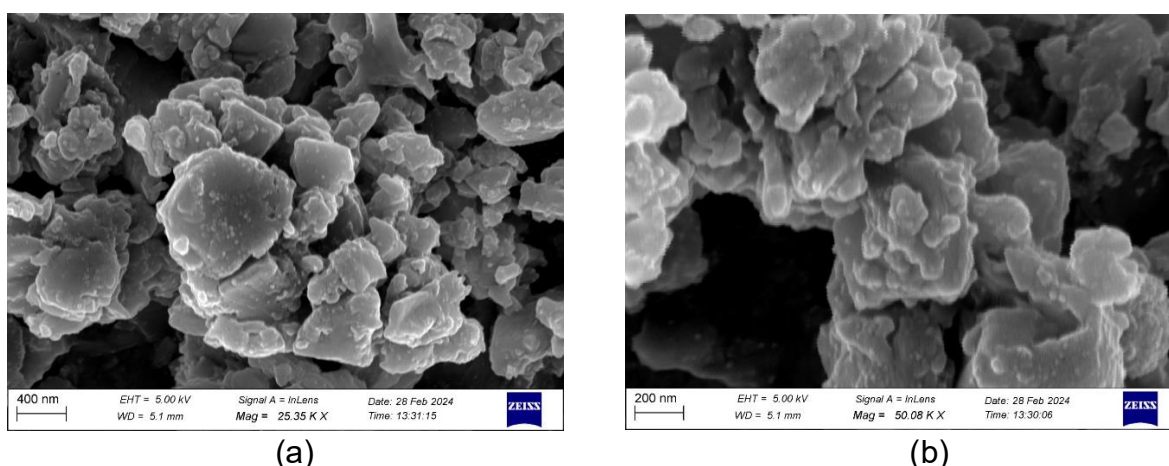


Fig.5.2 FE-SEM images silver nanoparticles synthesized using 10ml fresh turmeric extract on different magnification levels a) 400nm b) 200 nm

FE-SEM images taken at various magnification levels of silver nanoparticles derived from dried turmeric extract unveil a nearly successful reduction of silver nitrate.

However, at approximately 25KX magnification (400nm), the presence of larger reducing agent molecules suggests an inadequate reduction of silver nitrate by the 5ml fresh turmeric extract. Upon further magnification, the trend persists: there is a scarcity of nanoparticles, and the presence of larger reducing molecules from the fresh turmeric extract results in an uneven distribution of nanoparticles on the reducing turmeric. This ineffective dispersion, coupled with larger reducing agent molecules, indicates a potential deficiency in well-synthesized nanoparticles. Consequently, these nanoparticles are susceptible to rapid destabilization and agglomeration, rendering them unsuitable for applications requiring robust nanomaterials with exceptional properties. Essentially, 10 ml fresh turmeric proves to be a capable reducing agent, but the synthesized silver nanoparticles do not demonstrate much stability for utilization in the production of high-quality nanomaterials.

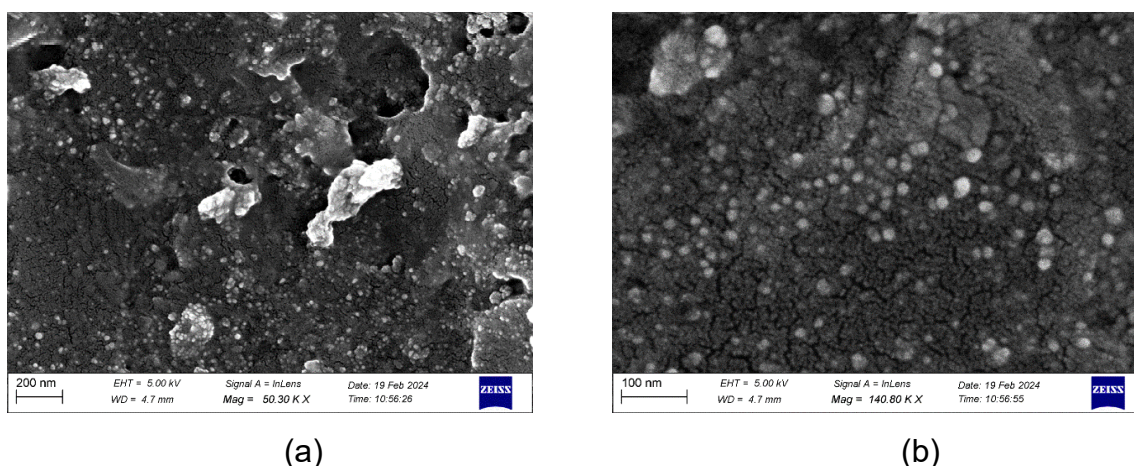


Fig.5.3 FE-SEM images silver nanoparticles synthesized using 5ml Fresh turmeric extract on different magnification levels a) 200nm b) 100 nm

FE-SEM images captured at various magnification levels of silver nanoparticles derived from fresh turmeric extract showcase the notably successful reduction of silver nitrate. At approximately 50KX magnification (200nm), the presence of well-defined nanoparticles indicates the efficient reduction of silver nitrate by the 5ml fresh turmeric extract, devoid of numerous larger reducing molecules. Upon closer inspection, the nanoparticles exhibit distinct and well-defined structures, alongside an even distribution, signifying the high stability of the synthesized nanoparticles. This observation positions 5ml fresh turmeric extract as a promising candidate for synthesizing nanomaterials, owing to its capability to produce stable nanoparticles with precisely defined properties.

FE-SEM images of silver nanoparticles derived from both 5ml and 10ml turmeric extracts offer valuable insights into their respective reduction processes. In the case of the 10ml extract, successful reduction of silver nitrate is observed, evident from the presence of nanoparticles at approximately 25KX magnification. However, upon closer examination, a concerning trend emerges: the nanoparticles exhibit notable agglomeration, leading to an uneven distribution across the reducing turmeric. This uneven dispersion indicates a potential lack of stability in the synthesized nanoparticles, limiting their suitability for applications requiring robust nanomaterials.

Conversely, FE-SEM images of silver nanoparticles derived from the 5ml turmeric extract present a different scenario. At similar magnification levels (50KX or approximately 200nm), well-defined nanoparticles suggest an effective reduction of silver nitrate. Unlike the nanoparticles from the 10ml extract, those from the 5ml extract exhibit clear and well-defined structures with an even distribution, indicative of high stability. This enhanced stability positions the 5ml turmeric extract as a superior choice for synthesizing nanoparticles, offering stable nanoparticles with precisely defined properties.

The comparison between the 5ml and 10ml turmeric extracts underscores the efficiency of the former in nanoparticle synthesis. Despite both extracts demonstrating proficiency as reducing agents, the stability and quality of the synthesized nanoparticles significantly differ. The stability and well-defined properties exhibited by nanoparticles derived from the 5ml turmeric extract highlight its superiority over the 10ml extract. Therefore, the 5ml turmeric extract emerges as the preferred option for synthesizing high-quality nanoparticles.

5.2 UV SPECTROSCOPY OF SILVER NP SOLUTION:

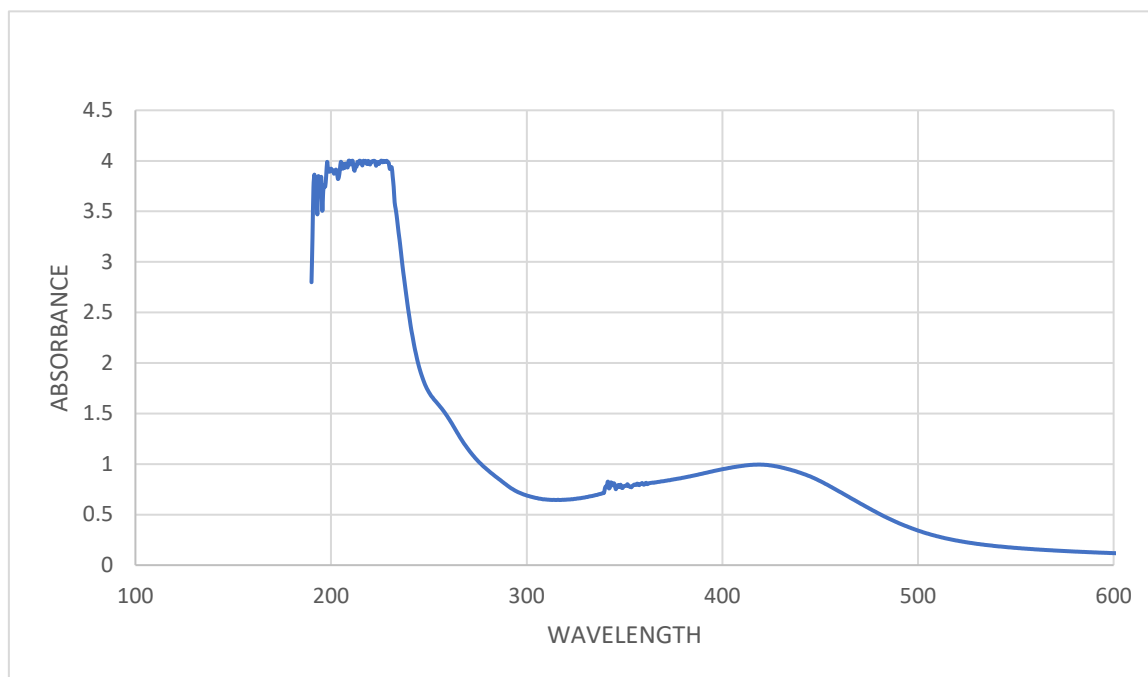


FIG 5.4 UV SPECTROSCOPY OF SILVER NP SOLUTION

After the synthesis process, the resulting solution containing silver nanoparticles derived from fresh *Curcuma longa* rhizome extract was subjected to further analysis. Specifically, a portion of the solution was sent for UV spectroscopy to confirm the presence of silver nanoparticles. UV spectroscopy is a valuable analytical technique commonly used to detect the presence of nanoparticles in solution based on their characteristic absorption spectra. Silver nanoparticles exhibit strong absorption in the UV-visible range due to surface plasmon resonance, making UV spectroscopy an ideal method for their detection. The solution containing the synthesized silver nanoparticles was carefully prepared for UV spectroscopy analysis. Spectrophotometric measurements were performed across a range of wavelengths to observe any characteristic absorption peaks associated with silver nanoparticles.

The successful detection of absorption peaks in the UV spectroscopy analysis, specifically in the range of 400 to 450 nm (as shown in Fig. 5), align closely with the findings mentioned in Alsammarraie et al.'s study from 2018, where they reported the maximum absorbance of UV-vis spectra to be at 432 nm, confirms the presence of silver nanoparticles in the synthesized solution. These absorption peaks are characteristic of silver nanoparticles and indicate their formation during the synthesis process. The confirmation of silver nanoparticle formation through UV

spectroscopy analysis provides additional validation of the effectiveness of the synthesis method employed. It further assures the quality and integrity of the synthesized nanoparticles, enhancing confidence in their potential applications. Overall, the successful detection of absorption peaks corresponding to silver nanoparticles reinforces the credibility of the synthesis process and underscores the suitability of the synthesized nanoparticles for various potential applications.

5.3 FTIR OF TURMERIC EXTRACT AND THE SILVER NP MIXTURE:

5.3.1 FTIR OF TURMERIC EXTRACT:

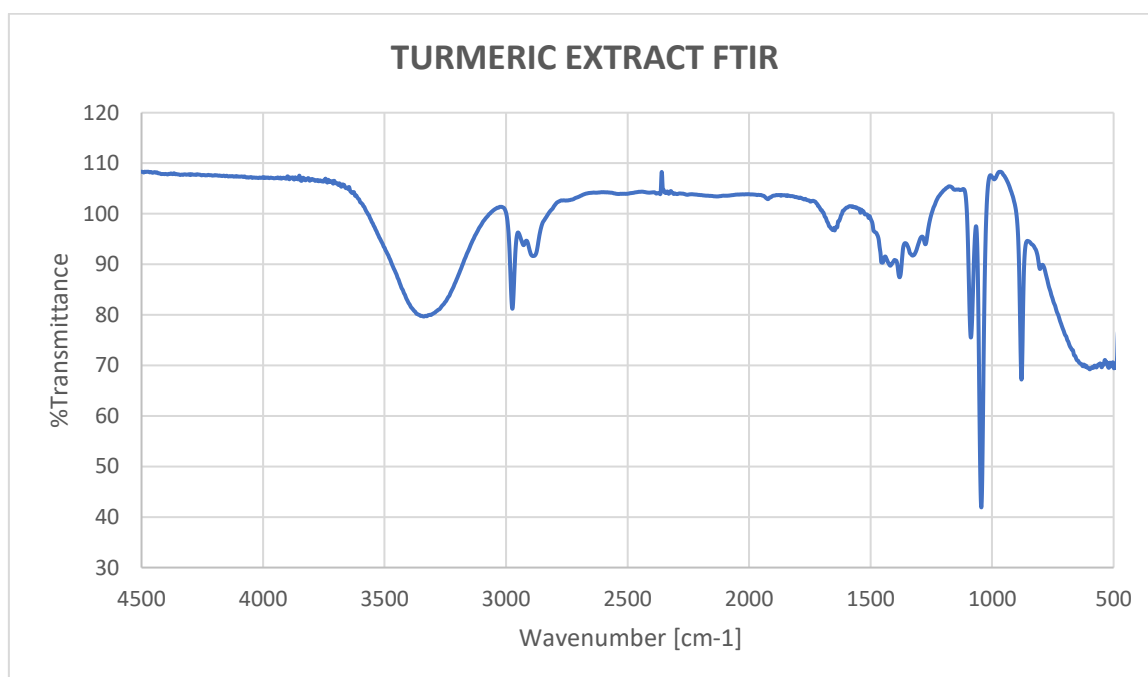


Figure 5.5. shows the analysis of to understand the appearance

peaks in the FTIR below, step-by-step process can be used. The results can be concluded as

follows:

Regarding the number of peaks, there are more than five peaks [Total 10 peaks], informing that the analysed chemical is not a simple chemical and can be a complex chemical. The peaks contained single bond area (2500-4000 cm⁻¹). Broad absorption band was found near 3500 cm⁻¹, informing there is hydrogen bond in the material. This band confirms the existence of hydrate (H₂O), hydroxyl (-OH), ammonium, or amino. Hydroxyl compound was confirmed, by the presence of spectra at frequencies of 1600–1300, 1200–1000 and 800–600 cm⁻¹. No other peaks between 3000 and 3200 cm⁻¹ were found, informing there is no aromatic

structure. Narrow bond at less than 3000 cm^{-1} responded to the C-H bond, showing aliphatic compounds. Absorption band for long chain linear aliphatic compounds is identified at 2935 and 2860 cm^{-1} . Confirmed by peaks at between 1470 and 720 cm^{-1} . No specific peak for aldehyde has been found at between 2700 and 2800 cm^{-1} . Peak at triple bond region (2000-2500 cm^{-1}) was detected, informing presence of $\text{C}\equiv\text{C}$ bond in the material. Regarding the double bond region (1500-2000 cm^{-1}), a peak was detected at about 1700 cm^{-1} . This informs some carbonyl double bond, which can be from ketones, aldehydes, esters, or carboxyl. -Since there is no specific peak for aldehyde at between 2700 and 2800 cm^{-1} (as described in the previous step), the prospective peak for carbonyl should be from ketone. Peak at about 1600 cm^{-1} , informing there is no $\text{C}=\text{C}$ bonding in the material.

Based on above interpretation, several conclusions can be obtained, including the analysed material has a hydroxyl component. This material has ketones-related component, aliphatic and not aromatic. Has double & triple bonds in the material. Since the peaks were only about 10 peaks, the material should be a small organic compound.

It can be concluded that the analysed material exhibits characteristics of a small organic compound. These include the presence of a hydroxyl component, ketone-related components, and aliphatic nature, indicating the absence of aromatic structures. Additionally, the material contains double and triple bonds. These features align well with major organic compounds found in turmeric extract, particularly curcuminoids like curcumin, desmethoxycurcumin, and bisdemethoxycurcumin. This suggests that the analysed material is likely a member of the curcuminoid family, commonly found in turmeric.

5.3.2 FTIR OF SILVER NP MIXTURE:

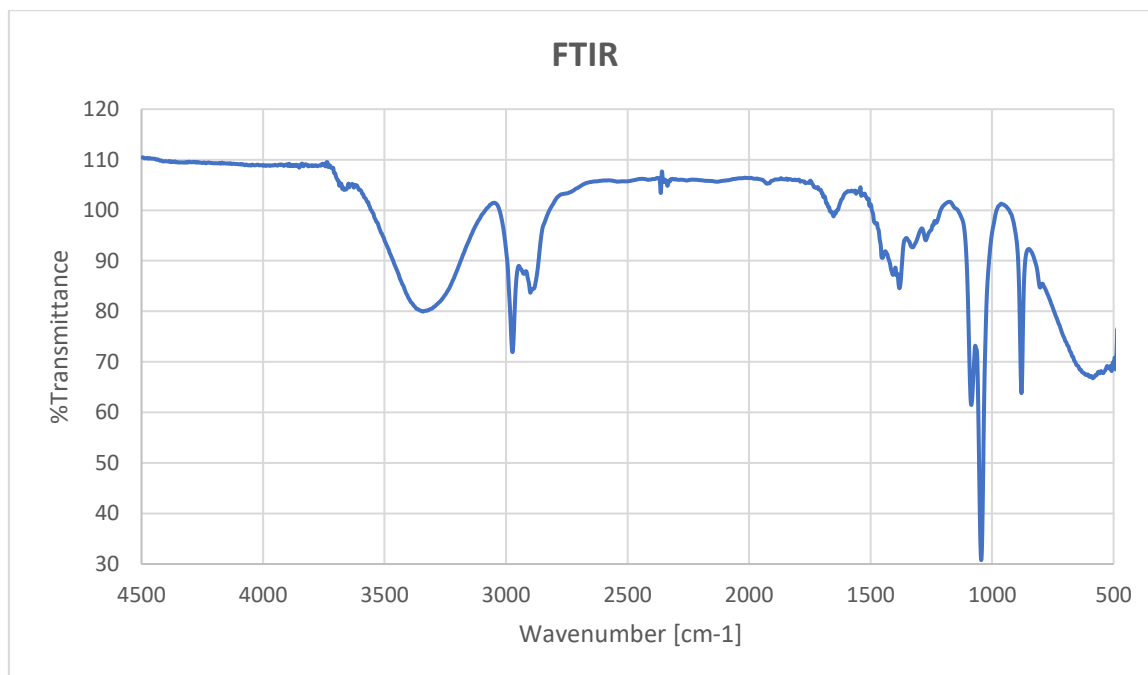


Fig.5.6 FTIR OF SILVER NP MIXTURE

peaks in the FTIR below, step-by-step process can be used. The results can be concluded as

follows:

Regarding the number of peaks, there are more than five peaks [Total 10 peaks], indicating that the analysed chemical is not a simple chemical and can be a complex chemical. The peaks contained single bond area (2500-4000 cm⁻¹). A broad absorption band was found near 3500 cm⁻¹, indicating the presence of a hydrogen bond in the material. This band confirms the existence of hydrate (H₂O), hydroxyl (-OH), ammonium, or amino. The presence of hydroxyl compound was confirmed by spectra at frequencies of 1600–1300, 1200–1000, and 800–600 cm⁻¹. No other peaks between 3000 and 3200 cm⁻¹ were found, indicating the absence of an aromatic structure. A narrow bond at less than 3000 cm⁻¹ responded to the C-C bond, showing aliphatic compounds. Absorption bands for long chain linear aliphatic compounds were identified at 2935 and 2860 cm⁻¹, confirmed by peaks between 1470 and 720 cm⁻¹. No specific peak for aldehyde was found between 2700 and 2800 cm⁻¹. A peak at the triple bond region (2000-2500 cm⁻¹) was detected, indicating the presence of a C≡C bond in the material. In the double bond region (1500-2000 cm⁻¹), a peak was detected at about 1700 cm⁻¹, indicating the presence

of some carbonyl double bond, possibly from ketones. Since there is no specific peak for aldehyde at between 2700 and 2800 cm^{-1} , the prospective peak for carbonyl should be from a ketone. A peak at about 1600 cm^{-1} indicates the absence of C=C bonding in the material. Based on the above interpretation, several conclusions can be drawn, including that the analysed material has a hydroxyl component, ketone-related component, aliphatic nature, and contains double and triple bonds. Since the peaks were only about 10 peaks, the material should be a small organic compound.

Based on the interpretation of FTIR peaks observed in the sample, which underwent reduction of silver nitrate using turmeric extract, The observed reduction in peaks within the range of 1500 to 1000 cm^{-1} and 4000 to 3500 cm^{-1} corresponds to functional groups such as ketones, aldehydes, esters, hydroxyl, or carboxyl. Previous FTIR analysis has confirmed that curcuminoids are the major compounds present. Curcuminoids, constituents of turmeric extract, are known to contain both ketone and hydroxyl phenol groups. The presence of ketone groups in curcuminoids indicates their potential involvement in the reduction reaction, alongside the phenolic functionalities, presenting the complex nature of these compounds in mediating such chemical transformations. Additionally, these compounds have effectively acted as reducing agents in reducing silver ions to silver nanoparticle size, underscoring their effective role in nanoparticle synthesis and emphasizing their significance in green synthesis approaches.

5.4 XRD OF THE SILVER NP PELLET POWDER:

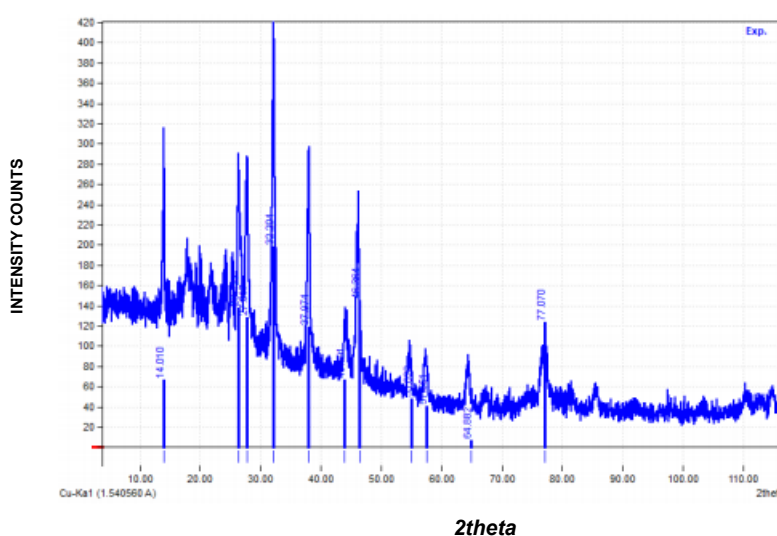


Fig. 5.7 XRD PATTERN OF SILVER NANOPARTICLES

The X-ray diffraction (XRD) graph of the sample was analysed in comparison with the XRD graph of silver nanoparticles to validate the crystalline nature and clear formation of silver nanoparticles. The XRD peak observed corresponds well with the standard XRD peaks of silver nanoparticles (Ag NPs), as reported in the study by Vanaja et al. in 2013. The presence of diffraction peaks at specific angles (33° , 44° , 64° , and 77°) in the 2θ range can be indexed to the (1 1 1), (2 0 0), (2 2 0), and (3 1 1) reflections, respectively, of a face-centered cubic (FCC) crystal structure. Therefore, the XRD pattern indicates that the crystalline structure of the metallic silver nanopowders is face-centered cubic (FCC) with a space group of Fm-3m (Bindhu MR et. Al 2015). Since the obtained graph displayed peaks analogous to those observed in the standard XRD graph of silver nanoparticles, affirming the presence of crystalline silver nanoparticles within the sample. This congruence in peak patterns corroborates the successful synthesis and crystallization of silver nanoparticles in the tested material.

5.5 CHITOSAN HYDROGEL WITH DIFFERENT CONCENTRATIONS

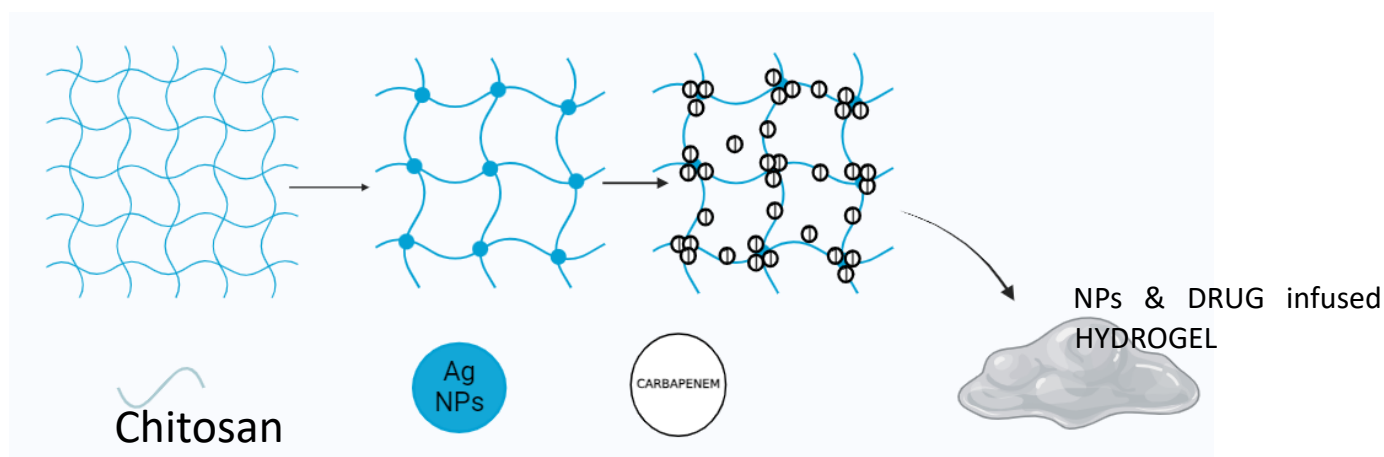
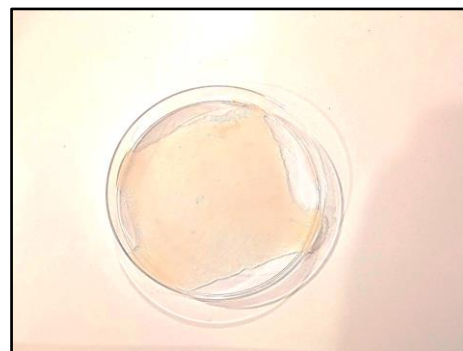


Fig 5.8 chitosan hydrogel preparation



Chitosan hydrogel prepared using cross linker glutaraldehyde



Chitosan hydrogel prepared without cross linker ,using agarose for gelation

Fig 5.9 Different preparations of Chitosan hydrogel (with and w/o Cross linker)

5.5.1 Statistical Analysis

For process optimization, a three level design was used by Box- Behnken design was used. A total of 15 experiments were performed to obtain different concentrations of silver nanoparticles and Imipenem incorporated chitosan hydrogel . As mentioned in table 5.5.3, the chitosan concentration, silver nps concentration, and Imipenem concentration varied and the results are represented in the table 5.5.1 where " X_1 " represents chitosan concentration, " X_2 " silver nps concentration, " X_3 " Imipenem concentration and " X_1X_2 ", " X_1X_3 " and " X_2X_3 " are the combination of interaction between the process parameters.

Table 5.5.1 Process parameters for Box Behnken design

Variables	Process Parameters	Levels		
		-1	0	+1
X1	DRUG $\mu\text{g/ml}$	10	20	30
X2	SILVER NPS $\mu\text{g/ml}$	50	100	150
X3	CHITOSAN mg	100	200	300

TABLE 5.5.3 Box-Behnken design for the experiment

RUNS	DRUG µg/ml	Ag NP µg/ml	CHITOSAN mg
1	10	50	200
2	10	150	200
3	30	50	200
4	30	150	200
5	10	100	100
6	10	100	300
7	30	100	100
8	30	100	300
9	20	50	100
10	20	50	300
11	20	150	100
12	20	150	300
13	20	100	200
14	20	100	200
15	20	100	200

5.6 ANTIMICROBIAL ACTIVITY OF PREPARED HYDROGELS:

Fifteen samples were precisely loaded into wells that were numbered accordingly. Each sample was carefully dispensed into its designated well to ensure accurate identification and tracking during subsequent analysis

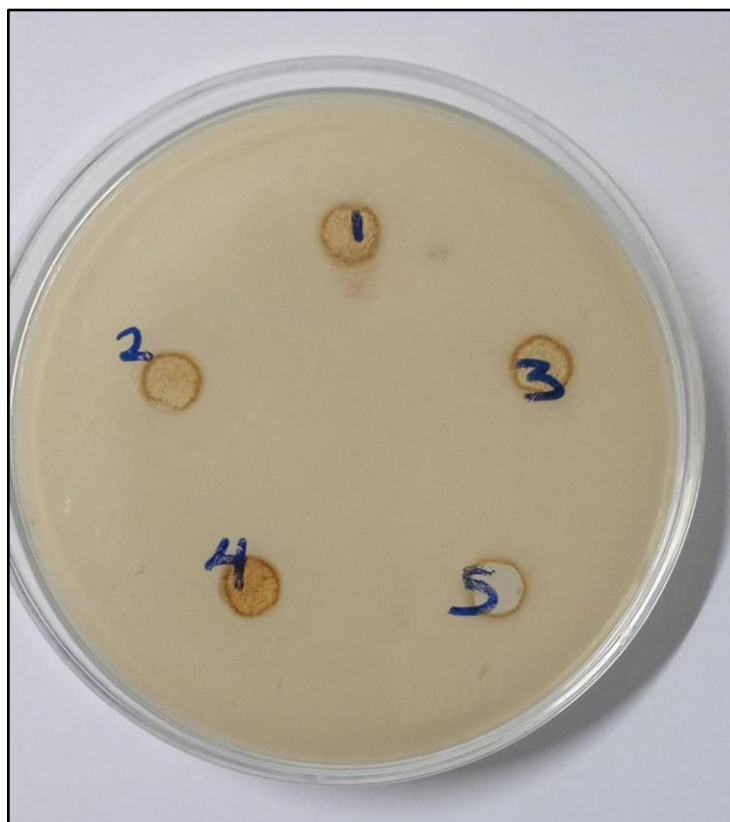


Fig. 5.10 *Numbered Wells on agar plate*

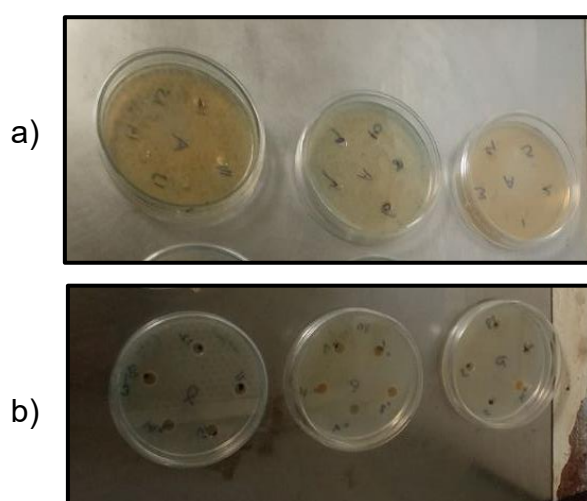
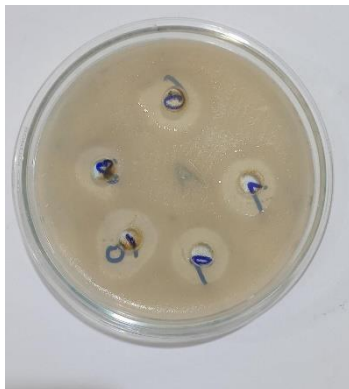
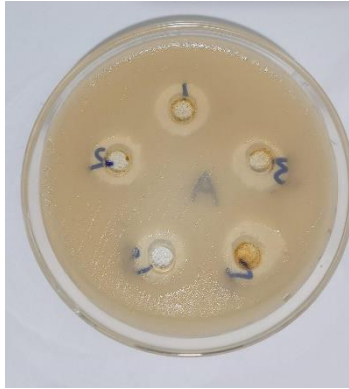


Fig 5.11 *Antimicrobial Susceptibility Assay Setup*

a) *Acinetobacter baumannii*



b) *Pseudomonas aeruginosa*

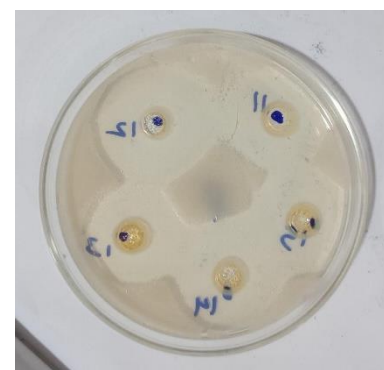
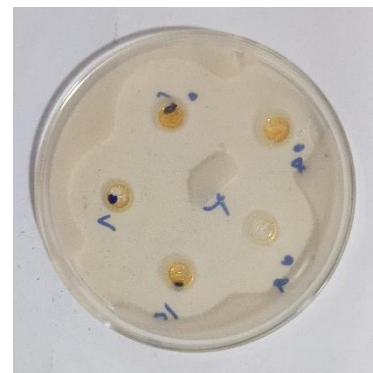
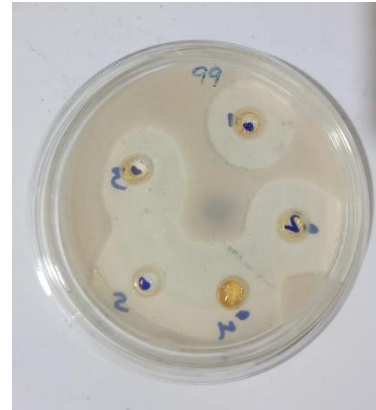


Fig 5.12 The setup for the antimicrobial susceptibility assay conducted using Muller Hinton agar plates. Three plates were prepared for each bacterium, a) *Acinetobacter baumannii* and b) *Pseudomonas aeruginosa*, totalling six plates. Each plate was designated for a specific bacterium and used for assessing the susceptibility of the bacteria to various samples.

ZONE OF INHIBITION

Table 5.6 Zone of inhibition exhibited by samples

SAMPLE LOADED	DRUG $\mu\text{g/ml}$	Ag NP $\mu\text{g/ml}$	CHITOSAN mg	<i>Acinetobacter baumannii</i> (cm)	<i>Pseudomonas aeruginosa</i> (cm)
1	10	50	200	1.7	3
2	10	150	200	1.7	3.2
3	30	50	200	1.8	2.4
4	30	150	200	2	3.2
5	10	100	100	1.5	3.8
6	10	100	300	2.4	3.4
7	30	100	100	2	3.2
8	30	100	300	1.7	2.4
9	20	50	100	2	3.6
10	20	50	300	2	2.8
11	20	150	100	0	2.8
12	20	150	300	0	3.6
13	20	100	200	1.7	2.4
14	20	100	200	1.7	2.4
15	20	100	200	1.7	2.4

a) Acinetobacter baumannii:

Here the highest zone of inhibition was present in 6th run with zone of inhibition measurement of 2.4

Imipenem concentration:

At a concentration of 10 µg/ml, the zone of inhibition ranges from 1.5 to 2.4.

At a concentration of 20 µg/ml, the zone of inhibition ranges from 1.5 to 2.0.

At a concentration of 30 µg/ml, the zone of inhibition ranges from 1.8 to 2.0.

Silver Nps concentration :

At a concentration of 50 µg/ml, the zone of inhibition ranges from 1.7 to 2.0.

At a concentration of 100 µg/ml, the zone of inhibition ranges from 1.5 to 2.4.

At a concentration of 150 µg/ml, the zone of inhibition ranges from 1.7 to 2.0.

Chitosan concentration :

At a concentration of 100 mg, the zone of inhibition ranges from 1.5 to 2.0.

At a concentration of 200 mg, the zone of inhibition ranges from 1.7 to 1.8.

At a concentration of 300 mg, the zone of inhibition ranges from 2.0 to 2.4.

From the ranges we can infer that the higher concentrations of Imipenem, silver Nps and chitosan seem to result in larger zones of inhibition range. There might be few exceptions or interactions between the inhibitors that influence the effectiveness.

Here the lowest zone of inhibition is found in sample of 5th run 1.5cm and highest in 6th run 2.4 cm. In the comparison of samples from the 5th and 6th runs, both had similar concentrations of imipenem and silver nanoparticles, set at 10 and 100 µg/ml, respectively. However, the chitosan content differed, with the 6th sample containing a higher amount at 300 mg compared to the 100 mg in the 5th sample. Despite the similarity in antimicrobial concentrations, the 6th sample exhibited the largest zone of inhibition at 2.4 cm, while the 5th sample showed the lowest at 1.5 cm. This difference can be attributed to the antibacterial effect of chitosan, which likely amplified the antimicrobial properties of the 6th sample, resulting in a larger zone of inhibition.

The data indicates an optimal concentration range for each inhibitor where the zone of inhibition is maximized.

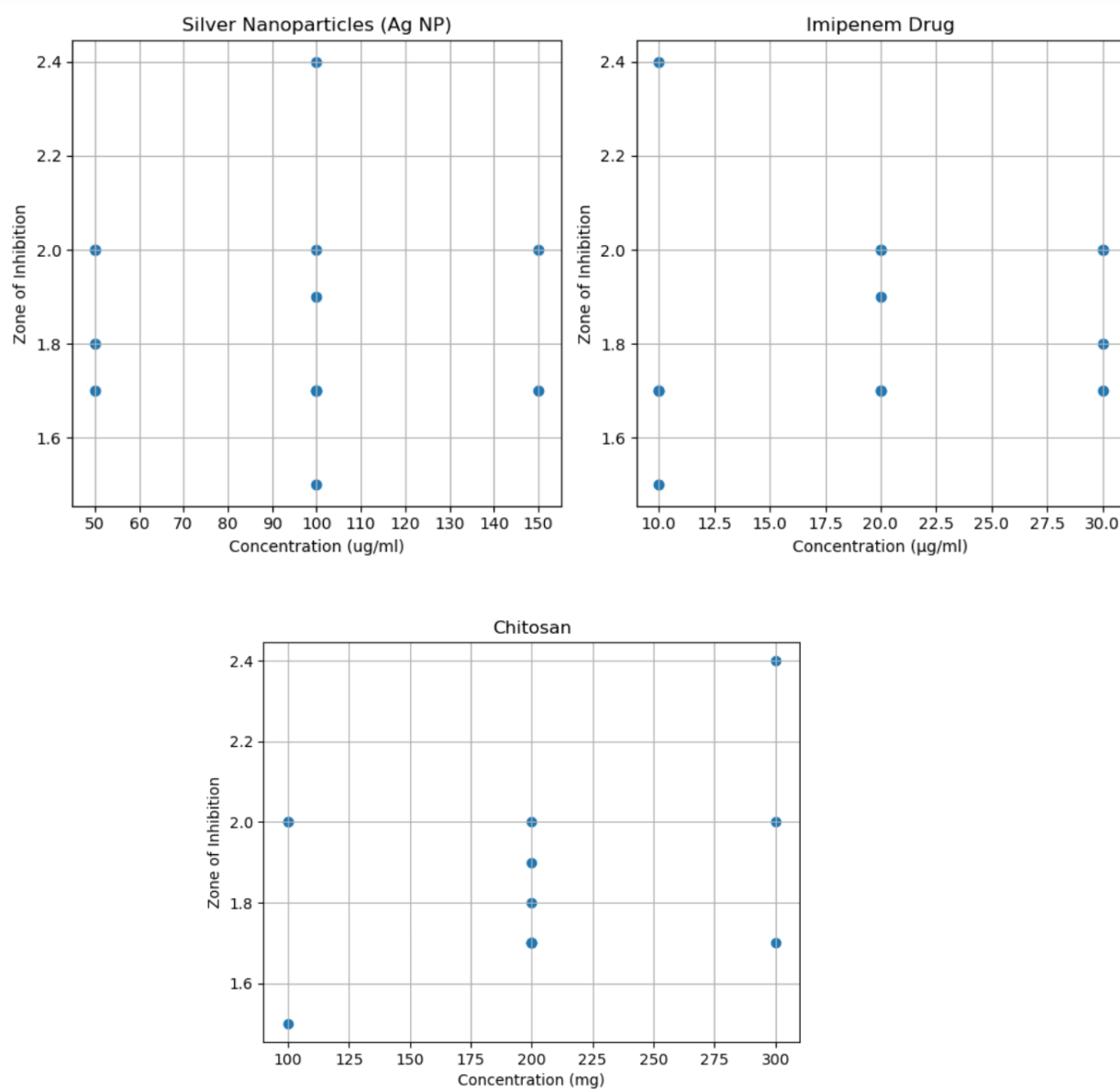


Fig. 5.13 Relationship between Concentration of Silver Nanoparticles (Ag NP), Imipenem Drug and Chitosan with Zone of Inhibition Values for the bacteria: *Acinetobacter baumannii*.

b) *Pseudomonas aeruginosa*:

For Imipenem:

- At a concentration of 10 µg/ml, the zone of inhibition ranges from 1.5 to 3.6.
- At a concentration of 20 µg/ml, the zone of inhibition ranges from 2.4 to 3.2.
- At a concentration of 30 µg/ml, the zone of inhibition ranges from 2.4 to 3.4.

For Silver Nanoparticles:

- At a concentration of 50 µg/ml, the zone of inhibition ranges from 2.4 to 3.2.
- At a concentration of 100 µg/ml, the zone of inhibition ranges from 3.0 to 3.8.
- At a concentration of 150 µg/ml, the zone of inhibition ranges from 2.4 to 3.8.

For Chitosan:

- At a concentration of 100 mg, the zone of inhibition ranges from 2.4 to 3.2.
- At a concentration of 200 mg, the zone of inhibition ranges from 2.4 to 3.6.
- At a concentration of 300 mg, the zone of inhibition ranges from 2.5 to 3.6.

Here the highest zone of inhibition was present in 5th run with zone of inhibition measurement of 3.8

Here the lowest zone of inhibition is found in sample of 13th run 2.4cm and highest in 5th run 3.8 cm. while the highest was in the 5th run at 3.8 cm. Both samples had similar concentrations of silver nanoparticles, set at 100 µg/ml. However, they differed in concentrations of imipenem and chitosan. In the 5th sample, the concentrations of imipenem and chitosan were 10 µg/ml and 100 mg, respectively, whereas in the 13th sample, these concentrations were doubled to 20 µg/ml and 200 mg, respectively. This divergence suggests that when the concentrations of imipenem and chitosan were changed, the 100 µg/ml silver nanoparticles may have undergone agglomeration, potentially leading to a loss of their antibacterial properties. Alternatively, the change in combinations due to the altered concentrations of imipenem and chitosan may have resulted in reduced antimicrobial properties in the 13th sample.

Generally, higher concentrations of both imipenem and Chitosan seem to result in larger zones of inhibition, but there might be exceptions or interactions between the inhibitors that influence the effectiveness against the bacteria *Pseudomonas aeruginosa*.

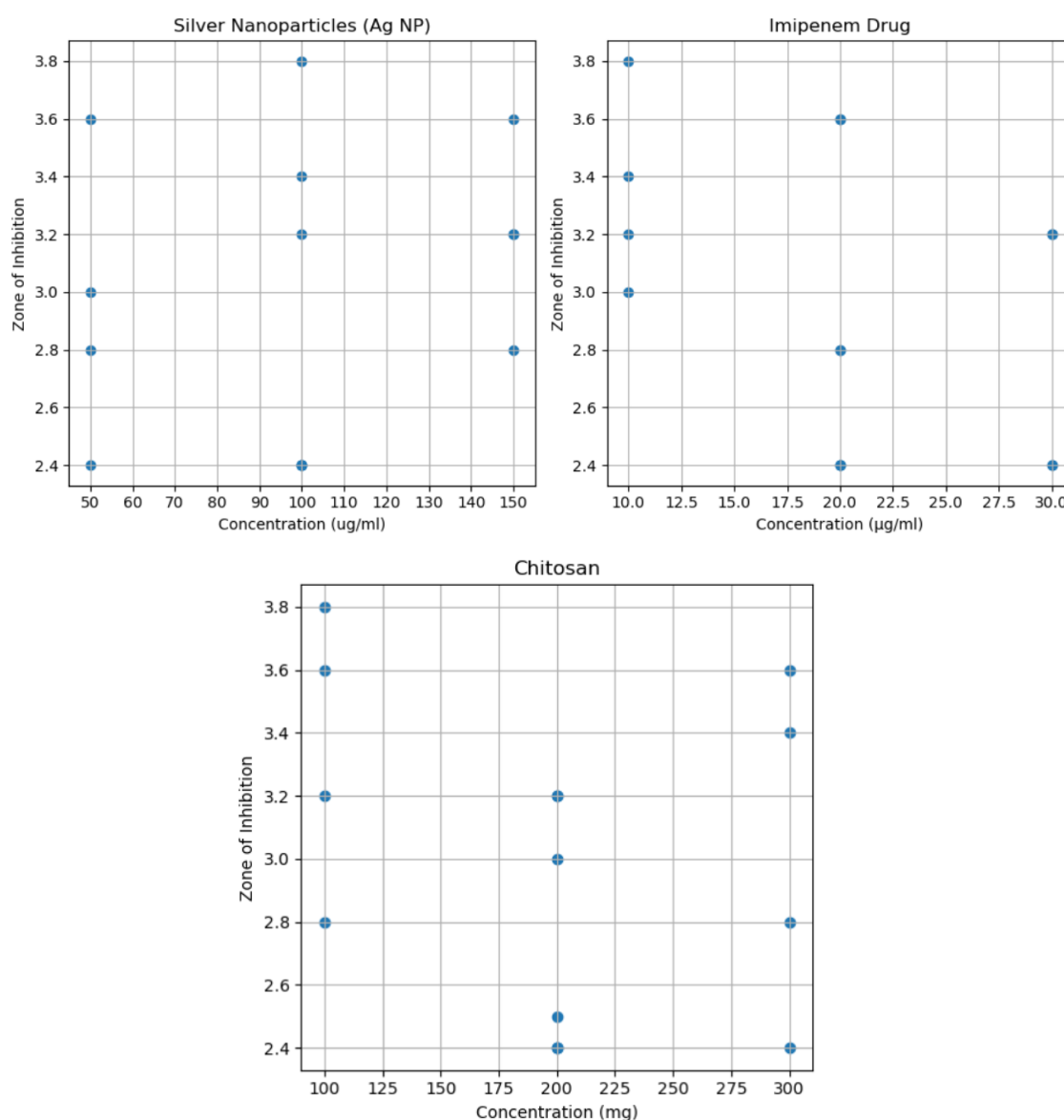


Fig. 5.14 Relationship between Concentration of Silver Nanoparticles (Ag NP), Imipenem Drug and Chitosan with Zone of Inhibition Values for the bacteria: *Pseudomonas aeruginosa*.

The zone of inhibition observed against *Pseudomonas aeruginosa* was greater compared to that against *Acinetobacter*. Specifically, in both studies, Sample 6 exhibited a notably higher antimicrobial potency. This suggests that Sample 6 possesses enhanced effectiveness against both *Pseudomonas aeruginosa* and *Acinetobacter* strains. These findings underscore the potential of Sample 6 as a promising candidate for combating infections caused by multidrug-resistant bacteria.

5.7 SWELLING STUDIES:

Table 5.7 Calculation of swelling ratio with respect to time

W (g)	SWELLING RATIO $= \frac{W - W_0}{W_0}$ (g) ($W_0 = 2.2g$)	TIME (Hrs)
2.2	0	0
2.7	0.2	2
3.6	0.6	4
4.1	0.8	6
4.5	1.04	8

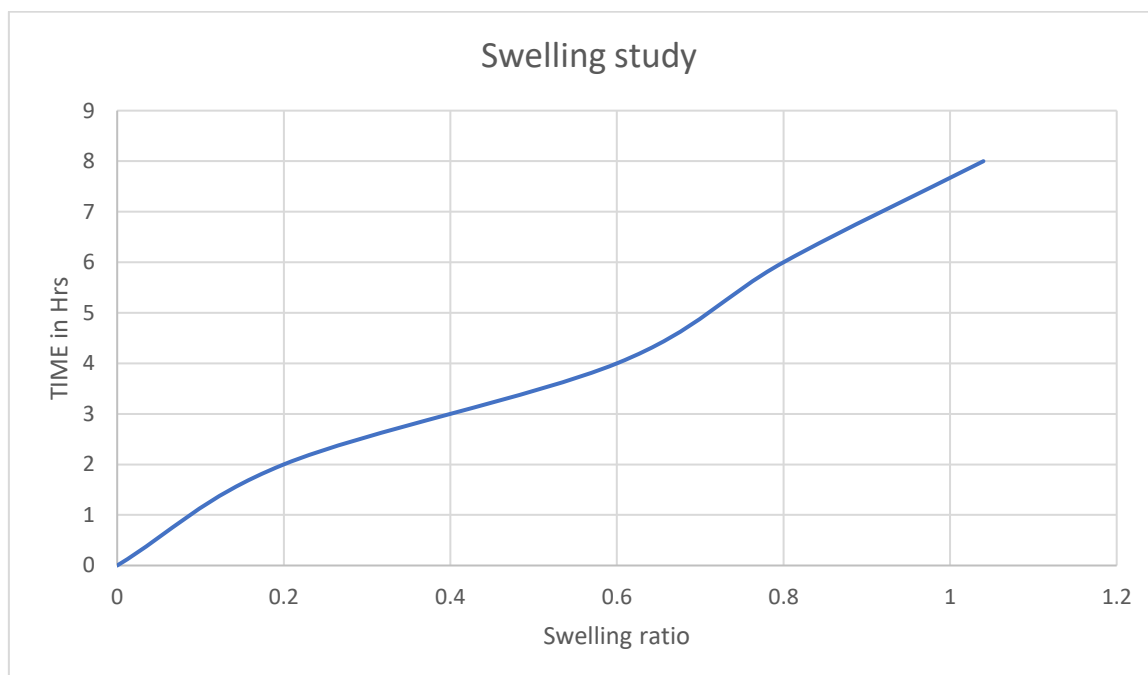


Fig 5.15 Swelling curve over time, showing the increase in mass swelling ratio over a period of 8hrs.

6.Summary and Conclusion

Our study aimed to encapsulate carbapenem into nanoparticles, optimize the synthesis process, characterize the synthesized nanoparticles using various techniques such as scanning electron microscope (SEM), x-ray diffraction (XRD), and FTIR, and evaluate the antimicrobial activity of the synthesized nanomaterial against multidrug-resistant microbial infections, with a specific focus on *Acinetobacter* & *Pseudomonas*.

We successfully synthesized silver nanoparticles (Ag NPs) and loaded them with imipenem using chitosan hydrogel as a carrier matrix. The optimization process allowed us to fine-tune the synthesis parameters, ensuring the production of nanoparticles with desirable characteristics.

Characterization of the synthesized nanoparticles revealed their morphology, crystal structure, and chemical composition. SEM imaging provided insights into the surface morphology, size, and shape of the nanoparticles. XRD analysis confirmed the crystalline nature of the nanoparticles, while FTIR spectroscopy helped identify functional groups and chemical bonds present in the nanomaterial.

Furthermore, our study explored the antimicrobial efficacy of imipenem-loaded chitosan hydrogel silver nanoparticles against microbial infections, with a particular focus on *Pseudomonas* species. While our investigation did not specifically involve usage of multidrug-resistant (MDR) bacteria, our findings demonstrate effectiveness against normal strains. This suggests that our formulation could be a promising candidate for future testing against MDR bacteria. Our results demonstrated significant antimicrobial activity, indicating the potential of the synthesized nanomaterial as an effective therapeutic agent against such infections.

Moreover, the positive results observed in the swelling studies suggest the suitability of the chitosan hydrogel matrix for drug delivery applications, highlighting its ability to absorb and retain fluids, which is crucial for sustained release formulations.

In conclusion, the successful synthesis and characterization of imipenem-loaded chitosan hydrogel silver nanoparticles, along with their promising antimicrobial activity against *Pseudomonas* infections, underscore the potential of this

nanomaterial for future therapeutic applications in combating antibiotic-resistant bacterial infections of *Pseudomonas* and *Acinetobacter* infections . Further research is necessary to explore the characterisation, clinical applicability and safety profile of these nano material for biomedical application.

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