DETERMINATION OF ANTIMICROBIAL ACTIVITY OF NANOPARTICLES AGAINST SELECTED GRAM NEGATIVE BACTERIA

A DISSERTATION SUBMITTED TO
ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM
IN PARTIAL FULFILMENT OF THE
REQUIREMENT FOR THE AWARD OF

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY
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2020 - 2022

CERTIFICATE

This is to certify that the project report entitled **"DETERMINATION OF ANTIMICROBIAL ACTIVITY OF NANOPARTICLES AGAINST SELECTED GRAM NEGATIVE BACTERIA"** submitted by Ms. Joys Mary, Reg. No. SM20ZOO004 in partial fulfillment of the requirements of Master of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Helvin Vincent and this is her original effort.

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1)

2)

DECLARATION

I, Ms. Joys Mary hereby declare that this project report entitled "**DETERMINATION OF ANTIMICROBIAL ACTIVITY OF NANOPARTICLES AGAINST SELECTED GRAM NEGATIVE BACTERIA"** is a bonafide record of work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Master of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

JOYS MARY

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I owe my deep gratitude to our project guide, Dr. Helvin Vincent having taken a keen interest in our research and guiding us along to build a good project work, by providing all the necessary information, the best available resources and moreover for having supported us throughout the study.

I thank all the teaching staff of the Department of Zoology, St. Teresa's college, Ernakulam who were always willing to encourage and help us in all our efforts.

My project work would not have been a success without the constant encouragement from my parents and friends.

Last but not the least, I thank God Almighty for showering his blessings upon me abundantly and for giving me the strength, ability, and opportunity to undertake and complete this project work.

JOYS MARY

LIST OF ABREVATIONS

1	µg	Microgram
2	µL	Microliter
3	A^{10}	Ampicillin
4	Ag2	Ag nanoparticle
5	AgNPs	Silver nanoparticles
6	C^{30}	Chloramphenicol
7	CS	Chitosan
8	CS1, CS7, CS8	Samples of Zinc Ferrite, AgCl calcined at 700°C, 800°C
9	CS2	Zinc Ferrite
10	CsAg	Zinc Ferrite/ Ag 600°C
11	CsZf	Zinc Ferrite/ AgCl
12	CX^{1}	Cloxacillin
13	CXM^{30}	Cefuroxime
14	D_1, D_2, D_3, D_4, D_5, D_6, D_7, D_8, D_9, D_{10}	Fe_2O_3, Gold, Silver and Platinum
15	DMSO	Dimethyl sulfoxide
16	E^{15}	Erythromycin
17	GEN^{30}	Gentamicin
18	LB	Luria Bertani Broth
19	MIC	Minimum Inhibitory Concentration
20	NA^{30}	Nalidixic acid

21	NC1	Composite of Zinc Ferrite and AgCl
22	NC2	Composite of Zinc Ferrite/ AgCl/ Ag and Ag3
23	NPs	Nanoparticles
24	OD	Optical Density
25	P^{10}	Penicillin-G
26	RMDA	Resazurin based Microtiter Dilution Assay
27	Rpm	Revolutions Per Minute
28	S^{25}	Streptomycin
29	TE^{30}	Tetracycline
30	ZF	Zinc Ferrite 600°C
31	ZF_3, ZF_4, ZF_5,ZF_6, ZF_7, ZF_8	Zinc Ferrite nanoparticles at different temperatures, 300°C to 800°C

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1. ABSTRACT

Nanotechnology using nanoscale materials is increasingly being utilized for clinical applications, especially as a new paradigm for infectious diseases. It has attracted much appreciation in recent years because of their improved synergistic properties like higher effective surface area, high electron mobility, stability, and biocompatibility. In the present study, antibacterial activity of 27 different nanoparticles was analysed against three gram negative bacteria - *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa.* Among the 27 samples tested, 18 nanoparticles exhibited antibacterial property against atleast one of the test organism. Out of the 18 positive nanoparticles, the Minimum Inhibitory Concentration (MIC) of five samples (D4, D10, CS1, CS2 and CsAg) were determined using Resazurin-based Microtitre Dilution Assay (RMDA). The MIC values of D4 against *E. coli* and *P. aeruginosa* were 7.8 μg. But it had no activity against *K. pneumoniae*. The MIC value of D10 was found to be 7.8 μg against *Escherichia coli* and 31.25 μg against *K. pneumoniae* and *P. aeruginosa.* CS1 has the MIC of 1.95 μg against *E. coli* and *P. aeruginosa* and 15.63 μg against *K. pneumonia*. The MIC value of CS8 and CSAg was found to be 3.91 μg against *E. coli* and *P. aeruginosa* and 15.63 μg against *K. pneumonia*. The antibacterial activity exhibited by nanoparticles was compared with 10 standard antibiotics out of which 4 antibiotics, Cefuroxime (CXM^{30}), Penicillin-G (P^{30}), Ampicillin (AMP^{10}) and Cloxacillin (CX^{1}), could not inhibit any of the test organism used. The present study becomes relevant as most of the nanoparticles inhibited the test organisms which were not inhibited by standard antibiotics. This study indicates that NPs exhibit a strong antimicrobial activity and thus might be developed as a new type of antimicrobial agents for the treatment of bacterial infection including multidrug resistant bacterial infection.

2. INTRODUCTION

For a long time, nanomaterials, especially metal nanostructures, have attracted much attention from researchers due to its versatility in applications. Nanotechnology encompasses the development, management, and application of structures in the nanometer size range and is a newly advanced discipline with great impact on diverse scientific fields (Farokhzad and Langer, 2006). It provides new tools for the molecular treatment and rapid detection of diseases, showing a great potential to transform pharmacy, biology, and medicine as well. Advanced materials with nanometric dimensions provide several means for innovative design of nanosized drug delivery systems to overcome biological barriers in order to direct the drug to specific targets. Diverse nanostructures such as nanotubes, nanoparticles, and nanofibers have been used as effective systems for drug delivery, becoming a part of nanomedicine (Faraji and Wipf, 2009; Brandelli et al., 2017).

Nanoparticles have attracted much interest because of their unique physical and chemical properties, which originate from the high area to volume ratio and elevated quantity of surface atoms. In fact, as the diameter decreases, the available surface area of the particle itself dramatically increases, and, consequently, there is an increase over the original properties of the corresponding bulk material. This feature makes nanoparticles superior and exceptional candidates for biomedical applications as a variety of biological processes occur at nanometer level (Sharma et al., 2009; Prabhu and Poulose, 2012). Thus, nanoparticles hold an incredible potential in various biomedical uses including effective drug delivery systems.

Since ancient times, heavy metals such as copper, silver, and gold have been widely used for the control and treatment of infectious diseases. Among these,

silver (Ag) is the principal metal that has been frequently used because of its recognized antimicrobial properties, and, being an antimicrobial agent, it is preferred in medical applications. Ag also shows potential activity against antibiotic-resistant organisms, which is one of the major concerns in public healthcare (Prabhu and Poulose, 2012). Currently, metallic Ag has been replaced by Ag nanoparticles (AgNPs). Metal nanoparticles are used in industry, agriculture, and healthcare. In addition, other metallic nanoparticles, including noble metal nanoparticles and metal oxide nanoparticles, have been also investigated for their antimicrobial potential or as effective antimicrobial drug carriers (Brandelli, 2012; Rai et al., 2015).

Zinc ferrite ($ZnFe_2O_4$) NPs synthesized by co-precipitation method exhibit potent antimicrobial activity against both Gram-positive and Gram-negative microbial strains at biocompatible concentration. The antimicrobial mechanisms that $ZnFe_2O_4$ NPs triggered is bacterial cell death via membranes disruption, protein leakage, and ROS generation for the bactericidal efficacy (Reihaneh, 2021). Zinc Ferrite has high electromagnetic performance, excellent chemical stability, mechanical hardness, low coercivity and moderate saturation magnetization. Zinc Ferrite nanoparticles exhibit unique structural morphological, opto-electrical, magnetic and photo catalytic activities due to their smaller particle size and higher surface area.

Gram-negative bacteria are bacteria that do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation. They are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane. Gram-negative bacteria are found in virtually all environments on Earth that support life. The gram-negative bacteria include the model organism *Escherichia coli*, as well as many pathogenic bacteria, such

as *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, and *Yersinia pestis*. They are an important medical challenge, as their outer membrane protects them from many antibiotics (including penicillin), detergents that would normally damage the inner cell membrane, and lysozyme, an antimicrobial enzyme produced by animals that forms part of the innate immune system. Additionally, the outer leaflet of this membrane comprises a complex lipopolysaccharide (LPS) whose lipid A component can cause a toxic reaction when bacteria are lysed by immune cells. This toxic reaction may lead to low blood pressure, respiratory failure, reduced oxygen delivery, and lactic acidosis - manifestations of septic shock. Silver nanoparticles showed significant antibacterial activity against the selected Gram-negative foodborne pathogens. Thus, AgNPs might be a good alternative to develop as antibacterial agent against the multidrug-resistant strains of bacteria.

3. AIM AND OBJECTIVES

Aim of the project work

The aim of the present study is to determine the antibacterial activity of 27 different nanoparticles against three Gram negative bacteria, *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa,* by using well diffusion method. The Minimum Inhibitory Concentration (MIC) of the nanoparticles was also determined using Resazurin based Microtitre Dilution Assay (RMDA) method.

Objectives of the project work

- To check the antibacterial activity of 27 nanoparticles against 3 gram negative bacteria using well diffusion method.
- To compare the antibacterial activity of nanoparticles with 10 standard antibiotics.
- To calculate the MIC value of nanoparticles against each bacterium employing Resazurin based Microtitre Dilution Assay (RMDA).

4. REVIEW OF LITERATURE

Metals have been used since ancient times to combat infectious diseases. With the introduction of nanotechnology, metal nanoparticles have gaining increased attention as antimicrobial agents due to their broad inhibitory spectrum against bacteria, fungi, and viruses. Although silver nanoparticles have been mostly investigated due to their recognized antimicrobial properties, while other metal nanoparticles have received increasing interest as antimicrobials. These include gold, zinc oxide, titanium oxide, copper oxide, and magnesium oxide nanoparticles, since their antibacterial effects have been described. Metal nanoparticles can exert their effect on microbial cells by generating membrane damage, oxidative stress, and injury to proteins and DNA. In addition, metal nanoparticles can be associated with other nanostructures and used as carriers to antimicrobial drugs, improving the array of potential applications.

Nanotechnology encompasses the development, management, and application of structures in the nanometer size range and is a newly advanced discipline with great impact on diverse scientific fields (Farokhzad and Langer, 2006). It provides new tools for the molecular treatment and rapid detection of diseases, showing a great potential to transform pharmacy, biology, and medicine as well. Advanced materials with nanometric dimensions provide several means for innovative design of nanosized drug delivery systems to overcome biological barriers in order to direct the drug to specific targets. Diverse nanostructures such as nanotubes, nanoparticles, and nanofibers have been used as effective systems for drug delivery, becoming a part of nanomedicine (Faraji and Wipf, 2009; Brandelli et al. 2017).

Nanoparticles possess antimicrobial activity that can overcome common resistant mechanisms, including enzyme inactivation, decreased cell permeability,

modification of target sites/enzymes, and increased efflux through over expression of efflux pumps, to escape from the antibacterial activity of antimicrobial agents (Mulvey and Simor, 2009; Baptista et al., 2018). Moreover, NPs conjugated with antibiotics show synergistic effects against bacteria, prohibit biofilm formation, and have been utilized to combat MDROs (Pelgrift and Friedman, 2013; Baptista et al., 2018).

Nanoparticles have attracted much interest because of their unique physical and chemical properties, which originate from the high area to volume ratio and elevated quantity of surface atoms. In fact, as the diameter decreases, the available surface area of the particle itself dramatically increases, and, consequently, there is an increase over the original properties of the corresponding bulk material. This feature makes nanoparticles superior and exceptional candidates for biomedical applications as a variety of biological processes occur at nanometer level (Sharma et. al. 2009; Prabhu and Poulose, 2012). Thus, nanoparticles hold an incredible potential in various biomedical uses including effective drug delivery systems.

Nanoparticles may enhance the inhibitory effects of antibiotics. Saha et al. (2007) demonstrated that gold NPs conjugated with ampicillin, streptomycin, or kanamycin could lower the minimum inhibitory concentrations (MICs) of the antibiotic counterparts against both gram-negative and gram-positive bacteria. Likewise, Gupta et al. (2017) demonstrated a synergistic effect of functionalized Au NPs and fluoroquinolone antibiotics for the treatment of multidrug-resistant *Escherichia coli* infections.

Nanoparticles are therefore regarded as next-generation antibiotics. In both *in vitro* and *in vivo* studies, NPs, mainly metallic, have been shown to exhibit activity against gram-positive and gram-negative bacteria (Zazo et al., 2016).

Though antimicrobial mechanisms that depend on the size, shape, ζ-potential, ligands, and material used are not well understood (Huh and Kwon, 2011; Singh et al., 2014; Zazo et al., 2016); currently accepted mechanisms include (1) direct interaction with the bacteria, leading to the disruption of membrane potential and integrity; (2) triggering of the host immune responses; (3) inhibition of biofilm formation; (4) generation of reactive oxygen species (ROS); and (5) inhibition of RNA and protein synthesis through the induction of intracellular effects (Pelgrift and Friedman, 2013; Beyth et al., 2015). NP coatings on implantable devices, wound dressings, bone cement, or dental materials can function as NP-based antibiotic delivery systems (Wang et al., 2017). Furthermore, NPs can be vectors to transfer drugs so that higher doses of antimicrobial agents can be delivered to infected sites (Pelgrift and Friedman, 2013). Thus, the combination of NPs and antimicrobial agents may be beneficial in fighting the ongoing crisis of antimicrobial resistance (Baptista et al., 2018). Clinical applications of NPs have recently been evaluated to highlight the *in vitro* antimicrobial activities of NPs and the potential adverse effects of NPs on human health.

Multidrug-resistant organisms (MDROs) are becoming a growing public health crisis and make many healthcare-associated infections difficult to treat with current antibiotics (Boucher et al., 2009; Peleg and Hooper, 2010). Globally, infections caused by MDROs are emerging causes of morbidity and mortality (Ismail et al., 2018; Kuo et al., 2018; Ting et al., 2018; Tsao et al., 2018). The development of new antibiotics requires tremendous economic and labor investment and is time-consuming (Huh and Kwon, 2011). For these MDRO infections, high doses of antibiotics will be administered and may generate intolerable toxic and adverse effects, which will prompt the development of alternative strategies. The application of nanoparticles (NPs) provides a potential strategy to manage infections caused by MDROs (Singh et al., 2014; Natan and Banin, 2017; Baptista et al., 2018; Muzammil et al., 2018). NPs exhibiting

antibacterial activities can target multiple biomolecules and have the potential to reduce or eliminate the evolution of MDROs (Slavin et al., 2017).

Nanoparticles with antimicrobial activity that combats *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species (Ansari et al., 2014; Dizaj et al., 2014; Beyth et al., 2015; Hemeg, 2017) include NPs containing Ag, Au, Zn, Cu, Ti, Mg, Ni, Ce, Se, Al, Cd, Y, Pd, or superparamagnetic Fe (Hemeg, 2017). Among various metallic NPs and their oxides already applied as active antimicrobial agents, silver or its ionic form is the most toxic to bacteria (Seil and Webster, 2012). This makes silver of particular interest. Silver NPs (Ag NPs) are used to a great extent since they have multiple mechanisms of antibacterial action (Cheng et al., 2016), high biocompatibility, and functionalized potential and are easy to detect (Baranwal et al., 2018). Although Ag NPs are difficult to functionalize with biomolecules and antibiotics, Ag–gold (Au) alloys provide another path, since they combine the antimicrobial effects of Ag with the effectiveness of functionalization and the stability of Au in the form of bimetallic NPs (Baptista et al., 2018).

Au–Pt bimetallic NPs have antibacterial activity against multidrug-resistant *E. coli* through the dissipation of bacterial membrane potential and the elevation of adenosine triphosphate (ATP) levels (Baptista et al., 2018). Cu–Ni bimetallic NPs have been utilized as coating agents but have been used less in antimicrobial applications (Baptista et al., 2018).

With biocompatibility and magnetic properties, iron oxide (FeO) is well known in the biomedical sector. Recently, the antibacterial properties of reduced iron and FeO NPs that damage bacteria cells through the disruption of the bacterial membrane and generation of oxidative stress inside the cell have been studied

(Baranwal et al., 2018). The characteristic compatibility and safety of ZnO NPs on human skin make them appropriate additives for cosmetics, fabrics, and surfaces in close proximity to human skin (Dizaj et al., 2014). Copper oxide (CuO) NPs have been shown to exhibit excellent bactericidal and fungicidal activity (Ren et al., 2009), whereas TiO_2 NPs possess spectacular antimicrobial properties, mainly related to ROS formation, particularly –OH free radicals (Baranwal et al., 2018).

To overcome antibiotic resistance, NPs can be tailored and packaged with diverse antimicrobial agents. NPs act on bacteria through multiple targets and/or a unique mechanism; thus, antimicrobial resistance is unlikely to develop if NPs are combined with antibiotics since multiple simultaneous mutations are required in the same microorganism (Fischbach, 2011; Zhao and Jiang, 2013). The functionalization of NPs with antibiotics can be a promising regimen to combat bacterial resistance. Moreover, NPs can deliver antimicrobial agents to or target the infected sites and reduce the dosage and toxicity of antibiotics (Hemeg, 2017). For example, the synergistic antibacterial efficiency of Ag NPs and antibiotics against *S. aureus*, beta-lactamase- or carbapenemase-producing *E. coli*, *P. aeruginosa*, and *A. baumannii* strains at extremely low concentrations has been found (Naqvi et al., 2013; Panacek et al., 2015; Scandorieiro et al., 2016), whereas synergistic antibacterial effects of Ag, Au, and ZnO NPs and antibiotics have been observed against *S. aureus*, *E. faecium*, *E. coli*, *A. baumannii*, and *P. aeruginosa* through the penetration of the bacterial cell membrane and the interference with important molecular pathways, formulating unique antimicrobial mechanisms (Hemeg, 2017). The efficacy of antibiotics combined with NPs was identical in both gram-positive and gram-negative bacteria, unlike the difficulty in killing MDROs with antibiotics alone (Hemeg, 2017). The combinations of antibiotics and functionalized Ag, Au, or ZnO NPs may promote the reversal of antimicrobial resistance and boost the antimicrobial effects of

several antibiotics, including polymyxin B, ciprofloxacin, ceftazidime, ampicillin, clindamycin, vancomycin, or erythromycin, against MDROs, including antibiotic-resistant *A. baumannii*, *P. aeruginosa*, *E. faecium*; vancomycin-resistant *Enterococcus* (VRE); and methicillin-resistant *S. aureus* (MRSA) (Hemeg, 2017).

The antimicrobial activity of NPs depends on several physicochemical properties, such as their size, shape, solubility, and ability to form free biocidal metal ions (Khan et al., 2016). Generally, smaller NPs show increased antibacterial activity compared to larger NPs (Lu et al., 2013). Gram-positive and gram-negative bacteria differ in terms of cell membrane components and structures and have different adsorption pathways for NPs (Lesniak et al., 2013). The susceptibility of bacteria to NPs depends on their biochemical composition since different NPs target different biomolecules (Khan et al., 2016). Moreover, rapidly growing bacteria are more susceptible to NPs or antibiotics than slow-growing bacteria. This may be due to the variable expression of stress-response genes between rapidly growing and slow-growing bacteria (Stewart, 2002; Khan et al., 2016).

Gram-negative bacteria are bacteria that do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation. They are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane. Gram-negative bacteria are found in virtually all environments on Earth that support life. The gram-negative bacteria include the model organism *Escherichia coli*, as well as many pathogenic bacteria, such as *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, and *Yersinia pestis*. They are an important medical challenge, as their outer membrane protects them from many antibiotics (including penicillin), detergents that would normally damage the inner cell membrane, and lysozyme, an antimicrobial enzyme produced by

animals that forms part of the innate immune system. Additionally, the outer leaflet of this membrane comprises a complex lipopolysaccharide (LPS) whose lipid a component can cause a toxic reaction when bacteria are lysed by immune cells.

The antibacterial effects of NPs have been noted to be more pronounced for gram-positive bacteria than for gram-negative bacteria. Such a finding may be related to the fact that the nonporous cell walls of gram-negative bacteria serve as penetration barriers for the entry of NPs (Zaidi et al., 2017). Cell walls of gram-positive bacteria with covalent links with neighboring proteins and components are relatively porous and allow the penetration of foreign molecules (Zaidi et al., 2017).

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations (MBCs) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media. MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the in vitro activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints.

Loo et al., (2018) determined the antibacterial activity of AgNPs against four species of Gram-negative foodborne pathogens *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13773, *S. typhimurium* ATCC 14028, and *S. enteritidis* ATCC 13076. The MIC values of AgNPs against the foodborne pathogens ranged from 3.9 to 7.8 µg/mL. *K. pneumonia, S. typhimurium* and *S. enteritidis* showed the MIC value of 3.9 µg/mL while E. coli showed the MIC value of 7.8 µg/mL.

Resazurin dye was used in the study as an indicator in the determination of cell growth, especially in cytotoxicity assays (McNicholl et al., 2007). Oxidoreductases within viable cells reduced the resazurin salt to resorufin and changed the color from blue non-fluorescent to pink and fluorescent. According to McNicholl et al. (2007), resazurin dye has been applied for decades to check for the bacterial and yeast contamination in milk. The MIC value was taken at the lowest concentration of antibacterial agents that inhibits the growth of bacteria (color remained in blue).

The results of in vitro antibiotic susceptibility testing can predict the clinical response to treatment and guide the selection of antibiotics. Bacteria are classified as sensitive, intermediate or resistant based on breakpoint MIC values that are arbitrarily defined and reflect the achievable levels of the antibiotic, the distribution of MICs for the organism and their correlation with clinical outcome. Broth dilution, agar dilution and gradient diffusion (the 'E test'), where twofold serial dilutions of antibiotic are incorporated into tubes of broth, agar plates or on a paper strip, respectively, are different methods to measure the MIC of an organism. The disk diffusion method defines an organism as sensitive or resistant based on the extent of its growth around an antibiotic-containing disk. MIC values are influenced by several laboratory factors. To ensure reproducible results, the laboratory must closely follow methods developed by the National Committee for Clinical Laboratory Standards, which defines standard growth media, incubation temperature and environment, the inoculum and quality control parameters (Smaill, 2000).

5. MATERIALS AND METHODS

5.1 MATERIALS

The aim of the project was to determine the antibacterial activity of 27 nanoparticles against three gram-negative bacterial strains.

The nanoparticles (5mg/ml DMSO) used for the study were designated as (D_1, D_2, D_3, D_4, D_5, D_6, D_7, D_8, D_9, D_{10} (Contains Fe_2O_3, Gold, Silver and Platinum), ZF_3, ZF_4, ZF_5, ZF_6, ZF_7, ZF_8 (Zinc Ferrite nanoparticles at different temperatures, 300°C to 800°C), CS1, CS7, CS8 (Composite samples of Zinc Ferrite, AgCl calcined at 700°C, 800°C) CS2 (contains Zinc Ferrite, AgCl and Ag), ZF (Zinc Ferrite 600°C), NC1 (Composite of Zinc Ferrite and AgCl), CsAg (Zinc Ferrite/ Ag 600°C), Ag2 (Ag nanoparticle), CsZf (Zinc Ferrite/ AgCl), Nc2 (Composite of Zinc Ferrite/ AgCl/ Ag) and Ag3.

The three test organisms used for the study include *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa* (ATCC 27853)

Standard antibiotics such as Cloxacillin (CX^1), Nalidixic acid (NA^{30}), Chloramphenicol (C^{30}), Penicillin-G (P^{10}), Ampicillin (A^{10}), Tetracycline (TE^{30}), Streptomycin (S^{25}), Cefuroxime (CXM^{30}), Gentamicin (GEN^{30}) and Erythromycin (E^{15}) were chosen to compare the antibacterial activity.

The MIC (Minimum Inhibitory Concentration) value of effective nanoparticles were determined using Resazurin based Microlitre Dilution Assay.

5.2 METHODS

DETERMINATION OF ANTIBACTERIAL ACTIVITY

Preparation of nutrient media

Nutrient broth was prepared by dissolving 1.3 gm of nutrient broth in 100 ml distilled water. Test tubes were filled with 5ml of nutrient broth and were sterilised using an autoclave.

Nutrient agar media was prepared by mixing 1.3gm of nutrient broth and 2gm of agar in 100 ml distilled water. The media was autoclaved and 20ml each poured into sterile petriplates under aseptic conditions.

Preparation of microbial cultures

The three-gram negative test organisms were inoculated into 5ml of sterilized nutrient broth and kept for overnight incubation at 37°C.

Well diffusion method (Mounyr Balouiri et al., 2016)

The petriplates were labelled and bacterial cultures were spread on the surface of sterile nutrient agar plates using sterile cotton swab. Wells of 6 mm diameter were cut into agar plates using sterile well cutter. The wells were labelled and 20µL of nanoparticles were loaded into corresponding wells. The petridishes were incubated overnight at 37°C. After 24 hours the zone of inhibition was measured.

Disc diffusion method (Mounyr Balouiri et al., 2016)

The antibacterial activity of nanoparticles were compared with standard antibiotics available. The antibiotics selected were Cloxacillin (CX^{1}), Nalidixic acid (NA^{30}), Chloramphenicol (C^{30}), Penicillin-G (P^{10}), Ampicillin (A^{10}), Tetracycline (TE^{30}), Streptomycin (S^{25}), Cefuroxime (CXM^{30}), Gentamicin (GEN^{30}) and Erythromycin (E^{15}). Sterile antibiotic discs of diameter 7mm were

used. The bacterial culture was spread on the surface of nutrient agar plates and sterile antibiotics were placed on the petri plates and incubated for 24 hours. After incubation the diameter of the zone of inhibition was measured.

Fig.A. Wells of 6 mm diameter cut into agar plates using sterile well cutter

Fig.B. Wells were labelled and 20µL of nanoparticles were loaded into corresponding wells.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The minimum inhibitory concentration is defined as the minimum amount of the sample needed to show bacteriostatic effect. It is the lowermost concentration of antimicrobial agent that completely inhibits visible growth of the microorganism in microtitre plate as detected visually after a period of incubation. The MIC of the samples were measured by Resazurin-based Microtitre Dilution Assay (RMDA).

Preparation of bacterial culture (Jose et al., 2020)

Using aseptic techniques a single colony of test organism was transferred into sterile 5ml LB broth. After 24hrs of incubation inoculated broth was transferred into 100 ml LB broth and kept in the incubator overnight for 12 -16 hours at

37°C. After incubation, the bacterial culture was centrifuged at 4000 rpm for 5minutes. The supernatant was discarded and the pellet was resuspended in 20mL of sterile water and re-centrifuged at 4000 rpm for 5 minutes. The pellet was dissolved in 20 mL sterile normal saline. The optical density of the bacterial culture was measured at 600 nm, and serial dilutions were carried out with appropriate aseptic techniques until the optical density was 1. The dilution factor was calculated and the dilution was carried out to obtain a concentration of $5x10^6$cfu/mL.

Preparation of Resazurin Dye Solution (RDS) (Sarker et al., 2007**)**

Resazurin dye (0.03gm) was dissolved in 4 mL sterile water. Vortex mixer was used to homogenize the solution. The solution was then referred as Resazurin dye solution. Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. It is purple non-fluorescent and non-toxic dye becomes pink and fluorescent when reduced to resorufin by oxidation reduction within viable cells. Resorufin is further reduced to hydroresorfin (uncoloured).

Resazurin based Microtiter Dilution Assay (RMDA)

Under aseptic conditions, 96 well microtitre plates were used for Resazurin based Microtitre Dilution Assay. The first row of microtitre plate was filled with 100 µl of test material in 10% DMSO or sterile water. All the wells of microtitre plates were filled with 100 µl of nutrient broth. Two-fold serial dilution (throughout the column) was achieved by starting transferring 100 µl test material from first row to the subsequent wells in the next row of the same column and so that each well has 100 µl of test material in serially descending concentrations. 10 µl of resazurin solution as indicator was added in each well. Finally, a volume of 10 µl was taken from bacterial suspension and then added to each well to achieve a final concentration of $5x\ 10^6$ CFU/mL. To avoid the dehydration of bacterial culture,

each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated.

Each microtitre plate had a set of 3 controls:

(a) A column with Gentamicin as positive control

(b) A column with all solutions with the exception of the nanoparticles

(c) A column with all solutions except bacterial solution replaced by 10 µl of nutrient broth.

The plates were incubated in temperature controlled incubator at 37° C for 24 h. The colour change in the well was then observed visually. Any colour change observed from purple to pink or colourless was taken as positive. The lowest concentration of NPs at which colour change occurred was recorded as the MIC value.

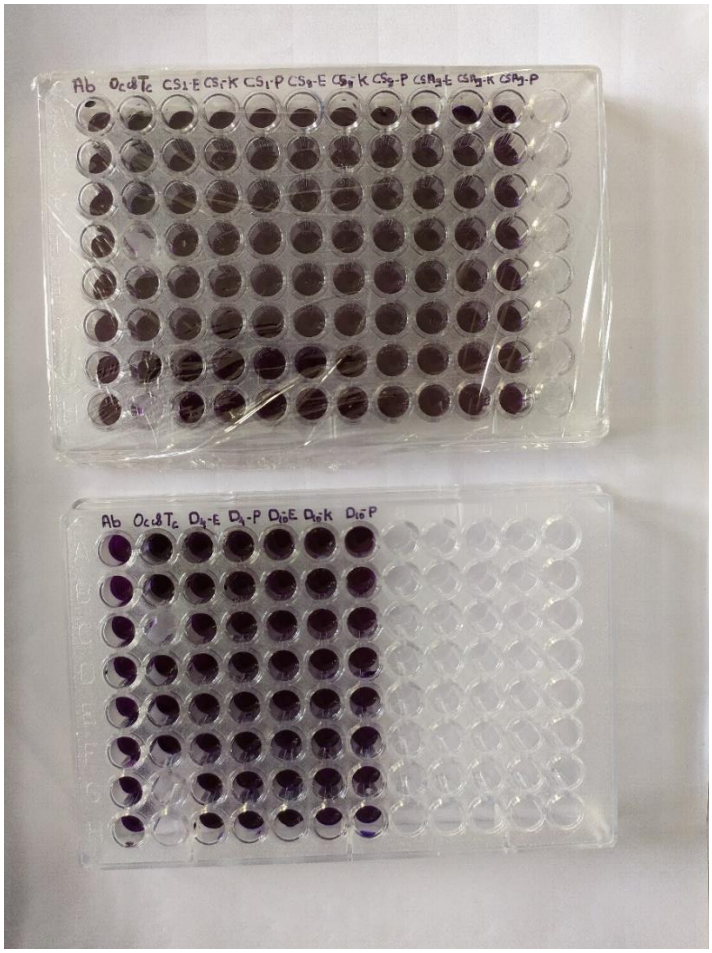


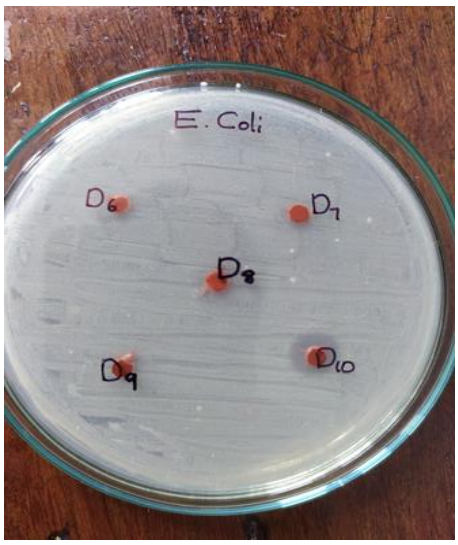
Fig.C. 96 well microtitre plates used for Resazurin based Microtitre Dilution Assay.

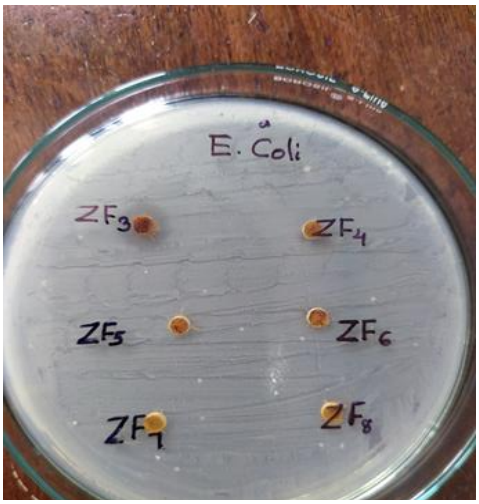
6. OBSERVATIONS AND RESULT

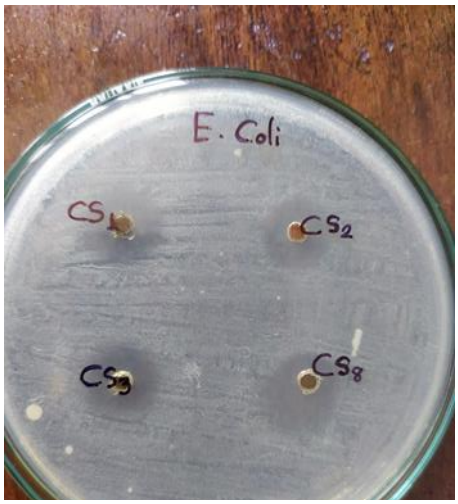
The aim of the study was to determine the antibacterial effect of 27 nanoparticles against three Gram negative bacteria.

DETERMINATION OF ANTIBACTERIAL ACTIVITY

The result of antibacterial activity of nanoparticles against *Escherichia coli* identified by well diffusion method is shown Figure 1.







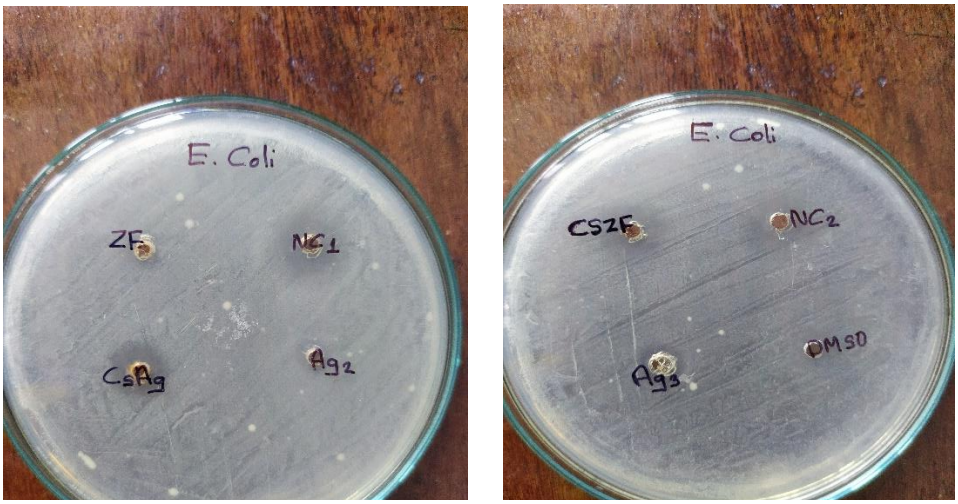
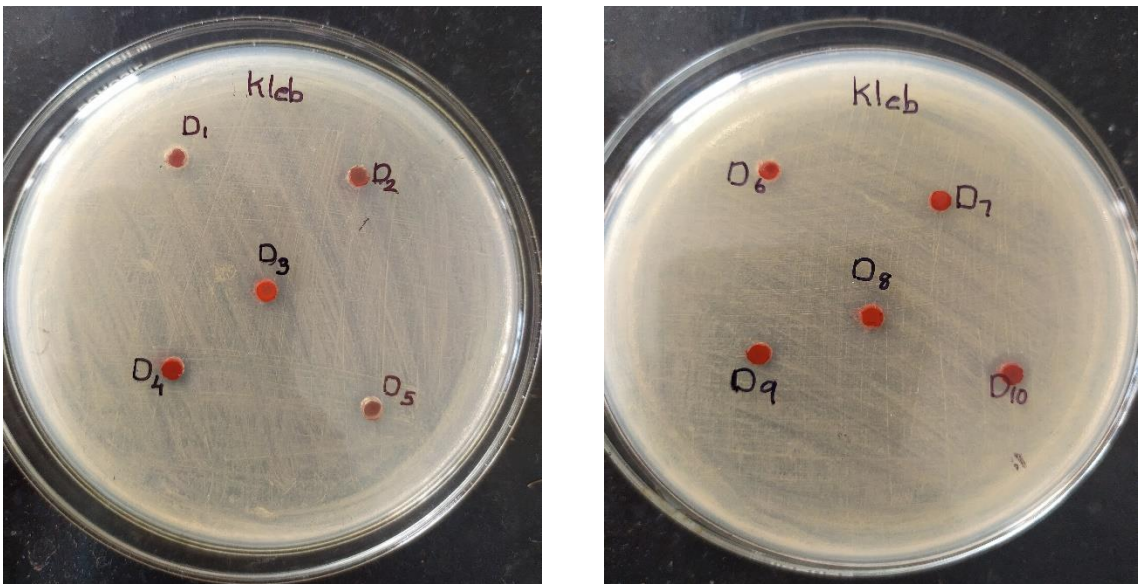


Figure 1 showing antibacterial activity of nanoparticles against *Escherichia coli*

The result showed that few of the NPs have good activity (CS1, CS2, CS7, CS8, D10, ZF3, NC1, CSAg, CSZF, NC2) while some others have less (Ag3, D4, D5, ZF4-ZF8, Ag2) and even no activity (D_1, D_2, D_3, D_6, D_7, D_8, D_9, ZF).

The result of antibacterial activity of nanoparticles against *Klebsiella pneumoniae* is shown Figure 2.



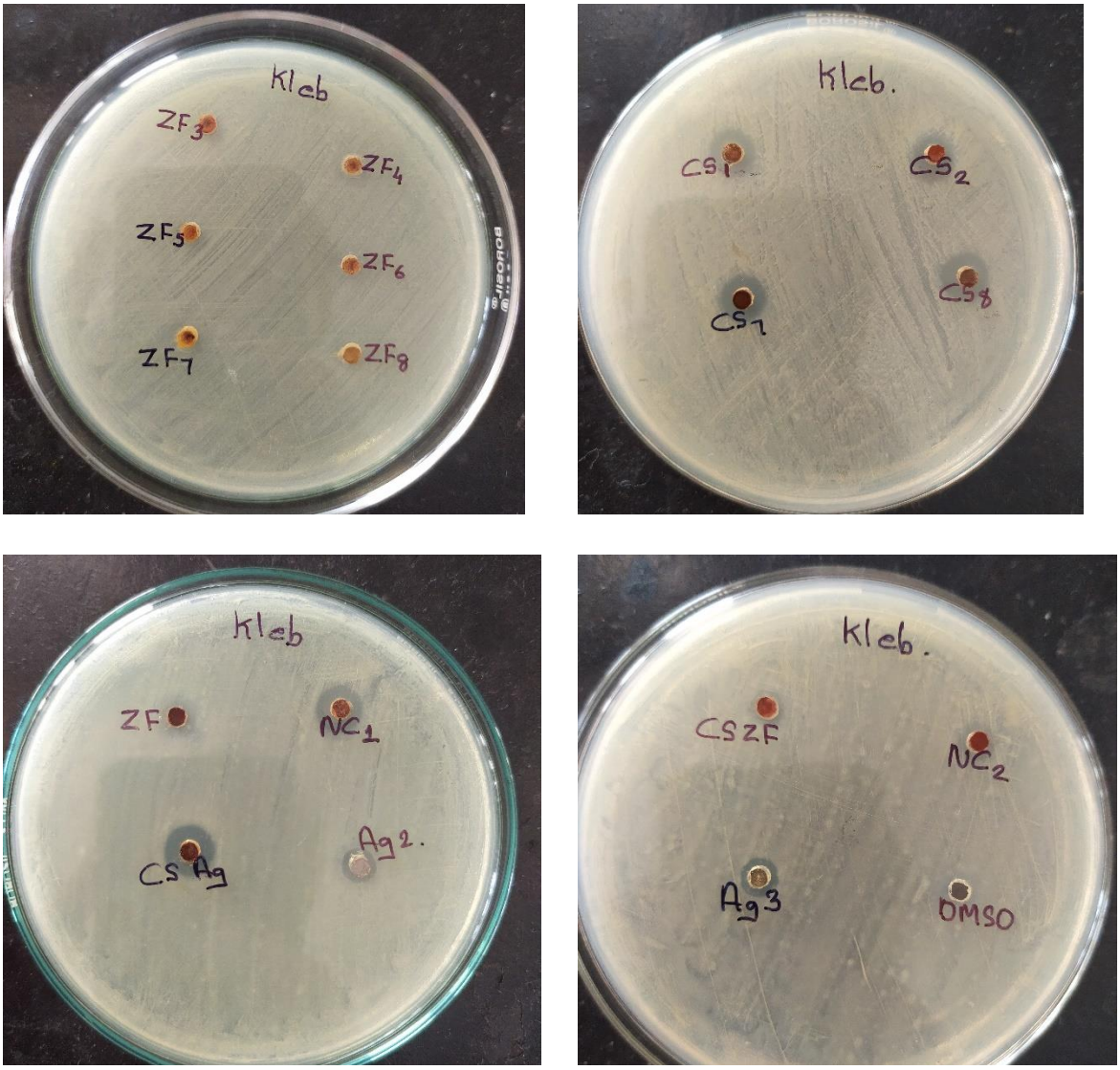


Figure 2 showing antibacterial activity of nanoparticles against *Klebsiella pneumoniae*

The result showed that the nanoparticles CS1 and CSAg have good activity while CS2, Ag3, CSZF, Ag2, D10, ZF, CS7, CS8, NC1 and NC2 have less activity and samples D1 – D9 and ZF3-ZF8 have no activity.

The result of antibacterial activity of nanoparticles against *Pseudomonas aeruginosa* is shown Figure 3.

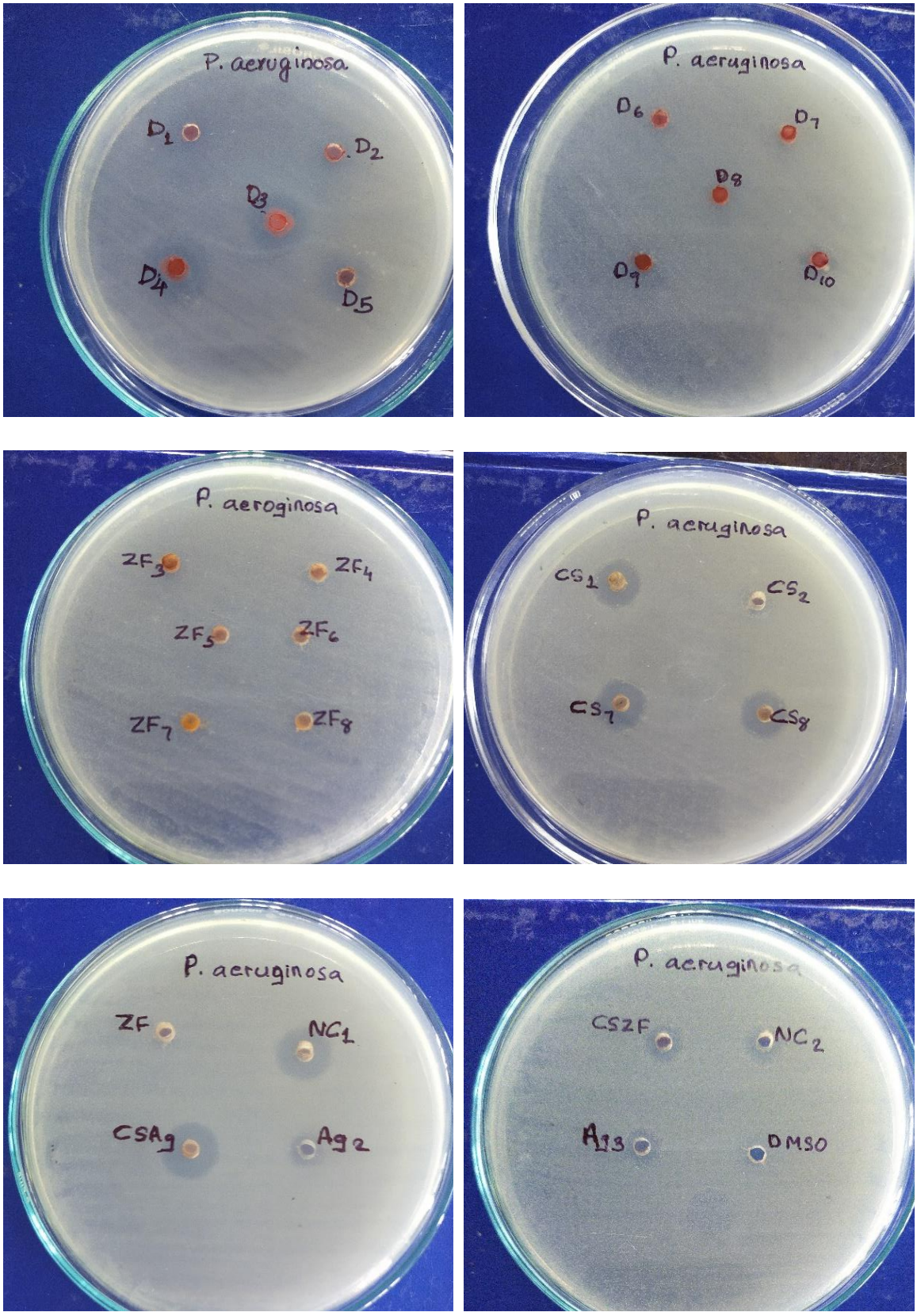


Figure 3 showing antibacterial activity of nanoparticles against *P. aeruginosa*

The result showed that the nanoparticles D1, D3, D4, D5, CS1, CS7, CS8, NC1, NC2 and CSAg have good activity while D2, D10, CS2, Ag2, CSZF and Ag3 have less activity and samples D6 - D9, ZF3 – ZF8, ZF had no activity against *Pseudomonas aeruginosa*.

The diameter of the zone of inhibition of the nanoparticles is given in Table 1.

Table 1 showing the diameter of zone of inhibition of nanoparticles against *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*.

Sl. No.	Nanoparticles	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
1	**D1**	-	-	**9**
2	**D2**	-	-	**8**
3	**D3**	-	-	**13**
4	**D4**	**7**	-	**14**
5	**D5**	**7**	-	**11**
6	**D6**	-	-	-
7	**D7**	-	-	-
8	**D8**	-	-	-
9	**D9**	-	-	-
10	**D10**	**9**	**7**	**6**
11	**ZF3**	**9**	-	-
12	**ZF4**	-	-	-
13	**ZF5**	-	-	-
14	**ZF6**	-	-	-
15	**ZF7**	-	-	-
16	**ZF8**	-	-	-
17	**CS1**	**13**	**10**	**12**
18	**CS2**	**11**	**7**	-
19	**CS7**	**13**	**7**	**12**
20	**CS8**	**14**	**7**	**11**
21	**ZF**	-	**6**	-
22	**NC1**	**13**	**8**	**12**
23	**CsAg**	**11**	**10**	**13**
24	**Ag2**	**3**	**5**	**8**
25	**CSZF**	**10**	**5**	**8**
26	**NC2**	**9**	**6**	**9**
27	**Ag3**	**7**	**5**	**7**

The results in Table 1 shows that 18 nanoparticles out of 27 samples tested exhibited antibacterial property against atleast one of the test organism. Out of

the 18 positive nanoparticles, five samples (D4, D10, CS1, CS2 and CSAg) were selected for the resazurin microtiter assay to find out the MIC value.

The antibacterial activity of antibiotics against *E. coli* is shown in Figure 4

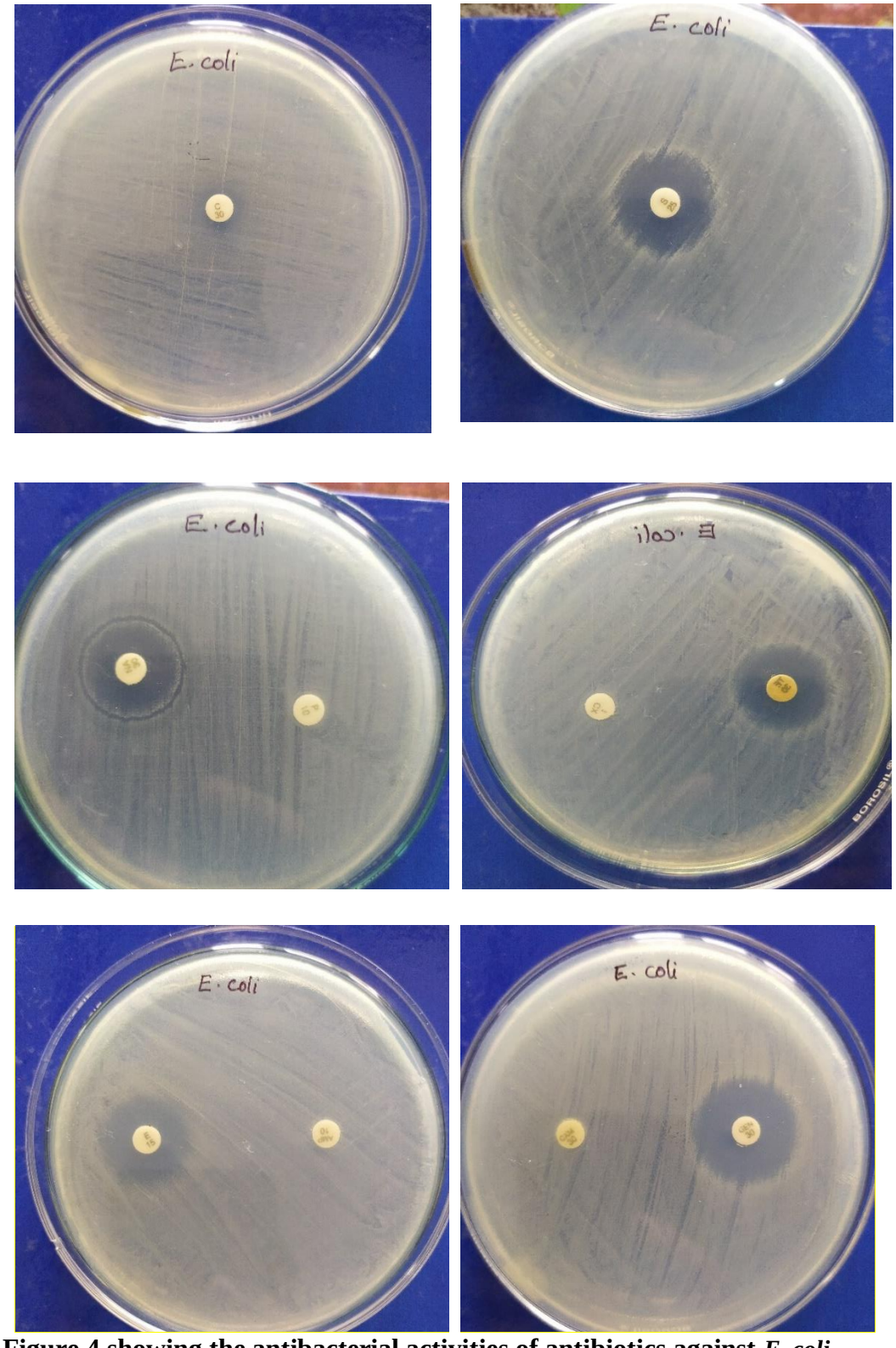


Figure 4 showing the antibacterial activities of antibiotics against *E. coli.*

The result showed that the antibiotics E^{15}, GEN^{30}, NA^{30}, TE^{30}, S^{25} and C^{30} produced visible clear zone against *E. coli* whereas CX^{1}, P^{10}, CXM^{30} and AMP^{10} showed no inhibitory zone.

The antibacterial activity of antibiotics against *Klebsiella pneumoniae* is shown in Figure 5

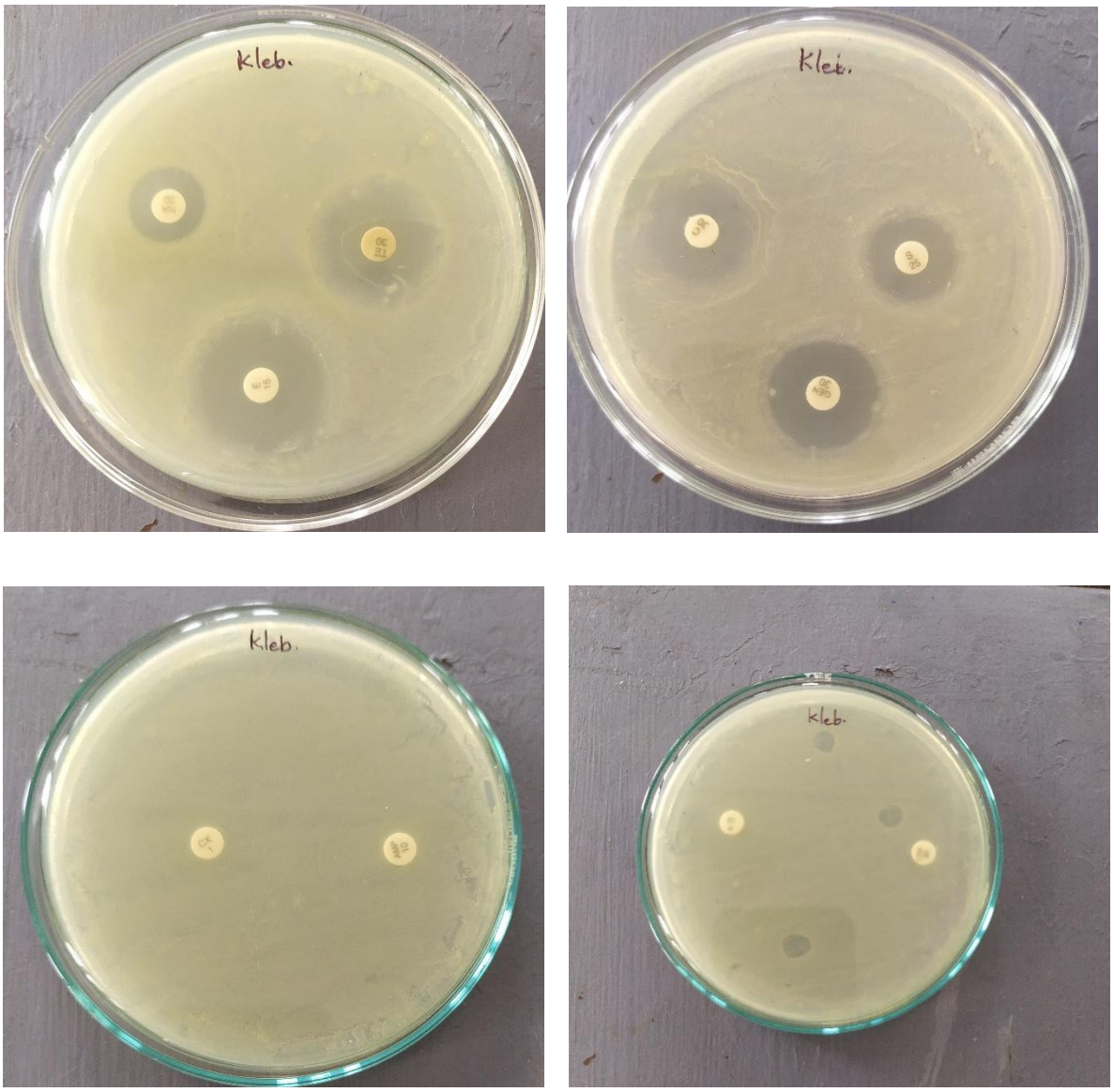
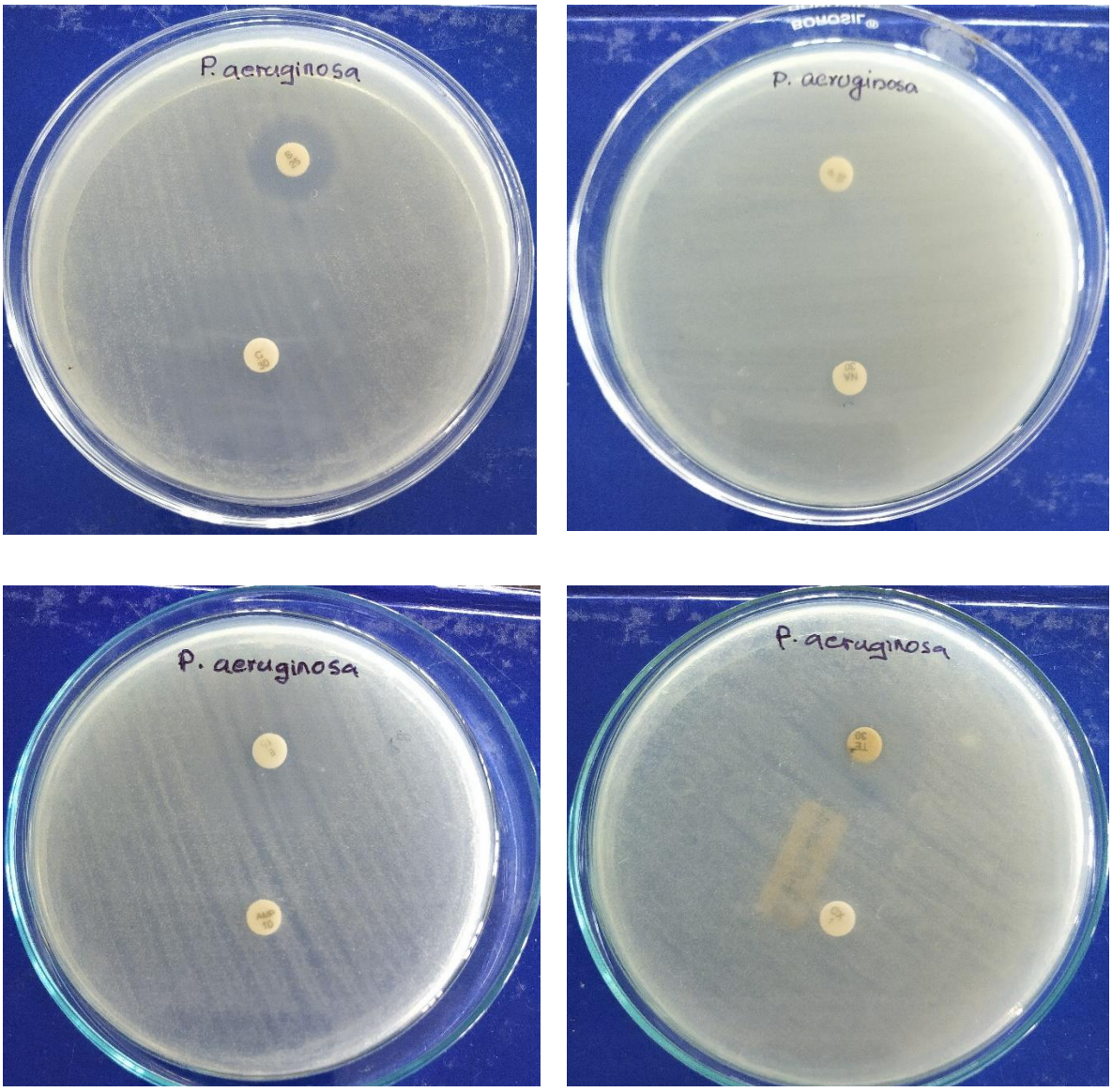


Figure 5 showing the antibacterial activities of antibiotics against *Klebsiella pneumoniae*

The result showed that the antibiotics E^{15}, GEN^{30}, NA^{30}, TE^{30}, S^{25} and C^{30} produced visible clear zone against *Klebsiella pneumoniae* whereas CX^{1}, P^{10}, CXM^{30} and AMP^{10} showed no inhibitory zone.

The antibacterial activity of antibiotics against *Pseudomonas aeruginosa* is shown in Figure 6



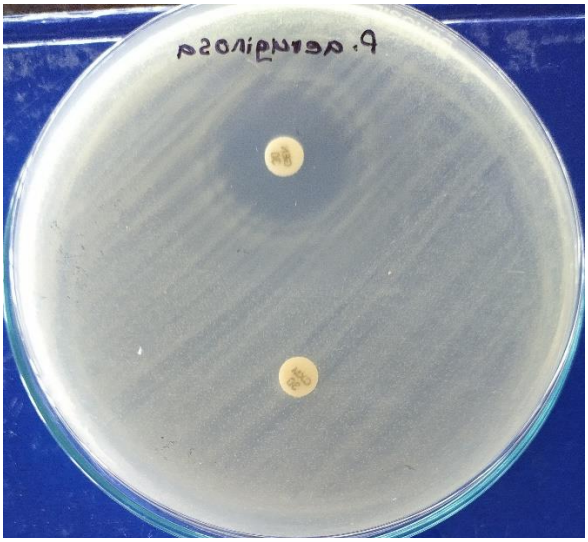


Figure 6 showing the antibacterial activities of antibiotics against *P. aeruginosa.*

The result showed that the antibiotics E^{15}, GEN^{30}, NA^{30}, TE^{30}, S^{25} and C^{30} produced visible clear zone against *Pseudomonas aeruginosa* whereas CX^{1}, P^{10}, CXM^{30} and AMP^{10} showed no inhibitory zone.

The diameters of the zone of inhibition of the antibiotics against three test organisms was measured and is shown in Table 2

Table 2 showing the diameter of zone of inhibition of antibiotics against *E. coli, K. pneumoniae and P. aeruginosa*

Sl. No.	Microorganisms	CXM^{30}	TE^{30}	P^{30}	AMP^{10}	CX^{1}	NA^{30}	GEN^{30}	E^{15}	C^{30}	S^{25}
1	***E. coli***	-	20	-	-	-	22	22	18	9	21
2	***K. pneumonia***	-	19	-	-	-	14	20	23	18	16
3	***P. aeruginosa***	-	23	-	-	-	9	23	30	37	19

From Table 2 it was noticed that the antibiotics TE^{30}, NA^{30}, GEN^{30}, E^{15}, C^{30} and S^{25} had antibacterial effect against the test organisms whereas the antibiotics CXM^{30}, P^{30}, AMP^{10} and CX^{1} had no effect against the test organisms.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The results of Resazurin Based Microtiter Dilution Assay are shown in Figures 7 and 8.

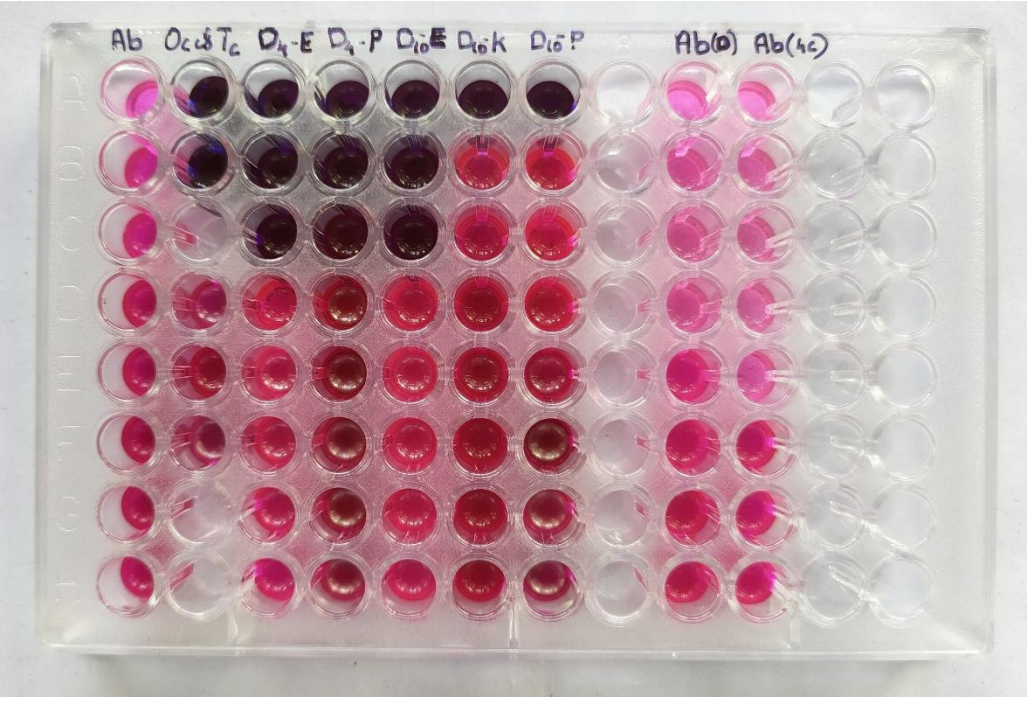


Figure 7 showing resazurin based microtiter dilution assay of D_4 and D_{10} against *E. coli, K. pneumoniae and P. aeruginosa*

Ab - positive control (Gentamicin in serial dilution+broth+bacteria+indicator), TC—negative control (broth+bacteria+indicator), OC—Organism control (broth+indicator+NPs), D_4, D_{10} - nanoparticles (in serial dilution in wells 1–12 +broth+bacteria+indicator), E, K, P – *E. coli*, *K. pneumonia*, *P. aeruginosa.*

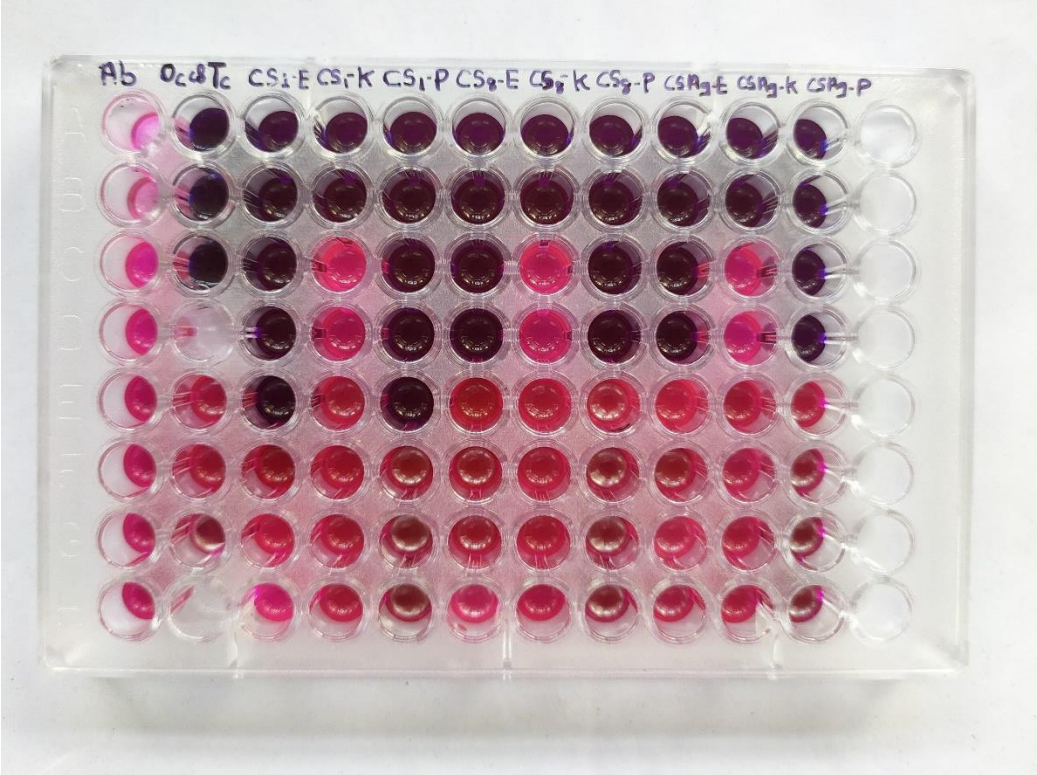


Figure 8 showing resazurin based microtiter dilution assay of CS_1, CS_8 and CSAg against *E. coli, K. pneumoniae and P. aeruginosa*

Ab - positive control (Gentamycin in serial dilution+broth+bacteria+indicator), OC - broth control (broth+indicator+NPs) TC - negative control (broth+bacteria+indicator); CS1, CS8, CSAg - prepared samples (NPs in serial dilution in wells 1–12+broth+bacteria+indicator), E, K, P – *E. coli, K. pneumonia, P. aeruginosa.*

Table 3 showing Concentration of nanoparticles in each wells of the column

Microtitre wells	Concentration of nanoparticles in each wells of the column
First well	62.5 µg
Second well	31.25 µg
Third well	15.625 µg
Fourth well	7.8125 µg
Fifth well	3.90625 µg
Sixth well	1.953125 µg
Seventh well	0.9765625 µg
Eighth well	0.48828125 µg

Table 4 showing MIC values (in µg/µL) of different nanoparticles against three Gram-negative bacteria.

Bacterial strains	D4	D10	CS1	CS8	CSAg
E. coli	7.81	7.81	1.95	3.91	3.91
K. pneumoniae	-	31.25	15.63	15.63	15.63
P. aeruginosa	7.81	31.25	1.95	3.91	3.91

From Table 4 it was noted that nanoparticle CS1 has the Minimum Inhibitory Concentration (MIC) of 1.95 µg against *Escherichia coli* and *Pseudomonas aeruginosa* which was the lowest MIC value obtained in the study. The MIC value of CS1 against *Klebsiella pneumoniae* was found to be 15.63 µg.

The MIC values of D4 against *E. coli* and *P. aeruginosa* are same (7.81). But it has no activity against *Klebsiella pneumoniae*.

The MIC value of D10 was found to be 7.81 µg against *Escherichia coli* and 31.25 µg against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

The MIC value of CS8 and CSAg was found to be was found to be 3.91 µg against *E. coli* and *P. aeruginosa* and 15.63 µg against *K. pneumonia*.

CS1 was the most effective NP retarding microbial growth with lowest concentration of 1.953125 µg/mL against *Escherichia coli* and *Pseudomonas aeruginosa* while D_{10} showed highest MIC value (31.25 µg/mL) against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The MIC value of the Antibiotic Gentamycin (taken as Ab control) against Enterococcus (Gram positive bacteria) was 0.09375 µg/mL, showed its high antibacterial activity. But the MIC of Gentamycin against *K. pneumonia* was not obtained from this microtiter dilution assay showed its low antibacterial activity. Other NPs showed variable antimicrobial activity against the test organisms studied. The results

revealed that all NPs were potentially effective in suppressing microbial growth of Gram negative bacteria with variable potency.

7. DISCUSSION

In this study, the application of nanoparticles as an antimicrobial agent was tested against selected Gram negative bacteria on agar plate medium. The results showed that the tested bacteria could be completely inhibited by NPs. 18 nanoparticles out of 27 samples tested exhibited antibacterial property against atleast one of the test organism.

Loo *et al.*, (2018) determined the antibacterial activity of AgNPs against four species of Gram-negative foodborne pathogens *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13773, *S. typhimurium* ATCC 14028, and *S. enteritidis* ATCC 13076. This study summarized the results for disk diffusion test, MIC and MBC of the AgNPs. For the disk diffusion test, the presence of clear zone around the AgNPs disk was noticed, suggesting that the AgNPs possessed antibacterial activity which is able to inhibit the growth of the Gram negative foodborne pathogens. The inhibition of bacterial growth was reported as affected by the concentration of AgNPs and bacteria used in the experiments (Sondi and Salopek-Sondi, 2004).

Out of the 18 positive nanoparticles, five samples (D4, D10, CS1, CS2 and CsAg) were selected for the resazurin microtiter assay to find out the MIC value. The nanoparticle CS1 has the Minimum Inhibitory Concentration (MIC) of 1.95 µg against *Escherichia coli* and *Pseudomonas aeruginosa* which was the lowest MIC value obtained in the study. D_{10} showed highest MIC value (31.25 µg/mL) against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

The MIC values of AgNPs against the foodborne pathogens ranged from 3.9 to 7.8 µg/mL. *K. pneumonia, S. typhimurium* and *S. enteritidis* showed the MIC value of 3.9 µg/mL while E. coli showed the MIC value of 7.8 µg/mL. Time-kill curves were used to evaluate the concentration between MIC and bactericidal

activity of AgNPs at concentrations ranging from 0×MIC to 8×MIC. The killing activity of AgNPs was fast acting against all the Gram-negative bacteria tested; the reduction in the number of CFU/mL was >3 Log10 units (99.9%) in 1–2 h. The bactericidal endpoint of AgNPs for E. coli was reached after 2 h of incubation at 4 × MIC (31.2 µg/mL) and 8 × MIC (62.4 µg/mL); while for *K. pneumoniae*, the bacteria was killed after 2 h of incubation at 2 × MIC (7.8 µg/mL), 4 × MIC (15.6 µg/mL), and 8 × MIC (31.2 µg/mL).

Guzman et al. (2012), reported that AgNPs employed antibacterial activity on Gram-negative bacteria. Bacterial live/dead imaging and zone of inhibition analysis demonstrated that $ZnFe_2O_4$ NPs showed dose-dependent bactericidal activities in various strains of Gram-negative and Gram-positive bacteria (Reihaneh Haghniaz *et al*., 2021).

In another study on the effect of same nanoparticles on gram positive bacteria ongoing in our laboratory, it was noticed that nanoparticles had low MIC value against Gram positive bacteria when compared to Gram negative bacteria indicating that Gram-positive bacterial strains are more sensitive in comparison to Gram negative strains towards the nanomaterials tested.

Our results are supported by Wang *et al*. (2014) who studied both Gram negative and Gram positive and reported ZnO as the most toxic nanomaterial among ten other nanomaterials. The bactericidal pattern of their synthesized nanomaterials against both Gram negative and Gram-positive bacterial strains was ZnO>CuO> Fe_2O_3. As previously observed with zone of inhibition studies, ZnO nanoparticles had 11% and 12% more bactericidal activity against Gram-positive *S. aureus* and *B. subtilis* than Gram-negative *E. coli* and *P. aeruginosa,* respectively. CuO nanoparticles were 12% and 21% more active against Gram-positive *S. aureus* and *B. subtilis* than Gram-negative *E. coli* and *P. aeruginosa*, respectively. It

should also be noticed that Gram-negative bacterial strains of *E. coli* and *P. aeruginosa* had inhibition-zone sizes that were 24% and 16% lower than Gram-positive bacterial strains of *B. subtilis* and *S. aureus* in the case of ZnO nanoparticles. And in the case of CuO nanoparticles, same Gram-negative bacterial strains of *E. coli* and *P. aeruginosa* had zone sizes that were 28% and 33% lower than Gram-positive *B. subtilis* and *S. aureus* bacterial strains, respectively. This observation could also be indicative of higher Gram-negative strain resistance/tolerance against such nanomaterials over Gram-positive bacterial strains. Azam *et al*. (2012) also studied that NPs have higher activity in Gram positive than Gram negative.

This may due to Gram-positive and gram-negative bacteria differ in terms of cell membrane components and structures and have different adsorption pathways for NPs (Lesniak *et al.*, 2013). Gram-negative bacteria are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane. They are an important medical challenge, as their outer membrane protects them from many antibiotics (including penicillin), detergents that would normally damage the inner cell membrane, and lysozyme, an antimicrobial enzyme produced by animals that forms part of the innate immune system. The antibacterial effects of NPs have been noted to be more pronounced for gram-positive bacteria than for gram-negative bacteria. Such a finding may be related to the fact that the nonporous cell walls of gram-negative bacteria serve as penetration barriers for the entry of NPs (Zaidi *et al.,* 2017). Cell walls of gram-positive bacteria with covalent links with neighbouring proteins and components are relatively porous and allow the penetration of foreign molecules (Zaidi *et al.*, 2017).

Interestingly, NPs showed antimicrobial activity through multiple mechanisms, such as cell membrane damage, protein leakage, and reactive oxygen species

generation, and were more effective against gram-positive bacteria. The exact mechanisms of AgNPs against bacteria still remain unknown. However, there are some researchers proposed that the action of AgNPs on bacteria may due to its ability to penetrate into the cell (Sondi and Salopek-Sondi, 2004), the formation of free radicals (Danilczuk *et al.,* 2006; Kim *et al.,* 2007), the inactivation of proteins in the cell by silver ions (Rai *et al.,* 2012) and the production of reactive oxygen species (ROS) (Dakal *et al.,* 2016). Besides that, there are also some factors in affecting the bactericidal mechanisms of AgNPs such as the concentration of AgNPs and bacteria class (Kim *et al*., 2007; Zhang *et al.,* 2014), shape (Pal *et al.,* 2007; Meire *et al.*, 2012), size (Martinez-Castanon *et al.*, 2008), and the combination of various antibiotics (Fayaz *et al.*, 2010; Singh *et al.*, 2013).

Resazurin dye was used in the study as an indicator in the determination of cell growth, especially in cytotoxicity assays (McNicholl *et al.,* 2007). Oxidoreductases within viable cells reduced the resazurin salt to resorufin and changed the color from blue non-fluorescent to pink and fluorescent. According to McNicholl *et al.* (2007), resazurin dye has been applied for decades to check for the bacterial and yeast contamination in milk.

8. CONCLUSION

In the present study, the antibacterial activity of 27 NPs was determined against three Gram-negative bacteria: *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa* employing well diffusion method. It was revealed that, several metal nanoparticles displayed antibacterial activity against different strains of gram negative bacteria. The presence of clear zone around the NPs suggested that the NPs possessed antibacterial activity. Well diffusion test was described as the preliminary study in screening the antibacterial activity of an antimicrobial agent; therefore, a further evaluation in determining the antibacterial activity of NPs using MIC value was needed.

Among the 27 samples tested, 18 nanoparticles exhibited antibacterial property against atleast one of the test organism. Out of the 18 positive nanoparticles, the Minimum Inhibitory Concentration (MIC) of five samples (D4, D10, CS1, CS2 and CsAg) were determined using Resazurin-based Microtitre Dilution Assay (RMDA). The composition of D_4 contains Fe_2O_3/2M Silver, CS1 and CS8 are Composite samples of Zinc Ferrite/Ag and Zinc Ferrite/AgCl respectively calcined at 100°C and 800°C respectively and CsAg contains Zinc Ferrite/ Ag calcinated 600°C.

The MIC values of D4 against *E. coli* and *P. aeruginosa* were 7.8 μg. But it has no activity against *K. pneumoniae*. The MIC value of D10 was found to be 7.8 μg against *Escherichia coli* and 31.25 μg against *K. pneumoniae* and *P. aeruginosa.* MIC value of D10 against *K. pneumonia* and *P. aeruginosa* were greater than that of *E. coli*. Hence small quantity of D10 is enough to inhibit the growth of *E. coli* when compared to other two test organisms. CS1 has the MIC of 1.95 μg against *E. coli* and *P. aeruginosa.* But a higher concentration of CS1 is required to inhibit the growth of *K. pneumonia* (15.63 μg/ μl). The MIC value

of CS8 and CSAg was found to be 3.91 μg against *E. coli* and *P. aeruginosa*. Here also a higher concentration of both is required to inhibit *K. pneumonia* (15.63 μg/ μl).

Ten common antibiotics were employed to compare the antibacterial activity of nanoparticles, and four of them—Cefuroxime (CXM^{30}), Penicillin-G (P^{30}), Ampicillin (AMP^{10}), and Cloxacillin (CX^{1})—were unable to inhibit any of the test organisms. The majority of the nanoparticles inhibited the test organisms, which were not inhibited by conventional antibiotics, making the current study pertinent.

This study indicates that nanoparticles exhibit a strong antimicrobial activity and thus might be developed as a new type of antimicrobial agents for the treatment of bacterial infection including multidrug resistant bacterial infection. Since the use of NPs in biomedical and pharmaceutical research is rapidly growing, we hope that this material can further be developed, so that it finds wide application both as a drug and as a detection tool for biomolecules.

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