Synergistic effect of iron-oxide NPs conjugated with antibiotics against gram-positive and gram-negative bacteria

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
Bachelor of Science in Microbiology and Biotechnology

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Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Abstract/ Executive Summary

Antimicrobial resistance (AMR) has become a worldwide public health issue. Pathogenic bacteria are gradually developing novel antibiotic resistance mechanisms. Enhanced treatment strategies, such as alternative drugs or combined therapy, should be developed to circumvent the resistance mechanisms. In this investigation, we propose a synergistic approach against pathogenic bacteria utilizing iron-oxide nanoparticles (Fe_3O_4 NPs) infused with various classes of antibiotic discs. Here, Fe_3O_4 NPs were physically infused with antibiotic discs (Fe_3O_4 +A). In addition, the antibacterial activity of bare Fe_3O_4 NPs, various classes of antibiotics, and (Fe_3O_4 NPs + A) was evaluated and contrasted against gram-positive and gram-negative bacteria using disc diffusion method. For instance, when 1000 µg/mL concentrated Fe_3O_4 was infused with a tetracycline antibiotic disc, it showed improved results compared to tetracycline alone. The exceptional results of Fe_3O_4 NPs + tetracycline was 25 ± 0.33 mm for *E. coli*, 20 ± 0.33 mm for *K. pneumoniae*, 19 ± 0.67 mm, for *S. aureus* and 28 ± 0.67 mm for *B. cereus*. Using Fourier transform infrared spectroscopy (FTIR), Fe_3O_4 nanoparticles were characterized. Furthermore, the hemocompatibility of Fe_3O_4 nanoparticles (NPs) in various concentrations was evaluated, and the NPs demonstrated remarkable hemocompatibility. To conclude, in this study a cost-efficient way was suggested to produce Fe_3O_4 NPs which was then conjugated with antibiotic disc displaying a synergistic effect against bacteria. This approach could be a potential therapeutic option for treating pathogenic bacteria.

Keywords: Antibiotic resistance, antibiotic disc, Fe_3O_4 NPs, Antibacterial susceptibility test (AST), minimum inhibitory concentration (MIC), hemocompatibility.

Dedication (Optional)

We dedicate our thesis to our parents, the kind and helpful faculty members and our loved ones for their unconditional love, support, and encouragement.

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List of Acronyms

AMR	Antimicrobial Resistance
MDR	Multi-drug Resistant
XDR	Extensive-drug Resistant
NPs	Nanoparticles
NA	Nutrient Agar
MHA	Muller Hinton Agar
PBS	Phosphate Buffered Saline
NA	Nutrient Agar
MHA	Muller Hinton Agar
LB	Luria Bertani Broth
PBS	Phosphate Buffered Saline
OD	Optical Density
RBC	Red blood cells
RPM	Revolutions per minute
BHI	Brain Heart Infusion broth
FTIR	Fourier transform infrared spectroscopy
Fe_3O_4	Ferrous ferric oxide
Fe_3O_4 NPs + A	Iron oxide nanoparticles conjugated with antibiotics

Chapter 1

Introduction

1.1 General background:

The discovery of antibiotics has altered the perspective of medical science over the past century. It is one of the major reasons for the declining rate of mortality and morbidity caused by pathogenic bacteria. In contrast, over the past few decades several pathogenic bacteria have developed antibiotic resistance mechanisms due to the widespread and unsupervised use of antibiotics. (Biggers 2022)Gradually, these pathogens have begun to manifest XDR and MDR which are responsible for the increased rate of mortality and morbidity. So, from a global aspect AMR is being a threat to the public health. Considering AMR, a threat to the public health, researchers all over the world are constantly working to find a solution(Ikuta et al. 2022). Several clinical therapies were proposed such as using broad spectrum antibiotics, combine antibiotic therapy, bacteriophage, probiotics, plant-based substances, magnetic nanoparticles, synergistic approach of NPs and antibiotics etc.(Łojewska and Sakowicz 2021) Among all those alternative therapeutic approaches nanoparticles and the synergistic approach of NPs has attracted the focus of the researchers. These proposed alternative therapies should be efficient enough to overcome the AMR. Firstly, the Magnetic nanoparticles (MNPs) consists of several unique characteristics that includes biocompatibility, non-cytotoxic, coercivity, high magnetic saturation, surface modification properties,(W. Wu, He, and Jiang 2008) photo-thermal properties, antimicrobial properties etc.(Gudkov et al. 2021). Due to its antimicrobial properties, it could act as an alternative compound instead of antimicrobial agents as they have a non-specific binding mechanism.(Sánchez-López et al. 2020) Hence, bacteria would not be able to identify the inhibition mechanisms as a result, pathogens would not be able to develop

resistance against MNPs. So, MNPs could alone be used to inhibit pathogenic bacteria but to achieve greater and more efficient results combine therapy or synergistic approaches should be embraced frequently. A synergistic approach refers to an interaction of two or more chemicals that results in a combined effect. There could be several types of synergistic effects depending on different sets of given conditions which would also influence the result of synergism. For instance, two drugs could have different mechanism or same mechanism, the drugs with same mechanism would function more efficiently than the different mechanisms.(Omoya et al. 2016)(Zou et al. 2021)

1.2 Pathogenic bacteria

Even though most bacterial species are beneficial for the host body, there are certain bacteria that could cause infectious diseases. The bacteria which could cause infectious diseases are called pathogenic bacteria. These pathogenic bacteria could invade the tissues of the host body by reducing the functions of the immune system. Also, the pathogens could be transmitted directly or indirectly to healthy another host but they require a portal of entry. But to exhibit their pathogenicity, bacteria express virulence factors. The virulence factors refer to molecules of bacteria such as capsules, endotoxins, adhere factors, invasion factors which helps the bacteria to invade the host, cause diseases and evade host immune system.(Maglangit, Yu, and Deng 2021)

Now, the first outer shell of the bacterial cell is the cell-wall which is associated with virulence factors. So, depending on the cell wall composition pathogenic bacteria could be separated into two classes one is the gram-positive bacteria and another is the gram-negative bacteria. The gram positive has thick peptidoglycan layer cell-wall and the gram-negative has thin peptidoglycan layer but also has a lipopolysaccharide (LPS) layer. This LPS layer could release endotoxin when it is ruptured. (Zhang et al. 1998)Moreover, the endotoxin are potent virulence

factors that play a vital role in pathogenesis.(Sharma et al. 2017) It could induce bacterial septic shock and cause multiple organ system failures. Observing the lethal virulence factor, WHO has declared majority of pathogenic bacteria to be gram-negative bacteria. Then again, there are other virulence factor such as adhere factors where pathogens use pili to adhere to host cell and exotoxin that could rupture host cell or disrupt cellular metabolism. Additionally, commonly known waterborne and foodborne pathogens like *E. coli, K. pneumoniae, S. aureus, B. cereus, S. pneumoniae, P. aeruginosa etc.* are responsible for causing significant infectious diseases like UTI, diarrhea, food poising, respiratory disfunction etc. which could also lead to death. In 2019, a study explained that 7.7 million deaths are associated with bacterial infections (Ikuta et al. 2022)Among them 54.9% of deaths were caused by only 5 pathogenic organisms and the all-aged death rate was 0.0996%.

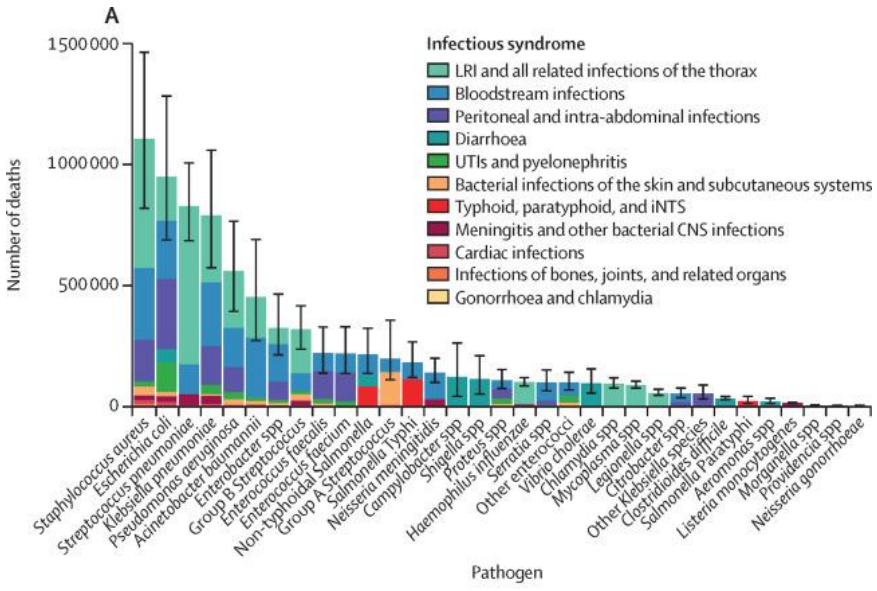


Figure 1: All age mortality rate due to pathogenic bacterial infections in 2019 (Ikuta et al. 2022)

1.3 Bacterial characterization

Gram-negative

Escherichia coli

The most common pathogenic bacteria *E. coli* belongs to the Enterobacteriaceae family. *E. coli* are motile, rod-shaped facultative anaerobic bacteria. These gram-negative strains could grow on selective media like MacConkey and EMB media which exhibit red and metallic green sheen colors respectively with colonies that are 2 to 3 mm in diameter. Additionally, they do not produce enterotoxin which could intoxicate the human body and cause gut related diseases.(Jnani and Ray 2022)

Klebsiella pneumoniae

K. pneumoniae is a gram-negative, rod-shaped, encapsulated, non-motile bacteria that which belongs to Enterobacteriaceae family and could be found mainly in aqueous environment. *K. pneumoniae* strains developed into yellow mucoid colonies on an agar medium containing ornithine, raffinose, and Koser citrate after 24 hours, and there was a little increase in colony size after 48 hours. This bacterium has been linked to pneumonia in patients with diabetes mellitus or alcohol use disorder. Additionally, *K. pneumoniae* typically colonizes the oropharynx and gastrointestinal (GI) tract mucosa of humans and. is widely present in the mouth, skin, intestines, hospital settings, and medical equipment. (Wang et al. 2020)

Gram-positive

Staphylococcus aureus

S. aureus is a gram-positive bacterium that can be frequently found in the environment (soil, water, and air), as well as in human skin and noses. *S. aureus* is a spherical, gram-positive, non-spore-forming member of the Staphylococcus genus of bacteria. It is an opportunistic pathogen that can cause illnesses like pneumonia, endocarditis, and bacteremia. BHI and tryptic soy broth (TSB) are the two most recommended media for this kind of bacteria. Moreover, they form a typical gold-colored colonies. *S. aureus* is non-spore-forming, non-motile, and typically

forms clusters. The colonies of *S. aureus* exhibit typical beta hemolysis and are spherical, large (1-3 mm), convex, opaque, and yellow. (Park and Seo 2022)

Bacillus cereus

A large, facultatively anaerobic, gram-positive, rod-shaped, motile, pathogenic, and opportunistic bacteria called Bacillus cereus is found in soil, vegetation, and food. This species of bacteria belongs to the Bacillaceae family which can produce toxins. Additionally, these are well known opportunistic bacteria. (Buckley and Grotticelli 2022)

1.4 Antimicrobial resistance

Antimicrobial resistance (AMR) refers to a phenomenon that occurs when pathogenic bacteria are exposed to antibiotics in an inconsistent manner. As a result, when infectious pathogens develop AMR and cause infectious diseases then it becomes quite challenging to cure the patients. Even, some commonly known pathogens that cause infectious diseases like diarrhea, cholera, food poisoning are now becoming life threatening. Also, patients with a compromised immune system, undergone surgery, have cancer and taking radiation therapy, have an exposed wound, and have been admitted to the intensive care unit are constantly at risk due to AMR. Hence, there is an increase in the mortality and morbidity rate. Because of this reason, the naturally occurring phenomenon has converted to a global crisis.(Urban-Chmiel et al. 2022) In 2014, WHO considered AMR as a global crisis and threat to public health and reported this era as “post-antibiotic” era. (WHO n.d.)So, AMR appears to be a global burden to public health and even a minor infection caused by antibiotic resistant bacteria could prove to be fatal as infectious diseases are exceedingly challenging to treat. Moreover, to assess the severity of AMR a global survey was conducted 2019, which reported 1.27 million deaths have been caused antibiotic resistant bacteria.(Murray et al. 2022) To overcome this global crisis, several research are going on to discover a novel class of antibiotics with a different mechanism to

inhibit the bacterial growth. Some of that research, also proposed synergistic approach of two different chemical compound, MNPs, combine therapy of MNPs with antibiotics. As those MNPs and antibiotic have potential antibacterial capabilities.

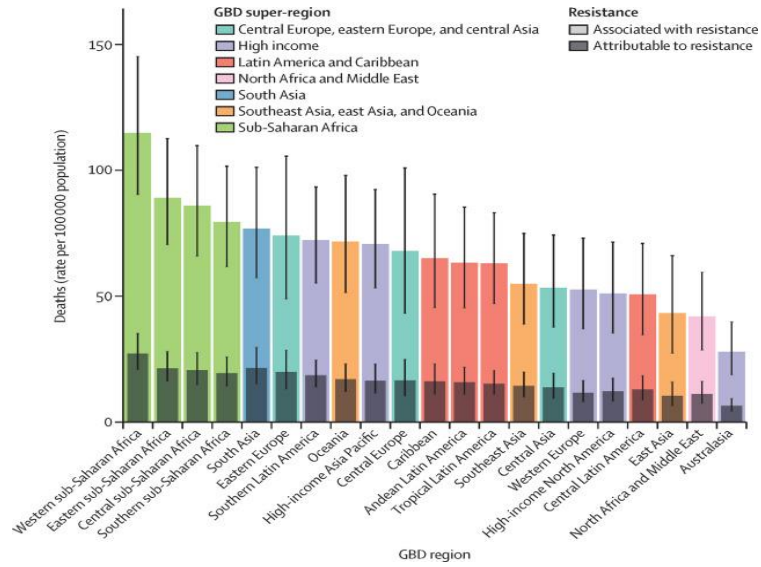


Figure 2: All age mortality rate due to Antimicrobial resistance, in 2019(Murray et al. 2022)

1.5 Magnetic nanoparticles

Since the early 1970s, when magnetic nanoparticles were synthesized and implemented in numerous disciplines, magnetic technology has developed rapidly. Developing techniques for identifying nanoscale structures. Additionally, nanoparticles have been studied. It is now the era of nanoparticle exploration. Not only are magnetic particles important in industrial technology, but also in environmental technology. Recently, magnetic particles have found widespread use in the disciplines of biology and medical diagnosis. Several varieties of iron oxides have been studied in the field of nanosized magnetic particles, with Fe_3O_4 NPs being one of the most significant. Magnetic nanoparticles are distinctive because of several unique properties. The average particle size of these magnetic nanoparticles is less than 100 nanometers. a saturation magnetization between 2 and 2000 emu/cm^2, a phase transition temperature between 40 and 200^o C, an average coherence length of less than 100 nanometers

between adjacent magnetic nanoparticles, and the magnetic nanoparticles being at least triatomic.(Lee, Isobe, and Senna 1996) (Shen et al. 2009)Using an external magnetic field, magnetic nanoparticles could be readily separated and demagnetized immediately after the field is removed. No residual magnetism remains. Magnetite nanoparticles tend to form larger concentrations because of their high specific surface energy. Aggregation of magnetic nanoparticles can drastically reduce their interfacial area, leading to a loss of magnetism and dispersibility.(Shen et al. 2009) Therefore, nanoparticle surface modification is essential, and the particle surface can be modified by grafting an inorganic or organic coating. Surface functionalization of MNPs confers significant properties to the particles. Surface modification of MNPs prevents particle aggregation, thereby stabilizing the colloidal system. (Shawon 2013) Functionalization of MNPs improves their solubility in water, biocompatibility, and nontoxicity. Significant efforts have been invested in modifying the surface of magnetic nanoparticles and creating organic–inorganic nanocomposites. Due to their magnetic characteristics and several other aspects, magnetic nanoparticles (MNP) have drawn more interest in recent years as a potential diagnostic tool. In addition, the demand for safe MNP for biomedical and agricultural applications is increasing due to their unique and tunable properties. MNP are utilized as drug delivery vehicles, drug candidates, contrast dye for magnetic resonance imaging (MRI), biosensors, separation of environmental contaminants, identification of pesticides, antimicrobial properties and nonfertilizer, among other applications.(Shakil et al. 2022)

1.6 Ferrofluids and its preparation

Iron oxides are iron and oxygen-based chemical compounds. Iron (2+, 3+) oxide (Fe_3O_4 NPs) or ferrous ferric oxide, is also referred to as magnetite. Ferrofluids consist of nanoscale particulates (usually 10 nanometers or less in diameter) of magnetite, hematite, or another iron-

containing compound. This is small enough for thermal agitation to uniformly disperse them throughout a carrier fluid and for them to contribute to the fluid's overall magnetic response. These fluids are colloidal mixtures of nanoscale ferromagnetic particles or ferrimagnetic, suspended in a carrier fluid, typically an organic solvent or water. A surfactant is applied to ferromagnetic nanoparticles to prevent their aggregation due to Van der Waals forces and magnetic forces ferrofluids do not display ferromagnetism, despite their name, because they do not maintain their magnetization in the absence of an externally applied field. Due to their high magnetic susceptibility and bulk-scale paramagnetic behavior, ferrofluids are frequently referred to as "superparamagnetic". Materials that are stable are ferrofluids. This shows that even in the presence of very strong magnetic fields, solid particles do not agglomerate or phase split. The nanoparticles will eventually aggregate, segregate, and stop contributing to the fluid's magnetic response as the surfactant deteriorates over time. (K. T. Wu et al. 2001)Liquids that coagulate in the presence of a magnetic field and resemble ferrofluids (FF) are referred to as magnetorheological fluids (MRF). In comparison to FF, the MRF have magnetic particles that are one to three orders of magnitude bigger on the micrometer scale. But ferrofluids lose their magnetic properties when the temperature is sufficiently high (Lee, Isobe, and Senna 1996). Depending on the substances employed to make the nanoparticles, a different temperature is needed. Ferrofluids can be made utilizing a variety of methods, including co-precipitation, decomposition, etc. The most popular approach, co-precipitation, is commonly used in laboratories. (Shawon 2013)

$FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ were mixed into deionized water, stirred to complete dissolution, and then used to make solutions of $FeCl_2$ and $FeCl_3$. Then an alkaline solution like NaOH or NH_4OH was used a precipitation agent. Also, to prevent oxidization nitrogen (N_2) was used throughout the reaction process. The Stirring was used to combine solutions that had been prepared at different concentrations. An hour of reaction period is allowed, and the reaction

temperature is maintained at 70°C. These blended solutions' final pH levels ranged from 12 to 14. Then the NaCl or NH_4Cl was washed with d.H_2O by using a centrifugation machine. With the distil water wash all the metal chloride would leave leaving Fe_3O_4 NPs as precipitating agent. Then Fe_3O_4 was dried at 70^o C for 18 hours in dry oven until all the moisture has been evaporated. It is possible to express the chemical process as follows:

$FeCl_2.4H_2O + 2FeCl_3.6H_2O + 8NH_4OH$ / 8 NaOH = $Fe_3O_4 + 8NaCl$ / 8 $NH_4Cl + 20H_2O$

1.7 Ferrous ferric oxide nanoparticles (Fe_3O_4 NPs)

Fe_3O_4 NPs possess cubic inverse spinel crystalline structure and consists of both octahedral and tetrahedral sites.(Jian et al. 2019) Fe_3O_4 NPs consists of a cubic closed structure of oxide ions where all the Fe^{2+} ions occupy half of octahedral sites while the Fe^{3+} are evenly distributed across the remaining octahedral sites and tetrahedral sites.(Salazar-Alvarez 2004) (Bock et al. 2020) Here, Fe^{3+} ions prefer the octahedral site and tetrahedral site and remains in the high spin complex, as it polarizes more water resulting in easy breakage of OH bond. This phenomenon produces a complex with a lower charge density and more stability and it has small ionic radius. On the contrary, Fe^{2+} prefers only octahedral positions and remains in high spin complex as it has lower charger density and wider ionic radius. (Salazar-Alvarez 2004; Nagashima, Ishida, and Akasaka 2006)

There are several ways to synthesize Fe_3O_4 NPs. For instance, chemical methods like co-precipitation,(Negrescu et al. 2022) hydrothermal, one-pot, sol-gel, mechanochemical etc., Another method could be biosynthesis where biological extracts such as plant, fruits, seeds, microbes could be used to synthesize nanoparticles. But all these methods could be classified into two different approaches one is the top-down approach that indicates large size particles must be shattered to synthesize nanoparticles (Amendola et al. 2011) and bottom-up method

indicates synthesis of nanoparticles from other small particles.(Galstyan et al. 2018) (Raut n.d.)The most used method is the chemical method named co-precipitation. Now, depending on the synthesis method the size and properties of Fe_3O_4 would vary. Moreover, Ferrous ferric oxide nanoparticles have some exceptional characteristics such Biocompatibility, magnetic properties that can be tuned, coercivity, high magnetic saturation, magneto-optic performance (W. Wu et al. 2015), anisotropy, magneto-crystalline anisotropy, (Clarke et al. 2022)surface modification properties, and photo-thermal properties. One of the most important characteristics of Iron-oxide nanoparticles are their physiochemical properties which could be modified using a versatile synthesis method, allowing for surface modification (Estelrich and Antònia Busquets 2018).

The surface modification properties of Fe_3O_4 NPs have unlimited potential. It could be used to enhance the stability of the particles, increase the biocompatibility, coating with polymers to enhance the stability as well as dispersibility; coating with antibiotics to enhance their antibacterial activity, using the surface modification for specific drug deliver, nanocarrier, nano enzymes, silica coating for various biomedical applications such as MRI and even bioconjugation where coating of protein, enzymes and nucleic acid could be conducted successfully. (Raghunath and Perumal 2017)(Arias et al. 2018)

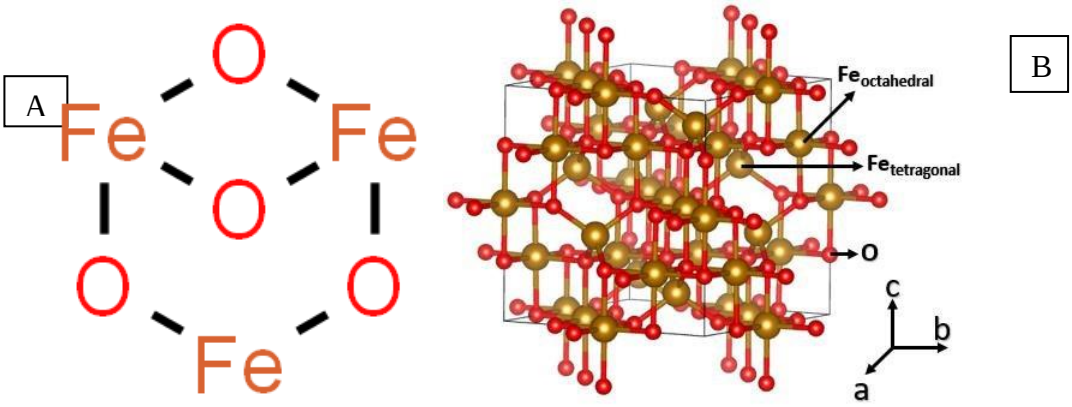


Figure 3: (A) Chemical structure of Fe_3O_4(Chemspider n.d.) (B) molecular crystalline structure of Fe_3O_4 (Arista et al. 2019)

1.8 Antibacterial activity of Fe_3O_4 NPs

Fe_3O_4 have diverse properties, which has great application in fields of biomedical, nanocarrier but the antibacterial properties currently stand out among them. The Antibacterial properties of Fe_3O_4 NPs depends on the size, concentration as well as synthesis method of Fe_3O_4.(Gudkov et al. 2021) The Fe_3O_4 NPs are positively charged particles and bacterial cell wall are negatively charged thereby they can create a repulsive electrostatic interaction which prevents the Fe_3O_4 from getting attached to the cell wall. But if the concentration is high enough then Fe_3O_4 NPs would show some antibacterial activity. Moreover, due to low cytotoxicity most of the time the surface of the Fe_3O_4 NPs must be modified so that it could exhibit antibacterial actions. There are several mechanisms of Fe_3O_4 NPs to inhibit bacteria such as Reactive oxygen species, cell wall disruption, cell membrane disruption, cell enzyme inhibition and protein and DNA damage. (Allafchian and Hosseini 2019; Gabrielyan et al. 2019a)

Generally, Fe_3O_4 at their bulk form do not show any antibacterial activity. However, if the size of the NPs is more than 40 nm then it is unable to penetrate through cell but if the size is not more than 10 nm then it would show efficient antibacterial activity. As the smaller NPs would have a larger surface to volume ratio therefore, penetration of cell membrane would be quite easy.(Liakos, Grumezescu, and Holban 2014) Again, the magnetic properties of Fe_3O_4 also enables the NPs to concentrate between the inner and outer membranes, and it binds with the FHL complex in the inner membrane of gram-negative bacteria and demonstrates substantial antibacterial activity. Surface modification is the fundamental functionalization method for Fe_3O_4 NPs. Therefore, other chemicals and antimicrobial agents might be added to the surface of Fe_3O_4 which would result in a synergistic approach against pathogenic bacteria that have

developed resistance to the drug's antimicrobial activity.(Gudkov et al. 2021; Amaro et al. 2021)

Fe_3O_4 NPs inhibits bacterial cell growth through a non-specific binding mechanism. Pathogens are unable to follow a pattern because they do not bind to specific bacterial receptors or proteins. As a result, bacteria cannot develop resistance to Fe_3O_4 NPs. Instead, Fe_3O_4 work as an adjuvant for antibiotics and have a synergistic effect on the destruction of pathogenic bacteria. Furthermore, Due to their biocompatibility and non-cytotoxicity against RBC, there has been reports that reveals Fe_3O_4 NPs have been employed as particular medication carriers and antibacterial agents.(Ezealigo et al. 2021)

1.9 Mechanism of Fe_3O_4 NPs

1.9.1 Generation of reactive oxygen species:

The reactive oxygen species (ROS) refers to a highly reactive chemical compound form from the diatomic oxygen (O_2). ROS is a potential antibacterial mechanism of Fe_3O_4 NPs. These ROS are powerful enough to induce oxidative stress that disrupt respiratory systems, inactivate cellular enzymes, impede the electron transport system, decrease membrane potential also, rupturing cell membranes by pore formation and lipid peroxidation. Although many other cell parts contribute to the production of ROS but mitochondria are the main source. (Dayem et al. 2017)Fe_3O_4 NPs diffuses and interact with mtDNA and damages it. As a result, Fe_3O_4 NPs interfere with the electron transport chain (ETC).(Tirichen et al. 2021) If the ETC is harmed during the oxidative phosphorylation, it would leak electrons because mtDNA is already in a susceptible position because it lacks a histone-like protein. In contrast, a small amount of oxygen was reacting with the electrons and NADH rather than being reduced because it was the final electron acceptor.(Zorov, Juhaszova, and Sollott 2014) As a result, ROS was created,

which led to oxidative stress, caused a series of malfunctions, and even led to cell death.(Zhao et al. 2019) The electron transport system (ETS) was rendered useless because ROS-induced mtDNA damage hindered mtDNA replication, transcription, and translation. (Canaparo et al. 2021)Lastly, the inducive oxidative stress could damage the cell membrane as well the whole genomic DNA which would ultimately, leading to cell death.(Gudkov et al. 2021) (Deshpande and Mohiuddin 2022)

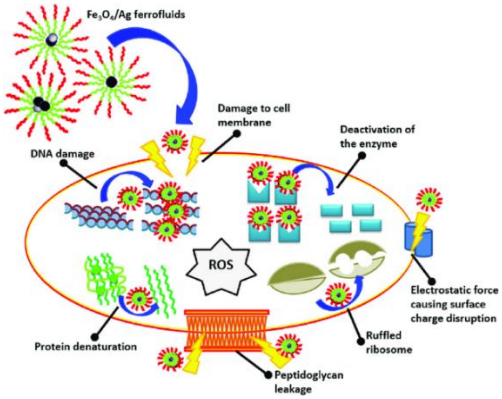


Figure 4: Reactive oxygen species mechanism of Fe_3O_4. (Taufiq et al. 2020)

1.9.2 Cell membrane or wall disruption:

The bacterial cell membrane contains glycoproteins such as peripheral proteins and transmembrane proteins that function as ion channels to transport molecules across the cytoplasmic membrane.(“4.1 An Overview of Membrane Functions 4.2 A Brief History of Studies on Plasma Membrane Structure 4.3 The Chemical Composition of Membranes 4.4 The Structure and Functions of Membrane Proteins 4.5 Membrane Lipids and Membrane Fluidity 4.6 The Dynamic Nature of the Plasma Membrane 4.7 The Movement of Substances Across Cell Membranes 4.8 Membrane Potentials and Nerve Impulses The Structure and Function of

the Plasma Membrane" 2007) Fe_3O_4 NPs containing positively charged iron ions may affect the integrity of cell membranes. After the iron ion disrupts the cell wall through electrostatic interaction, the metal ion attaches to the bacterial cell membrane or enters the intracellular space via protein channels. (Linklater et al., n.d.) The IONPs physically interact with the cell membrane and alter the membrane's structure with the cations, resulting in the disruption of the bacterial cell membrane. (Linklater et al., n.d.)Moreover, the mechanism of rupturing the cell membrane would depend on the size of the IONPs; when the IONPs are 10 nm in size, the released iron ions would be confined between the lipid bilayer, which could form a pore, and the clustered ions would enable translocation. Again, if the size is greater than 10 nm, they would deform the membrane by wrapping around it. Consequently, the cell permeability would increase and due to ionic imbalance, and the cell membrane would be compromised.(Gabrielyan et al. 2019b)(Ozdal and Gurkok 2022)

1.9.3 DNA and protein damage:

Once Fe_3O_4 have penetrated the cell membrane, they could interact with all elements of the cytoplasm. Reactive oxygen species are frequently the focus, but other studies tend to imply otherwise depending on size, shape, and concentration. The Fe_3O_4 NPs have shown that they interact with DNA and protein by generating ROS but they also, directly interact with the DNA and protein content.(Dissanayake, Current, and Obare 2015) By cleaving the DNA strands in the process Fe_3O_4 can inhibit bacterial growth. In addition, these ions may prevent ribosomes from performing their normal functions and from synthesizing proteins.(Karnwal et al. 2023) By gradually engaging with the cytoplasmic content, Fe_3O_4 NPs could show its antibacterial activity by hinder transcription, translation, and replication.(Radeloff et al. 2021)(Ansari et al. 2019; Ren et al. 2020)

1.10 Mechanism of conventional antibiotics

1.10.1 Inhibition of cell wall synthesis:

The most effective antibiotics for treating bacterial infections are those that prevent the formation of bacterial cell walls. Since neither eukaryotic nor mammalian cells have the characteristic structure that bacterial cell walls do, these medications have a great therapeutic efficacy. Peptidoglycan cell walls provide protection for bacterial cells. Various stages of peptidoglycan synthesis and cell wall formation are where antibiotics that damage bacterial cell walls work.(Bhattacharjee 2016) Examples include Beta Lactams, Bacitracin, Vancomycin, Penicillin, Cephalosporin, Ampicillin, and Methicillin. Basically, these antibiotics bind to the C-terminal d-Ala-d-Ala of the murein precursor, lipid II and immature peptidoglycan and inhibit the trans glycosylation during cell wall synthesis. So, these antibiotics could inhibit the synthesis of bacterial cell-wall. (Ghooi and Thatte 1995)

1.10.2 Inhibition of nucleic acid synthesis

Numerous antibiotics have exhibited antibacterial activity by inhibiting the synthesis of nucleic acids. These do not have the same specific side effects as other drugs. By binding to bacterial topoisomerase II, an enzyme that relaxes supercoil DNA during replication, many antibiotics prevent DNA synthesis.(Cambau and Guillard 2012) Others stop RNA polymerase from producing new RNA. In this example, DNA is synthesized using quinolone antibiotics, which primarily inhibit bacterial topoisomerase II. On the other hand, rifampicin reduces RNA synthesis by inhibiting bacterial RNA polymerase. Irinotecan, etoposide are examples of antibiotics that have an impact on human cells. (Foldesi 2021)

1.10.3 Inhibition of protein synthesis

Antibiotics could also eliminate bacterial growth by inhibiting protein synthesis. Although these medications have excellent therapeutic efficacy, they lack the strength to inhibit the cell-wall synthesis. However, because of these medications specific stages of protein synthesis could be blocked, they are also useful as research tools and treatment agents. The production of the 30S initiation complex and the construction of the 50S ribosomal subunit, combinedly forms the 70S ribosome, and elongation are all components of this process. Streptomycin, chloramphenicol, erythromycin aminoglycosides, tetracycline, and macrolides are a few examples. Most of the antibiotics in this class are selective, whilst the others react with human analogues of these enzymes. (Mccoy, Xie, and Tor 2011)

1.10.4 Disruption of cell membrane

There are certain antibiotics that disrupt the cell membrane permeability by binding to membrane phospholipids. As human cells have cell membranes, certain antibiotics that are administered comprehensively can be harmful to host cells. Consequently, their clinical utility is restricted to topical applications. Such significant antibiotics are polymyxins.

1.10.5 Inhibit metabolism and block pathway

There are certain antibiotics that eliminate the bacterial growth by inhibiting the metabolic pathways by acting as antimetabolites. The chemical compounds have structural similarity to metabolites but their functions are different. As these antimetabolites hinders the metabolic pathway therefore, bacteria would not be able to initiate metabolism resulting in bacterial cell death. They inhibit the enzymes that are responsible for bacterial metabolism. Sulfonamide and trimethoprim are examples of these types of antibiotics. (Stokes et al. 2019)

1.11 Synergistic effects of Fe_3O_4 NPs

Synergistic effects refer to a phenomenon where the combine effect of two or more compounds are greater than an individual.(Alavi et al. 2022) In this kind of interaction, the combine effects are more important than the simple effect of the individual component. Meaning, it is not necessary for each compound to work separately at the same time rather the combine actions of those compounds produce synergistic effect. Moreover, in each of the compounds could have same or different mechanism or they might have different target but as for synergistic effect when those compounds are incorporated, they would need to complement one another so that significant results could be shown. Now, there are several types of synergistic effects such as additive effect, potentiation effect and synergistic effect itself.(Roell, Reif, and Motsinger-Reif 2017) Firstly, additive effect refers to when two substances with similar mechanisms of action are combined, their effects sum up in a predictable manner. Then potentiation effect refers to an event when two substances are combined, one substance enhances the effect of the other without having an effect of its own.(Tekin et al. 2016)

1.12 Objective of the project

The overall objective of this research is to study the antimicrobial activity of the bare magnetic particles as well as observe the synergistic approach of biomolecules and Fe_3O_4 NPs. The desired goals of different procedures could be divided into the following:

1. Synthesis of Fe_3O_4 NPs is a cost-efficient way so that, it would not get oxidized and function for a longer period.
2. Characterization of magnetic nanoparticles.
3. Conjugate the different classes of antibiotic disc with different concentration of Fe_3O_4 NPs (Fe_3O_4 + A). Then observe their synergistic effect at two different concentrations (500µg/mL and 1000µg/mL) on both gram-positive and gram-negative organisms.
4. Observe hemolysis of the bare magnetic Fe_3O_4 NPs.

Chapter 2

Material and methods

2.1 Place of conducted experiment

This research was done at Biochemistry and Environmental Microbiology (BEM) lab, Department of Mathematical and Natural Sciences Department, BRAC University.

2.2 Bacterial strain

The following bacterial strains were used. *Escherichia coli* (ATCC- 25922), *Staphylococcus aureus* (ATCC-25925), *Bacillus cereus* (ATCC-14579) and *Klebsiella pneumonia* (ATCC-13883). The bacterial strains were collected from the laboratory's microorganism inventory. All microbes were preserved in a submerged state in T_1N_1 media with sterile liquid paraffin oil in the cryovial container. The bacteria were sub-cultured in the Nutrient agar media.

2.3 Apparatus and instruments

Table 1: List of Apparatus and instruments

Serial No.	Apparatus and instruments	Brand
1.	General Incubator	Incucell
2.	Shaker incubator	JSR
3.	Ultra-centrifugation machine	TOMY MX-307

4.	Autoclave machine	TOMY ES-315
5.	Laminar	Haier Biomedical
6.	Refrigerator	Samsung
7.	Petri dishes	N.A.
8.	Conical Flask	N.A.
9.	500 mL Beaker	Pyrex
10.	Screw- Capped bottles	N.A.
11.	Polypropylene screw-capped tubes	Falcon
12.	Glass test tubes	Pyrex
13.	Borosilicate vials with Cap	Pconlab
14.	Microcentrifuge tubes (1.5ml & 2.0ml)	Eppendorf
15.	Spirit lamp	N.A.
16.	Micropipette	Eppendorf
17.	Micropipette tips	NEST
18.	Inoculation loop	N.A.
19.	Inoculation needle	N.A.

20.	Cotton Swab	N.A.
21.	96- well ELISA microplates	N.A.
22.	Sonicator	N.A.
23.	ELISA machine	Thermofisher multiskan ex
24.	Vortex	DIGISYTEM VM- 2000
25.	Paraffin tape	Bemis Company, INC
26.	Syringe (5ml and 10 ml)	JMI
27.	Magnetic Stirrer	JSR
28.	Blank disc	Darwin biological
29.	Neodymium Magnet N52 (50*25*10mm)	Link up magnet
30.	Continuous power supplier	N.A.
31.	Teflon/ PTFE stirrer	N.A.
32.	DC motor (21000 rpm)	N.A.
33.	Regulator	N.A.
34.	FTIR	Thermofisher

2.4 List of media, reagent and chemical

Table 2: List of Media, reagents and chemicals.

Serial No.	Media, reagent and chemical	Brand
1.	Nutrient Agar (NA)	Himedia
2.	Muller Hinton Agar (MHA)	Himedia
3.	Brain Heart Infusion Broth (BHI)	Himedia
4.	NaCl	Himedia
5.	PBS	N.A.
6.	Hydrogen peroxide	N.A.
7.	Ferric Chloride hexahydrate ($FeCl_3.6H_2O$)	Merck, Germany
8.	Ferrous Chloride tetrahydrate ($FeCl2_3.4H_2O$)	SmartLab, Indonesia
9.	NH_4OH	Merck, Germany
10.	70% Ethanol	Sigma-Aldrich
11.	Spirit	Sigma-Aldrich

2.5 Preparation of Media and Reagents

2.5.1 Preparation Nutrient agar medium

The organisms were first grown on nutrient agar as it is a universal media, both gram-positive and negative bacteria would grow on it. Nutrient Agar Media consists of peptone, beef extract, and agar. To prepare the media 28 g of nutritional agar powder was mixed with 1000 mL of Distil water (d. H_2O). Heat this mixture until the compound fully dissolves when the compound dissolves and less turbidity is achieved. Autoclave the dissolved mixture for 15 minutes at 121^oC. After the nutritional agar has been autoclaved, let it cool down but do not let it solidify. Fill each petri dish with nutrient agar, then place the plates on the sterile surface to dry. Replace the lid of each Petri dish and store the plates in a refrigerator.

2.5.2 Muller Hilton Agar Composition & Preparation

Muller Hilton Agar A non-selective, non-differential media for bacterial growth. The agar consists of 2 g of beef extract, 17.50 g of acid hydrolysate of casein, 1.50 g of starch, 17 g of agar. To prepare MHA, 38 grams of the media should be dissolved in 1000 mL d.H_2O by using heat and continuous stirring. Then, it should be autoclaved for 15 minutes at 121 °C. later, when the heat comes down to tolerable level. It should be poured into the petri dishes on a level, horizontal surface to ensure equal depth and let it cool down at room temperature. At 25^o C, make sure the pH is 7.3-7.4. Store the plates between 2 and 8 degrees Celsius. The MHA agar also consists of starch that absorbs bacterial toxins, preventing them from interfering with the activity of antibiotics. Additionally, it regulates the rate of drug diffusion through the agar. Casein acid hydrolysate and beef extract contain essential nutrients such as nitrogen, vitamins, carbon, amino acids, and Sulphur. To determine how well bacteria respond to antibiotic or antimicrobial medications using the diffusion method (Kirby-Bauer method), it is

recommended to use a standardized solid medium called Mueller Hinton Agar (MHA) for a more appropriate inhibition zone is the result of enhanced diffusion.

2.5.3 Saline preparation

For the preparation of bacterial suspensions, physiological saline was utilized. Due to excessive alkaline conditions, all bacteria would perish if the saline contained more than 0.9% NaCl; therefore, 0.9g NaCl was dissolved in 100 ml of distilled water. Then, 5 mL of saline was pipetted into each test container using a micro pipette. The test containers were then autoclaved for 15 minutes at 121^{o}C before being stored at room temperature.

2.5.4 Preparation of PBS buffer

PBS buffer refers to phosphate buffer which is an isotonic solution with a pH of 7.4. This solution is being used to keep the pH of red blood cell (RBC) consistent when hemolysis test is being conducted. To prepare a 1x PBS buffer solution the following ingredients would be needed.

Table 3: Components of PBS

Component	**Amount**
Sodium Chloride	8g
Potassium Chloride	0.2g
Sodium phosphate	1.44g
Potassium Phosphate Monobasic	0.245g

800 mL distil water taken in a beaker then 8g of NaCl, 0.2g of KCl was added to the solution. Then, 1.44g of sodium phosphate dibasic and 0.245g of potassium phosphate Monobasic was

added to the solution. After adding every component to the solutions, the pH must be adjusted to 7.4. then distilled water added until the volume reached to 1000 mL.

2.6 Building a heat resistant non-reactive Teflon propellor

A Teflon coating 10 cm rod was ordered from chain. Then a 21000 rpm DC motor was bought and using welding the Teflon rod was welded on to the DC motor. Then a continuous power supplier with cooling fan and regulator box crafter indicating the speed of 1000 rpm. Two electrode clip was joined with positive and negative site where red one labeled as negative and black; one was positive. Later, the extended cable was joined with circuit of regulator and the off and on switch. The main wires were connected to the continuous power supplier. Thus, a non-magnetic, non-reactive and heat resistant Teflon propellor was built. This machine is built to stir and can be used to produce magnetic nanoparticles.

Figure 5: Using our own crafted propellor to synthesis MNPs.

2.7 Synthesis of bare iron-oxide oxide nanoparticles (Fe_3O_4 NPs)

Nanosized iron oxide nanoparticles were synthesized by chemical co-precipitation method. The synthesis methods required iron salts, distilled water and an alkaline precipitating agent. The molar ration between Fe^{3+} and Fe^{2+} salts should be 2:1 to synthesize Fe_3O_4 NPs.

Here, to synthesize 1 g of Fe_3O_4 NPs, 2.35 g of $FeCl_3.6H_2O$ and 0.86 g of $FeCl_2.4H_2O$ salts were used and dissolved in 40 mL of distilled water. Then the solution was kept under vigorous stirring at a speed of1000 rpm. As the solution was heated up to 70^o C, 11.5 mL of NH_4OH was added. Then the heat was raised up to 80^o C and the reaction was continued for another 1 hour with vigorous stirring. The black suspension was then cooled down at room temperature and washed with d.H_2O in the ultra-centrifuge machine at 14,000 rpm. This process must be repeated until the pH of the particles reaches 7. When the required pH 7 is met then the black particles need to wash with 70% ethanol to reduce the oxidation rate. (Ba-Abbad et al. 2022)After washing the particles twice with ethanol, they are transferred to a petri dish. Lastly, the particles are dried at 70^o C at hot oven drier for 18 hours until all the moisture is soaked. Then it was transferred into a glass vial with tightly sealed cap and preserved at -20^o C. This is how the bone iron-oxide nanoparticles or Fe_3O_4 NPs was achieved. (Shawon 2013) The following reaction has occurred throughout the process-

$$FeCl_2.4H_2O + 2FeCl_3.6H_2O + 8NH_4OH = Fe_3O_4 + 8\ NH_4Cl + 20H_2O$$

Figure 6: Synthesis of Bare Fe_3O_4 NPs

2.8 Sonication

In the laboratory, nanotubes are frequently distributed within the polymer matrix by sonication. Ultrasound radiation is used to agitate the nanoparticles in the polymer matrix during this procedure. Typically, an ultrasonic bath or horn/probe, also known as a sonicator, is used to conduct this procedure. For our experiment, an ultrasonic instrument was utilized. Ultrasonic probes, also known as transducers, convert the equipment's electric energy discharge into high-frequency sound waves that travel through the substance being tested. First, a bowl containing pulverized ice was obtained. Second, 1 mL of ethanol was withdrawn from two of the three vials, while 1 mL of distilled water was withdrawn from the third vial. The three vials were then placed in the ice basin. Then, set the spanner to 10 o'clock. Six distinct vials were then filled with NPs containing the following concentrations: 0.5 mg/mL, 5 mg/mL, 6 mg/mL, 1 mg/mL, 15 mg/mL, and 20 mg/mL. During sonication, the instrument was held with care, and the thumb switch was depressed each time. First, in the distilled water, the probe was depressed for three seconds, and then the thumb switch was depressed for three seconds. In the ethanol, the identical procedure was repeated. I repeated this process twice. Following this, tissue was used to clean the probe so that ethanol could not mix with the NPs, and the same procedure was repeated for the six distinct NP concentrations mentioned previously. Five identical repetitions of the sequence were performed for each concentration of particles. Each of the six particle-containing containers was vortexed properly following sonication.

2.9 Conjugation of Fe_3O_4 NPs on blank disc and antibiotic disc

Firstly, 500 µg and 1000 µg bone dry Fe_3O_4 NPs was taken into the MCT tube. Then 1 mL of distilled water was added and sonicated until the particles were fully suspended. With the process concentration like 500 µg/mL and 1000 µg/mL was achieved. Then a blank petri dish

cleaned with ethanol and blank disc as well as antibiotic disc were extracted from disc cartridge. The petri dish with disc were then placed onto a stove or hot plate with no magnetic properties. Subsequently, the sonicated NPs were vortexed and 40 µL was taken by using a 10-100 µL pipette and placed over one side of the blank disc or the antibiotic disc. Then the discs were flipped to the other side by using a stainless-steel tweezer which has no magnetic properties. The same process would be repeated once again. At mild temperature, the discs were allowed to soak the Fe_3O_4 NPs. This process was steadily followed for all the antibiotic and blank discs. Thus, the conjugation of Fe_3O_4 NPs within the antibiotics discs and blank disc were possible.

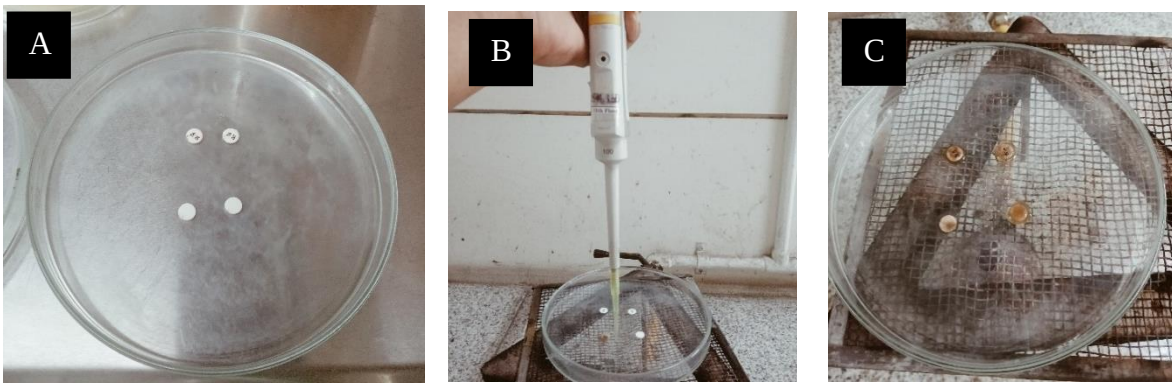


Figure 7: Conjugation of NPs with blank and antibiotic disc. (A) antibiotic and blank disc are taken into a sterile petri dish (B) adding 40µL of the concentrated particles (C) particles are physically being conjugated with the disc by using mild heat

2.10 Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared spectroscopy is a technique for determining the chemical bonding or molecular structure of organic or inorganic substances. Molecular structural information can be determined by using infrared spectroscopy. (Peak 2005)The vibrational movements, such as bending and stretching, of different types of bonds (O-H, Fe-O, etc.), correspond to the absorption of specific wavelengths in the infrared spectrum of electromagnetic radiation. Infrared spectroscopy is utilized for qualitative purposes rather than quantitative ones. (Shawon

2013) Typically, the region of the infrared spectrum acquired is between 400 and 4000 cm^{-1}. The region of the spectrum below 1500 cm^{-1} is the fingerprint region, in which each compound's bands are distinct. Therefore, two molecules are regarded as identical if they share the same 'fingerprint' in this region. The region above 1500 cm^{-1} where the vibrational movement of functional groups can be observed, is known as the functional group region.

2.11 Bacterial suspension preparation

In a test tube 5mL of saline solution was prepared. For each bacterial strains 3 test tubes containing 5mL of saline solution was prepared. So, there were 4 strains of bacteria a total of 12 test tubes were designed for three repetitive bacterial samples. Then, by using an inoculating loop, a single colony of bacteria were taken from the sub-cultured plate and inoculated into the saline solution. Lastly, the test tubes were vortexed until a smooth suspension was created the smooth suspension was compared with 0.5 McFarland solution. If the turbidity matches with the 0.5 reference of McFarland solutions, then it would mean the bacteria will be with a specified range. The cell density would not be very high or low.

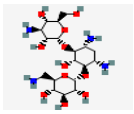
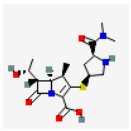
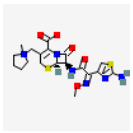
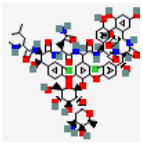
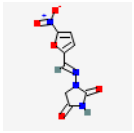
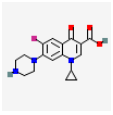
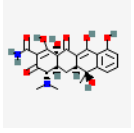
2.12 Creating the bacterial suspension lawn culture on MHA media:

Prior to antibiogram testing, bacterial samples must be grown on Nutrient agar media (NA) for 24 hours. Single colonies are extracted from the fresh culture medium one-two, suspended in 5mL of saline solution using a sterile loop. Then the bacteria were mixed by vortexing until 0.5 MacFarland standard is obtained. A sterile cotton swab is plunged into the saline solution containing bacteria and the excess solution was drained. Next, the sample was repeatedly swiped at a variety of angles to ensure that the bacteria were evenly distributed across the MHA surface.

2.13Experimented antibiotics for the zone of inhibition test and their measurements

Ten different classes antibiotics were selected to conduct the antibiogram test to observe the antibiotics susceptibility as well as resistance.

Table 4: List of antibiotics used in this study

Name of antibiotic Groups	**Used antibiotics**	**Manufacturer**	**Structure**
Aminoglycosides	Kanamycin	HiMedia	
Carbapenem	Imipenem	HiMedia	
Cephalosporin	Cefepime	HiMedia	
Glycopeptides	Vancomycin	HiMedia	
Monobactams	Aztreonam	HiMedia	
Quinolones/ Fluroquinolones	Levofloxacin	HiMedia	
Tetracyclines	Tetracycline	HiMedia	

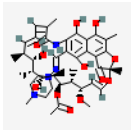
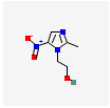
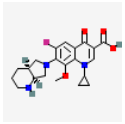
Phenicol	Chloramphenicol	HiMedia	
Penicillin	Penicillin-G	HiMedia	
Sulfonamides	Co-trimoxazole	HiMedia	
β- lactam	Ceftazidime	HiMedia	

Table 5 CLSI guideline standard disc diffusion measurements of sensitive, intermediate and resistant for different bacteria

Antibiotic group name	Antibiotic name	organism	Zone of inhibitons		
			S	i	R
Aminoglycoside	Kanamycin	*Enterobacteriaceae*	18	14-17	13
		Staphylococcus spp.	18	14-17	13
		Bacillus spp.	-	-	-
Peniciline	Penicillin	*Enterobacteriaceae*	-	-	-
		Staphylococcus spp.	29	-	28
		Bacillus spp.	-	-	-
Carbapenem	Imipenem	*Enterobacteriaceae*	23	20-22	19
		Staphylococcus spp.	16	14-15	13
		Bacillus spp.	>25	21-24	<20
Fluoroquinolone	Levofloxacin	*Enterobacteriaceae*	29	-	-
		Staphylococcus spp.	19	16-18	15
		Bacillus spp.	>19	16-18	<15

Tetracycline	Tetracycline	*Enterobacteriaceae*	15	12-14	11
		Staphylococcus spp.	19	15-18	14
		Bacillus spp.	>15	12-14	<11
Macrolides	Aztreonam	*Enterobacteriaceae*	21	18-20	17
		Staphylococcus spp.	-	-	-
		Bacillus spp.	-	-	-
Sulfonamidesz	Co-trimoxazole	*Enterobacteriaceae*	16	13-15	13
		Staphylococcus spp.	17	14-16	14
		Bacillus spp.	>16	11-15	<10
Phenicol	Chloramphenicol	*Enterobacteriaceae*	18	13-17	12
		Staphylococcus spp.	18	-	18
		Bacillus spp.	>18	13-17	<12
3rd generation cefalosporin	Ceftazidime	*Enterobacteriaceae*	22	19-21	19
		Staphylococcus spp.	-	-	-
		Bacillus spp.	-	-	-
Glycopeptides	Vancomycin	*Enterobacteriaceae*	-	-	--
		Staphylococcus spp.	>15	-	-
		Bacillus spp.	>16	-	-
4^{th} generation cephalosporin	Cefepime	*Enterobacteriaceae*	25	19-24	18
		Staphylococcus spp	18	15-17	14
		Bacillus spp.	-	-	-

2.14 Antibiotic disc and NPs conjugated disc placement on the bacterial lawn

Sterile tweezers were used to place the antibiotic disc, NPs conjugated antibiotics discs and only NPs conjugated discs. The discs made full contact with the bacterial lawn and they were

gently deposited and then pressed against it. For each bacterial sample, the process was repeated 3 times to observe the best outcome. After arranging all the discs properly over the bacterial lawn, the lead was closed and the plates were placed into the incubator for 24 hours at 37^{o} C. Then the zone of inhibition was measured. By using a millimeter ruler, the zone diameter was measured. The zone diameter was measured 3 times from different angles and the average results were noted and compared to antibiotic susceptibility chart.

2.15 Hemolysis assay

The hemocompatibility of Fe_3O_4 NPs is very high as its low cytotoxicity therefore, it is unable to lysis red blood cells (RBC). So, firstly, the hemolysis test was of bare Fe_3O_4 NPs was conducted with several lower concentrated particles. In this case, from 20-30 years old individual male and female donated 10mL of B+ blood. The blood was collected through the venipuncture method by a butterfly needle. In flacon tube prior 10% of EDTA was added that acts as an anticoagulant. Blood samples were centrifuged at room temperature for 5 minutes at 5000 RPM to separate RBCs from serum. After aspirating the serum, the RBCs were resuspended in 5 mL of phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 5000 RPM for 5 minutes. The RBCs were then rinsed twice for 5 minutes at 5000 RPM with 5 mL of phosphate-buffered saline (PBS, pH7.4) solution. The red blood cell suspension was then prepared in 11.5 mL of phosphate-buffered saline (pH 7.4). Subsequently, lower concentrated Fe_3O_4 NPs (500 µg/mL, 600 µg/mL, 700 µg/mL, 900 µg/mL, 1000 µg/mL and 1500 µg/mL) were sonicated. The RBC suspension (1 mL) was then combined with 200 µL of bare Fe_3O_4 NPs with different concentration and incubated in the shaker incubator with 120 rpm for 1 hour at 37^{o} C. After that, RBC with Fe_3O_4 NPs were centrifuged at 5000 rpm for 5 mins and the supernatant were collected and placed into the microtiter plater. Then, at 570nm the absorbance was measured. Here, the bare particles with distilled water were treated as negative control and RBC with PBS was treated as positive control.

Chapter 3

Results

3.1 Synthesis of bare Fe_3O_4 NPs

The synthesis of 1 g bare Fe_3O_4 NPs required mixing of 2.35 g of $FeCl_3.6H_2O$ and 0.86 g of $FeCl_2.4H_2O$ salts in 40 mL of distilled water. The iron (II) and iron (III) salt ratio should be 2:1 always. Then the solution was kept under vigorous stirring at a speed of1000 rpm. As the solution was heated up to 70^o C, 35 mL of NH_4OH was added. After several wash with d.H_2O when the pH of NPs lowered down to 7 then it was washed two time with 10mL of 70% ethanol. Generally, N_2 gas or inert gas must be applied during the synthesis of magnetic particles otherwise the NPs would be oxidized within 5-6 days. If the NPs are oxidized then it would lose their properties as well as efficacy.

During the synthesis of Fe_3O_4 nitrogen gas creates an inert atmosphere so that gas like oxygen do not take part in the process. If the O_2 gas takes part in the process the Fe_3O_4 (magnetite) would surlily convert into Fe_2O_3 (hematite) or other iron oxide. But by using ethanol as reducing agent to wash the Fe_3O_4 NPs, it prevents that oxidization and keeps the integrity of NPs for 4-5 months.

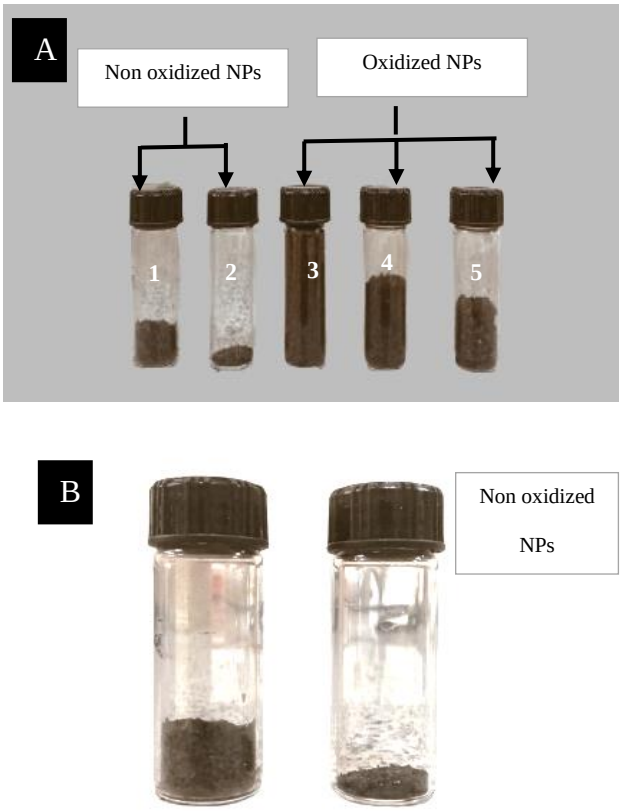


Figure 8: 1&2 no. vial consists of non-oxidized nanoparticles 3,4 & 5 consists of oxidized NPs (B) Non-oxidized NPs washed with ethanol.

Here, the NPs in vial no. 3, 4 and 5 oxidized within 7 days. The 3-no. vial oxidized within 2 day the 4-no. in 7days and 5^{th} oxidized after drying it in the oven for 18 hours. But the NPs washed in ethanol is functional for 5 months and continuing.

3.2 Fourier transform infrared spectroscopy (FTIR)

By using FTIR the chemical bonding or the molecular structure of a compound could be determined. The region of spectrum below 1500 cm^{-1} indicates to the unique bonds and spectrum above that region indicates the functional group region. Here, at the spectra the peak is at 580 cm^{-1} which corresponds to Fe-O bonding and another stretched peak is at 3206.83 which corresponds with O-H functional group. So, the FTIR determines that synthesis of Fe_3O_4

NPs was successfully conducted. This FTIR was conducted after 5 months of synthesizing the Fe_3O_4 NPs. The black color of iron oxide still intact after 5 months. But Fe_3O_4 NPs synthesized without using ethanol oxidized after 4 to 5 days and the black color changed to brown.

3.2.1 FTIR of bare Fe_3O_4 NPs

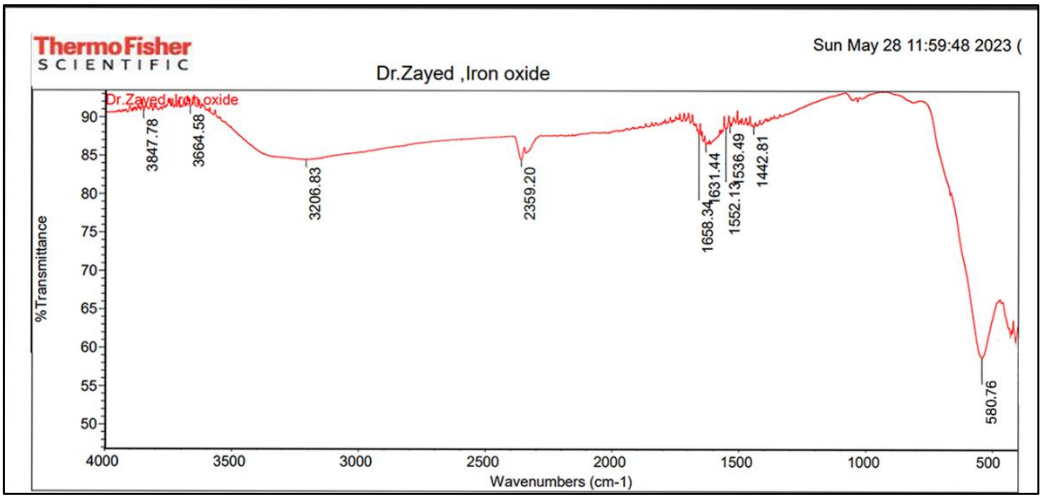


Figure 9: FTIR of Bare Fe_3O_4 NPs

3.2.2 FTIR of Fe_3O_4 conjugated antibiotic (aztreonam)

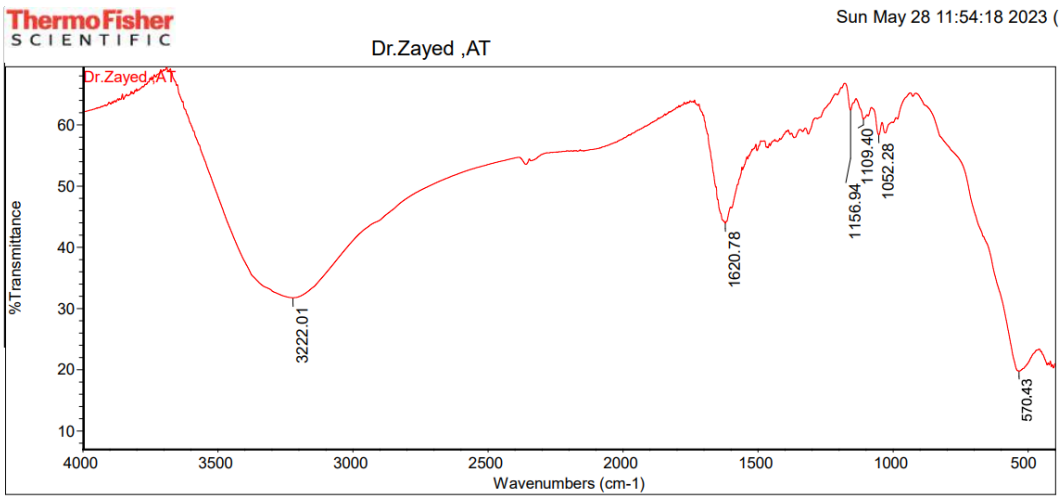


Figure 10: FTIR of Fe_3O_4 NPs conjugated within antibiotic disc

3.3 Antibiotic susceptibility testing on pathogenic strains:

Antibiotic susceptibility test was applied on 4 bacteria with 10 different class of antibiotics and Fe_3O_4 NPs conjugated antibiotics. Among these 4 bacterial strains 2 of the bacteria were multidrug resistance. *K. pneumonia* and *B. cereus* were MDR. Here, antibiotics discs and bare NPs with a concentration of 500µg/mL and 1000 µg/mL conjugated within the blank discs were used as a control group. The same concentration of Fe_3O_4 NPs was conjugated differently with 10 different antibiotics discs (Fe_3O_4 NPs + A) to observe their synergistic effect against both gram positive and negative bacteria. This Fe_3O_4 NPs + A is labeled as the experimental group.

Table 6: Control group and experimental group

Control group	1. 10 different class of Antibiotics 2. 500µg/mL concentrated Fe_3O_4 NPs soaked into blank disc 3. 1000 µg/mL concentrated Fe_3O_4 NPs soaked into blank disc
Experimental group (Fe_3O_4 NPs+A)	1. 500µg/mL concentrated Fe_3O_4 NPs conjugated with antibiotic discs. 2. 1000µg/mL concentrated Fe_3O_4 NPs conjugated with antibiotic discs.

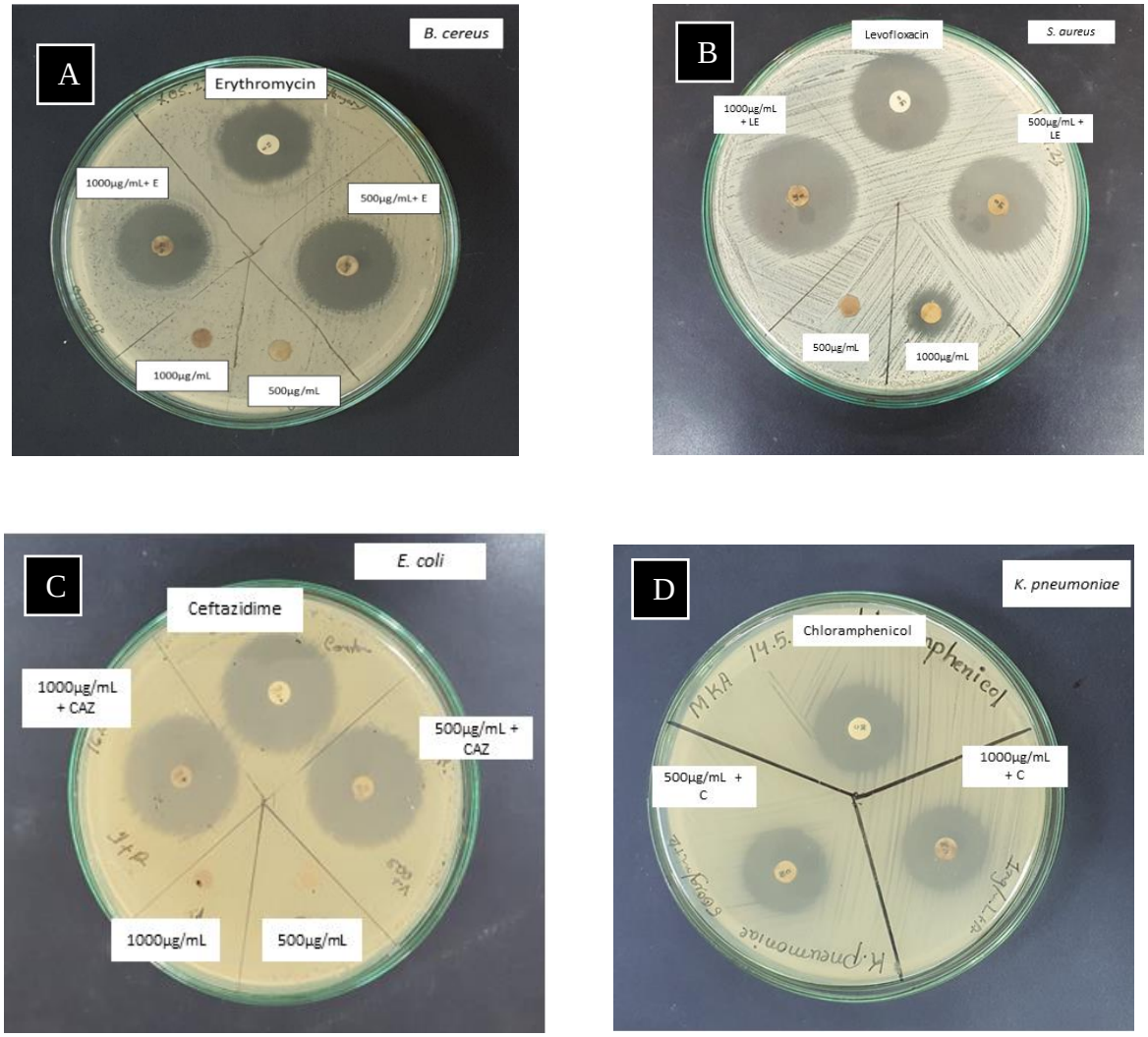


Figure 11: Zone of inhibition diameter (mm) observed (A) and (B) are gram-positive bacteria B. cereus *and* S. aureus. (C) and (D) are gram-negative bacteria *E. coli and K. pneumoniae*, respectively.

3.3.1 Zone of inhibition for gram negative bacteria in diameter (mm)

Table 7: *E. coli* and *K. pneumoniae*, gram-negative bacteria's Zone of inhibition in diameter (mm). Using control group and NPs conjugated antibiotic discs.

Gram negative											
	Antibiotic name	Tetracycline	Cefepime	Co-trimoxazole	Chloramphenicol	Kanamycine	Penecillin	Imepenum	Aztreonam	Levofloxacin	Ceftazidime
E.coli	Control group	17	26.33	26	24.67	17.67	0	35	33	25.67	20.33
	500 µg/ml + Antibiotic	22.33	29	29.67	27	23	0	37.67	37	30	34
	1000 µg/ml +Antibiotic	25	30	28	27.33	22	11	40	37	30.67	33
	500 µg/ml	0	0	0	0	0	0	0	0	0	0
	1000 µg/ml	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	Control group	16	18	25.67	24	10	0	23	11	22	0
	500 µg/ml + Antibiotic	20	20.33	27.67	26.33	10	0	15	10	24.33	0
	1000 µg/ml +Antibiotic	20.33	20	28	26.67	10	0	20	10	24	12
	500 µg/ml	0	0	0	0	0	0	0	0	0	0
	1000 µg/ml	0	0	0	0	0	0	0	0	0	0

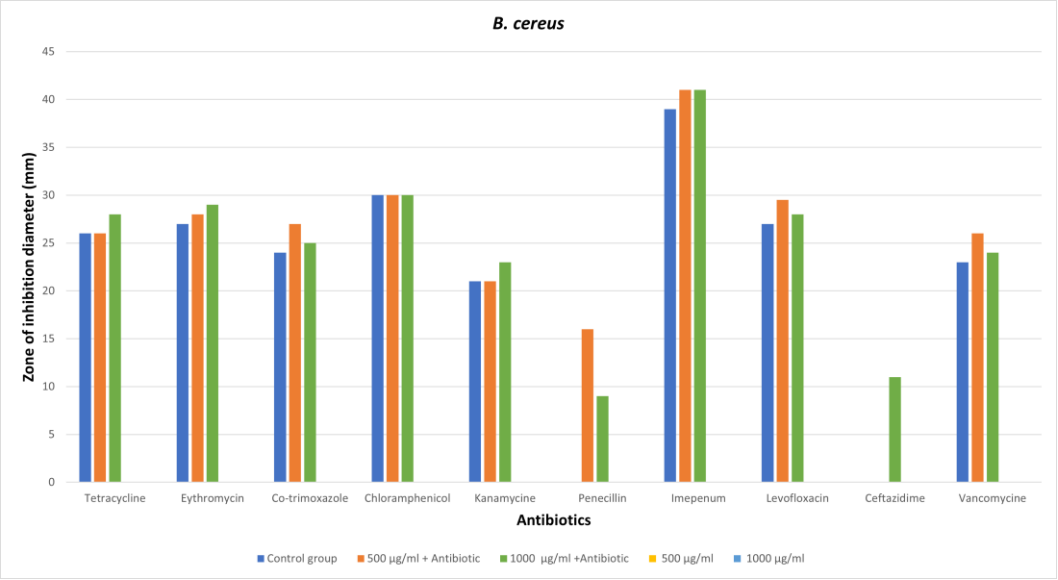
3.3.2 Zone of inhibition for gram-positive bacteria in diameter (mm)

Table 8: *S. aureus* and *B. cereus*, gram-positive bacteria's Zone of inhibition in diameter (mm). Using control group and NPs conjugated antibiotic discs.

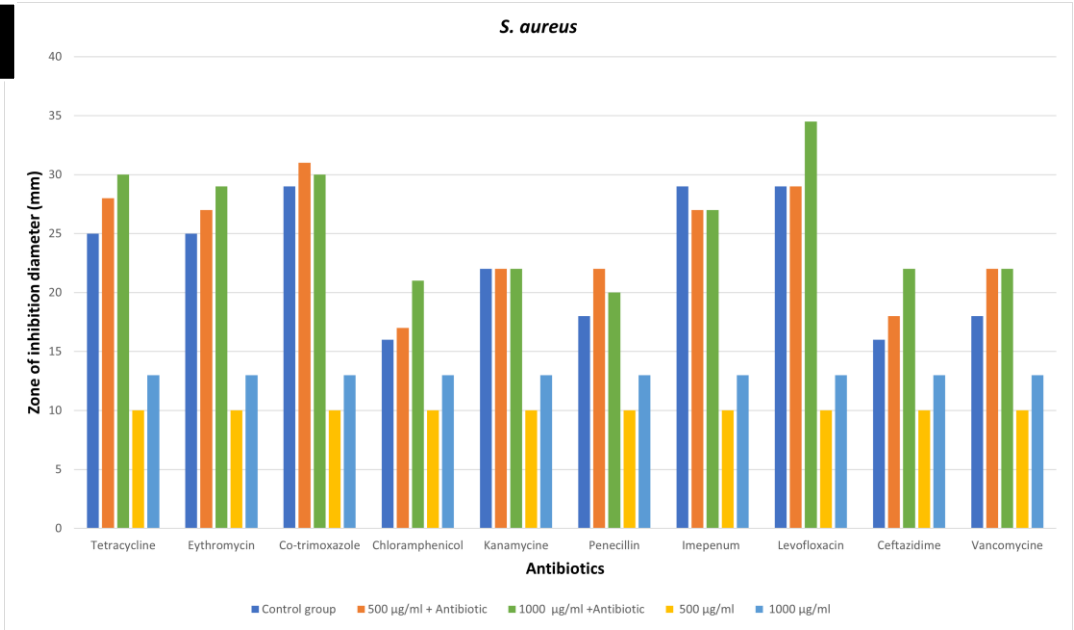
Gram Positive											
	Antibiotic name	Tetracycline	Eythromycin	Co-trimoxazole	Chloramphenicol	Kanamycine	Penecillin	Imepenum	Levofloxacin	Ceftazidime	Vancomycine
Staphylococcus aureus	Control group	25	25	29	16	22	18	29	29	15.33	18
	500 µg/ml + Antibiotic	28	27	31	17.33	22	22	27	29	18	22
	1000 µg/ml +Antibiotic	30	29	30.67	21	22	20	27	34.67	22	22
	500 µg/ml	0	0	0	0	0	0	0	0	0	0
	1000 µg/ml	13	13	13	13	13	13	13	13	13	13
Bacillus cereus	Control group	26	27	24.67	30	21	0	39	27.33	0	23
	500 µg/ml + Antibiotic	26	28.67	27	30	21.33	16	41	29.67	0	26
	1000 µg/ml +Antibiotic	28	29	25	30	23	9	41	28	11	24.67
	500 µg/ml	0	0	0	0	0	0	0	0	0	0
	1000 µg/ml	0	0	0	0	0	0	0	0	0	0

3.3.3 Comparison based on zone of inhibition between control groups and Fe_3O_4 NPs conjugated antibiotics

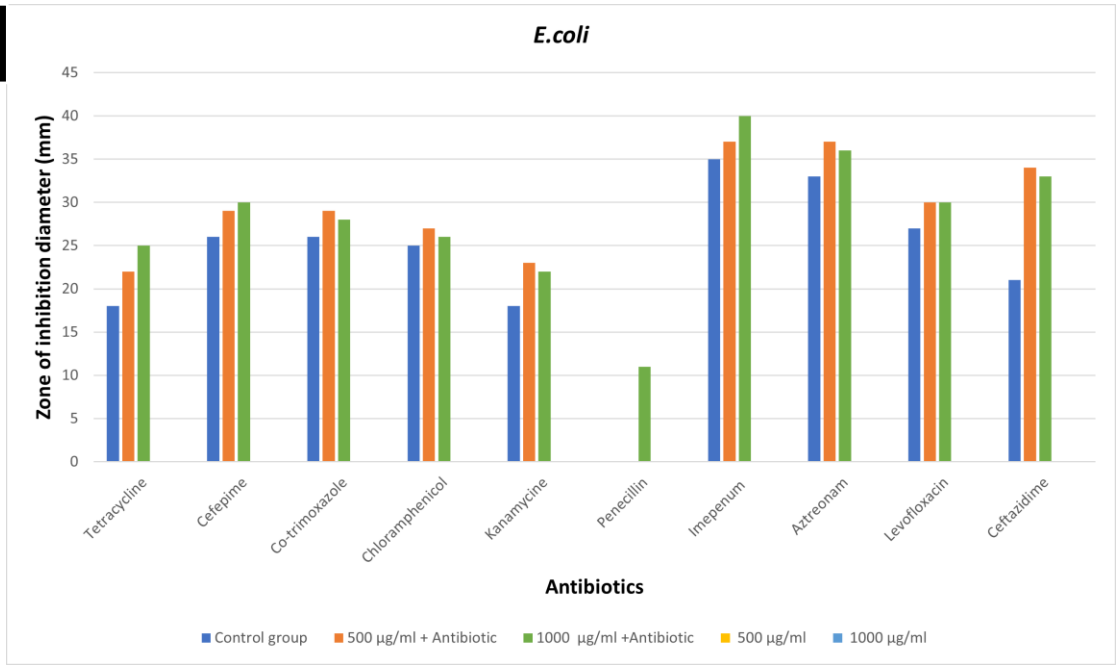
A



B



C



D

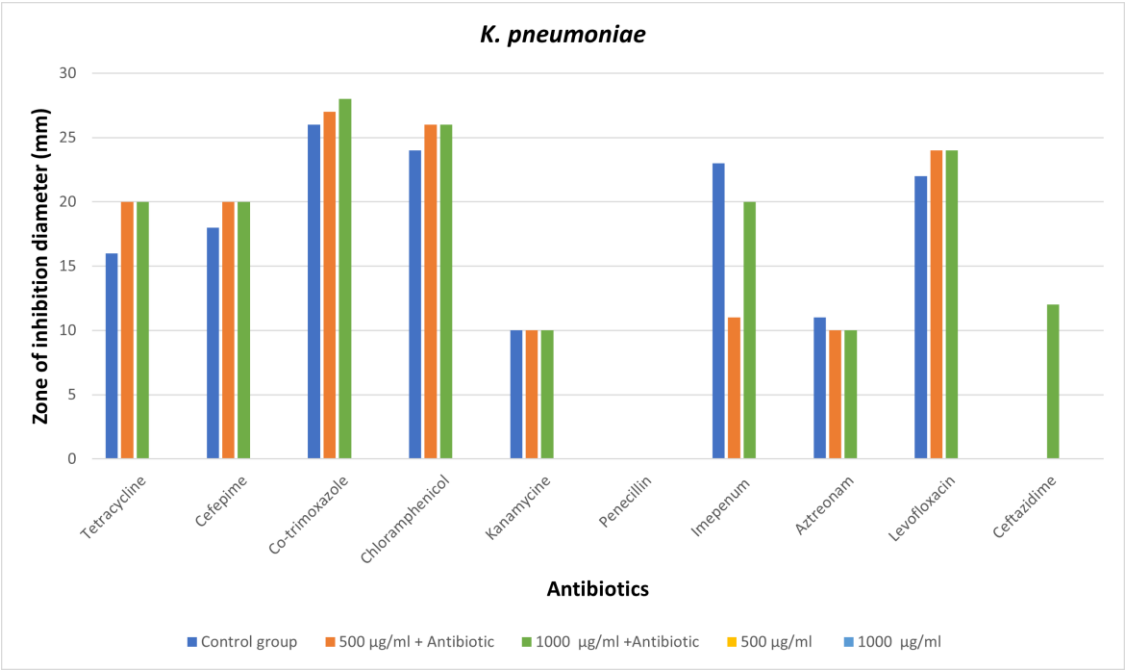


Figure 12: Antibiotic susceptibility pattern of 4 different bacteria using antibiotics as control groups and Fe_3O_4 NPs conjugated antibiotics as the experimental group.

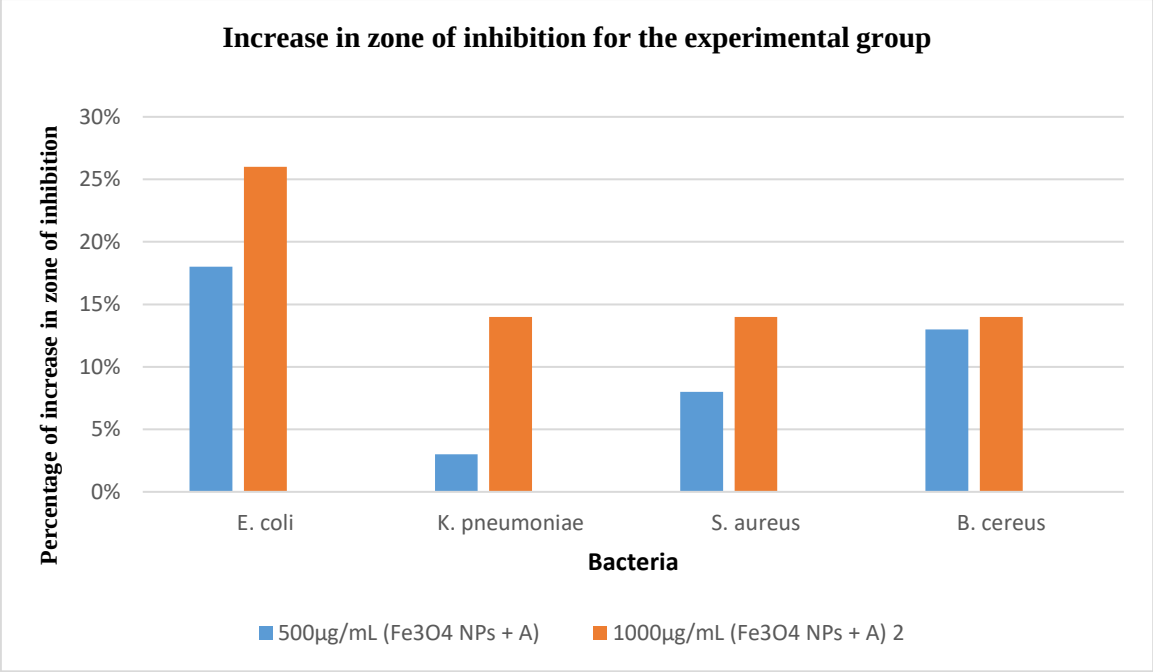


Figure 13: Percentage of increased zone of inhibition for each bacterium.

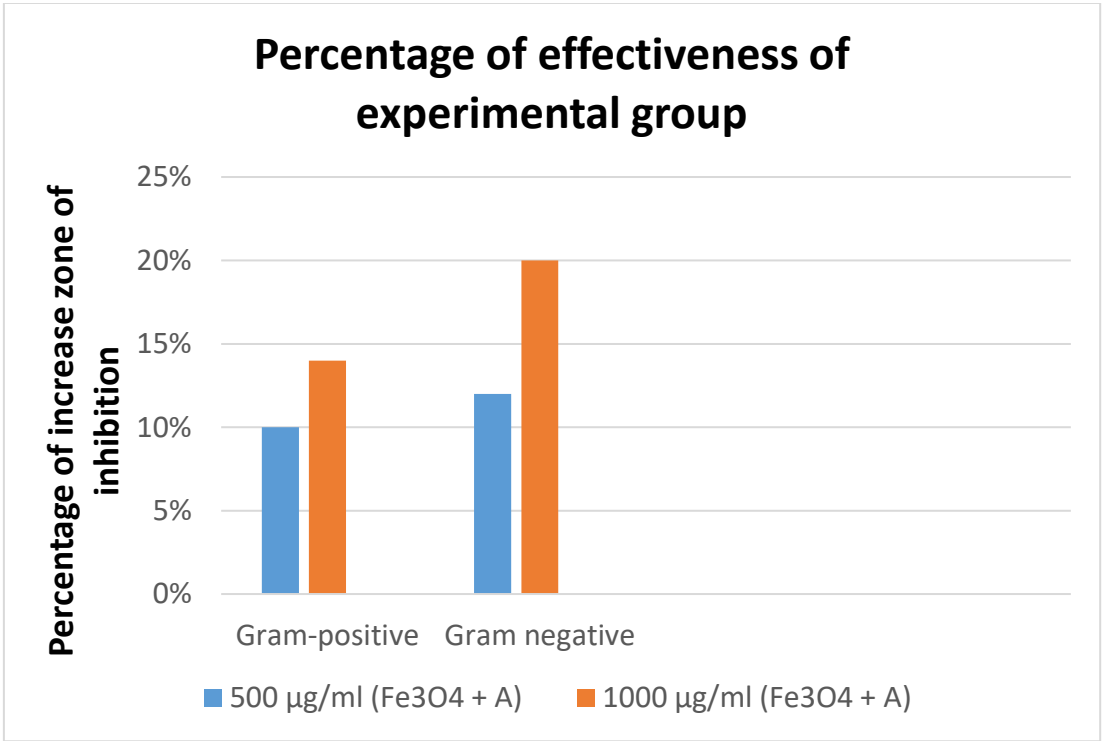


Figure 14: Percentage of increase in the zone of inhibition on gram-positive and gram-negative bacteria

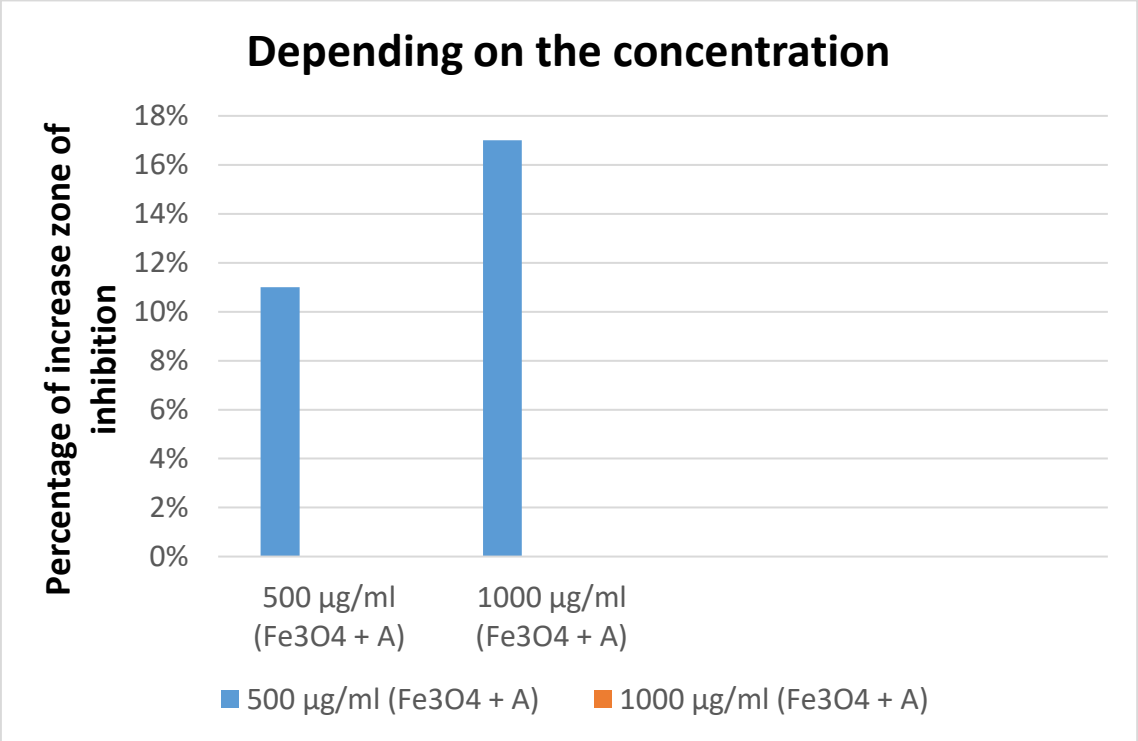


Figure 15: Depending on the concentration of experimental group percentage of zone of inhibition

The Antibiotic susceptibility was done using 10 different class of antibiotics which were conjugated with bare iron-oxide nanoparticles. For each of the bacteria, the experimental group showed better results than the control group. On an average, if the zone of inhibition in diameter (mm) of the control group is compared with the two experimental group (Fe_3O_4 NPs + A). Then 11% increase in zone of inhibition would be observed for the 1st experimental group and 17% increase in zone of inhibition regarding the 2nd experimental group. Depending on the concentration of the experimental group, the experimental group where 500µg/mL concentrated (Fe_3O_4 NPs + A) was used gave an average of 11% increase in the zone of inhibition. Then again, for the second experimental group where 1000µg/mL concentrated (Fe_3O_4 NPs + A) was used gave an average of 17% increase in the zone of inhibition compared to the antibiotic control group.

3.4 Hemolysis assay

In case of biomedical or clinical application biocompatibility is a huge issue. Biocompatible means if the compound is compatible with cell biology. Fe_3O_4 NPs is greatly known for its biocompatibility as it has lower toxic properties. Generally, red blood cell (RBC) is used test biocompatibility as every compound in a body is transferred through blood therefore, RBC is one of the potential candidates to test compatibility of Fe_3O_4 NPs in the in-vitro system. Regarding hemocompatibility test of Fe_3O_4 NPs the highest concentration 1500µg/mL and lowest concentration was 500 µg/mL. In total 6 different concentration (500µg/mL, 600 µg/mL,700 µg/mL,900 µg/mL,1000 µg/mL and 1500 µg/mL) was used to conduct the hemolysis assay. In the first 5 concentration there was no change of optical density but in the for the last concentration there a slight change in OD which could be neglectable. But if the OD with 15001000 µg/mL was compared with the positive control there was 2.85% hemolysis observed which is very low. To conclude, we could say that even at higher concentration the Fe_3O_4 NPs shows low hemolysis effects and higher hemocompatibility. Therefore, iron-oxide nanoparticles synthesized in this method are biocompatible and could be used in biomedical sector.

Chapter 4

Discussion

Antibiotic resistance of infectious disease-causing pathogenic bacteria has become a threat to the public health as all age mortality rate has increased due to this global issue. Infections caused by MDR pathogenic bacteria are getting very difficult to deal with. As there are scarcity of new class of antibiotics that could evade the resistance mechanism. So, effective medicines or an alternative treatment should be developed to secure the public health.

This study revealed a cost-efficient way to produce Fe_3O_4 NPs along with its antibacterial activity; the synergistic effect of Fe_3O_4 NPs with different class of antibiotics against 2 gram-positive and 2 gram-negative bacteria in the in-vitro system and hemolytic activity of Fe_3O_4 NPs.

Recently, in a study Abdulsada *et al.* (2022) reported the preparation of iron oxide nanoparticles was synthesized by co-precipitation methods under an inert environment and investigated it antibacterial activity. Furthermore, the Fe_3O_4 NPs was coated with polyethylene glycol and an antibiotic name gentamycin. The final product was used against 2 gram-negative and one 1 gram-positive bacteria. The final product Fe_3O_4 NPs+PEG+Gen showed greater results then bare particles and separate conjugation. (Abdulsada et al. 2022) Again, in another research by Sharaf *et al.* (2022) reported that Fe_3O_4 NPs was synthesized by using co-precipitation the rhamnolipid, gallic acid and coumaric acid was coated to observe its antibacterial activity (Sharaf et al. 2022). Then, in an article by Anush *et al. (2019)* reported the combine therapy of antibiotics and iron oxide. Here in the combine therapy antibiotic IV solutions were combined with iron oxide nanoparticles against *E. coli* bacteria. (Anush et al. 2019)

But this study revealed a cost-efficient way to produce Fe_3O_4 NPs along with its antibacterial activity; the synergistic effect of Fe_3O_4 NPs with different class of antibiotics against 2 gram-positive and 2 gram-negative bacteria in the in-vitro system and hemolytic activity of Fe_3O_4 NPs. Here, no coating material or IV solutions were used to observe the combine therapy. Rather, pre-impregnate antibiotic disc was used to conjugate the Fe_3O_4 NPs. Also, ten different classes of antibiotic discs were taken and infused with the NPs. Moreover, the experimental group was used against two gram-positive and two gram-negative bacteria.

In this study, Fe_3O_4 NPs was synthesized by co-precipitation method. But the challenge of this experimental work was to develop cost-efficient method to synthesize Fe_3O_4 NPs. Generally, to synthesize Fe_3O_4 NPs, iron salts, alkaline solution and continuous supply of nitrogen gas or inert gas is required to prevent oxidization. Also, a non-magnetic stirrer that could tolerate up to 80^o C. Due to the shortage of resources N_2 gas and non-magnetic stirrer was available. Therefore, a non-magnetic stirrer was made using Teflon propeller, DC motor, regulator and continuous power supplier. The regulator was basically used to control the rpm of the device as 1000 rpm was required to conduct the reaction. Then again, because of the non-availability of N_2 gas, other gas like O_2 was reacting during the synthesis of Fe_3O_4 NPs. So, as an alternative polar reducing agent (70% Ethanol) was used to wash the iron-oxide nanoparticles that would help prevent the oxidization of NPs. Now, ethanol being a polar solvent would form a layer around the nanoparticles which would create a steric barrier, this barrier limits its exposure to atmospheric oxygen.(Kharisov et al. 2014) Hence, oxidization can be prevented. But it also helps in stabilizing Fe_3O_4 NPs. Iron oxide NPs have surface charged group due to -OH groups, now ethanol is polar reagent could form a dielectric layer that would screen the electrostatic interaction between the particles and stop them from aggregating. Moreover, it could also forma dipole-dipole interaction. Thus, ethanol could help prevent oxidization as well as stabilizes the Fe_3O_4 NPs. Even after, 5 months the iron oxide NPs was stable.

In this study, antibiotic susceptibility test was done over four different types of bacteria with ten different classes of antibiotics, two different concentration of Fe_3O_4 NPs and two different concentrations of NPs conjugated with ten different classes of antibiotics. Here, the antibiotics and two different concentrations of Fe_3O_4 conjugated with blank discs are the 3 control groups. Again, the two different concentrations of Fe_3O_4 NPs conjugated with 10 different classes of antibiotics are the experimental group. The goal was to observe the zone of inhibition of the control groups and experimental groups. The two experimental group showed greater results than the control group. Now, the antibiotics have their own mechanism to inhibit bacteria like DNA, protein damage, inhibition of enzyme, cell membrane rupture etc. but the Fe_3O_4 NPs also has a unique mechanism called reactive oxygen species that could induce oxidative stress and lead to cell rupture, DNA damage and eventually cell death. Again, the Fe_3O_4 NPs exhibits non-specific binding therefore, getting resistance against Fe_3O_4 NPs is quite difficult. So, as antibiotics and Fe_3O_4 NPs have different inhibition mechanism therefore, when NPs was conjugated into the antibiotic disc it showed a synergistic effect which resulted in bigger zone of inhibition than the control groups.

For *E. coli* the diameter of zone of inhibition for Ceftazidime (CAZ) and levofloxacin (LE) was 20.33mm and 25.67mm. But when (Fe_3O_4 NPs + LE) was applied, it showed 16.86% enhanced zone and the diameter was 30mm for both the experimental groups. Then again, for (Fe_3O_4 NPs +CAZ) a 67.24% increase in zone was detected with a diameter of 34mm. To summarize, the antibiotic control group for *E. coli* showed an average zone 22.767 mm. The two experimental groups of 500µg/mL (Fe_3O_4 NPs + A) and 1000 µg/mL (Fe_3O_4 NPs + A) showed an average inhibition zone of 26.833mm and 28.2mm, respectively. So, for 500µg/mL (Fe_3O_4 NPs + A) and 1000µg/mL (Fe_3O_4 NPs + A) 18% and 26% increased zone of inhibition was observed.

For *K. pneumoniae,* all the experimental groups showed an increase in the zone of inhibition by 3-5mm except for imipenem which belongs to carbapenem group of antibiotics. For the experimental groups it showed zone of 20mm and for the control group the zone of inhibition of was 23mm. So, Fe_3O_4 NPS conjugated with imipenem showed an antagonistic effect rather than a Synergistics effect. But on an average, 500µg/mL (Fe_3O_4 NPs + A) and 1000µg/mL (Fe_3O_4 NPs + A) exhibited 3% and 14% increased zone of inhibition.

For *S. aureus* all the experimental groups showed an increase in the zone of inhibition by 3-7mm except for imipenem. On the contrary, the control group with 1000µg/mL concentrated bare particles showed a slight zone of inhibition, the diameter was 13mm. On an average, the two experimental group, 500µg/mL (Fe_3O_4 NPs + A) and 1000µg/mL (Fe_3O_4 NPs + A) respectively displayed 8% and 14% increased zone of inhibition when compared with the control group of antibiotics.

For *B. cereus* the average zone for all the antibiotic control was 21.8mm but when Fe_3O_4 NPs was conjugated with antibiotics they displayed an average inhibition zone range from 24-25mm. In this case, the imipenem antibiotics conjugated with the NPs showed greater zone compared to control group. The zone diameter increased 2mm for all the experimental group. Here, for the two experimental group, 500µg/mL (Fe_3O_4 NPs + A) displayed 13% and 1000µg/mL (Fe_3O_4 NPs + A) showed 14% increased zone of inhibition when compared with the control group of antibiotics.

In short, the first experimental group, consisting of a concentration of 500µg/mL (Fe_3O_4 NPs + A) displayed 11% increased zone of inhibition for all the bacteria and the second one with 100µg/mL (Fe_3O_4 NPs + A) displayed 17% increased zone of inhibition for all the bacteria.

By conducting the antibiotic susceptibility test over pathogenic bacteria, it was observed that iron-oxide NPs conjugated with antibiotic discs at different concentrations enhance the

antibacterial activity of the antibiotics. It represents a synergistic effect against those bacteria. But to determine whether the Fe_3O_4 NPs are compatible with any biomolecules or to ensure their biocompatibility as a medicine in the in vivo system, their hemolytic activity must be checked. Therefore, testing the cytotoxic effects of Fe_3O_4 NPs with human RBC was the most efficient way. As any medicine either oral or intravenous first gets into the blood stream and then effects on the designated destination. So, route of any medicine is blood. Therefore, a blood cytotoxic test was conducted to check the hemocompatibility of Fe_3O_4 NPs. The hemolytic activity was checked by using different concentration. Among all the concentration, the highest concentration 1500 µg/mL showed 2.85% hemolysis. So, the ultimate result showed that Fe_3O_4 NPs is highly hemocompatible.

Though the test were conducted in the in-vitro but with the given results combine therapy would be reasonable proposal in this post-antibiotic era. As the Fe_3O_4 NPs with its own non-specific antibacterial mechanism could enhance the antibacterial activity of antibiotics. The synergistic effect of nanoparticles with antibiotics could turn down global crisis of antibacterial resistant. Furthermore, the resistant antibiotics could again be functional with the help of Fe_3O_4 NPs

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