COMPARATIVE STUDY OF BACTERICIDAL ACTIVITIES OF ANTIBIOTICS AND PLANT-BASED NANOPARTICLES ON FISH PATHOGENS

BY

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APRIL, 2025

CERTIFICATION

This is to certify that this research work "**Comparative Study of Bactericidal Activities of Antibiotics and Plant-Based Nanoparticles on Fish Pathogens**" was carried out by **Asonye, Chinemerem Miracle, Reg. Number (20204256048)** in partial fulfillment of the requirements for the award of the degree of a Master of Science (M.Sc.) in Environmental Microbiology, in the Department of Microbiology of the Federal University of Technology, Owerri.

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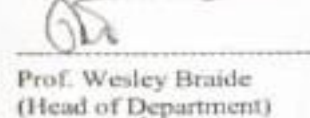
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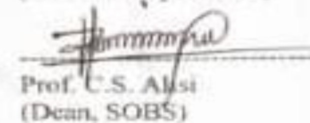
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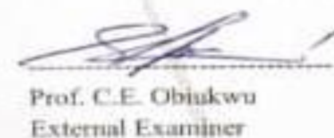
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DEDICATION

This research work is dedicated to God Almighty, for His abundant grace and strength all through the period of this programme.

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ABSTRACT

Bacterial fish pathogens cause diseases which result in a considerable economic impact on the aquaculture industry, necessitating the use of antibiotics for their control. However, intensive and indiscriminate use of antibiotics has led to increased occurrence of drug resistance in pathogenic bacteria as well as normal flora. This present study was conducted to determine the most prevalent bacterial fish pathogens, synthesize and characterize plant-based Silver and Zinc oxide nanoparticles extracted from *Azadirachta indica* (Neem leaves) and *Hibiscus rosa sinensis* (Hibiscus leaves), assess and compare the antibacterial activities of antibiotics and the plant-based nanoparticles on the most prevalent bacterial fish pathogens. The most prevalent bacterial fish pathogens namely, *Salmonella* sp., *Shigella* sp., *Enterobacter* sp., *Vibrio* sp. and *Bacillus* sp. were bacteriologically isolated from the liver, gills and intestines of fish samples gotten from three different fisheries in and around Owerri Metropolis which include Johnny Fish Farm, Umuodu Mbieri, Mbaitoli L.G.A., Chucky Fish Farm, 18 Norbert Nworieji Lane, New Owerri and an unnamed fish farm in Ohaji/Egbema, Imo State. The synthesized nanoparticles were characterized using techniques such as Fourier Transform Infra-Red (FTIR) Analysis, Scanning Electron Microscopy, UV-Visible Spectroscopy and Energy Dispersive X-Ray Spectroscopy. The isolated bacteria were identified by their colonial morphologies, Gram-Staining and different Biochemical reactions. Bacteria were mostly prevalent in the gills of the various fish samples. The mean heterotrophic bacteria had counts ranging from 1.15×10^6 to 970×10^6cfu/g while the mean *Vibrio* sp. had counts ranging from 0.087×10^6 to 5.42×10^6cfu/g. Disc diffusion method was employed to determine and compare the antibacterial activities of both the antibiotics and the different plant-based nanoparticles. In this study, it was observed that all the bacterial fish pathogens were resistant to most of the antibiotics (especially Tetracycline and Chloramphenicol) but were all susceptible to Gentamicin. It was also observed that the silver and zinc oxide nanoparticles synthesized from the plant leaves at different sizes and concentrations had inhibitory effects against the bacterial fish pathogens, mostly with the ones of higher concentrations having more inhibitory ability than the ones of lower concentrations although their efficacy did not surpass that of gentamicin which served as the control. The analysis has shown that farmed fish harbor potentially pathogenic bacteria which are resistant to some common antibiotics. This study also highlights the important benefits of using plant-based nanoparticles as potential antibacterial agents against bacterial fish pathogens to combat antibiotic resistance in aquaculture and maintain sustainable and hygienic aquatic environments.

Keywords: Bacterial fish pathogens, Antibiotics, Antibiotic resistance, Neem leaves, Hibiscus leaves, Silver nanoparticles, Zinc oxide nanoparticles, Antibacterial activities.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Food security has benefited greatly from aquaculture's contribution in recent decades to meeting the enormous need for animal protein. However, illness prevalence and environmental contamination are thought to be key issues facing the industry. Natural or artificial materials called antibiotics are used as antibacterial medications to either eradicate or stop the growth and spread of bacterial infections. In aquaculture, antibiotics are also used to promote fish growth. A variety of antibiotic classes are utilized in aquaculture, such as tetracyclines, aminoglycosides, quinolones, sulfonamides, macrolides, chloramphenicols, β-lactams, lincosamides and polymyxins (Sun, Chen, & Pan, 2020). Antibiotic resistance and indiscriminate use of antibiotics is as a result of unregulated use of antibiotics in the treatment of fish infections in many poor nations (Budiati, Rusul, & Wan-Abdullah, 2013).

Because of their extensive usage in humans and their enduring presence in the environment, antibiotics have been recognized as one of the most significant categories of emerging pollutants (Sodhi, Kumar, & Balan, 2021; Okoye, Okeke, Okoye, Echude, Andong, Chukwudozie, Okoye & Ezeonyejiaku, 2022a). Aquatic environments are contaminated by antibiotic-resistant microorganisms that come from humans and animals. These bacteria have the ability to transfer their genes, which contain resistance genes, to water-based microorganisms (Kraemer, Ramachadran & Perron, 2019). In order to properly address these difficulties, new technological avenues have been paved.

Among them, nanotechnology stands out as a cutting-edge instrument with a wide range of applications, including the preservation of aquaculture. It can offer novel medication management solutions, such as the release of vaccines, and so guarantee the civilized defense of farmed fish against pathogens that cause sickness. Because of their antibacterial action, metallic nanoparticles may one day replace some traditional antibiotics. The aim of this research was to create biologically synthesized silver and zinc oxide nanoparticles using leaf extracts from *Azadirachta indica* and *Hibiscus rosa sinensis*, compare their antibacterial efficaciousness against common fish pathogens to that of antibiotics, and determine their safety. The antibacterial activity of silver and zinc oxide nanoparticles against *Shigella* sp., *Salmonella* sp., *Bacillus subtilis*, *Vibrio* sp., and *Enterobacter* was evaluated, and the lowest inhibitory concentration was found.

1.2 Problem Statement

Antibacterial resistance of fish pathogens is a global concern and the quest to combat these phenomena is still ongoing. Antibiotics have been identified as one of the most important types of emerging contaminants due to their widespread use in humans; as well as long-term persistence in the environment (Sodhi et al., 2021; Okoye et al., 2022a). In searching for alternatives, nanoparticles have been found to be promising in combating antibacterial resistance mostly the plant-based nanoparticles.

1.3 Justification of the Study

This study hopes to compare the use of antibiotics and plant-based nanoparticles in bactericidal activities against fish pathogens. In this work, we hope to produce plant-based nanoparticles and show that plant extracts can have bactericidal effects on fish pathogens with almost the same efficacy as antibiotics and a lower chance development of resistance.

1.4 Aim of the Study

The aim of this study was to synthesize green metallic nanoparticles, determine and compare their antimicrobial efficacy with that of antibiotics on pathogenic bacterial isolates from fish.

1.5 Objectives of the Study

- To get the plant extracts from Hibiscus and Neem leaves.
- To synthesize the plant-based metallic nanoparticles (NPs); Silver and Zinc oxide Nanoparticles.
- To characterize the nanoparticles using UV-Visible Spectroscopy, Fourier Transform Infra-Red Analysis and Scanning Electron Microscopy.
- To discover and identify the most prevalent bacterial fish pathogens present in the organs of the diseased fish samples.
- To determine the antimicrobial activity of the nanoparticles against bacterial fish pathogens.
- To compare the bactericidal efficacy of antibiotics with that of plant-based silver and zinc-oxide nanoparticles synthesized from Hibiscus and Neem leaves on fish pathogens.

1.6 Significance of Study

Plant extract is easy to get and safe to handle where the reduction of metal is rapid and the procedure itself requires no specific conditions. This method of nanoparticles synthesis appear to be reproducible, environmentally friendly and is highly stable (Krishnaraj, Jagan, Rajasekar, Selvakumar, Kalaichelvan & Mohan, 2010). Nanoparticles exhibit specific features such as the large surface area to mass ratio, ultra-small size and high reactivity which show a lot of modified properties (Khan, Saeed & Idrees, 2019) and has great potential benefits to human health, environment and industries.

1.7 Scope of the Study

This study focuses on the green synthesis of silver and zinc oxide nanoparticles from *Azadirachta indica* (Neem leaves) and *Hibiscus rosa sinesis* (Hibiscus leaves) gotten from Federal University of Technology, Owerri premises. The characterization of the synthesized nanoparticles was done at Ahmadu Bello University, Zaria. Following the collection of diseased catfish samples from three different fisheries in and around Owerri Metropolis, isolation of the five most prevalent bacterial fish pathogens and comparing the bactericidal activity of the synthesized nanoparticles with that of conventionally used antibiotics by carrying out antibiotic susceptibility tests, Minimum Inhibitory Concentration tests and Minimum Bactericidal Concentration tests.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antibiotics Use and Residues in Aquaculture and Aquatic Ecosystems

Antibiotics are synthetic or natural substances that are used as antibacterial drugs to kill or inhibit the growth and spread of bacterial pathogens. They are used in aquaculture (Lozano, Diaz, Munoz & Riquelme, 2017; Schar, Klein, & Laxminarayan, 2020), as well as animal husbandry (Patel, Kumar, & Kishor, 2019; Okoye, Addey, Oderinde, Okoro, Uwamungu, Ikechukwu, Okeke, Ejeromedoghene & Odii, 2022b) prophylactically, therapeutically or metaphylactically. Antibiotics are also used to boost growth in aquaculture. Different classes of antibiotics that are used in aquaculture include aminoglycosides, quinolones, sulfonamides, tetracyclines, macrolides, chloramphenicols, β-lactams, lincosamides, nitrofurans and polymyxins (Sun et al., 2020).

Many underdeveloped countries have completely unregulated antibiotic usage in aquaculture, which encourages indiscriminate antibiotic use (Budiati et al., 2013). Even in those cases where laws do exist, strict adherence is usually non-existent. The regulatory bodies responsible for overseeing animal husbandry and aquaculture in many developed nations are tasked with approving and controlling the use of antibiotics in aquaculture, as well as making sure that strict adherence is maintained. It is impossible to ascertain the current levels of antimicrobial consumption in aquaculture worldwide due to the wide variations in nations' regulatory frameworks, amount of antibiotics used, and farming practices (Chen, Lin, & Fang, 2018).

Without determining the precise causes of fish infection, fish producers in a number of countries unintentionally utilize antibiotics (Rahman, Nielsen, & Khan, 2021). Combining antibiotics with aquaculture feed is the most popular way to provide antibiotics in this setting. Liu, Steele, & Meng (2017) reported that injectables and pond sprinkling are two other methods of administering

antibiotics. Aside from injection, every technique of administering antibiotics has an immediate effect on aquatic life and the aquatic ecosystem. Research has shown that fish cannot efficiently metabolize antibiotics (Sun et al., 2020), and it is anticipated that 75% of antibiotics given to fish are reabsorbed into the pond water (Burridge, Weis, & Cabello, 2010). Antibiotic residues can have detrimental effects on human and environmental health when they accumulate in pond water, sediments, animal tissues and aquatic products, as multiple studies have shown (Lulijwa & Kajobe, 2018; Chen et al., 2018). Antibiotics also accumulate in the aquaculture environment. Antibiotic residues in cultured aquatic goods have the potential to negatively disrupt the complex microbiota that resides in the human gastrointestinal tract and to be systematically hazardous to consumers (Monteiro & Andrade, 2018). Remaining chloramphenicol increases the risk of cancer and, at lesser quantities aplastic anemia. Additional adverse effects of leftover antibiotics include nephropathy and mutagenicity caused by gentamicin, as well as immunopathology and carcinogenic effects from sulfamethazine, oxytetracycline and furazolidone (Beyene, 2016).

Residual antibiotic-containing aquaculture effluents are frequently dumped into rivers or other bodies of water, where they end up as manure. These locations may be the source of leftover antibiotics that enter the food chain and have unfavorable effects. The variety of phytoplankton and zooplankton can also be impacted by residual antibiotics (Ferreira, Hawkins, & Bricker, 2007; Song, Zhang, & Fan, 2016). The disruption of phytoplankton chlorophyll production (Song et al., 2016) and zooplankton early developmental stages (Park and Kwak 2018) has also been linked to these antibiotics which include sulfonamides, tetracyclines and quinolones. This disturbance might then cause changes in the food chain, which would have an impact on the ecosystem as a whole. The remaining levels of various antibiotics in an aquaculture environment, aquatic animals and their products are influenced by a number of factors, including exposure concentration and

physiochemical characteristics. According to Tony et al., (2017), antibiotics that have poor water solubility, such as enrofloxacin, norfloxacin and ofloxacin, are considered to be “pseudopersistent” emerging pollutants because they tend to accumulate more in aquatic habitats and aquatic products.

Many studies have been conducted in an attempt to identify biological, chemical and physical ways that are efficient for eliminating leftover antibiotics from wastewater effluents and natural waters because residual antibiotics in aquatic environments and ecosystems are widely acknowledged as posing a growing threat to human health and the environment. Nevertheless, the most of these studies have concentrated on eliminating antibiotics from pharmaceutical companies, livestock farms and hospital effluent; very little research has specifically addressed treating aquaculture wastewater to eliminate leftover antibiotics (Senarathna, Abeysooriya, Vithushana, & Dissanayake, 2021). While aquaculture wastewater can be treated using the same methods, field-specific studies or research are still essential. Governments, relevant organizations and stakeholders must take a worldwide strategy to addressing the complicated issue of antibiotic residues in aquatic habitats, which is a growing concern due to the indiscriminate use of antibiotics in aquaculture. To lessen this issue, it is also crucial to put into place sustainable and efficient techniques for keeping an eye on the amount of antibiotics in the environment.

2.2 Toxicological Effects of Antibiotics in Aquaculture and Aquatic Organisms.

Antibiotics from various classes are used to treat a variety of illnesses, including trichomoniasis, dermatophytosis, urinary tract infections, HIV and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Becker, Lecerf, &, Claereboudt, 2020; Girijan, Paul, VJ, & Pillai, 2020; Trivedi, Mohan, & Byrareddy, 2020; Das 2021). However, persistent use of these medicines has both short and long-term consequences in humans, notably in the gut, which is home to a varied spectrum of bacteria, viruses, fungus and archae species.

Table 2.1 Toxicological Effect of Antibiotics on Different Biomarkers

Class of Antibiotics	Types	Effect	References
Cephalosporins	Ceftriaxone	Depletion of *Enterobacteriaceae*. Excessive growth of *Candida* and Enterococci species. *Clostridium difcile* infection.	Sullivan et al., (2001)
Nucleoside reverse transcriptase inhibitors (NRTIs)	Zidovudine, lamivudine	Inhibition of reverse transcriptase	Adriaenssens et al., (2011)
Synthetic nucleoside analogues	Acyclovir	Inhibition of DNA polymerase by virus	Adriaenssens et al., (2011) Bule et al., (2019)
Antimicrobials	Nitrofurantion	Inhibition of protein synthesis in DNA and RNA of bacterial cell wall.	Stewardson et al., (2015)
	B-Lactams	Increase in microbial load of fecal sample. Impairment of carbohydrate metabolism.	Hernandez et al., (2013) Panda et al., (2014)
Lincosamides	Clindamycin	Disruption of microbial in gut. *Clostridum difcile* infection (CDI)	Rashid et al., (2015)
Glycopeptides	Vancomycin	Reduction in species like Enterococcus, Clostridia, Bacteroides and Bafidobacteia. Enhances the growth of Lactobacillacaea, Enterobacteriaceae and Enterococci	Yin et al., (2015) Reijinders et al., (2016)
Macrolides	Erythromycin, azithromycin and clarithromycin	Reduction of Firmicutes in human gut Shift in the intestinal flora composition. Affects the metabolism of human gut	Korpela et al., (2016)
Glycopeptides-carbapenem	Vancomycin-Imipenem	High sugars and arabinitol in faeces.	Choo et al., (2017)

Aminoglycosides, glycopeptides, and carbapenem	Gentamicin, vancomycin, and meropenem	Increase in Enterobacteriaceae. Reduction in Bifidobacterium as well in species that produce butyrate.	Palleja et al., (2018)
Penicillins	Penicillin G	Inhibition of the synthesis of cell wall	Grenni et al., (2018)
Aminoglycosides and tetracycline	Gentamicin and oxytetracycline	Inhibition of synthesis of protein	Grenni et al., (2018)
Nitroimidazole	Metronidazole	Inhibition of DNA synthesis	Grenni et al., (2018)
Antihelminthes	Albenzole, praziquantel	Inhibition of microtubules polymerization. Enhances the permeability of parasite to calcium.	Grenni et al., (2018)
Quinolones	Ciprofloxacin	Loss of microbial diversity in human gut.	Grenni et al., (2018)
		Inhibition in nucleic acid synthesis which act on DNA gyrase.	Lulijwa et al., (2020)
Sulfonamides	Sulfamethoxazole	Interference with the synthesis of folic acid.	Grenni et al., (2018) Lulijwa et al., (2020)
Azole antifungal	Ketoconazole	Inhibition of the synthesis of ergosterol.	Sallach et al., (2021)
Antifungals	Amphotericin B, griseofulvin	Alters cytoplasmic membrane. Microtubule alteration	Sallach et al., (2021)
Antimicfrobials, macrolides, and protein-pump inhibitors	Clarithromycin, metronidazole, and omepreazole	Perturbation of microbiota in gut when used in treating infection caused by *Helicobacter pylori*.	Yang et al., (2021)
Penicillin , beta-lactamase inhibitors	Amoxicillin+clavulanic acid	Aerobic gram-positive cocci were wiped totally. High resistance of enterobacteriaceae.	Yang et al., (2021)
Polymyxin, penicillin	Colistin+amoxilin	Alteration in gut microbiota.	Li et al., (2021)
		Induction of antibiotics resistome	Yang et al., (2021)

2.3 Antibiotic-Resistant Bacteria

As a result of the growing need for antibiotics in treating various diseases, various antibiotic classes have been developed. In contrast, unethical and reckless antibiotic use has resulted in the development of resistant bacterial, fungal and viral strains (Zaman, Hussain, & Nye, 2017). In the past, production of novel antibiotics was directly linked to the development of resistant strains, but now the conventional method in disease-fighting is to modify existing antibiotics to combat evolving and re-evolving pathogenic resistance worldwide (Zaman et al., 2017).

The ability of a medication to effectively block the growth of microorganisms (viruses, fungi and bacteria) is known as antibiotic susceptibility. Antibiotics are always effective against these microorganisms, but in order to affect them, an antibiotic dosage must be larger than usual when the microorganisms exhibit resistance or low sensitivity (Zaman et al., 2017). A wide range of antibiotic-resistant microbial genes are found in human guts. It has been discovered that the human gut has genes resistant to various types of antibiotics, including tetracycline, cephalosporin, bacitracin and vancomycin (Field & Hershberg, 2015).

Human immune system function, body metabolism and overall health are all regulated by the microbiome found in the intestines of people (Chauhan 2017). Formulations containing antibiotics are used to treat and prevent microbial illnesses. Targeted microorganisms (viruses, fungi and bacteria) have grown immune to them as a result of their regular use (Yadav 2022). Gene mutation causes these bacteria to develop antibiotic resistance or immunity (Laxminarayan & Brown, 2001). Table 2.2 illustrates the strategies these bacteria use to withstand antibiotics as well as selective pressure.

Table 2.2: Mechanism of Antibiotic Resistance

Antibiotics	Resistance mechanism	References
Tigecycline, minocycline	Alteration of target Production of efflux Mono oxygenation	Dean et al., (2003) Zaman et al., (2017)
Synercid	Acetylation Carbon-Oxygen lyase Production of efflux pump Alteration of target	Werner et al., (2002) Zaman et al., (2017)
Penicillins, Monobacctams, penems, and Cephalosporins	Hydrolysis Efflux pump production Alteration of target	Zaman et al., (2017)
Cephalosporins	AmpC beta-lactamase ESBL$_s$	Zaman et al., (2017)
Clindamycin	Nucleotidylation production of efflux pump alteration target production	Zaman et al., (2017)
Linezolid	Pproduction of efflux pump alteration target production	Zaman et al., (2017)
Ciprofloxacin	Production of efflux pump acetylation alteration target production	Zaman et al., (2017)
Chloramphenicol	Acetylation production of efflux pump alteration target production	Zaman et al., (2017)
Streptomycin, spectinomycin, and gentamicin	Acetylation phosphorylation nucleotidylation efflux pump alteration target production	Zaman et al., (2017)
Sulfamethoxazole	Acetylation of target production of efflux pump over production of dihydropteroate synthase (DHFR) mutation of dihydrofolate (DHPS)	Zaman et al., (2017)
Teicoplanin, vancomycin	Reprogramming peptidoglycan biosynthesis	Zaman et al., (2017)
Daptomycim	Alteration on target	Zaman et al., (2017)
Erythromycin, azithromycin	Hydrolysis Phosphorylation Glycosylation Production of efflux pump Alteration on target Ribosomal methylation of binding sites	Sedaghat et al., (2017) Zaman et al., (2017)
Rifampin	Production of efflux ADP-ribosylation mutation in RNA polymerase gen alteration of target enzymatic degradation	Kakoullis et al., (2021)
Aztreonam	Extended – spectrum-B- lactamases (ESBLs)	Kakoullis et al., (2021)

On the human gut flora, these microorganisms' resistance does not come without consequences, either. Microbes' resistance to antibiotics has led to high mortality rates and illnesses that pose a hazard to human health (Zaman et al., 2017). For example, antibiotic resistance to bacterial diseases kills over 25,000 people in Europe, according to a 2011 study by Freire-Moran, Aronsson, & Manz.

The multidrug-resistant bacteria *Burkholderia cenocepacia*, *Staphylococcus aureus*, *Haemophilus influenza* and *Pseudomonas aeruginosa* cause the respiratory illness cystic fibrosis (Carmody, Gill, & Summer, 2010). The severity of pneumonia is caused by bacteria that are resistant to carbapenem class of antibiotics, including *Streptococcus pneumoniae, Klebsiella* sp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenza* and *Acinetobacter baumanii* (Singla, Harjai, Katare, & Chhibber, 2015; Martin, Cao, & Brisse, 2016). It has been found that bacteria resistant to rifampicin and isoniazid, which are used to treat tuberculosis patients, impede the effectiveness of tuberculosis treatment in those people (Yadav, 2022). Enteric infections are more likely when *Escherichia coli* is resistant to quinolone antibiotics, as reported by Chattaway, Aboderin, & Fashae, (2016). Antibiotic-resistant bacteria are the cause of a number of diseases including cholera, shigellosis, listeriosis, salmonellosis and gastrointestinal inflammation (Abdulamir, Jassim & Abu Bakar, 2014; de Vasconcelos, Hofer, Vallim & de Castro Almeida, 2016).

2.4 Antibiotic Resistance in Aquatic Organisms.

Microorganisms, plants, vertebrates and invertebrates are the four main groups of aquatic organisms. Each of these groupings is made up of complex species and subspecies that interact with one another at various trophic levels in the environment. The majority of wastewater treatment facilities are ill-equipped to remove antibiotics, which are considered emerging

contaminants in aquatic environments and pose a serious threat (Pal, Bengtsson-Palme, Kristiansson & Larsson, 2015; Gogoi, Mazumder & Tyagi, 2018; Michael-Kordatou, Karaolia & Fatta-Kassinos, 2018; Rodríguez-Molina, Mang & Schmitt, 2019; Liu, Tan & Zhang, 2021). According to recent studies, a large number of antibiotics are currently entering aquatic habitats, and little is known about how these drugs interact with aquatic life (Gogoi et al., 2018; Guha Roy 2019; Gomes, Maillard, & Simoes, 2020; Liu et al., 2021). As a result, it is critical to continue studying and expanding our understanding of the functions antibiotics perform in aquatic environments and their interactions with aquatic life.

In every setting, antibiotic resistance is seen as a major public health risk (Larsson and Flach 2021). Antimicrobial Resistance Genes (ARGs) have become more prevalent in aquatic environments due to the ongoing buildup of antibiotics in these settings (Richardson and Kimura 2019; Saima, Fiaz & Zafar, 2020; Liu et al., 2021). It has been observed that temporal and spatial distribution greatly influences the type of ARGs that are circulating in a certain area (Liu et al., 2021; Valdés, Santos & Rodriguez Castro, 2021). For instance, a study on different water bodies in China found that the kind of antibiotics present in a particular body of water was correlated with the industry infrastructures that were readily available locally, the way pharmaceutical companies disposed of their antibiotics and the kind of antibiotics that were utilized in the livestock industry in that particular area (Liu et al., 2021). The potential pathways that ARGs could use to interact with aquatic creatures are discussed in this section and are illustrated in Figure 2.1. Furthermore, domains where nanotechnology could be employed to disturb these exchanges are examined. Despite these solutions, it’s crucial to remember that research is still needed to thoroughly understand the potential destiny of nanomaterials in aquatic ecosystems before usage. If use is left

unchecked, continual use of nanomaterials in aquatic environments could become hazardous (Fajardo, Martinez-Rodriguez & Blasco, 2022).

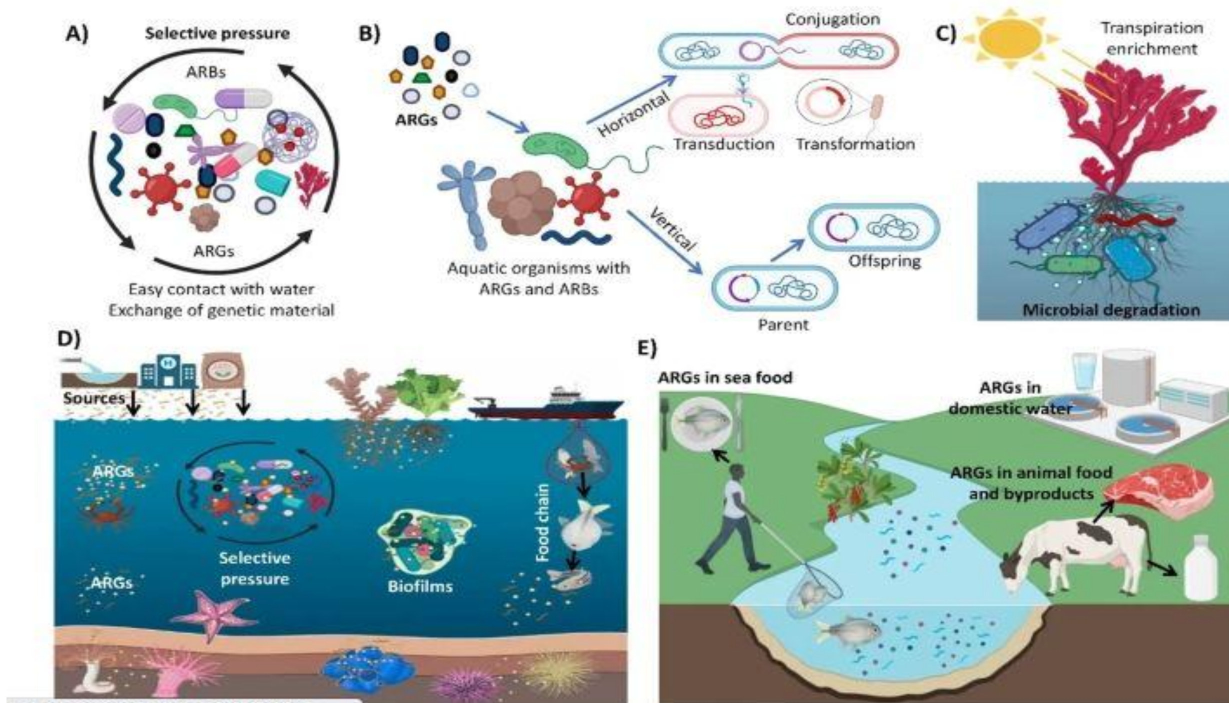


Fig 2.1: Antimicrobial resistance in aquatic organisms and possible fate.
Source: (Michael-Kordatou et al., 2018; Saima et al., 2020; Liu et al., 2021; Miranda et al., 2018; Sun et al., 2020).

The well-documented prevalence of antibiotics in aquatic environments causes aquatic bacteria to be exposed to them over time, gaining antibiotic-resistant genes (ARGs) that they then transfer to other species (Michael-Kordatou et al., 2018; Saima et al., 2020; Sun et al., 2020; Liu et al., 2021; Valdés et al., 2021). For instance, the selection pressure applied by antibiotics in aquatic environments facilitate the interchange and recombination of genetic material by promoting easy interaction between microbes and ARGs (Fig. 2.1A), Antibiotic-resistant bacteria (ARBs) or

microorganisms that can infect other aquatic microorganisms are formed as a result (Michael-Kordatou et al., 2018; Saima et al., 2020). Nanotechnology has the potential to address the selective pressure mechanism that results in ARBs. ARBs have recently been removed from aquatic ecosystems using nanotechnological methods (Fajardo et al., 2022). Nanomaterials can be utilized to remove contaminants that participate in selective pressure in addition to ARBs, such as those from the beauty and pharmaceutical industries (Hairom, Soon & Mohamed, 2021). The creation of customized nanomaterials to combat ARBs and other contaminants can be accomplished if the sources of ARBs or contaminants implicated in selective pressure can be found early on.

Under selective pressure or in addition to it, aquatic microorganisms can group together to create intricate microbial communities (biofilms, for example), where genetic material is transferred (Parrish and Fahrenfeld 2019; Fabra, Williams & Watts, 2021). Because of their complexity, these intricate microbial communities are more difficult to eradicate. As seen in Fig 2.1B (Michael-Kordatou et al., 2018), genetic material is transferred in these environments from one microorganism to another through a variety of channels, including horizontal transmission (such as conjugation, transduction and transformation) or vertical transmission to the next generation. Mutations that result in novel ARGs that can induce ARBs and create widespread bacterial resistance are a potential concern associated with the transfer of genetic materials (Wei, He & Zhang, 2019). Antimicrobial resistant biofilm communities can be disrupted by using nanotechnological techniques such as coupling nanomaterials with surfactants or antibiotics (Li, Wang & Gao, 2021). The extracellular polymeric substances (EPS) that hold environmental biofilms together, especially aquatic biofilms, are likely what allow them to function as effective binding matrices for nanomaterials, as demonstrated by recent study. This suggests that there is

potential for creating nanomaterials that have the ability to remove these biofilms, particularly those that in aquatic environments contain ARBs and ARGs.

ARGs persist across many trophic levels due to their ongoing bioaccumulation in aquatic environments. Antibiotics have been found in fish in aquatic food chains (He, Wang & Nie, 2014; Chen, Zhou & Cheng, 2017; Tang, Wang & Tai, 2020). Depending on their location in the aquatic environment, eating habits and place in the food chain, aquatic species can have different antibiotic concentrations. He et al., (2014) and Tang et al., (2020) conducted a study on fish that were caught in wild waters and found that the concentrations of antibiotics in fish increased gradually as the fish type changed from herbivorous to omnivorous to carnivorous. This finding suggests that carnivorous fish are the top consumers in the aquatic food chain. Antibiotics can build up in the roots of aquatic plants, where they will be further broken down by more bacteria that nourish the roots and aid in the plants' absorption of the antibiotics (Figure 2.1C) (Liu et al., 2021). Fish, plants and other aquatic species that are exposed to ARGs over an extended period of time become hazardous and provide a health concern to humans and other animals that eat them (Figure 2.1D,E) (Miranda, Godoy & Lee, 2018; Sun et al., 2020). In addition to directly consuming seafood, humans run the danger of being exposed to ARGs through the ingestion of meat or animal by-products that have been exposed to ARGs and ARBs from aquatic settings. Using nanotechnological methods to eliminate the dangers is one way to do so. For instance, research has demonstrated that ARB in fish can be removed in-vitro using nanoparticles (Shaalan, Mohamed & Mahdy, 2016; Nasr-Eldahan, Nabil-Adam & Shreadah, 2021). But it's important to ascertain how nanotechnology will affect fisheries and other aquatic species in the long run because they might contain poison (Fajardo et al., 2022). There could be additional dangers in the home water supply due to the emergence of novel ARGs that are difficult to eradicate using

standard waste disinfection techniques (Pal et al., 2015; Gogoi et al., 2018, Michael-Kordatou et al., 2018; Rodríguez-Molina et al., 2019; Liu et al., 2021). Therefore, in order to combat the changes in antimicrobial resistance that are occurring in aquatic habitats it will be helpful to continuously monitor the accumulation of antibiotics, ARGs, ARBs and mechanisms of transmission. The types of ARGs and ARBs in aquatic environments can help in the formulation of better nanotechnological water treatment procedures to reduce or completely eliminate their presence in both domestic and wild water. Nanotechnological approaches for cleaning aquatic systems already exist (Fajardo et al., 2022).

2.5 Nanotechnology, Aquaculture and Aquatic Ecosystems

2.5.1 Nanoparticles Synthesis

Synthesis of low-cost, high-yield nanomaterials has been a major problem since the dawn of nanoscience. For aquaculture and fish medicine, nanoparticles must be able to be produced in a variety of sizes, shapes, monodispersities and chemical compositions (Shah and Yoon 2017). There are several methods for creating metallic nanoparticles with the required characteristics, including top-down and bottom-up methods. Making synthetic nanoparticles and combining them into the final composite product is the bottom-up approach. Chemical synthesis or biological synthesis can be used for this kind of synthesis. One major advantage of the bottom-up technique is its ability to produce flawless metallic nanoparticles with uniform chemical composition. The "top-down" method shrinks the initial material to its size through the use of chemical or physical methods. The drawback of this process is the nonuniformity of morphology, which influences the physical and chemical properties of the created nanoparticles (Sakhare, Prasasvi & Palani, 2022). Biological nanoparticle synthesis is still in its infancy but physical and chemical approaches are frequently used.

2.5.1.1 Physical Synthesis

The two most significant physical methods are laser ablation and evaporation-condensation. The lack of solvent contamination in the thin films generated and the homogeneity of nanoparticle distribution are two benefits of physical synthesis over chemical methods. Silver nanoparticles have been produced by physical synthesis, which uses different microwave radiation frequencies to physically reduce silver. This procedure produced a higher concentration of silver nanoparticles and worked faster than a thermal approach using the same temperature and exposure. According to Jiang, Moon & Zhang (2006), the larger the particle obtained, the greater the concentration of silver nitrate used, the longer the reaction time and the higher the temperature.

2.5.1.2 Chemical Synthesis

The most common technique for creating nanoparticles is chemical synthesis using both organic and inorganic reducing agents. For instance, a number of reducing agents are used to reduce silver ions (Ag^+) in aqueous or non-aqueous solutions, including ascorbate, sodium citrate, N,N-dimethyl formamide (DMF), elemental hydrogen, Tollens reagent and sodium borohydride ($NaBH_4$). The proper metal ions can be combined with reduced polyoxometalates, which function as stabilizing and reducing agents to create nanoparticles at room temperature. Water-soluble polyoxometalates can carry out sequential multielectron redox reactions without undergoing structural disruption. Silver nanoparticles were formed when polyoxometalate/S/Ag^+ deaerated solution was illuminated. Additionally, it has been reported that mixed-valence polyoxometalates can be used to produce silver nanostructures in water at room temperature in a single step utilizing green chemistry (Iravani, Korbekandi, Mirmohammadi, & Zolfaghari, 2014).

2.5.1.3 Biological Synthesis

Finding economical and ecologically friendly ways to create nanoparticles is a popular issue. Utilizing biological methods is thought to be essential to this tactic. The three primary groups of organisms that generate biologically generated nanoparticles are plants, fungi and bacteria. Microbial enzymes or plant phytochemicals with reducing or antioxidant properties react with precursor molecules to form the necessary nanoparticles. The three fundamental parts of a biosynthetic nanoparticle system are a non-toxic stabilizing agent, an environmentally friendly/ acceptable reducing agent and a solvent medium for synthesis (Shaalan, Saleh, El-Mahdy & El-Matbouli, 2016).

Silver nanoparticles derived from *Origanum vulgare* leaf extract were found to be antibacterial and cytotoxic when tested against a human lung cancer cell line. Using cashew nut shell liquid, silver and gold nanoparticles were produced sustainably and effectively against a variety of fish diseases. It was discovered that juvenile *Feneropenaeus indicus* was susceptible to the bactericidal effects of tea leaf extract (*Camellia sinensis*) when it came to *Vibrio harveyi*. Zinc oxide nanoparticles (ZnO-NPs) were produced environmentally by using a broth derived from Aloe leaf extract, which showed greater antibacterial action than nanoparticles produced using conventional chemistry. The bacteria *Aeromonas hydrophilia* is employed as a reducing agent in a novel method to produce zinc oxide nanoparticles biologically. This process is economical and environmentally benign (Shaalan et al., 2017).

2.5.2 Nanoparticles Application in Aquaculture and Aquatic Organisms

2.5.2.1 Antimicrobial Activities of Nanoparticles.

One of the main problems in aquaculture has been controlling infections brought on by microbial pathogens. Since fish farming has been using antibiotics without restriction, many disease-causing (pathogenic) microbes in fish have developed resistance to common antibiotics. To address this issue, new therapeutic approaches have been suggested, and one of such approach is the use of nanoparticles as alternative antimicrobials to combat the emergence of antibiotic resistance by microbes in aquaculture (Swain, Nayak & Sasmal, 2014; Dar, Rashid & Mashid, 2020). Different metal nanoparticles have demonstrated promising antimicrobial effects against bacterial, viral and fungal pathogens (Table 2.3) through various mechanisms like rupturing the microbial cell membrane or cell wall, disrupting transports of protein, inactivation of essential enzymes and others (Figure 2.2) (Nasr-Eldahan et al., 2021).

Table 2.3: Studies of Nanoparticles Antibiotics Resistance in Aquaculture

Metal NPs	Purpose	Remark	References
CuO, ZnO, Ag and Ag-TiO_2	Antibacterial agent against aquaculture diseases	Broad spectrum of antibacterial antifungal activities against fungi like penicillium and mucor species	Swain et al., (2014)
Ag_3PO_4 loaded hydroxyapatite nanowires	Water treatment	Excellent antibacterial activities towards E. coli and *S.aureus* in water	Li et al., (2015)
γ-Fe_2O_3NPs	Oxytetracycline (OTC) administration in zebra fish	The dynamics related to OTC release is still unclear	Chemello et al., (2016)
MoS_2 nanofilms	Water disinfection and purification	Inactivation of bacteria under light irradiation via the generation of ROS	Liu et al., (2016)
Nanozyme	Antibiotics for drug resistant bacteria	Enhanced antibacterial function against E. coli and *S.aureus* in vitro and in vivo	Cao et al., (2019)
Se-NPs	Studies of the immune response and histopathological alterations induced by sublethal cadmium (Cd) toxicity in nile tilapia (*Oreochromis niloticus*)	Improvement of the growth immunity and antioxidant power of o. niloticus and alleviate the pathological disorders induced by Cd toxicity	Abu-Elala et al., (2021)
Algae-coated Se NPs	Antibacterial agent against vibrio harveyi in human and shrimp cell	High performance and nontoxic	Mansouri-Tehrani et al., (2021)

Cu_3P NPs	Impeding antibacterial resistance in fishery water	The multiple enzyme-like activities of the NPs and the action of ROS produced by their oxidase- and peroxidase-like activities facilitates their inherent activities for glutathione depletion and the lipid peroxidation	Chao et al., (2022)
Ag NPs	Antibiotics-resistant aeromonas veronii infection in nile tilapia, *Oreochromis niloticus* (L.)	The treatment increased fish survival; improved hematological, immunological, and antioxidant activities; and optimized liver and kidney function	Elgendy et al., (2022)

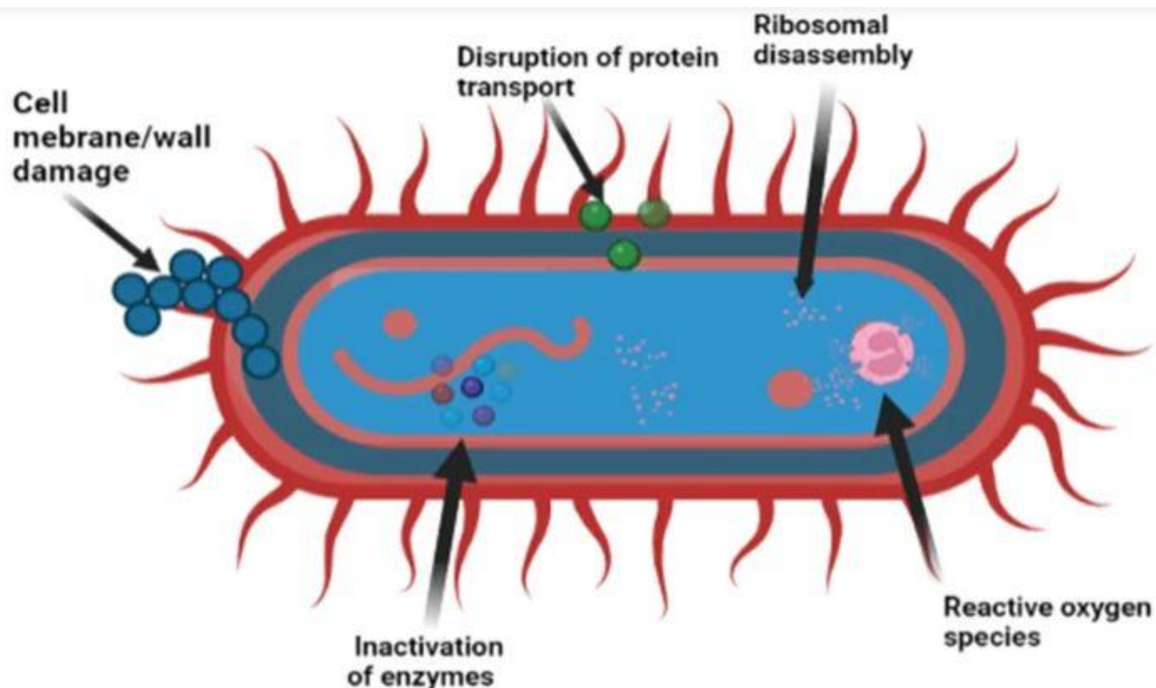


Fig 2.2: Action of metal nanoparticles against pathogenic bacteria

Source: (Nasr-Eldahan et al., 2021)

a) **Silver Nanoparticles (Ag-NPs)**

The most researched type of nanoantibacterial agent is silver nanoparticles. Silver nanoparticles work against disease-causing bacteria through a variety of processes, allowing them to overcome bacterial resistance in contrast to antibiotics, which often only function through one mechanism (Antony, Nivedheetha & Siva, 2013). It is unclear exactly how silver nanoparticles work as antimicrobials (Knetsch and Koole 2011). Due to the huge surface-to-volume ratio and shape of the nanoparticles, researchers have postulated mechanisms that could be connected to changes in the morphology and structure of the bacterial cells (Rai, Deshmukh, Ingle, Gupta, Galdiero & Galdiero, 2009). Silver nanoparticles' antibacterial activity is significantly influenced by these physiochemical factors (Beyth, Houri-Haddad, & Domb, 2015). Because of their small size, the

nanoparticles may interact with the bacterial cell more and penetrate the cell more easily (Rai et al., 2009). Silver nanoparticles are believed to adhere to the cell membrane by electrostatic charges, wherein the positive surface charge of the nanoparticles electrostatically binds to the negative charge of the cell membrane, thereby enhancing the adherence of the nanoparticles to the membrane. Ag^{+} ions are released when nanoparticles connect to the protein in the bacterial cell membranes changing the shape of the membrane and causing permeability and ultimately cell death (Sondi and Salopek-Sondi 2004). Additionally, they have the ability to bind to nucleonic acids and cytochrome, damaging and inhibiting cell division (Huang, Ma & Liu, 2012). It has been shown that silver nanoparticles have potent antibacterial activity against bacterial strains that are resistant to many drugs (Prokash, Pivin, & Swart, 2015). According to Swain et al., (2014), silver nanoparticles prepared with citrus lemon juice as reducing agent showed antibacterial and anticyanobacterial activity against *Oscillatoria* species as well as *Staphylococcus aureus* and *Edwardsiella tarda* bacteria. Silver nanoparticles like the commercial antifungal medication Amphotericin B (sanjemban), showed strong inhibitory effects against *Candida* species. Particularly about the medicinal application of silver nanoparticles with antifungal and antiviral qualities in aquaculture, there hasn't been much research.

b) Zinc Oxide Nanoparticles (ZnO-NPs)

Zinc oxide nanoparticles' antibacterial and antifungal qualities have garnered significant interest (Gunalan, Sivaraj & Rajendran, 2012; Sirelkhatim, Mahmud & Seeni, 2015). Zinc oxide nanoparticles' precise antibacterial toxicity mechanism is still up for debate though. Nevertheless, it has been postulated that the particles' breakdown of the bacterial cell membrane which allows cytoplasmic contents to sleep out of the cell, is what gives them

their antibacterial activity (Sirelkhatim et al., 2015). It has been demonstrated that in the aquaculture field, zinc oxide nanoparticles inhibit the growth of *Aeromonas hydrophila*, *Edwardsiella tarda*, *Citrobacter* spp., *Staphylococcus aureus, Vibrio* species, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Flavobacterium branchiophilum* (Swain et al., 2014). According to a study, zinc oxide nanoparticles have antibacterial action against the fish bacterium pathogen *Vibrio harveyi.* This activity grows when zinc oxide nanoparticle particle size decreases (Vaseeharan, Ramasamy & Chen, 2010). Another fascinating study (Jayaseelan, Rahuman & Kirthi, 2012) discovered that zinc oxide nanoparticles produced biologically utilizing *Aeromonas hydrophila* have antibacterial activity against *Escherichia coli, Candida albicans, Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Aspergillus flavus.* Zinc oxide nanoparticles have been shown in numerous studies to be fatal to microorganisms, harmless to human cells and to have good biocompatibility with human cells. These findings have made zinc oxide nanoparticles useful as antibacterial agents (Padmavathy and Vijayaraghavan 2008; Siddiq, Rahman, Tajuddin & Husen, 2018).

c) Titanium Dioxide Nanoparticles (TiO_2-NPs)

The application of titanium dioxide nanoparticles as therapeutic agents in aquaculture has not received much attention. Titanium dioxide nanoparticles were discovered to have a bactericidal effect against fish bacterial infections, including *Streptococcus iniae*, *Edwardsiella tarda* and *Photobacterium damselae,* after being activated by light (Cheng, Yao, & Yeh, 2011). When exposed to ultraviolet light, TiO_2-NPs can create highly active free radicals such as hydroxyl (-OH), superoxide ion (-O), peroxyl radical (-OOH) and others with a high capacity for oxidation. Through a series of chain reactions, these free radicals can interact with biomacromolecules found in microorganisms, including lipids,

proteins, enzymes and nucleic acid molecules, destroying their cell structures (Sonawane, Hedge, & Dongare, 2003). Nonetheless, research indicates that titanium dioxide nanoparticles may impact fish immune function by reducing fish neutrophils' antibacterial activity, hence reducing the fish's resistance against bacterial infections (Jovanoviç, Whitley, & Kimura, 2015).

d) Gold Nanoparticles (Au-NPs)

There is currently a lot of interest in researching the antibacterial properties of gold nanoparticles because of their minimal toxicity to eukaryotic cells (Li et al., 2014). According to Saleh et al., (2016), gold nanoparticles in aquaculture have the potential to be effective antibacterial agents and lower mortality from microbial illness. Gold nanoparticles have been studied as an antibacterial agent to stop the spread of common aquatic diseases including *Escherichia coli* and *Salmonella typhi*, which are growing resistant to bactericides (Lima et al., 2013). Gold nanoparticles have been the subject of numerous studies on their antibacterial properties in aquaculture (Vaseeharan et al., 2010). Fish bacterial isolates (Velmurugan et al., 2013) and other multidrug resistant bacterial isolates were shown to be slower to grow when gold nanoparticles were added (Li et al., 2014). Similar research has demonstrated the antifungal activity of gold nanoparticles against *Candida* species. The study also found that the efficacy of the nanoparticles was size-dependent, with smaller gold nanoparticles having stronger antifungal effects (Wani and Ahmad 2013). The mechanism by which gold nanoparticles are said to function against bacteria is thought to include disrupting the oxidative phosphorylation process and altering the potential of the bacterial cell membrane which lowers the activity of F-type ATP synthase and reduces ATP production and metabolism overall.

2.5.3 Nanotechnology in Preventing and Remediating Antibiotic Pollution and Antimicrobial Resistance in Aquaculture and Aquatic Ecosystems.

In order to address antibiotic resistance in aquaculture and the aquatic ecology, nanotechnology is an essential tool. Antibiotics and antibiotic resistance may be managed through the use of a number of current and new nanosystems (Nasr-Eldahan et al., 2021). We will continue to develop these nanosystems using two main strategies. In this initial strategy, we will talk about prophylactic nanosystems that can be used to stop the spread of antibiotic resistance and antibiotic contamination in aquaculture and the aquatic environment. The second method involved a thorough examination of potential remediation nanosystems for the management or treatment of an environment that was already contaminated with antibiotics and antibiotic resistance.

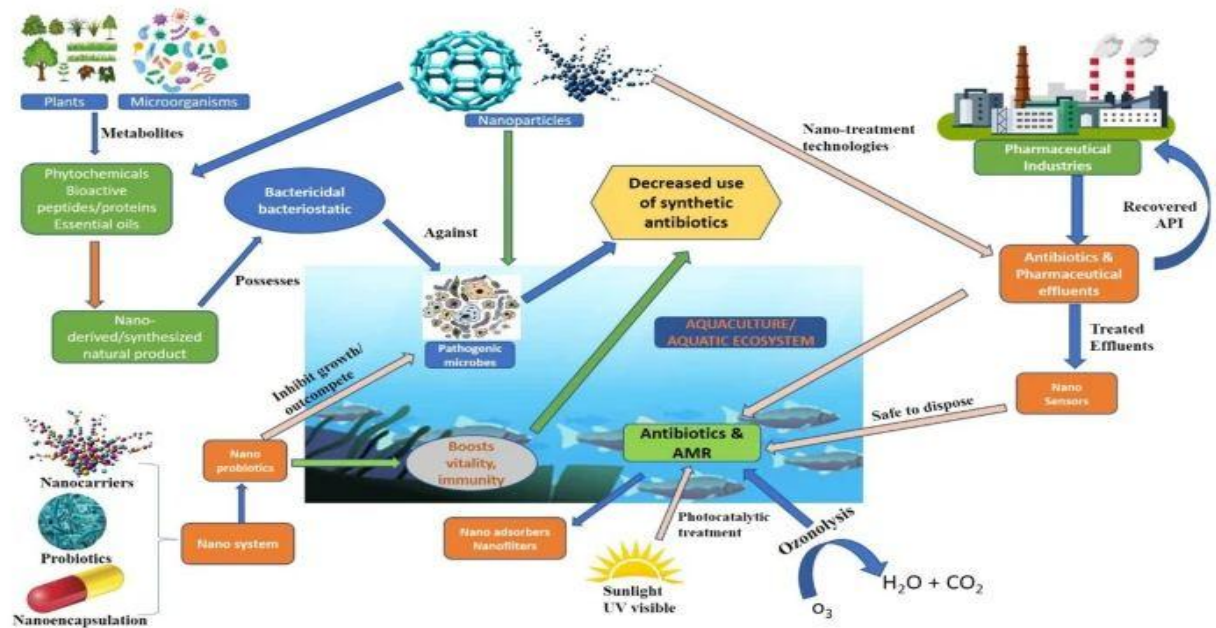


Fig 2.3: Application of nanotechnology in combating antimicrobial and antibiotic resistance in aquaculture and aquatic ecosystems

Source: Okeke E.S. et al., (2022)

2.5.4 Some Prophylactic/Preventive Nanotechnological Systems for Managing the Spread of Antibiotic Resistance.

Antibiotic pollution and the growth of antibiotic-resistant microbes in aquaculture and aquatic environments can be avoided with the aid of prophylactic nanosystems. This preventive strategy often uses different nanosystems to control the aquaculture's microbial load and stop the dishonest release of effluents high in antibiotics from the pharmaceutical manufacturing sector. A few of these systems include the application of nanoparticles to control the release of antibiotics in aquaculture, the use of nano-derived natural products as an alternative antimicrobial agent for aquaculture and the adoption of nanoprobiotics/ prebiotics to boost the vitality and immune resistance of aquaculture.

A) Use of Nano-derived Natural Products in Aquaculture

For the purpose of preventing or treating pathogenic assaults in aquaculture, natural products offer a sustainable substitute to synthetic antibiotic drugs. Natural antimicrobial compounds include bioactive peptides, phytochemicals, essential oils and metabolites from a wide range of plants, animals and microbes. For a variety of reasons, it has been demonstrated that nanoparticles, which can be produced from natural sources have more antibacterial power when it comes to battling infections and microbial colonization. Initially, nanoparticles work in concert with other antimicrobial processes to kill microorganisms. Secondly, when it comes to delivering these natural compounds specifically against microbial illness, nanoparticles enhance their specificity and accuracy. Lastly, the combination of natural ingredients with

nanoparticles guarantee that they have the same long-lasting antibacterial effects in aquatic settings and aquaculture as other synthetic antibiotics, but without encouraging multidrug or antimicrobial resistance. To be more precise, nanoparticles guarantee a programmable and regulated release of natural product to increase aquaculture's vitality and productivity (Fajardo et al., 2022). To create nano-derived natural products, a variety of physical and chemical techniques have been used including microemulsion, thermal breakdown, polyol, electrochemical synthesis, microwave synthesis, chemical vapour deposition, gamma radiation and others. Typically, the end result of these syntheses is nanoscale and comes in a variety of geometries including globular, cuboid, hexagonal, spherical, prismatic and irregular shapes.

Table 2.4: Studies on the use of nano-derived natural product (green synthesis) in aquaculture

Aquaculture	Nanparticles	Natural Products	Shape and Size	Aquaculture Pathogen	Bioactivities	References
Salmonid fish and cod	Chitosan Ag nanocomposites	Chitosan	Spherical (281nm)	Coldwater vibriosis caused by bacteria *Aliivibrio salmoicida*	Bacteriostatic and bactericidal with MIC and MBC of 50µg/ml and 100µg/ml respectively	Dananjaya et al., (2016)
Tilapia (*Oreochromis mossambicus*)	Au-NPs	Polysaccharide fucoidan from *Fucus vesiculosus*	Spherical and triangular (10-100nm)	*Aeromonas hydrophila*	Antibacterial and antibiofilm with a ZI of 23.2nm at 100µg/ml, greater than chloramphenicol (ZI 17.3mm)	Vijayakumar et al., (2017)
Cirrhinus mrigala	Ag-NPs	*Azadirachta indica* (Neem)	Spherical (35.4nm)	*Aeromonas hydrophila*	Antibacterial (with a 74% survival rate of treated fingerlings) and immune-modulatory	Rather et al., (2017)
Goldfish, *Carassius auratus*	Ag-NPs	Aqueous extract of garlic, *Allium sativum*	Spherical	*Bacillus licheniformis* and *Pseudomonas aeruginosa*	Antibacteria	Saha and Bandyopadhyay (2019)
Nile tilapia, *Oreochromis niloticus*	Chitosan nanoparticles (CNP)	Chitosan	Pentagon and hexagon (35nm)	Bacteria [*Aeromonas hydrophila, Aeromonas sobria* (at 0.156 to 2.5µg/ml dosage)] Fungi [*Aspergillus flavus, Mucor* sp., and *Candida* sp. (ZI 35mm at 20µg/ml dosage)]	-Bacterial cell wall destruction - Antifungal	Abdel-Razek (2019)
Labeo rohita fingerlings	Zinc oxide nanoparticles	*Spinacia oleracea* (Spinach)	Spherical (22.61nm)	*Saprolegnia* sp.	Feed additive growth and improved biochemical and hematological parameters	Thangapandiyan and Monika (2020)
Fish (not specific)	Zein nanoparticles	*Zein proteins, Eugenol* and garlic essential oils	Spherical (150nm)	*Aeromonas hydrophila*, *Edwardsiella tarda* and *Streptococcus iniaein*	Antibacteria	Luis et al., (2020)

Fish (not specific)	Ag-NPs	Red algae *Portieria hornemannii* extract	Spherical (70-75nm)	*Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio vunifucus* and *Vibrio anguillarum*	Antibacterial with ZI of 16-28mm	Fatima et al., (2020)
Longfin yellowtail	Au-NPs	Aqueous extracts of *Turnera diffusa* (oplopanone, γ-eudesmol, hydroquinone-β-d-glucoside (arbutin) and inositol)	Spherical (24nm)	*Vibrio parahaemolyticus* and *Aeromonas hydrophila*	Antimicrobial Antioxidant Immunomodulation	Reyes-Becerril et al., (2021)
Fish (not specific)	Cu(II) nanoflowers	Juice and peel of blood orange	Spherical	*Yersinia ruckeri* (causative for enteric mouth disease in fish)	Antibacterial with a ZI of 30.66-33.66mm at 0.5μg/ml	Demirbas (2021)
Fish (not specific)	Ag-NPs	Gum Arabic	Spherical (10nm)	*Aeromonas hydrophila* and *Pseudomonas aeruginosa* with ZI of 22 and 20mm and an MIC of 1.625μg/ml and 3.25μg/ml, respectively	Antibacteria and antibiofilm	El-Adawy et al., (2021)
Tilapia (*Oreochromis mossambicus*)	Ag-NPs	Polysaccharides of *Caulerpa racemose*	Spherical (88nm)	*Pseudomonas aeruginosa*	Antibacteria Antioxidant	Thanigaivel et al., (2022)
Nile tilapia and Sea bass	Ag-NPs	*Origanum vulgare* leaves extract	Irregular (100nm)	Bacteria (*Streptococcus agalactiae, Aeromonas hydrophila* and *Vibrio alginolyticus*) Fungi [*Aspergillus flavus, Fusarium moniliforme* and *Candida albicans*]	Antibacterial with a ZI of 23.7-31.3mm at 10μl/disk of the green AgNPs Antifungal (ZI 11-18mm at 10μl/disk)	Ghetas et al., (2022)

A spherical green synthesized nanoparticle from the solvent extraction of *Origanum vulgare* leaves was found to have remarkable antibacterial and antifungal activities against bacterial and fungal diseases of Nile tilapia and sea bass in a recent study by Ghetas et al., (2022). The antibacterial properties against *Aeromonas hydrophila, Vibrio alginolyticus* and *Streptococcus agalactiae* were documented as a zone of inhibition ranging from 23.7 to 31.3mm upon application of 10uL nanoparticles/disk. Similar to this, when treated with same concentration (10uL/disk), fungal pathogens such as *Aspergillus flavus*, *Fusarium moniliforme* and *Candida albicans* displayed an 11-18mm zone of inhibition (Ghetas et al., 2022). According to a different study, gold nanoparticles made from *Fucus vesiculosus* fucoidan polysaccharide shown noticeably superior antibacterial and antibiofilm properties against *Aeromonas hydrophila* (ZI 23.2mm) in comparison to chloramphenicol (ZI 17.3mm) (Vijayakumar et al., 2017). Additional research, which is compiled in Table 2.4, provides strong proof of the possibility of naturally occurring compounds obtained from nanotechnology as a substitute for antibiotics in aquaculture, hence reducing the emergence of antibiotic resistance.

b) Use of Nanoprobiotics to Boost the Vitality and Immune Resistance of Aquaculture.

An emerging application of nanosystems for effectively preparing and delivering probiotics to living things is called nanoprobiotics. Probiotics have been used for many years to improve the health and vitality of various host organisms when given in suitable numbers. In order to colonize the gut and outcompete pathogenic species, these probiotics are typically given orally through diet formulations (Hill et al., 2014; Varankovich et al., 2015). Probiotics are a better option than the usage of antibiotics and a more sustainable way to stop the evolution of antibiotic resistance since they may be utilized to provide protection against pathogenic bacteria through a variety of mechanisms (Watts et al., 2017).

Because probiotics increase overall production and resistance to infections through a variety of multifaceted methods, they have lately attracted attention in aquaculture and aquatic ecosystems. Probiotics increase aquacultures' body weight, digestive rate, fertility, antioxidant enzymes and stress tolerance among other things (Hasan and Banerjee 2020). In addition, it suppresses intestinal colonization, cortisol levels, viral infection and harmful bacteria. Probiotics' capacity to create siderophores, hydrogen peroxide, antibiotics, enzymes and bacteriocins-proteinaceous toxins is linked to their advantageous effects (Watts et al., 2017; Uma and Rebecca 2018). Strong antibacterial agents for aquaculture are among the fundamental processes of probiotics. The competitive exclusion of pathogenic bacteria, the establishment of unfavorable environments for these bacteria, the induction of host defense against infections and antiviral actions are these fundamental mechanisms. Probiotics have antibacterial properties, but they also improve water quality by promoting the breakdown of organic and inorganic pollutants in aquaculture and aquatic environments.

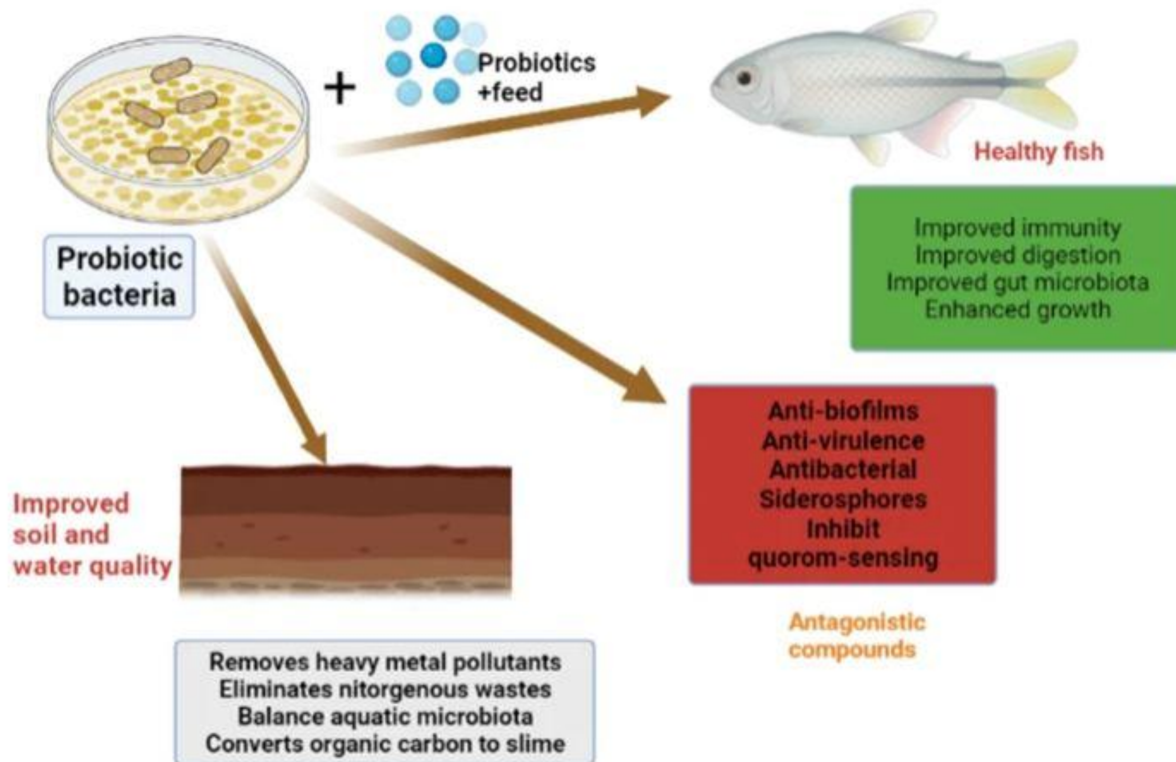


Fig 2.4: Beneficial action of probiotics to aquatic animals/ecosystems

Source: Okeke E.S. et al., (2022)

Probiotics have been given to humans and other animals via the use of nanosystems including nanoencapsulation, nanoparticles, nanobeads, nanofibers, nanoemulsion and nanolayers in recent years. However, because there are so few studies available, this approach is still novel for aquaculture (Fajardo et al., 2022). The adoption of nano-derived probiotics is a prophylaxis measure against the excessive use of antibiotics and the rise of antibiotic resistance in aquaculture. The nano-derived probiotics have improved the functionality, bioavailability, viability and stability of the beneficial microbes in the host systems. Moreover, nanoparticles associated with probiotics possibly improve their anti-oxidative properties for better management of cellular

damages due to the diseases state. Even with all these advantages of probiotic nanoformulation, if the formulations' concentration is not optimized, there could be a discernible rise in toxicity.

Probiotics have been used in aquaculture and aquatic environments for a number of years; nevertheless, there haven't been many investigations on their nanoformulations. Three microorganisms were identified from the stomach of a gigantic freshwater prawn by Sam-on and associates. *Bacillus subtilis*, *Bacillus velezensis and Bacillus pumilus*. *Bacillus velezensis* showed greater antibacterial activity against *Aeromonas hydrophila* (23.7mm) and *Aeromonas veronii* (25mm) after these bacteria were tested in-vitro as probiotics. *Bacillus* species are highly resistant to 0.3% bile salt and their protease, amylase and lipase activities further validate their usefulness as probiotics in aquatic settings (Sam-on et al., 2022). A third study looked at various *Bacillus* strains from mangrove habitats as probiotics for (Rohu) *Labeo rohita* diets. *Bacillus* species at varying concentrations (106cfu/g and 109cfu/g for *Bacillus amyloliquefaciens*, 106cfu/g and 109cfu/g for *Bacillus subtilis*, 106cfu/g and 109cfu/g for*Bacillus megaterium* and all three bacteria in the same proportion of 106cfu/g and 109cfu/g for all the same three bacteria and a control lacking probiotics) were cultivated on Rohu fingerlings with an average weight of 5.02+0.85g for 45days. Following this, the bacteria were exposed to *Aeromonas hydrophila* and the survival rate was monitored for 10days. Red blood cells, haemoglobin, serum superoxide dismutase activity, white blood cells, survival, protease and intestine alpha-amylase activities were all markedly enhanced by the combination of the three bacteria (106cfu/g). According to Saravanan et al., (2021), field tests with this combination of bacteria further demonstrated their potential as a reliable dietary probiotic with a range of advantages. On the other hand, a small number of probiotic studies produced from nanotechnology demonstrated the antibacterial and antioxidant properties of these formulations, which can be successfully used in aquaculture (Table 2.5).

Further targeted research on the use of probiotics derived from nanoparticles in aquaculture and aquatic environments is necessary, especially to combat the growth in antibiotic resistance and pollution.

Table 2.5: Potential Application of Nano-Derived Probiotics in Aquaculture

Probiotics	Sources	Nanoparticles	Shape	Size	Potential application in aquaculture	References
Lactobacillus delbrueckii	Probiotic curd	Ag-NPs	Spherical	54.3-112.7nm	Antimicrobial	Saravanan et al.,(2011)
Lactobacillus sporoogens	N/A	ZnO NPs	Hexagonal	145.70nm	Antimicrobial	Mishra et al., (2013)
Lactobacillus kimchicus DCY51T	Korean kimchi	AuNPs	Spherical	5-30nm	Antioxidant	Markus et al., (2016)
Lactobacillus casei ATCC 393	N/A	Selenium NPs	Spherical	50-80nm	Antimicrobial, increase intestinal cell proliferation and antioxidant	Xu et al., (2018a,b)
Lactobacillus casei subsp. *Casei*	N/A	CuO NPs	Spherical	30-75nm	Antimicrobial	Kouhkan et al., (2019)

c) Use of Nanosensors/Detectors for Estimating Antibiotic Pollution Level.

The first step in managing or preventing the spread of antibiotics and antimicrobial resistance bacteria in aquaculture or aquatic environments is the detection of these substances. In recent years, nanotechnology has proven useful in the development of highly sensitive biosensors for the detection of antibiotics and antibiotic resistance. According to Zeng et al., (2022), nanobiosensors are a component of the sophisticated method for detecting antibiotics. Several nanoparticles have

demonstrated high accuracy when fitted to several electrochemical, fluorimetric and colorimetric biosensors.

Colorimetric biosensors are nanosensitive sensors that exhibit colour gradients and observable colour changes in response to the concentration of antibiotics in an aquatic environment or aquaculture. Tetracycline (TCs) have a great affinity for Fe(II) and Fe(III), which is why TCs colorimetric biosensors have been made using Fe_3O_4 magnetic nanoparticles (MNPs). A TC-Fe_3O_4 combination is created when TCs are present in a solution and this complex promotes H_2O_2's oxidation of 3,3',5,'-tetramethylbenzidine (TMB) to create a dark visible solution. Tetracyclines and their derivatives in the medium can be measured using UV spectroscopy, with a detection limit (LOD) ranging from 12 to 48nm. In contrast to the TMB in the previous investigation, carbon quantum dots were utilized as the probes for fluorescence detection of the TCs-Fe_3O_4 combination in the colorimetric detection system.

2.5.5 Nanosystems for Some Remediation of Antibiotics Pollution and Antimicrobial Resistance in Aquaculture and Aquatic Ecosystems.

An overview of various recent nanosystems for controlling the pervasive antibiotic contamination in aquaculture and aquatic environments is provided in this section. Nanofiltration, nanoadsorbent, magnetic nanoparticles, nanocatalyst/nanodegrader, improved nano-oxidation process and microbial biofilm engineering are all components of this remediative nanosystem.

A. **Nanofiltration**

Antibiotics can be effectively removed from aquaculture and the aquatic environment with the use of nanofilters. To extract various organics and medications from water, nanofiltration methods use a moderately pressured membrane separation technique. It's operating position and general

principles fall somewhere in between reverse osmosis rejects or inhibits their passage. Conversely, especially for low molecular weight substances, nanofiltration traps or limits the flow of organic materials that would typically pass through an ultrafilter. As a result, other names for nanofilters include “tight ultrafiltration membranes” and “loose reverse osmosis membranes”. Because nanofillers require less operating pressure than reverse osmosis membranes to remove antibiotics from aquaculture, they are a more cost-effective method. According to a report, the majority of antibiotics fall under the nominal molecular weight cutoff (MWCO) range of 100-1000Da for the majority of currently available nanofiltration membranes. The range of organic molecular weights at which the nanofilter performs best is known as the MWCO. Using nanofiller to remove antibiotics from wastewater, aquaculture and even various aquatic ecosystems have been shown to remove over 90% of antibiotics in numerous recent studies.

Additionally, adding nanoparticles to the nanofiller membrane helps to overcome the severe biofouling restrictions, familiarizes oneself with convectional nanofilters and promotes increased flux movement for improved separation. The introduction of nanoparticles guarantees that the membrane filter may be readily adjusted to several properties inside the nanofilter membrane. Zeolites, carbon nanotubes, metal oxides (TiO_2) are common nanoparticles found in membrane filters. A hybrid carbon membrane with a tiny pore (between 3 and 10nm) may be able to remove tetracycline antibiotics from water, according to a study by Liu et al., (2017). A hybrid membrane was composed of thick graphene oxide and activated carbon with a thickness of 15um. Approximately 99% of the tetracycline was separated using vacuum filtering. Furthermore, compared to nanofilter membranes made of individual nanomaterials such as graphene oxide, activated carbon and carbon nanotubes, the hybridized nanomembrane demonstrated higher adsorption efficiency. Tetracycline, sulfamethoxazole and trimethoprim were effectively removed

from water using a carbon nanotube single-walled membrane adsorption, filtration and protraction technique. A single-wall of hydrophobic and hydrophilic layers was used to construct the carbon nanotubes and SiO_2 (743-1218mm^3/g) was added to change the pore diameters. Within 23 seconds of filtration and 5hours of adsorption, the modified carbon nanotubes were found to have removed 98.8% sulfamethoxazole, 95.5% trimethoprim and 87.0% tetracycline.

B. **Nanoadsorbents**

Recently, there has been an increased focus on nanoadsorption separation techniques due to its reduced cost, simplicity of usage and accessibility of nanoadsorbent. In aquaculture and aquatic environments, a variety of nanomaterials have been modified to serve as antibiotic and medication nanoadsorbents. Compared to other convectional and bulky particles (natural clay, zeolites and other polymeric substances), which are also less selective and have a lower surface area, nanoparticles' vast surface area offers additional advantages as adsorbents. Additionally, nanoadsorbents with great catalytic potential, reactivity, selectivity and flexibility might be inorganic or organic. Graphene and graphene oxides, carbon nanotubes, biochar, molybdenum disulphide nanosheet, boron nitride, silica and nanoactivated carbon are a few examples of nanoadsorbents. A number of intermolecular and atomistic interactions cause antibiotics from aquaculture and aquatic environment to become trapped in the pore space and surfaces of the adsorbents. Hydrogen bonding, pie-pie interaction and electrostatic and hydrophobic interaction are common interactions that have been documented in a large number of investigations. Surface complexation and covalent contact have been documented in several investigations, particularly with regard to carbon-based nanoparticles. To improve the efficiency of the adsorption process, functional groups or high-affinity chemicals can be added to the surfaces of nanomaterials.

A unique composite nanoadsorbent technology was created in a recent study by Wahab et al, (2019) to eliminate ofloxacin antibiotics from industrial liquid waste. The ofloxacin retention/adsorption effectiveness of the magnetic carbon-nanocomposite nanoadsorbents made from waste biomass (Dalbergia sisso sawdust) ranged from 57.8 to 99.57%. Similarly, Vu et al., (2020) showed that a nanocomposite made of laterite modified with polyanion had an 88% effectiveness in eliminating tetracycline from wastewater. The study's best conditions for the adsorption system were a pH of 4.0, 189 minutes of contact time and a solid-liquid ratio of 5mg/ml.

C. **Nanocatalytic Oxidation Processes**

By oxidative breakdown to more minor harmful metabolites, usually carbon dioxide and water, antibiotic pollution of aquaculture and aquatic ecosystems, along with other organic contaminants, can be removed. The two main alternate pathways for the nanocatalytic oxidation process are photocatalytic degradation and catalytic ozonation (Chen et al., 2021). Because of their huge surface area, ease of modification, more reactive surfaces and enhanced mass transfer, nanomaterials are the preferred catalysts in these processes and together they improve the overall kinetics of the reaction (Chankhanittha et al., 2021). In recent investigations on the catalytic ozonation or photocatalytic degradation of medicines and antibiotics, nanometal oxides, carbon nanotubes and graphene oxides have gained a lot of attention (Sukidpaneenid et al., 2023). Research has demonstrated that the combined action of ozonation and photocatalysis on nanosurfaces may effectively remove pharmaceuticals from aquacultures, leading to improved remediation effects.

2.5.6 Application of Nanoparticles in Aquaculture and Aquatic Organisms.

Recent biotechnological developments in aquaculture have been enhanced by the application of nanotechnology, which offers disinfection solutions which are more effective than those utilizing

conventional chemicals (Hjorth et al., 2017). The application of nanotechnology in aquatic ecosystem sustainability holds great promise for the administration of drugs, the transportation of nutrients and the repair of antibiotic resistance among other applications. The aquatic ecosystem's organic pollutants and other impurities have also been removed using nanoenabled approaches (Dar et al., 2019). After considerably absorbing up to 94.3mg/g of fluoride from an aqueous solution, TiO_2 and TiO_2- SiO_2 nanocomposites were discovered to show considerable potential for aquaculture purification (Zeng et al., 2017). According to reports, silver nanoparticles made from *Streptomyces* sp. cell filtrate may disinfect 1log10 CFU dangerous *Bacillus* endospores in around 20minutes (Gopinath et al., 2015). Silver nanoparticles were immobilized on silica beads in a water filter column to combat *Vibrio* sp. In a different investigation, Sarkheil et al., (2016) found that the post-larval stage of *P. vannamei* was raised in filtered seawater, which increased both the fish's growth performance and survival rate. A significant percentage reduction of nitrite, nitrates, ammonium and phosphates at 89.3%, 92.23%, 93.67% and 89.25% respectively was achieved in comparison to other parameters at the end of an investigation by Hesni et al., (2020) by using iron oxide nanoparticles and microalgae in tandem for the purification of aquaculture effluents in a constructed bioreactor. Aquaculture purification and treatment are thought to be key components of its sustainability and the development of nanotechnology offers exciting opportunities with cutting-edge nanotools to help realize this potential.

One of the primary issues with aquaculture systems is disease outbreak (Fajardo et al., 2022). Vaccination has emerged as a useful strategy for demonstrating immunity to diseases. There are three primary channels designated for the delivery of medications to aquaculture. The first approach involves bathing or immersing fish, which is more appropriate but necessitates huge dosages of medication and frequently results in fish stress that cannot be avoided. The second,

simplest approach is the in-feed formulation given orally during regular feeding without additional expense or stress. The third, injection, appears to be impractical for fish (Rather et al., 2021). DNA strands enclosed in nanocapsules have been utilized in the aquaculture sector to boost fish immune responses (Fajardo et al., 2022). Oral DNA vaccines against bacterial and viral infections in shrimps have been produced and are widely utilized as carriers, thanks to the development of poly-lactide-coglycolide acid and chitosan. They are quite successful in these applications because of their water solubility, biodegradability and non-toxic qualities (Chalamcherlla, 2015). Alginate particles are being considered as early candidates for the oral delivery of vaccinations to aquatic animals due to the development of several encapsulation techniques (Shah and Mraz 2020). According to several research (Bhattacharyya et al., 2015; Ogunkalu 2019), vaccinations encapsulated in nanoparticles have proven effective in protecting fish from bacterial and viral infections.

a. **Pathogen Detection in Fish**

The use of nanoparticles in pathogen diagnostics in aquaculture, especially in fish, has advanced recently and it has shown to be a quick and sensitive method. For instance, one of the most widely used nanoparticles in pathogen diagnosis is gold and it has several applications (Saleh et al., 2015). According to Chen et al., 2015, nanobiosensor devices are now being developed to identify contaminants and incredibly low concentrations of bacteria, viruses and parasites in aquatic environments. This is especially important when it comes to outbreaks that damage commercial aquaculture systems since it might take a long time for the causative agents to manifest symptoms and be identified, which can cause major delays in pathogen control measures and substantial financial losses. In this sense, early pathogen detection and eradication is one way that

nanotechnology can help solve this issue. Using *A. salmonicida* antibody-gold nanoparticles conjugated for the specific immunodiagnosis of fish furunculosis tissues, the application of gold nanoparticles also demonstrated notable good outcomes in the detection of infections in fish (Saleh et al., 2011). For the detection of *Alphanomyces invadans* in fish, an electrochemical DNA biosensor based on the coupling of gold nanoparticles with DNA reporter probe was used (Kuan et al., 2013). This assay could identify fungus at a lower level than PCR.

Carbon nanotube-based nanosensors have been discovered to have high sensitivity for finding residues of bacteria, viruses, parasites and other heavy metals in both water and food based on recent studies for fishpond clearance and stock inspection. Nanosensors, particularly those based on carbon nanotubes, are highly sensitive for the detection of traces of pathogens including viruses, parasites and bacteria as well as heavy metals, both from food and water (Dar et al., 2019). They are also used for cleaning fishponds and inspecting animals. Tracking nanosensors with trackers that transmit information about the location and health status of fish have been reported; these technologies, when combined with big data analysis, allow for the control of individual fish or the creation of intelligent cage systems (Singh Sekhon 2014; Dar et al., 2019).

b. **Nano-based Fish Vaccines**

One area of medical research that shows promise is the use of nanoparticles in vaccine compositions. The reason polymeric nanoparticles have drawn the most attention as vaccine delivery vehicles is their ability to preserve immunogenicity, provide long-term vaccine release and maintain antigen stability against enzyme degradation (Myhr and Myskja 2011; Zhao et al., 2014). Nanovaccines have been applied as targeted antigen delivery vehicles with slow antigen release, or as adjuvants for immunostimulants (Zhao et al., 2014). Chitosan nanoparticles have been used in the development of fish vaccines. One such vaccination is the inactivated virus

vaccine against the infectious salmon anaemia virus (ISAV), which has ISAV replicase DNA added as an adjuvant. This immunization demonstrated protection rates against ISAV of greater than 77% (Rivas-Aravena et al., 2015). Chitosan and chitosan/ tripolyphosphate nanoparticle was used to create an oral DNA vaccine against *Vibrio anguillarum* in an Asian Lates calcarifer (Vimal et al., 2012). Though the pathogen was only protected moderately by the nanovaccine.

To create an oral DNA vaccination, the outer membrane protein K (ompK) gene of *Vibrio parahemolyticus* was inserted into chitosan nanoparticles. The recombinant nanovaccine against *Vibrio parahemolyticus* generated a protective immunological response in black seabream (*Acanthopagrus schlegehi*) (Li et al., 2013). It was investigated whether recombinant DNA-chitosan nanoparticles could shield shrimps from the white spot syndrome virus (WSSV). When administered orally, the vaccine has been shown to increase shrimp immunity and produce a protective response against WSSV (Singh Sekhon, 2014). It has been demonstrated that PLGA nanoparticles as an adjuvant and carrier of DNA vaccine (Hølvold et al., 2014). Thus far, there hasn't been much use of PLGA nanoparticles in fisheries. PLGA and polymeric chitosan nanoparticles have been the most investigated nanoparticles in fish vaccination research so far.

c. **Seafood Processing**

It is now essential to extend the shelf-life of seafood items and such nanotechnology can be used in both the marketing of seafood and the breeding of aquaculture species. Because nanomaterials can provide additional benefits like decreased enzyme activity, product degradation, oxygen depletion, reduced antimicrobial and antifungal activities, improved product stability and toxin detection, their application in the food packaging industry has greatly increased research efforts (Adomako et al., 2012, Kuswandi 2016; Luis et al., 2019; Kumar et al., 2020). Many different types of nanostructures, such as nanofibers, nanoemulsions and nanostructures have shown great

efficacy in postponing food deterioration, preserving food quality as well as other sensory attributes including flavour and colour (Ozogul et al., 2017). Numerous studies have demonstrated the beneficial effects of using essential oils encapsulated in various nanostructures, including polymeric nanoparticles, zein nanoparticles, cyclic oligosaccharides and nanotubes (da Rosa et al., 2015; Abarca et al, 2016; Kim et al., 2016; Liakos et al., 2016; Chifiriuc et al., 2017; Pavela et al., 2017). This has also been applied in processing of seafood, shellfish such as *Oreochromis* sp. that has been preserved using chitosan (Tapilatu et al., 2016).

To create nanocomposite films, natural biopolymers like proteins, polysaccharides and lipids are utilized. Because these alternative packaging materials are edible, non-carcinogenic and environmentally benign, they are quickly replacing plastics made through petrochemical processes (Ogunkalu 2019). *Salmonella enteritidis, Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes* were the four well-known food pathogens that the nanocomposite films consisting of chitosan nanoparticles and gelatin with oregano essential oil added showed strong antibacterial action against the four food pathogens (Hosseini et al., 2016). The creation of edible films through the use of nanoemulsions is becoming more and more popular worldwide. To improve water dispersion and stop essential oil degradation, alginate films filled with nanoemulsions of essential oils have been created (Acevedo-Fani et al., 2015). According to recent studies, *Escherichia coli* grew in a nanoemulsion made with essential oils that have some antibiotic properties, such as thyme essential oil (Otoni et al.., 2014). It was recently demonstrated that lecithin nanoliposomes might encapsulate essential oils (Valencia-Sullca et al., 2016). Orange essential oil was encapsulated using rapeseed and soy lecithin to create remarkably, stable nanoliposomes, which were subsequently incorporated into caseinate starch films (Jiménez et al., 2014).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Collection of Plant Samples and Extraction of Plant Extracts:

Hibiscus rosa sinesis, and *Azadirachta indica* obtained from Federal University of Technology, Owerri premises and identified by a taxonomist from the Department of Botany, Federal University of Technology, Owerri were used to prepare the nanoparticles. To prepare leaf extract, the method described by Naseer, Aslam, Khalid, & Chen 2020 was used. Fresh leaves of the different plants were thoroughly washed with tap water, followed by distilled water to remove any contamination. The leaves were air-dried for a week at room temperature (~27°C) and blended to powdered form. About 5g of the powdered leaves was mixed in 500ml of distilled water and then heated at 70°C for 30 minutes, firstly filtered by muslin cloth and then using Whatman filter paper No.1, stirred with magnetic stirrer and then centrifuged at 1200rpm thrice for 5minutes to remove the impurities and heavy biomaterials. The extracts were stored at 4°C till use.

3.2 Synthesis of the Plant-based Nanoparticles:

Silver Nanoparticle (AgNP): Silver nanoparticle was prepared as described by Sohal, O'Fallon, Demokritou & Bello (2017). For 0.5M, 2.5g of silver nitrate was dissolved in 30ml of distilled water and 10ml of the plant extract was added (i.e. 3:1). For 0.1M, 0.5g of silver nitrate was dissolved in 30ml of distilled water and 10ml of the plant extract was added (i.e. 3:1). After which the mixture was continuously stirred with a magnetic stirrer for 15mins which led to colour change. Then the mixtures were centrifuged at 10,000rpm for 10mins thrice to remove impurities and dried in hot-air oven.

Zinc Oxide Nanoparticle (ZnONP): Zinc Oxide nanoparticle was prepared as described by Faisal, Jan, Shah, Khan and Tai (2021). For 1M, 8.9g of zinc nitrate hexahydrate ($Zn(NO_3)_2 .6H_2O$)

was dissolved in 30ml of distilled water and 10ml of the plant extract was added (i.e. 3:1). For 2M, 17.8g of Zinc nitrate hexahydrate was dissolved in 30ml of distilled water and 10ml of the plant extract was added (i.e. 3:1). After which the mixture was continuously heated and stirred with a magnetic stirrer at 60°C for 10mins which led to colour change from light brown to yellowish black indicating the synthesis of zinc oxide nanoparticles. Then, the mixtures were centrifuged at 10,000rpm for 15mins thrice to remove impurities and dried in hot-air oven.

3.3 Characterization of Plant-Based Nanoparticles

Scanning Electron Microscopy(SEM) Analysis: The plant nanoparticles were subjected to SEM analysis to study the surface morphology by employing the method described by Saqib, Munis, Zaman, Ullah & Nasar (2018).

UV-Visible Spectroscopy: The silver nanoparticles (AgNPs) and zinc oxide nanoparticles (ZnONPs) formation was observed through the UV-Visible spectrophotometer. The absorption spectrum and Surface Plasmon Resonance(SPR) of the formed AgNPs and ZnONPs were recorded using a UV-VIS. The UV-Visible spectrophotometer has a quartz cuvette with path length of 1cm. The nanoparticle sample obtained from the leaves was placed in the cuvette. The UV-Visible spectrum is produced between the wavelength ranges of 300-700nm (Ndikau, Noah, Andalaand & Masika, 2017).

Fourier Transform Infra-Red (FTIR) Analysis: The secondary metabolites present in the plant extracts and the functional groups on the AgNPs and ZnONPs were identified by using FTIR characterization technique as described by El-Belely et al., (2021).

X-Ray Diffraction (XRD) Analysis: The structure of the nanoparticles were studied by X-ray diffractometer with Cu K radiation at 25ºC and the structural assignments were made with reference to the JCP DS powder diffraction files as described by Saqib et al.,(2018).

3.4 Fish Sample Collection

Four diseased fish samples were collected each from three different fisheries and ponds in and around Owerri, Imo State which are Johnny Fish Farm, Umuodu Mbieri, Mbaitoli L.G.A., Chucky Fish Farm, 18 Norbert Nworieji Lane New Owerri and Ohaji/Egbema.

The various fish organs for investigation which included the liver, gills and intestines were aseptically separated from the various fishes using sterile dissecting scissors and scalpel blade. About 5g of each sample was weighed out, homogenized and dispensed into sterile bijoux bottles containing 50ml of sterile distilled water to serve as stock solution.

3.5 Isolation of Microorganisms:

Ten-fold serial dilution of each of the original stock solution was done and labelled 10^{-1} to 10^{-10}. Estimation of the bacterial load was determined by the Spread-plate technique as described by Kayser, (2005). An aliquot, 0.1ml of 10^{-5} dilution tube inoculant was assayed on Nutrient agar while 0.1ml of 10^{-3} dilution tube inoculant was assayed on other media and spread with a sterile glass spreader on the various solidified and sterilized agar plates and incubated for 24-48hours at 37°C. After which the number of colonies on the various plates was counted (Tsado, Adesina & Oyeleke, 2013).

3.6 Identification of Test Organisms:

The distinct and discrete colonies on the different media were isolated and purified on nutrient agar by repeated sub-culturing. Their various colonial, morphological characteristics and the following biochemical analyses were carried out on the five most prevalent bacteria test organisms to identify them using the following procedures as described by Cheesbrough (2003).

3.6.1. Gram Staining

Each colony was picked with a sterile wire loop, smeared on a sterile glass slide and fixed by gently heating on a Bunsen burner. Crystal violet solution was then applied on the smear to stain for 2 minutes and then washed off with running water. Lugol's iodine was then poured on the glass slide to act as a mordant for 1 minute and then washed again with running water. Acetone alcohol was then added to act as a decolourizer for 5 seconds. After washing with water, Safranine was added to counter stain and allowed to stain for 2 minutes. The slide was then washed again with running water, blotted and air-dried, after which it was examined under a microscope with high power objective (100X) using immersion oil.

3.6.2 Catalase Test

Aim: it was carried out to distinguish the isolates by their ability to produce catalase enzyme which breaks down hydrogen peroxide (H_2O_2) to produce water (H_2O) and liberate oxygen gas (O_2).

It was done using the Slide method. A sterile Pasteur's pipette was used to place a drop of sterile water on a glass slide. A colony of each isolate was transferred with a sterile wire loop into the water and used to make a thick smear of the culture. 1-2 drops of H_2O_2 were transferred on the

smear with a sterile Pasteur’s pipette and observed for production of gas bubbles. After which result of the observation was recorded.

3.6.3 Indole Test

Aim: it was carried out to distinguish the isolates by their ability to breakdown the amino acid, tryptophan with the enzyme tryptophanase, to produce indole.

The isolates were inoculated into Bijoux bottles containing 3ml of sterile tryptone broth and labelled. After which the Bijoux bottles were incubated at 37ºC for 48hours. Then 0.5ml of the Kovac's reagent was added and gently shook. The bottles were kept at room temperature for 5-10 minutes. The bottles were examined for the formation of a pink/red colour on the surface layer within 10minutes. After which the results were recorded.

3.6.4 Citrate- Utilization Test

Aim: To identify the isolates by their ability to use citrate as their only source of carbon and ammonia as their only nitrogen source.

Simon’s citrate agar slants were prepared in sterile test tubes and allowed to solidify. The Bunsen burner was lit and used to flame the wireloop until it was red hot and then it was allowed to cool. The sterile wireloop was used to take a loopful of each isolate and inserted into the test tubes containing the slant of Simon’s citrate agar but wasn’t allowed to touch the bottom. After which they were incubated for 24hours at 37ºC. They were observed for colour change from green to royal blue and their results were recorded.

3.6.5 Methyl-Red Test

Aim: To identify and differentiate the isolates by their ability to release gas in the fermentation of glucose.

Pure colony of each isolate was added to the 5ml of Methyl-Red Voges Proskaeur (MRVP) broth and incubated at 37°C for 48hours. Then 5-6 drops of Methyl-Red indicator was added. After which changes were observed (if red colour is retained, it is positive while a colour change from red to yellow indicated negative) and results were recorded.

3.6.6 Voges Proskaeur Test

Aim: To identify and differentiate the isolates by their ability to metabolize pyruvate and produce acetoin as the end product of glucose fermentation.

Well isolated colonies of the bacterial isolates were picked using sterile wireloops and inoculated into the broth. For 24hours the tubes were incubated aerobically at 37°C. After the incubation, 2ml of each broth was poured into sterile test tubes, shook and mixed well with 6 drops of 5% alpha-naphthol solution. After which another 2 drops of 40% potassium hydroxide solution was added. Then each of the tubes were shook vigorously and observed for a red-pink colour at the medium surface for 30 minutes after which the results were recorded.

3.6.7 Motility Test

Aim: To identify and differentiate the isolates by their ability to actively move from one place to another.

Motility agar was prepared in different test tubes and allowed to solidify, after which they were labelled. Each isolate was stab inoculated into each tube of the motility agar using sterile

inoculating needle and they were all incubated at 37^oC for 24hours. After which the results were observed and recorded.

3.6.8 Endospore Staining

Aim: To identify and differentiate isolates by their ability to form spores.

Clean grease free slides were used to make smears of the various isolates. After which they were heat-fixed, air-dried and covered with a blotting paper. The blotting paper was saturated with Malachite green stain solution and steamed for 5minutes over a conical flask of boiling water which kept the paper moist and more dye were added as required. The slide was washed with running water and counterstained with 0.5% Safranine for 30seconds. The stain was washed off with running water and the blot was dried. After which the slides were examined under microscope for the presence of endospores which are bright green in colour.

3.7 Inocula Preparation/ Standardization of Isolates:

The inoculum was prepared according to the procedures described by Hudzieki, (2009) and agrees with "National Committee for Clinical Laboratory Standards (NCCLS)" antimicrobial susceptibility testing procedure. The five most prevalent isolates which are *Bacillus* sp., *Enterobacter* sp., *Salmonella* sp., *Shigella* sp. and *Vibrio* sp. were standardized as follows; each organism was freshly prepared in a nutrient broth and left for 12-18 hours. After which the cloudish broth was put in tubes, spinned and washed twice using sterile water. 0.5 McFarland standard was prepared by adding 1%BaCl(0.05ml) in 1%H_2SO_4 (9.95ml) which results to 1.5×10^8 CFU/ml approximately in cell density which is 0.5MFU. Then the washed organism was compared visually with the McFarland standard by adding water until it they matched with the McFarland standard.

3.8 Antimicrobial Susceptibility Testing:

- **Kirby-Bauer Disk Diffusion**

The antibiotic susceptibility test of the bacterial isolates was done by modified Kirby-Bauer Disk Diffusion Susceptibility Test Protocol as described by Hudzieki, (2009) using Mueller Hinton Agar (MHA). Following disc diffusion method, Mueller Hinton Agar was prepared and poured in various sterile agar plates, allowed to solidify after which sterile pipettes were used to take 1ml of each of standardized inocula, fluid the agar surface and spread evenly using a sterile glass spreader and allowed to dry on agar surface. The sterile antibiotic discs were placed on the agar surface and incubated at 37°C for 24 hours. Using the application of the Clinical Laboratory Standard Institute (CLSI) guidelines, the susceptibility or resistance of the isolates to individual antibiotics was determined and recorded.

Determination of the Antibacterial Activity of the Nanoparticles

The antibacterial activity of the nanoparticles were determined by agar-well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) as follows; Mueller Hinton Agar was prepared. The nanoparticles were prepared at varying concentrations (500mg/ml, 250mg/ml, 125mg/ml and 62.5mg/ml) by a two-fold serial dilution where 1g of each nanoparticle was added to 1ml of distilled water creating 1000mg/ml and diluted serially to the concentration. The bacterial isolates were seeded on the Mueller Hinton Agar surface with sterile wire loop and allowed to solidify. After which sterile pipettes were used to create wells of uniform diameters on the agar. Sterile pipette was used to transfer the various concentration of the nanoparticles into the holes using Gentamicin as control. It was allowed on the table for 20-30minutes before being incubated for 24hours at 37°C. After which zones of inhibition were measured and recorded.

- **Minimum Inhibitory Concentration (MIC) using Agar Dilution**

The Minimum Inhibitory Concentration (MIC) of the Nanoparticles were determined as described by Hendriksen (2003) which agrees with NCCLS antimicrobial susceptibility testing procedure. 1ml of peptone water was prepared in sterile Bijoux bottles and 1ml of the nanoparticles at their four different concentrations were added. After which 0.5ml of my standardized inocula were added to all the bottles and incubated for 24hours at 37°C. After incubation, the growth of the bacterial isolates in the test tube were observed as turbidity using spectrophotometer between 100-600nm. The least concentration where no turbidity was observed was determined and noted as the MIC value.

- **Determination of Minimum Bactericidal Concentration (MBC) of the Nanoparticles.**

This was carried out as follows;

Nutrient agar was freshly prepared and poured into various agar plates. After which it was allowed to gel and surface-dried. The plates were divided into four different sections labelled A-D signifying the various concentrations of the nanoparticles. Sterile wireloop was used to pick a loopful of the suspension in each of the MIC bottle and streaked on the various sections of the agar plate according to label. After which the plates were incubated at 37°C for 24hours. Then the growth level was observed, recorded and the MBC was determined.

3.9 Statistical Analysis

The data obtained from the antibacterial activity assays and minimum inhibitory concentration (MIC) determinations were subjected to rigorous statistical analyses, including descriptive statistics, Analysis of Variance (ANOVA), post-hoc tests, regression analysis, and correlation

analysis, to compare the efficacy of plant-based nanoparticles with that of antibiotics against various fish bacterial pathogens.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Nanoparticle Characterization

4.1.1 Fourier Transform Infra-Red (FTIR) Analysis

A recorded spectrum gives the position of bands related to the strength and nature of bonds, and specific functional groups, providing the information concerning molecular structures and interactions. The Infra-Red (IR) spectral for the silver and ZnO nanoparticles prepared using aqueous leaf extracts of Hibiscus and Neem plant is presented in Figures 4.1 – 4.4, while the data and assignment are presented in Table 4.1.

Table 4.1 shows the IR spectra for the two plant leaves used to synthesize both ZnO and Ag nanoparticles. The peaks at > 3100 cm^{-1} are related to OH species with co-adsorbed H_2O on both ZnO and Ag surfaces. The characteristics bands at 2105.9 cm^{-1} and 2117.1 cm^{-1} were attributed to C≡CAlkyne stretch. Allene C=C=C functional group was observed at 1908.4 cm^{-1} for Hibiscus ZnO but not seen in Hibiscus Ag, Neem ZnO and Neem Ag nanoparticles. At wavelength 1625.1 cm^{-1}, 1636.3 cm^{-1}, 1625.1 $cm^{-1}$and 1636.61 cm^{-1} C=C unsaturated compound Alkene stretch functional group was observed for all plant synthesized ZnO and silver nanoparticles. At 1517.0 and 1520.8 bands respectively as observed in the IR for Neem ZnO and Hibiscus Ag represented a bend in the amide functional group (N-H bend), it was not seen in the IR of Neem plant Ag and Neem ZnO.

OH bend was observed at band 1412.7 cm^{-1} which was only seen in Hibiscus Ag nanoparticle. Amine C-N stretch functional group was seen in both neem prepared ZnO and Ag nanoparticle

and hibiscus ZnO respectively at 1341.8 cm^{-1}, 1353.0 cm^{-1} and 1379.1 cm^{-1} but it was totally absent in Hibiscus leaf extract Ag nanoparticle.

CN stretch of aromatic primary amine showed characteristic bands at 1263.6 cm^{-1} only observed in Hibiscus Ag nanoparticle. Alkyl halides were observed at wave length 1006.4 cm^{-1}, 1017.6 cm^{-1} and 1092.1 cm^{-1} for all nanoparticles with the exception of Neem plant Ag nanoparticles.

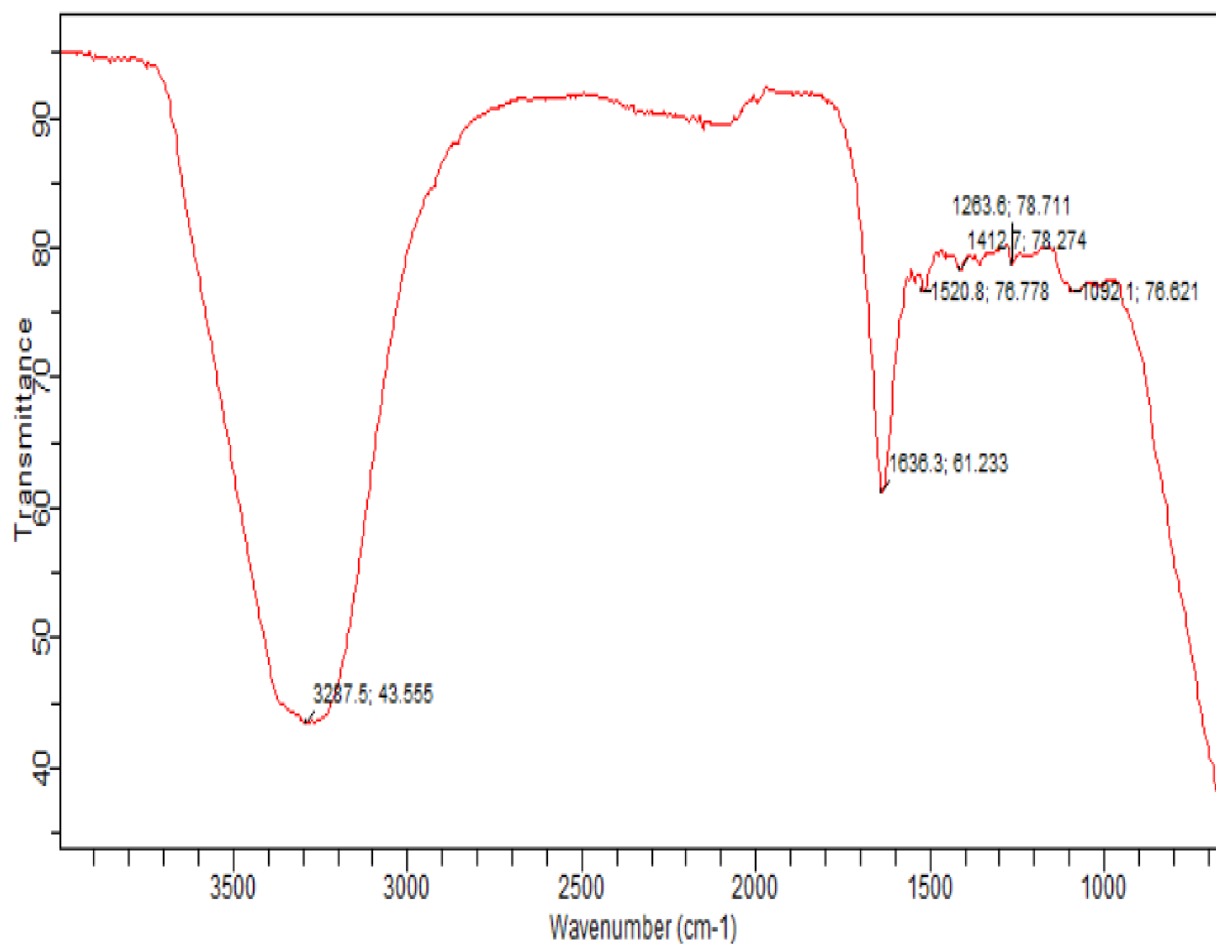


Figure 4.1: Infra-Red spectra for Hibiscus Leaves Silver Nanoparticle

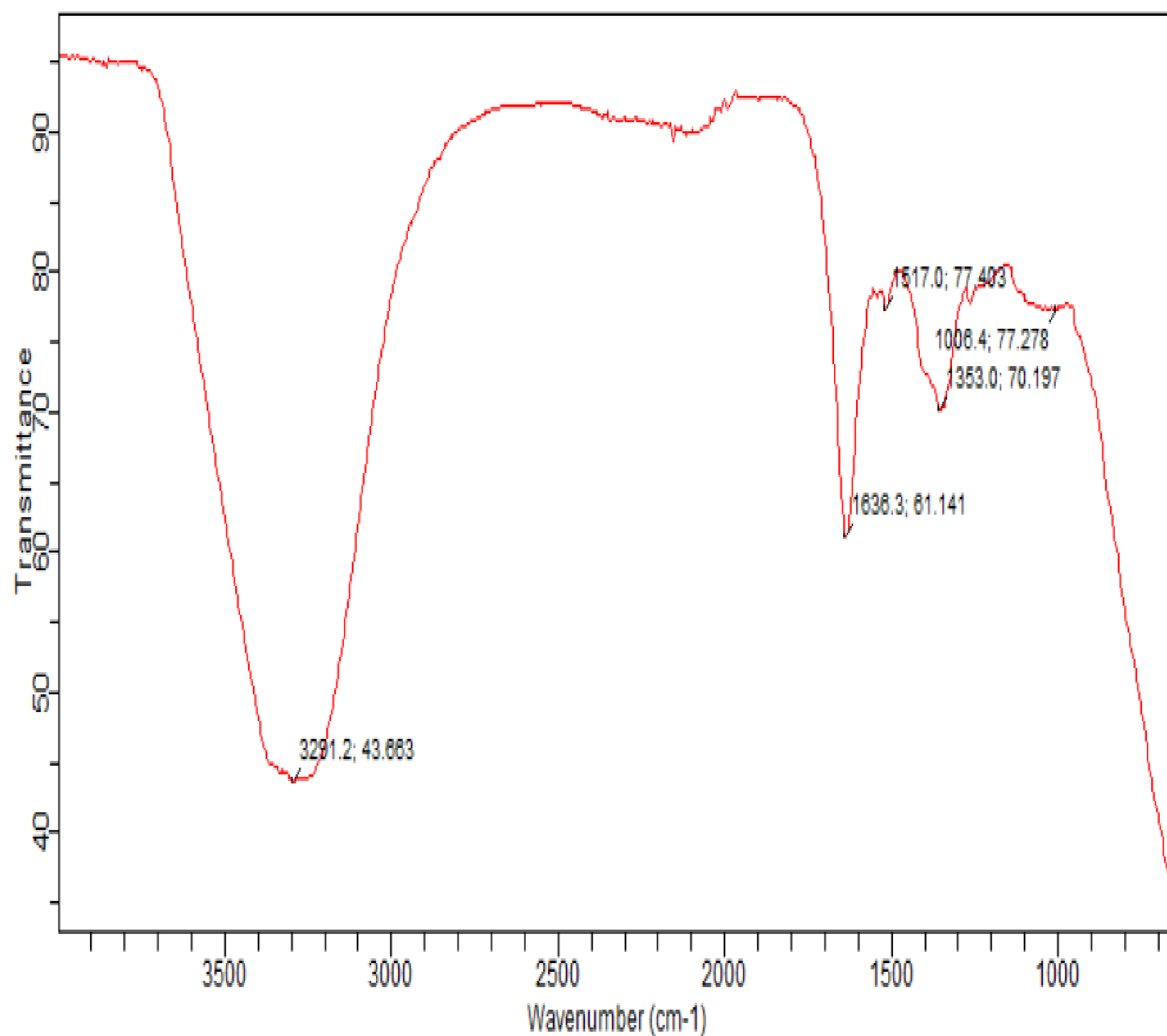


Figure 4.2: IR spectra for Neem Zinc oxide nanoparticle

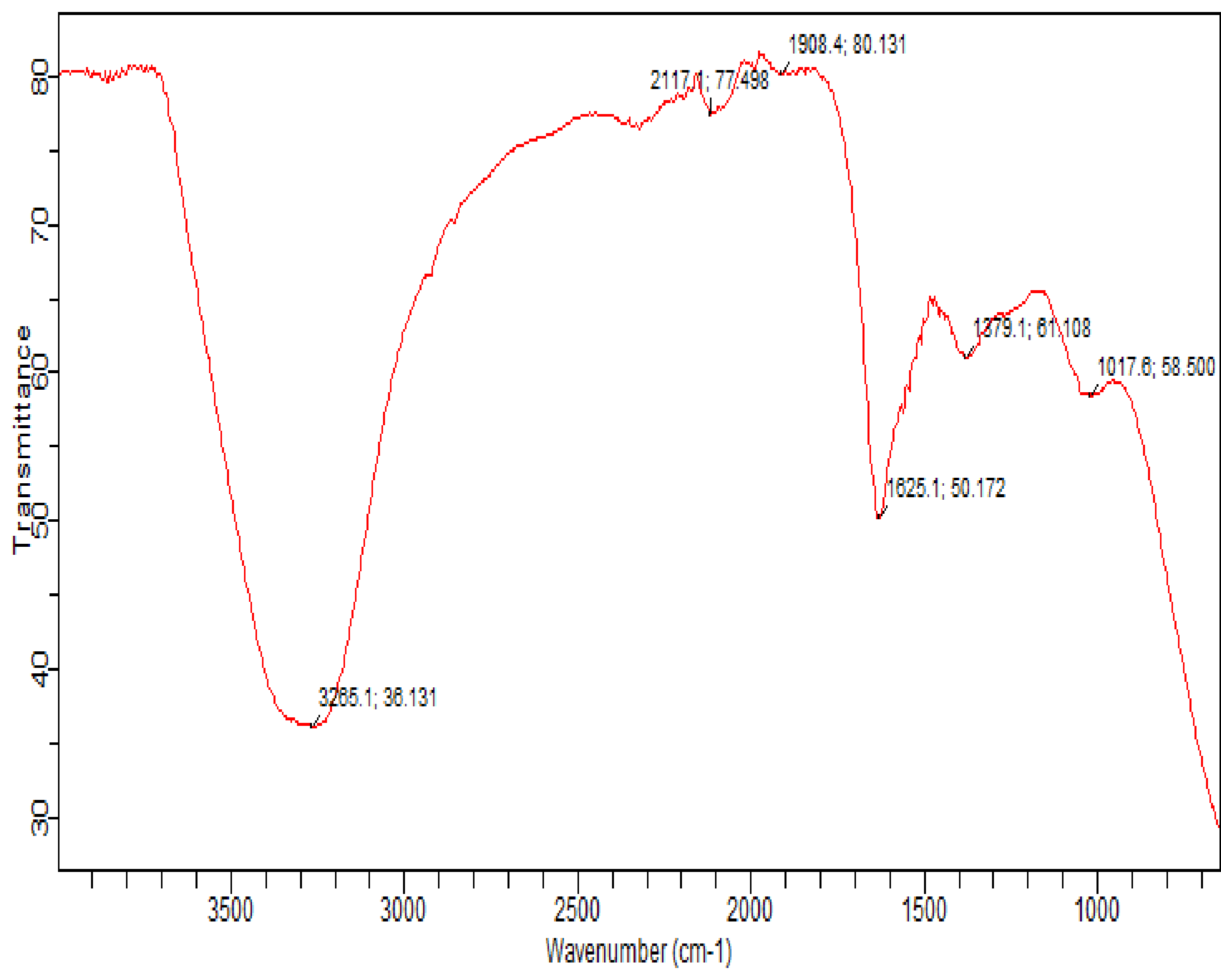


Figure 4.3: IR spectra for Hibiscus Leaves ZnO nanoparticle

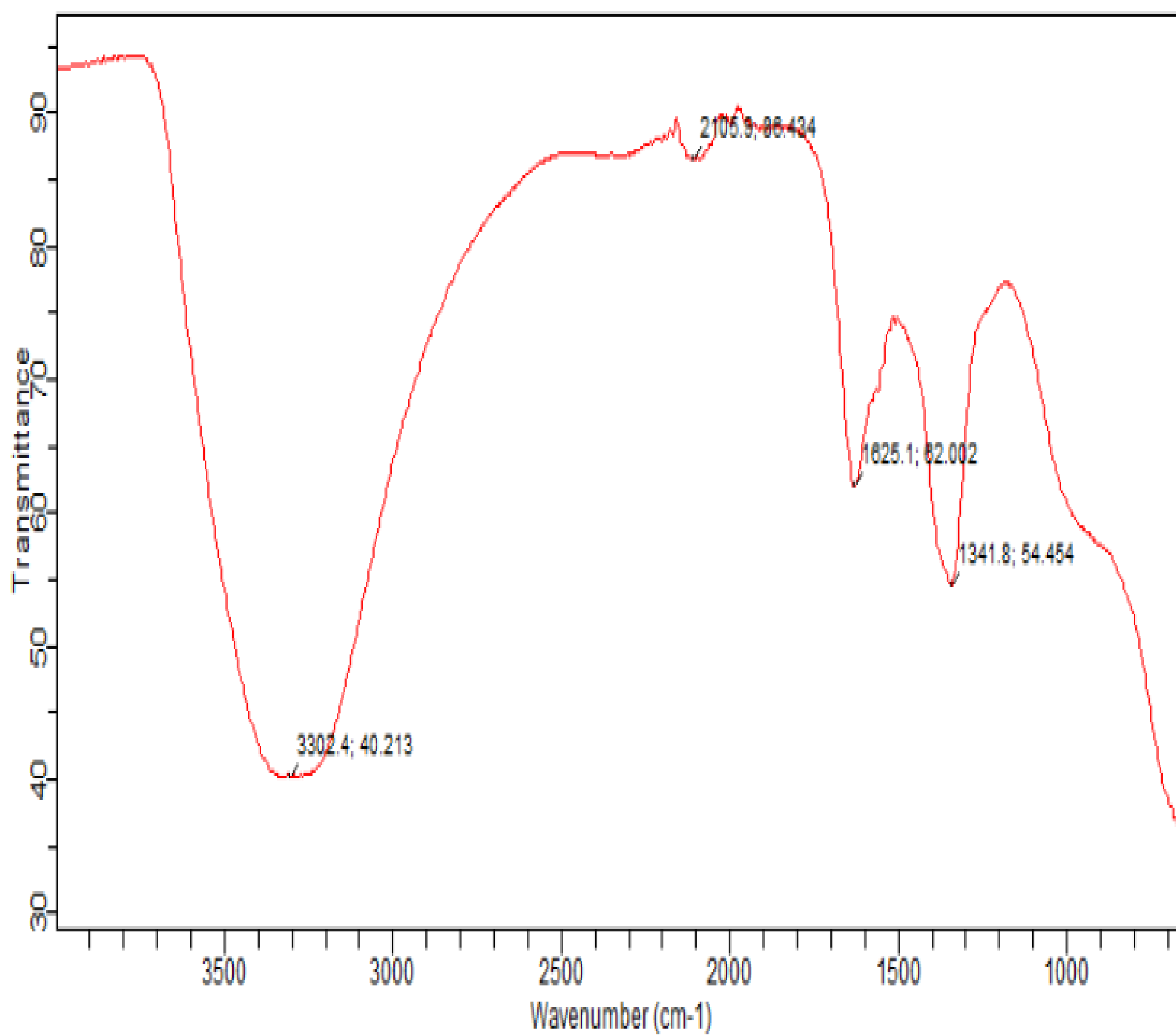


Figure 4.4: IR spectra for Neem Leaves silver nanoparticle

Table 4.1: IR spectral data for the different silver nanoparticle and zinc oxide nanoparticle for Neem and Hibiscus leaves extract

Assignment	Neem Ag	Neem ZnO	Hibiscus ZnO	Hibiscus Ag
Alkyl halides	-	1006.4	1017.6	1092.1
CN stretch	-	-	-	1263.6
amine C-N stretch	1341.8	1353.0	1379.1	-
OH bend Phenols or tertiary alcohol	-	-	-	1412.7
Amides N-H bend	-	1517.0	-	1520.8
C=O stretching vibration	1625.1	1636.3	1625.1	1636.61
H aromatic alkenes	-	-	1908.4	-
Alkynes	2105.9	-	2117.1	-
O-H stretch with H bonded	3302.4	3291.2	3265.1	3287.5

4.1.2 Scanning Electron Microscopy (SEM-EDX)

The surface morphologies of biosynthesized ZnO and Ag nanoparticles were studied by using SEM, and the results are presented in Figures 4.5 to 4.8. To gain further insight into the features of the biosynthesized Ag Nanoparticles, the analysis of the sample was performed using EDX techniques.

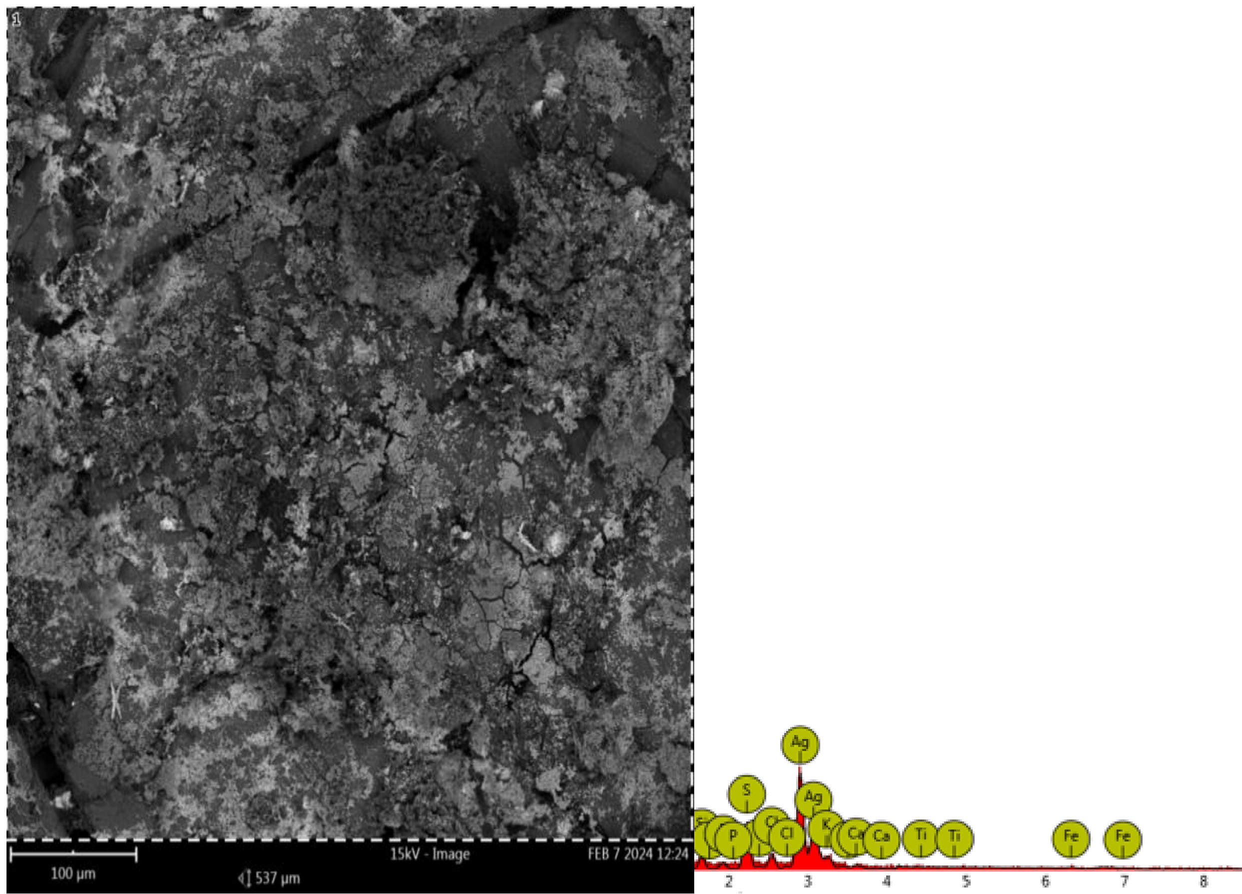


Figure 4.5: SEM-EDX for Ag nanoparticle Hibiscus leaves

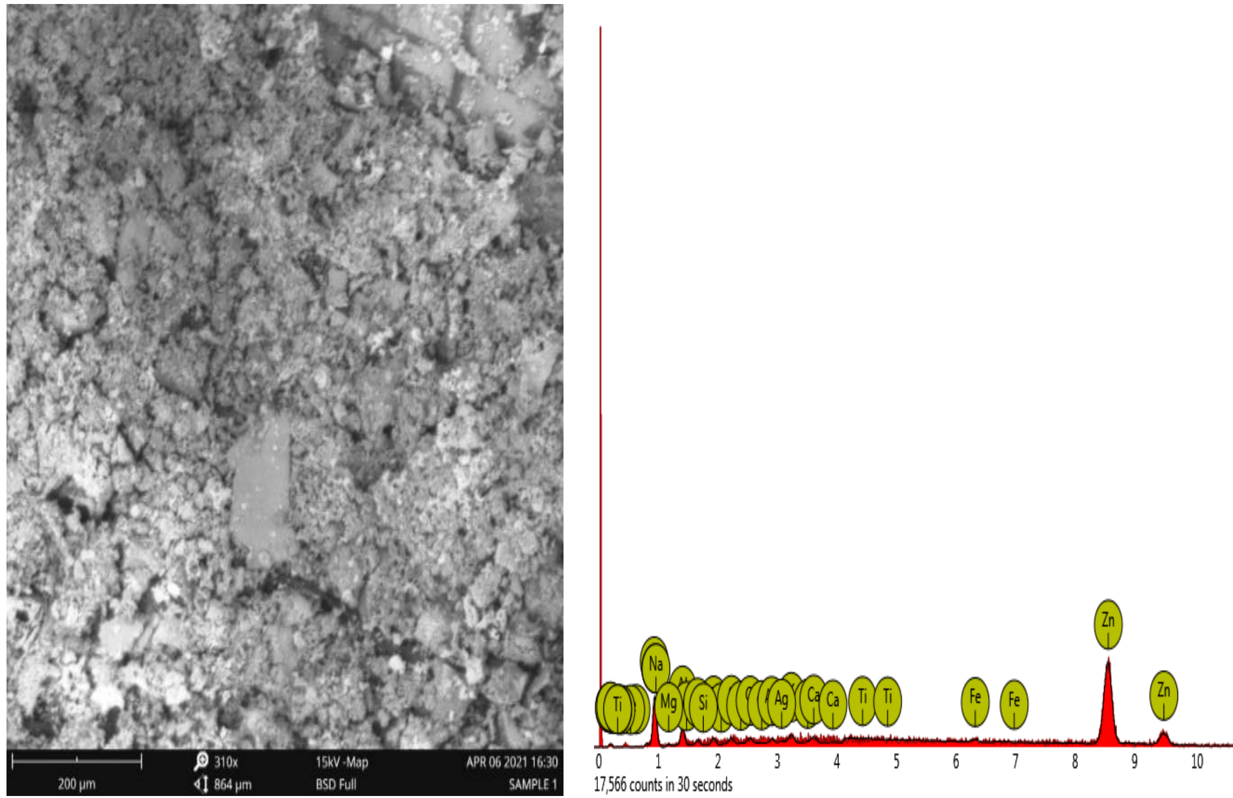


Fig 4.6: SEM-EDX for ZnO nanoparticle Hibiscus leaves extract

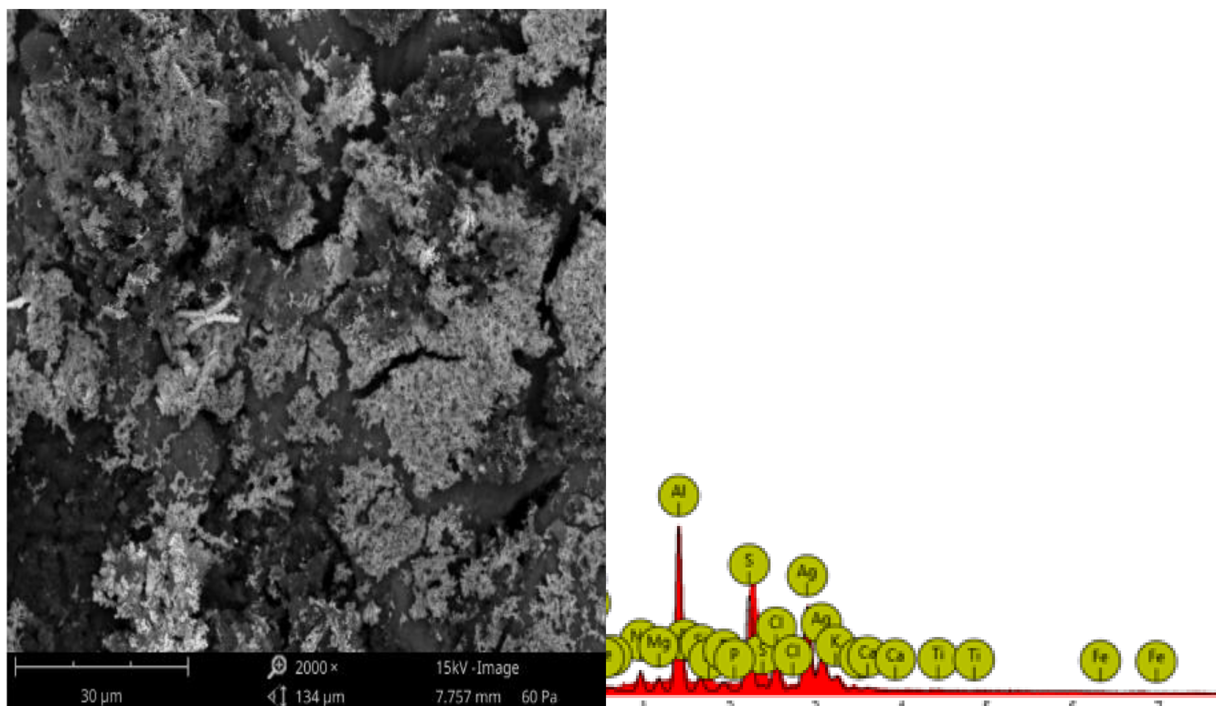


Figure 4.7: SEM-EDX for Silver nanoparticle on Neem leaves extract

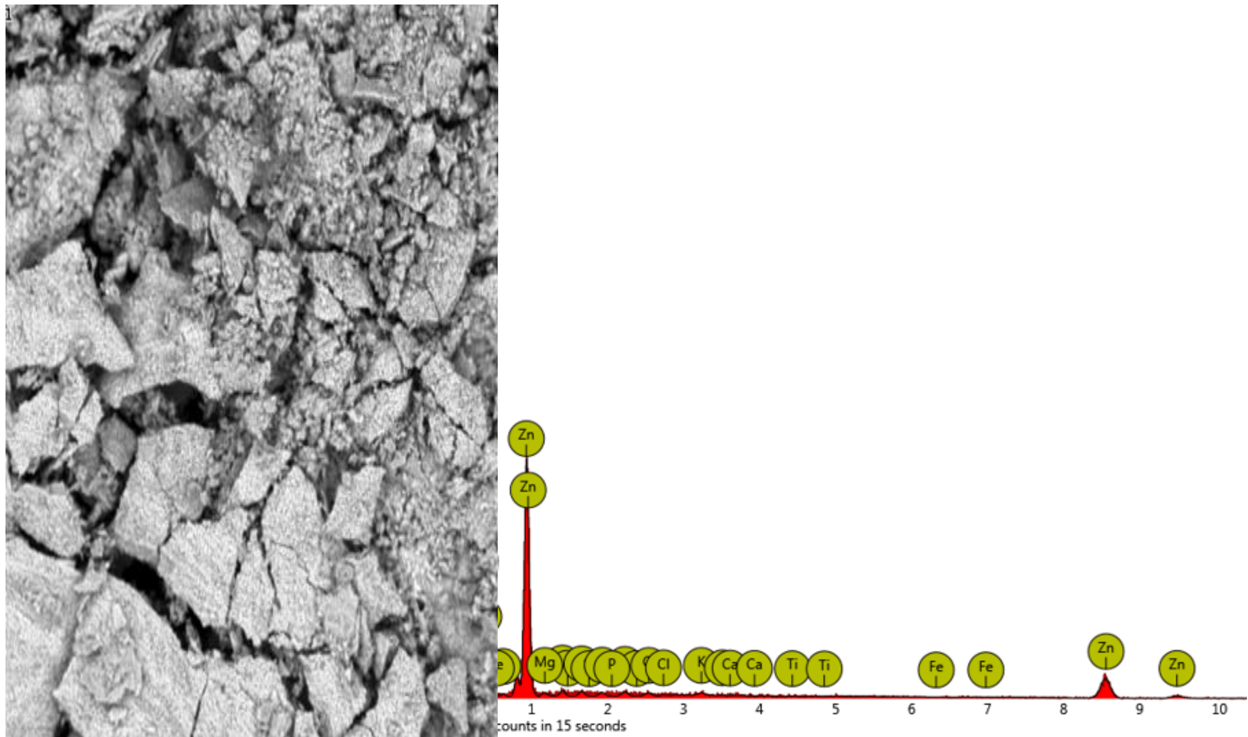


Figure 4.8: SEM-EDX for Zinc oxide nanoparticle using Neem leaves extract

4.1.3 X-ray diffraction analysis (XRD)

The XRD spectral for the different synthesized ZnO and silver nanoparticles are presented in Figures 4.9 to 4.12. from table 4.3, the synthesized silver nanoparticle from hibiscus plant extract, the diffraction peaks appeared at a 2θ value of ≈ 28.5478 °, 32.9773 °, 39.0551 ° and 46.9244 ° corresponding to (1 0 0), (1 0 1) and (1 1 1) crystal planes respectively. For the synthesized ZnO nanoparticle from hibiscus extract, the diffraction peaks appeared at a 2θ value of ≈ 6.7502 °, 16.4633 °, 30.4261 ° and 38.8336° corresponding to (1 0 0), (1 0 1) and (1 1 1) crystal plane. For the synthesized silver nanoparticle using neem plant extract the diffraction peaks appeared at a 2θ value of ≈ 34.7503 °, 47.8691 ° and 56.8981 ° which corresponded to (1 0 0), (1 0 1) and (1 1 1) crystal planes respectively. For the synthesized Zinc oxide nanoparticles using neem plant extract the diffraction peaks appeared at a 2θ value of ≈ 20.3510 °, 22.2353 ° and 31.0238 ° which corresponded to (1 0 0) and (1 0 1) crystal planes respectively.

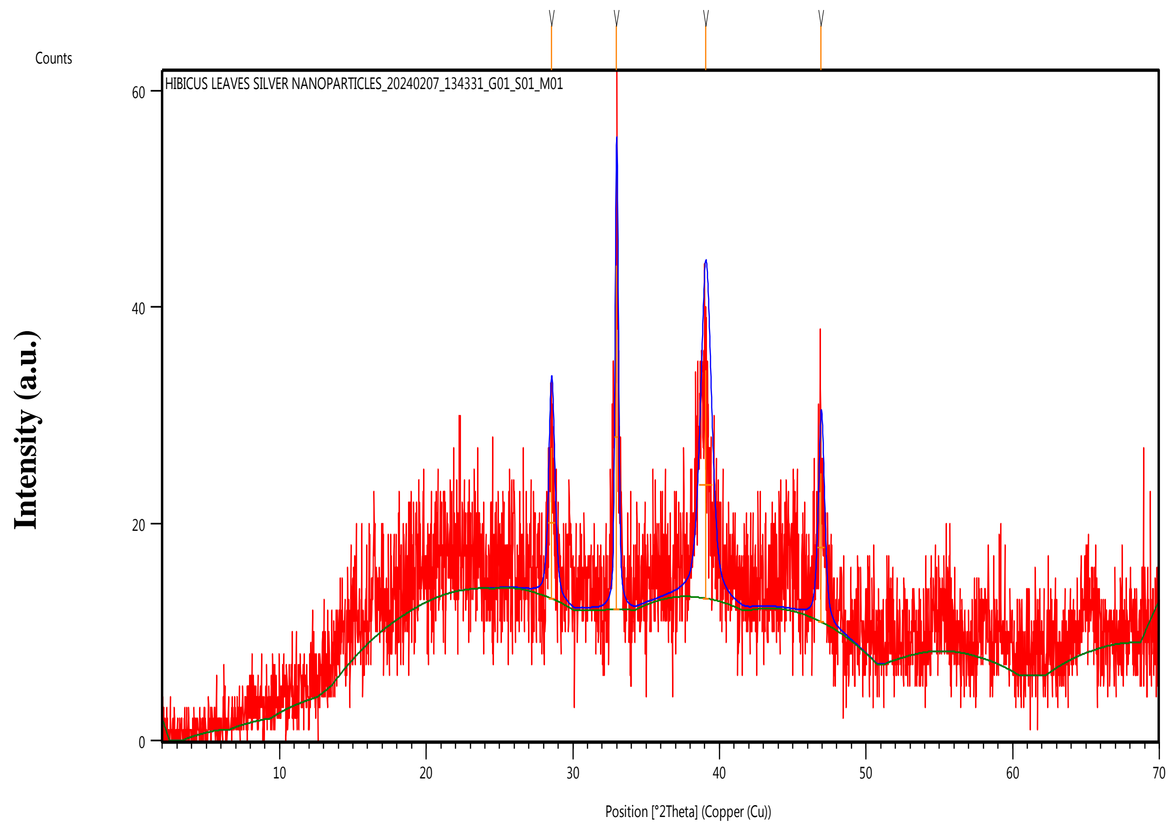


Figure 4.9: XRD spectra for Silver nanoparticle from Hibiscus plant leaf extract

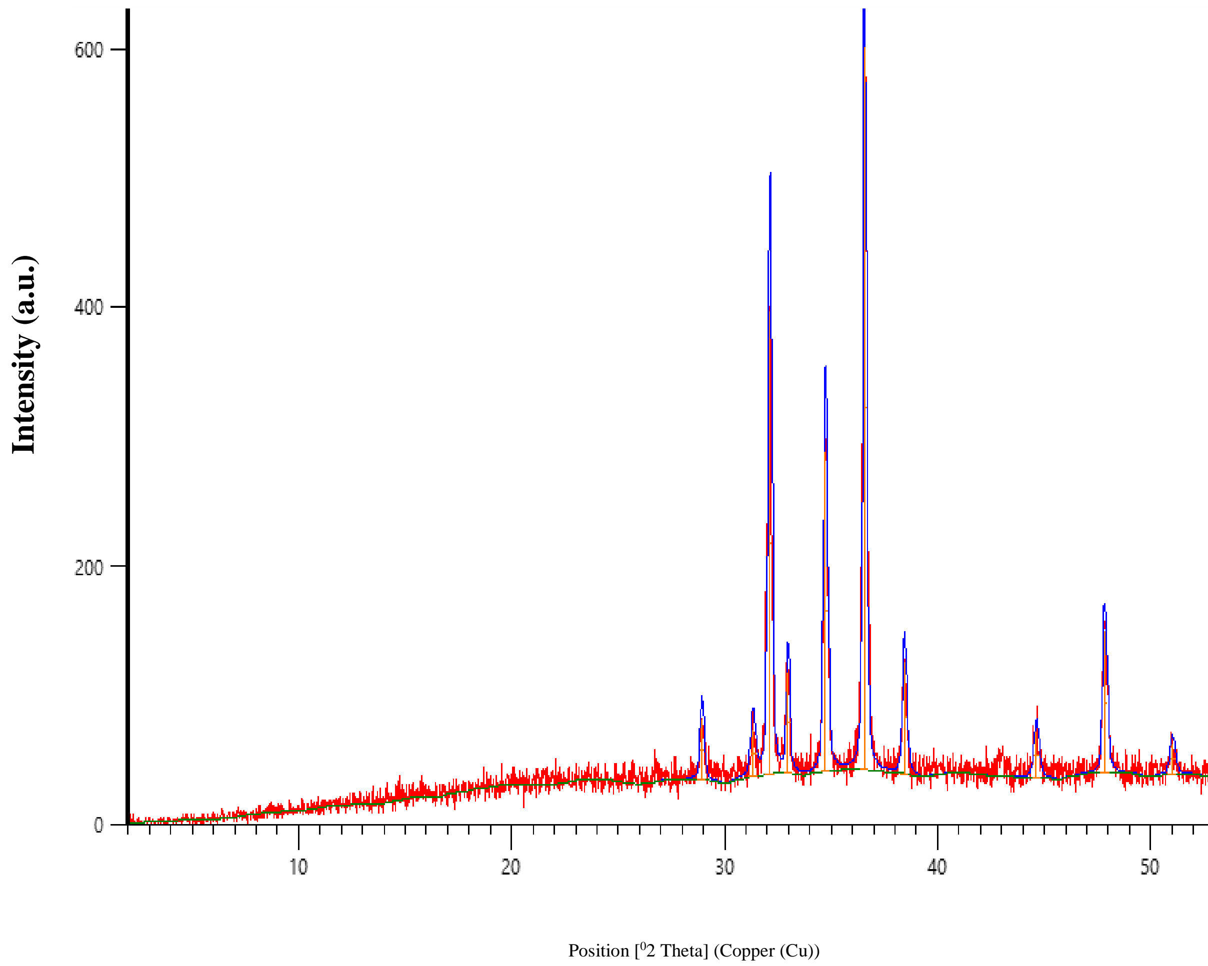


Figure 4.10: XRD spectra for ZnO nanoparticle from Hibiscus plant leaf extract

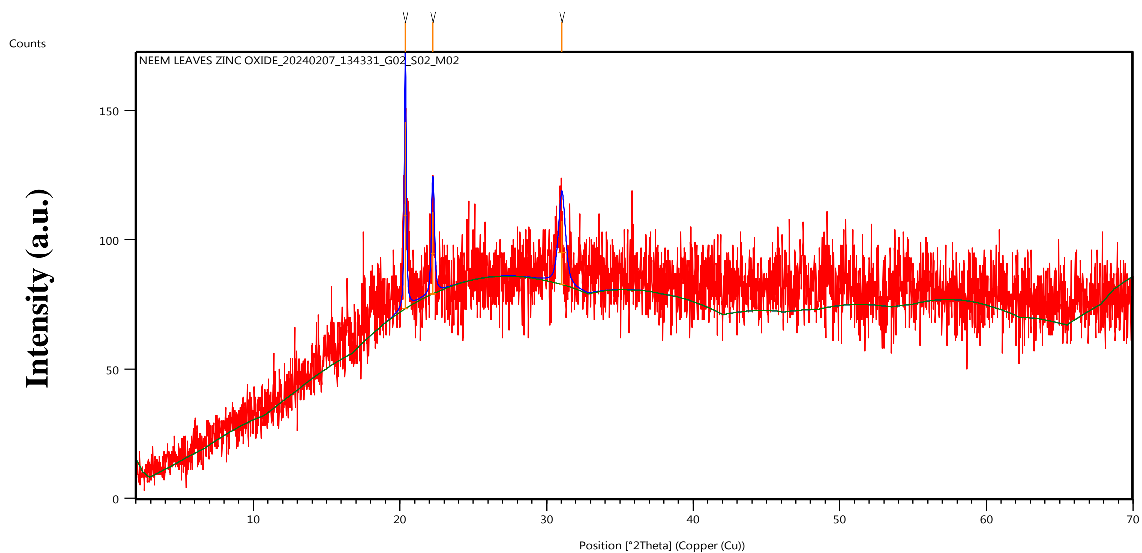
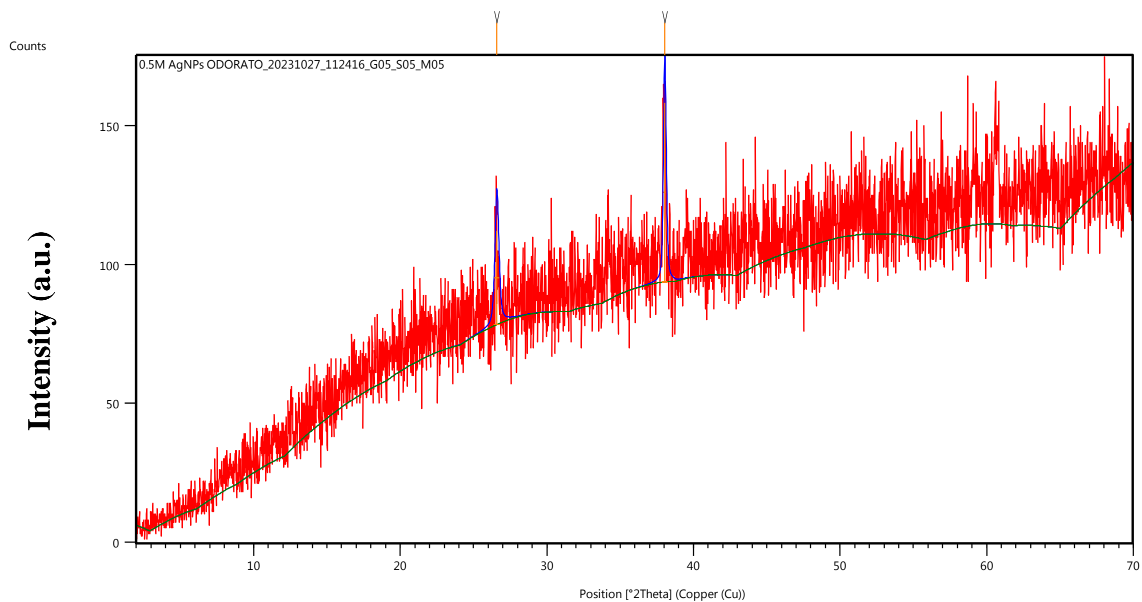


Figure 4.11: XRD spectra for ZnO nanoparticle from Neem leaves extract



4.12: XRD spectra for Ag nanoparticle from Neem plant leaves extract

Table 4.2: Full Width at Half Maximum (FWHM) values, average crystallite sizes calculated using Scherrer's formula, d-spacing, and Bragg's diffraction degree of ZnO NPs synthesized

h k l	Bragg's diffraction [°2Θ]	Full width at half maximum (FWHM) (degrees)	d-spacing [Å]
Silver + hibiscus plant leaf extract			
1 0 0	28.5478	0.4723	3.12680
1 0 1	32.9773	0.2362	2.71624
1 0 1	39.0551	0.8659	2.30640
1 1 1	46.9244	0.4723	1.93633
Average crystallite size = 15.40 nm			
ZnO + hibiscus plant leaf extract			
1 0 0	6.7502	0.3149	13.09505
1 0 0	16.4633	0.0590	5.38455
1 0 1	30.4261	0.1680	2.93549
1 1 1	38.8336	0.0960	2.32288
Average crystallite size = 64.85 nm			
ZnO + Neem plant leaf extract			
1 0 0	20.3510	0.1574	4.36387
1 0 0	22.2353	0.2362	3.99814
1 0 1	31.0238	0.5510	2.88267
Average crystallite size = 30.26 nm			

Ag + Neem plant leaf extract			
1 0 0	34.7503	0.0787	2.58161
1 1 0	47.8691	0.2362	1.90030
1 1 1	56.8981	0.2755	1.61833
Average crystallite size = 40.41 nm			

4.1.4 Ultra Violet-Visual analysis spectra of biosynthesied silver and ZnO nanoparticles

The UV-Vis analysis was used to determine the electronic transition in the synthesized silver and ZnO nanoparticles. The energy absorbed in the ultraviolet/ visible region produced changes in the electronic energy of the compound resulting from transition of valence electrons in the silver and ZnO nanoparticles. These transitions consist of the excitation of an electron from a filled molecular orbital (usually a non-bonding (n) or a bonding $\pi \rightarrow \pi^*$ molecular orbital of an unfilled molecular orbital. The UV-Vis spectra for the synthesized ZnO and silver nanoparticles are presented in Figures 4.13 to 4.16.

Strong absorbance was observed at 428.7 nm and 416.8 nm for silver nanoparticles synthesized from neem and hibiscus leaf extracts respectively. Also, Strong absorbance was observed at 366.40 nm, and 356.0 nm for ZnO nanoparticles synthesized from hibiscus leaf and neem extracts respectively.

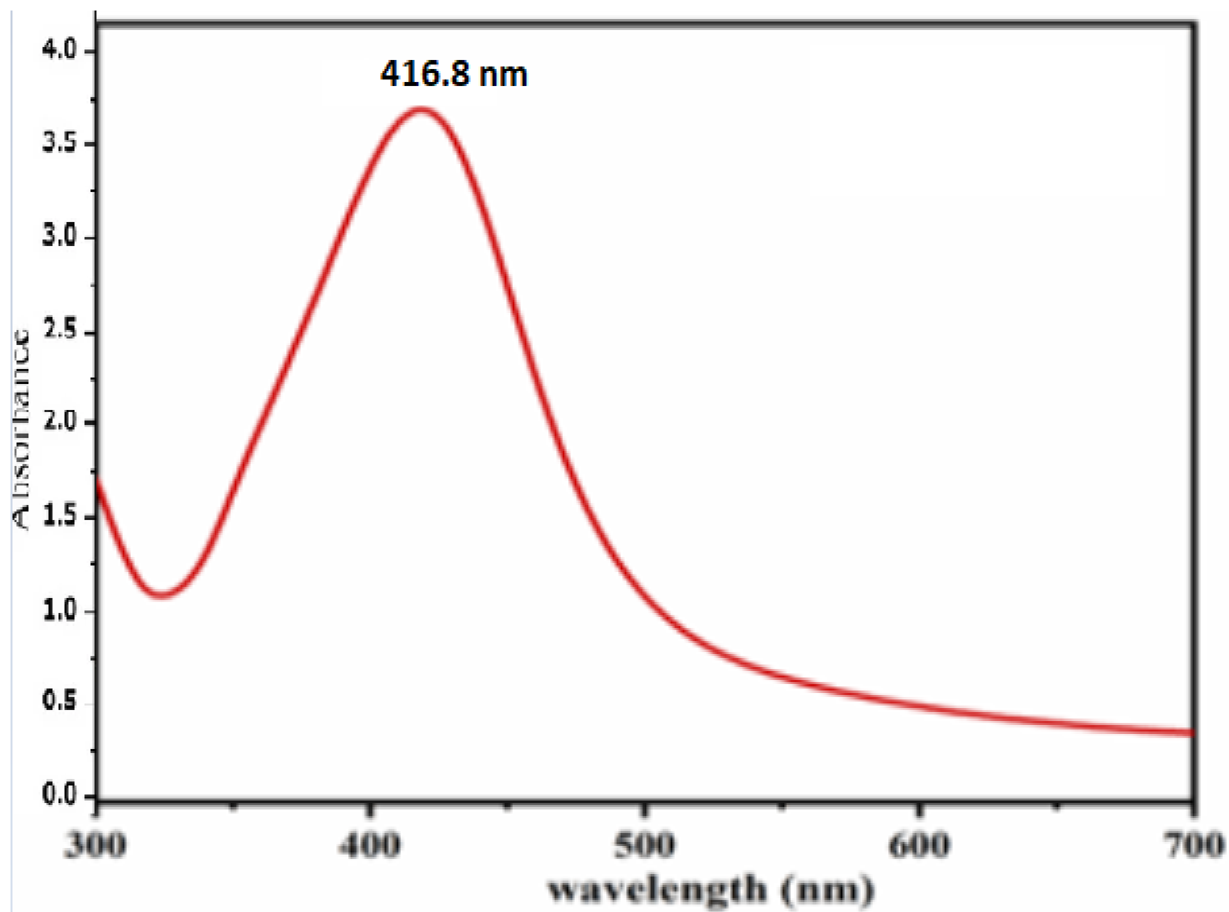


Figure 4.13: UV-Vis spectra for silver nanoparticle from Hibiscus plant leaves extract

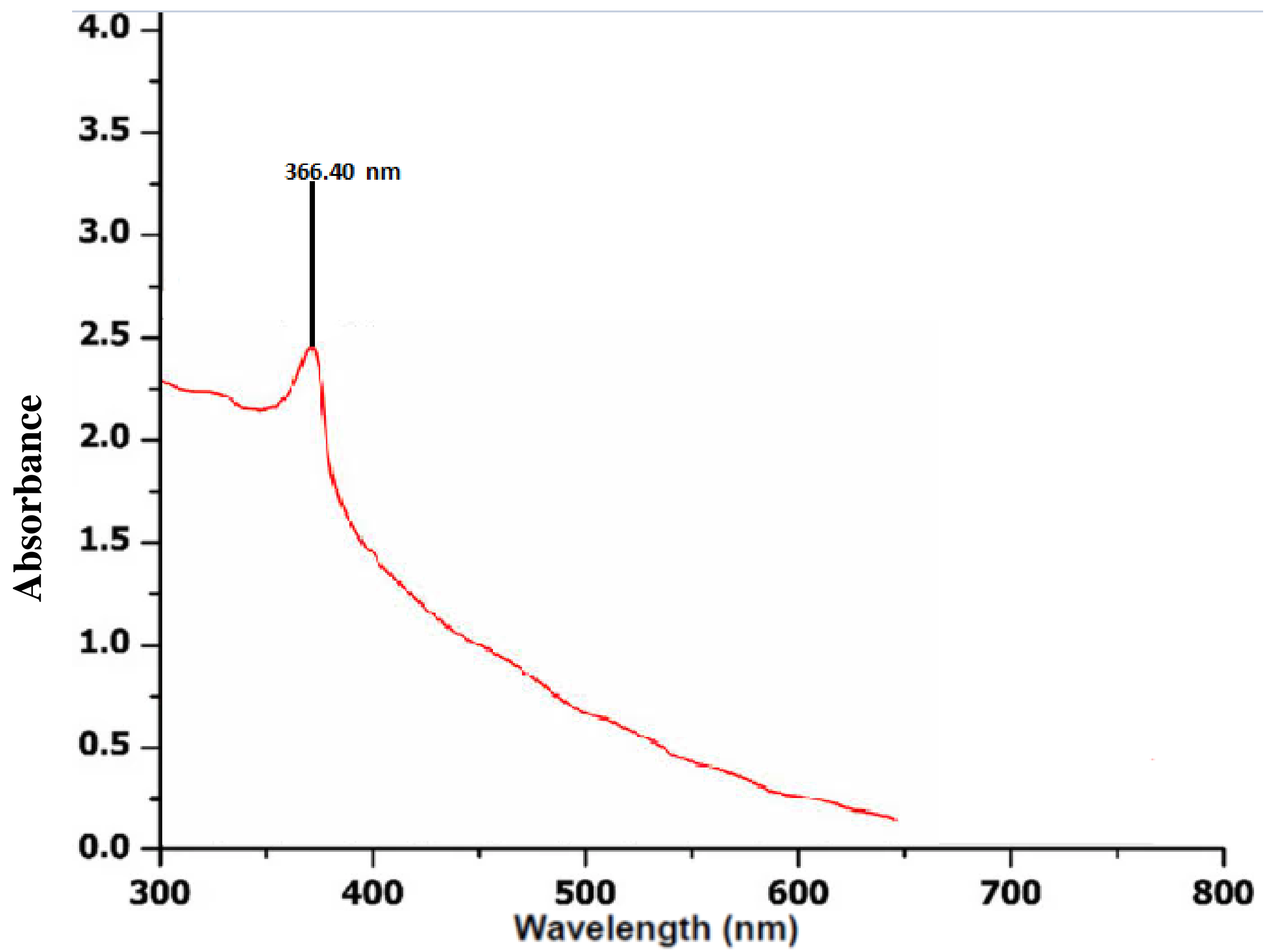


Figure 4.14: UV-Vis spectra for ZnO nanoparticle from Hibiscus plant leaves extract

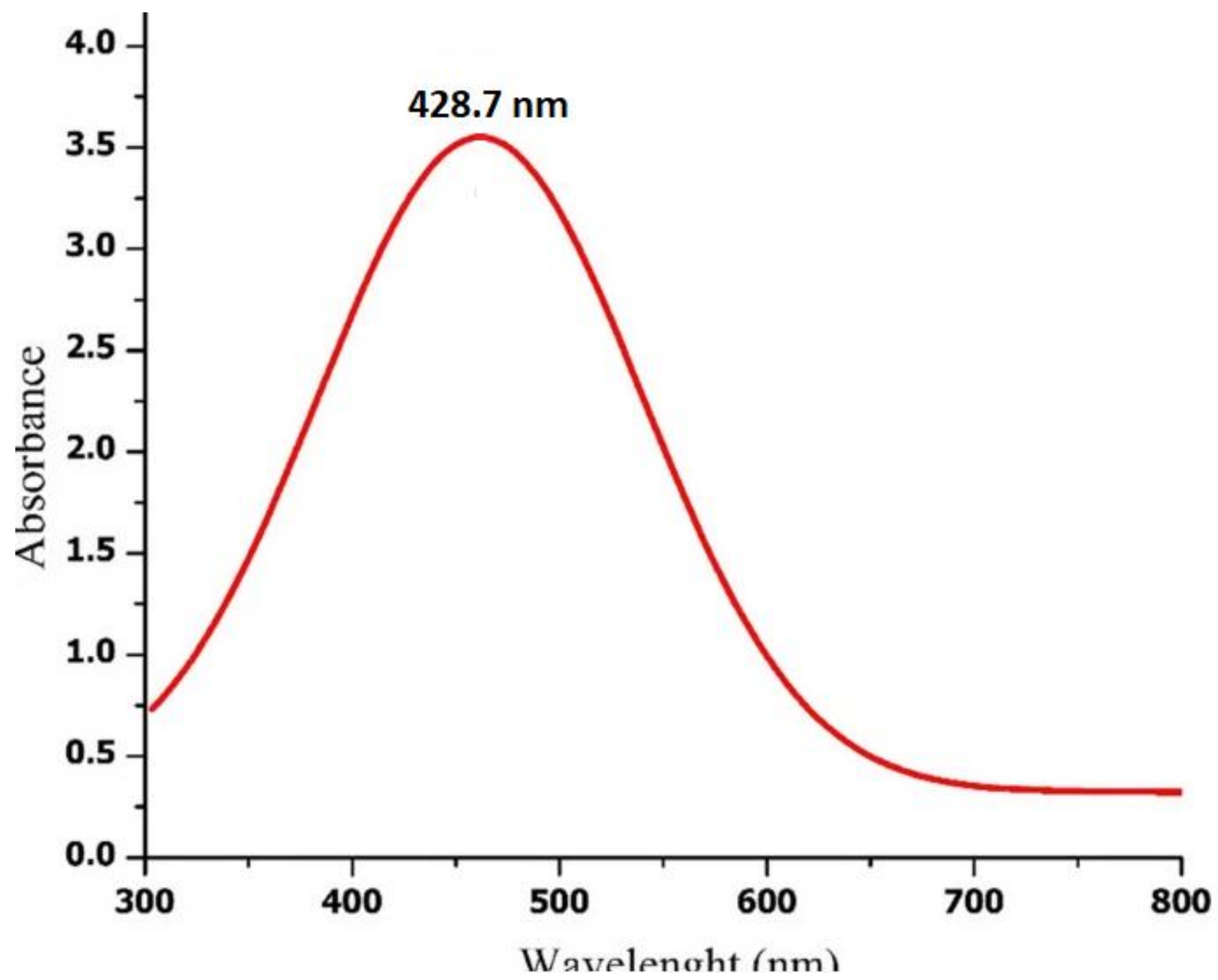


Figure 4.15: UV-Vis spectra for silver nanoparticle from Neem plant leaves extract

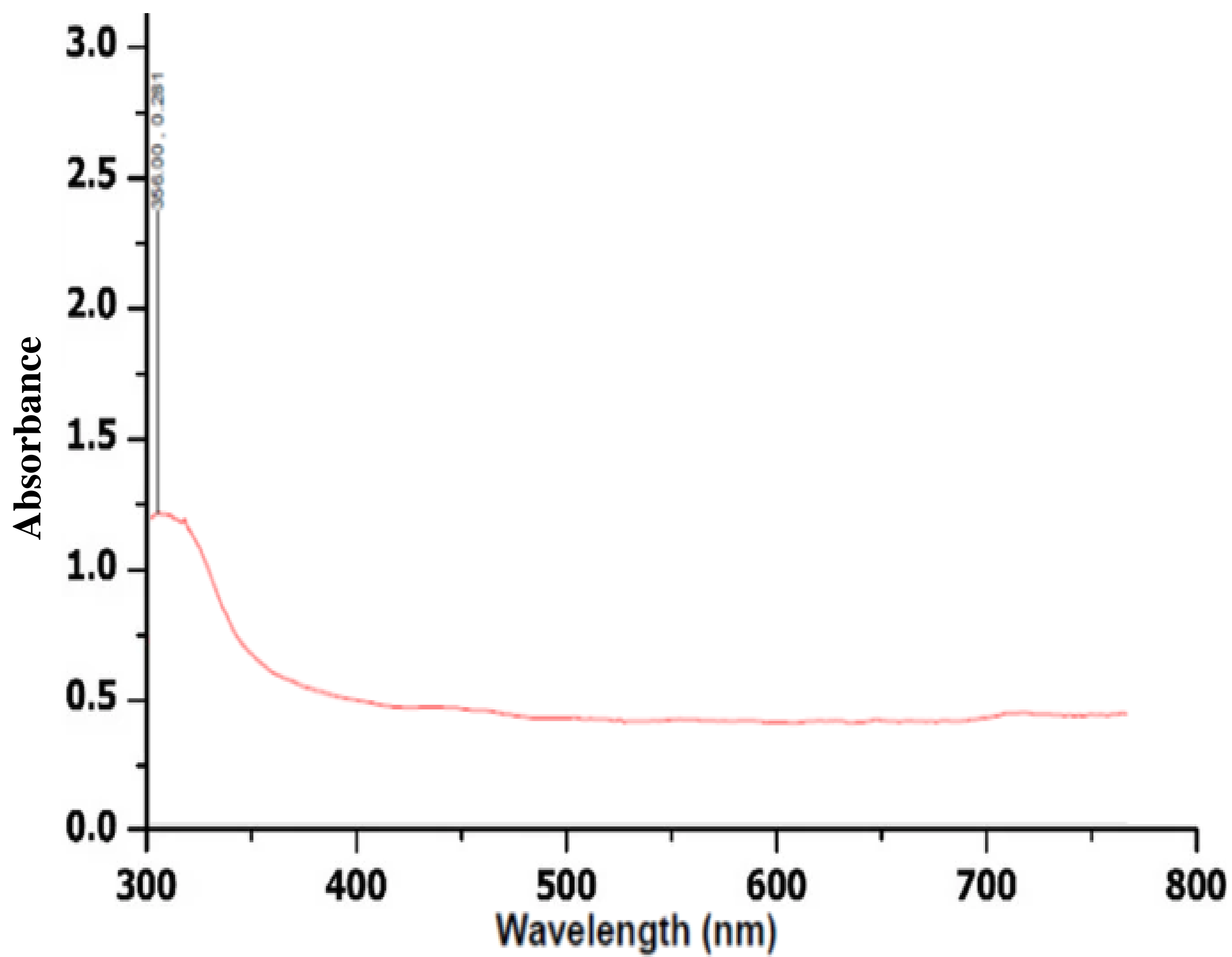


Figure 4.16: UV-Vis spectra for ZnO nanoparticle from Neem plant leaves extract

4.2 Morphological Characteristics of Bacterial Isolates

Based on the morphological characteristics of bacterial isolates observed on different selective and differential media (Salmonella Shigella Agar, Mannitol Salt Agar, MacConkey Agar, Eosin Methylene Blue Agar and Thiosulphate Citrate Bile Salt Agar), Gram-Staining, Endospore Staining and Biochemical reactions, *Salmonella* sp., *Shigella* sp., *Vibrio* sp., *Enterobacter* sp. and *Bacillus* sp. were isolated and identified as shown in Table 4.3.

Table 4.3 COLONIAL AND BIOCHEMICAL MORPHOLOGY OF ISOLATES FROM GILLS, INTESTINE AND LIVER OF DISEASED FISH SAMPLES

Colonial Characteristics	Gram-Staining Reaction	Motility/Spore Formation	MR	VP	Catalase	Indole	Citrate	Most Probable Identity
Tiny, light pink mucoid colonies	G-ve rods	+/-	-	+	+	-	+	*Enterobacter* sp.
Transparent colourless colonies	G-ve rods	-/-	-	-	+	+	-	*Shigella* sp.
Circular transparent colonies with black spots in their middle	G-ve rods	-/+	+	-	+	-	-	*Salmonella* sp.
Flat yellow colonies	G-ve rods	+/-	-	+	+	+	+	*Vibrio* cholerae
Pink colones with finger-like projections	G+ve rods	+/+	-	+	+	-	+	*Bacillus* sp.

Key: + = Positive**,** _ = Negative**,** G-ve = Gram negative**,** G+ve= Gram positive**,** MR= Methyl red**,** VP= Voges Proskauer

Bacterial Count

The Mean Salmonella and Shigella Count (cfu/g) of the different organs of catfish collected from the three different locations are shown in Table 4.5. The microbial count for *Salmonella* and *Shigella* on all the samples range from 0.102×10^6 to 3.25×10^6 cfu/g. The organs from location A had the highest *Salmonella* and *Shigella* count while the organs from location B had the lowest count.

The Mean Coliform Bacteria Count (cfu/g) of the different organs of catfish collected from the three different locations are shown in Table 4.4. The microbial count for coliforms on all the samples ranges from 0.105×10^6 to 855×10^6 cfu/g. The organs from location A had the highest coliform bacteria count compared to organs from location B and C.

The Mean Heterotrophic Bacteria Count (cfu/g) of the different organs of catfish samples collected from the three different locations are shown in Table 4.6. The heterotrophic bacteria count on all the samples ranges from 1.15×10^6 to 970×10^6cfu/g. The organs from location A had the highest heterotrophic bacteria count compared to organs from location B and C.

The Mean *Vibrio* sp. Count (cfu/g) of the different organs of catfish collected from the three different locations are shown in Table 4.7. The *Vibrio* sp. count on all the samples ranges from 0.087×10^6 to 5.42×10^6cfu/g. The organs from location B had the lowest *Vibrio* sp. count when compared to organs from location A and C.

Total bacterial count of the gills, liver and intestine of the various catfish gotton from three different locations

Table 4.4: Mean ± SD coliform bacteria count (cfu/g) of the different parts catfish

Parts of catfish	**Ohaji/Egbema fishery**	**Johnny fish farm**	**Chucky fish farm**
Gills	$855 \pm 577 \times 10^6$	$0.105 \pm 0.027 \times 10^6$	$2.06 \pm 0.51 \times 10^6$
Liver	$460 \pm 294 \times 10^6$	$0.987 \pm 0.034 \times 10^6$	$2.12 \pm 0.24 \times 10^6$
Intestine	$202 \pm 150 \times 10^6$	$0.188 \pm 0.042 \times 10^6$	$1.99 \pm 0.17 \times 10^6$

Table 4.5: Mean ± SD *Samonella* and *Shigella* count (cfu/g) of the different parts catfish

Parts of catfish	Ohaji/Egbema fishery	Johnny fish farm	Chucky fish farm
Gills	3.25 ± 2.39 $x10^6$	0.11 ±0.07 $x10^6$	1.78 ±0.55 $x10^6$
Liver	1.50 ± 1.15 $x10^6$	0.73 ±0.07 $x10^6$	0.85±0.49 $x10^6$
Intestine	1.26± 1.01 $x10^6$	0.12 ± 0.07 $x10^6$	1.24 ± 0.16 $x10^6$

Table 4.6: Mean Heterotrophic bacterial count ± SD (cfu/g) of the different parts of catfish

Parts of catfish	Ohaji/Egbema fishery	Johnny fish farm	Chucky fish farm
Gills	$970 \pm 320 \times 10^6$	$1.37 \pm 0.54 \times 10^6$	$259 \pm 46 \times 10^6$
Liver	$490 \pm 58 \times 10^6$	$1.15 \pm 0.49 \times 10^6$	$93 \pm 12 \times 10^6$
Intestine	$500 \pm 55 \times 10^6$	$1.57 \pm 0.84 \times 10^6$	$257 \pm 20 \times 10^6$

Table 4.7: Mean *Vibro* spp. count ± SD (cfu/g) of the different parts of catfish

Parts of catfish	Ohaji/Egbema fishery	Johnny fish farm	Chucky fish farm
Gills	$5.42 \pm 5.09 \times 10^6$	$0.013 \pm 0.007 \times 10^6$	$1.95 \pm 0.73 \times 10^6$
Liver	$1.60 \pm 0.72 \times 10^6$	$0.087 \pm 0.035 \times 10^6$	$1.32 \pm 0.58 \times 10^6$
Intestine	$1.70 \pm 1.69 \times 10^6$	$0.14 \pm 0.06 \times 10^6$	$2.03 \pm 0.50 \times 10^6$

4.3 Antibiotic profiles of the isolates

Table 4.8 shows varying susceptibility among the isolated organisms. The chi-square test did not reveal any statistically significant differences (p-value >= 0.05) in overall antibiotic sensitivity between the fish-isolated organisms. However, the data suggests some interesting trends: *Bacillus* isolates were susceptible only to ciprofloxacin (5µg) and gentamicin (30µg); *Enterobacter* was resistant to all antibiotics tested except gentamicin; *Salmonella* and *Vibrio* were sensitive to 60% of the tested antibiotics (ciprofloxacin, amikacin, and gentamicin), but resistant to chloramphenicol and tetracycline; *Shigella* was resistant to most antibiotics (chloramphenicol, tetracycline, and amikacin) but susceptible to ciprofloxacin (5µg) and gentamicin (30µg).

Table 4.8 Showing the mean result of Antibiotic Susceptibility Tests of three independent experiments (n=3) on the isolates according to Clinical Laboratory Standard International (CLSI) guideline.

Isolates	CIP (5ug)	AK (30ug)	C (30ug)	TE (30ug)	CN (30ug)
Bacillus sp.	S	R	R	R	S
Enterobacter sp.	R	R	R	R	S
Salmonella sp.	S	S	R	R	S
Vibrio sp.	S	S	R	R	S
Shigella sp.	S	R	R	R	S

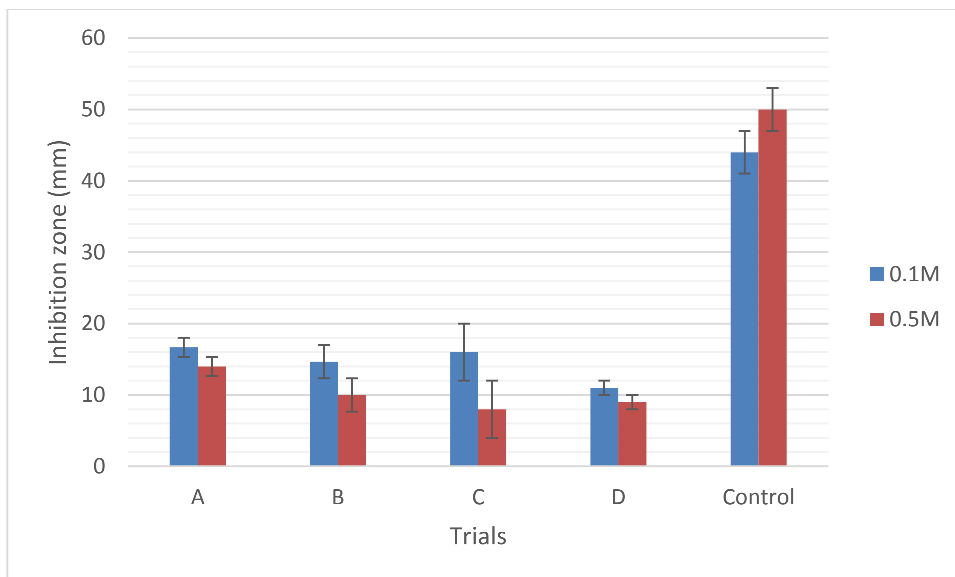
CIP = Ciprofloxacin, AK = Amikacin, C = Chloramphenicol, TE: Tetracycline, CN = Gentamicin

4.3.1 Antibacterial Activity and Minimum Inhibitory Concentrations and Minimum Bactericidal Concentration of the leaf extracts against the isolates

Figure 4.17 to 4.36 present the descriptive statistics for the antibacterial activity (inhibition zone diameter, mm) across various treatments and bacterial strains. These statistics offer insights into the central tendency and variability of the data, enabling a preliminary comparison of the effectiveness of different nanoparticle concentrations and the antibiotic control.

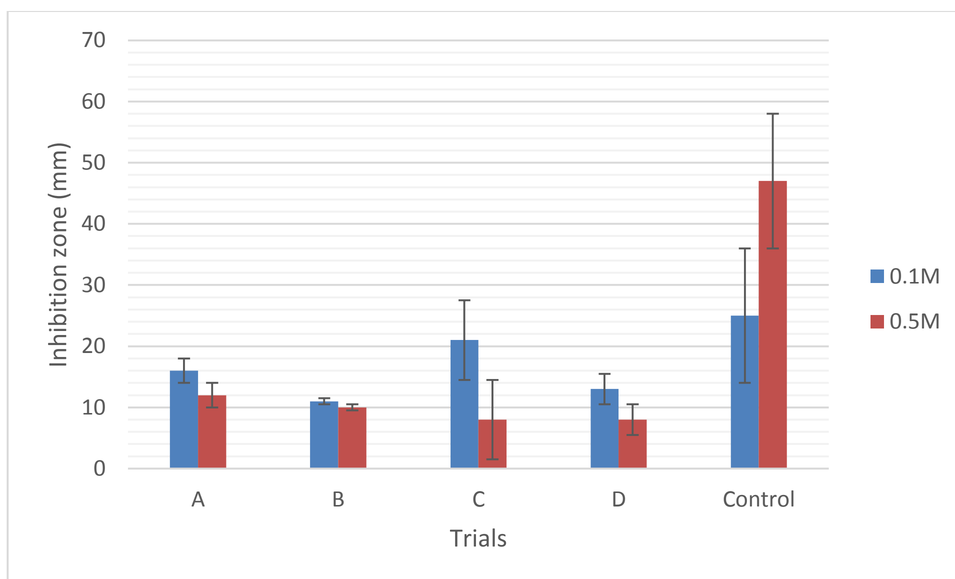
There are significant differences in antibacterial activity against *Shigella* sp, *Bacillus* sp, and *Enterobacter* sp among different concentrations of Neem leaves (p-value: 0.002). For *Vibrio* sp, and *Salmonella* sp, p-value: 0.001 (Significant) showing significant differences in antibacterial activity against *Vibrio* sp among different concentrations of Neem leaves.

The application of Hibiscus Leaf extract on *Shigella* sp, showed p-value: 0.006 meaning that there are significant differences in antibacterial activity against *Shigella* sp among different concentrations of Hibiscus leaves; also there are significant differences in antibacterial activity against *Vibrio* sp among different concentrations of Hibiscus leaves (p-value: 0.004), *Bacillus* sp (p-value: 0.021 (Significant), whilst *Enterobacter* sp recorded p-value: 0.113, and *Salmonella* sp (0.248) showing no significant differences in antibacterial activity against *Enterobacter* sp and *Salmonella* sp among different concentrations of Hibiscus leaves.



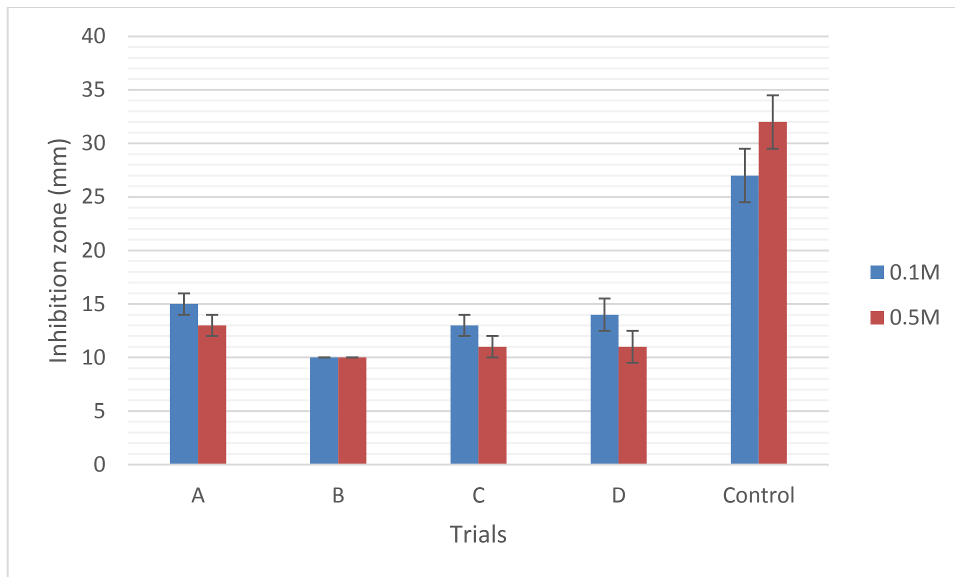
A: 500 mg/ml, B: 250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.17: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Azadirachta indica* (Neem leaves) at different concentrations on *Shigella* sp.



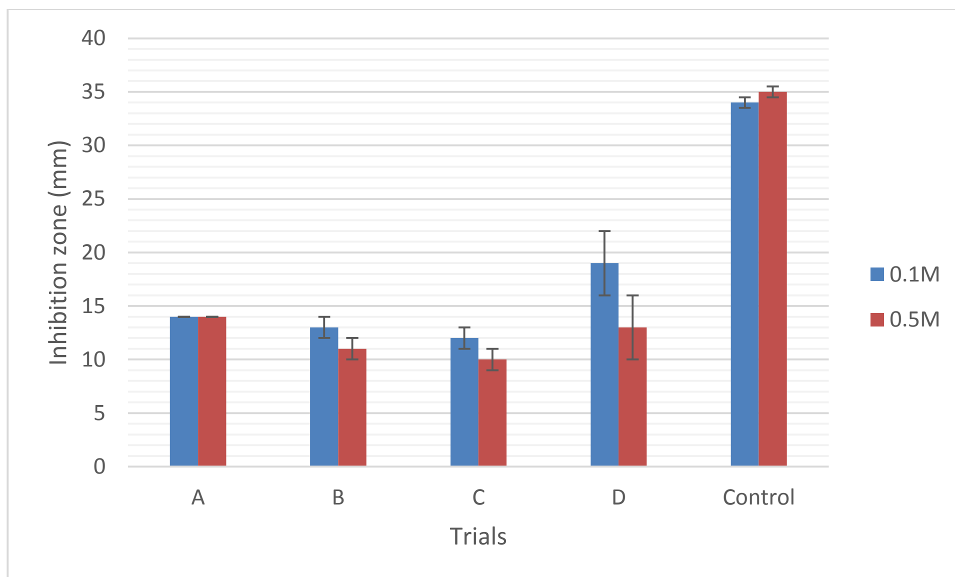
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.18: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Azadirachta indica* (Neem leaves) at different concentrations on *Vibrio* sp.



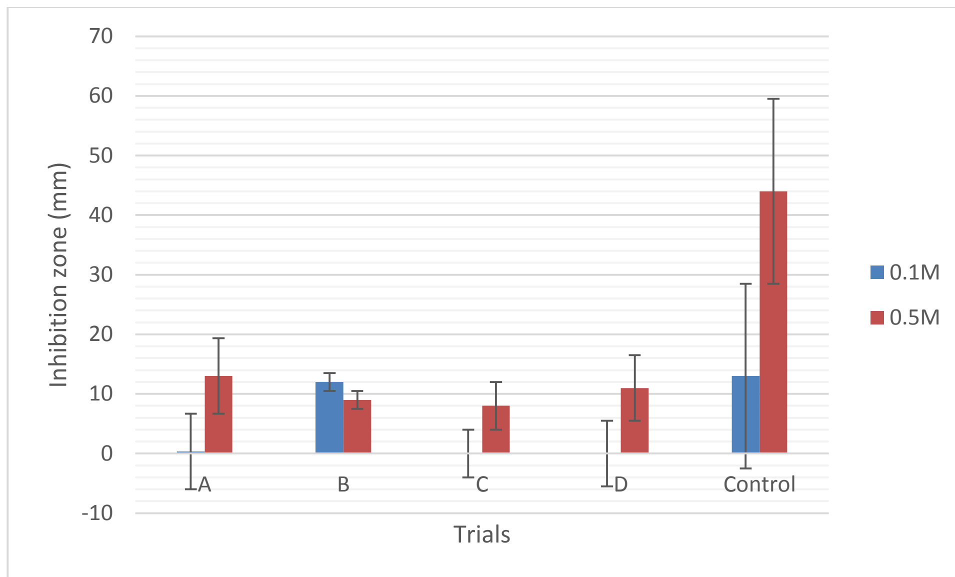
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.19: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Azadirachta indica* (Neem leaves) at different concentrations on *Bacillus* sp.



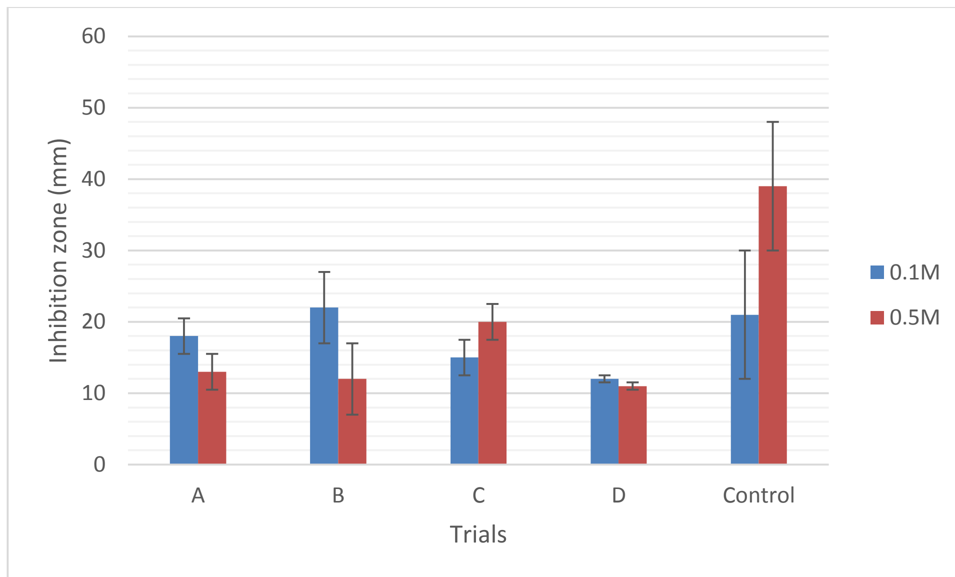
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.20: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Azadirachta indica* (Neem leaves) at different concentrations on *Enterobacter* sp.



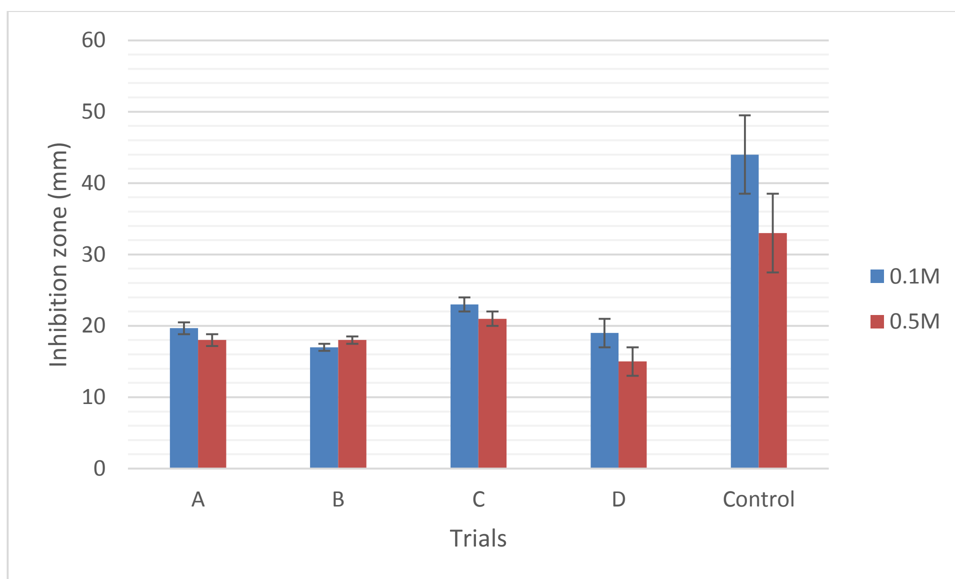
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.21: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Azadirachta indica* (Neem leaves) at different concentrations on *Salmonella* sp.



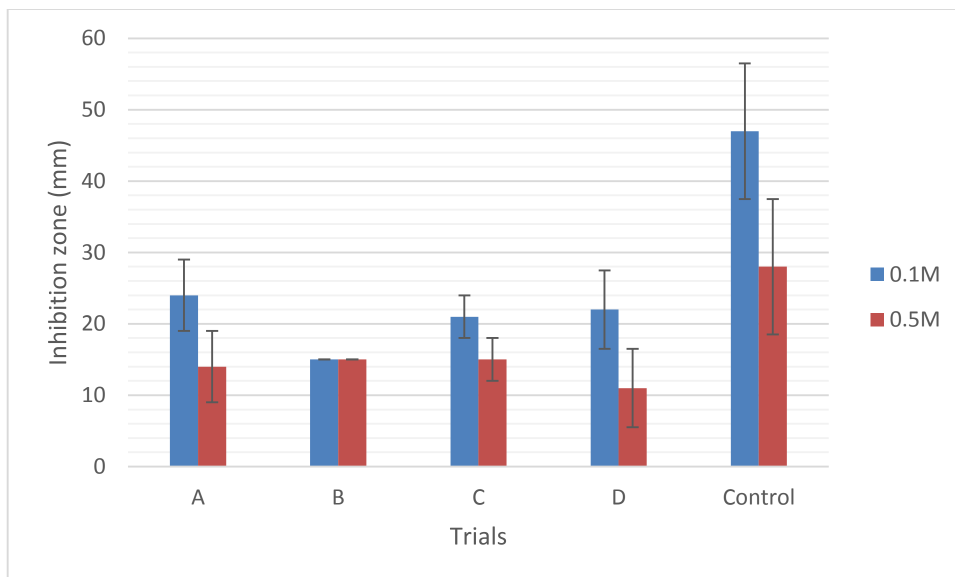
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.22: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Shigella* sp.



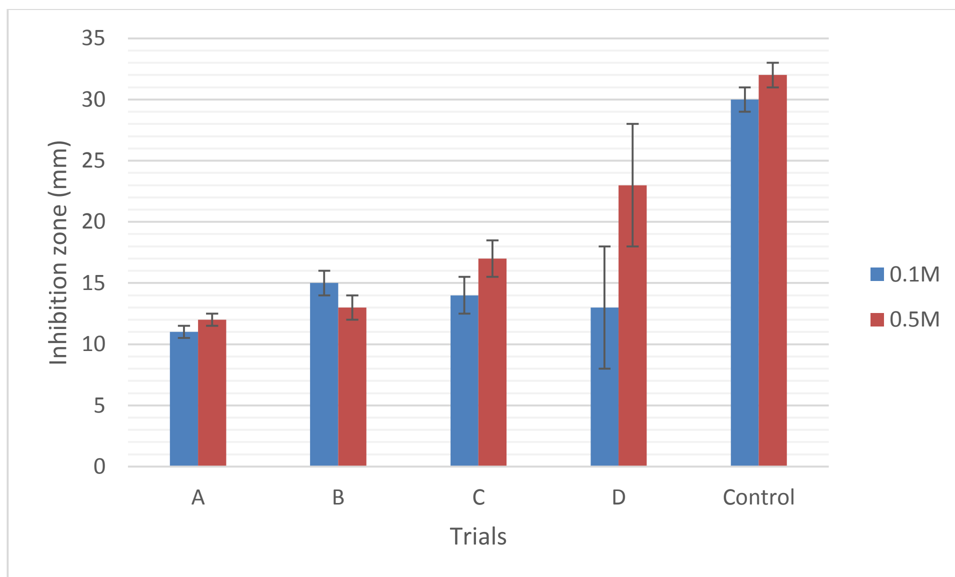
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.23: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Vibrio* sp.



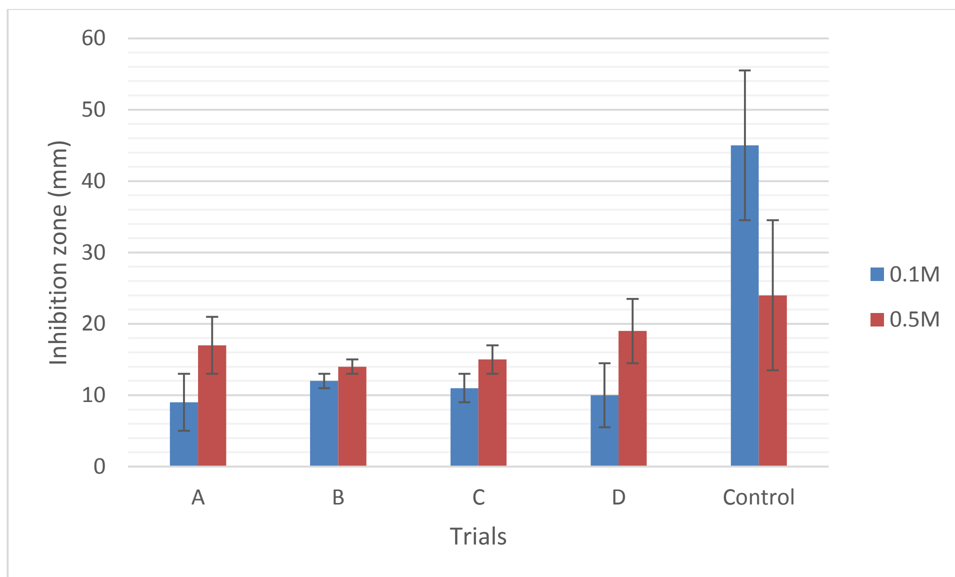
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.24: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Bacillus* sp.



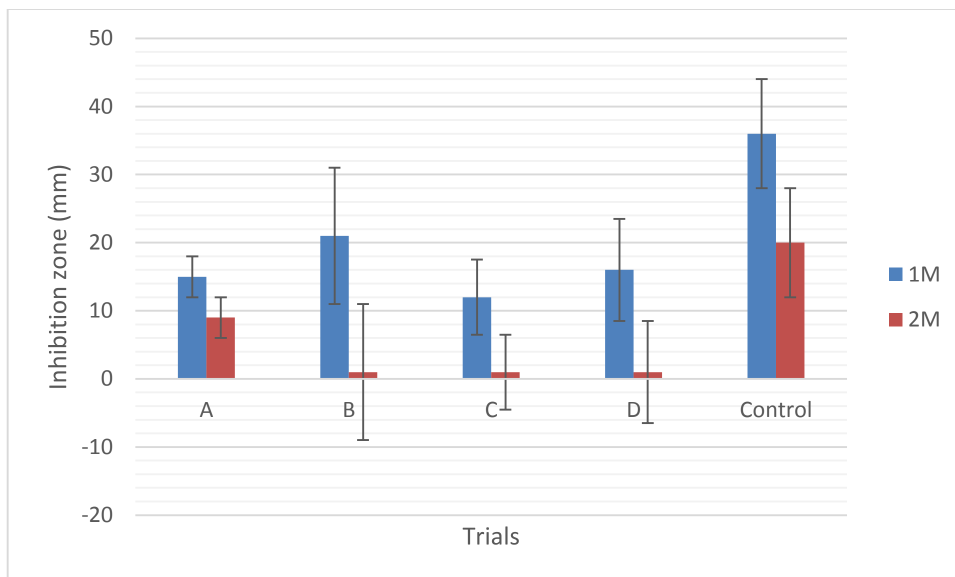
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.25: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Enterobacter* sp.



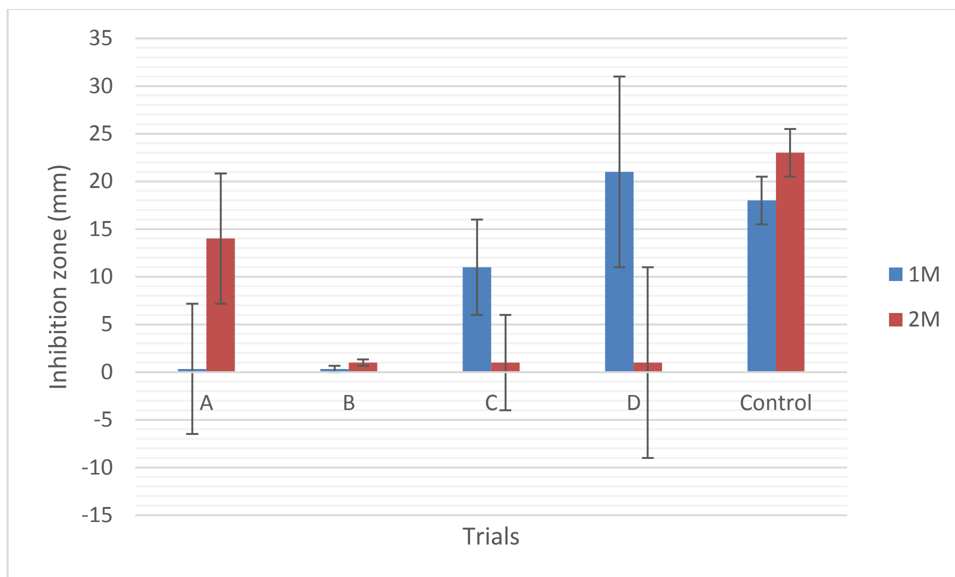
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.26: Mean antibacterial activity of three independent experiments of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Salmonella* sp.



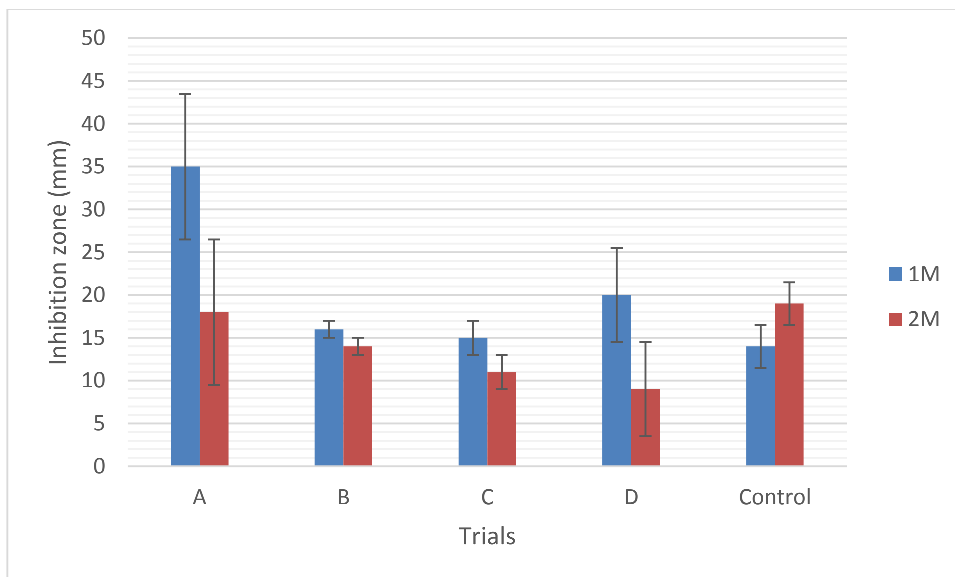
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.27: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Azadirachta indica* (Neem leaves) at different concentrations on *Shigella* sp.



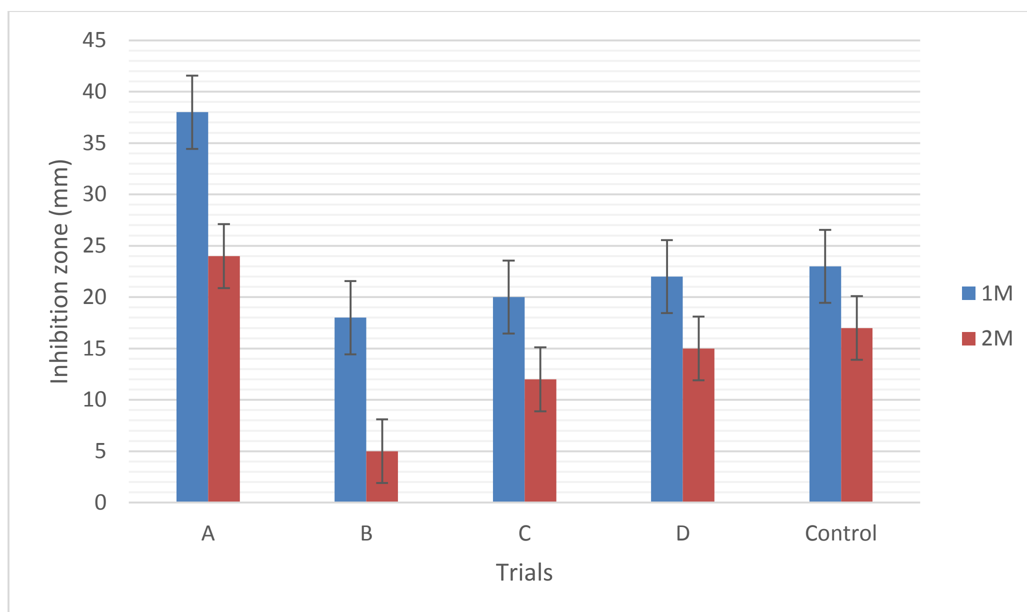
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.28: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Azadirachta indica* (Neem leaves) at different concentrations on *Vibrio* sp.



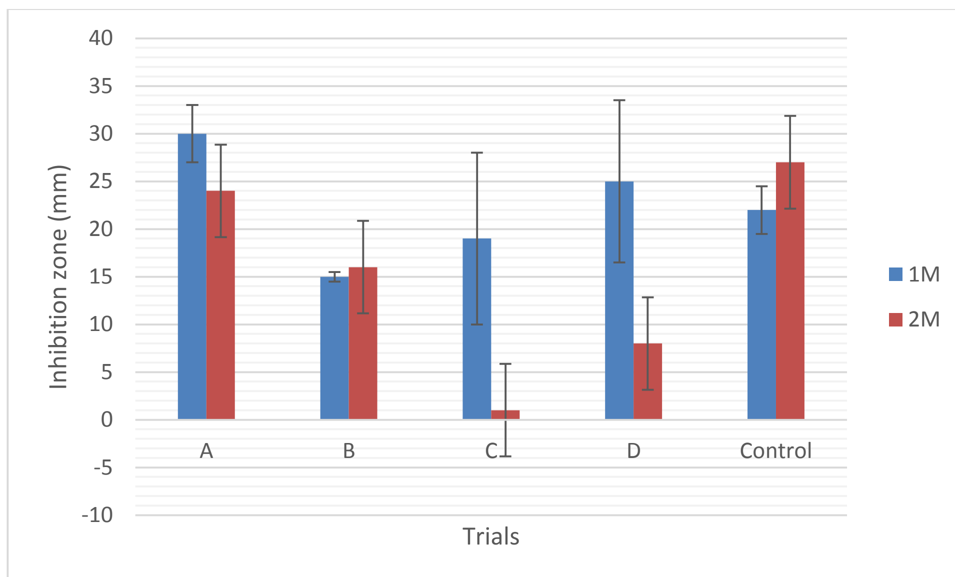
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.29: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Azadirachta indica* (Neem leaves) at different concentrations on *Bacillus* sp.



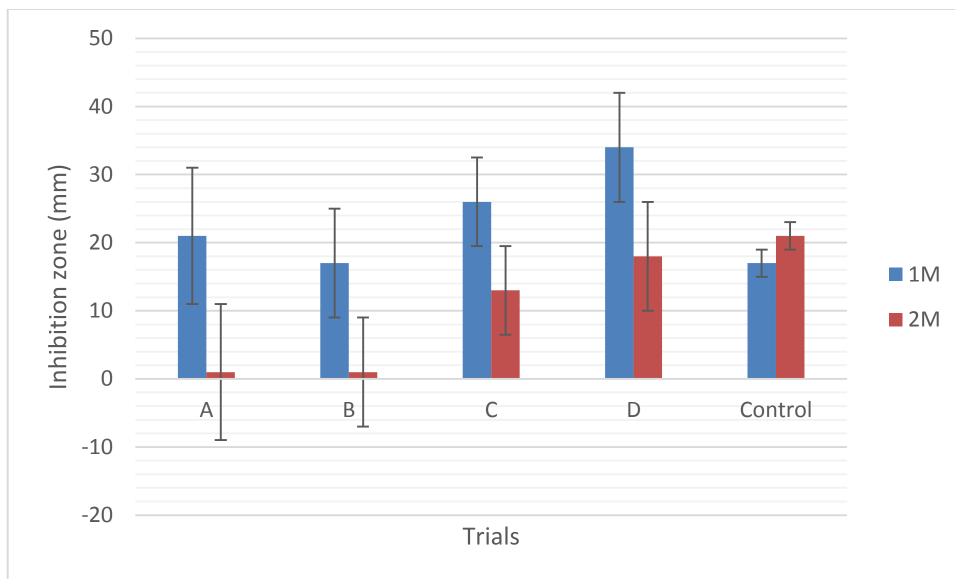
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.30: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Azadirachta indica* (Neem leaves) at different concentrations on *Enterobacter* sp.



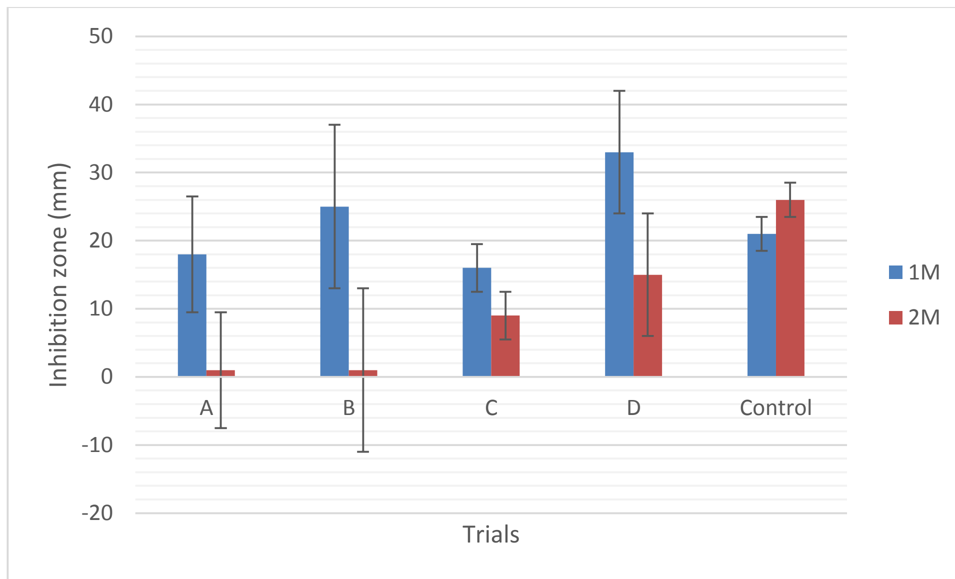
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.31: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Azadirachta indica* (Neem leaves) at different concentrations on *Salmonella* sp.



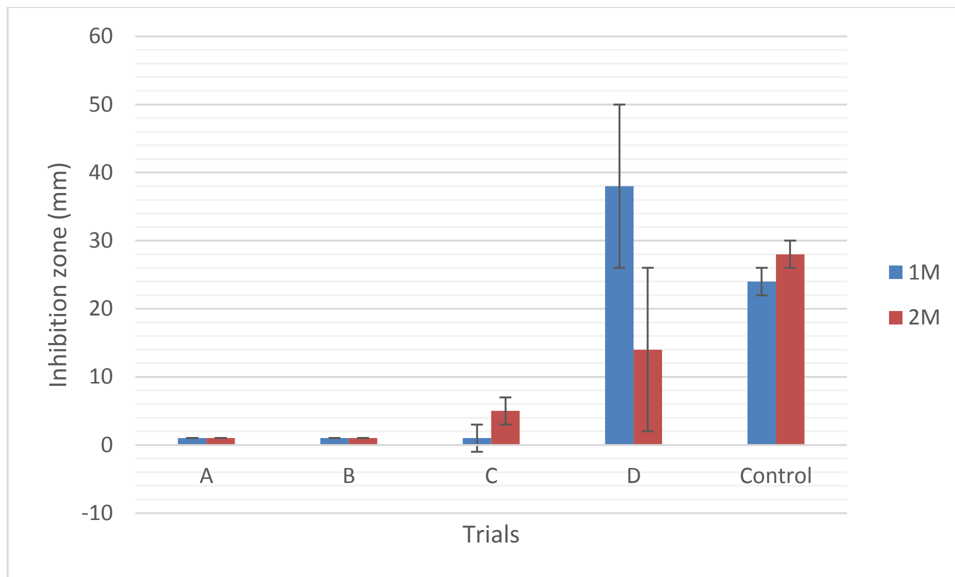
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.32: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Shigella* sp.



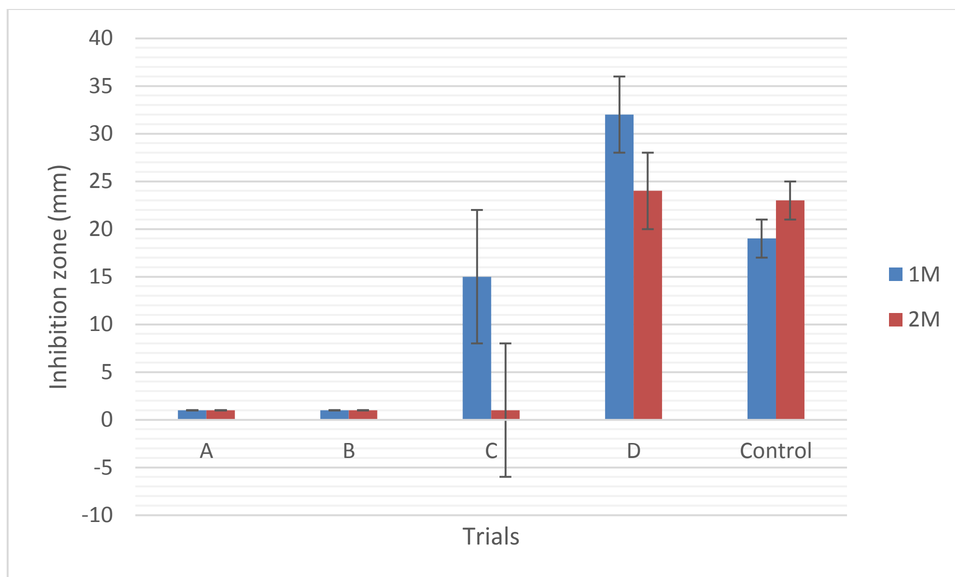
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.33: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Vibrio* sp.



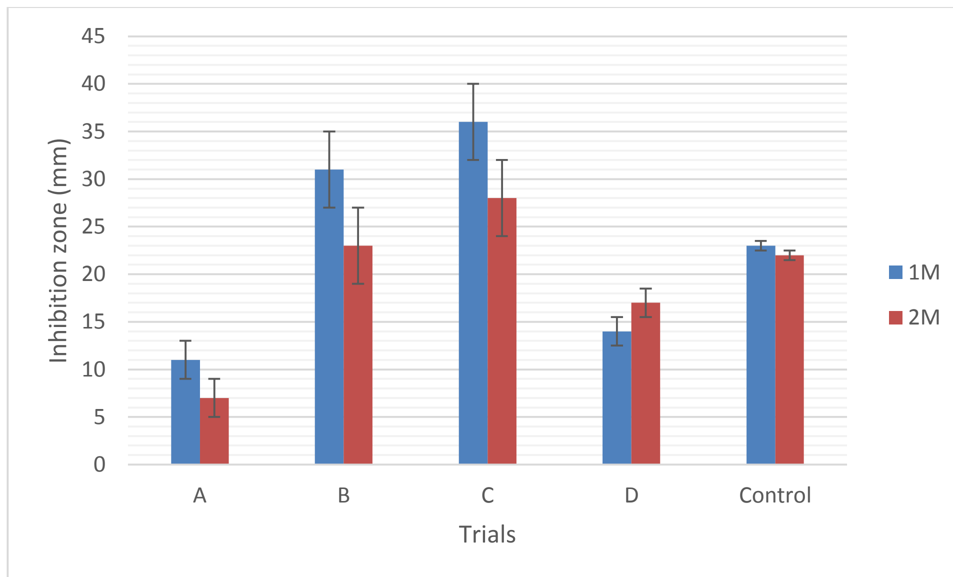
A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.34: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Bacillus* sp.



A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.35: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Enterobacter* sp.



A : 500 mg/ml, B :250 mg/ml, C :125 mg/ml, D :62.5 mg/ml, Control :80 mg/2ml gentamicin

Figure 4.36: Mean antibacterial activity of three independent experiments of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations on *Salmonella* sp.

Minimum Inhibitory Concentration of the Nanoparticles against the Isolates

Minimum Inhibitory Concentration (MIC) of Silver and Zinc Oxide Nanoparticles as shown in Tables 4.9-4.10 underwent ANOVA. The one-way ANOVA analyses showed significant differences in mean MIC values among the different nanoparticle concentrations (A, B, C, and D) for all bacterial strains (p-values < 0.05). Post-hoc tests revealed that higher nanoparticle concentrations (A and B) generally had significantly lower mean MIC values (indicating higher effectiveness) compared to lower concentrations (C and D).

For all bacterial strains, the one-way ANOVA analyses showed significant differences in mean MIC values among the different nanoparticle concentrations (A, B, C, and D) (p-values < 0.05). Post-hoc tests indicated that higher nanoparticle concentrations (A and B) had significantly lower mean MIC values compared to lower concentrations (C and D), suggesting higher effectiveness at higher concentrations.

Table 4.9 Mean Result for the Minimum Inhibitory Concentration (MIC) of three independent experiments ± S.D (n=3) of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) and *Azadirachta indica* (Neem leaves) at different concentrations.

Type	Molar Conc.	Trial	*Shigella* sp	*Vibrio* sp	*Bacillus* sp	*Enterobacter* sp	*Salmonella* sp
Neem	1M	A	$2.332{\pm}0.011^{a}$	$2.500{\pm}0.015^{a}$	$2.425{\pm}0.008^{a}$	$2.495{\pm}0.003^{a}$	$2.357{\pm}0.010^{a}$
		B	$2.352{\pm}0.007^{a}$	$2.458{\pm}0.006^{a}$	$2.451{\pm}0.006^{a}$	$2.479{\pm}0.003^{a}$	$2.414{\pm}0.005^{a}$
		C	$2.369{\pm}0.007^{a}$	$2.474{\pm}0.007^{a}$	$2.458{\pm}0.006^{a}$	$2.646{\pm}0.007^{a}$	$2.339{\pm}0.004^{a}$
		D	$2.584{\pm}0.005^{a}$	$2.351{\pm}0.007^{a}$	$2.742{\pm}0.005^{b}$	$2.623{\pm}0.007^{a}$	$2.323{\pm}0.005^{a}$
	2M	A	$1.445{\pm}0.013^{a}$	$1.968{\pm}0.008^{a}$	$2.515{\pm}0.009^{a}$	$2.281{\pm}0.003^{a}$	$2.241{\pm}0.004^{a}$
		B	$1.906{\pm}0.007^{b}$	$2.890{\pm}0.006^{b}$	$1.352{\pm}0.007^{b}$	$1.873{\pm}0.008^{b}$	$1.312{\pm}0.004^{b}$
		C	$1.786{\pm}0.007^{c}$	$1.527{\pm}0.007^{c}$	$1.791{\pm}0.008^{c}$	$2.351{\pm}0.006^{a}$	$1.643{\pm}0.005^{b}$
		D	$1.957{\pm}0.006^{b}$	$2.483{\pm}0.010^{d}$	$1.983{\pm}0.008^{c}$	$2.742{\pm}0.009^{c}$	$2.228{\pm}0.004^{a}$
Hibiscus	1M	A	$2.327{\pm}0.008^{a}$	$2.255{\pm}0.007^{a}$	$2.270{\pm}0.003^{a}$	$2.283{\pm}0.004^{a}$	$2.244{\pm}0.003^{a}$
		B	$2.236{\pm}0.005^{a}$	$2.257{\pm}0.004^{a}$	$2.542{\pm}0.006^{b}$	$2.239{\pm}0.004^{a}$	$2.229{\pm}0.003^{a}$
		C	$2.201{\pm}0.004^{a}$	$2.245{\pm}0.006^{a}$	$2.232{\pm}0.009^{a}$	$2.452{\pm}0.007^{a}$	$2.235{\pm}0.007^{a}$
		D	$2.326{\pm}0.004^{a}$	$2.482{\pm}0.004^{a}$	$2.624{\pm}0.009^{b}$	$2.281{\pm}0.003^{a}$	$2.306{\pm}0.006^{a}$
	2M	A	$1.939{\pm}0.008^{a}$	$2.251{\pm}0.004^{a}$	$2.381{\pm}0.007^{a}$	$2.629{\pm}0.004^{a}$	$2.131{\pm}0.003^{a}$
		B	$2.012{\pm}0.005^{a}$	$2.261{\pm}0.003^{a}$	$2.326{\pm}0.004^{a}$	$2.458{\pm}0.004^{a}$	$1.624{\pm}0.007^{b}$
		C	$2.321{\pm}0.006^{b}$	$1.983{\pm}0.004^{a}$	$1.926{\pm}0.006^{b}$	$2.477{\pm}0.008^{a}$	$1.241{\pm}0.003^{b}$
		D	$1.879{\pm}0.004^{a}$	$1.628{\pm}0.003^{b}$	$2.043{\pm}0.006^{b}$	$2.051{\pm}0.006^{b}$	$1.965{\pm}0.003^{a}$

Table 4.10 Minimum Inhibitory Concentration (MIC, µg/mL) of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) and *Azadirachta indica* (Neem leaves) at different concentrations.

Type	Molar Conc.	Conc.	*Shigella* sp	*Vibrio* sp	*Bacillus* sp	*Enterobacter* sp	*Salmonella* sp
Neem	0.1M	A	2.038±0.004^{a}	2.077±0.004^{a}	2.070±0.004^{a}	1.875±0.013^{a}	1.977±0.007^{a}
		B	2.022±0.004^{a}	2.413±0.007^{a}	1.991±0.001^{a}	1.552±0.005^{a}	1.835±0.009^{a}
		C	1.921±0.004^{a}	1.536±0.003^{b}	1.520±0.003^{b}	1.293±0.007^{b}	1.981±0.002^{a}
		D	2.256±0.003^{a}	1.492±0.007^{b}	1.921±0.007^{a}	1.313±0.008^{b}	1.568±0.009^{b}
Neem	0.5M	A	2.723±0.006^{a}	1.889±0.007^{a}	1.401±0.001^{a}	1.748±0.007^{a}	1.812±0.007^{a}
		B	3.225±0.006^{b}	2.269±0.009^{a}	2.128±0.003^{b}	1.955±0.009^{a}	1.951±0.005^{a}
		C	1.828±0.006^{c}	1.624±0.007^{b}	2.570±0.009^{c}	1.406±0.004^{b}	1.964±0.006^{a}
		D	1.505±0.008^{c}	1.191±0.007^{c}	1.964±0.009^{d}	1.282±0.003^{b}	2.548±0.005^{b}
Hibiscus	0.1M	A	1.426±0.003^{a}	2.419±0.002^{a}	1.989±0.003^{a}	1.993±0.007^{a}	1.977±0.004^{a}
		B	1.921±0.004^{b}	2.034±0.004^{a}	1.586±0.003^{a}	2.279±0.004^{a}	2.498±0.004^{b}
			1.857±0.008^{b}	2.141±0.007^{a}	2.371±0.008^{b}	1.564±0.006^{b}	3.259±0.006^{c}
		D	2.113±0.003^{c}	2.432±0.004^{a}	2.782±0.004^{b}	2.725±0.007^{c}	1.783±0.008^{a}
Hibiscus	0.5M	A	2.245±0.003^{a}	2.131±0.004^{a}	1.981±0.003^{a}	1.259±0.005^{a}	2.682±0.007^{a}
		B	2.682±0.004^{a}	2.052±0.005^{a}	2.230±0.005^{a}	2.726±0.006^{b}	2.723±0.007^{a}
		C	3.259±0.007^{b}	1.833±0.003^{a}	2.285±0.006^{a}	1.408±0.005^{a}	2.890±0.008^{a}
		D	1.682±0.003^{c}	1.417±0.007^{b}	2.196±0.007^{a}	1.742±0.006^{c}	1.352±0.007^{b}

From the tables (4.9- 4.10) and figures (4.17 - 4.36), it is apparent that higher concentrations of nanoparticles generally exhibited higher mean antibacterial activity and lower mean MIC values (indicating higher effectiveness) compared to lower concentrations. However, the trends varied across different bacterial strains and nanoparticle types (silver or zinc oxide). The standard

deviations and ranges also indicated substantial variability in the effectiveness of nanoparticle treatments compared to the antibiotic control.

One-way ANOVA analyses were conducted to determine if there were significant differences in antibacterial activity or MIC among the different treatment groups (nanoparticle concentrations and antibiotic control) for each bacterial strain. Significant differences were further explored using post-hoc tests, such as Tukey's HSD.

For all bacterial strains tested, the one-way ANOVA revealed significant differences in mean antibacterial activity among the treatment groups (p-values < 0.05). Post-hoc Tukey's HSD tests showed that the antibiotic control (Gentamicin) generally had significantly higher mean activity compared to the nanoparticle treatment groups. Additionally, higher nanoparticle concentrations (A and B) exhibited significantly higher mean activity compared to lower concentrations (C and D).

Antibacterial Activity of Zinc Oxide Nanoparticles (Figure 4.27-4.36) showed that for the bacterial strains Neem leaves (1M) and Hibiscus leaves (1M), the one-way ANOVA indicated significant differences in mean antibacterial activity among the treatment groups (p-values < 0.05). However, for Neem leaves (2M) and Hibiscus leaves (2M), no significant differences were found among the treatment groups (p-values > 0.05).

MINIMUM BACTERICIDAL CONCENTRATION (MBC) RESULTS

Table 4.11: Showing the Minimum Bactericidal Concentration (MBC) of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations.

Isolates	Hibiscus (0.1M)				Hibiscus (0.5M)			
	A	B	C	D	A	B	C	D
Salmonella sp	_ *	++	+++	+++	_	+	_ *	+
Vibrio cholerae	++	+++	+	_ *	_ *	+	++	+++
Shigella sp.	++	_ *	+	++	+	++	+++	_ *
Bacillus sp.	+	++	++	+++	_ *	+	+	++
Enterobacter sp	_	_	_ *	+	+	_	_ *	+

KEY: - = No growth, + = Scanty growth, ++ = Moderate growth, +++ = Copious growth, *= Minimum Bactericidal Concentration, A = 500mg/ml, B = 250mg/ml, C = 125mg/ml, D = 62.5mg/ml

Table 4.12: Showing the Minimum Bactericidal Concentration (MBC) of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Azadirachta indica* (Neem leaves) at different concentrations.

Isolates	Neem leaves (0.1M)				Neem leaves (0.5M)			
	A	B	C	D	A	B	C	D
Salmonella sp.	_ *	++	+	+++	_	_ *	+	+
Vibrio cholera	+	_ *	+++	++	_	_ *	++	+
Shigella sp.	++	_ *	+	++	+++	_ *	+++	++
Bacillus sp.	++	+	++	+++	+	+	++	_ *
Enterobacter sp.	_ *	++	+	+++	_ *	+	++	++

KEY: - = No growth, + = Scanty growth, ++ = Moderate growth, +++ = Copious growth, *= Minimum Bactericidal Concentration, A = 500mg/ml, B = 250mg/ml, C = 125mg/ml, D = 62.5mg/ml

Table 4.13: Showing the Minimum Bactericidal Concentration (MBC) of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) at different concentrations.

Isolates	Hibiscus leaves (1M)				Hibiscus leaves (2M)			
	A	B	C	D	A	B	C	D
Salmonella sp.	++	+	_ *	+	+++	+++	++	+
Vibrio cholerae	+++	++	+++	+	+++	+	+++	+++
Shigella sp.	+	++	+	_ *	+++	+++	+++	+++
Bacillus sp.	++	+++	++	_ *	+	+++	+++	+++
Enterobacter sp.	_ *	++	+++	+++	+	+++	+++	+++

KEY: - = No growth, + = Scanty growth, ++ = Moderate growth, +++ = Copious growth, *= Minimum Bactericidal Concentration, A = 500mg/ml, B = 250mg/ml, C = 125mg/ml, D = 62.5mg/ml

Table 4.14: Showing the Minimum Bactericidal Concentration (MBC) of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Azadirachta indica* (Neem leaves) at different concentrations.

Isolates	Neem leaves (1M)				Neem leaves (2M)			
	A	B	C	D	A	B	C	D
Salmonella sp.	+	+++	+++	++	+	+++	+++	++
Vibrio cholera	+++	+++	++	+	+	++	+++	+++
Shigella sp.	++	_ *	+++	++	+	+++	+++	+++
Bacillus sp.	_ *	++	+++	+	++	+++	+++	+
Enterobacter sp.	_ *	+++	++	+	+	+++	++	++

KEY: - = No growth, + = Scanty growth, ++ = Moderate growth, +++ = Copious growth, *= Minimum Bactericidal Concentration, A = 500mg/ml, B = 250mg/ml, C = 125mg/ml, D = 62.5mg/ml

4.3.2 Relationship between nanoparticle concentration and Antibacterial activity

Simple and multiple linear regression analyses were performed to model the relationships between nanoparticle characteristics (type and concentration), bacterial strain, and antibacterial activity.

Scatter plots with regression lines showing the relationship between nanoparticle concentration and antibacterial activity for selected bacterial strains. Simple linear regression analyses were conducted to model the relationship between nanoparticle concentration and antibacterial activity for each bacterial strain. The regression equations and coefficients of determination (R-squared values) were obtained, providing insights into the strength and direction of the linear relationships. These analyses allowed for the estimation of effective nanoparticle concentrations for specific antibacterial activity targets.

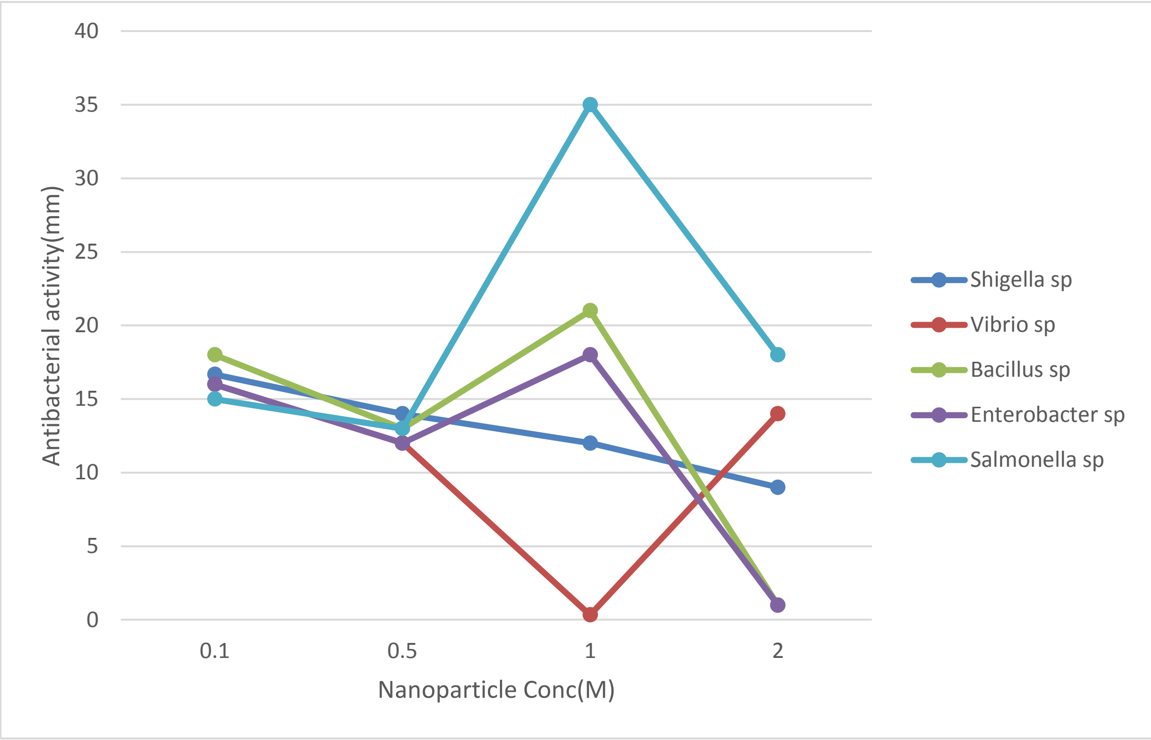


Fig. 4.37: Relationship between the Nanoparticle Concentration and Antibacterial Activity on the Bacterial Strains

Correlation analyses were conducted to investigate the relationships between the antibacterial activities of nanoparticles and nanoparticle concentrations across different bacterial strains. The correlation analyses revealed [positive/negative/mixed] correlations between the effectiveness of nanoparticles and antibiotics, suggesting [similar/different] mechanisms of action or potential [synergistic/antagonistic] effects. These findings contribute to the understanding of the potential use of plant-based nanoparticles as alternatives or adjuncts to antibiotics in combating bacterial pathogens affecting fish.

The results revealed that while the antibiotic control (Gentamicin) was generally more effective in terms of overall antibacterial activity, lower molar concentrations of nanoparticles exhibited comparable or even higher effectiveness in terms of their minimum antibacterial activities. The effectiveness of nanoparticles also seemed to depend on the specific bacterial strain and the type of nanoparticle (silver or zinc oxide).

It is important to note that these analyses assumed that the assumptions of normality and homogeneity of variances were met. In cases where these assumptions were violated, appropriate data transformations or non-parametric tests were employed to ensure the validity of the results.

Overall, the statistical analyses contributed to the understanding of the potential of plant-based nanoparticles as alternatives to antibiotics in combating bacterial pathogens affecting fish, while also highlighting the need for further investigations into the mechanisms of action, optimal concentrations, and potential synergistic or antagonistic effects with antibiotics for different bacterial strains.

4.4 DISCUSSION

The Fourier Transform Infrared Spectroscopy result revealed peaks at > 3100 cm^{-1} which are related to OH species with co-adsorbed H_2O on ZnO and Ag surfaces (Sharif, Ansari, Malik, Ali, & Khan, 2020; Li et al., 2019; Okewale and Olusoya 2019). The characteristics bands at 2105.9 cm^{-1} and 2117.1 which was observed in silver nanoparticles from Neem plant and ZnO nanoparticles from Hibiscus were attributed to the alkyne functional group (Devi, Sharma & Manhas, 2023). The characteristic peak at 1908.4 cm^{-1} as seen in ZnO nanoparticles synthesized using Hibiscus represents the presence of H- aromatic alkenes (Wyasu, Myek, George & Moses, 2020). Bands at 1625.1 cm^{-1}, 1636.3 cm^{-1}, 1625.1 cm^{-1} and 1636.6 cm^{-1} which represents C=O functional group was observed in both ZnO and silver nanoparticles synthesized using Hibiscus and Neem leaves respectively showing a stretching vibration in the ketone functional group and phytochemical compound (Sharif et al., 2020). At characteristic band of 1517.0 cm^{-1} and 1520.8 cm^{-1} as seen in ZnO synthesized using Neem plant and silver nanoparticle synthesized using Hibiscus represented N-O which was a stretch in nitro compound functional group of amides which was in the range 1550-1500 cm^{-1} (Sharif et al., 2020), while at 1412.7 cm^{-1} O-H functional group of bending alcohol as its frequency is between 1410-1310 cm^{-1}(Hemmalakshmi, Priyanga, & Devaki, 2017). At wavelength 1341.8 cm^{-1}, 1353.0 cm^{-1}, and 1379.3 cm^{-1} represents the amine C-N stretching functional group having a medium-weak peak.

The characteristics peak at around 1263.6 cm^{-1} in silver synthesized using Hibiscus represents a stretch in aromatic primary amine phytochemical group (Sharif et al., 2020). At band 1006.4 cm^{-1}, 1017.6 cm^{-1} and 1092.1 cm^{-1} a stretch of alkyl halides phytochemical group was observed for silver nanoparticles synthesized using Hibiscus and ZnO synthesized using both Neem plant and

Hibiscus respectively but was absent in silver nanoparticle synthesized using Neem plant (Sharif et al., 2020).

The X-Ray Diffractogram spectroscopy (XRD) shows the different synthesized nanoparticles which are in agreement with the previously reported work (Getie, Belay, Chandra & Belay, 2017; Demissie, Sabir, Edossa & Gonfa, 2020). The XRD spectra also showed that all the diffraction peaks fit well with the hexagonal wurtzite structure of silver and ZnO NPs (Getie et al., 2017; Demissie et al., 2020). The sharpness of the diffraction peaks related to the silver and ZnO structure indicates a polycrystalline nature of the nanoparticle. The findings for average crystallite size of the synthesized ZnO and silver nanoparticles are presented also. The sizes were 15.40 nm for silver nanoparticles synthesized from Hibiscus plant leaf extract, 64.85 nm for ZnO nanoparticles synthesized from Hibiscus plant leaf extract, 30.26 nm for ZnO nanoparticles synthesized from Neem leaf extract and 40.41 nm for silver nanoparticles synthesized from Neem plant leaf extract respectively. These findings are in agreement with the study of Ramamoorthy et al., (2019), Jain et al., (2009). The authors reported an average crystallite size in the range of 9 nm to 28 nm for papaya extract which act as reducing as well as capping agent in the green synthesis of silver. The particles which ranged from 18.12 to 60.98 nm were hexagonal wurzite in shape. This size range falls within the ranges reported when other plants were used in the biosynthesis of silver nanoparticle (Lateef et al., 2018). However, higher average crystallite sizes were obtained for ZnO nanoparticles from leaf of Hibiscus ZnO and Neem synthesized silver and ZnO nanoparticles which ranged from 30.26 nm to 64.85 nm. This was in accordance with zinc oxide nanoparticle synthesized using extracts of *Lippia adoensis* (Demissie et al., 2020). The obtained results are in a good agreement with previously reported (Mayekar et al., 2014). The variation in the average

crystallite size of ZnO NPs may be due to the variation of different precursors and high calcination temperature (Gei et al., 2017).

Scanning Electron Microscopy and Energy Dispersive X-Ray spectroscopy (SEM-EDX) were also performed and the observations generally showed hexagonal and rectangular shapes. Also in this study the particles are also found to be inclined together due to the presence of more capping agent that stabilizes the nanoparticles. Meruvu, Vangalapati, Chippada & Bammidi (2011) have reported similar results for ZnO nanoparticles synthesized by chemical methods.

The SEM for the ZnO nanoparticle synthesized using Hibiscus aqueous extract displayed a hexagonal like structure while Hibiscus silver nanoparticle synthesized using aqueous plant extract of Hibiscus in this study showed flake-type shapes in aggregated form. This was also observed in Neem synthesized silver nanoparticle. The aggregation/agglomeration may be caused due to polarity and electrostatic attraction of both silver and ZnO nanoparticles (Vijayakumar et al., 2016).

The Energy Dispersive X-Ray spectroscopy of the samples obtained from the SEM-EDX analysis show that the sample prepared by the different plants leaf extract has pure silver and ZnO phases.

The weight concentration of Zn in the different ZnO nanoparticles were 59.36 and 56.42 for Neem and Hibiscus plant leaf extract respectively. The findings corroborate with that reported for ZnO nanoparticle synthesized from leaf extract of *Lippia adoensis* (Koseret) (Demissie et al.., 2020) and indicated that the reaction product is composed of high purity zinc nanoparticles. The weight concentration of Ag in the different silver nanoparticles were 63.70 and 60.80 for Neem and Hibiscus plant leaf extract respectively.

The UV-Vis spectra strong absorbance was observed at 428.7 nm and 416.8 nm for silver nanoparticles synthesized from Neem leaf and Hibiscus leaf extracts respectively, this was in correspondence with work done by Aina et al., 2018 and Krishnaraj et al., 2010 showing the silver to be of pure product and fewer impurities. Also, strong absorbance was observed at 356 nm and 366.4 nm for ZnO nanoparticles synthesized from Neem leaf, and Hibiscus leaf extracts respectively. The synthesized ZnO nanoparticles confirmed, in this study by the UV – vis absorption spectra, at the wavelength which is the characteristic wavelength coupled with the absence of any other absorbance peak in the spectra confirms that the synthesized products are pure ZnO nanoparticles (Salahuddin et al., 2015; Mohammadian et al.., 2018; Demissie et al., 2020). These corroborate with ZnO nanoparticle synthesized from chamomile flower (*Matricaria chamomilla* L.), Olive leaves (*Olea europaea*), and red tomato fruit (*Lycopersicon esculentum* M.) (Ogunyemi et al., 2019) and from leaves of *Lippia adoensis* (Demissie et al., 2020). Furthermore, it is reported that the peak positions of UV-visible spectra are related with size of nanoparticles and blue shifted as the crystal size of the nanoparticles decreased (Pranjali et al., 2019).

4.5 Interpretation of the Bacterial Count Results

Results from this study show the presence of pathogenic bacteria in the various catfish samples purchased from fisheries in Owerri, Imo State. The most prevalent isolated organisms were *Salmonella* sp., *Shigella* sp., *Enterobacter* sp., *Vibrio* sp. and *Bacillus* sp. The highest level of bacterial load was recorded on the gills which agree with the findings of Elgendy, Soliman, Abbas & Ibrahim (2017) which stated that with the increase in water pollution and high organic loads in fish ponds, the fish gills and skin are usually attacked by water-borne bacteria.

High level of coliform bacteria on all samples (above 10^6cfu/g) could be as a result of unhygienic and poor sanitary practices by the fish farmers. The use of contaminated water for fish pond,

contaminated untreated feeds and poor sanitary habits have been reported as contributing factors to pathogens and diseases of fish (Hossain, 2015). Coliforms are indicator organisms and counts of 10^6cfu/g sample reported in this work are a cause for concern to fish vertenarians. Pre-treatment of livestock manure by exposing to sunlight is therefore recommended because it will lower the bacterial level.

The presence of *Salmonella* sp., *Shigella* sp. and *Vibrio* sp. in fish pose health risk to humans (Haenen et al., 2013 and Adeshina et al., 2016).

Some of the isolates such as *Bacillus* sp. have been reported to have beneficial health effects in fish and have been developed into fish probiotics (Capkin & Altinok (2009) and Oggioni et al., 2003). This may justify their occurrence in the fish organs. However, further studies are needed to assess their efficacy as probiotics.

4.5.1 Antibiotic Profiles of the isolates

The varying antibiotic susceptibility among the isolated organisms is consistent with findings in other studies. For instance, Mohd Fauzi et al, (2021) investigated the presence of *Aeromonas* spp., including its antibiotic resistance in various fish samples, *Oreochromis spp., Clarias gariepinus,* and *Pangasius hypophthalmus*, obtained from Kelantan and Terengganu, Malaysia against 14 antibiotics and found that all *Aeromonas hydrophila* isolates were susceptible to chloramphenicol and nitrofurantoin, but resistant to tetracycline with multiple antibiotic resistances index of the isolates ranging from 0.07 to 0.64.

The *Enterobacter* isolate displaying resistance to all antibiotics except gentamicin suggests potential multidrug resistance. This is a growing concern in aquaculture. The overuse of antibiotics in fish farms can create selective pressure, favoring the growth of resistant bacteria and so studies

have linked multidrug resistance in bacterial fish isolates to potential overuse of antibiotics in aquaculture (Wamala et al., 2018; Krahulcova et al., 2023). In addition, some *Bacillus* species are known for inherent resistance to various antibiotics, including chloramphenicol and tetracycline. Also, *Enterobacter* species can exhibit multidrug resistant plasmids, explaining the broad resistance observed in the present study whilst *Salmonella* and *Vibrio* often show variation in resistance profiles depending on the specific serotype and geographical location (Rensing et al., 2019; Nair, Venkitanarayanan & Kollanoor Johny, 2018).

4.5.2 Comparison of Antibacterial Activities

The statistical analyses revealed significant differences in the antibacterial activities of plant-based nanoparticles and the antibiotic control (Gentamicin) against various fish bacterial pathogens. While the antibiotic control generally exhibited higher overall antibacterial activity, as evidenced by the higher mean values and significant differences observed in the ANOVA and post-hoc tests (Figures 4.17-4.36), the nanoparticles demonstrated comparable or even superior effectiveness in terms of their minimum inhibitory concentrations (MICs) (Tables 4.9 and 4.10). These findings are consistent with previous studies that have reported the potential of nanoparticles as effective antibacterial agents (Ovais et al., 2018; Rai, Deshmukh, Ingle, Gupta & Galdiero, 2019).

Specifically, lower concentrations of silver and zinc oxide nanoparticles (concentrations 0.1M and 1M) exhibited significantly higher antibacterial activities compared to higher concentrations (0.5M and 2M), indicating their enhanced ability to inhibit bacterial growth at higher concentrations. This dose-dependent relationship was further supported by the negative linear trends observed in the simple linear regression analyses (Figure 4.37), where decreasing

nanoparticle concentrations were associated with higher antibacterial activity, corroborating the findings of similar studies (Kumar et al., 2020; Zhu et al., 2021).

It is noteworthy that the effectiveness of nanoparticles varied across different bacterial strains, suggesting potential strain-specific mechanisms or sensitivities. For instance, the zinc oxide nanoparticles exhibited significant lower MIC values (higher effectiveness) against Neem leaves (2M) and Hibiscus leaves (2M), but not against Neem leaves (1M) and Hibiscus leaves (1M) (Table 4.9) while the silver nanoparticles exhibited significant lower MIC values (higher effectiveness) against Neem leaves (0.1M) and Hibiscus leaves (0.1M) but not against Neem leaves (0.5M) and Hibiscus leaves (0.5M) (Table 4.10). Such variations underscore the importance of considering the potential differences in their modes of action and bacterial strain characteristics when evaluating the efficacy of nanoparticles as potential antibacterial agents, as highlighted by Rajendran et al., (2022) and Singh et al., (2023).

4.5.3. Potential Mechanisms of Action

While the specific mechanisms of action were not directly investigated in this study, the observed differences in antibacterial activities between nanoparticles and the antibiotic control, as well as the variations across bacterial strains, suggest potential differences in their modes of action, as discussed by Patra et al., (2018) and Zhang et al., (2022).

The antibiotic Gentamicin belongs to the aminoglycoside class and primarily acts by inhibiting protein synthesis in bacteria, leading to their death or growth inhibition (Krause et al., 2016). On the other hand, nanoparticles are known to exert antibacterial effects through various mechanisms, including disruption of cell membranes, generation of reactive oxygen species (ROS), and interference with cellular processes such as DNA replication and protein synthesis (Rajendran et al., 2022; Yan et al., 2023).

The correlation analysis provided insights into the relationships between the antibacterial activities of nanoparticles and the nanoparticle concentrations across different bacterial strains (Kumari et al., 2021; Zhu et al., 2021).

Additionally, the multiple linear regression analysis revealed significant effects of nanoparticle type (silver or zinc oxide) and bacterial strain on antibacterial activity, suggesting that the mechanisms of action may be influenced by the specific nanoparticle composition and the characteristics of the target bacterial strain, as highlighted by Singh et al., (2023) and Yan et al., (2023).

4.5.4 Implications of Findings

The emergence of antibacterial resistance in fish pathogens is a significant challenge in aquaculture, threatening the sustainability and productivity of the industry (Cabello et al., 2016; Watts, Schreier, Lanska & Hale, 2017). The development of resistance is often attributed to the overuse and misuse of antibiotics, highlighting the urgent need for alternative strategies to combat bacterial infections (Luby et al., 2021; Okocha, Nnabuife & Ozuah, 2022).

The findings of this study demonstrate the potential of plant-based nanoparticles as effective antibacterial agents against various fish bacterial pathogens. By exhibiting comparable or superior effectiveness to the antibiotic control, particularly in terms of their minimum inhibitory concentrations, nanoparticles offer a promising alternative for combating antibacterial resistance, corroborating the observations made by Kumar et al., (2020) and Yan et al., (2023).

Nanoparticles are less susceptible to the development of resistance mechanisms compared to traditional antibiotics, as their modes of action often involve multiple targets or non-specific mechanisms, such as physical disruption of cell membranes or generation of reactive oxygen

species (Patra et al., 2018; Rajendran et al., 2022). This multifaceted approach makes it more difficult for bacteria to develop resistance against nanoparticles, reducing the risk of resistance emergence, as discussed by Ovais et al., (2018) and Zhang et al., (2022).

Furthermore, the plant-based origin of the nanoparticles investigated in this study adds an additional advantage, as they are often derived from renewable and environmentally friendly sources, aligning with the principles of sustainable aquaculture practices (Rajendran et al., 2022; Yan et al., 2023).

A second implication is the potential application of nanoparticles in aquaculture. The findings of this study have significant implications for potential applications in aquaculture. Plant-based nanoparticles could be incorporated into various aspects of aquaculture practices, including:

a. Disease Prevention and Control: Nanoparticles could be used as prophylactic or therapeutic agents to prevent or treat bacterial infections in fish, reducing the reliance on traditional antibiotics and mitigating the development of resistance, as suggested by Luby et al., (2021) and Okocha et al., (2022).

b. Disinfection and Biosecurity: Nanoparticles may be utilized as disinfectants or sanitizers in aquaculture systems, helping to maintain biosecurity and prevent the spread of pathogens, as proposed by Kumari et al., (2021) and Rajendran et al., (2022).

c. Feed Additives: Nanoparticles could be incorporated into aquaculture feed formulations, potentially enhancing the immunity and overall health of the fish, while also providing antibacterial protection against potential pathogens, as suggested by Singh et al., (2023) and Yan et al., (2023).

d. Water Treatment: The application of nanoparticles in water treatment processes could help maintain water quality and reduce the risk of bacterial contamination in aquaculture systems, as discussed by Zhu et al., (2021) and Zhang et al., (2022).

However, it is crucial to conduct further research to assess the potential toxicity, environmental impact, and regulatory considerations associated with the use of nanoparticles in aquaculture settings before their widespread implementation, as emphasized by Cabello et al., (2016) and Watts et al., (2017).

4.5.5 Limitations of the Study

While this study provides valuable insights into the antibacterial activities of plant-based nanoparticles against fish pathogens, it is important to acknowledge certain limitations:

1. In-vitro Nature of the Study: The experiments were conducted in vitro, which may not fully represent the complex and dynamic conditions present in actual aquaculture systems or in vivo environments, as highlighted by Luby et al., (2021) and Okocha et al., (2022).

2. Limited Number of Bacterial Strains: The study focused on a selected set of bacterial strains relevant as fish pathogens. However, the efficacy of nanoparticles may vary against other bacterial species or strains not included in this study, as discussed by Rajendran et al., (2022) and Singh et al., (2023).

3. Potential Cytotoxicity and Environmental Impact: The potential cytotoxicity and environmental impact of the nanoparticles were not extensively investigated in this study, which are crucial considerations for their practical application in aquaculture, as emphasized by Cabello et al., (2016) and Watts et al., (2017).

4. Lack of Mechanistic Studies: While the study explored the antibacterial activities and effectiveness of nanoparticles, the specific mechanisms of action were not directly investigated, limiting the understanding of their modes of action and potential interactions with bacterial cells, as discussed by Patra et al., (2018) and Zhang et al., (2022).

4.5.6 Future Research Directions

Based on the findings and limitations of this study, several future research directions can be explored:

i. In vivo and field studies: Conducting in vivo studies using animal models or field trials in aquaculture settings would provide valuable insights into the efficacy, toxicity, and environmental impact of plant-based nanoparticles under real-world conditions (Luby et al., 2021; Okocha et al., 2022).

ii. Exploration of different nanoparticle types and sources: Investigating a wider range of nanoparticle types and sources, including different plant extracts or combinations, could lead to the identification of even more effective and sustainable alternatives (Rajendran et al., 2022; Singh et al., 2023).

iii. Mechanistic studies: Detailed investigations into the mechanisms of action of nanoparticles against different bacterial strains would enhance our understanding of their modes of action and potential resistance mechanisms, enabling the development of more targeted and effective nanoparticle-based antibacterial strategies (Patra et al., 2018; Zhang et al., 2022).

iv. Combination studies with antibiotics: Exploring the potential synergistic or antagonistic effects of nanoparticles in combination with traditional antibiotics could lead to novel therapeutic approaches, potentially reducing the required dosages and mitigating the development of resistance (Kumari et al., 2021; Zhu et al., 2021).

v. Optimization of nanoparticle synthesis and delivery: Research focused on optimizing the synthesis and delivery methods of nanoparticles could improve their stability, bioavailability, and targeted delivery, enhancing their efficacy and minimizing potential toxicity or environmental impacts (Yan et al., 2023).

By addressing these future research directions, the potential of plant-based nanoparticles as effective alternatives to antibiotics in combating bacterial pathogens in aquaculture can be further explored and realized, contributing to the development of sustainable and resilient aquaculture practices.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study has shown that farmed fish and their aquatic environments harbour potentially pathogenic bacteria that can cause severe illnesses and pose serious public health hazard to consumers and occupational hazards to fish harvesters in Owerri, Imo State. Through detailed antibiotic susceptibility tests carried out in this research, the emergence of antibiotic resistance posed by multidrug resistance (MDR) to the existing antibiotics among these prevalent bacterial fish pathogens (*Shigella* sp., *Salmonella* sp., *Vibrio* sp., *Bacillus* sp. and *Enterobacter* sp.) as a significant challenge in aquaculture, threatening the sustainability and productivity of the industry was discovered.

The findings of this study also demonstrate the potential of plant-based nanoparticles as effective antibacterial agents against these bacterial fish pathogens. Furthermore, the plant-based origin of the nanoparticles investigated in this study has added benefits, as they are often derived from environmentally friendly and renewable sources, aligning with the principles of sustainable aquaculture practices.

In conclusion, while this research explored the potential of biosynthesized silver and zinc oxide nanoparticles derived from Neem and Hibiscus leaf extracts in combating the most prevalent bacterial fish pathogens, it is essential to acknowledge that the efficacy of these nanoparticles did not surpass that of the antibiotic, Gentamicin which was used as control in the study. Several factors such as suboptimal dosage and concentration, lack of mechanistic studies, potential cytotoxicity, inadequate particle size and surface area may have contributed to this outcome.

5.2 Recommendation

Therefore, it is recommended that these challenges should be addressed by carrying out mechanistic studies, in-vivo field studies, optimization of nanoparticle synthesis and delivery and regulatory policy consideration. By so doing, the complexities of nanoparticle-based antimicrobial strategies will be reduced and the potential for plant-based nanoparticles to be used as effective alternative to antibiotics in combating fish pathogens will be explored and realized which will contribute to the development of sustainable and resilient aquaculture.

5.3 Contributions to Knowledge

To the best of my knowledge, this research contributed in the following ways

a) Green Synthesis Approach: The research demonstrated the additional advantage to Sustainable Development Goals derived from the green synthesis of nanoparticles as they are often derived from renewable and environmental friendly sources which does not pose any threats even from its wastes.

b) Nanoparticle Characterization: The study characterized silver and zinc oxide nanoparticles synthesized from Hibiscus and Neem leaf extracts using various techniques provided detailed information on the size, shape and composition of these green-synthesized nanoparticles.

c) Strain-Specific Sensitivity: The research revealed that the effectiveness of nanoparticles varies across different bacterial strains because Zinc oxide nanoparticles were most effective against *Salmonella* sp. and least effective on *Vibrio* sp. while it was vice-versa

for Silver nanoparticles, suggesting the potential strain-specific sensitivities or mechanisms of the nanoparticles.

d) Multidrug Resistance Insights: the study identified potential multidrug resistance in some bacterial isolates, particularly in *Enterobacter* species, highlighting the growing concern of antibiotic resistance in aquaculture.

e) Concentration-Dependent Effects: The study established a dose-dependent relationship between the nanoparticle concentration and the antibacterial activity showing the importance of following the right dosage when they are used because they vary.

These contributions expand our understanding of green-synthesized nanoparticles and their ability to serve as potential alternatives to conventional antibiotics for managing bacterial fish pathogens which can also lead to a sustained infection-free environment and serve as an asset for future aquaculture industry.

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APPENDICES

*Azadirachta indica (*Neem leaves) *Hibiscus rosa sinensis (*Hibiscus leaves)

Picture showing the steps used to synthesized the nanoparticles

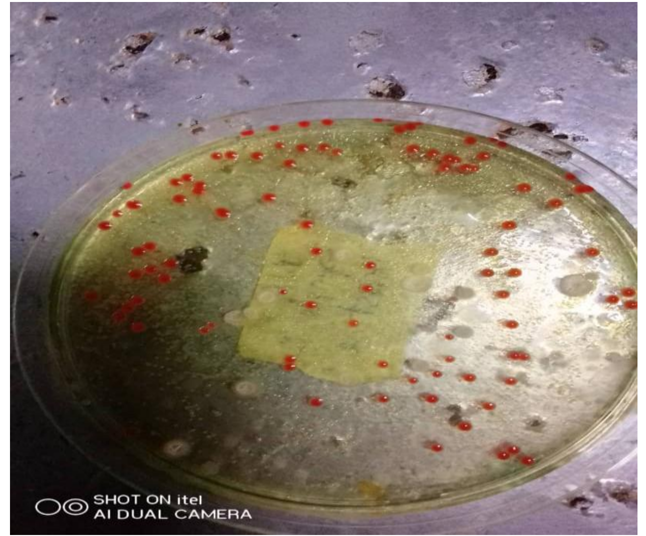
Diseased fish samples

Sluggishness/Skin Decolourization

Smooth circular and light pink colonies found on MacConkey agar

Smooth circular Opaque and tiny light pink colonies found on EMB agar

Circular colonies with dark spot in their middle found in SS agar

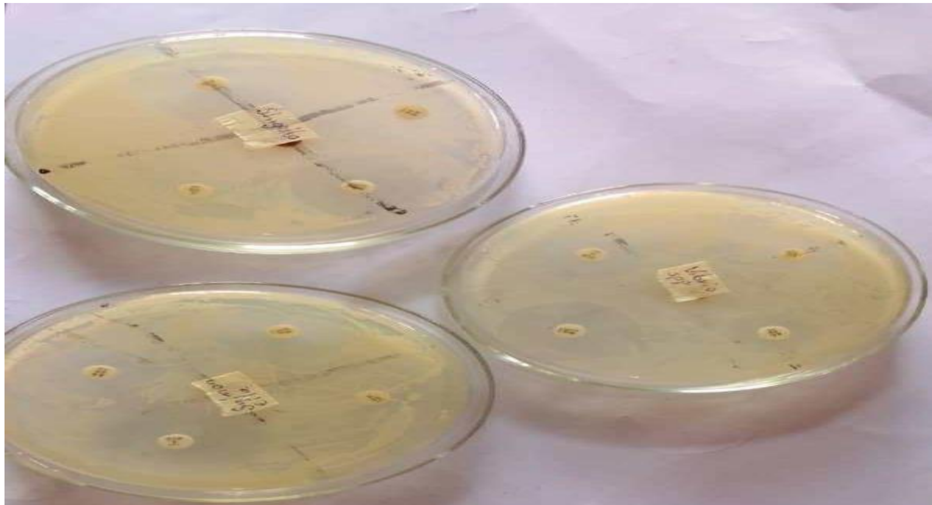


Pink colonies with finger like projections found in TCBS

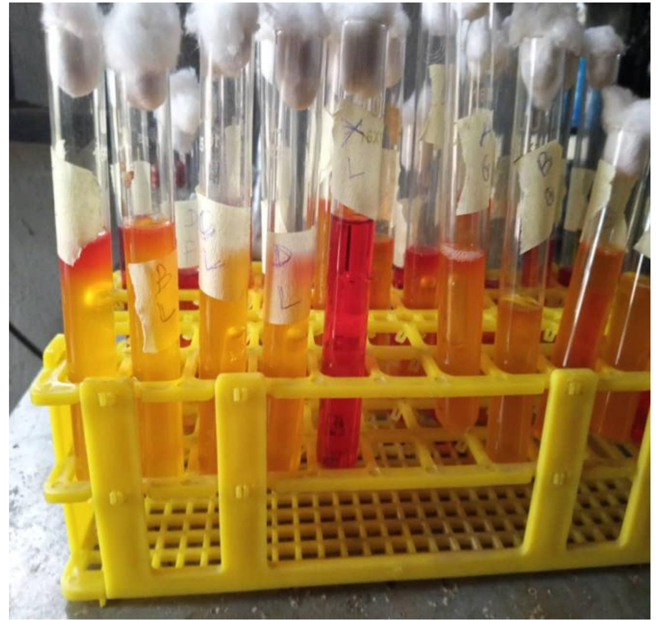
Colorless circular olonies with green spot in their middle found on TCBS agar

White circular colonies found on nutrient agar

Picture showing the antibacterial activity of Silver nanoparticles of H*ibiscus* on *Shigella* sp.



Picture showing antibiotic susceptibility of the various isolates



Result of citrate test

Result of indole test

Sterile collection of various organs of catfish using dissecting scissors

Various media used for bacterial isolation

Silver nitrate used for synthesizing silver nanoparticle   Spectrophotometer reading gotten during MIC test

Table 1: Mean of zone of inhibition (mm) of antibiotics on the various isolates

Isolates	CIP (5µg)	AK (30µg)	C (30µg)	TE (30µg)	CN (30µg)
Bacillus sp	19	14	10	13	21
Enterobacter sp	12	-	10	-	32
Salmonella sp	28	15	-	-	15
Vibrio sp	21	16	8	-	26
Shigella sp	28	-	-	-	25

CIP = Ciprofloxacin

AK = Amikacin

C = Chloramphenicol

TE = Tetracycline

CN = Gentamicin

Table 2: Mean antibacterial activity of three independent experiments ± S.D (n=3) of Silver nanoparticles (AgNps) from 0.1M and 0.5M of *Hibiscus rosa sinesis* (Hibiscus leaves) and *Azadirachta indica* (Neem leaves) at different concentrations.

Leaf Type	Molar Conc.	Trial	*Shigella* sp	*Vibrio* sp	*Bacillus* sp	*Enterobacter* sp	*Salmonella* sp
Neem	0.1M	A	16.67±0.58[a]	16.00±1.00[a]	15.00±1.00[a]	14.00±1.00[a]	0.33±0.58[a]
		B	14.67±0.58[a]	11.00±1.00[b]	10.00±1.00[b]	13.00±1.00[a]	12.00±1.00[b]
		C	16.00±1.00[a]	21.00±1.00[c]	13.00±1.00[a]	12.00±1.00[a]	0.00±0.00[a]
		D	11.00±1.00[b]	13.00±1.00[b]	14.00±1.00[a]	19.00±1.00[b]	0.00±0.00[a]
		Control	44.00±0.00[c]	25.00±0.00[d]	27.00±0.00[c]	34.00±0.00[c]	13.00±0.00[b]
	0.5M	A	14.00±1.00[a]	12.00±1.00[a]	13.00±1.00[a]	14.00±1.00[a]	13.00±1.00[a]
		B	10.00±1.00[b]	10.00±1.00[b]	10.00±1.00[b]	11.00±1.00[b]	9.00±1.00[b]
		C	8.00±1.00[b]	8.00±1.00[b]	11.00±1.00[b]	10.00±1.00[b]	8.00±1.00[b]
		D	9.00±1.00[b]	8.00±1.00[b]	11.00±1.00[b]	13.00±1.00[b]	11.00±1.00[a]
		Control	50.00±0.00[c]	47.00±0.00[c]	32.00±0.00[c]	35.00±0.00[c]	44.00±0.00[c]
Hibiscus	0.1M	A	18.00±1.00[a]	19.67±1.53[a]	24.00±1.00[a]	11.00±1.00[a]	9.00±1.00[a]
		B	22.00±1.00[b]	17.00±1.00[a]	15.00±1.00[b]	15.00±1.00[b]	12.00±1.00[b]
		C	15.00±1.00[c]	23.00±1.00[b]	21.00±1.00[c]	14.00±1.00[b]	11.00±1.00[b]
		D	12.00±1.00[c]	19.00±1.00[a]	22.00±1.00[c]	13.00±1.00[b]	10.00±1.00[a]
		Control	21.00±0.00[d]	44.00±0.00[c]	47.00±0.00[d]	30.00±0.00[c]	45.00±0.00[c]
	0.5M	A	13.00±1.00[a]	18.00±1.00[a]	14.00±1.00[a]	12.00±1.00[a]	17.00±1.00[a]
		B	12.00±1.00[a]	18.00±1.00[a]	15.00±1.00[a]	13.00±1.00[a]	14.00±1.00[b]
		C	20.00±1.00[b]	21.00±1.00[b]	15.00±1.00[a]	17.00±1.00[b]	15.00±1.00[b]
		D	11.00±1.00[a]	15.00±1.00[c]	11.00±1.00[b]	23.00±1.00[c]	19.00±1.00[a]
		Control	39.00±0.00[c]	33.00±0.00[d]	28.00±0.00[c]	32.00±0.00[d]	24.00±0.00[c]

KEY

Control = Gentamicin

A = 500mg/ml

B = 250mg/ml

C = 125mg/ml

D = 62.5mg/ml

(Values with different alphabets are significantly different across the different concentrations of Neem silver nanoparticle tested per isolate).

Table 3: Mean Result of Antibacterial Activity of three independent experiments + S.D.(n=3) of Zinc Oxide nanoparticles (ZnONps) from 1M and 2M of *Hibiscus rosa sinesis* (Hibiscus leaves) and *Azadirachta indica (*Neem leaves) at different concentrations.

Type	**Molar Conc.**	**Trial**	***Shigella* sp**	***Vibrio* sp**	***Bacillus* sp**	***Enterobacter* sp**	***Salmonella* sp**
Neem	1M	A	15.00±1.00[a]	0.33±0.58[a]	35.00±1.00[a]	38.00±1.00[a]	30.00±1.00[a]
		B	21.00±1.00[b]	0.33±0.58[a]	16.00±1.00[b]	18.00±1.00[b]	15.00±1.00[b]
		C	12.00±1.00[c]	11.00±1.00[b]	15.00±1.00[b]	20.00±1.00[b]	19.00±1.00[c]
		D	16.00±1.00[a]	21.00±1.00[c]	20.00±1.00[c]	22.00±1.00[b]	25.00±1.00[d]
		Control	36.00±0.00[d]	18.00±0.00[d]	14.00±0.00[b]	23.00±0.00[b]	22.00±0.00[d]
	2M	A	9.00±1.00[a]	14.00±1.00[a]	18.00±1.00[a]	24.00±1.00[a]	24.00±1.00[a]
		B	1.00±1.00[b]	1.00±1.00[b]	14.00±1.00[b]	5.00±1.00[b]	16.00±1.00[b]
		C	1.00±1.00[b]	1.00±1.00[b]	11.00±1.00[b]	12.00±1.00[c]	1.00±1.00[c]
		D	1.00±1.00[b]	1.00±1.00[b]	9.00±1.00[b]	15.00±1.00[c]	8.00±1.00[d]
		Control	20.00±0.00[c]	23.00±0.00[c]	19.00±0.00[a]	17.00±0.00[c]	27.00±0.00[a]
Hibiscus	1M	A	21.00±1.00[a]	18.00±1.00[a]	1.00±1.00[b]	1.00±1.00[b]	11.00±1.00[b]
		B	17.00±1.00[b]	25.00±1.00[a]	1.00±1.00[b]	1.00±1.00[b]	31.00±1.00[a]
		C	26.00±1.00[a]	16.00±1.00[b]	1.00±1.00[b]	15.00±1.00[b]	36.00±1.00[a]
		D	34.00±1.00[a]	33.00±1.00[a]	38.00±1.00[a]	32.00±1.00[a]	14.00±1.00[d]
		Control	17.00±0.00[c]	21.00±0.00[c]	24.00±0.00[a]	19.00±0.00[c]	23.00±0.00[a]
Hibiscus	2M	A	1.00±1.00[b]	1.00±1.00[b]	1.00±1.00[b]	1.00±1.00[b]	7.00±1.00[a]
		B	1.00±1.00[b]	1.00±1.00[b]	1.00±1.00[b]	1.00±1.00[b]	23.00±1.00[b]
		C	13.00±1.00[a]	9.00±1.00[b]	5.00±1.00[b]	1.00±1.00[c]	28.00±1.00[c]
		D	18.00±1.00[a]	15.00±1.00[b]	14.00±1.00[b]	24.00±1.00[c]	17.00±1.00[d]
		Control	21.00±0.00[c]	26.00±0.00[c]	28.00±0.00[a]	23.00±0.00[c]	22.00±0.00[a]

KEY

Control = Gentamicin A = 500mg/ml B = 250mg/ml

C = 125mg/ml D = 62.5mg/ml