

UNIVERSITY OF BAHRAIN

College of Science



Department of Biology

Eco-Friendly Solutions to Antibiotic Resistance in Wastewater: Integrating Plants, Nanoparticles, and Bacteriophages

A Thesis Submitted in Partial Fulfilment of the Requirements for the
M.Sc. Degree in Biological Sciences

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Abstract

Antibiotic resistance is a significant health issue that continues to rise due to the improper utilization of antibiotics across various sectors, leading to the emergence of a wide range of multidrug-resistant (MDR) bacteria. Therefore, an urgent need has been suggested to propose alternative strategies to combat the spread of antibiotic resistance. These non-traditional approaches involve innovative, safe, efficient, and environment-friendly methods. In this study, three methods were investigated against MDR bacteria isolated from wastewater: plants, nanoparticles, and bacteriophages.

The MDR bacteria were isolated from wastewater at different treatment stages and from the contaminated seawater. The bacterial susceptibility was examined against a group of antibiotics by the Kirby Bauer disc diffusion method, and those that demonstrated multidrug resistance were further analysed and identified. After that, the selected MDR bacterial isolates were treated by six plants crude ethanol extracts (*Hibiscus (H.) rosa-sinensis*, *Dodonaea (D.) viscosa*, *Cymbopogon (C.) citratus*, *Citrus (C.) aurantifolia*, *Olea (O.) europaea*, and *Manilkara (M.) zapota*), and three nanoparticles (silver, titanium dioxide and zinc oxide), by broth microdilution method. Furthermore, raw sewage samples were screened for MDR bacteria-specific bacteriophages by agar overlay plaque assay.

The study findings indicated that 30 bacterial isolates (73%) of the 41 tested ones showed multidrug resistance against three or more classes of tested antibiotics. Of those isolates, nine showed resistance to different antibiotic combinations, particularly to some showed effective action against most of the other isolates. These isolates involved three *Escherichia (E.) coli* strains: *Klebsiella (K.) pneumoniae*, *Pseudomonas (P.) putida*, two *Aeromonas (A.) veronii* strains, *Enterococcus (E.) faecalis*, and *Streptococcus* spp.

Plants' crude extracts showed great antimicrobial activity against all tested isolates at minimum inhibition concentrations ranging from 6.125 to less than 0.78 mg/mL. *C. aurantifolia* completely inhibited the growth of *P. putida*, *A. veronii*, *E. faecalis*, and *Streptococcus* spp. at all tested concentrations. *H. rosa-sinensis* and *D. viscosa* also inhibited the growth of *P. putida* and *E. faecalis* at all concentrations.

Silver nanoparticles showed the highest inhibition rates against all the bacterial isolates, particularly at a concentration of 1.0 mg/mL. There was a statistically significant difference in bacterial growth at different concentrations of silver nanoparticles.

One bacteriophage strain was isolated and purified from raw sewage. It successfully infected one of the MDR *E. coli* strains, indicating that its narrow host range is strain specific.

In conclusion, the screening of wastewater indicated the presence of a wide range of antibiotic-resistant bacteria. Plants crude extracts, nanoparticles and bacteriophages showed a remarkable antibacterial activity. These are locally sourced alternatives to antibiotics that hold promise in the fight against antibiotic-resistant bacteria.

Dedications

To my mother, whose persistent love has inspiring me in every journey.

To my father, whose unlimited support have shaped me into the person I am today.

To Dr. Essam Janahi, whose expertise and continuous guidance and encouragement
have been influential in shaping my academic journey.

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List of Abbreviations

ABC	ATP-binding cassette
ANOVA	Analysis of Variance
ARGs	Antibiotic resistance genes
CDC	Center for Disease Control
CFU	Colony forming units
CI	Confidence interval
CLSI	Clinical and Laboratory Standard Institute
EMB	Eosin methylene blue
ESBLs	Extended-spectrum B-lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GCC	Gulf Cooperation Council
GLASS	Global Antimicrobial Surveillance System
HGT	Horizontal gene transfer
IMP	Imipenemase
I	Intermediate sensitivity
KPC	Klebsiella pneumoniae carbapenemase
MATE	Multidrug and toxic compound extrusion
MDR	multidrug resistance
MENA	Middle East and North Africa
MFS	Major facilitator superfamily
MGEs	Mobile genetic elements
MHA	Muller Hinton agar
MIC	Minimum inhibition concentration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSA	Mannitol salt agar
NDM	New Delhi metallo B-lactamase
NPs	Nanoparticles
OD	Optical Density
OXA	Oxacillinase
PBP	Penicillin binding protein
PCR	Polymerase chain reaction

PFU	Plaque forming unit
R	Resistant
RND	Resistance nodulation cell division
ROS	Reactive oxygen species
S	Sensitive
SD	Standard deviation
SMR	Small multidrug resistance
SPSS	Statistical Package for Social Sciences
TSB	Tryptone soy broth
UAE	United Arab Emirates
VIM	Verona integron encoded metallo B-lactamase
WHO	World Health Organization
WPCC	Water Pollution Control Center
WWTP	Wastewater treatment plant
XLD	Xylose lysine deoxycholate
XRD	X-ray diffraction

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Chapter One

Introduction to the Study

- 1.1. Introduction
- 1.2. Research Problem
- 1.3. Research Questions
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- 1.5. Research Significance
- 1.6. The Study's Setting
- 1.7. Research Terminology

Introduction to the Study

1.1. Introduction

Bacterial infections have a severe effect on public health. In about a century, antibiotics were, and still are, the most effective pharmaceuticals to combat bacterial infections in both humans and animals, changing modern medicine and saving millions of lives (Arsene et al., 2022; Uddin et al., 2021). Antibiotics are low molecular weight substances that can be naturally produced or chemically synthesized. Unlike the antimicrobials, a general term for agents that kill a broad range of microorganisms, antibiotics act on essential components of bacterial cells to kill the microbe or block its growth (Arsene et al., 2022). Most known antibiotics were identified and clinically used in the Golden Age of antibiotics (1940s-1970s). However, the discovery and production of new classes of antibiotics dramatically declined later due to the rise of a significant, serious problem: antibiotic resistance (Hutchings et al., 2019).

Antibiotic resistance is a severe and complex health problem in which bacteria naturally adapt to the presence of antibiotics and evolve to become able to resist their inhibitory or damaging activity (Arsene et al., 2022). The capability of microbes for antimicrobial resistance could be due to the structural properties of their cells or the acquirement of some genes that encode proteins to attack the antimicrobial agent (Tiwari et al., 2022). The prevalence of this occurrence continues to rise due to the improper utilization of antibiotics across various sectors, including food industries, agriculture, and healthcare. Such antibiotic overuse has led to the transfer of resistance genes between different bacterial species (Raza et al., 2021). This has culminated in a grave and intricate health crisis, emerging as a

prominent global cause of mortality (Mancuso et al., 2021; Altayb et al., 2022).

There was once a belief that the issue of antibiotic resistance could be resolved through the continuous creation of novel antibiotics. Nonetheless, as bacteria have evolved various resistance mechanisms, complications have arisen in developing new potent molecules (Mancuso et al., 2021). Therefore, with the shortage of new antibiotics and other effective therapies and the lack of promising prevention measures, an urgent need has been suggested to propose alternative strategies to combat the spread of antibiotic resistance by establishing protocols that entail new bioactive materials (Frieri et al., 2017).

To overcome the crisis of antibiotic resistance, careful use of the available antibiotics, which should be prescribed by professionals, the use of antibiograms that show microbes' susceptibility to different antibiotics, and the development of new effective compounds to fight resistant microbes are required (Arsene et al., 2022). Among the alternative strategies being investigated, there is a substantial effort to find non-antibiotic antibacterial agents and unconventional techniques, which may show a promising solution to fight antibiotic-resistant organisms and treat the infections they cause.

Medicinal plants have been used for thousands of years in the treatment and protection from a wide range of diseases and infections worldwide (Almeida et al., 2013). Plant-derived substances are natural bioactive compounds in plants that act as a defence mechanism to protect from diseases. As the majority of these compounds are not well studied, the plant-derived compounds attract researchers' interest to investigate their antimicrobial activity as an alternative method to fight the antibiotic-resistant bacteria (Almeida et al., 2013).

Nanotechnology is one of the modern technologies that can provide new strategies in novel clinical applications and the treatment of infections. Nanoparticles (NPs) possess unique physical and chemical properties that are not known by the defence mechanisms of bacteria, thus show substantial antimicrobial effects to inhibit bacterial growth (Makabenta et al., 2021).

Bacteriophages, the bacterial viruses, have been used in the treatment of bacterial infections for a long time. Bacteriophages are non-conventional and natural antibacterial agents that can complement the conventional antibiotics. Their special characteristics of high specificity in their bactericidal effect, low toxicity and self-multiplication within their host cells allow them to be a good choice as an alternative strategy to combat antibiotic resistance (Ling et al., 2022).

1.2. Research Problem

Typically, antibiotics serve as agents to control bacterial growth. Nevertheless, the indiscriminate application of these antibiotics leads to the emergence of resistance mechanisms within bacteria, which subsequently disseminate across bacterial populations through genetic transfer. Consequently, numerous antibiotics have forfeited their efficacy against these bacteria. Antibiotic resistance represents a substantial challenge to both global health and the economy.

Studies revealed increased percentages of antibiotic resistance in the effluents of treated wastewater (Huang et al., 2012). Unlike developed countries, environmental studies about antibiotic resistance in the Middle East, typically in Bahrain, are few (Light et al., 2022). Antibiotic resistance can develop and spread through wastewater treatment plants, where human and animal pathogens, antibiotics, agricultural waste, and many other

agents are received through sewage. Therefore, it creates an effective environment for the bacteria to transfer and exchange their genetic material, which leads to mutated multidrug-resistant bacterial strains (Uddin et al., 2021; Dai et al., 2022).

1.3. Research Questions

- What varieties of bacteria are typically present in treated wastewater in Bahrain and exhibit antibiotic resistance?
- Among the plant species under examination in this research, which demonstrates the highest antibacterial effectiveness against antibiotic-resistant bacteria?
- Which nanoparticles slated for investigation in this study exhibit the most potent antibacterial properties against antibiotic-resistant bacteria?
- What types of phages are present in sewage and can infect antibiotic-resistant bacteria?

1.4. Research Objectives

The primary objective of this study is to conduct a comparative and analytical examination of the antimicrobial efficacy of three alternative approaches -plants, nanoparticles, and bacteriophages- against antibiotic-resistant bacteria in wastewater. The main objectives of this study are:

- To identify bacteria in wastewater samples that show resistance to multiple antibiotics.
- To determine the antimicrobial activity of plant extracts and nanoparticles against antibiotic-resistant bacteria.
- To detect bacteriophages that possess antimicrobial activity against antibiotic-resistant bacteria.

1.5. Research Significance

This study addresses a significant research gap in Bahrain, where limited studies on antibiotic resistance have focused primarily on clinical examinations. By shifting the focus to the environment, specifically wastewater, this study fills a significant knowledge gap and expands our understanding of antibiotic resistance dynamics in the region. This study holds particular significance for Bahrain as it investigates the efficacy of alternative methods to antibiotics using locally available sources. Understanding the potential of these alternative approaches to combat antibiotic-resistant bacteria is of great importance for the region, as it offers the opportunity to develop sustainable and locally relevant strategies for addressing the growing challenge of antibiotic resistance.

1.6. The Study's Setting

The study was conducted at the Department of Biology, College of Science, University of Bahrain, from October 2023 to April 2024. Wastewater samples were obtained from the Tubli Water Pollution Center (WPCC), Bahrain's largest wastewater treatment plant (WWTP), and from the effluent discharge area in Tubli Bay. All the experimental parts were performed at the College of Science laboratories, except for the bacterial identification part, which was done by ExpressMED laboratories, Bahrain. The study's timeframe is shown in Figure 1.

	October 2023	November 2023	December 2023	January 2024	February 2024	March 2024	April 2024	May 2024
Samples collection								
Experimental work								
Data analysis								
Thesis writing								

Figure 1: Timeframe of the study.

1.7. Research Terminologies

- **Antibiotics:** Compounds produced naturally by living organisms or chemically synthesized that have antimicrobial activity by either killing the microbe or inhibiting its growth. Antibiotics are used as medicines to fight bacterial infections in humans and animals (Lewis 2020).
- **Antibiotic resistance:** The inability of antibiotics to kill or inhibit the growth of bacteria because they evolve different resistance mechanisms against these antibiotics and become unresponsive to their effect. Unlike antimicrobial resistance, a broad term for all microorganisms, antibiotic resistance describes bacterial resistance against antibacterial agents (Arsene et al., 2022).
- **Antibiotic-resistance genes:** Genes that express the products (enzymes, protein pumps, etc.) that allow antibiotic resistance of the bacteria (Lerminiaux & Cameron, 2019).
- **Bacteriophages (phages):** A diverse group of viruses that specifically infect bacterial cells and use their machinery to replicate and multiply (Sieiro et al., 2020).

- **Horizontal gene transfer:** A non-reproductive method of gene transfer among bacterial cells, as the genetic information moves between bacteria that might be related (Tao et al., 2022).
- **Minimum inhibitory concentration:** The lowest concentration of an antimicrobial agent that causes complete inhibition of the bacterial growth (Parvekar et al., 2020).
- **Multidrug resistance:** Acquired non-susceptibility of microbes to at least one in three or more classes of antibiotics, based on the Clinical and Laboratory Standard Institute guidelines (Light et al., 2022).
- **Nanoparticles:** Particles with a size range of 1 to 100 nm possess unique physical and chemical properties (Murugaiyan et al., 2022).
- **Phage therapy:** The involvement of bacteriophages in the treatment of bacterial diseases (Sieiro et al., 2020).
- **Penicillin-binding proteins:** Enzymes involved in the bacterial cell wall synthesis and development of the peptidoglycan layer (Murugaiyan et al., 2022).
- **Raw sewage:** Domestic wastewater that mainly contain human waste (faecal matter) and other human practices waste such as laundry, kitchen, bathing, and other household activities (Chahal et al., 2016).
- **Selective pressure:** Pressure exerted by environmental stress factors that either reduce or enhance the survival of an organism (Holmes et al., 2016).
- **Wastewater:** Used water that has contaminants, can be domestic, commercial, industrial, agricultural, or rainwater that runoff from the surfaces of urban areas (Chahal et al., 2016).

Chapter Two

Theoretical Framework and Literature Review

- 2.5. Antibiotic resistance and current issues
- 2.6. Molecular mechanisms of antibiotic resistance
 - 2.6.1. Antibiotic mode of action
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- 2.7. Environmental antibiotic resistance
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- 2.8. Alternative strategies to combat antibiotic resistance
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Theoretical Framework and Literature Review

2.1. Antibiotic resistance and current issues

Antibiotic resistance of pathogenic bacteria is a global health challenge due to its association with high morbidity and mortality. As bacteria develop multidrug-resistant (MDR) patterns against the standard antibiotics, they cause untreatable infections or those that are difficult to treat by these antibiotics (Frieri et al., 2017). The World Health Organization (WHO) listed up to 12 bacterial families that cause significant health problems to humans (Mulani et al., 2019), and due to its severe implications for human health, antimicrobial resistance has become one of the top ten health threats globally (World Health Organization [WHO], 2019). Reports by the WHO (WHO, 2019) estimated difficulties treating some serious bacterial infections, such as pneumonia, tuberculosis, salmonellosis, and gonorrhea, if the disease-causing microbes become resistant to the conventional antibiotics used to treat these diseases (WHO, 2019). Such issues will result in extended hospitalization, medical costs, and increased mortality rates (Uddin et al., 2021; Arsene et al., 2022).

The antibiotic-resistant bacteria are categorized as critical, high, and medium priority by the WHO; based on their resistance pattern and the severity of infections they cause (De Oliveira et al., 2020). Bacterial pathogens, including *Pseudomonas*, *Acinetobacter*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter* spp. from the *Enterobacteriaceae* family, are among the critical priority group of pathogens. They cause severe infections such as pneumonia and infections related to the bloodstream and are resistant to multiple antibiotics, thus requiring urgent solutions to combat their infections. Other high-priority infectious bacteria, such as *Enterococcus faecium* and *Staphylococcus*

aureus, and medium-priority bacteria, including *Streptococcus pneumoniae* and *Shigella*, show resistance to some antibiotics and require serious attention (Breijyeh et al., 2020; Abdelaziz et al., 2021).

The resistance incidence rates of pathogenic bacteria and their implications have raised concern about the health problems over the years. Over two-thirds of known pathogenic bacteria resist at least one antibiotic in a short time (Economou & Gousia, 2015). Yearly, over 700,000 deaths occur due to resistant bacterial infections worldwide, and millions of deaths are expected by 2050 if this issue is not resolved correctly (Subramaniam & Girish, 2020). In 2019, the Center for Disease Control (CDC), reported by WHO, showed more than 2.8 million infections annually and more than 35000 deaths caused by antibiotic-resistant bacteria in the United States (WHO, 2022). Also, in 2019, Data reported in the Global Antimicrobial Surveillance System (GLASS) by WHO indicated bloodstream infections of methicillin-resistant *S. aureus* (MRSA) and *E. coli* in 25 and 49 countries, respectively (WHO, 2020). *S. aureus* developed resistance against penicillin after just one year of its discovery and against methicillin, a semi-synthetic antibiotic related to penicillin, within less than a year of its discovery (Dodds, 2017). In India, bacterial resistance to antibiotics causes one death among children every nine minutes and more than 50000 fatalities of newborns due to sepsis, an improper body response to bacterial infections (Subramaniam & Girish, 2020). These reports indicate the extent of antibiotic resistance and the urgent need for action for the containment of this global threat.

Bacterial resistance to antibiotics is a natural process; however, the inappropriate use of antibiotics caused the uncontrolled spread of bacterial resistance to antibiotics and led to such public health concerns. Several factors, collectively called socioeconomic determinants, lead to the

prevalence of antibiotic resistance. Such factors include inappropriate and overuse of antibiotics via inaccurate diagnosis or self-medication, poor sanitation and hygiene, poor hospital environment and clinical procedures to control infection, and extensive use of antibiotics in agriculture, food, and animal industries (Collignon & Beggs, 2019). These factors lead to the accumulation of antibiotics in the environment and raise public health concerns.

2.2. Molecular mechanisms of antibiotic resistance

2.2.1. Antibiotics mode of action

Antibacterial activity of antibiotics varies according to their chemical structure, composition, and type of the target microbe. Five major classes of antibiotics are identified based on their mechanism of action on the bacteria. Such mechanisms include inhibition of (i) bacterial cell wall synthesis, (ii) bacterial nucleic acid synthesis, (iii) different bacterial cell protein synthesis, (iv) specific cellular metabolic pathways, and (v) bacterial cell membrane and its deterioration (Uluseker et al., 2021).

The mechanism of bacterial cell wall synthesis inhibition involves specific interaction with its major components that leads to improper cell walls and, thus, damaged cells that cannot survive. As it is well known that the bacterial cell wall is composed of peptidoglycan, a rigid protective envelope that covers the bacterial cell, and it is composed of linear sugar chains cross-linked with peptides (Irazoki et al., 2019). β -lactam antibiotics, which are characterized by the 4-membered lactam molecule, block the last step of the peptidoglycan biosynthesis by acylating the enzymes (also known as penicillin-binding proteins (PBP)) that are involved in the assembly of the peptidoglycan chains, thus, the cell lyses due to osmotic pressure (Bush & Bradford, 2016). Glycopeptides are

another group of antibiotics inhibiting cell wall synthesis; however, they act on the peptide linkers by hindering its expansion (Wang et al., 2018).

The inhibition of bacterial protein synthesis is another mechanism of antibiotic action. The ribosomes are the main cellular structures involved in protein synthesis, where the genetic code carried in the messenger RNA (mRNA) is translated to the appropriate amino acids sequence of the protein, which is carried by the transfer RNA (tRNA) (Byrgazov et al., 2018). The 70S bacterial ribosome consists of ribosomal RNA (rRNA) and proteins, which form two subunits, the 30S and 50S subunits (Byrgazov et al., 2018). Antibiotics that act on the 30S subunit include aminoglycosides and tetracyclines. Aminoglycosides cause improper reading of the mRNA codons; thus, incorrect amino acids are added, which leads to improper synthesis of the proteins (Krause et al., 2016). Tetracyclines, on the other hand, bind to the tRNA binding site at the 30S subunit of the ribosome, preventing the addition of the amino acids to the expanding protein chain and thus stopping its synthesis (Grossman, 2016). Other antibiotics act on the 50S subunit of the ribosome, such as macrolides, chloramphenicol, and oxazolidinones (Uddin et al., 2021). Macrolides inhibit the protein translocation step during its synthesis; thus, incomplete protein chains are released (Parnham et al., 2014). Chloramphenicol inhibits the formation of peptide bonds between the amino acids, while oxazolidinones prevent the ribosomal assembly of the two subunits during translation initiation (Dinos et al., 2016; Papich, 2016).

The inhibition of bacterial nucleic acid synthesis is another method of action of antibiotics. Bacterial genetic information is encoded in DNA, and its expression is aided by the different RNA molecules (Holmes & Jobling, 1996). Fluoroquinolones and aminocoumarins act on the DNA gyrase that functions in the negative supercoiling of the DNA prior to replication, and

topoisomerase IV that segregates the daughter DNA strands after replication, thus hindering DNA synthesis (Helgesen et al., 2021; Nainu et al., 2021). RNA synthesis can also be inhibited by rifamycin, as it binds to the RNA polymerase enzyme that synthesizes RNA strands during transcription and blocks its elongation (Saito et al., 2017).

Bacterial metabolism, including the intracellular anabolic and catabolic processes, is crucial for bacterial cell growth and survival. Antibiotics such as sulfonamides and trimethoprim interfere with these metabolic processes by inhibiting the metabolic enzymes by competing with their substrates or functioning against the metabolites produced (Fernández-Villa et al., 2017; Wróbel et al., 2019; Akter et al., 2020).

The last group of antibiotics targets the bacterial cell membrane, such as polymyxins and daptomycin (Levin & Angert, 2015). The cytoplasmic membrane is one of the essential components of the bacterial cell. These antibiotics interfere with the lipopolysaccharides that make the cell membrane. Cell membrane disruption eventually causes cell lysis and death (Helgesen et al., 2021).

2.2.2. Antibiotic resistance mechanisms

Naturally, bacteria can develop resistance to antibiotics, either by frequently expressing the resistance genes available within the bacteria or by activating them only when mediated by certain antibiotics (Reygaert, 2018). However, bacteria can obtain resistance through specific genetic mutations or by attaining antibiotic resistance genes (ARGs) from their surroundings (Lerminiaux, & Cameron, 2019). Genetic mutations responsible for resistance can transfer to the bacterial generations via vertical inheritance through reproduction. Alternatively, bacteria can acquire resistance genes via horizontal gene transfer (HGT) (Tao et al., 2022). This method is effective and fast in disseminating ARGs among

different bacterial species. HGT is a non-reproductive method, as the mobile genetic elements (MGEs), such as plasmids and transposons, are transferred into the bacterial cells (Levin, & Angert, 2015; Tao et al., 2022). HGT can occur in three ways: conjugation, transformation, and transduction. First, in conjugation, the genetic material, usually plasmids, is transferred between two bacterial cells through cell-to-cell contact. Second, in transformation, the cell takes up external genetic material, usually from dead cells, and is incorporated into its genome. Last, the transduction method requires bacteriophages, the viruses that infect and reproduce in bacteria, as they replicate inside resistant bacterial cells and then carry the host DNA segments with their genome and transfer them to another cell while infecting it (Martínez et al., 2015).

Acquiring the ARGs allows the bacteria to develop several cellular mechanisms to withstand the presence of these drugs. These mechanisms include (i) reduced permeability of antibiotics, (ii) antibiotic efflux, (iii) cellular target modification, and (iv) antibiotic inactivation (Levin, & Angert, 2015; Martínez et al., 2015).

The mechanism of minimizing the intracellular concentration of antibiotics can be achieved by reducing its permeability to enter the cell or by ejecting it outside the cell. Most of the hydrophilic antibiotics enter the bacterial cell via porins, the water channel proteins; therefore, mutations in the porins alleles, their downregulation to reduce their expression, or substitution with more selective channels can reduce the penetration of these antibiotics, such as β -lactams, tetracyclines, and fluoroquinolones (Uddin et al, 2021). Recent studies have identified this mechanism in several *Enterobacteriaceae* bacteria, *Pseudomonas*, and *Acinetobacter* bacteria (Iredell et al., 2016).

The excretion of antibiotics to the outside of the cell is another resistance mechanism. Some bacteria, such as *Salmonella*, *Klebsiella*, and *Pseudomonas*, develop this mechanism using efflux pumps (Blair et al., 2015). A group of efflux pump families has been identified and classified according to their structure and energy requirements. The five efflux pump families include ATP-binding cassette (ABC), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), resistance nodulation cell division (RND), and major facilitator superfamily (MFS) (Reygaert, 2018; Uluseker et al., 2021). Some of these pumps are specific to certain antibiotics, while others are multidrug. For example, tetracyclines are ejected by the specific Tet efflux pump (Grossman, 2016). In addition, macrolides such as erythromycin are ejected through efflux pumps encoded by the *mef* genes (Munita, & Arias, 2016).

Changing the antibiotic target is another mechanism of antibiotic resistance. Upon antibiotic entry into the bacterial cell, it specifically binds to its target molecule, thus inhibiting bacterial growth and survival. As the target molecule's structure is modified to keep its normal function, it will prevent binding to the antibiotic and, therefore, ineffective action on the bacteria. Several studies discussed examples of target modification, which either takes place through gene mutations or target protection (Blair et al., 2015; Harris et al., 2023). In MRSA, the *mecA* gene encodes penicillin-binding protein (PBP2a) that are alternatives to the native PBPs that function in the cell wall synthesis but are insensitive to the β -lactam antibiotics (Harris et al., 2023). In addition, the *ermB* gene encodes a methyltransferase that methylates the 50S ribosomal subunit, thus changing the binding site of the antibiotics, such as macrolides (Harris et al., 2023). Moreover, the *qnr* genes encode proteins that help release

topoisomerase from the complex formed by the quinolones, thus reactivating them (Vetting et al., 2011).

In addition to their targets, bacteria can modify the antibiotics themselves in another mechanism of antibiotic resistance. Such modification may occur by hydrolyzing the antibiotic molecules or transferring some chemical groups from their molecules (Blair et al., 2015). A wide range of enzymes are involved in the degradation of different classes of antibiotics. Following the early β -lactamases, extended-spectrum β -lactamases (ESBLs) which are encoded by the *CTX-M* genes, and a range of carbapenemases such as imipenemase (IMP), Verona integron encoded metallo β -lactamase (VIM), *K. pneumoniae* carbapenemase (KPC), oxacillinase (OXA), and New Delhi metallo β -lactamase (NDM) have been identified in different bacteria around the world (Blair et al., 2015). As the β -lactam ring of the antibiotic is destroyed, its ability to bind to the PBP during cell wall synthesis is inhibited (Bush & Bradford, 2016). Other enzymes can chemically modify the antibiotics by altering their structure, thus preventing their binding to their target. For example, the aminoglycoside modifying enzymes can covalently alter the hydroxyl or amino groups of the aminoglycoside antibiotics, thus inactivating them (Munita & Arias, 2016). Chemical alteration of antibiotics can also involve other methods, such as acetylation, phosphorylation, and adenylation (Lin et al., 2015).

2.3. Environmental antibiotic resistance

Antibiotics are naturally produced by many organisms in the environment as a defence mechanism against other pathogenic organisms. However, due to the human impact in the massive production and use of antibiotics and improper release to the environment, the imbalanced situation has led to the involvement of the environment in the antibiotic resistance crisis

(Larsson & Flach, 2022). When certain environmental conditions gather, the microbe can survive and develop novel characteristics by undergoing genetic modifications, in a condition called selective pressure (Uddin et al., 2021). An intense selective pressure on a microbe occurs when it occurs inside a human or animal body and is treated with antibiotics, leading to mutations and, thus, developing resistance (Uddin et al., 2021). Although the external environment is less likely to contribute to the resistance evolution, some environmental bodies, particularly those that have been polluted with antibiotics, can play some role (Larsson & Flach, 2022). The environment receives antibiotics through human and animal excretions and their inappropriate disposal in the waste from municipal sewage, hospital effluents, or drug industrial sites (Larsson & Flach, 2022). Wastewater, including domestic, commercial, agricultural, industrial, or stormwater in urbanized areas, contains many different materials such as organic and inorganic materials, nutrients, metals, animal and plant waste, oil compounds, and other dirt particles (Chahal et al., 2016). In addition, varieties of pathogenic and non-pathogenic bacteria prefer growing in wastewater. Domestic sewage mainly contains fecal material, a significant source of human pathogenic microbes. In addition, waste from agriculture and livestock industries can be a source of animal microbes that cause zoonotic infections (Chahal et al., 2016). These combinations provide suitable conditions to perform selective pressure for microbial antibiotic resistance. Thus, studying antibiotic resistance in wastewater is of great significance, as it is one of the main sources of antibiotics spreading into the environment.

Monitoring antibiotic resistance in the environment, particularly wastewater, plays a vital role in large-scale surveillance efforts. It complements clinical surveillance by allowing the screening of a vast

number of bacteria present in a small sewage sample (Tiwari et al., 2022). As sewage receives diverse inputs, including fecal bacteria, agricultural waste, and other sources, it provides valuable insights into the prevalence and dynamics of antibiotic resistance in a population, and it reflects the mixed microbiome community of a population (Tiwari et al., 2022). Several studies highlighted the surveillance of antibiotic resistance using different methodologies, bioinformatics, and through opinions, critical or systematic reviews, and meta-analyses (Hendriksen et al., 2019; Huijbers et al., 2019; Pruden et al., 2021; Zaatout et al., 2021; Chau et al., 2022; Miłobedzka et al., 2022). They noted the different challenges and limitations. In a review paper, Larsson and Flach (2022) compared clinical resistance surveillance and sewage-based resistance surveillance through different attributes. Compared to gene-based methods, isolate-based sewage monitoring surveillance is better at identifying resistance phenotypes, rare resistance types, and multi-resistance patterns and linking resistance to specific species (Larsson & Flach, 2022). Furthermore, it is of low cost, requires no ethical issues with sampling, and simple sampling techniques, in which a single sample can reflect the resistance situation of the whole community because it contains bacterial pools from large populations; therefore, fewer resources are required (Larsson & Flach, 2022).

The prevalence of antibiotic resistance in the environment can be evaluated by different methods, which can be culture-dependent for phenotypic analysis, culture-independent for the analysis of ARGs, or shotgun metagenomics (Grehs et al., 2021). Each method has several advantages; the culture-based methods allow studying the viable cells and their phenotypic characteristics, while the culture-independent, or the polymerase chain reaction (PCR)-based methods permit the study of

specific genes and mutations and the abundance quantification in the environment. Metagenomics is a powerful and high throughput tool that provides a broader overview of the resistance trend in complex microbial communities and allows the identification of novel ARGs and other biomolecules (Grehs et al., 2021). In China, multiple antibiotic-resistant bacteria were isolated from the secondary effluents of a municipal WWTP, and their susceptibility was evaluated against six different antibiotics. The results show that three isolates (*Aeromonas*, *Enterobacter*, and *Escherichia*) were resistant to all the tested antibiotics (Huang et al., 2012). A study in Sweden showed that *E. coli* resistance rates from municipal sewage, although about half of the rates measured in primary health care, were strongly correlated. They were also more stable, suggesting a low-cost sewage-based surveillance system for antibiotic resistance (Hutinel, et al., 2019). Another study involved seven European countries, where 229 resistance genes and 25 mobile genetic elements of antibiotic resistance were analyzed by parallel quantitative PCR. This surveillance showed that the antibiotic resistance profiles from urban WWTP could mirror those observed in the clinical surveillance (Pärnänen et al., 2019). A recent study combined the culture-based methods for resistance pattern identification and molecular screening techniques for ARGs determination in wastewater. The most common resistance was against tetracycline and ampicillin, and the genes responsible for antibiotic resistance were present (Godinho et al., 2024). These studies reveal the prevalence of antibiotic resistance in wastewater, which various methods and techniques can determine.

2.3.1. Antibiotic resistance in the Middle East

In Europe and Central Asia, antibiotic resistance regional surveillance systems were established progressively as the problem arose. However, the

situation differed in low and middle-income countries, such as Africa and the Middle East (Truppa & Abo-Shehada, 2020). Recently, investigators conducted a group of studies to evaluate the prevalence of antibiotic resistance in this region. A systematic review studied the available data on the epidemiology of the resistant *S. pneumoniae* in the Middle East. It indicated the prevalence of antibiotic resistance over time in some countries, while no surveillance reports were found in other countries (El Moujaber et al., 2017). Another systematic review analyzed the epidemiology along with the genomic and microbiological characteristics of MDR *P. aeruginosa* in the Middle East and North Africa (MENA) region and revealed a wide variation in its antimicrobial characteristics, as well as an increase in its prevalence in most of the countries (Al-Orphaly et al., 2021). A recent retrospective study analyzed the prevalence of antibiotic resistance in *E. coli* and the other ESKAPE pathogens (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) in the Arabian region during the last 20 years. It reported its occurrence in 19 out of the 22 countries and indicated a significant increase in the resistance rates to various classes of antibiotics (Nasser et al., 2020). Another recent study included nine Arabian countries (Saudi Arabia, Bahrain, Kuwait, Qatar, United Arab Emirates (UAE), Oman, Iraq, Jordan, and Yemen). It showed that more than half of the mortalities from MDR infections were caused by *A. baumannii*, *M. tuberculosis*, and *S. aureus* in these countries (Borgio et al., 2021). A study in the Gulf Cooperation Council (GCC) countries indicated a high prevalence rate of MDR bacteria from *Enterobacterales*, particularly among intensive care unit patients and those with urinary tract infections (Hadi et al., 2023).

All the previously mentioned studies have covered the clinical surveillance of antibiotic resistance to evaluate its prevalence; however, information on environmental surveillance is limited in the Middle East. The inappropriate use and dispensing of antibiotics are shared every day in the Middle East, and many developing countries (Al Romaihi et al., 2018), so microbial resistance to antibiotics in the environment is expected. In 2022, a marine monitoring program was established to assess antibiotic resistance in the GCC region by analyzing seawater samples from high-risk areas of the Gulf countries (Light et al., 2022). High percentages of antibiotic resistance were detected against many different antibiotics, and the whole genome sequencing of the bacterial isolates revealed the presence of *qqnrS1* and *bla_{CTX-M-15}* genes that correlate with antibiotic resistance (Light et al., 2022). The importance of this study lies in establishing an environmental antimicrobial resistance surveillance system that involves the marine environment and using genome-based analysis techniques to monitor the antibiotic resistance patterns in the region (Light et al., 2022).

Moreover, another study identified antibiotic-resistant bacteria isolated from different locations on the Red Sea coast in Jeddah, Saudi Arabia (Alsalmi & Altayb, 2023). In Kuwait, sediment samples were collected from a coastline near the effluent discharge areas and recreational beaches and tested for antibiotic-resistant *E. coli* strains. Results showed resistance in 98% of the tested isolates detected in all the selected sites (Al-Sarawi et al., 2022). Such studies contribute to a broader understanding of antibiotic resistance and its extent in the environment.

In recent years, GCC region investigators have focused on determining antibiotic resistance in wastewater and WWTP. In UAE, culture-based methods and multiplex PCR techniques detected the occurrence of ESBL-producing bacteria in non-clinical sources like food and wastewater (Khan

et al., 2020). Also, in another study in UAE, both quantitative PCR and metagenomics shotgun sequencing methods were used to detect and quantify ARGs in wastewater, suggesting the possibility of establishing a more robust surveillance system to monitor ARGs in wastewater (Elbait et al., 2024). In Saudi Arabia, MDR clinically significant bacteria were isolated from hospital and municipal wastewater. In addition, different ARGs were detected, which indicated the presence of ESBL and carbapenemases-producing bacteria at significant rates (Irfan et al., 2023). These studies show that the Middle East and the GCC region are not an exception, and antibiotic resistance is also highly prevalent in this region, which is clearly documented.

2.3.2. Antibiotic Resistance in Bahrain

The Kingdom of Bahrain is a small archipelago composed of more than 33 islands with a dense and rapidly growing population (Al-Zubari et al., 2018; Janahi et al., 2020). In Bahrain, most studies have investigated antibiotic resistance in clinical isolates. Recent research has identified a cluster of bacteria within urinary pathogens exhibiting resistance to commonly used antibiotics. This finding underscores the presence of the problem within the local community. (Al Romaihi et al., 2018; Mohammed et al., 2022). Other studies also analyzed the prevalence of different antibiotic-resistant bacteria in clinical isolates, such as *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii*; their susceptibility was tested against different antibiotics via automated microbiological systems, and several resistance gene markers were identified (Joji et al., 2019; Shahid et al., 2022; Al-Rashed et al., 2023).

The environmental surveillance of antibiotic resistance in Bahrain is minimal. The main and largest WWTP is located in Tubli, which receives and processes most of the municipal wastewater. The treatment process

involves primary, secondary, and tertiary treatment stages, where the screening of the influent, chlorination, and ozonation processes take place, respectively (Aleisa & Al-Zubari, 2017). Currently, this WWTP produces secondary-treated effluent of nearly 150 million liters daily, of which only one-third is further processed through tertiary treatment, and the remaining is discharged into Tubli Bay (Abuhassan, 2017). Tubli Bay is a shallow, semi-enclosed bay located south of Manama, the capital city of Bahrain. It suffers from severe pollution due to the excessive and continuous discharge of wastewater effluents over the years (Naser, 2014).

In a study conducted in the GCC region involving Bahrain, seawater samples obtained from Tubli Bay and Nabih Saleh -areas close to the effluent discharge of the municipal WWTP- were tested against 14 different antibiotics, and antibiotic resistance phenotypes were elicited. Results show that Tubli Bay was one of the points where the highest percentages of MDR bacteria were detected (Light et al., 2022). In addition, another study in the Kingdom of Bahrain showed a high amount of fecal bacteriological indicators in the effluent samples of Tubli WWTP, such as *E. coli*, *Salmonella* spp., and *Shigella* spp. (Janahi et al., 2020). During the last few years, a densely populated residential area has been located along the coastline of Tubli Bay and near the wastewater discharge area, posing a serious health risk of being exposed to the microbes that would accumulate in this area, mainly if they were resistant to antimicrobials.

2.4. Alternative strategies to combat antibiotic resistance

One Health is an approach that recognizes the interconnection between human, animals, plants and the environment. Antimicrobial resistance is

one of the common health issues that threaten the health of people, animals and the environment (CDC, 2024). Bacteria develop antibiotic resistance in human and animal bodies and spread into the environment (soil or water), which then reach human and animals back from the shared environment. The control of this public health threat can be achieved by collaborating expertise in human, animal and environmental health, to determine the level of prevalence of antibiotic resistance. In addition, the use of environment-friendly alternatives to antibiotics as promising solutions to this problem, implementing the One Health approach.

Besides discovering new conventional antibiotics, the non-traditional strategies have attracted much of the researcher's interest. Alternative non-traditional approaches to combating microbial antibiotic resistance involve various innovative methods that are safe, efficient, and environmentally friendly methods. Such methods include antibodies, antimicrobial peptides, vaccines, cytokines, probiotics, plants, nanoparticles, and phage therapy (Uddin et al., 2021; Arsene et al., 2022). In addition, combinations of different strategies, along with the use of bioactive molecules, provide novel hybrid methods that result in promising integrated treatment solutions (Murugaiyan et al., 2022). These strategies can contribute to treating bacterial infections and limit the consequences of resistance. The following part reviews three alternative methods: plants, nanoparticles, and bacteriophages.

2.4.1. The use of plants

Using plant-derived compounds to control bacterial infection is one alternative method to tackle antibiotic resistance. Plants and medicinal herbs are essential in traditional therapeutics to treat and prevent various diseases and bacterial infections (Arsene et al., 2021). Of approximately 374,000 plants available, 28187 plant species are used for medicinal

purposes (Vaou et al., 2021). In addition, more than 1340 plants showed effective antimicrobial activity and are sources of more than 30000 antimicrobial compounds (Vaou et al., 2021).

As many bioactive compounds from plant extracts still need to be well studied, researchers move toward isolating and identifying new bioactive molecules that act against antibiotic-resistant bacteria. Recent studies showed the efficient antibacterial activity of plants, such as *Cymbopogon citratus*, *Lawsonia inermis*, and *Azadirachta indica*, against various types of bacteria (Nyamath & Karthikeyan, 2018; Pour & Farahbakhsh, 2020; Altayb et al., 2022). Several studies reveal that plant-derived bioactive compounds and phytochemicals possess antibacterial activity via different mechanisms, such as the destruction of bacterial cell membranes, inhibition of proteins and DNA synthesis, and inhibition of several enzymes involved in bacterial cell survival (Moloney, 2016; Barbieri et al., 2017; Vaou et al., 2021). Such compounds include alkaloids, phenolic compounds, sulfur-containing compounds, coumarins, and terpenes (Vaou et al., 2021). In a recent study, the in-silico analyses indicated high binding affinities between major plant bioactive compounds and some bacterial enzymes involved in different cellular functions (Aouf et al., 2022). These plant-derived antimicrobials are naturally available, safer than synthetic drugs, cost-effective, and easily administered; therefore, they offer a valuable alternative to treating antibiotic resistance (Vaou et al., 2021).

Plants can be used as a whole or a part of it, such as leaves, stems, roots, or flowers (Arsene et al., 2022). Different methods, such as maceration, infusion, and Soxhlet extraction, can extract plant-derived compounds (alkaloids, phenolics, flavonoids, and others). In addition, different alcoholic and organic solvents can be used based on the type of compounds to be extracted, such as methanol, ethanol, chloroform, or acetone

(Abubakar et al., 2020; Hikmawanti et al., 2021). In a study, crude ethanol extracts from *Pimenta pseudocaryophyllus* were obtained by cold maceration, and its antimicrobial activity was determined by agar well-diffusion method against different bacterial strains (de Paula et al., 2009). In another study, a microdilution assay tested methanolic extracts of *Callistemon viminalis* against different gram-positive and gram-negative bacteria (Salem et al., 2013). Overall, the different extraction protocols and the use of different solvents allow for obtaining a variety of plant bioactive compounds, which show significant antimicrobial activity.

2.4.2. The use of nanoparticles

Nanoparticles (NPs) are another promising alternative antibacterial agent against antibiotic-resistant bacteria. These smaller than 100 nm particulate substances show distinct characteristics, such as high surface area to volume ratio, multivalent interactions, and unique physical and chemical properties (Makabenta et al., 2021; Arsene et al., 2022). In addition, these synthetic agents are foreign to bacteria, thus able to avoid bacterial resisting mechanisms (Raza et al., 2021). Studies indicated the antibacterial activity of several metallic and metal oxide nanoparticles (Zain et al., 2014; Ikram et al., 2020; Mureed et al., 2021). A study revealed the antibacterial activity of silver nanoparticles (Ag NPs) against *S. aureus* at a minimum inhibitory concentration (MIC) of 0.625 mg/mL (Parvekar et al., 2020). In another recent study, copper-silver bimetallic nanoparticles (Cu: Ag NPs) were developed and tested against different bacterial strains (Mureed et al., 2021). The in-silico analyses indicated a possible inhibitory action of these bimetallic NPs against different bacterial enzymes (Mureed et al., 2021). Interestingly, NPs can be produced via green synthesis methods, a cost-effective and eco-friendly technique that uses living organisms and

biological extracts and has an acceptable yield and stability (Singh et al., 2020).

The ability of NPs to target multiple cellular pathways at a time, destroy cellular compartments, and inhibit DNA synthesis due to their easy penetration into the cell, allows to reduce the chance for bacteria to develop resistance mechanisms against them (Singh et al., 2020; Makabenta et al., 2021). The free silver ions released from Ag NPs act on the cell membrane disruption and DNA damage. In contrast, the copper ions from Cu NPs can produce reactive oxygen species (ROS) that destroy different amino acids and DNA synthesis processes. In addition, zinc and titanium ions from zinc oxide (ZnO) and titanium dioxide (TiO₂) NPs also cause the generation of ROS and damage to bacterial cell membranes (Hajipour et al., 2012; Miller et al., 2015; Gupta et al., 2019). This could provide viable and economical active therapeutic agents that are an alternative to conventional antibiotics in combating antibiotic resistance.

2.4.3. The use of bacteriophages

Bacteriophages, or phages, represent an environment-friendly solution to antibiotic resistance. Bacteriophages are ubiquitous viruses found abundantly in the environment as natural antibacterial agents. They specifically infect the target bacteria and use the bacterial cell machinery to replicate and release to the environment, destroying the host cell in their lytic cycle to infect another bacterial cell (Sieiro et al., 2020). On the other side, some bacteriophages integrate their genome after infection into the bacterial genome and remain there while the bacterial cell multiplies the viral genome along with its own genome until the lytic cycle is induced again to allow phage progeny propagation and release, leaving the bacterial cell destroyed (Sieiro et al., 2020). Due to its ability to destroy bacterial cells, phages can be a promising alternative to antibiotic resistance.

Phage therapy is the utilization of phages to treat bacterial diseases. It is not a new strategy; a century ago, these viruses that infect bacteria showed good efficacy as therapeutic agents to treat different bacterial infections. With the emergence of antibiotics, the researchers' interest in bacteriophages declined; however, it is currently regaining with the growing problem of antibiotic resistance and limitations of new antibiotic discovery (Sieiro et al., 2020; Raza et al., 2021; Ling et al., 2022). In addition, the availability of high throughput methods to screen large amounts of samples and the lower cost of modern techniques such as whole genome sequencing, compared to the past, allow the ability to characterize the phage's antibacterial effects and follow up the clinical investigations correctly (Kortright et al., 2019). Several studies reported isolating novel phages against drug-resistant bacteria (Mattila et al., 2015; Lehman et al., 2019; Ramadoss et al., 2022; Vera-Mansilla et al., 2022). In addition, studies reported the use of phage therapy in the treatment of different bacterial infections, such as those in the inner ear (Wright et al., 2009), urinary tract (Leitner et al., 2021), and multidrug-resistant bacteria (Aslam et al., 2020). Moreover, phage therapy can potentially make valuable contributions to veterinary medicine, the food industry, and bio-disinfection procedures (Raza et al., 2021; Garvey, 2022).

Compared to traditional antibiotics, phages have several advantages in treating antibiotic resistance. First, phages are naturally abundant everywhere in the environment. They can be rapidly isolated, mainly where the target pathogen is mostly available, such as in hospital wastewater and sewage effluents, fecal material, and the gastrointestinal tracts of humans and animals (Makabenta et al., 2020). Second, phages are host-specific; they target specific bacterial species or strains according to the cellular receptor they recognize for attachment and entry. Due to that, the typical

microbial community can be saved, unlike the antibiotics that damage a wide range of bacteria and cause further complications (Nobrega et al., 2015). In addition, phage can multiply inside the host cell, so a small dose is required for treatment. Furthermore, phages that recognize multiple cellular receptors can still infect bacteria that develop resistance by altering a specific cellular receptor (Silva et al., 2016).

The specificity of phages in targeting their host bacteria by recognizing specific receptor molecules on their cell surface is significant. Interestingly, phages that use cell receptors responsible for bacterial virulence or antibiotic resistance are very effective in phage therapy. For example, some phages use specific components of the bacterial capsules that cause bacterial virulence. To develop resistance against these phages, bacteria reduce the capsule formation, thus reducing its virulence by itself (Kortright et al., 2019). Similarly, other bacterial components that cause its virulence, such as pilli, adhesive, and secretive cell compartments, can be targets for phages as receptors, which cause reduced severity by reducing its expression to resist such phages and simultaneously reduce its pathogenicity (Kortright et al., 2019).

Similarly, phages can also use other cell compartments that bacteria express to resist antibiotics as receptors. For example, some phages recognize specific efflux pumps that expel antibiotics outside the cell. As the bacteria develop resistance against these phages by lowering or altering the efflux pump expression, they will also become sensitive to the antibiotics (Kortright et al., 2019). The latter is the main attractive situation for the researchers to implement in fighting antibiotic resistance.

The specificity of the phage to target its host is an essential factor in phage therapy and combating antibiotic resistance. Instead of using a single phage that recognizes one pathogen among the numerous bacteria that co-exist,

investigators apply phage cocktails, several phages to destroy several bacterial hosts, or polyvalent phage, a single phage that recognizes multiple bacterial hosts (Zhao et al., 2019; Brives & Pourraz, 2020). A study compared polyvalent phages and cocktails against antibiotic-resistant bacteria in soil systems. Data revealed that phage cocktails were more effective than polyvalent phages, and both were more effective than host-specific phages in reducing the abundance of the targeted pathogenic bacteria and their ARGs (Zhao et al., 2019). In another study, a polyvalent phage was isolated from slaughterhouse sewage and efficiently infected *E. coli* O157:H7, *Salmonella* spp., and *Shigella* spp., suggesting a suitable biocontrol against pathogenic bacteria is food (Kim et al., 2021). Such investigations suggest eco-friendly methods of using phages to control antibiotic resistance in the environment.

From the literature review demonstrated above, it is concluded that antibiotic resistance is a critical health problem that requires serious attention and urgent actions to minimize its implications worldwide, particularly in the Middle East and the Kingdom of Bahrain. The prevalence of antibiotic resistance is high, not only clinically but also environmentally. Antibiotic resistance is widely detected in wastewater effluents and WWTPs, which need regular screening and monitoring. Since antibiotics inhibit bacterial growth via different methods of action, antibiotic-resistant bacteria develop a variety of mechanisms to resist their action as well. Investigators tried a range of antibiotic alternatives to treat antibiotic-resistant bacterial infections. The diverse bioactive compounds in the plant extracts, the uniquely acting nanoparticles, and the bacteriophages that specifically infect bacteria are among those methods examined against antibiotic resistance bacteria and showed promising results to combat antibiotic resistance.

In the present study, a wide range of antibiotic-resistant pathogens are expected to be detected in the wastewater effluents. Therefore, the literature revealed the main culture-based methods for the isolation and detection of antibiotic-resistant bacteria and their specific bacteriophages and the differentiation of antibiotic-resistant bacteria from susceptible ones. Also, it explained methods of testing plant extracts and nanoparticles to determine their MIC and their inhibition pattern. Furthermore, it explained the efficiency of phages that specifically target their MDR-bacterial hosts as an alternative solution to antibiotic resistance.

Chapter Three

Methods and Procedures

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Methods and Procedures

3.1. Study methodology

Laboratory-based analysis of antibiotic resistance is one of the main components of antibiotic resistance surveillance systems. It involves receiving the samples, isolating bacteria, and performing antibiotic susceptibility tests (Amin et al., 2022). In this study, antibiotic resistance was investigated in wastewater. Tubli Water Pollution Control Center (WPCC), the main WWTP that receives around 70% of the wastewater in Bahrain, was selected as the source of wastewater samples. Although antibiotic resistance in raw sewage can represent its spread among the population (Larsson et al., 2023), secondary and tertiary treated wastewater was selected for investigation in this study because the marine environment mainly receives treated wastewater. Therefore, the secondary and tertiary treated water samples were obtained from the WWTP. In addition, wastewater samples discharged to the sea and seawater samples from areas about 500 m away from the discharge point were also examined. However, raw sewage was used as a source of bacteriophages infecting antibiotic-resistant bacteria.

Diverse and numerous bacterial strains were expected to be detected in wastewater. Therefore, in addition to the general media, different selective and differential culture media were used for bacterial isolation, including eosin methylene blue (EMB) agar medium, xylose lysine deoxycholate (XLD) agar medium, and mannitol salt agar (MSA) medium. The EMB agar medium differentiates lactose-fermenting from non-lactose fermenting coliforms and faecal coliforms with the use of two dyes, eosin and methylene blue. The color of these dyes changes due to the formation of dark purple products by the acids from lactose fermentation,

differentiating vigorous lactose fermenters (green metallic sheen color), and slow fermenters (dark color) from non-lactose fermenters (pink to translucent color) (Lal and Cheeptham, 2007). The XLD medium is used for *Salmonella* spp., *Shigella* spp. and other species of the *Enterobacteriaceae* family. The MSA medium mainly used for *Staphylococcus* spp. and other salt-tolerating Gram-positive bacteria, as it contains 7.5% sodium chloride (Shields and Tsang, 2006).

Usually, the antibiotic resistance test for phenotypic characterization of antibiotic resistance involves the disc diffusion method and broth microdilution method (McLain et al., 2016). Although many advanced methods have been evolved to determine antibiotic resistance, these conventional methods allow for determining viable antibiotic-resistant bacteria and quantitatively analyzing antibiotic resistance, for example, by determining the MIC (Oniciuc et al., 2018). This study used Kirby Bauer's disc diffusion method to screen and qualitatively determine antibiotic-resistant bacteria (Bauer et al., 1966). The selection of antibiotics was based on their mode of action from different antibiotics classes commonly used in clinical settings, so variable resistance mechanisms would be detected. The operating laboratory procedures should be standardized based on the guidelines provided by the standard organizations, such as the Clinical and Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Amin et al., 2022).

Quality control should be implemented in the phenotypic antibiotic susceptibility methods. To ensure this, the test was performed properly and in control of all the variables, efficient antibiotic activity occurred, and no contamination occurred, reference bacterial strains were used in line with

the environmental isolates, one representing Gram-negative bacteria (*E. coli*) and one representing Gram-positive bacteria (*S. aureus*).

After excluding the sensitive and intermediately sensitive bacteria and those resistant to less than three antibiotics, the remaining MDR strains that showed resistance to three or more classes of antibiotics were selected for further experimentation. An automated system was used to perform the microdilution method for antibiotic resistance testing against a broader range of antibiotics and to phenotypically identify the MDR bacterial species. Furthermore, these MDR bacteria were used to test the efficiency of the alternative antimicrobial agents investigated in this study.

In the current study, the antibacterial activity of three alternative methods to antibiotics was examined against the identified MDR bacteria. These methods include plant extracts, nanoparticles, and bacteriophages. The plants adopted in this study were selected based on their ability to grow locally, their availability, and their medicinal applications reported in the literature. The leaves of perennial plants can be a good and eco-friendly source of numerous bioactive compounds that could possess antimicrobial activity. Many different perennial plants require annual pruning, in which branches bearing leaves are removed for better renewal of the stems and leaves, while the removed branches and leaves are wasted (Chandrashekara, 2007). The bioactive compounds in the plant leaves were extracted using absolute ethanol as solvent, which is safe and effective in dissolving several molecules (Hikmawanti et al., 2021). Regarding the nanoparticles, pure metal, and metal oxide nanoparticles were examined for their antimicrobial activity at different concentrations.

Bacteriophages can be found widely in the environment. As mentioned, raw sewage was used as the source of phages infecting the wastewater-inhabiting bacteria. Here, two of the most popular methods in phage

experimentation were used: the spot test and plaque assay. One of the most accurate viral quantification and isolation methods is the plaque assay. The phages form plaques, the dead zones of bacterial cells, due to phage lysis (Baer & Kehn-Hall, 2014). Unlike the spot test that only determines the presence of phage, plaque assay can be used to determine the phage concentration or titer and to isolate pure phage since each plaque represents a single pure strain (Hyman, 2019).

The following flowchart demonstrates the workflow of the research protocol (Figure 2).

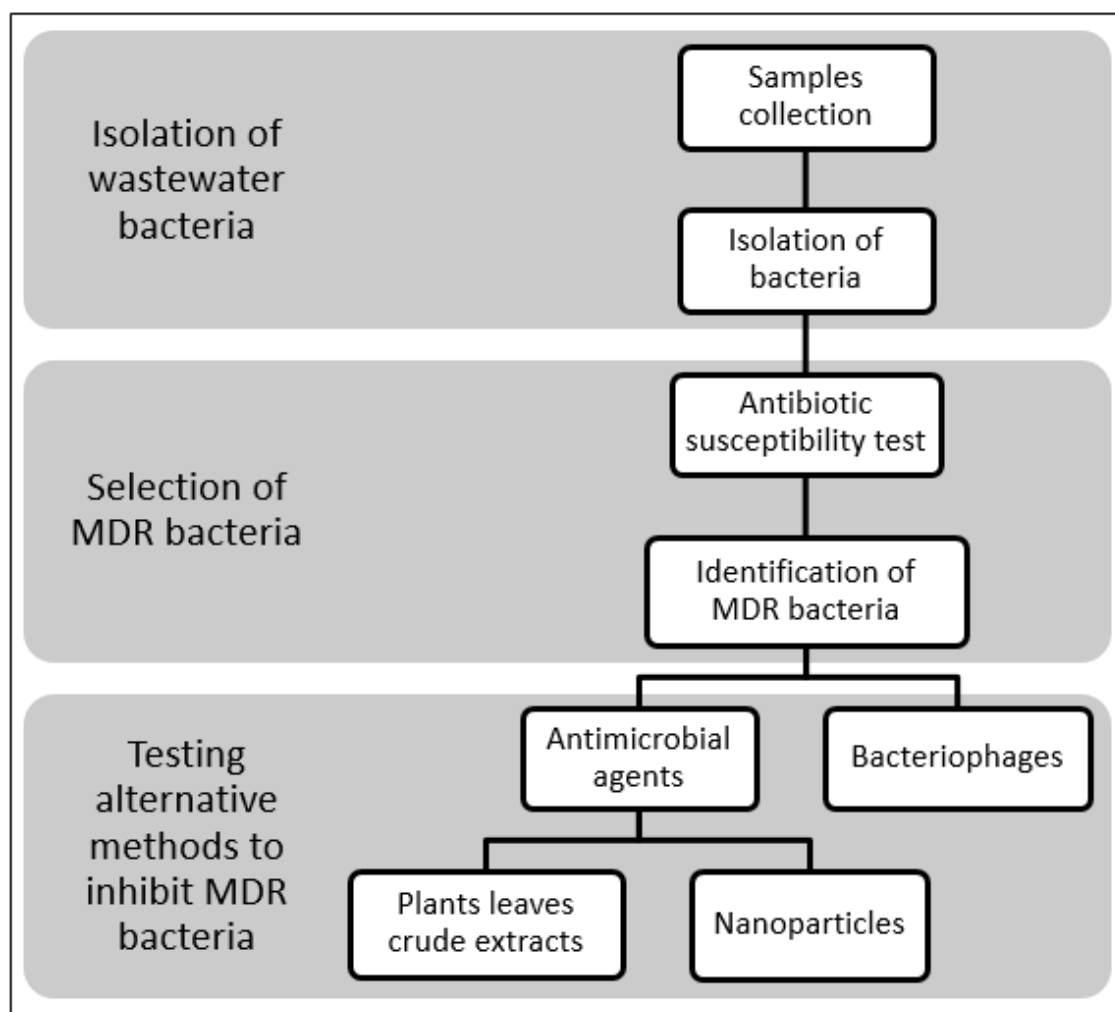


Figure 2: Flowchart of the research protocol.

3.2. Isolation of MDR bacteria from wastewater

3.2.1. Sample collection

The wastewater sampling process was done in November 2023. Triplicate wastewater samples (200 mL) at different treatment stages were obtained from Tubli WPC, Bahrain (secondary and tertiary treated water). In addition, effluent wastewater samples (200 mL) from the area were collected just before discharge to the sea and from the seawater of Tubli Bay, which was close to the discharge area (500 m distant from the effluent discharge point). The locations of the Tubli Bay and the sampling points are shown in Figure 3. The geographical coordinates of the effluent discharge point and the Tubli Bay seawater sampling point were (26°11'45.9"N 50°33'51.6"E) and (26°11'59.3"N 50°34'01.9"E) respectively. Wastewater samples were collected in sterile sampling bottles, kept on ice during transportation, and processed within four hours of the collection time.



Figure 3. Satellite map images of Bahrain showing the sampling area (a) Tubli Bay and the location of the sampling site (b) the sampling sites (A) Tubli WPCC (B) wastewater discharge point (C) seawater sampling about 500 m away from the discharge point.

3.2.2. Bacterial screening and isolation

Culture media

Eosin methylene blue (EMB) agar (Sigma-Aldrich, USA)

Xylose lysine deoxycholate (XLD) agar (Oxoid Ltd, England)

Mannitol salt agar (MSA) (Oxoid Ltd, UK)

Nutrient agar (Sigma-Aldrich, India)

Nutrient broth (Merk, USA)

Procedures

Immediately as received, the wastewater samples were 10-fold serially diluted with nutrient broth medium and cultured on different general (nutrient agar) and selective and differential (EMB agar, XLD agar, and MSA) media. The plates were incubated at 37°C for 24-48 hours. After that, bacterial growth was examined, and colonies were counted. In addition, their morphologies, shapes, colors, elevations, and margins were carefully observed. Different colonies were selected and sub-cultured to obtain pure cultures of the isolates, from which well-isolated colonies were picked, transferred to nutrient agar slants, and stored at 4°C until use.

Calculations

The colony plate count was used to determine the viable bacterial number in the samples. The following formula was used to calculate the viable bacterial count per mL of the sample.

$$\text{Viable bacterial count (CFU/mL)} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume of inoculated sample (mL)}} \quad (1)$$

3.2.3. Antibiotic susceptibility test by disc diffusion method

Culture media and reagents

Sodium chloride (NaCl) (Sigma-Aldrich, Germany).

Muller Hinton agar (MHA) (Oxoid Ltd, England).

Antibiotics set (penicillin (P), kanamycin (K), streptomycin (S), erythromycin (E), tetracycline (TE), novobiocin (NB), and chloramphenicol (C)): (Carolina, USA).

Bacterial strains

E. coli ATCC 11775 (MediMark, France).

S. aureus ATCC 12600 (MediMark, France).

Procedure

The Kirby-Bauer disc diffusion method evaluated bacterial isolates' susceptibility against antibiotics (Bauer et al., 1966). Saline suspensions of the bacterial isolates were prepared from their overnight cultures. Cell concentrations were adjusted to 10^8 colony forming units/mL (CFU/mL) (0.5 McFarland's standard) by measuring the optical density at 625 nm (OD_{625}) ranging from 0.08 to 0.1. Bacterial suspensions were spread on MHA plates, and seven different antibiotic discs were placed on the plates, then incubated at 37°C for 18 to 24 hours. Detailed information about the antibiotics used in this test is listed in Table 1. Two reference bacteria (*E. coli* ATCC 11775 and *S. aureus* ATCC 12600) were used for quality control.

Table 1. The antibiotics used for the susceptibility test of the bacterial isolates (Hutchings et al., 2019; Helgesen et al., 2021; Uddin et al., 2021).

Antibiotic	Dose/disc	Antibiotic class	Target	Mode of action
Penicillin	10 IU	β -lactams	PBPs	Disruption of cell wall synthesis
Kanamycin	30 μ g	Aminoglycosides	30S	Inhibition of protein synthesis
Streptomycin	10 μ g		ribosomal	
Tetracycline	30 μ g	Tetracyclines	subunit	
Erythromycin	15 μ g	Macrolides	50S subunits	
Chloramphenicol	30 μ g	Chloramphenicol		
Novobiocin	30 μ g	Aminocoumarins	DNA gyrase	Inhibition of DNA synthesis

The inhibition zones around the antibiotic discs (mm) were measured and recorded, and the bacterial susceptibility (S), intermediate susceptibility (I), or resistance (R) was determined according to the standards signified by the CLSI (Sarker et al., 2014; Wayne, 2020). The multidrug-resistant bacteria (resistant to three or more antibiotics) and those that showed unusual resistance pattern-stance against the tested antibiotics were selected for further investigations.

3.2.4. Microdilution test for antibiotic susceptibility and bacterial identification

Instrumentation

BD Phoenix M50

Procedure

The MDR bacterial isolates that showed antibiotic resistance patterns against three and more of the tested antibiotics, and to those commonly effective on most of the isolates (Table 1) were identified and simultaneously further tested against another range of antibiotics using the automated BD Phoenix system (Table 2).

Table 2. The antibiotics used for the Susceptibility test by BD Phoenix system (Walker, 1999; Scheinfeld, 2004; Hutchings et al., 2019; Mancuso et al., 2021; Uddin et al., 2021).

Antibiotic	Antibiotic class	Target	Mode of action
piperacillin-tazobactam	β-lactams- penicillins	PBPs	Disruption of cell wall synthesis
Oxacillin			
Amoxicillin			
amoxicillin-clavulanate			
Ampicillin			
Cefazolin			
Cefoxitin			
Cefuroxime			
Cefotaxime	Cephalosporins	PBPs	Disruption of cell wall synthesis
Ceftazidime			
Ceftriaxone			
Cefepime			
ceftolozane-tazobactam			
Ceftaroline	Carbapenems	PBPs	Disruption of cell wall synthesis
Ertapenem			
Meropenem			
Imipenem	Glycopeptide	Peptidoglycan polymerization	Disruption of cell wall synthesis
Teicoplanin			
Vancomycin	Aminoglycoside	30S subunit	Disruption of cell wall synthesis
Gentamycin			
Amikacin	Tetracyclines	50S subunit	Inhibition of proteins synthesis
Tigecycline			
Telithromycin	Ketolides	50S subunit	Inhibition of proteins synthesis
Clindamycin	Lincosamides		
Linezolid	Oxazolidinones		
Pristinamycin	streptogramins		
Mupirocin	Monoxycarbolic acid	Isoleucyl-transfer RNA	Inhibition of DNA synthesis
Ciprofloxacin	Fluoroquinolones	DNA topoisomerase and DNA gyrase	
Levofloxacin			
Moxifloxacin			
Rifampin	Rifamycins	DNA-dependent RNA polymerase	RNA synthesis inhibition
Nitrofurantoin	Azolidines	Intracellular flavoproteins	Metabolic pathways inhibition
Trimethoprim-sulphamethoxazole	Sulfonamides	Dihydropteroate synthase	
Daptomycin	Cyclic lipopeptides	Cell membrane	Cell membrane disruption
Colistin	Polymyxins	Phospholipids and lipopolysaccharides	

In the automated microdilution method, fresh cultures of the bacterial isolates were suspended in the identification broth to reach the 0.5 McFarland concentration. Then, 25 μ L of the suspension was transferred to the antibiotic susceptibility test broth, and an indicator drop was added to determine the bacterial growth. Both broth suspensions were loaded in the corresponding micro-well panels that contained specific reagents for the biochemical tests and the antibiotics to be tested against. The panels were incubated in the device for up to 16 hours, and dedicated software interpreted the results.

3.3. Plant extraction and antimicrobial activity

3.3.1. Plant material collection

Fresh, healthy leaves of 6 different perennial plants were collected during October and November 2023 from a private house garden at a time when plants' annual pruning was performed. These plants include Hibiscus (*Hibiscus rosa-sinensis*), Dodonaea (*Dodonaea viscosa*), Lime (*Citrus aurantifolia*), Lemongrass (*Cymbopogon citratus*), Olive (*Olea europaea*) and Sapodilla (*Manilkara zapota*) (Figure 4). The plant's leaves were collected in sterile plastic bags and processed within 24 hours of collection. A botanical specialist from the Department of Biology, College of Science, University of Bahrain performed the Taxonomical identification of these plant species.

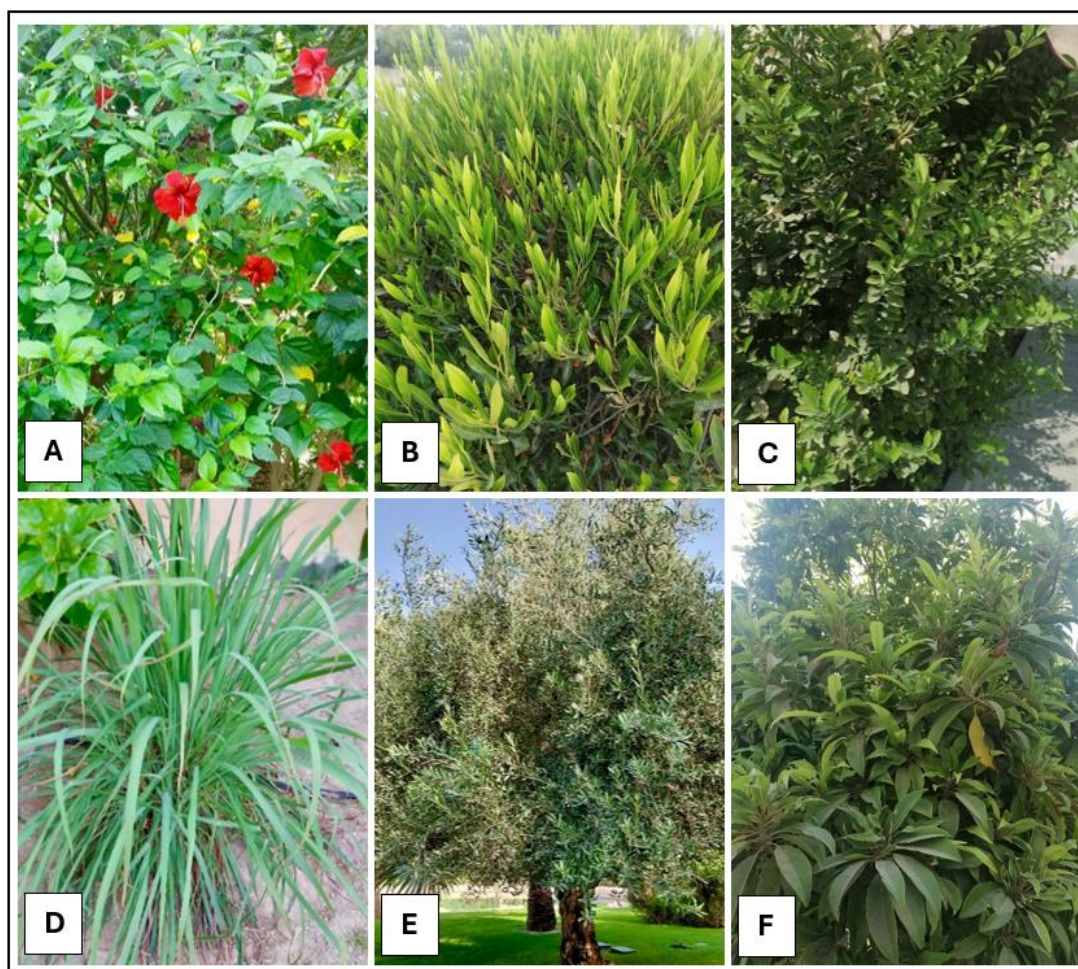


Figure 4. The plants involved in the study: (A) *H. rosa-sinensis*, (B) *D. viscosa*, (C) *C. aurantifolia*, (D) *C. cirtatus*, (E) *O. europaea*, (F) *M. zapota*.

3.3.2. Plant leaves crude extract preparation

Chemicals and Reagents

Ethanol absolute (Sigma Aldrich, USA)

Dimethyl sulfoxide (DMSO) (BDH, England)

Instrumentation

Orbital shaker (New Brunswick Scientific Innova 43)

Rotary evaporator (BUCHI Rotavapor® R-114, USA).

Procedure

The ethanol extraction of the plant leaves material was performed using Ferri's method as cited in de Paula et al. (2009) with some modifications. Firstly, the collected plant leaves were washed thoroughly by running tap water and distilled water to remove impurities and then air dried in the shade at room temperature for approximately two weeks. The leaf's weight was measured continually until no reduction was observed in the weight, indicating complete dryness. The dried leaves were ground by an electrical grinder into a coarse powder and collected into sterilized airtight containers. Subsequently, 50 grams of the prepared powder of each plant was soaked in absolute ethanol at a plant material-to-solvent ratio of 1:10 w/v. Maceration was performed for 72 hours at room temperature with shaking at 150 rpm. After that, the residues were removed by passing through cheesecloth and then filtering through Whatman filter paper No. 1. The filtrates were then further concentrated in a rotary evaporator at 47°C for 8 hours under reduced pressure, to remove the solvent and obtain thick crude extracts. The leaves crude extracts were then dissolved in DMSO to prepare 100 mg/mL stock concentrations and kept in a 4°C refrigerator for further use.

Calculations

The percentage yield of the plant's crude extracts was calculated using the following formula:

$$\% \text{ yield of plant extract} = \frac{\text{weight of the obtained extract}}{\text{weight of initial dry plant}} \times 100 \quad (2)$$

3.3.3. Antimicrobial activity of plants leaves crude extracts

Culture media and chemicals

Nutrient broth (Merk, USA)

DMSO (BDH, England)

NaCl (Sigma-Aldrich, USA)

Instrumentation

Microplate reader (Multiskan™ FC Microplate Photometer (Thermoscientific™))

Procedure

The broth microdilution method used in this study was based on a new assay developed by Green et al. (2020) to quantify the antimicrobial activity by using OD to determine the bacterial growth percentage (Green et al., 2020). Briefly, in sterile 96-well microplates, the stock plant crude extract solutions were diluted at 1:1 fold in sterile water to prepare extract concentrations of 6.25, 3.13, 1.56, and 0.78 mg/mL. Then, 100 µL of nutrient broth was added to all wells. After adding 10 µL of the saline MDR bacterial suspensions (adjusted to 0.5 McFarland standard) to all wells, the microplates were covered and incubated at 37°C for 18 hours. A plate reader at OD₆₂₅ was used to read the microplates, and the absorbance readings were recorded at time zero before and after incubation. The minimum concentration that showed no bacterial growth was determined

as the minimum inhibitory concentration (MIC). The positive controls involved bacteria grown in DMSO without plant extracts, and the negative control contained plant extracts without bacteria.

Calculations

To determine the bacterial percentage growth for each organism at each plant extract and each concentration, the OD readings of the microplate reader were used as shown in the following formula:

$$\text{Bacterial \% growth} = \frac{OD \text{ after incubation} - OD \text{ at time zero}}{OD \text{ of the positive control}} \times 100 \quad (3)$$

Negative values of the percentage growth were considered 0%, and values above 100% were considered normal 100% growth as the positive control without the presence of any antimicrobial agents.

3.4. Antimicrobial activity of nanoparticles

3.4.1. Nanoparticles Dissolution and Suspension

Preparation

Chemicals and reagents

Nanoparticles:

Silver nanoparticles (Ag NPs) (American Elements, USA)

Titanium dioxide nanoparticles (TiO₂ NPs) (Sigma Aldrich, USA)

Zinc oxide nanoparticles (ZnO NPs) (Sigma Aldrich, USA)

Instrumentation

Biorupture sonicator (COSMO BIO CO. Ltd, Japan)

X-ray diffractometer (Rigaku-Ultima IV)

Procedure

Based on their effectiveness and antimicrobial activity in the literature, three different nanoparticles were selected for this study: Ag, TiO₂, and ZnO. Before testing, the chemical compositions and crystalline phases of these NPs were confirmed by X-ray diffraction (XRD) analysis (Appendix 1) to ensure their stability and purification. After that, a stock concentration of 4 mg/mL was prepared for each nanoparticle by dissolution in water and sonication in a water bath-type sonicator on the high setting for 15 minutes with 30 seconds on/off intervals to obtain turbid suspensions of nanoparticles.

3.4.2. Broth microdilution test for antimicrobial activity

Culture media and chemicals

Nutrient broth (Merk, USA)

NaCl (Sigma-Aldrich, Germany)

Instrumentation

Microplate reader (Multiskan™ FC Microplate Photometer
(Thermoscientific™))

Procedure

Similar to the plant extracts microdilution test, the antimicrobial activity of the nanoparticles was determined by broth microdilution assay developed by Green et al. (Green et al., 2020). In 96-well microplates, the stock nanoparticle suspensions were diluted at 1:1 fold in sterile water to make final concentrations of 1.0, 0.5, 0.25, and 0.125 mg/mL. Then, 100 µL of nutrient broth and 10 µL bacterial suspensions (adjusted to 0.5 McFarland standard) were added to all wells. The microplates were examined for bacterial growth by reading them using the microplate reader at OD₆₂₅ before and after 18 hours of incubation at 37°C. The MIC of the

nanoparticles was determined by observing their lowest concentration, which showed no bacterial growth.

Calculations

Similar to the plant crude extracts, the bacterial percentage growth for each organism at each nanoparticle and each concentration was determined by the OD readings of the microplate reader as shown in the following formula:

$$\text{Bacterial \% growth} = \frac{OD \text{ after incubation} - OD \text{ at time zero}}{OD \text{ of the positive control}} \times 100 \quad (3)$$

As mentioned earlier, percentage growth values less than zero were considered 0%, and values above 100% were considered 100% average growth.

3.5. MDR bacteria-specific bacteriophage isolation

3.5.1. Phage enrichment and filtration

Culture media

Tryptone soy broth (TSB) (Oxoid Ltd, England).

Procedure

To enrich phages in the raw sewage samples, 5 mL of the overnight bacterial culture, 5 mL of TSB, and 45 mL of raw sewage were incubated overnight at 37°C with shaking at 150 rpm. On the next day, the enriched culture was centrifuged three times at 2500 rpm for 10 minutes, and the supernatant was collected. Finally, the supernatant was filtered through a 0.45 µm pore size membrane filter under vacuum, and the filtrate was kept in a sterile container at 4°C for further experimentation.

3.5.2. Phage titer determination

Culture media

TSB (Oxoid Ltd, England).

Agar (Sigma-Aldrich, Portugal)

Nutrient agar (Sigma-Aldrich, India)

Procedure

The titer of the phage was determined by plaque assay. A mid-log culture was prepared by adding 100 μ L of overnight bacterial culture to 3 mL TSB and measuring the absorbance at OD₆₀₀ to reach 0.1. The phage filtrate was diluted with TSB up to 10⁻⁶. Then, to a 3 mL tube of tryptone soy soft agar (TSB with 0.7% agar), 100 μ L of phage filtrate and 100 μ L of the mid-log culture was added, quickly mixed, and poured on a pre-warmed nutrient agar plate, and then spread with circular and swirling motions. This step was done for each phage dilution. The plates were allowed to solidify for 15 minutes and then incubated at 37°C for five hours until phage plaques appeared on the bacterial lawn. For broth clearing assay, 100 μ L of the mid-log culture was added to each tube of the phage dilutions and incubated at 37°C for three hours, after which the clearing of the broth and bacterial growth turbidity was observed. After five hours of plate incubation, the phage dilution that gave scattered plaques was selected, and the plaques were counted to determine the titer.

Calculations

The phage titer was determined by counting the plaques on the plate and expressed as plaque forming units (PFU)/mL of the sample by the use of the following formula:

$$\text{Phage titer (PFU/mL)} = \frac{\text{number of plaques} \times \text{dilution factor}}{\text{volume of sample (mL)}} \quad (4)$$

3.5.3. Phage purification

Culture media and chemicals

TSB (Oxoid Ltd, England).

Agar (Sigma-Aldrich, Portugal)

Nutrient agar (Sigma-Aldrich, India)

Tris Base (Sigma Aldrich, USA)

Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (Fluka, Switzerland)

NaCl (Sigma-Aldrich, Germany)

Gelatin (Fluka, Germany)

SM buffer preparation

In 200 mL deionized water, 5.8 g NaCl, 2.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mL of 1.0 M tris (pH 7.5), and 5.0 mL of 2% gelatin (w/v) were dissolved. The final volume was brought to 1.0 L with water.

Procedure

From the plate that showed scattered plaques, a well-isolated plaque was picked by a sterile Pasteur pipette and released in a vial containing SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% Gelatin (w/v)). As described earlier, the phage suspension was plated again, with mid-log bacterial culture in tryptone soy soft agar spread on a nutrient agar plate and incubated until plaques appeared. Then, a well-isolated plaque was picked again. The purification step was performed three times until even-sized plaques were observed, indicating pure phage.

3.5.4. Phage titer and volume amplification

Culture media and chemicals

TSB (Oxoid Ltd, England).

Agar (Sigma-Aldrich, Portugal)

Nutrient agar (Sigma-Aldrich, India)

Procedure

For further experimentations, a high volume and tittered phage was required; thus, it underwent a process of amplification. Briefly, the phage dilution, in which plaques showed a webbed pattern (plaques merged), was selected, and plaque assay was performed to prepare ten plates. The plates were incubated at 37°C for 24 hours to allow multiple rounds of infection and lysis. After incubation, plates were soaked with 6 mL of SM buffer for four hours at room temperature, with gentle occasional swirling. Then, the SM buffer was collected from all the plates into centrifuge tubes, which were centrifuged for 25 minutes at 5000 rpm, and the supernatant was filtered through a 0.45 µm pore size filter membrane and kept at 4°C until use. The titration step was performed for the amplified phage to determine the new titer by plaque assay.

3.5.5. Phage specificity and host range determination

A drop spot assay determined the phage's host range among the MDR bacterial isolates. Overnight cultures of the 10 MDR isolates were prepared in nutrient broth. To the 3 mL tryptone soy soft agar tube, 100 µL of the overnight culture bacteria was added, mixed, quickly poured on the pre-warmed nutrient agar plates, and allowed to solidify for 15 minutes. Next, drops of 10 µL phage suspension were placed on the surface of the solidified soft agar. Similar drops of SM buffer were added to serve as a negative control. The drops were allowed to absorb, and then the plates

were incubated at 37°C for 24 hours. After incubation, clear zones were determined in the drop places to determine the phage activity.

3.6. Statistical analysis

The statistical data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 21 and R version 4.2.2. Descriptive analyses for the inhibition zones and percentage bacterial growth values were presented as frequencies, percentages, and mean $\pm$ Standard deviation (SD). The mean difference of the antimicrobial activity between the antimicrobial agents at different concentrations was assessed and compared by One-way Analysis of Variance (ANOVA) and post hoc test using the Bonferroni method for the pairwise multiple comparisons, setting *P* values to less than 0.05 for statistical significance and confidence intervals (CI) at 95% for assessing the difference's strength.

Chapter Four

Results, Discussion, and Recommendations

- 4.4. The study's results
 - 4.4.1. Wastewater bacterial isolates
 - 4.4.2. Antibiotic susceptibility tests and bacterial identification
 - 4.4.3. Antimicrobial activity of plants
 - 4.4.4. Antimicrobial activity of nanoparticles
 - 4.4.5. MDR bacteria-specific phage detection
- 4.5. Discussion of the results
- 4.6. Study's recommendations

Results, Discussion, and Recommendations

4.1. The study's results

4.1.1. Wastewater bacterial isolates

Various bacterial species were detected in the different treated wastewater samples from the different treated wastewater samples. Gram-negative bacteria make up the majority of the isolates, as they thrive on media such as EMB and XLD agar, whereas Gram-positive bacteria are less common and grow on MSA.

Plate count analysis showed the average number of bacteria present in each type of the tested treated water. As shown in Table 3, the highest bacterial count was observed on the nutrient agar medium, a general medium on which different types of bacteria can grow. The secondary treated wastewater showed a higher number of bacteria compared to the discharging effluent and seawater. Comparatively, the bacterial count on MSA, which is primarily preferred by gram-positive bacteria, was the lowest of the samples taken from Tubli Bay and the discharging effluent. The bacterial count on the XLD medium was higher than that on EMB agar. Furthermore, no *Salmonella* or *Shigella* species were determined on the XLD agar medium. There was no statistical difference in the bacterial count grown on the different growth media between the three sampling locations. On the other hand, bacteria from tertiary treated water were too little to be counted.

Table 3. The mean total bacterial count present in wastewater grown on different culture media.

Wastewater Sample	Total bacterial count (Mean $\pm$ SD) (cells/mL)				<i>P</i> value*
	EMB agar	XLD agar	MSA	Nutrient agar	
Secondary treated	$3.4 \times 10^3 \pm 56.57$	$6.1 \times 10^3 \pm 50.91$	$1.9 \times 10^3 \pm 29.69$	$8.7 \times 10^4 \pm 32.53$	0.684
Tertiary treated	-	-	-	-	
Discharging effluent	$2.0 \times 10^3 \pm 28.28$	$4.0 \times 10^3 \pm 7.07$	$1.8 \times 10^3 \pm 25.46$	$1.2 \times 10^5 \pm 16.97$	
Tubli Bay	$1.88 \times 10^3 \pm 8.49$	$5.2 \times 10^3 \pm 42.43$	$1.58 \times 10^3 \pm 9.89$	$1.7 \times 10^4 \pm 22.63$	

**P* values < 0.05 indicates statistical significance.

EMB: Eosin methylene blue, XLD: Xylose Lysine Deoxycholate, MSA: mannitol salt agar.

4.1.2. Antibiotic susceptibility tests and bacterial identification

From the various bacterial colonies cultivated on the different growth media, and after thorough observation of the growth colonies' morphologies and characteristics, 41 different bacterial isolates were meticulously chosen and processed for the antibiotic susceptibility test. The code of the isolates was assigned based on their source and the growth media they were isolated from, i.e. SE1 accounts for isolate 1 from the secondary treated water grown on EMB agar media (Table 4). The antibiotic susceptibility test for the isolates was performed by Kirby Bauer disc diffusion method as described in the methodology against seven different antibiotics. Results were obtained based on the zone of inhibition breakpoint guidelines of the CLSI (Sarker et al., 2014; Wayne, 2020) (Table 4, Figure 5, Appendix 2).

Table 4. The inhibition zone measurements of bacteria against the tested antibiotics by Kirby Bauer method.

Isolate code	Inhibition zones mean ± SD (mm)													
	P10		K30		E15		S10		NB30		TE30		C30	
SE1	6 ± 0	R	17 ± 4.2	I	6.5 ± 0.7	R	6 ± 0.0	R	12 ± 1.4	I	7 ± 1.4	R	24 ± 0.0	S
SE2	6 ± 0	R	17 ± 4.2	I	6 ± 0.0	R	15 ± 1.4	S	6 ± 0.0	R	21 ± 1.4	S	19 ± 4.2	S
SE3	6 ± 0	R	17 ± 4.2	I	7 ± 1.4	R	12.5 ± 2.1	R	17.5 ± 3.5	S	23.5 ± 2.1	S	28.5 ± 2.1	S
SX1	6 ± 0	R	18 ± 2.8	I	13 ± 1.4	R	14.5 ± 0.7	I	11 ± 1.4	R	26 ± 1.4	S	33 ± 4.2	S
SX2	6 ± 0	R	18 ± 2.8	I	6 ± 0.0	R	13.5 ± 2.1	I	6 ± 0.0	R	23 ± 1.4	S	25 ± 1.4	S
SX3	6 ± 0	R	18 ± 2.8	I	9 ± 1.4	R	10 ± 2.8	R	10.5 ± 3.5	R	26.5 ± 2.1	S	26 ± 0.0	S
SX5	6 ± 0	R	19 ± 1.4	S	7 ± 1.4	R	13.5 ± 2.1	I	10.5 ± 3.5	R	25 ± 1.4	S	23.5 ± 2.1	S
SX6	6 ± 0	R	17 ± 4.2	I	6 ± 0.0	R	14 ± 0.0	I	9.5 ± 2.1	R	22.5 ± 2.1	S	23 ± 1.4	S
SN2	6 ± 0	R	15 ± 7.1	I	9 ± 4.2	R	8 ± 0.0	R	21.5 ± 19.1	S	23.5 ± 2.1	S	23 ± 1.4	S
SM1	24 ± 1.4	I	22.5 ± 3.5	S	27 ± 4.2	S	15 ± 0.7	S	24.5 ± 0.7	S	27 ± 4.2	S	27 ± 4.2	S
SM2	22.5 ± 3.5	I	21.5 ± 2.1	S	24.5 ± 0.7	S	12.5 ± 3.5	I	26.5 ± 2.1	S	27.5 ± 3.5	S	24.5 ± 0.7	S
SM3	29 ± 24.0	S	8 ± 0	R	6.5 ± 0.7	R	6 ± 0.0	R	23.5 ± 7.8	S	9.5 ± 2.1	R	23 ± 4.2	S
DE1	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	13.5 ± 0.7	I	8 ± 0.0	R	21 ± 1.4	S	20 ± 0.0	S
DE2	6 ± 0	R	19 ± 1.4	S	7 ± 1.4	R	9 ± 1.4	R	10 ± 0.0	R	6 ± 0.0	R	8 ± 0.0	R
DE3	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	14.5 ± 0.7	I	6 ± 0.0	R	23 ± 1.4	S	22.5 ± 0.7	S
DE4	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	16 ± 1.4	S	10 ± 0.0	R	22.5 ± 3.5	S	24.5 ± 0.7	S
DN1	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	15.5 ± 0.7	S	10 ± 0.0	R	25.5 ± 3.5	S	22.5 ± 3.5	S
DM1	20 ± 2.8	I	25 ± 0	S	25 ± 0.0	S	18 ± 0.0	S	28 ± 0.0	S	32.5 ± 2.1	S	15 ± 1.4	I
DM2	21.5 ± 2.1	I	22.5 ± 3.5	S	24 ± 1.4	S	16.5 ± 2.1	S	23 ± 4.2	S	30 ± 0.0	S	14.5 ± 0.7	I
DX1	6 ± 0	R	16.5 ± 2.1	I	13 ± 1.4	R	10.5 ± 0.7	R	15 ± 0.0	I	22.5 ± 3.5	S	30 ± 0.0	S
DX4	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	14 ± 1.4	I	9 ± 1.4	R	20 ± 2.8	S	23 ± 1.4	S
DX6	7 ± 1.4	R	20 ± 2.8	S	17 ± 1.4	I	13 ± 2.8	I	13.5 ± 2.1	I	23.5 ± 2.1	S	31 ± 1.4	S
DX7	6 ± 0	R	19 ± 1.4	S	7 ± 1.4	R	15 ± 1.4	S	8.5 ± 3.5	R	27 ± 4.2	S	27.5 ± 0.7	S
DX8	6 ± 0	R	18.5 ± 2.1	S	11 ± 0.0	R	6 ± 0.0	R	12.5 ± 0.7	I	24 ± 2.8	S	28 ± 2.8	S
DX9	6 ± 0	R	19 ± 1.4	S	11 ± 1.4	R	12.5 ± 3.5	I	10 ± 0.0	R	23.5 ± 2.1	S	28 ± 0.0	S

Table 4. Continued.

Isolate code	Inhibition zones mean ± SD (mm)													
	P10		K30		E15		S10		NB30		TE30		C30	
DX10	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	6 ± 0.0	R	9 ± 1.4	R	19 ± 1.4	S	21 ± 1.4	S
DX12	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	15 ± 0.0	S	9 ± 1.4	R	20 ± 0.0	S	25 ± 0.0	S
DX13	6 ± 0	R	18 ± 0	I	6 ± 0.0	R	14 ± 1.4	I	12 ± 0.0	I	20 ± 0.0	S	22 ± 2.8	S
DX14	6 ± 0	R	20 ± 0	S	9.5 ± 0.7	R	9 ± 1.4	R	13 ± 1.4	I	26 ± 2.8	S	30 ± 0.0	S
EE1	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	13 ± 2.8	I	10.5 ± 0.7	R	22 ± 4.2	S	23 ± 1.4	S
EE2	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	14.5 ± 0.7	I	7 ± 1.4	R	20 ± 2.8	S	25 ± 1.4	S
EE3	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	12 ± 0.0	I	10 ± 0.0	R	21 ± 1.4	S	24.5 ± 0.7	S
EN1	7 ± 1.4	R	16.5 ± 2.1	I	19 ± 1.4	I	13 ± 1.4	I	13.5 ± 2.1	I	22.5 ± 3.5	S	22.5 ± 3.5	S
EN2	6 ± 0	R	8 ± 0	R	15 ± 7.1	I	8 ± 0.0	R	35.5 ± 0.7	S	24.5 ± 0.7	S	27.5 ± 3.5	S
EM4	6 ± 0	R	20 ± 0	S	16.5 ± 2.1	I	17.5 ± 0.7	S	20 ± 0.0	S	20 ± 0.0	S	21 ± 1.4	S
EX1	6 ± 0	R	24 ± 0	S	6 ± 0.0	R	6 ± 0.0	R	12.5 ± 3.5	I	29 ± 1.4	S	13 ± 1.4	I
EX2	6 ± 0	R	21 ± 1.4	S	15.5 ± 0.7	I	14 ± 0.0	I	14.5 ± 2.1	I	24 ± 1.4	S	27.5 ± 3.5	S
EX3	6 ± 0	R	19 ± 1.4	S	10 ± 2.8	R	12.5 ± 0.7	I	6 ± 0.0	R	23.5 ± 4.9	S	29 ± 1.4	S
EX4	6 ± 0	R	20 ± 0	S	6 ± 0.0	R	11.5 ± 0.7	I	6 ± 0.0	R	22 ± 2.8	S	24.5 ± 0.7	S
EX5	6 ± 0	R	19 ± 1.4	S	6 ± 0.0	R	16 ± 2.8	S	15.5 ± 2.1	I	21 ± 4.2	S	25 ± 1.4	S
EX6	6 ± 0	R	18 ± 0	S	6 ± 0.0	R	13.5 ± 0.7	I	14.5 ± 0.7	I	20 ± 0.0	S	25.5 ± 0.7	S
<i>E. coli</i>	6 ± 0.0	R	18 ± 2.8	S	6 ± 0.0	R	12.5 ± 3.5	I	7 ± 1.4	R	20.5 ± 0.7	S	23 ± 4.2	S
<i>S. aureus</i>	17 ±1 5.6	R	15 ± 4.2	I	23.5 ± 2.1	S	11 ± 4.2	R	26.5 ± 2.1	S	27.5 ± 3.5	S	24.5 ± 0.7	S

R: resistant, I: intermediate sensitive, S: sensitive, P: penicillin, K: kanamycin, E: erythromycin, S: streptomycin, NB: novobiocin, TE: tetracycline, C: chloramphenicol.

Isolate code	Location	Growth media
S	Secondary treated	E EMB agar
D	Discharging effluent	X XLD agar
E	Environment (seawater)	M MSA
		N Nutrient agar

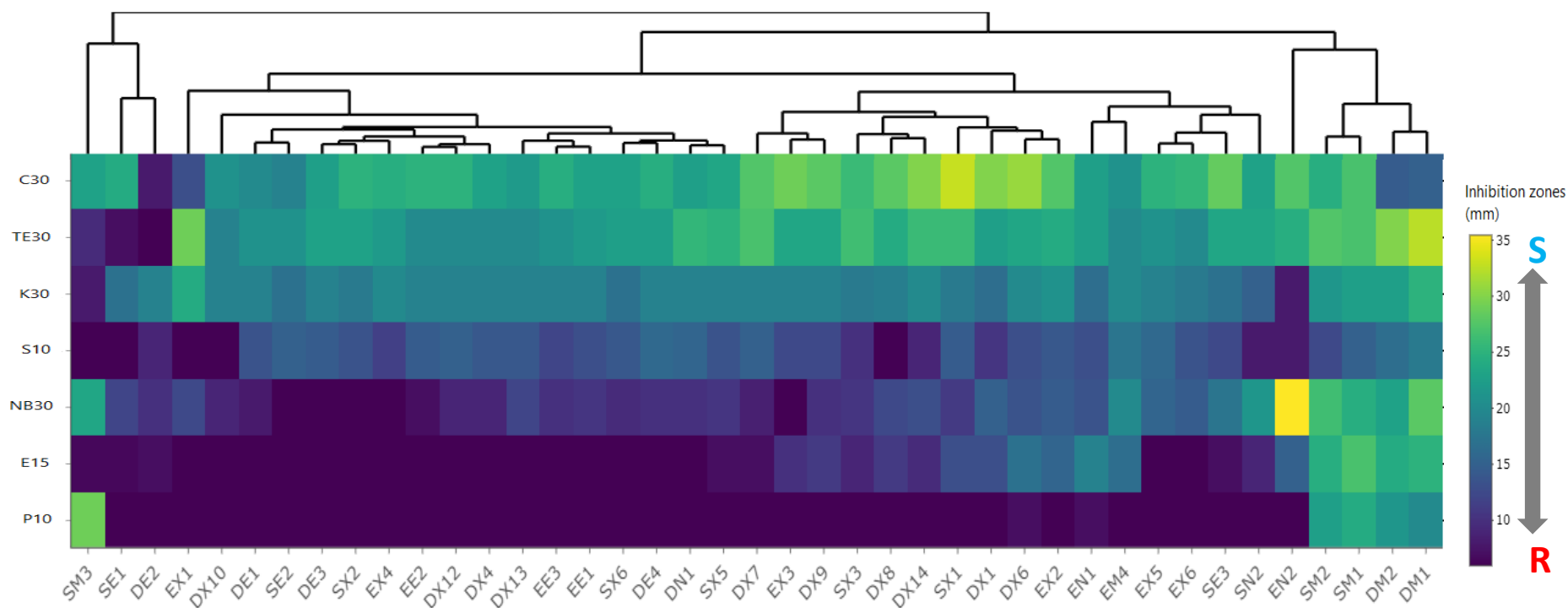


Figure 5. Heatmap showing the antibiotic susceptibility test results of all the tested isolates against the selected antibiotics. The darker color indicated resistance, with smaller inhibition zone diameter, while the lighter color to yellow indicates sensitivity, with larger diameter of inhibition zones. P10, penicillin; E15, erythromycin; NB30, novobiocin; S10, streptomycin; K30, kanamycin; TE30, tetracycline; C30, chloramphenicol.

According to the zones of inhibition data (Table 4, Figure 5), the majority of the examined bacterial isolates (73%) were resistant to three or more antibiotics, primarily penicillin, erythromycin, and novobiocin. Notably, one of the isolates found in the effluent discharged to the sea showed resistance to six of the seven tested antibiotics (Table 4 and Figure 6). On the other hand, four isolates, mainly gram-positive bacteria isolated from the MSA plates, showed no resistance to any antibiotics.

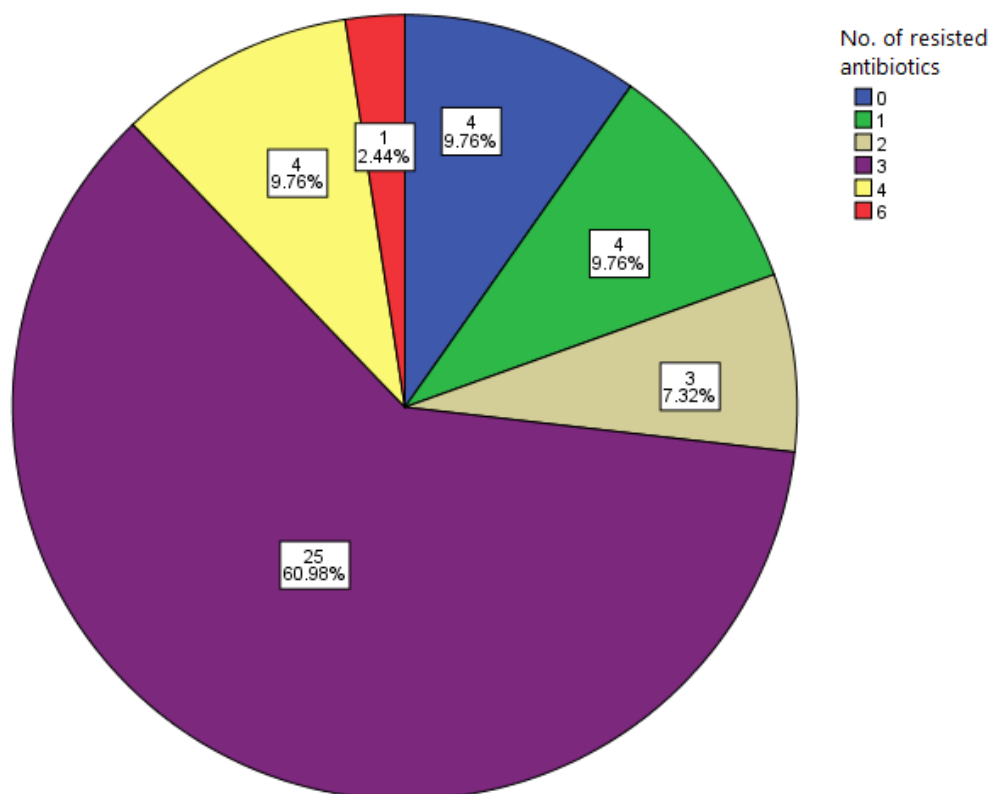


Figure 6. The number of bacterial isolates (and its percentages) that resisted a different number of the tested antibiotics. The colors indicate the number of resisted antibiotics by the tested isolates, from zero to six. The numbers and percentages on the chart are for the number of isolates resisted that number of antibiotics.

Table 5 shows the tested antibiotics and the number of susceptible (S), intermediately susceptible (I), and resistant bacteria (R). Most isolates showed resistance against penicillin, followed by erythromycin and novobiocin (87.8%, 78%, and 51.2%, respectively). The most sensitivity,

however, was found against tetracycline (92.7%), chloramphenicol (90.2%), and kanamycin (68.3%). There were no intermediately sensitive bacteria to tetracycline. Regarding streptomycin, most isolates (46.3%) showed intermediate sensitivity to it.

Table 5. Number and percentage (in parentheses) of bacterial isolates that are sensitive, intermediately sensitive, and resistant to the tested antibiotics.

Antibiotics	Frequency (%) of bacterial isolates		
	S	I	R
Penicillin	1 (2.4)	4 (9.8)	36 (87.8)
Kanamycin	28 (68.3)	11 (26.8)	2 (4.9)
Erythromycin	4 (9.8)	5 (12.2)	32 (78)
Streptomycin	10 (24.4)	19 (46.3)	12 (29.3)
Novobiocin	9 (22)	11 (26.8)	21 (51.2)
Tetracycline	38 (92.7)	0 (0)	3 (7.3)
Chloramphenicol	37 (90.2)	3 (7.3)	1 (2.4)

S: sensitive, I: intermediate sensitive, R: resistant.

Interestingly, nine bacterial isolates were found to exhibit a specific resistance profile, indicated in Table 6, as a result of the antibiotic susceptibility pattern among the tested isolates against the tested antibiotics. Those isolates that were particularly resistant to kanamycin, tetracycline, and chloramphenicol, in addition to streptomycin, or showed smaller zones of inhibition against the other antibiotics (Table 6) were identified and selected for further steps of identification and antibiotic susceptibility tests. Some showed similar resistance patterns but different colony morphologies, indicating a different bacterial species or a similar species isolated from different sampling sites (Table 4). They were also selected for further analysis. The further analysis involved the automated microdilution test for susceptibility against more of the clinically used antibiotics and phenotypic identification of the bacterial species. Detailed results of the microdilution tests are shown in Appendix 3.

Table 6. The identification of the isolates that were selected for further experimentation and their antibiotic resistance pattern

Isolate ID	Resisted antibiotics		Source	Identification
	Disc diffusion test	Microdilution test		
SE1	Penicillin, erythromycin, streptomycin, tetracycline	Cefazolin, cefuroxime, ceftazidime, ceftriaxone, cefepime, ampicillin, trimethoprim-sulphamethoxazole, ciprofloxacin, levofloxacin	Secondary treated wastewater	<i>E. coli</i> -1* ^{\$}
SM3	Kanamycin, erythromycin, streptomycin, tetracycline	Gentamycin, cefoxitin, cefotaxime, ceftaroline, trimethoprim-sulphamethoxazole, clindamycin, ciprofloxacin	Secondary treated wastewater	<i>E. faecalis</i>
SX3	Penicillin, erythromycin, streptomycin, novobiocin	Ertapenem, cefazolin, ampicillin, amoxicillin-clavulanate, trimethoprim-sulphamethoxazole	Secondary treated wastewater	<i>A. veronii</i> -1 ^{&}
DE2	Penicillin, erythromycin, streptomycin, novobiocin, tetracycline, chloramphenicol	Ampicillin, trimethoprim-sulphamethoxazole	Discharging effluent	<i>E. coli</i> -2 ^{\$}
DX4	Penicillin, erythromycin, novobiocin	Ampicillin	Discharging effluent	<i>K. pneumoniae</i>
DX10	Penicillin, erythromycin, streptomycin, novobiocin	Ampicillin	Discharging effluent	<i>E. coli</i> -3 ^{\$}
DX14	Penicillin, erythromycin, streptomycin	Ertapenem, cefazolin, ampicillin, amoxicillin-clavulanate, trimethoprim-sulphamethoxazole	Discharging effluent	<i>A. veronii</i> -2 ^{&}
EN2	Penicillin, kanamycin, streptomycin	Cefuroxime, cefepime, amoxicillin, clindamycin	Tubli Bay	<i>Streptococcus</i> spp.
EX1	Penicillin, erythromycin, streptomycin	Cefazolin, cefuroxime, ampicillin, amoxicillin-clavulanate, nitrofurantoin	Tubli Bay	<i>P. putida</i>

*This isolate showed an ESBL resistance marker.

^{\$}To differentiate between the *E. coli* strains, they were numbered *E. coli*-1, *E. coli*-2, and *E. coli*-3.

[&]To differentiate between the *A. veronii* strains, they were numbered *A. veronii*-1 and *A. veronii*-2.

As shown in Table 6, the selected isolates involved different bacterial species that exhibit considerable resistance patterns against the tested antibiotics. These isolates included *E. coli*, *K. pneumoniae*, *E. faecalis*, *A. veronii*, *P. putida*, and *Streptococcus* spp. The automated microdilution test detected resistance of a group of bacterial species against a diverse range of antibiotics. The antibiotics resistance pattern indicated the presence of three strains of *E. coli*, and the one from the secondary treated wastewater sample showed an ESBL resistance marker.

4.1.3. Antimicrobial activity of plants

This study tested crude extracts of six different plant leaves for their antimicrobial activity against the selected bacterial isolates. The plants' crude extracts were obtained using ethanol as solvent by cold maceration. Table 7 shows the crude extract yield for each plant.

Table 7. The percent yield of the obtained crude plant extracts.

Plant	Extract dry weight (g)	% yield
<i>H. rosa-sinensis</i>	1.2	4.4
<i>D. viscosa</i>	6.3	12.6
<i>C. aurantifolia</i>	1.75	3.5
<i>C. citratus</i>	2.35	4.7
<i>O. europaea</i>	7.45	14.9
<i>M. zapota</i>	5.65	11.3

As shown in Table 7, the highest yield of ethanol crude extracts was obtained from *O. europaea* leaves (14.9%), while the lowest yield was of *C. aurantifolia* (3.5%). *D. viscosa* and *M. zapota* also showed high extract yields (12.6% and 11.3%, respectively).

The antimicrobial activity of the crude extracts of plant leaves was performed using the broth microdilution method. The mean percentage of bacterial growth at different extract concentrations was calculated from the

OD₆₂₅ readings. The post hoc test of multiple comparisons was used to determine the plant extract which showed statistical differences from the other samples (Table 8, Figure 7). The results show that *C. citratus* was statistically different from all tested plants except *M. zapota*. Three plants including *H. rosa sinensis*, *D. viscosa*, and *C. aurantifolia* showed statistically significant differences from the other three plants.

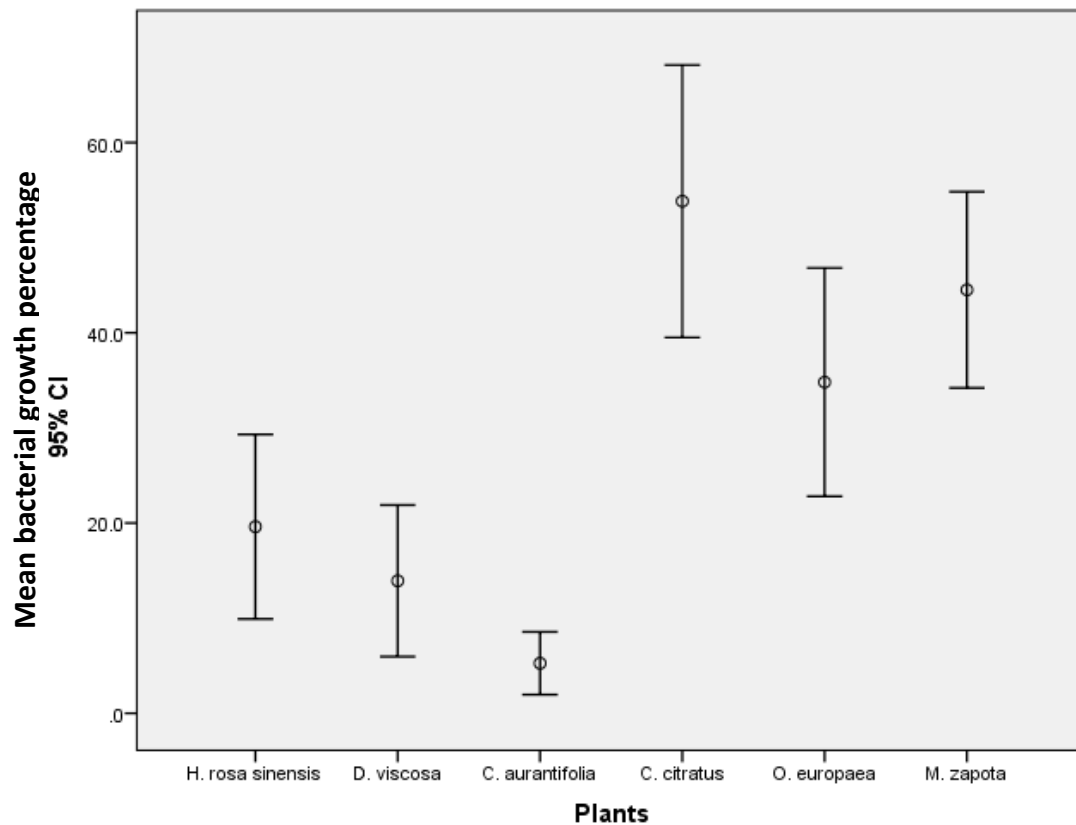


Figure 7: *Post Hoc* analysis of multiple comparisons of the plants crude extracts for the mean bacterial growth at 95% CI. No overlapping of the error bars indicates statistical significance.

Table 8. The pairwise multiple comparisons analysis of the different plant extracts regarding their antimicrobial activity.

Plants		Mean Difference	P value*	95% Confidence Interval
<i>H. rosa sinensis</i>	<i>D. viscosa</i>	5.685	1.000	-9.624, 20.995
	<i>C. aurantifolia</i>	14.356	0.088	-0.953, 29.666
	<i>C. citratus</i>	-34.221	0.000	-49.531, -18.912
	<i>O. europaea</i>	-15.200	0.053	-30.509, 0.109
	<i>M. zapota</i>	-24.904	0.000	-40.213, -9.594
<i>D. viscosa</i>	<i>H. rosa sinensis</i>	-5.685	1.000	-20.995, 9.624
	<i>C. aurantifolia</i>	8.671	1.000	-6.638, 23.980
	<i>C. citratus</i>	-39.906	0.000	-55.216, -24.597
	<i>O. europaea</i>	-20.885	0.001	-36.194, -5.576
	<i>M. zapota</i>	-30.589	0.000	-45.898, -15.280
<i>C. aurantifolia</i>	<i>H. rosa sinensis</i>	-14.356	0.088	-29.666, 0.953
	<i>D. viscosa</i>	-8.671	1.000	-23.980, 6.638
	<i>C. citratus</i>	-48.577	0.000	-63.887, -33.268
	<i>O. europaea</i>	-29.556	0.000	-44.865, -14.247
	<i>M. zapota</i>	-39.260	0.000	-54.569, -23.951
<i>C. citratus</i>	<i>H. rosa sinensis</i>	34.221	0.000	18.912, 49.531
	<i>D. viscosa</i>	39.906	0.000	24.597, 55.216
	<i>C. aurantifolia</i>	48.577	0.000	33.268, 63.887
	<i>O. europaea</i>	19.021	0.004	3.712, 34.331
	<i>M. zapota</i>	9.317	1.000	-5.992, 24.627
<i>O. europaea</i>	<i>H. rosa sinensis</i>	15.200	0.053	-0.109, 30.509
	<i>D. viscosa</i>	20.885	0.001	5.576, 36.194
	<i>C. aurantifolia</i>	29.556	0.000	14.247, 44.865
	<i>C. citratus</i>	-19.021	0.004	-34.331, -3.712
	<i>M. zapota</i>	-9.704	0.919	-25.013, 5.606
<i>M. zapota</i>	<i>H. rosa sinensis</i>	24.904	0.000	9.594, 40.213
	<i>D. viscosa</i>	30.589	0.000	15.280, 45.898
	<i>C. aurantifolia</i>	39.260	0.000	23.951, 54.569
	<i>C. citratus</i>	-9.317	1.000	-24.627, 5.992
	<i>O. europaea</i>	9.704	0.919	-5.606, 25.013

** P values < 0.05 were statistically significant.

Table 9 shows the mean percentage growth of each bacterial isolate at different concentrations of the tested plant extracts. The growth % was displayed as following: - for no growth, + for 1-24%, ++ for 25-49%, +++ for 50-74%, ++++ for 75-99%, and +++++ for 100% growth (Table 9).

Table 9. The growth percentage of the tested bacteria in the presence of different plants leaves crude extract concentrations.

Crude extract concentration (mg/mL)	% growth									P value*
	<i>E. coli</i> -1	<i>E. coli</i> -2	<i>E. coli</i> -3	<i>K. pneumoniae</i>	<i>P. putida</i>	<i>A. veronii</i> -1	<i>A. veronii</i> -2	<i>E. faecalis</i>	<i>Streptococcus</i> spp.	
<i>H. rosa-sinensis</i>										
6.25	-	-	+	+	-	-	-	-	-	0.000
3.13	-	+	+	+	-	-	-	-	-	
1.56	+	+	+++	+++	-	-	+	-	+	
0.78	+++	++++	++++	++++	-	+	++++	-	++	
<i>D. viscosa</i>										
6.25	-	-	-	-	-	-	-	-	-	0.000
3.13	-	-	-	-	-	-	-	-	-	
1.56	+	+	+	++	-	++	++	+	-	
0.78	+	++	++	++	-	+++++	+++++	++	+++	
<i>C. aurantifolia</i>										
6.25	-	-	+	+	-	-	-	-	-	0.042
3.13	-	+	+	+	-	-	-	-	-	
1.56	-	+	+	+	-	-	-	-	-	
0.78	++	++	++	++	-	-	-	-	-	
<i>C. citratus</i>										
6.25	+	-	-	++	++	+	+	-	+++	0.000
3.13	+	+	++	+++	++	+++	+++	-	+++	
1.56	++	++	+++	++++	+++	+++++	++++	+	+++	
0.78	+++	+++	++++	++++	+++++	+++++	+++++	++++	++++	
<i>O. europaea</i>										
6.25	-	-	+	+	-	-	-	-	-	0.000
3.13	-	+	++	++	+	-	+	-	-	
1.56	++	+++	+++	+++++	+	-	+	+++	++	
0.78	+++	+++	+++++	+++++	++++	++	+++	+++	+++	
<i>M. zapota</i>										
6.25	-	-	+	-	++	+	-	-	++	0.000
3.13	+	+	++	++	+++	+++	+	++	++	
1.56	++	++	++	++++	+++	+++++	++	++	++++	
0.78	+++	++	++++	+++++	++++	+++++	+++	++	++++	

*P values <0.05 are statically significant. [- = 0%, + = 1-24%, ++ = 25-49%, +++ = 50-74%, ++++ = 75-99%, +++++ = 100% growth].

All plant extracts showed a statistically significant difference ($P<0.05$) between the percentage of bacterial growth at different concentrations (Table 9). In all plants, the extracts' concentration of 6.25mg/mL showed almost no growth of all tested bacteria. As the crude extract concentrations reduced, more bacterial growth was observed.

The percentage growth of the bacteria varied among the different concentrations of the tested plant extracts. However, the difference was statistically non-significant (Table 9). The three *E. coli* strains showed variable growth patterns in the presence of the plant crude extracts. *E. coli*-1 was positively grown, although reduced compared to the average growth of the positive control, in all concentrations of *C. citratus*, while the highest growth was in the lowest concentration of *H. rosa-sinensis* and *M. zapota* (72.6% and 70.3%, respectively). *E. coli*-2, on the other hand, showed the least reduced growth in all concentrations of *D. viscosa* compared to the other plants. In contrast, the *E. coli*-3 strain grew in all plants except in concentrations of *D. viscosa* (6.25 and 3.13 mg/mL) and *C. aurantifolia* (6.25 mg/mL).

Unlike the *E. coli* strains, *E. faecalis* showed reduced growth in the presence of all plant extracts. No growth of *E. faecalis* was detected in the presence of any of the concentrations of *H. rosa-sinensis* and *C. aurantifolia*, and it had a maximum growth of 76% in 0.78 mg/mL of *C. citratus*. Similarly, in *Streptococcus* spp., no growth was detected in all concentrations of *C. aurantifolia*, and a maximum growth was found in the presence of 0.78 mg/mL of *C. citratus*.

The most reduced growth percentage was detected for *P. putida*. It did not show growth at any concentration of the three plants, *H. rosa-sinensis*, *D. viscosa*, and *C. citratus*, but the highest growth was at 0.78 mg/mL of *C. citratus* and *O. europaea*.

Both strains of *A. veronii*, on the other hand, did not exhibit any growth in any of the tested concentrations of *C. aurantifolia* but it showed 100% growth against 0.78 mg/mL of *D. viscosa* and *C. citratus*. However, the growth of *A. veronii*-1 strain isolated from the secondary treated water was more affected in the presence of *H. rosa-sinensis* and *O. europaea* compared to the second strain of this bacteria which was isolated from the discharging effluent.

The growth of *K. pneumoniae* was similar to that of *E. coli*. It showed growth in almost all concentrations of all plants, except in 6.25 mg/mL and 3.13 mg/mL of *D. viscosa*, and also in 6.25 mg/mL of *M. zapota*; it showed no growth.

The broth microdilution test determined the MIC values (mg/mL) for each plant extract. As shown in Table 9, the lowest MIC was found mainly in *C. aurantifolia* (less than 0.78 mg/mL), which was effective against more than 50% of the tested microbes. On the other hand, *C. citratus* had MIC mostly at 6.25 mg/mL or above for all bacteria except *E. faecalis*. Similarly, the MIC of *M. zapota* crude extract was 6.25 mg/mL or above for all of the bacteria except for the first strain of *E. coli*. Regarding the crude extract of *D. viscosa*, the MIC was 3.13 mg/mL for most bacteria and less than 0.78mg/mL for *P. putida* and *Streptococcus* spp. *H. rosa-sinensis*, however, had a more comprehensive range of MIC (Table 10).

Table 10. The MIC determined the antibacterial activity of the plant's crude extracts against different bacterial species.

Bacterial isolates	MIC (mg/mL)					
	<i>H. rosa sinensis</i>	<i>D. viscosa</i>	<i>C. aurantifolia</i>	<i>C. citratus</i>	<i>O. europaea</i>	<i>M. zapota</i>
<i>E. coli</i> -1	3.13	3.13	1.56	6.25	3.13	3.13
<i>E. coli</i> -2	6.25	3.13	6.25	6.25	6.25	6.25
<i>E. coli</i> -3	>6.25	3.13	> 6.25	6.25	> 6.25	> 6.25
<i>K. pneumoniae</i>	> 6.25	3.13	> 6.25	> 6.25	> 6.25	6.25
<i>A. veronii</i> -1	1.56	3.13	<0.78	>6.25	1.56	>6.25
<i>A. veronii</i> -2	3.13	3.13	< 0.78	> 6.25	6.25	6.25
<i>P. putida</i>	< 0.78	< 0.78	< 0.78	> 6.25	6.25	> 6.25
<i>E. faecalis</i>	< 0.78	3.13	< 0.78	1.56	3.13	6.25
<i>Streptococcus</i> spp.	1.56	< 0.78	< 0.78	> 6.25	6.26	> 6.25

4.1.4. Antimicrobial activity of nanoparticles

Like the crude plant leaf extracts, nanoparticle suspensions were also tested as alternative antimicrobial agents against MDR bacteria. One metal nanoparticle (Ag NPs) and two metal oxide nanoparticles (TiO₂ NPs and ZnO NPs) were mixed with the bacterial cultures, and OD₆₂₅ determined the percentage growth through the broth microdilution method.

The pairwise multiple comparison tests indicated a statistically significant difference between the TiO₂ NPs and the other two tested NPs, Ag and ZnO (Table 11, Figure 8).

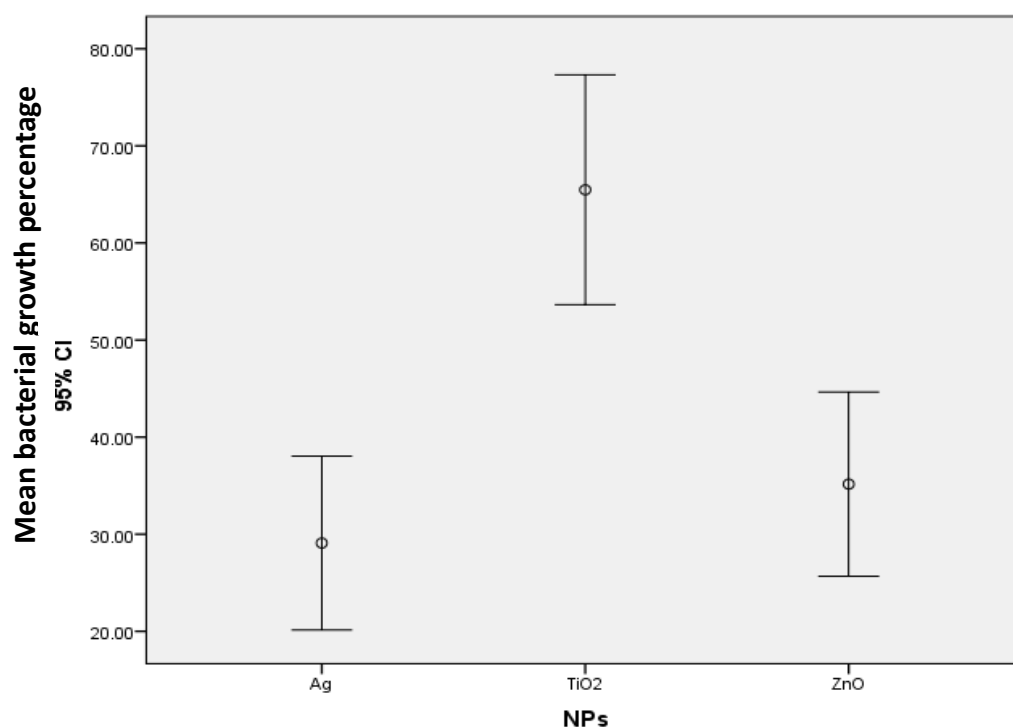


Figure 8: Post Hoc analysis of multiple comparisons of the tested nanoparticles for the mean bacterial growth at 95% CI. No overlapping of the error bars indicates statistical significance.

Table 11. Multiple comparisons between the tested NPs in regard to bacterial growth.

NPs		Mean Difference	P value*	95% Confidence Interval
Ag	TiO ₂	-36.37796	0.000	-53.6491, -19.1068
	ZnO	-6.06560	1.000	-23.3367, 11.2055
TiO ₂	Ag	36.37796	0.000	19.1068, 53.6491
	ZnO	30.31237	0.000	13.0412, 47.5835
ZnO	Ag	6.06560	1.000	-11.2055, 23.3367
	TiO ₂	-30.31237	0.000	-47.5835, -13.0412

*P values < 0.05 show statistical significance.

The following table (Table 12) shows the percentage growth of the bacterial isolates in the presence of different concentrations of NPs. As done for the plants extracts, the growth % was displayed as following: - for no growth, + for 1-24%, ++ for 25-49%, +++ for 50-74%, ++++ for 75-99%, and +++++ for 100% growth Overall, a statistically significant difference was found in the bacterial growth between the tested

concentrations of Ag NPs, while no statistical difference was found between TiO₂ NPs and ZnO NP concentrations. At a concentration of 1.0 mg/mL of Ag Nps, no bacterial growth was detected for any of the bacterial isolates. The maximum growth observed did not exceed 88.4% of *K. pneumoniae* at 0.125 mg/mL of Ag NPs. For TiO₂ NPs, the growth of almost all species of bacteria was detected in all concentrations, except *E. faecalis* and *A. veronii*-2, which showed no growth at 1.0 and 0.5 mg/mL concentrations. Regarding the bacterial growth in the presence of ZnO NPs, growth was detected in all concentrations for most bacterial species, such as *E. coli*-1, *E.coli*-2, *E. faecalis*, *P. putida*, and *Streptococcus* spp. *E. coli*-3 strain showed no growth in all concentrations except exhibited slight growth at 0.125 mg/mL (24.2%). *A. veronii*-2 showed no growth at 1 and 0.5 mg/mL concentrations, unlike the other strain which showed reduced growth at all concentrations. *K. pneumoniae* displayed growth in all concentrations except in 1.0 mg/mL.

Table 12. The growth percentage of the tested bacteria in the presence of different nanoparticles at different concentrations.

Nanoparticles concentration (mg/mL)	% growth									P value*
	<i>E. coli-1</i>	<i>E.coli-2</i>	<i>E. coli-3</i>	<i>K. pneumoniae</i>	<i>P. putida</i>	<i>A. veronii-1</i>	<i>A. veronii-2</i>	<i>E. faecalis</i>	<i>Streptococcus spp.</i>	
Ag NPs										
1.0	-	-	-	-	-	-	-	-	-	0.000
0.5	+	+	++	++	+	-	-	++	-	
0.25	++	++	+++	+++	++	+	++	+++	++	
0.125	+++	+++	++++	++++	+++	++	+++	+++	+++	
TiO ₂ NPs										
1.0	++	++	++	++	++++	-	-	-	++++	0.668
0.5	++++	+++	++++	++++	+++++	+	-	-	+++++	
0.25	++++	++++	++++	++++	+++++	+	++	+	+++++	
0.125	++++	++++	++++	+++++	+++++	++	+++++	+++	+++++	
ZnO NPs										
1.0	+	+	-	-	+	+	-	+	+	0.100
0.5	+	++	-	+	++	++	-	++	++	
0.25	+	++	-	++	++	++	+	++	+++	
0.125	+++	++	+	+++	++++	+++	++	+++	+++	

*P values <0.05 are statically significant. [- = 0%, + = 1-24%, ++ = 25-49%, +++ = 50-74%, ++++ = 75-99%, +++++ = 100% growth].

The MIC determined for the tested NPs is illustrated in Table 13. The MIC of Ag NPs was 1.0 mg/mL for all bacteria except for *A. veronii* and *Streptococcus* spp., which was 0.5 mg /mL. For TiO₂ NPs, the MIC was in a concentration higher than 1.0 mg/mL for all bacterial species except for *E. coli*-2, which was 1.0 mg/mL, and lower for *E. faecalis* and *A. veronii*-2 (0.5 mg/mL). Similarly, the MIC of ZnO was also above 1.0 mg/mL for most of the bacteria. However, among all tested NPs, the lowest MIC was found for ZnO against the *E. coli*-3 strain (0.25 mg/mL).

Table 13. The MIC of the NPs against the bacterial species.

Bacterial species	MIC (mg/mL)		
	Ag	TiO ₂	ZnO
<i>E. coli</i> -1	1.0	> 1.0	> 1.0
<i>E. coli</i> -2	1.0	1.0	> 1.0
<i>E. coli</i> -3	1.0	> 1.0	0.25
<i>K. pneumoniae</i>	1.0	> 1.0	1.0
<i>A. veronii</i> -1	0.5	1.0	> 1.0
<i>A. veronii</i> -2	0.5	0.5	0.5
<i>P. putida</i>	1.0	> 1.0	> 1.0
<i>E. faecalis</i>	1.0	0.5	> 1.0
<i>Streptococcus</i> spp.	0.5	> 1.0	> 1.0

4.1.5. MDR bacteria-specific phage detection

In this study, bacteriophage was studied as an alternative method to combat antibiotic resistance. A bacteriophage was isolated from a raw sewage sample using *E. coli* (ATCC 11775) as a reference host. The phage titer was determined to be 4.6×10^7 plaques forming units (PFU)/mL of sample. The broth-clearing assay initially indicated the presence of phage after three hours of incubation by showing a reduction of the bacterial growth turbidity as the concentration of the phage lysate increased.

After purification and amplifying its volume, the isolated phage was tested for host range determination among the wastewater MDR bacteria.

Table 14 shows positive results of a precise zone formation in the plate of only one *E. coli* strain.

Table 14. The specificity test of phage for host range determination.

Bacterial species	Clear zone formation*
<i>E. coli</i> -1	-
<i>E. coli</i> -2	+
<i>E. coli</i> -3	-
<i>K. pneumoniae</i>	-
<i>A. veronii</i> -1	-
<i>A. veronii</i> -2	-
<i>P. putida</i>	-
<i>E. faecalis</i>	-
<i>Streptococcus</i> spp.	-

*+, clear zone formed; -, clear zone not formed

4.2. Discussion of results

Environmental contamination has made a significant contribution to the prevalence of antibiotic resistance. Antibiotic resistance can spread in the environment through different human activities and improper antibiotic use. Studies indicated the possibility of MDR bacterial and ARG transfer between humans, animals, and the environment (Manaia, 2017; Oliveira et al., 2017). With the rising need for severe action to find solutions to combat antibiotic resistance, many different antimicrobial agents, techniques, and methods have been investigated to replace or complement conventional antibiotics. In this study, crude extracts of a group of plants and nanoparticles were tested for their antibacterial activity against MDR bacteria isolated from wastewater. In addition, MDR bacteria-specific phages were screened from raw sewage as a third alternative to antibiotics. The first stage of this study involved screening and isolating MDR bacteria from wastewater. The results show that a diverse group of gram-positive and gram-negative bacteria were found in the screened wastewater (Tables

3 and 4). The secondary treated wastewater contained various groups of MDR bacteria. The *E. coli*-1 strain detected in the secondary treated water showed ESBL resistance biomarkers. This isolate showed resistance to many tested antibiotics, including β -lactams, cephalosporins, tetracyclines, fluoroquinolones, and sulfonamides, indicating its possibility of acquiring various resistance mechanisms. In a study, culture-based methods were used to analyze the occurrence of ESBL-producing *E. coli* in urban community wastewater, and a bacterial load of 7.5×10^5 CFU/mL was detected (Brechet et al., 2014). The study demonstrated the possibility of the contribution of the general community to the prevalence of *E. coli* in the environment (Brechet et al., 2014). Similarly, in another study, ESBL-encoding genes were detected in different bacterial species, including *E. coli*, which were detected in wastewater effluents (Fadare & Okoh, 2021). In India, a study indicated that ESBL-producing MDR *E. coli* is present in livestock farm wastewater, hospital wastewater, and natural water sources (Satyaprakash et al., 2024). Hospital effluents can be a source of MDR bacteria and can spread through WWTPs to the environment (Lépesová et al., 2020). *E. coli* is a fecal coliform that commonly inhabits the human and animal gastrointestinal tract, and those ESBL-producing strains are of clinical importance because of the possibility that this class of β -lactamases to hydrolyze and resist most of the β -lactam antibiotics (Fadare & Okoh, 2021); thus infections caused by these microbes are severe and challenging to be treated (Schmiege et al., 2021; Satyaprakash et al., 2024).

In addition to the ESBL-producing *E. coli*, other MDR bacteria were also identified in the secondary treated water, such as *A. veronii*, and *E. faecalis*. The isolated *A. veronii*-1 strain showed resistance to different antibiotics, including some from the cephalosporins and carbapenems. Drk et al. (2023) found similar results in a study, as several *Aeromonas* species were

isolated from hospitals and treated municipal wastewater, including *A. veronii*. In another study, *A. veronii* strains and their ARGs were detected in wastewater effluents (Skwor et al., 2020). *A. veronii* is a rod-shaped Gram-negative bacterium that is usually found in aquatic environments, including seawater and sewage (Drk et al., 2023). Besides being a fish pathogen, it is also known to cause human infections, such as blood, skin, respiratory, and gastrointestinal infections (Liu et al., 2022; Drk et al., 2023). MDR *A. veronii* can cause global concern due to its harm to human health and aquaculture (Gao et al., 2020; Wang et al., 2021; Liu et al., 2022).

The *E. faecalis* resisted different antibiotics, mainly those from the aminoglycosides class, second-, third-, and fifth-generation cephalosporins, fluoroquinolones, and sulfonamides. Despite being the only one with penicillin sensitivity, it was one of only two isolates carrying kanamycin resistance and one of only three isolates with tetracycline resistance. This indicates the diverse combination of different resistance mechanisms this bacterial isolate develops. Many studies indicated the presence of *E. faecalis* in municipal wastewater (Sanderson et al., 2020; Velazquez-Meza et al., 2024). In a study, *E. faecalis* strains were isolated from hospital wastewater and showed resistance to tetracycline (Alduhaidhawi et al., 2022). In another study, kanamycin- and tetracycline-resistant *E. faecalis* strains were detected in different water sources, indicating the presence of fecal contamination (Motina & Rao, 2021). These Gram-positive bacteria live in the human and animal intestines, cause a range of urinary tract and bloodstream infections, and are considered one of the bacterial indicators of fecal contamination (Velazquez-Meza et al., 2024).

The tertiary treated water showed no viable bacterial growth and, thus, was undetectable by the conventional method. This might indicate the efficiency of the treatment process in eliminating the majority of the bacteria in the secondary treated water. In a study by Irfan et al. (2023), no bacterial growth was detected in hospital-treated wastewater; however, *Enterobacter* spp. and *Pseudomonas* spp. were isolated from municipal-treated wastewater (Irfan et al., 2023). In another study, different bacteria and ARGs were also detected in the treated wastewater (Godinho et al., 2024). The current study did not screen dead bacterial cells, ARGs, or mobile genetic elements. In addition, the growth conditions, such as the incubation temperature and time, can affect bacterial detection. Therefore, as the target was human pathogenic MDR bacteria, the incubation temperature was set at 37°C for a maximum of 48 hours, while other environmental bacteria might prefer lower temperatures and longer time to show visible growth, thus, they were not detected.

Despite no viable bacteria being found in the tertiary treated wastewater, a diverse group of bacteria was found in the effluents discharged to the sea and in seawater at an area 500 m away from the effluent discharging point. Two *E. coli* strains, *K. pneumoniae* and *A. veronii-2 strain*, were identified in the effluent before release to the sea. The *E. coli-2* strain showed more antibiotic resistance than the other species. Interestingly, it was the only species that conferred resistance to chloramphenicol among all the tested isolates. The other *E. coli-3* strain and *K. pneumoniae* showed similar antibiotic resistance patterns, except that *E. coli-3* was additionally resistant to streptomycin.

A recent study by Delgado-Blas et al. (2021) identified chloramphenicol-resistant *E. coli* strains in wastewater (Delgado-Blas et al., 2021). Karungamye et al. (2023) found antibiotic-resistant *E. coli* and *K.*

pneumoniae strains; the latter was predominantly detected in hospital wastewater effluents (Karungamye et al., 2023). Contrary to this study, other studies found *K. pneumoniae* strains that showed resistance to cephalosporins (Rocha et al., 2022), carbapenems (Loudermilk et al., 2022), and many other classes of antibiotics (Surleac et al., 2020). In contrast, in the current study, it was susceptible to most antibiotics.

Moreover, antibiotic resistance in seawater that receives wastewater effluents, different antibiotic-resistant isolates were detected, and two were identified: *Streptococcus* spp. and *P. putida*. The Gram-positive microbe, a strain of the *Streptococcus* genus is spherical bacteria, which resisted several antibiotics, including kanamycin, lincosamide, cephalosporins, and β -lactams. The *Streptococcus* genus has diverse species; some are pathogenic, such as *S. pneumonia* and *S. pyogenes*, and many are non-pathogenic (Al-Fahham et al., 2023). In a study, *S. pyogenes* was detected and quantified in wastewater samples (Shrestha et al., 2024). In a study by Gufe et al., MDR *Streptococcus pyogenes* was detected in wastewater effluents (Gufe et al., 2021). In another study, ARGs of *Streptococcus suis* were detected by culture-independent methods in municipal and hospital wastewater (Hutinel et al., 2022).

This study observed that *P. putida* is resistant to aminoglycosides, macrolides, first- and second-generation cephalosporins, and azolidines. Although *P. putida*, like many other *Pseudomonas* species, can be used in bioremediation processes in soil and water (John & Rajan, 2022), it can also cause severe infections in human and aquatic organisms. This Gram-negative bacillus was isolated from infected seabream fish (Aboyadak et al., 2024). In addition, Singh et al. (2021) reported a case of rapid severe sepsis due to infection of *P. putida* in the skin and soft tissues in humans. Therefore, MDR strains of *P. putida* could pose risks of nosocomial

infections, particularly in immunocompromised patients. In a recent study, *P. putida* was detected in all wastewater samples, with a group of ARGs identified in their genomes (Butiuc-Keul et al., 2021). Another study identified 51% of the tested isolates as *P. putida* in wastewater effluent samples (Govender et al., 2021).

Several researchers studied other MDR bacterial strains in the marine environment close to the wastewater release points. Light et al. (2022) studied antibiotic resistance in the GCC countries by analyzing the seawater samples near the wastewater effluent discharge areas. In Bahrain, bacterial isolates from samples from Tubli Bay showed resistance to a range of different classes of antibiotics, including ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline, and trimethoprim (Light et al., 2022). In Saudi Arabia, antibiotic-resistant bacteria were screened in seawater from the Red Sea coast. They found high resistance to many antibiotics, mainly cefotaxime, chloramphenicol, and gentamycin, indicating a high prevalence of antibiotic resistance in marine water, which serves as a reservoir for these bacteria (Alsalmi & Altayb 2023). In Kuwait, MDR *E. coli* strains were detected in marine sediments, particularly from areas near outfalls of local hospitals or near wastewater effluent discharge points, suggesting an urgent need to combat the prevalence of antibiotic resistance in the marine environment (Al-Sarawi et al., 2022). In another study in Kuwait, antibiotic-resistant *E. coli* strains were detected in the marine environment by whole genome sequencing and determination of ARGs, indicating the possibility of HGT of these ARGs in the marine environment (Al-Sarawi et al., 2023).

Based on the preceding discussion, it is evident that antibiotic resistance is highly prevalent, multifaceted, and requires significant action to address

the antibiotic resistance challenge. As a result, the second stage of the current study was to take an action and search for an effective solution.

This study evaluated the antibacterial activities of three alternative methods to inhibit the growth of the MDR bacteria isolated from wastewater. The first method involved a group of six plants, including *H. rosa sinensis*, *D. viscosa*, *C. aurantifolia*, *C. citratus*, *O. europaea*, and *M. zapota*. The antibacterial activity of their crude ethanolic extracts was tested, and the mean percentage growth of the wastewater MDR-bacteria was determined. The findings showed that all examined plants had a significant antibacterial activity at various doses. *H. rosa sinensis* crude extracts completely inhibited *P. putida* and *E. faecalis* growth at 6.25 mg/mL and less than 50% at their lowest tested concentration (0.78 mg/mL). The growth of *K. pneumoniae* and *E. coli*, two species belonging to the *Enterobacteriaceae* family, was reduced at higher concentrations despite their growth at low concentrations. Similar results were found in other studies, for example, the antibacterial activity of *H. rosa-sinensis* against several Gram-positive and Gram-negative bacteria was investigated by Ruban and Gajalakshmi (2012). In another study, Abate and Belay used *H. rosa-sinensis* leaves aqueous crude extracts against *E. coli* and *K. pneumoniae*, which showed the highest antibacterial activity and the highest yield of leaf extracts compared to bark and flower crude extracts (Abate & Belay, 2022). Nevertheless, a recent study indicated that ethanolic crude extracts express higher antibacterial activity than aqueous extracts (Udo et al., 2016). A recent study assessed the antimicrobial activity of *H. rosa sinensis* leaf extracts against MDR bacteria, including *E. coli* and *K. pneumoniae*, which was significant. The leaf extracts' phytochemical screening indicated several bioactive compounds, such as alkaloids, terpenoids, and flavonoids, contributing to the plant's

antimicrobial activity. However, these bioactive compounds were determined when methanol, benzene, and acetone were used as solvents for extraction (Neha, 2023). Another study indicated the potential antibacterial effect of *H. rosa-sinensis* leaf extracts against *P. putida* (Babu et al., 2020). Moreover, in another study, Farasayu et al. (2021) examined the antibacterial effect of ethanolic extracts of *H. rosa-sinensis* plant's flower against *Streptococcus* spp. They indicated a significant difference in the different concentrations of the extracts in the growth inhibition of the microbe (Farasayu et al., 2021). Moreover, the antibacterial activity of *H. rosa-sinensis* crude extracts was determined against other pathogenic *Aeromonas* species (*A. hydrophila*), in which 50% concentration was found to be the most effective (Paul et al., 2016).

The MIC of *D. viscosa* for all the *E. coli* strains and *K. pneumoniae* was 1.56 mg/mL. This plant's extracts are considered one of the two most powerful plant-source antibacterial agents against the tested bacteria in this study, suggesting the presence of bioactive compounds with antibacterial activity against various bacteria. None of the tested microbes grew at a 3.13 mg/mL concentration of *D. viscosa* leaf extracts. In a study by Priya et al. (2021), crude ethanol extracts of *D. viscosa* were tested against a range of human pathogenic bacteria. *Streptococcus* species showed significant inhibition of growth and reduced growth of other bacteria, including *E. coli* and *K. pneumoniae* (Priya et al., 2021). The main constituents of the ethanol crude extracts of *D. viscosa* leaves included alkaloids, flavonoids, terpenoids, glycosides, steroids, phenol, and tannin, which play a role in microbial growth inhibition and biofilm formation (Priya et al., 2021). In another study, *D. viscosa* leaf extracts were used in combination with other plant extracts and showed antibacterial activity against a range of bacteria, including *E. coli* and *K. pneumoniae*, at MIC of 16 and 8 mg/mL

respectively (Kadim & AL-Azawi, 2021), which were much higher than that of the current study (3.13 mg/mL). This evergreen plant's leaves have been traditionally used to treat different diseases (Alghamdi et al., 2022). Alghamdi et al. (2022) analyzed the ethanolic leaf extracts by gas chromatography-mass spectroscopy and found a wide range of antimicrobial compounds (Alghamdi et al., 2022). However, they found that the methanol and chloroform extracts had higher antimicrobial activity (Alghamdi et al., 2022).

Importantly, the *C. aurantifolia* leaf extracts exhibited significant antibacterial activity. Four of the tested MDR-bacterial isolates, which are *P. putida*, *A. veronii*, *E. faecalis*, and *Streptococcus* sp., showed complete inhibition at all tested concentrations. The other bacteria from the *Enterobacteriaceae* family, including *E. coli* strains and *K. pneumoniae*, showed reduced growth at 0.78 mg/mL, not exceeding 38% of the normal growth. This indicated that the bioactive compounds of *C. aurantifolia* leaves, even at a small concentration, possess a strong antibacterial effect against many bacteria. Among all tested plants, the lowest MIC against ESBL-producing *E. coli* was *C. aurantifolia* (1.56 mg/mL). Several studies investigated the antimicrobial activity of different *Citrus* species extracts on a diverse group of pathogenic bacteria. Similar to the present study, *C. aurantifolia* leaf extracts were tested against *E. coli*, indicating a significant inhibition of their growth (Pohan & Djojaputro, 2021). In addition, *C. aurantifolia* peel ethanolic extracts at concentrations ranged from 6.25 to 75% (w/v) positively inhibited the growth and biofilm formation of *E. faecalis* (Soraya et al., 2020). These concentrations were less than those were tested in the current study, which also indicated a MIC below 0.78 mg/mL, showing the similarity of the antimicrobial activity of the peel extracts and leaf extracts against *E. faecalis*.

Moreover, the essential oil of *C. aurantifolia* had vigorous antibacterial activity against *Streptococcus* spp. at a MIC of 20 µg/mL (Lemes et al., 2018) and against *P. putida* at MIC of 0.12 µg/mL (Pandey et al., 2012), which also agreed with the present study that indicated MIC at lower than 0.78 mg/mL. The leaf extract of *C. aurantifolia* was also tested against another species of *Klebsiella* and showed a similar antimicrobial effect at all tested concentrations from 40% to 100% (Zuraidah et al., 2023). The main active compounds found in the leaf extracts of this plant included alkaloids, saponins, and steroids (Zuraidah et al., 2023). Such phenolic compounds can penetrate the plasma membrane and cause cell damage by facilitating ion entry to the cell, thus causing ion imbalance and intracellular disruption (Soraya et al., 2020). On the other hand, crude extracts from other *Citrus* species positively affected the growth of different pathogenic bacteria (Budiarto et al., 2021; Budiarti et al., 2021; Nendissa & Nendissa, 2021). In a study, different concentrations, from 6.25% to 50% of methanol extracts of *Citrus* sp. were applied to two *Aeromonas* spp. isolated from fish samples and showed effectiveness against both isolates, indicating a similar effect as found against *A. veronii* in the current study (Lubis et al., 2023).

The antimicrobial activity of the aromatic perennial grass *C. citratus* has been widely studied against various bacteria (Aćimović et al., 2019). In the present study, crude extracts of *C. citratus* showed a variable effect on bacterial growth. On the one hand, *K. pneumoniae*, *P. putida*, *A. veronii*, and *Streptococcus* spp. showed almost average growth at 0.78 mg/mL concentration, indicating a lower antibacterial effect than the other plants at the same concentration. On the other hand, no growth was found at 6.25 mg/mL concentration of two *E. coli* strains and *E. faecalis*. Compared to the other tested plants, the MIC of *C. citratus* was the highest for almost

all tested bacteria. The variability of the antibacterial effect might be due to the extraction method; the bioactive compounds with higher antibacterial activity might not dissolved and extracted adequately by ethanol. In a similar method to this study, Ntulume et al. (2019) used ethanol as a solvent to obtain crude extracts from *C. citratus* leaves. The crude extracts showed antibacterial activity against *Streptococcus* sp. at MIC of 15.63 mg/mL (Ntulume et al., 2019), consistent with the current study, which showed MIC of > 6.26 mg/mL. In another study, Sakirigui et al. compared the antimicrobial activity of volatile extracts and non-volatile extracts of *C. citratus* (Sakirigui et al., 2019). The volatile extracts, the plant's essential oil, contained phytochemical compounds, which were not present in the non-volatile extracts from the powdered leaves. Notably, the antimicrobial activity of the volatile extracts was higher than that of the non-volatile ones for some microbes, particularly against *E. faecalis* and *S. oralis*. At the same time, the contrary was found against other microbes; the non-volatile extracts were more effective (Sakirigui et al., 2019). This indicates the participation of different compounds to develop the antimicrobial activity of a plant (Jantrapanukorn et al., 2017). Several studies evaluated the antimicrobial activity of *C. citratus* using its essential oil by applying different extraction methods or a different extraction solvent. Several studies have indicated effective inhibition of the growth of different bacteria, such as *Streptococcus* spp. (Almeida et al., 2013; Camilla et al., 2016; Toukourou et al., 2019), *E. coli* (Chouhan et al., 2017; Shendurse et al., 2021), and *Aeromonas* spp. (Starliper et al., 2015), *E. faecalis* and *K. pneumoniae* (Subramaniam et al., 2020). The phytochemical compounds from the secondary metabolites, such as tannins, alkaloids, and flavonoids, could play a role in the antibacterial effect of *C. citratus* (Nyamath & Karthikeyan, 2018).

The crude ethanolic extracts of *O. europaea* leaves showed significant antibacterial activity. All Gram-negative bacteria showed similar growth inhibition patterns, with no growth at the highest tested concentration and reduced growth up to 70% at the lowest tested concentration. In exception, the lowest concentration (0.78 mg/mL) did not affect *E. coli*-3 strain and *K. pneumoniae*. The Gram-positive bacteria also had a similar growth pattern: no growth at 3.13 mg/mL and above and reduced growth at the lowest concentration tested in the study. The antimicrobial activity of *O. europaea* leaf extracts was addressed by several studies against different bacteria: *E. coli* strains, *E. faecalis*, *K. pneumoniae* (Elnahas et al., 2021), *Streptococcus* spp. (Karygianni et al., 2019), and *P. putida* (Testa et al., 2019). Some studies indicated the same MIC (6.25 mg/mL against *Streptococcus* spp.) as the current study (Karygianni et al., 2019), some at higher MIC (12.5 mg/mL against *E. coli* and *E. faecalis*) (Karygianni et al., 2019) and some at lower concentrations (Elnahas et al., 2021). The leaves of *O. europaea* are an excellent source of different polyphenolic bioactive compounds of the same quantity and quality as those found in olive oil. Such compounds include oleuropein, verbascoside, rutin, tyrosol, and hydroxytyrosol (Sánchez-Gutiérrez et al., 2021). The antimicrobial activity lies in such bioactive compounds against the different MDR bacteria. The *Oleaceae* family is characterized by the presence of phenolic compounds, such as secoiridoids, oleuropein, demethyloleuropin, and lisuride, which accumulate in the leaves and fruits of this plant and are attributed to its antimicrobial activity (Khan et al., 2020).

Extracts from *M. zapota* evergreen tree leaves showed potential antibacterial activity at different concentrations. It completely inhibited the growth of two *E. coli* strains, *K. pneumoniae*, *A. veronii*, and *E. faecalis*, at a concentration of 6.25 mg/mL. At lower concentrations, all the bacteria

showed growth; however, it was reduced compared to average bacterial growth. Islam et al. (2013) determined the antibacterial activity of *M. zapota* leaves against *E. coli* and *Streptococcus* spp. and screened for the phytochemical content, which showed the presence of alkaloids, flavonoids, saponins, and tannins (Islam et al., 2013). In another study, methanol extracts of the leaves showed antibacterial activity against *E. coli* and *K. pneumoniae*, at MICs of 0.5 and 1.0 mg/mL (Ngongang et al., 2020), which were lower than those found in the present study. Usually, plant metabolites such as terpenoids and tannins are mainly responsible for the antibacterial activity against the microbes (Shahraki et al., 2023). Tannins are involved in the inhibition of oxidative phosphorylation, thus damage of the metabolic reactions, and growth inhibition (Shahraki et al., 2023). In addition, polyphenols also can be involved in the depolarization of the cell membrane, DNA and RNA inhibition, and disruption of synthesis of other macromolecules, leading to the antimicrobial effect (Shahraki et al., 2023).

The second alternative strategy to antibiotic resistance investigated in this study was using nanoparticles. A range of concentrations of three different NPs, including Ag NPs, TiO₂ NPs, and ZnO NPs, were examined for their antibacterial activity against the MDR bacteria isolated from wastewater. Overall, the highest effect was detected for the Ag NPs, followed by ZnO and then the TiO₂, which was statistically different from the other two NPs, indicating the difference in its effect on bacterial growth. Except for *A. veronii* and *E. faecalis*, which did not grow at concentrations of 0.5 mg/mL and above, all of the isolated bacteria had, although reduced, growth in the presence of TiO₂ NPs at all tested concentrations. In a study using green synthesized TiO₂ NPs, significant antimicrobial activity was exhibited against a group of bacteria, including *E. coli*, *K. pneumoniae*, *E. faecalis*,

and *Streptococcus* spp. (Subhapriya & Gomathipriya, 2018). In another study, green synthesized TiO₂ NPs showed efficient antibacterial activity against *E. faecalis* and *S. mutans*, suggesting that these NPs can increase cell permeability by causing tiny pores in the cell walls of the bacterial cells (Shamly & Jayalakshmi, 2022). Although no complete inhibition was observed in the current study, *E. coli* and *K. pneumoniae* showed reduced growth at higher concentrations of TiO₂ NPs. However, *P. putida* and *Streptococcus* spp. had higher growth percentages, generally at concentrations lower than 1.0 mg/mL. In a study, collagen nanocomposites, including TiO₂, showed variable activity against *P. putida*, as some loading levels showed no activity (Staneva et al., 2020). In another study, the MIC of TiO₂ NPs against *E. coli* was found at 1.0 mg/mL, completely inhibiting growth (Khashan et al., 2021). However, this concentration had the best antibacterial activity among all the tested organisms (Khashan et al., 2021). At the same time, in the current study, lower MICs were determined for *E. faecalis* and *A. veronii*-2 at 0.5 mg/mL. The TiO₂ MIC for *E. coli* and *K. pneumoniae* were found to be above 1.0 mg/mL. This finding was consistent with another study, which found the MIC for *E. coli* and *K. pneumoniae* at 1.489 and 1.166 mg/mL, respectively (Shawkat & Chehri, 2021). Jafari & Moslemzadeh (2024) suggested that TiO₂ NPs can inactivate bacterial cell proteins by targeting the –SH groups of the proteins (Jafari & Moslemzadeh, 2024). In addition, it can cause structural damage to the cell wall and plasma membrane and cell death, which is dependent on the size of the NPs (Jafari & Moslemzadeh, 2024). The antibacterial activity of TiO₂ NPs can be affected by the physiochemical factors of the NPs, which are linked to the shape, size, and crystalline structure of the NPs (Khashan et al., 2021).

All the bacteria exhibited a decrease in growth when ZnO NPs were present at the various tested concentrations. Interestingly, the third strain of *E. coli* showed no growth even at 0.25 mg/mL concentration, the lowest MIC reported among all the tested NPs. The antimicrobial activity of ZnO NPs against different bacteria was reported in several studies. Mwafy et al. (2023) demonstrated the MIC of ZnO NPs against MDR *E. coli* at 31.3 µg/mL. In another study, Hassan et al. (2020) found the MIC against *E. coli* at 50 µg/mL, while Rasha et al. (2021) determined the MIC at 0.7 mg/mL against *K. pneumoniae* (Rasha et al., 2021), which was almost consistent with the current study. In different studies, the antimicrobial activity was assessed against different *Streptococcus* species (Liang et al., 2020; Barma et al., 2021; Fadl et al., 2021), *A. veronii* (Das et al., 2020), and against *E. faecalis* (Djearmane et al., 2022) as well. Kim et al. (2020) suggested that the negatively charged bacterial cell wall components attract the positively charged Zn ions, leading to oxidative stress that affects cell integrity (Kim et al., 2020). Ouyang et al. (2020) indicated the role of ZnO NP concentration in the biofilm formation of *P. putida*, as at higher concentrations, it showed an inhibitory effect, but at low concentrations, it enhanced biofilm formation and cell growth (Ouyang et al., 2020). The ZnO mechanisms of antibacterial activity can be due to the cytotoxic amounts of Zn ions and the production of ROS (Ouyang et al., 2020). Furthermore, smaller NPs can easily penetrate the cell and intracellularly release Zn ions that can interact with different metabolic activities, causing toxicity (Demissie et al., 2020).

Compared to the TiO₂ NPs and ZnO, Ag NPs showed higher antibacterial activity against the tested MDR bacteria. Ag NPs are well known for their efficient antimicrobial activity. In this study, all tested bacteria had no growth in the presence of 1.0 mg/mL of Ag NPs, and growth was reduced

even at the lowest tested concentration. The MIC for all microbes was 1.0 mg/mL, except for *A. veronii* and *Streptococcus* spp., which was 0.5 mg/mL. A study reported the antibacterial effect of Ag NPs against two *Aeromonas* species (El-Shamy et al., 2023). Moreover, a study revealed that green synthesized Ag NPs showed complete inhibition of *E. coli* and *K. pneumoniae* at MICs (7.8 and 3.9 µg/mL, respectively) (Loo et al., 2018), which are lower than the present study. In addition, biosynthesized Ag NPs showed effective antimicrobial activity against *Streptococcus* spp. (Enan et al., 2021; Saliminasab et al., 2022). Acharya et al. (2021) found that spherical-shaped Ag NPs had faster and more efficient activity against *K. pneumoniae* compared to other shapes of NPs, suggesting that the shape of the NPs can have different antimicrobial effects (Acharya et al., 2021). The antibacterial activity of Ag NPs depends on their morphology, the total surface area, and the amount of Ag ions they release (Cheon et al., 2019). Pareek et al. (2021) found that the antibacterial activity of AgNPs against MDR-*K. pneumoniae* was due to its triclosan-like action and oxidative stress that led to its antibacterial activity (Pareek et al., 2021). Triclosan is a biocide that causes damage to the cell membrane and allows cell contents to leak out of the cell (Carey & McNamara, 2015). Similarly, Sarkar et al. (2019) suggested that the inhibitory effect of Ag NPs against *P. putida* might be due to the attachment of Ag ions released from the NPs to some proteins in the bacterial cell wall, resulting in their denaturation and death of the cell (Sarkar et al., 2019).

The third alternative method to combat antibiotic resistance was the use of bacteriophages. In phage therapy, bacteriophages are used to treat bacterial infections. Due to its efficient antibacterial effect, phage therapy can be a promising technique to replace or complement antibiotics to fight antibiotic resistance (Brives & Pourraz, 2020). In this study, MDR-

bacteria-specific phages were screened in the raw sewage, and their specificity and host range were determined. As shown in the results, one phage was successfully isolated and purified, indicating that sewage water can be a good source of bacteriophages that attack MDR bacteria. The reference host used was *E. coli*. The phage's titer was 4.6×10^7 PFU/mL, lower than that found in a previous study by Janahi et al. (2020), which was 3.2×10^{10} PFU/mL (Janahi et al., 2020). It might be due to seasonal changes at sampling time, as this study was conducted in cold weather. In summer, it is expected to get more bacteria and their corresponding phages (Janahi et al., 2020). The phage specificity test indicated that the isolated phage had a very narrow host range, as it was specific to only one strain of *E. coli* (*E. coli*-2), which was isolated from the discharging effluent. This indicates that the receptor the phage recognizes is specific to this strain, which the other strains do not express (Kortright et al., 2019). Studies used a similar method, using *E. coli* as a reference host, and, however, found phages that infect other strains and even other species. In a study, three phages were isolated from water samples and tested against a group of clinical isolates of *E. coli* strains and some other bacteria (Vera-Mansilla et al., 2020). One of the phages had a narrower host range, infecting only two strains, while the other two phages had a broader host range, though they were host-specific, not infecting other bacterial species (Vera-Mansilla et al., 2020). In another study, an *E. coli* was used as indicator strain to isolate phages from sewage water and specifically infected up to 12 *E. coli* strains; some were MDR (Fu et al., 2021). Kim et al. (2021) found a polyvalent phage using *E. coli* O157:H7 as an indicator strain, which was able to infect some *E. coli* strains, in addition to *Salmonella* and *Shigella* strains, suggesting it is an efficient biocontrol agent against food-borne pathogenic bacteria.

The isolated phage was unspecific in infecting the other MDR bacteria involved in this study. Therefore, other phage strains would be screened against those MDR-bacteria, using them as the reference bacteria during the phage enrichment step. Several studies used this protocol to isolate phages that are specific to the tested bacterial strains (Fanaei et al., 2021), including *Streptococcus* species (Chepchumba et al., 2024), *E. faecalis* (Mahgoub et al., 2020; El-Telbany et al., 2021), *A. veronii* (Elwafai et al., 2020), and *K. pneumoniae* (Peng et al., 2020; Gorodnichev et al., 2021; Balcão et al., 2022; Liang et al., 2022), suggesting a promising therapeutic tool for antibiotic resistance.

Overall, this study concludes that antibiotic resistance is prevalent in the environment through wastewater. The MDR involved a diverse group of gram-positive and gram-negative bacteria that show a wide range of antibiotic resistance patterns, suggesting the acquirement of several resistance mechanisms. This could be a threat to public health, the economy, and to the environment. The plant's crude extracts showed significant antibacterial activity. The plant-derived compounds are active against a wide range of harmful bacteria, indicating a diverse mechanism of action to inhibit their growth. The antimicrobial activity of the plants is mainly due to their bioactive compound constituents, which show varied degrees of antibacterial activity. Similarly, nanoparticles showed excellent antibacterial activity at different concentrations. Finally, the phage isolated from the municipal sewage showed a very narrow host range, infecting only one strain of MDR-*E. coli*. In addition to using conventional antibiotics, the potent combination of plants, nanoparticles, and phages is fighting antibiotic resistance. These strategies have the potential to completely transform our strategy, forging a strong barrier that defeats

resistant microorganisms and ushering in a new era of medical advancements.

4.3. Study's recommendations

The present study has a group of strengths and limitations. The study focused on using MDR bacteria isolated from the wastewater as an essential constituent of the environment. It showed the spread of antibiotic resistance in the environment rather than just finding it among the clinical cases. The environmental MDR bacteria face different parameters that allow them to develop mechanisms to survive. Therefore, these bacteria would also respond differently to the alternative methods that inhibit their growth, such as plants and nanoparticles, adding to the literature that already shows investigations of the effect of these alternative methods on the clinical isolates. However, the study has some limitations. Due to the time and resource limitations, only ten MDR isolates were identified. Identifying all isolates would give an idea about the most prevalent bacterial species available in the environment. In addition, the disc diffusion and agar well diffusion methods were not followed to test the antimicrobial activity of plant extracts and nanoparticles in this study. The inhibition zones were not well-formed because some plant extracts or nanoparticles were trapped in the discs or wells and did not diffuse in the agar media; thus, they did not give a clear idea about the qualitative determination of these agents' antimicrobial activity. However, the microdilution method and the calculation of percentage growth provided an excellent quantitative way to detect these agents' antimicrobial activity precisely. Nonetheless, in the microdilution method, higher concentrations of plant extracts and nanoparticles than those studied were challenging to investigate due to their higher color intensity, which was challenging to

measure by the microplate reader. Therefore, the following points are recommended to be involved in the future work:

- Widening the screening process and including as many isolates as possible to be identified and tested for antibiotic resistance will give a clear overview of the available MDR strains in the environment.
- The involvement of more areas away from the wastewater discharge point to determine the extent of the spread of antibiotic resistance in the environment.
- The detection of ARGs in the effluents and seawater close to the wastewater discharge area is done by culture-independent methods because the current method only detects viable cells. Dead cells and mobile gene elements could also be present in the area.
- Fractionation of the crude extracts using a range of solvents. This would allow obtaining a wide range of plant-derived bioactive compounds.
- Identification of these bioactive compounds and analyzing the type of their interactions with the bacterial cell compartments would allow a better understanding of the mechanisms of their action.
- A more comprehensive range of nanoparticles can be tested for their antimicrobial activity. In addition, combining these agents or the antibiotics would show better antibacterial activity.
- Isolation of a broader range of phages that infect MDR-bacteria and study of their characteristics to prepare cocktails that specifically treat a group of selected bacteria.

References

- Abate, T. A., & Belay, A. N. (2022). Assessment of antibacterial and antioxidant activity of aqueous crude flower, leaf, and bark extracts of Ethiopian *Hibiscus rosa-sinensis* Linn: geographical effects and Co2Res2 /Glassy carbon electrode. *International Journal of Food Properties*, 25(1), 1875–1889.
- Abdelaziz, S. M., Aboshanab, K. M., Yahia, I. S., Yassien, M. A., & Hassouna, N. A. (2021). Correlation between the antibiotic resistance genes and susceptibility to antibiotics among the carbapenem-resistant gram-negative pathogens. *Antibiotics*, 10(3), 255.
- Aboyadak, I. M., Abdel-Tawwab, M., & Ali, N. G. (2024). Identification and florfenicol-treatment of pseudomonas putida infection in gilthead seabream (*Sparus aurata*) fed on tilapia-trash-feed. *BMC veterinary research*, 20(1), 156.
- Abubakar, A. R., & Haque, M. (2020). Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. *Journal of pharmacy & bioallied sciences*, 12(1), 1–10.
- Abuhassan, N. (2017). *Assessment, control and management of the odor in Tubli sewage treatment plant*. (Master's Thesis). Engineering Management, University of Bahrain; Sakhir, Bahrain.
- Acharya, D., Pandey, P., & Mohanta, B. (2021). A comparative study on the antibacterial activity of different shaped silver nanoparticles. *Chemical Papers*, 75(9), 4907-4915.
- Aćimović, M., Čabarkapa, I., Cvetković, M., Jeremić, J. S., Kiproviski, B., Gvozdenac, S., & Puvača, N. (2019). *Cymbopogon citratus* (DC.)

- Staph: Chemical composition, antimicrobial and antioxidant activities, use in medicinal and cosmetic purpose. *Journal of Agronomy, Technology and Engineering Management*, 2(6), 344-360.
- Akter, T., Shahriar, A., Alo, M., Rahman, T., Emran, T., B. (2020). Survival assessment of pathogenic bacteria with antibiotic resistance traits from fresh summer royal grape: In vitro microbial challenge test. *Journal of microbiology, biotechnology and food sciences*, 10(3), 344-349.
- Al Romaihi, E., Al Khawaja, S., Saeed, N., Barakat, A., Azam, N., & Haji, S. (2018). Prevalence of antimicrobial resistance in uropathogens among patients visiting primary health centers: implications for empiric therapy. *Bahrain Medical Bulletin*, 40(4), 207-211.
- Alduhaidhawi, A. H. M., AlHuchaimi, S. N., Al-Mayah, T. A., Al-Ouqaili, M. T. S., Alkafaas, S. S., Muthupandian, S., & Saki, M. (2022). Prevalence of CRISPR-Cas systems and their possible association with antibiotic resistance in *Enterococcus faecalis* and *Enterococcus faecium* collected from hospital wastewater. *Infection and drug resistance*, 15, 1143–1154.
- Aleisa, E., & Al-Zubari, W. (2017). Wastewater reuse in the countries of the Gulf Cooperation Council (GCC): the lost opportunity. *Environmental Monitoring and Assessment*, 189, 1-15.
- Al-Fahham, B. M., Mohamed, R. A., Al-Talqani, J. M. T., Fahad, A. H., & Haider, J. (2023). Evaluating antimicrobial effectiveness of gold nanoparticles against *Streptococcus oralis*. *International journal of dentistry*, 9935556.

- Alghamdi, M. D., Nazreen, S., Ali, N. M., & Amna, T. (2022). ZnO nanocomposites of *Juniperus procera* and *Dodonaea viscosa* extracts as antiproliferative and antimicrobial agents. *Nanomaterials*, 12(4), 664.
- Almeida, R. B. A., Akisue, G., Cardoso, L. M. L., Junqueira, J. C., & Jorge, A. O. C. (2013). Antimicrobial activity of the essential oil of *Cymbopogon citratus* (DC) Stapf. on *Staphylococcus* spp., *Streptococcus mutans* and *Candida* spp. *Revista brasileira de plantas medicinais*, 15, (4), 474-482.
- Al-Orphaly, M., Hadi, H. A., Eltayeb, F. K., Al-Hail, H., Samuel, B. G., Sultan, A. A., & Skariah, S. (2021). Epidemiology of multidrug-resistant *Pseudomonas aeruginosa* in the Middle East and North Africa region. *mSphere*, 6(3), e00202-21.
- Al-Rashed, N., Bindayna, K. M., Shahid, M., Saeed, N. K., Darwish, A., Joji, R. M., & Al-Mahmeed, A. (2023). Prevalence of Carbapenemases in Carbapenem-Resistant *Acinetobacter baumannii* Isolates from the Kingdom of Bahrain. *Antibiotics*, 12(7), 1198.
- Alsalmi, M. A., & Altayb, H. N. (2023). Screening of antimicrobial resistance in bacterial isolates from Red Sea coast in Jeddah, Saudi Arabia. *Journal of Pharmaceutical Research International*, 35(24), 49–55.
- Al-Sarawi, H. A., Habibi, N., Uddin, S., Jha, A. N., Al-Sarawi, M. A., & Lyons, B. P. (2023). Antibiotic resistance mediated by *Escherichia coli* in Kuwait marine environment as revealed through genomic analysis. *Antibiotics*, 12(9), 1366.

- Al-Sarawi, H. A., Najem, A. B., Lyons, B. P., Uddin, S., & Al-Sarawi, M. A. (2022). Antimicrobial resistance in *Escherichia coli* isolated from marine sediment samples from Kuwait Bay. *Sustainability*, 14(18):11325.
- Altayb, H. N., Yassin, N. F., Hosawi, S., & Kazmi, I. (2022). In-vitro and in-silico antibacterial activity of *Azadirachta indica* (Neem), methanolic extract, and identification of Beta.d-Mannofuranoside as a promising antibacterial agent. *BMC plant biology*, 22(1), 262.
- Al-Zubari, W. K., El-Sadek, A. A., Al-Aradi, M. J., & Al-Mahal, H. A. (2018). Impacts of climate change on the municipal water management system in the Kingdom of Bahrain: Vulnerability assessment and adaptation options. *Climate Risk Management*, 20, 95-110.
- Amin, M. A., Pasha, M. H., Hoque, M. N., Siddiki, A. Z., Saha, S., & Kamal, M. M. (2022). Methodology for laboratory-based antimicrobial resistance surveillance in animals. *Veterinary world*, 15(4), 1066–1079.
- Aouf, A., Bouaouina, S., Abdelgawad, M. A., Abourehab, M. A. S., & Farouk, A. (2022). In silico study for algerian essential oils as antimicrobial agents against multidrug-resistant bacteria isolated from pus samples. *Antibiotics*, 11(10), 1317.
- Arsene, M. M. J., Viktorovna, P. I., Davares, A. K. L., Esther, N., & Nikolaevich, S. A. (2021). Urinary tract infections: Virulence factors, resistance to antibiotics, and management of uropathogenic bacteria with medicinal plants—A review. *Journal of Applied Pharmaceutical Science*, 11(07),1-12.

- Arsene, M. M. J., Jorelle, A. B. J., Sarra, S., Viktorovna, P. I., Davares, A. K. L., Ingrid, N. K. C., ... Carime, B. Z. (2022). Short review on the potential alternatives to antibiotics in the era of antibiotic resistance. *Journal of Applied Pharmaceutical Science*, 12(1), 29-40.
- Aslam, S., Lampley, E., Wooten, D., Karris, M., Benson, C., Strathdee, S., & Schooley, R. T. (2020). Lessons learned from the first 10 consecutive cases of intravenous bacteriophage therapy to treat multidrug-resistant bacterial infections at a single center in the United States. *Open forum infectious diseases*, 7(9), ofaa389.
- Babu, G. N., Srinivasan, G. R., Saravanan, S., & Shylaja, G. (2020). In vitro bacterial inhibitory effect of *Parthenium hysterophorus* and *Hibiscus rosa-sinensis* leaves. *Plant Cell Biotechnology and Molecular Biology*, 21(5-6), 65–68.
- Baer, A., & Kehn-Hall, K. (2014). Viral concentration determination through plaque assays: using traditional and novel overlay systems. Journal of visualized experiments. *Journal of Visualized Experiments*, (93), e52065.
- Balcão, V. M., Moreli, F. C., Silva, E. C., Belline, B. G., Martins, L. F., Rossi, F. P. N., ... da Silva, A. M. (2022). Isolation and molecular characterization of a novel lytic bacteriophage that inactivates MDR *Klebsiella pneumoniae* Strains. *Pharmaceutics*, 14(7), 1421.
- Barbieri, R., Coppo, E., Marchese, A., Daglia, M., Sobarzo-Sánchez, E., Nabavi, S. F., & Nabavi, S. M. (2017). Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. *Microbiological research*, 196, 44–68.
- Barma, M. D., Muthupandiyam, I., Samuel, S. R., & Amaechi, B. T. (2021). Inhibition of *Streptococcus mutans*, antioxidant property and

- cytotoxicity of novel nano-zinc oxide varnish. *Archives of oral biology*, 126, 105132.
- Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology*, 45(4), 493–496.
- Blair, J., Webber, M., Baylay, A., Ogbolu, D., O., & Piddock, J., V. L. (2015). Molecular mechanisms of antibiotic resistance. *Nature review. Microbiology*, 13, 42–51.
- Borgio, J. F., Rasdan, A. S., Sonbol, B., Alhamid, G., Almandil, N. B., & AbdulAzeez, S. (2021). Emerging status of multidrug-resistant bacteria and fungi in the Arabian Peninsula. *Biology*, 10(11), 1144.
- Bréchet, C., Plantin, J., Sauget, M., Thouverez, M., Talon, D., Cholley, P., Guyeux, C., Hocquet, D. and Bertrand, X., 2014. Wastewater treatment plants release large amounts of extended-spectrum β -lactamase-producing *Escherichia coli* into the environment. *Clinical Infectious Diseases*, 58(12), 1658-1665.
- Breijyeh, Z., Jubeh, B., & Karaman, R. (2020). Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules*, 25(6), 1340.
- Brives, C., & Pourraz, J. (2020). Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. *Palgrave Communications*, 6, 100.
- Budiarti, L. Y., Wydiamala, E., & Madani, R. A. (2021). Antibacterial activity of extract combination of leaves and peels kaffir lime (*Citrus hystrix* DC.) against some test bacteria. *Bioinformatics and Biomedical Research Journal*, 4(2), 39-47.

- Budiarto, R., Poerwanto, R., Santosa, E., Efendi, D., & Agusta, A. (2021). Preliminary study on antioxidant and antibacterial activity of kaffir lime (*Citrus hystrix* DC) leaf essential oil. *Applied Research in Science and Technology*, 1(2), 58-65.
- Bush, K., & Bradford, P. A. (2016). β -Lactams and β -lactamase inhibitors: An overview. *Cold Spring Harbor perspectives in medicine*, 6(8), a025247.
- Butiuc-Keul, A., Carpa, R., Podar, D., Szekeres, E., Muntean, V., Iordache, D., & Farkas, A. (2021). Antibiotic resistance in *Pseudomonas* spp. through the urban water cycle. *Current microbiology*, 78(4), 1227–1237.
- Byrgazov, K., Vesper, O., & Moll, I. (2013). Ribosome heterogeneity: Another level of complexity in bacterial translation regulation. *Current opinion in microbiology*, 16(2), 133–139.
- Camilla, B. R., Andreia, M. S., & Marco, A. B. P. (2016). In vitro antimicrobial activity of essential oil of *Cymbopogon citratus* (lemon grass) on *Streptococcus mutans* biofilm. *African Journal of Microbiology Research*, 10(31), 1224-1228.
- Carey, D. E., & McNamara, P. J. (2015). The impact of triclosan on the spread of antibiotic resistance in the environment. *Frontiers in microbiology*, 5, 780.
- Center for Disease Control. (2024). *One health*: Author. Available on: <https://www.cdc.gov/one-health/about/index.html#:~:text=One%20Health%20is%20a%20collaborative,plants%2C%20and%20their%20shared%20environment.>

- Chahal, C., van den Akker, B., Young, F., Franco, C., Blackbeard, J., & Monis, P. (2016). Pathogen and particle associations in wastewater: Significance and implications for treatment and disinfection processes. *Advances in applied microbiology*, 97, 63–119.
- Chandrashekara, U.M. (2007). Effects of pruning on radial growth and biomass increment of trees growing in homegardens of Kerala, India. *Agroforestry systems*, 69, 231–237.
- Chau, K. K., Barker, L., Budgell, E. P., Vihta, K. D., Sims, N., Kasprzyk-Hordern, B., Harriss, E., Crook, D. W., Read, D. S., Walker, A. S., & Stoesser, N. (2022). Systematic review of wastewater surveillance of antimicrobial resistance in human populations. *Environment international*, 162, 107171.
- Cheon, J. Y., Kim, S. J., Rhee, Y. H., Kwon, O. H., & Park, W. H. (2019). Shape-dependent antimicrobial activities of silver nanoparticles. *International journal of nanomedicine*, 14, 2773-2780.
- Chepchumba, B., Asudi, G. O., Katana, J., Ngayo, M. O., & Khayeli, J. A. (2024). Isolation of phages against *Streptococcus* species in the oral cavity for potential control of dental diseases and associated systemic complications. *Archives of microbiology*, 206(4), 175.
- Chouhan, S., Sharma, K., & Guleria, S. (2017). Antimicrobial Activity of Some Essential Oils-Present Status and Future Perspectives. *Medicines*, 4(3), 58.
- Collignon, P., & Beggs, J. J. (2019). Socioeconomic enablers for contagion: factors impelling the antimicrobial resistance epidemic. *Antibiotics*, 8(3), 86.

- Dai, D., Brown, C., Bürgmann, H., Larsson, D. G. J., Nambi, I., Zhang, T., ... Vikesland, P. J. (2022). Long-read metagenomic sequencing reveals shifts in associations of antibiotic resistance genes with mobile genetic elements from sewage to activated sludge. *Microbiome*, 10(1), 20.
- Das, S., Aswani, R., Midhun, S. J., Radhakrishnan, E. K., & Mathew, J. (2020). Advantage of zinc oxide nanoparticles over silver nanoparticles for the management of *Aeromonas veronii* infection in *Xiphophorus hellerii*. *Microbial pathogenesis*, 147, 104348.
- De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., ... Walker, M. J. (2020). Antimicrobial resistance in ESKAPE pathogens. *Clinical microbiology reviews*, 33(3), e00181-19.
- de Paula, J. A. M., de Paula, J. R., Pimenta, F. C., Rezende, M. H., & Freitas Bara, M. T. (2009). Antimicrobial activity of the crude ethanol extract from *Pimenta pseudocaryophyllus*. *Pharmaceutical Biology*, 47(10), 987–993.
- Delgado-Blas, J. F., Ovejero, C. M., David, S., Montero, N., Calero-Caceres, W., Garcillan-Barcia, M. P., ... Gonzalez-Zorn, B. (2021). Population genomics and antimicrobial resistance dynamics of *Escherichia coli* in wastewater and river environments. *Communications biology*, 4(1), 457.
- Demissie, M. G., Sabir, F. K., Edossa, G. D., & Gonfa, B. A. (2020). Synthesis of zinc oxide nanoparticles using leaf extract of *Lippia adoensis* (koseret) and evaluation of its antibacterial activity. *Journal of Chemistry*, 1-9.

- Dinos, G. P., Athanassopoulos, C. M., Missiri, D. A., Giannopoulou, P. C., Vlachogiannis, I. A., Papadopoulos, G. E., ... Kalpaxis, D. L. (2016). Chloramphenicol derivatives as antibacterial and anticancer agents: Historic problems and current solutions. *Antibiotics*, 5(2), 20.
- Djearamane, S., Loh, Z. C., Lee, J. J., Wong, L. S., Rajamani, R., Luque, P. A., ... Liang, S. X. T. (2022). Remedial Aspect of Zinc Oxide Nanoparticles Against *Serratia Marcescens* and *Enterococcus Faecalis*. *Frontiers in pharmacology*, 13, 891304.
- Dodds D. R. (2017). Antibiotic resistance: A current epilogue. *Biochemical pharmacology*, 134, 139–146.
- Drk, S., Puljko, A., Dželalija, M., & Udiković-Kolić, N. (2023). Characterization of third generation cephalosporin- and carbapenem-resistant *Aeromonas* isolates from municipal and hospital wastewater. *Antibiotics*, 12(3), 513.
- Economou, V., & Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infection and drug resistance*, 8, 49–61.
- El Moujaber, G., Osman, M., Rafei, R., Dabboussi, F., & Hamze, M. (2017). Molecular mechanisms and epidemiology of resistance in *Streptococcus pneumoniae* in the Middle East region. *Journal of medical microbiology*, 66(7), 847–858.
- Elbait, G. D., Daou, M., Abuoudah, M., Elmekawy, A., Hasan, S. W., Everett, D. B., ... Yousef, A. F. (2024). Comparison of qPCR and metagenomic sequencing methods for quantifying antibiotic resistance genes in wastewater. *PloS one*, 19(4), e0298325.

- Elnahas, R. A., Elwakil, B. H., Elshewemi, S. S., & Olama, Z. A. (2021). Egyptian *Olea europaea* leaves bioactive extract: Antibacterial and wound healing activity in normal and diabetic rats. *Journal of traditional and complementary medicine*, 11(5), 427–434.
- El-Shamy, O. A., El-Adawy, M. M., & Abdelsalam, M. (2023). Chemical synthesis of a polyvinylpyrrolidone-capped silver nanoparticle and its antimicrobial activity against two multidrug-resistant *Aeromonas* species. *Aquaculture Research*, 1-9.
- El-Telbany, M., El-Didamony, G., Askora, A., Ariny, E., Abdallah, D., Connerton, I. F., & El-Shibiny, A. (2021). Bacteriophages to control multi-drug resistant *Enterococcus faecalis* infection of dental root canals. *Microorganisms*, 9(3), 517.
- Elwafai, N. A., El-Zamik, F. I., Mahgoub, S. A. M., & Atia, A. (2020). Isolation of *Aeromonas* bacteriophage AvF07 from fish and its application for biological control of multidrug resistant local *Aeromonas veronii* AFs2. *Zagazig Journal of Agricultural Research*, 47(1), 179-197.
- Enan, E. T., Ashour, A. A., Basha, S., Felemban, N. H., & Gad El-Rab, S. M. F. (2021). Antimicrobial activity of biosynthesized silver nanoparticles, amoxicillin, and glass-ionomer cement against *Streptococcus mutans* and *Staphylococcus aureus*. *Nanotechnology*, 32(21).
- Fadare, F. T., & Okoh, A. I. (2021). The abundance of genes encoding ESBL, pAmpC and non- β -lactam resistance in multidrug-resistant *Enterobacteriaceae* recovered from wastewater effluents. *Frontiers in Environmental Science*, 9, 711950.

- Fadl, F. A., Atta, N. A. A., Abdelaziz, M. A., & Mohamed, A. H. (2021). In vitro assessment of antimicrobial activity of zinc oxide nanoparticles against pathogenic *Streptococcus parauberis*. *Advances in animal veterinary sciences*, 9(6), 913-918.
- Fanaei, V., Validi, M., Zamanzad, B., & Karimi, A. (2021). Isolation and identification of specific bacteriophages against methicillin-resistant *Staphylococcus aureus*, extended-spectrum beta-lactamases-producing *Escherichia coli*, extended-spectrum beta-lactamases-producing *Klebsiella pneumoniae*, and multidrug-resistant *Acinetobacter baumannii* in vitro. *FEMS microbiology letters*, 368(19), fnab139.
- Farasayu, R. D., Rachmawati, M. W., Ana, I. D., Syaify, A., & Listyarifah, D. (2021). The effect of Hibiscus flower extract (*Hibiscus rosa-sinensis* L.) on the growth of *Streptococcus sanguinis* bacteria. In *BIO Web of Conferences*, 41, (p. 07006). EDP Sciences.
- Fernández-Villa, D., Aguilar, M. R., & Rojo, L. (2019). Folic acid antagonists: Antimicrobial and immunomodulating mechanisms and applications. *International journal of molecular sciences*, 20(20), 4996.
- Frieri, M., Kumar, K., & Boutin, A. (2017). Antibiotic resistance. *Journal of infection and public health*, 10(4), 369–378.
- Fu, P., Zhao, Q., Shi, L., Xiong, Q., Ren, Z., Xu, H., ... Sang, M. (2021). Identification and characterization of two bacteriophages with lytic activity against multidrug-resistant *Escherichia coli*. *Virus research*, 291, 198196.
- Gao, X., Tong, S., Zhang, S., Chen, Q., Jiang, Z., Jiang, Q., ... Zhang, X. (2020). *Aeromonas veronii* associated with red gill disease and its

- induced immune response in *Macrobrachium nipponense*. *Aquaculture research*, 51(12), 5163-5174.
- Garvey M. (2022). Bacteriophages and food production: biocontrol and bio-preservation options for food safety. *Antibiotics*, 11(10), 1324.
- Godinho, O., Lage, O. M., & Quinteira, S. (2024). Antibiotic-resistant bacteria across a wastewater treatment plant. *Applied Microbiology*, 4(1):364-375.
- Gorodnichev, R. B., Volozhantsev, N. V., Krasilnikova, V. M., Bodoev, I. N., Kornienko, M. A., Kuptsov, N. S., ... Shitikov, E. A. (2021). Novel *Klebsiella pneumoniae* K23-specific bacteriophages from different families: similarity of depolymerases and their therapeutic potential. *Frontiers in microbiology*, 12, 669618.
- Govender, R., Amoah, I. D., Adegoke, A. A., Singh, G., Kumari, S., Swalaha, F. M., ... Stenström, T. A. (2021). Identification, antibiotic resistance, and virulence profiling of *Aeromonas* and *Pseudomonas* species from wastewater and surface water. *Environmental monitoring and assessment*, 193(5), 294.
- Green, K. J., Dods, K., & Hammer, K. A. (2020). Development and validation of a new microplate assay that utilises optical density to quantify the antibacterial activity of honeys including Jarrah, Marri and Manuka. *PloS one*, 15(12), e0243246.
- Grehs, B. W. N., Linton, M. A. O., Clasen, B., de Oliveira Silveira, A., & Carissimi, E. (2021). Antibiotic resistance in wastewater treatment plants: Understanding the problem and future perspectives. *Archives of microbiology*, 203(3), 1009–1020.

- Grossman T. H. (2016). Tetracycline antibiotics and resistance. *Cold Spring Harbor perspectives in medicine*, 6(4), a025387.
- Gufe, C., Ndlovu, M. N., Sibanda, Z., Makuvara, Z., & Marumure, J. (2021). Prevalence and antimicrobial profile of potentially pathogenic bacteria isolated from abattoir effluents in Bulawayo, Zimbabwe. *Scientific African*, 14, e01059.
- Gupta, A., Mumtaz, S., Li, C. H., Hussain, I., & Rotello, V. M., (2019). Combatting antibiotic-resistant bacteria using nanomaterials. *Chemical Society reviews*, 48(2), 415–427.
- Hadi, H. A., Al-Hail, H., Aboidris, L. E., Al-Orphaly, M., Ahmed, M. A. S., Samuel, B. G., ... Skariah, S. (2023). Prevalence and genetic characterization of clinically relevant extended-spectrum β -lactamase-producing *Enterobacterales* in the Gulf Cooperation Council countries. *Frontiers in Antibiotics*, 2, 1177954.
- Hajipour, M. J., Fromm, K. M., Ashkarran, A. A., de Aberasturi, D. J., de Larramendi, I. R., Rojo, T., ... Mahmoudi, M. (2012). Antibacterial properties of nanoparticles. *Trends in biotechnology*, 30(10), 499–511.
- Harris, M., Fasolino, T., Ivankovic, D., Davis, N. J., & Brownlee, N. (2023). Genetic Factors That Contribute to Antibiotic Resistance through Intrinsic and Acquired Bacterial Genes in Urinary Tract Infections. *Microorganisms*, 11(6), 1407.
- Hassan, A. A., Abo-Zaid, K. F., & Oraby, N. H. (2020). Molecular and conventional detection of antimicrobial activity of zinc oxide nanoparticles and cinnamon oil against *Escherichia coli* and *Aspergillus flavus*. *Advances in animal veterinary sciences*, 8(8), 839-847.

- Helgesen, E., Sætre, F., & Skarstad, K. (2021). Topoisomerase IV tracks behind the replication fork and the SeqA complex during DNA replication in *Escherichia coli*. *Scientific reports*, 11(1), 474.
- Hendriksen, R. S., Bortolaia, V., Tate, H., Tyson, G. H., Aarestrup, F. M., & McDermott, P. F. (2019). Using Genomics to Track Global Antimicrobial Resistance. *Frontiers in public health*, 7, 242.
- Hikmawanti, N. P. E., Fatmawati, S., & Asri, A. W. (2021, April). The effect of ethanol concentrations as the extraction solvent on antioxidant activity of Katuk (*Sauropus androgynus* (L.) Merr.) leaves extracts. In *IOP conference series: Earth and environmental science*, 755(1), p. 012060. IOP Publishing.
- Holmes R., K., & Jobling, M., G. (1996). Genetics. In: S., Baron, (Ed.), *Medical Microbiology*. (4th ed.). Galveston, USA.
- Holmes, A. H., Moore, L. S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., ... Piddock, L. J. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet*, 387(10014), 176–187.
- Huang, J. J., Hu, H. Y., Lu, S. Q., Li, Y., Tang, F., Lu, Y., & Wei, B. (2012). Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China. *Environment international*, 42, 31–36.
- Huijbers, P. M. C., Flach, C. F., & Larsson, D. G. J. (2019). A conceptual framework for the environmental surveillance of antibiotics and antibiotic resistance. *Environment international*, 130, 104880.

- Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: past, present and future. *Current opinion in microbiology*, 51, 72–80.
- Hutinel, M., Huijbers, P. M. C., Fick, J., Åhrén, C., Larsson, D. G. J., & Flach, C. F. (2019). Population-level surveillance of antibiotic resistance in *Escherichia coli* through sewage analysis. *Euro surveillance: European communicable disease bulletin*, 24(37), 1800497.
- Hutinel, M., Larsson, D. G. J., & Flach, C. F. (2022). Antibiotic resistance genes of emerging concern in municipal and hospital wastewater from a major Swedish city. *The Science of the total environment*, 812, 151433.
- Hyman, P. (2019). Phages for phage therapy: Isolation, characterization, and host range breadth. *Pharmaceuticals*, 12(1), 35.
- Ikram, M., Abbasi, S., Haider, A., Naz, S., Ul-Hamid, A., Imran, M., ... Ghaffar, A. (2020). Bimetallic Ag/Cu incorporated into chemically exfoliated MoS₂ nanosheets to enhance its antibacterial potential: in silico molecular docking studies. *Nanotechnology*, 31(27), 275704.
- Irazoki, O., Hernandez, S. B., & Cava, F. (2019). Peptidoglycan muropeptides: Release, perception, and functions as signaling molecules. *Frontiers in microbiology*, 10, 500.
- Iredell, J., Brown, J., & Tagg, K. (2016). Antibiotic resistance in *Enterobacteriaceae*: mechanisms and clinical implications. *BMJ*, 352, h6420.
- Irfan, M., Almotiri, A., & AlZeyadi, Z. A. (2023). Antimicrobial resistance and β -lactamase production in clinically significant gram-negative

- bacteria isolated from hospital and municipal wastewater. *Antibiotics*, 12(4), 653.
- Islam, M. R., Parvin, M. S., Banu, M. R., Jahan, N., Das, N., & Islam, M. E. (2013). Antibacterial and phytochemical screening of ethanol extracts of *Manilkara zapota* leaves and bark. *International Journal of Pharma Science*, 3, 394-97.
- Jafari, A. J., & Moslemzadeh, M. (2024). The effect of TiO₂ nanoparticles on bacterial growth: the effect of particle size and their structure - a systematic review. *International journal of environmental health research*, 34(2), 697–707.
- Janahi, E. M., Mustafa, S., Parkar, S. F. D., Naser, H. A., & Eisa, Z. M. (2020). Detection of enteric viruses and bacterial indicators in a sewage treatment center and shallow water bay. *International journal of environmental research and public health*, 17(18), 6483.
- Jantrapanukorn, B., Pongpraritt, S., Powthong, P., & Pheungphu, T. (2017). The study of antibacterial activity in enteric pathogens of Roselle (*Hibiscus sabdariffa* Linn.) by broth micro-dilution method. *Journal of Applied Pharmaceutical Science*, 7(5), 119-122.
- John, R., & Rajan, A. P. (2022). *Pseudomonas putida* APRRJVITS11 as a potent tool in chromium (VI) removal from effluent wastewater. *Preparative biochemistry & biotechnology*, 52(2), 163–170.
- Joji, R. M., Al-Rashed, N., Saeed, N. K., & Bindayna, K. M. (2019). Detection of VIM and NDM-1 metallo-beta-lactamase genes in carbapenem-resistant *Pseudomonas aeruginosa* clinical strains in Bahrain. *Journal of laboratory physicians*, 11(2), 138–143.

- Kadim, N. A., & AL-Azawi, A. H. (2021). Evaluation of the antibacterial activity of the polyherbal (*Conocarpus lancifolius* l., *Capparis spinosa* l. And *Dodonaea viscosa*) leaves extracts cultivated in Iraq. *Biochemical & Cellular Archives*, 21(2).
- Karungamye, P., Rugaika, A., Mtei, K., & Machunda, R. (2023). Antibiotic resistance patterns of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* isolated from hospital wastewater. *Applied Microbiology*, 3(3):867-882.
- Karygianni, L., Cecere, M., Argyropoulou, A., Hellwig, E., Skaltsounis, A. L., Wittmer, A., Tchorz, J. P., & Al-Ahmad, A. (2019). Compounds from *Olea europaea* and *Pistacia lentiscus* inhibit oral microbial growth. *BMC complementary and alternative medicine*, 19(1), 51
- Khan, H., Ahmad, W., Hussain, I., Imran, M., Afridi, M. S., & Ullah, S. (2020). Phytochemical composition, antioxidant and antimicrobial activities of leaves of *Olea europaea* wild variety. *Journal of Food Measurement and Characterization*, 14, 640-648.
- Khan, M. A., Thurgood, N. E., Faheem, S. M., Rais, N., Ansari, M. Z., Kaleem, S. M., & Khan, S. T. (2020). Occurrence of extended spectrum beta-lactamase gram-negative bacteria from non-clinical sources in Dubai, United Arab Emirates. *Water*, 12(9):2562.
- Khashan, K. S., Sulaiman, G. M., Abdulameer, F. A., Albukhaty, S., Ibrahim, M. A., Al-Muhimeed, T., & AlObaid, A. A. (2021). Antibacterial activity of TiO₂ nanoparticles prepared by one-step laser ablation in liquid. *Applied Sciences*, 11(10), 4623.
- Kim, I., Viswanathan, K., Kasi, G., Sadeghi, K., Thanakkasaranee, S., & Seo, J. (2020). Preparation and characterization of positively surface

- charged zinc oxide nanoparticles against bacterial pathogens. *Microbial pathogenesis*, 149, 104290.
- Kim, S. H., Adeyemi, D. E., & Park, M. K. (2021). Characterization of a new and efficient polyvalent phage infecting *E. coli* O157:H7, *Salmonella* spp., and *Shigella sonnei*. *Microorganisms*, 9(10), 2105.
- Kortright, K. E., Chan, B. K., Koff, J. L., & Turner, P. E. (2019). Phage therapy: A renewed approach to combat antibiotic-resistant bacteria. *Cell host & microbe*, 25(2), 219–232.
- Krause, K. M., Serio, A. W., Kane, T. R., & Connolly, L. E. (2016). Aminoglycosides: An overview. *Cold Spring Harbor perspectives in medicine*, 6(6), a027029.
- Lal, A., & Cheeptham, N. (2007). Eosin-methylene blue agar plates protocol. *American Society for Microbiology*.
- Larsson, D. G. J., & Flach, C. F. Antibiotic resistance in the environment. *Nature reviews Microbiology*, 20(5), 257–269.
- Larsson, D. G. J., Flach, C. F., & Laxminarayan, R. (2023). Sewage surveillance of antibiotic resistance holds both opportunities and challenges. *Nature reviews. Microbiology*, 21(4), 213–214.
- Lehman, S. M., Mearns, G., Rankin, D., Cole, R. A., Smrekar, F., Branston, S. D., & Morales, S. (2019). Design and preclinical development of a phage product for the treatment of antibiotic-resistant *Staphylococcus aureus* infections. *Viruses*, 11(1), 88.
- Leitner, L., Ujmajuridze, A., Chanishvili, N., Goderdzishvili, M., Chkonia, I., Rigvava, S., ... Kessler, T. M. (2021). Intravesical bacteriophages for treating urinary tract infections in patients undergoing transurethral resection of the prostate: a randomised, placebo-

- controlled, double-blind clinical trial. *The Lancet. Infectious diseases*, 21(3), 427–436.
- Lemes, R. S., Alves, C. C. F., Estevam, E. B. B., Santiago, M. B., Martins, C. H. G., Santos, T. C. L. D., ... Miranda, M. L. D. (2018). Chemical composition and antibacterial activity of essential oils from *Citrus aurantifolia* leaves and fruit peel against oral pathogenic bacteria. *Anais da Academia Brasileira de Ciencias*, 90(2), 1285–1292.
- Lépesová, K., Olejníková, P., Mackul'ak, T., Cverenkárová, K., Krahulcová, M., & Bírošová, L. (2020). Hospital wastewater-important source of multidrug resistant coliform bacteria with ESBL-production. *International journal of environmental research and public health*, 17(21), 7827.
- Lerminiaux, N. A., & Cameron, A. D. S. (2019). Horizontal transfer of antibiotic resistance genes in clinical environments. *Canadian journal of microbiology*, 65(1), 34–44.
- Levin, P. A., & Angert, E. R. (2015). Small but Mighty: Cell Size and Bacteria. *Cold Spring Harbor perspectives in biology*, 7(7), a019216.
- Lewis K. (2020). The science of antibiotic discovery. *Cell*, 181(1), 29–45.
- Liang, B., Zhao, W., Han, B., Barkema, H. W., Niu, Y. D., Liu, Y., ... Gao, J. (2022). Biological and genomic characteristics of two bacteriophages isolated from sewage, using one multidrug-resistant and one non-multidrug-resistant strain of *Klebsiella pneumoniae*. *Frontiers in microbiology*, 13, 943279.
- Liang, S. X. T., Wong, L. S., Lim, Y. M., Djearamanea, S., & Lee, P. F. (2020). Effects of zinc oxide nanoparticles on *Streptococcus*

- pyogenes*. *South African Journal of Chemical Engineering*, 34(1), 63-71.
- Light, E., Baker-Austin, C., Card, R. M., Ryder, D., Alves, M. T., Al-Sarawi, H. A., ... Lyons, B. P. (2022). Establishing a marine monitoring programme to assess antibiotic resistance: A case study from the Gulf Cooperation Council (GCC) region. *Environmental advances*, 9.
- Lin, J., Nishino, K., Roberts, M. C., Tolmasky, M., Aminov, R. I., & Zhang, L. (2015). Mechanisms of antibiotic resistance. *Frontiers in microbiology*, 6, 34.
- Ling, H., Lou, X., Luo, Q., He, Z., Sun, M., & Sun, J. (2022). Recent advances in bacteriophage-based therapeutics: Insight into the post-antibiotic era. *Acta pharmaceutica Sinica. B*, 12(12), 4348–4364.
- Liu, F., Yuwono, C., Tay, A. C. Y., Wehrhahn, M. C., Riordan, S. M., & Zhang, L. (2022). Analysis of global *Aeromonas veronii* genomes provides novel information on source of infection and virulence in human gastrointestinal diseases. *BMC genomics*, 23(1), 166.
- Loo, Y. Y., Rukayadi, Y., Nor-Khaizura, M. A., Kuan, C. H., Chieng, B. W., Nishibuchi, M., & Radu, S. (2018). In vitro antimicrobial activity of green synthesized silver nanoparticles against selected gram-negative foodborne pathogens. *Frontiers in microbiology*, 9, 1555.
- Loudermilk, E. M., Kotay, S. M., Barry, K. E., Parikh, H. I., Colosi, L. M., & Mathers, A. J. (2022). Tracking *Klebsiella pneumoniae* carbapenemase gene as an indicator of antimicrobial resistance dissemination from a hospital to surface water via a municipal wastewater treatment plant. *Water research*, 213, 118151.

- Lubis, N. D. A., Amelia, S., Yusraini, E., Rahmi, Z., & Balatif, R. (2023). Comparison of Antimicrobial Effectiveness of Orange and Lime's Extracts on *Aeromonas sobria* and *Aeromonas hydrophila*. *Journal of Pure and Applied Microbiology*, 17(4), 2431-2437.
- Mahgoub, S. A., Muhammad, M. I., Abd-Elsalam, S. T., Alkhazindar, M. M., & Abdel-Shafy, H. I. (2020). Isolation and characterization of *Pseudomonas aeruginosa* and *Enterococcus faecalis* lytic bacteriophages from wastewater for controlling multidrug resistant bacterial strains. *Plant Archives*, 20, 450-464.
- Makabenta, J., M., V., Nabawy, A., Li, CH., Schmidt-Malan, S., Patel, R., & Rotello, V., M. (2021). Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. *Nature Reviews Microbiology*, 19(1), 23–36.
- Manaia C. M. (2017). Assessing the risk of antibiotic resistance transmission from the environment to humans: Non-direct proportionality between abundance and risk. *Trends in microbiology*, 25(3), 173–181.
- Mancuso, G., Midiri, A., Gerace, E., & Biondo, C. (2021). Bacterial antibiotic resistance: the most critical pathogens. *Pathogens*, 10(10), 1310.
- Martínez, J. L., Coque, T. M., & Baquero, F. (2015). What is a resistance gene? Ranking risk in resistomes. *Nature review. Microbiology*, 13(2), 116–123.
- Mattila, S., Ruotsalainen, P., & Jalasvuori, M. (2015). On-demand isolation of bacteriophages against drug-resistant bacteria for personalized phage therapy. *Frontiers in microbiology*, 6, 1271.

- McLain, J. E., Cytryn, E., Durso, L. M., & Young, S. (2016). Culture-based Methods for Detection of Antibiotic Resistance in Agroecosystems: Advantages, Challenges, and Gaps in Knowledge. *Journal of environmental quality*, 45(2), 432–440.
- Miller, K. P., Wang, L., Benicewicz, B. C., & Decho, A. W. (2015). Inorganic nanoparticles engineered to attack bacteria. *Chemical Society reviews*, 44(21), 7787–7807.
- Miłobedzka, A., Ferreira, C., Vaz-Moreira, I., Calderón-Franco, D., Gorecki, A., Purkrtova, S., Jan Bartacek, Dziewit, L., Singleton, C. M., Nielsen, P. H., Weissbrodt, D. G., & Manaia, C. M. (2022). Monitoring antibiotic resistance genes in wastewater environments: The challenges of filling a gap in the One-Health cycle. *Journal of hazardous materials*, 424(Pt C), 127407.
- Moghadam, M. T., Amirmozafari, N., Shariati, A., Hallajzadeh, M., Mirkalantari, S., Khoshbayan, A., & Masjedan Jazi, F. (2020). How phages overcome the challenges of drug resistant bacteria in clinical infections. *Infection and drug resistance*, 13, 45–61.
- Mohammed, D., Isa, H. M., & Ali, M. F. (2022). Microbial etiology and antibiotic resistance patterns of urinary tract pathogens in hospitalized infants in Bahrain: A tertiary care center experience. *Cureus*, 14(9), e29236.
- Moloney M. G. (2016). Natural products as a source for novel antibiotics. *Trends in pharmacological sciences*, 37(8), 689–701.
- Motina, E., & Rao, J. M. (2021). Molecular detection of *Enterococcus* with special reference to *Enterococcus faecalis* from water sources and its antimicrobial resistance. *Journal of Entomology and Zoology Studies*, 9(2):287-294.

- Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., & Pardesi, K. R. (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Frontiers in microbiology*, 10, 539.
- Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology spectrum*, 4(2), 10.1128/microbiolspec.VMBF-0016-2015.
- Mureed, S., Naz, S., Haider, A., Raza, A., Ul-Hamid, A., Haider, J., ... Saeed, A. (2021). Development of multi-concentration Cu:Ag bimetallic nanoparticles as a promising bactericidal for antibiotic-resistant bacteria as evaluated with molecular docking study. *Nanoscale research letters*, 16(1), 91.
- Murugaiyan, J., Kumar, P. A., Rao, G. S., Iskandar, K., Hawser, S., Hays, J. P., ... van Dongen, M. B. M. (2022). Progress in alternative strategies to combat antimicrobial resistance: Focus on antibiotics. *Antibiotics*, 11(2), 200.
- Mwafy, A., Youssef, D. Y., & Mohamed, M. M. (2023). Antibacterial activity of zinc oxide nanoparticles against some multidrug-resistant strains of *Escherichia coli* and *Staphylococcus aureus*. *International journal of veterinary science*, 12(3): 284-289.
- Nainu, F., Masyita, A., Bahar, M. A., Raihan, M., Prova, S. R., Mitra, S., ... Simal-Gandara, J. (2021). Pharmaceutical prospects of bee products: Special focus on anticancer, antibacterial, antiviral, and antiparasitic properties. *Antibiotics*, 10(7), 822.
- Naser, H. A. (2014). Marine ecosystem diversity in the Arabian Gulf: threats and conservation. In: Grillo O. (Ed), *Biodiversity–The Dynamic Balance of the Planet*, (pp. 297-328). Croatia: InTech.

- Nasser, M., Palwe, S., Bhargava, R. N., Feuilloley, M. G. J., & Kharat, A. S. (2020). Retrospective analysis on antimicrobial resistance trends and prevalence of β -lactamases in *Escherichia coli* and ESKAPE pathogens isolated from Arabian patients during 2000-2020. *Microorganisms*, 8(10), 1626.
- Neha, P. (2023). Antimicrobial activity of Hibiscus and Murraya leaf extracts against a variety of multidrug-resistant samples. *Journal of BioScience and Biotechnology*, 12(2), 119-122.
- Nendissa, S. J., & Nendissa, D. M. (2021). Test for the antibacterial inhibition of kaffir lime leaf (*Citrus hysteric* DC) extract against pathogen bacteria in improving food safety. In *IOP Conference Series: Earth and Environmental Science*, 883(1), 012056. IOP Publishing.
- Ngongang, F. C., Fankam, A. G., Mbaveng, A. T., Wamba, B. E., Nayim, P., Beng, V. P., & Kuete, V. (2020). Methanol extracts from *Manilkara zapota* with moderate antibacterial activity displayed strong antibiotic-modulating effects against multidrug-resistant phenotypes. *Pharmacology*, 3(1), 37.
- Nobrega, F. L., Costa, A. R., Kluskens, L. D., & Azeredo, J. (2015). Revisiting phage therapy: new applications for old resources. *Trends in microbiology*, 23(4), 185–191.
- Ntulume, I., Victoria, N., Adam, A. S., & Aliero, A. A. (2019). Evaluation of antibacterial activity of *Cymbopogon citratus* ethanolic leaf crude extract against *Streptococcus pneumoniae* isolated from Kampala international university teaching hospital western campus, Uganda. *International Journal of Applied Science and Biotechnology*, 7, 31-38.

- Nyamath, S., & Karthikeyan, B. (2018). In vitro antibacterial activity of lemongrass (*Cymbopogon citratus*) leaves extract by agar well method. *Journal of Pharmacognosy and Phytochemistry*, 7(3), 1185-1188.
- Oliveira, P. H., Touchon, M., Cury, J., & Rocha, E. P. C. (2017). The chromosomal organization of horizontal gene transfer in bacteria. *Nature communications*, 8(1), 841.
- Oniciuc, E. A., Likotrafiti, E., Alvarez-Molina, A., Prieto, M., Santos, J. A., & Alvarez-Ordóñez, A. (2018). The present and future of whole genome sequencing (WGS) and whole metagenome sequencing (WMS) for surveillance of antimicrobial resistant microorganisms and antimicrobial resistance genes across the food chain. *Genes*, 9(5), 268.
- Ouyang, K., Mortimer, M., Holden, P. A., Cai, P., Wu, Y., Gao, C., & Huang, Q. (2020). Towards a better understanding of *Pseudomonas putida* biofilm formation in the presence of ZnO nanoparticles (NPs): Role of NP concentration. *Environment international*, 137, 105485.
- Pandey, A. K., Singh, P., Palni, U. T., & Tripathi, N. N. (2012). In-vitro antibacterial activities of the essential oils of aromatic plants against *Erwinia herbicola* (Lohnis) and *Pseudomonas putida* (Kris Hamilton). *Journal of the Serbian Chemical Society*, 77(3), 313-323.
- Papich, M. G. (2016). *Saunders handbook of veterinary drugs*. North Carolina, USA: Elsevier, 451-452.
- Pareek, V., Devineau, S., Sivasankaran, S. K., Bhargava, A., Panwar, J., Sri Kumar, S., & Fanning, S. (2021). Silver nanoparticles induce a

- triclosan-like antibacterial action mechanism in multi-drug resistant *Klebsiella pneumoniae*. *Frontiers in microbiology*, 12, 638640.
- Pärnänen, K. M. M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T. U., Cacace, D., Do, T. T., Elpers, C., ... Manaia, C. M. (2019). Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Science advances*, 5(3), eaau9124.
- Parnham, M. J., Haber, V. E., Giamarellos-Bourboulis, E. J., Perletti, G., Verleden, G. M., & Vos, R. (2014). Azithromycin: Mechanisms of action and their relevance for clinical applications. *Pharmacology & therapeutics*, 143(2), 225-245.
- Parvekar, P., Palaskar, J., Metgud, S., Maria, R., & Dutta, S. (2020). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles against *Staphylococcus aureus*. *Biomaterial investigations in dentistry*, 7(1), 105–109.
- Paul, C. A., Karpagam, B., & Krishnaveni, N. (2016). Phytochemical Screening and antibacterial evaluation of the aqueous extracts of *Hibiscus rosa-sinensis* leaves against *Aeromonas hydrophila*. *International Journal of Advanced Science and Research*, 1(8), 21-23.
- Peng, Q., Fang, M., Liu, X., Zhang, C., Liu, Y., & Yuan, Y. (2020). Isolation and characterization of a novel phage for controlling multidrug-resistant *Klebsiella pneumoniae*. *Microorganisms*, 8(4), 542.
- Pohan, D. J., & Djojoputro, M. (2021). Antibacterial effectiveness of extracts of lime (*Citrus aurantifolia* Swingle) and kaffir lime (*Citrus*

- hystrix* Dc) leaves against *Escherichia coli*. *International Journal of Modern Pharmaceutical Research*, 5(6), 29-36.
- Pour, A. P., & Farahbakhsh, H. (2020). *Lawsonia inermis* L. leaves aqueous extract as a natural antioxidant and antibacterial product. *Natural product research*, 34(23), 3399–3403.
- Priya, V. T., Balasubramanian, N., Shanmugaiah, V., Sathishkumar, P., Kannan, N. D., Karunakaran, C., ... Antonisamy, P. (2021). Partially purified lead molecules from *Dodonaea viscosa* and their antimicrobial efficacy against infectious human pathogens. *Journal of Infection and Public Health*, 14(12), 1822-1830.
- Pruden, A., Vikesland, P. J., Davis, B. C., & de Roda Husman, A. M. (2021). Seizing the moment: now is the time for integrated global surveillance of antimicrobial resistance in wastewater environments. *Current opinion in microbiology*, 64, 91–99.
- Ramadoss, R., Al-Marzooqi, F., Shomar, B., Ilyin, V. A., & Vincent, A. S. (2022). Genomic characterization and annotation of two novel bacteriophages isolated from a wastewater treatment plant in Qatar. *Microbiology resource announcements*, 11(1), e0109021.
- Rasha, E., Alkhulaifi, M. M., AlOthman, M., Khalid, I., Doaa, E., Alaa, K., ... Abdalla, M. (2021). Effects of zinc oxide nanoparticles synthesized using *aspergillus niger* on carbapenem-resistant *Klebsiella pneumonia* in vitro and in vivo. *Frontiers in cellular and infection microbiology*, 11, 748739.
- Raza, S., Matuła, K., Karoń, S., & Paczesny, J. (2021). Resistance and adaptation of bacteria to non-antibiotic antibacterial agents: Physical stressors, nanoparticles, and bacteriophages. *Antibiotics*, 10(4), 435.

- Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology*, 4(3), 482–501.
- Rocha, J., Ferreira, C., Mil-Homens, D., Busquets, A., Fialho, A. M., Henriques, I., ... Manaia, C. M. (2022). Third generation cephalosporin-resistant *Klebsiella pneumoniae* thriving in patients and in wastewater: what do they have in common? *BMC genomics*, 23(1), 72.
- Ruban, P., & Gajalakshmi, K. (2012). In vitro antibacterial activity of *Hibiscus rosa-sinensis* flower extract against human pathogens. *Asian Pacific journal of tropical biomedicine*, 2(5), 399–403.
- Saito, K., Warrier, T., Somersan-Karakaya, S., Kaminski, L., Mi, J., Jiang, X., ... Nathan, C. F. (2017). Rifamycin action on RNA polymerase in antibiotic-tolerant *Mycobacterium tuberculosis* results in differentially detectable populations. *Proceedings of the National Academy of Sciences of the United States of America*, 114(24), E4832–E4840.
- Sakirigui, A., Ladekan, E., Fagbonou, L., Chabi Sika, K., Assogba, M., & Joachim Djimon, G. (2019). Comparative antimicrobial activity of volatile and non-volatile extracts of *Cymbopogon citratus* leaves. *International Journal of Pharmacognosy*, 7, 234-239.
- Salem, M. Z., Ali, H. M., El-Shanhorey, N. A., & Abdel-Megeed, A. (2013). Evaluation of extracts and essential oil from *Callistemon viminalis* leaves: antibacterial and antioxidant activities, total phenolic and flavonoid contents. *Asian Pacific journal of tropical medicine*, 6(10), 785–791.
- Saliminasab, M., Jabbari, H., Farahmand, H., Asadi, M., Soleimani, M., & Fathi, A. (2022). Study of antibacterial performance of synthesized

- silver nanoparticles on *Streptococcus mutans* bacteria. *Nanomedicine Research Journal*, 7(4), 391-396.
- Sánchez-Gutiérrez, M., Bascón-Villegas, I., Rodríguez, A., Pérez-Rodríguez, F., Fernández-Prior, Á., Rosal, A., & Carrasco, E. (2021). Valorisation of *Olea europaea* L. olive leaves through the evaluation of their extracts: Antioxidant and antimicrobial activity. *Foods*, 10(5), 966.
- Sanderson, H., Ortega-Polo, R., Zaheer, R., Goji, N., Amoako, K. K., Brown, R. S., ... McAllister, T. A. (2020). Comparative genomics of multidrug-resistant *Enterococcus* spp. isolated from wastewater treatment plants. *BMC microbiology*, 20(1), 20.
- Sarkar, R., Kumar, C. A., Kumbhakar, P., & Mandal, T. (2019). Aqueous synthesis and antibacterial activity of silver nanoparticles against *Pseudomonas putida*. *Materials Today: Proceedings*, 11, 686-694.
- Sarker, M. M., Islam, K. N., Huri, H. Z., Rahman, M., Imam, H., Hosen, M. B., ... Sarker, M. Z. (2014). Studies of the impact of occupational exposure of pharmaceutical workers on the development of antimicrobial drug resistance. *Journal of occupational health*, 56(4), 260–270.
- Satyaprakash, K., Pesingi, P. K., Das, A., Vineeth, M. R., Malik, S. V. S., Barbuddhe, S. B., & Rawool, D. B. (2024). Occurrence of multidrug-resistant (MDR) extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in wastewater and natural water sources from the eastern part of Uttar Pradesh, India. *Water, Air, & Soil Pollution*, 235(2), 125.
- Scheinfeld N. (2004). Telithromycin: a brief review of a new ketolide antibiotic. *Journal of drugs in dermatology*, 3(4), 409–413.

- Schmiede, D., Zacharias, N., Sib, E., Falkenberg, T., Moebus, S., Evers, M., & Kistemann, T. (2021). Prevalence of multidrug-resistant and extended-spectrum beta-lactamase-producing *Escherichia coli* in urban community wastewater. *The Science of the total environment*, 785, 147269.
- Shahid, M., Ahmad, N., Saeed, N. K., Shadab, M., Joji, R. M., Al-Mahmeed, ... Dar, F. K. (2022). Clinical carbapenem-resistant *Klebsiella pneumoniae* isolates simultaneously harboring blaNDM-1, blaOXA types and qnrS genes from the Kingdom of Bahrain: Resistance profile and genetic environment. *Frontiers in cellular and infection microbiology*, 12, 1033305.
- Shahraki, S. H., Javar, F. M., & Rahimi, M. (2023). Quantitative and qualitative phytochemical analysis of *Manilkara zapota* (Sapodilla) extract and its antibacterial activity on some gram-positive and gram-negative bacteria. *Scientifica*, 5967638.
- Shamly, M., & Jayalakshmi, M. D. S. (2022). Evaluation of antimicrobial property of self-etch adhesive incorporated with green synthesized TiO₂ nanoparticles: An invitro study. *International Journal of Health Sciences*, (3), 2124-2130.
- Shawkat, S. J., & Chehri, K. (2021). Antimicrobial potential of Titanium dioxide nanoparticles in urinary tract infections: an experimental study on the growth inhibitory activity and biofilm inhibition. *Avicenna Journal of Clinical Microbiology and Infection*, 8(4), 123-129.
- Shendurse, A. M., Sangwan, R. B., Kumar, A., Ramesh, V., Patel, A. C., Gopikrishna, G., & Roy, S. K. (2021). Phytochemical screening and antibacterial activity of lemongrass (*Cymbopogon citratus*) leaves

- essential oil. *Journal of Pharmacognosy and Phytochemistry*, 10(2), 445-449.
- Shields, P., & Tsang, A., Y. (2006). Mannitol salt agar plates protocol. *American Society for Microbiology*.
- Shrestha, S., Malla, B., & Haramoto, E. (2024). Group A *Streptococcus pyogenes* in wastewater: Applicability of wastewater-based epidemiology for monitoring the prevalence of GAS pharyngitis during the late COVID-19 pandemic phase. *The Science of the total environment*, 928, 172447.
- Sieiro, C., Areal-Hermida, L., Pichardo-Gallardo, Á., Almuiña-González, R., de Miguel, T., Sánchez, S., ... Villa, T. G. (2020). A Hundred Years of Bacteriophages: Can phages replace antibiotics in agriculture and aquaculture? *Antibiotics*, 9(8), 493.
- Silva, J. B., Storms, Z., & Sauvageau, D. (2016). Host receptors for bacteriophage adsorption. *FEMS microbiology letters*, 363(4), fnw002.
- Singh, A., Gautam, P. K., Verma, A., Singh, V., Shivapriya, P. M., Shivalkar, ... Samanta, S. K. (2020). Green synthesis of metallic nanoparticles as effective alternatives to treat antibiotics resistant bacterial infections: A review. *Biotechnology reports*, 25, e00427.
- Singh, P., Montano, A., & Bostick, A. (2021). Rapid severe sepsis from *Pseudomonas fluorescens/putida* bacteremia due to skin and soft tissue infection - A case report. *Annals of medicine and surgery*, 70, 102845.
- Skwor, T., Stringer, S., Haggerty, J., Johnson, J., Duhr, S., Johnson, M., ... Stemme, M. (2020). Prevalence of potentially pathogenic antibiotic-

- resistant *Aeromonas* spp. in treated urban wastewater effluents versus recipient riverine populations: A 3-year comparative study. *Applied and environmental microbiology*, 86(3), e02053-19.
- Soraya, C., Mubarak, Z., & Gani, B. A. (2020). The growth and biofilm formation of *Enterococcus faecalis* in ethanol extract of *Citrus aurantifolia* Indonesian species. *Journal of Pharmacy & Pharmacognosy Research*, 8(6), 558-68.
- Staneva, A., Albu-Kaya, M., Martinov, B., Ivanova, I., & Vladkova, T. (2020). Preparation and antimicrobial activity of collagen/(RGO/ZnO/TiO₂/SiO₂) composites. *Journal of Chemical Technology & Metallurgy*, 55(5).
- Starliper, C. E., Ketola, H. G., Noyes, A. D., Schill, W. B., Henson, F. G., Chalupnicki, M. A., & Dittman, D. E. (2015). An investigation of the bactericidal activity of selected essential oils to *Aeromonas* spp. *Journal of advanced research*, 6(1), 89–97.
- Subhapriya, S., & Gomathipriya, P. (2018). Green synthesis of titanium dioxide (TiO₂) nanoparticles by *Trigonella foenum-graecum* extract and its antimicrobial properties. *Microbial pathogenesis*, 116, 215–220.
- Subramaniam, G., & Girish, M. (2020). Antibiotic resistance - A Cause for Reemergence of Infections. *Indian journal of pediatrics*, 87(11), 937–944.
- Subramaniam, G., Yew, X. Y., & Sivasamugham, L. A. (2020). Antibacterial activity of *Cymbopogon citratus* against clinically important bacteria. *South African Journal of Chemical Engineering*, 34, 26-30.

- Surleac, M., Czobor Barbu, I., Paraschiv, S., Popa, L. I., Gheorghe, I., Marutescu, L., ... Chifiriuc, M. C. (2020). Whole genome sequencing snapshot of multi-drug resistant *Klebsiella pneumoniae* strains from hospitals and receiving wastewater treatment plants in Southern Romania. *PloS one*, 15(1), e0228079.
- Tao, S., Chen, H., Li, N., Wang, T., & Liang, W. (2022). The Spread of Antibiotic Resistance Genes In Vivo Model. *The Canadian journal of infectious diseases & medical microbiology*, 3348695.
- Testa, B., Lombardi, S. J., Macciola, E., Succi, M., Tremonte, P., & Iorizzo, M. (2019). Efficacy of olive leaf extract (*Olea europaea* L. cv Gentile di Larino) in marinated anchovies (*Engraulis encrasicolus*, L.) process. *Heliyon*, 5(5), e01727.
- Tiwari, A., Kurittu, P., Al-Mustapha, A. I., Heljanko, V., Johansson, V., Thakali, O., ... Heikinheimo, A. (2022). Wastewater surveillance of antibiotic-resistant bacterial pathogens: A systematic review. *Frontiers in microbiology*, 13, 977106.
- Toukourou, H., Gbaguidi, F., & Quetin-Leclercq, J. (2019). Phytochemical composition, antibacterial activity against sore throat pathogens and toxicological evaluation of *Cymbopogon citratus* essential oil from Benin. *Journal of Pharmacognosy and Phytochemistry*, 8(4), 3258-3263.
- Truppa, C., & Abo-Shehada, M. N. (2020). Antimicrobial resistance among GLASS pathogens in conflict and non-conflict affected settings in the Middle East: A systematic review. *BMC infectious diseases*, 20(1), 936.
- Uddin, T. M., Chakraborty, A. J., Khusro, A., Zidan, B. R. M., Mitra, S., Emran, T. B., ... Koirala, N. (2021). Antibiotic resistance in

- microbes: History, mechanisms, therapeutic strategies and future prospects. *Journal of infection and public health*, 14(12), 1750–1766.
- Udo, I. J., Ben, M. G., Etuk, C. U., & Tiomthy, A. I. (2016). Phytochemical, proximate and antibacterial properties of *Hibiscus rosa-sinensis* L. Leaf. *Journal of Medicinal Plants Studies*, 4(5), 193-195.
- Uluseker, C., Kaster, K. M., Thorsen, K., Basiry, D., Shobana, S., Jain, M., ... Pala-Ozkok, I. (2021). A review on occurrence and spread of antibiotic resistance in wastewaters and in wastewater treatment plants: Mechanisms and perspectives. *Frontiers in microbiology*, 12, 717809.
- Vaou, N., Stavropoulou, E., Voidarou, C., Tsigalou, C., & Bezirtzoglou, E. (2021). Towards advances in medicinal plant antimicrobial activity: A review study on challenges and future perspectives. *Microorganisms*, 9(10), 2041.
- Velazquez-Meza, M. E., Galarde-López, M., Cornejo-Juárez, P., Carrillo-Quiroz, B. A., Velázquez-Acosta, C., Bobadilla-Del-Valle, M., ... Alpuche-Aranda, C. M. (2024). Multidrug-resistant *Staphylococcus* sp. and *Enterococcus* sp. in municipal and hospital wastewater: a longitudinal study. *Microorganisms*, 12(4), 645.
- Vera-Mansilla, J., Sánchez, P., Silva-Valenzuela, C. A., & Molina-Quiroz, R. C. (2022). Isolation and characterization of novel lytic phages infecting multidrug-resistant *Escherichia coli*. *Microbiology spectrum*, 10(1), e0167821.
- Vetting, M. W., Hegde, S. S., Wang, M., Jacoby, G. A., Hooper, D. C., & Blanchard, J. S. (2011). Structure of QnrB1, a plasmid-mediated

- fluoroquinolone resistance factor. *The Journal of biological chemistry*, 286(28), 25265–25273.
- Walker, R. C. (1999). The Fluoroquinolones. *Mayo Clinic Proceedings*, 74(10), 1030-1037. Elsevier.
- Wang, B., Mao, C., Feng, J., Li, Y., Hu, J., Jiang, B., ... Su, Y. (2021). A First report of *Aeromonas veronii* infection of the Sea Bass, *Lateolabrax maculatus* in China. *Frontiers in veterinary science*, 7, 600587.
- Wang, F., Zhou, H., Olademehin, O. P., Kim, S. J., & Tao, P. (2018). Insights into key interactions between vancomycin and bacterial cell wall structures. *ACS omega*, 3(1), 37–45.
- Wayne, P. A. (2020) Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, 30th ed., Supplement M100, CLSI, USA.
- World Health Organization. (2019). *World Health Organization Ten Threats to Global Health in 2019*: Author.
- World Health Organization. (2020). *Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2020*: Author.
- World Health Organization. (2022). *Antimicrobial resistance surveillance in Europe 2022–2020 data*: Author.
- Wright, A., Hawkins, C. H., Anggård, E. E., & Harper, D. R. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Official journal of Netherlands*

Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery, 34(4), 349–357.

Wróbel, A., Maliszewski, D., Baradyn, M., & Drozdowska, D. (2019). Trimethoprim: An Old Antibacterial Drug as a Template to Search for New Targets. Synthesis, Biological Activity and Molecular Modeling Study of Novel Trimethoprim Analogs. *Molecules*, 25(1), 116.

Zaatout, N., Bouras, S., & Slimani, N. (2021). Prevalence of extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* in wastewater: a systematic review and meta-analysis. *Journal of water and health*, 19(5), 705–723.

Zain, N. M., Stapley, A. G., & Shama, G. (2014). Green synthesis of silver and copper nanoparticles using ascorbic acid and chitosan for antimicrobial applications. *Carbohydrate polymers*, 112, 195–202.

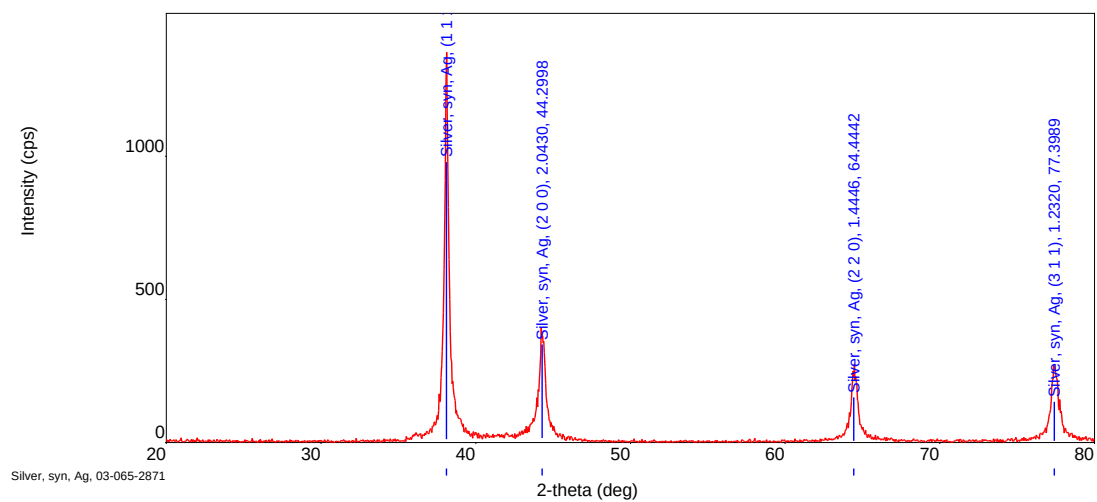
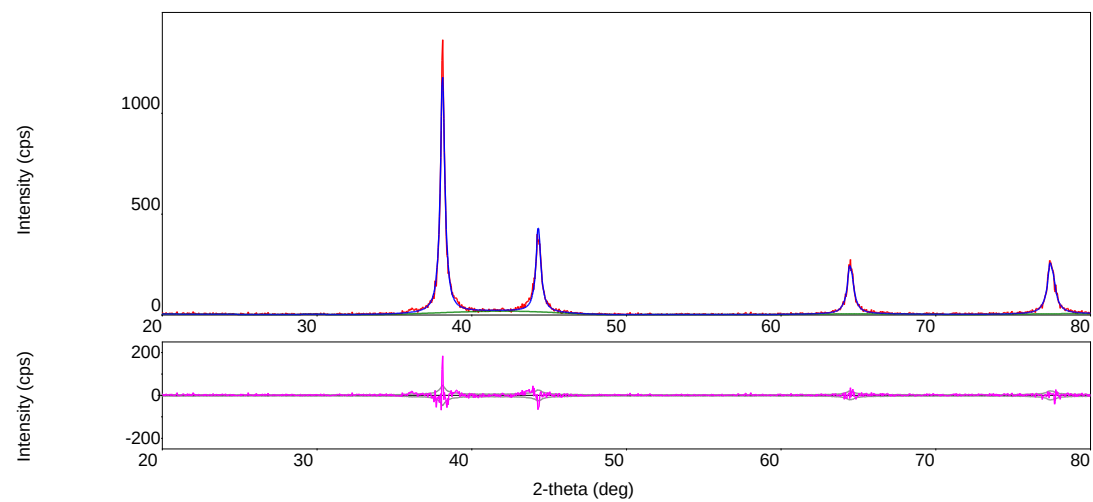
Zhao, Y., Ye, M., Zhang, X., Sun, M., Zhang, Z., Chao, H., ... Hu, F. (2019). Comparing polyvalent bacteriophage and bacteriophage cocktails for controlling antibiotic-resistant bacteria in soil-plant system. *The Science of the total environment*, 657, 918–925.

Zuraidah, Z., Widiyari, W., & Oviana, W. (2023). Antibacterial potential of leaf and fruit of citrus extract (*Citrus aurantifolia*) against *klebsiella oxytoca*. *Jurnal Pendidikan Biologi*, 8(1), 106-114.

Appendices

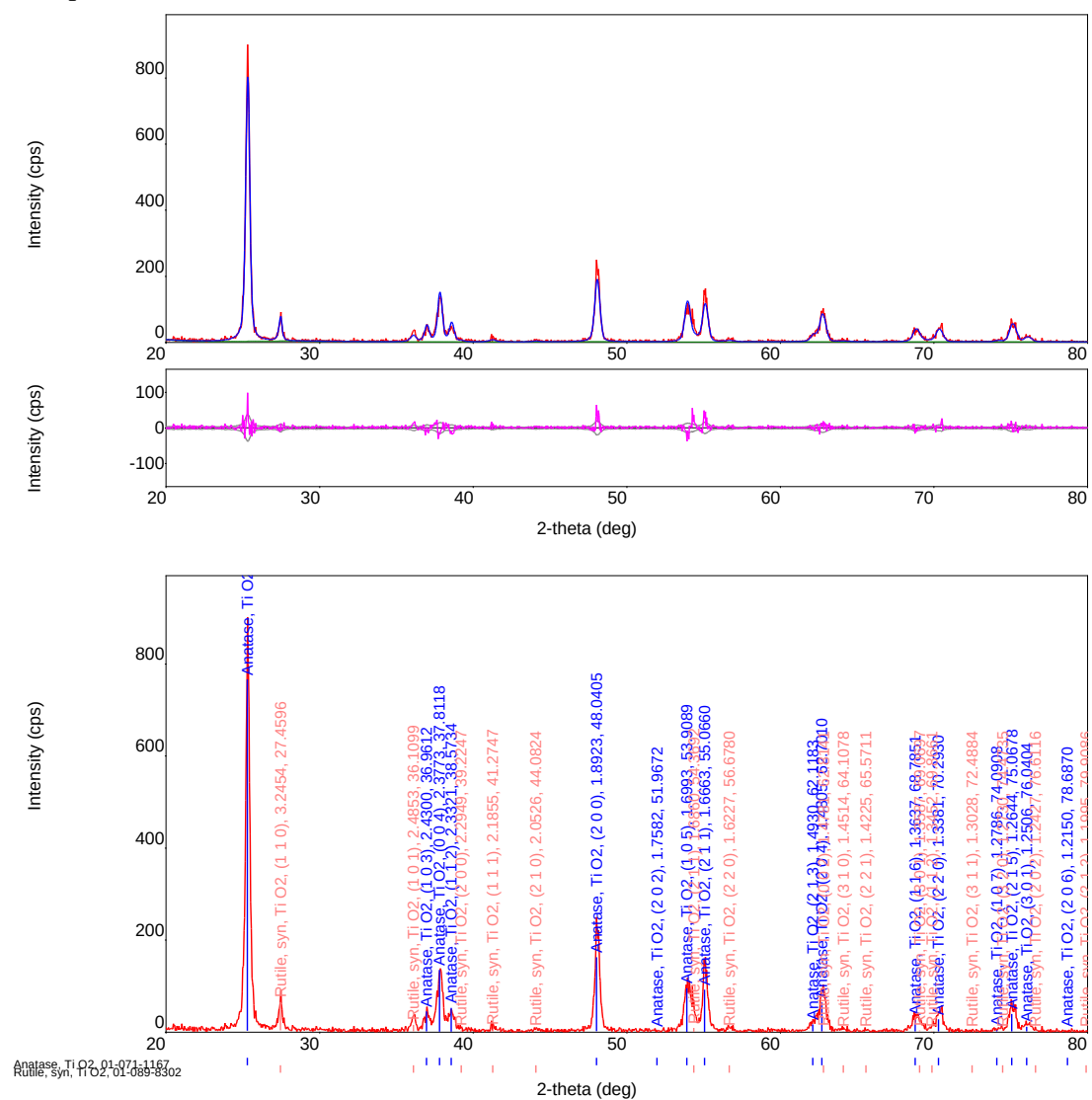
Appendix 1. XRD analysis of the nanoparticles involved in the study.

Sample: Ag



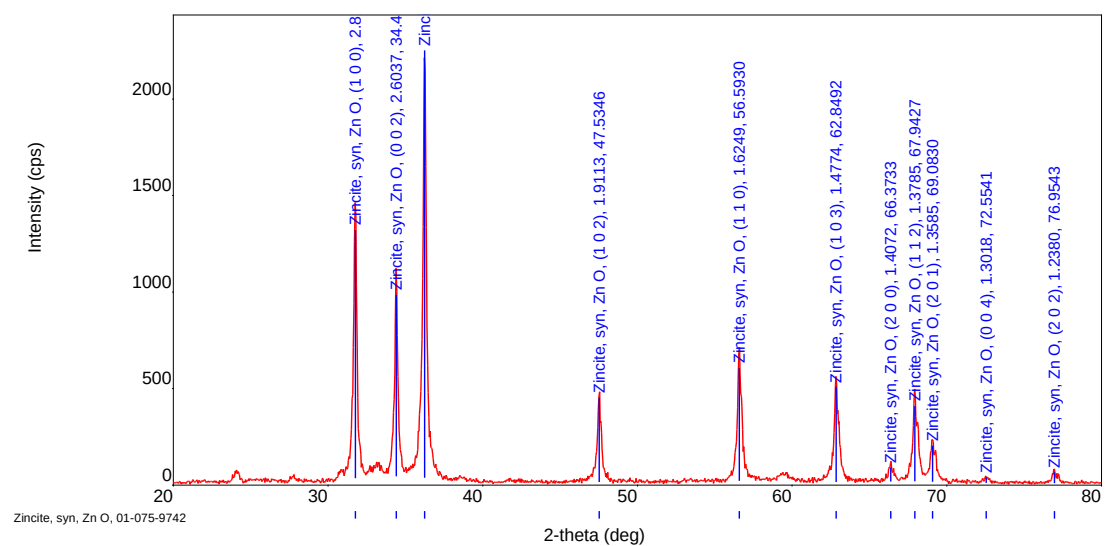
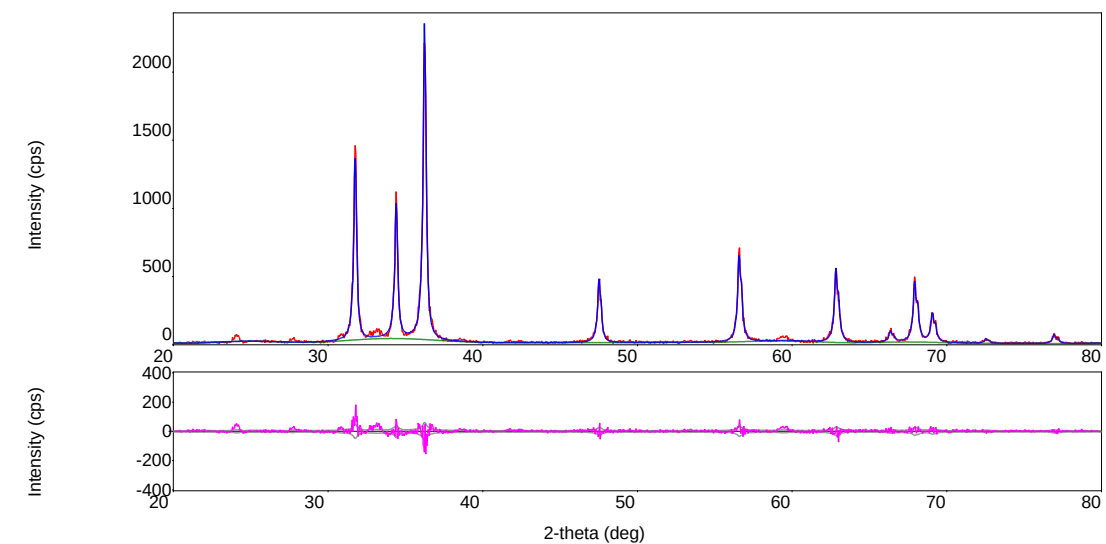
Ag: 100%

Sample: TiO₂



TiO₂ Anatase: 92%
TiO₂ Rutile: 8%

Sample: ZnO



ZnO: 100%

Appendix 2. The CLSI guideline for the inhibition zone diameter interpretive standard breakpoints for determination of bacterial sensitivity and resistance to antibiotics.

Name of antibiotics (dose)	Sensitive	Intermediate	Resistant
Kanamycin (30 µg/disk)	≥18	14–17	≤13
Streptomycin (10 µg/disk)	≥15	12–14	≤11
Erythromycin (15 µg/disk)	≥23	14–22	≤13
Tetracycline (30 µg/disk)	≥15	12–14	≤11
Chloramphenicol (30 µg/disk)	≥18	13–17	≤12
Novobiocin	≥16	12-15	≤11
<i>Enterococcus spp.</i>			
Tetracycline (30 µg/disk)	≥19	15-18	≤14
Penicillin	≥15	-	≤14
<i>Streptococcus spp.</i>			
Erythromycin (15 µg/disk)	≥21	19-22	≤18
Tetracycline (30 µg/disk)	≥23	19-22	≤15
Chloramphenicol (30 µg/disk)	≥21	16-20	≤15
Penicillin	≥24	-	-

Appendix 3. The bacterial identification and antibiotic susceptibility tests by BD Phoenix system.



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Document

في الختام، أظهر فحص مياه الصرف وجود مجموعة متنوعة من البكتيريا المقاومة لمضادات حيوية متعددة. إضافة إلى ذلك، أظهرت مستخلصات أوراق النباتات، والجسيمات النانوية والفيروسات العاثية فاعلية كبيرة للحد من نمو البكتيريا المقاومة للمضادات الحيوية، وهي بدائل محلية للمضادات الحيوية تعد واعدة في مكافحة البكتيريا المقاومة للمضادات الحيوية.

جامعة البحرين

قسم علوم الحياة



كلية العلوم

حلول صديقة للبيئة لمقاومة المضادات الحيوية في مياه الصرف: استخدام النباتات، الجسيمات النانوية والفيروسات العاثية

أطروحة مقدمة كجزء من متطلبات الحصول على درجة الماجستير في علوم الحياة

إعداد

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مايو 2024