School of Life Sciences

Department of Biological Sciences and Biotechnology

Antibacterial activities of potential antibiotic-producing bacteria and indigenous plants against antibiotic-resistant bacteria

By

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BSc (General) (University of Botswana), MSc Biological Sciences and Biotechnology

A Thesis submitted to the School of Life Sciences in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science in Biological Sciences of BIUST

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July, 2025

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ARB	Antibiotic-resistant bacteria
WHO	World Health Organisation
AMR	Antimicrobial resistance
ARGs	Antibiotic-resistant genes
RNA	Ribonucleic acid
tRNA	transfer ribonucleic acid
rRNA	ribosomal ribonucleic acid
DNA	Deoxyribonucleic acid
dsDNA	double-stranded Deoxyribonucleic acid
HGT	Horizontal gene transfer
MGEs	Mobile genetic elements
ISCR	Insertion sequence common region
ICE	Integrating conjugative elements
GTAs	Gene transfer agents
ROS	Reactive oxygen species
HMRGs	Heavy metal resistance genes

WWTPs	Wastewater treatment plants
MAC	MacConkey agar
R2A	Reasoner's 2A agar
NA	Nutrient agar
SE	Soil extract
AST	Antibiotic susceptibility testing
EUCAST	European Committee for Antimicrobial Susceptibility Testing
CLSI	Clinical and Laboratory Standards Institute
MDR	Multi-drug resistant
WGS	Whole-genome sequencing
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
API	Analytical profile index
antiSMASH	antibiotics & Secondary Metabolite Analysis Shell
DMSO	Dimethylsulfoxide
MIC	Minimum inhibitory concentration
INT	Iodonitrotetrazolium
CI	Confidence interval

ANOVA	Analysis of variance
CFU	Colony-forming units
ISEM	Intensive soil extract medium
NSE	New soil extract
CDS	Coding sequences
VF	Virulence factors
AMX	Amoxicillin
AMP	Ampicillin
PEN G	Penicillin G
AMK	Amikacin
KAN	Kanamycin
GEN	Gentamicin
TET	Tetracycline
RIF	Rifampicin
TMP	Trimethoprim
UTIs	Urinary tract infections
GC	Guanine-cytosine

IAA	indole-3-acetic acid
AAC	Aminoglycoside acetyltransferase
NRPS	Non-Ribosomal Peptide Synthetase
LAP	Lanthipeptide, Linear azol(in)e-containing peptide
T1PKS	Type-I Polyketide Synthase
TransAT-PKS	trans-acyltransferase polyketide synthases
RiPP	Ribosomally synthesized and post-translationally modified peptides
BGCs	Biosynthetic gene clusters
BLAST	Basic Local Alignment Search Tool
WGS	Whole genome sequencing

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DECLARATION

I, Katlego P. P. Makale, declare that the thesis titled "**Antibacterial Activities of Potential Antibiotic Producing Bacteria and Indigenous Plants Against Antibiotic-Resistant Bacteria**" represents the outcome of my original research. I confirm this study has not been submitted for a degree at another institution. All sources used have been properly acknowledged, and any assistance received has been credited.

Signature Date



_____ _____13/01/25_____

Dr T.O Rahube

As the candidate's main supervisor, I confirm that I have reviewed this thesis and believe it to be the candidate's original work.

Signature Date



_____ _____13/01/25_____

ACKNOWLEDGEMENTS

I want to express heartfelt gratitude to the Lord Jesus Christ for the unwavering strength, courage, and guidance He provided throughout this journey. His sustenance, which surpasses all human understanding, and the faith to persevere even on the most challenging days, have been my constant source of inspiration.

I appreciate my supervisors, Dr. T.O. Rahube, Dr. G. Rantong, and Prof. A. Makhzoum, for their invaluable guidance, constructive feedback, and support throughout my research project. Their dedication, selflessness, and tirelessness have shaped this research.

I am also grateful to the Plant Biotech Group, Ms. O. Dineo, Mr. B. Nduna, and Mr. W. Marenga, for their invaluable contributions and assistance during sampling and experimental work. The collaborative efforts and teamwork made the research process bearable.

To my friends and colleagues, Mr. R. Motswagole, Ms. P. Nthoiwa, Mr. F. Molapisi, Ms. K. Bannyaditse, and Ms. B. Lotshwao, thank you for your constant encouragement, invaluable feedback, and unwavering support throughout this journey. Your support and faith in me made challenges manageable and kept me motivated. I also thank my family for their love, care, prayers, and support.

I am also grateful to the Department of Biological Sciences and Biotechnology staff for their technical assistance and the conducive learning environment they provided. Finally, I extend gratitude to the Botswana International University of Science and Technology (BIUST) for allowing me to pursue my studies at the institution.

ABSTRACT

Antibiotic resistance is a significant global public health problem, intensified by anthropogenic activities. The resistance contributes to longer hospital stays, increased health care, and high death rates. Natural compounds from medicinal plants and bacteria are attractive alternatives for the development of novel antibiotics and ultimately combating antibiotic resistance. The study aimed to isolate, and screen crude plant extracts and bacteria for antibacterial properties. Selected test bacteria; *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli*, and *Proteus mirabilis* MN029 were used in the screening of antibacterial properties of selected plants and potential antibiotic-producing bacteria. Long-read PacBio® HiFi technology was used to sequence *Proteus mirabilis*-MN029 and the strain was used as a model for multi-drug resistant bacteria in evaluating the antibacterial properties of plant crude extracts. The *Proteus mirabilis*-MN029 genome comprised of a single chromosome, 38.5% G+C content and 3,988,563 bps in length, acquired and chromosomally encoded antimicrobial resistance genotypes to clinically relevant antibiotics, virulence factors, drug targets and transporters. Potential antibiotic-producing bacteria were also isolated from soil and water samples, the isolates that inhibited at least four test bacteria were subjected to Gram staining, biochemical testing, and antibiotic susceptibility testing. Four promising antibiotic-producing bacteria were sequenced using short-read sequencing technology on the Illumina platform. The potential antibiotic-producing bacteria were identified as *Paenibacillus elgii*-KM_B45, *Brevibacillus laterosporus*-KM_C76, *Paenibacillus oleatilyticus*-KM_D13 and *Paenibacillus oleatilyticus*-KM_D46, their genomes revealed 23 biosynthetic gene clusters associated with antibacterial properties. *Pterocarpus angolensis*, *Aloe zebrina* and *Aloe littoralis* were also tested

for antibacterial effectiveness against the test bacteria. Maceration was used to extract plant crude extracts using solvents of different polarities. The primary screening was conducted using Kirby Bauer Disc diffusion, while microdilution assay was employed as a secondary screening for determining the minimum inhibitory concentration of the medicinal plants against the test bacteria. Among the three tested medicinal plants, *P. angolensis* inhibited most of the test bacteria compared to *A. littoralis* and *A. zebirna.* The highest activity was demonstrated at MIC of 0.075 mg/ml by *A. littoralis* and *A. zebrina* ethylacetate root extracts against *S. aureus*. The results of antibacterial activities associated with the selected bacteria and plants provide a great opportunity for further research, which will focus on the purification of the secondary metabolites for use in the development of new antibiotics.

1.0 INTRODUCTION

Antimicrobial resistance (AMR) is a serious global and One Health challenge, impacting not only humans but also animals and the immediate environment (Tang et al., 2023). AMR reported as a major public health concern, has led to increased rates of illness and mortality (Munita and Arias, 2016; Gupta and Birdi, 2017; Reygaert, 2018). About 1.27 million deaths globally were attributed to AMR in 2019 and it is postulated to cause 10 million fatalities by 2050, globally (Godman et al., 2022; Kariuki et al., 2022; Tang et al., 2023). The estimated mortality rate projections in Africa alone will be 4,150,000 by 2050. The total expected costs are between US$300 billion to more than $1 trillion per year worldwide (Dadgostar, 2019). AMR also significantly increases healthcare costs, with the Centers for Disease Control and Prevention (CDC) estimating an additional $1,400 per patient in the U.S., potentially escalating to over $2 billion annually (Dadgostar, 2019). Sub-Saharan Africa bears a significant burden of AMR, largely because the region is made up of low- and middle-income countries (LMICs), where health systems often face resource constraints and high infectious disease rates (Dadgostar, 2019). In Botswana, recent health data show the urgency of addressing AMR. A national point-prevalence survey revealed that approximately 70% of inpatients received antibiotics, often without microbiological confirmation, and outpatient settings reported antibiotic prescription rates as high as 42.7%, exceeding the World Health Organization's (WHO) recommended threshold of 30% (Kitt et al., 2022; Seetswane et al., 2022). Moreover, inappropriate antibiotic use was observed in nearly half of pediatric prescriptions at tertiary hospitals, with a large proportion involving "Watch" category antibiotics as classified by WHO's AWaRe framework (Kitt et al., 2022; Seetswane et al., 2022). A study conducted in Botswana has identified methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical and

environmental sources, with resistance genes such as *blaTEM*, *fox*, and *tetO* frequently reported (Kitt et al., 2022; Seetswane et al., 2022), showing that AMR is a problem affecting Botswana as well.

In animals, AMR makes infections more difficult to treat, leading to loss of livestock, prolonged illness, higher veterinary costs, reduced productivity and economic setbacks for the agricultural sector (Ardakani et al., 2023; Radhouani et al., 2014). The animals also suffer and their welfare is compromised (Radhouani et al., 2014). Additionally, AMR in food-producing animals poses safety risks that can potentially impact human health, as resistant bacteria can enter the food chain (Ardakani et al., 2023; Radhouani et al., 2014). Human health risks also arise from exposure to contaminated soil and water (Stanton et al., 2022). Wildlife is also at risk, as exposure to contaminated environments can lead to difficult-to-treat infections, impacting biodiversity and wildlife populations (Radhouani et al., 2014). Antibiotic-resistant bacteria (ARB) disrupt natural microbial communities in the environment, affecting nutrient cycling and ecosystem health (Larsson & Flach, 2021; Radhouani et al., 2014; Stanton et al., 2022). The spread of ARB in the environment is primarily caused by human activities, including improper use and disposal of antibiotics, which leads to the high prevalence of ARB in the environment (Ayukekbong et al., 2017; Dadgostar, 2019; Manyi-Loh et al., 2018). The other anthropogenic activities include unregulated disposal of medications, unnecessary administration of antibiotics, insufficient enforcement of regulatory policies, non-adherence to Doctors' orders of completing the course of medication for prescribed antibiotic medications, and widespread availability of over-the-counter antibiotics (Ayukekbong et al., 2017; Barancheshme & Munir, 2018; Dadgostar, 2019;

Manyi-Loh et al., 2018). The agricultural sector also contributes to the prevalence of ARB by administering antibiotics to healthy animals as a preventive measure, to stimulate growth and increase feed efficiency (Ayukekbong et al., 2017; Dadgostar, 2019; Manyi-Loh et al., 2018).

Microbes including bacteria, viruses and fungi frequently and rapidly develop defenses against drugs, making these drugs ineffective in treating various infections (Gupta & Birdi, 2017; Munita & Arias, 2016; Reygaert, 2018). The microorganisms can express different genes based on external factors, including the presence of antimicrobials they are exposed to, enabling them to respond to survive and thrive in their presence (Munita & Arias, 2016). AMR genes evolve through mutations caused by selective pressures brought about by chemicals or antimicrobial agents. The selective pressure exerted by the antimicrobial agents provides a competitive advantage to strains with resistance genes (Munita & Arias, 2016). Bacteria can also develop resistance by acquiring mobile AMR genes through horizontal gene transfer (HGT) mechanisms like transduction, conjugation, and transformation (Gupta & Birdi, 2017; Tenover, 2006).

There are various mechanisms encoded by resistance genes enabling microorganisms to resist drugs. In bacteria, these mechanisms include the removal of drugs from the cell, restriction of drug uptake, drug inactivation, and modification of the drug's target (Reygaert, 2018). For instance, porin changes in bacteria with large outer membranes can limit drug uptake by reducing porin numbers or altering porin selectivity, affecting β-lactam antibiotics' effectiveness (Sekyere et al., 2015; Reygaert, 2018). Enterobacteriaceae bacteria, for example, use reduced porin numbers to resist carbapenems (Sekyere et al., 2015). Modifications to drug targets, such as mutations in RNA polymerase and DNA gyrase, prevent antibiotic binding (Peterson & Kaur, 2018;

Reygaert, 2018; Walsh, 2003). Bacteria also inactivate antibiotics through enzymatic degradation or chemical modification, as seen with beta-lactamase enzymes produced by bacteria to deactivate Penicillin G (Chandra et al., 2017; Khameneh et al., 2019; Lomovskaya et al., 2001). Additionally, bacteria use efflux pumps to expel antibiotics, with some increasing pump specificity to resist multiple drugs (McBain et al., 2003; Santajit & Indrawattana, 2016).

Traditional medicine is considered as an integration of medicinal knowledge and practice from different Indigenous cultural beliefs and experiences that are used in the diagnosis, prevention, and treatment of diseases (Ozioma et al., 2019). In traditional medicine, the knowledge of therapeutic uses of medicinal plants is passed down verbally through generations by different traditional/tribal healers (Gupta et al., 2017; Ozioma et al., 2019). In Africa, around 80% of people still rely on the therapeutic benefits of medicinal plants. These plants have gained momentum in countries such as Nigeria and other West African nations due to their affordability, accessibility, acceptance, and low toxicity (Ozioma et al., 2019). It is reported that extracts from these medicinal plants show various biological activities such as anti-inflammatory, antimicrobial, and antioxidant activities (Nguta et al., 2015). The therapeutic nature of the plants is because of the wide variety of secondary metabolites that they produce (Twaij & Hasan, 2022).

Microbes such as fungi and bacteria, are known to naturally produce antibacterial compounds for defense against other microbes in their environment (Chandra & Kumar, 2017). These antibacterial compounds treat bacterial infections and human diseases (Chandra & Kumar, 2017). Microbes manufacture these antibiotics as part of their secondary metabolites (Sengupta et al., 2013). Actinobacteria are producers of many bioactive compounds and have been crucial in the

development of antibacterial drugs (antibiotics) (Schneider, 2021). They are known for their large, linear genomes rich in biosynthetic gene clusters for non-ribosomal peptides and polyketides (Barka et al., 2016; Bérdy, 2005; Jose & Jha, 2016). By 2020 they served as the basis of over 70% of the clinically approved antibiotics (Delbari et al., 2023; Law et al., 2020). However, estimates suggest that only a few of their antibacterial compounds have been discovered (Schneider, 2021; Watve et al., 2001). This suggests plenty of unrealized potential for discovering novel antibacterial compounds from bacteria, including Actinobacteria.

Secondary metabolites produced by plants and bacteria continue to be considered promising candidates for developing new drugs, including antimicrobial drugs. The metabolites possess broad antimicrobial activity and can either kill the pathogens or inhibit their growth while having minimal toxicity to host cells (Nguta et al., 2015). They also display high degrees of bioavailability and have increased capacity to access sites of action within target cells, making them highly effective (Palomino & Martin, 2014). The modes of action of the metabolites against the microorganisms include interfering with DNA/RNA synthesis and function, disrupting their membrane function and structure (including the efflux system), interrupting normal cell communication, disrupting intermediary metabolism and inducing coagulation of cytoplasmic constituents (Gupta & Birdi, 2017).

Common antibiotics from microorganisms and antimicrobial compounds from plants include penicillin (from *Penicillium chrysogenum)*, gentamicin (from *Micromonospora purpurea)*, vancomycin (from *Streptomyces orientalis)*, quinine (from *Cinchona* spp bark), opium (from *Papaver somniferum*), eugenol (*Syzygium aromaticum*) and berberine (from *Berberis vulgaris*)

(Naga and Hakim, 2015; Khameneh et al., 2019; Mańdziuk and Kuchar, 2023). These are used in the treatment of various bacterial infections like bacterial meningitis, streptococcal pharyngitis, community-acquired pneumonia, urinary tract infections, diarrheal diseases and skin and soft tissue infections (Mańdziuk & Kuchar, 2023; Naga & Hakim, 2015).

Most pathogenic bacteria have unfortunately developed resistance to currently available antibiotics, and new antibiotics may quickly prove ineffective against the rapidly evolving multi-drug resistant bacteria (Gupta & Birdi, 2017). Compounds derived from plant and bacterial metabolites are a potential solution as they contain multiple and chemically diverse phytochemicals, thereby reducing the chances of resistance development and allowing therapeutic action through multiple pathways (Gupta and Birdi, 2017; Arip et al., 2022). These natural products also have less nitrogen, phosphorus, sulfur, and halogens. They are more complex, with a broader range of structures, more diverse ring systems, and a greater carbohydrate content (Arip et al., 2022). Natural products can also be used in synergy with antibiotics to improve their antibacterial effect (Arip et al., 2022).

Despite being sources of many novel biologically active compounds, few plant species and bacteria have been thoroughly investigated for their antibacterial properties. In Botswana, plants used to treat bacterial diseases include *Kigelia africana*, *Cassia abbreviata, Hibiscus calyphyllus*, *Aloe spp* and *Pterocarpus angolensis* (Moiketsi et al., 2023; Motlhanka & Makhabu, 2011). These plants may also be valuable in treating antibiotic-resistant strains (Vaou et al., 2021). Combating AMR requires dedicated research that addresses the significant impact of healthcare-associated infections and the development of new antimicrobials. Incorporating bioactive compounds

derived from both medicinal plants and bacteria into the strategy to combat AMR could be highly beneficial (Palomino & Martin, 2014).

1.1. PROBLEM STATEMENT

The rise of antibiotic resistance has led to a shortage of effective drugs, resulting in higher rates of illness and death worldwide. This leads to increased medical expenses, lengthier hospital stays, and ultimately, treatment failure and death.

Research and development efforts are needed to tackle antibiotic resistance and develop new treatments for bacterial infections. New antibiotic developments are considered expensive and time-consuming and because antibiotics are often used indiscriminately which leads to the rapid development and spread of resistance. In many regions of the world, there are countries such as Botswana, where there is a lack of specialized companies focusing on drug development.

It is also generally acknowledged that approximately 99% of bacteria cannot be cultured using traditional methods. Many bacterial species, especially those found in soil, are difficult to cultivate in laboratory settings, which makes their isolation a demanding task. This is because bacteria may have specific nutritional or environmental requirements that laboratory media do not provide. Limited research has been conducted to improve or develop new culture-based methods to maximize capturing the unculturable majority of soil bacterial communities. This makes isolating, identifying, and investigating their antibiotic qualities difficult. There is also the lack of genomic capacity and the use of innovative approaches towards understanding resistance mechanisms in Botswana.

1.2 JUSTIFICATION

The research emphasizes using bacteria and crude extracts from selected indigenous plants to counteract antibiotic-resistant bacteria. Botswana has a high prevalence of infectious bacterial diseases associated with resistance to virtually all important classes of antibiotics (Paramadhas et al., 2019). Research on local discovery of antibiotics can help combat this AMR threat and improve public health in Botswana, Africa, and globally. This research will aid in promoting the sustainable use of local biodiversity and the importance of preserving natural habitats and indigenous plants. Developing new antibiotics locally can have socio-economic benefits in the population leading to improved overall health by effectively managing infectious diseases, which ultimately reduce morbidity and mortality rates. This study aligns with United Nations sustainable development goals (SDG) specifically SDG3 (Good Health and Well-being) by promoting the use of indigenous plants and bacteria for One Health benefits.

1.3 HYPOTHESIS

Ponds, especially ones that have dried up, can support a variety of microorganisms because of their constantly changing surroundings. Because bacteria frequently compete for nutrients in pond habitats, these variances may result in the emergence of distinct bacterial species that could produce novel antibiotics. With harsh conditions like heavy metal contamination, low pH, and limited nutrients, mining areas may harbor unique bacteria with antibiotic-production capabilities as well. Studying these bacteria could show new antibiotic compounds, as mining areas are underexplored for microbial diversity, increasing the likelihood of discovering new antibiotic-producing bacteria.

Pterocarpus angolensis, *Aloe littoralis,* and *Aloe zebrina* are indigenous plants readily available in Botswana for various uses. In north-western Botswana, *Aloe spp* roots are used to dye palm fibers, which are then woven into baskets (Fayisa Diriba & Mirete Deresa, 2022). The powdered stem and leaf bases are used medicinally by women after childbirth, and the bitter juice is used for wound disinfection, worm expulsion, and skin treatment (Salehi et al., 2018; Diriba and Deresa, 2022). The bark of *P. angolensis* tree also has various uses, including stimulating lactation and treating nettle rash, and piles. It can be taken orally for multiple ailments, and its roots can be boiled for gonorrhea, malaria, and blackwater fever (Chipinga et al., 2018; Chowdhury et al., 2022). Due to their medicinal uses, the three plants were selected as potential producers of bioactive compounds against resistant bacteria.

In this study, I make the following predictions:

1. Soil from a dried pond site and a mining area, as well as pond water samples contains novel bacteria with potential antibacterial-properties
2. Selected indigenous plants contain bioactive compounds with varying concentrations, which can be extracted using various solvents, and exhibit antibacterial properties

1.3 AIMS AND OBJECTIVES

1.3.1 Aim

To evaluate the antibacterial activity of bacterial isolates and indigenous plant extracts against multi-drug resistant (MDR) bacteria

1.3.2 Specific Objectives

i. Design/optimize culture methods for isolating potential antibiotic-producing bacteria from soil and water samples

ii. Isolate, screen, and identify bacteria with potential antibacterial properties from soil and water samples

iii. Sequence the multidrug-resistant bacteria for use as a model in the evaluation of the antibacterial activity of Indigenous plant extracts

iv. Prepare different crude extracts from selected Indigenous plants and screen for potential antibacterial properties

2.0 LITERATURE REVIEW

Selman Waksman introduced the term "antibiotic" in 1941 to describe any microbe-produced molecule that inhibits the growth of other microorganisms (Clardy et al., 2009). Antibiotics are produced as part of secondary metabolites which are biologically active molecules that are not essential for survival but give the producing organism (bacteria or plants) a competitive advantage (War et al., 2012). Since microorganisms share niches, they produce antibiotics as a competitive tactic against other bacteria, as a defense mechanism or as signaling molecules to the same species, or to coordinate interactions with eukaryotic hosts such as insects and plants (Klassen, 2014; Seipke et al., 2012; Traxler & Kolter, 2015). Plants, however, produce these compounds for defense against insects, microorganisms, and herbivores, response to environmental stresses, and mediating organismal interactions (War et al., 2012).

1.2. Sources of antibiotic-producing bacteria

Most antibiotics used to treat infectious diseases come from microbial natural products, produced by microorganisms that can be found in various environments (Sethi Sonia et al., 2013). Studies on soil bacteria and fungi (Table 1) have shown that microorganisms are a rich source of unique bioactive substances. Many antibiotics that are used as of present were isolated from soil microorganisms, hence soil is always one of the first reservoirs investigated in the search for bioactive compounds that can be used as antibiotics (Sonia et al., 2013). Table 1 outlines microorganisms categorized by type (bacteria or fungi) and the commercial antibiotics they produce.

Table 1: Examples of Commercial Antibiotics Produced by Various Microbes from Soil and Water

Source	Type	Microorganism	Antibiotics Produced	Reference
Soil	Bacteria	*Streptomyces griseus*	Streptomycin	(Das et al., 2021; Schatz et al., 1944)
		Streptomyces venezuelae	Chloramphenicol	(Gottlieb et al., 1954; Hutchings et al., 2019)
		Streptomyces erythraeus	Erythromycin	(Das et al., 2021; Hutchings et al., 2019; Staunton & Wilkinson, 1997)
		Streptomyces nodosus	Amphotericin B	(Das et al., 2021; Donovick et al., 1955)
		Streptomyces noursei	Nystatin	(Hazen & Brown, 1950; Mehta et al., 2021)
		Streptomyces rimosus	Tetracycline	(Das et al., 2021; Duggar, 1948; Hutchings et al., 2019)
		Streptomyces clavuligerus	Clavulanic acid	(Reading & Cole, 1977)
		Streptomyces fradiae	Neomycin	(Das et al., 2021; Waksman &

				Lechevalier, 1949)
		Streptomyces aureofaciens	Chlortetracycline	(Duggar, 1948)
		Streptomyces kanamyceticus	Kanamycin	(Das et al., 2021; Umezawa et al., 1957)
		Streptomyces orientalis	Vancomycin	(Das et al., 2021; McCormick et al., 1955)
		Micromonospora purpurea	Gentamicin	(Mehta et al., 2021; M. J. Weinstein et al., 1963)
		Bacillus subtilis	Bacitracin	(Das et al., 2021; Johnson et al., 1945)
		Pseudomonas aeruginosa	Pyocyanin	(Baron & Rowe, 1981)
	Fungi	*Penicillium chrysogenum*	Penicillin	(Das et al., 2021; Fleming A, 1929; Hutchings et al., 2019)
		Cephalosporium acremonium	Cephalosporins	(Abraham & Newton, 1954; Das et al., 2021;

				Hutchings et al., 2019)
		Aspergillus terreus	Lovastatin	(Alberts, 1988)
Water	Bacteria	*Streptomyces marinensis*	Neomycin	(Das et al., 2021; Waksman & Lechevalier, 1949b)
		Micromonospora marina	Marinomycins	(Kwon et al., 2006)
		Salinispora tropica	Salinosporamide A	(Feling et al., 2003)
		Marinobacter hydrocarbonoclasticus	Hydrocarbonoclastin	(Gauthier et al., 1992)
		Alteromonas macleodii	Alterochromides	(Offret et al., 2016)
		Vibrio vulnificus	Vulnibactin	(Okujo et al., 1994)
		Pseudoalteromonas sp.	Alterins, Pseudoalterons	(Isnansetyo & Kamei, 2003)
		Pseudomonas aeruginosa	Pyocyanin	(Baron & Rowe, 1981)

Microorganisms in Table 1 above are predominantly derived from soil, signifying the soil's abundance of antibiotic-producing organisms (Ananbeh et al., 2020). Microbiota in soil have challenges due to the considerable variance in biotic and abiotic factors present in this extremely heterogeneous environment (Ananbeh et al., 2020). As a result, soil bacteria overcome

challenges by mechanisms like the production of antimicrobials to survive, soil has thus been the main source of bacteria that produce antibiotics (Ananbeh et al., 2020). The genus *Bacillus, Pseudomonas*, myxobacteria, and cyanobacteria are known for producing antibiotics (Pipite et al., 2022). However, antibiotics are primarily manufactured by the Actinomycetota bacteria, sometimes referred to as antibiotic makers (Subramani & Sipkema, 2019). These microorganisms produce the majority of the natural chemicals utilized in human medicines (Thirumurugan et al., 2018). Streptomyces, the biggest genus in Actinomycetota, provides two-thirds of all antibiotics (Pipite et al., 2022). Antibiotics can also be generated from less well-studied or rare Actinomycetes, the number of antibiotics generated by these rare Actinomycetes has grown by 25-30% over the previous two decades, because of advances in selective isolation and genetic characterization approaches (Subramani & Sipkema, 2019).

Actinomycetes, are widespread in soil and water ecosystems and are essential for antibiotic synthesis (Shrestha et al., 2021). Antibiotics such as tetracycline, macrolide, chloramphenicol, nucleosides, and polyenes have been extracted from them (Shrestha et al., 2021). Soil actinomycetes produce several antibiotics, including erythromycin, gentamycin, rifamycin, and streptomycin (Lim et al., 2008). The availability of antibiotic-producing actinomycetes varies according to soil texture and cultivation state (Lim et al., 2008). Despite being widespread, very few actinomycetes have undergone screening for antibiotic production. Actinomycetes from marine or terrestrial environments employ enzymes known as polyketide synthases (PKS) or non-ribosomal peptide synthetases (NRPS) to synthesize metabolically active substances (Rani et al., 2021).

Similarly, bacteria in mine-polluted areas might produce antibiotics as a survival mechanism or as a response to the stress and competition they face in their environment (Peltier et al., 2010). The production of antibiotics can provide these bacteria with a competitive advantage against other microorganisms, hence mine-polluted areas can be crucial sources of biotechnologically important microorganisms, including antibiotic producers, with a high potential for identifying new antibiotic-producing strains (Barancheshme & Munir, 2018; Kappell et al., 2018). Numerous factors, such as geographic location, waste content, and nutrient availability, might have an impact on microbial structure (Sitotaw et al., 2022). Furthermore, the ability of bacteria to produce antibiotics may vary significantly between different sites and may change significantly over time, therefore, due to their public health importance, microbes should be investigated for their antimicrobial production potential across different locations and time periods (Sitotaw et al., 2022).

The aquatic environment is also an attractive untapped reservoir for useful novel natural products from bacteria (Tawiah et al., 2012). The aquatic environment contains diverse bacterial groups with unique traits that allow them to survive in conditions that are extreme and potentially produce novel metabolites not found in terrestrial microorganisms. This is due to environmental differences (Tawiah et al., 2012). Aquatic environments like wastewater treatment plants (WWTPs) are interesting to study since they harbor different types of microorganisms from different anthropogenic sources (Kubera, 2021). Having a diverse group of microorganisms in the same niche leads to competition and hence a need to eliminate competition (Seipke et al., 2012). Hence one of the reasons WWTPs are studied for possible antibiotic-producing bacteria.

Besides soil and water, plants serve as a reservoir for bacteria capable of producing antibiotics (ALKahtani et al., 2020). Given the abundance of bacteria within plant ecosystems, competition for nutrients is inevitable, leading to the synthesis of potent secondary metabolites aimed at managing or eliminating competing organisms (ALKahtani et al., 2020). Medicinal plants present a compelling option in this context, as they are used in treating certain infectious diseases and contain secondary metabolites with antibacterial properties, these plants may host microorganisms that serve a similar antibacterial function (ALKahtani et al., 2020).

2.2 Isolation of antibiotic-producing bacteria

New methods are now being introduced to study microorganisms that were previously uncultivated. This is achieved through the usage of nutrient media containing specific substances while cultivating the microorganisms under different physical and chemical conditions (Cheptsov et al., 2019; Davis et al., 2005; Pham & Kim, 2012).

The choice of media is an important factor in isolating bacteria as it determines the bacteria that will grow on the specific medium (Nemr et al., 2021). For example, nutrient agar favors fast-growing bacteria, as they grow quickly and overcrowd the Petri plate. Since the usual incubation period is normally 24 hours, this doesn't give slow-growing bacteria enough time to grow (Nemr et al., 2021). Laboratory synthetic media also contains more nutrients than usual environments, for example, soil, where bacteria populate, which is another reason why fast-growing microorganisms are usually the most favored (Nemr et al., 2021; Sarhan et al., 2019). Media with different concentrations can be used to solve this, from low to high, and different incubation times, to cater to all kinds of microorganisms (Davis et al., 2005).

Another concerning issue is the usage of the same media for bacteria from different environments, either soil, water, or plants, as this does not create the diversity needed from the various environments, hence a little creativity is needed to at least mimic the environments they were isolated from (Nemr et al., 2021). In the case of soil for example, instead of using the usual media used in laboratories, the soil extract can be used as part of the preparation media (Nemr et al., 2021; Sarhan et al., 2019). In a study by Hamaki et al. (2005), novel bacteria were discovered using a soil-extract agar medium. Three strains were identified as Shinshu-th1, -th2, and -th3 and sequence analysis revealed high homology with unculturable bacteria and *Bradyrhizobium* sp., suggesting they belong to a new genus (Hamaki et al., 2005). A new medium, intensive soil extract medium (ISEM), was also used in the isolation of new taxonomic isolates and unculturable bacteria, accounting for 55% and 49% of the strains that were examined (Nguyen et al., 2018). The new strains belonged to seven phyla and contained a wider variety of low-molecular-weight organic compounds compared to two traditional soil extracts prepared with distilled water (Nguyen et al., 2018).

In addition to the above, the incubation conditions for the bacteria should also be adjusted to mimic the abiotic factors or conditions of their natural environment, these could include temperature, pH, and humidity of the environment (Chaudhary & Kim, 2019; Davis et al., 2005). Chaudhary et al., (2019) stated that the isolation of uncultured bacteria and novel strains was influenced by the cultivation media, sampling season and incubation duration. Bacterial recovery was enhanced by summer soil, extended incubation, and low-substrate modified media, indicating that replicating ecosystems allowed for the successful cultivation of unculturable isolates (Chaudhary et al., 2019). Slow-growing soil bacteria are often missed in cultivation

studies due to insufficient incubation periods (Chaudhary et al., 2019). To isolate these uncultured bacteria, extending the incubation period can increase the recovery of the isolates. A study by Chaudhary et al., (2019) supports this approach and achieved increased yields of novel and previously unculturable strains at extended incubation periods, demonstrating the success of this method. Davis et al., 2005 demonstrated that bacterial recovery increases with extended incubation time. Similarly, Qiu et al., (2022) observed that higher temperatures significantly enhanced bacterial growth, although ventilation was found to reduce the extent of this increase. Furthermore, the growth of both Bacillus subtilis and Bacillus tequilensis, along with their biosurfactant production, was found to increase progressively with longer incubation periods (Marajan et al., 2018).

2.3 Indigenous medicinal plants in Botswana

Botswana has a rich biodiversity of plant species, some traditionally used for many years for medical purposes by local communities (Motlhanka & Makhabu, 2011). The benefits of medicinal plants include their antioxidant capacity, anti-inflammatory activity, antitumor, antiviral, antimalarial, analgesic properties and antimicrobial activity (Aye et al., 2019; Motlhanka & Makhabu, 2011). Indigenous medicinal plants in Botswana include *Harpagophytum procumbens, Cassine transvaalensis, Bauhinia petersiana, Ozoroa paniculosa, Capparis tomentosa, Peltophorum africanum, Myrothamnus flabellifolius, Hoodia gordonii, Dichrostachys cinerea, Securidoca longipenduculata, Colophospermum mopane, Ziziphus mucronata, Cassia abbriviata, Clerodendrum uncinatum, Commiphora glandulosa, Elephantorhiza goetzei, Pterocarpus angolensis*, *Aloe zebrina,* and *Aloe littoralis* (Moiketsi et al., 2023; Motlhanka & Makhabu, 2011).

P. angolensis (known as Mukwa in Setswana; Figure 1), commonly known as the Bloodwood tree, is a deciduous tree that belongs to the Fabaceae family and is primarily found in the southern and eastern regions of Africa (Chipinga et al., 2018; Chowdhury et al., 2022). The tree (Figure 1A), possessing straight stems, a high canopy and a slightly flat crown, can reach up to 28 m in height. It has drooping leaves which are alternately arranged (Figure 1B), and orange-yellow flowers. Its fruits are round brown pods with pear-shaped papery wings (Figure 1C). The bark (Figure 1D) is rough with deep cracks, brown, grey or black and produces a red colored sap rich in colorants and organic compounds such as tannins (Mojeremane & Lumbile, 2016). The roots (Figure 1E) have nitrogen-fixing nodules and are long tap roots with lateral roots that spread widely (Amri, 2012; Bünger et al., 2021). The tree is well known for its quality timber, dye for fabric, and medicinal importance in treating several ailments such as ringworms, ulcers, urinary schistosomiasis and antimicrobial properties (Chipinga et al., 2018; Chowdhury et al., 2022). Scientists have also reported that *P.angolensis* extracts have anti-inflammatory activity and high inhibitory activity against bacteria such as *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli* (Chowdhury et al., 2022; Lotshwao et al., 2022).

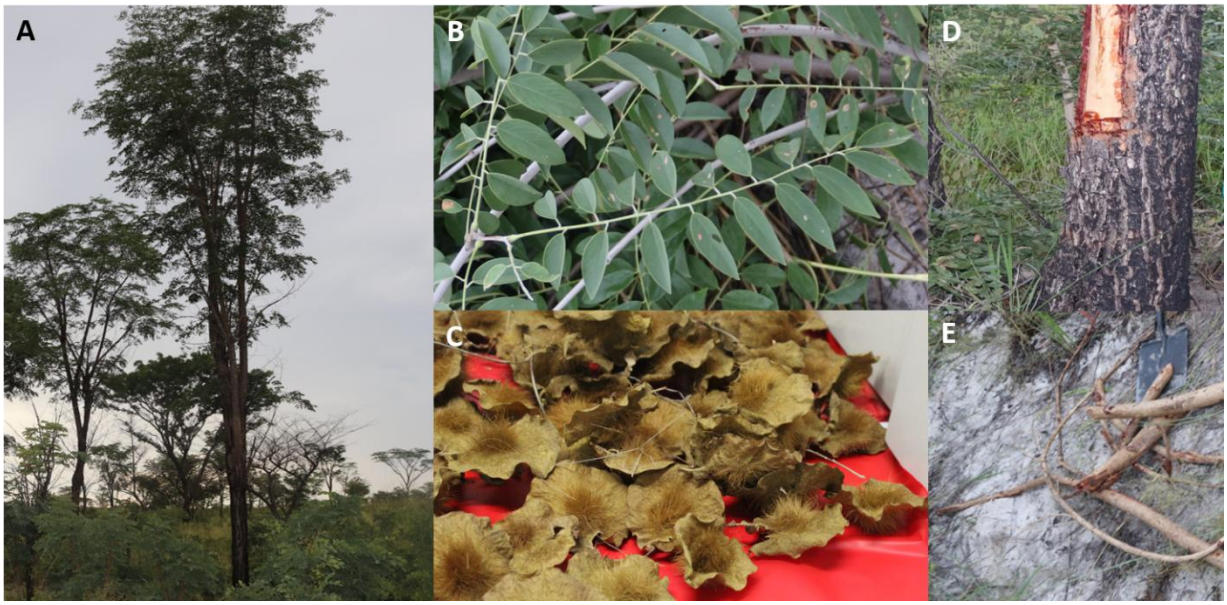


Figure 1*: P. angolensis* tree (A), leaves (B), fruits (C), bark (D) and roots (E).

Aloe plants have been extensively known and utilized for millennia, this is because of their numerous health, aesthetic, medical, and skin-care benefits (Diriba & Deresa, 2022). Species such as *Aloe zebrina* and *Aloe littoralis* (Figures 2 and 3, respectively) have also been used for a wide range of medicinal purposes by traditional healers from a variety of cultural groups in Africa including Botswana. These plants have also been reported to have various medicinal properties which include antibacterial, anticancer, antimicrobial, antidiabetic activities, antitumor, flushing out toxins and wastes from the body, anti-arthritic, anti-inflammatory, anti-rheumatoid, treating constipation, and promoting digestion (Salehi et al., 2018; Diriba and Deresa, 2022). For example, *A. littoralis* treats Bilharzia in Namibia's traditional health care system (Cheikhyoussef & Mapaure, 2011). Polysaccharides from *Aloe sp* have been reported to kill bacteria through phagocytic leucocyte stimulation (Salehi et al., 2018). This makes them good potential candidates for use against multidrug-resistant bacterial strains.

A. zebrina is an evergreen drought-tolerant plant with a compact circular arrangement of spiny succulent leaves. The leaves are typically green with elongated whitish spots and the leaf tips range from dark red to brown. Its inflorescence is made of scattered tubular, red flowers (Anywar et al., 2022; Huxley & Griffiths, 1999). The fruit is multi-seeded and dehisces. The seeds are dark-colored and have broad wings that assist in their dispersal. It typically grows to a height of 15-30 cm and is stemless although some may have a short stem (Anywar et al., 2022; Huxley & Griffiths, 1999).

A. littoralis, is a succulent herb or tree-like plant with elongated and pointed leaves (Hajhashemi et al., 2012; Walker, 2018). The leaves are greyish-green with numerous white spots and brown to reddish spines. It grows 2-4 meters tall and has a single stem, although they are sometimes stemless (Hajhashemi et al., 2012; Walker, 2018). The plant has a complex cluster of flowers that can branch into ten narrow and sparse flowered racemes. These flowers are tubular, up to 30 mm long, have a silvery sheen and vary in color from pink to red (Hajhashemi et al., 2012; Walker, 2018).

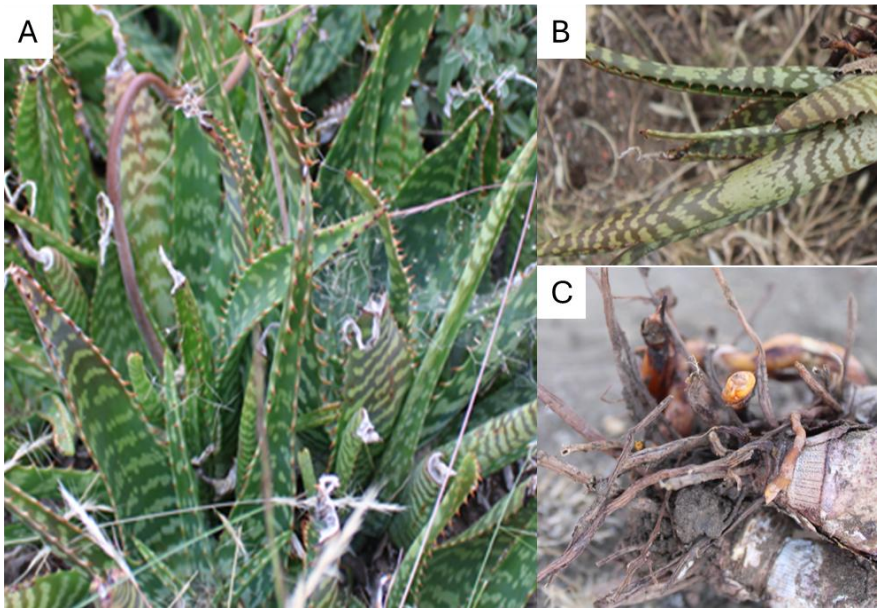


Figure 2: *A. zebrina* Whole plant (A), leaves (B) and roots (C)

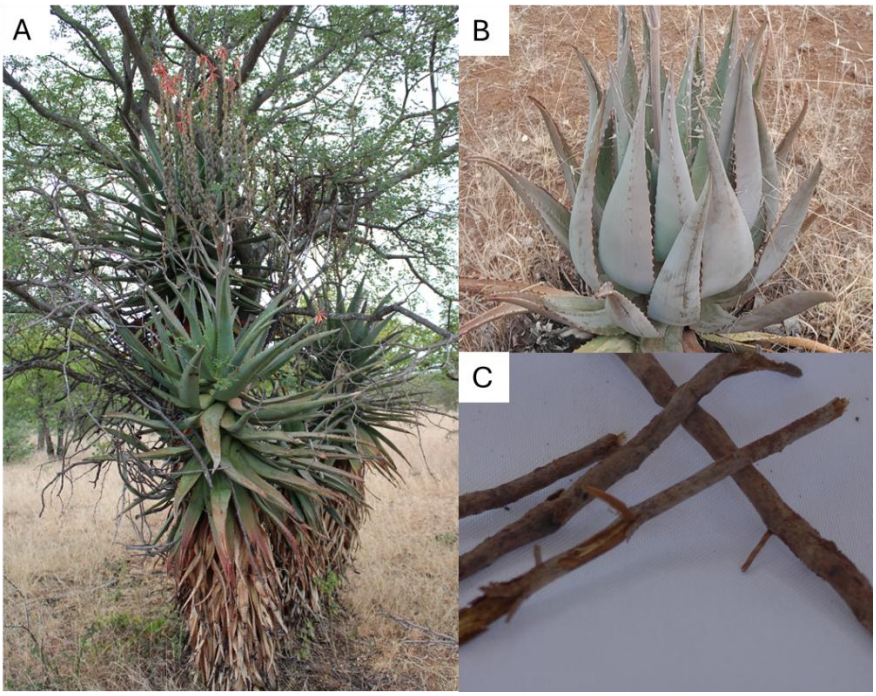


Figure 3: *A. littoralis* Whole plant (A), leaves (B) and roots (C)

2.4 Antibacterial plant secondary metabolites

More than 50,000 secondary metabolites have been discovered in the plant kingdom and are the basis for the therapeutic effects of medicinal plant extracts and modern medications (Teoh, 2016). These secondary metabolites include; lectins and polypeptides, terpenoids and essential oils, alkaloids, phenolic compounds, glycosides, organosulfur compounds and steroids (Keita et al., 2022; Seukep et al., 2023; Teoh, 2016; Thirumurugan et al., 2018). They do not constitute the organism's core molecular skeleton, and contrary to primary metabolites, their absence results in a greater degree of survival impairment rather than an immediate end to an organism's life (Thirumurugan et al., 2018). Nair et al., (2022) reported that of the approximately 1 million naturally occurring secondary metabolites, 25% are biologically active and plants contribute about 60% of these metabolites while microorganisms contribute the rest. People obtain a variety of secondary metabolites to benefit their health (antibiotics, antitumor agents, enzyme inhibitors, growth promoters and immunomodulators), expand nutrition (pigments and nutraceuticals) and increase agricultural productivity (pesticides, pheromones, effectors of ecological competition and symbiosis) (Thirumurugan et al., 2018).

Antibacterial activities of secondary metabolites and their proportions may vary from one species to another, which can affect their potency towards bacteria (A. J. Seukep et al., 2023). They may also have various mechanisms of action on different bacteria, including membrane damage, efflux pump inhibition, and protein inactivation (Keita et al., 2022; Seukep et al., 2023). Table 2 lists the different groups of secondary metabolites with antibacterial activities, names of the specific secondary metabolites, their source plants, examples of bacteria they inhibit and mechanisms of action.

Table 2: Antibacterial Plant Compounds and Their Mechanisms of Action Against Pathogens

Group	Secondary metabolite	Plant Source	Examples of Inhibited Bacteria	Mechanism of Action	Reference
Terpenoids	Thymol	*Thymus vulgaris, Thymus capitatus*	*P. aeruginosa, E. coli, Enterococcus faecalis*	Cell membrane disturbance	(Cowan, 1999; T. Liu et al., 2021)
	Carvacrol	*T. vulgaris, T. capitatus*	*P. aeruginosa, E. coli*	Disintegration of the outer membrane	(Ali-Shtayeh et al., 1997; Cowan, 1999)
Terpenes	Farnesol	*Vachellia farnesiana*	*E. coli, S. aureus*	Cell membrane disturbance	(Wang et al., 2011)
	Nerolidol	*Cannabis sativa*	*E. coli, S. aureus*	Cell membrane disturbance	(Chan et al., 2016; Krist et al., 2015)
Phenolics	Eugenol	*Syzygium aromaticum and Eugenia caryophillis*	*S. aureus, Helicobacter pylori*	Cell membrane disturbance	(Cowan, 1999; Mihaylova et al., 2025)
Alkaloids	Berberine	*Berberis vulgaris*	*E. coli*	Inhibition of the cell division protein FtsZ	(Cowan, 1999; N. C. C. Silva & Fernandes Júnior, 2010)

	Piperine	*Piper nigrum*	*S. aureus*	Efflux pump inhibitor	(Cowan, 1999; N. C. C. Silva & Fernandes Júnior, 2010)
	Conessine	*Holarrhena floribunda, Holarrhena antidysenterica, Funtumia elástica*	*P. aeruginosa*	Efflux pump inhibitor	(Chopra et al., 1927; Siriyong et al., 2017)
Flavonoids	Cinnamaldehyde	*Cinnamomum spp.*	*H. pylori*	Cell membrane disturbance	(Al-Bayati & Mohammed, 2009; Guo et al., 2024)
	Sakuranetin	*P. fruticosa*	*H. pylori*	Unknown	(Beutler et al., 1993; Junaid et al., 2023)
	Apigenin	*Matricaria chamomilla, Apium graveolens, P. fruticosa*	*H. pylori, E. coli, S. aureus*	Efflux pump inhibitors	(Panda, 2005; Siddiquee et al., 2025)
	Quercetin	*Capparis spinosa, P. fruticose*	*H. pylori, E. coli, S. aureus*	Unknown, Efflux pump inhibitor	(Cowan, 1999; Vohora et al., 1973)

	Sophoraflavanone B	*Desmodium caudatum*	*S. aureus*	Direct interaction with peptidoglycan	(Y. S. Lee et al., 2015; Mun et al., 2014)
	Genistein	*Glycine max*	*S. aureus*	Efflux pump inhibitor	(Abbas Al-Bayati, 2007; Lalouckova et al., 2021)
	Chrysoplenitin	*Artemisia absinthemum*	*S. aureus*	Efflux pump inhibitors	(Kaushik et al., 2024; Suberu, 2013)
	Kaempferol	*Moringa oleifera, Sambucus nigra, Aloe vera*	*S. aureus*	Unknown	(Kariyil & Sivapriya, 2022; Periferakis et al., 2022)
Organosulfur	Iberin	*Iberis spp.,*	*H. pylori*	Unknown	(Lawal et al., 2012)
	Erysolin	*Erysimum spp.*	*H. pylori*	Unknown	(Haristoy et al., 2005; Kjær, 1960)
	Hirsutin	*Rorippa sp., Nasturnium officinale*	*H. pylori*	Unknown	(Haristoy et al., 2005; Lawal et al., 2012)
	Allicin	*Allium sativum*	*Acinetobacter baumannii, P.*	DNA and protein	(Cowan, 1999;

			aeruginosa, E. coli, K. pneumoniae, E. faecalis, S. aureus	synthesis inhibitor	Vohora et al., 1973)
Coumarins	Aegelinol	*Ferulago campestris*	*S. aureus*	Unknown	(Basile et al., 2009; Dikpınar & Süzgeç-Selçuk, 2019)
	Agasyllin	*F. campestris*	*S. aureus*	Unknown	(Basile et al., 2009; Venugopala et al., 2013)
	Osthole	*Cnidium monnieri, Angelica archangelica and Angelica pubescens*	*K. pneumoniae, S. aureus*	DNA gyrase inhibitor	(Ansari et al., 2023; Venugopala et al., 2013)
Pyranocoumarin	Agasyllin	*F. campestris*	*Salmonella typhii*	DNA gyrase inhibitor	(Basile et al., 2009; Venugopala et al., 2013)
	Aegelinol	*Aegle marmelos, Ferulago asparagifolia Boiss*	*S. typhii*	Unknown	(Basile et al., 2009; Dikpınar & Süzgeç-Selçuk, 2019)

Diarylheptanoid	Curcumin	*Curcuma longa*	*E. coli*	Damaging of bacterial membrane	(Lee, 2006; Yun & Lee, 2016)
Naphthoquinones	Juglone derivatives	*Reynoutria japonica*	*H. pylori*	Unknown	(Khalil et al., 2019; Kong et al., 2008)
Polyphenol	Resveratrol	*Vitis vinifera*	*Campylobacter spp.*	Unknown	(Ma et al., 2018; Silvan & Martinez-Rodriguez, 2018)
Naphthoquinone	Plumbagin	*Plumbago zeylanica*	*S. aureus*	Unknown	(Jeyachandran et al., 2009; Periasamy et al., 2019)

2.5. Potential of medicinal plants to combat AMR

Medicinal plants have been studied for their potential to combat AMR. In studies by Tayel et al. (2018) and Chabán et al. (2019), plant extracts from *Lycium shawii, Lepechinia meyenii* and *Phyllanthus emblica* were highly effective against a methicillin-resistant *S. aureus* (MRSA) isolate. *Ehretia microphylla, Psidium guajava, Piper betle,* and *Phyllanthus niruri* leaf extracts also demonstrated antibacterial activity against vancomycin-resistant *Enterococcus* and MRSA (Valle et al., 2015). Their phytochemical analysis showed they were rich in flavonoids, alkaloids, tannins, and phenolic compounds (Tayel et al., 2018). Many plant extracts have also been shown to inhibit

various MDR bacterial strains. For instance, *P. angolensis* and *A. littoralis* extracts showed antibacterial activity against multidrug (amoxicillin, ampicillin, penicillin, gentamycin, tetracycline and trimethoprim) resistant *Proteus mirabilis*-MN029 (Makale et al., 2024). *K. pneumoniae*, resistant against tetracycline, amplicillin, aztreonam and cephalothin, was inhibited by extracts from *Entada abyssinica* (Tchana et al., 2014). *Oxalis corniculate* inhibited MDR *S. typhi* (Manandhar *et al.,* 2019). MDR *E. coli*, *K. pneumoniae* and *Klebsiella oxytoca* were inhibited *by Zanthoxylum alatum, Cinnamomum tamala* and *Ocimum sanctum* (Thapa *et al., 2019)*. These findings validate the use of plant extracts against ARB and their potential to be used as antibacterial drugs against MDR bacterial strains.

In addition to plant extracts alone, the association of antibiotics and plant extracts has been shown to have synergistic antibacterial activities against ARB. In Egypt, combinations of *Eucalyptus camaldulensis* leaves and antibiotics were evaluated for their effectiveness against multi-resistant strains of *E. coli, S. aureus*, and *P. aeruginosa* (Reda et al., 2017). The leaf extract showed significant antibacterial activity on their own, however, when combined with the antibiotics ceftriaxone and gentamycin, antibacterial activities increased significantly (Reda et al., 2017). Additionally, Triphala, a common indigenous medical product made up of fruits from *Emblica officinalis*, *Terminalia belerica* and *Terminalia chebula*, was investigated for its synergistic effects with gentamicin and oxacillin against MDR bacterial isolates (Manoraj et al., 2019). The study found that Triphala exhibited synergistic activities with gentamicin against selected MDR Gram-negative bacilli (*P. aeruginosa*, *K. pneumoniae*, *Enterobacter cloacae, Proteus spp and Serratia marcescens*) and with oxacillin against MRSA isolates (Manoraj et al., 2019).

Furthermore, *Pithecellobium clypearia* branch and leaf extracts, in combination with antibiotics (levofloxacin, tetracycline, polymyxin, cefoperazone, and ceftazidime) were found to be more effective against MDR *P. aeruginosa* and *Acitobacter baumannii*, targeting the cell walls and membranes of these bacteria (Liu et al., 2019). Other plant extracts that have shown synergistic effects with antibiotics against MDR bacteria include clove, thyme, pomegranate, and jambolana (Nascimento et al., 2000). Purified plant metabolites such as gallic acid have also shown synergistic effects with antibiotics against MDR bacteria (Jayaraman et al., 2010). These studies indicate the potential of using medicinal plant extracts and metabolites in combination with known antibiotics to combat AMR.

2.6 Mechanisms of action of antibacterial agents from bacteria and plants

Antibacterial agents can affect bacterial cells in various ways. Their main mechanisms of action include:

I. Cell-wall biosynthesis

Bi-functional enzymes with both transpeptidase and transglycosylase domains are effective targets for bactericidal antibiotics like penicillins and cephalosporins, (T. Schneider & Sahl, 2010). Glycopeptide antibiotics, like vancomycin, are known to target the peptidoglycan layer during cell wall assembly. They do this by binding to the peptide substrates of the peptidoglycan layer, which prevents reactions with enzymes that are necessary for cell wall construction, (T. Schneider & Sahl, 2010). Phenolic compounds and quinones, potential aromatic compounds, target microbial cell surface adhesins and membrane-bound polypeptides, potentially disrupting cell wall development and potentially leading to cell lysis (Ganesan & Xu, 2018).

II. Inhibiting nucleic acid synthesis

The antibiotic binds to the β-subunit of RNA polymerase within the DNA/RNA channel, preventing RNA synthesis by obstructing the elongating RNA's path when the transcript reaches a length of 2 to 3 nucleotides, (Jubair et al., 2021). Gyrase is a key target for antibiotics and antibacterial agents including fluoroquinolones such as ciprofloxacin and nalidixic acid (Lomovskaya et al., 2001b). Flavonoids like baicalein, morin, quercetin, and silymarin can hinder ATP synthesis, disrupting DNA replication (Chinnam et al., 2010; Gledhill et al., 2007). Apigenin, genistein, and myricetin are DNA gyrase inhibitors that block the ATP binding pocket (Ulanowska et al., 2006). These flavonoids can also interfere with the β subunit of DNA gyrase, thereby inhibiting both DNA replication and transcription by preventing the enzyme's role in DNA supercoiling (Gorlenko et al., 2020; Mickymaray, 2019). They can also inhibit the DNA separating and rearranging capacity of *E. coli* helicases (Xu et al., 2001).

III. Destruction of bacterial membrane

Different antibiotics, such as polymyxins, bind to the lipid A component of lipopolysaccharide causing structural alterations through phospholipid exchange that results in osmotic imbalance and rapid bacterial death (Tenover, 2006). Destruction of bacterial membranes can cause the coagulation of cytoplasm, leakage of intracellular constituents, and the loss of permeability (McBain et al., 2003). Flavonoids, such as sophoraflavanone, quercetin, tiliroside, naringenin, chalcone, and rutin, can form complexes with bacterial cell walls, causing decreased lipid bilayer thickness, fluidity levels, increased membrane permeability, and potential protein and ion

leakage in *S. aureus* and *S.* mutans (Ganesan & Xu, 2017; Sanver et al., 2016; Tsuchiya & Iinuma, 2000).

IV. Bacterial protein biosynthesis

Most antibiotics that block bacterial protein synthesis interfere with the processes at the 30S subunit or 50S subunit of the 70S bacterial ribosome, leading to misreading and premature termination of the mRNA translation (Walsh, 2003). Important classes of antibiotics such as macrolides, tetracyclines, aminoglycosides, and oxazolidinones show antibacterial activities through this mechanism (Walsh, 2003). Plant secondary metabolites like flavonoids and alkaloids can disrupt bacterial protein biosynthesis by binding to the 30S ribosomal subunit (Anjali et al., 2023; Ansar et al., 2016; Thirumurugan et al., 2018). They also affect translation initiation and disrupt elongation and RNA synthesis by interacting with ribosomes and DNA (Anjali et al., 2023; Ansar et al., 2016; Thirumurugan et al., 2018).

2.7. Methodologies for evaluating antibacterial activities

In the search for antimicrobial agents from both bacteria and plants, different methods are employed to get the best results, depending on the budget, the environment, and the knowledge of the researcher. Traditional methods for identifying bacteria and testing drug susceptibility usually rely on culture-based techniques that can take anywhere from two to seven days for results (Kelley, 2017).

1. Agar Diffusion Based

Agar diffusion-based assays, such as disk diffusion and well diffusion, are commonly employed in antimicrobial research to evaluate the antibacterial activity of test substances (Balouiri et al., 2016; Ezebialu et al., 2020; Hossain, 2024; Tawiah et al., 2012). In these techniques, antimicrobial chemicals are diffused into the agar medium through paper discs, wells, or plugs, inhibiting the growth of the test microorganism (Balouiri et al., 2016; Hossain, 2024). The resulting zone of inhibition can be measured, providing qualitative information on the effectiveness of specific substances against particular microbes (Matuschek et al., 2014; Balouiri et al., 2016; Atef et al., 2019). The agar diffusion assays are cost-effective, simple techniques that allow researchers to evaluate multiple test substances against a single microorganism, providing qualitative information and indicating the potency of the antimicrobial agent (Hossain, 2024;Weinstein & Lewis, 2020). They are versatile, allowing for the testing of antibiotics, plant extracts, and synthetic compounds against diverse microorganisms (Hossain, 2024). Results are typically available within 18-48 hours, and the assay has been validated by regulatory authorities like the Clinical and Laboratory Standards Institute (CLSI) (Weinstein & Lewis, 2020). As useful as these assays are for preliminary antimicrobial screening, they have several limitations. For instance, they are qualitative and only indicate the presence or absence of antimicrobial activity, without providing precise quantitative measurements (Hossain, 2024).

2. Cross/Perpendicular Streak

The cross-streak method, a variation of agar diffusion assays, involves streaking the test and indicator microbes across each other on agar (Ethica et al., 2019; Hossain, 2024). Antimicrobial production by the test microbe results in growth inhibition at the intersection points (Bizuye et

al., 2013; Gebreyohannes et al., 2013; Ghanbour, 2018; Singh et al., 2016). This method is used to assess antimicrobial activity and study microbial antagonism (Ethica et al., 2019). The cross-streak technique is a simple, fast, cost-effective primary antimicrobial activity screening tool. It requires minimal specialized equipment and resources, allows for direct visual observation of inhibition zones, and enables the evaluation of multiple microorganisms on a single agar plate(Balouiri et al., 2016). However, like other agar diffusion assays, it is limited by variations in diffusion properties, agent concentration, and target microorganism susceptibility, providing primarily qualitative data (Balouiri et al., 2016; Hossain, 2024). The subjective interpretation of inhibition zones can also introduce bias or variability.

3. Agar dilution and broth dilution

The agar dilution and broth dilution procedures are both used in determining the minimum inhibitory concentration (MIC) of antimicrobial drugs (Balouiri et al., 2016; Gonzalez-Pastor et al., 2023; Hossain, 2024; Valgas et al., 2007). The distinction is the medium, agar plates for agar dilution and liquid broth tubes for broth dilution (Balouiri et al., 2016). Both procedures require introducing a specified amount of microbial cells to the medium while varying the quantity of the antibacterial agent. The MIC is determined by monitoring the presence of microbial growth (Balouiri et al., 2016). These approaches are critical for determining the most appropriate antimicrobial therapy and monitoring changes in antimicrobial susceptibility. The agar dilution method visually detects bacterial growth on agar plates, simplifying the determination process compared to broth dilution. It allows simultaneous testing of multiple bacteria, while broth dilution tests multiple agents against a single microbe. Both methods provide quantitative data

through MIC determination, improving MIC endpoint identification and antibiotic concentration range (Ethica et al., 2019; Golus et al., 2016; Gonzalez-Pastor et al., 2023; Hossain, 2024; Khezripour et al., 2019). The broth microdilution method offers more flexibility, speed, cost minimization, and convenience. However, the tests are time-consuming and tedious due to the need to prepare a series of agar plates or tubes with different concentrations of antimicrobial agents (Golus et al., 2016; Khezripour et al., 2019). These tests may not be suitable for fastidious or slow-growing bacterial species or all sample types. MIC values obtained from these tests do not indicate the agent's mode of action or efficacy in vivo (Khezripour et al., 2019).

4. Bioautography

The bioautography technique uses a Thin-Layer Chromatography (TLC) plate to identify bioactive compounds in complex mixtures and allows for rapid and efficient identification of bioactive compounds (Gonzalez-Pastor et al., 2023; Hossain, 2024). This method is widely used to evaluate the antimicrobial activity of natural products, including essential oils, plant extracts, and bacterial secondary metabolites (Gonzalez-Pastor et al., 2023; Hossain, 2024). The three types used are direct bioautography, agar diffusion/contact bioautography, and immersion/overlay bioautography (Gonzalez-Pastor et al., 2023; Hossain, 2024). Direct bioautography involves spraying the TLC plate with microbial broth, agar diffusion/contact bioautography entails placing the plate on a petri dish with a pre-seeded layer of agar and immersion/overlay bioautography involves immersing the TLC plate with a seeded molten agar medium (Gonzalez-Pastor et al., 2023; Hossain, 2024). The presence of antimicrobial compounds inhibits the growth of the indicator organism, forming clear zones (bioautograms) on the agar surface.

TLC-bioautography is an effective method, known for its ability to pinpoint activity within complex mixtures (Suleiman et al., 2009). It enables the simultaneous detection and isolation of bioactive substances by incorporating a biological component into the analysis, allowing for direct visualization of these compounds (Nuthan et al., 2020). This assay is sensitive and can detect antimicrobial compounds at low concentrations. It provides a quick screening process and can be applied to a wide range of samples, including plant extracts, natural products, and synthetic compounds, making it a versatile tool for assessing antimicrobial activity (Horváth et al., 2011). However, bioautography is limited because it only provides qualitative data, making it hard to compare the activity levels of different compounds or determine their optimal concentration for activity (Patil et al., 2017). The sensitivity and reproducibility of the results depend on the microorganisms' growth conditions used in the assay (Patil et al., 2017; Wilkinson, 2006). Some antimicrobial compounds might not be detected if they are inactive under the assay conditions. Furthermore, bioautography relies on the extraction method and the choice of TLC solvent, which can limit its effectiveness (Hossain, 2024; Patil et al., 2017; Wilkinson, 2006).

Despite the variety of methods available, the aim of *in vitro* antimicrobial screening is to accurately predict how a microorganism will respond to an antimicrobial extract (Balouiri et al., 2016; Gonzalez-Pastor et al., 2023; Hossain, 2024). Hence, there is no universally best method for antimicrobial screening (Hossain, 2024). The selection of a method depends on the researcher's goals, the microorganism being studied, the experimental conditions, and the resources available (Balouiri et al., 2016; Gonzalez-Pastor et al., 2023; Hossain, 2024). Each technique has its unique advantages and limitations so the best choice will vary according to the specific needs of the study (Hossain, 2024).

Chapter Three

3.0 MATERIALS AND METHODS

3.1 Sampling

Soil and Water

Samples of soil and water were collected for the isolation of potential antibiotic-producing bacteria at Palapye and Selebi Phikwe, in Botswana. In Palapye, three soil samples were collected from a dried pond located in the BIUST campus [S 22°37’26.9"E027°07’36.0]. In Selebi Phikwe, seven soil samples were collected from the copper mine smelter area [2156'38.2''S 02751'19.6E]. Additionally, a sample of water was collected from a mine effluent stream [2156'03.0''S 02750'33.7E] situated in Selebi Phikwe.

Soil sampling procedure involved the removal of surface plant debris and a soil auger was utilized to collect soil samples from a depth of approximately 30 cm. Water from the effluent stream was aseptically collected using sterile 50 ml falcon tubes, with care taken to allow the water to settle beforehand to minimize disturbance and sedimentation, after collection, the tubes were then tightly sealed. All samples were collected into sterile containers, and transported to the laboratory at 4°C, for further processing.

Plants

Pterocarpus angolensis, *Aloe littoralis* and *Aloe zebrina* were selected based on the literature review and were collected from Mabolwe and Tsodilo areas in Botswana as described by Makale et al. (2024). *P. angolensis* was collected at 18°48'34.5"S 21°43'19.4.E in Tsodilo, while *A. littoralis* and *A. zebrina* were collected in Mabolwe at coordinates, 21°51'19.2"S28°44'10.6.E and 21°50'37.1"S 28°43'59.6.E respectively. For *P angolensis,* leaves, barks, seeds, and roots were collected while for *A. zebrina* and *A. littoralis* only leaves and roots were collected. The plant parts were taken to the laboratory where they were sun-dried for 72 hours before use.

3.2 Media preparation for isolation of antibiotic-producing bacteria

Six different types of media (MacCkonkey agar (MAC), Reasoner's 2A agar (R2A), Nutrient agar (NA), MAC + soil extract, R2A + soil extract, and NA + soil extract) were prepared for primary isolation of potential antibiotic-producing bacteria. MacConkey (Merck, Germany), Nutrient (Sigma-Aldrich, USA), and Reasoner's 2A (Conda, Spain) agar were prepared according to the manufacturer's instructions. To enhance diverse bacterial isolation, soil extracts (SE) were prepared and used as supplements in MAC, NA, and R2A media preparation. The soil extract was prepared following a protocol by (Chaudhary et al., 2019) with a few modifications. Briefly, 250 g of dried soil was mixed with one liter of distilled water and the mixture was shaken, left overnight to settle, and filtered. The filtrate was used to prepare the three different media. Cycloheximide (75 µL/mL) was added to all media preparations to prevent fungal growth.

The bacterial isolations were done using the 6 media described above, using the spread plating method. Briefly, the samples were homogenized and serially diluted to obtain 10-fold dilutions.

An inoculum of 100 µL of 10^{-1} – 10^{-3} dilutions was spread plated onto the media and the plates were incubated at three different temperatures 25°C, 30°C and 37°C for 96 hours (4 days). The cfu/mL was calculated for both 24 and 96 hours previously described by Brooks et al., (2023) to evaluate relative abundance in different media preparations. A total of 864 pure culture isolates were stored at -80°C in 20% glycerol from soil and water samples.

3.3 Description of the test bacteria and antibiotic susceptibility testing (AST)

The test bacteria used were the ATCC strains: *Staphylococcus aureus* 25923*, Escherichia coli* 33559 *,* clinical isolates; *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* obtained from the University of Botswana and an environmental isolate*:Proteus mirabilis-MN029* which was isolated from a polluted soil sample collected from a rural area of Palapye, Botswana*.* The agar dilution method was used to test for antibiotic susceptibility following a protocol outlined by Brooks et al., (2023). Briefly, MH agar supplemented with various antibiotics at different clinical breakpoint concentrations; gentamicin (16 µg/mL), ampicillin (32 µg/mL), rifampicin (30.7 µg/mL), tetracycline (16 µg/mL), erythromycin (8 µg/mL), amikacin (64 µg/mL), amoxicillin (32 µg/mL), and penicillin (16 µg/mL) were used. The plates were incubated at 37°C for 24 hours. The disc diffusion method was employed for AST following the European Committee for Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) (Igere et al., 2020; Kahlmeter et al., 2006; Weinstein & Lewis, 2020), to further verify the pattern of resistance of the isolates. The antibiotic disk panel comprised of Tetracycline (10 µg), Amikacin (30 µg), Penicillin G (10 µg), Rifampicin (30 µg), Ampicillin (10 µg), Trimethoprim (5 µg), Kanamycin (30 µg), and Amoxicillin (25 µg) and the plates were incubated at 37°C for 24 hours.

Table 3: Phenotypic characteristics of test bacteria

Antibiotic	*P. mirabilis-MN029*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*	*E. coli*
Amoxicillin	R	R	R	S	S
Amikacin	S	S	S	S	S
Ampicillin	R	R	R	S	S
Erythromycin	R	S	R	S	S
Penicilin	R	R	R	S	S
Gentamicin	R	R	R	S	S
Tetracycline	R	R	R	S	S
Rifampicin	S	S	R	S	S
Kanamycin	S	S	S	S	S
Trimethoprim	R	R	R	S	S
Gram reaction	-	-	-	+	-

Key: -; negative, + positive

3.4 Genomic sequencing of *P. mirabilis*-MN029

Proteus mirabilis-MN029 was selected as a model bacterium to evaluate the antibacterial activity of medicinal plant species due to its origin, clinical significance, and distinctive phenotypic and genomic traits. Long read HiFi PacBio technology was used to sequence *P. mirabilis*-MN029 at Inqaba Biotec, South Africa (Makale et al., 2024). Briefly, whole-genome sequencing (WGS) was performed using the PacBio SMRTbell® prep kit 3.0, adhering to the manufacturer guidelines. Genomic DNA was sheared using the Megaruptor 3 system to obtain a target size range of 15–18 kb for the long-read sequencing. SMRTbell libraries were prepared using PacBio's Microbial Multiplexing workflow, quantified with the Qubit HS dsDNA Assay, and evaluated for quality

using Genomics Screen Tape on a TapeStation. Sequencing libraries were constructed following the online SMRTlink-guided protocol. De novo assembly of PacBio HiFi long reads was conducted using PacBio SMRT Analysis software (SMRTLINK v.11.1). For bioinformatics analysis, two online platforms were employed: the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) for the genome analysis and ResFinder 4.1 for predicting phenotypic traits from genetic data.

3.5 Screening potential antibiotic-producing bacteria for antibacterial properties

3.5.1 Perpendicular Streak

Nutrient agar plates were used for primary screening of bacteria with potential antibacterial properties following protocol by Girma & Aemiro, (2021 and Sitotaw et al., (2022). Briefly, a line of the bacterial isolate (bioactive compound producing) was streaked twice in one plate to make duplicates of pure individual isolates (Figure 2), and this was incubated for 96 hours at 37°C, after incubation the test bacteria were streaked perpendicular or at a 90◦ angle to the isolates and incubated for 24 hours at 37°C. After the incubation period, the zones of inhibition were measured and recorded (Figure 4).

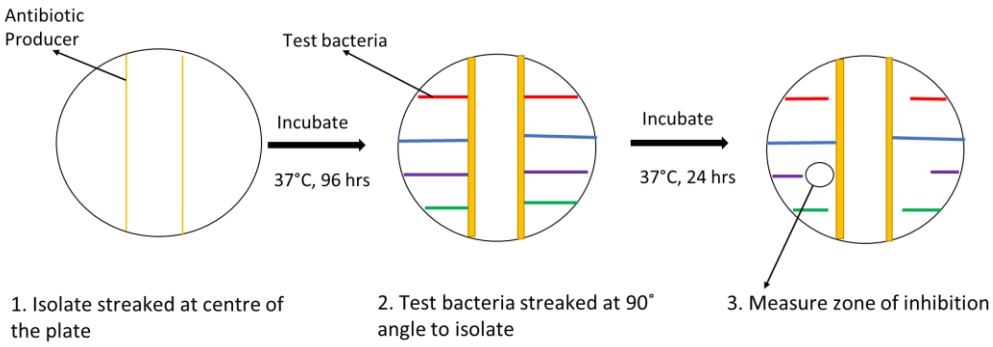


Figure 4: Illustration of Perpendicular Streak

3.5.2 Biochemical Tests

Gram staining and biochemical analyses were conducted on selected antibiotic-resistant bacteria and potential antibiotic-producing bacteria. Biochemical tests were done using API 20 A strips following the protocol by (Nwokoye et al., 2012). Briefly, inoculums of the bacteria were prepared to obtain a final turbidity equal to 3 McFarland, which was used immediately after preparation. The second step involved preparing a humid atmosphere within an incubation box using distilled water. Next, a bacterial suspension was inoculated onto an API 20 A strip, which was then placed within the incubation tray. After 24 hours at 37 °C of anaerobic incubation, the strip was carefully read and interpreted. Ultimately, the bacterial strain was identified by comparing the numerical profile of positive reactions to a comprehensive database.

3.5.3 Whole genome sequencing of antibiotic-producing bacteria

3.5.3.1. Genomic DNA extraction,

Four potential antibiotic-producing bacteria were subjected to genomic DNA extraction and sequencing. The genomic DNA was extracted using a Zymo research Quick-DNA Bacteria Miniprep kit according to the manufacturer's instructions. The genomic DNA was checked for purity using a nanodrop (Thermofisher Scientific, USA) at 260 and 280 nm wavelengths.

3.5.3.2. Next-generation sequencing and bioinformatics analysis

The antibiotic-producing bacteria were sequenced using the short-read Illumina technology in the Department of Biological Sciences and Biotechnology, BIUST. WGS was conducted following the laboratory standard operating procedure on Illumina iSeq100 (https://www.aphl.org/programs/food_safety/Documents/PNL40_WGS_on_iSeq.pdf). The

sequencing libraries were prepared using the Illumina DNA prep kit following the Illumina protocol, and quantified using the Qubit HS dsDNA Assay. BV-BRC platform was then used for genome assembly and identification of the bacterial isolates. The genomic sequences were then run on the antiSmash software(https://antismash.secondarymetabolites.org) for the identification of biosynthetic gene clusters (BGCs).

3.6 Screening plants for antibacterial properties

The selected medicinal plants used for screening were *P. angolensis , A. zebrina and A. littoralis.* Their crude extracts were tested against the test bacteria; *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*, *Klebsiella pneumoniae and Proteus mirabilis*-MN029*.*

3.6.1 Preparation of plant crude extracts

The preparation of crude extracts was done through maceration using a procedure outlined by (Mohamed, Muddathir and Osman 2020), with modifications. In brief, the different plant parts were sun-dried for 72 hours and crushed/ground into powder. Thirty grams of the powdered material was used for maceration per 100 ml of solvent (ethyl acetate, distilled water, *n*-hexane and methanol) at room temperature for 72 hours with constant shaking at 160 rpm. At the end of extraction, the micelle was separated from the marc by filtration using filter paper, and the micelle was separated from the menstruum by evaporation.

3.6.2 Kirby-Bauer Disc Diffusion Susceptibility Test

The disk diffusion method followed the guidelines established by the National Committee for Clinical Laboratory Standards (Mohamed et al., (2020; Haile & Jiru, 2022), with a few

moderations. Oxytetracycline (30 µg) and Kanamycin (30 µg) were used as positive controls, while negative controls were methanol, DMSO, distilled water, ethyl acetate and *n*-hexane alone. For the screening assay, crude extracts were prepared at varying concentrations; 40 mg/ml, 20 mg/ml, 10 mg/ml, and 5 mg/ml. The disk diffusion test was performed in triplicates and the diameter was measured for the plates that showed inhibition zones.

3.6.3 Micro-dilution assay

The determination of minimum inhibitory concentration (MIC) was carried out according to the protocol described by (Haile & Jiru, 2022), with some modifications. The modifications included changes in the concentration of the extract (ranging from 5 mg/mL to 0.039 mg/mL) and incubation time (24 hours). To serve as controls, DMSO (negative), Amikacin (positive), and MH broth (medium) were used. After incubation, a solution of Iodonitrotetrazolium (INT) (0.2 mg/ml) was added to all wells, followed by an additional 3-hour incubation. A pink color indicated bacterial growth, while a colorless appearance indicated the absence of growth.

3.7. Statistical Analysis

The perpendicular streak was conducted four times using the perpendicular streak method. The average value and the standard error (mean±SEM) were calculated from the results. To determine statistical significance at a 95% confidence interval (CI), a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed using GraphPad Prism 8.0.1 (244). A P-value of less than 0.05 was considered significant.

3.8. Data availability

The genome sequences generated during the study were deposited in the NCBI GenBank repository and are publicly available. The sequence data of MDR bacteria is available at http://www.ncbi.nlm.nih.gov/bioproject/860342, with a nucleotide sequence accession; NZ_CP102086.1. The sequence data of antibiotic-producing bacteria is available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1155603, *P. elgii*-KM_B45, *B. laterosporus*-KM_C76, *P. oleatilyticus*-KM_D13 and *P. oleatilyticus*-KM_D46. Their WGS accession numbers were JBHJMN01, JBHDLM01, JBHDLN01 and JBHDLO01 respectively.

Chapter Four

4.0 RESULTS AND DISCUSSION

4.1 Abundance of potential antibiotic-producing bacteria in prepared media and incubation conditions

The heatmaps reveal high colony-forming units (CFUs) recorded at temperatures between 30-37 °C, compared to 25 °C which showed lower CFUs. A Longer incubation time of 96 hours compared to 24 hours also revealed high abundance (Figure 5). The abundance of the bacteria was higher at 37 °C than at 30 °C and 25 °C and for the incubation time, abundance was higher after 4 days (96 hours) compared to one day (24hrs). Reasoner's 2A agar (R2A) media showed more CFUs, followed by nutrient agar (NA) and the least was MacConkey (MAC) agar. The addition of SE to the media increased the CFUs in some samples and media, notably in soil sample 5 in NA, while in soil sample 1, NA without SE resulted in higher CFU counts than NA with SE. The addition of SE increased the CFUs in R2A for the majority of the samples, it decreased the CFUs in NA and and had no effect on MAC CFUs for most samples. The extract hindered growth in samples 1, 6, and 8, did not affect samples 2, 7, and 9, and enhanced growth in samples 3, 4, and 5.

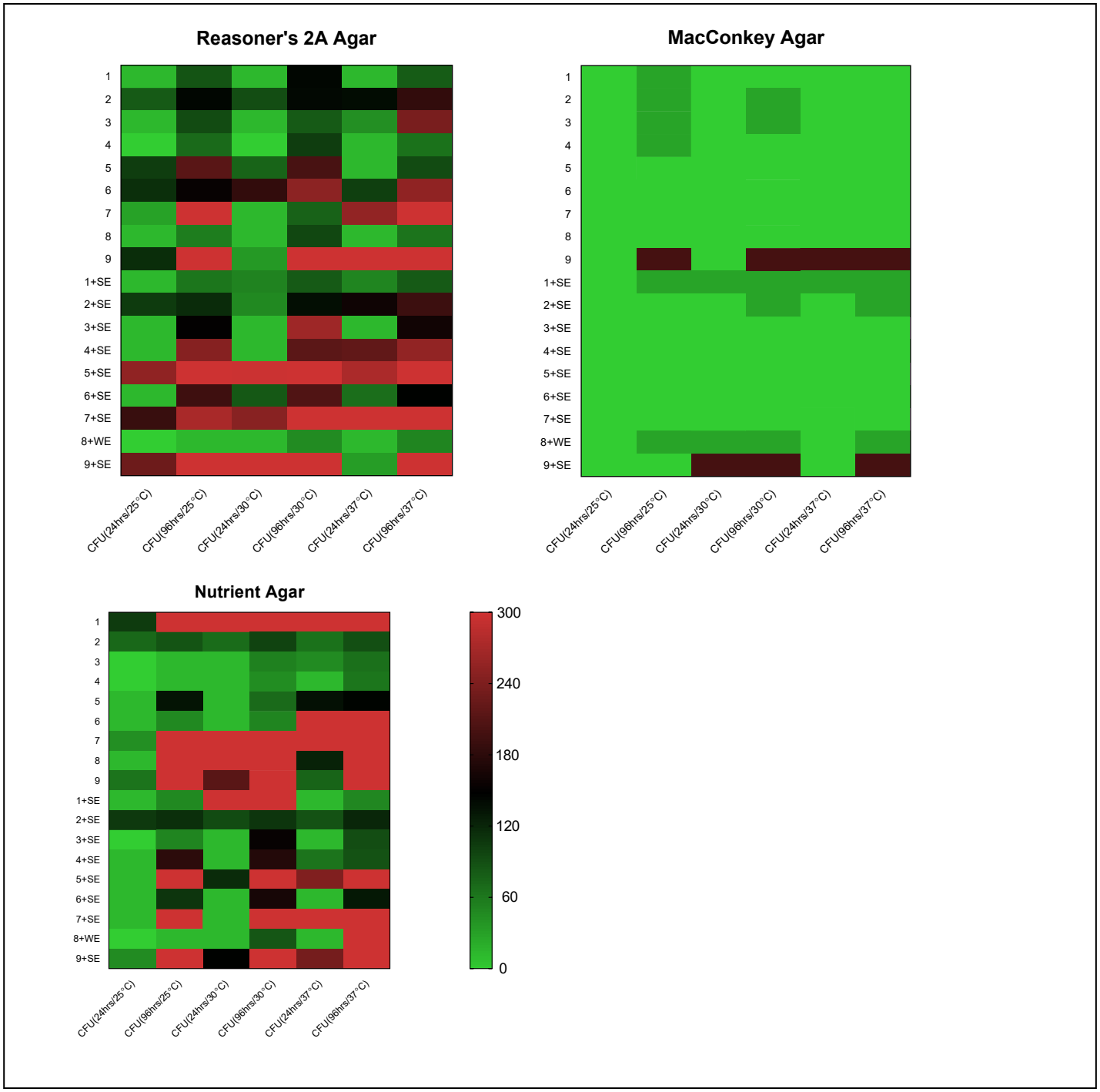


Figure 5: Heatmaps showing the abundance of bacteria at different incubation times (24hrs, 96hrs), temperatures (25, 30, 37 °C), and media (NA, R2A, MAC) for 9 samples without and with soil extract (SE)

Enabling the cultivation of most unculturable microorganisms requires implementing innovative culture systems. This involves the development of new approaches for media, with optimized culture conditions, and the identification of microbial colonies. There is evidence of bacterial abundance in the samples analyzed resulting from alteration of incubation periods. In this study, most bacteria appear to be most abundant at temperatures of 30 and 37 °C over 25 °C. According to Chikere & Udochukwu, (2014), longer incubation time increased colony count on different growth media, which correlates with the results in Figure 5. Chikere & Udochukwu, (2014), continued explaining that increasing the incubation time enables the isolation of some members of groups that may be known as unculturable. Extending the incubation period to three months facilitated the emergence of visible colonies from rarely isolated groups, which required a longer duration to manifest compared to members of commonly isolated groups (Chikere & Udochukwu, 2014).

Introducing extract to the media also revealed varying effects on bacterial growth and abundance: increasing CFUs for some samples, decreasing CFUs for others, and having no effect on others. This shows that bacteria from different environments prefer different conditions for growth. Microbes, particularly bacteria, exhibit remarkable tolerance to diverse environmental conditions compared to other organisms (Isnawati & Trimulyono, 2018). Despite their adaptability, each microbial species thrives within a specific range of conditions optimal for growth and reproduction, certain microorganisms can flourish at extremely low temperatures, reaching as low as -10°C, while others thrive in environments with temperatures as high as 100°C or even beyond (Isnawati & Trimulyono, 2018). This emphasizes the wide spectrum of conditions

under which different microbial species can successfully grow and reproduce (Isnawati & Trimulyono, 2018).

Novel discovery of bacterial strains have been reported from an isolation technique that involved selecting bacteria thriving on soil-extract agar medium compared to using conventional media (Hamaki et al., 2005). A similar technique employed in this study offers promising prospects in the isolation of novel bacteria, as shown in the results showing increased CFUs after the addition of soil extract. In our study, the soil extract improved growth for only 3 samples, while other results did not show much difference, interestingly, the other results revealed reduced CFUs. A study by Nguyen et al.,(2018) states that a novel medium, termed intensive soil extract medium (ISEM), was introduced, utilizing a new soil extract (NSE) prepared with 80% methanol, and it proved highly effective in isolating previously uncultured bacteria and identifying new taxonomic candidates and the isolates accounted for 49% and 55% of the total examined isolates. Our study also suggests that the impact of the SE may be context-dependent, and influenced by specific factors in our experimental conditions. Nguyen et al., (2018)'s success with the ISEM may stem from its unique composition, including 80% methanol in the NSE, which led to the isolation of a significant proportion of previously uncultured bacteria and new taxonomic candidates. This shows that soil extracts may be beneficial in isolating unculturable bacteria, depending on the type of sample.

4.2 Genotypic and phenotypic characterization of *P. mirabilis*-MN029

The MDR model strain was confirmed as *P. mirabilis* through phylogenetic and genomic analysis of the WGS data, as previously reported by Makale et al., (2024). Briefly, the complete genome

sequence of *P. mirabilis*-MN029 was characterized by a single chromosome, with an average G+C content of 38.5 %, a length of 3,988,563 base pairs, and 3,649 protein-coding sequences (CDS). The genome also included 22 ribosomal RNA (rRNA) genes and 84 transfer RNA (tRNA) genes. The genome annotation of the MDR strain shows numerous CDS homologous to known proteins associated with virulence factors, antimicrobial resistance (AMR) phenotypes, transporters, and drug targets (Figure 6).

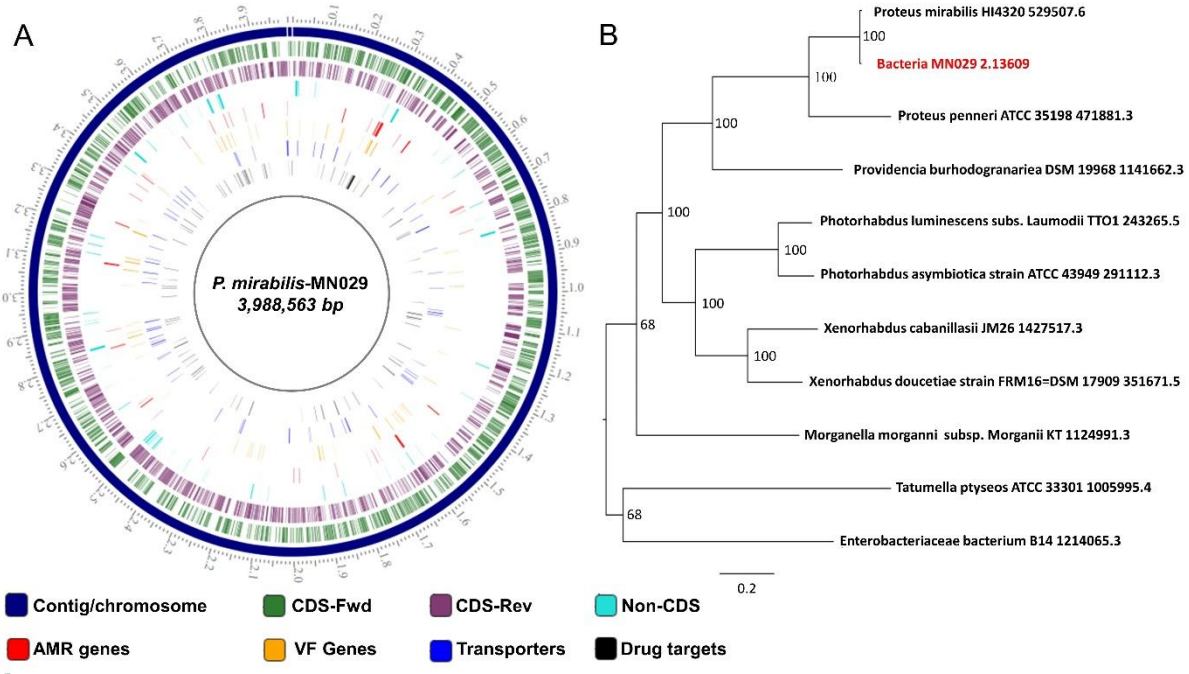


Figure 6: Genome Characterization of P. mirabilis-MN029. A. A circular graphical display illustrating the distribution of genome annotations. The outermost ring represents the chromosome, followed by rings for coding sequences (CDS) on the forward strand (CDS-Fwd) and the reverse strand (CDS-Rev). The next ring shows non-coding sequences (Non-CDS), while the innermost rings indicate CDS that are homologous to known antimicrobial resistance (AMR)

genes, transporters, virulence factors (VF), and drug targets. B. Phylogenetic analysis and identification (MDR) bacterial strain. *(Makale et al., 2024).*

The acquired antibiotic resistance genes; *mac*A, *mac*B (macrolide), *cat*A (chloramphenicol), Dfr (trimethoprim) and *tet* (J) (tetracycline) were predicted, along with various chromosomally encoded resistance genes, linked to multidrug-resistance mechanisms (Makale et al., 2024)(Table 4, 5).

Table 4: Antibiotic resistance pattern of *P. mirabilis*-MN-029 *(Makale et al., 2024)*

Antibiotic Class	**Antibiotic agar dilution**	**Antibiotic disk**	***P. mirabilis*-MN029**
Penicillin	AMX AMP PEN G	AML25 AP10 PG10	R R R
Aminoglycoside	AMK KAN GEN	-- K30 --	S S R
Tetracycline	TET	TE30	R
Rifamycin	RIF	RD30	S
Trimethoprim	TMP	W5	R

Keys: S, susceptible; R, resistant; --, not tested; Tetracycline (TE30, TET), Amikacin (AMK), Trimethoprim (W5, TMP), Ampicillin (AP10, AMP), Gentamicin (GEN), Rifampicin (RD30, RIF), Kanamycin (K30, KAN), Penicillin G (PG10, PEN G), Amoxicillin (AML25, AMX).

Table 5: Summary of Predicted AMR Genes and Resistance Mechanisms in *P. mirabilis*-MN029 *(*Makale et al., 2024*).*

AMR Mechanism	Antimicrobial Resistance Genes
Antibiotic inactivation enzyme	CatA1/CatA4 family
Antibiotic target protection protein	BcrC
Efflux pump conferring antibiotic resistance	AcrAB-TolC, AcrZ, EmrAB-TolC, MacA, MacB, MdtABC-TolC, MdtL, MexHI-OpmD, SugE, Tet(J), TolC/OpmH
Gene conferring resistance via absence	gidB
Protein-altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Regulator modulating expression of antibiotic resistance genes	AcrAB-TolC, EmrAB-TolC, H-NS, OxyR

Proteus mirabilis is a Gram-negative, highly motile and facultative anaerobic, rod-shaped bacterium belonging to the Enterobacteriales family, known to cause various symptomatic infections, especially urinary tract infections (Yuan et al., 2021) The clinical significance of *P. mirabilis* stems from its ability to form biofilms, facilitating its colonization of urinary tract epithelium and urinary catheters (Yuan et al., 2021). Once established, these biofilms can make *P. mirabilis* infections challenging to treat, as they protect host immune responses and antibiotic therapies (Yuan et al., 2021). Additionally, *P. mirabilis* possesses intrinsic resistance mechanisms and can acquire resistance genes through horizontal gene transfer, further complicating treatment strategies (Armbruster et al., 2018). *P. mirabilis* poses significant health risks as a

common causative agent of UTIs, with potential complications arising from recurrent infections and antibiotic resistance (Armbruster et al., 2018; Cloeckaert et al., 2024; Yuan et al., 2021). Understanding its phenotypic and genomic characteristics is important for developing effective strategies to combat *P. mirabilis* infections and minimize their impact on public health.

Our study focused on *P. mirabilis*-MN029, which exhibited a single 3,989 Mb chromosome with no plasmid, with an average G+C content of 38.5%, and 3,649 protein-coding sequences (CDS). *P. mirabilis*-MN029 is closely related to *P. mirabilis* HI4320, isolated from a patient with a urinary catheter, had a 4.063 Mb genome with a G+C content of 38.88% and a single plasmid and annotation identified 3,685 coding sequences (Pearson et al., 2008). In another study *P. mirabilis* PM1162 also contained one chromosome and one plasmid, the chromosome was larger, at 4,202,509 bp, with a G+C content of 38.96% and harbored 4,033 genes, including 15 ARGs located on the chromosome (Yang et al., 2023). Additionally, *P. mirabilis* PrK 34/57 had a genome size of 3,970,593 bp, a GC content of 38.7%, and 3,722 genes (Czerwonka et al., 2021).

The variation in genome size, plasmid content, and ARG profiles among the studied strains reflects the nature of *P. mirabilis* genomes and may suggest different evolutionary pathways. The difference in virulence factors and metabolic pathways may also highlight the strains' adaptation to specific host niches especially because they were isolated in different areas (Czerwonka et al., 2021; Pearson et al., 2008; Yang et al., 2023). The presence or absence of plasmids may influence the strain's ability to acquire and disseminate resistance genes, which may potentially impact its virulence and clinical outcomes (Czerwonka et al., 2021; Pearson et al., 2008; Yang et al., 2023). Comparing the genomic characteristics of MDR *P. mirabilis* strains across studies shows

differences in genome size, plasmid content, GC content, and ARG profiles. *P. mirabilis*-MN029, lacked a plasmid, while PM1162, PrK 34/57, and HI4320 harbored one or more plasmids. The absence of a plasmid in *P. mirabilis*-MN029 further makes it a good model for our study, to understand the mechanisms of antibiotic resistance located only in the chromosome, thus reducing the complexity in tracking gene expressions if studied *in vitro* for analysis against different antimicrobials including those from plant extracts.

The antibiotic resistance profiles of *P. mirabilis*-MN029 and the other strains (PM1162, PrK 34/57, and HI4320) show similarities and differences. *P. mirabilis*-MN029 exhibited resistance to penicillin and tetracycline, primarily mediated by antibiotic inactivation enzymes (CatA1/CatA4 family) and an efflux pump (Tet(J)), respectively. Surprisingly, despite possessing mechanisms for aminoglycoside resistance via target alteration, *P. mirabilis*-MN029 showed susceptibility to aminoglycosides (AMK, KAN, GEN), which suggests a potential complexity in the expression or regulation of the resistance mechanisms. Additionally, *P. mirabilis*-MN029 displayed resistance to rifamycin and trimethoprim and harbored genes conferring resistance via absence (gidB) and regulators modulating expression of resistance genes (AcrAB-TolC, EmrAB-TolC, H-NS, OxyR). In contrast, PM1162 exhibited multidrug resistance, including resistance to extended-spectrum cephalosporins mediated by blaCTX-M-14, along with resistance to aminoglycosides, quinolones, and sulfonamides (Yang et al., 2023). Similarly, PrK 34/57 also harbored genes conferring resistance to aminoglycosides and beta-lactams, however, the specific resistance mechanisms and the degree of resistance in PrK 34/57 were not specified (Czerwonka et al., 2021). While *P. mirabilis*-MN029 shares some similarities in resistance mechanisms with PM1162 and PrK 34/57,

such as beta-lactam resistance, the differences in aminoglycoside susceptibility highlight the variance in resistance profiles among the *P. mirabilis* strains.

4.3 Antibacterial activity of isolates against test bacteria

Out of the 864 isolates stored, a significant proportion were isolated from the Selebi-Phikwe area, constituting 88% of the total isolates, while the remaining 11% were isolated from soil in Palapye. Among the isolates from Selebi-Phikwe, 12.5% were isolated from water, while the majority, comprising 87.5%, were isolated from soil. Among all stored isolates, a subset of 35(4%), demonstrated potential antibacterial activity against at least one of the tested bacterial strains. Nine (26%) potential antibiotic-producing bacterial isolates inhibited one test bacteria, while twelve (34 %) inhibited two, two (6%) inhibited three, seven (20%) inhibited four and lastly, five (14%) inhibited five test bacteria respectively. *E. coli* test bacteria was inhibited by at least 19 potential antibiotic-producing isolates, 16 antibiotic-producing isolates inhibited *P. aeruginosa*, 10 inhibited *P. mirabilis*-MN029, 18 inhibited *K. pneumoniae,* and 27 inhibited *S. aureus*. The most inhibited bacterium was *S. aureus* the only Gram-positive bacteria, followed by *E. coli*, then *K. pneumoniae* and *P. aeruginosa,* and lastly, *P. mirabilis*-MN029, the least inhibited.

K. pneumoniae was most effectively inhibited by the 9 isolates tested, while *P. mirabilis*-MN029 and *P. aeruginosa* showed the least inhibition (Figure 7a). Interestingly, potential antibiotic-producing bacterial isolates B41 and B45 were both highly effective in inhibiting the majority of the test bacteria, with B41 inhibiting 4 and B45 inhibiting 5 of the test bacterial strains. B41 displayed a particularly larger inhibition zone of 22.55 ± 0.2630 mm against *P. mirabilis*-MN029, although it was less effective against *K. pneumoniae* with a zone of only 9.188 ± 0.1875 mm.

Conversely, B45 had the smallest inhibition zone against *P. mirabilis*-MN029 at 9.375 ± 0.3750 mm but was highly effective against *P. aeruginosa* with a zone of 21.75 ± 0.8539 mm.

The nine isolates (from soil) tested were mostly effective against *K. pneumoniae,* while *P. mirabilis-MN029* was the least inhibited (as shown in Figure 7b). Both B64 and C76 inhibited 4 test bacteria each. B64 inhibited *P. mirabilis*-MN029 with an inhibition zone of 17.23 ± 0.7642 mm and showed the lowest zone against *E. coli* with 10.88 ± 0.4270 mm. On the other hand, C76 was not active against *P. mirabilis*-MN029 but had the highest zone against *E. coli* at 23.50 ± 0.6455 mm and lowest against *S. aureus* at 17.50 ± 0.6455 mm.

Out of the nine isolates , most were effective against *S. aureus* while *P. mirabilis*-MN029 was the least inhibited (Figure 7c). D25 inhibited 4 out of the 5 test bacteria, whereas D13, D29, D40, and D46 were able to inhibit all 5 test bacteria. The zone of inhibitions for D13, D29, D40, and D46 against *P. mirabilis*-MN029 were 4.375 ± 0.2394, 8.025 ± 0.4289, 13.50 ± 0.5000, and 12.75 ± 0.4787 mm, respectively, which was the lowest zone for all except D40. In contrast, D25 was not effective against *P. mirabilis*-MN029 but had the highest inhibition zone against *K. pneumoniae* at 22.63 ± 0.3750 mm and the lowest zone against *S. aureus* at 17.00 ± 0.4082 mm.

S. aureus was most effectively inhibited by the nine isolates tested, while *K. pneumoniae* showed the least inhibition (Figure 7d). E14, E47, and E87 were effective in inhibiting the majority (4) of the test bacteria. E47 and E87 showed inhibition against *P. mirabilis*-MN029 with inhibition zones of 9.500 ± 0.2887 and 7.625 ± 0.2394 mm respectively, while E14 was not effective against *P. mirabilis*-MN029. All the 3 isolates showed a high inhibition zone against *P. aeruginosa*, ranging

from 13.75 ± 0.4787 to 15.50 ± 0.8539 mm. Isolates inhibiting at least four of the test bacteria are presented in Figures 2 and 3 below.

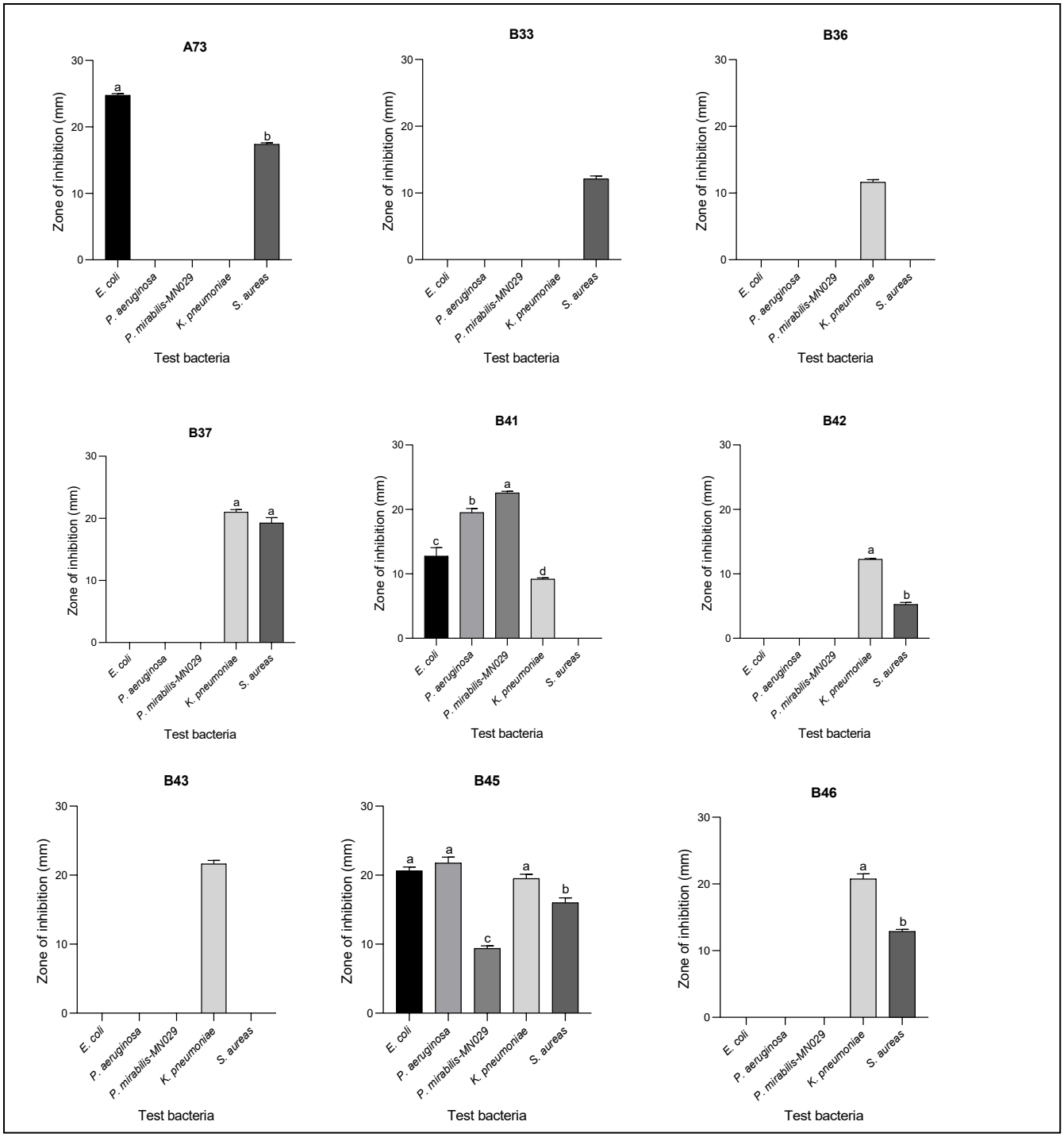


Figure 7 a: Antibacterial activity of selected potential antibiotic-producing isolates (A-F) against test bacteria

The lower case letters on the graph indicate whether the means are statistically similar or different.

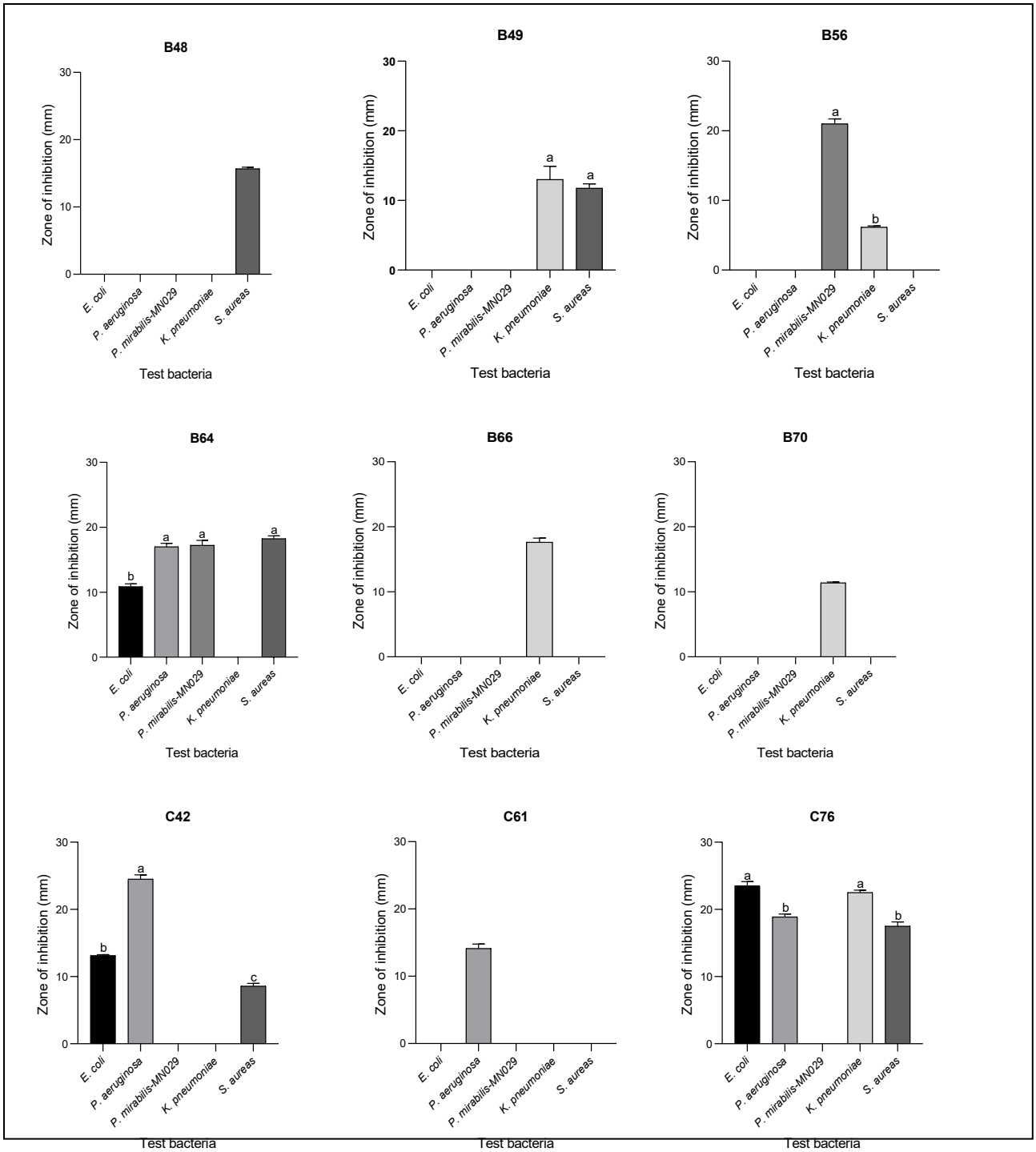


Figure 7 a: Antibacterial activity of selected potential antibiotic-producing isolates (A-F) against test bacteria

The lower case letters on the graph indicate whether the means are statistically similar or different.

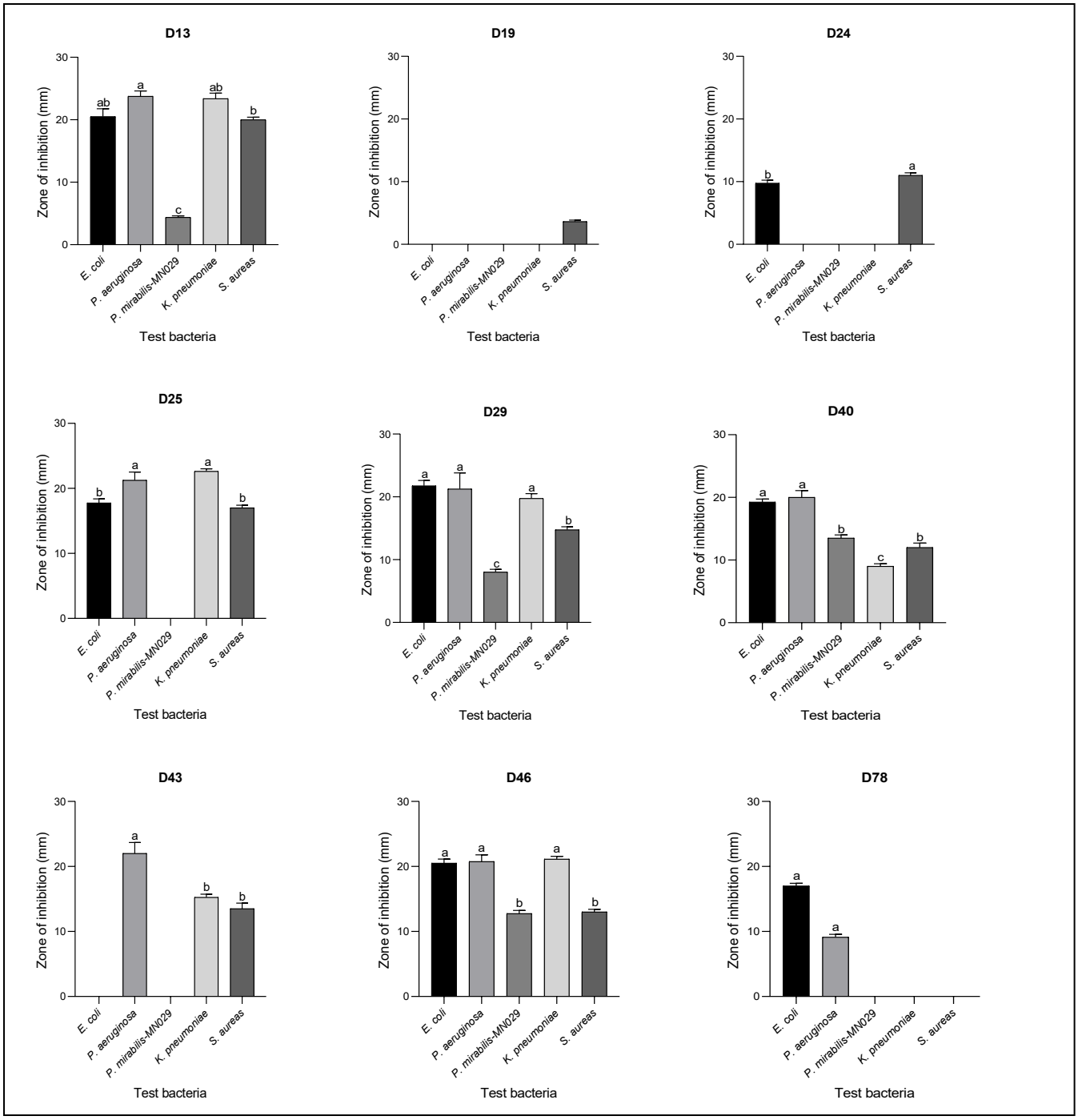


Figure 7 b: Antibacterial activity of selected potential antibiotic-producing isolates (A-F) against test bacteria

The lower case letters on the graph indicate whether the means are statistically similar or different.

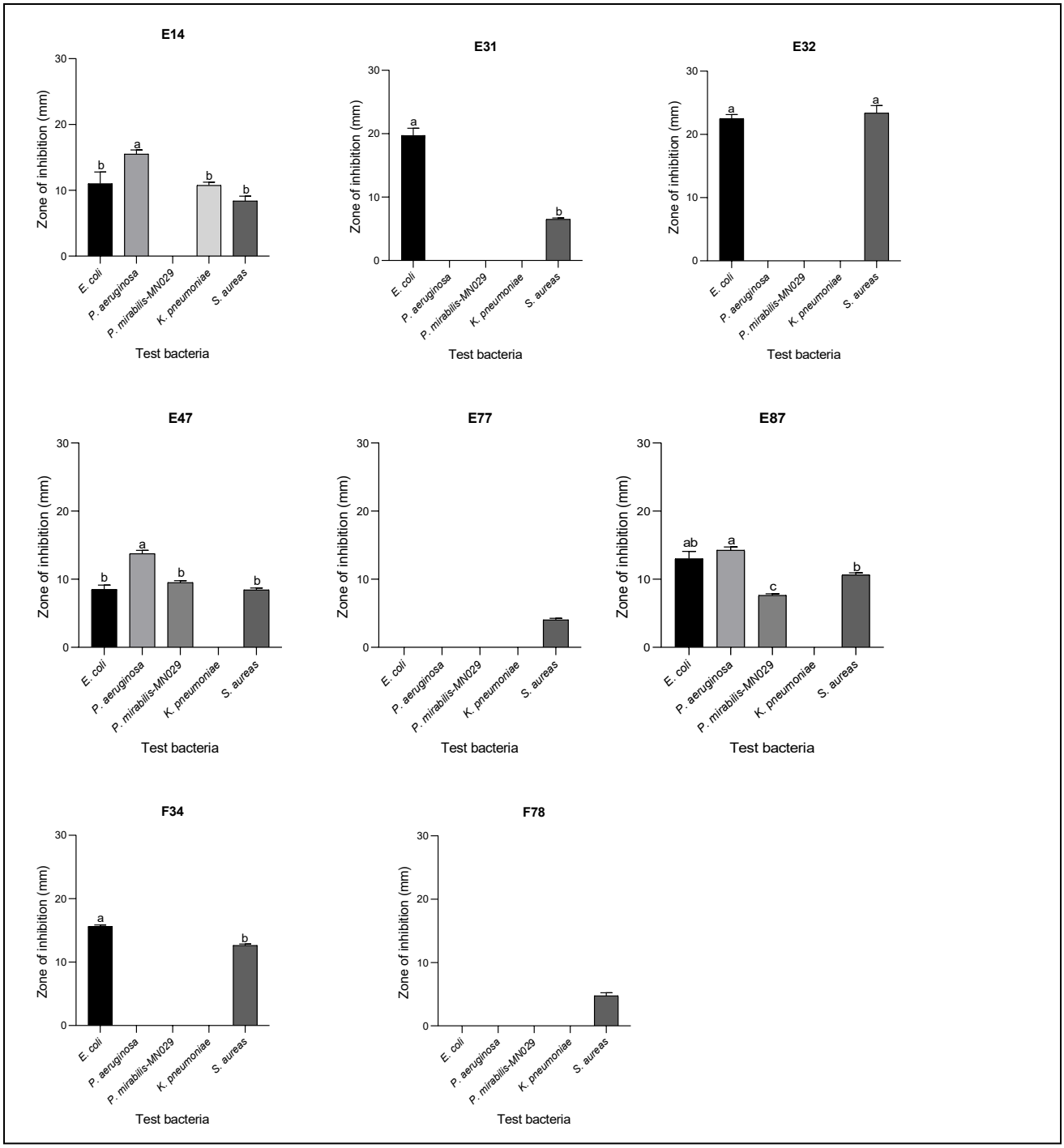


Figure 7 c: Antibacterial activity of selected potential antibiotic-producing isolates (A-F) against test bacteria

The lower case letters on the graph indicate whether the means are statistically similar or different.

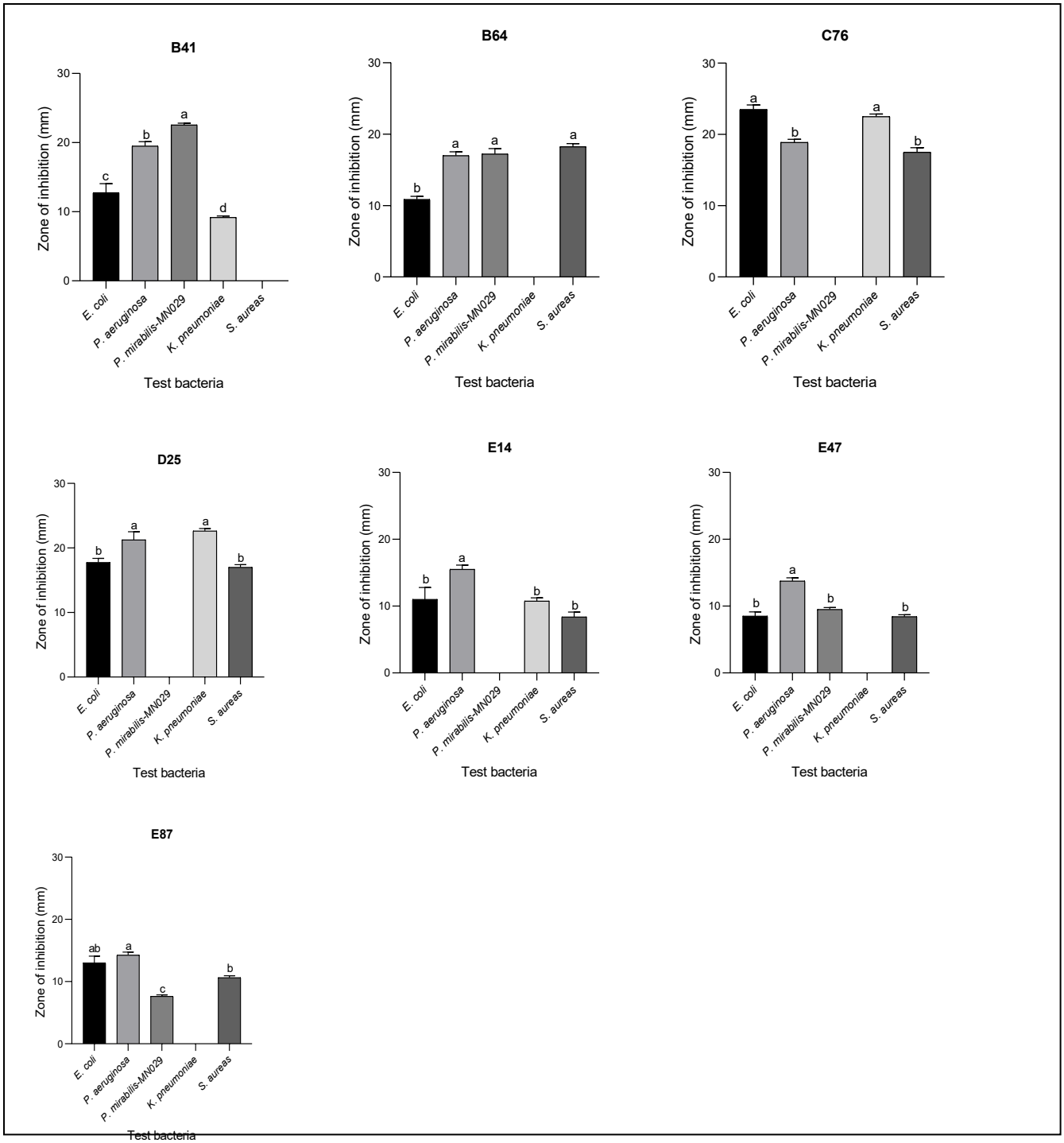


Figure 8: Antibacterial activity of isolates inhibiting four test bacteria

The lower case letters on the graph indicate whether the means are statistically similar or different.

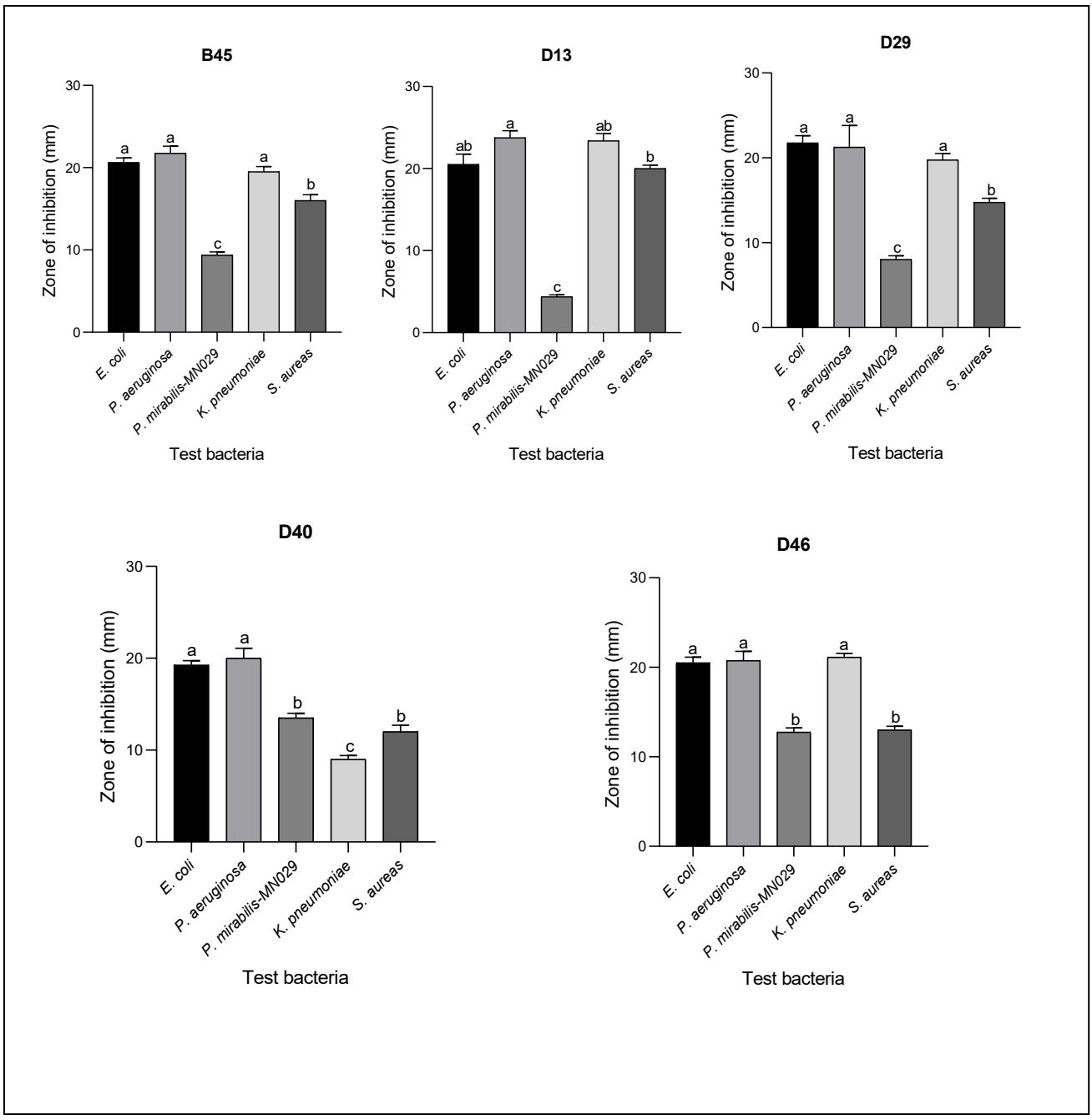


Figure 9: Antibacterial activity of isolates inhibiting five test bacteria

The lower case letters on the graph indicate whether the means are statistically similar or different

Among the tested potential antibiotic-producing isolates, different levels of inhibition were observed against the test bacteria, specifically, 2% of isolates expressed antibacterial activity against *E. coli*, 2% against *P. aeruginosa*, 1% against *P. mirabilis*-MN029, 2% against *K. pneumoniae*, and 3% against *S. aureus*. The inhibition zones ranged from 3.63 ± 0.24 mm to 24.75±0.25 mm across the 35 potential antibiotic-producing bacteria. In a study by Prashanthi et al., (2021), a total of 263 bacterial isolates underwent screening for antibacterial activity against pathogenic bacteria, *S. aureus, E. coli, P. aeruginosa, and K. pneumoniae,* and the results showed only 1% of the isolates exhibited antibacterial activity against all tested bacteria, the highest zone of inhibition ranged from 10.00 mm to 15.50 mm against the respective pathogenic bacteria. Compared to our experiment, the inhibition zones varied from 3.63 ± 0.24 mm to 24.75 ± 0.25 mm across the 4% potential antibiotic-producing bacteria. Out of the 97 strains studied by Girma & Aemiro, (2021), 10% displayed antibacterial activities against at least two of the tested bacteria, with their inhibition zones ranging from 5.00 to 16.00 mm, which is relatively small compared to the current study. The difference in the inhibition zones between the studies shows variations in the antibacterial activity of isolates amongst the different studies, possibly influenced by various experimental conditions or the genotypes of the bacterial strains used. The findings in this study suggest the broad-spectrum antibacterial potential of the screened bacterial isolates from different areas in Botswana, highlighting their potential and promise to work against bacterial pathogens including MDR bacteria.

4.4 AST testing of potential antibiotic-producing bacteria

Twelve potential antibiotic-producing strains that showed antibacterial activity were selected for further identification. These were chosen based on their ability to inhibit at least 80% of the test bacteria. All twelve selected potential antibiotic-producing bacteria showed resistance to penicillin and chlorotetracycline, nine showed resistance to ampicillin, seven to amikacin and only six were resistant to amoxicillin. All the potential antibiotic-producing isolates were susceptible to gentamicin, erythromycin, rifampicin, and tetracycline (Table 6).

Table 6: Resistance pattern of antibiotic-producing bacteria

Isolate	CTC	PEN G	AMP	AMX	AMK	GEN	ERY	RIF	TET
KM_B41	R	R	S	S	S	S	S	S	S
KM_B45	R	R	S	S	R	S	S	S	S
KM_B64	R	R	S	S	S	S	S	S	S
KM_C76	R	R	R	S	R	S	S	S	S
KM_D13	R	R	R	R	R	S	S	S	S
KM_D25	R	R	R	R	R	S	S	S	S
KM_D29	R	R	R	R	R	S	S	S	S
KM_D40	R	R	R	S	R	S	S	S	S
KM_D46	R	R	R	S	R	S	S	S	S
KM_E14	R	R	R	R	S	S	S	S	S
KM_E47	R	R	R	R	S	S	S	S	S
KM_E87	R	R	R	R	S	S	S	S	S

R- resistant S- susceptible

Ampicillin (AMP), gentamicin (GEN), tetracycline (TET), amikacin (AMK), rifampicin (RIF), erythromycin (ERY), penicillin (PEN G), chlortetracycline (CTC) and amoxicillin (AMX)

The resistance of isolates to antibiotics in a shared environment, like soil reflects the outcome of natural selection, where bacteria with inherent or acquired resistance gain a survival advantage (Bengtsson-Palme et al., 2018). This resistance can arise through mechanisms such as horizontal gene transfer, which enables the spread of resistance traits. In competitive settings, bacteria with antibiotic resistance genes have a selective advantage, outcompeting susceptible strains (Bengtsson-Palme et al., 2018). Coexistence with antibiotic-producing bacteria may also drive neighboring bacteria to develop resistance for survival, additionally, pre-existing resistance mechanisms in bacterial genomes contribute to the overall prevalence of antibiotic-resistant strains (Bengtsson-Palme et al., 2018). This may explain why the potential antibiotic-producing isolates were resistant to some antibiotics and understanding this is crucial to address antibiotic resistance and develop effective management strategies. It is a widely observed phenomenon that organisms that produce antibiotics also possess self-resistance mechanisms against the antibiotics they produce (Davies & Davies, 2010; Mak et al., 2014). Antibiotic-producing bacteria possess self-defense mechanisms to counteract their antibiotics, these mechanisms are frequently utilized simultaneously to provide defense (Peterson & Kaur, 2018). The expression of the genes involved in antibiotic production and the genetic determinants of self-resistance are co-regulated, and the genes are nearly always clustered together (Mak et al., 2014).

4.5 Biochemical analysis of antibiotic-producing bacteria

The Gram stain reactions and biochemical profiles of the twelve isolates with potential antibiotic production were determined as shown in Table 7. Out of the twelve, 67% (eight) were Gram-positive, 25% (three) were Gram-variable, and 8% (one) were Gram-negative. The biochemical test results of the 12 bacterial isolates show a wide range of metabolic characteristics. All but two isolates (KM_E47 and KM_E87) showed distinct metabolic patterns or outcomes in the biochemical testing. In particular, indole synthesis was uniformly negative across all the tested strains, and there was no utilization of xylose, arabinose, cellobiose, melezitose, raffinose, or rhamnose. Furthermore, all isolates exhibited the ability to hydrolyze esculin. Isolate KM_D25 was an exception, as it was the only strain positive for sorbitol, lactose, and mannitol. Glucose appeared as the most common substrate used by most strains.

Table 7: Biochemical tests of antibiotic-producing bacteria

Tests	KM_B41	KM_B45	KM_B64	KM_C76	KM_D13	KM_D25	KM_D29	KM_D40	KM_D46	KM_E14	KM_E47	KM_E87
Gram	+	+	+	+	-/+	-	-/+	+	-/+	+	+	+
INDole	-	-	-	-	-	-	-	-	-	-	-	-
UREase	-	-	+	-	+	-	+	+	-	-	-	-
GLUcose	-	+	-	+	+	+	+	+	+	+	+	+
MANnitol	-	-	-	-	-	+	-	-	-	-	-	-
LACtose	-	-	-	-	-	+	-	-	-	-	-	-
SACcharose	-	+	-	+	-	+	-	-	+	-	-	-
MALtose	-	+	-	+	+	+	-	+	+	+	+	+
SALicin	-	-	-	-	-	+	-	-	-	+	-	-
XYLose	-	-	-	-	-	-	-	-	-	-	-	-
ARAbinose	-	-	-	-	-	-	-	-	-	-	-	-
GELatin	+	+	-	+	+	-	+	+	+	+	+	+
ESCulin	+	+	+	+	+	+	+	+	+	+	+	+
GLYcerol	+	-	-	-	-	-	+	-	-	-	-	-
CELlobiose	-	-	-	-	-	-	-	-	-	-	-	-
ManNosE	-	-	-	-	-	+	-	-	-	+	-	-
MeLeZitose	-	-	-	-	-	-	-	-	-	-	-	-
RAFfinose	-	-	-	-	-	-	-	-	-	-	-	-
SORbitol	-	-	-	-	-	+	-	-	-	-	-	-
RHAmnose	-	-	-	-	-	-	-	-	-	-	-	-
TREhalose	-	+	-	+	-	+	+	-	+	+	+	+

Studies have reported that most of the soil isolates are Gram-positive in nature (Mandal et al., 2019), which might explain why most (67%) of our isolates were Gram-positive. Performing biochemical tests on potential antibiotic-producing bacteria is essential because the tests aid in identifying and differentiating strains, (Shoaib et al., 2020). None of the strains demonstrated indole production, indicating that the organisms do not possess the enzyme tryptophanase, which is necessary for the conversion of tryptophan to indole (Shoaib et al., 2020). Urease production varied, with some strains showing positive reactions, while the utilization of sugars exhibited a mixed pattern, with most strains able to metabolize glucose, while only select strains demonstrated the ability to utilize mannitol, lactose, saccharose, and maltose. Gelatin and esculin hydrolysis were predominantly positive across the strains and glycerol utilization showed variable results. Among the tested sugars (xylose, arabinose, cellobiose, mannose, melizitose, raffinose, sorbitol, and rhamnose), most strains exhibited no utilization whereas trehalose utilization was observed in several strains. These patterns demonstrate that the investigated potential antibiotic-producing bacteria have unique substrate preferences and metabolic capabilities. Gram-positive bacteria are identified using biochemical tests such as catalase, coagulase, starch hydrolysis, and nitrate, whereas Gram-negative bacteria are identified using oxidase, urease, indole, sulfur, and methyl red/Voges Proskauer tests (Shoaib et al., 2020).

4.6 Genomic characterization of potential antibiotic-producing bacteria

WGS and a comprehensive genome bioinformatics analysis were performed on four selected potential antibiotic-producing bacteria. The study of the isolates showed distinctive trends in their genomic characteristics (Table 8). Isolates KM_D13 and KM_D46, exhibited longer total genome lengths (7,663,567 bp and 7,666,303 bp respectively), with isolate KM_D46 slightly longer than isolate KM_D13. In contrast, isolates KM_B45 and KM_C76 displayed shorter genome lengths (6,116,533 bp and 5,551,567 bp). A lower G+C percentage was noted for KM_C76 at 40.67%, whereas KM_B45, KM_D13 and KM_D46 showed higher G+C content, at 52.60%, 53.36% and 53.38% respectively. A total of 7,606 protein-coding sequences (CDS) were KM_D13, 7,575 CDS for KM_D46, 6,224 for KM_B45, and 5,574 CDS for KM_C76 were also found in the isolates. In terms of hypothetical and functional proteins, the highest counts were 3,393 and 4,213 for KM_D13 and the lowest were 2,562 and 3,012 for KM_C76.

Table 8: Genome Analysis of Potential antibiotic-producing Isolates

	KM_B45	KM_C76	KM_D13	KM_D46
Contigs	211	129	56	56
GC content	52.60%	40.67%	53.36%	53.38%
Genome Length	6,116,533 bp	5,551,567 bp	7,663,597 bp	7,666,303 bp
CDS	6,224	5,574	7,606	7,575
Hypothetical Proteins	2,792	2,562	3,393	3,368
Functional Protein	3,432	3,012	4,213	4,207

The isolate KM_B45 was identified as *Paenibacillus elgii,* KM_C76 as *Brevibacillus laterosporous* while KM_D13, and KM_D46 were identified as *Paenibacillus oleatilyticus*, based on the BLAST and phylogenetic analysis of the WGS (Figure 10). Bacteria KM_D13 and Bacteria KM_D46 show close relatedness to *P. elgii* B69 and *P. oleatilyticus* at a bootstrap value of 100%. Bacteria KM_C76 shows close relatedness to *Brevibacillus laterosporus* at a confidence level or bootstrap value of 100%. Bacteria KM_B45 shows close relatedness to *Paenibacillus elgii* B69, at a bootstrap value of 100%.

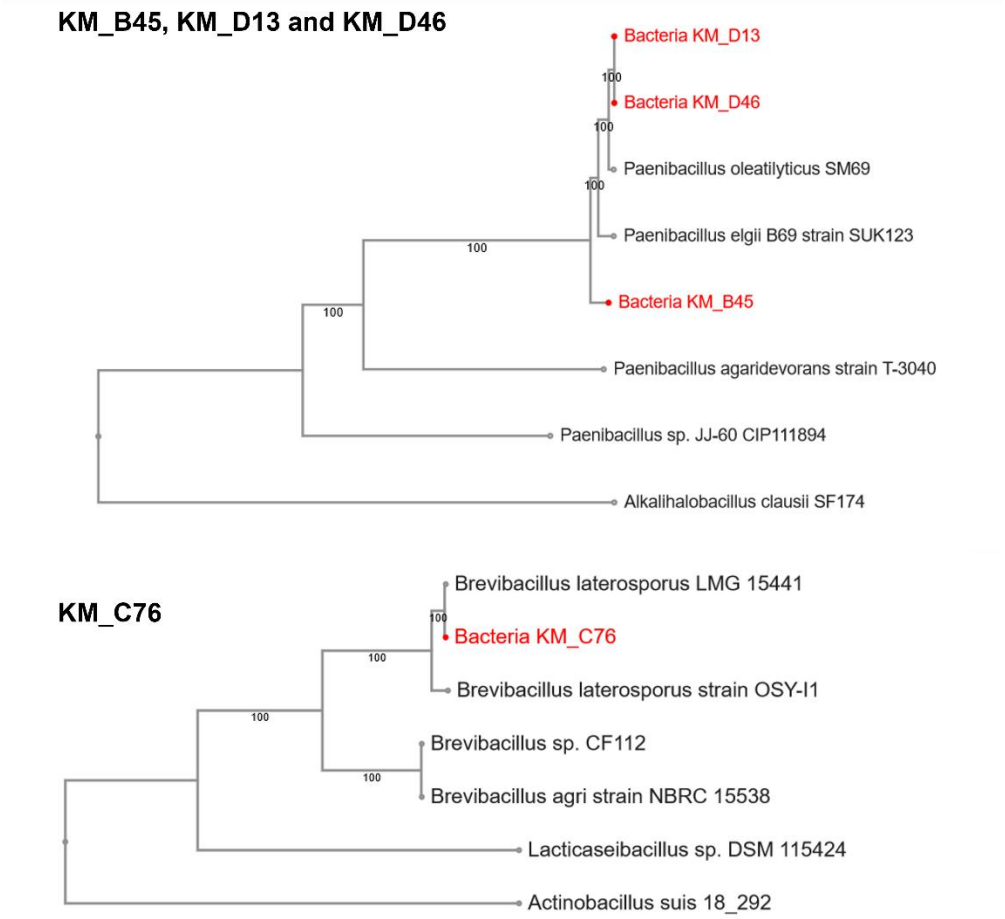


Figure 10: Phylogenetic Analysis of antibiotic-producing bacteria

Genome graphical display maps reveal high similarities among the three *Paenibacillus* strains (KM_B45, KM_D13, and KM_D46) (Figure 11). *P. elgii*-KM_B45 genomic sequence has equal distribution of CDS on both strands, with less similarity to resistance genes and fewer spikes in RNA genes. There are slight fluctuations in skew and GC content. Annotations of CDS with higher density are more prominent in some locations of *B. laterosporus*-KM_C76. RNA and resistance genes are more sporadic, with greater variance and pronounced peaks in GC content and skew. *P. oleatiliticus*-KM_D13 has more equally distributed CDS annotations, while resistance genes and RNA genes have a modest distribution. *P. oleatiliticus*-KM_D46 has more frequent RNA genes

and unique high-density regions of CDS annotations. Across the genomes *P. elgii*-KM_B45, *B. laterosporus*-KM_C76, *P. oleatiliticus*-KM_D13 and *P. oleatiliticus*-KM_D46, a consistent pattern is shown regarding the occurrences of subsystems. Metabolism represents the largest category across all isolates. Both *P. oleatiliticus*-KM_D13 and *P. oleatiliticus*-KM_D46 show the highest number of metabolism-related genes (749 genes-92 subsystems), followed by *B. laterosporus*-KM_C76 (710 genes-83 subsystems), and *P. elgii*-KM_B45 with the least (697 genes-87 subsystems). Protein Processing is the second largest category across all isolates. *B. laterosporus*-KM_C76 has the highest count (229 genes-41 subsystems), followed by *P. elgii*-KM_B45 (225 genes-42 subsystems), *P. oleatiliticus*-KM_D46 (209 genes-40 subsystems), and lastly *P. oleatiliticus*-KM_D13 (207 genes-39 subsystems). *P. oleatiliticus*-KM_D13 and *P. oleatiliticus*-KM_D46 have more genes related to stress response and defense compared to *P. elgii*-KM_B45 and *B. laterosporus*-KM_C76. Additionally, *B. laterosporus*-KM_C76 exhibits higher counts for most subsystems (energy, cellular processes, DNA processing, RNA processing, and Regulation and cell signaling) than the other isolates. *P. oleatiliticus*-KM_D13 and *P. oleatiliticus*-KM_D46 display similar trends with slight variations in Protein Processing.

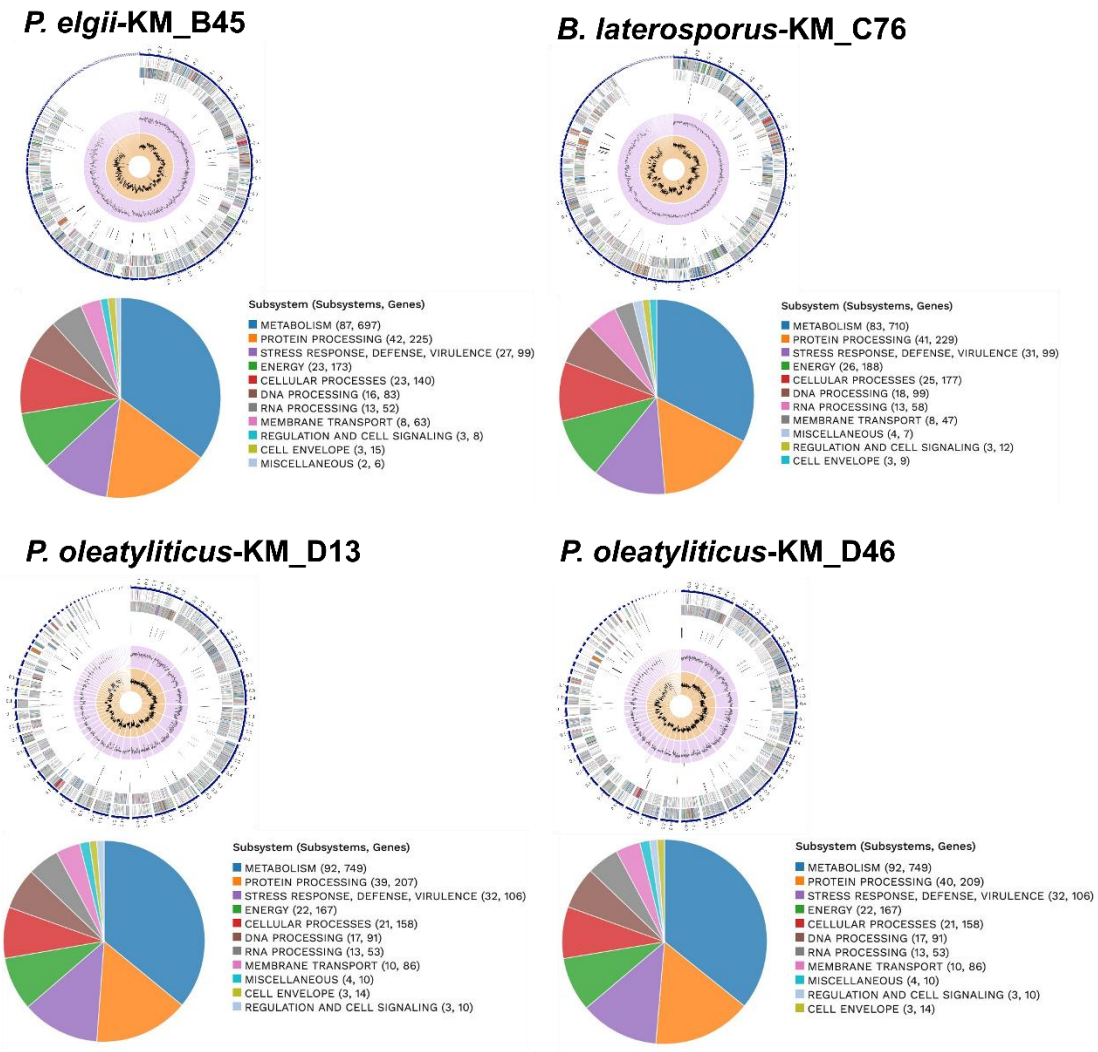


Figure 11: Graphical maps and venn diagrams showing similarities and differences in genome structure and subsystems/genes of four potential antibiotic-producing bacteria (*P. elgii*-KM_B45, *B. laterosporus*-KM_C76, *P. oleatyliticus*-KM_D13 and *P. oleatyliticus*-KM_D46). Circular graphical map displays the distribution of the genome annotations: from outer to inner rings corresponds to; the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content, and GC skew. The colors of the CDS on the forward and reverse strands correspond to the subsystem to which the genes belong.

WGS enables more thorough comprehensive genomic analysis compared to 16S rRNA gene sequencing, a gene-focused technique for examining microbial communities (Peterson et al., 2021; Ranjan et al., 2016). However, because of gene conservation and short amplicon lengths, 16S rRNA sequencing offers only a restricted level of taxonomic resolution (Peterson et al., 2021; Ranjan et al., 2016). On the other hand, WGS promotes gene prediction, diversity detection, and bacterial species identification from assembled contigs (Peterson et al., 2021; Ranjan et al., 2016). For preliminary taxonomic classification and wide comparisons, 16S rRNA sequencing is a good choice, however, some of its drawbacks include the short amplicon products, the fact that a single organism can have many copies of the 16S rRNA gene, the high sequence identity across closely related species, and the fact that the 16S rRNA gene is conserved, which limits its ability to resolve taxonomy (Peterson et al., 2021; Ranjan et al., 2016). Bacterial phylogenetic analyses are frequently conducted to investigate the interrelationships among different bacterial species and genera, mostly relying on the sequencing of their 16S rRNA genes (Nakano et al., 2022). The phylogenetic trees illustrate the relationships among four identified isolates and their associations with other related bacteria. The phylogenetic trees imply that these bacterial isolates share common ancestors and have varying degrees of genetic relatedness (Nakano et al., 2022). The bootstrap values indicate the statistical support for the inferred relationships. High bootstrap values generally suggest support for the depicted branching patterns, while lower values may indicate some level of uncertainty or genetic diversity in the inferred relationships (Nakano et al., 2022). The three isolates (KM_B45, KM_D13, and KM_D46) are the same genus as revealed by the phylogenetic tree, circular graphical map and the pie chart for the subsystems and genes. The slight differences were noted in the number of contigs, G+C content, the

distribution of proteins and genes throughout the genomes. Hence why, the three isolates (Bacteria KM_B45, KM_D13, and KM_D46) whose bootstrap values are 100, were identified as *P. elgii and P. oleatilyticus respectively* and Bacteria-KM_C76 whose bootstrap was 100% as *B. laterosporus*.

Many *Paenibacillus* species live in the soil and the rhizosphere of various plants. Due to their ability to produce a wide range of antibacterial chemicals and long-lasting spores, some strains within this genus have been considered as potential biological control agents (Wu et al., 2010). These microbes are believed to control plant diseases mainly by producing antibiotics. Paenibacillus species are known to produce numerous antimicrobial compounds that have a broad spectrum of action against various bacteria and fungi (Wu et al., 2010). The main antibacterial substances produced by these species are peptide antibiotics, such as polymyxins, jolipeptin, gavaserin, saltavalin, fusaricidin A–D, and gatavalin (Wu et al., 2010). Strain B69, identified as *Paenibacillus elgii*, is a spore-forming, rod-shaped, motile, Gram-negative bacterium that was isolated from soil (Wu et al., 2010). The strain demonstrated great effectiveness against fungus and other gram-positive and gram-negative bacteria and was the source of antibiotics Pelgipeptins A and B, which are members of the polypeptin family (Wu et al., 2010). In this study *P. elgii*-KM_B45 showed close relatedness to *P. elgii*-B69, at a bootstrap value of 100%.

The strains in this study differ in genome size, G+C content, and protein composition, which shows their genetic diversity and potential functional distinctions. In research by (Kim et al., 2004) the *Paenibacillus* members displayed versatility in their preference for anaerobic or aerobic conditions, featuring a rod-shaped morphology with the ability to produce ellipsoidal spores

within enlarged sporangia. Their genomic G+C content ranged from 45 to 54. Certain members of the genus are also known for synthesizing antibacterial compounds, including polymyxin, octopytin, and baciphelacin (Kim et al., 2004). The G+C content of *P. elgii*-KM_B45 was 52.60% which is in range with the numbers reported by (Kim et al., 2004) for *P. elgii*.

In their work, Kim et al., (2004), *Paenibacillus* exhibited positive results in biochemical tests for indole, glucose, xylose, and esculin while Bacteria *P. elgii* -KM_B45 was negative for both xylose and indoles tests and positive for esculin and glucose utilization. These positive reactions in biochemical assays contribute to the characterization of *Paenibacillus*, which is valuable for understanding the physiological traits of *Paenibacillus* species (Kim et al., 2004). Paenibacillus species are also known to play a pivotal role in enhancing crop growth through various mechanisms, they contribute directly by engaging in biological nitrogen fixation, solubilizing phosphate, producing the phytohormone indole-3-acetic acid (IAA), and releasing siderophores that facilitate iron acquisition (Grady et al., 2016). Additionally, these species protect against insect herbivores and phytopathogens, which include bacteria, fungi, nematodes, and viruses, this protective function is achieved through the synthesis of a diverse array of antimicrobials and insecticides (Grady et al., 2016).

The G+C content of *P. oleatilyticus* SM69, a new bacterium isolated from Indian soil, was reported to be 53.1% (Chauhan et al., 2022). The G+C contents of *P. oleatilyticus*-KM_D13 and *P. oleatilyticus*-KM_D46 also isolated from soil in this study, were found to be 53.36% and 53.38%, respectively. The genome size of *P. oleatilyticus* SM69 was 7,848,017 bp compared to 7,663,597 and 7,666,303 bp of *P. oleatilyticus*-KM_D13 and *P. oleatilyticus*-KM_D46 respectively. The

analysis of the *P. oleatilyticus* SM69 genome showed genes linked to phosphonate solubilization, antibiotic resistance, nitrogen fixation, and plant growth promotion, which highlights its biotechnological potential (Chauhan et al., 2022).

B. laterosporus is a bacterium that has many applications in biological control, environmental protection, agriculture, and therapeutic therapy (Hamze & Ruiu, 2022; Liu et al., 2024). The facultative anaerobe, which is recognized for its rod-shaped cells and endospore production, is commonly found in soil (Yang et al., 2016; Miljkovic et al., 2019). *B. laterosporus* is a probiotic bacterium with insecticidal, antibacterial, anticancer, and biodegradation properties, making it beneficial in a range of fields such as crop cultivation, animal husbandry, therapeutic applications, and environmental cleanup (Liu et al., 2024). Its potent antibacterial action is attributed to the production of peptides and antibiotics. Various strains of *B. laterosporus* can create antibacterial and antifungal compounds, including the newly identified strain *B. laterosporus* OSY-I1, which produces brevibacillin, a potent antimicrobial lipopeptide effective against specific Gram-positive bacteria (Yang et al., 2016; Miljkovic et al., 2019).

Identified as *B. laterosporus*, isolates BGSP7, BGSP9, BGSP11, and BGSP12 demonstrated inhibitory effects, presenting the largest zones of inhibition against all four pathogens in the investigation (Miljkovic et al., 2019). As reported by Yang et al., (2016), the biochemical tests revealed that OSY-I1 demonstrates positive reactions for catalase and oxidase activities, as well as the fermentation of glucose, fructose, mannose, and mannitol. Additionally, OSY-I1 exhibited esculin hydrolysis, while it tested negative for sorbitol and lactose fermentation. *B. laterosporus*-KM_C76, in comparison to the Brevibacillus mentioned above, demonstrated similar biochemical

characteristics, it tested positive for glucose and esculin while yielding negative results for mannitol, sorbitol, and lactose. However, specific tests for catalase, oxidase, fructose, and mannose were not done for *B. laterosporus*-KM_C76. These shared traits in carbohydrate utilization contribute to the comparative analysis of metabolic profiles between OSY-I1 and *B. laterosporus*-KM_C76.

4.7 Antibiotic Resistance Genes in potential antibiotic-producing bacteria

B. laterosporus-KM_C76 and *P. elgii*-KM_B45, *P. oleatiliticus*-KM_D13 and *P. oleatilyticus*-KM_D46) have several common resistance mechanisms, but also significant differences, particularly in efflux pumps, cell wall structure proteins, and regulatory genes (Table 9). All four strains contain the same antibiotic targets, which include enzymes, efflux pumps, the gidB resistance gene, and proteins that influence cell wall charge. However, *P. elgii*-KM_B45 is characterized by the presence of an additional antibiotic inactivation enzyme-resistance encoding genes (Vat(D)/Vat(H)). *P. oleatiliticus*-KM_D13 and *P. oleatiliticus*-KM_D46 share extra cell wall structural proteins-resistance encoding genes(VanH, VanI, and VanX), as opposed to *P. elgii*-KM_B45, which only has VanXY-unclassified genes. For regulators, *P. oleatiliticus*-KM_D13 and *P. oleatiliticus*-KM_D46 have VanA/I/Pt-type and VanR-unclassified, which are absent for *P. elgii*-KM_B45. All *Paenibacillusi* strains lack the BcrA and Tet(45) efflux pumps found in *B. laterosporus*-KM_C76, but they share antibiotic targets and modifying enzymes. *B. laterosporus*-KM_C76 also has cell wall structural proteins-encoding resistance genes (VanH, VanM, VanX, and VanXY-unclassified) and regulators modulating expression of antibiotic resistance genes (LiaR, LiaS, VanA/I/Pt-type, VanF/M-type, VanR-unclassified).

Table 9: Antibiotic resistance genes and mechanisms identified from potential antibiotic-producing bacterial isolates

AMR Mechanism	*B. laterosporus*-KM_C76	*P. elgii*-KM_B45	*P. oleatiliticus*-KM_D13)	*P. oleatiliticus*-KM_D46
Antibiotic inactivation enzyme		ANT(9)-I, CatA15/A16 family, Vat(D)/Vat(H)	ANT(6)-I, CatA15/A16 family	ANT(6)-I, CatA15/A16 family
Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target modifying enzyme	Cfr, RlmA(II)	Cfr, RlmA(II)	Cfr, RlmA(II)	Cfr, RlmA(II)
Efflux pump conferring antibiotic resistance	BcrA, BcrB, Tet(45)	BceA, BceB, BcrB	BceA, BceB, BcrB	BceA, BceB, BcrB

Gene conferring resistance via absence	gidB	gidB	gidB	gidB
Protein-altering cell wall charge conferring antibiotic resistance	GdpD, PgsA	GdpD, PgsA	GdpD, PgsA	GdpD, PgsA
Protein-altering cell wall structure conferring antibiotic resistance	VanH, VanM, VanX, VanXY-unclassified	VanXY-unclassified	VanH, VanI, VanX, VanXY-unclassified	VanH, VanI, VanX, VanXY-unclassified
Regulator modulating expression of antibiotic resistance genes	LiaR, LiaS, VanA/I/Pt-type, VanF/M-type, VanR-unclassified	BceR, BceS, LiaR, LiaS, VanF/M-type	BceR, BceS, LiaR, LiaS, VanA/I/Pt-type, VanF/M-type, VanR-unclassified	BceR, BceS, LiaR, LiaS, VanA/I/Pt-type, VanF/M-type, VanR-unclassified

Table 10 shows antibiotic susceptibility results of the identified potential antibiotic-producing strains corresponding to the antibiotic classes, including acquired resistance genes and antibiotic resistance-conferring chromosomal mutations identified from WGS. All the isolates showed resistance to Penicillin, Amikicin and chlortetracycline and were susceptible to gentamicin, erythromycin and tetracycline. *P. oleatilyticus*-KM_D13 was the only isolate resistant to

amoxicillin while the other three were resistant and *P. elgii*-KM_B45 was the only isolate susceptible to ampicillin. All isolates were sensitive to tetracycline, despite carrying the acquired (tet (45)) resistance gene and chromosomal (EF-G, EF-Tu) genes that the antibiotic targets. Chromosomal mutations linked to resistance to penicillin antibiotics (Alr and Ddl) were identified in all isolates. Aminoglycoside resistance is linked to acquired resistance genes (ANT(9)-I, ANT(6)-I) and chromosomal mutations (EF-G, EF-Tu, S10p, S12p), as well as gidB mutations. Macrolide resistance is associated with chromosomal mutations (EF-G, EF-Tu). Chromosomal mutations (rpoB, rpoC) are responsible for rifamycin resistance, while folA, Dfr, and folP mutations characterize trimethoprim-derivative resistance. Phenicol resistance is associated with the acquired resistance genes CatA15 and CatA16. Many acquired resistance genes contribute to polypeptide resistance, including BceA, BceB, BcrB, BceR, BceS, and several Van-type genes.

Table 10: Antibiotic susceptibility of antibiotic producing bacteria and predicted genotypic characteristics.

Antibiotic Class	Antibiotic tested	*P. oleatilyticusi* -KM_D13	*P. oleatilyticus*-KM_D46	*B. laterosporus* -KM_C76	*P. elgii* -KM_B45	Acquired resistance genes	Chromosomal mutations
Penicillin	AMX AMP PEN G	R R R	S R R	S R R	S S R	-- -- --	Alr, Ddl Alr, Ddl Alr, Ddl
Aminoglycoside	AMK GEN	R S	R S	R S	R S	ANT (9)-I, ANT (6)-I, --	EF-G, EF-Tu, S10p, S12p gidB, S10p, S12p
Macrolide	ERY	S	S	S	S	--	EF-G, EF-Tu
Tetracycline	TET CTC	S R	S R	S R	S R	*tet* (45) --	EF-G, EF-Tu --
Rifamycin	RIF	S	S	S	S	--	rpoB, rpoC
Trimethoprim-derivative	TMP	--	--	--	--	--	folA, Dfr, folP
Phenicol	CHL	--	--	--	--	*cat*A15/ *cat*A16	--
Polypeptide	BAC VAN	-- --	-- --	-- --	-- --	BceA, BceB, BcrB BceR and BceS, VanXY-unclassified, VanH, VanM, VanX, VanI, VanA/I/Pt-type, VanF/M-type, VanR-unclassified	-- --

Keys: Amoxicillin (AMX), Ampicillin (AMP), Penicillin G (PEN G), Amikacin (AMK), Kanamycin (KAN), Gentamicin (GEN), Erythromycin (ERY), Tetracycline (TET) Rifampicin (RIF), Trimethoprim (TMP), Chloramphenicol (CHL), Chlortetracycline (CTC), Bacitracin (BAC) and Vancomycin (VAN)., R;Resistant, S; Susceptible , --; not detected

All four isolates share similar antimicrobial resistance (AMR) mechanisms, encompassing various aspects such as antibiotic inactivation enzymes, target modification enzymes, efflux pumps, gene absences, and alterations in cell wall charge and structure, as well as regulation of resistance gene expression. Despite this overall similarity, there may be subtle differences in the specific genes or proteins involved in these mechanisms, indicating potential strain-level variations. Additionally, certain isolates may possess unique or additional AMR mechanisms not present in others, suggesting a strain-specific adaptation to certain antimicrobial agents. For example, the presence of specific antibiotic inactivation enzymes like Cfr and RlmA(II) in one isolate of *P. elgii* (KM_B45) shows strain-specific adaptations.

According to Jian et al., (2021) resistance genes can be categorized according to the classes of antibiotics they confer resistance to, these classes encompass tetracyclines (tet), sulfonamides (sul), β-lactams (bla), macrolides (erm), aminoglycosides (aac), fluoroquinolones (fca), colistin (mcr), vancomycin (van), and multidrug resistance (mdr). Hence the genes above represent the antibiotics that the isolates may be resistant to, which shows the importance of not only relying on phenotypic resistance patterns as stated by (Rahube et al., 2022). It is also critical to note that the expression and manifestation of resistance extend beyond the existence of resistance genes (ARGs) (Yamin et al., 2023). This encompasses factors such as the specific location of resistance genes within the bacterial genome, their proximity to other genetic elements, and their integration into mobile genetic elements(MGEs) (Yamin et al., 2023). The presence of resistance genes on MGEs, such as plasmids or transposons, can facilitate their horizontal transfer between bacterial strains and species, impacting the spread of resistance in diverse microbial populations (Yamin et al., 2023).

4.8 Predicted Secondary metabolite gene clusters in antibiotic-producing bacteria

Twenty-three secondary metabolite clusters were predicted across four bacterial isolates using antiSMASH. Non-ribosomal peptide Synthetase (NRPS), NRPS-like, Lanthipeptide, Linear azol(in)e-containing peptide (LAP), TransAT-PKS, Type-I Polyketide Synthase (T1PKS), Cyclic-lactone-autoinducer, Terpene and Phosphonate clusters were found to be present in all four isolates. Thiopeptide clusters are present in *P. elgii* and *P. oleatilyticus* isolates while NRP-metallophore clusters were present in *P. elgii*-KM_B45, *P. oleatilyticus*-KM_D13 and *B. laterosporus*-KM_C76. Various other clusters are present in only some isolates and not others, such as Opine-like metallophore (*P. oleatilyticus*-KM_D13 and *P. oleatilyticus*-KM_D46), HR-T2PKS Redox cofactor (*P. oleatilyticus* -KM_D13 and *P. oleatilyticus* -KM_D46), and RiPP-like clusters (*B. laterosporus*-KM_C76). The stacked graph (Figure 12) shows the frequency of the occurrence of Biosynthetic gene clusters (BGCs). NRPS is shown as the highest predicted clusters and present in all isolates with varying counts, the highest in *P. oleatilyticus*-KM_D13 followed by *P. elgii*-KM_B45 and the least being *B. laterosporus*-KM_C76. NRPS-like clusters also appear in all isolates, although in smaller numbers than the NRPS. The transAT-PKS gene clusters are also consistently present in all isolates, similarly, Type-I-PKS and various peptide clusters, such as thiopeptide, Linear azol(in)e-containing peptides (LAP), and cyclic-lactone-autoinducer, are distributed across the isolates. The *Paenibacillus* species (KM_D46, KM_D13, KM_B45) exhibit similarities in their BGCs profiles, with all three showing high frequencies of NRPS, terpene, lanthipeptide class I and V, PKS-like, Type-I PKS, and transAT-PKS BGCs. Additionally, they all contain RRE-containing, LAP, and cyclic-lactone autoinducer BGCs. However, there are minor variations, for instance, *P. oleatilyticus*-KM_D13 shows slightly higher frequencies of certain

unique BGCs (NRPS) compared to *P. oleatilyticus*-KM_D46 and *P. elgii*-KM_B45. *P. elgii*-KM_B45 also lacks the RRE-containing, redox cofactor, opine-like metallophore, and HR-T2PKS BGCs which are present in both *P. oleatilyticus*-KM_D46 and *P. oleatilyticus*-KM_D13.

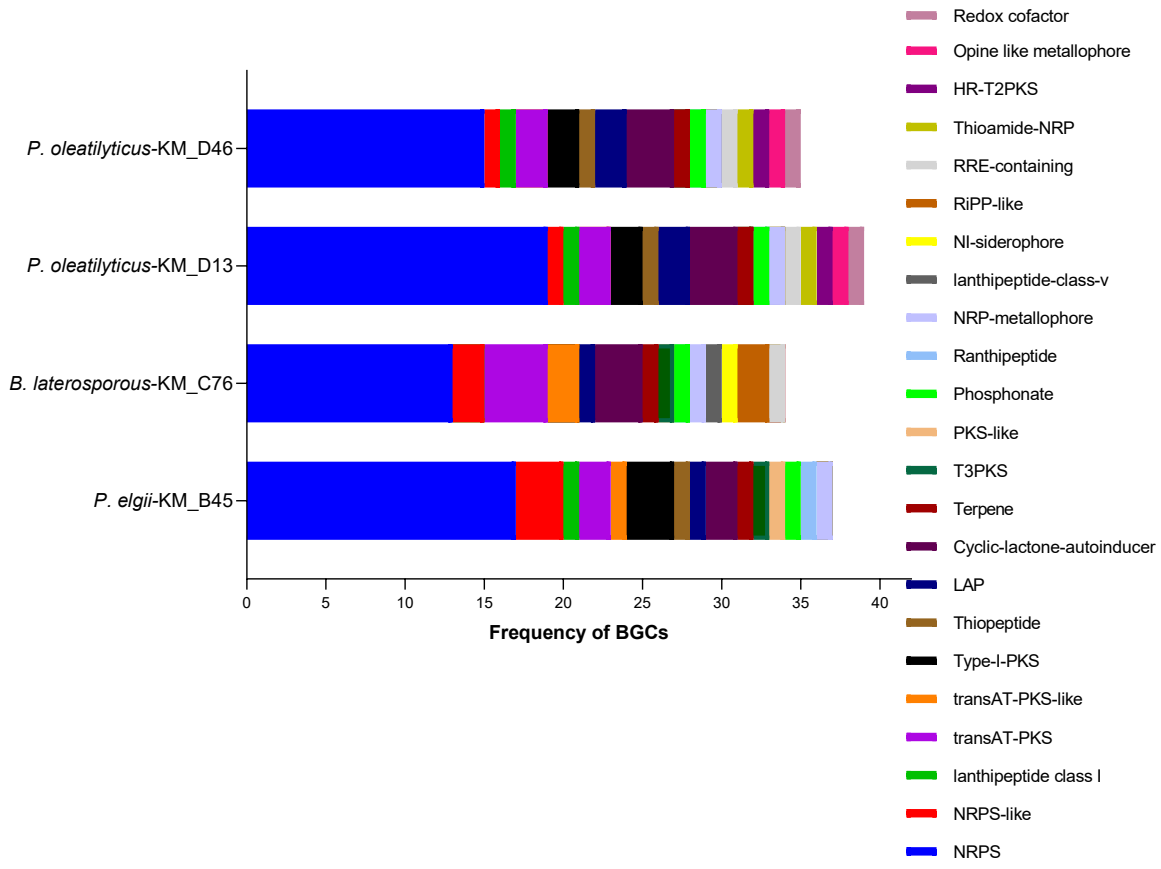


Figure 12: Frequency of identified biosynthetic gene clusters in potential antibiotic-producing bacteria

antiSMASH is a unique tool not only for its innovative integration of different tools ClusterFinder, ClustScan and BLAST but also for its accuracy in the annotation of individual BGCs (Medema et al., 2011; Naughton et al., 2017). Its strength lies in novel analytical approaches, such as employing BLAST-based gene cluster alignments and constructing secondary metabolite COG

phylogenetic trees for accessory genes, contributing to comprehensive and reliable identification of BGCs related to secondary metabolites (Medema et al., 2011). According to Naughton et al., 2017, BGCs are involved in the production of various bioactive molecules, such as antibiotics, antifungals, and other compounds with pharmaceutical or ecological significance.

BGCs and their associated biosynthetic pathways, depicting possible potential functions and roles of these metabolites within the four isolates are presented. The presence of NRPS, NRPS-like, Lanthipeptide, LAP, TransAT-PKS, T1PKS, Cyclic-lactone-autoinducer, Terpene, and Phosphonate clusters in all four isolates indicates possible shared functions of biosynthetic pathways across the microbial community. They show the abundance and diversity of secondary metabolite potential in the specified isolates. The commercial value of NRPs and polyketides lies in their diverse applications, particularly in the pharmaceutical industry because many antibiotics, anticancer drugs, immunosuppressants, and other clinically relevant compounds are derived from these classes of molecules (Naughton et al., 2017). NRPs were frequently predicted in all four isolates, and polyketides were predicted among the gene clusters, which suggests that these bacterial strains may biosynthesize commercially valuable and structurally diverse bioactive molecules.

In a related research by Naughton et al., (2017), to identify secondary metabolite gene clusters in the genus *Pseudovibirio*, the authors state that trans-AT PKS systems are involved in the biosynthesis of many pharmacologically important polyketides, such as the antibiotic mupirocin, hence it is exciting that the strains isolated in this study were also predicted to have these clusters, proving their potential pharmacological importance (Naughton et al., 2017). The study's

findings are similar to prior research, with NRPS being the most common class of BGCs (Shi et al., 2022; Meesil et al., 2023), suggesting their potential ecological importance. On the other hand, PKS, RiPPs, and terpene BGCs were less prevalent across all genomes (Shi et al., 2022; Meesil et al., 2023). These results align with the current study, proving the ecological significance of NRPS and the diverse biosynthetic pathways present in bacteria.

Streptomyces thinghirensis strain HM3 demonstrated inhibitory effects activity against pathogenic fungal species and animal pathogenic Gram-positive bacteria (Rehan et al., 2023). The SM-BGCs in antiSMASH predicted 16 clusters, including 1 siderophore, 1 lantipeptide, 2 PKS, 2 NRPS and NRPS-like fragments, two RiPP/RiPP, 2 butyrolactone and CDPS (tRNA-dependent cyclodipeptide synthases) in the genomic sequence of the bacterium (Rehan et al., 2023). The BGC-encoded secondary metabolites (32) that *Streptomyces formicae* (KY5) displayed included two PKS that might potentially encode formicamycins, eleven NRPS, and six terpenes (two clusters predicted to encode siderophore-like desferrioxamine, while another terpene was predicted to potentially produce the antibiotic albaflavenone) (Shah et al., 2019).

The antiSMASH analysis of *Streptomyces* sp. MP131-18 also revealed 36 gene clusters responsible for producing secondary metabolites (Paulus et al., 2017). The genome contained five predicted terpene three NRPS, a large group of RiPPs, and two siderophore molecules (Paulus et al., 2017). Additionally the draft genome sequence of *Streptomyces* sp. MWW064 contained three type I PKS, seven NRPS, and four hybrid PKS/NRPS gene clusters involved in rakicidin synthesis (Komaki et al., 2016). NRPS serve various purposes such as drugs, toxins, pigmentation agents, and siderophores while PKS metabolites play a significant role in medicine, commonly used to treat

delicate and degenerative diseases (Shah et al., 2019). Type 1 polyketide synthetase is organized into components that repetitively catalyze the elongation and synthesis of reduced PKS, as exemplified by erythromycin (Shah et al., 2019). Additionally, bacteria-produced terpenes are known for their antimicrobial properties and these properties make them effective in combating various pathogens, contributing to their importance in the pharmaceutical and biotechnological industries (Yamada et al., 2015; Shah et al., 2019). The findings in our study are in line with the studies above suggesting potential for developing new drugs and valuable products by studying bioactive compounds produced by these isolates.

4.9 Plant crude extraction

Different solvents (distilled water, methanol, ethyl acetate and *n*-hexane) were used to generate crude extracts from different plant parts (leaves, roots, bark and seeds) from three different plant species (*P. angolensis, A. littoralis*, and *A. zebrina)* (Figure 13). Generally, from all the samples, distilled water and methanol, which are polar solvents, seemed to extract more crude extracts, followed by ethyl acetate (intermediate polar), and lastly *n*-hexane (non-polar). The distilled water extract masses ranged from 0.514 - 3.642 grams (1.71 % - 12.14 %), methanol ranged from 0.851 - 3.348 grams(2.84 % - 11.16 %), ethyl-acetate 0.103 - 1.166 grams (0.34 % - 3.89 %), and *n*-hexane 0.03-0.367 grams (0.10 % - 1.22 %). The highest amount of extracts seemed to be from *P. angolensis* bark and *A. littoralis* leaf distilled water extract at 3.642 grams and 3.540 grams, respectively. These were followed by *A. littoralis* leaf, *P. angolensis* root and *P. angolensis* bark methanol extracts at 3.348 grams, 3.113 grams, 3.080 grams, respectively. The least amount

extracted appeared to be *n*-hexane *A. zebrina* and *A. littoralis* root extracts at 0.039 grams and 0.035 grams, respectively.

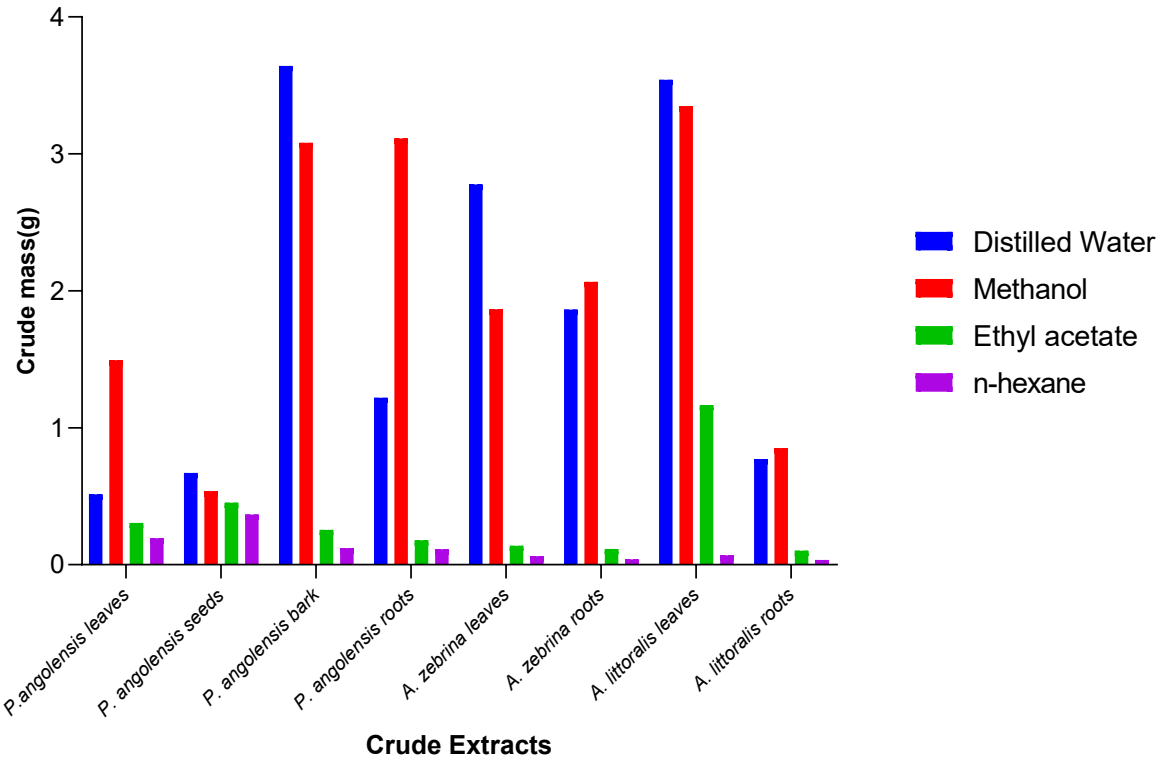


Figure 13: Mass of crude extracts from different plants and plant parts generated using different solvents

In a study by Chipinga et al., (2018), sequential extraction of the leaf, root, bark and fruit of *P. angolensis* was conducted using *n*-hexane, dichloromethane, methanol, and water to maximize the extraction of bioactive compounds of varying polarities. In this study, methanol was the most effective solvent and it yielded 13.76% from the leaf, 8.79% from the root, 16.00% from the bark, and 19.04% from the fruit. The values were generally comparable with our study where methanol yielded 4.98 % from leaves, 10.38% from roots, 10.27% from bark and 1.79% from fruits. In their

study, however, they did not report their yields from *n*-hexane and did not measure the weight of their water extracts since they had a liquid texture (Chipinga et al., 2018). The intermediate polarity solvent they used was dichloromethane while we used ethyl-acetate. However, the extraction yields were still comparable. They extracted 2.37% from the leaf, 3.03% from the root, 1.26% from the bark, and 1.64% from the fruit while we obtained 1.02% from the leaves, 0.6% from the root, 0.85% from the bark, and 1.51% from the fruit (Chipinga et al., 2018). The comparability of the findings attests to the extraction efficiency in our study and the variability may be due to plant properties associated with location since the study was done in Malawi.

Efficient extraction of bioactive compounds from plant materials involves a multifaceted process influenced by factors such as the chemical nature of phytochemicals, composition of the sample, extraction methods, sample particle size, solvent selection, extraction time, and the presence of interfering substances (Do et al., 2014). The solvent properties influencing extraction yield include its polarity, compatibility with the target compounds, pH, and temperature (Do et al., 2014). The choice of solvents must align with the polarity of target compounds, the extraction method and sample preparation techniques (Do et al., 2014). Based on data in Figure 12, generally, methanol and distilled water appeared to be more effective in extracting larger masses of crude extracts compared to ethyl acetate and *n*-hexane. For specific plant parts, the effectiveness of solvents in extracting mass seemed to vary, suggesting that the choice of solvent depends on the targeted compounds. For example, in *P. angolensis,* methanol appeared to be the most effective when using leaves. When extracting from the seeds and bark, however, distilled water seemed to be the most effective solvent. These trends show the importance of

selecting appropriate solvents based on the specific plant part and the desired compounds for extraction.

Other studies have generally shown that polar solvents such as methanol were the most effective in extracting higher yields of crude plant extracts (Do et al., 2014; Truong et al., 2019). For example, in a study by Truong et al., (2019), the extraction yield of *Severinia buxifolia* showed significant variations among different solvents. In this study, methanol was the most effective, yielding 33.2%, followed by distilled water at 27.0%, ethanol (12.2%), acetone (8.6%), chloroform (7.2%), and dichloromethane (4.9%). This aligns with the general trend in the literature, where polar solvents like methanol and water yield higher extraction efficiencies than less polar solvents (Do et al., 2014; Truong et al., 2019; Nawaz et al., 2020; El Mannoubi, 2023; Tourabi et al., 2023). The differences in extraction yields can be attributed to the composition of the plant material, with polar compounds being more soluble in solvents with higher polarities (Do et al., 2014; Truong et al., 2019).

4.10 Antibacterial activity of crude extracts on test bacteria

Twenty (out of thirty two) plant extracts were able to inhibit at least one of the five test bacteria (Table 11). Most (seventeen) of the plant extracts inhibited *S. aureus*, a gram-positive bacterium than the other 4 gram-negative bacteria. *P. mirabilis* was the most inhibited (by six extracts) gram-negative bacteria while *K. pneumoniae (*inhibited by two extracts*)* and *E. coli* (only one extract) were the least inhibited gram-negative bacteria. *P. mirabilis* was also the most inhibited (by six extracts) antibiotic-resistant isolate while *K. pneumoniae (*inhibited by two extracts*)* was

the least inhibited antibiotic-resistant isolate. A total of eleven crude extracts inhibited the three antibiotic-resistant bacteria (*P. mirabilis, P. aeruginosa* and *K. pneumoniae*) while eighteen crude extracts inhibited the two susceptible bacteria (*E. coli* and *S. aureus*). Generally, ethyl acetate and methanol crude extracts from the different plant species inhibited more test bacteria than the water and *n*-hexane extracts. A total of twelve extracts (*P. angolensis* water and *n*-hexane fruit extracts, *P. angolensis* ethyl-acetate and *n*-hexane bark and root extracts, *A. zebrina* and *A. littoralis* water leaf extracts, water and *n*-hexane root extracts) did not inhibit any of the test bacteria.

The effectiveness of plant extracts varied widely depending on the bacterial strain, the solvent used, the plant part used and the extract concentration. *P. angolensis* demonstrated the broadest antibacterial activity, its various parts (fruits, leaves, bark, and roots) inhibited a total of four of the five test bacteria. The leaf extracts exhibited the broadest efficacy, inhibiting all the bacteria except *E. coli*. Methanol and *n*-hexane extracts inhibited two test bacteria each (*P. aeruginosa* and *K. pneumoniae* for methanol, and *K. pneumoniae* and *S. aureus* for *n*-hexane*)*, while water and ethyl acetate extracts inhibited one test bacteria each (*P. aeruginosa and P. mirabilis,* respectively*)*. The root extracts inhibited a total of three test bacteria (*P. aeruginosa*, *K. pneumoniae*, and *S. aureus*). These three bacteria were all inhibited by the water extract while the methanol extract inhibited only *P. mirabilis* and *S. aureus.* The water and methanol bark extracts inhibited two (*P. mirabilis* and *S. aureus*) and methanol and ethyl acetate fruit extracts inhibited only one test bacteria (*S. aureus*).

A. littoralis was the second most effective plant, particularly with ethyl acetate root extracts which inhibited three (*E. coli*, *P. mirabilis*, and *S. aureus*) of the five test bacteria. Its methanol, ethyl acetate and *n*-hexane leaf extracts were effective against only *S. aureus*. *A. zebrina* inhibited the least variety of bacteria, inhibiting only *S. aureus* with its methanol, ethyl acetate, and *n*-hexane extracts from both leaves and roots.

Various crude extracts inhibited test bacteria at varying MICs. *P. mirabilis*-MN029 was inhibited by *P. angolensis* water bark and root extracts at MICs of 20 and 40 mg/ml, respectively, and *A. littorallis* ethyl acetate root extracts at an MIC of 10 mg/ml (Table 11). *K. pneumoniae* was inhibited by *P. angolensis* methanol and *n*-hexane leaf extract at MICs of 40 mg/ml. *E. coli* was only inhibited by the *A. littoralis* ethyl acetate root extract and at MIC of 20 mg/ml. *P. aeruginosa* was inhibited by the water root extract and the water leaf extracts of *P. angolensis* at MICs of 10 mg/ml and 20 mg/ml respectively.

S. aureus was inhibited by the following extracts from *P. angolensis*: water root extracts with an MIC of 20 mg/ml, *n*-hexane leaf extracts with an MIC of 20 mg/ml, ethyl acetate fruit extracts with an MIC of 10 mg/ml, and both water and methanol bark extracts, which also had MICs of 10 mg/ml. It was also inhibited by *A. zebrina* methanol, ethyl acetate, and *n*-hexane leaf extracts (MICs of 40 mg/ml), *A. zebrina* methanol root extract (MIC 40 mg/ml), *A. littoralis* methanol and *n*-hexane leaf extracts (MICs of 40 mg/ml) and *A. littoralis* methanol root extracts (MIC of 40 mg/ml). Nine (out of thirty two) plant extracts were able to inhibit at least one of the test bacteria at a concentration of down to 5 mg/ml, which was the lowest concentration used in disc diffusion (Table 11). For these plant extracts, MIC was determined.

Table 11: Zones of inhibition (mm) of crude extracts on test bacteria

Plant part	Solvent	Conc (mg/ml)	Zone of inhibition (mm)				
			E. coli	*P. aeruginosa*	*P. mirabilis*	*K. pneumoniae*	*S. aureas*
***P. angolensis* Fruits**	Methanol	40	-	-	-	-	9.6 ± 0.94
		20	-	-	-	-	9.5 ± 0.0
		10	-	-	-	-	8.5 ± 0.41
		5	-	-	-	-	7.6 ± 0.94
	Ethyl acetate	40	-	-	-	-	8.5 ± 0.41
		20	-	-	-	-	6.8 ± 0.24
		10	-	-	-	-	7.0 ± 0.0
***P. angolensis* Leaves**	DH_2O	40	-	6.7 ± 0.24	-	-	-
		20	-	6.3 ± 0.24	-	-	-
	Methanol	40	-	8.8 ± 0.24	-	7.5 ± 0.41	-
		20	-	7.7 ± 0.24	-	-	-
		10	-	6.7 ± 0.24	-	-	-
		5	-	6.2 ± 0.24	-	-	-
	Ethyl acetate	40	-	-	12.3 ± 0.47	-	-
		20	-	-	9.9 ± 1.6	-	-
		10	-	-	7.9 ± 1.5	-	-
		5	-	-	7.0 ± 0.8	-	-
	n-hexane	40	-	-	-	8.2 ± 0.24	8.2 ± 0.24
		20	-	-	-	-	5.6 ± 0.47
***P. angolensis* Bark**	DH_2O	40	-	-	11.3 ± 0.9	-	9.5 ± 0.71
		20	-	-	7.5 ± 0.41	-	7.3 ± 0.24
		10	-	-	-	-	6.2 ± 0.24
	Methanol	40	-	-	12.2 ± 0.24	-	9.5 ± 0.41
		20	-	-	11.5 ± 0.0	-	8.7 ± 0.24
		10	-	-	9.0 ± 0.0	-	6.6 ± 0.47
		5	-	-	9.0 ± 0.0	-	-
***P. angolensis* Roots**	DH_2O	40	-	7.3 ± 0.47	7.6 ± 0.94	-	7.2 ± 0.24
		20	-	6.7 ± 0.24	-	-	6.3 ± 0.24
		10	-	6.2 ± 0.24	-	-	-
	Methanol	40	-	-	12.2 ± 0.62	-	10.3 ± 0.47
		20	-	-	9.8 ± 0.24	-	9.2 ± 0.24
		10	-	-	9.0 ± 0.0	-	7.0 ± 0.0

		5	-	-	7.8 ± 0.24	-	6.6 ± 0.47
***A. zebrina* Leaves**	Methanol	40	-	-	-	-	9.0 ± 0.82
	Ethyl acetate	40	-	-	-	-	7.9± 1.41
	n-hexane	40	-	-	-	-	10.2 ± 0.24
***A. zebrina* Roots**	Methanol	40	-	-	-	-	10.0 ± 0.82
	Ethyl acetate	40	-	-	-	-	12.8 ± 0.62
		20	-	-	-	-	11.9 ± 1.08
		10	-	-	-	-	9.1 ± 1.03
		5	-	-	-	-	8.0 ± 0.82
***A. littoralis* leaves**	Methanol	40	-	-	-	-	7.0 ± 0.82
	Ethyl acetate	40	-	-	-	-	9.7 ± 0.47
		20	-	-	-	-	8.0 ± 0.0
		10	-	-	-	-	6.6 ± 0.47
		5	-	-	-	-	6.6 ± 0.47
	n-hexane	40	-	-	-	-	9.3 ± 0.94
***A. littoralis* Roots**	Methanol	40	-	-	-	-	10.5 ± 0.41
	Ethyl acetate	40	15.7 ± 2.9	-	13.8 ± 0.24	-	15.7 ± 0.47
		20	11 ± 0.8	-	9.2 ± 0.24	-	14.3 ± 0.47
		10	-	-	6.2 ± 0.24	-	12.5 ± 0.71
		5	-	-	-	-	11.3 ± 1.25

S. aureus was inhibited by six crude extracts at concentrations below 5 mg/ml (Table 12). The extracts that showed the lowest MIC were *A. littoralis* and *A. zebrina* ethylacetate root extracts at 0.075 mg/ml. These were followed by *A. littoralis* ethylacetate leaf extract (0.625 mg/ml), *P. angolensis* root and fruit methanol extracts (1.25 mg/ml) and *P. angolensis* fruit ethyl acetate extract (2.5 mg/ml). *P. mirabilis* was inhibited by three crude extracts. The lowest MIC (1.25 mg/ml) was obtained from *P. angolensis* root and bark methanol extracts while *P. angolensis* leaf ethyl acetate extract had an MIC of 2.5 mg/ml. *P aeruginosa* was inhibited by only one extract below 5 mg/ml (*P. angolensis* leaf methanol extract) at an MIC of 2.5 mg/ml.

Table 12: Minimum inhibitory Concentrations of extracts on test bacteria

Test bacteria	Crude Extract	MIC (mg/ml)
S. aureus	*P. angolensis* fruits (ethyl acetate)	2.5
	P. angolensis fruits (methanol)	1.25
	P. angolensis roots (methanol)	1.25
	A. littoralis leaves (ethyl acetate)	0.625
	A. zebrina roots (ethyl acetate)	0.075
	A. littoralis roots (ethyl acetate)	0.075
P. mirabilis	*P. angolensis* leaves (ethyl acetate)	2.5
	P. angolensis bark (methanol)	1.25
	P. angolensis roots (methanol)	1.25
P. aeruginosa	*P. angolensis* leaves (methanol)	2.5

Certain plant parts, such as *A. littoralis* leaves and roots, demonstrated activity at concentrations as low as 5 mg/ml, emphasizing their efficacy even at lower concentrations. Distilled water and *n*-hexane inhibited the least isolates, both failing to inhibit any bacterium down to 5 mg/ml, showing the importance of specific plant part and solvent choices for extracting bioactive compounds with potential antimicrobial applications.

The data provided in Table 12 contains information on the minimum inhibitory concentrations (MIC) of crude plant extracts against various test bacteria. The results indicate that *P. angolensis* seeds have antimicrobial properties, particularly their methanol extracts, with MIC values of 1.25

mg/ml against *S. aureus*. Furthermore, *P. angolensis* bark methanol extract demonstrated potent inhibitory effects against *P. mirabilis* (MIC: 1.25 mg/ml), highlighting its potential as a source of bioactive compounds. Similarly, *P. angolensis* root methanol extracts demonstrated broad-spectrum activity by inhibiting both *P. mirabilis* and *S. aureus* at 1.25 mg/ml. *A. zebrina* roots also displayed potency with a MIC of 0.075 mg/ml against *S. aureus*, demonstrating its high antimicrobial efficacy while *A. littoralis* leaves and roots also showed significant activity against *S. aureus*, with MIC values of 0.625 mg/ml and 0.075 mg/ml, respectively. The MIC values ranged from the lowest of 0.075 mg/ml for *A. zebrina* and *littoralis* to the highest of 2.5 mg/ml for *P. angolensis* (leaves and fruits).

The investigation into the antibacterial activity of *P. angolensis* extracts depicts promising results, suggesting the potential medicinal value of its different plant parts. The fruit extracts, particularly methanol and ethyl acetate extracts, displayed significant inhibition against *S. aureus*, showcasing specificity in antimicrobial effects while bark extracts, both in water and methanol, exhibited broad-spectrum activity by inhibiting *P. mirabilis* and *S. aureus*. Leaf extracts on the other hand demonstrated diverse inhibitory effects against various bacteria, with different solvents showing efficacy against specific strains; water extracts against *P. aeruginosa*, methanol extracts against *P. aeruginosa* and *K. pneumoniae*, ethyl acetate extracts against *P. mirabilis*, and *n*-hexane extracts against *K. pneumoniae* and *S. aureus*. Water and methanol root extracts, displayed antimicrobial activity against *P. aeruginosa, P. mirabilis*, and *S. aureus*. The MIC values ranging from 40 mg/ml to 1.25 mg/ml indicate concentrations at which bacterial growth was inhibited. The study on *P. angolensis* by Chipinga et al., (2018), evaluated its antimicrobial properties against resistant strains, including *Candida krusei, Staphylococcus aureus,*

Streptococcus agalactiae, and *Escherichia coli*. The findings indicated that most extracts exhibited maximum inhibition at a concentration of 0.166 g/ml *against S. aureus, S. agalactiae*, and *C. krusei*. The dichloromethane root extract showed minimum inhibition as low as 0.01042 g/ml for *S. agalactiae*, while leaf methanolic extracts inhibited the same organism at the lowest concentration of 0.0208 g/ml. The study indicated that the observed antibacterial and antifungal properties were attributed to the presence of tannins, flavonoids, and saponins detected during phytochemical screening and acknowledged that the use of crude extracts might reduce the effectiveness of active compounds due to impurities and potential antagonistic effects of other constituents (Chipinga et al., 2018). All extracts tested did not exhibit activity against *E. coli*, which was also consistent with the findings in our study (Tables 11 and 12). The variability in MIC values for both studies may be influenced by factors such as the bioactive compound composition of the extracts, the extraction efficiency, and the inherent resistance or susceptibility of the bacterial strains (Chipinga et al., 2018). These findings highlight the potential of *P. angolensis* as a source of natural antimicrobial agents, but further studies are needed to isolate and identify specific bioactive compounds and explore potential synergistic effects for enhanced efficacy. Findings from research by Samie et al., (2009) also show the broad-spectrum antimicrobial potential of *P. angolensis*, particularly its acetone extract with MIC values of 2 mg/ml to 0.0156 mg/ml. The ability of the extract to effectively inhibit the growth of both Gram-positive (*S. aureus*) and Gram-negative (*Enterobacter cloacae*) bacteria suggests a versatile antimicrobial activity (Samie et al., 2009).

The investigation into the antibacterial activity of *A. zebrina* and *A. littoralis* extracts showed promising results, showcasing their potential as natural sources of antibacterial agents. Both

plant species exhibited inhibitory effects against *S. aureus*, indicating their efficacy in combating *S. aureus* bacterial infections. *A. littoralis*, however, demonstrated a broader spectrum of activity, with its root extracts showing inhibition not only against *S. aureus* but also against Gram-negative bacteria such as *E. coli* and *P. mirabilis*. This broader efficacy suggests its potential application in addressing a wider range of bacterial infections. The choice of solvents during the extraction process, including methanol, ethyl acetate, and n-hexane, adds a layer of complexity, influencing the spectrum and potency of the bioactive compounds extracted (Truong et al., 2019). In a study by Athiban et al. (2012), the antimicrobial effectiveness of *Aloe vera*, a close relative, was evaluated based on the presence of zones of inhibition, where *E. coli, Enterococcus faecalis*, and *S. aureus* exhibited inhibition zones measuring 24 mm, 21 mm, and 24 mm, respectively. The findings imply that *Aloe vera* demonstrates substantial antimicrobial efficacy, particularly against *E. coli* and *S. aureus*. The significant size of the inhibition zones suggests noteworthy potential for practical applications, such as the formulation of antimicrobial agents or incorporation into products designed to hinder bacterial growth (Athiban et al., 2012). Moreover, given the specificity of the inhibitory effects against both Gram-negative and Gram-positive bacteria, the bioactive compound(s) may exhibit a broad-spectrum antimicrobial activity, enhancing its versatility in potential applications.

In a study by Arbab et al., (2021), the antibacterial activity of *Aloe vera* gel extracts was assessed against common skin infection pathogens, including *E. coli, Shigella, Salmonella spp*., and *S. aureus*, all of which exhibited positive results. At a volume of 100 µl, both *A. vera* leaf and root extracts demonstrated similar sensitivity compared to standard antibiotics against all bacterial isolates, encompassing both Gram-negative and Gram-positive strains (Arbab et al., 2021). The

investigation into the microbial activity of *Aloe vera* gel aimed to determine the most effective solvent for extraction and identify the organism most susceptible to the crude *Aloe vera* gel extract (Ifeanyi & Eziokwu, 2014). Compared to aqueous and methanol extracts, the ethanol extract exhibited superior MIC against *Escherichia coli, Staphylococcus aureus*, and *Candida albicans* (0.125, 0.125, and 0.40 mg/ml, respectively). In our research, the lowest MIC value was recorded at 0.075 mg/ml against *S. aureus*, highlighting the potential properties of *Aloe sp*., particularly against specific bacterial strains (Ifeanyi & Eziokwu, 2014). This suggests the presence of active compounds in the plant that contribute to its antimicrobial properties. A Silva et al. (2013) report establishes a criterion for considering plants to exhibit antimicrobial activity based on their MICs. According to their findings, plants with MICs below 8 mg/mL are generally recognized to possess some level of antimicrobial activity. The study emphasizes the significance of species with MICs below 1 mg/mL, indicating a higher potential for antimicrobial efficacy. In our study the *A. littoralis* and *A. zebrina* had MIC values ranging from 0.075 to 0.625 mg/mL against *S. aureus* aligning with this criterion, further supporting the notion that these plant species exhibit notable antimicrobial properties, thus warranting further investigation into their specific bioactive compounds and mechanisms of action. Our MICs also align with a criterion by Seukep et al. (2016) for significantly active antibacterial agents, reinforcing the potential of *Aloe sp.* as an effective natural antimicrobial agent. According to this classification, crude extracts are deemed significantly active antibacterial agents when their MIC values are below 100 µg/mL for crude extracts or 10 µg/mL for isolated compounds. Activity is considered moderate if the MIC falls within the range of 100 to 625 µg/mL for crude extracts or 10 to 100 µg/mL for individual molecules, this offers an approach to assess the potency of phytochemicals in inhibiting bacterial

growth, enabling consistent evaluation and comparison of their antimicrobial efficacy (Seukep et al., 2016). In our study, the significantly active crude extracts were *A. zebrina* and *A*. *littoralis* ethyl acetate root extracts at an MIC of 0.075 mg/ml while the moderate crude extracts was *A. littoralis* ethyl acetate leaf extract at an MIC value of 0.625 mg/ml.

Chapter Five

5.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

The study concludes that soil and water samples from the dried pond site and mining area harbor diverse bacterial species, including novel ones, with potential antibacterial properties. This is supported by the identification of four bacterial isolates in this study, two of which are understudied bacterial species, importantly, the first to be isolated and sequenced in Botswana and Africa. The soil environments are recognized for their extreme and fluctuating conditions, likely select bacteria with unique adaptations, possibly including the production of new antibacterial compounds.

Four antibiotic-producing bacteria (*P. elgii*-KM_B45, *B. laterosporus*-KM_C76, *P. oleatilyticus*-KM_D13 and *P. oleatilyticus*-KM_D46) were isolated and identified in this study as promising sources for new treatments. The isolates also exhibited 23 BCGs in their genomes, previously shown to aid in producing clinical antibiotics. The first *P. oleatilyticus* was discovered in soil in India using Illumina MiSeq shotgun sequencing. There is now three genome assemblies of *P. oleatilyticus* on NCBI database, including the two isolated from this study. In addition, there is only one complete genome assembly of *P. elgii* and nine assemblies available on NCBI. With regards to the *B. laterosporus strains*, there are 8 complete genomes, 21 scaffolds, and 24 contig assemblies. The complete genome records in the NCBI database were mostly achieved via long-read sequencing using PacBio technology, while the incomplete genomes were mostly from short-read sequencing using different Illumina platforms, 454, and Sanger. The sequence data of antibiotic-producing bacteria from this study was generated using Illumina, which provides only

the short reads which are challenging in terms of the assembly process. Therefore, future work should look into complementing the sequence data with long-read technologies sequencing like Pacific Biosciences and Oxford Nanopore Technology to obtain complete WGS assembly. Complete WGS of these antibiotic-producing bacteria could provide comprehensive data which is more accurate in the representations of whole bacterial genomes compared to short-read sequencing.

Nonetheless, this discovery of antibiotic-producing bacteria provides great opportunity and potential for further research into their genomics related to antibacterial activity, which could be valuable in addressing the problem of antibiotic resistance. The availability of these genome sequences in NCBI database is also important for the scientific community to explore further using bioinformatics tools. These sequences can be evaluated for genes responsible for bioactive compounds, which aids the search for novel antibiotics. Publicly accessible genome sequences also enable other researchers to validate and improve on previous discoveries, thus improving the search for new antibacterial drugs. Additionally, secondary metabolites from the isolates should be extracted and studied using *in vitro* techniques to further study their broad bioactivity against MDR isolates.

From the three indigenous plants *P. angolensis*, *A. littoralis*, and *A. zebrina,* polar solvents extracted more crude extracts than the intermediate and non-polar solvents. The crude extracts from these plants have been found to possess antibacterial properties due to the presence of bioactive compounds. These compounds were able to inhibit the growth of both MDR and susceptible bacterial isolates. Of all the crude extracts, twenty were able to inhibit at least one

test bacteria while 12 did not inhibit any test bacteria. *P. angolensis* inhibited most of the MDR bacteria, followed by *A. littoralis* while *A. zebrina* did not inhibit any of the MDR isolates. *P. angolensis* exhibited broad-spectrum activity, inhibiting most of the test bacteria, while *Aloe spp* showed the lowest MICs for specific bacteria. The ethyl acetate root extracts of *A. littoralis* and *A. zebrina* were the most effective, with lowest MIC of 0.075 mg/ml against *S. aureus*. The lowest MIC (1.25 mg/ml) for MDR bacteria was observed with *P. angolensis* root and bark methanol extracts. Generally, the most effective extracts were those obtained using methanol and ethyl acetate compared to water and n-hexane. The variations in antibacterial effectiveness among the plants were likely due to differences in the concentrations and types of bioactive compounds, which were extracted using different solvents.

Further studies should be conducted on the crude extracts to determine the specific secondary metabolites present, such as phenolics and flavonoids. Gas chromatography/mass spectrometry should be utilized to analyze the components of each crude extract, particularly those with lower MICs towards MDR. These extracts can potentially be used as alternative drugs or in synergy with existing antibiotics.

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