ANTIMICROBIAL STUDIES AND GC-MS ANALYSIS OF AQUEOUS EXTRACT OF *Azadirachta indica* STEM BARK

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY, FACUTY OF PHARMACY, UNIVERSITY OF BENIN, BENIN CITY IN PARTIAL FUFILMENT OF THE REQUIREMNTS FOR THE AWARD OF THE DOCTOR OF PHARMACY (PHARM.D) DEGREE

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CERTIFICATION

This is to certify that this work carried out by **Lawal Abiola Afeez** with Matriculation Number **PHA1808400** as an undergraduate final year project work, in partial fulfillment of the requirement of the requirement for the award of the Doctor of Pharmacy (Pharm.D) degree and submitted to the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

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DEDICATION

This project work is dedicated to the Almighty for seeing me through this phase of my life, and to my lovely family; The Lawals and Badrus for their support throughout my journey in school of Pharmacy.

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ABSTRACT

This study evaluated the antimicrobial activity and chemical constituents of the aqueous extract of *Azadirachta indica* stem bark using standard antimicrobial methods and GC-MS analysis. The antimicrobial activity was tested against clinical isolates, including *Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Candida albicans, Klebsiella spp.,* and *Aspergillus niger*, using the agar-well diffusion method. The extract, a dark brown substance with a yield of 12%, exhibited inhibition zones ranging from 13 to 40 mm. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified nine bioactive compounds, with 9-Octadecenoic acid (Z)-, methyl ester being the most abundant (64.56%). Other identified compounds include Pyrazine, tetramethyl-; 9-Tetradecenal, (Z)-; Di-n-octyl phthalate; Hexadecanoic acid, methyl ester; Ethanone, 1-cyclododecyl-; Methyl stearate; *N,N*-bis[2-trimethylsiloxyethyl] ethanamine; and Hexadecanoic acid, 1-[(2-aminoethoxy) hydroxy] derivatives. Some of these compounds have documented antimicrobial properties, which align with the observed inhibitory activity against Gram-positive and Gram-negative bacteria, as well as fungi. These findings support the ethnomedicinal use of *Azadirachta indica* in managing infections caused by the tested clinical isolates.

KEYWORDS: *Azadirachta indica*, Aqueous extract, Antimicrobial, Stem bark, Fungi, Bacteria

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CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Microorganisms, including bacteria, viruses, fungi, and microbial parasites, are fundamental to life on Earth, driving essential ecological processes such as nutrient cycling and decomposition. While many microorganisms play beneficial roles, certain pathogenic species have historically contributed to significant morbidity and mortality, particularly in regions with inadequate healthcare access (Almatrood *et al.*, 2024). The structural diversity of these microorganisms is a critical factor influencing their survival and pathogenicity, particularly in the context of infection. Bacteria are primarily classified into two categories based on their cell wall composition: Gram-positive bacteria (GPB) and Gram-negative bacteria (GNB). Gram-positive bacteria, characterized by a thick peptidoglycan layer, exhibit resilience to physical damage and can thrive in extreme conditions, with notable examples including *Staphylococcus aureus* and *Streptococcus pneumoniae*. Conversely, Gram-negative bacteria possess a thinner peptidoglycan layer and an additional outer membrane composed of lipopolysaccharides, which not only acts as a protective barrier against antibiotics but also contributes to their heightened resistance to treatment (Davati *et al.,* 2024). This structural complexity is exemplified by *Escherichia coli and Pseudomonas aeruginosa*, both of which pose significant challenges in clinical settings due to their resistance profiles. The advent of antimicrobial agents, including antibiotics, antifungals, antivirals, and antiparasitics, has transformed the landscape of infectious disease management, significantly reducing mortality rates and facilitating complex surgical medical procedures. Nevertheless, the misuse and overuse of these agents have precipitated a global crisis of antimicrobial resistance (AMR), wherein microorganisms evolve mechanisms to evade the effects of treatments designed to

eradicate them (Chaurasia, 2022; Mohanty *et al.,* 2023). This phenomenon has resulted in increased treatment failures, prolonged hospitalizations, and rising healthcare costs, underscoring the urgent need for innovative therapeutic strategies. In response to this growing threat, medicinal plants have emerged as a promising alternative due to their bio-active compounds with inherent antimicrobial properties. With a rich history in traditional medicine, these natural resources hold significant potential for developing novel treatments capable of addressing the challenges posed by antimicrobial resistance (AMR), thereby safeguarding public health for future generations. Research into the antimicrobial potential of medicinal plants is rapidly advancing, with numerous studies demonstrating their efficacy against resistant strains and paving the way for groundbreaking drug discovery.

1.2 GLOBAL BURDEN AND EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE (AMR)

Antimicrobial resistance (AMR) represents one of the most significant global health threats of the 21st century, posing a grave challenge to public health, the economy, and health systems worldwide. While AMR is a global issue, its impact is especially pronounced in low- and middle-income countries (LMICs), where healthcare system is mostly underdeveloped, and regulations on drug use are less stringent, and as such frequent disposal of antibiotics from community practice without proper laboratory test, or indication . The World Health Organization (WHO) estimates that over one million deaths annually are attributed to AMR, with projections indicating this could rise to 8.2 million deaths per year by 2050 if no action is taken. The majority of these deaths occur in regions where AMR has been left unchecked, compounding the burden on already fragile healthcare systems. (Kariuki *et al*, 2024; Sakalauskienė & Radzevičienė, 2024)

In high-income countries, AMR has been observed in hospital-acquired infections, with resistant pathogens such as Methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-resistant Enterococci (VRE), and Carbapenem-resistant Enterobacteriaceae (CRE) becoming increasingly common in healthcare settings. These infections often result in longer hospital stays, more intensive treatments, and higher healthcare costs, while also contributing to higher rates of morbidity and mortality. In low-income regions, the situation is more severe due to limited access to effective healthcare, overuse and misuse of antibiotics, and the absence of robust infection prevention and control measures. A report by the Centers for Disease Control and Prevention (CDC) highlights that over two million AMR infections occur annually in the United States alone, leading to approximately 29,000 deaths and more than $4.7 billion in medical expenses. Similarly, in the European Union and European Economic Area (EU/EEA), AMR results in over 670,000 infections and 33,000 deaths each year, creating significant economic burdens as consequence. (De oliveira *et al.,* 2022)

In Africa, the rise of AMR is particularly concerning due to a combination of factors such as the widespread use of antibiotics without prescription, the consumption of substandard or counterfeit medicines, and inadequate infection control practices in hospitals. Resistant bacterial strains like *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* have been increasingly reported across the continent, leading to treatment failures and more severe infections. In many African countries, over-the-counter availability of antibiotics, poor hygiene, and inadequate sanitation contribute to the spread of resistance. The African Union (AU) has expressed concern about the growing impact of AMR on public health and economic development. The World Bank has warned that AMR could cost the global economy as much as $3.4 trillion annually by 2030, disproportionately affecting developing countries. The projected economic losses are expected to be driven by reduced productivity, longer hospital stays, and higher medical costs (McDonnell *et al.,* 2024). For African nations,

the impact could be devastating, as many rely on affordable antibiotics for treating common infections.

Agriculture also plays a key role in the AMR crisis in Africa, as antibiotics are often used in livestock farming to promote growth and prevent disease. This practice not only accelerates the emergence of resistant bacteria but also results in the transmission of these resistant strains to humans via the food chain. Additionally, limited access to diagnostic tools means that antibiotics are often prescribed indiscriminately for conditions that do not require them, further fueling resistance. (Abou-Jaoudeh et al., 2024)

Given the growing burden of AMR globally, particularly in regions like Africa, as previously discussed, there is an urgent need for concerted efforts to curb the spread of resistance. These efforts include strengthening infection prevention and control measures, improving the regulation and rational use of antibiotics, enhancing surveillance systems, and developing new treatments and alternatives to current antibiotics. With traditional antibiotics becoming less effective, the search for novel antimicrobials, particularly those from under-explored sources like medicinal plants, has become a crucial priority. Furthermore, this also requires a comprehensive approach, including the exploration of alternative drug sources, improved healthcare practices, and global collaboration to combat this pressing health crisis. (Mendonca, 2023)

1.3 RESISTANCE TO CONVENTIONALLY AVAILABLE ANTIMICROBIAL AGENTS

The advent of the antimicrobial era, particularly with the discovery of penicillin in the 1920s, was a significant relief for the public health, by revolutionizing the treatment of infectious diseases and drastically reducing mortality rates. However, this golden age of antibiotics has

been met with a rising tide of antimicrobial resistance (AMR), which poses a growing challenge to global health. As the use of antibiotics has become widespread, microorganisms have evolved mechanisms to evade the effects of these drugs, rendering previously treatable infections difficult or impossible to manage. (Bhardwaj *et al.,* 2024)

Antimicrobial resistance is not limited to one class of drugs but extends across multiple classes, with certain pathogens exhibiting resistance to an array of antibiotics. This phenomenon, known as cross-resistance, occurs when resistance to one antimicrobial drug leads to resistance to other drugs within the same class or even across different classes of antibiotics. A prime example is aminoglycosides, a class of antibiotics that includes drugs such as gentamicin and amikacin. Once a pathogen develops resistance to one aminoglycoside, it often becomes resistant to all drugs in this class, making treatment options severely limited. The mechanism of cross-resistance in aminoglycosides involves the modification of the antibiotic by enzymes that are capable of modifying several aminoglycoside molecules, rendering them ineffective. (Baruah *et al.,* 2024)

An alarming trend is the emergence of superbugs, which are bacteria that have developed resistance to multiple antibiotics, sometimes from different classes. These superbugs include well-known pathogens such as Methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-resistant Enterococci (VRE), and Carbapenem-resistant Enterobacteriaceae (CRE) (Abdelazeem et al 2023; Kumar et al 2023). These bacteria have acquired resistance through various mechanisms, such as the production of enzymes that break down antibiotics For alterations to their cellular structure that prevent antibiotics from entering or binding to the bacteria. Superbugs pose a serious threat to public health because they often result in infections that cannot be treated with existing antibiotics, leading to prolonged illness, longer hospital stays, increased healthcare costs, and higher mortality rates. (Kumari, 2023)

*Escherichia col*i (*E. coli*), a common bacterium that can cause a range of infections including urinary tract infections and sepsis, has demonstrated resistance to several classes of antibiotics. One example is resistance to β-lactam antibiotics, including penicillins and cephalosporins. *E. coli* can produce β-lactamases, enzymes that break down the β-lactam ring structure of these antibiotics, rendering them ineffective. Additionally, *E. coli* has also developed resistance to ciprofloxacin, a fluoroquinolone antibiotic commonly used to treat urinary tract infections. Resistance to ciprofloxacin in *E. coli i*s often mediated by mutations in the bacterial DNA that alter the target enzyme of the drug, preventing the antibiotic from effectively inhibiting bacterial replication (Safitri et al., 2024). Similarly, *Pseudomonas aeruginosa*, a notorious pathogen known for its resistance to multiple antibiotics, has become increasingly resistant to commonly used treatments, including β-lactams and aminoglycosides. This pathogen can develop resistance through various mechanisms, such as the production of efflux pumps (Using the MexX and MexY gene) which expels antibiotics from the bacterial cell, the alteration of antibiotic targets, and the production of β-lactamases that degrade β-lactam antibiotics (Ali & Ghassan, 2024). *Pseudomonas aeruginosa* infections are particularly concerning in immunocompromised patients, where treatment options are limited and infection control becomes more challenging. (Algammal *et al.,*2023)

1.4 PLANT BIOMASS IN THERAPEUTIC MANAGEMENT OF DISEASE

Plants have long been central to the therapeutic management of diseases, with their use in traditional medicine spanning millennia. Early civilizations relied heavily on plant-based remedies, harnessing the natural bioactive compounds contained within them, such as alkaloids, flavonoids, terpenoids, and polyphenols. These compounds are responsible for the plants' antimicrobial, anti-inflammatory, analgesic, and antioxidant properties, making them effective treatments for a wide range of ailments. A recent study also revealed their

therapeutic potential in treatment of various types of cancer, most especially breast cancer. (Azam *et al.,* 2023)

However, before they are available for their therapeutic benefits they normally undergo the collection and harvesting processes. The collection of plant materials for medicinal use is a meticulous process which requires harvesting various parts of the plant, including leaves, roots, stems, flowers, and seeds. The timing of collection is crucial, as it influences the potency of the bio-active compounds. For instance, some plants are most effective when harvested during specific seasons or times of day. This is seen in Eucalyptus and Neem with their alkaloid content highest in the fourth Quarters of the year (Akiode *et al* 2021). The preference for herbal medicine and plant-based treatment is rooted in their accessibility, affordability, and the relatively few side effects they produce when used appropriately. This is especially evident in regions with limited access to modern healthcare, where plant remedies often serve as valuable alternatives to synthetic drugs. Among the many plants with therapeutic potential, *Azadirachta indica*, commonly known as neem, stands out. Renowned for its potent medicinal properties, neem has become a cornerstone of traditional medicine, especially in India and Africa. Neem is recognized for its broad spectrum of antimicrobial, anti-inflammatory, anti-diabetic, and antimalarial activities. Its widespread use and documented therapeutic benefits have established neem as a vital part of various healing traditions, particularly in tropical and subtropical regions. (Devi & Sharma, 2023)

In Africa, neem holds significant cultural and medicinal importance. In Nigeria, for example, the plant is commonly referred to as "Dongoyaro" among the Yoruba people, while the Igbo call it "Ayo" or "Onunu". These names highlight the deep-rooted history and reverence for neem in local medicinal practices. Various parts of the neem tree—such as the leaves, bark, seeds, and oil—are used to treat a variety of health conditions(Sidat & Chauhan, 2023). The

diversity of its uses speaks to the plant's versatility and the wealth of knowledge accumulated over centuries about its therapeutic benefits. Neem is widely documented for its broad antimicrobial spectrum. It is used to treat skin diseases, wounds, and respiratory infections. Neem leaves, often used in topical applications or as decoctions, are effective in cleansing wounds and preventing infection. The plant's antimicrobial properties extend to bacteria, fungi, and viruses, making it a valuable treatment for a wide range of infections. (Singh *et al*., 2021).

Neem's anti-inflammatory properties are another key benefit, making it a potent remedy for conditions such as arthritis. The compound nimbidin, found in neem, is known for its ability to reduce swelling and pain, providing relief to those suffering from inflammatory diseases. In addition to these effects, neem has been traditionally used as an antimalarial agent, particularly in parts of Africa where it is believed to help combat the Plasmodium parasite that causes malaria. Infusions or extracts from neem leaves and bark are commonly used to manage malaria symptoms (Ismail *et al.*, 2024). Beyond its role in fighting infections and inflammation, neem also has anti-diabetic properties. Studies have shown that neem can help regulate blood sugar levels, improving insulin function and supporting overall pancreas health. Furthermore, neem is a popular detoxifying agent, particularly for liver and kidney health. It is believed to help cleanse the body of toxins, strengthen the immune system, and promote general well-being (Brai et al., 2024; Mandi *et al.,* 2022).

Neem is also widely used in the treatment of various skin conditions, such as acne, eczema, and psoriasis. Its combination of antimicrobial and anti-inflammatory properties makes it highly effective in promoting skin health and speeding up the healing process for wounds (Dipika *et al.,* 2023). In Nigeria, particularly among the Yoruba, neem has been used for centuries to treat a wide array of conditions. Traditional uses include boiling the leaves and

bark to create decoctions for internal consumption or topical application. Neem oil, extracted from the seeds, is frequently used to treat scabies, ringworm, and other fungal infections. Additionally, neem has been employed in managing digestive disorders, fever, and as a general tonic for overall health improvement. (Aundhia *et al.,* 2024).

In other African countries, neem has similarly been revered for its therapeutic properties. In Kenya, Uganda, and Tanzania, neem is used to manage conditions like malaria, typhoid, and bacterial infections. In these regions, the bark and leaves are often utilized for medicinal purposes, underscoring neem's widespread acceptance as an antibacterial agent—particularly in rural areas with limited access to modern healthcare. (Efoli-Bam *et al.,* 2024; Sidat & Chauhan, 2023).

Plant biomass, particularly neem, continues to play a vital role in the therapeutic management of diseases. Its rich history of use across Africa and other parts of the world highlights its significance in traditional medicine, while its documented therapeutic benefits demonstrate its potential in addressing contemporary health challenges (Maiyo *et al.,* 2023). In particular, the antimicrobial, anti-inflammatory, and anti-diabetic properties of neem offer promising alternatives to synthetic drugs, especially in the context of the growing issue of antimicrobial resistance (AMR). Despite extensive exploration of its benefits, the full potential of neem remains underutilized, particularly in this era of AMR. As resistance to conventional antibiotics continues to rise, neem presents a natural source of novel antimicrobial agents, offering an alternative to combat resistant pathogens and improving healthcare access in underserved regions. Beyond its therapeutic applications, the sustainable cultivation of neem plays a crucial role in ensuring its availability for medicinal and agricultural uses.

1.5 CULTIVATION

Building on its historical and therapeutic significance, the cultivation of neem is a critical component in realizing its full potential as a natural remedy. Neem thrives in tropical and subtropical climates, requiring well-drained soil and ample sunlight for optimal growth (Farouq, 2022). In regions like Nigeria, farmers have successfully adopted inter-cropping methods, such as the taungya system, where groundnuts and millet are planted alongside neem to maximize land use and improve productivity (Ogazie *et al.,* 2022). Regular weeding is essential in neem cultivation to prevent competition for nutrients and moisture, ensuring healthy tree growth . In terms of propagation, tissue culture techniques have been developed to produce neem plantlets with enhanced metabolite content, especially Azadirachtin, which is vital for pest management (Omar *et al.,* 2024) . Additionally, neem trees are known for their coppicing ability, meaning they can regenerate from stumps after being cut, providing resilience to environmental stresses like fire or storms. Economically, neem cultivation offers significant benefits. It has the potential to substantially increase farmers' incomes through carbon credits and the sale of neem derived products, promoting sustainable agriculture (Farouq, 2022). Also, its natural insecticidal properties also help reduce reliance on chemical pesticides, making it an effective tool for sustainable pest management (Gelen et al., 2024). However, challenges such as inadequate infrastructure and regulatory barriers need to be addressed to fully realize the potential of neem in agriculture and its broader economic benefits (Datta, 2024).

1.6 EXTRACTION

This process involves separating the medicinally useful parts of a plant from the inert or inactive parts to isolate bioactive compounds. This is achieved through various techniques, with the choice of solvent playing a critical role in determining the efficiency and quality of the extracted compounds. Solvents such as water, alcohol, ether, chloroform, light petroleum, and ethyl acetate are commonly used, depending on the nature of the compounds being extracted and the plant properties. (Ramesh *et al.,* 2024; Mondal *et al.,* 2024). Water is ideal for extracting water-soluble compounds, alcohols (like ethanol) are useful for polar compounds, while ethers, chloroform, and light petroleum are more suited for non-polar compounds like essential oils and fatty acids (Salvi, 2024). Ethyl acetate is also often used for For extracting bioactive alkaloids and terpenes due to its ability to dissolve a range of organic compounds.

The principle of extraction relies on the selective solubility of plant metabolites in chosen solvents. This process works on the basic concept that different compounds within the plant tissue can be dissolved into the solvent, leaving behind the unwanted or inert parts. The solvent's polarity and its interaction with the target compounds drive the efficiency of this separation.

Conventional extraction techniques like Soxhlet extraction use solvents such as petroleum ether to achieve a high oil yield from neem (Azadirachta indica). Soxhlet extraction involves continuous extraction, where the solvent circulates through the plant material, ensuring thorough extraction of lipophilic compounds. According to a study by (Mustapha *et al.,* 2024), this method results in a maximum oil yield of around 52% at 125°C over 150 minutes. While the unconventional techniques, like Ultrasonic-Assisted Extraction (UAE), enhance energy efficiency and extraction yield, yielding 32.5% neem oil and 0.19% azadirachtin-A under

specific conditions (Saha *et al.,* 2022). Another environmentally friendly method is Aqueous Two-Phase Separation (ATPS), which efficiently extracts oleic acid and other bioactive compounds, achieving a total phenolic content of 8.033 mg GAE/g (Gawade *et al.*, 2022).

Fig 1A: *Azadirchta indica*- Dongoyaro Tree

1.7 BOTANICAL DESCRIPTION OF *AZADIRACHTA INDICA*

Azadirachta indica (neem) is native to the Indian subcontinent and is widely distributed across tropical and subtropical regions. In India alone, an estimated 20 million neem trees grow across various states. They thrive in regions with sub-arid to sub-humid climates, receiving an annual rainfall of 400–1200 mm. Their drought-resistant nature allows them to survive in areas with annual rainfall as low as 400 mm; however, under such conditions, they often rely on supplementary irrigation or deep-rooted access to groundwater. This adaptability makes neem trees a prominent species in arid and semi-arid landscapes. (Kirschner et al., 2021)

1.7.1 Taxonomy of Azadirachta indica

- Kingdom: Plantae
- Division: Magnoliophyta
- Class: Dipsacales
- Order: Rutales
- Sub-order: Rutinae
- Genus: *Azadirachta*
- Species: *indica*

1.7.2 Common names

In India, neem (*Azadirachta indica*) is called "Neem" in Hindi, "Vempu" in Tamil, and "Arishta" in Sanskrit. In Africa, it goes by different local names, such as "Dogonyaro" in Nigeria (Hausa), "Nim" or "Nimb" in Ghana, "Muarubaini" in Kenya (Swahili), and "Olweny" in Uganda. In South Africa, it is simply referred to as "Neem," while in Ethiopia, it is known as "Arsina" or "Azadirachta." This wide variety of names reflects the global distribution and cultural significance of the neem tree across different regions.

1.7.3 The Habits and Habitat of *Azadirachta indica* (Neem) include the following:

Leaves

The leaves of neem are bright green and vibrant when young, gradually darkening to a rich green as they mature. Each leaf is pinnate, comprising 20–30 lance-shaped leaflets that measure between 3 and 8 cm in length. The leaflets are smooth, glossy, and have serrated edges, giving the tree a lush and lively appearance. They are arranged alternately along the branches, providing the tree with its characteristic dense foliage. These leaves are evergreen in most conditions, though in severe droughts, the tree may shed its leaves to conserve water.

Flowers

The flowers of neem are small and delicate, with a cream to pale yellow hue that contrasts beautifully with the green foliage. Each flower is star-shaped and arranged in axillary clusters or panicles that can reach up to 25 cm in length. These flowers are fragrant, emitting a sweet, honey-like aroma that attracts pollinators such as bees and butterflies. Being hermaphroditic, neem flowers possess both male and female reproductive organs, enabling effective pollination. The flowering season varies based on climate, but it typically occurs during late winter to early spring.

Bark

Neem bark is dark grayish-brown with a rugged and cracked texture that exudes a sense of resilience and strength. The outer bark is rough and fissured, while the inner layers are fibrous and reddish in color. The trunk is usually straight and sturdy, providing a robust support system for the tree's expansive canopy. The bark plays an important role in shielding

the tree from environmental stressors and is often a distinguishing feature of mature neem trees.

Seeds

Neem seeds are enclosed within the tree's fruit and are oval or ellipsoidal in shape. The seed comprises a hard, fibrous endocarp surrounded by a thin, yellowish-white testa. Each seed contains a single kernel, which is the most vital reproductive part of the tree. These seeds are lightweight yet durable, aiding in their dispersal through natural agents like wind and water. The seeds are known to germinate readily in suitable conditions, ensuring the propagation of the species.

Roots

The root system of neem is extensive and deeply penetrating, allowing it to thrive in arid and semi-arid regions with poor soil quality. The primary taproot anchors the tree firmly into the ground, while a network of lateral roots spreads outward, stabilizing the soil and preventing erosion. This robust root system enables the tree to access underground water sources, making it highly drought-resistant. The roots also play a significant ecological role by improving soil fertility and promoting microbial activity.

Fruit

The fruit of neem is a drupe, oval to oblong in shape, and measures 1.4–2.8 cm in length. When unripe, the fruit is smooth and green, gradually turning yellow to yellowish-brown upon ripening. The fruit has a fleshy pericarp surrounding a hard endocarp that encases the seed. The ripened fruit is mildly sweet and is often consumed by birds and other animals,

aiding in seed dispersal. Fruits typically appear during late spring to early summer, following the flowering season.

Size and Structure

Neem is a medium to large-sized tree, reaching heights of 15–20 meters on average, though some specimens grow up to 30 meters. The tree's crown is wide-spreading and forms a dense, umbrella-like canopy that offers ample shade and supports various ecosystems. The trunk is straight and robust, often measuring up to 1.2 meters in diameter. The branching pattern is symmetrical, with branches spreading horizontally in a layered arrangement, enhancing the tree's majestic and stately appearance.

1.8 GAS SPECTROMETRY MASSSPECTROMETRY ANALYSIS

Gas Chromatography-Mass Spectrometry (GC-MS) is an advanced analytical technique that integrates the separation capabilities of Gas Chromatography (GC) with the identification and quantification power of Mass Spectrometry (MS). GC is highly efficient at resolving volatile and semi-volatile compounds within complex mixtures but cannot identify them *(*Cao *et al., 2024)*. Conversely, MS provides detailed structural information, enabling precise identification and quantification of compounds, though it lacks the ability to separate mixtures. The synergy between these two techniques was recognized early on, leading to the development of GC-MS as a unified analytical too (Shahdeo & Kumar, 2024). Both techniques operate with samples in the vapor phase and handle minute quantities (typically less than 1 ng), making them inherently compatible.

The principles of GC-MS are based on their the differential partitioning of analytes between a mobile gas phase (carrier gas) and a stationary phase within a chromatographic column. In GC, the sample is injected into a heated port, where it vaporizes and is transported through the column by an inert gas, such as helium or nitrogen. The stationary phase, which can be a solid or a liquid film coated on a solid support, interacts with the sample components based on their volatility and polarity. Compounds with higher volatility or weaker interactions with the stationary phase elute faster, while those with lower volatility or stronger interactions take longer. This separation process is optimized by the stationary phase composition and a controlled temperature gradient in the column oven (Jwaili, 2019; Laajimi et al., 2022).

Once separated by GC, the compounds are transferred to the mass spectrometer for ionization and analysis. In MS, ionization is typically achieved through electron ionization (EI), where high-energy electrons bombard the molecules, causing them to fragment into ions. These ions are then sorted by their mass-to-charge ratios using a mass analyzer, such as a quadrupole, ion trap, or time-of-flight (TOF) system. The resulting mass spectrum, which plots ion intensity against mass-to-charge ratio, serves as a molecular fingerprint, enabling the identification and quantification of the compounds (Leppert, 2022).

The instrumentation of GC-MS consists of two main components: the GC unit and the MS unit. The GC system includes an injection port, a capillary column housed in a temperature-controlled oven, and a carrier gas supply. The sample is introduced into the injector, vaporized, and carried through the column, where separation occurs based on the analytes' physicochemical properties. The MS unit, connected to the GC outlet, comprises an ionization source, a mass analyzer, and a detector. The transfer of analytes from GC to MS occurs in a vacuum to prevent interference during mass analysis. (Tian et al., 2023)

Despite its strengths, GC-MS has limitations. The high temperatures required for sample vaporization and column separation (up to 300°C) can cause thermal degradation of heat-sensitive compounds, potentially leading to the analysis of decomposition products rather than the intact analytes (Masoud et al., 2024). Additionally, highly polar or non-volatile compounds may require chemical derivatization to enhance their volatility, adding complexity to sample preparation. Matrix effects in complex samples, such as those from biological or environmental sources, can also interfere with ionization efficiency and spectral interpretation.(Lu et al., 2024)

GC-MS remains a cornerstone technique in analytical chemistry, offering high-resolution separation and precise identification of volatile and semi-volatile compounds. Advances in column technology, ionization methods, and mass analyzers continue to expand its applications, making it indispensable for addressing complex analytical challenges in fields such as environmental analysis, pharmaceuticals, and biochemical research.

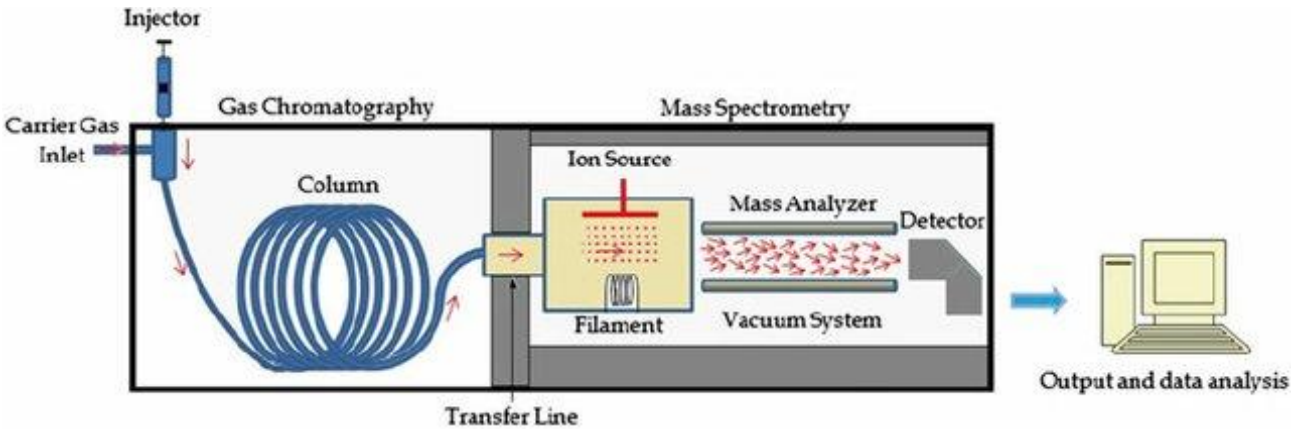

Fig 1B: Schematic plot of the main GC-MS Instrumentation (Emwas et al., 2015)

1.9 JUSTIFICATION OF STUDY

Azadirachta indica is a plant of immense historical and cultural significance, widely utilized in traditional medicine for centuries. Its therapeutic benefits have been extensively explored in folklore, and modern studies have confirmed the presence of important phytochemicals with potential antimicrobial properties. However, many bioactive compounds within *Azadirachta indica* remain unidentified and underexplored, highlighting the need for further scientific investigation.

This study is particularly significant in the context of the growing global challenge posed by antibiotic resistance. The overuse and misuse of conventional antibiotics have contributed to the emergence of multidrug-resistant bacterial strains, rendering many infections increasingly difficult to treat. Exploring alternative sources of antimicrobial agents, such as plant-based extracts, is essential to address this crisis. By investigating the antimicrobial potential of *Azadirachta indica*, this study aims to contribute to the search for effective, natural alternatives to conventional antibiotics, offering a sustainable and innovative approach to combating resistant infections.

1.10 AIMS AND OBJECTIVES

This study aimed to investigate the antimicrobial properties of the aqueous extract of Azadirachta indica (Neem) stem bark.

The specific objectives were:

To determine the antimicrobial properties of the stem bark extract of Azadchta indica against selected bacterial and fungal isolates using agar well diffusion method.

To determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the stem bark extract against the test microorganism.

To determine the chemical constituents present in the extract using Gas Chromatography Mass Spectrometry (GC-MS).

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Microbiological Media

Sabouraud dextrose agar (Titan Biotec)

Nutrient agar (Titan Biotec)

Mueller Hinton agar (Titan Biotec)

2.1.2 Equipment

Portable autoclave (Model YX-2803, Lincoln Mark Medical, England)

Weighing balance

Hot air oven (Gallenkamp, England)

Mortar and pestle

Mechanical grinding machine

Micropipette (P-1000)

Incubator

2.1.3 Glassware

Conical flask (Pyrex, England)

McCartney universal bottles

Test tubes (Pyrex, England)

Pipettes (Pyrex, England)

Glass stirrers (Pyrex, England) Glass funnels (Pyrex, England) Petri dishes (Pyrex, England)

Beakers (Pyrex, England)

Measuring cylinders

Porcelain dish

Pestle

Maceration jars

2.1.4 Clinical Isolates

Clinical isolates include;

Pseudomonas aeruginosa

Bacillus subtilis

Escherichia coli

Staphylococcus aureus

Klebsiella spp.

Candida albicans

Aspergillus niger

2.2 METHOD

2.2.1 Collection and Identification

The stem bark of *Azadirachta Indica* was collected from the whole tree found in Mrs. Ike's Compound, Senior Staff Quarters (SSQ), University of Benin and further identified and authenticated by Dr.(Mrs) Babaiwa, (BSc. Botany).

2.2.2 Preparation of Crude Extract

The collected stem bark of the *Azadirachta indica* were chopped into sizeable pieces, sun dried for three days and was later dried completely in the laboratory oven. The dried stem bark was pulverized using a commercial grinding machine. 395 g of the powdered stem bark was macerated using 2L of aqueous solvent in a maceration jar for 12 hours. During this period, the mixture was stirred at intervals to allow for proper permeation of extraction solvent into the bark. A double filtration using Whatman filter paper was carried out on the resultant mixture so as to ensure that no bark particles or residue was in the filtrate, after which the filtrate was exposed to air to evaporate. A dark brown-coloured extract was obtained which was then weighed and kept at room temperature in an airtight container until required for use.

2.2.3 Preparations Used for Antimicrobial Activity Test

2.2.3.1 Specimen Collection

Microorganisms used in this study were clinical isolates obtained from the University of Benin Teaching Hospital, Benin City, Edo state, Nigeria.

Gram positive used; *Staphylococcus aureu, Bacillus subtilis.*

Gram negative bacteria used; *Pseudomonas aeruginosa, Escherichia coli, klebsiella spp.*

Fungi species used; *Candida albicans, Aspergillus niger*

2.2.4 Determination of Antimicrobial Succeptibility Test

The antimicrobial susceptibility test was conducted using the agar well diffusion method. Seven Petri dishes were prepared, each labeled with the name of a specific test organism. Mueller Hinton agar and Sabouraud dextrose agar were sterilized in an autoclave, and 30 ml

of Mueller Hinton agar was dispensed into five Petri dishes for *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, and *Bacillus subtilis*. Similarly, 30 ml of Sabouraud dextrose agar was poured into two Petri dishes for *Candida albicans* and *Aspergillus niger*. Excess moisture was removed from the agar plates by drying them in a hot air oven at 40°C for 10 minutes.

The test organisms were standardized by preparing suspensions in 1 ml of sterile water, adjusting their turbidity to match the 0.5 McFarland standard. The standardized suspensions were used to inoculate the agar plates by streaking the surface evenly with a sterile cotton swab under aseptic conditions. Wells with a diameter of 10 mm were created in the agar using a sterile cork borer, and the well bases were sealed with molten Mueller Hinton agar to prevent leakage.

Each well was filled with 0.2 ml of *Azadirachta indica* extract using a calibrated micro-pipette. Ciprofloxacin was used as the positive control for bacterial isolates, while ketoconazole was used as the control for fungal isolates. The plates were allowed to stand for 30 minutes at 37°C to facilitate diffusion of the extract, followed by incubation in an inverted position for 24 hours. After incubation, the plates were observed for zones of inhibition, which were measured and recorded accurately to evaluate the antimicrobial activity of the extract.

2.2.5 Determination of Minimum Inhibitory Concentration (MIC)

The agar dilution method was employed in this study to determine the Minimum Inhibitory Concentration (MIC) of the extract. Serial dilutions of the extract were prepared to obtain concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, and 3.125

mg/ml, while the agar was prepared as per the manufacturer's instructions. Twelve Petri dishes were labeled with the names of the test organisms and the corresponding concentrations. Mueller Hinton agar was used for *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., *Bacillus subtilis*, and *Pseudomonas aeruginosa*, whereas Sabouraud dextrose agar was used for *Candida albicans* and *Aspergillus niger*. A specified amount of the extract and agar was aseptically combined, poured into 30 ml Petri dishes, gently mixed, allowed to solidify, and dried in a hot air oven for 10 minutes. The plates were then streaked with the respective microorganisms using a sterile wire loop on clearly marked sections of each dish. After incubating the plates at 37°C for 24 hours, they were examined for microbial growth at the inoculated spots, and the results were documented.

2.2.6 Determination of Minimum Bactericidal/ Fungicidal Concentration (MBC/MFC)

The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined using the agar dilution method. This involved subculturing from the agar plates used in the MIC test onto fresh agar plates without the test agent. Plates without visible growth were swabbed and streaked onto fresh Mueller Hinton agar for *Staphylococcus aureus, Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa*, and *Bacillus subtilis*, and onto Sabouraud dextrose agar for *Candida albicans* and *Aspergillus niger.*

The inoculated plates were incubated at 37°C for bacterial isolates and at room temperature for fungal isolates for 24 hours. Following incubation, the plates were examined for growth at the inoculation sites, and the results were recorded.

2.2.6 Gas Chromatography - Mass Spectroscopy (GC-MS) Analysis

The GC-MS analysis of the plant extract was conducted at Leedex Laboratories, Lagos, using a GC-MS QP2010 (version 5.0) instrument equipped with a DB-3S-MS capillary standard non-polar column (30 mm × 0.25 mm ID × 1 μm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector operated at 250°C, and the oven temperature was programmed to start at 60°C for 15 minutes, followed by a gradual increase to 280°C at a rate of 3°C per minute. Component identification was achieved using the AOC-20i autosampler and by comparing retention indices with the reference data in the Leedex Laboratories computer library attached to the GC-MS instrument. The results were tabulated based on these comparisons.

diameter) and 1 um (film thickness) in an Agilent 6890N gas chromatograph and Agilent Technologies 5973 Network Mass Selective Detector.

CHAPTER THREE

3.0 RESULTS

3.1 PERCENTAGE YIELD OF EXTRACT

Following the removal of the extractive solvent, the aqueous extract of *Azadirachta Indica* (Neem) stem bark revealed a thick, dark brown coloured fluid extract with a yield of 12% while the other properties are shown below in table 3.1.

3.2 ANTIMICROBIAL SUSCEPTIBILITY TEST

The susceptibility of a microorganism to an antimicrobial agent is evident by the presence of growth of inhibitory zone on seeded agar plates. This one is measured in millimetre as an index of the inhibitory or killing action of the test against a given organism. In this study, three (3) out of seven (7) test microorganisms were susceptible to the inhibitory effect of aqueous extract of neem stem bark, as shown in Table 3.2.

3.4 THE MINIMUM INHIBITORY CONCENTRATION (MIC), MINIMUM BACTERICIDAL/ FUNGICIDAL CONCENTRATION (MBC/MFC) OF AQUEOUS NEEM STEM BARK EXTRACT

The MBC results of Azadirachta indica extract shows that *Escherichia coli* and *Staphylococcus aureus* showed no growth at 50mg/mL. *Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella spp.,Candida albicans,* showed no growth at 100mg/mL. This is shown in Table 3.3

Table 3.1 properties and occurrence of aqeous neem extracts

Properties	Occurrence
Solvent	Aqueous (Water)
Extract Texture	Jelly-like
Color	Dark brown
Consistency	Semi-solid
Odor	Aromatic
Percentage Yield	12 %

Table 3.2: **Inhibition zone diameter (IZD) of clincal isolates against aqueous extract of *Azadirachta indica* bark**

Test Microorganism	**Inhibitory Zone Diameter (IZD)**	**Observations**	**Ciprofloxacin**	**Ketoconazole**
Staphylococcus aureus	13 mm	Susceptible	40 mm	NA
Escherichia coli	13 mm	Susceptible	40 mm	NA
Klebsiella spp.	NZ	Not Susceptible	35 mm	NA
Pseudomonas aeruginosa	NZ	Not Susceptible	15 mm	NA
Bacillus subtilis	15 mm	Susceptible	40 mm	NA
Aspergillus niger	NZ	Not Susceptible	NA	20mm
Candida albicans	NZ	Not Susceptible	NA	NA

KEY: NA: Not Applicable
NZ: No Zone of Inhibition

Table 3.3: Minimum Inhibitory Concentration of Aqueous extract of Neem Stem Bark, and the Minimum Bactericidal Concentration/ Fungicidal Concentration of Aqueous Extract of *Azadirachta indica* Stem Bark

Concentration (mg/mL)	***Escherichia coli***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Bacillus subtilis***	***Klebsiella spp.***	***Candida abicans***	***Aspergillus***
100	NG	NG	NG	NG	NG	NG	G
50	NG	NG	NG	NG	G	G	G
25	G	G	G	G	G	G	G
12.5	G	G	G	G	G	G	G
6.25	G	G	G	G	G	G	G
3.125	G	G	G	G	G	G	G
MBC							
100	NG	NG	NG	NG	NG	NG	NG
50	NG	NG	G	G	G	G	G

KEY: NG- No Growth
G- Growth

CHAPTER FOUR

4.0 DISCUSSION

4.1 PHYSICAL PROPERTIES AND PERCENTAGE YIELD

4.1.1 Physical properties

The aqueous extract of neem bark exhibited a characteristic deep brown color, consistent with the presence of tannins, flavonoids, and other phenolic compounds known to be abundant in neem. The semi-viscous consistency of the extract further suggests a moderate concentration of these phytoconstituents. Such properties align with literature reports on neem, particularly when water is used as the solvent for extraction (Bhagat *et al.,* 2024; Mudenda *et al*., 2024).

The extract was highly miscible in water, reflecting its hydrophilic nature and the predominance of polar compounds, such as saponins, glycosides, and alkaloids, within the extract. This miscibility is a critical factor for its potential bioactivity, especially for applications in aqueous environments or biological systems. Additionally, the extract showed good stability, with no visible precipitation or phase separation observed, reinforcing the suitability of the aqueous solvent for maintaining the solubility of bio-active components.

4.1.2 Percentage Yield

The extraction process yielded 12% (w/w) of aqueous Neem bark extract relative to the dry weight of the raw material. This relatively low yield highlights the selective recovery of water-soluble polar phytoconstituents while excluding non-polar compounds that are more effectively extracted with organic solvents. This results hence shows the action of the aqueous extraction process for isolating hydrophilic bioactive compounds aligning with previous studies (Aisosa, 202 4).

Several factors could contribute to the lower yield, including the nature of the plant material, particle size, duration of extraction, and temperature. Neem bark is known to contain a complex mixture of bioactive compounds, and the choice of aqueous solvents limits the extraction primarily to polar compounds. The absence of significant quantities of non-polar constituents, such as essential oils or terpenoids, could also account for the observed yield.

Despite the low yield, the aqueous extract remains significant due to its selective bioactivity against the clinical isolates, as demonstrated in the susceptibility tests. This yield serves as a reference point for optimizing extraction protocols in future studies or for comparisons with alternative solvents or methods.

4.2 SUSCEPTIBILITY TEST

The antimicrobial susceptibility test conducted in this study aimed to evaluate the inhibitory effects of the aqueous extract of *Azadirachta indica* bark on a range of microorganisms, including Gram-positive and Gram-negative bacteria, as well as fungi. This was compared to the reference antimicrobial agents Ciprofloxacin (for bacteria) and Ketoconazole (for fungi).

At a concentration of 100 mg/mL, the aqueous extract of *Azadirachta indica* bark exhibited inhibitory activity against *Staphylococcus aureus* and *Bacillus subtilis*, with inhibitory zones of 13 mm and 15 mm, respectively. These results indicate that the bio-active compounds present in the bark of *Azadirachta indica* show significant antibacterial potential, particularly against Gram-positive bacteria. The presence of a measurable zone of inhibition suggests that the extract possesses compounds capable of disrupting the bacterial cell wall or interfering with cellular processes, leading to bacterial growth inhibition (Mali *et al.,* 2024).

However, no inhibitory zones were observed for *Escherichia coli, Pseudomonas aeruginosa, Candida albicans, and Aspergillus niger* at any of the tested concentrations. The absence of antimicrobial activity against these microorganisms may be attributed to several factors. For Gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa*, the outer membrane, rich in lipopolysaccharides, acts as a physical barrier, preventing the bioactive compounds from penetrating the cell wall effectively. Additionally, the presence of efflux pumps in these bacteria could further reduce the effectiveness of the extract by actively expelling antimicrobial compounds.

In the case of *Candida albicans and Aspergillus niger*, the lack of activity may be due to the unique composition of fungal cell walls, which are made up of chitin and β-glucans. Plant-derived antimicrobial agents typically target bacterial cell walls or metabolic pathways, and unless the plant extract contains specific antifungal compounds that can target components like ergosterol in fungal membranes, activity against fungi is less likely. This is consistent with the performance of Ketoconazole, which showed effective inhibition against *Candida*

albicans (23 mm) and *Aspergillus niger* (20 mm), as this drug targets ergosterol synthesis, a critical component of fungal cell membranes.

The observed antimicrobial activity of the Azadirachta indica extract against Staphylococcus aureus and Bacillus subtilis is likely related to the presence of bioactive compounds such as alkaloids, flavonoids, and saponins, which are known for their antimicrobial properties. The activity was also concentration-dependent, with higher concentrations producing larger zones of inhibition, which suggests that the Minimum Inhibitory Concentration (MIC) for these microorganisms is above 100 mg/mL but still within the range where the plant extract shows efficacy.

When compared to the reference antibiotics, Ciprofloxacin demonstrated superior antibacterial activity, with larger zones of inhibition against both Staphylococcus aureus (26 mm) and Escherichia coli (25 mm), highlighting its broad-spectrum activity. Similarly, Ketoconazole was effective against the fungal strains tested, producing zones of inhibition of 23 mm for Candida albicans and 20 mm for Aspergillus niger. These results confirm the established effectiveness of Ciprofloxacin and Ketoconazole against their respective targets.

4.3. MINIMUM INHIBITORY CONCENTRATION (MIC), MINIMUM BACTERICIDAL/FUNGICIDAL CONCENTRATION (MBC/MFC)

4.3.1 MIC Determination

The MIC values obtained in this study indicate that *Azadirachta indica* extract demonstrates limited antimicrobial activity under the conditions tested. The MIC for Gram-positive bacteria, which are Staphylococcus aureus and Bacillus subtilis, was determined to be 50 mg/mL, while Gram-negative bacteria, including Escherichia coli and *Pseudomonas aeruginosa*, also exhibited an MIC of 50 mg/mL, suggesting comparable sensitivity across these groups. Klebsiella spp. and Candida albicans showed an MIC of 100 mg/mL, whereas Aspergillus niger exhibited growth at all tested concentrations, indicating an MIC exceeding 100 mg/mL.

These findings challenge the conventional view that Gram-positive bacteria are more susceptible to plant-derived antimicrobials due to their simpler cell wall structure, which

lacks the outer membrane of Gram-negative bacteria. The identical MIC values for both Gram-positive and Gram-negative bacteria suggest either a broad-spectrum but weak effect of the bioactive compounds or an insufficient concentration of active components to reveal significant differences. For Gram-negative bacteria, structural defenses such as the outer membrane enriched with lipopolysaccharides and efflux pumps likely contribute to their survival at these concentrations, presenting a well-documented challenge for plant-derived antimicrobials.

The results for fungal strains, *Candida albicans* and *Aspergillus niger*, emphasize the limitations of plant-based antimicrobials against fungi. While *Candida albicans* showed an MIC of 100 mg/mL, *Aspergillus niger* exhibited growth at all tested concentrations, with an MIC greater than 100 mg/mL. Fungal cell walls, primarily composed of chitin and β-glucans, present structural barriers that many plant-based antimicrobials cannot easily penetrate. Furthermore, the absence of specific antifungal compounds, such as those targeting ergosterol synthesis, may explain the extract's limited antifungal activity in line with previous study (Nahar *et al.*, 2024).

The implications of these findings extend to agriculture as well. Neem's antibacterial activity suggests potential applications in managing plant diseases caused by bacterial pathogens. However, its limited antifungal efficacy highlights the need for complementary treatments to address fungal threats effectively. This aligns with a study that neem extract enhances soil microbial activity at low concentrations but exhibits inhibitory effects at higher levels, underscoring the importance of dose management to prevent harm to beneficial microorganisms (Jiang & Liu., 2024).

These results also highlight the need to optimize the extraction process to concentrate bioactive compounds. Techniques such as bioassay-guided fractionation could help isolate specific antimicrobial components and improve efficacy. Additionally, combining *Azadirachta indica* extract with conventional antibiotics or antifungals may yield synergistic effects to overcome the observed limitations. Hence, while the MIC values reveal limited antimicrobial activity of *Azadirachta indica* extract, they indicate a potential for broad-spectrum applications, this is in line with previous study (Mahmoud et al., 2024). Further research should focus on testing higher concentrations, refining extraction methods, and exploring synergistic combinations to fully harness the antimicrobial potential of this plant across clinical, agricultural, and environmental applications.

4.3.2 MBC/MFC Determination

The minimum bactericidal concentration (MBC) values of *Azadirachta indica* extract showed moderate antimicrobial effects. Both *Escherichia coli* and *Staphylococcus aureus* had an MBC of 50 mg/mL, indicating effective bactericidal activity at this concentration. For *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella spp.*, and *Candida albicans*, the MBC was 100 mg/mL, showing these organisms required higher concentrations of the extract for bactericidal or fungicidal action. *Aspergillus niger* showed no reduction in growth at 100 mg/mL, indicating an MBC of >100 mg/mL.

As explained similarly for MIC, the extract demonstrated bactericidal effects across a range of pathogens but showed limited activity against fungi and some bacteria. The higher MBC values for *Klebsiella spp.* and *Candida albicans* suggest stronger defense mechanisms, while *Aspergillus niger*'s resistance underscores the extract's weak antifungal properties.

These results highlight the need for optimizing the concentration of active compounds in the extract to improve its bactericidal and fungicidal efficacy.

4.4 GCMS DISCUSSION

The GC-MS analysis of the aqueous Neem extract methyl revealed a complex phytochemical profile consisting of nine major compounds, each with diverse biological and industrial relevance. The dominant compound, 9-Octadecadenoic acid (Z), methyl ester, accounted for 64.56% of the extract. It is a polyunsaturated fatty acids and it is widely recognized for its antimicrobial, anti-inflammatory, and bio-diesel potential. This aligns with findings by (Harwood *et al.,* 2023), which reported its efficiency in bio-diesel production and its therapeutic applications in lipid modulation. Also, it is not only known to exhibit a direct antimicrobial effects but also reduce biofilm formation, which further underscores their

activity (Spiegel, 2022.). Their mechanism of action with antimicrobial activity is due to their ability to integrate into microbial membranes, altering their fluidity and permeability, which leads to cell lysis and death (Seabra *et al.,* 2023) . Furthermore, The presence of 9-Tetradecenal (8.63%), an aldehyde with documented insecticidal and antimicrobial properties. More specifically, it is a fatty aldehyde which works by inducing oxidative bursts in pathogens, destabilizing their cellular structures and functions responsible for their antimicrobial activity (Olaniyan *et al.,* 2023).

Other compounds, including Di-n-octyl phthalate (DOP) (6.62%) and Methyl stearate (6.55%), exhibit significant industrial and antimicrobial relevance. Di-n-octyl phthalate (DOP). It is reported to have a synergistic effect with other neem phytochemical components, enhancing antimicrobial efficacy, particularly against Gram-positive bacteria and yeasts. Additionally, they exhibit broad-spectrum antimicrobial activity, effectively targeting various microorganisms, including resistant strains (Mali *et al.,* 2024) . Compounds like Hexadecanoic acid derivatives, although present in smaller proportions, hold promise for applications in surfactant chemistry and pharmaceuticals, contributing to neem's versatility. These findings correlate with (Almowallad and Alqahtani 2024), who emphasized neem's broad-spectrum antimicrobial efficacy, particularly when incorporated into advanced delivery systems like chitosan nanoparticles to combat multi-drug resistant pathogens.

The findings from this study substantiate neem bark's potential as a source of bioactive compounds with antimicrobial properties with other multifaceted applications. The non-toxic nature of these phytochemicals, coupled with their ability to modulate biological targets, aligns with documented work (Nagesh *et al;* 2023), demonstrating their suitability for preclinical studies. The use of alcohol should further be explored as means of extraction, as alcohol yield more products components as compared to aqueous solvent ((Veerendrakumar

et al., 2023) (Hashim *et al.,* 2021). Future research should focus on elucidating the molecular mechanisms of action and optimizing formulations for clinical and industrial applications, thereby bridging traditional knowledge with modern scientific advancements. This work hence therefore shows the potential of neem, not only as a potential antimicrobial agent but its unique usefulness to bring sustainable solution in medicine, agriculture, and industry, offering a promising pathway for future innovations.

The limitation of the study is the use of an aqueous solvent for the extraction, which may not fully represent the complete spectrum of bioactive components present in the extract. Non-polar solvents, such as alcohol and ether, are known to extract additional bioactive compounds that may enhance antimicrobial activity. Future studies should consider employing these solvents to identify and evaluate a broader range of components.

Another limitation is in the organism Selection; the clinical isolates tested in this study may not comprehensively represent the diversity of microorganisms or their varied behaviors in different environments. Further research should include a wider range of microbial species, including both clinical and environmental isolates, to provide a more accurate assessment of the extract's antimicrobial potential.

And lastly is the concentration range, where for organisms like *Aspergillus niger*, which showed no inhibition even at the highest tested concentration of 100 mg/mL, higher concentrations should be explored. Testing beyond the current concentration limits may help establish more accurate MIC and MBC values and reveal potential efficacy against resistant strains.

CHAPTER FIVE

5.0 CONCLUSION

From the results and discussion in the study, it has established *Azadirachta indica* (Neem) stem bark contain active secondary metabolites responsible for their active against bacterial clinical isolates. However with limited activity against fungal isolates.

Furthermore, the Gas Chromatograpy-Mass Spectrometry showed the prescence of Nine main compounds with known antimicrobial properties and which may have have been responsible for their activity.

The results of this study has shown *Azadirachta indica,* holds promise as a potential source of natural compunds with therpaeutic potential and the since plant extract showed considerate levels of antimicrobial activity, it may be concluded that it is potential candidate for a further research as lead compounds from the analysis could be further evaluated and tested so it could be used in treatment of ailments caused by bacteria.

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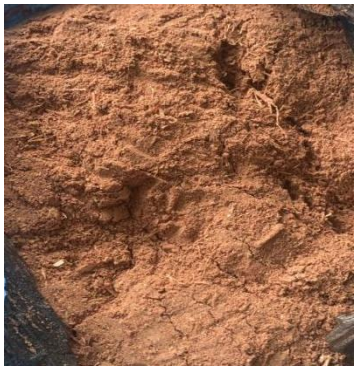
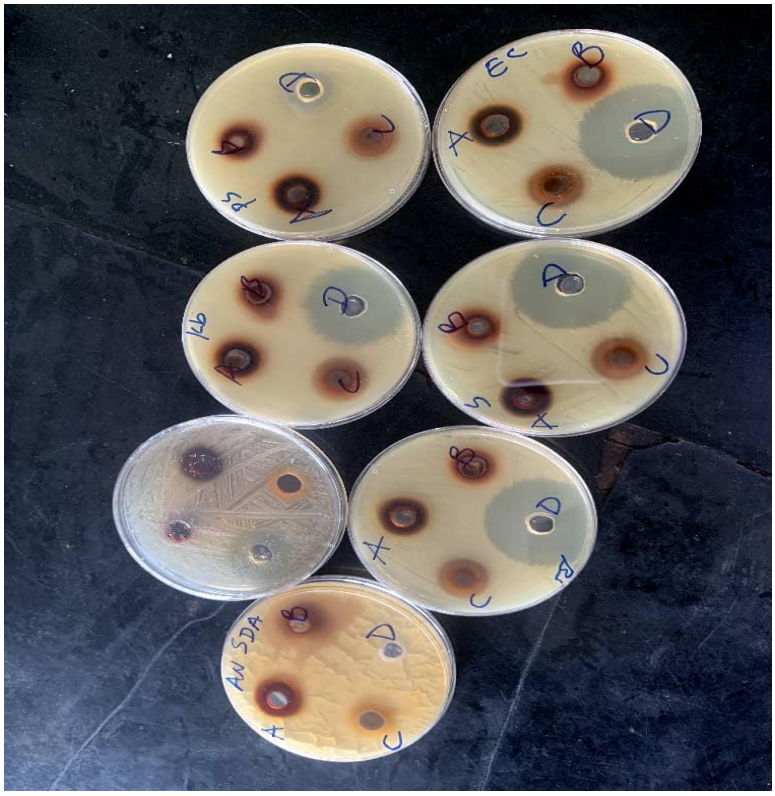
APPENDIX

1. Dongoyaro stem bark



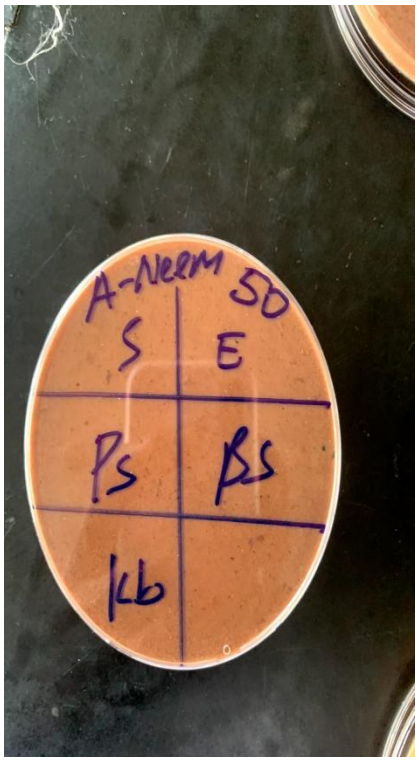
2. Powdered Dongoyaro bark

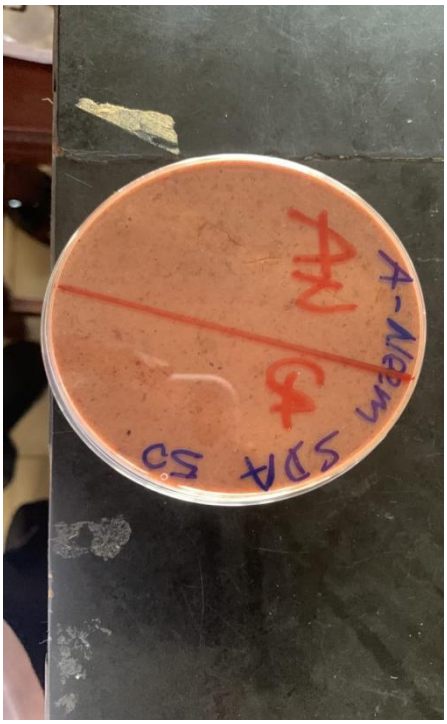
3.Inoculation Process



4. **Observing the Inhibitory Zone Diameter of AN SDA (Code Label on Petri Dish) of the Clinical Isolates. KEY:** ***EC- E. Coli, PS- Pseudomonas sp., Kb- Klebsiella sp., B- Bacillus sp., S- Staphylococcus aerueus.***

5.Measuring the Imhibitory Zone Diameter of the Neem Extract Against the Clinical Isolates





6.Observing the Growth after inoculation

4.Working in the Pharmaceutical Microbiology and Biotechnology Department (PMB)