ISOLATION AND IDENTIFICATION OF BACTERIA IN HERBAL MIXTURES SOLD AT SOME MARKET IN BENIN CITY, EDO STATE.

BY:

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DEPARTMENT OF MEDICAL LABORATORY SCIENCE,

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SEPTEMBER, 2025.

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**A PROJECT WORK SUBMITTED TO THE
DEPARTMENT OF MEDICAL LABORATORY SCIENCE,
SCHOOL OF BASIC MEDICAL SCIENCES,
UNIVERSITY OF BENIN,
BENIN CITY, EDO STATE**

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE AWARD OF BACHELOR OF MEDICAL LABORATORY SCIENCE DEGREE (BMLS) IN MEDICAL LABORATORY SCIENCE

SUPERVISED BY

DR. (MRS) A.O. ITEMIRE

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CERTIFICATION

This is to certify that this research work reported in this project work was carried out by **IKEM, KAMSOCHUKWU FAVOUR** with the matriculation number **BMS2009050** under the supervision of Dr. (Mrs.) A. O. Itemire in partial fulfillment for the award of Bachelor of Medical Laboratory Science (B.MLS) Degree from the University of Benin, Benin City, Edo State, Nigeria.

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DEDICATION

This project work is dedicated to God Almighty for His Faithfulness and to my family for their great support all round.

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ABSTRACT

Herbal mixtures are widely consumed in many developing countries due to their perceived therapeutic benefits, affordability, and accessibility. However, poor preparation and storage practices increase the risk of microbial contamination. This study aims to isolate and identify the microorganisms present in selected herbal mixtures sold at Uselu, New Benin and Ring Road Market in Benin City, Edo state. A cross-sectional analysis was conducted on herbal preparations collected from the three major markets. Microbiological assessment was performed to determine bacterial load and isolate species. Antibiotic susceptibility testing was carried out using standard disc diffusion techniques, and statistical analyses were applied to evaluate differences across dosage forms and locations. The overall mean bacterial load was 7.92×10^7 CFU/ml. Powdered forms exhibited the highest mean load (1.33×10^8 CFU/ml), followed by liquid forms (6.53×10^7 CFU/ml), while paste formulations showed no detectable growth. Variations across dosage forms ($p = 0.331$) and market locations ($p > 0.05$) were not statistically significant. *Bacillus subtilis* was the most prevalent isolate (8 occurrences), followed by *Klebsiella spp.* (3), *Bacillus cereus* (2), and *Staphylococcus aureus* (2), with *Pseudomonas aeruginosa* identified once. Antibiotic susceptibility revealed complete sensitivity to Azithromycin and Levofloxacin (100%) and high sensitivity to Pefloxacin (76.5%). Moderate sensitivity was recorded for Gentamycin, Rifampicin, and Erythromycin (52.9%), while reduced activity was observed for Zidovudine (29.4%) and Ampicillin (41.2%). Amoxicillin showed the lowest effectiveness, with resistance in 82.4% of isolates, confirmed by its minimal inhibition zone (4.41 ± 2.38 mm). The strongest inhibition zones were produced by Levofloxacin (19.06 ± 1.32 mm) and Azithromycin (16.94 ± 1.68 mm). In Conclusion, Locally marketed herbal mixtures in Benin City were found to harbor diverse bacterial contaminants, predominantly *Bacillus subtilis*. Although no significant differences were observed across dosage forms or market locations, the presence of pathogenic bacteria and high resistance to commonly used antibiotics such as Amoxicillin emphasizes it's potential health risks.

CHAPTER ONE

INTRODUCTION

1.1 Background of The Study

Phytomedicine, also known as herbal medicine, is a fast-growing field with a long tradition that makes use of natural plant products to treat diseases (Fazly Bazzaz *et al*., 2021).

The World Health Organization has beliefs, skills, knowledge of specific societies around the world, in the maintenance of health and in the prevention, defined herbal medicine as medicine based on the all inclusive utilization of theories, diagnosis, improvement or treatment of physical and mental illness. Over the past infection, typhoid fever, and skin disease choice, used to treat various types of years, herbal medicines have been promoted aliments, including diarrhoea, urinary tract as natural and safe, therefore, the preferred (Fazly Bazzaz *et al*., 2021).

Herbal medicine, derived from plant sources, has been used for centuries in many cultures around the world as a primary or complementary form of healthcare. In Nigeria, especially in urban and peri-urban areas like Benin City, the use of herbal mixtures is widespread due to cultural beliefs, accessibility, affordability, and perceived efficacy. These mixtures are commonly sold in local markets such as Uselu Market, New Benin and Ring Road Market, often without stringent regulatory oversight (Adesegun *et al*., 2020).

Microbial contamination of herbal mixtures can pose serious public health risks. Pathogenic microorganisms such as *Escherichia coli, Salmonella spp., Staphylococcus aureus, and Pseudomonas aeruginosa,* among others, can cause gastrointestinal disturbances, skin infections, and systemic illnesses, especially in immunocompromised individuals.

Additionally, the presence of spoilage organisms may reduce the shelf-life and therapeutic efficacy of these herbal mixtures (De Sousa Lima *et al*., 2020; Derbal, 2023)

Despite the popularity of these products in Benin City, limited research has been conducted to assess their microbiological quality. The lack of regulatory frameworks and quality control mechanisms makes it important to scientifically investigate the safety of these preparations. Isolation and identification of microorganisms in herbal products can provide essential data for public health monitoring and help improve the quality and safety standards of herbal medicine production and sales (Oshoma *et al*., 2017).

1.2 Statement of Problem

Herbal mixtures are widely consumed in Benin City, particularly in bustling markets like Uselu, New Benin and Ring Road, due to their perceived health benefits and affordability. However, these herbal preparations are often produced and sold under unhygienic conditions, with little to no regulatory oversight. As a result, they may serve as potential reservoirs for bacterial contamination, posing significant health risks to consumers. Despite the popularity of these remedies, there is a lack of scientific data on the bacterial load and specific bacterial species present in these mixtures. This knowledge gap makes it difficult to assess the safety of these products and to implement appropriate public health interventions. Therefore, it is crucial to isolate and identify the bacteria present in herbal mixtures sold at Uselu, New Benin and Ring Road markets to evaluate their microbiological quality and potential risk to public health.

1.3 Justification of the Study

1. Widespread Use of Herbal Mixtures: With increasing reliance on herbal treatments, understanding their safety is essential.
2. Potential Health Risks: Microbial contamination poses risks of infection and toxicity.

3. Public Health Relevance: Data from this study can help inform health officials and guide safer production practices.
4. Lack of Regulation: The informal nature of herbal medicine markets necessitates empirical surveillance.

1.4 Aim of Study

The aim of this study is to isolate and identify the microorganisms present in selected herbal mixtures sold at Uselu, New Benin and Ring Road Market in Benin City, Edo state.

1.5 Specific Objectives

The specific objectives of the study are;

1. To collect and analyze herbal mixture samples from the selected markets.
2. To isolate and culture microorganisms present in these mixtures.
3. To identify the microbial species using biochemical and possibly molecular techniques.
4. To assess whether the levels of identified microorganisms exceed acceptable standards.

1.6 Research Questions

1. What types of bacteria are present in herbal mixtures sold at Uselu, New Benin and Ring Road markets in Benin City?
2. Do these bacterial isolates indicate potential health risks to consumers?
3. Are there any differences in contamination levels between the three market locations?

1.7 Research Hypothesis

1.7.1 Null Hypothesis (H_0)

There is no significant presence of pathogenic bacteria in herbal mixtures sold at Uselu, New Benin and Ring Road markets in Benin City.

1.7.2 Alternative Hypothesis(H_1)

Herbal mixtures sold at Uselu, New Benin and Ring Road markets in Benin City contain significant levels of pathogenic bacteria that may pose health risks to consumers.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Overview of Herbal Medicine and Mixtures

Herbal products, botanical products, or phytomedicines are derived from plants or botanicals to maintain health or treat diseases. Herbal supplements are products specifically used for internal use. A large number of prescription drugs and over-the-counter medications originate from plant derivatives (Furhad *et al.,* 2024). Herbal medicines (herbals, botanicals, phytomedicines) are products that contain parts of plants, plant materials, plant preparations, or finished products that use plant-derived material as the active ingredient and are used with therapeutic, preventive, or health-promoting intent. This term covers everything from raw plant parts to standardized extracts and finished formulations (capsules, teas, tinctures, topical preparations) (Wang *et al*., 2023).

Herbal medication products have been utilized for centuries as a primary form of healthcare in many cultures worldwide (Motti, 2021).

2.1.1. Traditional Vs. Modern Use of Herbal Medicine- Concise Evidence-Based Comparison

Origin and Epistemology

Traditional herbal use is rooted in long-standing cultural knowledge, systems of diagnosis and practice (e.g., Ayurveda, TCM, Unani), and transmission by apprenticeship and community practice rather than controlled experimental testing (World Health Organization, 2019).

Purpose and Approach to Illness

Traditional systems view health holistically and often aim to restore balance (e.g., yin–yang, doshas) using individualized herbal formulas; therapeutic effects are interpreted within those conceptual systems rather than by single-molecule pharmacology (Wang *et al*., 2023; Balkrishna *et al*., 2024).

Materials and Preparations

Traditional preparations frequently use whole plant parts (decoctions, poultices, powders, combined multi-herb formulas) where multiple constituents are intended to act together; dosage and composition vary by region, practitioner and harvest/processing conditions (Wang *et al*., 2023).

Knowledge source and Evidence standard

Traditional claims are primarily supported by empirical experience, text-based tradition, and ethnobotanical records rather than randomized controlled trials (RCTs); this historic evidence is valuable but differs from the modern hierarchy of evidence used in clinical pharmacology (Balkrishna *et al*., 2024).

Modern Scientific Validation

Modern use emphasizes phytochemical characterization, standardization (marker compounds, fingerprints), preclinical pharmacology, and RCTs when developing phytopharmaceuticals or botanical drugs — moving from tradition-based anecdotes toward reproducible, mechanism-based evidence (Wang *et al*., 2023; Balkrishna *et al*., 2024).

Regulation and Legal Status

Modern regulatory frameworks treat herbal products heterogeneously: some products are regulated as dietary supplements/foods (with lower premarket data requirements), others as traditional-use medicines with simplified pathways, and a few as botanical drugs subject to full drug-approval processes the regulatory label depends on claims, evidence and jurisdiction (WHO, 2019; Furhad and Bokhari, 2024).

Safety Concerns and Pharmacovigilance

Traditional use is not synonymous with safety: risks include intrinsic plant toxicity, contamination, adulteration, misidentification, and herb–drug interactions. Modern practice emphasizes active pharmacovigilance, reporting systems, and laboratory testing to identify hepatotoxicity, nephrotoxicity and clinically important interactions (Gamil *et al*., 2025).

Herb–drug Interactions

As patients commonly combine herbal remedies with prescribed medicines, modern clinical attention focuses on pharmacokinetic and pharmacodynamic interactions (enzyme induction/inhibition, transporter effects), which can alter drug concentrations and outcomes, a major safety driver for integrating herbal use into routine medical records and clinical decision making (Gamil *et al*., 2025).

Accessibility and Market Forces

Traditional herbal use historically relied on local availability and community practitioners; modern markets have globalized herbal products (retail supplements, standardized extracts, OTC phytomedicines), increasing access but also creating supply-chain and quality challenges that modern regulation attempts to address (Balkrishna *et al*., 2024; WHO, 2019).

Integration with Conventional Healthcare

Modern integrative approaches aim to combine best evidence from both domains: preserving useful traditional knowledge while subjecting promising remedies to modern scientific validation, safety testing, and regulatory oversight so that safe, effective products can be recommended within conventional care (WHO, 2019; Wang *et al*., 2023).

Research and Development Trends

Recent research and development focuses on:

(a) standardizing extracts and analytical fingerprints;

(b) isolating active constituents or developing multi-constituent standardized phytopharmaceuticals;

(c) improving clinical trial design for complex botanical interventions; and

(d) applying genomics/metabolomics and in-silico methods to predict interactions and mechanisms (Wang *et al*., 2023; Balkrishna *et al*., 2024).

Practical Clinical Implications

Clinicians should ask patients about herbal use, document it in medical records, consider interaction and toxicity risks, and when possible prefer products with standardized composition and independent quality testing; patients should be counselled that "traditional" does not automatically mean "safe" or "efficacious" by modern standards (Gamil *et al*., 2025).

2.1.2. Popularity and usage in Nigeria (Benin City and other parts of Nigeria) and Africa.

Across Africa, traditional herbal medicine remains a mainstream source of care. WHO reports that up to 80% of people in some African countries rely on traditional medicine for basic health needs, with many governments creating policies and regulatory frameworks to integrate it into health systems. (WHO, Regional Office for Africa 2022; WHO,2023). In 2024, a Pan African Medical Journal commentary again underscored that herbal therapies are the most common form of traditional medicine in Africa and are used by as much as 80% of the population, citing affordability, cultural acceptance, and gaps in access to conventional care as key drivers (Ikhoyameh *et al*., 2024).

In Nigeria, population-based studies consistently show high use of herbal remedies. A cross-sectional study in Southwest Nigeria found 85% had used herbal medicine in the previous two years, and non-hospital care was common, reflecting cost, convenience, and perceived effectiveness (Aina *et al*., 2020). Among rural residents in Lagos State, 95.7% had ever usedherbal medicine and 87.4% reported use within six months of the survey; two-thirds preferred it as first-line treatment (Oyeleye *et al*., 2022). In Benin City (Edo State) specifically, herbal mixtures are widely sold in informal markets and as ready-to-drink products. A 2024 study evaluating such ready-to-drink herbal mixtures sold in Benin City documented their availability and assessed microbiological quality, highlighting active consumer use alongside safety concerns typical of unregulated products (Omoruyi *et al*., 2024). Separately, a 2023 ethnopharmacology study investigated “agbo-iba,” a commercially sold Benin-City antimalarial decoction, reflecting its popularity for self-care of malaria; the work examined experimental efficacy while noting the need for standardization (Erhunse *et al*., 2023).

Overall, drivers of use in Nigeria and across Africa include accessibility, affordability, cultural fit, and dissatisfaction with or limited access to orthodox services; common forms include decoctions (e.g., agbo), bitters, and proprietary herbal mixtures. At the same time, WHO and regional authorities emphasize the importance of regulation, quality control, and evidence-based integration given risks such as contamination, drug–herb interactions, and inconsistent dosing (WHO Regional Office for Africa, 2022; WHO, 2023).

2.2 Microbial Contamination of Herbal Mixtures.

2.2.1. Sources of Microbial Contamination

Contamination often begins upstream—during cultivation and harvesting—when soil, animal manure, and irrigation water inoculate plant material with enteric and environmental microbes. Downstream points of entry include poor washing, grinding and extraction on unclean surfaces, use of contaminated processing water, inadequate heating/drying, re-use of containers, and hand-to-product transfer during retail dispensing or hawking. Storage in warm, humid conditions and prolonged transport without hygienic controls further amplifies microbial loads (Darkwah *et al*., 2022). Country studies in West and East Africa repeatedly trace contamination to raw materials, processing/handling, storage and transport lapses, and non-adherence to regulatory good-manufacturing or vendor hygiene guidelines (Ahiabor *et al*.,2024).

2.2.2. Types of Microorganisms Commonly Found

Across African surveys, the most frequently reported bacteria in herbal mixtures are *Escherichia coli, Staphylococcus aureus, Bacillus spp., Klebsiella spp., Pseudomonas aeruginosa, Enterobacter spp., Salmonella spp., and Shigella spp.*; fungi include *Aspergillus spp., Penicillium spp., and Candida spp*. A 50-study systematic review (2000–2024) found bacterial contaminants in 98% of studies—*E. coli* (62%), *S. aureus* (57%), *Bacillus spp.*

(55%)—and fungi in 70%, led by *Aspergillus* (40%) and *Penicillium* (27%) (Ahiabor *et al*., 2024). City-level data echo this pattern: in Kampala, 32% of commercial products yielded clinically important bacteria (notably *Klebsiella pneumoniae, E. coli, S. aureus*) (Walusansa *et al*., 2022), while in Accra, *Bacillus spp., P. aeruginosa, and E. coli* were common (Darkwah *et al*., 2022); In Benin City, Nigeria*, Klebsiella pneumoniae* was the most frequent isolate in unregulated products (Omoruyi *et al*., 2024).

2.2.3. Implications for public health

Microbial contamination has three major public-health consequences:

Infectious Disease Risk: Enteric pathogens *(E. coli, Salmonella, Shigella*) signal fecal contamination and can cause gastroenteritis and invasive disease; *S. aureus and B. cereus* are linked with toxin-mediated food-poisoning syndromes; *Klebsiella and P. aeruginosa* add opportunistic infection risks—especially for infants, older adults, and the immunocompromised (Walusansa *et al*., 2022).

Failure to meet safety limits: Many street-vended or locally prepared remedies exceed accepted microbial limits (e.g., TAMC/TYMC and zero-tolerance for specified pathogens such as *E. coli and Salmonella*). In Kampala, 36% exceeded WHO permissible total viable loads (TVL), 42% exceeded total coliform limits, and 9% exceeded *E. coli* limits (Walusansa *et al*., 2022); Benin City work similarly reports higher non-compliance among unregulated vendors (Omoruyi *et al*., 2024).

Toxin and Spoilage Hazards: Fungal contamination (e.g., Aspergillus) raises concerns for mycotoxin exposure (e.g., aflatoxins) and product spoilage, undermining potency and stability, with system-level reviews urging stronger regulation and routine microbiological testing (Ahiabor *et al*., 2024). Pharmacopoeial compendia and WHO-aligned guidance

specify absence of key pathogens and set TAMC/TYMC ceilings for internal-use herbal products (Yadav *et al*., 2022).

2.3 Overview of Bacteria

Bacteria are single-celled, prokaryotic microorganisms that lack a membrane-bound nucleus and complex organelles, distinguishing them from eukaryotic cells (Madigan *et al*., 2021). They are found in virtually all environments, including soil, water, air, and within or on other organisms, showcasing their remarkable adaptability (Willey *et al*., 2023). Bacterial cells exhibit diverse morphologies, such as cocci (spherical), bacilli (rod-shaped), and spirilla (spiral), with typical sizes ranging from 0.5 to 5 micrometers (Madigan *et al*., 2021). Their cellular structure includes a cell wall primarily composed of peptidoglycan, a plasma membrane, cytoplasm, and genetic material organized in a single circular chromosome, often supplemented by plasmids (Willey *et al*., 2023). Bacteria reproduce asexually through binary fission and can exchange genetic material via processes like conjugation, transformation, and transduction (Hug and Thomas, 2022). They play essential roles in ecological processes, such as nutrient cycling and decomposition, and have significant impacts on human health, with some species acting as pathogens and others providing beneficial functions like aiding digestion or producing antibiotics (O'Toole and Flemming, 2024). Recent advances in metagenomics and CRISPR technologies have deepened our understanding of bacterial diversity, metabolic versatility, and their interactions with hosts and environments (Hug and Thomas, 2022).

2.3.1 Bacteria Classification

Bacteria are first classified by cell shape into cocci (spherical), bacilli (rod-shaped), curved/comma-shaped vibrios, rigid helices (spirilla), and flexible helices (spirochetes) (Ojkic *et al*., 2022). Transitional shapes such as coccobacilli and genuinely pleomorphic

forms also occur and are recognized as part of shape-based classification (Ojkic *et al*., 2022). Cell arrangement driven by division planes and post-division adhesion adds a second tier: cocci appear as diplococci, streptococci, tetrads, sarcinae, or staphylococci, whereas bacilli appear singly, in chains (streptobacilli), or in palisades (LibreTexts, 2023). Envelope morphology underlies the classic Gram dichotomy: Gram-positive bacteria have a single membrane with a thick peptidoglycan wall (monoderm), while Gram-negative bacteria are diderm with a thin peptidoglycan layer and an outer membrane rich in lipopolysaccharide (Sun *et al*., 2021). Acid-fast bacteria (e.g., Mycobacterium) form a third, diagnostically distinct envelope category due to mycolic-acid–rich cell walls that retain carbol-fuchsin after acid-alcohol decolorization (Jacobo-Delgado *et al*., 2023; Morton *et al*., 2023). Peptidoglycan architecture and its spatiotemporal insertion patterns are the principal determinants and maintainers of cell shape across most bacteria (Sun *et al*., 2021; Kelvin *et al*., 2024).Rod-shaped bacteria typically maintain elongation via MreB-guided lateral cell-wall synthesis, whereas cocci grow primarily through septal (divisome-focused) synthesis, yielding characteristic spherical morphology (Zhydzetski *et al*., 2024).Spirilla and spirochetes are both helical, but spirilla are rigid, whereas spirochetes are flexible helices; both are treated as distinct morphotypes in shape-based schemes. Prosthecate/stalked and budding morphologies (e.g., Caulobacter and Hyphomonas) broaden morphology-based classification by adding polar stalks or buds that increase surface area and mediate attachment (Pöhl *et al*., 2024; Sreepadmanabh *et al*., 2024). Surface layers (S-layers) are geometric protein lattices that sheath some cells (e.g., Caulobacter), contributing to envelope architecture that is morphologically distinctive under EM and can be treated as a morphological trait (Sreepadmanabh *et al*., 2024). Colony-level morphology size, form, color, margin, elevation, texture, hemolysis is routinely combined with Gram-stain morphology as a practical, morphology-led step in preliminary identification. Morphology is plastic: antibiotics, nutrient

conditions, and physical confinement can drive filamentation, swelling, or aspect-ratio shifts, so modern classification uses morphology alongside molecular/physiologic data (Ojkic *et al*., 2022; Sreepadmanabh *et al*., 2024).

Bacterial classification based on morphology categorizes prokaryotic microorganisms primarily by their cellular shape, size, arrangement, and structural features observed under microscopy (Aryal, 2024). The fundamental morphological shapes of bacteria include cocci (spherical or ovoid forms), bacilli (rod-shaped or cylindrical forms), vibrios (comma- or curved rod-shaped), and spirilla (helical or spiral-shaped) (Editors of Encyclopaedia Britannica, 2025). Cocci bacteria are typically 0.5 to 2.0 micrometers in diameter and can arrange in pairs (diplococci), chains (streptococci), irregular grape-like clusters (staphylococci), or cubic packets of four or eight cells (tetrads or sarcinae) depending on their planes of division (Kaiser, 2023). Bacilli, often measuring 0.3 to 1.5 micrometers in width and 1 to 10 micrometers in length, may appear singly, in pairs (diplobacilli), in chains (streptobacilli), or occasionally in palisade formations resembling stacked rods (Fattah, 2021). Vibrios are short, curved rods resembling a comma, typically motile with a single polar flagellum, and measure around 0.5 to 0.8 micrometers in width and 1.5 to 3 micrometers in length (Wikipedia contributors, 2025). Spirilla are rigid, helical bacteria with multiple twists, often 0.2 to 0.5 micrometers in diameter and up to 60 micrometers long, distinguished from flexible spirochetes by their external flagella (Wikipedia contributors, 2025). Some bacteria exhibit pleomorphic morphology, varying shapes like rods to cocci under different environmental conditions, as seen in certain actinomycetes or mycoplasmas lacking rigid cell walls (Aryal, 2022). Bacterial size generally ranges from 0.2 to 5 micrometers, though exceptions like the large Epulopiscium fishelsoni (up to 600 micrometers long) highlight variability in morphological classification (Kaiser, 2023). Morphological classification is often integrated with staining methods, such as Gram staining, which reveals cell wall

differences: thick peptidoglycan in Gram-positive (retaining violet stain) versus thin in Gram-negative (staining pink), aiding shape-based identification (Aryal, 2024). Acid-fast staining further classifies bacteria like mycobacteria, which resist decolorization due to high lipid content in their cell walls, appearing rod-shaped with waxy morphology (Fattah, 2021). Flagellar arrangements contribute to morphological typing, including monotrichous (single flagellum), lophotrichous (tuft at one pole), amphitrichous (flagella at both poles), or peritrichous (flagella over the surface), influencing motility and shape perception (Aryal, 2022). Capsule presence, a gelatinous layer surrounding some bacteria, enhances morphological distinction by creating halos in stained preparations, often seen in cocci or bacilli like *Streptococcus pneumoniae* (Kaiser, 2023). Endospore formation, characteristic of certain bacilli like *Bacillus* and *Clostridium*, results in swollen, spore-containing morphologies resistant to environmental stress, visible as refractile bodies (Kadner *et al*., 2025). Modern computational tools, including machine learning, analyze morphological features for automated classification, achieving high accuracy in distinguishing shapes like cocci from bacilli using image data (Mai and Ishibashi, 2021).Despite advances in genomic taxonomy, morphology remains a foundational, rapid method for initial bacterial grouping in clinical and environmental microbiology (Ease, 2025).

2.3.2 Gram-Positive and Gram-Negative Bacteria

Bacteria, classified based on their cell wall characteristics, are categorized into two primary groups; Gram-positive and Gram-negative.

Gram-positive bacteria are characterized by a thick peptidoglycan layer in their cell wall, which allows them to retain the crystal violet dye during Gram staining, resulting in a purple appearance (Buckley and Palmer, 2021). Gram-negative bacteria possess a thin peptidoglycan layer surrounded by an outer membrane containing lipopolysaccharides, causing them to lose

the crystal violet dye and take up the counterstain, appearing pink (Buckley and Palmer, 2021). The cell wall of Gram-positive bacteria lacks an outer membrane and periplasmic space, making it more permeable to certain antibiotics compared to Gram-negative counterparts (Rebelo *et al*., 2023). Gram-negative bacteria have a three-layer envelope including an outer phospholipid membrane that acts as a selective barrier, contributing to intrinsic antibiotic resistance by restricting drug access (Buckley and Palmer, 2021). Gram-positive bacteria often rely on efflux pumps like those encoded by the tcrYAZB operon for metal tolerance, such as copper, which can be plasmid-mediated and transferable (Rebelo *et al*., 2023). In contrast, Gram-negative bacteria utilize additional mechanisms like the CusCBA system for copper efflux in the periplasm, particularly under anaerobic conditions, due to their outer membrane (Rebelo *et al*., 2023). Treatment of Gram-negative infections is more challenging because of their less permeable cell walls, production of beta-lactamases, efflux pumps, and ability to modify shapes favoring resistant strains (Buckley and Palmer, 2021). Gram-positive bacteria generally have higher cell wall permeability, making them easier to treat, though resistance to drugs like methicillin and vancomycin still poses issues (Buckley and Palmer, 2021). For arsenic tolerance, both Gram-positive and Gram-negative bacteria employ ars operons, but Gram-negative species like *Escherichia coli* show minimum inhibitory concentrations ranging from 0.5-4 mM without genes to 8-≥128 mM with them (Rebelo *et al*., 2023). Mercury tolerance in Gram-negative bacteria often includes the merB gene for broad-spectrum resistance within mer operons, while Gram-positive may have distinct MerA sequences indicating different evolutionary paths (Rebelo *et al*., 2023). Examples of Gram-positive bacteria include Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus*, prevalent in long-term acute care with high infection rates (Buckley and Palmer, 2021). Examples of Gram-negative bacteria include *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and*

Enterobacter spp., which account for four of the six most problematic multidrug-resistant hospital pathogens (Buckley and Palmer, 2021). Gram-positive *Enterococcus spp.* serve as examples with tcrYAZB for copper tolerance and merR/merA for mercury (Rebelo *et al*., 2023). Gram-negative *Escherichia coli and Salmonella* exemplify copper management via CusCBA and CueP systems, as well as arsenic tolerance genes like arsA and arsB (Rebelo *et al*., 2023). Pipeline drugs show limited activity against resistant Gram-negative bacteria, with superiority trials difficult due to ethical and recruitment issues (Buckley and Palmer, 2021).

2.4. Common Bacteria Associated with Herbal Products

Herbal mixtures in African markets frequently harbor some bacteria which includes;

Staphylococcus aureus, an opportunistic pathogen responsible for skin infections, food poisoning, and systemic illness through heat-stable enterotoxin production (Ahiabor *et al*., 2024).

Pseudomonas aeruginosa is another common isolate, associated with urinary tract, respiratory, and wound infections, and noted for its intrinsic antibiotic resistance (Walusansa *et al*., 2022).

Klebsiella pneumoniae, a Gram-negative enterobacterium, is regularly recovered from herbal mixtures in Nigeria and East Africa, raising concern due to its role in pneumonia, bloodstream infections, and antibiotic resistance gene carriage (Omoruyi *et al*., 2024).

Bacillus subtilis is often detected as a non-pathogenic environmental contaminant but serves as an indicator of inadequate cleaning and high spore contamination (Darkwah *et al*., 2022).

Bacillus cereus is more clinically relevant, capable of producing emetic and diarrheal toxins leading to foodborne illness (Ahiabor *et al*., 2024).

2.4.1. Pathogenic vs. Non-pathogenic Species

Pathogenic species in herbal mixtures include *S. aureus, P. aeruginosa, K. pneumoniae, and B. cereus*, which directly threaten consumer health through infection or toxin-mediated disease (Walusansa *et al*., 2022).

Non-pathogenic or opportunistic species, such as *B. subtilis*, generally do not cause illness in healthy individuals but can spoil products, reduce shelf life, and indicate poor hygiene (Darkwah *et al*., 2022).

The pathogenic potential also depends on host immunity; for example, opportunistic bacteria like *P. aeruginosa and K. pneumoniae* become more harmful in immunocompromised patients (Ahiabor *et al*., 2024).

2.5. Factors Affecting Microbial Load in Herbal Mixtures

2.5.1. Preparation and Handling Practices:

- Microbial load is strongly influenced by the hygiene of processors, the cleanliness of utensils, and the quality of water used in preparation (Walusansa *et al*., 2022).
- Grinding, extraction, and bottling in open-air markets without sanitary controls introduce both environmental and fecal contaminants (Omoruyi *et al*., 2024).
- Use of raw plant material without adequate washing or heat treatment increases the risk of survival of spore-forming bacteria like *Bacillus spp.* (Darkwah *et al*., 2022).
- Different communities in Benin city, Nigeria have their way of preparing herbal mixtures and the method used in the preparation influences the presence or absence of microorganisms like bacteria which are significant to their safety. Lack of standard methods may indirectly affect the difference in the efficacy of plants from various communities (Tugume *et al*., 2019).

2.5.2. Storage Conditions:

- High humidity and ambient temperatures accelerate microbial growth in stored herbal products, especially when preservatives are absent (Ahiabor *et al*., 2024).
- Prolonged storage without refrigeration supports the proliferation of Enterobacteriaceae and molds capable of producing mycotoxins (Darkwah *et al*., 2022).
- Storage in reused, improperly cleaned containers compounds contamination risk (Omoruyi *et al*., 2024).

2.5.3. Packaging and Preservation Techniques:

Packaging in sterile, airtight containers reduces microbial ingress and moisture absorption (Yadav *et al*., 2022).

Opaque bottles limit light exposure, preventing photodegradation of bioactive compounds and reducing microbial activation (Walusansa *et al*., 2022).

Addition of natural or chemical preservatives can extend shelf life, but absence or inadequate concentration allows microorganisms to survive and multiply (Ahiabor *et al*., 2024).

2.5.4. Inadequate Quality Control on Herbal Mixtures:

Although some have shown promise, the majority of herbal products sold in stores or used by villagers are prepared without any consideration for quality. As a result, there is a high risk of microbial contamination and subsequent negative effects on people. Because there are no regulations or standards in place for the manufacturing of herbal medicines in African villages, these goods frequently contain microbial contamination from the production stage and packaging. As a result, the level of herbal products' safety determined by the quality of the ingredients used in their manufacture (De Lima Sousa *et al*., 2020).

2.6. Previous Studies on Microbial Contamination in Herbal Products

2.6.1. Studies in Nigeria and Africa

A systematic review of 50 studies on microbial contamination in herbal medicines across Africa from 2000-2024 found that Nigeria contributed the highest number of studies (50%), followed by Ghana (18%) and Kenya (8%) (Ahiabor *et al*., 2024).

The highest bacterial load of 3.54 × 10^12 cfu/mL was reported in unregulated herbal medicines from Nigeria (Ahiabor *et al*., 2024).

In Kenya, 72 out of 86 herbal medicinal products marketed in Nairobi for chronic diseases were contaminated with bacteria such as *Escherichia coli, Salmonella typhi, Salmonella paratyphi, and Enterobacteriaceae* (Hassan *et al*., 2021).

In Lesotho, all 5 herbal preparations sold in Maseru were contaminated with bacteria including *Pseudomonas aeruginosa* and coliforms, with bacterial loads ranging from 5.6 × 10^4 cfu/mL to 3.6 × 10^8 cfu/mL, and fungi like yeasts and moulds with loads from 3.0 × 10^5 cfu/mL to 6.0 × 10^8 cfu/mL (Mautsoe *et al*., 2021).

In Uganda, all 140 herbal medicines sampled in Kampala were contaminated with bacteria such as *Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Klebsiella oxytoca, Bacillus cereus, Pseudomonas aeruginosa, and Enterobacter spp*., with bacterial loads ranging from 0.0 to 1.42 × 10^7 cfu/mL for liquids and 1.8 × 10^3 cfu/g to 1.67 × 10^7 cfu/g for solids (Walusansa *et al*., 2022).

In Ghana, all 30 herbal preparations from Accra markets were contaminated with bacteria including *Escherichia coli, Pseudomonas aeruginosa, Bacillus spp., Citrobacter spp., Staphylococcus aureus, Staphylococcus spp., Enterobacter spp., Shigella sonnei, Moraxella*

catarrhalis, and Serratia marcescens, with coliform counts ranging from 3.1 × 10^1 cfu/mL to 1.7 × 10^5 cfu/mL, and fungi like *Candida spp.* (Darkwah *et al*., 2022).

In Nigeria, all 7 herbal products sampled were contaminated with bacteria such as *Staphylococcus aureus, Proteus spp., Pseudomonas spp., and Streptococcus spp.,* with bacterial loads ranging from 1.8 × 10^6 cfu/mL to 7.5 × 10^6 cfu/mL, and fungi including *Candida spp., Aspergillus niger, and Aspergillus flavus* (Anie *et al*., 2022).

Across African studies, common bacterial contaminants included *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella spp., Klebsiella spp., and Enterobacter spp*., while fungal contaminants were predominantly *Aspergillus spp., Candida spp., Penicillium spp.*, and yeasts (Ahiabor *et al*., 2024).

2.6.2. Studies in Other Developing Countries

A systematic review of 91 studies on contamination in herbal medicinal products from low-and-middle-income countries, including India, Brazil, Pakistan, and Bangladesh, found that 27.5% of articles reported microbial contaminants, with bacteria (73.1%) and fungi (50.0%) predominant (Opuni *et al*., 2023).

In Asia, including India and Pakistan, microbial contaminants included 57 different bacterial species such as *Escherichia coli, Salmonella spp., Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella spp.*, and fungi like *Aspergillus spp. and Candida spp.,* with 46.4% of samples exceeding regulatory limits for microbial load (Opuni *et al*., 2023).

In South America, including Brazil, microbial contaminants were primarily bacteria, with 16.4% of total samples across LMICs exceeding regulatory limits, and microbial contaminants showing the highest proportion (46.4%) above limits (Opuni *et al*., 2023).

In Bangladesh, herbal medicines showed microbial contamination with bacteria and fungi, contributing to the overall 16.4% of samples exceeding regulatory limits in LMICs (Opuni *et al*., 2023).

Across LMICs, microbial contamination levels exceeded regulatory limits in 482 out of 1039 samples tested, highlighting high risks in countries like India, Brazil, Pakistan, and Bangladesh (Opuni *et al*., 2023).

2.6.3. Gaps in Current Literature

The literature on microbial contamination in herbal medicines in Africa is limited by the low number of studies from countries outside Nigeria, Ghana, and Kenya, indicating a need for more research in underrepresented regions (Ahiabor *et al*., 2024).

There is a gap in studies addressing parasitic contaminants in herbal medicines, with only one study reported, suggesting a need for expanded investigation into this type of contamination (Ahiabor *et al*., 2024).

The literature search in LMICs was limited to English-language studies, potentially missing relevant publications in other languages (Opuni *et al*., 2023).

Few articles addressed organochlorine pesticides and residual solvents, limiting generalizations on these contaminants (Opuni *et al*., 2023).

Further studies using risk assessment models are needed to evaluate consumer risks from microbial contamination in LMICs (Opuni *et al*., 2023).

There is limited information on the microbiological safety of herbal preparations in Ghana, indicating a research gap that requires more comprehensive studies (Darkwah *et al*., 2022).

2.7. Regulations and Quality Control of Herbal Products

2.7.1 WHO Guidelines

The World Health Organization defines traditional medicine practitioners as community-recognized providers using prevalent practices and beliefs (Mbachu *et al*., 2024).

The World Health Organization supports scientifically proven traditional medicine, with potential treatments for diseases like HIV/AIDS and COVID-19 (Mbachu *et al*., 2024).

The World Health Organization notes that 60% of children with high fever from malaria are first treated with herbal medicine and documents effective medicinal plants for various diseases (Mbachu *et al*., 2024).

The World Health Organization recommends integrating palliative care into health systems, which is still developmental in Nigeria (Chukwunyere *et al*., 2023).

The World Health Organization established 2016 global mitigation strategies for drug shortages, implemented in Nigeria, including policy changes and training (Okeke *et al*., 2023).

The World Health Organization prescription guide is used for clinical audits to assess prescription quality (Okeke *et al*., 2023).

The World Health Organization prequalified pharmacies are mentioned for malaria medicine procurement (Okeke *et al*., 2023).

The World Health Organization's 2000 guidelines for research and evaluation of traditional medicine apply, potentially covering herbal products (Okeke *et al*., 2023).

The World Health Organization African Region Framework of Actions sets expectations for integrated health systems, potentially including traditional medicines (Onwujekwe *et al*., 2025).

2.7.2. Nigerian Regulatory Bodies (e.g., NAFDAC)

The National Agency for Food and Drug Administration and Control was established under the National Agency for Food and Drug Administration and Control Act Cap N1 LFN 2004, regulating manufacture, importation, exportation, distribution, marketing, and use of medical products, including traditional medicines (Okeke *et al*., 2023).

The National Agency for Food and Drug Administration and Control oversees clinical trials, inspects facilities, controls advertisements, conducts pharmacovigilance, performs post-market surveillance, and manages import/export control and quality labs (Okeke *et al*., 2023).

The National Agency for Food and Drug Administration and Control coordinates with other agencies for enforcement, ensuring compliance with national policies (Onwujekwe *et al*., 2025).

The National Agency for Food and Drug Administration and Control manages disposal of expired drugs through incineration and enforces compliance despite poor adherence (Okeke *et al*., 2023).

The National Agency for Food and Drug Administration and Control introduced pharmaceutical traceability using Global Standards 1 in 2022 for supply chain security (Okeke *et al*., 2023).

The National Agency for Food and Drug Administration and Control regulates herbal products under the 2007 Traditional Medicine Policy, including registration and safety monitoring (Okeke *et al*., 2023).

The National Agency for Food and Drug Administration and Control grants approval for packaged traditional medicines such as tablets, capsules, and syrups after laboratory tests (Mbachu *et al*., 2024).

The National Agency for Food and Drug Administration and Control's Pharmacovigilance/Food and Drug Information Centre is affiliated with the World Health Organization Collaborating Centre for International Drug Monitoring (Okeke *et al*., 2023).

The National Agency for Food and Drug Administration and Control receives budget envelopes from the Federal Ministry of Health for regulatory functions and is involved in allocation and expenditure processes (Etiaba *et al*., 2024).

The National Agency for Food and Drug Administration and Control serves as a purchasing agency for medicines and health commodities based on approved lists and operational guidelines (Uguru *et al*., 2024).

The National Agency for Food and Drug Administration and Control has established Good Manufacturing Practice guidelines to enhance the production of pure and high-quality herbal drugs (Onyeukwu *et al*., 2024).

The National Agency for Food and Drug Administration and Control regulations from 2019 mandate that herbal medicine labeling must include a quantitative list of ingredients in either botanical or common names under the declaration of ingredients section (Onyeukwu *et al*., 2024).

2.7.3. Challenges in Implementation

Implementation of herbal product regulations faces incomplete standardization and integration of traditional medicine into the health system, with poor implementation and auditing of national policies (Onwujekwe *et al*., 2025).

Partial implementation of the 2007 Traditional Medicine Policy and inadequate monitoring leads to the sale of unapproved herbal products (Okeke *et al*., 2023).

The Five Plus Five-Year Validity (Migration to Local Production) policy from 2019 faces challenges, with most medicines not licensed for local raw ingredient production (Okeke *et al*., 2023).

Poor compliance with expired medication disposal guidelines persists despite enforcement threats (Okeke *et al*., 2023).

Coordination and regulation mechanisms for non-state actors, including traditional medicine providers, remain inadequate (Etiaba *et al*., 2024).

Lack of structures for policy evaluation, absence of strict punishment for defaulters, and lack of funds for training hinder the World Health Organization's drug shortage mitigation strategies (Okeke *et al*., 2023).

Weak accountability, law enforcement at all governance levels, and regionally devolved health sector complexity exacerbate implementation issues (Etiaba *et al*., 2024).

Parallel use of traditional and orthodox medicines poses hazards, lack of dosage standardization, and treatment failures occur due to poor training of practitioners (Mbachu *et al*., 2024).

Coexistence of traditional and contemporary medicine risks patient safety, needing better regulation, which is currently hindered by incomplete integration (Onwujekwe *et al*., 2025).

Stock shortages and circulation of fake products arise due to poor policy implementation (Onwujekwe *et al*., 2025).

Chronic underfunding, inadequate information and communications technology infrastructure, weak data culture, limited capacity for data collection, lack of dedicated health records

officers, and poor coordination of health information systems affect implementation (Onwujekwe *et al*., 2025).

Corruption, weak accountability mechanisms, delays in budget approvals, and inefficiencies in public financial management impact implementation (Uguru *et al*., 2024).

Chaotic drug distribution occurs due to stakeholders ignoring responsibilities, including community pharmacies and patent medicine vendors (Okeke *et al*., 2023).

The Mobile Authentication Service for verifying medicine authenticity faces implementation challenges (Okeke *et al*., 2023).

Challenges include complex mixtures with unidentified active principles, lack of selective analytical methods or reference compounds, and variability in chemical composition due to chemo-varieties and harvesting methods (Onyeukwu *et al*., 2024).

Significant labeling irregularities, such as missing National Agency for Food and Drug Administration and Control numbers and batch numbers, indicate non-compliance with regulations (Onyeukwu *et al*., 2024).

High microbial contamination rates and lack of antimicrobial activity despite claims pose health risks (Onyeukwu *et al*., 2024).

The presence of pathogenic microorganisms like *Escherichia coli and Pseudomonas aeruginosa* suggests inadequate sterilization and potential fecal contamination (Onyeukwu *et al*., 2024).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out at the Faculty of Pharmacy, University of Benin, Benin City, Edo State. The Samples were gotten from the selected markets which include New Benin market, Ring Road Market and Uselu Market in Benin City, Edo State, South-South of Nigeria .

3.2. Sample Size

The Sample Size of this study was calculated using the standard sample-size formula ;

$n=Z^2\ P(1-P)\backslash d^2$

$= 1.96^2 \times 0.9\ (1-0.9)/\ 0.131^2$

$=20.14$

A Total of sample size of 20 would be used for this study to ensure validity of results.

where Z is the standard normal deviate for the desired confidence level (1.96 for 95%), $p$is the anticipated prevalence, and d is the desired half-width (precision) of the two-sided 95% confidence interval (CI).

(Naing *et al*., 2022).

3.3. Inclusion Criteria

Herbal Mixtures within the New Benin, Ring Road and Uselu Market were used for this research.

3.4. Exclusion Criteria

Herbal Mixtures outside the New Benin, Ring Road and Uselu Market were excluded in this research.

3.5. Ethical Approval

Ethical Approval was sought and obtained from the Ethical Committee of the College of Medical Sciences, University of Benin, Benin City, Edo State. Confidentiality of vendors was maintained by anonymizing market sources and vendor identities during data analysis and reporting.

3.6. Materials

Equipment used includes;

Microscope, Portable autoclave (Gallenkamp and Co. Ltd., England), Incubator (Gallenkamp, England), Hot air oven (Gallenkamp, England), Sterile wire loop, Refrigerator (Super Deluxe), Weighing balance, Cotton wool, Scissors, Petri dishes, Aluminum foil, Masking tape, rack, Bunsen burner, Wire gauze.

Glassware

Glass spreader, measuring cylinders, universal bottles, glass slides, cover slips, beakers, pipettes, infusion bottles (for melting and sterilizing agar), bijou bottles.

Reagents and Chemicals

Crystal violet, Lugol's iodine, Acetone, Neutral red, Citrate Agar,Kovacs reagent, Oxidase Reagent, Immersion oil, Distilled water, Quarter-strength Ringer’s solution, Peptone water.

Microbiological Media

Nutrient Agar, MacConkey Agar, Blood Agar, Mueller Hilton Agar.

Other Materials

Disposable hand gloves, permanent marker.

3.7. Sample Collection

A total of twenty (20) different herbal mixtures were purchased from the New Benin, Ring Road and Uselu Market in Benin City. The samples were collected in a sterile container and stored in the refrigerator at 4°C before the analysis.

Table 3.1: Sample form and Therapeutic claim of Herbal Mixtures

SAMPLE CODE	SAMPLE NAME	THERAPEUTIC CLAIM	SAMPLE FORM	LOCATION
S1	IBA	FOR FEVER	POWDER	USELU
S2	INFECTION (1)	FOR INFECTION.	POWDER	USELU
S3	KOKORONU-EJÉ	FOR INFECTION ON MEN'S PRIVATE PART.	POWDER	USELU
S4	GBOBONISHÉ	FOR DIFFERENT TYPE OF INFECTION.	POWDER	USELU
S5	SUGAR	FOR REGULATING SUGAR LEVEL.	POWDER	USELU
S6	AGBÀRÀ	FOR MAN POWER .	POWDER	USELU
S7	MAN POWER (WHITE)	FOR MAN POWER.	POWDER	USELU
S8	ARARIRO	FOR BODY PAIN.	POWDER.	NEW BENIN
S9	INFECTION (2)	FOR RASHES.	LIQUID.	NEW BENIN
S10	IBA	FOR FEVER.	LIQUID	NEW BENIN

SAMPLE CODE	SAMPLE NAME	THERAPEUTIC CLAIM	SAMPLE FORM	LOCATION
S11	JEDI (1).	FOR REGULATING SUGAR LEVEL.	LIQUID.	NEW BENIN.
S12	RHEUMATISM (1)	FOR RHEUMATISM.	LIQUID	NEW BENIN
S13	IDAKOLÉ	FOR MAN PERFORMING ENHANCER AND REDUCTION OF SUGAR LEVEL.	POWDER	NEW BENIN
S14	RHEUMATISM (2)	FOR BODY PAIN.	PASTE	NEW BENIN
S15	JEDI ATI INFECTION	FOR INFECTION AND WAIST PAIN.	LIQUID	RING ROAD
S16	INFECTION (3)	FOR INFECTION.	LIQUID	RING ROAD
S17	TYPHOID	FOR TYPHOID.	LIQUID	RING ROAD
S18	JEDI (2)	FOR DYSENTERY.	LIQUID	RING ROAD
S19	AWON KPA	FOR FEVER.	LIQUID	RING ROAD
S20	DOGOYARO	FOR MALARIA.	LIQUID	RING ROAD

3.8. Sample Examination

3.8.1. Preparation of Media

Culture media used for analysis were Nutrient Agar, Blood Agar, Mueller Hinton Agar and MacConkey Agar. They were all prepared according to the manufacturer's instructions.

3.8.2 Sample Preparation

The herbal product (powder, liquid and paste forms) was placed in sterile bottles and labeled S1–S20. A total of 20 products were collected (9 dried/powdered products, 1 Paste Product and 10 liquid products).

1 g of the powdered herbal products was weighed and dissolved into 10 ml of sterile water to prepare a stock solution. The mixture was shaken thoroughly to obtain a homogeneous suspension. From this suspension, 1 ml was aseptically transferred into 9 ml of sterile diluent (One-Quarter Strength Ringer's solution) to begin serial dilution.

For liquid herbal products, 1 ml of each product was aseptically transferred into 9 ml of sterile diluent to prepare the stock solution. Serial dilution was then performed from this stock in the same manner as for the powdered products.

Procedure for Serial Dilution

Six sterile universal bottles were prepared for each sample, each containing 9 ml of One-Quarter Strength Ringer's solution, and labeled A–F.

A 1 ml aliquot of the stock solution was aseptically transferred into bottle A (10^{-1} dilution).

From bottle A, 1 ml was transferred into bottle B (10^{-2} dilution).

From bottle B, 1 ml was transferred into bottle C (10^{-3} dilution).

From bottle C, 1 ml was transferred into bottle D (10^{-4} dilution).

From bottle D, 1 ml was transferred into bottle E (10^{-5} dilution).

From bottle E, 1 ml was transferred into bottle F (10^{-6} dilution).

At each transfer, the solution was gently shaken to ensure even distribution of the inoculum.

This procedure was repeated for all 20 herbal products.

3.8.3 Inoculation of Serially Diluted Samples onto Nutrient Agar

20 ml of molten Nutrient Agar (NA) was aseptically poured from sixty sterile universal bottles into sterile Petri dishes and appropriately labeled. After the agar had solidified, Excess surface moisture was reduced at 40 °C for 5 minutes.

A micropipette was used to dispense 0.5 ml of the diluent from tube B (10^{-2} dilution) aseptically and was inoculated evenly and spread over the agar surface. This procedure was repeated for all samples (S1–S20), each in triplicate.

3.8.4. Bacterial Count

One millilitres of diluted samples were dispensed onto the centre of the petri dish containing the medium to be used. It was incubated at 37°C for 24 hours and observed for bacteria growth. Colonies were counted after incubation.

CFU/mL= Total number of colonies counted × dilution factor/Volume of specimen used (aliquot).

Bacterial Tests

Colonies were characterized by morphology, pigmentation, and hemolytic patterns, Gram staining and biochemical tests (catalase, oxidase, citrate utilization, indole production, lactose sugar fermentation profiles) (Adebayo *et al*., 2022; Ezeudu *et al*., 2024).

3.8.5. Antimicrobial Susceptibility Testing

The Kirby–Bauer disc diffusion method on Mueller-Hinton agar was used (CLSI, 2022). Zones of inhibition are measured in millimeters after 18–24 hours of incubation at 37 °C to classify organisms as susceptible, intermediate, or resistant (Chukwu *et al*., 2023).

3.8.6. Determination of Inhibitory Zone Diameter (IZD)

The inhibitory zone diameter was determined using the agar diffusion method with slight modifications. Sterile Nutrient Agar (NA) was prepared, and 20 ml was aseptically poured into Petri dishes after incorporating a concentration of 3g of the herbal product into the nutrient agar, giving a final concentration of 150 mg/ml. The infused nutrient agar was then allowed to solidify (Andrade *et al.,* 2022).

Excess moisture was removed from the surface of the infused nutrient agar at 40 °C for about 5 minutes using hot air oven. A Sterile wireloop was used to pick a discrete colony from the test organisms obtained from the herbal products were then introduced by streaking on the infused Nutrient Agar.

The inoculated plates were incubated at 37°C for 24 hours. The procedure was repeated for all bacteria isolates obtained from the different herbal products. Plates without herbal product incorporated into the medium served as experimental controls. The experiment was performed in triplicate.

3.9. Statistical Analysis

Data collected from laboratory examinations were entered into Microsoft Excel and subsequently exported to the Statistical Package for the Social Sciences (SPSS) software, version XX (IBM Corp., Armonk, NY, USA) for analysis. Descriptive statistics such as means, standard deviations, frequencies, and percentages were computed where appropriate. Inferential statistics, including one-way analysis of variance (ANOVA) and chi-square tests, were employed to compare bacterial loads, microbial distributions, and antibiotic susceptibility patterns across different groups.

CHAPTER FOUR

4.1. RESULTS

Table 4.1 illustrates bacterial load across all herbal mixtures. Powdered herbal preparations recorded the highest mean bacterial count of 1.33×10^8 CFU/ml, followed by liquid preparations with a mean count of 6.53×10^7 CFU/ml, while paste preparations had no detectable bacterial growth. The difference in bacterial load between the sample was not statistically significant ($p > 0.05$). This finding as illustrated in Figure 4.1, which shows that although powdered forms harbored higher levels of bacteria compared to liquid and paste forms, the variation did not reach significance.

Table 4.2 showed bacterial load varied among the three market locations where the herbal mixtures were collected. The mean bacterial load was highest in samples from New Benin (1.12×10^8 CFU/ml), followed by those from Uselu (6.93×10^7 CFU/ml), and the lowest load was recorded in samples from Ring Road (5.50×10^7 CFU/ml). Despite the differences in mean counts, statistical analysis indicated no significant variation across market locations ($p > 0.05$).

Table 4.3 details the distribution of microorganisms isolated from the herbal mixtures across the three market locations. The predominant organism was *Bacillus subtilis* (8 isolates) across all locations. *Klebsiella spp.* was the second most frequent, with 3 isolates, while *Bacillus cereus* and *Staphylococcus aureus* were each identified in 2 isolates. *Pseudomonas aeruginosa* was detected only once, and one sample showed a mixed growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In addition, three samples yielded no bacterial growth. Statistical analysis revealed no significant association between the distribution of isolates and market location ($p > 0.05$). Same was illustrated in figure 4.3 with most frequently isolated organism being the *Bacillus subtilis.*

The antibiotic susceptibility profile of the isolates presented in Table 4.4, showed to different antibiotics. The isolates demonstrated complete sensitivity to Azithromycin (100%) and Levofloxacin (100%), indicating that all tested organisms were inhibited by these antibiotics. High sensitivity was also observed with Pefloxacin (76.5%). Moderate levels of sensitivity were recorded with Gentamycin (52.9%), Rifampicin (52.9%), and Erythromycin (52.9%), suggesting that about half of the isolates were susceptible to these drugs. On the other hand, reduced sensitivity was observed with Zidovudine (29.4%) and Ampicillin (41.2%), while the lowest was with Amoxicillin (17.6%), to which the majority of isolates (82.4%) were resistant. Despite these variations, statistical analysis showed that the differences in susceptibility patterns were not significant (p-values > 0.05).

Table 4.5 presents the mean zones of inhibition produced by the antibiotics against the bacterial isolates. The largest inhibition zones were observed with Levofloxacin (19.06 ± 1.32 mm) and Azithromycin (16.94 ± 1.68 mm), followed by Pefloxacin (14.06 ± 2.43 mm), showing strong antibacterial effects. Moderate inhibition zones were recorded with Ciprofloxacin (12.71 ± 2.71 mm), Gentamycin (11.71 ± 2.87 mm), and Rifampicin (11.18 ± 2.69 mm), indicating intermediate levels of antibacterial activity. Smaller inhibition zones were observed with Zidovudine (6.24 ± 2.57 mm), Ampicillin (8.41 ± 2.71 mm), and Erythromycin (10.24 ± 2.72 mm). Amoxicillin produced the lowest inhibition zone of 4.41 ± 2.38 mm, demonstrating the weakest effect against the isolates. Statistical analysis revealed no significant difference in the zones of inhibition among the antibiotics tested (p-values > 0.05).

Table 4.1. Bacterial Load of Herbal Mixtures (CFU/ml)

Sample Form	N	Bacterial count mean (cfu/ml)
Powder	7	1.33×10^8 CFU/ml
Liquid	10	6.53×10^7 CFU/ml
Paste	1	0.00×10^0 CFU/ml

significance when $p > 0.05$, N = number of herbal mixtures

Table 4.2 Bacterial Load by Market Location

Market	N	Bacterial count mean (Cfu /ml)
Uselu	7	6.93×10^7 CFU/ml
New Benin	7	1.12×10^8 CFU/ml
Ring Road	6	5.50×10^7 CFU/ml

significance $p > 0.05$, N = number of isolates.

Table 4.3. Distribution of Microorganisms Isolated by Market Location

Organism	Uselu	New Benin	Ring Road	Total
No Growth	1	1	1	3
Bacillus subtilis	3	3	2	8
Bacillus cereus	1	1	0	2
Staphylococcus aureus	1	0	1	2
Pseudomonas aeruginosa	0	1	0	1
Klebsiella spp.	1	1	1	3
Mixed (*Staph* + *Pseudomonas*)	0	0	1	1

Level of significance $p > 0.05$

Table 4.4. Antibiotic Sensitivity Pattern of Bacterial Isolates

Antibiotic	Sensitive (%)	Resistant (%)
Rifampicin	52.9	47.1
Amoxicillin	17.6	82.4
Zidovudine	29.4	70.6
Ampicillin	41.2	58.8
Gentamycin	52.9	47.1
Pefloxacin	76.5	23.5
Erythromycin	52.9	47.1
Azithromycin	100	0
Levofloxacin	100	0

significance when $p > 0.05$

Table 4.5. Zones of Inhibition of Bacterial Isolates Against Common Antibiotics

Antibiotics	Mean ± SEM
Azithromycin	16.94 ± 1.68
Ciprofloxacin	12.71 ± 2.71
Rifampicin	11.18 ± 2.69
Amoxicillin	4.41 ± 2.38
Zidovudine	6.24 ± 2.57
Ampicillin	8.41 ± 2.71
Gentamycin	11.71 ± 2.87
Pefloxacin	14.06 ± 2.43
Erythromycin	10.24 ± 2.72
Levofloxacin	19.06 ± 1.32

significance when $p > 0.05$. Mean ± SEM indicates mean ± standard error of mean

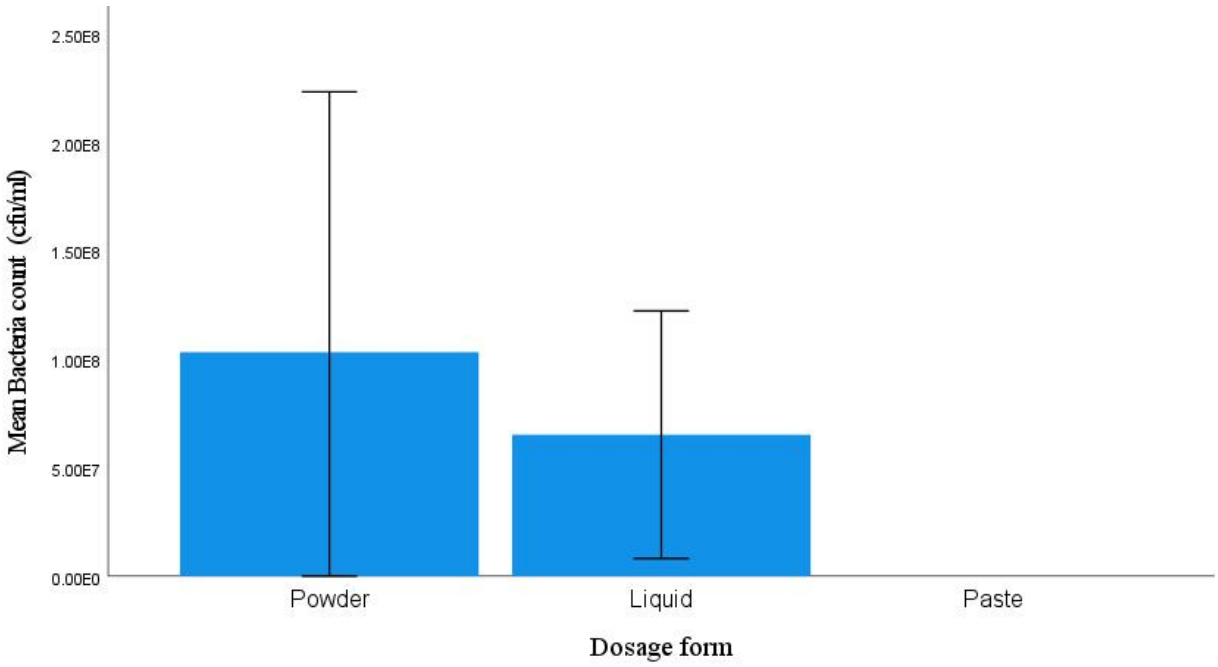

Figure 4.1. Bacterial load of herbal mixtures (CFU/ml) by sample form.
Error bars represent standard error of the mean (SEM); no significant differences were observed between dosage forms ($p > 0.05$).

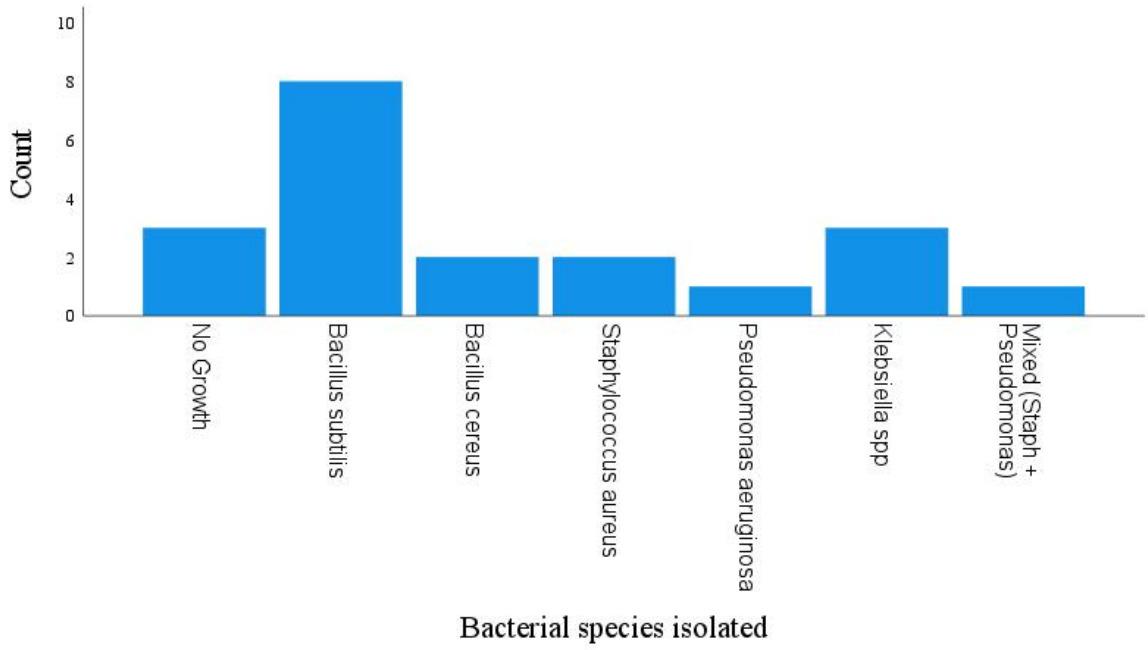

Figure 4.2. Frequency Distribution of Bacterial Species Isolated from Herbal Mixtures
Footnote: The most frequently isolated organism was *Bacillus subtilis* (n = 8), followed by *Klebsiella spp*. (n = 3), *Bacillus cereus* (n = 2), *Staphylococcus aureus* (n = 2), *Pseudomonas aeruginosa* (n = 1), and mixed growth (*Staphylococcus aureus* + *Pseudomonas aeruginosa,* n = 1). No growth was recorded in three samples. Chi-square test showed no significant difference in isolate distribution across market locations. ($p > 0.05$)

CHAPTER FIVE

5.1. DISCUSSION

Herbal medicines remain an integral part of healthcare in many African communities, including Nigeria, where they are widely sold in open markets without adequate regulation (Ahiabor and Darkwah, 2024). However, the safety of these herbal preparations has been questioned due to frequent reports of microbial contamination, which may ***compromise*** their therapeutic value and expose consumers to serious health risks which exceeds World Health Organization's permissible limit of 1.0×10^5 CFU/ml for herbal products (Opuni *et al.,* 2023 ; Omoruyi *et al.,* 2024). Studies across Nigeria and other African countries have shown that herbal mixtures often harbor pathogenic microorganisms such as *Escherichia coli*, *Salmonella spp.*, and *Staphylococcus aureus*, which are linked to gastrointestinal and systemic infections (Chinakwe and Ngumah, 2023). The presence of such contaminants is attributed to poor hygienic practices during preparation, use of contaminated water, and storage under unsuitable conditions in local markets (Darkwah *et al.,* 2022). In Edo State and other regions of Nigeria, the unregulated sale of herbal mixtures continues to pose significant public health challenges, as microbial loads in some products have been reported to exceed acceptable safety limits (Anie *et al.,* 2022). Previous findings have also shown that microbial contaminants not only reduce the potency of herbal remedies but may also introduce antibiotic-resistant strains into communities, thereby worsening the problem of antimicrobial resistance (Ideh and Ogunkunle, 2019).

Given these concerns, assessing the microbial quality of herbal mixtures sold in Benin City markets is crucial for understanding the potential risks to consumers and for providing evidence-based recommendations on safe usage and regulation.

The overall mean bacterial load of 7.92×10^7 CFU/ml observed in this study indicated that herbal mixtures sold in Benin City markets harbor microbial counts well above the World Health Organization's permissible limit of 1.0×10^5 CFU/ml for herbal products (Opuni *et al.,* 2023). This finding is consistent with previous reports across sub-Saharan Africa, where herbal medicines are frequently contaminated due to poor hygienic handling, unregulated production, and lack of microbial quality control (Yeboah, 2023).

When comparing dosage forms, powdered herbal preparations had the highest bacterial load (1.33×10^8 CFU/ml), followed by liquids (6.53×10^7 CFU/ml), while paste preparations showed no detectable growth. This sample type variation aligns with earlier studies, which reported that powdered herbal remedies tend to accumulate higher microbial loads due to increased surface area exposure, extended shelf-life, and susceptibility to contamination during grinding, packaging, and storage (Ideh and Ogunkunle, 2019). Similarly, herbal powders sold in Ogbomoso, Nigeria, were found to harbor multiple bacterial contaminants, including coliforms and *Staphylococcus* species, with counts surpassing recommended safety thresholds (Ideh and Ogunkunle, 2019). Liquid preparations in this study recorded relatively lower bacterial counts compared to powders but were still significantly contaminated. Previous investigations in Kenya and Ghana have shown that aqueous or liquid herbal formulations often support microbial proliferation because of their high moisture content and lack of preservatives, making them prone to spoilage during storage (Hassan, Njogu, and Njuguna, 2021; Darkwah, Agbettor, and Codjoe, 2022). Nevertheless, the lower bacterial count in liquids compared to powders in this study may suggest differences in handling or formulation practices within Benin City markets. Interestingly, paste formulations showed no detectable bacterial growth, a finding that diverges from common reports of microbial contamination in herbal medicines. This could be due to inherent preservative agents within the paste formulation, differences in moisture activity, or processing techniques that limit

microbial survival (Opuni *et al*., 2023). Paste-based herbal remedies may contain high concentrations of phytochemicals with antimicrobial properties, which could suppress bacterial proliferation more effectively than in powders and liquids (Yeboah, 2023).

Although powdered preparations had higher bacterial loads than liquids, the difference between dosage forms was not statistically significant 0.05. This suggests that, regardless of form, herbal mixtures sold in Benin City are generally at risk of contamination, corroborating findings from other regions in Nigeria where both solid and liquid formulations were consistently above acceptable microbial limits (Darkwah *et al*., 2022). This uniformity across forms underlines the role of systemic factors such as poor hygienic conditions, inadequate regulation, and improper storage, rather than dosage form alone, in determining microbial quality.

The variation in mean bacterial loads across the three sampled markets New Benin (1.12×10^8 CFU/ml), Uselu (6.93×10^7 CFU/ml), and Ring Road (5.50×10^7 CFU/ml) indicated differences in the microbial quality of herbal mixtures depending on point of sale. Although numerical differences were observed but was not statistically significance ($p > 0.05$) suggests that microbial contamination is a widespread issue across all locations rather than being confined to specific markets. Similar trends have been reported in Nigerian and African markets, where herbal medicines consistently exhibit high microbial counts regardless of their source location (Chinakwe *et al.,* 2023). The elevated bacterial load in New Benin samples may be attributed to preparation method and possible exposure to unhygienic environmental conditions such as dust and improper storage, which promote bacterial contamination (Dabo *et al.,* 2024). This aligns with the studies from Makurdi and Owerri, Nigeria, where herbal medicines from more crowded and unregulated markets showed higher

microbial loads than those from less congested areas (Chinakwe *et al.,* 2023; Dabo *et al*., 2024;).

Despite these numerical variations, the absence of significant differences across locations is consistent with findings from other Nigerian states, where herbal products, irrespective of their point of sale, were shown to harbor microbial counts above the World Health Organization's permissible limits (Opuni *et al.,* 2023). This indicates that contamination is largely systemic, driven by factors such as poor production practices, inadequate preservation, and lack of regulatory enforcement, rather than by the specific market environment alone (De Sousa Lima and Fujishima, 2020). Moreover, the uniform contamination pattern supports the argument that most herbal mixtures in Nigeria are prepared and distributed under similar unhygienic conditions, with limited quality control from production through to sale. Comparable studies from Ogbomoso and Lagos also reported high microbial loads across different local outlets, reinforcing the notion that location-based variation is minimal when contamination originates at the production stage (Ideh and Ogunkunle, 2019; Dabo *et al*., 2024).

The predominance of *Bacillus subtilis* in this study, accounting for 8 isolates, reflects its ubiquity in soil, dust, and air, which makes it a common contaminant in herbal medicines. This finding is consistent with earlier reports across Nigeria and Africa, where *Bacillus* species have frequently been identified as the dominant bacterial contaminants in herbal mixtures and other plant-based remedies (Ndukwu *et al.,* 2021; Ahiabor and Darkwah, 2024). While *Bacillus subtilis* is generally considered non-pathogenic, its presence in herbal medicines indicates poor handling or environmental contamination and raises concerns about product quality and hygiene (Esimone *et al.,* 2002). *Klebsiella spp.* was found in 3 isolates. It can indeed be found in herbal mixtures, especially where hygienic practices are poor. While

sometimes harmless, their presence poses a safety concern because of the risk of infections . Their isolation from herbal preparations has been reported in recent Nigerian studies, with authors warning that contaminated herbal products may serve as reservoirs for multidrug-resistant *Klebsiella* species (Olaniran *et al.,* 2022; Agada and Mohammed, 2024). This emphasizes the public health risk posed by such contaminants, especially in communities where herbal medicines are widely consumed without microbial quality checks. *Bacillus cereus* is known for its ability to produce enterotoxins that cause food poisoning, while *Staphylococcus aureus* is a well-recognized pathogen associated with skin, respiratory, and systemic infections. Previous Nigerian studies have similarly identified these organisms in herbal remedies, linking their presence to poor sanitary conditions during preparation and storage (Inoma and Evbuomwan, 2022; Chiegeiro, Ejiro, and Nnenna, 2022). In fact, *Staphylococcus aureus* has been repeatedly highlighted as one of the most frequent pathogens in herbal medicines sold across West Africa, posing risks of toxin production and antimicrobial resistance (Olaniran *et al*., 2022).

Pseudomonas aeruginosa was detected only once in this study, including in a mixed growth with *Staphylococcus aureus*. Although less frequent, its presence is significant given its role as a highly resistant opportunistic pathogen often linked with hospital-acquired infections. Previous work from Abuja and Port Harcourt similarly reported occasional isolation of *Pseudomonas* species in herbal preparations, warning of its potential to introduce drug-resistant infections into community settings (Ndukwu *et al*., 2021; Agada and Mohammed, 2024). The absence of growth in three samples may suggest that certain formulations contained natural antimicrobial compounds, or that handling and storage practices were comparatively more hygienic. Similar findings have been noted in studies where some herbal samples showed no microbial contamination, likely due to phytochemical constituents with antibacterial activity (Chiegeiro *et al*., 2022). The non-statistically significant association

between isolate distribution and market location ($p > 0.05$) highlights that the contamination pattern is widespread and not limited to specific outlets. This aligns with regional studies indicating that microbial contamination of herbal medicines is systemic across Nigerian markets, driven by shared practices of unregulated production, poor sanitation, and inadequate quality control (Inoma and Evbuomwan, 2022; Ahiabor and Darkwah, 2024).

The antibiotic susceptibility profile of isolates revealed complete sensitivity to Azithromycin and Levofloxacin , indicating strong efficacy of macrolide and fluoroquinolone classes against the tested organisms. This aligns with reports that these antibiotics remain effective against many bacteria recovered from herbal medicines, with fluoroquinolones in particular showing consistent broad-spectrum activity (Walusansa *et al.,* 2022). The high sensitivity to Pefloxacin further confirms the role of fluoroquinolones as reliable agents against potential contaminants in herbal preparations. Moderate sensitivity levels observed with Gentamycin, Rifampicin, and Erythromycin suggest partial effectiveness. This reflects trends in Nigeria and other African countries, where aminoglycosides and older macrolides have shown declining effectiveness due to emerging resistance among *Staphylococcus*, *Klebsiella*, and *Pseudomonas* isolates from herbal remedies (Asoso *et al.,* 2021; Turay *et al*., 2024). Such findings highlight a gradual shift toward resistance in common pathogens contaminating herbal mixtures, potentially linked to the misuse of antibiotics in both human medicine and agriculture.

Reduced sensitivity to Zidovudine and Ampicillin, and particularly the high resistance to Amoxicillin, underscores the declining utility of β-lactam antibiotics. Several studies from Nigeria and West Africa have reported widespread resistance to penicillin derivatives in bacteria isolated from herbal medicines, with resistance rates often exceeding 70% (Asoso *et al*., 2021; Turay *et al*., 2024). This pattern is consistent with global trends where β-lactam

resistance is driven by overuse, poor-quality drugs, and horizontal gene transfer of resistance mechanisms (Walusansa *et al*., 2022).

The absence of statistically significant differences in susceptibility patterns (p-values > 0.05) suggests that resistance is broadly distributed across isolates rather than being confined to specific species. This agrees with meta-analyses showing that antimicrobial resistance in bacteria from herbal products is not species-restricted but instead reflects widespread environmental and human-mediated selection pressures (Walusansa *et al*., 2022).

The mean inhibition zones observed in this study demonstrated that Levofloxacin and Azithromycin were the most effective antibiotics against the bacterial isolates. This strong activity of fluoroquinolones and macrolides corresponds with previous findings across Africa, where these classes have consistently shown the largest inhibition zones against bacteria isolated from herbal preparations (Walusansa, Asiimwe, and Nakavuma, 2022). Pefloxacin also demonstrated robust inhibition, reinforcing the efficacy of fluoroquinolones as reliable therapeutic options against contaminants of herbal medicines.

Moderate inhibition zones were recorded with Ciprofloxacin, Gentamycin, and Rifampicin. This pattern suggests intermediate antibacterial effects, similar to reports from Nigeria where aminoglycosides and rifamycins produced inhibition zones in the 10–15 mm range against *Staphylococcus aureus* and *Klebsiella* spp. recovered from herbal mixtures (Adeyemi *et al.,* 2021). The reduced inhibitory effect may reflect emerging resistance, consistent with recent evidence showing gradual decline in susceptibility of common pathogens to these antibiotics (Ugboko *et al.,* 2020). Smaller inhibition zones were observed with Zidovudine , Ampicillin , and Erythromycin. Amoxicillin, with the lowest inhibition zone, demonstrated the weakest antibacterial effect. These results agree with studies showing that penicillin derivatives often exhibit very limited inhibition zones against bacterial isolates from herbal medicines, with

many strains exhibiting resistance (Walusansa *et al*., 2022; Adeyemi *et al*., 2021). This highlights the declining effectiveness of β-lactam antibiotics in treating infections associated with contaminated herbal products.

The absence of statistically significant differences among the inhibition zones (all p-values > 0.05) suggests that although numerical variations exist, the general trend of reduced efficacy in older antibiotics compared to newer ones is consistent across isolates. This uniformity across test organisms mirrors findings in West African studies, where inhibition profiles showed broad similarities, reflecting widespread antimicrobial resistance pressures rather than organism-specific differences (Walusansa *et al*., 2022).

Collectively, these findings indicate that while newer antibiotics such as Levofloxacin and Azithromycin maintain strong activity against microbial contaminants of herbal mixtures, older and widely used antibiotics such as Amoxicillin and Ampicillin show poor performance, raising concerns about treatment failure and the propagation of resistant strains in the community.

5.2. CONCLUSION

The findings from this study revealed that herbal mixtures sold in markets across Benin City are widely contaminated with bacteria, with mean counts exceeding the World Health Organization's permissible limits. Although powdered formulations exhibited the highest bacterial loads, no significant differences were observed across samples form or market locations, underlining systemic contamination likely originating from poor hygienic practices and unregulated production. The predominance of *Bacillus subtilis*, alongside pathogenic isolates such as *Staphylococcus aureus*, *Klebsiella* spp., and *Pseudomonas aeruginosa*, highlights both environmental contamination and potential public health risks.

Antibiotic susceptibility testing further demonstrated that while modern agents such as Levofloxacin and Azithromycin remain highly effective, resistance to commonly available β-lactam antibiotics, especially Amoxicillin, was widespread.

5.3. RECOMMENDATIONS

1. Government agencies such as NAFDAC should intensify surveillance and enforce strict quality control measures on the production, packaging, and sale of herbal medicines to ensure compliance with microbial safety standards.

2. Vendors and producers of herbal mixtures should be educated and trained on proper hygienic practices, including the use of clean water, sterile containers, and sanitary handling during preparation and storage.

3. Herbal products intended for public consumption should undergo mandatory routine microbial analysis to monitor contamination levels and prevent the sale of unsafe products.

4. Consumers should be sensitized to the potential health risks associated with unregulated herbal mixtures and encouraged to seek products from certified and standardized sources.

5. Policies should be developed to promote standardization of herbal mixtures in terms of formulation, dosage forms, and preservation methods, reducing variability and minimizing contamination risks.

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APPENDIX I:

MATERIALS USED FOR SAMPLE EXAMINATION

The following materials, media, reagents, and equipment were employed during the examination of herbal products:

1. Culture Media

- Nutrient Agar (NA)
- Blood Agar
- Mueller Hinton Agar (MHA)
- MacConkey Agar

2. Sample Collection and Preparation Materials

- Sterile universal bottles (for sample collection and dilution)
- Sterile water (for preparation of stock solutions)
- One-Quarter Strength Ringer's solution (diluent for serial dilution)
- Micropipettes and sterile tips
- Analytical balance (for weighing powdered products)
- Sterile bottles labeled S1–S20 (sample storage)

3. Reagents and Biochemical Test Kits

- Gram staining reagents (crystal violet, iodine, decolorizer, neutral red)
- Catalase reagent (3% hydrogen peroxide)
- Coagulase reagent (plasma)
- Oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride)
- Citrate utilization medium (Simmons citrate agar)
- Indole test reagent (Kovac's reagent)
- Carbohydrate fermentation media (with lactose sugar)

- Distilled water

4. Serial Dilution and Inoculation Materials

- Sterile test tubes (15 mL, 50 mL)
- Pipettes (1 mL, 5 mL, 10 mL)
- Petri dishes (sterile, disposable)
- Wire loops (sterile, nichrome)
- Glass slides and cover slips
- Sterile spreaders

5. Antimicrobial Susceptibility Testing (AST)

- Kirby–Bauer antibiotic discs
- Mueller Hinton Agar plates
- Ruler (for measuring zones of inhibition)

6. Equipment

- Autoclave (for sterilization)
- Laboratory incubator (set at 37 °C)
- Hot air oven (for drying plates at 40 °C)
- Laboratory centrifuge
- Light binocular microscope (×10, ×40,×100 objectives lens,)
- Refrigerator (for media/reagent storage)

7. Safety and Protective Materials

- Laboratory coats
- Disposable gloves
- Face masks
- Hand sanitizers
- Biohazard/sharps disposal containers

APPENDIX II

RESEARCH ETHICS COMMITTEE

COLLEGE OF MEDICAL SCIENCES
UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

Chairman: Prof. F. A Imarhiagbe
MBChb, FMCP
Cert Clin Res and ethics (NIH), MD.
0803449092

P.M.B 1154, BENIN CITY
Email: researchethics.cms@gmail.com

Our Ref: CMS/REC/01/VOL.2/786 **Date:** 18th September, 2025

Re: ISOLATION AND IDENTIFICATION OF MICROOGANISM PRESENT IN HERBAL MIXTURE SOLD AT SOME MARKET IN BENIN CITY, EDO STATE

Name of Principal Investigator: **IKEM KAMSOCHUKWU FAVOUR**
Department Of Medical Laboratory Science,
School of Basic Medical Science
College of Medical Sciences,
University of Benin

REC Approval No: **CMS/REC/2025/786**

This is to inform you that the research described in the submitted proposal, the Informed Consent Forms and other participant information materials have been reviewed and approved by the College Research Ethics Committee, University of Benin.

This approval dates from **18th September, 2025 to 19th September, 2026.** In multi-year research, Endeavour to submit your annual report to the REC early in order to obtain renewal of your approval and avoid disruption of your research.

The National Code of Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the code including ensuring that all adverse events are reported promptly to the REC. No, changes are permitted in the research without prior approval by REC except in circumstances outlined in the code. REC reserves the right to conduct compliance visit to your research site without prior notice. Thank you.

PROF. F.A IMARHIAGBE
Chairman, REC

Promoting best ethical & scientific standard for research in Nigeria

APPENDIX III

Pictures showing Nutrient Agar in Triplicate.

Picture showing preparation of Quarter - Strength Ringer's solution and dispensed into sterile bijou bottles.