ISOLATION AND IDENTIFICATION OF FUNGI IN HERBAL MIXTURES SOLD AT SOME MARKETS IN BENIN CITY, EDO STATE, NIGERIA.

BY

OYEWUMI, GBENGA SAMUEL
BMS1900382

DEPARTMENT OF MEDICAL LABORATORY SCIENCE,
SCHOOL OF BASIC MEDICAL SCIENCES,
COLLEGE OF MEDICAL SCIENCES,
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THIS PROJECT IS SUBMITTED TO:
THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE,
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SUPERVISOR:

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 OYEWUMI, GBENGA SAMUEL with the matriculation number BMS1900382 under the supervision of Dr. (Mrs.) A. O. Itemire in partial fulfillment for the award of Bachelor of Medical Laboratory Science (B.MLS) Degree.

DR. (MRS) ANNE O. ITEMIRE	**DATE**
(Project Supervisor)	
DR. (MRS) Z. OMORUYI	**DATE**
(Head of Department)	
PROF. O. P. OMOSIGHO	**DATE**
(External Examiner)	

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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I give all glory to the Almighty God, the source of wisdom and knowledge. I sincerely appreciate the professional guidance and supervision of my supervisor, Dr. (Mrs.) Anne O. Itemire, whose invaluable support and direction greatly contributed to the success of this work. My special gratitude also goes to the Head of Department, Dr. (Mrs.) Z. Omoruyi, for constantly pushing us towards greatness

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ABSTRACT

Herbal mixtures are widely consumed in Nigeria for their perceived therapeutic benefits, yet concerns about microbial safety, particularly fungal contamination, persist. This study investigated the fungal load and diversity of herbal mixtures sold at selected markets in Benin City, Edo State. A total of 20 samples (powder, liquid, and paste formulations) were collected from Uselu, New Benin, and Ring Road markets. Fungal isolation was performed using Sabouraud Dextrose Agar, and identification was based on cultural and microscopic characteristics. Antifungal sensitivity was assessed by incorporating herbal mixtures into culture media. Results showed that 65% of samples were contaminated, with fungal loads ranging from 3.08×10^3 to 1.72×10^6 CFU/ml. The predominant isolates were *Mucor* (20%), *Fusarium* (20%), *Aspergillus niger* (15%), and *Penicillium* (10%). Powdered samples showed the highest contamination levels, while Ring Road samples recorded the least growth. Antifungal assays revealed only partial inhibitory effects at high concentrations (3000 mg), indicating limited intrinsic antifungal activity of the herbal mixtures tested. These findings highlight significant fungal contamination in locally sold herbal mixtures, with potential public health risks including opportunistic infections and mycotoxin exposure. Stronger regulation, quality control, and public health awareness are recommended to improve the safety of herbal medicines in Benin City.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Herbal mixtures are widely consumed as alternative remedies in Nigeria, yet concerns about their microbiological safety are mounting, particularly with respect to fungal contamination (Shitu *et al*., 2024). Studies across Nigeria report alarming fungal loads in herbal products, ranging from 10^5 to 10^{12} CFU/mL, with particularly high counts recorded in some regions, highlighting substantial risk to consumers (Ahiabor *et al*., 2024) . In North-West Nigeria, all evaluated powdered herbal preparations exceeded the acceptable fungal limit (2×10^2 CFU/g), with *Aspergillus (*e.g., *A. flavus*, *A. niger*) and *Penicillium* being the most frequently isolated genera (Abba *et al*., 2021).

In Southeast Nigeria, powdered herbal preparations from Enugu State were universally contaminated, with fungal counts reaching up to 10^4 CFU/g; dominant isolates included *Aspergillus, Penicillium, Mucor, and Candida* (Anyanwu, 2010). Similarly, herbal concoctions sold in Benin City revealed fungal loads ranging between 6.0×10^3 and 1.8×10^4 CFU/mL, with *Aspergillus flavus, A. niger***,** *Penicillium sp***.,** and *Rhizopus sp***.** as prevalent contaminants (Oshoma and Dijeh, 2017).

Mycotoxin contamination further compounds safety concerns. In samples from Ebonyi State, high prevalence rates were reported namely ochratoxin A (89.5%), fumonisin (82.5%), and aflatoxin (82.2%) with levels in some mixtures exceeding Nigerian and EU permissible thresholds (Ukibe *et al*., 2022). Broadly across Africa, herbal medicine fungal loads varied significantly, with Nigeria among the countries registering the highest bioloads (Ahiabor *et al*., 2024). The co-occurrence of toxigenic fungi and mycotoxins significantly jeopardizes consumer health, particularly for immunocompromised individuals. Despite these

documented risks, data specific to fungal contamination in herbal mixtures sold at markets in Benin City remain scarce. Although a related study on *Irvingia gabonensis* (ogbono) seeds in Benin City identified seven fungal species *Aspergillus flavus, A. fumigatus*, *A. niger*, *Mucor* sp., *Neurospora* sp., *Penicillium sp*., and *Rhizopus sp.* the survey did not target herbal mixtures commonly sold for therapeutic use (Okobiebi and Ezennia, 2021). This gap underscores the need for targeted research on mycological contamination in herbal mixtures circulating in Benin City's markets.

1.2 Statement of the Problem

Herbal mixtures remain an integral part of primary healthcare in Nigeria due to their affordability, accessibility, and cultural acceptance. However, the safety of these products is increasingly questioned as several studies across the country have reported significant levels of microbial contamination, particularly fungi such as *Aspergillus*, *Penicillium*, *Mucor*, and *Candida*. These fungi are not only opportunistic pathogens but are also known producers of mycotoxins, which have been implicated in carcinogenic, hepatotoxic, nephrotoxic, and immunosuppressive effects.

Despite the popularity and widespread consumption of herbal mixtures in Benin City, little is known about the specific fungal species contaminating these preparations or their potential health risks. This poses a serious public health concern, especially for immunocompromised individuals such as people living with HIV/AIDS, tuberculosis, diabetes, and other chronic conditions, who frequently consume these herbal products alongside or in place of conventional medicines.

Furthermore, the informal and poorly regulated nature of herbal medicine production and distribution in Nigeria increases the risk of contamination during harvesting, processing,

packaging, and storage. Regulatory bodies such as NAFDAC often face challenges in enforcing strict quality control due to the large number of unregistered vendors and the cultural reliance on traditional medicine. Without clear data on the extent and identity of fungal contaminants in herbal mixtures sold in Benin City, consumers remain exposed to potential health hazards.

1.3 Justification of the Study

Herbal medicines remain an essential part of healthcare in Nigeria due to their affordability and cultural acceptance (Shitu *et al*., 2024). However, evidence from recent studies shows that these products are often contaminated with fungi, including *Aspergillus*, *Penicillium*, and *Mucor* species, which not only reduce product quality but also pose serious public health risks (Chinakwe *et al*., 2023). Such contamination is of particular concern because these fungi are capable of producing mycotoxins such as aflatoxins, fumonisins, and ochratoxins, which are linked to liver cancer, nephrotoxicity, and immunosuppression (Ukibe *et al*., 2022).

High fungal loads have been reported in herbal preparations across Nigeria, sometimes exceeding internationally acceptable safety limits (Ahiabor *et al*., 2024). In Ebonyi State, mycotoxins including ochratoxin A, fumonisin, and aflatoxin were found at levels above both Nigerian and European Union permissible thresholds, indicating the scale of the problem (Ukibe *et al*., 2022). Toxicological studies further confirm that contaminated herbal mixtures can cause adverse biological effects, including weight loss and altered hematological parameters, in experimental animals (Terna *et al*., 2025).

Despite these growing concerns, limited research has been conducted in Benin City, Edo State, where herbal mixtures are widely consumed and traded. Given the high patronage of herbal medicine in this region, coupled with poor regulation and quality assurance, there is a

pressing need to evaluate the extent of fungal contamination in locally sold herbal mixtures (Shitu *et al*., 2024).

1.4. Aim of study

The study aimed to isolate and identify fungi present in herbal mixtures sold at selected markets in Benin City, Edo State, Nigeria.

1.5. The Specific Objectives

The Specific objectives of this study are to;

1. **determine the fungal load of herbal mixtures sold at selected markets in Benin City.**
2. isolate and identify fungal species associated with these herbal mixtures using cultural, morphological, and microscopic methods.
3. compare the frequency of occurrence of different fungal species across sampled herbal mixtures.
4. assess the potential health risks associated with identified fungi in relation to their Known pathogenicity and mycotoxin production.

1.6 Research Questions

1. What is the fungal load of herbal mixtures sold at selected markets in Benin City?
2. Which fungal species are present in herbal mixtures sold in Benin City?
3. How frequently do the isolated fungal species occur across different herbal mixtures?
4. What potential health risks are associated with the identified fungi in terms of pathogenicity and mycotoxin production?

1.7 Research Hypotheses

1.7.1 Null Hypotheses (H_0)

1. H_{01}: There is no significant fungal load in herbal mixtures sold at selected markets in Benin City.
2. H_{02}: There are no fungi present in herbal mixtures sold at selected markets in Benin City.
3. H_{03}: There is no significant difference in the frequency of occurrence of fungal species across herbal mixtures sold in Benin City.
4. H_{04}: The fungi isolated from herbal mixtures sold in Benin City do not pose any potential health risks to consumers.

1.7.2 Alternative Hypotheses (H_1)

1. H_{11}: There is a significant fungal load in herbal mixtures sold at selected markets in Benin City.
2. H_{12}: Fungi are present in herbal mixtures sold at selected markets in Benin City.
3. H_{13}: There is a significant difference in the frequency of occurrence of fungal species across herbal mixtures sold in Benin City.
4. H_{14}: The fungi isolated from herbal mixtures sold in Benin City pose potential health risks to consumers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Background of Fungal Contamination in Herbal Mixtures

Herbal medicines continue to play a vital role in healthcare globally, particularly in developing countries, where they are often more accessible and affordable than conventional pharmaceuticals (Shitu *et al*., 2024). In Nigeria, herbal mixtures are widely consumed for the management of diverse ailments ranging from malaria and gastrointestinal disorders to chronic diseases such as hypertension and diabetes (Chinakwe *et al*., 2023). However, despite their popularity, concerns have been raised about the microbiological safety of these products, particularly regarding fungal contamination (Ahiabor *et al*., 2024). Fungi are common contaminants of herbal products because of their ubiquitous nature and ability to grow on plant materials under poor storage, processing, and packaging conditions (Ukibe *et al*., 2022). Several species including *Aspergillus*, *Penicillium*, *Mucor*, *Fusarium*, and *Candida* have been isolated from herbal preparations in Nigeria and other African countries (Chinakwe *et al*., 2023). These fungi are of significant public health concern, as many are opportunistic pathogens capable of causing infections in immunocompromised individuals such as people living with HIV/AIDS and tuberculosis (Shitu *et al*., 2024).

Beyond direct infection risks, fungal contamination is also problematic because of the ability of certain species to produce mycotoxins, secondary metabolites that are toxic to humans and animals. Mycotoxins such as aflatoxins, ochratoxins, and fumonisins have been detected in herbal medicines sold in Nigeria at concentrations that often exceed national and international permissible limits (Ukibe *et al*., 2022). A recent systematic review reported that fungal loads in African herbal medicines can reach up to 10^{12} CFU/mL, with Nigeria among the countries recording the highest levels of contamination (Ahiabor *et al*., 2024). Such findings highlight

the dual threat of fungal colonization and mycotoxin contamination in herbal preparations. Toxicological studies have demonstrated that consumption of herbal mixtures contaminated with fungi and mycotoxins can lead to adverse biological effects including weight loss, organ damage, and hematological alterations in experimental animals (Terna *et al*., 2025). This evidence reinforces the need for continuous monitoring of fungal contamination in herbal mixtures to safeguard public health.

2.1.1 Historical Use of Herbal Mixtures in Traditional Medicine

The use of herbal mixtures in traditional medicine dates back thousands of years, with documented evidence from ancient civilizations such as Egypt, China, and India where plants were used for therapeutic purposes (World Health Organization [WHO], 2023). In Africa, herbal medicine has long been a central aspect of healthcare, often intertwined with cultural and spiritual practices (Ahiabor *et al*., 2024). Before the introduction of modern pharmaceuticals, communities relied heavily on herbal mixtures for the treatment of common ailments, infectious diseases, and chronic conditions (Chinakwe *et al*., 2023).

In Nigeria, herbal medicine continues to thrive as an important component of primary healthcare, largely due to affordability, accessibility, and cultural acceptance (Shitu *et al*., 2024). Surveys indicate that more than 70% of the Nigerian population uses herbal remedies either exclusively or in combination with orthodox medicine (Ukibe *et al*., 2022). These mixtures are commonly prepared by traditional healers, vendors, or households, and are used for treating malaria, gastrointestinal disorders, skin infections, and reproductive health issues (Terna *et al*., 2025).

The historical reliance on herbal mixtures has been reinforced by limited access to orthodox healthcare services in rural communities and the high cost of conventional drugs in urban

areas (Ahiabor *et al*., 2024). This reliance persists despite the lack of standardized formulations, quality control, and safety assessments, raising concerns about microbial contamination and toxicological risks (Ukibe *et al*., 2022).

2.1.2 Global Concerns on Safety and Quality of Herbal Products

The global consumption of herbal medicines has increased significantly over the past two decades, with estimates suggesting that up to 80% of the population in developing countries rely on herbal remedies for primary healthcare (World Health Organization [WHO], 2023). Despite this widespread use, concerns regarding the safety and quality of herbal products remain prevalent, particularly due to issues of contamination, adulteration, and lack of standardized quality control (Ahiabor *et al*., 2024).

One of the primary concerns is microbial contamination, especially by fungi capable of producing harmful mycotoxins. Studies across Africa, Asia, and Europe have reported that herbal medicines are frequently contaminated with fungal genera such as *Aspergillus*, *Penicillium*, and *Fusarium*, which pose risks of hepatotoxicity, nephrotoxicity, and immunosuppression in humans (Ukibe *et al*., 2022). A systematic review found that fungal loads in African herbal medicines often exceed international safety standards, with Nigeria recording some of the highest levels of contamination (Ahiabor *et al*., 2024).

In addition to microbial contamination, chemical adulteration and mislabeling of herbal products present further risks. Many herbal preparations sold in low- and middle-income countries lack adequate regulation and quality assurance, resulting in products with inconsistent composition, potency, and safety profiles (Shitu *et al*., 2024). In some cases, herbal products have been found to contain heavy metals, pesticide residues, or pharmaceutical adulterants, which significantly increase health risks (Chinakwe *et al*., 2023).

Globally, regulatory agencies such as the WHO and the European Medicines Agency (EMA) have emphasized the need for stricter quality control, pharmacovigilance, and safety assessments of herbal medicines (WHO, 2023). In Nigeria, the National Agency for Food and Drug Administration and Control (NAFDAC) has initiated steps to monitor herbal products; however, enforcement remains challenging due to the informal nature of herbal medicine markets and the high level of unregistered products (Ukibe *et al*., 2022).

2.2 General Characteristics of Fungi

Fungi are a diverse group of eukaryotic organisms that occupy a wide range of ecological niches, playing critical roles in decomposition, nutrient cycling, and symbiotic associations with plants and animals (Hyde *et al*., 2021). They are distinguished from other microorganisms by their chitin-containing cell walls, heterotrophic nutrition through absorption, and the production of spores as reproductive units (Zhang *et al*., 2023). Fungi exist in various morphological forms, including unicellular yeasts and multicellular filamentous molds, with some species capable of dimorphic transitions depending on environmental conditions (Ameen *et al*., 2022).

Most fungi reproduce both sexually and asexually. Asexual reproduction commonly occurs through spore formation, budding, or fragmentation of hyphae, whereas sexual reproduction involves the fusion of compatible mating types to form genetically diverse spores (Hyde *et al*., 2021). This reproductive versatility contributes to their resilience and widespread distribution in terrestrial, aquatic, and even extreme environments (Zhang *et al*., 2023).

Medically important fungi often belong to genera such as *Aspergillus*, *Penicillium*, *Candida*, *Mucor*, and *Fusarium* (Ameen *et al*., 2022). These organisms are opportunistic in nature, infecting immunocompromised hosts such as HIV/AIDS patients, transplant recipients, and

individuals undergoing chemotherapy. Beyond direct infections, many fungi produce secondary metabolites known as mycotoxins, including aflatoxins, ochratoxins, and fumonisins, which pose significant risks to human health through contaminated food and herbal medicines (Ukibe *et al*., 2022).

Table 2.1: Taxonomic Classification of Medically Important Fungi

Phylum	Representative Genera/Species	Medical Importance
Ascomycota	*Aspergillus* spp., *Candida* spp., *Fusarium* spp., *Pneumocystis jirovecii*	Cause of systemic and superficial mycoses; invasive aspergillosis, candidiasis, fusariosis, and pneumocystis pneumonia
Basidiomycota	*Cryptococcus neoformans*, *Cryptococcus gattii*	Opportunistic pathogens causing cryptococcosis affecting lungs and CNS, especially in immunocompromised patients
***Mucor*omycota**	*Rhizopus sp*p., *Mucor* spp., *Lichtheimia* spp.	Cause of *Mucor*mycosis (rapidly progressive, angioinvasive fungal infection) often in diabetes and immunosuppression
Chytridiomycota	Rare species occasionally pathogenic	Rarely associated with human infections but important for evolutionary understanding of fungi

2.2.2 Morphological and Physiological Characteristics of Fungi

Fungi exhibit diverse morphological and physiological traits that contribute to their ecological success and medical significance. Structurally, they are eukaryotic organisms with cell walls composed primarily of chitin and glucans, a feature that distinguishes them from bacteria and plants (Hyde *et al*., 2021). These rigid cell walls provide structural stability, facilitate pathogenicity, and influence antifungal drug susceptibility (Ameen *et al*., 2022).

In terms of morphology, fungi may exist as unicellular yeasts, multicellular filamentous molds, or exhibit dimorphism, switching between yeast and mold forms depending on environmental conditions (Zhang *et al*., 2023). Dimorphic fungi such as *Histoplasma capsulatum* and *Blastomyces dermatitidis* are medically important because morphological switching is linked to virulence, enabling adaptation to host environments (Ameen *et al*., 2022).

Fungal reproduction is both sexual and asexual, with asexual reproduction involving budding, fragmentation, or spore production, while sexual reproduction generates genetic diversity through the fusion of compatible mating types (Hyde *et al*., 2021). This versatility enhances survival and contributes to the rapid dissemination of pathogenic species in clinical and environmental settings (Zhang *et al*., 2023).

Physiologically, fungi are heterotrophic organisms that absorb nutrients from organic substrates through extracellular enzyme secretion (Ahiabor *et al*., 2024). This characteristic allows them to colonize plant materials, foods, and herbal mixtures, especially under poor hygienic conditions. Their ability to tolerate a wide range of environmental conditions including low moisture, acidic or alkaline pH, and elevated temperatures further explains their persistence in stored herbal medicines (Shitu *et al*., 2024).

Moreover, many fungi produce secondary metabolites, some of which are beneficial (e.g., antibiotics such as penicillin) while others are toxic mycotoxins. Toxigenic species like *Aspergillus flavus* and *A. niger* produce aflatoxins and ochratoxins, respectively, which are linked to liver cancer, nephrotoxicity, and immunosuppression (Ukibe *et al*., 2022).

2.2.3 Ecological Niches and Survival Strategies of Fungi

Fungi are cosmopolitan organisms that occupy diverse ecological niches ranging from soil and water to plants, animals, and decaying organic matter (Hyde *et al*., 2021). Their ecological success is attributed to remarkable adaptability, allowing them to thrive in environments with fluctuating temperature, moisture, and nutrient availability (Zhang *et al*., 2023). In particular, fungi are well-suited for colonizing herbal materials, which provide abundant organic substrates rich in carbohydrates, proteins, and secondary metabolites (Ahiabor *et al*., 2024).

A key survival strategy of fungi is spore production. Fungal spores are highly resistant to environmental stressors such as desiccation, ultraviolet radiation, and nutrient scarcity, enabling them to persist in harsh conditions and disperse widely through air, water, and vectors (Ameen *et al*., 2022). This explains the ubiquitous presence of fungal contaminants in herbal mixtures and plant-based foods, even after processing or storage.

Fungi also rely on extracellular enzyme secretion to degrade complex organic matter into simpler nutrients that can be absorbed (Shitu *et al*., 2024). This saprophytic mode of nutrition makes them important decomposers in ecosystems, while simultaneously predisposing poorly handled herbal mixtures to contamination and spoilage. Opportunistic pathogenic fungi exploit this ability by invading human tissues, particularly in immunocompromised hosts,

causing infections ranging from superficial mycoses to life-threatening systemic diseases (Ameen *et al*., 2022).

Another important adaptive trait is fungal biofilm formation. Pathogenic species such as *Candida albicans* and *Aspergillus fumigatus* can form biofilms on biotic and abiotic surfaces, which protect them against antifungal agents and immune defenses, thereby enhancing persistence and resistance (Zhang *et al*., 2023). This trait has clinical implications and may contribute to the survival of fungi in herbal preparations exposed to varying storage and environmental conditions.

2.3 Sources and Routes of Fungal Contamination in Herbal Mixtures

Fungal contamination of herbal mixtures commonly occurs along critical stages of the production and supply chain, including harvesting and post-harvest handling, processing, storage, and marketing. Each stage presents unique opportunities for fungal colonization, especially under substandard sanitary conditions (Ahiabor *et al*., 2024).

2.3.1 Contamination during Harvesting and Post-Harvest Handling

Fungal contamination of herbal mixtures often begins at the harvesting stage, where plants are exposed to environmental spores from soil, water, and dust (Aliyu, 2024). Herbs collected under humid tropical conditions, such as those in Nigeria, are particularly prone to colonization by *Aspergillus* and *Fusarium* species due to favorable moisture levels (Ilozue and Okoye, 2024). Handling with contaminated tools or bare hands also introduces fungi onto freshly harvested herbs (Chinakwe *et al*., 2023).

Post-harvest drying practices contribute significantly to contamination, especially when herbs are dried directly on the ground or in open-air environments where airborne spores can settle

easily (Aliyu, 2024). Inadequate sorting of harvested materials allows decayed or damaged plant parts, which serve as fungal substrates, to remain within mixtures (Shitu *et al*., 2024). Bulk packaging in non-sterile containers and transportation in unsanitary conditions further increase the microbial load (Ilozue and Okoye, 2024).

Market-level practices, such as exposing herbal mixtures in open containers during sale, exacerbate the contamination risk, as demonstrated in studies where fungal counts exceeded permissible limits for microbial safety (Aliyu, 2024). Frequent isolation of *Aspergillus flavus, A. niger*, and *Penicillium sp*ecies from herbal mixtures across Nigerian markets confirms that poor harvesting and post-harvest handling remain critical points of entry for fungal contaminants (Shitu *et al*., 2024).

2.3.2 Processing, Storage, and Packaging as Risk Factors

The processing of herbal mixtures is a major contributor to fungal contamination, particularly when unhygienic environments are used for grinding, mixing, or extraction (Aliyu, 2024). Studies in Ilorin revealed that herbal products prepared without sterilized water or equipment often contained *Candida albicans* and *Scedosporium* spp., suggesting direct contamination from the processing stage (Aliyu, 2024). Similarly, a survey in Owerri reported that improper processing practices, including inadequate washing of raw materials, resulted in fungal loads above acceptable microbial safety standards (Chinakwe *et al*., 2023).

Storage is another critical risk factor because fungi thrive in warm and humid environments common in Nigeria (Ilozue and Okoye, 2024). Herbal mixtures stored in non-airtight containers or in damp markets were more frequently contaminated with *Aspergillus* and *Penicillium sp*ecies (Shitu *et al*., 2024). Evidence from Anambra State showed that fungal

growth was significantly higher in herbal concoctions stored in reused plastic containers compared to those stored in sealed glass bottles (Ilozue and Okoye, 2024).

Packaging materials also influence the extent of fungal contamination. Products packaged in polythene bags or reused bottles often harbor residual spores, which serve as sources of re-infection (Shitu *et al*., 2024). Aliyu (2024) demonstrated that herbal mixtures packaged under open-air conditions had microbial counts exceeding 10^3 CFU/mL, highlighting packaging as a significant determinant of safety.

2.3.3 Market Environment and Hygienic Practices of Vendors

Herbal products sold in open market environments are frequently exposed to airborne fungal spores, dust, and insect vectors due to inadequate structural protection and sanitation (Aliyu and Abdulhamid, 2025). In Kaduna's crowded markets, herbal mixtures displayed in unsealed containers were found to contain fungal isolates like *Aspergillus niger*, *A. flavus*, *Mucor* sp., and *Rhizopus sp*. in over half of the samples tested (Aliyu and Abdulhamid, 2025). Vendors often handle these products without gloves and use unwashed utensils to dispense mixtures, thereby increasing the risk of fungal contamination (Adomi and Enwa, 2022). In Delta State, unregulated herbal products packaged by street vendors showed significantly higher microbial loads compared to regulated products, implicating poor hygiene and packaging practices in contamination (Adomi and Enwa, 2022).

Furthermore, powdered herbal medicines sampled across North-Western Nigeria including Kaduna and Kebbi were heavily contaminated with *Aspergillus flavus, A. niger*, and *Penicillium sp*p., a likely result of unhygienic market handling and inadequate vendor practices (Abba *et al*., 2024). Similarly, in Makurdi and Adikpo, recurring detection of

Aspergillus niger in herbal samples was linked to substandard vendor storage and display conditions, reinforcing the role of market hygiene in fungal contamination (Dabo *et al,* 2024).

2.4. Common Fungal Species Isolated from Herbal Mixtures

2.4.1 *Aspergillus* spp. (*A. flavus, A. niger, A. fumigatus)*

Aspergillus species are among the most frequently encountered fungal contaminants in herbal mixtures across Africa; as a systematic review shows, *Aspergillus* spp. appeared in 40% of studies evaluating herbal medicine contamination (Ahiabor *et al*., 2024). *Aspergillus flavus* is particularly concerning due to its production of aflatoxins potent hepatocarcinogens that are linked to liver cancer, immunosuppression, and growth impairment (Olatunbosun and Oluwafemi, 2023). In a study of herbal remedies sold at Itoku Market in Ogun State, *A. flavus* was directly isolated from local herbal mixtures alongside detected aflatoxin levels (Olatunbosun and Oluwafemi, 2023).

Aspergillus niger is another prevalent species**,** commonly recovered from powdered and liquid herbal preparations in markets throughout Nigeria (Dabo *et al.,* 2024). For instance, a comprehensive microbial assessment of powdered herbal medicines from Makurdi and Adikpo revealed *A. niger* as the most frequently isolated fungus accounting for nearly 30% of total fungal isolates followed closely by *A. flavus* ((Dabo *et al*., 2024)).

Although less prevalent, *Aspergillus fumigatus* remains clinically significant due to its ability to cause invasive aspergillosis in immunocompromised individuals. While data on its presence in herbal mixtures is limited, the overall dominance of the *Aspergillus* genus suggests ongoing risk of exposure to *A. fumigatus* in environments with high fungal contamination (Ahiabor *et al*., 2024).

2.4.2. *Penicillium* spp.

*Penicillium sp*p. are widely recognized as important fungi due to their dual role as both beneficial organisms in biotechnology and potential threats through mycotoxin production (Fernandez-Bunster, 2021). Their diversity and adaptability allow them to colonize a wide range of substrates, making them crucial for industrial, agricultural, and pharmaceutical applications (Fernandez-Bunster, 2021).

One of the primary concerns associated with *Penicillium sp*p. is their ability to produce toxic secondary metabolites, or mycotoxins, which pose significant risks to food safety and human health (Martínez-Culebras *et al*., 2021). These toxins contaminate agricultural produce, contributing to postharvest losses and health hazards in both humans and animals (Godana *et al*., 2023). Recent advances in biotechnological approaches have highlighted the potential of *Penicillium sp*p. for use in disease management strategies, including the development of bio-based controls that mitigate fungal pathogens and their associated toxins (Godana *et al*., 2023).

Beyond pathogenicity, *Penicillium sp*p. have also been explored for their beneficial metabolites, such as antimicrobial peptides and proteins that can be harnessed for biocontrol purposes against toxigenic fungi (Martínez-Culebras *et al*., 2021). This dual capacity underscores the genus's significance in both food security and biotechnological innovation. Moreover, molecular and phylogenetic profiling has improved species identification, which is essential for distinguishing pathogenic strains from those with industrial potential (Fernandez-Bunster, 2021).

2.4.3. *Fusarium* spp.

Fusarium spp. represents one of the most destructive groups of plant pathogenic fungi, causing widespread diseases in cereals and other crops worldwide (Shabeer *et al*., 2021). Their pathogenicity is largely associated with species such as *F. solani*, which is responsible for up to 50% of *Fusarium*-related plant diseases (Shabeer *et al*., 2021).

These fungi are notorious for producing a wide array of mycotoxins, including trichothecenes, fumonisins, and zearalenone, which not only compromise crop yields but also pose significant risks to food safety and public health (Ekwomadu and Mwanza, 2023). Mycotoxin biosynthesis is strongly influenced by environmental conditions, which in turn modulate the severity of plant diseases caused by *Fusarium* spp. (Xue *et al*., 2023). Moreover, these secondary metabolites act as virulence factors, enhancing the fungi's ability to colonize plant tissues (Shabeer *et al*., 2021).

Management of *Fusarium*-induced diseases remains challenging, as conventional chemical fungicides are often insufficient, leading researchers to explore alternative strategies such as plant-derived antifungal extracts and biological control agents (Xue *et al*., 2023). For instance, natural products like black spruce extract have shown promising antifungal effects against potato dry rot caused by *Fusarium* spp. (Xue *et al*., 2023). Similarly, beneficial microbes are being investigated as biocontrol agents to suppress *Fusarium* growth and reduce mycotoxin contamination (Shabeer *et al*., 2021).

2.4.4. *Candida* spp. and Other Yeasts

Candida spp. remains among the most clinically significant opportunistic fungal pathogens, responsible for both superficial and systemic infections in immunocompromised individuals (Czajka *et al*., 2023). The increasing prevalence of non-*albicans Candida* species, such as *C.*

glabrata, *C. tropicalis*, and *C. parapsilosis*, reflects a global shift in candidiasis epidemiology and highlights the need for updated surveillance (Silva *et al*., 2012).

The emergence of multidrug resistance in *Candida* spp. is a growing concern, with mutations in ergosterol biosynthesis and efflux pump overexpression contributing to reduced susceptibility to azoles and echinocandins (Czajka *et al*., 2023). Resistance development not only limits therapeutic options but can also enhance virulence, thereby exacerbating clinical outcomes (Bohner *et al*., 2022). This is particularly evident in *Candida auris*, a multidrug-resistant yeast that has rapidly become a major global health threat due to its persistence in healthcare environments and high mortality rates (Du *et al*., 2020).

Other yeasts, beyond *Candida*, also play emerging roles in opportunistic infections, with some species exhibiting intrinsic antifungal resistance and causing invasive disease in vulnerable patient populations (Farmakiotis and Kontoyiannis, 2017). These trends underscore the critical need for molecular diagnostics to rapidly identify resistant strains and guide targeted antifungal therapy (Czajka *et al*., 2023).

2.5. Mycotoxins and Their Public Health Implications

Mycotoxins are toxic secondary metabolites produced by fungi such as *Aspergillus*, *Penicillium*, and *Fusarium*, which contaminate food and feed commodities both pre- and post-harvest, posing serious risks to human and animal health (Ekwomadu *et al*., 2021). Chronic exposure to mycotoxins has been associated with carcinogenic, genotoxic, hepatotoxic, and immunosuppressive effects, with aflatoxins, fumonisins, and ochratoxin A identified as major contributors to global disease burden (Adeyeye and Ashaolu, 2022).

Food insecurity exacerbates the public health impact of mycotoxins, particularly in low- and middle-income regions where poor storage and food handling practices increase

contamination risk (Adeyeye and Ashaolu, 2022). In such contexts, populations may be highly vulnerable to both acute mycotoxicoses and long-term health consequences due to insufficient monitoring and control systems (Omotayo *et al*., 2019). Furthermore, indirect exposure occurs through the consumption of animal products such as milk and meat, where residues of mycotoxins accumulate from contaminated feed (Ekwomadu *et al*., 2021).

Public health authorities face significant challenges in addressing mycotoxin risks, as detection, regulation, and control require integrated approaches across agriculture, food safety, and health sectors (Adeyeye and Ashaolu, 2022). Recent research underscores the importance of implementing surveillance programs, strengthening laboratory capacities, and applying early warning systems to mitigate the impact of mycotoxins on food systems (Ekwomadu *et al*., 2021).

2.5.1. Types of Mycotoxins (Aflatoxins, Ochratoxins, Fumonisins, etc.)

Mycotoxins represent a chemically diverse group of fungal metabolites, with aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone being among the most studied due to their prevalence and toxicological impact (Awuchi *et al*., 2021). Aflatoxins, particularly aflatoxin B1, are highly potent hepatocarcinogens commonly produced by *Aspergillus* species and frequently contaminate maize, peanuts, and other staple crops (Awuchi *et al*., 2022). Ochratoxin A (OTA), produced by *Aspergillus* and *Penicillium sp*ecies, is nephrotoxic and implicated in kidney-related disorders, with evidence also pointing to genotoxic and carcinogenic properties (Awuchi *et al*., 2022).

Fumonisins, mainly fumonisin B1 produced by *Fusarium* spp., disrupt sphingolipid metabolism and have been associated with esophageal cancer and neural tube defects in humans (Qu *et al*., 2022). Trichothecenes, a large group of sesquiterpenoid mycotoxins, exert

strong immunosuppressive and cytotoxic effects, while zearalenone exhibits estrogenic activity, leading to reproductive toxicity (Awuchi *et al*., 2021). Co-occurrence of multiple mycotoxins in food commodities is an additional concern, as synergistic toxic effects can exacerbate their public health burden (Awuchi *et al*., 2021).

2.5.2. Mechanisms of Mycotoxin Toxicity in Humans

The mechanisms of mycotoxin toxicity involve diverse molecular pathways, including oxidative stress, genotoxicity, and disruption of cellular signaling. Aflatoxin B1 undergoes metabolic activation in the liver, producing reactive intermediates that form DNA adducts, thereby promoting hepatocellular carcinoma (Awuchi *et al*., 2022). Ochratoxin A contributes to toxicity by generating oxidative stress, impairing mitochondrial function, and forming DNA adducts, which together drive nephrotoxicity and carcinogenesis (Awuchi *et al*., 2022). Fumonisins exert their toxic effects by inhibiting ceramide synthase, leading to sphingolipid imbalance, membrane dysfunction, and apoptosis, processes linked to esophageal cancer and neural tube defects (Qu *et al*., 2022). Trichothecenes inhibit protein synthesis by binding to the 60S ribosomal subunit, triggering apoptosis and immune suppression, while zearalenone mimics estrogen and interacts with estrogen receptors, thereby altering endocrine signaling (Awuchi *et al*., 2021).

2.5.3. Mycotoxin Contamination in Herbal Mixtures and Food Products

Herbal mixtures and medicinal plants are increasingly consumed worldwide for their therapeutic and nutritional benefits, yet they are frequently contaminated with mycotoxins due to poor handling, storage, and environmental conditions (Yu *et al*., 2022). Aflatoxins, ochratoxins, and fumonisins are among the most commonly detected toxins in herbal medicines and dietary supplements, raising concerns for consumer health and safety (Pallarés

et al., 2022). Studies have shown that herbal infusions and teas, widely consumed globally, are particularly vulnerable to contamination by mycotoxins and other natural contaminants such as heavy metals (Caldeirao *et al*., 2021). In low- and middle-income countries, mycotoxin contamination of herbal medicinal products (HMPs) poses a significant public health hazard due to weak regulatory frameworks and limited surveillance (Opuni *et al*., 2023). Systematic reviews reveal frequent reports of aflatoxin and ochratoxin contamination in herbal preparations, often exceeding international safety thresholds (Opuni *et al*., 2023). Similarly, assessments of medicinal herbs and supplements across markets have identified varying levels of aflatoxin B1 and total aflatoxins, highlighting consumer exposure risks through products perceived as "natural" and safe (Pallarés *et al*., 2022).

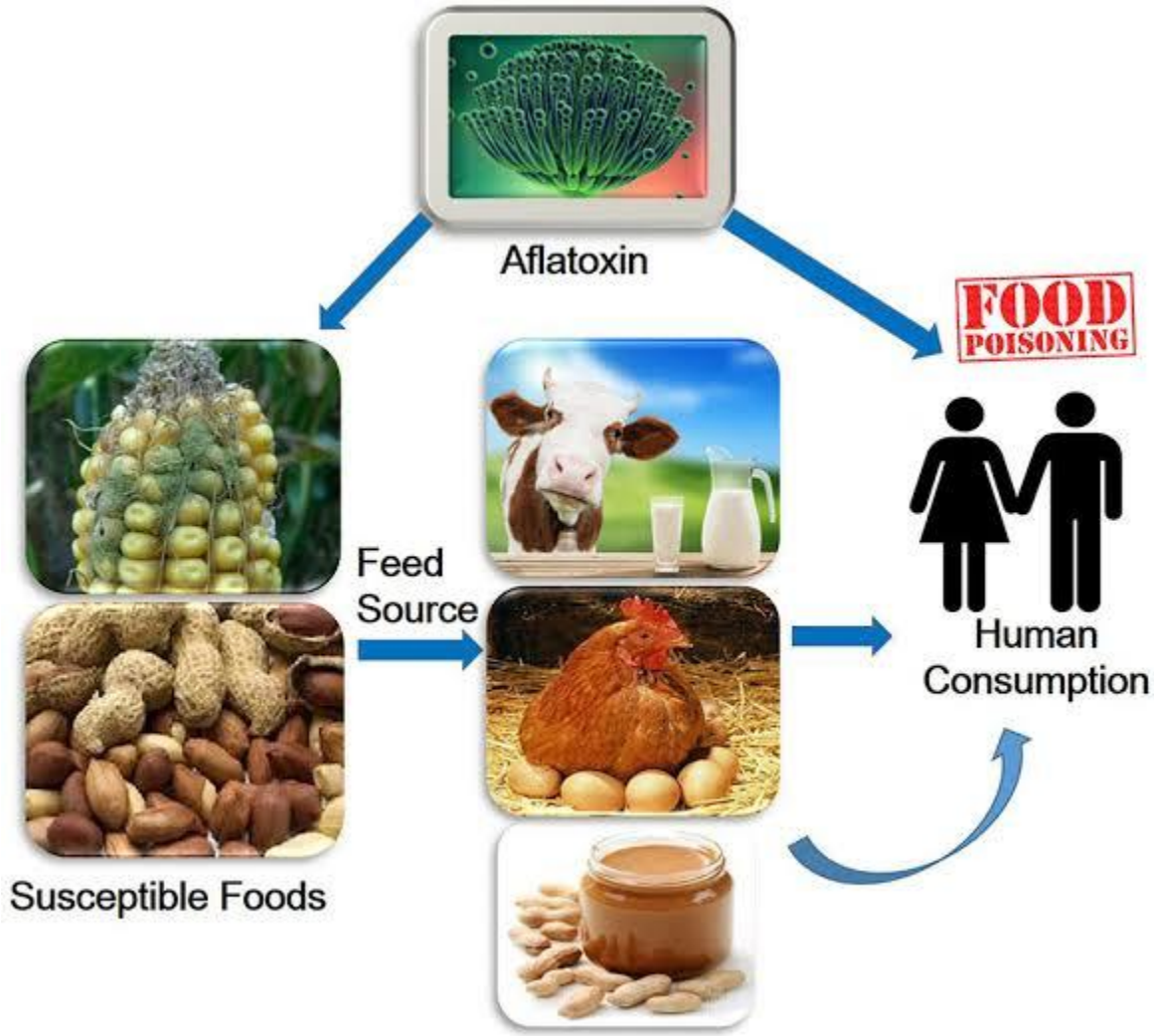

Figure 2.1 Mycotoxin Contamination in Food Products (Yu *et al.*, 2022).

2.6. Health Risks Associated with Fungal Contamination in Herbal Mixtures

2.6.1. Opportunistic Infections in Immunocompromised Patients

Fungal contamination in herbal mixtures poses serious risks to immunocompromised patients, who are highly susceptible to opportunistic infections from species such as *Aspergillus*, *Candida*, and *Cryptococcus* (Garvey and Rowan, 2023). Immunodeficiency due to conditions like cancer therapy, HIV, and organ transplantation increases vulnerability to invasive fungal infections, which may complicate existing diseases or treatments (Summerbell and Scott, 2025). Polyherbal medicines used in tuberculosis management have also been shown to harbor pathogenic fungi, raising the risk of co-infections in already compromised patients (Famewo *et al*., 2018).

2.6.2. Allergic Reactions and Respiratory Disorders

Inhalation or ingestion of contaminated herbal mixtures can trigger allergic responses, respiratory irritation, and asthma-like symptoms due to spores and fungal metabolites (Famewo and Afolayan, 2018). Certain fungi, such as *Alternaria* and *Cladosporium*, are known aeroallergens capable of worsening chronic respiratory diseases (Ndoro, 2023). Moreover, secondary metabolites from *Stachybotrys* species can exacerbate pulmonary inflammation and contribute to the development of chronic respiratory disorders (Summerbell and Scott, 2025).

2.6.3. Carcinogenic, Hepatotoxic, and Nephrotoxic Effects of Mycotoxins

Mycotoxins frequently detected in herbal products, such as aflatoxins and ochratoxin A, are associated with hepatotoxicity, nephrotoxicity, and carcinogenesis (Ndoro, 2023). Chronic consumption of contaminated herbal preparations increases the risk of liver cancer, kidney disease, and immunosuppression, particularly in populations relying heavily on herbal

remedies for primary healthcare (Garvey and Rowan, 2023). The multimycotoxin contamination observed in medicinal plants sold in markets underscores the cumulative toxic burden from long-term use (Ndoro, 2023).

2.6.4. Implications for HIV/AIDS and Tuberculosis Patients

HIV/AIDS and tuberculosis patients represent particularly high-risk groups due to their immunocompromised status and high reliance on traditional herbal medicines (Tonui, 2022). Studies from sub-Saharan Africa have shown fungal contaminants in herbal remedies marketed for TB, raising concerns about exacerbated co-infections and drug interactions (Famewo *et al*., 2018). In HIV patients, fungal co-infections such as cryptococcosis and histoplasmosis remain major contributors to morbidity and mortality, and contaminated herbal mixtures may serve as additional sources of exposure (Joao *et al*., 2020). The overlap between fungal infections, TB, and HIV further complicates treatment, demanding stricter monitoring of herbal formulations for fungal contamination (Tonui, 2022).

2.7. Methods of Isolation and Identification of Fungi in Herbal Mixtures

2.7.1. Conventional Culture Techniques (Sabouraud Dextrose Agar, PDA, etc.)

Conventional culture techniques remain the cornerstone of fungal isolation and identification in herbal mixtures, as they provide a simple, cost-effective, and widely applicable method for detecting contamination (Acharya and Hare, 2022). Sabouraud Dextrose Agar (SDA) is one of the most frequently used selective media, optimized with acidic pH and high glucose content to support fungal growth while suppressing bacterial contamination (Acharya and Hare, 2022). This medium is particularly suitable for isolating pathogenic yeasts such as *Candida albicans*, as well as filamentous fungi contaminating herbal products (Saeed and Saadullah, 2019).

Potato Dextrose Agar (PDA) is another standard medium commonly used for culturing a wide range of fungi from herbal medicines, as it promotes abundant sporulation and pigmentation, which aids in morphological identification (Wang *et al*., 2024). PDA has been successfully applied in surveys of herbal products to quantify fungal load and identify dominant genera, including *Aspergillus*, *Penicillium*, and *Fusarium* (Wang *et al*., 2024). In addition, PDA facilitates macroscopic colony characterization, which remains a critical first step in fungal taxonomy before molecular confirmation (Naji *et al*., 2023).

Studies investigating herbal medicines in China and other regions have demonstrated the consistent use of SDA and PDA in isolating fungi and subsequently identifying mycotoxigenic species that pose risks to consumer safety (Zheng *et al*., 2017; Wang *et al*., 2024).

2.7.2. Microscopic and Morphological Identification

Microscopic and morphological techniques remain fundamental for preliminary identification of fungi in herbal mixtures, relying on colony characteristics, spore morphology, and hyphal structures (Malhotra *et al*., 2023). These methods provide rapid, low-cost screening, especially in resource-limited settings, although they may lack the specificity needed for distinguishing closely related species (Atalay *et al*., 2016). Direct microscopic observation often serves as the first diagnostic step before applying molecular or biochemical confirmation (Malhotra *et al*., 2023).

2.7.3. Biochemical and Serological Approaches

Biochemical and serological assays complement morphological methods by targeting fungal metabolites, enzymes, or antigens. Commercial biochemical kits allow differentiation of clinically and environmentally relevant yeasts based on metabolic activity (Zhao *et al*., 2018).

Serological approaches, including antigen and antibody detection, can provide rapid identification, particularly in invasive infections, although cross-reactivity sometimes reduces specificity (Wanger *et al*., 2017). These methods are particularly useful for screening herbal mixtures suspected of contamination with pathogenic fungi when rapid results are required.

2.7.4. Molecular Techniques (PCR, DNA Sequencing, MALDI-TOF)

Molecular methods have become the gold standard for fungal identification, offering high specificity and sensitivity. PCR and sequencing of ribosomal DNA regions such as ITS and 18S rRNA are widely used to identify fungi in complex herbal products, providing precise taxonomic resolution (Malik *et al*., 2021). Real-time PCR and multilocus sequencing have further enhanced detection of mycotoxigenic and pathogenic fungi in food and herbal samples (Mitra *et al*., 2024).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a powerful tool for rapid fungal identification, with studies demonstrating its reproducibility and speed in distinguishing diverse fungal isolates, including *Aspergillus* and yeasts (Bader, 2013; Sánchez-Juanes and Calvo Sánchez, 2022). MALDI-TOF MS, when combined with PCR or sequencing, offers even greater diagnostic accuracy, making it particularly valuable for monitoring fungal contamination in herbal mixtures (Singhal *et al*., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in the Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria. The study was done to isolate and identify fungi present in herbal mixtures sold at selected markets in Benin City, Edo State, Nigeria. The markets included Uselu Market, New Benin Market, and Ring Road Market,

3.2 Research Design

The study involves the collection of herbal mixture samples from local vendors in major markets within Benin City, Edo State, in 2025. Samples were obtained from Uselu Market, New Benin Market, and Ring Road Market to ensure representation of common trading areas. The design allowed for the assessment of fungal contamination in products that are routinely consumed by the population, in order to determine the presence of fungal contamination in the samples.

3.3 Ethical Approval

Ethical approval for this study was obtained from the Research Ethics Committee, College of Medical Sciences, University of Benin, Benin City, Nigeria with Approval number **CMS/REC/2025/787**. The study adhered to institutional and national ethical guidelines for research involving human-related products and public health investigations 1979. Confidentiality of vendors was maintained by anonymizing market sources and vendor identities during data analysis and reporting.

3.5 Sample Size

To align with peer-reviewed precedent, the sample size was rounded and fixed at 20 herbal mixture samples. A similar study conducted in Kaduna metropolis, Nigeria, successfully employed 20 herbal mixture samples for the isolation and identification of fungal species including *Aspergillus, Saccharomyces, Fusarium,* and *Penicillium* (Shitu *et al*. 2024). This provides a valid scientific justification for the adoption of 20 samples in the present study in Benin City, Edo State.

3.6 MATERIALS

Equipment

Portable autoclave (Gallenkamp & Co. Ltd., England), incubator (Gallenkamp, England), hot air oven (Gallenkamp, England), iron wire loop, refrigerator (Super Deluxe), microscope, weighing balance, cotton wool, scissors, forceps, Petri dishes, aluminum foil, masking tape, rack, Bunsen burner, wire gauze, Cork borer, tripod stand.

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

Lactophenol cotton blue, immersion oil, distilled water, quarter-strength Ringer's solution, peptone water.

Microbiological Media

Sabouraud Dextrose Agar (SDA) (Guangdong Huankai Ltd).

Other Materials

Disposable hand gloves, permanent marker.

3.7 METHOD

3.7.2 Sample Collection

A total of twenty (20) samples was purchased from Herbal vendors across Uselu (7), New Benin (7), and Ring Road (6) Market in Benin metropolis. Sterilized bottles with covers were used to collect the samples. The samples were then transported to the laboratory for analysis to determine the presence of fungi.

Samples Collected include:

Table 3.1: Herbal Mixtures Collected from Markets in Benin City and Their Claimed Therapeutic Uses

S/N	LOCAL NAMES OF SAMPLES	THERAPEUTIC CLAIM	TYPES OF SAMPLES	MARKET
S1	FEVER(IBA)	FEVER	POWDER	USELU
S2	INFECTION 1	INFECTION	POWDER	USELU
S3	KOKORO EJE	FOR MENS PRIVATE PARTS	POWDER	USELU
S4	GBOGBONISHE	DIFFERENT TYPES OF INFECTIONS	POWDER	USELU
S5	SUGAR	TO REGULATE SUGAR LEVEL	POWDER	USELU
S6	AGBARA	TO ENHANCE PERFORMANCE	POWDER	USELU
S7	MAN POWER	TO ENHANCE PERFORMANCE	POWDER	USELU
S8	ARARIRO	FOR BODY	POWDER	NEW BENIN

		PAIN		
S9	INFECTION 2	FOR INFECTIONS AND RASHES	LIQUID	NEW BENIN
S10	IBA	FOR FEVER	LIQUID	NEW BENIN
S11	JEDI 2	TO REGULATE SUGAR LEVEL	LIQUID	NEW BENIN
S12	RHEUMATISM 1	FOR RHEUMATISM	LIQUID	NEW BENIN
S13	IDA-KOLE	TO ENHANCE PERFORMANCE	POWDER	NEW BENIN
S14	RHEUMATISM 2	FOR RHEUMATISM	PASTE	NEW BENIN
S15	JEDI ATI INFECTION	FOR INFECTIONS	LIQUID	RING ROAD
S16	INFECTION 3	FOR INFECTIONS	LIQUID	RING ROAD
S17	TYPHOID	FOR TYPHOID FEVER	LIQUID	RING ROAD
S18	JEDI 2	TO REGULATE SUGAR LEVEL	LIQUID	RING ROAD
S19	AWON KPA	AGAINST DIFFERENT TYPES OF INFECTIONS	LIQUID	RING ROAD
S20	DOGOYARO	FOR MALERIA	LIQUID	RING ROAD

3.7.3 Sample preparation

Each herbal product (both powder and liquid forms) was placed in sterile bottles and labeled S1–S20. (14 dried/powdered products 1 Paste and 5 liquid products).

1 g of 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. One milliliter was aseptically transferred into 9 ml of sterile diluent (One-Quarter Strength Ringer's solution).

1ml of Liquid product was aseptically mixed with 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. (Cheesbrough, 2022)

3.7.4 Inoculation of Serially Diluted Samples onto SDA

20 ml of molten Sabouraud Dextrose Agar (SDA) were aseptically poured into sterile Petri dishes and appropriately labeled. After the agar had solidified, the plates were dried at 40 °C for 5 minutes to reduce excess surface moisture.

The mixture (Diluent and Organism) was aseptically dispensed onto the surface of the set SDA plate labeled S1. The inoculum was evenly spread over the agar surface. This procedure was repeated for all samples (S1–S20), each in triplicate.

The inoculated plates were incubated at room temperature for 18–72 hours and observed daily for fungal growth.

3.7.5 Calculation of number of fungi present in the herbal products

Formula Used

Colony count (CFU/ml) = (Number of colonies × Dilution factor) ÷ Volume plated (ml)

Calculation (Sample S1, Plate A, dilution 10^{-1}):

- Number of colonies = 2 CFU
- Volume plated = 0.5 ml

Colony count in 1 ml = (2 × 1) ÷ 0.5 = 4× 10^0 CFU/ml

- Dilution factor used = 10^2

Total fungi in S1 = $4 \times 10^2 = 4.0 \times 10^2$ CFU/ml

Since 1 g of the S1 sample was suspended in 10 ml of sterile water, then:

- 1 ml of suspension corresponds to 1g of the sample.
- Therefore, 1 g of sample S1 contains 4.0×10^2 CFU/g.

Note: The same calculation was applied to all dried herbal samples. For liquid herbal products, the total fungal load was expressed as CFU/ml. (Cheesbrough, 2022)

3.8 PROCEDURE FOR IDENTIFICATION OF THE ORGANISMS

The isolates were identified based on their cultural characteristics and Microscopy as follows:

3.8.1 Observation of Colonial Morphology of Fungi (Moulds)

The Macroscopic (colonial) characteristics of mould grown on culture media were studied and described for preliminary identification.

Procedure

The inoculated plates were properly labeled and incubated at room temperature (25–28 °C) for 3–7 days. Fungal colonies were observed daily for growth and development and the Size, Texture, Surface colour and Topography of each colony were observed. The colonial characteristics were recorded systematically for each isolate. (ScienceDirect, 2023)

3.8.2 Microscopic Examination (Lactophenol cotton blue staining procedures)

The morphological structures of fungi (such as hyphae, spores, and conidia) were demonstrated and identified for microscopic examination.

Procedure

A drop of lactophenol cotton blue stain was placed at the center of a clean glass slide. A small portion of fungal mycelium was picked using a sterile inoculating needle. The mycelium was transferred into the drop of stain and was gently spread out to avoid clumping. A clean cover slip was carefully placed over the preparation without introducing air bubbles. The slide was examined under the microscope, first with the ×10 objective lens to locate the specimen. The ×40 objective lens was then used for detailed observation of hyphae, spores, and conidia. (Mokobi, 2022)

3.9 Antifungal sensitivity testing

Agar diffusion method was used and fungi mycelia growth was measured using a meter rule on a daily basis

3.9.1 Determination of Inhibitory Zone Diameter (IZD) by Measuring Mycelial Growth

The inhibitory zone diameter was determined using the agar diffusion method with modifications. Sterile Sabouraud Dextrose Agar (SDA) was prepared, and 20 ml was aseptically poured into Petri dishes after incorporating 3g of the herbal product into the medium, giving a final concentration of 150 mg/ml. The medium was then allowed to solidify.

The Petri dishes were dried in a hot air oven at 40 °C for about 5 minutes to reduce excess surface moisture. The test organisms isolated from the herbal products were cultured, and a suspension of each was prepared in sterile normal saline. The turbidity of the suspension was adjusted to match the 0.5 McFarland standard. Using a sterile wire loop, the standardized suspension was evenly spread over the surface of the dried agar plates to obtain a uniform lawn of growth. The test substances were then carefully applied onto the surface of the inoculated agar for subsequent evaluation of antimicrobial activity.

The inoculated plates were incubated at room temperature, and mycelial growth was measured daily for 7 days using a meter rule. Growth diameters were expressed in millimeters. The procedure was repeated for all fungal 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. (Techaoei *et al.,* 2020)

CHAPTER FOUR

RESULTS

4.1 Market distribution of Herbal mixtures

The distribution of herbal mixtures presented in Table 4.1. Out of the 20 samples analyzed, 45% were powders, 50% were liquids, and 5% were paste preparations. With respect to market distribution, New Benin and Uselu each contributed seven samples (35%), while Ring Road contributed six samples (30%).

4.2 Occurrence of Fungal Isolates

Fungi were isolated from 13 out of 20 samples (65%), whereas 7 samples (35%) yielded no growth. The fungal isolates identified included *Mucor* (20%), *Fusarium* (20%), *Aspergillus niger* (15%), and *Penicillium* (10%) as shown in Table 4.2.

Figure 4.1 illustrates powdered samples yielded the highest number of isolates (7), 53.9%, followed by liquid samples (5), 38.5 while the paste sample yielded only one isolate (7.65%). Although powdered samples accounted for fewer total collections than liquids, they contributed more isolates overall.

4.3 Distribution of Fungal Isolates by Market Location

The distribution of fungal isolates across market locations is shown in Table 4.3. New Benin and Uselu markets recorded six positive isolates each, whereas Ring Road accounted for only one.

4.4 Distribution of Fungal Isolates by Sample Type

Comparison of fungal occurrence by sample type is presented in Table 4.4. Powdered samples yielded seven isolates, liquid samples five, and the paste sample one. The chi-square

analysis showed no significant association between sample type and fungal occurrence ($p = 0.124$). This observation corroborates the distribution shown in Figure 4.1, where powders appear as the dominant source of fungal isolates.

4.5 Fungal Loads of Herbal Mixtures

The fungal loads of the herbal mixtures are presented in Table 4.5. Powdered samples recorded a mean microbial load of $5.18 \times 10^5 \pm 3.63 \times 10^5$ CFU/ml, liquid samples $9.75 \times 10^3 \pm 5.14 \times 10^3$ CFU/ml, and the single paste sample 5.17×10^4 CFU/ml. Although the paste preparation exhibited measurable microbial load, this result should be interpreted with caution as it was derived from only one sample ($n = 1$). Statistical analysis revealed no significant variation in microbial load across sample types ($p = 0.275$). The distribution of microbial loads by sample type is further illustrated in Figure 4.3a, where the higher variability observed in powdered samples is evident.

4.6 Microbial Loads by Market Location

A statistically significant variation in microbial loads was observed across market locations ($p = 0.019$). Samples obtained from Uselu had the highest microbial load (2.18×10^5 CFU/ml), followed by New Benin (1.08×10^5 CFU/ml), while samples from Ring Road showed no detectable growth. This trend is represented in Figure 4.3b, which highlights the consistently higher microbial loads observed in Uselu compared with the other markets.

4.7 Microbial Loads by Fungal Isolates

Further comparison of microbial loads among different fungal isolates is presented in Table 4.6. *Penicillium* exhibited the highest mean microbial load ($1.72 \times 10^6 \pm 1.67 \times 10^6$ CFU/ml), followed by *Fusarium* ($2.18 \times 10^5 \pm 1.08 \times 10^5$ CFU/ml), *Mucor* ($1.08 \times 10^5 \pm 8.66 \times 10^4$

CFU/ml), and *Aspergillus niger* ($2.51 \times 10^4 \pm 1.33 \times 10^4$ CFU/ml). The Kruskal–Wallis test indicated that this variation was statistically significant ($p = 0.005$).

4.9 Growth Pattern of Fungal Isolates on SDA Infused with Herbal Mixtures

When SDA medium was infused with herbal mixtures claiming therapeutic efficacy, fungal growth was observed at varying levels, as shown in Tables 4.8 and 4.10. At 3000 mg concentration, some mixtures showed partial inhibitory effects.

In the control SDA medium without herbal extracts, Day 5 growth reached 90 ± 2.0% for *Penicillium*, 79 ± 1.5% for *Aspergillus niger*, and 78 ± 1.8% for *Fusarium*, as shown in Tables 4.7 and 4.9.

For herbal products S15–S20 infused into SDA medium, Day 5 growth ranged between 72 ± 1.5% and 90 ± 2.1%, as shown in Table 4.8.

For selected herbal mixtures at 3000 mg concentration, Day 5 growth was 80–90 ± 2.3% for *Penicillium*, 55–63 ± 1.8% for *Aspergillus niger*, and 35–65 ± 2.0% for *Fusarium*, as shown in Table 4.10.

Table 4.1**:** Prevalence and Distribution of Fungal Isolates Obtained from Herbal Mixtures in different Market Locations, in Benin City

Variable	Category	Frequency (n)	Percentage (%)
Sample type	Powder	9	45
	Liquid	10	50
	Paste	1	5
Market location	New Benin	7	35
	Uselu	7	35
	Ring Road	6	30
Fungal isolate	*Mucor*	4	20
	Fusarium	4	20
	Penicillium	2	10
	Aspergillus niger	3	15
	No growth	7	35

N = 20 herbal mixture samples analyzed.

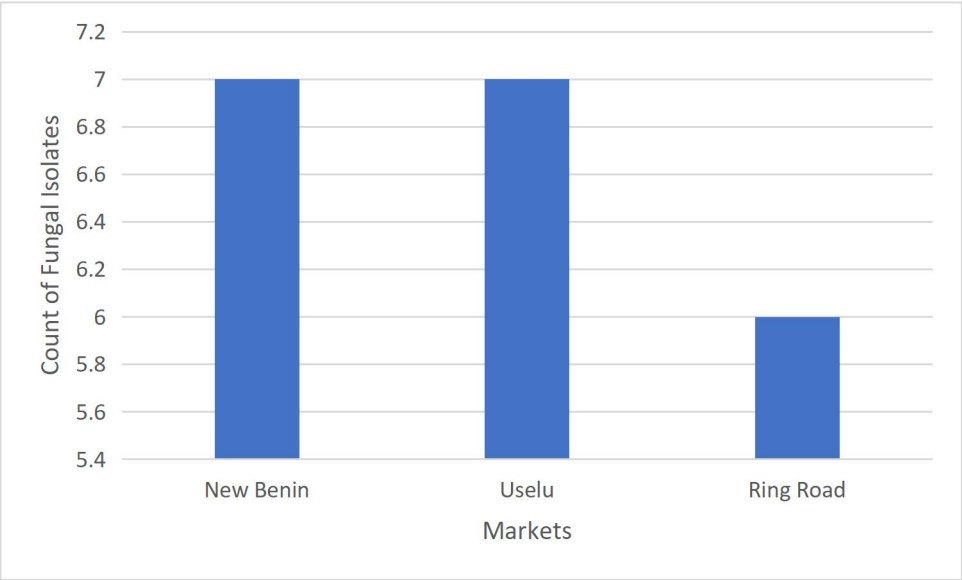

Figure 4.1: Distribution of fungal isolates by market location

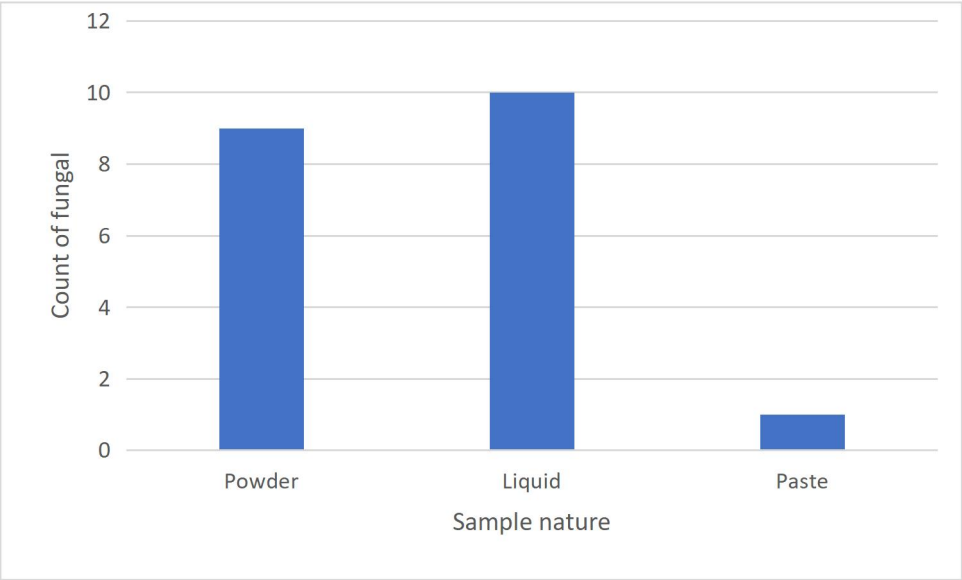

Figure 4.2: Distribution of fungal isolates by Sample nature

Table 4.2: Prevalence of Fungal Isolates Identified from Herbal Mixtures in Benin City

Fungal Isolate	Frequency (n)	Percentage (%)
Mucor	4	20
Fusarium	4	20
Penicillium	2	10
Aspergillus niger	3	15
Total fungal isolates	13	65
No growth	7	35

Fungi were isolated from 13 out of 20 samples.

Table 4.3: Distribution and Association Between Fungal Isolates and Market Location

Organism Identified	New Benin	Uselu	Ring Road	Chi square
Mucor	2 (28.6%)	1 (14.3%)	1 (16.7%)	11.888
Fusarium	1 (14.3%)	3 (42.9%)	0 (0.0%)	
Penicillium	1 (14.3%)	1 (14.3%)	0 (0.0%)	
Aspergillus niger	2 (28.6%)	1 (14.3%)	0 (0.0%)	
No growth	1 (14.3%)	1 (14.3%)	5 (83.3%)	

Significance < 0.05

Table 4.4: Distribution and Association of Fungi Isolates by Sample Nature

Organism Identified	Powder	Liquid	Paste	Chi square
Mucor	2 (22.2%)	2 (20.0%)	0 (0.0%)	12.653
Fusarium	3 (33.3%)	1 (10.0%)	0 (0.0%)	
Penicillium	1 (11.1%)	0 (0.0%)	1 (100.0%)	
Aspergillus niger	1 (11.1%)	2 (20.0%)	0 (0.0%)	
No growth	2 (22.2%)	5 (50.0%)	0 (0.0%)	

Significance < 0.05

Table 4.5: Microbial Loads (CFU/ml) of Herbal Mixtures Collected from Benin City Markets

Market	N	Mean ± SEM (CFU)
New Benin	7	$7.10 \times 10^4 \pm 4.99 \times 10^4$
Uselu	7	$6.14 \times 10^5 \pm 4.66 \times 10^5$
Ring Road	6	$3.08 \times 10^3 \pm 3.08 \times 10^3$
Sample Nature		
Powder	9	$5.18 \times 10^5 \pm 3.63 \times 10^5$
Liquid	10	$9.75 \times 10^3 \pm 5.14 \times 10^3$
Paste	1	$5.17 \times 10^4 \pm$ –

Significance < 0.05

N= number of fungi isolates

Table 4.6: Comparison of Microbial Loads of Fungal Isolates Identified in Herbal Mixtures in Benin city

Organism Identified	N	Mean ± SEM (CFU)
Mucor	4	$1.08 \times 10^5 \pm 8.66 \times 10^4$
Fusarium	4	$2.18 \times 10^5 \pm 1.08 \times 10^5$
Penicillum	2	$1.72 \times 10^6 \pm 1.67 \times 10^6$
Aspergillus niger	3	$2.51 \times 10^4 \pm 1.33 \times 10^4$

N = number of samples

Table 4.7: Growth of fungi on SDA medium without herbal extracts (Control for Table 8).

Fungus	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Penicillium	0	10	23	50	80	90
Aspergillus niger	0	22	50	72	76	74
Fusarium	0	20	33	55	66	78

Table 4.8: Growth of fungi on SDA medium infused with herbal products that did not yield growth but had therapeutic claims against infections (S15–S20).

Fungus	Sample	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Penicillium	S15	0	21	24	29	52	74
	S16	0	11	20	35	55	74
	S17	0	9	20	25	48	72
	S19	0	10	21	27	52	76
	S20	0	11	19	32	63	80
Aspergillus niger	S15	0	22	26	32	56	79
	S16	0	13	28	60	70	79
	S17	0	13	25	39	62	85
	S19	0	12	17	24	60	79
	S20	0	14	26	41	69	85
Fusarium	S15	0	12	23	33	60	78
	S16	0	10	20	25	50	72
	S17	0	12	18	29	51	90
	S19	0	15	27	40	62	88
	S20	0	10	20	23	55	77

Table 4.9. Growth of fungi on SDA medium without herbal extracts (Control for Table 10).

Fungus	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Penicillium	0	12	21	29	40	82
Aspergillus niger	0	14	24	32	39	79
Fusarium	0	13	21	35	53	62

Table 4.10: Growth of fungi on SDA medium infused with selected herbal mixtures with therapeutic claim against infections (3000 mg concentration).

Fungus	Mixture	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Penicillium	Mix 1	0	13	30	42	72	90
	Mix 2	0	13	33	36	62	90
	Mix 3	0	12	42	52	69	80
Aspergillus niger	Mix 1	0	14	33	39	41	55
	Mix 2	0	15	26	32	46	57
	Mix 3	0	13	31	41	52	63
Fusarium	Mix 1	0	15	27	30	32	35
	Mix 2	0	12	25	32	39	65
	Mix 3	0	12	31	37	40	43

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

This study investigated fungal contamination in herbal mixtures sold across Benin City markets, with particular emphasis on distribution by sample type, market location, microbial loads, and antifungal activity of the mixtures. Fungi were isolated from 65% of the samples, while 35% yielded no growth. The most prevalent fungal genera were *Mucor* and *Fusarium*, followed by *Aspergillus niger* and *Penicillium*. Powdered preparations accounted for 45% of the samples but yielded 7 isolates, compared to 5 isolates from liquid preparations and 1 isolate from the single paste sample. Statistical analysis revealed no significant association between sample type and fungal occurrence ($p > 0.05$), although powders had consistently higher contamination. These findings are consistent with (Chinakwe and Ngumah 2023), who reported that powdered herbal preparations in Owerri exhibited higher fungal counts due to their wide surface exposure and poor packaging. Similarly, (Oladeji *et al*.,2024) observed higher fungal diversity in dried herbal medicines in South Africa compared to aqueous formulations, attributing the differences to post-processing contamination. However, Archibong *et al*. (2017) noted higher microbial counts in liquids from Awka, Anambra State, highlighting that both formulation type and handling practices significantly influence contamination levels. New Benin and Uselu markets each contributed 35% of samples and recorded six positive isolates each, whereas Ring Road contributed 30% of samples but recorded only one positive isolate. Statistical analysis showed no significant association between market location and fungal occurrence ($p > 0.05$). However, microbial load analysis revealed significant variation across markets ($p > 0.05$), with Uselu recording the highest mean microbial load followed by New Benin while Ring Road samples had no detectable

growth. These findings align with Shitu *et al*. (2024), who reported significant differences in microbial contamination of herbal mixtures across Kaduna markets, attributing this to variations in environmental hygiene and vendor practices. Darkwah *et al*. (2022) similarly observed higher microbial loads in herbal preparations sold in unregulated open markets in Accra, Ghana, compared to better-controlled outlets. The predominance of *Mucor* and *Fusarium* is consistent with earlier findings in Owerri herbal remedies (Chiegeiro *et al*., 2022) and in South African medicinal plants (Oladeji *et al*., 2024). *Aspergillus niger* and *Penicillium* were also detected, both of which are important due to their potential to produce mycotoxins. Notably, *Penicillium* had the highest microbial load, followed by *Fusarium*, *Mucor* (1.08×10^5 CFU/ml), and *Aspergillus niger* (2.51×10^4 CFU/ml). The Kruskal–Wallis test confirmed this variation was statistically significant ($p < 0.05$). Similar observations were made in a systematic review by (Ahiabor and Darkwah 2024), which reported frequent detection of these genera across African herbal products, emphasizing their mycotoxigenic and public health significance. Powdered samples showed a mean microbial load of 5.18×10^5 CFU/ml, liquids 9.75×10^3 CFU/ml, and the single paste sample 1.72×10^6 CFU/ml. Although the paste had the highest load, this should be interpreted cautiously due to its limited sample size ($n = 1$). The chi-square analysis showed no significant difference in microbial loads across sample types ($p > 0.05$). Similar microbial ranges have been reported in Nigerian herbal mixtures, with (Chinakwe and Ngumah 2023) observing fungal loads exceeding pharmacopeial standards in more than 60% of products According to the World Health Organization (WHO, 2007) and the United States Pharmacopeia (USP, 2023), the acceptable limit for total yeast and mold count in herbal medicines is generally not more than 10^3 CFU/g, with complete absence of pathogenic fungi such as *Aspergillus flavus*. This suggests that poor post-harvest handling, coupled with improper packaging, remains a widespread issue. Herbal mixtures infused into SDA showed only partial inhibitory effects on

fungal growth at higher concentrations (3000 mg), with fungi such as *Fusarium* exhibiting growth reduction but not complete inhibition. This indicates that while bioactive compounds may be present, they are insufficient to eliminate contamination under market conditions. (Meshram *et al*., 2025) formulated a herbal antifungal ointment using neem, ginger, garlic, and turmeric. While the ointment showed significant antifungal activity against *Aspergillus niger* and *Candida albicans*, the inhibition zones were comparable to standard drugs but did not indicate complete fungal eradication. The authors recommend further clinical studies to confirm therapeutic potential, highlighting that current results are promising but not definitive for total inhibition under real-world conditions. On the contrary, Oladeji *et al*. (2024) documented strong antifungal effects of *Acacia nilotica* extracts against *Aspergillus flavus,* suggesting variability in antifungal efficacy depending on plant species, extraction method, and dosage. The significant presence of fungi, particularly *Mucor*, *Fusarium*, *Aspergillus*, and *Penicillium*, coupled with microbial loads exceeding 10^5 CFU/ml in most positive samples, poses serious public health concerns. Chronic exposure to aflatoxins, ochratoxins, and fumonisins from these genera has been associated with carcinogenic, hepatotoxic, and nephrotoxic effects (Ahiabor and Darkwah, 2024). Immunocompromised individuals, especially those with HIV/AIDS or tuberculosis, are at higher risk of opportunistic infections when consuming contaminated herbal mixtures (Opuni *et al*., 2023). The persistence of contamination despite antifungal claims highlights the gap between traditional therapeutic beliefs and actual microbiological safety.

5.2 Conclusion

In summary, the results from Benin City corroborate earlier reports of widespread fungal contamination in herbal products across Nigeria and Africa, though the relative contribution of sample types and market locations varied. The persistence of fungal isolates such as *Mucor*,

Fusarium, *Aspergillus*, and *Penicillium* aligns with regional studies, while the partial antifungal activity of herbal mixtures raises doubts about the reliability of their therapeutic claims. Comparative evidence suggests that contamination is influenced not only by product form but also by market hygiene, regulatory oversight, and handling practices. Addressing these issues requires strengthened regulatory frameworks, improved quality control, and public sensitization to reduce health risks associated with contaminated herbal medicines.

5.3 Recommendations

The findings of this study highlight the urgent need for coordinated action to address fungal contamination in herbal mixtures marketed in Benin City. To ensure public safety and promote the credibility of herbal medicine, regulatory authorities such as NAFDAC should strengthen their oversight role by enforcing strict microbial standards in line with international pharmacopeial guidelines. Regular inspection and laboratory analysis of marketed herbal products would help in identifying unsafe formulations and reducing consumer exposure to harmful fungi. Consumer education also plays a vital role in mitigating health risks. The public should be sensitized to the dangers associated with consuming contaminated herbal mixtures, particularly vulnerable groups such as immunocompromised patients, individuals living with HIV/AIDS, and those with chronic conditions like tuberculosis. Awareness campaigns can help consumers make informed choices and demand safer products from vendors.

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APPENDIX

APPENDIX I

Preparation of Media and Reagents

The following media were used

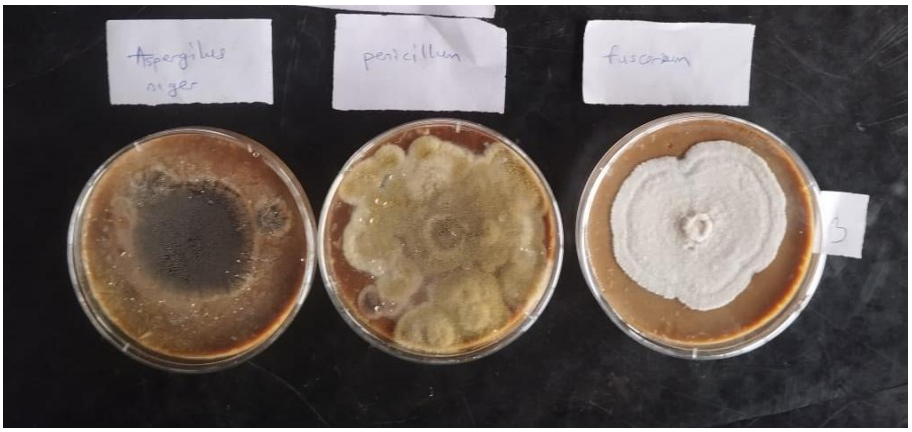
Sabouraud Dextrose Agar (SDA)

Twenty-eight grams (28 g) of SDA powder were dissolved in 1 L of distilled water in a sterile conical flask. The mouth of the flask was covered with aluminum foil and secured with masking tape. The mixture was brought to a boil to dissolve completely and then autoclaved at 121 °C for 15 minutes. After autoclaving, it was allowed to cool to 45 °C, mixed well, and dispensed aseptically in 20 ml volumes into Petri dishes. The medium was allowed to solidify and was used thereafter.

Quarter-strength Ringer's Solution

Two tablets of quarter-strength Ringer's solution were dissolved in 1 L of distilled water. The solution was dispensed into appropriate containers and sterilized in an autoclave at 121 °C for 15 minutes.

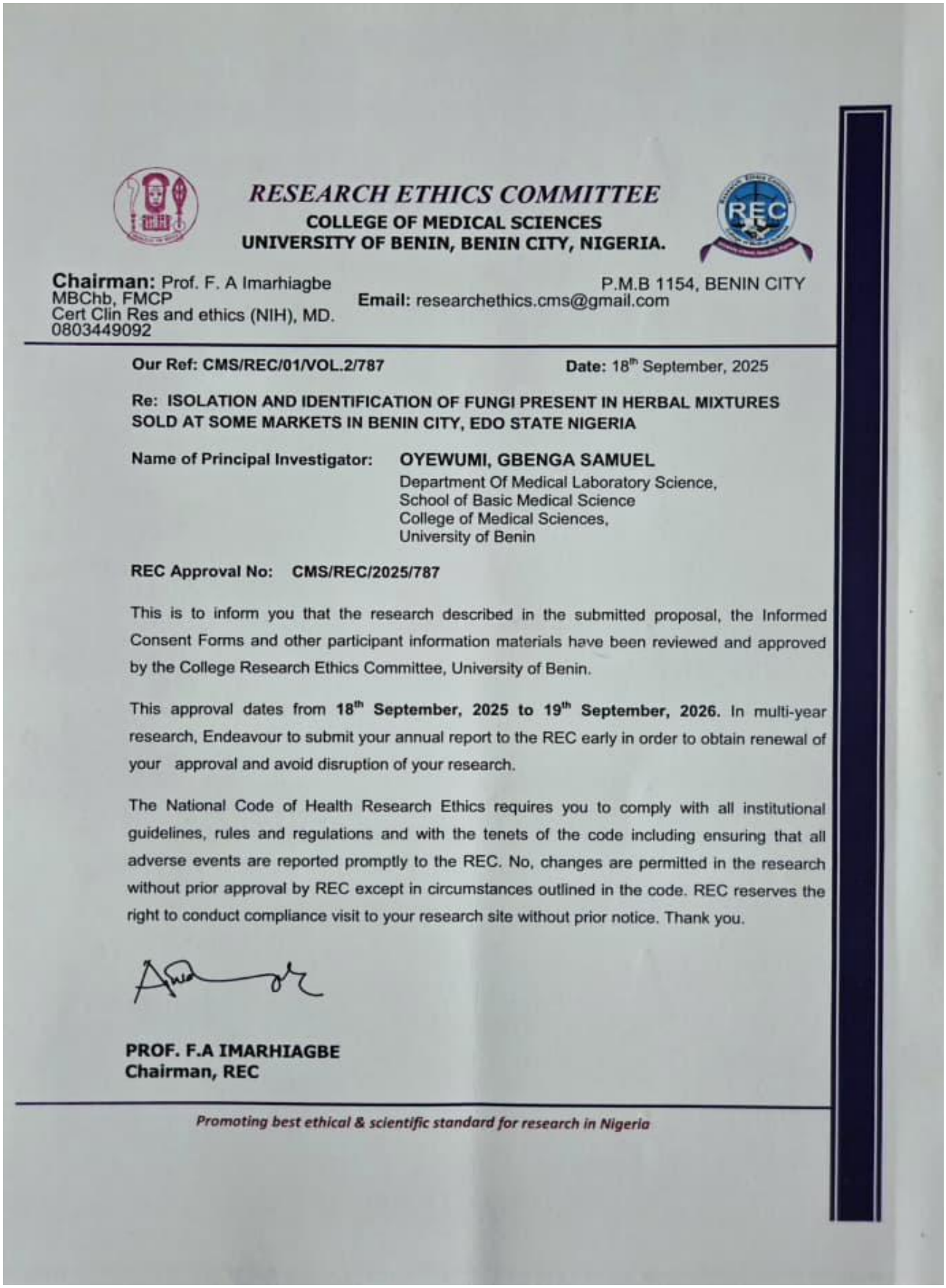
APPENDIX II



Picture showing Mycelial growth on SDA plate infused with herbal mixture after 5 days

Picture showing Preparation of Quarter-strength Ringer's Solution & dispensing into sterile glass bottles

APPENDIX III



RESEARCH ETHICS COMMITTEE

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0803449092

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

Our Ref: CMS/REC/01/VOL.2/787 **Date:** 18th September, 2025

Re: ISOLATION AND IDENTIFICATION OF FUNGI PRESENT IN HERBAL MIXTURES SOLD AT SOME MARKETS IN BENIN CITY, EDO STATE NIGERIA

Name of Principal Investigator: **OYEWUMI, GBENGA SAMUEL**
Department Of Medical Laboratory Science,
School of Basic Medical Science
College of Medical Sciences,
University of Benin

REC Approval No: CMS/REC/2025/787

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.

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