INVESTIGATING THE EFFECTIVENESS OF ESSENTIAL OIL EXTRACTED FROM SELECTED SPICES (POLYHERBAL MIXTURE) AS AN ANTI-FUNGAL AND ANTI-AFLATOXIN ON INFESTED GROUNDNUTS

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BENIN CITY

NOVEMBER, 2025.

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY IN PARTIAL FULFILLMENT FOR THE AWARD OF BACHELORS OF SCIENCE DEGREE (B.Sc.) IN SCIENCE LABORATORY TECHNOLOGY

(MICROBIOLOGY TECHNIQUES)

NOVEMBER, 2025.

CERTIFICATION

This is to certify that this research titled " INVESTIGATING THE EFFECTIVENESS OF ESSENTIAL OIL EXTRACTED FROM SELECTED SPICES (POLYHERBAL MIXTURE) AS AN ANTI-FUNGAL AND ANTI-AFLATOXIN ON INFESTED GROUNDUTS was carried out by "**Oreoluwa Favour MALOMO**" with matriculation number "**LSC2009892"** and presented to the Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin City; in partial fulfillment of the requirements for the award of Bachelor of Science (B.Sc.) in Science Laboratory Technology. It was conducted under suitable conditions and was carefully supervised and subsequently approved as having met the requirements for the award of Bachelor of Science degree in Science Laboratory Technology.

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DEDICATION

This project is dedicated to God Almighty, whose grace, strength, and wisdom made this research possible.

ACKNOWLEDGEMENTS

My heartfelt appreciation goes to my beloved mother, MALOMO Justina Onomeh, for her unwavering love, sacrificial financial support, and daily encouragement that kept me going. To my dear sister, Opeoluwa MALOMO, thank you for your constant motivation, prayers, and for always believing in me. I sincerely thank my uncles, Mr Francisco Saduwa and Mr Efetobor Saduwa and special appreciation to my aunt, Mrs Rita for their generous financial assistance and kind words that lifted my spirit during challenging times.To my supervisor, Prof E. O Oshomoh, thank you for your guidance, patience, and valuable insights that shaped this work. Special thanks to my friends, Rhodamae Ese-Edosomwan, Adewale Folorunsho, Prince David and David Olatawura, your support, late-night discussions, and shared resources made this journey lighter and enjoyable.I also appreciate the laboratory staff and department for providing the facilities to carry out this research.

Finally, to everyone who contributed in ways big and small — thank you. This project is a testimony of collective love and support.

ABSTRACT

Groundnut (*Arachis hypogaea* L.) is a vital food and oilseed crop in Nigeria, contributing significantly to dietary protein and economic livelihoods. However, long-term storage in sealed plastic or glass bottles applicable in a common household and small-scale trader practice creates a microaerobic, high-humidity environment conducive to fungal proliferation and aflatoxin contamination. This study aimed to evaluate the antifungal and antiaflatoxigenic efficacy of a ternary essential oil blend derived from cloves (*Syzygium aromaticum*), thyme (*Thymus* vulgaris) and bay leaves (*Laurus nobilis*) which are all Generally Recognized as Safe (GRAS) botanicals against fungal isolates from heavily infested groundnut samples. Fungal enumeration on Potato Dextrose Agar revealed a mean contamination level of 1.42×10^7 CFU/g (replicates: 1.38, 1.42, 1.46×10^7 CFU/g), exceeding the International Commission on Microbiological Specifications for Foods (ICMSF) safety threshold ($<10^4$ CFU/g) by 1,420-fold. Morphological and microscopic characterization identified six fungal species, with *Aspergillus parasiticus* (30.6%), *A. flavus* (28.2%), and *Emericella astellata* (18.8%) dominating the mycoflora. Coconut Extract Agar (CEA) under UV 365 nm and Ammonia Vapor Test (AVT) confirmed 88.2% of isolates as aflatoxigenic, indicating high risk of B- and G-group aflatoxin synthesis. The methanol extract of the spice mixture exhibited strong, concentration-dependent antifungal activity in disc diffusion assays, producing inhibition zones of 7.7–23.2 mm across 25–100% concentrations, with maximum efficacy (23.2 mm) against *E. astellata* at 100%. The essential oil blend demonstrated complete fungicidal action, with minimum inhibitory concentrations (MIC) ranging from 1.0% (*E. astellata*) to 7.0% (*A. parasiticus*) and minimum fungicidal concentrations (MFC) from 3.0% to 9.0%. The MFC/MIC ratio (≤ 4) confirmed fungicidal rather than fungistatic activity. Mechanistically, the blend disrupts fungal cell membrane integrity, inhibits ergosterol biosynthesis, and suppresses aflatoxin pathway genes. The GRAS-compliant essential oil blend presents a promising, safe, and natural antifungal and antiaflatoxigenic agent for groundnut preservation. Future research should focus on in-situ validation and quantitative aflatoxin reduction using high-performance liquid chromatography (HPLC).

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The global food system is perpetually challenged by the dual crises of food security and food safety. Among the most pressing safety concerns is the contamination of agricultural commodities with mycotoxins, toxic secondary metabolites produced by filamentous fungi. Aflatoxins, produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*, represent the most potent and hazardous class of these mycotoxins (Yohannis *et al*., 2025). Groundnut (*Arachis hypogaea*), a staple legume crop cultivated across tropical and subtropical regions, is exceptionally vulnerable to aflatoxin contamination due to its unique geocarpic growth habit and high oil content, which provides an ideal substrate for fungal proliferation (Dorner, 2008). The contamination can occur at any stage from pre-harvest under drought stress to post-harvest during improper drying, storage, and transportation under high humidity conditions (Hell and Mutegi, 2011).

The public health implications of aflato xin exposure are severe and multifaceted. Chronic dietary intake is unequivocally linked to an increased incidence of hepatocellular carcinoma (Liver cancer), as classified by the International Agency for Research on Cancer (Yohannis *et al*., 2025). Beyond carcinogenicity, aflatoxins are immunosuppressive, increasing susceptibility to infectious diseases, and are implicated in child growth impairment and kwashiorkor (Gong *et al*., 2016). Economically, aflatoxin contamination results in massive post-harvest losses, diminished market value, and trade barriers, disproportionately affecting smallholder farmers in developing nations (Udomkun *et al*., 2017).

For decades, the primary line of defense against mycotoxigenic fungi has been the application of synthetic chemical fungicides. While initially effective, their protracted use has unveiled significant drawbacks, including the emergence of resistant fungal strains, persistence of harmful

residues in food and the environment, and potential toxicity to non-target organisms, including humans (Dutta and Das, 2020; Snelders, 2012). This has catalyzed a paradigm shift in post-harvest protection strategies, steering research towards the exploration of natural, biodegradable, and safe alternatives.

1.1.1 The Critical Role of Essential Oils as Natural Preservatives

Essential oils represent complex mixtures of volatile secondary metabolites that plants produce for defense against pathogens and environmental stresses (Dhifi *et al*., 2016). These hydrophobic liquids contain 20 - 80 components at different concentrations, with two or three major compounds at relatively high concentrations that typically determine their biological properties. The growing concern over synthetic fungicide residues and the emergence of resistant fungal strains have revitalized interest in essential oils as natural alternatives for food preservation (Nazzaro *et al*., 2017). Their antimicrobial efficacy stems from multiple mechanisms of action, including membrane disruption, mitochondrial dysfunction, and enzyme inhibition, which collectively reduce the likelihood of resistance development. The lipophilic nature of essential oil components enables them to accumulate in lipid bilayers, increasing membrane permeability and causing leakage of cellular contents (Yammine *et al*., 2022). Furthermore, their ability to quench reactive oxygen species contributes to their antioxidant capacity, adding another dimension to their preservative potential. The Generally Recognized as Safe (GRAS) status of many essential oils by regulatory agencies like the United States Food and Drug Administration (U.S FDA) makes them particularly attractive for food applications, aligning with consumer preferences for clean-label products (Jackson-Davis *et al*., 2022).

1.1.2 The Fungal Threat: Growth and Aflatoxin Production

Aspergillus species, particularly *A. flavus* and *A. parasiticus*, present a formidable challenge to global food safety through their production of aflatoxins (Yohannis *et al*., 2025). These carcinogenic mycotoxins contaminate various agricultural commodities, with groundnuts being exceptionally vulnerable due to their geocarpic development and high oil content. Aflatoxin biosynthesis involves a complex regulatory network encoded by a 70 kb gene cluster, with aflR acting as the pathway-specific transcription activator (Khan *et al*., 2021). Environmental factors such as temperature, water activity, and oxidative stress significantly influence both fungal growth and toxin production. The health implications of aflatoxin exposure are severe, including hepatocellular carcinoma, immune suppression, growth impairment in children, and economic losses estimated at billions of dollars annually (Gemede *et al*., 2025). The stability of aflatoxins during food processing necessitates interventions that prevent fungal colonization rather than attempting to remove toxins post-production, making early prevention strategies crucial for food safety management (Balan *et al*., 2024).

1.1.3 Groundnut: A Vulnerable Staple Crop

Groundnut (*Arachis hypogaea*) serves as a crucial source of nutrition and income in tropical and subtropical regions, providing high-quality protein, healthy lipids, and essential micronutrients (United States Department of Agriculture, 2023). However, its susceptibility to aflatoxin contamination poses significant challenges to its utilization and trade. The crop's vulnerability begins in the field, where drought stress and insect damage compromise pod integrity, facilitating fungal invasion (*Sserumaga et ak., 2021*). Post-harvest handling further exacerbates the risk as improper drying and storage conditions create favorable environments for Aspergillus proliferation. Studies indicate that up to 40% of groundnut crops in developing countries may

exceed regulatory limits for aflatoxins, impacting both domestic consumption and export potential (Udomkun *et al*., 2017). The economic consequences include price penalties, rejection of shipments, and loss of market access, particularly to stringent markets like the European Union. Addressing aflatoxin contamination in groundnuts requires integrated management approaches that consider both pre- and post-harvest factors while meeting consumer demands for safe, high-quality products (Pandey *et al.*, 2019).

Plate 1.1: ***Arachis hypogaea*** **(Rao *et al*., 2013)**

1.1.4 The Synergistic Potential of Cloves, Bay Leaves, and Thyme

The selected botanicals, cloves (*Syzygium aromaticum*), bay leaves (*Laurus nobilis*), and thyme (*Thymus vulgaris*) offer complementary antimicrobial profiles that may produce synergistic effects when combined. Clove essential oil, rich in eugenol (70 - 90%), demonstrates potent antifungal activity against Aspergillus species through membrane disruption and inhibition of aflatoxin biosynthetic genes (Pinto *et al*., 2017). Bay leaf oil, characterized by 1, 8-cineole (30 - 50 %) and

other monoterpenes, contributes additional antimicrobial mechanisms and may enhance the penetration of other active compounds (Škrinjar and Nemet, 2019). Thyme oil, dominated by thymol and carvacrol, exhibits strong membrane-permeabilizing properties and has shown efficacy in suppressing aflatoxin production (Kosakowska et al., 2024). The concept of synergy in essential oil combinations suggests that the mixture could achieve greater efficacy at lower concentrations than individual components, potentially reducing sensory impacts on treated products (Tariq *et al*., 2019). This approach aligns with current trends in natural preservation that seek to maximize efficacy while minimizing usage levels, addressing both safety and quality concerns in food protection strategies.

1.3 Aim and objectives of the study:

Aim:

To investigate the effectiveness of the oil extracted from selected spices as an anti-fungal and anti-aflatoxin on infested groundnut.

Objectives of the study

The specific objectives of this research were to:

i. evaluate the minimum inhibitory concentration - MIC and minimum fungicidal concentration - MFC of the essential oil blend against the dominant aflatoxigenic *Aspergillus* species
ii. determine the in-vitro antifungal activity of methanol extract of spices mixture at different concentrations using the disc diffusion method

CHAPTER TWO

LITERATURE REVIEW

2.1: Global Prevalence and Impact of Mycotoxins

Mycotoxin contamination represents one of the most significant challenges in global food safety, with approximately 25 % of the world's agricultural commodities affected annually (Food and Agriculture Organization, 2021). These toxic secondary metabolites are produced by various filamentous fungi, primarily *Aspergillus*, *Fusarium*, and *Penicillium* species, under favorable environmental conditions. The economic impact is staggering, with annual losses estimated at $1.4 billion in the United States alone and significantly higher figures in developing nations where monitoring and control systems are less robust (World Bank, 2020). The health implications extend beyond acute toxicity to include chronic effects such as carcinogenicity, immunotoxicity, and endocrine disruption, making mycotoxins a critical public health concern worldwide (Eskola *et al*., 2020).

The global distribution of mycotoxin contamination follows distinct geographical patterns influenced by climate, agricultural practices, and socioeconomic factors. In tropical and subtropical regions, aflatoxins produced by Aspergillus species predominate, while in temperate climates, Fusarium toxins such as deoxynivalenol and zearalenone are more prevalent (Gruber-Dorninger *et al*., 2019). Climate change is altering these patterns, with rising temperatures and changing precipitation profiles expanding the geographical range of mycotoxigenic fungi previously confined to specific regions. Studies project that aflatoxin contamination risk in maize cultivation areas will significantly increase in Europe and North America by 2030, posing new challenges to food safety systems (Battilani *et al*., 2016).

2.1.1 Aflatoxins: Chemistry and Biosynthesis Pathways

Aflatoxins are difuranocoumarin derivatives classified into major types B1, B2, G1, and G2 based on their fluorescence properties and chemical structure. Aflatoxin B1 (AFB1) is the most potent natural carcinogen known, with an International Agency for Research on Cancer (IARC) classification as Group 1 (carcinogenic to humans) (Yohannis *et al*., 2025). The biosynthesis of aflatoxins involves a complex pathway encoded by a 70 kb gene cluster containing approximately 30 genes, which are coordinately regulated by environmental and developmental factors (Caceres *et al*., 2020). The pathway-specific transcription factor AflR plays a crucial role in activating the expression of structural genes, while AflS modulates AflR activity and ensures proper regulation of the cluster. AflR is a key activator of aflatoxin gene expression while AflS is a co-activator that works with AflR.

The biochemical pathway begins with the conversion of acetate to noranthrone through a series of steps involving polyketide synthases, fatty acid synthases, and various modifying enzymes. Key intermediates include norsolorinic acid, averantin, and versicolorin, leading to the formation of sterigmatocystin as the final precursor before aflatoxin B1 synthesis (Caceres et al., 2020). Understanding this biosynthetic pathway is crucial for developing targeted intervention strategies, as inhibition at specific enzymatic steps can effectively block toxin production without necessarily affecting fungal growth. Recent advances in molecular biology have enabled the identification of key regulatory nodes that could be targeted for novel control approaches, including RNA interference and gene editing technologies (Tani *et al*., 2024).

2.2 Groundnut Vulnerability and Aflatoxin Contamination Dynamics

2.2.1 Pre-harvest Contamination Factors

Groundnut's susceptibility to aflatoxin contamination begins in the field, where multiple factors interact to create favorable conditions for *Aspergillus flavus* infection and toxin production. The geocarpic nature of groundnut development, where pods mature underground, creates direct and prolonged contact with soil-borne inoculum (Dorner, 2008). This risk is significantly amplified by end-of-season drought stress, which compromises the plant's physiological defenses and creates microenvironments conducive to fungal growth. Research has shown that drought stress increases aflatoxin contamination by up to 400% compared to well-irrigated conditions (Bandyopadhyay *et al.*, 2016). The mechanism involves reduced synthesis of antifungal compounds like phytoalexins and increased seed permeability, facilitating fungal invasion.

Insect damage represents another critical factor in pre-harvest contamination. Insects such as the lesser cornstalk borer (*Elasmopalpus lignosellus*) and various storage pests create physical wounds that provide entry points for fungal spores while simultaneously acting as vectors for spore dispersal (Zorzetti *et al*., 2017). Studies employing molecular tracking techniques have demonstrated that insect-mediated dispersal can introduce new, highly toxigenic strains into field populations, significantly increasing contamination risks (Ortega-Beltra *et al*., 2019). Furthermore, the wound response in plants generates oxidative stress, which has been shown to stimulate aflatoxin biosynthesis in established infections, creating a vicious cycle of increasing contamination (Caceres *et al*., 2020).

2.2.2 Post-harvest Contamination and Management Challenges

Post-harvest handling represents the most critical phase for aflatoxin management in groundnuts. The period between digging and drying is particularly vulnerable, as high moisture content and

mechanical injuries during harvesting create ideal conditions for fungal proliferation (Dambolecha et al., 2019). Traditional sun-drying practices, while cost-effective, often result in incomplete drying and re-wetting events that can trigger aflatoxin production even in previously safe commodities. Research has shown that aflatoxin levels can increase by up to 500% during improper post-harvest handling, highlighting the importance of this phase in contamination control (Shabeer *et al*., 2022).

Storage conditions play an equally crucial role in aflatoxin dynamics. Factors such as temperature, relative humidity, grain moisture content, and oxygen concentration interact to determine fungal activity and toxin production. The critical moisture content for safe storage of groundnuts is 9 % or lower, corresponding to a water activity (aw) of 0.70, below which *Aspergillus flavus* growth is effectively inhibited (Shabeer *et al*., 2022). However, maintaining these conditions in tropical environments presents significant challenges, particularly for smallholder farmers with limited access to proper storage infrastructure. Recent innovations in hermetic storage technologies, such as Purdue Improved Crop Storage (PICS) bags, have shown promise in maintaining safe moisture levels and reducing aflatoxin accumulation during storage (Baoua *et al*., 2014).

2.3 Essential Oils as Antifungal Agents: Mechanisms and Efficacy

2.3.1 Chemical Diversity and Antimicrobial Properties

Essential oils represent complex mixtures of volatile secondary metabolites, primarily comprising terpenoids and phenylpropanoids, which plants produce as defence compounds against pathogens and herbivores (Dhifi *et al*., 2016). The chemical composition of essential oils varies significantly depending on plant species, geographical origin, harvest time, and extraction methods, resulting in substantial variations in antimicrobial potency. Gas chromatography-mass spectrometry (GC-MS) analysis has identified over 3,000 different compounds in various essential oils, with typically

20 - 60 components present in significant concentrations (Baser and Buchbauer, 2015). The major components, which often constitute 20 - 85% of the total oil, are primarily responsible for the biological activities, while minor components may contribute to synergistic effects or modify the overall properties.

The antimicrobial efficacy of essential oils depends on their chemical composition, with phenolic compounds generally exhibiting the strongest activity followed by alcohols, aldehydes, ketones, and hydrocarbons (Cabarkapa *et al*., 2016). The presence of specific functional groups, such as the hydroxyl group in phenols and alcohols, enhances antimicrobial activity by increasing solubility and reactivity with biological targets. Structure-activity relationship studies have revealed that the position and number of functional groups, the presence of double bonds, and molecular size significantly influence antimicrobial potency (Shamsudin *et al*., 2022). For instance, the presence of the phenolic hydroxyl group at the para position in carvacrol and thymol contributes to their superior antimicrobial activity compared to their structural isomers (Peter *et al*., 2024).

2.3.2 Mechanisms of Antifungal Action

The antifungal activity of essential oils involves multiple mechanisms of action that target various cellular structures and functions. The primary mechanism involves disruption of cell membrane integrity, where hydrophobic compounds partition into the lipid bilayer, causing increased membrane fluidity and permeability (Hyldgaard *et al*., 2012). This membrane damage leads to leakage of vital ions, AdenosineTriPhosphate, amino acids, and other cellular constituents, ultimately resulting in cell death. Studies using artificial membranes and fluorescence techniques have demonstrated that essential oil components can create transient pores and disrupt lipid packing, with phenolic compounds showing the most pronounced effects (Cooper *et al.*, 2015).

Beyond membrane disruption, essential oils interfere with multiple cellular processes, including energy production, enzyme activity, and genetic material function. Several essential oil components have been shown to uncouple oxidative phosphorylation or inhibit key enzymes in the electron transport chain, collapsing the proton motive force and depleting cellular ATP pools (Yammine *et al*., 2022). Recent research has revealed that some essential oil components can intercalate with DNA and inhibit nucleic acid synthesis, while others generate reactive oxygen species that cause oxidative damage to cellular components (Bouyahya *et al*., 2017).

2.3.3 Anti-mycotoxigenic Mechanisms

The ability of essential oils to inhibit mycotoxin production represents a crucial advantage over-conventional fungicides that primarily target fungal growth. Essential oils can suppress mycotoxin biosynthesis through multiple mechanisms, including downregulation of biosynthetic genes, inhibition of key enzymes, and modulation of environmental and physiological signals that trigger toxin production (Ismail *et al*., 2018). The antioxidant properties of many essential oils contribute to their anti-mycotoxigenic activity by quenching reactive oxygen species that serve as signaling molecules for mycotoxin biosynthesis (Mutlu- Inguk *et al*., 2020). Additionally, essential oils can interfere with the regulatory cascades that coordinate secondary metabolism in response to environmental cues. For instance, certain essential oil components have been shown to modulate the activity of protein kinases involved in stress response signaling, thereby disrupting the cellular communication network that activates mycotoxin production (Kumar *et al*., 2021). This multi-targeted approach to mycotoxin control reduces the likelihood of resistance development and provides more comprehensive protection compared to single-mechanism inhibitors.

2.4 Detailed Profile of Selected Botanicals

2.4.1 Clove (*Syzygium aromaticum*)

Syzygium aromaticum, commonly referred to as cloves is a native from Maluku islands in East Indonesia belong to *Myrtaceae* family and consists of about 1200 to 1800 flowering plants (Shahid *et al.*, 2017). Cloves are rich in eugenol, they exhibit strong antioxidant, antibacterial and anti-inflammatory effects, making them a cornerstone in traditional remedies and modern pharmacology (Batiha *et al.*, 2020). In culinary arts, cloves enhance dishes, from savory curries to sweet pastries, and are integral to spice mixes like garam masala (Milind and Deepa, 2011). Their applications extend to oral care products and essential oils, leveraging their antiseptic qualities (Batiha *et al.*, 2020). Indonesia leads global production, followed by countries like Madagascar and India, with cloves playing a significant role in international trade (Cortés-Rojas *et al.*, 2014).

2.4.2 Botanical description

Syzygium aromaticum, commonly referred to as cloves, is an evergreen tropical tree in the Myrtaceae family, native to the Maluku Islands, Indonesia (Cortés-Rojas *et al.*, 2014). It grows to a height of 8 to 12 meters, sometimes reaching a height of 20 meters with a cone-shaped covering of glossy, lanceolate leaves that are opposite and 8 to 12 cm long (Batiha *et al.*, 2020). The leaves are dark green with a leathery texture and aromatic oils. The tree produces clusters of reddish-brown flower buds, which are harvested before blooming and dried to become cloves, the spice (Milind and Deepa, 2011). These buds are small, 1 to 2 cm long, with a calyx and unopened petals forming a nail-like shape. The tree's bark is smooth and grayish, and its roots are extensive, supporting growth in well-drained, loamy soils in humid tropical climates (Cortés-Rojas *et al.*, 2014).

Plate 2.1: ***Syzygium aromaticum*** **(Mayekar *et al*., 2021)**

2.4.3 Taxonomy

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Myrtales

Family: Myrtaceae

Genus: *Syzygium*

Species: *Syzygium aromaticum*

2.4.4 Ethnomedicinal Uses

Cloves (*Syzy*gium *aromaticum)* has been utilized in traditional medicine, particularly in Indian and Chinese practices, for their warming and stimulating properties (Batiha *et al.*, 2020). It contains eugenol, which has anti-inflammatory properties, making them effective for treating acne, skin infections and hair loss (Martínez-Herrera *et al*., 2016) and also contains carminatives properties, which helps to relieve gas, bloating and indigestion (Milind and Deepa, 2011). Cloves possesses medicinal qualities to cure flatulence, loose motion, indigestion and nausea. It is used for relieving the symptoms of diarrhea, gastric irritability and vomiting (Milind and Deepa, 2011). Clove and clove oil boost the immune system by purifying the blood and help to fight against various diseases. Clove oil is also effective in curing Athlete's foot and nail fungus (Batiha *et al.*, 2020)

2.4.5 Pharmacology Properties

2.4.5.1 Anti-viral activity

Activity Against Herpes Simplex Virus (HSV)

Clove is a potent antiviral agent. Eugenin isolated from clove buds showed strong antiviral activity against herpes simplex virus (HSV) by inhibiting viral deoxyribonucleic acid (DNA) polymerase preventing replication at a concentration of 10 µg/mL (Jaiswal et al., 2021). In vitro, clove essential oil's (CEO) efficacy reduces HSV-1 plaques by up to 60% at 0.1–1% v/v, which is due to eugenol's virucidal action on viral envelopes (Kiki et al., 2023). In 2020, clove extracts synergized with acyclovir, lowering HSV-1 viral yields by 80% in Vero cells and reducing brain infection severity in mice by 50%, without toxicity at effective doses (Batiha *et al*., 2020). Topical CEO also delayed HSV-induced keratitis progression in animal models, mimicking eugeniin's protective effects (Reis *et al.*, 2022).

2.4.5.2 Mosquitoes repellent

Clove oil gave the longest duration of 100% repellency (2 - 4h) against three species of mosquitoes. A 2022 evaluation of undiluted essential oils reported clove oil providing high complete-protection times (120 – 360 minutes) against *Culex quinquefasciatus* with notable efficacy against *Aedes aegypti* (up to 270 minutes when combined with other oils) and *Anopheles dirus* (up to 180 minutes), surpassing citronella controls (Sutthanont *et al*., 2022). Nanoemulsions of clove essential oil (CEO) improved repellency, achieving 100% protection for over 3 hours against *Aedes aegypti* at lower concentrations (0.5% v/v), due to better skin adhesion and volatilization control (Mahrous *et al*., 2024). Field trials in dengue-endemic areas showed topical clove-based gels reducing *Aedes aegypti* bites by 85 - 95% for 2 - 3 hours, comparable to 10% N, N- Diethyl- meta- toluamide (DEET) but with fewer skin irritations (Sutthanont *et al.,* 2022).

2.4.5.3 Aphrodisiac

It has been found that ethanolic extract of clove(50 %) produced a significant and sustained increase in the sexual activity of normal male rats without any obvious gastriculceration and adverse effects (Yanuarty *et al.*,2024).

2.4.5.4 Anti-inflammatory activity

Cloves (*Syzygium aromaticum*), particularly their essential oil (CEO), are widely recognized for potent anti-inflammatory activity, attributed primarily to eugenol (70–90%), eugenyl acetate, β-caryophyllene, and other phenolic compounds. These bioactives inhibit key inflammatory pathways, reduce cytokine production, and modulate oxidative stress, making cloves valuable in managing acute and chronic inflammatory conditions such as arthritis, dermatitis, oral inflammation, and wound-related swelling (Batiha *et al.*, 2020).

2.4.6 Clove (*Syzygium aromaticum*) Essential Oil

Clove essential oil, derived from the flower buds of *Syzygium aromaticum* is renowned for its potent antimicrobial properties, primarily attributed to its high eugenol content (70 - 90%). Eugenol (4-allyl-2-methoxyphenol) is a phenolic compound characterized by strong antimicrobial activity against a wide spectrum of microorganisms, including fungi, bacteria, and viruses (Pinto *et al*., 2017). The mechanism of action involves disruption of cell membrane integrity, inhibition of ATPase activity, and suppression of quorum sensing in bacterial systems. Beyond eugenol, clove oil contains significant quantities of eugenyl acetate (up to 15%) and β-caryophyllene (5 - 12%), which may contribute to its overall bioactivity through synergistic interactions.

Research has demonstrated clove oil's exceptional efficacy against aflatoxigenic Aspergillus species. In vitro studies have shown complete inhibition of Aspergillus flavus growth at concentrations of 0.2-0.4 μL/mL, while sub-inhibitory concentrations 100(0.1 μL/mL) reduced aflatoxin B1 production by up to 95% (Mishra *et al*., 2020). The anti-mycotoxigenic effect is mediated through downregulation of key aflatoxin biosynthetic genes, particularly aflR and aflD, as revealed by reverse transcription Polymerase Chain Reaction (PCR) analysis. Additionally, clove oil exhibits strong antioxidant activity, with an ORAC (Oxygen Radical Absorbance Capacity) value of over 1,000,000 μmol TE/100g, which may contribute to its preservative effects by preventing lipid oxidation in food matrices (Shan *et al.*, 2005).

2.5 Bay Leaf (*Laurus nobilis* L.)

Laurus nobilis, often referred to as Bay leaf is an evergreen tree that has been used for 1000 years and is an essential ingredient in cooking and many traditional uses (Hanif *et al*., 2020). The leaves are used in fresh or dried form to flavor culinary preparations and scented aromatic essential oil in perfumery. Laurel has been traditionally used for years in traditional medicine due to its various pharmacological activities such as antimicrobial, antioxidant, anticancer, insecticide and

antifungal (Bendjersi *et al*., 2016; Nabila *et al*., 2022; Zibie *al*., 2022). This tree is native to Southern Mediterranean region. It is cultivated commercially for its leaves in Algeria, Morocco, Portugal, Spain, Türkiye, Italy, France and Mexico. It is widely cultivated in Europe and the United States as ornamental (Guenane *et al*., 2016).

2.5.1 Botanical description

Laurel is an evergreen shrub or tree up to 12 m tall in the wild and cultivation is usually pruned to 2 to 3 meters tall. The species has several trunks. The bark of the stem and branches is dark brown to almost black (Khaled Khodja *et al*., 2023). The foliage of *Laurus nobilis* is evergreen with a dark green color above and lighter below. The leaf shape is elongated, lanceolate with pointy tips and a short petiole. The blade is slightly thick and haa a wavy edge that curves inwards. The leaves are approximately 3 to 5 centimeters wide by 10 centimeters long. The leaves are hairy at first, then they take on a shiny and hairy appearance (Khaled Khodja *et al.*, 2023).

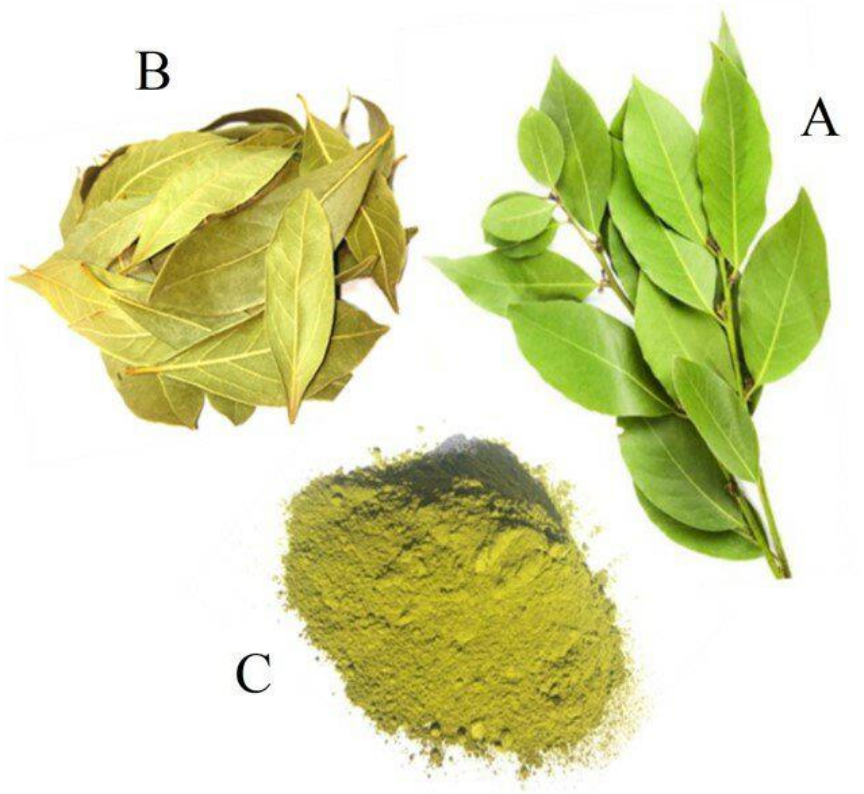

Plate 2.2: *Laurus nobilis* leaves: Green (A), dry (B) and powder (C) (Khodja *et al*., 2021)

2.5.2 Taxonomy

Kingdom: Plantae

Division: Angiosperm

Order: Laurales

Family: Lauraceae

Genus: Laurus

Species: *Laurus nobilis*

2.5.3 Ethnomedicinal uses

Bay (*Laurus nobilis*) is full of antioxidants and is a good source of minerals and dietary fibers. It compliments flavors and bay tea is used to treat stomachaches, clear up mucus in the lungs, colds and sore throat. Poultice of bay leaves is used for the treatment of rheumatism and neuralgia (Khaled Khodja *et al.,* 2023). Traditionally,it has been used as herbal medicine against number of diseases such as rheumatism,sprains,indigestion,earaches and to enhance perspiration(Fang *et al.,* 2005). Bay leaf is also used to treat arthritis, headache, fungal diseases, anorexia and diarrhea traditionally (Parthasarathy *et al.,* 2008). Laurel leaves are effective against many infections caused by fungi, viruses, bacteria and protozoa. They are also useful for inhibiting the growth of cancer cells and for fevers, coughs, flu, colds, bronchitis and asthma. Laurel juice is an effective drug for soothing sore eyes and night blindness usually caused by Vitamin A deficiency (Hanif *et al,,* 2020). Bay juice is an effective medication for sore eyes and night blindness which is generally caused by deficit of Vitamin A.

2.5.4 Pharmacology properties

2.5.4.1 Analgesic and anti-inflammatory activity

The antinociceptive and anti-inflammatory properties of *Laurus nobilis* (Lauraceae) leaf essential oil have been investigated in mice and rats. The essential oil was found to have significant analgesic and dose- dependent anti-inflammatory effects in the formalin induced edema test and the tail flick test. At the anti-inflammatory dosages, the essential oil also exhibited a mild sedative effect. The analgesic and anti-inflammatory effects of the essential oil were similar to those of morphine and piroxicam which are reference analgesic and non-steriodal anti-inflammatory drugs (Peixoto *et al.,* 2019). Tha analgesic effects of bay leaves is attributed to its ability to inhibit pain mediators such as prostaglandins and bradykinin (Kumar *et al.,* 2018).

2.5.4.2 Anti-viral activity

The essential oil of *Laurus nobilis* has been found to contain several components, including beta - ocimene, 1,8 - cineol, alpha - pinene and beta-pinene, that possess inhibitory effects against the replication of SARS-CoV and HSV-1 in vitro. The essential oil has demonstrated an IC50 value of 120mg/mL, along with a selectivity index of 4 approximately binding its efficacy in inhibiting the replication of these viruses (Kumar *et al.,* 2018).

2.5.4.3 Anti-convulsant activity

Laurus nobilis leaf essential oil showed anticonvulsant activity in mice. Essential oil components such as eugenol, pinene and methyleugenol are responsible for this activity (Sayyah *et al.,* 2015). The anticonvulsant effects may be due to its ability to modulate neurotransmitter and ion channels to the brain (Kumar *et al.,* 2018).

2.5.4.4 Hepatoprotective activity

The crude extract of *Laurus nobilis* leaves showed good hepatoprotective activity against paracetamol toxic effects on rats hepatocytes at a concentration of 40 µg/ml (Ayoub *et al*., 2013). Similarly, an experimental study performed on rats showed that the methanol extract of *Laurus* nobilis at 400 mg/kg acts on the liver as a potent scavenger of free radicals to prevent the hepatotoxicity induced by paracetamol (Ravindran *et al.,* 2013). Another in vivo study carried out by Gasparyan *et al.* (2015)revealed that some phenolic compounds such as flavonoids and eugenol of *Laurus nobilis* protect hepatocytes of rats against metabolic and histological abnormalities induced by tetrachloromethane.

2.5.4.5 Anti-oxidant activity

Laurus nobilis leaves demonstrated strong antioxidant properties. Elmastaş *et al.* (2006) showed that freeze dried extracts (water and ethanol) of bay leaves showed strong total antioxidant activity in the linoleic acid emulsion. The concentrations of 20, 40 and 60 µg/ml showed inhibition of 84.9, 95.7, 96.8 and 94.2, 97.7 and 98.6% of the lipid peroxidation of the emulsion of linoleic acid, respectively for aqueous and ethanolic extracts. The findings are comparable to 60 µg/ml of standard antioxidants including butylated hydroxyanisole, butylated hydroxytolene and α tocopherol with 96.6, 99.1, and 76.9% inhibition of lipid peroxidation, respectively. The alkaloid extract obtained from bay leaves expressed a high antioxidant activity with an IC50 of 63. 28 µg/ml higher than gallic acid (143.18 µg / ml) used as standard. The phenolic extract exhibited an antioxidant power with an IC50 of 317. 57 µg / ml, less than that of alkaloids extract (Khaled Khodja *et al*., 2021). Bay leaves contains antioxidants which protects against oxidative stress and inflammation (Kumar *et al*., 2018). The antioxidants properties contributes to its health benefits such as Anti-cancer and antidiabetic effects.

2.5.5 Bay Leaf (*Laurus nobilis*) Essential Oil

Bay leaf essential oil, extracted from the leaves of *Laurus nobilis*, possesses a distinct chemical profile dominated by 1,8-cineole (eucalyptol, 30-50 %), with significant contributions from other monoterpenes including α-pinene (3-10 %), sabinene (5-15 %), and linalool (2-7 %) (Škrinjar and Nemet, 2019). The antimicrobial activity of bay leaf oil is generally attributed to 1, 8-cineole, which exhibits multiple mechanisms of action including membrane disruption, inhibition of cellular respiration, and interference with enzyme systems. While less potent than phenolic-rich oils like clove and thyme, bay leaf oil demonstrates broad-spectrum antimicrobial activity and may enhance the efficacy of other essential oils through complementary mechanisms.

Studies on bay leaf oil's antifungal properties have shown moderate to strong activity against various food spoilage fungi, with minimum inhibitory concentrations ranging from 0.5-2.0 μL/mL for *Aspergillus* species (Marino *et al*., 2001). The oil's relatively high concentration of oxygenated monoterpenes contributes to its antimicrobial potency, while its hydrocarbon monoterpenes may enhance penetration through biological membranes. Beyond direct antimicrobial effects, bay leaf oil exhibits significant antioxidant activity, primarily attributed to its content of phenolic compounds and terpenoids with free radical-scavenging properties (Djenane *et al*., 2011). This dual functionality makes it particularly valuable in food preservation applications where both microbial control and oxidative stability are concerns.

2.6 Thyme (*Thymus vulgaris*)

Thymus vulgaris, commonly known as thyme, is a perennial flowering plants, member of the mint family Lamiaceae. Members of this family are greatly used in self-care products, flavoring, fragrance, scents, pesticide, and medicine industries (Özkan *et al.*, 2008). Globally, *Thymus vulgaris* has several common names. It is also known as Garden Thyme, French Thyme, Rubbed Thyme, Red Thyme, Folia Thyme depending upon their states in which they grow. In Romania, it is known as cimbru and avishan in Persia.

2.6.1 Botanical description

Thymus vulgaris, also known as Thyme, is a widely known aromatic herb. Thyme is a tiny perennial shrub that seldom grow 40cm tall with evergreen groundcover. With growing age, the stem becomes woody. *Thymus vulgaris* leaves are extremely small and fleshy, usually 2.5 cm to 5 cm with oval to rectangular form. *Thymus vulgaris* have greyish-green leaves and purplish or pinkish flowers in early summer. Thyme prefers a dry, sunny and temperate environment for its growth. Full exposure to sun is essential for thyme to grow to its full potential. Excessive moisture

will lead to disease development in thyme. Thyme has extremely small and round seeds that retain their germination capacity for around 3 years (Ahmad *et al.,* 2012).

Plate 2.3: *Thymus vulgaris* **(Escobar *et al*., 2020)**

2.6.2 Taxonomy

Kingdom: Plantae

Sub-Kingdom: Tracheophyta

Super division: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Sub-class: Asteridae

Order: Lamiales

Family: Lamiaceae

Genus: *Thymus*

Species: *Thymus vulgaris*

2.6.3 Ethnomedicinal uses

Since Romans and Greeks, thyme has been cultivated and used for various purposes. It is believed that consuming one cup of thyme extract provides freshness, lightness in the stomach and protects one against the morning cough. In traditional medicine, thyme was used for treating baldness (Arras *et al.*, 1992). It possesses ability to cure ulcer and various types of dermatitis, injuries and bruises. It is also used in medicines for treatment of sleeping disorders like insomnia. Gastro-intestinal troubles like dyspepsia, gastritis, diarrhea and gastric ulcers can also be treated by using thyme oil.

2.6. 3 Pharmacological properties

2.6.3.1 Antimicrobial activity

Thyme (*Thymus* vulgaris), is widely known for its broad-spectrum antimicrobial activity against bacteria, fungi, viruses and parasites. Thymol and carvacrol disrupt microbial cell membranes leading to leakage and cell death (Salehi *et al*., 2018). Thyme essential oil shows minimum inhibitory concentrations (MICs) of 0.05 - 0.2 % v/v against bacteria like *Staphylococcus aureus* and *Escherichia coli*. It also enhances the efficacy of antibiotics like gentamicin against resistant strains (Kowalska-Krochmal and Dudek-Wicher, 2021). For fungi, thyme oil inhibits *Candida albicans* and *Aspergillus niger* preventing biofilm formation with MICs around 0.03 – 0.1% v/v (Lorenzo et al., 2019). Antiviral effects such as inhibition of herpes simplex virus type 1, with thymol reducing viral plaques by 70% in cell cultures (Salehi *et al*., 2018).

2.6.3.2 Anti-cancer properties

Thyme compounds such as thymol and carvacrol, exhibit anticancer effects by inducing apoptosis and inhibiting proliferation. In cell lines like MCF-7 (breast) and A549 (lung), thymol triggers caspase activation and increases reactive oxygen species thereby reducing cell viability by 50 - 70% at concentrations of 50–100 μM (Al-Mamoori et al., 2023). Carvacrol inhibits tumor metastasis by downregulating matrix metalloproteinases gelatinase A (MMP-2) and matrix metalloproteinases gelatinase B (MMP-9) enzymes in colon cancer models (Salehi et al., 2018). In vivo, thyme extracts reduced tumor volume in breast cancer xenografts by 40%, showing synergistic effects with chemotherapeutic agents like cisplatin (Nikolić et al., 2014).

2.6.3.3 Anti-oxidant properties

Thymus vulgaris possesses strong antioxidant activity

Thyme exhibits strong antioxidant activity, countering oxidative stress linked to chronic diseases like cancer and cardiovascular disorders. In vitro, thyme extracts protect cells from hydrogen peroxide-induced damage by boosting superoxide dismutase and catalase levels (Lorenzo *et al* 2019). In vivo, thyme supplementation in diabetic rats reduces lipid peroxidation and restores glutathione levels in liver and kidney tissues providing protection against oxidative damage (Al-Mamoori *et al.*, 2023). These effects are due to the synergistic action of thymol, carvacrol, and flavonoids like apigenin (Nikolić *et al*., 2014).

2.6.4 Thyme (*Thymus vulgaris*) Essential Oil

Thyme essential oil, obtained from the aerial parts of *Thymus vulgaris*, is characterized by high concentrations of phenolic monoterpenes, primarily thymol (20-54 %) and carvacrol (1-25 %), along with their precursor p-cymene (10-30 %) and γ-terpinene (5-15 %) (Numpaque *et al*., 2011). The antimicrobial potency of thyme oil is primarily attributed to thymol and carvacrol, which are

among the most effective natural antimicrobial compounds known. These phenolic compounds exhibit strong membrane-disrupting activity, with studies showing rapid depletion of the transmembrane potential and ATP pool in treated fungal cells. Additionally, they demonstrate significant inhibition of ergosterol biosynthesis, further compromising membrane integrity and function (Ultee *et al*., 2002).

Research has consistently demonstrated thyme oil's exceptional efficacy against aflatoxigenic fungi. In vitro studies report minimum inhibitory concentrations of 0.1-0.3 μL/mL against *Aspergillus flavus*, with complete inhibition of aflatoxin production at sub-fungicidal concentrations (Rasooli and Owlia, 2005). The anti-mycotoxigenic mechanism involves significant downregulation of aflatoxin biosynthetic genes, particularly those encoding key enzymes in the early stages of the pathway. Thyme oil also exhibits strong antioxidant activity, with thymol demonstrating superior free radical-scavenging capacity compared to synthetic antioxidants like BHT in certain model systems (Yanishlieva *et al*., 1999). This combination of potent antimicrobial and antioxidant properties makes thyme oil particularly valuable for protecting high-lipid commodities like groundnuts from both microbial spoilage and oxidative rancidity.

2.7 Synergistic Interactions in Essential Oil Combinations

2.7.1 Scientific Basis of Synergy in Antimicrobial Formulations

Synergy in essential oil combinations occurs when the combined effect exceeds the additive effects of individual components, potentially through multiple mechanisms including multi-target action, enhanced bioavailability, and inhibition of degradation pathways (Bassolé and Juliani, 2012). The scientific basis for synergy lies in the complementary mechanisms of action of different essential oil components, which can create a more comprehensive antimicrobial effect than any single

compound. For instance, membrane-disrupting compounds like thymol can enhance the penetration of other active ingredients that target intracellular processes, while compounds that inhibit efflux pumps can increase the intracellular concentration of antimicrobial agents (Tariq *et al*., 2019).

Several mathematical models have been developed to quantify synergistic interactions, including the fractional inhibitory concentration (FIC) index, the sum of fractional inhibitory concentrations (ΣFIC), and isobologram analysis (Odds, 2003). These approaches allow for systematic evaluation of combination effects and identification of optimal ratios for maximum efficacy. Research has shown that carefully designed essential oil combinations can achieve equivalent antimicrobial effects at significantly lower concentrations (30-70% reduction) compared to individual oils, reducing potential sensory impacts and cost while maintaining efficacy. This approach is particularly valuable for food applications where strong flavors or aromas might limit the practical use of individual essential oils at effective concentrations (Gutiérrez *et al*., 2008).

2.7.2 Documented Synergistic Combinations and Mechanisms

Numerous studies have documented synergistic interactions between essential oil components with complementary mechanisms of action. The combination of thymol and eugenol has shown particularly strong synergy against various fungal pathogens, with FIC indices as low as 0.25-0.5 indicating 4-fold to 2-fold reductions in effective concentrations (Pei *et al*., 2009). The mechanism involves thymol's primary action on membrane integrity, which facilitates increased uptake of eugenol and enhanced inhibition of intracellular targets. Similarly, combinations of 1,8-cineole with phenolic compounds have demonstrated improved efficacy, possibly through improved distribution in biological systems or complementary effects on different cellular targets.

Research on ternary combinations is less extensive but shows promising results. Studies investigating three-component systems have revealed complex interactions that can yield superior antimicrobial effects compared to binary combinations (Bassolé and Juliani, 2012). The combination of thymol, eugenol, and 1, 8-cineole has shown particular promise, with hypothesized mechanisms involving sequential targeting of membrane integrity (thymol), intracellular enzymes (eugenol), and respiratory functions (1, 8-cineole). This multi-target approach not only enhances efficacy but also reduces the likelihood of resistance development, as multiple mutations would be required to confer resistance to all components simultaneously (Wagner and Ulrich-Merzenich, 2009).

2.8 Gaps in Current Research and Novelty of Present Study

2.8.1 Limitations of Existing Research

Despite substantial research on essential oils as natural antimicrobials, several significant gaps remain in the current literature. Most studies have focused on individual essential oils or simple binary combinations, with limited investigation of more complex multi-component systems that may offer superior efficacy through synergistic interactions (Tariq *et al*., 2019). Furthermore, the majority of research has emphasized in vitro effects without adequate consideration of performance in real food matrices, where factors like lipid content, pH, and water activity can significantly influence antimicrobial activity (Hyldgaard *et al*., 2012). There is also limited understanding of how essential oil combinations affect both fungal growth and mycotoxin production simultaneously, particularly under conditions relevant to commercial storage and distribution.

Another significant limitation concerns the application methods and formulation technologies for essential oils in food preservation. Many studies apply essential oils directly without considering

practical application methods, delivery systems, or potential impacts on food quality attributes (Donsì and Ferrari, 2016). The development of effective delivery systems that protect essential oil components from degradation, control their release, and minimize sensory impacts remains a critical challenge. Additionally, there is insufficient research on the economic feasibility of essential oil applications in commercial settings, particularly for small-scale operations in developing countries where aflatoxin contamination is most problematic.

2.9. Novel Contributions of the Present Study

This research addresses several critical gaps in the existing literature through its comprehensive approach to developing and evaluating essential oil combinations for aflatoxin control in groundnuts. The study's novelty lies in its systematic investigation of a ternary essential oil system (clove, bay leaf, and thyme) specifically designed to leverage complementary mechanisms of action against both fungal growth and aflatoxin production. Unlike previous studies that often focus solely on antimicrobial effects, this research examines multiple aspects including antifungal efficacy, anti-mycotoxigenic activity, impacts on product quality, and potential synergistic interactions.

The study employs advanced analytical techniques including GC-MS for chemical characterization, real-time PCR for investigation of molecular mechanisms, and sophisticated statistical models for synergy quantification. Furthermore, the research evaluates essential oil efficacy under conditions that closely mimic real-world storage scenarios, providing practical insights for implementation. By considering both efficacy and practical application aspects, this study aims to bridge the gap between laboratory research and commercial implementation, contributing valuable knowledge for developing effective, natural strategies for aflatoxin management in groundnuts and other susceptible commodities.

CHAPTER THREE

MATERIALS AND METHOD

3.1 MATERIALS

The following materials were used in this study:

3.1.1 APPARATUS AND EQUIPMENTS

Bench autoclave, binocular microscope, incubator, hot air oven, analytical weighing balance, centrifuge, pH meter, refractometer, refrigerator, water bath, spectrophotometer, soxhlet extractor, glass wares (pyrex burettes, pipettes, beakers, microscopic slides, petri dishes, measuring cylinders, flasks, separating funnels, bijou, bama bottles, universal and McCartney bottles), rotary evaporator, industrial blender, aluminum foil paper, plastic spoons, handkerchiefs, petroleum jelly, forceps, masking tape, stool.

3.1.2 Microbiological media

Nutrient Agar, MacConkey Agar, Mueller Hinton Agar, Nutrient Broth, Sabouraud Dextrose Agar, Sabouraud Dextrose Broth, Chromogenic Agar, Eosin Methylene Blue Agar, Mannitol Salt Agar, Citrate Agar, Peptone Broth.

3.1.3 Chemicals and Reagents

All chemicals used were of analytical grade and they include; Absolute methanol, Distilled water, N-Hexane, Hydrogen peroxide, 1% Tetramethyl-p-phenylenediamine hydrochloride (Oxidase reagent), Phenolphthalene, 0.1N Sodium Hydroxide (NaOH), Tween-80 (10%), Picric acid, Wagner reagent, Dragendroff's reagent, Methylated spirit, Crystal violet(0.5%w/v, BEMA), Lactophenol cotton blue (BEMA), Safranine (BEMA), Grams iodine, Plasma , 1% Barium chloride, 1% sulfuric acid (H2SO4), Dettol, Glycerol, Glycerin, Sodium chloride, Fehling's solution A and B, Ferric chloride, Sodium picrate, dilute ammonia solution.

All media and reagents used were prepared according to the manufacturer's instructions.

3.1.4 Antimicrobial agents and related products

Ketoconazole (Sigma-Aldrich Biochemika), Clove, Thyme and Bay leave oil extract.

3.1.5 Source of Test Microorganisms

The microorganisms used for the antimicrobial assay were Aflatoxigenic fungal isolates from stale groundnut isolated and stored in the Pharmaceutical Microbiology Laboratory of University of Benin. The reference microorganisms include *Aspergillus parasiticus, Aspergillus flavus, Emericella astellata, Penicillum puberulum.*

3.2 METHODS

3.2.1 Sources and Collection of Samples

The spices samples (Cloves, Thyme and Bay leave) were commercially sourced from Uselu Market in Benin City, Nigeria. The samples were identified and authenticated by Dr. H. A. Akinibosun from the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State, Nigeria prior to use while the stale groundnut samples for aflatoxigenic fungal isolation were obtained from previously stored bottled roasted Auchi groundnut (*Arachis hypogaea)* purchased around Benin metropolitan market. The state of staleness was authenticated by sensory evaluation by Prof. E. O. Oshomoh from the Department of Science Laboratory Technology and Dr. E. Dowe of Pharmaceutical Microbiology. All samples obtained were appropriately transported to the laboratory for analysis.

3.2.2 Preparation and Extraction of Spices Oil

The spices were sorted out, air dried at room temperature for 3 - 4 weeks until the spices were all dried and a maximum amount of water content has been lost. The dried spices were grinded into

fine powder with the aid of an industrial blender and stored in dried, clean labelled containers for easy identification and further analysis. Equal weights (7 g each) of cloves (*Syzygium aromaticum*), thyme (*Thymus vulgaris)* and bay leaves (Laurus *nobilis)* were measured accurately using the analytical weighing balance. The measured spices were mixed together to form a uniform blend. The extraction of the essential oil was carried out using a soxhlet extractor with N-Hexane as the solvent. The mixed spice blend was placed inside a clean white muslin fabric that was meticulously sewed together and placed in the extraction chamber, applying petroleum jelly at the base of the extraction chamber, making it easy for insertion or removal of the handkerchief. Extraction was performed continuously for an hour at the boiling point of the solvent (N-Hexane), until the solvent in the siphon tube becomes clear, indicating complete extraction. The resulting extract was concentrated using a rotary evaporator which means that the solvent which is the N-Hexane was evaporated from the sample leaving the oil.

3.2.3 Sample Treatment, Enumeration, Isolation, Characterization and Identification of Fungal Isolates

The groundnut samples was grinded into fine powder using oven sterilized laboratory mortar and pestle and the fungal load of the stale groundnut samples was enumerated by serial dilution and spread plate method. In the process, one gram (1g) of the grounded sample was introduced into 10 mL of sterilized normal saline, steadily agitated to homogenize and serially diluted in sterilized normal saline. Aliquots (100µL) of dilution factor 10^{-3} were aseptically plated into Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) containing 0.1 mg/mL ciprofloxacin using the spread plate method. The agar plates were incubated at 27 ± 2^{0}C (Ambient temperature) for 144 hours. After incubation, the fungal colonies that grew on the medium were enumerated, characterized and identified based on microscopic morphology and cultural characteristics. The emerging fungal isolates were repeatedly plated to obtain monosporic pure isolates maintained on

PDA slants containing containing 0.1 mg/mL ciprofloxacin, periodically sub-cultured and stored at 4^0C. Preparation of pure cultures for microscopy was done by degreasing a clean glass slide, adding drops of ethanol and fragment of pure culture was transferred into the slide with the aid of the sterile forceps, a drop of lactophenol cotton blue stain was added, it was made even on the slide with flamed blunted needle for about 60 seconds, slides were separately covered with a cover slip and viewed under the microscope (Cheesbrough 2000).

3.2.4 Preparation of Test Microorganisms

Prior to use, the test microorganisms were authenticated and subcultured from stock into sterile Potato Dextrose broth and incubated overnight at room temperature (27 ± 2^0C) for 72hours. After incubation, overnight broth culture was adjusted by one in hundred serial dilution (1:100) corresponding to 0.5McFarland standard to give an inoculum size of approximately 106 cfu/mL.

3.2.5 Preparations of Extract Dilutions and Stock Solutions

The stock concentration of the extracts previously constituted by addition of one parts of equal amount of the individual oil to form synergistic mixture (1:1:1). The mixture was constituted into varying concentrations/dilutions by adding 1mL (100%) stock of the sample and dissolving it in 10 mL of 10% tween-80 to obtain dilutions 50% and 25%. Other concentrations were made by appropriate dilutions and variation of specific volumes obtained from the stock concentrations. While the standard antifungal agent (Ketokonazole 10 µg/mL) was constituted by dissolving 1mg in 10mL of sterile distilled water to obtain a stock of 100 µg/mL. One in ten (1:10) serial diution of the stock ketoconazole was done to obtain 10µg/mL (Dowe *et al*., 2016).

3.2.6 Aflatoxigenic Screening of Isolates

1. Coconut Extract Agar (CEA) Aflatoxin Test

The Aflatoxin producing isolates were screened using the Culture plate (Coconut Extract Agar (CEA) screening) method and further confirmed using Ammonia (NH_4OH) vapor test method. In the process, the isolated fungal slants were reconstituted into Potato Dextrose Agar (PDA) broth, standardized to one in hundred 0.5 McFarland equivalent to obtain approximately 106cfu/mL of microbial suspension and 100µL of each suspension was spread plated into Coconut Extract Agar (CEA) and incubated at ambient temperature (27 ± 2^0C) for 48 - 96 hours. After incubation, growth and sporulation were observed, then used to point inoculate or plug the center of a fresh CEA prior to incubation in the dark at 27 ± 2^0C for 48hours undisturbed. After which plates are observed and viewed under Ultra Violet (UV) light at 365 nm and monitored daily for blue-green fluorescence on the observed and reversed side of the plates. Positive plates and their corresponding slants indicated by the presence of colored florescence on the agar reverse or surrounding colonies were selected and the intensity of coloration recorded as (strong +++/moderate ++/weak+/none-) test (Miklos *et al.,* 2020)

2. Ammonia (NH_4OH) vapor test

The Ammonia vapor test method described by Miklos *et al*. (2020) was used to further confirm screened isolates. Briefly in the process, the isolated fungal slants were reconstituted into PDA broth, standardized to one in hundred 0.5 McFarland equivalent to obtain approximately 106sfu/mL of microbial suspension and 100µL of each suspension was spread plated into Coconut Extract Agar (CEA) and incubated at ambient temperature (27 ± 2^0C) for 96 hours. After which culture plates were precautionary transferred to fume hood where plate lids were inverted with the introduction of 1.5 mL concentrated NH_4OH (ammonium hydroxide or aqueous ammonia), the lid lined with Bibulous absorbent paper and lids replaced with contact region sealed with parafilm for

8 - 16 minutes . After about 8 minutes, plates were observed for pink-red color development. The absence of pink-red coloration after 16 minutes is regarded as negative result for Aflatoxin production and color change after 20 minutes is regarded as false positive.

3.2.7 Determination of Antifungal Sensitivity to the Selected Antifungal Agents

Antifungal susceptibility of the isolates was assessed using the modified agar well diffusion method (Lalitha *et al*., 2004; Cheesbrough, 2006; Clinical and Laboratory Standard Institute (CLSI), 2010). In the process, wells of 6mm were made into Potato dextrose agar using flame-sterilized cork-borer. Sterile swab sticks was then dipped into the standardized microbial suspension and gently streaked on the surface of the agar plates in even strokes to obtain a uniform growth pattern across the entire surface of the plate. This was achieved by rotating the plate 90 degrees followed by 45 degrees with continuous streaking, and finally by streaking round the diameter of the agar. The 6 mm wells was filled with equal volumes of the stock concentration of the oil mixtures corresponding to 100%, 50% and 25% concentrations. The same quantity of 10% tween-80 served as negative controls while the standard antifungal drugs (10µg/ml of Ketoconazole) served as positive controls. The plates were left to stand for 1 hour on workbench to allow diffusion of extract before incubating plates at room temperature (27 ± 2^0C) for 48 - 72 hours. The diameter of clear zone was observed and measured in mm (millimeters). The experiments including controls were done in replicates and the mean Inhibition zone diameters (IZDs) were calculated and recorded.

3.2.8 Determination of Minimum Inhibitory Concentrations (MICs) of the Extracts (Oils) Against the Isolates

The modified broth dilution method described by Firas *et al.* (2008), was used to determine the MICs of the oil mixture against susceptible isolates. Varying concentrations of the sample mixture (oils) ranging from 0.05 - 16% were constituted in 10 ml of Potato dextrose broth in screw capped tubes from the 100% stock. The standardized (approximately 106 cfu/mL) colony or spore suspensions was used to inoculate the varying concentration of antifungal oils dilutions in the broth tubes. In each round of experiment, a tube without the antifungal agent but with same volume of broth and inoculum served as controls. All tubes were appropriately incubated. After incubation, tubes were observed for growth/turbidity. In all cases, the lowest concentration of the antimicrobial substance at which there was no observable fungal growth was recorded as the MICs.

3.2.9 Determination of Minimum Fungicidal Concentrations (MFCs) of the Antifungal Oils

The broth tubes with no visible growth following MIC determination were plated or inoculated into fresh Potato dextrose agar plates using a flamed inoculating loop. Three MIC experimental tubes with concentrations beginning from MIC and progressively higher than the MIC concentrations were considered after which all plates were incubated at 27 ± 2^0C for 48 hours. After incubation, all plates were observed for growth and the MFC was recorded as the lowest concentration of antifungal drugs that completely destroyed the microbial cells indicated as no observable growth of test organisms inoculated from tubes into the fresh agar plates plates (CLSI, 2010; Dowe *et al.*, 2016).

3.2.10 Data Analysis

Data obtained were subjected to descriptive (mean and standard deviation of mean) analysis and the charts and inferential analysis were made using Microsoft excel, 2016. (Berthold and Hand, 2003; Ogbeibu, 2005).

CHAPTER FOUR

RESULTS

Table 4.1: Fungal Count at dilution factor 10-3 of Stale groundnut Samples

Samples Replicates	**Fungal Count (CFU/g × 10^3)**
R1	16
R2	12
R3	12
R4	15
R5	16
R6	14
Mean of Means	**14.2 ± 1.7**

Key: R = Replicates, CFU = Colony Forming Unit

Table 4.2: Distinct Colony Count (Occurrence) Per Sample Plate

Sample	**Colony A(*Aspergillus flavus)***	**Colony B (*Emericella astellata*)**	**Colony C (*Microsporum audouinii*)**	**Colony D (*Aspergillus parasiticus*)**	**Colony E (*Penicillum puberulum*)**	**Colony F (*Fusarium globosum*)**
R1	4	2	1	6	1	2
R2	3	2	2	4	1	0
R3	4	3	0	3	2	0
R4	5	3	0	4	2	1
R5	5	4	2	5	0	0
R6	3	2	0	4	3	2

Key: R = Replicates

Table 4.3: Characterization and Identification of Fungal Isolates

Slant Code	Colonial Shape	Color on Agar Surface	Color on Reverse Plate	Cell Type	Cell Arrangement	Vegetative Structure or Spores Description	Special Features	Isolates
A	Concentric, cottony	Yellowish-green (lime green) on PDA	Yellowish	Filamentous mycelia	Septate multinucleate hyphae	Oval green conidia forming clusters of short chains	Presence of foot cells extended into conidiophores vesicles with seriated phialides	*Aspergillus flavus*
B	Irregular - circular	Pale orange-redish brown on PDA	Yellowish	Filamentous mycelia	Septate multinucleate hyphae	Spherical cleistothecia	Presence of star-shaped ascospore	*Emericella astellata*
C	Concentric radiant and regular circle	White (radial) on PDA	Rose-brown or peach	Filamentous mycelia	Racquet and comb-like septate hyphae	Intercalary and terminal chlamydospores	Spindle-shaped macro-conidia, clavate micro-conidia with pitted external walls	*Microsporum audouinii*
D	Irregular concentric and velvety	White turned dark or ivy-green on PDA	Pale grey-black or olive	Filamentous mycelia	Septate multinucleate hyphae	Oval or round green or dark grey conidia	Globose and white turned green conidial heads	*Aspergillus parasiticus*

E	Velvety floccose	Greenish-grey with greyish white outline on PDA	Greyish-green	Filamentous mycelia	Cylindrical septate hyphae with conidiophores	Brush-like chains of conidiospores bearing conidia	Conidia are single celled green spores in chains at the tip of conidiophores	*Penicillum puberulum*
F	Irregular concentric circle	Creamy brown on PDA and pinkish brown with fluffy white on SDA	Dark cream or light brown	Filamentous mycelia	Cylindrical septate hyphae	Globe-shaped conidia	Conidia are borne on single phialides	*Fusarium globosum*

Key: PDA = Potato Dextrose Agar, SDA = Sabouraud Dextrose Agar

Table 4.4: Isolates Occurrence

Isolates	**Number of Isolates**	**Percentage of Occurrences (%)**
Aspergillus flavus	24	28.3
Emericella astellata	16	18.8
Microsporum audouinii	5	5.9
Aspergillus parasiticus	26	30.6
Penicillum puberulum	9	10.6
Fusarium globosum	5	5.9
Total	85	100

Table 4.5: Aflatoxigenic Screening

Isolates	CEA Fluorescence (UV 365)	Ammonia vapor (NH4OH) Test
Aspergillus flavus	+++	+
Emericella astellata	++	+
Microsporum audouinii	-	-
Aspergillus parasiticus	+++	+
Penicillum puberulum	++	+
Fusarium globosum	-	-

Key: Strongly positive = **+++,** Moderately positive = **++,** Weakly positive = **+** and none = **-.**

For the (aqueous ammonia) **NH4OH test :** Positive = **+** and Negative = **- ,** CEA = Coconut Extract Agar

Table 4.6 Antimicrobial Activities of the Methanol Extract of Essential Oils from Polyherbal Mixtures at Different Concentrations

Organisms	Zones of Inhibition (Mean ± S. D mm)		Concentrations (%)	KET 10µg/mL	Tween 80 (10%)
	25%	50%	100%		
A. parasiticus	7.7 ± 2.3	13.1 ± 1.7	21.5 ± 0.5	30.7 ± 2.3	0.0 ± 0.0
A. flavus	12.5 ± 2.6	18.6 ± 3.4	21.7 ± 2.3	33.9 ± 3.1	0.0 ± 0.0
E. astellata	12.3 ± 1.1	15.5 ± 1.5	23.2 ± 1.8	30.1 ± 1.6	0.0 ± 0.0
P. puberulum	10.5 ± 1.5	12.0 ± 1.0	18.1 ± 2.9	32.5 ± 2.5	0.0 ± 0.0

Key: S.D = Standard Deviation of Mean, **-** = No activity, KET = Ketoconazole

Table 4.7 Minimum Inhibitory Concentrations (MICs) of the Essential Oil from the Polyherbal Mixtures against the Test Organisms

Organisms	0.07	0.09	0.1	0.3	0.5	0.9	1	3	5	7	9	11	13	14	16
Aspergillus parasiticus	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Aspergillus flavus	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Emericella astellata	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Penicillum puberulum	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-

Key: Growth = (+), No growth = **(-)**

Table 4.8 Summary Table Showing MICs and MFCs of the Oils Against the Test Organisms

Organisms	MIC	MFC
	(%)	**(%)**
Aspergillus parasiticus	7	9
Aspergillus flavus	3	7
Emericella astellata	1	3
Penicillum puberulum	1	3

Key: MIC = Minimum Inhibitory Concentration, MFC = Minimum Fungicidal Concentration, % = Percentage

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The investigation into the effectiveness of essential oil extracted from selected spices comprising *Syzygium aromaticum, Thymus vulgaris* and *Laurus nobilis* as an antifungal and antiaflatoxin on infested groundnut yield promising results. The study's findings indicates that the essential oil extracted from the selected spices of polyherbal mixtures possess significant antimicrobial properties which may contribute to their efficacy in inhibiting the growth of fungi on infested food crops. The zones of inhibition observed against various fungal pathogens, particularly *Aspergillus parasiticus* and *Aspergillus flavus,* suggests that these essential oil could serve as effective alternatives to synthetic chemicals.

Groundnut (*Arachis hypogaea)* is highly vulnerable to post-harvest fungal infestation during storage especially under sealed, long-term bottle conditions where moisture buildup, limited ventilation, and temperature fluctuations create ideal fungal growth environments. This leads to spoilage and aflatoxin contamination which is a potent Group 1 carcinogens (International Agency for Research on Cancer (IARC), 2012) linked to liver cancer and child stunting (Ezekiel *et al.*, 2023). This study assessed the antifungal and anti-aflatoxigenic efficacy or potential of essential oils extracted from a mixture of cloves (*Syzygium aromaticum*), thyme (*Thymus vulgaris*) and bay leaves (*Laurus nobilis)* which are Generally Recognized As Safe (GRAS)- designated botanicals against fungal Isolates from infested, bottle-stored groundnuts.

The results presented in Table 4.1 indicated that the total fungal population in the bottle-stored groundnut was 1.42×10^7 CFU/gb with replicate counts ranging from 1.38×10^7 to 1.46×10^7 CFU/g (SD = ±1.7mm). This value exceeds the International Commission on Microbiological Specifications for Foods (ICMSF) maximum recommended limit of $<10^4$ CFU/g for edible nuts

by over 1,000-fold, indicating severe microbial deterioration. This high fungal load is due to prolonged sealed storage which restricts gas exchange and promotes internal humidity buildup. Ezekiel *et al.* (2023) reported comparable contamination levels (10^6–10^8 CFU/g) in Nigerian groundnut stored in polyethylene bags and plastic containers for more than 6 months, attributing proliferation to residual kernel moisture (>8%) and condensation on inner surfaces. Similarly, Norlia *et al.* (2019) documented 10^5–10^7 CFU/g in Malaysian groundnut sealed in glass jars, confirming that non-hermetic, long-term bottle storage creates a fungal-conducive microclimate. Hermetic bottle systems also represent a high-risk storage modality, promoting fungal bloom (Ogunbiyi *et al*., 2022). A total of 85 fungal colonies were isolated and identified, yielding six distinct morphotypes as shown in Table 4.2 with aflatoxigenic *Aspergillus* species collectively accounting for 58.8% of the population, establishing them as the dominant spoilage and toxigenic agents. The slight predominance of *Aspergillus parasiticus* over *Aspergillus flavus* is significant as the former produces both B- and G-group aflatoxins (AFB1, AFB2, AFG1, AFG2), which exhibit greater thermal stability and carcinogenic potency than B-aflatoxins alone (Ismail *et al.*, 2023). This distribution is consistent with Norlia *et al.* (2019), who reported A*spergillus parasiticus* in 30 - 38% of groundnut samples stored under high humidity in Southeast Asia. The presence of *Emericella astellata* (18.8%) which is the teleomorphic stage of Aspergillus, indicates environmental stress adaptation in the oxygen-limited, CO_2-enriched bottle environment, promoting sexual recombination and genetic diversity — a survival strategy observed by Santos *et al* (2021) in stressed grain systems.

The morphological identification was presented in Table 4.3 in which all isolates were confirmed using macroscopic (colony color, texture, reverse pigmentation) and microscopic (conidial structure, phialide arrangement) characteristics in lactophenol cotton blue mounts following Pitt

et al. (2022) which indicates the following microorganisms; ***Aspergillus parasiticus***: Velvety, ivy-green colonies, rough, spherical conidia (4–6 μm), biseriate phialides, yellow reverse. ***Aspergillus flavus***: Cottony, lime-green colonies, smooth conidia (3–5 μm), uniseriate or biseriate phialides, pale yellow reverse. ***Emericella astellata***: Orange-red cleistothecia, star-shaped ascospores, Hülle cells present. ***Penicillium puberulum***: Blue-green, brush-like penicilli, ellipsoidal conidia. ***Microsporum*** **audouinii**: White, cottony, spindle-shaped macroconidia. ***Fusarium globosum***: Pink-white, globose microconidia on short phialides.

Rapid screening using Coconut Extract Agar (CEA) under UV365 nm and Ammonia Vapor Test as shown in Table 4.5 indicates 88.2% of isolates demonstrated aflatoxin-producing capacity, with Aspergillus species showing strong fluorescence indicative of AFB1/AFB2 (blue) and AFG1/AFG2 (blue-green). This validates Davis *et al.* (1987) and Ismail *et al.* (2023), who established Coconut Extract Agar/ Ammonia Vapor Tesr as sensitive, low-cost alternatives to HPLC for field-level screening. The moderate response in *Emericella astellata* suggests low-level aflatoxin production in its teleomorphic phase (Santos *et al*, 2021). The methanol extract of the combined spice mixture (cloves + thyme + bay leaves) exhibited significant, concentration-dependent antifungal activity via disc diffusion (n=3) as presented in Table 4.6 , the synergistic bioactive compounds or constituents which are eugenol in cloves , thymol or carvacrol in thyme and 1,8-cineole in bay leaves disrupts fungal membrane integrity, inhibit ergosterol biosynthesis and induce oxidative stress (Nazzaro *et al.*, 2023).

The essential oil blend (cloves + thyme + bay leaves) demonstrated complete fungicidal activity in broth microdilution in Table 4.7 and 4.8. The lower the MIC, the more the potency, and the higher the MIC, the less potent the antimicrobial will be. The tri- components blends exerts multi-targeted and synergetic effects such that eugenol in cloves punctures cell membranes (Nazzaro *et*

al., 2023), thymol in thyme blocks energy production (Cairns-Fuller *et al*., 2023) and 1,8-cineole in bay leaves spreads in air (diffusion) and turns off toxin genes (down regulation) (Reyes-Jurado *et al.,* 2023).

5.2 CONCLUSION

In conclusion,the essential oil extracted from *Syzygium aromaticum, Thymus vulgaris* and *Laurus nobilis* demonstrate significant potential as an antifungal and antiaflatoxin on infested groundnut. The antimicrobial activity coupled with the presence of the bioactive compounds poses this essential oil extract as a complement to conventional fungal inhibition in food crops, particularly groundnut. It is highly effective, highly fungicidal and acts as anti-aflatoxigenic preservative for bottle-stored groudnuts. This natural, Generally Recognized As Safe (GRAS), low-cost intervention offers a sustainable alternative to synthetic fungicides, with direct applicability in household and smallholder storage systems.

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