ANTIFUNGAL EFFECTS OF *Syzygium aromaticum* (CLOVES) ON FUNGI ISOLATES

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THIS PROJECT IS SUBMITTED TO:
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SUPERVISOR:

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CERTIFICATION

This is to certify that this project work was carried out by **NNABUENYI VALENTINE ONYEKACHI** with matriculation number **BMS2005042** in partial fulfilment of the requirements for the award of Bachelor of Medical Laboratory Science (BMLS) from the University of Benin, Benin City, Edo State, Nigeria.

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DEDICATION

This work is dedicated to my God who is the source of all knowledge and wisdom and to my wonderful parents and siblings for their unwavering love and support.

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I acknowledge the all-wise, all-knowing, all-intelligent, and Almighty God from whom wisdom and knowledge come. I recognise the professional supervision given to me by my supervisor, Dr. (Mrs) Anne o. Itemire, during this work. Special gratitude to the Head of Department, Dr. (Mrs) Z. Omoruyi for creating a conducive environment for learning. To all my wonderful lecturers, it was indeed a privilege to learn under your supervision. I appreciate the Head of the Department of Pharmaceutical Microbiology's permission to use the laboratory. I sincerely appreciate Mr John Uwadia, who guided and assisted me during this research work in the Laboratory. I want to specially appreciate my parents Mr and Mrs Wilfred and Beatrice Nnabuenyi for their unwavering support, encouragement and belief in me, from their sacrifices to their words of wisdom, I am grateful for their guidance and patience throughout this research work. I also want to extend my heartfelt thanks to my siblings, Chidera, Ifeoma, Uchenna and Kelechi for their support in both big and small ways, and for their constant encouragement, love and understanding. I also want to appreciate Rev. Fr. Okigbo, Mr. Sunday Nnabuenyi, Mr. Cyprain Nnabuenyi, Mr. Chiedozie Nnabuenyi, Mr. Obiora Nnabuenyi, Chisom Eze, my Grandparents and everyone who has stood by me throughout this Journey. Special appreciation goes to all my friends, Nathan, Prosper, Taiwo, Victoria, Abundance,Noble, Laura, Success, Chiamaka, Chinedu, Sunday, Shedrack and all my coursemates, May God continue to bless and protect you all

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ABSTRACT

The emergence of antifungal resistance necessitates the exploration of plant-based alternatives. This study investigated the antifungal activity of *Syzygium aromaticum* (clove) extracts prepared by Soxhlet and maceration methods. Clove buds were authenticated, extracted using ethanol–water (1:1), and tested against 12 fungal isolates, including *Candida albicans*, *Aspergillus niger*, *Penicillium* spp., and *Fusarium* spp. Antifungal susceptibility was assessed using agar well diffusion, while minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined by agar dilution. Ketoconazole served as the standard reference. Results showed concentration-dependent inhibition, with activity evident from 50 mg/ml. *Candida albicans* was the most sensitive organism, with inhibition zones up to 17.66 mm at 400 mg/ml, while *Penicillium* showed the least susceptibility. Maceration extracts consistently demonstrated higher efficacy than Soxhlet, yielding larger inhibition zones and lower MIC values (41.66 ± 12.12 µg/ml vs. 200.00 ± 145.5 µg/ml for *Candida albicans*). MFC assays confirmed fungicidal activity of maceration extracts at 50 µg/ml for *Candida albicans*, compared to higher concentrations required for other fungi. These findings indicate that *Syzygium aromaticum* possesses notable antifungal properties, particularly against *Candida albicans*, with maceration proving the more effective extraction method. The results provide scientific support for clove's traditional use and suggest its potential as a natural antifungal agent.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Fungal infections remain a persistent global health challenge, particularly among immunocompromised individuals and in agricultural systems vulnerable to phytopathogenic fungi. The growing incidence of antifungal resistance, especially among pathogens such as *Candida*, *Aspergillus*, and *Fusarium*, has catalyzed a renewed interest in plant-based therapeutics. Among these, *Syzygium aromaticum*, commonly known as clove, has emerged as a significant natural remedy due to its high eugenol content and broad-spectrum antimicrobial properties (Hiwandika and Sudrajat, 2021). Recent studies have substantiated the fungicidal effects of clove essential oil against diverse fungal strains. Atif *et al*. (2024) demonstrated its strong inhibitory activity against *Fusarium oxysporum* f. sp. *lycopersici*, a major plant pathogen, emphasizing its dual role in both clinical and agricultural biosecurity. Complementing this, Syib'li and Abadi (2024) found clove extract effective against *Fusarium oxysporum* in tomato crops through cutinase enzyme inhibition, confirming its mode of antifungal action. These findings are particularly significant given the increasing resistance of such fungi to synthetic fungicides. Moreover, clove oil's mechanism of action involves the disruption of fungal membranes, interference with enzyme function, and inhibition of sporulation, which collectively contributes to its antifungal potency (Das *et al*., 2022). The bioactive compounds, especially eugenol, are known to generate oxidative stress within fungal cells, compromising their integrity and viability. In broader therapeutic contexts, these phytochemicals have also shown potential in synergistic formulations, potentially reducing the need for high-dose antifungal drugs and thereby mitigating adverse effects (Das *et al*., 2022).

1.2 Statement of the Problem

Fungal infections caused by species such as *Candida*, *Aspergillus*, and *Fusarium* have become increasingly problematic in clinical and agricultural settings (Hiwandika and Sudrajat, 2021). This concern is amplified among immunocompromised populations, including individuals with HIV/AIDS and cancer, where systemic mycoses contribute to high morbidity and mortality (Das *et al*., 2022). In agriculture, phytopathogenic fungi like *Fusarium oxysporum* continue to reduce crop yields and threaten food security (Syib'li and Abadi, 2024). While synthetic antifungal agents are widely used, their prolonged use has been linked to adverse effects, toxicity, and the development of resistant fungal strains (Atif *et al*., 2024). Additionally, environmental risks associated with chemical fungicides demand the exploration of eco-friendly alternatives (Das *et al*., 2022). This has led to increased interest in natural antifungal compounds, particularly those derived from medicinal plants such as *Syzygium aromaticum* (Hiwandika and Sudrajat, 2021). Clove oil, rich in eugenol, has shown promising antifungal properties against a variety of fungi including *Fusarium* and *Candida* spp., both in clinical and agricultural isolates (Atif *et al*., 2024.). Despite these findings, gaps remain in understanding its precise mechanisms of action, effectiveness across different fungal strains, and potential for integration into standardized treatment or agricultural protocols (Das *et al*., 2022).

1.3 Justification of the Study

The global rise in antifungal resistance, particularly among *Candida species*, *Aspergillus spp., and Fusarium spp.,* underscores the urgent need for alternative therapies derived from natural products (Biernasiuk *et al*., 2022). Clove essential oil (*Syzygium aromaticum),* due to its primary

active compound eugenol, has gained considerable attention for its broad-spectrum antifungal activity and low toxicity profile. Research has shown its fungicidal effect against *Candida spp*. both as a monotherapy and in synergistic formulations with conventional antimycotics, indicating its potential for clinical integration (Biernasiuk *et al*., 2022). Furthermore, in plant pathology, clove oil has shown effectiveness in combating fungal infections like wheat common bunt, supporting its utility in agricultural biocontrol and food preservation strategies (Valente *et al*., 2023). These findings establish a dual relevance of clove oil in both medical and environmental health contexts. Given the escalating clinical burden of fungal infections, particularly in immunocompromised populations, and the growing resistance to synthetic antifungal drugs, it is scientifically and ethically justified to investigate clove oil as a cost-effective, eco-friendly, and potent antifungal agent. This study seeks to fill the knowledge gap regarding the therapeutic application and mechanism of action of clove oil, contributing to the development of sustainable antifungal strategies.

1.4 Aim of the Study:

The primary aim of this study is to evaluate the antifungal activity of *Syzygium aromaticum* (Clove*)* essential oil on selected fungi isolates.

1.5 Specific Objectives:

The specific objectives of this study are;

1. to extract and prepare clove essential oil using appropriate distillation or commercial sourcing methods.
2. to assess the in vitro antifungal activity of clove essential oil against the isolated fungi using standardized assays such as the disc diffusion and broth microdilution methods.

3. to compare the antifungal effectiveness of clove essential oil with that of conventional antifungal agents.
4. to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of clove essential oil against each fungal isolate.

1.6 Research Questions

1. Does *Syzygium aromaticum* (Clove*)* essential oil exhibit antifungal activity against the isolated fungal species?
2. How does the antifungal activity of clove essential oil compare with that of conventional synthetic antifungal agents?
3. What are the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of clove essential oil for each fungal isolate?

1.7 Research Hypotheses

1.7.1 Null Hypotheses (H_0):

1. **H_0:** *Syzygium aromaticum* (Clove) essential oil does not exhibit antifungal activity against the isolated fungal species.
2. **H_0:** There is no significant difference between the antifungal activity of clove essential oil and that of conventional synthetic antifungal agents.
3. **H_0:** Clove essential oil does not exhibit specific or consistent minimum inhibitory concentration (MIC) or minimum fungicidal concentration (MFC) values against the fungal isolates.

1.7.2 Alternative Hypotheses (H_1):

1. **H_1:** *Syzygium aromaticum (Clove)* essential oil exhibits significant antifungal activity against the fungal species isolated from the samples.
2. **H_1:** Clove essential oil demonstrates significantly greater or comparable antifungal activity when compared to conventional synthetic antifungal agents.
3. **H_1:** The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of clove essential oil are significantly effective and vary across different fungal isolates.

1.8 Scope of the Study

The scope of the study is limited to in vitro experiments and does not extend to in vivo studies or clinical trials. It also does not include a toxicological assessment of clove oil or an in-depth phytochemical analysis of its components.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Origin and Distribution of *Syzygium aromaticum*

Syzygium aromaticum, commonly known as clove, is widely recognized as a spice of significant economic and cultural importance, with a rich historical and geographical origin. The primary origin of this species is traced to the Maluku Islands (historically known as the Spice Islands) in Indonesia. These islands remain a key center for clove biodiversity and genetic resources today. Recent research by Mahulette *et al*. (2022) identified three major distribution areas in Maluku Ambon, Seram, and Buru Islands highlighting substantial morphological diversity among local clove varieties. Supporting this, Marasabessy *et al*. (2024) conducted a phylogenetic study on clove populations in Haya Village on Seram Island, identifying five distinct accessions, including wild types. This suggests not only a historical but an ongoing diversification within its native habitat. Furthermore, Khan (2022) emphasized that while clove is native to Indonesia, its cultivation has expanded to tropical and subtropical regions worldwide, where it is known by various local names. This global dispersal is closely tied to the spice trade routes established as early as 300 BC, when Indonesia drew traders from Arabia, China, and Europe (Otunola, 2022).

2.1.1. Botanical Description of *Syzygium aromaticum* (Clove)

Syzygium aromaticum (L.) Merr. and L.M. Perry, commonly known as clove, is a medium-sized evergreen tree belonging to the family Myrtaceae. It typically grows to a height of 10–20 meters. The tree has a straight trunk with grayish bark and a dense canopy. The leaves are opposite, simple, and oblong-lanceolate in shape with a leathery texture and aromatic oil glands characteristics common to many Myrtaceae members (Bermawie *et al*., 2025). The clove tree is best known for its aromatic flower buds, which are the primary commercial product. These buds

are initially pale and gradually turn green and then bright red when ready for harvest. Botanically, the buds consist of a long calyx that ends in four spreading sepals and four unopened petals forming a small ball at the center. The flower buds are harvested before they bloom and are dried for use as a spice (Ayushi and Danish, 2021). In terms of reproductive structures, *S. aromaticum* flowers are actinomorphic, bisexual, and borne in terminal clusters. The fruit is a purple drupe, which contains a single seed. The essential oils responsible for its strong aroma and medicinal value are concentrated in the buds, leaves, and stems (Ramadan, 2022).

Figure 2 .1 Buds and leaves of *Syzygium aromaticum* (Ayushi and Danish, 2021).

2.1.2. Historical and Traditional Uses of *Syzygium aromaticum* (Clove)

According to Otunola (2022), clove has held cultural and medicinal significance in many civilizations for over 2,000 years. It was widely used in ancient diets and healing practices, often as a treatment for dental pain, respiratory infections, and gastrointestinal issues. Its essential oil, rich in eugenol, has historically served as a local anesthetic and antiseptic, particularly in traditional dentistry. Ramadan (2022) further documents clove's use in Ayurveda and Unani systems as a natural remedy for inflammation, nausea, and colds. The book also discusses its ceremonial and preservative functions, particularly in Islamic and South Asian cultures. Sarker and Islam (2022) conducted a comparative ethnomedicinal review of *Syzygium aromaticum* and *Ocimum sanctum*, validating the historical knowledge of clove as a remedy for oral, gastrointestinal, and respiratory ailments. This study also notes its integration into herbal teas and food preparations in modern traditional practices. In addition, Vicidomini *et al*. (2021) highlight clove's traditional use in flavorings, perfumery, and medicine, linking its historical usage with current biomedical interest, especially for antiviral and anti-inflammatory applications.

2.1.3. Global and Regional Distribution Patterns of *Syzygium aromaticum* (Clove)

The Maluku Islands remain a genetic hotspot for clove diversity. In a study of three distinct clove-growing zones in Maluku (Ambon, Buru, and Seram Islands), Mahulette *et al*. (2022) documented notable morphological variation, reflecting regional diversification and local ecological pressures. Clove cultivation is now widespread across tropical Asia, East Africa (notably Zanzibar and Madagascar), and parts of South America. In Indonesia, particularly East Java and South Sulawesi, clove remains an essential crop with both economic and ecological importance. A study by Setiawan *et al*. (2021) found that mixed-culture plantations in Java

support biodiversity while enhancing clove yield. Similarly, Yusuf *et al*. (2023) assessed land suitability in South Sulawesi, identifying high potential for expanding sustainable clove farming in humid tropics.

2.2. Ethnobotanical and Medicinal Importance of *Syzygium aromaticum* (Clove)

Clove has been widely used in traditional medicine for its analgesic, anti-inflammatory, antimicrobial, and digestive properties. It features prominently in the ethnopharmacological systems of North Africa, where it has long been applied as a remedy for asthma, dental infections, respiratory ailments, and diabetes (Fakchich *et al*., 2024). In Morocco, its continued use reflects a deep-rooted traditional knowledge base that persists even in modern herbal therapies. Otunola (2022) emphasized clove's role not only as a culinary spice but as a foundational medicinal plant in indigenous medical systems. Its applications span oral care (especially toothache relief), digestive enhancement, and use as a natural preservative. A broader review by Ramadan (2022) documented the pharmacological basis for clove's therapeutic uses, noting its efficacy in preventing oxidative stress, modulating inflammation, and combating microbial infections. Eugenol, the main bioactive compound, underlies much of clove's pharmacodynamic profile. Further ethnobotanical evidence was gathered in a 2023 study in Swat Valley, Pakistan, where clove was among the aromatic medicinal plants commonly used by traditional herbalists for respiratory issues and as an antiseptic (Ali *et al*., 2023). In a complementary report, Yadav *et al*. (2022) elaborated on clove's widespread integration in Indian traditional medicine, including its applications in Ayurveda for treating headaches, colds, and inflammatory disorders.

2.2.1. Cultural and Traditional Applications of *Syzygium aromaticum* (Clove)

Clove is widely utilized in ceremonial and religious contexts across Asia and North Africa. For instance, in Moroccan traditional medicine, clove is a culturally endorsed remedy for respiratory illnesses, digestive complaints, and even spiritual cleansing, demonstrating its dual role in health and cultural symbolism (Ramadan, 2022). Similarly, clove has long been incorporated into incense offerings and dental rituals in Southeast Asia, particularly in Indonesian and Indian cultures, where it is burned or chewed for purification and fresh breath (Otunola, 2022). A review by Sarker and Islam (2022) highlights how clove remains a staple in Ayurvedic and Unani practices, often blended into decoctions and pastes for pain relief, digestive support, and respiratory treatment. The same study notes clove's usage as a fumigant or crushed spice in cultural postpartum care and rituals. Moreover, in Armenian and Middle Eastern traditions, clove continues to be infused in hot beverages and desserts not only for its aromatic profile but also for its perceived protective properties. A 2025 review by Sargsyan *et al*. (2025) extends this understanding by linking clove's neuroprotective roles to its traditional reputation for enhancing memory and focus in folklore.

2.2.2. Therapeutic Uses of *Syzygium aromaticum* in Infectious Diseases

A study by Otunola (2022) emphasized clove's broad-spectrum antimicrobial efficacy, particularly in the treatment of oral infections, gastrointestinal disturbances, and respiratory pathogens. Eugenol was found to disrupt bacterial cell walls and inhibit enzymatic systems in *Staphylococcus aureus* and *Escherichia coli.* Sarker and Islam (2022) compared clove with *Ocimum sanctum*, showing that clove had superior antifungal activity against *Candida albicans* and dermatophytes, making it suitable for fungal skin infections and oral thrush. It also exhibited synergistic effects when combined with conventional antifungals. Further, Yadav *et al*. (2022)

reported that clove extract significantly inhibited the replication of RNA viruses, including influenza and SARS-related strains, through downregulation of viral protease activity supporting its traditional use in flu and cough treatments.

2.2.3. Antifungal Applications of *Syzygium aromaticum* (Clove) in Traditional Medicine

A key 2021 study by Hiwandika and Sudrajat (2021) confirmed that clove extracts exhibit substantial inhibitory effects against both fungal and bacterial strains, including *Candida albicans*. The authors noted that traditional applications such as decoctions or poultices retain practical value in rural healthcare systems, especially in Indonesia where clove is native. Further evidence was provided by Mostafa *et al*. (2023), who explored different solvent extracts of clove. Their phytochemical screening linked antifungal action to compounds like eugenol, flavonoids, and tannins, which are abundant in traditional preparations. They emphasized clove's effectiveness against dermatophytes and opportunistic fungal pathogens. In the context of resource-limited healthcare settings, clove's antifungal potential is even more valuable. Traditional healers and community herbalists often use clove infusions for treating fungal skin infections, athlete's foot, and candidiasis, typically by applying it as an infused oil or water-based rinse (El-Saber Batiha *et al*., 2020).

2.3. Phytochemistry and Bioactive Components of *Syzygium aromaticum* (Clove)

A comprehensive review by Xue *et al*. (2022) highlighted that the nutritional and bioactive richness of clove makes it a key functional spice. Eugenol accounts for over 70% of the essential oil, contributing to its antimicrobial, anti-inflammatory, and antioxidant activities. Similarly, Mostafa *et al*. (2023) conducted solvent-based extraction and found that ethanolic and acetonic extracts yielded the highest concentrations of eugenol (46.53% and 38.79%, respectively). These

extracts also showed strong antiproliferative and antifungal activity. In a 2025 study, Yang *et al*. (2025) examined the less-utilized floral parts of clove and identified a similar spectrum of phytochemicals, reinforcing that multiple parts of the plant have medicinal value. The research emphasized sustainability by proposing broader applications for these underutilized components. Benmakhlouf *et al*. (2022) applied GC-MS analysis to characterize clove's essential oil and confirmed the presence of over 20 volatile compounds. Notably, besides eugenol, significant levels of eugenyl acetate, α-humulene, and methyl salicylate were identified, all contributing to its therapeutic effects. The immunomodulatory and cytotoxic potential of clove extracts was further studied by El Faqer *et al*. (2022), who showed that aqueous and ethanolic extracts as well as essential oil modulated cytokine responses and exhibited selective cytotoxicity in vitro supporting their utility in inflammatory and infectious disease settings.

2.3.1. Key Phytochemicals in *Syzygium aromaticum* (Clove): Eugenol, Caryophyllene, and Others

Modern phytochemical investigations reinforce the central role of *eugenol* as the dominant active compound in *Syzygium aromaticum*, comprising approximately 70–85% of clove essential oil. Eugenol exerts strong antioxidant, anesthetic, antimicrobial, and anti-inflammatory effects, making it the cornerstone of clove's medicinal properties (Mostafa *et al*., 2023). In addition to eugenol, β-caryophyllene and eugenyl acetate are consistently identified as significant constituents. These sesquiterpenes and phenolic esters contribute to the oil's cytotoxic, antifungal, and anti-inflammatory activity. For example, El Faqer *et al*. (2022) reported that clove essential oil contained 78.67% eugenol, 11.77% eugenyl acetate, and 6.85% β-caryophyllene, based on GC-MS analysis. Gas chromatography-mass spectrometry (GC-MS) profiling conducted by Benmakhlouf *et al*. (2022) confirmed additional compounds like α-

humulene, methyl salicylate, and terpineol, which enhance the oil's therapeutic versatility. These phytochemicals collectively exhibit broad-spectrum antimicrobial effects and support immune modulation. Sarker and Islam (2022) emphasized eugenol's dual role in traditional and modern applications serving both as an analgesic and a disinfectant while β-caryophyllene also showed promise as a selective CB2 receptor agonist, giving it potential in managing inflammatory and neurodegenerative disorders.

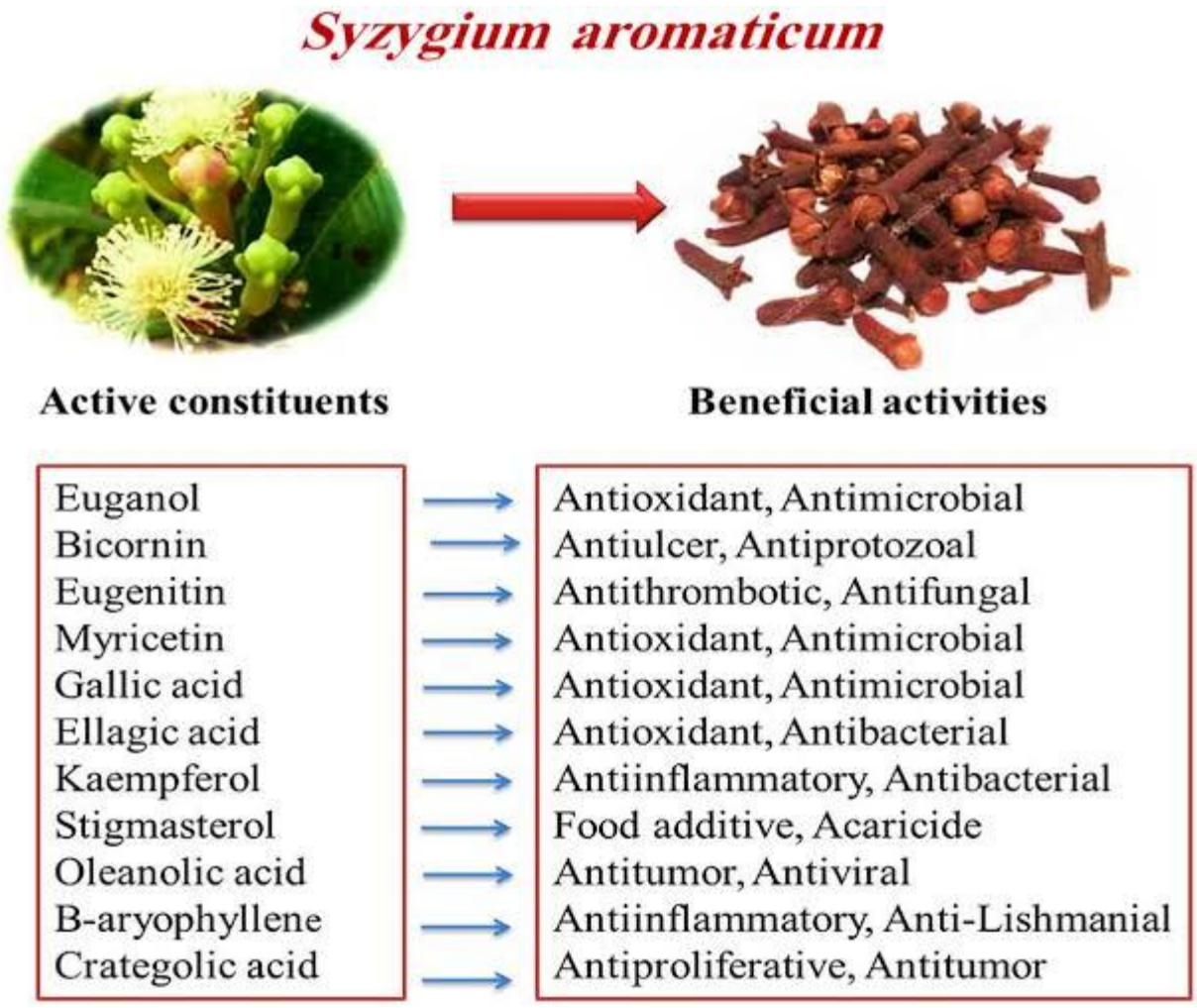

Figure 2 .1 Bioactive Components of *Syzygium aromaticum* (Mostafa *et al*., 2023).

2.3.2. Mode of Action of Eugenol on Microbial Cells

A 2021 study by Jeyakumar and Lawrence (2021) demonstrated that eugenol exerts its bactericidal effects primarily through membrane disruption. When applied to *Escherichia coli*, eugenol caused extensive damage to the bacterial cell membrane, increasing permeability and facilitating leakage of vital intracellular components. This interference disrupts cellular metabolism and ultimately leads to cell death. Complementary findings were published by Ulanowska and Olas (2021), who reviewed eugenol's multifaceted antimicrobial mechanisms. Besides its membrane-destabilizing role, eugenol was shown to inhibit bacterial biofilm formation a key factor in chronic and drug-resistant infections. This disruption impairs the pathogen's ability to adhere to surfaces and reduces its virulence. Furthermore, the non-specific mechanism of action makes eugenol effective across a wide range of microbes, including Gram-positive and Gram-negative bacteria. Its ability to interfere with the proton motive force and depolarize the membrane potential was also highlighted as a key feature in overcoming microbial resistance mechanisms (Ulanowska and Olas, 2021).

2.3.3. Extraction Techniques and Standardization of Clove Essential Oil (CEO)

Hydro-distillation remains one of the most commonly used traditional techniques for extracting clove essential oil. This method, despite its simplicity and cost-effectiveness, requires high energy input and extended processing time. However, it continues to be validated by researchers for its ability to yield high concentrations of eugenol. For instance, Benmakhlouf *et al*. (2022) reported that hydro-distillation produced clove oil with a eugenol concentration of approximately 78.67%, confirming its efficiency in preserving key volatile compounds essential to the oil's bioactivity. In contrast, solvent-based extraction methods particularly using ethanol, methanol, and acetone have been explored for their ability to recover a broader range of phenolic

constituents. Mostafa *et al*. (2023) demonstrated that ethanol and acetone were especially effective, yielding 46.53% and 38.79% eugenol, respectively. These methods are widely used in laboratory settings and traditional medicine, where the solvents can extract both volatile and non-volatile phytochemicals. However, solvent residues, toxicity, and environmental concerns limit their use in food and pharmaceutical applications without further refinement or purification steps. Emerging green technologies such as supercritical CO_2 extraction offer an eco-friendly alternative that preserves the integrity of heat-sensitive components while producing solvent-free oils. Although relatively underutilized in clove-specific studies, the technique shows great promise due to its selectivity, speed, and suitability for high-purity applications. Standardization of clove essential oil is a crucial step following extraction, especially given the variability in oil composition due to geographic, seasonal, and methodological factors. Gas Chromatography-Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC) are the primary tools used to analyze and quantify the chemical constituents. El Faqer *et al*. (2022) applied GC-MS to determine consistent ratios of eugenol, eugenyl acetate, and β-caryophyllene in different extracts, establishing chemical fingerprints necessary for product quality control. Similarly, Sarker and Islam (2022) emphasized that extraction efficacy should be correlated with pharmacological bioactivity during standardization to preserve the functional benefits of the oil.

2.4. Antifungal Properties of Clove Essential Oil (CEO)

One of the most recent findings was reported by Hiwandika and Sudrajat (2021), who evaluated the antifungal efficacy of CEO against *Candida albicans*, *Aspergillus niger*, and *Trichophyton mentagrophytes*. The study revealed minimum inhibitory concentrations (MICs) of 0.32% for CEO and demonstrated significant cell membrane disruption as the underlying mode of action. Lesions and leakage in fungal cell walls were attributed to eugenol's ability to alter membrane

integrity and interfere with ergosterol synthesis. In another study, Mostafa *et al*. (2023) evaluated solvent-extracted clove oil and showed potent antifungal activity against several mycotoxigenic fungi, including *Fusarium* and *Aspergillus* species. The ethanolic and acetonic extracts exhibited the highest efficacy, indicating that extraction method significantly influences antifungal potency. Further analysis by El Faqer *et al*. (2022) confirmed CEO's immunomodulatory and antifungal effects on human cell lines. Their GC-MS analysis revealed eugenol content above 70%, which correlated with strong inhibitory effects on fungal proliferation in vitro. They emphasized CEO's role in disrupting hyphal extension and spore germination key targets for antifungal therapeutics.

2.4.1. Overview of In Vitro and In Vivo Studies on the Antifungal Properties of Clove Essential Oil (CEO)

In Vitro Evidence

In vitro assays remain foundational in evaluating CEO's antifungal potential. Studies have consistently shown CEO to be effective against fungi such as *Candida albicans*, *Aspergillus flavus*, *Penicillium citrinum*, and *Rhizopus nigricans*. For example, Mostafa *et al*. (2023) reported potent growth inhibition of several pathogenic fungi using solvent-extracted CEO, highlighting the critical role of eugenol concentration and solvent polarity in determining activity. Minimum inhibitory concentrations (MICs) reported across studies range from 0.1% to 0.5% depending on fungal species and oil formulation.

In Vivo Studies

In vivo models have increasingly confirmed these findings, offering translational relevance for therapeutic applications. A pivotal 2023 study by Ali and Ibrahim (2023) tested CEO against

Candida albicans–induced skin infections in mice. Results showed significant reductions in fungal load, inflammation, and lesion size compared to untreated controls, confirming CEO's antifungal and anti-inflammatory potential in topical applications. Histopathological examination supported the observed clinical recovery. Earlier controlled studies, such as that by Muñoz Castellanos and Amaya Olivas (2020), demonstrated CEO's efficacy in reducing *Fusarium oxysporum* colonization in plant roots under greenhouse conditions, thus proving its agricultural value. These results were further echoed in vapor-phase studies like those by Aguilar-González *et al*. (2015), where CEO inhibited *Botrytis cinerea* in strawberries both in vitro and in storage environments.

2.4.2. Mechanisms of Fungal Inhibition by Clove Essential Oil (CEO)

Recent research between 2021 and 2025 has significantly advanced our understanding of the mechanisms by which clove essential oil (CEO), primarily composed of eugenol, exerts its potent antifungal effects. These mechanisms involve both structural and biochemical disruptions in fungal pathogens, leading to cell death and inhibition of virulence traits. One major mechanism is cell membrane disruption. Eugenol's lipophilic structure allows it to integrate into the fungal cell membrane, destabilizing its lipid bilayer and increasing permeability. This causes efflux of vital cellular components and disrupts ionic balance. Hiwandika and Sudrajat (2021) confirmed this by observing cytoplasmic leakage and lysis in *Candida albicans* treated with CEO, attributing it to severe membrane deformation. In addition to physical disruption, CEO affects ergosterol biosynthesis, a key target in fungal membranes. Ergosterol is the fungal counterpart to cholesterol and is essential for membrane integrity and fluidity. Inhibition of its synthesis weakens the membrane, making it more susceptible to external stressors. This was echoed in a 2024 review by Maggini *et al*. (2024), who also noted that CEO reduced ergosterol

content, collapsing membrane structure in pathogenic fungi. Another key antifungal mechanism is inhibition of fungal biofilms and spore germination. Biofilms are protective matrices that enable fungal persistence in host tissues or on surfaces. Eugenol disrupts quorum sensing pathways, preventing fungal cells from organizing into biofilms. It also blocks hyphal extension and germ tube formation, crucial steps in pathogenicity. These actions limit the spread and colonization of fungi, especially species like *Aspergillus* and *Candida*. Further, CEO has demonstrated the ability to alter enzyme activity involved in fungal metabolism. The work of Behbahani *et al*. (2019) found that CEO inhibited fungal dehydrogenase enzymes, disrupting energy production and leading to growth arrest.

2.4.3. Synergistic Effects of Clove Essential Oil (CEO) with Other Essential Oils and Antifungal Drugs

A significant 2022 study by Biernasiuk *et al*. investigated eugenol, the principal component of CEO, in combination with fluconazole and amphotericin B. The *in vitro* results revealed that the combination not only enhanced the inhibition of *Candida* spp., but also demonstrated a strong synergistic interaction, lowering the minimum inhibitory concentration (MIC) of standard antifungal drugs and improving their spectrum of action. Further evidence was provided by Parker *et al*. (2022), who explored the efficacy of CEO against *Candida auris* a multidrug-resistant pathogen. Their findings showed that CEO, when combined with fluconazole or amphotericin B, exhibited either synergistic or additive effects in all tested strains. This supports CEO's utility in combination therapies against resistant fungal infections. Additionally, Worawong *et al*. (2023) demonstrated partial synergy between CEO and azole drugs in combating *Microsporum gallinae*, a dermatophyte. Their study emphasized that the co-application of CEO significantly reduced the effective dosage of azoles, providing an

opportunity to minimize side effects in clinical settings. On the phytochemical synergy side, Sethunga *et al*. (2023) examined the combined antifungal and antibacterial activity of CEO with other oils like cinnamon and ginger. Their results showed enhanced activity against fungal strains due to complementary mechanisms of action, especially against *Aspergillus niger* and *Candida albicans*. These findings affirm that combining CEO with other botanicals can generate robust antimicrobial mixtures with potential applications in food safety and topical formulations.

2.5. Common Fungal Pathogens in Clinical and Environmental Samples

Candida species especially *Candida albicans*, *C. auris*, and *C. glabrata* remain among the most commonly isolated fungal pathogens. Their prevalence is associated with bloodstream infections, mucosal colonization, and urinary tract infections. According to Gisi (2022), Candida species are among over 300 fungi recognized as opportunistic pathogens, particularly dangerous for immunocompromised hosts such as those undergoing chemotherapy or organ transplantation. Aspergillus species, notably *A. fumigatus* and *A. flavus*, are the dominant airborne fungi in both hospital and general indoor environments. These fungi cause pulmonary infections such as aspergillosis, especially in patients with respiratory conditions or neutropenia. Their spores are ubiquitous and highly adaptable, with the potential to become pathogenic when inhaled by vulnerable individuals. From an environmental perspective, *Cladosporium* and *Penicillium* species are frequently encountered in air, dust, and soil samples. Though often regarded as non-pathogenic, these molds can trigger allergies, asthma, and occasionally invasive infections. A panfungal PCR-based evaluation by Camp *et al*. (2020) highlighted the presence of these fungi in both clinical and environmental samples, cautioning against underestimating their clinical relevance. In healthcare-associated waste and clinical solid waste environments, a 2016 study by Noman *et al*. found *Aspergillus*, *Fusarium*, and *Rhizopus* species dominating contaminated

samples. This underscores the dual risk of fungal exposure and environmental contamination in medical facilities.

2.5.1. Mycological Techniques for Isolation and Identification of Fungal Pathogens

Traditional and Selective Culture Media

One of the notable contributions in this domain is the development of FastFung**,** a specialized culture medium designed to isolate fastidious fungi from clinical specimens. As reported by Bittar *et al*. (2021), FastFung enhances the growth of uncommon fungal species that are typically underdiagnosed using conventional media. This medium provides higher sensitivity, especially for samples with polymicrobial populations.

Integration of Morphological and Molecular Techniques

Morphological analysis continues to be relevant in initial screening. However, its limitations in species-level identification have prompted the widespread integration of molecular techniques, particularly DNA sequencing and qPCR assays. Shinohara *et al.* (2021) compared culture-dependent morphological identification with culture-independent DNA sequencing methods, demonstrating that sequencing offered a more comprehensive and accurate profile of fungal communities in environmental dust samples. Similarly, a 2024 systematic review by Matys *et al*. (2024) focused on the detection of airborne fungi in healthcare environments. The review emphasized the precision of molecular tools like real-time PCR, ITS sequencing, and

metabarcoding in identifying airborne fungal pathogens, even when cultures fail due to non-viability or overgrowth by faster-growing species.

Environmental Mycology and High-Throughput Sequencing

Advancements in high-throughput sequencing (HTS) have facilitated the in-depth analysis of environmental fungal reservoirs. The study by Shinohara *et al*. (2021) also demonstrated that HTS can detect diverse fungal species directly from settled dust, highlighting its utility in ecological monitoring and hospital surveillance where infection risks are linked to airborne spores.

2.5.2. Clinical Relevance and Resistance Patterns of Fungal Pathogens

A comprehensive review by Fisher *et al*. (2022) emphasized the urgent global health threat posed by antifungal resistance, describing it as an emerging crisis comparable to bacterial antibiotic resistance. The review detailed how prolonged antifungal use in both clinical and agricultural settings has driven resistance in critical pathogens like *Candida auris* and *Aspergillus fumigatus*. *C. auris*, in particular, demonstrates intrinsic resistance to multiple drug classes, including azoles, polyenes, and echinocandins, complicating treatment protocols. In parallel, Vitiello *et al*. (2023) outlined the growing clinical burden of resistant fungal infections in hospital environments, where immunocompromised patients, including those with hematologic malignancies or undergoing organ transplantation, are especially vulnerable. These infections often require aggressive and prolonged therapy, yet the efficacy of antifungals like fluconazole and amphotericin B is waning. A systematic analysis by Alcázar-Fuoli and Mellado (2021) highlighted that *Aspergillus fumigatus*, one of the most clinically significant molds, has developed resistance to triazoles through environmental exposure to fungicides. This

phenomenon, known as "environmental resistance acquisition," threatens the utility of first-line antifungal agents even before patients are exposed to them.

2.6. Antifungal Testing Methods and Parameters

Between Current testing approaches combine standardized methods and molecular profiling to enhance diagnostic accuracy, therapeutic guidance, and surveillance of emerging resistance.

Standardized Methods: CLSI and EUCAST

The two most widely recognized antifungal testing standards are those issued by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Both provide protocols for determining minimum inhibitory concentrations (MICs) for antifungal agents. In their review, Lamoth *et al.* (2021) emphasize that CLSI and EUCAST methodologies continue to be the benchmark for antifungal management in invasive fungal infections. They guide clinicians on breakpoint values, interpretive criteria, and the relevance of susceptibility patterns to clinical outcomes.

Parameters: MIC, ECVs, and Breakpoints

A central parameter in AFST is the minimum inhibitory concentration (MIC) the lowest drug concentration that inhibits visible fungal growth. However, interpretation requires additional tools like epidemiological cutoff values (ECVs) and clinical breakpoints (CBPs). For newer or rare fungal pathogens where CBPs are unavailable, ECVs help distinguish wild-type from non-wild-type strains, aiding early resistance detection. Lamoth *et al*. (2021) further explain that MICs alone may be insufficient for treatment decisions. They propose incorporating dynamic

pharmacodynamic models and clinical outcome data to better predict therapeutic success, especially in severely immunocompromised patients.

2.6.1. Agar Diffusion and Broth Microdilution Techniques

Broth Microdilution Method

The broth microdilution (BMD) method is the gold standard for determining the minimum inhibitory concentration (MIC) of antifungal agents. This technique involves exposing fungal isolates to serial dilutions of antifungal drugs in liquid media. A major strength of BMD is its quantitative output, which provides exact MIC values used to interpret resistance or susceptibility according to defined breakpoints. Recent insights by Lamoth *et al*. (2021) emphasize that BMD remains the reference method for evaluating antifungal resistance, especially in *Candida* and *Aspergillus* species. However, the method is labor-intensive, time-consuming (requiring 24–48 hours of incubation), and sensitive to media composition and inoculum size.

Agar Diffusion Method

The agar diffusion technique**,** particularly disk diffusion and E-test formats, offers a simpler and more accessible alternative for many clinical laboratories. It involves placing antifungal-impregnated disks or strips on inoculated agar plates, where drug diffusion inhibits fungal

growth in a circular zone. The diameter of this inhibition zone is then measured and interpreted. Although less quantitative than BMD, disk diffusion methods have proven reliable for screening purposes, especially when automated BMD systems are unavailable. Studies comparing these two techniques have found good correlation for azoles and echinocandins, especially against *Candida* spp., as demonstrated in the multi-laboratory evaluations by Martos *et al*. (2012). Furthermore, E-tests, a form of gradient agar diffusion, bridge the gap between disk diffusion and BMD by offering semi-quantitative MIC data via an antifungal concentration gradient. They have been validated for use in testing amphotericin B, voriconazole, and caspofungin, particularly for molds and dermatophytes (Gupta *et al*., 2015).

2.6.2. Determination of MIC and MFC Values

The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) are core parameters in antifungal susceptibility testing that assess the efficacy of antifungal agents against fungal pathogens. MIC refers to the lowest concentration of an antifungal that inhibits visible growth, while MFC is the lowest concentration that kills ≥99.9% of the fungal inoculum. From 2021 to 2025, a limited but growing number of studies have investigated the practical relevance, methodological refinements, and interpretation of these values. In a 2024 study, Sasoni *et al*. (2024) highlighted the diagnostic and therapeutic value of MFC/MIC ratios in distinguishing fungistatic from fungicidal agents. They found that the inclusion of MFC values along with MICs provided better insight into drug efficacy against resistant or biofilm-forming isolates particularly for *Candida* species. This study advocated the joint application of the CLSI M27 document and MFC testing for improved resistance detection. The broth microdilution method remains the standard for determining both MIC and MFC values, though challenges persist in standardizing MFC endpoints. A notable limitation is that while MIC determination is

widely standardized, MFC protocols are more variable and depend on plating the contents of MIC wells onto drug-free media to assess regrowth, as reiterated by Lamoth *et al*. (2021). Moreover, new antifungals and combinations often exhibit discrepant MIC and MFC values, underscoring the need to interpret these parameters in tandem. Some echinocandins, for instance, may yield low MICs but high MFCs, suggesting a fungistatic rather than fungicidal profile against certain strains of *Candida* or *Aspergillus* (Sasoni *et al*., 2024).

2.6.3. Comparative Assessment with Synthetic Antifungal Agents

The comparative evaluation of natural versus synthetic antifungal agents has gained increasing importance between 2021 and 2025 due to the emergence of drug-resistant fungi and the global push for safer, eco-friendly alternatives. Recent investigations emphasize how plant-derived compounds and essential oils hold promise as adjuncts or alternatives to conventional antifungal drugs such as fluconazole, ketoconazole, and clotrimazole. In a notable 2024 review, Bibi *et al*. (2024) provided a comprehensive comparative analysis of natural and synthetic antimicrobials used in medical textiles and infection prevention. While synthetic agents demonstrated stronger immediate potency, natural agents like Aloe vera and clove oil showed significant antifungal activity with better biocompatibility and safety profiles. The authors emphasized that while synthetic drugs exhibit rapid fungistatic or fungicidal activity, long-term use often leads to resistance, toxicity, or side effects issues that botanical compounds may mitigate. Adding to this perspective, Jaiswal *et al*. (2023) conducted an experimental study comparing synthetic antifungal agents with powder extracts of *Lawsonia inermis* and *Withania somnifera* when incorporated into dental soft liners. Their results demonstrated that while synthetic agents like fluconazole maintained strong inhibition zones, the herbal extracts also significantly inhibited *Candida albicans*, suggesting potential for hybrid or plant-enhanced formulations.

2.7. Resistance Mechanisms in Fungal Pathogens

2.7.1. Multidrug Resistance in Fungal Species

Multidrug resistance (MDR) in fungal pathogens, particularly in *Candida auris* and *Aspergillus fumigatus*, has emerged as a significant global health concern between 2021 and 2025. These pathogens display resistance to multiple classes of antifungal agents, posing diagnostic and therapeutic challenges, especially in immunocompromised patients and nosocomial settings.

Candida auris: A Critical MDR Threat

C. auris has been classified as a high-priority fungal pathogen by public health authorities due to its ability to resist azoles, echinocandins, polyenes, and flucytosine. It has caused outbreaks in healthcare facilities worldwide, with mortality rates ranging from 20% to 50% (Wang *et al*., 2024). Molecular studies reveal resistance mechanisms that include mutations in ERG11 (azole target), FKS1 (echinocandin target), and overexpression of efflux pumps (Jangir *et al*., 2023). A landmark study by Carolus *et al*. (2021) used genome-wide analysis to identify novel MDR mechanisms in *C. auris*, including mutations conferring caspofungin resistance and compensatory stress adaptation. These findings underscore the evolutionary plasticity of this pathogen under antifungal pressure. The challenge of pan-resistance was further highlighted in a case series by Jacobs *et al*. (2022), which reported *C. auris* isolates resistant to all four major antifungal classes, a concerning milestone in antifungal resistance.

Aspergillus fumigatus: Azole Resistance and Beyond

For *A. fumigatus*, azole resistance is primarily linked to environmental exposure to agricultural fungicides. A 2023 study by de Moraes *et al*. (2024) reported transcriptional adaptations that support efflux pump regulation and biofilm formation as resistance strategies.

2.7.2. Potential of Clove Oil in Overcoming Resistance

In the context of mounting antifungal resistance, Clove Essential Oil (CEO) and its principal component, eugenol, have garnered substantial attention as promising agents to counteract multidrug-resistant fungal pathogens. Studies conducted from 2021 to 2025 underscore the broad-spectrum efficacy of eugenol, its synergistic potential, and its capacity to target biofilms and efflux-mediated resistance mechanisms two of the most challenging aspects of fungal resilience (Biernasiuk *et al*., 2022)

Mechanisms of Action and Antifungal Potency

Clove oil and eugenol demonstrate potent activity against both *Candida* spp. and *Aspergillus fumigatus*, functioning through membrane disruption, ergosterol binding, and ROS generation (Biernasiuk *et al*., 2022; Shariati *et al*., 2022). Eugenol's antifungal properties are particularly valuable against biofilm-forming strains, where traditional antifungals show reduced efficacy.

Overcoming *Candida auris* and MDR Biofilms

In 2024, Kowalczyk reviewed the synergistic effects of essential oils notably clove oil against *Candida auris*, suggesting that eugenol can inhibit growth and disrupt biofilm architecture, thereby enhancing the effectiveness of standard antifungals (Kowalczyk, 2024). Similarly,

Parker *et al*. (2022) showed that CEO exerted significant antifungal activity against drug-resistant *C. auris* isolates, comparable to fluconazole and amphotericin B.

Nanoemulsion and Drug Delivery Enhancements

An innovative formulation by de Almeida *et al*. (2025) developed a clove oil-based nanoemulsion loaded with amphotericin B, showing increased cellular uptake and reduced MIC values against *C. auris*. These delivery systems enhance bioavailability and target-specific delivery while minimizing toxicity, making them ideal for clinical applications.

Molecular Mechanisms: Efflux Suppression and Synergism

Beyond membrane targeting, eugenol has been reported to suppress efflux pump expression, which is often upregulated in resistant fungal species. According to Fernanda *et al. (*2024), eugenol derivatives exhibited structural modifications that enhanced their activity against efflux-mediated resistance pathways in both *Aspergillus* and *Candida* species. Additionally, clove oil has shown synergistic potential when combined with synthetic antifungal agents. Menotti *et al*. (2024) documented how co-application of CEO with azoles significantly reduced MIC values and inhibited clinical isolates of *C. auris* more effectively than either agent alone.

2.8. Toxicity, Safety, and Regulatory Aspects

2.8.1. Toxicological Assessment of Clove Essential Oil (CEO)

Clove essential oil (CEO), primarily composed of eugenol, has been extensively evaluated for its safety profile in recent years due to its growing applications in food preservation, pharmaceuticals, and medical formulations. Between 2021 and 2025, studies confirmed both the therapeutic promise and safety thresholds for CEO, while also warning of potential dose-related

toxicities. In a comprehensive risk assessment, the EFSA Panel on Additives and Products (2023) reviewed clove bud and leaf oils used in feed additives, emphasizing that toxicity is dose-dependent and can be reliably predicted using dose addition assumptions within defined toxicological assessment groups. The panel concluded that clove oils with standardized eugenol content are safe within regulated limits (EFSA Panel, 2023). Reinforcing this, Pires Costa *et al*. (2025) explored the cytotoxicity and anti-inflammatory activity of eugenol and bis-eugenol, confirming that at low concentrations, these compounds exhibit protective antioxidant effects without cytotoxicity. However, the study also noted that higher doses may induce phenoxyl radicals, necessitating controlled usage. A 2023 experimental study by Aryal *et al*. (2023) tested CEO on *Sitophilus zeamais* and reported low mammalian toxicity, supporting its GRAS (Generally Recognized as Safe) classification by regulatory bodies for food applications. Nevertheless, the authors emphasized the importance of species-specific toxicity profiles, as insecticidal potency doesn't equate to human safety. Toxicity screening has also extended into biomedical formulations. For instance, Laghari and Khan (2022) highlighted CEO's suitability in biocompatible delivery systems. Their assessments indicated negligible cytotoxicity and affirmed eugenol's pharmacological safety in nanoformulated and encapsulated forms.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin City, Edo state, Nigeria.

3.2 Material

3.2.1 Microbiological Media

Sabouraud Dextrose Agar (SDA)

3.2.2 Equipment and Apparatus

Portable autoclave, Rotary evaporator, Weighing balance, Hot air oven, Mortar and pestle Mechanical grinding machine, Refrigerator, Micropipette, Incubator, Whatman filter paper, Cotton wool, Pipette tip, Corkborer (10mm in diameter) Transparent millimetre rule, Grease pencil, Sterile swab sticks, Tripod stand, Bunsen burner, Foil paper, Heating Mantle, Water bath, Spatulas and Porcelain dishes.

3.2.3 Glassware

Conical flask, bottles (MacCartney, universal and Bijou) as well as test tubes, pipettes, glass stirrers, porcelain dish, pestle, maceration jars, glass funnels, beakers, measuring cylinders, and Petri dishes.

3.2.4 Chemicals and Reagents

All solvents used were of analytical grade.

Ethanol, Tween 80, Distilled water, Disinfectant: Purit and Distilled Water, Standard antifungal agent (ketoconazole), soap and detergent.

3.3 Sample Collection

Clinical isolates of *Candida albicans* were obtained from the Medical Microbiology Laboratory of the University of Benin Teaching Hospital (UBTH), Ugbowo, Benin City, Nigeria. *Penicillium Species*, *Aspergillus Niger* and *Fusarium Species* were obtained from the department of pharmaceutical microbiology laboratory University of Benin.

3.3.1 Sterilization of Materials

All materials used were adequately and appropriately sterilized before and after use. Glass wares, and metal equipment were thoroughly washed, rinsed, and autoclaved at 121°C for 20 minutes. New gloves were used for each sample analysis and media were prepared according to the manufacturer's instructions and autoclaved at 121°C for 15 minutes.

3.4 METHOD

3.4.1. FUNGI ISOLATES

A total of 12 fungal isolates was used, 3 *Candida albilicans*, 3 *Penicillium Species*, 3 Aspergillus Niger, 3 *Fusarium Species*

3.4.2 Collection and Identification of Syzygium aromaticum

The buds of *Syzygium aromaticum* was bought from New Benin market, Edo State, Nigeria in the month of August. The plant was officially identified at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State and, assigned Voucher numbers: *Syzygium aromaticum UBH-S385*

3.4.3 Preparation of Extract

Soxhlet Extraction of *Syzygium aromaticum*

Syzygium aromaticum(Clove buds) were first dried at room temperature and then ground into a fine powder using a laboratory mill. 300 grams of the weighed clove powder was soaked in a cellulose thimble and inserted into a Soxhlet apparatus. Ethanol /distilled water (ratio 1:1)L was used as the solvent . The extraction process was carried out for 3-4 hours under reflux conditions using a heating mantle maintained at 380^{o}C. The ethanol-aqueous extract was concentrated using a laboratory oven at 65^{o}C reduced temperature to obtain the crude clove essential oil. The oil was then stored in a dark glass vial at 4°C until further analysis. (*Aziz et al. 2023*)

Maceration Extraction of *Syzygium aromaticum*

300g of Syzygium aromaticum (Clove buds) was macerated using 2l ethanol aqueous solution (1:1). The mixture was sealed and kept at ambient room temperature (25 ± 2°C) for 72 hours with intermittent shaking to facilitate solubilization of the active compounds. The mixture was filtered using Whatman No. 1 filter paper. The filtrate was then concentrated at 60^{oC} using

laboratory oven to remove the ethanol, yielding the crude clove extract. The extract was collected and stored at 4°C in amber vials for subsequent use. (Adegbanke and Bada, 2024).

3.4.4 Preparation of Test Organisms

The isolates were subcultured onto sabouraud dextrose agar plates from the slants and incubated at room temperature for 24 to 72 hours to ensure purity and viability. 2 -3 colonies were aseptically picked into normal saline using sterile wire loop and shake vigorously for antifungal susceptibility testing

3.4.5 Determination of Extraction Yield

The percentage yield of the extract was calculated to determine the efficiency of the extraction for the solvent. The yield was calculated ethanol extract using the following formula:

The starting material weight for both is 300g

Soxhlet Extraction

Extract Yield (%) = (Weight of Soxhlet Extract / Weight of Starting Material) × 100

= (80 g / 300 g) × 100

= 26.67%

Maceration Extraction

Extract Yield (%) = (Weight of Maceration Extract / Weight of Starting Material) × 100

= (110 g / 300 g) × 100

= 36.67%

The extract yields are:

Soxhlet Extraction: 26.67%

Maceration Extraction: 36.67%

3.5 Antifungal Assay

3.5.1 Preparation of Test Organisms

Confirmed clinical isolates of *Candida albicans* obtained from the university of Benin teaching hospital and those from pharmaceutical microbiology department were used for antifungal testing. Each isolate was maintained in Sabouraud Dextrose agar (SDA) slant. Test organisms were subcultured and incubated at 37°C for 24–72 hours. After incubation, 2 to 3 well-isolated colonies was picked and suspended in sterile normal saline. The turbidity of the suspension were adjusted to match 0.5 McFarland standard, which corresponds to approximately 1×10^6 CFU/mL.

3.5.2 Preparation of Stock Solution of Ketoconazole (control)

Stock solution of 5ml Ketoconazole was prepared containing 25 mg/mL by dissolving 125 mg of Ketoconazole in 0.5ml of 10% DMSO (Dimethyl Sulfoxide) and 4.5ml of sterile water.

3.5.3 Preparation of McFarland Solution

A 0.5 McFarland standard solution was prepared by adding 0.05ml of 1.175% (weight/volume) Barium Chloride dihydrate salt ($BaCl_2 \cdot 2H_2O$) to 9.95ml of 1% Sulfuric acid (H_2SO_4). The solution was mixed completely to form a turbid suspension in a test tube which was then placed in a test tube rack and kept at room temperature before use.

3.6 Antifungal Susceptibility Tests

3.6.1 Determination of antifungal Inhibition Zone Diameters (IZD)

Antifungal Susceptibility test was carried out using agar well diffusion method (Murray, 2009) with some modifications. Sterile Sabouraud Dextrose Agar was prepared and poured into different petri dishes aseptically, each containing 30ml and allowed to set. The agar well were dried in hot air oven for about 5minutes at 40°C. Each of the dried plates were then streaked evenly with each test organism respectively using a swab stick aseptically. A sterile cork borer (10mm) was used to bore 6 wells (evenly spaced) in each agar plate, the disc was removed and each of the well was sealed with 2 drop of molten agar. Five of the wells were for both soxhlet and maceration extracts and were filled with 250ul in 25mg/ml, 50mg/ml,100mg/ml, 200mg/ml and 400mg/ml of the aqueous ethanonic extracts respectively and the sixth well was filled with the standard, Ketoconazole. The plates were incubated at 37°C for 24 hours. .The inhibition zone diameters (IZD) was measured and recorded.

3.6.2 Determination of Minimum Inhibitory Concentration (MIC)

Agar dilution method of Afyon and Meyer (1997) was used in this study for the determination of Minimum Inhibitory Concentration (MIC) of the extract and combination treatment. A 2 fold serial dilution of the extract was prepared to give concentrations of 12.5 mg/ml, 25 mg/ml, 50 mg/ml, 100 mg/ml, 200mg/ml and 400mg/ml. Double strength sabouraud dextrose agar was prepared according to manufacturer's instruction. Calculated volumes of the each extract concentration and double strength agar (1 gram of extract + 20ml molten agar) was poured into each petri dishes respectively and allowed to set. This procedure was repeated for both the soxhlet extract and maceration extract. Each of the test organisms prepared to a standard concentration was streaked with the aid of a sterile wire loop on well labeled different sections of

each plate. The dilution plates were incubated at room temperature for 24- 72 hours. After incubation, the plates were visually examined for growths in the inoculated spots. The lowest concentration of the extracts that inhibits growth was considered as the MIC. The experiment was done in triplicate

3.6.3 Determination of Minimum Fungicidal Concentration (MFC)

The MFC was determined from the agar dilution of the MIC tests by sub-culturing into sabouraud dextrose agar plates that did not contain any test extract. The dilution plates were then incubated at 37°C for 24 hours. After incubation, the plates were visually examined for growths in the inoculated spots. The lowest concentration of the extract that showed no growth were considered as the MFC respectively

3.7 Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). The data obtained from the experiments was recorded and analyzed using appropriate statistical methods, including analysis of various (ANOVA) followed by post-hoc tests for multiple comparisons. Results will be considered statistically significant at $p<0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1: Soxhlet extraction yield

The soxhlet extract had a weight of 80g and yield of 26.67%

4.2: Maceration extraction yield

The Maceration extract had a weight of 110g and yield of 36.67%.

4.3: Mean Zone of Inhibition (ZOI) for *Syzygium aromaticum* extract

The Maceration extract showed the higher zones of inhibition compared to soxhlet extract. At 400mg/ml, *Candida albicans* has the highest zone of inhibition (17.66mm) and the smallest inhibition was observed for *fusarium* (14.00mm). As concentration decreases, zone of inhibition decreases significantly across all fungal species ($p<0.05$) as shown in Table 4.1

4.4: Effects of extraction method on Zone of inhibition

The Maceration method produces significantly higher zones of inhibition compared to soxhlet

($p<0.05$). *Candida ablican* had the highest zone of inhibition in Maceration (11.77 ± 8.13mm) compare to soxhlet (5.11 ± 6.69 mm) . For *Aspergillus niger*, Maceration yielded (5.94 ± 6.93mm) while soxhlet yielded (5.38 ± 7.04mm). Similarly, for *Penicillum and Fusarium*, Maceration consistently produced higher zone of inhibition values as shown in Table 4.2

4.5: Minimum inhibitory concentration (MIC) for *Syzygium aromaticum* extract

Candida albicans has the lowest MIC in Maceration (41.66 ± 12.12µg/ml) compared to soxhlet (200.00 ± 145.5 µg/ml) while *Fusarium* recorded higher MIC values in soxhlet (266.66 ± 97.0 µg/ml) compared to maceration (116.66 ± 64.1 µg/ml) indicating greater resistance to both extraction methods shown in Table 4.3

4.6: Minimum Fungicidal Concentration (MFC) for Maceration

The Maceration method showed the lowest MFC for *Candida albicans* (50.00 ± 0.00 µg/ml) and *Penicillin* exhibited the highest MFC (200.00 ± 0.00 µg/mg) demonstrating its greater resistance to the extract as shown in Table 4.4

Table 4.1: Mean Zone of Inhibition (mm) of *Syzygium aromaticum* Extract Against Test Fungi at Different Concentrations

Concentration (mg/ml)	*Candida albicans*	*Aspergillus niger*	*Penicillium*	*Fusarium*
12.5[a]	0.00	0.00	0.00	0.00
25[a]	2.00	0.00	0.00	0.00
50[b]	6.33	0.00	1.83	1.83
100[b]	11.16	5.50	4.16	3.66
200[a]	13.50	13.16	13.16	9.83
400[b]	17.66	15.33	15.83	14.00

Values of Mean ± SEM.

KEY:

a: There's no statistical significance difference among the fungal species at the concentration

b: There's stastistical significance differences ($p<0.05$) among the fungal species at the concentration

Table 4.2: Effect of Extraction Method on Zone of Inhibition of *Syzygium aromaticum* Against Test Fungi

Organism	Soxhlet	Maceration
Candida albicans	5.11 ± 6.69	11.77 ± 8.13
Aspergillus niger	5.38 ± 7.04	5.94 ± 6.93
Penicillium	6.44 ± 7.64	5.22 ± 6.78
Fusarium	3.44 ± 5.75	6.33 ± 6.64

Values of Mean ± SEM

Table 4.3: Minimum Inhibitory Concentration (MIC, µg/ml) of *Syzygium aromaticum* Extract against Test Fungi

Organism	Soxhlet	Maceration
Candida albicans	200.00 ± 145.5	41.66 ± 12.12
Aspergillus niger	166.66 ± 48.5	133.33 ± 48.5
Penicillium	150.00 ± 72.7	166.66 ± 48.5
Fusarium	266.66 ± 97.0	116.66 ± 64.1

Values of Mean ± SEM

Table 4.4: Minimun Fungicidal Concentration (MFC) For *Syzygium aromaticum* Maceration Extraction Method

Isolates	400mg/ml	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	MFC mg/ml
Candida ablican 1	NG	NG	NG	NG	G	G	50
Candida ablican 2	NG	NG	NG	NG	G	G	50
Candida ablican 3	NG	NG	NG	NG	G	G	50
Aspergillus niger 1	NG	NG	NG	G	G	G	100
Aspergillus niger 2	NG	NG	G	G	G	G	200
Aspergillus niger 3	NG	NG	NG	G	G	G	100
Penicillium 1	NG	NG	G	G	G	G	200
Penicillium 2	NG	NG	G	G	G	G	200
Penicillium 3	NG	NG	G	G	G	G	200
Fusarium 1	NG	NG	NG	G	G	G	100
Fusarium 2	NG	NG	NG	G	G	G	100
Fusarium 3	NG	NG	G	G	G	G	200

STANDARD DRUG: KETACONAZOLE

NG= No Growth G= Growth

Table 4.5: Minimum Fungicidal Concentration (MFC, µg/ml) of *Syzygium aromaticum* Extract Against Test Fungi

Organism	Maceration
Candida albicans	50.00 ± 0.00
Aspergillus niger	133.33 ± 48.5
Penicillium	200.00 ± 0.00
Fusarium	133.33 ± 48.5

Values of Mean ± SEM

CHAPTER FIVE

5.0 DISCUSSION

5.1. Discussion

Syzygium aromaticum, commonly known as clove, is widely recognized as a spice of significant economic and cultural importance, with a rich historical and geographical origin. This study assessed the antifungal efficacy of *Syzygium aromaticum* extracts obtained by Soxhlet and maceration methods against *Candida albicans*, *Aspergillus niger*, *Fusarium spp*., and *Penicillium spp*. using zone of inhibition, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) assays. The findings highlight that maceration extracts generally exhibited stronger antifungal activity compared to Soxhlet extracts, with *Candida albicans* consistently emerging as the most susceptible organism, while *Fusarium* and *Penicillium spp*. displayed higher resistance. The non-significant overall difference in inhibition zones between Soxhlet and maceration ($p = 0.063$) suggests that both methods are effective, but maceration yielded higher inhibition against *Candida albicans*. Previous studies on *S. aromaticum* confirm that maceration often preserves volatile and thermolabile compounds such as eugenol and eugenyl acetate, which are responsible for antimicrobial activity (Benserradj, 2023). By contrast, Soxhlet involves prolonged heating, which can degrade sensitive bioactives and reduce antifungal potency. Similar findings were reported for spice extracts where maceration yielded broader inhibition zones than Soxhlet against molds such as *Aspergillus* and *Penicillium* (Pong, 2024). *Candida albicans* showed significantly larger inhibition zones and the lowest MIC (41.67 mg/mL) and MFC (50 mg/mL) under maceration. This confirms its high susceptibility to *S. aromaticum*, which has been widely documented. Phytochemical investigations show that clove oil constituents, particularly eugenol, disrupt fungal cell membranes and biofilm structures, leading to potent fungicidal effects (Neaz, 2019; Elamary *et al*., 2023). Earlier studies by Nigussie *et al*.

(2021) also showed MIC values of clove extracts against *Candida* in the range of 3–6 µg/mL when essential oils were used, indicating that crude extracts such as those tested here require higher concentrations but remain effective (Nigussie *et al*., 2021).

In contrast, *Fusarium spp*. and *Penicillium spp*. exhibited relatively higher MIC and MFC values, with *Fusarium* being most resistant under Soxhlet (266.67 mg/mL). This finding is consistent with prior reports that *Fusarium* species are notoriously resistant to plant extracts due to robust cell wall composition and efficient detoxification enzymes (Neelam, 2016). Similarly, *Penicillium spp*. showed high resistance, aligning with evidence that certain Penicillium strains require elevated concentrations of clove extract for inhibition compared to *Candida* and *Aspergillus* (Davidova *et al*., 2024). Although macerated *S. aromaticum* extracts demonstrated measurable fungicidal activity, their efficacy was lower than the standard drug ketoconazole, which showed complete inhibition at much lower concentrations. This contrast reflects the differences between crude plant extracts and purified antifungal agents. Nonetheless, the advantage of *S. aromaticum* lies in its safety profile and synergistic potential. Recent studies have reported synergism between *S. aromaticum* extract and conventional antifungals, enhancing activity against resistant *Candida* strains (Elamary *et al*., 2023).

While some reports emphasize Soxhlet as superior due to exhaustive extraction of non-volatile phytochemicals (Nigussie *et al*., 2021), our findings suggest that maceration is more effective for preserving antifungal compounds in *S. aromaticum*. This contrast underscores that the choice of extraction method should be tailored to the chemical profile of the target plant. Additionally, the consistent high sensitivity of *Candida albicans* compared to filamentous fungi confirms existing literature that yeasts are generally more susceptible to phytochemicals (Kabbashi, 2022).

5.2 Conclusion

The findings of this study confirm that *Syzygium aromaticum* extracts possess significant antifungal activity, with maceration being the preferred extraction technique. *Candida albicans* was the most sensitive organism, while *Fusarium* and *Penicillium* exhibited marked resistance. These results corroborate and extend previous literature, highlighting the potential of *S. aromaticum* as a natural antifungal agent, particularly in combination with conventional drugs. Future studies should isolate and quantify active phytochemicals such as eugenol, assess their synergistic effects, and evaluate their efficacy *in vivo.*

5.3 Recommendation

The findings of this study strongly suggest that maceration is a more effective extraction method for *Syzygium aromaticum* compared to Soxhlet extraction, particularly in preserving thermolabile bioactive compounds responsible for antifungal activity. It is therefore recommended that future investigations should focus on optimizing maceration parameters such as solvent polarity, extraction time, and temperature to further enhance the yield and potency of antifungal constituents. In addition, since this research was limited to crude extracts, it is essential that subsequent studies undertake detailed phytochemical characterization using advanced analytical techniques such as GC–MS and LC–MS. Isolating and quantifying compounds like eugenol and eugenyl acetate would provide deeper insight into the precise mechanisms of action and could open the way for developing standardized antifungal formulations.

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APPENDIX I

Preparation of Stock Solution of Ketoconazole (control)

Stock solution of 5ml Ketoconazole was prepared containing 25 mg/mL by dissolving 125 mg of Ketoconazole in 0.5ml of 10% DMSO (Dimethyl Sulfoxide) and 4.5ml of sterile water.

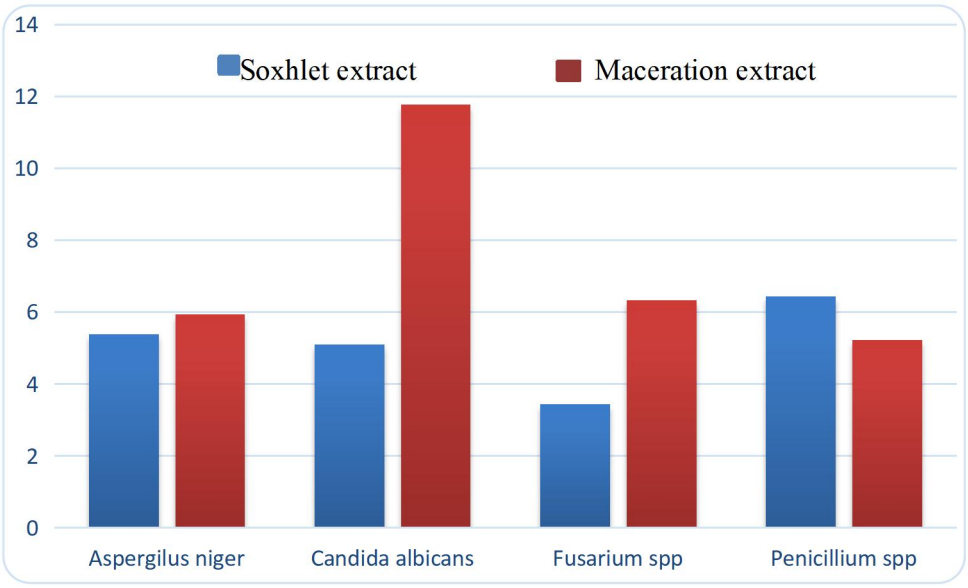
Preparation of McFarland Solution

A 0.5 McFarland standard solution was prepared by adding 0.05ml of 1.175% (weight/volume) Barium Chloride dihydrate salt ($BaCl_2 \cdot 2H_2O$) to 9.95ml of 1% Sulfuric acid (H_2SO_4). The solution was miixed completely to form a turbid suspension in a test tube which was then placed in a test tube rack and kept at room temperature before use.

APPENDIX II

Y- axis indicates the Concentrations of extract

X- axis indicates the Test organisms

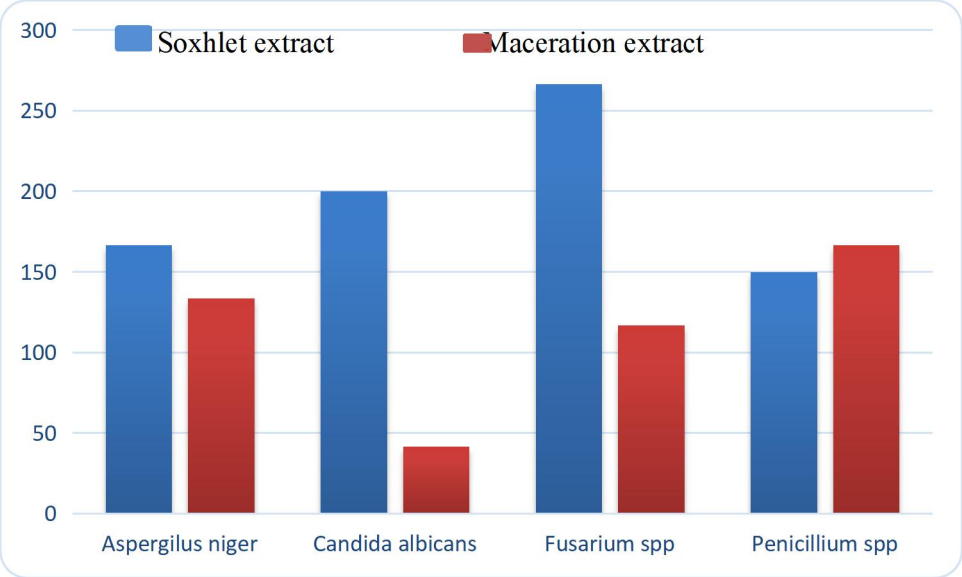


Zone of inhibition of isolates under Soxhlet and maceration extrsact.

APPENDIX III

Y- axis indicates the Concentrations of extract

X- axis indicates the Test organisms



Minimum inhibition concentration of isolates under Soxhlet extract and Maceration extract

APPENDIX IV

Prof. Akinnibosun Henry Adewale (FLS, MRSB; London)
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Department of Plant Biology and Biotechnology,
P. M. B. 1154 Ugbowo, 300283 Benin City,
Edo State, Nigeria.

University of Benin

Department of Plant Biology and Biotechnology

Herbarium Unit

Faculty of Life Sciences

University of Benin, Benin City, Edo State

Plant Name: *Syzygium aromaticum* (L.) Merr. & L. M. Perry

Family: Myrtaceae

Common Name: Clove

Voucher Number: UBH-S385

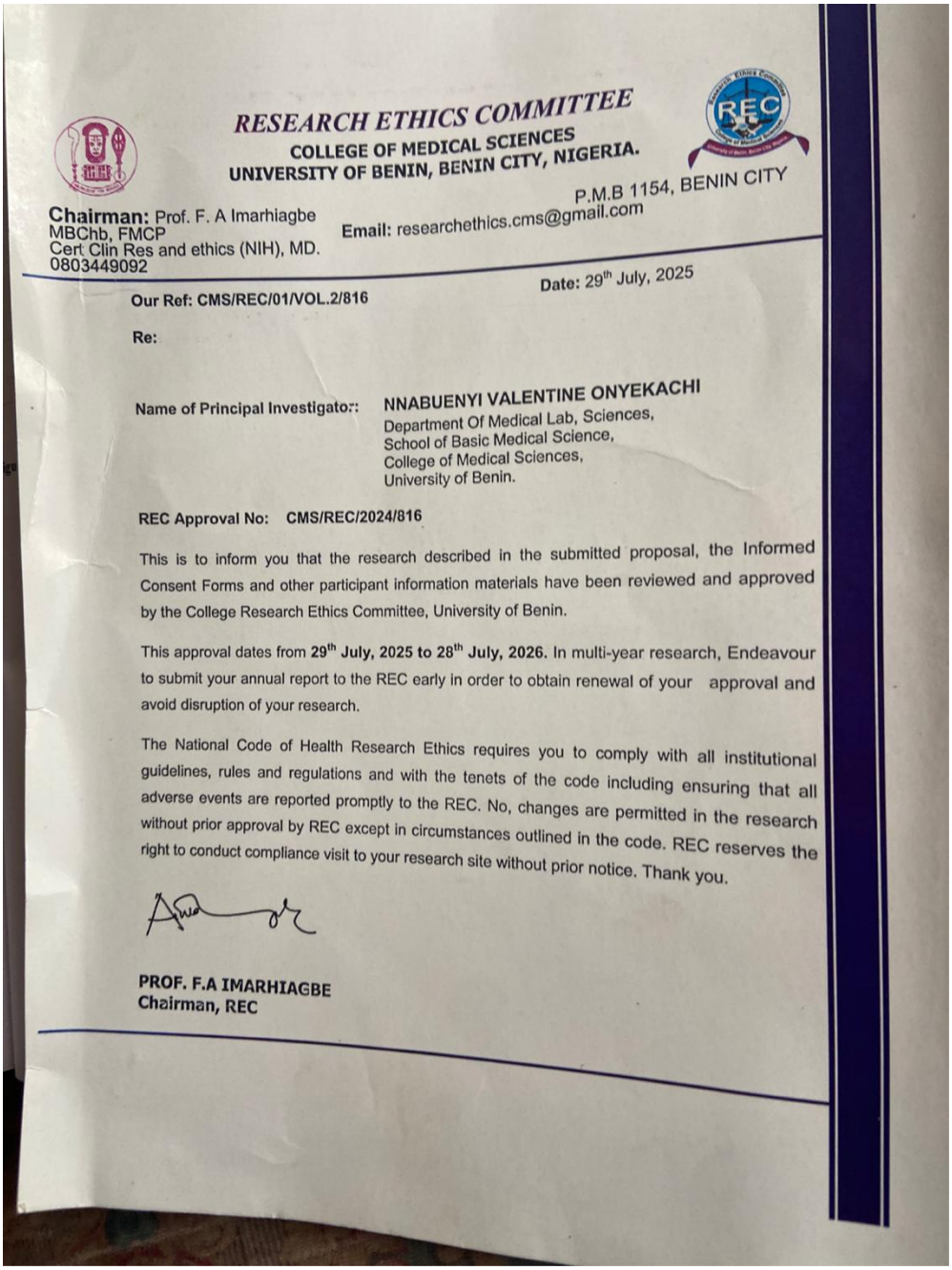
Student Name: Nnabuenyi Valentine Onyekachi

Plant Identification and Voucher Number Issued By:

19/08/2025

Prof. Akinnibosun Henry Adewale (FLS, MRSB; London, LMBOSON, MNES; Nigeria)

APPENDIX V



RESEARCH ETHICS COMMITTEE
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UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

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Chairman: Prof. F. A Imarhiagbe
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Our Ref: CMS/REC/01/VOL.2/816

Date: 29th July, 2025

Re:

Name of Principal Investigator: **NNABUENYI VALENTINE ONYEKACHI**
Department Of Medical Lab, Sciences,
School of Basic Medical Science,
College of Medical Sciences,
University of Benin.

REC Approval No: CMS/REC/2024/816

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 **29th July, 2025 to 28th July, 2026.** In multi-year research, Endeavour to submit your annual report to the REC early in order to obtain renewal of your approval and avoid disruption of your research.

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

PROF. F.A IMARHIAGBE
Chairman, REC