DEVELOPMENT OF CHITOSAN NANOENCAPSULATED CLOVE ESSENTIAL OIL AND ITS ANTIFUNGAL EFFICACY AGAINST *ASPERGILLUS NIGER*

AHMED ABDOU SAID ABDELMOATY ABDELWAHED

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Ahmed Abdou Said Abdelmoaty Abdelwahed

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ABSTRACT

This study focused on the development and characterization of chitosan nanoencapsulated clove essential oil (CEO-CSNPs) and its antifungal efficacy against *Aspergillus niger*, a common spoilage fungus. A preliminary screening was conducted to evaluate the antifungal activity of three plant essential oils, clove, cinnamon, and lemongrass using the disc diffusion method. Clove essential oil exhibited the highest inhibition zone (50.20 ± 0.51 mm), and was therefore selected for nanoencapsulation using the ionic gelation method with sodium tripolyphosphate (TPP) as the cross-linking agent. The CEO-CSNPs were characterized using Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Thermogravimetric Analysis (TGA), and Energy Dispersive X-ray Spectroscopy (EDS). These techniques confirmed the formation of spherical nanoparticles and the successful encapsulation of clove essential oil, as indicated by the presence of its characteristic functional groups within the chitosan matrix. Agar diffusion assays against *Aspergillus niger* demonstrated a concentration-dependent increase in antifungal activity of clove essential oil (CEO), with inhibition zones increasing by approximately 186% as the CEO concentration was raised from 5% to 100%. A similar trend was observed for nanoencapsulated CEO (CEO-CSNPs), where increasing the CEO-to-chitosan (CS) ratio from 1:1 to 1:2 resulted in an approximately 165% increase in the diameter of inhibition zones. These findings indicate that chitosan-based nanoencapsulation not only preserves but also enhances the antifungal efficacy of CEO. This encapsulation strategy offers a potential for the development of bio-based antifungal agents applicable in food preservation and shelf-life extension. supporting

its potential application as a natural antifungal agent for food preservation and shelf-life extension.

Keywords: Antifungal Activity, *Aspergillus niger*, Chitosan, Essential Oils, Clove Oil, Nanoencapsulation

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ABBREVIATIONS AND SYMBOLS

AFB1	Aflatoxin B1
AFs	Aflatoxins
AOAC	Association of Official Analytical Chemists
CFU	Colony Forming Unit
CS	Chitosan
CSNPs	Chitosan Nanoparticles
CEO	Clove Essential Oil
CEO-CSNPs	Clove Essential Oil-Loaded Chitosan Nanoparticles
CN	Chitosan Nanopolymer
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DTA	Differential Thermal Analysis
EDS	Energy Dispersive X-ray Spectroscopy
EO	Essential Oil
EOs	Essential Oils
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
GRAS	Generally Recognized As Safe
ITZ	Itraconazole
LGEO	Lemongrass Essential Oil
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
NP(s)	Nanoparticle(s)
PDI	Polydispersity Index
RT	Retention Time

CHAPTER 1

INTRODUCTION

1.1 Research Problem

Fungal contamination remains a persistent and significant challenge in global food production and storage, contributing to both public health concerns and substantial economic losses. Among the spoilage fungi, *Aspergillus* species particularly *Aspergillus niger* are especially concerning due to their resilience under diverse environmental conditions, their ability to colonize a wide range of food products, and their capacity to produce toxic secondary metabolites, including mycotoxins (Leyva Salas et al., 2017). *A. niger* has been frequently identified in cereals, nuts, fruits, and dairy items and has been associated with both food spoilage and allergic or respiratory complications, especially in immunocompromised individuals (Al-Maqtari et al., 2022; Allizond et al., 2023; Almeida et al., 2023).

Various strategies have been explored to mitigate *Aspergillus spp*. contamination in peanuts, ranging from chemical treatments to innovative nanotechnology applications. Allyl isothiocyanate vapor, dimethylformamide, and α-Fe2O3 nanorods show promise in inhibiting fungal growth and aflatoxin production (Okano et al., 2018; Pan et al., 2020). However, traditional methods such as chemical fungicides, nitrogen filling, and vacuum packaging come with drawbacks like high costs, alteration of food flavors, adverse health effects, environmental concerns, and potential pest resistance (Pokrzywa et al., 2020). Given these challenges, this thesis examines current strategies employed to mitigate *Aspergillus spp*. contamination in peanuts. It will evaluate methods from chemical treatments to nanotechnology applications and seek to identify areas where further research or novel approaches could enhance food safety and economic outcomes in the peanut industry. The use of plant-derived essential oils (EOs) has gained attention for their preservative properties against mycotoxigenic fungi in stored food commodities. EOs like clove essential oil (CEO) are composed of bioactive compounds with antifungal properties against pathogens

such as *Fusarium oxysporum* and *Aspergillus Niger* (Deepika et al., 2021; Somrani et al., 2020). Despite their volatility and susceptibility to degradation, encapsulation techniques have been developed to improve the practical application of EOs by enhancing stability and modulating release rates (Guo et al., 2020). These four EOs are not only environmentally friendly due to their rapid biodegradability but also offer broad-spectrum antifungal properties. For instance, garlic EO has been used on plums to inhibit *A. niger* and *A. niger* growth (Arasu et al., 2019), while clove, thyme, and cinnamon EOs have been effective against *A. niger* on peanut seeds (Achar et al., 2020). Additionally, the synergistic effect of combining EOs has been demonstrated in preventing mold growth and reducing aflatoxin production in stored maize grains. Traditional postharvest strategies used to mitigate fungal growth and aflatoxin production include chemical fungicides, nitrogen flushing, vacuum packaging, and irradiation. However, these methods are often costly, environmentally damaging, or ineffective in achieving long-term protection (Pokrzywa et al., 2020). Moreover, repeated use of synthetic fungicides contributes to the development of resistant fungal strains, raising the need for sustainable and eco-friendly alternatives. Residual toxicity and concerns over chemical residues in food further complicate the widespread acceptance of conventional chemical approaches (Sheikh et al., 2024; Hyldgaard et al., 2012). To address these limitations, researchers have increasingly turned toward plant-derived antifungal agents, particularly essential oils (EOs), which have been recognized for their natural origin, rapid biodegradability, and broad-spectrum antimicrobial activity (Reyes-Jurado et al., 2022; Donsì & Ferrari, 2016).

Common spoilage fungi such as *Aspergillus niger, Aspergillus flavas* and Penicillium spp. significantly decrease the shelf life and quality of a variety of food products such as bread and other bakery items (Safakas et al., 2025). Synthetic chemical preservatives and fungicides are commonly used for preventing fungus growth. Nonetheless, public health concerns regarding their potential toxicity and the emergence of resistant microbial strains are receiving heightened scrutiny (Leyva Salas et al., 2017; Rana et al., 2011). Consequently, there is an increasing demand from consumers and regulatory authorities for safer and natural preservation methods to propel the food industry towards sustainable solutions (Leyva Salas et al., 2017; Safakas et al., 2025; Al-Maqtari et al., 2022; Hyldgaard et al., 2012). An alternative to

synthetic preservatives is the use of natural antimicrobial agents derived from plants, including essential oils and plant extracts. Extracts and essential oils from spices such as clove, oregano, cinnamon, and thyme have demonstrated significant antibacterial efficacy against a range of pathogens. These natural compounds are increasingly favored for food preservation due to their safety, effectiveness, and consumer preference for clean-label ingredients (Saeed et al., 2019).

Essential oils (EOs) are complex mixtures of volatile compounds extracted from various parts of plants, including flowers, leaves, seeds, and roots. In recent years, they have attracted increasing attention for their potential as safe and effective antimicrobial agents, particularly in controlling fungal growth and mildew. EOs present several advantages over synthetic fungicides, including strong antifungal activity, low toxicity, and environmental compatibility (Lin et al., 2022). Their diverse chemical profiles contribute to a wide array of biological activities, making them valuable for applications in food preservation, pharmaceuticals, and cosmetics. The global search for sustainable antifungal agents has led researchers to focus on natural products, with essential oils emerging as particularly promising due to their broad-spectrum antimicrobial properties. These oils, derived from aromatic plants, are known to inhibit the growth of numerous pathogens, including fungi, bacteria, and viruses. Among these, *Aspergillus flavas* is especially concerning because of its ability to spoil food and produce aflatoxins—highly toxic and carcinogenic compounds that pose serious health risks to both humans and animals. The growing resistance of fungi to conventional fungicides, alongside consumer demand for natural preservatives, has emphasized the need to identify and develop effective natural antifungal agents (Usall et al., 2016; Amiri et al., 2008). In this context, EOs have been the subject of extensive research, especially for their effectiveness against fungi that commonly affect grains and other agricultural products. For instance, Reyes-Jurado et al. (2022) demonstrated that thyme essential oil significantly inhibited the growth of *A. niger* and *Penicillium expansum* in corn tortillas using a vapor-phase method. Similarly, Střelková et al. (2021) found that thyme, oregano, lemongrass, clove, and cajeput essential oils could completely inhibit the growth of five fungal strains, including *A. niger*, when used at 125 µL/L—except for cajeput EO, with thyme EO showing the strongest effect. Other studies have also highlighted the antifungal potential of EOs. Ju et al. (2018) reported that cinnamon and

clove EOs inhibited the growth of *Penicillium* and *Aspergillus* species and extended the shelf life of green bean cake by 9–10 and 3–4 days, respectively. Anžlovar et al. (2017) showed that thyme EO could suppress endophytic fungi in wheat without impairing seed germination. Raveau et al. (2022) also confirmed the potential of clary sage and coriander seed EOs in controlling postharvest fungal decay, specifically inhibiting *Zymoseptoria tritici* and *Fusarium culmorum*. These findings underscore the promise of essential oils as natural antifungal agents in food preservation and agriculture. Among the many EOs studied, cinnamon, clove, and lemongrass oils have emerged as particularly potent candidates due to their well-documented antifungal properties. Cinnamon essential oil, which contains high levels of cinnamaldehyde, has demonstrated strong antifungal activity against *A. niger* (Xiang et al., 2020; Achar et al., 2020). Clove essential oil, extracted from the dried flower buds of *Syzygium aromaticum*, is rich in eugenol (70–85%), a phenolic compound known to disrupt fungal cell membranes, inhibit ergosterol synthesis, and induce oxidative stress (Raut & Karuppayil, 2014; Ahmad et al., 2023). Additional components such as β-caryophyllene and α-humulene may contribute synergistically to its antimicrobial activity (Jiang et al., 2022). Clove EO is widely used not only in food preservation but also in pharmaceuticals, cosmetics, and packaging due to its antiseptic, analgesic, antioxidant, and insecticidal properties (Das et al., 2022; Aguilar-González et al., 2015). However, its direct use in food systems is limited by its volatility, susceptibility to degradation, and strong aroma, necessitating advanced formulation techniques for improved stability and consumer acceptability (Guo et al., 2020). Lemongrass essential oil (LGEO), rich in citral, also exhibits broad-spectrum antimicrobial activity and has shown strong inhibitory effects against *A. niger* even at low concentrations (Xiang et al., 2020; Achar et al., 2020).

Despite their high bioactivity, essential oils are frequently not used in food directly because they are volatile, have overpowering smells, and are susceptible to environmental factors. To address these challenges, the encapsulation of essential oils in biocompatible carriers such as chitosan-based nanoparticles has been investigated. Chitosan, a natural polymer obtained from chitin, is known for its ability to form films, biodegrade, and have antibacterial qualities (Rinaudo et al., 2006). Chitosan, when synthesized into nanoparticles, enables the controlled release of active chemicals and

protects sensitive essential oil components from degradation. Encapsulating essential oils in chitosan nanoparticles may enhance their effectiveness and durability as antifungal agents. Previous studies demonstrate that nano-encapsulation enhances the stability and antibacterial efficacy of essential oils (Hasheminejad et al., 2019).

Nanoencapsulation has emerged as a highly effective technology to address these challenges (Sánchez-García et al., 2020; El Asbahani et al., 2023; Al-Maqtari et al., 2022). By encapsulating EOs in nanocarrier systems, this technique can prevent the volatile compounds from degrading and enhance their solubility and dispersion, manage their release for long-term action, and possibly reduce their strong sensory impact (Al-Maqtari et al., 2022; Hosseini et al., 2013). The natural substance chitosan, which comes from chitin, is ideal for this purpose because it breaks down naturally, works well with other chemicals, is safe to use, and has natural antibacterial and antifungal qualities. Various studies have demonstrated that incorporating various essential oils, such as peppermint, cinnamon, oregano, and thyme, into chitosan nanoparticles (CSNPs) is effective. These changes make the oils more stable at high temperatures, control how much they release, and make them more effective at killing microbes than the oils when they are used alone (Barrera-Ruiz et al., 2020; El Asbahani et al., 2023; Hosseini et al., 2013; Shetta et al., 2019). Encapsulating the EO in chitosan may reduce the cytotoxicity of essential oils while enhancing their bioactivity (El Asbahani et al., 2023). Chitosan-encapsulated clove essential oil showed stronger antifungal activity compared to free clove essential oil (Hasheminejad et al., 2019; Saeed et al., 2019).

Chitosan nanopolymers emerge as an excellent candidate for encapsulation due to their unique properties. Chitosan, a naturally occurring biopolymer derived from chitin, is biodegradable, biocompatible, and non-toxic. It has been extensively studied for its film-forming ability and potential as a carrier for controlled release systems (Rinaudo, 2006). Moreover, chitosan exhibits intrinsic antimicrobial properties that could synergistically enhance the antifungal effect of CEO (Kumar et al., 2004). The positive charge of chitosan at acidic pH also facilitates its interaction with the negatively charged cell membranes of fungi, leading to increased permeability and disruption of cellular processes (Raafat et al., 2008). Nanopolymers derived from chitosan can be designed with nanoscale dimensions, significantly increasing their

surface area and enhancing their interaction with target pathogens (Huang et al., 2021). Encapsulating clove essential oil (EO) within chitosan nanoparticles is anticipated to shield the oil from environmental degradation while enabling a sustained release profile. This approach ensures the maintenance of effective clove EO concentrations over an extended duration (Yousefi et al., 2024).

To address these constraints, chitosan-based nanoencapsulation is used. Chitosan, a biopolymer derived from chitin, is biodegradable, nontoxic, and possesses natural antifungal activity. When used to form nanoparticles (CSNPs), chitosan offers controlled release, thermal protection, and improved bioavailability of encapsulated compounds. Encapsulation using ionic gelation with sodium tripolyphosphate (TPP) allows for efficient entrapment of hydrophobic EOs like CEO (Rinaudo, 2006; Raafat et al., 2008; Pitaloka et al., 2019). Numerous studies have shown that nanoencapsulation improves the stability and antimicrobial performance of EOs, including cinnamon, thyme, oregano, and peppermint (Hosseini et al., 2013; Barrera-Ruiz et al., 2020; El Asbahani et al., 2023). Specifically, CEO-CSNPs were found to have higher antifungal efficacy and stability than free EO, especially under stress conditions (Hasheminejad et al., 2019; Saeed et al., 2019).

While several studies have successfully demonstrated the encapsulation of essential oils such as peppermint, cinnamon, thyme, and oregano into chitosan nanoparticles (CSNPs) to enhance their antimicrobial efficacy, limited research has specifically addressed the nanoencapsulation of clove essential oil (CEO) using chitosan and its antifungal activity against *Aspergillus niger*, a common spoilage fungus. Therefore, the objective of this study was to formulate and characterize clove essential oil-loaded chitosan nanoparticles (CEO-CSNPs) using the ionic gelation method, and to evaluate their physicochemical properties, encapsulation efficiency, and antifungal activity through in vitro assays. This investigation aims to assess the potential of CEO-CSNPs as a natural and sustainable antifungal agent for food preservation applications, particularly in reducing fungal contamination.

To identify the most effective antifungal agent for further nanoformulation, a preliminary comparative study was conducted to evaluate the inhibitory effects of clove, cinnamon, and lemongrass essential oils against *Aspergillus niger*. The agar disc diffusion method was used to assess the inhibition zones, and clove EO exhibited the

strongest antifungal activity. This superior efficacy is attributed to eugenol, the primary component of clove EO, which is known to disrupt fungal membrane permeability, inhibit ergosterol biosynthesis, and generate oxidative stress in fungal cells (Raut & Karuppayil, 2014; Ahmad et al., 2023). Based on this preliminary screening, clove EO was selected for further development and encapsulation to enhance its application in food preservation. Its efficacy, availability, and generally recognized as safe (GRAS) status make it an ideal candidate for replacing synthetic antifungal preservatives in food storage systems (FDA, 2022).

1.2 Objective

1.2.1 To synthesize and characterize clove essential oil-loaded chitosan nanoparticles (CEO-CSNPs) using ionic gelation.

1.2.2 To evaluate the antifungal efficacy of CEO-CSNPs in comparison to free clove EO and Itraconazole.

1.3 Scope and Outcomes

The research is divided into two phases to ensure a comprehensive understanding and development of EO encapsulated in chitosan nanopolymers (EO-CN).

In Phase I, encompasses the identification of the most effective antifungal essential oil against *Aspergillus niger* from Cinnamon, Clove, and Lemongrass. then synthesis phase, our objective is to establish a reliable protocol for the creation of chitosan nanopolymers and optimize the encapsulation process of essential oil within these nanocarriers. This stage will involve a thorough characterization of the EO-CN particles, utilizing advanced techniques such as SEM, FTIR, XRD, and TGA to analyze their morphology, size distribution, and chemical structure. The focus will also be to identify the most effective formulation for antifungal activity.

Transitioning to Phase II, the evaluation phase, we will test the antifungal efficacy of EO-CN against *Aspergillus niger*.

CHAPTER 2

LITERATURE REVIEW

2.1 Aflatoxin Contamination

2.1.1 Understanding Aflatoxin Contamination

Recent scholarly investigations have significantly advanced our understanding of aflatoxin contamination and its extensive health risks, particularly emphasizing the pernicious effects of AFB1, a toxin produced by *Aspergillus flavus*. AFB1's carcinogenic properties have been well-documented, with its consumption through contaminated food products being strongly associated with acute hepatitis and heightened cancer risk in crucial organs like the stomach, lungs, and liver (Wang et al., 2024). This issue transcends geographical and demographic boundaries, presenting a global health challenge.

Aflatoxin contamination by *Aspergillus* fungi is a critical concern in peanut safety. The existing research identifies three primary mechanisms of resistance: shell infection resistance, seed infection resistance, and aflatoxin production resistance. However, there has been less effort to explore the differentiation and genetic relationship among these resistances in diversified peanut germplasm collections. This gap suggests a need for more comprehensive studies on the combined efficacy of these resistance mechanisms, especially concerning EO-CN's role.

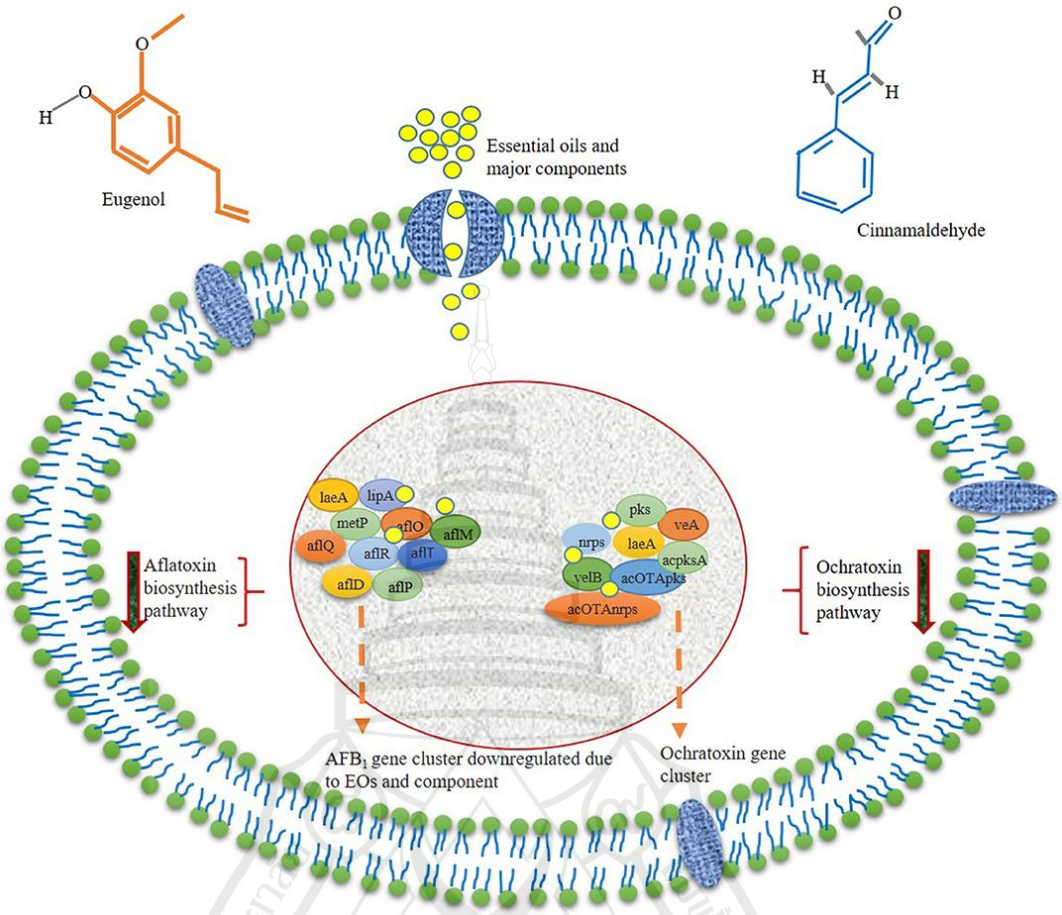
Beyond cancer, aflatoxin exposure has been implicated in teratogenic and immunosuppressive effects. The teratogenic impact can result in developmental anomalies in fetuses, a significant concern during pregnancy. Moreover, the immunosuppressive properties of AFB1 heighten vulnerability to infections and diseases (Li et al., 2019; Prakash et al., 2018). The widespread nature of fungal infections, especially in staple food items like groundnuts (*Arachis hypogaea L.*), accentuates the issue, as these crops are particularly prone to AFB1 contamination under certain storage conditions (Girardi et al., 2017). The implications extend to groundnut byproducts and vegetable oils, widely consumed globally, thus elevating

health concerns (Tan et al., 2015). Additionally, the link between high levels of AFB1 ingestion and acute hepatitis has been estimated to affect approximately 4 billion people worldwide (Williams et al., 2004), underlining the urgency of addressing this health hazard.

In the broader context, mycotoxins, a class of low-molecular weight chemical compounds produced as secondary metabolites by filamentous fungi, present significant risks to human and animal health (Bennett & Inamdar et al., 2015; Zain et al., 2011). These compounds are prevalent in environments conducive to mold growth, typically in warm and humid climates (Richard, et al., 2007). The adverse effects of mycotoxins, such as mycotoxicosis, are caused by dietary, respiratory, or dermal exposure. Research has primarily focused on various mycotoxins, including aflatoxins, citrinin, ergot alkaloids, and others, due to their severe health impacts (Hussein et al., 2001).

2.1.2 Aflatoxin Management and Regulatory Measures

Aflatoxins, notably, are classified as Group 1 carcinogens by the International Agency for Research on Cancer (IARC) (Richard, et al., 2007). The U.S. Food and Drug Administration (FDA) has set stringent limits on total aflatoxin concentration in food products and animal feed (Richard, et al., 2007; Bennett & Inamdar, et al., 2015). Developed countries allocate substantial resources for managing and mitigating aflatoxin contamination in crops, employing land management, cultivation, and storage techniques to minimize exposure risks (Khlangwiset & Wu et al., 2010; Mitchell et al., 2016; N'dede et al., 2012). Contrastingly, the situation in developing countries, particularly in Africa and Southeast Asia, is more challenging due to inadequate infrastructure and resources to combat aflatoxin contamination. This issue exacerbates liver disease incidence and hinders these nations' ability to participate effectively in international trade markets (Lauvergeat et al., 2001). Thus, there is an urgent need to deepen our understanding of aflatoxins, their biosynthesis, and strategies to reduce their presence in food crops.



Source Maurya et al. (2021)

Figure 2.1 Possible mechanisms of mycotoxin inhibition by interacting gene transcripts

2.2 Advancements in the Management of Fungal Contamination and Aflatoxin

2.2.1 Strategies for Controlling Fungal Contamination and Aflatoxins

Numerous methodologies, both physical and chemical, have been employed in the mitigation of fungal contamination and aflatoxins (AFs) in stored cereal grains. Aflatoxins, known for their heat stability, pose significant challenges in eradication through thermal treatments, with their decomposition temperatures ranging between 237 and 306 °C. Specifically, aflatoxin B1 remains stable under dry heat conditions below its decomposition point. However, heating at such high temperatures can degrade the nutritional value of the grains and produce an acrid odor, as noted by Lewis et al.

(2004). Alternative methods, such as ozone treatment, have been explored for AFs mitigation, as documented by Womack et al. (2014). Nevertheless, this approach may result in nutritional degradation of the cereals and raise additional safety concerns. Ultraviolet light application, another proposed method, encounters limitations due to its narrow wavelength range, limited penetration depth, and potential residual toxicity, as observed by Pankaj et al. (2018). Recent advancements include the use of cold atmospheric air plasma treatment for AFs reduction, but its long-term application in food industries is limited due to interactions between plasma gas reactive species and food components, a concern highlighted by Pankaj et al. (2014). Moreover, the precise mechanisms of AFs degradation in these methods remain insufficiently understood, precluding their recommendation for widespread industrial application.

While various strategies, including the use of biological agents and resistant cultivars, are employed to control aflatoxin contamination in peanuts, the effectiveness of these methods in different environmental conditions is a crucial area for further research. Understanding how EO-CN might integrate with these approaches, particularly in varied environmental conditions and stages of peanut growth, remains a significant research gap. Previous studies have identified peanut cultivars and germplasm with resistance to *A. flavus* infection and aflatoxin production. However, systematic evaluation and discovery of resistance for shell infection are less explored. Additionally, the inheritance of resistance to *A. flavus* infection and toxin production in peanuts has been studied through quantitative trait loci (QTL) mapping and genome-wide association studies (GWAS). These studies reveal different loci for seed infection and aflatoxin production resistance, indicating the potential for breeding strategies that combine different resistances. This area can be expanded upon to explore how EO-CN might interact with genetically identified resistance mechanisms (Ding et al., 2022).

2.2.2 Empirical Evidence Supporting the Efficacy of Essential Oils

In response to these challenges, there has been a growing interest in utilizing plant products, considered safer for humans, non-target organisms, and the environment. Essential oils (EOs), derived from plants through secondary metabolism, have garnered attention due to their traditional medicinal and culinary uses and general acceptance. As (Pavela & Benelli, 2016) noted, EOs protect plants against pathogenic microorganisms, environmental stress, and insects. Recognized as safe by the US-FDA

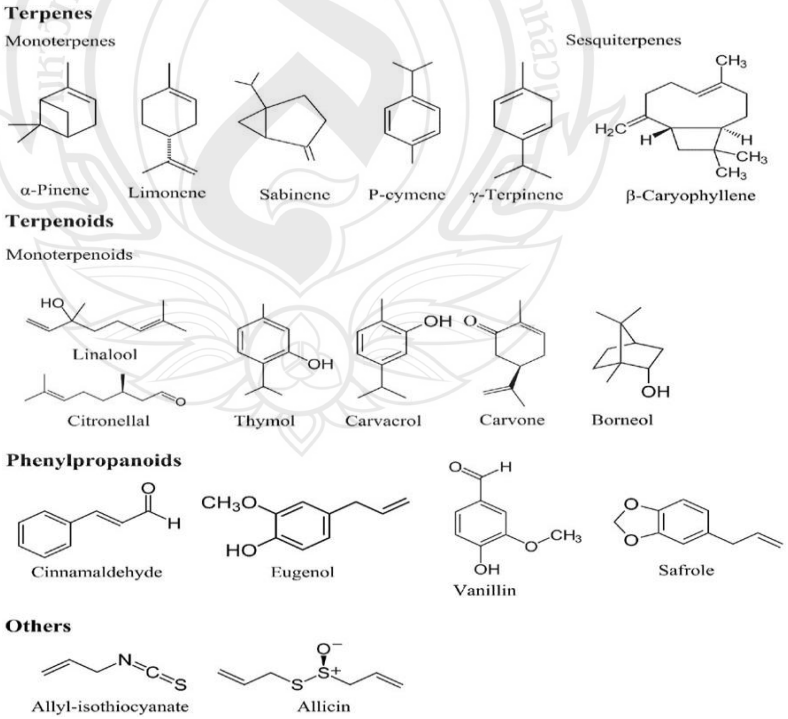
under the generally recognized as safe (GRAS) category, various EOs have been investigated for their antifungal and anti-mycotoxigenic properties, as reported by (Chaudhari et al., 2018; Chaudhari et al., 2021). These oils offer potential as preservatives for protecting cereal grains from fungi and AFs-induced deterioration. These oils offer potential as preservatives for protecting cereal grains from fungi and AFs-induced deterioration, as well as extending their shelf life.

Several studies exemplify the efficacy of EOs in this context. (Paranagama et al., 2003) demonstrated the anti-aflatoxigenic effectiveness of EO from *Cymbopogon citratus* against *A. flavus* in postharvest rice samples. (Razzaghi-Abyaneh et al., 2008; Kedia et al., 2014) further confirmed the potential of EOs from *Satureja hortensis L.* and *Cuminum cyminum L.*, respectively, in reducing aflatoxin synthesis. (Pavela & Benelli, 2016; Nerilo et al., 2020; Chaudhari et al., 2018; Chaudhari et al., 2020) have reported on the anti-aflatoxigenic activities of EOs from *Ziziphora clinopodioides Lam*., *Zingiber officinale Roscoe*., *Pimenta dioica*, and *Melaleuca cajuputi Powell*, respectively, in various cereal grains. Similarly, (Das et al., 2018; Das et al., 2019) explored the efficacy of *Myristica fragrans Houtt*. and a formulation comprising *Apium- graveolens L.* EO against AFB1 production in stored rice. These investigations affirm the potential of EOs as effective preservatives against hazardous mycotoxins in major stored cereal grains.

2.2.3 Essential Oils (EOs) and its Bioactive Compounds

Essential oils are primarily derived from aromatic plants, including lavender, lemon, cinnamon, clove, peppermint, and eucalyptus (Sharma et al. 2021). As a bioactive antibacterial agent, it presents superior antibacterial activity, inoxidizability, and unique aroma, giving it higher safety and acceptance than conventional chemical antibacterial agents such as benzoic acid, sorbate, and nitrate (Ozcan et al., 2023). Research efforts have been directed towards exploring the antifungal properties of EOs, especially against fungi commonly found in grains and agricultural products. For instance, Reyes-Jurado et al. (2022) investigated the efficacy of thyme EO in inhibiting the growth of *Aspergillus niger* and *Penicillium expansum* in corn tortillas using a vapor-phase approach, revealing promising results in slowing down the growth of *A. niger* (Reyes-Jurado et al., 2022). Cinnamon, Clove, and Lemongrass essential oils emerge as promising candidates due to their documented antifungal activities.

Cinnamon Essential Oil, rich in cinnamaldehyde, has garnered attention for its strong antifungal properties, particularly against *Aspergillus flavus*, highlighting its potential as a natural alternative to chemical fungicides (Xiang et al., 2020; Achar et al., 2020). Clove Essential Oil (Clove EO), with eugenol as its primary component, disrupts microbial cell membranes and exhibits a significant inhibitory effect on a variety of fungal pathogens, including *A. flavus* (Pei et al., 2009; Ahmad et al., 2023). Lemongrass Essential Oil (LGEO), known for its citral content, offers broad-spectrum antimicrobial activities, effectively inhibiting the growth of *A. flavus* at low concentrations (Xiang et al., 2020; Achar et al., 2020). This study aims to compare the antifungal efficacy of these four essential oils against *Aspergillus flavus* to identify the most potent EO. The selected EO will then be encapsulated using chitosan nanopolymer, a strategy anticipated to enhance the oil's stability, solubility, and controlled release properties. Encapsulation is expected to mitigate the limitations associated with the direct application of EOs, such as volatility and potential sensory impact on food products, thereby offering a novel approach to food preservation that leverages the natural antifungal capabilities of essential oils in a more effective and consumer-acceptable manner (Mukurumbira et al., 2022; Shi et al., 2022).



Source Guenther (1950)

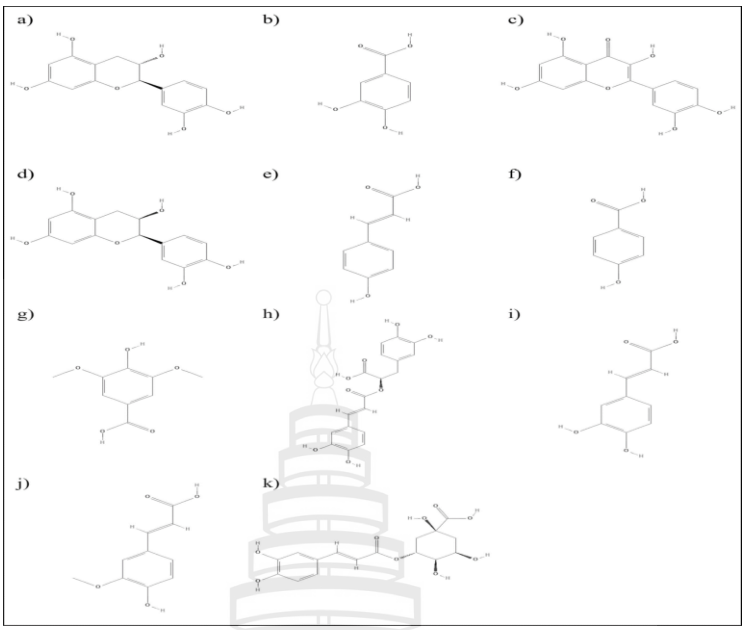
Figure 2.2 EOs representative chemical structures

2.2.3.1 Cinnamon Essential Oil

Cinnamon essential oil, extracted from the bark of *Cinnamomum verum*, has garnered attention for its potent antifungal properties, particularly against *Aspergillus flavus*, a notorious pathogen known for food spoilage and mycotoxin production. The efficacy of cinnamon EO in inhibiting fungal growth has been substantiated by various studies, which highlight its potential as a natural alternative to chemical fungicides in agricultural and food preservation applications. Cinnamon essential oil was identified as possessing a robust antifungal effect, especially notable against the AO-SLO-B-243 and AW-SLO-B-249 strains of *Aspergillus*, as evidenced by high R^2 values. This effect suggests a highly effective mechanism of action in inhibiting mycelial growth. Císarová et al. (2020) further observed that cinnamon oil required the lowest MID50 and MID90 values to inhibit growth on bread samples, particularly against the AF-SLO-B-201 strain, indicating its efficacy at relatively lower concentrations. Moreover, the oil achieved 100% inhibition of spore germination at 500 μL/L of air, highlighting its significant potential in preventing fungal development at the earliest stages. Research conducted by Xiang et al. (2020) demonstrated cinnamon EO's remarkable antifungal capabilities, reporting complete inhibition of *Aspergillus flavus* at minimal concentrations. The study noted that as little as 1 μL/disc of cinnamon EO was sufficient to halt fungal growth, showcasing its superiority over other essential oils tested. This robust efficacy is attributed to cinnamon EO's high content of cinnamaldehyde, which constitutes 89.33% of the oil shown in Figure 2.3. Cinnamaldehyde is renowned for its antimicrobial properties, capable of disrupting fungal cell membranes and inducing cell lysis and death. Such findings are echoed in the works of Achar et al. (2020), who observed significant antifungal activity of cinnamon EO against *A. flavus* in peanuts, with complete mycelial growth inhibition at concentrations ≤2000 ppm and at least 90% inhibition at 1000 ppm. This places cinnamon EO among the most effective essential oils in terms of antifungal efficacy, especially in the context of peanut preservation. The antifungal properties of cinnamon EO are attributed to a combination of active chemicals, including phenols, aldehydes, and terpenes. The synergy of these compounds contributes to the overall therapeutic properties of cinnamon EO, underscoring the importance of concentration and quality of these components (Attallah et al., 2020; Sun et al., 2021). Gas chromatographic

analysis has further identified cinnamaldehyde as the predominant compound in cinnamon EO, followed by benzyl alcohol and triacetin, among others, which aligns with the observed antifungal activity.

Research indicates that cinnamon EO can halt the growth of *A. flavus* mycelia at concentrations as low as 0.05 to 0.10 mg/mL, with the fungicidal concentration range being between 0.05 and 0.20 mg/mL (Ruolan et al., 2022; He, 2018). The antifungal efficacy of CEO is attributed to its major components, cinnamaldehyde and citral, which are known to cause considerable cellular damage to the fungus, including disruption of the fungal cell membrane and internal organelles, as observed under a scanning electron microscope (SEM) (Ruolan et al., 2022; He, 2018). In addition to its antifungal efficacy, cinnamaldehyde, the primary active ingredient in cinnamon EO, is associated with a range of health benefits. It has been reported to possess anti-inflammatory, anti-cancer, anti-mutagenic, hypoglycemic, and hypolipidemic properties (Koppikar et al., 2010; Tung et al., 2008). These multifaceted activities highlight the potential of cinnamon EO not only as an antifungal agent but also as a valuable component in health-related applications. Cinnamaldehyde, the predominant constituent of cinnamon essential oil, has been quantified to exhibit zones of inhibition measuring up to 38.66 ± 1.24 mm against *Aspergillus flavus* and 38.00 ± 0.81 mm against *Aspergillus* fumigatus, signaling its potent antifungal activity. These metrics not only illustrate the compound's ability to inhibit fungal growth but also its potential to serve as a natural antifungal agent.



Note a) catechin, b) protocatechuic acid, c) quercetin, d) epicatechin, e) p-coumaric acid, f) p-hydroxybenzoic acid, g) syringic acid, h) rosmarinic acid, i) caffeic acid, j) ferulic acid, and k) chlorogenic acid

Source Das et al. (2022)

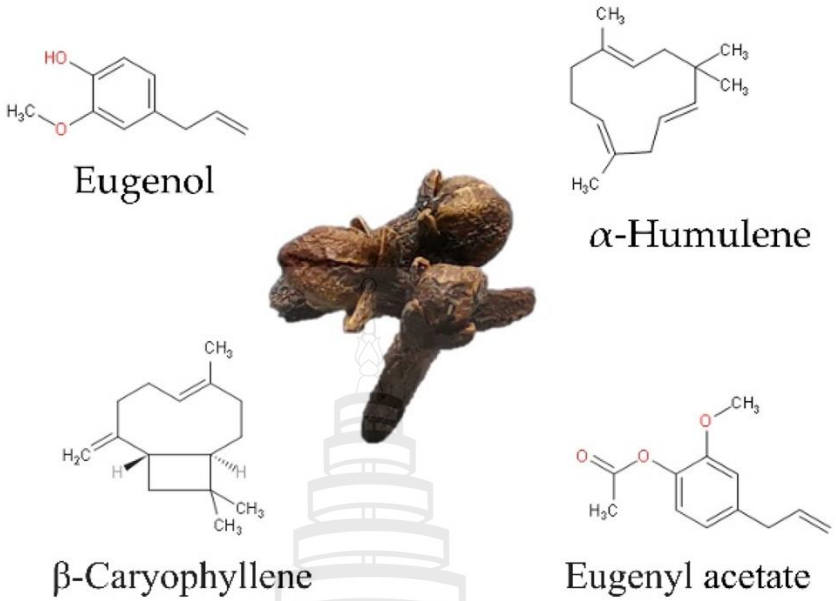
Figure 2.3 Most abundant bioactive compounds found in cinnamon bark

2.2.3.2 Clove Essential Oil (CEO)

Clove essential oil (Clove EO), which comes from *Eugenia caryophyllata*, is known for having strong antifungal properties, which are mostly due to its high eugenol content shown Figure 2.4. Eugenol, a phenolic compound, plays a pivotal role in the antimicrobial activity of clove oil, disrupting microbial cell membranes and precipitating proteins. The hydroxyl group in eugenol helps to penetrate and subsequently harm cell walls and membranes, which facilitates this disruption. Additionally, eugenol works better when combined with other parts of essential oils, such as carvacrol or thymol, which work together to break down cell membranes (Pei et al., 2009). Eugenol, the main component of clove oil, has similarly demonstrated formidable antifungal properties. Specifically, eugenol presented zones of inhibition of 31.33 ± 1.69 mm against *A. flavus* and 28.66 ± 0.94 mm against A. fumigatus. Such findings are indicative of eugenol's capacity to effectively disrupt fungal proliferation. Further, the minimal inhibitory concentration (MIC) for eugenol against *A. flavus*

stands at 320 μg/ml, with a minimum fungicidal concentration (MFC) of 640 μg/ml, denoting the concentration required to not only inhibit growth but also to eradicate the fungal cells. Clove EO is particularly effective against *Aspergillus flavus* due to its potent antifungal properties. Eugenol, the primary bioactive constituent of clove EO, is largely responsible for its effectiveness. By disrupting the fungal cell wall and membrane, eugenol induces the release of cellular contents and obstructs key enzymes for fungal growth (Ahmad et al., 2023). Due to its high antifungal properties, clove essential oil is an effective agent for preventing fungal contamination in a variety of environments. Achar et al., further observed that at a concentration of 500 ppm, clove oil exhibited complete inhibition of *A. flavus* mycelial growth, with more than 90% inhibition observed at 250 ppm. This efficacy surpasses that of the commercial fungicide, prothioconazole, used as a positive control in the study. The high antifungal activity of clove oil can be attributed to its major components, as revealed by GC-MS analysis. Eugenol, constituting 83.25% of the oil, alongside E-caryophyllene (13.36%) and α-humulene (2.18%), are the principal bioactive compounds that confer its robust antifungal properties (Achar et al., 2020).

The comprehensive analysis conducted by Allizond et al. (2023), along with contributions from Cano et al. (2017), Moritz et al. (2020), Wan et al. (2020), Purkait et al. (2020), Kurkina et al. (2020), and Achar et al. (2020), elucidates the antifungal prowess of clove essential oil, attributing it primarily to its eugenol content. These findings advocate for the broader application of Clove EO, from food preservation to potential health benefits, highlighting its significance as a natural, effective solution in combating fungal contamination and enhancing agricultural sustainability. Císarová et al. (2020) report on the inhibitory effects of clove essential oil, highlighting its moderate effectiveness against mycelial growth in *Aspergillus* strains. The study's regression data, indicating R^2 values, suggest a consistent antifungal action, underscoring the oil's capacity to inhibit fungal proliferation. The minimum inhibitory doses (MID) required for clove oil to affect growth in the vapor phase were found to be comparable to those of thyme and oregano oils, with an effectiveness threshold at 31.25 μL/L of air. Particularly noteworthy is clove oil's capacity to halt spore germination entirely at a concentration of 500 μL/L of air, suggesting its potent preventive capabilities against the initiation of fungal growth from spores.



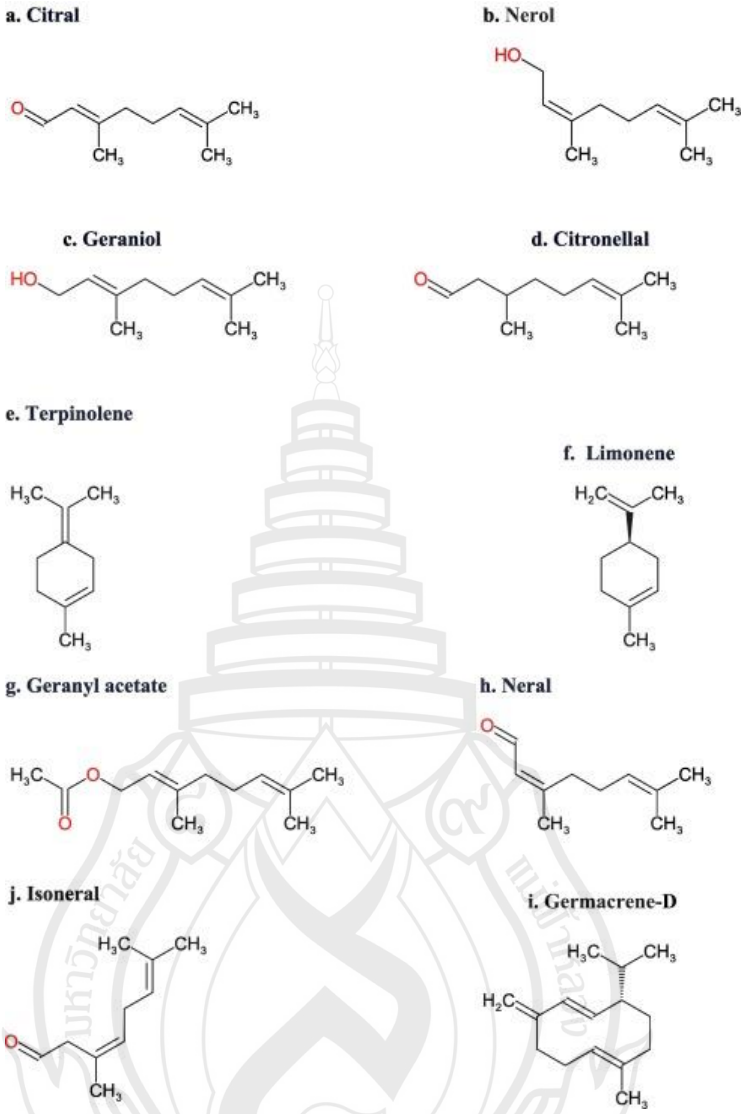
Source Haro-Gonzálezet al. (2021)

Figure 2.4 Chemical structure of main compounds of clove (S. aromaticum L.) essential oil

2.2.3.3 Lemongrass Essential Oil (LGEO)

Lemongrass essential oil (LGEO), which is extracted from the *Cymbopogon* species' leaves, is renowned for its powerful antibacterial and antifungal qualities. The potential natural agent's effectiveness against several fungal infections, with a specific focus on *Aspergillus flavus*, establishes it as a highly promising substance for medical and food preservation purposes. Lemongrass essential oil has been recognized for its strong antifungal capabilities, particularly against fungi such as *Candida albicans* and *Aspergillus niger*. Studies have shown that lemongrass EO's effectiveness improves with increased volumes, suggesting a dose-dependent mechanism of action (Mohamed et al., 2014). Further research has highlighted its potential to disrupt established fungal biofilms on silicone surfaces, commonly used in medical devices, underscoring its utility in medical settings to combat fungal infections (Natdhanai et al., 2021). Studies by Xiang et al. (2020) and Achar et al. (2020) have highlighted LGEO's potent antifungal activity, with complete growth inhibition of *A. flavus* observed at concentrations as low as 10 µL/disc. This remarkable efficacy is attributed to LGEO's primary components such as limonene, (Z)-citral, and (E)-citral, known for their strong

antimicrobial properties shown Figure 2.5. These compounds likely work in synergy to enhance LGEO's antifungal effects, providing a robust defense against fungal biofilms, which are particularly challenging in medical settings due to their resistance to conventional treatments. Lemongrass EO exhibited significant antifungal activity, with complete growth inhibition of *A. flavus* observed at 10 µL/disc. Its antifungal action is attributed to its main components—limonene and citrals ((Z)-citral and (E)-citral)—which possess antimicrobial properties. The ability of lemongrass EO to inhibit fungal biofilms, especially relevant in medical settings, highlights its broader applications beyond food preservation (Xiang et al., 2020). Lemongrass essential oil, similarly, to cinnamon oil, completely inhibited the mycelial growth of *A. flavus* at concentrations ≤2000 ppm. At 1000 ppm, lemongrass EO achieved at least 90% inhibition, showcasing its substantial antifungal activity and reinforcing its potential as an effective natural preservative agent (Achar et al., 2020). Císarová et al. (2020) examined lemongrass essential oil, revealing its substantial antifungal activity against *Aspergillus* mycelial growth, with regression analysis demonstrating significant variance in growth inhibition attributable to the oil's presence. Lemongrass oil exhibited remarkable antifungal strength, particularly in the vapor phase, where low MID values indicated high efficacy at minimal concentrations. Additionally, this oil was found to be exceptionally potent in spore germination inhibition, achieving 100% effectiveness across all concentrations and strains tested, underscoring its powerful preventive action against fungal growth initiation.



Source Ashaq al. (2024)

Figure 2.5 Chemical structures of bioactive compounds identified in lemongrass Essential oil

2.2.4 A Potent Antifungal Alternative with Diverse Mechanisms of Action

The bioactive components of Cinnamon essential oil is rich in cinnamaldehyde, eugenol, and linalool, which are responsible for its strong antifungal and antimicrobial properties. According to Bakr et al. (2024), cinnamon essential oil can effectively stop the growth of *Aspergillus flavus* because it contains a lot of cinnamaldehyde. The principal bioactive compound in cinnamon EO, cinnamaldehyde, is identified as the major constituent responsible for its antifungal and antimicrobial activities. Studies by

Ruolan et al. (2022) and He (2018) have shown that cinnamon EO can effectively halt the growth of *A. flavus* mycelia at concentrations ranging from 0.05 to 0.10 mg/mL, with fungicidal concentration ranges between 0.05 and 0.20 mg/mL. The mechanism of action involves considerable cellular damage to the fungus, including disruption of the fungal cell membrane and internal organelles, as confirmed through scanning electron microscopy (SEM) (Li et al., 2021). Further research highlights the synergy between cinnamaldehyde and other essential oils or their components, such as thymol, which enhances the antimicrobial effectiveness by increasing the permeability of bacterial membranes. This facilitates the entry of antimicrobial agents, leading to a significant reduction in microbial viability (Pei et al., 2009). Císarová et al. (2016) found that the oil had a strong fungicidal impact on a specific fungus strain at specified vapour phase concentrations. Cinnamaldehyde, the main component of cinnamon oil, is believed to disrupt the energy generation processes of fungal cells and prevent spore germination, thus limiting fungal growth. Císarová et al. (2016) revealed that the minimum inhibitory doses (MIDs) for cinnamon essential oil against *A. flavus* KM-202-LR were 31.5 µL/L of air.This suggests a high potency and efficiency of cinnamon oil as an antifungal agent, which could be utilized in lower concentrations to achieve desired outcomes. Manso et al. (2013) supported these results by showing that cinnamon oil is a potent antifungal agent when in direct contact with fungal cultures. This was demonstrated by its low Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values calculated using macrodilution techniques.

Eugenol, eugenyl acetate, and caryophyllene are the three main bioactive components of clove essential oil. These compounds contribute to its pronounced antifungal and antibacterial activities, particularly against *Aspergillus flavus*. The chemical composition of clove essential oil has been meticulously analyzed, revealing a predominance of eugenol (78.91% v/v), followed by eugenyl acetate (11.64% v/v) and β-caryophyllene (6.04% v/v), with minor components including α-humulene and α-copaene. Clove essential oil's strong antifungal effectiveness is supported by its diverse range of plant chemicals, particularly against *Aspergillus* strains. Studies have shown Minimum Inhibitory Concentration (MIC) values ranging from 0.56 to 2.25 mg/mL (Allizond et al., 2023). Eugenol's electrophilic nature, particularly its carbonyl

group, plays a key role in breaking down fungal cell walls, resulting in cellular and mitochondrial harm, finally leading to the death of the fungus (Allizond et al., 2023). The clove EO's potent inhibitory action with MIC values demonstrates its antifungal efficacy by showing its capacity to inhibit a variety of *Aspergillus* species. According to Minimum Fungicidal Concentration (MFC) values slightly above MIC values, clove EO has the ability to kill fungi, making it a complete solution for fungal infections. The disc-diffusion experiment proved that the clove EO has strong antifungal properties, showing that it stops fungus growth in a way that gets stronger as the oil concentration rises. (Allizond et al., 2023). Clove EO has been shown to be effective against *Aspergillus* species, particularly as demonstrated in a study by Císarová et al. (2016). After 14 days of exposure to an air concentration of 500 μL/L, the development of all fungi was halted. These results align with eugenol's mechanism of action against fungus, which includes disrupting cell membranes and inhibiting enzymes in fungal cells. Eugenol is a promising natural compound for controlling fungal growth, as confirmed by the literature on the antifungal properties of clove oil (Císarová et al., 2016). Debonne et al. (2019) demonstrated that a very small amount of clove oil (0.05%) significantly extended the lag phase of *Eurotium spp*. in their micro-dilution studies. Clove oil was found to have an inhibitory impact based on predictive growth/no-growth models. They demonstrated that it had the lowest inhibitory dosages required to halt development by 50% in all trials. In macro-dilution studies, clove essential oil has shown potent antifungal properties, with an inhibitory concentration range of 0.02% to 0.05%.

Lemongrass essential oil is primarily composed of citral (a mix of geranial and neral), myrcene, and limonene. Citral is mainly responsible for its antifungal effect on *Aspergillus flavus*. It breaks down fungal cell membranes and stops spores from germinating. The chemical composition of LGEO, as detailed by Valková et al. (2022), reveals a complex mixture of volatile compounds with citral (a combination of geranial and neral) being the predominant constituent. This composition is critical to LGEO's antimicrobial activity, with citral known for its ability to disrupt microbial cell membranes and inhibit enzymatic activities within pathogenic cells. The presence of geraniol and 1,8-cineole further enriches LGEO's antimicrobial spectrum, offering growth-inhibitory properties and the capability to compromise the integrity of

cytoplasmic membranes. These compounds do not act in isolation but may interact synergistically to enhance LGEO's overall antimicrobial effect. This synergistic interaction potentially allows for lower effective concentrations in applications, optimizing the use of LGEO in food preservation and therapeutic contexts while minimizing sensory impacts. LGEO's robust antioxidant potential, as demonstrated by Valková et al. (2022) using the DPPH radical scavenging assay, suggests strong free radical neutralizing abilities. This antioxidant activity underlines LGEO's potential therapeutic applications beyond its antimicrobial effects, contributing to its appeal as a natural compound with multifaceted health benefits. Lemongrass EO, on the other hand, demonstrated remarkable inhibitory activity, with MICs spanning from 0.56 to 1.12 mg/mL across all tested strains of *Aspergillus spp*. This EO was particularly effective against *A. flavus*, as well as some strains of A. fumigatus and A. niger, indicating its broad-spectrum antifungal activity. The lowest MIC recorded at 0.56 mg/mL suggests lemongrass EO's strong potential as a natural antifungal agent, likely due to its citral content, a known antimicrobial agent. (Allizond et al., 2023). Lemongrass essential oil showed antifungal properties, but required larger concentrations compared to clove oil. Debonne et al. (2019) found that concentrations over 0.5% were required in the micro-dilution experiment to inhibit the development of Eurotium spp. Cross-well effects demonstrated a potent antifungal impact on these levels. Apricot agar diffusion experiments indicated that lemongrass oil concentrations of 0.200% were required to inhibit fungal growth efficiently (Debonne et al., 2019).

2.2.5 Innovative Applications

Cinnamon essential oil is highly effective as an antifungal agent, often requiring lower concentrations to inhibit growth compared to other plant-derived compounds. This underlines its potential application as a natural preservative in the food industry, especially in products susceptible to *Aspergillus flavus* contamination (Debonne et al. 2019). Beyond its antifungal capabilities, CEO is known for its broad spectrum of health benefits. Cinnamaldehyde, its main active ingredient, exhibits anti-inflammatory, anti-cancer, and anti-mutagenic properties, among others (Koppikar et al., 2010; Tung et al., 2008). These multifaceted health benefits position CEO as a valuable natural product with applications extending beyond food preservation to include potential therapeutic uses. Based on their safety profile, these compounds'

hemolytic properties suggest that eugenol and cinnamaldehyde do not significantly break down red blood cells in mammals at the concentrations needed to kill fungi. For instance, eugenol exhibited a relatively low percentage of hemolysis of red blood cells (RBCs), at 1.65 ± 0.14% at 36 μg/ml, gradually increasing to 4.78 ± 0.25% at 576 μg/ml. This gradation reflects a measure of biocompatibility that is essential for therapeutic use, hinting at the feasibility of these compounds to be employed in medical treatments without adverse hemolytic effects.

Clove essential oil is widely used in dental care products for its analgesic and antiseptic properties. It also finds applications in food preservation and as an antifungal agent in agricultural practices. Beyond its antifungal capabilities, Clove EO is known for its array of health benefits, including anti-inflammatory, antioxidant, and analgesic properties. These benefits, coupled with its antifungal activity, make Clove EO a versatile natural product with potential applications in food preservation, pharmaceuticals, and as a natural remedy for various ailments. The broad-spectrum antimicrobial activity of Clove EO, particularly against fungi and bacteria, further enhances its utility as a natural preservative and protective agent in the food and beverage industry (Pei et al., 2009; Ahmad et al., 2023). Studies have highlighted the synergistic effects of clove oil with other natural compounds, enhancing its antifungal effectiveness. This synergy, along with CEO's rich eugenol content, positions it as a valuable alternative to synthetic fungicides in agricultural practices and food safety applications. Clove EO has shown promising results in reducing fungal contamination in food products, particularly cereal grains susceptible to fungal contamination and mycotoxin production. Its application extends the shelf life of perishable food items by inhibiting mold growth, underscoring its utility in food preservation (Cano et al., 2017; Moritz et al., 2020; Wan et al., 2020; Purkait et al., 2020; Kurkina et al., 2020; Achar et al., 2020).

Lemongrass essential oil application extends to aromatherapy for its calming effects, as a natural fungicide in agriculture, and as a food preservative due to its antimicrobial properties. LGEO demonstrates significant antifungal activity, making it a promising candidate for food preservation applications. Allizond et al. (2023) investigated LGEO's antifungal efficacy against *Aspergillus spp*., highlighting its broad-spectrum activity against various strains. The study reported remarkable

inhibitory activity, particularly against *A. flavus, A. fumigatus, and A. niger,* underscoring LGEO's potential as a natural antifungal agent. Moreover, Valková et al. (2022) explored LGEO's effectiveness as a natural preservative against Penicillium species in diverse food matrices. Their research revealed LGEO's concentration-dependent antifungal activity, with optimal inhibitory effects achieved at higher concentrations. Notably, LGEO's antifungal potency varied across different food substrates, suggesting the influence of food matrix characteristics on its preservative efficacy.

The studies conducted on clove and lemongrass essential oils demonstrate their potential as effective antifungal agents. Clove oil, in particular, stands out for its ability to inhibit fungal growth at lower concentrations compared to lemongrass, across various assays. These findings are significant for applications in food preservation, where the use of natural antifungals is increasingly desirable. The data and models provided by Debonne et al. (2019) serve as a basis for understanding the efficacy of these oils and their potential utility in preventing food spoilage caused by *Eurotium* spp. Comparatively, clove essential oil was more effective than lemongrass oil in inhibiting *Eurotium* spp. growth, suggesting its superior suitability as a natural preservative in food products. The predictive models and assays indicate that clove oil has the potential to be used in lower concentrations, which may be advantageous in maintaining the organoleptic properties of food while providing adequate protection against fungal spoil (Debonne et al., 2019).

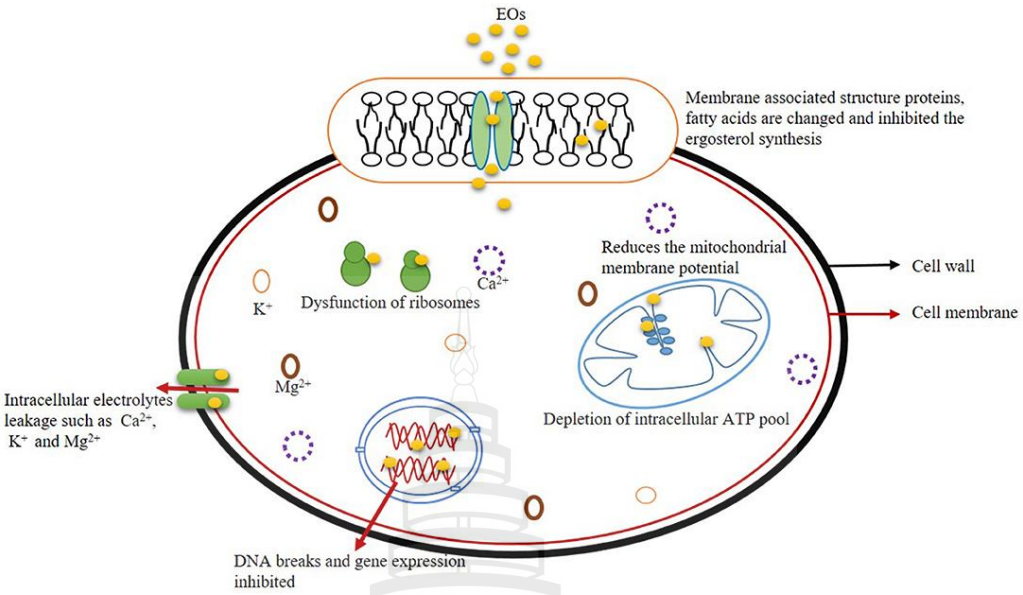
2.2.6 EO's Antifungal Mechanisms

The antifungal properties of cinnamon essential oil are attributed to its rich content of cinnamaldehyde, which disrupts fungal cell membranes, leading to cell lysis and death. The compound's mechanism involves interfering with the fungus's ability to synthesize essential components of its cell wall, thereby inhibiting growth and reproduction. Research by Attallah et al. (2020); Sun et al. (2021) supports the notion that the therapeutic properties of CEO, including its antifungal effects, are largely due to the synergistic action of its components, particularly phenols and terpenes. This synergy enhances CEO's ability to penetrate fungal cell walls and disrupt cellular functions, leading to the inhibition of fungal growth. Cinnamaldehyde, the main active component in cinnamon essential oil, has shown strong antifungal properties due to its

complex methods of action. Cinnamaldehyde has a synergistic impact with fluconazole against *Aspergillus fumigatus*, a major human disease. The synergy is demonstrated by a significant decrease in fluconazole's minimum inhibitory concentration (MIC) from 200 μg/mL to 25 μg/mL in vitro, indicating an improved effectiveness of the antifungal therapy when paired with cinnamaldehyde. This discovery suggests that cinnamaldehyde might weaken fungal defenses, making A. fumigatus more vulnerable to fluconazole. Cinnamaldehyde has been shown to stop important enzymes, like chitin synthase and β-1,3-glucan synthase, from building the cell walls of fungi. These enzymes are needed to keep the structure of fungus cells intact (Bang et al., 2000). The primary antifungal mechanism of cinnamon essential oil is the disruption of the fungal cell membrane, leading to cell disintegration. Cinnamaldehyde has been demonstrated to disrupt the electron transport chain and cellular respiration in fungal cells (Bakr et al., 2024). The efficacy of CEO against *A. flavus* is concentration-dependent, with studies indicating complete mycelial growth inhibition at concentrations as low as ≤2000 ppm (Achar et al., 2020). This suggests that CEO can be effectively utilized in minimal amounts, making it a cost-effective solution for controlling fungal contamination in various substrates, including food products like peanuts. Moreover, the fungicidal concentration range of CEO has been identified to be between 0.05 and 0.20 mg/mL, highlighting its potent antifungal activity at relatively low concentrations (Ruolan et al., 2022; He, 2018).

The primary bioactive constituent of Clove EO, eugenol, plays a pivotal role in its antifungal mechanism. Eugenol's efficacy is largely due to its ability to penetrate and disrupt the fungal cell wall and membrane, leading to the leakage of cell contents and eventual cell death. This action not only inhibits the growth of *A. flavus* but also prevents the formation of aflatoxins, enhancing the safety and shelf life of food products. The mechanism of action of eugenol and, by extension, Clove EO, highlights the importance of targeting the structural integrity of fungal cells as a means of controlling fungal contamination (Ahmad et al., 2023). Eugenol, a main component of clove oil, has been shown to damage the structural integrity of the fungal cell wall in *Aspergillus flavus.* Thymol, carvacrol, and eugenol, phenolic chemicals present in several essential oils, demonstrate antifungal effects by disrupting ion balance in the fungal cell membrane. This imbalance occurs due to the dissipation of H+ and K+ ion

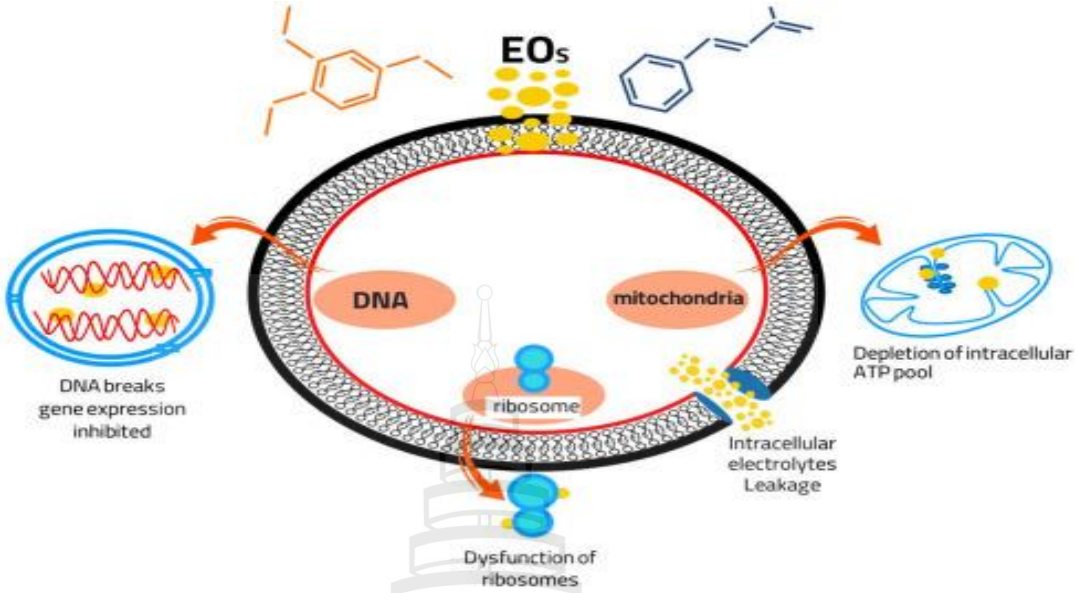
gradients, causing the leaking of essential cellular components and resulting in water stress, as well as intracellular ATP depletion (Dwivedy et al., 2016). The pH gradient across the cytoplasmic membrane is lowered by these chemicals, which work as proton exchangers. This breaks down the proton motive force and lowers the ATP pool. These actions can result in the release of iron and other components from inside the cell, leading to cell death (Ultee et al., 2002). Eugenol works by damaging the integrity of the fungal cell wall and membrane. This lets the contents of the cell leak out and stops enzymes from doing important jobs that are needed for the fungal to grow. Additionally, it demonstrates antioxidative characteristics that enhance its effectiveness against microorganisms (Ahmad et al., 2023). The antifungal mechanisms of clove oil are primarily attributed to its high eugenol content, which disrupts fungal cell membranes and interferes with enzyme function (Debonne et al., 2019). Future research should explore the potential synergistic effects of combining clove and lemongrass oils with other natural antifungal compounds to enhance their efficacy and reduce the concentrations needed, which could lead to cost-effective and consumer-acceptable food preservation solutions. Clove essential oil's profound impact on the ultrastructure of *Aspergillus flavus* has been vividly depicted through electron microscopy studies conducted by Achar et al. (2020). These studies have shown that clove oil, at a concentration of 500 ppm, causes severe cellular damage, disrupting the integrity of fungal conidia and hyphae. TEM imaging revealed significant alterations such as disintegration of conidial surfaces, hyphal wall thinning, vacuolization, and destruction of organelles, leading to a loss of fungal viability. SEM analysis supported these findings, illustrating blistering of hyphae and deformation of conidiophores, indicative of clove oil's penetration and disruption of cellular structures vital for fungal growth and reproduction. According to Allizond et al.'s (2023) chemical analysis, eugenol makes up the majority of Clove EO's composition, making up 78.91% v/v. Eugenyl acetate and β-caryophyllene are the following two main components. This composition is believed to contribute to the EO's antifungal properties. Khan et al. (2020) discovered that clove essential oil has antibacterial effects by potentially affecting the structure and function of pathogenic cells. Essential oils with phenolic components like thymol and carvacrol have comparable antibacterial effects. These compounds have the potential to enhance membrane permeability, resulting in the release of cell contents.



Source Maurya et al. (2021)

Figure 2.6 Possible target site for antifungal mechanisms of action

Lemongrass oil's active compounds, including citral, exhibit similar disruptive effects on fungal cells, although at higher concentrations (Debonne et al., 2019). The main constituents of lemongrass essential oil include citral (a combination of geranial and neral), myrcene, and limonene. The primary cause of its antifungal activity against *Aspergillus flavus* is citral. It disrupts the integrity of fungal cell membranes and inhibits the germination of spores (Da Cruz et al., 2023). The primary components of LGEO, including citral (a mixture of geranial and neral), are believed to contribute to its antifungal effects. These compounds likely disrupt the integrity of fungal cell membranes, leading to cell death. Citral is a monoterpene that comes from plants. It kills fungi very effectively by decreasing the activity of genes like erg7, erg11, erg6, erg3, and erg5 that are involved in making ergosterol (Zeng et al., 2024). This activity undermines the structural integrity of the fungal cell membrane, which is crucial for the survival of the fungus. Citral also changes the shape of mitochondria by causing the matrix to lose its shape and fall apart, which shows that it is very effective at killing fungi (Zheng et al., 2015). The antifungal properties of citral make it an attractive option for creating novel antifungal medications.



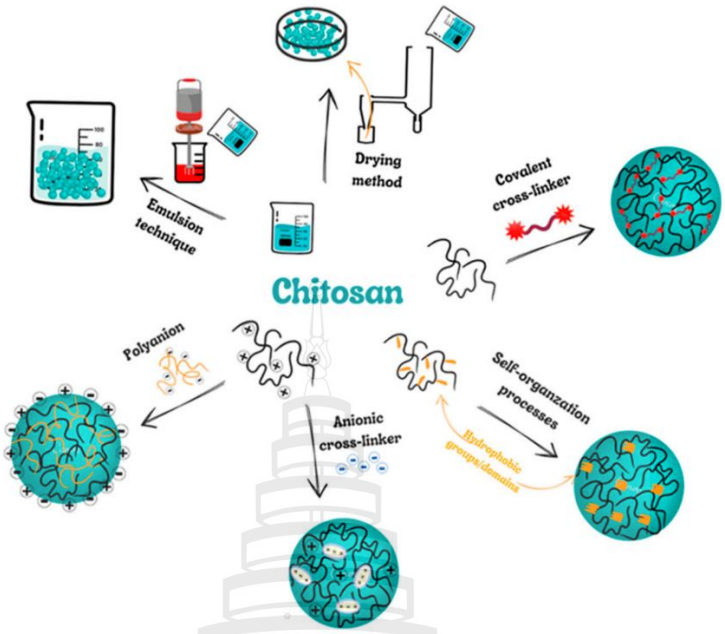
Source Abdi -Moghadam et al. (2023)

Figure 2.7 The effects of EOs on cell membranes, cell walls, and genetic materials

2.3 Chitosan Nanopolymers and Encapsulation Technologies

Chitosan nanopolymers, derived from chitin, exhibit several properties that make them an ideal candidate for encapsulating essential oil (EO) and enhancing its stability and controlled release. Chitosan is renowned for its biocompatibility, biodegradability, and non-toxicity, establishing it as a favorable nanocarrier for various bioactive substances, including essential oils (Zhang et al., 2021, Yu et al., 2021).

A study reported by (Shetta et al., 2024) outlines the strategic methods for encapsulating essential oils in chitosan nanosystems Figure 2.8 This review discusses the challenges with essential oils (EOs), such as their volatility, toxicity, and hydrophobicity, and how nano-encapsulation in biodegradable polymers like chitosan (CS) offers a solution. Various encapsulation techniques and applications, including their antimicrobial properties, are highlighted. The review emphasizes the significance of using chitosan nanocarriers for encapsulating EOs due to their biodegradability and efficacy in biomedical and food industries.



Source Jafernik et al. (2023).

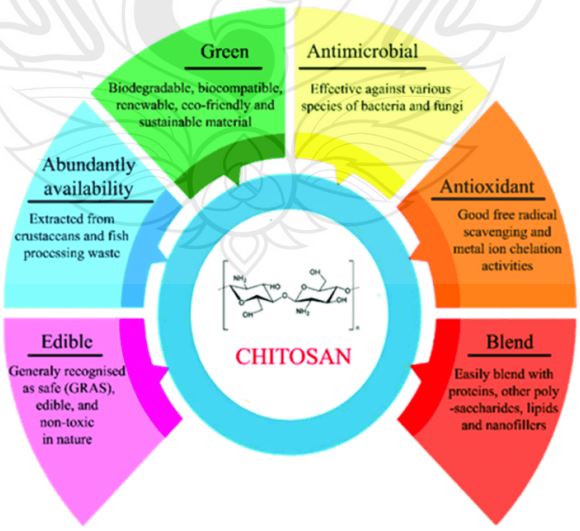
Figure 2.8 Scheme presenting the most popular methods of chitosan nanoparticles (ChNPs) producing

2.3.1 Unique Properties of Chitosan

The innovative application of chitosan in food preservation and agricultural practices has been increasingly recognized for its ability to enhance the stability and controlled release of essential oils, thereby boosting their antimicrobial effectiveness. As detailed in the study by Mondéjar-López et al. (2022), chitosan is an ideal encapsulation material, owing to its abundance, hydrophilicity, biodegradability, and film-forming capabilities. Its antifungal activity, particularly noted by Muzzarelli et al. (2000), further contributes to its utility in managing plant diseases and crop preservation.

The synergistic effects of chitosan nanocarriers and essential oils in antimicrobial applications are underscored in the research by Yang et al. (2023). This study highlights the enhanced antimicrobial, antioxidant, and anticancer properties achieved when essential oils are encapsulated in chitosan nanocarriers, particularly at the nanoscale. Such advancements suggest significant potential for these composites in the agricultural and food industries. Kalagatur et al. (2018) further illustrated this potential through their investigation of the antifungal activity of *Cymbopogon martinii*

essential oil (CMEO) encapsulated in chitosan nanoparticles. The results showed that chitosan-encased CMEO nanoparticles (Ce-CMEO-NPs) are more effective at killing fungi than CMEO alone. They are also more stable and have a controlled release mechanism. This finding indicates that chitosan nanocarriers can effectively enhance the antifungal properties of essential oils, making them suitable for use as mycobiocides in agricultural commodities. Additionally, the study by Hossain et al. (2019) delves into the development of chitosan-based antifungal films, which are reinforced with cellulose nanocrystals (CNCs) and encapsulate essential oil nanoemulsions. These films, containing various essential oil mixtures, exhibited significant antifungal activity against several fungi, including *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus*, and *Penicillium chrysogenum*. The study emphasizes the films' ability to gradually release volatile components while maintaining the stability of the chitosan matrix, thus presenting promising applications for the preservation of agricultural commodities. Gomez et al. (2018) further emphasize the environmental significance of the ionic gelation method, considering its solvent-free nature and the use of chitosan—a biopolymer that is both biodegradable and non-toxic to non-target organisms. The paper calls for comprehensive assessments of long-term soil and environmental health impacts, advocating for ongoing research to secure the method's place in future sustainable agriculture figure 2.9. Scheme presenting the most popular methods of chitosan nanoparticles (ChNPs) producing (Jafernik et al., 2023).



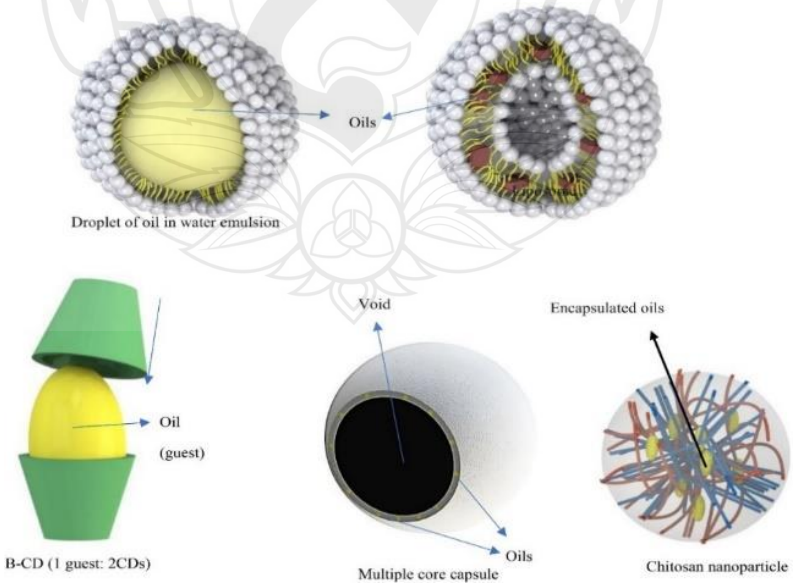
Source Basumatary et al. (2021)

Figure 2.9 Chemical characteristics and functional properties of chitosan

2.4 Encapsulation Technologies

Encapsulation is a widely-utilized process in the food industry, primarily aimed at enclosing various materials within matrices of liquid, gas, or solid. This technique chiefly serves to coat sensitive compounds, wherein these compounds form the core, and are enveloped by suitable wall materials. The encapsulation process is pivotal in protecting bioactive oils from detrimental factors such as oxygen exposure, high temperatures, and light, thereby enhancing their stability during thermal processing like pasteurization and throughout the storage period (Bagheri Darvish et al., 2020).

After ingestion, encapsulated volatile essential oils (V-EOs) within food products are safeguarded during mastication and the acidic environment of the stomach. This encapsulation system meticulously controls the release of oil molecules in the small intestine, thus improving the bio-accessibility and bioavailability of bioactive oil compounds. Encapsulated V-EOs can efficiently permeate into the bloodstream, traversing the mucus layer and intestinal epithelium, leading to enhanced bioavailability. Furthermore, encapsulation plays a crucial role in masking the undesirable flavors of certain oils and preventing their interaction with other food components, notably lipids (Bahrami et al., 2019; Esfanjani et al., 2018; Marques, 2010).



Source Delshadi et al. (2020)

Figure 2.10 Common nanoencapsulation method for vegetable and essential oils

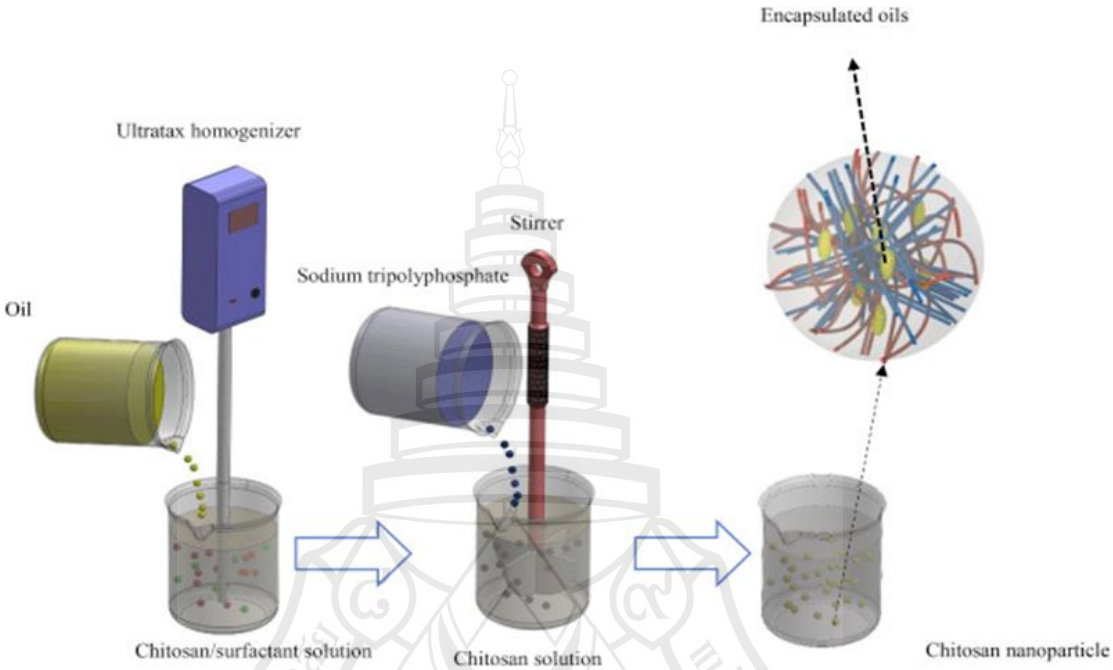
2.4.1 Ionic Gelation

Encapsulation technologies have significantly evolved as pivotal elements in modern agriculture, providing substantial advancements in crop management. A notable development in this realm is the ionic gelation method, which has gained prominence due to its capacity to create stable nanoparticles without resorting to toxic substances. This method involves a reaction between anionic cross-linking agents, such as sodium tripolyphosphate, and cationic polymers like chitosan, effectively safeguarding the bioactive properties of essential oils. The solvent-free technique is especially beneficial in agricultural contexts, prioritizing crop safety and minimal environmental impact (Agnihotri et al., 2004).

The foundational aspect of the ionic gelation method in nanoencapsulation is well documented by Agnihotri et al. (2004). This study elucidates the basic mechanism behind this process, where anionic cross-linking agents like sodium tripolyphosphate react with cationic polymers such as chitosan as shown in figure 2.11. This reaction results in the formation of stable nanoparticles, which are ideal for encapsulating bioactive compounds. emphasize the biocompatibility and biodegradability of the nanoparticles produced, highlighting their suitability for use in agriculture, especially for delivering nutrients and pesticides effectively. Chaudhary et al. (2021) provides a contemporary perspective on the advancements in the ionic gelation method. Their research sheds light on recent innovations that have enhanced the stability and efficiency of encapsulation. These advancements are crucial in adapting the method to a range of agricultural needs, such as controlled-release fertilization and targeted pest control. The study underscores improvements in nanoparticle formulation, which have led to more effective and sustainable agricultural practices.

In their study, Ngo et al. (2015) discuss the practical applications of ionic gelation in the context of agriculture. They focus on the technical aspects crucial for the successful implementation of this method, such as controlling particle size and preventing aggregation. These factors directly affect the release rate and overall efficacy of the encapsulated substances. The study provides insights into the application of ionic gelation for encapsulating a variety of agricultural inputs, thereby enhancing crop yield and protection. Gómez et al. (2018) delve into the environmental implications of the ionic gelation method, highlighting its alignment with eco-friendly

agricultural practices. The solvent-free nature of this technique minimizes environmental impact and ensures crop safety. The study emphasizes the method's contribution to sustainable agriculture by avoiding the use of harsh chemicals or toxic substances in the encapsulation process.



Source Delshadi et al. (2020)

Figure 2.11 Preparation of chitosan NPs by ion gelation technology

2.4.2 Nanoemulsions and Recent Advances

Nanoemulsions have also made a significant impact in agriculture, offering controlled release, high stability, and enhanced bioavailability. Stabilized by surfactants, these nanoemulsions have shown promise in protecting crops from microbial threats, thus extending the shelf life of agricultural products. Chitosan-essential oil films and coatings derived from these systems inhibit microbial growth and ensure food safety, while minimizing environmental impacts. This development aligns well with the increasing demand for sustainable agricultural practices (Zhang et al., 2021; Yu et al., 2021).

The formulation of emulsions is a prevalent method for encapsulating bioactive compounds, comprising two immiscible liquids mixed with surfactants. Oil in water (O/W) and water in oil (W/O) are two common systems employed for this purpose. The

O/W emulsion, particularly suitable for volatile essential oils (V-EOs), disperses oil molecules in water-containing emulsifiers. In the food industry, these emulsions are commonly stabilized using food-grade biopolymers like proteins and carbohydrates due to their emulsification activity, non-toxicity, and cost-effectiveness (McClements, 2015).

O/W nanoemulsions have captivated food researchers for encapsulating V-EOs, reducing the oil droplet size to below 1 μm. This decrease in size enhances the surface-to-volume ratio, leading to superior properties such as increased solubility, adsorption, and bioavailability. High-pressure homogenization and ultrasound are two industrial methods utilized for producing fine nanoemulsions (McClements, 2015). Addressing the challenge of oxidation in nanoemulsions containing V-EOs, the incorporation of antioxidants has proven effective. Studies by Sharif et al. (2017) demonstrated the enhanced oxidative stability of nanoemulsions with α-tocopherol and β-Carotene, while Sotomayor-Gerding et al. (2016) improved the stability of lycopene nanoemulsions and their bio-accessibility. Furthermore, the oxidative stability of flaxseed oil nanoemulsions was augmented using eugenol as a natural antioxidant (Williams, et al., 2004). These findings emphasize the efficacy of O/W nanoemulsions as efficient systems for encapsulating V-EOs in food products, which can be formulated using food-grade biopolymers and industrial production methods, with their oxidative stability further enhanced by natural antioxidants.

2.4.3 Nano-liposomes

Liposomes, characterized by their unique amphipathic properties, are formed based on the hydrophilic-hydrophobic interactions between phospholipid compounds and hydrophilic agents. In aqueous solutions, the polar heads of phospholipids orient towards the water, while their aliphatic chains interact with each other, forming a membranous structure. This configuration allows liposomes to act as carriers for both lipophilic and hydrophilic compounds (Pu & Tang, 2016).

The thin-film hydration method stands as a standard technique for liposome production. This process involves evaporating a solvent solution containing cholesterol, phospholipid, and hydrophobic compounds, followed by the addition of an aqueous phase with hydrophilic materials. The application of mechanical or thermal energy leads to the formation of multilamellar vesicles (MLVs), which are particularly

effective for encapsulating oil molecules. When subjected to ultrasonication or high-pressure homogenization, the size of these MLVs can be reduced to below one μm, thereby enhancing their functionality in producing volatile essential oils (V-EOs) nanoparticles (Reza Mozafari et al., 2008).

Recent research by Gulzar & Benjakul et al. (2020) focused on the production of nanoliposomes loaded with shrimp oil, utilizing techniques like ultrasonication and microfluidization. Their findings indicated that ultrasonication yields nanoliposomes with higher stability and smaller sizes. Furthermore, these nanoliposomes were effective in improving the retention of shrimp oil and masking its undesirable flavor. This advancement illustrates the potential of liposome technologies in the food industry, specifically in enhancing the delivery and preservation of bioactive compounds while optimizing sensory properties.

Table 2.1 Techniques for encapsulation of essential oils with their advantages and disadvantages

Technique	Advantages	Disadvantages	References
Ionic-Gelation	1. Easy and simple synthesis process	1. Larger size of particles	Benavides et al. (2016)
	2. Less involvement of chemical solvents	2. Heterogeneity in particle distribution	Odjo et al. (2022b)
	3. High loading capacity and entrapment efficiency		
	4. Mild laboratory condition		
Coacervation	1. Maximum reproducibility in synthesis process	1. Complex process with high cost	Bastos et al. (2020) Chen et al. (2022)
	2. High loading capacity	2. Caution in glutaraldehyde application	Tavares and Noreña (2020)
	3. Controlled release of bioactive constituents	3. Aggregation of nanoparticles	
Ultrasonication and High-Speed Homogenization	1. Simple and convenient method	1. Physical instability due to agglomeration	Turasan et al. (2015)
	2. Cost-effective	2. Low entrapment efficiency	
	3. Very fewer organic solvents and surfactants	3. Broader distribution of particle size	
Hot High-Pressure Homogenization	1. Easy and reproducible method	1. Input of very high energy due to heat	Aouf et al. (2020)
	2. Scalable	2. Degradation of some encapsulated materials	Garcia et al. (2012)
	3. Homogeneity in particle distribution		Khezri et al. (2020)

Table 2.2 (continued)

Technique	Advantages	Disadvantages	References
Cold High-Pressure Homogenization	1. Easily scalable	1. Requirement of high energy	Ahmad et al. (2016)
	2. Dispersion of particles	2. Broader particle size distribution	
Liposomes	1. High loading efficiency	1. Low chemical and physical stability	Gharib et al. (2017) Siyadatpanah et al. (2023)
	2. Formation of subcellular particles	2. Need for sophisticated post-treatment technology	
	3. Controlled delivery of volatile components	3. Cumbersome and high-cost process	
Spray Drying	1. Maximum entrapment efficiency	1. High temperature affects components degradation	Fernandes et al. (2017) Mehran et al. (2020)
	2. Cost-effective		Radünz et al. (2020) Shahidi Noghabi and Molaveisi (2020

2.5 Contemporary Strategies for Aflatoxin Contamination

Contemporary strategies for managing aflatoxin contamination in peanuts have become imperative, considering the significant health risks and economic impacts associated with aflatoxin exposure. Chemical treatments, such as fungicides and pesticides, are commonly employed to control aflatoxin-producing fungi. However, their effectiveness is often limited, and concerns arise regarding the environmental impact and potential development of resistant strains (Kumar et al., 2022).

2.5.1 Limitations of Chemical Treatments

Moreover, the use of chemical agents raises questions about food safety and the long-term effects on human health. Cultural practices, such as proper drying and storage, are fundamental in preventing aflatoxin contamination. However, these methods may be challenging to implement consistently, particularly in regions with variable climates and limited resources. Additionally, cultural practices alone may not provide sufficient protection against aflatoxin contamination (Mariana et al., 2019).

A variety of strategies have been investigated to curtail the growth of *Aspergillus spp*., a predominant mold in peanuts. Research has highlighted the efficacy of allyl isothiocyanate vapor in inhibiting the growth of *Aspergillus parasiticus* and the subsequent production of aflatoxin in stored peanuts. Another notable approach involves the application of dimethylformamide, which impedes the growth of *A. flavus* and aflatoxin B1 synthesis by obstructing glucose and amino acid metabolism (Pan et al., 2020). Additionally, the utilization of α-Fe2O3 nanorods, activated by sunlight, has demonstrated potential in halting hyphae and spore germination of *A. flavus* on peanuts. Despite these advancements, traditional methods such as chemical fungicides, nitrogen filling in the storage headspace, and vacuum packaging remain prevalent. However, these conventional techniques are not without limitations, including the high costs associated with equipment, potential alteration of food flavors, adverse health effects from chemical fungicides, environmental concerns, and the risk of developing pest resistance over time. This landscape underscores the imperative need to explore natural, effective, and safe alternatives to combat mold proliferation during peanut storage.

2.5.2 Biological Control Methods

Recent advancements in biological control methods have shown considerable potential in combating aflatoxin contamination in agricultural produce. These methods predominantly involve employing non-aflatoxigenic strains and various biocontrol agents to competitively inhibit aflatoxin-producing fungi. (Tan et al, 2021) highlight that while promising, the effectiveness of these strategies is often contingent upon environmental conditions, presenting challenges in developing efficient formulations.

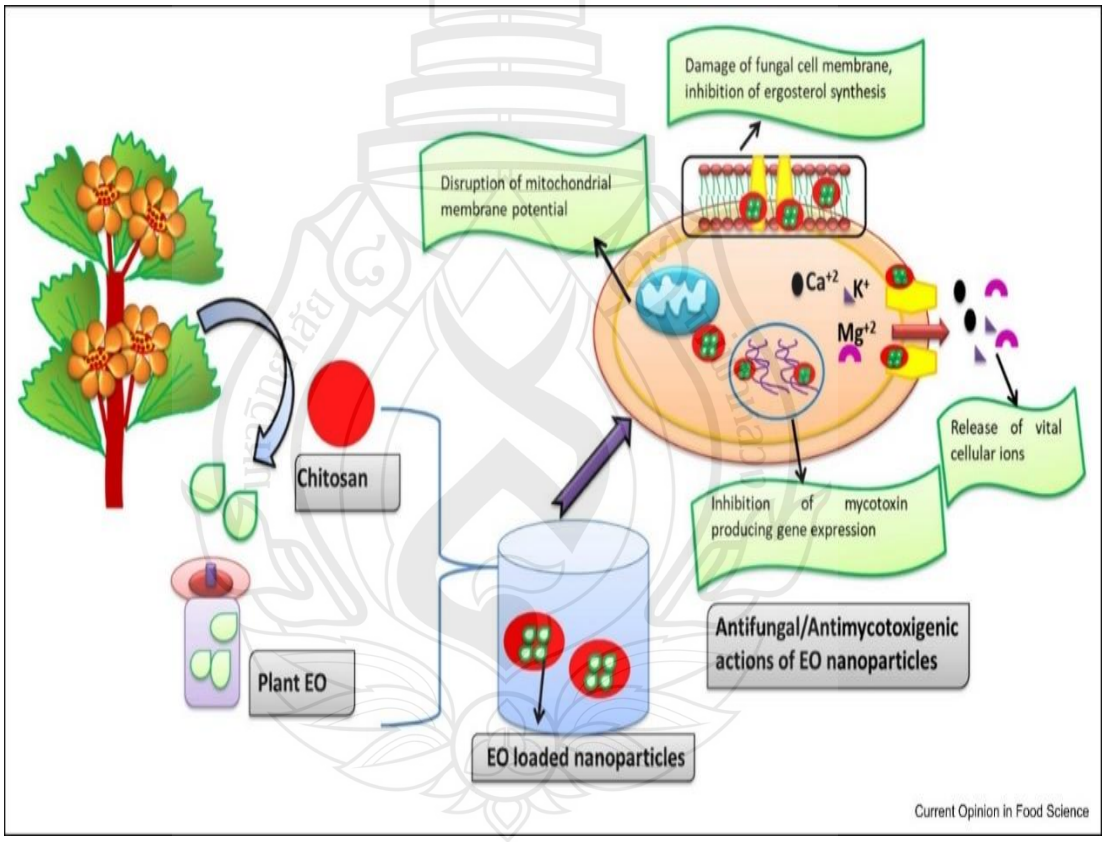
A pivotal aspect of biological control technologies is the use of selected microorganisms, such as bacteria, yeasts, and non-toxic molds, aimed at reducing aflatoxin contamination across both pre- and post-harvest stages. The mechanism of action of these microorganisms often involves spatial and nutrient competition, as well as biological interactions like antibiosis, to suppress aflatoxin production. For instance, in maize-producing regions, non-toxic strains of *A.flavus* (Mytoolbox Af01) have been utilized as a biological control method. This approach leverages the competitive interaction between atoxigenic and toxigenic strains, which has been effective in reducing aflatoxin levels in maize kernels by 51–83%.

Further illustrating the potential of biological control, Ali et al. (2021) investigated the use of various bacterial species, including *Enterococcus sp*., *Bacillus sp*., *Stenotrophomonas sp*., and *Pseudomonas sp*., to mitigate aflatoxin levels. Their study revealed that *Pseudomonas fluorescens* MN256402.1 could reduce AFB1, AFB2, and AFG2 levels by 99%, and AFG1 by 100%. Notably, this research marked the first reported instance of *Enterococcus casseliflavus* SA21, *Bacillus haynesii* SA22, *Bacillus tequilensis* S18, and *Bacillus amyloliquefaciens* S8C demonstrating aflatoxin degradation capabilities.

2.5.3 Shortcomings and the EO-CN Approach

The battle against aflatoxin contamination highlights a critical concern within global food safety and agricultural practices. Traditional strategies, including the use of chemical treatments and advanced nanotechnology, have encountered several shortcomings. Chemical fungicides, despite their widespread use, present environmental risks and health concerns. The potential for developing resistant strains of *Aspergillus spp*. further diminishes their long-term effectiveness (Kumar et al., 2022). Moreover, these methods often entail significant costs, which may not be feasible for small-scale farmers or in developing countries where the burden of aflatoxin contamination is highest (Mariana et al., 2019). Cultural practices such as crop rotation, proper drying, and storage techniques, while beneficial, cannot fully eliminate the risk of aflatoxin contamination. These methods require strict adherence to be effective and are dependent on climatic and environmental conditions that may not always be controllable (Tan et al., 2021). The limitations of current strategies underscore the urgent need for innovative approaches that are both effective and sustainable. The EO-CN (Essential Oils encapsulated in Chitosan Nanopolymers) approach emerges as a promising alternative, addressing the drawbacks of traditional methods. The encapsulation of essential oils like Cinnamon, Clove, and Lemongrass in chitosan nanopolymers aims to leverage their antifungal properties in a controlled and enhanced manner figure 2.12. This novel strategy not only targets the inhibition of *Aspergillus niger* growth but also offers a potentially eco-friendly solution to aflatoxin management (Yang et al., 2021; Sobolev et al., 2018). The encapsulation process is designed to overcome the volatility and degradation of essential oils, ensuring their stability and sustained antifungal activity. By optimizing the encapsulation conditions,

EO-CN aims to maximize the loading capacity and efficiency, enhancing the overall efficacy of the antifungal treatment (Guo et al., 2020). Advanced analytical techniques such as SEM, XRD, TGA, and FTIR provide critical insights into the morphology, size distribution, and chemical structure of EO-CN particles, allowing for the precise tailoring of the nanopolymers to meet the specific needs of peanut storage (Achar et al., 2020). Furthermore, the EO-CN approach aligns with the growing demand for environmentally sustainable and health-conscious food preservation methods. By mitigating the reliance on chemical fungicides and reducing the potential for environmental contamination, EO-CN represents a step forward in the safe and sustainable management of aflatoxin in peanuts (Wang et al., 2024).



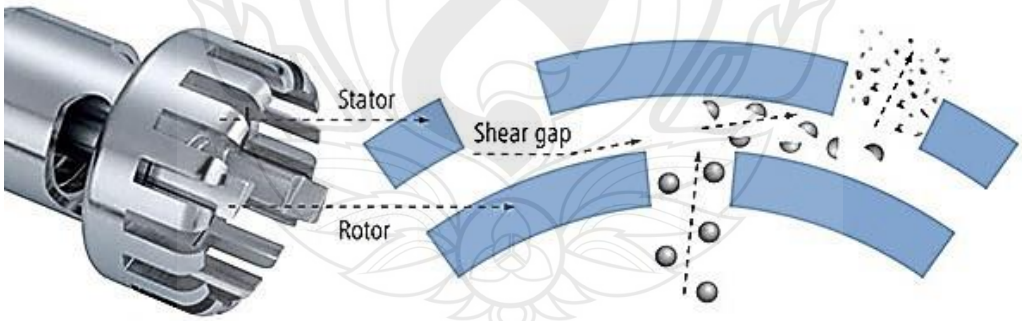
Source Tiwari and Dubey (2023)

Figure 2.12 Showing different antifungal actions of EO Nanoformulation

2.6 Theoretical Background

2.6.1 Rotor-Stator Homogenizer

A rotor-stator homogenizer is a type of mechanical homogenizer equipped with cutting blades, used extensively for reducing particle size and creating emulsions or dispersions (Sinico et al., 2005). The mechanism of action involves a high-speed rotor that spins at elevated revolutions per minute (rpm), producing intense shear forces within a narrow gap between the rotor and the surrounding stationary stator. This design generates circumferential force that propels material through the rotor-stator space, causing cavitation and turbulence. As a result, large particles are rapidly broken down into nanoscale materials, as illustrated in figure 2.13. The primary application of this homogenization system is the production of nanosized emulsions and dispersions (Wen et al., 2010). To ensure optimal performance, several process parameters must be carefully controlled, including the design and size of the rotor-stator probe, the tip speed, duration of homogenization, vessel geometry, and the positioning of the homogenizer. Additionally, sample-related factors such as initial particle size, viscosity of the medium, volume, and concentration also influence the homogenization efficiency (Wen et al., 2010).



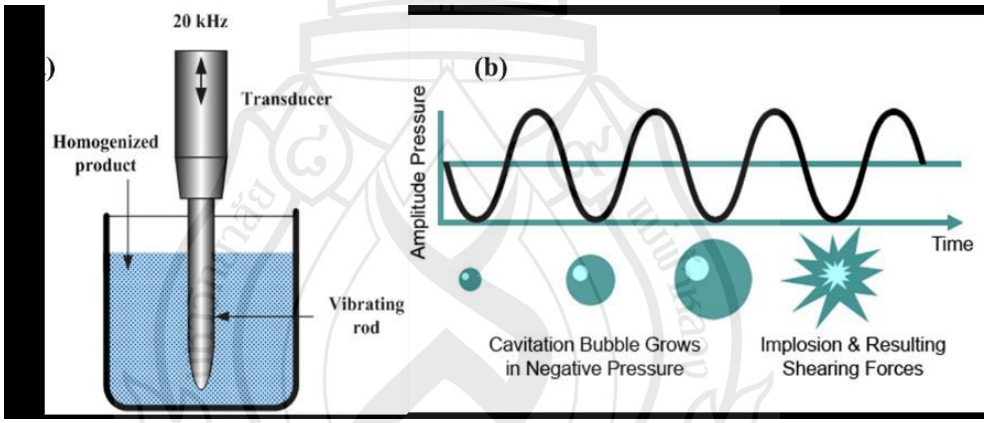
Source Sinico et al. (2005)

Figure 2.13 Rotor-stator type homogenizer

2.6.2 Probe Sonicator

Probe sonication, often referred to as ultrasonication, is a widely used technique for particle dispersion and size reduction in liquid media. As depicted in Figure 2.14a,

this method relies on high-frequency ultrasonic waves that induce cavitation, the rapid formation, growth, and collapse of microscopic bubbles in a liquid. These dynamic processes release intense localized energy, leading to the breakdown of particle aggregates and disruption of covalent bonds, as shown in Figure 2.14b (Wen et al., 2010). To optimize the effectiveness of probe sonication, key variables such as the amplitude and intensity of the probe, temperature control, sample concentration, and vessel dimensions must be considered. The typical probe consists of a piezoelectric horn that produces vibrations within the range of 15 to 25 kHz, with power settings varying from 10 to 375 watts. As a direct sonication method, the probe is immersed directly into the sample, providing more efficient energy transfer compared to indirect methods, where energy is transmitted through a surrounding liquid bath (Wen et al., 2010).



Source Wen et al. (2010)

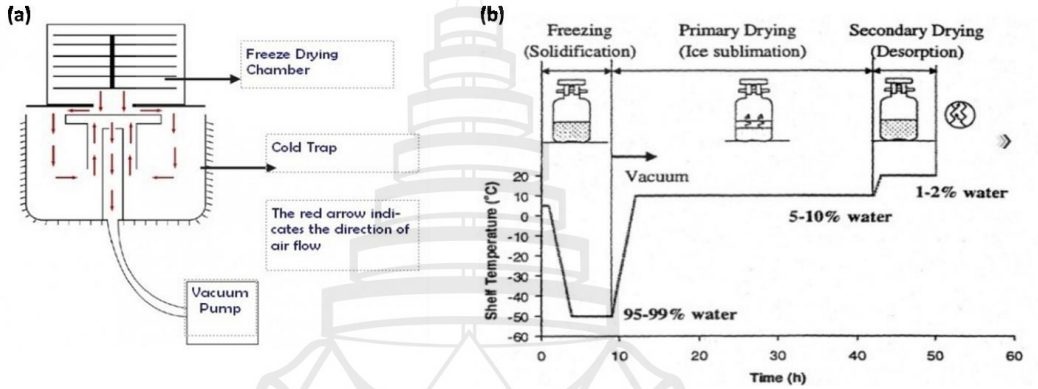
Figure 2.14 A typical probe sonicator (a), The phenomena of cavitation (b)

2.6.3 Lypholizer (Freeze Dryer)

Freeze-drying, or lyophilization, is a critical technique used to enhance the stability of colloidal systems such as liposomes, nanoemulsions, and nanoparticles. A standard lyophilizer setup includes a temperature-controlled drying chamber, a condenser unit, and a vacuum pump figure 2.15a. This method helps preserve particle size distribution, drug bioactivity, and extends shelf life. The lyophilization process is typically divided into three stages: (i) freezing, (ii) primary drying (sublimation), and (iii) secondary drying (desorption), as shown in figure 2.15 b. Initially, water in the colloidal system is frozen, increasing suspension viscosity and concentration. Then,

under reduced pressure, ice sublimates directly into vapor, creating a porous matrix. Finally, unfrozen bound water is removed during the desorption phase.

Despite its advantages, lyophilization can induce stress, such as particle aggregation, phase separation, or structural changes, that compromise stability. To address this, cryoprotectants like trehalose, sucrose, glucose, or mannitol are added. These agents stabilize nanoparticles by forming a glassy matrix that immobilizes them during freezing (Liolios et al., 2009).



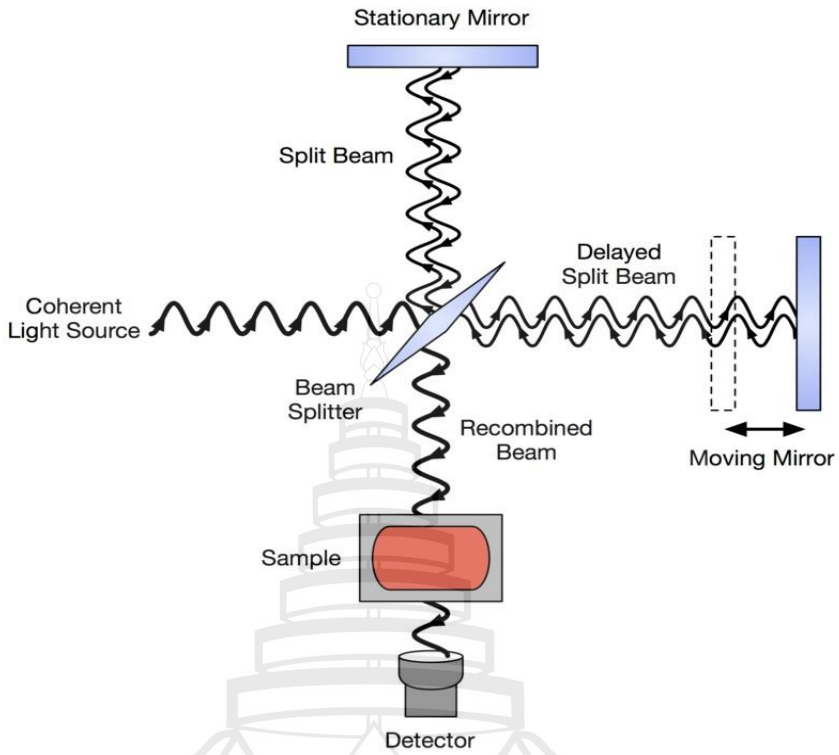
Source Liolios et al. (2009)

Figure 2.15 A typical benchtop freeze dryer (a), the lypholization cycle (b)

2.6.4 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared (FTIR) spectroscopy is a powerful analytical tool for identifying chemical structures through their vibrational spectra. This technique detects the stretching and bending of molecular bonds when exposed to mid-infrared radiation, typically between wavelengths of 2.5 μm and 25 μm. The resulting IR absorption peaks provide a molecular fingerprint of the sample.

FTIR instruments often employ a Michelson interferometer setup, as illustrated in figure 2.16. In this configuration, a He-Ne laser beam is split into two paths, one directed toward a fixed mirror and the other toward a moving mirror. When the reflected beams recombine, they form an interference pattern based on path length differences. This time-domain interferogram is mathematically converted into a frequency-domain spectrum using a Fourier transform function. The resulting data offer insights into the presence of specific functional groups and overall chemical composition (Chen et al., 2009).



Source Wei et al. (2009)

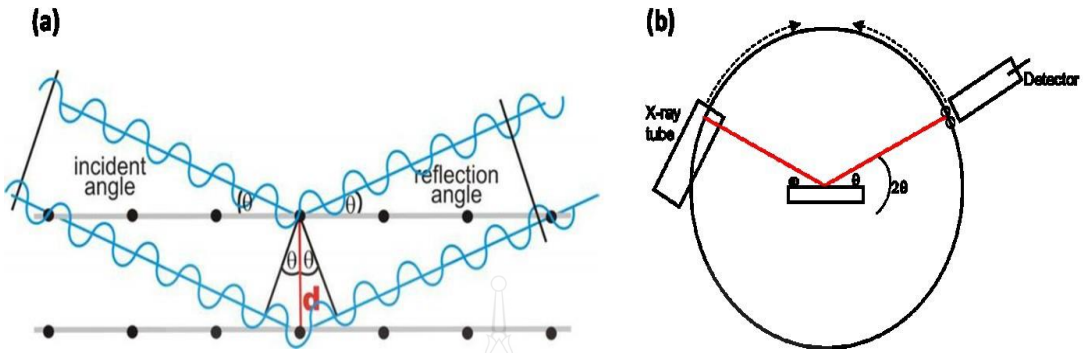
Figure 2.16 Schematic diagram of Michelson interferometer

2.6.5 X-ray Diffraction Analysis (XRD)

X-ray Diffraction (XRD) analysis provides critical insights into the packing characteristics and crystallinity of a material through the interpretation of diffraction peaks, specifically their shape, intensity, and position. This technique is fundamentally based on Bragg's law, expressed by the equation below:

$$n\lambda = 2d \sin \theta \qquad \text{(Equation 1)}$$

In this equation, n is the order of reflection, λ represents the wavelength of the incident X-rays, d is the spacing between the crystal planes, and θ is the incident angle. Figure 2.17 (a, b) illustrates that when a fixed X-ray wavelength is employed at a specific incident angle onto a set of atomic planes with spacing d, a diffraction peak is produced at a corresponding angle. The resulting diffraction pattern, typically recorded as the detector reading at an angle of 2θ, allows the estimation of the interplanar spacing. Additionally, the identity and arrangement of atoms in the diffracting planes may be inferred from the peak intensities (Maa & Hsu, 1996).



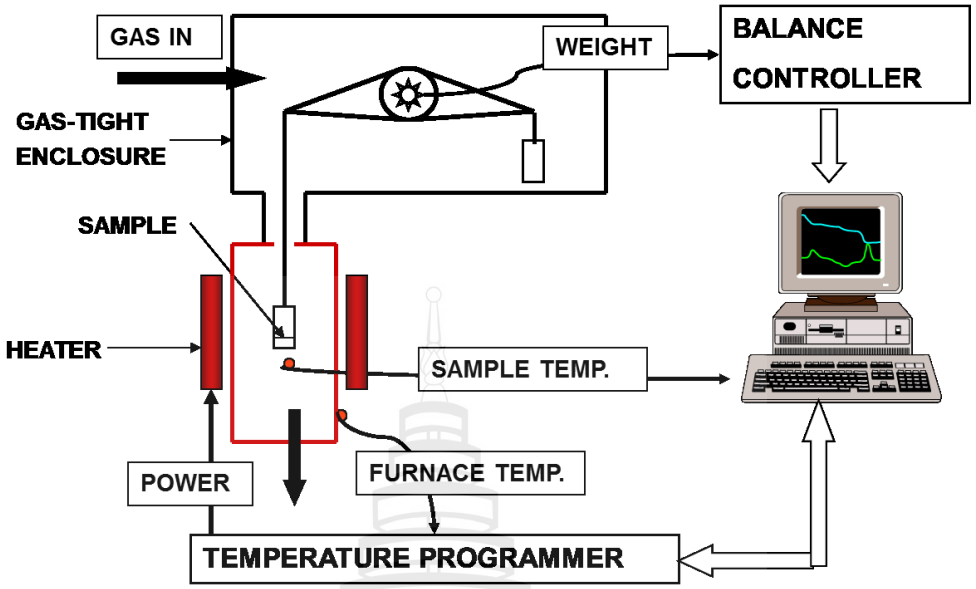
Source Maa and Hsu (1996)

Figure 2.17 Bragg's hypothesis (a), Schematic diagram of a typical XRD system (b)

2.6.6 Thermal Gravimetric Analysis (TGA)

Thermal Gravimetric Analysis (TGA) is an essential technique used to evaluate the thermal stability and composition of materials by monitoring weight changes under controlled heating conditions. Figure 2.18 illustrates that during TGA, a sample is placed in a precisely controlled oven that may operate under various gas atmospheres, and its weight is continuously recorded as the temperature increases. The output, known as a thermogram, graphs the weight change as a function of temperature, providing insights into phenomena such as decomposition, oxidation, or loss of volatile components.

A typical TGA apparatus comprises an oven equipped with a platinum thermocouple for accurate temperature measurements and a microgram-sensitive balance. The temperature is monitored via the platinum thermocouple, while changes in weight are determined by the corresponding beam deflection of the balance, ensuring that even minute weight losses can be detected (Dhankhar, 2014).



Source Dhankhar (2014)

Figure 2.18 Schematic diagram of TGA

CHAPTER 3

METHODOLOGY

3.1 Raw Material

The fungal strain *Aspergillus niger* (strain 3281) used in this study was obtained from the Biotechnology Laboratory at Mae Fah Luang University, Chiang Rai, Thailand. The strain was cultured on Potato Dextrose Agar (PDA) and maintained in darkness at 25 °C, with periodic sub-culturing to ensure viability.

Itraconazole (ITZ), a standard antifungal agent, was obtained in powder form (≥98% purity by HPLC) from (Tokyo Chemical Industry Co., Ltd. Thailand). Stock solutions of ITZ were prepared using 5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) and stored at −20 °C. ITZ served as the positive control in antifungal sensitivity evaluations.

Commercial-grade essential oils of Cinnamon, Clove, and Lemongrass were purchased from (Bangkok Chemical Co., Ltd. Thailand). Chitosan, with a molecular weight of 50–190 kDa and a degree of deacetylation between 75–85%, was purchased from (Bangkok Chemical Co., Ltd. Thailand) for use in nanoparticle formulation. Other reagents included glacial acetic acid, dichloromethane (CH_2Cl_2), sodium tripolyphosphate (TPP), and Tween® 80, all procured from Sigma-Aldrich. Deionized water and DMSO were used as solvents.

3.2 Methods

3.2.1 Fungal Inoculum Preparation

The fungal inoculum was prepared by cultivating *Aspergillus niger* on PDA plates at 25 ± 2 °C for seven days to promote optimal sporulation. Mature cultures were treated with approximately 5 mL of sterile 0.05% (v/v) Tween 80 solution to aid in the release of conidia. Using a sterile inoculating loop, the surface of the culture was gently

scraped to collect spores, which were transferred to a sterile tube and vigorously vortexed for 1–2 minutes to disrupt conidial clumps and ensure uniform suspension.

To eliminate any residual mycelial fragments, the conidial suspension was filtered through sterile glass wool. Spore concentration in the resulting filtrate was determined using a hemocytometer under a light microscope. The final suspension was standardized to 2×10^4 conidia/mL by dilution with sterile 0.05% Tween 80, following established protocols for antifungal susceptibility testing of *Aspergillus* species (Allizond et al., 2023; Tullio et al., 2007).

3.2.2 Antifungal Activity Assessment (Agar Disc Diffusion)

The antifungal efficacy of Cinnamon, Clove, and Lemongrass essential oils (EOs) against *A. niger* was initially evaluated using the agar disc diffusion method. This widely adopted technique enables rapid screening of EO activity and identification of the most potent candidate for nanoparticle encapsulation.

Sterile PDA plates (90 mm diameter) were prepared and uniformly inoculated with 100 µL of the standardized conidial suspension (2×10^4 conidia/mL) using a sterile L-shaped spreader. EO stock solutions were prepared in 5% DMSO containing 0.5% Tween 80, functioning as both solvent and emulsifier to enhance dispersion. Six EO concentrations were tested: 100%, 75%, 50%, 25%, 10%, and 5% (v/v).

Sterile filter paper discs (6 mm diameter, Whatman No. 1) were impregnated with 20 µL of each EO concentration. The discs were carefully placed on the inoculated agar surface and incubated in an upright position at 25 ± 2 °C for 5–7 days. Zones of fungal growth inhibition were measured in millimeters (mm) to evaluate antifungal potency.

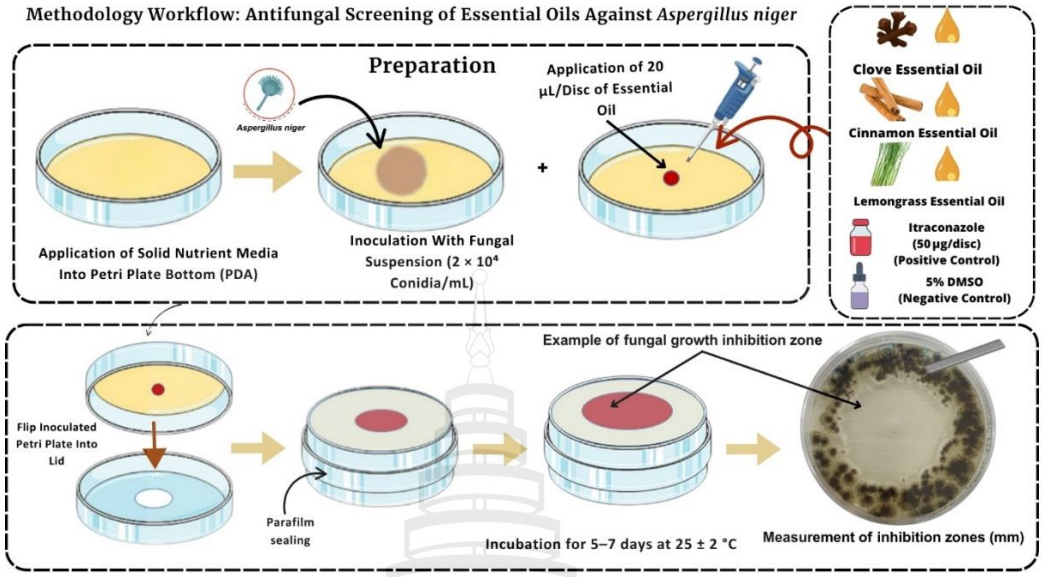


Figure 3.1 Schematic illustrations the screening of EO encapsulation Against *Aspergillus niger*.

3.2.3 Selection of Essential Oil

To identify the most effective essential oil for antifungal application, a preliminary screening was conducted using three plant-derived essential oils: clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum zeylanicum*), and lemongrass (*Cymbopogon citratus*). Each oil was tested against *Aspergillus niger* using the disc diffusion method at an equivalent concentration of 150 µg/disc. The antifungal activity was assessed by measuring the diameter of the inhibition zones after 72 hours of incubation at 28 ± 2 °C.

3.2.4 Preparation of EO-CSNPs

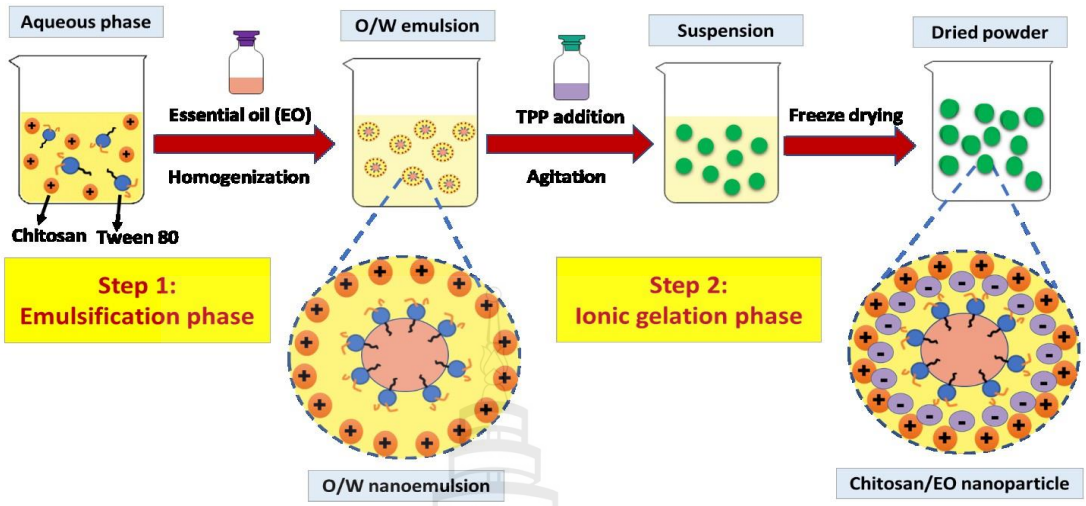
The encapsulation of essential oils into chitosan nanoparticles (EO-CSNPs) was performed using a modified two-step method based on protocols by Hosseini et al. (2013) and Shetta et al. (2019), with adaptations to optimize emulsification and cross-linking efficiency. This method, schematically represented in figure 3.2, involves the formation of a nanoemulsion followed by ionic gelation.

In the first step, an oil-in-water (O/W) emulsion was prepared by homogenizing an aqueous chitosan solution with an organic EO-containing phase. The aqueous phase consisted of 1% (w/v) chitosan dissolved in 1% (v/v) glacial acetic acid and mixed overnight at room temperature to ensure complete solubilization. The pH of the chitosan

solution was carefully adjusted to 4.6 using 1 M NaOH solution. This acidic condition is critical, as chitosan is only soluble in acidic environments due to the protonation of its amino groups ($–NH_2 \rightarrow –NH_3^+$). This protonation enhances the electrostatic interaction between the positively charged chitosan and negatively charged crosslinking agent (tripolyphosphate, TPP), facilitating nanoparticle formation via ionic gelation. The pH was monitored and confirmed using a calibrated digital pH meter to ensure reproducibility and optimal nanoparticle formation. An aqueous solution of TPP (0.1%, w/v) was then added dropwise into the chitosan solution under constant stirring. The resulting CEO-CSNPs were formed spontaneously due to ionic crosslinking between the chitosan and TPP. The formulation was allowed to stir for an additional 1 hour at room temperature to stabilize the nanoparticles. The mixture was then centrifuged using a laboratory centrifuge for 30 min at 10000 rpm then the supernatant was removed.

Tween 80, a nonionic surfactant with an HLB value of 15.9, was added in a weight ratio of 1:1.12 (chitosan: Tween 80) (0.5 g) to (50 mL) of the solution and stirred at 45 °C for 2 hours to obtain a homogeneous mixture and form the continuous aqueous phase.

Simultaneously, the CEO oil phase was prepared by dissolving varying concentrations of essential oil (EO) in 5 mL of dichloromethane (CH_2Cl_2), different amounts of CEO (0.25, 0.5, 0.75 and 0.5 g) were added corresponding to CS:EO ratios of 1:0.5, 1:1, 1:1.5, and 1:2 (w/w). The oily phase was introduced dropwise into the aqueous phase under high-shear homogenization at 14,000 rpm for 10 minutes in an ice bath using a rotor-stator homogenizer. This process produced a fine and stable O/W nanoemulsion.



Source Shetta et al. (2019)

Figure 3.2 Schematic illustrations of EO encapsulation two-step process

3.2.4.1 Ionic Gelation and Nanoparticle Recovery

The formation of EO-CSNPs was achieved through ionic gelation, wherein a negatively charged cross-linker, sodium tripolyphosphate (TPP), was gradually added to induce electrostatic interaction with the positively charged chitosan molecules in the emulsion. Specifically, 50 mL of 0.4% (w/v) aqueous TPP solution was added dropwise to the nanoemulsion under continuous magnetic stirring at 500 rpm (Phoenix RSM-01SH, Germany) for 40 minutes at room temperature.

Following gelation, the formed nanoparticles were separated by centrifugation at 10,000 rpm for 40 minutes at 4 °C. The resulting pellets were washed repeatedly with deionized water to remove residual reagents. To obtain a uniform suspension, the nanoparticles were redispersed in distilled water and subjected to ultrasonication using a probe sonicator (Sonics VCX 750, Sonics & Materials, INC., Newtown, CT, USA) for 4 minutes (2 seconds on, 1 second off) in an ice bath.

The suspensions were freeze-dried at −85 °C for 72 hours using a freeze dryer (CHRIST Beta 2-8 LSC basic, Germany). Both chitosan nanoparticles and supernatant were stored at 4 °C until further analysis. The experimental design included multiple CS:EO ratios, as summarized in Table 3.1.

Table 3.1 List of CS NPs samples with their CS: EOs weight ratios

Sample No	Weight ratios (w/w)
1	CS NP (1:0)
2	CS/CEO (1:0.50)
3	CS/ CEO (1:1.00)
4	CS/ CEO (1:1.50)
5	CS/ CEO (1:2.00)

3.2.5 Characterization of EO-CN

3.2.5.1 Surface Morphology and Size Analysis using (SEM/ EDS)

The surface morphology and particle size of both Clove EO-loaded CSNPs (CEO-CSNPs) and blank chitosan nanoparticles were assessed using Scanning Electron Microscopy (SEM) (TESCAN MIRA, Brno, Czech Republic). Approximately 1 mg of each freeze-dried nanoparticle sample was dispersed in 20 mL of deionized water and sonicated for 4 minutes in an ultrasonic bath to ensure adequate dispersion.

A drop of the diluted suspension was placed onto a clean glass slide and allowed to air dry at room temperature. The dried sample was then mounted on an SEM stub and sputter-coated with a thin layer of gold under high vacuum to improve conductivity and image clarity. Micrographs were captured at appropriate magnifications under an accelerating voltage of 10 keV. Elemental composition was further analyzed using Energy Dispersive X-ray Spectroscopy (EDS), with the relative weight percentages (W%) of carbon (C), oxygen (O), nitrogen (N), and phosphorus (P) recorded and presented as mean ± standard deviation (n = 3) (Hosseini et al., 2013).

3.2.5.2 Fourier Transform Infrared (FTIR)

FTIR analysis was conducted to identify functional groups and confirm the interaction between Clove EO and chitosan within the nanoparticle matrix. Spectra were recorded for pure chitosan powder, pure clove essential oil, unloaded CSNPs, and CEO-CSNPs using a PerkinElmer FTIR Spectrometer (Lambda 850+, USA), equipped with a Standard PMT and 100 mm PMT sphere detector. Each sample was scanned across the 400–4000 cm^{-1} wavenumber range with a resolution of 4 cm^{-1}, and 32 scans

were performed per sample to enhance spectral accuracy (Hosseini et al., 2013; Hasheminejad et al., 2019).

3.2.5.3 Powder X-ray Diffraction (XRD)

XRD was employed to investigate the crystalline structure of the formulated nanoparticles and detect any structural changes following encapsulation. Patterns were obtained using a PANalytical Empyrean diffractometer (Malvern Panalytical Ltd., UK) over a 2θ range of 5°–50°, with a step size of 0.013° and a measurement time of 38 seconds per step. This setup enabled high-resolution detection of crystal lattice reflections, providing insight into the material's crystallinity and potential interaction between chitosan and EO (Hosseini et al., 2013).

3.2.5.4 Thermal Properties (TGA)

Thermal behavior and stability of the samples were evaluated using Thermogravimetric Analysis (TGA) combined with Differential Thermal Analysis (DTA). Measurements were performed using a TGA/DSC 3+ analyzer (Mettler Toledo, Columbus, OH, USA). Samples (10 mg) of pure clove essential oil, blank CSNPs, and CEO-CSNPs were heated from 20 °C to 600 °C at a constant rate of 10 °C/min under a nitrogen atmosphere. The resulting thermograms provided insight into degradation patterns, thermal stability, and potential protective effects of encapsulation (Shetta et al., 2019).

3.2.5.5 Antifungal activity of EO-CSNPs against *A. niger*

To evaluate the antifungal potential of the prepared CEO-CSNPs, freeze-dried nanoparticles were reconstituted in sterile deionized water to concentrations corresponding to their original CS:EO weight ratios (1:0.5, 1:1, 1:1.5, and 1:2). Sterile paper discs (6 mm diameter) were impregnated with 20 µL of each nanoparticle suspension.

Positive control discs were loaded with 10 µg of Itraconazole (10 µL of a 1 mg/mL stock), while negative controls contained 20 µL of the solvent mixture (5% DMSO with 0.5% Tween 80). All discs were placed gently onto PDA plates inoculated with *A. niger* conidial suspension, ensuring firm contact between disc and agar.

Plates were incubated at 25 ± 2 °C in an upright position for 5–7 days. After incubation, the diameter of the inhibition zone around each disc was measured in millimeters using a ruler or digital caliper. All tests were conducted in triplicate, and

results were expressed as mean ± standard deviation (SD) (Allizond et al., 2023; Xiang et al., 2020; Aimad et al., 2022).

3.3 Statistical Analysis

All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine significant differences between treatment groups at different concentrations. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics (Version 26.0), and graphs were generated using MS Excel.

CHAPTER 4

RESULTS

4.1 Antifungal Activity

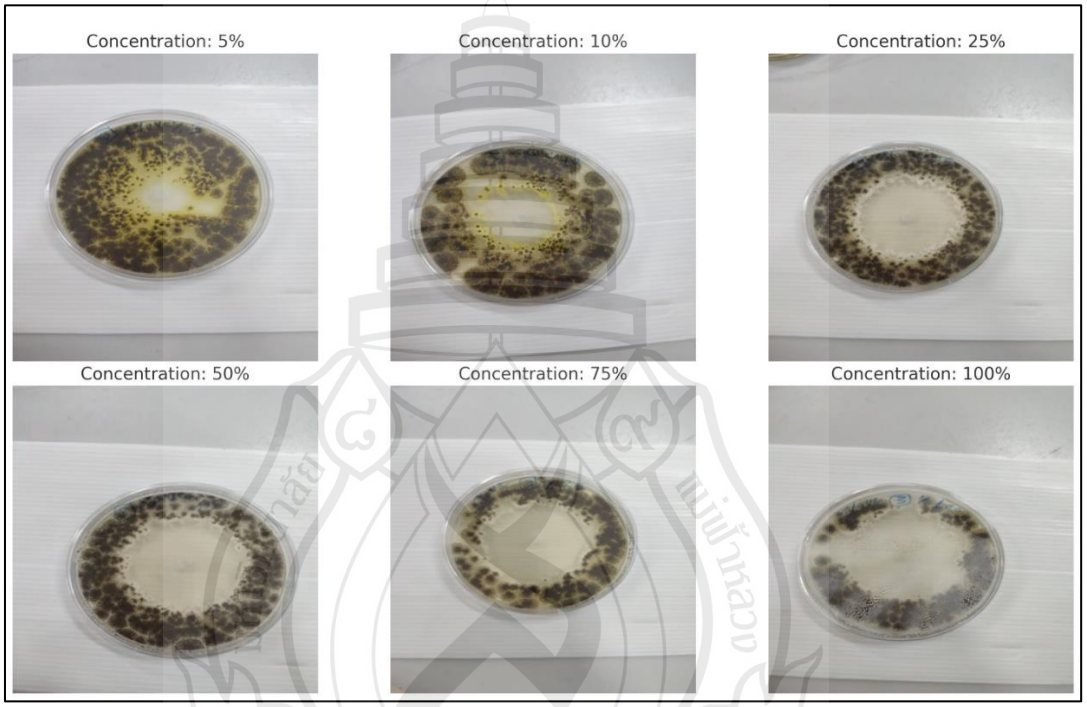


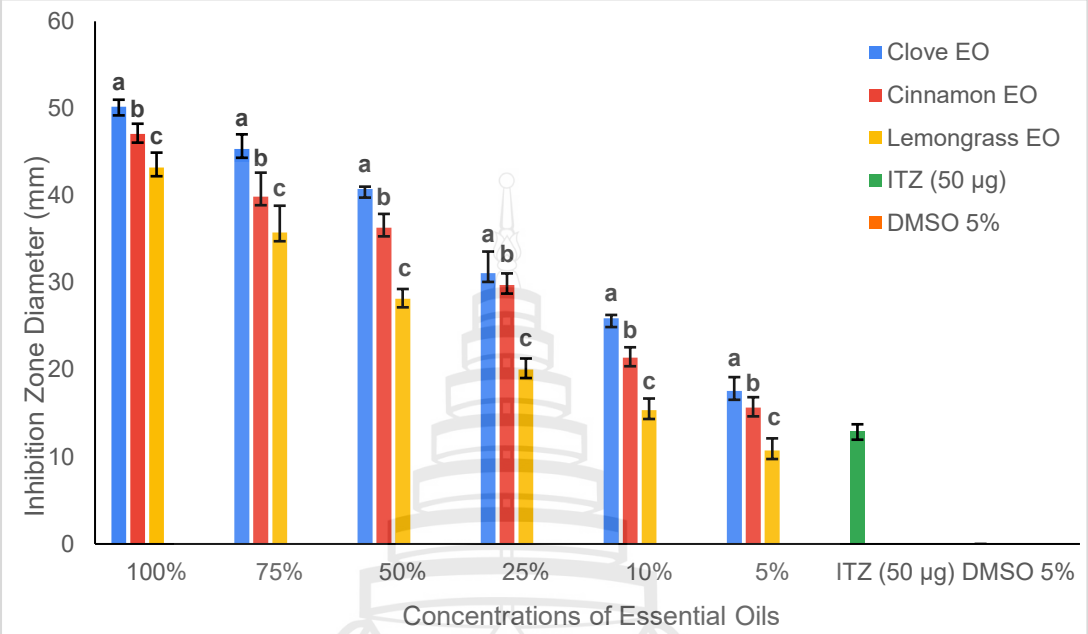
Figure 4.1 Inhibitory activity in agar medium of clove EOs 5% to 100% concentrations against *A. niger*

The antifungal activity of Clove, Cinnamon, and Lemongrass essential oils (EOs) against *Aspergillus niger* was evaluated at six concentrations (100%, 75%, 50%, 25%, 10%, and 5%). The mean inhibition zones (mm) and standard deviations for each treatment are summarized in (Figure 4.1 and 4.2). Statistical analysis was conducted using one-way ANOVA followed by Tukey's HSD post hoc test and independent t-tests to compare EOs with the controls (Itraconazole and DMSO). Clove essential oil demonstrated the most potent antifungal activity across all concentrations, with inhibition zones ranging from 50.20 ± 0.51 mm at 100% to 17.58 ± 0.81 mm at 5%. Even at lower concentrations (25% and 10%), Clove EO maintained relatively high

efficacy (31.08 ± 2.49 mm and 25.90 ± 0.39 mm, respectively), significantly exceeding the positive control, Itraconazole (ITZ), which exhibited a mean inhibition of 12.97 ± 0.77 mm. Notably, Clove EO at 25% was more than twice as effective as ITZ ($p < 0.0001$). Cinnamon EO showed moderate antifungal activity, with inhibition zones ranging from 46.40 ± 0.62 mm at 100% to 15.65 ± 1.13 mm at 5%. Although Cinnamon EO was significantly less effective than Clove EO at higher concentrations ($p < 0.05$), it still demonstrated superior activity compared to ITZ at all tested levels ($p < 0.01$). Lemongrass EO displayed the weakest activity among the three oils, with inhibition zones decreasing from 43.22 ± 1.71 mm at 100% to 10.80 ± 1.23 mm at 5% less than ITZ. At 5% concentration, the antifungal effect of Lemongrass EO is statistically comparable to that of Itraconazole. This suggests that at very low concentrations, Lemongrass EO may not offer a significant advantage over the positive control. The DMSO negative control exhibited no antifungal activity (0 mm across all replicates), confirming that the observed inhibition zones were due to the bioactive components of the EOs. Tukey's HSD test identified statistically significant groupings among the three EOs at each concentration. Clove EO was grouped as 'a', Cinnamon EO as 'b', and Lemongrass EO as 'c', indicating distinct efficacy levels ($p < 0.05$). These patterns were consistent across most concentrations. The data indicate a dose-dependent antifungal response, with Clove EO emerging as the most promising natural antifungal agent among the three, showing efficacy that surpasses the antifungal positive control Itraconazole at all tested concentrations.

These findings support previous literature that highlights CEO's broad-spectrum antimicrobial properties, largely attributed to its high eugenol content. Eugenol has been shown to disrupt fungal cell membranes, inhibit ergosterol biosynthesis, and cause oxidative damage to fungal cells (Raut & Karuppayil, 2014). The effectiveness of CEO at higher concentrations, particularly in comparison to Itraconazole, suggests its potential application as a natural antifungal agent in pharmaceutical or food preservation contexts. Similar results have been reported by Muñoz Castellanos et al. (2020), who confirmed the strong antifungal effects of CEO in food systems. While these results demonstrate CEO's strong in vitro efficacy, further research is warranted to explore it's in vivo performance, optimal dosing, and

formulation strategies to enhance stability and sustained release such as encapsulation in chitosan nanoparticles.



Note Bars represent mean values of three replicates. Significant group labels (a, b, c) indicate statistically different groups based on Tukey's HSD test ($p < 0.05$).

Figure 4.2 Mean inhibition zones (mm) of Clove, Cinnamon, and Lemongrass essential oils at varying concentrations (100%–5%) compared to controls (ITZ and DMSO) against *Aspergillus niger*

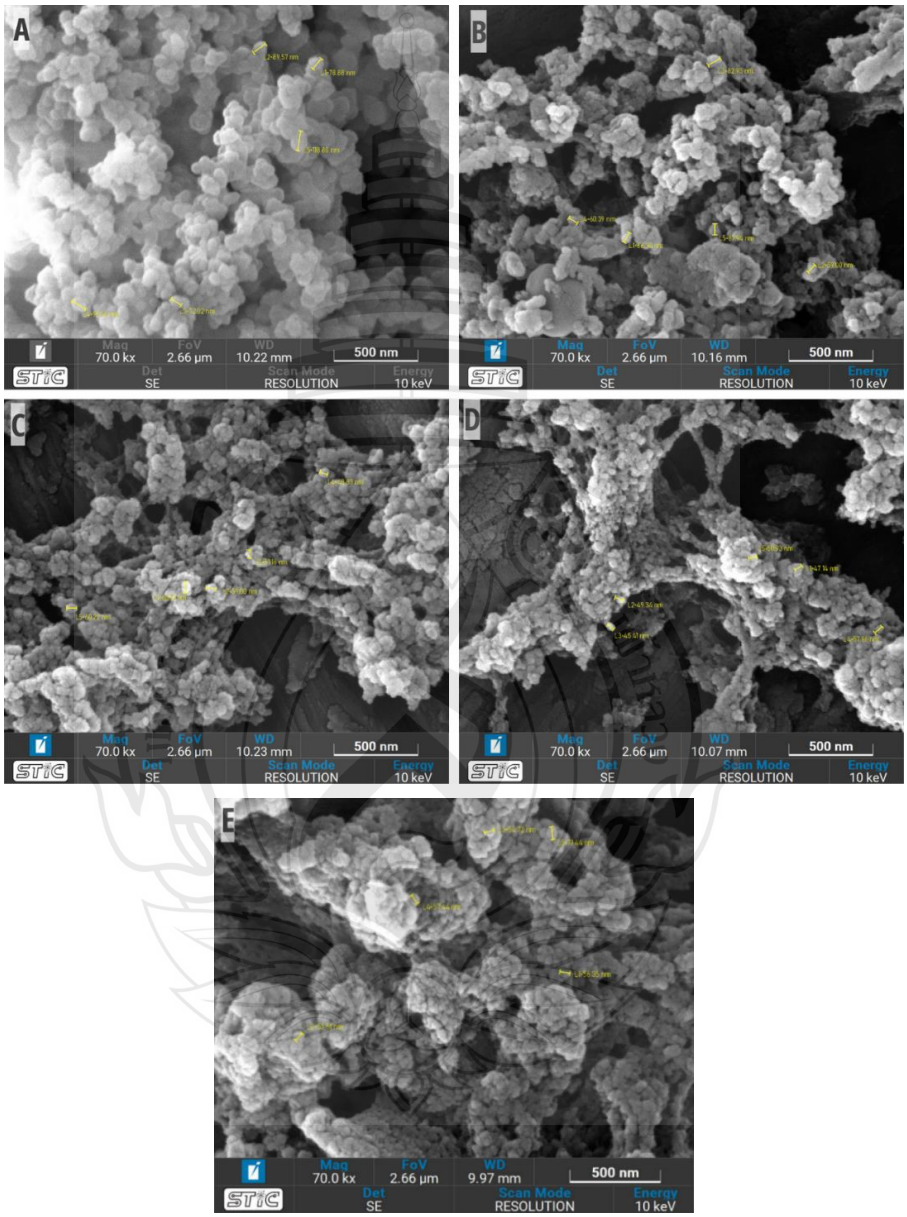
4.2 Nanoparticle Characterization

Physicochemical characterization provided insights into the structure and properties of the prepared nanoparticles and confirmed successful EO encapsulation.

4.2.1 Morphology (SEM/EDX)

Scanning Electron Microscopy images provided visual evidence of nanoparticle formation and morphology. Figure 4.3.A shows the empty chitosan nanoparticles (CSNPs), which appear as somewhat aggregated, roughly spherical or pseudo-spherical particles, typical for CSNPs prepared by ionic gelation. Figure 4.3 B-E display the CEO-loaded chitosan nanoparticles prepared with increasing initial CEO ratios (1:0.5, 1:1, 1:1.5, and 1:2, respectively). These loaded nanoparticles generally retained a similar

spherical morphology, although some variations in surface texture, aggregation state, or apparent particle size might be observed depending on the formulation. The scale bars (500 nm) suggest that the individual or aggregated structures are within the sub-micron or nanometer range. The images indicate successful particle formation across the different formulations (Tunma, 2021).



Note A (chitosan nanoparticles), B (1:0.5), C (1:1), D (1:1.5), and E (1:2). All images were acquired at 70,000× magnification using scanning electron microscopy at 10 kV. The scale bar in each image represents 500 nm

Figure 4.3 SEM images of clove essential oil-loaded chitosan nanoparticles, prepared with varying initial weight ratios of chitosan to clove essential oil

4.2.1.1 Particle Size Analysis

Quantitative particle size analysis was performed using measurements derived from SEM. The mean particle sizes (± standard deviation) calculated for each formulation are presented in Table 4.1.

Table 4.1 Mean particle size (nm) ± standard deviation of clove essential oil-loaded chitosan nanoparticles (CEO-CSNPs) prepared at various CS:CEO ratios, based on SEM analysis

Formulation (Image Label)	Chitosan: Clove EO Ratio	Mean Particle Size ± SD (nm)*
A	Chitosan Nanoparticles (CSNPs)	90.45 ± 17.48 a
B	1:0.5	67.31 ± 9.10 ab
C	1:1	57.44 ± 7.47 bc
D	1:1.5	50.15 ± 4.69 c
E	1:2	58.69 ± 6.90 bc

Note Values represent the average of five independent measurements (n = 5). Different superscript letters (a–c) indicate statistically significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's HSD test

Particle size analysis indicated a significant reduction in mean particle size with increased clove essential oil loading within chitosan nanoparticles (CEO-CSNPs) (Table 4.1). ANOVA confirmed statistically significant differences among the formulations ($F = 11.08$, $p < 0.0001$). Post-hoc Tukey's test revealed the largest particle size in unloaded CSNPs (90.45 ± 17.48 nm), significantly higher than nanoparticles with higher EO loadings (D (1:1.5): 50.15 ± 4.69 nm, and E (1:2): 58.69 ± 6.90 nm). Intermediate ratios (B (1:0.5) and C (1:1)) exhibited particle sizes with overlapping significance, suggesting moderate size reduction. These findings demonstrate that increased essential oil loading effectively reduces nanoparticle size, potentially enhancing EO encapsulation efficiency and stability.

The effect of essential oil loading on chitosan nanoparticle size reported in the literature varies. Some studies report an increase in particle size upon essential oil incorporation, such as with lemongrass oil (Nehad et al., 2024), and similar increasing trends have been noted in other referenced works (Hadidi et al., 2020). Conversely, other studies have observed a decrease in particle size, for instance, when encapsulating cinnamon or thyme essential oils (Barrera-Ruiz et al., 2020). The initial decrease in particle size observed in our study from (A to D) aligns with findings where encapsulation led to smaller particles (Hosseini et al., 2013). Similarly, Zhang et al. (2020) observed that loading essential oils into chitosan nanoparticles resulted in reduced particle sizes and improved morphological characteristics. However, the subsequent increase in size at the highest oil ratio (E) suggests a more complex relationship, potentially involving factors like altered particle packing or aggregation due to increased hydrophobicity, which differs from the simple increasing (Nehad et al., 2024, Ziaee et al., 2023) or decreasing (Barrera-Ruiz et al., 2020, Hosseini et al., 2013) trends reported elsewhere. A study specifically encapsulating clove essential oil (CEO) in chitosan nanoparticles reported sizes ranging from 223 to 444 nm (Hadidi et al., 2020), considerably larger than the 50-90 nm range observed here, possibly due to differences in chitosan molecular weight, preparation methods, or CEO concentration ranges studied (Hadidi et al., 2020). Zhang et al. (2020) saw size increase again at a 1:4 ratio after a minimum at 1:3. Hadidi et al. (2020) reported that EO-loaded chitosan nanoparticles exhibited superior antioxidant and antibacterial activities compared to free EO, attributing this to the improved physicochemical properties conferred by nanoencapsulation. Additionally, Zhang et al. (2020) found that chitosan nanoparticles loaded with plant essential oils demonstrated enhanced antibacterial activity against multi-drug resistant bacteria, further supporting the benefits of EO encapsulation.

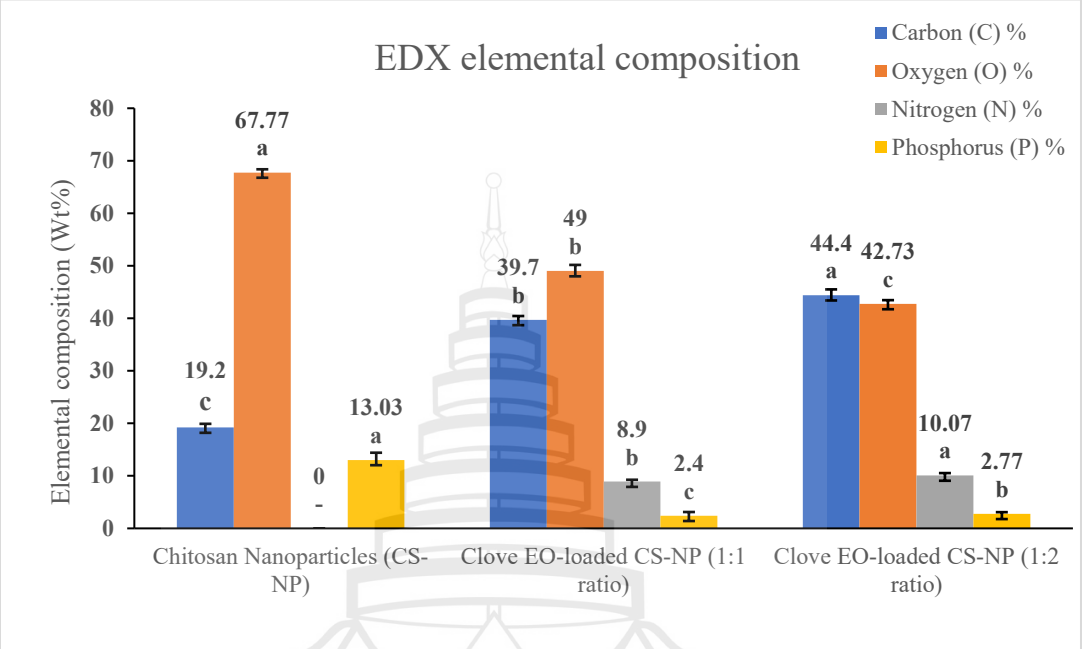
4.2.1.2 Elemental Composition via EDS Analysis

Energy Dispersive X-ray Spectroscopy (EDS) was performed to determine the elemental composition of the synthesized nanoparticles and confirm the successful encapsulation of clove essential oil (EO) within chitosan nanoparticles (CS-NPs). The results in figure 4.4 The EDS spectrum of the unloaded chitosan nanoparticles exhibited high weight percentages of oxygen (67.77 ± 0.61%), carbon (19.2 ± 0.71%), and phosphorus (13.03 ± 1.38%), with calcium also visually detected (10–15%) and nitrogen

qualitatively observed but not quantified. These findings, particularly the C, N, O, and P signals, are indicative of a successful ionic crosslinking process between chitosan and sodium tripolyphosphate (TPP) (Divya & Jisha., 2017; Furtado et al., 2018). The presence and significant percentage of phosphorus align with the coordination of TPP's phosphate groups with the protonated amine groups in chitosan during ionic gelation (Nayila et al., 2024; Shenvi et al., 2014). Adding clove essential oil (EO) at a 1:1 (v/v) ratio changed the surface's elemental composition a lot. The carbon content went up to 39.7 ± 0.74%, the oxygen content went down to 49.0 ± 1.17%, and the phosphorus content dropped to 2.4 ± 0.72%. 8.9 ± 0.36%, reflecting the retained chitosan matrix. The marked increase in carbon content is attributed to the introduction of eugenol, the major, carbon-rich component of clove EO (Shetta et al., 2019). The simultaneous drop in the surface phosphorus signal is probably because the hydrophobic EO layer is covering or shielding the TPP crosslinker, making it harder to see during EDS analysis. These compositional shifts upon loading are consistent with prior reports encapsulating various essential oils and extracts in CS-TPP nanoparticles (Salman et al., 2023). By adding more chitosan to the 1:2 (v/v) mixture, the carbon and nitrogen contents went up even more (44.4 ± 1.11% and 10.07 ± 0.46%, respectively), while the phosphorus content went up by 2.77 ± 0.32% and the oxygen content went down to 42.73 ± 0.73%.

The elevated nitrogen signal confirms the formulation's increased chitosan fraction. These elemental trends are supported by studies that show changes in EO loading can change the surface composition found by EDS (El-Naggar et al., 2023; Shenvi et al., 2014). Also, the small rise in phosphorus compared to the 1:1 sample suggests that there may be more TPP crosslinking points available compared to the surface EO coverage at this higher chitosan ratio. The presence of TPP within the nanoparticles, as confirmed by EDS, supports the fundamental structure formed via ionic gelation (Shetta et al., 2019). The presence of carbon (C) and oxygen (O) in high proportions aligns with the organic nature of both chitosan and clove essential oil, which are primarily composed of carbon-oxygen-based structures such as polysaccharides and phenolic compounds (e.g., eugenol). The detection of nitrogen (N) further supports the incorporation of chitosan, as it contains amino groups that are unique to its glucosamine backbone. The absence of metallic elements and the clean spectrum further confirm the purity of the bio-based formulation. These findings complement the SEM observations

and confirm that the CEO was successfully loaded into the chitosan matrix without the presence of contaminants.



Note Data are presented as mean ± SD (n = 3). Superscript letters(a,b,c) above bars indicate statistically significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's HSD test

Figure 4.4 Elemental composition (W%) of Chitosan Nanoparticles (CS-NP), Clove Essential Oil-loaded CS-NP at 1:1 and 1:2 ratios, as determined by Energy Dispersive X-ray Spectroscopy (EDS)

4.2.2 Chemical Interaction (FTIR)

Fourier Transform Infrared (FTIR) spectroscopy was employed to investigate the chemical structures and potential interactions among chitosan, clove essential oil (CEO), and the components formed during nanoparticle fabrication. As presented in Figure 4.5, the FTIR spectrum of pure chitosan exhibited characteristic peaks, including a broad absorption band around 3400 cm^{-1} (typically ranging between 3500–3300 cm^{-1}), which corresponds to the overlapping O–H and N–H stretching vibrations and indicates strong hydrogen bonding within the chitosan matrix (Dahmane et al., 2014). Additionally, peaks around 2900 cm^{-1} were attributed to C–H stretching vibrations. Prominent bands associated with the amide groups were observed near 1650 cm^{-1} (Amide I, due to C=O stretching of residual acetyl groups) and between 1590–1600 cm^{-1}

(Amide II, corresponding to N–H bending of primary amines), confirming the typical chemical structure of chitosan (Mohammadpour Dounighi et al., 2012).

The FTIR spectrum of pure CEO revealed distinctive peaks corresponding to eugenol, the major bioactive compound in clove oil. Aromatic C=C stretching vibrations were observed between 1600–1500 cm^{-1}, while a broad band within the 3500–3200 cm^{-1} range indicated phenolic O–H stretching. Additional peaks around 1270 cm^{-1} and 1150 cm^{-1} were attributed to C–O stretching, signifying the presence of aromatic and oxygenated functional groups characteristic of CEO (Prajapati & Parmar, 2024).

Significant spectral differences were observed in the FTIR spectra of blank chitosan nanoparticles (CSNPs), synthesized via ionic gelation with sodium tripolyphosphate (TPP), compared to those of pure chitosan. Notably, the amine and amide bands exhibited shifts and reduced intensity. For instance, the N–H bending peak shifted from approximately 1600 cm^{-1} to 1540 cm^{-1}, while the Amide I band moved from 1650 cm^{-1} to 1630 cm^{-1}. The O–H/N–H stretching region also broadened and shifted. These spectral changes indicate interactions between the positively charged amino groups ($–NH_3^+$) of chitosan and the negatively charged phosphate groups in TPP, confirming successful cross-linking essential for nanoparticle formation and stability. Moreover, the presence of phosphate-related peaks, particularly the P=O stretching band near 1170 cm^{-1}, further supports the incorporation of TPP (Mohammadpour Dounighi et al., 2012).

In the CEO-loaded CSNPs, additional changes were detected in the FTIR spectra. Characteristic peaks corresponding to CEO were still evident, indicating successful encapsulation. Slight shifts in these peaks relative to the spectra of pure CEO suggest that the essential oil was not merely physically mixed but chemically or electrostatically interacting with the chitosan matrix. These interactions likely contribute to the stability of the CEO-loaded nanoparticles (Beyaz et al., 2025; Bidooki et al., 2024).

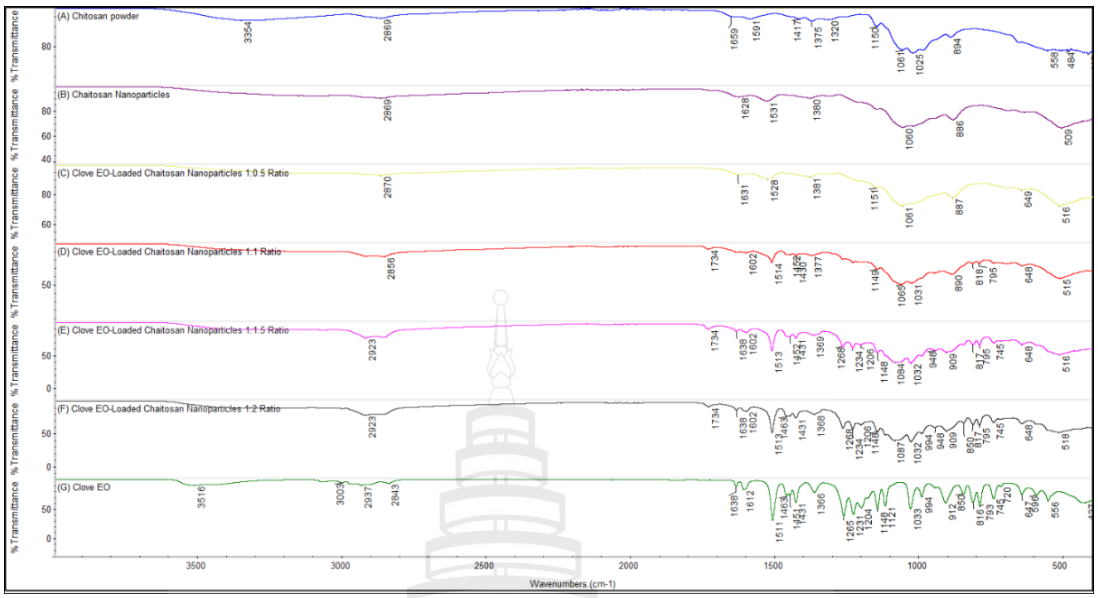


Figure 4.5 FTIR of (A) CS powder (Blue), (B) CS NPs (Purple), (C) CS/CEO NPs Ratio 1:0.5 (Yellow), (D) CS/CEO NPs Ratio 1:1 (red), (E) CS/CEO NPs Ratio 1:1.5 (Pink) and (F) CS/CEO NPs Ratio 1:2 Black), Pure CEO (Green)

4.2.3 Crystallinity (XRD)

The crystallographic structures of CS powder, CS NPS, CS/CEO NPs were determined by XRD. Figure 4.6 The pure chitosan powder (Red) showed a broad peak at around 20 degrees, suggesting semi-crystalline characteristics and the presence of overlapping crystal planes within the chitosan polymer matrix (Ali et al., 2024). The change to chitosan nanoparticles (Brown) was indicated by a sharper peak at the same angle, suggesting higher crystallinity from a more organized molecular structure (Shetta et al., 2019) and. This enhancement indicating that the process of creating nanoparticles leads to increased crystallinity in chitosan molecules, potentially enhancing the material's properties (Tang et al., 2003). As the ratios of CEO increased, significant changes were observed in the XRD patterns, with peak intensities decreasing and peaks becoming wider. This disturbance is likely due to CEO molecules interacting with the chitosan matrix, changing the typical arrangement of chitosan chains and increasing the amorphous regions within the nanoparticles (Shetta et al., 2019). These alterations not only demonstrate successful encapsulation but also imply chemical interactions that could enhance the flexibility and bioavailability of the matrix. The transition to less

defined structures in nanoparticles with CEO could significantly benefit applications that require controlled release mechanisms. This structural state may facilitate a more sustained and efficient release of active components (Adel et al., 2023). The absence of a defined structure in these nanoparticles renders them appropriate for drug delivery systems, where accuracy in release and material flexibility are vital for successful treatment. The XRD test shows that adding clove essential oil substantially alters the crystalline structure of chitosan nanoparticles, reducing crystallinity and enhancing their amorphous characteristics. These adjustments are vital for utilizing these nanoparticles in targeted drug delivery applications, where controlling the release of active compounds and the attributes of the delivery system are paramount.

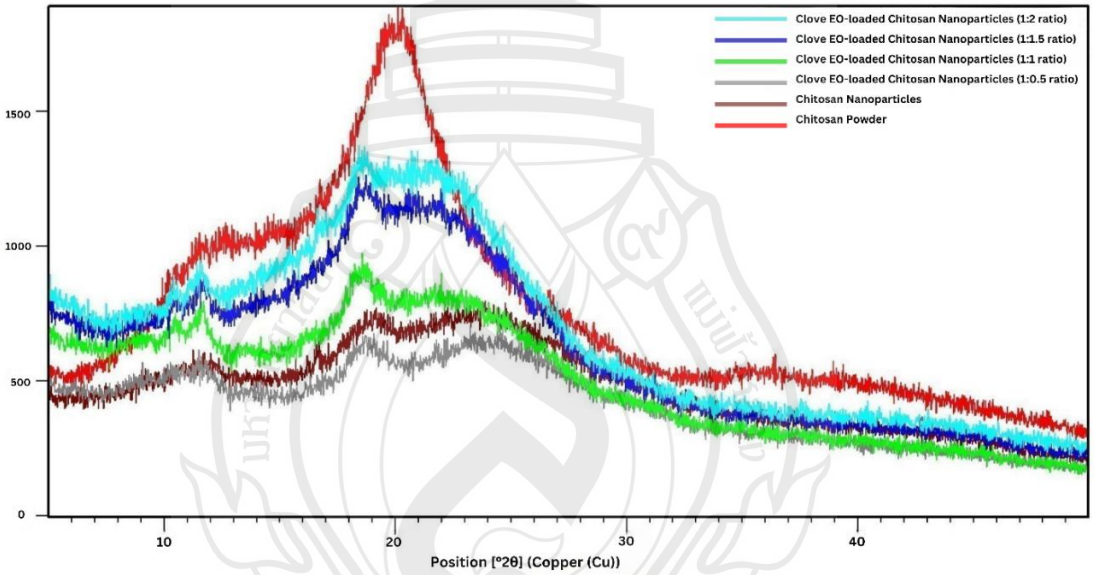


Figure 4.6 XRD of Chitosan powder (Red), Chitosan nanoparticles (Brown), and clove essential oil-loaded chitosan nanoparticles in various ratios (CS/EO): cyan (1:2), blue (1:1.5), green (1:1), and gray (1:0.5)

4.2.4 Thermal Stability (TGA/DTA)

TGA and DTA analyses assessed the thermal behavior and stability of the samples (Figure 4.7 and 4.8). The TGA curve for pure CEO (Blue, Figure 4.7) showed a rapid and almost complete weight loss occurring at relatively low temperatures (starting below 100°C and mostly complete by 230°C), reflecting its high volatility. Empty CSNPs (Green, Figure 4.7) displayed a typical multi-stage degradation pattern:

an initial weight loss below 100°C due to moisture evaporation, followed by major polymer decomposition at higher temperatures (e.g., starting around 250-300°C). The TGA curves for CEO-loaded CSNPs (Red for 1:0.5 ratio, Black for 1:1 ratio, Figure 4.7) showed patterns intermediate between empty CSNPs and pure CEO. Importantly, the significant weight loss attributed to CEO volatilization occurred at notably higher temperatures compared to free CEO, indicating that encapsulation within the chitosan matrix provided a protective barrier, enhancing the thermal stability of the oil.

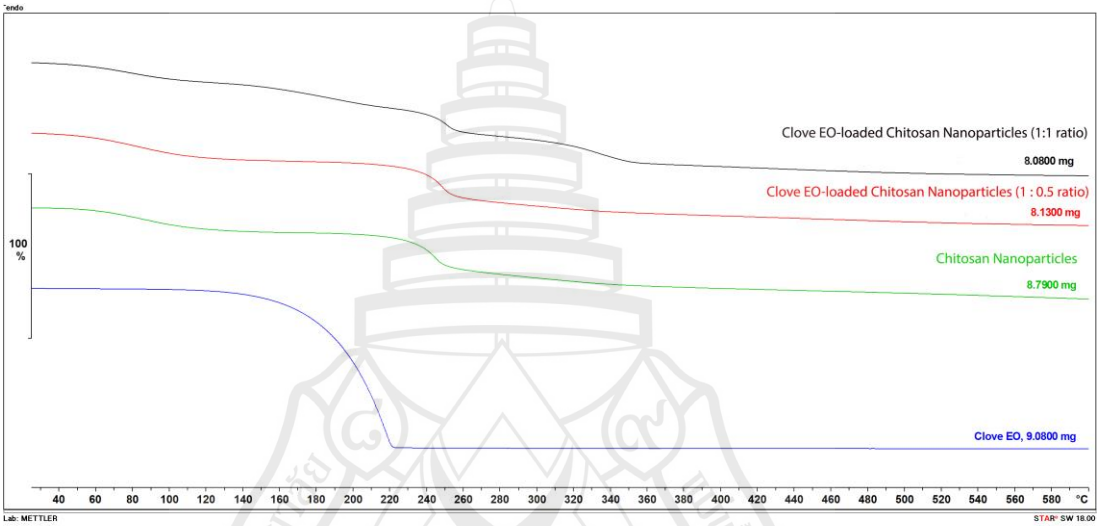


Figure 4.7 TGA thermogram of Pure EO (Blue), CS NP (Green) and Clove EO-loaded Chitosan Nanoparticles in different ratios (CS/EO): 1:0.5 (Red), 1:1 (Black)

DTA curves (Figure 4.8) complemented TGA, showing endothermic peaks corresponding to water evaporation (below 100°C) and CEO volatilization (peak around 220°C for free CEO, shifted to higher temperatures or merged with polymer degradation peaks for encapsulated CEO), and typically exothermic peaks related to chitosan decomposition at higher temperatures. These results collectively confirm successful encapsulation and improved thermal stability of CEO within the CSNPs.

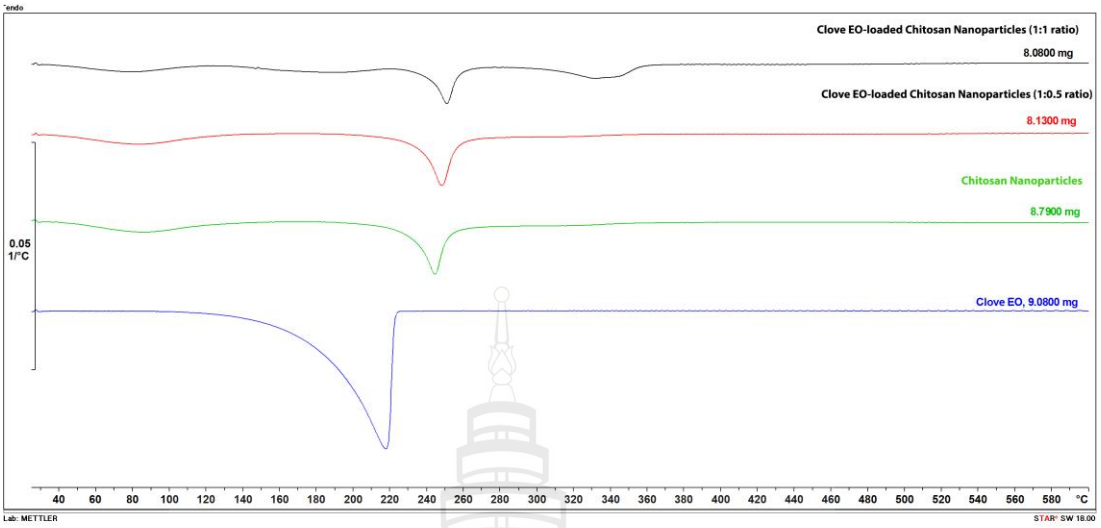


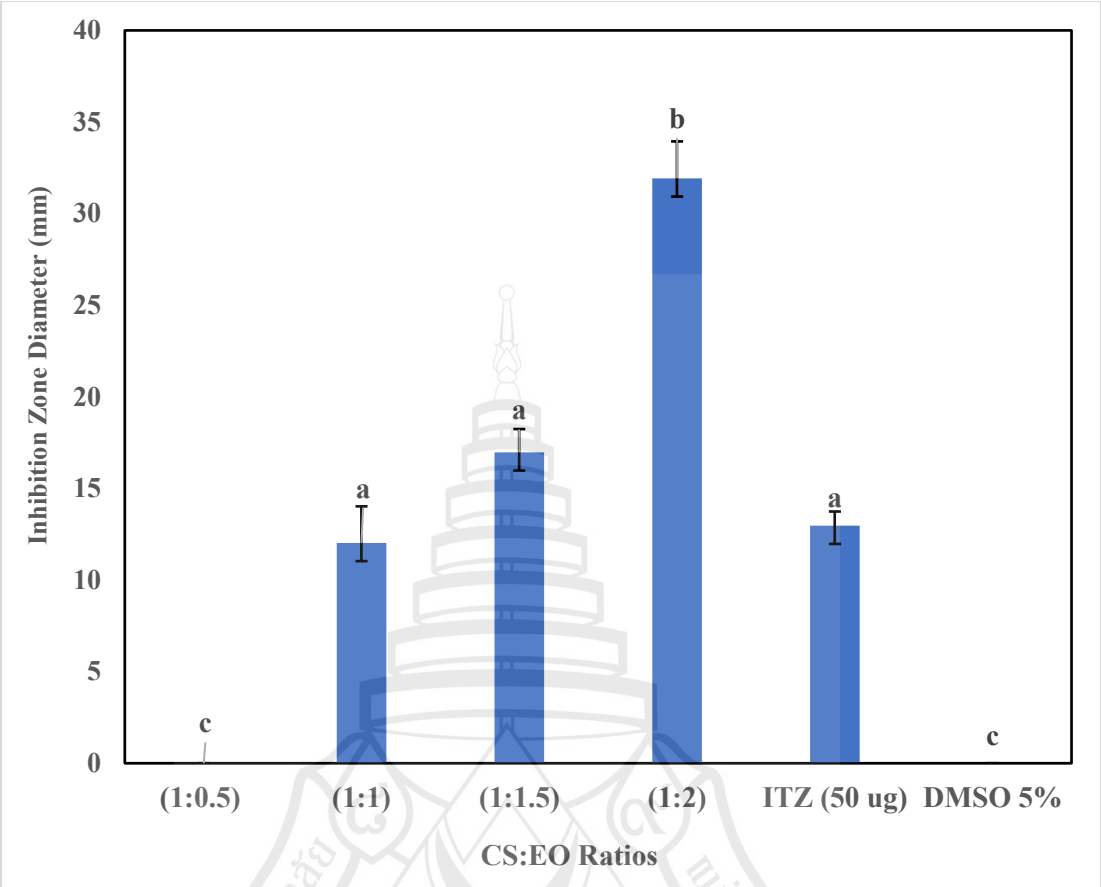
Figure 4.8 DTA thermogram of Pure EO (Blue), CS NP (Green) and Clove EO-loaded Chitosan Nanoparticles in different ratios (CS/EO): 1:0.5 (Red), 1:1 (Black)

4.3 Antifungal Activity of Clove EO Chitosan Nanoparticles

The antifungal activity of clove essential oil (CEO)-loaded chitosan nanoparticles (CEO-CSNPs) was evaluated against *Aspergillus niger* using the disc diffusion method across various chitosan to CEO ratios (1:0.5, 1:1, 1:1.5, and 1:2). A one-way ANOVA revealed statistically significant differences among the treatment groups ($F = 159.04$, $p < 0.0001$), as shown in figure 4.9 Among all formulations, the 1:2 ratio exhibited the largest inhibition zone (31.93 ± 2.01 mm), significantly outperforming all other formulations and the positive control, Itraconazole (12.97 ± 0.77 mm, $p < 0.05$). The 1:1.5 formulation (16.97 ± 1.27 mm) also demonstrated significantly greater antifungal activity than Itraconazole ($p < 0.05$). In contrast, the 1:1 formulation (12.03 ± 1.99 mm) showed no statistically significant difference from Itraconazole ($p > 0.05$), and the 1:0.5 formulation displayed no inhibition (0.00 ± 0.00 mm), equivalent to the negative control (5% DMSO). These findings indicate a clear dose-dependent enhancement in antifungal efficacy with increasing CEO loading. Tukey's HSD post hoc test confirmed that the 1:2 CEO-CSNPs group was significantly more effective than all other treatments ($p < 0.001$), while the 1:1 and 1:1.5 groups were comparable to the positive control. The DMSO control, as expected, exhibited no

antifungal activity. This improved antifungal performance at higher CEO loading ratios suggests that encapsulation enhances both the delivery and bioavailability of active compounds like eugenol. Sustained release and improved interaction with the fungal cell membrane may contribute to the observed efficacy. Statistical analysis using one-way ANOVA followed by Tukey's HSD test, the p-value ($p < 0.001$) is a statistically significant difference between the groups. The one-way ANOVA performed on this dataset indicates a highly statistically significant difference between the means of the different ratio concentrations, the ITZ group, and the DMSO control group. The 1:2 ratio CEO-CSNPs group was significantly more effective than all others, while the 1:1 and 1:1.5 formulations showed comparable efficacy to the positive control (Itraconazole), and the negative control (DMSO) demonstrated no effect.

These results suggest that encapsulation enhances the delivery and performance of clove EO, especially at higher loading ratios. Encapsulation likely promotes sustained release and improves bioavailability of active compounds like eugenol, leading to enhanced antifungal activity. These results align with previous studies (Allizond et al., 2023) and support the use of biopolymer-based encapsulation systems such as chitosan nanoparticles to enhance the stability, solubility, and bioefficacy of essential oils.



Note Bars represent mean inhibition zones ± SD (mm) (n = 3). The p-value ($p < 0.001$) is a statistically significant difference between the groups. Different letters (a–c) indicate significant differences ($p < 0.05$), determined by one-way ANOVA and Tukey's HSD post-hoc test. ITZ (50 µg) served as the positive control; DMSO 5% served as the negative control

Figure 4.9 Inhibitory antifungal activity of clove EO encapsulated within chitosan nanoparticles (CEO-CSNPs) at various ratios against *Aspergillus niger*

Free clove essential oil (CEO) and its nano-encapsulated form clove EO-loaded chitosan nanoparticles (CEO-CSNPs) were evaluated for antifungal activity against *Aspergillus niger*. The results, summarized in Table 4.2, demonstrated a dose-dependent antifungal response for free CEO. Even at 5%, free CEO produced measurable inhibition (17.55 ± 1.60 mm), with activity increasing substantially at higher concentrations. At 10%, inhibition (25.90 ± 0.39 mm) already surpassed that of the positive control, Itraconazole (ITZ, 12.97 ± 0.77 mm), and all CEO concentrations

≥10% exhibited statistically greater activity than ITZ ($p < 0.05$). The highest zone was observed at 100% CEO (50.20 ± 0.51 mm), highlighting its potent antifungal properties even at low doses.

Encapsulation of CEO into chitosan nanoparticles altered its antifungal profile, with efficacy highly dependent on the CS:CEO ratio. The 1:0.5 formulation showed no activity (0.00 ± 0.00 mm), while the 1:1 ratio produced moderate inhibition (12.03 ± 1.99 mm), not significantly different from ITZ ($p > 0.05$). Higher EO loadings, namely 1:1.5 (16.97 ± 1.27 mm) and 1:2 (31.93 ± 6.03 mm), resulted in significantly enhanced activity compared to ITZ ($p < 0.05$), with the 1:2 formulation performing comparably to free CEO at 25%.

These findings underscore the influence of formulation on antifungal efficacy. Free CEO demonstrated robust, dose-dependent inhibition, consistent with previous reports attributing its activity to eugenol the major bioactive component, which disrupts fungal membranes and inhibits ergosterol biosynthesis (Raut & Karuppayil, 2014). Recent studies confirmed CEO's potency, with MIC values ranging from 0.56 to 2.25 mg/mL against various *Aspergillus* species (Allizond et al., 2023). Nanoencapsulation influenced both the release kinetics and bioavailability of CEO. Lower EO loadings (e.g., 1:0.5) likely resulted in sub-threshold release, while optimized ratios (1:1.5 and 1:2) enabled controlled release with enhanced activity. Encapsulation stabilizes volatile components, prolongs efficacy, and enhances delivery, especially when the EO is well-distributed within the carrier matrix (Yang et al., 2023; Ravikumar et al., 2018). Furthermore, chitosan itself exhibits antimicrobial properties due to its polycationic structure, which disrupts negatively charged fungal membranes (Jafernik et al., 2023), contributing synergistically to the observed antifungal effect. In conclusion, free CEO is highly effective at low concentrations, offering rapid inhibition suitable for immediate antifungal applications. CEO-CSNPs, when properly formulated, offer controlled release and sustained activity, making them ideal for long-term protection. These results support the use of both free and encapsulated CEO as natural alternatives or complements to synthetic antifungal agents.

Table 4.2 Comparison of antifungal activity (zone of inhibition) of Free Clove Essential Oil (CEO), CEO-loaded chitosan nanoparticles (CEO-CSNPs), and control treatments against *Aspergillus niger*

Sample Type	CEO Conc/Ratio	Inhibition Zone (mm ± SD)
Free CEO	100%	50.20 ± 0.51 a
	75%	45.33 ± 1.70 b
	50%	40.76 ± 0.26 c
	25%	31.08 ± 2.49 d
	10%	25.90 ± 0.39 e
	5%	17.55 ± 1.60 f
CEO-CSNPs	1:2	31.93 ± 6.03 d
	1:1.5	16.97 ± 1.27 f
	1:1	12.03 ± 1.99 g
	1:0.5	0.00 ± 0.00 h
Positive Control	ITZ	12.97 ± 0.77 g
Negative Control	5% DMSO	00 ±00 h

Note Values represent mean ± standard deviation (n = 3). Different letters in the significance column indicate statistically significant differences between treatments based on one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Treatments sharing the same letter are not significantly different.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

The preliminary study shows that Clove essential oil (EO) exhibits the most potent antifungal activity against Aspergillus niger, followed by Cinnamon and Lemongrass EOs, corroborating findings from previous research that have highlighted the superior efficacy of Clove EO among commonly used plant-derived oils (Al-Maqtari et al., 2022). At 100% concentration, Clove EO achieved an inhibition zone of 50.20 ± 0.51 mm, which substantially exceeded the 13 mm zone observed for the positive control, Itraconazole. This aligns with prior studies reporting inhibition zones for Clove EO in the range of 40-55 mm against Aspergillus spp., depending on test conditions and fungal strain (Leyva Salas et al., 2017; Okano et al., 2020). The high antifungal potency of Clove EO is largely attributed to its major active compound, eugenol, which disrupts fungal cell membranes, induces cytoplasmic leakage, and inhibits ergosterol synthesis (Almeida et al., 2023). These mechanisms may explain its consistent activity across concentrations, including substantial inhibition at 25% and 10%, where the oil still outperformed Itraconazole, a synthetic antifungal widely used in clinical settings. Eugenol, the phenolic compound in CEO, exerts its antifungal effect by disrupting cell membrane integrity, altering membrane potential, inhibiting ergosterol biosynthesis, and increasing membrane permeability (Wang et al., 2024; Didehdar et al., 2022; Gupta et al., 2024). ITZ also compromises fungal membranes by inhibiting lanosterol 14α-demethylase, essential for ergosterol synthesis (Kurn & Wadhwa, 2023). The CEO was successfully encapsulated using the emulsification-ionic gelation technique, addressing the practical limitations of free essential oils—namely, volatility, low water solubility, and strong sensory impact (Hyldgaard et al., 2012; Sheikh et al., 2024). Chitosan was chosen as the encapsulant for its biocompatibility, biodegradability, and inherent antimicrobial properties (Rinaudo et al., 2006), potentially enhancing the oil's bioactivity. The ionic gelation method using

tripolyphosphate (TPP) offers advantages such as simplicity, mild reaction conditions, and solvent-free processing (Pitaloka et al., 2019).

Cinnamon EO also demonstrated considerable antifungal efficacy, albeit to a lesser extent than Clove EO. The observed inhibition zone of 46.40 ± 0.62 mm at 100% concentration falls within the range reported by earlier studies (30-48 mm) against A. niger and other spoilage fungi (Allizond et al., 2023). Cinnamon EO is rich in cinnamaldehyde, a phenylpropanoid known to interfere with fungal enzyme systems and cell wall integrity. While effective, its lower potency compared to Clove EO may be due to a less aggressive mode of action or lower diffusion efficiency in agar-based assays (Wang et al., 2024). Lemongrass EO, while demonstrating measurable antifungal effects, showed significantly reduced activity, particularly at lower concentrations. The inhibition zone at 100% (43.22 ± 1.71 mm) is consistent with previous findings where Lemongrass EO showed moderate antifungal activity attributed to citral and limonene, which possess membrane-disruptive and oxidative properties (Pascu et al., 2025). However, its steep drop in activity at 25% and below suggests a concentration-dependent threshold effect. This observation aligns with earlier reports indicating that Lemongrass EO becomes ineffective below 10% concentration in in vitro assays (Kodchasee et al., 2021).

Then we evaluated the antifungal potential of clove essential oil (CEO) encapsulated in chitosan nanoparticles (CSNPs) against Aspergillus niger, a common food spoilage mold. Free CEO demonstrated potent antifungal activity consistent with existing literature. The agar disc diffusion assay showed a dose-dependent effect, corroborating previous observations of CEO against Aspergillus species (Muñoz Castellanos et al., 2020). Inhibition zones at lower concentrations (5–10 %) were comparable to or greater than those of the standard antifungal drug Itraconazole (ITZ), suggesting CEO's promise as a natural fungicide. Recent findings affirm CEO's efficacy against clinical Aspergillus isolates and its superiority over some standard antifungals like ITZ in treating species such as A. fumigatus (Allizond et al., 2023; Naeem et al., 2023).

Physicochemical characterization confirmed successful CEO encapsulation. SEM analysis revealed spherical nanoparticles, consistent with previous studies (Keawchaoon & Yoksan, 2011; Hadidi et al., 2020). FTIR spectra displayed shifts in

O–H, N–H, and amide bands, indicating interactions between CEO and the chitosan matrix (Nasiri-Jahrodi et al., 2024). XRD analysis showed reduced crystallinity post-ionic gelation, a common observation that enhances CEO entrapment and controlled release (Hosseini et al., 2013; Hadidi et al., 2020; Mohammadi et al., 2015). Thermal analysis (TGA/DTA) revealed that encapsulated CEO exhibited greater thermal stability, evidenced by slower evaporation and higher decomposition temperatures, confirming the chitosan matrix's protective effect (Hadidi et al., 2020; Hosseini et al., 2013; Yang et al., 2023).

Antifungal performance of CEO-CSNPs correlated with EO loading levels. At the lowest ratio (1:0.5), CEO-CSNPs showed no antifungal effect, likely due to insufficient EO release. Increased ratios (1:1, 1:1.5, and 1:2) yielded progressively larger inhibition zones, indicating retained bioactivity (Hasheminejad et al., 2019; Hadidi et al., 2020). The highest ratio (1:2) achieved a 32 mm inhibition zone, approaching the free CEO's effect (31–50 mm for 25–100% concentrations). However, caution is needed when comparing free and encapsulated forms due to differing release kinetics and diffusion properties (Derguini et al., 2024). Free CEO diffuses rapidly due to volatility, while encapsulated CEO offers slower, sustained release (Yang et al., 2023; Kalagatur et al., 2018).

Encapsulation offers advantages like controlled release and prolonged antifungal protection, especially important in food and pharmaceutical applications. The smaller initial zones in disc diffusion assays for CEO-CSNPs do not diminish their long-term efficacy. Differences in diffusion and solubility between nanoparticle formulations and DMSO-dissolved free oil impact agar assay outcomes (Essid et al., 2023). The antifungal activity of CEO is attributed to eugenol's ability to disrupt cell membranes and inhibit vital fungal pathways (Hossain et al., 2022; Sharma et al., 2021). Encapsulation enhances this activity by stabilizing the oil, improving solubility, and enabling sustained release, making CS: EO formulations well-suited for applications in food preservation, agriculture, and pharmaceuticals (Donsì & Ferrari, 2016).

5.2 Conclusion

In this study, Clove Essential Oil (CEO) was effectively encapsulated within Chitosan Nanoparticles (CSNPs) using a two-step process comprising oil-in-water (O/W) emulsification followed by ionic gelation, wherein the chitosan polymer was cross-linked with sodium tripolyphosphate (TPP). This encapsulation strategy was designed to enhance the thermal stability and antifungal performance of the essential oil. Comprehensive physicochemical characterization of the CEO-CSNPs was carried out using advanced analytical techniques, including Fourier Transform Infrared Spectroscopy (FTIR), Powder X-ray Diffraction (XRD), and Thermogravimetric Analysis (TGA). Scanning Electron Microscopy (SEM) revealed that the nanoparticles possessed a uniform spherical morphology, with sizes ranging from 50.15 to 90.45 nm. TGA data confirmed that encapsulation significantly improved the thermal resistance of CEO. Notably, the CEO-CSNPs prepared at a 1:1 chitosan-to-EO ratio exhibited a thermal degradation range of 260–390 °C, underscoring the protective role of the chitosan matrix. The antifungal efficacy of CEO-CSNPs was assessed against *Aspergillus niger* using the agar disc diffusion method, with formulations tested at four different CS:EO ratios (1:0.5, 1:1, 1:1.5, and 1:2). One-way ANOVA revealed statistically significant differences among the groups ($p < 0.0001$). The 1:2 CEO-CSNP formulation exhibited the most potent antifungal activity, with an inhibition zone of 31.93 ± 2.01 mm significantly exceeding that of Itraconazole, the positive control (12.97 ± 0.77 mm, $p < 0.05$). The 1:1.5 formulation also demonstrated superior inhibition (16.97 ± 1.27 mm, $p < 0.05$), while the 1:1 formulation (12.03 ± 1.99 mm) was comparable to Itraconazole ($p > 0.05$). In contrast, the 1:0.5 formulation exhibited no measurable antifungal effect, similar to the negative control (5% DMSO). These findings demonstrate that increasing EO content significantly enhances antifungal performance, with the 1:2 ratio showing the highest efficacy, even higer than the standard antifungal. The encapsulation of CEO in chitosan nanoparticles effectively protected its bioactive components, enhanced its thermal stability, and scientifically improved its antifungal properties. These results highlight the promise of chitosan based nanocarriers as a sustainable and efficient delivery system for essential oils, with

potential application in natural food preservation. This approach aligns with current industry trends favoring safer, biodegradable, and environmentally friendly alternatives to synthetic preservatives. It offers a compelling strategy for extending shelf life and mitigating fungal spoilage in food products. However, it is important to note that this investigation was limited to in vitro evaluations. While the results are promising, they may not fully reflect the nanoparticles' performance under real-world conditions. Critical factors such as the behavior of CEO-CSNPs in complex food matrices, long-term storage, and environmental variability such as temperature and humidity, were not assessed. Additionally, the study did not examine efficacy in multi-strain or multi-species fungal systems.

5.3 Future Research Directions

To translate these findings into practical applications, future research should explore the use of CEO-CSNPs in real food systems. This includes in situ evaluation of antifungal performance, as well as assessing their influence on organoleptic properties, product shelf life, and overall consumer safety.

Further studies should also investigate the nanoparticles' behavior under a variety of environmental conditions such as changes in pH, temperature, and moisture to determine their stability and robustness. Broader-spectrum antifungal testing across different fungal genera and species is necessary to confirm their commercial viability and functional versatility.

Moreover, long-term stability studies and comprehensive toxicological evaluations are essential to ensure safety for human consumption and to meet regulatory standards. Exploring the encapsulation of other essential oils and comparing delivery formats such as nanofibers or liposomes could broaden potential applications and provide insights into how nanostructure influences bioactivity.

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