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Clove essential oil-loaded chitosan nanoparticles: Development, characterization and antifungal activity

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ABSTRACT

Clove essential oil (CEO), rich in eugenol, offers strong natural antimicrobial properties as an alternative to synthetic preservatives. This study aimed to encapsulate CEO into chitosan using ionic gelation, characterize the physical and chemical properties of the resulting nanoparticles, and evaluate their antifungal efficacy against *Aspergillus niger*. The nano-encapsulated CEO was characterized by using various techniques such as scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and X-ray Diffraction (XRD) confirmed the formation of spherical nanoparticles and the successful incorporation of CEO functional groups within the matrix. Initial assessments confirmed the dose-dependent antifungal activity of free CEO. Agar diffusion assays against *A. niger* demonstrated a substantial increase in antifungal efficacy, with inhibition zones increasing by approximately 186% as the concentration of clove essential oil (CEO) increased from 5% to 100%. The antifungal activity of the encapsulated CEO also increased with higher loading concentrations, with CEO-CSNPs at a 1:2 (CEO:CS) ratio exhibiting inhibition zones approximately 165% larger than those at the 1:1 ratio. These findings demonstrate that CEO encapsulated in chitosan nanoparticles significantly enhances antifungal activity and provides a promising strategy for natural food preservation by extending shelf life and reducing fungal spoilage.

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Introduction

Essential oils are aromatic and volatile compounds extracted from plants, widely appreciated for their antimicrobial potential. They are considered favorable alternatives to synthetic preservatives due to generally low toxicity and environmental friendliness (El Asbahanii et al., 2015; Bilia et al., 2014). Food safety concerns arise from fungal contaminants like *Aspergillus niger*, which can lead to spoilage and the production of toxic mycotoxins. Recent investigations confirm that various essential oils effectively inhibit foodborne fungi, supporting their application in controlling fungal growth (Almeida et al., 2023). Studies have demonstrated that essential oils such as clove, cinnamon, and lemongrass can significantly reduce fungal contamination and prolong the shelf life of bakery products and fruits (Ju et al., 2018). Clove

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essential oil has a lot of eugenol and has been shown to be very effective at killing fungi, including *Aspergillus* species, by breaking down their cell membranes (Jiang et al., 2022). Cloves, derived from the dried flower buds of the clove tree, contain multiple active compounds with strong antioxidant and antimicrobial properties. There are only small amounts of β-caryophyllene and α-humulene chemicals in clove essential oil (El Asbahani et al., 2015). Phenylpropanoids like eugenol and its derivatives are abundant in clove essential oil, enabling it to destroy fungal species such as *Aspergillus* by compromising their cell membrane integrity. Owing to its antioxidant, antibacterial, antiseptic, pesticidal, analgesic, and anticarcinogenic properties, CEO is utilized across food, healthcare, pharmaceutical, packaging, and cosmetic industries (Das et al., 2022). The use of CEO as a natural preservative, colorant, and spice in various food products is well-documented (Aguilar-González et al., 2015).

Common spoilage fungi such as *Aspergillus niger* and *Penicillium* spp. significantly decrease the shelf life and quality of a variety of food products such as bread and other bakery items (Darwish 2019, Abed et al. 2020, 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).

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; Essid 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; Essid 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 Essid 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).

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.

Materials and Methods

Materials

The fungal strain *Aspergillus niger* (strain 3281) utilized in this study was sourced from the Biotechnology Laboratory at Mae Fah Luang University, Chiang Rai, Thailand. It was maintained on Potato Dextrose Agar (PDA) medium and routinely sub-cultured in darkness at 25 °C. Itraconazole (ITZ), used as the antifungal standard, was provided in powdered form with a purity exceeding 98% (HPLC) and obtained from Tokyo Chemical Industry Co., Ltd., Thailand. Stock solutions of ITZ were prepared by dissolving the powder in 100% dimethyl sulfoxide (DMSO) and stored at −20 °C, serving as the positive control in fungal susceptibility assessments. Commercial-grade clove essential oil (*Eugenia caryophyllus* bud) was acquired from Bangkok Chemical Co., Ltd. (Bangkok, Thailand). For the encapsulation process and experimental procedures, low molecular weight chitosan (50–190 kDa; 75–85% deacetylation) was similarly obtained from Bangkok Chemical Co., Ltd. Additional chemicals and reagents included glacial acetic acid, Tween® 80, dichloromethane (CH_2Cl_2), sodium tripolyphosphate (TPP), and deionized water. DMSO was employed as a solvent for both ITZ and essential oils.

Fungal Inoculum Preparation

A. niger cultures were incubated on PDA plates at 25 ± 2 °C for seven days to promote abundant sporulation. Conidia were collected by gently scraping the surface of mature cultures with a sterile inoculating loop after adding approximately 5 mL of sterile 0.05% (v/v) Tween 80 solution to assist spore release. The resulting suspension was transferred into a sterile tube and vigorously vortexed for 1–2 minutes to disperse conidial clumps. Subsequently, the suspension was filtered through sterile cheesecloth or glass wool to eliminate mycelial fragments. Conidial concentration in the filtrate was measured using a hemocytometer under a light microscope. The suspension was adjusted with sterile 0.05% Tween 80 solution to a final concentration of 2×10^4 conidia/mL, following established protocols for *Aspergillus* susceptibility testing (Allizond et al., 2023; Tullio et al., 2007).

Antifungal Activity Assessment (Agar Disc Diffusion)

The antifungal activity of free CEO and CEO-CSNPs against *A. niger* was initially assessed using the agar disc diffusion method, a common screening technique for EO activity. Sterile PDA plates (90 mm diameter) were prepared. A volume of 100 µL of the standardized *A. niger* conidial suspension (2×10^4 conidia/mL) was evenly spread onto the surface of the agar plates using a sterile L-shaped spreader. For testing free CEO, stock solutions were prepared at concentrations of 100%, 75%, 50%, 25%, 10%, and 5% (v/v) using a solvent mixture of 5% DMSO and 0.5% Tween 80 to enhance dispersion. This solvent system was used exclusively for free CEO. Sterile filter paper discs (Whatman No. 1, 6 mm diameter) were impregnated with 20 µL of each CEO solution. For CEO-loaded chitosan nanoparticles (CEO-CSNPs), freeze-dried powders were reconstituted in sterile deionized water. The suspensions were prepared at concentrations corresponding to the original CEO loading ratios during synthesis (i.e., CS: CEO mass ratios of 1:0.5, 1:1, 1:1.5, and 1:2). Sterile paper discs were then loaded with 20 µL of each CEO-CSNP suspension. Sterile paper discs were impregnated with 20 µL of each nanoparticle suspension. Control discs were also prepared: a positive control disc containing 50 µg of itraconazole (ITZ) (prepared by applying 50 µL of the 1 mg/mL stock solution) and a negative control disc containing 20 µL of the same solvent mixture (5% DMSO with 0.5% Tween 80). The prepared discs (sample and controls) were carefully placed onto the surface of the inoculated PDA plates, ensuring firm contact with the agar. Plates were incubated in an upright position at 25 ± 2°C for 5–7 days, allowing for fungal growth and diffusion of the active compounds. Antifungal activity was determined by measuring the diameter (in millimeters, mm) of the clear zone of growth inhibition around each disc using a caliper or ruler. All tests were performed in triplicate, and results were expressed as the mean inhibition zone diameter ± standard deviation (SD). (Allizond et al., 2023; Xiang et al., 2020; Aimad et al., 2022).

Preparation of Clove EO-loaded Chitosan Nanoparticles (EO-CSNPs)

Essential oil-loaded chitosan nanoparticles (EO-CSNPs) were synthesized using a two-step ionic gelation technique, based on the method described by Hosseini et al. (2013), with slight modifications to optimize encapsulation efficiency and nanoparticle stability. Chitosan (medium molecular weight, 75–85% deacetylated) was dissolved at a concentration of 1% (w/v) in 1% (v/v) acetic acid solution. The solution was stirred continuously using a magnetic stirrer at room temperature (23–25 °C) for 24 hours to ensure complete dissolution. The resulting viscous solution was centrifuged at 10,000 rpm for 30 minutes to remove any

insoluble residues. The clear supernatant was collected and used as the polymer matrix. To enhance emulsion stability and minimize droplet aggregation, Tween 80 (0.5 g) was added as a non-ionic surfactant to 50 mL of the chitosan solution. The mixture was stirred at 45 °C for 2 hours to ensure homogeneous dispersion of the surfactant within the polymer matrix. Clove essential oil (CEO) was dissolved in 5 mL of dichloromethane (CH_2Cl_2). To evaluate the effect of CEO concentration on nanoparticle characteristics, varying amounts of oil (0.25, 0.5, 0.75, and 1.0 g) were used to achieve chitosan-to-oil mass ratios of 1:0.5, 1:1, 1:1.5, and 1:2, respectively. The oil phase was slowly added dropwise into the aqueous chitosan-surfactant mixture under high-speed homogenization at 14,000 rpm for 10 minutes using a homogenizer (T25 digital ULTRA-TURRAX®, IKA-Werke, Germany), maintained in an ice bath to prevent overheating. This step facilitated the formation of a fine and stable oil-in-water (O/W) emulsion. A freshly prepared 0.4% (w/v) sodium tripolyphosphate (TPP) aqueous solution (50 mL) was added dropwise to the emulsion under constant magnetic stirring (1000 rpm) at room temperature. This crosslinking process was continued for 40 minutes to allow electrostatic interactions between the cationic amino groups of chitosan and the anionic phosphate groups of TPP, resulting in the spontaneous formation of CEO-loaded nanoparticles via ionic gelation. The nanoparticles were collected by centrifugation at 10,000 rpm for 30 minutes at 4 °C, followed by triple washing with deionized water to remove unencapsulated oil and residual TPP. The resulting suspension was subjected to ultrasonication (SONICS VCX 750, Sonics Materials, Inc., Newtown, CT, USA) in an ice bath for 4 minutes, with alternating pulses of 2 seconds on and 1 second off, to reduce particle aggregation and enhance dispersion uniformity. The final nanoparticle suspension was frozen at −85 °C and lyophilized for 72 hours using a freeze dryer (CHRIST Beta 2–8 LSC basic, Germany). The dried CEO-CSNPs were stored in airtight containers at 4 °C until further characterization and bioactivity assays. Supernatants were also preserved for encapsulation efficiency analysis (Hosseini et al., 2013; Shetta et al., 2019).

Characterization of Nanoparticles

Surface Morphology and Size Analysis (SEM/EDS)

The surface morphology and particle size estimation of freeze-dried CEO-CSNPs and empty CSNPs were examined via Scanning Electron Microscopy (SEM) (TESCAN MIRA, Brno, Czech Republic). Approximately 1 mg of nanoparticle powder was dispersed in 20 mL of deionized water and briefly sonicated for 4 minutes to ensure adequate dispersion. A drop of the diluted suspension was placed onto a clean glass slide and allowed to air-dry completely at room temperature. The dried sample was subsequently mounted onto an SEM stub and coated with a thin layer of gold under high vacuum to improve conductivity and imaging quality. The coated samples were then visualized under SEM at appropriate accelerating voltages (10 keV), and representative images were recorded. The elemental composition (W%) was analyzed, reporting the mean ± standard deviation (n = 3) for carbon (C), oxygen (O), nitrogen (N), and phosphorus (P) (Hosseini et al., 2013).

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of pure chitosan powder, pure clove EO, unloaded CS NPs, and Clove EO-CSNPs were obtained using a PerkinElmer FTIR Spectrometer (Lambda 850+ by PerkinElmer, USA) equipped with a Standard PMT and Sphere 100 mm PMT. Samples were analyzed over a wavenumber range of 400 to 4000 cm^-1 with a resolution of 4 cm^-1, performing 32 scans per sample. (Hosseini et al.,2013; Hasheminejad et al., 2019).

Powder X-ray Diffraction (XRD)

XRD patterns were recorded using an X-ray diffractometer (PANalytical Empyrean, Malvern Panalytical Ltd., Malvern, UK) over a 2θ range of 5–50° with a step size of 0.013° and an integration time of 38 s per step, as described previously (Hosseini et al.,2013).

Thermal properties (TGA)

Thermogravimetric Analysis (TGA) and Differential Thermal Analysis (DTA) were performed using a TGA/DSC analyzer (TGA/DSC 3+, Mettler Toledo, Columbus, Ohio, U.S.A.). Samples (10 mg) of pure clove EO, unloaded CS NPs, and Clove EO-CSNPs were heated from 20 °C to 600 °C at a rate of 10 °C/min under a nitrogen atmosphere (Shetta et al.,2019).

Statistical Analysis

All experiments were conducted in triplicate, and results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to determine significant differences between treatment groups across various concentrations. A p-value below 0.05 ($p < 0.05$) was considered statistically significant. Statistical analyses were carried out using IBM SPSS Statistics software (Version 26.0), and graphical illustrations were generated using Microsoft Excel.

Results

Antifungal Activity

The antifungal activity of Free Clove Essential Oil (CEO) against *Aspergillus niger* was assessed using the disc diffusion method. The results revealed a distinct dose-dependent pattern, with inhibition zones increasing proportionally with CEO concentration (Fig. 1 and Fig. 2). At 100% concentration, CEO produced the largest zone of inhibition (50.20 ± 0.51 mm), followed by 75% (45.33 ± 1.70 mm), 50% (40.76 ± 0.26 mm), and 25% (31.08 ± 2.49 mm). Moderate activity was observed at 10% (25.90 ± 0.39 mm), while minimal inhibition was recorded at 5% (17.55 ± 1.60 mm). The positive control, Itraconazole (50µg/disc), produced a significantly smaller zone (12.97 ± 0.77 mm), whereas the negative control (5% DMSO) exhibited no inhibitory effect (0.00 ± 0.00 mm), confirming the specificity of CEO's antifungal action. Statistical analysis using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) confirmed significant differences among treatments. Each CEO concentration produced statistically distinct inhibition profiles, as indicated by the assigned significance letters in Fig. 2. Notably, all CEO concentrations at or above 25% showed significantly greater antifungal activity than Itraconazole, underscoring the oil's superior efficacy.

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 marked 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 documented strong antifungal effects of CEO in food systems. Moreover, the absence of inhibition in the DMSO control highlights that the observed antifungal effect is attributable solely to CEO. While these results demonstrate CEO's strong in vitro efficacy, further research is warranted to explore its in vivo performance, optimal dosing, and formulation strategies to enhance stability and sustained release such as encapsulation in chitosan nanoparticles. These results confirm a statistically significant difference between CEO-CSNPs and the control group ($p < 0.001$), consistent with the ANOVA and Tukey's post hoc analysis. This indicates that there is a statistically significant difference between the groups (concentrations, ITZ means of the different, and DMSO).

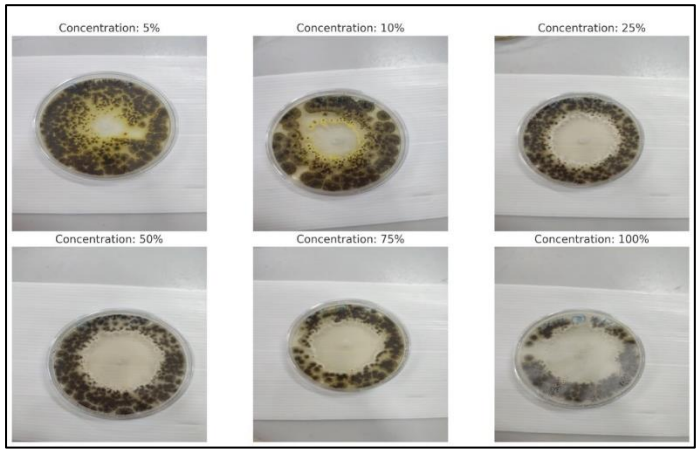

Fig 1. Inhibitory activity of clove essential oil (EO) at concentrations ranging from 5% to 100% against *Aspergillus niger* on agar medium. The diameter of the inhibition zone surrounding each disc reflects the antifungal effectiveness of the EO at the respective concentrations.

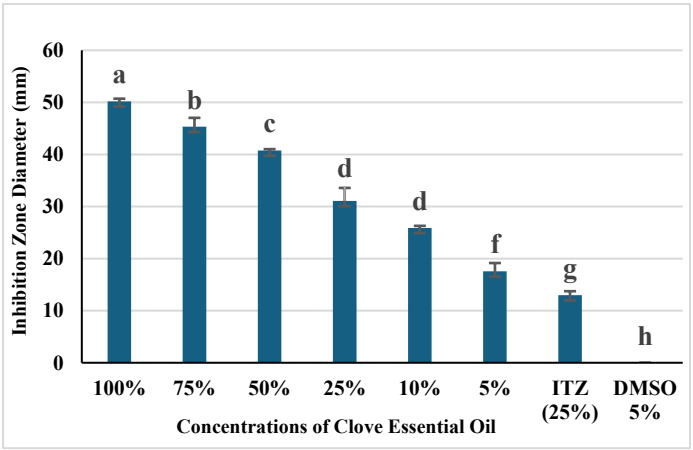

Fig 2. Zone of inhibition (mean ± SD, mm) of *Aspergillus niger* against varying concentrations of Free Clove Essential Oil (CEO) compared to Itraconazole (ITZ, 50 µg) as a positive control and DMSO 5% as a negative control. The p-value ($p < 0.001$) is a statistically significant difference between the groups. Different letters (a–h) indicate significant differences ($p < 0.05$), determined by one-way ANOVA followed by Tukey's HSD test.

Nanoparticle Characterization

Morphology (SEM/EDX)

Scanning Electron Microscopy images provided visual evidence of nanoparticle formation and morphology. Fig. 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. Fig. 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).

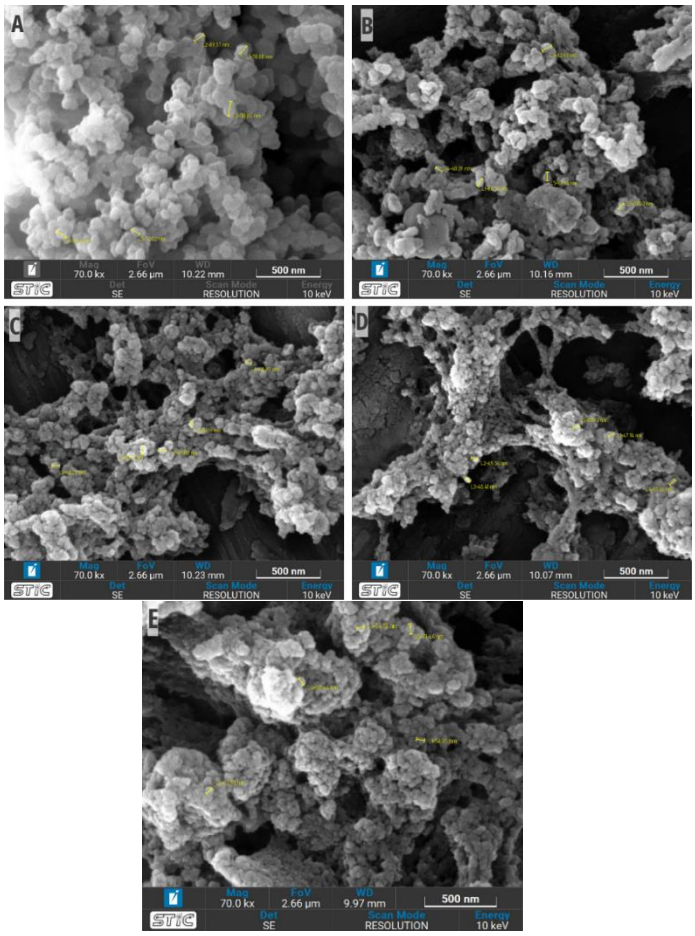

Fig 3. SEM images of clove essential oil-loaded chitosan nanoparticles, prepared with varying initial weight ratios of chitosan to clove essential oil: A (chitosan nanoparticles), B (1:0.5), C (1:1), D (1:1.5), and E (1:2). All images were acquired at 70,000× magnification using scanning electron microscopy at 10 kV. The scale bar in each image represents 500 nm.

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 1.

Table 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	1:0	90.4 ± 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

*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 particle size diameter with increasing (CEO-CSNPs) concentration, as shown in Table 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 reported effect of essential oil (EO) loading on chitosan nanoparticle size varies across the literature. Some studies have found that encapsulating essential oils, such as lemongrass oil, leads to an increase in particle size due to droplet expansion and matrix swelling (Abdelaziz et al., 2024; Hadidi et al., 2020). Conversely, other studies have shown that encapsulating EOs like cinnamon or thyme can result in a reduction in particle size, possibly due to enhanced emulsion stability or tighter packing of the polymer matrix (Barrera-Ruiz et al., 2020; Hosseini et al., 2013). In our study, the initial decrease in particle size from formulation A to D aligns with this latter trend. However, Zhang et al. (2020) reported that EO loading into chitosan nanoparticles

resulted in smaller particle sizes and improved morphological characteristics. However, the increase in size observed at the highest oil ratio (formulation E) suggests a more complex mechanism—possibly due to altered particle aggregation or reduced hydrophobic interactions leading to less compact nanoparticle formation. This biphasic trend contrasts with studies that observed only unidirectional changes, such as a consistent size increase (Abdelaziz et al., 2024; Ziaee et al., 2023) or decrease (Barrera-Ruiz et al., 2020; Hosseini et al., 2013). Hadidi et al. (2020) specifically investigated CEO-loaded chitosan nanoparticles and reported particle sizes ranging from 223 to 444 nm, significantly larger than the 50–90 nm range observed in our formulations. These differences could be attributed to variations in chitosan molecular weight, oil concentration, or preparation protocols. In addition to size effects, Hadidi et al. (2020) noted that CEO-loaded nanoparticles exhibited enhanced antibacterial activity and oxidative stability compared to free oils, highlighting the functional benefits of encapsulation. And, Zhang et al. (2020) further demonstrated that chitosan nanoparticles loaded with plant EOs improved antimicrobial performance against multidrug-resistant bacteria, emphasizing the therapeutic potential of nanocarrier-based EO delivery systems.

Elemental Composition by EDS Analysis

Energy Dispersive X-ray Spectroscopy (EDS) was used to analyze the elemental composition of the synthesized nanoparticles and to confirm the successful encapsulation of clove essential oil (CEO) within chitosan nanoparticles (CSNPs). As shown in Fig. 4, the EDS spectrum of unloaded chitosan nanoparticles revealed major elemental peaks for oxygen (67.77 ± 0.61%), carbon (19.20 ± 0.71%), and phosphorus (13.03 ± 1.38%), with calcium (10–15%) also detected. Nitrogen was qualitatively observed but not quantified. These elements reflect the composition and chemical integrity of the chitosan matrix. The strong signals for carbon, nitrogen, oxygen, and phosphorus indicate successful ionic crosslinking between chitosan and sodium tripolyphosphate (TPP). In particular, the phosphorus signal corresponds to the interaction of TPP's phosphate groups with the protonated amine groups of chitosan during nanoparticle formation (Nayila et al., 2024; Shenvi et al., 2014). Upon loading clove EO at a 1:1 (v/v) ratio, the surface elemental composition changed notably: carbon increased to 39.70 ± 0.74%, oxygen decreased to 49.00 ± 1.17%, and phosphorus dropped to 2.40 ± 0.72%. Nitrogen was measured at 8.90 ± 0.36%, indicating retention of the chitosan matrix. The marked rise in carbon is attributed to the incorporation of eugenol, the carbon-rich major component of clove EO (Shetta et al., 2019). The decrease in surface phosphorus is likely due to masking or shielding of the TPP crosslinker by the hydrophobic EO layer, limiting its surface detectability during EDS analysis. These compositional changes are consistent with previous studies involving EO encapsulation in CS-TPP nanoparticles (Salman et al., 2023). Further increasing the chitosan concentration in a 1:2 (v/v) formulation led to additional increases in carbon (44.40 ± 1.11%) and nitrogen (10.07 ± 0.46%), while oxygen content decreased to 42.73 ± 0.73%, and phosphorus slightly increased to 2.77 ± 0.32%. The elevated nitrogen content confirms the greater proportion of chitosan in the formulation. The modest rise in phosphorus compared to the 1:1 ratio suggests enhanced TPP crosslinking, possibly due to increased availability of amine groups from the higher chitosan content. Overall, the EDS data support successful encapsulation of CEO within a chitosan–TPP matrix, with surface elemental trends confirming structural and compositional modifications induced by EO loading. These findings align with earlier reports describing EO-dependent shifts in surface composition as detected by EDS (El-Naggar et al., 2023; Shenvi et al., 2014; Shetta et al., 2019).

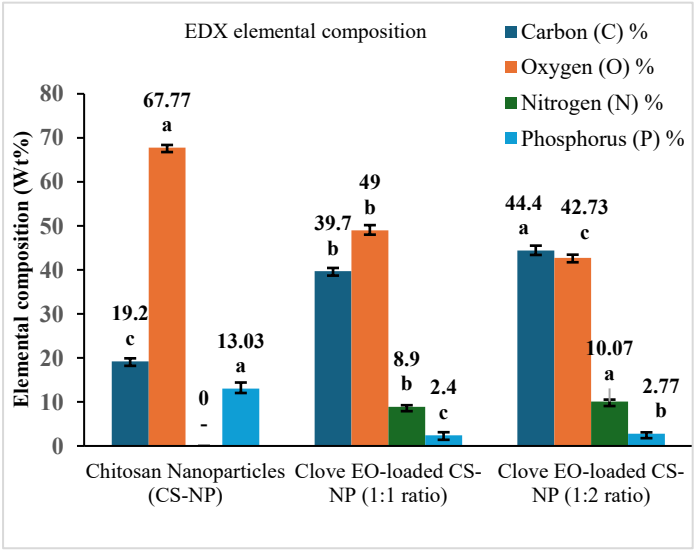

Fig 4. Elemental composition (W%) of Chitosan Nanoparticles (CS-NP), Clove Essential Oil-loaded CS-NP at 1:1 and 1:2 ratios, as determined by Energy Dispersive X-ray Spectroscopy (EDS). Data are presented as mean ± SD (n = 3). Superscript letters(a,b,c) above bars indicate statistically significant differences between groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's HSD test.

Chemical Interaction using Fourier transform infrared (FT-IR) spectroscopy analysis

Fourier Transform Infrared (FTIR) spectroscopy was employed to analyze the chemical structure and investigate interactions among chitosan, clove essential oil (CEO), and the components involved in nanoparticle formulation. As shown in Fig. 5, the spectrum of pure chitosan powder exhibited distinctive peaks, including a broad band around 3400 cm^{-1} (often described between 3500 and 3300 cm^{-1}), attributed to overlapping O–H and N–H stretching vibrations and hydrogen bonding within the polymer matrix (Dahmane et al., 2014). Additional peaks near 2900 cm^{-1} correspond to C–H stretching vibrations. Characteristic amide bands were also observed at approximately 1650 cm^{-1} (Amide I, C=O stretching of the residual acetyl group) and 1590–1600 cm^{-1} (Amide II, N–H bending of primary amines), confirming the chemical structure of chitosan (Mohammadpour Dounighi et al., 2012). The spectrum of pure CEO showed several distinct peaks corresponding to eugenol, its principal active compound. Aromatic C=C stretching was detected between 1600–1500 cm^{-1}, alongside a broad phenolic O–H stretching band typically appearing between 3500–3200 cm^{-1}. Additional peaks near 1270 cm^{-1} and 1150 cm^{-1}, attributed to C–O stretching, indicate the presence of aromatic and oxygen-containing functional groups typical of clove oil (Prajapati, 2024). In comparison, the spectra of unloaded chitosan nanoparticles (CSNPs) synthesized via ionic gelation with TPP displayed notable changes relative to pure chitosan. Amine and amide peaks showed reduced intensity and shifting: for instance, the N–H bending band shifted from ~1600 cm^{-1} to ~1540 cm^{-1}, and the Amide I band shifted from ~1650 cm^{-1} to ~1630 cm^{-1}. The broad O–H/N–H band also exhibited peak broadening and shifting. These spectral modifications indicate electrostatic interactions between the positively charged amino groups ($–NH_3^+$) in chitosan and the negatively charged phosphate groups in TPP during ionic crosslinking, a key mechanism for nanoparticle formation and stabilization. The emergence of a phosphate-related peak near 1170 cm^{-1} (P=O) further confirms the incorporation of TPP (Mohammadpour Dounighi et al., 2012). Additional spectral shifts were observed in CEO-loaded CSNPs. Characteristic peaks corresponding to CEO were present, confirming its successful incorporation within the nanoparticle matrix (Beyaz et al., 2025; Bidooki et al., 2024). Slight shifts in these peaks compared to the pure CEO spectrum suggest that the encapsulation process involves molecular interactions between CEO components and chitosan chains, rather than simple physical entrapment. These interactions contribute to the structural stability and integrity of the loaded nanoparticles.

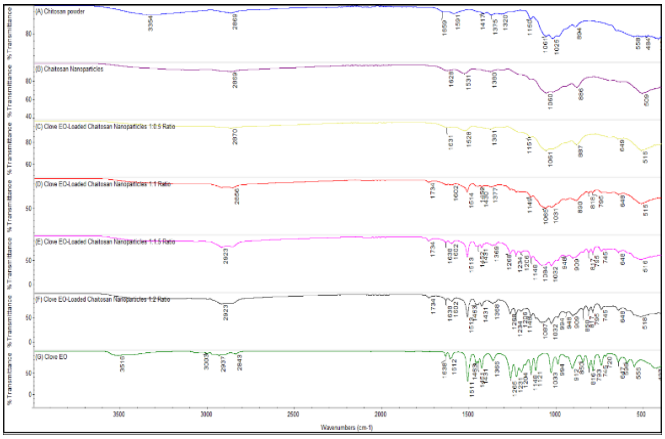

Fig 5. FTIR spectra of (A) CS powder (Blue), (B) CS NPs (Purple), (C) CS/CEO NPs Ratio 1:0.5 (Yellow), (D) CS/CEO NPs Ratio 1:1 (red), (E) CS/CEO NPs Ratio 1:1.5 (Pink) and (F) CS/CEO NPs Ratio 1:2 Black), Pure CEO (Green).

Powder X-ray Diffraction (XRD)

The crystallographic structures of chitosan (CS) powder, chitosan nanoparticles (CSNPs), and CEO-loaded CSNPs were determined using X-ray diffraction (XRD) analysis (Fig. 6). The pure chitosan powder (red) exhibited a broad peak at approximately 20°, indicating its semi-crystalline nature (Ali et al., 2024). The conversion to chitosan nanoparticles (brown) resulted in a sharper peak at ~20°, consistent with increased crystallinity due to particle size reduction and ionic crosslinking (Shetta et al., 2019; Tang et al., 2003). As the CEO loading ratios increased, notable changes were observed in the XRD patterns—specifically, peak intensities decreased and peaks became broader. These changes likely reflect interactions between clove essential oil (CEO) molecules and the chitosan matrix, which disrupt the native chain organization and increase the amorphous character of the nanoparticles (Shetta et al., 2019). These structural shifts confirmed successful encapsulation and suggest molecular interactions that may enhance the flexibility and bioavailability of the matrix. The transition to a more amorphous structure in CEO-loaded nanoparticles could be advantageous for controlled release applications, potentially improving the delivery of active compounds (Adel et al., 2023). The reduction in crystalline order makes these nanoparticles suitable for drug delivery systems, where controlled and sustained release is essential for therapeutic efficacy (Mohammed et al., 2020). Supporting this, XRD analysis demonstrated that the incorporation of clove essential oil

reduced the crystallinity and enhanced the amorphous properties of chitosan nanoparticles. These structural modifications are critical for the development of targeted drug delivery platforms, where the fine-tuning of release kinetics and carrier performance is essential (Hadidi et al., 2020).

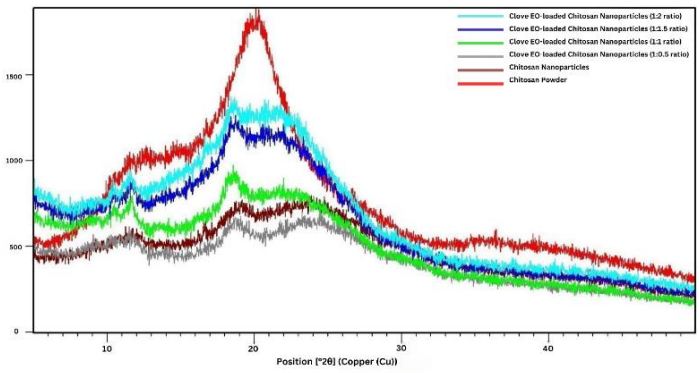

Fig 6. XRD of Chitosan powder (Red), Chitosan nanoparticles (Brown), and clove essential oil-loaded chitosan nanoparticles in various ratios (CS/EO): cyan (1:2), blue (1:1.5), green (1:1), and gray (1:0.5).

Thermal Stability (TGA/DTA)

TGA and DTA analyses assessed the thermal behavior and stability of the samples (Fig. 7 and Fig. 8). The TGA curve for pure CEO (Blue, Fig. 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.

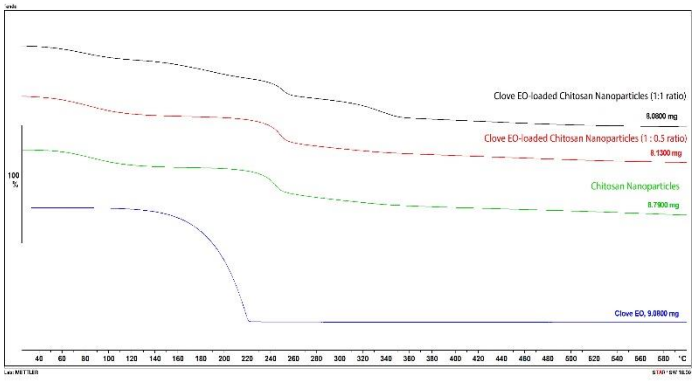

Fig. 7 TGA thermogram of Pure EO (Blue), CS NP (Green) and Clove EO-loaded Chitosan Nanoparticles in different ratios (CS/EO): 1:0.5 (Red), 1:1 (Black).

Empty CSNPs (Green, Fig. 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, Fig. 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.

DTA curves (Fig. 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.

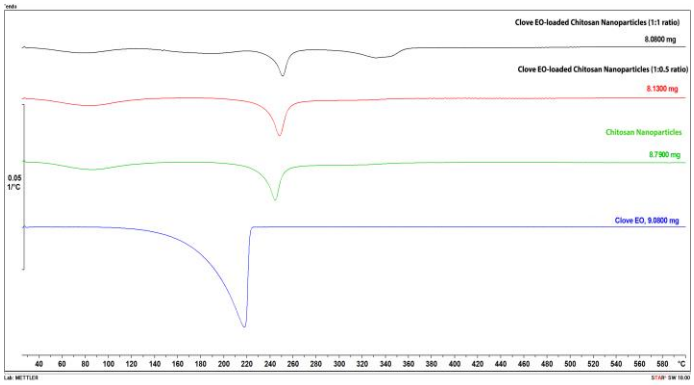

Fig 8. DTA thermogram of Pure EO (Blue), CS NP (Green) and Clove EO-loaded Chitosan Nanoparticles in different ratios (CS/EO): 1:0.5 (Red), 1:1 (Black).

Antifungal Activity of clove CEO 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 Fig. 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. 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.

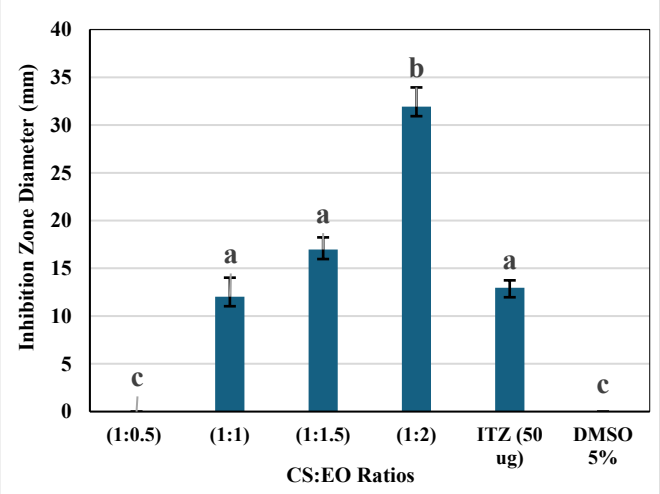

Fig 9. Inhibitory antifungal activity of clove EO encapsulated within chitosan nanoparticles (CEO-CSNPs) at various ratios against *Aspergillus niger*. Bars represent mean inhibition zones ± SD (mm) (n = 3). The ($p < 0.001$) indicates a statistically significant difference between the groups. Different 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.

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 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 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 Conc %	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 (CS:CEO ratio)	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	0.00 ± 0.00 h

* 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.

Discussion

This study evaluated the antifungal potential of clove essential oil (CEO) encapsulated in chitosan nanoparticles (CSNPs), specifically targeting *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).

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).

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).

Conclusion

In conclusion, this study successfully synthesized and characterized clove essential oil-loaded chitosan nanoparticles (CEO-CSNPs) using a straightforward emulsification-ionic gelation method. The investigation highlighted several key findings. Firstly, CEO-CSNPs with higher loading ratios (1:2 CS:EO) exhibited the strongest inhibition…" could be shortened by removing "(CS: EO). Secondly, both free CEO and the CEO-CSNPs demonstrated potent, concentration/loading-dependent antifungal activity against the common food spoilage mold *Aspergillus niger* in agar disc diffusion assays. Notably, CEO-CSNPs with higher loading ratios (1:2 CS: EO) exhibited the strongest inhibition against *A. niger*, with an inhibition zone of 31.93 ± 2.01 mm, significantly outperforming other formulations and the positive control, Itraconazole. Free CEO also showed strong dose-dependent antifungal activity, with 100% concentration yielding a 50.20 ± 0.51 mm inhibition zone. The practical applications of these findings are promising, particularly for the food industry. The developed CEO-CSNPs present a potential natural antifungal agent for food preservation, aligning with the increasing consumer demand for safer alternatives to synthetic preservatives. This nanoencapsulation strategy offers a method to extend the shelf life and ensure the safety of food products susceptible to fungal contamination by improving the stability and applicability of clove essential oil. The enhanced thermal stability and controlled release capabilities make these nanoparticles suitable for integration into food packaging or direct application to food surfaces.

Limitations

This study primarily focused on the in vitro antifungal efficacy of clove essential oil (CEO) and CEO-loaded chitosan nanoparticles (CEO-CSNPs) against *Aspergillus niger*. While promising, the results are limited to laboratory conditions and may not fully predict performance in complex food matrices or under real storage and environmental conditions. Additionally, the long-term stability of the nanoparticles and their behavior in multi-strain or multi-species fungal systems were not assessed.

Future Work

Future research should explore the in-situ application of CEO-CSNPs in real food systems, evaluating not only antifungal activity but also effects on sensory properties, product shelf life, and consumer safety. Investigating nanoparticle behavior under different environmental stressors (e.g., pH, temperature, humidity) and validating their efficacy against a broader range of fungal species will help support their commercial use. Long-term toxicological and environmental impact assessments are also necessary to confirm safety for widespread application.

Conflict of interest

The authors declare that there are no conflicts of interest related to the research, authorship, or publication of this article.

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Data availability

All data obtained from this study are included in the current manuscript.

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