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Chitosan Nanoparticle-Encapsulated Essential Oils as a Novel Strategy for Managing Bean Charcoal Rot

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Abstract

Charcoal rot, caused by the soil-borne fungus *Macrophomina phaseolina* (Holliday & Punithalingam 1988), is a devastating disease affecting many hosts, including beans. This pathogen persists in the soil for extended periods, primarily as microsclerotia, posing significant challenges for effective management. To address this issue, the present study aimed to enhance the antifungal properties of essential oils (EOs) by encapsulating them within chitosan nanoparticles. Specifically, EOs derived from five plant species—*Carum carvi* (L. 1753), *C. copticum* ((L.) C. B. Clarke 1879), *Mentha aquatica* (L. 1753), *M. piperita* (L. 1753), and *Satureja khuzistanica* (Jamzad 1994) —were nanoencapsulated to improve their stability and efficacy against *M. phaseolina*. The performance of these nanoencapsulated EOs was compared with their free counterparts. Chitosan nanoparticles containing EOs were synthesized using the ionic gelation method. Characterization analyses revealed that the nanoparticles were spherical, positively charged, and ranged in size from 45 to 232.6 nm. In vitro assays demonstrated that nanoencapsulated EOs—*C. carvi* essential oil (CEO), *C. copticum* essential oil (CcEO), *M. aquatica* essential oil (MaEO), *M. piperita* essential oil (MEO), and *S. khuzistanica* essential oil (SEO)—at a concentration of 500 ppm significantly inhibited the growth of *M. phaseolina* compared to their free forms. These findings suggest that nanoencapsulated EOs represent a promising strategy for managing *M. phaseolina*. Future research should focus on field trials to evaluate the efficacy of these formulations under real agricultural conditions. Additionally, exploring the synergistic effects of combining different EOs or nanoparticle formulations, and investigating the mechanisms underlying the enhanced efficacy of chitosan nanoparticles could pave the way for developing more effective biopesticides.

Keywords: Macrophomina phaseolina; Nanoencapsulation; Chitosan nanoparticles; Essential oils; Antifungal activity

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اسانس‌های کپسوله شده با نانوذرات کیتوزان به عنوان یک استراتژی جدید برای مدیریت پوسیدگی ذغالی لوبیا

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چکیده

پوسیدگی زغالی، ناشی از قارچ خاک‌زی *Macrophomina phaseolina* (Holliday & Punithalingam 1988)، بیماری مخربی است که طیف وسیعی از میزبان‌ها، از جمله لوبیا، را تحت تأثیر قرار می‌دهد. این عامل بیماری‌زا به‌صورت میکرواسکلروتیا برای مدت طولانی در خاک باقی می‌ماند و مدیریت مؤثر آن را با چالش‌های جدی مواجه می‌کند. این مطالعه با هدف ارتقای خواص ضدقارچی اسانس‌های گیاهی (EOs) از طریق کپسوله‌سازی آن‌ها در نانوذرات کیتوزان انجام شد. اسانس‌های استخراج‌شده از پنج گونه گیاهی شامل *Carum carvi* (L. 1753)–Clarke 1879)، *Mentha aquatica* (L. 1753)، *M. piperita* (L. 1753) و *Satureja khuzistanica* (Jamzad 1994) - نانو کپسوله شدند تا پایداری و کارایی آن‌ها در برابر *M. phaseolina* بهبود یابد. عملکرد اسانس‌های نانوکپسوله‌شده با نمونه‌های آزاد آن‌ها مقایسه شد. نانوذرات کیتوزان حاوی اسانس‌ها با روش ژلاسیون یونی سنتز شدند. آنالیز ذرات بدست آمده نشان داد که نانوذرات کروی، دارای بار مثبت و اندازه‌ای بین ٤٥ تا ٢٣٢/٦ نانومتر بودند. نتایج آزمایش‌ها نشان داد که اسانس‌های نانوکپسوله‌شده شامل اسانس *C. carvi* (CEO)، *C. copticum* (CcEO)، *M. aquatica* (MaEO)، *M. piperita* (MEO) و *S. khuzistanica* (SEO) در غلظت ۵۰۰ پی‌پی‌ام، در مقایسه با اشکال آزاد، به‌طور معنی‌داری رشد قارچ *M. phaseolina* را مهار کردند. این یافته‌ها نشان می‌دهند که اسانس‌های نانوکپسوله‌شده استراتژی نویدبخشی برای مدیریت پوسیدگی ذغالی لوبیا *M. phaseolina* ارائه می‌دهند. تحقیقات آتی باید بر آزمایش‌های مزرعه‌ای برای ارزیابی کارایی این فرمولاسیون‌ها در شرایط واقعی کشاورزی متمرکز شوند.

کلیدواژه ها: Macrophomina phaseolina، نانو کپسوله، نانو ذرات کیتوزان، اسانس، ضد قارچی

دبیر تخصصی: دکتر معصومه ضیائی

Introduction

Macrophomina phaseolina (Holliday & Punithalingam 1988), a devastating soil-borne fungal pathogen, is responsible for significant crop losses worldwide. This pathogen exhibits a broad host range, infecting over 500 plant species across more than 100 families, including economically important crops such as soybean, sunflower, cotton, sesame, eggplant, melon, maize, and strawberry (Gao et al., 2021; Sun et al., 2013; Majumder et al., 2018; Khan & Javaid, 2020). The fungus can cause various diseases in its host plants, including charcoal rot, stem rot, root rot, collar rot, damping-off, and seedling blight (Babu et al., 2007; Sun et al., 2013; Balamurugan, 2014). The disease can lead to severe yield reductions under favorable environmental conditions—such as high temperatures and dry soils (Hutton et al., 2013; Gomez et al., 2020). *M. phaseolina* persists in the soil as sclerotia, compact masses of hardened mycelial structures capable of remaining dormant for extended periods. These sclerotia germinate to produce hyphae that infect host plants (Balamurugan, 2014; Reddy et al., 2022). Additionally, the fungus secretes cell wall-degrading enzymes that contribute to its pathogenicity (Reddy et al., 2022).

Essential oils (EOs) are complex mixtures of volatile and semi-volatile organic compounds derived from secondary metabolites produced by plants in specialized secretory cells, glands, and ducts located in various plant parts, such as leaves, flowers, fruits, buds, roots, and branches (Rapper et al., 2023). These compounds are synthesized in response to stress arising from primary metabolic processes within plants (Orchard & Vuuren, 2017). EOs comprise a variety of bioactive compounds, including monoterpenoids, sesquiterpenoids, phenylpropanoids, and flavonoids, which confer unique biological properties such as antimicrobial, antifungal, antiviral, anti-parasitic, anti-tumor, and antioxidant effects (Orchard & Vuuren, 2017). Due to their wide range of biological activities—including antimicrobial, antioxidant, and anti-inflammatory properties—EOs have attracted significant attention for applications in the food, cosmetic, and pharmaceutical industries (Donsì et al., 2011; Froiio et al., 2019).

Chitosan-based nanoparticles, derived from the deacetylation of chitin, have been extensively studied for their diverse applications, including antimicrobial and antifungal activities, pest control, pharmaceutical uses, and food and cosmetic applications (da Silva Maia, 2021). Chitosan nanoparticles have emerged as a promising platform for the encapsulation, delivery, and controlled release of therapeutic agents due to their unique properties, such as biocompatibility, biodegradability, and the ability to form polyelectrolyte complexes with anionic polysaccharides (Froiio et al., 2019; Aftab, 2023).

The encapsulation of active compounds within nanoparticles enhances their stability, solubility, and targeted delivery, thereby improving their efficacy in diverse applications (da Silva Maia, 2021). For instance, encapsulating drugs, proteins, and essential oils within chitosan-based nanoparticles can enhance their stability, bioavailability, and targeted delivery (Schütz et al., 2011; Sadhukha & Prabha, 2014; Chenthamara et al., 2019). The physicochemical properties of nanoparticles, such as size and surface charge, can be fine-tuned by adjusting formulation parameters, including chitosan concentration, crosslinking agent concentration, chitosan molecular weight, and solution pH (Wang et al., 2018).

Chitosan nanoparticles are typically formed through ionic crosslinking with anionic counterions such as tripolyphosphate (TPP) (Schütz et al., 2011; Chenthamara et al., 2019). Chitosan-TPP nanoparticles

effectively encapsulate EOs, protecting them from degradation and enhancing their antimicrobial and insecticidal activities (Sadhukha & Prabha, 2014). Various encapsulation techniques, including ionic gelation, emulsion-based methods, and electrospraying, can be employed depending on factors such as the physicochemical properties of the EOs, desired nanoparticle characteristics, and intended application (Jenjob et al., 2019).

Researchers have explored various antifungal agents, including plant extracts, oils, and biocontrol agents, as potential control measures against *M. phaseolina* (Ugulino et al., 2018; Salman et al., 2022). Among these strategies, nanoparticles and essential oils have demonstrated promising antifungal activity. For example, silver and copper nanoparticles have proven effective against this pathogen, while essential oils from sources such as clove, *Citrus aurantifolia*, and *Senecio amplexicaulis* have shown significant inhibitory effects on the growth of *M. phaseolina* (Ugulino et al., 2018; Salman et al., 2022). However, conventional chemical pesticides pose substantial risks to environmental safety and human health, complicating management efforts (Pathak et al., 2022). Moreover, resistant cultivars face challenges due to the limited availability of effective resistance genes, low resistance levels, and the complexities of multi-gene inheritance (Manzoor et al., 2024). Given these challenges, *M. phaseolina* remains a formidable threat to numerous crops.

This research focuses on encapsulating chitosan-tripolyphosphate nanoparticles containing essential oils derived from five distinct plant species - *Carum carvi* (L. 1753, Family: *Apiaceae*), *C. copticum* ((L.) C. B. Clarke 1879, Family: *Apiaceae)*, *Mentha aquatica* (L. 1753, Family: *Lamiaceae)*, *M. piperita* (L. 1753, Family: *Lamiaceae*), and *Satureja khuzistanica* (Jamzad 1994, Family: *Lamiaceae*) - to inhibit the radial growth of *M. phaseolina*. The study compares the effectiveness of nanoencapsulated EOs with their free forms under laboratory conditions, representing a safer and more sustainable approach to managing this prevalent pathogen.

Materials and Methods

Isolation of Pathogenic Fungus

Bean plants exhibiting symptoms of charcoal rot such as green wilting, yellowing of leaves, and discoloration of root vascular tissue from brown to black were sampled from a bean farm in Iran. The samples were transported to the laboratory, where infected and healthy tissue segments were separated after surface disinfection with 5% sodium hypochlorite for one minute, followed by three rinses with sterile distilled water. After drying on sterile paper, the segments were cultured on potato dextrose agar (PDA) and incubated at 27°C for 7 days. Purification was performed using the hyphal tip method, and the isolates were stored at 4°C for further analysis.

DNA Extraction and Polymerase Chain Reaction (PCR)

For molecular identification, the mycelial mass of fungi cultivated in potato dextrose broth (PDB) was harvested from seven-day-old cultures. Genomic DNA extraction was performed using the method described by Alizade et al. (2022), with minor modifications. PCR amplification was conducted using the primers MpKF1 (5'-CCGCCAGAGGACTATCAAAC-3') and MpKRI (5'-CGTCCGAAGCGAGGTGTATT-3') (Babu et al., 2007). The PCR reaction mixture (25 µl total volume) consisted of 2.5 µl of 10X PCR buffer, 0.5 M Tris–HCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.04 Units/µl Taq DNA polymerase, 0.1 µM of each primer, and 10 pg of template DNA. The thermal cycling conditions were as follows: initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 30

seconds, annealing at 62.4°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 5 minutes. PCR products were sequenced by Codon Genetics (Iran), and the sequences were compared with those in the GenBank database.

Pathogenicity Test

The pathogenicity of *M. phaseolina* on bean plants was evaluated as follows: Bean seeds were surface-disinfected with 5% sodium hypochlorite and rinsed with distilled water. The seeds were then planted in pots containing a sterilized (121°C, 1 atm, 30 minutes) mixture of farm soil, leaf compost, peat, and perlite in a ratio of 1:1:2:2. At planting, three pieces of rice colonized with *M. phaseolina* were placed near the seeds (Rusuku et al., 1997). After three weeks, the external symptoms of the seedlings were examined. The germination percentage of bean seeds was calculated using the formula:

$GP = \sum Gi / N \times 100$

where *GP* is the germination percentage, *Gi* is the number of germinated seeds, and *N* is the total number of seeds. Positive control: Bean seeds inoculated with sterile rice seeds. Negative control: Distilled water.

Plant Samples for Essential Oil Preparation

The plant species used in this study were collected as follows: *M. piperita* was sourced from the vicinity of the Faculty of Agriculture at Tarbiat Modares University, while *M. aquatica* was obtained from its natural habitat in Malekan City, Iran. Due to the unavailability of fresh aerial organs of plant samples, *C. carvi, C. copticum, and S. khuzistanica* were purchased from Jahan Saderat Sepanj Company. All plant species were identified at the Research and Natural Resources Center of Tehran Province.

Specifications and preparation of chitosan solution

For this research, chitosan extracted from shrimp shells, characterized by a molecular weight of 810 kDa and a degree of acetylation exceeding 75%, was obtained from Sigma-Aldrich® (Germany).

Chitosan (0.3 g) was initially dissolved in 100 mL of 1% (v/v) acetic acid solution (Merck KGaA, Germany), followed by serial dilutions with sterile distilled water to prepare working concentrations. These dilutions were performed to determine the minimum inhibitory concentration (MIC) required to suppress 50% mycelial growth (MIC50). A 1% (v/v) acetic acid solution served as the negative control. The pH of the chitosan-acetic acid mixture was adjusted to 5, as reported by Kheiri, et al. (2016).

Preparation of Essential Oils (EOs)

Essential oils were extracted via hydro distillation using a Clevenger apparatus. For this purpose, 50 g of shade-dried aerial parts of each plant were ground and placed in the flask of the Clevenger apparatus. A total of 500 ml of distilled water was added, and the mixture was distilled for 3 hours (Negahban et al., 2006). The chemical composition of the essential oils was analyzed using gas chromatography-mass spectrometry (GC-MS).

Preparation of Chitosan Nanoparticles Containing Eos

Chitosan nanoparticles were synthesized using a modified version of the method described by Matshetshe et al. (2018). A chitosan solution (1% w/v) was prepared by dissolving chitosan in 1% (v/v) aqueous acetic acid under magnetic stirring at room temperature. To remove impurities, the solution was centrifuged at 10,000 rpm for 15 minutes after 24 hours. The resulting supernatant was collected, and its pH was adjusted to 5.5 using a NaOH solution. Tween 80 was added to the chitosan solution at a weight ratio of 1:10 under continuous stirring. The essential oil of each plant was then added dropwise to the chitosan solution with vigorous stirring for 30 minutes. Subsequently, a 0.2% (w/v) tripolyphosphate

(TPP) solution (cross-linking agent) was slowly added to the emulsion with gentle stirring at room temperature for 30 minutes. Finally, the nanoparticles were isolated by centrifugation at 14,000 rpm for 15 minutes at 18°C. Chitosan with a molecular weight of 810 kDa and a degree of deacetylation exceeding 75% was obtained from Sigma-Aldrich (Iran).

Physicochemical Characterization of Chitosan Nanoparticles

The particle size and zeta potential (ZP) of the synthesized nanoparticles were determined using Dynamic Light Scattering (DLS) (Malvern Nano-Series Zetasizer, UK). The morphology of the nanoparticles was examined using Scanning Electron Microscopy (SEM) (JSM-7610F).

In Vitro Antifungal Activity of Nanoencapsulated EOs and Free EOs

The antifungal activity of nanoencapsulated essential oils (NEOs) and free essential oils (EOs) was evaluated in vitro. To assess their effect on the radial growth of *M. phaseolina*, NEOs and EOs were incorporated into PDA medium at 125, 250, and 500 ppm concentrations. A fungal plug was placed at the center of each Petri dish, and the plates were incubated at 28°C in the dark for 3 days. The colony diameter was measured daily. Positive control: The fungicide chlorothalonil (CLT); Negative control: Distilled water. The percentage of mycelial growth inhibition was calculated using the formula:

$$I = (C - T) / C \times 100$$

where C is the mycelial growth in the control plate, T is the mycelial growth in the treated plate, and I is the percentage of mycelial growth inhibition.

Statistical Analysis

The experiment was conducted using a Factorial in Completely Randomized Design (FCRD). The first factor consisted of different plant species as sources of essential oils, while the second factor involved varying concentrations of these oils. Data were analyzed using factorial analysis of variance (ANOVA) in SAS statistical software (version 9.4). Significant differences among treatment means were determined using Tukey's Honestly Significant Difference (HSD) post-hoc test at a significance level of $P \leq 0.05$.

Results

Identification of Pathogenic Fungus

Morphologically, the isolated fungus produced white, fluffy mycelium that transitioned from brown to gray over time and eventually turned black. The fungus exhibited a high growth rate and formed a substantial number of microsclerotia in the culture medium. These characteristics were consistent with those reported for *M. phaseolina* in the literature (Dhingra & Sinclair, 1978; Beas-Fernandez et al., 2006). To confirm the morphological identification, specific primers (MpKF1 and MaKR1) were used for molecular analysis. These primers amplified a 350 bp fragment, confirming the isolate as *M. phaseolina*. The profile of the PCR products is shown in Figure 1. BLAST search results revealed a 99.81% identity with the ITS sequence (KF234552) of *M. phaseolina*.

Pathogenicity Test

The germination test indicated 100% seed germination under controlled conditions (Figure 2A). The virulence of the *M. phaseolina* isolate was evaluated on bean plants grown in pots. Four weeks post-inoculation, disease symptoms became evident. Initially, the plants appeared healthy with green foliage during the early growth stages. However, as the disease progressed, symptoms such as leaf yellowing, wilting, and blackening of the crown and stem were observed (Figure 2B and E). Approximately two weeks after the initial appearance of these symptoms, the infected plants ultimately withered. No such symptoms were observed in the control plants (Figure 2C and D).

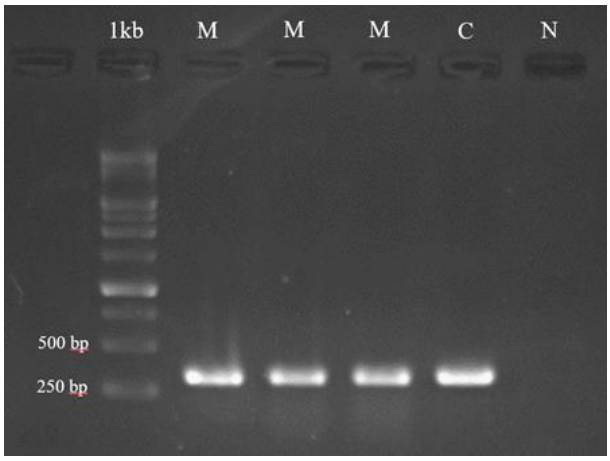


Figure 1. Agarose gel electrophoresis of PCR products. 1Kb: Ladder, M: *M. phaseolina*, C: positive control, and N: negative control.

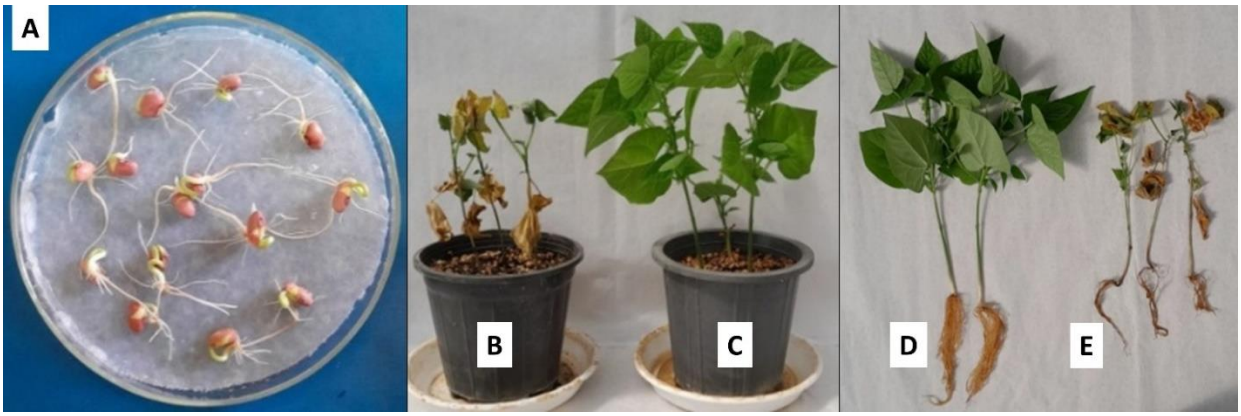


Figure 2. A: Seed germination test, C and D: Observer seedlings, B and E: Seedlings inoculated with *M. phaseolina*.

Chemical Composition of Essential Oils (EOs)

Gas chromatography-mass spectrometry (GC-MS) analysis of the essential oils from *C. cyminum* (CCEO), *C. carvi* (CEO), *M. piperita* (MPEO), *M. aquatica* (MEO), and *S. khuzistanica* (SEO) revealed distinct chemical profiles. In *S. khuzistanica*, carvacrol was the predominant compound (91.33%), followed by α-Pinene (2.67%). For *M. aquatica*, β-Pinene constituted 49.81%, with another component recorded at 2.4%. In *C. carvi*, benzaldehyde and γ-Terpinene were present at 20.58% and 21.86%, respectively. The essential oil of *M. piperita* was characterized by menthol (39.81%) and menthone (19.55%). Finally, *C. cyminum* showed significant levels of p-cymene (34.1%) and thymol (29.7%) (Table 1).

Characterization of Chitosan Nanoparticles Loaded with EOs

In this study, chitosan nanoparticles were synthesized to encapsulate the essential oils of five plant species. Scanning electron microscopy (SEM) images revealed that the nanoparticles were nearly spherical. Dynamic light scattering (DLS) analysis indicated particle sizes ranging from 45 to 232.6 nm. Specifically, the particle sizes for MPNEOs, MENEOs, SENEOs, CENEOs, and CCNEOs were recorded as 45 nm, 65.79 nm, 232.6 nm, 54.69 nm, and 150.3 nm, respectively (Table 2). All chitosan-coated nanoparticles exhibited a positive zeta potential, with values ranging from 2.69 mV to 39.6 mV.

Figure 3 presents the SEM image of CCNEOs, confirming their spherical

morphology and size range of 45–232.6 nm. The SEM images obtained in this study were consistent with those reported in previous studies.

Table 1. Chemical composition of essential oils (Eos). GC-MS analysis of essential oils from *C. cyminum* (CCEO), *C. carvi* (CEO), *M. piperita* (MPEO), *M. aquatica* (MEO), and *S. khuzistanica* (SEO) revealed distinct chemical profiles

plant species	Compound	(%)Percentage
	Menthol	39.81
	Mentone	19.55
	Neomenthol	8.83
	Menthyl acetate	8.64
	1,8-Cineole	5.81
M. piperita	Trance-beta-caryophllene	2.76
	Germacrene-d	2.73
	Viridiflorol	1.49
	Limonene	1.12
	Isomenthol	0.96
	Menthofuran	49.81
	Benzene, 1-methyl-2-(1- methylethyl)	0.51
	2-β-Pinene	2.4
	1,3,6-Octatriene,3,7-dimethyl	2
M. aquatic	Viridiflorol	1.92
	α-Pinene	1.70
	1-Octene-3 Ol	0.77
	Sabinene	0.68
	γ-Terpinene	0.65
	Carvacrol	91.33
	γ –Terpinene	1.7
	para-cymene	1.35
	alpha-terpineol	0.56
S. khuzistanica	α-Pinene	2.67
	α-Thujene	2.34
	β-Myrcene	2.44
	thymol acetate	1.82
	2-beta-pinene	1.81
	Beta- bisabolene	0.70
	Thymol	29.7
	Alpha-Pinene	0.51
	gamma-terpinene	18.7
C. copticum	p-Cymene	34.1
	Dillapiole	1.6
	2-cyclohexen-1-one	3.8
	β-Pinene	2.1
	5-hydroxyndole	1.9
	α.–Pinene	1.05
	γ-Terpinene	21.86
	Benzaldehyde, 4- (1- methyl ethyl) – (c(	20.58
	α-Thujene	11.66
	p-Cymene	7.11
C. carvi	1-limonene	3.53
	Hydrocinnamyl acetate	7.28
	β-Pinene	1.32
	Decane(1, 1-diethoxy)	0.86
	Sabinene	0.75

Table 2. The mean particle size and zeta potential (mV) values of chitosan nanoparticles (CNPs) and EOs-loaded chitosan nanoparticles (NEOs).

Type of particles	Z-average (nm)	Z-Potential (mv)
CNPs	101.1	21.2
MPNEOs	45	9.49
CCNEOs	150.3	2.69
SENEOs	232.6	10.4
MENEOs	65.79	17.6
CENEOs	54.69	39.6

In Vitro Antifungal Activity

The antifungal effects of essential oils (EOs) and chitosan nanoparticles loaded with EOs (NEOs) derived from five plant species were evaluated against *M. phaseolina* under in vitro conditions. Three concentrations (125, 250, and 500 ppm) were tested for each treatment, with results presented in Figure 4 and Table 3. Variance analysis revealed significant differences among treatments ($P \leq 0.01$) (Table 3).

The comparison of mean mycelial growth inhibition by EOs revealed that among the free essential oils (EOs), *C. copticum* at 250 ppm and 500 ppm exhibited inhibition rates of 96.06% and 94.94%, respectively. *S. khuzistanica* at both 500 ppm and 250 ppm showed an inhibition rate of 96.47%. Within its statistical group, *C. carvi* at 500 ppm demonstrated the highest inhibition rate of 87.66%. In contrast, the lowest inhibition rates were observed for *M. aquatica* at 125 ppm and 250 ppm, with inhibition rates of 0% and 11.38%, respectively. Additionally, *C. carvi* at 125 ppm showed a low inhibition rate of 11.7%, while *M. piperita* at 125 ppm and 250 ppm exhibited inhibition rates of 17.29% and 16.07%, respectively.

Regarding nanoencapsulated essential oils (NEOs), the highest antifungal activity was observed for *C. copticum* and *S. khuzistanica* at 500 ppm, both showing 100% inhibition. *C. carvi* at 500 ppm and 250 ppm exhibited inhibition rates of 100% and 95.29%, respectively, while *M. piperita* at 500 ppm displayed an inhibition rate of 95.29%. On the other hand, the lowest inhibition rates among NEOs were recorded for *M. aquatica* at 125 ppm and 250 ppm, with values of 0% and 16.08%, respectively, and for *C. copticum* at 125 ppm, which showed an inhibition rate of 15.87%.

Overall, nanoencapsulated essential oils (NEOs) demonstrated superior antifungal activity compared to their free EO counterparts, except for *C. copticum* and *S. khuzistanica* at lower concentrations (125 and 250 ppm). These findings suggest that nanoencapsulation significantly enhances the antifungal efficacy of essential oils.

Discussion

In the present study, essential oils (EOs) derived from *C. carvi, C. copticum, M. aquatica, M. piperita,* and *S. khuzistanica* demonstrated significant antifungal activity against *M. phaseolina*. Additionally, encapsulating these EOs in chitosan nanoparticles enhanced their stability, bioavailability and significantly improved their antifungal efficacy. This innovative approach highlights the potential for developing effective, natural antifungal treatments for agricultural applications.

M. piperita, commonly known as peppermint, has been extensively studied for its antifungal properties. The primary active compounds in *M. piperita* essential oil, such as menthol and menthone, exhibit potent antifungal activity against various fungal strains, including *Candida* species and *Aspergillus niger* van Tieghem, 1867 (Carretto et al., 2011; Ateş, 2024). These compounds disrupt fungal cell membranes, leading to increased permeability and eventual cell death (Tullio et al., 2019). Furthermore, studies have demonstrated that combining *M. piperita* essential oils (EOs) with other antifungal agents, such as silver ions, can generate synergistic effects, significantly enhancing their efficacy against resistant fungi like *Candida albicans* and *Aspergillus niger.* Notably, the antimicrobial activity of these combinations is influenced by storage conditions, with oils stored at -20°C exhibiting heightened inhibitory effects compared to those stored at +4°C. This synergy is likely attributed to the dominant bioactive constituents of *M. piperita* EO, menthol and menthone, which disrupt fungal cell membranes and enhance the penetration of co-applied antifungal agents (Yeşil et al., 2021).

Similarly, *C. carvi* essential oil has demonstrated notable antifungal activity in vitro, particularly against food spoilage molds and plant pathogens. In a disc diffusion assay conducted at 25°C for 48 hours on Sabouraud Dextrose Agar (SDA), concentrations of 0.5–10 µL per disc effectively inhibited growth of eight fungal species, including *Eurotium herbariorum* (F.H.Wigg.) Link, 1809 (complete inhibition at 10 µL) and *A. niger* (inhibition zone: 28 mm at 10 µL). The strongest antifungal effect was observed against *E. herbariorum*, while the weakest activity occurred against *A. niger*. These findings highlight the dose-dependent efficacy of caraway oil, with higher concentrations (≥5 µL) showing broader-spectrum activity compared to lower doses (0.5–1 µL). Notably, the oil's performance aligns with its traditional use in food preservation, though it exhibited lower potency than *Mentha piperita* oil in direct comparisons (Plavšić et al., 2017). Its mechanism of action involves disrupting fungal cell wall integrity and interfering with metabolic processes within fungal cells. When combined with other EOs, such as those from *M. piperita*, the antifungal efficacy of *C. carvi* is further amplified, providing a broader spectrum of activity against fungal pathogens (Plavšić et al., 2017).

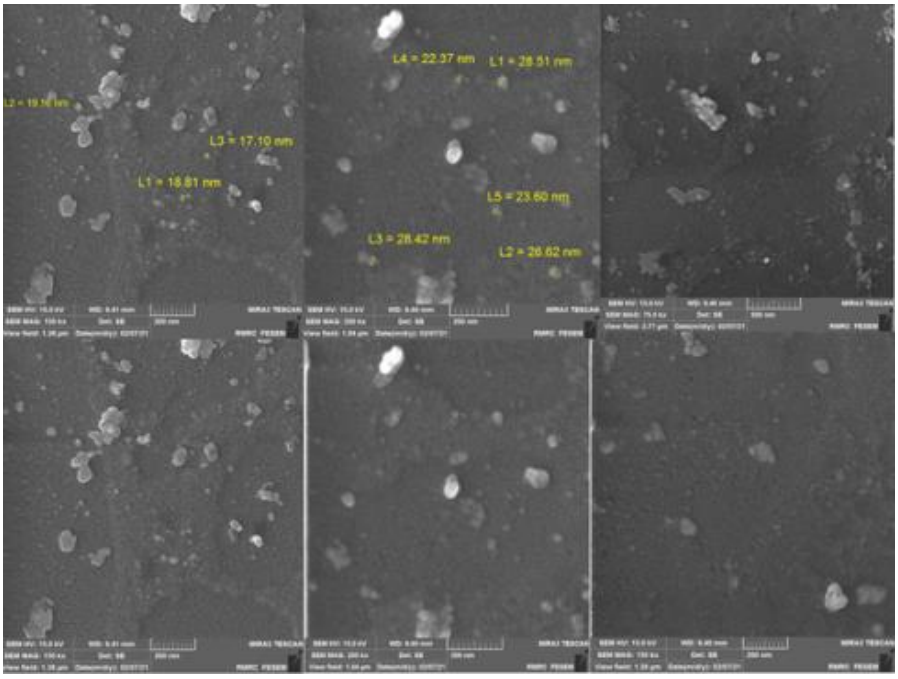


Figure 3. FE-SEM image of *C. cyminum* essential oil (CcEO)-loaded chitosan nanoparticles. The SEM image of CCNEOs confirms their spherical morphology and size range of 45–232.6 nm.

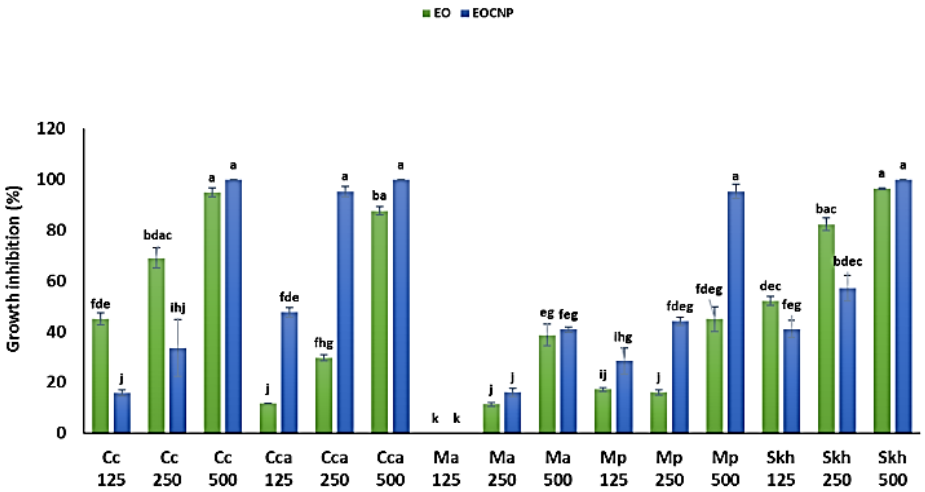


Figure 4. Mean (± SE) inhibition effect of five plant-derived essential oils (EO) and their nanoparticle formulations (NEOs) at three concentrations (125, 250, and 500 ppm) on the *in vitro* growth of *Macrophomina phaseolina*.

Table 3. Variance analysis of three concentrations of essential oils of five plant species encapsulated and not encapsulated with chitosan nanoparticles on the biological control of *M. phaseolina*.

Treatment	Degree of Freedom	F	Sig**
Type of particles (EOCNP and EO)	1	1851.50	**
Essential oil	4	10898.86	**
Concentration	2	29844.16	**
Essential oil × Type of particles	4	3815.26	**
Essential oil ×Concentration	8	395.46	**
Type of particles ×Concentration	2	443.99	**
Essential oil ×Type of particles × Concentration	8	781.41	**
Error	89	40.78	
CV	-	12.66	

**: Indicates significance at the 1% probability level.

The essential oils of *S. khuzistanica* and *C. copticum* have been less extensively studied compared to other aromatic oils but demonstrate promising antifungal activity, particularly against mycotoxigenic molds such as *E. herbariorum* and *A. niger* (Plavšić et al., 2017). In preliminary studies using disc diffusion assays, concentrations ranging from 0.5–10 µL per disc effectively inhibited fungal growth, with complete inhibition observed at 10 µL for *E. herbariorum*. Storage conditions, such as refrigeration at 4°C or freezing at −20°C, may further enhance their efficacy, as seen in related essential oils like *M. piperita*, where low-temperature storage preserved bioactive compounds like carvacrol and thymol (Yesil et al., 2021). These effects are likely mediated by membrane disruption and inhibition of spore germination, mechanisms observed in analogous essential oils. However, systematic research into optimal application conditions and synergistic interactions remains limited. Their essential oils contain active compounds such as carvacrol and thymol, which are known for their strong antifungal effects comparable to conventional antifungal agents (Achar et al., 2020). For instance, *C. copticum* has demonstrated effectiveness against a variety of fungal species, including those causing postharvest decay (Murtiastutik et al., 2022). Similarly, *M. aquatica* has been reported to possess significant antifungal properties, particularly against dermatophytes and other pathogenic fungi (Nikšić et al., 2012).

Studies have shown that EOs rich in monoterpenes, such as eugenol, thymol, and cinnamaldehyde, exhibit potent antifungal activity against various fungal pathogens, including those affecting plants (Youssef et al., 2016). These compounds disrupt fungal cell membranes, leading to increased permeability and eventual cell death (Fialho et al., 2017). Additionally, they induce oxidative stress in fungal cells by generating reactive oxygen species (ROS), which damage cellular components (Yu et al., 2019). The dual action of EOs—disrupting membrane integrity and inducing oxidative stress—not only enhances their antifungal efficacy but also reduces the likelihood of resistance development, a significant concern with conventional antifungal agents (Benzaid et al., 2019; Tančinová et al., 2021).

Encapsulation of EOs in chitosan nanoparticles offers several advantages. First, it protects volatile compounds from degradation, ensuring their stability during storage and application (Labib & Aldawsari, 2015). Second, it enables controlled release, prolonging the antifungal activity of the EOs (Mohammadi et al., 2015). For example, cinnamon essential oil (CEO) encapsulated in chitosan nanoparticles (CSNPs) demonstrated enhanced antifungal activity against *Botrytis cinerea* Pers., 1801 (gray mold) and *Penicillium expansum* Link, 1809 (blue mold), with minimum inhibitory concentrations (MICs) reduced by up to 50% compared to free CEO. The nanoparticles were prepared via ionic gelation using tripolyphosphate (TPP) as a crosslinker, yielding spherical particles of 125–175 nm diameter, as confirmed by

transmission electron microscopy (TEM). Encapsulation efficiency ranged from 45.24% to 3.26%, depending on the initial CEO-to-chitosan ratio (0.25–1 g/g). Antifungal assays, conducted at concentrations of 188–1500 ppm, revealed that CEO-loaded CSNPs (ZEO@CSNPs) completely inhibited mycelial growth of *B. cinerea* at 750 ppm, whereas free CEO required 1500 ppm for similar efficacy. This improved activity was attributed to sustained release of CEO from the chitosan matrix and synergistic membrane-disrupting effects of CSNPs, as observed in in vitro potato dextrose agar assays over 5–7 days of incubation at 25°C (Mohammadi et al., 2015).

Similarly, chitosan nanoparticles loaded with *M. piperita* essential oil (MEO) exhibited enhanced antifungal activity against *A. flavus* Link, 1809 and *E. herbariorum*, as demonstrated by disc diffusion assays at concentrations of 0.5–10 µL per disc, with complete inhibition observed at 10 µL (Beyki et al., 2014). The nanoparticles were synthesized via ionic gelation of chitosan with tripolyphosphate (TPP), yielding spherical particles of 125–175 nm diameter and encapsulation efficiencies ranging from 45.24% to 3.26%, depending on the initial MEO-to-chitosan ratio (0.25–1 g/g). Notably, storage at −20°C further enhanced antimicrobial potency, likely due to sustained release of bioactive compounds like menthol and menthone, which disrupt fungal cell membranes. This approach highlights the potential of nanoencapsulation for improving the stability and efficacy of essential oils in agricultural applications, particularly for controlling postharvest pathogens. (Beyki et al., 2014).

Zeta potential plays a crucial role in determining the stability of nanoparticles. Nanoparticles with a surface charge exceeding 31 mV are more stable, as this prevents particle aggregation and enhances stability (Yien et al., 2012). In a study by Izadi et al. (2021), chitosan nanoparticles encapsulating *C. copticum* essential oil and *Peganum harmala* L., (1753) extract demonstrated complete inhibition of *Alternaria alternata* (Fr.) Keissl., (1912) growth at a concentration of 200 ppm, surpassing the effects of the free essential oils and extracts at higher concentrations (Izadi et al., 2021; Kheiri et al., 2016).

The findings of the current study align with previous research demonstrating the enhanced antifungal activity of nanoencapsulated EOs. For instance, chitosan nanoparticles containing oregano essential oil exhibited uniform distribution and spherical morphology, ranging from 40 to 80 nm, as validated by scanning electron microscopy (SEM) and atomic force microscopy (AFM) (Hosseini et al., 2013). Similarly, encapsulation of *Phoenix dactylifera* plant extract in chitosan nanoparticles showed notable inhibitory effects against *Candida albicans* (Sahyon & Al-Harbi, 2020). These results underscore the potential of chitosan nanoparticles as carriers for enhancing the antifungal activity of EOs.

A study showed that chitosan/copper/thyme essential oil nano-complexes have strong antibacterial activity against *Pseudomonas syringae pv. syringae*, suggesting their potential as an eco-friendly option for controlling bacterial canker in fruit trees (Azarakhsh & Gerivani, 2025).

The use of nanoencapsulated EOs represents a sustainable and environmentally friendly approach to managing fungal pathogens in agriculture. By improving the stability, bioavailability, and controlled release of EOs, this method addresses key limitations associated with the use of free EOs, such as volatility and rapid degradation. Furthermore, the reduced likelihood of resistance development makes nanoencapsulated EOs a viable alternative to conventional chemical fungicides (Mane et al., 2022).

Conclusion

The findings of this study demonstrate the potential of chitosan nanoparticles to

enhance the antifungal properties of essential oils (EOs) against *M. phaseolina*, a major soil-borne pathogen responsible for charcoal rot disease. Nanoencapsulation of EOs derived from *S. khuzistanica, C. copticum*, and *C. carvi* resulted in stable formulations with particle sizes confirmed by dynamic light scattering (DLS) and field emission scanning electron microscopy (FE-SEM). Stability assessments under in vitro conditions revealed sustained antifungal activity, attributed to the controlled release of bioactive compounds like carvacrol and thymol at 500 ppm, these nanoencapsulated EOs achieved complete inhibition (100%) of fungal growth, surpassing the efficacy of their free (unencapsulated) forms. Notably, *S. khuzistanica* EO-loaded nanoparticles exhibited the strongest inhibitory effect, followed by *C. copticum* and *C. carvi*, while *M. piperita* and *M. aquatica* showed lower activity. The nanoformulations' enhanced bioavailability and prolonged stability highlight their promise as targeted biopesticides for sustainable agricultural practices, particularly for managing charcoal rot in bean crops.

Future research should focus on field trials to evaluate the performance of these nanoparticle-encapsulated EOs under real-world agricultural conditions. Such studies will provide critical insights into their practical applicability and effectiveness in diverse environments where *M. phaseolina* thrives. Additionally, exploring the synergistic effects of combining different EOs or nanoparticle formulations could further enhance antifungal activity and expand the range of effective treatments. Investigating the mechanisms by which chitosan nanoparticles improve the efficacy of EOs is also essential for optimizing biopesticide formulations. A deeper understanding of these interactions could pave the way for targeted strategies to manage soil-borne diseases more effectively.

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