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Assessment of physical properties and biological activity of chitosan beads with *Citrus hystrix* essential oil

Evaluación de las propiedades físicas y la actividad biológica de las perlas de quitosano con aceite esencial de *Citrus hystrix*

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ABSTRACT

The production and application of chitosan (CS) beads containing *Chúc* essential oil (CB-EO) is a new advanced technique to address the growing demand for natural and effective food preservation solutions, offering a sustainable alternative to synthetic preservatives. CB-EO was created at different *Chúc* essential oil (ChEO) concentrations of 0, 0.5, 1, and 2% (v/v). The CB-EO quality indicators were also evaluated in the experiments, such as yield and size, swelling ratio, antioxidant and antibacterial activity, Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), and scanning electron microscopy (SEM). Results showed that as ChEO concentration increased, antioxidant capacity rose from 33.5 to 40.8%, and the antibacterial inhibition zone diameter expanded from 3.67 to 8.33 mm. Bead collection efficiency changed insignificantly, from 31 to 34 beads per 2 mL of solution. Sphericity was not truly perfect, only reaching 59.60–66.60%. The swelling ratio did not depend on the presence of ChEO. The CB-EO was observed to be in the shape of a bean, with a smooth and wrinkled surface. FTIR analysis showed no new bonds or differences formed between the samples. Moreover, the crystallization ability of the beads showed a decrease in the diffraction intensity at the 28° position, which changed depending on the ChEO concentration. These findings demonstrate CB-EO's significant potential as a natural, highly effective agent for extending food shelf life and enhancing safety. Further research is needed to optimize encapsulation efficiency and evaluate practical application in various food matrices. Overall, the obtained results are promising and suggest good potential for future development and application.

Keywords:
Biopolymer beads
Food preservation
Free radical scavengin
Swelling ratio
SEM

RESUMEN

La producción y aplicación de perlas de quitosano (CS) que contienen aceite esencial de *Chúc* (CB-EO) es una nueva técnica avanzada e innovadora para satisfacer la creciente demanda de soluciones naturales y efectivas en la conservación de alimentos, ofreciendo una alternativa sostenible a los conservantes sintéticos. Para reemplazar gradualmente los conservantes sintéticos actuales, se creó CB-EO en diferentes concentraciones de aceite esencial de *Chúc* (ChEO) de 0, 0,5, 1 y 2% (v/v). En el experimento también se evaluaron los indicadores de calidad del CB-EO, como el rendimiento y el tamaño, la relación de hinchamiento, la actividad antioxidante y antibacteriana, la espectroscopia de infrarrojo por transformada de Fourier (FTIR), la difracción de rayos X (XRD) y la microscopía electrónica de barrido (SEM). Los resultados mostraron que, a medida que aumentaba la concentración de ChEO, la capacidad antioxidante se incrementó del 33,5 al 40,8%, y el diámetro del halo de inhibición antibacteriana se amplió de 3,67 a 8,33 mm. La eficiencia de recolección de perlas cambió de manera insignificante, de 31 a 34 perlas por 50 mL de solución. La esfericidad no fue realmente perfecta, alcanzando solo el 59,60–66,60%. La relación de hinchamiento no dependió de la presencia de ChEO. Se observó que el CB-EO tenía la forma de un frijol, con una superficie lisa y arrugada. El análisis FTIR no mostró nuevos enlaces ni diferencias formadas entre las muestras. Además, la capacidad de cristalización de las perlas mostró una disminución en la intensidad de difracción en la posición 28°, que cambió dependiendo de la concentración de ChEO. Estos hallazgos demuestran el gran potencial del CB-EO como agente natural y altamente efectivo para prolongar la vida útil de los alimentos y mejorar su seguridad. Se necesitan más investigaciones para optimizar la eficiencia de encapsulación y evaluar su aplicación práctica en diferentes matrices alimentarias.

Palabras clave:
Perlas de biopolímero
Conservación de alimentos
Eliminación de radicales libres
Índice de hinchamiento
SEM

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Food preservation has always been a top priority for researchers. Current preservation methods must not only ensure the safety and quality of food during storage but also be of natural origin, safe for humans, and environmentally friendly. Under these circumstances, the edible coating method is widely employed in fruit preservation (Pham et al. 2022; Pham et al. 2023b) and has also been applied to extend the shelf life of animal products such as eggs (Pham et al. 2023a). Among the materials used for edible coatings, chitosan is regarded as one of the most extensively studied biopolymers due to its broad-spectrum antibacterial, anti-inflammatory, and biofilm-forming properties (Mouhoub et al. 2023); however, its direct antibacterial efficacy remains limited. It is more effective when combined with other bioactive agents, and while not used to directly kill microbes, it can be combined with other agents to increase food preservation effectiveness (Mouhoub et al. 2025). In previous reports, Nguyen et al. (2023) used CS to preserve Hoa Loc mangoes, while Çoban (2021) combined CS and propolis extract to preserve shrimp. In addition, CS is also made into beads combined with lavender EO to help stabilize the color of tilapia fillets during storage (Junca et al. 2019).

Chúc EO (ChEO) is extracted from the waste of *Chúc* (peel), which is a valuable natural resource with high biological value, owing to its antibacterial, antioxidant, anti-cancer, anti-inflammatory, and anti-insect properties (Long and Quoc 2023). For example, Lertsatitthanakorn et al. (2006) reported the anti-inflammatory properties of ChEO, demonstrating its ability to inhibit Propionibacterium acnes and suppress 5-lipoxygenase activity. Additionally, Othman et al. (2023) found that the essential oil exhibited significant anti-proliferative activity against the HeLa cervical cancer cell line. Additionally, other reports have shown that ChEO at a concentration of 220–300 μg mL^{-1}, has potential anti-melanoma effects on three human cell lines, WM793, A375, and HTB140, by inhibiting hyaluronidase and tyrosinase (Kulig et al. 2022). Furthermore, many studies have also indicated that ChEO compounds, such as citronellol, limonene, pinene, sabinene, and terpinene-4-ol, act against bacteria and fungi, including *Saccharomyces cerevisiae*, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. These compounds also have a positive impact, like mosquito repellents and fly repellents. ChEO has also been shown to have stronger antibacterial activity than EOs from other citrus species (Long and Quoc 2023). Therefore, ChEO is widely used, especially in food preservation. However, EOs are not usually used directly but can be combined with other ingredients, such as CS, starch, etc. The most common application is to create a protective coating. For example, ChEO can be combined with cassava starch to form an edible coating that prolongs the shelf life of fresh beef (Utami et al. 2017). Additionally, ChEO can be combined with fish skin gelatin to enhance its antioxidant capacity (Tongnuanchan et al. 2012). However, creating particles containing EOs is less common but is suitable for products that are not affected by the taste and smell of the EOs (Long and Quoc 2023).

The current food preservation methods often rely on synthetic chemicals, raising concerns about safety and sustainability. There's a growing need for natural, eco-friendly alternatives. While chitosan (CS) and *Chúc* (*Citrus hystrix*) essential oil (ChEO) have been individually studied for their antimicrobial and antioxidant properties, their combination in the form of encapsulated beads has not yet been explored. Most previous studies have focused on film or coating applications, but the development of CS beads loaded with ChEO represents a novel strategy that allows better control of essential oil release, minimizes sensory impact, and enhances preservation efficiency.

Therefore, this study introduces a new approach by formulating chitosan beads incorporated with *Chúc* (*Citrus hystrix*) essential oil and evaluating their physical and biological properties. This formulation not only leverages the synergistic effects of two natural agents but also offers a promising and sustainable alternative to synthetic preservatives in food storage applications. The improved chitosan bead system has the potential to be adapted to a variety of foods, promoting safer and longer-term preservation. Future research may focus on optimizing scalability and exploring controlled release mechanisms to maximize efficacy and consumer acceptance.

MATERIALS AND METHODS

Materials

The chitosan (CS) sample, provided by Nha Trang University, is white, scale-shaped, and has a degree of deacetylation of 90%. The raw material for essential oil (EO) extraction was *Chúc* (*Citrus hystrix*) peel,

collected in Tinh Bien District, An Giang Province, Vietnam (coordinates: 10°37'55.6"N, 104°59'34.8"E), and processed via steam distillation. Before extraction, the fruit was washed, air-dried at room temperature, peeled, and chopped into small pieces.

As detailed in the previous work (Long et al. 2023), ChEO was extracted via a 3 h steam distillation process at 100 °C. The characteristics of this ChEO were determined to be an approximate yield of 2.8% per batch (from 50–60 kg peel), with acid, ester, and saponification values of 0.561±0, 10.66±1.405, and 11.22±1.405 mg KOH g^{-1} EO, respectively. Its refractive index was 1.4699±0.0002. The primary chemical components of this ChEO as analyzed by gas chromatography-mass spectrometry (GC-MS). The gas chromatography-mass spectrometry analysis method (GC-MS) was used to determine the EO's chemical makeup. First, 1 mL of methanol was created by dissolving 20 µL of the sample in methanol. Next, at an injection temperature of roughly 220 °C, 1 mL of EO was added to an Agilent DB-5MS gas chromatograph equipped with a capillary column (30 m×0.25 mm, 0.25 µm). After 1 minute, the temperature was raised to 70 °C, then by 12 °C each minute to 280 °C and finally maintained for 10 minutes. The range of mass was 29–650 atomic mass units (amu). The carrier gas, helium, had a steady flow rate of 1.2 mL min^{-1}. The relative abundance of each compound, expressed as percentage area, was determined and identified as: β-pinene (30.19%), D-limonene (22.15%), sabinene (21.37%), citronellal (6.17%), terpinen-4-ol (4.88%), and α-pinene (3.14%) (Long et al. 2023). The ChEO was then preserved in a dark glass bottle at room temperature.

A total of four bacterial strains were used in this study: two Gram-positive strains, *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778), and two Gram-negative strains, *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 13311). All strains were provided by the Institute of Biotechnology and Food Technology at the Industrial University of Ho Chi Minh City. The chemicals used included lactic acid and Tween 20 (Xilong, China), as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma, USA). The culture media consisted of Nutrient Broth and Mueller–Hinton Agar (MHA) (HiMedia, Thane, India), along with other analytical-grade reagents.

Preparation of chitosan beads with *Chúc* peel essential oil

For the production of CB-EO, the procedure of Sangsuwan et al. (2016) was followed with some appropriate modifications. A total of 0.75 g of CS was dissolved in 50 mL of 2% lactic acid solution at 55 °C and stirred for 30 min. The mixture was then mixed with ChEO in the following ratios: 0, 0.5, 1, and 2% of the solution volume (v/v) and Tween 20 (0.2% v/v). The 0% ChEO sample was considered the control sample. Stirring continued for 15 min. Then the solution was dripped through a syringe into 50 mL of 2 M NaOH. The beads were washed with distilled water five times and dried in a drying oven at 45 °C for 5 h. For monitoring and research, the CB-EO samples collected were stored in closed plastic boxes at room temperature and labeled according to the ChEO ratios added: control, CB-EO (0.5), CB-EO (1), and CB-EO (2).

Determination of bead collection efficiency

This process was performed by counting the number of beads comprising a unit volume of the resulting solution.

Determination of bead size after drying

Length (L), width (W), and thickness (T) were determined with a specialized stainless hardened digital caliper (Mitutoyo, model 500-182-30, USA). To determine the equivalent diameter (ED) (Equation 1) and sphericity (S) (Equation 2), the following Equations were used (Thakur and Nanda 2018):

$$ED = (L \times W \times T)^{\frac{1}{3}} \ (mm) \qquad (1)$$

$$S = \frac{ED}{L} \times 100 \ (\%) \qquad (2)$$

Where L, W, and T are length (mm), width (mm), and thickness (mm), respectively.

Determination of the swelling ratio of CB-EO

The swelling ratio was determined by the weight measurement method of Anchisi et al. (2006), which has been adjusted to suit the experimental conditions. Dry bead samples weighing 1 g were soaked in physiological water and stirred at 50 rpm at different time intervals (30, 60, 90, and 120 min). Then, the beads were drained and weighed.

The swelling ratio of the bead samples was calculated using Equation (3):

$$Swelling\ ratio = \frac{W_t}{W_0} \quad (3)$$

Where W_0 is the dry bead weight (g) and W_t is the wet bead weight (g).

Determination of the antioxidant activity of CB-EO

Antioxidant activity was measured via DPPH free radical scavenging capacity ($DPPH_{RSC}$) (Equation 4). This study evaluated the antioxidant activity according to the research procedure of Su et al. (2020), which has been adjusted to suit the experimental conditions. One gram of CB-EO was continuously stirred in 15 mL of alcohol for 2 h. Then 1 mL of this solution was added to 2 mL of the 0.1 mM DPPH reagent to create a reaction for 30 min; the samples were measured at a wavelength of 517 nm. The results were calculated by the percentage (%) inhibition of DPPH.

$$DPPH_{RSC}\ (\%) = \frac{A_0 - A_i}{A_0} \times 100 \quad (4)$$

Where A_0 represents the absorbance of the blank solution (DPPH solution) and Ai indicates the absorbance of the sample solution (DPPH solution and extract).

Determination of antibacterial activity of CB-EO

The antibacterial activity of CB-EO was determined using the disc diffusion method according to the study of Junca et al. (2019), which has been adjusted to match the experiment. About 0.1 mL of the bacterial solution (concentration of 1.5×10^8 CFU mL^{-1}, equivalent to 0.5 McFarland) was spread on the surface of MHA in a petri dish. CB-EO was also placed in petri dishes containing a Gentamicin (10 µg per plate) positive control and a dimethyl sulfoxide (DMSO 5%) negative control. The petri dishes were incubated for 24 h at 37 °C, and the resulting diameter of the inhibition zones of CB-EO were read in millimeters (mm).

Fourier transform infrared analysis

The Fourier transform infrared (FTIR) analysis aimed to identify the functional groups present in the chitosan beads and detect possible interactions between chitosan and essential oil components. FTIR analysis was performed on a Bruker Tensor 27 FTIR spectrometer (Bruker Optik GmbH, Germany). Spectra were recorded in the range of 4,000–400 cm^{-1} with a resolution of 1 cm^{-1} transmittance mode using a KBr beam splitter. The measurement was conducted in transmittance mode using the KBr pellet method, where approximately 2 mg of sample was finely ground and mixed with 200 mg of dry KBr powder, then pressed into a pellet.

X-ray diffraction analysis

X-ray diffraction analysis (XRD) was performed using a Bruker AXS D8 Advance ECO Xray diffractometer (Karlsruhe, Germany). During the analysis, the voltage and current were set to 40 kV and 25 mA, respectively. The analysis involved varying the angle within the 2θ range, and the scanning speed was set to 0.2 rad s^{-1}.

Scanning electronic microscopy of CB-EO

Scanning electron microscopy (SEM) analysis was conducted using a JSM-IT200 scanning electron microscope (JEOL, Japan). This versatile instrument allowed for magnifying samples at a range of ×40 to ×10,000, utilizing an accelerating voltage of 5 kV.

Before analysis, the samples underwent natural drying to remove any surface moisture. To enhance their conductivity and enable high-resolution imaging, a thin layer of platinum was applied using an auto-fine coater (JEC-3000FC, JEOL, Japan). This technique ensures clear and detailed images of the sample's surface morphology.

Data analysis

The experiment involved three independent measurements, and the results are reported as the mean ± standard deviation. Statistical analysis was carried out using Statgraphics Centurion XV software. A one-way analysis of variance (ANOVA) was used to assess overall differences among the groups, followed by Fisher's least significant difference (LSD) test to identify statistically significant pairwise differences. A significance level of $P<0.05$ was applied.

RESULTS AND DISCUSSION

Size and yield of CB-EO

Based on the results in Table 1, the yield in the process of creating beads in the same unit volume among the EO

Table 1. Size and yield of the beads.

Sample	Control	CB-EO (0.5)	CB-EO (1)	CB-EO (2)	Level of significance
Bead number/2 mL solution	34±1.15^{a}	33±0.58^{ab}	32±2^{b}	31±0.58^{b}	*
Length (mm)	4.37±0.33^{a}	4.24±0.29^{a}	4.45±0.35^{a}	3.77±0.30^{b}	*
Width (mm)	2.67±0.22^{a}	2.58±0.26^{a}	2.51±0.18^{a}	2.49±0.16^{a}	*
Thickness (mm)	1.64±0.03^{a}	1.65±0.02^{a}	1.66±0.02^{ab}	1.68±0.02^{b}	*
Equivalent diameter (mm)	2.67±0.12^{a}	2.62±0.12^{a}	2.64±0.12^{a}	2.50±0.10^{b}	*
Sphericity (%)	61.23±2.56^{a}	61.96±3.53^{a}	59.60±2.79^{a}	66.60±3.76^{b}	*

* Significant at $P<0.05$. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively. Significant differences ($P<0.05$) between samples are indicated by different letters within a row (a–b).

samples has a negligible deviation; the bead count only fluctuates from 31 to 34 per 2 mL.

Bead size also shows negligible fluctuations. However, the length of the beads tends to decrease at higher EO concentrations (4.37 mm for the control and 3.77 mm for CB-EO (2)), while width (2.49–2.67 mm) and thickness (1.64–1.68 mm) remain relatively constant.

The observed sphericity of the beads, ranging from 59.60 to 66.60%, suggests a non-spherical, bean-shaped morphology for the CB-EO beads. The sphericity in this study is significantly lower than that of other reported materials, such as bee pollen beads (76.55%) (Quoc 2021) or green bean beads (84.1–84.18%) (Thong et al. 2010).

Furthermore, the equivalent diameter of the beads is relatively low, ranging only from 2.50 to 2.67 mm. These results tend to be lower than those observed in other CB-EO studies. For instance, Lich et al. (2018) reported bead diameters ranging from 1.96 to 3.10 mm for CS combined with SiO_2. Determining the size and production yield of CB-EO can make their packaging in food products more reasonable. The variation in size is likely attributable to the material used, the initial degree of deacetylation in the CS, and the ability of CS to interact with other materials. Notably, prior research has yet to evaluate the sphericity of CB-EO beads.

Swelling ratio of CB-EO

Analyzing swelling ratio data for chitosan (CS) beads coated with *Chúc* essential oil (ChEO) reveals their water absorption properties (Table 2). Every type of bead, including the control and ChEO-containing ones, showed a notable ability to absorb water, swelling by 1.3 to 1.6 times their initial weight. This unequivocally demonstrates that the main factor influencing the beads' ability to absorb water and create a hydrogel is chitosan's intrinsic hydrophilic character, which is ascribed to its abundance of hydroxyl and amine groups.

The impact of integrating ChEO leads to a more sophisticated view. At most time intervals, there was a minor tendency of slightly decreased swelling ratios in the ChEO-loaded beads when compared to the control (ChEO 0%), even if the overall swelling capacity stayed high. This implies that even though the essential oil is hydrophobic, its presence may have a subtle effect on the structural dynamics of the hydrogel. One possible mechanism is the pore-filling effect, in which ChEO molecules occupy part of the empty spaces within the chitosan polymer network, thereby reducing the volume available for water molecules to penetrate and bind. This is consistent with previous findings, such as those of Anchisi et al. (2006), who also reported variations in the swelling behavior of chitosan when combined with different essential oils.

These CB-EO particles demonstrate strong structural integrity and stability in aqueous environments, as indicated by the relatively constant swelling ratio throughout the immersion period and the minimal impact of ChEO. This property ensures the effective retention of encapsulated active compounds over time without rapid degradation of the carrier, making them well-suited for applications in controlled release systems or food preservation.

Table 2. Swelling ratio of beads versus time.

Time (min)	30	60	90	120	Level of significance
Control	1.52±0.03	1.55±0.06	1.57±0.07	1.57±0.08	NS
CB-EO (0.5)	1.49±0.15	1.53±0.15	1.58±0.09	1.54±0.1	NS
CB-EO (1)	1.34±0.07	1.41±0.07	1.45±0.05	1.43±0.06	NS
CB-EO (2)	1.39±0.11	1.48±0.14	1.53±0.12	1.43±0.13	NS

NS Not significant at $P<0.05$. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively.

Antioxidant activity of CB-EO

The antioxidant activity of CB-EO is presented in Table 3. Results show a clear trend: higher EO content corresponds to increased antioxidant activity, ranging from 33.5% in the control to 40.8% in CB-EO (2). Compared to the control, the antioxidant activity of EO-containing samples was 14–21% higher. These findings indicate that chitosan (CS) possesses inherent antioxidant capacity, which is further enhanced by the presence of EO. This can be illustrated in the study of Su et al. (2020), on CS combined with cinnamon EO, where the antioxidant activity increased from 20.6 to 56.9% when the EO content increased. CB-EO shows strong antioxidant properties, making it a potential candidate for food preservation.

In comparison, sodium alginate gel beads encapsulating curcumin/gum Arabic/gelatin microcapsules exhibited significantly higher antioxidant activity, with DPPH and ABTS radical scavenging capacities of 95.59 and 87.65%, respectively (Lin et al. 2024). While CB-EO's antioxidant activity is lower, the encapsulation of ChEO in chitosan beads enhances stability and controlled release, similar to the hydrogel beads' role in protecting curcumin, thereby minimizing undesirable flavor impacts in food applications (Lin et al. 2024). However, while increasing the concentration of EO enhances its antioxidant effect, it can also introduce undesirable flavors to the food. Therefore, depending on the type of food product, users can adjust the appropriate essential oil concentration to exploit CB-EO properties optimally.

Table 3. Antioxidant activity of chitosan beads.

Antioxidant activity	Control	CB-EO (0.5)	CB-EO (1)	CB-EO (2)	Level of significance
$DPPH_{RSC}$ (%)	$33.54±0.73^a$	$38.34±0.07^b$	$39.43±0.08^c$	$40.86±0.07^d$	*

*Significant at $P<0.05$. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively. Significant differences ($P<0.05$) between samples are indicated by different letters within a row (a–d).

Antibacterial activity of CB-EO

The antibacterial activity of CB-EO was determined based on the ability to inhibit the growth of bacteria, through the diameter of the bacterial inhibition zone, as shown in Table 4 and Figure 1. The negative control, DMSO 5% did not exhibit a bacterial inhibition zone, while the positive control, Gentamicin, had a wide bacterial inhibition zone with all four test strains. Antibacterial activity ranked in the following order: *B. cereus* > *S. typhimurium*> *P. aeruginosa* > *S. aureus*.

For CB-EO, the control sample showed low antibacterial activity from 3.67 to 4.33 mm, but it also showed that CS has antibacterial activity. When combined with ChEO, the antibacterial activity increased significantly from 5.3 to 8.33 mm. The highest antibacterial effect was shown in the CB-EO (2) sample for the *S. aureus* and *B. cereus* strains, with a diameter of 8.33 mm. All of these results are similar to the results of the study by Junca et al. (2019). The EO kept inside CS beads can inhibit EO's evaporation and improve food storage time. Besides, CB-EO also has less influence on the product's taste than pure EOs.

Similar to the findings with chitosan beads incorporated with *Thymus capitatus* essential oil (CB-TCEO),

Table 4. Antibacterial activity of chitosan beads.

Bacteria	Bacterial inhibition zone (mm)					
	Control	CB-EO (0.5)	CB-EO (1)	CB-EO (2)	Gentamicin	Level of significance
P. aeruginosa	3.67 ± 0.58^{aA}	5.67 ± 0.58^{bA}	6.33 ± 0.58^{bA}	6.67 ± 0.58^{bA}	17.00 ± 1.00^{cA}	*
S. aureus	3.67 ± 0.58^{aA}	5.33 ± 0.58^{bA}	6.67 ± 0.58^{cA}	8.33 ± 0.58^{dB}	16.33 ± 0.58^{eA}	*
S. typhimurium	3.67 ± 0.58^{aA}	5.33 ± 0.58^{bA}	6.67 ± 0.58^{cA}	7.67 ± 0.58^{cAB}	17.33 ± 0.58^{dA}	*
B. cereus	4.33 ± 0.58^{aA}	5.33 ± 0.58^{aA}	6.67 ± 0.5^{bA}	8.33 ± 0.58^{cB}	20.67 ± 1.15^{dB}	*

* Significant at $P<0.05$. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively. Significant differences ($P<0.05$) between samples are indicated by different letters within a row (a–e) or a column (A–B).

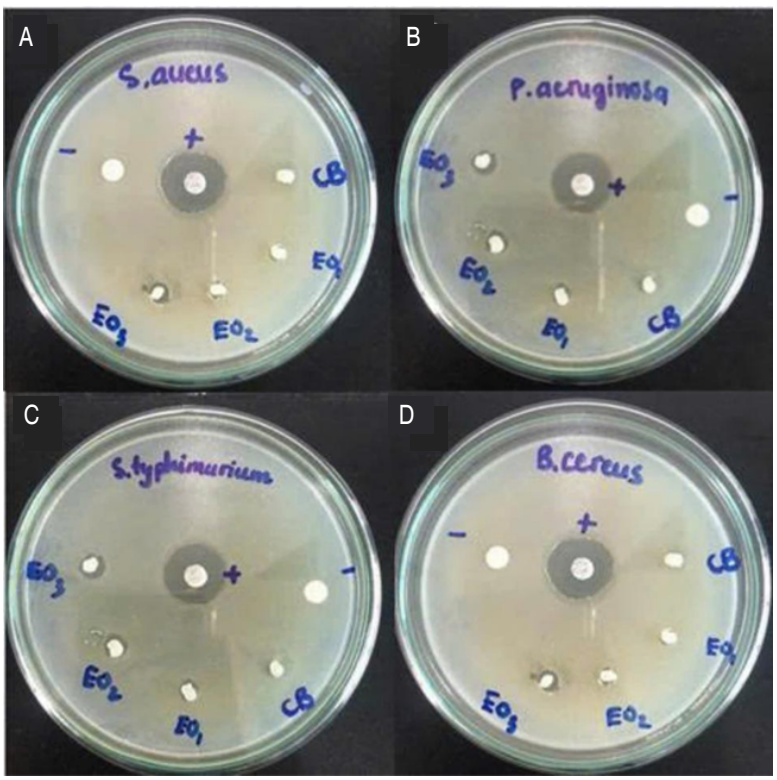

Figure 1. Antibacterial activity of CB-EO beads against **A**. *S. aureus*, **B**. *P. aeruginosa*, **C**. *S. typhimurium*, and **D**. *B. cereus*. -, +, CB, EO1, EO2, and EO3 correspond to negative control, positive control, control sample, CB-EO (0.5), CB-EO (1), and CB-EO (2), respectively.

CB-EO exhibited stronger inhibition against Gram-positive bacteria (*B. cereus*) compared to Gram-negative bacteria (*S. typhimurium* and *P. aeruginosa*), likely due to the thinner cell wall of Gram-positive bacteria allowing easier penetration of active compounds, with limonene, one of the key antibacterial components in ChEO, acting as an alkylating agent, binding to nucleophilic groups and causing protein denaturation and cell lysis (Millezi et al. 2014). Chitosan interacts electrostatically with bacterial cell membranes, disrupting their structure and facilitating the penetration of key components of the essential oil. Chitosan beads combined with Citrus hystrix essential oil exhibit antibacterial activity through a synergistic effect between chitosan and the major constituents of the essential oil.

Based on the experimental results, the synergistic combination of CS and ChEO in antibacterial properties will be an alternative method to synthetic antibacterial additives in food preservation as well as other applications in practice.

SEM analysis of CB-EO

SEM images at ×40 magnification show that CB-EO is bean-shaped, with a sphericity of 59.6 to 66.6%. As shown above, the beads have a smooth surface (Figure 2). The small wrinkles on the outer surface may be due to shrinkage during the drying process. This shape is completely similar to that observed in the study of CS combined with sodium tripolyphosphate by Yousefi et al. (2019). When CS is combined with different materials, it may create different shapes.

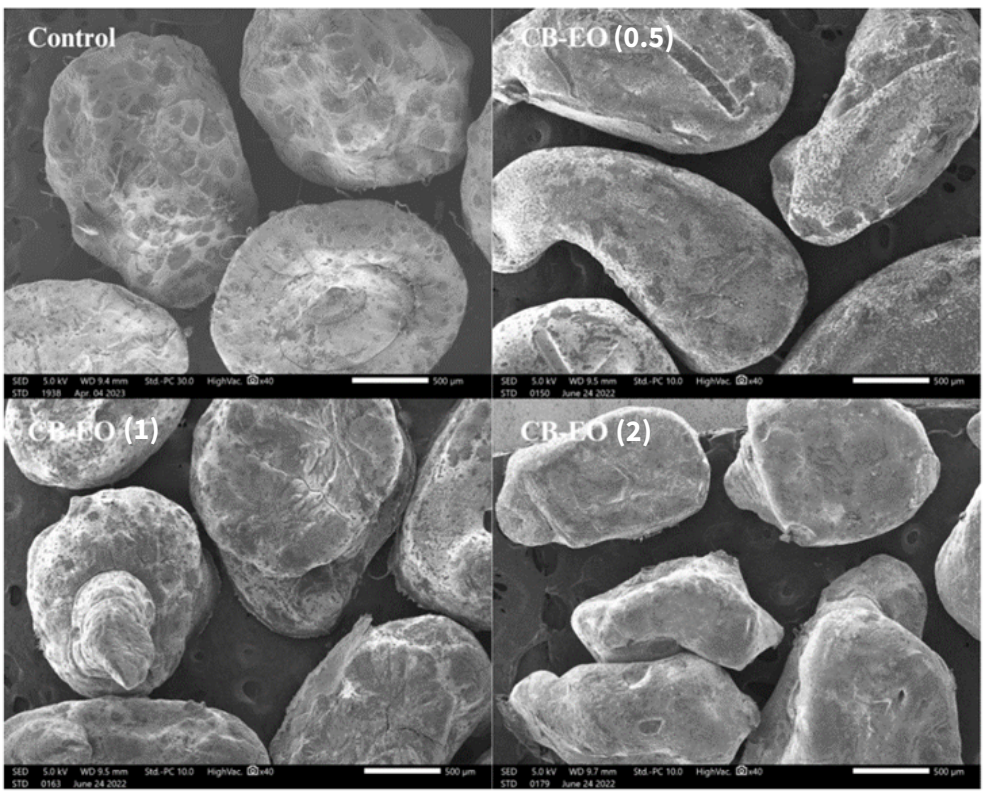

Figure 2. Morphological structure analysis of the beads at ×40 magnification. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively.

At high magnification, the microstructure of the samples and its changes with different essential oil concentrations can be observed. At ×10,000 magnification, the surface of the control sample appeared rough and had fewer wrinkles compared to the EO-containing samples (Figure 3). This may be due to the tendency of ChEO to aggregate during the drying process. This study is also similar to that by Lich et al. (2018), where CS was combined with SiO_2 to create a smooth surface with numerous pores. Compared with the sodium caseinate-sorbitol biofilm containing citral microparticles, CB-EO did not show obvious micropores, possibly due to the lower efficiency of air bubble removal by sonicating probe than by ultrasonic bath (Yoplac et al. 2022). The results show that differences in shape, size, and surface state can affect the release of EOs during processing and preservation.

However, beyond structural description, it is important to emphasize why morphology matters: in food preservation applications, the surface characteristics of the

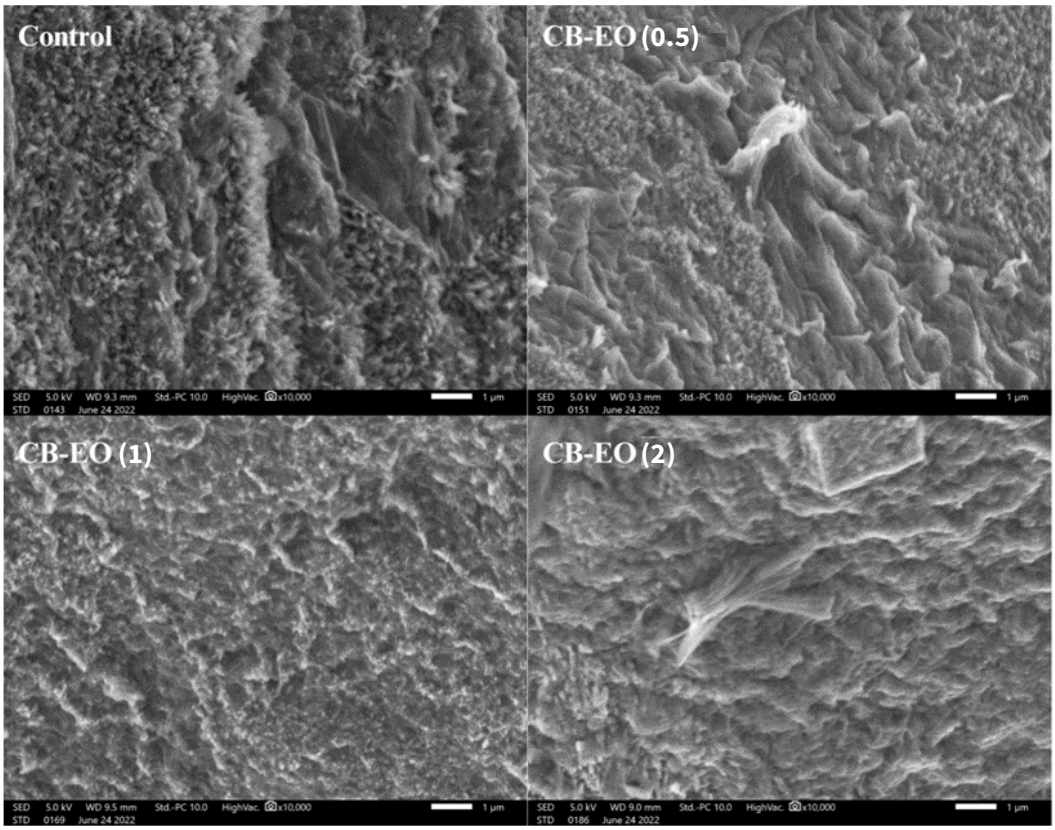

Figure 3. Morphological structure analysis of the beads at ×10,000 magnification. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively.

beads—such as smoothness, presence of pores, or wrinkles—play a crucial role in the release kinetics of active compounds. A smoother surface may reduce premature diffusion of essential oils, thereby enabling a more controlled and prolonged release, which is beneficial for extending the shelf-life of food products. On the other hand, excessive wrinkles or porosity could lead to rapid initial release, reducing long-term effectiveness. Therefore, analyzing SEM images is not merely for morphological documentation but to help optimize bead structures for practical use in food packaging or coating systems (Yoplac et al. 2021).

FTIR analysis of CB-EO

FTIR analysis is one of the preliminary evaluation methods for the presence of compounds and functional groups in the sample, and this evaluation has been carried out in many previous studies on EOs combined with other types of coatings, such as CS combined with *Thymus capitutus* EO (Junca et al. 2019) and CS combined with *Cinnamomum* EO (Su et al. 2020). As shown in Figure 4, all four samples exhibit the presence of several similar functional groups. Specifically, absorption bands were observed at 3,450; 1,640; 1,380; and 1,076 cm^{-1}, corresponding to OH- groups, C=O groups, S=O groups, and pyranose C–O–C groups, respectively. In addition, at the wavelength of 2,928 cm^{-1}, it shows the presence of the C-H group, which is similar to the results of Su et al. (2020), who previously researched *Cinnamomum* EO encapsulated in CS nanoparticles. At the wavelength of 1,586 cm^{-1}, it proves the presence of the N-H group, similarly to the study of Hosseini et al. (2013), who studied oregano EO in CS nanoparticles.

Following the results, there is an interaction of some compounds in ChEO with CS. Specifically, compared to the control sample, the sample containing ChEO can interact with CS to reduce the intensity of wavelengths such as 3,450; 1,640; and 1,380 cm^{-1}. Some other wavelengths do not change significantly, such as 1,586;

2,928; and 1,076 cm^{-1}. The presence of biologically active compounds with different EO content can interact with CS to create different properties of CB-EO product.

These interactions are vital for the stability and integrity of the encapsulated essential oil within the chitosan matrix, directly influencing its release profile and ultimately its effectiveness as an antibacterial and antioxidant agent in food. Stronger interactions suggest better retention of the active compounds, leading to prolonged antimicrobial and antioxidant activity and thus extended shelf life of the food product (Yoplac et al. 2022).

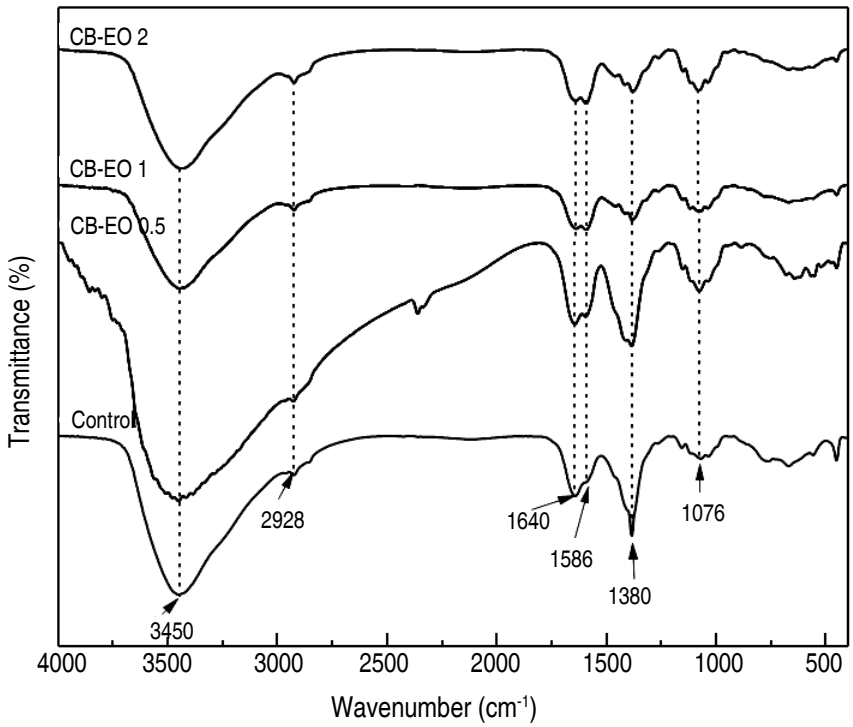

Figure 4. Fourier transform infrared (FTIR) spectra of the beads. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively.

X-ray diffraction analysis of CB-EO

CS particles combined with EO can change crystallinity, as shown in the study of Yoncheva et al. (2021), in which there were two strong diffraction peaks at 20 and 42° when CS particles were combined with oregano EO. This contrasts with the results of this study, in which the control, CB-EO (0.5), CB-EO (1), and CB-EO (2) samples have the appearance of forming peaks mainly at two positions of 20 and 29° (Figure 5). The diffraction peaks of the samples with ChEO are lower than those of the control, typically at an angle of 29°. The addition of EO may change the interaction between CS and ChEO, leading to a decrease in the crystallinity of the samples. This also shows that the change in crystallinity is related to the nature of CS, such as origin, deacetylation level, and the compounds present in different EOs. At the same time, the interaction between CS and different types of EO contributes to influencing XRD diffraction peaks.

These results suggest that the developed CB-EO system holds strong potential for real food preservation applications. Similar approaches have been successfully employed by Junca et al. (2019), who demonstrated extended shelf life of red tilapia fillets using chitosan beads loaded with *Thymus capitatus* essential oil, and by Sangsuwan et al. (2016), who effectively inhibited *Botrytis cinerea* and reduced fruit decay in strawberries

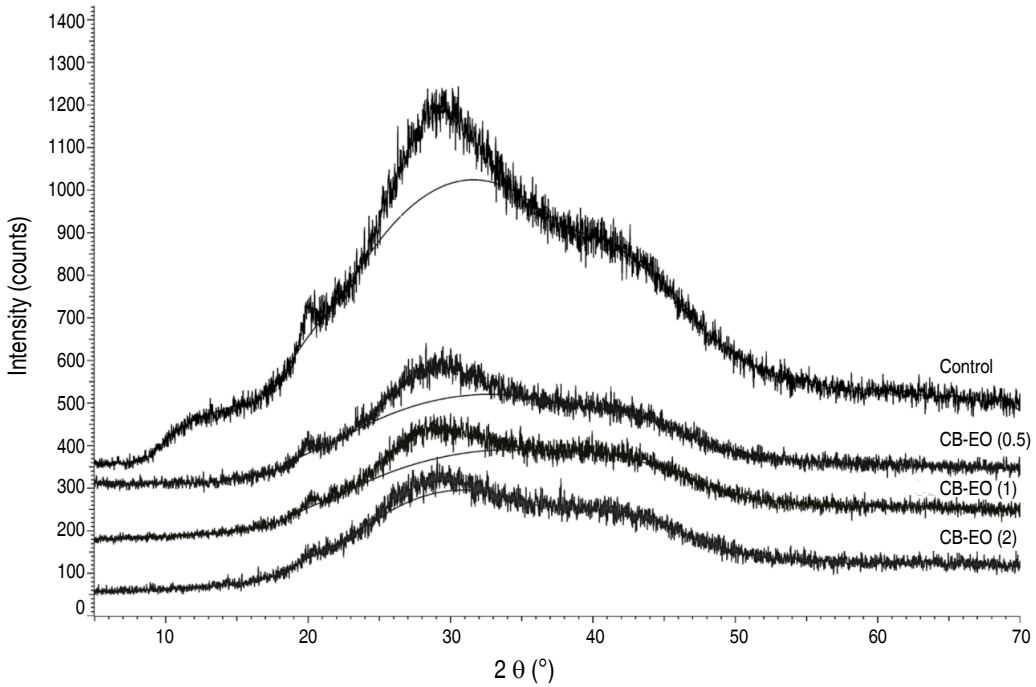

Figure 5. X-ray diffraction (XRD) patterns of the beads. Control, CB-EO (0.5), (1), and (2) are the chitosan beads containing *Chúc* essential oil at concentrations of 0, 0.5, 1, and 2% (v/v), respectively.

using chitosan beads combined with lavender or red thyme oils. In this context, the present study reinforces the practical feasibility of using CB-EO as a safe, natural, and biodegradable alternative to synthetic preservatives—particularly in perishable food systems—aligning with current demands in the food industry for green, sustainable technologies. These findings are further supported by the work of Mouhoub et al. (2024), in which chitosan coatings enriched with essential oils significantly extended the postharvest life of strawberries while maintaining sensory and physicochemical quality.

CONCLUSION

This study successfully developed chitosan beads (CB-EO) by combining chitosan (CS) and *Citrus hystrix* essential oil (ChEO), both sourced from manufacturing waste. The formulation enhanced antioxidant activity by 14–21% and significantly improved antimicrobial properties compared to the control, demonstrating strong potential for food preservation. Its natural origin ensures safety for human health and environmental compatibility, making it a promising alternative to synthetic preservatives. Despite these promising results, the study was primarily limited to in vitro physicochemical characterization, without validation in real food systems. Therefore, CB-EO may be most suitable for products with an outer layer that is typically removed before consumption, such as many fruits, or where the essential oil's aroma is acceptable. Future studies should evaluate its performance in real food products, conduct sensory assessments, and explore scalability and economic feasibility to support industrial application.

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CONFLICT OF INTERESTS

The authors declare no potential conflicts of interest.

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