RESEARCH PAPER

Preparation, Characterization, and Antibacterial Effects of *Ferula Gummosa* Essential Oil–Chitosan (CS-FEO) Nanocomposite

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ABSTRACT

Nowadays, the antimicrobial properties of herbal essential oils (EO) have received extensive attention due to their considerable antimicrobial potential against multidrug-resistant pathogens. However, the volatility and sensitivity of OEs limit their traditional use in aquatic environments. Specialized nanocarriers as additives can improve their chemical stability and solubility, delay degradation and curb rapid evaporation. With this aim, chitosan *Ferula gummosa* EO-nanocomposite (CS-FEO) was prepared through a facile method. In addition, the antibacterial properties were studied by observing the inhibition zone against *Escherichia coli, Staphylococcus aureus,* and *Bacillus cereus* using the agar well diffusion assay method. Further, a transparent and flexible CS-FEO biopolymer film was prepared and characterized. The results showed significant antibacterial activity of the prepared CS-FEO nanocomposite. The fabricated CS-FEO nanocomposite was characterized by Fourier transform infrared spectroscopy (FTIR), field-emission scanning electron microscopy (FESEM), and X-ray diffraction (XRD) techniques. The FTIR results revealed the interaction between chitosan and FEO, and the XRD pattern for pure CS nanoparticles indicated a high degree of crystallinity. In contrast, CS-FEO nanoparticles exhibited a reduction in crystallinity peaks, indicating that the loading of CS may further reduce its crystallinity. The obtained results demonstrates that the prepared CS-FEO nanocomposite could be a potential candidate for food and biomedical applications as they hold the promising capability of proper interactions and advanced features.

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INTRODUCTION

Bacteria, viruses, fungi, and parasites are the main pathogenic agents in various environments (1,2). Many commercial antibiotics are recently known to be ineffectual due to the emergence of many antibiotic-resistant pathogens. Frequently indiscriminate prescription of commercial antimicrobial medicines for treating infectious illnesses has resulted in the emergence of multiple drug-resistant human pathogenic microorganisms (3). For example, essential oils (EOs) are becoming increasingly popular as natural antimicrobial agents for several applications such as food preservation, complementary medicine, and traditional therapies. However, their potential use as a natural anti-inflammatory agents, antioxidants, antitumors, and antibiotic agents has received less attention (4).

The FDA (Food and Drug Administration) and the EPA (Environmental Protection Agency) have designated EO as GRAS (generally regarded as safe)

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chemicals (5). They include between 20 and 60 low molecular weight components (alkaloids, phenols, and terpenes) (5). Recently, many researchers have focused on the antimicrobial properties of medicinal plants and their EOs. For example, *Ferula gummosa or Barijeh* is an aromatic plant of the *Apiaceae* family that is well known for its therapeutic properties (6). The antibacterial activity of *F. gummosa* EO, in both liquid and vapor phases, against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Shigella dysenteriae*, *Klebsiella pneumonia*, *Salmonella typhi*, and *Pseudomonas aeruginosa* bacteria was investigated (7). The investigated EO showed strong antibacterial efficacy in vapor phase against all tested bacteria. The volatile EO obtained from *F. gummosa* is rich in monoterpenes such as α- pinene and β-pinene. Regardless of the potential of EOs, the volatility and sensitivity to many environmental factors limit their traditional use in the medical industry.

Physical and chemical features, such as their inability to dissolve in water (the lipophilic components in EO) and their short half-life, further hampered using EO as a therapeutic aid (8). This essential oil's bioavailability might be hindered by including hydrophobic and nonpolar active components, but specific additives such as nanocarriers could solve this problem. Transporting and loading EOs using nanoencapsulation technology has the potential for boosting bioactive component absorption in cells by increasing their penetration into deeper tissues. It is possible to regulate and modulate the release of active chemicals in the appropriate places and improve biological activity.

Nanoencapsulation generally has the potential to improve the solubility of EO in water. Selecting proper nanocarriers could significantly overcome the instability issue. To meet this challenge, chitosan is one of the best natural nanocarriers; it is a linear polysaccharide made by treating chitin shells of shrimps which manifests antimicrobial properties (9). The antibacterial activity of the synthesized NPs-lime EO on food-borne pathogens (*S.aureus, L.monocytogenes, Shigella dysenteriae,* and *E.coli)* were investigated; the results showed that incorporating lime EO into chitosan NPs enhances the antibacterial activity (10). In another study, clove EO was encapsulated by chitosan nanoparticles (CNP) using the emulsion ionic gelation method and the fungicidal activity was performed on *Aspergillus niger* isolated from sour pomegranate; the nano-encapsulated CEO showed higher performance, compared with ChNPs and pure EO (11). Recently, *Eryngium campestre* EO encapsulated in chitosan nanoparticles were coated on cherries, and it was observed that microbial counts and weight loss were reduced (12).

There is no previous report on the characterization and antibacterial properties of chitosan- *F. gummosa* essential oil (CS-FEO) in the literature. In this study, the synthesis and characterization of CS-FEO are reported. TPP was selected as a crosslinker and CS-FEO nanoparticles, and a biopolymer was synthesized using a simple ionic gelation method. Furthermore, the structural and antibacterial properties of the prepared nanocomposite were investigated.

EXPERIMENTAL WORKS AND CHARACTERIZATIONS

Chitosan & Ferula gummosa Essential Oil

Chitin was converted to chitosan (CS) using a thermo-chemical method. To prepare CS, ground particles of shrimp and crab shells were exposed to sequential treatment processes of demineralization, discoloration, deproteinization, and deacetylation (13).

The oleo-gum-resin from *F. gummosa* was collected from Semnan (north central Iran) during July 2020. FEO were prepared by the hydro-distillation method using Clevenger apparatus (Ashke-shisheh Co., Iran). 15 g of the oleo-gum resin was soaked in 200 mL of water for about 12 h. Then, they were poured into a balloon (1 L) and 600 cc of distilled water were added to it. The mixture was boiled for 3 h, and the collected EOs were dried with anhydrous sodium sulphate. The EO with a pleasant smell was then stored in dark dropper containers and kept at 4-6 °C until their use.

CS-FEO Nanocomposite

CS (0.1 gr) was added to an aqueous acetic acid solution (1% v/v, 25 cc) and stirred for 30 min. Then, 0.5 gr of FEO was added to the homogeneous mixture. TPP was dissolved in distilled water (0.002 mol.% or 0.04 % $\frac{w}{v}$, 10 mL), and added dropwise to the homogeneous emulsion and stirred for 30 min to form suspensions. The ratio of FEO to CS was 5 w/w. Nanopowders (NPs) were collected by centrifugation at 4,000 rpm for 10 min and dried at 80 °C. Both the CS-FEO nanocomposites and their suspensions were stored at 4 °C. Fig. 1 illustrates

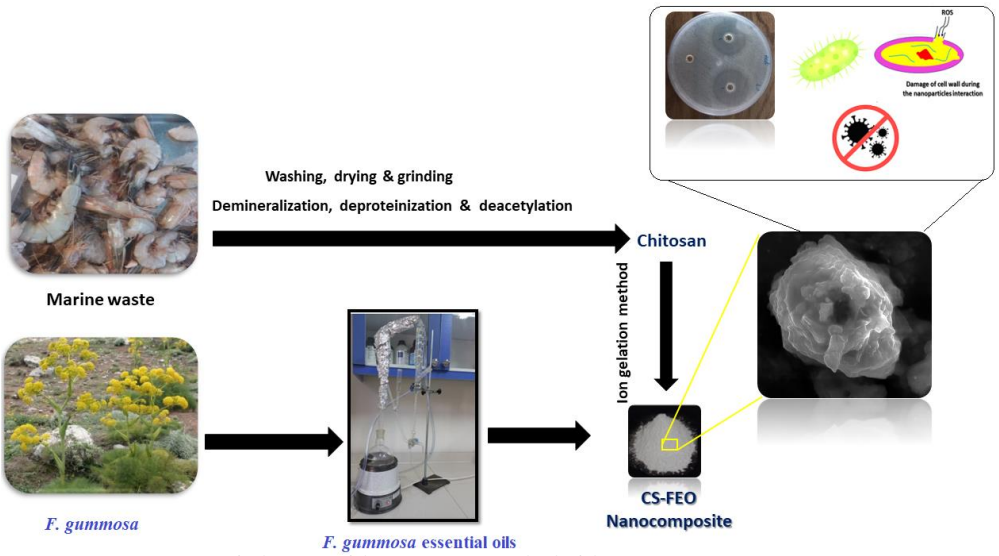

Fig. 1. A Brief schematic of the preparation method of the CS-FEO nanocomposite.

the preparation of CS-FEO.

Antimicrobial Activity Assay

The bacterial strains *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* were used from Semnan University to evaluate the antibacterial properties of the samples. The bacteria were grown in an incubator at 37 °C for 24 h. The antimicrobial activity of the FEO and CS-FEO nanocomposite was determined using agar well diffusion assay. The nutrient broth agar plates were used to check the antibacterial activity in this study. All bacterial strains were sub-cultured on nutrient broth media at 37°C for 24. After complete solidification of nutrient agar in the plates, 5 mm wells were made aseptically using sterile pipette pasteur. A volume of 50 µl of the CS and CS-FEO nanocomposite were loaded in each well, and 50 µl of growing culture were then streaked on agar plate. The plates were incubated at 37°C for 24 h. At the end of incubation, the diameter of the inhibition was measured in millimeters for each group (repeated three times).

Analytical methods

Using a PANalytical PW3050/60 X-ray diffractometer with a Cu-Kα radiation source (λ= 0.15418 nm), the X-ray diffraction (XRD) pattern of the samples was studied. The morphology of the samples was investigated using field emission scanning electron microscopy (FE-SEM; Mira3 Tescan), and Fourier transformed infrared (FTIR) spectroscopy was used to examine the functional and chemical groups. The optical properties were investigated using a UV-visible spectrophotometer (PerkinElmer).

RESULTS AND DISCUSSION

Structural properties (XRD & FTIR)

The results of the XRD studies of pure CS and the prepared CS-FEO are shown in Fig. 2. The XRD pattern of pure chitosan powder exhibited peaks at ~2θ = 10° and 2θ = 20°, indicating a high degree of crystallinity. However, the peak observed for CS-FEO nanocomposite at 2θ = 10° disappeared and a broad peak at 2θ = 24° became weak. These results suggest that chitosan has good compatibility and displays an amorphous form, which may be used in biomedical applications (14,15). In the diffraction spectrum of the prepared nanocomposite, the characteristic peak at 2θ =24° confirms the presence of FEO within chitosan nanoparticles. This

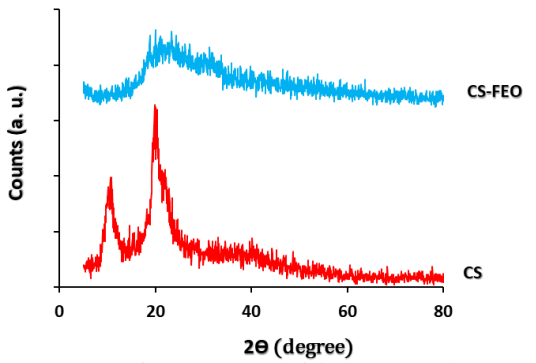

Fig. 2. X-ray Diffraction patterns of the pure CS and CS-FEO nanocomposite.

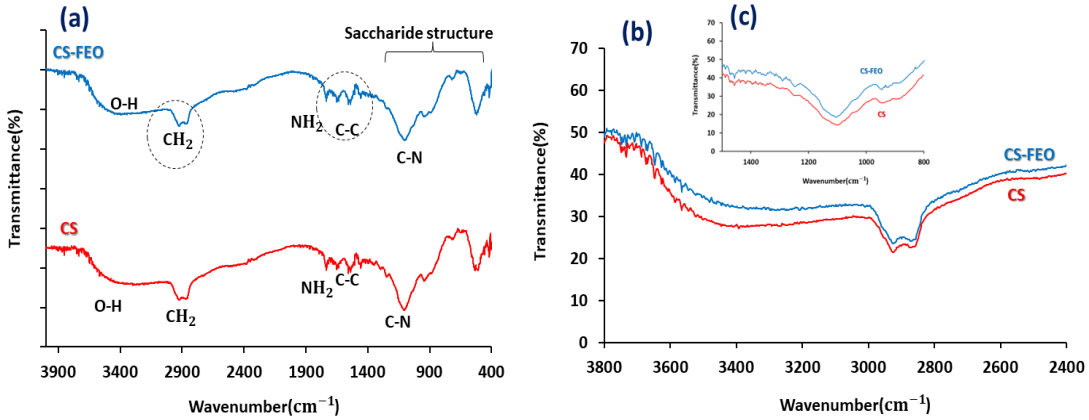

Fig. 3. (a-c) FTIR spectra of the pure CS and CS-FEO nanocomposite.

indicates that the incorporation of FEO resulted in a change in the chitosan–TPP packing structure.

Fig. 3 (a-c) shows the FTIR spectra of chitosan powder and the prepared CS-FEO nanocomposite. The FTIR spectrum of CS displayed strong peaks at 3200-3450 owing to O-H and N-H_2 stretching, 2850 cm^{-1} due to C-H stretching, 1700 cm^{-1} due to C=O stretching of amide I, 1580 cm^{-1} due to N-H_2 bending of amide II, and 1375 cm^{-1} due to C-N stretching [15]. In both samples, the peaks located at 2850-2950 cm^{-1} are due to C–H stretching and 1400 cm^{-1} related to the C=C/C-C stretching vibration of aromatic rings (16). Moreover, in comparison with the FTIR spectrum of CS, the addition of FEO resulted in a markedly increase in the intensity of the CH stretching peak at 2800–2950 cm^{-1} at 3400-3500 cm^{-1}, indicating an increase in the content of ester/hydroxyl groups, which might come from OEO molecules (Fig. 3b). Additionally, the appearance of several small peaks, at 820, 980, 1233, 1300 cm^{-1} in the nanocomposite sample may be related to the spectrum of FEO and may indicate the presence of FEO in the CS matrix (Fig. 3c).

Morphological investigation (FESEM)

The FESEM images of the pure CS and prepared CS-FEO nanocomposite is shown in Fig. 4. The FESEM image of pure CS exhibited a nonporous and smooth membranous surface. In contrast, prepared CS-FEO nanocomposite exhibited a morphology of flower shape containing near-spherical nanoparticles with the diameter size of 180 nm. A similar behavior was reported in Chitosan-Chromone Derivative for Biomedical Applications (15)

Antibacterial properties

Antibacterial properties of the FEO and CS-FEO nanocomposite were investigated using the agar well diffusion assay. According to Fig. 5 and Table 1, the prepared nanocomposite exhibited more antibacterial activity over three bacteria compared with the pure CS. Elevated antibacterial properties was seen in *B. cereus*. Generally, the nano-encapsulated FEO showed 37.5% higher

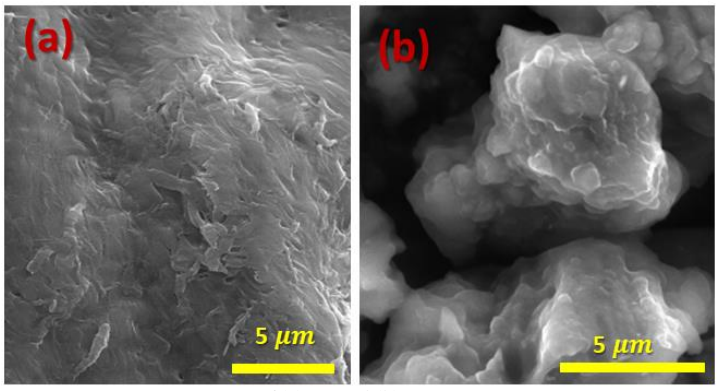

Fig. 4. FESEM images of (a) CS and (b)FEO-CS nanocomposite.

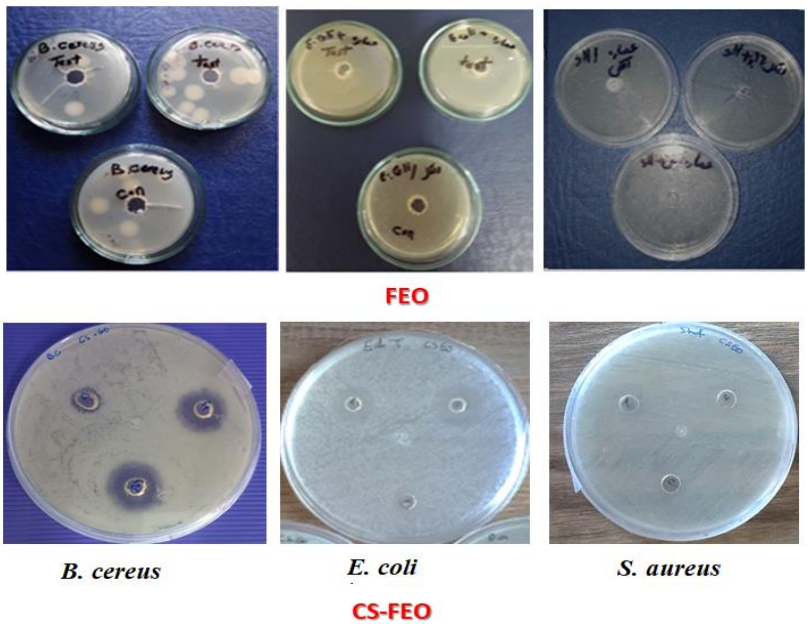

Fig. 5. Antibacterial activity of the prepared nanocomposite using agar well diffusion assay method.

antibacterial efficiency, compared with pure FEO.

It was previously observed that direct implementation of FEO was hard to measure the zone of inhabitation in the agar well diffusion assay method (7), because FEOs are concentrated hydrophobic liquids that readily evaporate at room temperature due to their volatile chemical composition. CS was employed as an additive to address these issues. Nanoencapsulation could protect EOs from evaporation and oxidation. Furthermore, it improves the water-solubility and bioactivity, and enhances the stability during food processing.

EOs are efficient against various microbes due to their aldehydes, phenolics, terpenes, and other antimicrobial components. Composition, active component functional groups, and synergistic interactions determine EO activity (17–19). The antimicrobial mechanism differs by EO type or microbe strain. It is reported that Gram-positive bacteria are more EO-sensitive than Gram-negative (17–20). The observation of the present study confirms this phenomenon; the largest inhibition zone belonged to *B. cereus*. The average inhibition zone recorded for the Gram-positive bacteria was higher than the other. Gram-negative bacteria have a complex outer membrane rich in lipopolysaccharide that limits the diffusion of hydrophobic compounds; however, Gram-positive bacteria have a thick peptidoglycan wall that is not dense enough to resist small antimicrobial molecules, allowing access to the cell membrane (20). Gram-positive bacteria may ease hydrophobic EO penetration due to lipophilic ends of lipoteichoic acid in cell membranes.

In our previous work, *F. gummosa* EO showed dramatic antibacterial efficacy in the vapor phase, although the direct-contact assays have faced problems due to the hydrophobic and volatility characteristics (7). This challenge was solved

Table 1. Obtained results of the antibacterial test (agar well diffusion assay method).

Sample Name	Zone of Inhabitation(mm)		
	E. coli	*B.cereus*	*S.aureus*
Control (Acetic acid)	0	0	0
CS	10±1	14±2	9±1
FEO-CS nanocomposite	14±1	18±2	11±1

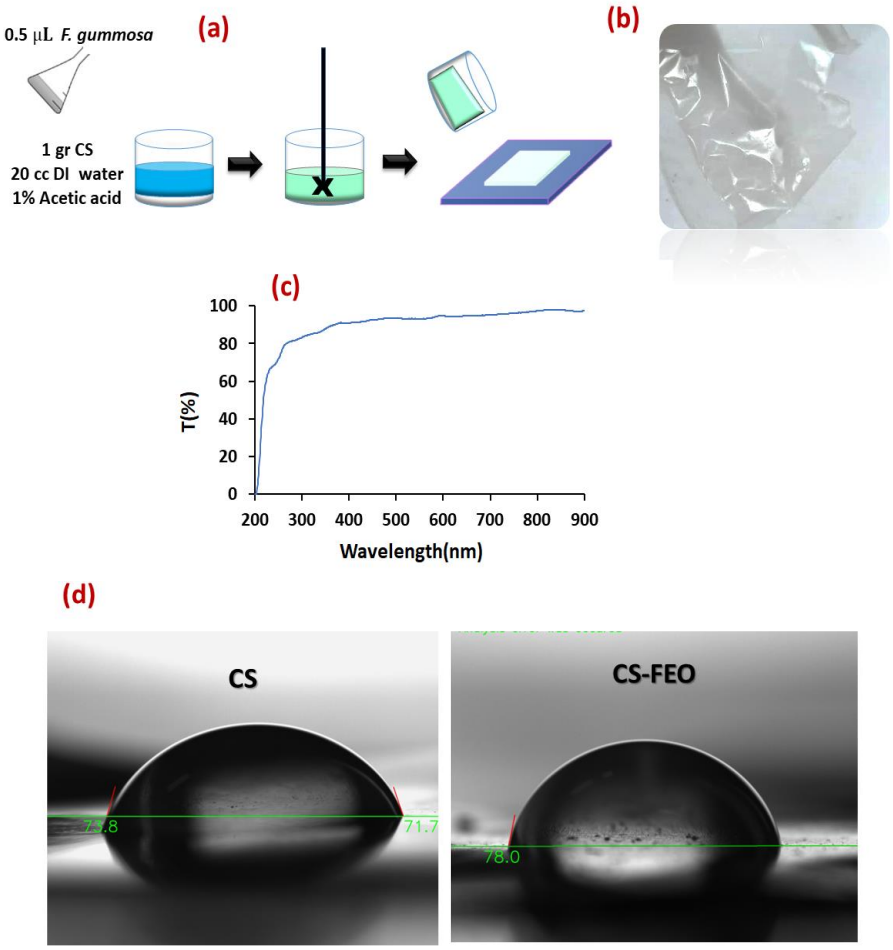

Fig. 6. (a) The schematic image of the preparation process of the FEO-CS biopolymer, (b)Visual transparency of the flexible FEO-CS biopolymer, (c) The transmittance spectrum of the prepared biopolymer, and (d) Contact angle of the CS and FEO-CS nanocomposite films.

in the present work using chitosan, and the antibacterial activity of pure CS was also increased by incorporating *F. gummosa* EO.

Investigating the chemical composition has shown that terpenes and terpenoids are dominant chemical compounds presented in EO extracted from oleo-gum-resin of *F. gummosa*. β-pinene and α-pinene are the other major compounds of FEO [8]. The functional groups (–C–O–C–, –C–O–, –C=C–, and –C=O–) in flavones, alkaloids, phenols, and anthracenes can create new antibacterial features.

Basically, plant extracts and/or EOs contain a variety of active biomolecules that help to reduce and stabilize NPs. Due to the large availability of OH groups, polyphenols can act as reducing agents in the synthesis of NPs for various applications. They also prevent the generation of toxic secondary products.

Flexible FEO-CS biopolymer characterizations

The plastics industry is looking for a multifunctional alternative to traditional polymers. Chitin and its byproduct CS are the second most prevalent biopolymer in the world. In addition, CS is biocompatible and biodegradable. Accordingly, as shown in Fig. 6a, a feasibility investigation for the preparation of CS-FEO nanocomposite biopolymer was performed. As is observed in Fig. 6b, the prepared biopolymer film has good transparency and flexibility, and is free of pores or bubbles. Fig. 6c shows the transmittance spectrum of the prepared nanocomposite. This biopolymer displayed above 85% transparency in the visible region. Fig. 6d shows the water contact angles of the CS and FEO-CS nanocomposite films. The contact angle of the neat CS film was ~72.8 degrees, while the CS-FEO film had a contact angle of ~78 degrees, making it more hydrophobic than the neat film.

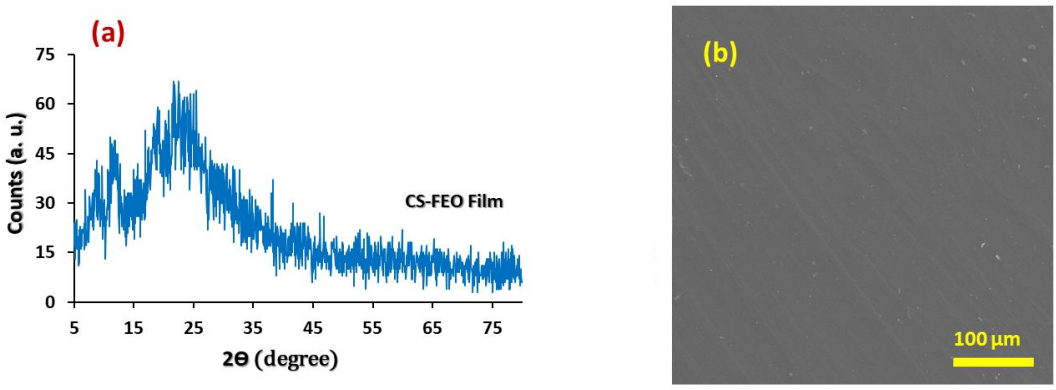

Fig. 7. (a) XRD pattern and (b) FESEM image of the surface of the CS-FEO biopolymer.

The hydrophobicity of FEO is responsible for the increase in the water contact angle of the film. In a similar study, it was observed that incorporating clove/melaleuca EOs into chitosan improved the properties of produced films for wound-healing applications (26). Based on the obtained results in the present study, it was found that the prepared CS-FEO nanocomposite film could be introduced as a good candidate for packaging application.

The XRD pattern of the prepared CS-FEO nanocomposite biopolymer is shown in Fig. 7a. The characteristic peaks at 2θ:7–25° agree with the assumption that CH is a molecular polysaccharide (21, 22, 27). This result is in agreement with previously published studies (24). It is worth mentioning that the characteristic peaks for the CS film were near 2θ = 10° and 20° (24,25). However, the positions of these characteristic peaks of the CS-FEO nanocomposite film were slightly shifted, indicating that by adding FEO, the crystalline structure of CS was decreased (25). Fig. 7b shows the FESEM image of the CS-FEO nanocomposite film; the prepared CS-FEO biopolymer exhibited a smooth and uniform surface.

CONCLUSION

In this study, FEO-CS nanocomposite powders with their flexible and transparent biopolymer were synthesized using a straightforward and cost-effective method. The samples were characterized by different analytical methods. The addition of FEO to CS revealed better antibacterial performance in the liquid phase. The interaction of FEO with CS was confirmed by FTIR and XRD measurements. FESEM images obtained from CS-FEO nanocomposite powder and its biopolymer showed spherical shapes (with the size of around 180 nm) and a uniform surface, respectively. These findings probably show that FEO could be effectively loaded in CS to further facilitate its utilization in the food or medical industries.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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