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ORIGINAL ARTICLE

Encapsulation of *Zataria multiflora* Boiss L. Essential Oil in Chitosan Nanoparticles: Characterization and Antifungal Properties in Culture Media and UF-Feta Cheese

Aso Kaboodi[1], Hamid Mirzaei[*1,2], Farzad Katiraee[3], Afshin Javadi[1,2], Mohammad Reza Afshar Mogaddam[4,5]

[1]*Department of Food Hygiene, Faculty of Veterinary Medicine, Tabriz Medical Sciences, Islamic Azad University, Tabriz, Iran*

[2]*Department of Food Biotechnology, Biotechnology Research Center, Tabriz Branch, Islamic Azad University, Tabriz, Iran*

[3]*Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran*

[4]*Food and Drug Safety Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

[5]*Pharmaceutical Analysis Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

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KEYWORDS

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ABSTRACT: This work explores the antifungal effect of *Zataria multiflora* essential oil (ZEO) and its nanocapsules (ZEO-NCs) on the culture medium and UF-Feta cheese. After selecting ZEO among the three desired EOs (based on MIC and MFC assays), chitosan nanoparticles were prepared in optimal conditions. Based on GC-MS analysis, the main constituents of ZEO were thymol (20.09%) followed by cymene (19.88%), and gamma-terpinene (18.58%). According to the response surface method, the optimum amounts of chitosan, tween 80, and ZEO were 7.97% w/v, 1.3% v/v, and 0.87% v/v, respectively, to produce the proper nanoparticles with the minimum particle size and the maximum values of zeta potential and encapsulation efficiency. A sharp peak at 2θ of 20◦ in the XRD spectrum of chitosan and its broadening in the spectrum of ZEO-NCs exhibited a high degree of crystallinity and entrapment of ZEO within nanocapsules. Also, the SEM images showed clusters of accumulated particles with relatively spherical nano-sized capsules. The formulated nanocapsules showed a stronger antifungal effect on *Penicillium citrinum* and *Aspergillus niger*, while the free form of ZEO caused more inhibition of *Aspergillus flavus*. Also, the lowest Antifungal activity among all treatments was recorded for Non-loaded NCs.

INTRODUCTION

Food safety is a fundamental issue from the perspectives of both food consumers and legal authorities. Accordingly, considering the evidence related to the numerous cases of foodborne diseases, food safety issues and the provision of methods for longer storage of food is expanding. Therefore, a wide range of synthetic preservatives are used in the preparation of various food products and without their use, the production and consumption of many food products becomes almost impossible [1]. Due to the negative effects of chemical preservatives on health, investigating safe substitute options is strongly necessary [2] Hence, the tendency to use essential oils from different plant sources instead of chemical additives in food is increasing [3,4]. Ultrafiltered (UF)-Feta cheese is one of the most consumed types of cheese in Iran [5, 6]. The presence of

*Corresponding author: hmirzaei@iaut.ac.ir (H. Mirzaei)
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nutrients in cheese provides a suitable environment for the growth of various microorganisms. Members of the genus *Penicillium* and *Aspergillus* are important causes of contamination and spoilage of cheese during storage, which may constitute a health risk due to the potential for mycotoxin production [7, 8]. Fungal control in the food industry is usually done using conventional fungicides such as potassium sorbate and sodium benzoate, which have side effects such as carcinogenicity and teratogenicity [9].

Concerns about the use of synthetic compounds have encouraged food consumers to use natural products in food. The essential oils derived from plants which have long been used in food for various purposes such as flavoring, antioxidants and antimicrobials, can be considered a valid alternative that better meets the consumer's wishes [4]. It has been shown that the effect of plant essential oils against microorganisms, including molds, are due to the ability of hydrophobic compounds to penetrate into the cell membrane lipid of the microorganism and disrupt cellular function [10, 11]. Thyme (*Zataria multiflora* Boiss L.), Basil (*Ocimum basilicum* L.), and Rosemary (*Rosmarinus officinalis* L.) are all three plants of the mint family Lamiaceae that have proven antimicrobial effects [10, 12, 13].

Despite the mentioned benefits, the stability of the active ingredients of the essential oils in different environmental conditions is usually low and is lost through physical and chemical interactions during processing, packaging, and storage [14]. Therefore, using a method that can protect these compounds against environmental factors, as well as increase the antimicrobial potential and control its release in food systems is very important. Nanoencapsulation referred to as nanocarriers for coating substances or bioactive molecules has been proposed as one such process that can achieve these goals [15].

Although the antifungal properties of various plant essential oils have been studied in the culture media [16], but due to the complexity of the relationships between the various components in foods, the effects observed in culture media and food samples may often be different. This study aims to determine the antifungal activity of thyme, rosemary and basil essential oils against the mycelial fungi of *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus niger*. Also, the most effective essential oil is selected and the antifungal effects of its nanocapsules in UF-Feta cheese and culture medium are investigated.

MATERIALS AND METHODS

Preparation of fungal strains

The fungal suspensions were prepared according to the method described previously [17]. Briefly, the strains of *P. citrinium*, *A. flavus*, and *A. niger* were inoculated in a slant Malt extract agar medium (MEA) in the glass test tube and incubated for 7 days at 26°C to produce spores. Then the surface of the culture was washed using 5 mL of saline solution and the suspension was transferred into another sterile tube. To remove the mycelium fragments from the suspension, a nylon filter was used and then a Neubauer lam (Azma Tajhiz, Iran) was used to count fungal spores. Fungal suspensions were adjusted to 10^6 conidia per mL in Malt extract broth.

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The fungistatic effect was determined by dilution in tube method using concentrations ranging from 0.05 to 1% of food-grade thyme (*Zataria multiflora* Boiss L.), rosemary (*Rosmarinus officinalis* L.), and basil (*Ocimum basilicum* L.) essential oils (Avat Shimi Pezhvak, Iran) in Malt extract broth (MEB) medium. The tubes were incubated at 26°C for 7 days and the lowest concentration of essential oils that prevented mold growth and visible turbidity was determined as MIC. To determine the fungicidal effect, an aliquot of the concentrations (0.1 ml) in which no turbidity was observed was surface cultured in MEA medium. The concentrations did not show any growth after 7 days of incubation at 26°C were considered the MFC [15].

Chemical composition of ZEO

Identification of the ZEO composition was performed by an Agilent (Santa Clara, CA, USA) GC (6890) /MS (5890) system. The GC injector temperature was set at 300˚C and it was worked in split mode at a ratio of 1:50. The analytes were separated on an HP- MS capillary column with the length of 30 m. The column film

thickness and inner diameter were 0.25 µm and 0.25 mm, respectively. The system carrier gas was helium with purity of 99.999% and its flow rate was 1.0 mL min^{-1}. The column oven temperature was initially adjusted at 80˚C and it was increased up to 300˚C at a rate of 8˚C min^{-1}. Ionic source temperature was set as 280˚C. Transfer line temperature, detector voltage, and electron ionization energy were 300˚C, -1700 v, and 70 eV, respectively. The active components of the oil were identified by analysis of the mass spectra. The peak area measurement (reported as area percentage) was used for quantitative analysis of constituents of the EO [18].

Preparation of ZEO-NCs

Twenty *Zataria multiflora* essential oil nanocapsule samples (ZEO-NCs) were prepared (Table 1) as previously described [19] and then the process was optimized based on the RSM method. Briefly, a 50 mL of medium molecular weight chitosan (Merck, Germany) solution at different concentration (w/v) in acetic acid (1% v/v) was prepared by stirring K the solution for 24 h at 250 rpm at room temperature. Then, to create a Tween solution with various concentrations (v/v), a proper volume of Tween 80 (Merck, Germany) was gently added to the solution and mixture was transferred into an ultrasonic bath (Backer, Vcleaner, Iran) adjusted at 50˚C and sonicated for 2 h. The essential oil was subsequently added to the mixture to obtain a solution with different concentrations of ZEO (v/v). After that, 1 mg per mL of trisodium polyphosphate (TPP) solution (Merck, Germany) was added to the solution drop wisely and the mixture was homogenized for 15 min at 5200 rpm. The mixture was then centrifuged (Eppendorf, 5810R) for 10 min at 13000 rpm. The supernatant was removed and the sediment phase was eluted by tween 80 solution to remove the un-recated substances. The ZEO-NCs stored at 4 ◦C until use.

Characterization of ZEO-NCs

Encapsulation efficiency

The efficiency of ZEO entrapment in chitosan nanoparticles was determined using ultraviolet-visible spectrophotometer (Varian Cary 500, USA). Aliquots of 10 mg ml^{-1} of ZEO-NCs dispersions and EO-free nanocapsules (as blank samples) were lysed by boiling in 5 mL HCl (1 M) at 95◦C for 30 min. Then 1 ml of ethanol (99–100%) was added after cooling down the mixture to room temperature and the resulting solution centrifuged at 10000 rpm for 5 min at 25°C. Subsequently, the EO content in supernatant was measured by UV–vis spectrophotometry at 270 nm (maximum measured wavelength). Finally, total amount of loaded ZEO was calculated by a standard curve of changes in the concentration of ZEO against its absorption changes and EE was calculated through the equation1. The experiments were done in triplicate [20].

$$EE = \text{Amount of loaded ZEO in ZEO-Ns} / \text{Initial amount of ZEO} \times 100 \quad (1)$$

Particle Size, Polydispersity and Zeta Potential

The mean particle size, the polydispersity index (PDI) and the zeta potential of freshly prepared ZEO-NCs were determined by dynamic light scattering (DLS) method [21] using a Nanotrac Wave (DKSH, Shanghai, China). The measuring range was from 0.8 to 6500 ng. Temperature control of the DLS system was ranged from 5 to 90˚C. The system laser wavelength was 780 nm. The electrophoretic mobility was<5 mS cm^{-1}. All of the spectrophotometric determinations were done by a UV 1800 Shimadzu system (Kyoto, Japan).

Optimization of ZEO-NCs

In order to choose the best formulation for ZEO-NCs preparation, the effects of three independent variables including A) chitosan percent, B) tween 80 amount, and C) the EO percent (at five levels), on particle size, Zeta potential and encapsulation efficiency were investigated. Optimization was achieved by central composite design (CCD)-response surface methodology (RSM) using Design Expert 11 software (Stat-Ease Inc, USA) with total of 20 experimental runs.

Fourier-transform infrared (FTIR) spectroscopy

Using FTIR spectra, interactions between chemical structural elements of ZEO-NCs were investigated. For this, about 20 mg of the powdered nanoparticles were mixed with 100 mg of potassium bromide powder and pressed into a thin tablet. The tablets were then exposed

to infrared light (FTIR spectrophotometer, Bruker, Germany) and FTIR spectra were recorded from a wavelength of 4000 to 400 cm^{-1}.

X-ray diffraction (XRD) pattern

X-ray diffraction was used to study the crystalline structure of chitosan and ZEO-loaded chitosan nanoparticles. For this purpose, the samples were fixed on holder and exposed to X-rays (X-Ray diffractometer, Rigaku, USA). The X-ray generator was set at 40 kV, 40 mA and 2200 W. The reflected beams of the sample were measured in the angle range of 2θ from 5° to 80° with a speed of 0.04°/min.

Morphology properties by SEM

Scanning electron microscope (TESCAN MIRA3, Czech Republic) was used to observe the surface morphology of the prepared nanoparticles. The samples were coated with a thin layer of gold-palladium using a sputtering device (SC 7620, UK) and photographed at different magnifications.

Antifungal activity

Evaluation of spore production

This test was performed according to previously described method [7]. Briefly, aliquots of 0.1 ml of spore suspension (10^6 spores mL^{-1}) of three selected fungal strain was spread on the MEA agar surface containing 1 mg mL^{-1} of ZEO, formulated ZEO-NC and Non-loaded NC. The negative controls (without experimental samples) also prepared. After incubation at 26 °C for 7 days, the produced spores were transferred to a flask containing 50 ml 0.05% Tween 80 and the suspension was shaken vigorously. Then the number of spores per cm^2 of the plate was counted using a neubauer lam and the inhibition of spore production was calculated by the equation 2.

$$\text{Inhibitory percentage (IP)} = [(Nc - Ns) / Nc] \times 100 \quad (2)$$

Nc and Ns are the number of spores in control and treated sample, respectively.

Radial growth analysis on culture media

For radial mycelial growth analysis of three selected fungal strain, amounts of 1 mg mL^{-1} of ZEO, formulated ZEO-NC and Non-loaded NC were added into the sterile petri dishes containing molten PDA medium at 45°C. The negative controls (without experimental samples) also prepared. After solidification, all plates were seeded with 0.5 cm diameter Whatman No. 1 filter paper disc on center and inoculated with 10 µl of spore suspension (10^6 spores mL^{-1}). Finally, the means of two perpendicular diameters of the fungal colony was measured after incubation of the plates in a dark place (7 days at 26°C). The inhibitory percentage (IP) was determined from the equation3 [7].

$$IP = [(Dc - Ds) / Dc] \times 100 \quad (3)$$

Dc and Ds are the mycelium diameters in the control and treated samples, respectively.

Direct growth analysis on UF-Feta cheese

For this, slices about 5 mm thick were prepared from the UF-Feta cheese (previously exposed to UV light for 60 min) to be fit into the 8-cm diameter sterile petri dishes, using a sterile knife. Then, cheese slices were immersed into a solution containing 1 mg mL^{-1} of ZEO, formulated ZEO-NC and Non-loaded NC and after 10 min, aliquots of 10 µl of fungal spore suspension (10^6 spores mL^{-1}) were inoculated onto the center of each plate. The negative controls (without experimental samples) were also prepared. Finally, the means of two perpendicular diameters of the fungal colony was measured after 7 days of incubation of the plates at 26°C in a dark place [21]. The Inhibitory percentage was determined as described for radial growth analysis on the PDA medium.

Statistical analysis

The one-way analysis of variance (ANOVA) was performed through SPSS Statistics version 23 (IBM Inc., USA). Tukey's multiple range test was used to determine the difference among the means with a 95% significance level. All analyses except GC-MS measurements were performed in three replicates. Design Expert 11 software (Stat-Ease Inc, USA) was used to create surface plots for optimization experiments.

RESULTS

MIC and MFC assays

Samples with minimum MIC and MFC values were considered to exhibit stronger antifungal effects. Based on the results, the highest antifungal properties of three selected essential oils against three types of molds tested were recorded for ZEO (data not shown), so subsequent analyses were performed only on ZEO.

Chemical composition of ZEO

The GC-MS chromatogram of ZEO along with the mass spectra of its main identified chemical components revealed that the most common constituent compounds were thymol (20.09%) followed by cymene (19.88%), gamma-terpinene (18.58%), linalool (14.52%) and carvacrol (12.23%).

Selection of optimum ZEO-NCs

The design of the CCD and the obtained results for the analytes are summarized in Table 1. For evaluation of these data, the ANOVA results were obtained for each analyte (data not shown). The results indicate that all of the studied factors are significant. The obtained results confirm that there is a quadratic relationship between the results and three factors through a second order polynomial equation. Also, comparison of coefficients of determination (R^2, R^2-predicted and R^2-adjusted against zeta potential, particle size and EE%) specify that the results are in good agreement with the equations and the model has acceptable reliability and accuracy. Also, the effect of diverse factors on the method efficacy was investigated as 3D-response surface and contour plots (data not shown), which in summary the optimum amounts of chitosan, tween 80, and the essential oil percent were 7.97% w/v, 1.3% v/v, and 0.87% v/v, respectively.

Table 1. The CCD design and the results obtained from the experiments for ZEO-NCs formulation.

Std	Run	A*	B*	C*	Average determinations obtained from three repeated analysis		
					Zeta potential	Particle size	EE%
2	1	8.0	0.3	0.3	111	959	34.4636
5	2	2.0	0.3	1.0	53	1888	52.0058
9	3	0.0	0.6	0.6	362	109	45.0519
20	4	5.0	0.6	0.6	49	447	78.408
8	5	8.0	1.0	1.0	155	377	75.383
19	6	5.0	0.6	0.6	49	447	81.185
1	7	2.0	0.3	0.3	97	758	28.7823
16	8	5.0	0.6	0.6	49	447	75.383
7	9	2.0	1.0	1.0	212	424	57.718
10	10	10.0	0.6	0.6	321	947	57.6565
14	11	5.0	0.6	1.3	9	1619	79.013
3	12	2.0	1.0	0.3	193	2242	18.5239
4	13	8.0	1.0	0.3	158	574	31.823
15	14	5.0	0.6	0.6	49	447	85.2203
17	15	5.0	0.6	0.6	49	447	80.5904
6	16	8.0	0.3	1.0	96	4050	60.6849
11	17	5.0	0.0	0.6	72	1968	46.6756
13	18	5.0	0.6	0.0	51	734	22.4939
18	19	5.0	0.6	0.6	49	447	82.9334
12	20	5.0	1.3	0.6	156	1049	39.325

* A, B and C: Chitosan, Tween 80 and essential oil percents, respectively.

Fourier-transform infrared (FTIR) spectroscopy

The FTIR spectra of chitosan (Ch) and formulated ZEO-NCs are shown in Figure 1(a, b). As depicted in Figure 1-b, the addition of EO to the ZEO-NCs structure did not cause significant changes in the peak intensity or shift in the chitosan wave numbers.

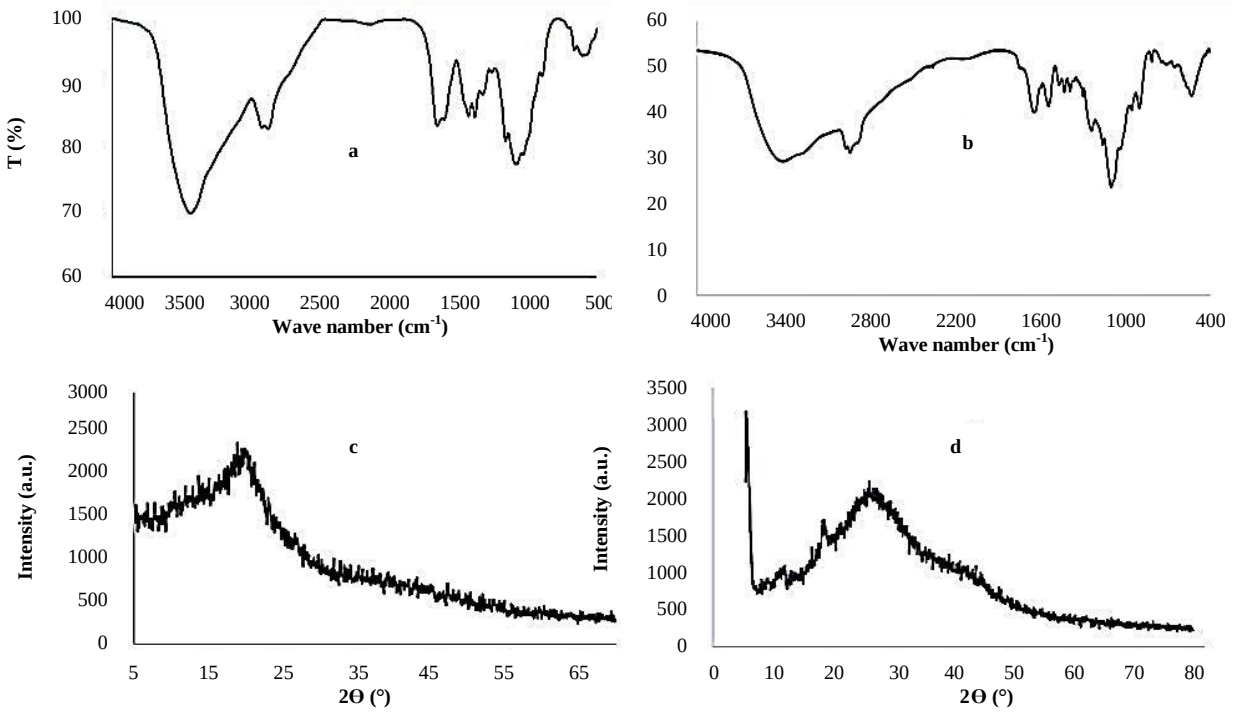

Figure 1. FTIR spectra (a,b) and XRD patterns (c,d) of chitosan and formulated ZEO-NCs.

X-ray diffraction (XRD) pattern

The XRD was performed to compare the impact of the addition of ZEO to NCs on the crystalline profile of chitosan. As shown in Figure 1-c, the diffraction spectrum of chitosan powder showed a sharp peak at 2θ of 20◦, which became broader in ZEO-loaded chitosan diffractogram and observed at 2θ value between 25◦ and 35◦.

Morphology properties by SEM

The obtained images showed nano-size spherical nanocapsules with relatively smooth surfaces in large clusters of accumulated particles (Figure 2).

Antifungal activity of ZEO-NCs

In this study, the results of spore production, radial growth in the PDA medium and direct growth of *Penicillium citrinum*, *Aspergillus niger*, and *Aspergillus flavus* on UF-Feta cheese are shown in Table 2. Based on these tests, all three forms of pure ZEO, Non-loaded NCs, and ZEO-NCs showed antifungal properties, and the lowest inhibitory activities were recorded for blank samples.

Table 2. Antifungal activity of pure ZEO, ZEO-NC and Non-loaded NC against fungi.

Treatment	Spore production		Growth on PDA medium		Growth on UF cheese	
	Spore count (10^6 spores/cm^2)	Inhibition (%)	Colony diameter (mm)	Inhibition (%)	Colony diameter (mm)	Inhibition (%)
			Penicillium citrinum			
Control	75.3±1.69^{d}	0	43.0±0.00^{b}	0	35.6±0.94^{c}	0
Pure ZEO	46.0±0.81^{b}	38.9	26.6±1.24^{a}	38.1	23.0±2.16^{ab}	35.4
ZEO-NC	33.3±1.69^{a}	55.7	21.3±2.05^{a}	50.4	19.6±1.69^{a}	44.9
Non-loaded NC	63.0±1.41^{c}	16.3	41.6±1.24^{b}	3.2	30.3±2.62^{bc}	14.9
			Aspergillus flavus			
Control	182.0±6.68^{b}	0	75.0±4.08^{c}	0	54.6±3.85^{b}	0
Pure ZEO	92.3±3.09^{a}	49.3	35.0±0.81^{a}	53.3	36.3±1.69^{a}	33.5
ZEO-NC	108.3±8.17^{a}	38.3	44.0±4.96^{a}	41.3	37.0±1.41^{a}	32.2
Non-loaded NC	161.0±2.94^{b}	11.5	59.6±2.05^{b}	20.5	48.0±0.00^{b}	12.1
			Aspergillus niger			
Control	176.6±5.43^{c}	0	77.6±3.68^{b}	0	56.0±3.55^{b}	0
Pure ZEO	73.0±3.26 ^{a}	58.6	34.3±3.39^{a}	57.8	28.6±3.29^{a}	48.9
ZEO-NC	69.3±1.24 ^{a}	60.7	33.0±0.00^{a}	57.5	22.0±2.94^{a}	60.7
Non-loaded NC	148.6±3.39^{b}	15.8	69.0±7.48^{b}	9.9	48.6±2.35^{b}	13.2

Data (n=3) are represented as means ± SD. Different letters in each column are significantly different at P<0.05 according to the Tukey's test.

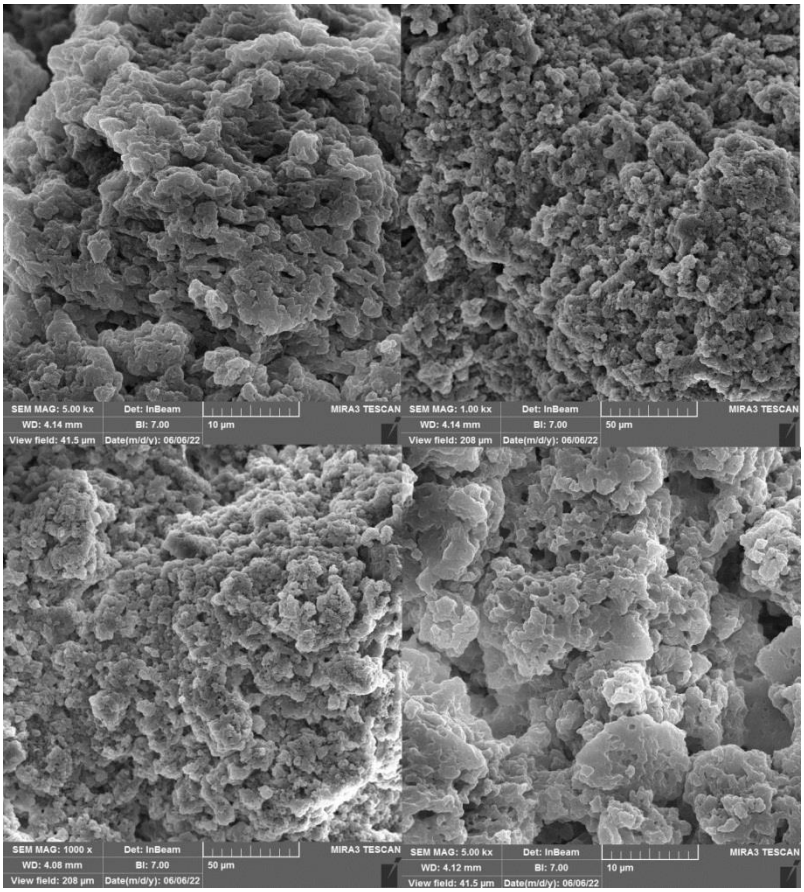

Figure. 2. SEM images of the surface of formulated ZEO-NCs.

DISCUSSION

It has been claimed that due to the hydrophobicity and volatile nature of EOs, they may interact with the cell membrane of microorganisms, allowing the leakage of cytoplasmic content and ions and ultimately causing cell

death [16]. Also, ZEO owing to its high contents of thymol and carvacrol shows enhanced biological activity [23]. The antifungal effect of ZEO has been confirmed by several studies, obtaining different degrees of inhibition [7, 14]. This antifungal activity is highly dependent on factors such as geobotanical conditions, extraction technique of essential oil, and fungal genus [24].

In various studies, thymol and carvacrol have been reported as the most abundant compounds in ZEO [23]. It should be emphasized that several factors including regional, seasonal, and experimental conditions, can affect the composition of each essential oil and alter its biological activity [25].

In general, zeta potential higher than 30 mV is considered physically stable [26]. Higher (positive or negative) values of zeta potential indicate the stronger surface electrical charge leading to particle repulsion, which results in their physical stability [27]. In addition, the lowest size particles with the greater surface-to-volume ratio, result in greater release of bioactive compound from ZEO-NCs and consequently better antifungal effect [20]. Therefore, the goal of optimization was to achieve the most appropriate nano-carrier formulation with the smallest particle size and the highest value of zeta potential and encapsulation efficiency.

FTIR technique provides useful information about the chemical structure and possible interactions between the major components and subsequently the entrapment of EO within nanocapsules. In the FTIR spectra, the peaks in regions 3700-3000 cm-1 and 600-450 cm^{-1} were attributed to inter-molecular hydrogen bonding of chitosan structure [28]. The band at position 2800–2900 cm-1 is denoted by the C-H group. The bands appeared at wave numbers 1500 and 1600 corresponding to C=O stretching of amide and N-H stretching of amine groups, respectively. Also, the peaks at 1300 cm-1 and 1150 cm-1 are linked to C–H and C–O bonds, respectively [29]. Further, the addition of EO to the ZEO-NCs structure did not cause significant changes in the FTIR spectrum, which is similar to the results of other studies [30]. Accordingly, these slight changes between the two FTIR spectra can be related to the non-covalent interaction or surface adsorption between chitosan and ZEO [28].

The sharp peak observed in the diffraction spectrum of chitosan powder and ZEO-loaded chitosan, indicates a high degree of crystallinity. Other researchers have reported the occurrence of a characteristic XRD peak in the chitosan structure at $2\theta = 20^\circ$ (15) and $2\theta = 25^\circ$ [31]. The broadening of the sharp peak of the X-ray diffractogram of chitosan powder and its appearance at 2θ value between 25° and 35° implies the entrapment of ZEO within ZEO-NCs (Figure 1-d). Previous researches on the encapsulation of various EOs have also revealed a shift or broadening of the peaks in the XRD spectrum [26, 27].

The spherical nanocapsules may represent the stability of the nanoparticles during the preparation. Moreover, the sphericity of the ZEO-NCs causes them to have the highest ability for controlled-release essential oil [20]. The ZEO-NCs exhibited large clusters of accumulated particles, which was also confirmed in other studies [27, 31]. The Formation of clustered aggregates might have resulted from the presence of minor amounts of EO on the surface of ZEO-NCs [32].

Species of Aspergillus and Penicillium are among the common causes of cheese spoilage [33], which have the potential to produce mycotoxins such as aflatoxin and citrinin [7, 22]. Determining the inhibitory effect of EOs against sporulation of different molds, in-vitro growth on the culture media, and direct growth on several types of cheese are the most widely used methods of investigating the antifungal properties of fungi [7, 22, 34].

Based on the results, the antifungal effects of the ZEO-NCs against *P. citrinum* and *A. niger* were higher than that of EO alone, while the free EO showed a better inhibitory effect on *Aspergillus flavus*, although the differences were not significant except in two cases ($P>0.05$). Similar to the results obtained in this work, the stronger antifungal effect of other essential oil-loaded nanoparticles have been proven in previous researches [14, 35]. Also, the stronger antimicrobial effects of the free form of EOs have been shown compared to the nano-encapsulated forms in some studies [27, 34].

The antifungal effects of ZEO can be attributed to phenolic compounds, especially thymol and carvacrol. Previous reports about the antimicrobial activity of these compounds are consistent with the findings of this research [14, 23]. Chitosan itself has also antimicrobial

properties due to its polycationic nature [36, 37] so; this may explain the slight antifungal effect of unloaded NCs. Also, the superior inhibitory effect of ZEO-NCs against *P. citrinium* and *A. niger* over free EO, might be due to the protection of NCs against environmental factors and enzymatic degradation by the pathogens [38] or due to the controlled release of bioactive compounds from NCs as well as the inhibitory effect of unloaded NCs itself [35]. Moreover, the stronger inhibitory effects of all treatments on the growth of fungi in the culture medium compared to the surface of the cheese can be attributed to the degradation of bioactive compounds through the interaction with organic substances in the cheese [39].

In conclusion, the antifungal properties of *Zataria multiflora* essential oil (ZEO) and its nanocapsules (ZEO-NCs) were investigated in UF-Feta cheese and culture medium. Thymol, cymene, gamma-terpinene, linalool, and carvacrol were identified as the main chemical constituents of ZEO by the GC-MS study. *Zataria multiflora* essential oil-loaded chitosan nanoparticles were synthesized by emulsification ionic gelation technique. According to the RSM, the optimized concentrations of chitosan, tween 80, and ZEO to produce the proper nanocapsule formulation were 7.97% w/v, 1.3% v/v, and 0.87% v/v, respectively. A successful encapsulation of ZEO within chitosan nanocapsules was confirmed by FTIR, XRD, and SEM images. The antifungal effect of the encapsulated ZEO on *A. flavus* was lower than that of the pure essential oil, and also, the higher antifungal effect of the ZEO-NCs on *P. citrinum* and *A. niger* did not show a significant difference ($P>0.05$) with the free ZEO. Of course, it should be noted that the encapsulation process has other advantages, such as the entrapping of the strong taste of essential oils or the slow releasing of the bioactive compounds and their longer-term effects of them. Overall, it seems that deciding on the efficacy of nanoencapsulation for enhancing pure ZEO activity in UF-Feta cheese requires more detailed studies including the examination of organoleptic properties of cheese or the study of antimicrobial effects in longer periods (during the cheese storage).

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Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

1. Serra S., Fuganti C., Brenna E., 2005. Biocatalytic preparation of natural flavours and fragrances. Trends Biotechnol. 23(4), 193-198.

2. Smith-Palmer A., Stewart J., Fyfe L., 2001. The potential application of plant essential oils as natural food preservatives in soft cheese. Food Microbiol. 18(4), 463-470.

3. Singh S., Chaurasia P.K., Shashi Lata Bharati S.L., 2022. Functional roles of Essential oils as an effective alternative of synthetic food preservatives: A review. J Food Process Preserv. 46(8), e16804.

4. Javan A.J., Salimiraad S., Khorshidpour B., 2019. Combined effect of Trachyspermum ammi essential oil and propolis ethanolic extract on some foodborne pathogenic bacteria. Vet Res Forum. 10(3), 235-240.

5. Soltani M., Sahingil D., Gokce Y., Hayaloglu A.A., 2019. Effect of blends of camel chymosin and microbial rennet (*Rhizomucor miehei*) on chemical composition, proteolysis and residual coagulant activity in Iranian Ultrafiltered White cheese. J Food Sci Technol. 56(2), 589-598.

6. Hassanzadazar H., Ehsani A., Mardani M., 2014. Antibacterial activity of Enterococcus faecium derived from Koopeh cheese against *Listeria monocytogenes* in probiotic ultra-filtrated cheese. Vet Res Forum. 5(3),169-175.

7. Gandomi H., Misaghi A., Basti A.A., Bokaei S., Khosravi A., Abbasifar A., Javan A.J. 2009. Effect of *Zataria multiflora* Boiss. essential oil on growth and aflatoxin formation by *Aspergillus flavus* in culture media and cheese. Food Chem Toxicol. 47(10), 2397-2400.

8. Mohammadiani E., Aliakbarlu J., Ownagh A., Kaboudari A., 2021. Antifungal interactions of Persian shallot (Allium hirtifolium) extracts and potassium

sorbate against Aspergillus flavus and Penicillium citrinum. Flavour Fragr J. 36(3), 332-338

9. Mpountoukas P., Vantarakis A., Sivridis E., Lialiaris T., 2008. Cytogenetic study in cultured human lymphocytes treated with three commonly used preservatives. Food Chem Toxicol. 46(7), 2390-2393.

10.Burt S., 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol. 94(3), 223-253.

11. Fathollahi M., Aminzare M., Mohseni M., Hassanzadazar H., 2009. Antioxidant capacity, antimicrobial activities and chemical composition of *Pistacia atlantica* subsp. kurdica essential oil. Vet Res Forum. 10(4), 299-305.

12. Langroodi A.M., Tajik H., Mehdizadeh T., 2018. Preservative effects of sumac hydro-alcoholic extract and chitosan coating enriched along with *Zataria multiflora* Boiss essential oil on the quality of beef during storage. Vet Res Forum. 9(2), 153-161.

13. Moradi M., Tajik H., Rohani S.M.R., Oromiehie A.R., Malekinejad H., Aliakbarlu J., Hadian M., 2012. Characterization of antioxidant chitosan film incorporated with Zataria multiflora Boiss essential oil and grape seed extract. LWT - Food Sci. Technol. 46(2), 477-484

14. Nasseri M, Golmohammadzadeh S, Arouiee H., Jaafari M.R., Neamati H., 2016. Antifungal activity of *Zataria multiflora* essential oil-loaded solid lipid nanoparticles in-vitro condition. Iran J Basic Med Sci.19(11), 1231-1237.

15. Kapustová M, Granata G., Napoli E., Puškárová A., Bučková M., Pangallo D., Geraci C., 2021. Nanoencapsulated essential oils with enhanced antifungal activity for potential application on agri-food, material and environmental fields. Antibiotics. 10(1), 31.

16. Basak S., Guha P.A., 2018. Review on antifungal activity and mode of action of essential oils and their delivery as nano-sized oil droplets in food system. J Food Sci Technol. 55(12), 4701-4710.

17. Déziel E., Comeau Y., Villemur R., 2001. Initiation of biofilm formation by Pseudomonas aeruginosa 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. J Bacteriol. 183(4), 1195-1204.

18. Saei-Dehkordi S.S., Tajik H., Moradi M., Khalighi-Sigaroodi F., 2010. Chemical composition of essential oils in Zataria multiflora Boiss. from different parts of Iran and their radical scavenging and antimicrobial activity. Food Chem Toxicol. 48(6), 1562-1567

19. Chaudhari A.K., Singh A., Das S., Dubey N.K., 2021. Nanoencapsulated *Petroselinum crispum* essential oil: characterization and practical efficacy against fungal and aflatoxin contamination of stored chia seeds. Food Bioscience. 42, 101117.

20. Hasheminejad N., Khodaiyan F., Safari M., 2019. Improving the antifungal activity of clove essential oil encapsulated by chitosan nanoparticles. Food Chem. 275, 113-122.

21. Soltanzadeh M., Peighambardoust S.H., Ghanbarzadeh B., Mohammadi M., Lorenzo J.M., 2021. Chitosan nanoparticles as a promising nanomaterial for encapsulation of pomegranate (*Punica granatum* L.) peel extract as a natural source of antioxidants. Nanomaterials. 11(6), 1439.

22. Vazquez B.I, Fente C., Franco C.M., Vázquez M.J., Cepeda A., 2001. Inhibitory effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. Int J Food Microbiol. 67(1-2), 157-163.

23. Ebadi Z., Ghaisari H,. Tajeddin B., Shekarforoush S.S., 2022. Evaluation of the properties and antibacterial activity of microchitosan film impregnated with Shirazi thyme (*Zataria multiflora*) and garlic (*Allium sativum*) essential oils. Iran J Vet Res. 23(1), 53-60.

24. Falcone P., Speranza B., Del Nobile M.A., Corbo M.R., Sinigaglia M., 2005. A study on the antimicrobial activity of thymol intended as a natural preservative. J Food Prot. 68(8), 1664-1670.

25. Lang G., Buchbauer G., 2012. A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals. Flavour. Fragr J. 27(1), 13-39.

26. Shetta A., Kegere J., Mamdouh W., 2019. Comparative study of encapsulated peppermint and green tea essential oils in chitosan nanoparticles: Encapsulation, thermal stability, in-vitro release, antioxidant and antibacterial activities. Int J Biol Macromol. 126, 731-742.

27. Soltanzadeh M., Peighambardoust S.H., Ghanbarzadeh B., Mohammadi M., Lorenzo J.M., 2021.

Chitosan nanoparticles encapsulating lemongrass (*Cymbopogon commutatus*) essential oil: Physicochemical, structural, antimicrobial and *in vitro* release properties. Int J Biol Macromol. 192, 1084-1097.

28. Salehi F, Behboudi H., Kavoosi G., Ardestani S.K., 2020. Incorporation of *Zataria multiflora* essential oil into chitosan biopolymer nanoparticles: A nanoemulsion based delivery system to improve the in-vitro efficacy, stability and anticancer activity of ZEO against breast cancer cells. Int J Biol Macromol. 143, 382-392.

29. Ardekani N.T., Khorram M., Zomorodian K., Yazdanpanah S., Veisi H., Veisi H., 2019. Evaluation of electrospun poly (vinyl alcohol)-based nanofiber mats incorporated with *Zataria multiflora* essential oil as potential wound dressing. Int J Biol Macromol. 125, 743-750.

30. Rubilar J.F, Cruz R.M., Silva H.D., Vicente A.A., Khmelinskii I., Vieira M.C., 2013. Physico-mechanical properties of chitosan films with carvacrol and grape seed extract. J Food Eng. 115(4), 466-474.

31. Rajkumar V., Gunasekaran C., Paul C.A., Dharmaraj J., 2020. Development of encapsulated peppermint essential oil in chitosan nanoparticles: Characterization and biological efficacy against stored-grain pest control. Pestic Biochem Physiol. 170, 104679.

32. Yoksan R., Jirawutthiwongchai J., Arpo K., 2010. Encapsulation of ascorbyl palmitate in chitosan nanoparticles by oil-in-water emulsion and ionic gelation processes. Colloids Surf B Biointerfaces. 76(1), 292-297.

33. Banjara N., Suhr M.J, Hallen-Adams H.E., 2015. Diversity of yeast and mold species from a variety of cheese types. Curr Microbiol. 70(6), 792-800.

34. Bedoya-Serna C.M., Dacanal G.C, Fernandes A.M., Pinho S.C., 2018. Antifungal activity of nanoemulsions encapsulating oregano (*Origanum vulgare*) essential oil: *in vitro* study and application in Minas Padrão cheese. Braz J Microbiol. 49, 929-935.

35. Beyki M., Zhaveh S., Rahmani-Cherati T., Abollahi A., Bayat M., Tabatabaei M., Mohsenifar A., 2014. Encapsulation of Mentha piperita essential oils in chitosan-cinnamic acid nanogel with enhanced antimicrobial activity against *Aspergillus flavus*. Ind Crops Prod. 54, 310-319.

36. Al-Hetar M., Zainal Abidin M., Sariah M., Wong M.Y., 2011. Antifungal activity of chitosan against *Fusarium oxysporum* f. sp. cubense. J Appl Polym Sci. 120(4), 2434-2439.

37. Hassanzadeh P., Moradi M., Vaezi N., Moosavy M.H., Mahmoudi R., 2018. Effects of chitosan edible coating containing grape seed extract on the shelf-life of refrigerated rainbow trout fillet. Vet Res Forum. 9(1), 73-79.

38. Donsì F., Annunziata M., Sessa M., Ferrari G., 2011. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. LWT – Food Sci Technol. 44(9), 1908-1914.

39. Bagamboula C., Uyttendaele M., Debevere J., 2004. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*. Food Microbiol. 21(1), 33-42.