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Biopolymeric Nanoparticles, Pickering Nanoemulsions and Nanophytosomes for Loading of *Zataria multiflora* Essential Oil as a Biopreservative

Mansoureh Taghizadeh[1], Seid Mahdi Jafari[2,3,4], Kianoush Khosravi Darani[5], Mahmood Alizadeh Sani[6] Saeedeh Shojaee Aliabadi[1], Nader Karimian Khosroshahi[7], Hedayat Hosseini[1,8*]

[1] Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Department of Food Materials and Process Design Engineering, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[3] Universidade de Vigo, Nutrition and Bromatology Group, Department of Analytical Chemistry and Food Science, Faculty of Science, E-32004 Ourense, Spain
[4] College of Food Science and Technology, Hebei Agricultural University, Baoding, 071001, China
[5] Department of Food Technology Research, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Science, Tehran, Iran
[6] Division of Food Safety and Hygiene, Department of Environmental Health Engineering, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[7] Food and Beverages Department, Food and Drug Administration, Ministry of Health and Medical Education, Tehran, Iran
[8] Food Safety Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Background and Objective: Essential oils include low solubility, poor bioavailability and rapid release, which may limit their use as bioactive compounds in foods and medicine. Nanoencapsulation can preserve inherent qualities of essential oils and improve their physicochemical characteristics and health benefits. Focus of the present study was on the loading of essential oils from *Zataria multiflora* in pickering nanoemulsions, nanoparticles and nanophytosome. In addition, the present study assessed how these systems affected their physicochemical characteristics and antioxidant and antimicrobial activities, compared to free-essential oils.

Material and Methods: Encapsulation of *Zataria multiflora* Boiss essential oil in nanocarriers as a novel phytoconstituents delivery system was carried out using three various methods. Physicochemical characterization of nanocarriers was studied using dynamic light scattering, Fourier transform infrared spectroscopy, field emission scanning electron microscope, confocal laser scanning microscopy, optical microscope and antioxidant activity. The minimum inhibitory and bactericidal concentration assessment effects against *Listeria monocytogenes* at 24 h and temperatures (10, 25 and 37 °C) were investigated. Encapsulated *Zataria multiflora* Boiss essential oil with subinhibitory concentrations (0.25, 0.5 and 0.75) in hamburger formulation was selected as a food model for chemical, microbiological and sensory evaluation.

Results and Conclusion: In general, this study compared three types of biocarriers with free essential oils. Primarily, nanophytosome showed promising results in delaying oxidation and in antimicrobial and sensory assessments, compared to two other nanocarriers. In conclusion, essential oil nanophytosomes of *Zataria multiflora* Boiss include the potential as an efficient natural food preservative.

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***Corresponding author:**

Hedayat Hosseini*

Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran,
Food Safety Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Tel: + 98 -9123007655
E-mail: hedayat@sbmu.ac.ir

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1. Introduction

Zataria multiflora Boiss, a Lamiaceae plant, is the major source of phenolic monoterpenes such as carvacrol and thymol, which include antibacterial activity against diverse pathogens [1]. However, their chemical and biological degradation, limited water solubility, volatility and instability in food storage and processing have made their use and development difficult [2]. Nanocarriers can prevent degradation of essential oils (EOs) and their bioactive compounds such as antimicrobials and antioxidants and control their release, increasing bioavailability and efficiency in natural food industries [3]. Pickering emulsions (PEs) stabilized by colloidal particles are surfactant-free and stable against coalescence, unlike conventional emulsion complicated structures [4]. Dispersed colloid particles form jointing layers thicker than surfactants on oil-water droplets. These are irreversibly anchored under static states, forming stable PEs [5].

In particular, PEs composed of protein particles are evolving as potential stabilizers in food industries due to surface activity, biocompatibility, high biodegradability and excellent nutritious values [6]. Zein as an amphiphilic in combination with a hydrophilic material such as chitosan, hydrogel, pectin nanoparticles (NPs) and gum Arabic (GA), results in materials that stabilize PEs, offering strong storage stability [7]. Technically, GA is an amphiphilic hydrocolloid with valuable characteristics that enable it to widely be used in food industries as an emulsion stabilizer at a wide pH range, providing stability in oil-in-water emulsions and high thermal resistance [8].

Phytosome is an emerging technique for vesicular drug delivery systems, which contains plant constituents surrounded and bound by at least one lipid molecule [9]. The major interaction that occurred during phytosome construction was the complex of hydrogen bonds between the plant components and the polar heads of phospholipids [10]. Thus, providing higher bioavailability than non-encapsulated EOs. Other benefits of nanophytosomes (NPHs), is their ability to increase the antimicrobial activity of EOs and their protection during heat treatment and storage [11].

Investigation for a potential inhibitory of foodborne pathogens has recently become a significant challenge and development of novel antibacterial agents is urgently needed to treat infections by these pathogens [12]. Juan Li et al. reported that NPs loaded with carvacrol inhibit growth of *Pseudomonas syringae* and *Fusarium oxysporum* with minimum inhibitory concentrations (MIC) of 135 and 270 µg ml^{-1}, respectively. Another study by [13] on preparation of Zein/GA stabilized PEs for antimicrobial activity against *Escherichia coli* showed excellent inhibition rates.

Significantly, use of antimicrobial activity EO-loaded NPHs as a novel method of encapsulation in food systems has less been studied. Innovation of the current study involves preparation of edible ZPNE, NPs and NPHs as promising carriers for improving *Zataria multiflora* EO (ZEO) characteristics and description of their physic-chemical characteristics. Furthermore, their appropriateness for antimicrobial activity was compared with that of normal EO using MIC and the minimum bactericidal concentration (MBC) methods. Additionally, capability of these compounds as support structures to sustain antibacterial effects, chemicals and sensory quality of nanocarriers in hamburgers (as a food model) was assessed.

2. Materials and Methods

2.1. Chemicals, cultures and microorganism

Zein (No. Z3625, Sigma-Aldrich, USA), GA (No. 1.04228, Sigma-Aldrich, USA), 99% phosphatidylcholine (PC) (Art.5331, Merck, Germany), rhodamin B (No. 620014, Merck, Germany), 96% ethanol (No. 1.00983, Merck, Germany), Tween 80 (No. 822187, Merck, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, USA), 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, USA), fluorescein isothiocyanate (FITC, No. Z3625, Sigma-Aldrich, USA) were used in this study. Olive oil was purchased from local markets in Tehran, Iran. ZEO was purchased from Tabib Daru, Kashan, Iran. For antibacterial assessments, *Salmonella-Shigella* agar, plate count agar, violet red bile glucose agar (VRBA), eosin-methylene blue agar, Baird-Parker agar, Mueller-Hinton agar, Mueller-Hinton broth, brain-heart infusion (BHI) broth and Sabouraud dextrose agar were purchased from Merck, Germany. *Listeria* (*L.*) *monocytogenes* ATCC 19112 was provided by the collection of Iranian microorganisms.

2.2. Analysis of Zataria multiflora Boiss essential oil using gas chromatography-mass spectrometry

Chemical composition of ZEO was assessed using gas chromatography (GC, model 7890) combined with mass spectrometry (MS, model 5975) system (Agilent Technologies, Santa Clara, CA, USA) fitted with an HP-5Ms capillary column of 30-m length, 250-µm diameter and 0.25-µm film thickness [14].

2.3. Fabrication of Zataria multiflora essential oil nanoparticles

The ZEO-GA-Zein NPs (ZGZNPs) were modified versions of an antisolvent sedimentation technology [13]. To achieve a zein-ethanol ratio of 1:20, zein (1 g) was first dissolved in 20 ml of ethanol 80% (v v^{-1}) and agitated for 60 min. zein- ZEO-ethanol stock was prepared by blending 10 ml of ZEO with 20 ml of zein-ethanol and stirring magnetically at 700 rpm for 40 min at ambient temperature. The GA stock solution was then prepared by combining 1 g of GA in 10 ml of DDW at a ratio of 1:10 for 90 min using magnetic stirrer. To prepare ZGZNP solution, zein- ZEO-ethanol stock (800 ml) and GA stock solution (300 ml) were quickly

injected into DDW (40 ml). Solution was then continuously stirred using magnetic stirrer under ambient temperature for 60 min. Then, pH of the mixtures was maintained at 4.0 during preparation using 0.5 mol l^{-1} HCl. Size of the solution first decreased using rotor-stator homogenizer (IP20, Germany) at 5,000 rpm for 5 min and then ultrasonic processor (CTchromtech-UP-100, Taiwan) for 5 min with 1-min breaks between the cycles.

2.4. Preparation of Zataria multiflora essential oil pickering nanoemulsion

In preparing NPs (ZNPs), half of the ZNPs was prepared as ZPNE using olive oil at 30% ratio. Olive oil was gradually added to the particle suspension using quick homogenizer at 10,000 rpm. Then, ZPNE was produced by homogenizing the mixture for further 5 min after the olive oil was completely included. The resulting solutions were stored at 4 °C for future analysis.

2.5. Preparation of Zataria multiflora essential oil nanophytosome

The NPHs were prepared using thin-layer hydration technique with a few minor modifications [15]. Briefly, ZEO and PC were encapsulated in equal quantities (2:2) and dissolved in 10 ml of 96% ethanol for 30 min. To set for potential interactions, this combination was maintained at 4 °C for 24 h. Organic solvents were eliminated from the mixture by vacuum-assisted rotary evaporator (Hahn shin Scientific, South Korea) at 50 °C, forming a thin layer that encircled the flask. Generally, 10 ml of DDW were used to hydrate the corresponding thin layer. Using homogenizer (Heidolph, Germany) set to 5,000 rpm for 5 min and probe sonicator (Heidolph, Germany) with 10 cycles of 2-min active time and 2-min rest, size of the phytosome decreased. The NPHs were stored at 4 °C for further analysis.

2.6. Particle size, polydispersity index and zeta potential assessments

Using dynamic light scattering (DLS) method, polydispersity index (PDI), particle size (PS) and surface charge study were calculated (Zeta sizer Nano ZS, Malvern Instruments, UK). All assessments were carried out at the quoted 25 °C.

2.7. Morphological characteristics

Morphology of the ZNPs and ZNPH was investigated using field emission scanning electron microscope (FESEM) (TESCAN MIRA 3 LMU, China). Nanostructure of ZPNE was imaged using CLSM (Leica TCS SPE, Germany). To stain, 1 ml of ZPNE and 40 µl of each color of FITC and rhodamine B (0.1% v v^{-1}) were added to a 1.5-ml tube and mixed well. Then, 20 µl of dyed ZPNE were transferred on the slide and covered with a coverslip. Sample was studied under fluorescence using objective lens of 20 and 40 and excitation and emission wavelengths of 488 and 633 nm. In addition, microstructures of the prepared ZPNE were studied using optical microscope (MEDLINB-CETI-MAGNUM, Algeria). Then, 10 µl of the sample were transferred onto a microscope slide and covered with a cover slip. Image was recorded at 10× (confocal). Appearance, sedimentation rate and biphasing of ZNPs, ZPNE and ZNPH were assessed after storing in refrigerator for 1, 15 and 30 d.

2.8. Fourier transform infrared spectroscopy

Fourier transform infrared spectrophotometer recorded the sample chemical structures (TENSOR 27, Bruker Optics, MA, USA). Each sample (2 mg) with calibration was carried out as a blank (200 mg). Spectra were captured between 4000-250 cm^{-1}. Wave number was affected by signal averaging of 32 scans and the scope was received as transmittance (%) versus wave number.

2.9. Encapsulation efficiency and loading capacity

Quantity of ZEO loaded into ZNPs, ZPNE and ZNPH was assessed using UV-visible spectroscopy (Shimadzu UV-VIS 1601, Japan). To estimate the maximum absorbance, calibration curve was prepared using various free ZEO concentrations ranging 20-200 µg ml^{-1}. At 275 nm, ZEO showed a sharp peak. For the preparation of NPs, 400 ml of the sample and 4 ml of 50% ethanol were centrifuged (Hettich Gmbh, Germany) at 9,000 rpm for 10 min under ambient temperature. After centrifugation, the solution serum layer was filtered using 8-layer silica filter [16]. To assess concentration of ZEO in the core oil droplets of PEs [17], a 500 µg ml^{-1} sample was diluted with 10 ml of 50% ethanol before setting into a sonication bath for 10 min to demulsify the produced emulsion. Then, fluid was centrifuged at 9,000 rpm for 15 min. The resulted serum layer was filtered through an 8-layer silica filter. After absorbance reading, the absorbance was replaced in the linear regression of the calibration curve using $Y = 76.049X + 25.79$ and $R^2 = 0.9638$. The equations 1 & 2 were used to calculated encapsulation efficiency (EE) and loading capacity (LC):

$$\text{EC}\ (\%) = \frac{\text{Total quantity of loaded ZEO} - \text{quantity of free ZEO}}{\text{Quantity of total ZEO used}} \times 100 \quad \text{Eq(1)}$$

$$\text{LC}\ (\%) = \frac{\text{Quantity of encapsulated ZEO}}{\text{Weight of total primary material dried}} \times 100 \quad \text{Eq (2)}$$

2.10. Antioxidant activity

In a nutshell, a 50-µl volume of various dilutions of each of the four samples was mixed with 500 µl in 99% ethanolic solution of DPPH (0.1 M), followed by 30 min incubation at 25 °C in dark. Then, sample absorbance was recorded against the control at 517 nm.

Antioxidant activity of the test samples in a concentration providing 50% inhibition was reported as IC_{50} (µg ml^{-1}) [18]. A lower IC_{50} value meant a higher antiradical activity. Each experiment was carried out in triplicate and data were present as a mean of the three values. The IC_{50} values were calculated using linear regression. The absorbance was replaced in $Y =$

1.98355x + 48.108 (R^2 = 0.8882), Y = 0.4999x + 10.695 (R^2 = 0.8674), Y = 0.3123x + 34.394 (R^2 = 0.7909), Y = 1.3655x - 12.693 (R^2 = 0.843) curves from ZEO, ZNPH, ZNPs and ZPNE, respectively. The equation (3) was used:

$$\text{Reduction } \% = \frac{Abs\ blank - Abs\ sample}{Abs\ blank} \times 100 \qquad \text{Eq (3)}$$

2.11. Antimicrobial activity

2.11.1. Minimum inhibitory concentration and minimum bactericidal concentration assessments

L. monocytogenes was refreshed in 10 ml of BHI broth at 37 °C for 24 h before antimicrobial susceptibility assays. Bacterial cell pellets were separated by centrifuging at 5,000 rpm for 10 min. Then, particles were redissolved in physiological serum after removing the supernatants. Suspensions were modified with 1×10^4 CFU ml^{-1} to assess MICs and MBCs using spectrophotometer. The MIC values of ZEO were reported using microbroth dilution method in 96-well plates, which were prepared by dissolving the ZEO in Tween 80 and adding Muller-Hinton broth until each serial concentration was reached. A total of 200 µl, 20 µl of ZEO suspension, 20 µl of bacterial cell suspensions (nearly 1×10^4 CFU ml^{-1}) and 160 µl of Muller-Hinton broth were transferred into each well of the 96-well plates. Plates were incubated at 10, 25 and 37 °C for 24 h. Moreover, TTC assay was used to investigate if bacteria were propagating or not. Each well received 20 µl of TTC (5 mg ml^{-1}) and plates were incubated for 30 min at the specific temperature [19]. Then, MICs and MBCs were calculated to include the lowest concentration of ZEO, which was able to inhibit development of red dye. The final assessment of the colorless wells was carried out on the plates containing Mueller-Hinton agar. All assays were carried out for ZNPs, ZPNE and ZNPH using 96-well plate microdilution method and then plates were incubated at 10, 25 and 37 °C for 24 h with positive and negative controls. Plates were then investigated using TTC assay.

2.12. Use of encapsulated Zataria multiflora essential oil in hamburger formulation

2.12.1. Hamburger preparation

Hamburgers were prepared in laboratory conditions according to Iranian national standard no. 2304. Hamburgers were prepared using a recipe that included 60% meat, 17.3% dehydrated onion, 1.2% salt, 1.5% spices, 8% wheat flour and 12% rusk flour. Raw materials were purchased from food supply stores in Tehran, Iran, and the samples were formulated.

2.12.2. Chemical and microbiological analyses of the formulated hamburgers

To assess antibacterial total count of the microorganisms and carry out chemical assays [pH, thiobarbituric acid index (TBA)], groups were formed. Treatments in this study included 1- hamburgers without EOs as control treatment; 2- hamburgers containing subMIC (0.25, 0.5 and 0.75) ZEO; 3- hamburgers containing subMIC (0.25, 0.5 and 0.75) ZNPs; 4- hamburgers containing subMIC (0.25, 0.5 and 0.75) ZPNEs and 5- hamburgers containing subMIC (0.25, 0.5 and 0.75) ZNPHs.

2.12.3. Sensory assessment of the formulated hamburgers

Hamburger samples were pan fried in oil at 100 °C for 20 min to assess their organoleptic quality. Then, random 3-digit number was used to code each sample. The 40-person trained panel was asked to rank the hamburger samples for general liking, taste, smell, color and texture using 5-point hedonic method. Between the assessments, the panel members were told to eat low-salt crackers and drink water to cleanse their palates [20].

2.13. Statistical analysis

Analysis of variance was carried out using SAS software v.9.3. Moreover, means were compared using Tucky's multiple range test at the significance level of $p \leq 0.05$. All functional characteristic experiments were carried out in three replicates and data for each experiment were reported as an average of three measurements.

3. Results and Discussion

3.1. Chemical components of Zataria multiflora Boiss essential oil

Table 1 enlists a total of 20 chemical compounds, which account for 98.79% of ZEO composition. The ZEO was mostly constituted of carvacrol (44.81%), thymol (17.76%) and spathulenol (9.110%). Chemical composition of ZEO in this study was similar to that of other studies; however, quantities of each component varied [21,22].

3.2. Characterization of ZEO-loaded nanocarriers

Based on the findings in Table 2, significant differences were reported in size and distribution of NPs within the samples ($p \leq 0.05$). However, size of NPHs was mildly smaller, which could be affected by various factors.

According to Khosh Manzar et al. [11], the NPH particle size decreased by increasing concentration of cumin EO, which was most likely due to the physicochemical characteristics of solubility, polarity and size of EO molecules. Probe sonication produced smaller particles than that homogenization did and combination of the two methods might result in even smaller particles with a transparent suspension [23]. As shown in Table 2, PDI was less than 0.9 in all samples, possibly due to the type of sonicator probe. Therefore, this element significantly affected physical stability of the nanocarriers and narrow size distribution was necessary for the stability over time [24]. A lower PDI (0.337) in NPHs suggested a monodispersed population.

Table 1. Chemical components of *Zataria multiflora* Boiss essential oil

Peak No	Compound	Retention time (min)	Percentage (%)
1	α-Pinene	6.20	0.10
2	β-Pinene	7.09	0.29
3	β-Myrcene	7.34	0.06
4	γ-Terpinene	8.09	5.08
5	Terpinolene	8.18	0.22
6	Carene	8.82	7.21
7	d-Limonene	9.46	0.06
8	Eucalyptol	9.50	0.07
9	p-cymene	9.69	0.87
10	α-Terpinene	10.01	2.83
11	Spathulenol	11.09	9.11
12	Carvacrol	11.29	44.81
13	Aromandrene	12.74	6.84
14	Thymol	13.73	17.76
15	Phenol	13.92	2.32
16	Caryophyllene	17.33	0.16
17	B-Limonene	20.19	0.07
18	Naphthalenone	23.19	0.22
19	Octasiloxane	25.23	0.581
20	Heptasiloxane	30.40	0.03

It seemed that the positive charge of the major component of ZEO interacted with the negative charge of the phosphate group in PC, providing a smaller negative charge to the produced NPHs. This led to improvements in NPH physical stability. In general, negative zeta values could preserve nanocarriers from sticking together, increasing their electrostatic repulsion [25]. Furthermore, the negative charge might increase interactions between the nanocarriers and the bacterial compounds, resulting in cell death.

Findings from the EE and LC values (Table 2) showed that ZEO concentration in NPH structure was associated to the high LC of NPHs (44.48%). The high EE (80.08%) might be associated to several factors, including manufacturing process, ZEO lipophilicity, interaction with lecithin and significant phytosome particle size ($p \leq 0.05$). However, it could be concluded that efficient encapsulation of the EO enclosed in the phytosome improved its antibacterial effects because released doses of the EO could be adjusted based on the bacterial pathogenicity, leading to the release of the minimum quantity of EO. Increased absorption and surface size resulted in a greater proportion of EE for ZPNE, compared to ZNPs. Therefore, zein and GA covered the olive oil and formed hydrogen bonds between ZEO and zein or GA in ZPNE. The high proportion of EE in ZPNE indicated that ZEO was encapsulated in GA and zein NPs. When the quantity of encapsulation increased, PE resistance to emulsification and oxidation increased, making it further stable in harsh environments [26]. Recent similar studies reported high EE in Pes, high EE (94.07%) in whey protein isolate nanofiber PEs [27] and PEs with loading efficiency and cinnamon EO retention ranging 79.49-81.13% [28]. These were similar to the composition of wall encapsulation materials and particle size characteristics. In the current study, EE% and LC% in ZNPs were lower than those in ZNPH and ZPNE (Table 2). However in other studies, EE of curcumin-loaded NPs was higher (94%) and encapsulated curcumin was further bioavailable, environmentally stable and antioxidant active [29]. By comparing the antioxidant characteristics, this study reported that NP was the lowest but relatively good for antibacterial characteristics.

3.3. Morphology of Zataria multiflora essential oil-loaded nanocarriers

Confocal laser scanning microscopy (CLSM), optical microscopy and FESEM were carried out to investigate morphological characteristics of the nanocarriers (Fig. 1). Olive oil droplets were green labeled with FITC (Fig. 1a). As shown, zein and GA complex NPs were red stained with rhodamine B (Fig. 1b). The ZPNE clearly formed a network structure (Fig. 1c). Zein and GA were integrally wrapped around the oil phase to establish stable oil-water interactions. These findings might be attributed to zein and GA, creating compact thick interface layers on the surface of spherical oil droplets which resulted in stringent barriers against coalescence for PEs [13]. Optical microscopy (Fig. 1d) could be used to study the droplet shape, size and emulsion state of ZPNE. As shown in Fig. 1d, emulsion droplets were dispersed with the lowest particle sizes with an oil concentration of 30%. The PE droplet sizes were homogenous and spherical. As a result, zein and GA NPs could be used to create Pickering high internal-phase emulsions [30]. Once the oil proportion reached a value of 0.3, droplets were uniformly distributed with no signs of agglomeration. At an oil fraction of 0.6, bridge flocculation phenomenon and occurrence of Ostwald ripening within excessive unstable oil droplets caused bigger droplets of the PEs. This occurred because the oil droplets became further unstable. In addition, FESEM analysis (Fig. 1) demonstrated the particle shape and surface morphology and verified particle sizes from DLS analysis. Vesicles of ZNPs (Fig. 1e) and ZNPH (Fig. 1f) included uniform spherical structures and were homogeneous in size and shape.

As seen in Fig. 2, a little changes were seen in size of the ZPNE droplets even after a storage time of 1 m and only a little aggregation occurred. It seemed that ZNPH included high degrees of storage stability against droplet aggregation, while ZNPs showed further improved stable potentials.

Table 2. Characterization of ZNPs, ZPNE and ZNPH

Nanocarrier	Particle size (nm)	Zeta Potential	PDI	EE (%)	LC (%)
ZPNE	100.2 ± 22.90	-12.3mv ±1.02	0.87	67.42± 1.40	48.71±2.40
ZNPs	80.6±39.00	-16.1mv ±1.24	0.68	55.74 ±2.11	45.741±2.22
ZNPH	60.2±30.90	-17.1mv ±1.33	0.33	80.08±2.01	56.48±2.02

ZEO Pickering Nanoemulsions (ZPNEs), ZEO Nanoparticles (ZNPs) and ZEO Nanophytosome (ZNPH). Data were presented as mean ± standard deviation (n>3).

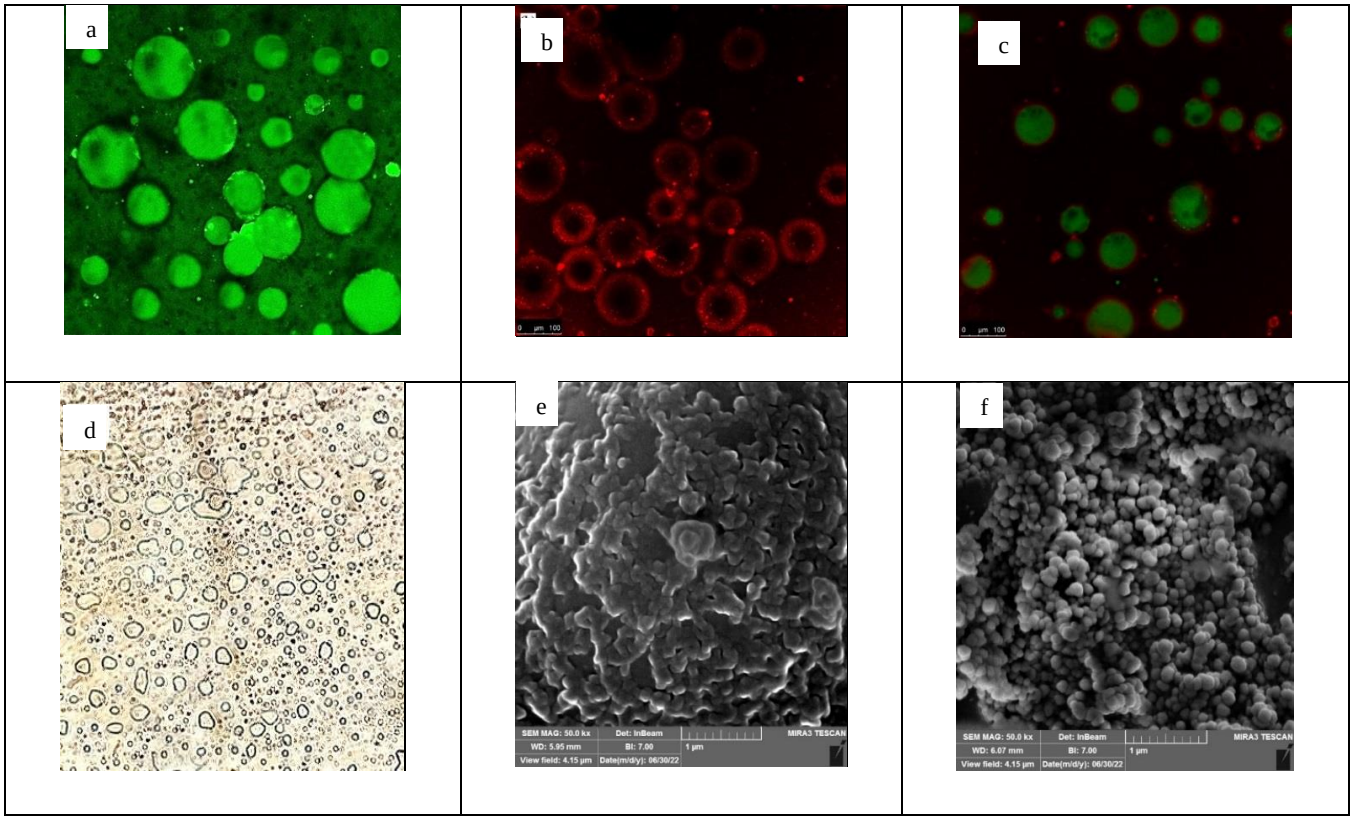

Figure 1. (a,b,c) CLSM and (d) optical microscope of the ZEO Pickering nanoemulsion (ZPNE). Scale bar is 100 µm and (e) FE-SEM of ZEO nanoparticles (ZNPs) and (f) FE-SEM of ZEO nanophytosome (ZNPH) scale bar is 1 µm.

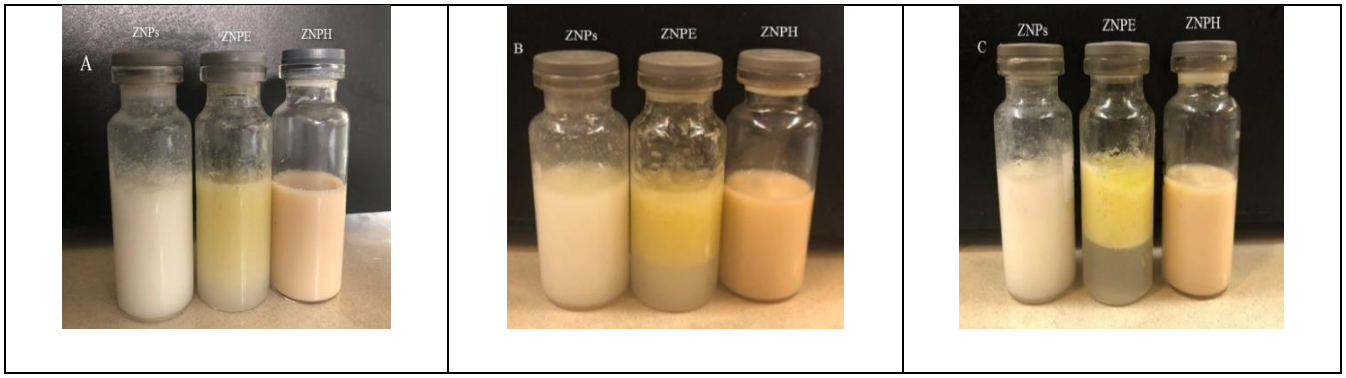

Figure 2. Effect of storage time on stability of ZNPs, ZPNE & ZNPH. (A) Fresh, (B) stored for 15 d; and (C) stored for 30 d.

3.4. Molecular interactions in Zataria multiflora essential oil-loaded nanocarriers

The characteristic peak in 3100-3400 cm^{-1} band (Fig. 3) was identified as hydrogen bond [31]. Hydrogen bond peaks in the spectra of ZPNE (Fig. 3a) were located at 3376.97 and 2959.51 cm^{-1}. Additionally, formation of hydrogen bonds between zein and GA was verified by changes in the peak of hydrogen bonds in the spectrum of ZPNE to 3011.59 cm^{-1}. The spectra were similar in ZNPH (Fig. 3b) and ZNP (Fig.3c) samples between 3354.60 and 2959.51 cm^{-1}. However, ZNPH demonstrated a broad peak at 3354.60 cm^{-1}, corresponding to free O-H group stretching of alcoholic esters, showing increases of hydrogen bands by NPH formation. Similar results were achieved by studying NPHs

containing cumin EO [11]. The peak at 3793 cm^{-1} was caused by binding of the polar head of lecithin to possibly active groups. Additionally, a peak at 1640.03 cm^{-1} might be linked to carbonyl (C=O) groups in the structure of lecithin [32].

Since ZEO is found in nanocarriers, peaks at 1453.66-1585.87cm^{-1} were due to asymmetric and symmetric stretching of C=C groups in esters and aromatic cyclohexenes compounds [33]. Peaks at 1617.66 cm^{-1} on ZPNE (Fig. 3a) and ZNPs (Fig. 3C) were respectively weak and sharp and matched to the carbonyl stretch of amides in proteins, which was caused by the presence of zein. Analysis of the zein spectra revealed two strong absorption peaks at 1658.17 and 1532.73 cm^{-1}, which were identified as amide I (C=O stretching) and amide II (C-N stretching) bonds, respectively [34]. Furthermore, peaks at 1230.02-1289.66 to 1088.38, 857.29–805.10 and 805.13 cm^{-1} (Fig. 3a, b, c) were caused by the stretching of alkane groups (CH_2), phenols and carbonyl groups (C=O). Another study discovered carbonyl groups, phenols and alkene-linked peaks in FTIR spectra of mace-arils manufactured NPs, supporting the current findings [35].

3.5. Antioxidant activity

The least scavenging activity against DPPH was detected in the free form of ZEO (IC_{50} = 147.283 µg ml^{-1}). Of the tested nanocarriers, ZPNE showed a low scavenging activity ((IC_{50} = 55.585 µg ml^{-1}). This slight antioxidant activity has previously been reported [12] whereas the highest value belonged to ZPNH (IC_{50}=35.69 µg ml^{-1}). In other studies, phytosome technology enhanced the antioxidant activity of rutin [36]; similar to that of the current study. Moreover, the antioxidant activity was seen in ZNPs (IC_{50}=50.007 µg ml^{-1}). Differences in the structure of phenolic compounds such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups and free carboxylic groups affected their functions [15].

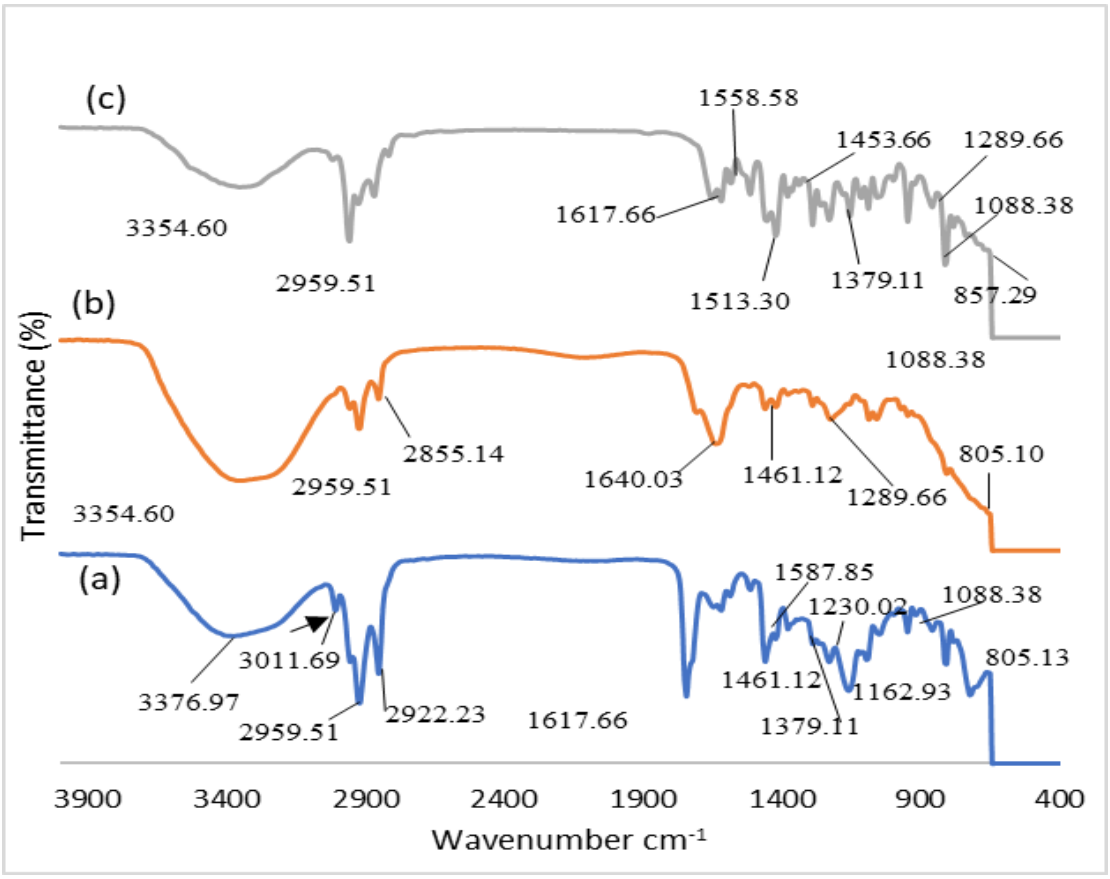

Figure 3. Fourier transform infrared spectroscopy spectra of (a) ZEO Pickering nanoemulsion (ZPNE), (b) ZEO nanophytosome (ZNPH) and (c) ZEO nanoparticles (ZNPs).

3.6. Antimicrobial analysis (minimum inhibitory concentration and minimum bactericidal concentration)

In this study, nanocarriers in combination with ZEO included stronger antimicrobial activities than those the pure ZEO did and the low temperature of 10 °C included significant contributions to the antimicrobial activity, compared to two other temperatures (Table 3).

In this study, ZNPH included the most inhibitory MIC at 37 °C (1.562 µl ml^{-1}) and 25 °C (0. 781 µl ml^{-1}) and ZNPs included the highest MIC at 10 °C (0. 390 µl ml^{-1}). Furthermore, ZEO included the lowest inhibitory effect at the

three temperatures. In another study, ZnO NPs and oregano EO were used as PEs on the film. Results showed exceptional antibacterial activity against *L. monocytogenes*. The inhibition rate increased to 89.61% [37]. Technically, size of NPs is a key factor in how antimicrobial agents are delivered to destroy pathogens. Results of a study by Memar *et al.* showed that decreasing size of the particles up to the nano-sized range made rutin more effective in killing bacteria [38]. As previously stated, MIC for nanocarriers at 10 °C is lower than that at 25 and 37 °C. Agglomeration of NPs at high temperatures included substantial agglomeration, resulting in bigger particle sizes, which prevented them from penetrating into the bacterial cell membranes and decreased their antibacterial effectiveness [39].

3.8. Analyses of chemical and microbiological parameters of the treated hamburgers

Initial pH values (Table 4) were in the range of 5.3–5.5 ($p \leq 0.05$). Generally, pH increased from the initial day until the end of storage. However, a slower increasing of pH was seen in hamburgers formulated with ZEO-loaded nanocarriers, compared to that in the control and ZEO ($p \leq 0.05$). A similar result was reported in fresh ground beef patties containing encapsulated ZEOs [40]. Increases in pH values might be resulted from the production of alkaline byproducts through the multiplication and stationary phases of microorganisms as well as deamination of proteins.

The slow trend of increasing pH for nanocarriers-treated samples could be associated to the inhibitory effects of phenolic compounds on bacterial growth and subsequently disintegration of amino compounds during the storage [41]. On Day 5, significant differences were observed between the treated samples and the control sample ($p \leq 0.05$). Sample with free EO showed significance with nanoencapsulated samples, indicating that they could partially prevent hamburger spoilage until Day 5 in refrigerator. On Day 10, no significant differences ($p > 0.05$) were seen between the samples of ZEO, ZPNE and ZNPs and the control sample; hence, significant differences could be seen in ZNPH in all three sub-MICs (0.25, 0,5, 0.75) ($p \leq 0.05$). It could be concluded that ZNPH included greater effects than those the other nanocarriers did in preventing spoilage on Days 5 and 10 at all the three sub-MICs (0.25, 0.5 and 0.75). It might be due to better inhibitory effects of EO in the form of ZNPH on microbial growth, which delayed formation of basic nitrogen compounds. Similar to those of this study, pH values were reported for the meat packaged with soybean films incorporated into cinnamon EO in other studies; for which, lower pH values were achieved by cinnamon EOs in the form of nanoemulsion [42]. Regarding TBA index, studies showed that aroma and flavor caused by oxidation began from 0.2 to 0.5 degrees and range of TBA changes within one year of meat storage varied between 0.47 and 6.47. In several cooked meat products such as sausages and raw hamburgers, greater values were reported and the index reached 9 in high-fat products, [43].

No significant differences were reported in ZEO samples after Day 1 of storage in refrigerator, compared to the control samples ($p > 0.05$). In other nanocarriers, significant decreases were reported in TBA index ($p \leq 0.05$). On Day 5 of analysis, increases were observed in the control and ZEO samples with no significant differences ($p > 0.05$). Increase in ZEO samples was low, compared to the control sample, revealing that antioxidant effects of EOs included the important antioxidant potential of thyme extract due to its high concentrations of phenolic compounds such as carvacrol, thymol and *p*-cymene [44].

Samples with nanocarrier demonstrated significant decreases, compared to the control samples and ZEO. On Day 10, no significant differences were reported between the control and ZEO samples ($p > 0.05$). Unlike effects of sub-MIC of 0.25, sub-MICs of 0.5 and 0.75 included significant differences in ZNP samples as well as decreases in TBA index and ZPNE samples; however, no significant differences were seen in all three subMICs ($p > 0.05$). In ZNPH, it caused significant decreases in TBA index in the three sub-MICs ($p \leq 0.05$). In Table 4, the total count of microorganisms was achieved on Day 10 for the control sample. Moreover, the lowest value was achieved for the ZPN treatment with sub-MIC of 0.75 and for ZPNE with the three sub-MICs (0.25, 0.5 and 0.75). on Day 1, significant differences were demonstrated between various times and treatments ($p \leq 0.05$).

Table 3. Minimum inhibitory concentration and minimum bactericidal concentration of ZEO, ZNPs, ZPNE and ZNPH against *L. monocytogenes* at 10, 25 and 37 ºC for 24 h.

ZEO						ZNPs					
MIC[a] (µl ml^{-1})			MBC[b] (µl ml^{-1})			MIC[a] (µl ml^{-1})			MBC[b] (µl ml^{-1})		
10 ºC	25 ºC	37 ºC	10 ºC	25 ºC	37 ºC	10 ºC	25 ºC	37 ºC	10 ºC	25 ºC	37 ºC
1.56	3.12	6.25	3.12	6.25	6.25	0.39	1.56	3.12	0.78	3.12	6.25
ZPNE						ZNPH					
MIC[a] (µl ml^{-1})			MBC[b] (µl ml^{-1})			MIC[a] (µl ml^{-1})			MBC[b] (µl ml^{-1})		
10 ºC	25 ºC	37 ºC	10 ºC	25 ºC	37 ºC	10 ºC	25 ºC	37 ºC	10 ºC	25 ºC	37 ºC
0. 78	1.56	6.25	1.56	3.12	6.25	0. 78	0. 78	1.56	1.56	1.56	3.12

MIC [a:] minimal inhibitory concentration, MBC [b]: minimal bactericidal concentration

Table 4. Mean comparison of the interactions between sub-MICs of ZEO, ZNPs, ZPNE and ZNPH at times on chemical and microbiological characteristics of the hamburger samples.

Treat-ment		Control	ZEO			ZNPs			ZPNE			ZNPH		
Level (µl ml^{-1})		0	0.25	0.5	0.75	0.25	0.5	0.75	0.25	0.5	0.75	0.25	0.5	0.75
pH	D1	5.50±0.02^{b}	5.42±0.05^{b}	5.42±0.01^{b}	5.32±0.01^{b}	5.45±0.01^{b}	5.41±0.01^{b}	5.33±0.02^{b}	5.31±0.01^{b}	5.33±0.01^{b}	5.37±0.03^{b}	5.20±0.03^{b}	5.54±0.04^{b}	5.50±0.04^{b}
Population ± SD	D5	5.80±0.02^{b}	5.66±0.02^{a}	5.52±0.01a	5.44±0.01^{a}	5.48±0.01^{a}	5.46±0.01^{a}	5.40±0.05^{a}	5.40±0.01^{a}	5.38±0.02^{a}	5.45±0.01^{a}	5.40±0.02^{a}	5.58±0.01^{a}	5.55±0.01^{a}
(log CFU m^{-1}) [a*]	D10	6.40±0.06^{ab}	6.30±0.01^{ab}	6.29±0.03^{ab}	6.28±0.04^{ab}	6.20±0.02^{ab}	6.19±0.03^{ab}	6.18±0.03^{ab}	6.17±0.03^{ab}	6.22±0.03^{ab}	6.23±0.017^{a}	6.11±0.02^{ab}	6.10±0.01^{b}	6.0±0.01^{ab}
TBA	D1	5.50±0.02^{b}	5.42±0.05^{b}	5.42±0.01^{b}	5.32±0.01^{b}	5.45±0.01^{b}	5.41±0.01^{b}	5.33±0.02^{b}	5.31±0.01^{b}	5.33±0.01^{b}	5.37±0.03^{b}	5.2±0.03^{b}	5.54±0.04^{b}	5.50±0.04^{b}
(mg MDA/kg)	D5	3.32±0.02^{a}	3.28±0.15^{a}	3.25±0.06^{a}	3.24±0.15^{a}	2.54±0.11^{c}	2.52±0.08^{c}	2.46±0.07^{cd}	2.3±0.07^{d}	2.44±0.05^{cd}	2.39±0.05^{cd}	2.56±0.10^{c}	2.52±0.07^{c}	2.50±0.08^{c}
	D10	3.62±0.02^{ef}	3.60±0.01d^{ef}	3.58±0.005^{e}	3.57±0.04^{e}	3.43±0.11^{bcde}	2.51±0.08^{bc}	2.39±0.05c^{d}	2.25±0.08^{f}	2.35±0.12d^{ef}	2.32±0.04^{ef}	2.51±0.02^{bc}	2.46±0.04^{bcd}	2.48±0.10^{bc}
Total count	D1	2.04±0.01^{a}	1.70±0.07^{bcd}	1.69±0.04^{cde}	1.72±0.02^{bc}	1.76±0.04^{bc}	1.72±0.01^{bc}	1.62±0.05^{e}	1.78±0.03^{b}	1.77±0.03^{b}	1.72±0.02^{bc}	1.47±0.04^{cde}	1.40±0.03^{de}	1.42±0.03^{e}
Population ± SD	D5	2.37±0.07^{a}	1.80±0.05^{b}	1.77±0.03^{bc}	1.78±0.02^{bc}	1.71±0.08^{bcde}	1.64±0.04^{efg}	1.55±0.06^{g}	1.76±0.02^{bcd}	1.75±0.04^{bcd}	1.68±0.02^{cdef}	1.66±0.05^{def}	1.59±0.05^{gf}	1.58±0.06^{gf}
(log CFU ml^{-1})	D10	2.45±0.04^{a}	1.91±0.02^{b}	1.90±0.02^{b}	1.83±0.03^{bc}	1.69±0.08^{ed}	1.63±0.04^{ef}	1.41±0.03^{g}	1.76±0.03^{cd}	1.75±0.04^{cd}	1.65±0.04^{ef}	1.63±0.07^{ef}	1.56±0.06^{gf}	1.56±0.05^{gf}
Yeasts and molds	D1	-	-	-	-	-	-	-	-	-	-	-	-	-
Population ± SD	D5	1.13±0.04^{a}	0.59±0.0.1^{bc}	0.41±0.10^{bc}	0.51±0.07^{bc}	0.59±0.11^{bc}	0.51±0.07^{cd}	0.20±0.17^{e}	0.59±0.11^{bc}	0.41±0.04^{cd}	0.41±0.10^{de}	0.25±0.24^{bc}	0.66±0.05^{bc}	0.56±0.07^{cd}
(log CFU ml^{-1})	D10	1.78±0.07^{a}	1.24±0.14^{b}	1.14±0.09^{bc}	1.07±0.03^{cd}	1.10±0.03^{cd}	1.11±0.05^{cd}	1.04±0.03^{cd}	1.04±0.03^{cd}	1.06±0.02^{cd}	1.06±0.02^{cd}	1.06±0.02^{cd}	1.06±0.05^{d}	1.01±0.02^{e}
Salmonella	D1	-	-	-	-	-	-	-	-	-	-	-	-	-
Population ± SD	D5	0.20±0.17^{a}	-	-	-	-	-	-	-	-	-	-	-	-
(log CFU ml^{-1})	D10	0.75±0.04^{a}	0.35±0.10^{bc}	0.47±0.01^{b}	0.35±0.10^{bc}	0.30±0.01^{bc}	0.25±0.24^{cd}	0.35±0.10^{bc}	0.30±0.01^{e}	0.35±0.10^{cde}	0.10±0.17^{d}	0.30±0.01^{bc}	0.30±0.01^{bc}	0.30±0.01^{bc}
Coliform	D1	-	-	-	-	-	-	-	-	-	-	-	-	-
Population ± SD	D5	-	-	-	-	-	-	-	-	-	-	-	-	-
(log CFU ml^{-1})	D10	-	-	-	-	-	-	-	-	-	-	-	-	-
S. aureus	D1	-	-	-	-	-	-	-	-	-	-	-	-	-
Population ± SD	D5	0.46±0.15^{ab}	0.59±0.11^{a}	0.47±0.01^{ab}	0.20±0.17^{de}	0.41±0.10^{abc}	0.20±0.17^{cde}	0.40±0.10^{abc}	0.41±0.10^{abc}	0.35±0.10^{bc}	0.10±0.17^{cde}	0.35±0.10^{bc}	0.41±0.10^{abc}	0.30±0.01^{bcd}
(log CFU ml^{-1})	D10	0.86±0.03^{a}	0.80±0.03^{a}	0.66±0.05^{b}	0.47±0.01^{e}	0.60±0.01^{bc}	0.41±0.10^{e}	0.47±0.01^{de}	0.56±0.07^{bcd}	0.66±0.10^{b}	0.66±0.05^{de}	0.63±0.05^{b}	0.63±0.05^{b}	0.51±0.07^{cde}
E. coli	D1	-	-	-	-	-	-	-	-	-	-	-	-	-
Population ± SD	D5	-	-	-	-	-	-	-	-	-	-	-	-	-
(log CFU ml^{-1})	D10	-	-	-	-	-	-	-	-	-	-	-	-	-

[a*] Populations in represent the number of microorganisms out of the number analyzed in three replicates trials ($p \leq 0.05$). TBA; Thiobarbituric acid, MDA; Malondialdehyde, ZEO; *Zataria multiflora* EO, ZPNE; ZEO Pickering Nanoemulsion, ZNPH; ZEO Nanophytosome and ZNPs; ZEO Nanoparticles

Based on a report, pads derived from emulsions containing EOs of thyme slightly induced (0.3-0.8 Log 10 CFU g^{-1}) with the reproducible decreases of microbiota in minced meats and hamburgers stored for 12 and 15 days at 4°C [45]. On Day 1, yeasts and molds were not seen in the treatments and the highest number of the microorganisms was achieved on Day 10 for the control sample. However, the lowest number of the microorganisms was reported for ZPN treatments with sub-MIC of 0.75 on Day 1. Furthermore, significant differences were reported between various times and treatments ($p\leq0.05$). On Day 1, no *Salmonella* was observed in the treatments and the control sample. On Day 5, no *Salmonella* was observed in treatments of the nanocarriers. The highest number was reported for the control sample on Day 10 and the lowest number for the ZPNE treatment with sub-MIC on 0.75 on Day 10. Moreover, significant differences were seen between various times and treatments ($p\leq0.05$). In this study, antimicrobial activity of the active films on ground beef samples with the thymol-loaded nanoemulsions carriers was assessed, including the longest protection against the microorganisms [46]. On Day 1, *Staphylococcus aureus* was not detected in the control samples. On Day 5, ZEO with sub-MIC of 0.75, ZPN with sub-MIC of 0.75, ZPNE with sub-MICs of 0.5 and 0.75 and ZNPH with sub-MICs of 0.25, 0.5 and 0.75 included the minimum numbers of *Staphylococcus aureus* with significant differences ($p\leq0.05$). Unlike 0.25 sub-MIC of ZEO, significant differences were recorded between various treatments on Day 10 ($p\leq0.05$).

3.9. Sensory analysis of the treated hamburgers

Based on Fig. 4, the highest score in color of the samples belonged to ZNPH between all the treatments and the lowest score belonged to the ZEO sample followed by the control with no significant differences ($p>0.05$). The highest score belonged to the flavor of samples treated with ZNPs and ZNPH without significant differences and the least score to ZEO samples with no significant differences between the control and nanocarriers ($p>0.05$). In other words, use of nanocarriers even in higher concentrations could positively affect flavor of the produced hamburgers due to the encapsulation of EO processed with nanocarriers that includes a better flavor, compared to the control sample [47]. The highest score of the appearance of the samples in all treatments belonged to ZNPs at sub-MICs of 0.5 and 0.75 and the lowest score belonged to the control sample ($p>0.01$). In other words, use of NPs in hamburgers could better affect appearance of the hamburgers, compared to the control sample. The lowest score of taste in all samples belonged to ZEO treatments and the highest score to ZNPH treatments. Additionally, no significant differences were observed between the treatments containing EO and nanocarriers and the control treatment. In other words, use of ZNPH and their higher consumption levels could positively affect improvement and increase of taste score of the samples. Generally, ZNPH traps flavor compounds in its structure. Therefore, improvement of the taste of samples might be due to the entrapment of the highlighted compounds in their matrices. Treatment of nanocarriers in all sub-MICs included the highest overall acceptance score with significant differences with the control and ZEO treatments ($p\leq0.05$). Results were similar to the results of the studies that reported that use of nanocarriers increased the shelf life, improved the aroma and smell and caused the overall acceptance [48].

4. Conclusion

In this study, ZEO with three various types of preparation methods was developed. In general, ZEO Pickering nanoemulsion and ZEO NPs stabilized by ZGZNPs and ZEO NPHs were successfully fabricated to enhance their antibacterial ability. Systems showed stability during storage and controlled release effects of the antibacterial activity. The system with antimicrobial activity against *L. monocytogenes*, one of the most widespread foodborne pathogens, showed potentials as a possible natural food preservative. Based on the current findings, ZEO nanocarriers (especially ZEO NPHs) can effectively include appropriate physicochemical characteristics, particularly in meat food products such as hamburgers.

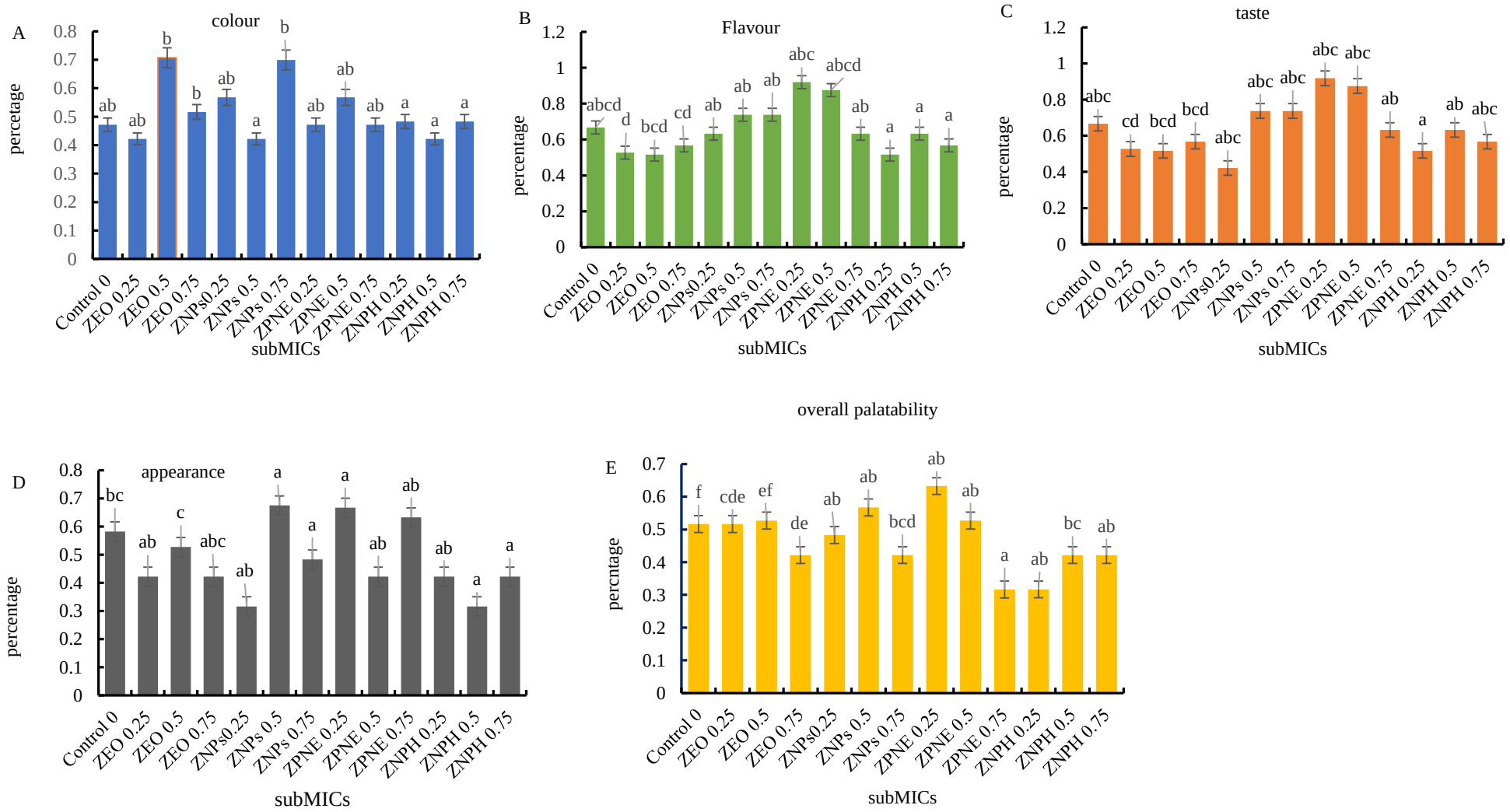

Figure 4. Effects of sub-MICs of ZEO, ZNPs, ZPNE and ZNPH on sensory characteristics of the hamburger samples

5. Acknowledgements

The Ethics Committee of the National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, approved the study (ethical code: IR.SBMU.nnftri.Rec.1400. 089).

6. Conflict of Interest

The authors report no conflicts of interest.

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نانوذرات بیوپلیمری، نانومولسیون های جمع کننده و نانوفیتوزوم ها برای بارگیری اسانس آویشن شیرازی[1] به‌عنوان نگهدارنده زیستی

منصوره تقی‌زاده[1]، سید مهدی جعفری[2، 3 و 4]،کیانوش خسروی دارانی[5]، محمود علیزاده ثانی[6]، سعیده شجاعی-علی‌آبادی[1]، نادر کریمیان خسروشاهی[7]، هدایت حسینی[1و8*]

۱- گروه علوم و صنایع غذایی، انستیتو تحقیقات تغذیه‌ای وصنایع غذایی کشور، دانشکده علوم تغذیه و صنایع غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران
۲- گروه مهندسی طراحی مواد غذایی و فرآیند، مهندسی طراحی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران
۳- دانشگاه د ویگو، گروه تغذیه و بروماتولوژی، گروه شیمی تجزیه و علوم غذایی، دانشکده علوم، E-32004 اورنس، اسپانیا
۴- کالج علوم و صنایع غذایی، دانشگاه کشاورزی هبی، بائودینگ، ۰۷۱۰۰۱، چین
۵- گروه تحقیقات صنایع غذایی، انستیتو تحقیقات تغذیه‌ای وصنایع غذایی کشور، دانشکده علوم تغذیه و صنایع غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران
۶- گروه ایمنی و بهداشت مواد غذایی، گروه مهندسی بهداشت محیط، دانشکده بهداشت، دانشگاه علوم پزشکی تهران، تهران، ایران
۷- گروه مواد غذایی و نوشیدنی‌ها، سازمان غذا و دارو، وزارت بهداشت درمان و آموزش پزشکی، تهران، ایران
۸- مرکز تحقیقات ایمنی مواد غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران

چکیده

سابقه و هدف: اسانس‌های روغنی حلالیت کم، زیست‌فراهمی ضعیف و رهایش سریع دارند که می‌تواند کاربرد آنها را به عنوان ترکیبات زیست فعال در مواد غذایی و داروها محدود کند. نانوریزپوشانی[2] می‌تواند کیفیت ذاتی اسانس‌های روغنی را حفظ کند و ویژگی‌های فیزیکوشیمیایی و مزایای آنها را بهبود بخشد. مطالعه حاضر بر بارگذاری اسانس روغنی آویشن شیرازی در نانومولسیون جمع کننده ، نانوذرات و نانوفیتوزوم متمرکز بود. علاوه بر این، مطالعه حاضر چگونگی تاثیر این سامانه‌ها بر ویژگی‌های فیزیکوشیمیایی و فعالیت‌های ضداکسایشی و ضد میکروبی آن‌ها در مقایسه با اسانس روغنی آزاد را بررسی می‌کند.

مواد و روش ها: ریزپوشانی اسانس‌روغنی آویشن شیرازی به‌عنوان سامانه‌ای نوین انتقال ترکیبات گیاهی با استفاده از سه روش گوناگون انجام شد. خصوصیات فیزیکوشیمیایی نانوحامل‌ها با استفاده از پراکندگی دینامیکی نور، طیف بینی مادون قرمز تبدیل فوریه[3]، میکروسکوپ الکترونی روبشی گسیل میدانی، میکروسکوپ روبشی لیزری هم‌کانونی[4]، میکروسکوپ نوری و فعالیت آنتی اکسیدان مورد مطالعه قرار گرفت. اثرات تعیین حداقل غلظت بازدارندگی و حداقل غلظت کشندگی در برابر *لیستریا مونوسیتوژنز* در ۲۴ ساعت و درجه‌حرارت‌های (۱۰°C، ۲۵ و ۳۷) مورد بررسی قرار گرفت. اسانس‌روغنی ریزپوشانی آویشن شیرازی غلظت‌های زیربازدارنده (۰/۲۵، ۰/۵۰ و ۰/۷۵) در فرمولاسیون همبرگر به عنوان مدل غذایی برای ارزیابی ویژگی‌های شیمیایی، میکروبیولوژیکی و حسی انتخاب شد.

یافته‌ها و نتیجه‌گیری: به‌طور کلی، این مطالعه سه نوع حامل زیستی را با اسانس آزاد مقایسه کرد. نانوفیتوزوم در مقایسه با دو نانوحامل دیگر، نتایج امیدوارکننده‌ای را در فعالیت های آنتی اکسیدانی ، ارزیابی‌های ضد میکروبی و حسی نشان داد. در نتیجه، نانوفیتوزوم‌های اسانس آویشن شیرازی دارای پتانسیل کارآمد به عنوان یک نگهدارنده طبیعی در مواد غذایی هستند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- نانوحامل‌های نوظهور
- نانوفیتوزوم
- نانومولسیون های جمع کننده۵
- تجزیه و تحلیل شیمیایی
- عمرانباری
- فرآورده‌های گوشتی

*نویسنده مسئول

هدایت حسینی
گروه علوم و صنایع غذایی، انستیتو تحقیقات تغذیه‌ای وصنایع غذایی کشور، دانشکده علوم تغذیه و صنایع غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران، مرکز تحقیقات ایمنی مواد غذایی، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران
تلفن: ۹۱۲۳۰۰۷۶۵۵-۹۸+
پست الکترونیک:
hedayat@sbmu.ac.ir

[1] *Zataria multiflora*
[2] Nanoencapsulation
[3] Fourier transform infrared spectroscopy or FTIR
[4] Confocal laser scanning microscopy
[5] Pickering