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The Antimicrobial Efficacy of Chitosan Nanoemulsion Coating Incorporating *Ziziphora Clinopodioide* Essential Oil and Nisin against *Listeria Monocytogenes* Inoculated in Vacuum-Packed Chicken Breast Fillet

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ABSTRACT

Background: The present study aimed to determine the chemical composition and antibacterial activity of essential oil extracted from *Ziziphora clinopodioide* (ZCEO) *in vitro*. Furthermore, the study aimed to evaluate the effectiveness of chitosan nanoemulsion coating containing ZCEO and nisin in inhibiting the growth of *Listeria monocytogenes* inoculated on chicken breast fillets that were inoculated and vacuum-packaged, and stored at a temperature of 4±1 °C for 16 days.

Methods: The chemical composition and *in vitro* antibacterial activity of ZCEO were determined by gas chromatography-mass spectrometry (GC-MS) and microdilution method, respectively. The chicken breast fillet samples were divided into seven groups, namely, the control group (uncoated) without vacuum, control group with vacuum, chitosan nanoemulsion without vacuum, chitosan nanoemulsion with vacuum, chitosan nanoemulsion with 0.5% ZCEO and vacuum, chitosan nanoemulsion with nisin and vacuum, and chitosan nanoemulsion with 0.5% ZCEO, nisin, and vacuum. All the samples were stored in a refrigerator, and the population of *L. monocytogenes* was enumerated on days 0, 1, 2, 4, 8, 12, and 16.

Results: the chemical analysis of ZCEO revealed that carvacrol, thymol, and p-cymene were the three main compounds present in the oil. Application of chitosan nanoemulsion coatings, specifically chitosan nanoemulsion + ZCEO 0.5% + vacuum and chitosan nanoemulsion + nisin + vacuum, resulted in a significant reduction in the growth rate of *L. monocytogenes* in chicken breast fillet samples during storage. In addition, the chitosan nanoemulsion coating containing the combined ZCEO and nisin was found to be more effective in reducing the growth of *L. monocytogenes* during the storage period.

Conclusion: Based on the results of this study, chitosan nanoemulsion + ZCEO 0.5% + nisin + vacuum coating can be used to reduce the risks that might be associated with *L. monocytogenes* in chicken breast fillets.

1. Introduction

Foodborne illnesses resulting from the consumption of products contaminated with *Escherichia coli*, salmonella, and

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listeria species, pose a significant threat to public health, causing fatalities and economic losses each year. Foods of animal origin, including poultry, are a valuable source of essential nutrients for human consumption, and their consumption is consistently increasing globally [1]. Poultry meat, in particular, is highly regarded for its affordability, availability, and nutritional value. It is an excellent source of high-quality protein, essential amino acids, B complex vitamins, and unsaturated fatty acids, while also exhibiting low levels of fat and cholesterol [2]. Despite its nutritional benefits, poultry meat is highly perishable and vulnerable to both bacterial and chemical spoilage. Multiple factors, such as gut and skin microbiota, water, utensils, and air contribute to the contamination of bird carcasses. Microorganisms, especially bacteria, can degrade chicken protein by secreting proteolytic enzymes, thereby releasing ammonia and volatile nitrogen, which can lead to spoilage [3]. *Listeria monocytogenes* is a gram-positive, rod-shaped, facultative anaerobic bacterium that cannot produce spores. Among the 6 species of Listeria, *L. monocytogenes* is recognized primarily as a human pathogen [4, 5]. This bacterium is capable of surviving in harsh environmental conditions such as low pH, high salt concentration, and low temperatures. *L. monocytogenes* is both a pathogenic and opportunistic organism, with global significance due to its ability to cause food-borne infections, particularly in meat, poultry, and dairy products as well as gastrointestinal diseases such as listeriosis [6]. Chitosan is a biocompatible polymer derived from natural renewable sources, and it has diverse applications in many fields, including the production of edible films and coatings. Chitosan has antibacterial and antifungal properties that are useful for food preservation [7]. The antimicrobial activity of chitosan is influenced by several factors, including the degree of acetylation and polymerization, the type of bacteria, and environmental conditions such as temperature, pH, and the composition of food nutrients [8]. The antimicrobial and other beneficial properties of plant essential oils have been proven and widely used since ancient times. In the food industry, these oils have become increasingly popular as preservatives, owing to the growing consumer preference for natural coatings and additives. Among the essential oils widely used for preserving food and leveraging their antimicrobial and anti-pathogenic properties is *Ziziphora clinopodioide* essential oil (ZCEO), which belongs to the Lamiaceae family. *Ziziphora clinopodioide* is an annual herbaceous plant with a short stem, 5-15 cm in height, and pointed and narrow leaves, that is distributed across various regions of Iran. The components of *Ziziphora clinopodioide* demonstrate anti-tumor properties, with the potential to reduce the growth of malignant tumors by 32.6%. Despite the widespread use of mint family plants as flavoring agents in Iran, they are not yet extensively utilized for food preservation and inhibiting spoilage microorganisms [9]. Preservatives of microbial origin are another type of natural preservative that are used to preserve food quality. Nisin, a bacteriocin produced by *Lactococcus lactis*, exhibits antibacterial activity against a wide broad range of gram-positive bacteria and some gram-negative bacteria [10]. The United States Food and Drug Administration (FDA) has recognized nisin as Generally Recognized as Safe (GRAS). Owing to its potent antibacterial activity against a broad spectrum of pathogens, nisin is used as a preservative in different food products, including meat, dairy, and aquatic products [11-13]. A nanoemulsion is an oil-in-water emulsion with droplet sizes ranging from 100-600 nm, making it also known as a miniemulsions. The small droplet size of nanoemulsions allows for even distribution on the surface and facilitates the connection of active components in the protective layer [14, 15]. Nanoemulsions are used as delivery systems for lipophilic compounds, such as nutrients, drugs, flavors, antioxidants, and antimicrobial agents [16]. Vacuum packaging is a highly effective system for the distribution and long-term storage of fresh meat, as it eliminates substantially all the air before sealing the package. This type of packaging can decrease fat oxidation and bacterial growth [17]. There have been numerous studies concentrating on the application of chitosan nanoemulsion coating in food preservation [18]. Moreover, a few studies have investigated the effect of chitosan nanoemulsion containing ZCEO against food-borne pathogenic in food models [19]. However, to the best of our knowledge, no study has been conducted on the efficacy of chitosan nanoemulsion coating containing ZCEO and nisin against food-borne pathogens in chicken meat. Therefore, this study was designed to evaluate the effectiveness of chitosan nanoemulsion coating containing ZCEO and nisin against *L. monocytogenes* bacteria in vacuum-packed chicken breast fillets.

2. Materials and Methods

The ZCEO used in this study was obtained from the Academic Jihad Research Center in Karaj, Alborz Province, Iran. The *Ziziphora clinopodioide* plants were collected from the mountains of Bojnord city in North Khorasan province, Iran. Chitosan with a low molecular weight with 91% deacetylation was purchased from Sigma-Aldrich (St. Louis, USA). All culture media were purchased from Merck (Darmstadt, Germany), while nisin and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Four reference strains of *L. monocytogenes* (ATCC: 7644, 19115, and 19111 and CIP: 7834) were obtained from the Department of Food Hygiene, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

2.1 Chemical composition of ZCEO

The present study employed gas chromatography-mass spectrometry (GC-MS) to identify the components of ZCEO. The GC analysis was performed by a gas chromatograph (Agilent 6890, Swindon, UK) equipped with an HP-5MS capillary column (30 × 0.25 mm ID × 0.25 mm film thickness). The temperature program involved an initial temperature of 50 °C, followed by a temperature ramp of 5 °C per min, and a final temperature of 240 °C min to 300 °C (holding for 3 min), with an injector temperature of 290°C. Helium was used as the carrier gas, and the split ratio was set at 0.8 mL/min. The

ZCEO sample was subjected to gas chromatography-mass spectrometry (Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass selective detector; Agilent, Swindon, UK) to reconfirm the results of the previous step. The mass spectrometer was run in electron-ionization mode with an ionization energy of 70 eV and a library of Wiley-VCH 2001, Weinheim, Germany [20] was employed.

2.2 Preparation of L. monocytogenes bacteria

Four reference strains of *L. monocytogenes* were used in this study. All strains were incubated in brain heart infusion (BHI) broth at 37 °C for 18 h. After incubation, a volume of 1 mL from the 18-hour culture was transferred to 10 mL of sterile BHI broth and incubated again at 37 °C for 24 h. The bacterial suspension was obtained from 24-h culture, and its turbidity was adjusted to 0.5 McFarland turbidity standard, which contained 1.5×10^8 CFU/mL. Further, the suspension was diluted in a 1:10 ratio to reach 1.5×10^7 CFU/mL [20].

2.3 Antimicrobial Activity of emulsion and nanoemulsion of ZCEO

2.3.1 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The microdilution method was used to evaluate the antibacterial activity of the emulsion and nanoemulsion of ZCEO. First, 80 mg of ZCEO was weighed and 40 mg of it was dissolved in 2 mL of tween 80 to prepare an emulsion with a concentration of 20 mg/mL. The remaining 40 mg was mixed with 2 mL of tween 80, and subjected to Ultra Turrax for 3 min at 3000 rpm, followed by ultrasonic emulsification sonicator (power: 750 W, pulse; 45 s and rest; 15 s) for 6 min to obtain a nanoemulsion with a concentration of 20 mg/mL [14]. Then both types of essential oils were serially diluted (1:2) to obtain a concentration range of 20-0.156 mg/mL in sterile distilled water. To each well of the microplate, 160 µL of BHI broth, 20 µl of emulsion or nanoemulsion of ZCEO, and 20 µL of bacterial suspension were added, with the concentration of emulsion or nanoemulsion of ZCEO ranging from 0.156-20 mg/mL. Positive control wells contained 180 µL of BHI broth and 20 µL of bacterial suspension, while negative control wells contained 180 µL of BHI broth and 20 µL of emulsion or nanoemulsion of ZCEO. The microplate was then incubated at 37 °C for 24 h, and the minimum inhibitory concentration (MIC) was determined as the lowest concentration of each type of essential oil that inhibited bacterial growth. The minimum bactericidal concentration (MBC) was determined by inoculating 10 µLfrom wells with no visible bacterial growth on BHI agar using the drop method and incubating at 37 °C for 24 h. The minimum concentration without any bacterial growth was regarded as the MBC represented as [21].

2.4 Preparation of coating solutions

In this study, an edible coating was prepared using chitosan. To prepare a 2% chitosan solution, 2 g of chitosan powder was mixed with 100 mL of 1% aqueous acetic acid solution and stirred for 10 min. Then, glycerol (1.5% w/v) was added as a plasticizer, and the pH of the solution was adjusted to 5.9 using 0.1 normal sodium hydroxide solution. In the next step, ZCEO (at a concentration of 0.5% v/w) and Tween 80 (0.2% of ZCEO) were added to the chitosan solution and mixed. In addition, a stock solution of nisin was prepared by dissolving 50 mg of nisin powder in 5 mL of hydrochloric acid (0.02 mol/L), and then, 200 IU/g of nisin was added to the chitosan solution. The coating solutions were homogenized using an Ultra Turrax for 3 min at 3000 rpm and then subjected to an ultrasonic emulsification sonicator (power sonic 505 W, 50 °C, pulse; 45 s and rest; 15 s) for 6 min to form nanoemulsion [22].

2.5 Preparation of treatments

Chicken breast fillets were bought from a daily market and instantly transported in insulated polystyrene ice flasks to the laboratory. The fillets were divided into 10 g samples and immersed in 70% ethanol for 3 min to eliminate the natural microbial flora. The samples were then placed on sterile plates to allow the ethanol to evaporate. Subsequently, 100 µL of a bacterial cocktail containing 4 strains of *L. monocytogenes* at a concentration of 10^7 CFU/mL was inoculated onto the surface of the fillets to gain a final concentration of 10^5 CFU/g. The inoculated samples were immersed in the prepared coating solutions (Table 1) for 3 min and drained. Except for the Con and Nano CH groups, all samples were vacuum-packaged using a packaging machine (Henkelman 200 a, Helmond, Netherlands) and stored at 4±1 °C for microbiological analyses on days 0, 1, 2, 4, 8, 12, and 16.

Table 1: Treatments used in this study for chicken breast fillets inoculated with *L. Monocytogenes* cocktail

Row	Treatments	Description
1	Con	Uncoated chicken breast fillets
2	Con + Vac	Uncoated chicken breast fillets with vacuum
3	Nano CH	Chicken breast fillets coated with 2% chitosan nanoemulsion
4	Nano CH + Vac	Chicken breast fillets coated with 2% chitosan nanoemulsion and vacuumed
5	Nano CH + ZCEO + Vac	Chicken breast fillets coated with 2% chitosan nanoemulsion containing 0.5% (w/v) ZCEO and vacuumed
6	Nano CH + N + Vac	Chicken breast fillets coated with 2% chitosan nanoemulsion containing nisin 200 IU/g and vacuumed
7	Nano CH + ZCEO + N + Vac	Chicken breast fillets coated with 2% chitosan nanoemulsion containing 0.5% (w/v) ZCEO and nisin 200 IU/g and vacuumed

2.6 Microbial analysis

To determine the microbial count, each sample was mixed with 90 mL of 0.1% peptone water using a stomacher (Seward Medical, London, UK) for 2 min. Then, 100 µL of the prepared suspension was taken and serially diluted (1:10) using 0.1%

peptone water. Appropriate dilutions were cultured by surface method on plates containing Palcam agar for the enumeration of *L.monocytogenes*, and incubated at 37 °C for 24-48 h. The microbial count was expressed as the logarithm of the number of colony-forming units per gram (log CFU/g).

2.7 Statistic analysis

The data, obtained in triplicate, were expressed as the mean ± standard deviation (Mean ± SD). The trend of changes in the logarithm of the number of bacteria in different groups during the 16-day period was statistically analyzed by repeated measure ANOVA. All statistical analyses were performed by SPSS (version 21) and a significance level of $P < 0.05$ was considered significant.

3. Results and Discussion

3.1 Chemical composition of ZCEO

According to Table 2, 18 compounds were identified in the ZCEO by the GC-MS method. These compounds accounted for 99.65% of the total ZCEO. The most abundant compounds in ZCEO were carvacrol (65.2%), thymol (19.5%), and p-cymene (4.8%). This finding is in agreement with the result of Shavisi et al. (2017) and Shahbazi et al. (2016) who also identified cavacrol, thymol, and p-cymene as the three main compounds of ZCEO [23, 24]. However, other studies by Baygan et al. (2022), Aghababaei et al. (2021), and Khanzadi et al (2019) reported that the main ingredient in ZCEO was pulegone [25-27]. Similarly, Shakour et al (2021) found that thymol was the main component of ZCEO [28]. The differences in the amount and type of essential oil compounds can be attributed to several factors such as the geographical location of the plant, seasonal and environmental conditions, harvest time, extraction and drying techniques, essential oil extraction from different organs, and plant genetic difference of the plant [28].

Table 2: Chemical composition of ZCEO by GC-MS

Row	Components	%	RT	KI
1	*α-Thujene*	0.2	11.33	927
2	*α-Pinene*	0.2	11.71	934
3	Camphene	0.1	12.61	952
4	Myrcene	0.5	14.62	992
5	*α-Phellandrene*	0.1	15.58	1010
6	*α-Terpinene*	0.7	16.11	1021
7	*p-Cymene*	4.8	16.62	1030
8	Limonene	0.1	16.77	1033
9	*β-Phellandrene*	0.1	16.89	1036
10	*γ-Terpinene*	4.6	18.31	1063
11	Linalool	0.1	20.5	1105
12	Borneol	0.6	24.36	1183
13	Terpinene-4-ol	0.4	24.7	1190
14	Thymol	19.5	29.61	1293
15	Carvacrol	65.2	30.57	1315
16	*E-Caryophyllene*	1	35.47	1427
17	Spathulenol	0.1	42.10	1590
18	Caryophyllene oxide	0.3	42.30	1595
		99.65		

3.2 Antimicrobial Activity of emulsion and nanoemulsion of ZCEO

Table 3 presents the results of the antibacterial activity of the emulsion and nanoemulsion of ZCEO against four strains of *L. monocytogenes* using the microdilution method. The MIC and MBC values of ZCEO nanoemulsion were lower than those of ZCEO emulsion. This finding is consistent with the findings of Shahbazi et al. (2016), who indicate the effect of particle size reduction on enhancing the antimicrobial activity of ZCEO [24]. Similarly, Khanzadi et al. (2019) evaluated the MIC and MBC of emulsion and nanoemulsion of ZCEO against *E. coli*. and found that the *Ziziphora clinopodioides* nanoemulsion was more potent than the *Ziziphora clinopodioides* emulsion, which is in agreement with the results of the present study [27].

Table 3: MIC and MBC results of emulsion and nanoemulsion of ZCEO against *L. monocytogenes* by microdilution method

Listeria monocytogenes strains	Emulsion		Nanoemulsion	
	MIC mg/mL	MBC mg/mL	MIC mg/mL	MBC mg/mL
ATCC 7644	0.5	1	0.062	0.125
ATCC 19111	0.5	1	0.125	0.25
CIP 7834	0.25	2	0.062	0.125
ATCC 19115	0.5	2	0.125	0.25

3.3 Enumeration of L. monocytogenes bacteria in chicken breast fillets

Table 4 shows the growth of *L. monocytogenes* inoculated on chicken breast fillets during refrigerated storage. The Con and Con + Vac groups had the highest number of *L. monocytogenes* on day zero. While the number of *L. monocytogenes* was significantly lower in other treatments on the same day. During the storage period, the number of *L. monocytogenes* rapidly increased in both the Con and Con + Vac groups, which is in line with the result of Keykhosravy et al. (2020) [29]. Psychotropic bacteria such as *L. monocytogenes* can grow on chicken breast fillets under refrigeration conditions. The most effective treatment was the Nano CH + ZCEO + N + Vac, resulting in a 1.3 logarithmic reduction of *L. monocytogenes* compared to the Con group on day zero. This finding is consistent with the results reported by Shahbazi et al. (2016) and Azizian et al. (2019), who reported that the combination of nisin and essential oil had the highest reduction in the number of tested bacteria compared to each coating of nisin and essential oil alone [19, 24]. The number of *L. monocytogenes* in the samples treated with Nano CH was 0.2 log lower than the Con on day zero, which is in agreement with Hashemi et al. (2022), who found that the number of *L. monocytogenes* in chicken fillets coated with nanochitosan was 0.3 log lower than the control [30]. Furthermore, Keykhosravy et al. (2020) also reported that nanochitosan coating alone caused a 0.5 log decrease in *L. monocytogenes* count in turkey meat samples on day zero [29]. In the present study, the number of *L. monocytogenes* in the samples coated with Nano CH + Vac was 5.1 log CFU/g

on day 16 of storage, while the number of this bacteria in the samples treated with Nano CH + ZCEO + Vac was 3.8 CFU/g on the same day. Similarly, Hasan et al. (2019) found that the chitosan coating + ZCEO was more effective than chitosan alone to reduce the growth of bacteria in chicken Fillets [31]. The *L. monocytogenes* counts in the samples treated with Nano CH + ZCEO + Vac and Nano CH + N + Vac were 5 and 4.8 log CFU/g, respectively, on day zero. In another study, Azizian et al. (2019) reported that the number of *E. Coli* O157:H7 in beef samples coated with nanochitosan + ZCEO and nanochitosan + nisin was 4.84 and 5.12 log CFU/g, respectively, on day zero [19]. The difference between these two studies may be due to the meat species (beef and chicken breast fillet) or the type of bacteria (*L. monocytogenes* as gram-positive bacteria and *E. coli* as gram-negative bacteria) [32].

Table 4: Changes in *Listeria monocytogenes* count (log CFU/g) of chicken breast samples in different treatments during 12 days of storage at 4±1 °C (mean ± SD)

Days	Con	Con+Vac	Nano CH	Nano CH + Vac	Nano CH + ZCEO + Vac	Nano CH + N + Vac	Nano CH + ZCEO + N + Vac
0	5.8 ± 0.4^{Ba}	5.7 ± 0.2^{Aab}	5.6 ± 0.1^{BCab}	5.2 ± 0.2^{Dbc}	5 ± 0.3^{Dcd}	4.8 ± 0.1^{Ccd}	4.5 ± 0.4^{Cd}
1	5.2 ± 0.5^{Abc}	5.9 ± 0.4^{Aa}	5.4 ± 0.1^{Bab}	5 ± 0.2^{BCDbc}	4.8 ± 0.3^{CDcd}	4.4 ± 0.1^{BCde}	4.2 ± 0.2^{BCe}
2	6.2 ± 0.5^{BCa}	6 ± 0.05^{Aa}	4.7 ± 0.2^{Ab}	4.5 ± 0.2^{ABbc}	4.3 ± 0.1^{ABCbc}	4.2 ± 0.2^{ABbc}	4 ± 0.2^{ABCc}
4	6.3 ± 0.6^{BCa}	6.1 ± 0.1^{Aa}	4.7 ± 0.2^{Ab}	4.4 ± 0.5^{Abc}	4.4 ± 0.1^{BCbc}	4.1 ± 0.1^{ABc}	3.9 ± 0.6^{ABc}
8	6.7 ± 0.1^{Ca}	6.2 ± 0.3^{Aa}	4.6 ± 0.1^{Ab}	4.5 ± 0.4^{ABb}	4.2 ± 0.1^{ABbc}	3.8 ± 0.1^{Acd}	3.6 ± 0.1^{Ad}
12	7.7 ± 0.2^{Da}	7 ± 0.4^{Bb}	5.4 ± 0.1^{Bc}	4.6 ± 0.4^{ABCd}	4 ± 0.2^{ABe}	3.9 ± 0.3^{ABe}	3.5 ± 0.4^{Ae}
16	7.8 ± 0.1^{Da}	7.7 ± 0.2^{Ca}	6 ± 0.5^{Cb}	5.1 ± 0.1^{CDc}	3.8 ± 0.1^{Ad}	3.7 ± 0.1^{Ad}	3.5 ± 0.2^{Ad}

* The same lowercase letters in the same column are not significantly different at $p < 0.05$ level.
* The same upper case letters in the same row are not significantly different at $p < 0.05$ level.

4. Conclusion

In summary, the main compounds in ZCEO were carvacrol, thymol, and p-cymene, and the nanoemulsion form of ZCEO showed stronger antimicrobial activity against *L. Monocytogenes* than the emulsion form. The combination of nanoemulsion containing, ZCEO, and nisin was found to be the most effective treatment in reducing the growth of *L. monocytogenes* in chicken fillet samples during refrigerated storage. Therefore, chitosan nanoemulsion containing ZCEO and nisin can be a promising natural preservative to reduce *L. monocytogenes* in chicken meat. However, further studies are needed to evaluate the effectiveness of this combination on other foodborne pathogens in meats.

Authors' Contributions

Vahid BehManesh: Investigation; Methodology; Writing-original draft. Saeid Khanzadi: conceptualization; funding acquisition; project administration; supervision; validation; writing-review & editing. Mohammad Hashemi: methodology; resources; visualization; writing-review & editing. Mohammad Azizzadeh: data curation; formal analysis; software; validation. Fatemeh Mollaei: Writing-original draft; writing-review & editing.

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Conflicts of Interest

The authors report no conflict of interest.

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