Comparing the Antibacterial Properties of *Ziziphora clinopodioides* Essential Oil in Free and Encapsulated States in Minced Beef Contaminated with *Salmonella typhimurium*

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ABSTRACT: *Salmonella, is among the most common foodborne pathogens that affect millions of people annually, sometimes with severe and fatal outcomes. In recent years, significant efforts have been made to develop natural antibacterial compounds, such as essential oils. Based on GC/MS analysis, Pulegone (33.10 %), Carvacrol (10.60 %), Piperitenone (9.33 %), Eucalyptol (8.01 %), γ-Terpineol (5.46 %) and L-Menthone (4.79 %) were the major components of phytochemicals of Ziziphora clinopodioides essential oil (ZEO). Encapsulation of ZEO using maltodextrin and gum arabic as wall in ratio of 1:1 with ZEO concentrations of 30 % (w/w) and 2.5 % (w/w) was done by spray drying method. The research results showed that, if the concentration of essential oil increased, the antimicrobial properties increased. The microbial population in the encapsulated ZEO treatment was lower than in the free ZEO treatment. The lowest inhibitory effect of ZEO was related to the concentration of 0.25 % (w/w) in the free state. The highest inhibitory effect of ZEO was related to the concentration of 1% (w/w) in the encapsulated state. The bacterial inhibitory property at the concentration of 0.5 % ZEO (w/w) in the free state was approximately equal to the concentration of 0.25 % ZEO (w/w) in the encapsulated state. The inhibitory properties of bacteria at concentration of 1 % (w/w) in the free state were better than the concentrations of 0.25 and 0.5 % (w/w) in the encapsulated state. Based on the results, formulation of ZEO in minced beef can prolong its shelf life and control microbial changes during storage at 4 °C. ZEO is insoluble in water, but a water-soluble microcapsule can be produced using this method. ZEO can be used as a natural and effective preservative for reducing pathogenic bacteria and increasing the shelf life of food.*

KEYWORDS: *Antibacterial; Ziziphora clinopodioides; Essential oil, Encapsulation; Salmonella typhimurium; Minced beef.*

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INTRODUCTION

Food poisoning and foodborne illnesses are major problems in the world and, despite new improvement in food production technique, food safety remains as a widespread public health issue. Common foodborne causative agents for the outbreak include *Salmonella enterica*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Clostridium botulinum*, *Campylobacter jejuni*, *Escherichia coli O157:H7* and *Lysteria monocytogenes* [1].

Salmonella bacteria cause the foodborne illness salmonellosis and have been known to make people sick since 1885. Symptoms in people start within 6 hours to 6 days after ingesting the bacteria and include fever, diarrhea (which may be bloody), nausea, vomiting and stomach pain. There is a group of *Salmonella*, called serotypes. So far, more than 2,500 *salmonella* serotypes have been identified, but less than 100 cause the most cases of "Salmonellosis" in humans. *Salmonella* infection often causes gastroenteritis, which can range from mild to severe. Symptoms are more severe in children under 5 years of age, pregnant women, the elderly and those with weakened immune systems [2].

In recent years, various chemical and synthetic compounds as preservative, such as antibiotics for controlling and eliminating foodborne pathogens, have been used. Because of increasing concern regarding food safety containing chemical additives, remarkable attempts have been made to develop natural antibacterial compounds such as Essential Oils (EOs) [3].

Research shows that one way to control food hygiene and increase the shelf life of food products is to use essential oils. As the concentration of essential oils increases, the logarithm of the number of microorganisms decreases [4]. Extracts and essential oils of various plants are used as preservatives in various food products. Essential oils are acceptable to the consumer due to their natural composition as well as antimicrobial and antioxidant properties for various foods [5].

Ziziphora clinopodioides is a genus of *Ziziphora*, a family of *Lamiaceae*, which grows in Iran and Turkey. The genus of *Ziziphora* consists of four species, including *Z. persica*, *Z. tenuior L*., *Z. capitata L*., and *Z. clinopodioides lam*. *Ziziphora clinopodioides* is known in Iran by the local name of "Mountain Kakoty" and is a perennial plant with stemmy bushes, short stems between 5 and16 cm as well as thin, sharp leaves [6]. Previous studies have identified its major phenolic compounds such as pulegone, 1,8-cineole, neomenthol, 4-terpineol, 1-terpineol, neomenthyl acetate and piperitenone [7].

Generally, in Iranian folk medicine, the healing properties of this plant have been used for flu disease, wounds, carminative effects, treatment of stomach tonic and its other effects such as antibacterial, anthelmintic, antifungal and antiviral properties [8]. Traditionally, in different areas of Iran, *Ziziphora clinopodioides* has been powdered and used in many food products such as meat, dairy products, especially yoghurt and doogh (a yogurt-based Iranian drink) to enhance the aroma and flavor [9].

Encapsulation is a rapidly expanding technology with numerous potential applications pharmaceutical and food industries. The encapsulation method was employed to protect bioactive compounds such as polyphenols, micronutrients, enzymes, antioxidants, etc. [10]. Materials used for designing the protective shell of encapsulates must be food-grade, biodegradable, and able to form a barrier between the internal phase and its surroundings. Polysaccharides are most commonly used for encapsulation in food industries. Proteins and lipids are also appropriate for encapsulation. Spry drying is the most extensively applied encapsulation technique in the food industries because it is flexible, continuous and has an economical operation [11].

Encapsulation is a process, in which active substances are coated by extremely small capsules. It is a new technology that has been used in the cosmetic industry as well as in the pharmaceutical, agrochemical, and food industries, being used in flavors, acids, oils, vitamins, and microorganisms, among others. The success of this technology is due to the correct choice of the wall material, the core release form, and the encapsulation method [12]. Microcapsules are particles having a diameter between 3 and 800 μm [13].

There are various techniques for encapsulation such as spray drying, freeze drying, spray cooling, coacervation, centrifugation, liposomal systems, fluidized bed systems, and ultrasonic waves. Among the various encapsulation methods, the spray drying method has been preferred due to its economical, high operating speed, high reliability, and high flexibility for aromatic substances and essential oils. Encapsulation by spray drying apparatus in this research

included various steps such as aqueous phase preparation, emulsion preparation, homogenization, and, finally, spray drying which is described in the materials and methods section [14].

Nanoparticles (NPs) are increasingly used to target bacteria as an alternative to antibiotics. Nanotechnology may be particularly advantageous in treating bacterial infections. The antibacterial mechanisms of NPs are poorly understood, but the currently accepted mechanisms include oxidative stress induction, metal ion release, and non-oxidative mechanisms. The multiple simultaneous mechanisms of action against microbes would require multiple simultaneous gene mutations in the same bacterial cell for antibacterial resistance to develop; therefore, it is difficult for bacterial cells to become resistant to NPs.

Bacterial infections are a major cause of chronic infections and mortality. Antibiotics have been the preferred treatment method for bacterial infections because of their cost-effectiveness and powerful outcomes. However, several studies have provided direct evidence that the widespread use of antibiotics has led to the emergence of multidrug-resistant bacterial strains. In fact, super-bacteria, which are resistant to nearly all antibiotics, have recently developed due to abuse of antibiotics [15].

The antibiotic metronidazole is used to treat *Trichomonas gallina* parasites, which can lead to drug resistance. In a new study, a biocompatible nanocomposite including chitosan nanocapsules and cellulose nanofibrils was utilized to evaluate their anti-trichomonal activity. The most efficient chitosan nanocapsules were 241.5 nm in diameter and the thickness of their skins was around 80.61 nm [16].

In another study, a new procedure to lower side effects of the drug molecules and enhance the treatment of disease was used. In this method, whisker-formed SBA-15 nanoparticles were utilized for the first time. They had mesoporous structures, in which metronidazole molecules could be trapped. Additionally, these crystalline nanowhiskers were modified with tannic acid to improve the release process. Whisker-like SBA-15 nanocrystals had a mesopore volume of 0.5931 cm^3/g, pore diameter of 6.06 nm, and surface area of 491.38 m^2/g [17].

Research has shown that ZEO has antimicrobial and bactericidal properties in fish burgers during refrigerated storage and can be used as a natural preservative in food. Also, results of another study showed that microencapsulation of ZEO improved sensorial scores of the treated samples during 20-day storage at 4 ^oC and the samples containing microencapsulated ZEO demonstrated the strongest effects on preserving the sensorial quality of fish burger [18]. Other research has shown that the microbial population is decreased significantly with the addition of ZEO in milk and causes an increase in the shelf life and control of *Salmonella typhimurium* [19]. Research has shown that ZEO in combination with nisin can be applied as an alternative antimicrobial agent in Doogh (Iranian yogurt drink) to inhibit the growth of *E. coli O157:H7* [20].

This study aims to compare the antibacterial effect of *Ziziphora clinopodioides* essential oil (ZEO) at 0.25, 0.5 and 1 % (w/w) concentrations in free and encapsulated states on *Salmonella typhimurium* in minced beef during storage at 4 ^oC for 20 days. The innovation of this research is in the use of ZEO to control the population of pathogen bacteria *Salmonella typhimurium* in minced beef using an encapsulation technique by spray drying apparatus.

EXPERIMENTAL SECTION

Materials

Maltodextrin with DE = 18-20 from ROQUETTE company in France and Arabic gum from Kian Shimi company in Iran were prepared (Table 1). Deionized water was used to prepare the solutions and emulsions. The complete sample of the plant including the aerial parts, stems and roots of *Ziziphora clinopodioides* in the flowering stage in early summer 2019 was prepared from the mountains of Bojnourd in North Khorasan Province and their leaves were used.

Salmonella typhimurium was purchased with ATTC code 10708 from "Iranian Biological Resource Center". A master culture was prepared from *Salmonella typhimurium* in Müller-Hinton broth and was kept in an incubator at 37 ° C for 24 h. Firstly, 0.5 McFarland turbidity standard was prepared from *Samunella typhimurium*. The absorption rate of the 0.5 McFarland turbidity standard was measured by using a spectrophotometer (model RAY LEIGH UV-2601) at the wavelength of 625 nm (0.08 – 0.13). The 0.5 McFarland turbidity standard contained 1.5×10^8 colony-forming units of bacteria (CFU/mL) [21].

Table 1: Material specifications.

Material	Structure	Purity	Vendor	CAS NO.
Maltodextrin	Mixture of glucose, disaccharides and polysaccharides, obtained by the partial hydrolysis of starch	99.9 %	ROQUETTE company	9050-36-6
Arabic gum	Mixture of glycoproteins and polysaccharides	99 %	Kian Shimi company	-

Essential Oil Extraction and Analysis

After identifying and confirming the plant, the samples were dried in the shade and 20 kg of leaves were separated from the stem [8]. The leave sample of *Ziiziphora clinopodioides* is shown in Fig. 1. The percentage of moisture in the plant was measured by gravimetric method and its amount was 5 % [14]. The essential oil was extracted from the leaves by using the distillation method in a Clevenger balloon for 3 to 4 h [22, 23]. The percentage of essential oil extraction was 1.1 % [14]. Ohmic-Assisted HydroDistillation (OAHD) is an advanced HydroDistillation (HD) technique utilizing an ohmic heating process which could be considered as a novel method for extracting essential oils [24]. After separating the essential oil, a small amount of anhydrous sodium sulfate was added to absorb water and residual moisture. To protect the essential oil, the lid of the storage container was closed and kept in the refrigerator at 4 °C [25].

The chemical composition of the essential oil was determined by Gas Chromatography and coupled with Mass Spectrometry (GC-MS) [26]. Chemical analysis of essential oil was performed by the Agilent 6890 gas chromatography device connected to mass detector Agilent 5973 N mass spectrometer with BPX 5 column, column length 30 m, column inner diameter 0.25 mm, layer thickness 0.25 µm, initial column temperature 50 °C, final column temperature 300 ° C and Helium carrier gas.

Fig. 1 :Leave of Ziiziphora clinopodioides.

ZEO Encapsulation

At first, the aqueous phase of the emulsion was prepared to produce microcapsules. To produce ZEO encapsulated, a suitable ratio of wall materials including maltodextrin and gum arabic at the ratio of 1:1 was prepared. Then, wall materials (maltodextrin and gum arabic) with the concentration of 30 % w/w were added to deionized water. The aqueous phase was kept to 4 °C for 24 h for complete dehydration. The next day, ZEO with 2.5 % w/w was added to the aqueous phase at 25 °C. Then, it was mixed properly by a mechanical stirrer (PZR2102 Control, Heidolph company, Germany) for 5 min. The above suspension was completely homogenized by an Ultratorax homogenizer (Model T25, IKA, Germany) at 18,000 rpm for 10 min [27].

Final homogenization was performed using an ultrasonic device (model VCX750, SONICS company, USA) with 100 % control amplitude and frequency of 20 kHz for 1 min at 45 °C. In the last step, the final emulsion was pulverized by the spray dryer [28]. Emulsion inlet flow was 15 ml/min, airflow velocity 70 km/h, inlet air temperature 180 $\pm$ 10 °C , outlet air temperature 90 $\pm$ 10 °C and time was 4 $\pm$ 1 s [14]. To protect the volatile essential oil, encapsulated powder was packed in a plastic container and kept at -18 °C [29].

Emulsion Properties

The emulsion brix number was measured by using the refractometer (model RX-5000α, ATAGO company, Japan). Emulsion particle size was measured by a particle size measuring device (Zetasizer Nano ZS model, Malvern, UK). The zeta potential of the emulsion was measured using a zeta potential measuring device (Zetasizer Nano ZS model, Malvern, UK). The polydispersity index (PDI) was measured by using Zetasizer Nano ZS model, Malvern, UK [30].

Microcapsule properties

First, a 10 % solution (w/w) was prepared from the encapsulated powder [29]. To measure the moisture of the microcapsules, the vacuum gravimetric method was used. For this purpose, 1 g of the microcapsules was placed in the vacuum oven at 70 °C and pressure of 1 bar for 6 h. The moisture content was obtained from the following equation [31]:

$$\text{Moisture content} = (\text{Mass of moisture} / \text{Mass of microcapsules}) \times 100 \quad (1)$$

The particle size of the encapsulated powder was measured by using a particle size measuring device (Zetasizer Nano ZS model, Malvern, UK). The zeta potential of the encapsulated powder was measured using a zeta potential measuring device (Zetasizer Nano ZS model from Malvern, UK).

To evaluate the solubility of the microcapsules, a 0.4 % w/v solution of the microcapsules with distilled water was prepared. It was then stirred at 25 °C on a magnetic stirrer. The dissolution time of the microcapsules in distilled water should not be more than 5 min [32].

SEM images of the encapsulated powder particles were prepared (SEM model VEGA3 TESCAN).

Sample Preparation

Beef was transported to the laboratory in the ice box immediately after slaughter. At first, the beef was washed, then minced in a sterilized meat mincer with a pore size of 1 mm and stored in a sterilized glass container in the refrigerator (4 °C). Firstly to inoculate, all of the bacteria in the beef must be killed and, for this purpose, the minced beef was transferred to a 2000 cc glass container with the lid closed and placed in an autoclave at 121 °C for 15 min. To ensure that the beef was free of bacteria, a culture of it was performed in Müller-Hinton broth (MHB) and kept in an incubator at 37 °C for 24 h. Lack of colony growth after 24 h indicated the absence of bacteria in beef [33].

Inoculation of Salmonella Typhimurium into Beef

After autoclaving the beef, 10^3 bacteria of *Salmonella typhimurium* should be inoculated per gram of beef. For this purpose, 350 g of beef was weighed by a digital scale and transferred into a sterile glass container. Then, 350 µL of 0.5 McFarland diluted (containing 1.5×10^6 CFU/mL) of *Salmonella typhimurium* was added to the beef container and mixed thoroughly with a sterilized steel spoon. To repeat the experiments, the samples were divided into 3 separate containers and stored in the refrigerator (4 °C).

After the inoculation, the initial bacterial population was counted by culturing in the plate count agar (PCA) by using the pour plate method. For this purpose, dilutions of 1/10, 1/100, and 1/1000 of inoculated beef were prepared and 1 cc of each dilution was poured on the PCA surface. After 30 min, the plate lids were closed and placed in an incubator at 37 °C for 24 h. The test was repeated 3 times for 3 separate containers and, after 24 h, the number of colonies was counted and recorded [33].

Preparation of Treatments

The inoculated beef was divided into seven equal parts, as shown in Table 2. In 7 Erlenmeyer 250 cc (previously autoclaved), 50 g of inoculated beef was weighed by a digital scale. According to Table 2, ZEO in the free state and at 3 levels (0.25 %, 0.50 %, and 1 % w/w) was added to treatments 1, 2, and 3. Also, ZEO in the encapsulated state and 3 levels (0.25 %, 0.50 %, and 1 % w/w) was added to treatments 4, 5, and 6. Treatment 7 was the control and without ZEO. Then, all the treatments were stored in the refrigerator (4 °C) and were analyzed periodically on days 0, 5, 10, 15, and 20 for *Salmonella typhimurium* count [29].

Microbiological Analysis

On days 0, 5, 10, 15 and 20, at first, the dilution of 1/10 of each treatment was prepared. For this purpose, 4 g of the samples from each treatment was weighted into sterile Erlenmeyer (250 cc) containing 36 mL sterile Ringer's solution and homogenized for 5 min. Then, different dilutions of the sample including 1/100, 1/1000 and 1/10.000 were prepared. For the microbial counting, the culture was performed on Plate Count Agar (PCA) using pour plate method. The experiment was repeated 3 times for each treatment. *Salmonella typhimurium* counts were determined after incubation at 37 °C for 24 h [33].

Data Analysis

The number of bacteria counted on days 0, 5, 10, 15 and 20 for seven treatments with three replications was recorded. Then, it was analyzed using SAS-9.2

Table 2: List of treatment formulations.

Treatment	Treatment	Description
1	Addition of ZEO (in free state)	0.25 % (w/w)
2	Addition of ZEO (in free state)	0.50 % (w/w)
3	Addition of ZEO (in free state)	1 % (w/w)
4	Addition of ZEO (in encapsulated state)	0.25 % (w/w)
5	Addition of ZEO (in encapsulated state)	0.50 % (w/w)
6	Addition of ZEO (in encapsulated state)	1 % (w/w)
7	Control	Without ZEO

statistical software. In this study, first, the descriptive statistics of the data were extracted and the initial statistical distributions of the data were examined. Normal data distribution and variance independence tests were performed using *Bartlett* test. Then, ANOVA procedure was performed to analyze the variance of the data. The mean values of each treatment were extracted for each trait and the comparison between the means was performed by *Duncan's* multiple range comparison test. In *Duncan* grouping, those treatments with different letters were significantly different from each other.

RESULTS AND DISCUSSION

Maltodextrin is a non-ionic compound and hydrophilic, but Arabic gum is a compound with a dual structure of hydrophilic and hydrophobic; a new wall composition with good performance for ZEO encapsulation can be achieved by combining these two substances. ZEO is insoluble in water, but ZEO encapsulated in a spray dryer is water soluble. A spray dryer is a suitable apparatus for ZEO encapsulation. The performance (oil retention) and encapsulation efficiency of the spray dryer were 60 % and 96.65 %, respectively [14].

Investigation of Chemical Analysis of ZEO

In this study, the yield of essential oil extraction was 1.1 %. The chemical composition of ZEO was determined by using GC-MS analysis. According to Table 3 and based on gas chromatography, 24 compounds were identified and Pulegone (33.10 %), Carvacrol (10.60 %), Piperitenone (9.33 %), Eucalyptol (8.01 %), γ-Terpineol (5.46 %), L-Menthone (4.79 %), tranc-Menthone (3.45 %), Piperitone (2.52 %) and para-Cymene (2.05 %) were the major components of phytochemicals of ZEO, which was consistent with the results of other studies [34].

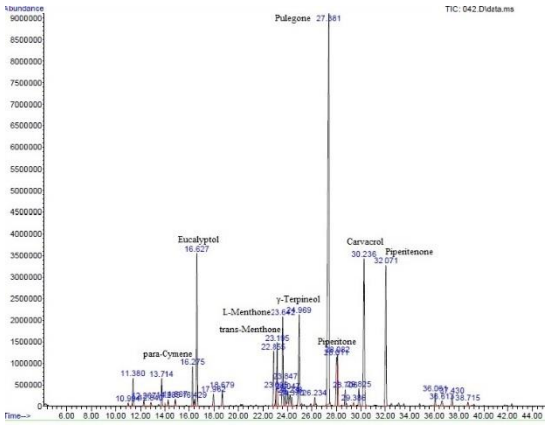

Fig. 2: GC/MS analysis of ZEO.

The spectrum of chemical analysis of ZEO is shown in Fig. 2.

Another study showed that Pulegone, Menthone, and Isomenthone were identified as the major components of ZEO [8] and another study showed that Carvacrol, Thymol, p-Cymene, and γ-Terpinene were the main chemical components of ZEO [19]. In another research, the essential oil extract was obtained from aerial parts of *Ziziphora clinopodioides Lam.* In the Eastern part of Turkey, GC–MS analyses allowed 18 compounds to be determined; the main constituents of the essential oils were Pulegone (31.86 %), 1,8-cineole (12.21%), Limonene (10.48%), Menthol (9.13%), β–pinene (6.88%), Menthone (6.73%), Piperitenone (5.30%) and Piperitone (4.18%) [35]. Results of the research showed that essential oils have antioxidant and antimicrobial properties on Gram-positive and Gram-negative bacteria [36].

Emulsion Properties

Fig. 3 shows that ZEO is spherically and evenly distributed inside the emulsion. There is also no agglomeration inside the emulsion which indicates complete

Table 3: Chemical composition of Ziziphora clinopodioides essential oil by GC/MS analysis.

NO.	Retention time (RT)	%	Components	Kovats index (KI)	Type
1	11.00	0.15	α-Thujene	928	MH
2	11.38	1.34	α-Pinene	935	MH
3	12.27	0.29	Camphene	953	MH
4	13.71	1.40	β-Pinene	982	MH
5	14.28	0.30	Myrcene	993	MH
6	14.87	0.34	3-Octanol	1004	Other
7	16.27	2.05	*para*-Cymene	1032	MH
8	16.43	0.37	Limonene	1035	MH
9	16.63	8.01	Eucalyptol	1038	MO
10	17.96	0.61	γ-Terpinene	1064	MH
11	18.68	0.86	*p*-Mentha-3,8-diene	1078	MH
12	23.19	3.45	*tranc*-Menthone	1168	MO
13	23.65	4.79	*L*- Menthone	1178	MO
14	23.85	1.39	*neo*-Menthol	1182	MO
15	24.05	0.95	Borneol	1186	MO
16	24.24	0.88	Isopulegone	1190	MO
17	24.37	0.47	Terpinen-4-ol	1192	MO
18	24.97	5.46	γ-Terpineol	1205	MO
19	27.38	33.10	Pulegone	1255	MO
20	28.08	2.52	Piperitone	1270	MO
21	29.82	1.02	Thymol	1307	MO
22	30.24	10.60	Carvacrol	1317	MO
23	32.07	9.33	Piperitenone	1358	MO
24	38.72	0.21	β-Bisabolene	1515	SH
		89.89	Total Identified		

MH: Monoterpene Hydrocarbons, MO: Oxygenated Monoterpenes, SH: Sesquiterpene Hydrocarbons

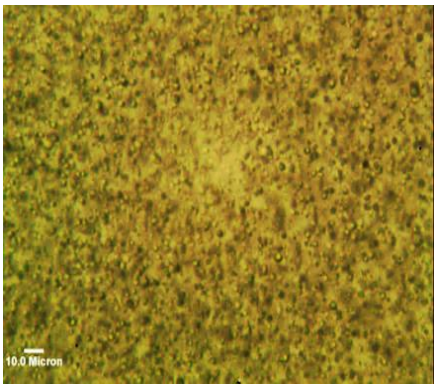

Fig. 3: Microscopic image of emulsion particles.

homogeneoity. The results of physicochemical properties of emulsion are reported in Table 4.

Microcapsules Properties

Fig. 4 shows that the shape of all particles is almost spherical and looks like a soccer ball. Also, all of the particles are separated from each other and do not stick to each other. These results demonstrate that the encapsulation process of *Ziziphora clinopodioides* Essential Oil (ZEO) is completely correct.

The final microcapsule with pH = 4.7, moisture of = 0.9 %, particle size <20 µm, total essential oil content 4.655 %, surface oil 0.018 %, oil retention 60 %, encapsulation efficiency 96.65 %, zeta potential = -22.7 mV, polydispersity index (PDI) = 0.375 and water-soluble property with light yellow color and without sediment was produced [14]. ZEO is insoluble in water, but a water-soluble microcapsule with a light yellow color and without

Table 4: Physicochemical properties of emulsion.

Property	Description
Solvent	Deionized water
Materials of aqueous phase	Maltodextrin and Gum arabic with ratio of 1:1
Concentration of wall	30 % (w/w)
Concentration of ZEO	2.5 % (w/w)
pH	3.875
Brix	30.44 %
Particle size	429 (nm)
Zeta potential	-25.8 (mV)
Polydispersity Index (PDI)	0.681

Table 5: Physicochemical properties of microcapsules [14].

Property	Description
Core	*Ziziphora clinopodioides* Essential oil (ZEO) (2.5 % w/w)
Wall	Maltodextrin and Gum Arabic (ratio 1:1) (30 % w/w)
pH	4.7
Moisture content	0.9 %
Particle size	<20 µm
Total essential oil content	4.655 %
Surface oil	0.018 %
Oil retention	60 %,
Encapsulation efficiency (EE)	96.65 %
Zeta potential	-22.7 (mV)
Polydispersity index (PDI)	0.375
Indicator solubility	Water soluble (1 min)
Color powder	Light yellow
Sediment	No sediment

sediment can be produced using this method. Results of physicochemical properties of microcapsules are reported in Table 5.

Zeta potential (ζ) is a scientific term for electrokinetic potential in colloidal dispersions and shows the potential difference between the phase boundaries between solids and liquids. Zeta potential is a way to determine the electrical charge of a particle. The particle load is always very important and the high particle load (negative or positive) leads to the stability of the particles in liquids and their non-deposition. The usual units are volts (V) or, more commonly, millivolts (mV). The stability of a colloid depends on the amount of zeta potential.

In most of the cells, this measured potential is between -10 and -20 mV. Usually, the higher the zeta potential, the more stable the colloidal particles would be. The results of this study with the value of -25.8 (mV) for the emulsion showed that the electric charge of the cell membrane surface in the emulsion is near moderate stability, which is an important factor in the stability of colloidal materials. Zeta potential of ±30 to ±40 mV has moderate stability [37].

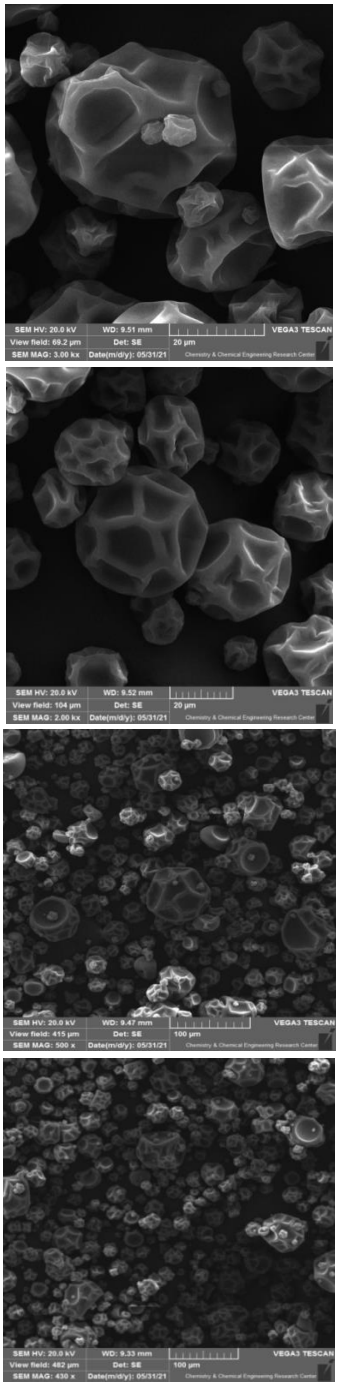

Fig. 4: SEM images of particle size encapsulation of Ziziphora clinopodioides Essential Oil (ZEO).

Microbial Counts

In this study, the initial number of bacterial cells on day zero was the same for all the treatments. On day 5, the highest number of bacteria belonged to treatments 7, 1, 4, 2, 5, 3 and 6, respectively. Each of the treatments 7, 1, 3 and 6 had significant differences from other treatments ($p < 0.01$). Also, treatments 4, 2 and 5 did not have significant differences with each other and had significant differences with other treatments ($p < 0.01$).

On day 10, the highest number of bacteria belonged to treatments 7, 1, 2, 4, 5, 3 and 6, respectively. There was also a significant difference between all the treatments ($p < 0.01$).

On day 15, the highest number of bacteria belonged to treatments 7, 1, 2, 4, 5, 3 and 6, respectively. Each of the treatments 7, 1, 5, 3 and 6 had significant differences with other treatments ($p < 0.01$). Also, treatments 2 and 4 did not have significant differences with each other and had significant differences with other treatments ($p < 0.01$).

On day 20, the highest number of bacteria belonged to treatments 7, 1, 2, 4, 5, 3 and 6, respectively. There was also a significant difference between all the treatments ($p < 0.01$). The results of bacterial count analysis in different treatments are recorded in Table 6. The final comparison of *Salmonella typhimurium* growth in different treatments is shown in Fig. 5.

Results of this study showed that, with increasing the concentration of ZEO, the microbial population decreased significantly. The microbial population in the encapsulated ZEO treatment was lower than that in the free state. The lowest inhibitory effect of ZEO was related to the concentration of 0.25 % (w/w) in the free state. The highest inhibitory effect of ZEO was related to the concentration of 1% (w/w) in the encapsulated state. The bacterial inhibitory property at the concentration of 0.5 % ZEO (w/w) in the free state is approximately equal to the concentration of 0.25 % ZEO (w/w) in the encapsulated state. The inhibitory properties of bacteria at the concentration of 1 % (w/w) in the free state were better than the concentrations of 0.25 and 0.5 % (w/w) in the encapsulated state.

In another study, to determine the antibacterial activity of *Ziziphora clinopodioides* (ZEO) essential oil to increase the shelf life and control *Salmonella typhimurium* in milk stored at the refrigerator temperature was performed. Results showed that the microbial population decreased significantly with adding ZEO, increasing their concentrations and longer storage time [3].

In another study, the effect of *Ziziphora clinopodioides* (0.1 and 0.2 %) and nisin (250 and 500 IU/g), both separately and in combination, was evaluated on

Table 6: Population changes in Salmonella typhimurium in minced beef in different treatments over a period of 20 days at the refrigerator temperature (Log CFU/g).

Treatment	Day 0	Day 5	Day 10	Day 15	Day 20
1	a 4.9996 ± 0.0217	b 4.9912 ±0.0044	b 5.6742 ±0.0065	b 5.5571 ±0.0030	b 5.5448 ±0.0043
2	a 4.9996 ±0.0217	c 4.8470 ±0.0141	c 5.6020 ±0.0039	c 5.5323 ±0.0048	c 5.4292 ±0.0049
3	a 4.9996 ± 0.0217	d 4.5179 ±0.0263	f 5.2031 ±0.0056	e 4.8632 ±0.0119	e 4.6643 ±0.0446
4	a 4.9996 ± 0.0217	c 4.8611 ±0.0139	d 5.5828 ±0.0028	c 5.5258 ±0.0052	c 5.4007 ±0.0114
5	a 4.9996 ± 0.0217	c 4.8301 ±0.0183	e 5.2544 ±0.0050	d 5.2261 ±0.0039	d 5.1816 ±0.0142
6	a 4.9996 ±0.0217	e 4.1130 ± 0.0334	g 4.5677 ±0.0235	f 4.5430 ±0.0373	f 4.5002 ±0.0211
7	a 4.9996 ± 0.0217	a 5.2040 ± 0.0071	a 5.6992 ±0.0050	a 5.8721 ±0.0055	a 5.8654 ±0.0041
P-Value	p > 0.05	p < 0.01	p < 0.01	p < 0.01	p < 0.01

The values given in the table are averaged ± standard deviation.
Uneven English letters indicate significant difference.

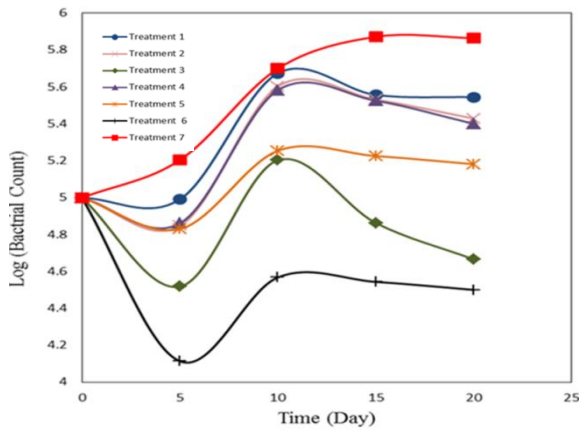

Fig. 5: Comparison of Salmonella typhimurium population changes in treatments 1 to 7 during 20 days at 4 °C.

mesophilic, psychrotrophic and Enterobacteriaceae microorganisms as well as on *Staphylococcus aureus* and *Escherichia coli O157:H7* in raw beef patty during storage at refrigerated temperature for 9 days. Both the essential oil and nisin significantly ($P < 0.05$) affected the growth of psychrotrophic, Enterobacteriaceae and mesophilic bacteria as well as *S. aureus* and *E. coli O157:H7*. Among the experimental groups, the samples treated with 0.2 % essential oil + 500 IU/g nisin showed the significant rapid decrease ($P < 0.05$) in a number of the tested microorganisms [38].

In another work, the ZEO inhibited the growth of *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli O157:H7*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* at MIC values between 0.03 and 0.04 % [39].

Another study was conducted to preserve the chemical and microbial quality of trout fish burgers during storage using *Ziziphora clinopodioides* Essential Oil (ZEO) individually and in combination with nisin. Different treatments of trout fish burger were formulated using ZEO and nisin as natural preservatives, stored in refrigerator for 20 days. According to the obtained results, the combination of ZEO and nisin had the strongest effect on chemical and microbial quality of fish burger; however, their individual use had significant effects on preserving the chemical and microbial quality of fish burger as well [8].

CONCLUSIONS

Encapsulation is one of the most important methods to protect essential oils from evaporation, degradation and preservation of antimicrobial properties for reducing the number of microorganisms in food. The highest chemical composition of ZEO in North Khorasan Province was Pulegone (33.10 %). This research results showed that t he application of ZEO resulted in extending the shelf life of minced beef and can be usefully applied in food industries. Spray drying method is a suitable method for encapsulation of ZEO. Microbiological characteristics of minced beef treated with ZEO in free and encapsulated states were better than those of control samples. Microbiological characteristics of minced beef treated with ZEO encapsulated were better than those treated with ZEO in the free state. Due to the antioxidant and

antimicrobial properties of ZEO, it can be used as a natural and effective preservative for reducing pathogenic bacteria in minced beef and increasing the shelf life of food. ZEO is insoluble in water, but a water-soluble microcapsule can be produced using this method. ZEO extends the shelf life of minced beef, milk, and fish burgers, and can be used as natural antibacterial compounds in food products.

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