Journal of Human, Environment, and Health Promotion. 2019; 5(2): 94-97

Journal of Human, Environment, and Health Promotion

Journal homepage: www.zums.ac.ir/jhehp

Chemical Composition and Antibacterial Activity of the Emulsion and Nano-emulsion of *Ziziphora clinopodioides* Essential Oil against *Escherichia coli* O_{157}:H_7

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ARTICLE INFO

Article type:
Short Communication

Article history:
Received 20 December 2018
Revised 13 February 2019
Accepted 9 March 2019

DOI: 10.29252/jhehp.5.2.8

Keywords:
Essential oil
Antibacterial activity
Nanotechnology
Escherichia coli O_{157}:H_7

ABSTARACT

Background: The present study aimed to determine the chemical composition and *in-vitro* antibacterial activity of *Ziziphora clinopodioides* essential oil (ZCEO).
Methods: The chemical composition of ZCEO was determined using gas chromatography-mass spectrometry (GC-MS) analysis. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were calculated using the microwell dilution assay to assess the antibacterial effects of ZCEO emulsion and nano-emulsion on *E. coli* O_{157}:H_7.
Results: A yield of 1% (w/w) was identified for ZCEO isolation and pulegone (58.78%), menthone (1.15%), and isomenthone (9.91%) as the key components of ZCEO phytochemicals. The MIC values of the ZCEO emulsion and nano-emulsion were 0.8 and 0.025 mg/ml, respectively, and the MBC values were estimated at 1.6 and 0.05, respectively.
Conclusion: It is recommended that the nano-emulsion of ZCEO be applied as a potential source of natural preservatives in food industries.

1. Introduction

Numerous antibacterial agents are available to control the growth of various bacteria, fungi, and yeasts in food products [1]. Preservatives are applied through various methods in food industries, such as direct augmentation into food products [2], incorporation of sachets into food packages [3], direct application on food surfaces [4], and incorporation into packaging materials [5].

Essential oils are aromatic, oily liquids that are obtained from plants using various methods and contain numerous phenolic compounds, such as phenolic acids and flavonoids. In addition, essential oils exhibit antimicrobial effects on various microorganisms [6, 7].

Ziziphora clinopodioides belongs to the genus *Ziziphora* and *Lamiaceae* family, which mainly grows in Iran and Turkey [8]. This plant is widely used as a food preservative since it has potent antioxidant and antimicrobial properties, produces aroma, and enhances food flavor. Furthermore, *Ziziphora clinopodioides* is carminative, appetitive, and stomach tonic and could improve hypertension, fever, edema, cardiac diseases, neurasthenia, insomnia, tracheitis, lung abscess, and hemorrhoids. This plant has also been reported to have antiseptic and expectorant properties [9], which could be attributed to its bioactive constituents, such as phenolic compounds (e.g., pulegone, thymol, 1, 8-cineole, carvacrol, limonene, p-cymene, and cis-caran-trans-2-ol) [8].

How to cite: Khanzadi S, Azizian A, Hashemi M, Azizzadeh M. Chemical Composition and Antibacterial Activity of the Emulsion and Nano-emulsion of *Ziziphora clinopodioides* Essential Oil against *Escherichia Coli* O_{157}:H_7. *J Hum Environ Health Promot.* 2019; 5(2): 94-7.

Nano-emulsions are applied in food products owing to their unique properties, such as the ease of preparation, high-grade functions, and fine particle size, which enhance the interactions of active compounds and biomembranes, as well as their transfer [10]. Nano-emulsions could be produced using multiple methods, including high-energy and low-energy techniques [10]. For instance, ultrasonic emulsification is a high-energy technique effectively applied to prepare nano-emulsions with small droplet diameters and low size distribution [11]. Today, emulsion-based systems are developed by food-grade components and easily distributed in various food products to control the growth of various microorganisms [12].

The present study aimed to assess the chemical composition and antibacterial properties of the emulsion and nano-emulsion of *Ziziphora clinopodioides* essential oil (ZCEO) on *Escherichia coli* O_{157}:H_7. Our findings could lay the scientific groundwork for the application of the nano-emulsion form of ZCEO as a potential agent against pathogenic microorganisms in food industries.

2. Materials and Methods

2.1. Experimental Materials

Ziziphora clinopodioides were purchased from the Iranian Institute of Medicinal Plants in Karaj, Alborz province, Iran. *E. coli* O_{157}:H_7 (NCTC 12900) was obtained from the Department of Food Hygiene at the School of Veterinary Medicine at Ferdowsi University of Mashhad in Mashhad, Iran. In addition, all the culture media were obtained from Merck (Darmstadt, Germany).

2.2. Essential Oil Extraction and Analysis

The isolation and analysis of ZCEO were performed using hydro distillation based on the method proposed by the European Pharmacopoeia [14]. The Clevenger-type apparatus was utilized to extract the essential oil of the dried aerial parts of the plant, which were collected during the flowering stage from the mountains in Bojnurd county, located in North Khorasan province (Iran) and identified by the herbarium of the Iranian Institute of Medicinal Plants in Alborz province.

The plants were distilled with water for four hours in accordance with the method proposed by Ehsani et al. (2016) [13], and the yield of the essential oil was calculated following the exposure of 350 grams of the dried plants to extraction by the hydro distillation and quantification of the obtained essential oil. The obtained ZCEO was dehydrated using sodium sulfate, filtrated, and stored at the temperature of 4°C until further analysis. The composition of the essential oil was determined via gas chromatography-mass spectrometry (GC-MS) as described previously based on the method proposed by Ehsani et al. (2016) [13].

2.3. Preparation of E. coli O_{157}:H_7

The bacterium was cultured in nine milliliters of brain heart infusion (BHI) broth, incubated at the temperature of 37 °C for 24 hours, and re-incubated for 18 hours at the temperature of 37 °C. Afterwards, the applied bacterial suspension was obtained from an 18-hour culture in order to prepare 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml) and diluted (1:10) to the density of 1.5×10^7 CFU/ml [13].

2.4. Preparation and Characterization of the Emulsion and Nano-emulsion of ZCEO

At this stage, ZCEO (0.5% w/v) was dissolved in sterile distilled water containing TWEEN 80 (0.2% w/w ZCEO) as the emulsifier. This process was followed by constant stirring for 10 minutes to achieve a stable, uniform, clear emulsion. The emulsion of ZCEO was formulated in accordance with the protocol proposed by Ghosh et al. (2013) with slight modifications. Afterwards, the emulsion were subjected to ultra turrax (OPTIMA, XL100K, Clausthal, Germany) for three minutes at 3,000 rpm and ultrasonic emulsification sonicator (50 °C; pulse: 45 s; rest: 15 s) for six minutes (probe diameter: 15 mm) [11]. Particle size was measured using a dynamic light scattering (DLS) device (Nanophox Sympatec GmbH, Clausthal, Germany).

2.5. MIC and MBC Values of the Emulsion and Nano-emulsion of ZCEO

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of both forms of ZCEO were determined using the microwell dilution assay [13]. To do so, two stock solutions of the emulsion and nano-emulsion of ZCEO were prepared at the concentration of 32-0.125 mg/ml. The wells containing 160 microliters of the BHI broth were filled with 20 microliters of the inoculum (1.5×10^7 CFU/ml) and 20 microliters of various concentrations of the emulsion and nano-emulsion of ZCEO. The bacteria-free wells (180 μL of BHI broth and 20 μL of ZCEO nano-emulsion) and ZCEO-free wells (180 μL of BHI broth and 20 μL of inoculum) were considered as the negative and positive controls, respectively.

At the final volume of the wells (200 μL), the concentration of the bacterial suspension was approximately 1.5×10^6 CFU/ml, and the concentration of ZCEO was within the range of 0.0125-3.2 mg/ml. Incubation was performed in a shaker incubator (GFL 3031, Kiev, Ukraine) at the temperature of 37 °C for 18-24 hours, along with continuous shaking at 50-100 rpm. The MIC values were determined based on the lowest concentration without visible bacterial growth. In addition, the contents of the turbidity-free wells were cultured on the BHI agar and incubated at the temperature of 37 °C for 24 hours in order to obtain the MBC values based on the lowest concentrations without visible bacterial growth on the BHI agar.

3. Results and Discussion

3.1. GC-MS Analysis of ZCEO

The contents of the essential oil obtained from the aerial parts of the plant were obtained in the yield of 1% (w/w) based on the method proposed by Morteza-Semnanis et al. (2005) (0.98%) [8], while the results obtained by Bahmani et al. (2014) differ in this regard [15]. According to

the results of the present study, pulegone (58.78%), menthone (1.15%), and isomenthone (9.91%) were the most frequent components of ZCEO (Table 1). Similar results have been reported by Morteza-Semnanis et al. (2005) [8], while other studies have denoted different compounds (P menth-3-en-8-ol: 14% and pulegone: 46.8%, pulegone: 61.67% and cis-caran-trans-2-ol: 12.66%) to be dominant in ZCEO [15, 16]. The differences in the chemical composition of ZCEO in various studies could be due to the differences in the climate change, extraction methods, changes in standardized or applied hydro distillation, used plant parts, cultivation conditions, genetic factors (e.g., cultivar and maturity of the plants), and geology and regions of plant growth [16, 17].

3.2. Characterization of the Emulsion and Nano-emulsion

Table 2 shows the mean droplet size in the emulsion and nano-emulsion and their polydispersity index (PDI) in the study groups. Accordingly, the mean droplet size in the ZCEO emulsion was 1,116 nanometers, which decreased to approximately 75.12 nanometers after the preparation of the nano-emulsion. This was the first study to apply the nano-emulsion of ZCEO; in another research in this regard, Noori et al. (2018) observed that the droplet size of the nano-emulsion of ginger essential oil (GEO) decreased to 57.4 nanometers [14]. The nano-emulsion of essential oils has been fabricated in some other studies as well, all of which have reported the droplet size to be less than 490 nanometers[11, 14].

According to the information in Table 2, the PDI decreased after the fabrication of the nano-emulsion. PDI was recorded to be 0.898 for the conventional emulsion of ZCEO and decreased to 0.288 after the preparation of the nano-emulsion. In this regard, Noori et al. (2018) reported the PDI to be 0.222 for GEO nano-emulsion, which increased to 0.584 after preparing the conventional emulsion. On the other hand, some studies have reported this value to be less than 0.500 . In the present study, the lower PDI of the nano-emulsion confirmed the efficiency of the ultrasonication method in the formation of a nano-emulsion with uniform size distribution [14].

3.3. MIC and MBC Values of the ZCEO Emulsion and Nano-emulsion

According to the information in Table 3, the examined bacteria showed significant sensitivity to the ZCEO. The MIC value of ZCEO emulsion against E. coli O157:H7 was estimated at 0.8 mg/ml, while the MIC value of ZCEO nano-emulsion was recorded to be 0.025 mg/ml. In a similar research, Anzabi et al. (2013) reported the MIC of ZCEO to be 250 µg/ml for most of the gram-negative bacteria [19].

The current research was the first comparative study to report the MIC values of ZCEO emulsion and nano-emulsion. However, Moghimi et al. (2016) reported that the MIC value of the nano-emulsion of Thymus daenensis essential oil against *E. coli* O_{157}:H_7 was 0.4 mg/ml, which could be due to the increased antibacterial activity of the nano-emulsion compared to its conventional form; the droplet size reduced in the nano-emulsion form, which in turn enhanced the antibacterial activity of the essential oil [10].

Table 1: Chemical composition of essential oil from Ziziphora clinopodioides by GC-MS

No	RT	%	Components	KI	No
1	11.33	0.21	α-Thujene	927	1
2	11.72	1.13	α-Pinene	934	2
3	12.62	0.14	CampHene	958	3
4	13.80	0.89	Sabinene	975	4
5	14.06	1.60	β-Pinene	981	5
6	14.32	0.19	1-Octen-3-ol	986	6
7	14.63	0.55	Myrcene	992	7
8	15.18	0.45	3-Octanol	1003	8
9	16.62	0.67	p-Cymene	1030	9
10	16.78	0.99	Limonene	1033	10
11	16.97	6.79	1,8-Cineole	1037	11
12	18.31	0.65	γ-Terpinene	1063	12
13	19.03	0.16	p-Mentha-3,8-diene	1077	13
14	23.16	2.04	p-Mentha-3-en-8-ol	1159	14
15	23.53	1.15	Menthone	1166	15
16	23.99	9.91	iso-Menthone	1175	16
17	24.17	0.63	neo-Menthol	1179	17
18	24.37	0.30	Borneol	1183	18
19	24.56	0.82	neoiso-Isopulegol	1187	19
20	24.71	0.19	Terpinen-4-ol	1190	20
21	25.27	3.33	iso-Menthol	1201	21
22	27.46	0.27	Thymol methyl ether	1248	22
23	27.70	58.78	Pulegone	1253	23
24	28.39	1.08	Piperitone	1267	24
25	29.74	0.23	Methyl acetate	1296	25
26	30.05	1.43	Carvacrol	1303	26
27	32.31	2.32	Piperitenone	1354	27
28	38.10	0.60	Germacrene D	1489	28
29	40.39	0.11	Z-β-Bisabolene	1547	29
		97.61	Total Identified		

Table 2: Particle size and distribution of the ZCEO emulsion and Nano-emulsion

Group	z-average (d.nm)	PDI
Emulsion of ZCEO	1116	0.898
Nano-emulsion of ZCEO	75.12	0.288

Table 3: Antibacterial properties (MIC, MBC) of ZCEO emulsion and Nano-emulsion against *E. coli* $O_{157}H_7$

Group	MIC (mg/mL)	MBC (mg/mL)
Emulsion of ZCEO	0.8	1.6
Nano-emulsion of ZCEO	0.025	0.05

4. Conclusion

According to the results, ultrasonication was an effective method for the preparation of *ZCEO* nano-emulsion with reduced droplet size to approximately 75 nanometers. Furthermore, the growth inhibitory effect of *ZCEO* and *ZCEO* nano-emulsion against *E. coli* O_{157}:H_7 as a foodborne pathogen was confirmed, indicating the efficacy of *ZCEO* nano-emulsion in the reduction of the bacterial activity compared to the pure essential oil of the plant. Therefore, it could be concluded that the nano-emulsion of *ZCEO* could be utilized as a natural preservative in various food products.

Authors' Contributions

S.Kh., performed laboratory works, A.A., designed the study as M.H., revised the manuscript, and M.A., performed statistical analysis.

Conflict of Interest

There is no conflict of interests in this study.

Acknowledgments

Hereby, we extend our gratitude to Ferdowsi University of Mashhad in Mashhad, Iran for the financial support of this study (Grant No. 3/46031). We would also like to thank Mrs. S. Khajenasiri for assisting us in this research project.

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