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Investigating the Potential of the Essential Oil Nanoemulsion of *Zataria multiflora* Boiss. on the Gene Expression of *stx1A* and *stx2A* in *Escherichia coli* as a Foodborne Pathogen

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Abstract

Background: Several occurrences of infections and intoxications have been globally announced as a result of the *Escherichia coli* contamination of foodstuffs. In addition, the emergence of antibiotic resistance in different geneses of bacteria is becoming a major concern for public/medical health authorities and researchers.

Objective: Accordingly, this study determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the essential oil (EO) nanoemulsion (NEO) of *Zataria multiflora* Boiss against *E. coli*. Finally, different sub-MIC concentrations of the NEO of zataria EO on the growth rate and the gene expression of *stx1A* and *stx2A* were investigated as well.

Materials and Methods: Oil in water NEO was formed by the phase inversion technique. The mean diameter of droplets and the zeta potential of NEO were determined, and then the MIC and MBC of EO and NEO were estimated using the broth microdilution method. Eventually, the growth rate and expressions of the *stx1A* and *stx2A* genes of *E. coli* were evaluated after exposure to various sub-MICs.

Results: Based on the results, carvacrol was the main constituent of the EO, and NEO droplets had an average size of 61.5 nm and a zeta potential of -27 mV. Further, the MIC values of EO and NEO were 0.45 ± 0.17 and 0.25 ± 0.10 mg/mL, and their MBCs were found 0.55 ± 0.20 and 0.30 ± 0.05 mg/mL, respectively. Furthermore, NEO expressed a stronger inhibitory effect against *E. coli* growth compared to pure EO. At 75% MIC of EO, the transcriptional rate of *stx1A* and *stx2A* decreased 2.24 and 2.66 times at the end of the 72-hour period compared with the control, respectively. After 72 hours, treatment with 75% MIC of NEO resulted in the down-regulation of *stx1A* and *stx2A* as 4.75 and 4.80 fold, respectively.

Conclusion: The greater activity of the NEO of *Z. multiflora* Boiss. in comparison with pure EO for slowing down the growth of *E. coli* and Shiga toxin production shows its potential as a novel 'green' food-grade preservative.

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Background

Escherichia coli O157:H7 is reported as the most prevalent serotype of enterohemorrhagic *E. coli* (EHEC), and several outbreaks of food-borne infections and intoxications have been announced throughout the world due to the *E. coli* contamination of foodstuffs.[1,2] One of the pathogenicity mechanisms of EHEC is related to Shiga toxins (e.g., *Stx1* and *Stx2*) which are the most potent known biological poisons.[2] These toxins enhance the expression of nucleolin (i.e., the major nucleolar protein of growing eukaryotic cells) by enterocytes, helping *E. coli* adhere and colonize on the colon wall. On the other hand, Shiga toxins disrupt the mechanism of protein synthesis in eukaryotic cells and cause hemolytic uremic syndrome and hemorrhagic colitis.[3,4] According to previous studies, *Stx2* toxin shows heat-resistance and survives at a usual cooking process (even when the central temperature increases up to 72°C) and heat industrial processing. It is further reported that *Stx2* toxin represents 400 times more virulence compared to *Stx1*.[5]

Due to the growing consumer awareness of the harmful effects of synthetic additives and concerns about occurring microbial resistance toward conventional preservatives, applying natural antimicrobials as food preservatives has been one of the most important subjects of food industry studies. Many herbs and spices possess antimicrobial potentials. The genus of zataria (*Zataria multiflora* Boiss.) from the Lamiaceae family has many medicinal properties, and the fresh or dried leaves are commonly used in marinades, yogurt, ready-to-

eat foods, and barbecues. Among the major ingredients in its essential oil (EO), carvacrol and thymol have the highest antimicrobial activity. In addition, Zataria EO is extensively applied as a disinfectant, antioxidant, and flavoring agent in the food and pharmaceutical industries.[6] Despite the considerable potential of herbal EOs as antioxidants and antimicrobials, some problems cause restrictions in their usage, including high volatility of phenolic compounds and low solubility in water.

To protect hydrophobic bioactive compounds against environmental adverse conditions and improve their solubility and stability, different encapsulation methods are used, including nanoencapsulation, microemulsion, and nanoemulsifying. Nanoemulsions (NEOs) are optically transparent or semi-transparent due to their small droplet size and show high thermodynamic stability against precipitation and accumulation.[7]

Further, NEO particles are thermodynamically driven away toward organisms which have a lipid structure in their membranes, and this attachment improves by electrostatic gravity between the cationic loads of NEO particles and the anionic loads of the outer membranes of microorganisms. The energy of NEO is released when it is sufficiently attached to the pathogen thus the lipid membrane of the pathogen becomes unstable and causes cell digestion and death. This is one of the main antimicrobial mechanisms of the NEOs.[8]

Several studies have investigated the formulation and antimicrobial effects of NEOs. For instance, Sundararajan et al showed that the NEO of *Ocimum basilicum* L. leaves exhibited significantly higher antibacterial activity against pathogens compared to pure EO.[9] Moreover, Li et al formulated a NEO of finger citron (*Citrus medica* L. var. *sarcodactylis*) and concluded that it had high physical stability and antibacterial activity.[10] Similarly, Zhang et al reported that blended tarragons/cinnamon NEOs had good structural, thermal, and storage stability compared with nonencapsulated EO and implied higher antimicrobial activity against the common microorganisms.[11]

Thus, the aim of this work was to measure the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the NEO of zataria against *E. coli* O157:H7. Furthermore, the study sought to investigate different sub-MICs of zataria NEO on the growth rate and evaluate the effect of different sub-MICs on the gene expression of *stx1A* and *stx2A*.

Materials and Methods

Materials

Zataria multiflora Boiss. was harvested in Mid-June-July from zataris plants in Shiraz (Fars, Iran). The taxonomic identification of the plant was confirmed by the Institute of Medicinal Plants, Medical University of Tehran, Iran. Additionally, Tween® 80 (polyethylene sorbitan monooleate, 822187) synthetic grade and Span® 80 (sorbitan monooleate, 840123) synthetic grade were ordered from Merck-Millipore (Darmstadt, Germany). All chemicals were obtained from Merck (Darmstadt, Germany).

Essential Oil Analysis

The zataria EO was analyzed by gas chromatography (GC, Thermo Quest® 2000, UK) equipped with a DB-5 capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness, Agilent Technologies, USA). A certain condition was used to acquire the data. The initial temperature was 50˚C, and then it was increased by a rate of 2.5˚C/minutes to reach the final temperature of 265˚C, and finally, the injector temperature was adjusted at 250˚C. Additionally, the injection volume was chosen as 0.5 µL and employed by an autosampler (autosampler 7693-100 positions, Agilent Technologies, USA). In addition, helium was utilized as the carrier gas, the split ratio was 120, and the pressure of the column head was recorded as 24.9 kPa. Further, a flame ionization detector (Agilent 6890, Agilent Technologies, USA) was applied at 200 Hz, and gas chromatography-mass spectroscopy (Thermo Quest Finnigan®, UK) was conducted with the same capillary column in order to analyze the EO. Furthermore, the MS was done in the electron ionization mode (ionization energy: 70 eV). Moreover, EO constituents were identified based on the comparison of relative retention times and mass spectra with the standards.[12] Relative retention indices were calculated using N-alkanes (C_8-C_{20}) as reference points, and the reported data in reference books and standard libraries (Wiley 275.L and 7n.L).[13]

Preparation of Nanoemulsion

The oil in water emulsion (O/W) of zataria EO was formed at ambient temperature by the emulsion phase inversion method through blending 5, 10, and 15 wt% of zataria EO with 90, 85, and 80 wt% water, respectively, and 5 wt% surfactant (Tween® 20). The EO and Tween 20 were mixed with a magnetic stirrer (Favorit Company, Malaysia) at 800 rpm for 30 minutes at room temperature. Then, sodium phosphate buffer (5 mM, pH=7) was added at the rate of 3.5 mL/min and mixed at 800 rpm for 60 minutes.[14]

Measurement of NEO Droplet Size and Zeta Potential

The dynamic light scattering technique was applied to determine the mean droplet size of zataria NEO by considering a particle size and a zeta potential analyzer (Nanotrac Wave II, Nanotrac Company, USA) apparatus. All samples were dispersed with ultrapure water (1:100 v/v) and run ten times at 25˚C. Additionally, measurements were performed at 25˚C and each experiment was done in triplicate.

Bacterial Strain

The lyophilized cells of *E. coli* O157:H7 (ATCC 35218), with the potential of producing *Stx1* and *Stx2* toxins, was kindly donated by the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Determination of MIC and MBC

The MIC value of different concentrations of EO and NEO was measured through the broth micro-dilution technique. In addition, the test was conducted with a brain-heart infusion (BHI) broth medium blended with 5% (v/v) dimethylsulfoxide (DMSO) as the emulsifying agent and different amounts of EO and NEO. Further, the bacterial inoculum level in each microwell was 6 $\log_{10}$cfu/mL, and the same amount of the BHI broth with DMSO and without EO or NEO was used as blank. The inoculated microplates were incubated at 37°C for 18-24 hours, and MIC was considered as the lowest concentration in which no visible growth was observable. Furthermore, MBC was measured by a subculture of 50 µL from each well with no visible bacterial growth on BHI agar plates after incubating at 37°C.[15]

Growth Evaluation

EO and NEO were added at various sub-MICs (i.e., 25, 50, and 75% MIC) to the tubes of the BHI broth and DMSO (5% v/v). They were then well mixed with inoculums at about 10^6 cfu/mL, and the blank culture only contained DMSO. Moreover, colony counting was done preparing serial dilutions from the BHI cultures kept at 35°C for 24, 48, and 72 hours and spread on BHI agar petri dishes.

RNA Extraction

Escherichia coli was cultured in a BHI broth with the sub-MIC amounts of EO and NEO at 37°C for 72 hours, and RNA was extracted using the TriPure reagent (Roche Applied Science, Germany) at the intervals of 24, 48, and 72 hours of incubation. Briefly, cells were precipitated in polypropylene centrifuge tubes by centrifugation at 12 000×*g* for 5 minutes at 4°C. Then, 1 mL of TriPure was transferred to the tubes containing the cell pellet, and homogenized samples were stored for 5 minutes at 25°C to let the dissociation of nucleoprotein complexes. Next, 0.2 mL chloroform was added to each tube and the mixture was shaken for about 15 seconds before incubation at 25°C for 15 minutes. Then, the tubes were centrifuged at 12 000×*g* for 15 minutes (2-8°C) and three separate phases were obtained accordingly. The following method was applied to precipitate RNA from the colorless aqueous phase.

About 0.5 mL of isopropanol was mixed with the aqueous phase, and then the mixture was shaken and incubated for 10 minutes at 25 °C. Next, the samples were centrifuged (12 000×*g*, for 10 minutes at 2-8°C) to obtain the RNA pellet. The RNA precipitate was washed twice with 1 mL of 75% ethanol and then treated with RNase-free DNase I (Qiagen, Hilden, Germany) in order to remove contaminating DNA. Additionally, RNA was quantified by reading the absorbance at 260 nm. Likewise, nucleic acid purity was evaluated through measuring the A_{260nm}/A_{280nm} ratio by applying a NanoDrop spectrophotometer 2000 (Thermo Scientific, USA). In addition, the quality and integrity of RNA were assessed by the electrophoresis of RNA on a 1% agarose gel and staining with ethidium bromide. Finally, the obtained DNA-free RNA was dissolved in DEPC water (diethyl pyrocarbonate treated double-distilled water) and kept at -70°C.

cDNA Synthesis

The reverse transcription of RNA into cDNA was performed using the OmniScript Reverse Transcription kit (Qiagen, Hilden, Germany) and the procedure was provided by the manufacturer. Eventually, cDNA was stored at -20°C.

Real-Time Polymerase Chain Reaction (RT-PCR)

PCR reactions were conducted using Power SYBR Green (Applied Biosystems) according to the procedure of the manufacturer and applying the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). Table 1 presents the primer pairs that were used in the reaction. Thermal cycling conditions were adjusted as one cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The mRNA levels of the target genes (i.e., *stx1A* and *stx2A*) were normalized against the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene, and normalized values were obtained to calculate the ratios of expression levels (relative fold changes) in EO or NEO treated against untreated (control) samples according to the $2^{-\Delta\Delta CT}$ method described in Applied Biosystems User Bulletin No. 2.[16]

Given that SYBR Green detects any double-stranded DNA including contaminating DNA, primer dimers, and PCR products from misannealed primers, and a dissociation (melting) curve were run following the RT-PCR.

Statistical Analysis

All data (i.e., MIC and MBC determination, growth experiments, and PCR experiments) were obtained in triplicate assays and statistically analyzed by SPSS 22.0 (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance and a two-sample *t* test. Further, data were shown as the

Table 1. The Applied Primer Pairs in Real-time PCR[3,17]

Target Gene	Size (Base Pair)	Sequence (5′-3′)
Glyceraldehyde 3-phopshate dehydrogenase	299	Forward: TCCGTGCTGCTCAGAAACG Reverse: CACTTTCTTCGCACCAGCG
Shiga toxin 1 Subunit A (*stx1A*)	296	Forward: CCATTCTGGCAACTCGCG Reverse: GCAAGAGCGATGTTACGGT
Shiga toxin 2 Subunit A (*stx2A*)	184	Forward: TGCTGTGGATATACGAGGGC Reverse: TCCGTTGTCATGGAAACCG

Note. PCR: Polymerase chain reaction.

mean ± standard deviation, and significant differences were determined at the 95% level.

Results

Chemical Composition of Zataria multiflora Boiss. EO

Based on the GC-MS analysis, 12 components were identified for zataria EO (Table 2) and the main components included the carvacrol (71.12%) as a phenolic monoterpene, gamma-terpinene (7.34%), alpha-pinene (4.26%), and eucalyptol (3.37%).

Size and Zeta Potential of Nanoemulsiom Droplets

In the present study, zataria NEO droplets were found to have a mean diameter of 61.5 nm and a zeta potential of -27 mV.

MIC and MBC

The MICs of zataria EO and NEO against *E. coli* were 0.45 ± 0.17 and 0.25 ± 0.10 mg/mL, and their MBCs were found as 0.55 ± 0.20 and 0.30 ± 0.05 mg/mL, respectively.

Growth Rate of Escherichia coli

As shown in Figure 1A, the 75% MIC of zataria EO decreased the final population of *E. coli* by 2.7, 3.16, and 3.35 $\log_{10}$ cfu/mL after 24, 48, and 72 hours of incubation at 35°C compared to the control culture, respectively. For NEO (Figure 1B), 75% MIC reduced the final cell density of the *E. coli* up to 4.04, 4.24, and 4.78 $\log_{10}$ cfu/mL for the same time length. The reductions in the growth rate were statistically significant compared to the control for both EO and NEO ($P<0.05$), and NEO expressed stronger inhibitory effects against *E. coli* growth in comparison to pure EO ($P<0.05$).

Table 2. Chemical Components of *Z. multiflora* Boiss. EO Identified by GC/MS

Compounds	Amount (%)	Retention Index
Thujene	0.19	930
Alpha-pinene	4.26	937
β-Pinene	0.43	976
Beta-myrcene	0.85	985
Eucalyptol	3.37	10.24
Gamma-terpinene	7.34	1055
Linalool	0.68	1090
Thymyl methyl ether	0.47	1236
Carvacrol methyl ether	0.46	1243
Carvacrol	71.12	1299
trans-caryophyllene	0.41	1418
Globulol	2.32	15.82
Total	91.9	

Note. EO: Essential oil; GC/MS: Gas chromatography-mass spectroscopy.

Effect of Zataria multiflora Boiss. EO and NEO on the Transcription of stx1A and stx2A Genes

According to the melting curve data, no primer dimer or contaminating DNA was observed in the reactions. The melting temperatures of *stx1A, stx2A*, and *GAPDH* were 81.2, 79.5, and 83.5°C, respectively. The results showed that a dose-dependent reduction in *stx1A* and *stx2A* transcription was observed in *E. coli* upon treatment with EO and NEO (Figure 2). As depicted in Figure 2, the transcriptional levels of *stx1A* and *stx2A* decreased 0.32 and 0.25 fold after 24 hours, as well as 2.24 and 2.66 fold after 72 hours when cultured with 75% MIC of EO in comparison with the control, respectively. Based on the results, treatment with 75% MIC of NEO resulted in the down-regulation of *stx1A* and *stx2A* as 0.50 and 0.47 fold after 24 hours and 4.75 in addition to 4.80 fold after 72 hours compared to the control, respectively.

Discussion

Herbal EOs and extracts have long been used as flavoring agents in foods and beverages although following the attention of food regulatory departments and consumers toward natural food grade preservatives, their use has increased considerably in recent years. As mentioned earlier, the main components of zataria EO in this study are composed of terpenes and terpenoid phenols and other aromatic and aliphatic constituents. According to previous studies, the presence of some constituents (e.g., carvacrol) and oxygenated carvacrol derivatives (e.g., carvacrol methyl ether and thymol methyl ether) and terpenes (e.g., γ-terpinene, eucalyptol, p-cymene, and α-pinene) might improve the antimicrobial activity of EOs.[18,19]

Furthermore, the findings of another study, the physicochemical features of NEOs (e.g., surface charge, particle size, shape, and homogeneity) are key factors in determining the rheological properties of nano-colloidal systems, the release of the core material within droplet walls, and the cellular absorption of droplets by microorganisms.[20]

To exert antimicrobial effects, emulsion particles should attach onto the cell membrane and efficiently penetrate through the cell wall layer. It should be noted that surface charge and electrostatic or charge repulsion/attraction between the particles, which is defined as the zeta potential, are considered as determining factors in the attachment of droplets to the outer membrane of the microbial cell wall. The zeta potential shows the potential difference between the dispersion and the stationary phase of the fluid.[21] In our study, zataria NEO particles were indicated to have a zeta potential of -27 mV. It is stated that higher negative zeta potential of emulsion nanoparticles leads to the higher occurrence of cellular uptake compared with other nanoformulations with less negative charge or positive surface charge.[22] It seems that the zeta potential of zataria NEO particles in this study (-27 mV) resulted in a considerable penetration of EO into *E. coli* cells and exerting antimicrobial effects.

As stated by Huang et al, the size of nanoparticles can considerably affect their biophysical properties and

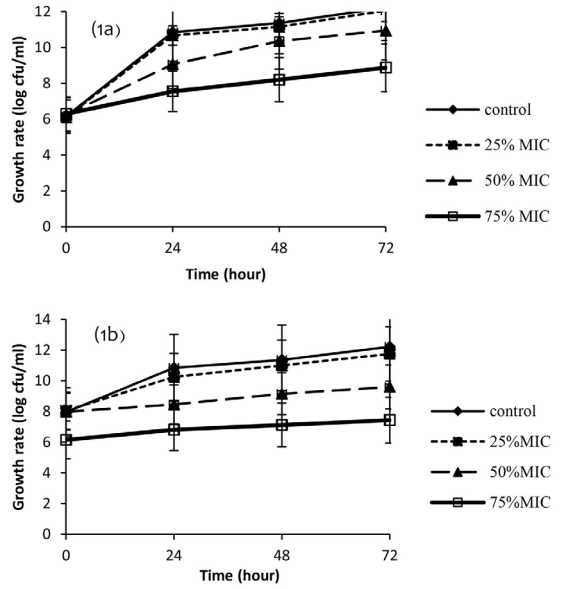

Figure 1. Inhibitory Effect of the Sub-MIC Levels of *Zataria multiflora* Boiss. EO and NEO on *Escherichia coli* O157:H7 Growth at 35 °C
Note. MIC: Minimum inhibitory concentration; EO: Essential oil; NEO: Nanoemulsion.

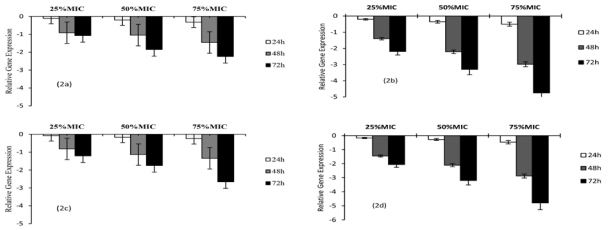

Figure 2. Relative Expression of *stx1A* and *stx2A* Genes Treated With the Sub-MIC Levels of the EO and NEO of *Zataria multiflora* Boiss. in *Escherichia coli* O157:H7.
Note. MIC: Minimum inhibitory concentration; EO: Essential oil; NEO: Nanoemulsion. The housekeeping gene GAPDH was used to normalize the expression level of *stx1A* and *stx2A*. Data are presented as the mean ± standard deviation, and the deviation for three independent experiments is significant at $P<0.05$.

activities.[23] In their study, the antibacterial activity of selenium nanoparticles was strongly size-dependent and 81 nm particles showed the maximal growth inhibitory and killing effect on methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. The data from several previous studies indicated that nano-particle size is a key parameter for maximal antimicrobial activity and smaller particles cause further penetration into the microbial cell.[23,24] In the present study, zataria NEO droplets had a mean diameter of 61.5 nm, which seems to provide considerable penetration of droplets throughout the microbial cell wall.

In this research, the MIC and MBCs of zataria EO and NEO were lower than those of Masoomi et al, indicating 2.5 mg/mL.[25] In addition, Zataria NEO expressed a considerable level of the inhibitory effect against *E. coli* with MIC and MBC of 0.25 and 0.30 mg/mL, respectively, which was significantly stronger compared to pure EO. The obtained data are closely in line with the results of Khatibi et al representing the MIC of 0.30 and 0.15 mg/mL for zataria EO and NEO, respectively.[26]

The data further revealed that zataria EO and NEO slow down the growth rate and the shigatoxin production of *E. coli* in a dose-dependent trend. The results (Figure 1) also showed that zataria NEO had a greater inhibitory effect on *E. coli* in comparison with pure EO. Similar results were obtained in studies reported by Donsi and Ferrari, Zhang et al, and Li et al, demonstrating significant increases in the antimicrobial activities and growth inhibitory effects of EOs by nanoemulsification.[10,11,27,28] The results of another study by Feiz Langaroudi and Kazemi concluded that the NEO of mint and the parsley aqueous extract with a nanometer size reduced the growth rate of *E.coli* highly stronger compared to pure EO and the extract.[29] Similarly, Sundararajan et al demonstrated that the NEO of *O. basilicum* L. had a lower MIC value in comparison to the free EO against all the tested pathogens, and an enhanced inhibitory effect was observed against the growth of microorganisms.[9] Likewise, Moghimi et al reported that the inhibitory activity of *Thymus daenensis* EO against *E. coli* improved significantly when it was emulsified into nano-sized particles. According to their results, nanoemulsification provides easier access to bacterial cells for the EO and improves its ability to disrupt cell wall integrity.[30] Moreover, Shahavi et al concluded that the antibacterial activity of clove EO significantly improved following nanoemulsification since NEO represented higher stability in comparison to pure EO.[31]

Our results showed that the sub-MIC levels of zataria NEO significantly reduced the expression of *stx1A* and *stx2A* genes and even the low sub-MIC levels of NEO demonstrated a greater inhibitory effect as compared to the high sub-MIC levels of pure EO. Similar findings were obtained by Mahmoudzadeh et al reporting the strong effect of the sub-MIC levels of *Carum copticum* EO on decreasing the transcription of the *stx1A* and *stx2A* genes of *E. coli* in the ground beef in a refrigerator.[32] Likewise, Azizkhani et al, Husain et al, and Landau and Shapira concluded that the exposure of *Staphylococcus aureus, Aeromonas hydrophila, E. coli,* and *Pseudomonas aeruginosa* to the sub-MICs levels of herbal EOs or their compounds down-regulated the expression of virulence factors.[15,33,34]

Additionally, Prateeksha et al developed a NEO of *Gaultheria fragrantissima* wall against *E. coli* and reported that the expression of genes including Shiga-like toxins, curli, type I fimbriae, quorum sensing, and *ler*-controlled toxins, which are required for pathogenicity, attachment, and biofilm formation, reduced based on the transcriptional analysis. In their study, the expression of LEE genes and the *ler* gene, which encode and controls *stxs*, respectively, was down-regulated as well.[35] In our study, the inhibitory effects of the sub-MIC levels of NEO against *stxs* expression were about 2-times higher than its bulk form (free EO). Therefore, these results confirmed that the process of nanoemulsification enhances anti-*E. coli* activity of zataria EO and its bioactive

compounds.

The main component of zataria EO in the present study was carvacrol, which has been among the most active compounds against *E. coli* in a study by Xu et al.[36] Carvacrol is a plant secondary metabolite with the monoterpenoid phenol structure. Several studies have focused on the antibacterial activity of carvacrol and reported that it expresses considerable antibacterial activity. It is noteworthy that the mode of the action of carvacrol may be rather related to its high hydrophobic character. In a study by Khan et al, carvacrol completely reduced the growth rate of *E. coli*, causing an increase in the reactive oxygen species level, and the depolarization of bacterial membrane resulted in *E. coli* cell death at its MIC. Moreover, carvacrol showed a significant effect on bacterial membrane disruption and cellular material release. Furthermore, at sub-MICs, a strong effect of carvacrol was reported on the motility of *E. coli* cells and the invasion of human colon cells.[36,37]

Based on the results, the NEO exerted greater inhibitory effects against the expression of *stxs* genes in comparison to pure zataria EO. The ability of the nano-sized droplets of the EO to penetrate effectively into the bacterial cell, interact with cell components, and interrupt its mechanisms is the main reason for NEO efficiency in down-regulating virulence genes.

A modern strategy in the food preservation system is targeting bacterial pathogenicity and virulence factors such as adhesion factors, along with different types of hemolysins and enterotoxins.[38] Considering that the pathogenicity of *E. coli* considerably relies on the production of several extracellular virulence factors and the potential of biofilm formation, the inhibitory effect of antimicrobial agents is not only determined by their growth inhibitory or bactericidal capacity but also by their influence on producing and releasing virulence factors.

Conclusion

Totally, the results of this research indicated the potential of natural antimicrobial agents which show inhibitory effects against virulence factors. The great activity of the NEO of zataria for inhibiting the growth and Shiga toxin production in *E. coli* represents its potential as a novel 'green' food-grade preservative. In addition, the application of high-efficiency delivery systems for EOs (as NEO) can be expected to decrease the required EO concentration. Converting EOs to nano-sized forms provides better dispersion in the food, controls the release rate, slows the evaporation rate, partly masks the odor and taste of EOs, and prevents interaction with other food components. Accordingly, further studies on diverse food models will develop the application of *Z. multiflora* Boiss. NEO as a natural substitute for synthetic harmful food preservatives.

Authors' Contributions

MA: Conception and design, experiment accomplishment, manuscript drafting; FT: Experiment accomplishment, along with data analysis and interpretation; FD: Data acquisition.

Ethical Approval

Not applicable.

Conflict of Interests Discosure

The authors declare that they have no conflict of interests.

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