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Physicochemical, antioxidant, antibacterial and antibiofilm activity of *Carum copticum* essential oil nanoemulsion on *Escherichia coli* O157:H7 and *Listeria monocytogenes*

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Abstract

Carum copticum essential oil (CEO) is used to prevent the growth of food-borne pathogens. The *Carum copticum* essential oil nanoemulsion (CEON) was prepared using low energy sonication at 0, 2.50, 5.00 and 10 min based on surfactant to-oil ratio (SOR=1). Chemical composition, antimicrobial and antibiofilm properties of CEON were examined. Our data showed that the average diameter of the droplets of CEON was between 46.89 and 120.90 nm. The MICs of CEON and CEO against *E. coli* O157:H7 and *L. monocytogenes* were tested. *L. monocytogenes* was more sensitive than *E. coli* O157:H7. The sonication time and the total viable bacteria (TVC) in the study were inversely related to each other. Furthermore, CEON at the 4.00 × MIC concentration and contact time of 20 min caused 77.14% and 67.03% reduction of *E. coli* O157:H7 and *L. monocytogenes* biofilms, respectively. The antibiofilm activity of CEO was significantly lower than CEON and caused a 62.60% and 43.86% reduction of *E. coli* O157: H7 and *L. monocytogenes* biofilms, respectively. The results showed that CEON produced by low energy sonication would have a higher antibacterial efficiency than non-encapsulted essential oil.

Introduction

Today, there is an increasing need for safe food, which does not allow the growth of harmful microbes or chemical changes that can have negative effects on its shelf life. This is usually done by adding antibiotics and antioxidants.[1] Efforts are also made to expand the use of plant essential oils to reduce defects caused by the use of traditional methods.[2,3] Plant essential oils are generally safe to use in food systems. These anti-microbial compounds are used as an active packing system to increase the shelf life of food throughout the storage phase and to prevent the growth of pathogenic bacteria causing diseases that can infect or even contaminate food.[4,5] There are many scientific resources showing their role as an antioxidant,[6,7] antimicrobial,[8] antispasmodic,[9] antibiofilm,[10] antifungal and anti-carcinogenic factors.[11] Phenolic compounds are generally the main factor of antimicrobial properties of essential oils (EOs).[12]

Carum copticum is a plant growing in arid and semi-arid regions of Central Europe, India, Iraq, Azerbaijan, Afghanistan, Pakistan and Iran (Sistan and Baluchestan) which is called Zenyan in Persian. Its essential oils include α-pinene, paracymene, terpinene, β-pinene as well as carvacrol and thymol compounds which can be used in traditional medicine.[7] The components of this plant were identified as six main chemical compositions, including thymol 49.00%, gamma terpinene 30.80%, ρ-cymene 15.70%, β-pinene 2.10%, myrcene 0.80% and limonene (0.70%).[13] However, it was also found that EOs was consisted of 15 different components and three unknown components. It was also found that 95.44% of its components were thymol, ρ-cymene and γ-terpinen.[14]

It has been used to reduce or eliminate many pathogens transmitted through food, most notably *E. coli* O157:H7 and *L. monocytogenes*.[15,16] The *L. monocytogenes* is a food-borne pathogen responsible for many serious human diseases.[17]

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Additionally, *E. coli* O157:H7 is a food-borne pathogen that has serious implications for the public health and economy of the global community.[15]

Ultrasound is one of the ways to obtain nano-emulsions (oil/water).[18] In particular, low-power ultrasound (LPU), which is currently an effective and inexpensive method and, is used in the study of physicochemical properties for food emulsifiers based on spontaneous emulsification.[19] The emulsion inversion point (EPI) method consists of two phases, aqueous phase and oil phase. Most previous studies have focused on inappropriate materials for food applications using the EPI Method to create nanoemulsions. Emulsions contain relatively large droplets that scatter light very strongly, therefore, tend to be opaque or very opaque which is desirable for some applications and undesirable for others.[20,21] Nanoemulsions are inversely related to sound time, emulsifier concentration, energy level, and the concentration and type of the applied surfactant (Tween 80, Span 80).[20]

The current work was carried out to promote the use of the low-energy ultrasound method in production of *Carum copticum* essential oil nanoemulsion (CEON) and to evaluate of the antioxidants, antimicrobial and antibiofilm properties.

Materials and Methods

Chemicals. Tween 80 (Sigma-Aldrich, St. Louis, USA) was used as a non-ionic emulsifier due to its wide range of hydrophilic characteristics. It is frequently used in "oil-in-water" emulsions as an emulsifying agent. Sodium phosphate solution (5.00 mM, pH 7.00), was purchased from Sigma-Aldrich Company. Double distilled water (DDW) was used to prepare almost all solutions used in the study.

Preparations of CEO. Seeds of *Carum copticum* were purchased from local markets in Mashhad, Khorasan Razavi. The collected materials were dried at room temperature (~ 25.00 ˚C) and powdered using a laboratory blender. The studied plant was identified as *Carum copticum* by a botanist. Powdered sample (100 g) were mixed with 500 mL distilled water and subjected to hydrodistillation using a Clevenger-type extractor for 3 hr until total extraction of oil. The obtained oils were dried over sodium sulfate and stored at 4.00 ˚C until use.[22]

Chemical composition of CEO. The components of CEO were determined by flame-ionization detection gas chromatography (GC-FID, 6890; Agilent Technologies Inc., Santa Clara, USA). A capillary column was used and interfaced directly into the ion source of the mass selective detector. Helium was used as the carrier gas at a flow rate of 0.80 mL per min. The column temperature was programmed from 50.00 to 240˚C at 4.00 ˚C per min, then from 240 to 300 ˚C at 17.00 ˚C per min and finally held isothermal for 4 min. The temperatures for ion source were 220 ˚C and for GC injector was 290 ˚C. The components were identified based on gas chromate-graphic retention indices, mass spectra from Wiley MS Chemstation Libraries (Hewlett-Packard, Palo Alto, USA) and the literature.[23]

Preparation of CEON. A Tween 80 (10.00% w/w) was poured drop wise on sodium phosphate buffer solution as an aqueous solution and stirred by a magnetic stirrer for 30 min at 750 g. The CEO (10.00% w/w) was added progressively into the aqueous solution and mix for 30 min. The obtained emulsion was sonicated at (0, 2.5, 5 and 10 min) using a SONOPULS ultrasonic Homogenizer (HD2200; 150 W, 20.00 KHz with a VS 70 T probe; Bandelin, Berlin, Germany). The samples were placed in a bowl containing ice cubes during the sonication procedure**.** The obtained CEON nanoemulsions were poured in an amber glass vials and sealed with a tight lid, then stored under dark conditions at 4.00 ˚C.[21]

Particle and droplet size measurement. The VASCO™ nanoparticle size analyzer (Cordouan Technologies, Pessac, France) was used to detect the weighted average diameter of the density at room temperature and scattering angle 90.00°, based on the dynamic light scattering. The span was determined using the formula:

$$Span = (d90–d10) / d50$$

where, *d10*, *d50* and, *d90* represent the relative cumulative particle sizes corresponding to the intensity of 10.00, 50.00 and 90.00% of the distribution curve. The physical stability of nanoemulsion, prepared after 10 min sonication, was assigned by measuring the droplet size to see the effect of the time factor. To ensure the possibility of phase separation, the centrifuge was applied to the Nano samples at 4,000 *g* and 25.00 ˚C for 30 min.

Zeta potential test. Zeta potential voltage was carried out using a Zetasizer Nano (Malvern Instruments Inc., Westborough, USA) and indicated the degree of electrostatic dissociation between the neighboring particles charged with dispersion based on the size of these particles and their resistance to assembly or their easy compliance with dispersion.[24,25]

Antioxidant activity of CEON. Different concentrations of CEON including 6.25, 12.50, 25.00, 50.00, 100, 200, 400 and 800 µg mL^{-1} were prepared. The 2,2-diphenyl-1-picyrylhydrazil (DPPH) solution was prepared by dissolving 4 mg of powder DPPH in 100 mL of methanol (Merck, Darmstadt, Germany). The optical absorbance of samples was read at 517 nm for a period of 30 min compared to methanol.[26] The percentage of free-radical inhibition was calculated using the following equation:

$$I\,(\%) = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

where, A_{blank} represents the absorbance of the control (comprising all reagents except the test compound) and A_{sample} refers to the absorbance of the test compound. Extract concentration providing 50.00% inhibition (IC_{50}) refers to the effective concentration at which DPPH radicals are removed from 50.00%. This was achieved through interpolation and utilizing linear regression analysis. Butylated hydroxy toluene (BHT; Merck) was applied as a control.

Bacterial strains. Two types of food-borne pathogenic bacteria were used, including *E. coli* O157:H7 (Gram-negative bacteria) and *L. monocytogenes* ATCC 7644 (Gram-positive bacteria) from the Food Hygiene and Aquaculture Department at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. Bacterial strains were kept in the food safety laboratory until test time in Brain Heart Infusion (BHI; Merck) and glycerin at 25.00% at – 80.00 ˚C.[27]

Preparation of microbial suspension. Microbial suspensions were prepared from 24 hr culture of bacteria using a physiological serum and its opacity was compared to the McFarland 0.50 standard.

Disc diffusion method. To determine the antimicrobial properties of the CEO and CEON, the disc diffusion method using Muller Hinton agar (MH; Merck) was adopted.[28] Blank paper disks (6.00 mm) were placed at a given distance from each other and on the edge of the plate on the agar. The amount of 20.00 µL of various concentrations of essential oil was added to the disks. Gentamicin disks (10.00 µg per disk) were used as positive controls.

Broth microdilution method. This test was performed using a 96-well polystyrene flat-bottomed microtiter plate (MTP; Merck). The outcomes of the broth microdilution way were reported in MIC using a 0.01% triphenyltetrazolium eye coloring method. Then, 100 µL of the wells where the growth of bacteria was stopped (no opacity) were transferred to plates containing the medium MH and incubated for 24 hr at 37.00 ˚C, and the lowest condensation of CEON, in which 99.90% of the bacteria did not grow was considered MBC. All the tests were performed in three replications and the average of the data acquired was presented as MBC and MIC outcomes.[29]

Broth macrodilution method. The grading dilution method was used to assess the effect of the sonication and the CEON concentration on the antimicrobial properties of CEON. The serial dilutions of CEON were prepared as follow: 0.00, 500, 1,500, 2,000, 2,500, 3,000 and 3,500 mg mL^{-1}. Then they were poured into sterile test tubes containing 500 µL soybean broth (Merck).[29]

Time-kill assays. One milliliter of 1.00×10^6 CFU mL^{-1} bacterial cultures was added to the tubes containing 9.00 mL of BHI medium with 0.50 × MIC, 1.00 × MIC and 2.00 × MIC from CEON. The positive control was prepared using 10.00% (w/v) of CEO. Non-nanoemulsion tubes were used as negative controls. All the tubes were shaken at 150 rpm at 37.00 ± 1.00 ˚C and the aliquots of samples were collected at 0, 30, 60, 90, 120, 150, 180 and 210 min.[30] A tenfold preparation was prepared with ten levels of dilution in 0.1% of peptone water and was plated on Palcam agar (Oxoid, Wesel, Germany) and sorbitol MacConkey agar (Oxoid) to enumerate the number of *L. monocytogenes* and *E. coli* O157:H7.[31]

Biofilm assays. This test was performed using 24-well flat-bottomed polystyrene microtiter plates (Merck). 200 µL stationary phase suspension of tested bacteria (OD600 = 0.10) was inoculated to each well filled previously with 1800 µL of BHI broth using four wells per isolates to reach a suspension with 1.00×10^7 CFU mL^{-1} per well. The MTP plates were incubated for 24 hr at 37.00 ˚C. The planktonic cells in wells were then removed and washed three times with phosphate-buffered saline (PBS; Sigma-Aldrich) and air-dried for 20 min at 23.00 ± 2.00 ˚C. A volume of 2,000 µL of 0.50 × MIC, 1.00 × MIC, 2.00 × MIC and 4.00 × MIC concentrations of CEON and CEO (10.00%) were gently poured in the wells and incubated for 15 min at ambient temperature.[32] The solution was then removed, and MTP plates were washed three times with PBS and air-dried for 20 min at 23.00 ± 2.00 ˚C. Then, biofilms were stained with 2.00 mL of 1.00% w/v crystal violet (CV, Merck) for 30 min and washed twice with tap water. Then they were left to dry. Biofilm was determined by eluting CV with 2.00 mL 95.00% ethanol and by measuring the optical absorbance of the eluted dye at 540 nm with a Microtiter plate reader (Bio-Rad, Richmond, USA). Wells containing only BHI broth and, BHI and bacteria without any treatment were designed as controls. The biological percentage was determined by applying this formula.[32]

$$Reduction\ (\%) = (C–B) – (T–B) / (C–B) \times 100$$

where, C = OD_{540} nm of control wells, B = OD_{540} nm of negative controls and T = OD_{540} nm of treated wells.

Statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) by Tukey test using SPSS (version 22.0; IBM, Armonk, USA). A probability level of $p < 0.05$ was used in testing the statistical significance of all experimental data. All the experiments were replicated three times and data were expressed as mean values ± standard deviation (SD).

Results

The GC-MS essential oil analysis of *Carum copticum* showed that it was consisted of 12 oil compounds. The ratios of the EO components were measured by the peak area using the FID detector. The basic components of this essential oil were thymol (44.18%), γ-terpinene (28.50%) and o-cymene (19.55%), (Table 1).

Table 1. Chemical composition of *Carum capticum* essential oil.

Components	Retention index (min)	Sum area (%)
2-thujene	12.04	0.73
α-pinene	12.50	0.31
Camphene	13.33	0.94
β-pinene	14.79	0.43
β-myrcene	15.16	0.34
α-phellandrene	16.28	0.09
α-terpinene	16.89	0.31
o-cymene	17.46	19.55
Limonene	17.67	3.05
γ-terpinene	19.51	28.50
Thymol	29.53	44.18
Carvacrol	29.84	0.524
Total	-	98.96

The diameter of the droplets of CEON was calculated at 25.00 ˚C for all the tested samples. The average diameter of the droplets was between 46.89 and 120.90 nm.

Our results showed that zeta values ranged from – 5.00 to – 60.00 mV, confirming that CEON stability was high and could maintain stability of the properties and the size of the droplets for a long time. This was not only related to the Tween 80, but also on the type of EO used, method of sonication as well as preservation method. However, Tween 80 was a non-ionic surface material, but the change in zeta values could be due to changes in factors such as definite stability or storage conditions such as light, temperature and packaging.

Based on the results, the CEO and CEON showed radical scavenging activity with IC_{50} of 1.80 µg mL^{-1} and 1.07 µg mL^{-1} respectively. The antioxidant activities of CEON was lower than BHT (0.77 µg mL^{-1}).

The inhibitory effects of gentamycin and CEON on *E. coli* O157:H7 and *L. monocytogenes* were estimated and the diameter of inhibition zones according to the concentrations of CEO and bacterial type were different. The results showed that the diameter of the bacterium prevented the growth of the bacteria varied significantly ($p < 0.05$) based on the difference in the type of bacteria research resource and the concentration of CEON compared to gentamycin used to study commercial and natural antibiotics.

The MICs of CEON and CEO against *E. coli* O157:H7 were 75.00, 25.00 mg mL^{-1} respectively, while those against *L. monocytogenes* were 37.5 and 6.25 mg mL^{-1}, respectively. The MBCs of CEON and CEO against *E. coli* O157:H7 were 150 and 50.00 mg mL^{-1}, respectively, while those against *L. monocytogenes* were 75.00 and 12.50 mg mL^{-1}, respectively.

The study results of the influence of time of the sonication and concentration of CEON (0.00, 500, 1000, 1500, 2000, 2500, 3000 and 3500 mg mL^{-1}) on the antimicrobial properties against TVC (log CFU mL^{-1}) were measured after incubation for 18 hr at 37.00 ˚C (Fig. 1).

The results showed that A, B, and C of the CEON solution, during the first half-hour of incubation, and the bacterial number were decreased slightly, but gradually increased with increasing the incubation period (Fig. 2). The differences between them were significant ($p < 0.05$). The differences were clearer and more significant compared to previous inhibition of bacterial groups to NC (non-CEON) and positive control (PC) 10.00% CEO (w/v) groups. In this case, the PC showed more inhibitory activities against *L. monocytogenes* compared to other groups and with *E. coli* O157:H7 in BHI broth. Therefore, we could state that 10.00% CEO was more efficient in inhibiting the growth of both *E. coli* O157:H7 and *L. monocytogenes* compared to nanoemulsions. The reason for *E. coli* O157:H7 may be higher resistance to CEON than *L. monocytogenes* in BHI broth due to poor bacterial growth rate in the BHI broth.

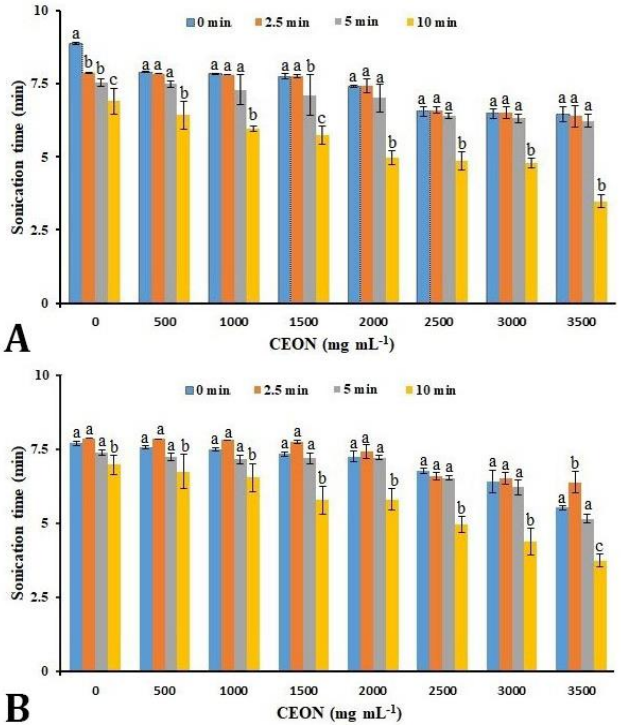

Fig. 1. Effect of sonication time (min) and CEON concentration (mg mL^{-1}) on **A)** *E. coli* O157:H7 (log CFU mL^{-1}) count and **B)** *L. monocytogenes* (log CFU mL^{-1}) count.
[abc] Different letters indicate significant differences at $p < 0.05$.

The biofilm reduction (%) increases in CEON from 1.00 × MIC concentration to 4.00 × MIC concentration for *E. coli* O157:H7 (Fig. 3A) and *L. monocytogenes* (Fig. 3B). CEON at the 4.00 × MIC concentration and contact time of 20 min caused 77.14% and 67.03% reduction of *E. coli* O157:H7 and *L. monocytogenes* biofilms, respectively. On the contrary, the biofilm reduction (%) was decrease in CEO from 1.00 × MIC concentration to 4.00 × MIC concentration for *E. coli* O157:H7 and *L. monocytogenes*, and CEO at the 4.00 × MIC concentration caused 62.60% and 43.86% reduction of *E. coli* O157:H7 and *L. monocytogenes* biofilms, respectively. On the contrary

for CEO, the ratio of reduction always was increased exponentially with MIC increase for CEON. Moreover, the antibiofilm activity of CEO concentrations was significantly lower than that of CEON ($p < 0.05$) and the activity was decreased with the increase of CEO concentration.

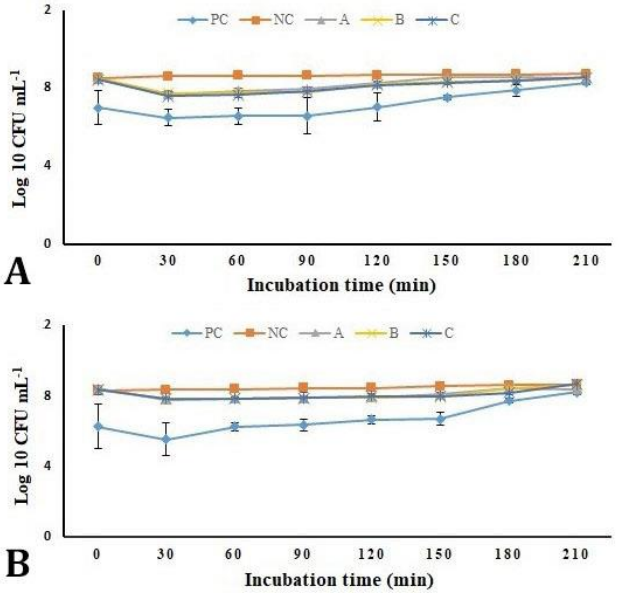

Fig. 2. Bacterial growth (kinetic) curves of **A)** *E. coli* O157:H7 and **B)** *L. monocytogenes* after exposure to different concentrations of CEON. PC: positive control with CEO 10.00%; NC: negative control without CEON; A: 0.50 × MIC; B: 1.00 × MIC; C: 2.00 × MIC.

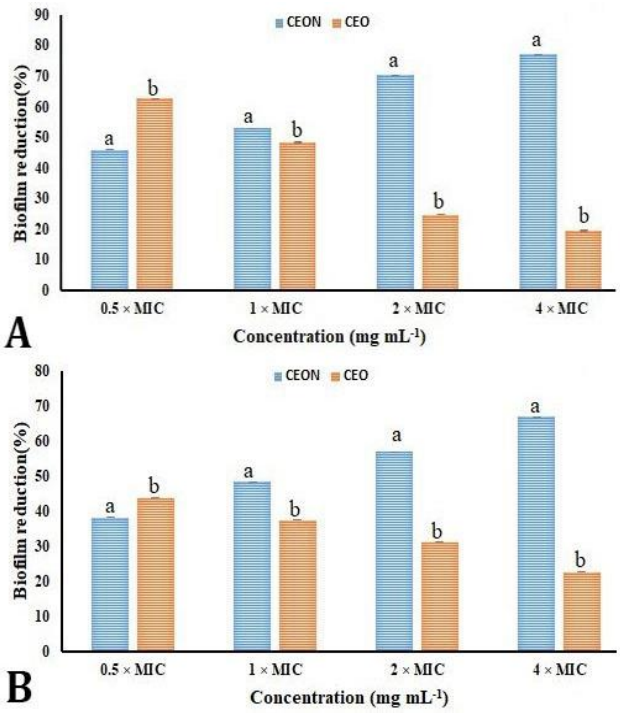

Fig. 3. The effect of CEON and CEO concentrations on biofilm reduction (%) of *E. coli* O157:H7 (A) and *L. monocytogenes* (B). [ab] Different letters indicate significant differences at $p < 0.05$.

Discussion

It has been found that the main component of *Carum copticum* oil was thymol (40.25%), α-terpinene (38.70%) and ρ-cymene (15.80%), which were the same to those obtained by others on EO of *Carum copticum* types found in Iran.[33,34]

Obviously, the longer the duration of exposure to ultrasound, the lower the diameter of droplets, being consistent with what was stated that the rise in the sonication time (to 10 min) reduced the partial size of the droplets to less than 100 nm and it was directly proportional, where the average droplet size was 210.50, 168.90, 114.70 and 90.90 nm at the sonication time 0, 2.5, 5 and 10.00 min, respectively.[35] Furthermore, it significantly increased the inhibition zones against the tested organisms. Moreover, it was found also that the average particle size of *Zataria multiflora* Boiss essential oils nanoemulsion prepared by the inversion phase was 66.50 nm.[36] It might be due to the surfactant-to-oil ratio (SOR ≥ 0.70), type of surfactant (Tween 80), essential oil type and the low or high energy method.[21] In addition, the concentration of essential oil can play an important role in influencing diameter droplets.[37]

To the best of our knowledge, very few reports regarding the DPPH radical scavenging activity of CEON were available. The results in our study were consistent with those who found that the principal components of CEO was thymol (40.25%), α-terpinene (38.70%) and IC_{50} for radical scavenging were between 40 to 60 μg mL^{-1}.[33] In addition, it was found that the CEO reduced the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) with a 50.00% inhibition concentration (IC_{50}) of 20.30 ± 0.90 μg mL^{-1}.[38] In the DPPH assay, CEON exhibited remarkable antioxidant activity. It seems that antioxidant activity of the CEO is associated with high thymol content.[38] Therefore, the CEO can be used as natural antioxidants safely and effectively contributing to preservation of fatty foods during storage by enhancing oxidative stability and activating its activity against food-borne pathogens.[39] The main reason may be the presence of phenolic compounds, which have often been referred to as the vital sieve of free radicals.[40] In this regard, many articles have confirmed the possession of carvacrol, thymol, and c-terpinene for antioxidant properties.[41]

Our findings were consistent with those who stated that the diameter of the inhibition zones of CEON was significantly ($p < 0.05$) lower than that of gentamycin against both types of the bacteria studied.[29] This could be due to a low concentration (10.00% w/v) of CEO in CEON, however, they were not consistent with the results of many other recent studies confirming the application of the high-energy ultrasound method to oregano oil to improve its anti-bacterial activity.[42] In addition, it was indicated that the method of preparation of nano

emulsions of essential oils had a significant effect on the rise in inhibition zones from 12.00 to 20.00 mm against *E. coli* O157:H7 and from 11.00 to 21.00 mm against *B. cereus*.[35] The reason could be attributed to the different type bacteria of the research resource, the type of essential oils used or even their composition as well as the method used in the preparation of nanoemulsions.[43]

However, a number of researchers have confirmed that essential oils containing high condensations of thymol and carvacrol, like thyme, usually inhibit gram-positive bacteria more than Gram-negative bacteria.[44] Our data were in agreement with the above study data since *E. coli* was less sensitive to CEO and *L. monocytogenes* was more sensitive. Although gram-negative bacteria were usually less sensitive to essential oils than Gram-positive bacteria.

What was characterized by nanoemulsion from long-term stability showed that the higher water solubility enhanced ability to penetrate across the biological membranes, and may reach the target organ more easily owing to their small size. However, conversion of EO to nanoemulsion does not necessarily improve its antibacterial efficiency.[29] It was confirmed that the MICs and MBCs of ZEO and ZEON were 5,000 and 2,500 μg mL^{-1} against *S.* Typhimurium, and *L. monocytogenes*, respectively.[29] However, it was mentioned that conversion of essential oils to nano-emulsions increased their properties against bacteria tenfold compared to the normal emulsifier of EO.[45] The main component of the CEO used in this study is Thymol and γ-terpinene, and high MICs of nano-emulsion might be attributed to the low solubility in BHI broth. Some researchers have argued that the use of high-pressure homogenization on anise oil provides stronger antibacterial properties.[46] The MIC was for *Salmonella typhi* (78.00 ± 8.00 μg mL^{-1}), *E. coli* O157:H7 (65.00 ± 7.00 μg mL^{-1}), *Staphylococcus aureus* (14.00 ± 3.00 μg mL^{-1}), *Bacillus subtilis* (5.00 ± 2.00 μg mL^{-1}), *Aspergillus niger* (5.60 ± 1.30 μg mL^{-1}), and *Candida albicans* (8.80 ± 2.20 μg mL^{-1}) of CEO.[33] Furthermore, *Carum copticum* was rich in phenolic monoterpenes (carvacrol and thymol), where its antimicrobial activity was due to hydroxyl groups at different positions around the phenolic ring and it destroyed the cytoplasmic membrane of the cell leading to a bug in the ions and loss in ATP.[47]

We believed that the reason was the smaller size of the nano-droplet at a time of higher resonance than the other. The same was true for CEON's focus where the relationship between them and TVC was an inverse relative relationship. We strongly agree that the effect of sonication time (min) and concentration of ZEON (500, 1000 and 2,000 ppm) on the antimicrobial features versus the whole viable biomass (log CFU mL^{-1}) resulted in a decrease in total viable bacteria around 2.00 CFU mL^{-1} for both positive and negative bacteria.[35] The antibiofilm activity of the ZEO concentrations was significantly lower than that of nanoemulsion ($p < 0.05$) and the activity was decreased with the increase of ZEO concentration from 1.00 × MIC to 4.00 × MIC. This difference in effect was between CEO and CEON, which might be attributed to a phase separation that usually was accelerated when the CEO was increased and caused contact with CEON with biofilm. It was also varied according to the type of emulsion or nanoemulsion or by type of positive or Gram-negative bacteria.[48]

In conclusion, CEON using the low energy method to produce nano-size droplets would have higher antibacterial activity than pure oil alone. These findings clearly indicated that nanoemulsion played an important role in eliminating pathogens. Further study is required for adding CEON to installation of edible films and using it as antioxidants against bacteria to preserve food and to increase shelf life.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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