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(Research Paper)

Nanoemulsification of *Zataria multiflora* essential oil and its antibacterial and anti-biofilm activity against foodborne pathogen *Salmonella typhimurium*

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Abstract:
Although *Zataria multiflora* essential oil (ZEO) has good antimicrobial properties like other EOs, it cannot be used in food systems and commercial products due to its high volatility and low water solubility, and its bioactive compounds can be easily degraded under the influence of environmental factors. In this case, their nanoemulsification may be an efficient and practical approach to overcome this problem. In addition, such delivery systems can improve the stability, bioactivity and quality properties of antimicrobial EOs in food systems. The optimized formulation, prepared using a low energy production method, containing 4% w/w ZEO and 12% w/w mixed surfactant (SDS+Tween 80), produced a transparent and stable nanoemulsion for 90 days with a mean particle diameter of 120.1 nm. The antibacterial activity of pure ZEO (PZEO) and its nanoemulsions (ZEON) against the tested strain was evaluated using solid diffusion, vapour phase and broth assays. Minimum inhibitory concentration (MIC) and bacteriostatic concentration (MBC), dynamic time-kill, anti-biofilm activity and morphological changes in *Salmonella* were determined. The strong antibacterial activity of PZEO and ZEON was demonstrated with MIC values of 2000 ppm and 4000 ppm, respectively. The killing kinetics study showed that there was a rapid and widespread decrease in CFU during the first 15 min of exposure to ZEON at MIC concentration. In addition, ZEON had significant anti-biofilm activity , reducing 82% of the one-day-old biofilm of *S. typhimurium* at the MIC concentration. The SEM micrographs taken at MIC showed significant morphological damage in the ZEON treated bacterial cells and no viability. The data presented, taking into account the optimal performance of antimicrobials against foodborne pathogens, can help in the rational design of delivery systems based on nanoemulsion of essential oils in the food industry.

Keywords: Antibiofilm activity; *Zataria multiflora*; Nanoemulsion

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Introduction:

The increasing prevalence of foodborne diseases in developing and even developed countries has made food safety a major concern for consumers and the food industry (1). To date, more than 250 foodborne diseases have been reported, most of them infections caused by a wide range of bacteria and viruses. According to the World Health Organization (WHO), more than two million people die each year from diarrhoeal diseases, mainly caused by consumption of contaminated food (2). Foodborne pathogens impose a significant burden of infection on populations in different countries of the world. Among food-borne pathogens, *Salmonella typhimurium* is one of the most common causes of food poisoning and related infections, and various methods have been used to control it (1).

Lately, there has been a lot of focus on natural antimicrobials, such as plant-derived essential oils (EOs), which have proven to have antimicrobial properties against foodborne pathogens. Moreover, EOs are generally-recognized-as-safe (GRAS) food additives by the US Food and Drug Administration (FDA) (3), making them a favorable alternative to synthetic additives. Consequently, the food industry has shown great interest in the use of EOs for food preservation. Numerous research studies have investigated the antibacterial properties of EOs and their specific volatile components against microorganisms that cause food spoilage and foodborne illness, both under laboratory conditions and on actual food samples (4).

In this context, essential oils derived from *Zataria multiflora* Boiss (ZEO), which belongs to the *Lamiaceae* family and is found in many parts of the Mediterranean region, have been used in the food industry as flavouring agents, antimicrobials and antioxidants, as well as in the cosmetic industry (5). With a rich source of biologically active compounds such as sesquiterpenes, monoterpenes and their oxygenated derivatives (e.g. aldehydes, ketones, ethers, esters and phenols), it is known as a promising natural compound for reducing or eliminating pathogenic bacteria (4). However, like other EOs, its use in food systems and commercial products is often limited due to its high volatility and low water-solubility (6). Its bioactive compounds are susceptible to degradation by environmental factors such as pH, heat, moisture, light, pressure and oxygen (6). In this case, encapsulation of EOs in well-designed colloidal delivery systems such as nanoemulsions can be an efficient and practical approach to overcoming this problem. Furthermore, these delivery systems may also improve the stability, bioactivity and quality properties of antimicrobial EOs in food systems (6,7).

Oil in water (O/W) nanoemulsions are colloidal systems containing small emulsifier-coated oil droplets as the internal phase dispersed in a continuous aqueous phase as the external phase (8). They are one of the types of emulsions with droplet sizes below 200 nm that are known to be one of the best and most promising colloidal transfer systems for encapsulating EOs (9). Nanoemulsions are important components of many commercial products, including dietary supplements, cosmetics, foods, agrochemicals, pharmaceuticals and personal care products (9).

Methods of producing nanoemulsions can be categorized into low-energy and high-energy methods, each with its own set of pros and cons. Low-energy methods have the advantage of producing smaller droplet sizes compared to high-energy methods (4). Due to the small size of the droplets, nanoemulsions appear translucent or transparent and are more consistent and stable in terms of Ostwald ripening, creaming, flocculation, and coalescence than macroemulsions (10). This property can improve the physical stability, appearance and biological activity of EOs in food and drink products compared to the larger size droplets in conventional emulsions, making them an excellent candidate in the food and pharmaceutical industries as agents for the delivery of drugs and bioactive compounds (11). In terms of antimicrobials, nanoemulsion delivery systems with incorporated compounds significantly increase their antimicrobial activity compared to non-encapsulated systems. Such frameworks can increase the concentration of bioactive mixtures in regions where microorganisms ideally resid (10).

The results of some studies on the antimicrobial activity of nanoemulsions prepared by different methods show that the antimicrobial properties of nanoemulsions are directly related to their preparation methods (7). This study was therefore undertaken: (i) to investigate the optimal conditions for the preparation of ZEO nanoemulsions (ZEON) using spontaneous emulsification as a low-energy method with mixed surfactants and (ii) to investigate the antimicrobial activity of the optimized ZEON and its comparison with pure ZEO (PZEO) against an important foodborne pathogen, *S. typhimurium.*

Materials and Methods:

Material

The ZEO (98%) used in this study was supplied by Tabibdaru Co (IRAN). Its quality parameters such as appearance, color, odor, solubility, purity and chemical properties including Brix, pH and acidity

were described in an accompanying technical report. The chemical composition of the ZEO used was thymol (51.34%), p-cymene (10.37%), γ-terpinene (17.69%), carvacrol (13.76%), linalool (5.20%) and together with other minor compounds.

Tween 20, Tween 80, and Sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Deionized water was used in the preparation of all solutions and emulsions. The culture media consisted of Müller-Hinton broth (MHB), Nutrient agar (NA) and MHA agar supplied by Merck (Germany). All chemicals used in this study were of analytical grade and were used as received.

Preparation of nanoemulsions

ZEONs were prepared according to the method of Anton & Vandamme (2009) (12), with some slight modifications, from a mixture of surfactants (including Tween 20 or 80 and SDS), ZEO (4% v/v), with different surfactant/oil weight ratios (SOR) and deionized water. The oil phase was gradually added to the stirring mixture (~700 rpm) of the aqueous phase (mixture of surfactant and deionized water, previously homogenise with a Vortex® mixer for 10 min at 700 rpm) for 3 h to obtain an oil-in-water emulsion. Nanoemulsions were then prepared using a probe sonicator (MPI, Dattwil, Switzerland) at 20.5 KHz frequency and 30% of maximum power (250 W) for 10 min.

Nanoemulsion properties

The polydispersity index (PDI) and mean droplet size distribution of the nanoemulsions were measured using a Dynamic Light Scattering (DLS) instrument (Zetasizer NanoZS, Malvern Instruments Ltd, Malvern, UK) after dilution with deionized water to avoid multiple scattering effects. The surface charge and Zeta potential were measured using an electrophoresis method on the same instrument. In addition, the stability of the ZEONs was evaluated by determining the change in mean particle diameter and by visual observation to monitor for any phase separation or creaming on days 1 and 90 at room temperature.

Evaluation of antibacterial activity

Agar well diffusion test

The antimicrobial evaluation of PZEO and ZEON on the test bacterium was first performed using the agar well-diffusion test (13). In this assay, 100µl of indicator strain at 0.5 McFarland's concentration were incubated on MHA medium. Agar wells of 10mm diameter were made on preincubated sterile Petri dishes (PD) by gel puncture. Different concentrations of treatments (125 to 4000 ppm) were prepared and added to wells (30µl). The plates were then incubated for 24 hours at the optimum temperature of the indicator strain; the zone of inhibition was observed and measured. The experiment was repeated five times.

Vapor Phase Test (VPT)

The *in vitro* VPT was based on the modified method described by Lopez et al, 2005. Briefly, 14 and 6ml of medium were poured into 90mm plastic PDs and their lids, respectively. After solidification, 20µl of the test bacterial suspension (containing 10^5 CFU/ml) was inoculated into the medium in the dish part. Then, 20µl of each treatment was distributed on the surface of 6 mm sterile filter paper discs (Oxoid) and placed on the medium in the lid of each PD. All PDs were sealed with parafilm and incubated at 37°C for 24h. All assays were performed in triplicate (14).

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

Antibacterial activity (%), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to the broth microdilution test (BDT) recommended by the Clinical and Laboratory Standard Institute (15). It involved the preparation of a series of two-fold dilutions of the test nanoemulsion over the range 125 to 4000ppm in a 96-well microtiter plate (Nunc, Copenhagen, Denmark) filled with cation-adjusted of Mueller-Hinton Broth (CAMHB) (pH 5.9) as a growth medium. Bacteria were inoculated into each well to achieve an initial bacterial count of approximately $1 \approx 2 \times 10^5$ CFU/ml. The contents of the wells were mixed by vortexing and incubated at 35°C. The optical density (OD) of each well was recorded every 2 hours using a microreader (infinite M200 Pro, TECAN, Switzerland) at a wavelength of 600nm (OD600). The antibacterial activity of the treatments was evaluated according to the following equation:

Antibacterial activity (%) = $(OD_c\text{-}OD_s)/ OD_s \times 100$

OD_S: optical density of the sample, OD_C: optical density of the control

The MIC was determined as the lowest treatment concentration in the well without turbidity after 24 hours of incubation. For the determination of MBC, 100µl of wells without growth were transferred to MHA and incubated overnight at 35°C. MBC was recorded as the lowest sample concentration in the well corresponding to the agar plate where no microbial growth was observed. Each test was performed in triplicate (16).

Dynamic Time-Kill Assays

Dynamic time-kill assays of ZEON against *Salmonella* were performed using the modified method of Liang et al. (2012) (17). Briefly, tubes containing MHB supplemented with 0.1, 0.01 and 0.001 dilutions of ZEON and its MIC value were inoculated with a bacterium to a density of ≈ 10^6 CFU/ml in 10ml final volume. They were then incubated at 37°C in a shaking bath for 15, 30, 45 and 60 minutes. At each time point, 100µl were removed and their dilution series were inoculated onto MHA and incubated for 24 hourse to determine viable counts. Experiments were performed in triplicate.

Anti-biofilm activity

Inhibition of *S. typhimurium* biofilm formation was performed according to the procedure described by Hassan et al. (2015), with minor modifications (18). Briefly, bacteria were inoculated into microtiter plates previously filled with MHB broth and sublethal concentrations of ZEON and PZEO to obtain initial numbers of approximately 1≈ 2×10^5 CFU/ml per well. Untreated bacteria were used as negative control. After 24 hours incubation at 35ºC, the supernatant was discarded and the intact biofilm was stained with 1% crystal violet and the inhibition of ZEON and PZEO in the biofilm control was recorded at 570nm (OD_{570} treated wells). The anti-biofilm activity was calculated using the formula (19):

$$\text{Anti-biofilm activity (\%)}: \frac{(\mathrm{N}-\mathrm{C})-(T-TC)}{(\mathrm{N}-\mathrm{C})} \times 100$$

C = $OD_{540\ nm}$ of control (medium only), N = $OD_{540\ nm}$ of the negative control (broth with bacteria), T = $OD_{540\ nm}$ of the treated wells (broth with bacteria and treatment), and TC = $OD_{540\ nm}$ of the treatment control (broth and treatment without bacteria)

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to evaluate the morphological changes in *S. typhimurium* after exposure to ZEON. ZEON at MIC concentration was added to the *S. typhimurium* culture and untreated controls were also performed. After incubation at 35ºC for 10 min and 60 min, 1ml of each sample was removed and centrifuged at 4000g for 10 min at 4ºC. The sections were then prepared for SEM according to the method previously described by Becerril and co-authors (20). *S. typhimurium* morphologies were observed using a field emission gun scanning electron microscope (SEM, Hitachi, Tokyo, Japan).

Statistical Analysis:

GraphPad Prism statistical software, version 6.02 (GraphPad Software, Inc., San Diego, CA, USA) was used. Statistical analysis was performed using one-way analysis of variance and comparison of means using Tukey's test at a significance level of 0.05 ($p \leq 0.05$).

Results and discussion

Effect of oil phase composition

In this study, the low-energy production method was used because it is a fast and relatively simple method for producing nanoemulsions with a narrow size distribution and small droplet size (9). This production method also has real advantages in terms of potential industrial scale-up, formulation efficiency and non-aggressive properties (e.g. against encapsulated fragile active molecules) (12).

Initially, ZEONs containing 4%v/v oil phase (ZEO), 4-12% v/v Tween 20/80 as surfactant and the remaining weight percent aqueous phase (deionized water) were prepared using the spontaneous emulsification method. The results showed that between the two Tweens 80 and 20, the nanoemulsions made with Tween 80 at the same concentration had a better transparency in appearance. In addition, the nanoemulsions made with Tween 20 became duller after a few minutes, but the nanoemulsions made with Tween 80 did not changeIt was therefore concluded that Tween 80 is a more suitable surfactant than Tween 20.

Tween 20 has a relatively linear structure of saturated lauric acid compared to the more complex structure of the unsaturated oleic acid fraction in Tween 80. Tween 20 has a relatively linear structure of saturated lauric acid compared to the more complex structure of the unsaturated oleic acid fraction in Tween 80 (6). According to Wang et al (2000), the introduction of a double bond in the hydrophobic part of non-ionic surfactants leads to the production of smaller and more stable nanoparticles (21).

In a series of tests, the effect of the amount of Tween 80 and SDS surfactants on the formation and stability of ZEONs was determined. Ideally, due to economic issues and cost concerns, as well as taste and toxicity, we sought to produce clear and stable nanoemulsions with the lowest level of surfactant that appeared without phase separation. To this end, eight series of nanoemulsions consisting of ZEO at a constant concentration of 4% v/v (determined on the basis of preliminary studies), 0.25% v/v SDS as co-surfactant and Tween 80 in different ratios were tested to find the best production conditions for the desired nanoemulsions (Table 1).

Table 1: The effect of the amount of Tween 80 and SDS surfactants on the formation and stability of ZEONs.

No.	ZEO (%V/v)	Tween80(%V/v)	SDS(%V/v)	Property of nanoemulsions
1	4	4	-	**Opaque, unstable**
2	4	8	-	**Opaque, unstable**
3	4	12	-	**Translucent, unstable**
4	4	16	-	**Transparent, unstable**
5	4	4	0.25	**Translucent, unstable**
6	4	8	0.25	**Translucent, unstable**
7	4	12	0.25	**Transparent, stable**
8	4	16	0.25	**Transparent, stable**

The results obtained showed that the variety of surfactants and their different ratios cause differences in turbidity, color, and stability of nanoemulsions. The ZEONs obtained from 4 and 8% v/v Tween 80 alone appear opaque (white). In addition, increasing the surfactant (Tween 80) concentration while keeping the ZEO concentration constant slightly reduced the turbidity of the nanoemulsions. The addition of the cosurfactant SDS also had the same effect. As shown in Table 1, 12% v/v Tween 80 alone,and 4% and 8% Tween 80 with SDS resulted in the production of translucent nanoemulsions. When first prepared, these nanoemulsions showed phase separation at room temperature. However, when the concentration of Tween 80 was further increased and a co-surfactant was added at higher levels, the nanoemulsions became completely transparent. The results showed that 16%v/v Tween 80 alone, as well as 12% and 16% Tween 80 with SDS, led to the production of fully transparent nanoemulsions. For further studies, two optimized nanoemulsions were selected based on their transparency and physical stability: Nos. 4, 7and 8.

Properties of nanoemulsions

Particle diameter is an important and fundamental feature that can determine the *in vivo* fate and efficiency of nanoemulsions, so determining the particle size of nanoemulsions is important. Based on the DLS results, the average droplet size (Z-average) and the PDI value for ZEONs are determined. It should be noted that only clear nanoemulsions without phase separation, worming or cracking were evaluated by DLS.

In our study the Z-averages of ZEOs prepared with Tween 80 alone and in combination with SDS were determined to be 222.09 and 121.34 nm, respectively. Shahabi et al. (2017) obtained a similar result for nanoemulsions of ZEOs formulated by a low-energy method using a non-ionic surfactant (polysorbate 20). Their result represents a droplet size of <200 nm (4). In another study by Yazdi et al (2020), ZEON was prepared by mixing oleic acid, ZEO (1:1 v/v), then adding normal saline containing Tween 80 and sonicating with an ultrasonicator. They reported that the droplet size of ZEON was 80 ± 2 nm (22). According to Huang et al. (2010), reducing the droplet diameter in nanoemulsions can be effective in reducing aggregation and creaming, phenomena that can also be prevented by using appropriate surfactants (23,24). In our study, the initial size of ZEON droplets in the presence of SDS as co-surfactant was significantly smaller than in its absence, indicating that the co-surfactant material contributes more to the formation of nanoemulsions.

The PDI values in our formed nanoemulsions using Tween 80 alone and in combination with SDS were around 0.38 and 0.23 respectively, indicating acceptable quality results for ZEO nanoemulsions. It should be noted that low PDI values (PDI < 0.3) indicate a somewhat narrow distribution and droplet size uniformity of EOs in an aqueous system (25), which was observed in the sample prepared with a combination of Tween 80 and SDS. In contrast, the higher the PDI value, the lower the droplet size uniformity. Similarly, Shahabi et al. (2017) reported the development of ZEONs with PDI values around 0.2 (4).

The net surface charge of the ZEON droplets was -7.16 mV. According to the information available, this negative surface charge can cause sufficient electrostatic repulsion between particles. In this regard, Mendes et al. (2018) and Artiga-Artigas et al. (2017) have hypothesized that droplets with a potential ζ below -30 mV have good colloidal stability due to inter-droplet electrostatic repulsion (24,26). The zeta potential obtained in our

study also indicates good electrostatic stability of ZEO nanoemulsions.

Stability

The results showed that between the two nanoemulsions made with Tween 80 alone and in combination with SDS, although both were clear on the first day, the nanoemulsions made with Tween 80 became cloudy after one day and oil particles appeared at the bottom of the container. However, the nanoemulsified ZEOs using Tween 80 + SDS were stable after 90 days and did not change in appearance (Figure. 1).

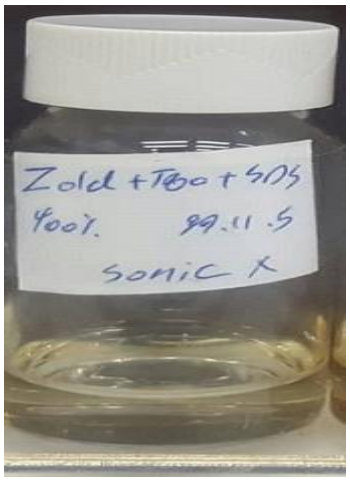

Figure 1. Evaluation of the stability of Zataria multiflora nanoemulsion prepared by spontaneous emulsification method with Tween 80 and SDS surfactants after 90 days.

In fact, no turbidity or phase separation was observed in these samples when stored at ambient temperature for 90 days. In addition, the changes in particle size over this period were not statistically significant. Similar results were reported by Shahabi et al. (2017), who observed that the ZEONs were stable during 21 days of storage at 23 ± 2 °C (4). The lower stability of the ZEON was published by Yazdi et al. (2020), who previously mentioned their preparation method. Their nanoemulsions were stable for 3 days at 5 ± 3°C (22). Compared to the mentioned works, our nanoemulsions prepared with combinations of Tween 80 + SDS had high stability and maintained this stability at normal room temperature. It was therefore concluded that this combination leads to the production of more suitable nanoemulsions. Among these, ZEON produced with a combination of 12% v/v Tween 80 and SDS is preferred due to the use of lower surfactant concentrations, which would be an advantageous in terms of ingredient cost. On this basis, this nanoemulsion was selected for all subsequent studies.

Antimicrobial activity

3.5. Agar well diffusion test

The antibacterial effect of ZEO and its optimized nanoemulsion on *S. typhimurium* is shown in Table 2. Amoxicillin and ampicillin were used as positive controls. The diameter of the inhibition zone also indicates the degree of antibacterial activity of the product or sample in question; in such an assessment, the larger the inhibition zone, the more effective it is against the target bacterium. PZEO oil showed high antibacterial activity against *S. typhimurium* with an inhibition zone diameter of 20 mm, While its nanoemulsified form showed a lower inhibitory effect (13mm). This finding is consistent with previous studies showing that ZEO nanoemulsion has lower antibacterial activity against *Listeria monocytogenes* and *Salmonella typhimurium* compared to its pure form (4).

Table 2: Agar well diffusion and Vapor phase assay results of Salmonella with pure ZEO, ZEO nanoemulsions, and controls.

	Inhibition zone (mm)	
	Agar well diffusion	**Vapor phase test**
Pure ZEO	20.00 ± 0.30[a]	60.00 ± 0.3[f]
ZEO nanoemulsion	13.00 ± 0.50[b]	0.00 ± 0.00
Tween 80	0.00 ± 0.00[c]	0.00 ± 0.00
Amoxicillin	25.00 ± 0.60[d]	0.00 ± 0.00
Ampicillin	10.00 ± 0.20[e]	0.00 ± 0.00

[a-e] Means with different letters within the same column differed references significantly ($p < 0.05$). Values are mean ± standard deviation (n=3)

The results also showed that amoxicillin and ampicillin, as references, exhibited antimicrobial activity against *S. typhimurium*. PZEO and ZEON were more inhibitory than ampicillin.

Tween 80 at the concentration used had no effect on the *Salmonella* tested. ZEO and its nanoemulsion showed good antibacterial activity against the tested bacterium. However, compared to the nanoemulsion form, the results showed that bulk ZEO had a more effective inhibitory effect on *Salmonella*. This result is completely consistent with the published results of some studies, which have shown the antimicrobial properties of EOs do not necessarily increase with the conversion to emulsion or nanoemulsion form (27,28). In contrast, some studies have reported an increase in the antibacterial activity of emulsified EOs compared to their bulk form (29–31). This difference in results can be attributed to differences in the composition of EOs, emulsion formulations, particle diameters and tested bacterial. Another reason for these differences is the presence of the carrier oil, which can reduce the antibacterial activity of nanoemulsions by acting as a solvent for the EOs, thus reducing the amount of EOs available to interact with the bacterium (28).

Vapor phase test (VPT)

The diameters of the growth inhibition zones formed in the VPT for PZEO and ZEON solutions were measured using a caliper and the amount of growth inhibition zone is given in Table 2. ZEON showed no antibacterial activity against selected bacteria when used in the vapor phase. In contrast, PZEO showed strong antibacterial activity in the vapor phase, similar to that seen in the solid phase antimicrobial test. This finding contradicts the results of the study by Ghaderi et al . (2017), who reported stronger potent antimicrobial properties for sage oil nanoemulsions in VPT (29). This is in agreement with the results of Shahabi et al. (2017), who showed that ZEO nanoemulsions showed no inhibition zone against the bacteria tested in VPT compared to pure oil (4). This was attributed to the low concentration of ZEO in the nanoemulsion and the lipophilic EO encapsulated in an oil-water nanoemulsion, which prevents the ZEO from evaporating, thus preventing the EO from coming into contact with the bacterial grass (4).

Determination of MIC and MBC concentrations

Figure 2 shows the results of the inhibition percentage of the antibacterial properties of pure *Zataria multiflora* and its nanoemulsion solution prepared by the microdilution method against the target bacterium.

The solutions tested at increasing concentrations significantly reduced the OD values of the bacterium compared to the control during the incubation period ($p < 0.05$). The antibacterial activity increased with increasing concentrations of PZEO and ZEON from 125 to 4000 ppm ($p < 0.05$). Wells containing PZEO and ZEON at concentrations ≤1000 and ≤2000 ppm were cloudy after 24 hours of incubation, indicating bacterial growth. In contrast,culture media containing bacterial suspension and dilutions of ≥2000 ppm for PZEO and ≥4000ppm for ZEON were completely clear, indicating complete inhibition of bacterial growth. As a result, the MIC for ZEON was proposed to be 4000 ppm, while this value was reduced to 2000ppm for PZEO. Similarly, the MBC values for PZEO and ZEON against *Salmonella* were tested at concentrations higher than the MIC values, which were ≥4000ppm for PZEO, but ZEON was not bactericidal at these concentrations. The result suggested that PZEO was 2 times more antibacterial than ZEON, suggesting that the conversion of these EOs to nanoemulsion form greatly reduces their bactericidal activity. These results are in line with other recent studies showing that conversion of EOs into nanoemulsion forms reduces their antibacterial activity, as mentioned above.

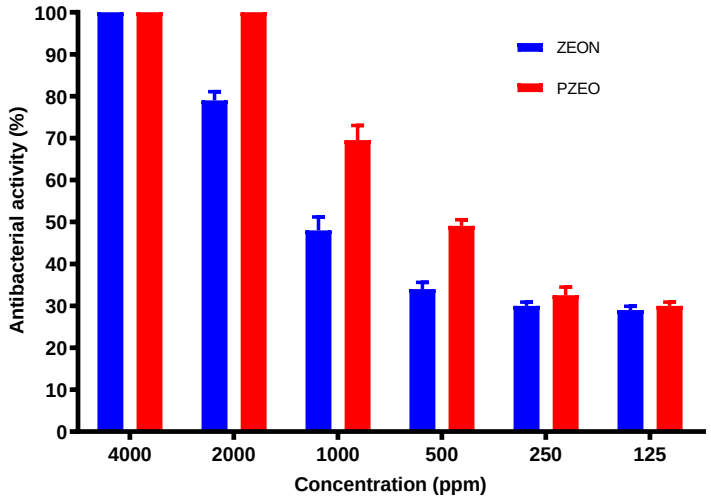

Figure 2. The antibacterial activity of pure ZEO (PZEO) and nanoemulsified ZEO (ZEON) was measured on S. typhimurium after 24 h of cultivation. For each treatment, the vertical bars represent the standard deviations of three replicates.

Killing kinetics assay

A killing kinetics assay was used to explore the possible mechanism of action and to determine changes in the viability of *S. typhimurium* upon interaction with different dilutions of nanoemulsion (ZEON) and its MIC value for 60 min. Killing kinetics studies revealed a change in the viability of *S. typhimurium* upon interaction with ZEON over a short period of time. Figure. 3A shows the killing rate of *S. typhimurium* observed upon treatment with different dilutions of ZEON. For the 40-fold diluted ZEON (MIC concentration), there was a rapid and widespread decrease in the number of viable microorganisms during the first 15 minutes, i.e., from about 10^6 to 100 CFU/ml. This result is consistent with the previous report showing that nanoemulsions can cause a rapid reduction in viable bacterial cells (32). A reduction in CFU was also observed after interaction of other ZEON dilutions, but this reduction was much less dramatic, i.e. from around 10^6 to $10^{1.5}$ (10-fold), $10^{3.6}$ (100-fold), and $10^{5.2}$ (1000-fold) CFU/ml after 60 min.

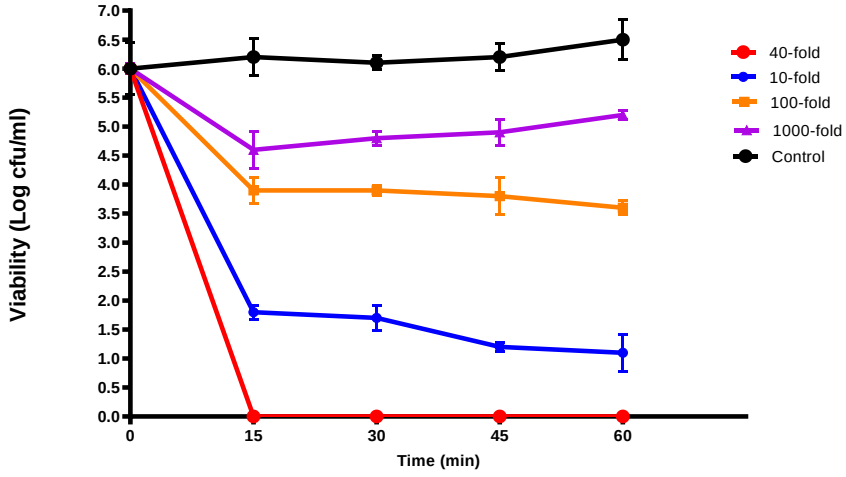

Figure 3.A

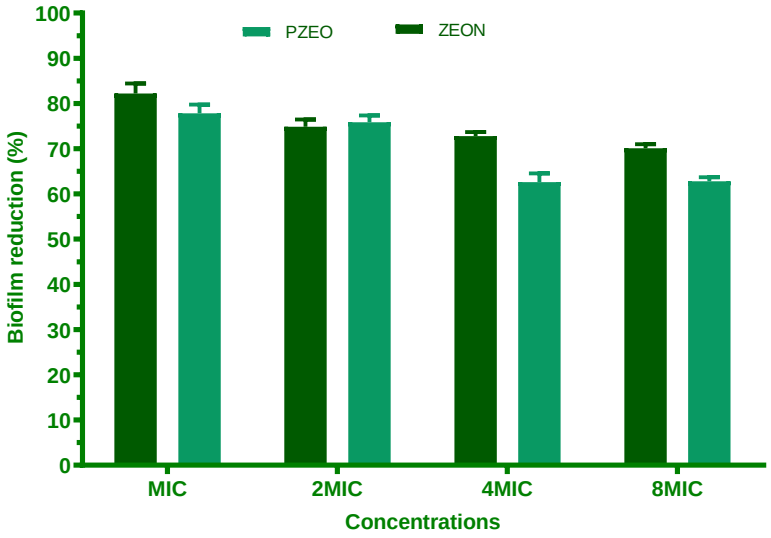

Figure 3.B

Figure 3. A) Surviving cells of S. typhimurium in MHB after exposure of 106 CFU/mL to different dilutions of nanoemulsion (ZEON) for 1 h at 35 °C. Tubes without nanoemulsion were used as control. Differences between all treatments and control were statistically significant ($p < 0.001$). B) Antibiofilm activity of pure ZEO (PZEO) and nanoemulsified ZEO (ZEON) on S. typhimurium after 24 h of cultivation. In each treatment, vertical bars represent standard deviations of three replicates.

Biofilm

The anti-biofilm properties of ZEON and PZEO at MIC dilution against *S. typhimurium* were performed in a 96-cell plate and the results are summarized in Figure 3B. The ZEON concentrations of MIC, 2MIC, 4MIC and 8MIC resulted in biofilm inhibition of 92.21%, 84.88%. 82.80% and 80.10%, respectively. Its anti-biofilm activity for MIC, 2MIC, 4MIC and 8MIC of PZEO is 87.84%, 85/85%, 72.62% and 72.81%, respectively. The results showed that the antibiofilm activity of both treatments decreased as their concentration increased from MIC to 8MIC.

This finding is consistent with the results of Shahabi et al. (2017), who showed that the antibiofilm activity of pure ZEO decreased with increasing concentration. However, it contradicts the results of their nanoemulsions because in their study the anti-biofilm activity of ZEON increased with increasing concentration from MIC to 4MIC (4). This reduction in anti-biofilm properties may be due to phase separation of ZEON and PZEO, which occurs more rapidly at higher concentrations of two treatments reducing their contact with the biofilm. Further studies are needed to investigate the mechanisms of interactions between suspended cells, emulsions and biofilms.

Study of morphology by SEM

In order to obtain information on the morphological changes of the microorganisms after treatment with ZEON, SEM analysis was used. A suspension of *S. typhimurium* bacteria was treated with an MIC concentration of ZEON and the microstructure was then evaluated (Figure. 4). The untreated bacterial cells were typically rod-shaped, and remained intact with no visible change in cell surface integrity (Figure 4A). In contrast, SEM micrographs taken with MIC showed significant morphological damage to the bacterial cells treated with ZEON and no viability (Figure 4B). However, there were still microbes able to survive on the basis that it was a bacteriostatic but not bactericidal concentration**.** In contrast to the control cells (Figure 4A), the microbial cells treated with ZEON (Figure 4B) showed varying degrees of disruption and deformation. This observation was in agreement with the data of the antibacterial activity and killing kinetics assay**.**

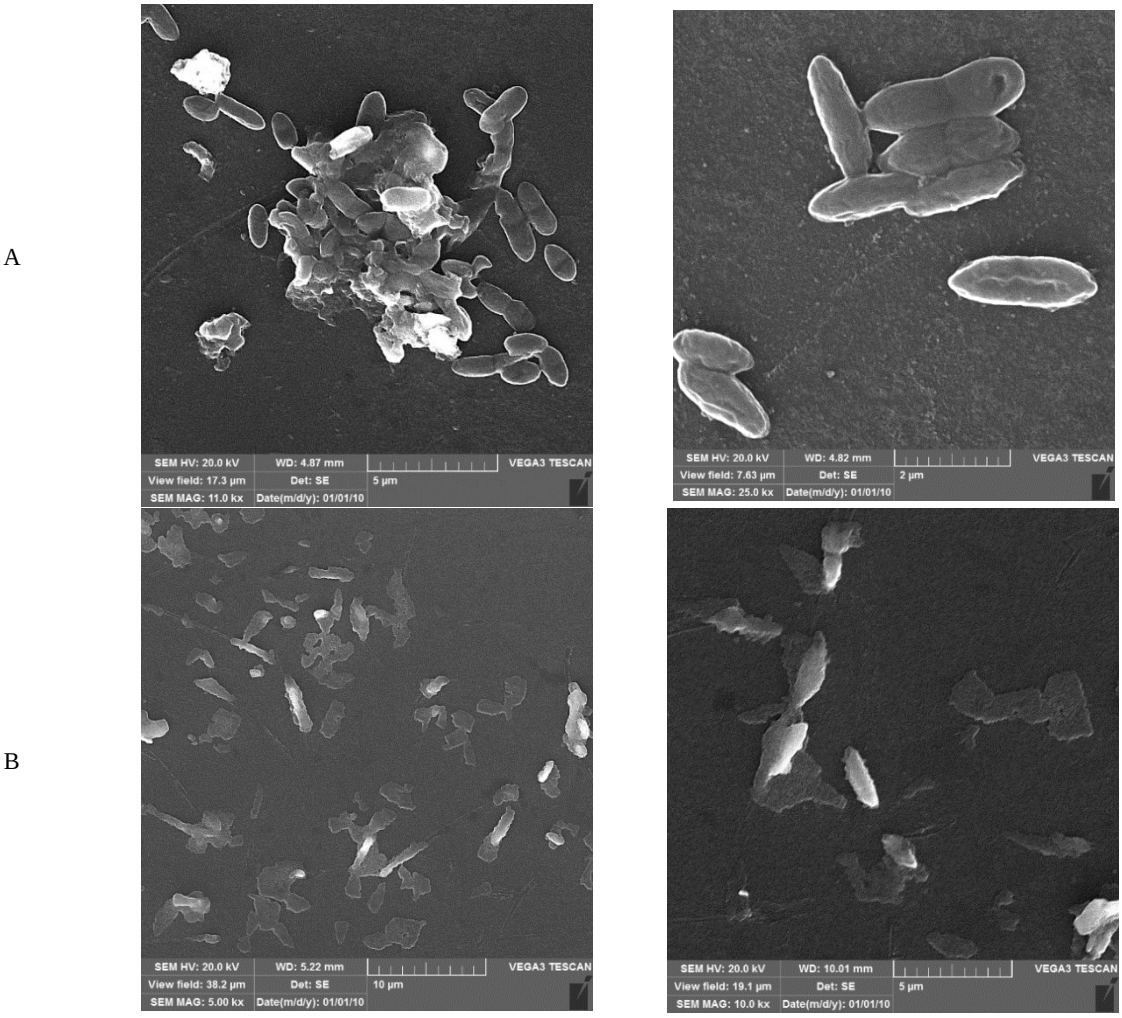

Figure. 4. Scanning electron micrographs of S. typhimurium cells: Control (A), treated with ZEO nanoemulsion at the MIC concentration (B). The magnification of the samples was either 30,000 or 50,000 times.

Conclusion

Our research study evaluated the physical, antibacterial and anti-biofilm properties of *Zataria multiflora* essential oil and its nanoemulsion. The optimized formulation resulted in a nanoemulsion whose long-term stability was satisfactory based on its small size distribution (polydispersity index < 0.3), small droplet size (121.34 nm), and acceptable zeta potential (7.16 mV) during 90 days of storage. The data obtained showed that the *Zataria* nanoemulsion had acceptable antimicrobial activity, but the conversion of ZEO to nanoemulsion did not improve and enhance its antibacterial activity. However, its antibiofilm properties were greatly enhanced. The antibacterial and anti-biofilm activity, as well as the high bacterial killing rate observed in the current study, suggest that nanoemulsions containing ZEO are also exceptionally potent antimicrobial agents that may have great potential as natural food preservatives.

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