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Original Article

Effect of Environmental Stress on Physical Properties and Antibacterial Activity of Atlas Cedar (*Cedrus atlantica*) Oil-in-water Emulsion

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ABSTRACT

Synthetic preservative compounds can prevent pathogenic bacterial growth, but they cause other concerns related to the adverse effect on human health. Essential Oil (EO), which possesses antibacterial activity, have potential replacers for synthetic preservatives. This study was conducted to develop Atlas cedar EO antibacterial activity, physical properties and sustainability against environmental stress via emulsification. Firstly, screening to select the most potent EO among various EOs (i.e. anise, Atlas cedar, curry leaf and onion) was done. As Atlas cedar was the most efficient antibacterial agent, emulsions containing Atlas Cedar EO were subsequently prepared using different concentrations of Polysorbate20 via a solvent-displacement technique. The physical properties (droplets size, stability, lightness and turbidity) and antibacterial activity (agar disk diffusion) of emulsions were determined. Results showed that emulsion containing 7% (wt) of Polysorbate20 was the most desirable sample in terms of physical properties of antibacterial activity. Henceforth, it was selected for environmental stresses study (i.e. thermal processing, freeze-thaw cycle and ultraviolet exposure). Results revealed that all types of environmental stresses had a significant ($p< 0.05$) effects on physical properties. Environmental stress treatments showed antibacterial activity enhancement against Gram-positive bacteria. Thus, the present work proved the potential use of emulsion as the delivery system of EO as antibacterial agent for applications in the food industry.

INTRODUCTION

Nowadays, rising consumers concern cause their demand for high-quality food free of chemical additives and food-borne pathogens. Other than that, synthetic preservatives have side effects on consumer health [1]. For instance, the nitrates substance can cause loss of consciousness and death, especially in infants while increased levels of nitrates can increase deaths from Alzheimer's, Parkinson's and Type 2 diabetes [2]. In this regard, natural preservation such as essential oils (EOs), which have antimicrobial capability, has the potential to control and reduce foodborne pathogens. Antimicrobial activity is linked to the composition, configuration, amount, and ability of interaction of EOs, which are complex combinations containing a wide variety of components [3]. As an example, Atlas cedar (*Cedrus atlantica* (Endl.) Manetti ex. Carrière) oil, which is a tall tree where the range from 40m to 60m of height mostly found in the mediterrano-himalayan region [4], has a high content in α-pinene and manool in their seeds EO [5]. Those compounds decrease membrane integrity and enhanced microbial influx to kill or inhibit microbial [4]. Previous studies by Derwich and co-workers (2010) have reported that Atlas cedar EO from leaves active against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus intermedius*, *Enterococcus faecalis* and *Bacillus sphericus* [6].

Although EOs able as an alternative to chemical preservatives, they have some limitations that must be overcome before applying in food systems. Low water solubility, high volatility, and strong odour are the main properties that cause the limitation of EOs to apply in the food systems [7]. Hydrophobicity properties of EOs cause non-

uniform distribution in food matrices and reduce their antimicrobial activity when directly integrated in foods, such as proteins and lipids, due to the hydrophobic binding with food components. Also, a high concentration of EOs affects the organoleptic properties of food. It is due to the application of higher concentration of EOs is required for inhibitory bacterial effect in situ system. In another word, higher concentration of EOs is needed to cause similar effects in real foods [8]. Hence, the emulsification process can help to solve this problem [9].

Emulsions are made by mixing two immiscible phases in the presence of stabiliser molecules [10, 11]. Emulsification of EOs able to overcome the limitation of EOs, and increasing the antibacterial activity of EOs. According to Topuz *et al*. (2016), the emulsified EO of anise showed better and long-term physicochemical stability and antimicrobial activity compared to bulk anise oil [12]. Moreover, in the other study emulsified EOs also showed higher antimicrobial activity even at far lower concentrations [13]. Furthermore, food product during storage and process undergoes various environmental stress such as sunlight, heat, freezing etc. Therefore, there is a growing emphasis on gaining a better knowledge of the effect of environmental stresses and conditions on the performance of the stabiliser system [11] and as a result on the stability of the emulsion system. Thus, the aim of this study is evaluation of physical properties and antibacterial activity of the most efficient emulsified EOs concluded the screening of selected EOs compounds and also investigation about the environmental stress of the best emulsion formulation on physical properties and antibacterial activity. To the best of our knowledge, there is no study on the characterization of emulsified Atlas cedar wood in terms of antibacterial activity and physical properties and investigation of related character after undergoes environmental stress treatments.

MATERIALS AND METHODS

Materials

The EOs of onion and curry leaf were provided from Soule brand, Malaysia. The EO of anise (seed part) and Atlas cedar (wood part) were provided from NOW Foods Essential Oils Brand, USA. Polysorbate 20 were purchased from BIO-RAD Laboratories Inc. (US). Muller Hinton Broth (MHB) and Muller Hilton Agar (MHA) were supplied from Merck, (Berlin, Germany). Blank Paper Disk and sterile swab that use for antimicrobial properties were purchased from Bioeconomy Co. (Kuala Lumpur, Malaysia). *Listeria monocytogenes* ATCC 19114, *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 19585 and *Escherichia coli* ATCC 25922 were target bacteria in this study.

Fabrication of EO Emulsions

The EO emulsions were prepared according to the solvent displacement technique reported by Ribeiro *et al*. (2008) [14]. The organic phase was prepared by dissolving 5% w/w EO in acetone (25% w/w), and the aqueous phase contained Tween 20 (3, 5, 7% w/w) and distilled water (67, 65, 63% w/w) respectively. The organic phase was added to the aqueous phase, under continuous magnetic stirring. Finally, the resulting emulsion was subjected to rotary evaporation (Eyela NE-1101, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at a temperature of 40 °C under reduced pressure (250 mbar) to remove the organic solvent (acetone), which was added earlier stage. 25% w/w of distilled water was added to each fabricated formula. The formulation of EO emulsions with different concentration of Tween 20 is shown in **Error! Reference source not found.**

Average Droplet Size Measurement

An optical microscope with a 60x objective lens and 10x eyepiece was utilized to measure of droplet size. The measurement of the droplet size was taken by software LOGO (size 240x60) installed in Android Tablet attached with the optical microscope. Hence, the measurement of the droplet size was recorded as an image with three replications.

Color Measurement

The prepared emulsion sample in the glass petri dish bowl was attached with Chroma Meter CR-400 Konica Minolta Sensing, Inc. (Japan). Color profile L* was measured by passing the light across emulsion samples. The detector of colorimeter has measured the light which passes through the solution. The emulsion sample was repeated for different concentration in three replicates [3].

Table 1 The formulation of EO emulsions with different concentration of Tween 20

Formulation	Atlas cedar (w/w%)	Emulsifier (Tween 20, w/w%)	Acetone (w/w%)	Distilled water (w/w%)
Formulation 1	5	3	25	67
Formulation 2	5	5	25	65
Formulation 3	5	7	25	63

Turbidity Measurement

The turbidity of emulsions was measured according to Komaiko (2016) [15]. In this regard, a Thermo Spectronic GENESYS 20 Visible Spectrophotometer (US line cord) at 600 nm was used. The concentrated emulsion of Atlas cedar was diluted with 60 ml distilled water (sample: distilled water, 1:60). Turbidity measurement test was performed in triplicate and the mean of the three individual trials was taken for data analysis.

Stability measurement

The stability of fabricated EO emulsions were measured by using Mid Bench Centrifuge (Hettich Universal 32R, UK) at 4000 RPM for 10 minutes. The emulsion stability index (ESI) was calculated as follows (Eq. (1)):

$$ESI\ \% = \frac{He - (Hc + Hs)}{He} * 100 \quad (1)$$

Where H_e is the initial emulsion height, H_c is the height of the cream layer and H_s is the height of the sedimentation phase. The emulsion stability index test was performed in triplicate and the mean of the three individual trials was taken for data analysis.

Preparation of Bacterial Cultures

In order to the preparation of bacterial culture, one to five colonies were transferred into a sterile universal bottle containing MHB and incubate overnight at 37 °C for 24 hours. Then, the overnight culture diluted with MHB to 0.5 McFarland standard and checked with a visible spectrophotometer (Thermo Fisher Scientific, GENESYS 20, CA, USA). The turbidity of the 0.5 McFarland standard solution was 0.132 OD at 600 nm [16, 17]. The viability of 0.5 McFarland standard solution for each strain was approximately equal to 10^8 cells per millilitre.

Agar Disk Diffusion

The agar disk diffusion (ADD) was performed according to the methods reported by Choo *et al*. [18], with minor modifications. Briefly, the culture was diluted 1:100. Then, the inoculum was spread evenly on MHA in a Petri dish using a sterile cotton swab. Paper disks contain positive control, negative control and vary of EOs and EOs emulsion was placed on the surface of the agar. The distance between disks is no closer than 24 mm from center to center. The plates were incubated at 37°C for 24 hours. The zone of inhibition was measured by using sliding calipers after 24 hours of incubation and recorded as millimeter (mm). The results represented the net zone of inhibition including the diameter (6 mm) of the paper disk. The positive and negative control were streptomycin and 10% w/w Dimethyl sulfoxide (DMSO), respectively. Also, bulk Eos was dissolved in 10% w/w DMSO. 10% w/w DMSO was sterilized by filtration through a 0.2 μm membrane filter. The experiment was done in three replicates.

Environmental Stress

The best sample among the three formulations in terms of Physical Properties and Antibacterial Activity undergo environmental stress. Thermal processing (pasteurization time and temperature), UV radiation, and freeze-thaw cycle were selected as the environmental stress.

Thermal Processing Stability

Thermal processing stability was evaluated according to Galvão et al. (2018) [19]. Bottles containing the emulsions were incubated in a water bath at 63 °C for 30 min. After that, the samples were allowed to cool for 30 minutes at room temperature. The physical and antibacterial properties of the emulsion were evaluated.

UV Radiation Stability

Proper amounts of emulsion (35 mL) were transferred into small beakers according to Sheng *et al*. (2018) [20], and then it was treated under UV light of laminar flow hood (CFM series, ERLA, ERLA Technologies (M) Sdn. Bhd. Malaysia) at room temperature for 20 minutes.

Freeze-thaw Stability

The study of freeze-thaw stability was according to Aoki *et al*. (2005) with modification [21]. First, EO emulsion was transferred into the bottle and

incubated in a -18 °C freezer for 24 hours. Then, thawed by incubating in a water bath at 30 °C for 2 hours. The physical and antibacterial properties of the emulsion were evaluated.

Statistical Analysis

The data were analysed by one-way analysis of variance (ANOVA). Fisher multiple comparison tests at the confidence level of $p < 0.05$ was applied for finding any significant difference among the samples. Statistical analyses were performed with the MINITAB version 16 package (Minitab 16, Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Screening of antibacterial activity of EOs against pathogens

The antibacterial activity of target compounds including anise, Atlas cedar, curry leaf and onion against four different bacteria namely *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes* was assessed with the disk diffusion method. The results are presented in clear zone diameters (**Error! Reference source not found.**). Main compositions of anise seed EO obtained by the hydro-distillation method, as a research carried out by Jafari, Zandi [22], were trans-Anethole (55.36%), D-Carvone (12.35%), (E)-4-Methoxy-2-(prop-1-en-1-yl)phenyl2-methylbutanoate (5.89%), γ-Himachalene (4.9%), D-Limonene (4.5%) and Estragole (4.30%). As reported by Kačániová, Galovičová [23] The chemical constitutions of atlas cedar wood EO by hydro-distillation method were δ-cadinene (36.3%), (Z)-β-farnesene (13.8%), β-himachalene (9.4%), viridiflorol (7.3%), and himachala-2,4-diene (5.4%), α-calacorene (3.0%). Also, Major compounds detected in the Curry leaf oil were Linalool (32.83%), Linalyl acetate (16%), Elemol (7.44%), Geranyl acetate (6.18%), Myrcene (6.12%), Allo-Ocimene (5.02), α-Terpinene (4.9%), and (E)-β-Ocimene (3.68%) and Neryl acetate (3.45%) [24]. Furthermore, the bioactive properties and characteristic flavor of onion have been attributed to sulfur-containing compounds, which are the main constituents of its EO. (Dipropyl disulphide (21.31-60.4%), Dipropyltrisulphide (17.1-21.92%), Methyl 5- methylfuryl sulphide (18.3%), Methyl 3,4-dimethyl-2-thienyl disulphide (11.75%), Methyl 1-propenyl disulphide (13.14%), Methyl 1-propenyl trisulphide (13.02%), Methyl propyl trisulphide (7.05-14.95%), Methyl propyl disulphide (9.5%), Propyl *trans*-propenyl disulphide (7.87%), Allyl propyl disulphide (3.56%), Dipropyl tetrasulphide (3.04%), Dimethyl trisulphide (1.14-16.64%), Propyl *Cis*-propenyl disulphide (4.67-9.72%), Dimethyl disulphide (1.31%), Dimethyl tetrasulphide (0.46-7.24%), Isopropyl disulphide (0.31%)). The water-insoluble extractive obtained onion EO consists of a complex mixture of volatile sulfur compounds, mostly mono-, di-, tri-, and tetra-sulphides with different alkyl groups [25].

The inhibition zone of the target EOs ranged from 6.17 ± 0.144 to 12.83 ± 1.155 mm. There was no antibacterial activity for anise, curry leaf and onion EOs against *E. coli*. Also, anise and curry leaf did not show any antibacterial activity against *S.* Typhimurium. Similarly, there was no inhibitory activity for curry leaf and onion EOs against *S. aureus*. No inhibition zones were produced by a control negative (10% DMSO). The lowest zone inhibition diameter was 6.17 ± 0.144 mm for Atlas cedar and onion EOs against *S.* Typhimurium. The highest zone inhibition diameters were determined as 6.63 ± 0.177, 6.17 ± 0.144, 12.83 ± 1.155 and 11.50 ± 1.323 mm for Atlas cedar against *E. coli*, *S.* Typhimurium, *S. aureus* and *L. monocytogenes*, respectively. Atlas cedar among all selected EOs showed the best antibacterial activity compared to other EOs. The antibacterial activity of Atlas cedar might be due to the presence of δ-cadinene in Atlas cedar wood EO. δ-cadinene is bicyclic sesquiterpenes [26]. Sesquiterpenes are a subclass of terpenes that have been described to display a large range of biological and pharmaceutical activities that include effects on the central nervous system, anti-tumor actions, and antimicrobial [27]. Our obtained result is the same line as previous researcher that claim that cedar EO, obtained by hydrodistillation from cedar wood, have been showed antimicrobial activity against Gram-positive (*Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella* enterica subsp. enterica, *Serratia marcescens*) via disc diffusion method [23].

In addition, There is proof that terpenes modify the physical properties of membranes due to their insertion between the lipid bilayer's fatty acyl chains [28,29]. This disrupts the van der Waals interactions between acyl chains [28,30], disrupting lipid packing and decreasing lipid order [28,31]. Terpene

molecules accumulate in the bilayer, the lipid volume increases, causing swelling and increase thickness of the membranes [28,29]. The expansion of membrane decreases the membrane integrity and ultimately leads to the loss of intracellular compounds. As such, changes in membrane fluidity could be among the initial effects of EO treatment [28]. Therefore, Atlas cedar EO from wood was selected for the next step of our study.

Physical Properties of Atlas Cedar EO Emulsions

Error! Reference source not found.a presents the average droplet size of fabricated Altas cedar emulsion (ACE) with various concentrations of Tween 20. The droplet size of the ACE ranged from 40.00 ± 10.000 to 93.33 ± 0.547 µm. The largest droplet size was obtained with 3% w/w of Tween 20. The 7% concentration of Tween 20 showed the lowest size of droplet compared to the 3% and 5% w/w concentration of Tween 20. However, for concentration 5% and 7% w/w, there is no significant difference between them. The chart (**Error! Reference source not found.**a) shows that there has been a decrease in the droplet size by increasing the concentration of Tween 20. These results probably occurred due to sufficient concentrations of the surfactant, which were sufficient to fully cover the newly formed droplets of oil and quickly adsorb at the interface [32]. Previous studies by Srinivasan *et al*. in 2002 have shown that the increase in sodium caseinates as emulsifier decrease the size of droplet size. Higher protein concentration in the aqueous phase improves the availability of emulsifier to encapsulate oil droplets eventually smaller droplet size was produced [33].

The stability of ACEs was tested by the emulsion stability index (ESI) method (**Error! Reference source not found.**b). The highest ESI value was recorded as the most stable emulsion since it can resist coalescence [34].

Stability study showed that concentration had a significant ($p < 0.05$) effect on the stability of the emulsion. ACE containing 7% and 3% w/w Tween 20 were the most and least stable emulsions, respectively. In **Error! Reference source not found.**b, there is a clear trend of increasing stability by increasing emulsifier concentration.

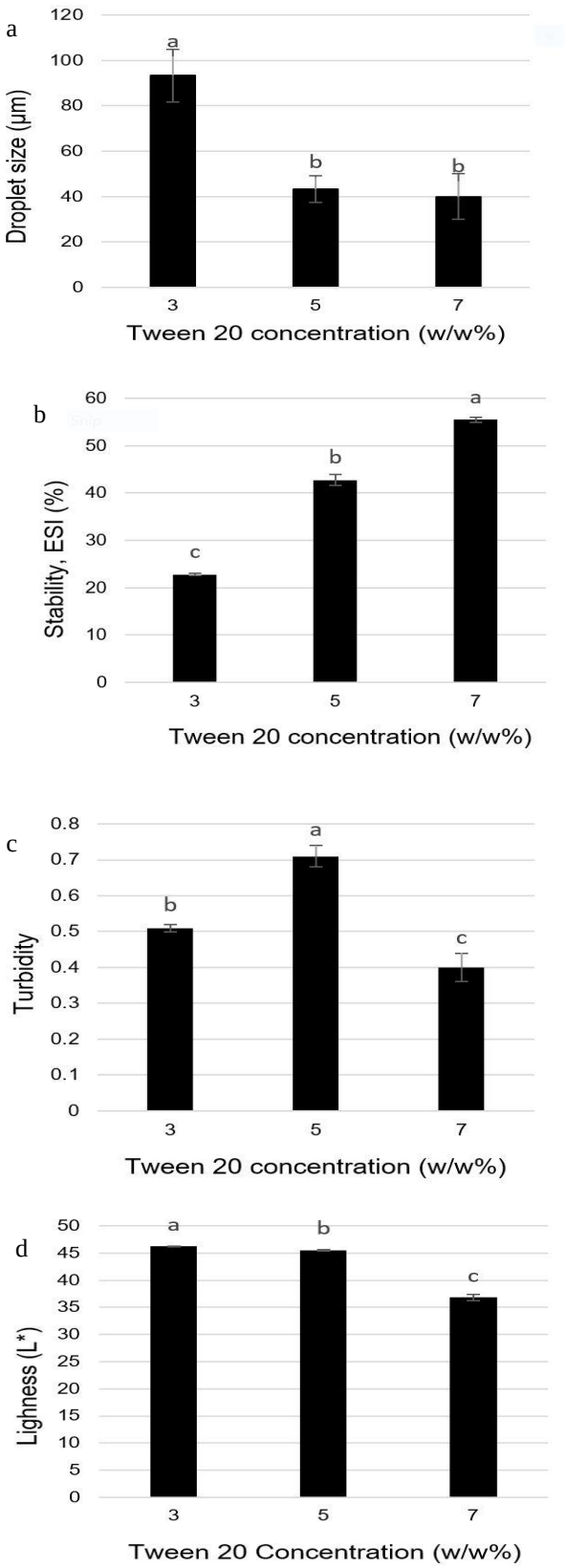

Fig. 1 The effect of concentration (various concentration of Tween 20 as emulsifier) on droplet size (a), emulsion stability (b), lightness (c) and turbidity (d), Values are means ± standard deviations (n=3), Different letters show statistically significant differences between values ($p < 0.05$)

Thus, emulsions prepared by higher concentration emulsifier are more resistant to coalescence. Coalescence of emulsions is an irreversible process by which two or more droplets merge during contact to form a single droplet. This reduction of the average droplet size cause increment in the stability of the emulsion [34]. This finding is in agreement with Xue in 2015 that it was proven that a higher concentration of emulsifiers more stable than the lower concentration of emulsifiers [34]. Another possible explanation is that many tiny droplets and other little structures appeared to surround the larger droplets. This can be due to the fact that large droplets cream faster, and that droplets in the cream layer pack tightly together, which might facilitate coalescence that can cause the emulsion to reduce its stability [15,35]. Thus, increasing the concentration of the emulsifier can reduce the droplet size of emulsion stability.

In terms of practical application, the optical properties of an emulsion-based delivery system are extremely important. It is crucial to have an optically transparent delivery system in some applications (e.g., clear beverages), while it is not necessary in others (e.g., opaque food). A turbid delivery mechanism might even be desirable (e.g., cloudiness in soft drinks). In most cases, the emulsion is fabricated by aiming to have a much smaller droplet and a clear appearance. However, it may not be attainable to make a highly clear emulsion in some cases. This issue is more obvious if the emulsion contains a high concentration of oil compound and high droplet size. Optical properties (i.e. lightness and turbidity) of the ACE were measured (**Error! Reference source not found.**c and **Error! Reference source not found.**d). The results in our study have indicated that ACE contained 7% w/w emulsifier had the lowest lightness and turbidity among the other fabricated sample. This could be due to the significant changes in the average droplet size of the ACE emulsion. Smaller droplets scatter the light less efficiently than larger droplets. The decreased turbidity of the emulsions containing smaller droplets is due to this [36].

Antibacterial Properties of Atlas Cedar EO Emulsions

The antibacterial activity of fabricated emulsions including the various concentration of the Tween 20 as emulsifier against four different bacteria, which had been selected in the screening part, was tested via the disk diffusion method. The results are presented as clear zone diameters (**Error! Reference source not found.**). The inhibition zone of the target EOs ranged from 6.17 ± 0.289 to 8.42 ± 0.144 mm. There was no antibacterial activity for all fabricated ACE against *E. coli*. Similarly, ACE contain 5% w/w emulsifier did not show any antibacterial activity against *S.* Typhimurium. Also, no inhibition zones were produced by a control negative (10% DMSO, emulsions contain no EO). It is crucial to mention that 5% w/w Atlas cedar EO in free form and streptomycin consider control positive. The lowest zone inhibition diameter was 6.17 ± 0.289 mm for 3% and 7% w/w ACE against *S.* Typhimurium, 5% w/w ACE against *S. aureus* as well as 3% and 7% w/w ACE against *L. monocytogenes*. The highest zone inhibition diameters were determined as 8.33 ± 0.318 and 8.42 ± 0.144 mm for ACE with 3% and 7% w/w emulsifier against *S. aureus*, respectively. Overall ACEs have better antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria. This finding is in agreement with other researchers [37] who concluded the EO that they studied *(Leonotis leonurus and L. ocymifolia*) were more active against the Gram-positive bacteria than the Gram-negative ones.

In addition, the effect of emulsification on the antibacterial activity of Atlas cedar EO is clearly shown in **Error! Reference source not found.** It can be achieved by comparing the free form of Atlas cedar EO with emulsified forms. Generally, the antibacterial activity of Atlas cedar EO in emulsified forms are either decreased or maintained constant. In other words, there is no antibacterial activity improvement after the emulsification process. It might be due to the fact that the emulsion system may limit the contact of the antibacterial compound with the membrane of bacteria [38]. It might be a consequence of stronger binding as the emulsifier is present. Terjung *et al*. (2012) studied the effect of the average droplet size on the efficacy of oil-in-water emulsions loaded with carvacrol and eugenol as phenolic antimicrobials. They reported that binding between Tween 80 and EO components after nanoemulsification was correlated to the reduction of antimicrobial activity. This could be explained by the fact that the emulsified antimicrobials had lower availability than the free

antimicrobial [39]. Among all fabricated emulsions with different concentrations of emulsifier, ACE contained 7% w/w Tween 20 showed lower droplet size, lightness and turbidity with higher stability. Also, it has better antibacterial activity against all selected target bacteria. Therefore, it was selected as the best formulation in terms of physical properties and antibacterial activity. Hence, the further step of this study (i.e. environmental stress section) was continued with ACE contained 7% w/w Tween 20.

Effect of environmental stress on physical properties of Atlas cedar EO emulsion

The best ACE which contained 7% w/w tween 20 went undergo the environmental stress treatment to characterize the physical properties (**Error! Reference source not found.**). The best ACE (BACE), which was contained 7% w/w tween 20, consider as the control in this section of our study.

As shown in **Error! Reference source not found.**a, the droplet size of the BACE after undergoing all types of environmental stresses (i.e. thermal processing, UV radiation and Freeze-thaw) were decreased significantly ($p < 0.05$).

Thermal processing and freeze-thaw had the largest and the smallest droplet size, respectively. In the other word, among the environmental stress treatments, thermal processing treatment shows the highest droplet size (31.33 ± 1.155 µm) compared to UV radiation and freeze-thaw treatment (19.33 ± 1.155 and 10.67 ± 1.155 µm, respectively). The overall decrement in droplet size in thermal processing and UV radiation might be due to the loss of EO after environmental stresses. Atlas cedar EO, as the target antibacterial, has volatile compounds.

Therefore, the loss of volatile compounds in the target oil might be happening. It might be a consequence of the decrease in the concentration of the organic phase and then a decrease in the droplet size. Another possible explanation for the droplet size reduction after all types of environmental stresses is releasing of EO from the capsule to the continuous phase. This fact is correlated to the loss in the emulsion system.

As shown in **Error! Reference source not found.**b, the stability of the BACE after undergoing all types of environmental stresses (i.e. thermal processing, UV radiation and Freeze-thaw) significantly ($p < 0.05$) were increased.

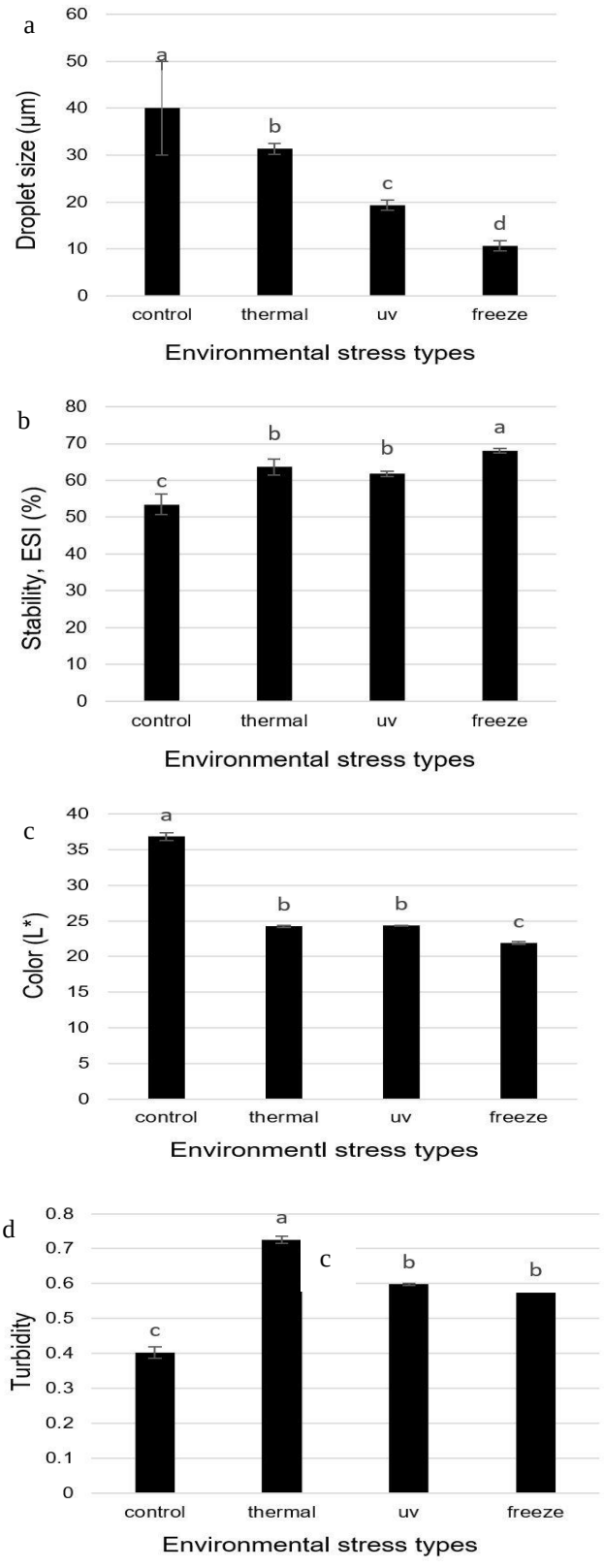

Fig. 2 The effect of environmental stress (thermal processing, UV radiation and freeze-thaw) on droplet size (a), emulsion stability (b), lightness (c) and turbidity (d) on the best selected emulsified sample (emulsion contained 7 w/w% Tween 20), Values are means ± standard deviations (n=3), Different letters show statistically significant differences between values ($p < 0.05$).

The highest and lowest emulsion stability index was belong freeze-thaw (68.10 ± 0.582%) and UV radiation (61.850 ± 0.733%), respectively. However, there is no significant difference between thermal and UV radiation stress treatment. The overall increment in the stability of emulsion after undergoing thermal processing, UV radiation and freeze-thaw might be due to the smaller droplet size in the contrast control sample. The reduction of the average droplet size cause increment in the stability of emulsion [34]. Therefore, freeze-thaw treatment showed the more stable emulsion due to the droplet shift towards smaller droplet sizes occurring as the processing conditions increase in the shear force and freezing temperature applied to the emulsion.

The optical properties of an emulsion-based delivery system are mostly essential in the measure to predict the visual property of application of it, especially during the storage. Optical properties (i.e. lightness and turbidity) of the BACE were measured (**Error! Reference source not found.**c and **Error! Reference source not found.**d). The results in our study have shown that the lightness of BACE after undergoing all types of environmental stresses (i.e. thermal processing, UV radiation and Freeze-thaw) significantly ($p< 0.05$) decreased. The highest and lowest lightness among all environmental stress treatments belong to UV radiation and freeze-thaw treatment, respectively. Also, the turbidity of BACE after undergoing all types of environmental stresses (i.e. thermal processing, UV radiation and Freeze-thaw) significantly ($p< 0.05$) increased. The highest and lowest lightness among all environmental stress treatments belongs to Thermal and freeze-thaw treatment, respectively. The increment of turbidity in the case of thermal processing stress might be due to the effect of the solubility of the dispersed phase in the continuous phase, making the absorbance becoming more increased.

Effect of environmental stress on antibacterial properties of Atlas cedar EO emulsion

The antibacterial activity of BACE went undergo the environmental stress treatments against four selected bacteria investigated through the disk diffusion method (**Error! Reference source not found.**). The inhibition zone of the BACE went to undergo the environmental stress treatments changed from 9.92 ± 0.382 to 12.25 ± 0.500 mm. The lowest and highest zone inhibition diameter was 9.92 ± 0.382 and 12.25 ± 0.500 mm against *L. monocytogenes* and *S. aureus,* respectively. There was no antibacterial activity against *E. coli* and *S.* Typhimurium after environmental stress treatments. Overall BACE, after environmental stress treatments, have better antibacterial activity against Gram-positive bacteria (**Error! Reference source not found.**). This finding is in the same line with previous researchers that claimed EOs with antibacterial properties are most effective against Gram-positive bacteria [40, 41]. It is stated by Delcour in 2009 that the Gram-negative outer membrane (OM) bacteria plays the crucial role in providing an extra layer of protection to the organism without compromising the exchange of material essential for survival [42]. As mentioned earlier, the poor antibacterial activity of Gram-negative bacteria in this study (i.e. *E. coli* and *S.* Typhimurium) were lost after environmental stress treatments. It might be due to the fact that EOs have high volatile compounds [7]. As Clarke (2008) suggested that Atlas cedar EO should be kept at a low room temperature which was no higher than 15 °C to prevent deterioration [43]. Low temperatures storage also can avoid EOs from evaporating and decomposition [44]. Besides, the antibacterial activity of Gram-positive bacteria (i.e. *S. aureus* and *L. monocytogenes*) were increased after environmental stress treatments.

Table 2 The clear zone inhibition diameter (mm) of selected bacteria against four type of essential oils

Bacteria \ Type of EOs	Anise	Atlas cedar	Curry leaf	Onion
E. coli	6.00±0.000 B	6.63±0.177 A	6.00±0.000 B	6.00±0.00 B
S. Typhimurium	6.00±0.000 A	6.17±0.144 A	6.00±0.000 A	6.17±0.144 A
S. aureus	6.97±0.630 B	12.83±1.155 A	6.00±0.000 B	6.00±0.000 B
L. monocytogenes	6.67±0.289 B	11.50±1.323 A	6.67±0.289 B	7.25±0.354 B

Note: Values are given as mean ± standard deviation (n=3). Inhibition zone includes diameter of disc (6 mm). Different letters indicate significant differences between samples in columns at $p< 0.05$.

Table 3 The clear zone inhibition diameter (mm) of Atlas cedar essential oil emulsions against selected bacteria

Con.TW20 / Bacteria	3 w/w%	5 w/w%	7 w/w%	Control (AC free form)
E. coli	6.00±0.000 A	6.00±0.000 A	6.00±0.000 A	6.63±0.177
S. Typhimurium	6.17±0.289 A	6.00±0.000 A	6.17±0.289 A	6.17±0.144
S. aureus	8.33±0.318 A	6.17±0.289 B	8.42±0.144 A	12.83±1.155
L. monocytogenes	6.33±0.289 A	6.17±0.289 A	6.17±0.289 A	11.50±1.323

Note: Values are given as mean ± standard deviation (n=3). Inhibition zone includes diameter of disc (6 mm). Different letters indicate significant differences between samples in rows (3, 5 & 7 w/w%) at $p < 0.05$. Con., Concentration; TW20, Tween 20; AC, Atlas cedar.

Table 4 The clear zone inhibition diameter (mm) of BACE undergoes environmental stress against selected bacteria

EST / MOs	Thermal processing	UV radiation	Freeze- thaw	Control (BACE)	Control (free form)
E. coli	6.00±0.000 A	6.00±0.000 A	6.00±0.000 A	6.00±0.000	6.63±0.177
S. Typhimurium	6.00±0.000 A	6.00±0.000 A	6.00±0.000 A	6.17±0.289	6.17±0.144
S. aureus	10.83±0.764 A	11.67±0.946 A	12.25±0.500 A	8.42±0.144	12.83±1.155
L. monocytogenes	9.92±0.382 A	11.33±1.443 A	11.5±0.901 A	6.17±0.289	11.50±1.323

Note: Values are given as mean ± standard deviation (n=3). Inhibition zone includes diameter of disc (6 mm). Different letters indicate significant differences between samples in rows (various environmental treatments i.e. thermal processing, UV radiation and freeze-thaw) at $p < 0.05$. MOs, Microorganisms; EST, Environmental stress type; BACE, the best Atlas cedar emulsion.

A possible explanation for this might be that releasing the antibacterial EO from encapsulated antibacterial emulsion was happened due to exposure to environmental stress treatments. Hence, free form EO in the system reason of the increment in the antibacterial activity compared to control (BACE).

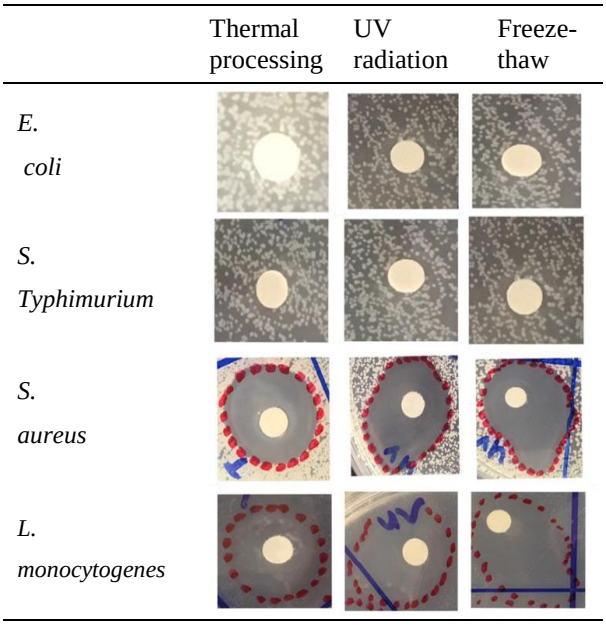

Fig. 3 The clear zone inhibition of environmental stress treatments against selected target bacteria

CONCLUSIONS

Atlas cedar EO showed the best antibacterial activity among selected EOs (i.e. anise, Atlas cedar, curry leaf and onion). The emulsification of Atlas cedar EO revealed that by increasing the concentration of emulsifier the droplet size and stability of emulsions decrease and increase, respectively. Also, the emulsification of Atlas cedar did not improve the antibacterial activity of Atlas cedar. In other words, Atlas cedar emulsions antibacterial activity either decrease or remain constant. Emulsion with 7% Tween 20 shown the best physical properties and antibacterial activity. Therefore, emulsion with 7% Tween 20 was subjected to study the effect of environmental stress on it. Emulsion system under thermal, UV radiation and freeze-thaw stress treatments, illustrated decrease in the droplet size and increased stability. Moreover, stress treatments of Atlas cedar presented better antibacterial activity compared to the ACE as control. However, the antibacterial activity of samples under environmental stress treatments did not display the same trend with the antibacterial activity of the free form of Atlas cedar oil. Overall, Atlas Cedar EOs more active against Gram-positive compared to Gram-negative bacteria. Further research is recommended studying the inhibitory activity of the antifungal emulsified and free form of

Atlas cedar while these antibacterial and antifungal could be examined in a real food system.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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