



# Superior antibacterial activity of nanoemulsion of *Thymus daenensis* essential oil against *E. coli*



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## ABSTRACT

Natural preservatives are being extensively investigated for their potential industrial applications in foods and other products. In this work, an essential oil (*Thymus daenensis*) was formulated as a water-dispersible nanoemulsion (diameter = 143 nm) using high-intensity ultrasound. The antibacterial activity of the essential oil in both pure and nanoemulsion forms was measured against an important food-borne pathogen bacterium, *Escherichia coli*. Antibacterial activity was determined by measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The antibacterial activity of the essential oil against *E. coli* was enhanced considerably when it was converted into a nanoemulsion, which was attributed to easier access of the essential oils to the bacterial cells. The mechanism of antibacterial activity was investigated by measuring potassium, protein, and nucleic acid leakage from the cells, and electron microscopy. Evaluation of the kinetics of microbial deactivation showed that the nanoemulsion killed all the bacteria in about 5 min, whereas only a 1-log reduction was observed for pure essential oil. The nanoemulsion appeared to amplify the antibacterial activity of essential oils against *E. coli* by increasing their ability to disrupt cell membrane integrity.

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## 1. Introduction

Food poisoning by *Escherichia coli* is a growing problem in both developing and developed countries. Many consumers are concerned about the potential adverse side effects of using synthetic antimicrobials in foods, and would prefer to use foods that are preserved using more natural approaches for preventing and controlling pathogenic microorganisms (Cakir, Kordali, Kilic, & Kaya, 2005; Goñi et al., 2009). Therefore, the development of safe and natural antibacterial agents is becoming increasingly important. Essential oils are aromatic volatile components, generated by the secondary metabolism of herbal plants, and can be used as natural antimicrobials (Diao, Hu, Feng, Li, & Xu, 2013). There are many reports on the antimicrobial activity of essential oils, and the use of essential oils as antimicrobial agents in food products (Burt, 2004; Sagdic, Karahan, Ozcan, & Ozkan, 2003; Salgueiro, Martins, & Correia, 2010). *Thymus daenensis* is a species of the genus

*Thymus* that is commonly found in Iran, which is used for both its aromatic and medicinal properties (Nickavar, Mojab, & Dolat-Abadi, 2005). The essential oil extracted from *T. daenensis* is an abundant source of thymol and carvacrol, both of which have been reported to have strong antioxidant activities (Ghasemi Pirbalouti, Hashemi, & Ghahfarokhi, 2013; Moazeni, Khajeali, Izadi, & Mahdian, 2014). It is also used as a food component, a herbal tea, and a medicinal plant for its reputed therapeutic properties (Alizadeh, Alizadeh, Amari, & Zare, 2013; Gourama & Bullerman, 1995). There are a number of potential technological challenges associated with incorporating essential oils into food products due to their low water-solubility, poor chemical stability, and volatile nature (Sugumar et al., 2013). Encapsulation of functional oils within nanoparticles has been investigated as a potential strategy for improving their utilization, stability, and efficacy (Donsì, Annunziata, Sessa, & Ferrari, 2011; Qian & McClements, 2011; Rao & McClements, 2011). Among the nanoencapsulation systems currently being utilized for the delivery of bioactive components, nanoemulsions have been reported to be especially appropriate for utilization in food products, due to their ease of preparation and desirable functional attributes (McClements & Rao, 2011; Silva, Cerqueira, & Vicente, 2012). The small particle size may

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increase interactions between the active compounds with biological membranes, as well as their transfer through them. Moreover, nanoemulsions can be designed to have a good kinetic stability and low turbidity, which is appropriate for a broad range of commercial applications (Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005), including as preservatives in foods, beverages (Rao & McClements, 2012), cosmetics, and pharmaceuticals (Quintão, Tavares, Vieira-Filho, Souza, & Santos, 2013). Several techniques have been applied for producing nanoemulsions, including various low-energy and high-energy methods. Ultrasonic emulsification is a high-energy method that is rapidly and efficiently capable of preparing nanoemulsions with small droplet diameters and narrow size distributions (Ghosh, Mukherjee, & Chandrasekaran, 2013; Manchun, Dass, & Sriamornsak, 2014). Essential oil nanoemulsions have previously been reported to be effective antibacterial treatments (Chang, McLandsborough, & McClements, 2012; Hamouda & Baker, 2000; Liang et al., 2012; Zhang, Vriesekoop, Yuan, & Liang, 2014; Ziani, Chang, McLandsborough, & McClements, 2011); however, knowledge of their mode of action against microorganisms is currently limited. The aim of the present work was therefore to produce stable nanoemulsions from *T. daenensis* using a sonicator, and then investigate their mechanism of action against *E. coli*. This goal was achieved by measuring the kinetics of bacterial deactivation, the release of cell constituents (proteins, nucleic acids and potassium), and changes in bacterial microstructure (scanning electron microscopy). This study provides important new information about the mode of action of antimicrobial nanoemulsions based on essential oils on an important bacterial species.

## 2. Material and methods

### 2.1. Plant materials and chemicals

Tween 80, Span 80 and glutaraldehyde were purchased from Merck Millipore (Darmstadt, Germany). The aerial parts of the cultivated *T. daenensis* were collected in April 2014 from the Dehloran region of Ilam in Iran.

### 2.2. Essential oil isolation

The essential oil of all air-dried samples was prepared using hydro-distillation for 3 h and a Lab HEAT Clevenger-type apparatus (SAF Wärmetechnik, Mörlenbach, Germany). The extracted essential oil was dried with anhydrous sodium sulfate and stored in vials under dark conditions at 4 °C prior to use.

### 2.3. Nanoemulsion preparation

Essential oil nanoemulsions were prepared from a mixture of *T. daenensis* oil (2% w/w), Tween 80 (2% w/w), water (96% w/w) and lecithin (0.001% w/w) as ripening inhibitor. This formulation was selected for study as it was the most stable preparation from a series of preliminary experiments.

### 2.4. Droplet size measurement

Measurement of droplet size and particle size distribution of the nanoemulsions was performed using a Dynamic Light Scattering (DLS) instrument (Nanophox Sympatec GmbH, Clausthal, Germany). The samples were diluted with deionized water to have a specified particle count range, between 200 and 2000 kCPS.

### 2.5. Nanoemulsion stability

Accelerated stability analysis of nanoemulsions were performed by using centrifugation at 3500 rpm for 30 min (Shafiq & Shakeel, 2010) and then measuring any phase separation and changes in particle size. Moreover, the particle size and size distribution were monitored over a six month period.

### 2.6. Antimicrobial activity

#### 2.6.1. Determination of MIC and MBC

The minimum inhibitory concentration (MIC) of pure and emulsified essential oils were investigated using the agar dilution method. Aliquots of samples were sequentially diluted in a 96 well plate containing Mueller Hinton Broth (MHB) medium to produce a concentration range of 0.0078–32 mg/ml for pure essential oil and 0.00078–3.2 mg/ml for emulsified essential oil. The final concentration of *E. coli* ATCC 25922 was adjusted to  $5 \times 10^6$  CFU ml<sup>-1</sup>. To evaluate the MIC for the pure essential oil, a 0.5% v/v Tween 80 solution was added to the medium. Plates were incubated at 37 °C for 24 h and the lowest concentration of additive that showed no visible microbial growth were determined. To determine the Minimum Bactericide Concentration (MBC), 100 µl of the broth from wells containing no growth were plated onto Mueller Hinton Agar (MHA) and were incubated at 37 °C for 24 h. The MBC was the lowest concentration that could kill 99.9% of treated cells of *E. coli*.

#### 2.6.2. Killing kinetics assay

The activities of the pure and emulsified essential oils against *E. coli* were investigated by measuring the reduction in the numbers of CFU per milliliter over 1 h. For this purpose, 5 ml cultures were grown for 24 h in nutrient broth (NB) medium and then transferred to 150 ml cultures and incubated at 37 °C under agitation until an OD<sub>600</sub> of the bacterial suspension reached 0.5–0.6 cm<sup>-1</sup>, which corresponds to the exponential phase of bacterial growth. Afterward, 4.5 ml of bacteria medium was added to 0.5 ml of sterile normal saline solution (0.085%) containing the MIC values of the essential oil nanoemulsion and equivalent amounts of pure essential oil. The normal saline for the pure essential oil contained 0.5% w/w Tween 80. All samples were maintained at 37 °C under agitation condition. After 0, 5, 10, 15, 30, 45, 60 min from the time of incubation, 100 µl portions were removed from each tube for analysis and diluted several times for colony counting.

#### 2.6.3. Integrity of the cell membrane

The cell integrity of *E. coli* strains was established by measuring the release of cell constituents into the supernatant according to the method described by Sugumar et al. (2013), with some modifications. 5 ml cultures were grown for 24 h in NB medium, and then additional NB medium was added to make 150 ml of culture. The system was then incubated at 37 °C under agitation until the OD<sub>600</sub> of the bacterial suspension reached 0.5–0.6 cm<sup>-1</sup> (exponential phase of bacteria). The medium was then collected and centrifuged for 10 min at 4000g, washed three times with sterile normal saline (0.085% NaCl), and finally re-suspended in 50 ml sterile normal saline. In this situation, the count of bacteria was approximately 10<sup>12</sup>–10<sup>13</sup> CFU/ml. 5 ml of microbial cell suspension (0.5 ml bacterial suspension + 4.5 ml normal saline) containing pure or emulsified essential oil were then incubated at 37 °C under agitation for 1 h. For the pure essential oil, 0.5% Tween was added to the normal saline to have a similar composition to the nanoemulsion. Examination was performed at MIC concentrations to find out the concentration of the constituents released. In order to compare the pure and emulsified essential oil, the equivalent concentration of essential oil that was present in the MIC

concentration of the nanoemulsion was used. This corresponded to using 1/10th the amount of essential oil as the nanoemulsion. Therefore, for the determination of cell constituent release in the presence of the essential oil was examined at 1/10th the MIC concentrations. The mixture was centrifuged for 10 min at 4000g and 10  $\mu$ l supernatant was used to measure the UV absorption at 260 nm using a UV–visible spectrophotometer (Bio-photometer, Eppendorf AG, Hamburg, Germany).

#### 2.6.4. Determination of protein release

For these experiments, all the steps for determining cell constituent release were repeated and the UV absorbance at 280 nm was measured using a UV–visible spectrophotometer (Bio-photometer, Eppendorf AG, Hamburg, Germany). The amount of protein released was calculated from a standard curve using BSA as a standard.

#### 2.6.5. Potassium release assay

The concentration of free potassium ions in the bacterial suspensions were determined as described previously (Bouhdi et al., 2010) with some modifications. 60 ml cultures were grown in NB medium and incubated at 37 °C under agitation until the OD<sub>600</sub> of bacteria reached about 0.5 cm<sup>-1</sup>. The medium was then collected and centrifuged for 10 min at 4000g, washed three times with sterile normal saline solution (0.085% NaCl), and resuspended in 6 ml NB culture. A cell suspension was prepared by mixing 1 ml bacterial suspension with 9 ml normal saline solution containing either pure or emulsified essential oil. In the case of the pure essential oil, 0.5% Tween was added to the normal saline. 10 ml of cell suspension was then incubated at 37 °C under agitation for 1 h, with an essential oil concentration equal to the control and the MIC. The potassium concentration in the supernatant was measured using an atomic absorption spectrophotometer (Analytik Jena, ContrAA700, Jena, Germany). The instrumental parameters were as follows: potassium hollow cathode lamp, wavelength of 766.5 nm, band pass of 0.5 nm, air-acetylene flame. Absorbance values were converted to potassium ion concentration (ppm) by reference to a curve previously established using standard potassium ion solutions at 0, 0.4, 0.8, 1.2, 2 ppm concentrations.

#### 2.6.6. Scanning electron microscopy (SEM)

To evaluate the effect of the pure or emulsified essential oil on the morphology of *E. coli*, SEM was performed. Pure or emulsified essential oil was added to working cultures at MIC concentrations of *E. coli*. After incubating for 5 min and 1 h at 37 °C, 1 ml portions were removed from each tube and centrifuged for 10 min at 4000g at 4 °C. The samples were then washed twice with PBS (pH = 7.4), and resuspended in 1 ml PBS. In the next step, 10  $\mu$ l of suspension was coated onto a glass slide (1 cm  $\times$  1 cm). Samples were then placed in a fixative (2.5% glutaraldehyde [v/v] in PBS) overnight and dehydrated in water–alcohol solutions at various alcohol concentrations (70%, 80%, 90% and 100%). Samples were then fixed on an SEM support and sprayed with Au–Pd prior to observation using a field emission gun scanning electron microscope (SEM, Hitachi, Tokyo, Japan).

#### 2.7. Statical analysis

Each experiment was performed in triplicate and all values are reported as mean  $\pm$  standard deviation (SD) by Microsoft Excel. The data was statistically analyzed by one-way analysis of variance (ANOVA). A *p* value of less than 0.05 was considered statistically significant.

### 3. Result and discussion

#### 3.1. Fabrication and characterization of nanoemulsions

The essential oil nanoemulsion was produced using 15 min of high-intensity sonication. Tween 80 was utilized as a surfactant to facilitate nanoemulsion formation and stability, and lecithin was used as a ripening inhibitor to prevent droplet growth. Ostwald ripening is a phenomenon that occurs in emulsions and nanoemulsions in which the oil phase has some solubility in the surrounding aqueous phase. Oil is transported from small to large droplets through the surrounding aqueous phase because the water-solubility of the oil surrounding a small droplet is higher than that surrounding a larger droplet (Tadros, Izquierdo, Esquena, & Solans, 2004). Ostwald ripening is a major problem in oil-in-water nanoemulsions containing essential oils because they have a relatively high water solubility (McClements, 2004). Droplet growth can be retarded by adding a ripening inhibitor to the oil phase (McClements & Rao, 2011). A ripening inhibitor works through an entropy of mixing effect, and may be a water-insoluble lipid (such as soybean, corn, or sunflower oils) or a water-insoluble surfactant (such as lecithin). The use of water-insoluble lipids as ripening inhibitors has been shown to be effect at retarding Ostwald ripening, but it may also reduce the antimicrobial activity of essential oils (Chang et al., 2012). Therefore, lecithin was investigated as a natural water-insoluble surfactant for inhibiting Ostwald ripening in this study. Measurements of the mean particle diameter for the nanoemulsions over a six-month period indicated a negligible increase from 143.2  $\pm$  2.6 nm to 149.6  $\pm$  1.0 nm that was not significant (*p* < 0.001). This suggests that the nanoemulsion was stable to coalescence and Ostwald ripening. In addition, no phase separation was observed after the nanoemulsions were centrifuged (35,000 rpm), indicating that they contained small particles that were stable to creaming.

#### 3.2. Antimicrobial activity of nanoemulsion

The measured MICs of the pure and emulsified essential oils showed different inhibitory effects against *E. coli* (Table 1). The nanoemulsion exhibited high inhibitory effects with MICs of 0.4 mg/ml. The essential oil nanoemulsion had 10 times more antibacterial activity than the pure essential oil, which suggested that converting the essential oil to nano-scale particles greatly improved its bactericidal activity. These results were in agreement with other recent studies that showed that conversion of flavor or essential oils into nanoemulsions greatly improved their antibacterial activity, e.g.  $\alpha$ -limonene (Zhang et al., 2014) and oregano oil (Bhargava, Conti, da Rocha, & Zhang, 2015). Experiments were performed using a model gram-negative bacteria (*E. coli*) to gain a better understanding of the antibacterial activity mechanisms of the essential oil.

##### 3.2.1. Killing kinetics assay

A killing kinetics assay was used to establish changes in the viability of *E. coli* upon interaction with pure or emulsified essential oils over a short time period (Fig. 1). No viable cells were observed

**Table 1**

Antibacterial activity of pure and emulsified essential oil (*Thymus daenensis*) against *E. coli*. The antibacterial activity is expressed as the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC).

| Samples      | MIC (mg/ml) | MBC (mg/ml) |
|--------------|-------------|-------------|
| Nanoemulsion | 0.4         | 0.4         |
| Pure oil     | 4.0         | 4.0         |

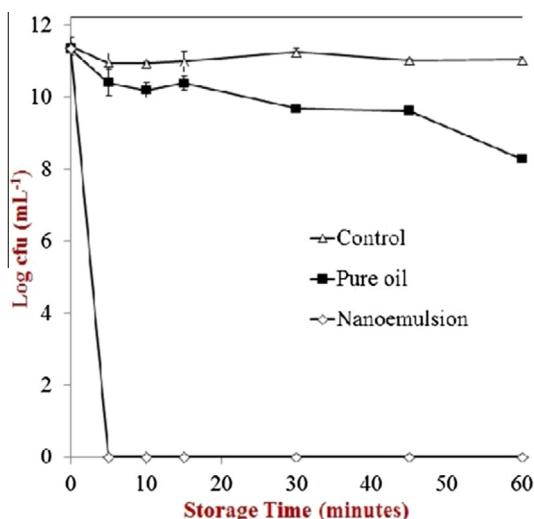


**Fig. 1.** Kinetics of bacterial killing after treatment with control sample, pure essential oil, and essential oil nanoemulsion over a 60 min period. The nanoemulsion was highly effective at killing the bacteria within a short time frame, whereas the same amount of pure essential oil was much less effective.

after 5 min interaction of the nanoemulsion with the *E. coli*. This result is in agreement with previous reports that show nanoemulsions are able to cause rapid reductions in viable bacteria cells (Ghosh et al., 2013; Sugumar et al., 2013). The antibacterial activity results clearly show that the essential oil nanoemulsion was more effective at inactivating the bacteria than the pure essential oil. This suggests that a nanoemulsion containing *T. daenensis* essential oil may have great potential as a natural preservative in food applications.

### 3.2.2. Release of 260 nm absorbing materials

One strategy for determining the mode of action of antimicrobials against food-borne bacteria is to measure the release of 260 nm absorbing materials from the filtrates of *E. coli* (Table 2). Substances that absorb at this wavelength include proteins and nucleic acids, and are an indication of cell membrane disruption and release of internal cell constituents. A significant increase in the cell constituents release compared to the control was observed when *E. coli* cells were treated with essential oil nanoemulsion at the MIC concentration. Following 60 min treatment with either pure or emulsified essential oil there was an approximately 2.8 and 10-fold increase in the optical density of the bacterial cell culture filtrates compared to the control sample, respectively.

### 3.2.3. Release of protein

Additional information about the potential disruption of the cell membranes and release of internal cell contents was obtained by measuring protein release (Table 3). The concentration of protein released for the essential oil nanoemulsion was much higher than for the control and the pure essential oil. For example, protein release from the control group was only 0.2 ppm, while it increased to  $5.20 \pm 0.09$  ppm for the nanoemulsion. There was an increase in

**Table 2**

Determination of nucleic acids release as determined by measuring the absorbance of the aqueous solutions surrounding the bacteria at 260 nm ( $OD_{260\text{ nm}}$ ).

| Samples      | $OD_{260\text{ nm}}$ |
|--------------|----------------------|
| Control      | $0.37 \pm 0.062$     |
| Nanoemulsion | $3.75 \pm 0.372$     |
| Pure oil     | $1.05 \pm 0.113$     |

Note: Difference between control and treatment group were significant ( $p < 0.005$ ).

**Table 3**

Measurements of protein release from bacteria after being treated for 60 min with control, essential oil nanoemulsion, or pure essential oil (used at their MICs).

| Samples      | $OD_{280\text{ nm}}$ | Protein concentration (ppm) |
|--------------|----------------------|-----------------------------|
| Control      | $0.12 \pm 0.002$     | $0.2 \pm 0.008$             |
| Nanoemulsion | $1.24 \pm 0.123$     | $2.0 \pm 0.131$             |
| Pure oil     | $0.65 \pm 0.064$     | $1.2 \pm 0.08$              |

Note: Difference between control and treatment group were significant ( $p < 0.005$ ).

optical density for the samples in the control group, which can be attributed to the death of some of the bacteria in their normal life cycle, since this causes protein release ( $OD_{280} = 0.12\text{ cm}^{-1}$ ) and nucleic acid release ( $OD_{260} = 0.37\text{ cm}^{-1}$ ). These results suggested that the cell membranes of *E. coli* were damaged, which led to losses of cell internal constituents thereby promoting cell death.

### 3.2.4. Determination of potassium leakage

Alterations in cell membrane permeability may lead to the leakage of potassium ions from the interior of the cells. Consequently, the extracellular concentration of potassium was measured using atomic absorption spectrophotometry (Fig. 2). The potassium concentration due to leakage from the control group was  $1.87 \pm 0.03$  ppm, but this value increased significantly to  $3.64 \pm 0.05$  ppm for the essential oil nanoemulsion. Some potassium leakage was observed in the control group, which is presumably associated with the death of some of the bacteria during their normal life cycle leading to cell lysis. The extracellular concentration of potassium in the bacterial suspensions treated with the essential oil nanoemulsion was significantly higher than for pure essential oil ( $p < 0.001$ ) and for the control group ( $p < 0.001$ ). These results suggest that increased membrane permeability is a determinant factor in the mechanism of antibacterial activity. Evaluation of potassium leakage from bacteria treated with nanoemulsions has not been reported in previous studies, and the results from the current study highlight the potential importance of potassium release on the antibacterial activity of essential oils.

### 3.2.5. SEM observation

Scanning electron microscopy (SEM) was used to provide information about changes in morphology of the bacteria after treatment with the pure or emulsified essential oils. A suspension of

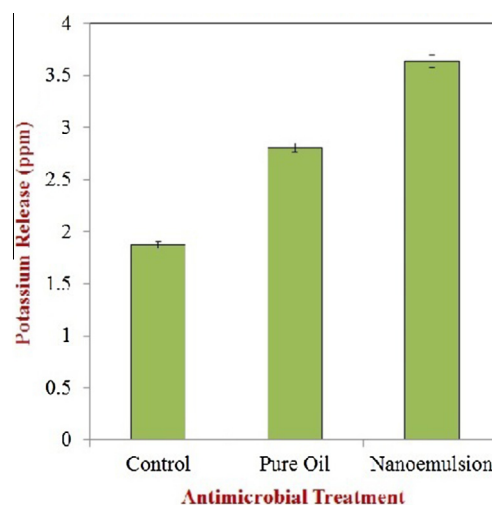


**Fig. 2.** Potassium leakage from cell suspension of *E. coli* after being treated for 60 min with control, essential oil nanoemulsion, or pure essential oil (used at their MICs). Note: Differences between control and the nanoemulsion were significant ( $p < 0.001$ ), and the difference between the pure and emulsified essential oil groups ( $p < 0.001$ ) were statistically significant.

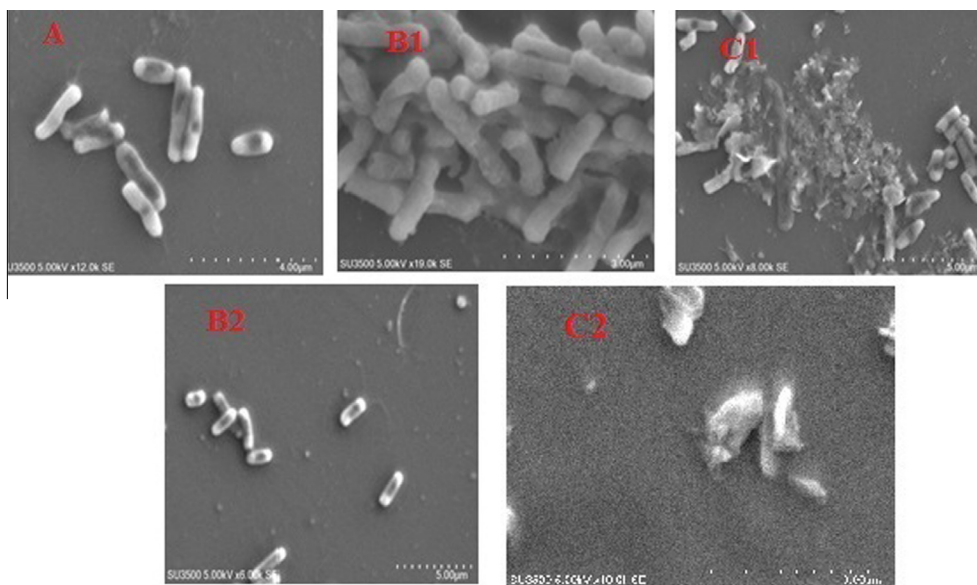


**Fig. 3.** Scanning electron micrographs of *E. coli* cells: Control (A), treated with pure essential oil (B), treated with essential oil nanoemulsion (C) used at a level equal to the MIC of the nanoemulsion (an equivalent amount of pure essential oil was used). Treatments were carried out for either 5 min (B1, C1) or 60 min (B2, C2). The magnification of the samples was either 30,000 or 50,000 times.

*E. coli* bacteria was treated with pure or emulsified essential oil at the MIC concentration, and then the microstructure was evaluated after 5 and 60 min treatment, respectively (Fig. 3). Significant morphological changes were observed in the microbial cells treated with the nanoemulsion (Fig. 3C) compared to the intact cell (Fig. 3A) and the cells treated with pure essential oil (Fig. 3B). Untreated bacterial cells had a rod-like shape with smooth intact surfaces (Fig. 3A), whereas bacterial cells treated with essential oil nanoemulsion for 5 min (Fig. 3C1) showed different degrees of deformation and disruption. No significant morphological alterations were observed on the surfaces of the treated strains after 5 min treatment with the pure essential oil (Fig. 3B1). Even after 60 min treatment (Fig. 3B2) the change in morphology was negligible, although some cells did exhibit shrinkage. This observation was in agreement with the killing kinetics assay and showed that nanoemulsions could kill all the bacteria in 5 min, and were much more effective than the pure essential oils.

#### 4. Conclusions

Based on the present study, the conversion of an essential oil (*T. daenensis*) into a nanoemulsion greatly enhanced its antibacterial activity against an important food-borne pathogenic bacterium (*E. coli*). The produced nanoemulsion exhibited good physical stability, with little evidence of phase separation or particle growth over a 6-month period. Based on our results, it can be proposed that the mechanism of action for the antimicrobial nanoemulsion against *E. coli* involved bringing the essential oil into close proximity with the cell membrane. This enabled the hydrophobic molecules within the essential oil to disrupt the cell membrane, possibly by altering the phospholipid bilayer integrity or by interfering with active transport proteins embedded in the phospholipid bilayer. The change in permeability of the disrupted cell membranes led to leakage of nucleic acids, proteins, and potassium from the cell interior, which led to cell death within 5 min. A comparison of the antibacterial activity of pure and emulsified essential oil showed that the nanoemulsion was much more effective. Presumably, this is because the small lipid particles within the nanoemulsion were able to bring the essential oil to the cell

membrane surface, whereas the pure oil (which has a low water solubility) could not easily interact with the cell membranes. The ability of the antimicrobial nanoemulsion to disrupt the morphology of the *E. coli* was clearly shown by electron microscopy. In conclusion, nanoemulsions may be particularly effective delivery systems for essential oils due to their ability to facilitate antimicrobial application and increase antimicrobial efficacy.

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