

Antimicrobial Activity and Physicochemical Characterization of Oregano, Thyme and Clove Leave Essential Oils, Nonencapsulated and Nanoencapsulated, Using Emulsification

Nancy Ruiz-Gonzalez, Aurelio Lopez-Malo, Enrique Palou, Nelly Ramirez-Corona, Maria Teresa Jimenez-Munguia*

Universidad de las Americas Puebla, Chemical, Food and Environmental Engineering Department, Ex hacienda Sta. Catarina Martir, S/N, San Andres Cholula, Puebla, Mexico. CP. 72810.

Abstract

Background and objective: Functional properties of essential oils are attributed to their components, many of which exhibit antimicrobial activity against pathogenic and spoilage microorganisms in a wide variety of foods. However, essential oils are unstable compounds; therefore, they can be encapsulated for a better protection and increase of functionality. In this work, antimicrobial activities of oregano, thyme and clove leave essential oils (non-encapsulated and nanoencapsulated) were assessed against *Escherichia coli* ATCC 29922, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923 using emulsification.

Material and methods: The essential oils were characterized based on their physicochemical properties. Nanoemulsions were prepared, using 5% (w w⁻¹) of essential oils, and then characterized based on their physical properties, stability and encapsulation efficiency. The microdilution antimicrobial assay was carried out to assess minimum inhibitory concentration and minimum bactericidal concentration of the essential oils and their nanoemulsions. Data from physical properties of the essential oils and physical properties, stability and encapsulation efficiency of the nanoemulsions were statistically analyzed.

Results and conclusion: Antimicrobial activity of the essential oils showed decreases in minimum inhibitory concentration by 27-60% for the nanoencapsulated oils, compared to nonencapsulated oils. Nanoencapsulated and nonencapsulated oregano essential oils exhibited the lowest minimum inhibitory concentration and minimum bactericidal concentration values. Based on the results, nanoencapsulated essential oils may further be used in various foods to avoid microbial contaminations.

Conflict of interest: The authors declare no conflict of interest.

How to cite this article

Ruiz-Gonzalez N, Lopez-Malo A, Palou E, Ramirez C, Jimenez-Munguia MT. Antimicrobial Activity and Physicochemical Characterization of Oregano, Thyme and Clove Leave Essential Oils, Nonencapsulated and Nanoencapsulated, Using Emulsification. *Appl Food Biotechnol* 2019; 6(4): 237-246. <http://dx.doi.org/10.22037/afb.v6i4.25541>

Article Information

Article history:

Received 27 May 2019
Revised 30 Jul 2019
Accepted 24 Aug 2019

Keywords:

- Antimicrobials
- Essential oils
- Nanoemulsions
- Nanoencapsulation
- Pathogens

*Corresponding author:

Maria Teresa Jimenez-Munguia,
Universidad de las Americas Puebla, Chemical, Food and Environmental Engineering Department, Ex hacienda Sta. Catarina Martir, S/N, San Andres Cholula, Puebla, Mexico.

Tel: +52-222 229 4353
E-mail: mariat.jimenez@udlap.mx

1. Introduction

Essential oils (EOs) are extracted from plants, including a mixture of several biosynthesized chemical substances [1]. There is an increasing interest in food industries for EOs because they are produced from natural sources, use of them is safe and they are friendly to environment and exhibit antioxidant and antimicrobial properties. Microbial controls using EOs have become one of the most important research areas with the aim of replacing synthetic preservatives [2]. The antimicrobial properties of EOs are based on their components; of which, phenolic compounds are one of the

most important groups. Studies have assessed the antimicrobial activity of components in EOs, including thymol, carvacrol and eugenol, against pathogenic and deteriorative microorganisms that are common in foods [3-5]. The action mechanisms of the antimicrobial compounds depend on the type of microorganisms to be inhibited or inactivated. These compounds can cause cellular damages or intervene in metabolic processes of the microorganisms [6]. Several studies have demonstrated that various terpenic compounds in EOs include synergistic effects as antimicro-

bials since they demonstrate interactive antibacterial effects on biochemical processes of the target bacteria [7]. However, EOs are chemically unstable when exposed to certain environmental conditions such as light, moisture, oxygen and elevated temperatures; all of which can cause the loss of their antimicrobial and antioxidant properties [8]. An alternative to protect EO properties against degradation and interaction with other food components is encapsulation. The encapsulation process involves formation of a multicomponent structure in form of composite particles; usually including two substances of core material and the encapsulating agent [9]. Using a diffusion process, oil can pass through the materials of capsule and be released in a controlled manner at required sites. Various encapsulation techniques such as emulsification, spray drying, coaxial electrospray system, freeze drying, coacervation, in-situ polymerization, extrusion, supercritical fluid technology and fluidized bed coating have been described. Study on this subject is broad since antimicrobial activity of EOs, encapsulated with different encapsulating agents or encapsulation techniques, includes various effects on pathogenic and deteriorative microorganisms in foods. The most common technique for EO encapsulation is spray drying [2]. In recent years, studies have been carried out to assess antimicrobial effects of oregano, coriander, cinnamon and garlic encapsulated EOs in model systems [6,10,11] against various microorganisms of interest in foods. Examples of these microorganisms include *Micrococcus luteus*, *Bacillus (B.) subtilis*, *B. cereus*, *Rhodospirillum rubrum*, *Candida utilis*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Penicillium glaucum*, *Geotrichum candidum*, *Alternaria alternata*, *Escherichia (E.) coli*, *Salmonella (S.) typhimurium* and *Staphylococcus (Staph.) aureus* [2]. Food industries constantly investigate efficient and cost-effective intervention strategies to control growth of common microorganisms to ensure microbial quality and safety of foods [12].

Encapsulation of EOs has been demonstrated as a good alternative to increase the oil antimicrobial activity and preservation time during storage. However, a few studies have been reported up to date on encapsulation of EOs and emulsification techniques [13,14]. In particular, many studies have focused on EO compounds as antimicrobials, which are generally extracted and purified before the encapsulation processes [15,16]. However, encapsulation of EOs may be less expensive than the encapsulation of specific compounds for practical uses. Various EOs must be studied to develop their uses in distinctive foods with sensory compatibility. Regarding nanoencapsulated EOs, studies have published on *Zataria multiflora* and oregano EOs [17,18]. However, there are a little information on clove leave EO because the herbal behavior is different from the clove flower EO. Nowadays, effects of various proportions of stabilizing and emulsifying agents and EOs

are described, as well as other homogenization methods. These together can completely change the physical properties, stability and encapsulation efficiency (EE) of the emulsions [17,18-20].

Therefore, the aim of this study was to characterize and compare antimicrobial activities of oregano, thyme and clove leave EOs, nonencapsulated or nanoencapsulated, against EE, *E. Coli* and *S. aureus* using emulsification. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were calculated for nonencapsulated and nanoencapsulated EOs against the bacteria in model systems using microdilution.

2. Materials and methods

2.1. The essential oils

Oregano essential oil (OEO), thyme essential oil (TEO) and clove leave essential oil (CLEO) were purchased from Laboratories Hersol S.A. de C.V., Mexico.

2.1.1 Chemical characterization of the essential oils

The EOs were analyzed using Agilent Technologies 6850N Gas Chromatographer (Palo Alto, CA, USA), equipped with Agilent 5975 C Mass-Spectrometer Detector (Palo Alto, CA, USA). The flow rate of the carrier gas (helium) was set at 1.1 ml min⁻¹. Derivatives were separated using HP 5 Fused Silica Column (Agilent, Palo Alto, CA, USA; 30-m length, 250 μ m and 0.25- μ m film thickness). The injector was set at 300°C and operated in a split mode of 10:1. The column oven was set at 60°C for 2 min and then ramped to 250°C at a rate of 10°C min⁻¹. The EO constituents were identified by comparing current mass spectra to those in National Institute of Standards and Technology Mass Spectral Database [21].

2.1.2 Physical properties of the essential oils

The EOs were characterized based on their color parameters (L, a, b) in Hunter scale using colorimeter (Model CR400, Konica Minolta, Japan) and refractive index using digital refractometer (PAL-BX / RI, ATAGO, Japan) according to A.O.A.C. 920.141 [22]. The EO viscosity was calculated using Cannon-Fenske Viscometer (Model 350, Thomas Scientific, USA) and the density was calculated using glass pycnometer according to A.O.A.C. 920.134 [23,24]. These experiments were carried out in triplicate.

2.2. Preparation and characterization of nanoemulsions

Nanoemulsions of 5% (w w⁻¹) EOs (dispersed phase) were prepared at 10% (w w⁻¹) of agave inulin as stabilizing (Fructagave PR95, Agaviotica, Mexico) and 3% (w w⁻¹) of polysorbate 80 as emulsifying agents (Tween 80, Sigma-Aldrich, USA) for the continuous phase. Nanoemulsions were homogenized using ultrasonic device (Ultrasonic Processor CP 505, Cole Parmer, USA) at 84 μ m of amplitude for 15 min [25].

2.2.1 Physical properties of nanoemulsions

Nanoemulsions were characterized based on their color parameters (L, a, b) in Hunter scale using colorimeter (Model CR400, Konica Minolta, Japan) and pH using potentiometer (PC45, Conductronic, Mexico) according to A.O.A.C. 981.12 [26]. Both equipments were calibrated. The EO viscosity was calculated using Cannon-Fenske Viscometer (350, Thomas Scientific, USA) [23] and density using glass pycnometer [24]. These calculations were carried out in triplicate.

2.2.2 Encapsulation efficiency (EE)

The EE of EOs was calculated using UV/VIS spectrophotometer (Cary 100, Varian, USA) according to protocols from literatures with modifications [27]. The maximum peak was calculated in spectrograms of the EOs dissolved in n-hexane (J. T. Baker, USA) at a wavelength range of 190-500 nm. The maximum peak found for OEO, TEO and CLEO included 271, 274 and 281 nm, respectively. The EE was calculated through a calibration curve achieved from five EO standard solutions diluted with n-hexane and prepared at various concentrations (0.5-5 $\mu\text{l ml}^{-1}$). To calculate the EE, extraction of the free essential oil from nanoemulsion was carried out by dissolving 700 μl of nanoemulsions in 9.3 ml of n-hexane with constant stirring (60 rpm) for 15 s. These assessments were carried out in triplicate. Then, absorbance was set on a specific wavelength according to the EO. The EE was calculated as a percentage using the following equation:

$$EE \% = \frac{EOM - FEOM}{EOM} \times 100 \text{ eq. 1}$$

Where, EOM was EO content of the nanoemulsion formulation fixed at 5% ($\text{ml}_{\text{EO}} (\text{ml}_{\text{nanoemulsion}})^{-1}$) and free essential oil from nanoemulsion was free EO from nanoemulsion ($\text{ml}_{\text{EO}} (\text{ml}_{\text{nanoemulsion}})^{-1}$).

2.2.3 Stability of nanoemulsions

Stability of the nanoemulsions was assessed using visual inspection and describing the first signs of development of creaming. For this assessment, 25 ml of each nanoemulsion were transferred into a graduated tube (by triplicate) and stored in a dark place at $23 \pm 1.0^\circ\text{C}$ for 28 days. Then, nanoemulsions were inspected and creaming was recorded after 7, 14, 21 and 28 days of storage according to Hebishy et al. with minor modifications [28]. Granulometric distribution of the EO droplets dispersed in nanoemulsions was assessed in triplicate using dynamic light scattering particle analyzer (Nanotracs Wave II, Microtrac, USA).

2.3 Antimicrobial activity of O/W essential oils and nanoemulsions

2.3.1 Microbial culture preparation

Strain cultures used in this study included *E. coli* ATCC 29922, *S. typhimurium* ATCC 14028 and *Staph. aureus* ATCC 25923 provided by the Food Microbiology

Laboratory of the Universidad de las Americas Puebla, Mexico. The bacterial strains were cultivated on tryptone soy agar (BD BIOXON, Mexico) and incubated at $37 \pm 1^\circ\text{C}$ for 24 h. These were then stored at 4°C for a maximum of three weeks. At the end of storage, the bacterial strains were reactivated and cultivated. Suspensions were prepared using two loopfulls of the bacterial cultures to inoculate tubes containing 10 ml of tryptone soy broth (BD BIOXON, Mexico). Tubes were incubated at $37 \pm 1^\circ\text{C}$ for 24 h.

2.3.2 Calculation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of EOs and nanoemulsions was evaluated using microdilution with mild modifications [29]. Briefly, serial dilutions of EOs and nanoemulsions were prepared and transferred into the wells of microplates. From a stock solution of 25 $\mu\text{l ml}^{-1}$ of EOs, 1-40 μl were added into the wells to achieve EO concentrations of 0.1-4 $\mu\text{l ml}^{-1}$. Nanoemulsions were prepared at 5% (w w^{-1}) of EOs, adding 0.5-10 μl of the nanoemulsions into the wells to achieve concentrations of 0.1-2 $\mu\text{l ml}^{-1}$ of EOs using tryptone soy broth. Then, 2.5 μl of the bacterial suspensions at 10^8 CFU ml^{-1} concentrations were added to each well to achieve a final concentration of 10^6 CFU ml^{-1} . Microplates were incubated at $37 \pm 1^\circ\text{C}$ for 18 h. After the incubation, 10 μl of resazurin (Sigma Aldrich, Germany) at a concentration of 0.015% were added to each well to assess the microbial growth. After incubation at $37 \pm 1^\circ\text{C}$ for 2 h, the MIC value was calculated as the concentration of EOs or nanoemulsions that did not change from blue resazurin to pink resorufin. Active microbial growths were detected by the reduction of blue resazurin to pink resorufin. The MBC of EOs and nanoemulsions was calculated using microdilution with mild modifications [29]. Briefly, 5 μl of solutions from negative wells were spread on tryptone soy agar plates. The MBC value corresponded to the lowest concentration of EOs or nanoemulsions with negative subcultures after incubation at $37 \pm 1^\circ\text{C}$ for 24 h. These calculations were carried out in triplicate.

2.4 Statistical analysis

Data were statistically analyzed using Minitab Software v.17 (LEAD Technologies, Sate College, PA, USA) and ANOVA and Tukey tests with a confidence level of 95%.

3. Results and discussion

3.1. Characterization of the essential oils

3.1.1 Chemical characterization

Differences in the chemical characterization of EOs is usually common due to the EO composition, which depends on various geographical and environmental factors [30]. Therefore, it was important to identify the major components of EOs used as antimicrobials in this study. The major components identified in the EOs are shown in Table

1. The OEO contains more than 30 compounds, including carvacrol that is usually one of the major components of OEO. In this study, carvacrol (60.76%) was detected as a major component of the OEO. Carvacrol is a phenolic compound and mainly responsible for the OEO antimicrobial activity [31]. Similarly, the antimicrobial activity of TEO is mainly attributed to carvacrol and thymol [32]. In the current study, the major component of TEO was thymol (30.76%). Clove EOs exhibit antimicrobial activity principally due to the eugenol content of the oil [33]. In this study, one of the major components found in CLEO was eugenol (25.72%).

Table 1. Chemical characterization of the essential oils

| Essential oil | Major component | Area (%) |
|---|------------------------|-------------------------|
| OEO | Carvacrol | 60.76 |
| | Syn carvacrol | 15.21 |
| | Syn m-cymene | 6.92 |
| | Syn δ -terpinen | 5.64 |
| | Caryophyllene | 3.53 |
| | Syn linalol | 2.52 |
| TEO | Thymol | 30.76 |
| | m-cymene | 21.77 |
| | 1r- α -pinene | 9.35 |
| | Syn-linalol | 6.16 |
| | Syn δ -terpinen | 6.14 |
| | δ -terpinen | 4.64 |
| | Caryophyllene oxide | 2.22 |
| | Caryiophyllene | 2.09 |
| | CLEO | α -caryophyllene |
| Eugenol | 25.72 | |
| Syn δ -cadiene | 12.43 | |
| 4-((1E)-3-hidroxy-1-propenyl)-2-methoxyphenol | 8.82 | |
| α -farnesene | 4.11 | |
| Caryophyllene oxide | 3.37 | |
| Diepicedrene-1-oxide | 3.28 | |

OEO: oregano essential oil; TEO: thyme essential oil; CLEO: clove leave essential oil. Analyses were carried out in triplicate.

3.1.2 Physical properties of the essential oils

Every EO includes different properties, which are linked to the type of source plants, as well as other factors. It is possible to find variations in EO properties from similar plant genera [30]. Therefore, physical properties of the EOs were differentiated before the nanoencapsulation process to show how their properties were affected. Due to properties such as density and viscosity of the dispersed phase, EO properties are better to characterized to allow formulation of stable emulsions. Physical properties of the EOs are presented in Table 2. Color is a physical property of the

EOs, which includes a large variation depending on the type of EOs. In this study, color parameters for OEO, TEO and CLEO were recorded based on the Hunter scale (Table 2). Moreover, high luminosity and tendency toward yellow and green were recorded for the EOs. A refractive index value of 1.4774 has been reported for OEO by other researchers, which is close to values from the current study (Table 2). The refractive index of TEO (1.499) was also similar to that of other studies [34,35]. In other studies, the refractive index for CLEO ranged 1.5330-1.5350, which was similar to that of the present study [36]. Viscosity is a characteristically physical property of EOs and varies depending on the type of EOs. In general, values for the OEO, TEO and CLEO from this study (Table 2) were similar to values from other studies [37]. These included 0.066 ± 0.006 P for clove flower and 0.041 ± 0.001 P for cinnamon EOs. In the current study, the density value of OEO (Table 2) was similar to that 0.92 g (cm³)⁻¹ in literatures [38]. Another study [39] reported a density value of 0.925 g (cm³)⁻¹ for TEO, which was similar to that the present study did. In the present study, the CLEO presented the highest value of density [1.046 - 1.053 g (cm³)⁻¹], as shown by other studies [36].

Table 2. Physical properties of the essential oils

| Property | OEO | TEO | CLEO |
|-------------------------------|---------------------|---------------------|---------------------|
| Color | | | |
| L | 80.490 ± 0.310^b | 81.440 ± 0.100^a | 81.600 ± 0.200^a |
| a | 2.230 ± 0.030^b | 1.060 ± 0.020^c | 3.920 ± 0.030^a |
| b | 8.370 ± 0.050^b | 3.920 ± 0.030^c | 16.430 ± 0.030^a |
| Refractive index | 1.510 ± 0.001^b | 1.502 ± 0.006^b | 1.535 ± 0.004^a |
| Viscosity (P) | 0.095 ± 0.004^a | 0.048 ± 0.003^c | 0.079 ± 0.004^b |
| Density (kg L ⁻¹) | 0.947 ± 0.041^a | 0.920 ± 0.071^a | 1.042 ± 0.020^a |

OEO: oregano essential oil; TEO: thyme essential oil; CLEO: clove leave essential oil. Different letters in the same row show significant differences ($P\leq 0.05$). Measurements were carried out in triplicate. Color parameters L, a, b are in Hunter scale.

3.2 Characterization of the nanoemulsions

Emulsions include different properties, according to the materials used in their preparation [40]. Therefore, it is necessary to characterize physical properties of the emulsions to show correlations with their stability and assess their use as antimicrobials in model systems. The physical properties and stability of the O/W nanoemulsions of EOs are shown in Table 3.

3.2.1 Physical properties of the nanoemulsions

In this study, color parameters were recorded for OEO, TEO and CLEO nanoemulsions based on the Hunter scale. High values of luminosity and tendency toward yellow and green were recorded (Table 3). In fact, characterization of color parameters was necessary to show if it was possible to carry out MIC assays without any interference using the color change test. The pH is an important parameter since a significant change in its value may suggest chemical changes of the components in emulsions, which affect its stability [40]. According to other studies, food emulsions

generally include a pH range of 2.5-7.5, similar to the current pH ranges of OEO, TEO and CLEO nanoemulsions (4.64-5.04) [41]. The pH of OEO nanoemulsion was reported as the highest pH, which could affect its stability since it was the most unstable nanoemulsion (Table 4). In stability of the emulsions, it was shown that a more viscous continuous phase could make the creaming process slower [42]. In this study, a nanoemulsion viscosity of 0.018 P was recorded. Generally, viscosity of emulsions is linked to the viscosity of continuous phase [41]. However, no significant differences ($P>0.05$) were recorded for the viscosity of OEO, TEO and CLEO nanoemulsions in this study (Table 3) possibly due to their low EO contents (5%). Density is an important parameter for the stability of emulsions [43]. If the continuous and dispersed phases include different densities, the great pressure on dispersed phase droplets drives a separation process called creaming [42]. In the

current study, density values of the OEO, TEO and CLEO nanoemulsions were similar (Table 3) with an average value of $1.033 \text{ g (cm}^3\text{)}^{-1}$. However, no significant differences ($P>0.05$) were seen in density values of the OEO, TEO and CLEO nanoemulsions; possibly due to their low EO contents (5%).

3.2.2 Encapsulation efficiency (EE)

Calculation of EE is important because this parameter demonstrates retention of the oil in the capsule [44]. In this study, EE was calculated in a range of 76.2-96.7% (Table 3); of which, the CLEO nanoemulsion presented the highest values of EE. Smaller particle sizes together with greater EE values could explain the greater stability of CLEO and TEO nanoemulsions, compared to that of OEO nanoemulsion.

Table 3. Physical property and stability of O/W nanoemulsions of the essential oils

| Property | OEO | TEO | CLEO |
|-------------------------------|----------------------------|----------------------------|----------------------------|
| Color | | | |
| L | 80.530±0.390 ^b | 81.750±0.280 ^a | 81.820±0.300 ^a |
| a | 0.750±0.010 ^b | 0.710±0.010 ^c | 0.780±0.010 ^a |
| b | 1.130±0.010 ^a | 0.550±0.010 ^c | 1.010±0.010 ^b |
| pH | 5.040±0.010 ^a | 4.730±0.040 ^b | 4.640±0.010 ^c |
| Viscosity (P) | 0.018±0.002 ^a | 0.018±0.002 ^a | 0.018±0.003 ^a |
| Density (kg l ⁻¹) | 1.034±0.021 ^a | 1.032±0.010 ^a | 1.034±0.032 ^a |
| D ₁₀ (µm) | 174.600±0.058 ^a | 97.700±0.045 ^b | 90.800±0.057 ^c |
| D ₅₀ (µm) | 232.500±0.058 ^a | 130.800±0.045 ^b | 116.400±0.057 ^c |
| D ₉₀ (µm) | 343.000±0.058 ^a | 237.800±0.045 ^c | 301.000±0.057 ^b |
| EE (%) | 76.243±0.001 ^c | 92.182±0.001 ^b | 96.709±0.001 ^a |

OEO: oregano essential oil; TEO: thyme essential oil; CLEO: clove leave essential oil; EE: encapsulation efficiency. Color parameters L, a, b are in Hunter scale. Different letters in the same row show significant differences ($P\leq 0.05$). Measurements were carried out in triplicate.

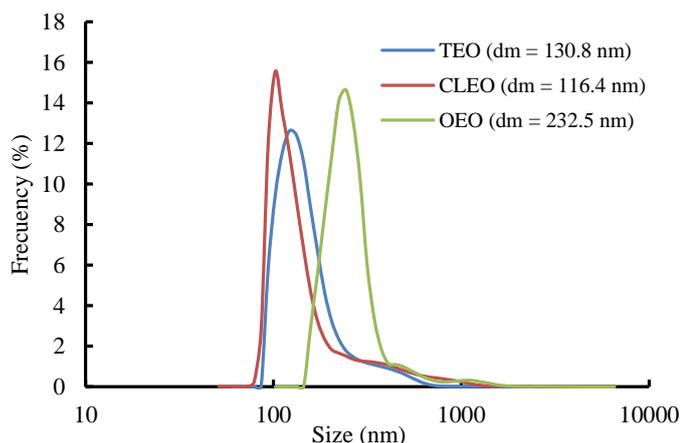
Table 4. Stability of essential oils nanoemulsions during storage at 23°C

| Nanoemulsions | Observation period (days) | | | |
|---------------|--|---|--|--|
| | 7 | 14 | 21 | 28 |
| OEO | 4-7 drops of oil were observed on the surface, without a total phase separation. | 8-10 drops of oil were observed on the surface, without a total phase separation. | 12-14 dispersed drops of oil were observed on the surface, without a total phase separation. | 14-16 dispersed drops of oil were observed on the surface, without a total phase separation. |
| TEO | No visible changes | No visible changes | No visible changes | No visible changes |
| CLEO | No visible changes | No visible changes | No visible changes | No visible changes |

OEO: oregano essential oil; TEO: thyme essential oil; CLEO: clove leave essential oil
Measurements were carried out in triplicate.

3.2.3 Stability of nanoemulsions

In this study, the most stable nanoemulsions belonged to TEO and CLEO with no visible changes during an observation period of 28 days (Table 4). The OEO nanoemulsion showed first signs of creaming with the presence of lipid droplets on the surface of nanoemulsion on Day 7. At the end of observation period, the nanoemulsion exhibited 14-16 dispersed oil drops on its surface with no total phase separation. Droplet size plays a critical role in determining long-term stability of the emulsions [45]. A very small droplet size of 116.4-232.5 nm (D_{50}) was reported for the nanoemulsions (Figure 1); of which, the smallest size belonged to CLEO nanoemulsion. The best description for an emulsion is through its droplet size distribution, which statistically provides records of the dispersed phase fragmentation [42]. Decreased average droplet sizes can prevent or minimize creaming [46]. In this study, the greater stability of CLEO nanoemulsion might be seen because it included the lowest droplet size values (Figure 1, Table 3).



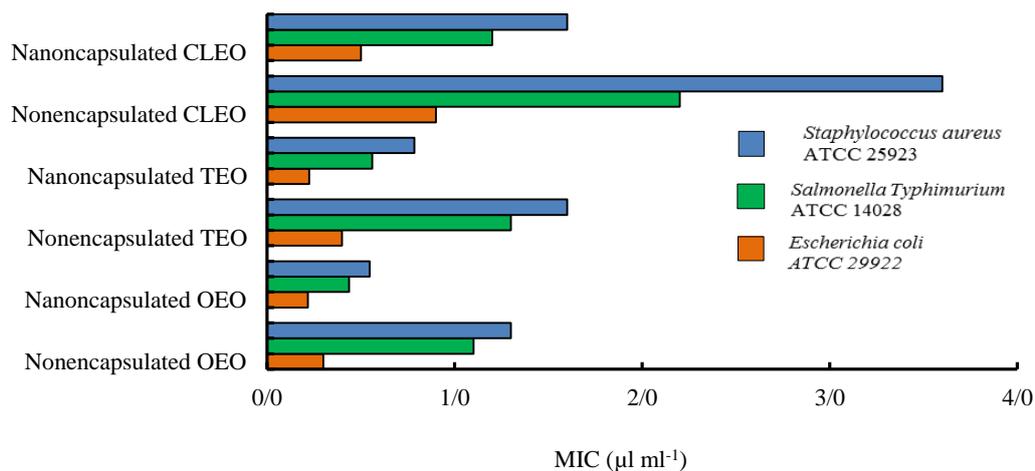
OEO: oregano essential oil; TEO: thyme essential oil; CLEO: clove leaf essential oil; dm: medium diameter. Analyses were carried out in triplicate.

Figure 1. Nanoemulsion droplet sizes

3.3 Antimicrobial activity of O/W essential oils and nanoemulsions

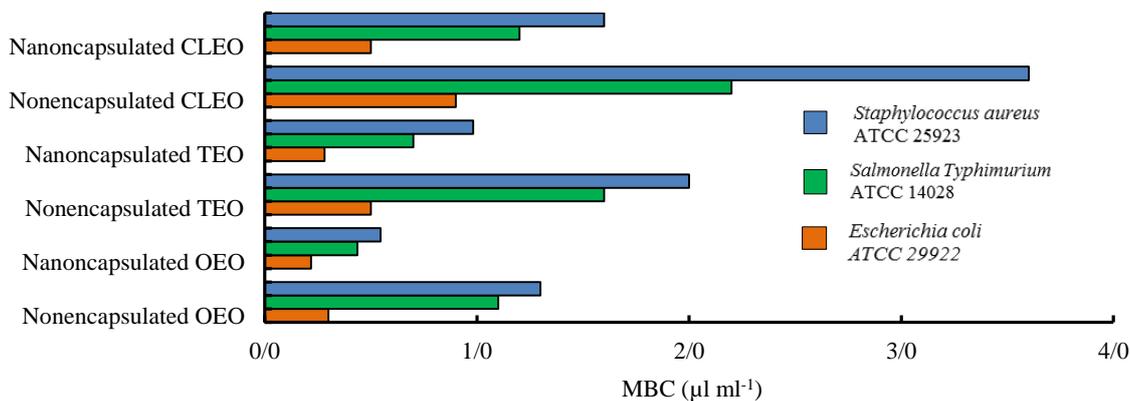
The antimicrobial activity of EOs depends mainly on three factors of their chemical components, type of the target microorganisms and the EO hydrophilic/hydrophobic characteristics [47,48]. Based on the results from chemical characterization, phenolic compounds with antimicrobial activity were included in OEO (60.76% of carvacrol), TEO (30.76% of thymol) and CLEO (25.72% of eugenol). Results from other studies [5] have shown that phenolic components are the major chemicals responsible for antimicrobial properties of the EOs. The chemical components of EOs act by altering cytoplasmic membrane structure of the microorganisms, inhibiting the transport of

electrons or acting as potential denaturing agents of proteins, solvents and dehydrators [6]. In antimicrobial activity assessment of the three EOs, the highest activity belonged to nonencapsulated and nanoencapsulated OEO since the lowest MIC and MBC values were seen against the bacterial strains (*E. coli*, *S. typhimurium* or *Staph. aureus*), compared to tested TEO and CLEO nonencapsulated or nanoencapsulated (Figures 2 and 3). These results can be due to the high content of carvacrol (60.76%) in tested OEO. The results obtained in this study agree with other studies [48], which reported that OEO is one of the most effective EOs for antimicrobial control; its phenolic components have the ability to alter the outer cell membrane, which causes the leakage of protons and potassium ions and results in the collapse of the membrane and inhibition of ATP synthesis. In contrast, it was observed that the least effective EO was tested CLEO (nonencapsulated or nanoencapsulated), since it showed the highest values of MIC and MBC, against the bacterial strains. These results were similar to those from other studies [49], showing antimicrobial efficiency of EOs in following order of oregano > clove flower > coriander > cinnamon > thyme > mint > rosemary > mustard > cilantro > sage. Regarding the target bacterial genus, nonencapsulated and nanoencapsulated EOs exhibited excellent antimicrobial activity against *E. coli*, including the lowest MIC and MBC values. Based on the data from literatures, MIC of the OEO (*Origanum vulgare*) against *S. typhimurium* includes 0.12-3.12 $\mu\text{l ml}^{-1}$ [30] and MIC of OEO (*Origanum compactum*) against *E. coli* and *Staph. aureus* includes 0.625 and 1.25 $\mu\text{l ml}^{-1}$, respectively [50]. Furthermore, MIC of TEO (*Thymus vulgaris*) against *S. typhimurium* includes 0.45-7.20 $\mu\text{l ml}^{-1}$ [30] and MIC of TEO (*T. serpyllum*) against *Staph. aureus* includes 2.5 $\mu\text{l ml}^{-1}$ [50]. Results from the present study are similar to those by other studies; however, composition of EOs depends on several factors including harvesting seasons and geographical sources. These can explain the differences in results by different studies [30]. Studies have documented that EO components include greater antimicrobial effects on Gram-negative bacteria [51]. In the current study, greater resistances generally belonged to *Staph. aureus* against nonencapsulated and nanoencapsulated EOs, compared to those belonged to *E. coli* and *S. typhimurium* (Figures 2 and 3). In comparison of Gram-negative bacteria in this study, a greater resistance to the EOs was detected in *S. typhimurium*, compared to that detected in *E. coli*. In this study, decreased MIC of 27-60% was seen in nanoencapsulated EOs, compared to that in nonencapsulated EOs. In characterization of the nanoencapsulated EOs, very small particle sizes of 116.4-232.5 nm (D_{50}) were detected (Table 3), which facilitated their easy diffusion into the media, as shown by other studies [52].



OEO: oregano essential oil; TEO: thyme essential oil; CLEO: clove leave essential oil. Analyses were carried out in triplicate, MIC: minimum inhibitory concentration.

Figure 2. Antimicrobial activity of the essential oils (nonencapsulated and nanoencapsulated) against the highlighted bacteria for minimum inhibitory concentration



OEO: oregano essential oil; TEO: thyme essential oil; CLEO: clove leave essential oil; analysis were in triplicate, MBC: minimum bactericidal concentration.

Figure 3. Antimicrobial activity of the essential oils (nonencapsulated and nanoencapsulated) against the highlighted bacteria for minimum bactericidal concentration

4. Conclusion

In conclusion, the nanoencapsulation process using emulsification was effective in preserving the major constituents of pure OEO, TEO and CLEO. This result was linked to low droplet sizes and high EE of the nanoencapsulated EOs. Antimicrobial activity of the EOs depended on the EO type and solubility and the bacterial strain. Nonencapsulated and nanoencapsulated OEO showed promises in controlling growth of the bacteria in model systems, where a complete growth inhibition was observed at EO concentrations of 0.218-0.300 µl ml⁻¹ for *E. coli*, 0.437-1.100 µl ml⁻¹ for *S. typhimurium* and 0.546-1.300 µl ml⁻¹ for *Staph. aureus*. In general, the nanoencapsulated EOs were more effective in inhibition of the bacterial strains, compared to that the nonencapsulated EOs were. Nanoemulsions, produced in this study, exhibited important antibacterial activities against the bacterial

strains. However, further studies are necessary to investigate interactions of EOs with constituents in real food systems and establish models for the optimization of EO antimicrobial effects. It is also necessary to establish maximum use quantities of the EOs to ensure safety of foods with no unfavorable effects on sensory characteristics.

5. Acknowledgements

The authors acknowledge financial supports from the National Council for Science and Technology (CONACyT) of Mexico (grant numbers CB-2016-01-283636 and SRE-CONACyT-278363) and Universidad de las Americas Puebla (UDLAP). Furthermore, Nancy Ruiz-Gonzalez gratefully acknowledges financial supports from CONACyT and UDLAP for her PhD in Food Science.

6. Conflict of interest

The authors declare no conflict of interest.

References

- Ait-Ouazzou A, Loran S, Bakkali M, Laglaoui A, Rota C, Herrera A, Pagan R, Conchello P. Chemical composition and antimicrobial activity of essential oils of *Thymus algeriensis*, *Eucalyptus globulus* and *Rosmarinus officinalis* from Morocco. *J Sci Food Agric*. 2011; 91(14): 2643-2651. doi:10.1002/jsfa.4505
- Bakry AM, Abbas S, Ali B, Majeed H, Abouelwafa MY, Mousa A, Liang L. Microencapsulation of oils: A comprehensive review of benefits, techniques, and applications. *Compr Rev Food Sci Food Saf*. 2015; 15(1):143-182. doi:10.1111/1541-4337.12179
- Sakkas H, Papadopoulou C. Antimicrobial activity of basil, oregano and thyme essential oils. *J Microbiol Biotechnol*. 2017; 27(3): 429-438. doi: 10.4014/jmb.1608.08024
- Gavaric N, Mozina SS, Kladar N, Bozin B. Chemical profile, antioxidant and antibacterial activity of thyme and oregano essential oils, thymol and carvacrol and their possible synergism. *J Essent Oil Bear Plants*. 2015; 18(4): 1013-1021. doi: 10.1080/0972060x.2014.971069
- Delaquis P. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int J Food Microbiol*. 2002; 74(1-2):101-109. doi: 10.1016/s0168-1605(01)00734-6
- Hernandez-Hernandez E, Regalado-Gonzalez C, Vazquez-Landaverde P, Guerrero-Legarreta I, Garcia-Almendarez BE. Microencapsulation, chemical characterization and antimicrobial activity of Mexican (*Lippia graveolens* H.B.K.) and European (*Origanum vulgare* L.) oregano essential oils. *Sci World J*. 2014; 14:1-12. doi: 10.1155/2014/641814
- Burt S. Essential oils: Their antibacterial properties and potential applications in foods-a review. *Int J Food Microbiol*. 2004; 94:223-253. doi: 10.1016/j.ijfoodmicro.2004.03.002.
- Misharina TA. Changes in the composition of the essential oil of marjoram during storage. *Appl Biochem Microbiol*. 2003; 39(3):311-316. doi: 10.1023/a:1023592030874
- Adamiec J. Moisture sorption characteristics of peppermint oil microencapsulated by spray drying. *Dry Technol*. 2009; 27(12): 1363-1369. doi: 10.1080/07373930903383695
- Ayala-Zavala JF, Soto-Valdez H, Gonzalez-Leon A, Alvarez-Parrilla E, Martin-Belloso O, Gonzalez-Aguilar GA. Microencapsulation of cinnamon leaf (*Cinnamomum zeylanicum*) and garlic (*Allium sativum*) oils in β -cyclodextrin. *J Incl Phenom Macrocycl Chem*. 2007; 60(3-4):359-368. doi: 10.1007/s10847-007-9385-1
- Dima C, Cotarlet M, Tiberius B, Bahrim G, Alexe P, Dima S. Encapsulation of coriander essential oil in β -cyclodextrin: antioxidant and antimicrobial properties evaluation. *Rom Biotechnol Lett*. 2014; 19(2): 9128-9140.
- Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol*. 2001; 91: 453-462. doi: 10.1046/j.1365-2672.2001.01428.x
- Lu WC, Huang DW, Wang CC, Yeh CH, Tsai JC, Huang YT, Li PH. Preparation, characterization, and antimicrobial activity of nanoemulsions incorporating citral essential oil. *J Food Drug Anal*. 2018; 26(1): 82-89. doi: 10.1016/j.jfda.2016.12.018.
- Sharif HR, Abbas S, Majeed H, Safdar W, Shamoan, M, Khan MA, Shoaib M, Raza H, Haider J. Formulation, characterization and antimicrobial properties of black cumin essential oil nanoemulsions stabilized by OSA starch. *J Food Sci Technol*. 2017; 54(10): 3358-3365. doi: 10.1007/s13197-017-2800-8
- Donsi F, Annunziata M, Sessa M, Ferrari G. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *J Food Sci Tech*. 2011; 44: 1908-1914. doi: 10.1016/j.jbiotec.2010.08.175
- Bejrappa P, Choi MJ, Surassmo S, Chun JY, Min SG. Formulation and antimicrobial activity on *Escherichia coli* of nanoemulsion coated with whey protein isolate. *Korean J Food Sci Anim Resour*. 2011; 31(4): 543-550. doi: 10.5851/kosfa.2011.31.4.543
- Emamjomeh L, Imani S, Talebi KH, Moharrampour S, Larijani K. Preparation of nanoemulsion formulation of essential oil of *Zataria Multiflora* and comparison of contact toxicity with pure essential oil on *Ephestia Kuehniella*. *J Food Saf*. 2012; 32(3): 326-332. doi: 10.1111/j.1745-4565.2012.00384.x
- Moraes-Lovison M, Marostegan LFP, Peres MS, Menezes IF, Ghiraldi M, Rodrigues RAF, Fernandes AM, Pinho SC. Nanoemulsions encapsulating oregano essential oil: production, stability, antibacterial activity and incorporation in chicken pate. *J Food Sci Technol*. 2017; 77: 233-240. doi: 10.1016/j.lwt.2016.11.061
- Pathania R, Khan H, Kaushik R, Khan MA. Essential oil nanoemulsions and their antimicrobial and food applications. *Curr Res Nutr Food Sci*. 2018; 6(3): 626-643. doi: 10.12944/CRNFSJ.6.3.05
- Kumari S, Kumaraswamy RV, Choudhary RC, Sharma SS, Pal A, Raliya R, Biswas P, Saharan V. Thymol nanoemulsion exhibits potential antibacterial activity against bacterial pustule disease and growth promotory effect on soybean. *Sci Rep*. 2018; 8(1): 6650. doi:10.1038/s41598-018-24871-5.
- National Institute of Standards and Technology (NIST). Automated mass spectral library with search program (NIST 11, software, version 2.0g).
- A.O.A.C. Official Method 920.141 - Refractive Index of Lemon and Orange Oils. In: Official Methods of Analysis A.O.A.C International, 17th Edition. Gaithersburg, Md, Association of Official Analytical Chemists, 2000: 1-25.
- Tadros T. Handbook of colloid and interface science. De Gruyter, Berlin, 2018.
- A.O.A.C. Official Method 920.134 - Specific Gravity of lemon, orange and lime extracts - Pycnometer Method. In: Official Methods of Analysis A.O.A.C International, 17th Edition. Gaithersburg, Md, Association of Official Analytical Chemists, 2000: 1-25.
- Davila-Rodriguez M, Lopez-Malo A, Palou E, Ramirez-Corona N, Jimenez-Munguia MT. Antimicrobial activity of nanoemulsions of cinnamon, rosemary, and oregano essential

- oils on fresh celery. *LWT Food Sci Technol.* 2019; 112:108247.
doi: 10.1016/j.lwt.2019.06.014
26. A.O.A.C. Official Method 981.12–pH of acidified foods. In: *Official Methods of Analysis A.O.A.C International*, 17th Edition. Gaithersburg, Md., Association of Official Analytical Chemists, 2000: 1-14.
 27. Xiao Z, Li W, Zhu G, Zhou R, Niu Y. Study of production and the stability of styrallyl acetate nanocapsules using complex coacervation. *Flavour Fragr J.* 2016; 31(4):283-289.
doi: 10.1002/ffj.3306
 28. Hebishy E, Buffa M, Guamis B, Trujillo JA. Stability of sub-micron oil-in-water emulsions produced by ultra-high pressure homogenization and sodium caseinate as emulsifier. *Chem Eng Trans.* 2013; 32: 1813-1818.
doi: 10.3303/CET1332303
 29. Haba E, Bouhdid S, Torrego-Solana N, Marqus AM, Espuny MJ, Garcia-Celma MJ, Manresa A. Rhamnolipids as emulsifying agents for essential oil formulations: antimicrobial effect against *Candida albicans* and methicillin-resistant *Staphylococcus aureus*. *Int J Pharm.* 2014; 476(1-2):134-141.
doi: 10.1016/j.ijpharm.2014.09.039
 30. Boskovic M, Zdravkovic N, Ivanovic J, Janjic J, Djordjevic J, Starcevic M, Baltic MZ. Antimicrobial activity of thyme (*Tymus vulgaris*) and oregano (*Origanum vulgare*) essential oils against some food-borne microorganisms. *Proc Food Sci.* 2015; 5:18-21.
doi: 10.1016/j.profoo.2015.09.005
 31. Olmedo R, Nepote V, Grosso NR. Antioxidant activity of fractions from oregano essential oils obtained by molecular distillation. *Food Chem.* 2014; 156:212-219.
doi: 10.1016/j.foodchem.2014.01.087
 32. Navarrete P, Toledo I, Mardones P, Opazo R, Espejo R, Romero J. Effect of *Thymus vulgaris* essential oil on intestinal bacterial microbiota of rainbow trout, *Oncorhynchus mykiss* (Walbaum) and bacterial isolates. *Aquac Res.* 2010;41:667-678
doi: 10.1111/j.1365-2109.2010.02590.x
 33. Bhuiyan MNI, Begum J, Nandi NC, Akter F. Constituents of the essential oil from leaves' and buds of clove (*Syzygium caryophyllatum* (L.) Alston). *Afr J Plant Sci.* 2010; 4(11): 451-454.
 34. Alabado-Plaus E, Saez-Flores G, Grabiell-Ataucusi S. Composicion quimica y actividad antibacteriana del aceite esencial del *Origanum vulgare* (orégano). *Rev Med Hered.* 2001; 12(1):16-19.
doi: 10.20453/rmh.v12i1.660
 35. Haddouchi F, Chaouche T, Lazouni HA, Benmansour A. Physicochemical study essential oils of *Thymus fontanesii* according to its conservation. *Der Pharma Chemica.* 2011; 3(2):404-410.
 36. Nowak K, Ogonowski J, Jaworska M, Grzesik K. Clove oil properties and applications. *Sci Tech.* 2012; 66(2):145-152.
 37. Siddiqui N, Ahmad A. A study on viscosity, surface tension and volume flow rate of some edible and medicinal oils. *Int J Sci Environ Technol.* 2013; 2(6):1318-1326.
 38. Busatta C, Mossi AJ, Rodrigues MRA, Cansian RL, De Oliveira JV. Evaluation of *Origanum vulgare* essential oil as antimicrobial agent in sausage. *Braz J Microbiol.* 2007; 38(4):610-616.
doi: 10.1590/s1517-83822007000400006
 39. Shabnum S, Wagay MG. Essential oil composition of *Thymus Vulgaris L.* and their uses. *J Res Dev.* 2011; 11: 1-12.
 40. Campelo PH, Junqueira LA, Resende JV, Zacarias RD, Fernandes RV de B, Botrel DA, Borges SB. Stability of lime essential oil emulsion prepared using biopolymers and ultrasound treatment. *Int J Food Prop.* 2017; 20(1):5564-5579.
doi: 10.1080/10942912.2017.1303707
 41. McClements DJ. *Food Emulsion: Principle, Practices, and Techniques*, 2nd Edition. CRC Press, Boca Raton, FL, USA, 2005: 1-609.
 42. Salager JL. Emulsion Properties and Related know-how to attain them. In: Nielloud F, Marti-Mestres G. *Pharmaceutical Emulsions and Suspensions*. New York, Marcel Dekker Inc., 2000: 72-125.
 43. McClements DJ. *Food Emulsions: Principles, Practice and Techniques*. CRC Press, Florida, 1999:1-374.
 44. Jafari SM, Assadpoor E, He Y, Bhandari B. Encapsulation efficiency of food flavours and oils during spray drying. *Dry Technol.* 2008; 26(7):816-835.
doi: 10.1080/07373930802135972
 45. McClements DJ, Rao J. Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Crit Rev Food Sci Nutr.* 2011; 51(4):285-330.
doi: 10.1080/10408398.2011.559558
 46. Barbosa-Canovas GV. *Food Engineering, Vol. II. EOLSS, USA, 2009:1-334*
 47. Lopez-Malo A, Palou E, Leon-Cruz R, Alzamora SM. Mixtures of natural and synthetic antifungal agents. *Adv Food Mycol.* 2006; 261-286.
doi: 10.1007/0-387-28391-9_18
 48. Fisher K, Phillips C. Potential antimicrobial uses of essential oils in food: is citrus the answer?. *Trends Food Sci Technol.* 2008; 19(3): 156-164.
doi: 10.1016/j.tifs.2007.11.006
 49. Rodriguez-Garcia I, Silva-Espinoza BA, Ortega-Ramirez LA, Leyva JM, Siddiqui MW, Cruz-Valenzuela MR, Gonzalez-Aguilar GA, Ayala-Zavala JF. Oregano essential oil as an antimicrobial and antioxidant additive in food products. *Crit Rev Food Sci Nutr.* 2015; 56(10):1717-1727.
doi: 10.1080/10408398.2013.800832
 50. Ouedrhiri W, Balouiri M, Bouhdid S, Moja S, Chahdi FO, Taleb M, Greche H. Mixture design of *Origanum compactum*, *Origanum majorana* and *Thymus serpyllum* essential oils: Optimization of their antibacterial effect. *Ind Crops Prod.* 2016; 89:1-9.
doi: 10.1016/j.indcrop.2016.04.049
 51. Moraes-Lovison M, Marostegan LFP, Peres MS, Menezes IF. Nanoemulsions encapsulating oregano essential oil: Production, stability, antibacterial activity and incorporation in chicken pate. *J Food Sci Technol.* 2016; 77: 233-240.
doi: 10.1016/j.lwt.2016.11.061
 52. Weiss J, Gaysinsky S, Davidson M, McClements J. Nanostructured Encapsulation Systems: Food Antimicrobials. In: Barbosa-Canovas G, Mortimer A, Lineback D, Spiess W, Buckle K, Colonna P. *Global Issues in Food Science and Technology*. New York, NY, USA, Academic Press, 2009:425-479.
doi: 10.1016/b978-0-12-374124-0.00024-7

فعالیت ضد میکروبی و ویژگی‌های فیزیکوشیمیایی اسانس روغنی برگ پونه کوهی، آویشن و میخک، ریزپوشانی نشده و نانوریزپوشانی شده به روش امولسیون سازی

رویز-گونزالز نانسی، لویز-مالو آریلیو، پائولو انریکه، رامیرز کرنا، جیمز-مانگویا ماریا ترزا*

دانشگاه آمریکایی پوئبلا، گروه مهندسی شیمی، محیط زیست و مواد غذایی، هاسیندا، کاتارینا مارتیر، سان آندرس کولولا، پوئبلا، مکزیک کدپستی ۷۲۸۱۰.

چکیده

سابقه و هدف: خواص فراسودمندی اسانس‌های روغنی به ترکیباتشان مربوط می‌شود، بسیاری از این ترکیبات فعالیت ضد میکروبی در برابر میکروارگانیسم‌های بیماریزا و مولد فساد در طیف گسترده‌ای از مواد غذایی دارند. با این حال، اسانس‌های روغنی ترکیبات ناپایداری می‌باشند؛ بنابراین، به منظور محافظت بهتر و افزایش عملکرد ریزپوشانی می‌شوند. در این تحقیق، فعالیت ضد میکروبی اسانس روغنی برگ پونه کوهی، آویشن و میخک (ریزپوشانی نشده و نانوریزپوشانی شده) در برابر *شرشیا کلی* ATCC 29922، *سالمونلا تیفی موریوم* ATCC 14028 و *ستافیلوکوکوس اورئوس* ATCC 25923 با استفاده از امولسیون سازی بررسی شد.

مواد و روش‌ها: اسانس‌های روغنی بر اساس خواص فیزیکوشیمیایی طبقه بندی شدند. نانوامولسیون‌های حاوی ۵ در صد وزنی/وزنی اسانس روغنی تهیه و سپس بر اساس خواص فیزیکی، پایداری و کارایی ریزپوشانی طبقه بندی شدند. اثر ضد میکروبی با روش ریز رقت سازی برای تعیین حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی اسانس‌های روغنی و نانوامولسیون‌های آنها بررسی شد. اطلاعات به دست آمده در مورد خواص فیزیکی اسانس‌های روغنی و خواص فیزیکی، پایداری و کارایی ریزپوشانی نانوامولسیون‌ها از نظر آماری مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: فعالیت ضد میکروبی اسانس‌های روغنی با کاهش حداقل غلظت مهارکنندگی تا ۶۰-۲۷ در صد برای اسانس‌های نانوریزپوشانی شده، در مقایسه با انواع ریزپوشانی نشده نشان داده شد. اسانس روغنی ریزپوشانی شده و ریزپوشانی نشده پونه کوهی پایین‌ترین حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی را نشان داد. بر اساس نتایج به دست آمده، اسانس‌های روغنی ریزپوشانی شده برای جلوگیری از آلودگی میکروبی در مواد غذایی گوناگون می‌توانند مورد استفاده قرار گیرند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۲۷ می ۲۰۱۹
داوری ۳۰ جولای ۲۰۱۹
پذیرش ۲۴ اگوست ۲۰۱۹

واژگان کلیدی

- ترکیبات ضد میکروبی
- اسانس‌های روغنی
- نانوریزپوشانی
- میکروارگانیسم‌های بیماری‌زا

*نویسنده مسئول

جیمز-مانگویا ماریا ترزا،
دانشگاه آمریکایی پوئبلا، گروه
مهندسی شیمی، محیط زیست و
مواد غذایی، هاسیندا، کاتارینا مارتیر،
سان آندرس کولولا، پوئبلا، مکزیک
کدپستی ۷۲۸۱۰.

تلفن: ۴۳۵۳ ۲۲۹ ۲۲۲-۵۲+

پست الکترونیک:
mariat.jimenez@udlap.mx