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Synthesis and characterization of lemon essential oil nanoliposomes as potential antimicrobial agents

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Abstract

Lemon essential oil (LEO) has antimicrobial efficiency and other biological activities, but its exposure to deterioration from environmental circumstances restricts its application in the food industry. In this work, LEO nanoliposomes were formulated to enhance LEO characteristics, using the modified heating method (Mozafari method). Four different concentrations of LEO were synthesized and characterized. For the first day of preparation, the size ranged from 124.4 to 215.7nm and zeta potential ranged from -69.35 to -80.0 mV, depending on LEO content, and polydispersity index (PDI; < 0.6). After 60 days of storage at 4 °C, the results indicated high stability. High-resolution transmission electron microscopy (HRTEM) analysis confirmed the spherical structure of the nanoliposomes. Encapsulation efficiency (EE) was determined by GC–MS. LEO was successfully encapsulated in nanoliposomes with EE (> 96 %). Free LEO and encapsulated LEO were tested as antimicrobial agents in a commercial chicken soup as a food system. The antimicrobial effect was investigated against Staphylococcus aureus and Escherichia coli as food-borne pathogens for 4 days at 20 °C. The results revealed that LEO nanoliposomes have higher activity than free LEO, especially against E. coli. From the previous findings, encapsulated LEO is a promising approach that could be applied in the food industry.

Keywords: Lemon essential oil; nanoliposomes; antimicrobial activity; Mozafari method; chicken soup.

Introduction

In the last few years, there has been an increasing interest toward food preservation and its shelf life extension. The efficiency of essential oils provides novel and safer alternatives for traditional chemical preservatives against food-borne pathogens as antimicrobial agents [1].

Essential oils contain a variety of natural volatile compounds produced from plants as secondary metabolites such as terpenoids, alcoholic compounds, acidic compounds, aldehyde, ketonic bodies, and phenols. Therefore, essential oils could have a various biological properties as antibacterial, antifungal, antioxidant, antiviral, antiparasitic, and antiseptic activities [2].

Lemon essential oil (LEO) is one of the most effective essential oils that exhibit antimicrobial activity, and it's obtained from *Citrus limon* species that belong to *Rutaceae* family. Lemon is consumed throughout the world as it is considered an important source of valuable components such as ascorbic acid, flavonoids, and phenolic compounds [3].

However, poor bioavailability, low aqueous solubility, and stability against environmental conditions of essential oils have restricted their applications in the food industry. Therefore, nanoencapsulation systems might effectively overcome these limitations and improve their properties [4]. Nanoliposomes, nanoemulsions, nanolipid carriers, nature-inspired nanocarriers, and biopolymer nanoparticles represent different formulations for nanoencapsulation applied in the food industry [5, 6].

Nanoliposomes are enclosed spherical arrangements formed of a lipid bilayer containing part of the solvent inside it. Nanoliposomes are composed of amphiphilic phospholipid with a water-soluble hydrophilic head portion and a lipid-soluble hydrophobic tail portion. The feature of self-assembling into sphere, when placed in an aqueous medium, constitutes a system able to entrap hydrophilic molecules in their interior

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and hydrophobic molecules in the lipid bilayer [7]. Therefore, encapsulation of essential oils by nanoliposomes is efficient in enhancing their bioavailability by shielding them against harsh environments and processing conditions, and regulating their release.

Various techniques are used to prepare nanoliposomes for encapsulating essential oils and other bioactive constituents; thin-film hydration, solvent injection, reverse phase evaporation, microfluidisation, modified technique of rapid expansion of supercritical solutions (RESS) and heating method [8]. Rosemary, lemon [9], Zanthoxylum tingoassuiba [10], and Anethum graveolens [11] essential oils were prepared by thin-film hydration procedure. Cardamom [12] and clove [13] oils were encapsulated by solvent injection method. Most preparation techniques involve using toxic solvents or applying high shear force; also, they are time and cost-consuming. In addition, the hazardous solvent residue may be retained in the final product leading to toxicity and instability of the nanoliposomes [14].

The study aimed to prepare encapsulated LEO in nanoliposomes by Mozafari method, which reduces the limitations of the other methods. The physicochemical properties and the EE were investigated. Then the nanoliposomes were tested as an antimicrobial agent in a food model.

Materials and methods

Materials

Phosphatidyl-choline soya lecithin was provided from Carlo Erba Reagents (France). Cholesterol was supplied from Bio Basic Inc (USA). LEO of Citrus limon species was obtained from Purity Company (Egypt). Other chemicals of analytical grade were purchased from Sigma-Aldrich. The study was carried out in Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Egypt, during February 2021 till February 2022.

Methods

GC-MS analysis of LEO components

To identify the active components of LEO, GC–MS was used for the analysis. GC (Agilent 7890A, Germany) was supplied with HP-5MS 5% Phenyl Methyl Silox capillary column having the following features; 30 m length x 250 µm internal diameter x 0.25 µm film thickness. GC was paired with inert MS with triple-axis detector (Agilent 5975, Germany). Temperature program of GC oven was set as follows: firstly, 80 °C (held for 3 min), then temperature raised with rate of 4 °C/min to 180 °C for 6 min, finally, 230 °C with rate of 6 °C/min. High purity helium was passed through the column as a carrier gas with a flow rate of 1 mL/min. Other temperature parameters were set as follow: injector was 250 °C, mass source and mass quadrupoles were 230 and 150 °C, respectively. LEO was diluted with hexane and injected with splitless mode. Data were obtained by MS working in electron-impact ionization (EI) mode at 70 eV, with a mass scan range m/z 50 to 550.

Preparation of nanoliposomes

Nanoliposomes were formed according to the modified heating method by Mozafari [15]. First, cholesterol was hydrated in deionized water and stirred at 1000 rpm at a temperature of 120 °C under N2 gas for 30 min. A mixture of LEO and glycerol was heated to 30 °C (3 % final concentration, v/v), and added to the liposomal components (lecithin, cholesterol with a ratio (8:1). All components were stirred at 1000 rpm for 1 h on a hotplate stirrer while the temperature was kept at 30 °C. Then the solution was sonicated at 30 °C for 30 min in a sonication bath to reduce the size of the liposomes. After that, the prepared nanoliposomes remained under N2 at room temperature for 60 min to be stabilized. Different formulations were prepared according to LEO concentrations; 4, 8, 12, and 24 ug/mL.

Hydrodynamic diameter, size distribution and zeta potential measurements

Hydrodynamic diameter, size distribution (polydispersity index; PDI) and zeta potential of LEO nanoliposomes were estimated by a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, UK).

High-resolution transmission electron microscopy (HRTEM)

To examine the microstructure of the nanoliposomes, transmission electron microscope analysis was performed. The liposomal suspension was photographed with JEOL- JEM 2100 (Japan) operating at an acceleration voltage of 200 kV, using uranyl acetate negative staining.

Encapsulation efficiency determination

The amount of the encapsulated oil into liposomes was detected according to Sebaaly et al. [13] with some modification. The liposomal dispersion was ultracentrifuged at 100,000 x g for 2 h (Sorvall

by GC-MS to calculate the amount of D-limonene content as a main constituent of LEO [16]. EE % was evaluated using this equation [17].

$$(EE\ \% = \frac{(Amount\ of\ total\ oil\ in\ liposomal\ dispersion - amount\ of\ free\ unencapsulated\ oil)}{Total\ oil\ amount} \times \times 100\ \ )$$

Antimicrobial activity

The antimicrobial activity was performed against *Staphylococcus aureus* (ATCC 25913) as a Gram-positive and *Escherichia coli* (ATCC 25922) as a Gram-negative bacteria. These strains were obtained from Cairo Microbiology Research Center. The bacterial suspension was prepared for each strain by cultivation on nutrient agar at 35 ℃ for 24 hours. The colonies were transferred into test tubes containing sterile saline to reach 1.5×10^8 CFU/mL. The turbidity of the bacterial suspensions was adjusted by using 0.5 McFarland standard as a reference (0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2\cdot2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4), using a spectrophotometer to determine the optical density) [2].

Preparation of chicken soup

Food model in this work was a commercial chicken soup (Maggi, Egypt). According to the producer's instructions, the soup cube was dissolved in one liter of distilled water and heated. The soup was then split up into tubes and autoclaved at 121 ˚C for 15 min.

Chicken soup inoculation

The initial concentration of the bacterial suspension was serially diluted and seeded into the soup to a final concentration of 1.5×10^3 CFU/mL of soup. Soup with inoculum is considered as control. LEO was diluted in 5 % DMSO (v/v), this concentration is found to be non-lethal [2]. The free and loaded LEO were filtered and added to inoculated soup tubes to get a final concentration of 2.4 µL/mL soup for each one. The tubes were incubated at 20 ˚C while shaking [16]. Then each tube was examined by plate counting method to estimate bacterial count by serial dilution at days 0, 1, 2, 3, and 4.

Statistical analysis

A one-way analysis of variance (ANOVA) and Tukey's post hoc test were used to determine significant differences P <l 0.05 between means by IBM SPSS Statistics version 20. Data were expressed as mean ± standard deviation.

Results and discussion

Components of LEO

Figure 1 displays the chromatogram of the analyzed sample of LEO by GC-MS, and **Table 1** shows the percent of various components of the analyzed sample of LEO displayed by the total ions chromatogram obtained by GC-MS. These components are mixture of aromatic hydrocarbons, monoterpenes, and their oxygenated derivatives of alcohols and aldehydes [18]. The identified components have antimicrobial activity [19]. The most common are *D*-limonene (25.9 %), geranial (16.17 %), neral (13.56 %), dihydromercenol (10.31 %), and linalool (6.82%).

Table 1. Components of lemon essential oil as revealed by GC-MS

No.	Components	RT	Area %
1	beta-pinene	4.88	0.44
2	*D*-Limonene	5.8	25.9
3	Dihydromyrcenol	6.85	10.31
4	Linalool	7.64	6.82
5	2-Phenylethanol	8.1	0.63
6	Acetic acid, phenylmethyl ester	9.5	2.75
7	alpha-Terpineol	10.42	1.96
8	Decanal	10.81	1.01
9	3-Carene	11.57	1.85
10	Neral	11.9	13.56
11	Geraniol	12.4	3.76
12	Geranial	12.94	16.17
13	Camphene	15.42	0.52
14	Undecanal, 2-methyl-	15.93	1.06
15	alpha-Pentylcinnamaldehyde	24.3	3.33

Hydrodynamic diameter, PDI and zeta potential

The measurements of hydrodynamic diameter, PDI, and zeta potential are displayed in **Figure 2.** The diameter of all the formulae ranged from 124.4±1.76 to 215.7±4.94 nm on the first day of preparation and from 152.6±17.6 to 172.6±2.96 nm after 60 days of storage at 4 ˚C. This increase in size is still in adequate nano scale that is satisfactory for antimicrobial activity. Lipid type and concentration, encapsulated material, stabilizing agent, and method of preparation are parameters that affect the particle size [11, 18]. The distribution of bioactive materials in the lipid bilayer of nanoliposomes raises the particle size.

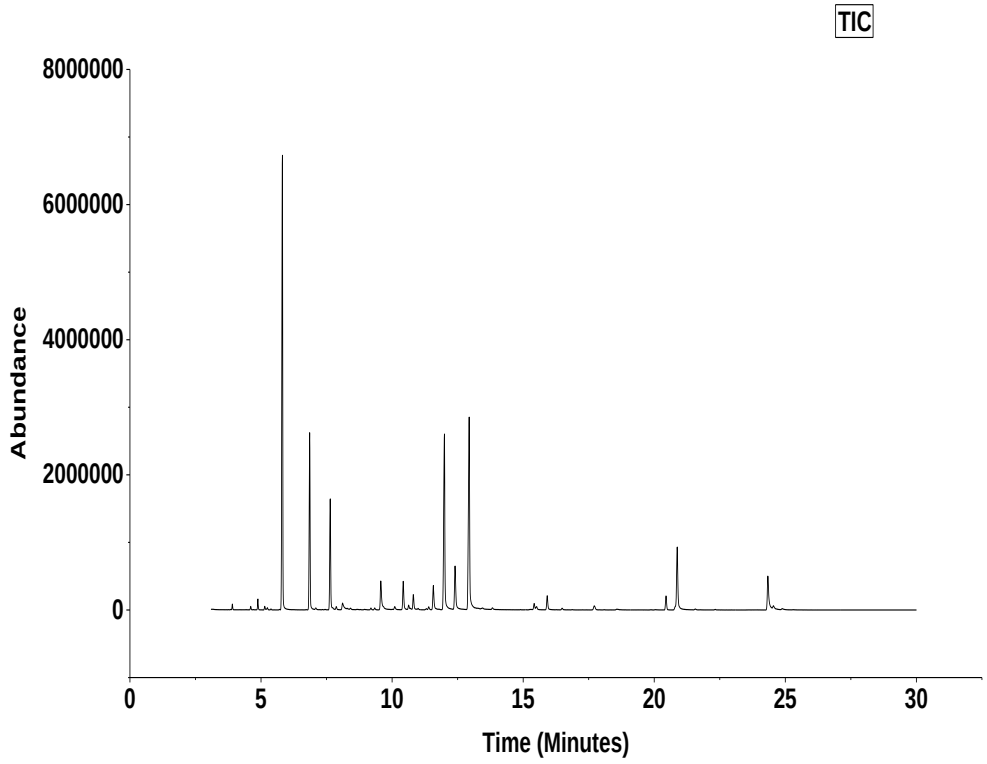


Figure 1: GC-MS chromatogram of lemon essential oil.

This is attributed to interactions between the hydrophobic material and acyl chains phospholipid bilayer, producing enlargement of the membrane [21]. However, a reduction in particle size would be obtained if the hydrophobic compound tends to achieve ultimate packing in the membrane bilayer [22].

PDI indicates particle size distribution, it is important parameter for physical stability of the nanoliposomes. All prepared formulae exhibited PDI values below 0.4 on the first day of preparation, except for the formula of 8 ug/mL LEO content, which has PDI value 0.55. The lower values of PDI indicating large homogeneity for the nanoliposomes [23]. After 60 days of storage, the obtained PDI values were less than 0.5, revealing satisfying physical stability [24].

The surface charge of the nanoliposomes is affected by composition and concentration of liposomal ingredients, bioactive material and the medium. The more positive value of zeta potential than +30 mV and the more negative value than -30 mV produce repulsive forces between particles and consequently, better physical stability [25]. The zeta potential of the preparations was ranged from -69.35±2.33 to -80.0±0.84 mV on the first day and from -56.40±3.11 to -72.80±2.40 mV after 60 days of storage, which indicate high physical stability for the nanoliposomes.

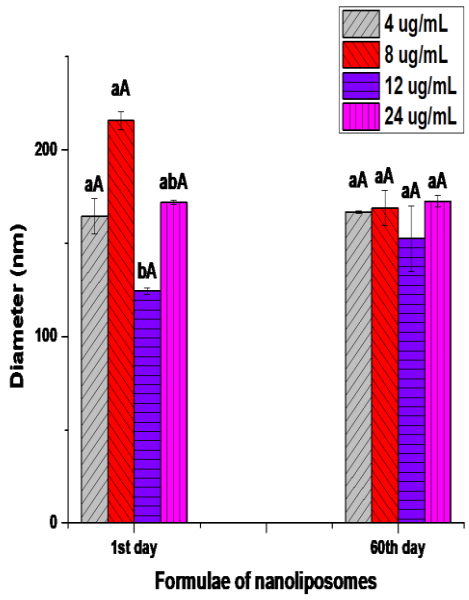
Nanoliposomes microstructure

Micrographs of the prepared nanoliposomes are shown in **Figure 3.** The nanoliposomes were found to be spherical in shape with bilayer membrane, indicating encapsulation of LEO without alteration in liposome structure. The result is comparable with that obtained by DLS.

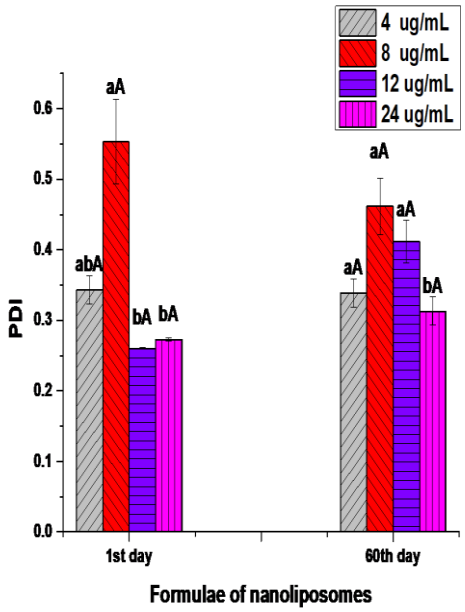
Encapsulation efficiency

EE % was evaluated according to the concentration of *D*-limonene component of LEO that was entrapped into the different formulations of the nanoliposomes. GC-MS analyzed various concentrations of LEO to create a calibration curve between the concentration of limonene and the area ratio **(Figure 4).** All formulae exhibited EE of more than 96% **(Table 2)**. EE% increased from 96.35±0.19 to 99.62±0.02 when the concentration of LEO increased from 4 to 8 ug/mL. However, EE slightly decreased from 99.41±0.02 to 98.46±0.06 when LEO concentration increased from 12 to 24 ug/mL, indicating that higher concentrations would decrease EE. EE is affected by a diversity of parameters; method of preparation, phospholipid concentration and type, EO/lipid ratio, cholesterol concentration, hydrophobicity of encapsulated oil, and stabilizing agent [4, 19]. It is also affected according

to the used nanoencapsulation system. The high EE in this study may be related to the phospholipid bilayer, which represents the hydrophobic portion of the nanoliposomes, and this portion constitutes a storage of LEO. Some studies reported that EE of LEO ranged from (85-94) % [24, 25]. Our findings; preparation of encapsulated LEO by a suitable and safe method with higher EE than that reported by the previous studies.



(a)



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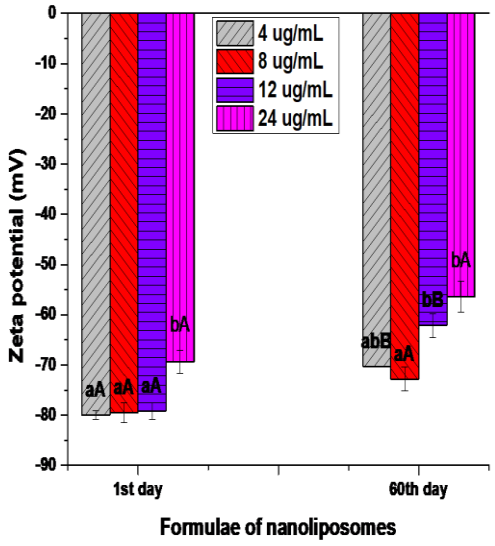


(c)

Figure 2: (a): Hydrodynamic diameter (nm), (b): polydispersity index (PDI) and (c): zeta potential (mV) of the nanoliposomes formulae of LEO. Different lowercase letters indicate significant differences ($p < 0.05$) between all formulae and different uppercase letters indicate significant differences ($p < 0.05$) between times for each formula.

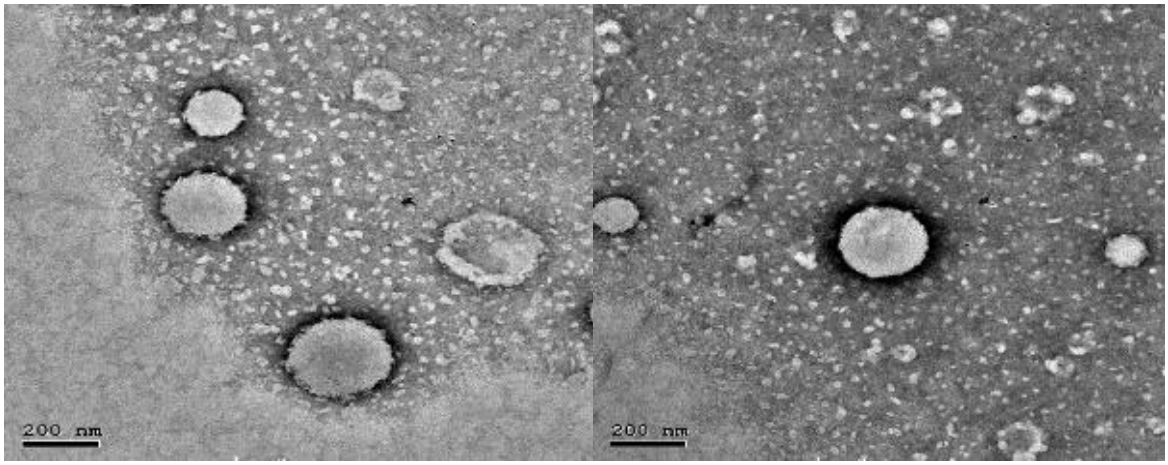


Figure 3: TEM micrographs of lemon essential oil nanoliposomes.

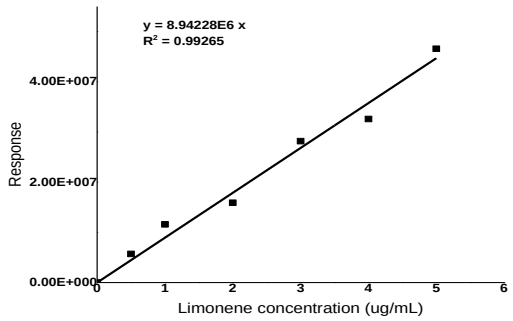


Figure 4: Calibration curve of limonene using GC-MS.

Table 2 EE % of the formulae of loaded nanoliposomes with different concentrations of LEO.

LEO (ug/mL)	EE %
4	96.35±0.19[a]
8	99.62±0.02[b]
12	99.41±0.02[b]
24	98.46±0.06[c]

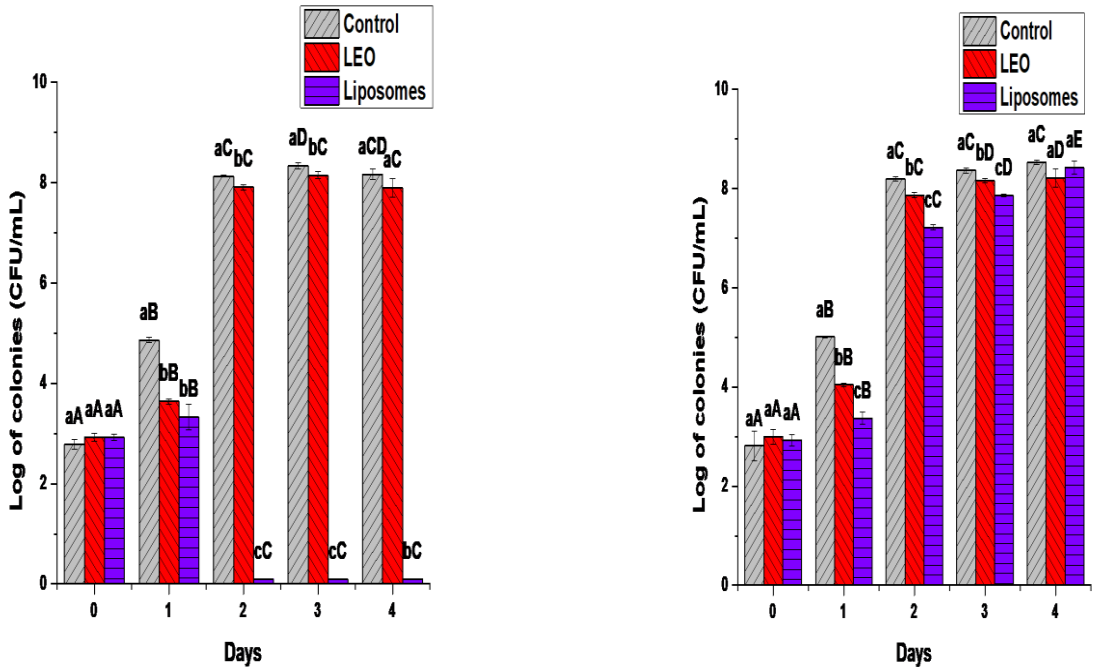
Values are reported as mean ± standard deviation.
Different lowercase letters indicate significant differences in column ($p < 0.05$).

Antimicrobial activity

In this work, the antimicrobial activity of the free and loaded LEO in the chicken soup was estimated from day 0 to day 4 at 20 ˚C. **Figure 5a** reveals the log of colonies count for *E. coli.* The colony count in control was increased constantly from day 0 to day 4. *E. coli* count in the soup with free and loaded LEO were significantly ($P<0.05$) lower than that of the control. Encapsulated LEO caused a reduction in the growth rate of *E. coli* in commercial soup than free LEO on day 1, the number of colonies was reduced by 1.22 logs when treated with free LEO, and by 1.53 logs when treated with encapsulated LEO. . From day 2 to day 4, colonies were not detected in the treatment with encapsulated LEO, while number of colonies reduced by 0.22, 0.19, and 0.27 logs on days 2, 3, and 4 respectively, when treated by free LEO; indicating more efficient activity of encapsulated LEO than the free one. **Figure 5b** reveals the log of colonies count of *S. aureus*, which shows the continuous increase in *S. aureus* count in control from day 0 to day 4. The nanoliposomes of LEO and free LEO exhibited a significantly ($P<0.05$) reduction of *S. aureus* growth than the control one from day 1 to day 3. The number of colonies after 1 day was reduced by 0.97 log when treated with free LEO, and by 1.64 logs when treated with encapsulated LEO. The antimicrobial effect is more efficient for the nanoliposomes than the free LEO. However, there was no significant difference between the control, the free and loaded LEO on day 4, this may be attributed to the availability of nutrients in the food system that can repair damaged cells [28]. The antimicrobial effect of LEO was a result of the presence of terpenes and their derivatives [19]. These compounds, with their lipophilicity, interact with the bacterial cell membrane, leading to membrane deformation and subsequently increasing their permeability and loss of cell contents [27, 28]. Antimicrobial activity of EO of *Citrus limon* and other essential oils (EOs) were reported in other studies. The response of *S. aureus*, *E. coli* and other microorganisms toward *C. limon* essential oil was active [19]. Lemongrass EO was found to be efficient against *E. coli* and *S. aureus* by agar diffusion method [31]. The growth of *S. aureus* in barley soup as a food model was reduced by the effect of lemon peel EO [28].

Our results displayed the enhancement of antimicrobial efficiency by encapsulated LEO than

free LEO; this is related to better stability of the EO by liposome encapsulation. Encapsulation overcomes the obstacle of instability and degradation by environmental factors such as light, oxygen, and temperature [32], also, improving dispersion of EO in food systems [33]. Nanoemulsion of LEO exhibited antimicrobial efficacy better than free oil against *S. aureus* and other food-borne pathogens [29]. The growth of *E. coli* was diminished by the treatment of nanoliposomes containing *D*-limonene; the activity of the encapsulated form was more than the non-encapsulated one [34]. Researchers also reported the influence of other encapsulated EOs on enhancing antimicrobial potency; garlic essential oil nanophytosomes exhibited antimicrobial effects against *S. aureus* and *E. coli*. Liposomes encapsulated curry EO showed a better antimicrobial effect in rice than the EO alone [1]. The previous studies were in agreement with our results of developing the antimicrobial activity of EOs by encapsulation. Further surveys would be of importance to evaluate LEO and other essential oils as an antimicrobial agents against other food-borne pathogens, in different food systems and scaling up to food preservatives.



(a) (b)

Figure 5: Colony counting of (a) *Escherichia coli* and (b) *Staphylococcus aureus*, inoculated in free chicken soup as a control, soup with LEO and soup with nanoliposomes, for 4 days of storage at 20 ˚C. Different lowercase letters indicate significant differences ($p < 0.05$) between all groups and different uppercase letters indicate significant differences ($p < 0.05$) between times for each group.

Conclusion

In this study, LEO nanoliposomes were successfully prepared by the modified heating method, a non-toxic, appropriate, and time saving method. The nanoliposomes were prepared with different concentrations of LEO and the same liposomal ingredients. The method efficiently produced nanoliposomes with particle size less than 215 nm and a high negative value of zeta potential of more than -69 mV, indicating high stability. EE was estimated according to a calibration curve of *D*-limonene constructed by analysis on GC-MS, and results revealed EE more than 96 %. The antimicrobial activity of the encapsulated LEO was studied in a commercial chicken soup against *E. coli* and *S. aureus* as food-borne pathogens. The bacterial count in the food system was estimated by plate count method from day 0 to day 4 at 20 ˚C. The LEO nanoliposomes exhibited an antimicrobial effect greater than that of the free LEO in both microorganisms. Furthermore, the activity was more effective in *E. coli* than *S.*

aureus, as a complete inhibition occurred from day 2 in the case of *E. coli*. These findings present a promising natural and safe food preservative.

Author contributions

The authors contributed the experimental design, analysis and data interpretation; took part in writing the manuscript or revising it critically; agreed to submit to the current journal.

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