

RESEARCH PAPER

The preparation, characterization, and antibacterial activity evaluation of nanoliposomes incorporated with terebinth extract

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Abstract

Nanoliposomes are drug release systems that increase bioavailability and are used for encapsulation of therapeutic active ingredients. Terebinth, which is a medicinal plant that grows in many parts of Türkiye, has antibacterial, antioxidant, and anti-inflammatory activity. The antibacterial activity of nanoliposomes incorporated with ethanol extract of terebinth leaves (TLE) was investigated to determine the effects of formulation.

The nanoliposome formulation was prepared in two steps which were high pressure and high intensity homogenization techniques. Characterization parameters (zeta potential, particle size and distribution, polydispersity index, and encapsulation efficiency) were determined. After third cycle of microfluidization, the zeta potential charge of nanoliposome dispersion was measured -66.6 mV and 91.13 nm in size. The PDI was 0.231. Also, the nanoliposome encapsulation efficiency was calculated as 91.90%. The TLE was encapsulated with nanoliposomes and their antibacterial activity was examined by disk diffusion and minimum inhibition concentration tests against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Nanoliposome encapsulated TLE (NLTLE) has antibacterial activity against *S. aureus* ATCC 25923. While NLTLE has less active substance, it displays the same antibacterial activity as TLE.

Introduction

Liposomes are tiny spherical structures made of amphipathic lipids organized in one or more bilayers. It is also less immunogenic, biodegradable, and biocompatible. (Ulrich, 2002, Vemuri & Rhodes, 1995; Vuilleumard, 1991). Liposomes have many advantages with their structural features, compositions, types and preparation methods. Today, it can be used in many different fields such as food, cosmetics, medicine, pharmacy, and textile (Banerjee, 2001; Betz et al., 2005;

[Cui et al., 2015](#); [Martí et al., 2012](#); [van Balen et al., 2004](#)).

The material being evaluated can be enclosed in a liposome and delivered at the desired place when liposomes are used as medication delivery systems (Pierre & Costa, 2011; Puglia & Bonina, 2012). Similar to this, liposomes are used to encapsulate the substances such as peptides, antibiotics, bioactive chemicals, extracts, and essential oils. Studies indicate that raising the antibiotic concentrations (either passively or actively targeted) near the infection site increases the bactericidal activity, particularly when

liposomes are chosen as antimicrobial drug carriers. It can also lessen the toxicity of the potentially harmful antibacterial active ingredient, which is another contribution (da Silva Malheiros et al., 2010; Engel et al., 2017; Lagacé et al., 1991; Low et al., 2013). Nowadays, nano-sized liposomes have been formed, and their efficacy has been investigated (Tometri et al., 2020). Nanostructures enhance the activity of the encapsulated active material when utilized as a drug carrier. Especially, the microfluidization process is preferred for encapsulation. This technique has several benefits, including high encapsulation efficiency, ability to encapsulate large molecules, to obtain liposome dispersions in nano sizes and long-term stability (Gibbs et al., 1999; Hintz et al., 2015; Hughes, 2005; Torchilin, 2005; Zoghi et al., 2018; Zylberberg & Matosevic, 2016).

The liposomal encapsulation is commonly used with extracts and essential oils from medicinal plants. One of them is *Pistacia terebinthus* L. (Terebinth), which grows naturally in Türkiye, and is used in various fields due to their antibacterial, anti-inflammatory, and cytotoxic activities. The aromatic feature and various medicinal properties of the terebinth are due to the fact that it is a plant rich in phenolic compounds, especially in tannins (Baytop, 1984; Bozorgi et al., 2013; Pelvan & Demirtaş, 2018).

In this study, high pressure and high intensity homogenization (microfluidization) procedures were used to develop a nanoliposome formulation. The physical characteristics of the nanoliposome system were evaluated. Then, the formulation was used to encapsulate the ethanol extract of the terebinth, and its antibacterial activity against gram (+) and gram (-) bacteria were examined.

Materials and Methods

Materials

Terebinth leaves used in the study were obtained from Gaziantep Pistachio Research Institute. The chemicals used in the experiments had analytical purity. Ethanol (99.9%) was obtained from ISOLAB (Wertheim, Germany), L- α -phosphatidylcholine (soybean lecithin, $\geq 30\%$ enzymatic), and sodium carbonate from Sigma-Aldrich (Missouri, USA).

Preparation of Terebinth Extracts and Their Nanoliposomal Forms

In the first step, microemulsions were made to produce nanoliposomes. For initial homogenization, 200 mL of distilled water and 4 g (2% w/v) of L-phosphatidylcholine material were mixed. For the next 5 min, a direct-end sonicator (IKA- T-18 Digital Ultra-Turrax Homogenizer, a high-speed homogenizer, Staufen, Germany) was used at 70% power (Hafner et al., 2011). The high-pressure homogenizer microfluidizer (M-110EH-30 Microfluidizer High Pressure Homogenizer, Westwood, USA) was used to mechanically prepare liposomes as secondary

homogenization. The microfluidization technique was applied on this mixture for three cycles, under a 15000 psi pressure (Macit et al., 2021). After that, to improve its stability and eligibility for encapsulation, the resultant liposome dispersion was lyophilized (Christ Alpha 1-4 LSC Freeze-Dryer, Harz, Germany). For this process, liposome dispersion was kept at $-80\text{ }^{\circ}\text{C}$ for overnight. Then, the frozen liposome was lyophilized.

The method used by Doğan et al. (2017) was modified and used in the ethanol extraction of the terebinth leaves. The leaves were dried in an oven at $40\text{ }^{\circ}\text{C}$ for 12 h. Then, 200 mL of 90% ethanol solution was added to 20 g of terebinth leaves and stirred at room temperature for 24 h (Stuart/UC 152, Staffordshire, United Kingdom). Subsequently, the mixture obtained by passing through ordinary filter paper was centrifuged at 4500 rpm for 15 min at room temperature (Beckman-Coulter Allegra X30R, Indiana, USA). The mixture obtained after centrifugation was separated from the ethanol part with the help of a rotary evaporator (BUCHI rotary evaporator/R360-E, Flawil, Switzerland). The extract was lyophilized (Toros / TRS 2/2V, Istanbul, Turkey). After lyophilization, powdered TLE weighed as 5.7 g. The lyophilized process was carried out in order not to deteriorate the structures of the TLEs stored for use in the studies.

For encapsulation, lyophilized nanoliposomes were dissolved with autoclaved water at a ratio of 1:10 (w:v) using direct-end sonicator (Bandelin/GM4200, Berlin, Germany) in 5 min under ultrasonic conditions, with an ultrasonic power of 70 W. The nanoliposomes were stored at $+4\text{ }^{\circ}\text{C}$ till their characterization analysis and antibacterial experiments.

Analysis of Vesicle Size, Zeta Potential (Z-Potential), and Encapsulation Efficiency (EE)

The mean diameter, zeta potential and PDI values of liposomes were measured by dynamic light scattering (DLS) performed on Malvern Zetasizer (Malvern brand ZS 501).

The encapsulation efficiency analysis was modified and carried out by taking the studies of Kamra et al. (2005) and Nii & Ishii (2005) as an example. The nanoliposome formulations were centrifuged at 18,000 rpm at $5\text{ }^{\circ}\text{C}$ for 60 min. The sediment portion of the mixture was separated and fragmented using ethanol. After this, 1 mL of ethanol-lyzed nanoliposome solution was taken and compared with a UV visible spectrophotometer (VWR/UV-1600PC, Pennsylvania, USA) at 340 nm wavelength.

For this process, lyophilized TLE was dissolved in water instead of ethanol, which is its main solvent. After adding 1.0 g of powdered TLE into 50 mL autoclaved water, it was allowed to dissolve well in the mixer for 90 min. The resulting precipitates were centrifuged at 4500 rpm for 30 min at $26\text{ }^{\circ}\text{C}$ and separated from their residues. Since the main solvent of TLE is not water, only a part of the first 1.0 g of TLE was dissolved. To calculate the weight of the solute fraction, it was obtained by

subtracting the amount of TLE that precipitated in the water after centrifugation from the initial powdered TLE. The amount of solute dissolved in 50 mL of distilled water was found to be 0.19 g.

Scanning Electron Microscope (SEM)

The characterization of the nanoliposome-encapsulated terebinth leaf ethanol extract (NLTLE) was performed via an SEM instrument (GeminiSEM 300, Carl Zeiss, Germany). 5 μL of NLTLE was first added to one side of carbon tape, which was adhesive on both sides and placed on an aluminum stub for drying at ambient temperature. Then, the samples were coated with gold under argon vacuum conditions. Gold sputtering thickness was 3.6 nm, obtained in 50 seconds. The accelerating voltage was 5 kV for all experiments. The mean particle sizes of NLTLE were measured using the ImageJ software with the data obtained from the different SEM images of samples.

Antibacterial Activity Determination

Gram (-) bacteria for antibacterial activity; *E. coli* ATCC 25922 and gram (+) bacteria *S. aureus* ATCC 25923 were chosen. Bacterial cultures were obtained from the culture collection unit in the Department of Bioengineering at Bursa Technical University, Bursa, Türkiye. Disc diffusion method (Bauer, 1966) and minimum inhibition concentration method were used to determine the inhibition effect of NLTLE prepared in the study on test bacteria. In the disc diffusion test, a nutrient liquid (NB) (Merck) medium was used for the growth of bacteria. *E. coli* and *S. aureus* were incubated in NB medium at 37 °C for 24 h. A nutrient agar was used in the disc diffusion test. The cultures obtained were adjusted according to the McFarland turbidity standard no. 0.5 and 100 μL of each was spread on separate media with the help of the Dragalski loop. Then, sterile discs with a diameter of 6 mm (OXOID, Antimicrobial Susceptibility Test Discs) were placed on the surface of the media at certain intervals. 18 μL of NLTLE, TLE, and nanoliposome formulation (NLF) were added to the placed discs with micropipettes and impregnated. Ofloxacin and distilled water were used for a positive control (PC) and negative control (NC) on the empty disc, respectively. At the end of the application, the petri dishes were incubated at 37 °C for 48 h. At the end of the incubation, the diameter of the zones formed around the disc was measured. The disc diffusion test was performed in three replicates for each bacteria and test substance. Test groups were evaluated statistically using the Kruskal-Wallis rank sum test.

The minimum inhibition concentration (MIC) values were determined by the broth microdilution method according to the CLSI M7-A7 (2006) protocol. Nine dilutions were made for NLTLE, TLE, NLF, and ofloxacin in the value range of 100-0.781 $\mu\text{L}/\text{mL}$. Bacterial suspensions were adjusted to 0.5 McFarland standard turbidity. In brief, the wells of 96-well plates were dispensed with 95 μL of nutrient broth and 5 μL of

the bacteria. 100 μL of nine different TLE concentrations were added to it. The same procedures were used with nine different concentrations of NLTLE. The last well was used as a NC. The 96-well microplate was held for 40 sec at 150 rpm on a microplate shaker and incubated in a 37 °C for 24 h. After incubation, each microplate was read in a microplate reader at 600 nm absorbance (Biotek EPOCH microplate spectrophotometer, Vermont, USA). The MIC was evaluated as the lowest concentration of the compounds to inhibit the growth of microorganisms. The MIC test was performed in three replicates for each bacteria and test substance.

Results

Characterization of Nanoliposomes

Particle sizes and zeta potential were determined for each step of the liposome dispersions prepared in two steps. The mean value of the zeta potential of the dispersion prepared in the first stage was measured as -55.0 mV, and the mean size value was measured as 440.6 nm.

Microfluidization, which is a high-pressure homogenization technique, was used as the second homogenization to obtain nanoliposomes. According to the zeta potential data, the mean value of the zeta potential charge was found to be -25.7 mV in three measurements of liposome charges obtained from the first cycle of the microfluidization technique. It has been understood that the first cycle liposomal dispersion with a zeta potential of less than -25 mV is stable

for storage processes, but the number of cycles should be increased for better stability. The average size of the obtained product was 144.9 nm, and the polydispersity index was 0.671 nm. The mean value of the zeta potential charge of the same product of the liposomes after the second cycle was measured as -56.0 mV, and the mean size value was 107.0 nm. The PDI was found to be 0.235. In the second cycle, the size of the liposome vesicles began to approach the nanoscale. Finally, the mean value of zeta potential charge after the third cycle was measured as -66.6 mV and the mean size value was 91.13 nm. The PDI was found to be 0.231 (Table 1).

Table 1. Analysis of nanoliposomal forms

Formulations	Zeta Potential (mV)	Average Size (nm)	Polydispersity Index (PDI)
DES	- 55.0	440.60	0.626
MFT (Cycle 1)	- 25.7	144.90	0.671
MFT (Cycle 2)	- 56.0	107.00	0.235
MFT (Cycle 3)	- 66.6	91.13	0.231

DES: Direct-End Sonicator, MFT: Microfluidization Technique

The liposome encapsulation efficiency was calculated for ten different nanoliposomal formulation, and the highest value was reported as 91.90%. NLF2 formulation (0.9 mL TLE), which has the highest value in the formulation prepared with this technique, was used

in characterization studies (Table 2). Besides, considering the amount of encapsulated substance according to the selected nanoliposome formulation (NLF2), the amount of encapsulated terebinth leaves in 1.5 mL was calculated as 3.14 mg. We used almost the same amount of TLE all experiments with encapsulated or unencapsulated.

Table 2. Encapsulation efficiency of nanoliposome formulations

Nanoliposome Formulation	Absorbance Amount (nm)	Encapsulation Efficiency (%)
NLF1 (1 mL TLE)	2.280	77.24
NLF2 (0.9 mL TLE)	2.713	91.90
NLF3 (0.8 mL TLE)	2.475	83.84
NLF4 (0.7 mL TLE)	2.238	75.81
NLF5 (0.6 mL TLE)	2.520	85.37
NLF6 (0.5 mL TLE)	2.319	78.56
NLF7 (0.4 mL TLE)	1.941	65.75
NLF8 (0.3 mL TLE)	1.525	51.66
NLF9 (0.2 mL TLE)	1.488	50.41
NLF10 (0.1 mL TLE)	1.441	48.81

The resulting formulation was used for morphological characterization and visualized by SEM. The sizes of NLTLE particles were measured between 130 and 340 nm with the Image-J program on the SEM image. Considering the size difference between the liposome particles compared to the initial nanoliposomes (91.13 nm), it was observed that the nanoliposome increased in size after encapsulation with TLE. Figure 1 shows the SEM image of the NLTLE.

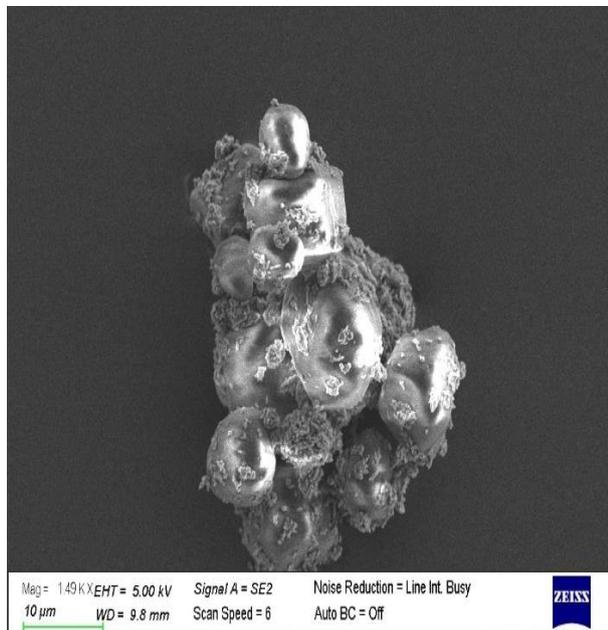


Figure 1. SEM image of nanoliposome-encapsulated terebinth leaf ethanol extract (NLTLE).

Determination of Antibacterial Activity

In terms of bacterial groups, the more antibacterial effect was observed in *S. aureus*. The highest effect was determined at the 48th h for both bacterial groups (Table 3). The amounts used for disc diffusion were calculated from the prepared solutions. It was calculated that there was 68.4 µg of TLE in 18 µL of extract taken for the test. The amount of TLE encapsulated in NLTLE was

calculated as 37.7 µg. When evaluated together with the control groups, a statistically significant difference was found between the groups only in *S. aureus* at the 16th h, and *E. coli* at the 24th and 48th h ($p < 0.05$) (Figure 2).

Table 3. Comparison of the antibacterial activity with the disc diffusion test

Bacteria Type	Hour	Zone Diameters (mm)				
		NLTLE	TLE	NLF	PC	NC
<i>E. coli</i> ATCC 25922	16	10.67 ±3.21 ^a	9.3 ±0.58 ^b	-	38.01 ±1.00 ^{ac}	-
	24	11.67 ±2.51 ^a	9.3 ±0.58 ^b	-	38.67 ±1.15 ^c	-
	48	12.67 ±2.08 ^a	11.01 ±0.00 ^b	-	39.01 ±1.00 ^c	-
<i>S. aureus</i> ATCC 25923	16	15.01 ±2.00 ^a	16.67 ±1.15 ^b	-	31.33 ±1.52 ^c	-
	24	15.67 ±2.52 ^a	16.67 ±1.15 ^b	-	33.33 ±0.00 ^c	-
	48	17.01 ±2.65 ^a	18.01 ±1.00 ^b	-	33.33 ±0.58 ^c	-

NLTLE: Nanoliposome-encapsulated terebinth leaf ethanol extract; TLE: Terebinth leaf extract; NLF: Nanoliposome formulation; PC: Positive control (Ofloxacin); NC: Negative control (UV distilled water); -: no microbial growth was observed; ac: Different lowercase letters in the same row indicate a statistically significant difference ($P < 0.05$) between the data

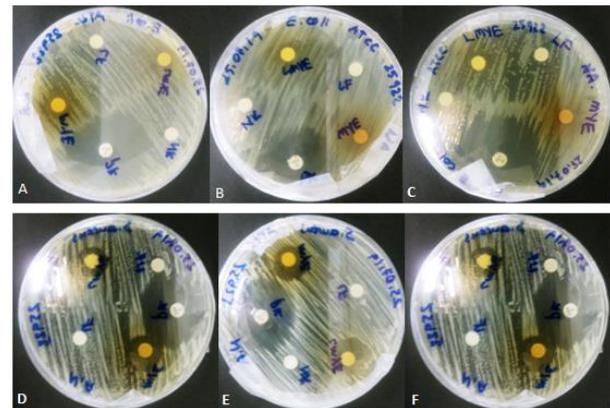


Figure 2. The results of disc diffusion test for *E. coli* ATCC 25922 (A, B, C) and *S. aureus* ATCC 25923 (D, E, F).

The results of the MIC values are presented in Table 4. The amount of encapsulated terebinth leaf for *E. coli* was 2.69 µg and 3.77 µg for *S. aureus*. In addition, the minimum value for TLE was found to be 0.152 mg for *E. coli* and 0.228 mg for *S. aureus*. Results were determined by the broth dilution method with standards approved by the Clinical and Laboratory Standards Institute (CLSI, 2006).

Table 4. Antibacterial activity results for tested samples as MIC

Bacteria Type	Test Samples	MIC (mg)
<i>E. coli</i> ATCC 25922	TLE	0.152
<i>E. coli</i> ATCC 25922	NLTLE	0.114
<i>S. aureus</i> ATCC 25923	TLE	0.228
<i>S. aureus</i> ATCC 25923	NLTLE	0.0838

NLTLE: Nanoliposome-encapsulated terebinth leaf ethanol extract; TLE: Terebinth leaf extract

Discussion

There are many studies on formulations that will both contribute to the effectiveness of antimicrobial agents and controlled release by encapsulation ([Engel et al., 2017](#); [Ferreira et al., 2021](#); [Lopes et al., 2019](#)) Principally, with the use of nano-sized liposomes, there is a potential to increase bioavailability after encapsulation ([Kirtil & Öztop, 2014](#)). In this study, the preparation stages of nanoliposomes, their characterization, and their effects on the antimicrobial activity of terebinth plant extract were determined.

The study of liposome average size and its distribution is of interest because of its impact on the liposome stability ([Were et al. 2004](#)). The effect of preparation methods for the encapsulation of liposomes on the average size and size distribution are shown in Table 1. According to the results, the average particle size of nanoliposomes after three cycles of microfluidization was 91.13 nm. The particle size was reduced by microfluidization process. These results show that microfluidization operating conditions used were adequate for the nanoliposomes ([Kumar et al. 2022](#)).

Moreover, the zeta potential is usually used as an indicator of accessible charges in the liposome surface ([Awad et al., 2005](#); [De Mello et al. 2013](#)). When the zeta-potential is more than ± 61 mV, the solution is highly compatible with water and the particles are kept very stable ([Duman et al., 2014](#); [Heo et al. 2020](#)). The zeta potentials of nanoliposomes were -66 mV, which indicates that they are very compatible for encapsulation.

Looking at the PDI, the measured value indicates that the sample has a very wide particle size distribution if it is greater than 0.7 nm. If this value is less than 0.05 nm, it is called monodisperse. It shows that such dispersions have particles of the same size. Therefore, different algorithms for size distribution are studied with PDI value data among 0.05-0.7 nm. In table 1, the values of the physical properties (zeta potential, size distribution, and surface charge) of the obtained liposomes were compared. It is seen that the dimensions of the liposomes prepared in the first step are 440.6 nm. Looking at the zeta potential graph, it was determined that the liposomal dispersion obtained in the first cycle of the microfluidization technique was stable for storage processes. However, the number of cycles has been increased for better stability and reduced size. In the second cycle, the size of the liposome vesicles began to approach the nanoscale. When the zeta potential and size distribution of the liposome prepared in the third cycle are examined, it is seen that nanoliposomes are formed 91.13 nm, 0.231 PDI, and the liposome load -66.6 mV is stable. Therefore, nanoliposomes taken in the third cycle were used for encapsulation.

In the encapsulation stage, it was dissolved in water instead of ethanol, which is the main solvent of

lyophilized TLE. This process was done hence the solvent of the nanoliposomal formulation was water. In addition, the emergence of ethanol-induced effects in antibacterial activity tests was also prevented. Encapsulation efficiency is determined by using organic solvents to release any lipophilic substance trapped between the liposomes bilayer and decompose the liposomal bilayer. The double layer is dissolved in the presence of organic solvents and detergents ([Mozafari, 2008](#)). However, not all organic solvents and detergents dissolve lipids with the same efficiency.

In most of the studies, various chemicals such as methanol, isopropanol, Triton-X 100, and Tween-20 are used as lipid solvents. In addition, few studies mention the need to investigate the use of ethanol as a less toxic alternative ([Carugo et al., 2016](#); [Demircan, 2016](#)). In this study, ethanol was preferred as the lipid solvent because it is less toxic. The liposome encapsulation efficiency was calculated, and the highest value was reported as 91.90%. In table 1, it is thought that the main reason for the decrease in yield after a certain rate is due to aggregation and deterioration in the liposome structure.

When antibacterial assays were compared, it was observed that TLE and NLTLE were more effective on gram (+) bacteria than gram (-) bacteria (Table 3). It has been observed that two times less concentration of the formulation compared to the extract provides higher antimicrobial activity against *S.aureus*. These results obtained in our study are similar to the antimicrobial activities in the literature ([Durak et al., 2015](#); [Tohidi et al., 2011](#), [Sethi et al., 2011](#)). The study of [Kavak et al. \(2010\)](#) supports these results. According to the study, the ethanol extract of the leaf of the terebinth plant showed antibacterial activity against *S. aureus*. On the other hand, the extract had no antibacterial effect on *E. coli*.

In another study conducted by [Cui et al. \(2015\)](#), antimicrobial effects of clove oil containing liposomes were tested against *S.aureus* ATCC 25923 and *E.coli* ATCC 25922. As a result, the liposomal formulation has shown antibacterial activity against only *S.aureus* ATCC 25923 which is gram (+) bacteria. Because, *S.aureus* ATCC 25923 has pore-forming toxins, it can form pores in liposomes. Therefore, toxins can increase the release of substances and interact more easily with active substances. Accordingly, encapsulated TLE has more antibacterial activity on to *S. aureus*.

Overall, this study showed that nanoliposomal formulation increased the bioavailability of TLE at antibacterial tests.

Conclusion

Results presented in this study indicate that nanoliposomes may be good candidates for the encapsulation of bioactive compounds such as TLE. However, data presented in this study also show that preparation of liposomes plays a critical role in terms of

formulation. Here, nanoliposomal formulation with microfluidization allowed significantly decreased liposome size and displayed the best stability of liposomes. According to the antibacterial tests results of NLTLE showed similar activity with TLE although that contains 50% less TLE. These results showed that the decrease in the amount of active substance in encapsulation with nanoliposome did not decrease in the activity, on the contrary, it increased it (Table 5).

Table 5. Effect of TLE and NLTLE on bacteria by amount

Type	Amount of TLE	Bacteria	
		<i>E. coli</i>	<i>S. aureus</i>
TLE	68.4 µg	11.01 ± 0.00 ^b	1.00 ± 18.01 ^b
NLTLE	37.7 µg	12.67 ± 2.08 ^a	17.01 ± 2.65 ^a

As a conclusion of this study, it has been demonstrated that nanoliposome prepared with microfluidization as a drug carrier is more economical in terms of reducing the amount of active substance and can be used by increasing the bioavailability and antibacterial properties at the same time.

Author Contributions

First Author: Data Curation, Formal Analysis, Investigation, Methodology; Second Author: Data Curation, Formal Analysis, Investigation, Methodology; Third Author: Data Curation, Formal Analysis; Fourth Author: Supervision, Writing - review and editing; and Fifth Author: Investigation, Conceptualization, Writing - review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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