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RESEARCH PAPER

# *In vitro* evaluation and comparison of anticancer, antimicrobial, and antifungal properties of thyme niosomes containing essential oil

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## ABSTRACT

***Objective(s):*** Due to the inefficiency of current treatment methods in the treatment of various cancers, as well as increasing antibiotic resistance in bacteria and fungi, attention to the use of medicinal plants and their essential oils is increasing. However, leading barriers to the use of plant essential oils, such as rapid oxidation and high volatility, highlight the need for a drug delivery system to increase their efficiency.

***Materials and Methods:*** Therefore, this study aimed to investigate the antimicrobial, antifungal, and cytotoxic effects of free thyme essential oil (TEO) and compare it with its nanoniosomal form. Initially, the chemical component of TEO was analyzed by the gas chromatography method. Then, to improve biopharmaceutical properties and enhance the stability of TEO in light and volatility, nanoniosomes containing thyme essential oil synthesized by thin-film hydration method and their physicochemical properties such as size and zeta potential, morphology, encapsulation efficiency (EE%), and profile of *in vitro* release were investigated.

***Results:*** The results showed that the nanoparticles had an average size of 97 nm with a zeta potential of -37 mV. Also, in the optimal formula, the EE% of essential oil in nanoniosomes and the maximum release rate of the TEO from nanosystem were 81% and 57%, respectively. On the other hand, it was found that the antibacterial and antifungal activity of TEO remarkably increased after encapsulation. Also, the cytotoxicity assay of TEO on cancer cells showed that blank nanoniosome had no cytotoxicity and besides, the $IC_{50}$ of TEO in encapsulated form decreased by 1.75 times on MCF-7 cancer cells compared with its free form, which indicates an increase in its anti-cancer properties.

***Conclusion:*** Overall, encapsulation of thyme essential oil in optimal synthesized nano systems improved its anticancer and antimicrobial properties, which could be the beginning of a revolution in the treatment of cancer and microbial diseases using nano-encapsulated herbal remedies.

***Keywords:*** *Antimicrobial; Essential oils; Herbal drug; Niosome; Thymus*

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## INTRODUCTION

Dietary plant products occasioned protection against the development and progression of cancers, cardiovascular diseases, diabetes, and bacterial, and fungal infections (1). Cancer, bacterial and fungal diseases have become some of the most critical health care concerns of the 21st century. Meanwhile, treating breast cancer as common cancer with nearly 1.5 million new cases and nearly 500 thousand dead annually, has become an unresolved challenge. Application of

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chemotherapy and surgery to remove the tumor is also ineffective in the treatment of many cases, in addition to side effects and the possibility of recurrence. On the other hand, improper use of antibiotics has led to the development of resistant strains of bacteria and fungi, which, in addition to the loss of life and mortality, have led to high costs for governments in the field of health. Due to the above and the treatment challenges ahead, the need for a new treatment system with fewer side effects and higher efficiency is highlighted (2). Currently, many studies have investigated the replacement of antibacterial, antifungals, and chemical preservatives with their natural variants mainly in herbal medicine. Further, numerous studies have shown the practical effectiveness of medicinal plants in treating various types of cancer. Also, herbal polyphenols-rich drugs could be excellent sources of natural antioxidants (3, 4). Essential oils (EOs), as secondary plant metabolites, are volatile liquids that possess a wide range of biological activity, including antioxidant and antibacterial activity (5, 6). EOs are composed of many chemical compounds and each of them has a substantial health benefit. Many studies have investigated the bioactivity and benefits of EOs and their possible applications in the medicine and the food industry (7). Generally recognized as a safe list (GRAS) provides many, EO-based formulations that are not only approved by the Food and Drug Administration but also are commercially available as food preservatives and agricultural supplements (8). Thyme Essential oil (TEO) extracted from Thymus vulgaris of the Lamiaceae family, is one of the most famous genera of medical plants. The possible anticancer, antibacterial, antifungal, and antioxidant potential of thyme essential oil was attributed to several compounds such as thymol, carvacrol, p-cymene, and 3–terpinene (9). Despite these attractive properties, there are also some considerable obstacles to implementing essential oils. Free EO is not resistant enough and is rapidly oxidized in environmental conditions. The volatilization, hydrophobicity, and poor solubility in water reduce its antimicrobial and antioxidant properties (10). Encapsulation stabilizes the bioactive compounds during the chemical processes and prevents adverse reactions. Several novel methods can be implemented to increase the stability, bioactivity, and solubility of essential oils and, in turn, preserve their therapeutic properties. One of these methods is encapsulating them into nano-carriers such as nanoniosomes. Nano-niosome-based carrier system, as a bilayer non-ionic surfactant vesicle, is a novel drug delivery system (11). The advantages of nanotechnology in medical areas, chiefly in drug delivery, have been investigated largely over the past years. Controlled drug release in target tissues, increasing solubility and half-life of the drug in the circulatory system, use for combination therapies, and facilitating the antioxidant, antimicrobial and antifungal activities of drugs are among the advantages of this technology (12-17). Nano-metric scale size, biodegradability, low toxicity, remarkable stability, high storage time, and low synthesis cost have enabled nanoniosomes to be one of the most important types of nano-carriers (18, 19). Nanoniosomes are nanoparticles organized from the aggregation of non-ionic surfactants in the aqueous medium. Therefore, our aim in this study is to identify the active compounds in thyme EO by loading them into nanoniosomes and characterizing them to overcome some of the obstacles in using the essential oil of this plant. For this purpose, after loading the EO into the nanosystems, the anticancer and antimicrobial properties of the synthesized system are compared with the free form of TEO (free-TEO).

## MATERIALS AND METHODS

### *Materials*

Tween-60 and cholesterol were purchased from DaeJung Chemicals & Metals (South Korea) and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), respectively. Chloroform solvent, 2-Propanol, dialysis bag (MW = 12 kDa), PBS tablets, DMSO (dimethyl sulfoxide), MTT (3 – (4, 5-dime-thylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) and paraformaldehyde solution were procured from Sigma-Aldrich (St. Louis, MO). All of the organic solvents and other chemicals were analytical grade.

### *Essential oil extraction and analysis of the chemical constituents of thyme essential oil by gas chromatography-mass spectrometry (GC-MS)*

The leaves of thyme plants were collected and dried away from sunlight in August 2019 from the south of Iran and approved by the State Veterinary and Food Administration of the Slovak Republic (Accreditation No. Ro-3239/15-221 and Ro-1640/17-221). The essential oil was extracted from the collected thyme leaves using a clevenger

apparatus. Using Chinese mortar, 25 g of the dried leaves were semi-crushed and transferred to a flat bottom balloon with a volume of 500 ml. After adding 250 ml of distilled water, the essential oil extraction process started. After extracting the essential oils of the thyme, the components of essential oil were analyzed and identified by the Gas chromatography-mass spectrometry (GC/MS) analytical method. For this purpose, Varian 3400 Gas Chromatograph with a mass detector and DP-5 column (30 m × 0.25 mm × 0.25 µm) was implemented. The initial temperature of the system was set to 50 °C. Then the temperature was increased to up to 220 °C at the rate of 3 °C/min. An electron ionization system with ionization energy of 70 eV was used. Helium, as the carrier gas, was injected with a velocity of 31.5 cm/s. The sample flow velocity was 1.1 mm/min and the scan duration was 1 sec with an AMU of 40–350. The temperature of the injection port was set to 230 °C.

***Preparation of thyme essential oil-loaded nanoniosomes***

The nanoniosomes containing thyme essential oil (Nio-TEO) were synthesized by a thin film hydration method. Briefly, tween 60 was mixed with cholesterol at different molar ratios based on Table 2 in chloroform as solvent. Then, the essential oil was dissolved in methanol and was added to the mixture in a round flask. To, prepare niosomes containing thyme essential oil, the lipid-formed film was hydrated with phosphate-buffered saline (PBS) for 60 min at 60 °C using a rotary instrument (Heidolph, Germany). To produce small vesicular units, nanoparticles were sonicated over an ice bath for 15 min using a microtip probe sonicator (E – Chrom Tech Co, Taiwan). Then, unencapsulated thyme essential oils (unentrapped) were separated from encapsulated TEO by dialysis bags (cut-off of 12 kDa) (2).

***Investigating encapsulation efficiency, loading capacity, and in vitro drug release profile***

To investigate Encapsulation Efficiency (EE%) and Loading Capacity (LC%), first, the maximum absorbance wavelength (λ max) of TEO was obtained, and the standard curves of TEO in phosphate-buffered saline (PBS) and isopropyl buffers were plotted. After that, the membrane of Nio-TEO was lysed with isopropanol, and the quantity of encapsulated TEO in them was measured using a UV/Vis spectrophotometer (model T80+, PG Instruments, United Kingdom) at λ max = 274 nm. The EE% and LC% of niosome containing thyme EO were calculated using the following equation:

EE%= Entrapped thyme essential oils concentration (mg/ml)/primary thyme essential oils (mg/ml) ×100

LC%= mass of drug in nanocarrier (mg)/mass of nanocarrier (mg) × 100

Investigating the drug release pattern from niosomes was carried out by a dialysis tube and the medium contained phosphate-buffered saline (PBS) at 37 °C and pH=7.4 (physiological conditions of the body), under gentle vibration (rpm=75). Then, the drug release pattern was calculated by reading the absorbance of obtained samples from the sampling of PBS medium at λmax= 274 nm using a UV/Vis spectrophotometer. Based on higher Encapsulation Efficiency (EE%) and Loading Capacity (LC%), the optimum formula was selected for further analysis (2, 15).

***Morphological characterization of the optimized formulation***

To determine the particle size, polydispersity index (PDI), and zeta potential of nanoniosomes containing thyme essential oils (Nio-TEO), dynamic light scattering (DLS) technique, by a particle size analyzer and ZetaPALS zeta potential Brookhaven Corp Instruments (Holtsville, NY, USA) were used. Scanning Electron Microscope (SEM) (EM3200, KYKY, China) was used to analyze the surface morphology of the nanoniosome. For this purpose, 5 µl of suspension was used to obtain a thin layer of the film by pouring it on the glass plate and drying it in the air. The sample was coated with a layer of gold to maintain electrical conductivity. Also, the shape and the 3-D morphological analysis of blank nanoniosomes and drug-loaded nanoniosomes were obtained using an atomic force microscope (AFM) (Nanowizard II; JPK instruments; Germany). For preparation of the sample for imaging by AFM, the first sample was diluted with water (1;1000), and to decrease size, sonication was performed for 15 min and after that placed on a mica sheet, and imaging was done (2, 15).

***Functional group characterization***

Fourier transform infrared (FTIR) spectroscopy (Model 8300, Shimadzu Corporation, Tokyo, Japan) was applied in the wavelength range of 400–4,000 $cm^{-1}$ to investigate the chemical interaction between thyme essential oil and nano-carrier for

pure TEO, Nio-TEO and blank nano-noisome (2, 15).

***In vitro antibacterial studies***

In this study, the antibacterial activity of TEO and TEO-NIO against *Escherichia coli* bacteria and Staphylococcus aureus bacteria were assayed and compared. After, procuring these bacteria from Tehran Pasteur Institute, a concentration of 1.5 × 106 CFU/mL from each bacterium was cultured on Nutrition Agar medium (Merck, Germany) and incubated under aerobic conditions at 37 °C, separately. They were then cultured on a Müller-Hinton agar medium (Merck, Germany) using a sterile swab. Then an empty disk was placed at the bottom of each well and 20 µl of TEO, and TEO-NIO were added to the respective wells. Ampicillin antibiotic disk (Padtan Teb, Iran) as the positive control and distilled water as the negative control were used and then incubated at 37 °C for 24 hr. Then, from the non-growth zone around each sample the antibacterial activity of each antimicrobial agent was evaluated (2).

***Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determinations***

The lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism is known as, MIC. MIC testing could be performed on agar or an intermediate fluid. The usual method for determining MIC is the sequential dilution technique which also was used in this study. First, different dilutions of TEO and NIO-TEO were prepared and added separately to tubes containing 0.5 ml of Müller-Hinton culture medium. Then 0.5 ml of bacterial suspension was added to the tubes (in each tube, the concentration of antibacterial agent was half of the previous tube, but the concentration of bacteria in all tubes was the same) and was incubated for 24 hr at 37 °C. The concentration of the last tube in which no bacterial growth was observed was reported as MIC (2). MBC refers to the lowest concentration of an antibacterial agent that can reduce the bacterial population by 99.9% after 24 hr. In other words, reducing the initial population by a thousand times. After determining the MIC concentration for TEO and NIO-TEO, 50 ml of each tube on which bacterial growth was not visible, was seeded on BHI agar plates (Merck, Germany), and incubation was performed for 24 hr at 37 °C. When 99.9% of the bacterial population was killed at the lowest concentration of antimicrobial agent (TEO, NIO-TEO), that concentration was reported as MBC concentration, which was figured out by observing the agar plates before and after incubation for the presence or absence of bacteria. Each test tube contains different concentrations of the antimicrobial agent, and the same amount of microorganisms was inoculated. After proper incubation, the lowest concentration of antibacterial material at which microbial growth was visible was considered MIC and the result was reported as µg/ml. Eleven test tubes were used for determining the MIC. First, 0.5 ml of culture medium entered tubes No. 2 to 11. 2.5 ml of antibacterial agent (Streamed-C, surfactant vesicle, essential oil, and surfactant vesicle containing essential oil) was introduced into tubes No. 1 and 2. Then, 0.5 ml of solution in tube 2 was transferred to tube 3, and this process continued until tube number 9. Afterward, 0.5 ml from tube number 9 was removed and discarded. Then, 0.5 ml of *Escherichia coli* bacterial suspension was injected into tubes 2 to 10. Thus, the concentration of antibacterial agent from tubes 2 to 9 was diluted exponentially (by a factor of one-half). Still, the number of bacteria inoculated into the test tubes was the same. Tube No. 10 contained the culture medium and bacterial suspension and was considered a control tube. In addition, MBC refers to the lowest concentration of antimicrobial agent that destroys 99.9% of the inoculated microbial population. In the standard procedure, after dilution test and MIC determination, 100 µl of suspension were removed from each tube that showed non-growth and then cultured on the surface of TSA. Afterward, it was incubated for one night at 37 °C, and, the number of colonies grown was counted. If the colonies are reduced by one-thousandth of the amount in the bacterial suspension, that concentration was reported as MBC (2).

***Antifungal analysis***

In order to study the antifungal properties of NIO-TEO and compare them with TEO, *Candida Albicans, Trichophyton. Rubru*, and *Trichophyton. mentagrophytes* were purchased from Pasteur Institute in Tehran, Iran and cultured according to ATCC protocol. To analyze the antifungal activity of the synthesized nanosystem, we proceeded as mentioned above, with the difference that here we used Sabouraud Dextrose Broth culture medium as a liquid culture medium and Sabouraud Dextrose

Agar culture medium as a solid culture medium (2).

***Nano-system stability evaluation***

One of the main challenges in using niosomes as a drug delivery system is their weak stability. To evaluate this figure, synthesized niosomes were maintained at 4 °C for 90 days, and their stability in terms of EE%, size, zeta potential, and PDI was based on what was previously described and investigated in alternating and regular periods (2).

***Toxicity and cell viability assessments***

MTT assay was performed to investigate the cytotoxicity of the niosomal form of TEO on MCF-7 cancerous cells and compared with the free form of TEO, as well as investigating the non-toxicity of blank niosomes on cells. Briefly, MCF7 cells were provided by the Pasteur Institute (Tehran, Iran) and cultured with DMEM and 10% FBS. Then seeded in 96-well plates and incubated to reach 70%–80% confluence. After that, cells were treated with empty niosome (blank niosome), free-TEO, and niosomal TEO at various concentrations (15, 30, 60, 125, and 250 µg/ml) and incubated. After 48 hr the contents of the wells were removed and incubated with MTT solution (5 mg/ml) for 4 hr to allow the production of formazan crystal. Then 150 µl DMSO was added in order to dissolve formazan crystals. The cell viability rate was assessed using an EPOCH microplate spectrophotometer (Synergy HTX, Bio-Tek, Winooski, VT) at 570 nm. In order to determine Half Maximal Inhibitory Concentration ($IC_{50}$, a concentration of drug at which 50% of the cells were alive was considered as $IC_{50}$ (2).

***Statistical analysis***

The statistical analysis was calculated via GraphPad Prism version 9 (Graph Pad, San Diego, CA, USA). The experimental values were computed three times and expressed as mean±SD. The Statistical analysis was performed using Student's t-test and ANOVA test when comparing two independent groups and multiple samples, respectively. Differences were considered statistically significant at $P< 0.05$.

Table 1. List of biochemical constituents of thyme essential oil.

| Components | Ratio (%) | Retention indices |
|---|---|---|
| α- pinene | 2.73 | 934.8 |
| Camphene | 0.27 | 950 |
| β-pinene | 0.48 | 974 |
| 3-Octanone | 0.9 | 981 |
| Myercene | 1.23 | 991 |
| α- phellandrene | 0.23 | 1009 |
| p-Cymene | 7.74 | 1024 |
| 1,8 Cineole | 2.73 | 1034 |
| γ-Terpinene | 3.33 | 1058 |
| t-Linalool oxide | 0.61 | 1069 |
| α- Terpinolene | 0.28 | 1081 |
| Linalool | 16.87 | 1091 |
| Borneol | 0.32 | 1160 |
| 4-Terpineol | 1.65 | 1170 |
| α- Terpineol | 2.3 | 1183 |
| Thymol methyl ether | 0.15 | 1225 |
| Carvacrol methyl ether | 2/66 | 1238 |
| Thymol | 7.35 | 1283 |
| Carvacrol | 35.56 | 1290 |
| Carvacrol acetate | 1.21 | 1365 |
| t- caryophyllene | 6.46 | 1399 |
| Aromadendrene | 1.45 | 1453 |
| α- Caryophyllene | 0.3 | 1462 |
| Ledol | 1.57 | 1559 |
| Spathulenol | 0.43 | 1570 |
| Caryophyllene oxide | 0.78 | 1578 |

**RESULTS**

***Chemical composition of thyme essential oil***

The chemical components of thyme essential oil were detected using GC-MS methods, which was demonstrated in Table 1. It identified that Carvacrol was the significant component with a ratio of 35.56% of the total amount. The other main components included Linalool (16.87%), p-Cymene (7.74%), Thymol (7.35%), t- caryophyllene (6.46%), and γ-Terpinene (3.33 %).

***Characterization of Nio-TEO formulation***

The results of the percentages of the entrapment efficiency (% EE) and Loading Capacity (% LC) of five different formulations of the thyme essential oil (TEO) niosomes with various molar ratios of Cholesterol: tween-60 were presented in Table 2. It was observed that the Entrapment Efficiency and Loading Capacity of TEO niosomes increased with rising Tween 60 (F5). Based on high Entrapment Efficiency and Loading Capacity, the F5 formula has been selected as the optimal

Table 2. Molar ratio of lipid and cholesterol used for synthesizing each formulation of thyme EO-Containing niosome and their effect on Entrapment Efficiency (EE%) of niosomes. *: Optimal formula

| Formula | Cholesterol:Tween 60 (molar ratio) | Entrapment Efficiency (EE%) |
|---|---|---|
| 1 | 35:65 | 34 |
| 2 | 45:55 | 39 |
| 3 | 55:45 | 45 |
| 4 | 65:35 | 59 |
| *5 | 75:25 | 81 ± 3.5 |

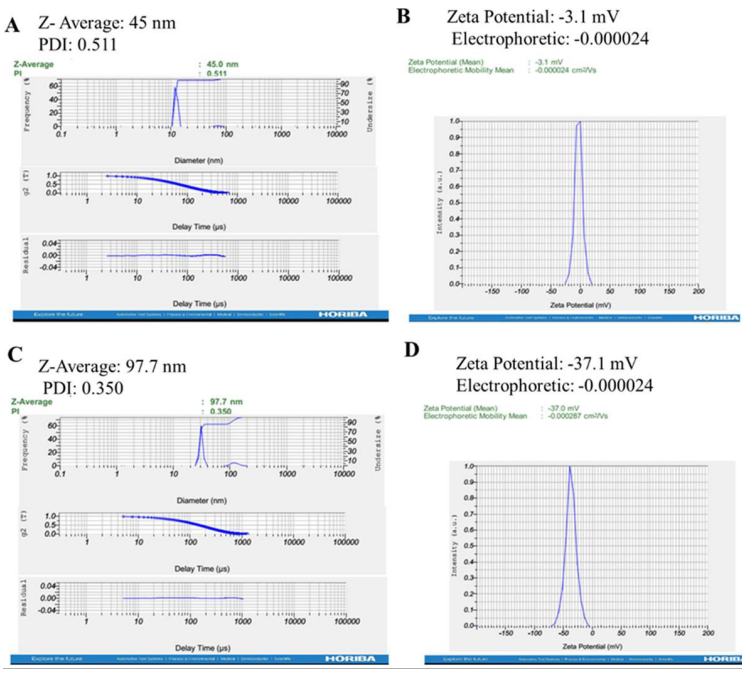


Fig. 1. Average size (A) and surface charge (B) of nanoparticles before TEO loading and average size (C) and surface charge (C) of nanoparticles after TEO loading, obtained from DLS

formulation for the next studies. As seen in Fig. 1, the particle size and zeta potential of optimal blank niosome (F5) were 45 nm and -3.1 mV, respectively. The size of the optimal formula of TEO-Nio (F5) was increased to 97.7 nm when loaded with thyme Essential Oil and zeta potential pulled towards the negative charge -37 mV. The particle size and the morphological features of the blank noisome and Nio-TEO were characterized by AFM and SEM analysis (Fig. 2). The microscopic studies revealed the spherical shape with narrow size distribution and smooth surface of the nanoniosomes. On the other hand, by comparing the images obtained from SEM, it is clear that the size of nanoparticles increased after loading the essential oil in them, which indicates the appropriate placement of the essential oil inside the bilayer vesicles. Overall, these data demonstrate the appropriate morphology of nanoparticles for delivering essential oil to target tissues. All results were consistent with the findings of the DLS analysis, and no aggregates were observed. Also, SEM analysis (Fig. 2C, D) depicts the mean diameter coordination of Nio-TEO with DLS results.

### *Release profile of TEO from niosomes*

The release profile of TEO from nanoniosomes in physiological conditions (pH=7.4 and 37 °C) and cancerous conditions (pH= 4.5 and 42 °C) was investigated, and the results of this study are shown in Fig. 3. After 48 hr, the total release rate of TEO from niosomes at 37 °C and 42 °C at pH 7.4 was 35% and 42%, respectively. Whereas, the total release rate of TEO from niosomes was 68% and 84% at 37 °C and 42 °C at pH 4.5, respectively. It reveals that lower pH could enhance the release rate of essential oil from niosome. Moreover, the release rate of TEO increased with an increase in

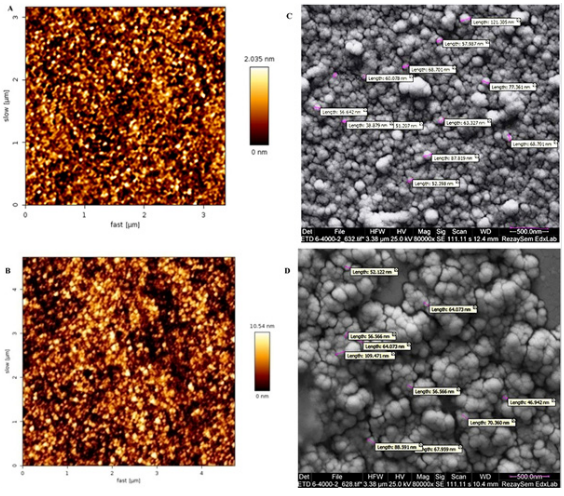

Fig. 2. Morphological evaluation. Images obtained using atomic force microscope (AFM) before (A) and after (B) loading of thyme essential oil. Images obtained using scanning electron microscope SEM before (C) and after (D) TEO loading

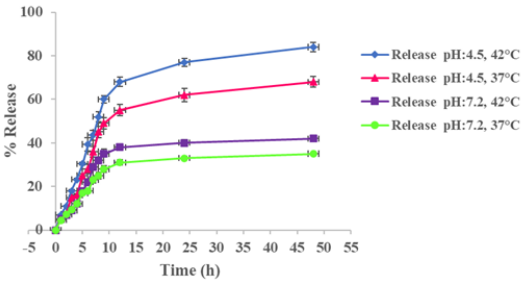


Fig. 3. Different release patterns TEOs from nanoniosomes in response to different stimuli (pH and temperature)

temperature. Based on these results, it could be concluded that the TEO-Nio system is a thermo-pH sensitive system and therefore, could increase the cellular uptake of the drug by cancer cells compared with normal cells and, in turn, reduce adverse side effects of the drug. Also, the maximum TEO release rate from the optimal formula at 42 °C and pH =4.5 was 84% in 48 hr.

### *Fourier transform infrared spectroscopy (FT-IR)*

Fig. 4 represents the FTIR spectrums of the free form of TEO and blank niosome and TEO-Nio. The FTIR spectra obtained for blank niosome (without drug) demonstrate various characteristic peaks of Tween-60 and cholesterol in the range of 4000–500 $cm^{-1}$. The peak observed at 3459 $cm^{-1}$ was assigned to cholesterol and Tween-60 due to the presence of OH stretching. The C=O and C-O-C stretching vibration exhibits an absorption band in the range of 1633 $cm^{-1}$ and 1148 $cm^{-1}$, which belongs to Tween-60. All index peaks repeated in the FTIR spectrum of TEO-Nio which indicates there is not any chemical interaction between the essential oil and the niosome, and the essential oil has retained its chemical nature. The FTIR spectra display several characteristic bands of various compositions of TEO as a complex mixture system. According to GC-MS results, The TEO contained significant amounts of Carvacrol and thymol (35.5 and 7.3%, respectively). As exhibited in Fig. 4, the index peaks in the range of 3,350–3,450 $cm^{-1}$ are due to the presence of functional groups of O-H and N-H. The peaks in the range of 3,415 $cm^{-1}$ and 2,966 $cm^{-1}$ have verified the existence of stretching around the OH axis of the hydroxyl group and vibration around the C-H axis of methyl and isopropyl groups on the phenolic rings of thymol. Several characteristic bands in the ranges of 2858 $cm^{-1}$ ($–CH_2$ asymmetric and symmetric stretching), 1,640 $cm^{-1}$ (conjugated double bond of the ring), 1,459 $cm^{-1}$ (CH deformation), and

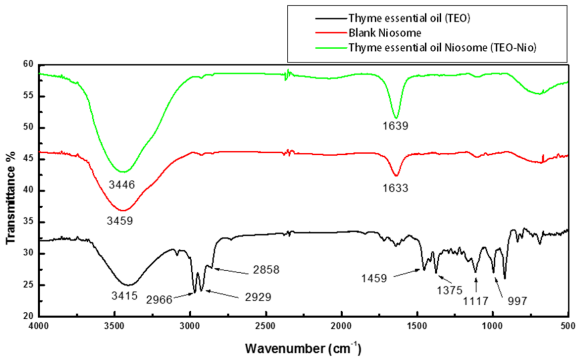


Fig. 4. Comparison of FTIR spectrum obtained from the optimal formula, blank niosomes, and TEO-NIOs

809 $cm^{-1}$ (CH wagging vibrations) also verify the existence of pure TEO.

### *Stability analysis*

The stability of synthesized niosomes is depicted in Fig. 5. It is clear that after 90 days, the size of nanoparticles increased and reached above 120 nm, however, this increase is very small compared with freezing time. The electrical charge of nanoparticles and their size followed an upward trend, and it has moved toward being more positive. On the other hand, over time, the amount of essential oil leakage from niosomes has risen and the amount of loaded TEO in nanoparticles has slumped, which may be due to the chemical nature of the essential oil and its volatility. In term of PDI, the nanoparticles fluctuated, and the changes in the PDI of nanoparticles was negligible.

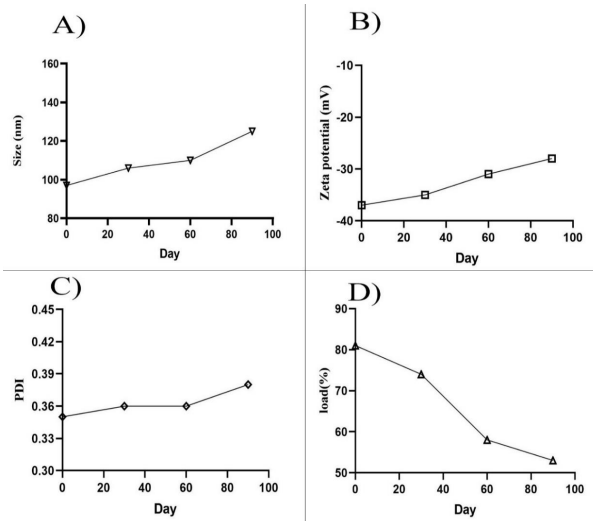


Fig. 5. Stability of synthesized niosomes over 180 days in terms of A) size (nm), B) zeta potential (mV), C) PDI, and D) drug release

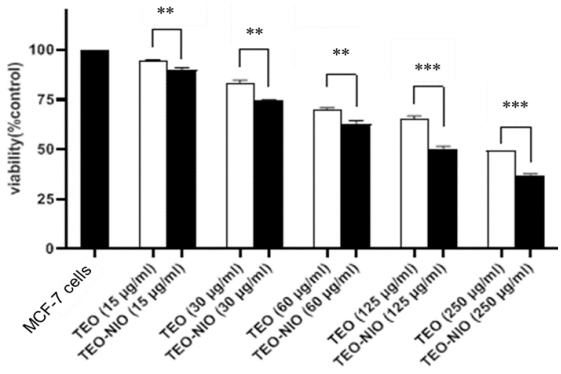


Fig. 6. MTT assay result on MCF-7 cells after treatment with various concentrations of Nio-TEO and TEO in 48 hr. ***: *P*<0.05

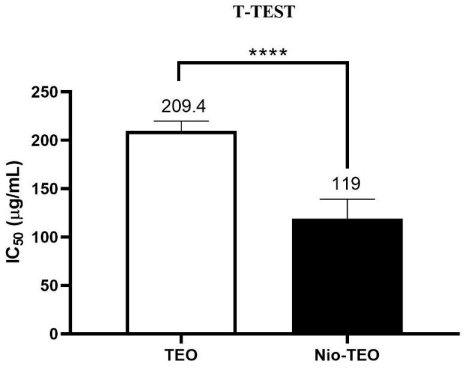


Fig. 7. Comparing $IC_{50}$ values of Nio-TEO and free form of thyme essential oil on MCF-7 cells in 48 hr. ***, ****: *P*<0.05

### *Cytotoxicity assay*

As depicted in Fig. 6, the response of MCF-7 cells to essential oil is a dose-dependent response that manifests itself as inhibition of cell growth. Also, it could be concluded that TEO-Niosomes had higher cytotoxicity compared with the free form of TEO. The $IC_{50}$ value for the free form of TEO is 209.4 µg/ml in 48 hr, while the $IC_{50}$ value for TEO-NIO is 119 µg/ml in 48 hr (Fig. 7). These findings revealed that the usage dose of essential oil in the nisomal form is lower than its free form, from which can be concluded that encapsulation of TEO improves its anticancer properties.

### *MIC and MBC results*

In this study, a spectrophotometer was used to measure the optical absorption of each test tube due to the turbidity arising from vesicle particles. The device was tuned to a wavelength of 600 nm, and the optical absorption of the solutions was determined. The more dilute solution, which was more transparent (less light absorption), compared with the next solution with a lower concentration of the solution, was considered MIC. For determining the MBC, due to the turbidity of the surfactant vesicle colloidal solutions, 100 µl of all test tubes were removed and surface culture was performed. After one night of incubation at 37 °C, a Petri dish containing the most dilute colloidal solution and whose number of colonies had reached one-thousandth was considered MBC. As can be interpreted from Table 3, the inhibitory effect of the thyme niosomal solution is increased compared with the free form of TEO. The MBC, MIC, and diameter of zone of inhibition for thyme essential oil and thyme niosomal solution are illustrated in Table 3. The zone of inhibition for thyme EO-Containing niosome was 15 mm and less than 10 mm, for *Escherichia coli* and *Staphylococcus aureus,* respectively.

### *Antifungal experiment*

For antifungal results, the values of MBC and MIC, and diameter of the zone of inhibition for both thyme essential oil and thyme niosomal solution are shown in Table 4. The zone of inhibition for *Candida albicanse, Trichophyto rubrum, and Trichophyto mentagrophytes* in thyme EO-Containing niosome were 30, 22, and 20, respectively.

## DISCUSSION

Due to the well-known properties of thyme EOs, which can be used to treat microbial and fungal diseases, they can also be used to repair food industry deterioration caused by

Table 3. MIC, MBC, and inhibition zone (mm) of thyme EO-Containing noisome

| Microorganisms | Antibacterial components | MIC (µg/ml) | MBC (µg/ml) | Zone of inhibition (mm) |
|---|---|---|---|---|
| *E. coli* | Thyme EO | 12.5 | 25 | 38 |
| | Thyme EO-Containing niosome | 12.5 | 25 | 15 |
| *S. aureus* | Thyme EO | 25 | 50 | 70 |
| | Thyme EO-Containing niosome | NA | NA | <10 |

Table 4. MIC, MBC tests, and the inhibition zone (mm) of thyme EO-Containing noisome

| Microorganisms | Antifungal components | MIC (µg/ml) | MFC (µg/ml) | Zone of inhibition (mm) |
|---|---|---|---|---|
| *Candida albicans* | Thyme EO | 25 | 50 | 50 |
| | Thyme EO-Containing niosome | 6.25 | 12.5 | 30 |
| *Trichophyton rubrum* | Thyme EO | 12.5 | 25 | 70 |
| | Thyme EO-Containing niosome | 6.25 | 12.5 | 22 |
| *Trichophyton mentagrophytes* | Thyme EO | 3.125 | 6.25 | 40 |
| | Thyme EO-Containing niosome | 1.56 | 3.125 | 20 |

microorganisms and fungi. Plant-based foods, like thyme, tend to be rich in polyphenols which are known as antioxidants that are shown to reduce the risk of cancer and microbial diseases. It was reported that TEO is a mixture of monoterpenes.In a study by Boukhatem *et al*., the phenol content for TEO was determined as 56.79% Carvacrol (20). The variety in the amount of these contents would be due to differences in production method, gathering seasons, and geographical origins (21). Carvacrol has high antimicrobial, antifungal, and anticancer activity which have been reported in many kinds of research (22-24). Our results proved the presence of active biological compounds in the chemical composition of TEO and it seems that the antimicrobial and anticancer properties of TEO are because of these polyphenols. In this study, thyme EO was incorporated into surfactant vesicles, and their characterization and biological efficacy were evaluated *in vitro*. To reach the highest amount of drug loading, the different molar ratios of cholesterol and tween 60 for synthesizing TEO niosomes were applied. Although low cholesterol (F5) concentrations increase membrane permeability, which increases drug loading, they also increase drug leakage during preparation and reduce the final concentration of the encapsulated drug. The high concentration of cholesterol (F1) also restricts the movement of the acyl chain, which leads to greater penetration into the inner layers of the vesicles, thereby reducing drug accumulation capacity and encapsulation efficiency. Also, the type of surfactants used in synthesizing niosome play a key role in EE%. Tween-60, which was used in synthesizing this study's niosomes, because of larger hydrophobic heads, shows a greater tendency to amine bonding with phenolic compounds of essential oils compared with span-60. Moreover, the ability of Tween-60 increases in trapping essential oil due to having a longer hydrophobic tail, which makes it superior to span-60 in this term (25, 26). The EE% of synthesized niosome in this study was 81 ± 3.5 which is in the acceptable range. The particle size of the TEO nanoniosome was also larger than that of the empty vesicle indicating the portion of the TEO in the bilayer vesicle and the interaction between essential oil and components of niosome. The size of niosomes is another important factor in using them as a drug delivery nano-carrier which depends on many factors like the type of surfactant used for their synthesis. Minor hydrophobic-lipophilic balance (HLB) compared with Span-60, and a shorter hydrocarbon chain compared with the hydrophilic surface make Tween-60 an appropriate surfactant for synthesizing niosomes with suitable size. Decrement in surface energy of niosomes and subsequently reduction of their size is a characteristic of using the proper content of cholesterol in the synthesis of niosomes. The size of synthesized niosomes in this study was 97.7±6.8, which is in a suitable range in addition to easy absorption by the studied pathogenic microorganisms, it is also easily absorbed, decomposed, and excreted by the human body. (27, 28). The zeta potential of the selected formula was -37 ± 8.4 mV, which has been shown to have a higher zeta potential (< -30 mV and > +30 mV) bringing a stronger repelling force between particles and making a more stable system. However, it has been proven that particles with different electrical charges than the electrical charge of body cells are quickly detected and removed by macrophages. Prevention of undesired interaction with human cells, blood cells specifically, and reducing the risk of being swallowed by macrophages are among the advantages of nano-systems with electric charge in the range of electric charge of body cells compared with nano-systems far from this limit (18). As mentioned before, the result of the TEO release pattern at different pH and temperatures

shows TEO- NIO are thermo-pH sensitive. Also, the continuous drug release rate of the optimum nanoniosome is significant to diminish the cytotoxicity of TEO-NIO on normal cells. This is one of the most important factors in reducing the adverse side effects of cancer treatment agents on normal cells because normal cells have a higher temperature and lower pH than cancer cells. pHs below 7 (acidic) protonize the amino groups in the essential oil compounds and make a proton gradient in the membrane of the niosomes, which in turn can increase the membrane's permeability, and thus enhance the release of essential oils from the niosomes. On the other hand, increasing the temperature can enhance the permeability of the niosomes' membrane as mentioned earlier, summating the permeability of the membrane can cause increment drug release and thus improve drug performance. However, the increase in the permeability of the niosomes' membrane by temperature strongly depends on the type and amount of surfactants and lipids used in its synthesis (26, 29). One of the novel approaches to cancer therapy is using herbal agents, especially in a synergistic manner with chemotherapeutic agents. Tabatabaei *et al*. reported that the $IC_{50}$ for *T. vulgaris* was about 100 µg/mL for 48 hr, and in our study, it was 209.4 µg/ml. This difference resulted from essential oil compounds, extraction method, and geographical area of the plant (30). Salari *et al*. developed nanoliposomal rosemary essential oil and evaluated the anti-cancer properties of both free and nano-formulation of essential oil on the MCF7 cell line. Their results demonstrated that encapsulation of essential oil in nano-carrier had higher toxicity than its free form on cancer cells, which was consistent with our research (31). Our study proved that the $IC_{50}$ of TEO decreases after encapsulation in niosomes from 209.9 µg/mL to 119 µg/mL, which indicates the improvement in the anticancer properties of TEO. Nano-system stability analysis also showed that although the studied indices have changed over 90 days, the extent of these changes is small and negligible, which in turn indicates that the synthesized nanosystem has relative stability. By investigating the MIC and MBC results, it can be understood that thyme essential oil has an inhibitory effect on the growth of *E. coli* and *S. aureus*. After 24 hr of incubation at 37 °C, the minimum inhibitory concentration for both types of bacteria was evaluated, which was 12.5 µg/ml for *E. coli* and 25 µg/ml for *S. aureus*. Also, the minimum bactericidal concentration was 25 and 50 µg/ml for *E. coli* and *S. aureus,* respectively. Several studies have investigated the antibacterial effect of thyme EO (32-34). By encapsulating the TEO into niosomes, MBC and MIC were the same for the *E. coli* compared with its free form. However, TEO-NIO did not have any effect on *S. aureus*. Similar investigations were done for studying the effect of encapsulating EOs into nano-carriers and evaluating their antibacterial activity in this form (35). The researches show the enhanced antibacterial activity of EOs loaded in nanosystems. As observed in Table 3, the diameter of the zone of inhibition in the nanoniosomal thyme EO system is lower than the free form of it. The lower diameter of the zone of inhibition in thyme niosoml solution is due to the sustained and slow release of the EO from the niosomes. The results showed that 24 hr, is not enough time for thyme EO-loaded niosomes (TEO-NIO) to be released thoroughly, while free EO is most effective at the beginning of the test. On the other hand, the loading efficiency of the niosomes is not 100%. So at the start of the test, the free TEO has its most effect on the sample, whereas the niosomes loaded with thyme EO have sustained and slow release. Therefore, if the duration of the test for evaluating the diameter of the zone of inhibition was longer, then the effect of TEO-NIO would be more than its free form. Table 4 shows the antifungal effect of thyme essential oil for both free form and niosomal form. By investigating the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), it was revealed that thyme EO has a perfect inhibitory effect on the growth of fungi such as *Candida albicanse*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes.* The MIC for *Candida albicanse*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes* were 25, 12.5, and 3.125 µg/ml, respectively, for the free form of EO, and were 6.25, 6.25, and 1.56 for niosomal form of EO. Rasooli *et al*. studied thyme essential oil's effect on fungal growth. They reported a strong inhibitory effect of this essential oil against the growth of the fungi and recommended the substitution of such natural EO with currently used antifungal and inhibiting chemicals (36). By encapsulating thyme EO into niosomes, the MIC and MFC were reduced clearly and it shows that the antifungal effect of thyme increased by loading in the nano-carriers.

Garcia-Diaz reported a new formulation of EO-loaded niosomes, which has been successfully applied to reduce fungal growth (35). Other research indicates the antifungal activity of thyme essential oil and its potential ability to substitute similar chemicals (37). Similar to the antibacterial results, the diameter of the zone of inhibition for antifungal tests for the encapsulated EO is lower than the free form. As discussed earlier, it is due to the slow and sustained release of essential oil from nanocarriers (by noting that the current duration is 24 hr). If the test duration was longer, the inhibition zone diameter would be larger. Other reasons for improving the antimicrobial properties of encapsulated essential oils compared with free essential oils include the interaction of synthesized nanoparticles with microorganisms. These interactions include the passive absorption of niosomes by the membranes of microorganisms due to their small size, which increases the efficiency of the encapsulated drug in them by increasing uptake. Structural similarities, as well as the constituents of niosomes with biological membranes, cause the biological activity of these nanoparticles with microorganisms. For example, fusion, intermembrane transfer, and phagocytosis are biological interactions of niosomes with microorganisms that improve the antimicrobial properties of the encapsulated drug (2).

## CONCLUSION

This study is one of the few studies, in which, after identifying the biochemically active compounds of thyme essential oil, different formulations of nanosystems containing thyme essential oil were synthesized and its anticancer and antimicrobial properties were measured in comparison with the free form of the essential oil. The introduction of a new niosomal formulation to deliver the TEO to the target tissue and the studied microorganisms was an achievement in this study. In the next step, using DLS, SEM, FTIR, AFM, and spectrophotometric methods, the synthesized nanosystem was also characterized. Finally, the antimicrobial and anticancer activity of nanosystems containing thyme essential oil were examined and compared with the free form of thyme essential oil and it was found that encapsulation of thyme essential oil can not only increase its anticancer properties but could enhance its antimicrobial activities, in other words, encapsulation of thyme essential oil improves its function. The result of this study was introduction of nanoniosomes containing thyme essential oil as effective agents in the treatment of cancer, as well as nanoparticles with enhanced antimicrobial properties that can be used commercially and therapeutically in the future with some additional analysis.

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## CONFLICTS OF INTEREST

None.

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