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RESEARCH PAPER

Synthesis of nano-liposomes containing *Mentha piperita* essential oil and investigating their stability, antimicrobial, antioxidant, and antiproliferative properties

Mohammad Majdizadeh [1], Sanaz Akbarzadeh [2, 3], Hussein H Al-Turnachy [4], Mahdie Hemati [5, 6], Milad Akhlaghi [6], Fatemeh Haghiralsadat [6, 7*], Fatemeh Oroojalian [8, 9*]

[1]Nano-Biotech Foresight Company Biotechnology Campus, Science & Technology Park of Yazd, Yazd, Iran
[2]Department of Chemistry, Yasouj University, Yasouj, Iran
[3]Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, ON M5S 3H6, Canada
[4]Department of Biology, Faculty of Science, University of Kufa, Iraq
[5]Department of Clinical Biochemistry, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[6]Biotechnology Research Center, International Campus, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[7]Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[8]Natural Products and Medicinal Plants Research Center North Khorasan University of Medical Sciences Bojnūrd, Iran
[9]Department of Medical Nanotechnology, School of Medicine, North Khorasan University of Medical Sciences, Bojnūrd, Iran

ABSTRACT

Objective(s): Considering the problems of using medicinal plants in the treatment of diseases and the role of nanotechnology in reducing these challenges, the present research was conducted with the aim of preparing nano-liposomes containing *Mentha piperita* essential oil and investigating their physicochemical characteristics.

Materials and Methods: Four nano-liposome formulations containing essential oil were prepared using cholesterol and phosphatidylcholine by thin layer method. Encapsulation efficiency, size, zeta potential, and essential oil release were measured in all formulations. The appropriate formulation was selected to investigate the morphology of the particles, their interaction between nano-liposomes and essential oil, toxicity, and antioxidant, antibacterial, and antifungal properties. Then, the stability of the selected formulation was checked for 120 days.

Results: Formulation F1 was selected with an encapsulation efficiency of 62.12%, nano-particle size of 121 nm, and zeta potential of -21.8 mV. In this formulation, no interaction between nano-liposomes and essential oil was observed, and the spherical shape and two-layer nature of the nanoparticles were confirmed. Nano-liposomes with and without essential oil caused little toxicity to normal HFF cells and in all concentrations compared to free essential oil, they had more toxicity on MCF-7 cancer cells and higher antioxidant properties. The anti-proliferative effects of nano-liposomes on some microorganisms were higher than the free essential oil. Also, there were slight changes in some physicochemical properties of nanoparticles during 120 days.

Conclusion: Considering the suitable physicochemical properties of nano-liposomes containing essential oil and their anti-proliferative effects, these nano-systems can be suggested for further research in the field of cancer and microbial diseases.

Keywords: *Antioxidant, Antibacterial, Breast cancer, Essential oil.Mentha piperita, Nano-liposomes*

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* *Corresponding authors: Emails: fhaghirosadat@gmail.com; f.oroojalian@ut.ac.ir; Oroojalian.f@gmail.com*
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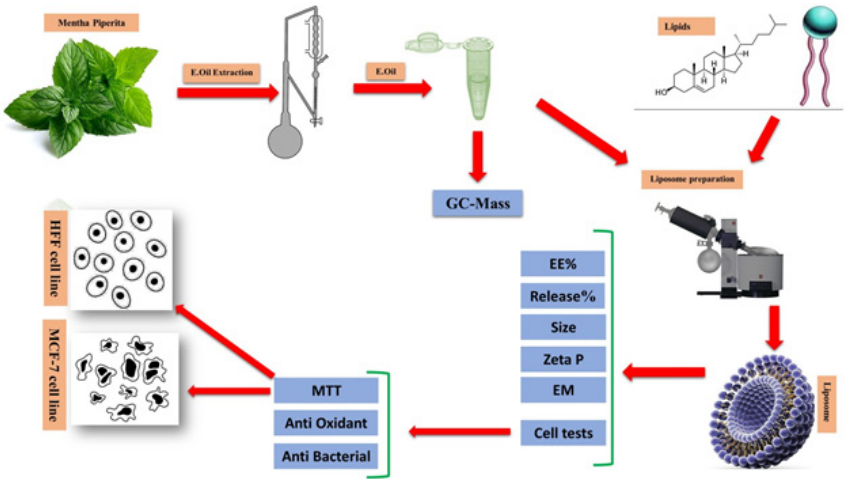
INTRODUCTION

Cell division is a complex process that causes the production of new cells in the human body. This process may be carried out in an uncontrolled manner in some organs of the body, which eventually causes the production of a mass of cells called a tumor. Tumor cells continue to grow and multiply regardless of the mechanisms that control the cell cycle, and because of their high ability to change their metabolic conditions, they win the competition with normal cells and cause damage to normal tissues [1]. The prevalence of cancers has increased greatly in recent years, with more than 1,950,000 new cases of cancer and more than 600,000 cancer deaths expected in the United States in 2023 [2]. Breast cancer is one of the deadly malignancies among women, which affects a large number of women every year. This heterogeneous disease has become a global concern with about 2.26 million new cases each year and 15.5% of annual cancer deaths in women [3]. Nanotechnology is showing successful and beneficial uses in the fields of diagnostics, disease treatment [4, 5]. Today common strategies such as chemotherapy, surgery, and radiotherapy are used to treat cancer [6-8]. Although with the progress of these methods, many successes have been achieved in cancer treatment, but their use causes many side effects for patients, which reduces their quality of life during treatment [9, 10] For example, in breast cancer chemotherapy, drugs such as cisplatin, paclitaxel, doxorubicin, and 5-fluorouracil are used, which cause many side effects of nausea, vomiting, severe myelotoxicity, peripheral neuropathy, and hair loss [11, 12]. Therefore, it is necessary to use natural compounds, especially compounds extracted from plants that have high antitumor effects and low side effects. Medicinal plants have long been of interest to specialists due to their bioactive compounds and the interest in medicinal plants has increased recent years, and the pharmaceutical industries use plant compounds to make a large number of chemical drugs [13-16]. Hence, it is essential to conduct more research on the role of these natural compounds in improving human health. *Mentha piperita*, known as a peppermint, is a perennial, herbaceous plant belonging to the *Lamiaceae* family, used in traditional medicine to treat colds, fever, digestive disorders, and inflammation of the throat and mouth. Today, it is known that this popular medicinal plant has antimicrobial, antiviral, antioxidant, and antitumor effects due to its bioactive compounds such as flavonoids, limonene, and carvone [17-19]. For example Saidi et al. showed in 2019 that *M. piperita* extract has an inhibitory effect on antibiotic-resistant bacteria [20]. Also, Saravanan et al. reported in 2021 that the chloroform extract of *M. piperita* leaves has antibacterial and antioxidant effects, and it induces an anti-proliferative effect on MCF-7 breast cancer cells [21]. Raeisi et al. reported in 2019 that *M. piperita* essential oil contains compounds such as alpha-pinene, beta-pinene, menthone, and menthol. Also, their research showed that *M. piperita* essential oil has antibacterial and antioxidant effects [22]. Although the products extracted from plants have various bioactive compounds, but their use faces problems of high oxidizability and non-targeted effects, which have limited their use [23, 24]. So, it is needed to use new methods for targeted delivery of plant compounds to target tissues. It seems that nanotechnology can solve some problems of delivering herbal compounds to target cells by improving drug delivery [25-27]. Liposomes are colloidal particles with two or more layers of phospholipid membrane. based on their high importance as drug carriers in modern drug delivery systems, it is focused to engineer a wide range of them with different sizes, phospholipid composition, and characteristics surface. These vesicles consist of self-assembling lipid molecules that enclose a part of the aqueous phase in which they are dispersed. Having hydrophilic and hydrophobic parts, liposomes have the ability to carry and deliver a variety of hydrophilic and hydrophobic drugs. Being biocompatible, biodegradable, and non-toxic are among the features that have made liposomes a popular system in drug delivery [14, 28-30]. The purpose of this research is to prepare and characterize the different formulation of nano-liposomes containing *M. piperita* essential oil, compare its antibacterial, antifungal, and antioxidant effects with non-liposome essential oil and investigate its toxicity on breast cancer MCF-7 cell line and healthy skin HFF cell line (Scheme 1).

MATERIALS AND METHODS

Materials

For the present study, *M. piperita* plant was collected from Barabad farms in Khaf city of Iran. Soybean phosphatidylcholine 60 (SPC60), cholesterol, dimethyl sulfoxide (DMSO), 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DIL), 4′,6-diamidino-2-phenylindole (DAPI),



Scheme 1. A schematic overview of the present study.

and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) were supplied by Sigma-Aldrich Company. Isopropyl, chloroform, MüllerHinton agar medium, Nutrition Agar medium and phosphate buffered saline (PBS) were purchased from Merck, Germany. Ampicillin antibiotic disk was purchased from Padtan Teb Iran.

Essential oil extraction

M. piperita plant was collected from Barabad farms in Khaf city of Iran during the season of flower production and its type species was confirmed by the experts of Ferdowsi University of Mashhad Plant Research Institute under herbarium number 1469. First, *M. piperita* aerial parts were dried in a dry environment away from sunlight. For the essential oil extraction of *M. piperita* aerial parts, the method of distillation in water was used by clevenger device. For this purpose, 25 gr of dried and powdered *M. piperita* were transferred to a 500 ml balloon with 250 mL of distilled water; the balloon was connected to the Clevenger, and essential oil was extracted for 4 h. Finally, the produced essential oil was taken out of the clevenger machine and collected for the next steps [31].

Investigating composition of M. piperita essential oil

Identification of the essential oil compounds was done by gas chromatography method. For this purpose, the gas chromatograph column (Shimadzu, model 9A) was set at 50 for 5 m. Then, the temperature increased to 250and both injection and detection were done at 290. Helium gas with a linear velocity of 32 cm/s was the carrier of the column. The composition of the essential oil was identified using retention index and mass spectra of the compounds and their comparison with the standard mass spectra available in computer libraries and authoritative sources [32].

Determining maximum absorption wavelength (λ_{max}) and plot calibration curves

Spectrophotometry was used to determine the maximum absorption wavelength of *M. piperita* essential oil and draw a calibration graph. In this method, a stock solution of essential oil with a concentration of 1 mg/mL was prepared in PBS and isopropyl. Then, the different concentrations of essential oil were prepared in PBS and isopropyl solvents using stock solution. After that, the absorption spectrum was recorded by a spectrophotometer (Epoch, USA) in the range of 200 to 800 nm for all concentrations. The wavelength at which the maximum absorption occurred at all concentrations was considered as the maximum absorption wavelength. Then, the calibration curve of essential oil was drawn in PBS and isopropyl buffer using the absorption spectrum obtained from the different concentrations at the maximum absorption wavelength. The normalized equation of the line in PBS and isopropyl was calculated using the calibration curve [12, 31]. Experiments at this stage were repeated three times.

Preparation of nano-liposomal system containing M. piperita essential oil

The liposomal system containing *M. piperita* essential oil was prepared by a thin layer hydration method using SPC60 and cholesterol according to the concentrations in Table 1 along with *M. piperita* essential oil with a concentration of 1

Table 1. Chemical compounds used in the structure of liposomal formulations

Formulation Number	Lipid/Drug (molar ratio)	Cholesterol (molar ratio %)	SPC60 (molar ratio %)
F_1	10	10	90
F_2	10	80	20
F_3	10	70	30
F_4	10	60	40
F_5	10	50	50

mg/mL. First, SPC60, cholesterol, and *M. piperita* essential oil were dissolved in chloroform solvent. To assess the cellular uptake, fluorescent label DIL (0.1% mol) was added to lipid mixture. Then, thin film was prepared under vacuum using rotary evaporator (Heidolph, Germany) at 45°C under reduced pressure. After that, hydration was performed by adding the specific volume of distilled water for 60 min at 55oC. The prepared nanoparticles were then reduced in size using bath sonicate (Grant XB6, England) for 40 min. Finally, the nanoparticles were passed through 0.45 and 0.22 µm filters to homogenize [19]. The prepared formulations were maintained at 4 °C. In this study, the dose of essential oil was 1 mg/mL for all of the formulations and the L/D ratios were kept at 10.

Calculating encapsulation efficiency and release rate of essential oil from Liposomal systems

To calculate the efficiency of essential oil encapsulation, unencapsulated essential oil (free essential oil) was removed by dialysis bag method [33]. Then, the liposomal system was dissolved in isopropyl with a ratio of 20:1 to break the nano-liposome membrane. The absorption of liposomal essential oil was measured with a spectrophotometer at lambda max 220 nm, and the encapsulation efficiency of each formulation was calculated according to the calibration chart of the essential oil in isopropyl and using equation 1.

$$Entrapment\ Efficiency(\%EE) = \frac{Encapsulated\ Drug\ Concentration\ (mg/ml)}{Primary\ Drug\ Concentration\ (mg/ml)} \times 100 \qquad \text{Eq (1)}$$

The essential oil release from all formulations was investigated at a temperature of 37°C, pH=7.4 (similar to the environment of a normal cell) and 42°C, pH=5.4 (similar to the environment of a cancer cell). Dialysis bag method was used to evaluate essential oil release. In this method, a certain amount of liposome containing essential oil was poured into the dialysis bag. The dialysis bag was placed next to PBS for 72 hr and at specific intervals of 0.5, 1, 2, 3, 5, 6, 7, 8, 9, 12, 24, 48, 60, and 72 h, a certain amount of PBS was removed around the bag and its absorption was measured by a UV/Vis spectrophotometer. At the end, the release rate of essential oil was calculated at the different times by using the obtained absorptions and referring to the calibration chart of essential oil in PBS [31].

Physical characterization of *M. piperita*- loaded nano-liposome and selection of appropriate formulation

The particle size, poly dispersity index (PDI), and zeta potential of the nanoparticles obtained from the selected formulation were measured using a zeta sizer device (model: HORIBA) at 25 °C, an angle of 90 degrees, and a wavelength of 657 nm.

By measuring the encapsulation efficiency, essential oil release pattern, particle size, PDI, and zeta potential of the particles, a formulation with appropriate physicochemical characteristics was selected as optimum formulation and used for further studies.

Physicochemical characteristics of selected formulation containing M. piperita essential oil

Examining morphology of nanoparticles

The shape and morphology of synthesized nanosystem were investigated using advanced imaging techniques. Micrographs obtained via Field Emission Scanning Electron Microscopy (FESEM) were employed to visualize the synthesized nanoparticles. These nanoparticles were prepared from a diluted sample (0.1 mg/mL) coated with a thin layer of gold. Additionally, atomic force microscopy (AFM) using Nanowizard II instrument (JPK instruments, Germany) was utilized to assess the three-dimensional structure of the nanosystem. Prior to imaging, the sample

were further diluted (1:1000) with distilled water and homogenized through sonication for 30 min. Finally, the prepared samples were placed on mica sheets for imaging.

Functional group analysis

Fourier transform infrared (FTIR) spectroscopy technique was used to investigate the interaction between the synthesized liposome and *M. piperita*. In this method, FTIR spectra of free form of *Mentha piperita*, liposome containing *M. piperita*, and blank synthesized liposome were obtained separately. For this purpose, 1 mg of each sample in a ratio of 1 to 100 was added to potassium bromide (KBr), and then, the sample was located in a hydraulic press to form the pellets. Each sample was analyzed by FT-IR spectrum instrument (Brucker, Germany) at a wavelength of 400-4000 cm^{-1} and its functional groups were identified [34].

Cytotoxicity and anti-oxidant assay of nano-liposomes obtained from selected formulation

Cytotoxicity study on MCF-7 and HFF cell lines

The purpose of this step was to investigate the toxicity of selected formulation containing essential oil and free essential oil (blank liposomes) on MCF-7 and HFF cell lines by MTT method. The MCF-7 and HFF cell lines were prepared from the Pasteur Institute of Iran and cultivated in sterile flasks with DMEM medium, containing 10% FBS along with penicillin and streptomycin antibiotics in an incubator with a temperature of 37 °C and 5% carbon dioxide. In the MTT test, HFF and MCF-7 cells were cultured separately with a concentration of 104 in each well in a 96-well plate for 48 h. MCF-7 cells were treated with 16, 32, 64, 125, 250, 500, and 1000 µg/mL of free essential oil and liposomes containing essential oil for 48 hr. Also, HFF cells were treated similarly. After the desired treatments, 20 µL of MTT solution with a concentration of 5 mg/mL was added to each well and incubated for 4 h. After that, the supernatant was removed and 150 µL of DMSO was added to dissolve the formazan crystals; and the absorbance was recorded at 570 nm wavelength using ELISA reader [35-37]. Finally, the percentage of cell viability was calculated according to equation 2.

$$Cell\ viability\ \% = \frac{Average\ absorption\ in\ the\ test\ group - Average\ absorption\ in\ culture\ medium}{Average\ absorption\ in\ the\ control\ group - Average\ absorption\ in\ culture\ medium} \times 100 \qquad \text{Eq (2)}$$

Measurement of antioxidant properties

1 mL of DPPH (0.1 mM) solution was added separately to 1 mL of free essential oil and to 1 mL of nano-liposomes containing essential oil (obtained from selected formulation) with the concentrations of 0.08, 0.2, 0.5 and 0.7 and 1 mg/mL. The solutions were kept for 30 min at 25°C and away from light. Then, the absorbance of the solutions was measured at 517 nm wavelength with the blank sample of methanol and DPPH solution, and the rate of inhibition of free radicals was measured according to equation 3 [38].

$$\%\ free\ radical\ inhibition = \frac{Control\ absorption - Sample\ absorption}{Control\ absorption} \times 100 \qquad \text{Eq (3)}$$

In-vitro antibacterial studies

In this study, the antibacterial activity of free *M. piperita* essential oil and nano-liposomes containing *M. piperita* essential oil (obtained from selected formulation) were tested on *Escherchia. Coli*, *Enterococcus faecalis*, and *Staphyloccus. aureus* bacteria. A concentration of 1.5×10^6 CFU/mL from each bacterium was cultured on Nutrition Agar medium and incubated under aerobic conditions at 37 °C, separately. They were then cultured on a MüllerHinton agar medium using a sterile swab. An empty disk was placed at the bottom of each well and 20 µl of free essential oil and nano-liposomes containing essential oil were added to the respective wells. Ampicillin antibiotic disk as the positive control and distilled water as the negative control were used and incubated at 37 °C for 24 hr. The antibacterial activity of each antimicrobial agent was evaluated from the non-growth zone around each sample [39].

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

Eleven test tubes were used for determining MIC. First, 0.5 mL of Mueller–Hinton broth entered the tubes No. 2 to 11, and 2.5 mL of antibacterial agent (free essential oil and nano-liposome containing essential oil) was introduced into the tubes No. 1 and 2. Then, the serial dilutions of free or liposome-encapsulated essential oil were prepared in the tubes No. 2 to 9. After that, 0.5 mL of bacterial suspension was injected into the tubes 2 to 10. Tube No. 10 contained the culture medium and bacterial suspension, considered as control tube. The tubes were incubated for 24 hr

at 37 °C. The concentration of the last tube which inhibits the visible growth of a microorganism was reported as MIC. In addition, MBC refers to the lowest concentration of antimicrobial agent that destroys 99.9% of the inoculated microbial population. In the standard procedure, 100 µL of the suspension was removed from each tube after the dilution test and MIC determination that showed no growth, and then, was 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 [36, 40].

Antifungal analysis

To study the antifungal properties of free essential oil and nano-liposomes containing essential oil (obtained from selected formulation), *Penicillium expansum* and *Aspergillus niger* were prepared 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.

Investigating entry of nano-liposomes containing essential oils into cancer cells

To determine the distribution of nano-liposome containing essential oil in cancer cells, the fluorescence intensity was recorded. Briefly, the tumor cells were trypsinized, and about 105 cells/mL of the MCF-7 cell suspension along with 3 mL of culture medium were transferred to the 6-well plates which had a cover-glass at the bottom of each well and incubated for 24 hr. Then ,10 µL of DIL-labeled nano-liposome (obtained from selected formulation) containing essential oil was added and incubated for 3 hr. Next, the cells were washed twice with cold PBS and fixed with 150 µL of 95% ethanol, and about 15 µL of DAPI dye was spread on the slide to stain the nucleus with a concentration of 1 µg/mL. Finally, the samples were examined for the cellular uptake process by using a fluorescence microscope (Olympus, Japan).

Stability examination

During 120 days, the selected nano-liposomes (obtained from selected formulation) containing essential oil were kept at a temperature 4°C to prevent the evaporation of the essential oil from the nano-liposomes. To investigate the physicochemical stability of the nano-liposomes containing essential oil, their encapsulation efficiency, release of essential oil from nano-liposomes, size, zeta potential, morphology, and toxicity were investigated during 120 days. For this purpose, the particle size, zeta potential, and encapsulation efficiency was examined every 30 days. At the end of 120 days, the morphology of nano-liposomes, the release of essential oil from nano-liposomes at temperature of 37 °C, pH=7.4 and 42 °C , pH=5.5, and the toxicity of the nano-liposomes on MCF-7 cell line were analyzed.

Statistical software

In this research, Excel 2016 software was used to calculate the mean and standard deviation, and Graphpad Prism version 9 software was used to perform the two-way ANOVA test.

RESULTS

Chemical composition of M. piperita essential oil

The chemical compounds in *M. piperita* essential oil were identified using GC-Mass, reported in Table 2. (±)-Pulegone with 61.84%,

Table 2. Chemical compounds in M. piperita essential oil

Name of the compound	Percent	RT
α-Pinene	0.45	7.10
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	0.24	7.99
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.48	8.06
D-Limonene	11.60	9.19
cis-Menthone	16.68	13.41
trans-Isopulegone	1.28	12.2
.(±)-Pulegone	4.27	11.77
(-)-Carvone	16.68	13.47
2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl	0.29	13.66
Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	1.76	15.2
(-)-β-Bourbonene	0.23	15.99
Caryophyllene	0.45	16.56
β-Cubebene	0.43	17.54
Total	100	-

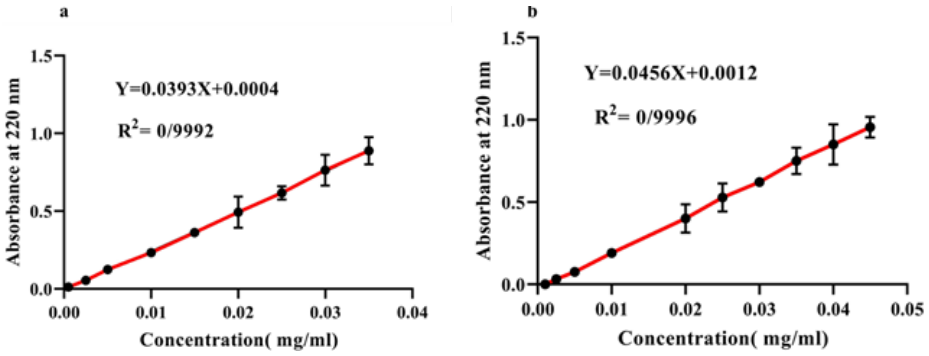


Fig 1. M. piperita essential oil calibration curve a) in isopropyl & b) in PBS.

carvone with 16.68%, and D-Limonene with 11.60% are the most abundant compounds in *M. piperita* essential oil.

Determination of Lambda max and standard curve of M. piperita essential oil in isopropyl and PBS

Absorption spectrum of *M. piperita* essential oil at wavelengths of 200 to 800 nm showed that the essential oil has the highest absorption at the wavelength of 220 nm. To calculate the encapsulation efficiency, the calibration chart M. piperita essential oil was drawn in isopropyl (Fig. 1a). The line equation was obtained Y=0.0393X + 0.0004 with a regression coefficient of R2=0.9992. Also, the calibration chart of M. piperita essential oil in PBS was drawn to calculate the amount of essential oil release, and its line equation and regression coefficient were Y=0.0456X + 0.0012 and R2=0.9996, respectively (Fig. 1b).

Encapsulation efficiency (EE%), vesicular size, PDI and zeta potential of nanoliposomal formulations

According to Table 3, the encapsulation efficiency varies between 42.33±2.76% (formulation F5) to 62.12 ± 3.06% (formulation F1). The largest particle size is related to F5 formulation (166.3 nm) and the smallest one is related to F1 formulation (121 nm). Also, the difference between the most negative amount of zeta potential (F1: -21.8 mV) and the most positive amount of zeta potential (F5: -19.1 mV) is -2.1 mV. The PDI in all formulations is less than 0.150, and formulation F1 has the lowest amount of PDI.

Release of M. piperita essential oil from formulations F1 to F5

Essential oil release from nano-liposomes F1-F5 was obtained in the similar conditions to healthy cells (pH = 7.4 and 37 °C) and cancer cells (pH = 5.4 and 42°C). According to Fig 2, the amount of essential oil release in F1 formulation is higher compared to other formulations within 72 hr. Also, the amount of essential oil released from F5 formulation within 72 hr is less compared to other formulations. The maximum release of essential oil from formulation F1 within 72 hr in the similar to the conditions of the cancer and normal cells are 56.74±1.14% and 50.06±0.41%, respectively. The maximum release of essential oil from formulation F2 within 72 hr in the similar to the conditions of cancer and normal cells are 51.04±0.11 and 47.46+0.76, respectively. These are 46.92±0.16 and 43.10±0.52 for F3 formulation within 72 hr in the similar to conditions cancer and normal cells, respectively. For formulation F4 within 72 hr, they are 46.37±0.54 and 41.62±0.35, respectively. For formulation F5 within 72 hr, they are 34±0.10 and 41.16±0.56, respectively. Also the release of essential oil from all formulations is slow and continuous, and the rate of release from liposome systems is high in the early hours, but with the passage of time, the rate of release of essential oil from nano-liposomes decreases.

Table 3. EE%, size, PDI, and zeta potential of nanoliposomal formula.

Formulation number	EE%	Size (nm)	PDI	Zeta potential (mV)
F_1	62.12 ± 3.06	121.0	0.131	-21.8
F_2	56.38 ± 3.38	134.4	0.137	-21.1
F_3	46.69 ± 3.19	146.9	0.141	-20.3
F_4	45.26 ± 4.09	157.6	0.147	-20.1
F_5	42.33 ± 2.76	166.3	0.149	-19.7

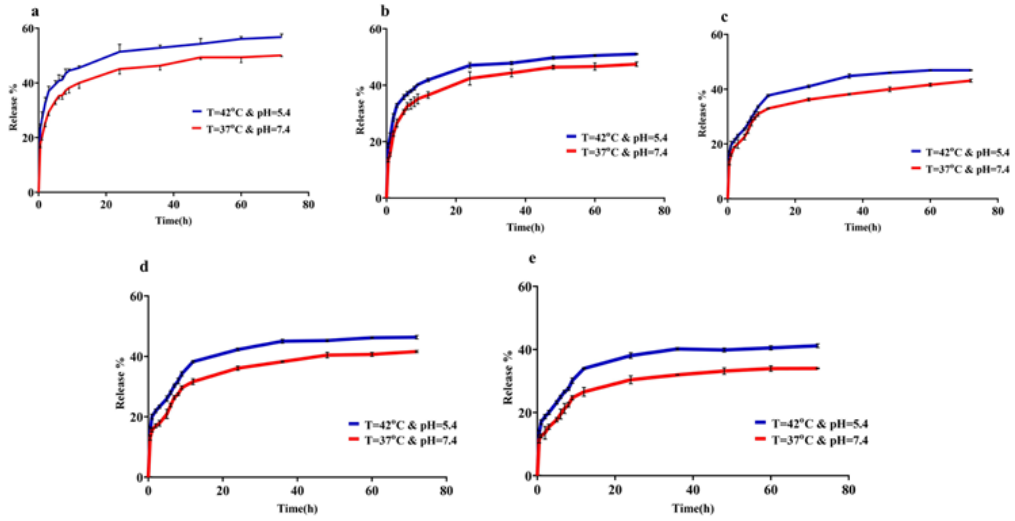


Fig 2. The release pattern of essential oil from nano-liposomes during 72 hr; a) F1 formulation, b) F2 formulation, c) F3 formulation, d) F4 formulation, and e) F5 formulation.

Also, the significance of the release rate at the different times in the similar conditions of cancer and normal cells is shown in Fig 3.

Choosing a suitable formulation to perform further tests

Considering that liposomes obtained from F1 formulation have higher encapsulation, appropriate release, more negative zeta potential, and smaller size than other formulations, F1 formulation was chosen for further experiments.

Release of M. piperita essential oil from F1 formulations

The release of essential oil from the liposomes

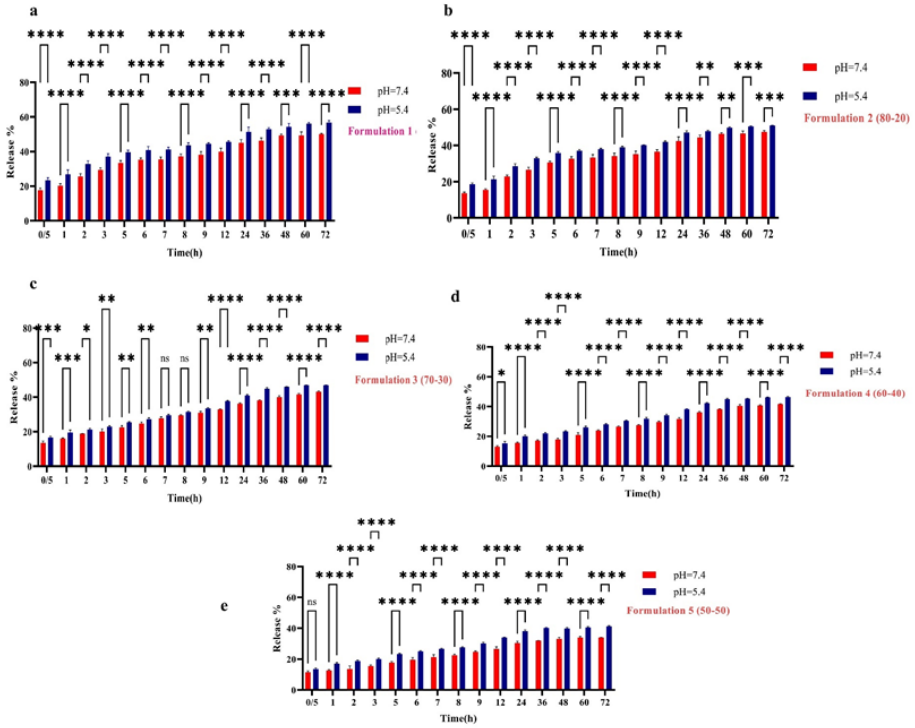


Fig 3. The significance of the release rate at the different times in similar conditions of cancer (pH=5.4) and normal (pH=7.4) cells; a) F1 formulation, b) F2 formulation, c) F3 formulation, d) F4 formulation, and e) F5 formulation (Significance: * P<0.05,** P<0.01, *** P<0.001 and ns = non-significance)

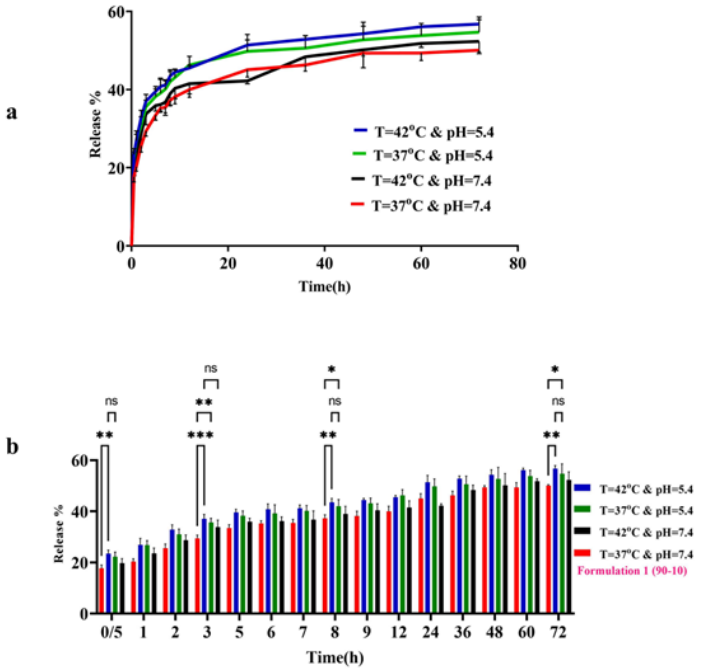


Fig 4. a) The release pattern of essential oil from F1 formulation at the different pH values and temperatures, b) The significance of essential oil release rate from F1 formulation at the different pH values and temperatures (Significance: * P<0.05,** P<0.01, *** P<0.001 and ns = non-significance)

obtained from the selected formulation (F1) was also investigated at 37 °C, pH=5.4 and at 42°C, pH=7.4. As shown in Fig. 4, the release of essential oil is slow and continuous, and the maximum release of essential oil is 54.68±3.89% at 37°C and pH=5.4 and 52.32±3.13% at 42 °C and pH=7.4.

Morphology of nano-liposomes obtained from F1 formulation

Fig. 5 confirms the formation of nano-liposomes obtained from formulation F1. Also, the nano-liposomes have a spherical shape and a smooth surface. The size of nano-liposomes has

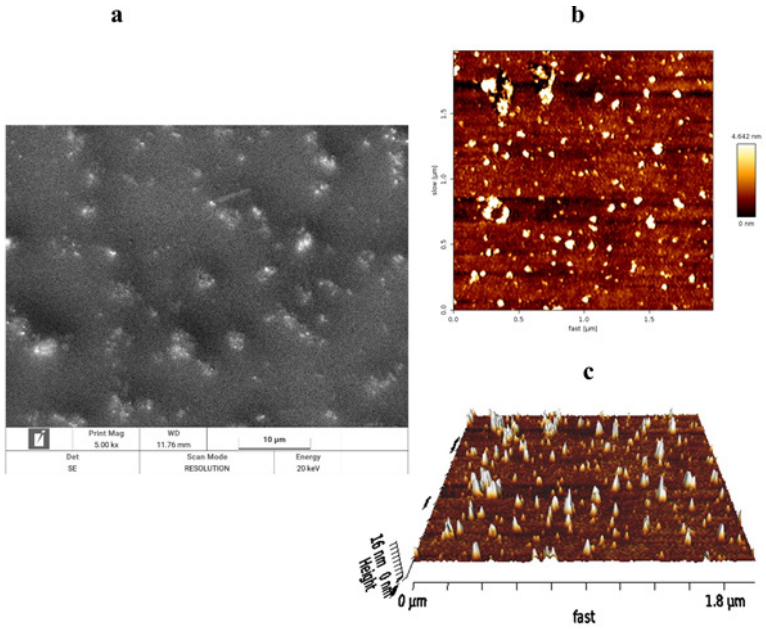


Fig. 5. Electron microscope images of the nano-liposomes obtained from formulation F1 by a,b) Fe-SEM microscope c) AFM

been determined, which is close to the results of the Zeta Sizer device.

FT-IR analysis

FT-IR graph of nano-liposomes without essential oil obtained from F1 formulation, nano liposomes containing essential oil obtained from F1 formulation, and M. piperita essential oil are shown in Fig. 6a, b, and c, respectively. Examining the FT-IR graph of essential oil in Fig. 6c shows that the broad peak at 3500 cm^{-1} is characteristic of the OH group, phenols, and alcohols, and the peak at 2924.96 cm^{-1} is characteristic of CH_3 stretching vibration. Also, the peak at 1662.49 cm^{-1} is the characteristic of stretching vibration of C=O; the other at 1377.87 cm^{-1} is related to nitro groups. The peak at 1443.55 cm^{-1} belongs to bending movement of CH3. The peaks in the region of

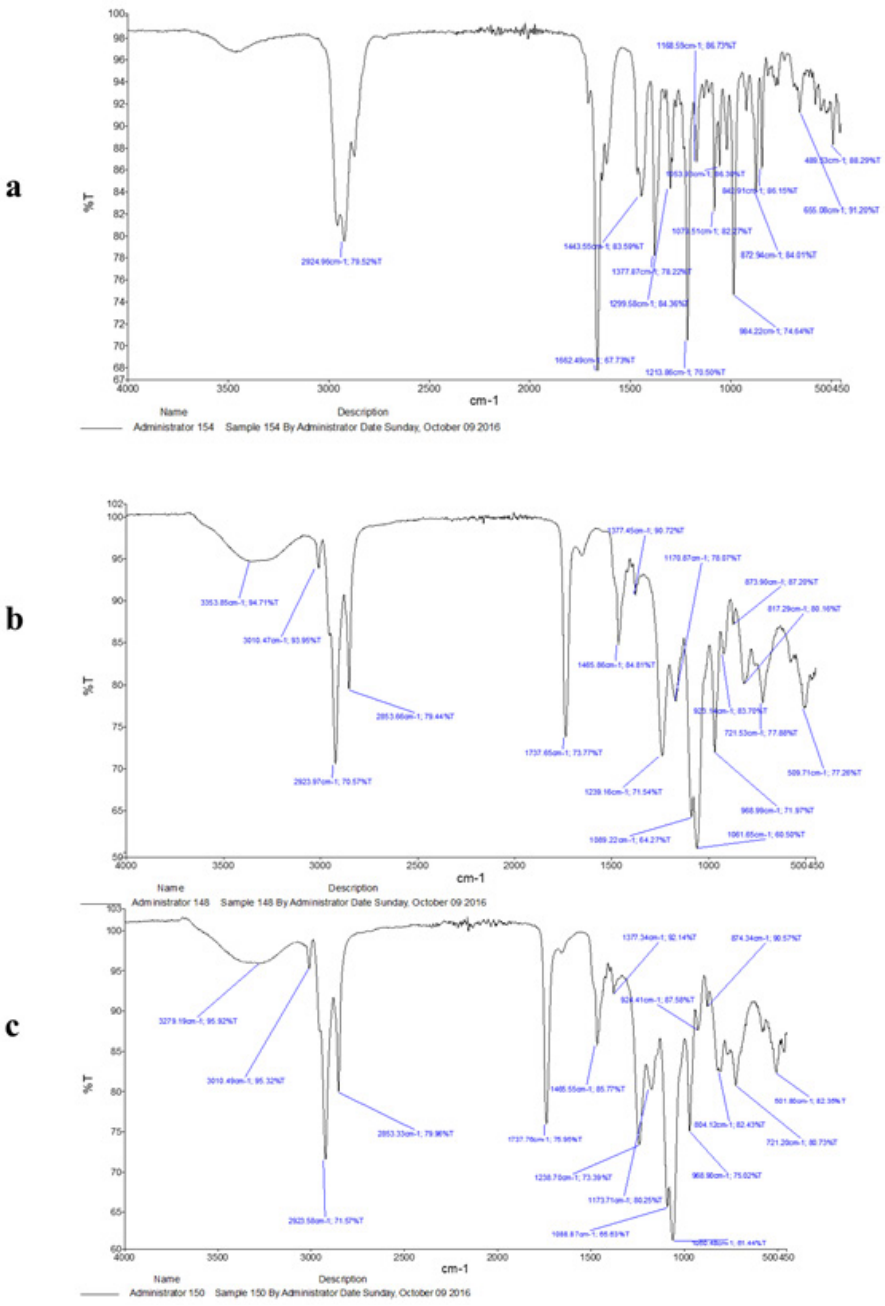


Fig. 6. FT-IR graphs of a) Nano-liposomes without essential oil, b) Nano-liposomes containing essential oil, and c) *M. piperita* essential oil

1000-1300 cm^{-1} are related to the C-O group. Ones at 984.22 and 872.94 cm^{-1} are characteristics of P-OR and P-O. Examining the FT-IR graphs of nano-liposomes without essential oil and nano-liposomes containing essential oil show that the peak at 3279.19 cm^{-1} is corresponded to the OH functional group and the others at 3010.4919 cm^{-1} and 2923.58 19 cm^{-1} are characteristic of stretching vibration of CH3. The peaks of 2853.33 and cm^{-1} 1737.76 are related to stretching vibration of CH2and C=O bond, respectively. the wavelength of cm^{-1} is characteristic of bending motion. Also, the peaks at 1465.55 and 1377.34 cm^{-1} are characteristic of CH_2 and CH_3 bending motions. The peaks in the region of 1000-1300 cm^{-1} are related to the C-O group. The peaks at 968.90 and 924.41 cm^{-1} are related to P-OR. Ones at 874.34, 1238.70, and 721.20 cm^{-1} belong to P-O, P=O, and bending motion of CH_2 in an open chain (long chain band). Comparison of the FTIR graphs of the liposomal system before loading the essential oil and after loading the essential oil shows that these two spectra have similar peaks and with essential oil loading, no additional peaks are seen in the liposomal system containing the essential oil, proving no chemical interaction between liposomal system and essential oil. On the other hand, the peaks created in the FT-IR spectrum of the system containing essential oil compared to the system without essential oil have undergone slight changes in location, which is a proof of the confinement of the essential oil inside the liposome.

Investigating antioxidant properties and toxicity of nano liposomes from F1 formulation

Comparison of the antioxidant power of nano-liposomes containing M. piperita essential oil and free essential oil is reported in Fig. 7. Based on Fig. 7a, nano-liposomes containing essential oil and free essential oil have dose-dependent antioxidant properties, and with the increase in the concentration of each component, its antioxidant power increases. Also, in all concentrations, the antioxidant power of nano-liposomes containing essential oil is higher than that of free essential oil.

The results of the MTT test show that the liposomal system without essential oil and liposomal system containing the essential oil caused very little toxicity to the healthy HFF cells (Fig. 7b, 7c). The comparison of the toxicity of the liposomal system containing essential oil and free essential oil in Fig. 7d shows that free essential oil and liposomal system containing essential oil have concentration-dependent toxicity on MCF-7 cancer cells, such that at a concentration of 16 µg/mL, they have the lowest toxicity and at a concentration of 1000 µg/mL, they have the highest toxicity. Likewise, in all concentrations, the toxicity of liposomal system containing essential oil on MCF-7 cell line is higher than that of free essential oil. This toxicity difference in the concentrations of 250, 500, and 1000 µg/mL is

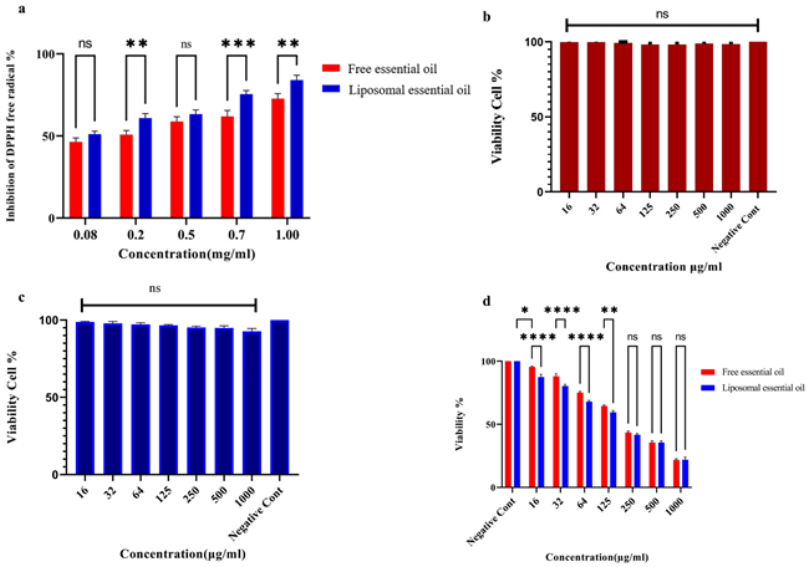


Fig 7. a) Comparison of the power of nano liposomes containing essential oil and free essential oil in inhibiting DPPH free radical; b) Toxicity of blank nano-liposomes on HFF cell line; c) Toxicity of nano-liposomes containing essential oil on HFF cell line; d) Comparison of the toxicity of nano-liposomes containing essential oil and free essential oil on MCF-7 cell line. (Significance: * P<0.05,** P<0.01, *** P<0.001 and **** P<0.0001 and ns = non-significance)

Table 4. Inhibitory effect of nano-liposomes containing *M. piperita* essential oil and free M. piperita essential oil on microorganisms

Type of microorganism	Type of used material	MIC (µg/mL)		Diameter of Inhibition Zone (mm)
Escherchia.Coli	free essential oil	50	50	9
	nano liposomes containing essential oil	25	25	13
Enterococcus faecalis	free essential oil	25	50	10
	nano liposomes containing essential oil	12.5	12.5	15
Staphyloccus.aureus	free essential oil	25	50	11
	nano liposomes containing essential oil	12.5	12.5	17
Penicillium expansum	free essential oil	25	50	10
	nano liposomes containing essential oil	12.5	25	15
Aspergillus niger	free essential oil	50	50	9
	nano liposomes containing essential oil	25	50	14

small and not significant. It can be concluded that the toxicity of the liposome system containing essential oil is higher compared to free essential oil in concentrations less than 250 µg/mL. Besides, the amount of IC_{50} of free essential oil and nano-liposomes containing essential oil was calculated as 155.1 and 148.8 µg/mL, respectively.

Comparing antimicrobial properties

The results of comparing the antimicrobial effects of nano-liposomes containing essential oil and free essential oil are reported in Table 4. In all tested microorganisms, the inhibitory effect of nano-liposomes containing essential oil is higher than free essential oil. The lowest amount of MIC is related to bacteria *Enterococcus faecalis* and *Staphyloccus. aureus* and fungus *Penicillium expansum*, and the lowest amount of MBC is related to bacteria *Enterococcus faecalis* and *Staphyloccus aureus*.

Investigating entry of nano-liposomes containing essential oils into cancer cells

Fig. 8 (rows A and B) shows the treatment with liposomal systems containing essential oil, and the system without essential oil (blank liposome). Columns numbers (1) and (2) represent the nucleus stained with DAPI dye under the blue filter and the liposomal system stained with DIL under the green filter, respectively. Column (3) is also the fluorescent image recorded with the Merge filter. The images clearly indicate the entry of the nanosystem into the cell nucleus (Fig. 8).

Stability examination

The stability of optimized nano liposomes obtained from F1 formulation was measured during 120 days. Zeta potential, particle size, and encapsulation efficiency were measured every 30 days, shown in Table 5. During 120 days, the

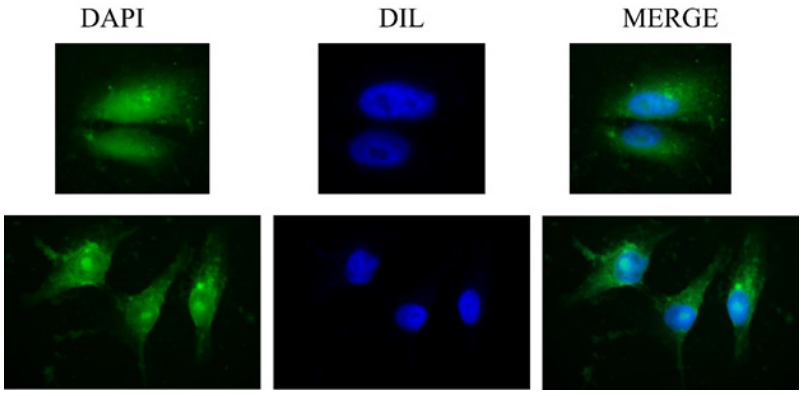


Fig 8. Entry of nano-liposomes containing essential oil into MCF-7 cells

Table 5. Changes in size, zeta potential, and encapsulation efficiency during 120 days

Formulation number	After 30 days			After 60 days			After 90 days			After 120 days		
F1	EE%	Size (nm)	Zeta (mV)	EE%	Size (nm)	Zeta (mV)	EE%	Size (nm)	Zeta (mV)	EE%	Size (nm)	Zeta (mV)
	54.53±1.60	124.6	-20.2	50.79±1.55	129.7	-18.8	45.53±1.04	132.0	-18.1	43.46±3.21	136.5	-17.5

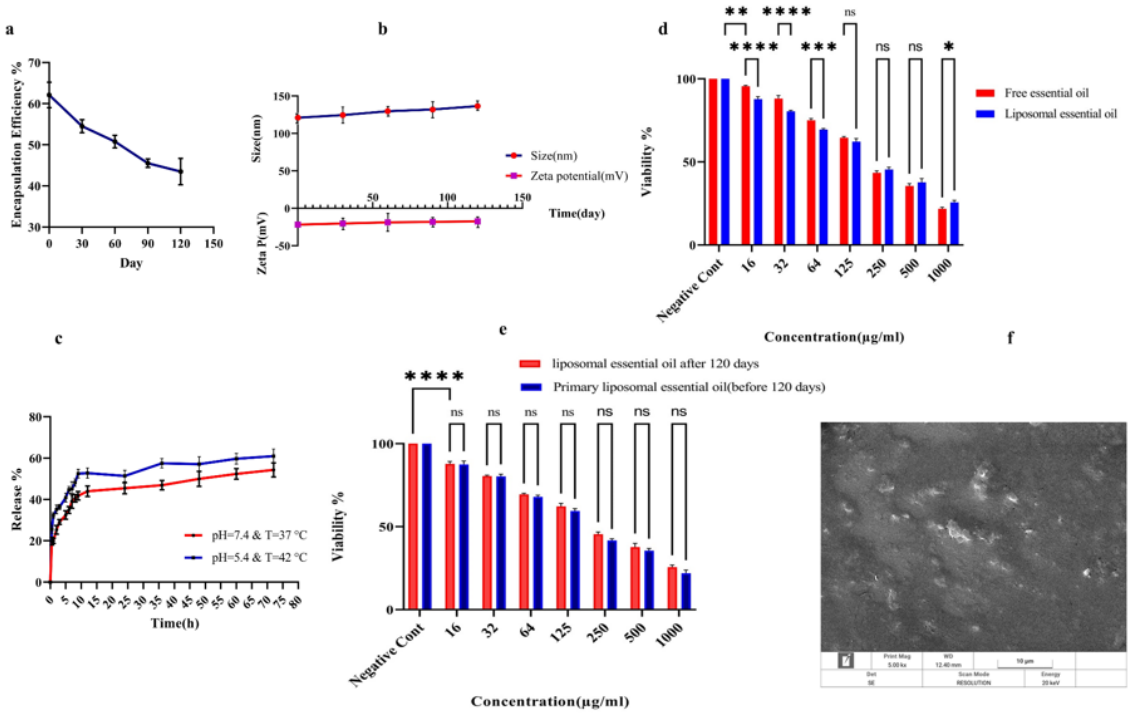


Fig. 9. Sustainability results: a) Encapsulation efficiency changes; b) Size changes and zeta potential changes; c) Release pattern; d) Comparison of the toxicity of nano-liposomes containing essential oil and non-liposomal essential oil on MCF-7 cell line; e) Comparison of the toxicity of nano liposomes containing essential oil before and after stability testing on MCF-7 cell line; f) SEM image of nano-liposomes containing essential oil

encapsulation efficiency decreases by 18.66% (Fig. 9a). The zeta potential of nanoparticles becomes more positive by 4.3 mV, and the size of the particles increases by 15.5 nm, which is a small change considering the storage of nanoparticles for 4 months (stability time) ((Fig. 9b). The release of M. piperita essential oil from nano-liposomes has been investigated after 120 days (Fig. 9c). According to Fig. 9c, the pattern of essential oil release at pH=7.4, T=37 and pH=5.4, T=42 after 120 days is similar to the release of essential oil before the stability measurement. Besides, the amount of essential oil release has increased compared to that was before the stability measurement. The electron microscope image shows that the nanoparticles have maintained their spherical shape and flat surface after 120 days (Fig. 9f). The toxicity of nano-liposomes containing *M. piperita* essential oil on MCF-7 cell line was evaluated after 120 days, and the results are reported in Fig. 9d. According to Fig. 9d, at the concentrations of 125, 64, 32, and 16 µg/mL, the toxicity of nano-liposomes containing essential oil is significantly higher than that of free essential oil. In the concentrations of 250, 500, and 1000 µg/mL, the toxicity of free essential oil is higher than that of nano-liposomes containing essential oil, and this toxicity difference is not significant in the concentrations of 250 and 500 µg/mL. Comparing the toxicity of nano-liposomes containing essential oil on MCF-7 cell line, before and after the stability test, shows that the toxicity of nano-liposomes containing essential oil has decreased after the stability test, but this difference is not significant (Fig. 9e). Moreover, the amount of IC_{50} of nano-liposomes containing essential oil after 120 days is 165.5 µg/mL.

DISSCUTION

Our research has two parts. In the first part, the chemical compounds in *M. piperita* essential oil have been identified, and in the second part, the preparation and investigation of nano-liposomes containing *M. piperita* essential oil have been discussed. In the first part, thirteen chemical compounds with the different percentages were identified in *M. piperita* essential oil, some of them have various essential properties. As an example, alpha-pinene is a type of monoterpene that has anti-tumor, anti-metastasis, antimicrobial,

and antioxidant effects. Some studies show that this chemical compound has antifungal effects as well [41-43]. Also, D-limonene is another monoterpene identified in *M. piperita* essential oil, for which antibacterial, antioxidant, and antitumor properties have been reported [44, 45]. In 2020, Ye et al. proved that limonene provides high antitumor capacity to induce cell cycle arrest, suppression of cell migration, invasion, and apoptosis [46]. The most common chemical compound known in the current research is Pulegone with antibacterial and antioxidant properties. Farhanghi et al. in 2022 showed that Pulegone has an anti-Staphylococcus aureus effect [47]. Carvone is another compound identified in *M. piperita* essential oil as a monoterpene ketone with antioxidant and anti-inflammatory properties [48]. Patel et al. reported that L-carvone can stop MCF 7 cells in the S phase of the cell cycle and induce apoptosis in cancer cells [49]. There are also some promising reports of anti-Staphylococcus aureus for carvone [50]. Among other compounds identified in *M. piperita* essential oil is menthone. This chemical compound has antimicrobial effects. For example, Zaia et al. confirmed that herbal medicine containing 14-32% menthone can reduce infection caused by S. Mansoni [51]. In the second part, nano-liposomes containing *M. piperita* essential oil were prepared according to formulations F1 to F5, in which the best formulation (F1) had a particle size of 121 nm, zeta potential of -21.8 mV, entrapment efficiency about 62%, and the release of essential oil was continuous and slow. One of the important indicators in the study of lipid nanocarriers is the zeta potential, which depends on the various factors such as pH environment and type of materials used in the structure of nanoparticles. Zeta potential actually refers to the electric charge around nanoparticles, which is also observed around cells [52]. Zeta potential plays an important role in the stability of lipid nanoparticles. Increasing the surface charge of nanoparticles increases the repulsive force between particles, which prevents deposition and accumulation of nanoparticles and ultimately increases the stability of nanoparticles [53, 54]. Research has shown that the zeta potential more negative than -30 mV and more positive than +30 mV, is indicating the high stability of nanoparticles against aggregation [55, 56]. In the present study, considering the nano-liposomes obtained from formulations F1 to F5 have high and negative zeta potential, it seems that these nano-liposomes, especially in formulation F1, have acceptable stability against sedimentation and aggregation. In 2018, Majdizadeh et al. prepared nano-liposomes containing *M. piperita* essential oil for which they reported a zeta potential of -34.54 mV [31]. Najlah et al., reported negative zeta potential for all liposomal formulations in their research (0.1 to 0.3) [57]. In the present study, the size of nano-liposomes obtained from formulations F1 to F5 varies between 121 nm for formulation F1 and 166.3 nm for formulation F5. Considering that nano-liposomes with a size between 50 and 200 nm are suitable for drug delivery [58], it seems that the size of liposome particles in all formulations are suitable for drug delivery (essential oil). Another factor affecting drug delivery by nanocarriers is the encapsulation efficiency. Various factors such as the type and molar percentage of the compounds used in the structure of the nanocarrier, the type and nature of the material loaded in the nanocarrier, and the method of manufacturing the nanocarrier are effective on the encapsulation efficiency of the nanocarriers [59, 60]. In the present study, the encapsulation efficiency of nanocarriers obtained from F1 to F5 formulations varies between 62.12 ± 3.06% for F1 formulation and 42.33 ± 2.76% for F5 formulation. This difference in the percentage of encapsulation efficiency can be justified due to the different molar percentage of cholesterol in formulations F1 to F5. Although the use of cholesterol increases the stability of lipid nanoparticles, the encapsulation efficiency decreases by increasing its amount in the structure of lipid nanoparticles. Actually cholesterol occupies the space of hydrophobic drugs by being in the vicinity of the hydrophobic tails of lipids that make up lipid nanocarriers, and thus, it can reduce the encapsulation efficiency of hydrophobic drugs, including essential oils [61, 62]. To approach the in-vivo conditions, the release of essential oil at pH and temperature similar to that of normal and cancer cells was investigated within 72 h in PBS buffer. The pattern of essential oil release from nano-liposomes in all formulations is slow and continuous, and the amount of essential oil release from nano-liposomes obtained from F1 formulation is higher compared to the other formulations. In fact, the reduction of cholesterol in the F1 formulation compared to other formulations has led to a decrease in the ability of

the liposomes from this formulation to retain essential oil, as a result of which the amount of essential oil released from this formulation has increased compared to other formulations. This part of the results in the present study is consistent with Deniz et al.'s research in 2010 [63]. The examination of the release pattern of essential oil in the different formulations shows that the release of essential oil from nano-liposomes in the environment similar to cancer cells is more than in the environment similar to normal cells. It can be concluded that the nano-liposomes obtained from formulations F1 to F5 more effectively release the essential oil in the environment similar to cancer cells. In 2017, Naderinejad et al. designed Lipo-Niosomal systems containing doxorubicin and curcumin and, like the present study, reported slow and continuous drug release from this lipid carrier and showed that the drug release rate is higher in acidity similar to cancer cells [64]. Moreover, Haghiralsadat et al. designed liposomal systems containing doxorubicin, which reported slow and continuous release and greater delivery of the drug in conditions similar to cancer cells [64]. The use of blood plasma instead of PBS buffer in the release section could bring the release process closer to the in-vivo scenario, which was used due to the researchers' lack of access to blood plasma. Therefore, we recommend the use of blood plasma instead of PBS buffer in the release process to future researchers. In our study, the toxicity of liposomes containing *M. piperita* essential oil was measured on HFF and MCF-7 cell lines. The MTT results showed that blank liposome and liposome system containing essential oil has very little toxicity for healthy HFF cells. Siyadatpanah et al. and Mirzaei et al. clarified that liposomal systems containing plant essential oils have little toxicity on HFF cell lines [59, 65]. Also, the MTT results indicate that the toxicity of the liposomal system containing *M. piperita* essential oil on MCF-7 cell line is higher in all concentrations than the free *M. piperita* essential oil. This proves that the liposomal system increases the anti-proliferative effects of *M. piperita* essential oil. Taebpour et al. reported in 2022 that lipid nanocarriers can enhance the anti-proliferative effects of curcumin on MG-63 cell line [12]. Ebrahimpour et al. also reported that lipid nanocarriers containing Thyme essential oil are more toxic to MCF-7 cancer cells than free Thyme essential oil [39]. AIn the present study, it was found that the liposomal system containing *M. piperita* essential oil has higher antimicrobial effects on some microorganisms than the free essential oil. Other scientists reported that lipid nanocarriers containing plant compounds have more antimicrobial effects than free essential oil and free extract in some cases [38, 39]. In this research, the physicochemical stability of liposomal nanoparticles containing *M. piperita* essential oil was investigated for 120 days. During 120 days, the liposomal systems containing essential oil were kept at a temperature below 4°C to prevent the evaporation of the essential oil from the liposomal system. The results illustrate that the liposome system had relatively high stability during 120 days. Compared to the initial state, the zeta potential of nanoparticles decreased while the particle size increased; but these changes are minor. The encapsulation efficiency significantly decreased within 120 days, which led to a decrease in the toxicity of the liposomal system on the MCF-7 cell line. The volatile nature of the essential oil [66] can be one of the factors that reduces the encapsulation of the liposomal system during 120 days.

CONCLUSION

In summary, the chemical compositions of *M. piperita* essential oil were first investigated. Pulegone (61.84%), carvone (16.68%), and D-Limonene (11.60%) included three major compounds of *Mentha piperita*. An optimum and well-organized nano-liposomes containing *M. piperita* essential oil was constructed for anti-proliferative effects against microbial infection and cancer cells. The results exhibited the formulation had nano scale size, spherical-shaped vesicles with the negative zeta potential and high EE values and loading rates. Entrapment of *M. piperita* into nano-liposome enhanced bioavailability and stability, and also managed the release behavior of volatile components. Nano-liposomes were stable after 4 months of storage at 4°C. Entrapped *M. piperita* essential oil induces enhanced microbial toxicity against bacteria and fungi in comparison with free form. The results prove that nano-liposomes containing *M. piperita* essential oil have suitable physicochemical properties and have little toxicity on healthy cells, but at the concentrations more than 250 µg/mL, they possess no significant anti-proliferative effects on MCF-7 cell lines compared to free essential oil. Consequently, the use of *M.*

piperita liposome essential oil for anti-cancer research requires more and more comprehensive research.

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CONFLICT OF INTEREST

There is no conflict of interest.

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