

# Lavandula Angustifolia Essential Oil Loaded in Liposomal Nano-Carriers Regulate the HER2 and CASP3 Genes in MCF-7 and SK-BR-3 Cell-Lines

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## Research

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## Abstract

Cancer drug delivery has recently focused on using novel carrier systems due to their high drug-loading capacity, release rate properties, and minimum side effects. Finding novel chemotherapeutic agents would be essential for the improvement of integrated treatment planning. In this study, the effect of constructed nano-liposomal particles of *Lavandula angustifolia* essential oil (LAEQ) was evaluated on cell toxicity and the expression of *HER-2* and *Caspase-3* genes in two *MCF-7* and *SK-BR-3* cell lines. Our results showed a high entrapment efficiency ( $50 \pm 3.34$ ), and a low average size ( $68.3 \pm 7.28$  nm) of nano-liposomes under suitable conditions. The surface charge of particles was at a range of -2 to -4.5 mV. The release rate of nanoparticles was estimated to be 63.98% ( $37^\circ\text{C}$ ; pH = 7) and 87.63% ( $42^\circ\text{C}$ ; pH = 5) in *MCF-7* and *SK-BR-3* cell lines after 48 hours, respectively. The stability of nanoparticles without a great increase in particle sizes and spherical shape was observed using force atomic microscopy. The degree of cell growth in response to nanoliposomal LAEO was significantly decreased in comparison with free essential oil ( $p$ -value < 0.01) when assayed on both cell lines. The analysis of gene expression showed that the treatment of cancer cell lines with nanoliposomal LAEO significantly elevated the relative expression of the *caspase-3* gene while reducing the expression of *HER-2* ( $P < 0.05$ ). Based on the obtained results, it seems that LAEO in the form of nanoparticles could have more efficiency on the growth inhibition of cancer cell lines in comparison with the free form of the essential oil. Further studies are required to assess the *in vivo* effects of LAEO nanoliposomes with refined formulations against various types of cancer (Fig. 1).

## Introduction

Breast cancer is one of the most common types of cancer diagnosed in adult women over the world (1). It is estimated that 1.3 million women are annually diagnosed with breast cancer, according to the reports announced by the World Health Organization (2). In Iran, a high prevalence and mortality rate of breast cancer is considered as one of the major healthcare problems (3).

Despite significant efforts made for the treatment of breast cancer, such as surgical procedures and prescribing chemotherapeutic agents, the recurrence of the disease remains an unresolved dilemma in patients, especially those who are in advanced stages. To date, numerous chemotherapeutic agents have been designed for breast cancer therapy that can induce cell death or lower the cell growth rate in tumors; however, the usage of these chemicals is usually accompanied by several adverse effects (4). Thus, designing and the development of novel anticancer compounds would be requisite for the enhancement of integrated treatment plans (5).

*Lavandula angustifolia* Mill. is a widely distributed aromatic herb, belonging to the *Lamiaceae* family. This herb contains a rich source of oleanolic acid, uric acid, and betulinic acid. Analytical studies have shown that the active compounds of the Lavandula essential oil comprised of geranyl acetate (5%), alpha-terpineol (6.7%), lavandulyl acetate (15.9%), linalyl acetate (17.6%), and linalool (32.8%). Plants belonging to the *Lamiaceae* family have been shown to possess a rich source of phytochemicals, such

as carotenoids, phytosterols, terpenoids, and flavonoids, which are able to scavenge a free radical agent as a result of its antioxidant potential and stimulate the immune response. These phytochemicals can due to DNA adduct and lead to induce suppressing carcinogenesis activity (6).

The strategy by which a substance is delivered to its target can profoundly affect its adequacy. Some drugs have an optimal concentration range in which maximum efficiency is achieved, and higher/lower concentrations of the optimal range can be lethal or ineffective, respectively (7). In other words, due to the low efficiency of some drugs scientists have been interested to develop an interdisciplinary approach for the delivery of drugs on their targets with high efficiency. Nowadays, drug delivery systems have focused on using novel carrier systems because of their high drug-loading capacity, controlled-release properties, and minimum side effects (8). Nanoparticles can be organized with an incredible set of materials and structures (9). Lipid-based drug delivery systems exhibited promising results when applied in different studies and showed their proper characteristics in controlled and targeted drug delivery. Liposomes are bilayer vesicles that are made of amphiphilic lipids with well-organized structures that can carry hydrophilic, hydrophobic, and amphiphilic compounds. Nanoliposomes can have different shapes and sizes depending on the purpose for which they are made, the target tissue, and the materials loaded on them. The etiology of breast cancer is multifactorial in which age, late menopause, using oral contraceptives, hormone therapy, genetics, history of benign breast diseases, obesity, dietary status, and exposure to carcinogenic compounds are involved (10). The molecular feature of breast cancer could be described by the absence or presence of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor-2 (HER-2). Among these receptors, HER-2-positive breast cancer cells are more common in women with breast cancer as HER-2 positivity accounts for 20% of cases diagnosed with breast cancer in which the HER-2 gene is remarkably overexpressed (11). Studies indicated that *HER-2* is upregulated in 25–30% of all cases diagnosed with breast cancer, and its expression rate is even higher in those patients who are in advanced stages of the disease (12). It has been well-accepted that impaired apoptosis is one of the features of cancer cells. The process of apoptosis is mediated by two major pathways, namely extrinsic pathway and intrinsic pathway. The extrinsic pathway is activated upon binding of death ligands to their cognate death receptors (DRs) superfamily members, leading to the activation of caspase-3, while intrinsic pathway is initiated by the release of cytochrome-c from the inner membrane of mitochondria, leading to the activation of caspase-9 and subsequently caspase-3. *Caspase-3* is a significant regulator of the apoptosis process which interacts with the Calpain family, a group of cysteine protease enzymes that are activated during tumorigenesis.

The aim of the current study was to characterize the constructed nanoliposomal particles loaded with LAEO in terms of the size and morphology of particles, zeta potential, mean electrophoretic mobility, dispersion medium, viscosity, and entrapment efficiency, as well as examining the cytotoxicity effects of PEGylated liposomal loaded with LAEO on breast cancer cell lines. The relative expression levels of *HER-2* and *Caspase-3* genes were also analyzed in cell lines treated with LAEO-loaded nanoliposomes.

## Materials And Methods

# **Essential Oil Extraction, Preparation of Nanoliposomes, and Encapsulation Process**

*Lavandula angustifolia Mil.* species was collected, and 200 grams of dried plant samples were homogenized in 1 liter of distilled water for four hours using by means of a Clevenger apparatus. The essential oil was extracted and stored at 4 °C until future use (7).

Afterward, soybean phosphatidylcholine (SPC), cholesterol, and polyethylene glycol (PEG) at molar ratios of 69:30:1 were mixed in 15 ml chloroform as a solvent. Then, the essential oil was dissolved in ethanol and added to the mixture that was poured in a round flask. At this stage, organic solvents were evaporated under reduced pressure using a rotary evaporator (Heidolph, Germany) at 35 °C until a thin film was formed on the walls. Then, 5 ml of distilled water was added to the flask, and the lipid film was hydrated in a rotary evaporator (in the absence of vacuum) at 40 °C for 45 minutes. In order to decrease the size of particles, the obtained liposomal solution was sonicated by a probe sonicator (Misonix, USA) in an ice bath at 60% amplitude for 10 minutes (10-sec sonication along with 15-second intervals to allow cooling the samples). Next, the free form of the herb, LAEO, was separated from nanoliposomes loaded with LAEO using a cellulose membrane (dialysis method) with the cut-off of 10 KDa against distilled water at 4 °C. In order to increase the purity of liposomal solution (e.g., removing titanium particles formed during sonication) of the liposomal solution, samples were centrifuged at 5000 rpm for 5 minutes. For the separation of larger particles from smaller particles and homogenization of the resulting suspension, a 0.45 µm filter was used, while for sterilizing the resultant suspension, a 0.22 µm filter was utilized (13).

## **Physicochemical Characterization of LAEO-loaded nanoliposomes**

The components of the essential oil were determined by means of gas-mass chromatography (14). For this aim, nano-vesicles were assessed by multiple analytic methods, including size, zeta potential, encapsulation efficiency, the release kinetic profile, and surface morphology (15).

## **Gas-Mass Chromatography (GC-mass)**

The analysis of the prepared essential oil was performed by a GC apparatus (Agilent 7890B), equipped with a mass detector (Agilent 5977B MSD) and a capillary column (HP-5MS). The temperature of the injector was 50 °C, and the samples were initially heated at 60 °C for 3 minutes. Afterward, the temperature was gradually increased up to 250 °C at a rate of 8 °C/ minute. Following that, the temperature of the apparatus was set at 220 °C at a rate of 20 °C/minute. The specimens were maintained at 230 °C for 3 minutes.

# **Atomic Force Microscopy (AFM)**

The surface morphology of nanoliposomes was analyzed by means of the atomic force microscopy (AFM) (Nanowizard II; JPK instruments; Germany). For this aim, nanoliposomes were diluted with deionized water at a ratio of 1:1000 and then sonicated for 20 minutes in an ultrasonic agitator (E-Chrom Tech Co, Taiwan). Then, the obtained solution was deposited onto a mica sheet and observed by AFM (16).

## **Dynamic Light Scattering (DLS)**

Dynamic light scattering (DLS) (Brookhaven Instruments Ltd, Brookhaven, USA) was applied for the determination of the size of particles, polydispersity index (PDI), and zeta potential using a Brookhaven Zeta Potential Analyzer (Brookhaven Instrument Corp., Holtsville, NY).

## **Fourier-Transforms Infrared (FTIR)**

The pure forms of the essential oil, blank liposomes, and liposomes loaded with the essential oil were assessed by the Fourier-transforms infrared (FTIR) method to analyze the chemical interactions between nanoliposomes and the essential oil. Samples were mixed and pressed with KBr pellets to form tablet-like shapes and then FTIR spectra of samples were recorded at a wavelength range of 400–4000 cm<sup>-1</sup>.

## **Entrapment efficiency**

For the determination of the essential oil contents in liposomes, the unentrapped component was separated from liposomes containing the essential oil by the membrane dialysis method against PBS at 4 °C. Thus, liposomes were lysed with isopropanol to measure the concentration of the essential oil. Then, the entrapment efficiency was determined by a UV/VIS spectrophotometer (Beckman, DU 530, Switzerland) at a wavelength of 270 nm. Finally, the essential oil concentration entrapped in nanoliposomes was calculated using the following formula (17):

$$\text{EE\%} = \frac{C}{C_0} \times 100$$

where C is the amount of loaded drug in liposomes (mg/ml), while C<sub>0</sub> is the concentration of the total essential oil.

## ***In-vitro* Release Profile**

About 500 µL of the nanoliposomes loaded LAEO were transferred into a 12 kDa cut-off dialysis tube and incubated in tube containing phosphate-buffered saline (PBS), as a medium, for 72 h at 37 °C and 42 °C (for cancer cells), and the pH values of 7.4 and 5.4 (tumor microenvironment) under gentle vibration. Then, the drug release rate was calculated by recording the optical absorbance of samples at a wavelength of 270 nm using a UV/VIS spectrophotometer (Beckman, DU 530, Switzerland) (16).

## **Cell toxicity assay**

MCF-7 and SK-BR-3 cells were procured from the Pasteur Institute of Iran (Tehran, Iran) and then cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco). Next, the two cell lines were incubated at 37 °C in a 5% CO<sub>2</sub> incubator under a humidified air atmosphere.

The cytotoxicity of *Lavandula angustifolia* essential oil and PEGylated nanoliposomal *Lavandula angustifolia* essential oil were investigated against MCF-7 and SK-BR-3 cell lines by MTT assay in a 96-well plate reader. MCF-7 cells were cultured in 10% DMEM (containing 10% FBS and 1% penicillin/streptomycin) at 37 °C in 5% CO<sub>2</sub> under saturated humidity. The SK-BR-3 cells were cultured in the same condition, except using RPMI-1640 instead of DMEM. The medium was replaced every two or three days.

Samples were sterilized by means of UV radiation and then completely dispersed in cell culture media by ultrasonic waves to determine the antitumor activity of specimens.

Following 24, 48, and 72 hours of incubation, the resulting supernatants were collected, and 20 µl of the MTT solution was added to each well. Cells were incubated at 37 °C for an additional four hours. Afterward, the supernatants were replaced by dimethyl sulfoxide (DMSO) by adding 150 µl to each well. Then, the wells were agitated on a shaker at 130 rpm for 15 minutes to dissolve the formed formazan crystals. Finally, the optical absorbance of a 96-well plate was read at 570 nm using a UV-vis spectrophotometer. The percentages of cell viability and cytotoxicity were calculated in accordance with the below formula (18).

$$\text{Cytotoxicity} = [(\text{Control cells} - \text{Treated cells})/\text{Control}] \times 100$$

$$\text{Cell viability} = (\text{Treated cells}/\text{Control cells}) \times 100$$

## RNA extraction, cDNA production, and real-time PCR

Total RNA was isolated from the whole blood samples using the Total RNA Extraction Kit (Parstous biotechnology, Iran). RNA concentration was measured by a NanoDrop™ Spectrophotometer (NanoDrop™ 2000/c Spectrophotometers, UK) and then reverse-transcribed using First-Strand cDNA Synthesis Kit (Amplicon, USA), according to the manufacturer's instructions. Real-time PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems, USA) using qPCR Master Mix Green-High Rox (Amplicon, South Korea). PCR reactions were conducted at a final volume of 20 µl containing 4 µl of cDNA, 10 µl of Master Mix (2×), 2 µL of related primers (0.25 µM), and 4 µl of sterile distilled water. Data obtained from the expression levels of *caspase3* and *HER-2* genes were normalized against the expression of the *GAPDH* gene as a housekeeping gene. The primer sets were designed by Beacon Designer 4.2 software based on the sequences available in the NCBI database. The sequences and characteristics of primers are summarized in Table 1. The PCR program was set at the following conditions: initial denaturation stage at 95 °C for 15 minutes, followed by 40 cycles with denaturation at 95 °C for 15 seconds, annealing at 59.5 °C for 15 seconds, and extension at 72 °C for 20 seconds.

Relative changes in gene expression were calculated according to the  $2^{-\Delta\Delta Ct}$  method. Figure 1 shows a schematic diagram of the experimental procedures.

## Statistical Analysis

The analysis of the obtained data was carried out by SPSS software. For the comparison between the variables in response to different treatments T-student test, one-way ANOVA, and Pearson correlation coefficient were performed where appropriate. Variables were examined at least three times, and the experiments were conducted twice.

## Results

### Physicochemical characterization of LAEO

The analysis of gas-mass chromatography of LAEO showed that the essential oil contained 14 different chemical compounds, and the most significant of which were three components: cineol, 2-brennanone (de-camphor) and endo-breneol. The percentages of each compound are shown in Table 2 and Fig. 2.

The analysis of vesicles performed by DLS showed that the mean size of nanoliposomes loaded with LAEO was  $68.3 \pm 7.28$  nm, and it was almost equal to the size of blank nanoliposomes (Fig. 2). Zeta-potential values of particles exhibit the surface electrical charge. Blank and LAEO-loaded nanoliposomes showed zeta potential values in a range of -2 to -4.5 mV, indicating the neutral surface charge (Fig. 2). The values of the mean electrophoretic mobility and dispersion medium viscosity were –  $0.000015 \text{ cm}^2/\text{Vs}$  and  $0.891 \text{ mPa.s}$ , respectively.

The standard curve of the lavender essential oil was plotted against 2-isopropanol solvent and PBS buffer, and the equations of these curves were calculated. Then, the standard load curve of the liposomal essential oil was estimated. The entrapment efficiency of LAEO was nearly  $50 \pm 3.34\%$ . LAEO-loaded nanoliposomes were incubated in PBS buffer, under two pH values and temperatures, as follows, pH = 7.4 at 37 °C and pH = 5.4 at 42 °C. The *in vitro* release behavior of nanoliposomes was evaluated for 72 hours, and the results were presented as a cumulative release percentage in Fig. 3. The cumulative release profiles under both conditions were 63.98% and 87.63%, respectively. These profiles imply desirable conditions of nanoliposomes in terms of stability and storage of loaded components. It would be crucial for liposomes to maintain the loaded components inside their cargo before reaching their target sites.

Images obtained from AFM demonstrated that LAEO-loaded nanoliposomes exhibited spherical shape and homogenous structure with a smooth surface (Fig. 2).

The analysis of the FTIR spectra of the essential oil demonstrated distinct peaks at 3660.41 cm<sup>-1</sup>, 2955.84 cm<sup>-1</sup>, 1743.1 cm<sup>-1</sup>, 1463.04 cm<sup>-1</sup>, and 1374.89 cm<sup>-1</sup> assigning to –OH, stretching vibration of –

CH, aliphatic ester (stretching vibration of C = O), bending vibration of –CH<sub>2</sub>, and –CH<sub>2</sub>CH<sub>3</sub> functional groups. Stretching vibration of the functional groups P = O and P-O-C showed peaks at frequencies of 1214.88 cm<sup>-1</sup> and 982.51 cm<sup>-1</sup>. The comparison of the FTIR spectra between LAEO-loaded nanoliposomes and blank nanoliposomes indicated that some distinct peaks at 3372.41 cm<sup>-1</sup>, 2295.87 cm<sup>-1</sup>, 1641.68 cm<sup>-1</sup>, 1058.15 cm<sup>-1</sup>, corresponding to –OH, -PH, C = O, and C-O functional groups shifted to the peaks at frequencies of 3328.19 cm<sup>-1</sup>, 2297.72 cm<sup>-1</sup>, 635.87 cm<sup>-1</sup>, and 292.38 cm<sup>-1</sup> in nanoliposomes loaded with LAEO. Such shifts denote the encapsulation process of the essential oil into nanoliposomes. Considering no additional peaks emerged or disappeared, it would be conceivable that no chemical interactions exist between the essential oil and nanocarrier system, and both maintained their former nature and did not undergo chemical modifications (19) (Fig. 4).

## Cell toxicity assay

The antitumor effect of *Lavandula angustifolia* essential oil and nanoliposomes loaded with LAEO on MCF-7 and SK-BR-3 cells were assessed by MTT assay at 24, 48, and 72 hours. These cell lines were treated with multiple concentrations of the essential oil at a dose range of 5 to 1000 µg/ml. The results showed a negative correlation between cell growth and LAEO concentrations. The IC<sub>50</sub> values for the essential oil for MCF-7 cells were estimated to be 425 µg/ml, 355 µg/ml, and 305 µg/ml for 24 h, 48 h, and 72 h, respectively. Furthermore, as shown in Fig. 5, the essential oil at concentrations of 550 µg/ml, 450 µg/ml, and 400 µg/ml induced cell death in 50% of SK-BR-3 cells after 24 h, 48 h, and 72 h, respectively. The comparison of the obtained IC<sub>50</sub> values suggested that SK-BR-3 cells were relatively more resistant to the essential oil than MCF-7 cells. The induction of cell death in MCF-7 and SK-BR-3 cell lines by LAEO occurred in a time and dose-dependent manner.

The survival rate of MCF-7 and SK-BR-3 cells in response to treatment with different concentrations of liposomes in the absence of the essential oil is more than 90 percent, implying that liposomes had no toxicity against the cells. The results demonstrated that the inhibition of the cell growth process in response to nanoliposomes loaded with LAEO was more pronounced compared with the free form of the essential oil (*p*-value < 0.01). On the other hand, the toxicity of the free form of the essential oil and encapsulated essential oil showed that the induction of cell death is time-dependent for both forms, and the inhibition of cell proliferations has increased markedly from 24 h to 72 hours (Fig. 6).

## Gene expression assay

As depicted in Fig. 7, the impact of LAEO on the expression changes of *CASP3* and *HER-2* genes was determined on MCF-7 and SK-BR-3 cell lines treated with both the free form of the essential oil and encapsulated essential oil after 24 h, 48 h and 72 h. The analysis of real-time qPCR indicated that LAEO significantly induced the expression of *caspase-3*, while it suppressed the expression of *HER-2* in both cell lines (*P* < 0.05).

## Discussion

Produced nanoliposomes containing LAEO were shown to have acceptable stability and physicochemical characteristics. As mentioned earlier, these types of nanoliposomes are classified as non-ionic surfactant vesicles that may form sphere-shape strictures when emulsified in aqueous media and act as a carrier for hydrophilic and hydrophobic compounds. The production of liposomes for the encapsulation of essential oils (EOs) is a new method to maintain their physicochemical properties and high antioxidant activity.

In the last few years, there has been a growing interest in providing natural antioxidants. Numerous studies indicated that the spices derived from the *Lamiaceae* family, along with their extracts and essential oils, have considerable antioxidant activity (20). According to the results of DLS, the majority of vesicles were separately dispersed. Neutral charge on liposome has also been shown to reduce their bio-distribution between tumor interstitium and microvasculature without causing a disturbance in total tumor uptake. The in-vitro release behavior of the essential oil could be fine-tuned by changing the solubility, pH, and zeta-potential of the formulation process. Some studies demonstrated that phosphatidylcholine (PC) is capable of interacting with the negatively charged (acidic) compounds when incorporated into films, leading to the adverse effects on the drug release profile when used in the tumor microenvironment. On the other hand, incorporation of polyethylene glycol (PEG) into the surface of liposomes has been demonstrated to be useful in increasing the stability of formulations, controlled release rate, prolonging the half-life of the drug in the bloodstream, and preventing the uptake of nanocarriers by the reticuloendothelial system (21, 22).

Bioactive nutrients, such as LAEO, show promising results in the induction of cell death in cancer cells. In the current study, the treatment of MCF-7 and SK-BR-3 cells with LAEO showed that the herb extracts significantly decreased the cell viability in a time and dose-dependent manner when compared with untreated cells. Nanoliposomes were shown to be safe for the transportation of LAEO into cancer cells by which the essential oil induces cell death, and liposomes facilitate the delivery of that to cancer cells.

Our findings demonstrated that the expression of *caspase-3* was elevated in response to the treatment of cells with PEGylated LAEO-loaded nanoliposomes. It is now known that caspase-3 plays a critical role in the initiation and termination of apoptosis, and any strategies to overexpress this gene/protein could pave the way for the elimination of cancer cells.

Numerous studies have shown that the *HER-2* gene is upregulated in cancer cells, and the downregulation of this gene/protein could be a good candidate for the treatment of cancer. We indicated that the application of LAEO-loaded nanoliposomes reduced the expression of this gene in both cell lines. In cells that are subject to undergo apoptosis, caspase-3 is activated via two major cell death pathways, namely extrinsic and intrinsic pathways.

The zymogenic characteristics of caspase-3 would be requisite since the dysregulated expression of caspase-3 can indiscriminately induce cell death even in normal cells. Caspase-3 is normally found in the cells as a proenzyme that has no activity unless receiving death signals from the inside or outside of the cells. Upon the activation of *caspase-3*, the proenzyme is cleaved by initiator caspase enzymes (23). On the other side, the activation of HER-2 can trigger the cell signaling of the phosphatidylinositol kinase

3/Akt (PI3K/AKT) pathway, which is one of the main downstream signaling pathways of HER2. Also, Akt signaling has been demonstrated to lower the activity of caspase-9, which can form a complex protein called apoptosome. The formed apoptosome is the downstream effector of caspase-3. Various investigations indicated the impairment of the intrinsic apoptotic pathways in a variety of cancer cells (24).

Previous studies showed that LAEO could be considered a potential chemotherapeutic agent for the induction of apoptosis in cancer cells.

In one study, it was shown that *Lavandula angustifolia* extracts (ethanol, ether de petrol, water) and its essential oil were able to induce apoptosis in Hela cells and human blood lymphocytes. They showed that the growth of malignant cells was suppressed in response to the essential oil and the herb extracts in a dose- and time-dependent manner. The apoptosis of the Hela cell was further confirmed by the accumulation of DNA at the sub-G1 phase of the cell cycle. Also, the expression level of Bax, as well as the cleavage of PARP, was induced by the treatment of Hela cells with herb extracts when compared with control cells (25).

Zhao *et al.* showed that lavender essential oil is capable of oppressing the proliferation of human prostate cancer cells that were xenografted into nude mice. They indicated that linalool, but not linalyl acetate, is responsible for the antitumor effects (26).

In another study conducted by Cerchiara *et al.*, they revealed the anticancer potential of two different solutions of linalool on *NCTC 2544* normal keratinocyte and *RPMI 7932* human melanoma cell lines. They demonstrated that linalool acts as a selective inhibitor on the proliferation of melanoma cells in a dose-dependent manner. The administration of linalool induced remarkable changes in the morphology of cancer cells, as analyzed by TEM and SEM methods. The expression of caspase-3 is increased in melanoma cells while it is almost absent in normal keratinocytes. These findings showed that linalool could be used as a therapy or a lead compound for developing potential therapeutic agents for the cure of melanoma (27).

## Conclusion

Our study demonstrated that *Lavandula angustifolia* essential oil could be a potential candidate for further investigations in the field of cancer therapy. Nowadays, drug delivery systems provide a platform for the development of anticancer agents, as they have higher solubility, stability, and bioavailability when applied in-vitro and *in vivo*. The inhibitory effects of LAEO-loaded nanoliposomes on the cell proliferation of cancer cells were more pronounced than the free form of the essential oil. The application of both forms of LAEO increased the expression of *caspase-3*, while suppressed the expression of *HER-2* in both MCF-7 and SK-BR-3 cell lines. Altogether, *in vivo* studies are warranted to identify the precise cytotoxicity mechanisms of the essential oil. Also, refining the formulation of the essential oil is seemingly needed to achieve maximum efficiency.

## **Declarations**

## **Acknowledgments**

Not applicable.

## **Competing interest**

The authors declare no competing interest.

## **Availability of data and materials**

All data generated or analyzed during this study are included in the manuscript.

## **Consent for publication**

Not applicable.

## **References**

1. Singh SK, Singh S, Lillard Jr JW, Singh R. Drug delivery approaches for breast cancer. International journal of nanomedicine. 2017;12:6205.
2. Duo J, Ying G-G, Wang G-W, Zhang L. Quercetin inhibits human breast cancer cell proliferation and induces apoptosis via Bcl-2 and Bax regulation. Molecular medicine reports. 2012;5(6):1453-6.
3. Enayatrad M, Amoori N, Salehiniya H. Epidemiology and trends in breast cancer mortality in Iran. Iranian journal of public health. 2015;44(3):430.
4. Yap TA, Carden CP, Kaye SB. Beyond chemotherapy: targeted therapies in ovarian cancer. Nature Reviews Cancer. 2009;9(3):167.
5. Deng X-H, Song H-Y, Zhou Y-F, Yuan G-Y, Zheng F-J. Effects of quercetin on the proliferation of breast cancer cells and expression of survivin in vitro. Experimental and therapeutic medicine. 2013;6(5):1155-8.
6. Tabatabaei SM, Kianinodeh F, Nasiri M, Tightiz N, Asadipour M, Gohari M. In Vitro Inhibition of MCF-7 Human Breast Cancer Cells by Essential Oils of Rosmarinus officinalis, Thymus vulgaris L., and Lavender x intermedia. Archives of Breast Cancer. 2018;81-9.
7. Devi VK, Jain N, Valli KS. Importance of novel drug delivery systems in herbal medicines. Pharmacognosy reviews. 2010;4(7):27.
8. Senapati S, Mahanta AK, Kumar S, Maiti P. Controlled drug delivery vehicles for cancer treatment and their performance. Signal transduction and targeted therapy. 2018;3(1):7.

9. Arabi MH, Chabok H, Mirzapour A, Shafiee M. Preparation of nanoliposomes containing Rosmarinus officinalis L essential oil: A comparative study. *Med Commun Biosci Biotechnol Res Comm.* 2017;10(1):103-8.
10. Abdulkareem IH. Aetio-pathogenesis of breast cancer. *Nigerian medical journal: journal of the Nigeria Medical Association.* 2013;54(6):371.
11. Dai X, Xiang L, Li T, Bai Z. Cancer hallmarks, biomarkers and breast cancer molecular subtypes. *Journal of Cancer.* 2016;7(10):1281.
12. Carlsson J, Nordgren H, Sjöström J, Wester K, Villman K, Bengtsson NO, et al. HER2 expression in breast cancer primary tumours and corresponding metastases. Original data and literature review. *British journal of cancer.* 2004;90(12):2344.
13. Begum K, Sarker A, Shimu IJ, Chowdhury MMI, ul Jalil R. Characterization of Nanoemulsion Prepared from Self-emulsifying Rifampicin and its Antibacterial Effect on *Staphylococcus aureus* and *Stap. epidermidis* Isolated from Acne. *Dhaka University Journal of Pharmaceutical Sciences.* 2015;14(2):171-7.
14. Haghirsadat F, Azhdari M, Kalantar SM, Naderinezhad S, Teymourizadeh K, Yazdani M, et al. Strategy of Improvements in the therapeutic index of medicinal herbs of Iranianin digenous: Synthesis and characterization of phospholipid lipid-based vesicles in corporated *Trachyspermum copticum*. *SSU\_Journals.* 2016;24(6):468-78.
15. Hasan M, Elkhoury K, Kahn CJ, Arab-Tehrany E, Linder M. Preparation, Characterization, and Release Kinetics of Chitosan-Coated Nanoliposomes Encapsulating Curcumin in Simulated Environments. *Molecules.* 2019;24(10):2023.
16. Hemati M, Haghirsadat F, Jafary F, Moosavizadeh S, Moradi A. Targeting cell cycle protein in gastric cancer with CDC20siRNA and anticancer drugs (doxorubicin and quercetin) co-loaded cationic PEGylated nanoniosomes. *International Journal of Nanomedicine.* 2019;14:6575.
17. Amoabediny G, Haghirsadat F, Naderinezhad S, Helder MN, Akhoudi Kharanaghi E, Mohammadnejad Arough J, et al. Overview of preparation methods of polymeric and lipid-based (níosome, solid lipid, liposome) nanoparticles: A comprehensive review. *International Journal of Polymeric Materials and Polymeric Biomaterials.* 2018;67(6):383-400.
18. Yang R, An Y, Miao F, Li M, Liu P, Tang Q. Preparation of folic acid-conjugated, doxorubicin-loaded, magnetic bovine serum albumin nanospheres and their antitumor effects in vitro and in vivo. *International journal of nanomedicine.* 2014;9:4231.
19. Mohammadpanah M, Mojodi E, Ehsani R. The Synthesis and Characterization of Liposomal Nano-Carriers Loading *Lavandula angustifolia* Essential Oil to Affect Breast Cancerous Cell-Lines. *Yafteh.* 2020;22(1).
20. Stagos D, Portesis N, Spanou C, Mossialos D, Aligiannis N, Chatita E, et al. Correlation of total polyphenolic content with antioxidant and antibacterial activity of 24 extracts from Greek domestic Lamiaceae species. *Food and Chemical Toxicology.* 2012;50(11):4115-24.

21. Haeri A, Alinaghian B, Daeihamed M, Dadashzadeh S. Preparation and characterization of stable nanoliposomal formulation of fluoxetine as a potential adjuvant therapy for drug-resistant tumors. Iranian journal of pharmaceutical research: IJPR. 2014;13(Suppl):3.
22. Chenthamara D, Subramaniam S, Ramakrishnan SG, Krishnaswamy S, Essa MM, Lin F-H, et al. Therapeutic efficacy of nanoparticles and routes of administration. Biomaterials Research. 2019;23(1):1-29.
23. Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, et al. Apoptosis and cancer: mutations within caspase genes. Journal of medical genetics. 2009;46(8):497-510.
24. Pu X, Storr SJ, Zhang Y, Rakha EA, Green AR, Ellis IO, et al. Caspase-3 and caspase-8 expression in breast cancer: caspase-3 is associated with survival. Apoptosis. 2017;22(3):357-68.
25. Amiri A, Tayarani-Najaran Z, Karimi G, Mousavi S. Evaluation of *Lavandula angustifolia* cytotoxic and apoptotic effects on human cervical cancer cell line (HeLa) in compare with normal cells. Research in Pharmaceutical Sciences. 2012;7(5):141.
26. Zhao Y, Chen R, Wang Y, Qing C, Wang W, Yang Y. In vitro and in vivo efficacy studies of *Lavender angustifolia* essential oil and its active constituents on the proliferation of human prostate cancer. Integrative cancer therapies. 2017;16(2):215-26.
27. Cerchiara T, Straface S, Brunelli E, Tripepi S, Gallucci MC, Chidichimo G. Antiproliferative effect of linalool on RPMI 7932 human melanoma cell line: ultrastructural studies. Natural product communications. 2015;10(4):1934578X1501000401.

## Tables

Table 1  
Primers set used for RT-PCR.

Gene Name	Gene ID	Cytogenetic Location	Primer Sequence
HER-2	NM_001005862.2	17q12	F (5'- CCTCTGACGTCCATCATCTC - 3') R (5'- ATCTTCTCGTGCCGTCGCTT - 3')
CASP3	NM_001354777.1	4q35.1	F (5'- AAGCGAATCAATGGACTCTGG - 3') R (5'- CTGTACCAGACCGAGATGTC - 3')
GAPDH	NM_001256799.3	12p13.31	F (5'- CCATGAGAAAGTATGACAAC - 3') R (5'- GAGTCCTTCCACGATACC - 3')

**Table 2**  
 Chemical compositions *Lavandula angustifolia* essential oil identified by gas chromatography-mass spectrometry.

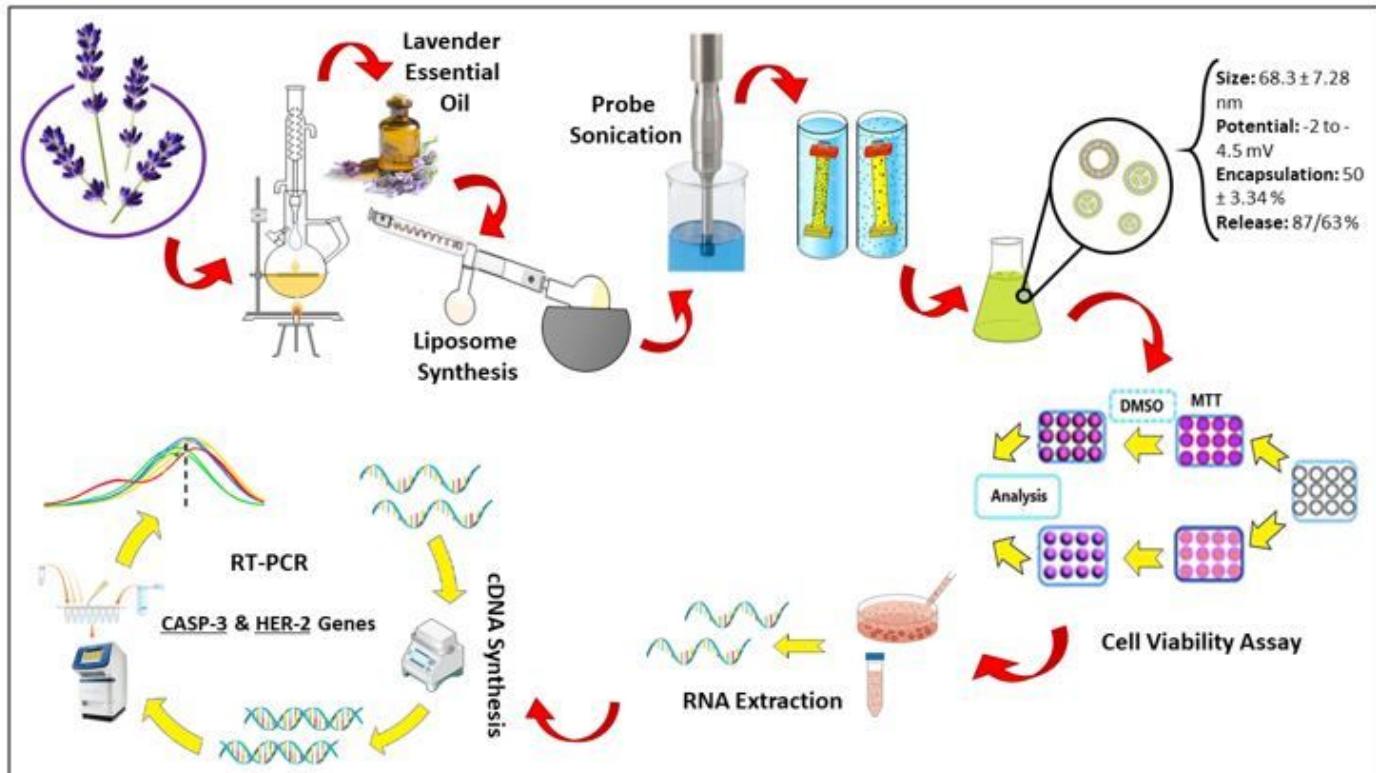
Compound	Composition (%)
α-Pinene	1.41
Camphene	0.65
β-Pinene	0.43
o-Cymene	1.47
Eucalyptol	30.5
(+)-2-Bornanone	30.74
endo-Borneol	29.2
Terpinen-4-ol	1.43
Cyclohexene, 1-methyl-4-(1-methylethyl)-	1.74
Bicyclo[3, 1, 1]hept-2-ene-carboxaldehyde,6,6-dimethyl-	0.44
2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis	0.25
Acetic acid, 1,7,7-trimethyl-bicyclo[2, 2, 1]hept-2-yl ester	0.15
Carvone	0.35
p-Cymen-7-ol	0.24

Table 3  
The IC<sub>50</sub> values of *Lavandula angustifolia* essential oil when applied in the forms of free essential oil and liposomal forms on MCF-7 and SK-BR-3 cells

Treatment type	IC <sub>50</sub> values (μg/ml)	
	MCF-7 cells	SK-BR-3 cells
Free essential oil (24 h)	425 ± 0.45	550 ± 0.32
Free essential oil (48 h)	355 ± 0.36	450 ± 0.41
Free essential oil (72 h)	305 ± 0.29	400 ± 0.39
Encapsulated essential oil (24 h)	240 ± 0.42	350 ± 0.44
Encapsulated essential oil (48 h)	170 ± 0.52	225 ± 0.13
Encapsulated essential oil (72 h)	100 ± 0.34	175 ± 0.12

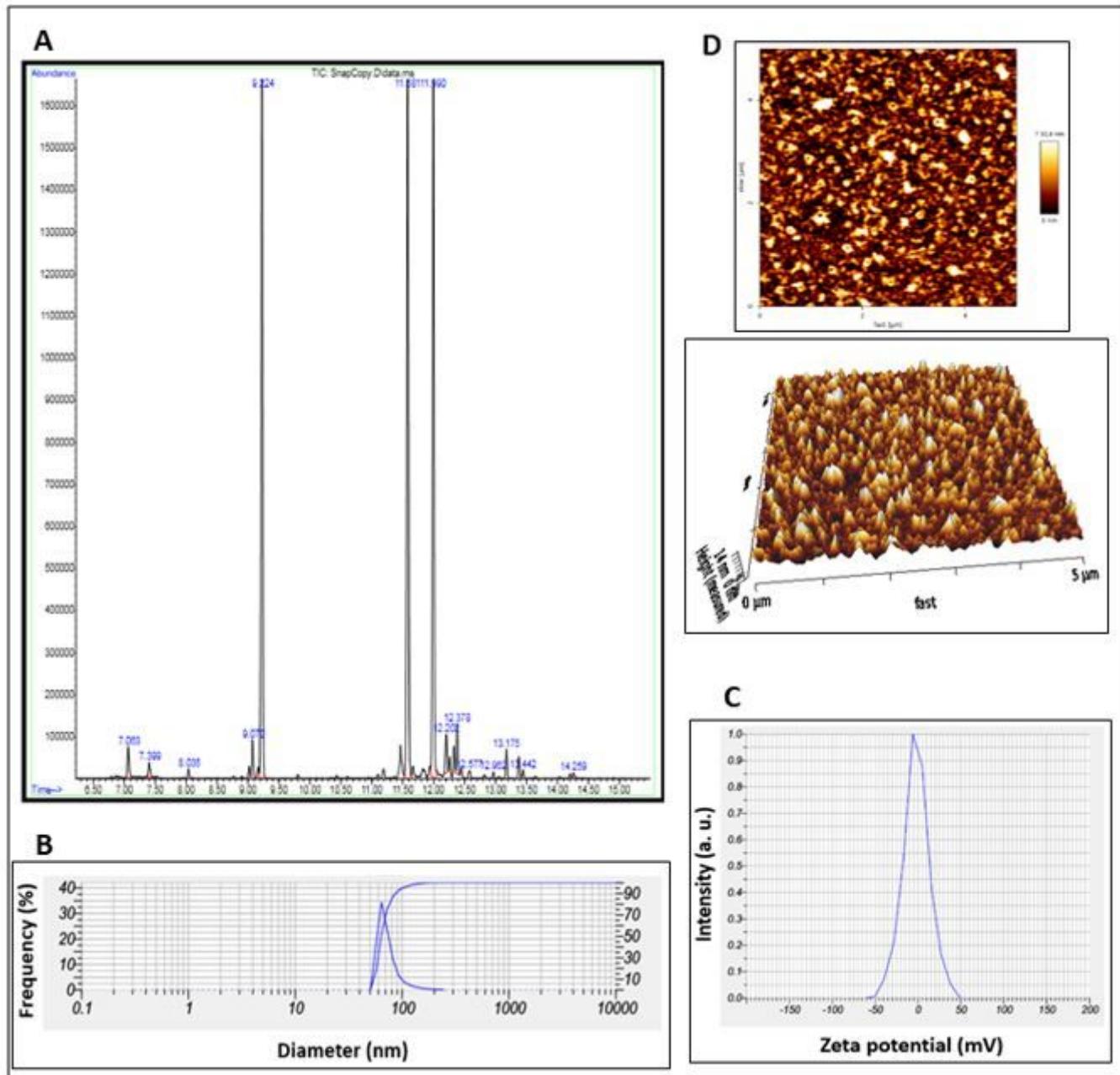
Data are expressed as the mean ± SD.

## Figures



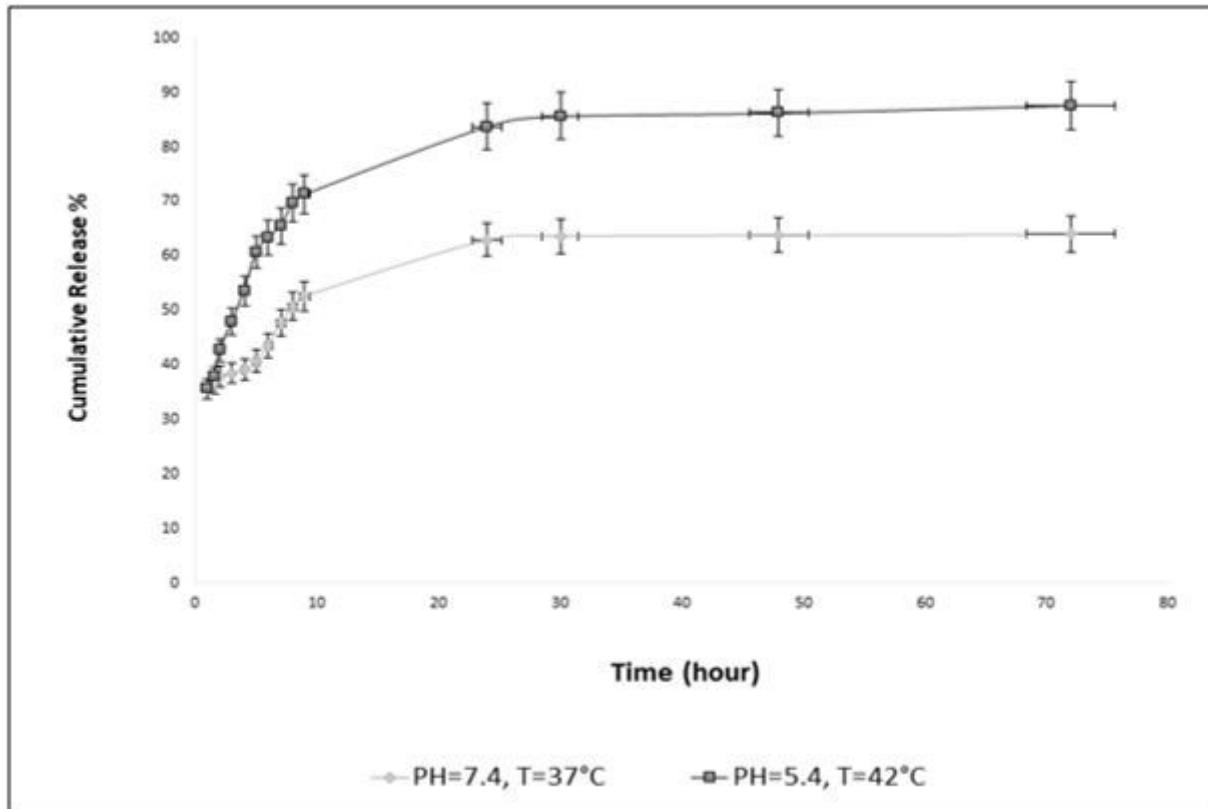
**Figure 1**

Graphical abstract.



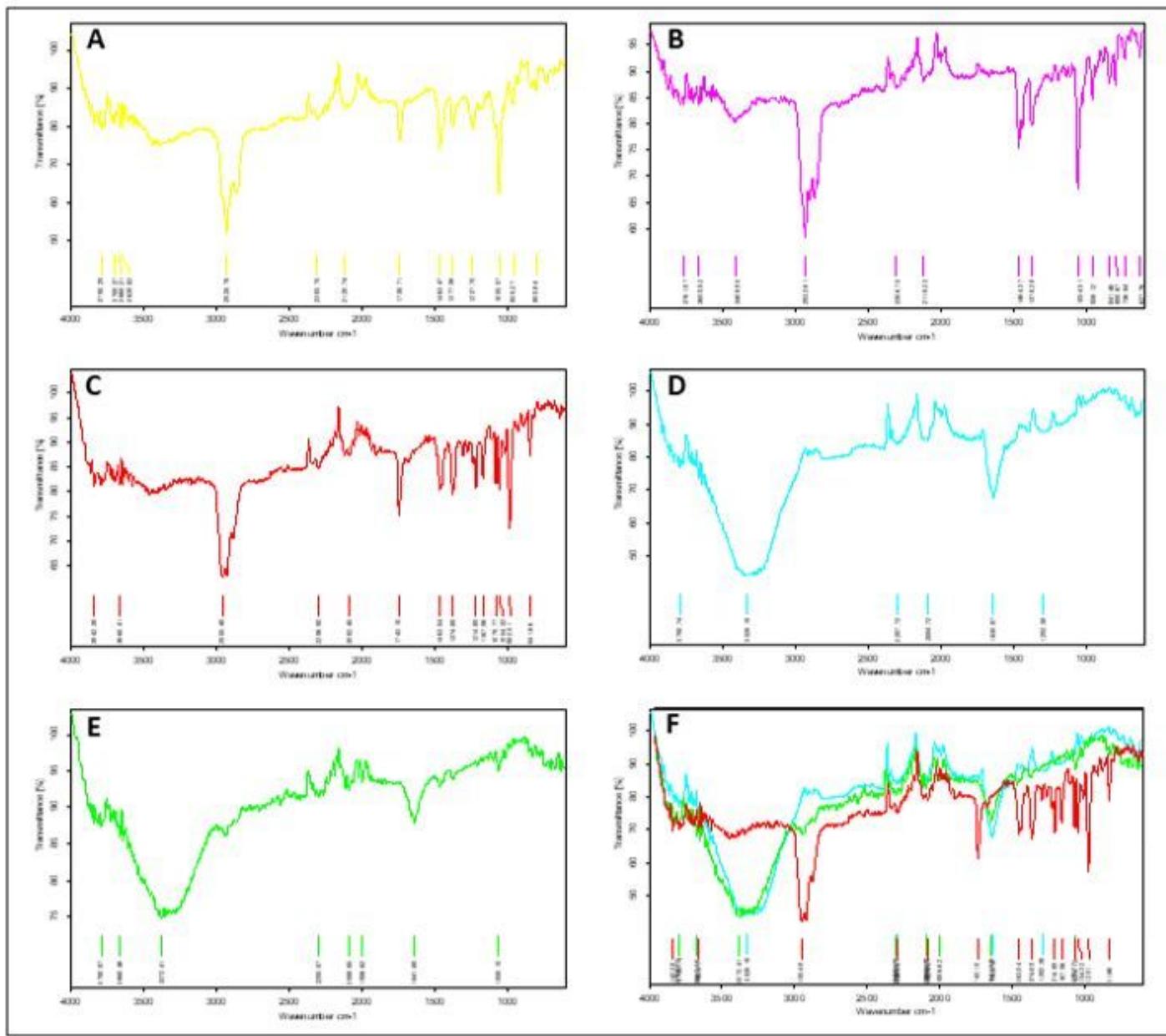
**Figure 2**

(A) High-performance GC-mass chromatogram of LAEO (B) Particle size distribution profile of LAEO-loaded PEGylated liposomes, (C) Zeta potential values of PEGylated liposomal LAEO, (D) Two-dimensional and three-dimensional AFM images of PEGylated liposomal LAEO



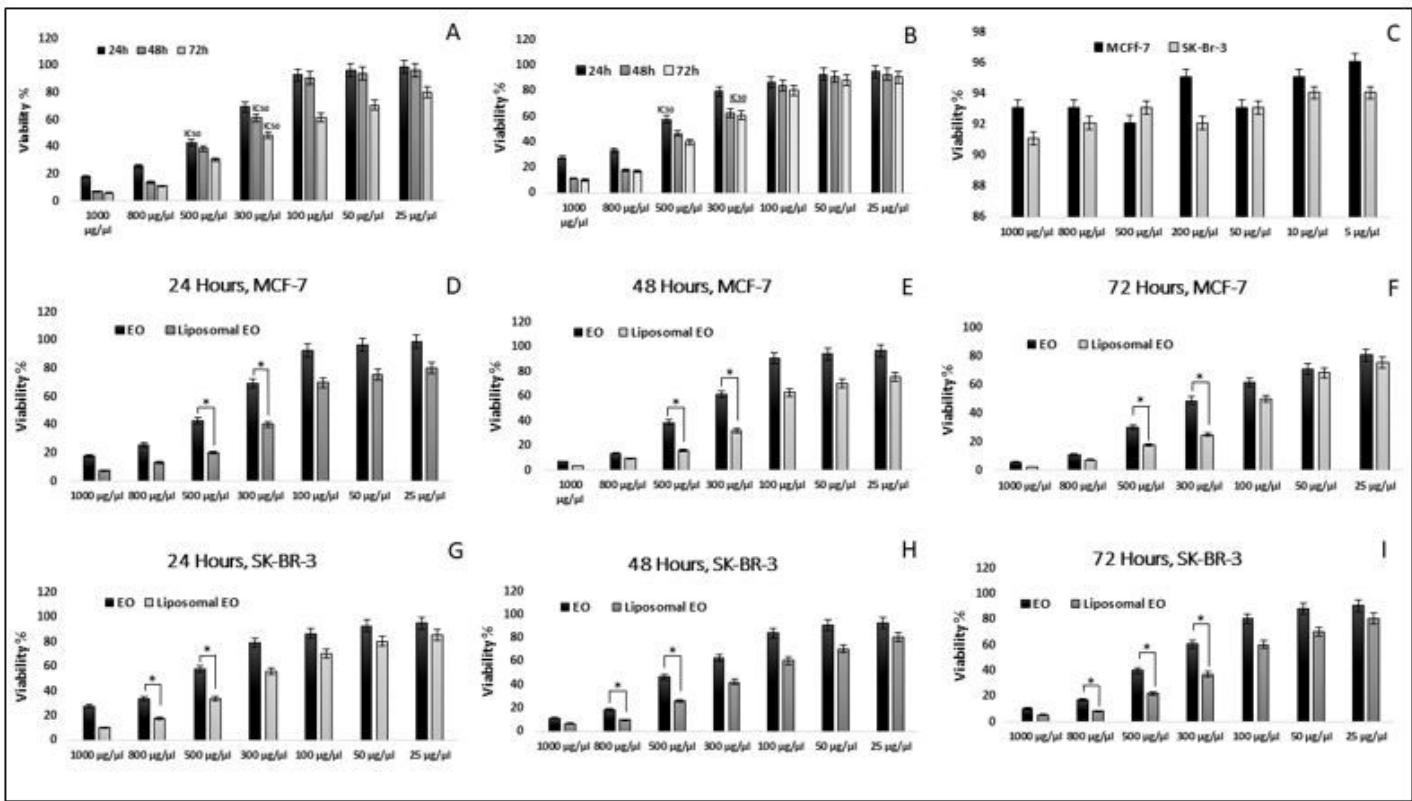
**Figure 3**

In-vitro cumulative release profile of nanoliposomes loaded with *Lavandula angustifolia* essential oil in PBS solution (pH = 7.4, T = 37 °C; pH = 5.4, T = 42 °C).



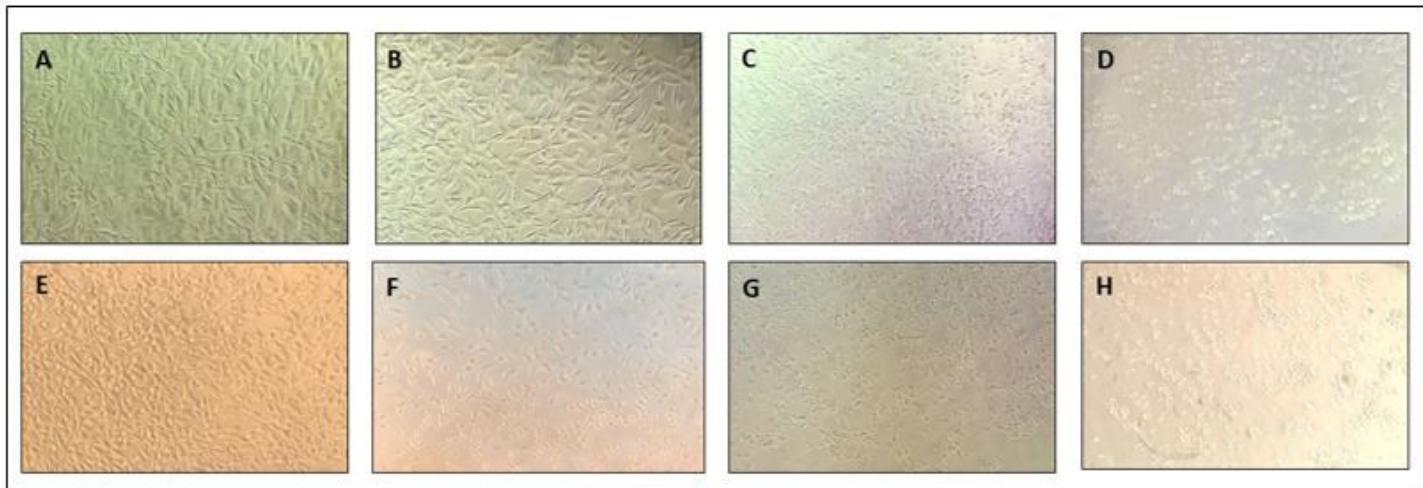
**Figure 4**

Infrared spectra of (A) SPC, (B) Cholesterol, (C) LAEO, and (D) blank nanoliposomes, (E) Nano-liposomes containing essential oil and (F) The comparison among blank liposomes and liposomal LAEO and free essential oil.



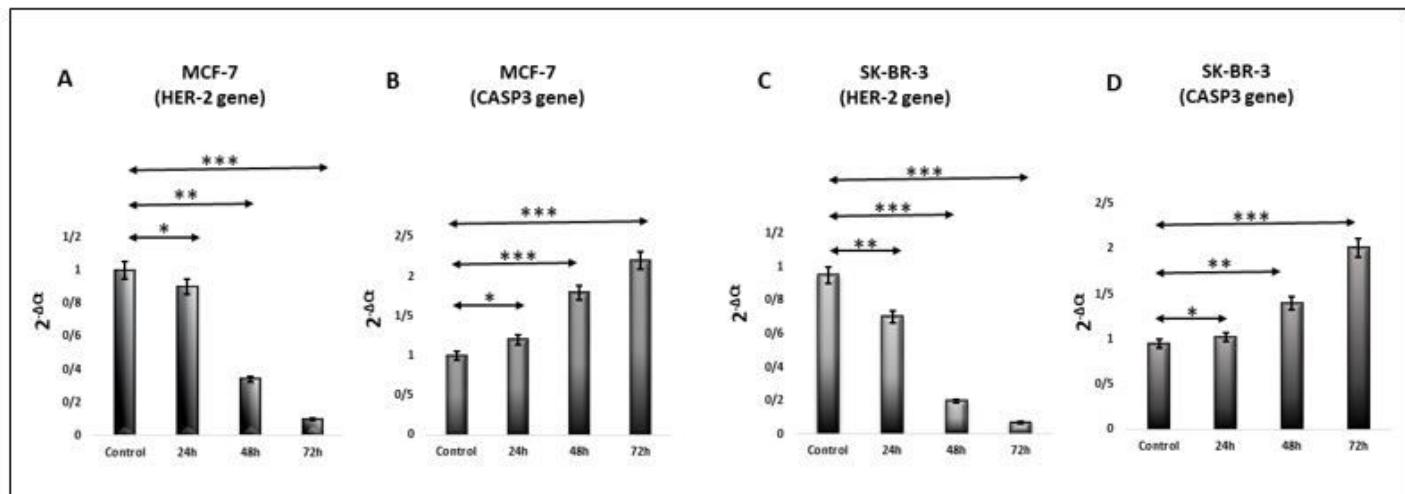
**Figure 5**

The cytotoxicity of LAEO on cancer cells. MCF-7 and SK-BR-3 cells were treated with different concentrations of LAEO: (A) MCF-7 (25–100  $\mu\text{g}/\text{ml}$ ) for 24h, 48h, and 72h, (B) SK-BR-3 (25–1000  $\mu\text{g}/\text{ml}$ ) for 24h, 48h, and 72h, (C) The effects of blank nanoliposomes on MCF-7 and SK-BR-3 cells (0–45  $\mu\text{g}/\text{ml}$ ) for 48h, (D) The impact of free essential oil and liposomal essential oil on MCF-7 for 24h, (E) The effect of free essential oil and liposomal essential oil on MCF-7 for 48h, (F) The impact of free essential oil and liposomal essential oil on MCF-7 for 72h, (G) The effect of free essential oil and liposomal essential oil against SK-BR-3 cells for 24h, (H) The impact of free essential oil and liposomal essential oil on SK-BR-3 cells for 48h, (I) The effect of free essential oil and liposomal essential oil on SK-BR-3 cells for 72h (\* $P < 0.05$ ).



**Figure 6**

Microscopic images of MCF-7 and SK-BR-3 cells treated with the IC<sub>50</sub> values of LAEO-loaded nanoliposomes: (A) MCF-7 control cells, (B) MCF-7 treated with LAEO-loaded nanoliposomes for 24h, (C) 48h, and (D) 72h; (E) SK-BR-3 control cells, (F) SK-BR-3 cells treated with LAEO-loaded nanoliposomes for 24h, (G) 48h, and (H) 72h. The treatment of both cell lines with liposomes loaded with LAEO showed a significant reduction in cell viability compared with untreated cells in a time- and dose-dependent manner (200× Magnification).



**Figure 7**

The treatment of cancer cell lines with LAEO-loaded nanoliposomes downregulated the expression of the HER-2 gene (A and C). It also upregulated the expression of caspase-3 in both cell lines (B and D). Bars represent the mean and standard error of four independent experiments. Control: untreated sample (\*P>0.05, \*\* P<0.05, \*\*\*P<0.01).