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Processing of liposome-encapsulated natural herbs derived from Silybum marianum plants for the treatment of breast cancer cells

A. Ramedani[a], O. Sabzevari[b,c,*], and A. Simchi[a,d,*]

a. *Institute for Nanoscience & Nanotechnology (INST), Sharif University of Technology, Azadi Avenue, 14588 Tehran, P.O. Box 11155-9466, Iran.*
b. *Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.*
c. *Toxicology and Poisoning Research Centre, Tehran University of Medical Sciences, Tehran, Iran.*
d. *Department of Materials Science and Engineering, Sharif University of Technology, Azadi Avenue, 14588 Tehran, P.O. Box 11155-9466, Iran.*

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Abstract. It has long been known that Silibinin, a nature-derived herbal phytochemical, is an effective drug for treating toxic thyroid damage; however, its role in cancer treatment is still not approved and is under investigation. In this work, a protocol to prepare Liposome-Encapsulated Silibinin (LES) is proposed with the potential to produce Reactive Oxygen Species (ROS) to treat MCF-7 breast cancer cells. Spherical-shaped LES nanoparticles with an average size of 60 nm and narrow particle size distribution (PDI = 0.11) were prepared through the hydration of thin films. Studies of the pharmacokinetics illustrated that a burst release occurred during the first 12 h, followed by a sustained release over the next 12 days. MTT assays and the analysis of the drug effect determined that LES nanoparticles displayed a significant cytotoxic effect in killing breast cancer cells. IC_{50} values for LES nanoparticles were experimentally determined to be 20 μM which was significantly lower than that of the pristine drug (38 μM). It was also found that LES could remarkably change the expression levels of Caspase-3, Caspase-9, Bax, Bcl-2, and hTERT genes compared to the pristine drug. Therefore, encapsulating Silibinin into liposomes is an effective strategy to enhance the effectiveness of breast cancer treatment dramatically.

1. Introduction

Cancer, a disease involving abnormal cell growth, is caused by a combination of risk factors that change genes [1]. Among various cancers, breast cancer is the most common type in women than any other variety in the world [2]. To treat breast cancer, phototherapy (PT), Immunotherapy (IT), radiotherapy (RT), stem cell transplant, chemotherapy (CT), hormone therapy (HT), and surgery are the mainstays [3]. The size and location of the tumor are essential factors to consider when choosing a treatment approach [4]. Regulatory organizations have approved various chemo drugs for breast cancer treatment; however, there are limitations associated with these drugs that restrict their success in the treatment of cancer [5]. The primary limitations include the insensitivity of breast cancer cells to the available chemotherapy and the development of drug

*. *Corresponding authors.*
E-mail addresses: omid@tums.ac.ir (O. Sabzevari); simchi@sharif.edu (A. Simchi)

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resistance during treatment [6,7]. The refraction mechanism is attributed to the limited drug absorption and inactivation inside the cells [8]. The oral administration of anticancer agents also has toxicity and adverse effects. Besides, risks of degradation by the acidic environment of pre-systemic metabolism and the lack of exclusivity with common carriers are high. In contrast to synthetic chemotherapeutic agents, herbal phytochemicals are safe and effective in the treatment of cancers [9].

Silibinin, known as flavonolignan, is extracted from the Silybum marianum plant and possesses a wide variety of activities, including antioxidant, hepatoprotective, and anti-inflammatory [10]. The anticancer activity of Silibinin is also under investigation [11]. Recent studies have shown that the expression level of telomerase is reduced in prostate and breast cancer cells [12]. The drug's potential to inhibit the proliferation of tumor cells by modulating specific pathways, including STAT, PI3K/Akt, NF-kB, and MAPK, has also been demonstrated [13]. The US Food and Drug Administration has considered Silibinin a safe drug without significant adverse effects [14,15]. However, oral administration of this medicine is not practical because of its low bioavailability associated with its low aqueous solubility, poor uptake into the epithelial cells, hepatic metabolism, and systemic elimination [16]. Therefore, intravenous administration is the only suitable option to improve clinical effectiveness.

Nanotechnology is a crucial solution to improving administrated drugs' pharmacokinetic and pharmacodynamic profiles. Various studies have revealed that drugs' pharmacological efficacy and bioavailability can be tailored by nano-formulation, encapsulation, or incorporation in the nanocarriers [17–20]. Besides, the solubility, tissue distribution, and body retention time can be adjusted. So far, several nanoplatforms, such as organic (e.g., liposomes and polymers) and inorganic carriers (e.g., carbon and gold nanostructures), have been examined [21]. For instance, Ebrahimnezhad et al. [22] prepared Silibinin-loaded PLGA-PEG-Fe_3O_4 nanoparticles and compared the effect of these nanoparticles on the viability of lung cancer cells and the level of hTER gene expression in these cell lines. The effect of Silibinin on the liver cancer cell cycle was investigated by Varghese et al. [23] through the fabrication of solid lipid nanoparticles. They illustrated a significant growth inhibition of both hepatocellular and carcinoma cell lines. Therefore, encapsulation of Silibinin in liposomes is promising to control pharmacokinetic profiles while achieving long-term stability with reduced side effects. However, it is crucial to formulate lipid nanoparticles to attain a controlled and prolonged release. Enhanced therapeutic efficacy and reduced frequency with which the drug must be administered are also imperative. This research aims to synthesize liposome-encapsulated Silibinin to treat breast cancer cells. We employed a facile chemical procedure to prepare solid lipid nanoparticles and studied the kinetics of drug release. The cytotoxicity of the nanoparticles on breast cancer cells was evaluated. The results of this research might be used to develop liposome-based nanocarriers for controlled delivery of herbal medicines for cancer research.

2. Materials and methods

2.1. Materials and cell lines

The following chemicals were purchased from Sigma-Aldrich (USA): Silibinin, dimethyl sulfoxide (DMSO, 99%), (3, 4, 5-dimethyl thiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT, 99%), and chloroform. The products of Avanti Polar Lipids (USA), including 1, 2- distearoyl-sn-glycero-3-phosphocholine (DSPC), 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid (DOTA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), and cholesterol, were utilized. 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-poly (ethylene glycol) (DSPE-PEG) was purchased from NOF Co. (Japan). The Pasteur Institute of Iran provided MCF-7 breast cancer cells. The SYBR Green PCR Master Mix was obtained from Roche company (Germany). RPMI-1640, streptomycin, penicillin, and Fetal Bovine Serum (FBS) (10270) were provided by Gibco (Life Technologies, Carlsbad, CA, USA).

2.2. Preparation of liposome-encapsulated Silibinin nanoparticles

Hydration of thin lipid films was utilized for the synthesis of LES nanoparticles according to the procedure explained in [24]. DSPC, cholesterol, DSPE-DOTA, and DSPE-PEG (molar ratio: 49:44:4:3) were mixed in chloroform/methanol (4:1 v/v) and homogenized. Afterward, the organic solvent was removed in a rotovap (rotary evaporator) at 40°C and 50 rpm. The flask was then purged with nitrogen, and the lipid film was saturated by a citrate buffered solution (10 mL; pH = 4) in a bath sonicator for 10 min. In the following, a bicarbonate buffer (pH = 0.5) was dropwise added to the mixture to attain a pH of 7. The addition of Silibinin at a ratio of 1:10 (relative to the lipid) was carried out at 25°C and incubated for 1 day. A high-pressure homogenizer was employed for the lipid extrusion and repeated 10 times. Finally, the product was dialyzed against DI water for 24 h and lyophilized at 0.1 mbar/–40°C.

2.3. Materials characterizations

The hydrodynamic size distribution and surface charge (ζ-potential, mv) of LES nanoparticles were measured by a Malvern nano-ZS instrument (UK). Sample preparation includes dissolving the liposomes in

DI water (0.5 mg/mL) and sonication for 10 min at 27°C. A scanning electron microscopy (FE-SEM, TESCAN-Mira3) was used to investigate the morphology and shape of the LES nanoparticles. Using an ABB/Bomem MB-100 spectrometer, FTIR spectra of Silibinin and LES nanoparticles on KBr were recorded in the range of 4000 to 400 cm^{-1}.

2.4. In vitro drug release study

A UV spectrophotometer (Perkin Elmer - Lambda 35 UV/Vis Spectrophotometer) was applied to determine the amount of drug loaded at the absorbance maximum of Silibinin (288 nm). The Encapsulation Efficiency (EE) and Drug Loading (DL) were calculated using the following equations:

$$EE = \frac{\text{weight of silibinin in liposomes}}{\text{weight of the initial silibinin}} \times 100, \quad (1)$$

$$DL = \frac{\text{weight of silibinin in liposomes}}{\text{weight of liposomes}} \times 100. \quad (2)$$

The dialysis technique was utilized so that researchers could investigate the release of silibinin from LES nanoparticles. LES nanoparticles (25 mg) were dispersed in 5 mL phosphate buffer solution (PBS; pH = 7.4), and the mixture was stirred at 100 rpm and incubated at 37°C. Sampling was carried out at different time intervals. After sampling, an equal volume of freshly prepared PBS was added to the buffer solution. The amount of released drug was analyzed by UV spectrophotometry at 288 nm according to the constructed calibration curve.

2.5. In vitro assays

2.5.1. Cell viability

The MTT assay was utilized to evaluate the cytotoxic potential of Silibinin and LES nanoparticles. MCF-7 breast cancer cell lines (2×10^4 cells) were cultivated in a 96-well plate. The plate was incubator at 37°C for 24 h under a humidified atmosphere containing 5% carbon dioxide. In the following, the cells were subjected to different concentrations of Silibinin and LES nanoparticles, incubated for 72 h, and finally subjected to 200 μL PBS containing 0.5 mg/mL MTT. The plates were then covered with aluminum foil and incubated at 37°C for 4 h. The produced formazan crystals were extracted by adding 200 μL DMSO and 25 μL Sorensen's glycine buffer, followed by incubating for 20 min. An ELISA-microplate reader was used to determine cell viability at a wavelength of 570 nm. There were three separate runs of each experiment.

2.5.2. ROS measurements

MCF-7 cells with a density of 4×10^5 cells per well were incubated in 6-well plates containing varying concentrations of LES. As a control group, incubated cells in a medium containing no serum (RPMI-1640) were prepared. After 4 h, the cells were washed 3 times with the buffer solution and then, incubated with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) at a concentration of 10 M for 20 min at 37°C in the dark. MCF-7 cells were collected after washing with PBS by centrifugation (1000 rpm for 5 min). The samples were then dispersed in 0.5 mL PBS for the flow cytometer. The excitation wavelength was 488 nm, and the emission wavelength was 525 nm.

2.5.3. Real-time RT-PCR

The cells were exposed to drug-free and drug-loaded nanoparticles for 72 h. The total cellular RNA was extracted using a Trizol reagent based on the manufacturer's protocol. A Nanodrop spectrophotometer (ND-1000) was employed to determine the concentration and purity of the extracted RNA by measuring the OD260/OD280 ratio. The integrity of the isolated RNA was also assessed by agarose gel electrophoresis at a concentration of 1.5% agarose. A first-strand cDNA synthesis kit was utilized as well. Besides, the quantitative polymerase chain reaction (qPCR) method with the qPCR mix containing EvaGreen dye and HotStart Taq DNA Polymerase was used to analyze the levels of expression of the hTERT gene. The parameters were: 10 min holding step at 95°C; 15 s denaturation step at 95°C (1 cycle); 30 s annealing step at 60°C (40 cycles); 30 s extension step at 72°C (40 cycles); and the melting step at 65-95°C. The relative amount of hTERT expression was normalized per the housekeeping gene and calculated by the 2-$\Delta\Delta$CT formula [25,26].

2.6. Statistical analysis

The OriginPro 2018 software carried out the analysis of the data. The average values were reported with the standard deviation (±SD). The statistically significant P-value was considered ≤ 0.05. All experiments were replicated 3 times.

2.7. Codes of ethics

Tehran University of Medical Sciences issued a license for all biological experiments and these studies were carried out in compliance with the regulations.

3. Results and discussion

3.1. Characterization of Silibinin-loaded nanoliposomes

In this study, a hydrophobic herb derived from Silybum marianum plants was loaded into liposome nanoparticles by the thin-film hydration method, a common and well-characterized technique for synthesizing liposome-encapsulated drugs [27,28]. However, the synthesis protocol should be controlled carefully to attain sufficiently small particles to avoid detection and destruction by the immune system [29–32]. We measured the

Table 1. Size and surface charge characteristics of liposomes and LES nanoparticles.

Nanoparticles	Particle size (nm)	PDI	Zeta potential (mV)
Liposome	55 ± 8	0.16	-7.3 ± 0.5
LES	60 ± 5	0.11	-4.2 ± 0.4

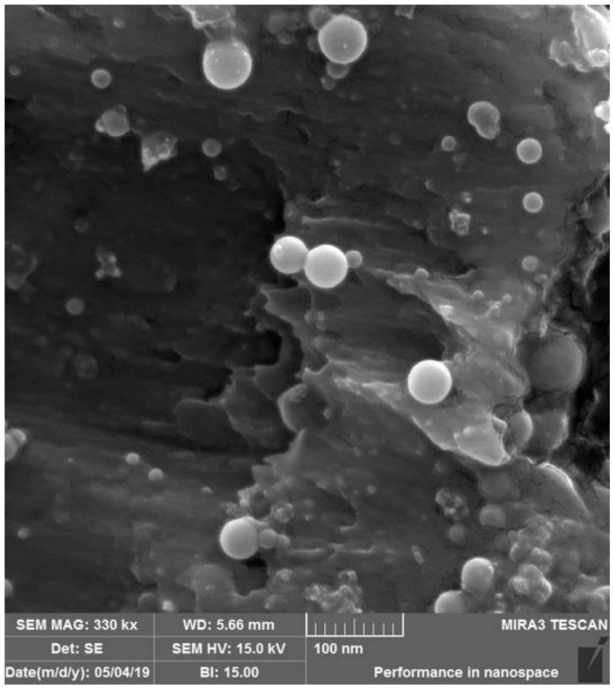

Figure 1. FE-SEM micrograph of LES showing spherical nanoparticles with an average diameter of about 50 nm.

hydrodynamic size and size distribution of nanoparticles using a zeta potential analyzer equipped with the Dynamic Light Scattering (DLS) method. Table 1 determines that the average particle size of liposomes is 55 ± 7 nm with a polydispersity index (PDI) of 0.16. The zeta potential is -7.3 ± 0.5 mV. After drug loading, the average particle size slightly increases to 60± nm with lower polydispersity. The surface charge was also decreased to -4.2 ± 0.4 mV. It should be noted that the surface charge of nanoparticles plays a fundamental role in determining their short- and long-term biostability and their interaction with cells [33]. The value of zeta potential is negative because deprotonation of free hydroxyl groups of PEG results in a negatively charged polymer chain [34].

FE-SEM studies determine that LES nanoparticles have spherical morphology with a mean diameter of about 50 nm (Figure 1). The slight discrepancy between the results of DLS and FE-SEM is attributed to the effect of surface charge and the assay condition, i.e., dehydrated for SEM and hydrated for DLS [35]. We believe that the size of LES nanoparticles is small enough to penetrate tumor cells and exert anti-cancer actions. It has been known that nanoparticles with diameters less than 400 nm enable cell uptake [36]. On the other hand, nanoparticles of sufficiently large size are required to ensure efficient endocytosis into cancer cells and to arrest macrophages avidly. Therefore, LES nanoparticles appear to be a promising candidate for treating cancer cells [35].

Figure 2(a) shows the FTIR spectrum of pristine Silibinin and LES nanoparticles. The bonds located at 3362 cm^{-1} (–OH =C–H), 2962 cm^{-1} (O–H), 1510 cm^{-1}, 1614 cm^{-1}, 1633 cm^{-1} (aromatic C=C rings and ketone C=O rings), 1269 cm^{-1} (C–O–C), 1126 cm^{-1} (C=C), 1161 cm^{-1}, 1182 cm^{-1} (aldehyde C=O), 1029 cm^{-1}, 1510 cm^{-1} (aromatic alkene), and 995 cm^{-1} (O–H) [37] were characterized. The characteristic peaks of Silibinin and LES are similar, although the peak intensity of Silibinin in LES is reduced because of lower concentration. We have experimentally found that the encapsulation efficiency is about 75%; thus, the loading capacity is $\sim 12\%$. We have also uncovered that the structure of the encapsulated drug has not been altered and that a new complex between the components of the formulation has not been formed. Figure 2(b) shows the UV-Vis spectrum of LES compared to liposome and Silibinin. The absorption peak of Silibinin is observed at 288 nm, which is associated with the $\pi - \pi^*$ electron transition in aromatic groups [38,39]. LES nanoparticles also exhibit the absorption edge of the drug but with lower intensity due to lower concentration.

3.2. In vitro release profiles

The dialysis membrane method was utilized to determine the release profile of the drug from the LES nanoparticles in PBS at pH = 7.4. Figure 3 shows the results. An initial burst release is observed at the initial stage that lasts for about 12 h. About 35% of the drug is released in this stage. The fast initial release is attributed to the adsorbed drug molecules and weakly bound to the surface of the nanoparticles [40–42]. After the burst release, the rate decades, and about 65% of the loaded Silibinin is released within the first 4 days. Afterward, a steady release is observed for up to 12 days. The release pattern of LES nanoparticles is similar to other liposomes reported in the literature [43,44].

3.3. Toxicity potential toward cancer cells

The MTT assay was used to assess the cytotoxic potential of Silibinin and LES nanoparticles. MCF-7

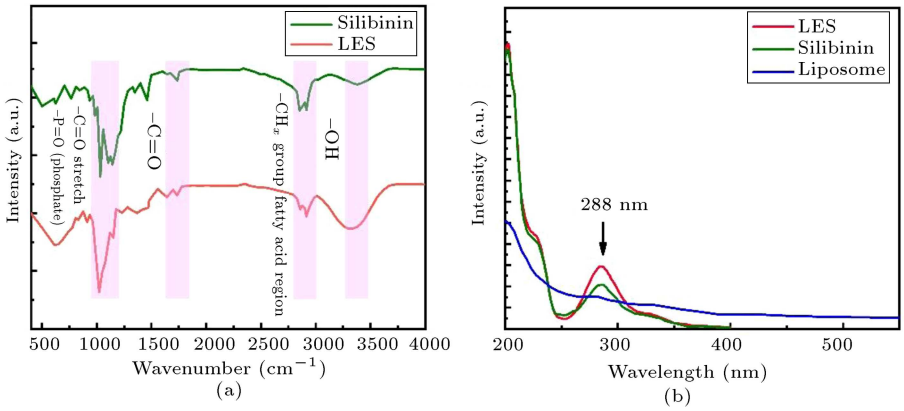

Figure 2. Spectroscopic spectra of LES nanoparticles as compared to their constituents: (a) FTIR spectra and (b) UV–Vis spectra.

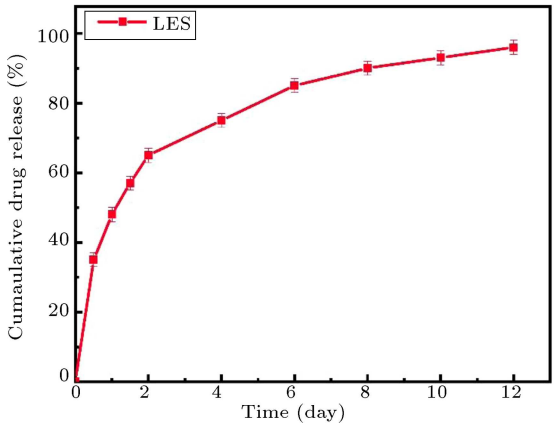

Figure 3. In vitro release profile of Silibinin from LES nanoparticles in PBS at 37°C and pH = 7.4.

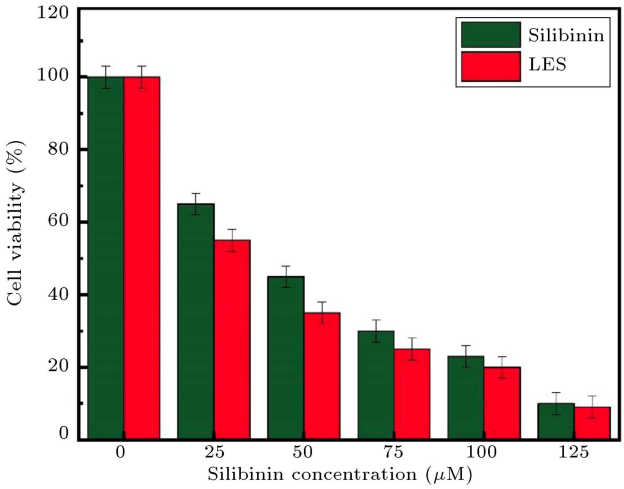

Figure 4. Cytotoxicity assay showing the dose-dependent effect of Silibinin and LES nanoparticles on MCF-7 breast cancer cells.

breast cancer cells were used. As shown in Figure 4, both the free drug and LES exhibit cytotoxic effects on MCF-7 cells, depending on the dosage. Half-maximal inhibitory concentration (IC_{50}) values were determined to be 38 μM (for Silibinin) and 20 μM (for LES nanoparticles), which are comparable to those in previous reports [45,46]. It is also notable that after 72 h incubation of the cancer cells with the materials, LES nanoparticles exhibit more LES triggered by a very efficient process, i.e., the drug is delivered into the cell efficiently. In other words, the enhanced cytotoxic effects of LES can be attributed to enhanced cellular absorption, resulting in higher intracellular concentrations and an increase in ROS levels [47,48]. Other studies on human cancer cell lines (COLO 205) have demonstrated boosted PTEN expression, decreased Akt phosphorylation, and inhibited mTOR phosphorylation via increasing AMPK phosphorylation [49].

3.4. Gene expression analysis

The expression of apoptotic genes was analyzed using real-time PCR as part of investigations into the molecular mechanisms behind the anticancer activity of LES nanoparticles. MCF-7 cells were incubated with LES nanoparticles at the IC_{50} level and compared with the pristine drug. The incubation time was 72 h. The results are shown in Figure 5(a). It is known that the anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) genes, as well as those that control the mitochondria apoptosis pathway (Caspase-3 and Caspase 9), play a significant role in the survival of different types of cancer [50–56]. A change in the amount of these genes affects the apoptotic process and reduces the proliferation of cancer cells [57]. Besides, cancer cells have a capacity for self-renewal, which is achieved through the preservation of telomeres [58]. Therefore, inhibiting hTERT activity, which is the subunit of telomerase [59], makes cancer cells more susceptible to apoptosis [59]. Our results indicate that significant changes occur in the expression levels of genes after treating breast cancer cells with the LES nanoparticles. Interestingly, the nanoparticles possess much more significant inhibition of hTERT and Bcl-2 and overexpression of Bax, Caspase-3, and Caspase-9 than the pristine drug. The results of this

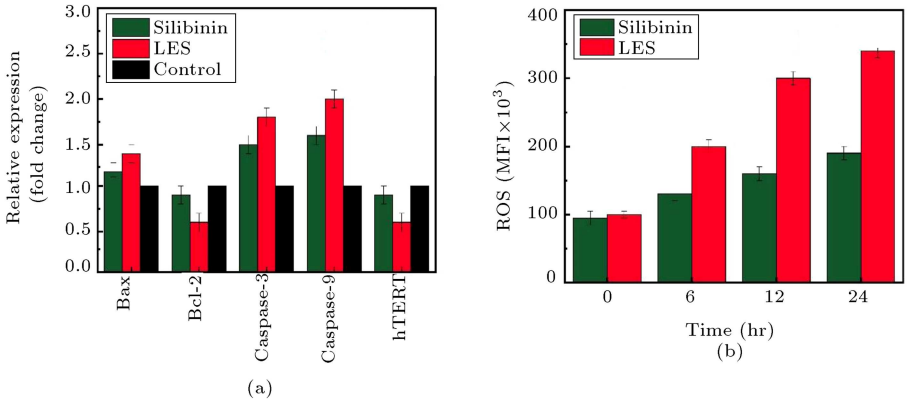

Figure 5. (a) Levels of gene expression in MCF-7 cell lines treated with Silibinin and LES nanoparticles for 72 h. (b) Mean Fluorescence Intensity (MFI) of ROS produced by Silibinin and LES nanoparticles. Data are displayed as mean ± SD ($n = 3$).

study concur with the MTT assay, showing that LES administration triggers apoptosis in MCF-7 breast cell lines. Previous studies have shown synergistic antiproliferative effects of LES through the downregulation of Bcl-2 and hTERT [45].

We have also investigated the intracellular ROS that influences the beginning stages of apoptotic signaling [47,60]. Figure 5(b) reveals that the levels of intracellular ROS in MCF-7 breast cancer cells after treatment with Silibinin and LES nanoparticles dramatically increase in a time-dependent manner. We have hypothesized that LES deeply penetrates the cell to trigger a strong ROS generation that lasts up to 24 h.

4. Conclusions

In the current study, spherical liposome-encapsulated Silibinin nanoparticles with a diameter of 50-60 nm was prepared via the thin-film hydration method. The nanocarrier containing nature-derived anticancer agent was delivered to MCF-7 breast cancer cells. It was shown that the LES nanoparticles were more effective than the pristine drug in preventing the growth of MCF-7 cells. The release profile revealed a burst release for up to 12 h followed by a reduced rate for up to 12 days. In vitro assays determined that the nanoparticles significantly impacted cell viability inhibition and apoptosis by modifying the expression of hTERT, Bcl-2, Bax, Caspase-3, and Caspase-9. Therefore, delivering medical herbs via liposome nanoparticles is an effective strategy in treating breast cancer.

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Biographies

Arash Ramedani received his BSc degree in Chemistry in 2010 from the Ferdowsi University of Mashhad and his MSc degree in Biomedical Engineering in 2012 from the University of Tehran. Currently, he is a PhD candidate at the Institute for Nanoscience and Nanotechnology at Sharif University of Technology. Her research interests include drug delivery, microfluidics, optical biosensors, organ-on-a-chip systems, and tissue engineering.

Omid Sabzevari is a Professor in Mechanistic Toxicology and Pharmacology, Cancer, Xenobiotics Analysis, and Food Safety at Tehran University of Medical Sciences (TUMS). He was a Temporary Adviser of WHO, a scientific member of the Expert Panel on Food Safety, JECFA, and the Iranian Society of Toxicology (IranTox) president. He is currently the President of the Iranian Association of Pharmaceutical Scientists (IranAPS), a member of the Board of Pharmaceutical Sciences (BPS), FIP, and the Head of the Basic and Clinical Toxicology Research Centre at TUMS.

Abdolreza (Arash) Simchi is a Professor of Materials Science and Engineering at Sharif University of Technology. He is a fellow of the Alexander von Humboldt Foundation (Bonn, Germany) and holds the 2002 Khwarizmi International Award (the highest national award) and World Intellectual Property Award (as the Best Young Inventor, United Nations Organization). Dr. Simchi's interest lies in broad areas of nanostructured materials, metal matrix composites and nanocomposites, nanoparticles, nanoceramics, biomaterials, and functional structures.