

Nanoencapsulation of *Hirudo medicinalis* proteins in liposomes as a nanocarrier for inhibiting angiogenesis through targeting VEGFA in the breast cancer cell line (MCF-7)

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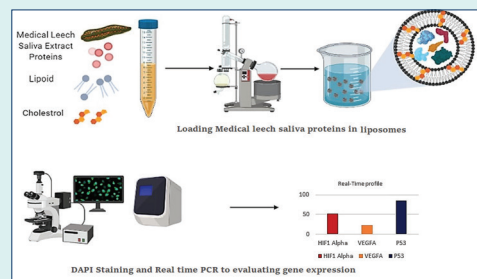
Abstract

Introduction: Breast cancer is the most serious cause of women's death throughout the world. Using nanocarrier vehicles to the exact site of cancer upgrades the therapeutic efficiency of the drugs. Capsulation of active proteins in the vesicular liposomes' hydrophilic core is essential to develop a therapeutic protein carrier system. We aimed to encapsulate the medicinal leech saliva extract (LSE) and assess the inhibition of angiogenesis of breast cancer cells by targeting vascular endothelial growth factor A (VEGFA).

Methods: In this research, enhanced formulation of liposomal protein was determined by zeta potential analysis, droplet size, drug release assay, and transmission electron microscopy (TEM). Furthermore, a cytotoxicity assay of liposomal LSE was performed to determine the cytotoxic activity of components. For assessing the expression of VEGFA, P53, and hypoxia-inducible factor subunit alpha (HIF1a) genes, Real-Time PCR was applied.

Results: Nano liposome was chosen as an enhanced formulation due to its much smaller size (46.23 nm). Liposomal LSE had more practical actions on the MCF-7 cells. As noticed by DAPI staining, apoptosis was extensively greater in treated MCF-7 cells. Wound healing assay demonstrated that MCF-7 cells could not sustain growth at the presence of liposomal LSE and expression of the VEGFA gene was declined in treated cells. Downregulation of VEGFA was evaluated with western blotting technique.

Conclusion: It can be concluded that our investigation of the tests confirmed the fact that nano liposomal LSE is a novel promising formulation for anticancer drugs and can significantly improve the penetration of protein drugs to cancer cells.



Introduction

The advanced delivery systems are highly demanded since they are appropriate for delivering different active pharmaceutical ingredients (APIs), particularly systems with high efficiency, low risk, low toxicity, and cost.^{1,2} Over the last few decades, the targeted intracellular delivery of drugs attracted remarkable attention for improving biological activity. There are different biomaterials, and it has been shown that their usage is a milestone in treating diseases with high mortality.^{3,4} Proteins play a crucial role in maintaining all cellular functions.⁵ Therefore,

malfunctioning or inadequate protein expression in the cell is the origin of most genetic and many acquired diseases. Replacing dysfunctional macromolecules via in vitro or in vivo delivery of proteins can be the most secure and unambiguous method for treating diseases, especially cancer.⁶ Therapeutic materials with a protein basis play a crucial role in the medical field for treating different disorders like cancer, diabetes, and inflammatory diseases.⁷

Various APIs can be utilized well via dynamic light scattering (DLS), Nano-size drug delivery systems

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designed to improve the APIs delivery with poor biodistribution and pharmacokinetics.⁸ For example, most of the chemotherapeutic medications include the characteristics of low pharmacokinetic profiles along with non-specific distribution in the body organs and tissues, which result in extreme systemic toxicity and side effects.⁹ Therefore, better treatment of the APIs is demonstrated by nano-sized structure-based pharmaceutical formulations (e.g., liposomes,¹⁰ polymeric nanoparticles,¹¹ electro sprayed particles,¹² and nano suspension¹³). Furthermore, in cancer treatment, effective penetration of anti-cancer agents encapsulated within a nanocarrier is the chief challenge owing to the solid tumors complexity.^{14,15} Liposomes as the most frequently assessed nanostructures are utilized in the developed drug delivery initially found by Alee Bangham, in 1963.¹⁶ Liposomes are artificially spherical vesicles originated from a phospholipid naturally. They involve one or more lipid bilayers with discrete aqueous space. Liposomal carriers are widely used for the delivery of therapeutic proteins, antibodies, and enzymes.^{17,18} The lipid bilayers of liposomes inhibit access of extracellular and endosomal proteases in order to maintain protein stability.¹⁹ The main advantage of nano liposome as the drug carrier is capability in dissolving other hydrophilic and hydrophobic drugs, protecting drugs against degradation, increasing bioavailability, and drug solubilizing with long term stability. Adequate interfacial space is provided by small size droplets for drug absorbing and penetrating the skin. Nano liposome becomes attractive by these features in different types of drug delivery systems including ocular, topical, intranasal, oral, and intravenous delivery systems. Recently, by nanotechnology, it is possible to utilize various definite compounds from medicinal leeches in manufacturing cosmetic and pharmaceutical products.²⁰⁻²² Based on the use of medicinal leeches in modern medicine, it is indicated that these substances are advantageous in the treatment of different skin disorders.

The medical applications of leeches are dated back to the beginning of evolution. Conventionally, in several countries with medical leech, it had been utilized for numerous human body disorders. Furthermore, several reports have revealed the uses of leech in skin sicknesses, nervous system irregularities such as brain congestion, reproductive, and urinary system difficulties. Dental problems, ocular inflammation, and hemorrhoids are also treated by leech therapy.^{23,24} Medical leech salivary glands include over 100 bioactive proteins and enzymes with antiedematous, analgesic, and bacteriostatic effects; they have resolving activity, cause microcirculation disorders elimination, restoring the hurt vascular permeability of organs and tissues, hypoxia elimination, blood pressure reduction, improved immune system activity, detoxified organism, relieving it from the hostile complications like strokes and infarct, and the organism's bioenergetic status improvement.²⁵ Saliva secretions of the leech

contain antimetastatic agents. A protein labeled antistatic is involved in the leech saliva which prevents lung cancer colonization. There are anti-proteolytic, platelet aggregation inhibitors, and anticoagulant enzymes. In addition, anti-tumor activity is involved in other elements like hyaluronidase. It is considered that by destroying the hyaluronic acid-CD44 interaction, hyaluronidase antitumor activity may occur to some extent through pro-tumorigenic immune cell inhibition into the tumor stroma.²⁶ Concerning metastasis and cancer therapy, numerous scientists defined the practical applications of leech saliva extract (LSE) as an anti-metastatic and Anti-Cancer agent.²⁷ Other explorations defined successful synthetic hirudin preparing as an effective metastasis inhibitor of various cancer cells like pulmonary carcinoma, osteosarcoma, leukemia, and breast carcinoma. Recent studies exploring the compounds and therapeutic potential of LSE have recognized many peptides and proteins with multiple therapeutic properties containing anti-thrombotic, antimicrobial, and anti-metastatic. In vitro anti-cancer effects were revealed in breast, prostate, and lung cancer cell lines. In vivo anticancer activity of the LSE was shown in multiple breasts and prostate cancer.²⁸ Hirudin extracted from *Hirudo medicinalis* demonstrates practical anti-metastatic activity in various malignancies like pulmonary carcinoma, osteosarcoma, breast carcinoma, and leukemia.²⁹

Activating tumor-associated macrophages (TAMs) is the impact of hyaluronic acid (HA) accumulation throughout tumorigenesis.³⁰ For instance, the stromal compartments shaped in HA-high tumors are especially supplemented with TAMs, expedited by HA-CD44 interaction. HA is degraded by hyaluronidase and HA-CD44 contact is disturbed, preventing leucocyte and tumor growth in multiple HA-high cancer variations.³¹ That is clear that a part of the tumoral hyaluronidase activity could occur through inhibition of HA-CD44 interaction.^{32,33} HA fragments are generated by highly aggressive bladder cancers within the 30–50 saccharide size range, incredibly mutagenic for endothelial cells, and thus terribly antigenic.³⁴

In numerous studies, different bioactive molecules of leeches were found in their secretions. Over 100 molecules and their action modes were recognized, however, numerous more awaiting explorations exist. These molecules contain anti-tumor activity. It is believed that further indications may be emerged within more investigations owing to newly clarified impact mechanisms. In this study, a nano liposome was designed with a lipid bilayer around an aqueous phase. This system is the most widely assessed nanocarrier for transporting anticancer drugs. Embedding biocompatible substances reactive to definite stimuli in the bilayer can obtain a controlled release of drugs from liposomes. Furthermore, it is possible to use the aqueous core of liposome in loading therapeutic drugs; Thus, LSE was loaded into liposomes.

Novelty of this study is encapsulating medical leech saliva proteins as a carrier for MCF7 breast cancer cells, which can affect VEGFA gene expression. Moreover, LSE has anti-cancer activity with a decrease in the expression of VEGFA via HIF1 α activation pathway, for transporting these proteins inside cell liposomes. The drug release from liposomes is assessed, and the impacts of the combined liposomal-LSE treatment for cancer cells are evaluated. Hence, this paper provides a vision, and takes widely into account the action modes.

Materials and Methods

Materials

The main substances in our research were purchased as follows: Ethanol 96%; chloroform, glycerin, and cholesterol from Merck (Germany); Medical Leeches from Hakim Razi Herbal Medical Clinic (HRHMC) and soybean lecithin from Lipoid. Phosphate buffered saline (PBS, 1X sterile solution) was acquired from AMERCO; LDH kit and 4',6-diamidino-2-phenylindole (DAPI) from Sigma-Aldrich, and sterile double-distilled water utilized in all the tests.

Protein extraction of leech saliva

Over the investigation, the leeches were kept in starvation. The saliva extraction procedure was initiated by rinsing the leeches with distilled water, followed by a slow addition of 8 % (v/v) ethanol solution. Since the leeches are sensitive to ethanol, vomiting in response to this sensitivity causes saliva secretion followed by 15 minutes. For obtaining pure saliva and eliminating other impurities, multi-stages of centrifugation and filtering were accomplished.³⁵ Protein concentration was calculated by spectrophotometer utilizing absorption at 280 nm.³⁶

Preparation of liposome

Briefly, cholesterol and lecithin were dissolved at a molar ratio of 70:30 (21 mol:9 mol) in 5 mL of chloroform and thoroughly mixed. Adding cholesterol structural rigidity was provided for the phospholipid bilayers.³⁷ Via rotary evaporation at 35°C, the chloroform was eliminated from the mixture to create thin lipid layers over the flask wall. The flask with the lipid films was freeze-dried for 12 hours for complete removal of the residual chloroform. Prior to addition to the flask comprising the lipid films, 6 mg of the protein being encapsulated was dissolved in 6 mL of 10 mM PBS (pH 7.4) for 5 minutes at 37°C. Then, the combination was completely hydrated in the rotary evaporator at ambient temperature for 30 minutes and sonicated 3 times for 10 seconds each for entirely detaching the lipid layers from the flask wall. The protein-liposome mixture was exposed to 10 freeze-thaw cycles in liquid nitrogen and a 45°C water bath. Ultimately, to create liposomes with a predefined diameter, extruding the mixture was performed 15 times via a mini-extruder armed with a 100 nm, 200 nm, or 400 nm polycarbonate membrane filter. In

this stage, MLVs (multi-lamellar vesicles) were converted to LUVs (big unilamellar vesicles) with relatively narrow, monodisperse diameter distributions near the selected pore size.³⁸ Over extruding, the temperature was kept over the lecithin's phase transition temperature cautiously to avoid pollution.³⁹ Each test group had continuous lipid film extrusion and formation processes. Created liposomes were kept at 4°C (Fig. 1).⁴⁰

Nano liposome droplet size analysis

The size distribution, surface charge, and mean particle size measurements of the zeta potential of polyampholyte-modified and unmodified liposomes were analyzed through DLS analysis utilizing a Zeta sizer 3000 (Malvern Instruments, Worcestershire, UK) with a 135° scattering angle. The polydispersity index (PDI) and mean particle diameter (Z-average) were determined based on the particle size distribution. The liposomes' colloidal suspension diluted with PBS and the particle size were analyzed at a temperature of 25°C and a scattering angle of 135°C. The liposomes were dispersed in PBS. Data were attained as the mean of over three measurements on various specimens.^{41,42}

Liposome pH determination

The pH (25°C) values of liposomes were measured via a pH meter (HACH digital pH meter, Loveland, NY, USA).⁴³

Transmission electron microscopy

Transmission electron microscopy (TEM) was utilized for observing the morphology of droplets (Zeiss, Jena, Germany) at 100 kV. Before the analysis, the optimized liposomes were diluted ten times with distilled water. A drop of the diluted specimen was placed over a 200-mesh film grid for drying at room temperature. Using a 2% phosphotungstic acid solution, the specimen was stained. It was then left to dry for 2 minutes prior to observing with the electron microscope.⁴⁴

Scanning electron microscopy

Utilizing a scanning electron microscope (SEM; Hitachi S-510, Hitachi-High Technologies Europe GmbH, Krefeld, Germany), the tests were performed. Conductive carbon tabs were used to pipet the liposome containing LSE onto SEM pin stubs, and a laminar airflow hood to dry it. After fixing the samples with 2% glutaraldehyde for one hour, they were immersed in distilled water three times and stained with 2% osmium tetroxide for the following hour. Then, they were re-immersed three times in distilled water, dehydrated with increasing concentrations of ethanol, and then thoroughly dehydrated through supercritical drying utilizing a Bal-Tec CPD 030 Critical Point Dryer (Bal-Tec AG, Balzers, Liechtenstein). The specimens were ultimately sputtered thrice with gold at 10 mA and 13.3 Pa Argon by the use of an Edwards S150 sputter coater (Edwards Vacuum, Crawley, UK), and evaluated under

5.3×10^{-4} Pa vacuum by the use of a scanning electron microscope with an emission current of 30 A and a 5 kV accelerating voltage. A DISS 5 digital image gaining system (Point Electronic GmbH, Halle, Germany) was used to record the micrographs digitally.⁴⁵

In vitro drug release assay

Placing 5 mL of liposomal LSE suspensions into dialysis bags (Sigma) with a 10 kDa molecular weight cut off, the solution was dialyzed in 50 mL of PBS (pH 7.4, 37°C) in a closed container. Dialysis solution aliquots were taken at various time intervals and the same PBS volume was replaced. Ultimately, the samples drug concentration was calculated utilizing UV-visible spectroscopy. The following equation calculated the cumulative drug release percentage:

$$M_{Total}(n) = \frac{V_b \times C_n + V_r \times \sum_{1}^{n-1} C_m}{M_{Total}} \times 100$$

in which M_{total} shows the overall quantity of trapped LSE in the nano liposome, V_b represents the release media's volume (50 mL), V_r shows the sample volume eliminated at various time intervals ($V_r = 1$ mL), and C_n is the LSE concentration in the eliminated sample each time.

Cancer cell culture and cytotoxicity assay of liposomal LSE

The human breast adenocarcinoma cell line (MCF-7) was attained from American Type Cell Collection (ATCC). The cells were cultured at a primary inoculum cell concentration of 104 cells/cm² in 15 mL Roswell Park Memorial Institute medium (RPMI) with 10% FBS (v/v) strep in Corning® 75 cm² cell culture flask. Incubation was performed for the cultured cells at 37°C in a 5% CO₂ humidified atmosphere.^{46,47} The result of the mixture of liposomal LSE and Etoposide treatment on cell ability of MCF-7 cells was quantified utilizing an LDH assay. One hundred microliters of diluted liposomal LSE at 50 µg/mL was inserted into the treated plate. Then 10 µL of diluted Etoposide was added to control positive plate. After 24 and 48 hours of incubation, the LDH solution was introduced into wells in the 96-well plate. Ultimately, a Quant ELISA Reader (Bio-Tek Instruments, Maharashtra, India) was used to read the optic density of the plate at 630 nm and 570 nm as the reference wavelength.⁴⁸ The test was repeated at least for three times with triplicate specimens for each test. The proliferation percentage was determined as:

$$\text{Cell viability percentage} = (\text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100\%$$

DNA fragmentation assay

DAPI could be a DNA-binding fluorescent coloring used to envision DNA fragmentation. First of all, using 4% paraformaldehyde cells were fixed within the microfluidic channels for 10 minutes at room temperature, after washing

process with PBS, 0.3% Triton X-100 permeabilized the cells for 10 minutes, they were stained by DAPI for 15 minutes. Apoptosis was noticeable by morphology, chromatin concentration, DNA strand damage, apoptotic bodies, or nuclear DNA fragmentation.⁴⁹

Cell migration assay

A scratch test was utilized to assess cell migration capability. First, MCF-7 and HUVEC cells were planted into 1.5 ml medium in each well of a 24-well plate. Then, the cells were grown over a confluent layer (48 hours), and a scratch was then created in each well utilizing a pipette tip. Afterward, rinsing the cells was slightly performed with PBS, then liposomal LSE was inserted into the respective wells. An image was captured at time point 0. Then, the cells were incubated in 5% CO₂ at 37°C, and the images were attained after 24 hours. To reduce the potential effect of proliferation over the scratch closing, the 24-hour time point was selected.⁵⁰

Real-time fluorescent quantitative polymerase chain reaction

TRIzol was used to extract the overall RNA of the two types of cells based on the protocol of the manufacturer and was reverse transcribed based on the instruction of the manufacturer. The reaction system of real-time fluorescent quantitative PCR contained 1 µL sense primer, 10 µL SYBR, 1 µL cDNA, 7 µL dH₂O, and 1 µL antisense primer. The specimens were examined in triplicate for the internal control and target gene. Table 1 shows the designed PCR primers. The quantitative test was performed 40 cycles under the circumstances of 95°C for 20 seconds, 60°C for 30 seconds, and 95°C for 3 minutes. By determining the average of each sample group, the relative transcript level of Vascular Endothelial Growth Factor A (VEGFA), P53, hypoxia-inducible factor Subunit Alpha (HIF1a) was examined as $\Delta Cq = \text{target gene } Cq - \text{reference gene } Cq$; $\Delta\Delta Cq = \text{target gene } \Delta Cq - \text{standard values } \Delta Cq$; and the relative copy of target gene = $2^{-\Delta\Delta Cq}$, and GAPDH gene was applied as an interior control for analysis of genes.

Western blot analysis

RIPA buffer was used for lysing the cells (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM NaF). Protein concentrations were determined using a Bradford assay. Protein samples (50 µg) were analyzed by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA). Membranes were washed in Tris-buffered saline containing Tween 20 (TBST) and 5% skim milk was applied to block PVDF for 30 minutes at room temperature, and incubated with the primary antibody at 4°C overnight. The membranes were incubated in secondary antibodies for 1 hour at room temperature. After washing, proteins were detected using enhanced chemiluminescence (Millipore Corporation). Finally, gel

Table 1. Primers used for Real time PCR of human breast cancer cells (MCF-7)

Gene	GeneBank No	Primer	
VEGFA	NM-001171627	Forward	AGGGCAGAATCATCACGAAGT
		Reverse	AGGGTCTCGATTGGATGGCA
P53	NM_001126117	Forward	TCAAGACTGGCGCTAAAAGT
		Reverse	TTTCAGGAAGTAGTTTCCATAGGT
HIF1a	NM-181054	Forward	ATCCATGTGACCATGAGGAAATG
		Reverse	TCGGCTAGTTAGGGTACACTTC
GAPDH	NM-001256799	Forward	AATGAATGGGCAGCCGTTAG
		Reverse	TTGGAACATGTAAACCATGTAGTTG

was analyzed with Image J software to evaluate protein bands and their expression.⁵¹

Statistical analyses

One-way analysis of variance was applied via Prism 9 software. $P < 0.05$ was selected as a statistically significant variance. Triplicate tests were done in all experiments and all samples were analyzed in duplicate. The data were expressed as mean \pm SD.

Results

Protein extraction

The existence of proteins in leech extracted saliva was studied through SDS-PAGE analysis. Over 25 types of proteins and peptides with a molecular weight within the range of 10-170 kDa were stained with Coomassie blue and silver nitrate (Fig. 1).

Analysis of liposome size and stability

DLS determines the particles' hydrodynamic radius in a solution. Based on the findings, the various liposomes have the same sizes of almost 100 nm, which is an outstanding size value for a nanocarrier for intravenous administration. The sizes do not essentially rely on the lipid character or the presence or absence of the proteins.⁵² The outcomes of DLS indicated that Z- Average was 49.26, and PDI was 0.145 nm (Fig. 2). The homogeneity grade in a particle solution size is determined by the PDI. A specimen is regarded homogeneous by PDI lower than 0.3. The attained PDI values are ≤ 0.3 , representing the prepared liposomes' homogeneity based on their sizes.⁵³ The particles with zeta potentials of < -30 mV are generally stable, hence, they overcome the natural tendency to aggregate. By low zeta potential, the repulsion may be exceeded by the attractive forces, and liposome droplets probably break and flocculate.

Stability studies

To assess the impact of gravity on nano liposome stability, a centrifugation test was performed. Followed by centrifugation for 30 minutes at 3500 g, no sign of phase separation was found in all nano liposome specimens indicating that nano liposome had excellent stability over the gravitational separation force. The storage temperature is a key factor in the quality of protein since they normally

have high sensitivity to heat and are unstable and rapidly degradable in high temperatures. Hence, it is essential to store the nano liposome at low temperatures (around 4°C). The optimized nano liposome is stored at 4°C. The rate of hydrolysis of nano liposome is considerably affected by pH value. Mainly, hydrolysis can be prevented by the pH value ranges within 6.5-8.5. Fig. 3 displays the pH variations of improved nano liposome stored at 4°C, over a 30-day storage period. The primary pH of the optimized nano liposome stored at 4°C on day 0 was 6.5. On day 30, only a slight alteration in pH was found at 4°C. At 4°C, it is possible to minimize both lipid hydrolysis and oxidation. Consequently, nano liposome droplets can be protected from oxidation and hydrolysis. Hence, the prepared nano liposome was physically stable. No considerable alteration was found in the optimized particle size of nano liposome at temperatures up to 40°C, being under 200 nm (Fig. 3B).

Transmission electron microscopy

Based on the TEM analysis, it was observed that formulation droplets were homogeneous and spherical shape. It appeared that droplets in the optimized liposome were dark, and in the bright background (Fig. 3C). Based on these findings, it was confirmed that numerous droplets were in the nano size range (less than 200 nm). In conclusion, droplets of nano liposomes were well-dispersed without droplet aggregation. According to the morphological investigation utilizing TEM, it was found that liposome was spherical with some deviations. It characteristically implied the use of the routine technique for preparing the samples in TEM. Based on the measurements of droplet size, the droplets measured over 200 nm. Nevertheless, there was no uniform size distribution of liposomes. Theoretically, by adding a drop of nano liposome to a TEM copper grid and letting it dry, particles may be rearranged over drying.

Scanning electron microscopy

SEM micrographs were very helpful with the structural establishment of these new liposomes. The micrographs showed that these liposomes are mostly spherical, and they are 50–100 nm in size, as confirmed by the DLS measurements. Given the affinity of lecithin phospholipids, identifying the stained structures under an electron microscope is easier even at a larger magnification.⁵⁴ The

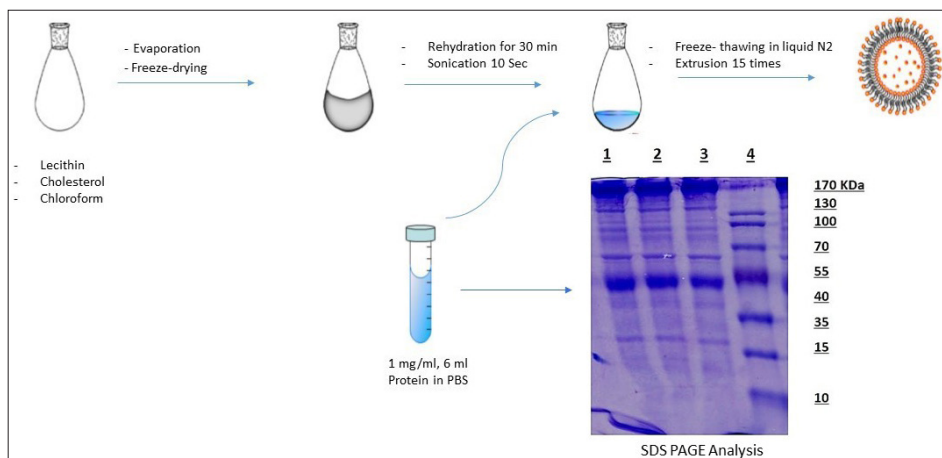


Fig. 1. Schematic representation of the liposome preparation process. SDS-PAGE analysis of leech saliva extract (Coomassie blue staining). Lanes 1-3: medical leech saliva extract. Lane 4: Molecular Weight Size marker.

liposomes scaled in the micrographs resembled in size those in the DLS measurements (Fig. 3D).

In vitro drug release

Fig. 4 represents the profile of the drug release of the LSE. Around 50% of the total LSE loaded on liposome was released in 150 hours in a sustained manner implying the relative stability of the liposomes at 37°C. Moreover, it was found that the LSE is more slow-releasing, which could be caused by the interactions between LSE proteins and the hydrophobic region of phosphatidylcholine in liposomes. All data were compared with LSE and PBS as a control.

Cytotoxicity assay

Results demonstrated that LSE had a significant anti-proliferation activity against MCF-7 cell line. The concentration of the total protein of LSE inhibiting the growth of 50% of the treated cells after 24 and 48 hours of incubation was 80.52 µg/mL. On the other hand, the anti-proliferation effect of nano liposomal LSE was compared to LSE effect, and all of them was compared with Triton X 100 that kills cells. Nano liposome LSE kills 97% of cancer cells, but leech saliva was about 50%. Besides that, Leech saliva and liposomal LSE had about 10% anti-proliferation activity against the HUVEC cell line as a normal cell (Fig. 5).

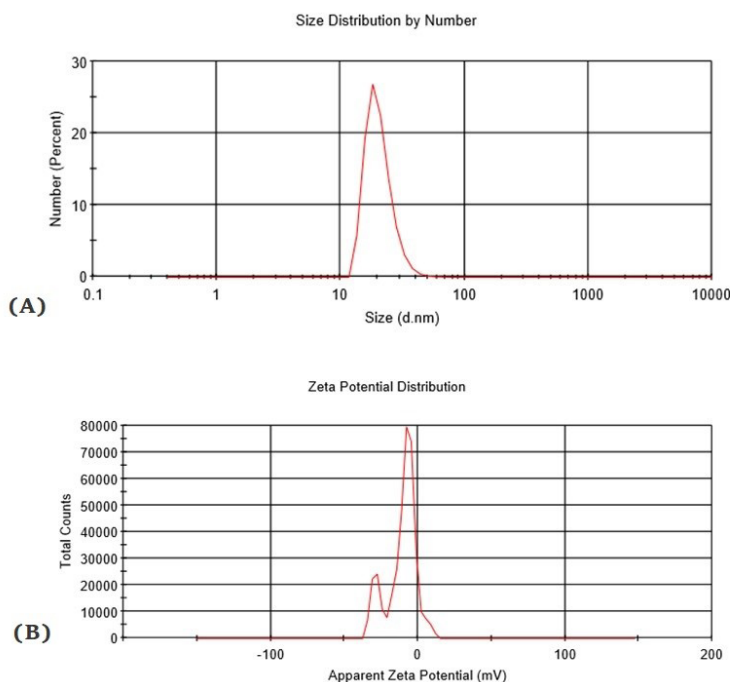


Fig. 2. DLS analysis of liposomes. Particle size distribution of liposomal protein were analyzed by DLS [size distributions by intensity] at 0 day. The graph (A) demonstrated that Z-Average (d. nm) is about 49.26, PDI is 0.145 and zeta potential represented in graph (B) result quality is good.

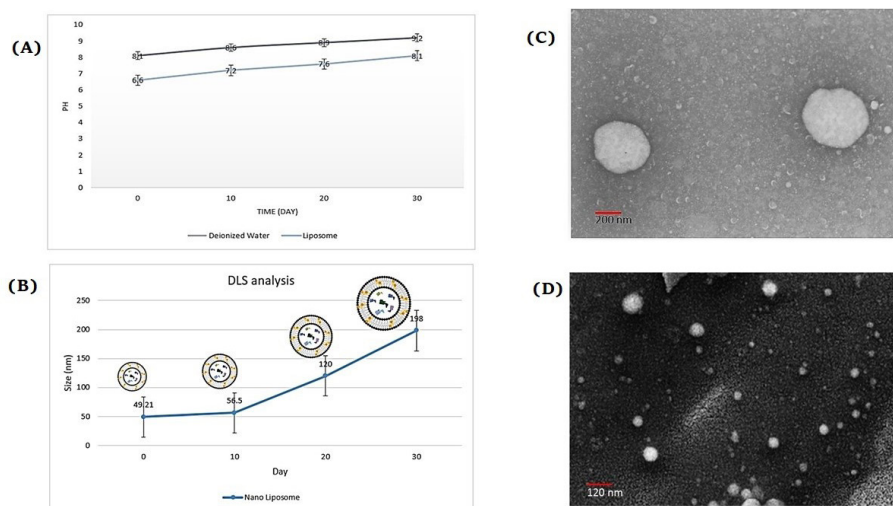


Fig. 3. Stability studies of liposomes. (A) pH changes of optimized nano liposome at 4°C. (B) Droplet size of nano liposome, duration: 30-day period of storage. (C) TEM photograph of stable formulation displays measurement of droplet size less than 50 nm. (D) SEM analysis of nano liposome at 120 nm. Photograph demonstrated that morphology of liposomes is spherical standard. The white spheres are to guide the eyes.

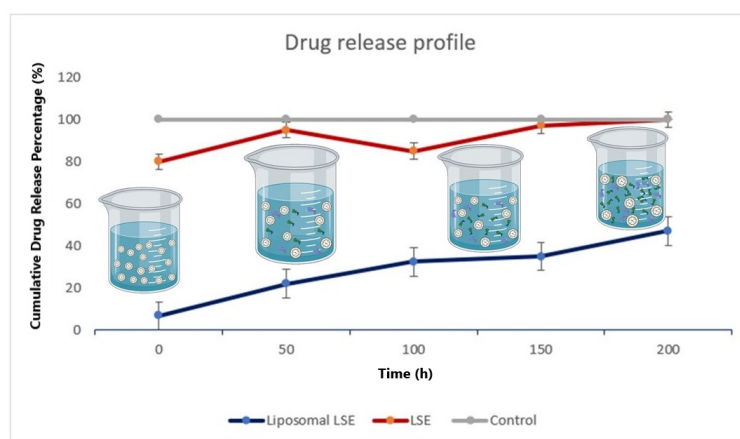


Fig. 4. Drug release assay. In vitro drug release assay for liposomal protein in different periods of time. Line blue indicated that after 50 hours, 40% of drug was released.

DAPI staining

Over morphological examination, a high cell death incidence was found, particularly with 50 µg/mL and 12.5 µg/mL treated cells. DAPI staining was performed for evaluating the impact of liposomal LSE on the cell nucleus of the treated and control cells. Dosages of 10, 25, and 50% of liposomal LSE for 24 hours post-treatment were used to perform DAPI staining. Based on treating with a 10% dose, it was shown slight nuclear disintegration in comparison with control cells; however, treating with 25% revealed a considerable nuclear death quantity. The dose of 50% indicated a remarkable significant quantity of cell death and nuclear disintegration (Fig. 6).

Cell migration assay

The ability of MCF-7 and HUVEC cells to migrate was examined at three time points (4, 24, and 48 hours); through wound healing assays indicated that the scratches'

width was progressively decreased (Fig. 7). Based on multiple comparisons of the 48-hour scratch repair rates, it was found that the rates in the experimental groups were considerably lower compared to the control group (all P values were <0.05 ; 1-way ANOVA). The scratch band healed almost entirely without treatment. Conversely, the migration of MCF-7 treated with liposomal LSE was predominantly inhibited. These results showed that liposomal LSE could suppress the migration of MCF-7 cells (Fig. 8A).

Detection of VEGFA, Hif1a, P53 expression in the cells via real-time fluorescent quantitative PCR

Utilizing the $2^{-\Delta\Delta C}$ analysis technique, the data were analyzed. Based on the results, it was indicated a remarkable reduction in the VEGFA mRNA expression level in treated MCF7 cells in comparison to the negative control ($*P < 0.05$). No statistically significant difference

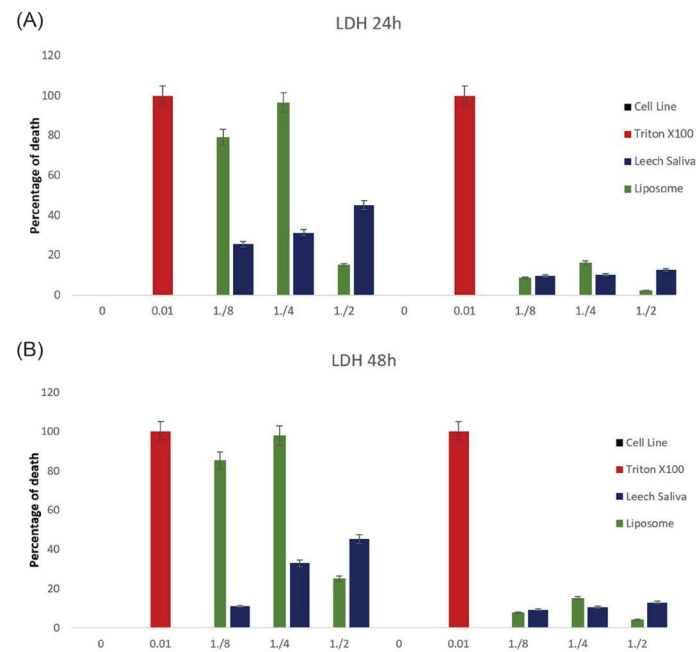


Fig. 5. LDH Test for MCF-7 as breast cancer cells and HUVEC cell line as control group. (A) Treatment after 24 hours. (B) Treatment after 48 hours. In two times confirmed that 97% of cancer cells were destroyed by nano liposomal LSE and 50% of cancer cells were destroyed only by LSE with no effect on HUVEC cell line.

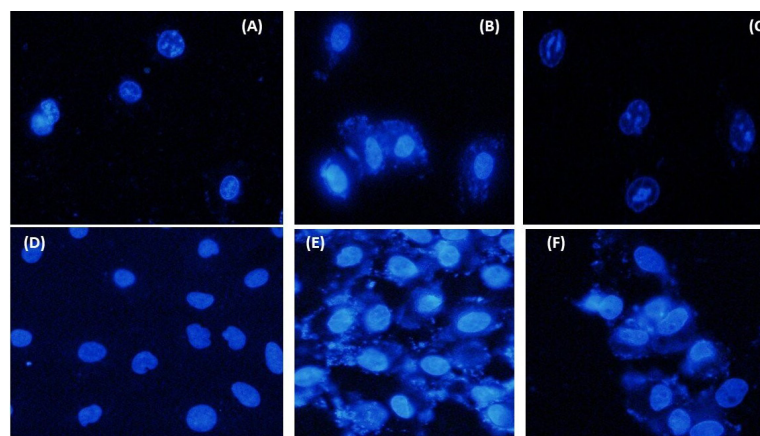


Fig. 6. Fluorescent microscopic images of MCF-7 cells stained with 4',6-diamidino-2-phenylindole (DAPI) (blue color); (1) MCF-7 cells were treated with liposomal LSE, (2) MCF-7 cells without treatment, and (3) positive control treated with Etoposide. (4) HUVEC treated with liposomal LSE, (5) without treatment as a negative control, and (6) positive control treated with Etoposide.

was found in expressing VEGFA between black control MCF-7 cells and negative control cells. Based on the expression of VEGFA in MCF-7 cells, mRNA expression of VEGFA inhibition rate was 53%. Our data indicated that VEGFA expression was decreased in breast cancer cells (MCF7). Fig. 8B shows a comparison between different cells.

Expression of VEGFA in cells

To evaluate the expression of the VEGFA protein, we examined the levels of VEGFA in different cells by Western blot analysis. Our data indicated that VEGFA protein was present at high levels in the MCF-7 (negative control), at

moderate levels in the HUVEC cells (negative control), and at low levels in the treated MCF-7 with liposomal LSE. GAPDH was also used as a control protein and graph demonstrated that in treated cells with liposomal LSE, expression of VEGFA is a responsible factor to upregulate cancer cells (Fig. 9).

Discussion

Protein delivery into cells is a powerful strategy for the development of new therapeutics since it can replace poorly expressed or dysfunctional proteins, minimizing off-target effects. However, efficient delivery of protein is essential to achieve this goal. First and foremost, we need

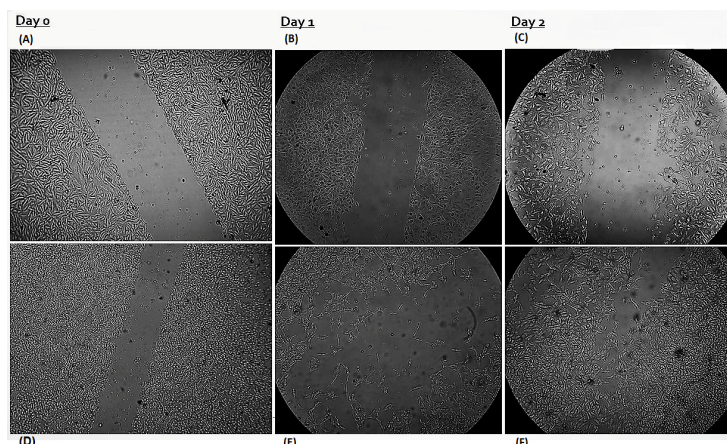


Fig. 7. The inhibitory effect of liposomal LSE on the migration of MCF-7 and HUVEC cells. MCF-7 cells were treated with or without liposomal LSE for 24 or 48 hours. Wounds were created in the cultured cells and images were taken with a microscope. (A) MCF-7 cells were treated with liposomal LSE. (B) and (C) shows no migration activity in 48 hours. (D) HUVEC cells as a negative control. (E) and (F) The untreated control cells grew and migrated more rapidly in 24 and 48 hours

to focus on what happens once proteins are inside the cell.⁵⁵

Leech therapy is a key method that is clinically utilized for patients with different kinds of cancers; however, tumor cell resistance to this agent is still the key obstacle in effective cancer treatment. Assessing the molecular mechanisms included in signaling the VEGFA can contribute to designing novel strategies in cancer

treatment.⁵⁶ Our approach is further corroborated by our promising results, hence, the anti-cancer activity of medical LSE can be obtained through incorporating into liposomes. Here, a protein extracted from medicinal leech saliva is studied for formulating a kind of vital and original nano liposome. Further detailed investigations and optimization are needed for clarifying the mechanism of incorporation of liposomes to enhance absorption proteins, to allow targeting and further enhancements to increment the formulation stability. Hence, it will be possible to develop an effective nature-derived anti-cancer formulation to offer a substitute for the presently available therapy.⁵⁷ The present study was the first work studying the use of medical leech saliva proteins inside liposomes as an anticancer drug. In each traditional and trendy therapeutic approaches, leeches are promising within the treatment of varied diseases. Except for the recognized anti-metastatic activity of leech saliva, here, we initially reported that the liposomal LSE achieved from the *H. medicinalis* had an anti-proliferative activity against the human breast adenocarcinoma cell line (MCF-7). DAPI staining of cells proved that liposomal LSE had an anti-tumor activity, and the Scratching test demonstrated that drugs had an anti-proliferative activity.

Furthermore, expression of VEGFA, HIF1a, and P53 illustrated that liposomal LSE has an anti-cancer effect. Hence, additional works still are required in this regard to separate and determine the active principle, to check the mechanism of action, and to judge its effect on different cell line varieties. We first applied liposomal LSE for breast cancer cells (MCF-7 cell line). Followed by determining the cytotoxicity in treated cells and control cells, the impacts of high concentrations of liposomal LSE were assessed on treated-MCF-7 cells for investigating mRNA expression levels of VEGFA, HIF1a, and P53. Our results revealed that low expression of VEGFA in MCF-7 cells

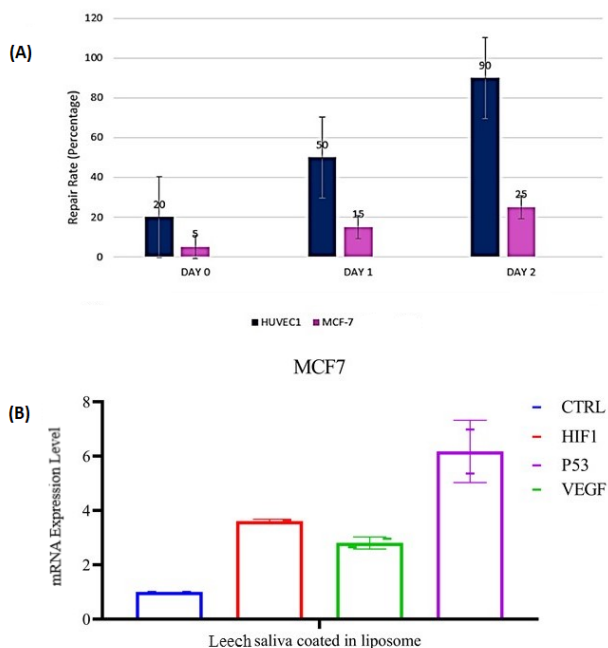


Fig. 8. (A) Wound healing assays. Images of cells taken at 0, 24, and 48 hours after scratching in wound healing assays; quantitative analysis indicated that a liposomal LSE inhibited MCF-7 cell migration. **(B)** Gene expression patterns of VEGF, HIF1a, and P53 genes in MCF-7 and HUVEC cells. GAPDH was used as a housekeeping gene. Effects of liposomal LSE on pro/anti-apoptotic genes: Levels of mRNA of P53 and HIF1a were increased significantly in MCF-7 ($P < 0.05$) compared with HUVEC cell line. Level of VEGFA reduced significantly in MCF-7 cell line.

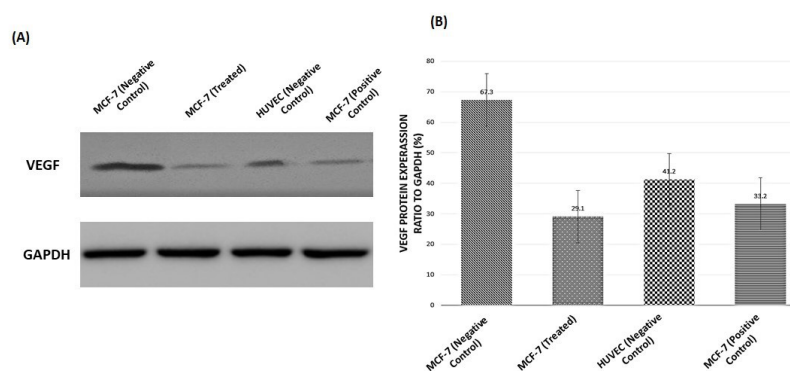


Fig. 9. VEGFA expression in MCF-7 and HUVEC cells. (A) VEGFA protein levels were analyzed using western blotting. GAPDH levels were calculated as a loading control. (B) Graph demonstrated VEGFA protein expression relative to that of GAPDH. All data corresponded to the mean \pm SD of three independent experiments.

reduced cell migration, which is evident from wound healing assay, and also western blotting showed that expression of VEGFA gene was declined and HIF1 α and P53 increased in treated cells.

Conclusion

Our study proposes that mechanisms of liposomal leech saliva proteins for downregulation of VEGFA are special. Comprehensive data and optimization to enable active targeting and further developments to raise the stability of the drug are essential to enable the development of an operative nature anti-cancer formulation, which can provide an alternative to the accessible antitumor agents. Furthermore, our data add new illustration of biological activity of leech saliva in the expression of VEGFA to tumor angiogenesis. This also highlights the position of studying leech therapy with other features. Present data together with the previous results demonstrate an impressive impact of medical leech saliva proteins inhibition on tumor growth and angiogenesis. This is one of the first studies demonstrating the *in vitro* antitumor activity of leech saliva proteins in MCF-7 cells. The anti-cancer effect of leech saliva can be further improved by the use of combined therapy, thus making it a hopeful remedy for administration of breast cancer patients.

Research Highlights

What is the current knowledge?

✓ Nowadays medical leech therapy is well known all around the world and majority of people apply medical leech for a wide range of diseases without any information about its saliva proteins.

What is new here?

✓ In this paper we aimed to evaluate medical leech proteins as cancer inhibitor by using nano liposome as the carrier to cells for the first time. Nano drug was evaluated on MCF7 cells and expression of VEGFA, HIF1 α , and P53 were recognized by real-time PCR and western blotting.

Future perspective

Protein delivery into cells is a potentially powerful strategy for developing new therapeutics since it can replace poorly expressed or dysfunctional proteins, minimizing off-target effects. However, efficient delivery of protein is essential to achieve this goal. First and foremost, we need to focus more on what happens once proteins are inside the cell. Improved methods for endosomal escape as well as mechanisms that avoid endocytosis altogether provide the promise of considerable improvement beyond current vehicles. Thinking further ahead, efficient access to the cytosol will enable intracellular targeting, providing another strategy that will further increase the efficacy of protein-based therapy. The various secretions of LSE are individually been extracted and synthesized by recombinant techniques. These compounds have been studied for various aspects like molecular structure, cellular and molecular activity, physiological functions, and their specific therapeutic actions on various disease conditions. Hopefully, this may assist in the development of various specific therapeutic agents for a variety of diseases.

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Ethical statement

The Ethics Committee of Drug Applied Research Center of Tabriz University of Medical Sciences has approved this study (Reference Number: 421799/AS).

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

Authors' contribution

All authors contributed to the study conception and design. Material

preparation, data collection and analysis were performed by AS, HK. The first draft of the manuscript was written by AS and all authors commented on previous versions of the manuscript and HH and JA were supervisors of this project. All authors read and approved the final manuscript.

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