RESEARCH PAPER

Design and manufacturing of novel smart pegylated nano-liposome co-encapsulating two herbal compounds: Silibinin and Glycyrrhizic acid with HAb18 monoclonal antibody for targeted co-delivery to liver cancer cells

Mohammad Mahdi Ochi[1,2*], Ghasem Amoabediny[2,3*], Seyed Mahdi Rezayat[4], Azim Akbarzadeh[2,5], Bahman Ebrahimi[6]

[1] *Assistant professor of the Department of Nano biotechnology & Biomimetic, School of Life Science Engineering, College of Interdisciplinary Science and Technology, University of Tehran, Tehran, Iran*
[2] *Department of Nano Biotechnology, Research Center for New Technologies In Life Science Engineering, University of Tehran, Tehran, Iran*
[3] *Department of Chemical Engineering, School of Engineering, University of Tehran, Tehran, Iran*
[4] *School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[5] *Pasteur Institute of Iran (IPI), Tehran, Iran*
[6] *Department of Nano biotechnology & Biomimetic, School of Life Science Engineering, College of Interdisciplinary Science and Technology, University of Tehran, Tehran, Iran*

ABSTRACT

Objective(s): Herbal nano-liposomes, also referred to as nano-Phytosomes, are formed through hydrogen bonding interactions between the phospholipids of lipid membranes and phytomolecules. This structure enhances the delivery efficiency of therapeutic agents. This study focuses on the use of PEGylated nano-liposomes co-loaded with two anti-cancer compounds derived from herbs, silibinin and glycyrrhizic acid, to target liver cancer cells.

Materials and Methods: The co-encapsulated nanoscale liposomes were synthesized using the thin-layer film hydration method with HEPES buffer, followed by sonication. The vesicles encapsulating silibinin and glycyrrhizic acid consisted of DPPC, cholesterol, and DSPE-mPEG2000 in a molar percentage ratio of approximately 61.5:35:3.5. A fluorescent label (DIL) was incorporated into the lipid bilayer at a concentration of 0.1 mol%. The multilamellar vesicles were then sonicated and filtered to produce the nano-liposomes. To enhance targeting, these co-encapsulated the nano-liposomes were conjugated with the monoclonal antibody HAb18. The resulting PEGylated nano-liposome formulation demonstrated a narrow size distribution, with an average particle diameter of approximately 45 nm. The zeta potential of the co-encapsulated nano-Phytosome was measured at -23.25mV. The encapsulation efficiencies of silibinin and glycyrrhizic acid were approximately 24.37% and 68.78%, respectively.

Results: The study revealed that the mean diameter of the nano-Phytosome increased upon targeting. Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) images confirmed that the average diameters of the targeted co-encapsulated nano-liposomes were approximately 84 nm and 81 nm, respectively, while the monoclonal antibody HAb18 exhibited an average diameter of around 16.1 nm and 15 nm.

Conclusion: This targeted nano-system offers efficient delivery of herbal drugs to liver cancer cells.

Keywords: *Targeting, Nano-liposome, Glycyrrhizic acid, Silibinin, Liver cancer*

How to cite this article

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INTRODUCTION

Cancer remains one of the deadliest diseases worldwide. Among its various forms, hepatocellular carcinoma (HCC) stands out as the sixth most prevalent tumor worldwide. Because of its poor prognosis, it remains the third leading cause of cancer-related death [1]. Conventional anticancer drugs often cause numerous adverse effects due to their lack of specificity, leading to widespread toxicity and unintentional targeting of healthy cells [2]. Nano liposomes are nanoscopic vesicles made up of lipid membranes and are being rigorously studied as advanced drug delivery systems aimed at enhancing the transport of therapeutic compounds. Coating their surface with hydrophilic polymers such as poly(ethylene)glycol (PEG), these liposomes can extend the time these liposomes circulate

**Corresponding author(s) Email: mmojie@ut.ac.ir, amoabediny@ut.ac.ir*

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within the body, thereby improving their effectiveness as carriers [3]. Nanophytosomes are nanoscale liposomal vesicles formed as a result of hydrogen bonding between phytoconstituents and the phospholipid components of the lipid bilayer. These structures are designed to enhance the delivery of therapeutic agents. Most phytosomal studies, particularly on planterosomes, have predominantly focused on the Iranian medicinal plant *Silybum marianum* (L.), also referred to as milk thistle (Figure 1). This plant is notable for its silymarin mixture, a primary flavonoid recognized for its liver-protective properties [4].

Fig. 1. *Silybum marianum* (Milk Thistle) (left) & *Glycyrrhiza glabra* (right)

Silymarin has many beneficial properties, including membrane stabilization, anti-fibrotic, anti-oxidative, anti-inflammatory, immunomodulatory, and liver-regenerating effects, which are crucial in the management and study of experimental liver diseases [5]. The components of silymarin are recognized for their hepatoprotective properties; however, they exhibit poor water solubility and low bioavailability. Studies on humans and laboratory animals have shown that oral administration of powdered extracts results in plasma concentrations only at the nanogram per milliliter level [6, 7]. Dried extracts of milk thistle seeds typically contain around 60% silymarin, which is a complex consisting of four primary flavonolignans: These include silibinin (50–60%), silichristin (20%), isosilibinin (5%) and silidianin (10%). Among these, silibinin stands out as the most significant antioxidant flavonolignan and serves as the principal active component of silymarin (Figure 2) [8].

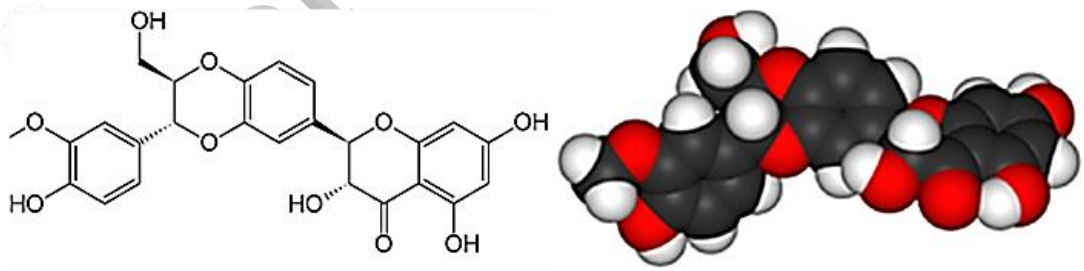

Fig. 2. Chemical structure of Silibinin.

Silibinin is recognized as an anti-cancer drug, and its effectiveness against tumors is largely attributed to its ability to reduce N-nitrosodiethylamine levels in hepatocellular carcinoma cells [9, 10].

However, silibinin exhibits minimal oral absorption and is unsuitable for direct use in parenteral formulations due to its limited solubility in both aqueous and lipid environments [11]. An in-depth knowledge of the role of the specific hydroxyl groups and components of the Silibinin molecule has made it possible to rationally select suitable sites for the derivatization of Silibinin without losing the biological activity of the resulting conjugates [12]. Silymarin has demonstrated a range of clinical and pharmacological effects, including the ability to inhibit cancer cell metastasis. Silymarin is a low solubility drug with high permeability; its main biological active component is silibinin, which is largely responsible for its antihepatotoxic activity.

Unfortunately, the poor solubility of silymarin in both aqueous and lipid media limits its effective permeation across the intestinal epithelial membrane [13]. Accordingly, the development of sophisticated drug delivery approaches is essential for overcoming the poor solubility and limited bioavailability of silymarin. This study focuses on targeting of two herbal drugs of Silibinin and Glycyrrhizic acid loaded Nano liposome system (Nano phytosome) for delivery to liver cancer cells. Notably, the antitumor efficacy of the drugs tends to improve with the reduction in vesicle size. Encapsulating of silibinin and glycyrrhizic acid in nanoliposomes can significantly enhance the biological activity of silibinin, improve its stability in the bloodstream, and potentiate the therapeutic effects of glycyrrhizic acid in the treatment of HCC. Silibinin has demonstrated a synergistic effect in boosting the therapeutic impact of doxorubicin in advanced-stage DU145 human prostate cancer cells [14]. Another herbal drug in this study was Glycyrrhizic acid (Figure 3), which is obtained from the plant *Glycyrrhiza glabra* (L.) (Figure 1). The ability of the herbal drug Glycyrrhizic acid to inhibit tumor growth is related to its inhibition of matrix metalloproteases (MMPs) and its protection of DNA in cancer cells.

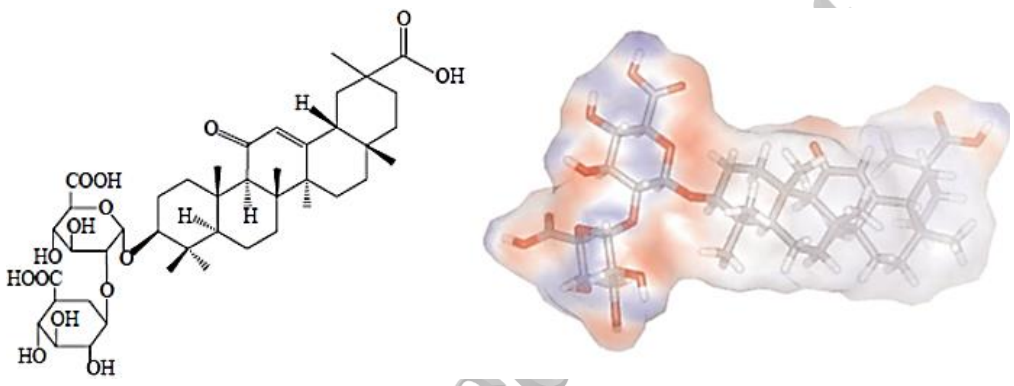

Fig. 3. Chemical structure of Glycyrrhizic acid.

Liposomal anticancer agents that are modified with targeting ligands often demonstrate superior binding efficiency and lower toxicity than their non-targeted counterparts, resulting in better therapeutic efficacy. A recently developed approach for preparing ligand-targeted liposomes is known as the post-insertion technique [15]. In this study, co-encapsulated pegylated nano-liposomes are targeted by monoclonal antibody HAb18. In this method, the monoclonal antibody HAb18 is conjugated to the terminal end of polyethylene glycol (PEG) lipid derivatives incorporated within the micellar structure. Then, through a simple time- and temperature-dependent incubation process, the derivatives are incorporated into the lipid bilayer of pre-formed, drug-loaded liposomes.

MATERIALS AND METHODS

Materials

The hepatocarcinoma-specific monoclonal antibodies HAb18 and anti-CD147 were provided by Abcam plc (USA). DPPC and mPEG2000-DSPE (methoxy polyethylene glycol–modified distearoyl phosphatidylethanolamine) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHOL), the fluorescent dye DIL, HEPES buffer (10 mM, pH 5.5) and silibinin were obtained from Sigma-Aldrich (St. Louis, MO). NHS-activated PEG2000-DSPE (NHS-PEG2000-DSPE) was custom synthesized by Nanocs, Inc. (USA). Glycyrrhizic acid was sourced from a local supplier in Shiraz, Iran; maltose and ethanol were purchased from standard commercial sources.

Co-encapsulation process of nano-liposomes

Non-targeted co-encapsulated nanoliposomes were formulated via the thin-film hydration technique, employing HEPES buffer for hydration followed by sonication. Initially, multilamellar vesicles (MLVs) entrapping silibinin and glycyrrhizic acid were formulated. Then, a mixture of DiPalmitoyl PhosphatidylCholine (DPPC), cholesterol, and DSPE-mPEG2000 in a molar ratio of approximately 61.5:35:3.5, along with silibinin and glycyrrhizic acid as the lipophilic phase at a specific molar ratio, was dissolved in absolute ethanol [16, 17]. To facilitate confocal microscopy analysis, a fluorescent label (DIL) was integrated into the lipid bilayer at a concentration of 0.1 mol%. The organic solvent was removed via a rotary evaporator to form a thin lipid film. Lipid hydration was achieved by adding 6 mL of a HEPES buffer solution (10 mM, pH 5.5) containing 0.28 g of maltose as the hydrophilic phase, followed by heating above 50°C degrees [18]. The resulting liposomes were sonicated to reduce their mean diameter. Specifically, MLVs were sonicated at 60 percent amplitude for 10 minutes using an S-4000 Misonix

Sonicator, followed by filtration to produce small unilamellar vesicles (SUVs) or nanoliposomes.

Preparation of micelles

Lipid mixtures consisting of mPEG2000-DSPE and NHS-PEG2000-DSPE at a molar ratio of 1:4 were dissolved in a HEPES buffer solution (10 mM, pH 5.5) at concentrations above respective critical micelle concentrations (CMCs) and then incubated in a water bath 60°C for 10 minutes with occasional gentle vortexing.

Formation of HAb18 coupled to micelles

In brief, HAb18 monoclonal antibody and NHS-PEG2000-DSPE were mixed at a molar ratio of 1:10 (HAb18:NHS- PEG2000 - DSPE) and dissolved in HEPES buffer (10 mM, pH 5.5), followed by continuous stirring at ambient temperature for 24 hours (Figure 4) [15, 19].

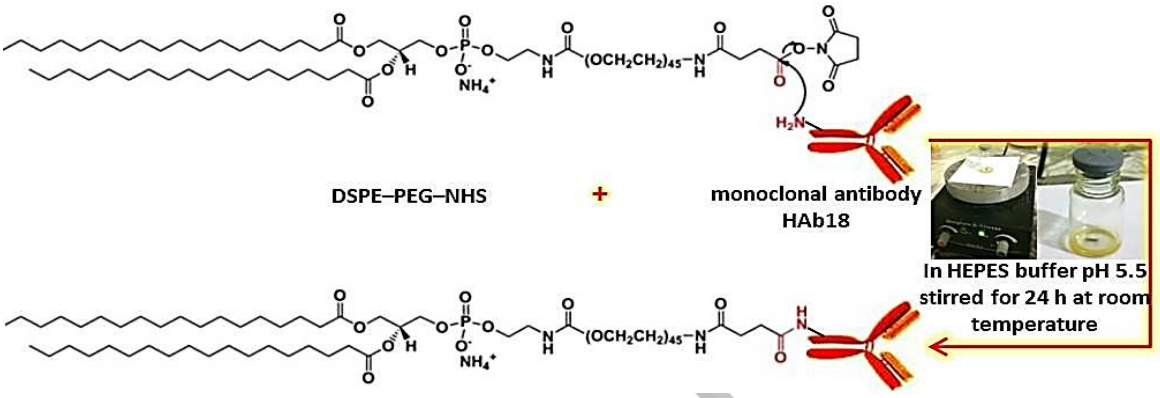

Fig. 4. Models for the conjugation of monoclonal antibody HAb18 with NHS-PEG2000-DSPE in micelles

Subsequently, the micelles were incubated at 60°C for 1 hour with preformed liposomes incorporating 3 mol% PEG. The resulting antibody incorporation efficiency was 16.30 µg of antibody per µmol of liposome.

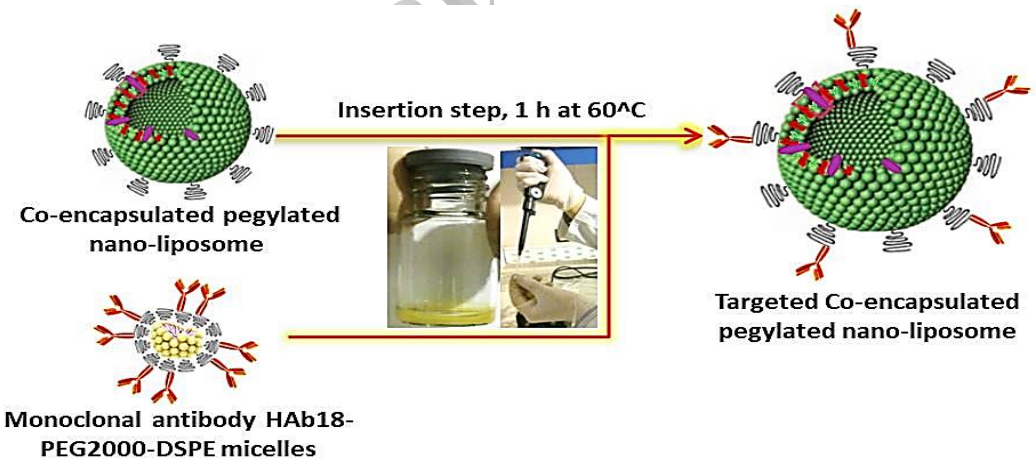

Fig. 5. Models for the incorporation of the monoclonal antibody HAb18 into liposomes after its release from micelles.

Analysis of the morphological features of multilamellar vesicles

The structural characteristics of multilamellar vesicles were analyzed using fluorescence microscopy techniques.

Analysis of particle size and zeta potential of co-encapsulated nanoliposomes

In this study, measurements of zeta potential and dynamic light scattering (DLS) were performed to determine the particle size and surface charge of both targeted and non-targeted co-encapsulated nanoliposomes, using the particle size and zeta potential analyzer (BI-90 model, manufactured by Brookhaven Instruments, USA).

Assessment of encapsulation efficiency for silibinin and glycyrrhizic acid within PEGylated nanoliposomes

Encapsulation efficiency of nano-liposomes that entrapping of Silibinin and glycyrrhizic acid

was determined by a high-performance liquid chromatography (HPLC) method. UV/Vis spectrophotometric analysis of silibinin and glycyrrhizic acid was carried out in the 200–350 nm wavelength range in conjunction with the HPLC method. To remove the unencapsulated drug molecules, the co-loaded nanoliposomes were dialyzed against a 10 mM HEPES buffer solution at pH 5.5 using a Spectra/Por® dialysis membrane with a molecular weight cutoff between 12,000–14,000 Da. Encapsulation efficiency (EE) evaluates the ability of liposomes to encapsulate drugs by comparing the amount of drug incorporated during production to the amount of drug within the liposome.

Assessment of Silibinin and Glycyrrhizic Acid Release by HPLC

The in vitro release profiles of silibinin and glycyrrhizic acid were evaluated using HPLC. The release capability of glycyrrhizic acid and silibinin was also evaluated at pH 7.4 and pH 5.5. To do so, 1 mL of the liposomal suspension was then transferred into a cellulose dialysis bag with a molecular weight cut-off (MWCO) ranging from 12,000-14,000 Da (Membrane Filtration Products, Inc.). The dialysis bags were immersed in 50 mL of HEPES buffer and maintained at 37°C with continuous stirring at 100 rpm using a magnetic bar. At predetermined time intervals (1, 2, 4, 6, 15, 18, 21, 24, 48, 66, 69, 72, and 75 hours), 200-µL samples aliquots were withdrawn and analyzed by HPLC at a detection wavelength of 240 nm.

Morphology of targeted micelles and co-encapsulated pegylated nano-liposomes

SEM and TEM were utilized to characterize the morphology and determine the size of the nanoliposomes. The shape and size of liposomes are mainly characterized using negative-stain TEM and SEM.

Liposome formation was confirmed by evaluating their morphology using a TEM (TEM; ZEISS EM 10, Germany). Approximately 10 µL of the liposomal aqueous dispersion was applied onto copper grids. After allowing it to stand for one minute, excess liquid was blotted off, and the sample was negatively stained with a 1% aqueous solution of uranyl acetate for 30 seconds. The prepared grids were then examined under a TEM operating at an accelerating voltage of 80kV. The morphological characteristics of the micelles were further analyzed using both TEM and SEM.

The conjugation of the monoclonal antibody with PEG was analyzed using Analytical techniques including SEM, TEM, and ATR-FTIR were employed for characterization, while HPLC was used to confirm the results.

Cytotoxicity assay

In the experimental setup, HepG2 and fibroblast cells were cultured, and 100 µL of a suspension containing 10,000 cells was dispensed into each well of a 96-well plate. The plates were then incubated separately at 37 °C in a 5% CO_2 atmosphere. After 24 hours of initial incubation, the culture medium was removed and the cells were treated with different formulations: (A) Targeted PEGylated nanoliposomes co-loaded with glycyrrhizic acid (75% w/v) and silibinin (25% w/v) and functionalized with the monoclonal antibody HAb18; (B) Non-targeted PEGylated nanoliposomes containing the same drug combination; and (C) Free silibinin and glycyrrhizic acid at identical concentrations. Control groups were also included. The treated cells were then incubated for an additional 48 hours. After this period, the supernatant was discarded and 100 µL of MTT solution (0.5 mg/mL) was added to each well. After 3 hours of incubation, the development of a purple color indicating formazan formation was observed. The mixture was then dissolved in 100 µL of isopropanol in living cells. Absorbance was measured at 540 nm using a PowerWave XS spectrophotometer (BioTek Instruments, USA), and the IC_{50} values were determined using the Pharm PCS (Pharmacologic Calculation System) statistical software (Springer Verlag, USA).

Investigating cellular binding and uptake of targeted nanoliposomes through fluorescence microscopy imaging

The surface antigen of the liver cancer cell line HepG2, CD147, is of the internalizing type. By binding the anti-CD147 antibody to the surface of the nanoliposome, the drug system can be transferred into the cancer cell. To investigate the binding and internalization of nanoliposomes bound to the surface antigen of the cancer cell line, first 200 µL of cells suspension containing an appropriate number of cells from the HepG2 and fibroblast cell lines (liver cancer) in culture medium (RPMI-1640) was added to the wells of 24-well culture plates. After 48 hours, an appropriate number of cells adhered to the bottom of the well and reached their stable state. Then, the excess culture medium was removed from each well with a sampler, and the wells were washed with cold saline buffer (PBS). Then, 200 µL of targeted drug-carrying nanoliposomes at the appropriate concentration were added to each well and incubated for 3 hours at 37°C to

affect the desired samples. Then, the specific transfer of targeted drug-carrying liposomes into liver cancer cells was examined using fluorescent microscopy on cells that had absorbed fluorescent pigment-containing nanoliposomes with absorption wavelengths of 550 and 567 nm in phosphate buffer.

Statistical analysis

The impact of formulation variables was analyzed by using one-way ANOVA, with a significance level set at $p<0.05$. Data processing and statistical evaluations were performed using SAS software (version 9.1; SAS Institute, Cary, NC, USA).

Ethical considerations

All the cell models used in this study are established cell lines; therefore, no specific ethical considerations were required.

RESULTS

Morphology of silibinin and glycyrrhizic acid multilamellar vesicles

In vitro light microscopy studies of glycyrrhizic acid and silibinin multilamellar vesicles (Figure 6):

In vitro fluorescence microscopy studies of glycyrrhizic acid and silibinin multilamellar vesicles (Figure 7):

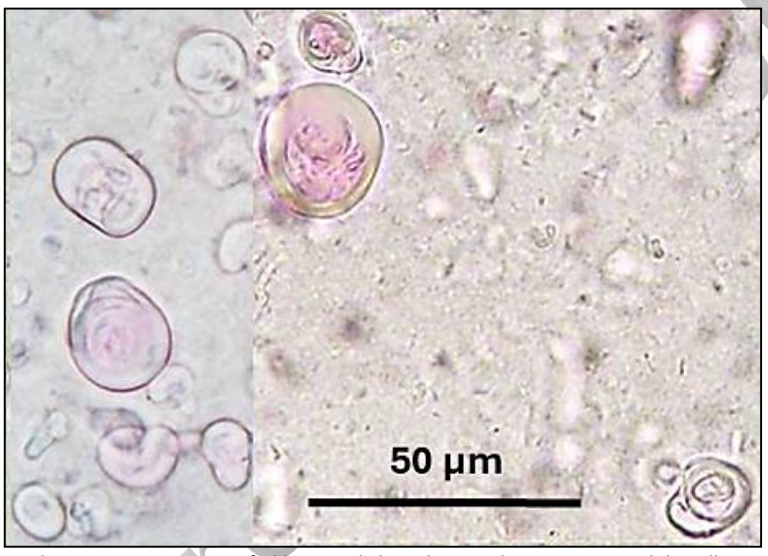

Fig. 6. Light microscopic images of silibinin and glycyrrhizic acid micrometric multilamellar vesicles

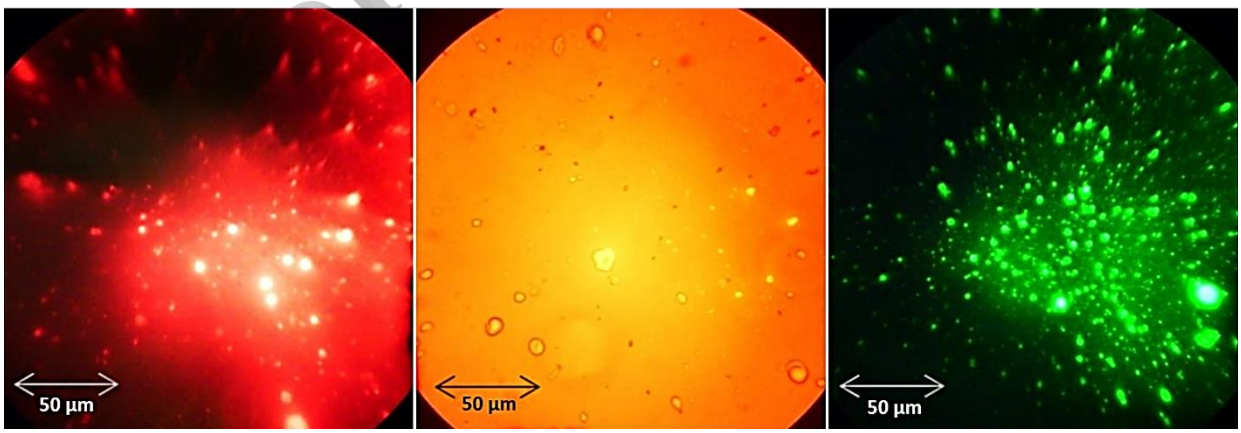

Fig. 7. Fluorescence microscopic images of silibinin and glycyrrhizic acid multilamellar vesicles

Characterization of particle size and surface charge in co-encapsulated nanoliposomes

The PEGylated nano-liposomes prepared by sonication at 60% amplitude exhibited a uniform and narrow particle size distribution with an average diameter of approximately 46.3 nm. The co-encapsulated nano-liposomes exhibited a zeta potential of -23.25 mV, suggesting a moderate surface charge and stable colloidal dispersion (Figure 8).

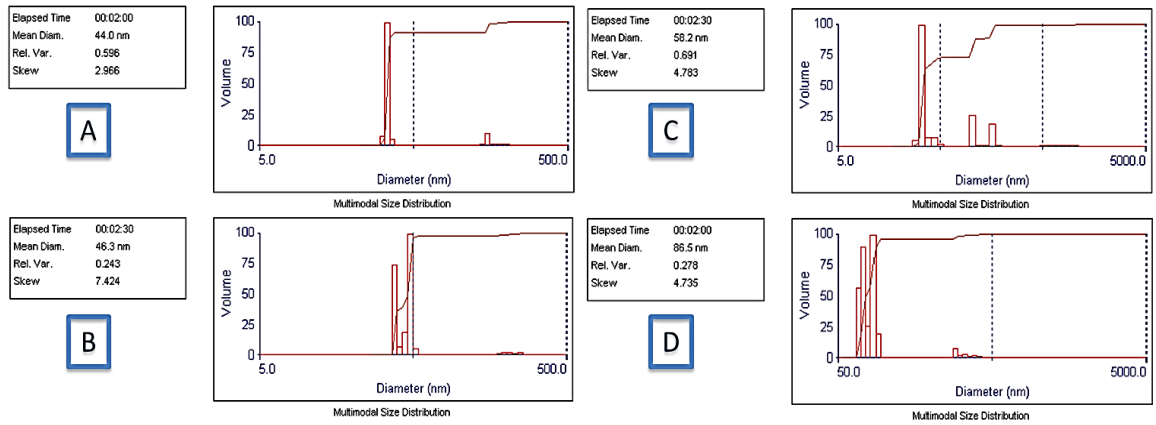

Fig. 8. Particle sizes were evaluated for different formulations: (A) plain nanoliposomes without herbal compounds, (B) co-encapsulated nanoliposomes after 3 days, (C) the same formulation after 3 months of storage, and (D) targeted co-encapsulated nanoliposomes with Hab18 monoclonal antibody after dialysis.

The results indicated that the loading of glycyrrhizic acid and silibinin led to an increase in the mean diameter and a reduction in the negative zeta potential of the nano-liposomes, respectively (Table 1).

Table 1. Effect of entrapment & targeting of botanicals on the size & zeta potential of nanoliposomes.

Sonication with 60 percent amplitude	Mean Particle size (nm)	Mean zeta potential
Nano-liposome without herbal drugs	44	-31.66
Nano-liposome co-encapsulating two herbal drugs after a period of 3 days	46.3	-23.25
Nano-liposome co-encapsulating two herbal drugs after a period of 3 months	58.2	-25.52
Targeted co-encapsulated nano-liposomes with Hab18	86.5	-0.74

Micelles prepared with mPEG2000-DSPE and NHS-PEG2000-DSPE at a 4:1 molar ratio were initially measured to be approximately 11-12 nm in diameter. After conjugation with HAb18 monoclonal antibody, their size increased to approximately 33-59 nm.

Efficiency of the encapsulation of silibinin and glycyrrhizic acid in PEGylated nano-liposomal formulations

Encapsulation efficiency was measured by HPLC method. The glycyrrhizic acid and silibinin peaks were found at 240 nm. The encapsulation efficiencies of silibinin and glycyrrhizic acid in the PEGylated nanoliposomes were about 24.37% and 68.78%, respectively.

Evaluation of Silibinin and Glycyrrhizic acid release in an in vitro setting

Drugs release rate was measured by HPLC method (Figure 9).

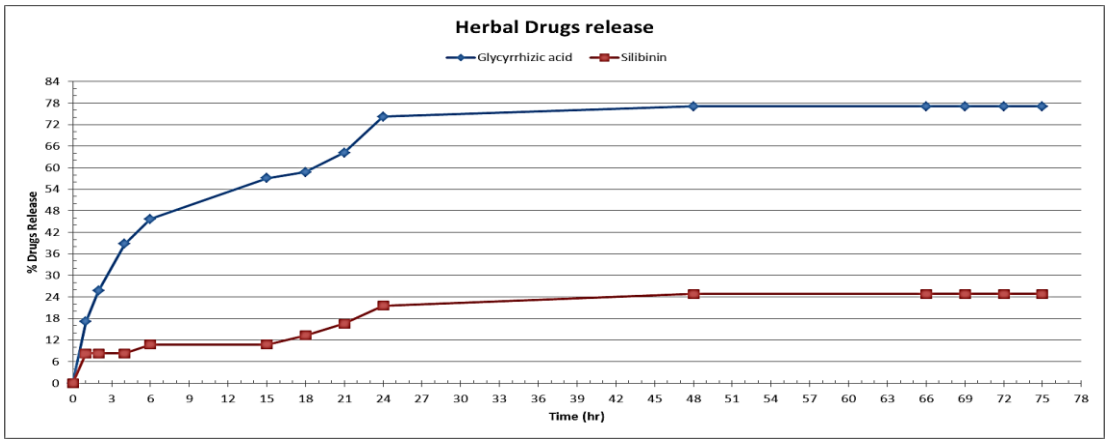

Fig. 9. In vitro release of silibinin and glycyrrhizic acid

Morphology of targeted micelles and co-encapsulated pegylated nano-liposomes

The morphological features of the nanoliposomes were examined using TEM and SEM. SEM analysis revealed an average diameter of 43 nm for the co-encapsulated nanoliposomes.

Results of SEM analysis after 3 months showed that the mean diameter of 8 co-encapsulated nano-liposomes was 55.36 nm (Figure 10).

The morphology of the micelles was analyzed by both TEM and SEM. The results of the SEM analysis showed that the mean diameter of 7 targeted micelles was 42 nm.

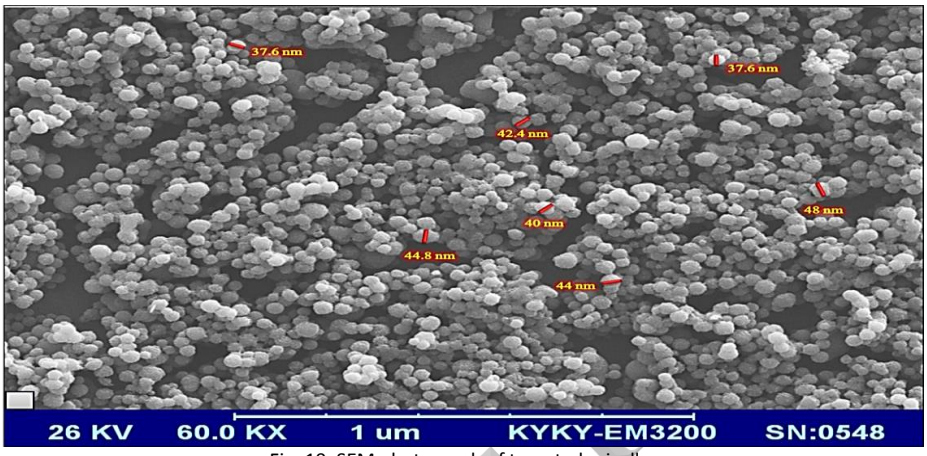

Fig. 10. SEM photograph of targeted micelle.

SEM analysis following targeting revealed that the average diameter of the six HAb18-conjugated co-encapsulated nanoliposomes was approximately 84 nm, while the mean diameter of the eight individual monoclonal HAb18 antibodies was around 16.1 nm (Figure 11).

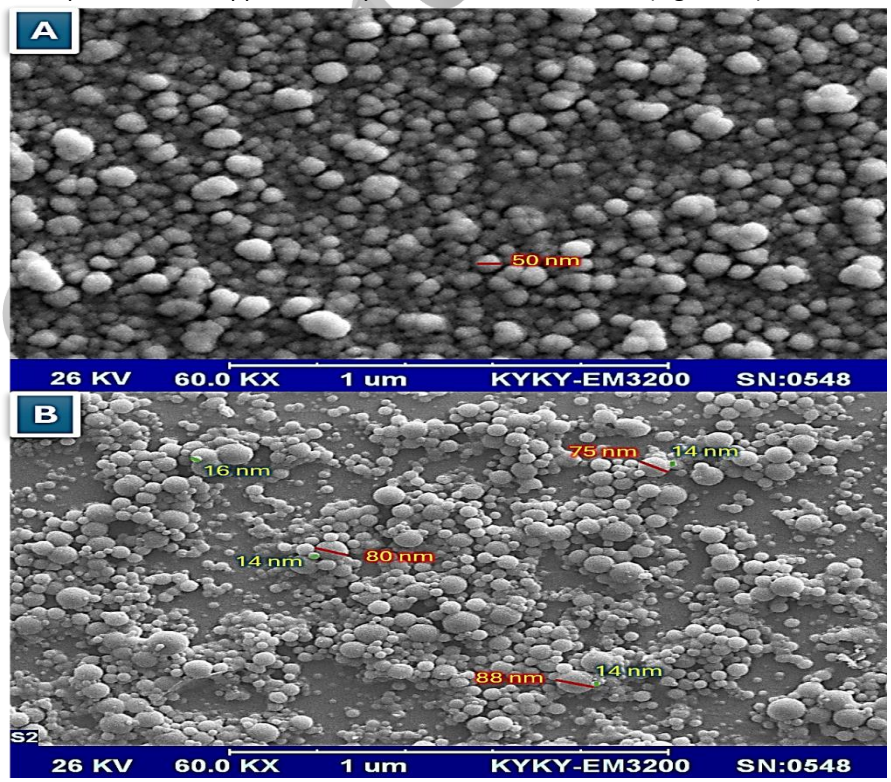

Fig. 11. SEM photograph of A. Non-targeted co-encapsulated nano-liposomes, B. Targeted co-encapsulated nano-liposomes (red line) with HAb18 (green line).

The pre-targeting TEM results indicated that the diameter range of the co-encapsulated nanoliposomes was approximately 40 to 60 nm (Figure12).

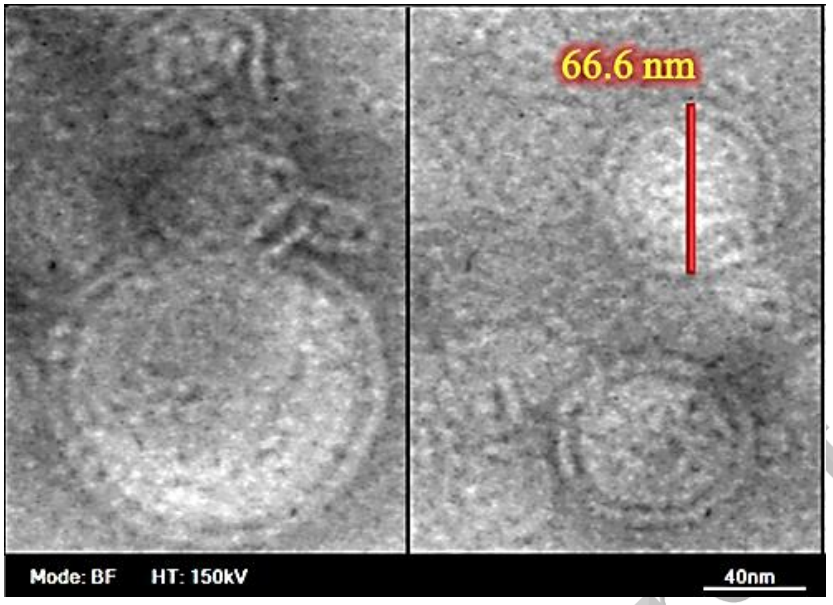

Fig. 12. TEM photograph of non-targeted co-encapsulated nano-liposomes

TEM photographs taken after targeting showed that diameter of targeted co-encapsulated nano-liposomes was about 81 nm, They also showed that 15 nm (Figure 13).

the monoclonal antibody HAb18 had a Y shape and a diameter of about

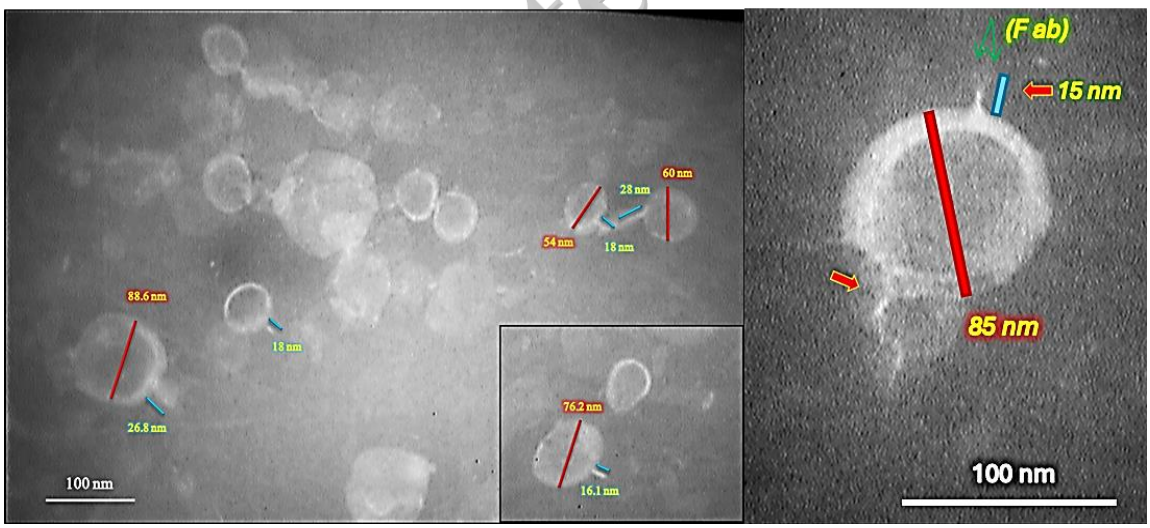

Fig. 13. TEM photograph of Targeted co-encapsulated nano-liposomes with HAb18

Spectroscopic evaluation of PEGylated antibody conjugation and its assembly into nanoliposomes via ATR-FTIR

Monoclonal antibody conjugation to PEG2000-DSPE was analyzed by ATR-FTIR (Figure14, 15, 16, 17).

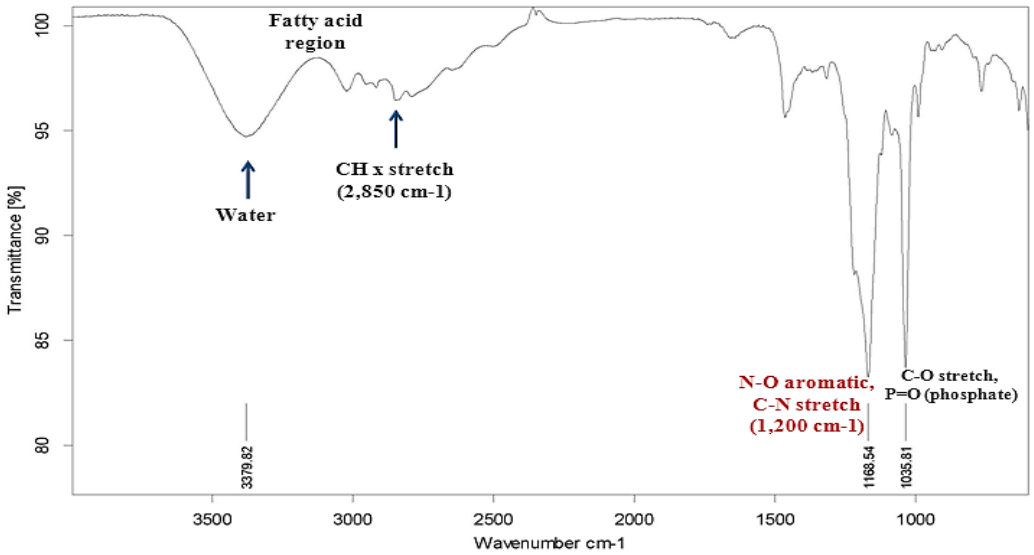

Fig. 14. ATR-FTIR of non-targeted micelle.

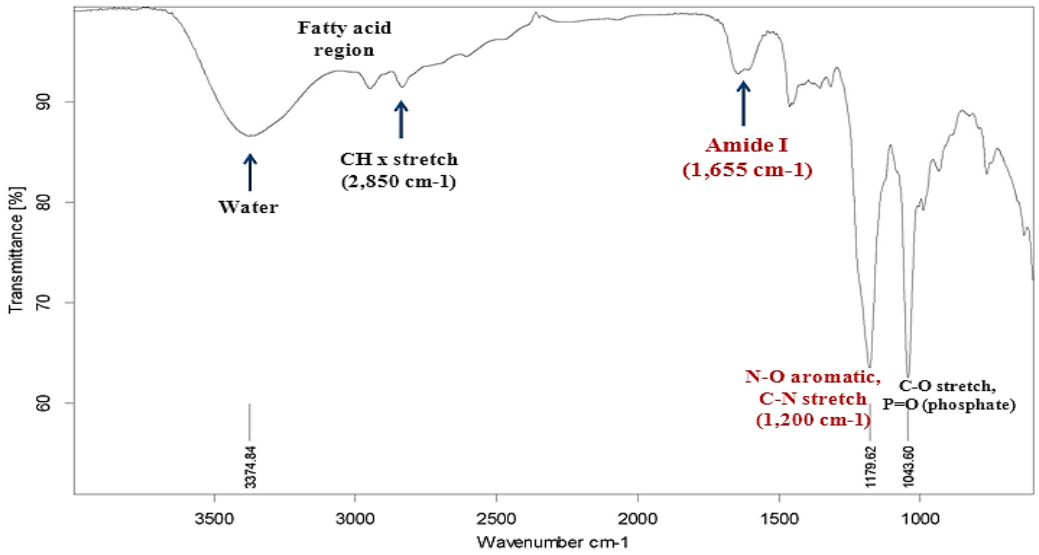

Fig. 15. ATR-FTIR of targeted micelle.

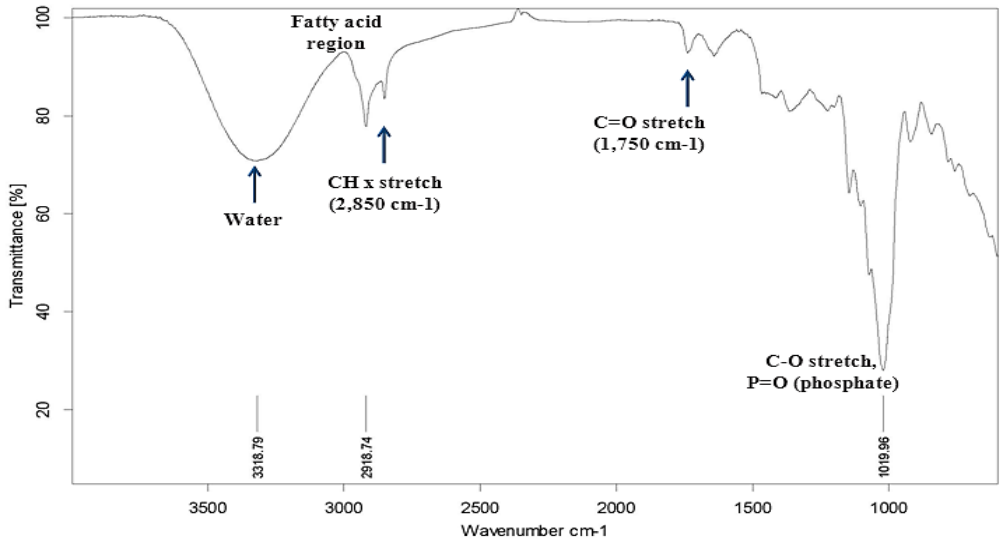

Fig. 16. ATR-FTIR of non-targeted co-encapsulated nano-liposomes

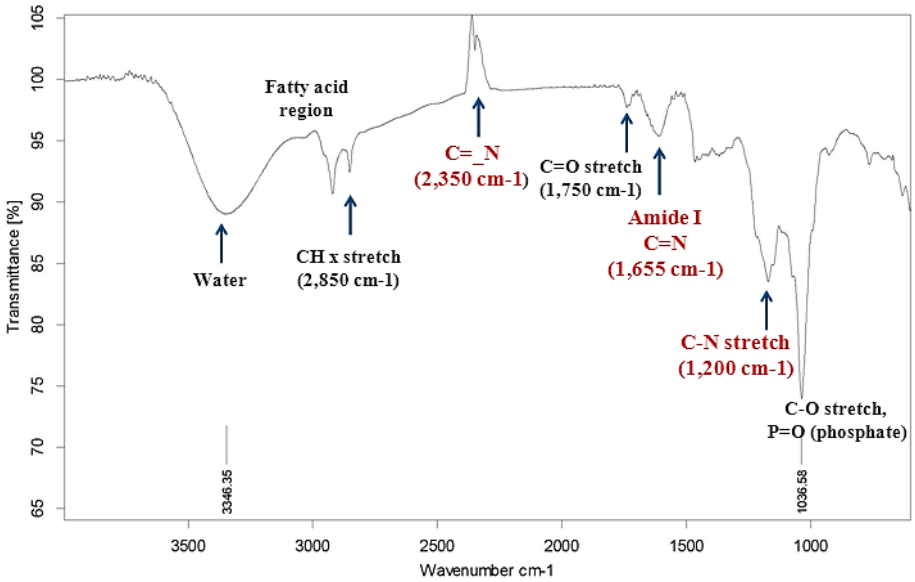

Fig. 17. ATR-FTIR of targeted co-encapsulated nano-liposomes with HAb18

HPLC method study on the conjugation of monoclonal antibody with PEG2000-DSPE and insertion into nano-liposome

The conjugation of monoclonal antibody with the PEG2000-DSPE was confirmed by HPLC method (Figure 18).

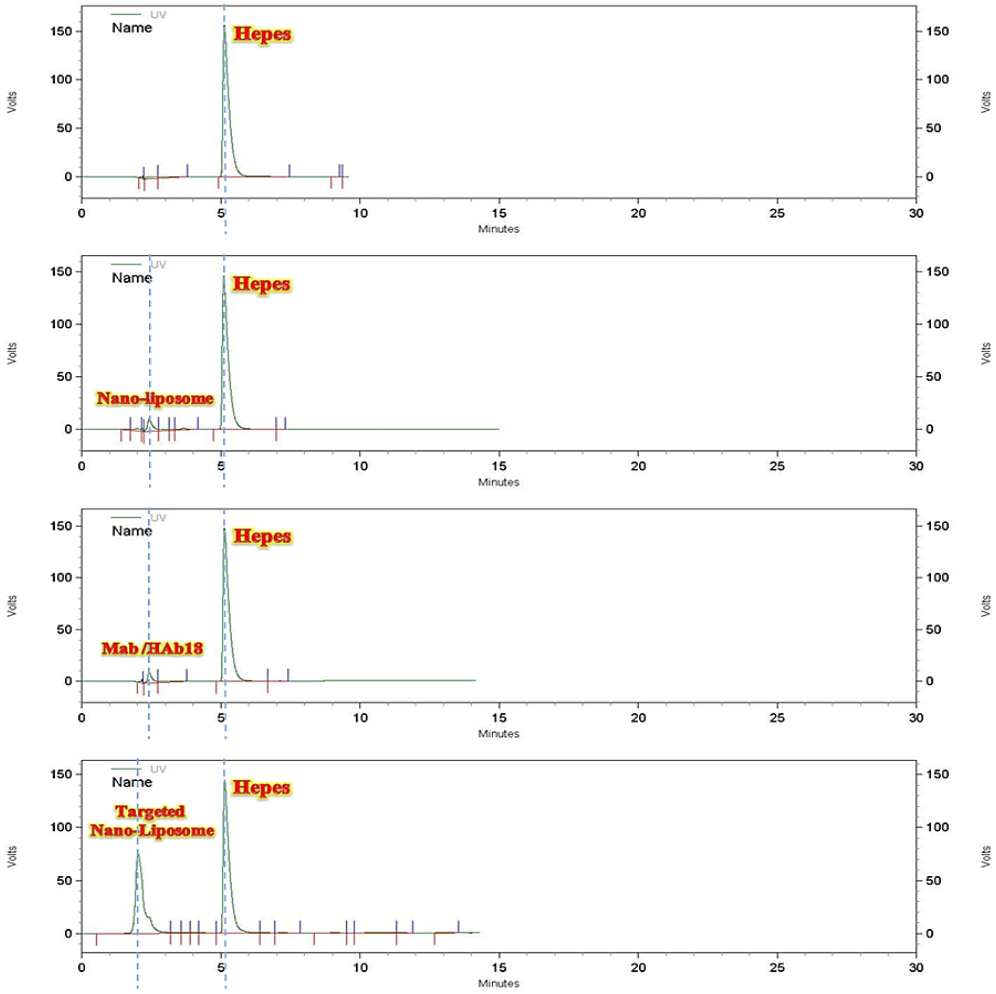

Fig. 18. HPLC peaks of monoclonal antibody conjugation and insertion to nano-liposome

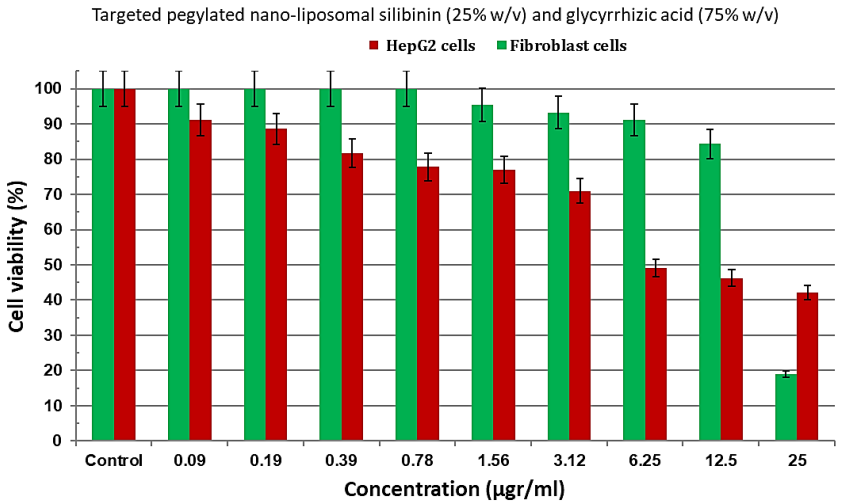

Fig. 19. The cell viability of targeted co-encapsulated pegylated nano-liposomal glycyrrhizic acid (75% w/v) and silibinin (25% w/v) drugs conjugated with HAb18 monoclonal antibody on both HepG2 and fibroblast cell lines. Data are presented as mean ± standard deviation (SD).

Cytotoxicity

The cytotoxic effects of the drug at different concentrations were evaluated using the MTT assay. The highest level of drug release was observed within the first 48 hours, which coincided with the peak in cytotoxic activity. Figure 19 presents a comparative analysis of cell viability between HepG2 and fibroblast cell lines treated with the targeted co-encapsulated PEGylated nanoliposomes containing glycyrrhizic acid (75% w/v) and silibinin (25% w/v), conjugated with the HAb18 monoclonal antibody.

The IC50 values for the HepG2 cell line were as follows:

- Targeted co-encapsulated pegylated nano-liposomal formulation of silibinin and glycyrrhizic acid: 13.7 µg/mL
- Non-targeted pegylated nano-liposomal formulation: 48.67 µg/mL
- Free drug combination: 485.45 µg/mL On the fibroblast cell line, the IC50 values were 22.45, 105.45, and 244.2 µg/mL for the targeted co-encapsulated pegylated nano-liposomal formulation, the non-targeted pegylated nano-liposomal formulation, and the free drug combination, respectively (Figure 20). The PEGylated nanoliposomal formulation containing silibinin and glycyrrhizic acid exhibited nearly double the cytotoxicity against HepG2 cells compared to fibroblast cells.

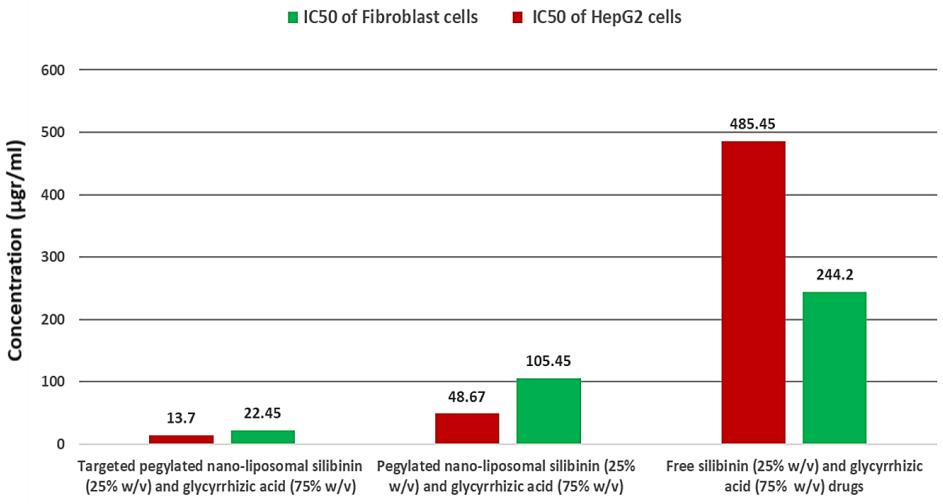

Fig. 20. The half maximal inhibitory concentration (IC50) of targeted co-encapsulated pegylated nano-liposomal silibinin and glycyrrhizic acid drugs with HAb18 monoclonal antibody and co-encapsulated nano-liposomal silibinin and glycyrrhizic acid drugs and free silibinin and glycyrrhizic acid drugs on HepG2 and fibroblast cells. Values are expressed as mean ± standard deviation (SD).

A reduction in the toxicity of targeted liposomes on fibroblast cells was observed compared to their toxicity on cancer cells, so that the IC50 of targeted nanoliposomes containing two drugs on fibroblast cells was determined to be 22.45 µg/ml and on cancer cells was 13.70 µg/ml (with a ratio of 75% glycyrrhizic acid and 25% silibinin). The results show that the cytotoxicity of targeted nanoliposomes containing two drugs on cancer cells is about 1.6 times greater than the cytotoxicity of targeted nanoliposomes containing two drugs on fibroblast cells. A cytotoxicity assessment test on the HepG2 liver cancer cell line shows that the cytotoxicity of the targeted nanoliposome containing two drugs is about 3.5 times the cytotoxicity of the non-targeted nanoliposome containing two drugs and 35 times the cytotoxicity of the free form of the two drug combinations together.

Investigating the binding and cellular uptake of targeted nanoliposomes using fluorescent microscopy:

Given that the surface antigen of the liver cancer cell line HepG2, called CD147, is of the internalizing type, it can bind to the antibody on the surface of the fluorescently labeled nanoliposome and transport it into the cell. Images taken with light and fluorescence microscopy of HepG2 cells after treatment with targeted nanoliposomes carrying two drugs are shown in the figure below.

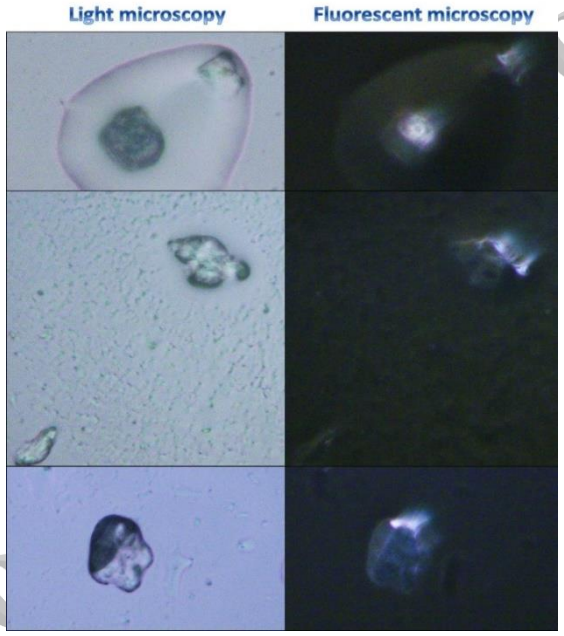

Fig. 21. Images of HepG2 cells after treatment with targeted nanoliposomes carrying two drugs (From left to right: light microscopy, fluorescent microscopy)

The intensity of fluorescent light in the images of HepG2 cells is much higher compared to that in fibroblast cells, indicating that the absorption and penetration of the targeted drug-carrying nanoliposome into HepG2 cells, which have an antigen that binds to the antibody, was higher, which also confirms the evaluations obtained in determining cytotoxicity (Figure 22).

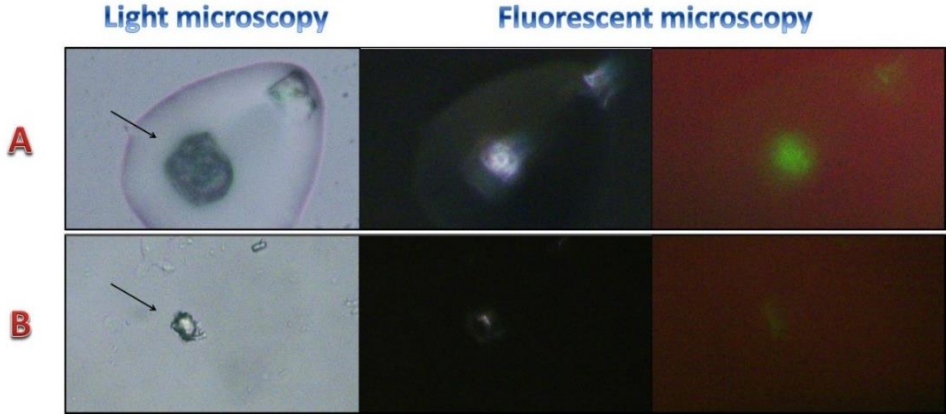

Fig. 22. Comparison of images of HepG2 (A) and fibroblast (B) cell lines after treatment with targeted nanoliposomes carrying two drugs, (From left to right: light microscope, fluorescent microscope, fluorescent microscope with green filter)

DISCUSSION

The produced liposomal nanosystem containing two herbal compounds has suitable physicochemical properties such as: 1- Having an appropriate size of the system, which was about 50 nanometers before targeting and about 85 nanometers after targeting, 2- Having a surface charge of less than –23 mV, 3- Using PEG polymer in the membrane structure has increased biocompatibility and prevented the agglomeration of the system and maintained its stability well for up to three months. Also, the appropriate size of the system and the selection of the appropriate molar ratio of drug to lipid have resulted in this liposomal nanosystem having an appropriate loading efficiency of the two drug compounds and a slow release curve, so that the release rate of the two drug compounds in the produced nanoliposomes for up to 52 hours in an environment with pH = 7.4 (blood plasma pH) at 37 degrees Celsius (body temperature conditions) was measured simultaneously with three replicates by HPLC. The results showed that the highest release of the two drug compounds from the liposomal system occurred in the first 48 hours.

Chu et al. demonstrated that silybin inhibits the invasion and motility of highly metastatic A549 cells while having minimal impact on cell adhesion. Their findings revealed that silymarin achieves these effects by downregulating-expression of MMP-2 and urokinase-type plasminogen activator (u-PA) while upregulating the expression of tissue inhibitor of metalloproteinase-2 (TIMP-2). Silybin was shown to regulate u-PA and MMP-2 at the transcriptional level, while TIMP-2 expression was influenced at either the translational or post-translational level [20]. Moreland and colleagues observed that silymarin helped mitigate certain adverse effects caused by prenatal ethanol exposure in a rat model [21]. Throughout the gestational period, pregnant Fisher/344 rats were fed a liquid diet in which ethanol contributed 35% of the total caloric intake. In parallel, they received co-administration of a silymarin–phospholipid complex containing 29.8% silybin. Beyond its chemopreventive properties, silymarin has shown antitumor activity against human cancers, such as those of the prostate and ovary, in rodent models. Clinical trials have consistently demonstrated that silymarin is both bioavailable and pharmacologically safe. Ongoing research is now focused on validating its clinical efficacy in combating various forms of cancer [22]. Given that both silibinin and glycyrrhizic acid compounds have anticancer properties, and on the other hand, glycyrrhizic acid and its salts also have antiviral and liver-protective properties, encapsulating silibinin with glycyrrhizic acid simultaneously in nanoliposomes has increased the stability and enhanced the anticancer properties of these two compounds. Liposome lipid membranes are also effective in repairing liver tissue membranes damaged by cancer activity. Targeting of nanoliposomes has also been performed to increase silibinin accumulation by delivering and binding precisely to liver cancer cells. This targeting reduces the dosage of the two drug compounds used to treat cancer cells, which is also confirmed by the results of cytotoxicity evaluation and fluorescent microscope images of drug delivery to liver cancer cells.

Targeting the HAb18G/CD147 molecule on HCC cells with the HAb18 monoclonal antibody resulted in a similar inhibitory effect on MMP secretion and cell invasion, while showing no significant influence on cell proliferation. In an orthotopic HCC model using nude mice, HAb18 treatment significantly inhibited tumor growth and metastasis while also reducing the expression of key factors in the HCC microenvironment including vascular endothelial growth factor (VEGF), MMPs, and fibroblast surface protein in the surrounding tissues. These findings highlight the crucial role of HAb18G/CD147 in facilitating HCC invasion and metastasis, primarily by modulating both fibroblasts and HCC cells to disrupt the tumor microenvironment [23].

In 2002, the hexapeptide antagonist G was chemically conjugated to the terminus of PEG through a thioether bond. This modification was carried out within micelles made from Mal-PEG-DSPE, which were subsequently transferred into preformed Stealth liposomes. Across multiple experimental trials, the introduction of antagonist G conjugates led to a marginal increase in the average liposome size, by approximately 3–4 nm. The resulting liposome diameters ranged between 94 and 99 nm [24].

This study's regression analysis revealed that the critical micelle concentration (CMC) for mPEG2000-DSPE mixed with NHS-PEG2000-DSPE was approximately 16.4 μM. The transfer of HAb18 micelle conjugates into nano-liposomes was assessed using nano-liposomes containing 3.51 mol% mPEG. Micelles formed from a 4:1 molar ratio of mPEG2000-DSPE to NHS-PEG2000-DSPE exhibited diameters of 11–12 nm. After coupling with HAb18, the micelle diameter significantly increased, ranging between 33 and 59 nm.

A 2011 investigation focused on targeted micellar systems composed of doxorubicin–poly

(d,l-lactic-co-glycolic acid)–poly (ethylene glycol) (DOX–PLGA–PEG), developed with specificity toward HCC. These micelles were functionalized with the bivalent fragment HAb18 F(ab')2 to enhance their therapeutic efficacy against the disease [1]. The human hepatocarcinoma- specific monoclonal antibody HAb18 was effectively attached to the surface of custom-engineered liposomal microbubbles via electrostatic interactions, leading to the formation of targeted liposomal microbubbles. To achieve this, the HAb18 antibody was proportionally introduced into a suspension of liposome microbubbles and mixed thoroughly for 2 hours under controlled conditions of pH 4.0 and a temperature of 4°C. The resulting liposome microbubbles exhibited an average diameter ranging between 2 and 5 µm [25]. Targeted liposome microbubbles with remarkable biological specificity have been successfully developed. These microbubbles demonstrate a strong affinity for human hepatocarcinoma cells while exhibiting no cytotoxic effects on hepatocytes. The findings suggest that these liposome microbubbles hold great potential as an ultrasound contrast agent specifically for hepatocellular carcinoma (HCC). By improving ultrasound imaging, this innovation could significantly enhance the early detection and diagnosis of HCC [25].

The method of attaching the antibody to the liposome in this study was the (Post-Insertion) method by attaching the functional phospholipid with an NHS ring to the free amine groups of the Ab18 antibody against the CD147 antigen. This method has the least destructive effect on the functional phospholipid and monoclonal antibody compared to other methods. In this study, considering that the amino acid lysine is an alkaline diamine amino acid with a free amine group, if its free amine group is located in the spatial structure adjacent to water molecules and forms a hydrogen bond, it can react with the functional phospholipid NHS ring and be connected form a covalent bond. In this study, the use of the Anti-CD147/HAb18 antibody served to precisely deliver the drug to the cancer cell and to block the activity of the cancer cell antigen, and two herbal medicines are also effective in controlling liver cancer cells according to the results obtained. This study revealed through SEM and TEM imaging that the average diameters of the targeted co-encapsulated nano-liposomes measured approximately 84 nm and 81 nm, respectively, while the monoclonal antibody HAb18 exhibited diameters of around 16.1 nm and 15 nm. Additionally, for Y-shaped antibody biomolecules, slight variations in the lateral dimensions of the Fab or Fc domains were observed across different techniques, with measurements of 8 nm by AFM and 6–7 nm by X-ray and TEM [26]; and through NAMA–STM measurements, the values obtained were approximately 10–11 nm [27]. The measurements of our Fab or Fc domain, as determined by TEM analysis, were approximately 8 nm.

CONCLUSIONS

This study focused on the development of silibinin and glycyrrhizic acid encapsulated nano-liposomes (Nano-Phytosomes) for targeted delivery to liver cancer cells. In this study, the targeting of a liposomal nanosystem containing two herbal active ingredients was carried out using the monoclonal antibody HAb18. Given the specificity of this antibody for the liver cancer cell antigen marker (CD147), this liposomal system can facilitate and direct the delivery of two herbal drug compounds to liver cancer cells in a completely targeted manner. The findings revealed that the average diameter of the Nano-Phytosomes increased when conjugated with the HAb18 monoclonal antibody. Additionally, the results indicated that the targeted co-encapsulated pegylated nano-liposomes and the non-targeted co-encapsulated pegylated nano-liposomes (containing these two herbal compounds) exhibited cytotoxicity levels approximately 35-fold and 10-fold higher, respectively, than the free-form herbal drugs when tested on the HepG2 cell line.

CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relations hips that could have appeared to influence the work reported in this paper.

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