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Nanoliposomal Auraptene: A Comprehensive Study on Preparation, Characterization, Cytotoxicity, and Anti-Angiogenic Potential

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ABSTRACT

Aim: To suppress angiogenesis, auraptene is used in the form of liposome to enhance solubility and effectiveness.

Background: Nanoliposomes are spherical nano-sized capsules enclosed by lipid membranes, serving as a biocompatible vehicle to enhance the delivery of therapeutic agents. **Objective**: The objective of this research is to prepare and characterize nanoliposome-encapsulated auraptene and compare its cytotoxic and anti-angiogenic effects to non-liposomal auraptene.

Methods: Liposomal auraptene was formulated using DSPC/ DSPG/ Cholesterol (molar ratio of 4:1:2) in combination with two different molar ratios of auraptene (0.1 and 0.05). The entrapment efficiency was evaluated using High- Performance Liquid Chromatography (HPLC). Various parameters, including Dynamic Light Scattering (DLS), zeta potential, stability, and release kinetics, were investigated. Subsequently, both liposomal and non-liposomal auraptene, along with bare liposomes, were applied to the MDA-MB-231 cell line for duration of 72 hours at 37°C at varying concentrations. Cytotoxicity was assessed using the MTT assay. Additionally, the study examined the anti-angiogenic effects on the vessels in the chorioallantoic membrane of chick embryos.

Results: The entrapment efficiency of auraptene was found to be satisfactory at 50%. The liposome size ranged from 85 to 241 nm, with a Z-Average of 190.9 nm. The zeta potentials for all formulations were consistently around -55.7, and the Polydispersity Index (PDI) was less than 0.3 for all formulations. The release profile demonstrated approximately 80% drug release over a period of 130 hours. Notably, liposomal auraptene exhibited a significantly lower IC_{50} value (38.61 (95% Confidence Interval: 30.56 to 48.78)) compared to non-liposomal auraptene (50.36 (95% Confidence Interval: 43.58 to 58.19)) ($p = 0.0240$).

Conclusion: Moreover, the administration of 2.5 and 5 µM of liposomal auraptene led to a reduction in the vessels within the chorioallantoic membrane at the injection site when compared to the control group.
In summary, the use of biodegradable nanoliposomal carriers improved the solubility, release profile, and stability of auraptene while demonstrating anticancer and anti-angiogenic properties.

INTRODUCTION

Cardiovascular diseases rank as the leading cause of mortality, with cancer following closely as the second most deadly ailment (1). Consequently, cancer treatment has gained significant prominence in recent decades (2). The natural compound known as auraptene (7-geranyloxycoumarin) made its debut in research in 2011 (3). Auraptene boasts a diverse array of pharmacological properties, including anti-inflammatory effects (4, 5), antioxidant attributes (6), and anticancer potential (7). Studies have demonstrated that auraptene can inhibit transformation, proliferation, angiogenesis, and metastasis in cancer cells while activating apoptosis pathways, particularly in breast and gastric cancer cells (8, 9). However, the clinical

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utility of auraptene has been hampered by its poor water solubility, limited bioavailability, short half- life, rapid metabolism, and chemical instability (10).

Nanoparticles offer a promising avenue for addressing these challenges in drug delivery and cancer detection (11, 12). The objective of nanoparticle- based drug delivery systems is to enhance the bioavailability of lipophilic drugs, achieve a more effective therapeutic index with reduced side effects (13-15), and facilitate drug transport across biological barriers (16). Maintaining a consistent therapeutic concentration of medication in blood plasma within the therapeutic window is typically desired. Nanoliposomes represent a potential drug delivery system capable of regulating drug release profiles. The discovery of liposomes by Bangham and Horne in 1964 marked a pivotal moment in this field (17). Nanoliposomes exhibit biodegradability, biocompatibility, and safety characteristics. Among various nanoparticle platforms, liposomes are suitable for delivering both hydrophilic and lipophilic compounds (18, 19). Their small size allows nanoliposomes to navigate through vascular capillaries and accumulate within tumor tissues, making them a subject of considerable research interest (13, 20). By encapsulating drugs within liposomes, drug distribution can be finely tuned, reducing access to normal tissue while enhancing access to tumor tissue through the enhanced permeability and retention effect. Thus, in our investigation, nanoliposomes were employed to enhance the cytotoxicity of auraptene (21). Clinical studies have consistently shown that liposomes improve the pharmacokinetics and biodistribution of therapeutic drugs (14). The primary objective of this study is to evaluate whether nanoliposomal auraptene addresses the drug's solubility challenge, enhances its cytotoxicity against cancer cell lines, and suppresses angiogenesis.

MATERIALS and METHODS

Materials

Auraptene was obtained from the Department of Pharmacognosy at Mashhad University of Medical Sciences in Mashhad, Iran, through the Pharmaceutical Research Center (PRC). Distearoylphosphatidylcholine (DSPC), distearoylglycerophosphoglycerol (DSPG), and cholesterol were supplied by Avanti Polar Lipid in Alabaster, USA. Fetal bovine serum (FBS), RPMI 1640 (Gibco, Carlsbad, CA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT), chloroform (Merck, Darmstadt, Germany), and HPLC grade methanol (Scharlau, Barcelona, Spain) were utilized in this study. The human colon cancer cell line MDA-MB-231 was provided by the Pasteur Institute of Iran. Specific pathogen-free (SPF) embryonated chicken eggs were supplied by the Razi Vaccine and Serum Research Institute. All other materials and solvents employed were of analytical grade.

Preparation of liposomal auraptene

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved this study under protocol number IR.SBMU.RETECH.REC.1398.595. Liposomal auraptene formulations were prepared using the solvent evaporation method, as described in reference (22).

In this method, two different molar ratios of auraptene (0.1 M and 0.05 M) were dissolved in a mixture of chloroform and methanol (2:1) along with lipids, including DSPC, DSPG, and cholesterol in a 4:1:2 ratios. The organic solvent was then removed using a rotary evaporator (Heidolph, Germany). The resulting lipid film was rehydrated with 10 mM HEPES buffer (pH = 7.0), which was maintained at a temperature between 50-60°C, exceeding the transition temperature of phospholipids. To ensure the uniformity of nanoliposomes, the liposome suspension was extruded through a nucleopore polycarbonate membrane with pore sizes of 400 and 200 nm, using an Avantipolar extruder. Subsequently, the liposomes were subjected to centrifugation at 14,000 rpm for 15 minutes to separate any unencapsulated auraptene.

Particle Size and zeta potential characterization

The particle size and zeta potential of the liposome formulations were assessed using a dynamic light scattering (DLS) spectrophotometer (Zetasizer, Malvern, UK) at room temperature.

HPLC Analysis

Quantitative analysis was conducted via High-Performance Liquid Chromatography (HPLC) using a SHIMADZU system with a C18 column (Shimpack, Tokyo, Japan). The quantification was performed at a detection wavelength of 322 nm. The mobile phase comprised 95% HPLC grade methanol and 5% deionized water containing 0.1% formic acid, with a flow rate of 1 ml/min and an injection volume of 25 µl.

Entrapment Efficiency of Auraptene

To assess the entrapment efficiency of auraptene, following the centrifugation of liposomal formulations, 100 µl of the supernatant was combined with 900 µl of methanol. This step aimed to disrupt the liposomal structure and release the drug into the organic phase. Subsequently, the resulting mixture was injected into the HPLC system, and the quantity of auraptene was determined using a standard curve analysis. The entrapment efficiency of auraptene was computed using the following formula:

$$\%\text{Entrapment Efficiency} = \frac{concentration\ of\ Aurapten\ in\ liposomes}{initial\ concentration\ of\ the\ Auraptene} \times 100$$

In Vitro Drug release study

To investigate the drug release profile, a dialysis sac with a molecular weight cut- off of 12 KD was employed. Liposomes containing auraptene were enclosed in a sealed dialysis bag and immersed in a volume of phosphate buffer solution containing 0.1% tween 80 (pH = 7.4), which was 80 times the sample volume. Subsequently, the setup was placed in a water bath set at 37 °C and stirred magnetically. At specific intervals, samples from the buffer surrounding the dialysis bag were collected. The concentrations of the released drug at different time points were then determined using HPLC and analyzed using the calibration equation for auraptene in PBS buffer. A graphical representation of the drug release kinetics was constructed using the following equation:

$$\% \text{ Cumulative Auraptene Release} = \frac{\text{Auraptene Release}}{\text{Total Entrapped Auraptene}} X\ 100$$

Cell viability assay

The MDA-MB-231 cell line was subjected to treatments involving both liposomal and non-liposomal auraptene, as well as bare liposomes, and 0.25% dimethyl sulfoxide (DMSO) as control groups. MDA-MB-231 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml), and incubated at 37°C in an atmosphere with 95% humidity and 5% carbon dioxide.

Cell viability was assessed using the color density of formazan dye generated through the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) by viable cells. Initially, cell lines were seeded at an initial density of 5 × 10^3 cells per well and incubated for 24 hours at 37°C. Subsequently, the cultured cells were treated for 72 hours with a medium containing auraptene at concentrations ranging from 100 to 5 µM in both liposomal and non-liposomal forms. Additionally, control groups received medium containing only bare liposomes. Following the incubation period, MTT solution at a final concentration of 5 mg/ml was added to all wells, and the cells were further incubated at 37°C for 3 hours. After removing the culture medium, 200 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the insoluble formazan. The absorbance was then measured at 570 nm (with 630 nm as a reference) using an ELISA reader (Bio Tek Inc.).

Angiogenesis study

ROSS eggs with 10-day-old embryos, obtained from the Razi Vaccine and Serum Research Institute, were subjected to disinfection using 70% ethanol. Subsequently, they were placed horizontally in a 37°C environment with 70% humidity within an incubator for duration of 48 hours. Fourteen eggs were divided into seven distinct groups as follows: control (n=2), liposome control (n=2), ethanol (95%) control (n=2), 2.5 µM liposomal auraptene (n=2), 5 µM liposomal auraptene (n=2), 2.5 µM ethanolic auraptene (n=2), and 5 µM ethanolic auraptene (n=2).

On the second day of incubation, a square window measuring 8 x 8 mm was carefully cut into the horizontal surface of the eggs using a razor. Sterilized discs (6.4 mm in inner diameter) were then soaked in 50 microliters of each solution corresponding to the respective egg group. These discs were gently placed through the window created on the chick's chorioallantoic membrane using forceps. After six days, the eggs were removed from the incubator for examination. The seals were broken, and the windows were photographed using a research stereomicroscope. ImageJ software was employed to analyze the captured images, enabling the quantification of the number and length of vascular branches within a 1.5 cm frame surrounding each disc.

Statistical analysis

The IC_{50} values were calculated and subjected to statistical analysis using the log (inhibitor) vs. response variable slope (four parameters) least squares fit method within GraphPad Prism (version: 9.0). Additionally, in the SPSS software, the ANOVA statistical test was utilized to compare the number and length of vascular branches among the different experimental groups.

RESULTS

Characterization of liposomal auraptene

The physicochemical characterization of the liposomal formulations included measurements of size (in nanometers), polydispersity index (PDI), and zeta potential. The liposomes exhibited a size range spanning from 85 to 241 nm, with an average size (Z-Average) of 190.9 nm. The zeta potentials for all formulations were consistently -55.7, and the polydispersity index (PDI) was maintained below 0.35 for all formulations.

Entrapment Efficiency

The entrapment efficiency (EE) of auraptene loaded into nanoliposomes was determined to be 50%, as depicted in Figure 1.

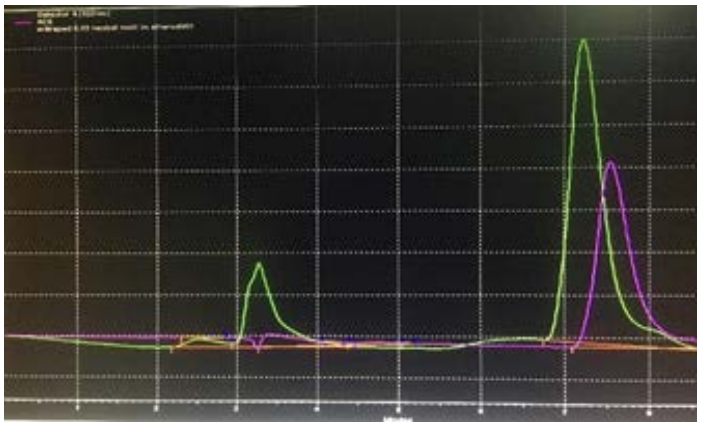


FIGURE 1. Comparison of HPLC Spectra between Liposomal Auraptene and Non-Liposomal Auraptene. The green chromatogram represents non- liposomal auraptene, while the purple chromatogram depicts liposomal auraptene with 50% encapsulation efficacy.

Stability tests

In this study, we examined the physical stability of the liposomal formulations over storage durations of 2 and 4 months at 4°C. The evaluation encompassed visual appearance, size, zeta-potential, polydispersity index (PDI), and encapsulation efficacy (EE), as summarized in Table 1.

Table 1. Stability of Auraptene Nanoliposomes over a 4-Month Period

	Formulation date	After 2 months	After 4 months
Size	190.9	265.1	295.7
PDI	0.344	0.447	0.356
Zeta potential	-55.7	-38.9	-35.5
Encapsulation efficacy (EE)	70	67.5	65.2

PDI: Poly dispersity index

Release profile

The characteristics of the in-vitro release study of the liposomes were examined, as illustrated in Figure 2. It was observed that after 130 hours, approximately 80% of the auraptene had been released into the PBS buffer (pH 7.4). These findings indicate that the liposomal formulations exhibited a sustained release profile.

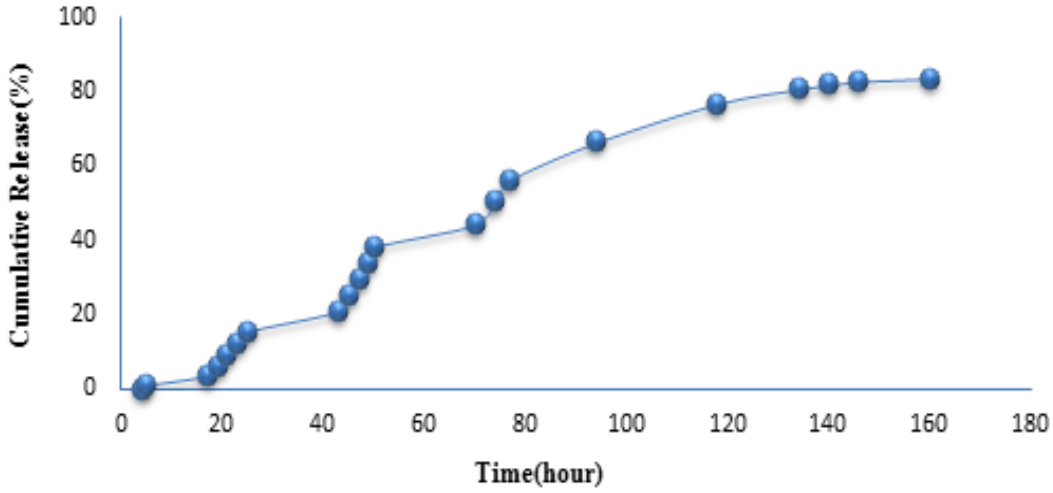


FIGURE 2. The release profile of liposomal auraptene, conducted using the dialysis method, is detailed in the Method section.

In- vitro cytotoxicity study

The cytotoxicity of liposomal auraptene was notably higher in a concentration- dependent manner compared to non-liposomal auraptene, which was dissolved in DMSO (0.25%). Specifically, the IC50 value for liposomal auraptene was calculated to be 38.61 (95% Confidence Interval: 30.56 to 48.78), whereas the IC50 value for non-liposomal auraptene was 50.36 (95% Confidence Interval: 43.58 to 58.19). This difference in IC50 values was statistically significant ($p = 0.0240$), as illustrated in Figure 3.

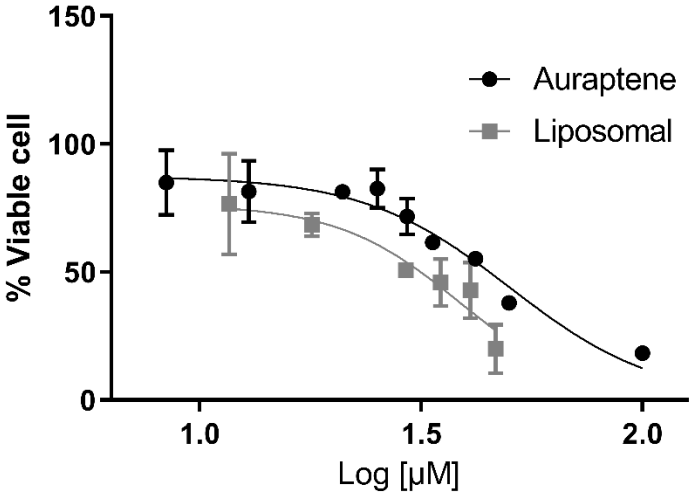


FIGURE 3. Impact of Auraptene on the Viability of the MDA-MB-231 Cell Line. Cells were subjected to treatment with varying concentrations of auraptene (non- liposomal) and liposomal auraptene for duration of 72 hours.

Angiogenesis

Angiogenesis was assessed using the Chicken Chorioallantoic Membrane (CAM) assay. Our findings suggest that auraptene inhibits angiogenesis in a manner dependent on its concentration. Specifically, the length and average number of vessels were significantly reduced in the groups treated with 2.5 and 5 µM liposomal auraptene when compared to those treated with the bare liposome carrier. Similar results were observed in the groups treated with 2.5 and 5 µM ethanolic auraptene in comparison to their respective ethanol solvent controls, as depicted in Figures 4 and 5.

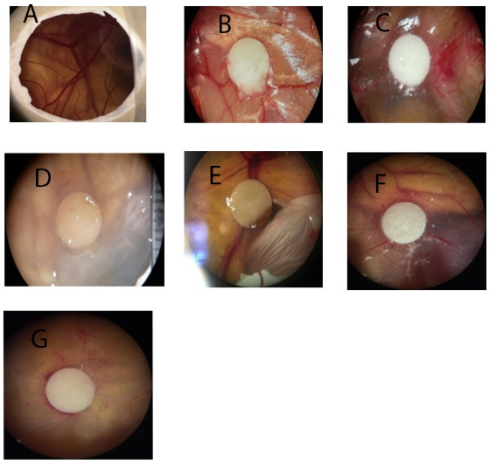
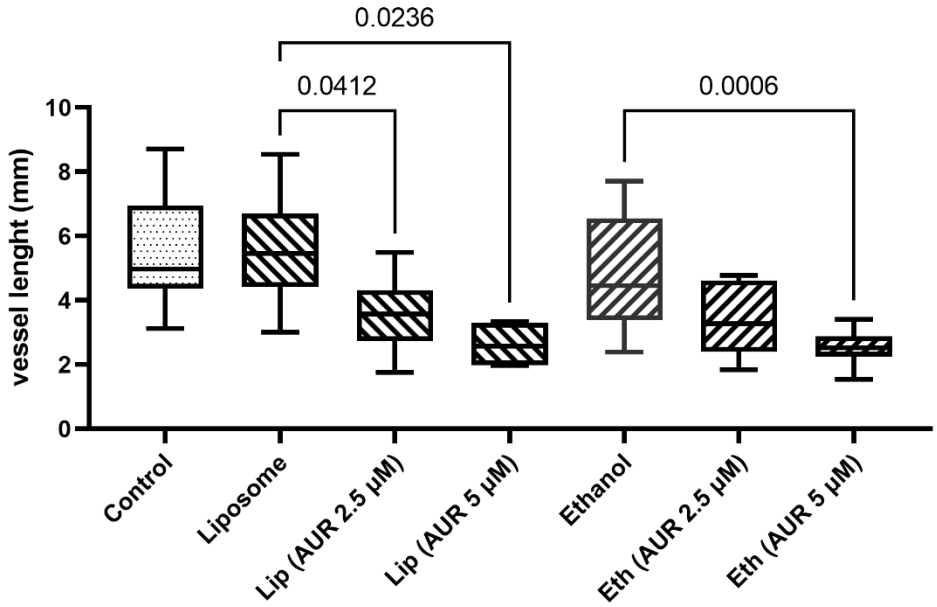


FIGURE 4. This figure presents a comparison of the length of vascular branches at various concentrations of Auraptene in comparison to the solvents and the control group. Additionally, the lower section of the figure displays the average number of vascular branches.



Group	Control	Liposome	Lip (AUR 2.5 µM)	Lip (AUR 5 µM)	Ethanol	Eth (AUR 2.5 µM)	Eth (AUR 5 µM)
Average number of vascular branches	10	9	6	5	8	8	10

Figure 5. A. Control. B. Liposome. C. Aurapten 2.5 µM in liposome solvent. D. Aurapten 5 µM in liposome solvent. E. Ethanol. F. Aurapten 2.5 µM in ethanol solvent. G. Aurapten 5 µM in ethanol solvent.

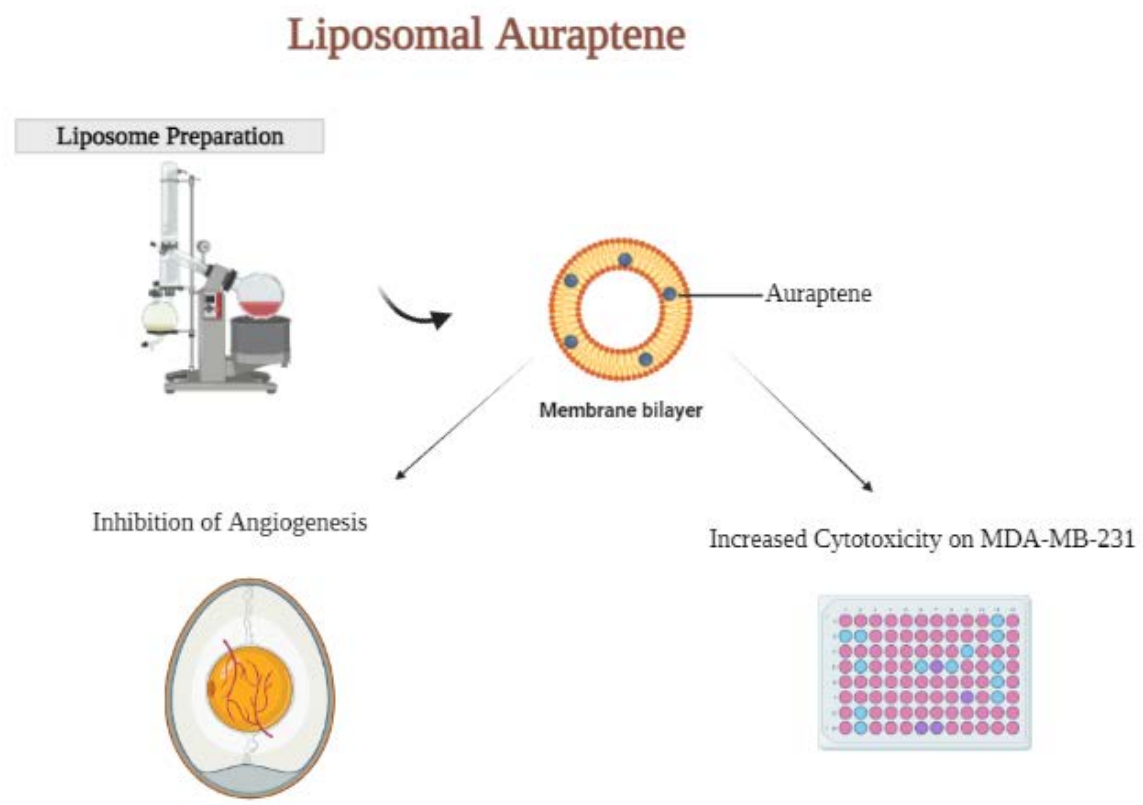


FIGURE 4. Liposomal formulations effectively address the challenge of limited solubility of this compound in water and hydrophilic environments like extracellular fluid and plasma. This enables faster drug uptake by cells and antiangiogenic effects at lower drug concentrations when formulated in liposomal form. Moreover, the stability and release profile of auraptene nanoliposomes proved to be excellent in this study. Our findings indicate that the use of auraptene nanoliposomes enhances cytotoxicity against the MDA-MB-231 cancer cell line while reducing angiogenesis in chorioallantoic membrane capillaries.

DISCUSSION

This study has established that nanoliposomal auraptene, formulated using a combination of DSPC, DSPG, and cholesterol, exhibits favorable characteristics in terms of size within the nanoscale range, stability, entrapment efficiency, and release profile. Notably, liposomal auraptene demonstrated significant inhibitory effects on angiogenesis and a more pronounced suppression of the growth of MDA-MB-231 cancer cells in culture media when compared to nonliposomal auraptene in the bioassay.

Cancer has long been a pressing concern for humanity, and chemotherapy stands as one of the most commonly employed treatments. Salari et al. (23) found that auraptene could serve as an adjunctive therapy in cancer treatment. Their research indicated that the combination of auraptene and ionizing radiation enhanced apoptosis in cultured CT26 cells. Additionally, auraptene, which inhibits the PI3K-Akt-mTOR signaling pathway and caspase-3, was shown to reduce the expression of Cyclin D1 and CD44. However, one limitation of auraptene lies in its limited solubility, a challenge effectively addressed through the use of nanoparticles (10).

Nanotechnology, an interdisciplinary field, holds immense potential in various aspects of cancer biology, encompassing tumor diagnosis, biomarker discovery, and the development of novel therapeutics (24). This rapidly evolving field offers promise for the discovery of innovative cancer therapy approaches (25). The designations of nanoparticles as "targeted" depend on their drug loading capacity, particle and drug stability, and drug release rate. Nanoparticles come in various sizes, shapes, and materials (26). Nanocarriers have demonstrated their ability to efficiently deliver drugs to target cells, and nanoliposomes represent one class of lipid nanocarriers. Liposomes serve to protect drugs from degradation and thereby minimize adverse effects (27).

In this study, the liposomes employed had a size below 250 nm, a dimension previously shown to enhance permeability and durability (21). Notably, tumor blood vessels exhibit larger gaps (100-600 nm) compared to normal endothelium, typically only 6 nm wide. As a result, the synthesized liposomal auraptene may possess the capacity to target a greater number of tumor cells within malignant tissues (28).

Nanoliposomes with particle sizes around 190 nm offer a loading capacity of approximately 70% and follow a stepwise release mechanism (29). The nanoliposome containing auraptene displayed a prolonged blood half-life compared to the drug in its pure state, with the data indicating a 50% loading efficiency.

In the bioassay, liposomal auraptene exhibited superior efficacy compared to nonliposomal auraptene in terms of cell toxicity. The concentration-response curve shifted to the left, underscoring the heightened potency of liposomal auraptene.

Finally, owing to auraptene's poor solubility in water and hydrophilic media such as extracellular fluid and plasma in vivo, demonstrating its antiangiogenic effects on cell-level angiogenesis becomes challenging (9). However, in our investigation, the liposomal formulation of auraptene displayed a concentration-dependent anti-angiogenic impact, significantly reducing chorioallantoic vessels when compared to both the control group and the unencapsulated liposomal carrier.

CONCLUSION

This study has shown that utilizing nanoliposomes as carriers for auraptene effectively addresses the issue of drug solubility while simultaneously enhancing its size, stability, entrapment efficiency, release profile, and its biological effects, including cytotoxicity on cancer cell lines and the suppression of angiogenesis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. Financial support for this study was provided by Shahid Beheshti University of Medical Sciences School of Medicine. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Conceptualization: (Seyed Ali Ziai); Methodology: (Tara Emami, Roghaieh Tarasi, Shiva Ghafghazi, Mohammad-Abbas Sheikholeslami, Fatemeh Kalhor, Seyed Ali Ziai); Formal analysis and investigation: (Roghaieh Tarasi, Fatemeh Kalhor, Seyed Ali Ziai); Writing- original draft preparation: (Tara Emami, Roghaieh Tarasi, Shiva Ghafghazi,); Writing - review and editing: (Seyed Ali Ziai); Funding acquisition: (Seyed Ali Ziai); Resources: (Mohammad-Abbas Sheikholeslami, Seyed Ali Ziai); Supervision: (Seyed Ali Ziai)

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