doi.org/10.36721/PJPS.2024.37.1.REG.139-145.1

Formulation design and characterization of silymarin liposomes for enhanced antitumor activity

Zhongcheng Ke[1,2,3], Xiaoling Cheng[4], Huimin Yang[1,2], Yimin Niu[1,2], Xu Cheng[1,2], Ting Ye[1,2], Guangying Sun[1,2], Ziyang Cheng[1,2] and Yinyu Sun[1,2]*

[1]College of Chemistry and Chemical Engineering, Huangshan University, Huangshan, Anhui, China
[2]Research Center of Chinese Medicine Efficacy and Health Technology, Huangshan University, Huangshan, Anhui, China
[3]College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China
[4]Health Supervision Institute, Tunxi District Health Bureau, Huangshan, Anhui, China

Abstract: Liposomes, a nanoscale carrier, plays an important role in the delivery of drug, affects the in vivo efficacy of drugs. In this paper, silymarin(SM)-loaded liposomes was optimized using the response surface method (RSM), with entrapment efficiency (EE%) as an index. The formulation was optimized as follow: lecithin (7.8mg/mL), SM/lecithin (1/26) and lecithin/cholesterol (10/1). The optimized SM liposomes had a high EE (96.58±3.06%), with a particle size of 290.3±10.5nm and a zeta potential of +22.98±1.73mV. *In vitro* release tests revealed that SM was released in a sustained-release manner, primarily via diffusion mechanism. *In vitro* cytotoxicity studies demonstrated that the prepared SM liposomes had stronger inhibitory effects than the model drug. Overall, these results indicate that this liposome system is suitable for intravenous delivery to enhance the antitumor effects of SM.

Keywords: Silymarin, liposomes, response surface method, entrapment efficiency, cytotoxicity.

INTRODUCTION

According to the GLOBOCAN 2020 data, approximately 1.76 million deaths from lung cancer occur worldwide each year and the treatment of lung cancer faces enormous challenges (Global Cancer Observatory. 2022). Searching for new compounds to treat cancer has been the goal of numerous studies but is a time-consuming and low-success process; therefore, exploring new uses of existing drugs has become a novel approach for drug development (Parvathaneni *et al.,* 2019; Aggarwal *et al.,* 2021).

Silymarin (SM), an extract isolated from the plant *Silybum marianum*, has been widely applied in the hepatitis treatment (Kiruthiga *et al.,* 2014; Lim *et al.,* 2022; Zaidi SNF *et al.,* 2017). Previous researches have confirmed that it has good anticancer activity against lung cancer (Xu *et al.,* 2022; Wu *et al.,* 2016). Regrettably, oral bioavailability is limited owing to its poor solubility. Accordingly, relevant studies on solid dispersions (Lim *et al.,* 2022), nanoemulsions (Ahmad *et al.,* 2018), nanosuspensions (Chi *et al.,* 2022) and liposomes (Elmowafy *et al.,* 2013) have been conducted. Liposomes are nano-bimolecular structures formed by the directional arrangement of phospholipid molecules. There are a few reports on the use of silymarin liposomes for liver protection (Mohsen *et al.,* 2017; El-Samaligy *et al.,* 2006), but thus far, the anti-tumor application of silymarin liposomes has not been studied.

Owing to the enhanced permeability and retention (EPR) effect, liposomes can passively accumulate in tumors to increase anti-tumor activity. In addition, owing to the advantages of the lipid bilayer for internally encapsulating water-insoluble drugs and providing external water compatibility, the application of liposomes is becoming increasingly popular. For excellent liposome performance, a high encapsulation efficiency of the drug is essential and is always used as a dependent variable for prescription optimization (Hwang *et al.,* 2012; Yang *et al.,* 2012). Compared with the linear mathematical model fitting of an orthogonal design, the advanced RSM utilizes experimental data to solve multivariate equations to optimize formulations or processes (Khan Q *et al.,* 2021; Chmiel *et al.,* 2017). RSM has been widely used to optimize various liposome formulations, including glycyrrhiza polysaccharide liposomes (Wu *et al.,* 2017), madecassoside liposomes (Wang *et al.,* 2014), and quercetin liposomes (Jangde *et al.,* 2016).

Base on this, a novel SM-loaded liposome was designed for anti-tumor application using RSM. The physicochemical properties of SM liposomes, such as particle size, zeta potential, entrapment efficiency (EE(%)) and *in vitro* release profile, were evaluated. In addition, *in vitro* anti-tumor effect was also evaluated.

MATERIALS AND METHODS

Materials

Silymarin (Beijing Institute for Food and Drug Control), lecithin (Macklin Co., Ltd.), cholesterol (Macklin Co., Ltd.), and anhydrous ethanol (AR; Bohr Chemical Reagent Co., Ltd.) were of analytical grade.

**Corresponding author:* e-mail: yysun_hsu@163.com

Preparation of SM liposomes

SM-loaded liposomes were prepared using the ethanol injection method. In brief, SM, lecithin, and cholesterol were accurately weighed and dissolved in quantitative ethanol. Under magnetic stirring at 35ºC, quantitative ethanol solution was slowly injected into a 50mL buffer system. After stirring for 30 min, ethanol was removed by rotary evaporation and rapidly cooled to obtain SM-loaded liposomes.

Determination of entrapment efficiency

The SM concentration was measured by UV-visible (UV-Vis) spectroscopy (Kandimalla *et al.,* 2017). In brief, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0mL of standard solution were accurately transferred to 10mL flasks and diluted to the scale. Then, absorbance (A) was determined at 288 nm, and the concentration (C) was calculated using standard equation A= 0.045C + 0.0058 (R^2=0.9998).

The EE (%) of SM liposomes was determined as follows (Tran *et al.,* 2019). Diluted silymarin liposomes (5mL) were transferred to ultrafiltration tubes and centrifuged at 5,000r/min. After 12min, quantitative supernatant was taken out to measure the absorbance and calculate the EE (%) of SM.

Preliminary preparation experiments

A pre-experiment was conducted using a single-factor set up. The values of three factors were fixed and the range of the other factor was optimized. For example, silymarin, cholesterol, and buffer solution pH were fixed at 0.10g, 0.20g, 7.30, respectively; only lecithin (g/mL) was changed to 2, 4, 6, 8 and 10g/L. The experiments were conducted according to the parameters in table 1.

Box-Behnken design

Based on the preliminary experiments, the Box–Behnken design was used to optimize the formulation of SM-loaded liposomes (Khatib *et al.,* 2021). SM, cholesterol, and lecithin were set as arguments, each factor was set at three levels, with code values of -1, 0 and 1 and the EE (%) was used as the dependent variable. The actual operating values represented by the code values are shown in table 2 and the experimental design and related results are also shown in table 2.

Determine of particle sizes and zeta-potentials

Using a Malvern Zetasizer Nano ZS instrument, particle size and zeta potentials of SM liposomes were determined and each assay was performed in triplicate.

Morphology

Transmission electron microscopy (TEM, JEOL-2100, JEOL, Japan) was applied to observe the morphology, Prior to analysis, the diluted solution of SM liposomes were pasted on a copper grid and dried in the presence of UV lamp, followed by negatively staining with phosphotungstic acid.

Storage stability

The storage stability was evaluated by storing the SM liposomes at 4ºC for 30 d and the particle size changes was monitored throughout this entire period.

In vitro drug release

*In vitro r*elease of SM was determined using the dialysis method (Ke *et al.,* 2018). Briefly, an aliquot of 1mL of SM-loaded liposomes was transferred into a dialysis bag (MW 10 kDa), which was then placed in 50mL buffer solution at pH 7.4 and the whole set was placed in a 37 incubator shaker with a rate of 100 rpm and the samples were withdrawn at preset time to determine the percentage of drug released.

In vitro cytotoxicity study

A549 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After incubation of 24h, the culture medium was replaced with free SM or SM liposomes, and the cells were incubated for another 24h. After washing with PBS three times, 100μL of MTT solution was added into each well and incubated for 4h. Finally, medium was removed, and 100μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a CMax Plus Molecular Devices (Molecular Devices, USA).

STATISTICAL ANALYSIS

All data are expressed as mean ± standard deviation. Statistical significance was determined using Student's t-test using SPSS 21.0 software, with statistical significance set at $p<0.05$.

RESULTS

Preliminary experiments

To obtain the range of the factors, a single-factor experiment with four factors was conducted. Throughout these experiments, all factors were held constant, except for one, which was manipulated.

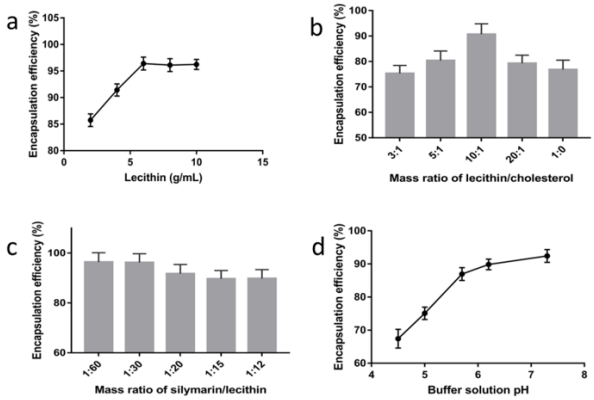

Fig. 1: Single factor experiments for the preparation of SM-loaded liposomes (a, lecithin; b, cholesterol; c, mass ratio of SM/lecithin; d, buffer solution pH).

Table 1: Preliminary experiments for the preparation of SM-loaded liposomes.

Factors	Mass ratio of SM/lecithin	Lecithin (mg/mL)	Mass ratio of lecithin/cholesterol	Buffer pH
levels	1:60	2	3:1	7.3
	1:30	4	5:1	6.2
	1:20	6	10:1	5.7
	1:15	8	20:1	5.0
	1:12	10	1:0	4.5

Table 2: Comparison of code values and actual values for factors and levels.

levels	Mass ratio of SM/lecithin	Lecithin (mg/mL)	Mass ratio of lecithin/cholesterol
1	1:40	4	8:1
0	1:30	6	10:1
-1	1:20	8	12:1

Table 3: Box–Behnken design and experimental data for preparation of silymarin-loaded liposomes.

Run	SM/lecithin	Lecithin (mg/mL)	lecithin/cholesterol	EE (%)
1	1/40	6	8	78.25 ± 3.17
2	1/30	8	8	93.52 ± 3.65
3	1/30	6	10	96.87 ± 4.02
4	1/20	8	10	90.13 ± 3.87
5	1/30	6	10	91.88 ± 4.14
6	1/30	6	10	92.62 ± 3.43
7	1/30	6	10	95.12 ± 3.51
8	1/20	6	12	85.28 ± 3.62
9	1/20	6	8	80.72 ± 2.73
10	1/30	4	8	82.91 ± 3.64
11	1/30	8	12	90.19 ± 3.83
12	1/30	4	12	88.17 ± 3.10
13	1/40	6	12	76.73 ± 2.86
14	1/40	4	10	78.54 ± 3.34
15	1/20	4	10	92.41 ± 3.61
16	1/30	6	10	93.24 ± 3.19
17	1/40	8	10	88.22 ± 2.89

Table 4: ANOVA analysis and statistical parameters of the model.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	627.68	9	69.74	21.29	0.0003
X_1	89.98	1	89.98	27.46	0.0012
X_2	50.00	1	50.00	15.26	0.0058
X_3	3.09	1	3.09	0.9424	0.3640
$X_1 X_2$	35.94	1	35.94	10.97	0.0129
$X_1 X_3$	9.24	1	9.24	2.82	0.1369
$X_2 X_3$	18.45	1	18.45	5.63	0.0494
X_1^2	238.93	1	238.93	72.93	< 0.0001
X_2^2	3.56	1	3.56	1.09	0.3319
X_3^2	160.19	1	160.19	48.89	0.0002
Residual	22.93	7	3.28		
Lack of Fit	6.48	3	2.16	0.5252	0.6881
Pure Error	16.45	4	4.11		
Cor Total	650.61	16			

Table 5: Release kinetics of SM from the optimized liposomes.

Model	Equation	Correlation coefficient
Zero-order	Mt/M=0.0219 t + 0.2146	R^2 = 0.7386
First-order	ln(1- Mt/M) = -0.384 t -0.2348	R^2 = 0.8774
Higuchi	Mt/M=0.1252 $t^{1/2}$ + 0:0933	R^2 = 0.9395

As shown in fig. 1a, the drug EE (%) augmented as lecithin level increased. when the content of lecithin was 6g/mL, the EE (%) was the highest at 93.40±1.23%. Increasing lecithin concentration had little effect on the EE (%) of liposomes; therefore, the phospholipid concentration range was set at 4-8mg/mL.

As shown in fig. 1b, the EE (%) increased with cholesterol concentration. When the mass ratio of lecithin/cholesterol was 10:1, the EE (%) was the highest at 90.68±4.12%. The further increase in cholesterol content has a negative impact on the EE (%) of SM liposomes. Therefore, the lecithin/cholesterol mass ratio was set to 8:1-12:1.

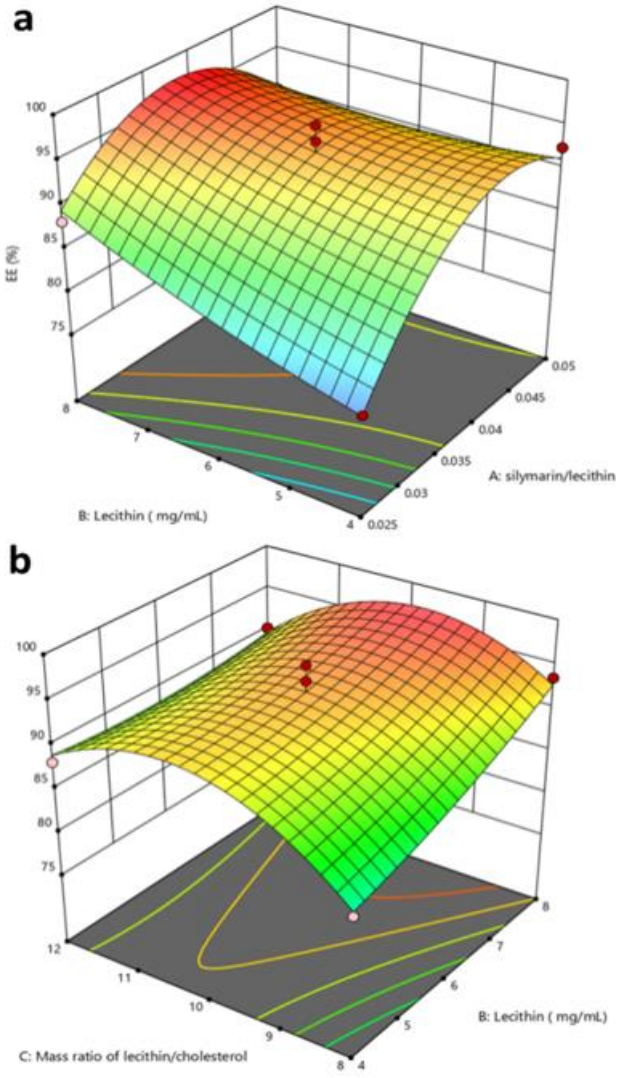

Fig. 2: 3D-response surface plots for the graphical optimization of SM-loaded liposomes, illustrating the interaction of (a) the mass ratio of SM/lecithin and lecithin (mg/mL) on EE(%) and (b) lecithin (mg/mL) and the mass ratio of lecithin/cholesterol on EE(%).

As shown in fig. 1c, the EE (%) was greater than 96% when the mass ratio of SM/lecithin was less than 1:30. However, the EE (%) decreased with a further increase in the mass ratio of SM/lecithin. Therefore, the mass ratio of SM/lecithin was set at 1:20-1:40.

As shown in fig. 1d, the drug EE (%) gradually augmented with an increase in pH of the buffer solution. When the pH was higher than 6.0, a minimal influence was observed, and the EE (%) was 92.41±1.93% when the pH was 7.3. Therefore, pH of the buffer solution was set to 7.3.

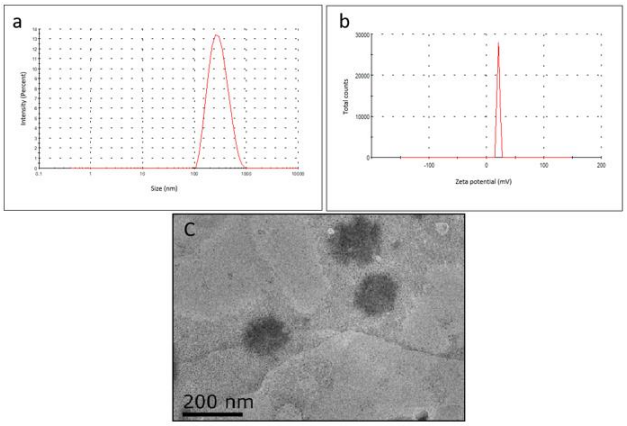

Fig. 3: Size (a), zeta-potential (b) morphology (c) of the optimized SM-loaded liposomes.

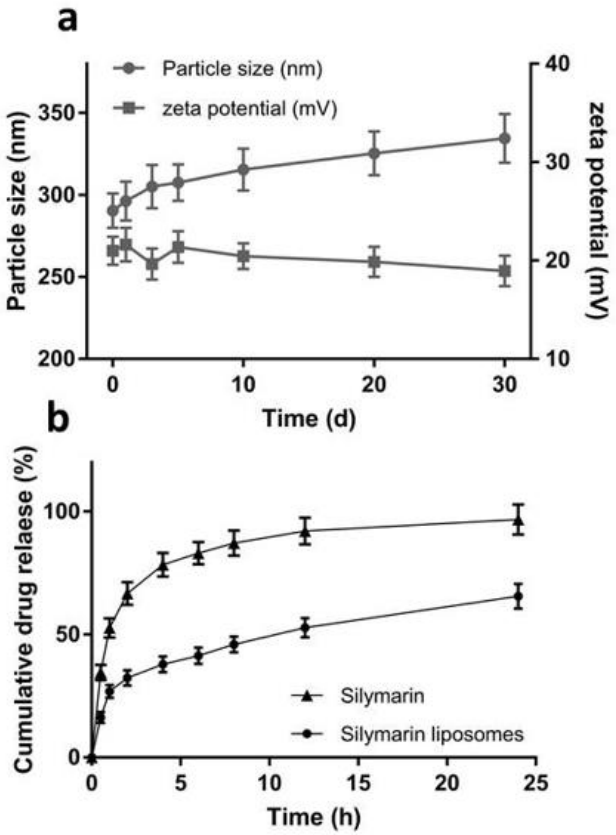

Fig. 4: Stability profile (a) and in vitro release behavior (b) of SM-loaded liposomes at 4ºC.

Box-Behnken design of SM-loaded liposomes

Table 3 shows the 17 experimental runs performed for the optimization of SM-loaded liposomes, together with the observed EE (%). The responses (Y) were fit to a multiple regression analysis to obtain the following quadratic polynomial equations (table 3):

$Y = 93.95 + 3.35\ X_1 + 2.50\ X_2 + 0.6213\ X_3 - 3\ X_1X_2 + 1.52\ X_1X_3 - 2.15\ X_2X_3 - 7.53\ X_1^2 + 0.9195\ X_2^2 - 6.17\ X_3^2$

The model F-value of 21.29 demonstrated that the suggested quadratic model was significant. A p-value less than 0.05 indicates that the model factor was significant, so EE (%) was significantly influenced by X_1 (mass ratio of SM/lecithin) and X_2 (concentration of lecithin), whereas the effect of X_3 (mass ratio of lecithin/ cholesterol) was not statistically significant (table 4). Of the three independent variables, X_1 showed the most prominent positive effect on the EE (%) of SM-loaded liposomes. In addition, the interaction between X_1 and X_2 had a significant negative impact on EE (%), as well as the interaction between X_2 and X_3.

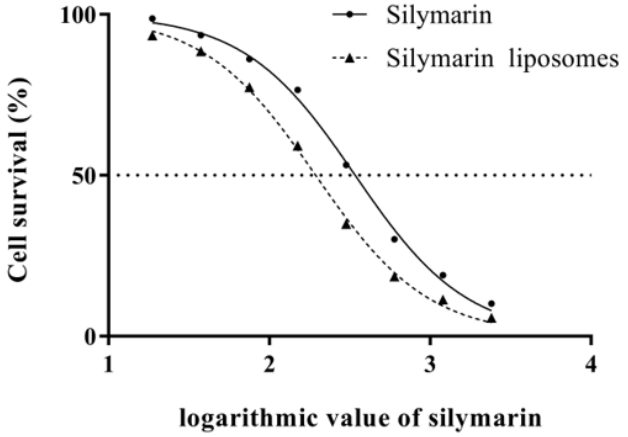

Fig. 5: *In vitro* cytotoxicity of SM-loaded liposomes and free SM.

Using this mathematical model, it was predicted that the optimal combination was lecithin (7.77mg/mL), SM/lecithin (0.03837), lecithin/cholesterol (9.926), and maximum EE (%) of 96.90%. Based on the principle of convenient operation, the process conditions were adjusted to lecithin (7.8mg/mL), SM/lecithin (1/26) and lecithin/cholesterol (10/1) for validation experiments. The EE (%) was determined to be 96.58±3.06%. Therefore, the established mathematical model has reliable predictability and the selected process conditions have high reproducibility.

Particle sizes and zeta-potentials

Size and zeta potential of SM-loaded liposomes were measured in sequence. As shown in fig. 3a and fig. 3b, SM-loaded liposomes had a zeta potential of +22.98± 1.73 mV and a particle size of 290.3±10.5 nm.

Morphology analysis

Through TEM observation, SM liposomes are sphere like particles, with a size about 250nm (fig. 3c), which was consistent with the result of size distributions.

Storage stability

During a 30-day test period, no apparent turbidity was observed in the SM-loaded liposomes stored at 4ºC. Besides, no obvious changes in particle size or zeta potential indicated that the SM liposomes possessed satisfactory stability. Additionally, the zeta potential was greater than 20mV (fig. 4a), indicating that SM liposomes exhibit a low aggregation tendency, which is due to the electrostatic repulsion between particles.

In vitro drug release

For investigating in vitro release behavior of SM in medium of pH 7.4, 5% sodium dodecyl sulfate was used to facilitate drug release. Data showed that 96.74±6.11% of SM was released from the suspension in 24h. In comparison, the optimized SM liposomes presented 41.32 ±3.31% (6h) and 65.57±4.90% (24h) of cumulative release at pH 7.4 (fig. 4b), which meets the needs of long-term blood circulation and passive tumor targeting (Yang *et al.,* 2015; Zhang *et al.,* 2019).

Various mathematical models were used to evaluate the mechanisms of SM released from liposomes. As shown in Tab 5, using mathematical models such as Zero order, First order, and Higuchi to fit time and cumulative drug release, the correlation coefficient (R) ²were 0.7386, 0.8774 and 0.9395, respectively.

In vitro cytotoxicity of SM liposomes

A MTT assay was performed to explore the cytotoxicity of SM liposomes. The IC_{50} values of SM, SM liposomes in A549 cells were 341.6±14.7μg/mL and 193.9±8.8 μg/mL, which indicates that the designed liposomes obviously improve the cytotoxicity of SM against A549 cells.

DISCUSSION

The lipid liposome bilayer was mainly composed of lecithin, and the addition of cholesterol improves membrane fluidity and liposome stability (Kaddah *et al.*, 2018; Kaddah *et al*., 2021). SM, an insoluble substance, was expected to insert into the lipid bilayer to form a uniform liposome system. When the dosages of drugs, lecithin, and cholesterol were appropriate, stable liposomes with high EE (%) were obtained (Maherani *et al.*, 2012; Huang *et al*., 2014).

Besides, no obvious changes in particle size or zeta potential indicated that the SM liposomes possessed satisfactory stability in a 30-day test period. Liposomes with a particle size of around 300 nm are

beneficial for passive tumor targeting based on EPR effect, improving the in vivo therapeutic effect of drugs. Additionally, the zeta potential was greater than 20 mV (fig. 4a), indicating that SM liposomes exhibited a low aggregation tendency, which was due to the electrostatic repulsion between particles.

Various mathematical models were used to evaluate the mechanisms of SM released from liposomes, and the best fit was obtained with Higuchi model, indicating that the encapsulated SM was mainly released by diffusion pathway (Mehanna *et al*., 2009; Kala *et al*., 2022). The IC50 value of SM liposomes in A549 cells is almost half that of the SM group, due to the hydrophobic bilayer structure of liposomes and the cell biocompatibility of phospholipids, which increases the solubility and cellular uptake of SM liposomes, resulting in a significant increase in anti-tumor activity.

CONCLUSION

SM-loaded liposomes with a high EE (%) was developed using Box–Behnken method. The predicted and experimental values of EE (%) were basically equivalent, which suggests that the response surface methodology was reliable. The formulations were as follows: lecithin (7.8mg/mL), SM/lecithin (1/26) and lecithin/cholesterol (10/1). *In vitro* release profile revealed that SM was released in a sustained manner, primarily via a diffusion mechanism. Finally, *in vitro* toxicity test revealed that the SM liposomes possess stronger inhibitory effects than the free drug. Therefore, the SM-loaded liposomes developed in this study have good application potential and can serve as a reference for the anti-tumor applications of hydrophobic drugs.

ACKNOWLEDGEMENTS

This work was financially supported by Anhui Provincial Natural Science Foundation (2008085MH269); National College Student Innovation Training Program, Natural Science Research Project of Anhui Educational Committee (2022AH051946, 2022AH051954, KJHS2021B03).

REFERENCES

Aggarwal S, Verma SS, Aggarwal S and Gupta SC (2021). Drug repurposing for breast cancer therapy: Old weapon for new battle. *Semin Cancer Biol.,* **68**: 8-20.

Ahmad U, Akhtar J, Singh SP, Ahmad FJ and Siddiqui S (2018). Silymarin nanoemulsion against human hepatocellular carcinoma: development and optimization. *Artif. Cells Nanomed. Biotechnol.,* **46**(2): 231-241.

Chi C, Zhang C, Liu Y, Nie H, Zhou J and Ding Y (2022). Phytosome-nanosuspensions for silybin-phospholipid complex with increased bioavailability and hepatoprotection efficacy. *Eur. J. Pharm. Sci.,* **144**: 105212

Chmiel T, Kupska M, Wardencki W and Namieśnik J (2017). Application of response surface methodology to optimize solid-phase microextraction procedure for chromatographic determination of aroma-active monoterpenes in berries. *Food Chem.,* **221**: 1041-1056.

Elmowafy M, Viitala T, Ibrahim HM, Abu-Elyazid SK, Samy A, Kassem A and Yliperttula M (2013). Silymarin loaded liposomes for hepatic targeting: in vitro evaluation and HepG2 drug uptake. *Eur. J. Pharm. Sci.,* **50**(2): 161-171.

El-Samaligy MS, Afifi NN and Mahmoud EA (2006). Evaluation of hybrid liposomes-encapsulated silymarin regarding physical stability and *in vivo* performance. *Int. J. Pharm.,* **319**(1-2): 121-129.

Global Cancer Observatory (2022). Cancer. [accessed 2022 Feb 3]. https://www.who.int/news-room/fact-sheets/detail/cancer.

Huang Y, Wu C, Liu Z, Hu Y, Shi C, Yu Y, Zhao X, Liu C, Liu J and Wu Y (2014). Optimization on preparation conditions of Rehmannia glutinosa polysaccharide liposome and its immunological activity. *Carbohydr. Polym.,* **104**: 118-126.

Hwang SY, Kim HK, Choo J, Seong GH, Hien TB and Lee EK (2012). Effects of operating parameters on the efficiency of liposomal encapsulation of enzymes. *Colloids Surf. B Biointerfaces,* **94**: 296-303.

Jangde R and Singh D (2016). Preparation and optimization of quercetin-loaded liposomes for wound healing, using response surface methodology. *Artif. Cells Nanomed. Biotechnol.,* **44**(2): 635-41.

Kaddah S, Khreich N, Kaddah F, Charcosset C and Greige-Gerges H (2018). Cholesterol modulates the liposome membrane fluidity and permeability for a hydrophilic molecule. *Food Chem. Toxicol.,* **113**: 40-48.

Kaddah S, Khreich N, Kaddah F, Charcosset C and Greige-Gerges H (2021). Pentacyclic triterpenes modulate liposome membrane fluidity and permeability depending on membrane cholesterol content. *Int. J. Pharm.,* **610**: 121232.

Kala SG and Chinni S (2022). Bioavailability enhancement of vitamin E TPGS liposomes of nintedanib esylate: Formulation optimization, cytotoxicity and pharmacokinetic studies. *Drug Delv. Transl. Res.,* **12**(11): 2856-2864.

Kandimalla R, Dash S, Bhowal AC, Kalita S, Talukdar NC, Kundu S and Kotoky J (2017). Glycogen-gold nanohybrid escalates the potency of silymarin. *Int. J. Nanomedicine,* **12**: 7025-7038.

Ke Z, Yang L, Wu H, Li Z, Jia X and Zhang Z (2018). Evaluation of *in vitro* and *in vivo* antitumor effects of gambogic acid-loaded layer-by-layer self-assembled micelles. *Int. J. Pharm.,* **545**(1-2): 306-317.

Khan Q, Shah SNH, Arshad MS, Usman F, Khalil R, Ul-Haq Z, Siddiqui FA, Hussain T, Yousaf AM, Rizvi SA and Shahzad Y (2021). Formulation and optimization of dimenhydrinate emulgels for topical delivery using response surface methodology. *Pak. J. Pharm. Sci.,* **34**(1): 245-255.

Khatib I, Chow MYT, Ruan J, Cipolla D and Chan HK (2021). Modeling of a spray drying method to produce ciprofloxacin nanocrystals inside the liposomes utilizing a response surface methodology: Box-Behnken experimental design. *Int. J. Pharm.,* **597**: 120277.

Kiruthiga PV, Karutha Pandian S and Pandima Devi K (2014). Silymarin prevents the toxicity induced by benzo (a) pyrene in human erythrocytes by preserving its membrane integrity: An *in vitro* study. *Environ. Toxicol.,* **29**(2): 165-175.

Lim DY, Pang M, Lee J, Lee J, Jeon JH, Park JH, Choi MK and Song IS (2022). Enhanced bioavailability and hepatoprotective effect of silymarin by preparing silymarin-loaded solid dispersion formulation using freeze-drying method. *Arch. Pharm. Res.,* **45**(10): 743-760.

Maherani B, Arab-tehrany E, Kheirolomoom A, Reshetov V, Stebe MJ and Linder M (2012). Optimization and characterization of liposome formulation by mixture design. *Analyst.*, **137**(3): 773-786.

Mehanna MM, Elmaradny HA and Samaha MW (2009). Ciprofloxacin liposomes as vesicular reservoirs for ocular delivery: Formulation, optimization, and *in vitro* characterization. *Drug Dev. Ind. Pharm.,* **35**(5): 583-593.

Mohsen AM, Asfour MH and Salama AAA (2017). Improved hepatoprotective activity of silymarin via encapsulation in the novel vesicular nanosystem bilosomes. *Drug Dev. Ind. Pharm.,* **43**(12): 2043-2054.

Parvathaneni V, Kulkarni NS, Muth A and Gupta V (2019). Drug repurposing: A promising tool to accelerate the drug discovery process. *Drug Discov. Today,* **24**(10): 2076-2085.

Tran BH, Yu Y, Chang L, Tan B, Jia W, Xiong Y, Dai T, Zhong R, Zhang W and Le VM (2019). A novel liposomal S-propargyl-cysteine: A sustained release of hydrogen sulfide reducing myocardial fibrosis via TGF-β1/Smad pathway. *Int. J. Nanomedicine.,* **14**: 10061-10077.

Wang H, Liu M and Du S (2014). Optimization of madecassoside liposomes using response surface methodology and evaluation of its stability. *Int. J. Pharm.,* **473**(1-2): 280-285.

Wu T, Liu W, Guo W and Zhu X (2016). Silymarin suppressed lung cancer growth in mice via inhibiting myeloid-derived suppressor cells. *Biomed. Pharmacother.,* **81**: 460-467.

Wu Y, Yi L, Li E, Li Y, Lu Y, Wang P, Zhou H, Liu J, Hu Y and Wang D (2017). Optimization of glycyrrhiza polysaccharide liposome by response surface methodology and its immune activities. *Int. J. Biol. Macromol.,* **102**: 68-75.

Xu S, Zhang H, Wang A, Ma Y, Gan Y and Li G (2020). Silibinin suppresses epithelial-mesenchymal transition in human non-small cell lung cancer cells by restraining RHBDD1. *Cell Mol. Biol. Lett.,* **25**: 36.

Yang S, Chen J, Zhao D, Han D and Chen X (2012). Comparative study on preparative methods of DC-Chol/DOPE liposomes and formulation optimization by determining encapsulation efficiency. *Int. J. Pharm.,* **434**(1-2): 155-160.

Yang Z, Liu J, Gao J, Chen S and Huang G (2015). Chitosan coated vancomycin hydrochloride liposomes: Characterizations and evaluation. *Int. J. Pharm.,* **495**(1): 508-515.

Zaidi SNF, Mahboob T (2017). Prevention of liver cirrhosis by Silymarin. *Pak. J. Pharm. Sci.,* **30**(4): 1203-1211.

Zhang ZQ, Kim YM and Song SC (2019). Injectable and quadruple-functional hydrogel as an alternative to intravenous delivery for enhanced tumor targeting. *ACS Appl. Mater. Interfaces,* **11**(38): 34634-34644.

Zorzi GK, Schuh RS, Maschio VJ, Brazil NT, Rott MB and Teixeira HF (2019). Box Behnken design of siRNA-loaded liposomes for the treatment of a murine model of ocular keratitis caused by Acanthamoeba. *Colloids Surf. B Biointerfaces,* **173**: 725-732.