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Inhibitory activity of Gypensapogenin D against α-glucosidase and preparation of its liposomes

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Abstract

Gynostemma pentaphyllum is a functional food that is commercially available worldwide. Triterpenoid saponins are the main active components in *Gynostemma pentaphyllum*, which are usually hydrolyzed into triterpene sapogenins in organisms to play a pharmacological role. Gypensapogenin D is a triterpene sapogenins with the highest content in the hydrolysate of gypenoside. In addition, it was found that Gypensapogenin D had a certain inhibitory effect on α-glucosidase. In view of the poor solubility of Gypensapogenin D, which affects its bioavailability, Gypensapogenin D was prepared as a liposomal formulation in this paper, which provides valuable data for the later in-depth study and drug development and utilization of this compound.

Keywords: Gypensapogenin D; α-glucosidase inhibition; molecular docking; liposomes.

Practical Application: The preparation of liposomes provides useful information for the research and development of *Gynostemma pentaphyllum* Gypensapogenin D.

1 Introduction

In recent years, the extraction of pure and natural ingredients from plants has received increasing attention (Tan et al., 2023; Wang et al., 2022b, 2022c). *Gynostemma pentaphyllum* (Thunb.) Makino (Cucurbitaceae), usually called "Jiaogulan" in China, is also widely distributed in Asia including Japan, Korea and other southeast Asian countries (Zhang et al., 2019). In China, *G. pentaphyllum* has been prescribed in Chinese medicines for a long history from Ming Dynasty (1368-1644 AD) (Xie et al., 2022) and mainly cultivated and became an important income resource for local farmers in Shaanxi, Guangxi, Fujian, Guizhou and Hubei province, today (Zhang et al., 2015). And it has hypoglycemic, hypolipidemic, anti-inflammatory, antioxidant, immunomodulatory and hepatoprotective effects (Gao et al., 2016; He et al., 2015; Niu et al., 2014; Zhang et al., 2017), but no side effects at regular doses (Chiranthanut et al., 2013).

Previous studies have found that the active ingredients in *G. pentaphyllum* are mainly dammarane type triterpenoid saponins, and about 180 triterpenoid saponins have been discovered from *G. pentaphyllum* (Shen et al., 2020; Shi et al., 2017). The literature reports that saponins have low bioavailability when administered orally and are usually absorbed as sapogenins after hydrolysis in the gastrointestinal tract to exert their effects (Song et al., 2018). And it has been reported that the hydrolyzed sapogenins of gypenoside have shown good activity in anti-tumor and hypoglycemia. In our study of the hydrolysis products of gypenosides, we found that Gypensapogenin D was the most abundant sapogenin element, and the only pharmacological effects reported on Gypensapogenin D were antitumor activity (Li et al., 2012). However, whether Gypensapogenin D has the same hypoglycemic effect as *G. pentaphyllum* has not been reported. α-Glucosidase is an important target for lowering postprandial blood glucose, and inhibition of α-glucosidase activity can lower postprandial blood glucose by causing less carbohydrate absorption in the upper part of the small intestine (Jagadeesan et al., 2022). Therefore, the inhibition rate of α-glucosidase is often used to evaluate the hypoglycemic potential of drug candidates. In this paper, we found that Gypensapogenin D has some inhibitory effect on α-glucosidase by molecular docking and α-glucosidase inhibition assay. However, it was also found that Gypensapogenin D is a chemical composition that is insoluble in water, which determines its low oral bioavailability and limits its clinical therapeutic efficacy. Liposomes, as a new drug carrier, have the advantages of high bioavailability, good biocompatibility, facilitated transport and improved drug stability. Therefore, in this study, we next used the thin film dispersion method for the preparation of Gypensapogenin D liposomes and evaluated the quality of Gypensapogenin D liposomes using entrapment efficiency(EE), mean particle size, zeta potential, polydispersity index (PDI) and drug loading capacity as evaluation indexes (Wang, 2017).

2 Materials and methods

2.1 Materials

Gypensapogenin D (Isolation and preparation by our laboratory); soya bean lecithin (Solarbio); Egg Yolk Lecithin

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(Solarbio); Cholesterol (Adamas); Trichloromethane (Chuandong Chemical); α-glucosidase (100 U, Solarbio), 4-nitrophenyl-α-D-glucopyranoside (PNPG), Acarbose (Solarbio), Phosphate buffer solution (PBS, PH = 6.8 and PH = 7.2 ~ 7.4, Qingdao Nishui);

2.2 a-Glucosidase inhibition assay

Determination of α-glucosidase inhibitory activity by microtiter plate method (Han et al., 2020; Liu et al., 2017). In a 96-well plate, add 50 μL phosphate buffer solution (PBS, pH 6.8), 50 μL sample solution and 10 μL α-glucosidase solution (1 U/mL) were mixed and incubated for 15 min at 37 °C. Then add 40 μL of 5 mM PNPG, mix well, and keep the reaction at 37 °C for 15 min, for which 40 μL of sodium carbonate solution was added to terminate the reaction, and the absorbance value was measured at wavelength 405 nm using an enzyme standardization instrument. Three times in parallel, acarbose was used as a positive control. The inhibition rate was calculated according to Equation 1.

$$\%\text{Inhibitionrate} = \left(1 - \frac{A4 - A3}{A2 - A1}\right) \times 100\% \quad (1)$$

Where A1 is the absorbance of the blank group, A2 is the absorbance of the control group, A3 is the absorbance of the sample blank group and A4 is the absorbance of the sample test group. Statistical analysis was performed with GraphPad Prism 9.0 (Harvey Motulsky, Los Angeles, CA, USA) software to calculate the IC50 values.

2.3 Molecular docking

To investigate the interaction of Gypensapogenin D compounds with α-glucosidase, active site analysis was used in molecular docking studies (Hawash et al., 2021). The crystal structure was obtained from the online protein database (PDB), and the α-glucosidase (PDB code: 1MFU) was pre-docked by Autodock software. The 3D structure of Gypensapogenin D was constructed using ChemBioDraw Ultra 14.0 software and then converted to PDBQT file format using AutoDockTools. The rotatable bonds in the ligands were assigned with AutoDock Tools and ligand docking was done with AutoDock Vina. Two-dimensional maps of protein-ligand interactions were constructed using LigPlot software to analyze the interaction forces of protein-ligand binding. 3D maps of protein-ligand interactions were constructed using PyMOL software to view the binding sites between proteins and ligands.

2.4 Chromatographic conditions

In this experiment, the content of Gypensapogenin D in liposomes was determined by HPLC (Ren et al., 2019). For the separation of the analytes, Hypersil ODS2-C18 column (4.6 × 250 mm) with a particle size of 5 μm was used. A mixture of acetonitrile and water at a ratio of 90:10 was used as mobile phase. The mobile phase was eluted in isocratic manner at a flow rate of 1.0 mL/min for 10 min. A total of 20 μL of each sample was injected for the analysis. And was detected at the wavelength of 202 nm. The column was kept at 30 degrees Celsius.

2.5 Method validation

The HPLC method was validated as per ICH guidelines. The following validation characteristics were explored: specificity, linearity, repeatability, precision, stability, and recovery rate (Kamal & Nazzal, 2020).

Specificity. Specificity is one of the significant features of HPLC, and it refers to the ability of the analytical method to discriminate between the analyte and the other components in the complex mixture (Batrawi et al., 2017). Specificity of the method was evaluated by injecting 10 μL solutions of standard, sample, and blank liposome separately.

Linearity. The linearity of the method was evaluated by injecting the mixture samples with concentrations ranging from 5 to 400 μg/mL of each drug. To construct the calibration curve, the drug concentrations were plotted against corresponding peak areas. The linearity was evaluated by linear regression analysis of the calibration curve. All samples were injected in triplicate.

Repeatability. A proper amount of Gypensapogenin D liposome was diluted and demulsified with methanol, and 6 samples were prepared in parallel. The samples were analyzed according to the chromatographic conditions of "2.4", and the repeatability was investigated.

Precision. Precision and accuracy of the method were assessed by injecting six replicates of samples with Gypensapogenin D concentrations of 5, 40 and 100 μg/mL. The samples were injected in six independent series in the same day for determining intra-day precision and three consecutive days for inter-day precision. Precision was expressed as the %RSD of the analyte concentration.

Stability. The sample to be tested was put on the sample tray of high performance liquid chromatograph and analyzed at 0, 2, 4, 8 and 12 h. The peak areas were counted and the RSD values were calculated to investigate the stability.

Sample recovery rate. Accurately measure 3 parts of Gypensapogenin liposomes prepared in the same batch, and detect the drug content in the samples by HPLC. Add Gypensapogenin reference substance at three levels of 80%, 100% and 120% of the known content, and suck 20μL of the sample to detect the total drug content. Each group of experiments is conducted in parallel to three times. The obtained peak area value is substituted into the standard curve, and the amount of drugs in the liposome sample and the actual measured the total amount of drugs is calculated. Calculate the sample recovery rate according to the recovery rate formula (Equation 2):

$$\text{percent recovery} = \frac{\text{The total amount of drug was actually measured}}{\text{Amount of drug in the sample + Add the amount}} \times 100\% \quad (2)$$

2.6 Prescription optimization

By consulting the relevant literature, we found that the main factors influencing the encapsulation rate of liposomes were: drug to lipid ratio, lecithin: mcholesterol, ultrasonic time, ultrasonic power, etc (Wang et al., 2022a). In this study, drug entrapment efficiency was used as the evaluation index, and the above indexes were investigated by single factor.

2.7 Preparation and characterization of liposomes

For liposome preparation the method outlined by Li & Wang (2016) performed with minor modification. Gypensapogenin D liposomes were prepared by thin film dispersion method. Lecithin, cholesterol and Gypensapogenin D (1:8:1) were accurately weighed and dissolved in chloroform-methanol (v/v = 3:1). In a dry eggplant-shaped bottle, after vacuum spinning in a constant temperature water bath at 40 °C, 10 min was blown by nitrogen to completely remove the organic solvent, and a uniform and transparent film was formed on the bottle wall. Add proper amount of phosphate buffer to pH 7.4, hydrate at 40 °C for 1 ~ 1.5 h at atmospheric pressure, shake fully until hydration is complete. After ultrasonic treatment with probe, 25% ultrasonic power and ultrasonic 20 min (ultrasonic 3 s, interval 5 s, ice bath cooling), Gypensapogenin D liposomes were finally obtained and stored in a refrigerator at 4 °C. Preparation of blank liposomes by the same method (Didar, 2021).

2.8 Gypensapogenin D encapsulation efficiency of liposomes

Encapsulation rate is the ratio of the amount of drug encapsulated in liposomes to the total amount of encapsulated and unencapsulated drug in the liposome suspension, and is one of the important indicators for evaluating the quality of liposomes, which is used to reflect how much drug is encapsulated and is a key indicator for screening the best process (Patil & Jadhav, 2014) . Methods for the determination of encapsulation rate include low-speed centrifugation, ultrafiltration centrifugation, dialysis, and dextran gel methods, each of which has its advantages and disadvantages. Therefore, it is crucial to choose a suitable method to determine the encapsulation rate. Pre-experiments were conducted at the beginning of this experiment to test different separation methods separately and the obtained separation results were compared. Based on the results of the pre-experiments, the low-speed centrifugation method was finally chosen, which can effectively separate the encapsulated drug from the free drug in liposomes.

In this experiment, low-speed centrifugation was used to separate Gypensapogenin D liposomes and unencapsulated free drug, which has the advantages of simple operation method and a low requirement for equipment. Because the lipid-soluble drug is insoluble in the aqueous phase and has the property of aggregating into small particles and settling easily. The low-speed centrifugation method is suitable for the determination of the encapsulation rate of the lipid-soluble drug. The key to this method is the correct choice of centrifugation speed and centrifugation time. Large centrifugation speed and a long time will make the liposome settle; small centrifugation speed and a short time will make the free drug settling incomplete. The encapsulation rate of gynostemma saponin meta-liposomes determined by this method was stable and reliable, which proved that low-speed centrifugation is an effective method to determine the encapsulation rate of liposomes of fat-soluble drugs.

It is an effective separation of the unencapsulated drug by centrifugation, where the unencapsulated drug settles to the bottom and the encapsulated drug is dispersed in the supernatant. Then the encapsulation rate was determined by HPLC. The entrapment efficiency (EE) of Gypensapogenin D liposome was calculated by the formula (Equation 3):

$$Encapsulation\ efficiency = (1 - W_f / W_t) \times 100\% \quad (3)$$

Where W_f is the amount encapsulated in the microparticle formulation and W_t is the total drug amount encapsulated versus unencapsulated in the microparticle formulation.

Experimental method: The liposome of Gypensapogenin D was removed precisely and centrifuged at 1500 r/min for 10 min. The supernatant was discarded and the precipitate was dissolved by ultrasonication with an appropriate amount of methanol and filtered through a 0.22 μm micro porous membrane and placed in a liquid phase vial. Determine the amount of free drug according to the high performance liquid chromatography conditions under "2.4". In addition, 1 mL of Gypensapogenin D liposome suspension was measured in a 10 mL flask, methanol was added to the scale, the emulsion was broken by vortexing, filtered by 0.22 μm micro porous membrane, and placed in a liquid phase vial, and the total amount of drug in the liposome was determined according to the "2.4" high performance liquid chromatography conditions. The encapsulation rate of liposomes was calculated according to the above formula.

2.9 Quality evaluations of Gypensapogenin D liposome

The characterization of liposomes is essential to assess the quality of the formed liposomes and their applications. By determining their particle size distribution, zeta potential, polydispersity coefficient (PDI), encapsulation rate and drug loading capacity, thus preparing liposomes of stable quality (Didar, 2021).

3 Results and discussion

3.1 Inhibition of α-glucosidase by Gypensapogenin D

The inhibitory effect of Gypensapogenin D on α-glucosidase activity was studied. It was found that Gypensapogenin D had a strong inhibitory effect on α-glucosidase activity. Further study found that the inhibition rate increased with the increase of concentration, showing a strong concentration-dependent relationship. Through calculation, IC50 value is 1.50 mmol/L. The results are shown in Table 1.

3.2 Molecular docking

To further confirm whether Gypensapogenin D can directly interact with the core target of α-glycosidase (PDB code:1MFU), the binding energy and dominant pattern with the key target

Table 1. α-Glucosidase inhibitory activity of Gypensapogenin D.

Concentration (mg/mL)	α-Glucosidase Inhibition (%) ± SD	IC50 (mM)
2.99	76.20 ± 2.66	1.50
1.50	61.92 ± 1.88	
0.75	56.21 ± 1.03	
0.38	39.97 ± 0.60	
0.25	28.03 ± 1.25	

compound were verified by molecular docking assessment. Molecular docking of Gypensapogenin D about α-glycosidase showed a binding energy of -9.0 kcal/mol, indicating strong binding activity of both, and the molecular docking results are shown in Figure 1.

3.3 Methodological validation of chromatography for Gypensapogenin D determination

Specific experiment

The experimental results are shown in Figure 2. It can be seen that the separation of Gypensapogenin D and solvent components is good. The peak shape is sharp, and there is no interference peak in the negative control group, which indicates that the method has good specificity.

Linearity and range

The regression equation of Gypensapogenin D concentration to absorption peak area was obtained as follows: Y = 31219X + 10804, R^2 = 0.9999. The results were shown in Figure 3, which indicated that there was a good linear relationship between Gypensapogenin D concentration and peak area in the range of 5 - 400 μg·mL^{-1}.

Repeatability test

The repeatability results are shown in Table 2, with RSD of 2.30% (n = 6). The relative standard deviation was within the allowable range, indicating that the test was good repeatability.

Precision test

The intra-day precision and inter-day precision were obtained in Table 3. The results showed that the intra-day and inter day precision of high (100 μg·mL^{-1}), medium (40 μg·mL^{-1}) and low (5 μg·mL^{-1}) Gypensapogenin D were less than 2.0%, indicating that the precision of the test was good and met the requirements of methodology.

Stability test

The results were shown in Table 4, and the relative standard deviation was less than 3.0%, indicating that the test has good stability.

Sample recovery test

The results are shown in Table 5. The average recoveries were above 90% and the relative standard deviations were less than 3.0%. It shows that the method has good recovery.

Table 2. repeatability for the determination of Gypensapogenin D (n = 6).

Sample	Peak area	RSD (%)
Gypensapogenin D	30613	2.30
	33114	
	31591	
	32413	
	31193	
	34236	

Table 3. Intra-day precision and day-time precision of Gypensapogenin D (n = 6).

Concentration (μg/mL)	Intra-day precision (RSD/%)	Day-time precision (RSD/%)
5	1.95	1.92
40	1.14	1.59
100	1.21	1.73

Table 4. Results of stability experiments of Gypensapogenin D.

Time (h)	Peak area	RSD (%)
0	34872	2.74
2	34146	
4	36102	
8	36103	
12	36866	

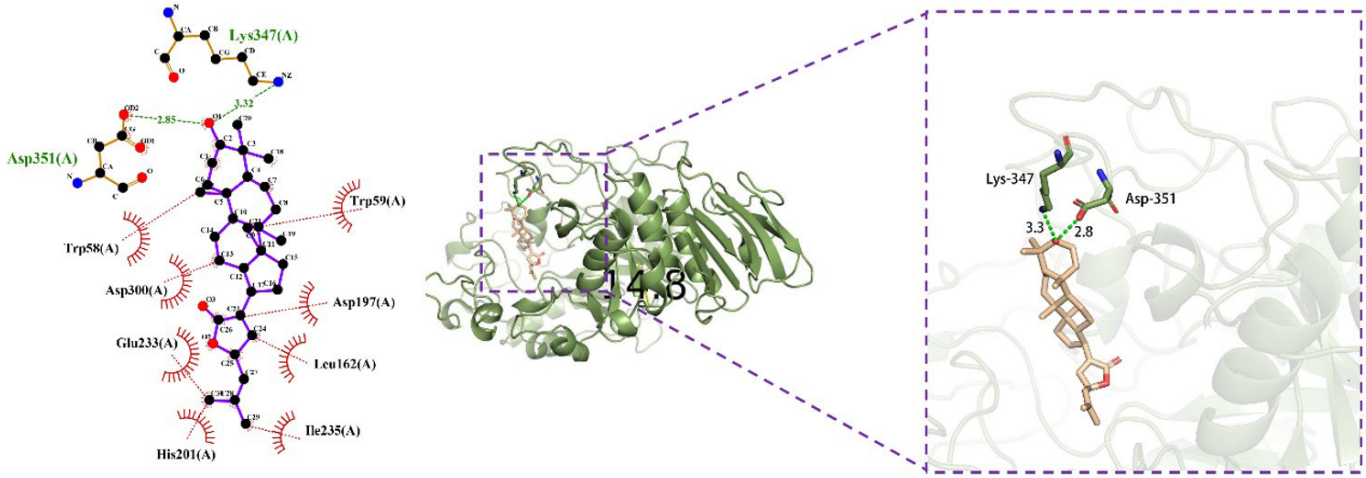

Figure 1. Molecular docking of Gypensapogenin D with α-glucosidase.

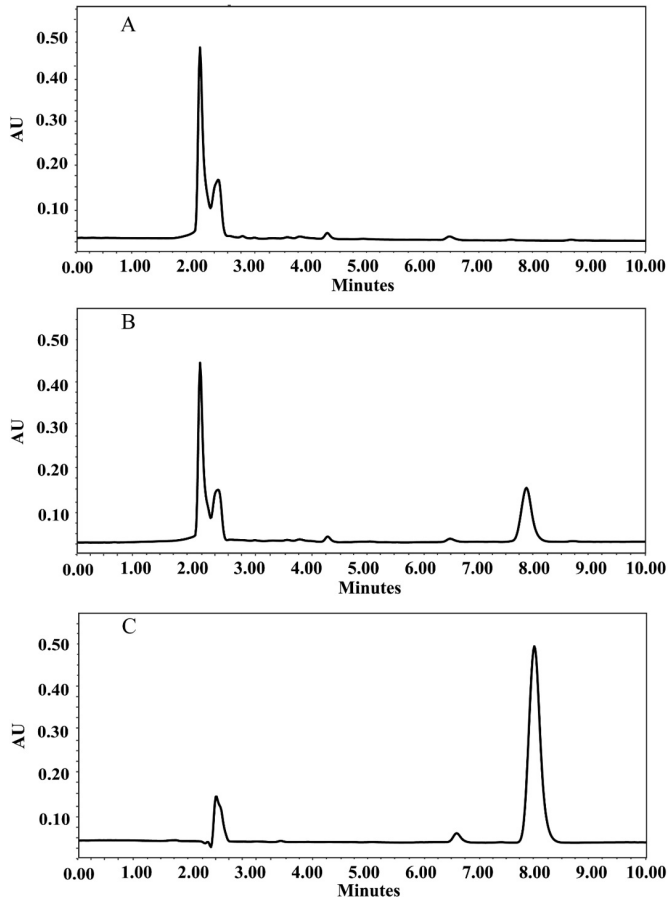

Figure 2. HPLC chromatogram of (A) the blank liposome; HPLC chromatogram of (B) Gypensapogenin D liposome; HPLC chromatogram of (C) the control solution.

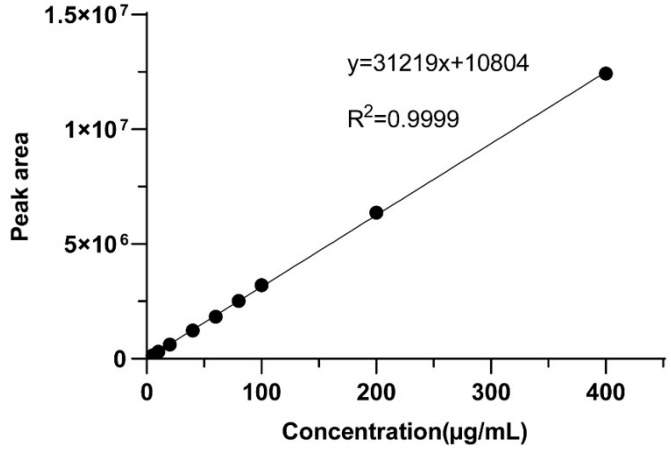

Figure 3. Standard curve of Gypensapogenin D.

3.4 Measurement of entrapment efficiency

Establishment of low-speed centrifugation method: The experimental results showed that the change of separation effect was no longer obvious when the centrifugation time was 10 min and the speed exceeded 1500 rpm/min, so the centrifugation intensity was chosen to be 1500 rpm/min; when the centrifugation intensity was 1500 rpm/min, the separation effect did not change much after the centrifugation time exceeded 10 min, and too long time might lead to the sedimentation of liposomes. Therefore, the centrifugation time was chosen to be 10 min.

Table 5. Results of sample recovery experiments.

Sample	Control ratio added	Recovery rate	Average recovery rate	RSD (%)
Gypensapogenin D	80%	98.99	99.42	1.49
		101.41		
		97.87		
	100%	101.59	102.08	2.04
		99.82		
		104.84		
	120%	100.77	102.55	1.72
		104.95		
		101.91		

3.5 Prescription optimization

Effect of phospholipid type on liposome EE

Phospholipids are the main membrane materials in liposome preparation. Phospholipids that can form a bilayer in water or can form a bilayer structure after mixing can be used as membrane materials for liposome preparation, mainly natural phospholipids and synthetic phospholipids. In this study, encapsulation rates of soy lecithin and egg yolk lecithin were (96.02 ± 0.008)% and (98.49 ± 0.001)%, respectively. Therefore, entrapment efficiency of Gypensapogenin D liposomes prepared from egg yolk lecithin was higher than that of soy lecithin, and therefore egg yolk lecithin was chosen for the preparation of Gypensapogenin D liposomes.

Effect of probe sonication time on liposomes EE

The results of the examination of the sonication time are shown in 4A. It is seen that the entrapment efficiency is higher when the sonication time is at 20 min, so the sonication time is chosen to be 20 min.

Effect of soy phospholipid to drug mass ratio on liposome encapsulation rate

The results of the drug-lipid ratio were examined as shown in Figure 4B. The encapsulation rate was higher when the drug-lipid ratio was 1:8, so drug:phospholipid = 1:8 was chosen for the preparation of Gypensapogenin D liposomes.

Effect of soy phospholipid to cholesterol mass ratio on liposome encapsulation rate

The results of the examination of phospholipid to cholesterol mass ratio are shown in Figure 4C. The encapsulation rate was higher when phospholipid: cholesterol = 8:1, so phospholipid:

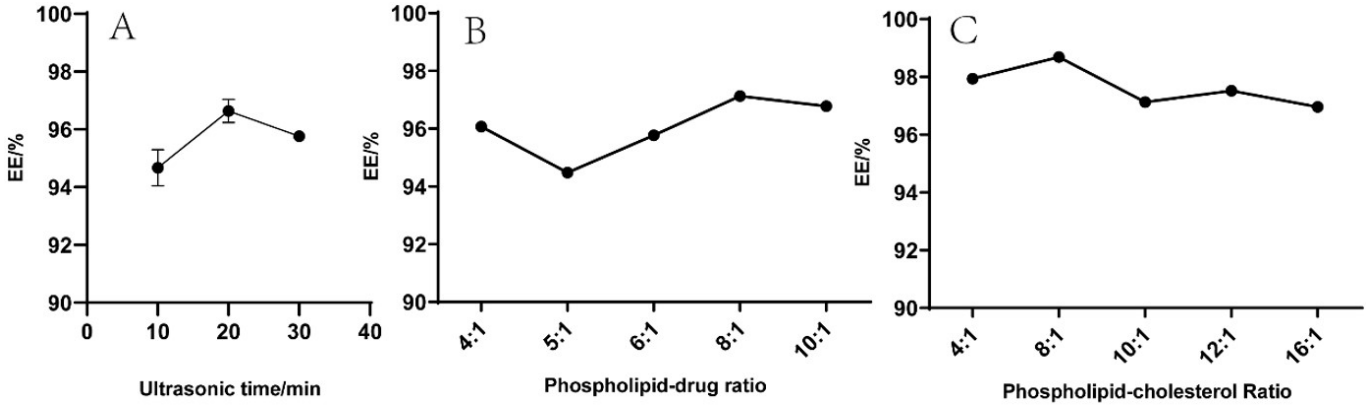

Figure 4. Results of prescription optimization. A: effect of ultrasonic time on entrapment efficiency; B: Selection of mass ratio of phospholipid to drug; C: Selection of mass ratio of phospholipid to cholesterol.

Table 6. Physical and chemical parameters of liposomes ($\bar{x} \pm S$, n = 3).

Sample	Size /nm	PDI	Zeta potential /mV	EE /%	Drug loading capacity /%
Gypensapogenin D	199.31 ± 1.73	0.354 ± 0.007	17.58 ± 3.29	98.72 ± 0.03	7.61 ± 0.18

cholesterol = 8:1 was chosen to prepare Gypensapogenin D liposomes.

3.6 Quality evaluations of Gypensapogenin D liposomes

Three Gypensapogenin D liposomes were prepared in parallel according to the optimized prescription, and their encapsulation rate, particle size, PDI value and zeta potential were measured, and the results are shown in Table 6. The mean particle size distribution of Gypensapogenin D liposomes was 199.31 ± 1.73nm and the PDI value was 0.354 ± 0.007, indicating that the Gypensapogenin D liposomes prepared according to the optimized prescription had a uniform particle size and good dispersion.

4 Conclusion

Gypensapogenin D is a monomer sapogenins isolated from the hydrolysate of gypenoside. In this study, Gypensapogenin D was found to have certain inhibitory activity on α-glucosidase. However, the solubility of Gypensapogenin D is very poor, which seriously affects its efficacy. For this purpose, Gypensapogenin D liposome was successfully prepared by thin film dispersion method. The average diameter of the prepared liposome was 199.31 ± 1.73nm, PDI was 0.354 ± 0.007, zeta potential was -17.58 ± 3.29 mV, EE was (98.72 ± 0.03)%, and drug loading rate was (7.61 ± 0.18)%. This study provides valuable data for the later in-depth research and drug development and utilization of this compound.

Conflict of interest

All authors have no conflicts of interest to declare.

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