Preparation and Evaluation of Repaglinide Loaded Liposomes by Ether Injection Method

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ABSTRACT: Repaglinide is a commonly used antidiabetic drug to treat type 2 diabetes mellitus. However, it does not always support a suitable dose regimen due to its low bioavailability and half-life. The purpose of this study was to develop and analyze repaglinide loaded control release liposomes to provide suitable dosage form to treat diabetes. Repaglinide loaded liposomes were prepared by modified ether injection method. According to the USP-II paddle method, *in vitro* dissolution studies of liposomes were performed for 8 hours in phosphate buffer (pH 7.4). The stability study was executed according to ICH guidelines under three different environmental conditions for 14 days. Additionally, the presence and size of liposomal vesicles was confirmed by microscopic observation. The formulations containing phosphatidylcholine and cholesterol at a molar ratio of 1:0.4 revealed the highest drug entrapment efficiency of 73.1% with an optimum stirring rate of 300 RPM. The pegylated liposomes (PEG400/1500) prolonged the drug loading capacity for the formulations compared to non-pegylated liposomes. On the contrary, the addition of nigella oil caused a decrease in entrapment efficiencies of the liposomes. Most of the formulations followed zero order kinetic model and supper class II release mechanism. *In vitro* dissolution showed controlled release pattern of the liposomes and maximum drug release after 8 hours was 92.64%. Additionally, all the liposomal formulations were found to be more stable at refrigeration temperature (5 ± 2ºC) where pegylated liposomes were most stable over 14 days at three different environmental conditions. Visual observation under an optical microscope, the vesicular structure of liposomes was confirmed.

Key words: Repaglinide, liposomes, ether injection method, drug entrapment efficiency, controlled release.

INTRODUCTION

Repaglinide is an important oral insulin secretagogue used to treat type 2 (non-insulin dependent) diabetes mellitus.[1] Scott described that repaglinide has low water solubility, 56% bioavailability and a short half-life but high permeability. Culy & Jarvis[2] mentioned that repaglinide does not always approve a suitable dose regimen due to low bioavailability because of the extensive hepatic metabolism. Additionally, tablet dosage form of repaglinide is mostly available in the market and used to manage diabetes. So, the development of a dosage form with improved bioavailability and extended drug delivery has become very essential to obtain optimized drug therapy. Research works have proved that suitable dosage forms can control pharmacokinetics, pharmacodynamics, non-specific toxicity and efficacy of drugs. The novel drug delivery system can improve solubility, therapeutic indices, drug stability and efficacy with reduced side effects.[3]

Liposomes, one of the most effective and advanced drug delivery system has improved patient compliance, bioavailability, drug solubility with reduced dosing frequency and side effects. It has been reported that a liposomal drug delivery system is a controlled and ideal drug-carrier system which can encapsulate both hydrophilic and lipophilic drug molecules.[4] In our study, the modified ether injection method was used to prepare repaglinide loaded liposomes where the most common phospholipid,

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lecithin (also known as phosphatidylcholine) was used to form stable formulations. Additionally, cholesterol was used to improve the liposomal delamination, fluidity and stability of the layers. It also reduced the permeability of water-soluble molecules through membranes.[5]

So, considering the advantages of liposomes and limitations of the drug repaglinide, the study aims to formulate a repaglinide loaded liposomal drug delivery system as well to analyze the effect of various excipients and carriers on the dosage form in order to determine the optimum process variable to develop an effective dosage form.

METHODS AND MATERIALS

Materials. Different types of materials and solvents were used to prepare repaglinide loaded liposomes. Lecithin, cholesterol, diethyl ether, methanol, nigella oil, PEG 400, PEG 1500 etc. were the major ingredients that were used in different ratio and composition to get desired liposomal formulations.

Formulation design. Ten (10) mg repaglinide as an active pharmaceutical ingredient was used in each formulation. Different formulations were prepared having varying ratios of the formulation ingredients in order to observe the influence of independent formulation variables on dependent variables (Table 1).

Table 1. Dependent and independent variables with their range in experimental design.

Independent variables	Minimum	Maximum	Dependent variables
Lecithin (phosphatidylcholine)	50 mg	150 mg	Drug entrapment efficiency (%)
Cholesterol	30 mg	75 mg	
PEG 400	10 ml	20 ml	
PEG 1500	10 mg	20 mg	
Nigela Oil	2.5 ml	10 ml	
Stirring rate	100 rpm	500 rpm	

Preparation of repaglinide loaded liposomes by ether injection method (EIM). Lecithin (phosphatidylcholine) and cholesterol were dissolved in 10 ml diethyl ether containing 10 mg of repaglinide. The resulting solution was slowly injected by using a micro syringe at a rate of 0.5ml/min into 20 ml of the hydrating solution of phosphate buffer (pH 7.4). At 45-50ºC temperature, the solution was stirred continuously on magnetic stirrer at a rate of 300 RPM. Due to temperature differences between phases ether was vaporized quickly to cause spontaneous vesiculation to form liposomes. All the formulations as per experimental design were prepared using a similar procedure by the addition of various quantities of formulation ingredients.[6]

Standard curve preparation. Stock solution of repaglinide (100 µg/ml) was prepared in phosphate buffer (pH 7.4). After serial dilution of the stock solution, different working solutions were prepared having concentration ranging from 5µg/ml to 90µg/ml. Absorbance of the solutions were recorded at 279 nm by using UV spectrophotometer and a standard curve was constructed by taking concentration versus absorbance.[7] The standard curve obtained was a straight line with the R^2 value of 0.9993 and equation of $y = 0.0106x + 0.005$

Determination of percentage of drug encapsulated in the liposomes. Entrapment efficiency was measured by measuring the un-entrapped (free) drug remaining in the liposomal dispersion. The free drug was determined by subjecting the liposomal formulation to centrifuge at 4000 RPM for 2 hours to separate the free drug. After centrifugation, the supernatant was collected and analyzed spectrophotometrically to determine drug

content at 279 nm. The percent of drug entrapment efficiency was determined by the following formula.[8]

$$(\%)\text{Entrapment efficiency} = \frac{(\text{Total drug} - \text{Unentrapped drug})}{\text{Total drug}} x100$$

Stability studies of liposomes. The stability of liposomes was performed according to ICH guidelines. The liposomal dispersions were kept in the air-tight glass vials and stored at refrigeration temperature (5 ± 2°C), room temperature (25 ± 2ºC, 60 ± 5 RH) and at elevated temperature (40 ± 2ºC, 75 ± 5 RH) for 14 days. Samples were withdrawn on the 7^{th} and 14^{th} days for checking their physical appearance and evaluating the stability of formulation by measuring drug entrapment efficiency.[9]

Evaluation of liposomes

Optical microscopy. The vesicular structure of liposomes was confirmed by optical microscopy in 1200x resolution. The liposomal suspension was placed over a glass slide and fixed over by drying at room temperature for observation under a microscope.[10]

***In vitro* dissolution study of liposomal dispersions.** *In vitro* release study of repaglinide loaded liposomes was done according to the USP II paddle method. A total 3 ml of the liposomal suspension was centrifuged for 2 hours at 4000 RPM. The sedimented liposomes were separated and diluted with 2 ml buffer media (pH 7.4). At 37^{o} C temperature, the suspension was directly poured into dissolution vessel containing 900 ml of phosphate buffer (pH 7.4) with 75 RPM. At predetermined intervals, 10 ml of the sample was withdrawn for 8 hours from each basket and replenished by fresh 10 ml medium. The absorbance of each sample was recorded by using UV spectrophotometer at 279 nm to determine the drug release from the formulations.[11]

Interpretation of dissolution profile of liposomes. Absorbance values obtained from the dissolution studies were converted into percent release of drug from the formulations of liposomes. This was done by comparing the absorbance values with the standard curve of repaglinide.[12]

Release kinetics. Data obtained from *in vitro* release studies were fitted to various kinetic equations to find out the mechanism of drug release from the liposome. Zero order, first order, Higuchi, and Korsmeyer-Peppas model were used to describe the release kinetics of prepared liposomal formulations. Successive fractional dissolution time like T25%, T50%, T80% and MDT values were also calculated.

RESULTS AND DISCUSSION

The liposomal formulation containing repaglinide were prepared by modified ether injection method (EIM) using different proportion of phosphatidylcholine, cholesterol, nigella oil and PEG-400/1500 as drug retarding agents in the formulations.

Percent drug entrapment efficiency (%DEE) of repaglinide loaded liposomes. Percent of repaglinide entrapment efficiency of various formulations containing 10 mg of repaglinide with different ratios of excipients was evaluated.

At a constant stirring rate, the percent of drug entrapment efficiency varied from 40.6% to 71.1% for the formulations (F-1 to F-5) having a constant amount of phosphatidylcholine with varying ratios of cholesterol. It was seen that drug entrapment efficiency increased with an increasing amount of cholesterol in the formulations. Additionally, a molar ratio of 1:0.4 (phosphatidylcholine: cholesterol) encapsulated the highest percentage of repaglinide by the liposomes. At a fixed level of cholesterol and RPM, drug entrapment efficiency (formulations F-6 to F-10) decreased ranging from 71.7% to 60.4% with a gradually decreasing amount of phosphatidylcholine. At different stirring rates (100 to 500), the percent of drug entrapment efficiency was found to decrease or increase with a certain level of stirring rates in the formulations (F-11 to F-20).

The entrapment efficiency decreased with increasing of nigella oil content in the formulations. Formulations containing PEG-400 and PEG-1500 as drug retarding agent revealed higher drug entrapment efficiency than the formulations without PEG.

***In vitro* drug release study of repaglinide loaded liposomes.** *In vitro* dissolution study was done for the best fitted liposomal formulations based on their percent of repaglinide entrapment efficiency. So, dissolution data of the selected formulations are discussed here.

Data obtained from in-vitro drug release studies of liposomes were treated by zero order, first order, Higuchi and Korsmeyer- Peppas model. The formulations F-12, 13, 21, 22, 25, 26, 27, 29, 30, 31 & 32 were best fitted with zero order while F-17 & 28 with first order and F-18 with Higuchi model due to having highest correlation coefficient (R^2). Additionally, all of the formulations followed super class-II drug release mechanism except F-18, F-21 & F-28 (non-Fickian) (Table 6). Successive fractional dissolution times of the liposomal formulations showed that F-13, 22, 27 & 31 formulations have MDT values of 3.59, 4.20, 4.79 & 5.11 hours respectively which are comparatively higher than other formulations and indicating better drug retardation capacity.

Additionally, the cumulative percent release was increased consistently with course of time that proved a controlled release pattern of the liposomes. Figure 1 shows formulations (F-13, 22, 27 and 31) revealed on an average 85% drug release after 8 hours.

Stability studies of liposomes. According to the ICH guidelines[13] physical & chemical stability and MDT values were evaluated for the repaglinide liposomes (F-13, F-22, F-27 & F-31) having highest percent of drug entrapment efficiency. Drug entrapment efficiency was calculated for a period of 14 days at refrigerator (5 ± 2ºC, ambient), room temperature (25 ± 2ºC, 60 ± 5 RH) and an elevated temperature (40 ± 2ºC, 75 ± 5 RH). Among all the formulations, F-13 and F-31 were found most stable over 14 days at three different conditions. Table 8 shows the stability studies data of repaglinide loaded liposomes (F-13, 22, 27 & 31).

Table 6. Release rate constants and R^2 values for liposomal formulations of repaglinide.

Formulation code	Zero order		First order		Higuchi		Korsmeyer-peppas		Release mechanism of liposomes
	K_0	R^2	K_1	R^2	K	R^2	N	R^2	
F-12	11.53	**0.983**	-0.142	0.842	35.83	0.966	0.961	0.912	Super class -II
F-13	11.75	**0.991**	-0.160	0.972	37.85	0.964	1.07	0.950	Super class -II
F-17	11.86	0.912	-0.137	**0.972**	37.70	0.960	0.865	0.960	Super class -II
F-18	10.49	0.940	-0.118	0.929	33.49	**0.979**	0.708	0.942	Non – Fickian
F-21	11.88	**0.982**	-0.134	0.931	37.44	0.962	0.735	0.950	Non – Fickian
F-22	11.78	**0.990**	-0.111	0.967	35.61	0.941	1.083	0.973	Super class-II
F-25	11.12	**0.981**	-0.099	0.973	33.91	0.949	1.063	0.954	Supper class-II
F-26	11.85	**0.988**	-0.117	0.965	36.22	0.951	0.958	0.971	Super class-II
F-27	11.06	**0.996**	-0.097	0.976	34.05	0.953	1.287	0.967	Super class-II
F-28	10.34	0.929	-0.094	**0.978**	32.60	0.960	0.715	0.911	Non – Fickian
F-29	11.95	**0.979**	-0.117	0.949	36.04	0.936	1.08	0.942	Super class-II
F-30	11.83	**0.967**	-0.127	0.949	36.78	0.951	0.934	0.921	Super class-II
F-31	11.95	**0.994**	-0.109	0.980	36.36	0.939	1.172	0.981	Super class-II
F-32	11.26	**0.983**	-0.102	0.954	32.97	0.881	0.873	0.942	Super class-II

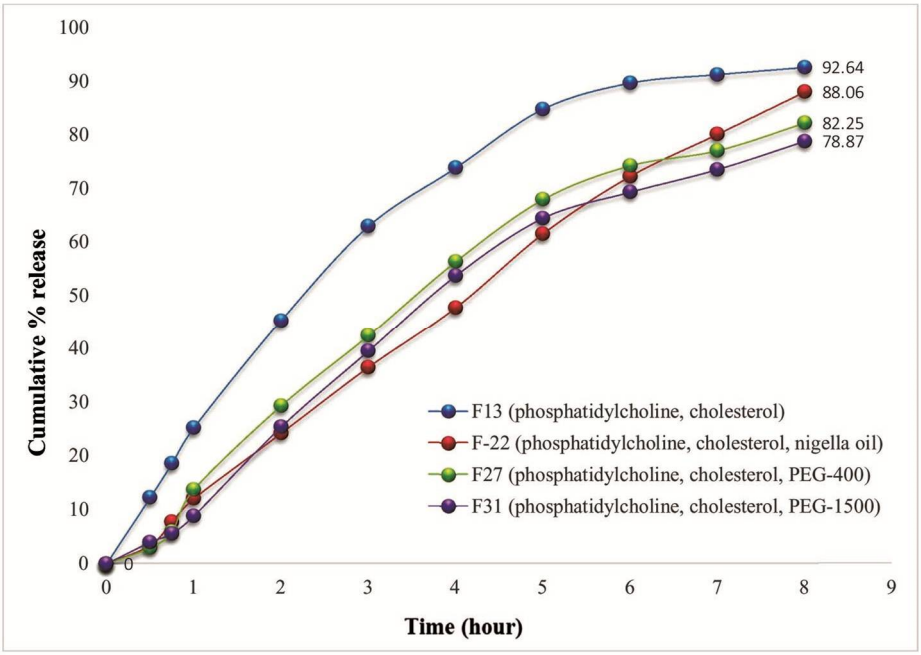

Figure 1. Cumulative % release of formulations F-13, F-22, F-27 and F-31

Table 8. Stability studies of liposomes (F-13, 22, 27 and 31).

Formulation Code	25 ± 2 ºC, 60 ± 5 RH			5 ± 2 ºC, ambient			40 ± 2 ºC, 75 ± 5 RH		
	7 days	14 days	SD	7 days	14 days	SD	7 days	14 days	SD
F-13	69.8	69.0	**0.423**	68.6	67.5	**0.779**	66.7	68.0	**0.217**
F-22	63.2	61.41	1.17	59.2	57.9	1.291	61.9	59.7	2.09
F-27	75.4	72.1	2.330	69.25	68.5	1.127	70.8	67.8	2.121
F-31	77.7	77.2	**0.314**	76.2	75.5	**0.459**	74.6	73.9	**0.194**

Optical microscopic evaluation of liposomes. The morphology of the liposomal formulations was determined by optical microscope (1200X) equipped with digital camera. These photomicrographs confirmed the formation of vesicular structures of liposomes. Figure 2a and 2b shows the microscopic images of liposomal formulations F-13 and F-17 respectively. The liposomes in F-17 (molar ratio 1:0.48 of phosphatidylcholine & cholesterol) were seen slightly smaller in size with thicker bilayer than F-13 (molar ratio 1:0.4 of phosphatidylcholine & cholesterol). Liposomal dispersions (F-21, 22, 23 & 24) contained nigela oil in different ratios. Figures 2c and 2d represent the liposmes of F-21 and F-24 repectively. The figures showed the size of the vesicles were decreasing and became thicker with the increase of nigela oil in the formulations. It was also observed that liposomes were becoming thicker with the addition of PEG-400 and 1500 in the formulations (Figure 2e and 2f).

Statistical analysis. The data of percent drug entrapment efficiency (DEE), successive fractional dissolution time and mean dissolution time were expressed as the mean ± standard error of the mean (mean ± SEM). Statistical analysis was done using the statistical software package SPSS version 23.0 (IBM Corp., Armonk, NY).

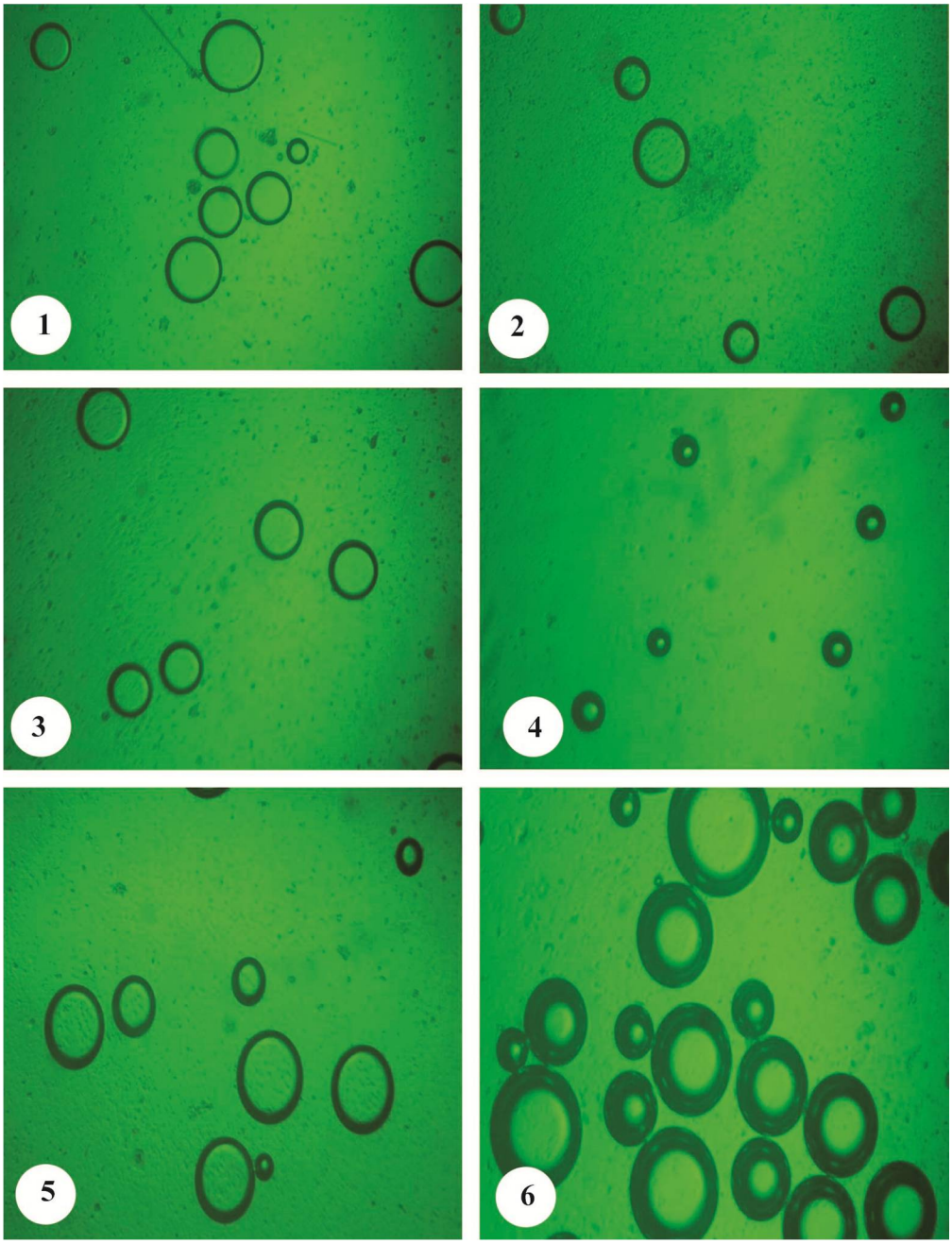

Figure 2. Microscopic images of different liposomal formulations.

In this study, 10 mg repaglinide containing different liposomal formulations were prepared to study the effect of independent variables (phosphatidylcholine, cholesterol, PEG-400/1500, nigella oil and stirring rate) on two dependent variables (percent drug entrapment efficiency and percent cumulative drug release) respectively.

It was seen that with an increment of cholesterol in the liposomal formulation drug retarding capacity was increased gradually due to the stabilization of phospholipid bi-layer of liposomes by cholesterol.[14]

On the other hand, drug entrapment efficiency also decreased upon the addition of cholesterol after a certain level. It was reported that incorporation of cholesterol into vesicular structure reduced fluidity of vesicles and increased the rigidity of liposomal bilayers.[15] It was found that at 1:0.4 molar ratio of phosphatidylcholine and cholesterol, liposomes exhibited optimized drug retarding capacity. Phosphatidylcholines are used as a major component for liposome formation and found that drug entrapment decreased upon decreasing its amount for a given amount of cholesterol and constant RPM.

For a lesser amount of phosphatidylcholine, less number and smaller vesicles were formed that lead to less amount of active drug incorporation into liposomes. Decreasing or increasing drug entrapment efficiency at varying stirring rates proved that stirring rates played an important role in liposomal formation. Stirring rates intensified the micro-mixing between two phases that lead to the formation of varying particle sizes of liposomes.[16] Maximum drug retardation by liposomes was found at 300 RPM which suggest the optimum for micro-mixing of two phases. Incorporation of increased amount of nigella oil as a drug retarding agent resulted in the decreased drug entrapment efficiency by liposomes due to smaller vesicle formation. Nigella oil contains the antioxidant thymoquinone[17] which led to aggregate liposomes and reduction of oxidation of phospholipids to form more rigid smaller liposomal vesicles. It can be observed that addition of both the PEG-400 & 1500 influenced the drug encapsulation efficiency of liposomes. PEG increased in drug entrapment within the bilayer of liposomes due to the presence of hydrophobic long chains of the PEG that acted as a barrier against drug diffusion and slowed the release rate of the drug from vesicles.[18]

A higher value of mean dissolution time (MDT) indicated a higher retaining ability of liposomes. The overall results of the MDT value showed that if the amount of phosphatidylcholine and cholesterol level was high, the retarding affinity of formulations also increased. Additionally, successive fractional dissolution time was used to characterize the drug release rate from the liposomes. Most of the liposomes followed super class-II transport mechanism[19] because the diffusion exponent (n) was greater than 0.85.

A 1:0.4 molar ratio of phosphatidylcholine and cholesterol along with the PEG revealed most stability over 14 days at three different conditions due to less oxidation and aggregation of the liposomes. On the contrary, other formulations became less stable due to leakage of the vesicular bilayers.[20] It was observed that vesicular size shrinked due to increased amount of cholesterol in the liposomal formulations. The addition of cholesterol made more rigid and thick vesicles that led to reduction of the size of liposomes. Moreover, addition of PEG in the liposomes became thicker due to the presence of the hydrophobic long alkyl chains of the PEG that made the bilayer more rigid.[21]

CONCLUSION

This study was designed to develop best fitted repaglinide loaded controlled release liposomal formulation. Formulations containing a 1:0.4 molar ratio of phosphatidylcholine and cholesterol at 300 RPM revealed maximum drug encapsulation capacity. Additionally, pegylated liposomes revealed the highest drug retarding capacity than non-pegylated liposomes which extended drug release for more than 8 hours. It can be concluded that repaglinide loaded liposomes prepared by the ether injection method could be an effective dosage form with increased bioavailability and better efficacy. Finally, it should be addressed that further work is needed to characterize the physicochemical properties of prepared liposomes such as particle size distribution, charge through SEM (scanning electron microscopy) and zeta potential tests. These formulations can also be considered as candidate for performing *in vivo* studies in the animal model to establish an *in-vitro in-vivo* correlation.

ETHICAL APPROVAL

It is not applicable.

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Competing interests

The authors have declared that no competing interests exist.

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