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Original Research Article

The Optimization of Encapsulation Mangosteen (*Garcinia mangostana* L.)-Gotu Kola (*Centella asiatica* L. Urban) Fraction Combination in Soybean Liposome by Response Surface Methodology

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ABSTRACT

A recent study reported that the combination of mangosteen-gotu kola fractions showed lots of bioactivities, such as strong antioxidant, anti-bacterial, and immunomodulatory activities. However, the solubility and bioavailability of the mixture combination of mangosteen-gotu kola fraction were low. Encapsulation of the mixture in a liposome was believed to increase its solubility in water, bioavailability, and medical applications due to the similar structure between a liposome and biological cell membranes. However, liposome system has a weakness, such as low encapsulation ability which are influenced by the manufacture, and the composition of the constituents, namely cholesterol, phospholipids, and encapsulated active substances. Therefore, it was necessary to optimize liposomal formulations that produce liposomes with the best encapsulation capabilities. This study was designed to optimize the encapsulation of mixture combination of mangosteen-gotu kola fraction in liposomes using thin film hydration method and response surface methodology (RSM). The RSM optimization consisted of 13 runs with two experimental factors: mass of the mixture (5.00-15.00 mg) and mass of cholesterol (11.30-22.60 mg) as independent variables. The mass of phospholipid (113.00 mg) was used as a dependent variable. The observed parameters were encapsulation efficiency (EE) and loading capacity (LC). As a result, quadratic models were used for response prediction. The maximum response was obtained using 15 mg of mixture combination, 22.60 mg of cholesterol, and 113 mg of phospholipid. It was shown that EE and LC were 82.42% and 2.18%, respectively. The liposome-loaded mixture combination obtained had a monodispersity form with a particle size and surface charge of 912.7 nm and -24.3 mV, respectively.

***Keywords*:** encapsulation, gotu kola (*Centella asiatica* L. Urban), liposome, mangosteen (*Garcinia mangostana* L.), response surface methodology (RSM)..

Introduction

Mangosteen (*Garcinia mangostana* L.) and gotu kola (*Centella asiatica* L. Urban) have been known worldwide to have lots of bioactivities such as anti-cancer, anti-bacterial, antioxidant, and immunomodulatory activities.[1–13] The combination of mangosteen-gotu kola fraction 1:3 ratio from their ethyl acetate fractions showed synergistic interactions in antioxidant activities.[14] However, research reported that α-mangostin which is the main chemical constituent in mangosteen, is mostly present in the semi-polar phase and had low solubility in water, and for medicinal purposes, the drug must be easily dissolved in water.

Encapsulation of the drug is believed to increase its solubility, bioavailability, and pharmacology. Encapsulation of the drug or bioactive compound in a liposome has been widely reported.[15–24]

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Liposomes are believed to maintain the pharmacology of the drugs, have lots of bioactivities such as being biodegradable, non-toxic, and biocompatible, and could load two different phases of drugs (lipid-soluble drugs and water-soluble drugs) at the same time. However, the liposome system has weaknesses such as low efficiency of encapsulation (EE) and low loaded capacity (LC), so the best formulation of a liposome-loaded mixture combination of mangosteen-gotu kola should be well considered. Liposomes consist of phospholipids, cholesterol, and loaded drugs or bioactive compounds, so considering the composition of liposomes in formulation is necessary to obtain the maximum EE and LC of liposomes.

One of the experiment methods to develop optimization of liposome formulation is using response surface methodology (RSM). RSM is one of the methods employed to design experiments, including optimization of the experimental processes.[25–27] The urgency of using RSM as a tool for generating liposome formulation because it provides several advantages, such as minimizing unnecessary trial runs, taking into account the estimated interaction between factors in the experiment, generating mathematical models that describe the relationship between variables and experimental responses, performing statistical analysis, and optimizing the desired response criteria. Those output responses of liposome formulation using RSM could give us the best prediction of the formula of the scientist's desired responses as well as the formula of any further desired responses.

This study's most significant finding is the formulation of liposome loaded combination of active fractions mangosteen and gotu kola

which exhibited great efficiency of encapsulation (EE) and loading capacity (LC) using response surface methodology (RSM). In this research, we optimized the liposomal formulation of the combination of mangosteen-gotu kola fractions using RSM to determine which formulation obtained the best EE and LC. The factors in this formulation optimization were the mass of the mixture combination of mangosteen-gotu kola and the mass of cholesterol as dependent factors, and mass of phospholipids as independent factors. We also determined the characteristics of the liposome obtained, namely its particle size and zeta potential (surface charge).

Materials and Methods

Materials and chemicals

Mangosteen peel powder (voucher number 8/3/2020), gotu kola leaves powder (voucher number 7/3/2020). The plant materials were collected from Java Plant, Karanganyar, Central Java, Indonesia, in March 2020, -7.628789079463784, 111.09187549480963, and stored in the biological pharmacy laboratory of Diponegoro University, Phospholipid soybean (Sigma Aldrich, USA), Cholesterol (Sigma Aldrich, USA), chloroform (Merch, Germany), methanol (Merck, Germany), disodium hydrogen phosphate (Merck, Germany), sodium dihydrogen phosphate (Merck, Germany), demineralized water. Ultrasonic Homogenizer (Model 300 V/T), Digital dry bath (Hangzhou Miu Instruments. Co. Ltd. Model NK200-1B), Vortex (MX-S DLAB made in China Ser No VB223AR0000113), Centrifuge (Hettich Zentrifugen EBA 200), Centrifuge tubes (Bio-seen), Spectrophotometer UV-Vis (Genesys 10S), nanoparticle and zeta potential analyzer (Horiba Scientific SZ-100).

Preparation of mixture combination of mangosteen-gotu kola fraction 3:1

Each of the mangosteen and gotu kola crude ethanolic extracts was dissolved in ethanol 1:4 (w/v), partitioned by n-hexane 1:1 (v/v), and then separated. The ethanolic phase was next given a 1:1 (v/v) addition of distilled water, and subsequently partitioned by ethyl acetate 1:1 (v/v) to produce ethyl acetate. The residue of the last partition is known as the ethanolic fraction. Finally, all the fractions are filtered, and their solvent is removed using a rotary evaporator. The ethyl acetate fraction of mangosteen peel and an ethanolic fraction of gotu kola were combined (3:1) by dissolving each of the ethyl acetate fraction of mangosteen as much as 7.5 grams, and 2.5 grams of the ethanol fraction of gotu kola in 20 mL of methanol until dissolved, then mixed and homogenized, the methanol solvent is then evaporated using a water bath. The mangosteen-gotu kola ratio in the combination was 3:1. The choice of mangosteen peel ethyl acetate fraction, gotu kola ethanol fraction, and combination ratio was based on unpublished preliminary bioactivities experiments of anti-microbial and immunomodulator.

Preparation of mixture combination of mangosteen-gotu kola fraction 3:1 standard solution curve

The standard solution concentration is made based on the concentration of the combined fractions to be encapsulated. Concentrations of 0, 5, 20, 35, 50, and 65 ppm were achieved. A 35 ppm concentration was then examined over a 200-600 nm wavelength range. The maximum wavelength measured was 316 nm.

Simulated Intestinal fluid solution preparation

First, dissolved as much as 8.9 g of anhydrous NaH_2PO_4 with 500 mL of demineralized aqua to make a 0.1 M X solution. Second, dissolved as much as 7.8 g of $Na_2HPO_4.7H_2O$ with 500 mL of demineralized aqua to make a Y 0.1 M solution. Third, as much as 81 mL X solution is mixed with 19 mL of Y solution and demineralized aqua is added to a solution until the volume of final solution reaches 100 mL. Then, the pH of the solution adjusted to 7.4 using a pH meter.

Preparation of mixture combination of mangosteen-gotu kola 3:1 loaded liposome using thin film method hydration

Each formulation was weighed and dissolved in 50 mL of 9:1 chloroform/methanol until completely dissolved, after which the colloid was filtered. Second, 10 mL of colloid was transferred to a large test tube, and nitrogen was introduced until the solvent evaporated and a thin layer formed at the bottom of the tube. Third, 10 mL of phosphate buffer solution 7.4 was added. Fourth, it was frozen and thawed at 4 ˚C and 50 ˚C then homogenized using a vortex (5 minutes each) until the colloid was homogeneous and there were no residues or particulates. Fifth, the colloid was sonicated at a power of 10 for 3 minutes. The sixth step was centrifuged for 2 hours (adjusted) at 6000 rpm. Supernatant and pellet (liposomes) were separated. Diluted with the solvent used to create the standard calibration curve.

Optimization of formulation condition by Response surface methodology

Liposomes are consisting of cholesterol, phospholipids, and loaded bioactive compounds, so that cholesterol and loaded bioactive compounds were used as the main factors influencing the efficiency of encapsulation, whereas phospholipid was used as constant factor. Experiments were carried out according to CCD. The CCD level of the experiment can be seen in Table 1. The formulating design using RSM can be seen in Table 2.

To predict optimized conditions, the quadratic equation was used to generate response surfaces. The sequential *p*-value, lack of fit *p*-value, and adjusted coefficient determination (R^2_{Adj}) were evaluated to investigate the adequacies of the model. The analysis of variance (ANOVA) was selected to evaluate the statistical significance of the regression coefficient after selecting the most accurate model. Design expert software 11 was used to spot the graph of response surfaces. The optimum condition was verified by performing supplementary experiments at these conditions. The *p*-value of 0.05 is considered statistically significant.

Encapsulation efficiency (EE) and loading capacity (LC) evaluation

The experiment will be carried out by centrifuging the colloid for 2 hours at 3.461x g (6000 rpm), collecting the supernatant, and diluting the supernatant of each formula with absolute methanol according to the concentration on the standard curve. A UV-Vis spectrophotometer was used to test absorbance at a known wavelength (316 nm). The blanks were empty liposomes (only phospholipids and cholesterol were present).

Particle size analyzer

The test conditions were as follows: scattering angle 173°, holder temperature 25°C, dispersing medium viscosity 0.887 mPa.s., transmission intensity before measurement 9979 for blank liposomes, and 7 for liposomes loaded with mixture combinations.

Zeta potential analyzer

The test conditions were as follows: holder temperature of 25°C, dispersion medium viscosity of 0.887 mPa.s, the conductivity of 12.592 $mS.cm^{-1}$ for empty liposomes, and 13.005 $mS.cm^{-1}$ for liposomes loaded with mixture combination, and electrode voltage of 1.3 V. Scattering angle: 90° with a He/Ne laser source λ= 633 nm.

Table 1: Levels and code of variables chosen for central composite design (CCD)

Factors	Code	Level and range				
		axial	-1	0	+1	axial
Mass of Cholesterol (mg)	*X1*	8.96	11.30	16.95	22.60	24.94
Mass of loaded mangosteen-gotu kola fraction 3:1 combination (mg)	*X2*	2.93	5.00	10.00	15.00	17.07

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Table 2: Liposomal formulation of mixture combination of mangosteen-gotu kola fraction 3:1 optimization using response surface methodology (RSM) with central composite design (CCD)

Runs	Factors		
	Soybean phospholipids (mg)	Cholesterol (*X1*, mg)	Mixture combination of mangosteen-gotu kola fraction 3:1 (*X2*, mg)
1.	113.00	22.60	15.00
2.	113.00	24.94	10.00
3.	113.00	11.30	15.00
4.	113.00	22.60	5.00
5.	113.00	16.95	2.93
6.	113.00	16.95	10.00
7.	113.00	11.30	5.00
8.	113.00	16.95	10.00
9.	113.00	16.95	17.07
10.	113.00	8.96	10.00
11.	113.00	16.95	10.00
12.	113.00	16.95	10.00
13.	113.00	16.95	10.00

Result and Discussion

The formulation optimization comprised of 13 runs to investigate the effect of cholesterol mass, mixture combination mass, and phospholipid mass on encapsulation efficiency (EE) and loading capacity (LC). Table 3 displays the optimization outcome.

Based on the preceding optimization results, for EE responses in the range 12.1361% - 81.0612% and for LC responses in the range 0.147797% - 2.152068%. In this study, the phospholipid value was fixed at 113 mg and constituted a dependent variable. Cholesterol levels ranged is chosen from 10% to 15% to 20% of total phospholipids to evaluate which portion give the best EE and LC. The expansion of the value of each limit in the RSM CCD design was 8.96 mg and 2.93 mg, respectively, for the expansion of the lower limit of cholesterol and the mixture combination. Meanwhile, The expansion of the upper limit of cholesterol and the mixture combination was 24.94 mg and 17.07 mg, respectively. Those values are obtained from the software.

Based on the optimization outcomes, the response data were assessed to identify the appropriate model and mathematical equation. The response model for each response was used to examine it. Table 4 shows the results of the response model analysis for EE responses and Table 5 shows the results for LC responses.

Based on the results of the model selection, it was clear that the quadratic model was the best fit for the experimental data for the response of the EE model. Because it was significant ($p<0.05$), the quadratic model was adequate, and the lack of fit was not significant ($p>0.05$). The model must be significant, yet its imprecision must not be considerable. An identical LC response was obtained, showing that the quadratic model provided the best fit. Analysis was continued with the analysis of variance of the best model. The analysis of variance of these responses showed in Table 6 and Table 7.

Based on the findings of ANOVA testing on EE and LC responses. The model value was significant, indicating that the model was appropriate. The value of the model's inaccuracy was not substantial, indicating that the model is appropriate and fits. Mathematical models were y= 2.46503 - 0.086465(A) - 0.171886(B) - 0.000613(AB) + 0.003714(A2) + 0.009747(B2) for EE and y= -0.058163 - 0.090097(A) - 0.062600(B) -0.000613(AB) + 0.003821(A2) + 0.006830 + 0.009747(B2) for LC. Constants with negative or positive signs suggest a direct or inverse relationship between the variable or factor and the response. Figures 1 and Figure 2 show the 3D model graphs for the EE and LC responses, respectively.

The two responses showed a quadratic model with a parabolic curve, according to the 3D graphic illustration above. The absence of red colour on the curve and the absence of a stationary peak on the curve indicate that optimization has not yet achieved the maximum optimal position. The blue hue indicates that the result was still low; the green colour was one level higher. However, the highest response point of the variable may still be seen from the curve.

All of the criteria, in this case, were reported to be within the range or at specified positions. Because a maximal EE and LC response was sought, the maximum criterion was used. Table 8 displays the optimization outcome from software prediction. Table 9 shows the result verification from the predicted optimum formula.

The characterization carried out on liposomes were the measurement of particle size and the determination of their surface charge. Liposomes whose particle size and surface charge were measured were empty liposomes and liposomes loaded with active substances. The results of particle size analysis can be seen in Table 10.

The presence of the active chemical causes the liposomes to grow in size, according to the analysis results. This is due to the presence of active molecules in both the lipid bilayer and the aqueous core of liposomes. The active ingredient encapsulated into liposomes in this study was a combination of mangosteen peel and gotu kola, both of which were known to possess the primary components α-mangostin and asiaticoside. α[EF1]-mangostin is a semi-polar molecule that is most likely encased in a lipid bilayer. However, in addition to α-mangostin, other compounds, specifically polar molecules, may be enclosed in the aqueous core of the liposome.

A liposome's surface charge is closely connected to its stability. Liposomes with the same surface charge will repel each other, preventing aggregation and precipitation.[20,28] Liposomes whose surface charge was measured were both blank and loaded with active compounds. Table 11 shows the surface charge analysis results.

Based on the result above, it may be concluded that the active ingredient encapsulated in the liposome also serves to stabilize the liposome. The surface charge value of loaded liposomes is lower than the surface charge value of empty liposomes. This is due to the influence of the OH group of α-mangostin, which leads to the surface in the lipid bilayer system, resulting in a negative charge effect.[29] Ayumi Yoshida and colleagues conducted research.[29] It was also discovered that the surface charge of liposomes loaded with α-mangostin became increasingly negative. Ayumi Yoshida also used the fluorescence approach to determine the position of α-mangostin in the lipid bilayer system.

Cholesterol is a substance that makes up the framework or structure of biological cell membranes. The presence of cholesterol in the lipid bilayer membrane system provides rigidity and regulates the fluidity of the membrane.[30]

the influence of the amount of cholesterol on the ability of liposomes to maintain their active substances or on their efficiency and encapsulation capacity. The results of the influence of the amount of cholesterol on the EE and KE of liposomes can be seen in Figure 3.
According to the graph above, at the amounts of phospholipids of 113 mg and the mixture concentration of 200 ppm, an increase in the amount of cholesterol in the range of 7.92% to 22.07% increases the EE and LC of liposomes. Cholesterol can increase EE and LC because cholesterol helps stabilize the double-layer lipid system so that it is more rigid and absorbs more active substances. It also allows synergistic interactions in the form of hydrophobic interactions between cholesterol and α-mangostin and other substances so that the presence of α-mangostin and other substances is more retained in the liposome system.

Conclusion

The formulation using the RSM optimization formula showed that the formula composition of phospholipids: cholesterol: active substance concentration of 113 mg: 22.60 mg: 300 ppm produced the highest EE and LC, namely 82.42% and 2.18%, where the results were in accordance with the results predicted by the software. The liposomes produced with this formula have the following characteristics particle size and surface charge of 912.7 nm and -24.3 mV, respectively.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 3: Liposomal formulation optimization result of a mangosteen-gotu kola fraction 3:1 using response surface methodology (RSM) with central composite design (CCD)

Runs	Factors			Responses	
	Soybean Phospholipids (mg)	Cholesterol (*X1*, mg)	Mixture of combination of mangosteen-gotu kola fraction 3:1 (*X2*, mg)	Efficiency of encapsulation (EE) (%)	Loading capacity (LC) (%)
1.	113.00	22.60	15.00	81.06	2.15
2.	113.00	24.94	10.00	56.63	1.00
3.	113.00	11.30	15.00	39.70	1.05
4.	113.00	22.60	5.00	37.96	0.34
5.	113.00	16.95	2.93	62.09	0.32
6.	113.00	16.95	10.00	25.06	0.44
7.	113.00	11.30	5.00	15.85	0.14
8.	113.00	16.95	10.00	12.14	0.15
9.	113.00	16.95	17.07	43.29	1.31
10.	113.00	8.96	10.00	14.99	0.27
11.	113.00	16.95	10.00	14.72	0.26
12.	113.00	16.95	10.00	16.08	0.28
13.	113.00	16.95	10.00	17.17	0.30

Table 4: Statistical analysis and model fitting for the response of encapsulation efficiency (EE) of mangosteen-gotu kola fraction mixture combination 3:1

Model	Sequential *p*-value	Lack of fit *p*-value	Adjusted R^2	
Linier	0.1337	0.0379	0.1976	
2FI	0.8992	0.0286	0.1101	
Quadratic	**0.0097**	**0.1637**	**0.6956**	**Suggested**
Cubic	0.0584	0.8382	0.8682	Aliased

Table 5: Statistical analysis and model fitting for the response of loading capacity of mangosteen-gotu kola fraction mixture combination 3:1

Model	Sequential *p*-value	Lack of fit *p*-value	Adjusted R^2	
Linier	0.0015	0.0843	0.6715	
2FI	0.8750	0.0647	0.6360	
Quadratic	**0.0220**	**0.2415**	**0.8427**	**Suggested**
Cubic	0.0933	0.9620	0.9147	Aliased

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Table 6: Analysis of variance (ANOVA) test results for the response to encapsulation efficiency (EE) of a mangosteen-gotu kola fraction mixture combination 3:1

Model	Sum of square	df	Mean square	F-value	*p*-value	
Model	**0.7829**	**5**	**0.1566**	**6.48**	**0.0147**	**Significant**
A-Cholesterol	0.2833	1	0.2833	11.73	0.0111	
B-mixture combination of mangosteen-gotu kola	0.0321	1	0.0321	1.33	0.2869	
AB	0.0012	1	0.0012	0.0479	0.8300	
A^2	0.0978	1	0.0978	4.05	0.0841	
B^2	0.4131	1	0.4131	17.11	0.0044	
Residual	0.1690	7	0.0241			
Lack of fit	**0.1154**	**3**	**0.0385**	**2.87**	**0.1637**	**Not Significant**
Pure error	0.0536	4	0.0134			
Cor total	1.51	12				

Table 7: Analysis of variance (ANOVA) test results for the response to loading capacity (LC) of a mangosteen-gotu kola fraction mixture combination 3:1

Model	Sum of square	df	Mean square	F-value	p-value	
Model	**1.37**	**5**	**0.2743**	**13.86**	**0.0016**	**Significant**
A-Cholesterol	0.2833	1	0.2833	14.31	0.0069	
B-mixture combination of mangosteen-gotu kola	0.8133	1	0.8133	41.09	0.0004	
AB	0.0012	1	0.0012	0.0606	0.8126	
A^2	0.1035	1	0.1035	5.35	0.0561	
B^2	0.2033	1	0.2033	10.27	0.0150	
Residual	0.1386	7	0.0198			
Lack of fit	**0.0849**	**3**	**0.0283**	**2.11**	**0.2415**	**Not Significant**
Pure error	0.0536	4	0.0134			
Cor total	1.51	12				

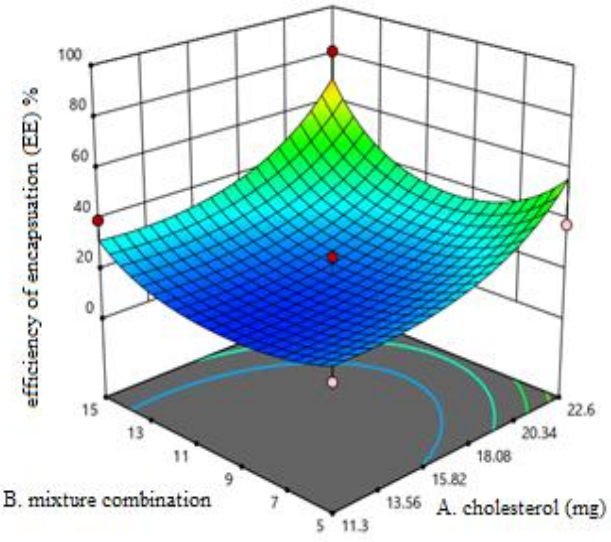

Figure 1: 3D graphical model of the encapsulation efficiency (EE) response of the mangosteen-gotu kola fraction liposomal mixture combination 3:1

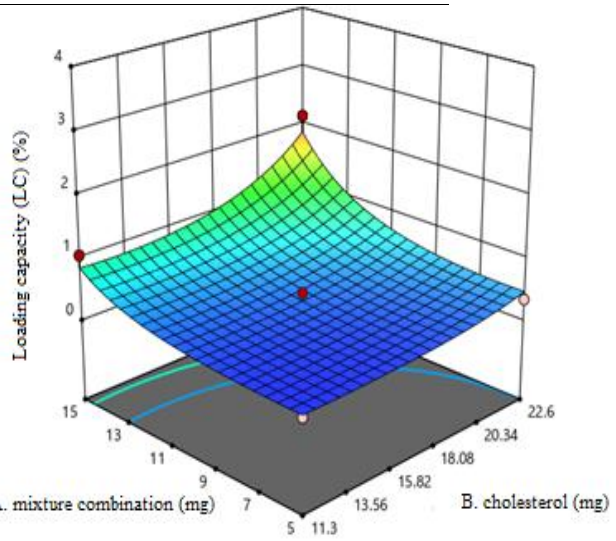

Figure 2: 3D graphical model of the loading capacity (LC) response of the mangosteen-gotu kola fraction liposomal mixture combination 3:1

Table 8: Predicted results in liposome formula with maximum responses

No.	Cholesterol (mg)	Mixture combination of mangosteen-gotu kola 3:1 (mg)	Efficiency of encapsulation (EE) (%)	Loading capacity (LC) (%)	Desirability	
1.	**22.60**	**15.00**	**65.29**	**1.77**	**0.91**	**Selected**
2.	22.60	14.87	63.30	1.71	0.89	
3.	22.350	15.00	62.67	1.70	0.89	
4.	22.60	14.81	62.43	1.68	0.89	
5.	22.60	5.00	52.82	0.44	0.57	
6.	22.60	10.78	34.54	0.67	0.56	
7.	11.30	15.00	29.73	0.81	0.50	
8.	11.598	15.00	29.51	0.80	0.55	
9.	22.60	5.68	46.52	0.44	0.54	
10.	22.60	5.80	45.49	0.44	0.54	
11.	11.30	5.00	20.50	0.17	0.14	

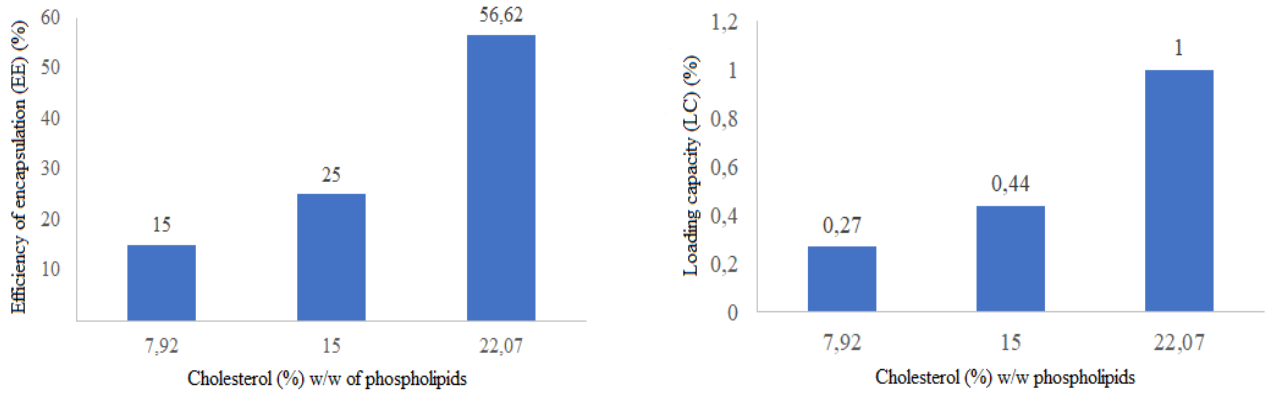

Figure 3: Effect of total cholesterol (%) on encapsulation efficiency (% EE) and encapsulation capacity (% LC) in liposomes containing a combination of mangosteen- Gotu Kola fraction in concentration of 200 ppm

Table 9: Predicted and experimental values of the responses at maximum conditions

Response (%)	Prediction range (%)	Observation value (%)
Efficiency of encapsulation (EE)	26.6139 – 160.181	**82.42**
Loading capacity (LC)	0.787323 – 3.99814	**2.18**

Table 10: Particle size values of blank liposomes and loaded liposomes of the combination of mangosteen-gotu kola fraction 3:1

Samples	Particle size (nm)	Dispersity form	Poly dispersity
Blank liposome	206.5	monodispersity	0.360
Loaded liposome	912.7	monodispersity	0.395

Table 11: Surface charge values of blank liposomes and loaded liposomes of the mangosteen peel-gotu kola fraction combination 3:1

Samples	Zeta potential (mV)	Stability classification*
Blank liposome	-9.5	Very unstable
Loaded liposome	-24.3	Fairly stable

Note: * = [20]

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