

DEVELOPMENT AND OPTIMIZATION OF METHOTREXATE-LOADED GLYCEROSOMES USING BOX-BEHNKEN DESIGN FOR ENHANCED TOPICAL DRUG DELIVERY

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Abstract

Methotrexate is an effective disease-modifying antirheumatic drug (DMARD) widely used in the management of rheumatoid arthritis and psoriasis; however, its conventional administration is associated with poor patient compliance and systemic adverse effects. The present study aimed to develop and optimize methotrexate-loaded glycosomes for enhanced topical drug delivery using a Box–Behnken response surface design. The formulation was optimized by evaluating the effects of phosphatidylcholine, stearylamine, and cholesterol on entrapment efficiency and vesicle size. Thirteen experimental formulations were prepared and statistically analyzed using Design-Expert® software. Among all formulations, the optimized formulation (F7) exhibited a high entrapment efficiency of 89.85%, a small vesicle size of 160.74 nm, and a zeta potential of -39.65 mV, indicating excellent colloidal stability. The optimized glycosomes demonstrated sustained in vitro drug release, achieving 98.78% cumulative drug release over 12 hours. Release kinetic analysis revealed that drug release predominantly followed the Higuchi diffusion model ($R^2 = 0.9843$), indicating diffusion-controlled release from the lipid matrix. The close agreement between predicted and experimental responses confirmed the validity and robustness of the optimization model. The optimized methotrexate-loaded glycosomal formulation exhibited desirable physicochemical characteristics, prolonged drug release, and excellent stability, suggesting its potential as an effective topical drug delivery system for improving therapeutic efficacy while minimizing systemic side effects in the treatment of inflammatory skin disorders.

Keywords: Methotrexate, Glycosomes, Box–Behnken design, Response surface methodology, Entrapment efficiency, Vesicle size, Zeta potential, Sustained drug release, Topical drug delivery, Rheumatoid arthritis.

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Introduction

Methotrexate (MTX) is a folic acid antagonist widely used as a first-line disease-modifying antirheumatic drug (DMARD) for the treatment of rheumatoid arthritis, psoriasis, and several autoimmune and inflammatory disorders (Cipriani et al., 2014). Despite its excellent therapeutic efficacy, oral and parenteral administration of methotrexate is frequently associated with dose-dependent systemic adverse effects, including hepatotoxicity, nephrotoxicity, gastrointestinal disturbances, bone marrow suppression, and poor patient compliance during long-term therapy (Shen et al., 2012). Moreover, the low permeability of methotrexate across biological membranes limits its effectiveness when administered through conventional dosage forms. Therefore, the development of an efficient topical delivery system capable of providing localized and sustained drug release while minimizing systemic toxicity has become an important area of pharmaceutical research (Abolmaali et al., 2013). Topical drug delivery offers several advantages over systemic administration, including avoidance of first-pass metabolism, reduced systemic exposure, improved patient compliance, and direct

delivery of the drug to the site of inflammation (Zhu et al., 2025). However, the highly organized structure of the stratum corneum acts as a major barrier to the permeation of hydrophilic drugs such as methotrexate, resulting in limited therapeutic effectiveness. Consequently, the development of novel vesicular carrier systems has attracted significant attention for improving dermal and transdermal drug delivery (Prasad and Koul, 2012).

Glycosomes are advanced phospholipid-based nanovesicular systems containing a high concentration of glycerol within the aqueous phase. The incorporation of glycerol enhances the elasticity, deformability, and fluidity of the phospholipid bilayer, enabling improved penetration through the skin compared with conventional liposomes (Abdallah et al., 2025). In addition, glycosomes provide high drug-loading capacity, enhanced physicochemical stability, prolonged drug release, and improved localization of drugs within the skin. These characteristics make glycosomes highly suitable carriers for topical delivery of anti-inflammatory and

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antipsoriatic agents such as methotrexate (Touitou and Natsheh, 2024).

The physicochemical characteristics of glycerosomes, including vesicle size, entrapment efficiency, surface charge, and drug release behavior, are strongly influenced by formulation variables such as phosphatidylcholine, cholesterol, and charge-inducing agents (Devi et al., 2026). Therefore, systematic optimization of these formulation components is essential to obtain a stable vesicular system with desirable therapeutic performance. Traditional trial-and-error methods are time-consuming and often fail to identify the optimal combination of formulation variables. In contrast, statistical experimental designs provide a scientific and economical approach for formulation optimization while reducing the number of experimental trials (Dangeti et al., 2023).

Among response surface methodologies, the Box–Behnken design (BBD) is one of the most efficient optimization techniques for pharmaceutical formulations. It enables simultaneous evaluation of the individual and interactive effects of multiple formulation variables on critical quality attributes through a limited number of experimental runs (Yadav et al., 2020). Furthermore, BBD facilitates the development of predictive mathematical models, response surface analysis, and desirability-based optimization, thereby improving formulation robustness and reproducibility.

In the present study, methotrexate-loaded glycerosomes were developed and optimized using a three-factor, three-level Box–Behnken design. Phosphatidylcholine, stearylamine, and cholesterol were selected as independent formulation variables, while entrapment efficiency and vesicle size were considered the critical responses. The optimized glycerosomal formulation was further characterized for zeta potential and in vitro drug release behavior, and release kinetics were evaluated using different mathematical models. The objective of this study was to develop a stable glycerosomal carrier capable of enhancing topical delivery of methotrexate through improved drug encapsulation, nanosized vesicles, sustained drug release, and enhanced therapeutic potential for the management of inflammatory skin disorders.

Material and Methods

Material

Methotrexate was used as the model drug for the preparation of glycerosomes. Phosphatidylcholine served as the primary phospholipid, stearylamine was employed as a charge-inducing agent, and cholesterol was incorporated to improve vesicle stability and membrane rigidity. Glycerol was used as the hydration medium to impart flexibility to the vesicles. Chloroform and ethanol were used as organic solvents for lipid dissolution, while phosphate buffer (pH 7.4) served as the dissolution

medium during in vitro drug release studies. All chemicals and reagents used in the study were of analytical grade and were utilized without further purification.

Methods

Experimental Design

The optimization of glycerosome formulation was carried out using Design-Expert® Software (Version 11.0.4.0) employing a three-factor, three-level Box–Behnken response surface design (BBD). A total of 17 experimental runs were generated based on a quadratic model in a randomized order without blocking. Three independent formulation variables were selected: Phosphatidylcholine (A: 50–150 mg), Stearylamine (B: 10–30 mg), and Cholesterol (C: 5–15 mg). The effects of these variables on the dependent responses, namely Entrapment Efficiency (%) and Vesicle Size (nm), were evaluated. The experimental data were fitted to a quadratic polynomial equation, and response surface analysis along with desirability function optimization was performed to identify the optimum formulation with maximum entrapment efficiency and minimum vesicle size (Salem et al., 2018).

Table 1: Independent Variables and Their Levels Used in the Box–Behnken Experimental Design for Optimization of Glycerosome Formulation

Factor	Name	Units	Minimum	Maximum	Mean	Standard Dev.
A	Phosphatidylcholine	mg	50.00	150.00	100.00	35.36
B	Stearylamine	mg	10.00	30.00	20.00	7.07
C	Cholesterol	mg	5.00	15.00	10.00	3.54

Preparation of Glycerosomes

Glycerosomes were prepared using the thin-film hydration technique. Initially, Methotrexate (50 mg) was dissolved in ethanol in a round-bottom flask. Separately, phosphatidylcholine (50 to 150mg), stearylamine (10 to 30mg), and cholesterol (5 to 15 mg) were dissolved in chloroform (10ml). The solution were combined thoroughly, and the organic solvents were removed under reduced pressure using a rotary vacuum evaporator until complete evaporation,

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resulting in the formation of a thin, uniform lipid film on the inner wall of the flask.

The dried lipid film was subsequently hydrated with phosphate buffer (pH 7.4) containing different concentrations of glycerol. Hydration was continued with gentle agitation to facilitate vesicle formation. The resulting multilamellar vesicular dispersion was further subjected to probe ultrasonication for a predetermined duration to reduce vesicle size and obtain nanosized glycosomes with a uniform size distribution (Aati et al., 2024).

Optimization of Glycosomal Formulation Using Full Factorial Design

The glycosomal formulations were optimized using a 2^3 full factorial design, in which two formulation variables were evaluated at three levels each. A total of nine experimental formulations were prepared according to the factorial design. The independent variables selected for optimization were Phosphatidylcholine (X_1) and Stearylamine (X_2) and Cholesterol (X_3). Their effects were investigated on two critical quality attributes, namely entrapment efficiency (Y_1) and Vesicle size (Y_2). The experimental design facilitated systematic evaluation of the influence of formulation variables and their interactions, enabling identification of the optimized glycosomal formulation with desirable physicochemical characteristics and drug release behavior (Salem et al., 2018).

Table 2: Composition of Methotrexate-Loaded Glycosome Formulations Prepared According to the Box-Behnken Experimental Design

F. C o d e	Met hotr exate (mg)	Phosph atidylc holine (mg)	Stea ryla mine (mg)	Cho leste rol (mg)	Chl orof orm (ml)	Et ha no l (ml)
F 1	50	150	30	10	10	q.s
F 2	50	50	20	15	10	q.s
F 3	50	150	10	10	10	q.s
F 4	50	100	10	15	10	q.s
F 5	50	100	10	5	10	q.s

F 6	50	100	20	10	10	q.s
F 7	50	150	20	5	10	q.s
F 8	50	150	20	15	10	q.s
F 9	50	50	10	10	10	q.s
F 10	50	50	30	10	10	q.s
F 11	50	100	30	15	10	q.s
F 12	50	100	30	5	10	q.s
F 13	50	50	20	5	10	q.s

Final Equation in Terms of Coded Factors

Entrapment Efficiency (%) = $88.91 + 5.96A + 1.30B + 0.9138C + 0.2650AB - 0.3500AC + 0.3375BC - 2.76A^2 - 4.17B^2 - 1.85C^2$.

Final Equation in Terms of Actual Factors

Entrapment Efficiency (%) = $38.44000 + 0.343480$ (Phosphatidylcholine) + 1.67928 (Stearylamine) + 1.66755 (Cholesterol) + 0.000530 (Phosphatidylcholine $\times$ Stearylamine) - 0.001400 (Phosphatidylcholine $\times$ Cholesterol) + 0.006750 (Stearylamine $\times$ Cholesterol) - 0.001105 (Phosphatidylcholine²) - 0.041748 (Stearylamine²) - 0.073990 (Cholesterol²).

Final Equation in Terms of Coded Factors

Vesicle Size (nm) = $171.57 - 26.60A + 8.02B + 12.62C - 1.74AB + 0.5375AC - 4.14BC + 23.38A^2 + 13.11B^2 + 5.28C^2$.

Final Equation in Terms of Actual Factors

Vesicle Size (nm) = $329.19000 - 2.35435$ (Phosphatidylcholine) - 3.26425 (Stearylamine) - 0.26050 (Cholesterol) - 0.003485 (Phosphatidylcholine $\times$ Stearylamine) + 0.002150 (Phosphatidylcholine $\times$ Cholesterol) - 0.082750 (Stearylamine $\times$ Cholesterol) + 0.009353 (Phosphatidylcholine²) + 0.131063 (Stearylamine²) + 0.211250 (Cholesterol²).

Characterization of Methotrexate Loaded Glycosomes Formulations

Vesicle Size Analysis

The mean vesicle size of the prepared methotrexate-loaded glycosome formulations was determined using a Dynamic Light Scattering (DLS) Particle Size Analyzer. Prior to analysis, 1 ml of the glycosomal suspension was diluted with 10 ml of distilled water to minimize multiple light scattering effects. The diluted sample was subjected to bath sonication for 1 minute to ensure uniform dispersion of the vesicles. The sample was then analyzed using the particle size analyzer, and the average vesicle size was recorded in nanometers (nm) (Moolakkadath et al., 2020).

Zeta Potential Measurement

The surface charge of the prepared glycosome formulations was determined by measuring the zeta potential using a Zeta Sizer based on the principle of Laser Doppler Electrophoresis (LDE). An appropriate amount of the glycosomal suspension was diluted with distilled water and transferred into a zeta potential cell. The electrophoretic mobility of the vesicles was measured, and the corresponding zeta potential was calculated automatically by the instrument software. The zeta potential values were expressed in millivolts (mV), and all measurements were carried out in triplicate. The obtained zeta potential values were used to assess the colloidal stability of the glycosome formulations (Aati et al., 2024).

Entrapment Efficiency

The entrapment efficiency of methotrexate-loaded glycosomes was determined by the ultracentrifugation method. A quantity of glycosomal formulation equivalent to 5 mg of methotrexate was transferred into ultracentrifuge tubes and centrifuged at 15,000 rpm for 30 minutes. Following centrifugation, the untrapped (free) methotrexate remained in the supernatant, whereas the drug-loaded glycosomes formed sediment at the bottom of the tube. The supernatant containing the free drug was carefully separated without disturbing the sediment and analyzed using a UV-Visible spectrophotometer at 294 nm to determine the concentration of untrapped methotrexate. The amount of entrapped drug was calculated by subtracting the amount of free drug present in the supernatant from the total amount of drug initially added (Moolakkadath et al., 2020). The percentage entrapment efficiency (%EE) was calculated using the following equation:

$$\% \text{ Entrapment Efficiency} = \frac{\text{Total Drug} - \text{Free Drug}}{\text{Total Drug}} \times 100$$

In Vitro Drug Release Study

The in vitro drug release of methotrexate from the prepared glycosome formulations was evaluated using the dialysis bag diffusion technique with a HiMedia Dialysis Membrane. A quantity of glycosomal suspension equivalent to 5 mg of

methotrexate was placed inside the dialysis membrane, and both ends of the membrane were securely tied. The dialysis bag was immersed in 200 mL of phosphate buffer saline (PBS, pH 7.4), which served as the receptor medium.

The release medium was maintained at $37 \pm 0.5^\circ\text{C}$ and continuously stirred at 100 rpm using a magnetic stirrer to simulate physiological conditions. At predetermined time intervals of 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 12 hours, 5 ml aliquots were withdrawn from the receptor medium and immediately replaced with an equal volume of fresh preheated PBS to maintain sink conditions and a constant diffusion volume. The collected samples were analyzed using a UV-Visible spectrophotometer at 294 nm to determine the cumulative amount of methotrexate released (Patil et al., 2024). All experiments were performed in triplicate ($n = 3$), and the cumulative percentage drug release was calculated and expressed as mean $\pm$ standard deviation (SD).

In Vitro Drug Release Kinetic Study

The release kinetics of methotrexate from the prepared glycosome formulations were investigated to determine the mechanism governing drug release. The cumulative percentage drug release data obtained from the in vitro release study were analyzed by fitting them into various model-dependent mathematical models, including the Zero-order, First-order, Higuchi and Korsmeyer-Peppas models. These kinetic models provide valuable information regarding the release pattern and the underlying mechanism of drug transport from the vesicular carrier system.

The suitability of each kinetic model was evaluated by calculating the regression coefficient (R^2) values for the corresponding plots. The model exhibiting the highest R^2 value was considered to best describe the drug release behavior of the formulation. Furthermore, the release exponent (n) obtained from the Korsmeyer-Peppas model was used to characterize the mechanism of drug release. Depending on the value of the release exponent, the release mechanism was classified as Fickian diffusion, anomalous (non-Fickian) transport or Case-II transport, thereby providing insight into whether the release was governed predominantly by diffusion, polymer relaxation, or a combination of both mechanisms (Kharbanda et al., 2026).

Results and Discussion

The present investigation successfully optimized methotrexate-loaded glycosomes using the Box-Behnken response surface design, which enabled systematic evaluation of the effects of phosphatidylcholine, stearylamine, and cholesterol on the critical quality attributes of the formulation. The selected independent variables and their respective ranges are presented in Table 1, while

the composition of all glycosomal formulations prepared according to the experimental design is summarized in Table 2. The application of Design-Expert® software facilitated the identification of the optimum formulation by minimizing the number of experimental runs while maximizing formulation performance.

The experimental responses obtained for the thirteen glycosomal formulations (Table 3) revealed considerable variation in both entrapment efficiency and vesicle size, confirming that lipid composition significantly influenced the physicochemical characteristics of the vesicles. Entrapment efficiency ranged from 74.86% to 90.42%, whereas vesicle size varied from 160.74 to 244.34 nm. The optimized formulation (F7) exhibited an entrapment efficiency of 89.85% with the smallest vesicle size (160.74 nm), suggesting that the selected proportions of phosphatidylcholine, stearylamine, and cholesterol were ideal for efficient drug encapsulation and nanosized vesicle formation. The higher phosphatidylcholine concentration provided a larger lipid bilayer for drug incorporation, while an optimum cholesterol concentration stabilized the bilayer without excessively increasing vesicle rigidity.

The adequacy of the developed quadratic models was confirmed through statistical diagnostic plots. The normal probability plots of externally studentized residuals for both responses (Figure 1a and Figure 2a) demonstrated that the residuals closely followed a straight line, indicating normal distribution of experimental errors. Likewise, the residuals versus predicted plots (Figure 1b and Figure 2b) exhibited random dispersion around zero without any systematic pattern, confirming model adequacy, homoscedasticity, and absence of significant experimental bias.

The contour plots and three-dimensional response surface plots clearly illustrated the influence of formulation variables on the measured responses. The contour and 3D plots for entrapment efficiency (Figures 1c and 1d) indicated that increasing phosphatidylcholine concentration significantly enhanced drug encapsulation, whereas excessive cholesterol slightly reduced entrapment owing to increased membrane rigidity. Similarly, the contour and response surface plots for vesicle size (Figures 2c and 2d) demonstrated that phosphatidylcholine contributed to the formation of smaller vesicles, while higher concentrations of cholesterol and stearylamine increased vesicle diameter because of enhanced membrane thickness and lipid packing. These response surface plots effectively demonstrated the interaction among formulation variables and aided in identifying the optimal formulation region.

The predictive capability of the optimization model was further verified by comparing the predicted and experimental values of the optimized formulation (Table 4). The predicted entrapment efficiency (89.69%) closely agreed with the experimental value (89.85%), showing a minimal residual error of 0.1588. Likewise, the predicted vesicle size (160.47 nm) was in excellent agreement with the experimentally observed value (160.74 nm), producing a residual of only 0.2700 nm. Such negligible differences indicate the high accuracy, robustness, and reliability of the mathematical model developed using the Box- Behnken design.

The desirability-based optimization was further supported by the overlay plots (Figures 3a–3c), which defined the optimized design space where both responses simultaneously satisfied the optimization criteria. These plots identified the ideal combination of phosphatidylcholine, stearylamine, and cholesterol concentrations that yielded glycosomes possessing maximum drug entrapment with minimum vesicle size. Such graphical optimization provides a practical approach for formulation development and future scale-up.

The optimized glycosomal formulation (F7) exhibited a zeta potential of -39.65 mV, as shown in Table 5. Zeta potential values greater than ± 30 mV are generally considered indicative of excellent colloidal stability because sufficient electrostatic repulsion prevents vesicle aggregation during storage. Therefore, the observed negative surface charge suggests that the optimized glycosomal dispersion possesses good physical stability and is suitable for long-term storage.

The *in vitro* drug release profile of the optimized formulation (Table 6) demonstrated a sustained release pattern extending over 12 hours. Approximately 18.42% of methotrexate was released within the first 30 minutes, followed by a gradual increase to 98.78% at the end of 12 hours. The initial burst release may be attributed to drug molecules adsorbed on the vesicle surface, whereas the subsequent controlled release resulted from diffusion of encapsulated drug through the phospholipid bilayer. This sustained release profile is advantageous for topical drug delivery because it prolongs drug residence time at the application site, reduces dosing frequency, and improves therapeutic efficacy.

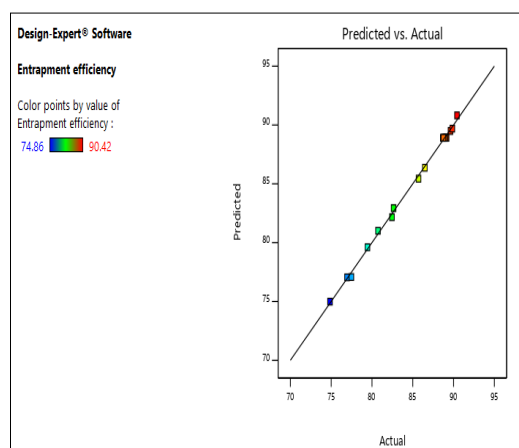
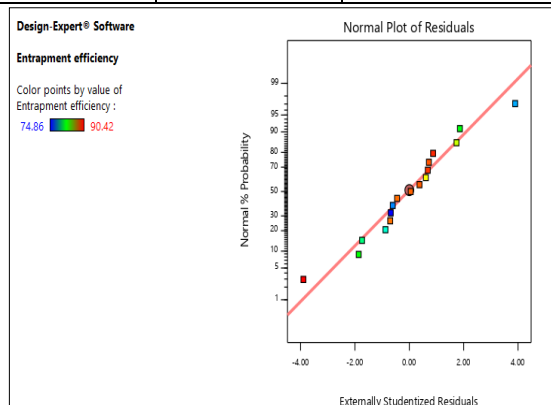
Release kinetic evaluation (Table 7) indicated that the optimized glycosome formulation best fitted the Higuchi diffusion model ($R^2 = 0.9843$), suggesting that diffusion through the lipid matrix was the primary mechanism governing methotrexate release. The Korsmeyer–Peppas model also showed an excellent correlation coefficient ($R^2 = 0.9834$), indicating diffusion-controlled release with possible contribution from

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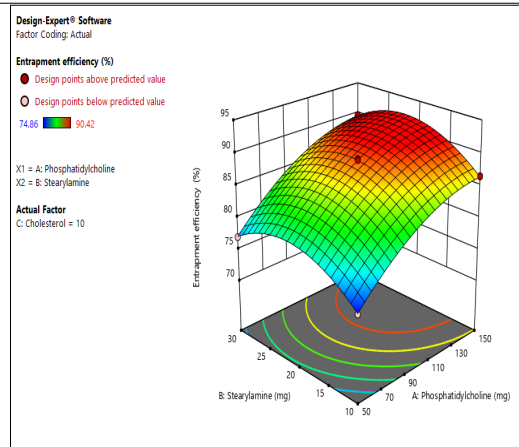
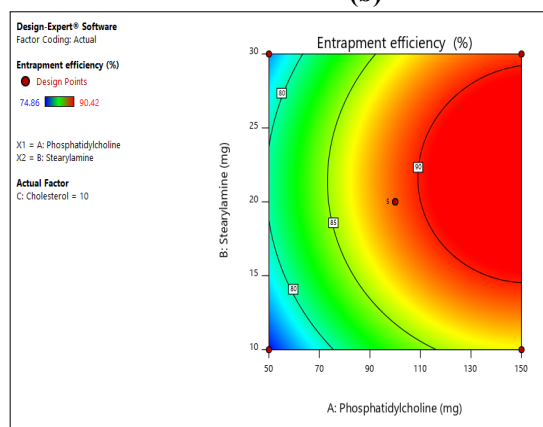
matrix relaxation. In contrast, the zero-order ($R^2 = 0.9187$) and first-order ($R^2 = 0.9573$) models exhibited comparatively lower regression coefficients, confirming that the Higuchi model most accurately described the release kinetics of the optimized glycosomal system.

Table 3: Experimental Values of Entrapment Efficiency (%) and Vesicle Size (nm) of Glycosome Formulations Prepared Using the Box-Behnken Design

F. Code	Response 1	Response 2
	Entrapment efficiency (%)	Vesicle size (nm)
F1	89.62	187.24
F2	79.45	238.65
F3	86.48	175.26
F4	82.45	198.47
F5	80.74	164.82
F6	88.76	171.53
F7	89.85	160.74
F8	90.42	186.93
F9	74.86	225.39
F10	76.94	244.34
F11	85.71	206.82
F12	82.65	189.72
F13	77.48	214.61



(a) (b)

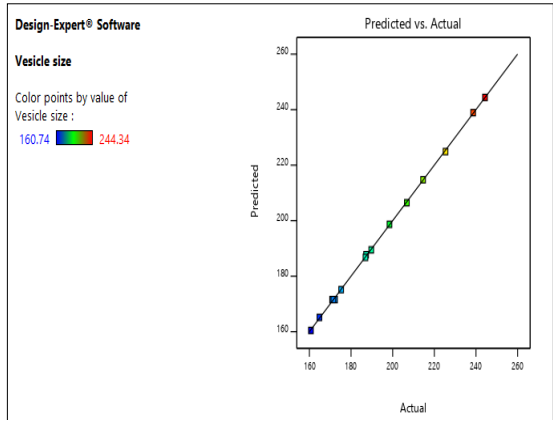
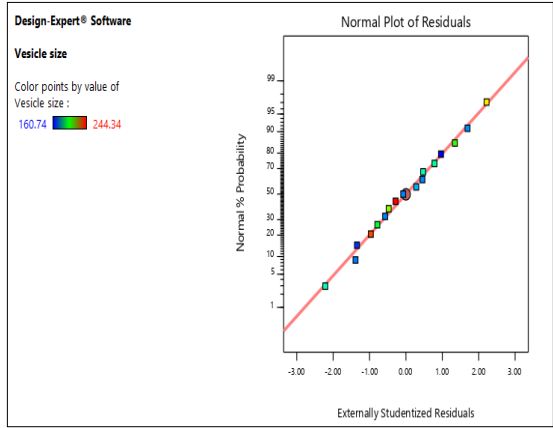


(c) (d)

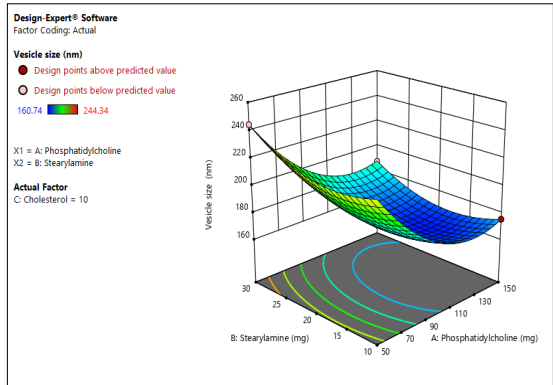
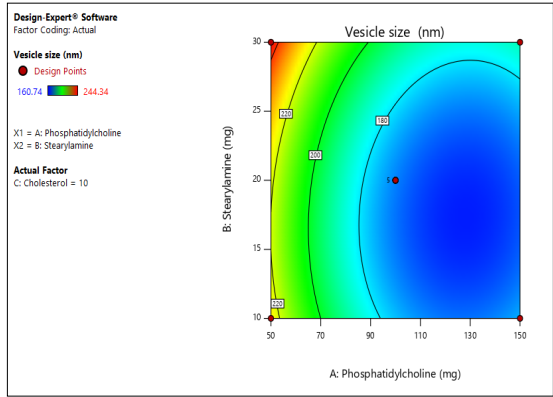
Figure 1a-d: (a) Normal Probability Plot of Externally Studentized Residuals for Entrapment Efficiency of Glycosome Formulation (b) Residuals versus Predicted Plot for Entrapment Efficiency of Glycosome Formulation (c) Contour Plot Showing the Effect of Phosphatidylcholine and Stearylamine on Entrapment Efficiency of Glycosome Formulation at a Fixed Cholesterol Concentration (10 mg) (d) 3D Plot Showing the Effect of Phosphatidylcholine and Stearylamine on Entrapment Efficiency of Glycosome

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Formulation at a Fixed Cholesterol Concentration (10 mg).



(a)
(b)



(c)

(d)

Figure 2a-d: (a) Normal Probability Plot of Externally Studentized Residuals for Vesicle size of Glycosome Formulation, (b) Residuals versus Predicted Plot for Vesicle size of Glycosome Formulation Contour Plot Showing the Effect of Phosphatidylcholine and Stearylamine on Vesicle size of Glycosome Formulation at a Fixed Cholesterol Concentration (10 mg), (c) Contour Plot Showing the Effect of Phosphatidylcholine and Stearylamine on Vesicle size of Glycosome Formulation at a Fixed Cholesterol Concentration (10 mg), (d) 3D Plot Showing the Effect of Phosphatidylcholine and Stearylamine on Vesicle size of Glycosome Formulation at a Fixed Cholesterol Concentration (10 mg).

Table 4: Comparison of Experimental and Predicted Responses for the Optimized Glycosome Formulation (F7)

Optimiz ed Formul ation	Respon se	Experim ental Value	Predic ted Value	Resid ual
F7	Entrap ment Efficie ncy (%)	89.85	89.69	0.158 8
	Vesicle Size (nm)	160.74	160.47	0.270 0

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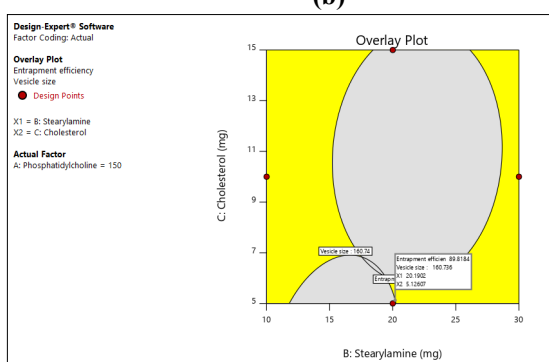
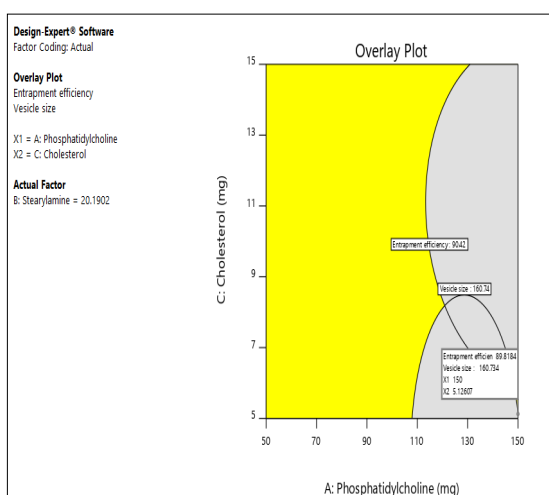
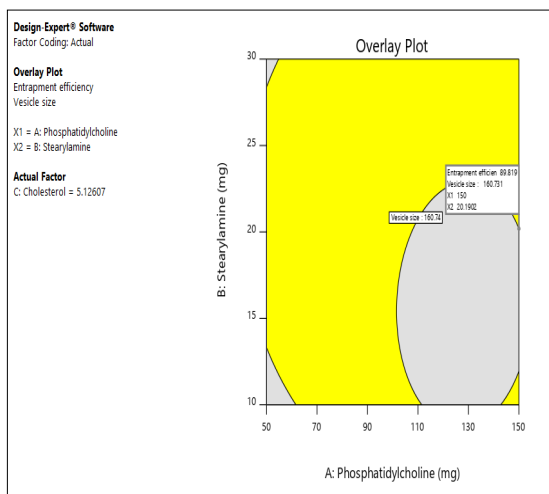


Figure 3a-c: (a) Overlay Plot Showing the Optimized Design Space for Entrapment Efficiency and Vesicle Size of Glycosome Formulation as a Function of Phosphatidylcholine and Stearylamine at a Fixed Cholesterol Concentration (5.13 mg), (b) Overlay Plot Showing the Optimized Design Space for Entrapment Efficiency and Vesicle Size of Glycosome Formulation as a Function of Phosphatidylcholine and Cholesterol at a Fixed Stearylamine Concentration (20.19 mg), (c) Overlay Plot Showing the Optimized Design

Space for Entrapment Efficiency and Vesicle Size of Glycosome Formulation as a Function of Stearylamine and Cholesterol at a Fixed Phosphatidylcholine Concentration (150 mg).

Table 5: Results of Zeta Potential of Optimized Formulation F7

S. No.	Formulation Code	Zeta Potential (mV)
1	F7	-39.65

Table 4: In Vitro Drug Release Profile of the Optimized Methotrexate-Loaded Glycosome Formulation (F7)

S. No.	Time (h)	Cumulative Drug Release (% $\pm$ SD)
1	0.5	18.42 $\pm$ 0.24
2	1.0	29.85 $\pm$ 0.31
3	1.5	38.76 $\pm$ 0.28
4	2.0	47.92 $\pm$ 0.35
5	4.0	64.85 $\pm$ 0.29
6	6.0	78.43 $\pm$ 0.33
7	8.0	88.76 $\pm$ 0.27
8	10.0	94.82 $\pm$ 0.25
9	12.0	98.78 $\pm$ 0.21

Table 6: Release Kinetics Data of the Optimized Methotrexate-Loaded Glycosome Formulation (F7)

S. No.	Time (h)	$\sqrt{t}$	Log Cumulative Drug Release (%)	% Drug Remaining	Log Drug Remaining	Cumulative Drug Released (%)	Log Cumulative Drug Released (%)
1	0.5	0.707	1.265	18.42	1.265	18.42	1.265

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2	1.000	1.000	0.000	29.85	70.15	1.846	29.85	1.475
3	1.55	1.225	0.176	38.76	61.24	1.787	38.76	1.588
4	2.0414	1.4101	0.3011	47.92	52.08	1.717	47.92	1.681
5	4.000	2.000	0.002	64.85	35.15	1.546	64.85	1.812
6	6.0449	2.4478	0.7038	78.43	21.57	1.334	78.43	1.894
7	8.0828	2.9803	0.9033	88.76	11.24	1.051	88.76	1.948
8	10.0162	3.1602	1.0000	94.82	5.18	0.714	94.82	1.977
9	12.0409	3.4647	1.1079	98.78	1.22	0.086	98.78	1.995

Table 7: Release Kinetic Model Fitting of the Optimized Methotrexate-Loaded Glycerosome Formulation (F7)

S. No.	Kinetic Model	Plot	Regression Coefficient (R ²)
1	Zero-order	Cumulative % Drug Release vs Time	0.9187

2	First-order	Log % Drug Remaining vs Time	0.9573
3	Higuchi	Cumulative % Drug Release vs $\sqrt{\text{Time}}$	0.9843
4	Korsmeyer–Peppas	Log Cumulative % Drug Release vs Log Time	0.9834

Conclusion

The present study successfully developed and optimized methotrexate-loaded glycerosomes using the Box–Behnken design. The optimized formulation (F7) exhibited high entrapment efficiency, nanosized vesicles, excellent colloidal stability, and sustained drug release over 12 hours. The optimized glycerosomal system demonstrated favorable physicochemical characteristics and diffusion-controlled drug release, indicating its potential as an effective topical carrier for methotrexate with improved therapeutic efficacy, prolonged drug action, and reduced systemic side effects.

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