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DEVELOPMENT AND OPTIMIZATION OF POLYHERBAL PHYTOSOMES CONTAINING *CITRUS LIMON*, *PONGAMIA PINNATA*, AND *PHYLLANTHUS NIRURI* USING BOX–BEHNKEN DESIGN

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ABSTRACT
The present study aimed to develop and optimize a phytosomal formulation containing the ethanolic extracts of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* using a Quality by Design (QbD) approach with Box–Behnken Design (BBD). Three formulation variables, namely phospholipid concentration, cholesterol concentration, and sonication time, were optimized to achieve minimum particle size and maximum entrapment efficiency. Thirteen experimental formulations were prepared and evaluated. Among them, the optimized formulation (PF10) exhibited a particle size of 158.0 nm, entrapment efficiency of 87.12%, and zeta potential of −38.25 mV, indicating the formation of stable phytosomal vesicles. Transmission electron microscopy confirmed the formation of spherical and uniformly distributed phytosomes. The optimized formulation exhibited sustained in vitro drug release, achieving 93.5% cumulative drug release within 12 h. Drug release kinetics showed the best fit to the Higuchi model ($R^2 = 0.9987$), indicating diffusion-controlled release. Stability studies conducted for three months demonstrated only minimal changes in particle size and entrapment efficiency under refrigerated and room-temperature storage conditions, confirming the good stability of the formulation. Overall, the optimized phytosomal formulation showed desirable physicochemical characteristics, high encapsulation efficiency, sustained drug release, and excellent stability, suggesting its potential as an effective phytosomal delivery system for improving the therapeutic performance of the selected herbal extracts.

KEYWORDS: *Citrus limon*, *Pongamia pinnata*, *Phyllanthus niruri*, Phytosomes, Box–Behnken Design, Design of Experiments (DoE), Particle size, Entrapment efficiency, Drug release, Stability study.

INTRODUCTION

Herbal medicines have gained considerable attention in recent years owing to their therapeutic efficacy, safety, affordability, and wide availability (Barkat et al., 2021). Numerous medicinal plants contain bioactive phytoconstituents such as flavonoids, phenolics, alkaloids, terpenoids, and tannins that exhibit antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, and immunomodulatory activities. However, the clinical application of many herbal extracts is often limited by poor aqueous solubility, low bioavailability, instability, and inadequate absorption across biological membranes (El-Saadony et al., 2025).

Phytosomes are an advanced lipid-based drug delivery system developed to overcome these limitations by complexing plant phytoconstituents with phospholipids. Unlike conventional herbal formulations, phytosomes improve membrane permeability, enhance gastrointestinal absorption, increase bioavailability, and provide better protection of phytochemicals against degradation. Owing to these advantages, phytosomal technology has emerged as a promising approach for enhancing the therapeutic efficacy of herbal medicines (Barani et al., 2021). *Citrus limon* is a rich source of flavonoids, vitamin C, and phenolic compounds with well-documented antioxidant, antimicrobial, and anti-inflammatory properties (Ben Hsouna et al., 2023). *Pongamia pinnata* contains bioactive constituents such as karanjin, pongamol, and flavonoids, which exhibit antimicrobial, antioxidant, wound-healing, and anti-inflammatory activities (Jakkannavar et al., 2025). *Phyllanthus niruri* is widely recognized for its hepatoprotective, antioxidant, antiviral, nephroprotective, and anti-inflammatory effects, primarily due to the presence of lignans, flavonoids, tannins, and polyphenolic compounds (Narendra et al., 2012). The combination of these three medicinal plants is expected to produce complementary and synergistic therapeutic effects by targeting multiple biological pathways simultaneously.

The quality and performance of phytosomal formulations are significantly influenced by formulation and process variables, including phospholipid concentration, cholesterol concentration, and sonication time (Kumar et al., 2025). Traditional formulation approaches require numerous experimental trials and often fail to identify interactions among variables. In contrast, Design of Experiments (DoE) provides a systematic and statistically efficient method for optimization by simultaneously evaluating multiple formulation factors and their interactions. Among the available DoE approaches, the Box–Behnken Design (BBD) is widely employed

because it requires fewer experimental runs while providing reliable mathematical models for optimization (Türkdoğan et al., 2026).

Therefore, the present study was undertaken to develop and optimize a polyherbal phytosomal formulation containing ethanolic extracts of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* using the Box–Behnken Design. The influence of phospholipid concentration, cholesterol concentration, and sonication time on particle size and entrapment efficiency was investigated to obtain an optimized formulation with desirable physicochemical characteristics. The optimized phytosomes were further characterized for particle size, zeta potential, morphology, in vitro drug release, release kinetics, and stability to evaluate their suitability as an efficient herbal drug delivery system.

Material and Methods

Material

The materials used in the present study included the ethanolic extracts of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* as the active herbal ingredients. Phospholipid (soya lecithin) and cholesterol were used for the preparation of phytosomes. Ethanol served as the solvent for dissolving the phospholipid and plant extracts, while distilled water was used as the aqueous phase. All chemicals and reagents employed in the study were of analytical grade and were procured from standard commercial suppliers.

Methods

Design of Experiment (DoE) procedure for optimization of phytosomal formulation

The optimization of phytosomal formulation of the ethanolic extracts of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* was carried out using Design of Experiments (DoE) with a Box–Behnken Design (BBD). This statistical design helps in evaluating the effect of formulation variables and their interactions on the selected responses while reducing the number of experimental runs.

Three independent formulation variables were selected based on preliminary studies: phospholipid concentration (A), cholesterol concentration (B), and sonication time (C). Each factor was studied at three levels: low (-1), medium (0), and high (+1). The levels were selected to obtain optimal vesicle formation and stability. The selected independent variables and their levels were phospholipid concentration (1–3 % w/v), cholesterol concentration (0.5–1.5 % w/v), and sonication time (5–15 min).

Selection of Independent Variables (Factors) for DoE

Table 1: Selection of Independent Variables (Factors) for DoE

Factor Code	Independent Variable	Low Level (-1)	Medium Level (0)	High Level (+1)
A	Phospholipid concentration (% w/v)	1	2	3
B	Cholesterol concentration (% w/v)	0.5	1	1.5
C	Sonication time (min)	5	10	15

Preparation of combined phytosomal formulation of the ethanolic extracts of *Citrus limon*, *Pongamia pinnata* and *Phyllanthus niruri*

The phytosomal formulation of the ethanolic extracts of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* was prepared by the solvent evaporation technique. Initially, the required quantity of the combined ethanolic extracts was dissolved in ethanol to obtain a clear extract solution. Separately, the lipid phase was prepared by dissolving soya phosphatidylcholine and cholesterol in an organic solvent such as dichloromethane or chloroform. The extract (500 each) was then gradually added to the lipid phase under continuous stirring to facilitate the formation of a phytoconstituent–phospholipid complex. The resulting mixture was subjected to rotary evaporation at a temperature of 40–45°C to remove the organic solvent under reduced pressure. During this process, a thin lipid film containing the phytoconstituent–phospholipid complex was formed on the inner wall of the round-bottom flask. The formed thin film was subsequently hydrated with distilled water or phosphate buffer (pH 7.4) with gentle agitation to obtain a phytosomal suspension. The dispersion was further sonicated for a specific period to reduce the vesicle size and to obtain uniformly distributed phytosomes. Finally, the prepared phytosomal suspension was collected and stored at 4°C until further characterization and evaluation studies (Jagtap et al., 2023).

Table 2: Experimental design and composition of phytosomal formulations (PF1–PF13) prepared using Box–Behnken Design showing levels of phospholipid concentration, cholesterol concentration, sonication time, and constant amount of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* extracts.

F. Code	Std	Run	Factor 1 A:Phospholipid % w/v	Factor 2 B:Cholesterol % w/v	Factor 3 C:Sonication time min	*Citrus limon* Extract (mg)	*Pongamia pinnata* Extract (mg)	*Phyllanthus niruri* Extract (mg)
PF1	2	1	3	0.5	10	250	250	250
PF2	7	2	1	1	15	250	250	250
PF3	1	3	1	0.5	10	250	250	250

PF4	4	4	3	1.5	10	250	250	250
PF5	5	5	1	1	5	250	250	250
PF6	15	6	2	1	10	250	250	250
PF7	3	7	1	1.5	10	250	250	250
PF8	10	9	2	1.5	5	250	250	250
PF9	6	10	3	1	5	250	250	250
PF10	8	12	3	1	15	250	250	250
PF11	11	13	2	0.5	15	250	250	250
PF12	12	16	2	1.5	15	250	250	250
PF13	9	17	2	0.5	5	250	250	250

Final Equation in Terms of Coded Factors

Particle size = $174.00 - 0.5000A + 7.13B - 38.88C + 5.50AB + 6.50AC + 1.25BC + 12.87A^2 + 13.62B^2 + 4.63C^2$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors

Particle size = 416.00000 − 76.00000 (Phospholipid) − 121.75000 (Cholesterol) − 14.57500 (Sonication time) + 11.00000 (Phospholipid × Cholesterol) + 1.30000 (Phospholipid × Sonication time) + 0.500000 (Cholesterol × Sonication time) + 12.87500 (Phospholipid²) + 54.50000 (Cholesterol²) + 0.185000 (Sonication time²).

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

Final Equation in Terms of Coded Factors

Entrapment Efficiency $= 79.70 + 8.82A + 1.94B + 1.06C - 0.6950AB + 0.3725AC + 0.3300BC - 1.03A^2 - 0.4217B^2 - 1.20C^2$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors

Entrapment Efficiency = 45.47000 + 13.58075 (Phospholipid) + 8.70900 (Cholesterol) + 0.894650 (Sonication time) − 1.39000 (Phospholipid × Cholesterol) + 0.074500 (Phospholipid × Sonication time) + 0.132000 (Cholesterol × Sonication time) − 1.02925 (Phospholipid²) − 1.68700 (Cholesterol²) − 0.048170 (Sonication time²).

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

Characterization of Phytosomal Formulation

Determination of Particle Size

The particle size and polydispersity index (PDI) of the prepared phytosomal formulations were determined using Dynamic Light Scattering (DLS) technique. The analysis was carried out using a particle size analyzer (Malvern Zetasizer). The phytosomal dispersion was diluted appropriately with distilled water to avoid multiple scattering effects. The diluted sample was transferred into a disposable cuvette, and measurements were performed at 25°C with a detection angle of 90°. The instrument software calculated the average particle size (nm) and polydispersity index, which indicates the uniformity of vesicle size distribution. All measurements were performed in triplicate and the average values were reported (Sasongko et al., 2019).

Determination of Zeta Potential

The zeta potential of the phytosomal formulations was measured to evaluate the surface charge and stability of the vesicular system. The analysis was carried out using a zeta potential analyzer based on electrophoretic light scattering (Malvern Zetasizer). A small amount of the phytosomal suspension was diluted with deionized water and placed in a zeta potential measurement cell. The sample was analyzed at 25°C, and the electrophoretic mobility of the particles was measured. The zeta potential values were calculated automatically by the instrument software and expressed in millivolts (mV). Higher absolute zeta potential values indicate better stability of the phytosomal dispersion (Sasongko et al., 2019).

Determination of Entrapment Efficiency

The entrapment efficiency (EE%) of phytosomes was determined by the centrifugation method. The phytosomal dispersion was centrifuged at 15,000 rpm for 30 minutes at 4°C using a high-speed refrigerated centrifuge to separate the free (unentrapped) extract from the phytosomal vesicles.

After centrifugation, the supernatant containing free extract was carefully collected and analyzed using UV–Visible spectrophotometry at the previously determined λmax of the extract. The amount of entrapped extract was calculated by subtracting the amount of free extract from the total extract used in the formulation (Rahman; 2021).

The entrapment efficiency was calculated using the following equation:

$$EE(\%) = \frac{Total\ extract - Free\ extract}{Total\ extract} \times 100$$

Where:

- Total extract = Initial amount of extract used in formulation
- Free extract = Amount of extract present in the supernatant

Morphological Analysis (Transmission Electron Microscopy)

The morphology and shape of phytosomes were examined using Transmission Electron Microscopy (TEM)**.** A drop of the diluted phytosomal suspension was placed on a carbon-coated copper grid and allowed to dry at room temperature. The sample was then stained with phosphotungstic acid (1%) to enhance contrast. The grid was observed under a TEM instrument, and images were captured to evaluate the shape, size, and vesicular structure of the phytosomes (Rani et al., 2022).

***In-vitro* release study**

The in-vitro release of phytoconstituents from phytosomes was evaluated using a dialysis bag diffusion method. A known quantity of phytosomal suspension equivalent to a fixed amount of extract was placed in a pre-soaked dialysis membrane bag**.** The dialysis bag was then immersed in phosphate buffer (pH 7.4) maintained at 37 ± 0.5°C under constant stirring using a magnetic stirrer**.**

At predetermined time intervals of 0, 1, 2, 4, 6, 8, 10 and 12 Hrs**,** 5 ml aliquots of the release medium were withdrawn and replaced with an equal volume of fresh buffer to maintain sink conditions**.** The withdrawn samples were filtered if necessary and analyzed using UV–Visible spectrophotometry at the λmax of the extracts**.** The cumulative percentage drug release was calculated and plotted against time to evaluate the release profile of the phytosomal formulation (Vaghela et al., 2024).

Drug Release Kinetic Study

The drug release kinetics of the optimized phytosomal formulation were evaluated to determine the mechanism of drug release. The in-vitro drug release data obtained from the release study were fitted into different kinetic models including Zero-order, First-order, Higuchi, and Korsmeyer–Peppas models**.**

Stability Study

The optimized phytosomal formulation was subjected to stability studies to evaluate its physical stability. The formulation was stored at 4°C and room temperature for a period of three months. Samples were withdrawn at regular intervals and analyzed for particle size, entrapment efficiency, and visual appearance to assess any changes in the formulation characteristics (Wanjiru et al., 2022).

RESULTS AND DISCUSSION

The phytosomal formulations containing the ethanolic extracts of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* were successfully optimized using a Box–Behnken Design (BBD) with phospholipid concentration, cholesterol concentration, and sonication time as the independent variables. The experimental design efficiently evaluated the influence of these formulation variables on particle size and entrapment efficiency while minimizing the number of experimental runs (Table 2). Statistical analysis and diagnostic plots demonstrated a good agreement between the experimental data and the predicted responses, confirming the adequacy and reliability of the optimization model (Figure 1 and Figure 2).

The prepared phytosomal formulations exhibited particle sizes ranging from 145.0 to 238.0 nm, whereas the entrapment efficiency varied between 66.34% and 88.76% (Table 3). Increasing the phospholipid concentration generally improved entrapment efficiency due to enhanced formation of the phospholipid bilayer; however, excessive phospholipid concentration also increased particle size. Cholesterol contributed to membrane rigidity and vesicle stability, while prolonged sonication effectively reduced vesicle size by breaking larger vesicles into smaller and more uniform particles. The contour and three-dimensional response surface plots clearly illustrated the interaction between phospholipid concentration and cholesterol concentration on both particle size and entrapment efficiency (Figure 1 and Figure 2).

Numerical optimization identified PF10 as the optimized phytosomal formulation, containing 3% w/v phospholipid, 1% w/v cholesterol, and 15 min sonication time (Table 4). The predicted responses closely matched the experimental values, with a predicted particle size of 158.63 nm compared to an experimental value of 158.00 nm, and a predicted entrapment efficiency of 87.72% compared to the experimentally obtained 87.12%, confirming the validity of the optimization model (Table 5).

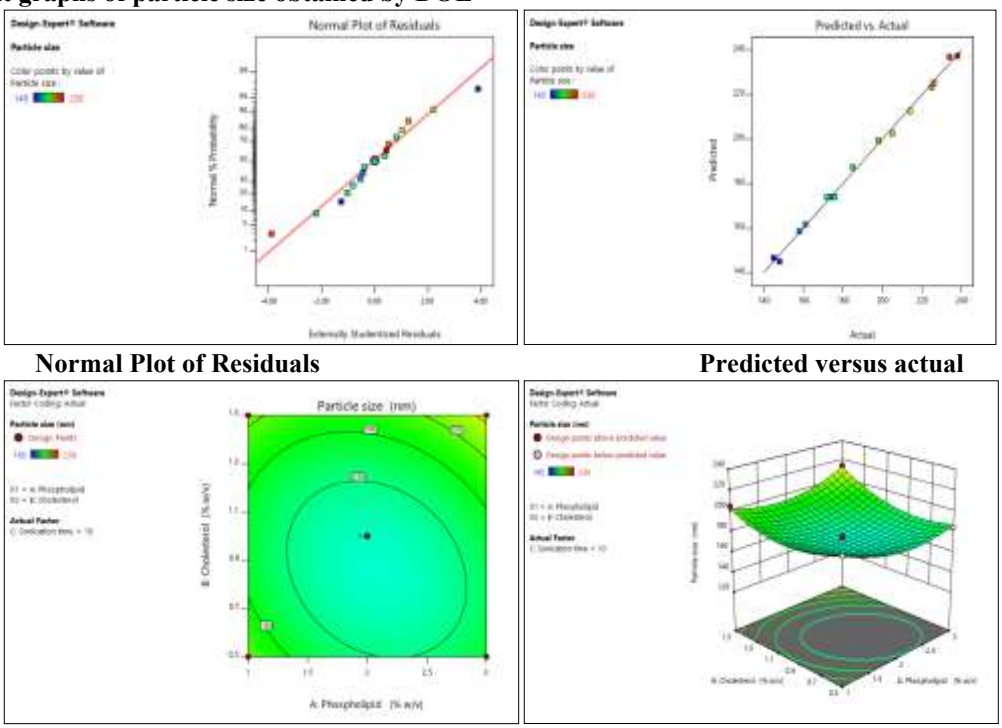
The optimized formulation (PF10) exhibited a particle size of 158.0 nm and a zeta potential of −38.25 mV, indicating the formation of nanosized phytosomes with excellent colloidal stability due to sufficient electrostatic repulsion between vesicles (Table 6). The TEM image further confirmed the formation of uniformly distributed, nearly spherical phytosomal vesicles (Figure 3).

The in vitro drug release study demonstrated a sustained release pattern from the optimized formulation, with cumulative drug release reaching 93.5% after 12 hours (Table 7). Release kinetic analysis showed that the formulation best followed the Higuchi model ($R^2 = 0.9987$), followed closely by the Korsmeyer–Peppas model ($R^2 = 0.9959$), indicating that drug release predominantly occurred through diffusion from the phytosomal matrix (Table 8).

The stability study demonstrated that the optimized phytosomal formulation remained stable over a period of three months under both refrigerated (4°C) and room temperature conditions. Only a slight increase in particle size and a minimal decrease in entrapment efficiency were observed during storage, indicating good physical stability and retention of the encapsulated phytoconstituents (Table 9).

Overall, the Box–Behnken Design successfully optimized the phytosomal formulation, and the optimized formulation (PF10) exhibited desirable particle size, high entrapment efficiency, sustained drug release, and excellent stability, making it a promising carrier system for the combined delivery of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* extracts.

Different graphs of particle size obtained by DOE

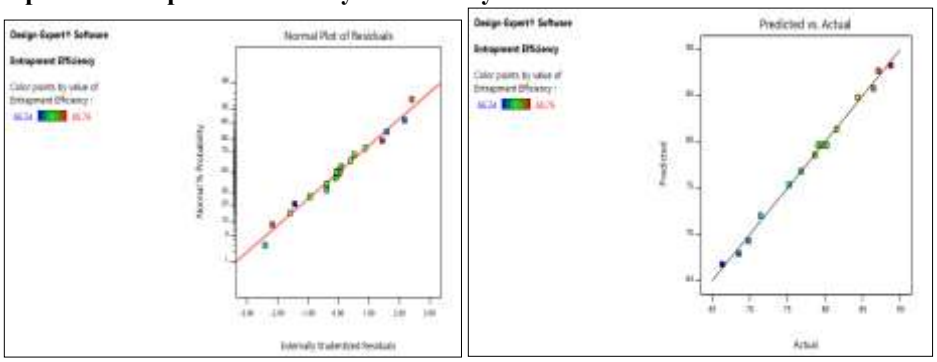


Normal Plot of Residuals **Predicted versus actual**

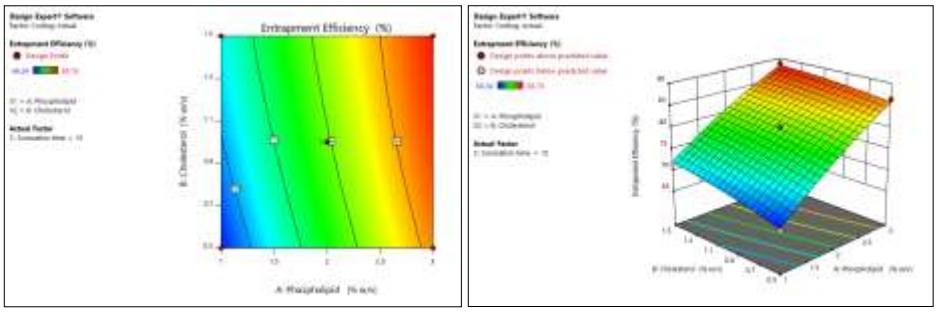
Contour plots between Phospholipid and Cholesterol **3D plots between Phospholipid and Cholesterol**

Figure 1: Different graphs of particle size obtained by DOE

Different graphs of Entrapment efficiency obtained by DOE



Normal Plot of Residuals **Predicted versus actual**



Contour plots between Phospholipid and Cholesterol **3D plots between Phospholipid and Cholesterol**

Figure 2: Different graphs of entrapment efficiency obtained by DOE

Table 3: Results of Particle size and Entrapment Efficiency

F. Code	Response 1	Response 2
	Particle size	Entrapment Efficiency
	(nm)	(%)
PF1	185.0	86.45
PF2	145.0	69.82
PF3	198.0	66.34
PF4	214.0	88.76
PF5	238.0	68.55
PF6	176.0	79.12
PF7	205.0	71.43
PF8	234.0	78.65
PF9	225.0	84.36
PF10	**158.0**	**87.12**
PF11	148.0	76.83
PF12	161.0	81.54
PF13	226.0	75.26

Table 4: Optimization Data Showing Predicted Responses for Optimized Phytosomal Formulation

Factor	Optimized Value
Phospholipid (% w/v)	3
Cholesterol (% w/v)	1
Sonication Time (min)	15

Table 5: Predicted and Experimental Responses

Response	Predicted Value	Experimental Value
Particle Size (nm)	158.63	158.00
Entrapment Efficiency (%)	87.72	87.12

Table 6: Results of particle size and zeta potential of Optimized formulation PF10

Formulation Code	Zeta Potential (MV)	Particle Size (nm)
PF10	-38.25	158.0

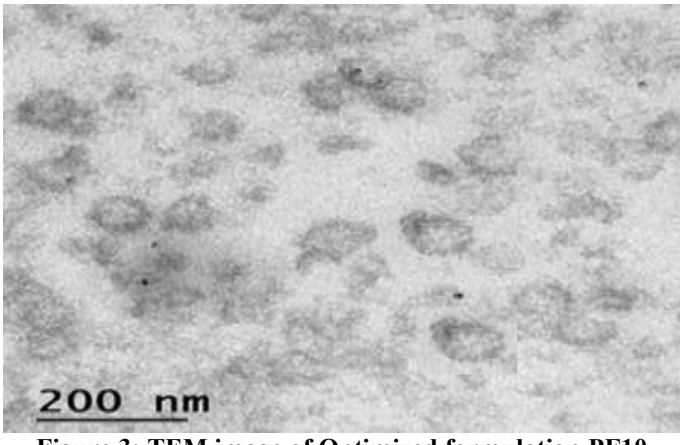


Figure 3: TEM image of Optimized formulation PF10

Table 7: In-vitro drug release data for the optimized formulation PF10

Time (h)	Cumulative Drug Release (%)
0	0
0.5	8.5
1	15.2
2	28.4
4	45.7
6	61.3
8	74.6
10	85.2
12	93.5

Average of Three determinations (n=3±SD)

Table 8: In-vitro Drug Release Kinetics of Optimized Formulation PF10

Kinetic Model	Regression Coefficient (R^2)
Zero-order	0.9760
First-order	0.9645
Higuchi	0.9987
Korsmeyer–Peppas	0.9959

Table 9: Results of Stability study

Time (Months)	Particle Size (nm)		Entrapment Efficiency (%)	
	-4 °C	Room Temp	-4 °C	Room Temp
0	158.0	158.0	87.12	87.12
1	160.2	161.0	86.85	86.70
2	162.5	163.8	86.50	86.35
3	164.1	166.5	86.20	85.90

CONCLUSION

The present study successfully optimized a phytosomal formulation containing the ethanolic extracts of *Citrus limon*, *Pongamia pinnata*, and *Phyllanthus niruri* using the Box–Behnken Design. The optimized formulation (PF10) exhibited a particle size of 158.0 nm, high entrapment efficiency (87.12%), excellent colloidal stability (−38.25 mV), and sustained drug release following the Higuchi model. The formulation also remained stable during three months of storage with minimal changes in particle size and entrapment efficiency. The optimized phytosomal formulation demonstrated desirable physicochemical characteristics and sustained release behavior, indicating its potential as an effective phytosomal drug delivery system for enhanced therapeutic performance.

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