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RESEARCH ARTICLE

Phytoconstituents Loaded Liposomes Fabricated Based on Box Behnken Design for Metabolic Syndrome: *In Vitro* and *In Vivo* Characterization

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Abstract

The global use of secondary metabolites like flavonoids, plant sterols, and alkaloids has been increasing due to their therapeutic benefits and fewer side effects compared to modern medicines. However, despite promising *in vitro* results, many herbal drugs and extracts demonstrate limited effectiveness *in vivo* due to their low lipid solubility and poor bioavailability. To address this issue, novel formulation strategies, particularly lipid-based delivery systems, are being proposed as carriers to enhance their bioavailability. This study focuses on the pharmaceutical development of liposomes that encapsulate three phytoconstituents, namely quercetin, berberine, and phytosterol, using the Quality by Design (QbD) concept. The Ishikawa diagram was utilized to identify the key factors affecting formulation quality, and the statistical experiment design concept was employed to optimize these factors. The liposomes were designed using the screening with the Placket-Burman approach and further optimized using the Box-Behnken method. The optimized liposomes exhibited an ideal size and achieved high entrapment efficiencies of 80.6%, 81.3%, and 80.35% for quercetin, berberine, and phytosterol, respectively. These liposomes were prepared using Phospholipon 90 G and cholesterol through the thin film hydration method. The resulting liposomes were thoroughly characterized and evaluated for morphology, % drug release, pharmacodynamic investigation, and stability studies.

Keywords: Berberine, Metabolic Syndrome, Phytosterol, Quercetin

1. Introduction

Metabolic syndrome, alternatively referred to as syndrome X and insulin resistance syndrome[1], encompasses a cluster of metabolic abnormalities. These dysfunctions include insulin resistance, type 2 diabetes, obesity, high blood pressure, and dyslipidemia, which is characterized by abnormal blood fat levels[1].

The development of obesity-related metabolic disorders is attributed to underlying mechanisms such as insulin resistance, elevated plasma-free fatty acids, chronic inflammation, and oxidative stress[2]. In obese individuals, the elevated level of free fatty acids leads to insulin resistance by suppressing insulin clearance. To compensate for this, the pancreas secretes more insulin, resulting in hyperinsulinemia[2,3].

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By encouraging the aberrant production of adipocytokines such as TNF-, IL-1, IL-6, leptin, and plasminogen activator inhibitor-1, chronic inflammation, which is linked to visceral fat, worsens insulin resistance[4-6]. Due to this vicious loop, inflammation and metabolic dysfunction get worse and insulin resistance and hyperinsulinemia get worse[7,8].

One of the main approaches used to increase medicine safety and effectiveness is the use of nano-based drug delivery systems[9].

The high dosage, poor efficacy, poor bioavailability, lack of target specificity, and dose-dependent side effects are a few of the key problems of conventional drug delivery techniques.

Despite demonstrating significant potential in *in vitro* studies, many herbal drugs and extracts fail to produce the same effects *in vivo* due to factors such as poor lipid solubility or improper molecular size. As a result, these compounds are poorly absorbed and have low bioavailability, ultimately leading to limited or no *in vitro* activity[9].

Liposomes are regarded as a versatile platform for effectively delivering pharmaceutical drugs and active substances in a range of applications in the fields of biomedical and nanomedicine[9,10]. These liposomes possess controllable properties such as lipid composition, size, structure, morphology, surface charge, and the ability to modify their surfaces with polymers or ligands. One notable feature is their capacity to encapsulate both hydrophilic and lipophilic active compounds, as well as diverse biomolecules like carbohydrates, proteins and peptides, DNA, or imaging compounds. The structure of liposomes is regulated by soft interactions and self-assembly phenomena, which govern their structural characteristics and stability in biological tissue environments[10-13].

Incorporating drugs within the nanostructure of vesicles enhances the solubility of active chemicals in solution and safeguards them against chemical and biological degradation. Furthermore, the utilization of liposome nanoformulations significantly enhances their therapeutic effectiveness[11]. Flavonoids, a group of naturally occurring compounds found widely in plants as secondary metabolites, possess noteworthy clinical properties, including anti-inflammatory, anti-allergic, anti-viral, anti-bacterial, and anti-tumor activities. Among these flavonoids, QE (3,5,7,3',4'-pentahydroxyflavone)[4], depicted in Figure 1(a), stands out for its ability to prevent oxidant-induced damage and cell death through multiple mechanisms. These mechanisms include scavenging oxygen radicals, protecting against lipid peroxidation, and chelating metal ions[4-8].

Berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium), represented in Figure 1(b), is a benzylisoquinoline alkaloid that holds significance in the fields of pharmacology and medicinal chemistry. It is recognized as a highly significant natural alkaloid for synthesizing numerous bioactive derivatives through the condensation, modification, and substitution of functional groups at strategic positions. These synthetic strategies aim to design novel, selective, and potent drugs[14-20].

Phytosterol (shown in Figure 1(c)) on the other hand is a primary plant sterol (mixture of B sitosterol, stigmasterol and campesterol), containing cyclopentano perhydro phenanthrene ring as in cholesterol. Therefore it is likely that it competes for cholesterol absorption in the lower intestine and in turn, reduces the levels of cholesterol in the blood[21-24].

Figure 1(a). Chemical structure of Quercetin.

Figure 1(b). Chemical structure of Berberine.

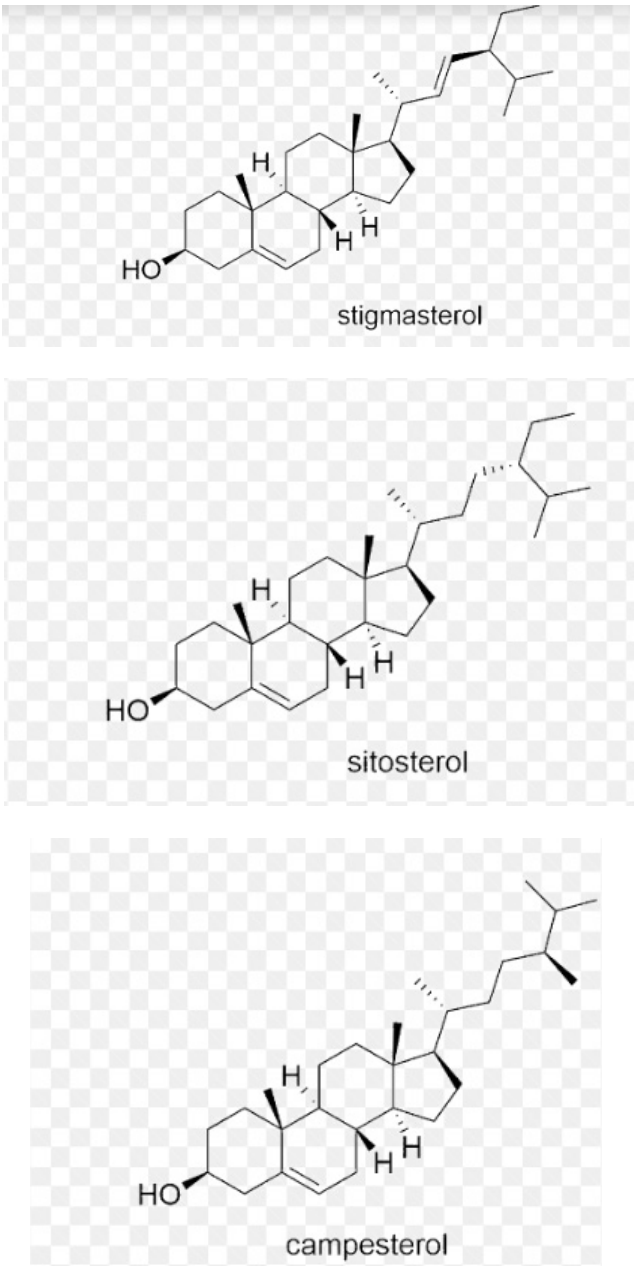


Figure 1(c). Chemical structure of phytosterol consisiting stigmatserol, sitosterol and campesterol.

Since the three Phytoconstituents exhibit antidiabetic, antihypertensive, and anti-hyperlipidemic effects individually, they have been chosen to explore their combined synergistic effects.

Currently, there are numerous allopathic formulations available for treating specified diseases. However, these formulations often bring about various side effects. Hence, the objective of the proposed research is to create a singular oral prophylactic formulation that effectively addresses these diseases while minimizing significant side effects.

The primary goal of this study is to develop a single oral dosage form comprising liposomes loaded with Quercetin, Berberine, and Phytosterol. The study focuses on assessing the impact of different formulation and process variables on the creation of a prophylactic single oral dosage form specifically designed for treating metabolic syndrome resulting from modern lifestyle and dietary habits.

In this study, the concept of Quality by Design (QbD) has been employed to achieve high-quality Liposome formulation[26]. QbD is a systematic, risk-based approach that promotes the development of pharmaceuticals in a methodical and optimistic manner[25,26]. It involves a thoughtful design and development process that considers the relationship between dependent and independent variables, as well as their impact on product performance[25-28].

The objective of QbD is to identify Critical Quality Attributes (CQAs) and critical process parameters by establishing a Quality Target Product Profile (QTPP) and Conducting a Quantified Risk Assessment (QRA) and Risk Analysis (CPPs)[29]. The subsequent step in the design process involves experimental design (DoE), where critical process parameters are selected based on their significance in determining product quality. This approach ensures the selection of an efficient process and the achievement of desired product quality parameters[29,30].

The distinctive aspect of this study lies in the utilization of Quality by Design (QbD) methodologies for the design of a Liposome formulation, which involves the identification and mitigation of risk factors associated with achieving a high-quality product. This unique approach enables the formulator to validate the formulation with fewer experiments within the study design.

The study was specifically designed to identify critical independent factors in the Liposome formulation through the application of Placket-Burman's design. Subsequently, the optimization of Critical Process Parameters (CPPs) was carried out using QbD principles to determine Critical Quality Attributes (CQAs), such as particle size and % entrapment efficiency. This optimization process was conducted using a 3-factor, 3-level Box-Behnken approach[31,32].

Furthermore, the study aims to evaluate the effectiveness of the developed formulation through both *in vivo* and *in vitro* assessments[30-32].

2. Materials and Methods

2.1 Materials

Quercetin (99.7%), Berberine (98%) and Phytosterol (94%) were purchased from Yuccca laboratories in Mumbai. Cholesterol and Phospholipon 90 G were gifted from Lipoid Germany. Methanol and chloroform were purchased from SD fine.

2.2 Instrumentation

The production of the liposome involved the utilization of specific equipment. A rotary evaporator (IKA RV 10 digital, Staufen, Germany) and a probe sonicator (Frontline Sonicator, Mumbai, India) were employed. The estimation of particle size was conducted using the Malvern Zetasizer (Nano ZS 90, Malvern Instruments, UK).

2.3 Animal

Male albino Wistar rats were chosen as the subjects for the *in vivo* studies. These experimental rats were acclimated to the test site, which was appropriately situated in a well-ventilated animal house maintained at a temperature of 25 ± 2 °C and relative humidity of 75%.

2.4 Methods

2.4.1 Experimental Design

2.4.1.1 Examining of Variables[29]

The Ishikawa diagram (Figure 2) was used in the current research to identify the potential risk variables for product quality (CQAs, such as particle size and % entrapment efficiency), which were then further screened. The screening has been done by using Placket and Burmann design considering 7 factors (Amount of lipid, Amount of cholesterol, Hydration volume, Speed of rota evaporator, temperature, sonication time, and hydration time) at 2 levels as mentioned in Table 1.

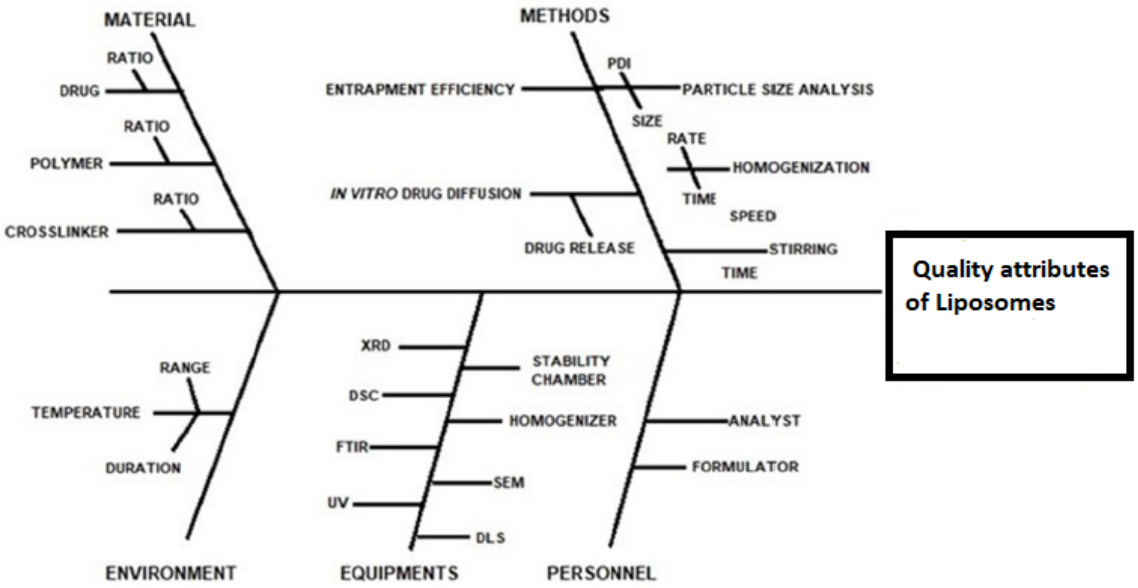


Figure 2. An Ishikawa diagram was created to visually represent the different formulation and process variables that impact the expected excellence of Liposomes.

$$Y = A0 + A1X1 + A2X2 + A3X3 + A4X4 + A5X5 + A6X6 + A7X7 + A8X8 + A9X9$$

Where Y is the response, A0 is the constant, A1 to A7 are the coefficients of response values, X1 to X8 are the independent variables.

Table 1. Placket and Burman screening design

Preparation of Liposomes on the basis of Placket and Burman design									Response
Formulation Code	Amount of Lipid (mg)	Amount of Cholesterol (mg)	Hydration Volume (ml)	Speed of Rota evaporator (rpm)	Temperature (C°)	Sonication time (min)	Hydration time (min)	Particle size (nm)	%EE
F1	60	12	50	120	45	10	90	115.6	75.00
F2	90	12	50	60	60	5	90	113.7	85.43
F3	90	18	50	60	45	10	60	91.31	80.66
F4	60	18	100	60	45	5	90	112.3	80.00
F5	90	12	100	120	45	5	60	141.8	76.66
F6	60	18	50	120	60	5	60	80.39	77.30
F7	60	12	100	60	60	10	60	108.9	78.30
F8	90	18	100	120	60	10	90	128.7	88.30

2.4.1.2 Optimization of formulations (Box Behnken Design)

Design of experiments (DoE) has been employed as a powerful strategy to reduce discrepancy in the formulation development and ultimately produce Liposomes with high product yield and constant particle size. Optimization of the formulations was done using Box - Behnken design by taking three independent variables as Amount of lipid, Amount of cholesterol and hydration volume which are screened by Placket and Burmann design. The identified independent variables and their values to formulate each 15 formulations for Quercetin, Berberine and Phytosterol are given in Table 3. The polynomial equation engendered for Box Behnken design is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + X_1^2 + X_2^2 + X_3^2 + X_4^2 + b12X1X2 + b13X1X3$$

2.4.2 Formulation of Liposomes[29,30]

Liposomes were prepared using the thin layer hydration method, employing various molar ratios of Phytoconstituent, Phospholipid 90G, and cholesterol. Firstly, the Phytoconstituent and Phospholipid 90G were dissolved in methanol, while cholesterol was dissolved in chloroform. The resulting mixture was then transferred to a round bottom flask and subjected to evaporation in a rotary evaporator at 45 °C, leading to the evaporation of all solvents and the formation of a thin dry film in the flask. Vacuum drying was performed to ensure the complete removal of organic solvents.

Subsequently, the dry film was hydrated with distilled water in a rotary apparatus at 45 °C. To further reduce the size of the liposome particles, the suspension was subjected to sonication using a probe sonicator.

2.4.3 Morphological Examination[27-30]

The diameters of the solid particles in the formulation are referred to as the particle size. Nanometers (nm) are the size unit for nanoparticles. Malvern Zetasizer (Malvern ZS90) was used to measure the optimised liposome formulation's particle size, polydispersity index, and zeta potential.

2.4.4 Physicochemical Evaluation by FTIR and DSC[27-30]

The FTIR spectrum of the samples was acquired using the Perkin Elmer system in ATR mode. Pure Phytoconsytituents, lipid, cholesterol and its synthetic mixture was scanned for FT IR by ATR mode.

2.4.5 % Entrapment Efficiency[27-30]

The entrapment efficiency of the liposomes loaded with all three phytoconstituents was determined by centrifuging the liposomes in methanol (Remi, India) at 1000 rpm for 10 minutes. The concentration of the drug in the supernatant was then measured using a UV spectrophotometer (Shimadzu, 1900) at wavelengths of 380 nm, 349 nm, and 207 nm for Quercetin, Berberine, and Phytosterol, respectively.

2.4.6 In Vitro Drug Release Studies[27-30]

For *in vitro* drug release studies, a Franz diffusion cell with a volume of 5 mL and an internal diameter of 1 cm was utilized. The liposomes loaded with phytoconstituents were permeated through a dialysis membrane with a molecular weight cutoff of 12-14 KDa[30]. In the donor compartment, 100 mg of the formulation was placed, while the receptor medium consisted of phosphate buffer with a pH of 7.2, continuously stirred using a small magnetic bead.

To mimic human conditions, the temperature during the experiment was maintained at 37 ± 0.5 °C. At predetermined time points (0, 1, 2, 3, 4, 5, 6, 7, and 24 hours), 1 mL of samples was withdrawn from the receptor medium and replaced with fresh receptor solution. The withdrawn samples were then spectrophotometrically analyzed at wavelengths of 380 nm, 349 nm, and 207 nm for Quercetin, Berberine, and Phytosterol, respectively.

The amount of drug released was calculated, and the percentage of drug released was plotted against time to evaluate the drug release profile.

2.4.7 Lyophilization[28-31]

Liposomal suspension of all individual phytoconstituents were lyophilized by adding mannitol as cryoprotectant. The amount of mannitol was added (lipid: Cryoprotectant -1:3) to the Liposomal suspension and were frozen at -80 °C for 24 hours using an ultra-cold freezer (RevcoTM, ThermoScientific, Waltham, MA, USA). Subsequently, the samples were freeze-dried for 48 hours using a Flexi-DryTM MP Freeze Dryer (SP Scientific, Stone Ridge, NY, USA) under conditions of -90 °C and 380 mT of pressure, resulting in the production of a dry powder[31].

2.4.8 Stability Studies[28-31]

The optimized formulation underwent stability studies in accordance with ICH guidelines. These studies were conducted at room temperature (25 °C ± 2 °C / 60 ± 5 % RH) and accelerated conditions (40 °C ± 2 °C / 75 ± 5 % RH) for a period of 6 months. The formulation was assessed for particle size, entrapment efficiency, and drug release to evaluate its stability over time.

2.4.9 Animal Studies[28-31]

Institutional Animal Ethics Committee gave its approval to the animal experiment. Parul university (Protocol No. CPCSEA 921/PO/ReBi/S/05/CPCSEA/PIPH 04/21). Wistar rats were chosen for the activity. 24 rats were divided in four groups as I) Normal control group II) High fat diet group III) high fat diet and streptozotocin induced diabetes group IV) high fat diet and streptozotocin induced diabetes group. Group I animals were control group and treated with normal saline. Group II and Group III animal were on high fat diet (mixture of normal pellet diet+raw cholesterol + vanaspati ghee) to induce metabolic syndrome for 28 days and received streptozotocin (40 mg/kg body wt, i.p. dissolved in 0.01 M citrate buffer) for 3 days to produce diabetes, hyperlipdemia and hypertension. After 28 days Group II did not receive any treatment while Group III animals received standard treatment Metformin, Atorvastatin, and Atenolol for 28 days. While Group IV has received a Novel liposomal formulation containing all three Phytoconstituents as per dose 30 mg/kg for 28 days. Evaluation has been done by measuring body weight weekly, blood pressure has been measured weekly by tail-cuff method, blood glucose level weekly by strip glucose oxidase method, and lipid profile has been measured after 28 days withdrawing blood by Retro orbital vein.

3. Result and Discussion

3.1 Screening of Process and Formulation Variables

Based on the initial experiments conducted using the Placket-Burman design, it was evident that the Amount of lipid, Amount of cholesterol, and Hydration volume significantly influenced the design of the Liposome formulation. These preparation variables were considered in relation to the particle size and entrapment efficiency, which were determined using the Placket-Burman design.

The relationship between the independent factors and the quantitative outcomes of particle size and entrapment efficiency can be described by the following non-linear equation.

Particle size = 143.12 + 7.2 (Amount of lipid) - 8.32(Amount of cholesterol) + 11.4 (Hydration volume) + 5.12 (speed of rota evaporator) - 3.75 (temperature) - 0.46(sonication time) + 5.98 (Hydration time)

The precision of the estimation of coefficient value for particle size was determined to be 6.04. All factors that showed a standardised effect value of more than 6.04 were deemed significant. Identifying dependent and

independent factors for the final formulation is supported by the Pareto chart (Figure 2.). The Pareto graphic illustrates the relationship between the independent factors and their plausible influence on particle size.

Entrapment Efficiency = 86.1+ 2.55 (Amount of lipid) - 8.72(Amount of cholesterol) - 18.5 (Hydration volume) – 0.89 (speed of rota evaporator) + 2.12 (temperature) + 0.35 (sonication time) + 1.97 (Hydration time).

The precision of estimation of coefficient value for the determined entrapment efficiency was observed to be 2.20. All influences displayed standardized effect values greater than 2.20 were considered as significant variables. The amount of lipid, amount of cholesterol and Hydration volume shows a significant effect and all other variables were non-significant as observed from the Pareto chart shown in Figures 3 and 4.

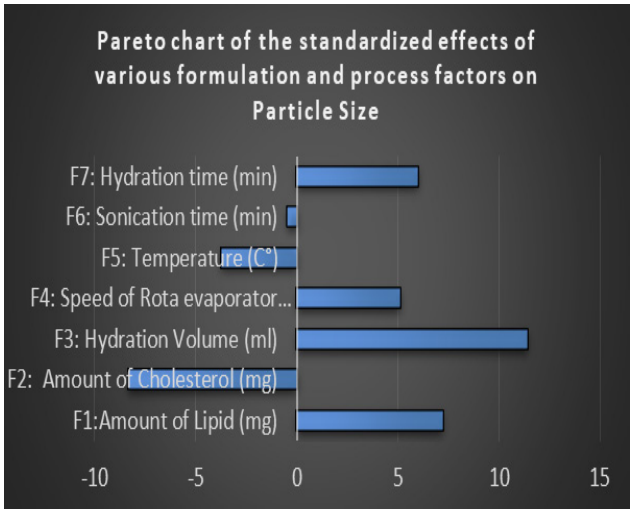


Figure 3(a). Pareto chart for Screening of formulation and process variables by Placket and Burman design.

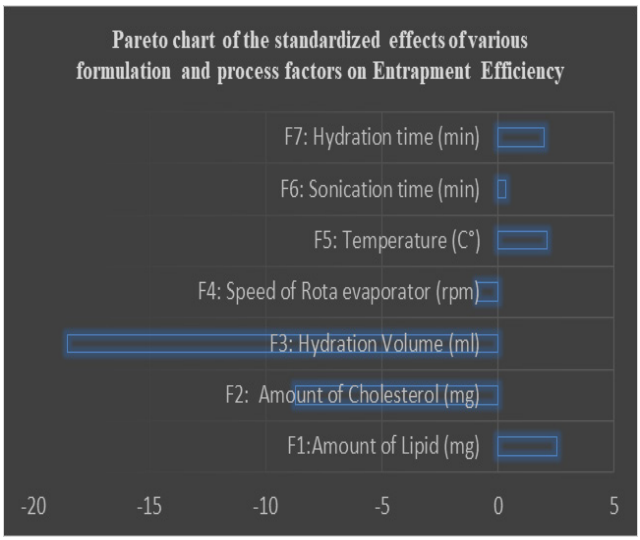


Figure 3(b). Pareto chart for Screening of formulation and process variables by Placket and Burman design.

3.2 Optimization of the Formulation by Box-Behnken Design

Through the implementation of the Placket Burman Design, three factors, namely the Amount of lipid, Amount of cholesterol, and Hydration volume, were identified as having a significant impact on both particle size and entrapment efficiency. Subsequently, employing a 3-factor, 3-level Box Behnken Design, a total of 15 liposome formulations loaded with Quercetin, Berberine, and Phytosterol were optimized and evaluated for their particle size and entrapment efficiency. The obtained response data for Quercetin, Berberine, and Phytosterol are presented in Tables 2, 3, and 4, respectively, following the layout of the Box-Behnken design.

Table 2. Box Behnken design for optimization of Independent variables on Quercetin loaded Liposomes

No. of Runs	Independent Variables			Dependant Variables	
	X1	X2	X3	Y1(nm)	Y2(%)
1	75	12	50	98.54	62.1
2	75	15	75	101.5	67.2
3	75	18	50	111.5	71.3
4	60	15	50	98.7	48.6
5	75	18	100	227.8	69.1
6	75	15	75	165	66.9
7	90	18	75	226	83.2
8	90	15	100	187	76.3

Table 2 continued...

No. of Runs	Independent Variables			Dependant Variables	
	X1	X2	X3	Y1(nm)	Y2(%)
9	75	12	100	105.7	64.3
10	60	12	75	99.3	46.9
11	90	15	50	210	73.4
12	90	12	75	179.1	75.77
13	75	15	75	102.7	66.3
14	60	18	75	100.3	50.2
15	60	15	100	91.3	46.8

Table 3. Box Behnken design for optimization of independent variables on Berberine loaded Liposomes

No. of Runs	Independent Variables			Dependent Variables	
	X1	X2	X3	Y1(nm)	Y2(%)
1	90	15	50	227.8	80.3
2	60	15	100	104.9	44.7
3	60	12	75	94.8	42.1
4	75	18	50	130.2	61.9
5	75	15	75	123.9	57.1
6	75	12	50	114.6	52.6
7	90	15	100	210.9	82.9
8	60	15	50	107.3	46.2
9	75	15	75	122.8	58.4
10	75	12	100	119.7	53.3
11	75	15	75	120.7	55.9
12	90	12	75	213.3	78.6
13	60	18	75	110.4	49.3
14	90	18	75	237.7	84.1
15	75	18	100	131.8	60.7

Table 4. Box Behnken design for optimization of independent variables on Phytosterol loaded Liposomes

No. of Runs	Independent Variables			Dependent Variables	
	X1	X2	X3	Y1(nm)	Y2(%)
1	60	15	100	97.9	50.7
2	75	15	75	131.5	65.88
3	75	15	75	133.4	64.11
4	75	15	75	138.7	62.23
5	90	15	100	190.4	80.51
6	90	12	75	182.1	75.88
7	75	12	50	124.9	61.1
8	90	18	75	207.6	83.9

Table 4 continued...

No. of Runs	Independent Variables			Dependent Variables	
	X1	X2	X3	Y1(nm)	Y2(%)
9	60	15	50	99.8	52.89
10	90	15	50	213.2	76.43
11	60	12	75	93.6	49.78
12	60	18	75	110.6	55.9
13	75	18	50	147.8	68.72
14	75	18	100	151.2	70.23
15	75	12	100	121.2	58.6

Using Design Expert® software, the data were statistically analysed by the application of ANOVA. The model with the greatest F value was regarded as the most effective.

The influence of independent variables on the particle size of Quercetin loaded liposomes

The particle size of the Quercetin-loaded liposome batches, determined using the Box-Behnken design, ranged from a minimum of 91.3 nm to a maximum of 227.8 nm. Through response surface analysis, a statistical equation was derived to illustrate the relationship between particle size and the independent variables. The equation is presented below as a reduced polynomial equation.

$PS = -266.76400+3.437\ A^2$

The resulting quadratic model's F-value of 7.85 indicates that it is appropriate for use with the particle size results. The model's R2 value was discovered to be 0.6817, indicating a strong connection between predicted and actual particle size outcomes. Additionally, the "Pred R-Squared" value of 0.4063 was found to align reasonably well with the "Adj R-Squared" value of 0.5948, indicating that the chosen model is suitable for predicting particle size. In order to examine the combined effects of two independent factors on a single response, 3D response surface plots were analyzed as depicted in Figure 4.

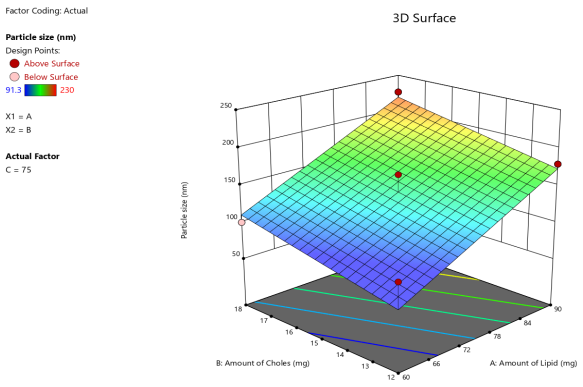


Figure 4. Independent variables influence on the particle size of Quercetin loaded Liposomes.

The influence of independent variables on the particle size of Berberine loaded liposomes

The particle size of the Berberine-loaded liposome batches, determined using the Box-Behnken design, ranged from a minimum of 94.8 nm to a maximum of 237.7 nm. Through response surface analysis, a statistical equation was derived to illustrate the relationship between particle size and the independent variables. The equation is presented below as a reduced polynomial equation.

$PS = +818.05-22.81A-4.85972B+0.178296\ A^2$

The resulting quadratic model's F-value of 123.57 indicates that it is appropriate for use with the particle size results. The model's R2 score, which indicates a strong connection between anticipated and actual particle size findings, was discovered to be 0.9914. Furthermore, the "Pred R-Squared" value of 0.8839 was found to be in agreement with the "Adj R-Squared" value of 0.9804, indicating that the selected model is suitable for accurately predicting particle size. To establish the mutual effects of two separate influences on a single response, 3D response surface frames were analysed (Figure 5).

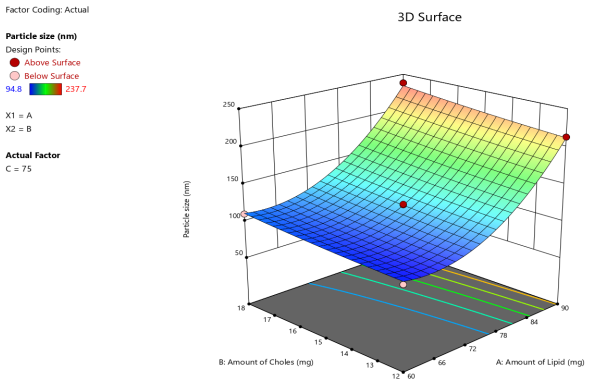


Figure 5. Independent variables influence on the Particle size Berberine loaded Liposomes.

The influence of independent variables on the particle size of Phytosterol loaded Liposomes.

The particle size of the Phytosterol-loaded liposome batches, as determined using the Box-Behnken design, exhibited a range from a minimum of 94.8 nm to a maximum of 237.7 nm. Through response surface analysis, a statistical equation was derived to depict the relationship between particle size and the independent variables. The equation, presented below, represents a reduced polynomial equation.

$PS = +818.05-22.81A-4.85972B+0.178296\ A^2$

The quadratic model obtained demonstrated a significant F-value of 70.30, indicating its suitability in predicting particle size. The R2 value of 0.9922 indicated a strong correlation between the predicted and observed results for particle size. Additionally, the "Pred R-Squared" value of 0.9780 was found to be in reasonable agreement with the "Adj R-Squared" value of 0.8924, further supporting the appropriateness of the selected model for predicting particle size. (Figure 6).

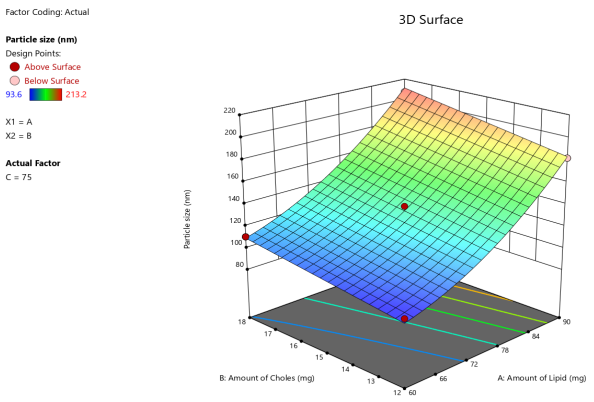


Figure 6. Independent variables influence on the Particle size Phytosterol loaded Liposomes.

The influence of independent variables on the % Entrapment Efficiency of Quercetin loaded Liposomes.

The % Entrapment Efficiency of the Quercetin-loaded liposome formulations exhibited a range from a minimum of 46.8 % to a maximum of 83.2%. The quadratic model provided the following expression, illustrating the relationship between Entrapment Efficiency and the independent variables defined in the study.

% Entrapment Efficiency = -111.6911 + 2.9947A + 0.447 B - $0.013A^2$

The F-value of 116.80 obtained for the quadratic model signifies its statistical significance in predicting % Entrapment Efficiency. Additionally, the "Pred R-Squared" value of 0.9953 aligns reasonably well with the "Adj R-Squared" value of 0.9867, indicating the appropriateness of the selected model in fitting the % Entrapment Efficiency results. The correlation coefficient of 0.9270 further confirms the goodness of fit of the model (Figure 7).

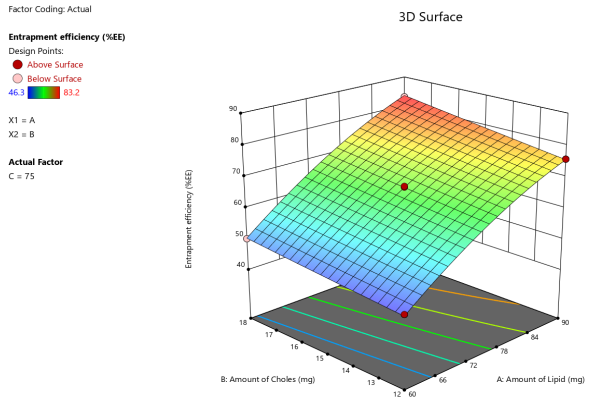


Figure 7. Independent variables influence on the Entrapment efficiency of Quercetin loaded Liposomes.

The influence of independent variables on the % Entrapment Efficiency of Berberine loaded Liposomes.

The % Entrapment Efficiency of the Berberine-loaded liposome formulations exhibited a range from a minimum of 42.1% to a maximum of 82.9%. The quadratic model proposed the following expression, illustrating the relationship between Entrapment Efficiency and the independent variables defined in the study.

% Entrapment Efficiency = + 106.1625 - 3.130A + 2.422B + 0.028A^2

The obtained F-value of 283.61 indicates the statistical significance of the proposed quadratic model for the % Entrapment Efficiency. Furthermore, the "Pred R-Squared" value of 0.9980 aligns well with the "Adj R-Squared" value of 0.9945, supporting the suitability of the selected model in accurately predicting the % Entrapment Efficiency results. The correlation coefficient of 0.9838 demonstrates a strong level of fit for the model, indicating its goodness of fit (Figure 8).

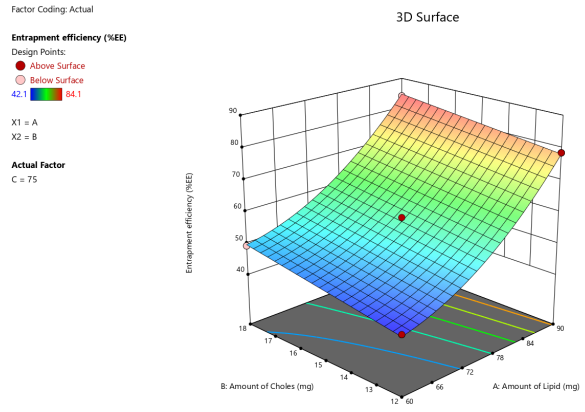


Figure 8. Independent variables influence on the Entrapment efficiency of Berberine loaded Liposomes.

The influence of independent variables on the % Entrapment Efficiency of Phytosterol loaded Liposomes.

The % Entrapment Efficiency of the formulated batches of Phytosterol-loaded liposomes was found to vary within a minimum of 49.78% and a maximum of 80.51%. The quadratic model suggested the following expression depicting the correlation between the Entrapment Efficiency and the defined independent variables:

% Entrapment Efficiency = -111.6911+2.9947A+0.447 B-0.013A^2

The obtained F-value of 160.71 indicates the statistical significance of the proposed quadratic model for the % Entrapment Efficiency. Additionally, the "Pred R-Squared" value of 0.9777 aligns well with the "Adj R-Squared" value of 0.9716, indicating the suitability of the selected model in fitting the % Entrapment Efficiency results. The correlation coefficient of 0.9584 demonstrates a strong level of fit for the model, indicating its goodness of fit (Figure 9).

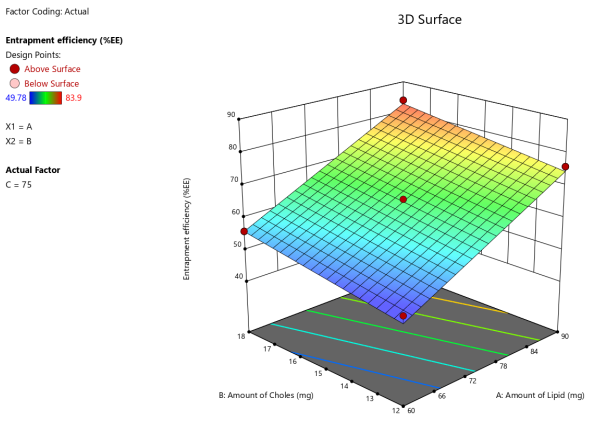


Figure 9. Independent variables influence on the Entrapment efficiency of Phytosterol loaded Liposomes.

3.3 Optimization of Quercetin, Berberine and Phytosterol-Loaded Liposomes Individually

After gathering response data from all batches, numerical optimization was implemented to simultaneously optimize the process parameters in order to generate Quercetin, Berberine, and Phytosterol-loaded liposomes with desired characteristics. Design ExpertVR software was utilized, setting specific limits for all three process parameters, aiming to achieve liposomes with minimal particle size and maximal % entrapment efficiency. The optimized batch was designed based on the predicted values of the independent variables, thereby assessing the predictability of the Box-Behnken Design, as presented in Table 5. The effectiveness of BBD for statistical optimization was confirmed by the low percentage bias values observed between the predicted and experimental response data, establishing its authenticity.

Table 5. Comparison of experimental results with predicted responses of the optimized formulation

Batch code	Response	Predicted value	Experimental value	Percentage error
Quercetin	Particle size (nm)	216	217.9	1.9
	% EE	81.3	80.6	0.7
Berberine	Particle size (nm)	210.5	212.3	1.8
	% EE	80.2	81.3	1.1
Phytosterol	Particle size (nm)	201.48	202.1	0.62
	% EE	80.35	80.6	0.25

3.4 Particle Size

Particle size is a critical parameter that plays a significant role in the drug release and *in vivo* absorption of a formulation, ultimately impacting its bioavailability. The optimized liposomes demonstrated average particle sizes of 141.62 nm, 142.92 nm, and 144.72 nm for Quercetin, Berberine, and Phytosterol-loaded liposomes, respectively.

3.5 FT IR Study

FTIR analysis was conducted on Quercetin, Berberine, Phytosterol, Phospholipon 90 G, cholesterol, physical mixture, and lyophilized liposomes to investigate potential physical and chemical interactions between the drug and excipients. The results showed no significant alterations or interactions observed in the spectra, indicating the absence of any adverse effects. Notably, there were noticeable changes in the stretching frequency of the phenolic O-H group of the drug, shifting from 3,430.41 cm^{-1} to 3,416.95 cm^{-1}. This can be attributed to molecular interactions during the liposome formulation process. Furthermore, alterations in the absorption peaks of phospholipids were observed at 1,226.66 cm^{-1} and 1,061.61 cm^{-1}, suggesting molecular changes due to liposome formation. The retention of peaks in both the physical mixture of excipients and the formulated liposomes suggests a harmonious physicochemical compatibility between the drug and other ingredients.

3.6 Morphological Characterization

The morphological characterization using SEM micrographs established that liposomes of Quercetin, Berberine and Phytosterol were needle in shape, discrete and distributed uniformly throughout. The SEM micrograph is depicted in Figures 10(a-d).

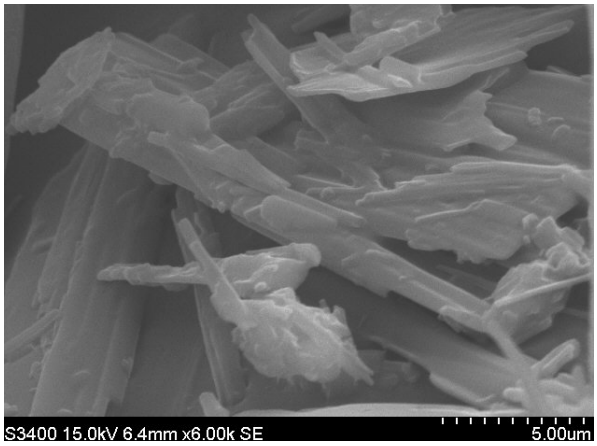


Figure 10(a). SEM image of Berberine Liposomes.

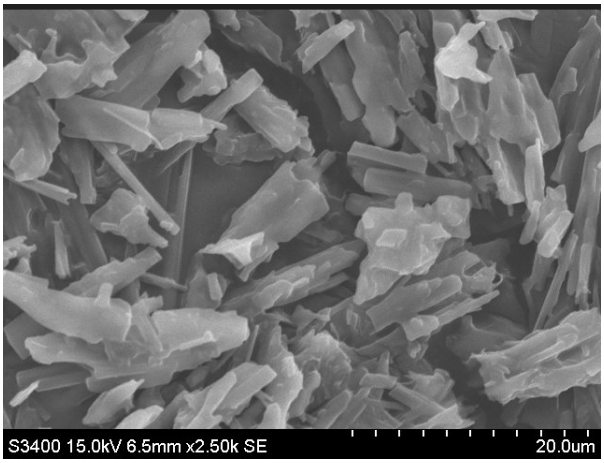


Figure 10(b). SEM image of Quercetin Liposomes.

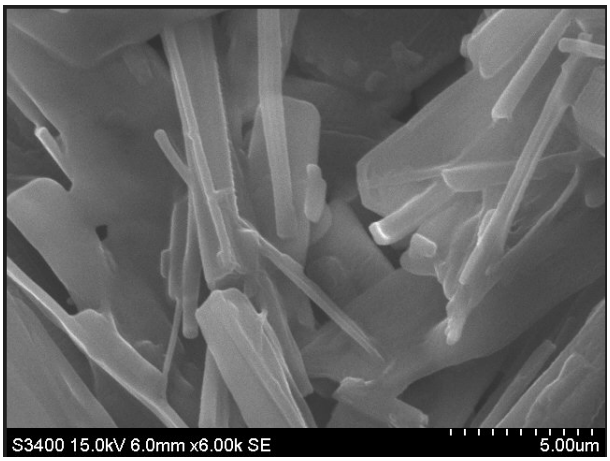


Figure 10(c). SEM image of Phytosterol loaded liposomes.

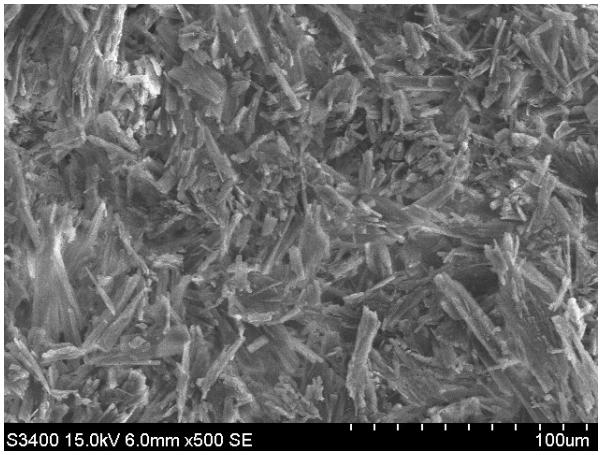


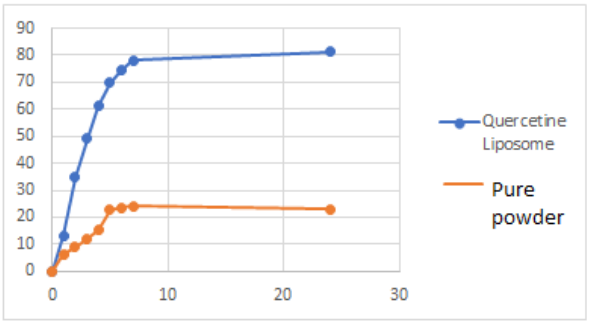
Figure 10(d). SEM image of mixure of all three Phytoconstituents loaded liposomes.

3.6.1 % Entrapment Efficiency of Prepared Liposomes

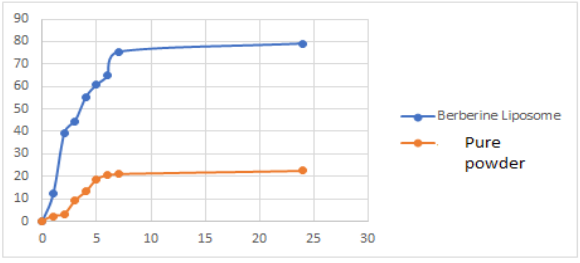
The % Entrapment Efficiency (% EE) values of Quercetin, Berberine and Phytosterol-loaded liposomes are presented in Table 1. It is evident that the linear effect of Phospholipon 90G had a significant impact. The independent variables had an influence on the liposomes loaded with phytoconstituents, whereby higher lipid concentration led to increased EE. This resulted in a greater amount of phytoconstituents being encapsulated within the vesicles. The % EE values for the optimized batch of Quercetin, Berberine, and Phytosterol loaded liposomes were determined to be 80.6 %, 81.3 %, and 80.35 % respectively. The nature of the drug itself also played a crucial role in determining the entrapment efficiency since it is encapsulated within the lipid phase.

3.7 *In vitro* Release Study

Figure 11 illustrates the *in vitro* release profile of the optimized Quercetin, Berberine, and Phytosterol-loaded liposomal suspension in a phosphate buffer with pH 7.4 at a temperature of 37 ± 0.5 °C. The cumulative % drug release (% DR) values for the aforementioned liposomal suspension over a 24-hour period were determined as 81.2%, 78.97%, and 80.2% respectively. The release pattern depicted in the *in vitro* release curve indicates that approximately 40% of the drug was released within the first two hours, followed by sustained release facilitated by the tailored liposomes. The comparison between the % drug release of liposomal formulations containing phytoconstituents and the respective free drugs is presented in Figures 11(a), 11(b) and 11(c).

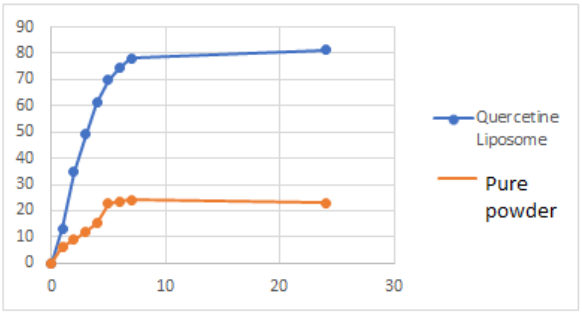


(a)



(b)

Figure 11 to be continued...



(c)

Figure 11. *In vitro* release study **(a).** Quercetin **(b).** Berberine **(c).** Phytosterol.

3.8 Pharmacodynamic Evaluation

The rats in the normal control group which were treated with normal saline acted as the negative control and the mean glucose levels, lipid profile, body weight and blood pressure of rats in this group were found to be in the normal range. On the other hand, Group II rats' high-fat diet shows HDL 46.59 mg/dL LDL 168.37 mg/dL and TG 176.81mg/dL, and blood pressure 150 mmHg. Group III and group IV rats also show high values in lipid profile, an increase in blood glucose level around 250 mg/dl. The glucose levels in group III standard drug and IV (test formulation containing three phytoconstituents) were significantly lower than in group II. Moreover, a comparison was made between group III and group IV on day 7 and day 14, and it was found that the rats in group IV exhibited significantly lower blood glucose levels. Lipid profiles such as (HDL, LDL and TG), blood pressure and body weight were estimated and a comparison was done between groups II, III and IV as shown in Figures 12((a), (b)) and 13((a), (b)).

Hence, indicating that test formulation was therapeutically equivalent and effective as a standard treatment of metabolic syndrome.

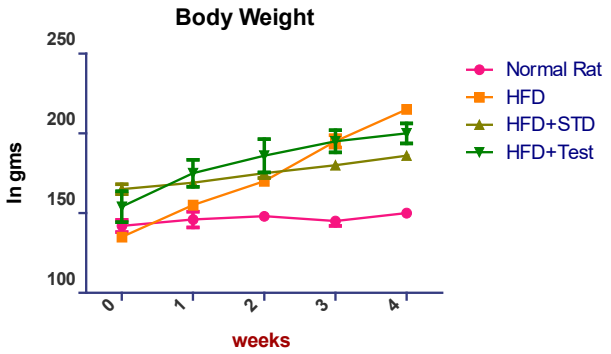


Figure 12(a). Body weight of animals in all four groups.

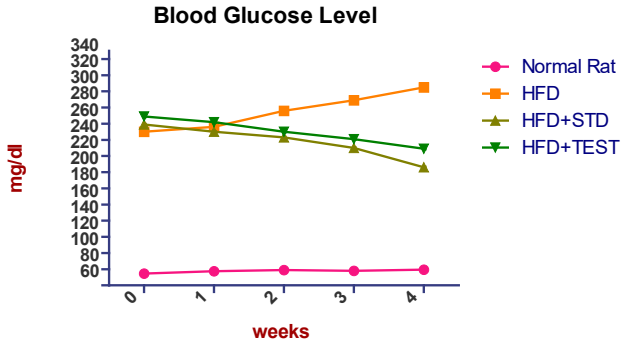


Figure 12(b). Body weight of animals in all four groups.

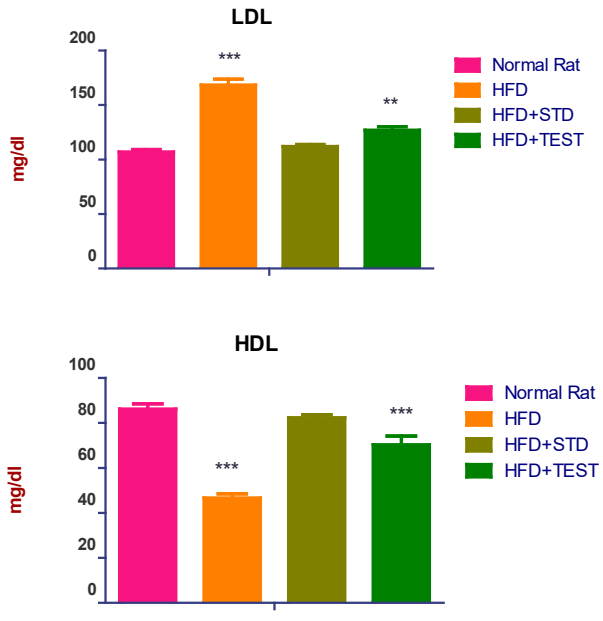


Figure 13 to be continued...

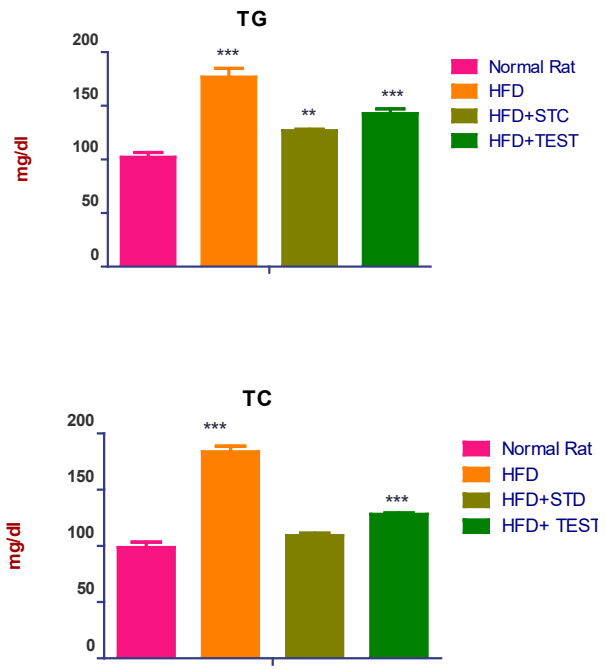


Figure 13(a). Lipid profile in all groups of all animals.

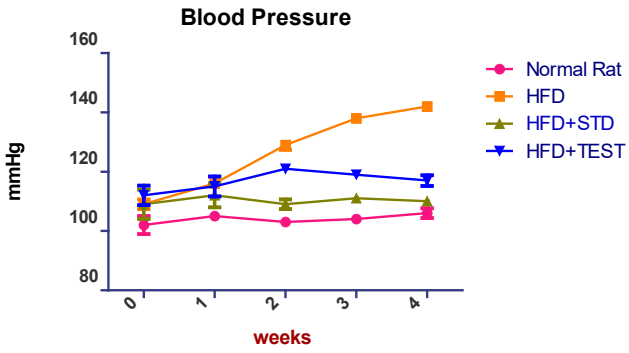


Figure 13(b). Blood pressure measurement in all groups of animals.

3.9 Stability Studies

Stability studies were conducted on capsules containing lyophilized liposomal suspension following ICH Q1C guidelines. The selected batch of capsules loaded formulation was stored in glass bottle at room temperature (25 ± 2 °C/60 ± 5 % RH) and accelerated storage condition at 40 ± 2 °C/75 ± 5 % RH.

4. Discussion

Numerous studies have focused on the possibility of specific phytoconstituents in giving symptomatic relief for metabolic syndrome, a lifestyle illness impacted by dietary practices. In this study, liposomes were created using the thin film hydration technique, and Plackett-Burman and Box-Behnken designs were used to optimise their formulation. The selection of lipids and other ingredients was based on their compatibility with the selected phytoconstituents. The screening phase utilized a Plackett-Burman design, considering seven factors (Amount of lipid, Amount of cholesterol, Hydration volume, Speed of rota evaporator, temperature, sonication time, and Hydration time) at two levels, focusing on dependent variables such as entrapment efficiency (EE %) and liposome vesicle size. Three significant factors (amount of lipid, amount of cholesterol, and hydration volume) were further optimized using a Box-Behnken design with three levels while maintaining the same dependent variables.

The optimized liposomal formulations underwent comprehensive evaluations including Fourier Transform Infrared Spectroscopy (FTIR) to assess compatibility, determination of liposomal vesicle size, drug entrapment, drug content, surface morphology studies using SEM and inverted microscopy, *in vitro* drug release analysis, and stability evaluation. FTIR compatibility studies confirmed that the formulated liposomes were compatible with the other ingredients used in their fabrication, as no shifting, stretching, or bending of peaks were observed. The sustained drug release achieved by the optimized formulations can potentially reduce the frequency of drug administration. Additionally, the lyophilized mixture of the three developed phytoconstituents was successfully evaluated for pharmacodynamic investigation.

5. Conclusion

In recent times, liposomal vesicular drug delivery has gained significant attention due to its ability to enhance the delivery of lipophilic drugs. In this study, liposomes loaded with phytoconstituents were successfully prepared

using a Plackett-Burman design, and an optimized formulation was achieved through the implementation of a Box-Behnken design. The formation of liposomes was confirmed using techniques such as FTIR, SEM, and optical microscopy. The drug release rate was notably improved in the optimized formulation.

Stability studies were conducted on the lyophilized powder for a period of 6 months, and the results demonstrated consistent vesicle size, drug content, and % entrapment efficiency without any deviations. The study aimed to develop a prophylactic single oral dosage form utilizing phytoconstituents and conducted *in vitro* characterization and *in vivo* of the formulation. However, further extensive studies are necessary to validate the potential outcomes of the developed formulation.

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