Research article

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DEVELOPMENT AND FACTORIAL DESIGN OF GLYCERYL TRISTEARATE BASED SOLID LIPID NANOPARTICLES (SLNs) CONTAINING BERBERINE

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ABSTRACT

Berberine hydrochloride (BH) is an isoquinolin alkaloid with favorable antioxidant, cardiovascular protective, anti-depressant, neuroprotective, anti-diabetic, anti-obesity, hepatoprotective, anti-rheumatic, antiangiogenic, anticlastogenic and anti-cancer activities. However, further development and application of this compound had been troubled by its poor aqueous solubility, low gastrointestinal absorption, and rapid metabolism in the body. In this paper, a solid lipid nanoparticle (SLN)-based system was developed for efficient incorporation and persistent release of BH. In this work, we present a formulation of these nanoparticle materials by a hot solvent evaporation method. A 3-factor, 2-level Box-Behnken design was used to optimize the process parameters including Triglyceride (A), Tween 20 (B) and Span 40 (C). Four dependent variables particle size, % CDR, % entrapment efficiency and viscosity of aqueous phase were measured as responses. Mathematical equations and response surface plots were used to relate the dependent and independent variables. The optimization model predicted particle size of about 0.6772 µm, CDR at 12th hours 64.05% and entrapment efficiency 65.4% with A, B and C levels of 125, 300 and 300 respectively. The observed responses were in close agreement with the predicted values of the optimized process.

Keywords: Berberine hydrochloride, Solid lipid nanoparticles, Glyceryl tristearate, Tween 20, RP HPLC

INTRODUCTION

Berberine (Ber) is an isoquinoline derivative alkaloid isolated from many medicinal herbs, such as Hydrastis canadensis (goldenseal), Cortex phellodendri (Huangbai) and Rhizoma coptidis (Huanglian) [1]. Berberine has a long history of medicine application, where it has been used to treat diarrhea, microbial infection, and gastrointestinal pain for many years owing to its strong antipyretic, antidotal and antibacterial properties [2,3]. In recent decades, much focus has been put on its other significant bioactivities, such as antioxidant, cardiovascular protective, anti-depressant, neuroprotective, anti-diabetic, anti-obesity, hepatoprotective, anti-rheumatic, antiangiogenic, anti-clastogenic and anti-cancer activities [4-8]. Despite the promising biological effects, Berberine is poorly absorbed, resulting in low bioavailability after oral administration.

In the past decade, lipid-based colloidal carriers have developed as a technique to deliver poorly water-soluble drugs, particularly when oral delivery is needed. Colloidal carriers can include of solid or liquid lipids or a mixture of both in numerous ratios and display a wide range of particle sizes. The enhancements of lipid-based formulations include a wide spectrum of applications, the use of biodegradable physiological lipids accepted as safe or having a regulatory acceptance rank, and the risk of scaling up to an industrial production level.[9-11] Lipid-based colloidal carriers have also demonstrated a boundless ability to improve the oral absorption of lipophilic drugs, the mechanisms of which include lipid digestion by components of the gastrointestinal tract and selective uptake by the lymphatic system.[12]

In this studies, our research group prepared lipid-based colloidal dispersions via hot solvent diffusion method with the aim of carrying berberine (Ber), a lipophilic natural drug that shows strong antipyretic, antidotal and antibacterial properties but has a very low oral bioavailability. Moreover, nanostructured lipid carriers (NLCs) were prepared by mixing triglyceride and surfactant in different ratios.

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MATERIALS AND METHODS

Berberine hydrochloride and cholesterol was purchased from Himalaya Healthcare, India. Tween 20, Span 20 and cholesterol were purchased from R&M marketing; Essex, All other solvents and chemical used were of HPLC and analytical grades.

PREPARATION OF GTS BASED (SLN_S)

GTS based nanocarriers were prepared by a hot solvent evaporation method [13]. Briefly, 200 mg of glyceryl tristearate (GTS) were completely dissolved into dichloromethane. The resulting organic solution was added drop wise into 25 mL of an aqueous solution containing tween 20, span 40 and 200 mg of drug maintained under magnetic stirring at 82±1ºC for 2 hours (table 2). During the homogenization process, the temperature was maintained around 82°C to guarantee that the lipid material does not solidify. Then, the obtained SLNs were transferred to glass vials and cooled down immediately to room temperature to maintain the stability of SLNs.

***INVITRO* EVALUATION OF BERBERINE (SLN_S)**

Particle Size:

The average particle size and size distribution of berberine (BH) loaded SLNs were determined by Zetasizer 4000S, Japan at room temperature. After being diluted 10 times with double-distilled water, samples were measured to obtain the data of its particle sizes.

Drug entrapment efficiency:

Encapsulation efficiency (EE) of berberine (BH) loaded SLNs was calculated by determining the amount of free berberine using centrifugal ultrafiltration technique. Firstly, 2-ml berberine (BH) loaded SLN colloidal solution was placed into a centrifuge tube matched and centrifuged for 45 min at 4,500 rpm. The liquid supernatant was filtered by 0.45-μm microporous filtering membrane and then kept as unencapsulated berberine (BH) sample for further detection. Secondly, the total berberine content in berberine loaded SLNs was determined as follows: aliquots of 2-ml berberine (BH) loaded SLN dispersion were dissolved and diluted appropriately by methanol to dissolve the SLNs. After the same centrifugal method, the obtained suspension was allowed to filter through 0.45-μm membrane filters and kept as total berberine in berberine loaded SLNs. Thirdly, the ultrafiltrates containing the unencapsulated berberine and total BH contents of BH-loaded SLNs were determined by HPLC method.

$$EE = \frac{W_B - W_U}{W_B} \times 100\ \%$$

$$DLC = \frac{W_B - W_U}{W_B - W_U + W_S} \times 100\%$$

In detail, WB represents for the amount of berberine used for each sample, WU represents for the amount of unencapsulated berberine after centrifugation, and WS was the weight of lipid added during the whole system.

In vitro drug release studies:

Berberine (BH) release from SLNs was performed using the dialysis bag method. Phosphate buffer (PBS, pH 7.4) with 0.2% (w/v) sodium dodecyl sulfate (SDS) was served as dissolution medium. The dialysis bag (12 kDa molecular weight, Sigma-Aldrich) could retain nanoparticles and allow the diffusion of free drug into dissolution media. The bags were soaked in double-distilled water for 8 h before use. Berberine (BH) loaded SLN dispersion, 2 ml was poured into the bag with the two ends fixed by clamps. Then, the bags were placed in a 50-ml centrifuge tube, and 25 ml fresh dissolution media was added into it. The centrifuge tubes were placed into a thermostatic shaking incubator at 37°C at a rate of 120 rpm. At 0.5, 1, 2, 4, 6, 8, 10 and 12 h of the time points, 0.5 ml of the mediums in the conical flask were removed for analysis and same amount of fresh dialysis medium was then added to maintain the conditions. The drug contents in samples were analyzed by detailed of HPLC chromatogram was shown in figure 37. All the operations were carried out in triplicate, and the cumulative percentages of the release profiles of free berberine and (BH) loaded SLNs were calculated.

RP HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50μL loop volume. The LC solution version 1.25 was used for data collecting and processing (Shimadzu, Japan). The HPLC was carried out at a flow rate of 1.0 ml/min using a mobile that is phase constituted of acetonitrile, 10 mM ammonium acetate (pH 4.5) (70:30, v/v), and detection was made at 254 nm. The mobile phase was prepared daily, filtered through a 0.45μm membrane filter (Millipore) and sonicated before use. A Thermo C18 column (25cm × 4.6mm i.d., 5μ) was used for the separation [14].

Particle size analysis:

Particle size of berberine (BH) loaded SLNs was determined using Malvern particle size analyzer (Zetasizer 4000S, Japan).

Viscosity Studies:

The rheological studies were performed by using Brookfield viscometer (DVII+ Model pro II type-USA). The viscosity of SLNs medium was determined at 0.3 rpm and means of two readings were used to estimate the viscosity [15].

EXPERIMENTAL DESIGN

In this work, we report the successful effect on the formulation of berberine (BH) solid lipid nanoparticles (SLNs). Through preliminary experiments the Triglyceride (A), Tween 20 (B) and Span 40 (C) were identified as the most significant variables influence the particle size, % CDR, % entrapment efficiency and viscosity of aqueous phase. Among various design approaches, the Box-Behnken (BBD) has good design properties, little collinearity, rotatable or nearly rotatable; some have orthogonal blocks, insensitive to outliers and missing data. Does not predict well at the corners of the design space. Use when region of interest and region of operability nearly the same. This Box-Behnken design is appropriate for exploring quadratic response surfaces and constructing second order polynomial models. The BBD consists of simulated center points and the set of points lying at the midpoint of each edge of the multi-dimensional cube.

Seventeen runs were essential for the response surface methodology based on the BBD. Based on the experimental design, the factor combinations produced different responses as presented in Table 1. These results clearly indicate that all the dependent variables are strongly dependent on the selected independent variables as they show a wide variation among the 17 runs [16]. Data were analyzed using Stat-Ease Design-Expert software (DX9) to obtain analysis of variance (ANOVA), regression coefficients and regression equation.

RESULTS AND DISCUSSION

These equations represent the quantitative effect of Triglyceride (A), Tween 20 (B) and Span 40 (C) and their interaction on Particle size (R1), % CDR (R2), % EE (R3) and Viscosity of aqueous phase (R4). The values of the coefficient A, B and C are related to the effect of these variables on the responses R1, R2, R3 and R4.Coefficients with more than one factor term and those with higher order terms represent interaction terms and quadratic relationship respectively (table 2). A positive sign represents a synergistic effect, although a negative sign specifies an antagonistic effect. A backward elimination procedure was espoused to fit the data to the quadratic model. Both the polynomial equations were found to be statistically significant ($P < 0.01$), as determined using ANOVA, as per the provision of Design Expert software (DX9).

Table-1: List of Independent variable and Dependent variables in Box-Behnken design

Independent variable			**Levels**		
Variable	Name	Units	Low	Middle	High
A	Triglyceride	mg	50	125	200
B	Tween 20	mg	200	300	400
C	Span 40	mg	200	300	400
Dependent variable			**Goal**		
R1	Size	nm		Minimize	
R2	CDR	%		Maximum	
R3	EE	%		100	
R4	Viscosity of aqueous phase	cps		---	

Table-2: Factorial design of berberine (SLNs) formulations

Run	Factor 1 A:Triglyceride mg	Factor 2 B:Tween 20 mg	Factor 3 C:Span 40 mg	Response 1 Size micrometer	Response 2 CDR at 12 ho... %	Response 3 EE %	Response 4 Final viscosit... cps
1	125	200	400	0.672	62.8	68.4	1200
2	200	400	300	0.825	68.5	74.6	1999
3	50	200	300	0.467	60	55.8	1200
4	200	300	200	0.831	68.3	73.4	1920
5	50	400	300	0.466	60.7	59.4	1100
6	125	300	300	0.677	63.9	65.1	1495
7	125	300	300	0.676	63.6	65.6	1496
8	125	400	200	0.672	65.4	66.2	1497
9	125	300	300	0.676	63.6	65.8	1495
10	200	300	400	0.827	67.1	75.9	1350
11	125	400	400	0.675	64.1	69.7	1850
12	125	300	300	0.676	64.3	65.2	1499
13	200	200	300	0.829	66.9	74.3	1929
14	50	300	200	0.465	60.7	55.8	499
15	50	300	400	0.471	60.1	59.8	999
16	125	300	300	0.678	64.4	65.3	1490
17	125	200	200	0.674	64.6	63.5	1560

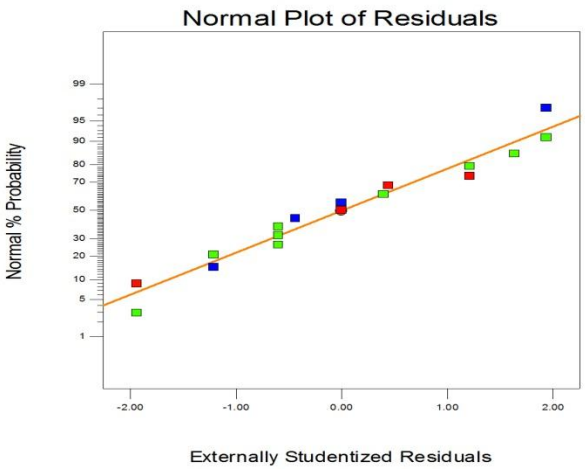

Figure-1: Normal % probability plot of the externally studentized residuals (R1)

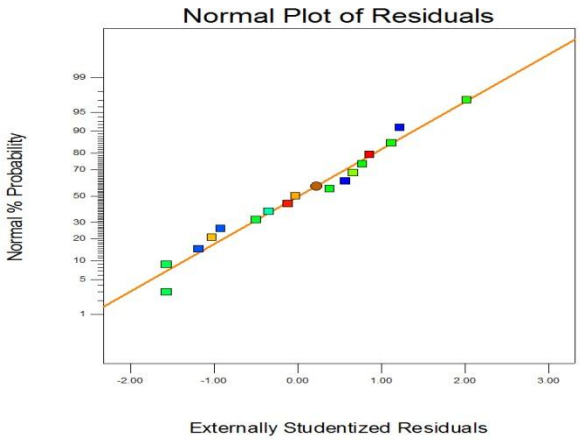

Figure-2: Normal % probability plot of the externally studentized residuals (R2)

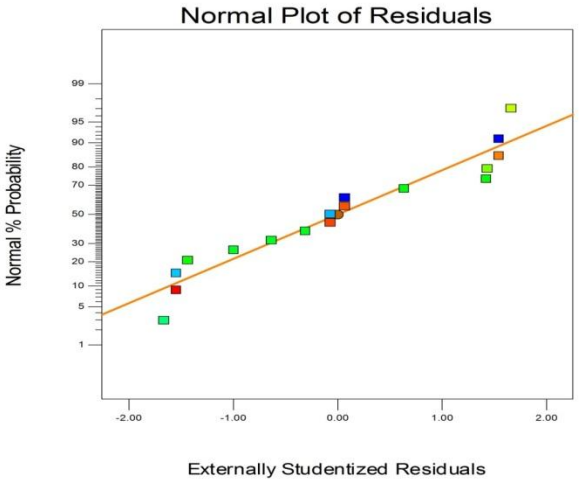

Figure-3: Normal % probability plot of the externally studentized residuals (R3)

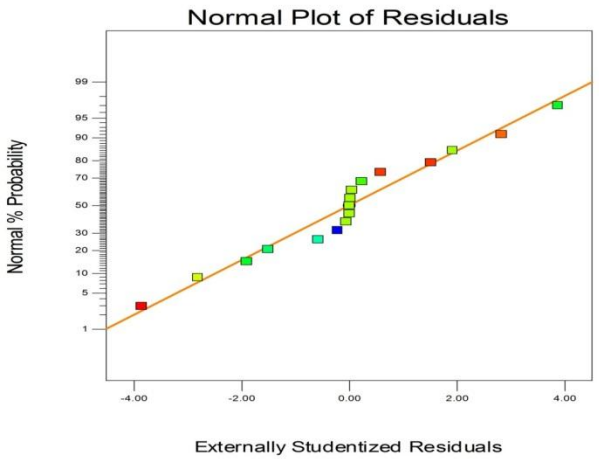

Figure-4: Normal % probability plot of the externally studentized residuals (R4)

The normality of the data could be proved through the normal % probability plot of the externally studentized residuals. If the points on the plot lie on a straight line, the residuals are normally distributed as confirmed in Fig. 1, 2, 3 and 4.

The assumption of constant variance was tested by plotting externally studentized residual versus predicted values as illustrated in above figures. The studentized residuals are located by dividing the residuals by their standard deviations. According to evident from this figure 5, 6, 7 and 8, the points are scattered randomly between the outlier detection limits -3.5 to +3.5 and - 4.5 to + 4.5.

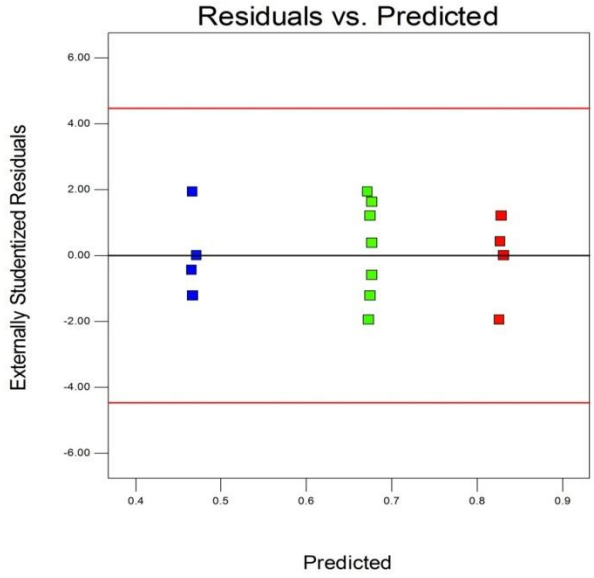

Figure-5: Residuals vs. Predicted (R1)

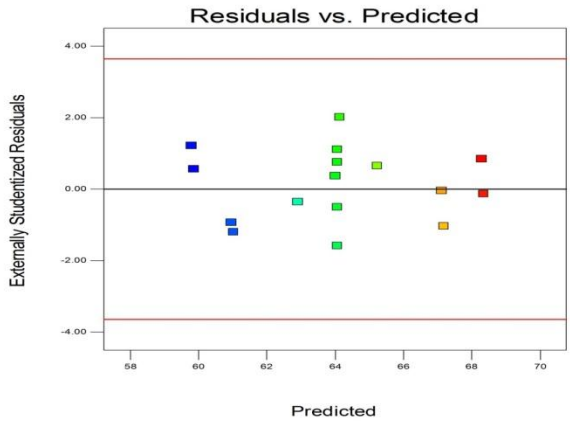

Figure-6: Residuals vs. Predicted (R2)

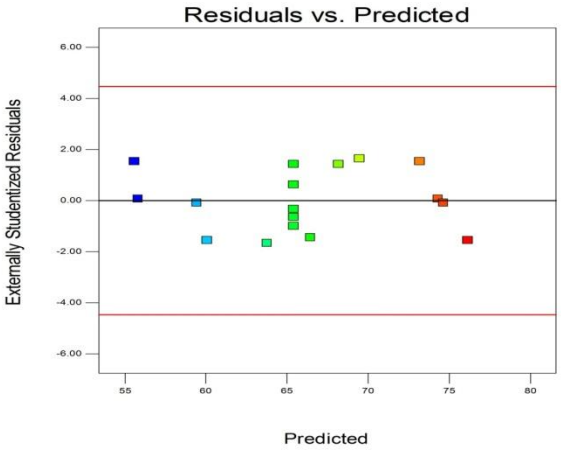

Figure-7: Residuals vs. Predicted (R3)

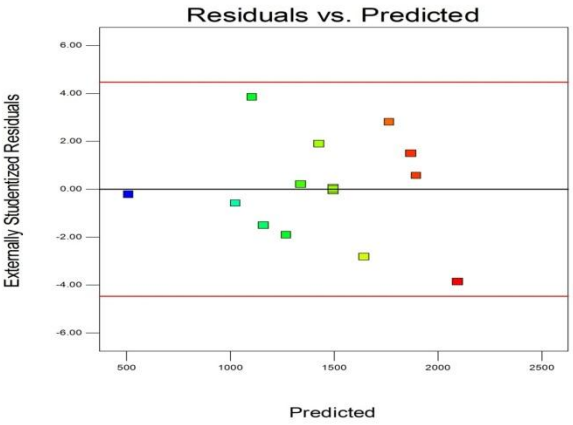

Figure-8: Residuals vs. Predicted (R4)

The Residuals vs. Predicted and Residuals vs. Run were scattered randomly (figure 9 to 12). From the results it can therefore be seen that the model is suitable for use and can be used to identify the optimal parameters.R1, R2, R3 and R4 results are quite satisfactory. Also, a high correlation between observed and predicted data indicates their low discrepancies.

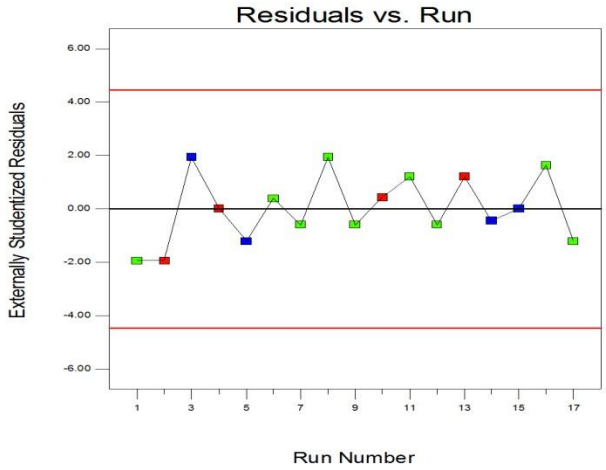

Figure -9: Residuals vs. Run (R1)

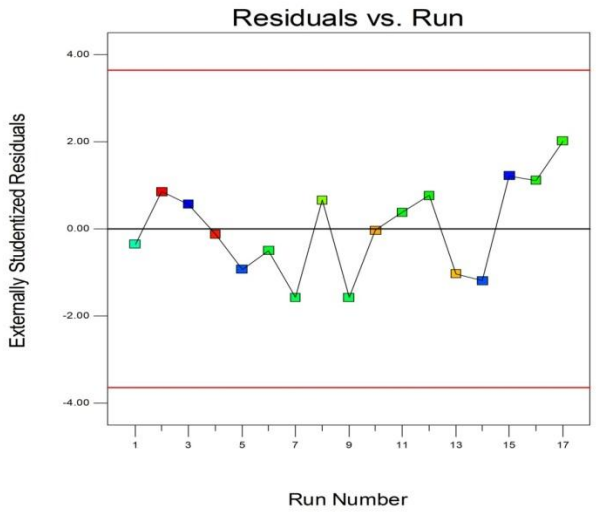

Figure -10: Residuals vs. Run (R2)

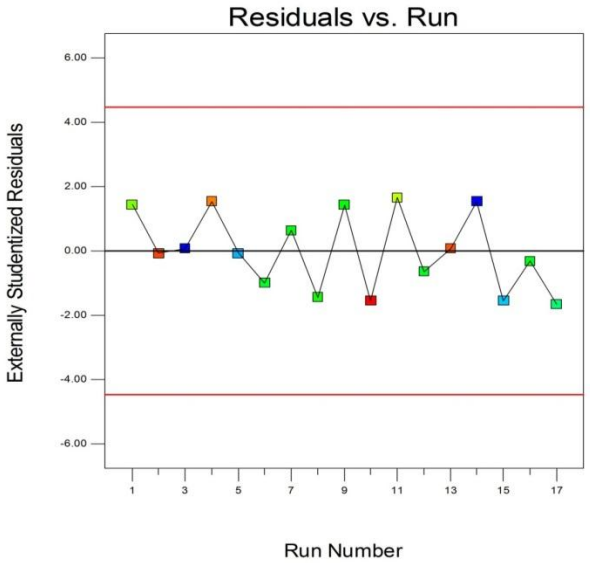

Figure -11: Residuals vs. Run (R3)

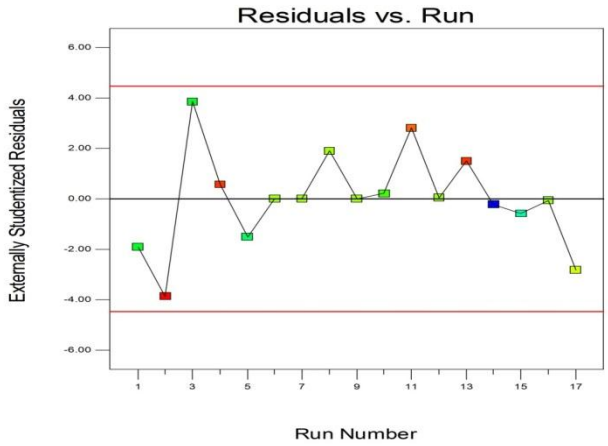

Figure -12: Residuals vs. Run (R4)

The plot of predicted response versus actual responses performs the same function, albeit graphically and also helps to detect the points where the model becomes inadequate to predict the response of the system. This is the simplest graph which shows that the selected model is capable of predicting the response satisfactorily within the range of data set as shown in the Figure 13, 14, 15 and 16.

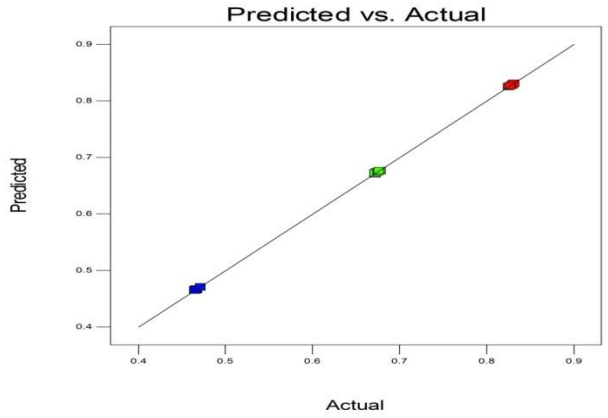

Figure -13: Actual Response vs. Predicted (R1)

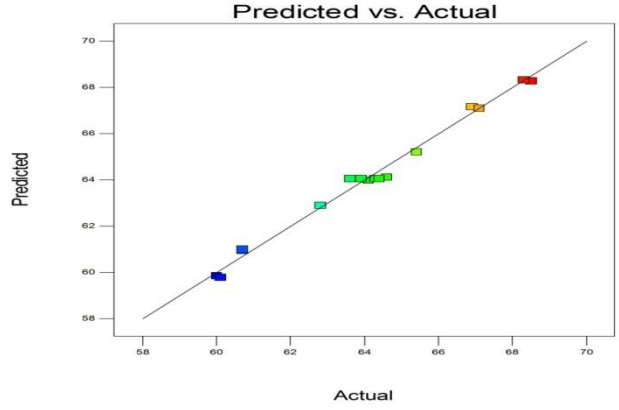

Figure -14: Actual Response vs. Predicted (R2)

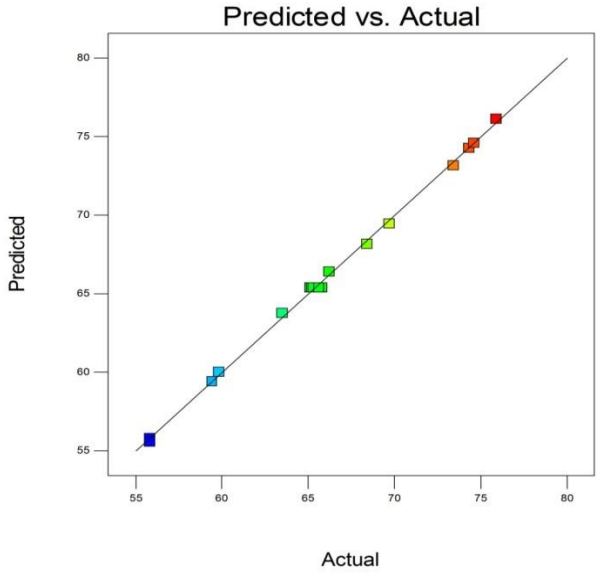

Figure -15: Actual Response vs. Predicted (R3)

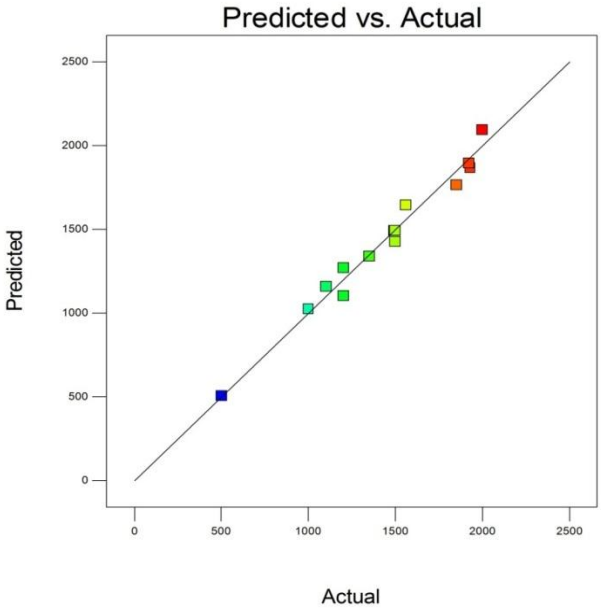

Figure -16: Actual Response vs. Predicted (R4)

The transformation parameter, λ, is chosen such that it maximizes the log-likelihood function. The maximum likelihood estimate of λ agrees to the value for which the squared sum of errors from the fitted model is a minimum. This value of λ is determined by fitting a numerous values of λ and choosing the value corresponding to the minimum squared sum of errors. t can also be chosen graphically from the Box-Cox normality plot. Value of λ = 1.00 indicates that no transformation needed and produces results identical to original data shown in Figure 15 to 18.

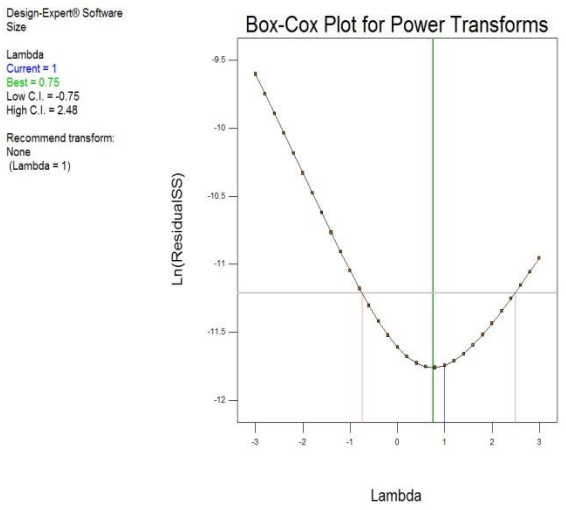

Figure -17: Box-Cox Plot (R1)

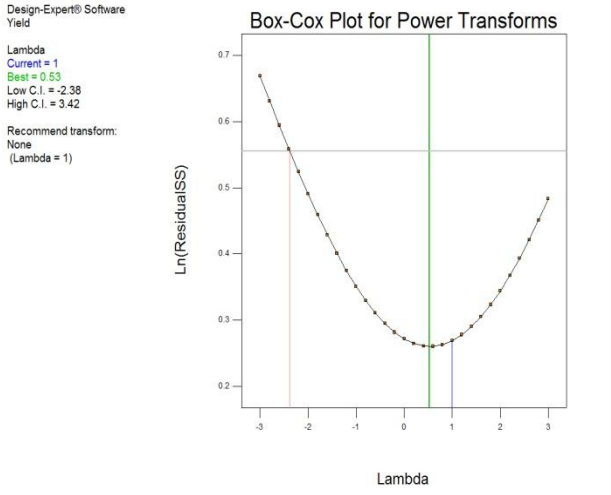

Figure -18: Box-Cox Plot (R2)

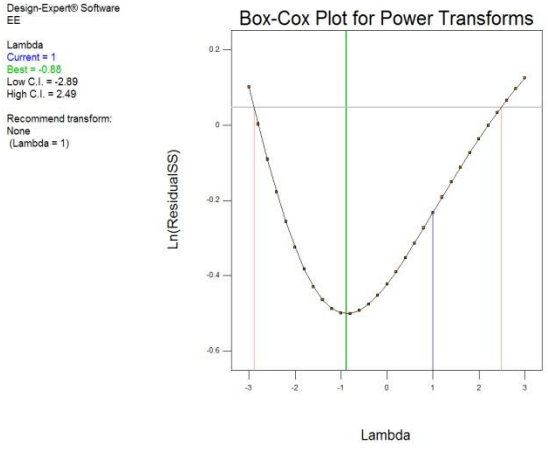

Figure -19: Box-Cox Plot (R3)

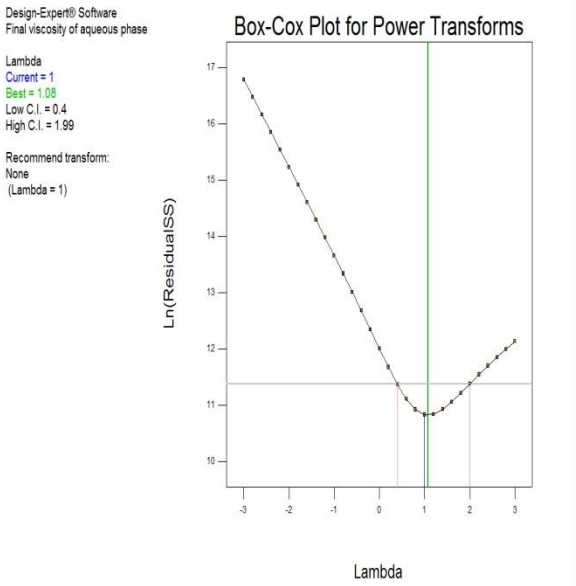

Figure -20: Box-Cox Plot (R4)

Particle size analysis of berberine (SLNs) was found to be in the range of 0.467 – 0.831 µm as shown in figure 38. The factorial equation for particle size exhibited a good correlation coefficient (1.000) and the Model F value of 19359.27 which implies the model is significant. Values of "Prob> F" less than 0.0500 indicate model terms are significant. In this case A, AC, BC, A^2, B^2, C^2 are significant model. Results of the equation indicate that the effect of (A) is more significant than B and C. The influence of the main and interactive effects of independent variables on the particle size was further elucidated using the perturbation and 3D response surface plots. The individual main effects of A, B and C on particle size are as shown in perturbation plots Figure 21. It is found that all the variables are having interactive effects for the response R1. The 2D contour plots and 3D response surfaces of the response R1 are shown in figure 22 & 23 to depict the interactive effects of independent variables on response R1, one variable was kept constant whereas the other two variables diverse in a certain range. The shapes of response surfaces and contour plots reveal the nature and extent of the interaction between different factors. The interaction between A and B on particle size at a fixed level of C is shown in Figure 23. At low levels of A, R1 reduced from 0.471 to 0.465 µm. Similarly at high levels of A, R1 reduced from 0.831 to 0.825 µm. The 3-D cube plots of Box-Behnken design are as shown in Figure 24.

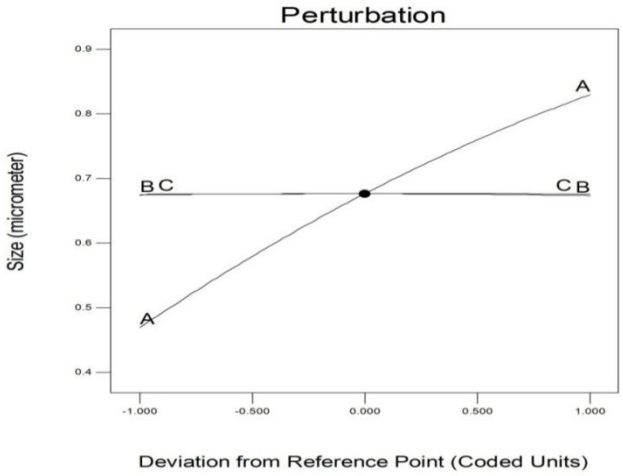

Figure-21: Perturbation plot showing the main effect of Triglyceride (A), Tween 20 (B) and Span 40 (C) on particle size (R1)

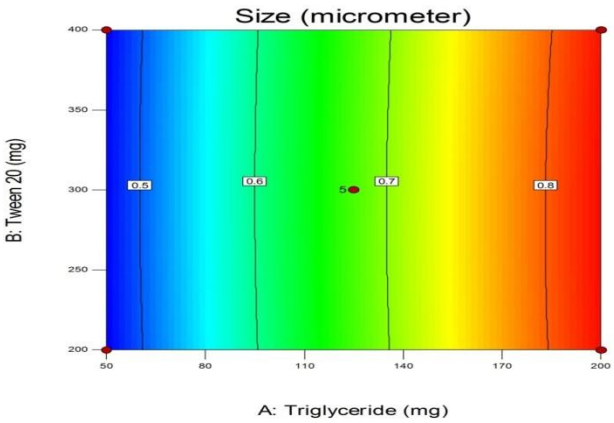

Figure- 22: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the particle size at constant span 40 concentration.

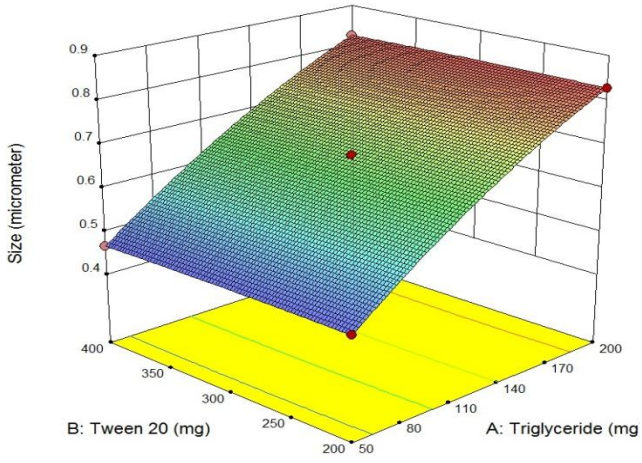

Figure -23: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the particle size at constant span 40 concentration.

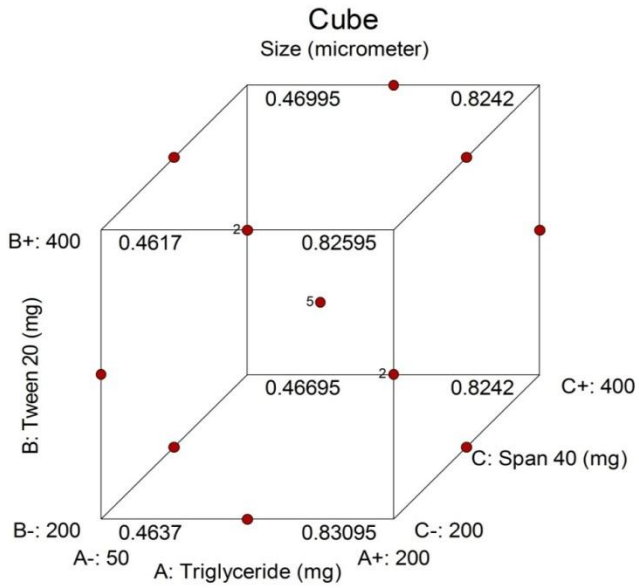

Figure -24: 3-D cube plot of Box-Behnken design

The coefficient of determination, R-squared, is a measure of the fraction of the total squared error that is explained by the model. By definition the value of R^2 varies between zero and one and the closer it is to one, the better. However, a large value of R^2 does not necessarily imply that the regression model is good one. Adding a variable to the model will always increase R^2, regardless of whether the additional variable is statistically significant or not. Thus it is possible for models that have large values of R^2 to CDR poor predictions of new observations or estimates of the mean response. To avoid this confusion, an extra statistic called the Adjusted R-squared statistic is needed; its value decreases if unnecessary terms are added. These two statistics can, when used together, imply the existence of extraneous terms in the computed model which is indicated by a large difference, usually of more than 0.20, between the values of R^2 and Adj-R^2. The amount by which the output predicted by the model differs from the actual output is called the residual. Predicted Residual Error Sum of Squares (PRESS) is a measure of how the model fits each point in the design. It is used to calculate predicted R^2. Here, the "Pred R-Squared" of 0.9994 is in reasonable agreement with the Adj R-Squared of 0.9999. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. "Adeq precision" showed was 389.135 indicates an adequate signal respectively. This model can be used to navigate the design space. These statistics are used to prevent over fitting of model.

The mathematical model generated for % CDR (R2) was found to be significant with F-value of 373.28 ($p < 0.0001$) and R^2 value of 0.9885. Results of the equation indicate that the effects of A, B, C are more significant model. Here, the "Pred R-Squared" of 0.9812 is in reasonable agreement with the Adj R-Squared of 0.9859. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. "Adeq precision" showed was 55.554 indicates an adequate signal respectively. The influence of the main and interactive effects of independent variables on the % CDR was further elucidated using the perturbation plots and 3D response surface plots. The perturbation plot (Figure 25) showing the main effects of A, B and C on the percentage CDR (R2) of berberine (SLNs). This figure clearly shows that A, B and C has the main and the major effect on R2. The relationship between the dependent and independent variables was further elucidated using 2D response surface plots; 3D response surface plot and 3-D cube plot are shown in (Figure 26, 27 and 28). Figure 27 shows the interactive effect of A and B on the % CDR (R2) at fixed level of C. At low levels of A (Triglyceride), R2 decreases from 60.7% to 60.0%. Similarly, at high levels of A, R2 increases from 66.9% to 68.5%.

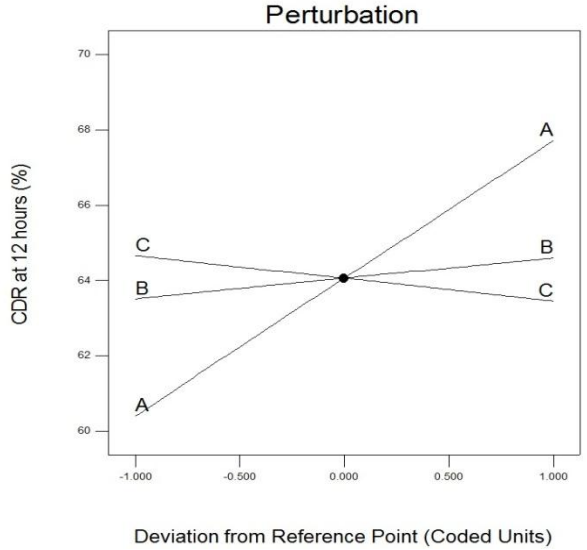

Figure-25: Perturbation plot showing the main effect of Triglyceride (A), Tween 20 (B) and Span 40 (C) on % CDR at 12 hours (R2)

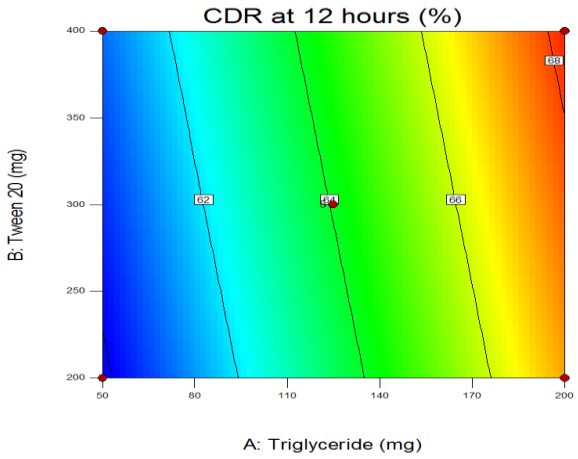

Figure- 26: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the % CDR at 12 hours at constant span 40 concentration.

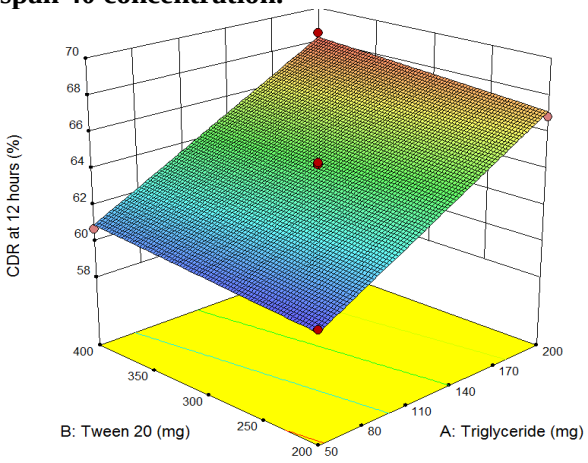

Figure -27: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the % CDR at 12 hours at constant span 40 concentration.

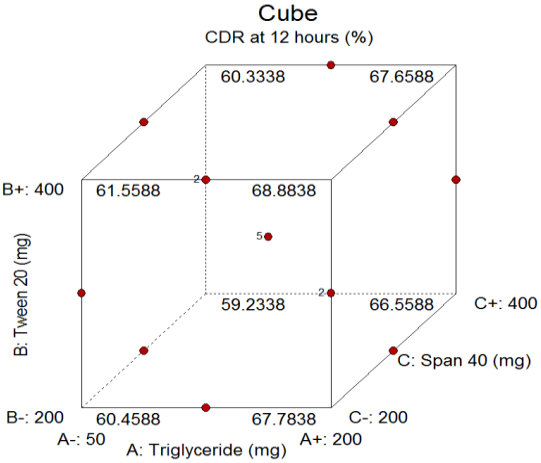

Figure -28: 3-D cube plot of Box-Behnken design

The accurate model produced for % EE (R3) was found to be significant with F-value of 601.22 ($p < 0.0001$) and R^2 value of 0.9987. The independent variables A, B, C has significant effects on the % EE, since the P-values less than 0.0500 represent the significant model. Here, the "Pred R-Squared" of 0.9873 is in reasonable agreement with the Adj R-Squared of 0.9970. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. "Adeq precision" showed was 79.729 indicates an adequate signal respectively. The perturbation plot (Figure 29) showing the main effects of A, B and C on the % EE (R3) of berberine (SLNs). The correlation among the dependent and independent variables was further elucidated using 2-D response surface plots; 3-D response surface plot and 3-D cube plot are shown in figure 30, 31 and 32. At low levels of A (Triglyceride), R3 decreases from 59.8% to 55.8%. Similarly, at high levels of A, R3 increases from 73.4% to 74.6%.

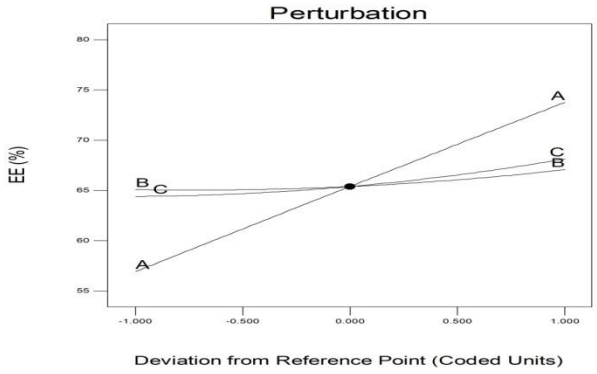

Figure-29: Perturbation plot showing the main effect of Triglyceride (A), Tween 20 (B) and Span 40 (C) on % EE (R3)

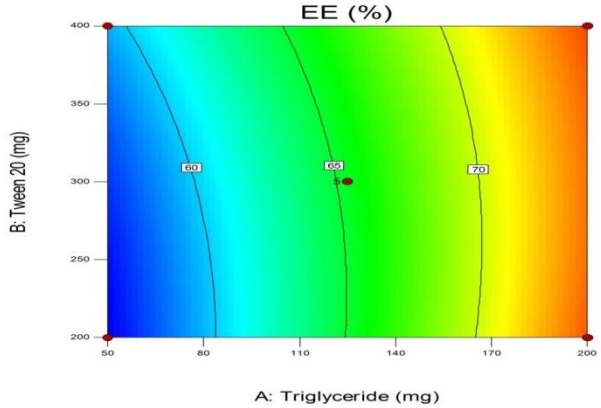

Figure-30: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the % EE at constant span 40 concentration.

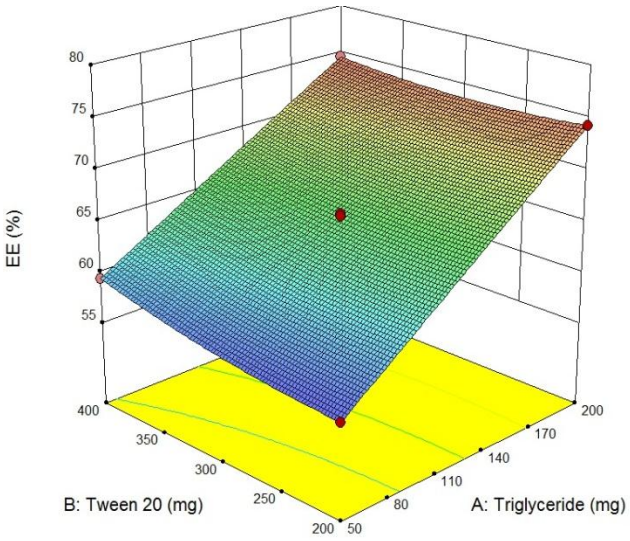

Figure-31: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the % EE at constant span 40 concentration.

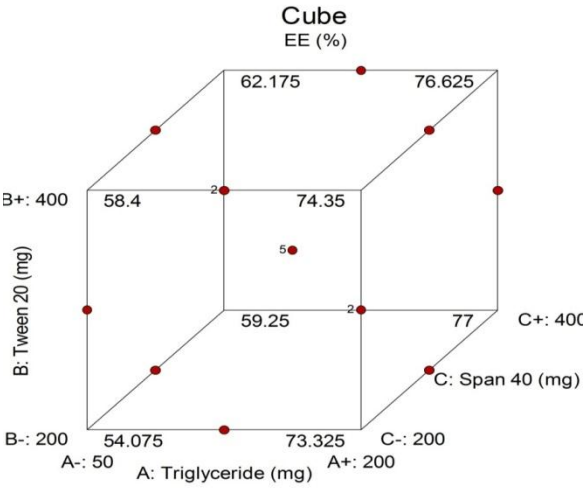

Figure-32: 3-D cube plot of Box-Behnken design

The accurate model produced for viscosity of aqueous phase (R4) was found to be significant with F-value of 34.51 ($p < 0.0001$) and R2 value of 0.9780. Since the P-values less than 0.0500 represent the significant model. In this case A, AC, BC, A^2, B^2, C^2 are significant model. The perturbation plot (Figure 31) showing the main effects of A, B and C on the viscosity (R4) of berberine (SLNs). The correlation among the dependent and independent variables was further elucidated using response surface plots, response surface plot and 3-D cube plot are shown in Fig. 32, 33 and 34. Figure 33 shows the interactive effect of A and B on the viscosity (R4) at fixed level of C.

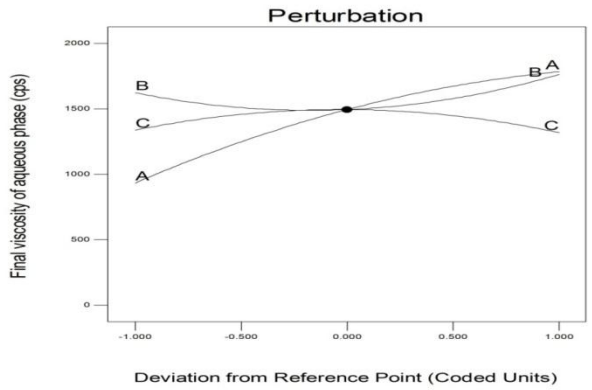

Figure-33: Perturbation plot showing the main effect of Triglyceride (A), Tween 20 (B) and Span 40 (C) on viscosity of aqueous phase (R4)

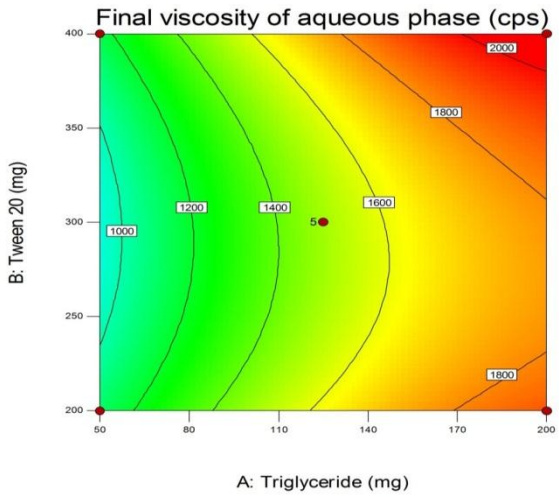

Figure-34: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the viscosity of aqueous phase at constant span 40 concentration.

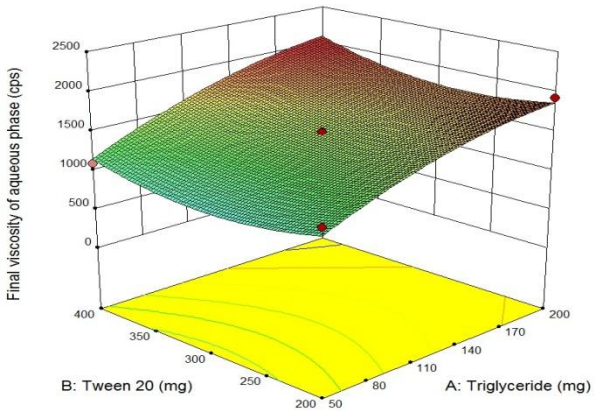

Figure-35: Response surface plot presenting the interaction between the triglyceride and tween 20 affecting the viscosity of aqueous phase at constant span 40 concentration.

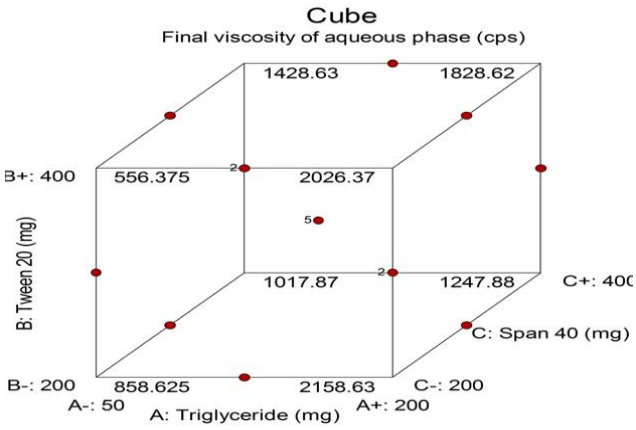

Figure-36: 3-D cube plot of Box-Behnken design

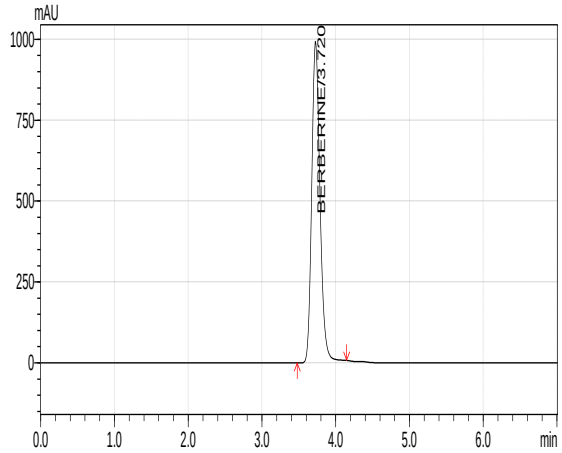

Figure-37: Chromatogram of sample

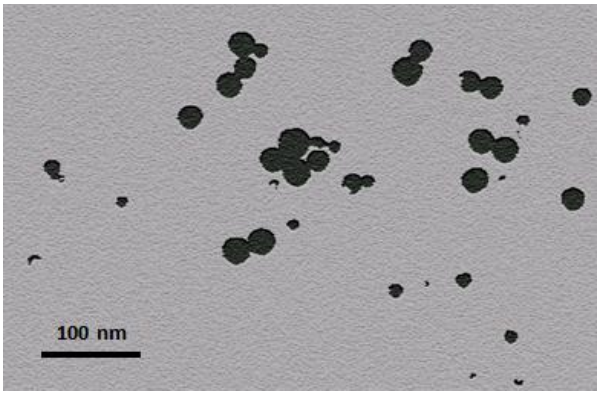

Figure-38: TEM photograph of SLNs

Three batches of berberine (SLNs) were prepared according to these optimized levels. Observed responses were in close agreement with the predicted values of the optimized process was shown in confirmation report, thereby demonstrating the feasibility.

CONCLUSIONS

The results of the present work demonstrated that lipid nanodispersions made from glyceryl tristearate. The developed BH-loaded SLNs demonstrated good stability with small nanoparticle size and drug good release rate compared with free BH. the "Pred R-Squared" of 0.9812 is in reasonable agreement with the Adj R-Squared of 0.9859. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. "Adeq precision" showed was 55.554 indicates an adequate signal. The Residuals vs. Predicted and Residuals vs. Run were scattered randomly.

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