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DEVELOPMENT AND OPTIMIZATION OF LIPOSOMAL KETOTIFEN FUMARATE DRY POWDER USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT: The aim of this study was to develop and optimize nano-liposomally entrapped ketotifen fumarate (KTF) loaded dry powder inhalation (DPI) formulation using response surface methodology (RSM). Based on the 3^3 full factorial design (3^3FFD), the mass ratio of KTF solution to soybean phosphatidylcholine (SPC_{94}), KTF/ SPC_{94} to 0.5 M lactose solution that after lyophilization produce lyophilized liposomal powder (LLP) and LLP to coarse lactose carrier (CLC) were selected as independent variables with KTF liposomal encapsulation efficiency (%EE), liposomes particle size (PS) and fine particle fraction (%FPF) as dependent variables. The aerosolized liposomal dry powder was prepared utilizing vesicular phospholipid gel technique followed by lyophilization after incorporating it into lactose solution as a cryoprotectant. A full and reduced second-order polynomial models were developed for each response using multiple linear regression analysis. Applying a desirability function method, the optimum parameters were: KTF: SPC_{94} of 0.045, KTF/ SPC_{94}: 0.5 M lactose of 0.389 and LLP: CLC of 0.080. At this optimum point, the %EE, PS and %FPF were found to be 52.68%, 444.00 nm and 20.46%, respectively. The powder bulk density of (0.31 gm/cm^3) is within the acceptable range for pulmonary delivery, whereas the angle of repose of (30.14 θ) indicates good powder flowability. The in vitro release study of KTF from the optimize liposome suspension revealed that Korsmeyer – Peppas model of release gives the best fitness and the mechanism of release was non-Fickian, whereas Weibull 3 model was the best for fitting the data obtained indicating that the curve of release was parabolic (b<1, case 3). Thus, response surface methodology aid in developing in situ generated sphere liposomal vesicles on lactose carrier particles that will impart a continued drug release for more than 12 hours without a significant burst effect.

INTRODUCTION: By comparing pharmaceutical aerosol formulations to formulations intended for conventional routes of administration (e.g.; oral and parenteral), they are typically more sophisticated and less efficient.

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A therapeutic aerosol with a sustained release capability can prolong the residence of an administered drug in the airways or alveolar region, improving patient compliance by reducing dosing frequency and adverse effects [2].

In contrast to the rapid drug onset of action being pulmonary administered, the duration of this action is unfortunately short due to the pulmonary two main clearance players; the mucociliary system and the alveolar macrophages [3]. Although drug choice is the first step in prescribing inhaled therapy, the

next will be choosing of an appropriate inhaler device. Despite of there is more than 100 inhaled devices currently available for the treatment of asthmatic patients categorized under five types; pressurized metered-dose inhalers (pMDIs), breath-actuated metered-dose inhalers (BApMDIs), dry powder inhalers (DPIs), nebulizers and soft mist inhalers (SMIs), most patients require dosing four to six times in some diseases and dose increment with time. This is due to the lung clearance mechanisms, incorrect use of inhaler and underestimation of disease severity [4].

Improving pulmonary drug delivery systems still an area of increasing interest. Liposomal aerosols as carrier systems for pulmonary delivery offer many advantages such as solubilizing poorly soluble dugs, provide a pulmonary sustained release reservoir prolonging local and systemic therapeutic drug level, facilitate intracellular delivery of drugs especially to alveolar macrophages, tumour cells or epithelial cells, prevent local irritation of lung tissue and reduce the drug's toxicity, target specific cell populations using surface bound ligands or antibodies and be absorbed across the epithelium to reach the systemic circulation intact [5].

Conventional dry powder formulations (DPFs) fulfill the requisites concerning drug delivery to the right site in the lung, at the required dose and at an optimum frequency but fail in drug stays for the required period of time. Liposomal drugs enhance the drug residence time in the lungs, prevents enzymatic degradation of the drug and the nano-size prevents its rapid removal through the clearance mechanism, alleviating the limitations of plain drug or conventional liposomal drug. Drug encapsulated nano-liposomes (NLs) can be processed into DPF form using freeze-drying, spray-drying, and spray freeze-drying to achieve long-term stability, and overcome problems associated with the suspension form of liposomes [6].

Ketotifen fumarate (KTF) is an antihistaminic drug given orally in a dose equivalent to 1 mg of ketotifen twice daily with food in the prophylactic management of asthma due to its stabilizing action on mast cells analogous to that of sodium cromoglycate. It is completely absorbed from the gastro-intestinal tract, but the bioavailability is only about 50% due to the hepatic first pass metabolism. It belongs to tricyclic compound of benzocyclohepatathiophene class, is a non-specific, oral mast cell stabilizer. Its usefulness in allergic/atopic asthma prophylaxis related to its biochemical and pharmacological activities that include H_1 antagonism, phosphodiesterase inhibition and inhibition of calcium flux in smooth muscles [7-8].

The low dose therapeutic, substantial biotransformation and a prolonged period sustained blood level of the drug required for controlling allergic asthma makes routes other than pulmonary route (e.g.; oral and transdermal) incapable of achieving the epic goals when KTF locally administered. An earlier effort was made for developing an ideal liposomal formulation for KTF utilizing lipid film hydration and sonication followed by lyophilization after blending liposomal dispersion with an appropriate cryoprotectant [9].

In this research a 3^3 full factorial design was employed to study the effect of different variables on the development and optimization of an ideal aerosolized liposomal dry powder formulation utilizing vesicular phospholipid gel technique followed by lyophilization after incorporating it into lactose solution as a cryoprotectant at an optimal strength. In vitro investigations include KTF encapsulation efficiency, liposomes particle size, fine particle fraction, flow properties and release profiles.

MATERIALS AND METHODS:

Materials: Pure ketotifen fumarate (KTF) and lactose were produced from (SDI/Iraq). Soybean phosphatidylcholine, purity>94% (SPC_{94}) (Lipoid® S 100) was a gift from (Lipoid GmbH/Germany). All other chemicals/solvents used were of analytical grade.

Methods:

Preparation and Dilution of Vesicular Phospholipid Gels / Lyophilized Liposomal Powder / Dry Powder Inhalation: Different mass ratios (1:15, 1:20 and 1:25) of aqueous KTF solution (10 mg/ml) to SPC_{94} were mixed and allowed to swell in a water bath at 60°C for 2 h.

Then the mixtures were stirred by a homogenizer (SilentCrusher M-Heidolph/Germany) for 5 min until semisolid vesicular phospholipid gels (VPGs) were formed. The VPGs were kept hydrating in a water bath after dilution with different mass ratios (1:9, 2:8 and 3:7) of KTF/SPC_{94} to 500 mM lactose solution in deionized water to form liposome suspension at 60 °C for 12 h. Afterward; the liposome suspensions were frozen at – 20 °C and then lyophilized for 48 h using (VirTis Freeze Dryer-Virtis Co./USA) to produce lyophilized liposomal powder (LLP).

The resulting porous cakes were sieved through 400-mesh sieve (Retsch/Germany) manually. Further; the effect of formulation parameters mentioned earlier together with the different mass ratios (1:10, 1:12.5 and 1:15) of LLP to coarse lactose carriers (CLC) (57 – 108 µm sieved α-lactose monohydrate + 0.5% magnesium stearate as a lubricator) on the fine particle fraction was also studied after filling in capsules (size 2) with 200 mg dry powder [10].

Determination of Liposomes Encapsulation Efficiency: The encapsulation efficiency was determined as the percentage of KTF encapsulated in liposome to the original amount of KTF added. Liposomes suspension was centrifuged at 18000 rpm for 30 min at 25 °C using (High Speed Refrigerated Centrifuge VS18000M-VisionSci. Co. Ltd., Korea), the KTF content of the supernatant was determined spectrophotometrically at 300 nm using (Shimadzu UV–1800/Japan). Then the liposomes after removing the supernatant was ruptured using sufficient volume of 70% ethanol and the amount of KTF was also determined spectrophotometrically [11]. Encapsulation efficiency was calculated using the following equation:

$$\% \text{ EE} = \left(\left[\frac{T-F}{T}\right]\right) \text{X } 100 \text{ ….. (Eq. 1)}$$

Where, EE is encapsulation efficiency, T is total KTF for encapsulation and F is free drug in sample.

Liposomes Morphology and Particle Size Determination: The morphological features of liposomes before lyophilization were examined by (Biological Microscope-Meiji Techno Co. LTD/Japan). Furthermore; the liposomes particle size distribution and mean diameter were determined by a particle size analyzer (Malvern/UK) [12].

Determination of Fine Particle Fraction: The fine particle fraction (FPF) was determined using the Andersen Cascade Impactor (ACI) (Graseby-Andersen/USA) to evaluate the in vitro deposition profiles of KTF. A Rotahaler® was used as a delivery device at a flow rate of 60±5 L/min for 5 s [13]. The FPF, which is total percentage deposited at stage 2-7 of the ACI was used to evaluate aerosol performance using the following equation:

$$\% \text{ FPF} = \frac{FPD}{TD} \text{ X } 100 \text{ ….. (Eq. 2)}$$

where FPF – fine particle fraction, FPD – fine particles dose (i.e.; total weight of the particles≤5 µm) and TD – total dose weight of the particles delivered from the mouthpiece of the inhaler into the apparatus.

Experimental Design and Optimization: Response surface methodology (RSM) was employed to optimize the liposomal KTF dry powder preparation and the 3^3 full factorial design (3^3FFD) matrix was chosen and built by the statistical software package using (Design-Expert® Software Version 8.0.7.1- Stat-Ease Inc., MN/USA).

Based on the preliminary experiments, three formulation parameters which included KTF:SPC_{94} (1:15, 1:20 and 1:25)(X_1), KTF/SPC_{94}: 0.5M lactose (1:9, 2:8 and 3:7) (X_2) and LLP: CLC (1:10, 1:12.5 and 1:15) (X_3) at 3 different levels as low (-1), medium (0) and high (1) were identified as independent variables responsible for the responses which included KTF liposomal encapsulation efficiency (%EE [Y_1]), liposomes particle size (PS (nm) [Y_2]) and fine particle fraction (%FPF [Y_3]).

Table 1 show the independent variables range whereas **table 2** shows the 3^3FFD experimental runs.

A full second-order polynomial model relates the response to the selected variables through the equation below:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{123} X_1 X_2 X_3 \ldots\ldots \text{(Eq. 3)}$$

where: Y – predicted reposnse(s), b_0 – intercept coefficient, b_1, b_2 and b_3 – linear coefficients, b_{11}, b_{22} and b_{33} – squared coefficients, b_{12}, b_{13}, b_{23} and b_{123} – interaction coefficients and X_1, X_2 and X_3 – independent variables. A full and reduced model for each response was established by putting regression coefficients values in equation 3. The linear, quadratic and interactive effects of the independent variables on the responses appropriately can be evaluated by using the equation 3 and analysis of variance (ANOVA) through Fischer's test. The *P*-value less than 0.05 was considered to be statistically significant. Statistical analysis and graphing were performed utilizing Design-Expert Software by varying levels of two factors and keeping the third factor at fixed levels at a time. Multiple correlation coefficient (R^2) and adjusted (R^2) were employed as quality indicators for evaluating the second-order polynomial equation fitness. Three-dimensional plots were used to demonstrate the relationship and interaction between the coded variables and the responses. Eight optimum checkpoints were selected, prepared and evaluated for response parameters, i.e.; %EE, PS (nm) and %FPF in order to validate the experimental design and the derived polynomial equation in optimizing the liposomal dry powder preparation. A statistical comparison was employed between predicted and experimental values for deriving percentage error and evaluating significant difference. The optimal points were determined by solving the equation derived from the final quadratic model and grid search in RSM plots regarding the constraints in which the liposomes PS (nm) is in its minimum whereas the %EE and %FPF both at maximum levels [14].

TABLE 1: CODED VALUES OF THE FORMULATION PARAMETERS

Coded Value	Actual Value		
	X_1	X_2	X_3
-1	1:15	1:9	1:10
0	1:20	2:8	1:12.5
1	1:25	3:7	1:15

TABLE 2: THE 3^3 FULL FACTORIAL DESIGN IN VARIOUS RUNS AND ENCAPSULATION EFFICIENCY (%EE [Y_1]), PARTICLE SIZE (PS (nm) [Y_2]) AND FINE PARTICLE FRACTION (%FPF [Y_3]) AS THE RESPONSES

Run no.	X_1	X_2	X_3	X_1^2	X_2^2	X_3^2	$X_1 X_2$	$X_2 X_3$	$X_1 X_3$	$X_1 X_2 X_3$	Y_1 %EE	Y_2 PS(nm)	Y_3 %FPF
1	0	0	-1	0	0	1	0	0	0	0	69.69±1.363	540.00±18	14.68±1.040
2	0	0	0	0	0	0	0	0	0	0	68.23±0.833	520.00±17	22.50±1.630
3	0	0	1	0	0	1	0	0	0	0	59.60±0.851	590.00±16	29.48±0.600
4	0	-1	-1	0	1	1	0	1	0	0	71.73±0.805	885.00±14	12.63±1.400
5	0	-1	0	0	1	0	0	0	0	0	70.53±0.737	879.00±22	24.57±0.646
6	0	-1	1	0	1	1	0	-1	0	0	65.74±1.013	859.00±27	27.70±0.641
7	0	1	-1	0	1	1	0	-1	0	0	65.12±0.846	472.00±25	16.35±0.660
8	0	1	0	0	1	0	0	0	0	0	63.22±1.011	452.00±23	21.50±0.374
9	0	1	1	0	1	1	0	1	0	0	51.22±0.527	477.00±11	32.45±0.483
10	-1	0	-1	1	0	1	0	0	1	0	50.33±0.775	482.00±13	14.50±0.731
11	-1	0	0	1	0	0	0	0	0	0	48.00±0.658	404.00±15	24.43±0.604
12	-1	0	1	1	0	1	0	0	-1	0	41.41±0.840	424.00±12	33.23±0.301
13	-1	-1	-1	1	1	1	1	1	1	-1	55.14±0.660	573.00±8	12.80±0.517
14	-1	-1	0	1	1	0	1	0	0	0	52.51±0.623	529.00±9	26.50±1.104
15	-1	-1	1	1	1	1	1	-1	-1	1	45.52±0.713	538.00±7	31.61±1.002
16	-1	1	-1	1	1	1	-1	-1	1	1	42.40±0.611	429.00±10	11.54±0.043
17	-1	1	0	1	1	0	-1	0	0	0	41.16±0.647	407.00±13	22.51±1.541
18	-1	1	1	1	1	1	-1	1	-1	-1	34.05±0.585	468.00±12	31.65±0.503
19	1	0	-1	1	0	1	0	0	-1	0	69.55±0.824	648.00±16	15.44±1.403
20	1	0	0	1	0	0	0	0	0	0	68.28±0.877	626.00±19	27.50±0.654
21	1	0	1	1	0	1	0	0	1	0	58.33±0.726	663.00±15	34.56±0.741
22	1	-1	-1	1	1	1	-1	1	-1	1	73.00±1.009	812.00±11	18.23±0.670
23	1	-1	0	1	1	0	-1	0	0	0	72.15±1.040	850.00±21	22.81±0.763
24	1	-1	1	1	1	1	-1	-1	1	-1	65.22±1.030	878.00±20	35.64±0.373
25	1	1	-1	1	1	1	1	-1	-1	-1	63.68±0.873	590.00±26	13.34±0.841
26	1	1	0	1	1	0	1	0	0	0	62.24±0.741	560.00±23	22.44±0.607
27	1	1	1	1	1	1	1	1	1	1	50.00±0.635	520.00±28	29.71±0.301

Bulk Density and Angle of Repose Determination: A pre-weighted amount of the powder was filled into a 10 ml graduated cylinder and the bulk density was calculated from the initial volume. The powder volume was noted to be the same after 500 taps and the following equation was employed for bulk density calculation [15]:

$$\rho = \frac{m2 - m1}{V} \quad \text{..... (Eq. 4)}$$

The funnel method was used for the angle of repose determination. An accurately weighted amount of the powder was taken in funnel and its height was adjusted where its tip barely touched the powder apex. The powder allowed to flow freely on the surface, the formed cone diameter was measured and angle of repose (θ) was calculated using the following equation.

$$\theta = \tan^{-1}\frac{h}{r} \quad \text{..... (Eq. 5)}$$

Where, h and r are the height and radius of powder cone [16].

In Vitro Release Study: A dialysis method was employed for studying the in vitro release of KTF from the optimize liposome suspension. A liposome suspension equivalent to 10 mg KTF was put into the dialysis membrane-70 (Himedia/India) pre-treated with phosphate buffer pH 7.4. Then, placed in a container with 300 ml of phosphate buffer pH 7.4 (dialysis medium) in a shaking water bath (100 rpm) at 37°C. The same approach was done on solution of free KTF with an initial concentration of 4.7 mg/ml. Because, liposome suspension volume equivalent to 10 mg show a KTF EE of about 53 %, 4.7 mg/ml free KTF was used to determine the KTF non-entrapped amount migrating across the dialysis membrane.

A 3 ml aliquot of the dialysis medium was withdrawn at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 hr. At the same time, the withdrawn solution replaced with an equal volume of phosphate buffer pH 7.4. The percentage of drug released was determined spectrophotometically at 300 nm according to the equation below:

$$R = \left[\frac{(Cdl \times Vdl) - (Cds \times Vds)}{(Cl - Clf)} \times V_l\right] \times 100\ \% \quad \text{..... (Eq. 6)}$$

Where, C_{dl} and C_{ds} are the concentrations of KTF in the dialysis medium for liposome suspension and free KTF solution (4.7 mg/ml, representing the non-entrapped KTF), respectively; V_{dl} and V_{ds} (300 ml) are the volumes of dialysis medium used for liposome suspension and free KTF solution (4.7 mg/ml), respectively; C_l and V_l are the KTF concentration and volume of liposome suspension, respectively; C_{lf} is the concentration of dissolved KTF not encapsulated in liposome vesicles [10, 17].

Release Kinetic Models and Mechanism: The in vitro drug release data were plotted in various kinetic models: zero-order (cumulative amount of drug released vs. time), first-order (log cumulative percentage of drug remaining vs. time), Higuchi's model (cumulative percentage of drug released vs. square root of time) and Korsmeyer-Peppas model (log cumulative percentage of drug released *vs*. log time) as in equations 7, 8, 9 and 10 respectively, in order to find the best fitted line to predict the mechanism of drug release using DDSolver software (Zhang, China) [18].

$$Q_t = Q_o + K_o t \quad \text{..... (Eq. 7)}$$

$$\ln (Q_o + Q_t) = K_1 t \quad \text{..... (Eq. 8)}$$

$$\frac{Qt}{Qo} = K_H\sqrt{t} \quad \text{..... (Eq. 9)}$$

$$\frac{Qt}{Qo} = K_{KP}t^n \quad \text{..... (Eq. 10)}$$

where: Q_t – amount of drug released in time t, Q_0 – initial KTF amount in the liposomes, K_0, K_1, K_H and K_{KP} – zero order, first order, Higuchi and Korsmeyer-Peppas release rate constants respectively and n – release exponent. Furthermore; Weibull dissolution model equation below can be successfully applied to almost all kinds of dissolution curves [19]. It expresses the accumulated fraction of the drug, Q, in solution at time, t, by:

$$Q = 1 - \exp\left[\frac{-(t - Ti)^b}{a}\right] \quad \text{..... (Eq. 11)}$$

where: a – scale parameter that defines process timescale, Ti – lag time before the onset of dissolution or release process and will be zero in most cases and b – shape parameter that characterizes the curve as either exponential when b=1 (case 1), sigmoid/S-shaped/with upward curvature followed by a turning point when b>1 (case 2) or parabolic/with a higher initial slope and after that consistent with the exponential when b<1 (case 3). Therefore; the properly selected optimized formula was further fitted into Weibull dissolution model equation.

RESULTS AND DISCUSSIONS:

Model Fitting: Table 2 showed the combined effects of KTF: SPC94 ratio, KTF/SPC_{94}: 0.5M lactose ratio and LLP: CLC ratio on the %EE, PS and %FPF. A full model for each response was established by putting the values of intercepts and regression coefficients in polynomial equation, i.e.; equation 3 as shown in table 3 and as follows:

$$Y_1EE = 68.09 + 9.55 X_1 - 5.47 X_2 - 4.98 X_3 - 9.84 X_1^2 - 1.23 X_2^2 - 3.38 X_3^2 + 0.093 X_1 X_2 - 0.48 X_1 X_3 - 1.04 X_2 X_3 - 0.90 X_1 X_2 X_3 \ldots \text{(Eq. 12)}$$

$$Y_2PS = 564.59 + 105.17 X_1 - 134.89 X_2 - 0.78 X_3 - 52.61 X_1^2 + 76.89 X_2^2 + 21.89 X_3^2 - 44.50 X_1 X_2 + 5.42 X_1 X_3 - 2.58 X_2 X_3 - 26.25 X_1 X_2 X_3 \ldots \text{(Eq. 13)}$$

$$Y_3FPF = 23.64 + 0.61 X_1 - 0.61 X_2 + 8.70 X_3 + 1.37 X_1^2 - 1.04 X_2^2 - 0.78 X_3^2 - 0.40 X_1 X_2 + 0.11 X_1 X_3 + 1.37 X_2 X_3 - 0.29 X_1 X_2 X_3 \ldots \text{(Eq. 14)}$$

F-values of 272.75, 17.96 and 27.11 respectively for EE, PS and FPF indicate that the model is significant as shown in table 4. For EE, PS and FPF, there is only 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Probability value > F" less than 0.0500 indicate model terms are significant. For EE X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_{23} and X_{123}, for PS X_1, X_2, X_1^2, X_2^2 and X_{12} and for FPF X_3 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Therefore, by omitting the insignificant model terms, i.e., model reduction will improve the model as follows:

$$Y_1EE = 68.09 + 9.55 X_1 - 5.47 X_2 - 4.98 X_3 - 9.84 X_1^2 - 1.23 X_2^2 - 3.38 X_3^2 - 1.04 X_2 X_3 - 0.90 X_1 X_2 X_3 \ldots \text{(Eq. 15)}$$

$$Y_2PS = 564.59 + 105.17 X_1 - 134.89 X_2 - 52.61 X_1^2 + 76.89 X_2^2 - 44.50 X_1 X_2 \ldots \text{(Eq. 16)}$$

$$Y_3FPF = 23.64 + 8.70 X_3 \ldots \text{(Eq. 17)}$$

In the same sense for EE, the "Predicted R^2" of 0.9793 is in reasonable agreement with the "Adjusted R^2" of 0.9905, for PS, the "Predicted R^2" of 0.7325 is in reasonable agreement with the "Adjusted R^2" of 0.8671 and for FPF; the "Predicted R^2" of 0.8236 is in reasonable agreement with the "Adjusted R^2" of 0.9094. Generally, R^2 values greater than 0.80 indicate the suitability of the regression models for the behavior explanation but in the same time a larger R^2 value does not always imply the model adequacy. Thus, using an adjusted R^2 is better for the model adequacy evaluation. Generally, a CV greater than 10% indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model.

Therefore, the CV value was 1.88, 9.8 and 9.74 for EE, PS and FPF respectively which indicates a better reproducibility and reliability of the conducted experiments. Also, a comparison between the full model and reduced model was done through the F-statistic to check the omission effect of the statistically insignificant coefficients from the full model as shown in **table 4**.

Liposomes Encapsulation Efficiency: All parameters shown in table 3 have significant ($p<0.05$) effect on the KTF EE except that of the interactive parameters of KTF: SPC_{94} by KTF/SPC_{94}: 0.5M lactose, i.e., X_1 x X_2 and KTF: SPC94 by LLP: CLC, i.e., X_1 x X_3 was not significant. Independent variables effect on KTF liposomes is shown in figure 1a, 1b and 1c. As KTF: SPC_{94} ratio increased, there will be an increment in the EE due to that more KTF was encapsulated into the vesicles. In contrast a negative relation and the reverse occur when the ratio is reduced as the liposomes vesicles become densely packed due to the increment in the SPC_{94} head groups interactions [20].

TABLE 3: STATISTICAL REGRESSION ANALYSIS

Parameters	Y_1EE		Y_2PS		Y_3FPF	
	Coefficient	P – Value	Coefficient	P – Value	Coefficient	P – Value
Intercept	68.09	< 0.0001	564.59	< 0.0001	23.64	< 0.0001
X_1 (1:15, 1:25)	9.55	< 0.0001	105.17	< 0.0001	0.61	0.2752
X_2 (1:9, 3:7)	-5.47	< 0.0001	-134.89	< 0.0001	-0.61	0.2710
X_3 (1:10, 1:15)	-4.98	< 0.0001	-0.78	0.9556	8.70	< 0.0001
$X_1 \times X_1$	-9.84	< 0.0001	-52.61	0.0421	1.37	0.1584
$X_2 \times X_2$	-1.23	0.0144	76.89	0.0053	-1.04	0.2806
$X_3 \times X_3$	-3.38	< 0.0001	21.89	0.3717	-0.78	0.4151
$X_1 \times X_2$	0.093	0.7748	-44.50	0.0177	-0.40	0.4588
$X_1 \times X_3$	-0.48	0.1486	5.42	0.7519	0.11	0.5549
$X_2 \times X_3$	-1.04	0.0046	-2.58	0.8800	1.37	0.8720
$X_1 \times X_2 \times X_3$	-0.90	0.0351	-26.25	0.2213	-0.29	0.7207
R^2	0.9942		0.9182		0.9443	

TABLE 4: ANALYSIS OF VARIANCE (ANOVA) OF FULL AND REDUCED MODELS OF MULTIPLE LINEAR REGRESSIONS

Response	Model		Degree of Freedom	Sum of Squares	Mean of Squares	F Value	Adjusted R^2	Predicted R^2	C.V. %	ANOVA Comparison	
										F Calculated	F Tabulated
Y_1EE	Regression	FM	10	3307.87	330.79	272.75	0.9905	0.9793	1.88	1.1942	3.63
		RM	8	3304.98	413.12	333.16					
	Residual (Error)	FM	16	19.40	1.21						
		RM	18	22.29	1.24						
Y_2PS	Regression	FM	10	611263	61126.30	17.96	0.8671	0.7325	9.80	0.519	5.96
		RM	5	602432.71	120486.54	40					
	Residual (Error)	FM	16	54443.30	3402.71						
		RM	21	63273.59	3013.03						
Y_3FPF	Regression	FM	10	1401.42	140.14	27.11	0.9094	0.8236	9.74	0.868	241.88
		RM	1	1361.03	1361.03	276.63					
	Residual (Error)	FM	16	82.72	5.17						
		RM	25	123.11	4.92						

By using lactose as a cryoprotectant sugar at 500 mM in order to make sure that its existence on the liposomal bilayer both sides would decrease the liposomes permeability and hence enhance the KTF EE. Results revealed that a higher lactose ratio has a significant-negative effect on the KTF EE due to the lyophilization process will impart liposomal vesicles stability through their polar head groups hydrating with the hydroxyl groups of lactose that replaces water molecules. Thus, the vesicles will retain their contents and do not re-encapsulate due to an optimum surface of crystallized sugar by which liposomes can constrict and get coated on [21].

Liposomes Particle Size: Photomicrographs (1000x) before dehydration and after rehydration under plain are shown in **figure 2a and 2b**. The effect of independent variables on KTF liposomes PS is shown in **figure 3a, 3b and 3c**.

Based on the sum of squares shown in table 4, both KTF: SPC_{94} (X_1) and KTF/SPC_{94}: 0.5M lactose (X_2) have significant ($p<0.05$) effect on the KTF liposomes PS, whereas LLP: CLC (X_3) was not significant. As SPC_{94} concentration increase the PS increase and this is due to the fact that phospholipids constitute the liposome membrane and their concentration have a direct effect on the liposomes PS [22].

Lactose ability to preserve the liposomal structural integrity during hydration is due to liposome polar head groups hydration with the lactose hydroxyl groups that maintains the liposome stability. Therefore; an optimal cryoprotectant concentration is essential in order to provide an adequate surface area for the adherence of the condensed liposome bilayer [23].

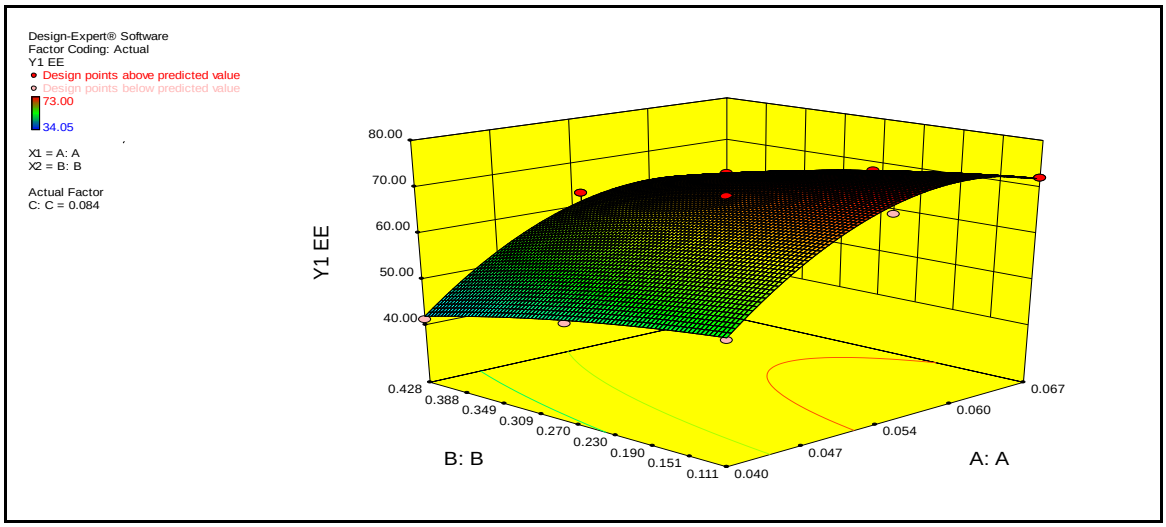

FIGURE 1a: RESPONSE SURFACE FOR THE EFFECT OF KTF: SPC_{94} (X_1) AND KTF/SPC_{94}: 0.5M LACTOSE (X_2) ON THE KTF LIPOSOMAL EE (Y_1)

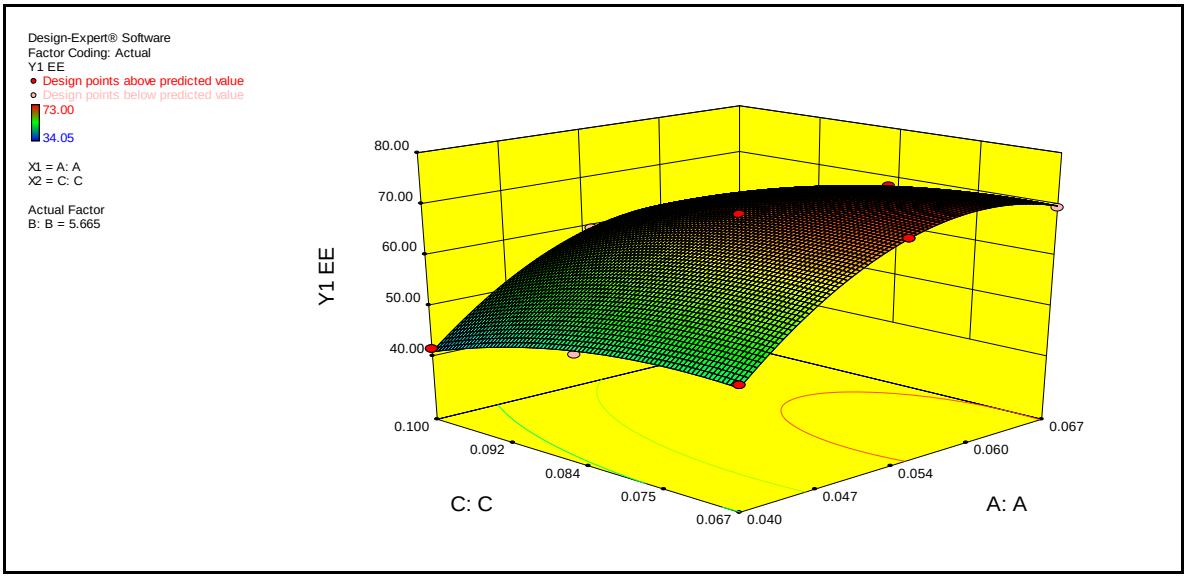

FIGURE 1b: RESPONSE SURFACE FOR THE EFFECT OF KTF: SPC_{94} (X_1) AND LLP: CLC (X_3) ON THE KTF LIPOSOMAL EE (Y_1)

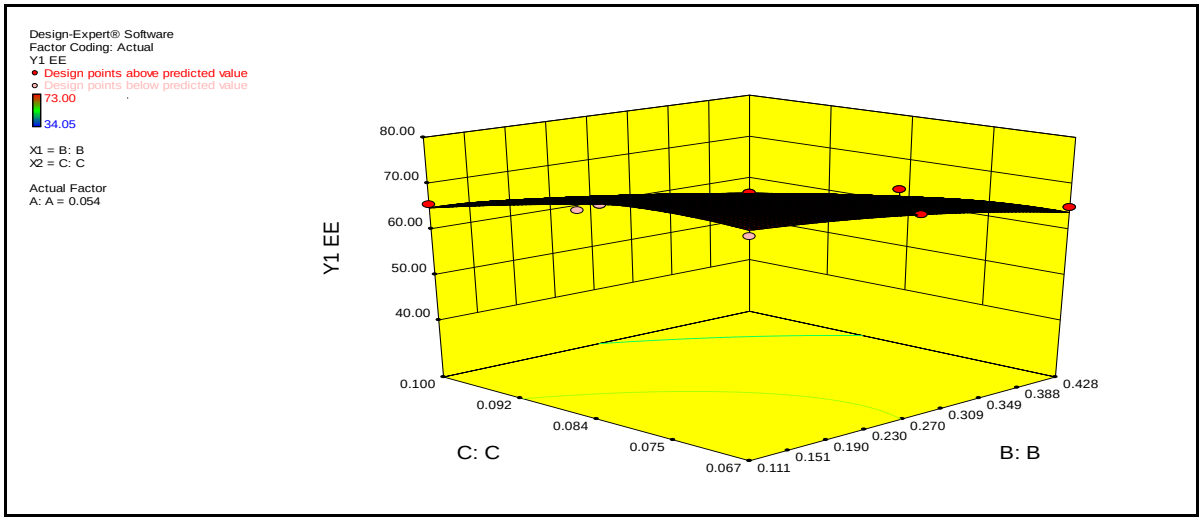

FIGURE 1c: RESPONSE SURFACE FOR THE EFFECT OF KTF/SPC_{94}: 0.5M lactose (X_2) AND LLP: CLC (X_3) ON THE KTF LIPOSOMAL EE (Y_1)

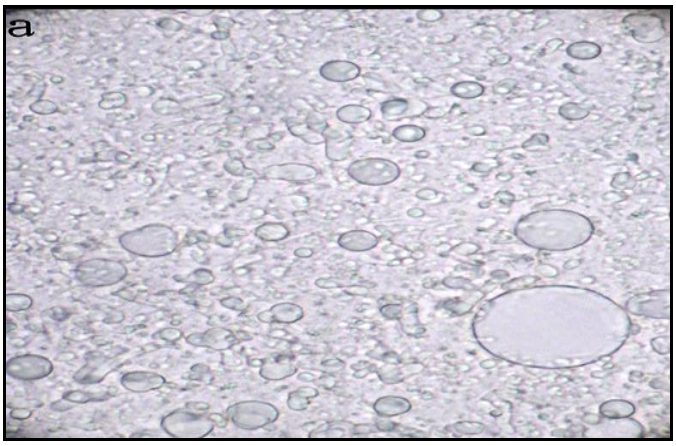

FIGURE 2a: PHOTOMICROGRAPH AT 1000 X MAGNIFICATION PLAIN BEFORE DEHYDRATION

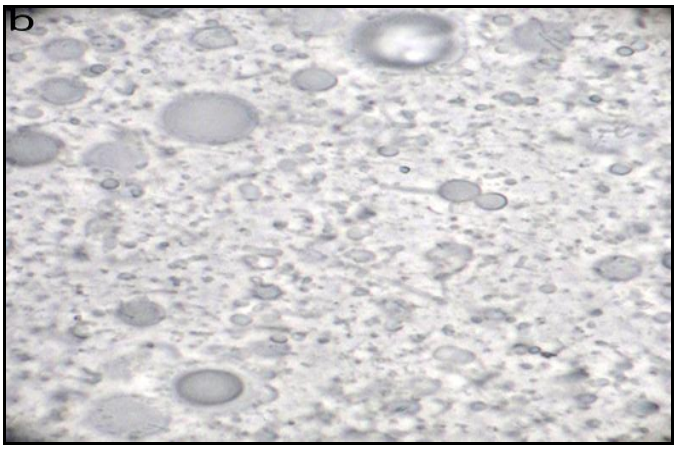

FIGURE 2b: PHOTOMICROGRAPH AT 1000 X MAGNIFICATION PLAIN AFTER DEHYDRATION – REHYDRATION

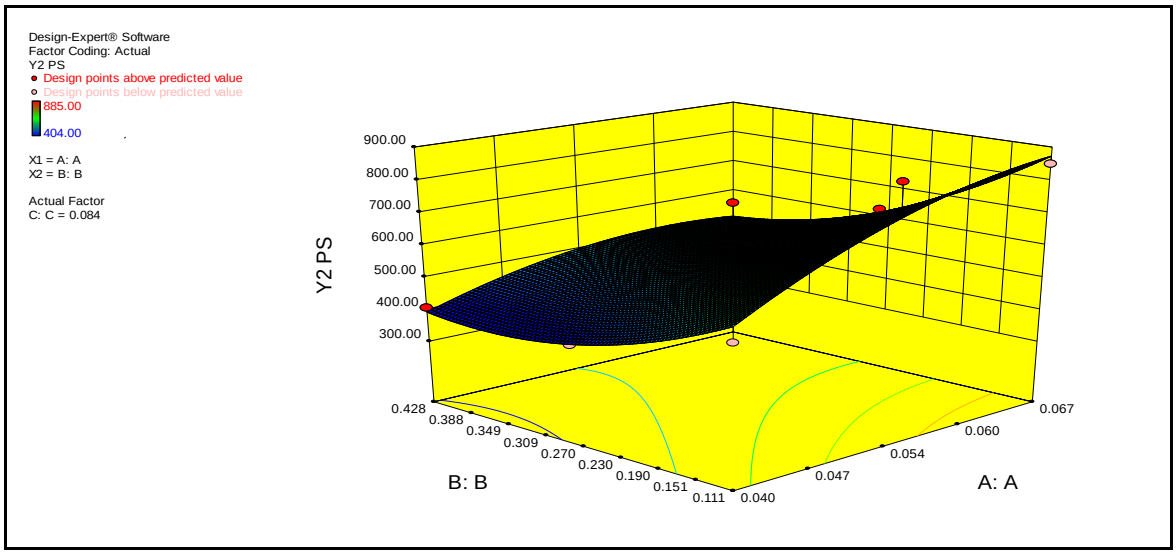

FIGURE 3a: RESPONSE SURFACE FOR THE EFFECT OF KTF: SPC_{94} (X_1) AND KTF/SPC_{94}: 0.5M lactose (X_2) ON THE KTF LIPOSOMAL PS (Y_2)

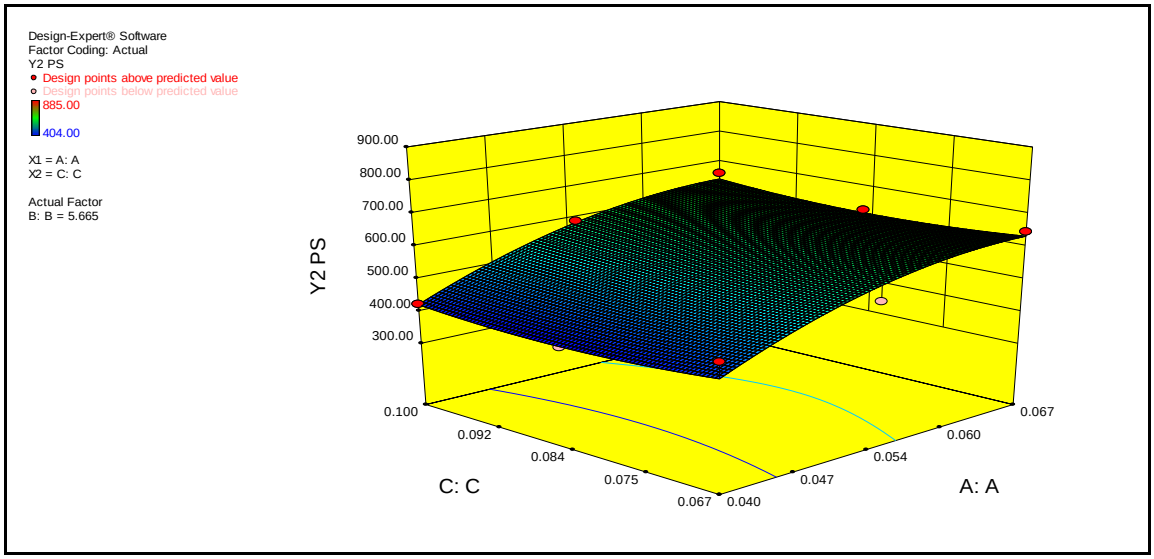

FIGURE 3b: RESPONSE SURFACE FOR THE EFFECT OF KTF: SPC_{94} (X_1) AND LLP: CLC (X_3) ON THE KTF LIPOSOMAL PS (Y_2)

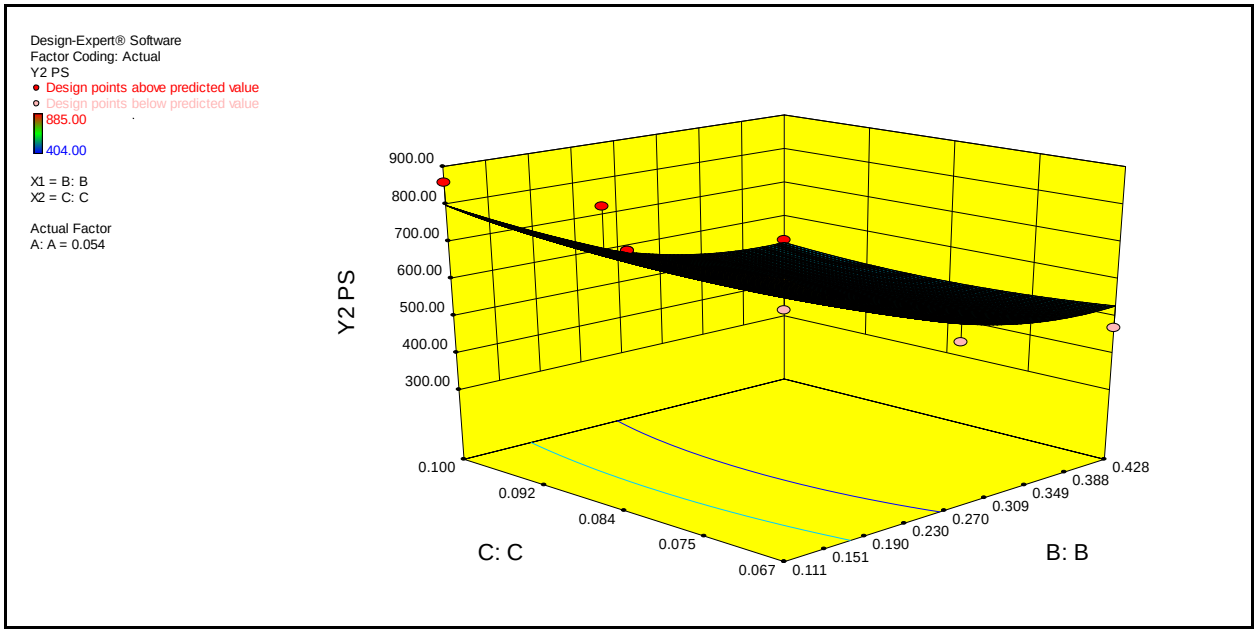

FIGURE 3c: RESPONSE SURFACE FOR THE EFFECT OF KTF/SPC$_{94}$: 0.5M lactose (X_2) AND LLP: CLC (X_3) ON THE KTF LIPOSOMAL PS (Y_2)

Fine Particle Fraction: At minor formulation parameters ratios, there will be an approximate strength of 113.636 µg of entrapped drug per 200 mg of the formulation. The only parameter with a significant effect on the FPF shown in **table 3** is LLP: CLC (X_3). Generally, an optimum amount of lactose carrier is required for improving FPF and optimizing the in vitro deposition of liposomal dry powders. **Figure 4a, 4b and 4c** shows the effect of the independent variables on the FPF. Figure 4b and 4c shows an ascending from the lactose carrier side, i.e., C: C which proof that as lactose carrier ratio increase there will be an increase in the FPF.

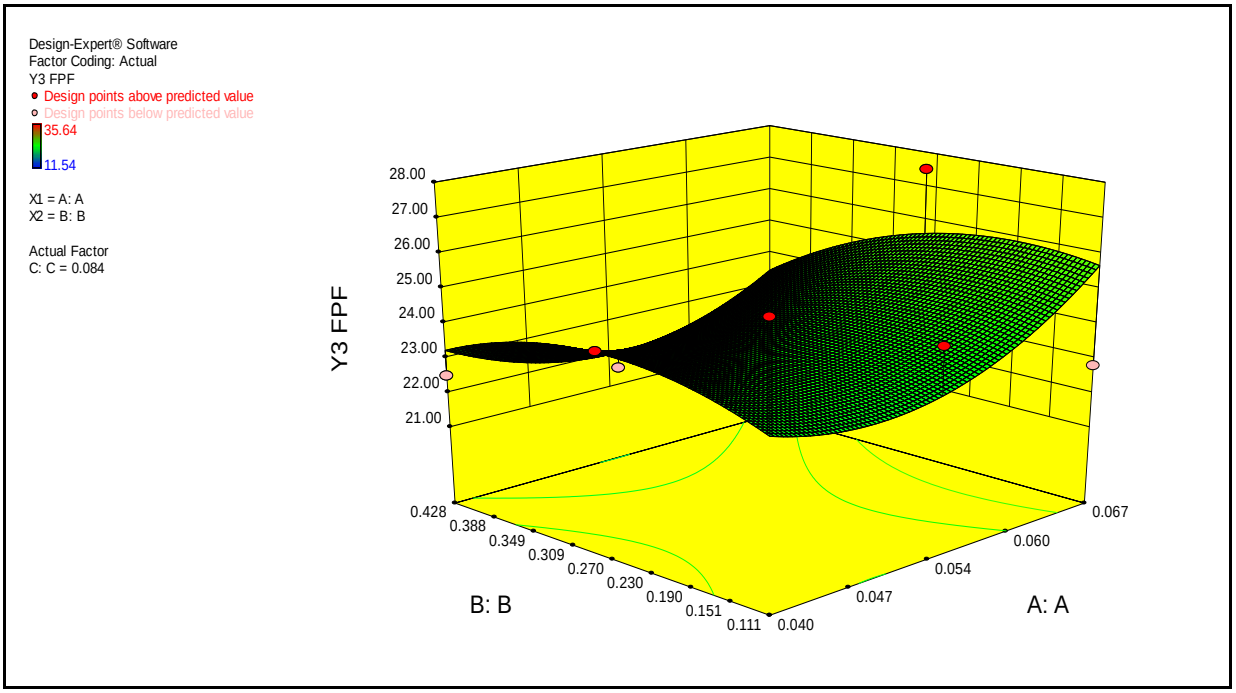

FIGURE 4a: RESPONSE SURFACE FOR THE EFFECT OF KTF: SPC$_{94}$ (X_1) AND KTF/SPC$_{94}$: 0.5M lactose (X_2) ON THE FPF (Y_3)

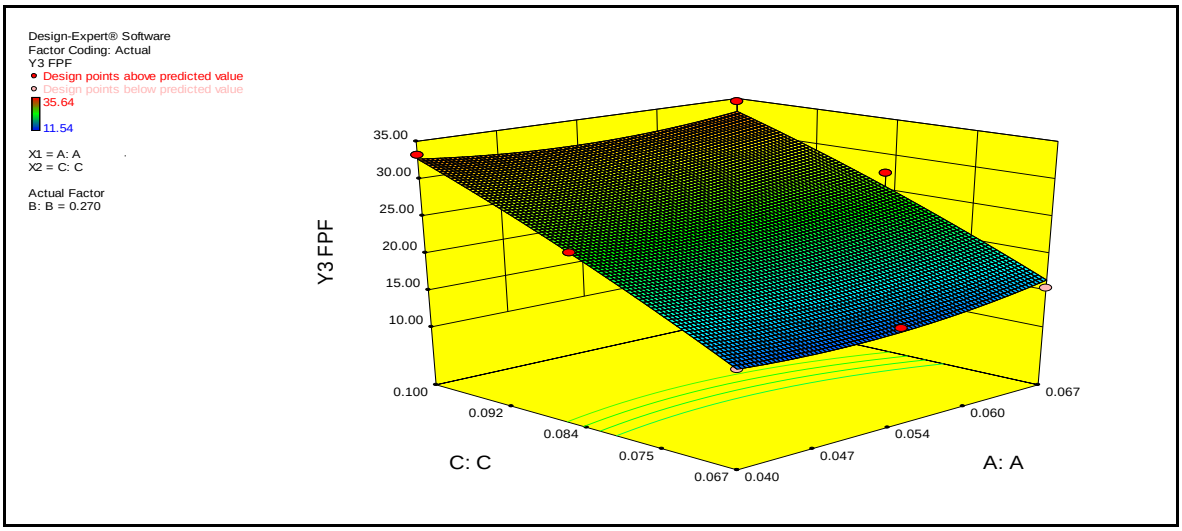

FIGURE 4b: RESPONSE SURFACE FOR THE EFFECT OF KTF: SPC_{94} (X_1) AND LLP: CLC (X_3) ON THE FPF (Y_3)

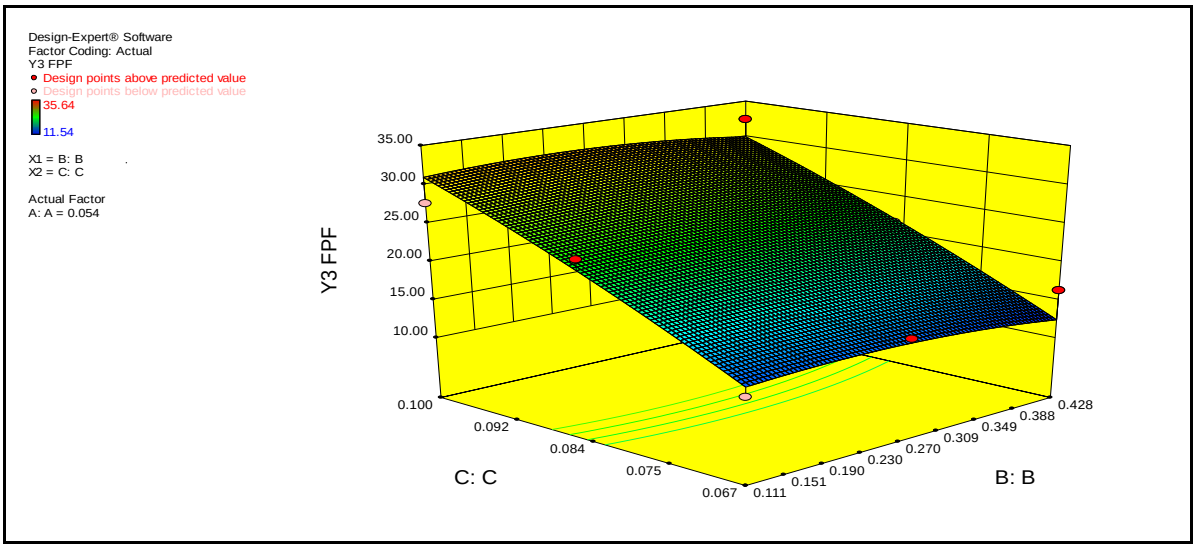

FIGURE 4c: RESPONSE SURFACE FOR THE EFFECT OF KTF/SPC_{94}: 0.5M lactose (X_2) AND LLP: CLC (X_3) ON THE FPF (Y_3)

Check Point Analysis: Eight preparations were prepared and evaluated for the response variables. The compositions, predicted and experimental values, percentage error and p-value are listed in **table 5**. For each variable, i.e., EE, PS and FPF, the linear correlation between the observed and predicted response is plotted along with R^2 values as shown in **figure 5a, 5b and 5c** respectively. The differences between the predicted and experimental values considered to be insignificant when the p-value>0.05. Also, higher R^2 values (0.936, 0.949 and 0.922 for EE, PS and FPF respectively) of the linear correlation plots indicate the fitness and RSM with a high predictive capability.

TABLE 5: CHECKPOINT ANALYSIS

Sr. No.	Formulation Composition			Comparison											
				Y_1 EE				Y_2 PS				Y_3 FPF			
	X_1	X_2	X_3	Pred. Value	Expert. Value	% Error	p-Value	Pred. Value	Expert. Value	% Error	p-Value	Pred. Value	Expert. Value	% Error	p-Value
1	1:16.5	2.4:7.6	1:13	66.35	69.40±0.751	-4.596	p>0.05 non-significant	534.99	544.00±16	-1.684	p>0.05 non-significant	24.04	25.00±1.410	-3.993	p>0.05 non-significant
2	1:18	2.2:7.8	1:11.75	66.47	65.12±0.611	2.030		537.66	530.00±13	1.424		24.15	23.04±0.500	4.596	
3	1:24.5	1.7:8.3	1:14	66.90	64.28±0.77	3.916		544.04	534.00±15	1.845		24.11	25.16±0.550	-4.355	
4	1:23.5	1.5:8.5	1:11.25	66.99	67.72±0.962	-1.089		547.30	540.00±17	1.333		24.29	23.74±1.043	2.264	
5	1:21	1.2:8.8	1:13.75	67.36	66.44±0.913	1.365		552.27	554.00±19	-0.313		24.19	23.95±1.520	0.992	
6	1:18.5	2.8:7.2	1:10.5	65.72	68.17±0.966	-3.727		528.37	524.00±14	0.827		24.09	23.84±1.021	1.037	
7	1:15.5	2.9:7.1	1:14.5	65.83	65.62±0.906	0.319		527.67	535.00±12	-1.389		23.84	23.59±0.760	1.048	
8	1:16	1.3:8.7	1:11	67.30	66.97±1.063	0.490		552.19	550.00±15	0.396		24.34	24.62±0.403	-1.150	

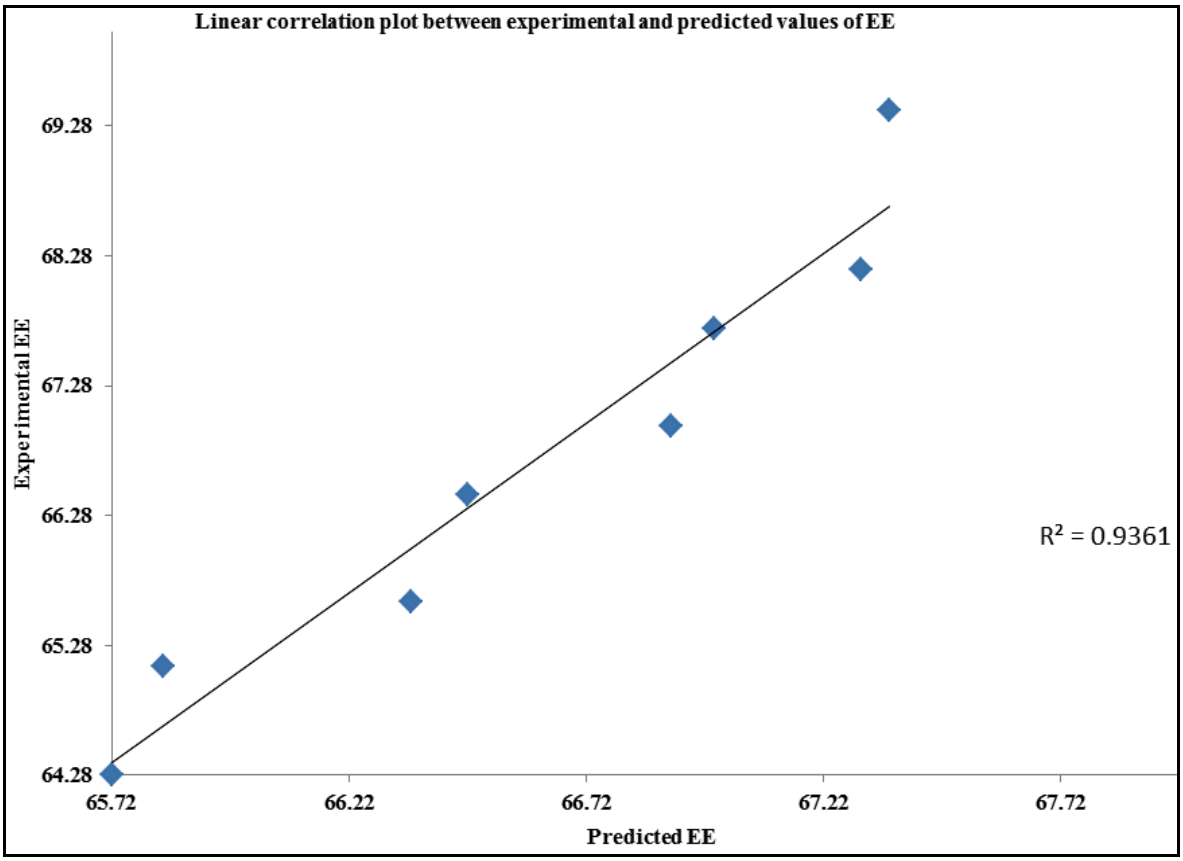

FIGURE 5a: LINEAR CORRELATION PLOT OF THE EXPERIMENTAL RESPONSE VALUES VERSUS THE PREDICTED RESPONSE VALUES FOR EE

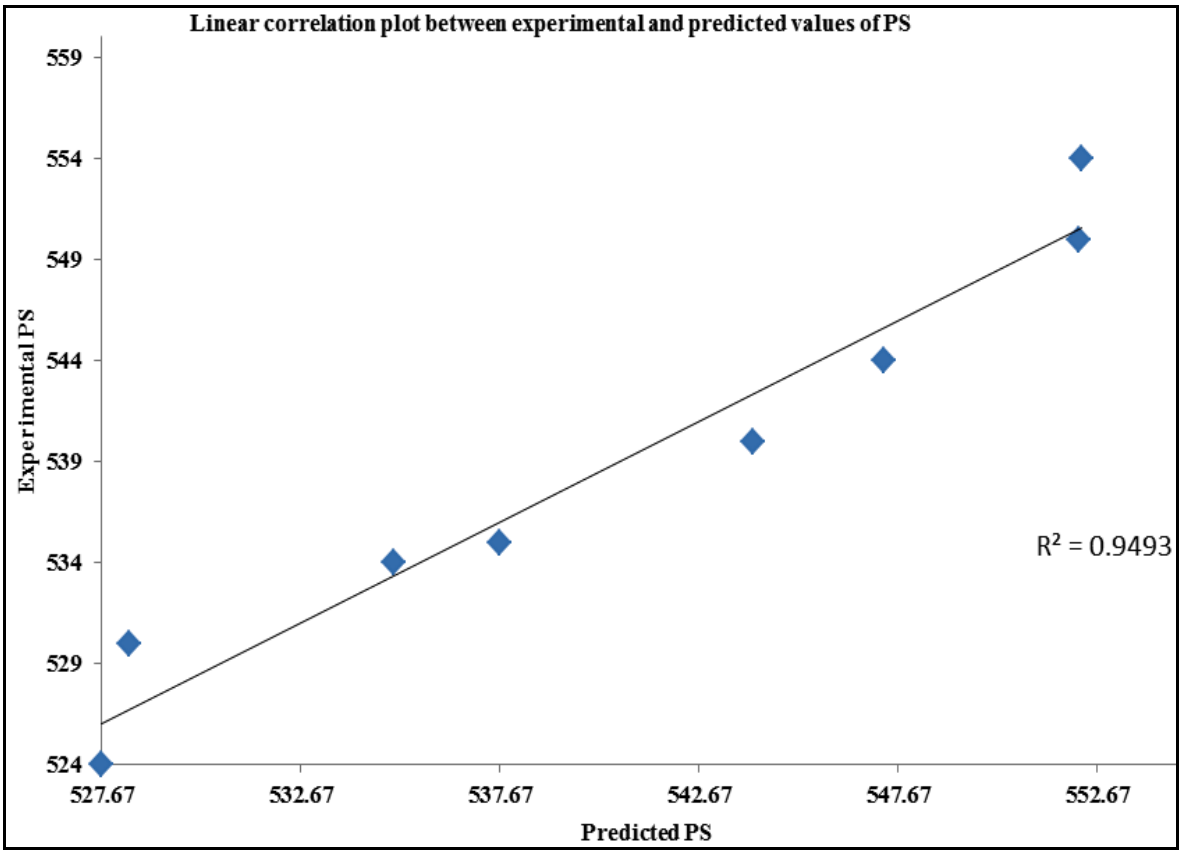

FIGURE 5b: LINEAR CORRELATION PLOT OF THE EXPERIMENTAL RESPONSE VALUES VERSUS THE PREDICTED RESPONSE VALUES FOR PS

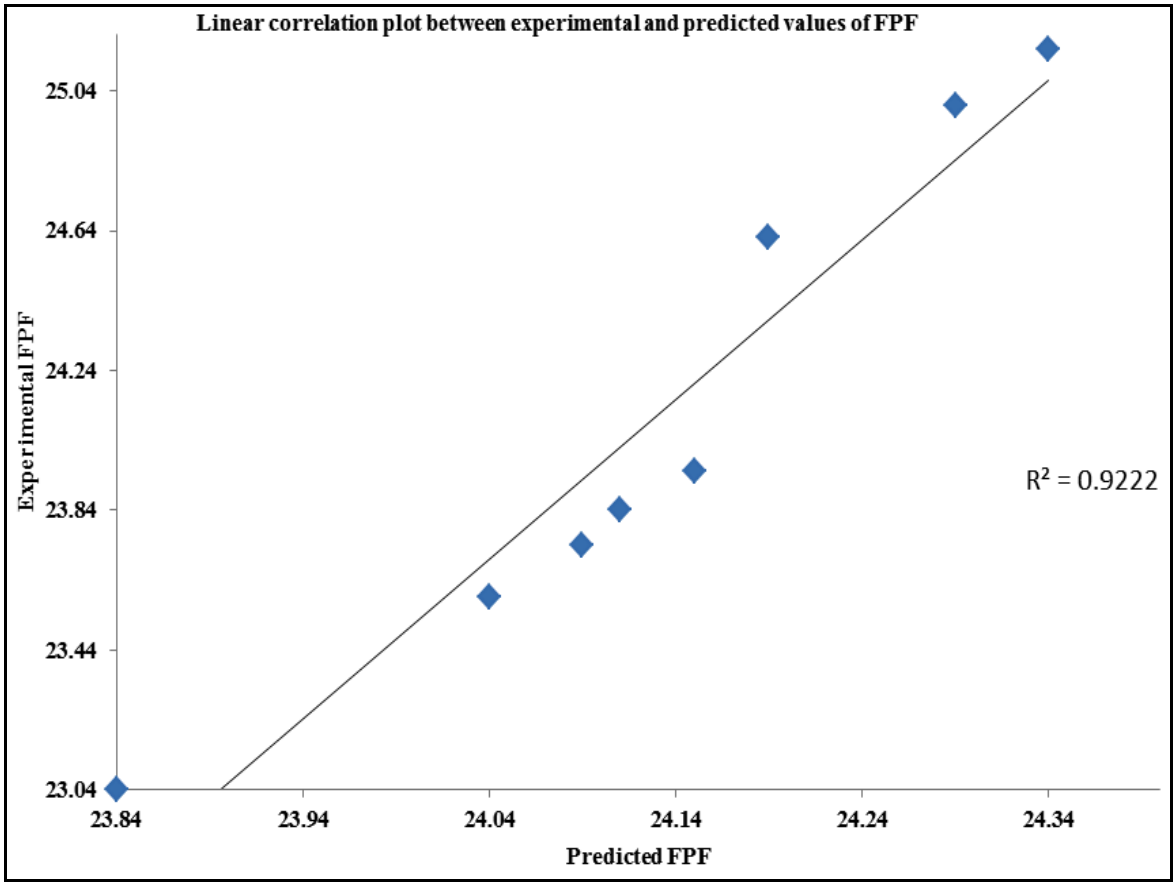

FIGURE 5c: LINEAR CORRELATION PLOT OF THE EXPERIMENTAL RESPONSE VALUES VERSUS THE PREDICTED RESPONSE VALUES FOR FPF

Optimization: By putting the aim and the importance of each variable and response, the optimum formula can be fulfilled. Table 6 shows the optimized batch (KTF: SPC_{94} ratio = 0.045; KTF/SPC_{94}: 0.5M lactose ratio = 0.389 and LLP: CLC ratio = 0.080) that was prepared and the comparison between the experimental and predicted values. P>0.05 indicates the insignificant differences between the experimental and predicted values.

TABLE 6: THE OPTIMIZED FORMULA DERIVATION

Constrains			Lower Limit	Upper Limit	Predicted Solution [A]	Experimental Results [B]	Comparison of A and B (P Value)
Name	**Aim**	**Importance**					
KTF: SPC_{94}	Minimize	3	-1	1	0.045	0.045	
KTF/SPC_{94}: 0.5M lactose	Maximize	3	-1	1	0.389	0.389	
LLP: CLC	Minimize	3	-1	1	0.080	0.080	
EE	Maximize	3	34.05	73.00	54.53	52.68±0.749	P > 0.05, Non-significant
PS	Minimize	3	404	885	442.32	444.00±9	P > 0.05, Non-significant
FPF	Maximize	3	11.54	35.64	21.03	20.46±0.697	P > 0.05, Non-significant

Bulk Density and Angle of Repose: The obtained optimized liposomal KTF dry powders formulation bulk density was (0.31 gm/cm^3) and become (0.53 gm/cm^3) when added to coarse lactose carrier (CLC) because of the mixture boarder particle size, whereas the angle of repose was (30.14 θ) which indicates a good powder flowability [24].

***In-vitro* Release:** The standard curve of KTF was determined to be y=0.028x (R^2=0.999, where x is the concentration of KTF and y is the absorbance of KTF), with concentrations ranging from 5 to 30 µg/ml. In case of free KTF solution at 4.7 mg/ml all of the KTF had emptied within approximately 11 min into the receptor compartment through the dialysis membrane. Therefore, KTF encapsulated in liposomes (10 mg/ml) will take about more than 12 hr to permeate the membrane. Thus, the non-entrapped KTF (47% of the total KTF) in the liposome suspension (10 mg/ml) rapidly emptied through the membrane within the first 0.183 hr, corresponding to the rate of KTF transport observed for the 4.7 mg/ml solution, i.e., 11 min. This is due to that the liposome vesicles were unable to cross the dialysis membrane and the entrapped KTF must first release from the liposomes prior to migration through the membrane. Noticeably, around 69% of the entrapped KTF was able to be released from the liposomes after 12 hr, proofing liposomes as a feasible delivery system for sustaining KTF release. **Figure 6** shows the release profile of KTF from the liposome suspension, taking into account only the entrapped amount of KTF crossing the dialysis membrane after being released from the liposomes.

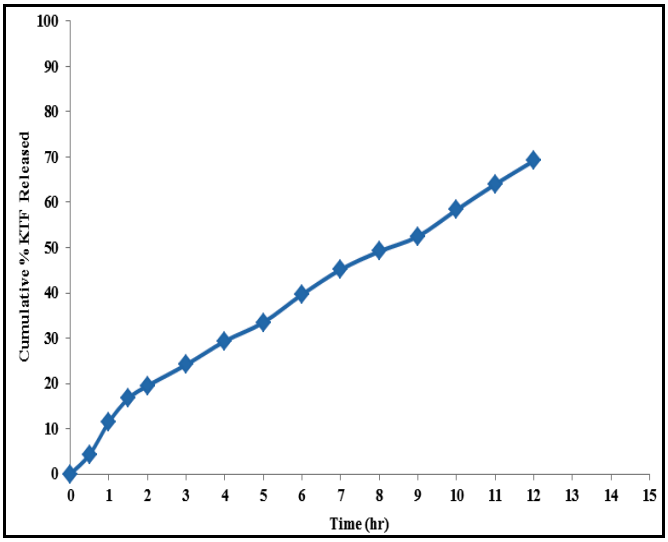

FIGURE 6: *IN VITRO* CUMULATIVE RELEASE PROFILE OF KTF FROM OPTIMIZED KTF LIPOSOME SUSPENSION (ONLY ENTRAPPED KTF) IN PHOSPHATE BUFFER pH 7.4 AT 37 ± 0.5°C

Release Kinetic Models and Mechanism: The data shown in table 7 revealed that Korsmeyer – Peppas model of release gives the best fitness of all models, whereas the mechanism of release was non-Fickian (anomalous) due to non-swellable liposomal sphere vesicles, i.e., the mechanism of release is mainly mediated by diffusion and erosion ($0.43 < n < 0.85$).

TABLE 7: THE RELEASE KINETIC DATA

Zero-Order Model		First-Order Model		Higuchi-Matrix Model		Korsmeyer-Peppas Model		
R^2	K_0 (%h^{-1})	R^2	K_1 (h^{-1})	R^2	K_H (%$h^{-1/2}$)	R^2	n	K_{KP} (%h^{-n})
0.9932	6.088	0.9951	0.089	0.9863	17.345	0.9981	0.745	10.587

Weibull 3 model was the best for fitting the data obtained as shown in equation 18 [25]:

Weibull 3 Dissolution Model:

$$F_t = F_{max} \, x \left[1 - e^{-\frac{t^b}{a}} \right] \quad \text{..... (Eq. 18)}$$

The release kinetic data were; F_{max}=69.17%, a=3243942.813, b=0.749 and R^2=0.9982. Therefore; the overall KTF release equation will be:

$$F_t = 0.6917 \, x \left[1 - e^{-\frac{t^{0.749}}{3243942.813}} \right] \quad \text{..... (Eq. 19)}$$

CONCLUSIONS: The mass ratio of KTF solution to soybean phosphatidylcholine (SPC_{94}), KTF/ SPC_{94} to 0.5 M lactose solution and lyophilized liposomal powder (LLP) to coarse lactose carrier (CLC) effects on preparing KTF nano-liposomes were studied. As KTF: SPC_{94} ratio increased, there will be an increment in the KTF liposomal encapsulation efficiency, whereas a higher lactose ratio has a significant-negative effect. As SPC_{94} concentration increase the liposomes particle size increase, whereas an optimal cryoprotectant concentration is essential in order to provide an adequate surface area for the adherence of the condensed liposomal bilayer. An optimum amount of lactose carrier is required for improving fine particles fraction and optimizing the in vitro deposition of liposomal dry powder.

Applying a desirability function method, the optimum parameters were: KTF: SPC_{94} of 0.045, KTF/ SPC_{94}: 0.5 M lactose of 0.389 and LLP: CLC of 0.080. By employing response surface methodology, the development of optimum KTF nano-liposomal vesicles with a continued release were possible and pulmonary targeting via loading on lactose carrier can be fulfilled.

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