Original Article

Development, Characterization and Validation of Silymarin Loaded Solid Lipid Nanoparticles for the Treatment of Liver Cirrhosis

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Abstract

Cirrhosis can arise in consequence of an exogenous/ toxic, infectious, toxic/allergic, immunopathological/ autoimmune, or vascular process or an inborn error of metabolism**.** The commonest causes of cirrhosis in Germany are alcoholic and non-alcoholic fatty liver disease and viral hepatitis (B or C). Among these causes, the most common of all is alcoholic fatty liver disease, which caused 8619 deaths in Germany (8.9 deaths per 100 000 population) in 2009 and thus ranks among the country's top 20 causes of death. Cirrhosis is rising in importance as a public health problem: the number of deaths from cirrhosis per 100 000 population doubled from 5 in 1980 to 9.9 in 2005. Autopsy studies have revealed fatty liver disease in 70% of overweight persons and in 35% of persons of normal weight. They have also revealed cirrhosis in 18.5% of overweight diabetics. 0.5% of the German population are chronically infected with the hepatitis B virus, and 0.5% with the hepatitis C virus. The aim of this study is to develop, characterize silymarin loaded Solid lipid nanoparticles for the treatment of psoriasis.

INTRODUCTION

Cirrhosis is the last stage found by various chronic liver diseases after years or decades of slightly slow progression. There are, however, method to prevent cirrhosis, because the diseases that most showed it progress slowly, and measures are available to prevent and treat them. Moreover, most cases of hepato - cellular carcinoma (HCC) occur in a cirrhotic liver, so cirrhosis hampering is, in fact, also HCC prevention. The risk of developing HCC depends on the underlying disease: It is low, for example, when the underlying disease is autoimmune hepatitis (2.9% in 10 years) (1), and high when the underlying disease is chronic hepatitis B with a viral burden greater than 107 copies/mL (19.8% in 13 years) **[1].** Aside from chronic viral hepatitis, fatty liver disease due to any of the very common underlying disorders (obesity, diabetes, alcohol abuse) commonly progresses to cirrhosis and thus merits both specialized medical treatment and close follow-up by the primary-care physician. This article, based on a selective review of the literature, deals with approaches to cirrhosis prevention that involve the proper diagnostic work-up for early detection of chronic liver disease, followed by risk-adapted treatment **[2].** Silymarin showed low solubility and low permeability. It is an extract of *Silybum marianum*, or milk thistle, and its major active compound is silybin, which has a remarkable biological effect. It is majorly used in different liver disorders, particularly chronic liver diseases, cirrhosis, and hepatocellular carcinoma, because of its antioxidant, anti-inflammatory and antifibrotic power **[3].** Silymarin, an extract of the milk thistle (Silybum marianum), is a mixture of flavonoids and polyphenols; Silibinin is its major bioactive constituent. Silymarin is a well-known hepatoprotective drug which has been shown to have antioxidant, anti-inflammatory/immunomodulatory, and antifibrotic properties in various in vitro and in vivo animal models. Its antioxidant activity is most likely to

attenuate the pathologic effects initiated by oxidative stress in the liver, which influence pathways of inflammation, necrosis, and fibrosis in chronic liver disease. Silymarin is therefore used to treat numerous liver disorders characterized by degenerative necrosis and functional impairment. Silymarin, however, has a very poor aqueous solubility, improper tissue distribution, and gastric degradation. Recent studies developed silymarin in nanoscale were evaluated on paracetamol-induced and CCl4-induced hepatotoxicity. Those and others proved an improvement in pharmacokinetics and bioavailability of silymarin and improvement in liver functions with no attention given to the resolution of fibrosis **[4].** However, silymarin coated gold nanoparticles promoted extracellular matrix (ECM) degradation, hepatic stellate cells (HSCs) inactivation with strong enhancement of hepatic regenerative capacity in CCl4-induced liver injury and cirrhosis.

MATERIALS AND METHODS

Silymarin was obtained as gift sample from Organic India Company, New Delhi, and sodium hydroxide from Central Drug House (P) Ltd. New Delhi, Ethanol was purchased from Loba Chemie Pvt. Ltd., Mumbai and All other reagents and chemicals used were of analytical grade.

PREFORMULATION STUDIES

Silymarin was evaluated for its organoleptic properties viz. nature, color, odour and taste.

Partition Coefficient

The partition coefficient of Silymarin was determined by shake flask method.10 mg of drug was placed in octanol and water (1:1) in a separating funnel. The funnel was kept in a mechanical shaker for 24 h to attain equilibrium. The aqueous layer was separated from octanol it was filtered through Whatman filter paper (0.45µm) and dilution were made for analyzing spectrophotometric analysis **[5]**

The formula for calculating log P is as follows:

$$\textbf{Log P} = \frac{\textbf{Concentration in organic phase}}{\textbf{Concentration in aqueous phase}}$$

Solubility study:

To determine the solubility of Silymarin in different solvent systems, the saturated solution of drug was obtained by placing an excess amount of drug in various solvents at 25℃ in a mechanical shaker for 72 h to attain the equilibrium **[6]**. The samples were withdrawn and filtered with whatman filter paper (0.45µm) and analyzed by UV spectrophotometer **[7, 8].**

IDENTIFICATION OF DRUG

To confirm the identity of Silymarin procured before proceeding to its formulation in to Solid Lipid Nanoparticles formulation system, UV spectroscopy and DSC were employed.

UV spectroscopy analysis:

The UV spectrum of Silymarin was obtained in ethanol and phosphate buffer saline (PBS 7.4) using Shimadzu UV-1601 UV spectrophotometer (Shimadzu crop. Kyoto, Japan) Sample were scanned over a wavelength range of (200-400mn**) [5].**

IR spectroscopy analysis:

Accurately weigh amount of KBr (0.3g) previously dried 250℃ for 1 hour, cooled and powdered. Silymarin 1-2 mg was added in to it mixed and grinded to a uniform mixture. Small quantity of mixture was taken and compressed in to a thin semi-transparent pellet by applying pressure. The IR spectrum of the pellet was recorded using FT/IR-4100 IR Spectrometer (Bruker) **[8].**

Analytical methodology:

Analytical methodology using UV spectrometer was employed for the purpose of quantification of Silymarin at various stages.

Preparation of calibration curve in ethanol

To prepare stock solution accurately weighed 10 mg of Silymarin was placed in 100 ml volumetric flask and ethanol was added to dissolve the drug and make up the volume. From this stock solution of 100µg/ml serial dilution were prepared in concentration range of 2-10µg/ml using the same solvent. The absorbance values at 284nm corresponding to each concentration were recorded. A graph plot between concentration (X-axis and (Y-axis) was plotted.

Preparation of calibration curve in Phosphate buffer saline (PBS) of pH 7.4

Calibration plot was constructed from 2-10µg/ml in PBS pH 7.4. To prepare stock solution accurately weighed 10 mg of Silymarin was placed in 100 ml volumetric flask and PBS 7.4 pH was added to dissolve the drug and make up the volume. From this stock solution of 100µg/ml serial dilution were prepared in concentration range of 2-10µg/ml using the same solvent. The absorbance values at 284nm corresponding to each concentration were recorded. A graph between concentration (X-axis and (Y- axis) was plotted **[7].**

Differential scanning calorimetry analysis:

The DSC thermogram of Drug was obtained. Nitrogen gas flow was 60 ml per minute. Sample were weighed about 1-2 mg, sealed in a aluminum pan of 40 µL capacity and equilibrated at 10℃, was subjected to the DSC run over the temperature range of 10 to 100℃ at a heating rate of 10℃. The temperature was calibrated using pure drug sample with a melting point of 225.℃ **[9].**

PRELIMINARY STUDIES

Selection of Lipid and Surfactant:

Solid Lipid was selected based on solubility of drug in lipid and surfactant was selected based on percentage transmission.

Formulation development:

Optimization of formulation is very necessary to obtain a desirable product. Along with concentration of Lipid, the method for formulation should also be optimized as it finally affects the formulation **[10].**

PREPARATION OF SOLID LIPID NANOPARTICLES

High Sheer Homogenization Method

Lipid Phase (100 mg stearic acid) was taken and 200 µl each oil, Peppermint oil, Eucalyptus and Lavender oils were also taken in the same Beaker and required amount of drug was also added in to it.

Aqueous Phase Surfactant was used as stabilizer 1 ml Tween 80 and 1 ml Co-Surfactant Labrasol was used when both the Phases prepared. Oil phase was added in to aqueous phase at homogenization speed 20000 rpm and sonicate the SLN to get desire Nano Size [10, 11].

DESIGN OF EXPERIMENTAL RESULTS OF SILYMARIN LOADED SLN NANOPARTICLES

Experimental design

In this study a 17 run, 3 factors, 3 level Box-Behnken design was employed to construct polynomial models for the optimization of process because it requires few runs with 3or 4 variables. This design was suitable for the investigating the quadratic response surface and for constricting second order polynomial using Design- Expert Software, the design consisted of replicated center point and a set of point lying at the mid points of edge of the multi-dimensional cube, which defined the region of interest used to evaluate the main effects, interaction effects, and quadratic effects of formulation ingredients, and to optimize the formulation. The linear quadratic model generated by the design **[12].**

Table: 1 Independent and dependent variables range lower to higher.

Factors	Coded Levels		
Independent variable	Low (-1)	Medium (0)	high(+1)
X1= Stearic Acid (mg)	50	500	1000
X2= Tween : Transcutol P (%)	1	2.5	5
X3=Sonication time (min) 25000	5000		12500
Dependent variables	Constraints		
Y1=Size (nm)	(100-400)		
Y2= Polydispersity Index (PDI)	Minimum		
Y3= Entrapment Efficiency	100%		

Particle size, polydispersity Index (PDI)

Solid Lipid Nanoparticles were characterized by Zetasizer ® (Nano ZS, Malvern Instrument, UK) with respect to size, polydispersity index (PDI) and. The zeta sizer was equipped with a red laser of wavelength λ0=633 nm (He-Ne, 4.0 MW). Particle and polydispersity index (PDI) were analyzed by photon correlation spectroscopy (PCS) Sample was diluted in MilliiQ- water to measure the size, polydispersity index ((PDI) **[13].**

Drug Encapsulation Efficiency (EE) and Loading Capacity (LC)

The fabricated Solid Lipid Nano particles was centrifuged in a cooling centrifuge at 15000 rpm at 4℃ for 15 min. The amount of Drug was calculated as the difference between the total amounts used to prepare the SLN and amount present in supernatant. Freeze dried Solid Lipid Nanoparticles ware used for determining drug loading capacity. SLN. (50 mg) were dissolved in distilled water by stirring for 24 h at 1000 rpm and ultrasonicated for 10 min at 60 magnitude and 0.8 cycles, this colloidal dispersion was filtered by 0.2µm membrane filter. The drug concentration was calculated by UV spectrophotometer by observing the absorbance at 288nm **[14]**.

$$\textbf{Entapment efficiency}(\textbf{EE}) = \frac{\textbf{Total drug}-\textbf{Free drug}}{\textbf{Total amount of drug}} \textbf{x 100} \quad \text{…. Equation}$$

$$\textbf{Loading Capacity } (\textbf{LC}) = \frac{\textbf{Total drug}-\textbf{free drug}}{\textbf{Nanoparticles weigh}} \textbf{x 100} \quad \text{……. ..Equation}$$

CHARACTERIZATION OF OPTIMIZED NANOPARTICLES

Particle Size and Morphology

The particle size of selected SLN formulation were observed using Transmission Electron Microscopy (TEM) Tecnai G2 S-twin, FEI, Netherland operating at 120 KV at the scale of 500nm which capable of point-to-point resolution. For preparing TEM samples a small amount of NPs were dispersed in phosphate buffer of pH 6.8. Samples dropped on to copper grids, dried at room temperature and examined. Images were captured and analyzed using Keen View FM and Olympus Soft Imaging Viewer respectively with 5000 X magnification **[11, 13]**.

IN VITRO DRUG RELEASE STUDIES OF SILYMARIN SOLID LIPID NANOPARTICLES

Dialysis membrane specification

The dialysis membrane used in the study was cellulose membrane (Sigma USA), tubing as such without treatment is stored at room temperature. Its capacity was 60ml/feet; average flat width was 23mm. its molecular weight cut of was 12000g/mol. Before the experiment the dialysis bag was treated as mentioned in the reference **[9]**

IN VITRO STUDIES

Dialysis bag technique

In vitro release of drug from dialysis membrane (Molecular weight cut-off 12000-14000 Da, sigma) was done in PBS pH 7.4, 37±2℃. 5ml of the Silymarin Loaded SLN and Silymarin Suspension to 5 ml was placed in dialysis bag sealed at both the end and then placed in 900 ml PBS maintained over magnetic stirrer at 100 rpm. 3 ml of sample was withdrawn at selected time interval and were replaced with fresh dissolution medium. Samples were then analyzed spectrophotometrically after suitable dilution at 288nm. Cumulative amount of drug release was calculated using equation **[15, 16]**

$$\textbf{\% Drug Release} = \frac{\textbf{Conc.}\left(\mu\frac{\textbf{g}}{\textbf{ml}}\right)\times\textbf{vlume of release medium(ml)x dilution factor}}{\textit{intial amount oof drug taken } \mu g} \boldsymbol{x}\ \textbf{100} \quad \text{… equation}$$

EX -VIVO PERMEATION STUDY

Wistar Rat skin was obtained from animal house. It was kept deep freeze -80℃ and then fatty layer was remove carefully. Rate skin was then stabilized in Franz diffusion cell (Logan instrument Corporation, NJ USA) containing phosphate buffer pH 6.4 in both donor and accepter compartment. Phosphate buffer from both the side was removed and then 3ml of SLN formulation and 3ml Silymarin suspended in PBS pH 6.4 as a controlled group containing drug equivalent was placed in donor compartment of capacity 5 ml and acceptor compartment capacity 11ml was filled with phosphate buffer pH 6.4. Drug was permeating through an area of 0.785cm^2. The 1 ml of samples from acceptor compartment was removed past appropriate time intervals and was replaced with equal volume of phosphate buffer pH 6.4 media to maintain sink condition. Samples were analyzed at 288nm using UV –visible spectrophotometer [17, 18].

Amount of drug permeated through wistar Rat skin 1calculated using following formula:

Amount of drug

$$\textbf{Permeated} = \frac{\textbf{Conc.}\left(\mu\frac{\textbf{g}}{\textbf{ml}}\right)\textbf{x dilution factorX Vol.of release meium (ml)}}{\textbf{Permeation area(cm2)}}$$ …… equation

RESULTS AND DISCUSSION

Physicochemical characterization of Silymarin drug.

Evaluation of Organoleptic properties

The organoleptic properties of Silymarin was studied and the results are shown in **[Table 2]**

Table 2. Organoleptic properties of Silymarin

S.No	Parameters	Inferences
1	Nature	Crystalline
2	Color	Brown powder
3	Odour	Odorless

Partition coefficient

Partition coefficient of Silymarin was performed and it was found to be 1.45 and 1.72. (Reported 1.5) Which shows that the drug is lipophilic in nature [Table 3].

Table 3. Partition coefficient.

Medium	Partition coefficient (log P)
n- octanol : distilled water	1.75
n- octanol : PBS 7.4	1.92
Partition coefficient is >1 Hence the drug is lipophilic	

Determination of Melting point

Melting point of Silymarin was found to be 225℃ by capillary method. The melting point was found to be in agreement with the previous reported data 228℃ [19].

Solubility determination

The result of solubility is shown in table 4 indicating that the drug is freely soluble in ethanol and soluble in phosphate buffer saline PH 7.4 [Table 4].

Table 4. Solubility studies of Silymarin in various solvents

Solvent system	Descriptive Term
Water	3.5
Ethanol	154
Phosphate Buffer 7.7 pH	43
Chloroform	57

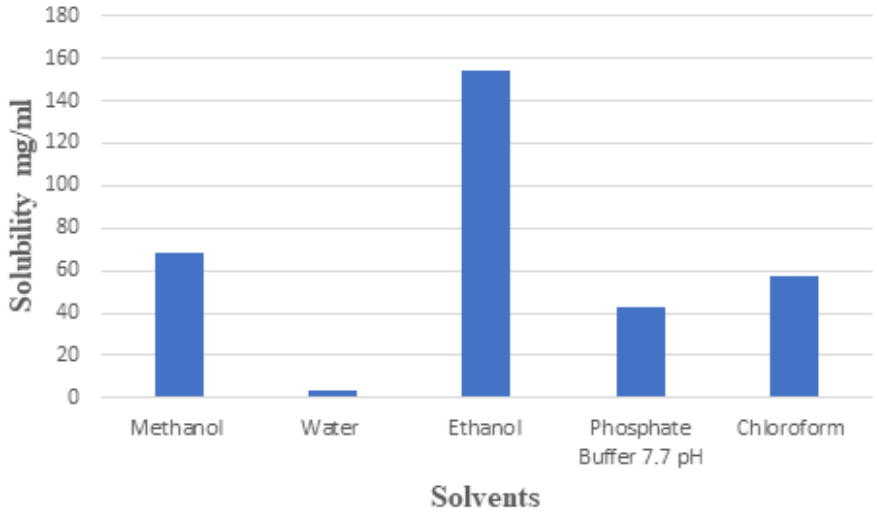


Figure 1. Solubility graph

IDENTIFICATION OF DRUG

UV spectroscopy

UV absorption spectra of stock solution (10µg/ml) were scanned for absorbance in the region of 400-200 nm **[Table 5]**

Table 5. UV absorption maxima of Silymarin.

Solvent system	**Maximum Wavelength (nm) (observed)**	**Maximum wavelength (nm) (reported)**
Ethanol	288nm	287nm
PBS of pH 7.4	288nm	287nm

Table 6. Calibration curve data of Silymarin in Ethanol at 288 nm

S.No.	**Concentration µg/ml**	**Absorbance µg/ml**
1	2	0.416
2	4	0.476
3	6	0.561
4	8	0.613
5	10	0.702

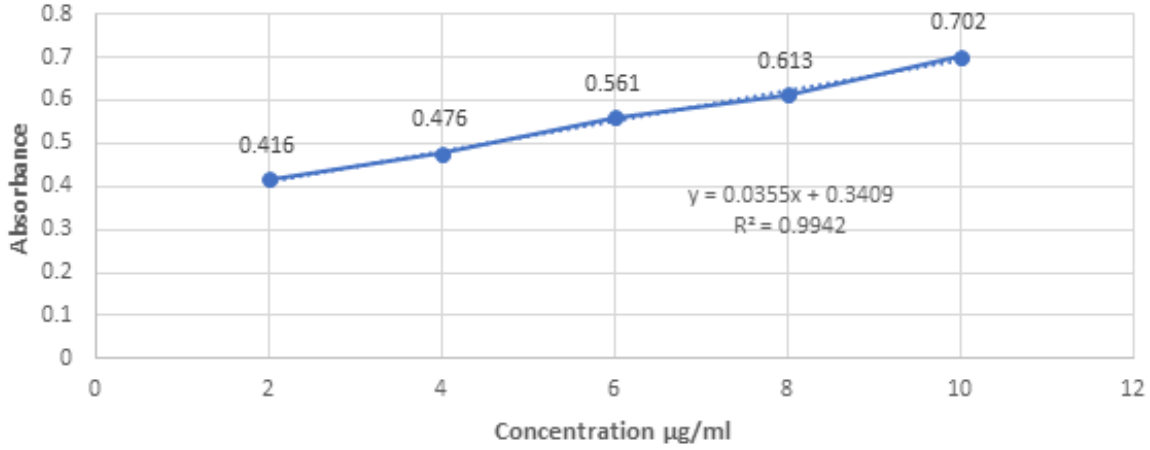


Figure 2. Calibration of Silymarin in Ethanol

IR Spectroscopy

The FTIR spectrum showing percentage transmission (T %) versus wave number of Silymarin in figure. It shows the main Peaks, indicated by the appearance of skeletal vibration in the fingerprint region, 1600 cm and 500 cm [20]. Silymarin API showed same FTIR Spectra as reference compound this indicates that using API is pure and authentic

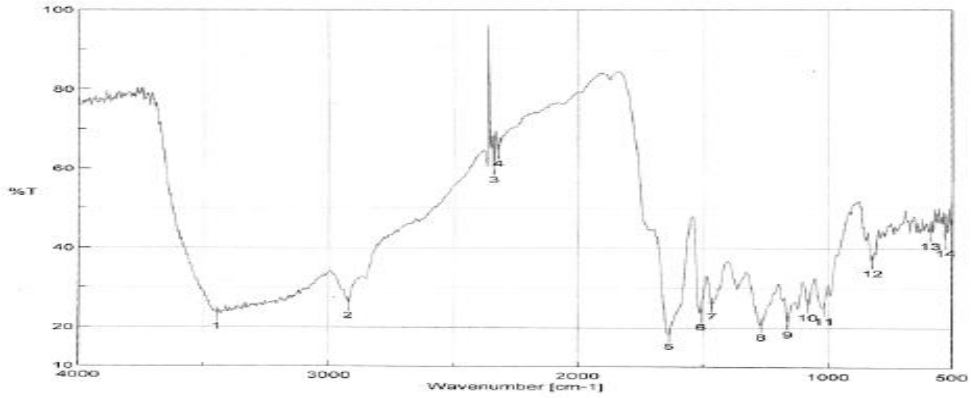


Figure 3. Scan of Sample Reference Compound

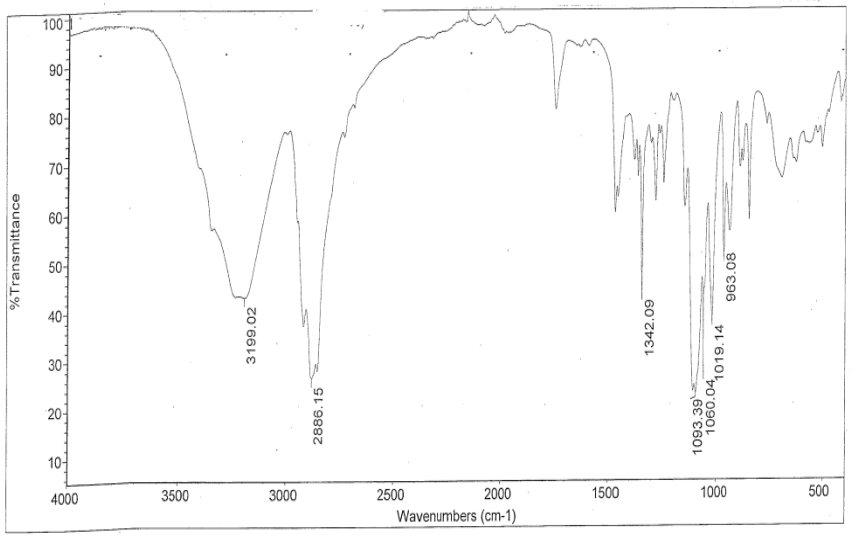


Figure 4. Scan of Sample Silymarin

Differential Scanning Calorimetry Analysis

Differential Scanning Calorimetry Analysis (DSC) of the drug was done to determine its physical state. The thermogram showed that Silymarin is crystalline substance with sharp endothermic peak at 225℃. It was found in agreement with reported data (228℃) [21].

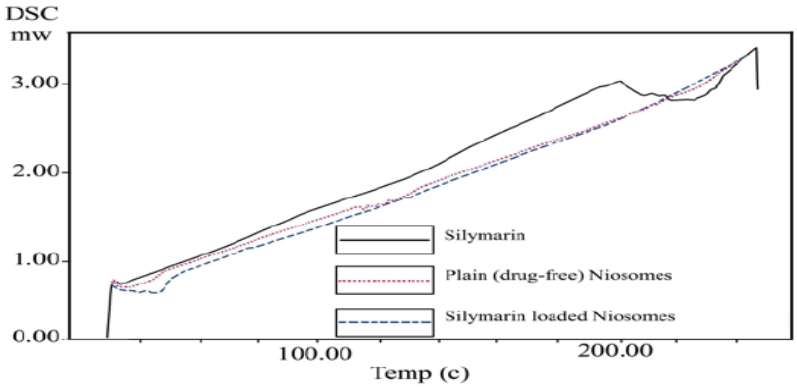


Figure 5. Differential Scanning Calorimetry Analysis for Reference compound

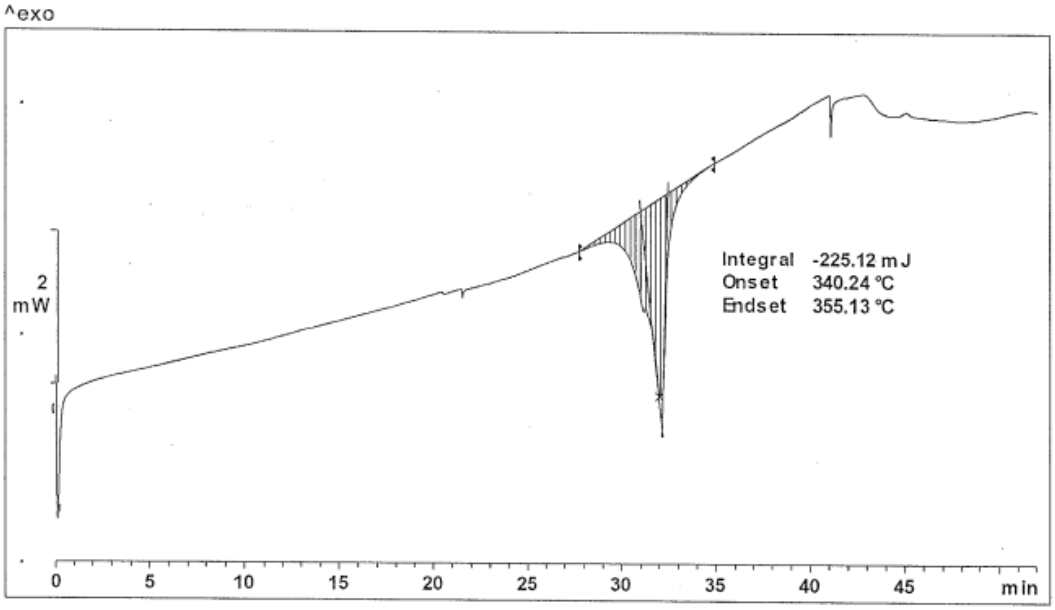


Figure 6. Differential Scanning Calorimetry Analysis for Reference compound

FORMULATION DEVELOPMENT

Optimization of nanoparticles

In this study a 17 run, 3 factors, 3 level Box-Behnken design was employed. This design was suitable for the investigating the quadratic response surface and for constricting second order polynomial using Design- Expert Software, the design consisted of replicated center point and a set of point lying at the mid points of edge of the multi-dimensional cube, which defined the region of interest used to evaluate the main effects, interaction effects, and quadratic effects of formulation ingredients, and to optimize the formulation. The linear quadratic model generated by the design **[22,23]**

Table 7. Independent and dependent variables range lower to higher.

Factors Coded Levels			
Independent variable	**Low (-1)**	**Medium (0)**	**high(+1)**
X1= Stearic Acid (mg)	**50**	**500**	**1000**
X2= Tween : Transcutol P (%)	**1**	**2.5**	**5**
X3=Sonication time (min)	**5000**	**12500**	**25000**
Dependent variables	**Constraints**		
Y1=Size (nm)	**(100-400)**		
Y2= Polydispersity Index (PDI)	**Minimum**		
Y3= Entrapment Efficiency	**100%**		

Particle Size analysis by Malvern Particle size Analyze

Particle size was found 124nm and Poly-dispersibility was found to be 0.452

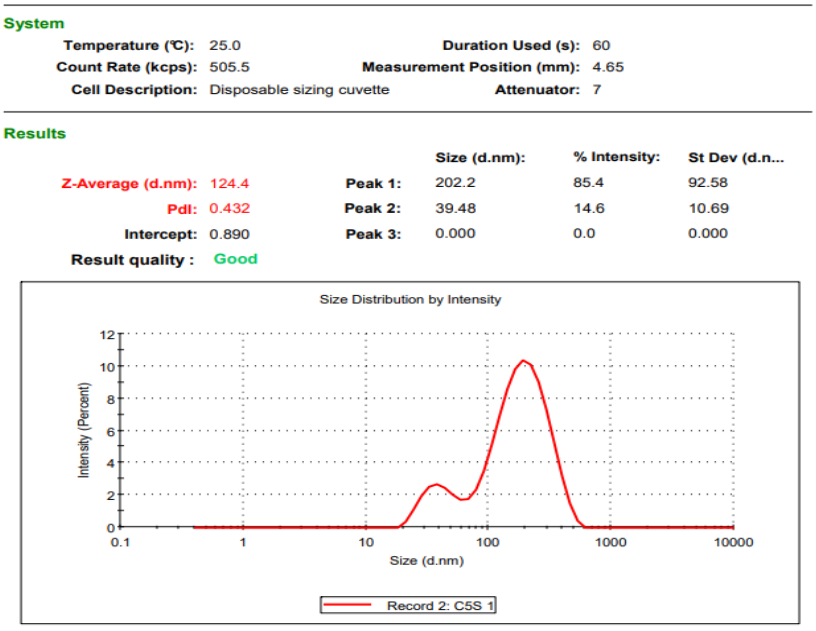


Figure 7. Particle size distribution study

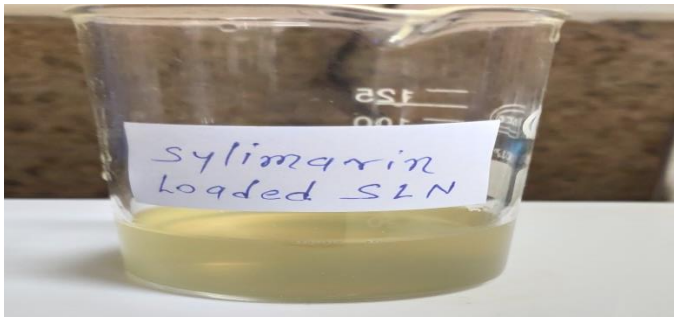


Figure 8. Silymarin loaded SLN

Figure 9. Formulation Optimization by Box-Behnken Design

File Edit View Display Options Design Tools Help

Change View | Round Columns | Augment Design | Hide/Show Columns

Navigation Pane

- Design (Actual)
- **Information**
- Notes
- Summary
- Graph Columns
- Evaluation
- Constraints
- **Analysis [+]**
- Particle Size
- PDI
- % EE
- **Optimization**
- Numerical
- Graphical

Design Properties

Run 1	
Comment	
Row Status	Normal

Std	Run	Factor 1 A:Stearic Acid mg	Factor 2 B:Tween 80 : Tra... %	Factor 3 C:Homogenizati... rpm	Response 1 Particle Size nm	Response 2 PDI	Response 3 % EE %
16	1	525	3	15000	124.4	0.242	81
7	2	50	3	25000	174.6	0.51	42
12	3	525	5	25000	150	0.323	87
2	4	1000	1	15000	177.8	0.295	63
10	5	525	5	5000	196	0.188	81
11	6	525	1	25000	124.4	0.242	56
8	7	1000	3	25000	163.7	0.556	96
4	8	1000	5	15000	178.9	0.282	70
17	9	525	3	15000	124.4	0.242	81
14	10	525	3	15000	124.4	0.242	81
1	11	50	1	15000	178.9	0.282	47
3	12	50	5	15000	178.9	0.482	44
15	13	525	3	15000	124.4	0.242	81
6	14	1000	3	5000	196	0.188	59
13	15	525	3	15000	124.4	0.242	81
5	16	50	3	5000	196	0.688	55
9	17	525	1	5000	196	0.388	62

Solid Lipid Nanoparticles were prepared according to the High Sheer Homogenization Method. To carry out the optimization of Solid Lipid Nanoparticles, first various concentration of drug solubility in solid lipid was performed. They were optimized for particle size, polydispersity Index (PDI) entrapment efficiency.

Statistical Analysis of Experimental Data by Design Expert Software

The results of experimental design were analyzed using Design-Expert software, which provided considerable useful information and reaffirmed the utility of statistical design for conducting experiments. The selected independent variables including Lipid, surfactant, and Homogenization speed significantly influenced the observed responses for particle size polydispersity index (PDI) entrapment efficiency [24]. Which are presented in Polynomial equations involving the main effect and interaction factors were determined based on estimation of statistical parameters such as multiple correlation coefficient, adjusted multiple coefficient correlation, and predicted residual sum of squares generated by Design-Expert Software. The statistical validation of the polynomial equation was established by ANOVA provision available in the Software

Fitting of Data of the Statistical Models

The response observed for 15 formulations were simultaneously fitted to linear, second order, Quadratic and Cubic models using Design-Expert Software. Among all, the best fit model suggested by Software was found to the Quadratic model.

Fit Summary

Response 1: Particle Size

	Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
	Linear	0.2545		0.0898	-0.1078	
	2FI	0.9790		-0.1618	-0.9007	
	Quadratic	**< 0.0001**		**0.9058**	**0.3405**	**Suggested**
	Cubic			1.0000		**Aliased**

ANOVA for Quadratic model

Response 1: Particle Size

	Source	Sum of Squares	df	Mean Square	F-value	p-value	
	Model	13895.79	9	1543.98	18.09	0.0005	significant
	A-Stearic Acid	18.00	1	18.00	0.2109	0.6600	
	B-Tween 80 : Trancutol P	89.11	1	89.11	1.04	0.3409	
	C-Homogenization Speed	3667.96	1	3667.96	42.98	0.0003	
	AB	0.3025	1	0.3025	0.0035	0.9542	
	AC	29.70	1	29.70	0.3480	0.5738	
	BC	163.84	1	163.84	1.92	0.2084	
	A^2	5187.41	1	5187.41	60.78	0.0001	
	B^2	1540.07	1	1540.07	18.04	0.0038	
	C^2	2241.92	1	2241.92	26.27	0.0014	
	Residual	597.44	7	85.35			
	Lack of Fit	597.44	3	199.15			
	Pure Error	0.0000	4	0.0000			
	Cor Total	14493.23	16				

Fit Summary

Response 2: PDI

	Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
	Linear	0.4758		-0.0226	-0.6227	
	2FI	0.1665		0.1815	-1.1437	
	Quadratic	**0.0030**		**0.8226**	**-0.2416**	**Suggested**
	Cubic			1.0000		**Aliased**

ANOVA for Quadratic model

Response 2: PDI

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.3051	9	0.0339	9.25	0.0039	significant
A-Stearic Acid	0.0514	1	0.0514	14.01	0.0072	
B-Tween 80 : Trancutol P	0.0006	1	0.0006	0.1576	0.7032	
C-Homogenization Speed	0.0040	1	0.0040	1.09	0.3307	
AB	0.0113	1	0.0113	3.09	0.1220	
AC	0.0745	1	0.0745	20.33	0.0028	
BC	0.0197	1	0.0197	5.38	0.0534	
A^2	0.0907	1	0.0907	24.73	0.0016	
B^2	0.0121	1	0.0121	3.29	0.1127	
C^2	0.0394	1	0.0394	10.75	0.0135	
Residual	0.0257	7	0.0037			
Lack of Fit	0.0257	3	0.0086			
Pure Error	0.0000	4	0.0000			
Cor Total	0.3307	16				

Fit Summary

Response 3: % EE

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0822		0.2515	-0.1020	
2FI	0.3645		0.2820	-0.5949	
Quadratic	**0.0065**		**0.8043**	**-0.3699**	**Suggested**
Cubic			1.0000		**Aliased**

ANOVA for Quadratic model

Response 3: % EE

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3935.38	9	437.26	8.31	0.0054	significant
A-Stearic Acid	1250.00	1	1250.00	23.74	0.0018	
B-Tween 80 : Trancutol P	364.50	1	364.50	6.92	0.0338	
C-Homogenization Speed	72.00	1	72.00	1.37	0.2805	
AB	25.00	1	25.00	0.4749	0.5129	
AC	625.00	1	625.00	11.87	0.0108	
BC	36.00	1	36.00	0.6839	0.4355	
A^2	1181.32	1	1181.32	22.44	0.0021	
B^2	286.58	1	286.58	5.44	0.0524	
C^2	6.58	1	6.58	0.1250	0.7341	
Residual	368.50	7	52.64			
Lack of Fit	368.50	3	122.83			
Pure Error	0.0000	4	0.0000			
Cor Total	4303.88	16				

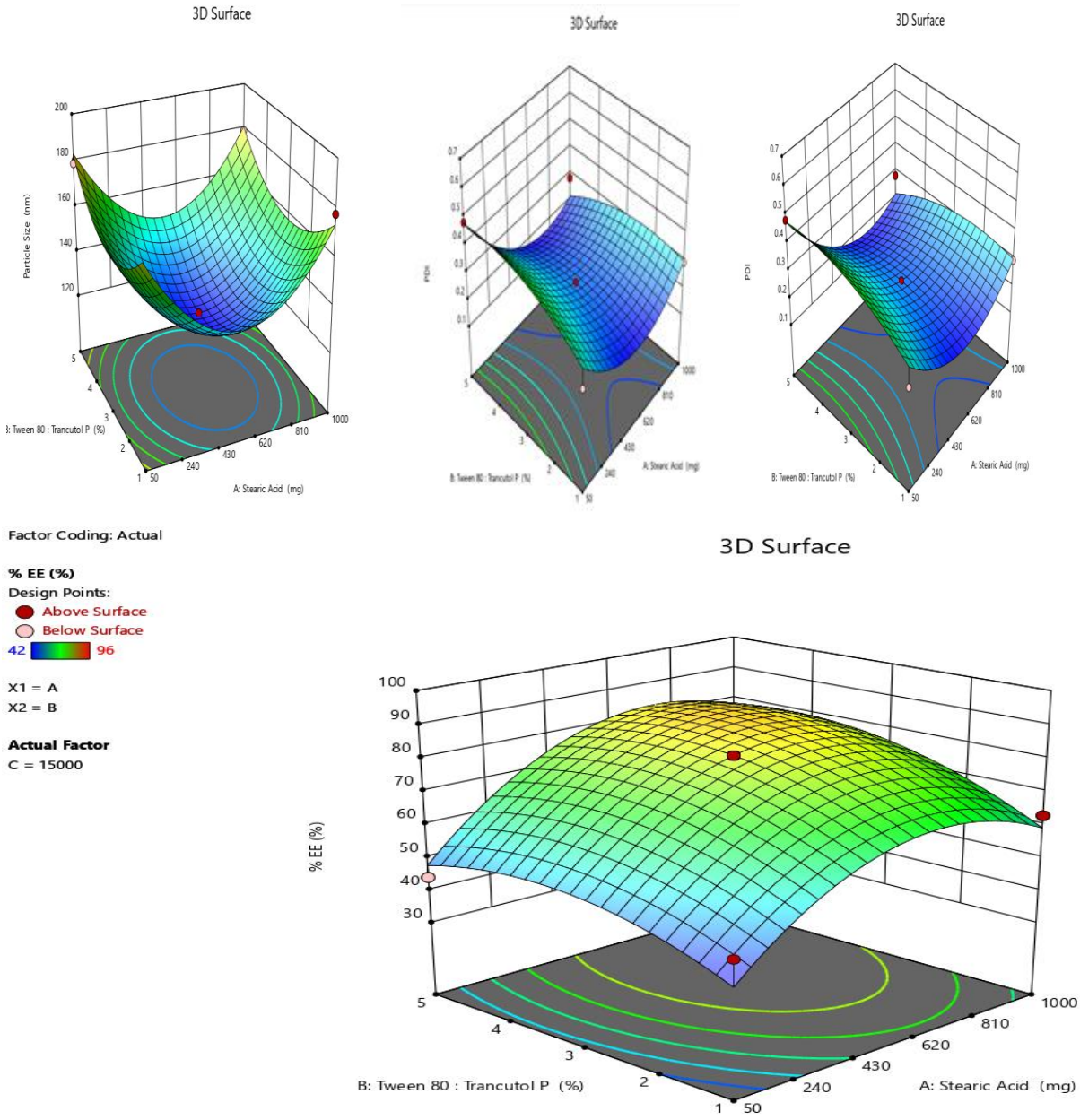


Figure 10. 3D Surface Diagram

QbD approach for optimization of Formulation

The first step in the QbD based optimization process is to establish Quality Target Product Profile (QTPP) of the final formulation. QTPP defines the critical quality attributes (CQA) i.e., the attributes that critically defines the quality standards of the final formulation [25]. Thus, after initial experimentation and literature search, the QTPP of lignin microparticles was established as given in Table 8.

Table 8. Quality Target Product Profile of final formulation

Quality Attribute (Range of Acceptance)	**Reason for criticality**
Particle size (µm)	For sustained drug delivery system
PDI (<0.3)	To ensure uniformity of size.
Drug Loading (<25.0%)	Dose minimization and patient compliance.

Initial risk assessment

This involves identification and assessment of various parameters that affects the critical quality attributes of the final formulation. These parameters involve both Critical Material Attributes (CMAs) and Critical Process

Parameters (CPPs). Both CMAs and CPPs affect greatly to the quality of the formulation, initial risk assessment of the intensity of their effect on the critical quality attributes is required to finalize the high risks parameters that can greatly affect the quality of the final formulation [26]. So, from the literature search and initial experimentation various CMAs and CPPs were identified and listed in table 9.

Table 9. Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs)

Critical Material Attributes (CMAs)	**Critical Process Parameters (CPPs)**
Silymarin concentration (mg/mL)	Amount of dialysis medium (mL
Drug concentration (mg/mL)	Dialysis time (h)

Table 10. Entrapment Efficiency& Drug Loading

% Entrapment Efficiency	**81%**
% Drug Loading	**16%**

Transmission Electron Microscopy Image

Particle size of SLN was found to be spherical and below 200 nm.

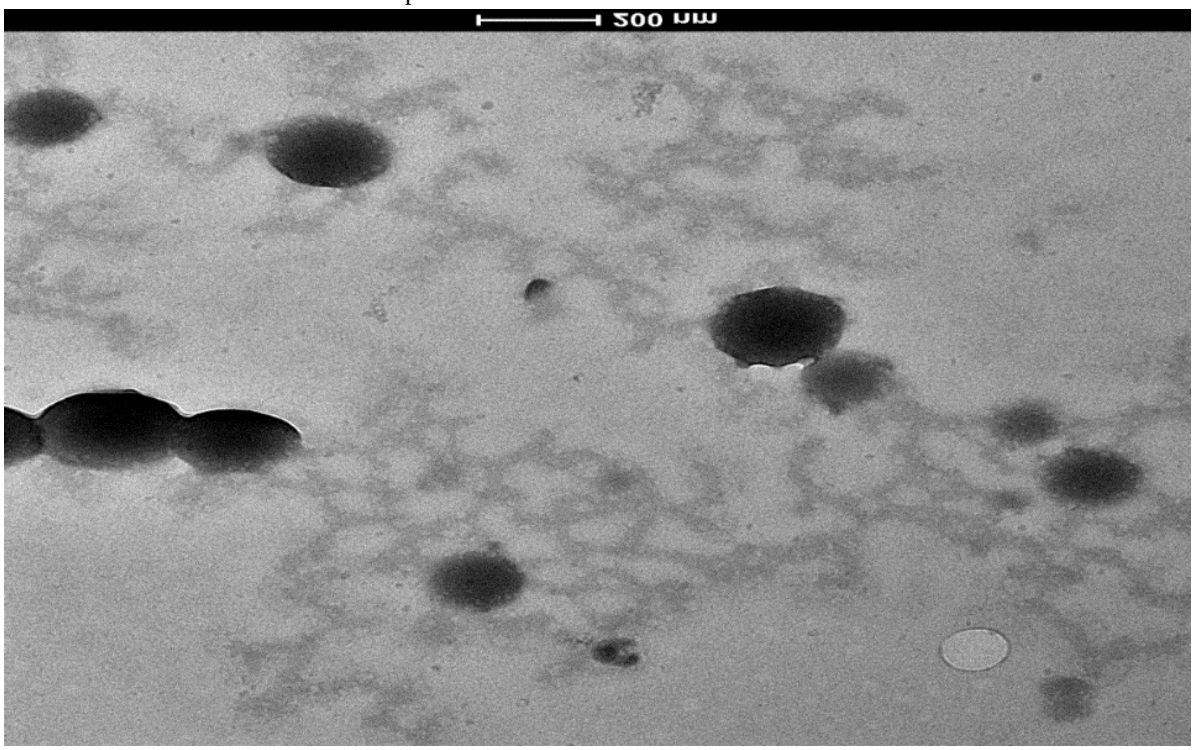


Figure 11. Particle size analysis of nanoparticles

In- vitro drug release and kinetics

In different time interval drug release kinetics study of formulation NPs1and Carmustine was performed in phosphate buffer (pH 7.4). Release of Carmustine from nanoparticles showed biphasic type it might be initial rapid release of Carmustine absorbed to the NPs, surface or weakly interacted with nanoparticles. This followed by slower sustained and continuous release of Carmustine stored in the core of nanoparticles.

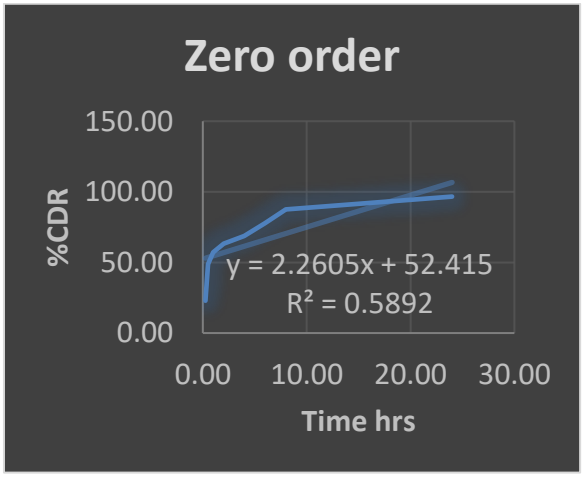
Drug release profile calculated the data via Zero order, First order, Higuchi, and Peppas Korsmeyer. Results show that the drug release followed Peppas Korsemeyer, and related correlation coefficients were superior to other kinetics [Table 13]

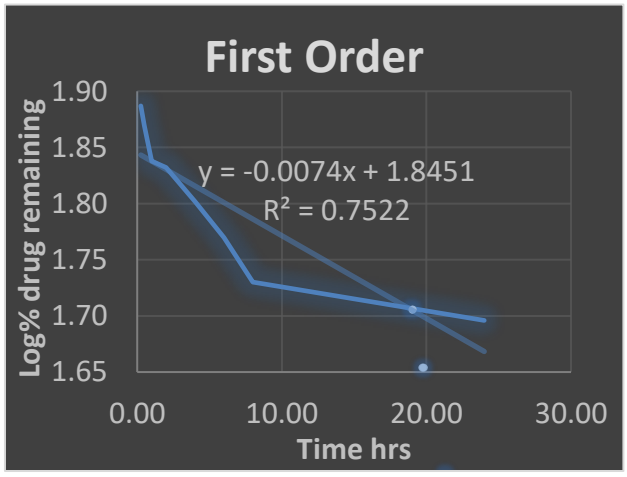
Table 11. In- Vitro drug release study (Silymarin Loaded SLN)

Time (hr.)	SQRT	Log time	ABS	Conc. µg/ml	%DR	%CDR	Log % CDR	% drug remaining	Cube root of %drug remaining	Log% drug remaining
0.25	0.50	-0.60	0.11	1.27	22.91	22.91	1.36	77.09	4.26	1.89
0.50	0.71	-0.30	0.17	1.44	25.96	48.87	1.41	74.04	4.20	1.87
1.00	1.00	0.00	0.24	1.65	29.66	55.61	1.47	70.34	4.13	1.85
2.00	1.41	0.30	0.28	1.75	31.53	61.19	1.50	68.47	4.09	1.84
4.00	2.00	0.60	0.35	1.95	35.13	66.67	1.55	64.87	4.02	1.81
6.00	2.45	0.78	0.36	1.98	35.59	70.72	1.55	64.41	4.01	1.81
8.00	2.83	0.90	0.37	2.01	36.15	71.74	1.56	63.85	4.00	1.81
24.00	4.90	1.38	0.40	2.09	37.67	73.82	1.58	62.33	3.96	1.79

Table 12. In- Vitro drug release study (Silymarin suspension)

Time (hr.)	SQRT	Log time	ABS	Conc. µg/ml	%DR	%CDR	Log % CDR	% drug remaining	Cube root of %drug remaining	Log% drug remaining
0.25	0.50	-0.60	0.11	1.27	22.91	22.91	1.36	77.09	4.26	1.89
0.50	0.71	-0.30	0.17	1.44	25.96	48.87	1.41	74.04	4.20	1.87
1.00	1.00	0.00	0.27	1.73	31.18	57.13	1.49	68.82	4.10	1.84
2.00	1.41	0.30	0.29	1.78	32.04	63.22	1.51	67.96	4.08	1.83
4.00	2.00	0.60	0.38	2.04	36.65	68.69	1.56	63.35	3.99	1.80
6.00	2.45	0.78	0.47	2.29	41.17	77.82	1.61	58.83	3.89	1.77
8.00	2.83	0.90	0.57	2.57	46.29	87.45	1.67	53.71	3.77	1.73
24.00	4.90	1.38	0.65	2.80	50.34	96.63	1.70	49.66	3.68	1.70





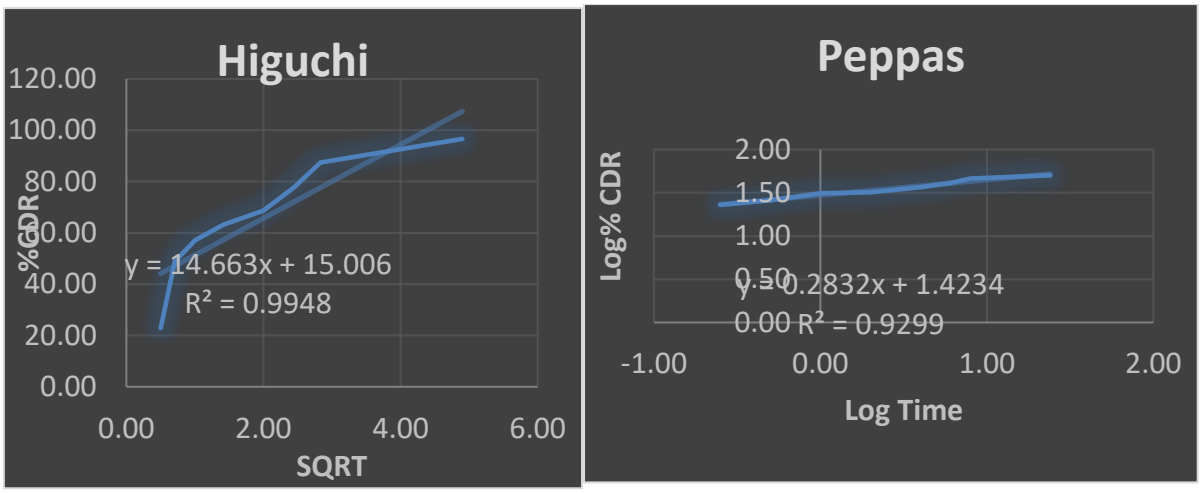


Figure 12. Graphs for the kinetic models are given below.

Table 13. Various kinetic models for optimized formulation Silymarin loaded SLN.

Formulation	Zero order (R^2)	First order (R^2)	Higuchi plot (R^2)	Peppas Korsemeyer (R^2)	Bet fit model
NPs1	0.5892	0.7522	0.9948	0.9299	**Higuchi plot**

The best fit model for the nanoparticles was found by **Higuchi model** showing that the drug released from the particle's diffusion mechanism.

Table14. Ex-Vivo Permeation study of Silymarin Loaded SLN

Silymarin Loaded SLN						
S.NO	**Time**	**ABS**	**Con.µg/ml**	**Amt. of Drug Permeated**	**%Permeability**	**Flux µg/cm2/h**
0	0	0	0	0	0	967.58
1	0.25	0.18	6.02	2057.58	20.58	
2	0.50	0.28	8.65	2957.26	29.57	
3	1.00	0.48	13.77	4708.95	47.09	
4	2.00	0.66	18.52	6331.08	63.31	
5	4.00	0.74	20.83	7122.81	71.23	
6	8.00	0.89	24.57	8400.36	84.00	

Table 15. Ex-Vivo Permeation study of Silymarin Loaded SLN

Silymarin Suspension						
S.No	**Time**	**ABS**	**Con.µg/ml**	**Amt. of Drug Permeated**	**%Permeability**	**Flux µg/cm2/h**
	0	0	0	0	0	610.8
1	0.3	0.1	4.0	1364.8	13.6	
2	0.5	0.2	6.8	2309.5	23.1	
3	1.0	0.3	9.3	3173.2	31.7	
4	2.0	0.3	9.8	3353.1	33.5	
5	4.0	0.4	12.3	4217.7	42.2	
6	8.0	0.6	15.7	5377.4	53.8	

Table 16. Comparative Ex-Vivo Permeation study of Silymarin Loaded SLN and Silymarin Suspension

Time	**%Permeability Silymarin Loaded SLN**	**%Permeability Silymarin Suspension**
0.25	0.0	0.0
0.50	20.6	13.6
1.00	29.6	23.1
2.00	47.1	31.7

4.00	63.3	33.5
8.00	71.2	42.2
0.25	84.04	53.74

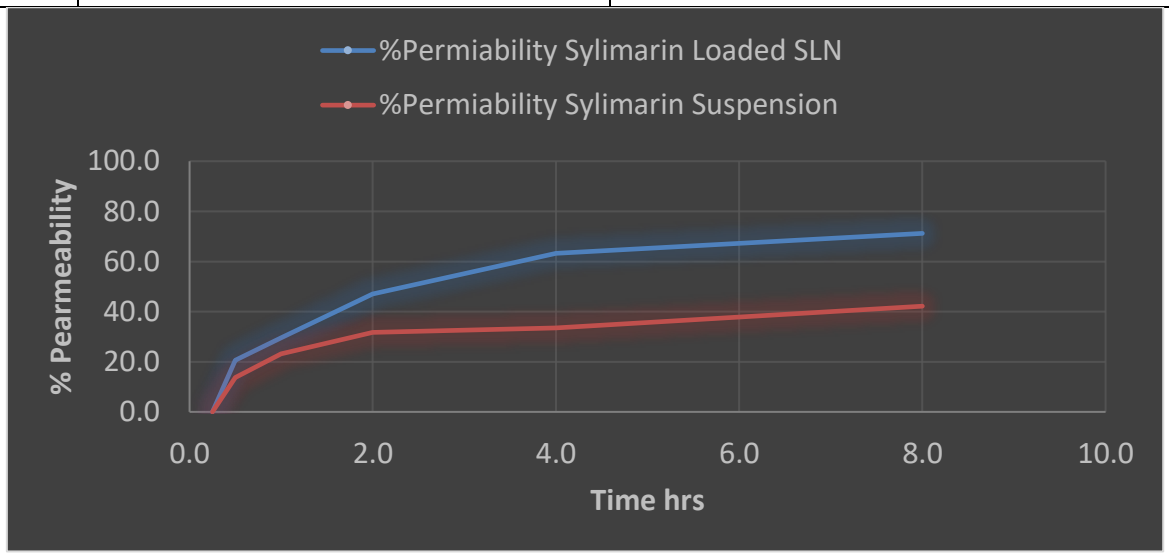


Figure 13. Comparative %Permeability Silymarin Loaded SLN and %Permeability Silymarin Suspension

The increased permeation of SLN formulation compared to Silymarin suspension is due to nano size of formulation.

CONCLUSION

Liver cirrhosis is a life-threatening disease all over the world. It is mainly caused by different reasons like infections, genetically, high alcohol consumption and non-alcoholic fatty liver. In this study, the main focus was to develop and characterize silymarin loaded Solid lipid nanoparticles for the treatment of psoriasis. Silymarin is an extract of *Silybum marianum*, or milk thistle, and its major active compound is silybin, which has a remarkable biological effect. It is majorly used in different liver disorders, particularly chronic liver diseases, cirrhosis, and hepatocellular carcinoma. Various analytical techniques *viz.* UV spectroscopy, DSC, FTIR etc were used to characterize the drug and prepared formulations. Box-Behnken design was employed to optimize the study. *In vitro* drug release study exhibited an enhanced drug release profile of solid lipid nanoparticles. Particle size analysis revealed the particle size of less than 200 nm. An increased permeation of SLN formulation compared to Silymarin suspension is due to nano size of formulation. Further in vivo and toxicity profile studies could be performed to explore the benefits of nanotechnology technique in the treatment of liver cirrhosis, psoriasis and other drug delivery systems.

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REFERENCES

1. Schuppan D, Afdhal NH. Liver cirrhosis. The Lancet. 2008 Mar 8;371(9615):838-51.
2. Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. The Lancet. 2014 May 17;383(9930):1749-61.
3. Pinzani M, Rosselli M, Zuckermann M. Liver cirrhosis. Best practice & research Clinical gastroenterology. 2011 Apr 1;25(2):281-90
4. Lingayat VJ, Zarekar NS, Shendge RS. Solid lipid nanoparticles: a review. Nanoscience and Nanotechnology Research. 2017 Apr;4(2):67-72.
5. Grattagliano I, Ubaldi E, Bonfrate L, Portincasa P. Management of liver cirrhosis between primary care and specialists. World journal of gastroenterology: WJG. 2011 May 14;17(18):2273.
6. Mehnert W, Mäder K. Solid lipid nanoparticles: production, characterization, and applications. Advanced drug delivery reviews. 2012 Dec 1;64:83-101.

7. Chaurasia G. A review on pharmaceutical preformulation studies in formulation and development of new drug molecules. International journal of pharmaceutical sciences and research. 2016 Jun 1;7(6):2313.
8. Chaurasia G. A review on pharmaceutical preformulation studies in formulation and development of new drug molecules. International journal of Pharmaceutical sciences and research. 2016 Jun 1;7(6):2313.
9. Lin CH, Chen CH, Lin ZC, Fang JY. Recent advances in oral delivery of drugs and bioactive natural products using solid lipid nanoparticles as the carriers. Journal of food and drug analysis. 2017 Apr 1;25(2):219-34.
10. Silva AC, Kumar A, Wild W, Ferreira D, Santos D, Forbes B. Long-term stability, biocompatibility, and oral delivery potential of risperidone-loaded solid lipid nanoparticles. International journal of pharmaceutics. 2012 Oct 15;436(1-2):798-805.
11. Zur Mühlen A, Schwarz C, Mehnert W. Solid lipid nanoparticles (SLN) for controlled drug delivery–drug release and release mechanism. European journal of pharmaceutics and biopharmaceutics. 1998 Mar 1;45(2):149-55.
12. Yadav N, Khatak S, Sara US. Solid lipid nanoparticles-a review. Int. J. Appl. Pharm. 2013;5(2):8-18.
13. Sanna V, Gavini E, Cossu M, Rassu G, Giunchedi P. Solid lipid nanoparticles (SLN) as carriers for the topical delivery of econazole nitrate: in-vitro characterization, ex-vivo and in-vivo studies. Journal of pharmacy and pharmacology. 2007 Aug;59(8):1057-64.
14. Sanna V, Gavini E, Cossu M, Rassu G, Giunchedi P. Solid lipid nanoparticles (SLN) as carriers for the topical delivery of econazole nitrate: in-vitro characterization, ex-vivo and in-vivo studies. Journal of pharmacy and pharmacology. 2007 Aug;59(8):1057-64.
15. Scioli Montoto S, Muraca G, Ruiz ME. Solid lipid nanoparticles for drug delivery: Pharmacological and biopharmaceutical aspects. Frontiers in Molecular Biosciences. 2020:319.
16. Cengiz M, Kutlu HM, Burukoglu DD, Ayhancı A. A comparative study on the therapeutic effects of silymarin and silymarin-loaded solid lipid nanoparticles on D-GaIN/TNF-α-induced liver damage in Balb/c mice. Food and Chemical Toxicology. 2015 Mar 1;77:93-100.
17. Naseri N, Valizadeh H, Zakeri-Milani P. Solid lipid nanoparticles and nanostructured lipid carriers: structure, preparation, and application. Advanced pharmaceutical bulletin. 2015 Sep;5(3):305.
18. Kong WH, Park K, Lee MY, Lee H, Sung DK, Hahn SK. Cationic solid lipid nanoparticles derived from apolipoprotein-free LDLs for target specific systemic treatment of liver fibrosis. Biomaterials. 2013 Jan 1;34(2):542-51.
19. Jawahar N, Meyyanathan SN, Reddy G, Sood S. Solid lipid nanoparticles for oral delivery of poorly soluble drugs. Journal of Pharmaceutical Sciences and Research. 2012 Jul 1;4(7):1848.
20. Gupta S, Kesarla R, Chotai N, Misra A, Omri A. Systematic approach for the formulation and optimization of solid lipid nanoparticles of efavirenz by high pressure homogenization using design of experiments for brain targeting and enhanced bioavailability. Biomed research international. 2017 Jan 23;2017.
21. Silva AC, Kumar A, Wild W, Ferreira D, Santos D, Forbes B. Long-term stability, biocompatibility, and oral delivery potential of risperidone-loaded solid lipid nanoparticles. International journal of pharmaceutics. 2012 Oct 15;436(1-2):798-805.
22. Jawahar N, Meyyanathan SN, Reddy G, Sood S. Solid lipid nanoparticles for oral delivery of poorly soluble drugs. Journal of Pharmaceutical Sciences and Research. 2012 Jul 1;4(7):1848.
23. Ferreira SC, Bruns RE, Ferreira HS, Matos GD, David JM, Brandão GC, da Silva EP, Portugal LA, Dos Reis PS, Souza AS, Dos Santos WN. Box-Behnken design: an alternative for the optimization of analytical methods. Analytica chimica acta. 2007 Aug 10;597(2):179-86.
24. Aslan NE, Cebeci YA. Application of Box–Behnken design and response surface methodology for modeling of some Turkish coals. Fuel. 2007 Jan 1;86(1-2):90-7.
25. Muthukumar M, Mohan D, Rajendran M. Optimization of mix proportions of mineral aggregates using Box Behnken design of experiments. Cement and Concrete Composites. 2003 Oct 1;25(7):751-8.
26. Bhaskar K, Anbu J, Ravichandiran V, Venkateswarlu V, Rao YM. Lipid nanoparticles for transdermal delivery of flurbiprofen: formulation, in vitro, ex vivo and in vivo studies. Lipids in health and disease. 2009 Dec;8(1):1-5.