Nanotechnology Perceptions
ISSN 1660-6795
www.nano-ntp.com

Quality-By-Design (Qbd) Approach To Fabricate Methotrexate-Loaded Lyotropic Liquid Crystal-Based Gel For Breast Cancer

Swapnil S Chopade [1,2], Dr. Sachinkumar V. Patil [3]*

[1,2]Research Scholar, Ashokrao Mane College of Pharmacy, Peth-Vadgaon 416112, Maharashtra, India.
[2]Assistant Professor, Tatyasaheb Kore College of Pharmacy, Warananagar 416113, Maharashtra, India.
[3] Principal & Professor, Dr. Ashok Gujar Institute of Pharmacy, Karad: 415110, Maharashtra, India.
Dr. Sachinkumar V. Patil
Principal & Professor, Dr. Ashok Gujar Institute of Pharmacy, Banawadi Ext, Vidhyanagar-Karad: 415110, Maharashtra, India. Email id: sachinpatil.krd@gmail.com

Methotreaxte (MTX) is a type of chemotherapy, BCS class-II drug having poor aqueous solubility, inadequate oral bioavailability along with gastrointestinal side effects. Thus, the novelty behind this research work was to develop a novel topical gel loaded with MTX-LLCs prepared with top-down method and optimized using Box-behnken design. The optimized formulation was then converted into gelusing carbopol as gelling agent. Herein, MTX-LLCs were prepared using PLGA (X_1), Pluronic F-127: PVA (X_2) and Sonication time (X_3) as independent variables, and PS (Y_1) and EE (Y_2) are dependent variables. The optimized formulation had satisfactory PS (181.8) and, % EE (68.58 %). Furthermore, the gel was analyzed for differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FTIR) and percent drug release (81.87%). The formulated gel were examined physically and tested for viscosity, homogeneity, and stability criteria; the findings got satisfactory results. This study has demonstrated the considerable promise of an innovative medication for treating breast cancer through a topical route, which overcomes the limitations of an oral route and provides a sustained, targeted release of the drug free from accumulation and toxicity.

Keywords: Breast cancer, Box-Behnken design, Top-down method.Ex-vivo permeation study

Introduction

Breast cancer (BC) is the most prevalent malignancy diagnosed in women and the second most common cause of mortality, accounting for one-fourth of all female cancers globally [1]. The global burden of breast cancer highlights the need for ongoing research into its pathogenesis

and treatments [2]. Genes, environmental factors, and lifestyle choices all contribute to the risk of developing breast cancer (BC). There are different types of the disease, including hormone receptor-positive (HR+), human epidermal growth factor receptor 2-positive (HER2+), and triple-negative breast cancer (TNBC). Each has a different outlook and reaction to treatment. Unique receptor profiles identify these groups, aiding doctors in treatment decisions and future prediction [3].

According to a study from GLOBOCAN 2020, there were 19.3 million new cases of cancer in 2020, and by 2040, there will be 28.4 million cases, a 47% increase from 2020. Although BC is commonly associated with women, it can also affect men. A precise and prompt diagnosis, together with efficacious therapy, is crucial for maximizing survival prospects. Standard treatment includes locoregional and systemic medicines, with chemotherapy being a primary option. The principal treatment techniques for BC are chemotherapy, radiation therapy, and surgical intervention [4].

Methotrexate (MTX) is a chemotherapeutic agent commonly used to treat certain types of cancer such as breast, lung, and bladder tumors. However, MTX has represented a significant potential against breast cancer [5], and it is an FDA-approved folic acid antagonist, {(2 S)–2-[[4-[(2,4-diaminopteridin-6-yl)-methyl ethyl amino] benzoyl] amino] pentanedioic acid. MTX acts as a competitive inhibitor of dihydrofolate reductase, necessary for DNA synthesis. MTX has exhibited excellent anti-cancer activity in the cells that over express folate receptors. MTX has some problems, such as a low permeability (Clog P = 0.53), a low solubility (0.01 mg/mL), a short plasma half-life (~2–10 h), and a high level of non-specificity [5-6].

Transitioning from the oral route to the topical route for dermatological conditions not only improves the medication's bioavailability but also inhibits systemic circulation by preserving a greater quantity of the drug inside the dermis and epidermis. The topical route enhances the administration of highly lipophilic medicines and facilitates focused treatment for many dermatological conditions [7].

Recent studies have found that nanotechnology-based strategies, such as nano- or microparticles, enhance the pharmacokinetic behaviour of drugs in cancer therapy [8]. Lipid-based self-assembled nanoparticles provide an excellent platform for encapsulating both hydrophobic and hydrophilic drugs. Lytotropic liquid crystals (LLCs) are one type of lipid nanocarrier that has gotten a lot of attention because they work really well for topical delivery and let active molecules get deeper into the skin [9].

Lipid nanocarriers systems have been found to be a suitable choice for topical delivery due to their increased resemblance to the skin, and enhanced penetration by polarity and fluidization [RP]. LLCs systems, are thermodynamically stable systems of amphiphilic molecules with polar solvents. Additionally, they exhibit fluidity, highly ordered structural alignment [9], and distinctive characteristics, including limitless swelling, prolonged drug release, and protection of the drug against physical and enzymatic destruction [9-10]. PLGA Poly (lactic-co-glycolic acid) Polymer, Resomer RG 752Hand Pluronic F-127 combine to form LLCs in a surplus aqueous medium. The former refers to the self-assembling biocompatible lipid, while the latter denotes the often-used surfactant in the formulation of LLCs. The scattered phase incorporates surfactants at concentrations up to 20%. LLCsenhance the biopharmaceutical properties of cutaneous medication delivery [10].

Materials and methods

Materials

Methotrexate (MTX) was obtained from Ramdev Chemicals, Pvt. Ltd. Boisar, Pune, as a gift sample. PLGA Poly (lactic-co-glycolic acid) Polymer, Resomer RG 752H (lactide: glycolide: 75:25, inherent viscosity 0.14-0.22 dL/g, Mw = 4000-15,000) was obtained as a gift sample from Evonik Industries, Mumbai). Methylparaben, Propylparaben, Propylene glycol, Pluronic F-127, Triethanolamine (TEA), and Dimethyl sulphoxide (DMSO) was procured from fine chemical Ltd., Mumbai. Carbopol 940 was kindly provided by Lubrizol Corporation, Mumbai. The study was conducted with analytical-grade materials and reagents.

Screening and optimization using DoE

Selection of CPPs and CQAs by BBD

To achieve desired results from formulations, we implement a quality by design (QbD) approach. With the aid of Design-Expert® stat 13.0 DoE ++ software, QbD can be used to examine the critical process parameters (CPPs) and critical quality attributes (CQAs). Using a response surface methodology, the optimization principle was used to develop and analyse second-order polynomial models[11].

Optimization of CQAs using BBD

A three-factor, three-level Box Behnken design (BBD) was used according to the initial research findings and the literature works available, in which independent variables (factors) are PLGA (X_1), Pluronic F-127: PVA (X_2) and Sonication time (X_3). These factors were varied at 3 levels; high (+1), medium (0) and low (-1) were obtained as critical factors affecting on PS (Y_1) and EE% (Y_2) of MTX-LLCs. A total number of 15 runs were generated by the Design-Expert®stat 13.0 DoE ++ software (Table 1)[12]

1**.** The BBD statistical (experimental) design displays the polynomial equation is as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_{21} + b_{22}X_{22} + b_{33}X_{23}$$

Where Y is the dependent variable (response), b_0 is the intercept, b_1 to b_{33} are the coefficients of regression derived from the experimental runs of the determined experimental values of Y; X_1, X_2, and X_3 are the independent variables (factors) chosen from the preliminary screenings.

$X_1 = (A-X_0)/\Delta X$; X_1 = Coded value of the variables A; X_0 = Value of A at the centre point; ΔX = Step change and so on where A, B, etc., are the input or independent variables.

Table 1: Coded matrix of formulation variables as per BBD for MTX-LLCs

Run order	Independent Variables (X_1)	(X_2)	(X_3)	Dependent Variables (Y_1)	(Y_2)
1	1	0	1	309	44.36
2	-1	0	-1	354	39.16
3	0	0	0	253	51.25

4	-1	0	1	367	37.56
5	-1	-1	0	402	37.61
6	0	-1	-1	91.21	26.5
7	0	0	0	253	51.25
8	**1**	**0**	**-1**	**181.8**	**68.58**
9	0	1	1	240	59.11
10	1	1	0	310	42.36
11	0	1	-1	224	65.21
12	0	0	0	253	51.25
13	-1	1	0	510	30.26
14	0	-1	1	392	35.56
15	1	-1	0	484.01	35.1

Independent variables (Factors)	**Levels**		
	Low	**Medium**	**High**
X_1= PLGA (mg)	100	200	300
X_2= Pluronic F127: PVA (%)	2	4	6
X_3= Sonication time (min)	3	5	7
Dependent variables (Response)			
Y_1= Particle size	Minimum		
Y_2= Entrapment efficiency	Maximum		

Data are expressed as the mean ± S.D. (n=3).

Data analysis

The optimized formulation was; calculated using the Design-Expert® stat 13.0 DoE ++ software. The polynomial equation was developed for the responses of PS and EE with the independent variables. The suitable level (concentration) (X_1, X_2, and X_3) of independent variables plays a crucial role in achieving the minimum PS and maximum % EE of MTX-LLCs and optimization as well[12-13].

Fabrication of drug-loaded MTX-LLCs

The MTX- MTX-LLCswere prepared using atop-down method with slight changes. In brief, PLGA (50 mg) and MTX (5 mg) were separately dissolved on ultrasonic bath for 15 minutes in 5 mL of organic solvents (acetone, dichloromethane [DCM]), and a mixture of acetone and DCM). The mixtures of Pluronic F-127 and PVA at different ratio were prepared in water. For emulsification, the organic phase was added dropwise into the aqueous solution of PVA. Further, the dispersion was sonicated (1-5 min) with ultra-probe sonication (60 W/cm^3, Hielscher, Ultra-sonics, Germany). A magnetic stirrer was used to stir the formulation at 1500 rpm for 6 hours. Finally, the resulting mixture was agitated for 24 h at 25^0C±2^0C to ensure that the organic solvent was completely evaporated. The prepared LLCs were centrifuged at 40°C for 20 minutes at 15,000 rpm (Remi, Mumbai, India). LLCs were separated and lyophilized using cryoprotectant (PEG 0.2%) and stored for further study [14].

Characterization of drug-loaded MTX-LLCs

The prepared drug-loaded MTX-LLCs were analysed for their particle size distribution, zeta potential, and entrapment efficiency.

Particle size, polydispersity index (PDI), and zeta potential

The mean particle size (z-ave), PDI and zeta potential were analysed by dynamic light scattering method using Malvern zeta sizer, ZEM5002 (Ver. 6.20 Malvern Instruments Ltd.) at room temperature (27±0.5°C). Prior to the study 1 mL of drug-loaded MTX-LLCs was diluted ten-fold (10 mL) with double distilled water to a suitable scattering intensity, and average particle size, PDI and zeta potential was measured [15].

Entrapment efficiency (EE) and drug loading capacity (DLC)

The % EE and DLC were determined by analysing free drug which is not entrapped in LLCs. The drug-loaded MTX-LLCs (10 mL) was taken with the help of pipette, and transferred it into a centrifuge tube and centrifuged at 15000 rpm for 20 min at room temperature (REMI R8C), the lipid portion was isolated, and the absorbance of the drug in the supernatant was determined spectroscopically using UV-vis spectrophotometer (Carry 60, 2100, Agilent Technology, Germany) at 260 nm. The concentration of drug was calculated from the calibration curve. The % EE and %DL was calculated by using the following equation: [16-17].

$$\text{EE}\ (\%) = \frac{\text{Total drug content} - \text{free drug content}}{\text{Total drug content}} \times 100$$

$$\text{DL}\ (\%) = \frac{\text{Total drug content} - \text{free drug content}}{\text{Total weight of LLCs used}} \times 100$$

Preparation of MTX-LLCs-loaded gel complex

To prepare gel-based network systems, 30 mg of prepared and optimised MTX-LLCs was mixed with 1 to 2% w/v carbopol 940P and stored in a cool and dark place for 24 hours with continuous magnetic stirring at 100 rpm. The mixture of methyl and propyl paraben (0.03% w/w) in a 2:1 ratio added in propylene glycol to the aforesaid dispersion. To obtain a yellowish transparent gel, the pH of the produced gel was neutralised with 1.0 mL triethanolamine before being agitated again. In addition, the gel dispersion was stirred at 12000 rpm for 15 minutes to form a uniform gel[18].

Characterization of prepared MTX-LLCs loaded gel

Homogeneity and pH

The homogeneity of the prepared MTX-LLCs loaded gel was checked visually after settling in a small container. The pH was measured in triplicate at room temperature (25^0C) using a digital pH meter (METTLER TOLEDO S400). To avoid irritation, the pH of the gel should be as close to the skin's pH as possible[19-20].

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of pure drug MTX, PLGA, Pluronic F-127, PVA, Carbopol 940, optimized MTX-LLCs and MTX-LLCs-loaded gel were analyzed. The spectra were obtained and interpreted [21].

Spreadability

The spreadability of MTX-LLCs loaded gel (0.5 g) was evaluated by striking a 1cm diametric circle strike on a glass plate. It was covered by a second glass plate weighing 250 g that was allowed to rest above for 1 minute. The diameter was extended due to the spreading of gel[21-22].

Rheological study measurements

A Brookfield RS/-CPS rheometer (Cone plate model, instrument) with a spindle C-75 was used to determine the viscosity of the formulation, with the results analysed using the software Rheo 3000 V1.2. With the help of a spatula, a small amount of sample was inserted on the bottom plate, and the spindle was started. The tests were conducted at room temperature (25°C). All the measurements were taken three times and the average value was reported [23].

In vitro drug release study

The release kinetics of pure MTX from optimized MTX-LLCs, MTX-LLCs loaded gel, and pure drug was investigated in phosphate buffer (PBS) pH 7.4 using a cellophane dialysis membrane (molecular weight cut off 8k Da) with the help of Franz diffusion cell. Briefly, in between donor and receptor compartments of the Franz diffusion cell, a cellophane dialysis membrane was arranged (previously soaked for 24 hours in PBS pH 7.4). The prepared formulations of optimized MTX-LLCs(1mL), MTX-LLCs loaded gel(1mL), and pure drug (10 mg) was added in the different setup to the receptor compartment containing PBS pH 7.4 (30mL) and maintained at 37±0.5˚Cwith 50 rpm. Thereafter a fixed time (0.5, 1, 2, and 3…….24 hours), 1mL of the sample was extracted from the receptor compartment and the same amount of freshly prepared PBS pH 7.4 was replaced. Finally, the collected samples were analyzed by using the UV-Vis spectrophotometer at 260 nm, and the % MTX release was measured and reported [24].

Ex vivo permeation study

The ex vivo permeation study was conducted on goat ear pinna skin. The freshly sacrificed animal goat ear pinna skin was purchased from the nearest slaughterhouse.The non-dermatome skin was removed using a scalpel after the skin had been cleaned with cold water. The MTX-LLCs loaded gel and marketed formulation Nanofast@ were evaluated on a Franz diffusion cell with an effective sectional area of 3.14 cm^2 and a receiver (receptor) compartment capacity of 30 mL, and the temperature was kept at 37±0.5˚C. In brief, the goat ear pinna skin was arranged between the donor and receiver (receptor) compartments. The receiving compartment was then filled with isotonic PBS pH 7.4. The formulation was added to the top surface of the goat ear pinna skin. The magnetic bead was put and constantly stirred at 50 rpm in the receptor compartment. An aliquot (1 mL) of the sample was taken at the regular time intervals of 1, 2, 3, 4……12 h, and an equivalent quantity of volume was replaced with fresh PBS pH 7.4. Finally, the absorbance of the collected samples was analysed at 260 nm using UV-vis

spectrophotometer. Additionally, % drug permeation with Steady-state (Jss) for ear pinna skin of goat and permeability coefficient (Kp), were determined by using Eqn. (1) and (2) respectively [26-27]

$$Jss = \frac{Kp}{Co} \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$Kp = \frac{Jss}{Co} \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Stability study

The stability study of the prepared and optimized MTX-LLCs and MTX-LLCs-loaded gel formulations was performed as per the ICH Q1A (R2) guidelines. For a period of three months, each formulation was kept at a different temperature of 25±2°C/60±5% RH and 40±2°C/75±5%. The samples were taken out after 1, 2, and 3 months, respectively and physical appearance, PS, and EE were analysed [28].

Statistical analysis

BBD was used for statistical analysis and Design-Expert® stat 13.0 DoE ++ software was used to perform multiple regression analysis on the 15 batches. All data was illustrated by the mean ± standard deviation (SD). At $p > 0.05$, the values were considered significant [29-30].

Results and Discussion

Screening and optimization of process parameters using DoE

Selection of CPPs and CQAs by BBD

The numeric factors were screened using the Design Expert stat. Ver. 13.0 software. Various independent variables (factors) such as drug (MTX), polymer (PLGA), surfactant (Pluronic F-127), co-surfactant (PVA), and process parameters (sonication time, temp., and speed) were tested. Among various factors, PLGA, Pluronic F127: PVA and sonication time were found to be a significant effect on PS and EE% [30].

Optimization of CQAs using BBD

The optimization of selected numeric factors (PLGA, Pluronic F127: PVA, and sonication time) from BBD was applied. The outcomes tabulated in Table 1. Following the polynomial equation in terms of coded values for a response was procured by the software.The positive impact of X_1 suggests an increase in the concentration of PLGA and has a directly proportional relationship with PS, and the same coefficient is observed with X_2, clearly indicating that the individual effects of X_1 and X_2 have a positive impact. However, in the case of X_3, it has a positive effect on PS at a certain time level then it changes.

Likewise, the interaction effect and the quadratic effect of X_1, X_2, and X_3 on PS showed a positive impact and the interaction effect of X1X3 and the quadratic effect of X2.

Particle size (Y_1) = $+173.82i+22.88A+55.60B+24.58C+55.25AB-82.26BC-30.50AC +107.02A^2+84.07B^2-78.06C^2$

The positive (sign) coefficient of X_1 indicates that a rise in PLGA will lead to an increase in EE, and the same coefficient is seen for X_2, demonstrating that each factor's positive influence on the response. In the case of X_3, it has a detrimental impact on EE. The interaction effect

and quadratic effect of X_1 and X_2 on EE also showed a positive coefficient for the effects of X_1, X_2, and the quadratic effect of X_3, respectively. However, X_3 indicates a negative impact on EE.

Entrapment efficiency (Y_2) = +62.34i+1.51A-1.09B-24.58C-5.72AB-2.90BC+6.15AC-10.79 A^2-16.46 B^2+12.54 C^2

Data analysis

The MTX-LLCs produced by all of the batches were assessed for PS and EE after being prepared in accordance with the BBD experimental runs. Table1 displays the conversion values for each batch. All chosen dependent variables (response) obtained at various levels of X_1, X_2, and X_3 were subjected to multiple regression to produce a second polynomial equation [31-32].

ANOVA, pure error, and lack of fit

The results of ANOVA demonstrate that the model is significant for all dependent variables [Table2&3]. Regression analysis was carried out to determine the regression coefficient, and all the independent variables were found to be significant for all response variables. The quadratic model was found to be significant for Y_1 and Y_2. So, the above results indicate that both factors are crucial in the formulation containing drug-loaded MTX-LLCs.

The significance of the model for each response variable was revealed by the ANOVA for the dependent variables. PVA was observed to be significant along with its quadratic and interaction terms for all the dependent variables, showing effects similar to the PLGA and Pluronic F127. Therefore, the aforementioned findings suggest that all independent variables were crucial, and that the ideal concentration in the formulation of LLCs produced the ideal PS and EE. The model is significant in terms of unfitness because the computed F-value was lower than the critical F-values.

The three replicated centre points in the Box-Behnken statistical design allowed the pure error of the tests to be assessed and the models to be examined for lack of fit. In this work, the model was checked for lack of fit for all the responses [33]. The mean square against estimates of the experimental error was used to determine the statistical significance of each effect. It was observed that X_1, X_2, and X_3 with their interaction effects other than X_1X_2 and quadratic effects had a P-value less than 0.050, indicating that the model has a significant impact on the variables in the prediction of X, as opposed to linear effect X_1 interaction effect X_1X_3 and quadratic effect of X_{22}, X_{33}, which indicates a significant impact of this variable in the prediction of response Y_2. The standard error shows the coefficient's standard deviation (SD).

Table 2: The ANOVA of the quadratic model for Particle size (PS)

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	1.055E+05	21095.43	3.66	**0.0438**	**Significant**
A-PLGA	4186.58	4186.58	0.7270	0.4160	
B-Pluronic F127: PVA	24729.77	24729.77	4.29	0.0681	

C-Sonication time	10023.13	10023.13	1.35	0.2756
AB	12210.25	12210.25	2.12	0.1793
BC	15753.63	15753.63	2.12	0.1796
AC	3721.61	3721.61	0.3995	0.5431
A^2	42543.05	42543.05	7.39	0.0237
B^2	26253.50	26253.50	4.56	0.0615
C^2	10974.85	10974.85	1.48	0.2554

(R^2 = 0.9378, adjusted R^2 = 0.7157, adequate precision = 7.7923, p >0.05 was considered to be significant)

Table 3: The ANOVA of the quadratic model for Entrapment efficiency (%EE)

Source	**Sum of Squares**	**Mean Square**	**F-value**	**p-value**	
Model	1510.04	302.01	4.14	**0.0314**	**Significant**
A-PLGA	18.27	18.27	0.2505	0.6287	
B-Pluronic F127: PVA	9.55	9.55	0.1309	0.7258	
C-Sonication time	231.85	231.85	2.76	0.1311	
AB	131.10	131.10	1.80	0.2129	
BC	19.63	19.63	0.2335	0.6405	
AC	151.41	151.41	1.89	0.2024	
A^2	432.43	432.43	5.93	0.0377	
B^2	1006.01	1006.01	13.79	0.0048	
C^2	584.20	584.20	7.30	0.0244	

(R^2 = 0.9652, adjusted R^2 = 0.6257, adequate precision = 10.3608, p >0.05 was considered to be significant.)

Optimum solution

In the desirability approach, the optimum solution suggested by Design-Expert stat Ver. 13.0 DoE ++ software was used for further study. The optimum solution predicted that PLGA (200 mg), Pluronic F127: PVA (4%) and sonication time (5 min), of independent variables showed a minimum particle size of 181.8 nm and maximum % EE of 68.58% from MTX-LLCs(Table 4)(Qindeel, Ullah, Fakhar Ud, Ahmed, & Rehman, 2020).

Table 4: The optimum solution showing values of variables suggested by the desirability approach

Factors	**Value**	**Responses**	**Value**
A: PLGA	200 mg	Particle size	181.8 nm

B: Pluronic F127: PVA	4%	Entrapment efficiency	68.58%
C: Sonication time	5 min		

Characterization of drug-loaded MTX-LLCs

Optimization of dependent variables (Response)

The BBD was used to study the contour plots (2D) and three-dimensional response surface plots (3D) based on the polynomial functions of the model to evaluate the change in the response surface. These plots can further help to understand the correlation between factors, and response.

Influence of Independent variables on PS (Y_1)

The ANOVA was used to measure the models and individual response parameters significance ($p>0.05$). The contour plots and response surface plots were analyzed to check the effect of independent variables on the PS. When the P-value is $p>0.0438$, the quadratic model with an F-value of 3.66 indicates that the model is significant for X_1-X_2, X_1-X_3 and X_2-X_3 respectively [Figure 3 (a) & 3 (b)]. The contour plot and response surface plot displayed the effect of factors on PS. It was found that the increase in PLGA concentration results in the decreased PS of drug-loaded MTX-LLCs but at a certain level, it gets increases. The possible reason behind this may be the viscosity of PLGA which appears to change the PS of LLCs due to interference in the rapid dispersion of higher viscosity PLGA solution into the aqueous phase leading to a decrease in the size of LLCs. The concentration of emulsifier Pluronic F127: PVA plays a crucial role in analyzing the PS of LLCs. It was observed that a decrease in the concentration of Pluronic F127: PVA significantly decreases the PS of LLCs which means factors X_1 and X_2 positively affect on the Y_1 response.

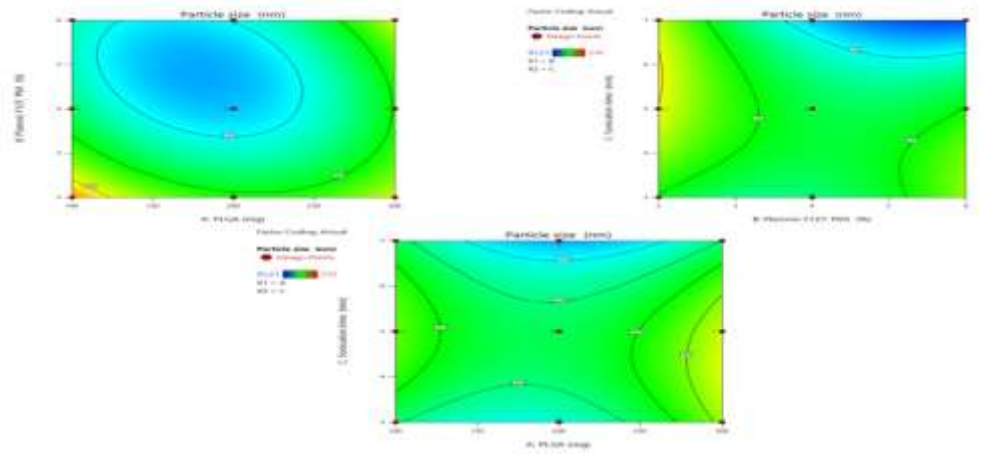

Figure 1 (a): 2 D or Contour plot showing the effect of PLGA(X_1), Pluronic F-127: PVA(X_2), and Sonication time(X_3)on particle size (Y_1)

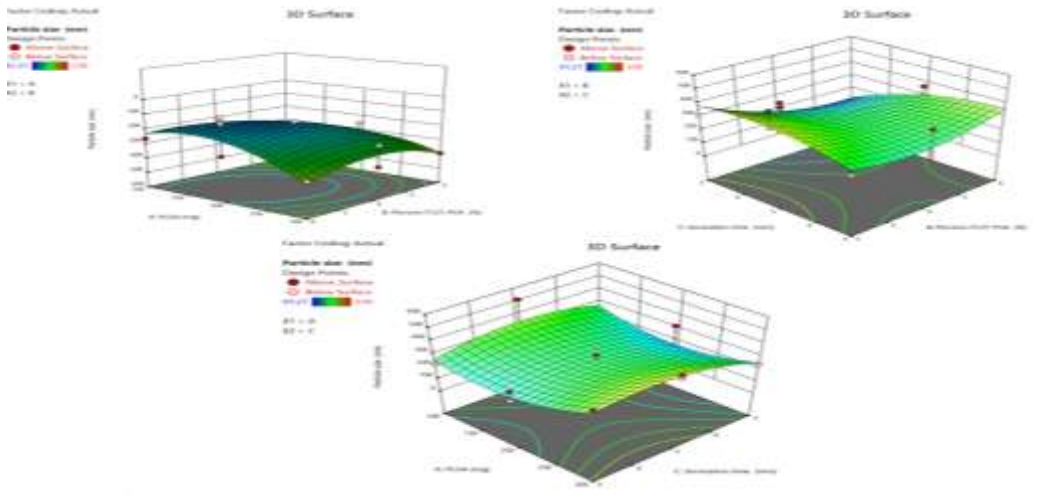

Figure 1 (b): 3D or Response surface plot showing the effect of PLGA (X_1), Pluronic F-127: PVA (X_2) and Sonication time(X_3)on particle size (Y_1)

Influence of Independent variables on EE (Y_2)

The ANOVA estimation showed the quadratic model of F-value 4.14 implies the model is significant where P-value is $p>0.0314$ for X_1-X_2, X_1-X_3 and X_2-X_3 respectively. The contour plot and response surface plot in [Figure4 (a) &4 (b)], showed the effect of all independent variables on EE. As per the results, the EE of drug-loaded-MTX-LLCs increases as the increase in concentration of PLGA, Pluronic F127: PVA and for same, the sonication time was kept at the centre level. Increasing the concentration of PLGA from 100 to 300 increases the EE due to the viscosity of the organic phase leading to less partitioning between the aqueous and organic phases. It helps to reduce the amount of drug diffuses to the aqueous phase. Furthermore, the second factor Pluronic F127: PVA also showed twin behaviour on EE. An increase in the Pluronic F127: PVA concentration leads to increases in EE. Further, an increase in Pluronic F127: PVA concentration up to 5% w/v the EE slightly decreases. The decrease in EE may be due to the hydrophobic property of drug molecules in the particles [34].

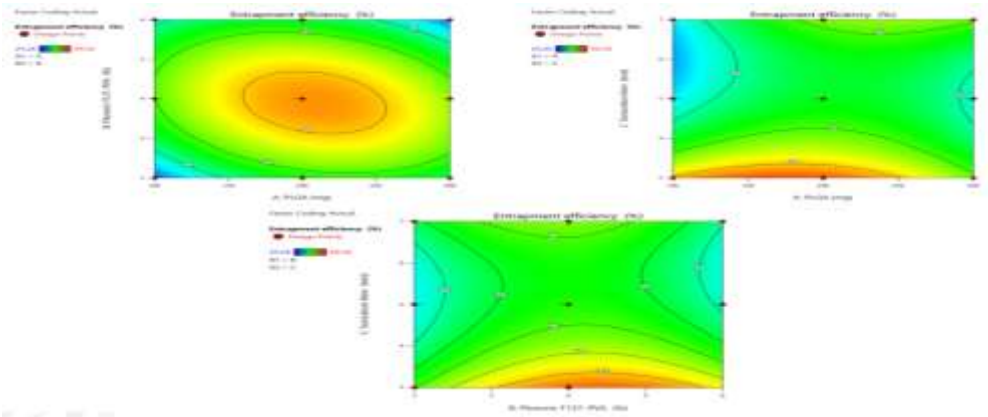

Figure 2 (a): 2 D or the Response surface plot showing the effect ofPLGA (X_1), Pluronic F-127: PVA (X_2), and Sonication time(X_3)on entrapment efficiency (Y_2)

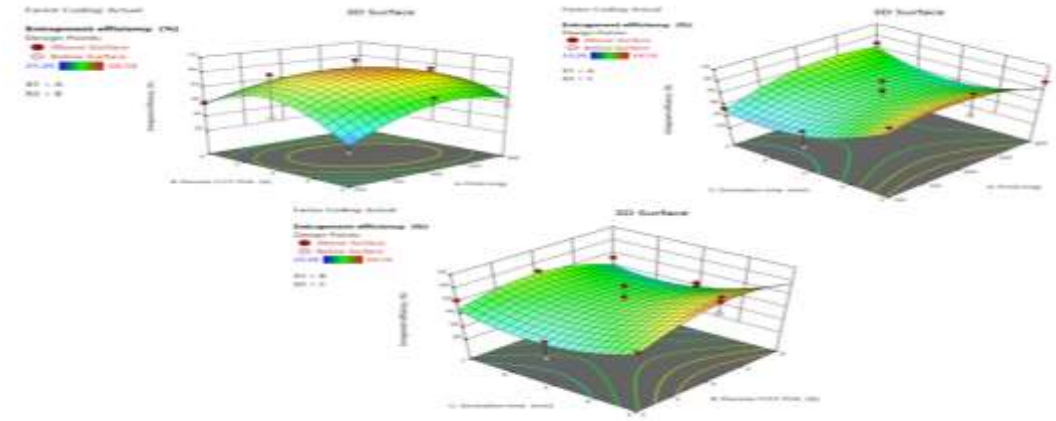

Figure 2 (b): 3 D or the Contour plot showing the effect of PLGA (X_1), Pluronic F-127: PVA (X_2), and Sonication time(X_3) on entrapment efficiency (Y_2)

Polydispersity index (PDI), and zeta potential

For better physical stability, the PDI (0.210), a parameter used to predict LLCs physical stability should be less than one. Additionally, the zeta potential was discovered to be -10.6 2.41 MeV [Figure5 (a) &5 (b)]. A higher value of the zeta potential results in greater colloidal stability because it describes the stability of the particles in the dispersion medium. Consequently, there is very low chance of particle aggregation [35].

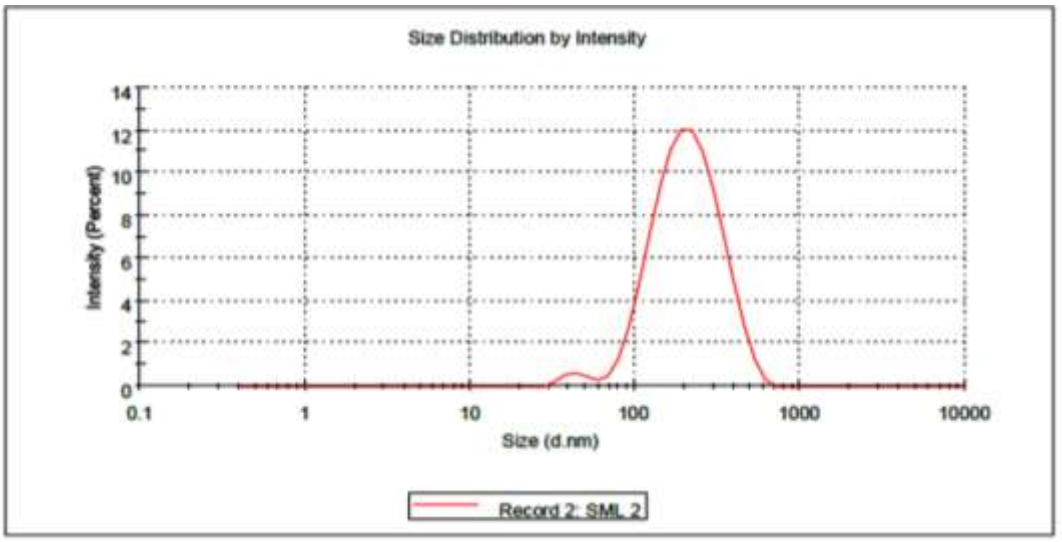

Figure 3 (a): Particle size of optimized batch of MTX-LLCs

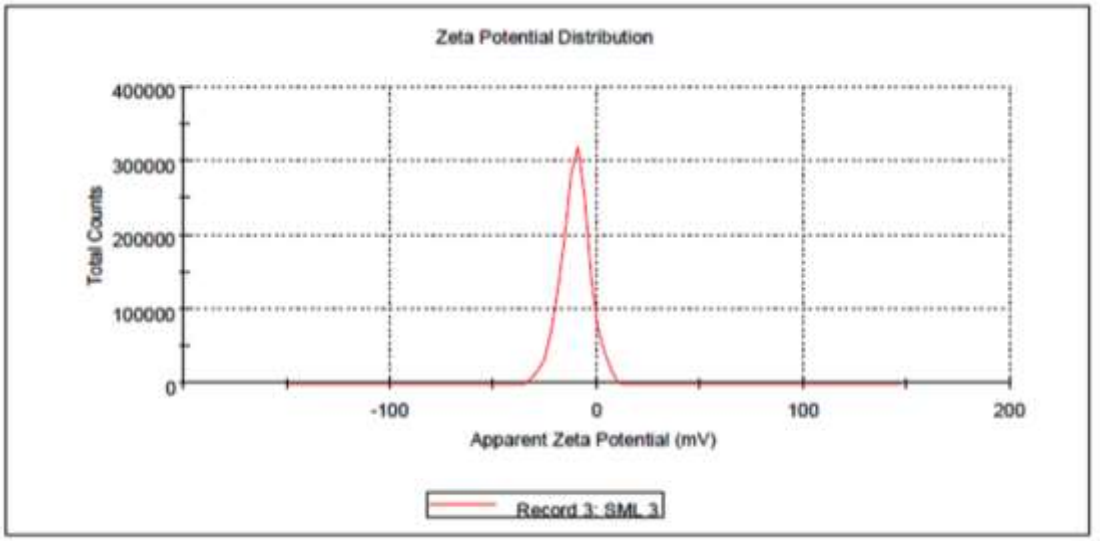

Figure 3 (b): Zeta potential of optimized batch of MTX-LLCs

Drug loading capacity (%DLC)

The % DLC of the optimized MTX-LLCs was found to be 4.32%. The amount of drug loading in MTX-LLCs was higher. The batch (F10) DLC was discovered to be 4.09%.

Characterization of prepared MTX-LLCs-loadedgel

Homogeneity and pH

Visual examination of a settled MTX-LLCs-loaded gel in a container demonstrated the formation of translucent and homogeneous gels with no indications of separation or precipitation. The pH of gel was found to be 6.1, which is compatible with the pH of the skin.

Spreadability

The spreadability of a gel formulation is an important characteristic that indicates the extent to which the gel readily spreads on the surface. The spreadability of gel tends to increase with distance travelled. The spreadability was measured, and the result was 5 to 8 cm, which is within the acceptable range [36].

Fourier transforms infrared spectroscopy (FTIR)

To confirm the successful fabrication of MTX-LLCs-loaded gel, the FTIR spectra of gel were used, similarly the spectra of pure drug MTX, PLGA, Pluronic F-127, PVA, was comparatively studied(Figure 1). The absorption peak at 3504.5 cm^{-1} was confirmed to be caused by terminal hydroxyl groups (-OH). The C-H stretches were assigned to the band observed at 2874 cm^{-1}. The peaks at 1751 cm-1 were associated with the carbonyl group (C14O). The C-O-C stretch is responsible for the bands at 1086 and 1182 cm^{-1}. The peak at 1453 cm^{-1} revealed a methyl group in the LA region. These findings supported the formulation of the MTX-LLCs loaded gel.

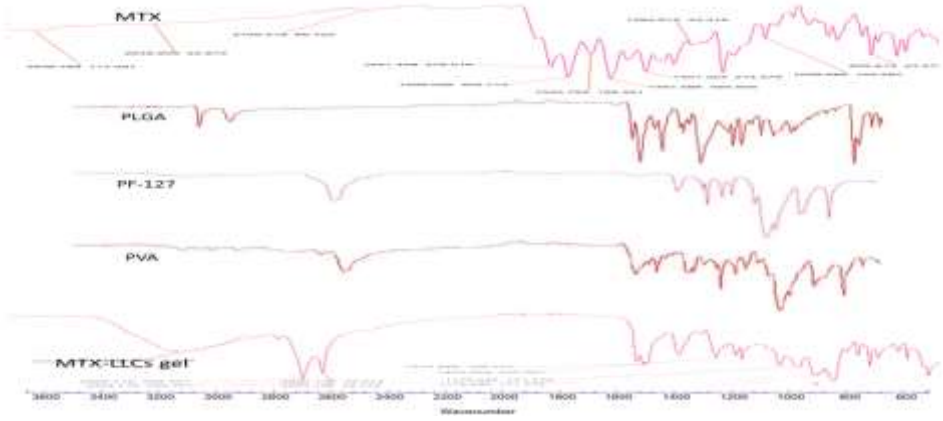

Figure 4: FT-IR spectroscopy of pure MTX, PLGA, PF-127, PVA, and optimized MTX-LLCs loadedgel

Rheological study measurements

The rheological profile of MTX-LLCs-loaded gel was measured using a Brookfield RS/-CPS rheometer (Rheo 3000+) with a spindle C-75 and results of optimized batch represented in Table 5 [37].

Table 5: Characterization of optimized MTX-LLCs -loaded gel

Batch Code	Homogeneity	pH	Spreadability	Viscosity (cps)	% Drug content
Batch F-4	Good	6.2±0.5	5-8	0.209±0.75	97.32%±1.2

Data are expressed as the mean ± S.D. (n=3).

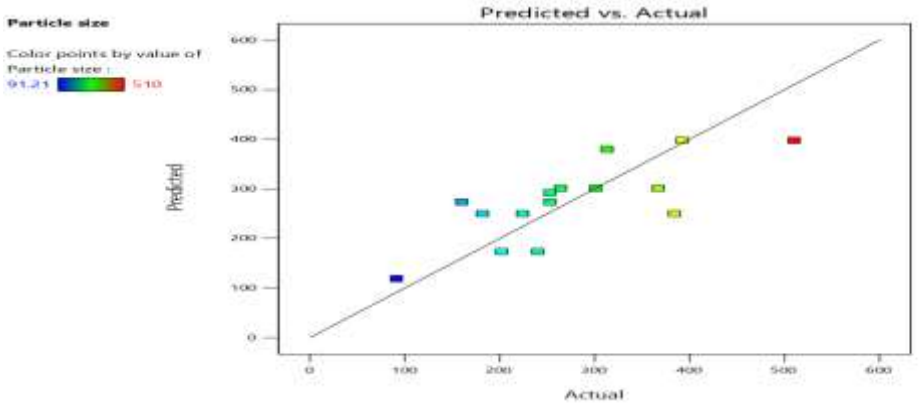

Figure 5 (a): Normal probability plot for particle size of drug-loaded MTX-LLCs

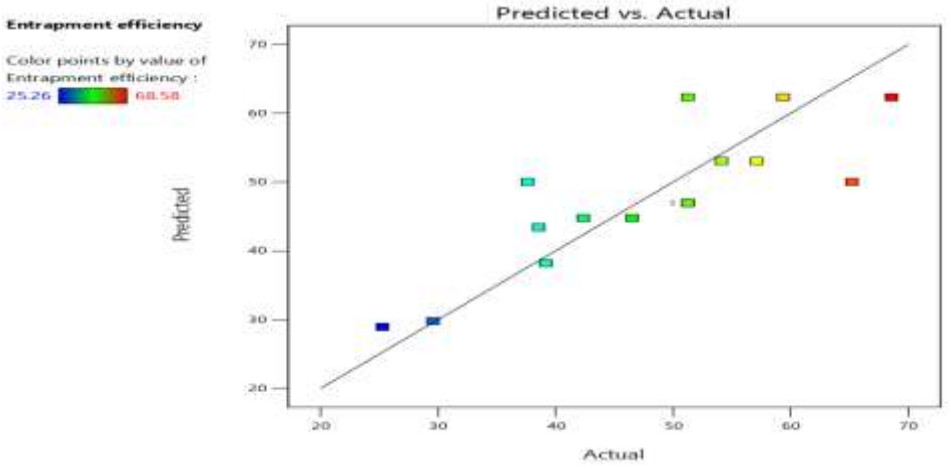

Figure 5 (b): Normal probability plot for entrapment efficiency of drug-loaded MTX-LLCs

In vitro drug release study

The comparative in vitro release study was carried out at 37±0.5˚Cin PBS pH 7.4 with the cellophane dialysis membrane (molecular weight 8k Da) by using a Franz diffusion cell. The results revealed significant differences in the drug release profile of MTX, MTX-LLCs, and MTX-LLCs-loaded gel. Obtained results showed 55.19 ± 0.94, 57.79 ± 0.79, and 81.87± 1.04 % respectively in the 12 h study. The sustain release pattern was found in MTX-LLCs-loaded gel because the release rate of drug was controlled by diffusionand degradation of the polymer matrix. In the present study, there was a small difference in the drug release pattern of MTX-LLCs and MTX-LLCs-loaded gel, mainly because of the obstructive effect of the gel matrix. From the above results, it is clear that the slow release of MTX from MTX-LLCs-loaded gel in the body, targets more pain and inflamed area in rheumatic conditions, and therefore, it meets the requirements for an effective drug delivery system [38].

Ex vivo permeation study

The exvivo permeation study was carried out to evaluate the permeation of MTX from MTX-LLCs-loaded gel and conventional gel using goat ear pinna skin. The results revealed that better permeation of MTX from prepared gel (69.25±7.32%) as compared to the marketed formulation after 12 h. The Jss and Kp values for drug-loaded MTX-LLCs-loaded gel were found to be highest for goat ear pinna skin i.e. 10.023± 1.12 mg/cm^2/h and 1.2014 ± 0.092 mg [39-40], respectively due to the presence of PLGA and Pluronic F-127-PVA.

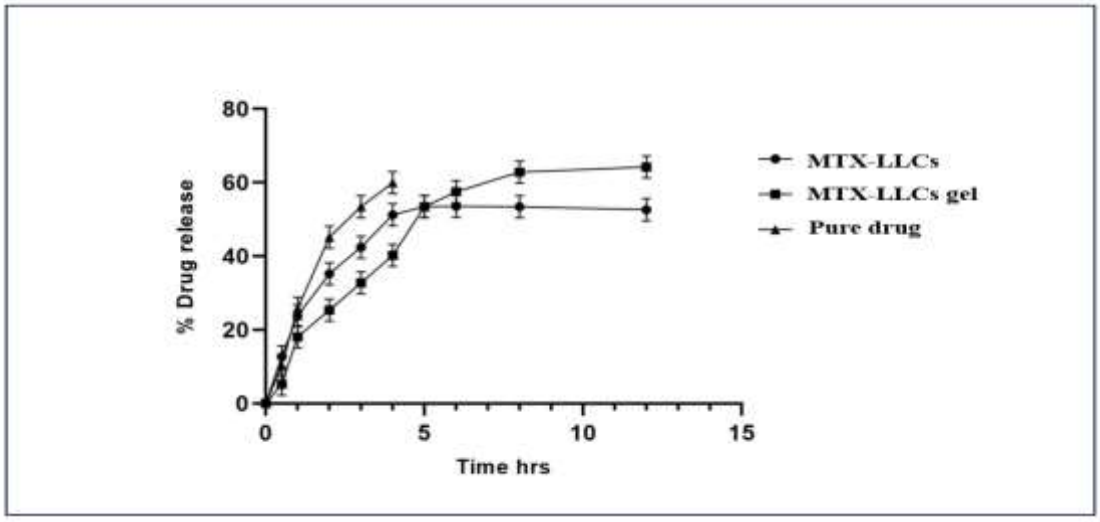

Figure 6: % CDR from pure drug MTX, MTX-LLCs and MTX-LLCs loaded gel

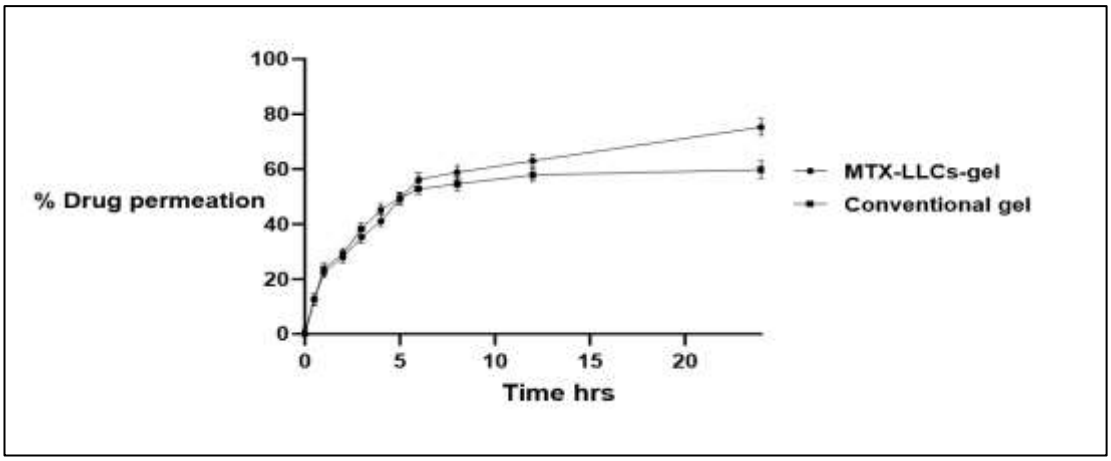

Figure 7: % Drug permeation fromMTX-LLCs-based gel and Conventional gel

Stability study

The stability study based on ICH guidelines of optimized MTX-LLCs and MTX-LLCs-loaded gel was assessed at different temperatures 25±2°C/60±5% RH, and 40±2°C/75±5% RH. No momentous alteration in the PS, EE and in vitro drug release of MTX-LLCs as well as in the appearance and clarity of MTX-LLCs-loaded gel was observed during stability studies (Table 6). The PS of the gel remained constant when stored at room temperature. The drug content, viscosity, and spreadability were found to be within the acceptable range. As a result, it is possible to conclude that the prepared formulations are stable in all storage conditions.

Table 6: The stability study results of optimized formulation

Temp.	Months	MTX-LLCs		MTX-LLCs-loaded gel	
		PS (nm)	EE (%)	PS (nm)	EE (%)
25±2°C/60±5%	1	181	68.58	179	71.25
	2	201	67.23	204	69.41
	3	223	65.17	230	66.09
40±2°C/75±5%	1	185	67.10	190	70.81
	2	205	68.80	200	68.26
	3	220	64.25	211	64.50

Data are expressed as the mean ± S.D. (n=3).

Conclusion

Gel is an impending approach for the delivery of MTX. The developed drug-loaded MTX-LLCs nanoparticles, using an emulsification sonication method with slight modification, which was then converted into a gel using the gelling agent carbopol 940 p and optimized using BBD. Moreover, the physico-chemical properties, particle size, rheological behaviour, in vitro drug release, ex vivo permeation study, and stability study of the drug-loaded MTX-LLCsgel were evaluated. The optimized drug-loaded MTX-LLCsgel was clear, homogeneous,

and compatible with the skin pH. The particle size of gel was observed in the nanometer so we could expect better absorption of MTX from the skin. Moreover, the optimized gel demonstrated sustained release of MTX for 12 h and was the best fit for zero-order kinetics. There were no significant changes in the physical properties or MTX content of the gel, indicating its good stability at 40°C/75% RH. Furthermore, the targeting efficiency of the topically applied drug is higher, which may provide an enhanced therapeutic index, resulting in a lower dose necessity and a decrease in dose-related systemic side effects.

Conflict of Interest Declared None

Acknowledgements
We greatly acknowledge the Ashokrao Mane College of Pharmacy, Peth-Vadgaon & Tatyasaheb Kore College of Pharmacy, Warananagar, for their kind support and motivation during research.

References

1. Zhang, Y., Ji, Y., Liu, S., Li, J., Wu, J., Jin, Q., Liu, X., Duan, H., Feng, Z., Liu, Y., Zhang, Y., Lyu, Z., Song, F., Song, F., Yang, L., Liu, H., & Huang, Y. (2025). Global burden of female breast cancer: new estimates in 2022, temporal trend and future projections up to 2050 based on the latest release from GLOBOCAN. Journal of the National Cancer Center. https://doi.org/10.1016/j.jncc.2025.02.002
2. Zhang, S., Jin, Z., Bao, L., & Shu, P. (2024). The global burden of breast cancer in women from 1990 to 2030: assessment and projection based on the global burden of disease study 2019. Frontiers in Oncology, 14. https://doi.org/10.3389/fonc.2024.1364397
3. Cardoso, F., Paluch-Shimon, S., Boyle, F., Cardoso, M., Carey, L., Cortés, J., Saghir, N. E., Elzayat, M., Eniu, A., Fallowfield, L., Francis, P., Winer, E. (2020a). 5th ESO-ESMO international consensus guidelines for advanced breast cancer (ABC 5). Annals of Oncology, 31(12), 1623–1649. https://doi.org/10.1016/j.annonc.2020.09.010
4. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA a Cancer Journal for Clinicians, 71(3), 209–249. https://doi.org/10.3322/caac.21660
5. Raut, J., Sarkar, O., Das, T., Mandal, S. M., Chattopadhyay, A., & Sahoo, P. (2023). Efficient delivery of methotrexate to MDA-MB-231 breast cancer cells by a pH-responsive ZnO nanocarrier. Scientific Reports, 13(1). https://doi.org/10.1038/s41598-023-49464-9
6. Chopade SS. Design and in-vitro evaluation of Tenoxicam nanogel containing noveon polymer. Int. J. of Pharm. Sci and Res. 2019;10(12): 5430-5434.
7. Ammara HO, Ghorabb M, El-Nahhasc SE, Higazya IM. Proniosomes as a carrier system for transdermal delivery of Methotrexate. Int J of Pharm. 2011; (405):142–152.
8. Chopade SS, Gaikwad E, Jadhav A, Patil N, Payghan SA. et al. Formulation and Evaluation of Fast Disintegrating Tenoxicam Tablets and the Comparison with Marketed Product. Asian Journal of Research in Pharmaceutical Sciences. 2010; 10(4): 257-263.
9. Muslu, H., Kalaycıoğlu, Z., Erdoğan, T., Gölcü, A., & Bedia Erim, F. et al. Synthesis, characterization, anti-inflammatory evaluation, molecular docking and density functional theory studies of metal based drug candidate molecules of Methotrexate. Results in Chemistry. 2021; 3. https://doi:10.1016/j.rechem.2021.100111

10. Kumar JA, Pullakandam N, Prabu SL, Gopal SL. Transdermal Drug Delivery System: An Overview, Int J of Pharm. Sci. Rev and Res. 2010;(3):49.
11. Negi LM, Chauhan M. Nano-appended transdermal gel of Methotrexate via ultra-deformable drug carrier system, J of experimental nanoscience. 2013;(8):657-669.
12. Kan, S., Lu, J., Liu, J., Wang, J., & Zhao, Y. (2014). A quality by design (QbD) case study on enteric-coated pellets: Screening of critical variables and establishment of design space at laboratory scale. Asian Journal of Pharmaceutical Sciences, 9(5), 268–278. https://doi.org/10.1016/j.ajps.2014.07.005
13. Sharaf, N. S., Shetta, A., Elhalawani, J. E., & Mamdouh, W. (2021). Applying Box–Behnken design for formulation and optimization of PLGA-Coffee nanoparticles and detecting enhanced antioxidant and anticancer activities. Polymers, 14(1), 144. https://doi.org/10.3390/polym14010144
14. Sopyan, I., Gozali, D., Sriwidodo, N., &Guntina, R. K. (2022). DESIGN-EXPERT SOFTWARE (DOE): AN APPLICATION TOOL FOR OPTIMIZATION IN PHARMACEUTICAL PREPARATIONS FORMULATION. International Journal of Applied Pharmaceutics, 55–63. https://doi.org/10.22159/ijap.2022v14i4.45144
15. Alshehri, S., & Imam, S. S. Formulation and evaluation of butenafine loaded PLGA-nanoparticulate laden chitosan nano gel. Drug Deliv. 2021; 28(1): 2348-2360. https://doi:10.1080/10717544.2021.1995078
16. Kim, D. H., Nguyen, T. N., Han, Y. M., Tran, P., Rho, J., Lee, J. Y., Park, J. S. et al. Local drug delivery using poly(lactic-co-glycolic acid) nanoparticles in thermosensitive gels for inner ear disease treatment. Drug Deliv. 2021; 28(1), 2268-2277. https://doi:10.1080/10717544.2021.1992041
17. Bawazeer, S., El-Telbany, D. F. A., Al-Sawahli, M. M., Zayed, G., Keed, A. A. A., Abdelaziz, A. E., & Abdel-Naby, D. H. et al. Effect of nanostructured lipid carriers on transdermal delivery of Methotrexate in irradiated rats. Drug Deliv. 2020; 27(1): 1218-1230. https://doi:10.1080/10717544.2020.1803448
18. Khaledi, S., Jafari, S., Hamidi, S., Molavi, O., & Davaran, S. et al. Preparation and characterization of PLGA-PEG-PLGA polymeric nanoparticles for co-delivery of 5-Fluorouracil and Chrysin. J Biomater Sci Polym Ed. 2020; 31(9), 1107-1126. https://doi:10.1080/09205063.2020.1743946
19. Kesharwani, P., Jain, A., Srivastava, A. K., & Keshari, M. K. Systematic development and characterization of curcumin-loaded nanogel for topical application. Drug Dev Ind Pharm. 2020; 46(9), 1443-1457. https://doi:10.1080/03639045.2020.1793998
20. Qureshi, M., Qadir, A., Aqil, M., Sultana, Y., Warsi, M. H., Ismail, M. V., & Talegaonkar, S. et al. Berberine loaded dermal quality by design adapted chemically engineered lipid nano-constructs-gel formulation for the treatment of skin acne. Journal of Drug Delivery Science and Technology. 2021; 66. https://doi:10.1016/j.jddst.2021.102805
21. Yang, M., Feng, X., Ding, J., Chang, F., & Chen, X. et al., (2017). Nanotherapeutics relieve rheumatoid arthritis. J Control Release. 2017;252:108-124. https://doi:10.1016/j.jconrel.2017.02.032
22. Zhao, J., Chen, X., Ho, K.-H., Cai, C., Li, C.-W., Yang, M., & Yi, C. et al. Nanotechnology for diagnosis and therapy of rheumatoid arthritis: Evolution towards theranostic approaches. Chinese Chemical Letters. 2021;32(1): 66-86. https://doi:10.1016/j.cclet.2020.11.048
23. Kesharwani, P., Jain, A., Srivastava, A. K., & Keshari, M. K. Systematic development and characterization of curcumin-loaded nanogel for topical application. Drug Dev Ind Pharm. 2020; 46(9), 1443-1457. https://doi:10.1080/03639045.2020.1793998

24. Gebreel, R. M., Edris, N. A., Elmofty, H. M., Tadros, M. I., El-Nabarawi, M. A., & Hassan, D. H. et al.Development and Characterization of PLGA Nanoparticle-Laden Hydrogels for Sustained Ocular Delivery of Norfloxacin in the Treatment of Pseudomonas Keratitis: An Experimental Study. Drug Des Devel Ther. 2021; 15: 399-418. https://doi:10.2147/DDDT.S293127
25. Mahtab, A., Rizwanullah, M., Pandey, S., Leekha, A., Rabbani, S. A., Verma, A. K., Talegaonkar, S. et al. Quality by design driven development and optimization of teriflunomide loaded nanoliposomes for treatment of rheumatoid arthritis: An in vitro and in vivo assessments. Journal of Drug Delivery Science and Technology. 2019; 51: 383-396. https://doi:10.1016/j.jddst.2019.03.008
26. Payghan SA, Kate V. Interactive mixture of piroxicam and polymers for development of mucoadhesive fast disintegrating sublingual tablet: In vitro in vivo evaluation. J Pharm Sci Tech. 2015;5:32-44.
27. Payghan SA, Mane YV, Kate VK, Tamboli ZJ. Anhydrous emulsion: Vehicles for topical delivery of ketoconazole. Invent Impact NDDS. 2015; (1): 43-53.
28. Sahu, P., Kashaw, S. K., Kashaw, V., Shabaaz, J. P., & Dahiya, R. et al. Synthesis and ex vivo evaluation of PLGA chitosan surface modulated double walled transdermal Pluronic nanogel for the controlled delivery of Temozolomide. Int J Biol Macromol. 2021; 187:742-754. https://doi:10.1016/j.ijbiomac.2021.07.129
29. Rabiei, M., Kashanian, S., Samavati, S. S., Derakhshankhah, H., Jamasb, S., & McInnes, S. J. P. et al. Nanotechnology application in drug delivery to osteoarthritis (OA), rheumatoid arthritis (RA), and osteoporosis (OSP). Journal of Drug Delivery Science and Technology.2021: 61. https://doi:10.1016/j.jddst.2020.102011
30. Taufik BN, Adhav AJ, Payghan SA. Composition of terbinafine HCL polymeric gel for mucosal drug delivery. Int J Biol Pharm Allied Sci. 2016;(5): 2146-68.
31. Kesharwani, P., Jain, A., Srivastava, A. K., & Keshari, M. K. Systematic development and characterization of curcumin-loaded nanogel for topical application. Drug Dev Ind Pharm. 2020; 46(9), 1443-1457. https://doi:10.1080/03639045.2020.1793998
32. Kriplani, P., Guarve, K., & Singh Baghel, U. Formulation optimization and characterization of transdermal film of curcumin by response surface methodology. Chin Herb Med. 2021;13(2), 274-285. https://doi:10.1016/j.chmed.2020.12.001
33. Nesseem, D. I., Eid, S. F., & El-Houseny, S. S. Development of novel transdermal self-adhesive films for Methotrexate, an anti-inflammatory drug. Life Sci 89(13-14): 430-438. https://doi:10.1016/j.lfs.2011.06.026
34. Klodzinska, S. N., Wan, F., Jumaa, H., Sternberg, C., Rades, T., & Nielsen, H. M. et al. Utilizing nanoparticles for improving anti-biofilm effects of azithromycin: A head-to-head comparison of modified hyaluronic acid nanogels and coated poly (lactic-co-glycolic acid) nanoparticles. J Colloid Interface Sci. 2019; 555: 595-606. https://doi:10.1016/j.jcis.2019.08.006
35. Raj AR. Formulation evaluation and in vitro permeation studies of transdermal nifedipine from matrix type patches. Int J Pharm Sci. 2014; 6 (1): 185-188.
36. Mahtab, A., Rizwanullah, M., Pandey, S., Leekha, A., Rabbani, S. A., Verma, A. K., Talegaonkar, S. et al. Quality by design driven development and optimization of teriflunomide loaded nanoliposomes for treatment of rheumatoid arthritis: An in vitro and in vivo assessments. Journal of Drug Delivery Science and Technology. 2019; 51: 383-396. https://doi:10.1016/j.jddst.2019.03.008

37. Avasatthi V, Pawar H, Dora PC, Bansod P, Gill MS, and Suresh S. A, et al. Novel nanogel formulation of methotrexate for topical treatment of psoriasis: optimization, in vitro and in vivo evaluation. Pharm Dev Technol. Early Online. 2019: 1-9.
38. Shen CY, Xu PH, Shen BD, Min HY, Li XR, Han J and Yuan HL et al. Nanogel for dermal application of the triterpenoids isolated from Ganodermalucidum (GLT) for frostbite treatment. Drug Delivery. 2016;23(2): 610-618.
39. Qureshi, M., Qadir, A., Aqil, M., Sultana, Y., Warsi, M. H., Ismail, M. V., & Talegaonkar, S. et al. Berberine loaded dermal quality by design adapted chemically engineered lipid nano-constructs-gel formulation for the treatment of skin acne. Journal of Drug Delivery Science and Technology. 2021; 66. https://doi:10.1016/j.jddst.2021.102805
40. Agrawal, M., Saraf, S., Pradhan, M., Patel, R. J., Singhvi, G., Ajazuddin, & Alexander, A. et al. Design and optimization of curcumin loaded nano lipid carrier system using Box-Behnken design. Biomed Pharmacother. 2021;141: 111919. https://doi:10.1016/j.biopha.2021.111919
41. Sadarani, B., Majumdar, A., Paradkar, S., Mathur, A., Sachdev, S., Mohanty, B., & Chaudhari, P. et al. Enhanced skin permeation of Methotrexate from penetration enhancer containing vesicles: In vitro optimization and in vivo evaluation. Biomed Pharmacother. 2019; 114: 108770. https://doi:10.1016/j.biopha.2019.108770