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Development and Optimization of Dihydrofolate-Targeted Methotrexate-Loaded Lyotropic Liquid Crystal-Based Gel for Localized Breast Cancer Therapy

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ABSTRACT: Breast cancer needs localized drug delivery strategies to improve therapeutic efficacy while minimizing systemic toxicity. This study aimed to develop and optimize methotrexate-loaded lyotropic liquid crystal nanoparticles (MTX-LLCs) and incorporate them into a topical gel for localized breast cancer therapy. MTX-LLCs were prepared by a top-down approach and optimized using a Box-Behnken design (BBD), with GMO (X1), Poloxamer 407 (X2), and probe sonication cycles (X3) as independent variables and entrapment efficiency (EE; Y1) and Vesicle size (Y2) as responses. The optimised formulation containing 2 mL GMO, 150 mg Poloxamer 407 and 20 cycles of sonication showed $92.14 \pm 0.57\%$ EE, 155.31 ± 2.41 nm vesicle size and 0.231 ± 0.07 PDI. The MTX-LLCs gel showed a prolonged release of drug ($30.75 \pm 4.25\%$ at 48 h) compared to the conventional MTX gel ($64.80 \pm 3.38\%$). Ex vivo permeation and improved anti-cancer efficacy, suggesting it was improved to 246.5 ± 5.32 $\mu\text{g}/\text{cm}^2$ at 8 h, compared to 65.8 ± 5.47 $\mu\text{g}/\text{cm}^2$ for conventional gel. The optimized gel also showed better cytotoxicity against MCF-7 cells with an IC₅₀ of 19.86 ± 2.18 $\mu\text{g}/\text{mL}$ as compared to plain MTX of 42.16 ± 3.92 $\mu\text{g}/\text{mL}$. The formulation was shown to be stable regarding physicochemical properties, especially when kept at refrigeration temperatures. In conclusion, the Box-Behnken design was an effective tool for the optimization of MTX-LLCs, and its formulation into a topical gel offered sustained release, increased permeation and improved anti-cancer efficacy, which suggests its potential for localized breast cancer treatment.

INTRODUCTION

Globally, breast cancer (BC) remains a major health challenge and is the most commonly diagnosed cancer among women. According to recent cases, approximately 2.4 million women were diagnosed with BC and 694,000 deaths were attributed to the disease in 2024, highlighting the continuing need for effective and safer therapeutic strategies [1]. Although advances in screening, molecularly targeted therapies, surgery, and radiotherapy have improved clinical outcomes, chemotherapy remains an important treatment modality, particularly for aggressive, advanced, and metastatic disease [2].

Methotrexate (MTX) is an established antifolate chemotherapeutic agent that exerts its anticancer activity primarily through inhibition of dihydrofolate reductase (DHFR). By competitively inhibiting DHFR, MTX prevents the conversion of dihydrofolate to tetrahydrofolate, resulting in depletion of reduced folate cofactors required for thymidylate and purine biosynthesis. Therefore, DNA synthesis and cellular proliferation are impaired, making rapidly dividing malignant cells particularly susceptible to MTX [3,4]. Recent studies in human breast cancer cells have demonstrated that MTX-induced DHFR inhibition is associated with disruption of folate-dependent metabolic pathways and inhibition of DNA and purine synthesis [4,5]. However, systemic MTX therapy can be associated with dose-limiting toxicities, including myelosuppression, mucositis, hepatotoxicity, and nephrotoxicity, which may restrict its therapeutic application [6,7].

These limitations have encouraged the development of localized drug-delivery approaches capable of increasing drug exposure at the tumor site while reducing unnecessary systemic distribution. Topical and transdermal delivery systems can provide prolonged drug release and potentially improve local drug concentration; however, effective penetration of anticancer agents through the skin remains challenging because of the highly restrictive stratum corneum [8]. Therefore, the development of nanostructured lipid-based systems capable of enhancing drug incorporation, controlling release, and improving skin permeation is of considerable interest.

Lytotropic liquid crystals (LLCs) are self-assembled nanostructured lipid systems formed by amphiphilic lipids in the presence of water. Depending on lipid composition and hydration, LLCs can form highly ordered cubic, spherical, hexagonal, or lamellar structures. Among these, glyceryl monooleate (GMO)-based cubic systems have attracted considerable attention because of their biocompatibility, ordered internal structure, drug-loading capacity, and ability to provide controlled drug release [9,10]. GMO-based LLCs nanoparticles have also demonstrated potential for incorporating anticancer agents and enhancing their biological activity against breast cancer cell lines [10]. Furthermore, the structural characteristics of LLCs can be exploited to develop localized delivery systems with prolonged drug retention and improved drug-tissue interaction.

Despite the therapeutic potential of MTX as a DHFR-targeting anticancer agent, limited research has investigated its incorporation into GMO-based LLCs followed by integration into a topical gel for localized breast cancer therapy. Therefore, the present study was designed to develop and optimize MTX-loaded lyotropic liquid crystal (MTX-LLCs)-based gel for topical breast cancer treatment. The developed system was comprehensively evaluated for its physicochemical characteristics, drug-release behavior, ex-vivo permeation, in-vitro anticancer activity, and in-vivo therapeutic efficacy, with the overall objective of enhancing localized MTX delivery while minimizing systemic exposure.

MATERIALS AND METHODS

Materials

Methotrexate (MTX) was generously provided as a gift sample by Cipla Ltd., India. Glyceryl monooleate (GMO) was procured from Mohini Organics Pvt. Ltd., India, and Poloxamer 407 (Pluronic® F127) was obtained from BASF, Germany. Isopropyl myristate (IPM), Carbopol® 940, triethanolamine, and other analytical-grade chemicals and solvents were purchased from Loba Chemical Pvt. Ltd., Mumbai, India, and Unique Biologicals, Kolhapur, India. All chemicals and reagents used in the study were of analytical reagent (AR) grade and were used without further purification. Double-distilled water was used throughout the study for formulation preparation and analytical investigations.

Design of experiments (DoE)

Design of Experiments (DoE) is an important Quality by Design (QbD) tool that enables the systematic and simultaneous evaluation of multiple formulation and process variables, thereby overcoming the limitations of conventional one-factor-at-a-time approaches [12]. In the present study, DoE was employed to investigate the effects of selected independent variables on the critical quality attributes and responses of the formulation. Response Surface Methodology (RSM) was utilized to establish

the relationship between the independent variables and measured responses [13]. Depending on the experimental objectives, designs such as Central Composite Design (CCD), Box-Behnken Design (BBD), and full factorial design can be employed. An appropriate experimental design was selected based on the formulation characteristics and study requirements to identify the optimal formulation conditions [14].

Preparation of MTX-Loaded LLCs

MTX-LLCs were prepared using a modified top-down emulsification approach based on previously reported methods for GMO-based liquid crystalline nanoparticles [15]. Briefly, accurately weighed quantities of GMO and Poloxamer 407 were heated to 80 °C until a homogeneous molten lipid phase was obtained. MTX, equivalent to 0.1% w/w, was subsequently incorporated into the molten lipid phase under continuous stirring to ensure uniform drug distribution. An aqueous phase, preheated to the same temperature, was gradually added to the lipid phase under continuous high-speed magnetic stirring to facilitate hydration and formation of the liquid crystalline dispersion. The resulting dispersion was maintained under stirring at 1200 rpm for 1 h to obtain a uniform and stable formulation. The prepared MTX-LLCs dispersion was then allowed to cool gradually to room temperature and stored under appropriate conditions until further characterization. The use of GMO as the lipid phase and Poloxamer 407 as a stabilizer is consistent with established approaches for the preparation of non-lamellar LLCs formulation [16, 17].

optimization using BBD

The MTX-LLCs formulation was systematically optimized using a three-factor, three-level Box-Behnken design (BBD) employing Design-Expert® software version 13.0 (Stat-Ease Inc., Minneapolis, USA). A total of 15 experimental formulations were generated and evaluated to investigate the individual and interactive effects of the selected formulation and process variables, namely GMO concentration (X_1), Poloxamer P-407 concentration (X_2), and probe sonication cycles (X_3), on entrapment efficiency (%EE; Y_1) and vesicle size (Y_2). The experimental design and corresponding observed responses are presented in **Table 1**. Similar BBD-based optimization approaches have been successfully applied for GMO-based LLCs systems, where lipid concentration, stabilizer concentration, and processing conditions significantly influence on drug entrapment and vesicle size [18,19].

The experimental data were subjected to analysis of variance (ANOVA) to determine the statistical significance and adequacy of the fitted models. The effects of the independent variables and their interactions on %EE and vesicle size were further evaluated using response-surface methodology. The optimized MTX-LLCs formulation was selected using the numerical desirability function, with the formulation variables and response constraints simultaneously optimized to obtain maximum entrapment efficiency and minimum vesicle size. This systematic optimization strategy is consistent with recent reports demonstrating the suitability of BBD and desirability-based numerical optimization for developing LLCs drug-delivery systems. [20]

Table 1 Formulation variables used with their coded and actual levels

Independent Variables	Levels		
	Low (-1)	Medium (0)	High (+1)
GMO (mL)	1	1.5	2
Poloxamer 407 (mg)	100	150	200
Probe cycle (Nos)	10	20	30
Dependent Variables	Criteria		
EE (%)	Maximize		
Vesicle Size (nm)	Minimize		

Characterization of prepared batches

Determination Entrapment efficiency

The entrapment efficiency (EE) of MTX-LLCs was determined using an ultrafiltration–centrifugation method. Briefly, 1 mL of the MTX-LLCs dispersion was transferred into an ultra-centrifugal filter unit (10 kDa molecular weight cut-off; Millipore, USA) and centrifuged at 10,000 rpm for 30 min to separate untrapped (free) MTX from the LLCs-associated drug. The filtrate containing free MTX was collected, passed through a 0.22 µm membrane filter, and quantified using a validated UV-visible spectrophotometric method. The entrapment efficiency was calculated using Eq. (1) [22]:

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100$$

Determination of vesicle size, zeta potential and PDI

The vesicle size, polydispersity index (PDI), and zeta potential of MTX-LLCs were determined using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) at 25 ± 1 °C. Samples were appropriately diluted with deionized water before analysis to minimize multiple-scattering effects. Vesicle size and PDI were measured by dynamic light scattering, while zeta potential was determined from electrophoretic mobility. All measurements were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD) [21].

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed for pure MTX, GMO, Poloxamer 407, and MTX-LLCs using an FTIR spectrophotometer (Bruker Alpha II, ATR technique, Japan) to evaluate the compatibility of MTX with the formulation components of the LLCs [22].

Powder X-ray diffraction (P-XRD)

Powder X-ray diffraction (P-XRD) analysis was conducted to evaluate the crystalline nature of pure MTX and the optimized MTX-LLCs formulation using a Rigaku MiniFlex-600 X-ray diffractometer. The samples were analyzed using monochromatized Cu K α radiation ($\lambda = 1.742$ Å) over a 2θ range of 5°–80°, at an operating voltage of 30 kV and current of 30 mA [23].

Transmission electron microscopy analysis (TEM)

For transmission electron microscopy (TEM) analysis, the MTX-LLCs dispersion was diluted 50-fold with distilled water. A drop of the diluted dispersion was placed onto a 200-mesh copper grid and negatively stained with 2% phosphotungstic acid for 1 min. The grid was then allowed to dry at room temperature before being mounted on the specimen holder. The sample was examined using a JEM-1200 transmission electron microscope (JEOL Co. Ltd., Japan) operated at an accelerating voltage of 200 kV. TEM images were obtained to assess the morphology and structural features of the MTX-LLCs [23].

Incorporation of optimized MTX-LLCs into gel matrix

The optimized MTX-LLCs were incorporated into a Carbopol 940 gel matrix. Briefly, Carbopol 940 (2% w/w) was gradually dispersed into the optimized MTX-LLCs formulation under continuous stirring at 1200 rpm for 1 h. The dispersion was then allowed to hydrate overnight to ensure complete polymer swelling and formation of a uniform gel. Subsequently, eucalyptus oil (1% w/w) was incorporated as a permeation enhancer and mixed thoroughly. The pH was adjusted to the physiological range using triethanolamine (0.05% w/w), resulting in a homogeneous MTX-LLC-based gel. A conventional MTX gel was prepared similarly by incorporating MTX directly into a 2% w/w Carbopol 940 gel base. This approach is consistent with recent reports describing the incorporation of optimized lipid/lyotropic nanocarriers into Carbopol-based topical gels for enhanced localized drug delivery [24].

Characterization of MTX-LLCs-based gel

Determination of pH

The pH of the MTX-LLCs-based gel and conventional MTX gel was determined using a calibrated digital pH meter. A suitable quantity of gel was dispersed in distilled water, and the pH was measured at room temperature [25].

Rheological behavior

The viscosity of the MTX-LLCs-based gel and conventional MTX gel formulations was determined using a Brookfield rheometer equipped with a cone-and-plate assembly. Measurements were performed at a rotational speed of 5 rpm using spindle no. 7, maintained at a controlled temperature of $25 \pm 2^\circ\text{C}$ [25].

Ex-vivo skin permeation study

Albino Wistar rat skin was obtained from the Central Animal House, Tatyasaheb Kore College of Pharmacy, Warananagar, India, following euthanasia of the animals. The study was approved by the Institutional Animal Ethics Committee (IAEC; Protocol No. TKCP/08/2023/08) and conducted in accordance with CPCSEA guidelines. The excised skin was carefully cleaned to remove adhering hair and subcutaneous fat and then mounted between the donor and receptor compartments of a Franz diffusion cell, with the stratum corneum facing the donor compartment [1,2]. The receptor compartment was filled with 10 mL of phosphate-buffered saline (pH 5.5): ethanol (7:3, v/v) and maintained under continuous stirring at 600 rpm. MTX-LLCs-gel and conventional MTX gel (1 mg/mL) were applied to the donor compartment. At predetermined intervals (1, 2, 4, 6, 8, 12, and 24 h), 1 mL of receptor medium was withdrawn and immediately replaced with an equal volume of fresh medium to maintain sink conditions. The collected samples were suitably diluted and analyzed for MTX content using a Uv-visible spectrophotometer [26].

In vitro drug release study

The *in vitro* release of plain MTX, MTX-LLCs, and MTX-LLCs-based gel and conventional MTX gel was evaluated using the dialysis bag method at pH 6.8 (tumor microenvironment) and pH 7.4 (physiological conditions). The gel formulations were additionally evaluated at pH 5.5 to simulate the acidic skin environment.

Formulations containing an equivalent amount of MTX were placed in pre-soaked dialysis membranes (MWCO 12-14 kDa) and immersed in the respective release media maintained at $37 \pm 0.5^\circ\text{C}$ under continuous stirring. At predetermined intervals (0, 1, 2, 4, 6, 8, 12, and 24 h), aliquots were withdrawn and replaced with an equal volume of fresh medium to maintain sink conditions. The samples were analyzed for MTX content using a suitable validated analytical method, and the cumulative percentage drug release was calculated. The release profiles were compared to assess the sustained and pH-responsive release behavior of MTX-LLCs-based gel and conventional MTX gel relative to plain MTX [27].

In vitro cytotoxicity study

The MTT assay was used to evaluate the MTX, MTX-LLCs, and MTX-LLCs-based gel in MCF-7 breast cancer cells. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated at 37°C for 24 h in humidified atmosphere containing 5% CO_2 . The cells were then treated with indicated formulations at MTX equivalent concentrations ranging from 0.01 to 500 $\mu\text{g/mL}$ for the indicated period of time. Control cells were untreated cells. After treatment, MTT solution was added and incubated to form insoluble formazan crystals in viable cells. Crystals were dissolved in DMSO and absorbance was read at 570 nm with a microplate reader. Cell viability was expressed as percentage of untreated control, and IC_{50} values were calculated from the concentration-response curves [28].

Stability study

The stability study of the optimized MTX-LLCs and MTX-LLCs based gel was assessed as per ICH stability guidelines. The formulations were packed in screw-capped glass containers and kept under refrigerated conditions ($4 \pm 2^\circ\text{C}$) and accelerated conditions ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$) using environmental test chamber (REMI Electrotechnik Ltd., Mumbai, India). Samples were collected at pre-determined time points (0, 1, 2, 3 and 6 months) and analysed for changes in particle size, PDI, zeta potential and entrapment efficiency (%EE). Physical appearance and gel stability were also assessed throughout the study period [32].

RESULTS AND DISCUSSION

Optimization of MTX-LLCS using BBD

To optimize the prepared MTX-LLCs and evaluate its effects of formulation and process variables on critical quality attributes, a Quality-by-Design (QbD) approach was employed. A three-factor, three-level Box-Behnken design (BBD) was selected to efficiently assess quadratic interactions with a limited number of experimental runs. Accordingly, 15 formulations were generated and analyzed using Design-Expert® software (Version 13.0; Stat-Ease Inc.). Similar BBD-based approaches have been widely applied for optimizing LLCs nanocarriers, particularly for controlling vesicle size and drug entrapment

efficiency (**Table 2**) [30]. The MTX-LLCs were successfully prepared by a top-down approach, yielding homogeneous nanosized dispersions. The effect of the concentration of GMO (X_1), the concentration of Poloxamer 407 (X_2), and cycles of probe sonication (X_3) on entrapment efficiency (%EE; Y_1) and vesicle size (Y_2) was studied. The experimental batches 1, 4, 6, 7, 8, 11 and 15 exhibited significant variation in both responses, thereby confirming the influence of the selected formulation and process variables. The experimental data were analysed by ANOVA, and the fitted quadratic models were found to be statistically significant ($p < 0.05$) with a satisfactory correlation between the predicted and experimental responses. Lack-of-fit was not significant, which further confirmed the adequacy of the models to describe the experimental data [23]. The concentration of GMO (X_1) showed a positive effect on %EE (Y_1) and vesicle size (Y_2), which may be due to the increase in the lipid content available for drug incorporation and LLCs formation. In contrast, the increase in the concentration of Poloxamer 407 (X_2) led to the reduction of vesicle size and better stabilisation of the dispersion due to the increase in the interfacial stabilisation. The increase in cycles of probe sonication (X_3) significantly decreased the vesicle size as larger aggregates were disrupted and finer LLCs dispersions were formed. However, excess sonication had a slight negative effect on %EE. Interaction effects, especially between GMO and Poloxamer 407, indicated that an optimal lipid-to-stabiliser ratio is required for favourable particle characteristics (**Figure 1a-b**). A desirability-function approach was then used for numerical optimization with the objectives of maximising %EE and minimising vesicle size. The optimised formulation was a relatively high concentration of GMO, moderate concentration of Poloxamer 407 and higher probe-sonication cycles. Then the optimised batch was prepared experimentally, and the observed responses were in close agreement with the predicted values, confirming the predictive ability and reliability of the developed models. In conclusion, the BBD-based QbD approach efficiently identified the critical formulation and process variables and enabled the development of a reproducible MTX-LLC formulation with desirable physicochemical characteristics [33].

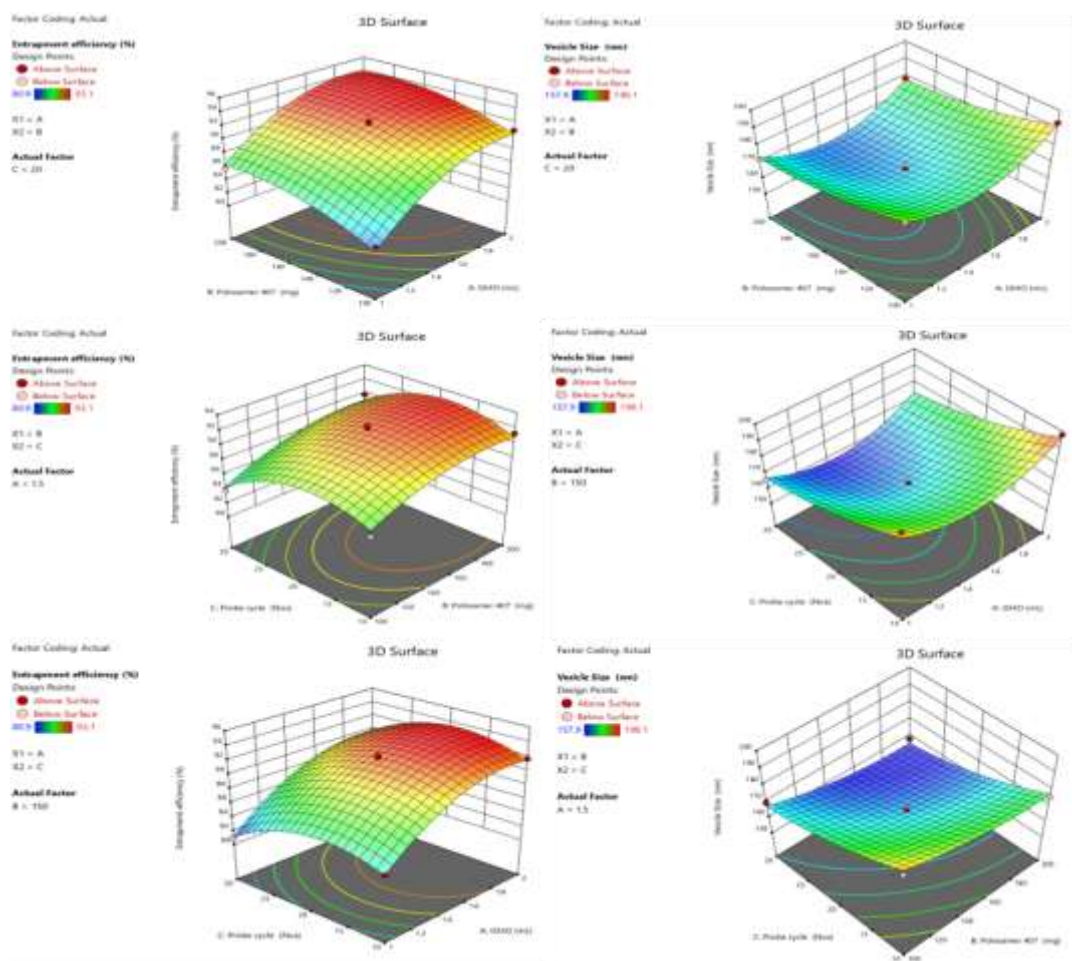


Fig. 1a. The response surface plots (3D) showing the effect of GMO (X_1), poloxamer-407 (X_2) and probe cycle (X_3) on entrapment efficiency (Y_1) and particle size (Y_2)

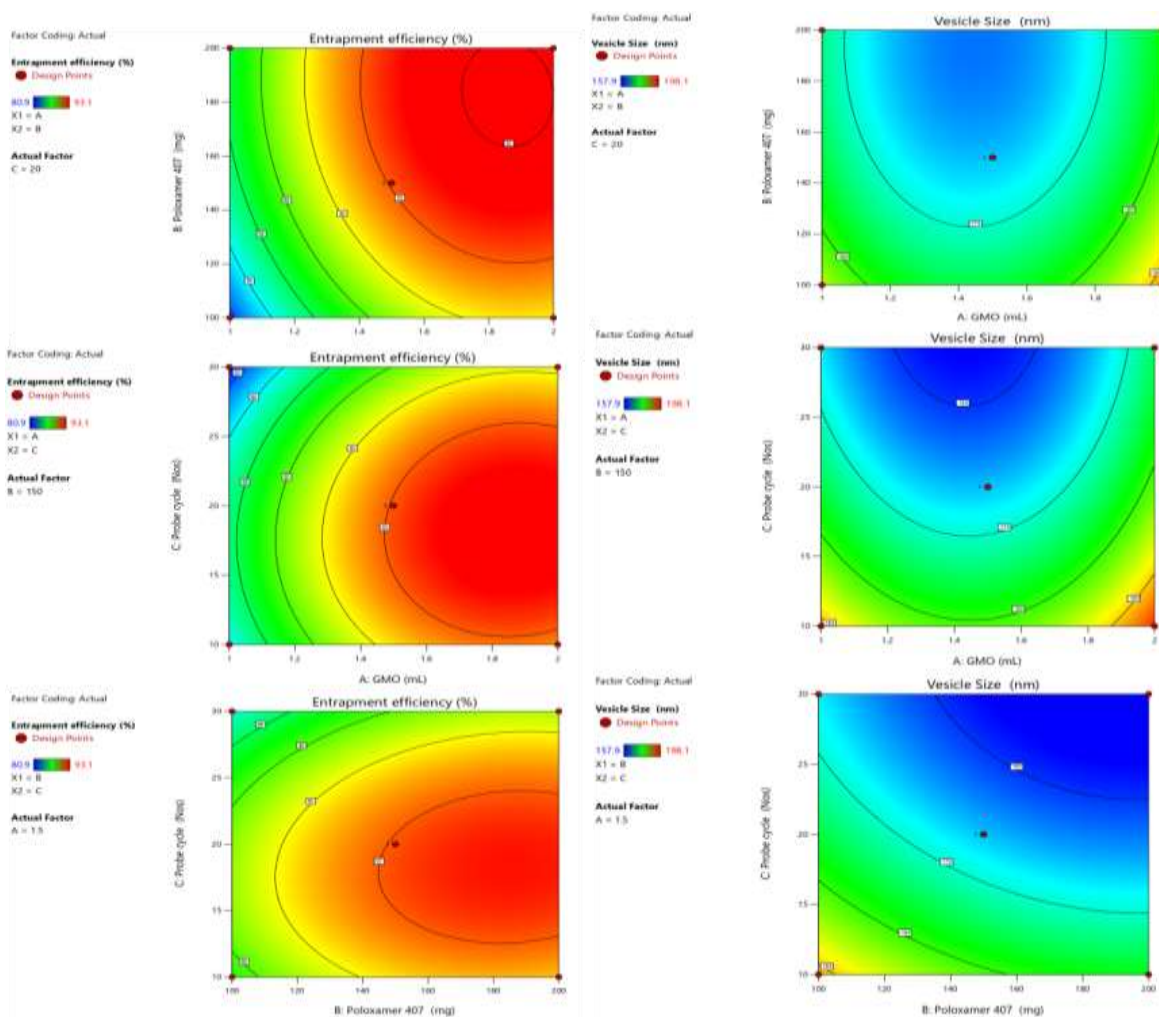


Fig. 1b. The contour plots (3D) showing the effect of GMO (X1), poloxamer-407 (X2) and probe cycle (X3) on entrapment efficiency (Y1) and particle size (Y2)

Table 2 Experimental runs generated using BBD runs

Formulation Code	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	GMO (mL)	PF-407 (mg)	Probe Cycle (Nos)	Entrapment efficiency (%)	Vesicle size (nm)
F1	1.0	100	20	82.40 ± 0.85	184.61 ± 3.21
F2	2.0	100	20	90.81 ± 0.72	193.23 ± 3.64
F3	1.0	200	20	85.73 ± 0.91	171.89 ± 2.87
F4	2.0	200	20	83.16 ± 0.76	178.55 ± 3.12
F5	1.0	150	10	84.12 ± 0.68	194.32 ± 3.48
F6	2.0	150	10	91.70 ± 0.63	198.16 ± 3.71
F7	1.0	150	30	80.92 ± 0.94	165.78 ± 2.65

F8	2.0	150	30	89.64 ± 0.81	171.24 ± 2.91
F9	1.5	100	10	86.50 ± 0.73	188.71 ± 3.25
F10	1.5	200	10	91.31 ± 0.69	175.45 ± 2.94
F11	1.5	100	30	83.82 ± 0.88	169.59 ± 2.76
F12	1.5	200	30	89.85 ± 0.75	157.98 ± 2.58
F13	1.5	150	20	92.14 ± 0.57	155.31 ± 2.41
F14	1.5	150	20	91.86 ± 0.64	166.22 ± 2.73
F15	1.5	150	20	90.47 ± 0.79	165.16 ± 2.69

Values are mean ± SD (n=3)

Desirability plot

Desirability plots (**Figure 2**) revealed a well-defined optimum region with overall desirability of about 0.96, representing a satisfactory compromise between maximisation of entrapment efficiency (%EE) and minimisation of vesicle size. Response surfaces showed that increasing GMO (X_1) from a low level to an intermediate-high level increased overall desirability, but too high GMO levels led to a less favourable response. Poloxamer 407 (X_2) showed an optimum at intermediate-to-high concentrations, indicating the significance of an appropriate lipid-to-stabiliser ratio for the formation and stabilisation of nanosized LLCs. Increasing probe-sonication cycles (X_3) generally improved desirability by decreasing vesicle size, with an optimum region found around 23-25 cycles. The combined response surface indicated an optimised region of approximately 1.6 mL GMO, 160-170 mg Poloxamer 407 and 23-25 sonication cycles with a desirability of ~0.96. These results are in agreement with recent studies suggesting that concentration of GMO, amount of Poloxamer 407 and processing conditions have significant influence on the vesicle size and entrapment efficiency of cubosomal/lyotropic liquid-crystalline systems [34].

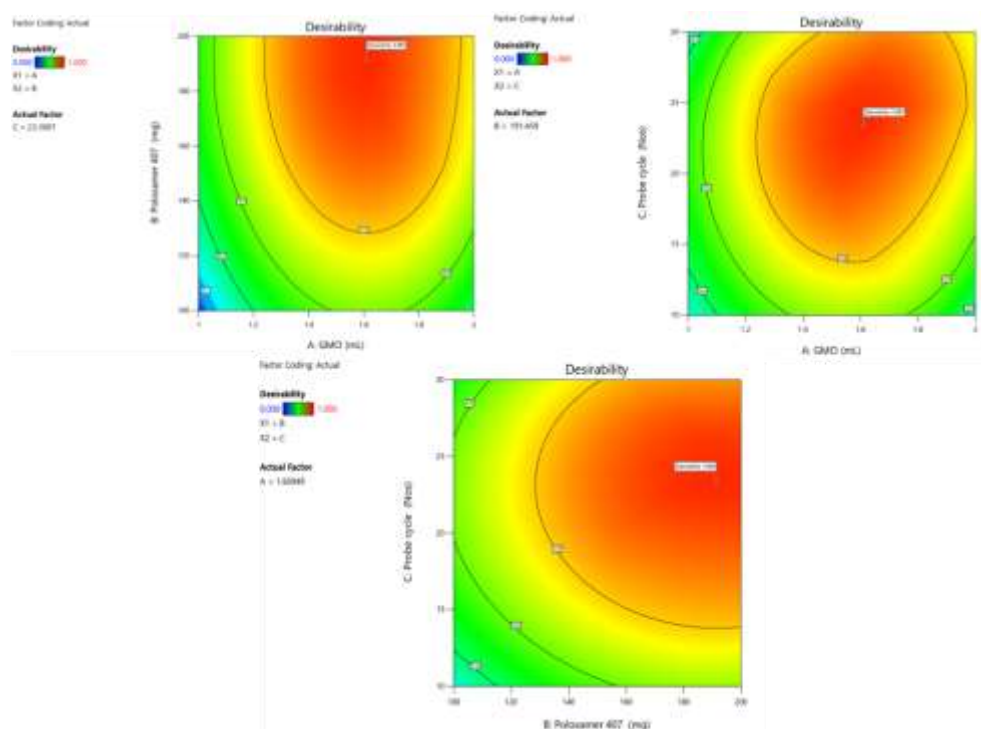


Fig. 2. Graphical representation of the desirability function and optimized levels of X_1 , X_2 and X_3 for % EE (Y_1) and particle size (Y_2)

Effect of independent variables on entrapment efficiency

Results of ANOVA analysis showed that the quadratic model had significant effect on the entrapment efficiency ($F = 34.27$, $p = 0.0006$). The maximum influence was observed for the concentration of the GMO (X_1) ($F = 172.08$, $p < 0.0001$) followed by the concentration of Poloxamer 407 (X_2 ; $F = 44.92$, $p = 0.0011$) and the probe sonication cycle (X_3 ; $F = 15.07$, $p = 0.0116$) indicating their significant contribution to drug entrapment. The positive effect of GMO could be related to the increase in the availability of lipids and drug incorporation in the LLCs matrix, while Poloxamer 407 could improve the interfacial stabilization and avoid drug leakage. The more sonication cycles, the better the particle disruption and homogenization and consequently the more efficient drug incorporation. The significant quadratic terms (X_1^2 , X_2^2 , and X_3^2 ; $p < 0.05$) indicated the nonlinear effect of the formulation variables. The interaction terms were non-significant ($p > 0.05$). The insignificant lack of fit ($F = 13.19$, $p = 0.0713$) confirms the suitability of the fitted model for prediction of entrapment efficiency (**Table 3**) [34].

Table 3 ANOVA for quadratic model of entrapment efficiency

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	230.86	9	26.56	34.27	0.0006	significant
Residual	3.74	5	29.73			
Lack of Fit	3.56	3	1.19	13.19	0.0713	Non-significant
Pure Error	0.1800	2	0.0900			
Cor Total	234.60	14				

Effect of independent variables on Vesicle size

The quadratic model had a highly significant effect on the vesicle size ($F=23.81$, $p=0.0014$). The concentration of Poloxamer 407 (X_2 ; $F = 34.23$, $p = 0.0021$) was the most influential formulation variable on the vesicle size followed by the concentration of GMO (X_1 ; $F = 7.54$, $p = 0.0405$) ($F = 105.98$, $p = 0.0001$). Increased sonication cycles should reduce the size of the vesicle due to increase in shear stress, particle breakup and homogenisation. Poloxamer 407 decreases the interfacial tension and stabilises smaller vesicles. (X_1^2 ; $F = 61.48$, $p = 0.0005$) A strong quadratic effect of GMO content was observed, indicating a non-linear effect on vesicle size. Other quadratic and interaction terms were not significant ($p > 0.05$). The non-significant lack of fit ($F = 54.42$, $p = 0.0681$) [35] further supports the suitability of the model to predict vesicle size (**Table 4**) [35].

Table 4 ANOVA for quadratic model of vesicle size

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2148.27	9	238.70	23.81	0.0004	significant
Residual	50.13	5	10.03			
Lack of Fit	49.52	3	16.51	54.42	0.0681	Non-significant
Pure Error	0.6067	2	0.3033			
Cor Total	2198.40	14				

PDI

The optimised MTX-LLCs showed low PDI of 0.231 ± 0.07 indicating narrow and uniform vesicle size distribution. PDI value less than 0.5 represents good homogeneity of the formulation and less particle aggregation. This confirms the efficacy of the optimised formulation to obtain a stable and homogeneously dispersed nano-sized system.

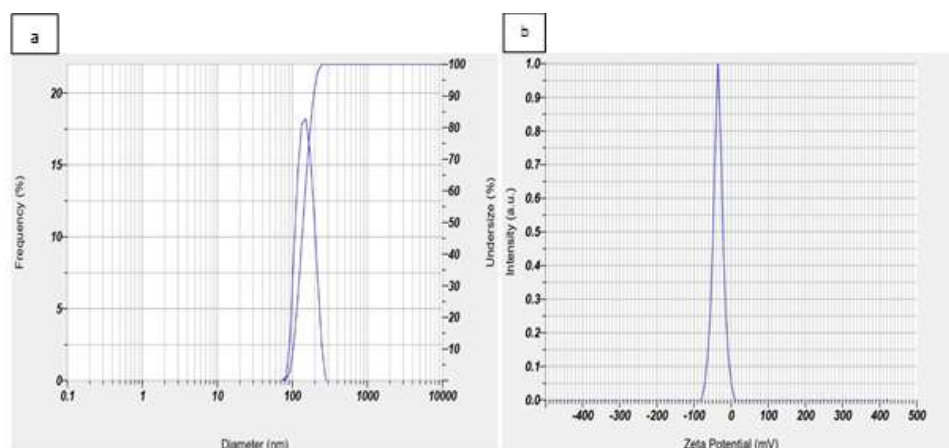


Fig. 3. Mean particle size of (a) Optimized MTX-LLCs, (b) Zeta potential of MTX-LLCs

FTIR

The FTIR spectra of pure MTX, GMO, Poloxamer 407 and optimized MTX MTX-LLCs are shown in **Figure 4**. Pure MTX showed characteristic bands at $3410\text{--}3450\text{ cm}^{-1}$ (O-H/N-H stretching), $2920\text{--}2940\text{ cm}^{-1}$ (aliphatic C-H stretching), $1650\text{--}1670\text{ cm}^{-1}$ (C=O stretching), $1600\text{--}1500\text{ cm}^{-1}$ (aromatic C=C/N-H vibrations) and $1300\text{--}1200\text{ cm}^{-1}$ (C-N stretching), confirming its characteristic functional groups. GMO showed sharp peaks at $\sim 3400\text{ cm}^{-1}$ (O-H), 2920 and 2850 cm^{-1} (CH_2 stretching), $1735\text{--}1745\text{ cm}^{-1}$ (ester C=O) and $1230\text{--}1050\text{ cm}^{-1}$ (C-O-C and glycerol backbone vibrations). Characteristic bands of Poloxamer 407 were observed at $3400\text{--}3450\text{ cm}^{-1}$ (O-H), $2880\text{--}2890\text{ cm}^{-1}$ (C-H stretching), and $1100\text{--}1060\text{ cm}^{-1}$ (C-O-C ether stretching). The characteristic bands of MTX, GMO, and Poloxamer 407 were preserved in the optimised MTX-LLCs with slight shifts and peak intensity changes. Major O-H, CH₂, ester carbonyl, MTX carbonyl and ether bands were preserved, indicating retention of the principal functional groups after formulation. The small spectral shifts and intensity decrease can be attributed to hydrogen bonding and hydrophobic interactions between MTX and the lipid-surfactant matrix. Therefore, the absence of new characteristic peaks or disappearance of major drug-related bands confirmed the absence of chemical incompatibility or degradation, supporting the successful incorporation of MTX into the LLCs matrix.

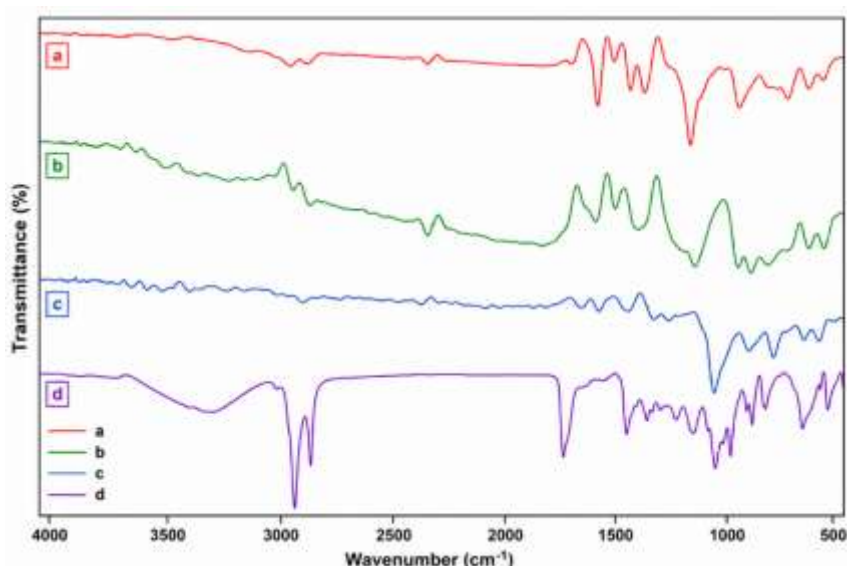


Fig. 4. FTIR spectra of (a) Pure MTX, (b) GMO (c) Ploxamer-407 (d) MTX-LLCs

P-XRD

Figure 5 shows the P-XRD patterns of pure MTX and optimized MTX-LLCs. The pure MTX showed many sharp and intense diffraction peaks at $2\theta \approx 9.8^\circ, 12.0^\circ, 13.4^\circ, 15.0^\circ, 16.8^\circ, 18.3^\circ, 19.9^\circ, 20.8^\circ, 22.4^\circ, 24.8^\circ, 26.2^\circ$ and 27.1° , which

confirm the highly crystalline nature of the MTX. The most intense reflection was at $2\theta \approx 9.8^\circ$ with intensity ~ 6500 counts, while other significant peaks in the $10-30^\circ$ range had intensities of $\sim 3000-4700$ counts. The intensity and the number of the characteristic diffraction peaks of MTX decreased significantly after incorporation into the LLCs system. The intensity of the main peak at $2\theta \approx 9.8^\circ$ was reduced to about 2000 counts, which corresponds to about 69% reduction. Similarly, the reflections in the $18-22^\circ$ region were reduced from about 3200-3400 counts to 1200-1900 counts. Several MTX peaks were significantly broadened or suppressed and only weak reflections (<800 counts) were seen at higher angles. These changes indicate a loss of crystallinity of MTX, which suggests a partial amorphization and/or molecular dispersion of the drug in the lipidic matrix. Therefore, only the expected diffraction peaks were observed, that is no detectable new crystalline phase or polymorphic transformation occurred during the formulation. The observed decrease in the crystallinity of MTX in the matrix of LLCs may be due to the increased solubilization of the drug, which may be advantageous for sustained release and improved drug permeation through the skin.

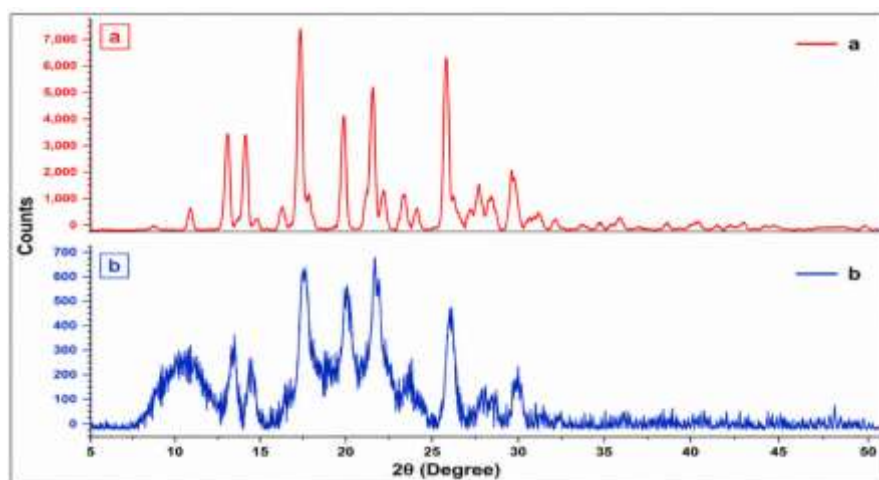


Fig. 5. XRD of (a) Pure MTX, (b) MTX-LLCs

TEM

TEM analysis (**Figure 6**) showed that the optimised MTX-LLCs were composed mainly of discrete, spherical to near-spherical nanostructures with relatively smooth and well-defined boundaries. The particles were dispersed individually with limited aggregation, which supports the nanoscale vesicle size and low PDI values obtained by dynamic light scattering. The observed morphology and the homogeneity of the dispersion can be explained by the stabilising effect of Poloxamer 407 and the homogenization/sonication process that permits efficient disruption and stabilisation of the lipid structures. The TEM results are consistent with the physicochemical characterisation results and confirm the successful formation of well dispersed nanosized MTX-LLCs.

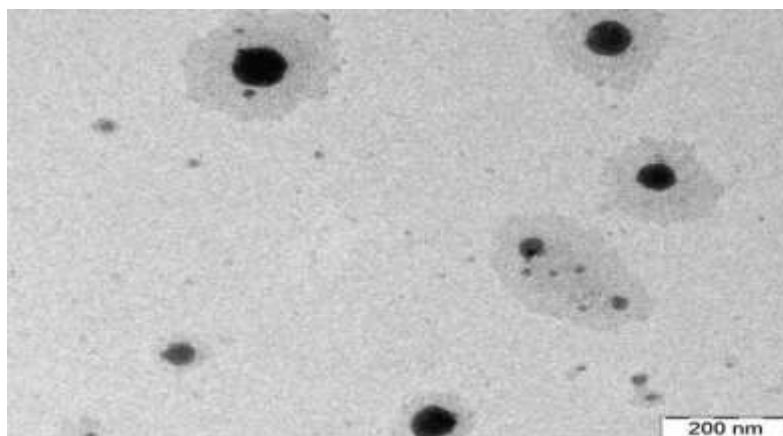


Fig. 7. TEM Morphology study of MTX-LLCs

Characterization of MTX-LLC-based gel

pH

The pH of the formulations was found to be in the range of 5.2-5.8, which is close to the natural pH of the skin. Such pH compatibility is advantageous for topical application, as it is expected to enhance MTX permeation through the skin while reducing the likelihood of skin irritation and ensuring better patient acceptability.

Vesicle size and zeta potential

The vesicle size of the MTX-LLCs based gel was found to be 195.6 ± 14 nm with PDI of 0.23 ± 0.02 whereas, the vesicle size of the MTX-conventional gel was comparatively larger, 274.5 ± 12 nm with PDI of 0.43 ± 0.04 . The values of zeta potential were -38.6 ± 4.2 mV for MTX-LLCs gel (Figure 8c), and -44.7 ± 5.8 mV for conventional gel. The smaller vesicle size and PDI of the MTX-LLCs-based gel is indicative of a relatively homogenous nanosized dispersion that can be useful for better drug deposition and penetration via the skin. The zeta potential values above ± 30 mV also exhibited good electrostatic stabilisation and good colloidal stability of both the formulations.

Rheological behavior

Viscosity is an important rheological parameter for topical formulations since it influences spreadability, drug release, application characteristics and formulation stability. The viscosity of MTX-LLCs gel and MTX-conventional gel were 6.45 ± 0.28 Pa·s and 9.68 ± 0.39 Pa·s, respectively. The relatively lower viscosity of the MTX-LLCs-based gel may allow a better spreadability and support-controlled drug release in comparison with the conventional gel. In conclusion, the optimised LLCs-based gel showed good rheological properties for topical application and could offer better formulation performance [31].

Ex-vivo skin permeation

The MTX-LLCs-based gel showed significantly higher membrane permeation compared to the conventional gel during the entire study (Figure 8). The cumulative amount permeated after 8 h was 249.3 ± 4.27 $\mu\text{g}/\text{cm}^2$ for the LLCs-based gel, while it was 69.4 ± 5.12 $\mu\text{g}/\text{cm}^2$ for the conventional gel. The increased permeation could be attributed to the nanostructured liquid-crystalline lipid matrix that enhances MTX solubilisation and favours drug diffusion due to its small vesicle size and high surface area. Furthermore, the sustained drug release from the LLCs may maintain a favourable concentration gradient across the membrane and support the enhanced permeation and the potential of the formulation for prolonged topical MTX delivery [35].

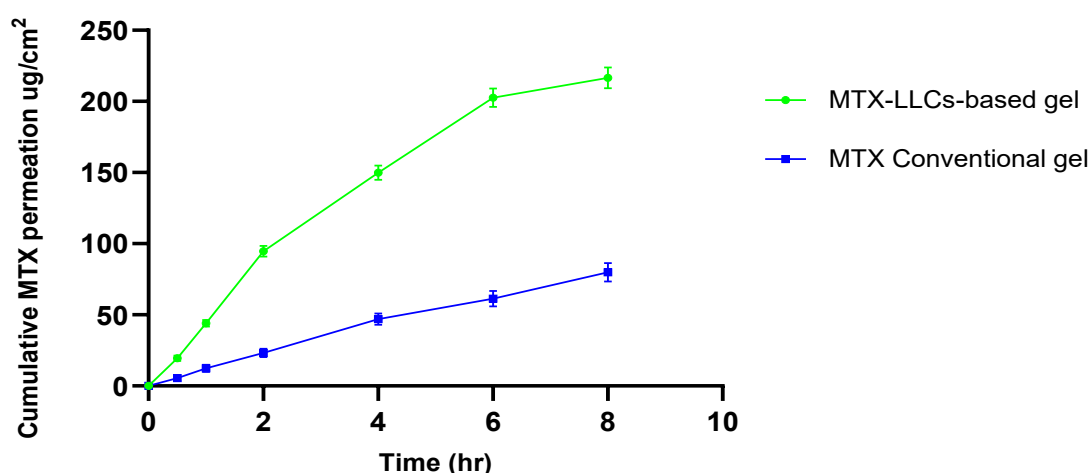


Fig. 8. In vitro release profile of MTX-LLCs-in various PBS a) in pH 7.4 b) in pH 6.8 c) in pH 5.5

In-vitro drug release study

In vitro release profiles of pure MTX dispersion, MTX-loaded LLCs, MTX-LLCs-based gel and MTX conventional gel were studied in PBS at pH 7.4, 6.8 and 5.5 (**Figure 9a-e**). Pure MTX dispersion provided a rapid drug release of nearly 91.52% in 6 h at pH 7.4 and 6.8. In contrast, MTX-LLCs showed a controlled release profile with $45.8 \pm 2.6\%$ and $48.6 \pm 3.1\%$ of drug release at 6 h at pH 7.4 and 6.8, respectively, and then gradually increased to about 91% after 48 h. The controlled release was because of effective entrapment of MTX in liquid crystalline lipid matrix which restricted the drug to diffuse rapidly and reduced the initial burst effect. The MTX-LLCs-based gel showed further prolongation of drug release, $31.9 \pm 4.1\%$ release at pH 7.4 after 48 h compared to $63.7 \pm 3.5\%$ for the conventional gel. The LLC gel showed an enhanced release at slightly acidic pH values, with $75.6 \pm 5.2\%$ at pH 6.8 and $81.8 \pm 4.7\%$ at pH 5.5, while the conventional gel showed $61.5 \pm 4.5\%$ and $45.8 \pm 5.5\%$, respectively ($p < 0.05$). The increase in the release in acidic conditions might be related to the pH-dependent modifications of the lipidic matrix facilitating the diffusion of MTX. The overall gel based on MTX-LLCs showed sustained release of the drug in physiological conditions and enhanced release in acidic medium suggesting its potential for localised delivery of the drug in a pH-responsive manner with prolonged therapeutic action and reduced systemic exposure [18-20,34-35].

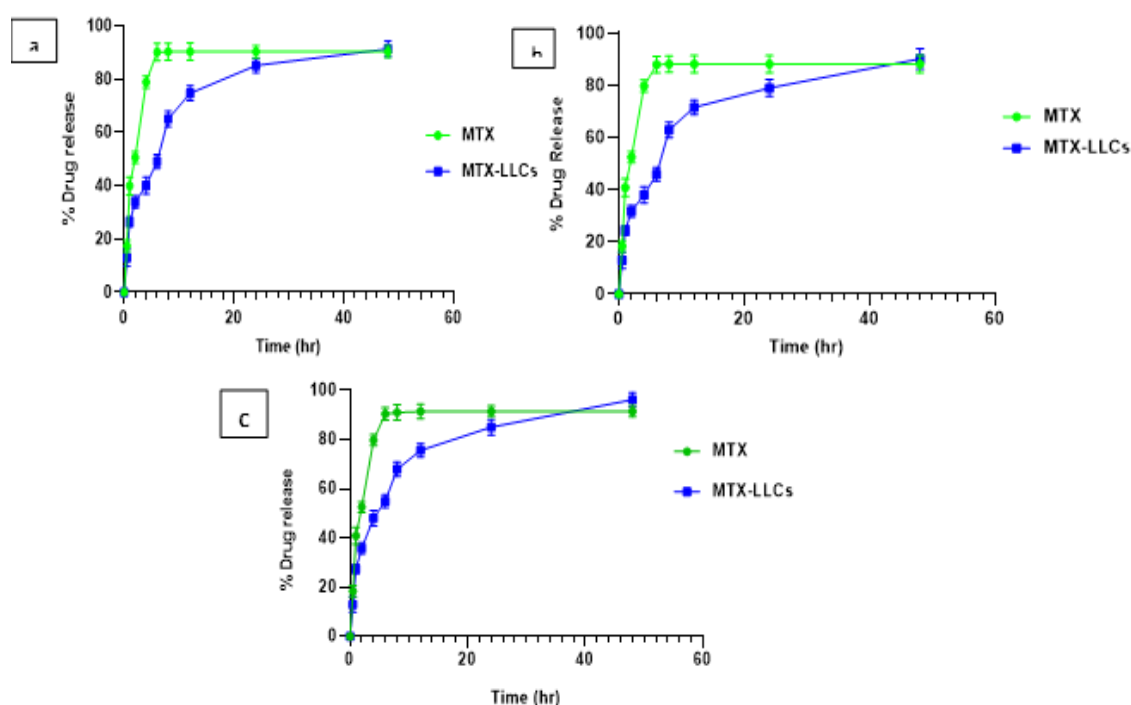


Fig. 9. In vitro release profile of MTX-LLCs-in various PBS a) in pH 7.4 b) in pH 6.8 c) in pH 5.5

In vitro cytotoxicity study

The plain MTX showed an IC_{50} value of $42.16 \pm 3.92 \mu\text{g/mL}$ in MCF-7 cells, while the IC_{50} value for MTX loaded LLCs was significantly lower ($24.85 \pm 2.06 \mu\text{g/mL}$), indicating an enhanced cytotoxic activity after incorporation of LLCs. The gel of MTX-LLCs showed the highest antiproliferative activity with the lowest IC_{50} value of $19.86 \pm 2.18 \mu\text{g/mL}$ (**Figure 10**). The gel based on LLCs showed nearly 2.1-fold more cytotoxic potency than plain MTX probably due to enhanced cellular uptake and sustained availability of MTX from the nanostructured lipidic system.

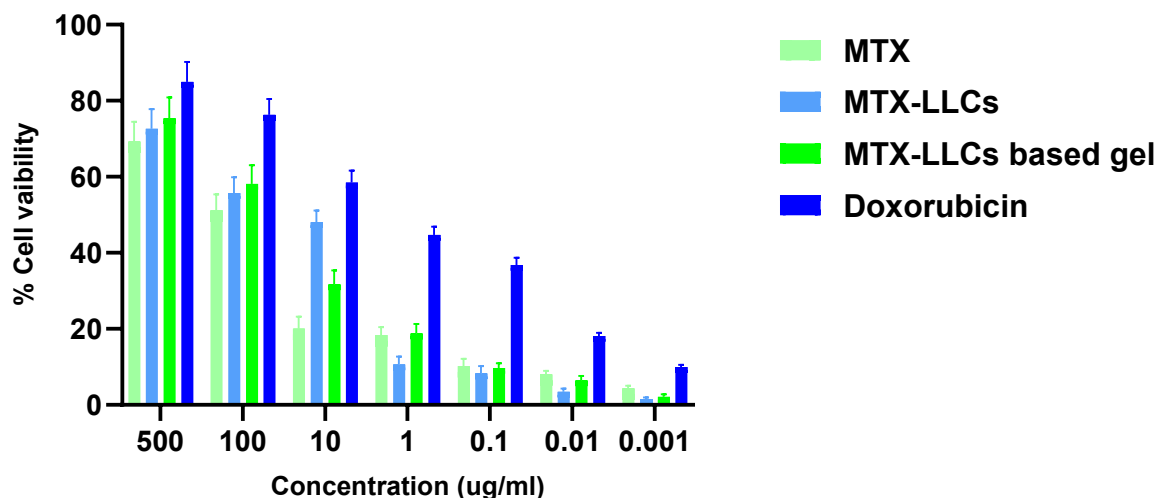


Fig. 10. *In vitro* cell viability graph of formulations against MCF-7 cells

Table 5 IC₅₀ values of MTX and MTX-LLCs against breast cancer sensitive (MCF-7) cells

Formulation	IC ₅₀ value (µg/mL) after 24 h
	MCF-7 Cells IC ₅₀ (µg/mL)
Plain MTX	42.16 ± 3.92
MTX-LLCs	24.85 ± 2.06
MTX-LLCs-based gel	19.86 ± 2.18

Values are expressed as mean ± SD (n = 3)

Stability studies

Stability studies of optimized MTX-LLCs and MTX-LLCs based gel were carried out under refrigerated (4 ± 2 °C) and accelerated (40 ± 2 °C/ $75 \pm 5\%$ RH) conditions. The entrapment efficiency of the MTX-LLCs under refrigeration remained almost unchanged after 6 months, from $92.1 \pm 3.70\%$ to $90.25 \pm 2.60\%$, with a small increase in vesicle size from 155.3 ± 3.5 to 170.8 ± 3.6 nm and PDI from 0.23 ± 0.02 to 0.29 ± 0.03 . The entrapment efficiency decreased to $87.6 \pm 2.9\%$ and a moderate increase in vesicle size (181.4 ± 4.2 nm) and PDI (0.34 ± 0.04) was observed under accelerated conditions indicating some particle growth during storage. The gel based on MTX-LLCs was homogeneous and did not show any visible phase separation, precipitation or syneresis under either of the storage conditions. The pH was in the acceptable range with slight changes from 5.5 ± 0.1 to 5.4 ± 0.1 under refrigerated storage and 5.3 ± 0.1 under accelerated storage. Little decrease in viscosity was observed during storage. Overall, the formulations showed better stability when refrigerated and the moderate changes obtained in the accelerated storage were within the acceptable limits, confirming the physicochemical stability of the optimized MTX-LLCs based gel during storage.

CONCLUSION

The present study was successfully developed MTX-LLCs and incorporated them into a Carbopol 940 topical gel for localized breast cancer therapy. The Quality-by-Design approach using Box-Behnken Design effectively optimized the formulation, producing $92.14 \pm 0.57\%$ entrapment efficiency, 155.31 ± 2.41 nm vesicle size, and 0.231 ± 0.07 PDI. FTIR, P-XRD, and TEM studies confirmed successful drug incorporation and formation of well-dispersed nanosized structures. The optimized LLCs-based gel exhibited suitable physicochemical and rheological properties, sustained and pH-responsive drug

release, and markedly improved ex-vivo skin permeation compared with conventional MTX gel. Furthermore, enhanced cytotoxicity against MCF-7 cells was observed, with an IC_{50} of $19.86 \pm 2.18 \mu\text{g/mL}$ compared with $42.16 \pm 3.92 \mu\text{g/mL}$ for plain MTX. The formulation also demonstrated acceptable stability, particularly under refrigerated conditions. Overall, the developed MTX-LLCs-based gel shows promising potential as a localized, sustained, and effective delivery system for breast cancer therapy.

DECLARATION OF COMPETING INTEREST

The authors declare no competing financial interests or personal relationships that could have influenced the research presented in this paper.

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