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Original Article

Development and Optimisation of Luteolin-Loaded Liposomal Hydrogel for Topical Drug Delivery Using Box-Behnken Design

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ABSTRACT

Background: The current work aimed to formulate and statistically optimise a luteolin-loaded liposomal hydrogel for topical medication administration to increase drug encapsulation, physicochemical stability, and controlled drug release. Luteolin is promising as an antioxidant and anti-inflammatory agent, but its pharmaceutical use is limited by low solubility in water and poor stability. The integration of luteolin into liposomes, and subsequently into a Carbopol 940 hydrogel, was studied to enhance its topical distribution and residence time at the site of application. Methods: The thin-film hydration approach was used to create luteolin-loaded liposomes, which were then added to a Carbopol 940 hydrogel matrix. Optimisation of the concentrations of phosphatidylcholine, cholesterol, and Carbopol 940, along with zeta potential, particle size, entrapment efficiency, and cumulative drug release as important responses, was carried out using a 3-factor, 3-level Box-Behnken design. The improved formulation was assessed for pH, viscosity, spreadability, extrudability, bioadhesive strength, drug content, zeta potential, particle size, PDI, morphology, *in vitro* drug release, release kinetics & accelerated stability. Results: The optimal preparation was LH8, with a particle size of 51.29 nm, a PDI of 0.287, a Zeta potential of -34.8 mV, an entrapment efficiency of 87.64%, and a cumulative drug release of 88.92%. The formulation displayed appropriate topical properties and essentially spherical vesicles with a well-defined bilayer structure. The accelerated stability study showed relatively small changes in the main formulation properties throughout a period of six months. Conclusion: The optimised luteolin-loaded liposomal hydrogel showed favourable physicochemical properties, effective drug encapsulation and regulated drug release with good accelerated stability. These results suggest its potential as a topical delivery method for luteolin, but more *in vitro*, *in vivo,* and clinical research are necessary to determine its effectiveness and safety.

Keywords: Box-Behnken design, Carbopol 940, Liposomal hydrogel, Liposomes, Thin-film hydration method, Topical drug delivery

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INTRODUCTION

As the main organ of human beings, the skin serves as the primary defense against microbiological, chemical, and physical harm. When the integrity of the skin is broken, a well-regulated series of biological processes begins, including inflammation, haemostasis, tissue remodeling & proliferation, all attempting to restore the skin's functional and physical integrity. This repair process can be effective depending on several intrinsic and extrinsic factors such as age, infection, metabolic disorders, and oxidative stress. As a result, the adoption of improved topically administered drugs that seek to both optimise drug administration and provide an environment conducive to tissue regeneration is becoming more and more common.[1] Therapeutic agents are still administered topically via such conventional formulations as creams, ointments, and gels. But these dosage forms often have drawbacks like poor penetration of the drug into the skin, insufficient retention at the application site, instability of the bioactive molecules, and uncontrolled release of the drug, which

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can lead to therapeutic failure and frequent dosing. However, recent developments in nanotechnology for pharmaceutical applications have enabled the development of alternative methods to overcome these shortcomings by improving drug solubility, protecting sensitive compounds from degradation, increasing the permeation of the skin, and allowing sustained drug release.[2,3]

Liposomes are composed mainly of phospholipid bilayers that closely resemble biological membranes, allowing them to interact efficiently with skin tissues. Although these vesicular systems have desirable characteristics for pharmaceutical applications in topical medicine, traditional liposomal dispersions tend to be low in viscosity and have a short duration of action at the skin's surface.[4,5] To overcome these drawbacks, incorporation of liposomes in hydrogel matrices is an effective formulation strategy. Hydrogels are hydrophilic polymeric networks, three-dimensional, that can hold a significant amount of H_2O and exhibit desirable rheological properties, prolonged residence time in the body, and patient-friendly application properties. Liposomal hydrogels are the combination of controlled-release liposomes and the structural stability and bioadhesive properties of hydrogels, which can be used as multifunctional delivery systems for topical administration.[6,7]

Many therapeutic fruits, herbs, and vegetables contain luteolin (3',4',5,7-tetrahydroxyflavone), a plant-based flavonol. Luteolin has been extensively studied in pharmacology and has been found to exhibit several cytoprotective properties, including anti-inflammatory, antioxidant, antimicrobial, and biological activities. Therefore, the encapsulation of luteolin in nanocarrier systems has proposed as a promising policy to enhance its physicochemical properties and enable its incorporation into topical formulations.[8]

Optimisation of drug product formulation is now a common approach in modern pharmaceutical product development and is increasingly performed systematically using statistical experimental designs. The QbD (Quality by Design) principles suggest using a structured experimental approach to identify critical formulation variables and their effects on critical quality attributes. Of these, using Response Surface Methodology (RSM) and Box–Behnken Design (BBD) is an effectual optimisation technique with a minimum number of experimental runs that can effectively estimate the individual, interaction, and quadratic effects of the formulation variables. This strategy is widely adopted for optimising nanocarrier-based drug delivery schemes due to its ability to improve drug delivery system robustness, reproducibility, and product quality.[9]

The present study successfully developed a luteolin-loaded liposomal hydrogel using the thin-film hydration technique and incorporated it into a Carbopol 940 hydrogel matrix. Cumulative drug release, zeta potential, entrapped efficiency, and particle size were selected as the essential quality responses. Physicochemical characteristics of the improved formulation, including pH, viscosity, spreadability, extrudability, biological adhesive strength, drug content, particle size distribution, standardised zeta potential, *in vitro* drug-release behaviour, release kinetics, and accelerated stability, were then assessed. This study offers a systematic formulation strategy that has been employed to create an optimised liposomal hydrogel for topical drug delivery, and that will serve as a basis for further studies in the field of biological and preclinical research.[10,11]

The present study intended to develop an effective liposomal luteolin-loaded hydrogel via the thin-film hydration method and subsequently incorporate it into a Carbopol 940 hydrogel matrix. A 3-factor, 3-level Box–Behnken Design was used to augment the concentration of phosphatidylcholine, cholesterol, and Carbopol 940, with the critical quality responses being cumulative drug release, the zeta potential, the particle size, and the entrapment efficiency. The optimised preparation was then assessed for physicochemical properties, including pH, viscosity, spreadability, extrudability, bioadhesive strength, drug content, particle-size distribution, zeta potential, release kinetics, *in vitro* drug-release behaviour and accelerated stability. In this study, a systematic formulation strategy is proposed for preparing an optimised liposomal hydrogel for the topical delivery of drugs, and a framework for further biological and preclinical studies is outlined.

MATERIAL AND METHODS

Soy lecithin, cholesterol, luteolin (BLD Pharma Tech (India) Pvt. Ltd., Hyderabad, Telangana, India), chloroform, ethanol, phosphate-buffered saline, triethanolamine, Carbopol 940, and glycerin (CDH).

Calibration Curve of Luteolin by Ultraviolet Spectrophotometry

100 milligrams of luteolin were weighed and dissolved in 100 mL of ethanol to create a main stock solution with a concentration of 1000 µg/mL. Further dilution was carried out using ethanol. To get an ultimate concentration of 100 µg/mL, dilute 1 mL of the main standard solution to 10 mL. To get the end result of 2.0 µg/ml, dilute 0.2 millilitres of a secondary standard solution to ten millilitres with ethanol. A 10-millilitre volumetric container was used, and 0.2 mL of the secondary standard solution was taken, with the volume made up to 10 mL to achieve a 2 µg/ml concentration. Initial spectral analysis in the 200-400 nm region showed the highest wavelength. The longest wavelength observed was 350 nm.[12]

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Drug excipients compatibility study by using FTIR

The FTIR (Fourier Transform Infrared spectroscopy) spectrophotometer was used to measure drug-excipient interaction. The analysis was conducted by preparing samples of the required powder mixed with KBr at a 1:100 ratio. Each cell was then scanned using an FTIR instrument (PerkinElmer Spectrum IR version 10.7.2) in the range of 4000 to 450 cm^{-1} wavenumbers, with transmittance spectra being recorded. The analysis focused on the detection of shifts in the drug spectra induced by polymer excipients, thereby aiding the evaluation of physical connections between the drug and the excipients.[13]

Experimental Design

Numerical approaches are an essential part of systematic formulation analysis, and response surface methodology (RSM) utilises them. It is easy to find the best settings for crucial process factors that affect the response surface while observing how they change at different levels of the parameters. This study used Design-Expert® (Version 12.0.3.0) to examine the main nonlinear effects and the interplay between both independent and dependent elements in a basic composite quantitative screening assay.

$$Y = b0 + b1X1 + b2\,X2 + b3\,X1X2 + b4\,X1^2 + b5\,X_2^2 + b6\,X1X_2^2 + b7X1^2X2 \quad (1)$$

To optimise the development of the luteolin-loaded liposomal hydrogel, a Box–Behnken design was used, with the number of levels of the III factors fixed. To optimise the formulation of the luteolin-loaded liposomal hydrogel, a Box–Behnken design with three levels for each factor was selected and completed. Phosphatidylcholine concentration (P), cholesterol concentration (Q), and Carbopol 940 concentration (R) were chosen as the independent formulation variables. The experimental design consisted of Fifteen runs, with Three replicates at the centre point, enabling the systematic assessment of the individual, interactive, and quadratic effects of the selected factors. The responses that were evaluated included cumulative drug release (Y_4), zeta potential (Y_2), particle size (Y_1), and entrapment efficiency (Y_3). It was necessary to perform fewer experimental trials than those required by conventional optimisation methods.[14] All the data obtained in the experiments were fitted to an appropriate second-order response surface model using Design-Expert® software (Version 12.0.3.0). Analysis of variance (ANOVA) was used to check the adequacy and statistical significance of each model. The F value and the associated p-value were used to assess model significance, while the coefficient of determination (R^2), adjusted R^2 and predicted R^2 were used to evaluate the goodness-of-fit. The lack-of-fit test was also used to assess whether the selected model adequately represented the experimental data relative to the experimental error. As an additional indicator of signal-to-noise ratio, we used adequate precision. We regarded models with a p-value < 0.05 as statistically significant. During numerical optimisation and confirmatory experiments, we also examined agreement between predicted and experimentally obtained responses.

Table 1: Design of Box-Behnken, Independent and Dependent Variables of Luteolin-Loaded Liposomal Hydrogel

Formulation Code	Coded Value Factors			Actual Value Factors		
	A	B	C	A	B	C
LH1	0	1	-1	3	1.5	0.5
LH2	0	-1	-1	3	0.5	0.5
LH3	0	0	0	3	1	1
LH4	-1	-1	0	2	0.5	1
LH5	-1	0	-1	2	1	0.5
LH6	1	0	1	4	1	1.5
LH7	1	-1	0	4	0.5	1
LH8	0	0	0	3	1	1
LH9	0	0	0	3	1	1
LH10	0	-1	1	3	0.5	1.5
LH11	-1	0	1	2	1	1.5
LH12	1	1	0	4	1.5	1
LH13	1	0	-1	4	1	0.5
LH14	-1	1	0	2	1.5	1
LH15	0	1	1	3	1.5	1.5

The Box–Behnken design generated fifteen experimental runs, including three centre-point replicates (LH3, LH8, and LH9), which were used to estimate experimental error & evaluate model reproducibility (as shown in **Table 1**).

Preparation of luteolin-loaded liposomal hydrogel

Soya lecithin, cholesterol, and luteolin were dissolved in ethanol. Hydrate the lipid film with an aqueous phase (e.g., PBS (Phosphate-Buffer Saline) or water) under agitation. Sonicate or extrude the dispersion to obtain uniform liposomes. Disperse the Carbopol in water or buffer, stirring continuously to avoid clumping. Adjust the pH to 6.5-7.5 using triethanolamine (TEA) if using Carbopol. As shown in **Table 2** Slowly add the prepared liposomal suspension to the gel, stirring gently.[15]

Luteolin was incorporated into liposomes by using the thin-film hydration method. To sum up: the amount of luteolin set in advance was dissolved mixed with phosphatidylcholine and cholesterol in the above-described organic solvent system. The amounts of phosphatidylcholine and cholesterol were changed based on Box–Behnken experimental design, while the amount of luteolin was kept constant. The organic phase was transferred to a

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Table 2: Preparation of luteolin-loaded liposomal hydrogel by using Box-Behnken Design

Component	LH1	LH2	LH3	LH4	LH5	LH6	LH7	LH8	LH9	LH10	LH11	LH12	LH13	LH14	LH15
Luteolin (% w/w)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Soya Lecithin (% w/w)	3	3	3	2	2	4	4	3	3	3	2	4	4	2	3
Cholesterol (% w/w)	1.5	0.5	1	0.5	1	1	0.5	1	1	0.5	1	1.5	1	1.5	1.5
Ethanol (% w/w)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Carbopol 940 (% w/w)	0.5	0.5	1	1	0.5	1.5	1	1	1	1.5	1.5	1	0.5	1	1.5
Triethanolamine (% w/w)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Glycerin (% w/w)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Distilled Water (g)(Q.S.)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

round-bottom flask and rotary evaporated to a uniform thin lipid film at a controlled temperature and reduced pressure. The film was then stored under vacuum to remove any residual solvents. The dried lipid film was then hydrated with the predetermined amount of aqueous phase at the predetermined hydration temperature and left to hydrate for the predetermined time period while being gently stirred. These multilamellar vesicles were then sonicated in a controlled manner to decrease the size of the vesicles and achieve a relatively uniform dispersion. If desired, the dispersion was filtered through the given membrane/extrusion system to obtain the desired vesicle-size distribution. The formulated luteolin-loaded liposome dispersion was then further incorporated into Carbopol 940 hydrogel.[16]

Appearance of the prepared liposomal hydrogel

The topical gel formulations were visually scrutinised for appearance/clarity, color, odor, and suspended particulate matter.[17]

Determination of pH

The compatibility and stability of the liposomal hydrogel with the skin are determined by measuring the pH of the prepared hydrogel. To do this, carefully weigh approximately 1 g of the liposomal hydrogel formulation and dissolve it in a suitable volume of distilled water or an appropriate buffer solution (e.g., 10 mL) to obtain a homogeneous solution. A pH meter is then used to measure the dispersion's pH. Before collecting readings, the pH meter is calibrated using normal buffer solutions (pH values of 4.0, 7.0, and 9.2) to guarantee accuracy. The pH is recorded and measured three times, usually to ensure the reliability of the measurement.

Evaluation of Viscosity

The Brookfield Viscometer was used to test the hydrogel's viscosity. The gels were rotated at 10–20 rotations per minute using spindle number 64 at a regulated temperature of 25 ± 1 °C, and values of viscosity were noted promptly after formulation and after storage for 24 hours at 25 °C.[18]

Determination of Homogeneity

All prepared hydrogels were visually examined for homogeneity after placement in the container. The appearance and existence of any aggregates were checked.[19]

Determination of Spreadability

The time it takes for two slides to slide off the gel is stated in seconds. The scattering value is significant for determining the therapeutic potency of a topical preparation. One gram of gel was sandwiched between two slides below the influence of a certain weight. The period at which the slides separated was recorded in seconds.[20]

The formula is:

$$S = \frac{M \times L}{T}$$

Whereas M - Weight in pan, S - Spreadability, T - Time (in seconds), and L - Length moved by the slide are used to isolate slides.

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Determination of Extrudability

A collapsible aluminium tube filled with liposomal gel is pressed under a **known weight (e.g., 500 g),** and the **length or weight of the gel extruded within a fixed time (10 s)** is measured. The extrudability study showed that the liposomal gel was extrudable to a good to excellent extent.

$$\text{Extrudability} = \frac{\text{Weight applied } (g)}{\text{Area of orifice } (\text{cm}^2)}$$

Extrudability was determined by gauging the amount of gel extruded from a collapsible tube under a fixed load within a specified time. Results are expressed as **g/cm².**[21]

Determination of Bioadhesive Strength

Individual hydrogel samples were placed on the base of an inverted glass container, secured with two-sided adhesive tape. The space between the 2 vials was adjusted. Pressure was exerted on both sides of the container for 10 seconds to ensure good adherence of the gel to the mucosa.

Bioadhesive strength (dynes/cm^2)

$$S = \frac{M \times g}{A}$$

Where, M = weight required for detachment in grams, g = acceleration due to gravity (980 cm/s^2), A = area of mucosa exposed.[22]

Drug Content of the prepared formulation

To assess the consistency of drug distribution inside the gel matrix, the drug content of the generated liposomal hydrogel preparation was ascertained. A volumetric flask with 100 mL of a standardized phosphate buffer (pH 7.4) was filled with a carefully weighed 100 mg sample of each formulation. The drug-liposomal hydrogel mixture was then shaken continuously for two hours on a mechanical shaker to certify complete extraction and dissolution of the drug from the liposomal hydrogel.[23] The final solution was filtered to remove any insoluble excipients and gel residue. The drug content was determined by subjecting the filtrate to UV-visible spectrophotometry with phosphate buffer (pH 7.4) as a blank.[24]

In-vitro dissolution study

The Franz diffusing cell was utilised to modulate the discharge profile of the medication from the liposomal-constituted hydrogel. The receptor chamber had a 24 mL capacity and contained a magnetic bead.[25] The whole assembly was preserved at room temperature and placed on a magnetic stirrer to gently stir. In the donor compartment, the formulations were placed across the membrane and agitated. At predefined intervals, usually 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 hours, samples were detached from the receptor compartment, and a similar volume of diffusion medium was added back. To prevent air from becoming trapped behind the diffusion membrane, the diffusion medium was carefully added to the receptor compartment. After appropriate dilutions, the samples were spectrophotometrically measured at 350 nm. To illustrate the drug-release mechanism of the optimised liposomal hydrogel, the in vitro release data were fitted to various kinetic models, including Korsmeyer–Peppas, first-order, zero-order and Higuchi, Hixson–Crowell. The model with the highest R^2 value was chosen as the finest fit. The drug release mechanism was determined using the release exponent (n) from the Korsmeyer–Peppas equation.[26]

Drug Release Kinetics Studies

The *in-vitro* %age cumulative drug release data were integrated into IV models Korsmeyer-Peppas models, zero-order, first-order, AND Higuchi to determine the drug release kinetics for solid dosage forms.

Optimisation using desirability function

Derringer and Suich's desirability function method, a numerical optimisation technique, was used to optimise all five responses concurrently. An individual response is linked to a partial desirability function, where an acceptable response has a value between 0 and 1, whereas an undesirable or unsatisfactory response has a value of 0.[27] The response's proximity to its target value, or minimum to the most desired, is represented by a value from 0 to 1.[28]

Evaluation of optimised formulation

These are the following parameters for evaluating the optimised formulation:

Size of particles & Zeta potential of the prepared/optimised preparation

The final liposomal mixture's zeta potential and particle size were ascertained using the dynamic scattering of light technique. Before measurement, the liposomal sample was appropriately diluted with distilled H_2O to avoid multiple scattering and ensure reliable readings. The instrument was employed to examine the mean particle size as well as the polydispersity index (PDI) of the particles, based on the dynamic light scattering principle at 25 ± 1 °C.[29] The instrument's software automatically calculated the zeta potential values based on the electrophoretic mobility. All parameters were performed three times, and the results were analyzed as the Mean ± SD. Determining particle size and zeta potential is essential for assessing the stability, uniformity, and external charge properties of the liposomal formulation.[30]

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The optimised formulation's transmission electron microscopy

TEM examination exposed that the prepared liposomes were round and had a clear bilayer structure with a uniform size distribution. Minimal aggregation was also observed, which demonstrated good structural stability and successful liposome formation.[31]

Stability studies of the prepared/optimised formulation

The accelerated stability test of the liposomal hydrogel formulation was conducted to evaluate its stability, both physical and chemical, under stressed conditions. The optimised liposomal hydrogel formulation was loaded into adequate airtight bottles and kept in a chamber of stability at 40 ± 2 °C with a relative humidity of 75 ± 5%.[32]

The samples were also taken at known intervals of 0, 1, 2, 3, and 6 months and tested for parameters such as appearance, *in vitro* drug release, viscosity, pH and drug content to determine whether there was any change in the samples during storage. The results obtained were then likened to the values to assess the stability of the liposomal hydrogel formulation using accelerated conditions. The formulation was considered stable when no significant changes were observed in its physicochemical properties during the study period.[33]

RESULTS

The results are as follows:

Calibration Curve of Luteolin

In the range of 0–10 µg/mL, the luteolin calibration curve was effectively constructed at 350 nm. The absorbance values, which ranged 0.148 ± 0.002 at 0 µg/mL to 0.891 ± 0.008 at 10 µg/mL, increased linearly with increasing luteolin concentration. The calibration plot showed a good linear relationship b/w concentration and absorbance, as shown by the equation for regression y = 0.0746x + 0.1478 and a correlation coefficient (R^2 = 0.9999) (as shown in **Figure 1**).

The precision and repeatability of the analytical approach were also demonstrated by the low standard deviation at each concentration. These findings support the established UV-visible spectrophotometric method's validity, reliability, and suitability for quantitative luteolin estimate in formulation creation, drug content evaluation, encapsulation efficiency, and in vitro drug release investigations.

Drug Excipients compatibility study by using FTIR

The FTIR spectrum of luteolin exhibits significant absorption bands that match with other previously reported FTIR spectra of flavonoids and authorize the presence of its key functional groups. As shown in **Figure S1**, a strong band in the ~3200–3500 cm^{-1} region of the spectrum indicates the presence of numerous phenolic hydroxyl groups engaged in hydrogen bonding. The weak to moderate bands between ~2920–2850 cm^{-1} are attributed to aliphatic/aromatic C–H stretching. A prominent absorption band near ~1650–1660 cm^{-1} is allotted to the conjugated C=O stretching vibration of the flavone carbonyl group, a defining feature of luteolin's backbone structure. Strong bands in the region of ~1600–1500 cm^{-1} arise from C=C extending vibrations of the aromatic rings, confirming the polyphenolic aromatic system. The peaks observed between ~1450 and 1300 cm^{-1} are linked with C–O stretching and O–H bending vibrations of phenolic groups.

FTIR spectrum of the luteolin-loaded liposomal hydrogel formulation reflects the typical functional groups of the drug and excipients without the appearance of any significant peaks, which means that the drug and the excipients have been successfully encapsulated and there is no incompatibility. As shown in **Table S1**, the wide absorption band in the range of about 3400 cm^{-1} is indicative of O-H stretching of luteolin and cholesterol, whereas its peaks at around 2924 and 2850 cm^{-1} are representative of CH_2 stretching of the lipid alkyl chains of soya lecithin and cholesterol. The C=O stretch of phospholipids can be seen at around 1735 cm^{-1}, and intense absorptions at 1650–1600 cm^{-1} are the conjugated C=O and aromatic C=C stretches of luteolin.[34]

Experimental Design

Y1= 57.91-0.1363A+2.30B-0.3313C-4.06AB-5.15AC+3.92BC (2)

Polynomial Eq. 2 states that Y1 represents particle size, A represents phosphatidylcholine concentration, B represents cholesterol concentration, and C represents carbopol 940 concentration. According to Eq. 1, phosphatidylcholine has a negative coefficient, meaning that particle size tends to decrease as phosphatidylcholine concentration increases. This implies that phosphatidylcholine might help stabilise smaller particles. Carbopol 940 has a marginally positive impact on particle size, and cholesterol has a coefficient that is positive, which means that increased cholesterol concentrations are linked to an enhancement in particle size. However, in comparison to phosphatidylcholine and cholesterol, the impact is negligible.

Y2= 35.74+1.14A+2.08B+1.39C+0.1500AB+4.08 AC+0.2000BC (3)

According to polynomial Eq. 3, the polynomial equation shows that all three formulation components, Phosphatidylcholine (A), Cholesterol (B), along with Carbopol 940 (C), improve

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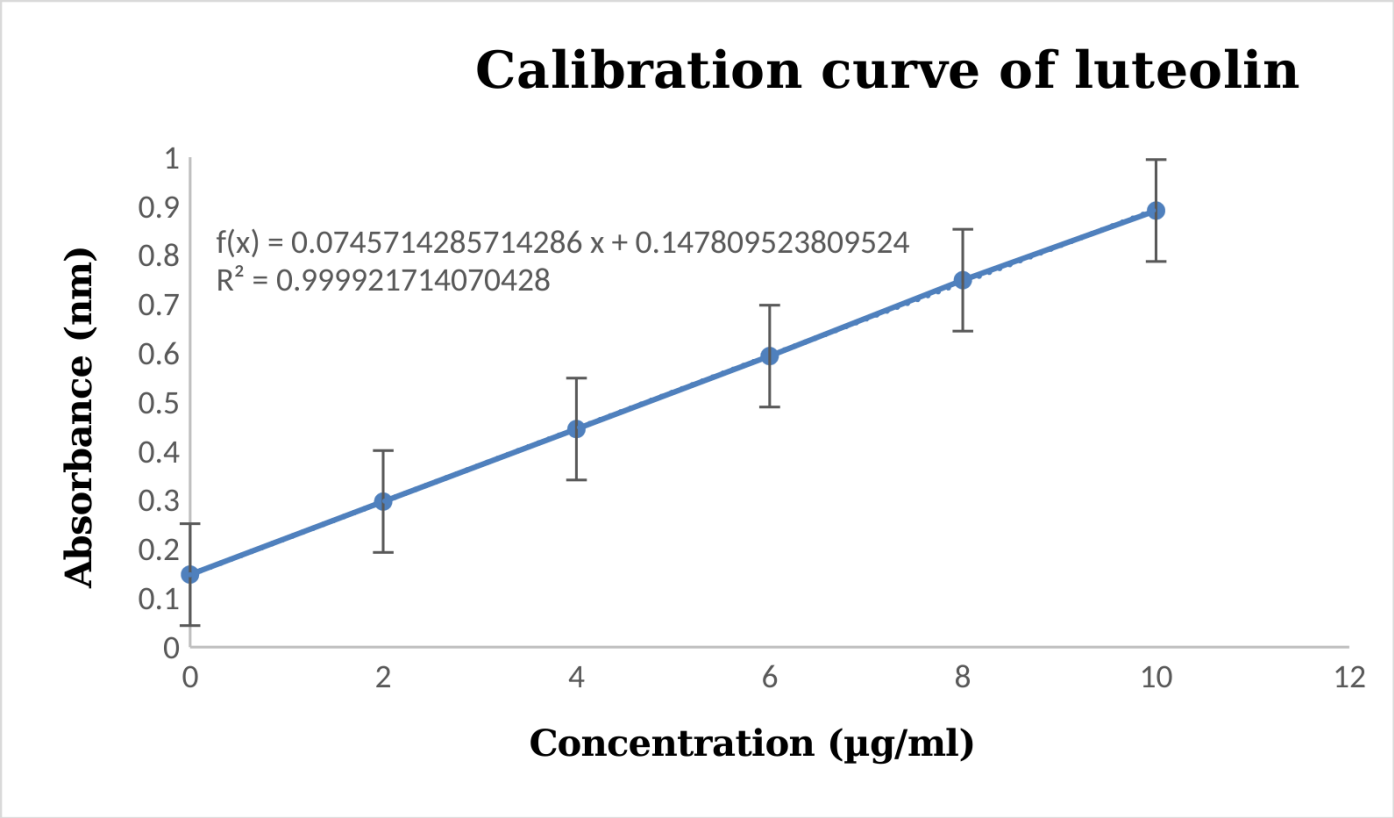


Figure 1: Calibration curve of Luteolin

the system's zeta potential. Higher absolute values, whether negative or positive, usually indicate better electrostatic repulsion between particles. This response surface model is a valuable tool for identifying the optimal ratios and amounts of phosphatidylcholine, cholesterol, and Carbopol 940 to achieve the desired stability characteristics in nanostructured formulations.

$$Y3= 69.15+1.0000A-4.65B+7.19C+8.60AB+1.22AC+9.23BC-3.68A^2+10.25B^2-4.43C^2 \quad (4)$$

All of the given %EE enhancers, phosphatidylcholine, cholesterol, and Carbopol 940, contribute to the encapsulation process according to polynomial Eq. 4. Phosphatidylcholine probably contributes significantly to the active ingredient's encapsulation. Although it has a lesser effect than phosphatidylcholine, cholesterol also improves entrapment efficiency. A negative coefficient indicates a minor reduction in entrapment efficiency with a growth in Carbopol 940 concentration.

$$Y4= 85.93+0.8625A+2.08B+3.63C+1.31AB-7.55AC-5.08BC-7.30A^2-6.38B^2-7.13C^2 \quad (5)$$

All of the specified %CDR enhancers, phosphatidylcholine, cholesterol, and Carbopol 940, align with polynomial Eq. 5. A key factor in lowering %CDR is phosphatidylcholine, which is advantageous for formulations with sustained release. Carbopol 940 has a subtle effect, slightly lowering %CDR, while cholesterol raises %CDR, as shown in **Table S2** and **Table S3**.

Response Surface Plots

The figure presents a set of 3-D response surface plots that display the combined effects of preparation variables: A, phosphatidylcholine (%); B, cholesterol (%); and C, Carbopol 940 (%), on key response parameters such as cumulative drug release, zeta potential, EE%, and particle size. The interaction plots AB, BC, and AC show the effect that the simultaneous change in two factors has on each response variable, while keeping the third factor fixed. As shown in **Figure 2** and **Table S3**, The compositional sensitivity of particle size and zeta potential is moderate, while the sensitivity of drug release & entrapment efficiency is pronounced, exhibiting clear optimal ranges at intermediate levels of the components.

The obtained response surfaces indicate that an optimal ratio of phosphatidylcholine, cholesterol, and Carbopol 940 is essential to obtain the desired characteristics of nanoparticles and controlled drug release.

Determination of pH, Viscosity

The pH evaluation of liposomal gel formulations (LH1-LH15) revealed values, all of which fall within the skin-compatible

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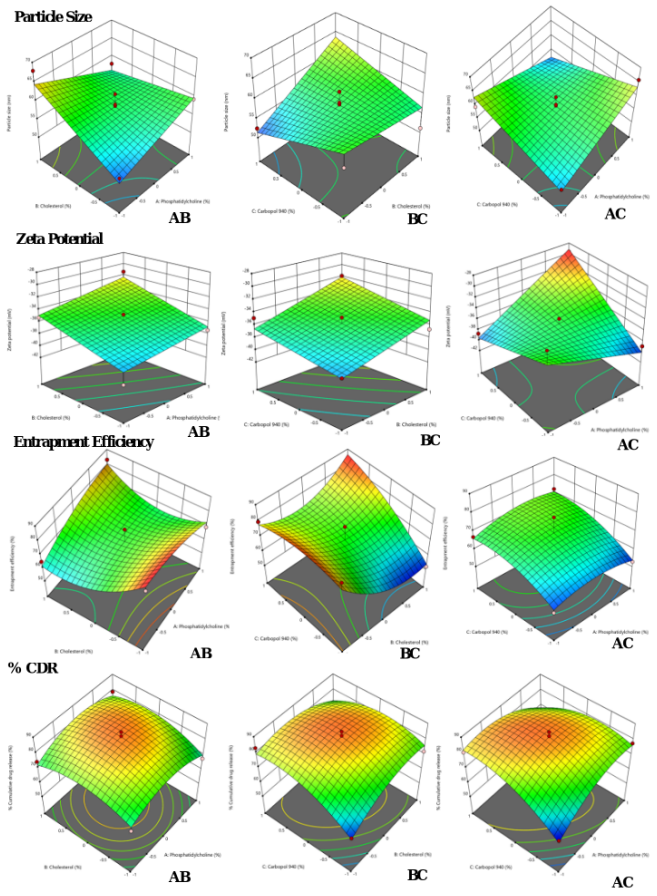


Figure 2: Response surface plots

range (6.5-7.0). This indicates that the gels are safe for topical application, non-irritating, and capable of maintaining a favourable environment for wound healing while ensuring formulation stability. In terms of appearance, all fifteen formulations (LH1-LH15) exhibited a yellowish-brown colour, which remained consistent across batches, indicating uniformity in the prepared gels with Mean±SD.

Due to its low flow resistance under high shear conditions, it is desirable to design a non-Newtonian flow system. Gel compositions displayed non-Newtonian flow in this investigation. The viscosities of liposomal hydrogel formulations ranged from 8,450 to 19,750 cP (LH1-LH15). The higher concentration of polymer, the greater the viscosity. As shown in **Table 3**, the topical applications LH8 and LH9 conformed to optimal viscosity, implying a favourable compromise of spreadability, extrudability, and bioadhesion. The colour of the produced gel LH2 was examined visually. The developed product was clear and transparent. The produced gel LH2 had good homogeneity and no lumps.

Spreadability, Extrudability of the formulation

The spreadabilities of liposomal hydrogel formulations (LH1-LH15) ranged between 21.8 and 42.1 g.cm/s. The lower the viscosity, the greater the spreadability. Formulations LH8 and LH9 exhibited the best spreadability as they were able to spread evenly across the surface of the skin and were easily applied. According to reports, the gels' ability to disseminate also affects their medicinal efficacy. Spreadability plays a momentous role in patient compliance and is helpful in the gel's consistent application to the skin.

The drug concentration in the liposome-based gel formulations ranged from 73.49% to 97.45%. Among all the formulations, F8 contained the highest drug content (95.43±1.73%), indicating good drug entrapment efficacy and minimal drug loss during formation. Formulations LH2, LH3, LH6, and LH8 have a drug content of more than 85%, which is beneficial for efficient therapeutic administration. LH1 and LH4 had the lowest drug contents, which could be a sign of insufficient drug loading or possible preparation-related degradation.

As shown in **Table 3**, the extrudability of liposomal hydrogel formulations (LH1-LH15) varied between 16.9 and 28.8 g/cm^2. Formulations LH8, LH10, and LH11 were more extrudable, which is a sign of the highest gel consistency and extrudability, while the lower values of LH4 and LH15 could be explained by greater viscosity.

The liposomal hydrogel formulations (LH1-LH15) exhibited bioadhesive strengths ranging between 24.3 and 53.8 g of bioadhesive strength. Formulation LH3 from the initial batches and formulations LH11-LH15 from the extended batches demonstrated greater bioadhesive strength, indicating improved adhesion and potentially longer residence time at the site of application.

In-vitro drug release

In-vitro drug release of all the formulated batches was accomplished, and it was observed that the dissolution data obtained for formulations LH1 to LH15 lie between 83.54 ± 0.288% and 96.88 ± 0.456%, as shown in **Figure 3**.

It was observed that formulation LH8 exhibited the highest *in vitro* drug release, whereas formulation LH3 showed the lowest.

Drug release kinetics

For luteolin-loaded liposomal hydrogels (LH1–LH15), the *in vitro* release data were fitted to Higuchi, first-order, Korsmeyer-Peppas and zero-order kinetic models. The evaluated models, the Korsmeyer-Peppas model exhibited the maximum coefficient of determination (R^2 = 0.9693-0.9986) across all formulations, indicating that it most accurately described the release behaviour of luteolin from the liposomal hydrogel system. As per the

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Table 3: Results for the spreadability, drug content, and values of liposomal hydrogel formulations (LH1–LH15) determined using the glass slide method

Formulation	Drug content	Spread-ability (g·cm/s)	Extrud-ability (g/cm²)	Bioadhesive Strength (g)	pH	Viscosity (cP)
LH1	73.49±2.21	21.8 ± 0.7	18.4 ± 0.6	28.6 ± 1.2	6.57± 0.208	8,450 ± 120
LH2	83.91±3.32	23.1 ± 0.8	21.2 ± 0.7	34.9 ± 1.4	6.53± 0.203	9,120 ± 135
LH3	93.98±2.91	31.6 ± 0.9	25.8 ± 0.8	35.74 ± 1.6	6.64± 0.209	13,760 ± 160
LH4	74.27±3.78	26.0 ± 1.0	16.9 ± 0.5	24.3 ± 1.1	6.55± 0.119	10,430 ± 155
LH5	77.44±3.36	27.4 ± 1.1	19.6 ± 0.6	26.8 ± 1.2	6.48± 0.212	11,250 ± 160
LH6	95.97±3.11	28.9 ± 1.2	22.1 ± 0.7	29.4 ± 1.3	6.52± 0.119	12,080 ± 175
LH7	73.6±2.28	30.3 ± 1.3	23.5 ± 0.8	32.1 ± 1.4	6.63± 0.211	12,950 ± 180
LH8	**94.33±1.73**	**31.7 ± 1.4**	**25.9 ± 0.9**	**35.68 ± 1.5**	**6.69± 0.112**	**13,780 ± 190**
LH9	94.44±3.36	31.1 ± 1.5	25.4 ± 1.0	35.29 ± 1.6	6.66± 0.802	13,770 ± 180
LH10	90.87±3.11	34.6 ± 1.6	28.8 ± 1.1	40.9 ± 1.7	6.57± 0.223	15,450 ± 225
LH11	73.61±2.28	36.0 ± 1.7	26.2 ± 0.9	43.5 ± 1.8	6.65± 0.215	16,320 ± 240
LH12	92.53±1.73	37.5 ± 1.8	24.1 ± 0.8	46.1 ± 1.9	6.58± 0.116	17,180 ± 255
LH13	78.49±2.21	39.0 ± 1.9	22.0 ± 0.7	48.7 ± 2.0	6.78± 0.209	18,050 ± 270
LH14	89.91±3.32	40.6 ± 2.0	20.3 ± 0.6	51.3 ± 2.1	6.54± 0.210	18,920 ± 290
LH15	93.28±2.91	42.1 ± 2.1	18.7 ± 0.5	53.8 ± 2.2	6.67± 0.207	19,750 ± 310

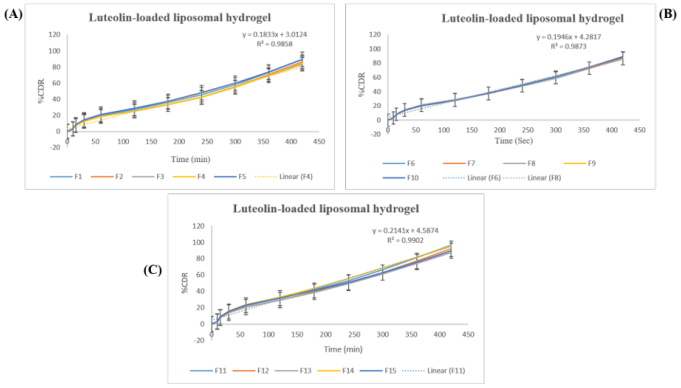


Figure 3: Graphs of In-vitro drug release from (A) LH1 to LH5 (B) LH6 to LH10 (C) LH11 to LH15

Table S6, Although the Higuchi model also showed high R^2 values (0.9418-0.9861), confirming a significant contribution of diffusion, the higher R^2 values found with the Korsmeyer–Peppas model indicate that the release mechanism is more complex than simple Fickian diffusion. To pinpoint the precise medication release mechanism.

Optimisation using desirability function

The desirability approach was utilized to optimise the luteolin-loaded liposomal hydrogel formulation by simultaneously considering all critical parameters, including zeta potential, %CDR, particle size and EE%. The optimisation criteria aimed to minimize particle size and PDI, and to maximize zeta potential, entrapment efficiency and drug release. Among all the prepared formulations, LH8 had the highest overall desirability and was designated as the optimised formulation. The optimised formulation exhibited a balanced combination of nanosized vesicles, good colloidal stability, high entrapment efficiency, enhanced drug release, and favourable topical characteristics as shown in **Table S4**. Therefore, formulation LH8 was considered the most suitable formulation for further characterisation and stability studies. To further confirm the optimised composition, independent centre-point batches (LH3, LH8, and LH9) were prepared, and the results showed low variability, which confirms the reliability of the optimisation model. The optimised formulation obtained through the desirability function approach demonstrated a significant desirability rating (D = 0.729), which validates the robustness and dependability of the optimisation model by showing good agreement between anticipated and experimental responses, as shown in **Table S5**.

For the improved formulation (n = 3), the anticipated and empirically observed responses were compared using a paired Student's t-test. The optimisation model's validity and dependability were confirmed by the lack of statistically significant changes in entrapment efficiency, particle size, cumulative drug release or zeta potential ($p > 0.05$).[35,36]

Evaluation of optimised formulation

The evaluated optimised results are as follows:

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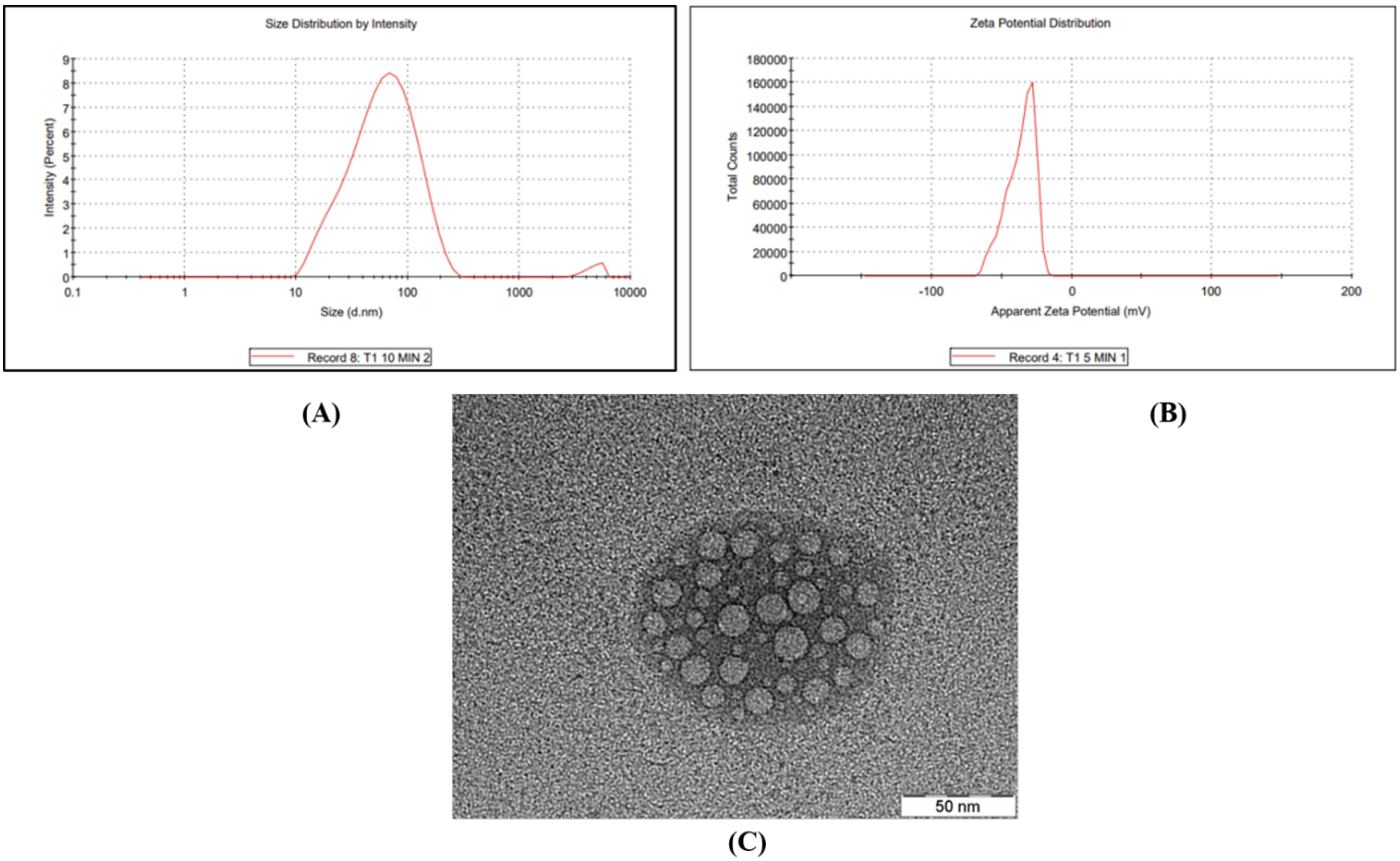


Figure 4: (A) Particle size of optimised formulation (B) Zeta potential of the optimised formulation (C) Transmission electron microscopy of optimised formulation

Zeta potential and Size of particles

The particle size analysis of the optimised luteolin-loaded liposomal hydrogel preparation was performed using the DLS technique. The optimised formulation exhibited an average particle size (approx.) 51.29 nm with a low polydispersity index (PDI), suggesting uniform vesicle dispersion and minimal aggregation within the formulation system (Figure 5). The intensity-based size distribution curve showed a predominant sharp peak within the nanometric range, confirming the presence of a monodisperse liposomal population. The nanoscale particle size of the optimised formulation is valuable for topical drug delivery as it enhances surface area, improves skin penetration, promotes uniform drug distribution, and facilitates sustained drug release as shown in **Figure 4A.**

The zeta potential analysis of the optimised luteolin-loaded liposomal hydrogel formulation was carried out to estimate the charge on the surface and the physical stability of the liposomal vesicles. The zeta potential of the improved formulation was about −34.8 mV, indicating the presence of a sufficiently high negative surface charge on the liposomal particles, as shown in **Figure 4B**.

The zeta potential distribution graph displayed a sharp and narrow peak within the negative potential region, confirming the uniform distribution of charged vesicles and the good colloidal stability of the formulation. The absence of multiple broad peaks suggested minimal aggregation and a homogeneous dispersion of liposomal particles throughout the system.

Transmission electron microscopy

TEM analysis was executed to examine the surface morphology, structural features, and vesicle distribution of the optimised luteolin-loaded liposomal hydrogel formulation. The TEM micrograph revealed that the prepared liposomes were mainly round in shape with well-defined boundaries and a uniform nanosized distribution. The vesicles appeared discrete and uniformly spread with minimal aggregation, indicative of the successful formation of stable liposomal nanoparticles as shown in **Figure 4C**.

The liposomes exhibited a distinct bilayer structure, which is characteristic of phospholipid-based vesicular systems and confirms the successful encapsulation of luteolin within the lipid matrix.

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Table 4: Stability Study of Optimised Luteolin-Loaded Liposomal Hydrogel Formulation

Stability Parameters	Initial	I Month	III Months	VI Months
Size of particles (nm)	51.29 ± 0.21	52.14 ± 0.28	53.06 ± 0.31	54.18 ± 0.36
Zeta Potential (mV)	−34.08 ± 0.18	−33.74 ± 0.21	−33.26 ± 0.24	−32.81 ± 0.29
Entrapment Efficiency (%)	87.64 ± 1.42	86.92 ± 1.36	85.84 ± 1.28	84.76 ± 1.21
% Cumulative Drug Release	88.92 ± 1.65	88.11 ± 1.52	87.26 ± 1.47	86.34 ± 1.39

Optimised Formulation Stability Study

Following ICH recommendations, an accelerated stability analysis of the optimised luteolin-loaded liposomal hydrogel composition was conducted for six months at 40 ± 2 °C & 75 ± 5% relative humidity. The optimised formulation was evaluated periodically at initial, I month, III months, and VI months intervals for EE%, particle size, cumulative drug release, and zeta potential as shown in **Table 4**. Although the accelerated stability study showed minimal changes in entrapment efficiency, drug-release particle size, and zeta potential characteristics, potential physical stability challenges during long-term storage should be considered. Liposomal systems may undergo vesicle fusion, aggregation, membrane reorganisation, or gradual leakage of the encapsulated drug during prolonged storage.

In the present formulation, the relatively high negative zeta potential (−34.8 mV) suggests sufficient electrostatic repulsion between vesicles, which may help reduce aggregation and fusion. Furthermore, incorporating liposomes into the Carbopol 940 hydrogel network may physically restrict vesicle mobility, thereby enhancing colloidal stability.

DISCUSSION

Optimisation of formulation stipulations is essential to ensure the progress of a stable liposomal hydrogel with desirable physicochemical properties without compromising the structure and stability of the encapsulated drug. Overall, the findings of the present study indicated successful formulation of an optimised luteolin-loaded liposomal hydrogel, validated by Fourier-transform infrared (FTIR) spectroscopy, particle-size analysis, the measurement of zeta potential, the Box–Behnken Design (BBD) for statistical optimisation, and the determination of entrapment efficiency, with the assistance of the transmission electron microscope (TEM). The characterisation studies are important since they directly affect the drug loading capacity, stability, and performance of nanocarrier-based TDDS.[37]

The present results showed that the excipients selected are chemically compatible with luteolin and can be used to obtain a stable nano-vesicular formulation. A systematic optimisation strategy is always the basic guideline for creating strong pharmaceutical formulations. The conventional trial-and-error method has been known as statistically inefficient, as it requires many experimental trials to assess several formulation variables, whereas response surface methodology (RSM) is an efficient method to evaluate several formulation variables simultaneously with fewer experimental runs.[38] The Box–Behnken Design is the most widely accepted RSM designs in the pharmaceutical formulation area because of its ability to evaluate linear, interaction, and quadratic effects of the formulation variables using fewer trial runs than full factorial designs. For this study, the concentrations of phosphatidylcholine, cholesterol, and Carbopol 940 were chosen as independent variables, as they are critical for the formation of vesicles, the stability of the membranes, and the rheology of the hydrogel.[39]

The polynomial equations derived from the developed model were found to be a good fit for describing the relationship among the preparation variables and critical eminence attributes, such as zeta potential, particle size, entrapment efficiency & cumulative drug release. The ANOVA results, which were statistically significant, and the satisfactory coefficient of determination values ($R^2 > 0.74$) indicate a good predictive ability and explain the variation in the experimental data reasonably well.[40] Various similar optimisation strategies have been successfully realised for liposomal formulations of plant-based bioactive compounds, which have also shown that statistical optimisation can enhance formulation reproducibility and decrease experimental cost and development time. Furthermore, the response surface graphs made the interplay between the formulation factors very evident, and it was found that simultaneous optimisation is better than sequential optimisation, changing one variable at a time.[41]

The size is one of the most critical quality parameters in liposomal formulations as it is related to drug encapsulation, colloidal stability, diffusion properties and topical performance. Dynamic light scattering analysis displayed that the optimised preparation had a mean particle size of about 51 nm, which was successful in preparing nanosized vesicles. Generally, liposomes smaller than 100 nm are most physically stable due to less sedimentation, greater surface area, and increased interaction with biological membranes. Moreover, nanosized vesicles are evenly distributed in the hydrogel matrix, which promotes uniform spreading of drugs and prolongs the release. The particle size is consistent with previous reports on phospholipid-based nanocarriers for topical applications, which fall in the range of 40-100 nm.[42]

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The response surface analysis showed that phosphatidylcholine concentration significantly reduced the particle size, with higher levels of phospholipid working to form smaller vesicles. Special care should be taken to ensure sufficient availability of phospholipids for a complete bilayer and to prevent vesicles from aggregating during hydration and sonication. The increases in cholesterol concentration, on the other hand, caused the particle size to grow, perhaps as a result of cholesterol intercalating between the phospholipid molecules, which contributes to the rigidity and thickness of the membrane bilayers. The results are consistent with earlier studies, which showed that cholesterol does stabilise the liposomal membranes but can also increase the size of the vesicles if present in a large enough amount. Polydispersity index (PDI) gives a lot of information about the consistency of the particle size and the homogeneity of the formulation.[43,44] The optimised liposomal hydrogel had a PDI less than 0.30, which means that the particles had a narrow size distribution, indicating a monodisperse vesicular population. In general, PDI values below 0.30 are considered to be representative of a homogeneous system of nanoparticles with low aggregation and good uniformity of the particle sizes. Uniform particle size distribution is especially relevant since the drug release behaviour and physical stability of a heterogeneous population of nanoparticles during storage can be different. The PDI values in the present study were low, which proves the uniform dispersion of liposomes obtained by the thin film hydration method in combination with probe sonication.[44]

The optimised formulation of liposomes was also physically stable, as confirmed by zeta potential analysis. The zeta potential was measured as about –35 mV, a high charge density which can produce sufficient electrostatic repulsion between vesicles to prevent aggregation and fusion during storing. The absolute values of the zeta potential of the nanocarrier systems are typically higher than +30 mV and -30 mV, and hence, according to the criteria of the absolute values of the zeta potential, the nanocarriers are generally considered to be physically stable due to the fact that the van der Waals attraction forces are less than the electrostatic repulsive forces. The negative surface charge found in the present study is primarily due to the phospholipid nature of the liposomal membrane and is in accordance with previous reports on phosphatidylcholine-based liposomes. Topical formulations are especially sensitive to a favourable zeta potential, which helps maintain colloid stability and a uniform therapeutic effect throughout the invention's shelf life.[41]

Another important parameter, which is related to the ability of liposomes to encapsulate and retain the drug in the lipid bilayer, is called entrapment efficiency. The optimised formulation had approximately 88% entrapment efficiency, which shows efficient loading of luteolin in the phospholipid vesicles. This high encapsulation efficiency could be explained by the lipophilic properties of luteolin, which aid in partitioning of luteolin into the lipophilic phospholipid bilayer during vesicle formation. Phosphatidylcholine led to a greater amount of lipid matrix for drug accommodation, increasing the EE, while cholesterol led to stabilisation of the membrane and decreased the leakage of the drug.[40]

Transmission electron microscopy allowed direct imaging of the tailored liposomes and validated the results from dynamic light scattering. TEM scans indicated that the vesicles were mostly spherical, with smooth surfaces and a well-defined bilayer structure, with no aggregation. The measured vesicle dimensions were in good agreement with the particle size estimated by DLS, indicating excellent agreement between the two characterisation methods. Uniform morphology and unbroken bilayer architecture suggest effective manufacture of structurally stable liposomes, which are capable of safeguarding the encapsulated medication throughout storage and topical delivery. Phosphatidylcholine-based liposomal formulations made by the thin-film hydration technique have been regularly reported with a spherical shape of the liposomes. To summarise, the physicochemical analysis validates the improved formulation with nanoscale narrow particle size distribution, particle size, high encapsulation efficiency, appropriate surface charge, and outstanding structural integrity. These properties are regarded as the key conditions for the successful creation of liposomal hydrogels for topical drug administration and offer a solid scientific foundation for the subsequent assessment of formulation performance, drug release behaviour, and long-term stability.[37]

The optimised LH8 formulation exhibited a nanoscale particle size along with a relatively narrow particle-size distribution and favourable surface characteristics. These physicochemical properties may offer potential advantages for topical formulation development; however, particle size alone cannot establish enhanced penetration or retention within biological skin. The Franz diffusion experiment performed in the present study used an artificial membrane and therefore primarily evaluated the in-vitro release/diffusion behaviour of luteolin from the formulation. Although the observed release profile was favourable, it should not be directly extrapolated to skin permeation or therapeutic wound-healing efficacy. Ex-vivo skin permeation and retention studies, together with appropriate cellular and in-vivo wound-healing investigations, are required to establish whether the formulation provides enhanced topical delivery and biological efficacy.

Overall, the optimised LH8 liposomal hydrogel demonstrated favourable physicochemical characteristics and in-vitro release behaviour, supporting its potential for further investigation as a topical delivery system. However, its wound-healing efficacy and ability to enhance skin permeation or retention have not been established in the present study and require confirmation through subsequent biological and in-vivo investigations.

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CONCLUSION

In the current work, a luteolin-loaded liposomal hydrogel was effectively developed and optimised via BBD. The improved preparations, LH8, demonstrated a nanosized particle size of 51.29 nm, high entrapment efficiency of 87.64%, prolonged drug release of 88.92%, favourable topical characteristics, and good stability up to 6 months. The created liposomal hydrogel was proven to be a suitable bioadhesive delivery solution for the localised approach in wound care. The improved luteolin-loaded liposomal hydrogel exhibited satisfactory physicochemical and stability features, prolonged drug release, and therapeutic potential. However, additional biological assessment is required to establish its therapeutic potential. *In vivo* wound models may be assessed for wound closure, tissue regeneration, collagen deposition, angiogenesis, and inflammatory responses by histological, immunohistochemical, and molecular analysis. Considering the well-known anti-inflammatory and antioxidant activity of luteolin and the lengthy duration of its release from liposomal hydrogels, the prepared hydrogel might be useful for topical administration in chronic and difficult wound healing. But before it can be tested in clinical trials, it must be rigorously tested for effectiveness and safety.

ABBREVIATIONS

ABTS – 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ANOVA – Analysis of Variance; BBD – Box–Behnken Design; CAT – Catalase; %CDR – Cumulative Drug Release percentage; DLS – Dynamic Light Scattering; DPPH – 2,2-Diphenyl-1-picrylhydrazyl; %EE – Entrapment Efficiency percentage; FRAP – Ferric Reducing Antioxidant Power; FTIR – Fourier-Transform Infrared Spectroscopy; GSH – Reduced Glutathione; IC_{50} – Half-Maximal Inhibitory Concentration; ICH – International Council for Harmonisation; LPO – Lipid Peroxidation; MDA – Malondialdehyde; PBS – Phosphate-Buffered Saline; PDI – Polydispersity Index; QbD – Quality by Design; R^2 – Coefficient of Determination; ROS – Reactive Oxygen Species; RSM – Response Surface Methodology; SOD – Superoxide Dismutase; TEM – Transmission Electron Microscopy; TDDS – Transdermal Drug Delivery System.

AUTHOR CONTRIBUTION

PV: Conceptualization, methodology, investigation, experimentation, data curation, analysis, and manuscript preparation. **PC:** Supervision, conceptual guidance, critical review, editing, and final approval of the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

ETHICAL APPROVAL

This study does not contain any studies with human or animal subjects performed by any authors.

FUNDING

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, P.V., upon reasonable request.

TABLES

(A = Phosphatidylcholine, B = Cholesterol, C = Carbopol 940)

FIGURES

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