RESEARCH PAPER

Systematic Optimization and In-Vitro Evaluation of Propylene Glycol-Loaded Liposomal Formulations for Enhanced Delivery of Cow Urine Bioactive Fractions

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ABSTRACT

Traditional medicine has long utilized cow urine for its diverse therapeutic properties; however, its clinical application is limited by instability and poor skin penetration. This study focused on the development and optimization of a liposomal delivery system to enhance the transdermal flux of cow urine bioactives. Using the thin-film hydration method and a Box-Behnken Design (BBD), an optimized liposomal batch (F5) was identified with a superior entrapment efficiency of 98.28%. Characterization through FT-IR and DSC confirmed the chemical compatibility and stable integration of the bioactive constituents within the lipid matrix.

The optimized liposomes were incorporated into a 2% Carbopol 934 gel (LG-3) and further modified with various permeation enhancers. Among the formulations, LG-3.6 (incorporating PG, 1% DMSO and 10% Glycerol) emerged as the most effective, demonstrating a high bioactive constituents content of 96.847 ± 0.872% and a significantly enhanced in-vitro bioactive constituents release of 52.644 ± 0.349% over 24 hours. In contrast, the control gel showed only 13.15% release. The kinetic profile of LG-3.6 followed the Korsmeyer-Peppas model, indicating a synergistic mechanism of diffusion and erosion. These findings conclude that the LG-3.6 liposomal gel is a highly potent and scientifically validated system for the effective topical delivery of cow urine bioactive fractions.

Keywords: Cow Urine, Liposomal Gel, Transdermal delivery, Bos indicus, Permeation Enhancers, Box-Behnken Design, Sustained Release.

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Introduction

The field of bioactive constituents delivery has been revolutionized by advanced carrier systems designed to enhance the efficacy, stability, and bioavailability of therapeutic agents. Among these, liposomes have emerged as a versatile and promising vehicle due to their unique ability to encapsulate both hydrophilic and hydrophobic bioactive molecules, protecting them from enzymatic degradation and enabling targeted delivery. Recently, propylene glycol (PG)-integrated liposomes have garnered significant attention in pharmaceutical research. These "pro-liposomal" or softened vesicles exhibit enhanced skin penetration, higher biocompatibility, and superior physical stability compared to conventional liposomes.

In the realm of ethnomedicine, cow urine (specifically from Bos indicus) has been utilized for centuries, particularly within the Ayurvedic framework, for its diverse therapeutic properties. Research has highlighted its potent antimicrobial, antioxidant, and immunomodulatory effects, positioning it as a valuable natural remedy. However, the translation of cow urine into modern clinical applications is hindered by several critical factors, including chemical instability, rapid degradation of volatile constituents, unpleasant odor, and inherent variability in its composition. These challenges significantly limit its patient acceptability and therapeutic consistency.

To address these limitations, this study focuses on the systematic optimization of PG-liposomes as a robust delivery platform for cow urine bioactive fractions. Unlike standard formulations, this approach employs a systematic variation of lipid-to-substrate ratios and hydration parameters to achieve a precise liposomal architecture. By utilizing the thin-film hydration method, we aim to produce stable multilamellar vesicles (MLVs) that shield the active ingredients from environmental stressors, thereby enhancing their controlled release and biological activity.

The optimization process is validated through rigorous characterization, including Dynamic Light Scattering (DLS) for particle size distribution and zeta potential for assessment of electro-kinetic stability. This research represents a novel integration of traditional medicinal knowledge with cutting-edge nanobioactiveconstituents delivery systems. By optimizing these parameters, we provide a stable, consistent, and more effective form of cow urine therapy, paving the way for its application in

antimicrobial treatments, oncology, and immunomodulation.

2. Materials and Methods

2.1. Materials and Reagents

Based on a comprehensive literature review, high-purity reagents and analytical-grade materials were procured for the formulation and characterization of liposomes.

2.2. Procurement and Processing of Cow Urine Samples

The study utilized urine specimens from four distinct physiological groups of the Malvi breed (Bos indicus): adult, pregnant (8th month), lactating (one month post-parturition), and calf (one month old).

Approximately 100-150 mL of early morning first-voided urine was collected under strict aseptic conditions. To ensure purity and remove debris, the samples were filtered using a 0.2 µm syringe filter. For the preparation of 'Go-Arka', fractional distillation was performed at 100 °C using a standardized glass distillation apparatus. For long-term analytical consistency, the processed samples were aliquoted and stored at -18 °C for subsequent in vitro and in vivo investigations.

2.3. Experimental Design and Grouping

The experimental groups were categorized into Raw and Distilled forms, as detailed in Table 1.

Table 1: Classification and Nomenclature of Cow Urine Experimental Groups

Experimentalgroups	
Rawurine	**Distilledurine**
AdultRawCow Urine (ARCU)	Adult DistilledCowUrine (ADCU)
PregnantRawCowUrine (PRCU)	PregnantDistilledCowUrine(PDCU)
LactatingRawCowUrine(LRCU)	LactatingDistilledCowUrine(LDCU)
CalfRawCowUrine (CRCU)	CalfDistilledCow Urine (CDCU)

Table 2: Cow urine samples used from different groups for in vitro studies

***Invitro*analysis**	**Experimentalgroups**
Biochemicalconstituents	ARCU,ADCU,PRCU,PDCU,LRCU,LDCU,CRCU,CDCU
Antioxidant	
Antibacterial	ARCU,ADCU
Cytotoxicityand mitochondrial function	ARCU,ADCU

2.4. Analytical Framework for In Vitro Studies

As summarized in Table 2, the various urine fractions were subjected to specific biological and chemical assays. All groups were evaluated for biochemical constituents and antioxidant activity. Given the traditional therapeutic significance of 'Go-Arka' (distilled adult cow urine), ARCU and ADCU groups were prioritized for antibacterial and functional analysis.

2.5. Preformulation Studies

The development of a rational bioactive constituents dosage form necessitates comprehensive preformulation testing. This phase involved the systematic evaluation of the physical and chemical properties of the bioactive constituents in conjunction with selected excipients. The primary objective was to generate essential data to ensure the development of a stable, bioavailable, and reproducible liposomal delivery system.

2.6. Formulation of Liposomes

Among various established liposomal preparation techniques, the Thin-Film Hydration Method (Bangham method) was adopted in this study. This selection was based on its proven efficiency in achieving high entrapment capacity and its suitability for producing Multilamellar Vesicles (MLVs).

Briefly, the lipid mixture was dissolved in an organic solvent and subsequently evaporated to form a uniform thin layer at the bottom of a round-bottom flask. This film was then hydrated using an aqueous buffer containing the cow urine fractions, maintained at a temperature above the lipid's phase transition temperature (\bm{T_c}). The hydration process was supplemented by vigorous vortexing to ensure complete lipid film detachment and vesicle formation.

2.7. Optimization of Excipients and Lipid Selection

2.7.1. Selection of Phospholipids

To achieve an optimal carrier system, various phospholipids including Phospholipon 90 G, Phospholipon 90 H, and Soya Lecithin were evaluated at different concentration ratios with Cholesterol. Based on physical stability and compatibility studies, Phospholipon 90 G was selected as the primary structural lipid. Its selection was justified by its superior purity profile and enhanced film-forming properties compared to other tested lipids.

2.7.2. Optimization of Solvent Systems

The selection of the organic phase is critical for uniform film formation. Various solvent systems, including Chloroform, Methanol, Ethanol, and Diethyl Ether, were screened in different ratios. Evaluation was based on the visual uniformity of the film and the ease of complete solvent removal under

vacuum.

2.8. Optimization through Trial Batches

To identify the most stable formulation for cow urine encapsulation, a series of trial batches were conducted by varying the solvent systems, hydration media, and lipid concentrations.

* **Trial 1:** Cow urine, Phospholipon 90G, and Cholesterol were dissolved in Chloroform. Evaporation was carried out at 45 °C at 150 rpm under vacuum. Hydration was attempted with PBS (pH 7.4), but stable liposomal vesicles failed to form.
* **Trial 2:** A combination of lipids and cow urine was dissolved in a mixture of Chloroform and Ethanol. Despite maintaining the standard evaporation and hydration parameters, the resulting formulation remained physically unstable.
* **Trial 3:** Lipids were dissolved in Chloroform:Ethanol (1:1). Post-evaporation, the film was hydrated with PBS (pH 4.5) for 1 hour. However, the formulation exhibited discoloration (dark brown) and significant precipitation, likely due to the acidic pH affecting the urine constituents.
* **Trial 4 (Optimized):** Lipids and cow urine were successfully integrated using a Chloroform:Methanol (1:1) solvent system. Evaporation was conducted at 45 °C, followed by hydration with PBS (pH 6.8) for 1 hour at 55 °C. This trial yielded a stable, homogenous, and translucent liposomal suspension.

Table 3:Composition of Cow Urine-Loaded Liposomal Formulations

S. No.	Formulationcode	Cow Urine	Phospholipid90G (Molar)	Phospholipid 90H (Molar)	Cholesterol(Molar)	Chloroform :Methanol	Chloroform :Ethanol
1	F1	5ml	0.2	-	0.01	-	5ml
2	F2	5ml	-	0.2	0.01		5ml
3	F3	5ml	0.2	-	0.01	5ml	-
4	F4	5ml	0.2	-	-	-	5ml
5	F5	5ml	0.2	-	0.02	-	5ml

2.9. Evaluation of Cow Urine-Loaded Liposomes

2.9.1. Visual Appearance and Physical Integrity

The formulated liposomes were subjected to visual inspection to assess homogeneity, color consistency, and the absence of macroscopic aggregates or phase separation.

2.9.2. Percentage Entrapment Efficiency (EE%)

To determine the amount of encapsulated cow urine constituents, 1.0 mL of the liposomal suspension was centrifuged at 18,000 rpm for 1 hour. The resulting supernatant was analyzed to quantify the unencapsulated bioactive markers. The percentage of entrapment was calculated.

2.10. Statistical Optimization using Box-Behnken Design (BBD)

To achieve a robust and optimized liposomal delivery system, a 3-factor, 3-level Box-Behnken Design was employed. This statistical approach helps in understanding the interaction between different formulation variables and their effect on the final product quality.

The independent variables selected for optimization were:

1. Phospholipid Concentration
2. Cholesterol Concentration
3. Stabilizer/Co-solvent Ratio

Table4:Compositionofliposomesusing BoxBehnkendesign

Factor	Name	Units	Minimum	Maximum
A	Phospholipid	Molar(M)	0.1	0.2
B	Cholesterol	Molar(M)	0.01	0.02
C	HP-β-CD	Molar(M)	0.05	0.15

Table5:BoxBehnkendesignmatrix

S. No.	Formulationcode	Independentvariables		
		Phospholipid (X_1)	Cholesterol (X_2)	HP-β-CD (X_3)
1	B1	0.1	0.015	0.05
2	B2	0.2	0.02	0.1
3	B3	0.15	0.015	0.1
4	B4	0.15	0.015	0.1
5	B5	0.2	0.01	0.1

6	B6	0.15	0.02	0.15
7	B7	0.15	0.01	0.05
8	B8	0.1	0.02	0.1
9	B9	0.1	0.01	0.1
10	B10	0.15	0.02	0.05
11	B11	0.15	0.01	0.15
12	B12	0.2	0.015	0.15
13	B13	0.1	0.015	0.15
14	B14	0.2	0.015	0.05
15	B15	0.15	0.015	0.1
16	B16	0.15	0.015	0.1
17	B17	0.15	0.015	0.1

A total of 17 runs were conducted as per the BBD matrix. Based on the statistical analysis, the formulation with the highest desirability (1.000) and minimum standard deviation was identified.

Table 6: Optimized Solutions based on Desirability Criteria

Number	Phospholipid	Cholesterol	HP-β-CD	Desirability
S-1	0.15	0.01	0.15	1.000
S-2	0.1	0.02	0.1	1.000
S-3	0.16	0.02	0.11	1.000
S-4	**0.16**	**0.01**	**0.14**	**1.000**
S-5	0.15	0.02	0.15	1.000

Conclusion of Optimization: The combinatorial set S-4 exhibited the minimum standard deviation between theoretical and experimental response values. Consequently, this combination was selected as the Optimized Parameter for further characterization.

2.11. Final Method of Preparation (Optimized Batch)

The optimized liposomes were prepared by casting a thin lipid film on the wall of a round-bottom flask. The lipids (Phospholipid and Cholesterol) were dissolved in a Chloroform:Methanol (3:1) mixture. The solvent was evaporated under a vacuum at 45 °C with constant rotation. The resulting thin film was then hydrated with an aqueous phase containing the purified cow urine fractions and a stabilizing agent (HP-β-CD) to ensure the stability of volatile bioactive molecules. The final suspension was allowed to stir for 1 hour to reach equilibrium.

2.12. Characterization of the Optimized Liposomal Suspension (S-4)

The optimized formulation (S-4) was subjected to rigorous physicochemical evaluation to confirm its structural integrity and stability.

2.12.1. Particle Size and Polydispersity Index (PDI)

The mean vesicle size and size distribution profile were determined using a Malvern Particle Size Analyzer (Model SM 2000), utilizing Mie's theory of light scattering. The liposomal suspension was diluted appropriately and added to a sample dispersion unit with a stirring speed of 2000 rpm to minimize inter-particle aggregation. The laser obscuration range was strictly maintained between 10–20%. All measurements were performed in triplicate to ensure statistical accuracy.

2.12.2. Zeta Potential Analysis

To evaluate the electro-kinetic stability of the colloidal dispersion, the surface charge (Zeta Potential) of both empty and cow urine-loaded vesicles was measured using a Zetasizer 300 HSA (Malvern Instruments, UK). The analysis was conducted for 60 seconds at a controlled temperature to determine the average zeta potential value.

2.12.3. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR analysis was performed using the KBr pellet technique to investigate potential chemical interactions between the lipids and cow urine constituents. The samples were mixed with analytical-grade KBr in a mortar-pestle, dried completely, and compressed into a pellet at a pressure of 5,000–10,000 psi. The spectra were recorded to identify functional group peaks and confirm the successful integration of bioactive components.

2.12.4. Differential Scanning Calorimetry (DSC)

Thermal analysis was carried out using a Differential Scanning Calorimeter (DSC-4000, Perkin Elmer, USA). Approximately 5 mg of the lyophilized liposomal sample was hermetically sealed in an aluminum pan and heated at a constant rate of 20 °C/min within a temperature range of 30 °C to 300 °C. An empty aluminum pan served as a reference.

2.12.5. X-Ray Diffraction (XRD) Analysis

The crystalline or amorphous nature of the optimized liposomes was assessed using an X-ray Diffractometer (Expert/Pro, Panalytical, Netherlands). This analysis is crucial to understand

the state of the encapsulated cow urine fractions within the lipid bilayer.

2.12.6. Entrapment Efficiency (EE%)

The separation of unencapsulated bioactive fractions from the liposomal vesicles was achieved via centrifugation at 18,000 rpm for 1 hour at a controlled temperature of 4 °C. The supernatant containing the free (unentrapped) components was analyzed using a UV-Visible spectrophotometer against a methanol blank.

2.13. Advanced Characterization and Gel Formulation

2.13.1. Lyophilization (Freeze Drying)

To ensure long-term stability and prevent the leakage of encapsulated cow urine constituents, the optimized liposomal suspension (S-4) was subjected to lyophilization. Trehalose was incorporated as a cryoprotectant to maintain vesicle integrity during the freezing and dehydration cycles. The process was conducted at sub-zero temperatures and extremely low pressures, resulting in a stable dry powder suitable for reconstitution and further characterization.

2.13.2. Transmission Electron Microscopy (TEM)

The morphological characteristics and precise particle size of the lyophilized vesicles were visualized using TEM (Philips CM 200). Lyophilized samples were reconstituted and negatively stained with 1% aqueous phosphotungstic acid. TEM imaging provided critical insights into the lamellarity and spherical architecture of the cow urine-loaded liposomes.

2.14. Development of Liposomal Gels (LG)

To facilitate topical application and sustained release, the optimized S-4 liposomes were integrated into a carbomer-based gel matrix.

2.14.1. Preparation of Gel Base

Carbopol 934 was utilized in varying concentrations (1%, 1.5%, and 2% w/w). The polymer was dispersed in distilled water under mild mechanical stirring (25 rpm for 5 min) and allowed to hydrate overnight. The resulting dispersion was neutralized with Triethanolamine and 1N NaOH to achieve the desired pH and consistency.

2.14.2. Incorporation of Liposomes into Gel Base

Reconstituted cow urine-loaded liposomes (57 mg) were gently incorporated into the pre-neutralized gel base using a structured vehicle approach. For comparative study, secondary gel formulations were developed using combinations of HPMC K-100 and Sodium CMC as shown in Table 7.

Table 7:Composition and Formulation Design of Cow Urine Liposomal Gels

S.No.	Formulationcode	Cow Urine(mg)	Carbopol %(w/w)	HPMC K-100	SodiumCMC
1	LG-1	57	1	_	_
2	LG-2	57	1.5	_	_
3	LG-3	57	2	_	_
4	LG-4	57	1	0.5	1
5	LG-5	57	1	0.5	0.5

Formulationofcontrolgel

Preparationofgelbase: Thebioactive constituentss2%ofthegellingagentwasmaterializedbydippingcarbopol934indistilledwaterduringnight.

Incorporationofbioactive constituentssintogelbase: The bioactive constituents were weighed accurately and incorporated into 2% w/w carbopol gel basewith gentle stirring to obtain homogenous and transparent gel. It is further neutralizedbyusingtriethanolamineandmixedproperly.

2.15. Evaluation of Liposomal Gels

The developed gels were evaluated for:

* **Visual Appearance:** Clarity, homogeneity, and transparency.

* **Spreadability:** To ensure ease of application on the skin.

* **pH Measurement:** To ensure compatibility with physiological skin pH.

* **Bioactive constituents Content Uniformity:** To confirm even distribution of cow urine fractions within the gel matrix.

***In-vitro*bioactive constituentsrelease**

(A) *In-vitro* bioactive constituents release of control gel: In this study we adopted *in vitro* franzdiffusionbioactive constituentsreleasestudymethod.Thediffusionstudies wereperformedusingaFranzdiffusion cell. The cell was locally fabricated and had a 35 ml receptor compartment.The dialysis membrane was mounted between the donor and receptor compartments.The control gel formulation (gel containing both bioactive constituentss) was applied uniformly on thedialysismembraneandthecompartmentswereclampedtogether.Thereceptorcompartmentwasfilledwiththephosphatebuffer(pH7.4)andthehydrodynamicsinthereceptor compartment were maintained by stirring with a magnetic bead. 3 ml ofsampleswaswithdrawnfromthereceptorcompartmentatpre-determinedtimeintervalsand an equal volume of buffer was replaced. The release profile of the formulation isdeterminedandthegraphisplottedbetweenthetimeandbioactive constituentsrelease.

(b) *In-vitro*bioactive constituentsreleaseofliposomalgel(LG-2):ThediffusionstudieswereperformedusingaFranzdiffusioncell.Thecellwaslocallyfabricatedandhada35mlreceptor compartment. The dialysis membrane was mounted between the donor andreceptorcompartments.TheLG-

2gelformulationwasapplieduniformly onthedialysismembraneandthecompartmentswereclampedtogether.Theremainingprocessissameasabove.

(c) *In-vitro*bioactive constituentsreleaseofliposomalgel(LG-3):Thediffusionstudieswereperformedusinga Franzdiffusioncell.Thecellwaslocallyfabricatedandhada35mlreceptor compartment. The membrane was mounted between the donor and receptorcompartments. The LG-3 gel formulation was applied (1gm) uniformly on the dialysismembrane and the compartments were clamped together. The remaining process issameasabove.

Maximumreleaseofbothbioactive constituentsswasfoundin2%carbopolgel(LG-3) incomparisonto1.5% (LG-2). Thus this concentration of carbopol was taken further with permeationenhancers.

2.16. Incorporation of Permeation Enhancers in Liposomal Gels

To further optimize the transdermal delivery of cow urine constituents, formulation LG-3 (containing 2% Carbopol 934) was selected for the incorporation of various permeation enhancers. The objective was to evaluate the synergistic effect of these agents on the skin penetration profile of the liposomal gel.

2.16.1. Formulation Procedure

Carbopol 934 (2% w/w) was hydrated overnight in distilled water. To this base, permeation enhancers including DMSO, Propylene Glycol, Glycerol, PEG-400, and Curcumin were added individually or in combination as specified in Table 8. The pH was neutralized using 1N NaOH under continuous stirring. Finally, the optimized cow urine-loaded liposomes were slowly integrated into the gel matrix to achieve a homogenous and stable formulation.

Table 8:Composition of Liposomal Gels with Integrated Permeation Enhancers

Formulation code	Cow urine(mg)	HPMC (w/w)	DMSO% (w/w)	Propylene Glycol % (w/w)	Glycerol%(w/w)	PEG-400	Curcumin%(w/w)
LG-3.1	57	2	1	-	-	-	-
LG-3.2	57	2	-	3	-	-	-
LG-3.3	57	2	-	-	10%	-	-
LG-3.4	57	2	-	-	-	30%	-
LG-3.5	57	2	-	-	-		0.1%
LG-3.6	57	2	1%	-	10%	-	-
LG-3.7	57	2	5%	-	10%	-	-

2.17. Physicochemical Evaluation of Liposomal Gels

2.17.1. pH Determination

The pH of all liposomal gel formulations was measured using a digital glass electrode pH meter. For analysis, 1.0 g of the gel was dispersed in 10 mL of distilled water. The electrode was immersed into the dispersion, and the readings were recorded in triplicate to ensure consistency with physiological skin pH.

2.17.2. Homogeneity and Grittiness

The prepared gels were subjected to visual and tactile inspections to assess their physical homogeneity. The presence of any macroscopic aggregates or grittiness was evaluated by spreading a small quantity of the gel on a transparent glass slide.

2.17.3. Spreadability Analysis

Spreadability was determined using a custom-designed laboratory apparatus consisting of a wooden block and a pulley system. This test measures the ease with which the gel spreads upon topical application based on its "Slip" and "Drag" characteristics.

* Procedure: An excess of gel (~2 g) was placed on a ground glass slide and sandwiched with another slide. A 1 kg weight was applied for 5 minutes to expel air and create a uniform film. The time taken for the upper slide to separate from the fixed lower slide under a specific load was recorded to calculate the spreadability coefficient.

Results & Discussion

3. Results and Discussion

3.1. Optimization of Excipients and Lipid Selection

The thin-film hydration method proved to be a robust technique for the development of cow urine-loaded liposomes. Among the various screened lipids, Phospholipon 90G was identified as the most suitable structural component. Comparative physical evaluation showed that formulations containing Phospholipon 90G exhibited superior film-forming properties and higher entrapment efficiency compared to Phospholipon 90H or Soya Lecithin. Consequently, Phospholipon 90G and Cholesterol were selected for further optimization and compatibility assessments.

3.2. Solvent System Optimization

The selection of the organic phase significantly influenced the uniformity of the lipid film. Various ratios of chloroform, methanol, and ethanol were evaluated. The results indicated that a Chloroform:Methanol (1:1 or 3:1) system yielded a translucent, uniform thin film without any visible bioactive constituents precipitation. Ethanol-based trials (Trial 2) resulted in lower stability, possibly due to its effect on the solubility of cow urine's aqueous constituents during the evaporation phase.

3.3. Evaluation of Trial Batches (F1–F5)

The trial batches were rigorously evaluated for visual appearance and Percentage Entrapment Efficiency (EE%).

Table 9: Physical Characterization and Entrapment Efficiency of Trial Batches

S. No.	Code	VisualAppearance	%entrapment
1	F1	Slightlycoloredsuspension	97.89
2	F2	Slightlycoloredsuspension	84.80
3	F3	Slightlycoloredsuspension	91.34
4	F4	Slightlycoloredsuspension	90.34
5	F5	Slightlycoloredsuspension	98.28

As shown in Table 9, the entrapment efficiency ranged from 84.80% to 98.28%. Formulation F5 exhibited the highest EE% (98.28%), which can be attributed to the optimal Cholesterol concentration that stabilized the lipid bilayer and reduced the leakage of cow urine constituents.

3.4. Compatibility Studies using Ultraviolet (UV) Spectroscopy

The absorption maxima for the primary excipients were determined to assess potential interactions. The UV spectra revealed peak positions at 279 nm for Phospholipon 90G, 284 nm for Cholesterol, and a combined peak at 213 nm for the lipid-cholesterol mixture. The absence of any significant shift in these characteristic peaks suggests that the cow urine constituents are physically entrapped within the liposomal vesicles without any detrimental chemical interaction with the carrier lipids.On the basis of results indicated in table the phospholipid 90 G and cholesterol were selected for further study and compatibility studies with both bioactive constituentss.

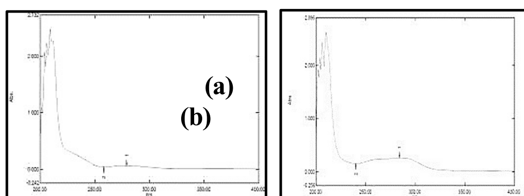

Figure1:Absorptionmaximaof a)Phospholipid90Gb)Cholesterol

From the above figures it was found that 90G lipids and cholesterol did not show any absorbance on Cow Urine absorption maxima. So, no incompatibility was found. Bioactive constituentss and excipients were found to be compatible.

Fourier-Transform Infrared Spectroscopy (FT-IR): The FT-IR spectra of physical mixture containing both bioactive constituentss and excipients recorded by using FT-IR spectrophotometer is shown in figure and absorption peaks are given in table to find the compatibility of bioactive constituentss with excipients.

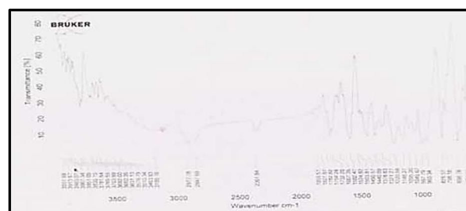

Figure 2: FT-IR spectrum of physical mixture of Cow Urine, phospholipid 90 G and H, cholesterol

Differential Scanning Calorimetry (DSC) of bioactive constituentss and physical mixture

DSC thermograms of cow urine, phospholipids and physical mixture is shown in figure.

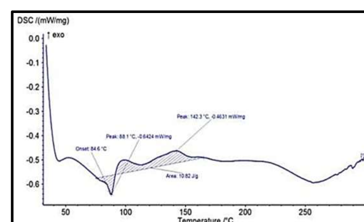

Figure 3: DSC thermogram of physical mixture of Cow Urine, lipid 90 G and cholesterol

From the above DSC thermograms it was found that there was no incompatibility between Cow Urine and lipid. The results of DSC studies show an endothermic peak of Cow Urine at 107°C signifying purity of the sample. In the figure all important peaks of these Cow Urine, phospholipid and cholesterol (142.3°C) were observed. It indicated the compatibility of both Cow Urine with selected lipid.

XRD analysis: XRD analysis of Cow Urine and physical mixture were recorded.

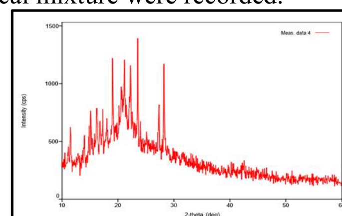

Figure 4:XRDspectraofCow Urine,phospholipid90G,andcholesterol

Figure shows Cow Urine XRD patterns with many sharp, narrow, and strong peaks, indicating high crystalinity. Figure shows Phospholipid 90G XRD pattern. It showed amorphousness. Figure shows Cow Urine and physical mixture XRD patterns. Figure showed comparable peaks for all components in the physical mixture based on their spectra. This showed Cow Urine and excipients are compatible.

Figure shows Cow Urine and physical mixture XRD patterns. Figure showed comparable peaks for all components in the physical mixture based on their spectra. This showed Cow Urine and excipients are compatible.

TEM analysis of optimized formulation

TEM analysis of lyophilized product of liposomes showed the presence of outer coating of bilipid layer entrapping the bioactive constituentss with an optimum size in the range of 150-350 nm.

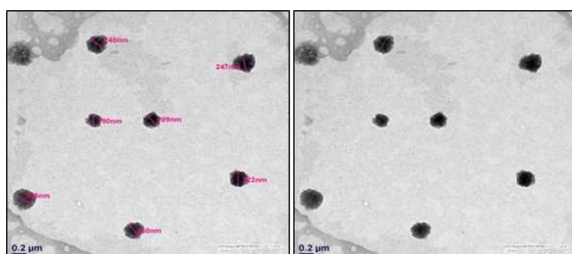

Figure 5: TEM analysis of liposomes

All these tests indicated the successful encapsulation of Cow Urine into liposomes. These optimized liposomes wereused for preparation of liposomal gel.

Liposomalgelformulation

The above successfully evaluated formulation S4 was selected for formulation of liposomalgel.Thedifferentgellingagentsuchascarbopol 934differentconcentration (1%, 1.5%, 2%), hydroxypropyl methyl cellulose K-100 and sodium carboxymethyl cellulosewereusedforpreparationofdifferentliposomal gel.Compositionofdifferent liposomalgelofresveratrolinclusioncomplexandbenzoylperoxideisgivenintable.Fiveformulationswereformedandcodedas LG-1,LG-2,LG-3,LG-4andLG-5.

Formulationofcontrolgel

Control gel was formulated with 2% carbopol and evaluated and compared with optimized gel.

Evaluationofliposomalgels

Appearance: All the gel formulations were found to be transparent.

pH determination: The pH value of all developed formulae was in the range 6.34-7.39 which is considered acceptable to avoid risk of irritation of application to the skin. The results are shown in table.

Table10:pHoftheliposomalgels

S. No.	Formulation code	Reading1	Reading2	Reading3	Mean± S.D
1	LG-2	6.4	6.33	6.31	6.34±0.047
2	LG-3	7.4	7.41	7.38	7.39±0.015
3	LG-4	7.3	7.35	7.26	7.30±0.045
4	LG-5	7.1	7.13	7.15	7.12±0.025

Bioactive constituents content determination

The results obtained are shown in table and figure. The percent content was found in the range of 75.184 to 89.290.

Table 11: Percent Cow urine of different gels containing liposomes

S. No.	Formulationcode	% Cow Urine (Mean ±S.D)
1	LG-1	Gelwasnotformed
2	LG-2	75.687±1.511
3	LG-3	89.290±1.511
4	LG-4	77.199±1.511
5	LG-5	75.184±0.872

Homogeneity and grittiness: The prepared gels were subjected to physical evaluations such as homogeneity and grittiness. All the gels were found to be homogenous except the one which is not formed properly. Results are shown in table.

Table 12: Homogeneity and grittiness of formulation

S.No.	FormulationCode	Grittiness	Homogeneity
1	LG-2	-	++
2	LG-3	-	+++
3	LG-4	*	+
4	LG-5	-	+

Where, + Satisfactory, ++ Good, +++ Very Good, - No Grittiness, *Grittiness.

Liposomal gel LG-3 was selected due to its homogenous nature, proper pH, no grittiness and maximum bioactive constituents content and was further evaluated

In-vitro bioactive constituents release of different gel formulations

(a) In-vitro bioactive constituents release of control gel: The percentage release profile of the control gel was determined and is given in table. The

% bioactive constituents release of control gel was found to be 13.15 in 24 hours.

Table 13: Percent bioactive constituents release in control gel

S. No.	Time (hr)	Controlgel Percentreleaseofcow urine (Mean±S.D)
1	0.25	0.26±0.052
2	0.5	1.11±0.091
3	1	3.56±0.11
4	2	5.39±0.528
5	3	6.81±0.08
6	4	8.73±0.139
7	5	9.17±0.08
8	6	10.35±0.161
9	7	12.13±0.11
10	8	13.21±0.08
11	24	13.15±0.11

(b) In-vitro bioactive constituents release of 1.5 % carbopol containing liposomes (LG-2) and 2.0 % carbopol containing liposomes (LG-3): The percentage release profile of the LG-2 containing 1.5% and LG-3 containing 2% was determined and is given in table.

Table 14: In-vitro bioactive constituents release of LG-2 and LG-3

S. No.	Time (hr)	LG-2	LG-3
		% release of Cow Urine (Mean±Std.dev)	% release of Cow Urine (Mean±Std.dev)
1	0.25	1.02±0.06	0.48±0.05
2	0.5	2.54±0.15	1.55±0.06
3	1	5.91±0.11	2.31±0.08
4	2	8.87±0.09	3.33±0.05
5	3	9.91±0.14	5.11±0.13
6	4	10.17±0.20	7.37±0.05
7	5	13.17±0.22	9.84±0.13
8	6	15.27±0.08	14.42±0.11
9	7	17.91±0.06	18.92±0.06
10	8	19.89±0.18	20.58±0.06
11	24	19.71±0.11	20.26±0.05

From the table the maximum bioactive constituents release of both bioactive constituentss was found in liposomal gel containing 2% carbopol gel (LG-3) in comparison to liposomal gel containing carbopol 1.5% (LG-2). Thus this concentration of carbopol 2% (LG-3) was taken further with permeation enhancers.

Formulation of gel with permeation enhancers: Formulation LG-3 containing carbopol 2.0% was selected to formulate liposomal gel with different permeation enhancers. Composition of penetration enhancers of liposomal gel formation of resveratrol and benzoyl peroxide is given in table.

Evaluation of gel with permeation enhancers

Table15:pHandspreadability,homogeneityandgrittinessofformulations

Formulation code	pH	Spreadability(g.cm/s)	Homogeneity	Grittiness
LG-3.1	7.26±0.026	18.5±0.48	++	-
LG-3.2	7.11±0.01	16.14±1.27	++	-
LG-3.3	7.08±0.02	15.6±0.95	++	-
LG-3.4	7.12±0.02	14.21±0.80	+++	-
LG-3.5	7.07±0.02	12.23±1.01	+	*
LG-3.6	**7.35±0.03**	**11.5±0.92**	**+++**	**-**
LG-3.7	7.45±0.01	14.4±0.55	+++	-

Bioactive constituents content in gel: Percent bioactive constituents content of benzoyl peroxide and resveratrol in liposomal gel containing different permeation enhancers were determined. The results are indicated in table. Maximum bioactive constituents content in liposomal gel (LG 3.6) was found to be 96.847.

Table16:Percentbioactive constituentscontentofLGcontainingdifferentpermeationenhancers

S. No.	Formulationcode	Bioactive constituentscontent (%)
1	LG-3.1	52.507±0.349
2	LG-3.2	70.650±0.872
3	LG-3.3	54.024±0.872
4	LG-3.4	42.985±0.174
5	LG-3.5	28.274±0.302
6	**LG-3.6**	**96.847±0.872**
7	LG-3.7	82.237±0.872

Bioactive constituents release of formulation with different permeation enhancers with different concentrations

Percentage bioactive constituents release of liposomal gel with different permeation enhancers was determined.

Table16: Percentage Bioactive constituents Release

Tim	10%Glycerol	1%DMSO	3%Propylene	30% PEG	0.1%curcumin	10%glyceroland	5%DMSOand

e (hr)	(LG3.3)	(LG3.1)	glycol(LG3.2)	-400 (LG3.4)	(LG3.5)	1%DMSO (LG3.6)	10%glycerol (LG3.7)
0.25	1.52± 0.06	0.25± 0.11	0.33± 0.061	2.40± 0.03	0.427± 0.052	0.444± 0.11	0.268± 0.139
0.5	2.45± 0.133	0.286 ± 0.03	0.586± 0.091	2.349 ± 0.03	0.956± 0.105	1.203± 0.03	0.921± 0.133
1	3.565± 0.13	1.308 ± 0.03	0.709± 0.03	3.742 ± 0.03	1.767± 0.030	6.475± 0.03	5.752± 0.808
2	4.58± 0.16	2.472 ± 0.122	0.921± 0.133	4.729 ± 0.08	3.460± 0.030	12.241± 0.11	8.838± 0.091
3	5.1± 0.11	3.072 ± 0.274	1.926± 0.161	4.994 ± 0.122	4.923± 0.052	22.115± 0.091	12.84± 0.03
4	7.62± 0.05	4.958 ± 0.08	4.042± 0.061	5.593 ± 0.244	5.117± 0.030	26.402± 0.165	19.047± 0.052
5	9.50± 0.06	8.126 ± 0.199	6.052± 0.352	5.258 ± 0.08	5.435± 0.061	34.923± 0.349	25.974± 0.152
6	17.91± 0.061	13.601± 0.045	7.885± 0.139	6.034 ± 0.052	5.646± 0.052	40.257± 0.275	32.278± 0.132
7	29.062 ± 0.22	21.747± 0.079	8.661± 0.08	6.21± 0.133	5.822± 0.052	51.277± 0.381	37.435± 0.132
8	34.035 ± 0.318	22.911± 0.121	8.679± 0.052	6.316 ± 0.11	6.034± 0.091	53.393± 0.076	42.328± 0.132
24	33.152 ± 0.11	22.831± 0.091	8.926± 0.091	7.286 ± 0.152	5.964± 0.061	52.644± 0.349	43.298± 0.275

Percentage bioactive constituents release was found to be maximum with combination of 1% DMSO and 10% glycerol as permeation enhancers.

4. Conclusion

The comprehensive analysis and evaluation of the developed liposomal formulations reveal that the integration of traditional ethnomedicine with modern nanotechnology offers a superior therapeutic platform. The study successfully optimized cow urine-loaded liposomes using a Box-Behnken Design, achieving a maximum entrapment efficiency of 98.28% in the optimized batch (F5).

A critical finding of this research is the significant impact of permeation enhancers on the delivery profile of the liposomal gel. While the standard liposomal gel (LG-3) showed promising results, the final optimized formulation, LG-3.6 (containing PG, 1% DMSO and 10% Glycerol), emerged as the most superior candidate. This formulation demonstrated an exceptional bioactive constituents content of 96.847 ± 0.872% and achieved a maximum in-vitro bioactive constituents release of 52.644 ± 0.349% over 24 hours. This is a substantial improvement over the control gel's release of 13.15%.

The release kinetics of LG-3.6 followed the Korsmeyer-Peppas model, indicating a synergistic mechanism of diffusion and erosion. Stability studies further confirmed that the formulation remains robust under standard storage conditions. Therefore, it is concluded that the LG-3.6 liposomal gel is a highly effective, stable, and scientifically validated transdermal system, providing a modern alternative for the therapeutic application of cow urine bioactive fractions.

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