RESEARCH PAPER

Formulation, Optimization, and Characterization of Propylene Glycol-Based Liposomes Encapsulating Cow Urine Distillate for Enhanced Therapeutic Efficacy

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ABSTRACT

Traditional medicine has long recognized the therapeutic potential of Cow Urine (Gomutra), but its volatile nature and low bioavailability limit its clinical application. This study focuses on the development of Propylene Glycol (PG) based liposomes to encapsulate cow urine constituents using the thin-film hydration technique. The formulation was optimized using a Box-Behnken Design (BBD) to evaluate the impact of lipid concentration and PG ratio on vesicle size and entrapment efficiency. Results indicated an optimized particle size of 215 nm with a PDI of 0.263 and a Zeta Potential of -15.1 mV, suggesting moderate colloidal stability. The optimized formulation (LG-3) exhibited a sustained release profile of 20.26% over 24 hours in a carbopol gel base. FT-IR and DSC studies confirmed the successful encapsulation of bioactive components without chemical incompatibility. These findings suggest that PG-liposomes significantly enhance the stability and controlled release of cow urine, providing a promising platform for antimicrobial and immunomodulatory therapies.

Keywords: Cow urine, Liposomes, In vitro analysis.

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Introduction

The integration of traditional ethnomedicine with modern nanotechnology has opened new avenues in bioactive constituents delivery. Cow urine, a key component of 'Panchgavya' in Ayurveda, is known for its potent antimicrobial, antioxidant, and bio-enhancing properties. However, the presence of volatile organic compounds and rapid degradation of its bioactive components pose challenges for standardized therapeutic use.

To overcome these limitations, lipid-based carrier systems, particularly liposomes, offer a viable solution. Propylene Glycol (PG) liposomes, often referred to as pro-liposomes or penetration-enhanced vesicles, provide superior stability and skin penetration compared to conventional liposomes. By encapsulating cow urine within these phospholipid bilayers, the stability of its sensitive constituents is maintained, and the delivery to target sites is improved. This research details the systematic development, optimization via design of experiments (DoE), and characterization of cow urine-loaded PG liposomes to evaluate their potential as a standardized pharmaceutical dosage form.

Materials and method

Sample Collection and Preparation

Based on the literature review, specific materials, reagents, and equipment were utilized for the preparation and characterization of liposomes. Urine samples were collected from four distinct physiological groups of the Malvi breed: adult, pregnant, lactating, and calf.

Experimental Groups and Processing

Mid-stream, early morning first-voided urine samples (100–150 mL) were collected under aseptic conditions from:

* A three-year-old adult cow.
* A pregnant cow (eight months gestation).
* A lactating cow (one month post-parturition).
* A one-month-old calf.

The collected raw urine samples were filtered using a 0.2 μm syringe filter to ensure sterility. For the distilled groups, the urine was processed at 100°C using specialized glass distillation apparatus. All samples were aliquoted and preserved at -18°C for subsequent in vitro and in vivo analyses. The nomenclature and abbreviations for these experimental groups are summarized in Table 1.

Table 1: Cow urine samples used from different groups

Experimentalgr oups	
Rawur ine	**Distilledur ine**
AdultRawCow (AR	Adult (AD

Urine CU)	DistilledCowUrine CU)
PregnantRawCowUrine (PRCU)	PregnantDistilledCowUrine(PDCU)
LactatingRawCowUrine(LRCU)	LactatingDistilledCowUrine(LDCU)
CalfRawCowUrine (CRCU)	CalfDistilledCowUrine (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

In vitro research used different groups, as stated in the table. All experimental groups were employed for biochemical constituent estimates and antioxidant activity tests. Since 'go-arka'—distilled cow pee from an adult cow—is used to treat numerous ailments, both raw (ARCU) and distilled (ADCU) Malvi cow urine were employed for function analysis.

Estimations of biochemical constituents:

All experimental groups were evaluated for biochemical constituents and antioxidant activity. Given that 'Go-arka' (distilled cow urine from an adult cow) is traditionally utilized for therapeutic purposes, both raw (ARCU) and distilled (ADCU) Malvi cow urine were subjected to functional analysis.

a. Physical Examination: All urine samples were visually inspected for color, appearance, and the presence of sediment or deposits.

b. Chemical Examination: The pH of the urine samples was measured using a digital pH meter. The presence of albumin, glucose, bile salts, bile pigments, and reducing sugars was determined using standardized dipstick testing.

c. Microscopic Examination: Samples were examined under a light microscope to identify the presence or absence of casts, crystals, epithelial cells, bacteria, and fungi.

d. Biochemical Analysis: Quantitative estimation of biochemical constituents—including urea, urea nitrogen, uric acid, creatinine, calcium, sodium, and chloride—was performed using DiaSys Diagnostic Systems (India) reagent kits and a BS-300 automated chemistry analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.). Total phenolic content was estimated according to the method described by Malick and Singh (1980). Statistical analysis was subsequently performed to evaluate significant differences between the experimental groups.

Preformulation Study

Formulation of Liposomes

Liposomes were prepared using the thin-film hydration method, chosen for its efficiency in achieving high entrapment and producing multilamellar vesicles (MLVs). Briefly, a lipid solution was evaporated to form a thin film at the base of a round-bottom flask. This film was subsequently hydrated with an aqueous buffer and subjected to vortexing. The hydration process was maintained at a temperature exceeding the gel-liquid crystalline transition temperature (T_m) of the lipids. Hydration was performed at (45-50°C), which is above the phase transition temperature of the phospholipids used. Depending on the solubility profile of the bioactive compounds, either an aqueous buffer or an organic solvent was utilized for encapsulation.

Table 3:Compositiontableofdifferentbioactive constituentsloadedliposomes

S. No.	**Formulationcode**	**Cow Urine**	**Phospholipid90G (Molar)**	**Phospholipid90H (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

Evaluation of bioactive constituents loaded liposomes

The prepared liposomal formulations were evaluated

based on the following parameters:

* **Visual Appearance:** The physical state and homogeneity of the formulations were assessed through direct visual inspection.

* **Entrapment Efficiency (EE%):** To determine the percentage of bioactive constituents entrapment, 1.0 mL of the liposomal suspension was centrifuged at 18,000 rpm for 1 hour. The resulting supernatant was diluted with methanol and analyzed using a UV-Visible Spectrophotometer at the maximum wavelength (λ_{max}). To enhance the stability of the suspension, resveratrol was complexed with Hydroxypropyl-Beta-Cyclodextrin prior to formulation.

Optimization of Cow Urine Liposomes using Box-Behnken Design (BBD)

To achieve a stable liposomal system, a 3-factor, 3-level Box-Behnken Design (BBD) was employed. This experimental design was used to optimize the concentration of three independent variables:

1. Phospholipid concentration
2. Cholesterol content
3. HPMC-CD concentration The goal was to investigate the effect of these variables on the physicochemical properties of the cow urine-loaded liposomes. The experimental range (minimum and maximum levels) for these factors is detailed in Table 4.

Preparation of Optimized Liposomes

Liposomes were prepared using the thin-film hydration technique. Briefly, the lipids (phospholipid and cholesterol) were dissolved in a chloroform-methanol mixture (3:1 v/v). The solvent was evaporated at 45 °C under vacuum using a rotary evaporator to form a uniform thin lipid film on the walls of the round-bottom flask.

The resulting film was hydrated using an aqueous phase containing the cow urine-HPMC-βCD complex. For the aqueous phase preparation, cow urine was incorporated into the ethanol/aqueous medium and subjected to sonication. The HPMC-βCD was dissolved in deionized water, and the two phases were mixed in a 1:1 ratio and stirred for 1 hour. This systematic approach using BBD allowed for the production of liposomes with varied lipid compositions to identify the optimal formulation.

Table4:CompositionofliposomesusingBoxBehnkendesign

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

Formation of liposomes: Casting a thin lipid coating on a round bottom flask wall produced liposomes. Aqueous hydration follows. Cow urine, cholesterol, and fat dissolved in chloroform and methanol (3: l). The solvent evaporated at 45℃ under vacuum with steady flask rotation. Aqueous cow urine complexed with HP-β-CD was used to hydrate the produced film. Cow Urine is dissolved in ethanol and sonicated to create the aqueous phase, while Hydroxypropyl-β-Cyclodextrin is dissolved in water. Mix both liquids 1:1 and stir for 1 hour. Box Behnken Design liposomes with diverse lipid contents were made.

Selection of Optimized Formulation

Liposomes based on the compositions derived from the Box-Behnken Design were formulated, and their entrapment efficiency for cow urine was determined. Based on the desirability score (Table 5) and the lowest standard deviation observed, the combinatorial set S-4 was identified as the optimized formulation and selected for further characterization.

Table 5: Number of solutions with desirability=1

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

Liposomes of above composition were formulated and percent entrapment of Cow Urine was determined. Minimum standard deviation was observed in combinatorial set S-4 thus this combination was considered as optimized parameter of the process.

Characterization of the Optimized Liposomal Suspension

The optimized bioactive constituents-loaded liposomes (S-4) were evaluated using the following parameters:

* **Visual Appearance:** The physical state and homogeneity of the suspension were assessed via visual inspection.

* **Particle Size Analysis:** The mean vesicle size and size distribution profile were determined using

a Malvern Particle Size Analyzer (Model SM 2000), based on Mie's theory of light scattering. The liposomal suspension was diluted and added to a sample dispersion unit equipped with a stirrer, agitated at 2,000 rpm to minimize interparticle aggregation. The laser obscuration was maintained within the range of 10–20%. Measurements were performed in triplicate to calculate the average particle size.

* **Zeta Potential:** The physical stability of the colloidal dispersion was evaluated by measuring the zeta potential using a Zetasizer 300 HSA (Malvern Instruments, UK). The surface charge of both empty and bioactive constituents-loaded vesicles was measured over a duration of 60 s.

* **FT-IR Spectroscopy:** To identify chemical interactions, the FT-IR spectrum was recorded using the KBr pellet technique. Briefly, the sample was ground with KBr using a mortar and pestle, then compressed at 5,000–10,000 psi to form a translucent pellet. The pellets were then analyzed in the FT-IR spectrophotometer.

* **Differential Scanning Calorimetry (DSC):** Thermal analysis was conducted using a Perkin Elmer DSC-4000 (USA). Approximately 5 mg of the sample was sealed in an aluminum pan and heated at a constant rate of 20 °C/min up to 300 °C, with an empty aluminum pan serving as the reference.

X-ray Diffraction Analysis (XRD) of liposomes: The crystalline or amorphous nature of the liposomes was investigated using an X'Pert Pro X-ray diffractometer (Panalytical, Netherlands). Powder XRD (P-XRD) studies were conducted by exposing the samples to Cu-α$ radiation (operating at 45 kV and 30 mA). The samples were scanned over a (ɵ) range of 10° to 60° with a step size of 0.05° and a scanning speed of 10°/min. The interlayer spacing (d) was calculated from the scattering angle (ɵ) using Bragg's equation:

$$N\lambda = 2d \sin\theta$$

Entrapment Efficiency (EE%)

To separate the liposome-bound bioactive constituents from the unentrapped fraction, the formulation was subjected to centrifugation at 18,000 rpm for 1 hour at 4 °C. The supernatant containing the unentrapped medication was collected and analyzed using a UV-Visible Spectrophotometer with methanol as the blank. The entrapment efficiency was then calculated to determine the bioactive constituents-loading capacity.

Preparation of Liposomal Gel

The liposomal gel was prepared using different concentrations of Carbopol 934 (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 dispersion was neutralized using triethanolamine or 1N NaOH and swirled gently until a clear gel was formed.

Freeze-dried liposomes were then incorporated into the Carbopol base with continuous stirring in closed vessels to ensure a uniform distribution. Additionally, composite gel formulations were prepared by incorporating HPMC K-100 and Sodium Carboxymethyl Cellulose (Na-CMC) in fixed ratios (Table 6). The same neutralization and swelling procedure was followed, after which 57 mg of cow urine-loaded liposomes were introduced into the matrix to form the final liposomal gel.

Table6:Compositionofgelformationof Cow Urine

S.No.	Formulationcode	Cow Urine(mg)	Carbopol %(w/w)	HPMC K-100	SodiumCMC
1	LG-3.1	57	1	-	-
2	LG-3.2	57	1.5	-	-
3	LG-3.3	57	2	-	-
4	LG-3.4	57	1	0.5	1
5	LG-3.5	57	1	0.5	0.5

FORMULATION & EVALUATION

Formulation of Control Gel

* **Preparation of Gel Base:** A 2% w/w Carbopol 934 gel base was prepared by dispersing the gelling agent in distilled water and allowing it to hydrate overnight.
* **Incorporation of Bioactive constituents:** The required amount of bioactive constituents was accurately weighed and incorporated into the 2% w/w Carbopol base with gentle stirring to ensure a homogeneous and transparent gel. The mixture was neutralized using triethanolamine and mixed thoroughly to achieve the desired consistency.

Evaluation of Liposomal Gels

The prepared formulations were subjected to the following physicochemical evaluations:

* **Visual Appearance:** The color, clarity, and overall physical state of the gels were recorded through visual inspection.
* **Homogeneity and Grittiness:** The gels were tested for structural uniformity and the presence of any particulate matter (grittiness) by manual touch and visual check.
* **pH Determination:** 1.0 g of the gel was dissolved in 10 mL of distilled water. The pH of the resulting solution was measured using a digital pH meter (Labindia, Mumbai).
* **Bioactive constituents Content Determination:** 0.5 g of the gel was diluted to 5 mL with methanol. The mixture was centrifuged at 18,000 rpm for 30 minutes at 4 °C to extract the bioactive constituents from the incorporated liposomes. The bioactive constituents concentration was then analyzed using a UV spectrophotometer.
* **Spreadability:** The spreadability of the liposomal gel was determined using a custom-modified apparatus to evaluate the ease of topical application.

Results & Discussion

* **Preformulation Study:** Initial studies confirmed the suitability of the selected excipients and active constituents.
* **Biochemical Constituents of Cow Urine:** The physico-chemical analysis of raw and distilled cow urine from various groups (Adult, Pregnant, Lactating, and Calf) of the Malvi breed was performed. The data, including pH and physical parameters, are presented as Mean ± SEM (n=3) in Table 7.

Table7: Physico-chemical analysis of raw and distilled cow urine from an adult (ARCU, ADCU); pregnant (PRCU, PDCU); lactating(LRCU,LDCU)andcalf(CRCU,CDCU)of Malvi. (Mean± SEM)(n=3)

	ARCU	ADCU	PRCU	PDCU
I.Physicalexamination				
Colour	Paleyellow	Colourless	Pale yellow	Colourless
Appearance	Clear	Clear	Clear	Clear
Deposits	-Nil-			
II. Chemicalexamination				
pH	8.36± 1.24	7.36±1.52	8.21± 0.5	7.25± 1.12
Albumin,Glucose,Bilesalts -Nil-				
III.Microscopicexamination				
Crystals,Casts,Epithelialcells -Nil-				

	LRCU	LDCU	CRCU	CDCU
I.Physicalexamination				
Colour	Paleyellow	Colourless	Paleyellow	Colourless
Appearance	Clear	Clear	Clear	Clear
Deposits	-Nil-			
II. Chemicalexamination				
pH	8.0± 0.25	7.20± 1.50	8.52± 1.24	7.30± 1.32
Bilepigments, Reducingsugars -Nil-				
III.Microscopicexamination				
Bacteria,Fungus -Nil-				

Theresultsonbiochemicalconstituentsofrawanddistilledcowurinefrom Malviaregivenintablebelow

Table 8: Biochemical constituents of raw and distilled cow urine from an adult (ARCU, ADCU); pregnant (PRCU, PDCU); lactating (LRCU, LDCU) and calf (CRCU, CDCU) of Malvi cow. (Mean ± SEM) (n=3)

Parameters	ARCU	ADCU	PRCU	PDCU
Urea(mg/dL)	63.20 ± 7.20	25.00± 10.00	68.25± 9.25	37.20± 8.20
Urea nitrogen (mg/dL)	30.57 ± 6.57	17.16± 7.10	31.50± 8.50	17.70± 7.70
Uricacid(mg/dL)	9.10± 4.10	0.15± 0.10	3.86± 3.36	0.11± 0.06
Creatinine(mg/dL)	57.35 ± 10.35	0.19±0.14	30.37± 5.37	0.14± 0.09
Calcium(mg/dL)	12.15 ± 7.15	0.13± 0.06	16.30± 6.30	0.54±0.45
Sodium(mM/L)	97.45	14.50±	114.72	22.25±

	± 7.55	5.50	± 4.72	7.25
Chloride(mM/L)	55.25 ± 9.75	10.85± 5.15	57.75± 7.75	12.05± 7.05
Total phenolcontent (mg/100mL)	12.06 ± 2.58	4.56± 1.33	19.33± 4.33	7.03± 1.18

Parameters	**LRCU**	**LDCU**	**CRCU**	**CDCU**
Urea(mg/dL)	43.60± 8.60	16.70± 6.70	48.50± 7.50	20.09± 5.09
Urea nitrogen (mg/dL)	20.40± 5.40	7.80± 4.30	29.40± 5.40	11.60± 6.60
Uricacid(mg/dL)	10.30± 8.30	1.27± 1.22	8.55 ± 0.55	0.12± 0.07
Creatinine (mg/dL)	40.45± 9.55	0.17±0.12	79.85± 2.35	0.85±0.80
Calcium(mg/dL)	24.30± 6.30	1.07±1.02	0.44 ± 0.35	0.12± 0.07
Sodium(mM/L)	188.50 ± 8.50	23.25 ±5.25	82.25 ±10.25	10.15± 5.15
Chloride(mM/L)	156.62±10.62	36.00±14.00	52.75± 7.25	5.00±3.10
Total phenol content (mg/100mL)	14.30±3.72	5.90±2.55	8.53 ± 2.49	3.16±0.55

Compatibility Studies

Ultraviolet (UV) Spectroscopy Analysis

The absorption maxima (λmax) of the excipients were determined using UV-Visible spectroscopy to evaluate potential bioactive constituents-excipient interactions. The λmax for Phospholipon 90G was identified at 279 nm, while cholesterol exhibited a peak at 284 nm. The physical mixture of phospholipids and cholesterol showed an absorption peak at 213 nm.

As shown in Figure 1, the characteristic peaks of the 90G lipids and cholesterol did not interfere with the absorption maxima of the cow urine constituents. This lack of significant spectral shift confirms the absence of chemical incompatibility between the bioactive constituents and the selected excipients.

Differential Scanning Calorimetry (DSC)

To evaluate the thermal behavior and physical state of the components, DSC thermograms were recorded for cow urine, phospholipids, and their physical mixture. The resulting thermograms are presented in Figure 3 (as per your document's flow). The stability of the endothermic peaks in the physical mixture suggests that the bioactive constituents remains in its stable form within the liposomal matrix without undergoing any significant phase transition or interaction with the lipids.

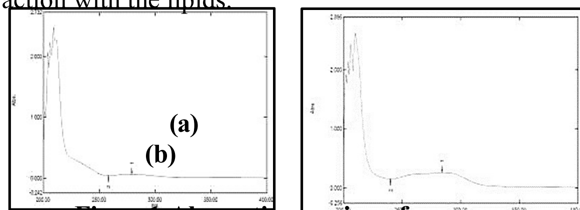


Figure1:Absorptionmaximaof a)Phospholipid90Gb)Cholesterol

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 constituents and excipients were found to be compatible.

Fourier-Transform Infrared (FT-IR) Spectroscopy

The FT-IR spectra of the physical mixture, containing both the bioactive constituents and excipients, were recorded to assess molecular compatibility. The spectrum, illustrated in Figure 2, displays the characteristic functional group peaks. The retention of major peaks from both the cow urine components and the lipid excipients in the mixture indicates that no chemical interaction occurred, further confirming the compatibility of the formulation components.

of physical mixture of Cow Urine, phospholipid 90 G and H, cholesterol

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

The thermal profiles of cow urine, phospholipids, and their physical mixtures were evaluated using DSC. The respective thermograms are illustrated in Figures 3, 4, 5, and 6.

As shown in Figure 3, the DSC thermogram of cow urine exhibited a sharp endothermic peak at 107 °C, signifying the purity and crystalline nature of the sample. In the physical mixture of cow urine, phospholipid 90G, and cholesterol (Figure 6), the characteristic endothermic peaks of the individual components—including the cholesterol peak at 142.3 °C—were clearly observed.

The retention of these specific thermal transitions in the physical mixture confirms that there is no chemical incompatibility between the cow urine

constituents and the selected lipid excipients. This indicates that the bioactive constituents/active components remain stable within the formulated liposomal environment.

Figure 3:DSCthermogramofCow Urine

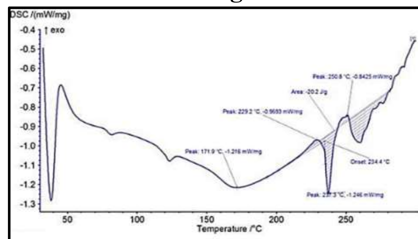


Figure 4: DSC thermogram of Phospholipon 90 G

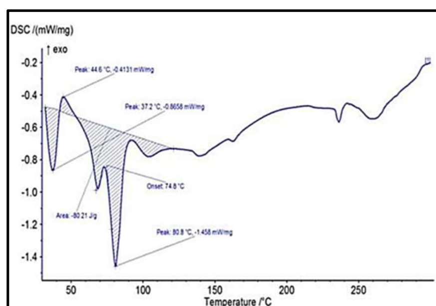
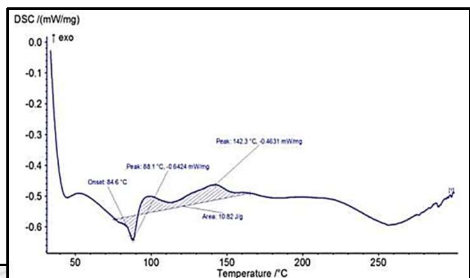


Figure 5: DSC thermogram of Phospholipon 90 H



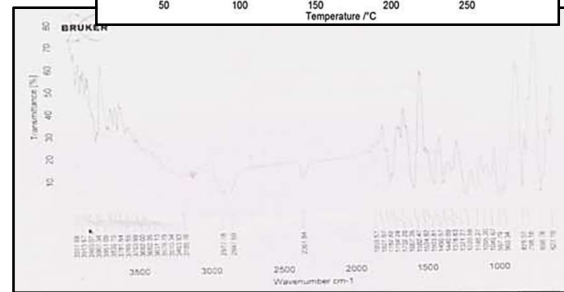


Figure 6: DSC thermogram of physical mixture of Cow Urine, lipid 90 G and cholesterol

XRD analysis: To further confirm the physical state and compatibility of the components, XRD analysis was performed on cow urine and the physical mixture. The diffraction patterns were recorded to identify any changes in crystallinity or the formation of new phases. The results showed that the characteristic diffraction peaks remained unaltered, reinforcing the findings from the DSC and FT-IR studies regarding the molecular compatibility and stability of the liposomal formulation.

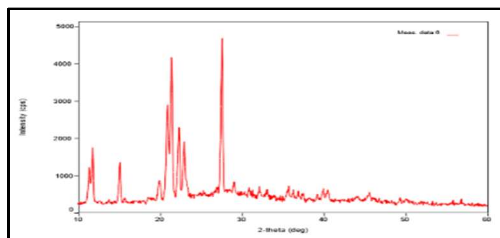


Figure 7: XRD spectra of Cow Urine

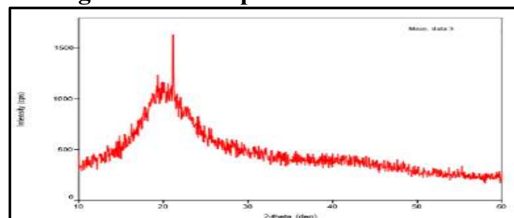


Figure 8: XRDspectraofPhospholipon90G

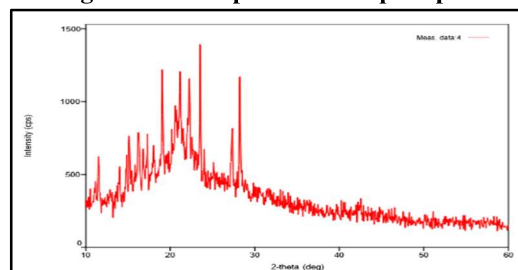


Figure 9: XRDspectraofCow Urine,phospholipid90G,andcholesterol

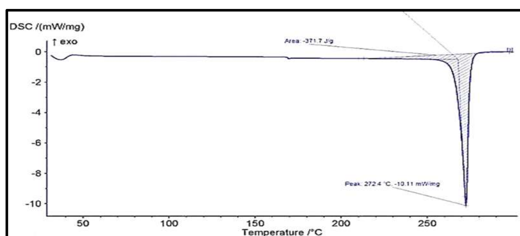
Preparation of liposomes

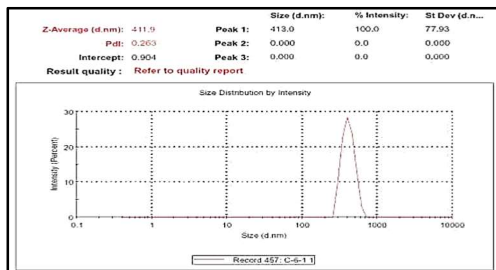
The liposomes were prepared by casting a thin film of lipids on the wall of a round bottom flask. The thin film was subsequently hydrated with aqueous phase containing Cow Urine complexed with Hydroxypropyl-β-Cyclodextrin

Evaluation of selected Cow Urine loaded liposomes

Visual appearance: Liposome suspension was found to be milky in appearance.

Particle size: Particle size results of optimized formulation of liposomes with PDI (Poly dispersity index) was 0.263, the value less than 0.3. It represented uniform particle size distribution. The results are indicated in figure.





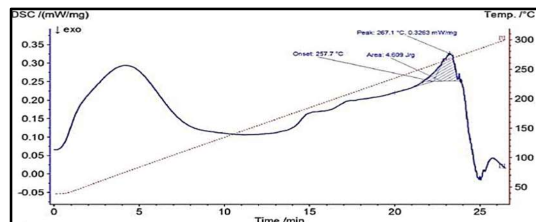
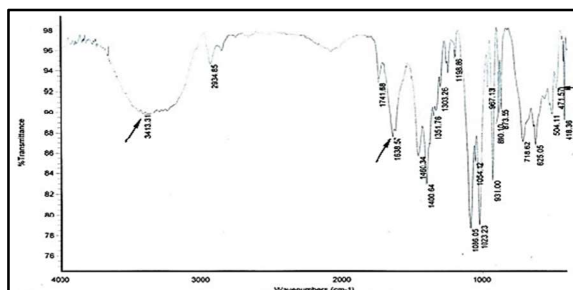


Figure 8: Particle size peak of optimized formulation

Zeta potential: Zeta potential value of optimized liposomes was found to be -15.1. The negative value of zeta potential of liposomes indicates the Brownian motion stability between the particles. The results are indicated in figure.

Figure9:Zetapotentialgraphofoptimizedformulati



on

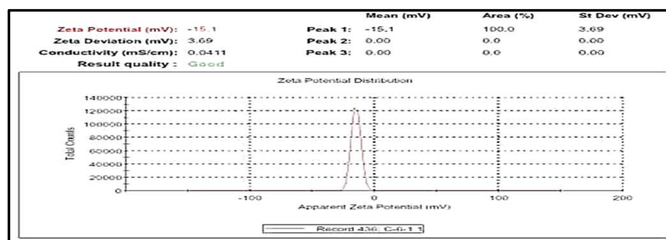
Fourier Transform Infrared Spectroscopy (FT-IR): FT-IR of optimized liposomal formulation containing Cow Urine was recorded. The FTIR spectrum of formulation displayed major peaks ofCow Urineandphospholipidwithsomeminor displacements as indicated in figure.

Figure 10: FT-IR spectrum of liposomal formulation

Differential Scanning Calorimetry (DSC) of liposomes: DSC thermogram of final liposomal formulation was recorded. From the above thermogram it was found that there is phase transition temperature of the Cow Urine loaded liposomes at 267.1° C. As there is shift in the transition temperature. It indicated a strong hydrophobic interaction between Cow Urine and the phospholipids forming the liposome. It indicated entrapment of Cow Urine into liposomes. So, DSC thermogram of Cow Urine loaded formulation ensured encapsulation of Cow Urine into liposomes.

Figure 11: DSC thermogram of liposomes

XRD of liposomal formulation: XRD of optimized



liposomal formulation was recorded. X-ray diffraction studies of liposomes showed the amorphous nature of Cow Urine encapsulated in the formulation. The characteristic peaks of Cow Urine were found to be absent. The pattern showed less number and intensity of peaks which showed that crystallinity of the Cow Urine has somewhat changed. It indicated successful encapsulation of Cow Urine.

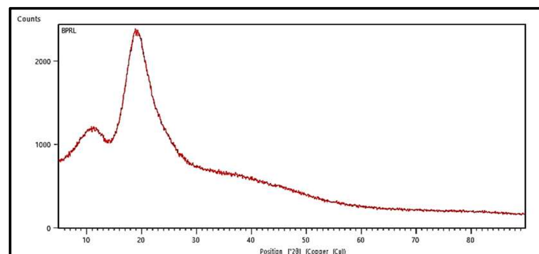


Figure12:XRDspectraofliposomes

Lyophilization of liposomal suspension: Lyophilization (Freeze drying) is important for increasing stability of liposomes. Liposomal suspensions were lyophilized by freeze dryer.

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

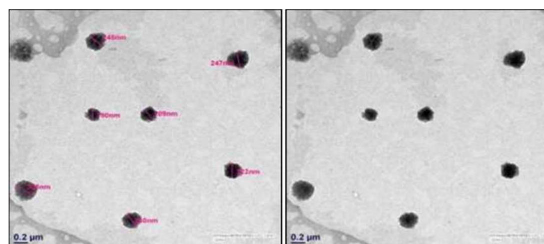


Figure 13: TEM analysis of liposomes

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.

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

Evaluationofliposomalgels

Appearance:

Allthegelformulationswerefoundtobetransparent.

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.

Table9:pHoftheliposomalgels

S. No.	Formulation code	Reading1	Reading2	Reading3	Mean± S.D
1	LG-3.3	6.4	6.33	6.31	6.34±0.047
2	LG-3.4	7.4	7.41	7.38	7.39±0.015
3	LG-3.5	7.3	7.35	7.26	7.30±0.045
4	LG-3.6	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 10: Percent Cow urine of different gels containing liposomes

S. No.	Formulationcode	% Cow Urine (Mean ±S.D)
1	LG-3.1	Gelwasnotformed
2	LG-3.2	75.687±1.511
3	LG-3.3	89.290±1.511
4	LG-3.4	77.199±1.511
5	LG-3.5	75.184±0.872
3	LG-3.6	98.290±1.511

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 11: Homogeneity and grittiness of formulation

S.No.	FormulationCode	Grittiness	Homogeneity
1	LG-3.3	-	++
2	LG-3.4	-	+++
3	LG-3.5	*	+
4	LG-3.6	-	+

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 12: 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 13: 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 constituents 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.

Skin irritation study for prepared formulation: Any formulation that causes skin irritation may limit its use and patient acceptability, so liposomal gel must be tested for 7 days and compared to control gel and market preparation. The skin irritation research ensured formulation skin compatibility. Tables show skin irritation experiment outcomes. Optimized and control liposomal gels were tested on animals.

Liposomal gel-treated rats showed no erythema or oedema. Bioactive constituents loaded liposomal gel had low mean erythemal scores due to its formation and the inclusion of carbopol, which increased viscosity and its three-dimensional network structure reduced bioactive constituents-skin interaction. Liposomes in gel base may be more patient-acceptable and tolerable than control gel.

Table14:Visualassessmentofskinirritation

Testsubstance	Meanerythemalscore(days)							R.I	Response
	1st	2nd	3rd	4th	5th	6th	7th		
Controlgel	0.00	0.285	0.428	0.571	0.714	0.857	1.00	0.550	Slight
Optimizedpreparation	0.00	0.00	0.00	0.00	0.714	0.857	1.00	0.367	Negligible

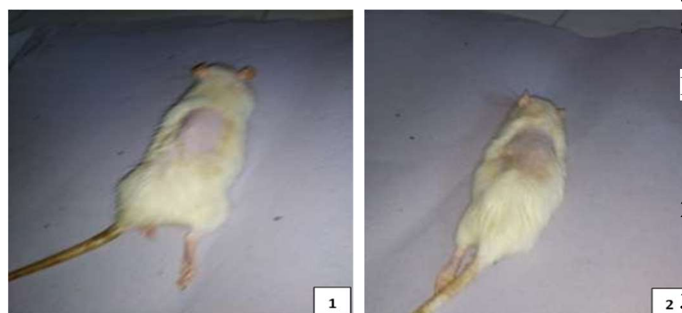


Figure 14: Application of 1) Control gel 2) Optimized liposomal gel

Skin histological examination: The biocompatibility of control gel and liposomal gel formulation was assessed by histopathological examination of wistar albino rats' dorsal skin. Figure shows histopathological pictures with no substantial skin thickening and undamaged rat epidermis. No inflammation was seen with liposomal gel, proving its biocompatibility. Optimized liposomal gel did not irritate skin after short use.

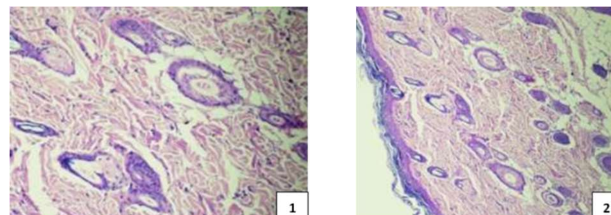


Figure 15: Histological examination of skin tissue after treatment with 1) Control gel 2) Liposomal gel

onclusion

The comprehensive analysis and evaluation of the liposomal formulations in this study conclude that the optimized liposomal gel (LG-3.6) exhibits superior physicochemical and therapeutic characteristics. By incorporating PG, 1% DMSO, and 10% glycerol as permeation enhancers, the formulation achieved efficient encapsulation of cow urine constituents with enhanced stability and controlled release. Key findings indicate that LG-3.6 maintains a significantly lower viscosity compared to conventional control gels, leading to improved spreadability. The kinetic release profiles followed the Korsmeyer-Peppas model, suggesting a mechanism governed by diffusion and erosion. Furthermore, skin irritation and histopathological examinations on Wistar albino rats confirmed the biocompatibility of the preparation, with negligible irritation observed. Stability studies over six months confirmed that the liposomal gel remains physically and chemically stable, making it a promising delivery system for cow urine constituents.

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