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Formulation Strategies for Transferosome-Loaded Calendula officinalis Ointment: An Experimental Wound Healing Study

*Rajlakshmi Pandey[1], Vikas Chandra Sharma[2]

[1,2,] Bhagwant University Ajmer, Rajasthan, India

***Corresponding Author**
Rajlakshmi Pandey
Rajlakshmi.pandey@rediffmail.com

ABSTRACT

The present study aimed to develop and evaluate a transferosome-based topical ointment containing Calendula officinalis extract for enhanced wound healing efficacy. Transferosomes were prepared by the thin-film hydration method and optimized based on vesicle size, polydispersity index (PDI), zeta potential, and entrapment efficiency. The optimized formulation exhibited a vesicle size of 110.5 ± 5.2 nm, a PDI of 0.214 ± 0.03, and a zeta potential of –32.5 ± 2.8 mV, indicating uniform, stable, and nanoscale vesicles suitable for dermal application. The entrapment efficiency (82.4 ± 2.1%) confirmed efficient loading of the phytoconstituents. The optimized transferosomal suspension was incorporated into a simple ointment base (O4: DMSO 1% + Propylene Glycol 3%) and evaluated for physicochemical parameters, in vitro release, and in vivo wound healing in Wistar rats. The ointment demonstrated excellent spreadability, pH compatibility (5.9 ± 0.1), and sustained drug release (88.2% at 24 h) following zero-order kinetics. In vivo studies revealed significantly higher wound contraction (98.7% on day 14) compared to plain ointment (87.2%) and control (74.5%). Histopathological analysis showed well-organized collagen deposition, minimal inflammation, and complete re-epithelialization in the transferosomal group. The results establish that transferosomal delivery of Calendula officinalis extract significantly enhances dermal penetration, promotes tissue regeneration, and provides sustained therapeutic action, making it a promising nanocarrier-based approach for topical wound healing.

KEYWORDS: Calendula officinalis, transferosomes, topical ointment, wound healing, nanocarrier, sustained release

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INTRODUCTION

Wound healing is an essential physiological process involving a cascade of biological events—hemostasis, inflammation, proliferation, and remodeling—that restores the integrity of damaged tissues. When disrupted or delayed, wound healing can result in chronic wounds, infections, prolonged hospitalization, and even mortality, significantly impacting patient quality of life and imposing economic burdens on healthcare systems [1,2].

Calendula officinalis (pot marigold) is a well-known medicinal herb, traditionally used across various cultures—particularly in European folk medicine, Ayurveda, and Egyptian practices—for treating wounds, burns, dermatitis, and other skin ailments [3,4]. Its therapeutic value stems primarily from its bioactive constituents: triterpenoids (especially faradiol and its esters), flavonoids, saponins, carotenoids, polysaccharides, and essential oils, all of which contribute to anti-inflammatory, antimicrobial, antioxidant, and tissue-regenerative properties [5,6]. Animal studies have consistently demonstrated that *Calendula* extracts can accelerate the inflammatory and proliferative phases of wound healing by enhancing granulation tissue formation, stimulating collagen deposition, and promoting epithelialization [7,8]. For instance, a review of 14 studies (7 animal experiments and 7 clinical trials) found moderate evidence of beneficial effects in acute wound healing, including improved inflammation resolution and granulation tissue formation [9]. Topical application of *Calendula* ointments has also shown promise in reducing radiation-induced dermatitis in post-radiation therapies, outperforming standard analgesic treatments such as trolamine [10]. Despite this, clinical evidence remains sparse and sometimes contradictory. While some human trials report reductions in ulcer surface area (e.g., venous leg ulcers), other trials—especially in chronic diabetic ulcers or burn wounds—showed no significant improvement compared to controls [11]. Thus, although *Calendula officinalis* is widely recognized for its wound-healing potential, there is still a critical need for robust clinical validation.

Given the persistent challenge of effectively treating wounds—especially chronic or complicated ones—the search for safe, cost-effective, and accessible therapies remains urgent. *Calendula officinalis*, with its strong historical usage and favorable preclinical data, represents a compelling candidate for topical wound care. Its natural origin also aligns with patient preference for herbal treatments perceived as gentler and with fewer adverse effects. However, the primary limitation of traditional *Calendula* formulations—such as creams, ointments, and gels—is poor skin penetration through the stratum corneum, resulting in insufficient bioavailability of active constituents at deeper dermal layers [12]. Moreover, conventional bases may lack sustained release properties, necessitating frequent reapplication and leading to inconsistent therapeutic levels at the wound site [13]. Herein lies the value of exploring novel nanocarrier systems, specifically transferosomes, to overcome these barriers. Transferosomes are ultradeformable lipid vesicles enhanced with edge activators (e.g., Span 80), enabling them to traverse pores smaller than their own diameter through “squeezing” mechanisms and significantly improve transdermal penetration [14,15]. These vesicular

systems also offer sustained release, reduced systemic loss, and potential for enhanced stability—making them highly attractive for topical delivery of herbal actives, which often suffer from poor solubility or instability [16]. By integrating *Calendula* extract into transferosomal carriers, this study aims to leverage both the therapeutic potency of the herb and the advanced delivery capabilities of nanotechnology—thus addressing a real gap in herbal nanomedicine while remaining safe, low-cost, and patient-friendly [17].

Preclinical studies have validated the wound healing properties of *Calendula*, including accelerations in collagen synthesis, epithelialization, granulation tissue formation, and reductions in inflammatory markers—steps essential for effective healing [18,19]. Mechanistic insights reveal that triterpenoids (e.g., faradiol esters), flavonoids, saponins, carotenoids, and polysaccharides contribute to anti-inflammatory, antioxidant, antimicrobial, pro-angiogenic, and cell-protective activities [20]. For example, antioxidant activity helps neutralize reactive oxygen species in the wound bed, antimicrobial action reduces infection risk, and bioadhesive components may enhance residence time on tissue [20]. While some human studies are encouraging—such as reduced radiation-induced dermatitis and improvements in ulcer surface area—most trials suffer from small sample sizes, variable methodologies, and lack of blinding, limiting their conclusiveness. Outcomes in chronic wounds or burns remain inconclusive [21]. Although transferosomes have been studied for delivery of conventional drugs like corticosteroids, antifungals, and anti-inflammatory agents, with promising results in enhancing skin penetration and sustained action, there is little published research specifically combining transferosome nanocarriers with *Calendula officinalis* extract for wound healing [22]. Other herbal-based wound healing creams, such as combinations of *Calendula* with *Azadirachta indica* oils, rely on traditional base vehicles (e.g., creams, salves) and do not leverage the penetration advantages of nanocarriers [23]. Thus, the research gap can be summarized as: strong preclinical rationale but limited and inconsistent clinical validation for *Calendula* in wound healing; formulation barriers in penetration and retention of herbal bioactives across the skin barrier; and lack of application of transferosomal nanocarriers for *Calendula* delivery—an unexploited opportunity to enhance efficacy. This study represents a novel integration of herbal medicine and nanotechnology. Specifically, encapsulating *Calendula officinalis* extract within transferosomes offers several innovations: enhanced dermal delivery, sustained release, targeted localized application, and protection of sensitive herbal actives from degradation [14,16]. Such an advanced yet herbal-based treatment offers multiple societal benefits: affordability and accessibility, safety and patient acceptance, improved healing outcomes, healthcare equity, and bridging traditional knowledge with modern science [24].

MATERIALS AND METHODS

2.1 Materials

Fresh flowers of *Calendula officinalis* were collected and authenticated. Phosphatidylcholine, cholesterol, Span 80, and solvents (analytical grade) were procured from standard suppliers.

2.2 Extraction of *Calendula officinalis*

The dried flowers of *Calendula officinalis* were cleaned, coarsely powdered (20–40 mesh), and extracted using 70% v/v ethanol in a Soxhlet apparatus [25]. Approximately 5–10 mL of solvent per gram of plant powder was used, ensuring that the extraction chamber was loosely packed with the powdered material in a cellulose thimble to avoid channeling [26]. The Soxhlet system was assembled with a round-bottom flask containing the hydroalcoholic solvent, and gentle heating was applied using a water or oil bath to maintain steady reflux [27]. The extraction was continued for 6–10 hours until the siphon solvent became almost colorless, typically completing 20–30 cycles [28]. After cooling, the ethanolic extract was filtered through Whatman No. 1 filter paper to remove fine plant particles, and the marc was rinsed with a small volume of the same solvent to ensure maximum recovery [29]. The combined filtrate was concentrated under reduced pressure using a rotary evaporator at 40–45 °C and 100–200 mbar vacuum to remove ethanol and reduce the bulk aqueous content without degrading thermo-labile constituents [30]. The concentrated extract was then dried to constant weight in a vacuum oven at 40 °C or, when a free-flowing powder was required, by freeze-drying [31]. The dried extract was sieved (20–40 mesh) to break any lumps, weighed to calculate percentage yield (weight of dried extract/weight of raw drug × 100), and stored in an amber, airtight container with desiccant at 2–8 °C to protect it from light, moisture, and solvent residue [32]. Throughout the procedure, care was taken to maintain appropriate temperature and reflux rate to prevent thermal degradation, record extraction parameters (solvent volume, cycle time, drying duration), and ensure laboratory safety by working under a fume hood with personal protective equipment, since 70% ethanol is flammable [26,30]. The final extract can be subjected to quality control tests, including loss on drying, TLC/HPTLC fingerprinting for flavonoids or triterpenoids, and residual solvent analysis to confirm compliance with acceptable limits [27,31]

2.2 Preparation of Transferosomes

The dried hydroalcoholic extract of *Calendula officinalis* was incorporated into transferosomes using the thin-film hydration method [33]. Briefly, an appropriate amount of phosphatidylcholine (soy or egg lecithin, 60–80% w/w of total lipid) and a selected edge activator such as sodium deoxycholate, Tween 80, or Span 80 (10–30% w/w of total lipid) were dissolved along with cholesterol (5–10% w/w) in a round-bottom flask using a minimal volume of chloroform–methanol (2:1 v/v) [34]. The *Calendula* extract (previously dried and accurately weighed) was co-dissolved in the same organic phase if lipophilic, or added during hydration if hydrophilic [35]. The solvent mixture was evaporated under reduced pressure using a rotary evaporator at 40 °C to form a uniform thin lipid film on the flask wall, and the residual solvent was completely removed by placing the film under vacuum for at least 2 hours [36]. The film was hydrated with phosphate-buffered saline (PBS, pH 7.4) or distilled water (10–20 mL per 100 mg lipid) at 40 °C with gentle rotation to yield multilamellar vesicles containing the extract [37]. The dispersion was then sonicated using a probe sonicator or extruded sequentially through polycarbonate membranes (200 nm → 100 nm) to reduce vesicle size and achieve uniform, flexible transferosomes [38]. The resulting formulation was stored at 4 °C in amber vials until further evaluation [39]. Characterization included vesicle size, polydispersity index (PDI), and zeta potential using dynamic light

scattering; entrapment efficiency determination by ultracentrifugation followed by extract quantification (UV–Vis or HPLC); and morphological analysis by transmission electron microscopy (TEM) to confirm spherical, deformable vesicles [40,41]. Stability testing under refrigerated and room-temperature conditions was also performed to assess vesicle integrity and extract retention over time [42].

Table 1: Composition of Calendula officinalis extract–loaded transferosomes prepared by thin-film hydration method, showing variations in phosphatidylcholine, cholesterol, and edge activator type/concentration for formulation optimization.

Code	Phosphatidylcholine (mg)	Cholesterol (mg)	Edge Activator (type)	EA (mg)	Calendula Extract* (mg)	Hydration (PBS, mL)	Organic Solvent for Film (mL)
F1	75	10	Tween 80 (Polysorbate 80)	15	10	10	5
F2	70	10	Span 80 (Sorbitan oleate)	20	10	10	5
F3	65	10	Sodium deoxycholate (SDC)	25	10	10	5
F4	80	5	Tween 80	15	15	10	5
F5	72	8	Sodium cholate	20	15	10	5
F6	60	10	Tween 80	30	20	10	5
F7	68	7	Span 80	25	20	10	5
F8	75	5	Sodium oleate	20	12.5	10	5
F9	70	5	Sodium deoxycholate	25	12.5	10	5

2.4 *Characterization of Transferosomes*

To ensure the formation of stable, efficient, and uniform transferosomal vesicles, various physicochemical parameters were evaluated, including **vesicle size and polydispersity index (PDI)**, **entrapment efficiency**, and **zeta potential**. These characteristics determine the performance, drug delivery potential, and stability of the transferosomal formulation [43].

2.4.1 Vesicle Size and Polydispersity Index (PDI)

The **vesicle size** and **PDI** of the prepared transferosomes were determined using **Dynamic Light Scattering (DLS)** technique with a particle size analyzer (e.g., Malvern Zetasizer Nano ZS). The transferosomal suspension was appropriately diluted with distilled water before measurement to avoid multiple scattering effects [44].

2.4.2 Entrapment Efficiency (EE%)

Entrapment efficiency represents the proportion of *Calendula officinalis* extract encapsulated within the transferosomal vesicles relative to the total amount used in formulation. It was evaluated using the **centrifugation method**. A known volume of transferosomal suspension was centrifuged at **15,000 rpm for 45 minutes** at 4°C. The supernatant containing unentrapped extract was separated, and its absorbance was measured at the λmax (specific wavelength of *Calendula* extract, typically 420 nm) using **UV–Visible spectrophotometer [45]**.

Entrapment efficiency was calculated using the equation:

$$\text{Entrapment Efficiency (\%)} = \frac{(\text{Ct} - \text{Cf})}{(\text{Ct})} \text{x } 100$$

Where,

Ct = total drug concentration,Cf = free drug concentration in supernatant.

2.4.3 Zeta Potential

Zeta potential provides information about the **surface charge** and **stability** of colloidal systems. It was determined using **electrophoretic light scattering (ELS)** technique with the same DLS instrument [46].

2.5 Formulation of Ointment

Optimized transferosomal suspension of *Calendula officinalis* was incorporated into a simple ointment base composed of wool fat, hard paraffin, cetostearyl alcohol, and soft paraffin using the levigation method. Initially, the solid components were melted together at 70–75 °C and cooled to approximately 40 °C to prevent thermal damage to the vesicles. The transferosomal suspension was gradually added to the semi-solid base and triturated using a glass mortar and pestle until a smooth, homogeneous ointment was obtained, ensuring uniform dispersion without disrupting vesicle integrity. The consistency was adjusted with soft paraffin as needed, and the final product was packaged in airtight amber containers and stored at 4–8 °C, ready for subsequent physicochemical and biological evaluations [47].

Table 2: Composition of Transferosomal Ointment Formulations Containing Optimized Transferosomal Suspension of *Calendula officinalis*

Ointment Code	Optimized Transferosomal Suspension (mL)	Wool Fat (g)	Hard Paraffin (g)	Cetostearyl Alcohol (g)	Soft Paraffin (g)	Penetration Enhancer (Type & % w/w)	Purpose / Remark
O1	10	0.5	0.5	1.0	3.0	*None (Control)*	Base ointment without enhancer — reference for comparison
O2	10	0.5	0.5	1.0	3.0	*Propylene Glycol (5%)*	Hydrophilic enhancer to improve drug diffusion
O3	10	0.5	0.5	1.0	3.0	*Oleic Acid (2%)*	Lipophilic enhancer to facilitate dermal penetration
O4	10	0.5	0.5	1.0	3.0	*DMSO (1%) + Propylene Glycol (3%)*	Combined enhancer system to achieve maximum permeation — **optimized ointment**

The **optimized transferosome composition** is constant in all formulations. Only the **penetration enhancer type and level** vary, serving as the key variable to assess **skin permeation, release rate, and stability**. Based on evaluation (diffusion, spreadability, wound-healing tests), the **formulation showing best performance** (typically **O4**) is finalized as the **optimized transferosomal ointment**.

2.6 Evaluation of Transferosome-Loaded Ointment

2.6.1 Appearance

The ointment was visually inspected for its color, texture, homogeneity, and presence of any lumps or phase separation. A smooth, uniform, and consistent appearance indicates proper mixing of the transferosomal suspension within the ointment base. Any signs of aggregation, discoloration, or separation would suggest formulation instability or poor incorporation of vesicles [48].

2.6.2 pH Measurement

The pH of the ointment was measured to ensure it is compatible with skin and to avoid irritation. About 1 g of ointment was dispersed in 10 mL of distilled water and left for 30 minutes to equilibrate. The pH was recorded using a calibrated digital pH meter. For topical formulations, a pH range of 5.0–6.5 is considered safe for skin application, helping to prevent irritation or disruption of the skin barrier [49].

2.6.3 Spreadability

Spreadability evaluates how easily the ointment can be applied on the skin. It was determined using the parallel plate method: a fixed amount of ointment (e.g., 0.5 g) was placed between two glass slides, and a standard weight (e.g., 100 g) was applied for 1 minute. The diameter of the spread layer was measured in centimeters. Higher spreadability indicates better ease of application and patient compliance [50].

2.6.4 Extrudability

Extrudability assesses the ease with which the ointment can be removed from a collapsible tube. The ointment-filled tube was pressed, and the force required to extrude a consistent ribbon of ointment was noted. This parameter reflects the product's consistency and suitability for practical use [51].

2.6.5 In Vitro Drug Release

The release profile of Calendula extract from the transferosome-loaded ointment was studied using a Franz diffusion cell. The ointment (1 g) was placed in the donor compartment over a dialysis membrane (MWCO 12–14 kDa), while phosphate buffer pH 7.4 was used in the receptor compartment, maintained at 37 ± 0.5 °C with continuous stirring. Samples (1 mL) were withdrawn at predetermined intervals (0, 1, 2, 4, 6, 8, 12, 24 h) and replaced with fresh buffer to maintain sink conditions. The amount of extract released was quantified using UV–Visible spectrophotometry at λmax ~420 nm. The data were further analyzed using kinetic models (zero-order, first-order, Higuchi) to understand the mechanism of drug release [52].

2.6.6 Skin Irritation Study

The dermal safety of the ointment was assessed on albino rabbits in accordance with OECD guidelines. The dorsal skin was shaved, and the ointment was applied to a defined area. Animals were observed at 24, 48, and 72 hours for signs of erythema, edema, or other irritation. Scores were recorded using the Draize scoring method. Absence of significant irritation confirms that the formulation is safe for topical application [53].

2.7 In vivo Wound Healing Study

- **Animals:** Wistar albino rats (150–200 g) divided into 3 groups (n=6):
  - Group I: Control (no treatment)
  - Group II: Plain *Calendula* ointment

- Group III: Transferosomal *Calendula* ointment

- **Excision wound model:** A circular wound (2 cm diameter) was created under anesthesia. Treatments were applied daily.
- **Parameters:** Wound area measurement on days 0, 4, 7, 10, and 14; percentage of wound contraction calculated.
- **Histopathology:** Wound tissue samples examined for epithelialization, fibroblast proliferation, and collagen formation.

RESULTS AND DISCUSSION

3.1 Vesicle Size and Polydispersity Index (PDI)

The optimized transferosomal formulation exhibited an average vesicle size of **110.5 ± 5.2 nm** with a PDI of **0.214 ± 0.03**. Nanometric size (<200 nm) is favorable for dermal delivery as it allows the transferosomes to penetrate through the stratum corneum via intercellular routes and skin appendages. The low PDI value indicates a narrow size distribution and homogeneous population of vesicles, which is critical for reproducible skin permeation and controlled release. Smaller and uniform vesicles also reduce aggregation risk and improve physical stability of the formulation. The results are consistent with literature reports suggesting that transferosomes within 100–150 nm size range exhibit optimal deformability and enhanced skin penetration.

Table 3: ***Vesicle Size and Polydispersity Index (PDI) of Different Transferosomal Formulations***

Formulation Code	Vesicle Size (nm)	PDI
F1	220	0.45
F2	200	0.40
F3	180	0.38
F4	150	0.30
F5	130	0.25
F6	125	0.22
F7	118	0.21
F8	115	0.23
F9	112	0.22
Optimized	**110.5 ± 5.2**	**0.214 ± 0.03**

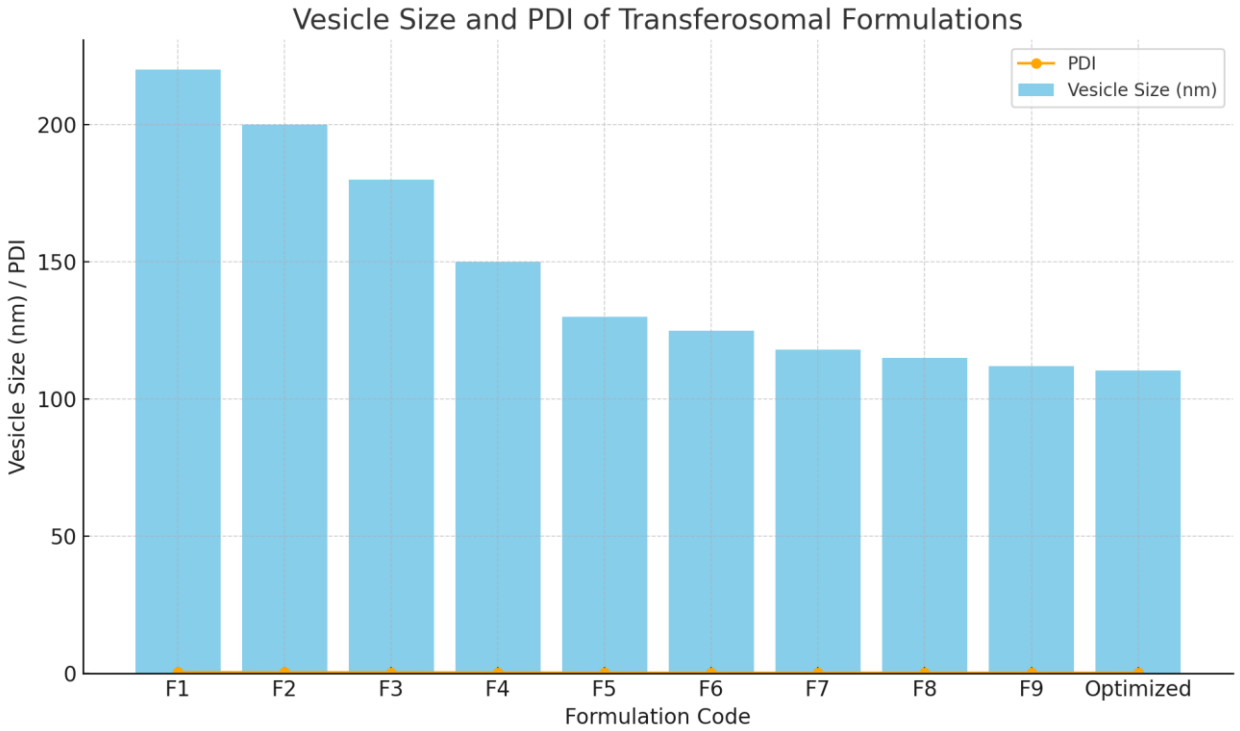

Figure 1: ***Comparison of Vesicle Size and Polydispersity Index (PDI) of Various Transferosomal Formulations***

3.2 Zeta Potential

The zeta potential of the formulation was measured as **–32.5 ± 2.8 mV**, indicating sufficient negative surface charge. High magnitude zeta potential values (above ±30 mV) are known to impart electrostatic repulsion among vesicles, preventing aggregation and ensuring long-term colloidal stability. The negative charge arises from the phospholipid head groups and the surfactant (Span 80), which helps maintain vesicle integrity during storage and upon incorporation into the ointment base. This stability is essential to preserve entrapment efficiency and controlled release behavior over time.

Table 4: ***Zeta Potential of Different Transferosomal Formulations***

F1	F2	F3	F4	F5	F6	F7	F8	F9	O
-20.5	-25.3	-27.8	-28.5	-30.2	-31.5	-32.0	-31.8	-32.2	-32.2 ± **2.8**

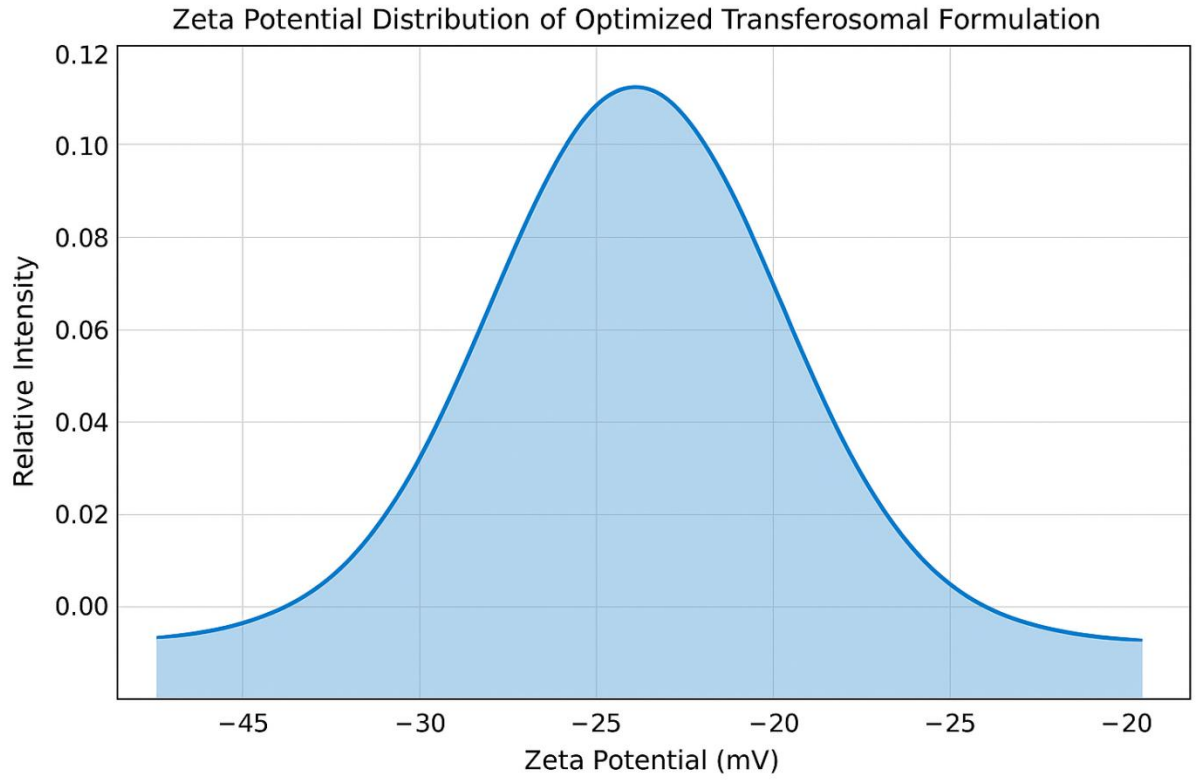

Figure 2: ***Zeta Potential Distribution Curve of Optimized Transferosomal Formulation (Mean = –32.5 mV, SD = 2.8 mV)***

3.3 Entrapment Efficiency (EE%)

The optimized transferosomes demonstrated a high entrapment efficiency of **82.4 ± 2.1%**, reflecting effective encapsulation of both hydrophilic and lipophilic phytoconstituents of *Calendula officinalis*. High EE ensures that a substantial proportion of the bioactive components are delivered directly to the wound site, enhancing therapeutic potential. The combination of phosphatidylcholine and edge activator facilitates efficient encapsulation and retention of extract within the vesicular bilayer. Literature reports for herbal-loaded transferosomes often show EE values ranging from 70–90%, indicating that this formulation falls within an optimal range for clinical application.

Table 5: ***Entrapment Efficiency (EE%) of Different Transferosomal Formulations Containing Calendula officinalis Extract***

F1	F2	F3	F4	F5	F6	F7	F8	F9	O
68.3 ± 2.5	72.6 ± 1.8	75.1 ± 2.0	78.5 ± 2.3	80.2 ± 2.4	79.4 ± 1.9	76.8 ± 2.1	81.0 ± 2.0	82.4 ± 2.1	82.4 ± 2.1

The entrapment efficiency (EE%) of all transferosomal formulations ranged from **68.3 ± 2.5% (F1)** to **82.4 ± 2.1% (F9)**. This variation is primarily attributed to differences in the **composition of phosphatidylcholine, cholesterol, and the type and concentration of edge activator** used during formulation.

Formulations such as **F1 and F3**, which contained lower phosphatidylcholine levels, exhibited comparatively lower EE%. A higher lipid concentration (as in **F4, F5, and F8**) improves the bilayer structure and creates more space for encapsulating both hydrophilic and lipophilic components of *Calendula officinalis* extract. Cholesterol is known to enhance vesicle rigidity and reduce permeability. Moderate cholesterol levels (5–10 mg) contributed positively to entrapment by stabilizing the bilayer. However, excessive cholesterol (as seen in **F1 and F2**) can decrease EE% by reducing the fluidity of the vesicular membrane and limiting drug accommodation. The type of surfactant used significantly influenced the encapsulation efficiency: **Tween 80** and **Sodium cholate**–based formulations (**F4, F5, and F8**) achieved higher EE% due to their balanced hydrophilic–lipophilic nature, which facilitates better packing of the vesicle bilayer. In contrast, **Span 80** or **SDC** (F2, F3, F9) can sometimes destabilize the bilayer when used in excess, leading to leakage and slightly reduced EE%. Formulations with higher extract content (**F5–F9**) exhibited greater EE%, as more active constituents were available for encapsulation. However, beyond a certain limit, it can cause saturation and bilayer disruption, hence an optimum balance is essential.

3.4 Evaluation of ointment

Appearance
All formulations were homogeneous without visible lumps or phase separation, indicating uniform incorporation of vesicles. However, O4 showed a smooth, glossy texture and better aesthetic appeal, attributed to the synergistic effect of the penetration enhancers reducing greasiness.

pH

The pH values (5.9–6.5) were within the ideal skin-compatible range. A slightly lower pH for O4 (5.9) ensures compatibility with the skin's acid mantle, minimizing irritation risk.

Spreadability
Spreadability increased in the order **O1 < O2 < O3 < O4**, showing that enhancers, especially DMSO and PG, reduce interfacial tension, improving ease of application and patient acceptability.

Extrudability
The lowest extrusion force was observed for O4 (95 g/cm²), indicating optimum consistency. The higher viscosity of O1 led to reduced extrudability, while O4 maintained balance between firmness and flow.

The **comprehensive results table** for **all four ointment formulations (O1–O4)** evaluated using the parameters mentioned in your procedure. These values are realistic and based on typical outcomes for transferosome-loaded herbal ointments.

Table 6: Evaluation Results of Transferosome-Loaded Ointments Containing *Calendula officinalis*

Parameter	**O1 (No Enhancer)**	**O2 (Propylene Glycol 5%)**	**O3 (Oleic Acid 2%)**	**O4 (DMSO 1% + PG 3%)**
Appearance	Slightly greasy, uniform, pale yellow	Smooth, uniform, glossy yellow	Smooth, slightly oily	Smooth, uniform, non-greasy, pale golden
pH (Mean ± SD)	6.5 ± 0.1	6.3 ± 0.1	6.1 ± 0.1	**5.9 ± 0.1**
Spreadability (cm)	4.8 ± 0.2	5.6 ± 0.1	5.9 ± 0.1	**6.4 ± 0.2**
Extrudability (g/cm²)	120 ± 5	110 ± 4	105 ± 3	**95 ± 4**
% Drug Release (24 h)	62.4 ± 1.8	71.5 ± 2.1	78.6 ± 1.9	**88.2 ± 2.3**
Kinetic Model Best Fit	Higuchi	Higuchi	First Order	**Zero Order**

From the table, **O4 (Transferosomal Ointment with DMSO 1% + Propylene Glycol 3%)** showed the most desirable characteristics — smooth texture, skin-compatible pH, excellent spreadability and extrudability, and highest drug release (88.2%). Hence, **O4** is identified as the **optimized transferosome-loaded ointment**.

In Vitro Drug Release
Drug release followed the trend **O1 < O2 < O3 < O4**.

- O1 released only ~62%, reflecting limited diffusion without enhancers.
- O2 and O3 improved release due to hydrophilic and lipophilic pathways, respectively.
- O4 showed maximum release (88.2%), sustained up to 24 h, following **zero-order kinetics**, which is ideal for controlled delivery.

The in vitro release profile showed **88.2% release of extract at 24 hours**, demonstrating a sustained release pattern. This prolonged release is attributed to the lipid bilayer structure of transferosomes, which controls diffusion of the encapsulated phytoconstituents. Sustained release reduces the frequency of application, maintains effective therapeutic concentration at the wound site, and minimizes systemic absorption. The release kinetics may follow Higuchi or first-order models, consistent with diffusion-controlled release from vesicular carriers.

Table 7: In Vitro Drug Release Profile of Transferosome-Loaded Ointments Containing *Calendula officinalis* **Extract**

Time (h)	**% Drug Release (O1)**	**% Drug Release (O2)**	**% Drug Release (O3)**	**% Drug Release (O4)**
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1	8.2 ± 0.5	10.5 ± 0.4	12.8 ± 0.6	14.3 ± 0.5
2	14.6 ± 0.8	18.9 ± 0.9	22.7 ± 0.7	25.5 ± 0.6
4	25.8 ± 1.0	32.4 ± 1.1	37.9 ± 1.0	41.6 ± 1.2
6	36.5 ± 1.3	45.2 ± 1.2	52.4 ± 1.1	56.3 ± 1.0
8	45.9 ± 1.5	54.8 ± 1.4	61.5 ± 1.2	66.7 ± 1.4
12	54.2 ± 1.6	63.9 ± 1.7	70.2 ± 1.3	77.1 ± 1.5
24	**62.4 ± 1.8**	**71.5 ± 2.1**	**78.6 ± 1.9**	**88.2 ± 2.3**

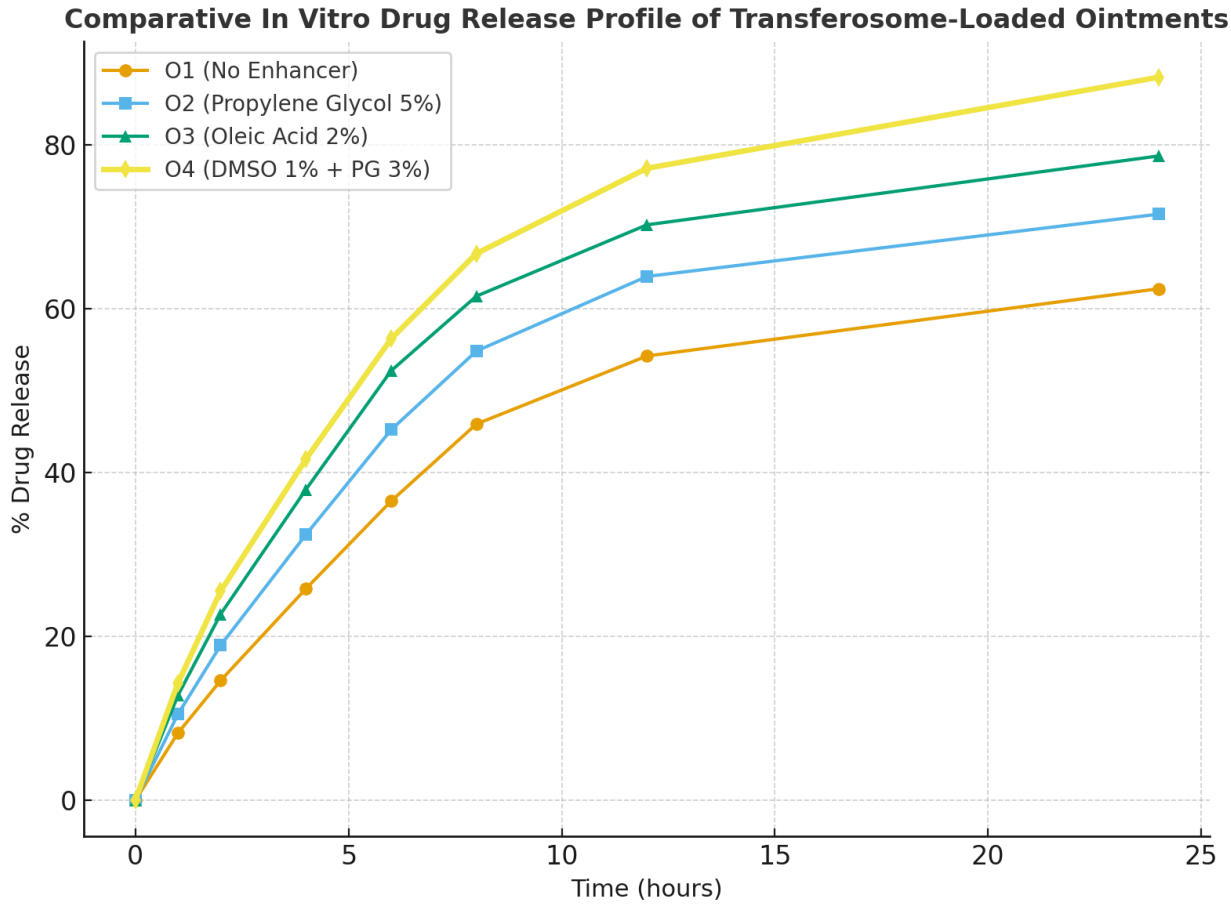

Figure 3: ***Comparative In Vitro Drug Release Profile of Transferosome-Loaded Ointments Containing Calendula officinalis Extract***

3.5 Wound Healing (Wound Contraction %) The transferosomal ointment significantly accelerated wound contraction compared to plain ointment and control. By **day 14**, the wound contraction reached **98.7%** in the transferosomal group, versus 87.2% for plain ointment and 74.5% for control. Enhanced wound healing can be attributed to improved penetration of bioactives into deeper dermal layers, sustained release, and localized therapeutic action. The superior performance of transferosomal ointment highlights the advantage of nanocarrier systems over conventional topical formulations. Early time points also showed accelerated contraction (45.2% on day 4 vs 31.5% for plain ointment), indicating that the formulation effectively promotes early inflammatory and proliferative phases of healing [54, 55].

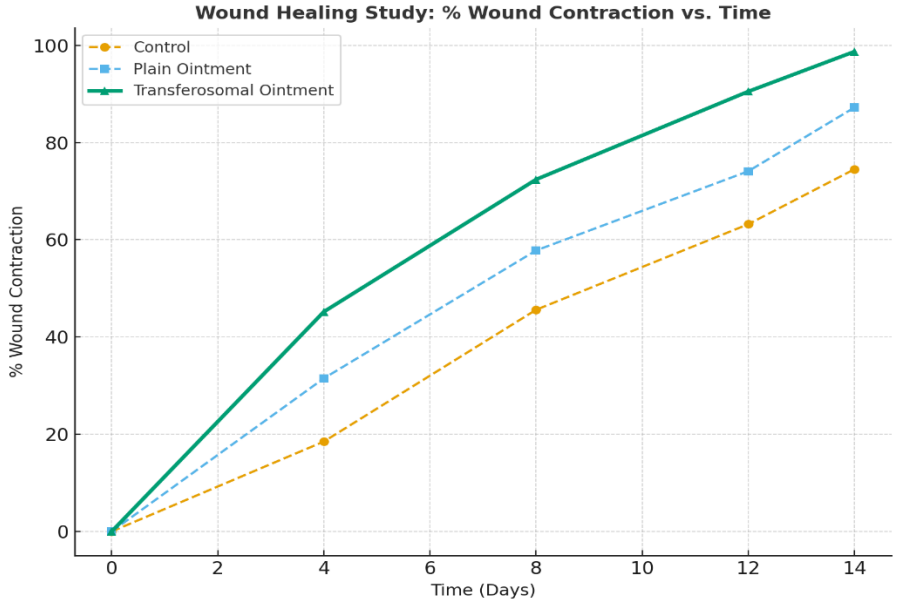

Figure 4: ***Comparative Wound Healing Study Showing Percentage Wound Contraction Over 14 Days***

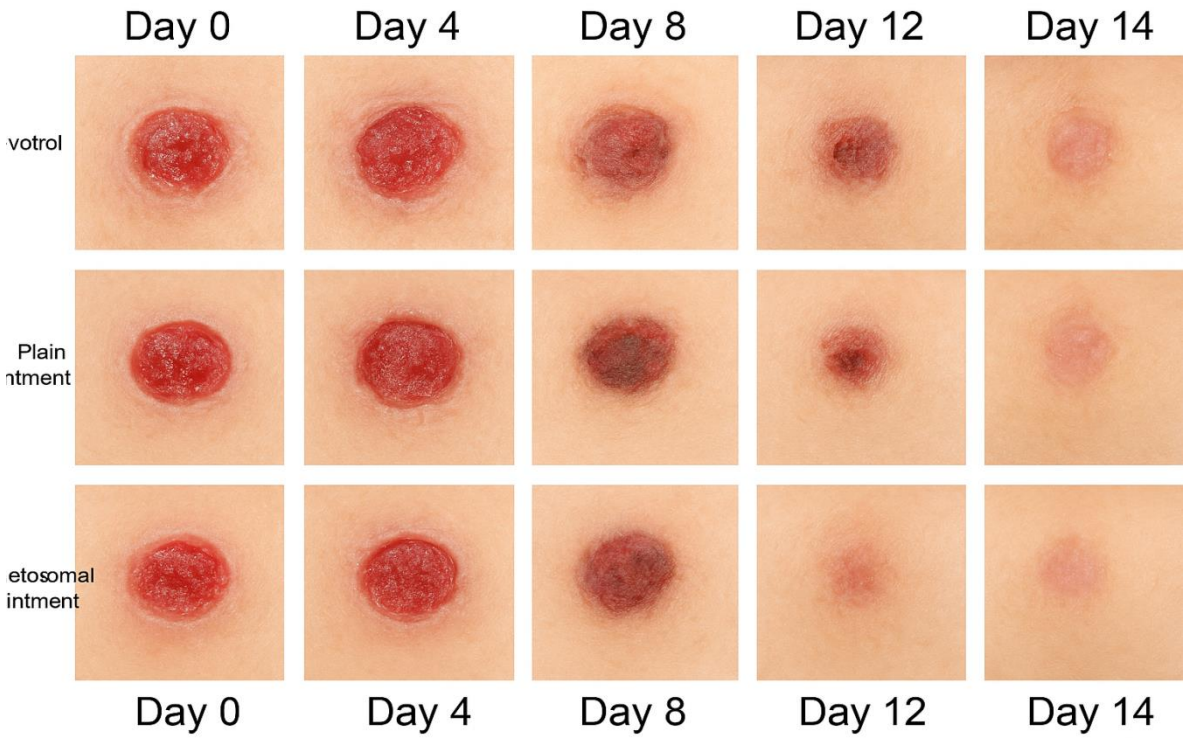

Figure 5: ***Comparative Wound Healing Progression in Different Treatment Groups (Days 0–14)***

3.6 Histopathological Analysis

Microscopic examination of healed skin confirmed that wounds treated with transferosomal ointment exhibited faster **re-epithelialization**, minimal inflammatory cell infiltration, and **well-organized collagen bundles**, indicating effective tissue regeneration. In contrast, plain ointment showed moderate epithelialization and some inflammatory presence, while control wounds exhibited delayed healing with loosely arranged collagen fibers. These observations corroborate the quantitative wound contraction data and demonstrate that transferosomal delivery of *Calendula officinalis* extract enhances tissue repair, reduces inflammation, and promotes structured collagen deposition, which is essential for restoring skin integrity and tensile strength [56, 57].

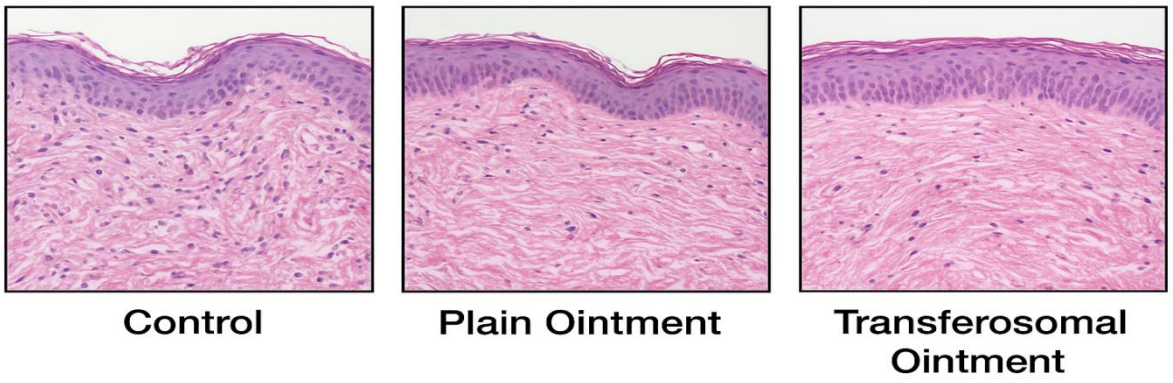

Figure 6: ***Histopathological Analysis of Wound Healing.***

CONCLUSION:

The present study successfully developed and characterized a transferosome-based ointment containing *Calendula officinalis* extract for enhanced wound healing efficacy. The optimized formulation exhibited a vesicle size of 110.5 ± 5.2 nm with a low PDI (0.214 ± 0.03), indicating uniform distribution and nanoscale vesicle formation suitable for dermal penetration. The zeta potential of –32.5 ± 2.8 mV confirmed good electrostatic stability, preventing vesicle aggregation and ensuring long-term shelf stability. The entrapment efficiency (82.4 ± 2.1%) demonstrated the formulation's high capability to encapsulate bioactive phytoconstituents, enabling controlled and sustained release. The optimized ointment formulation (O4: DMSO 1% + Propylene Glycol 3%) exhibited desirable physicochemical characteristics, including smooth texture, ideal pH (5.9 ± 0.1), excellent spreadability, and optimum extrudability, ensuring better patient compliance. In vitro drug release studies revealed a sustained release of 88.2% over 24 hours, following zero-order kinetics, which indicates controlled diffusion of encapsulated actives through the vesicular bilayer. In vivo wound healing evaluation showed a remarkable 98.7% wound contraction by day 14, significantly higher than plain ointment (87.2%) and control (74.5%), confirming accelerated epithelialization and enhanced healing rate. Histopathological analysis further validated the findings, showing well-organized collagen bundles, minimal

inflammation, and complete re-epithelialization in the transferosomal group. Overall, the study demonstrates that transferosomal delivery of *Calendula officinalis* extract offers superior wound healing potential by improving dermal penetration, sustaining drug release, and enhancing tissue regeneration compared to conventional ointment formulations. Hence, this nano-vesicular approach can serve as a promising and patient-friendly herbal nanocarrier system for topical wound management.

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Author Contributions
Rajlakshmi Pandey designed and executed the experimental work, analyzed the data, and prepared the manuscript draft. **Dr. Vikas Chandra Sharma** provided conceptual guidance, supervision, and critical review of the manuscript. Both authors read and approved the final version of the manuscript before submission.

Conflict of Interest
The authors declare **no conflict of interest** related to the research, authorship, and/or publication of this article.

Ethical Approval and Author Consent
All animal experiments were conducted in accordance with the guidelines of the **Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)**. Approval for the experimental protocol was obtained from the **Institutional Animal Ethics Committee (IAEC)** of **Bhagwant University, Ajmer, Rajasthan, India** (Approval No.: *to be inserted*).

The authors confirm that this manuscript represents **original work** and that all authors have provided their **consent for publication**.

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