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ORIGINAL RESEARCH

Development and Evaluation of Trans-Resveratrol-Loaded Transfersomes: Role of Cholesterol in Formulation Design for Dermal Delivery

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Purpose: This study investigated the development and characterization of trans-resveratrol-loaded transfersomes, with and without cholesterol, for potential non-irritating dermal applications.

Methods: Transfersomes were prepared using thin-film hydration combined with probe sonication, incorporating hydrogenated lecithin, cholesterol, and Tween® 60 in six different ratios. The formulations were characterized for their physicochemical properties, including particle size, polydispersity index, zeta potential, entrapment efficiency, morphology, in vitro release profiles, dermal permeation potential, and safety profile.

Results: All formulations exhibited particle sizes below 150 nm and zeta potentials below −30 mV, indicating favorable characteristics for dermal delivery. Cholesterol incorporation significantly increased particle size and enhanced zeta potential ($p<0.05$). Formulations containing 3–3.5% w/v hydrogenated lecithin achieved superior entrapment efficiency (>90%) compared to those with lower lecithin content ($p<0.05$), regardless of cholesterol incorporation. Transfersomes containing cholesterol displayed morphology with well-defined edges compared to cholesterol-free formulations. In vitro release studies revealed distinct release profiles, with cholesterol-free formulations releasing 70–83% of trans-resveratrol over 24 hours, compared to only 0–30% for cholesterol-containing formulations. Strat-M® membrane-based permeation studies confirmed enhanced trans-resveratrol delivery across all transfersomal systems compared to the saturated solution ($p<0.05$), though cholesterol showed no significant impact on permeation efficiency. These findings indicate that cholesterol influences release profile but has limited effect on permeation efficiency. Safety assessment using the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay classified the developed transfersomes as weak irritants, indicating their dermal safety. Notably, formulation F3, with a hydrogenated lecithin to cholesterol to Tween 60 ratio of 6:0:4, emerged as the optimal candidate, achieving the highest release rate (80.24% over 24 hours) while maintaining favorable permeation compared to control.

Conclusion: These findings feature the potential of transfersomal systems, particularly cholesterol-free variants, as promising carriers for the effective and safe dermal delivery of trans-resveratrol.

Keywords: trans-resveratrol, transfersomes, cholesterol, in vitro release, in vitro permeation, HET-CAM test

Introduction

In recent years, the demand for effective and safe skin-whitening agents in cosmetic formulations has increased substantially. However, delivering these active compounds through the stratum corneum, the skin's outermost barrier, remains a major formulation challenge. One promising candidate is trans-resveratrol, a natural polyphenol found in grapes, peanuts, and berries, known for its strong antioxidant, anti-inflammatory, anti-aging, and skin-whitening properties.[1] Its depigmenting effect is largely attributed to its ability to inhibit tyrosinase, the rate-limiting enzyme in

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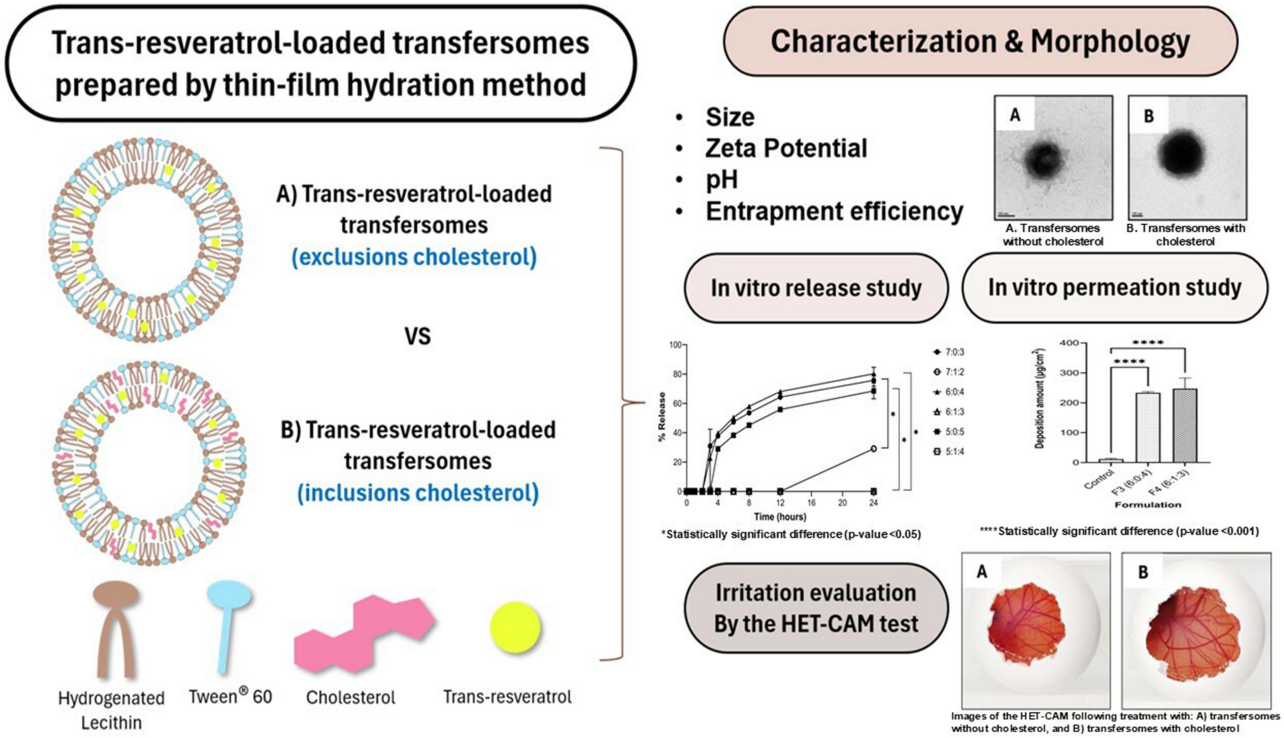
Graphical Abstract



melanin biosynthesis, by interfering with both monophenolase and diphenolase activities. Additionally, trans-resveratrol downregulates key melanogenesis-related proteins, including tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1), and TRP-2.[2,3] At a concentration as low as 0.1%, trans-resveratrol has demonstrated higher tyrosinase inhibition efficacy than kojic acid, a commonly used whitening agent.[4]

Beyond its skin-whitening effects, trans-resveratrol exerts remarkable anti-aging properties by inhibiting collagenase, elastase, and matrix metalloproteinases, which are key enzymes involved in skin aging and extracellular matrix degradation.[5] It effectively reduces reactive oxygen species production and inhibits UVB-induced activation of the nuclear factor kappa-B (NF-κB) transcription factor pathway,[6,7] thereby offering photoprotective effects. Trans-resveratrol also regulates inflammation by modulating signaling pathways involving NF-κB and Activator Protein-1, leading to reduced expression of inflammatory mediators.[8,9] Furthermore, it has demonstrated antibacterial activity against *Cutibacterium acnes*.[10] Altogether, these properties establish trans-resveratrol as a strong candidate for multi-functional cosmeceutical formulations. Its primary sites of action include the epidermis, where it exerts skin whitening and anti-acne effects,[11,12] and the dermis, where it contributes to anti-aging benefits through collagen preservation.[13]

Despite its promising biological activity, the clinical application of trans-resveratrol in topical formulations is limited by several physicochemical barriers. These include its hydrophobicity (log P ≈ 3.1), poor aqueous solubility, chemical instability (especially under light and oxidative conditions), and moderate molecular weight (228.25 Da), which collectively hinder its percutaneous absorption and bioavailability.[14,15] Consequently, the development of advanced and safe delivery systems is critical to enhance its stability, skin permeation, and therapeutic efficacy in cosmetic products.

Previous studies have investigated vesicular systems such as liposomes[16,17] and niosomes[18] for the delivery of trans-resveratrol. Liposomes, typically composed of phospholipids and cholesterol, and niosomes, formed from non-ionic surfactants and cholesterol, utilize cholesterol as a membrane stabilizer to enhance rigidity, reduce leakage, and improve

overall stability.[19] Moreover, both systems often suffer from drawbacks such as low entrapment efficiency and short half-life,[16,20] limiting their effectiveness for dermal delivery.

To overcome these limitations, transfersomes, second-generation, ultra-deformable vesicles composed of phospholipids and edge activators such as Tweens, Spans, or sodium deoxycholate, have been developed. Originally developed by Cevc and Blume in the 1990s, transfersomes possess highly flexible membranes that enable them to penetrate the stratum corneum by exploiting the skin's transepidermal water gradient.[21] Several studies have demonstrated that transfersomes outperform conventional liposomes in enhancing skin penetration and drug bioavailability, especially for targeting deeper skin layers.[22] Importantly, transfersomes are capable of encapsulating lipophilic compounds such as diclofenac diethylamine (DDEA) and curcumin with relatively high entrapment efficiencies of nearly 90%.[23,24] Additionally, Cevc et al (1996) reported that transfersomes could transport lipophilic fluorescent markers through murine skin at rates more than 50% higher than liposomes.

The performance of transfersomes is significantly influenced by both the type of edge activator used and the phospholipid-to-edge activator ratio, as these factors directly affect vesicle size, entrapment efficiency, and skin permeation.[25] Several studies have highlighted the superior encapsulation capacity of polysorbate-based edge activators, particularly Tween surfactants. For instance, Caddeo et al (2018) demonstrated that Tween 60 and Tween 80 achieved higher entrapment efficiency for hydrophobic compounds such as tocopherol.[26] Furthermore, stability studies indicate that Tween 60 provides greater vesicle stability compared to Tween 80, likely due to the absence of unsaturated double bonds in its structure.[27,28] Additionally, trans-resveratrol shows improved solubility in hydrophilic surfactants like Tween, compared to hydrophobic surfactants such as Span, making Tween 60 an ideal candidate for transfersome formulations.[26] However, the concentration of edge activators must be carefully optimized. Excessive edge activator can disrupt bilayer integrity by forming pores, thereby reducing drug retention and permeation efficiency.[29] On the other hand, insufficient surfactant can limit vesicle formation and maturation. Interestingly, smaller vesicles are often observed at higher phospholipid-to-surfactant ratios, due to restricted surfactant availability that prevents full bilayer expansion and vesicle maturation.[30] Therefore, optimizing the phospholipid-to-edge activator ratio is crucial to achieving a balance between vesicle stability, size, and drug delivery efficiency. The importance of this ratio was further demonstrated in a study by Wu et al (2019), which investigated the stability of trans-resveratrol-loaded transfersomes. In that study, a formulation composed of lecithin and edge activators (Tween 20 or Tween 80) at a 3:1 ratio exhibited enhanced stability, with no significant degradation observed over 14 days. The optimized formulation achieved an entrapment efficiency of approximately 59.93% and demonstrated improved skin accumulation (27.59%) over a 6-hour period.[31] These findings reinforce the potential of transfersomes as a promising vehicle for the topical delivery of trans-resveratrol.

Despite these advances, most transfersome formulations have focused primarily on optimizing lipid and surfactant components, often excluding cholesterol, a well-known membrane stabilizer in conventional liposomal systems. While cholesterol is recognized for its ability to enhance membrane integrity, reduce drug leakage, and extend shelf life, its role within transfersomal systems remains largely unexplored, presenting a valuable opportunity for innovation in vesicular drug delivery.

Therefore, this study systematically evaluates cholesterol's dual role in modulating structural integrity and functional performance within transfersomal systems, specifically for the delivery of trans-resveratrol. By varying phospholipid-to-edge activator ratios and incorporating cholesterol, key formulation attributes will be assessed: particle size, zeta potential, morphology, in vitro release, skin permeation, and irritation potential via HET-CAM assay. This comprehensive approach bridges the knowledge gap while establishing an optimized trans-resveratrol delivery platform for advanced cosmetic skin-whitening applications.

Materials and Methods

Materials

Trans-resveratrol was obtained from DSM Nutritional Product, Switzerland. Hydrogenated lecithin (Lecinol S-10M; PC 55–65%) was purchased from Nikko Chemicals Co., Ltd., Japan. Tween 60 was purchased from Croda Singapore Pte Ltd, Singapore. Cholesterol was purchased from Combi-Blocks, Inc., USA. Chloroform, methanol and acetonitrile were

bought from Thermo Fisher Scientific™, USA. Propylene glycol was bought from Tokyo Chemical Industry, Japan. Cellu·Sep® regenerated cellulose tubular membrane (MWCO: 12000–14000 Da) was obtained from Orhiyo Enterprise Inc., Taiwan. Strat-M membrane was purchased from Merck Millipore, Germany. Other chemicals and solvents used in the study were of analytical reagent grade.

Preparation of Trans-Resveratrol Loaded Transfersomes

The transfersomes were prepared using the thin-film hydration method followed by probe sonication downsizing. The components used for the preparation of the transfersomes are presented in Table 1. Briefly, hydrogenated lecithin, cholesterol, Tween 60, and trans-resveratrol were dissolved in a mixture of chloroform and methanol (4:1 v/v). The solution was transferred into a round-bottom flask and evaporated using a rotary evaporator (N-1000, Eyela, Taiwan) under vacuum conditions at a pressure ranging from 35–65 cmHg at 50°C to form a thin film. Nitrogen was introduced for 15 minutes to eliminate any remaining traces of solvent. The thin films were hydrated with double-distilled water using a rotary evaporator rotating without vacuum for 30 minutes. After hydration, 30 mL of each formulation underwent downsizing using a probe sonicator (Q125 Sonicator®, QSONICA, USA). The sonication was performed at 10% amplitude for 5 cycles, with each cycle lasting 120 seconds. The formulations were then kept at 25°C for 24 hours before measuring their size, zeta potential, entrapment efficiency (EE), and pH.

Characterization of Trans-Resveratrol-Loaded Transfersomes

Physical Appearance and pH

The physical appearance of the transfersomes was examined by visual inspection for sedimentation and phase separation. The pH was measured using a pH meter (SevenCompact™ pH/ion meter s220, Mettler Toledo, USA).

Particle Size, Polydispersity Index, and Zeta Potential

Briefly, before analysis, the samples were diluted 10-fold with double-distilled water to avoid multiple scattering effects and ensure optimal measurement conditions. The diluted samples were then equilibrated at 25 ± 1 °C for 5 minutes prior to measurement to allow temperature stabilization. Particle size and polydispersity index (PDI) as well as zeta potential were determined using dynamic light scattering (Otsuka Electronics, ELSZ-2000 Series, Japan) with a scattering angle of 90.

Morphology

The morphology of the trans-resveratrol-loaded transfersomes, both with and without cholesterol, was evaluated using transmission electron microscopy (TEM). The sample was diluted before being deposited on the copper grid. A diluted sample drop was placed on the copper grid and allowed to dry. The sample was then stained with 1% phosphotungstic acid and allowed to dry again. The copper grid with the dried sample drop was placed in a vacuum drying machine for at least 24 hours before being examined using the transmission electron microscope (JEOL, JEM 3100, Japan) operating at 200 kV.

Table 1 Components Used for the Preparation of Trans-Resveratrol-Loaded Transfersomes by the Thin-Film Hydration Method Followed by Probe Sonication

Formulation	Hydrogenated Lecithin: Cholesterol: Tween 60 (Mass Ratio)	% w/v			
		Trans-Resveratrol	Hydrogenated Lecithin	Cholesterol	Tween 60
F1	7:0:3	0.1	3.5	-	1.5
F2	7:1:2	0.1	3.5	0.5	1
F3	6:0:4	0.1	3	-	2
F4	6:1:3	0.1	3	0.5	1.5
F5	5:0:5	0.1	2.5	-	2.5
F6	5:1:4	0.1	2.5	0.5	2

Quantification of Trans-Resveratrol by High-Performance Liquid Chromatography

The amount of trans-resveratrol was analyzed using HPLC-UV (Pump; Chromaster 5110, Hitachi, Japan and Autosampler; Chromaster 5210, Hitachi, Japan) on a C18 column (Spherisorb ODS2 10 μm, 4.6×250 mm). The mobile phase was run as an isocratic elution, consisting of 0.1% acetic acid in water and acetonitrile (65:35, v/v), with a flow rate of 1 mL/min. The column temperature was maintained at 25 °C, and the eluent was detected at a wavelength of 306 nm by the UV-Vis Detector (Chromaster 5420, Hitachi, Japan). The overall run time was set at 12 minutes.

Entrapment Efficiency

Entrapment efficiency was determined using the ultracentrifugation method.[32] An ultracentrifuge (Hitachi, CS150 GXL, Japan) was employed to separate the free trans-resveratrol from the trans-resveratrol-loaded transfersomes. A 500 μL sample was loaded into an ultracentrifuge tube and subjected to centrifugation at 120,000 rpm at 4°C for 1 hour. The supernatant obtained after centrifugation was collected for subsequent analysis by High-Performance Liquid Chromatography (HPLC) to quantify the free (unentrapped) trans-resveratrol (C free). To determine the total trans-resveratrol concentration in the transfersomes, 50 μL of the sample was mixed with 50 μL of chloroform and 900 μL of methanol and the mixture vortexed for 1 minute before being centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatants were then analyzed by HPLC as previously described to quantify the total trans-resveratrol content in the formulations (C total).

The following equation was used to calculate the entrapment efficiency.

$$\text{Entrapment efficiency } (\%) = \frac{\text{C total} - \text{C free}}{\text{C total}} \text{x}100 \quad (1)$$

In vitro Release Study

An in vitro release study was conducted using a Franz diffusion cell (Model VTC-200, LOGAN Instruments Corp., USA) equipped with a cellulose membrane (Cellu·Sep regenerated cellulose tubular membrane; MWCO: 12,000–14,000 Da). The cellulose membrane was placed between the donor compartment and the receptor compartment of the Franz diffusion cell apparatus. Subsequently, the receptor compartment was filled with a medium consisting of pH 7.4 phosphate-buffered saline containing 20% propylene glycol and 20% ethanol.[33] The temperature was set at 37 ± 2 °C with circulation, ensuring that the membrane surface temperature remained near 32 °C, and a magnetic stirrer was employed at 600 rpm during testing. One milliliter of the sample was added to the donor compartment. Samples were withdrawn at predetermined time points (0.5, 1, 2, 3, 4, 6, 8, 12, 24 hours) and replaced with equal volumes of fresh buffer. The collected samples were then subjected to analysis by HPLC to obtain quantitative results. At the end of the 24-hour release study, the remaining formulation in the donor compartment was collected. A 50 μL aliquot of this was mixed with 50 μL of chloroform and 900 μL of methanol, vortexed for 1 minute, and centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant was analyzed by HPLC. The % release was calculated based on the amount of drug quantified in the receptor compartment at predefined time intervals (0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours), relative to the total initial amount of drug applied to the donor compartment. The percent recovery was also calculated as the sum of the drug recovered from the donor compartment, membrane, and receptor compartment, divided by the initial dose applied.

In vitro Permeation Study

An in vitro permeation study of trans-resveratrol (t-RES) loaded transfersomes was conducted using a Franz diffusion cell equipped with a Strat-M membrane (effective area: 0.95 cm^2) over a period of 24 hours. A Strat-M membrane is a synthetic membrane made up of multi-layers of polyester sulfone with a thickness of approximately 300 μm to mimic different layers of human skin. This membrane is widely used in early-stage topical delivery research as a non-biological alternative to excised human or animal skin. Strat-M consists of multiple layers that structurally and functionally mimic the skin's key layers. The uppermost dense layer, coated with synthetic lipids, simulates the stratum corneum, while the underlying porous layers represent the viable epidermis and dermis. Its low inter-batch variability, ease of handling, and absence of ethical concerns make it especially suitable for preliminary permeation screening. Additionally, several studies have shown a strong correlation between permeation data from Strat-M and from human or porcine skin,

particularly for lipophilic compounds like trans-resveratrol.[34–36] The Strat-M membrane was placed between the donor compartment and the receptor compartment of the Franz diffusion cell apparatus, with the shiny side in contact with the donor. Subsequently, the receptor compartment was filled with a medium consisting of pH 7.4 phosphate-buffered saline containing 20% propylene glycol and 20% ethanol. The receptor medium was maintained at 37 ± 2°C using a circulating water bath, ensuring that the membrane surface temperature remained near 32 °C, in alignment with physiological skin conditions.[37,38] The receptor medium was stirred continuously using a magnetic bar at 600 rpm. The test was conducted under non-occlusive conditions. Three hundred microliters of the sample were added to the donor compartment. The receptor medium was withdrawn from the receptor compartment at 24 hours. The amount of trans-resveratrol in the receptor medium at 24 hours (t-RES R_{24}) was then subjected to analysis by HPLC to obtain quantitative results, and the remaining amount of transfersomes in the donor compartment was taken out for the HPLC analysis to determine the remaining trans-resveratrol amount in the donor site (t-RES D_{24}) after 24 hours of testing. The deposited amount of trans-resveratrol in membrane was calculated according to the following equations:[35,39,40]

$$\text{Deposited t} - \text{RES in membrane} = \text{Added t} - \text{RESamount} - (\text{t} - \text{RES}R_{24} + \text{t} - \text{RES}D_{24}) \quad (2)$$

A saturated aqueous solution of trans-resveratrol was used as the control.

Hen's Egg Test – Chorioallantoic Membrane Assay

The HET-CAM assay was employed as a primary screening tool for irritation potential. HET-CAM is a well-established, validated alternative method recognized by regulatory agencies (eg, ECHA 2015, EU) for assessing acute irritation, particularly in early-stage dermal and ocular formulations.[41–43] Its ability to evaluate vascular responses such as hyperemia, hemorrhage, and coagulation makes it a valuable model for detecting irritant effects without the use of animal testing. Moreover, its use aligns with the 3Rs principle (Replacement, Reduction, and Refinement) in biomedical research. At this stage of development, HET-CAM assay was used to investigate the irritation potential of blank transfersomes and trans-resveratrol-loaded transfersomes by observing the effects on the chorioallantoic membrane (CAM) of the hen's egg. Hen eggs were purchased from the Animal Drugs Inspection Branch, Veterinary Research Institute, and the Ministry of Agriculture of Taiwan. On the 7th day, the eggshell was opened on the side of the air chamber, exposing the internal white membrane. The white membrane was moistened with 0.9% (w/v) sodium chloride (NaCl) for 30 seconds before removing it. Then, 300 µL of the formulation was applied to the chorioallantoic membrane. The irritation potential was assessed by visually monitoring and recording the onset time (in seconds) of three key reactions: hemorrhage (H), vasoconstriction (V), and coagulation (C), after application of the test formulation. These changes were observed directly on the chorioallantoic membrane for a total of 300 seconds (5 minutes) using a digital stopwatch and documented in real-time under standardized lighting conditions. The recorded onset times were then used to calculate the Irritation Score (IS) using the following equation:[44,45]

$$\text{Irritation Scores (IS)} = \frac{(301 - \text{H})\text{x}5}{300} + \frac{(301 - \text{V})\text{x}7}{300} + \frac{(301 - \text{C})\text{x}9}{300} \quad (3)$$

Where H represents hemorrhage: the start time of bleeding from the vessels (in seconds); V represents vasoconstriction: the start time of blood vessel disintegration (in seconds); C represents coagulation: the start time of intra- and extravascular protein denaturation, observed as a decrease or cessation of blood flow due to thrombosis (in seconds).

Positive controls I and II used were 0.1 M sodium hydroxide (NaOH) and 1% (w/v) sodium lauryl sulfate (SLS), respectively. The negative control used was 0.9% (w/v) NaCl. The irritation score (IS) of the tested formulations was classified according to the following scale (Table 2):[32,44]

Statistical Analysis

All measurement was performed in triplicate to ensure reproducibility and the results are presented as mean ± standard deviation. Differences between the formulations' characteristics and properties were assessed using one-way ANOVA followed by Tukey's post hoc test with the Statistical Package for the Social Sciences (SPSS) version 29.0 (USA). Statistical significance was considered for p values < 0.05.

Table 2 The Irritation Scores (IS) for the HET-CAM Assay and Their Corresponding Irritation Categories

IS	Irritation Category
0 – 0.9	Non - irritation
1.0–4.9	Weak or slight irritation
5.0–8.9	Moderate irritation
9.0–21.0	Strong or severe irritation

Results

Characterization of Trans-Resveratrol-Loaded Transfersomes

Physical Appearance and pH

Photographs depicting the physical appearance of all the formulations are presented in Figure 1. All the formulations appeared translucent and homogeneous, with pH values ranging from 5 to 5.5. No statistically significant differences in pH values were observed among the formulations.

Particle Size, Polydispersity Index, and Zeta Potential

Size-wise, all formulations exhibited particle sizes below 150 nm (Table 3). Comparative analysis of formulations with equivalent hydrogenated lecithin content (eg, F1 vs F2, F3 vs F4, and F5 vs F6) revealed a consistent trend: cholesterol-containing formulations produced significantly larger particles ($p < 0.05$). However, an exception was observed with F5 and F6, where F5 displayed a larger particle size despite lacking cholesterol. Further comparisons of formulations with similar total lipid content (hydrogenated lecithin combined with cholesterol) (F1 vs F4, F3 vs F6) confirmed that cholesterol-containing formulations consistently produced significantly larger particles. The polydispersity index of all formulations was in the range of 0.26–0.32. Zeta potential analysis indicated robust electrostatic stabilization across all formulations, with values exceeding −30 mV. Notably, cholesterol-containing formulations exhibited significantly more negative zeta potential values ($p < 0.05$).

As summarized in Table 3, the particle size, polydispersity index, and zeta potential of the resulting trans-resveratrol-loaded transfersomes highlight the dual role of cholesterol in both increasing particle size and enhancing electrostatic stability ($p < 0.05$). While the inclusion of cholesterol increases particle size, it also improves the surface charge (zeta potential) characteristics.

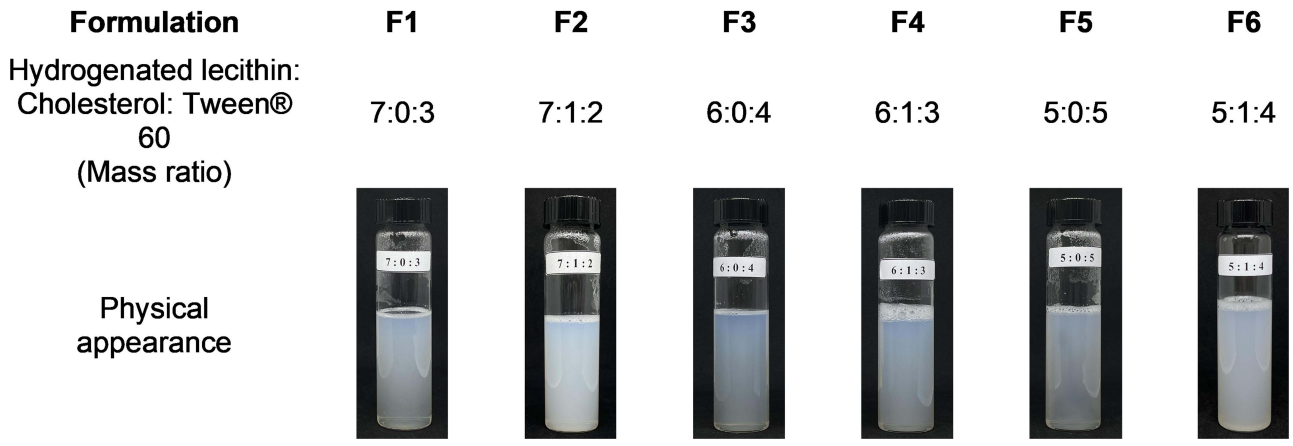


Figure 1 The physical appearance of freshly prepared trans-resveratrol-loaded transfersomes.

https://doi.org/10.2147/NSA.S529010

Table 3 Particle Size, Polydispersity Index, Zeta Potential of the Trans-Resveratrol Loaded Transfersomes

Formulation	Hydrogenated Lecithin: Cholesterol: Tween 60 (Mass Ratio)	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
F1	7:0:3	76.33 ± 1.88	0.30 ± 0.00	−39.88 ± 1.58
F2	7:1:3	108.40 ± 0.44	0.26 ± 0.01	−56.55 ± 0.92
F3	6:0:4	84.93 ± 0.96	0.27 ± 0.01	−43.96 ± 0.89
F4	6:1:3	110.0 ± 1.91	0.30 ± 0.01	−57.26 ± 1.14
F5	5:0:5	131.57 ± 1.31	0.32 ± 0.02	−41.67 ± 0.97
F6	5:1:4	109.23 ± 0.90	0.32 ± 0.01	−55.47 ± 0.38

Entrapment Efficiency

The EE of all formulations exceeded 70%. Formulations F1 (7:0:3), F2 (7:1:3), F3 (6:0:4), and F4 (6:1:4), which contained 3–3.5% w/v hydrogenated lecithin, achieved EE values of 90.95 ± 2.80%, 91.55 ± 4.63%, 90.83 ± 1.37%, and 95.24 ± 0.65%, respectively, indicating superior encapsulation performance. In contrast, formulations F5 (5:0:5) and F6 (5:1:4), which contained 2.5% w/v hydrogenated lecithin, exhibited lower EE values of 71.76 ± 5.41% and 72.78 ± 5.66%, respectively. When comparing formulations with and without cholesterol, no significant differences in EE were observed between F1 and F2 or F5 and F6 ($p > 0.05$). Interestingly, an exception was noted in F4 (6:1:3), where the inclusion of cholesterol significantly improved EE compared to F3 (6:0:4) ($p < 0.05$), despite both formulations having the same hydrogenated lecithin content. This observation suggests that the impact of cholesterol on EE was inconsistent across formulations.

Morphology

From the physicochemical characterization above, formulations F3 and F4 were selected as the representative formulation to examine the morphology of trans-resveratrol-loaded transfersomes, with and without cholesterol, respectively. TEM analysis revealed that both formulations exhibited a generally spherical shape, characteristic of well-formed vesicular structures (Figure 2). However, transfersomes containing cholesterol (F4) displayed a more uniform morphology with well-defined edges (Figure 2B) than the cholesterol-free formulation (F3), which exhibited slight irregularities (Figure 2A). Formulation F4 was also observed to be slightly larger than F3, aligned to the dynamic light scattering measurement earlier.

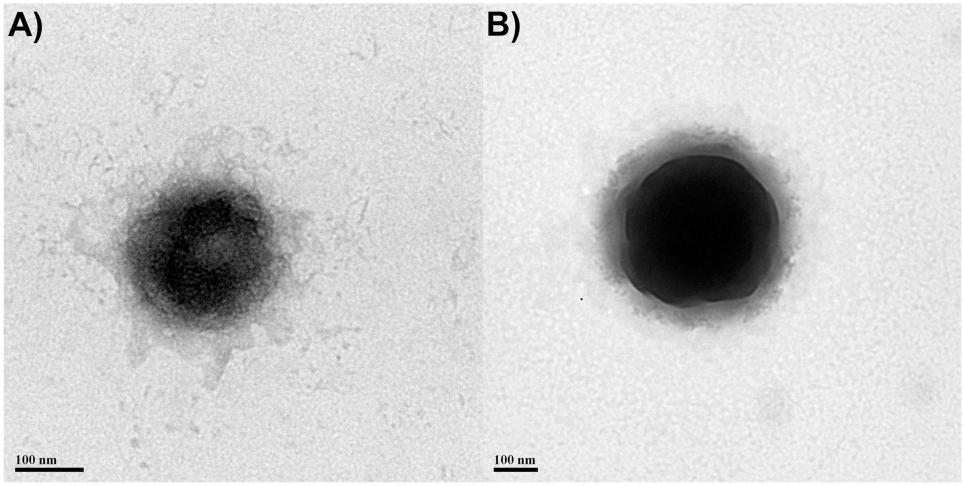


Figure 2 TEM images of trans-resveratrol loaded transfersomes: (**A**) Formulation F3 (6:0:4) and (**B**) Formulation F4 (6:1:3).

In vitro Release Study

The significance of cholesterol incorporation into the transfersomes was distinctly revealed by the in vitro release profiles of the formulations over 24 hours illustrated in Figure 3. Cholesterol-free formulations (F1, F3, and F5) exhibited substantial release rates, with trans-resveratrol release ranging from 68.53% to 80.24% over 24 hours. In contrast, cholesterol-containing formulations (F2, F4, and F6) demonstrated significantly reduced release rates, ranging from below the limit of quantification (0%) to 29.17%. Among all tested formulations, F3 (6:0:4), exhibited the highest release efficiency, achieving 80.24% release within 24 hours. The other two ratios (F1 and F5), which lack cholesterol, exhibit similar characteristics but slightly lower % release after 24 hours. Therefore, the 6:0:4 ratio represents an optimal formulation for achieving the desired release characteristics of trans-resveratrol. Drug recovery across all formulations was within acceptable limits, with less than 10% loss, consistent with criteria reported for in vitro release studies.[46,47]

Based on these promising release characteristics, F3 was selected for subsequent evaluation via permeation studies and HET-CAM irritation assessments. For comparative analysis, F4 was selected as a reference formulation, as it serves as a counterpart to F3 with identical hydrogenated lecithin content but includes cholesterol, allowing evaluation of cholesterol's specific effects on transfersome permeation and irritation.

In vitro Permeation Study

The permeation profiles revealed that both transfersomal formulations [F3 (6:0:4) and F4 (6:1:4)] achieved significantly higher permeation compared to the saturated aqueous solution of trans-resveratrol ($p < 0.05$) (Figure 4). After 24 hours, trans-resveratrol was not detected in the receptor compartment, simulating systemic circulation, indicating that the compound remained localized within the Strat-M membrane layers. Quantitative analysis showed that formulation F4 (with cholesterol) demonstrated a slightly higher permeation than that of F3 (without cholesterol). However, no significant difference was observed ($p > 0.05$), suggesting comparable deposition efficiency of trans-resveratrol within the skin-mimicking membrane.

The Hen's Egg Test – Chorioallantoic Membrane Assay for Potential Irritation Assessment

The irritation scores of the blank and trans-resveratrol-loaded transfersomal formulations (F3 and F4), as assessed using the HET-CAM assay, are presented in Table 4. Observations were conducted at 20 seconds, 2 minutes, and 5 minutes post-application, with positive controls I and II (0.1 N NaOH and 1% SLS) validating the assay's sensitivity. Positive control I, 0.1 N NaOH, demonstrated the highest irritation (12.74 ± 3.15), categorizing it as an "obvious irritant" (Irritation Score [IS] 9.0–21). Similarly, positive control II, 1% SLS, showed moderate irritation (IS 5.0–8.9) with a score of 5.01 ± 2.13. In contrast, the negative control (0.9% NaCl) exhibited no irritation, confirming its classification as non-irritating (IS 0–0.9). The results revealed that both F3 (6:0:4) and F4 (6:1:3) formulations and their blank counterparts

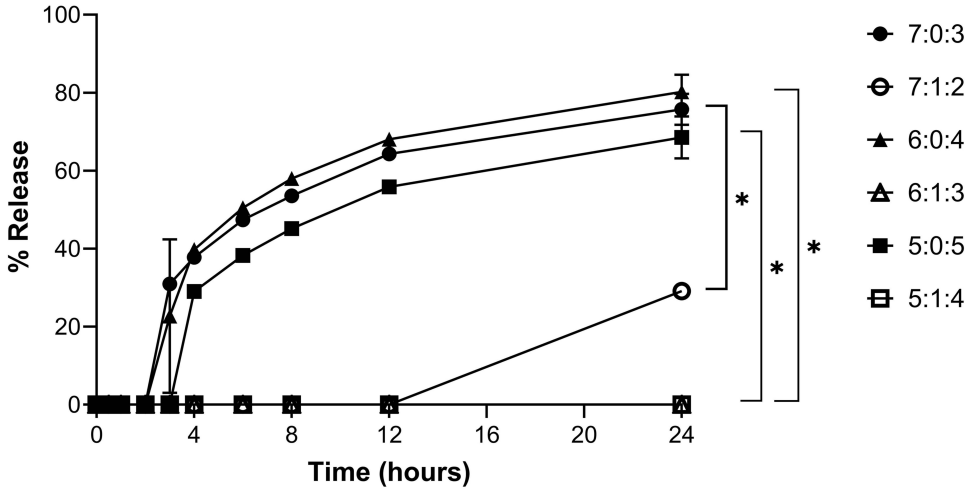


Figure 3 Release of trans-resveratrol-loaded transfersomes. Results are expressed as mean ± SD (n = 3). Statistically significant difference (*p-value <0.05).

https://doi.org/10.2147/NSA.S529010

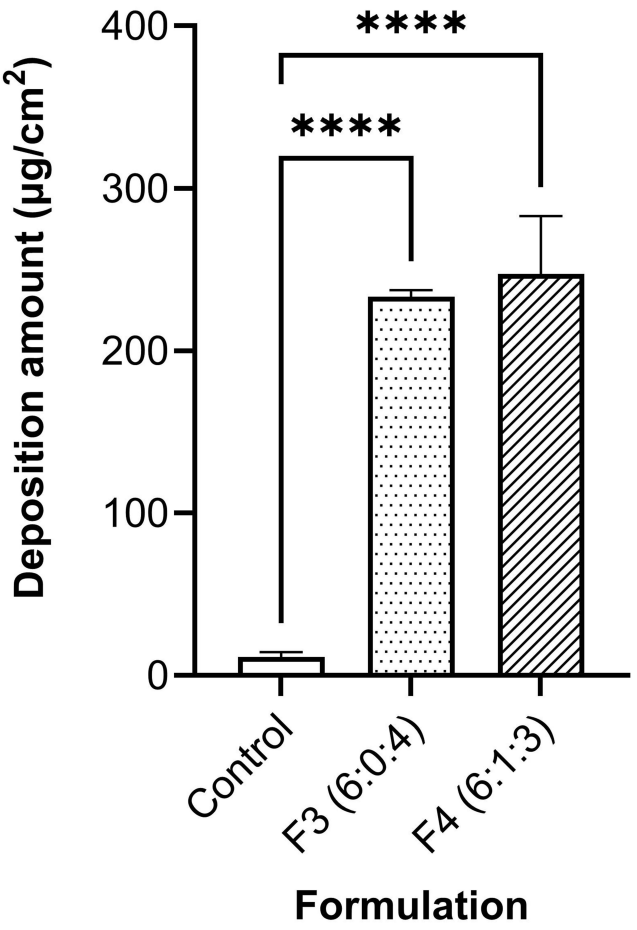


Figure 4 Deposition amounts of trans-resveratrol in Strat-M membrane after 24 hours. Results are expressed as mean ± SD (n = 3). Statistically significant difference (****p-value < 0.001).

demonstrated weak irritation potential, with irritation scores falling between 1.0 and 4.9. Figure 5 illustrates the appearance of the chorioallantoic membrane (CAM) in fertilized chicken eggs at 0 seconds, 20 seconds, 2 minutes, and 5 minutes after application, highlighting time-dependent irritation effects of the tested samples.

Table 4 Irritation Scores (IS) of the Chorioallantoic Membrane in Fertilized Chicken Eggs Following Treatment with Positive Control I (0.1 N NaOH), Positive Control II (1% SLS), Negative Control (0.9% NaCl), Blank Transfersomes and Trans-Resveratrol Loaded Transfersomes

Samples	IS (Mean ± SD) n = 3	Classification
0.1 N NaOH (Positive control I)	12.74 ± 3.15	Obvious irritation
1%SLS (Positive control II)	5.01 ± 2.13	Moderate irritation
0.9% NaCl (Negative control)	0	Non-irritation
Blank F3 (6:0:4)	1.01 ± 1.74	Weak irritation
F3 (6:0:4)	3.38 ± 0.64	Weak irritation
Blank F4 (6:1:3)	1.43 ± 1.24	Weak irritation
F4 (6:1:3)	2.57 ± 1.18	Weak irritation

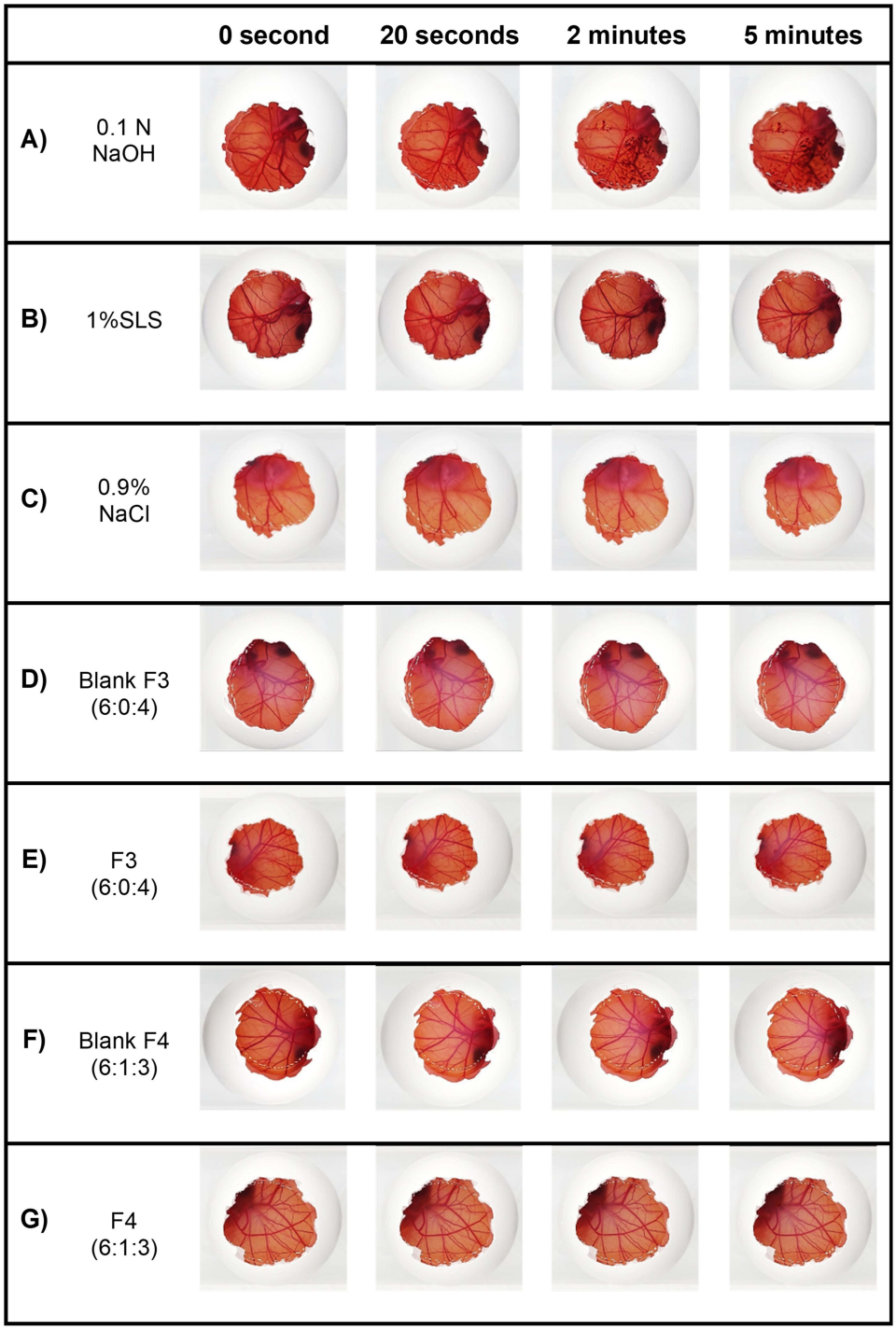


Figure 5 Images of the chorioallantoic membrane (CAM) in fertilized chicken eggs following treatment with: (**A**) positive control I (0.1 N NaOH), (**B**) positive control II (1% SLS), (**C**) negative control (0.9% NaCl), (**D**) blank F3, (**E**) F3, (**F**) blank F4, and (**G**) F4. Images were captured at 0 seconds, 20 seconds, 2 minutes, and 5 minutes post-application to observe time-dependent irritation effects.

Discussion

The pH values of all transfersome formulations, ranging from 5 to 5.5, were found to be compatible with human skin, ensuring skin tolerance and minimizing the risk of irritation.[48] This pH range also appears suitable for maintaining the stability of trans-resveratrol as degradation of the compound is known to increase exponentially above pH 6.8.[49] At pH 5,

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trans-resveratrol demonstrates its longest half-life,[50] suggesting that this pH range is optimal for preserving the stability of the active compound in the formulation.

The particle sizes of the prepared transfersome formulations, all under 150 nm, fall within the ideal range for transdermal delivery (<300 nm). Smaller particle sizes are crucial for efficient skin permeation while maintaining system stability.[51] Cholesterol-containing formulations (F2, F4, and F6) consistently produced larger particles than their cholesterol-free counterparts (F1, F3, and F5). This increase in particle size is likely attributable to cholesterol's stabilizing effect on the lipid bilayer, which enhances membrane rigidity and alters molecular packing. Choi et al (2004) reported that the incorporation of cholesterol into the lipid bilayer of liposomes increases membrane packing density, as confirmed by fluorescence anisotropy, resulting in larger vesicle sizes.[52] Although their findings were specific to liposomes, the underlying mechanism can be extended to transfersomes, as both systems share bilayer-based architectures. Thus, it is plausible that similar effects of cholesterol on bilayer structure and vesicle enlargement also apply to transfersomes. An exception was observed in the formulation containing a 1:1 ratio of hydrogenated lecithin and Tween 60 (F5), which displayed a larger particle size despite lacking cholesterol, compared to F6. This anomaly may be due to the simultaneous formation of transfersomes and micelles, disrupting particle uniformity and highlighting the complex interplay of formulation components in determining particle characteristics.[53] Further comparisons among formulations with similar total lipid content confirmed that cholesterol-containing formulations consistently yielded larger particles. However, the polydispersity index (PDI) of all formulations remained within a narrow range, suggesting that cholesterol's influence on particle size did not significantly affect size distribution. These findings indicate that cholesterol can modulate transfersome particle size while maintaining uniform particle distribution.

Cholesterol-containing formulations exhibited more negative zeta potential values. This enhanced surface charge is likely due to cholesterol's ability to reorganize the phospholipid arrangement, exposing negatively charged groups on the vesicle surface.[54] Additionally, zeta potential values exceeding −30 mV suggest that these formulations may help prevent particle aggregation, which likely contributed to the observed favorable polydispersity index values.

The %EE of all formulations exceeded 70%, confirming the effective encapsulation capacity of the transfersomal systems for trans-resveratrol. Among these, formulations with higher hydrogenated lecithin content (F1, F2, F3, and F4) achieved significantly greater %EE, suggesting the role of lecithin as a structural component in creating an optimal internal environment for drug entrapment. This may be due to lipids with a higher phase transition temperature, such as hydrogenated lecithin, contributing to the formation of more rigid bilayers that limit drug leakage and enhance stability,[55] which could improve the loading efficiency of the systems. In contrast, formulations with reduced lecithin content (F5 and F6) showed lower %EE, indicating insufficient bilayer integrity for stable drug entrapment. This may also reflect increased micelle formation due to higher concentrations of surfactants relative to phospholipids, leading to suboptimal vesicle structures.[53]

Interestingly, no consistent trend was observed regarding the influence of cholesterol on %EE across all formulations. However, formulation F4 (6:1:3) exhibited a significantly higher %EE than F3 (6:0:4), despite both containing the same amount of hydrogenated lecithin. This suggests that the presence of cholesterol can enhance entrapment efficiency in a composition-dependent manner. Cholesterol likely contributes to improved packing of lipid chains, which condenses the bilayer and reduces permeability. This condensing effect may increase structural integrity and internal volume while reducing leakage,[56] thus favoring higher entrapment of trans-resveratrol. This finding is not in agreement with the study by Khan et al (2021), which showed that formulations without cholesterol exhibited significantly higher entrapment of hydrophobic drugs compared to transfersome vesicles with cholesterol due to competition for accommodation in the concentric bilayers.[57] Notably, our study indicates that this benefit occurs only within specific ratios, emphasizing the importance of optimizing the ratios of phospholipids, cholesterol, and edge activators. These findings highlight the critical role of formulation optimization in maximizing entrapment efficiency in transfersomal systems.

According to Raffy and Teissié (1999), poor packing of lipid chains can lead to membrane defects, which may explain the formation of irregular vesicle shapes in the absence of cholesterol.[58] McMullen et al (2000) demonstrated that the incorporation of cholesterol into phospholipid bilayers reduces the temperature, enthalpy, and cooperativity of the gel-to-liquid crystalline phase transition, while increasing the order of hydrocarbon chains.[59] This supports the hypothesis that cholesterol has a membrane-rigidifying effect. Kaddah et al (2018) used TEM imaging to show that the addition of

cholesterol induces a transition from irregular to nanosized unilamellar vesicles,[60] which closely aligns with our observations. Similarly, Brocca et al (2004) reported cholesterol-induced stiffening of DPPC liposomes, as evidenced by shape fluctuations detected via Dynamic Laser Light Scattering.[61] In our study, transfersomes containing cholesterol (F4) exhibited enhanced morphological characteristics with well-defined boundaries when examined using transmission electron microscopy (TEM), compared to cholesterol-free transfersomes (F3). These findings highlight cholesterol's crucial role in shaping transfersome morphology—similar to its effects in liposomal systems.

The release kinetics demonstrated a clear distinction between formulations with and without cholesterol. Cholesterol-free formulations exhibited significantly higher release rates, while the inclusion of cholesterol resulted in reduced release profiles. This trend is consistent with cholesterol's known ability to intercalate within the lipid bilayer, increasing membrane order and rigidity, thus hinder the diffusion of encapsulated compounds, a phenomenon previously reported in vesicular systems.[62–64]

Among the tested formulations, F3 (6:0:4) showed the most favorable release behavior, achieving a release efficiency of 80.24% over 24 hours while maintaining a high entrapment efficiency (>90%). This superior performance can be attributed to a well-balanced ratio of hydrogenated lecithin and Tween 60, which together form a bilayer that is both structurally stable and sufficiently flexible. Hydrogenated lecithin offers membrane integrity due to its saturated fatty acid content and high transition temperature, while Tween 60, a non-ionic surfactant, acts as an edge activator that increases bilayer elasticity, promoting drug diffusion.[65,66] This suggests that the absence of cholesterol in this specific ratio allows Tween 60 to exert its fluidizing effect more effectively, facilitating controlled release without compromising vesicle stability.

While other cholesterol-free formulations such as F1 (7:0:3) and F5 (5:0:5) also showed extended-release behavior, their slightly lower release percentages compared to F3 highlight the sensitivity of vesicle performance to component ratios. In these cases, either an excess of lecithin (F1) or Tween 60 (F5) may have disrupted the optimal bilayer architecture, resulting in suboptimal release kinetics. Therefore, achieving an ideal formulation requires carefully balancing structural and functional components.

Previous permeation studies have established that vesicles containing phospholipids and cholesterol without surfactants typically achieve higher deposition of active compounds within the upper layer of skin, with limited subsequent movement, resulting in lower flux rates.[67,68] While conventional transfersomes, composed of phospholipid and edge activator, generally demonstrate deeper skin permeation, making transfersomes particularly suitable for targeted dermal delivery applications.[69] In this study, the combination of hydrogenated lecithin and Tween 60, with or without cholesterol, produces similar permeation effects in the Strat-M human skin mimic. The comparable permeation profiles of both formulations suggest that cholesterol incorporation, while known to enhance membrane rigidity, does not significantly impact the permeation-enhancing capabilities of transfersomes. However, this effect was not observed in the present study, possibly due to the low concentration of cholesterol (0.5% w/v) used in the formulations. This finding is particularly noteworthy when considered alongside the in vitro release studies, where cholesterol-containing formulations demonstrated substantially lower release rates. The apparent disconnect between release profile and permeation efficiency suggests that the rate-limiting step in trans-resveratrol delivery may not solely depend on vesicular release characteristics. Factors related to the drug itself such as the lipophilicity and molecular weight of trans-resveratrol, along with its affinity to the membrane may also play a role in its partitioning into and across the membrane.[70]

Since formulation F3 achieved comparable permeation efficiency while exhibiting superior release characteristics, incorporating cholesterol in transfersomal formulations may be unnecessary for optimal dermal delivery performance. These results have important implications for formulation optimization, as cholesterol-free transfersomes simplify the manufacturing process and maintain or enhance delivery efficiency, making them an attractive option for cost-effective and scalable production in cosmetic and dermatological applications.

F3 and F4 were selected for irritation potential assessment using the HET-CAM assay. While originally developed for ocular irritation testing, the HET-CAM assay has emerged as a widely accepted alternative method for evaluating the safety of dermatological formulations. This assay provides a sensitive and ethically sound alternative to traditional animal testing methods.,[32,71] making it particularly valuable for screening the biocompatibility of novel topical delivery systems. This method evaluates irritation by monitoring three key biological responses—haemorrhage (bleeding), lysis

(vessel disintegration), and coagulation (protein denaturation)—after applying test substances to the chorioallantoic membrane of fertilized hen eggs. The results revealed that both F3 (6:0:4) and F4 (6:1:3) formulations and their blank counterparts demonstrated weak irritation potential. This mild response can be attributed to the use of Tween 60 as a single-chain surfactant in the formulations. Specifically, F3 contained 2% w/v Tween 60, while F4 contained 1.5% w/v. Despite their surfactant content, the irritation levels observed were significantly lower than those of the positive controls, supporting the overall safety of the formulations for topical applications. The weak irritation potential of Tween 60 is consistent with its well-documented safety profile. According to the Cosmetic Ingredient Review (CIR), Tween 60, when diluted to 15% in water, has shown no irritation in traditional Draize tests, which assess chemical irritation on animal skin or eyes.[72] Polysorbates, including Tween 60, are widely used as emulsifiers and solubilizers in pharmaceuticals, cosmetics, and food products, further corroborating their safety. However, transient interactions between the surfactant molecules and biological membranes may induce minimal irritation even at lower concentrations (1.5–2% w/v) in transfersomal formulations. This mild response is also likely linked to the surfactant's role in enhancing membrane permeation, a critical function for the efficacy of transfersomal systems. The findings of this study reaffirm the reduced irritation potential of transfersomes for dermal delivery, supporting their suitability for applications such as skin whitening, where mild irritation may be an acceptable trade-off for enhanced skin permeation.[73,74] To build upon these promising results, future studies should include in vitro cytotoxicity assays using relevant human skin cell lines, as well as ex vivo permeation and skin retention studies with porcine or human skin. Furthermore, long-term stability assessments under various environmental conditions will be crucial for formulation robustness. Ultimately, in vivo evaluations of safety and clinical efficacy will be necessary to fully validate the potential of cholesterol-free transfersomes in cosmetic and pharmaceutical dermal applications.

Conclusion

This study provides novel insights into the role of cholesterol in trans-resveratrol-loaded transfersomes for dermal delivery. While cholesterol incorporation enhanced vesicle morphology and increased particle size by improving bilayer rigidity, it concurrently reduced the release rate of trans-resveratrol without significantly affecting skin permeation. Notably, cholesterol-free formulations, particularly F3, demonstrated superior release efficiency and comparable permeation profiles, indicating that cholesterol may not be essential for effective trans-resveratrol delivery in transfersomes at the tested concentrations. This challenges the conventional assumption that cholesterol is always beneficial in vesicular systems and suggests that omitting cholesterol can simplify formulation design, reduce production costs, and maintain or improve delivery performance. Consequently, this study advances the understanding of lipid component interactions in transfersomes and proposes a practical strategy for optimizing dermal delivery systems through selective exclusion of cholesterol. Furthermore, irritation assessment via the Hen's Egg Test–Chorioallantoic Membrane (HET-CAM) assay classified the developed transfersomes as weak irritants, supporting their potential suitability for topical applications.

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Disclosure

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