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RESEARCH PAPER

Tofacitinib Free Base-Loaded Transfersomal Gel for Follicular Delivery in Cyclophosphamide-Induced Alopecia: Formulation, Ex Vivo Skin Permeation, and In Vivo Preliminary Safety Evaluation

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ABSTRACT

Transdermal drug delivery offers significant advantages over conventional oral administration by avoiding hepatic first-pass metabolism and reducing systemic adverse effects. However, the stratum corneum constitutes a major barrier to drug permeation. Transfersomes, ultra-deformable lipid vesicles, have emerged as promising carriers for enhanced transdermal and follicular drug delivery. This study aimed to develop, characterize, and preclinically evaluate a Tofacitinib Free Base (TFB)-loaded transfersomal gel (TFB-LTFsG) for the treatment of cyclophosphamide-induced alopecia (CIA). TFB-loaded transfersomes (TFB-LTFs) were prepared using the thin-film hydration technique with Soya lecithin, oleic acid, and Tween 80. Four formulations were characterized for vesicle size, polydispersity index (PDI), zeta potential, entrapment efficiency (EE%), morphology (FESEM), and drug-excipient compatibility (FTIR). The optimized formulation was incorporated into a 0.5% Carbopol 934 gel matrix. Ex vivo permeation was evaluated using mouse skin in Franz diffusion cells. In vivo efficacy and preliminary safety were assessed in a BALB/c mouse model of cyclophosphamide-induced alopecia (n=6 per group; 14 days treatment), monitoring hair regrowth, biochemical (ALT, AST, urea, creatinine, CRP), and hematological (CBC) parameters. The optimized formulation TFB-LTFs3 exhibited spherical vesicles with a vesicle size of 139.90 nm, PDI of 0.406, zeta potential of -30.20 mV, and high entrapment efficiency (85.73 ± 2.07%). The transfersomal gel demonstrated colloidal stability (zeta potential -32.17 mV) and enhanced ex vivo skin permeation with a steady-state flux of 15.82 ± 1.7 μg/cm²/h and cumulative permeation of 68.27 ± 4.2% over 12 hours. FTIR confirmed drug-excipient compatibility. In vivo evaluation revealed substantial hair regrowth in the TFB-LTFsG group, with reduced CRP levels compared to the untreated model group. Biochemical markers showed no significant hepatic or renal toxicity, supporting preliminary systemic tolerability.

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INTRODUCTION

Transdermal drug delivery has emerged as a compelling alternative to conventional oral and parenteral routes, offering advantages such as bypassing hepatic first-pass metabolism, sustained therapeutic levels, and improved patient compliance [1]. The principal challenge in transdermal delivery is the formidable barrier posed by the stratum corneum, which limits the permeation of most drugs, especially those with higher molecular weights or poor lipophilicity. To overcome this limitation, many nanocarrier systems, including liposomes, ethosomes, and transfersomes, have been developed, demonstrating enhanced skin permeability [2,3]. Tofacitinib free base (TFB) is a Janus kinase (JAK) inhibitor that has been authorized to treat a number of inflammatory diseases, including rheumatoid arthritis. It has shown potential in treating skin diseases by blocking JAK-STAT signaling pathways. Oral administration of TFB may cause systemic side effects and inconsistent absorption, thus alternative routes of administration should be explored [4,5]. Using flexible vesicle systems to deliver TFB through the skin can effectively focus the treatment on the places that need it while reducing exposure to the whole body [6].

Among these, are ultra-deformable vesicles composed of phospholipids and edge activators, which may provide high flexibility and deformability known as transfersomes, these vesicles have the ability to penetrate narrow intercellular pathways within the skin more effectively than conventional liposomes [2,7]. Since they are highly flexible, they can fit through pores that are much smaller than their own diameter therefore facilitating deep skin penetration [8]. These characteristics make transfersomes especially suited for delivering both hydrophilic and hydrophobic chemical compounds through the skin [9]. Besides transfersomes, other nanocarrier systems, including cationic lipid nanoparticles and hybrid lipid carriers, have been explored for tofacitinib delivery, highlighting the broader utility of engineered vesicles in overcoming dermal barriers [10,11].

Previous studies have usually focused on the influence of particle size and carrier material for perifollicular distribution. Researchers have investigated the influence of surface charge, but they have come to different results because of the different carriers and animal models used [12,13]. Li et al. reported in their study that hydrophobicity-modified nanostructured lipid carriers (NLCs) showed higher follicular targeting in a nude mouse back skin model and that positively charged NLCs also exhibited higher follicular targeting [11], but Raber et al. found that positively charged poly (lactic-*co*-glycolic acid) nanoparticles coated with chitosan accumulated less in the hair follicles of pigs. Further investigations are ongoing to determine the optimal size, shape, and surface properties of vesicles to improve the efficacy of transdermal drug delivery. Recent research indicates that transfersomes penetration is primarily governed by membrane deformability and hydration-driven mechanisms, while surface charge may play a secondary role in influencing vesicle stability and skin interaction. This change in understanding highlights a transition from charge-dependent systems to mechanically driven delivery strategies, making transfersomes particularly promising for targeted drug delivery, including hair follicle applications [14].

Recent studies have demonstrated the utility of transfersomes in transdermal drug delivery. Such as, the study of mannose-decorated transferosomes loaded with tofacitinib citrate showed significantly enhanced skin permeation and flux compared to uncoated formulations, indicating the ability of modified transfersomes to improve drug delivery in arthritic models [15], thereby leveraging their unique physicochemical properties to improve permeation across the skin. Given these advances, the present study aims to develop and characterize Tofacitinib Free Base -loaded transfersomes (TFB-LTFs) and Tofacitinib Free Base -loaded transfersomes Gel (TFB-LTFsG). for treating cyclophosphamide-induced alopecia (CIA) by applying TFB topically instead of orally. To balance preliminary safety and efficacy, TFB transfersomes were designed using phospholipids and edge activators to achieve optimal vesicle deformability and follicular targeting. Most studies suggest that hair movement plays a crucial role in the penetration of nanomedicine into the hair follicle channel [16]. Therefore, it can be predicted that certain characteristics of nanoparticles are closely related to their delivery capability. However, due to the differences in hair follicle morphology in different animal models and skin areas, and the imperfection of in vitro research models, there is currently a lack of systematic research. other research has confirmed that nanomedicine can still effectively enhance

delivery in the hair follicles of alopecia areata, even after hair loss [17]. The formulation and optimization of transfersomes loaded with TFB represent a promising strategy for enhancing the targeted delivery of this therapeutic molecule.

MATERIALS AND METHODS

Study Design and Reporting Compliance

This study is an experimental laboratory-based investigation conducting at the College of Science, University of Misan (Maysan, Iraq) and comprising a formulation development phase, an ex vivo skin permeation study, and an in vivo preclinical animal study using a randomized controlled design with four parallel groups. The reporting of the in vivo animal experiments was conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) 2.0 guidelines, ensuring transparent and reproducible documentation of animal selection, housing, randomization, allocation, intervention, outcome assessment, and statistical analysis.

Ethical Considerations

The study protocol, including all in vivo and ex vivo procedures involving laboratory animals, was reviewed and approved by the Institutional Animal Ethics Committee of College of Science, University of Misan, Maysan, Iraq ID no. D.A./132 dated 19 August 2025.

Type of Sampling and Reasons for Selection

A purposive sampling strategy was employed for the in vivo experiment. Male BALB/c mice were selected because of their well-characterized hair cycle, defined dermal architecture, and established suitability as a preclinical model for CIA and follicular drug-delivery studies. A total of 24 healthy mice were recruited from the institutional animal house and randomly allocated, divided into four equal groups (n = 6 per group): normal control, cyclophosphamide-induced alopecia model, TFB-LTFsG-treated, and rosemary-oil-treated comparative groups.

Inclusion Criteria

In Vitro Evaluation (Transfersomes Characterization of Formulations containing tofacitinib successfully incorporated into Carbopol 934 gels. Vesicle size within the nanoscale range (generally <200 nm). Acceptable polydispersity index (PDI ≤ 0.5) Physicochemical stability during the evaluation period. For ex vivo skin-permeation studies, only intact dorsal mouse skin samples without visible damage, inflammation, lesions, or hair-follicle abnormalities were included.

In Vivo Animal Studies

Healthy male BALB/c mice aged 6–8 weeks and weighing 20–30 g, with intact dorsal skin, normal hair-coat appearance, and no visible signs of dermatological disease, systemic illness, wounds, or injury at the intended dorsal application site.

Exclusion criteria

Any transfersomes vesicle formulations that failed to meet the desired physicochemical properties or stability criteria were likely excluded from the study. Animals showing weight loss greater than 15% during the acclimatization period, observable behavioral abnormalities, skin lesions, infections, or any sign of dermatological disorder; animals that died or were euthanized for humane reasons before the end of the study; and skin samples showing macroscopic damage, perforations, or contamination after excision.

Accuracy, reproducibility, and quality control

In order to ensure maximum accuracy and study reproducibility, all analytical instruments were calibrated regularly in compliance with manufacturer specifications. Standard operating procedures were meticulously executed by certified personnel. Data consistency was safeguarded via double-data entry and systematic cross-verification. To further enhance methodological validity, an independent biostatistician oversaw the statistical auditing, and to eliminate inter-rater variability.

Materials

Tofacitinib Free Base (TFB) was obtained from Xi'an Sonwu Biotech, China. Soya lecithin and Carbopol 934 were purchased from Himedia, India. Tween 80 and oleic acid were purchased from Loba Chemie, India. Analytical-grade chloroform, methanol, and distilled water were obtained from Chemlab, UK. Phosphate-buffered saline (PBS, pH 7.4) was purchased from Sigma-Aldrich, Germany, while dimethyl sulfoxide (DMSO) was obtained from Alpha Chemika, India. Cyclophosphamide (CPA) was obtained from Baxter, Germany, and used for the induction of CIA in mice. Dialysis bags with a molecular weight cut-off of 8000-14000 Da

were obtained from Spectrum Labs, USA. Cellulose acetate syringe filters with a pore size of 0.45 µm were purchased from ALWSCI, China. All chemicals and reagents were of analytical grade and were used as received without further purification.

Laboratory Animals

Male BALB/c mice were maintained under standard laboratory conditions, including controlled temperature, humidity, and a regular light-dark cycle. Animals were provided with standard food and water ad libitum. Only healthy animals with normal skin appearance and no visible signs of dermatological disease, systemic illness, or injury at the dorsal application site were included in the in vivo experiment. For ex vivo skin permeation studies, excised dorsal mouse skin was obtained from ethically sacrificed animals. Only intact skin samples with no visible damage, wounds, inflammation, or disease were used. Skin samples were handled carefully and used under standardized experimental conditions. The study protocol was approved by the institutional animal ethics committee at the department of Biology/ University of Misan.

Preparation of Tofacitinib Free Base-loaded transfersomes

TFB-loaded transfersomes (TFB-LTFs) were prepared using the thin-film hydration method followed by size reduction. Briefly, TFB, soya lecithin, oleic acid, and Tween 80 were dissolved in a mixture of chloroform and methanol at a ratio of 2:1 v/v to form the organic phase. The mixture was stirred for 10 min until a clear solution was obtained. The organic solvent was then removed under reduced pressure using a rotary evaporator at 45 °C to form a thin lipid film on the wall of the flask. The film was further dried for 2 h to ensure complete removal of residual solvent. The dried lipid film was hydrated with PBS pH 7.4 to obtain multilamellar vesicles. Size reduction was performed by ultrasonication for 30 min using three cycles of 10 min, with 5 min cooling intervals in an ice bath to avoid thermal degradation. The resulting dispersion was filtered through a 0.45 µm membrane filter to remove aggregates and improve vesicle uniformity. The prepared formulations were stored at 4 °C until further characterization. Four transfersomal formulations were prepared by varying the amounts of lecithin, oleic acid, and Tween 80 while maintaining a constant TFB amount and hydration volume, as shown in Table 1.

Preparation of TFB-loaded transfersomal gel

The optimized transfersomal formulation, TFB-LTFs3, was incorporated into a Carbopol 934 gel matrix to prepare TFB-LTFsG. Carbopol 934 (0.5%) was gradually dispersed in distilled water under continuous stirring and allowed to hydrate completely for four hours. The optimized transfersomal dispersion was then added slowly to the hydrated Carbopol base under gentle stirring to ensure uniform distribution of the vesicles within the gel matrix. The final gel was adjusted to the desired consistency and stored at 4 °C until further evaluation. The neutralizing agent was triethanolamine (pH 6.5) [18].

UV-visible spectrophotometric analysis and calibration curve

UV-visible spectrophotometry was used for the quantitative determination of TFB. A solution of TFB was scanned in the range of 200-400 nm to determine the wavelength of maximum absorption. The λmax of TFB was identified at 286 nm. A calibration curve was prepared by measuring the absorbance of standard TFB solutions at different concentrations in PBS pH 7.4 containing 0.4% DMSO. The calibration curve was used for determining drug concentration in entrapment efficiency and ex vivo permeation studies [19,20].

Characterization of TFB-loaded transfersomes

The prepared TFB-loaded transfersomal formulations were characterized for vesicle size, polydispersity index, zeta potential, entrapment

Table 1. Composition of Tofacitinib Free Base-loaded transfersomal formulations.

Formulation code	Tofacitinib Free Base (mg)	Lecithin (mg)	Oleic acid (mg)	Tween 80 (mg)	PBS volume (mL)
TFB-LTFs1	50	300	25	50	10
TFB-LTFs2	50	350	30	60	10
TFB-LTFs3	50	350	40	75	10
TFB-LTFs4	50	350	50	75	10

efficiency, drug excipient compatibility, and morphology. Vesicle size, PDI, and zeta potential were measured using dynamic light scattering after suitable dilution of the samples with distilled water or PBS. Vesicle size and PDI were used to assess particle-size distribution and formulation homogeneity, while zeta potential was used to evaluate surface charge and colloidal stability. Entrapment efficiency was determined using the centrifugation method, 1 mL of TFB-LTFs3 was centrifuged at 5000 rpm for 25 min to separate free drug. The supernatant was filtered, and 100 µL was diluted to 10 mL using phosphate buffer (pH 7.4) containing 0.4% DMSO. Samples were analyzed spectrophotometrically at λmax 286 nm, and drug concentration was calculated using a calibration curve [19,20]. The concentration of the free drug (x) was calculated using the linear regression equation derived from the standard calibration curve.

Calculation of total free drug amount (W free) in mg

$$W_{free} = \frac{(x \times DF \times V)}{1000} \quad (1)$$

(Where DF = 100, and 1000 is the conversion factor from µg to mg)

The percentage of entrapment efficiency was calculated using the following formula:

EE% = [(Total amount of drug - Amount of free drug) / Total amount of drug] × 100

$$EE\ (\%) = \left(\frac{(W_{total} - W_{free})}{W_{total}}\right) \times 100 \quad (2)$$

FTIR spectroscopy was performed using an FTIR spectrophotometer to analyze the infrared spectra of (TFB, TFB-LTFs3. and TFB-LTFsG) This study aimed to identify potential physicochemical interactions between the drug and excipients used in the formulation. The samples were scanned in the spectral range of 400–4000 cm^{-1} [21].

Field-emission scanning electron microscopy was also performed to examine the morphology, vesicle shape, surface characteristics, and distribution of the optimized formulation within the gel matrix.

Ex Vivo Skin Permeation Study

Skin permeation of TFB-LTFsG was evaluated using excised mouse skin in a modified and fabricated Franz diffusion cell under approved ethical conditions. The dorsal skin was prepared, cleaned, and mounted between donor and receptor compartments, with phosphate buffer (pH 7.4) containing 0.4% DMSO used as the receptor medium maintained at 32 ± 1 °C under continuous stirring). The inclusion of 0.4% DMSO was essential to maintain the complete solubility of TFB without compromising the integrity of the mouse skin during the spectrophotometric analysis. The effective permeation surface area was 3.14 cm^2. A specified amount of the gel formulation (TFB-LTFsG gel) was applied on the skin surface, and samples were withdrawn at predetermined time intervals up to 12 h. The collected samples were analyzed spectrophotometrically at λmax 286 nm using the calibration curve to determine drug concentration [19,22–24]. The cumulative amount of drug permeated per unit area (µg/cm^2) was plotted against time (h). The permeation rate parameters, including steady-state flux (Jss) were calculated.

Corrected Concentration Equation:

$$C_{corr} = C_n \left(\frac{V_s}{V_t}\right) \sum_{i=1}^{n-1} C_i \quad (3)$$

Where: C (corr): The corrected concentration at the nth sampling time, Cn: The measured concentration at the nth sampling time, Vs: The volume of the sample withdrawn (3 mL), Vt: The total volume of the receptor medium (100 mL), Σ (Ci): The sum of concentrations measured at all previous sampling times.

B. Steady-State Flux (Jss): $Jss = dQ / (A \times dt)$

Where A = 3.14 cm^2.

In Vivo Study

An in vivo study was performed to evaluate the therapeutic efficacy of TFB-LTFsG using a mice model of cyclophosphamide-induced alopecia. A total of 24 male BALB/c mice (6–8 weeks, 20–30 g) were used due to their well-characterized hair cycle and suitability for assessing follicular drug

delivery [25–27]. Alopecia was induced by dorsal depilation to synchronize hair follicles into the anagen phase, followed by a single intraperitoneal injection of cyclophosphamide (CPA) (150 mg/kg) at day 9 post-depilation, resulting in follicular dystrophy and hair loss, establishing a clinically relevant model of CIA [28]. Animals were randomly divided into four groups (n = 6 in each group): G1 (normal control), G2 (CIA model group without treatment), G3 (CIA group treated with TFB-LTFsG), and G4 (CIA group treated with rosemary oil as a comparative standard. The optimized TFB-LTFsG formulation was applied topically to the depilated dorsal skin at a dose of 50 µL twice daily for 14 days. Rosemary oil was applied in the same manner as a comparative treatment. The normal control and model groups were handled under the same experimental conditions. On day 29 (14 days post-treatment initiation), animals were euthanized according to institutional ethical protocol prior to blood and tissue collection as summarized in (Fig. 1).

Evaluation of hair regrowth

Hair regrowth was monitored during the treatment period by visual observation and standardized photographic documentation. Images of the dorsal skin were captured under similar lighting conditions, distance, and angle to allow comparison among groups. Hair-regrowth activity was assessed based on the visible hair covered area, hair density, and restoration of skin appearance. Where applicable, image analysis software may be used to quantify the percentage

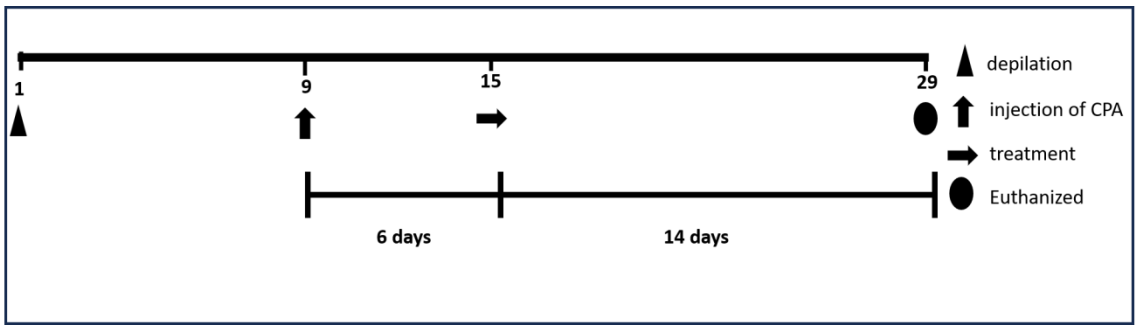

Fig. 1. Timeline of the experimental design and treatment schedule.

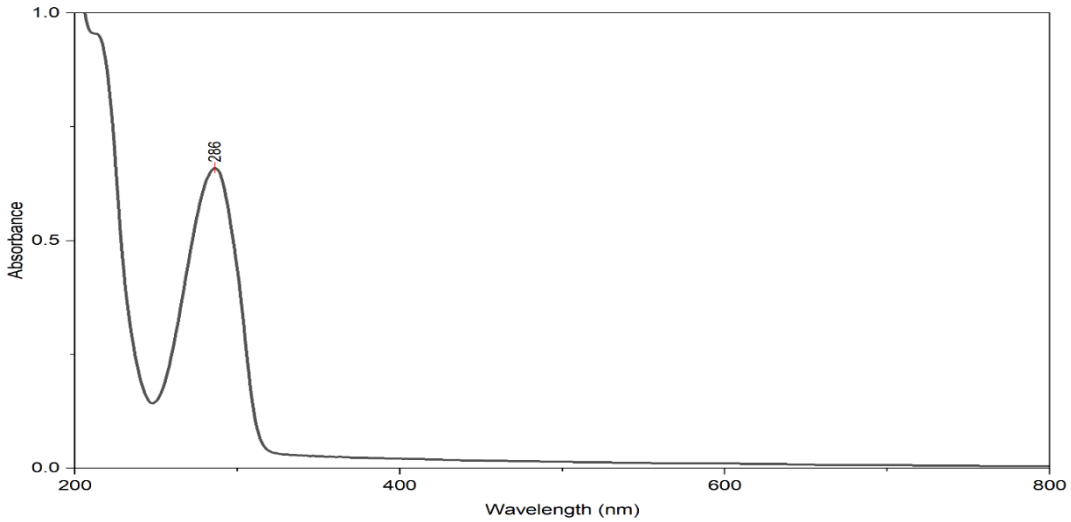

Fig. 2. UV-Visible absorption spectrum of TFB, showing the characteristic maximum absorption peak (λ_{max}) at 286 nm.

of hair-regrowth area.

Blood collection and biochemical analysis

At the end of the treatment period, animals were weighed and anesthetized using an approved anesthetic method according to the institutional ethical protocol. Blood samples were collected by cardiac puncture. The collected blood was divided into two portions. The first portion was transferred into EDTA tubes for complete blood count analysis. The second portion was allowed to clot and then centrifuged to separate serum. Samples were used to determine liver and kidney function biomarkers, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine. C-reactive protein (CRP) was also measured to evaluate systemic inflammatory status. Biochemical parameters were analyzed using a biochemistry analyzer according to the manufacturer's instructions.

Statistical Analysis

Experimental data were expressed as mean ± standard deviation. Data were initially organized using Microsoft Excel and then analyzed using GraphPad Prism 11. Comparisons among multiple groups were performed using one-way analysis of variance followed by Tukey's post hoc test. A p-value of less than 0.05 was considered statistically significant. With 95% confidence intervals reported where appropriate. The principal assumption underlying the parametric analysis was normality

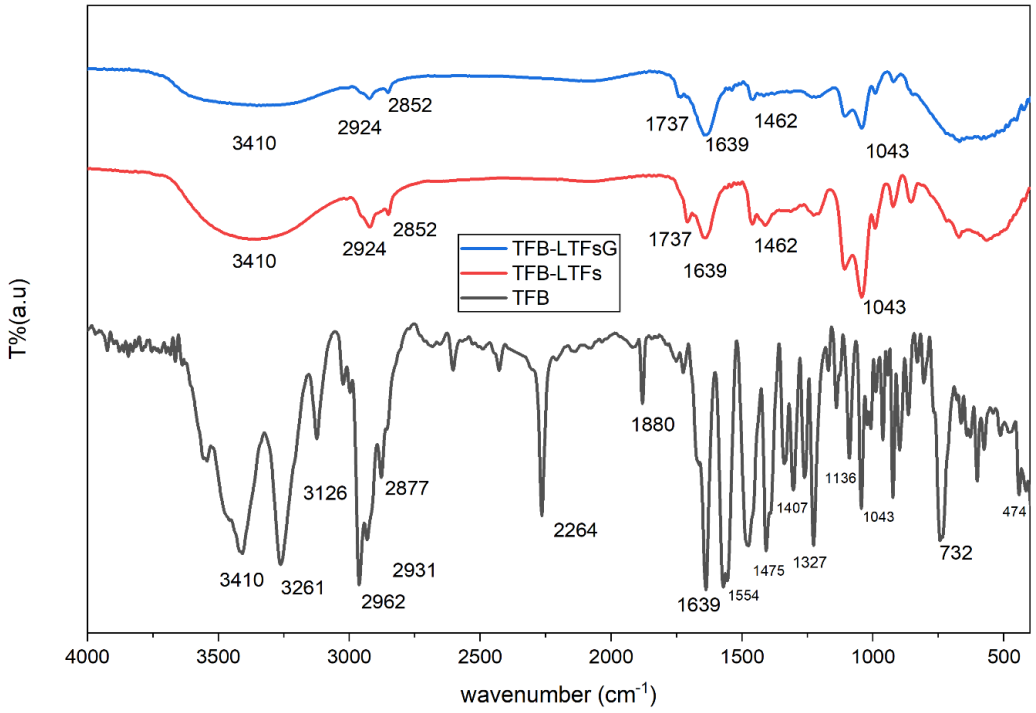

Fig. 3. FTIR Spectra Origin image files for TFB, TFB-LTFs, and TFB-LTFsG.

Table 2. Vesicle size, PDI, and Zeta potential of Tofacitinib transfersomes.

Formulation Code	Vesicle size (nm)	PDI	Zeta Potential (mV)
TFB-LTFs1	184.56	0.143	-41.56
TFB-LTFs2	186.19	0.138	-37.68
TFB-LTFs3	139.90	0.406	-30.20
TFB-LTFs4	179.62	0.152	-37.16

of the data distribution within each group; given the small sample size (n = 6 per group), results should be interpreted cautiously and considered preliminary, and additional confirmatory studies with larger sample sizes are recommended.

RESULTS AND DISCUSSION

UV–Vis analysis

Using a UV-Vis spectrophotometer, a spectral scan was performed in the range (200–400 nm) for a solution containing 10 µg/ml of TFB in PBS.

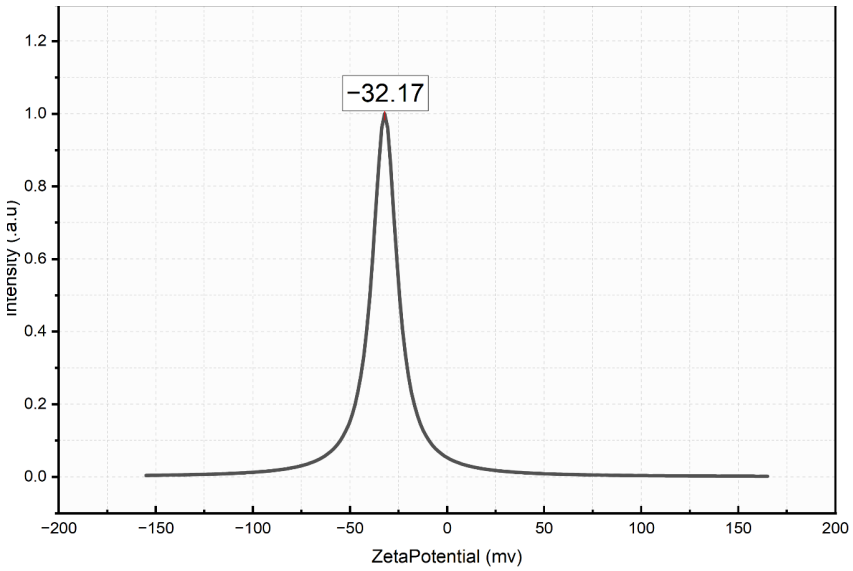

Fig. 4. Zeta potential spectra of the obtained of TFB-LTFsG.

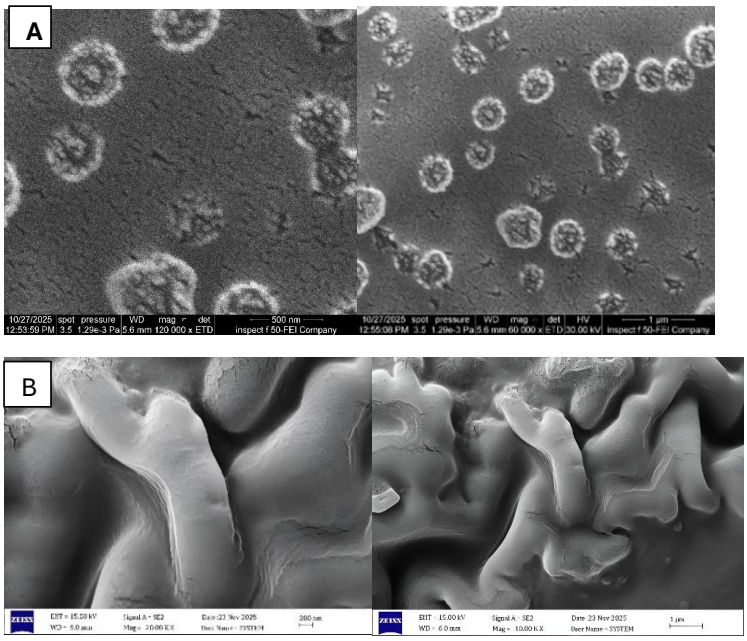

Fig. 5. (A) FESEM images showing the morphology and surface characteristics of optimized TFB-LTFs. (B) FESEM images showing the morphology and surface characteristics of optimized TFB-LTFsG. indicated the transfersomes dispersed in the 3D pore structure of Carbopol 934 gel.

The maximum absorption wavelength (λ_{max}) was identified at 286 nm, demonstrating close agreement with previously reported values in the literature [19], as summarized in (Fig. 2).

Characterization of TFB-loaded transfersomes

The FT-IR spectra were analyzed to verify the chemical structure of TFB, TFB-LTFs3, and TFB-LTFsG. To evaluate its compatibility with the various excipients used in the formulation stages. as shown (Fig. 3).

The DLS analysis revealed that the average hydrodynamic diameters for the four TFB-LTFs formulations ranged from 139.90 to 186.19 nm. PDI values were calculated for all samples, and the values were within an acceptable range (0.143–0.406). Among the tested formulations, TFB-LTFs3 was selected because it had the smallest vesicle size of all the formulations we tested. This helps with skin penetration and targeting. TFB-LTFs3 also had a good zeta potential value, which means it was stable, and subsequently incorporated into a Carbopol gel as shown in Table 2. After incorporation of TFB-LTFs3 into Carbopol 934 gel, the formulation was referred to as TFB-LTFsG. The zeta potential of the prepared gel was measured to evaluate stability, and the obtained value (-32.17 mV) was comparable to that of the original vesicular system, indicating preservation of vesicle stability after gel incorporation (Fig. 4).

FESEM micrograph (Fig. 5A) representing the surface morphology of the TFB-LTFs3. The image

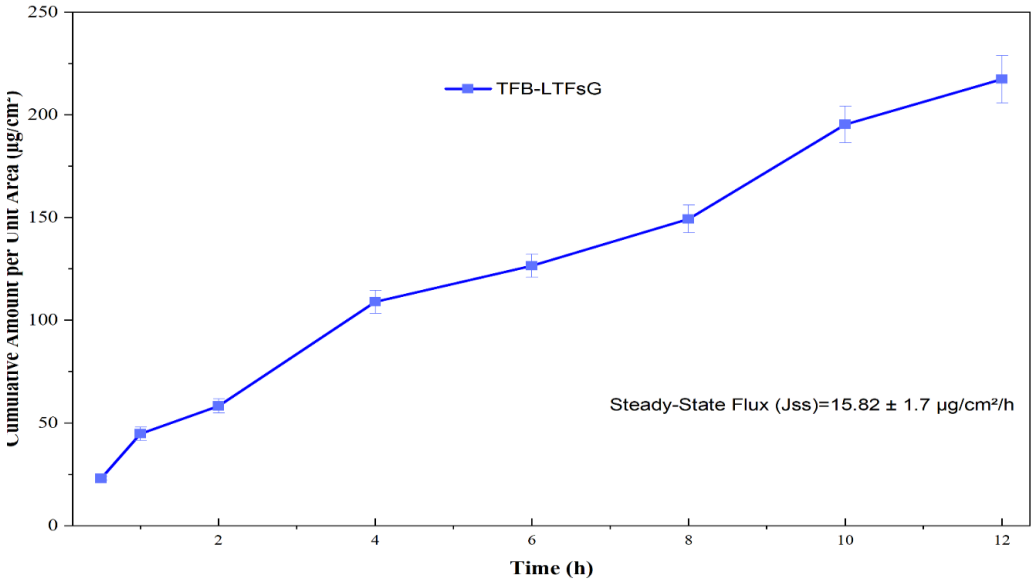

Fig. 6. Ex vivo cumulative permeated per unit area drug release profile of TFB-LTFsG.

Table 3. Biochemical parameters of liver and kidney functions across all experimental groups.

Group	ALT (U/L)	AST (U/L)	Creatinine (mg/dL)	Urea (mg/dL)
Normal control	57.60 ± 27.27	315.3 ± 106.4	0.1450 ± 0.01	38.39 ± 5.02
Model	78.25 ± 21.29	524.4 ± 126.4	0.2400 ± 0.04	59.14 ± 16.47
TFB-LTFsG	79.40 ± 12.60	464.5 ± 79.07	0.2417 ± 0.06	43.89 ± 6.00
Rosemary Oil	71.23 ± 16.60	361.0 ± 68.79	0.2983 ± 0.06	50.00 ± 6.44

reveals that the particles are spherical in shape and relatively uniform in distribution, with minor agglomerations. These observations confirm the successful synthesis of well-defined nanoscale vesicles with the expected morphological characteristics of transfersomes. Furthermore, (Fig. 5B) illustrates the morphology and surface characteristics of the optimized TFB-LTFsG, where the image reveals that the transfersomal vesicles are embedded within the dense Carbopol 934 gel matrix, although only a few vesicles were visibly distinguishable. This may be attributed to their embedding within the dense polymer network.

Ex vivo skin permeation

The developed method was employed for

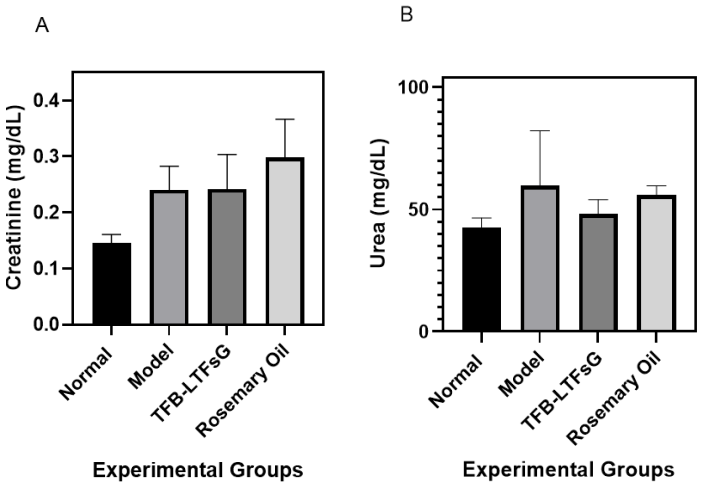

Fig. 7. Comparison of Urea Level and Creatinine Level in Serum Studied Groups. (A) Comparison of serum Creatinine levels between the control groups and the treated groups receiving TFB-LTFsG, and rosemary Oil over the 14-day study period in mice. No significant differences were observed among the groups (P > 0.05); (B) Comparison of serum Urea levels between the control groups and the treated groups receiving TFB-LTFsG, and Rosemary Oil over the 14-day study period in mice. No significant differences were observed among the groups (P > 0.05).

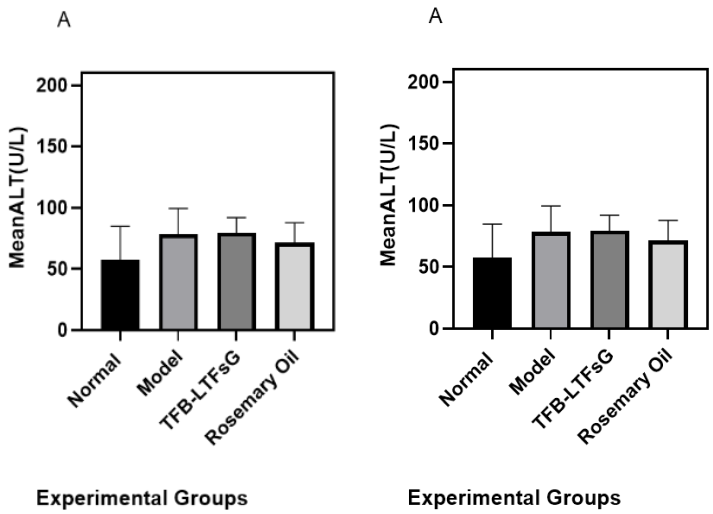

Fig. 8. Comparison of Liver Enzymes Level in Serum Studied Groups. (A) Comparison of serum ALT (SGPT) levels between the control groups and the treated groups receiving TFB-LTFsG, and rosemary Oil over the 14-day study period in mice. No significant differences were observed among the groups (P > 0.05); (B) Comparison of serum AST (SGOT) levels between the control groups and the treated groups receiving TFB-LTFsG, and Rosemary Oil over the 14-day study period in mice. No significant differences were observed among the groups (P > 0.05).

the determination of the amount of tofacitinib permeation through Mouse skin. Cumulative Amount per Unit Area of drug permeated through the skin tissue was found to be (Q12): 217.43 ± 1.24 µg/ cm² in 12 h. The ex vivo skin permeation profile of TFB-LTFsG from the skin is shown in (Fig. 6). The flux of the TFB-LTFsG was found to 15.82 ± 1.7µg/cm²/ at a percentage of 68.3% ± 4.2. The Tofacitinib Entrapment Efficiency (EE%) Data was 85.73± 2.07% when Free Drug Conc. (µg/ml) was 7.13 and the Total Free Drug (mg) was 0.713.

In vivo study

As illustrated in Table 3, the biochemical

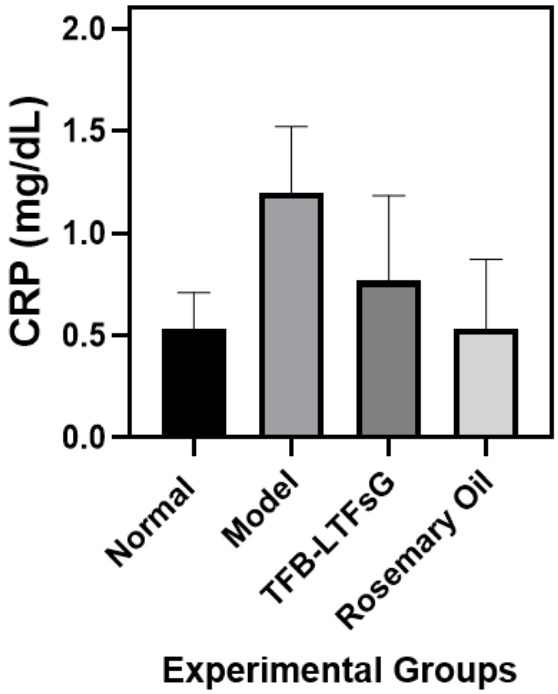

Fig. 9. Serum C-reactive protein (CRP) levels in the experimental groups. The CIA model group showed the highest numerical CRP value, while TFB-LTFsG treatment reduced CRP levels compared with the untreated model group. No statistically significant differences were observed among the groups (P > 0.05).

Table 4. Hematological parameters (CBC) for the experimental groups at the end of the treatment period.

Group	WBC (10^3 /µl)	RBC (10^6/µl)	Hb (g/dl)	PLT (10^3 /µl)
Normal control	6.71 ± 1.65	6.84 ± 0.59	9.66 ± 0.87	515.8 ± 129.6
Model	6.49 ± 1.14	6.68 ± 0.45	9.43 ± 0.57	563.0 ± 125.5
TFB-LTFsG	4.55 ± 1.22	5.09 ± 0.78	7.35 ± 1.11	408.3 ± 69.53
Rosemary Oil	8.91 ± 2.33	6.22 ± 0.79	8.95 ± 1.21	422.5 ± 148.1

analysis of liver and kidney functions showed no significant effects (P > 0.05) in the groups treated with Rosemary Oil and TFB-LTFsG compared to the control groups. These findings confirm the biocompatibility and systemic preliminary safety of the developed formulations.

Kidney-function markers are shown in (Fig. 7). Creatinine levels showed mild numerical increases in the model, TFB-LTFsG, and rosemary oil groups compared with the normal control group. Urea levels were highest in the model group, while the TFB-LTFsG group showed lower values than the untreated model group. However, no significant differences were observed among groups for creatinine or urea levels (P > 0.05). Liver-function markers are presented in (Fig. 8). ALT and AST levels were numerically elevated in the model and TFB-LTFsG groups compared with the normal control group, whereas rosemary oil showed lower values. These variations were not statistically significant (P > 0.05), suggesting no marked hepatic alteration during the treatment period. CRP levels are shown in (Fig. 9). The model group showed the highest numerical CRP level, while TFB-LTFsG reduced CRP compared with the untreated model group. No significant differences were observed among

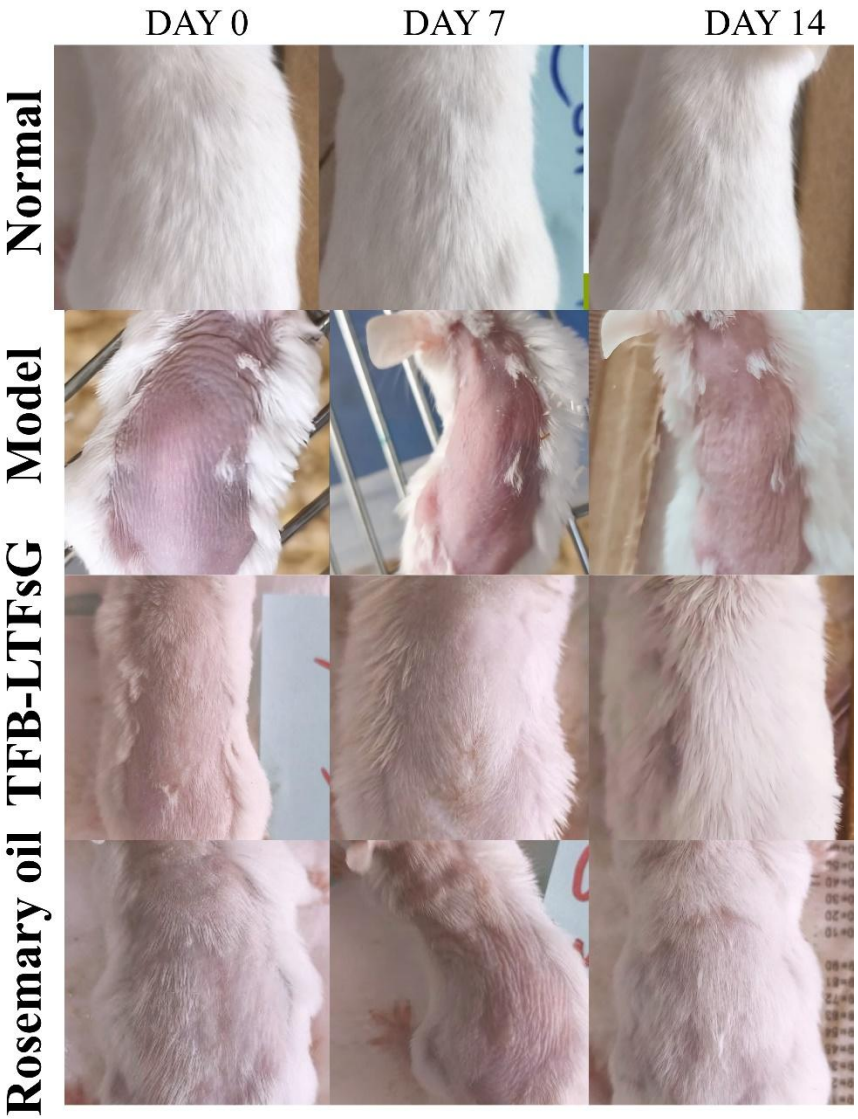

Fig. 10. Hair Regrowth Rate and Density Among the Four Groups.

groups (P > 0.05), indicating that TFB-LTFsG did not induce a significant systemic inflammatory response.

The hematological parameters, including WBC, RBC, Hb, and PLT levels, are presented in Table 4. No statistically significant differences were observed among the experimental groups (P > 0.05). However, the TFB-LTFsG-treated group showed numerical decreases in WBC, RBC, Hb, and PLT values compared with the normal control and model groups. Therefore, these findings suggest preliminary hematological tolerability of the formulation during the 14-day treatment period, although the observed numerical changes should be interpreted cautiously.

In the TFB-LTFsG group, marked hair regeneration and increased hair density were observed, which shows that the follicles were getting more stimulation and the hair cycle was moving along better, the group treated with rosemary oil showed a moderate rate of hair regrowth, however, the newly formed hair appeared less dense compared to the TFB-LTFsG group, indicating partial follicular stimulation without achieving full restoration of hair density. The group with alopecia model had little to no visible hair regrowth during the research, which showed that follicular damage had been successfully produced and the hair growth cycle had been stopped for a long time, The normal control group was left untreated without any intervention or hair cycle induction beyond physiological conditions, and therefore maintained its natural hair growth pattern throughout the study period as presented in(Figs. 10 and 11).

The current study aimed to develop and characterize a novel topical delivery system for Tofacitinib free base, utilizing transfersomes incorporated into a Carbopol 934 gel (TFB-LTFsG). This approach was designed to enhance

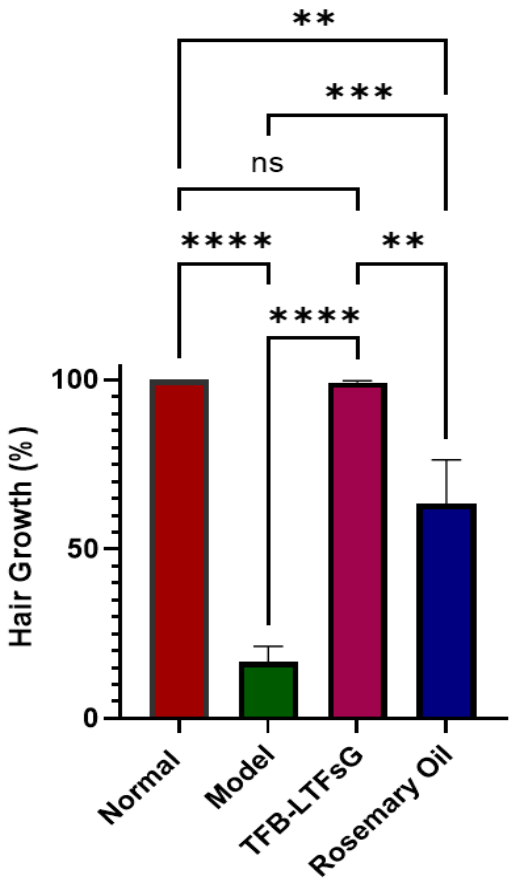

Fig. 11. Comparative in vivo hair regrowth activity of TFB-LTFsG and rosemary Oil in mice at day.

drug permeation, improve bioavailability, and provide controlled drug delivery for effective therapeutic outcomes. The full characterization of the developed formulation included a range of physicochemical and biological evaluations that gave us information about its structural integrity, drug loading capacity, and possible therapeutic potentialc the peak absorption wavelength (λmax) for Tofacitinib free base was identified at λmax = 286 nm and linear calibration curve was made for concentrations between [5 and 40] µg/mL. It had a good correlation coefficient (R^2) of (0.9943). This validated method ensured accurate and reliable quantification of Tofacitinib in various samples, including entrapment efficiency studies, and ex vivo Skin Permeation [20]. The thin-film hydration approach was chosen because it is easy to use and works well to make stable vesicular systems. Lecithin gave the structure stability and biocompatibility, while oleic acid and Tween® 80 worked as an edge activator and surfactant to make the vesicles more flexible and help them pass through the skin Following ultrasonication-mediated size reduction, filtration through a 0.45 µm membrane was performed to ensure homogeneity and remove aggregates.

Physicochemical Characterization of of TFB-LTFsG
Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was performed to confirm that Tofacitinib free base was successfully entrapped in the Transfersomes and that it worked well with the Carbopol 934 gel matrix. The FTIR spectra of the TFB-LTFs exhibited notable differences compared to the pure drug. The distinctive nitrile stretching peak at 2264 cm^{-1} and the overtone band at 1880 cm^{-1} of Tofacitinib were noted to decrease or vanish in the Transfersome formulation [29,30]. This behavior can be attributed to the chemical compatibility and the successful incorporation of the drug within the lipid core and the protective influence of the lipid bilayer and the edge activator (Tween 80), confirming the absence of any detrimental chemical interactions that could undermine the drug's stability. The polar nitrile group probably interacts weakly with the phospholipid heads or polyoxyethylene chains of Tween 80 through hydrogen bonding or dipole-dipole interactions. This may have caused broadening or disappearance of the peaks [29,31]. At the same time, a strong peak appeared at about 1737 cm^{-1}, which is typical of the ester carbonyl stretching (C=O) of Tween 80 and the phospholipids. This confirmed that the vesicular structure had formed successfully [29]. Moreover, the lack of new peaks and the persistence of the principal Tofacitinib peaks (e.g., amide C=O at 1639 cm^{-1} and aromatic ring vibrations at 1554 cm^{-1}) in the Transfersome-loaded Carbopol gel demonstrated exceptional chemical compatibility among the drug, Transfersomes, and the Carbopol 934 polymer. So This indicates that no detrimental chemical interactions transpired that could undermine the drug's stability or effectiveness [32].

Vesicle size, Polydispersity Index (PDI), and Zeta Potential

Dynamic light scattering (DLS) measurements revealed that the optimized formulation TFB-LTFs3 had a vesicle size of 139.90 nm with a PDI of 0.406. This compact, even size is very important for making drugs penetrate the skin better and go through biological membranes better [33]. The moderate PDI value reflects a relatively homogeneous distribution of vesicles, which is important for stable and consistent drug delivery. The zeta potential of the TFB-LTFsG was measured to be [-32.17] mV. This surface charge shows that the vesicles exhibit colloidal stability, preventing aggregation and ensuring longer shelf life. A sufficiently negative zeta potential provides electrostatic repulsion between vesicles, maintaining their discrete nature within the formulation [34]. The negative zeta potential of TFB-LTFsG is attributed to the phosphate groups present in the phospholipids used in the formulation [33].

Field Emission Scanning Electron Microscopy (FESEM)

FESEM images revealed the transfersomes had a correct shape and their integration within the Carbopol gel. The optimized TFB-LTFs3 images showed spherical, well-defined, and homogeneous vesicles, consistent with the expected morphology of transfersomesto look like. while The FESEM image of the gel formulation showed the polymeric matrix exhibited a glossy, highly porous morphology with minor cracking and moderate waviness, this was probably because the polymeric chains overlapped while the matrix was drying. Micrographic analysis confirmed the existence of these porous surfaces, which are

essential in drug loading, the controlled release of therapeutic agents, and water retention. Such porosity facilitates the uptake of physiological media, thereby promoting microparticle swelling. Also, the observation of whitish spots validates the successful incorporation of tofacitinib, indicating that the transfersomes were uniformly dispersed within the Carbopol 934 gel without significant aggregation or structural damage. This uniform distribution is vital for ensuring consistent drug release and effective topical application [29,31,34]. Entrapment efficiency (EE): TFB-LTFs3 have a high entrapment efficiency of (85.73± 2.07%). This high EE means that a lot of the drugs was successfully entrapped inside the transfersomes, which could reduce drug loss during production and increased the therapeutic dose. To acquire the right therapeutic concentration at the target location and lower the risk of systemic adverse effects, it is important to have a high entrapment efficiency [34].

Ex Vivo Skin Permeation Studies

The ex vivo permeation studies, conducted using Franz diffusion cells with excised mouse skin, demonstrated enhanced permeation of TFB from TFB-LTFsG. The cumulative amount of drug permeated and the flux across the skin were markedly higher for the TFB-LTFsG formulation. This enhanced permeation is directly attributable to the unique properties of transfersomes, including their ultra-deformability and small size, which allow them to squeeze through intercellular lipid lamellae of the stratum corneum. The presence of Tween 80 as an edge activator further contributes to this deformability, facilitating deeper penetration into the skin layers. The Carbopol 934 gel base, providing a suitable vehicle for topical application [20,29,35].

In Vivo Pharmacological Efficacy and Preliminary Safety in the CIA Model

In vivo studies, performed on a CIA mouse model, showed the enhanced therapeutic efficacy of the TFB-LTFsG [31]. The results showed that the treated areas had a lot of hair regrowth. ImageJ software was used to measure the affected dorsal area in CIA mice. A substantial reduction in the hairless area was observed, indicating effective hair regrowth in the treated BALB/c mice. These findings are consistent with the improved skin permeation observed in ex vivo studies, indicating that the optimized formulation effectively delivers Tofacitinib to the hair follicles and the target site in sufficient concentrations to exert its pharmacological action as a JAK inhibitor. Similar to conventional Carbopol 934 gel bases, the hydrogel phase in TFB-LTFsG can be considered a suitable topical vehicle because it provides appropriate consistency, prolongs contact with the dorsal skin, and helps maintain a hydrated local environment at the application site. This concept is supported by hydrogel-based and porous-gradient systems that were used to generate controlled local gradients and dynamic microenvironments for biological studies [36,37], as well as by site-specific porous hydrogel coatings designed for tunable local drug release [38].

The comparison among the four groups demonstrated an important gap in hair thickness. The normal control group had hair that was the same thickness as it was at the start, while the alopecia model group had hair that was much thinner because the follicles were damaged. The group that used rosemary oil saw a modest rise in hair thickness, which suggests that some of the follicles' activity has returned. The TFB-LTFsG-treated group, on the other hand, showed the most increase in hair thickness. This suggests that the follicles were more stimulated and that the drug was more effective to reach the hair follicles. Thus, the superior increase in hair thickness in the TFB-LTFsG group may be related to the combined effect of tofacitinib-mediated JAK inhibition and improved local residence of the nanovesicular hydrogel at the skin surface. This interpretation is consistent with external nanoparticle-based formulations in which iron oxide nanoparticles enhanced the local biological activity of active agents in a scalp-related pediculicidal model [42].

The results indicate that the liver and kidneys are primary targets of drug-induced toxicity. Hepatic injury is associated with elevated AST and ALT levels, while renal impairment is reflected by reduced glomerular filtration rate (GFR) [39]. These findings emphasize the clinical importance of drug toxicity and its impact on organ function. Transdermal drug delivery represents a promising strategy to minimize systemic exposure by delivering drugs locally to the target site. This may reduce drug distribution in the bloodstream and limits potential hepatic and renal adverse effects. Such an approach is particularly beneficial in alopecia treatment, where targeting hair follicles

enhances therapeutic outcomes while reducing systemic toxicity [34]. In this study, Liver and kidney function markers (ALT, AST, urea, and creatinine) were evaluated to assess the preliminary safety profile of the formulation. Although nanocarriers may enhance skin penetration, their primary role is to promote localized drug delivery rather than systemic absorption. Consistent with this, no significant changes were observed in these parameters, indicating minimal systemic exposure. It is also important to note that biochemical assessments were performed 21 days after a single cyclophosphamide injection, by which time the acute pharmacological and toxicological effects of cyclophosphamide would have already resolved due to its short half-life and rapid clearance. This confirms that the developed system is safe and does not induce hepatic or renal toxicity.

CRP as an acute-phase reactant, is a widely used clinical biomarker for monitoring inflammatory activity. A decrease in CRP levels may reflect enhanced regulation of immune-mediated inflammation caused by hair loss [40, 41]. In the present study, the decrease in CRP observed in the TFB-LTFsG group may be explained by the localized topical delivery of tofacitinib free base, which allows the drug to act at the affected site while minimizing unnecessary systemic exposure. This interpretation is supported most directly by studies showing that nanoscale formulations can enhance target-site biological activity. In a broader biomedical context, titanium nanoparticles and graphene oxide-based systems have also been investigated for antibacterial and anticancer activity, respectively [44,45]. Although these studies do not directly prove anti-alopecia efficacy, they support the general rationale that nanocarriers can modify biological responses when designed for a specific target application. Regarding the hematological assessment (CBC), Although the changes in WBC, RBC, Hb, and PLT levels in the TFB-LTFsG group were not statistically significant ($P > 0.05$), the observed numerical reductions warrant scientific consideration. Importantly, all hematological values remained within the established physiological reference ranges for healthy BALB/c mice [46], and no clinical signs of bleeding, anemia, weight loss, lethargy, or hematological abnormalities were observed in any treated animal throughout the study period. All animals also maintained normal grooming and activity behavior. The sample size (n = 6 per group), although adherent to the 3Rs principle of animal reduction in preclinical research, represents an intrinsic limitation common to small-cohort studies and may have contributed to the wider confidence intervals observed for some parameters. These findings support the preliminary hematological tolerability of TFB-LTFsG, while extended-duration studies with larger cohorts are recommended to fully characterize the long- term safety profile.

Because iron oxide nanoparticles have also been reported to interact with immune-related responses in other biomedical settings, including enhancement of vaccine antibody responses [43], hematological monitoring was important to exclude unwanted systemic immune or blood-related effects. In the present study, the absence of significant changes in WBC, RBC, Hb, and PLT suggests that topical TFB-LTFsG did not produce detectable systemic hematological disturbance under the tested conditions. White Blood Cell (WBC) count is an important biomarker for monitoring Alopecia, as the condition involves immune-mediated attack on hair follicles. The stability of WBC levels, along with reduced CRP levels, suggests an improvement in inflammatory status. This may be attributed to the localized effect of transdermal drug delivery, which targets the affected site while limiting systemic exposure. Based on the topical application route, this approach may help control the immune response at the affected site, potentially reducing the widespread immunosuppression associated with conventional systemic therapies [34]. Furthermore, micro/nano-engineered platforms, such as surface acoustic streaming-based microfluidic systems for rapid multicellular tumor spheroid generation, highlight the value of nanotechnology in developing advanced biological models [47].

CONCLUSION

This study successfully developed and characterized a novel topical delivery system for Tofacitinib free base, Among the tested formulations, TFB-LTFs3, exhibited the highest entrapment efficiency and smallest vesicle size, establishing it as the optimized formulation. integrating drug-loaded transfersomes within a Carbopol 934 gel matrix was designed to enhance skin targeting. Physicochemical characterization, including FTIR analysis, Vesicle size, zeta potential and FESEM imaging, demonstrated that the optimized formulation possesses ideal properties

for topical application. FTIR analysis confirmed the successful entrapment of the drug and excellent chemical compatibility among components, while the uniform vesicle size and colloidal stability of the transfersomes ensured their effective skin penetration.Furthermore, biological studies, both ex vivo on mouse skin and in vivo in CIA model, showed the enhanced therapeutic efficacy and preliminary safety of the developed system. The developed formulation exhibited superior skin permeation and enhanced drug delivery, leading to significant improvement in hair regrowth and reduction of inflammation in the CIA model. These findings confirm that the transfersome-in-Carbopol 934 gel (TFB-LTFsG) system represents a promising strategy for topical Tofacitinib delivery, offering an effective and safe therapeutic solution for alopecia.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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