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Analytical Characterization and Molecular Interaction Study of Quercetin-Loaded Transferosomes

Quercetin is a bioactive flavonoid that has potent anti-inflammatory activity. Its topical applications are limited due to poor solubility and permeability. The present study aims to develop and characterize quercetin-loaded transferosomes and to establish a validated RP-HPLC method for its quantification. Molecular docking studies revealed favourable binding of quercetin with key inflammatory targets. This included NF-kB (-8.66 kcal/mol), IgE (-7.22 kcal/mol), and TSLP (-6.54 kcal/mol). An RP-HPLC method was developed using C18 column with the mobile phase of acetonitrile and 0.3 % OPA (50:50 v/v). The method showed good linearity over the range of 20-120 µg/mL (R = 0.998). The retention time of quercetin was 4.303 min. Validation parameters demonstrated accuracy (99.89-100.11 %), precision (RSD <2 %), and sensitivity with LOD and LOQ of 0.57 µg/mL and 1.69 µg/mL, respectively. Transferosomes prepared using the thin-film hydration method exhibited a mean particle size of 190.4±1.6 nm, PDI of 0.324, and zeta potential of -22.4 mV. The entrapment efficiency was found to be 86.24 ± 1.61 %. FTIR, DSC, and SEM confirmed successful drug incorporation and vesicle formation. The transferosome exhibited sustained release (93.6 ± 0.9 % at 24 h) and Korsmeyer-Peppas kinetics. Hence, the developed system represents a promising platform for transferosome-loaded quercetin assessment.

Keywords: quercetin, transferosomes, drug delivery, molecular docking, RP-HPLC, method validation, anti-inflammatory activity, skin disorders

1 Introduction

Quercetin is a naturally occurring flavonoid widely distributed in fruits and vegetables [1]. It is known for its anti-oxidant, anti-inflammatory, and immunomodulatory properties. Due to these activities, quercetin has attracted considerable interest for applications in inflammatory skin conditions. However, its practical use is limited by poor aqueous solubility, low stability, and inadequate skin permeability [2]. Topical delivery offers a direct and non-invasive route for the management of localized skin disorders. However, effective delivery of poorly soluble compounds like quercetin through the skin remains a challenge. The stratum corneum works as a strong barrier, restricting drug permeation and reducing therapeutic efficiency. Therefore, there is a need for carrier systems that can improve drug solubility and enhance skin permeation.

Transferosomes are ultra-deformable vesicular systems composed of phospholipids and edge activators [3]. These vesicles could easily pass through the narrow pores of the skin due to their flexible membrane structure. The presence of surfactants destabilizes the lipid bilayer, allowing the vesicles to deform without

losing integrity. This property makes transferosomes suitable carriers for transdermal and topical drug delivery [4].

In addition to formulation development, understanding molecular interactions between drug molecules and biological targets is important. Molecular docking provides insight into binding affinity and interaction patterns, supporting the potential biological activity of the compound [5]. Furthermore, reliable analytical methods are required for accurate quantification of the drug during formulation and evaluation [6]. Although several studies have reported quercetin-loaded transferosomal formulations for topical and transdermal delivery, these investigations have primarily focused on formulation development and biological evaluation. In most of these studies, drug quantification has been performed using UV spectrophotometric methods. However, a formulation-specific HPLC method validated in accordance with ICH guidelines for quantitative estimation of quercetin encapsulated within transferosomes has not been adequately reported. Such a method offers reliable quantification with high specificity in the presence of formulation excipients and supports routine analytical evaluation of transferosomal formulations [6]. Therefore, the present study integrates formulation development, physicochemical characterization, validated RP-HPLC method development, and molecular docking to provide a thorough analytical and formulation approach for quercetin-loaded transferosomes.

2 Experimental

2.1 Materials

Quercetin was obtained from Xi'an Herb Bio-tech Co. Ltd (Xi'an City, China). Phospholipon ® 90G was procured from Lipoid (Ludwigshafen, Germany). Edge activator (Tween 80) was purchased from Sigma-Aldrich (USA). Chloroform, methanol, and acetone were obtained from HiMedia Laboratories (Mumbai, India). HPLC-grade methanol, acetonitrile, and water were used for chromatographic analysis (Sisco Research Laboratories Pvt. Ltd., Mumbai, India). Double-distilled water was prepared in-house and used throughout the study. All other chemicals and reagents used were of analytical grade and utilized without further purification.

2.2 *Methods*

2.2.1 In silico Molecular Docking Analysis

Molecular docking analysis was performed using Schrödinger Maestro software (Ver. 13.3) with default parameters to investigate the binding affinity and interaction profile of quercetin, hydrocortisone, and tacrolimus with selected protein targets relevant to inflammatory pathways. Hydrocortisone and tacrolimus were selected as reference drugs because they are widely used in the clinical management of localized skin disorders [7]. The two-dimensional (2D) structure of quercetin was retrieved from PubChem. The X-ray crystal structures of target proteins, including human IgE-Fc bound to its high-affinity receptor FC Epsilon RI (PDB: 2Y7Q), the NF-kB crystal structure of a higher-order complex of p50 (PDB: 3GUT), and TSLP, the pro-inflammatory cytokine (PDB: 5J11) were obtained from the RCSB PDB (Protein Data Bank: https://www.rcsb.org/). Protein structures were selected based on high resolution and acceptable R-free values [8]. Protein preparation (bond-order assignment, water-molecule removal, hydrogen-atom addition, etc.) was performed using the Protein Preparation Wizard in Maestro. Missing residues were corrected, followed by restrained energy minimization. Ligand preparation was performed using LigPrep, and the structures were energy minimized to generate the most stable conformers. A receptor grid was generated by defining the active site region using site-mapping. Using the Glide module, docking simulations were conducted. Selection of optimal binding poses was done based on docking scores and interaction analyses. Protein-ligand interactions were studied using Maestro visualization tools.

2.2.2 HPLC Method Development and Validation

Instrument Set-up Parameters and Run Conditions

A PDA-coupled RP-HPLC system (Shimadzu DGU-20A5R, Kyoto, Japan) with a C-18 column (Shim-Pack Solar 4.6 I.D.×250 mm, 5 µm) was utilized to quantify quercetin. The instrument included a Model LC-20AD binary pump, SIL-20AC HT autosampler with LabSolutions software, CTO-10AS column oven, SPD-M40 detector photodiode array (PDA), and a degassing unit model no. DGU-20A5R. The detection wavelength was set at 369 nm. The mobile phase was made up of ACN and 0.3 % (v/v) o-phosphoric acid (OPA) in water in the ratio 50:50, delivered in isocratic mode at a flow rate of 1.0 mL/min. Before use, the solvents were passed through a membrane filter (pore size:0.45 µm) and degassing was done by sonication. The column temperature was maintained at 25°C. The runtime was kept to confirm complete elution of the analyte,

and the injection volume was set to 10 µL. All chromatographic analyzes were carried out under optimized conditions to achieve adequate resolution and peak symmetry [9, 10].

Preparation and Dilution of Analytes

The preparation of quercetin stock was done by precisely weighing an appropriate amount of the drug and dissolving it using methanol to get a sample of 1000 µg/mL concentration. Suitable serial dilution was done for the stock in order to prepare various working standard solutions. The various sample solutions were made by dissolving a precisely measured amount of the transferosomal formulation in methanol, followed by sonication to ensure complete extraction of the drug. Before use, the samples were passed through a membrane filter (pore size:0.45 µm). Appropriate dilutions were made with methanol to bring the concentration within the linearity range of the method [11].

2.2.3 Method Validation

The developed RP-HPLC method was validated in accordance with ICH guidelines with respect to linearity, accuracy, precision, sensitivity, specificity, robustness, and ruggedness [12]. Linearity was assessed by analysing several quercetin standard solutions at different concentrations. Calibration curves were generated from plots of peak area and concentration. The correlation coefficient (R2) was thus determined. Different concentration levels (e.g., 80%, 100%, and 120%) were made for accuracy studies, and the percentage recovery was calculated. Intra-day and interday precision were evaluated. The results were expressed as percentage relative standard deviation (%RSD). Using the curve slope in addition to the standard deviation of response, the limit of detection and limit of quantification values were calculated. To ensure the absence of interference at the retention time of quercetin, both blank and sample solutions were assessed. Robustness of the method was studied by making deliberate small changes in study conditions, including flow rate, constituents of the mobile phase, and wavelength, and observing their effect on the results. Ruggedness was determined by performing the analysis under different conditions, such as different analysts and instruments and evaluating the consistency of the results. System suitability was assessed by repeated injection of standard quercetin solution (n=6) under optimized conditions. Parameters such as retention time, theoretical plate count, tailing factor, and peak area reproducibility were evaluated to ensure adequate performance of the chromatographic system [13].

2.3 Preparation of Quercetin-loaded Transferosomes

Quercetin-loaded transferosomes (QCT-TF) were prepared using the thin film hydration method [14]. Briefly, 120 mg of Phospholipid (Phospholipon 90G) and 20mg of Tween 80 (lipid-to-edge activator ratio of 6:1 w/w) were dissolved in a chloroform-methanol mixture (2:1 v/v). Quercetin (10% w/w of total lipid) was added to the same organic phase. Under vacuum, the solvent was evaporated in a rotary evaporator (REMI Equipment Pvt. Ltd., India) at 40 °C and 100 rpm. This led to the formation of a thin film on the round-bottom flask, which was further dried overnight. The dried film was hydrated with 10 mL of phosphate-buffered saline (PBS, pH = 6.0) under continuous stirring. This resulted in the formation of multilamellar vesicles. The sample was then probe sonicated for three cycles of 5s each to reduce vesicle size and improve uniformity. The prepared transferosomes were stored under refrigeration until further use [15].

2.4 Characterization of Transferosomes

*2.4.1 Dynamic Light Scattering (DLS) Studie*s

The DLS principle was used to determine transferosome size, zeta potential, and homogeneity/ PDI (Horiba, Kyoto, Japan). Samples were appropriately diluted with distilled water before analysis. Measurements were carried out at room temperature, and each sample was analyzed in triplicate [16].

2.4.2 Scanning Electron Microscopy

For analysis of the surface architecture of the prepared transferosomes, scanning electron microscopy was performed (Leo Supra, Germany). Samples were placed on a suitable stub and allowed to dry. A thin film of palladium was coated on dried samples to improve their conductivity. The samples were then observed under the microscope to evaluate vesicle shape, surface characteristics, and structural integrity [17].

2.4.3 Molecular Analysis Using ATR-FTIR and DSC

The molecular compatibility and solid-state characteristics of quercetin in the optimised transferosomal formulation were investigated using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) and Differential Scanning Calorimetry (DSC) [4]. Comparative analyses were performed using pure quercetin, Phospholipon® 90G and thequercetin-loaded transferosomes to evaluate potential drug-excipient

interactions, changes in the characteristic functional groups, and alterations in the physical state of quercetin following encapsulation.

FTIR analysis was carried out using an ATR-FTIR spectrophotometer (Shimatzu Corporation, Kyoto, Japan). The spectra of all samples were recorded over the wavenumber range of 4000- 400 cm^{-1} at a spectral resolution of 4 cm^{-1} with 32 scans per sample. The characteristic absorption bands were compared to assess molecular compatibility and confirm successful incorporation of quercetin into the transferosomal vesicles.

Thermal analysis was performed using a Differential Scanning Calorimeter (DSC, Mettler Toledo, Switzerland). Approximately 5mg of each sample was weighed into standard aluminium pans and hermetically sealed, while an empty sealed aluminium pan served as the reference. The samples were heated from 40 to 450 °C at a heating rate of 10 °C/min under a nitrogen purge of 50 mL/min. The obtained thermograms were comparatively analyzed to evaluate changes in the melting behaviour and thermal transitions of quercetin following encapsulation within the transferosomal system.

2.4.4 Drug Entrapment

Entrapment efficiency (*EE*, %) of quercetin in transferosomes was tested by ultracentrifugation. The transferosomal dispersion was centrifuged at high speed to separate the free drug from the entrapped drug. The supernatant containing unentrapped quercetin was collected and analyzed using the developed HPLC method [18]. The total quantity of entrapped drug was calculated by deducting the free-form drug from the total drug added. All the experiments were performed in triplicate, and results were expressed as mean ± SD. Entrapment efficiency (*EE*, %) was computed using the following equation:

$$EE = \left(\frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \right) \times 100\,\%$$

2.4.5 In vitro Drug Release and Release Kinetics Studies

The in vitro release was carried out using a six-station Franz diffusion cell apparatus (Orchid Scientific & Innovative India Pvt. Ltd., India) with a receptor compartment capacity of 12 mL and an effective diffusion area of approximately 1.77 cm^2. A pre-soaked dialysis membrane (LA395, Dialysis membrane-110 AV; HiMedia Laboratories, Mumbai, India) was mounted between the donor and receptor compartments [6]. The receptor compartment was filled with phosphate-buffered saline (PBS, pH 7.4) and maintained at 37 ± 0.5°C under continuous magnetic stirring at 100 rpm. An accurately measured quantity of the transferosomal dispersion equivalent to 10 mg of quercetin was placed in the donor compartment. At predetermined time intervals (0.5, 1, 2, 3, 4, 5, 6, 10, 12, and 24 h), 2 mL aliquots were withdrawn from the receptor compartment. This was immediately replaced with an equal volume of fresh pre-warmed release medium to maintain constant volume and sink conditions. The collected samples were filtered through a 0.45 µm membrane filter and analysed using the validated RP-HPLC method described previously. Drug release from a plain quercetin suspension equivalent to 10 mg of quercetin in PBS (pH 7.4) was evaluated under identical experimental conditions for comparison. All experiments were performed in triplicate, and results were expressed as mean ± SD. To investigate the release mechanism, the cumulative drug release data were fitted to zero-order, first-order, Higuchi, and Korsmeyer-Peppas kinetic models. The corresponding correlation coefficients (R^2) were determined. The model showing the highest R^2 value was considered to best describe the release kinetics, while the release exponent (n) obtained from the Korsmeyer-Peppas model was used to predict the mechanism of drug release [15].

2.5 Statistical Analysis

All experiments were executed thrice, and the outcomes are discussed as mean ± standard deviation (SD) and relative standard deviation (RSD). GraphPad Prism version 8.0.2 (GraphPad Software Inc., San Diego, CA, USA) was used to apply statistics to the results. Differences between experimental groups were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test. A value of $p<0.05$ was considered statistically significant.

3 Results and Discussion

3.1 Molecular Docking Analysis

Molecular docking was carried out to evaluate the binding affinity and interaction characteristics of quercetin against clinically established anti-inflammatory agents, hydrocortisone and tacrolimus. Docking was done towards selected protein targets involved in inflammatory pathways. This included NF-kB (PDB

ID: 3GUT), TSLP (PDB ID: 5J11), and IgE (PDB ID: 2Y7Q). The calculated binding energies (**Table 1**) indicate that quercetin exhibits favourable binding across all targets with a clear preference for NF-kB. Quercetin showed the highest binding affinity toward NF-kB (-8.66 kcal/mol), followed by IgE (-7.22 kcal/mol) and TSLP (-6.542 kcal/mol). The more negative binding energy for NF-kB suggests a thermodynamically more stable complex arising from optimal complementarity between the ligands pharmacophoric features and the binding pocket [19,20]. Hydrocortisone exhibited binding energies of -6.54, -5.26, and -6.54 kcal/mol, and tacrolimus showed binding energies of -6.34, -5.29, and -6.34 kcal/mol against NF-kB, TSLP, and IgE, respectively. The comparatively lower binding energies of quercetin suggest stronger stability towards these inflammatory mediators than the reference drugs under applied docking conditions (Supplementary **Figures S1-S6**).

Table 1

Binding energy values of quercetin with selected protein targets

S. No	Ligand/Drug	Protein/Receptor	Docking score, kcal/mol
1.	**Quercetin**	**NF-kB (PDB ID: 3GUT)**	-8.66
2.	**Quercetin**	**TSLP (PDB ID: 5J11)**	-6.54
3.	**Quercetin**	**IgE (PDB ID: 2Y7Q)**	-7.22
4.	**Tacrolimus**	**NF-kB (PDB ID: 3GUT)**	-6.54
5.	**Tacrolimus**	**TSLP (PDB ID: 5J11)**	-5.26
6.	**Tacrolimus**	**IgE (PDB ID: 2Y7Q)**	-6.54
7.	**Hydrocortisone**	**NF-kB (PDB ID: 3GUT)**	-6.34
8.	**Hydrocortisone**	**TSLP (PDB ID: 5J11)**	-5.29
9.	**Hydrocortisone**	**IgE (PDB ID: 2Y7Q)**	-6.34

3.1.1 Binding Interaction Profile

The chemical structure of quercetin is presented in **Figure 1A**. The 3D and 2D interaction maps (**Figure 1**) reveal that quercetin engages in a network of hydrogen bonding and hydrophobic interactions owing to its polyphenolic scaffold. In NF-kB (**Figure 1a and d**), quercetin establishes multiple hydrogen bonds through its phenolic hydroxyl groups with polar and charged residues. These include lysine, glutamine, and arginine. The aromatic rings of quercetin were also involved in hydrophobic interactions with surrounding residues. This led to a stable conformation. The presence of multiple interaction points supports the higher binding affinity observed for this target. In comparison, both hydrocortisone and tacrolimus also interacted through hydrogen bonding and hydrophobic interactions but exhibited fewer favourable contacts, resulting in comparatively lower binding affinities.

For TSLP (**Figure 1b and e**), quercetin showed moderate binding affinity. The interaction profile indicated hydrogen-bonding interactions involving hydroxyl groups with residues such as serine and threonine. This was along with hydrophobic contacts with leucine and isoleucine residues. Although stable interactions were observed, the number and strength of interactions were comparatively lower than those seen with NF-kB. This might explain the reduced binding energy. The reference compounds formed stable interactions with residues including Ser68, Arg153, Thr77, and Asn85, although their scores remained lower.

In IgE (**Figure 1c and f**), quercetin demonstrated good binding affinity with a combination of hydrogen bonding and hydrophobic interactions. The hydroxyl groups participated in hydrogen bonding with polar residues. The planar aromatic structure facilitated hydrophobic interactions within the binding pocket. These interactions contribute to the stability of the ligand-protein complex. The interaction network is sufficient to stabilize the complex, but it appears less extensive than that observed for NF-kB. Although hydrocortisone and tacrolimus occupied the same binding pocket and established hydrogen bond residues such as Pro12, Ser93 and Glu95, their interaction networks were comparatively less extensive than those observed for quercetin [21].

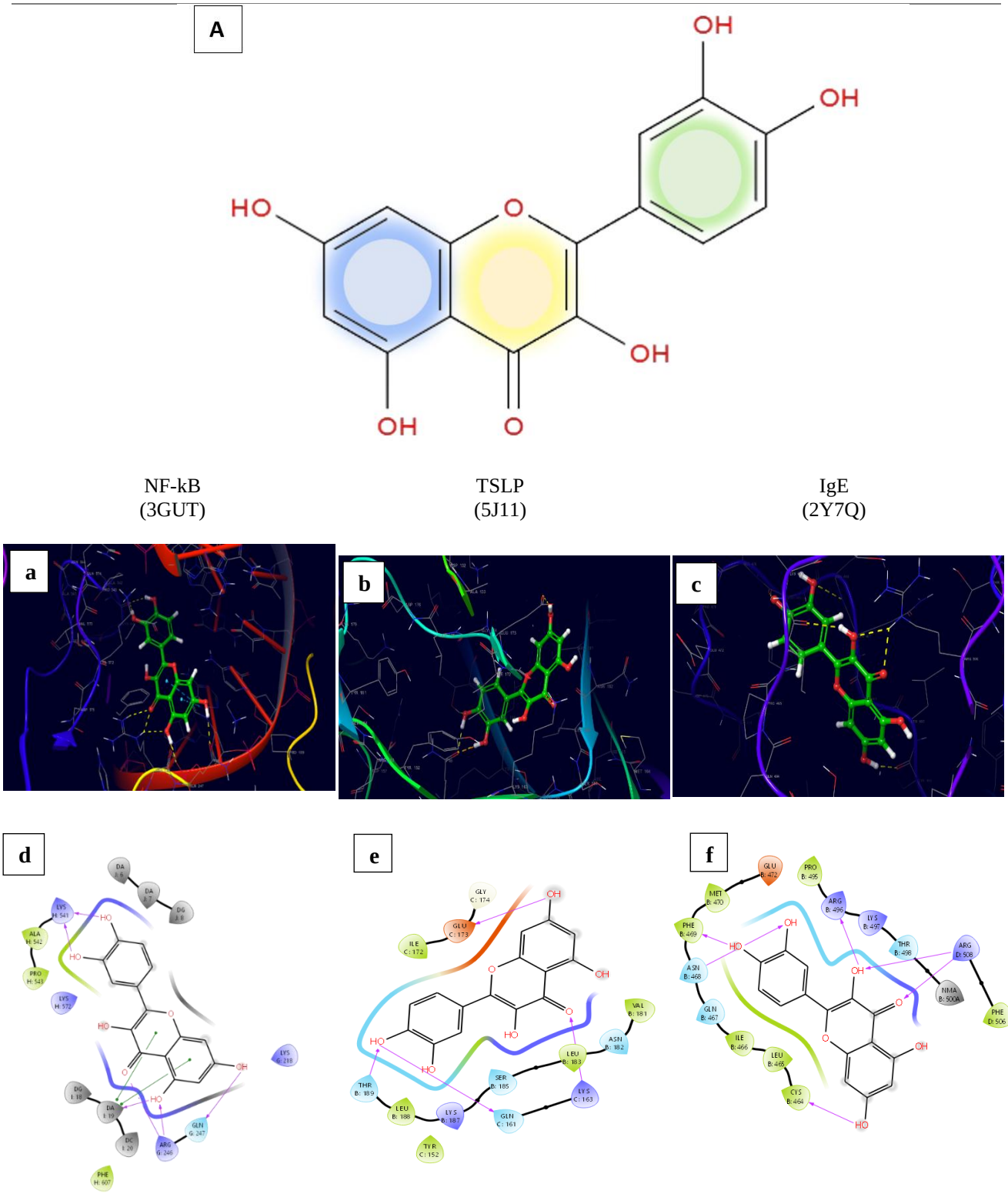

Figure 1 Structural representation and molecular docking interactions of quercetin with selected protein targets. The 2D chemical structure highlighting its functional groups is shown at the top (A). Molecular docking poses of quercetin within the active sites of (a) NF-kB (PDB ID: 3GUT), (b) TSLP (PDB ID: 5J11), and (c) IgE (PDB ID: 2Y7Q) are presented as 3D interaction views. Corresponding 2D ligand-protein interaction diagrams are shown in (d-f), illustrating hydrogen bonding and hydrophobic contacts between quercetin and key amino acid residues.

3.1.2 Pharmacophoric Considerations

The binding behaviour of quercetin could be rationalized based on its structural features. The phenolic hydroxyl groups (-OH) act as key hydrogen bond donors and acceptors, enabling strong polar interactions with amino acid residues. The carbonyl (C=O) serves as an additional hydrogen bond acceptor, enhancing interaction versatility. Due to its planar, aromatic nature, the molecule engages in hydrophobic and pi-pi stacking interactions. This facilitates stable accommodation within hydrophobic regions of the protein pocket. This combination of hydrogen bonding capacity and hydrophobic surface area represents a favourable pharmacophoric profile for interaction with protein targets involved in inflammatory signalling [22].

3.2 HPLC Method Development

3.2.1 Optimization of HPLC Method

The chromatographic conditions for the quantitative estimation of quercetin were systematically optimized to achieve adequate peak resolution, symmetry, and reproducibility. Several preliminary trials were performed. This included varying the mobile phase composition, solvent ratios, and chromatographic parameters. Initially, different ratios of aqueous phase (0.3% orthophosphoric acid) and organic phase (acetonitrile) were investigated. The mobile phase composition was systematically varied from 10:90 to 90:10 (v/v) in incremental ratios to achieve optimal separation. At higher aqueous compositions (>60%), quercetin exhibited increased retention with broadened and asymmetric peaks. This indicated strong interaction with the stationary phase. Conversely, higher organic content (>70% acetonitrile) resulted in rapid elution with poor peak resolution and reduced sensitivity. Optimization trials revealed that a 50:50 (v/v) ratio of 0.3% OPA (A) and acetonitrile (B) provided the most favourable chromatographic performance (**Figure 2**). Under these conditions, quercetin demonstrated a sharp and symmetrical peak with optimal retention.

3.2.2 Chromatographic Performance

Under the optimized conditions (mobile phase A:B = 50:50, flow rate 1.0 mL/min, injection volume 10µL, detection wavelength 369 nm), quercetin eluted at a retention time of 4.303 min (**Figure 2**). The peak was symmetrical and free from interference, indicating high specificity of the method. The chromatographic system exhibited excellent column efficiency, as evidenced by a theoretical plate count of 5941, which is significantly above the acceptable limit (>2000). This indicates efficient mass transfer and minimal band broadening during separation. Peak symmetry was evaluated using the tailing factor, which was found to be 0.887, indicating minimal peak distortion within the acceptance criteria.

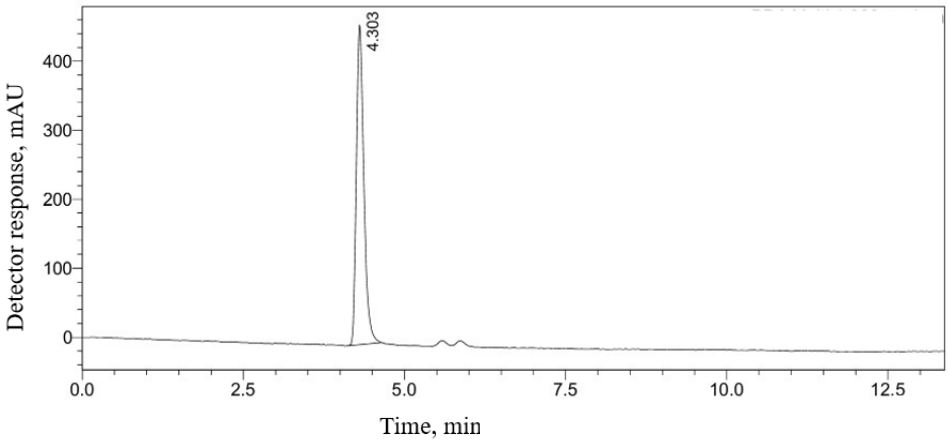

Figure 2. RP-HPLC chromatogram of quercetin under optimized conditions (0.3% OPA: ACN, 50:50 v/v), showing a sharp peak at 4.303 min with good peak symmetry and resolution. Chromatographic separation was achieved on a C18 column at a flow rate of 1.0 mL/min with detection at 369 nm

3.2.3 System Suitability and Method Reliability

The parameters for system suitability were evaluated prior to analysis to confirm the adequate working of the system under optimized conditions. A standard solution of quercetin was injected repeatedly (n=6), and key parameters, including retention time, theoretical plate count, tailing factor, and peak area consistency, were assessed. The chromatographic system produced a distinct and symmetrical peak for quercetin at a

retention time of 4.303 ± 0.01 min. Theoretical plate count was found to be 5941 ± 122, indicating satisfactory column efficiency, while the tailing factor (0.887 ± 0.03) confirmed acceptable peak symmetry. The consistency in retention time and peak area across replicate injections demonstrated stable system performance. These results show that the system was appropriate for subsequent method validation and quantitative analysis.

3.3 HPLC Method Validation

Method Validation of the optimized chromatographic conditions was done by following the ICH-guidelines. It was evaluated to ensure its suitability for analytical applications. The method was evaluated for key validation parameters indicating linearity, accuracy, precision, sensitivity, specificity, robustness, and ruggedness. Each parameter was assessed using appropriate experimental procedures under optimized chromatographic conditions. The results obtained were analyzed to confirm the reliability, consistency, and analytical performance of the method for the intended purpose [23].

3.3.1 Linearity and Range

The linearity of the developed RP-HPLC method for quercetin was evaluated over a concentration range of 20-120 µg/mL. Calibration standards were prepared at six concentration levels and analyzed under optimized chromatographic conditions. A strong linear relationship was observed between peak area and concentration, as depicted in the calibration curve (Figure 3). The regression equation was found to be:

$$y = 37567\,x - 15333\,.$$

The correlation coefficient (R^2) was found to be 0.998, indicating good linearity across the selected range. The linear response confirms that the process is fit for the quantitative assessment of quercetin within the studied concentration range. The corresponding calibration curve is provided in Supplementary **Figure S7**.

3.3.2 Sensitivity

The slope of the calibration curve and the standard deviation of the responses were used to determine the LOD and LOQ. Using the slope obtained from the regression equation (S= 37567) and considering the typical standard deviation of the response derived from calibration data, the LOD and LOQ were calculated as:

$$LOD = \frac{3.3\,x\,SD}{S}$$

$$LOQ = \frac{10\,x\,SD}{S}$$

The calculated LOD of 0.57 µg/mL and LOQ of 1.69 µg/mL directs method is sensitive enough to detect and quantify low concentrations of quercetin. Specifically, the low LOQ supports its application in routine analysis demanding high precision.

3.3.3 Accuracy

The developed RP-HPLC method's accuracy was evaluated using the standard addition technique at three concentration levels (80%, 100%, and 120%). Each level was analyzed in triplicate to assess the recovery of quercetin in the presence of the formulation matrix. The percentage recovery values obtained at all three levels ranged from 99.28 % to 100.96 % (**Table 2**).

Table 2

Accuracy data of quercetin determined by RP-HPLC using the standard addition method at 80%, 100%, and 120 % (n=3), expressed as mean % recovery ± SD and %RSD

S. No	Level, %	Amount added, µg/mL	Amount found, µg/mL	Recovery, %	Mean Recovery ± SD, %	RSD, %
1	80	80.0	79.42	99.28	99.89 ± 0.59	0.59
2		80.0	80.36	100.45		
3		80.0	79.95	99.94		
4	100	100.0	99.10	99.10	99.99 ± 0.86	0.86
5		100.0	100.82	100.82		
6		100.0	100.05	100.05		
7	120	120.0	118.96	99.13	100.11 ± 0.92	0.92
8		120.0	121.15	100.96		
9		120.0	120.28	100.23		

This indicates that the method is capable of accurately quantifying quercetin without interference from excipients present in the Transferosomal system. The %RSD values for all levels were observed to be less than 2%. The recovery values confirm the absence of significant matrix effects. This indicates that the method is both accurate and reliable for quantitative estimation of quercetin in the developed formulation.

3.3.4 Precision

The precision of the developed RP-HPLC method was evaluated in terms of intra-day (repeatability) and inter-day (intermediate precision) at three concentration levels. Each concentration was analyzed in triplicate under the same experimental conditions. For intra-day precision, the % RSD values were found to be 0.73, 0.77, and 0.76 % for 80, 100, and 120 µg/mL, respectively (**Table 3**). Similarly, inter-day precision showed %RSD values of 1.04, 0.98, and 0.94 % for the same concentrations. The observed %RSD values for both intra-day and inter-day studies were well below the acceptable limit of 2 %.

Table 3

Intraday and interday precision data for quercetin determined by RP-HPLC at 80, 100, and 120 µg/mL (n=3), expressed as mean % recovery ± SD and RSD

S. No	Concentration, µg/mL	Intraday Precision		Interday precision	
		Peak Area (Mean ± SD)	RSD, %	Peak Area (Mean ± SD)	RSD, %
1	80	2985911± 21850	0.73	2978450 ± 31200	1.04
2	100	3732389 ± 28950	0.77	3725600 ± 36800	0.98
3	120	4478867 ± 34200	0.76	4470200 ± 41950	0.94

3.3.5 Robustness

The robustness of the proposed method was investigated by deliberately applying slight changes to important parameters in chromatography (flow rate, mobile phase ratio, and detection wavelength). The influence of these variations on chromatographic responses such as retention time, peak area, tailing factor, and theoretical plate count was carefully analyzed. In all tested conditions, the RSD values for peak area remained below 2%, and the system suitability criteria were consistently met. These results indicate that the method maintains its performance even when minor adjustments are introduced (**Table 4**).

Table 4

Robustness evaluation of the RP-HPLC method under small deliberate variations in the chromatographic conditions, including Flow rate (mL/min), Mobile phase (OPA: ACN), and wavelength (nm)

Parameter	Condition	Retention time, min	RSD, %	Tailing factor	Theoretical plates
Flow rate, mL/min)	0.9 mL/min	5.82	0.88	1.21	5120
	1.0 mL/min	4.303	0.77	1.18	5941
	1.1 mL/min	3.965	0.95	1.23	5058
Mobile phase (OPA: ACN)	48:52	4.120	0.83	1.25	5190
	50:50	4.303	0.77	1.17	5283
	52:48	5.633	0.91	1.22	5210
Wavelength, nm	367	4.307	0.94	1.16	5220
	369	4.303	0.77	1.17	5285
	371	4.311	0.82	1.15	5175

3.3.6 Ruggedness

The method's ruggedness was evaluated by performing the analysis under varying conditions such as different analysts and on different days. The effect of these variations on chromatographic parameters was assessed. This included peak area, retention time (RT), and system suitability parameters. The RSD values obtained under all tested conditions were found to be less than 2%. The outcomes suggest that the method is rugged and reproducible irrespective of analysts or day-to-day experimental differences.

3.3.7 Specificity

The method's specificity was evaluated by analysing blank, standard, and sample solutions to assess potential interference from excipients and formulation components. The chromatograms obtained for the Transferosomal formulation showed no co-eluting peaks or interfering signals at the retention time of quercetin. A well-defined, sharp, and symmetrical peak was observed at the optimized retention time, indicating efficient chromatographic separation. The absence of interference from phospholipids, surfactants, or other excipients present in the Transferosomal system confirms that the method is highly specific for the detection and quantification of the drug. The validated RP-HPLC method demonstrated satisfactory analytical performance with respect to linearity, accuracy, precision, specificity, robustness and ruggedness. This supports its suitability for routine quantitative analysis and quality control of the developed transferosomal formulation.

3.4 Transferosome Evaluation

3.4.1 Dynamic Light Scattering (DLS) Studies

The particle size distribution of the optimized quercetin-loaded transferosome was determined using dynamic light scattering. The vesicles exhibited a mean particle size of 190.4 ± 1.6nm, indicating formation of nanosized carriers suitable for topical delivery (**Figure 3 a)**. The size is considered optimal for enhanced skin permeation. This is because nanoscale vesicles have the ability to efficiently traverse the stratum corneum. The obtained size falls within the range reported for previously developed quercetin-loaded transferosomal systems [6]. Minor differences in particle size among studies may be attributed to variations in phospholipid composition, edge activator concentration, drug-to-lipid ratio, and processing conditions as well as intended route of administration. The polydispersity index (PDI) values below 0.5 are generally indicative of uniform dispersion. Here, the PDI was found to be 0.324, suggesting a moderately narrow size distribution and acceptable homogeneity. This value is comparable with those reported by Pandit et al. [15] (0.333). The zeta potential was recorded as -22.4 mV (Figure 3 b). This reflects a stable colloidal system. The negative zeta potential observed in the present study is consistent with previously reported quercetin-loaded transferosomal systems [6, 15]. The negative surface charge may be attributed to the presence of phospholipids and edge activators. They contribute to the constant motion of the vesicles, thereby reducing aggregation via electrostatic repulsion [24].

3.4.2 Morphological Analysis (SEM)

The surface morphology of the optimized Transferosomal formulation was examined using scanning electron microscopy (SEM), as shown in **Figure 3 c**. The micrograph revealed the presence of a distinct vesicular structure with spherical morphology. The vesicles appeared well-formed with smooth surfaces and defined boundaries. The slightly deformed morphology is characteristic of ultra-flexible lipid-based carriers like transferosomes. The absence of crystalline drug deposits or sharp-edge particulates in the micrographs suggests that quercetin is well loaded [25, 26].

3.4.3 Molecular Analysis Using ATR-FTIR and DSC

The FTIR spectra of pure quercetin, Phospholipon® 90G, and the optimised quercetin-loaded transferosomes are presented in **Figure 3d**. Pure quercetin exhibited characteristic adsorption bands corresponding to the O-H stretching vibration (3275 cm^{-1}), aromatic C=C stretching (1605-1508 cm^{-1}), C=O stretching (1662 cm^{-1}), and C-O stretching vibrations within the 1310-1080 cm^{-1} region, confirming the identity of the drug. Phospholipon® 90G displayed its characteristic lipid peaks, including C-H stretching vibrations at 2917 and 2850 cm^{-1}, ester carbonyl (C=O) stretching at 1735 cm^{-1}, and phosphate-associated vibrations in the 1236-1061 cm^{-1} region [27, 28]. The FTIR spectrum of the optimised transferosomal formulation retained the characteristic adsorption bands of both quercetin and the phospholipid, with slight reductions in intensity and minor shifts in selected peaks. Importantly, no new adsorption bands or disappearance of characteristic functional groups were observed. This indicates the absence of chemical interactions between quercetin and the formulation excipients during transferosome preparation. These findings suggest that quercetin remained chemically stable following encapsulation and was successfully incorporated within the vesicles.

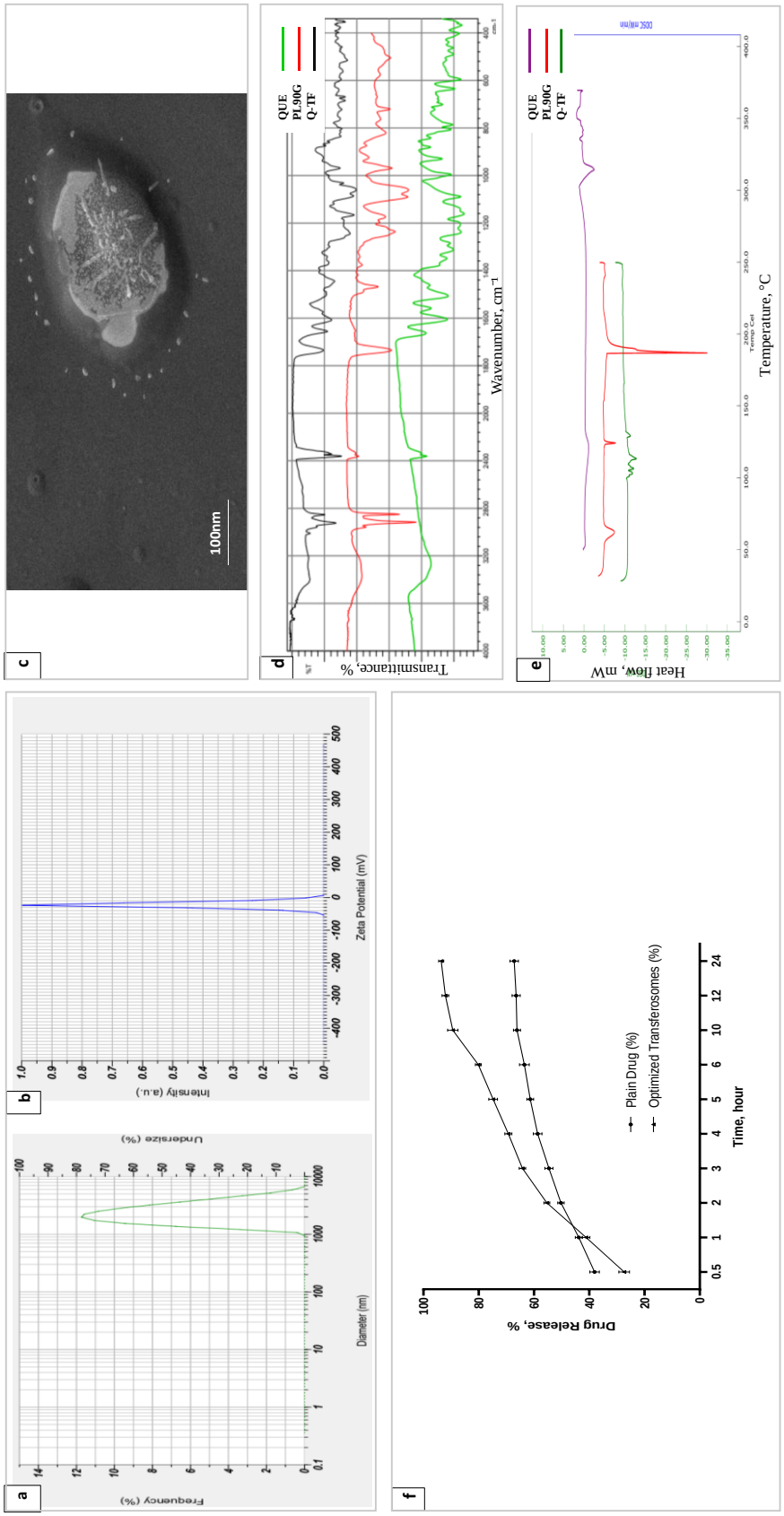

Figure 3 Physicochemical characterization of quercetin-loaded transferosomes: (a) particle size distribution, (b) zeta potential, (c) scanning electron microscopy (SEM) micrograph, (d) Fourier transform infrared (FTIR-ATR) spectra, (e) differential scanning calorimetry (DSC) thermograms, and (f) in vitro cumulative drug release profile.

The DSC thermograms of pure quercetin, Phospholipon® 90G, and the transferosomal formulation are shown in **Figure 3e**. Pure quercetin exhibited a distinct sharp endothermic peak at approximately 314^{o}C. This corresponds to its melting point and confirms its crystalline nature. In contrast, Phospholipon® 90G showed a characteristic endothermic transition around 187^{o}C. This represents the thermal behaviour of phospholipids. The DSC thermogram of the transferosome showed the absence of the characteristic melting endotherm of crystalline quercetin, while the thermal transitions associated with the lipid matrix remained evident [29]. The disappearance of the quercetin melting peak indicates that the drug was no longer present in its crystalline state but was molecularly dispersed or entrapped within transferosomes. Also, no additional thermal events indicative of drug degradation or incompatibility were observed.

3.4.4 Entrapment Efficiency

The entrapment efficiency (EE, %) of quercetin within the Transferosomal vesicles was determined using a previously developed and validated RP-HPLC method. Following ultracentrifugation, the supernatant containing unentrapped drug was collected. It was then quantified at 369nm under optimized chromatographic conditions. The entrapment efficiency was found to be 86.24 ± 1.61 %, indicating incorporation of quercetin into the lipid bilayer. The high entrapment could be attributed to the lipophilic nature of quercetin [30, 31]. The high entrapment efficiency achieved in the present study is comparable with values reported for quercetin-loaded transferosomes in the literature. Differences in drug loading and encapsulation efficiency may be influenced by lipid concentration, drug-to-lipid ratio, and preparation methodology [4,6,15].

3.4.5 In vitro Drug Release

The cumulative in vitro release profiles of plain quercetin and the optimised quercetin-loaded transferosomes are presented in **Figure 3f**. The plain drug exhibited a relatively rapid initial release, with approximately 38.1 ± 1.7% released within the first 30 min. This was followed by a gradual plateau, reaching 67.2 ± 1.5% cumulative release after 24 h. The limited release of the plain drug could be attributed to the poor aqueous solubility of quercetin. In contrast, the transferosomal formulation exhibited a characteristic biphasic release profile consisting of an initial burst release followed by a sustained release phase. Approximately 27.4 ± 1.9% of quercetin was released within the first 30 min, increasing to 80.2 ± 1.1% after 6 h. The cumulative drug release reached 93.6 ± 0.9% after 24 h, which was significantly higher than that observed for the plain drug ($p<0.05$). The reduced initial burst release from the transferosomes indicates efficient encapsulation of quercetin within the phospholipid bilayer. The subsequent sustained release could be attributed to the gradual diffusion of the drug through the hydrated lipid membrane. The incorporation of Tween 80 as an edge activator likely enhanced membrane flexibility and permeability. This facilitates controlled drug diffusion while maintaining vesicular integrity. In addition, the nanosized vesicles provide a large surface area that promotes efficient interaction with the release medium and improves drug dissolution [32].

3.4.6 Release kinetics

The release data were fitted to various kinetic models to investigate the mechanism of drug release from the developed transferosomes. Among the evaluated models, the Korsmeyer-Peppas model exhibited the highest correlation coefficient (R^2 =0.980), indicating that this model best describes the release behaviour of quercetin from the transferosomal system. The Higuchi model also demonstrated a good correlation (R^2 =0.923), suggesting that diffusion through the hydrated phospholipid matrix plays a significant role in drug release. The calculated release exponent (n=0.41) indicates Fickian diffusion. This demonstrates that quercetin release is primarily governed by concentration-driven diffusion from the lipid bilayer rather than by erosion or structural relaxation of the vesicles. The comparatively lower R^2 values obtained for the zero-order (0.611) and first-order (0.807) models further support that the release does not follow constant rate kinetics but is predominantly diffusion-controlled [33].

4 Conclusions

The present study successfully established and validated a robust RP-HPLC method for the quantitative assessment of quercetin. The analytical method demonstrated excellent linearity, accuracy, precision, and specificity, confirming its suitability for routine analysis. The optimized transferosomes exhibited nanoscale particle size, moderate polydispersity, and a negatively charged surface. FTIR and DSC analyses confirmed the compatibility and suggested successful incorporation of the drug. High entrapment efficiency confirmed effective incorporation of the drug within the vesicular system. Morphological analysis further supported the formation of deformable lipid vesicles. Additionally, molecular docking studies provided preliminary mech-

anistic insights into the interaction of quercetin with key inflammatory targets. The formulation exhibited sustained drug release over 24 h with release kinetics best described by the Korsmeyer-Peppas model. In conclusion, the study offers a promising strategy for enhanced topical delivery of quercetin that warrants further *ex vivo* and pre-clinical evaluations.

Supporting Information

The Supporting Information is available free at https://ejc.buketov.edu.kz/ejc/article/view/740/416

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Natasha Sudhir Akojwar** data curation, investigation, methodology, validation, visualization, writing-review & editing**; Nikhil Yadavrao Yenorkar** data curation, formal analysis, visualization**; Ayusha Omprakash Dondulkar** data curation, formal analysis, visualization**; Ankit Ganeshpurkar** data curation, investigation, visualization, writing-review & editing **Beauty Behera** data curation, formal analysis, resources, supervision; **Satyendra Kuldip Prasad** conceptualization, data curation, formal analysis, funding acquisition, resources, supervision, validation, writing-original draft, writing-review & editing.

Conflict of interest

The authors declare no conflict of interest.

Declaration

The authors declare that the AI-assisted tools were used only for language refinement and grammatical editing of the manuscript and not for data generation or analysis.

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