

Rapid and Stability-Indicating RP-HPLC Method for Taxifolin in Nanoethosomes: Development, Validation and Quality Control Application

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ABSTRACT

Purpose: Development and validation of a rapid, stability-indicating, reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of taxifolin (TAX) in nanoethosomal formulations and demonstrate its applicability in formulation evaluation.

Methods: The chromatographic analysis utilized a C18 column with an isocratic methanol–acetonitrile mobile phase and UV detection at 289 nm. Method validation was carried out by evaluating linearity, precision, accuracy, robustness, ruggedness, specificity, limit of detection, and limit of quantification. Stability-indicating capability of the method was confirmed by forced degradation studies under acidic, basic, oxidative, photolytic and thermal conditions. The validated method was applied to determine encapsulation efficiency and in vitro release of TAX from ethosomal formulations.

Results: The method showed satisfactory linearity, precision and recovery with high sensitivity and robustness against minor operational variations. The clear separation of degradation products from the TAX peak established the specificity of the developed method. Application to nanoethosomal formulations allowed reliable estimation of entrapment efficiency and drug release profiles.

Conclusion: The developed method is simple, rapid, precise, stability indicating and hence can be used for routine quality control of taxifolin loaded nanoethosomal formulations. To the best of our knowledge, this is the first validated HPLC method specifically developed for taxifolin within ethosomal formulations, filling an important analytical gap

Keywords: Taxifolin, RP-HPLC, Method validation, Nanoethosomes, Psoriasis, Quality control s

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INTRODUCTION

Taxifolin (TAX), also known as dihydroquercetin, is a natural compound that belongs to the class of flavonoids and occurs abundantly in different plant materials. There has been great interest in Taxifolin due to its various biological properties such as antioxidant, anti-inflammatory and others. TAX has been explored for its possible use in skin inflammatory diseases such as psoriasis. The pharmaceutical utility of TAX may however be hindered by its poor solubility in water and instability when exposed to certain environmental and chemical stresses. TAX has been

observed to be unstable under acidic, oxidative, and especially alkaline conditions (1, 2).

Quantitation of TAX in nanoethosomes must be accurate and precise for formulation optimization and stability assessment purposes. For quantitation of flavonoids, HPLC has been found to be among the most frequently utilized analytical methods due to its high sensitivity and specificity. Whereas several HPLC techniques for TAX have been developed, these have mostly been for plant extracts or standard solutions such as RP-HPLC and HPLC-PDA/UV techniques for taxifolin analysis in plant

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materials. Some of the methodologies utilized involve gradient elution techniques as well as lengthy chromatography operations, while stability-indication analysis using forced degradation techniques has been explored to a lesser degree. From the stability test by Stenger Moura et al., it can be seen that TAX is unstable in acidic, oxidative, and alkaline environments. Also, literature on the quantitation of TAX in liposomal drug delivery systems, for instance, ethosomes, is scanty, despite the possibility that formulation components might have an effect on the methodological procedure, thus affecting the accuracy of the analysis (2). Ethosomal drug delivery systems have phospholipids and a large amount of ethanol (3). In addition to these problems, the poor aqueous solubility and instability of TAX towards oxidation and hydrolysis call for an analysis technique which will have high sensitivity and selectivity (2). As far as we know, no stability-indicating HPLC method that is validated has been developed for the quantitative analysis of TAX from ethosomal formulations. It is for this reason that this study was undertaken.

2. Material and methods

2.1 Materials

All HPLC-grade solvents, including acetonitrile, methanol, and orthophosphoric acid, were obtained from Merck Pvt. Ltd., New Delhi, glacial acetic acid and orthophosphoric acid were obtained from Lobachemie Pvt. Ltd. Mumbai, India. The study utilized 0.45 μ m nylon membrane filters sourced from Whatman, UK. The Milli-Q water purification system installed in the laboratory was used to prepare distilled water. Soya phosphatidylcholine, absolute ethanol (99%) and propylene glycol were purchased from Sigma Aldrich Pvt. Ltd.

2.2 Analytical Instrumentation

Chromatographic analysis was carried out using a Shimadzu LC-20AD instrument (Shimadzu Corporation, Kyoto, Japan) with a gradient pump and photodiode array detection at 289 nm. This experiment was performed with a 20-microliter Hamilton syringe and an RP-HPLC Hyperclone C-18 column with a 20- μ L sample loop capacity. Data acquisition and system suitability were done through Lab solutions software.

2.3 Optimized chromatographic parameters

The amount of taxifolin was determined quantitatively by HPLC according to a modified method based on previously reported literature. For chromatographic separation, a Shimadzu C18 column (250 $\times$ 4.6 mm, 5 μ m particle size) was used, with detection at a wavelength of 289 nm. Standard and sample preparations were performed with a mobile phase of methanol and acetonitrile (45:55 v/v) in an isocratic mode. Before use, the mobile phase was degassed and passed through a 0.45 μ m nylon membrane filter. A 20 μ L injection volume was used at a flow rate of 1 mL/min, while the column temperature was maintained at 30 $^{\circ}$ C. Under these conditions, taxifolin showed a retention time of 2.551 min.

2.4 Selection of wavelength

A 100-ppm standard solution was prepared with the mobile phase. The UV absorbance spectrum of the solution was

measured from 190 to 800 nm. The sample was injected into the RP-C18 column using a Hamilton injector at a concentration of 20 μ g/mL. So, the maximum absorbance of taxifolin in the eluents was monitored by PDA detector working at 289 nm. Taxifolin purity peak index and a representative scan are shown in Figure 1.

2.5 Preparation of Standard and Sample Solutions

Initially, 10 mg of taxifolin was dissolved in the mobile phase in a 10 mL volumetric flask and diluted to volume with the same solvent to obtain a 1000 μ g/mL stock solution. For further dilution, 0.05, 0.1, 0.2, 0.3, and 0.4 mL portions of the stock solution were transferred separately into 10 mL volumetric flasks. Standard solutions at concentrations of 5, 10, 20, 30, and 40 μ g/mL were prepared by diluting the stock solution with the mobile phase to a final volume of 10 mL. The sample solutions were prepared for the study of in-vitro drug release and entrapment efficiency by developed RP-HPLC method (4).

2.6 Preparation of the Calibration Curve

For calibration, taxifolin solutions ranging from 5 to 40 μ g/mL were injected three times into the HPLC system, and the responses were recorded at 289 nm. The standard curve (Figure 1A) was plotted using the mean values of five measurements ($n = 5$). Linearity, accuracy, and precision of the calibration curve were evaluated through intra-day and inter-day validation studies.

2.7 RP-HPLC Method Optimization

The RP-HPLC method was systematically optimized for the accurate estimation of taxifolin. Various mobile phase compositions were tried, one of which was a mixture of water acidified with 2% v/v glacial acetic acid and acetonitrile (ACN) in 50:50 ratios which produced an irregular peak. The use of 0.1% orthophosphoric acid and acetonitrile (50:50 v/v) led to a longer retention time and poor peak symmetry. As presented in Table 8, the 45:55 (v/v) methanol-ACN composition resulted in optimal chromatographic parameters. Representative chromatograms of taxifolin are shown in Figure 2F and 2G

2.8 Method Validation Studies

The analytical method was validated according to the ICH Q2A and Q2B guidelines (4–7) using parameters such as linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection (LOD), and limit of quantification (LOQ) (5–8).

2.8.1 Specificity

Method specificity was assessed by analyzing taxifolin in nanoethosomes to confirm the absence of interference from excipients and degradation products. Potential interference at the analyte retention time was evaluated. The method specificity was assessed under hydrolytic and oxidative stress conditions to check for interference from degradation products.

2.8.2 System suitability parameters

System suitability was assessed through five replicate injections of a 10 μ g/mL taxifolin solution. The aim of the test was to assess the correct functioning of the analytical system and the reproducibility of the results (9). System suitability was determined by evaluating retention time, theoretical plates, peak resolution, HETP, and tailing factor (8).

2.8.3 Linearity Assessment

A series of taxifolin solutions (5–40 µg/mL) was prepared from the 1000 µg/mL stock solution to assess method linearity by HPLC. Each prepared solution was subjected to three injections of 20 µL each. The calibration curves were generated by plotting concentration versus peak area, followed by determination of the regression equation, slope, intercept, and R^2 value (8).

2.8.4 Accuracy Assessment

The developed method was evaluated for accuracy using the standard addition technique. This was done by spiking with pure drug at three different levels of concentration 80%, 100% and 120% in triplicates. The guidelines prescribed by ICH were followed. The peak area was used to obtain the remaining concentration percentage recovery and percentage RSD by using linearity equation. For each concentration, six replicate samples ($n = 6$) were prepared (8).

2.8.5 Precision

Precision was evaluated through repeatability and intermediate precision studies, and the results were expressed as relative standard deviation (%RSD). The method was tested with three selected concentrations 20, 30 and 40 µg/mL which were within the linear range. The intraday precision was determined on the similar day, although the interday precision was determined on three different days, with each test being performed in triplicate. The peak areas were then recorded for each concentration (8).

2.8.6 LODs and LOQs

The limit of quantification and limit of detection were calculated from the slope and deviation of the peak areas on the calibration curve, and were used to determine the sensitivity of the analytical method (8, 10).

2.8.7 Robustness

The method was tested for robustness by varying the flow rate, detection wavelength, and mobile phase ratio. The outcome of such variations was studied on the determinations of the drug as well as tailing factor and theoretical plates. When the percentage RSD is less than 2, the method is robust.

2.8.8 Ruggedness

Three analysts performed the analysis, and the peak locations were compared to assess robustness. The robustness of the procedure was indicated by the %RSD of the procedure, which was less than 2%.

2.9 Force degradation studies

The bulk samples were subjected to acidic, basic, oxidative damage, and photo and thermal degradation as per the ICH strategies to evaluate the stability and degradation of taxifolin. The aim was to assess the ability of the method to correctly quantify the analytes, taking into account the possible degradation products.

2.9.1 Degradation of acids and bases

Taxifolin solution (1 mg/mL, 1 mL) was added to 9 mL of 0.1 N sodium hydroxide or hydrochloric acid and heated under reflux for 2 h in a boiling water bath. The sample was cooled to room temperature and an equal volume of base or acid added to neutralise the solution. The solution was then

diluted to a concentration of 20 µg/mL, and the analysis was conducted via the described procedure.

2.9.2 Oxidative degradation

The drug solution (1 mg/mL, 1 mL) was treated with 9 mL of 30% hydrogen peroxide and refluxed for 2 h in a boiling water bath. After cooling and dilution to 20 µg/mL, a 20 µL aliquot was injected into the HPLC system.

2.9.3 Degradation by heat

The drug was subjected to thermal stress at 70 °C for 48 h. After cooling, 10 mg of the treated drug was dissolved in 10 mL of water to obtain a 1 mg/mL solution, followed by dilution and HPLC analysis using a 20 µL injection volume.

2.9.4 Photolytic degradation

The drug was treated with UV light (254 nm) for 48 h. The sample was prepared at a concentration of 1 mg/mL in 10 mL of Millipore water and filtered using a syringe filter. Afterwards UV exposure the solution was diluted to 20 µg/mL and analysed by advanced HPLC.

2.10 Application of the validated analytical method

Taxifolin loaded nanoethosomes were quantified using validated HPLC method. The developed nanoethosomes were subjected to percentage entrapment efficiency and cumulative drug release studies.

2.10.1 Preparation of taxifolin-loaded nanoethosomes

Nanoethosomes were prepared by simple cold technique as informed by Touitou et al. In brief, 3% of soy lecithin and 40 mg of drug were dissolved in 40% ethanol (v/v) in a totally closed vessel with magnetic stirrer at 30°C. Stirring was done by dropwise addition to the mixture of propylene glycol and agitation water from a separate vessel heated to 30°C with subsequent probe sonication for 5 min. The whole blend was agitated for 15 min at 700 rpm. The nanoethosomes were prepared by spontaneous (11-13).

2.10.2 Characterization of the prepared nanoethosomes

The prepared taxifolin-loaded nanoethosomes were evaluated for particle size and polydispersity index using a Zeta Sizer (Nano-ZS, Malvern, UK) at 25 °C and a detection angle of 90°(14). Morphology was envisioned by transmission electron microscope (TEM) (Philips CM 12 electron microscope), using a 100 kV stimulating voltage.

2.10.3 Entrapment efficiency:

The suggested procedure was used to calculate the taxifolin entrapment efficiency (EE) of the nanoethosomes. This method was derived from a previously published method with minor adjustments, in which an ultracentrifuge was used to centrifuge 2 mL of the ethosomal suspension for 20 minutes at a temperature of 4°C and 13,000 rpm. The supernatant (1 mL) was diluted to 10 µg/mL, filtered using a 0.45 µm syringe filter, and analyzed by HPLC at 289 nm (15).

Furthermore, the concentration of taxifolin in the nanoethosomes was assessed by preparing dilutions with the mobile phase, followed by analysis to control the total drug amount in the formulation. The entrapped taxifolin and overall drug content were quantified via HPLC with a detection wavelength of 289 nm, referencing a standard calibration curve.

2.10.4 *In vitro* drug release

Taxifolin release from the prepared nanoethosomes was determined using the dialysis membrane method(16). The

10mL nanoethosomal suspension was sealed in a dialysis membrane-60. It was placed in 800 mL of phosphate buffer (pH 7.4) and maintained at 37 °C with continuous stirring at 200 rpm. During the 24 h study, 1 mL of sample was periodically withdrawn and replaced to maintain sink conditions. The amount of taxifolin in the sample was determined by the well-established method of HPLC. A graph was made using the computed percentage of cumulative drug use (17).

3. Results and Discussion

3.1 Method development

This method was developed with an aim to obtain rapid, sensitive and reproducible quantification of taxifolin in nanoethosomal formulations. Different combinations of mobile phase were tried to improve the retention time, shape of the peaks and resolution. A mixture of

methanol:acetonitrile (45:55, v/v) provided symmetrical peaks, less tailing and a short retention time of 2.55 min which was better than other compositions tested. The chosen isocratic elution also facilitated analysis and decreased solvent consumption in comparison to previously reported gradient methods.

3.2 Method validation

3.2.1 Validation of System Suitability and Specificity

The chromatographic system was tested for suitability and reproducibility before sample analysis. The method showed acceptable values for tailing factor, theoretical plates, retention time, and regression coefficient, as specified by ICH Q2(R1) (Table 1). The method showed specificity, with no interference from excipients or degradation products at the taxifolin retention time. These results demonstrate the suitability of the method for stability assessment and analysis of complex matrices.

Table 1. System suitability parameters for taxifolin quantification by RP-HPLC

Parameter	Acceptance criteria*	Observed value	Compliance
Tailing factor	≤ 2.0	0.99	Yes
Theoretical plates (N)	≥ 4000	4904	Yes
Retention time (min)	Consistent ($\pm 2\%$)	2.55	Yes
Regression coefficient (R^2)	≥ 0.999	0.9995	Yes

3.2.2 Linearity, Accuracy, and Precision

A strong linear relationship was observed over the concentration range of 5–40 $\mu\text{g/mL}$, as indicated by the regression equation $y = 10288x - 43246$ and an R^2 value of 0.9995. The %RSD values for peak areas were within the acceptable limits with minimum variation, representing the accuracy and precision of the method in the tested range (Table 2). The method accuracy was checked by the standard addition technique at three concentration levels (80%, 100% and 120% of the nominal concentration). Percentage salvages were in the range of 98.75 to 99.70 % and the tailing factors were found to be near to 1.0 and the

counts of the theoretical plates were more than 4000 which were in accordance with the criteria of ICH Q2(R1) (Table 3). The established RP-HPLC method was validated for precision by intra-day and inter-day variability at three different concentration levels (20, 30 and 40 $\mu\text{g/mL}$). The %RSD for intraday studies was found to be between 0.211% to 0.944% and for interday studies was between 0.189% to 0.948 % which is below the limit of 2% as recommended by ICH Q2(R1). Retention times, tailing factors and theoretical plate counts were found to be consistent in all replicates, which confirm the high repeatability and intermediate precision of the method (Table 4).

Table 2. Linearity of calibration curve

Concentration ($\mu\text{g/mL}$)	Peak area
5	9162 ± 1.528
10	58469 ± 3.512
20	159996 ± 2.516
30	270597 ± 1.528
40	365838 ± 1.0

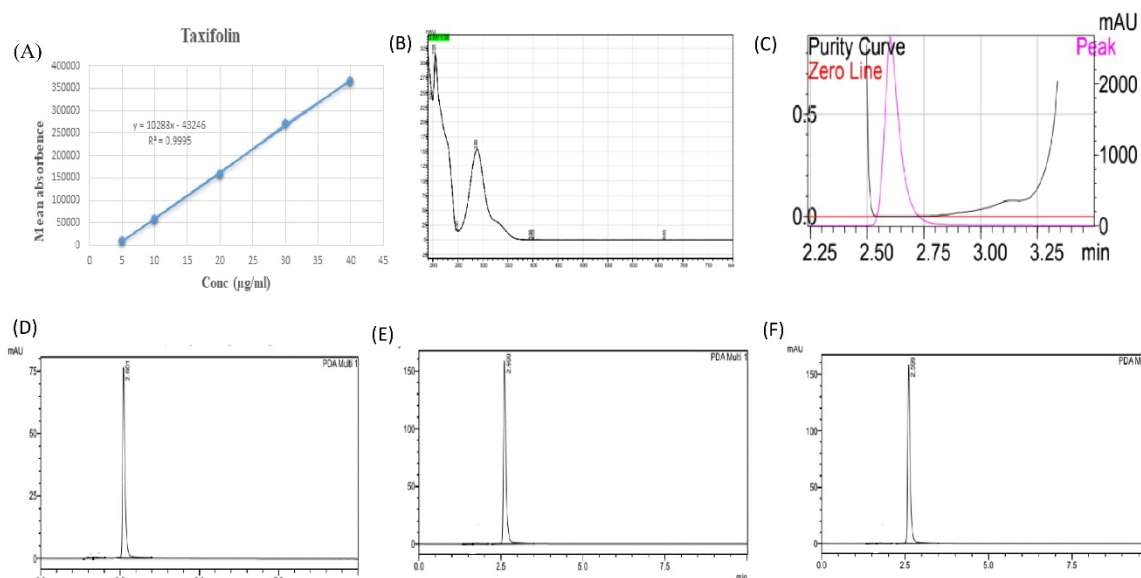


Figure 1. A) Calibration curve of taxifolin B) Spectrum of Taxifolin at 289 C) Peak purity at zero-line D) Chromatogram at 288 nm E) Chromatogram at 289 nm F) Chromatogram at 290 nm

Table 3. Accuracy studies of Taxifolin

Nominal concentration (µg/mL)	Amount added (µg/mL)	% Recovery level	Recovery (%)	Tailing factor	T. plates	% RSD
20	16	80	98.75	0.94	4904.50	0.51
20	20	100	99.4	0.99	5010.31	0.49
20	24	120	99.7	0.98	4883.42	0.67

All values are presented as mean ± SD (n = 3).

Table 4. Precision studies for taxifolin quantification by RP-HPLC

Precision type	Sample concentration (µg/mL)	Mean peak area (± SD)	Rt (min)	Tailing factor	Theoretical plates (N)	%RSD
Intraday	20	155445 ± 120	2.535	0.989	4543.74	0.835
	30	270490 ± 135	2.596	0.838	4353.57	0.5
	40	369676 ± 142	2.56	0.936	4384.29	0.495
	20	159447 ± 128	2.54	0.839	4677.47	0.349
	30	270795 ± 140	2.531	0.935	4578.85	0.444
	40	364500 ± 132	2.539	0.939	4347.68	0.211
	20	157880 ± 118	2.501	0.739	4845.67	0.486
	30	278386 ± 137	2.53	0.928	4873.55	0.944

	40	363963 ± 125	2.582	0.936	4336.02	0.284
Interday	20	154600 ± 130	2.535	0.989	4543.74	0.787
	30	279871 ± 145	2.596	0.838	4353.57	0.948
	40	369100 ± 150	2.56	0.936	4384.29	0.462
	20	158762 ± 122	2.54	0.839	4677.47	0.537
	30	271824 ± 139	2.531	0.935	4578.85	0.516
	40	367103 ± 148	2.539	0.939	4347.68	0.189
	20	157860 ± 127	2.501	0.739	4845.67	0.25
	30	272287 ± 142	2.53	0.928	4873.55	0.432
	40	360954 ± 133	2.582	0.936	4336.02	0.651

All values are presented as mean ± SD (n = 3). %RSD = Relative standard deviation.

Table 5. Robustness analysis

Condition	Level	Mean peak area (± SD)	Rt (min)	Tailing factor	Theoretical plates (N)	%RSD
Flow rate (mL/min)	0.8	264380 ± 265	2.475	0.923	4543.45	0.1
	1	279870 ± 280	2.534	0.921	4353.87	0.1
	1.2	265571 ± 266	2.599	0.976	4384.34	0.1
Wavelength (nm)	288	269351 ± 269	2.589	0.946	4677.78	0.1
	289	274696 ± 275	2.529	0.843	4578.82	0.1
	290	260974 ± 261	2.503	0.974	4347.23	0.1
Mobile phase ratio	50:50:00	264871 ± 265	2.563	0.673	4845.32	0.1
	55:45:00	270764 ± 271	2.559	0.987	4873.73	0.1
	60:40:00	263568 ± 264	2.434	0.897	4336.11	0.1

All values are presented as mean ± SD (n = 3).

3.2.3 Sensitivity, Robustness, and Ruggedness

The method was found to be sensitive with LOD and LOQ values of 1.194 µg/mL and 3.62 µg/mL, respectively, which enabled the reliable detection of low drug concentrations. Robustness (deliberate changes in flow rate, wavelength and mobile phase composition) was verified with %RSD < 2% which showed negligible effect on analytical performance (Table 6). The ruggedness test was performed for different analysts and gave consistent results, which supports the reliability of the method in different laboratory conditions.

Table 6. Ruggedness analysis

Analyst	Mean area under curve ($\pm$ SD)	Retention time (min)	Tailing factor	Theoretical plates (N)	%RSD
Analyst 1	278634 $\pm$ 225	2.419	0.834	4837.03	0.81
Analyst 2	273251 $\pm$ 45	2.55	0.678	4239.49	0.16
Analyst 3	270645 $\pm$ 173	2.47	0.478	3992.3	0.64

All values are presented as mean $\pm$ SD (n = 3).

3.2.4 Forced degradation study

Stress degradation studies were conducted using acidic, basic, oxidative, thermal and photolytic conditions. No significant degradation of taxifolin was observed under acidic, basic and oxidative stress conditions, and thermal and photolytic stress conditions resulted in additional minor peaks that were well resolved from the main peak (Table 7).

Table 7. % Recovery of Taxifolin under degradation condition

Degradation condition	Sample preparation	Percentage recovery ($\pm$ SD)
Acid hydrolysis	0.1 N HCl	94.67 $\pm$ 2.87
Alkaline hydrolysis	0.1 N NaOH	91.38 $\pm$ 1.98
Oxidation	30% H ₂ O ₂	100.56 $\pm$ 2.53
Thermal degradation	At hot air oven 70°C 48 hr.	90.22 $\pm$ 2.11
Photolytic degradation	Under UV 48 hr.	89.45 $\pm$ 2.46

All values are presented as mean $\pm$ SD (n = 3).

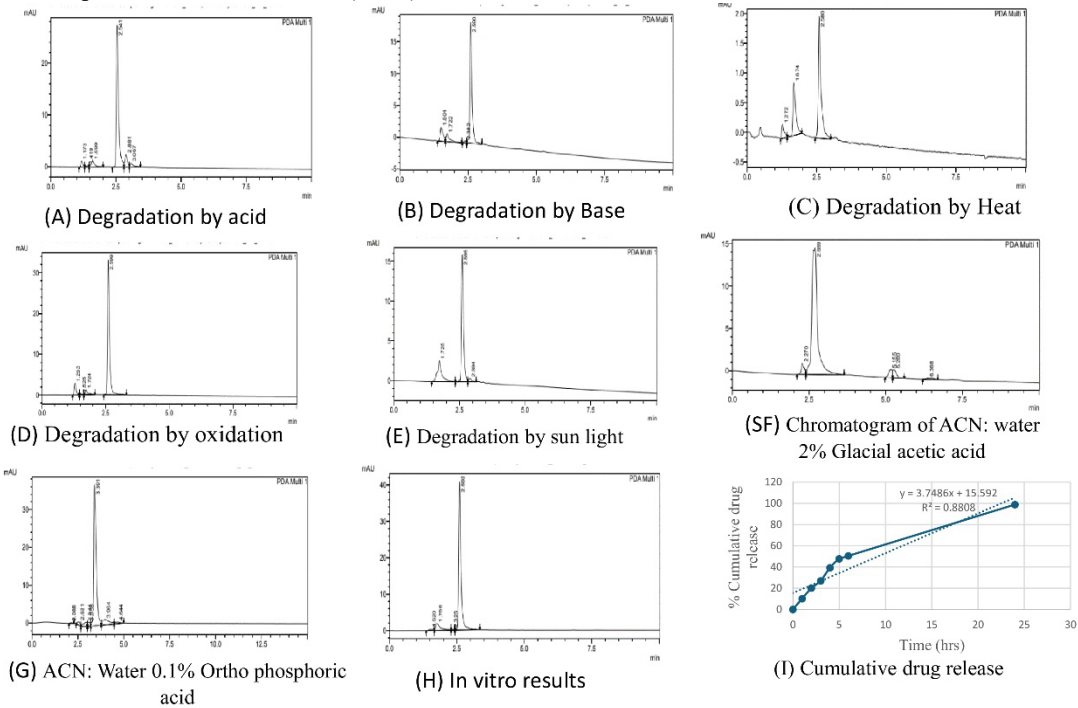


Figure 2. Chromatograms obtained from A) Degradation by 0.1 N NaOH B) by 0.1 N HCl C) by Heat D) by 30% H₂O₂ E) by sun light F&G) Chromatogram with trials mobile phases I) Cumulative drug release of taxifolin nanoethosomes

Table 8. Optimization of mobile phase

S. no	Trails taken	Observation	Remarks
1	ACN: water 2% Glacial acetic acid (50:50 v/v)	Irregular peak was obtained	Not satisfactory
2	ACN: water 0.1% Orthophosphoric acid (50:50 v/v)	Peak was found to be little asymmetrical and more retention time.	Not satisfactory
3	ACN: Methanol (55:45 v/v)	Good symmetrical peak	Satisfactory

3.3 Application to Nanoethosomal Formulation Evaluation

The established method was used to quantify taxifolin in nanoethosomal formulations. The entrapment efficiency was found to be 78.15 $\pm$ 1.34% and *in vitro* drug release

study demonstrated $98.86 \pm 2.11\%$ release within 24 h. The chromatograms obtained from these studies showed well resolved peaks with no interference indicating the effectiveness of the method for analyzing formulations

4. Comparison with Previously Reported Methods

Numerous chromatographic methods have been stated for quantification of taxifolin in plant extracts, biological matrices and commercial formulations. These include HPLC-UV, HPLC-PDA, UHPLC-MS and HPTLC techniques, using different stationary phases such as C18, CN and biphenyl columns and mobile phases from aqueous mixtures of phosphoric or trifluoroacetic acid to high ratios of methanol or acetonitrile. Although these techniques have shown satisfactory analytical performance, many of them present long retention times (up to 18-20 min.), complex gradient programs or depend on expensive and environmentally hazardous solvents.

The present method, in contrast, has a very short run time of only 2.5 min under simple isocratic conditions with methanol-acetonitrile mobile phase without the need of buffers or acidic modifiers and thus reduced solvent consumption and column maintenance problems. Forced degradation studies confirmed the stability-indicating nature of the method, as the degradation products were distinctly separated under acidic, alkaline, oxidative, thermal, and photolytic conditions. Another novel aspect is its direct application on nanoethosomal formulations to quantify taxifolin accurately for entrapment efficiency and drug release evaluation which was not dealt in earlier reports.

In addition to the previous reports, other stability-indicating HPLC methods for different flavonoids and nanocarrier systems have been reported very recently (2022–2025). Previous studies have described RP-HPLC methods for different pharmaceutical and botanical matrices. Mallya et al. (2024) used a gradient RP-HPLC method for curcumin and naringin in a co-amorphous system with retention times of 5–11 min, confirmed an HPLC-PDA method for phenolics in Pinus bark extract using buffer-containing mobile phases, (18) and used a sensitive method for a botanical matrix, not nanocarrier formulations (19). Carvalho et al. (2023) developed and validated an HPLC-DAD method for determining quercetin in synthetic nanoparticles, with a retention time of about 3.6 min and strong validation characteristics (20). Likewise, Chimagave et al. (2023) developed a stability-indicating RP-HPLC assay for hesperidin in nanotransfersomes (21), and Shamim et al. (2023) employed a Quality-by-Design framework for a bilosomal formulation containing ciprofloxacin and rutin (22).

Compared to these contemporary studies, the present method is distinguished by shorter run time (~2.5 min), buffer free binary mobile phase and direct application to taxifolin in nanoethosomes, a molecule especially susceptible to degradation. Unlike plant extract assays or gradient methods, this protocol is simple, fast and highly transferable to quality control laboratories. It is also not limited to quantification but to practical formulation parameters like entrapment efficiency and *in vitro* release.

This work provides a regulatory ready, cost-effective analytical platform that advances both phytopharmaceutical research and nanocarrier development by full compliance with ICH Q2(R2) guidelines for validation and by demonstrating stability-indicating capability under multiple stress conditions.

5. Discussion

In the present work, An RP-HPLC method offering simplicity and reliability was developed and validated for quantifying taxifolin in nanoethosomal formulations. The reported methods were mainly applied to plant extracts or bulk drug samples, whereas our method is faster (retention time ~2.55 min) and uses a simple isocratic mobile phase and follows the ICH Q2(R2) guidelines. These features make it economical, easy to reproduce and practical for routine quality control.

The validation results showed good performances. The method was linear with high correlation coefficient ($R^2=0.9995$), precise (% RSD < 1%) and accurate (recoveries of 98.75–99.7%). The stress studies showed that the method is stability-indicating as the degradation products were well resolved from the major TAX peak. This is important because flavonoids, such as taxifolin, are often unstable and can be degraded by light, heat, or acidic or alkaline conditions.

The method was successful in determining entrapment efficiency ($78.15 \pm 1.34\%$) and in following the drug release profile ($98.86 \pm 2.11\%$ within 24 h) when applied to TAX loaded nanoethosomes. This shows the sensitivity of the method to determine the important parameters of formulation. Therefore, the method can be used not only for analysis but also for formulation development and optimisation.

Our method is faster than previous HPLC methods, uses less solvent and does not require complex gradient elution systems. Previous studies reported longer retention times (> 6 min) and less convenient buffer systems for routine use. To our knowledge, this is the first HPLC stability-indicating method validated for taxifolin in ethosomal formulations. To conclude, the RP-HPLC method showed good simplicity, speed, accuracy, and stability-indicating performance. It can be employed in research and industry for routine quality control of taxifolin formulations and provides a valuable tool for further development of phytopharmaceutical nanocarriers.

6. Conclusion

The developed RP-HPLC method enabled rapid and reliable quantification of taxifolin in nanoethosomal formulations and demonstrated compliance with ICH Q2(R2) validation requirements. The method showed excellent sensitivity, precision, accuracy and robustness, with good separation of degradation products under forced-degradation conditions. Its applicability was verified by the reliable estimation of encapsulation efficiency and *in vitro* drug release. The present method is suitable for routine analysis and quality control of taxifolin based nanoformulations due to its simple mobile phase composition, stability-indicating property and short run

time. Importantly, this is the first validated HPLC method reported for taxifolin in ethosomal systems, providing a useful analytical tool for the advancement of phytopharmaceutical research and formulation development.

7. Conflict of Interest

The authors declare that there is no conflict of interest.

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9. Data Availability Statement:

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