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SIMULTANEOUS RP-HPLC QUANTIFICATION OF CURCUMIN, SILYMARIN, AND PIPERINE: METHOD DEVELOPMENT AND VALIDATION

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ABSTRACT

Background: Herbal formulations containing multiple phytoconstituents require analytical methods that can simultaneously determine structurally diverse compounds with adequate selectivity and precision. Curcumin (CUR), Silymarin (SLY), and Piperine (PIP) are widely incorporated into polyherbal preparations owing to their clinically studied pharmacological actions. Ensuring consistent quality of such formulations requires a validated, reliable chromatographic procedure. **Methodology:** A reverse-phase HPLC method was established for concurrent estimation of CUR, SLY, and PIP using an Agilent Zorbax Bonus RP column (250 × 4.6 mm, 5 µm). Separation was achieved with a mobile phase of 0.1% perchloric acid and acetonitrile (45:55, v/v) at 1.0 mL/min, with UV detection at 215 nm. The method was evaluated for specificity, precision, linearity, accuracy, and sensitivity using standard validation criteria aligned with internationally accepted analytical quality guidelines. **Results and Discussion:** The analytes were successfully separated within 11 minutes, with retention times of 4.09 min for Silymarin, 7.39 min for Piperine, and 10.25 min for Curcumin. System precision demonstrated %RSD values below 0.21% for all analytes. Recovery values across 80–120% concentration levels were close to 100%, with %RSD <0.5%, reflecting excellent accuracy. Calibration curves exhibited strong linearity (R^2 = 0.999). The method demonstrated high sensitivity with LOD values below 2 µg/mL and LOQ values below 6 µg/mL for each compound. Both intra-day and inter-day evaluations showed %RSD <0.4%, confirming robust reproducibility. Robustness testing with small, deliberate variations showed %RSD <1.5%, confirming the method's reliability. **Conclusion:** The developed RP-HPLC protocol offers a selective, reproducible, and sensitive analytical approach suitable for simultaneous quantification of SLY, PIP, and CUR in combined herbal matrices.

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INTRODUCTION

Herbal preparations often incorporate multiple bioactive phytochemicals that exert complementary therapeutic actions. Accurate quantification of these constituents is essential for ensuring batch-to-batch uniformity and maintaining product quality. Among the commonly used phytoconstituents, Silymarin, Piperine, and Curcumin have gained substantial attention due to their diverse biological effects and their frequent co-formulation in nutraceutical and herbal products [1-2].

Silymarin is a complex mixture of flavonolignans primarily isolated from *Silybum marianum* (milk thistle) [3]. Traditionally valued for hepatoprotective benefits, it has been reported to exhibit antioxidant, anti-inflammatory, and cytoprotective effects across various organ systems. Such actions are largely attributed to its ability to modulate oxidative stress and support endogenous antioxidant pathways.

Piperine, an alkaloid derived primarily from Piper nigrum and Piper longum, exhibits a distinctive pungency and plays pharmacological roles in metabolism, inflammation, and enhancing bioavailability. Its biopotential and absorption-modifying effects make it a frequent component in formulations containing other herbal actives [4].

Curcumin, the principal curcuminoid of *Curcuma longa*, is widely studied for its antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Its ability to interact with various molecular targets and modulate signaling networks contributes to its broad therapeutic profile.

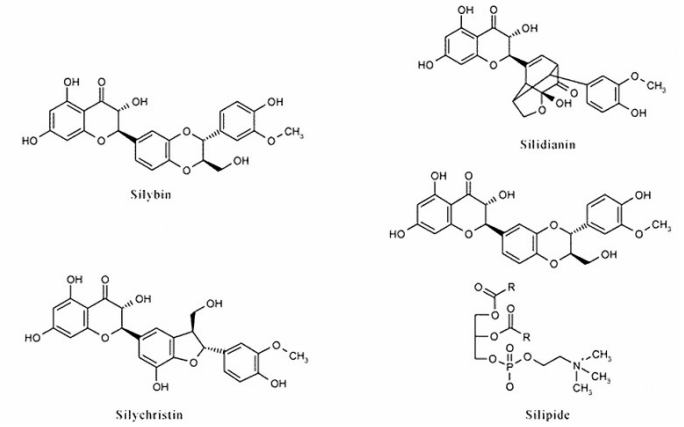

Figure 1: Chemical structure for Silymarin [6]

Although many analytical procedures individually quantify these compounds, fewer methods support the simultaneous determination of all three in a single chromatographic run [5]. A combined method is challenging due to substantial structural and polarity differences, requiring optimized conditions to achieve efficient separation, acceptable peak symmetry, and adequate resolution [6].

The present study aimed to develop a simple, rapid, and robust RP-HPLC method capable of accurately quantifying SLY, PIP, and CUR in blended samples. The method was systematically optimized and validated to establish its suitability for routine analytical applications in pharmaceutical and herbal product quality control (Figure 1).

Piperine is a naturally occurring alkaloid predominantly found in black pepper (Piper nigrum), long pepper (Piper longum), and other species in the family Piperaceae (Figure 2). It is the primary bioactive compound responsible for the distinct, sharp, or pungent flavor associated with black pepper [7].

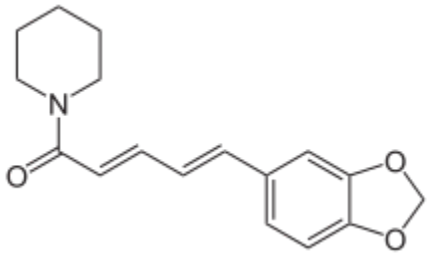

Figure 2: Chemical structure of Piperine [8]

Curcumin, chemically identified as *1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione* or diferuloyl methane, is a naturally occurring polyphenolic constituent predominantly isolated from the rhizomes of C. longa (turmeric) and other species within the genus Curcuma (Figure 3). This compound exhibits a broad spectrum of pharmacological properties, including antioxidant, anti-inflammatory, antibacterial, antimutagenic, and anticancer activities. Due to these diverse bioactivities, *C. longa* has been widely utilized in traditional medicine systems across Asia and has gained global recognition for its therapeutic significance [10–11].

Curcumin acts as a pleiotropic molecule capable of modulating multiple intracellular signaling cascades. Its interaction with numerous molecular targets underlies its extensive pharmacological versatility and health-enhancing properties. A substantial body of evidence indicates that curcumin plays a significant role in the management of metabolic disorders, inflammatory conditions, pain syndromes, and degenerative diseases [12–13]. Moreover, curcumin has been reported to exert

protective effects in ocular pathologies of inflammatory and degenerative origin by attenuating oxidative stress and suppressing pro-inflammatory mediators. Emerging studies have further demonstrated its nephroprotective efficacy, suggesting a capacity to preserve renal integrity and prevent toxin-induced kidney impairment [14].

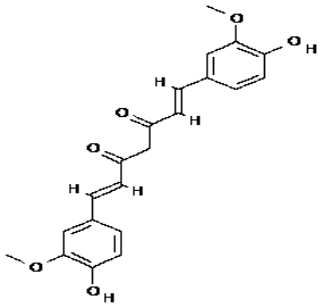

Figure 3: Chemical structure of Curcumin [15]

The literature reveals several distinct HPLC techniques available for the individual quantification of these drugs. However, there is an evident need for reliable methods tailored to the analysis of compounded formulations to ensure patient safety and therapeutic efficacy. Therefore, this study aimed to achieve the simultaneous estimation of Silymarin (SLY), Piperine (PIP), and Curcumin (CUR) in their blended form. Several nutraceutical and hepatoprotective formulations combine curcumin, silymarin, and piperine to enhance antioxidant potential and bioavailability. Piperine is widely recognized as a bioenhancer that improves systemic exposure of curcumin and silymarin. Such fixed-dose combinations are available in herbal capsules and dietary supplements marketed for liver protection and anti-inflammatory support. Therefore, a unified analytical method is essential for regulatory quality control of these multi-component formulations [16-20].

MATERIALS AND METHODS

Chemicals and Reagents

Certified reference standards for Silymarin were obtained from Sigma-Aldrich, while Curcumin and Piperine were sourced from Yucca Enterprises. HPLC-grade acetonitrile (Qualigens, India) and analytical-grade perchloric acid (Molychem, Mumbai) were used throughout the analysis. Water purified through a Milli-Q system served as the aqueous component.

Instrumentation

All chromatographic procedures were conducted on an Agilent 1260 Infinity II HPLC system equipped with a quaternary pump and diode array detector. Data acquisition and processing were performed using Agilent OpenLab EZChrom software. A Labman ultrasonicator was employed to facilitate dissolution of samples and standards, and an Aczet analytical balance was used for precise weighing.

Selection of the mobile phase

Preliminary trials were conducted using different proportions of aqueous phase (0.1% perchloric acid) and acetonitrile to identify a composition that adequately separates the three analytes. Ratios including 50:50, 45:55, 70:30, 60:40, and 55:45 (v/v) were tested. Optimization focused on achieving sharp peaks, reducing runtime, and sufficient resolution, considering the analytes' varied polarities. Alternative acidic modifiers such as orthophosphoric acid and formic acid were initially evaluated. However, these conditions resulted in peak broadening and a reduction in theoretical plate counts for silymarin. The use of 0.1% perchloric acid significantly improved peak symmetry and resolution between critical pairs. Although perchloric acid is corrosive, its low concentration and controlled usage ensured column compatibility during method validation. The column performance remained stable throughout analysis, with no observed deterioration in efficiency [21]. UV spectra from 190–400 nm were examined using the DAD detector. Among the tested wavelengths, 215 nm offered the most uniform and intense absorbance for all three analytes, enabling simultaneous monitoring [22].

Wavelength Selection

Individual UV spectra of Silymarin, Piperine, and Curcumin were recorded over the 190-400 nm range using a diode-array detector. Overlay spectral analysis was performed to identify a common wavelength suitable for simultaneous monitoring.

Method Development

Several chromatographic conditions were assessed to achieve acceptable system suitability parameters. Experimental trials included adjustments to the mobile-phase composition, flow rate, and organic content proportion to improve separation and peak characteristics. The polar-embedded amide functionality of the Zorbax Bonus RP column enhances retention and selectivity for moderately polar flavonolignan components of silymarin while maintaining separation efficiency for relatively nonpolar curcumin and piperine. Conventional C18 columns were evaluated in preliminary trials but led to co-elution and inadequate peak symmetry for silymarin isomers [23].

Preparation of Mobile Phase and Diluent

The optimized mobile phase consisted of 0.1% perchloric acid and acetonitrile (45:55, v/v). The mixture was filtered through a 0.45 µm nylon membrane and degassed by brief sonication. The diluent used for preparing standards comprised an equal proportion of the aqueous component and acetonitrile (50:50, v/v), similarly filtered and degassed before use [24].

Preparation of Standard Solutions

Stock solutions of Silymarin, Piperine, and Curcumin (1000 µg/mL each) were prepared in 10 mL volumetric flasks using the prepared diluent and sonicated to ensure complete dissolution. Equal volumes (1 mL each) of the stock solutions were combined and diluted to a final volume of 10 mL to obtain a mixed working standard containing 100 µg/mL of each analyte [25].

Quantification of Silymarin

Silymarin is a naturally occurring mixture of flavonolignans, predominantly comprising silybin A, silybin B, isosilybin A, isosilybin B, silychristin, and silydianin. In the present study, quantification was performed using the principal chromatographic peak corresponding to the major silybin isomer fraction obtained from the certified reference standard. The selected peak eluted at approximately 4.09min under the optimized chromatographic conditions and was used consistently for calibration, validation, and assay determinations. Quantitative evaluation was based on the integrated area of this peak, which represented the analytical marker for silymarin in the developed method.

Method Validation

Validation parameters, such as specificity, system suitability, precision, accuracy, linearity, LOD, LOQ & intermediate precision, were assessed to ensure methodological reliability and performance consistency.

Specificity: Individual injections of SLY, PIP, and CUR were compared with the chromatogram of their combined mixture to confirm retention-time uniformity and the absence of interference from the blank or diluent.

System Suitability: Indicators such as theoretical plate count, asymmetry, and resolution were evaluated to ensure the chromatographic system was performing consistently within acceptable limits [26].

Precision: Repeatability was assessed using six consecutive injections of the mixed standard solution. %RSD was calculated to determine instrument precision [27].

Accuracy: Recovery was assessed at 80%, 100%, and 120% of the target concentration for all analytes, with each level performed in triplicate [28].

Linearity: Linearity was evaluated over a broad concentration range extending from the limit of quantification (LOQ) to 150% of the target assay concentration. Calibration standards corresponding to LOQ, 25%, 50%, 75%, 100%, 125% & 150% levels were prepared and analyzed in triplicate. Calibration curves were generated by plotting peak area against analyte concentration, and regression statistics were calculated [29].

Sensitivity (LOD and LOQ): LOD and LOQ values were calculated using the response standard deviation and the calibration curve slope [30].

Intermediate Precision: Intra-day & inter-day variability were assessed using peak area measurements taken at different time intervals.

Robustness Study

The robustness of the developed RP-HPLC method was evaluated to assess its reliability under small, deliberate variations in chromatographic conditions. The study was performed in accordance with ICH guidelines to assess the influence of minor operational changes on analytical performance [31].

Forced Degradation Studies

To establish the stability-indicating nature of the developed RP-HPLC method, forced degradation studies were conducted under acidic, alkaline, oxidative, thermal, and photolytic conditions. Samples containing Silymarin, Piperine & Curcumin were subjected to stress conditions and analyzed after appropriate neutralization and dilution.

Sample Analysis of Commercial Formulation

Twenty capsules containing Silymarin, Piperine, and Curcumin were accurately weighed and powdered. An amount equivalent to one dose unit was transferred into a volumetric flask, extracted using diluent under sonication, filtered through a 0.45 µm membrane filter, and analyzed by the developed RP-HPLC method.

RESULTS

Wavelength Selection

The 215 nm wavelength was selected for analysis to ensure optimal detection and appropriate peak intensity for Silymarin. (SLY), Piperine (PIP) & Curcumin (CUR) as shown in Figure 4.

HPLC Method Development Trials

A series of trials was conducted to optimize the HPLC method for the simultaneous determination of Silymarin (SLY), Piperine (PIP), and Curcumin (CUR) using an Agilent Zorbax Bonus RP column (250 × 4.6 mm, 5 µm) with varying mobile-phase compositions and flow rates. The observations are summarized as follows (Table 1).

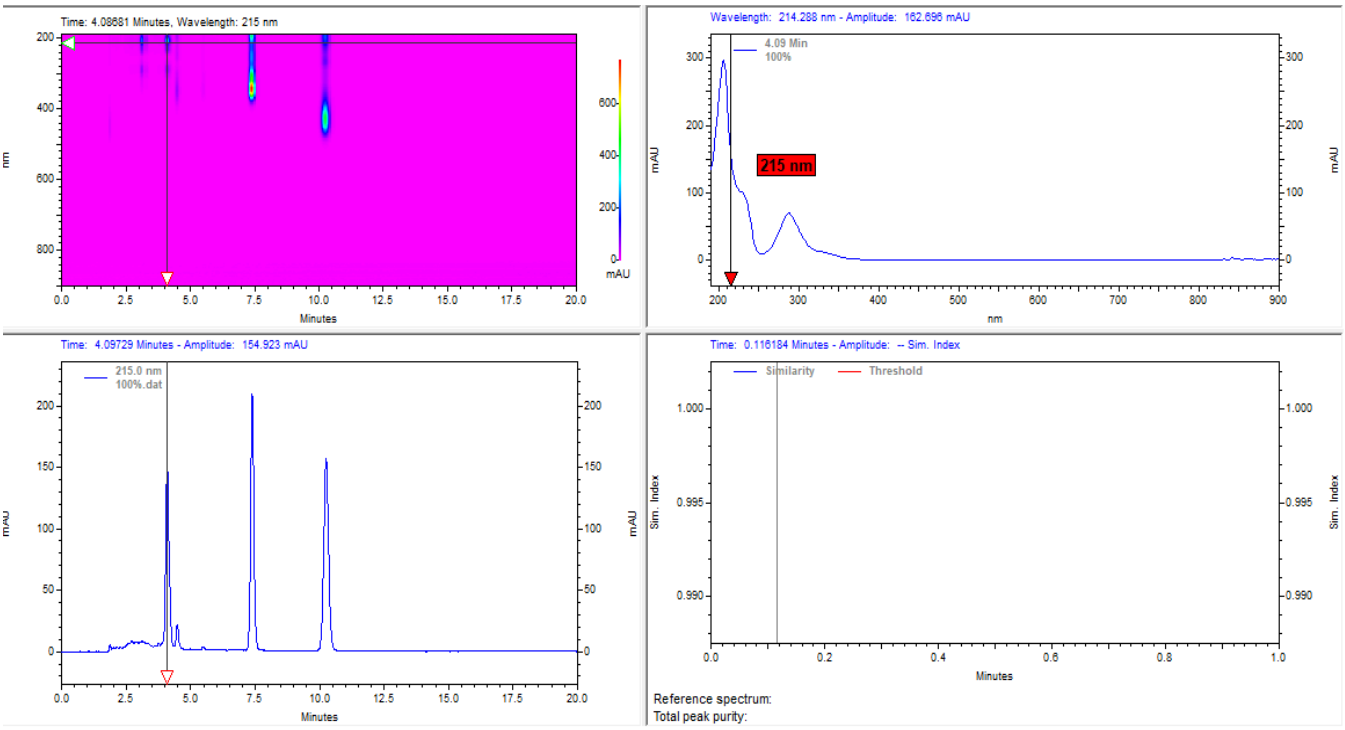

Figure 4: Wavelength selection of SLY, PIP, and CUR

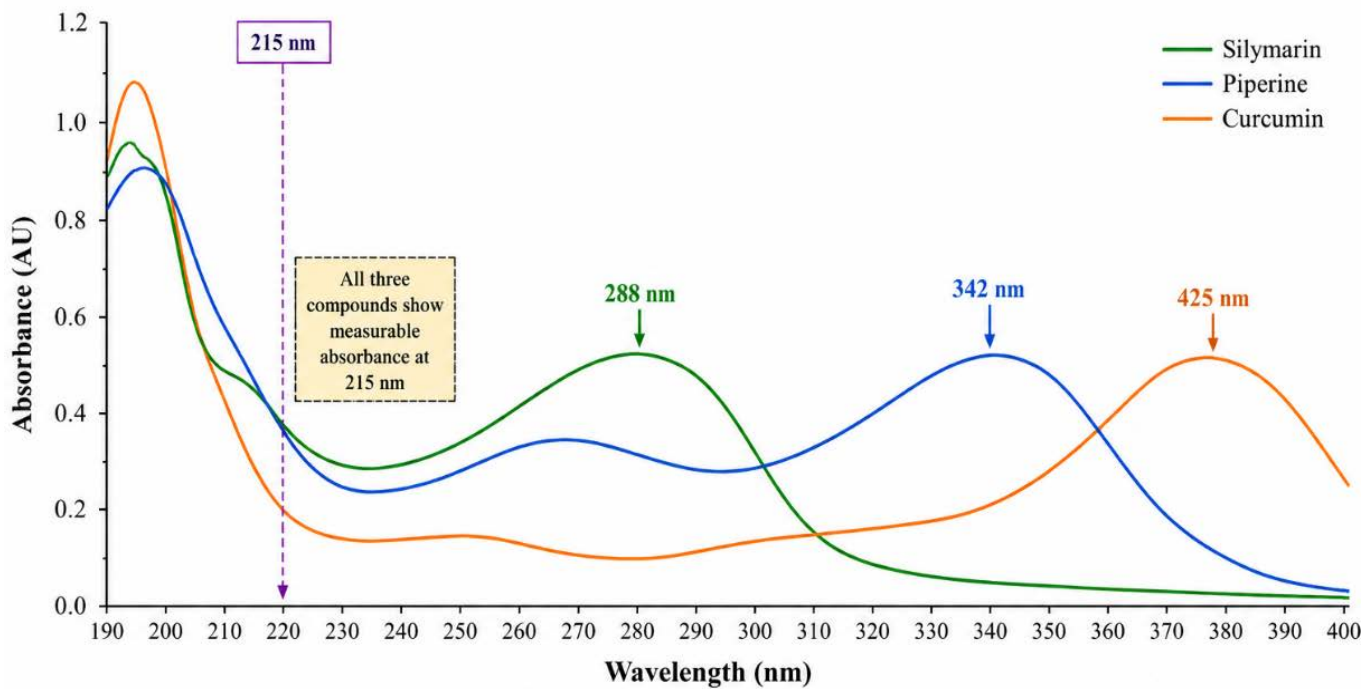

Figure 5: Overlay UV Spectra

Table 1: Method Development for SLY, PIP & CUR by HPLC

Trial	Mobile Phase	Ratio (v/v)	Observations
1	0.1% Perchloric acid: ACN	50:50	SLY peak did not elute; PIP and CUR eluted at 11.60 and 15.27 min, respectively. To facilitate SLY elution and reduce resolution, ACN was increased by 5% and the wavelength adjusted to 238 nm.
2	0.1% Perchloric acid: ACN	45:55	SLY, PIP, and CUR eluted at 4.07, 7.39, and 10.14 min, respectively. Resolution values were 15.13 for PIP and 9.61 for CUR. To enhance the theoretical plates of SLY, 0.1% perchloric acid was increased to 70%.
3	0.1% Perchloric acid: ACN	70:30	Injection of SLY alone resulted in elution at 14.59 min. This extended runtime prompted a reduction of 0.1% perchloric acid to 60%.
4	0.1% Perchloric acid: ACN	60:40	SLY peak eluted at 12.08 min; however, peak intensity decreased.
5	0.1% Perchloric acid: ACN	55:45	The SLY peak eluted at 7.64 min, but its shape was unsatisfactory.
6	0.1% Perchloric acid: ACN	45:55	Flow rate increased to 1.1 ml/min (based on trial 2). SLY eluted at 3.66 min; theoretical plates slightly decreased.
7	0.1% Perchloric acid: ACN	45:55	Flow rate decreased to 0.8 ml/min (based on trial 2). SLY eluted at 5.03 min; the peak was broad and not sharp.

These trials indicate that the mobile phase composition and flow rate significantly influence retention times, peak shapes, and theoretical plate counts, and were crucial in selecting the optimized chromatographic conditions for simultaneous estimation of SLY, PIP, and CUR (Table 2).

Table 2: Method Development results for SLY, PIP & CUR by HPLC

Trial No.	Silymarin				Piperine				Curcumin			
1	RT	TP	Asy	Res	RT	TP	Asy	Res	RT	TP	Asy	Res
2	-	-	-	-	11.60	16961	1.06	0.00	18.96	15561	1.03	15.27
3	4.07	6272	1.19	0.00	7.39	16108	1.05	15.13	10.14	14371	1.08	9.61
4	14.59	11608	1.10	0.00	-	-	-	-	-	-	-	-
5	12.08	11425	1.09	0.00	-	-	-	-	-	-	-	-
6	7.64	8451	1.01	0.00	-	-	-	-	-	-	-	-
7	3.66	5443	1.18	0.00	-	-	-	-	-	-	-	-
8	5.03	6774	1.25	0.00	-	-	-	-	-	-	-	-

RT – Retention time; TP – Theoretical plates; Asy – Asymmetry; Res - Resolution

Table 3: Final Chromatographic Conditions

Parameter	Condition
HPLC Instrument	Agilent 1260 Infinity II
Column	Agilent Zorbax Bonus RP (250 mm × 4.6 mm, 5 µm)
Wavelength	215 nm
Mobile Phase	0.1% Perchloric acid: Acetonitrile (45:55 % v/v)
Diluent	0.1% Perchloric acid: Acetonitrile (50:50 % v/v)
Run Time	20 minutes
Injection Volume	10 µL
Flow Rate	1.0 mL/min
Column Oven Temperature	30°C

Method Validation

Specificity

Specificity was assessed to determine whether interference from the blank or excipients occurred at the retention times of the active pharmaceutical ingredients (APIs). The analysis confirmed that the API peaks were well resolved and free of co-eluting impurities or matrix interference, demonstrating the method's ability to quantify each analyte (Table 4) selectively.

Table 4: Specificity results of SLY, PIP & CUR

Sample ID	Silymarin RT (min.)	Piperine RT (min.)	Curcumin RT (min.)
Silymarin	4.07	-	-
Piperine	-	7.39	-
Curcumin	-	-	10.23
Mixture	4.09	7.39	10.25

Based on specificity data, the Retention time of the individual working standard was similar to that of the mixture working standard, and there was no interference from the blank with the main peaks of SLY, PIP, and CUR. Placebo samples containing all formulation excipients were analyzed under optimized chromatographic conditions. The peak observed at approximately 4.09 min corresponded to the major silybin fraction of the silymarin reference standard and was selected as the quantitative marker throughout the study. No peaks were observed at the retention times corresponding to Silymarin, Piperine, or Curcumin, confirming the absence of interference from excipient components (Figure 6).

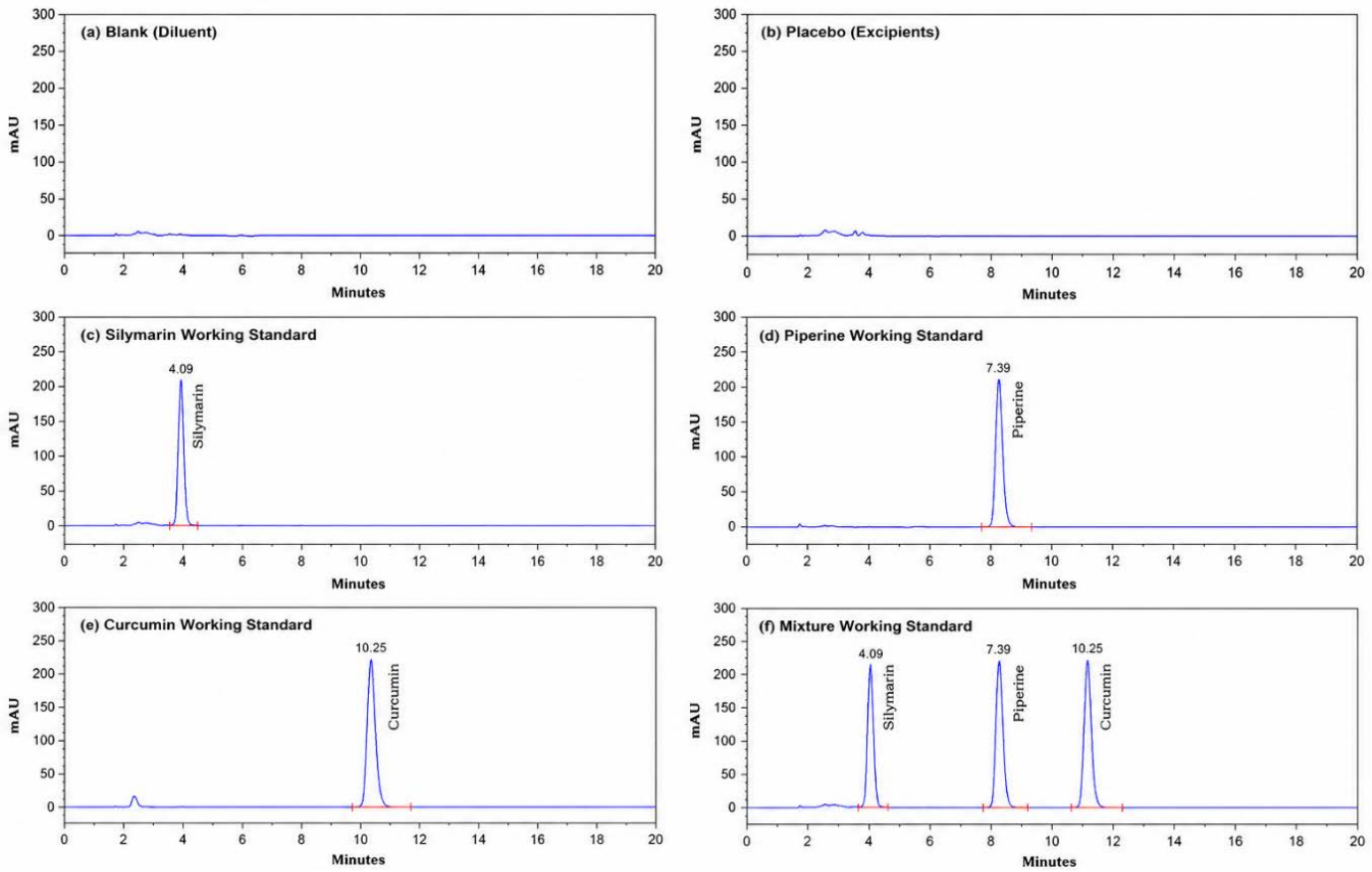

Figure 6: Chromatogram ID a] Diluent, b] Placebo (Excipients), c] Silymarin, d] Piperine, e] Curcumin, f] Mixture Working Standard

Instrument Precision & System Suitability

The appropriateness of the HPLC instrument was assessed as part of validation. According to the limits specified in Table 1, the equipment was deemed appropriate for conducting the validations. The precision test was conducted after assessing the system's applicability. The data presented in Tables 6 & 7 indicate that the % relative standard deviation for Instrument precision for SLY, PIP & CUR are 0.21%, 0.19%, and 0.19%, respectively. These % RSDs demonstrate that the approach is highly precise and reliable when used by various analysts and for multiple sample preparations with the same concentration. The data are displayed in Tables 5 & 6.

Table 5: Results of Repeatability

Sample ID	SLY Peak Area	PIP Peak Area	CUR Peak Area
Replicate 1	2670746	3870212	4224309
Replicate 2	2668541	3862454	4219456
Replicate 3	2669654	3882778	4235548
Replicate 4	2673569	3871671	4219745
Replicate 5	2679225	3862211	4229785
Replicate 6	2662124	3870298	4237754
Average	2670643	3869937	4227766
STDEV	5660.26	7533.795	7870.698
% RSD	0.21	0.19	0.19

Table 6: System Suitability Results for Silymarin (SLY), Piperine (PIP), and Curcumin (CUR)

Sample ID	Silymarin				Piperine				Curcumin			
	RT (min)	TP	Tailing	Rs	RT (min)	TP	Tailing	Rs	RT (min)	TP	Tailing	Rs
Rep 1	4.09	5941	1.19	—	7.39	15601	1.08	14.7	10.25	13674	1.05	7.7
Rep 2	4.09	5866	1.13	—	7.39	15748	1.03	14.7	10.25	13258	1.01	7.7
Rep 3	4.09	5872	1.12	—	7.39	15998	1.08	14.7	10.25	13778	1.02	7.7
Rep 4	4.09	5763	1.18	—	7.39	14928	1.04	14.7	10.25	13964	1.03	7.7
Rep 5	4.09	6017	1.11	—	7.39	15377	1.09	14.7	10.25	13757	1.04	7.7
Rep 6	4.09	6255	1.15	—	7.39	15845	1.05	14.7	10.25	13260	1.02	7.7

RT – Retention time; TP – Theoretical plates; Asy – Asymmetry; Res – Resolution

Accuracy

The accuracy of the method for Silymarin (SLY) was evaluated in triplicate at three concentration levels: 80%, 100% & 120% of the target concentration. The method demonstrated excellent accuracy, with % relative std. deviations (%RSD) of 0.24%, 0.04% & 0.30% for 80%, 100% & 120% conc., respectively. These results confirm that the method can reliably quantify varying concentrations of SLY in solution. The detailed accuracy data are presented in Table 7.

The accuracy of the method for Piperine (PIP) was assessed in triplicate at 80%, 100%, and 120% of the target concentration. The method exhibited high accuracy, with percent relative standard deviations (%RSD) of 0.29%, 0.26%, and 0.39% for the respective concentrations. These results confirm the method's ability to quantify PIP concentrations in solution accurately. The corresponding accuracy data are summarized in Table 8.

Table 7: Accuracy data for Silymarin

Silymarin working Standard peak Area = 2670643								
% Level	**Reps**	**Spiked Amount**	**Peak Area**	**Amount Recovered**	**% Recovery**	**Average**	**STDEV**	**% RSD**
80%	Rep 1	80 (ug/ml)	2141833	80.20 (ug/ml)	100.25	100.09	0.24	0.24
	Rep 2	80 (ug/ml)	2132585	79.85 (ug/ml)	99.82			
	Rep 3	80 (ug/ml)	2140957	80.17 (ug/ml)	100.21			
100%	Rep 1	100 (ug/ml)	2670746	100.00 (ug/ml)	100.00	99.96	0.04	0.04
	Rep 2	100 (ug/ml)	2668541	99.92 (ug/ml)	99.92			
	Rep 3	100 (ug/ml)	2669654	99.96 (ug/ml)	99.96			
120%	Rep 1	120 (ug/ml)	3205683	120.03 (ug/ml)	100.03	99.97	0.30	0.30
	Rep 2	120 (ug/ml)	3193365	119.57 (ug/ml)	99.64			
	Rep 3	120 (ug/ml)	3212145	120.28 (ug/ml)	100.23			

Table 8: Accuracy data for Piperine

Piperine Working Standard Peak Area = 3869937								
% Level	**Reps**	**Spiked Amount**	**Peak Area**	**Amount Recovered**	**% Recovery**	**Average**	**STDEV**	**% RSD**
80%	Rep 1	80 (ug/ml)	3101445	80.14 (ug/ml)	100.18	100.22	0.29	0.29
	Rep 2	80 (ug/ml)	3094585	79.96 (ug/ml)	99.96			
	Rep 3	80 (ug/ml)	3112545	80.43 (ug/ml)	100.54			
100%	Rep 1	100 (ug/ml)	3870212	100.01 (ug/ml)	100.01	100.05	0.27	0.26
	Rep 2	100 (ug/ml)	3862454	99.81 (ug/ml)	99.81			
	Rep 3	100 (ug/ml)	3882778	100.33 (ug/ml)	100.33			
120%	Rep 1	120 (ug/ml)	4658748	120.38 (ug/ml)	100.32	99.92	0.39	0.39
	Rep 2	120 (ug/ml)	4622584	119.45 (ug/ml)	99.54			
	Rep 3	120 (ug/ml)	4639252	119.88 (ug/ml)	99.90			

Accuracy for Curcumin (CUR) was evaluated in triplicate at 80%, 100%, and 120% of the target concentration. The method demonstrated excellent accuracy, with percent relative standard deviations (%RSD) of 0.43%, 0.20%, and 0.08% for the respective concentrations. These findings confirm the method's suitability for reliably quantifying varying concentrations of CUR in solution. The detailed accuracy data are presented in Table 9.

Table 9: Accuracy data for Curcumin

Curcumin Working Standard Peak Area = 4227766								
% Level	**Reps**	**Spiked Amount**	**Peak Area**	**Amount Recovered**	**% Recovery**	**Average**	**STDEV**	**% RSD**
80%	Rep 1	80 (ug/ml)	3367208	79.65 (ug/ml)	99.56	99.66	0.43	0.43
	Rep 2	80 (ug/ml)	3358545	79.44 (ug/ml)	99.30			
	Rep 3	80 (ug/ml)	3386678	80.11 (ug/ml)	100.13			
100%	Rep 1	100 (ug/ml)	4224309	99.92 (ug/ml)	99.92	99.97	0.20	0.20
	Rep 2	100 (ug/ml)	4219456	99.80 (ug/ml)	99.80			
	Rep 3	100 (ug/ml)	4235548	100.18 (ug/ml)	100.18			
120%	Rep 1	120 (ug/ml)	5103063	120.70 (ug/ml)	100.59	100.67	0.08	0.08
	Rep 2	120 (ug/ml)	5110850	120.89 (ug/ml)	100.74			
	Rep 3	120 (ug/ml)	5108094	120.82 (ug/ml)	100.69			

Linearity

Excellent linearity was observed across the evaluated concentration range. range. Correlation coefficients exceeded 0.999 for Silymarin, Piperine, and Curcumin, indicating a strong linear relationship between concentration and detector response across the analytical working range as summarized in Table 10. Representative calibration graphs are shown in Figures 7, 8, and 9.

Table 10: Linearity data of SLY, PIP & CUR

% Level	**Conc. (µg/mL)**	**Silymarin Peak Area**	**Piperine Peak Area**	**Curcumin Peak Area**
LOQ	5	151720	172114	102412
25%	25	682919	952547	971056
50%	50	1346917	1928088	2056862
75%	75	2010916	2903629	3142667
100%	100	2674914	3879171	4228473
125%	125	3338913	4854713	5314278
150%	150	4002911	5830254	6400084

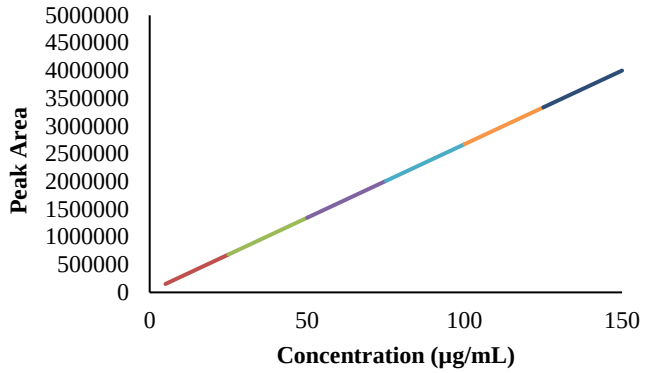

Figure 7: Linearity of Silymarin

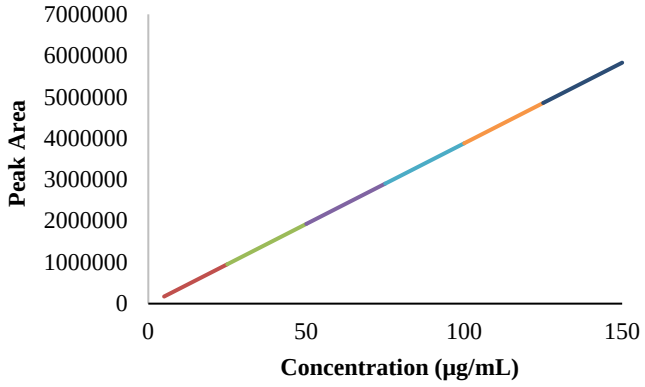

Figure 8: Linearity of Piperine

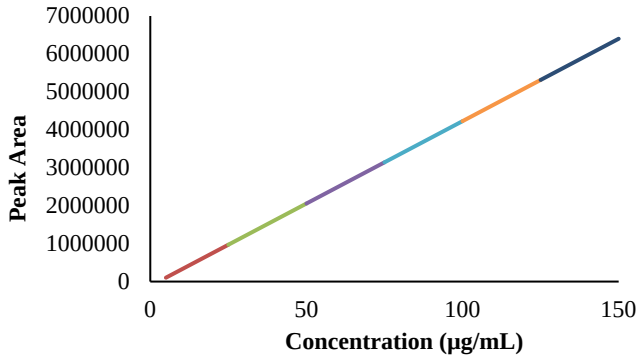

Figure 9: Linearity of Curcumin

LOD & LOQ

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for Silymarin (SLY), Piperine (PIP), and Curcumin (CUR) to evaluate the method's sensitivity. The results obtained for each analyte are summarized in Table 11.

Table 11: Results of LOD & LOQ for SLY, PIP & CUR

Name	**LOD (µg/ml)**	**LOQ (µg/ml)**
Silymarin	1.56	4.73
Piperine	1.84	5.57
Curcumin	1.73	5.26

The low LOD and LOQ values indicate that the developed method is highly sensitive and capable of accurately detecting and quantifying even minimal drug concentrations. Such sensitivity makes the method suitable for applications such as cleaning validation in pharmaceutical manufacturing, enabling the detection of residual API traces on equipment or in production vessels.

Inter and Intraday Precision

Intra-day and inter-day precision studies were conducted by analyzing API peak areas at different time points. The percent relative standard deviation (%RSD) was calculated to evaluate reproducibility. According to the method validation criteria, %RSD values for peak area should be less than 2%. The results of the precision study are summarized in Table 12

Table 12: Intra-day and Inter-day Precision of Silymarin, Piperine, and Curcumin

Condition	Interval	Silymarin		Piperine		Curcumin	
		RT	Peak Area	RT	Peak Area	RT	Peak Area
Intra-day	Morning	4.09	2670746	7.39	3870212	10.25	4224309
	Evening	4.09	2669475	7.39	3865483	10.25	4206785
Inter-day	Day 2	4.09	2663994	7.39	3851254	10.25	4192254
% RSD		0.00	0.13	0.00	0.26	0.00	0.38

The intra-day and inter-day precision for Silymarin (SLY), Piperine (PIP), and Curcumin (CUR) were evaluated, yielding %RSD values of 0.13%, 0.26%, and 0.38%, respectively. These low %RSD values indicate excellent reproducibility and confirm the method's precision.

Robustness Evaluation

The robustness study demonstrated that small, deliberate variations in chromatographic conditions did not significantly affect the system suitability parameter or quantification performance.

Effect of Flow Rate Variation

When the flow rate was reduced to 0.9 mL/min, the retention times of Silymarin (SLY), Piperine (PIP), and Curcumin (CUR) increased slightly due to a reduced mobile-phase velocity. Conversely, at 1.1 mL/min, a minor reduction in retention times was observed. However:

- %RSD of peak areas remained below 1.2%
- Resolution between SLY–PIP and PIP–CUR was maintained above 7.0
- Tailing factor values remained within 1.05–1.25

These results indicate that moderate flow rate variations do not compromise peak integrity or quantification accuracy.

Effect of Column Temperature Variation

Altering column oven temp. Temperatures of 28°C and 32°C caused negligible changes in retention time, with no distortion of peak shape.

- %RSD values were below 1.0%
- Resolution remained greater than 6.5
- Tailing factors were below 1.3 for all analytes

The minimal impact of temperature fluctuations confirms the thermal stability of analyte retention under the selected chromatographic conditions.

Effect of Organic Phase Composition (±2% ACN)

Modifying the acetonitrile composition to 53% and 57% resulted in slight shifts in retention time, consistent with changes in solvent strength. Increased organic content slightly reduced RT, while decreased organic content prolonged elution.

Forced Degradation Studies

Forced degradation studies were conducted under acidic, alkaline, oxidative, thermal, and photolytic stress conditions to evaluate the stability-indicating capability of the developed RP-HPLC method. The results presented in Table 13 demonstraed varying degrees of degradation in Silymarin, Piperine, and Curcumin under different stress conditions. Maximum degradation was observed under oxidative conditions (15.5%), followed by alkaline (11.6%) and acidic degradation (8.8%). Thermal and photolytic stress resulted in comparatively lower degradation of 5.8% and 7.4%, respectively. In all cases, the analyte peaks were well resolved from degradation products, confirming the specificity and stability-indicating nature of the developed RP-HPLC method. Peak purity analysis further verified the absence of co-eluting degradation impurities.

Sample Analysis of Commercial Formulation

The developed RP-HPLC method was successfully applied to the analysis of a commercial formulation containing Silymarin, Piperine, and Curcumin. The assay values obtained for all three analytes ranged from 99.4% to 99.5% of the labeled claim, demonstrating excellent agreement with the declared content

(Table 14). The absence of interfering peaks and the high assay values confirmed the method's suitability for routine quality-control analysis of pharmaceutical and nutraceutical dosage forms.

Table 13: Forced Degradation Results Demonstrating the Stability-Indicating Capability of the Developed RP-HPLC Method

Condition	% Assay Remaining	% Degradation
Control	100.0	0.0
Acidic Degradation	91.2	8.8
Alkaline Degradation	88.4	11.6
Oxidative Degradation	84.5	15.5
Thermal Degradation	94.2	5.8
Photolytic Degradation	92.6	7.4

Recovery studies performed at 80%, 100%, and 120% spiking levels demonstrated excellent extraction efficiency & analytical accuracy of the developed method. The recovery values ranged from 99.6% to 100.2%, indicating negligible matrix interference from formulation excipients (Table 15). These results confirm that the method is accurate, reliable, and suitable for quantitative estimation of Silymarin, Piperine, and Curcumin in finished pharmaceutical formulations.

Table 14: Assay Results of Commercial Formulation Containing Silymarin, Piperine, and Curcumin

Analyte	Label Claim (mg)	Found (mg)	% Assay
Silymarin	140	139.2	99.4
Piperine	10	9.95	99.5
Curcumin	250	248.7	99.5

Table 15: Recovery Study of Silymarin, Piperine, and Curcumin in Formulation Matrix

Recovery Level	Recovery (%)
80%	99.6
100%	100.2
120%	99.8

Table 16: Comparison of Proposed RP-HPLC Method with Reported Methods

Reference	Analytes	Run Time	LOD	LOQ	Remarks
Petrásková et al., 2020	Silymarin flavonolignans	18 (min)	3.2 (ug/ml)	9.8 (ug/ml)	Individual analysis
Bharati et al., 2022	Piperine	15 (min)	2.8 (ug/ml)	8.4 (ug/ml)	Single analyte
Narayanam et al., 2022	Curcuminoids	20 (min)	2.4 (ug/ml)	7.1 (ug/ml)	Curcuminoids only
Setyaningsih et al., 2021	Curcumin + Piperine	16 (min)	2.0 (ug/ml)	6.0 (ug/ml)	Dual analyte method
Present Study	**SLY + PIP + CUR**	**11** (min)	**1.56–1.84** (ug/ml)	**4.73–5.57** (ug/ml)	**Simultaneous determination of 3 phytoconstituents**

Compared with previously reported chromatographic methods, the proposed RP-HPLC method offers a shorter analytical runtime while maintaining excellent sensitivity and chromatographic resolution (Table 16). In addition, the method enables simultaneous quantification of three therapeutically relevant phytoconstituents within a single chromatographic run, thereby improving analytical efficiency and reducing solvent consumption. These advantages make the method particularly suitable for routine quality control of combined herbal and nutraceutical formulations.

DISCUSSION

The analytical wavelength of 215nm was selected for the simultaneous quantification of Silymarin (SLY), Piperine (PIP), and Curcumin (CUR), as it provided optimal absorbance and well-defined chromatographic peaks for all three compounds. This wavelength enhanced sensitivity and peak resolution, allowing accurate measurement of each analyte without interference. Silymarin exhibited a major absorbance band around 288 nm, Piperine near 342 nm & Curcumin near 425 nm. However, all three compounds exhibited sufficient absorbance in the lower-UV region. The wavelength of 215 nm was selected because it provided measurable and balanced detector response for all analytes simultaneously, thereby enabling single-wavelength quantification without compromising sensitivity.

Method development focused on optimizing mobile phase composition and flow rate using an Agilent Zorbax Bonus RP column (250 × 4.6 mm, 5 µm). Initial trials with a 50:50 acetonitrile-0.1% perchloric acid mixture resulted in non-elution of SLY and delayed retention of PIP and CUR. Increasing the organic component to 55% (mobile phase ratio of 45:55 acid: acetonitrile) produced retention times of 4.07, 7.39, and 10.14 minutes for SLY, PIP, and CUR, respectively, along with improved peak symmetry, theoretical plate counts, and resolution. Subsequent adjustments to perchloric acid concentration and flow rates led to the final optimized conditions: a 45:55 (v/v) mobile phase at a flow rate of 1.0

mL/min, yielding sharp, symmetric peaks and a reduced overall run time without compromising separation efficiency.

Although each analyte exhibits distinct λmax values at longer wavelengths, the overlaid spectra revealed a common absorbance region in the lower UV range. At 215 nm, all three compounds exhibited adequate molar absorptivity, enabling simultaneous detection in a single chromatographic run without wavelength switching. Additionally, baseline stability was confirmed using blank injections, which showed minimal solvent interference under optimized conditions. Specificity was verified by injecting the analytes individually and in combination. The retention times in the mixture matched those of individual injection & blank injections showed no peaks at these retention times, confirming the method's selectivity.

System suitability parameters, including theoretical plate number, peak asymmetry, and resolution, were within ICH-specified limits, indicating the reliability and precision of the chromatographic system for routine use. Instrument precision, assessed via six replicate injections of the same standard solution, yielded %RSD values of 0.21%, 0.19%, and 0.19% for SLY, PIP, and CUR, respectively, demonstrating excellent repeatability and minimal instrumental variability.

Accuracy was evaluated through recovery studies at 80%, 100% & 120% conc. levels. Recoveries were near 100% for all analytes, with %RSD values below 0.5%, confirming the method's capability to quantify analytes accurately across a range of concentrations. Linearity was established across a wide analytical range extending from the limit of quantification (LOQ) to 150% of the target assay concentration. Calibration standards at LOQ, 25%, 50%, 75%, 100%, 125%, and 150% showed a proportional increase in detector response with concentration across all analytes. Excellent linear relationships were observed for Silymarin, Piperine, and Curcumin, with correlation coefficients (R^2) greater than 0.999, confirming the method's suitability for quantitative analysis over the intended concentration range. The method exhibited high sensitivity, with LOD and LOQ values below 2 µg/mL and 6 µg/mL, respectively, for all analytes. Such sensitivity makes the method suitable for detecting trace amounts, supporting applications in pharmaceutical cleaning validation & contamination control. The obtained LOD values are lower than previously reported individual methods for curcumin & piperine, indicating enhanced sensitivity. The short retention times reduce solvent consumption compared to reported methods with 20–30 minute run times. Intra-day and inter-day precision studies confirmed the method's stability and reproducibility, with %RSD values consistently below 0.4%, demonstrating robustness under varying analytical conditions.

Across all intentionally introduced variations in flow rate, column temperature, and organic phase composition, the chromatographic performance remained consistent and within acceptable limits. The %RSD values for peak areas were below 2.0%, resolution between critical peak pairs remained well above the minimum acceptance criterion of 2.0, and tailing factors were maintained below 2.0 for all analytes. No meaningful changes in retention time, peak symmetry, or separation efficiency were observed. These results demonstrate that the developed RP-HPLC method is robust and maintains analytical reliability under minor operational fluctuations, supporting its suitability for routine QC applications.

The degradation products generated under stress conditions were chromatographically resolved from the analyte peaks without interference. Peak purity analysis confirmed homogeneity of analyte peaks. These findings establish the stability-indicating capability of the proposed RP-HPLC method. The successful assay of the commercial formulation & excellent recovery values confirmed the suitability of the developed method for routine quality control of pharmaceutical and nutraceutical dosage forms. Compared with reported individual methods, the present method achieves simultaneous separation within 11 minutes with acceptable sensitivity & resolution. The developed HPLC method is precise, accurate, selective, sensitive, and reproducible. It is suitable for the simultaneous quantification of Silymarin, Piperine, and Curcumin in pharmaceutical formulations, making it an effective tool for quality control laboratories.

CONCLUSION

The developed RP-HPLC method achieved efficient and reliable separation of curcumin, silymarin, and piperine, three structurally diverse phytoconstituents, within a total run time of approximately 11 minutes. The optimized chromatographic conditions ensured satisfactory peak symmetry, adequate resolution between critical peak pairs, and consistent system suitability performance. Validation results confirmed high sensitivity, precision, accuracy, and linearity across the evaluated concentration range, demonstrating excellent

reproducibility of the method. The robustness assessment further established that minor deliberate variations in chromatographic parameters did not significantly affect analytical performance, supporting the reliability of the method for routine application. Overall, the validated method satisfies essential quality control requirements for the simultaneous estimation of multi-component herbal combinations. Forced degradation studies demonstrated adequate separation of degradation products from the analyte peaks, confirming the stability-indicating capability of the developed RP-HPLC method.

FINANCIAL ASSISTANCE
NIL

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
Misbah Sultana Abdul Kausar Badewale and Varsha Siddheswar Tegeli collected data and performed experiments. Varsha Siddheswar Tegeli conducted the analysis. Misbah Sultana Abdul Kausar Badewale wrote the first draft of the manuscript, and all authors reviewed and revised previous versions. All authors contributed to the study's conception and design and gave final approval.

DECLARATION OF USE OF AI TOOLS
The authors used an AI-assisted tool (ChatGPT) to support manuscript preparation, including language refinement, editing, and improving clarity where appropriate. All scientific content, analyses, interpretations, and conclusions were critically reviewed, verified, and approved by the authors, who take full responsibility for the originality, accuracy, integrity, and final content of the manuscript.

REFERENCES
[1] Sayyad M, Sutar AD, Shivhare K, Shukla R, Flora SJS. Silymarin as a phytopharmaceutical agent: advances in mechanistic insights, formulation strategies, and pre-clinical applications. *Front Pharmacol*, **16**, 1711653 (2025) https://doi.org/10.3389/fphar.2025.1711653

[2] Selc M, Babelova A. Looking beyond silybin: the importance of other silymarin constituents in translational research. *Front Pharmacol*, **16**, 1637393 (2025) https://doi.org/10.3389/fphar.2025.1637393

[3] Rizwan M, Naseem S, Durrani AI. Pharmacokinetics and therapeutic potential of milk thistle extracted phytoconstituents: a leading role in cancer treatment. *J Adv Immunopharmacol*, **3**(2), e138889 (2023) https://doi.org/10.5812/tms-138889

[4] Satyam SM, Bairy LK, Rehman A, Nair AA, Farook M, Binu NN, et al. Dapagliflozin and silymarin ameliorate cisplatin-induced nephrotoxicity in female Wistar rats. *Sci*, **7**(2), 59 (2025) https://doi.org/10.3390/sci7020059

[5] Xie Y, Zhang D, Zhang J, Yuan J. Effects of silymarin on drug-metabolizing enzymes and transporters: implications for drug–drug interactions. *Molecules*, **24**(20), 3693 (2019) https://doi.org/10.3390/molecules24203693

[6] Sharma P, Asediya V, Kalra G, Sultana S, Purohit N, Kibitlewska K, et al. Hepatoprotective effect of silymarin herb in prevention and treatment of metabolic-associated steatotic liver disease: current evidence and future prospects. *Nutrients*, **17**(20), 3278 (2025) https://doi.org/10.3390/nu17203278

[7] Periferakis AT, Adalis GM, Periferakis A, Troumpata L, Periferakis K, Dragosloveanu CDM, et al. The multifaceted antimicrobial profile of piperine in infectious disease management: current perspectives and potential. *Pharmaceuticals*, **18**(10), 1581 (2025) https://doi.org/10.3390/ph18101581

[8] Akter K, Gul K, Mumtaz S. Revisiting curcumin in cancer therapy: recent insights into molecular mechanisms, nanoformulations, and synergistic combinations. *Curr Issues Mol Biol*, **47**(9), 716 (2025) https://doi.org/10.3390/cimb47090716

[9] Kotha RR, Luthria DL. Curcumin: biological, pharmaceutical, nutraceutical, and analytical aspects. *Molecules*, **24**(16), 2930 (2019) https://doi.org/10.3390/molecules24162930

[10] Zheng BW, Wang BY, Xiao WL, Sun YJ, Yang C, Zhao BT, et al. Different molecular weight hyaluronic acid alleviates inflammation response in DNFB-induced mice atopic dermatitis and LPS-induced RAW 264.7 cells. *Life Sci*, **301**, 120591 (2022) https://doi.org/10.1016/j.lfs.2022.120591

[11] Jamil SNH, Ali AH, Feroz SR, Lam SD, Agustar HK, Mohd Abd Razak MR, et al. Curcumin and its derivatives as potential antimalarial and anti-inflammatory agents: a review on structure-activity relationship and mechanism of action. *Pharmaceuticals (Basel)*, **16**(4), 609 (2023) https://doi.org/10.3390/ph16040609

[12] Kiskova T, Smajda B. The effects of bioactive compounds on human brain structures and diseases. *Int J Mol Sci*, **25**(6), 3326 (2024) https://doi.org/10.3390/ijms25063326

[13] Joshi P, Bisht A, Paliwal A, Dwivedi J, Sharma S. Recent updates on clinical developments of curcumin and its derivatives. *Phytother Res*, **37**(11), 5109–5158 (2023) https://doi.org/10.1002/ptr.7974

[14] Babich O, Larina V, Ivanova S, Tarasov A, Povydysh M, Orlova A, et al. Phytotherapeutic approaches to the prevention of age-related changes and the extension of active longevity. *Molecules*, **27**(7), 2276 (2022) https://doi.org/10.3390/molecules27072276

[15] Wilar G, Suhandi C, Fukunaga K, Shigeno M, Kawahata I, Abdulah R, et al. Effects of nanocurcumin supplementation on metabolic syndrome: a systematic review and meta-analysis of randomized controlled trials. *Pharmacol Res*, **213**, 107641 (2025) https://doi.org/10.1016/j.phrs.2025.107641

[16] Wang Z, Jones G, Winzenberg T, Cai G, Laslett LL, Aitken D, et al. Effectiveness of Curcuma longa extract for the treatment of symptoms and effusion–synovitis of knee osteoarthritis: a randomized trial. *Ann Intern Med*, **173**(11), 861–869 (2020) https://doi.org/10.7326/M20-0990

[17] Dowgiałło-Gornowicz N, Masiewicz A, Kacperczyk J, Lech P, Saluk S, Osowiecka K. Long-term outcomes of chronic cough reduction after laparoscopic Nissen fundoplication — a single-center study. *Medicina*, **58**(1), 47 (2022) https://doi.org/10.3390/medicina58010047

[18] Miserocchi E, Giuffrè C, Cicinelli MV, Marchese A, Gattinara M, Modorati G, et al. Oral phospholipidic curcumin in juvenile idiopathic arthritis-associated uveitis. *Eur J Ophthalmol*, **30**(6), 1390–1396 (2020) https://doi.org/10.1177/1120672119892804

[19] Lastra JM, Curieses Andrés CM, Munguira EB, Juan CA, Pérez Lebeña E. Silymarin as a redox-signalling and proteostasis modulator. *Neutraceu*, **6**(2), 25 (2026) https://doi.org/10.3390/nutraceuticals6020025

[20] Wu M, Xiong Y, Zhu L, ZeWeng YZ, Li R, Zhang J, et al. A comprehensive method for quality evaluation of Tibetan medicine Synotis solidaginea by integrating UHPLC-Q-Orbitrap MS chemical profiling and UHPLC-DAD multi-components quantification. *J Pharm Biomed Anal*, **240**, 115983 (2024) https://doi.org/10.1016/j.jpba.2024.115983

[21] Petrásková L, Káňová K, Biedermann D, Křen V, Valentová K. Simple and rapid HPLC separation and quantification of flavonoids and flavonolignans in silymarin. *Foods*, **9**(2), 116 (2020) https://doi.org/10.3390/foods9020116

[22] Amber B, Ravindra MK, Christine J. HPLC method development and validation for the quantification of related impurities in testosterone cypionate active pharmaceutical ingredient. *Indian J Pharm Educ Res*, **56**(1), 240–246 (2022) https://doi.org/10.5530/ijper.56.1.28

[23] Ha ES, Han DG, Seo SW, Kim JM, Lee SK, Sim WY, et al. A simple HPLC method for the quantitative determination of silybin in rat plasma: application to a comparative pharmacokinetic study on commercial silymarin products. *Molecules*, **24**(11), 2180 (2019) https://doi.org/10.3390/molecules24112180

[24] Kagawad P, Gharge S, Jivaje K. Quality control and standardization of quercetin in herbal medicines by spectroscopic and chromatographic techniques. *Futur J Pharm Sci*, **7**, 176 (2021) https://doi.org/10.1186/s43094-021-00327-y

[25] Parab GV, Mannur VK, Hullatti K. Quality assessment and analytical quality by design-based RP-HPLC method development for quantification of piperine in Piper nigrum L. *Futur J Pharm Sci*, **8**, 16 (2022) https://doi.org/10.1186/s43094-022-00405-9

[26] Chaudhari VS, Borkar RM, Murty US, Banerjee S. Analytical method development and validation of reverse-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous quantifications of quercetin and piperine in dual-drug loaded nanostructured lipid carriers. *J Pharm Biomed Anal*, **186**, 113325 (2020) https://doi.org/10.1016/j.jpba.2020.113325

[27] Setyaningsih D, Santoso YA, Hartini YS, Murti YB. Isocratic HPLC for simultaneous quantification of curcumin and piperine in a microparticle formulation containing Curcuma longa and Piper nigrum. *Heliyon*, **7**(3), e06541 (2021) https://doi.org/10.1016/j.heliyon.2021.e06541

[28] Rodriguez EL, Zhang C, Woolfork AG, Li Z, Bi C, Kaur H, et al. Analysis of curcumin and piperine in biological samples by reversed-phase liquid chromatography with multi-wavelength detection. *J Chromatogr B*, **1162**, 122487 (2021) https://doi.org/10.1016/j.jchromb.2020.122487

[29] Marcon MV, Schafka VM, Justus B, Nadal JM, Schnekenberg CM, Shirabayashi JB, et al. Co-quantification of curcuminoids and piperine by RP-HPLC/DAD from spray-dried microparticles for routine quality control. *Braz Arch Biol Technol*, **68**, e25240301 (2025) https://doi.org/10.1590/1678-4324-2025250760

[30] Noshadi B, Burgaz V, Pozharani LB. Development and validation of an RP-HPLC method for the simultaneous quantification of metformin and curcumin. *Cyp J Med Sci*, **10**(1), 4–7 (2025) https://doi.org/10.4274/cjms.2024.2024-132

[31] Desai NE, Momin M, Singh UP, Khan TA, Sherje A. Analytical method development and validation for simultaneous estimation of curcumin and cyclosporine by RP-HPLC. *Int J Pharm Pharm Sci*, **11**(2), 26–33 (2019) https://doi.org/10.22159/ijpps.2019v11i2.28975