RESEARCH PAPER

Development and Validation of RP-HPLC Method for Simultaneous Estimation of Resveratrol, Quercetin, and Ascorbic Acid in Combined Pharmaceutical Formulation

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ABSTRACT

The present research work describes the development and validation of a reliable Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method for simultaneous estimation of Resveratrol, Quercetin and Ascorbic Acid in combined pharmaceutical formulation. The method was optimized using a C18 column with an appropriate mobile phase composition (Methanol: Water, 50:50), flow rate of 0.800 mL/min and detection wavelength to achieve efficient separation and accurate quantification.

Detection was carried out at 210 nm, where all three analytes showed adequate absorbance. The chromatographic conditions provided well-resolved peaks with acceptable retention times and system suitability parameters. The developed method was validated as per ICH Q2 (R1/R2) guidelines. Linearity was established over selected concentration ranges 5-25 µg/mL of Resveratrol, 1-5 µg/mL Quercetin and 6-30 µg/mL Ascorbic Acid with correlation coefficients 0.9995 for three drugs. LOD and LOQ for Resveratrol, Quercetin and Ascorbic Acid was found to be 1.59, 0.32 and 1.91 and 4.83, 0.97 and 5.79 µg/mL respectively. Accuracy was confirmed through recovery studies, which results found to be 97.17-98.90 for Resveratrol, 97.50-98.77 for Quercetin and 95.73-97.88 for Ascorbic Acid. Percentage assay was found to be 98.07 for Resveratrol, 97.48 for Quercetin and 98.53 for Ascorbic Acid. Robustness studies confirmed that minor variations in analytical conditions did not significantly affect the performance of the method.

Overall, the developed RP-HPLC method is simple, accurate, and suitable for routine analysis and quality control of combined formulations containing Resveratrol, Quercetin, and Ascorbic Acid.

Keywords: Resveratrol, Quercetin, Ascorbic Acid, RP-HPLC, Analytical Method Development (AMD) and Analytical Method Validation (AMV), Simultaneous Estimation, Pharmaceutical Analysis

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INTRODUCTION

Analytical methods are essential tools for the qualitative and quantitative determination of chemical substances in pharmaceutical analysis, environmental monitoring, food safety, and biomedical research. The selection of an appropriate analytical technique depends on factors such as sample characteristics, sensitivity, accuracy, precision, and the intended application. Modern analytical techniques, particularly chromatographic and spectroscopic methods, provide high sensitivity, selectivity, and reproducibility, making them indispensable for quality control and regulatory compliance in the pharmaceutical industry.

Analytical method development is a systematic process aimed at establishing reliable, accurate, and robust procedures for the determination of target analytes. It involves selecting suitable chromatographic conditions, including stationary phase, mobile phase composition, flow rate, column type, detection wavelength, and operating temperature, followed by optimization to achieve adequate resolution, sensitivity, and analysis time. The development process often requires iterative modifications to overcome challenges associated with complex sample matrices, degradation products, and interfering impurities while ensuring consistent analytical performance. [1,2]

Method validation confirms that an analytical procedure is suitable for its intended purpose and consistently generates reliable and reproducible results. According to ICH guidelines, validation involves the assessment of key performance characteristics including specificity, accuracy, precision, linearity, range, limit of detection (LOD), limit of quantification (LOQ), and robustness. Accuracy reflects the closeness of measured values to the true value, whereas precision evaluates the repeatability and intermediate precision of the method. Specificity demonstrates the ability of the method to accurately measure the analyte in the presence of impurities, degradation products, or excipients. Linearity establishes a direct relationship between analyte concentration and detector response within a defined range, while LOD and LOQ determine the minimum detectable and quantifiable concentrations, respectively. Robustness assesses the method's capacity to remain unaffected by small but deliberate variations in analytical conditions. Collectively, these validation parameters ensure the reliability, consistency, and regulatory acceptability of analytical methods used in pharmaceutical research and quality assurance. [3,4,5,6]

Resveratrol is a natural polyphenolic compound with the IUPAC name 5-[(E)-2-(4-hydroxyphenyl) ethenyl] benzene-1,3-diol. It has the molecular formula $C_{14}H_{12}O_3$ and a molecular weight of 228.24 g/mol. It appears as an off-white powder and is soluble in ethanol, DMSO, and dimethylformamide (DMF), with limited solubility in phosphate-buffered saline (PBS). It has a melting point of 261–263 °C, a log P value of 3.10, pKa values of 8.8, 9.8, and 11.4, and a CAS number of 31100-06-8. Resveratrol is a natural antioxidant

with anti-inflammatory, cardioprotective, neuroprotective, and anticancer activities. It acts by activating SIRT1 and AMPK pathways while inhibiting NF-κB signaling, thereby reducing oxidative stress and inflammation. Common side effects at high doses include nausea, diarrhea, abdominal discomfort, headache, and dizziness. [7]

Quercetin is a naturally occurring flavonoid with the IUPAC name 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one. It has the molecular formula $C_{15}H_{10}O_7$ and a molecular weight of 302.23 g/mol. It appears as a yellow crystalline powder, is soluble in methanol, ethanol, acetone, and glacial acetic acid, but insoluble in water. It has a melting point of 316–318 °C, a log P value of 1.39, a pKa value of 9.73, and a CAS number of 117-39-5. Quercetin is a natural antioxidant with anti-inflammatory, antiviral, and anticancer activities. It acts mainly by inhibiting quinone reductase 2 (QR2), thereby reducing oxidative stress. Common side effects include headache, nausea, stomach upset, and tingling sensation. [8]

Ascorbic acid (Vitamin C) is a water-soluble vitamin with the IUPAC name (4R,5R)-5-[(1S)-1,2-dihydroxyethyl]-4-hydroxyoxolane-2,3-dione. It has the molecular formula $C_6H_8O_6$ and a molecular weight of 176.12 g/mol. It appears as a white to slightly yellow crystalline powder and is freely soluble in water. It has a melting point of 190–192 °C, a log P value of –1.07 to –1.2, pKa values of 4.2 and 11.6, and a CAS number of 50-81-7. Ascorbic acid is a water-soluble antioxidant used for immune support, collagen synthesis, wound healing, and prevention of oxidative damage. It acts as a reducing agent and enzyme cofactor. Common side effects of excessive intake include nausea, diarrhea, stomach cramps, heartburn, headache, and bloating. [9]

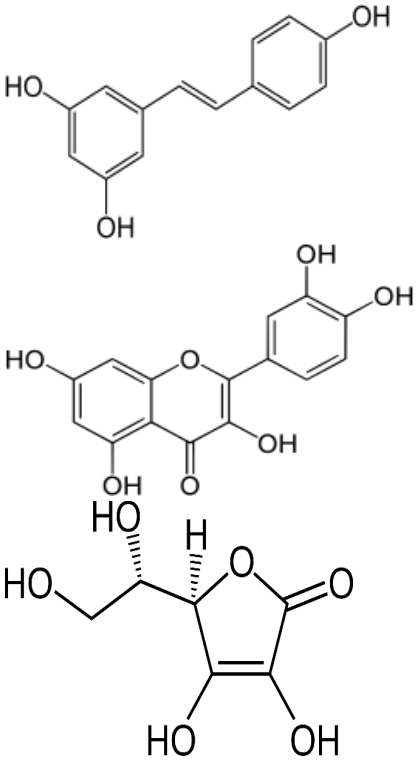

Figure 1 Structure of Resveratrol, Quercetin, Ascorbic acid

Although numerous research and review articles have reported analytical methods for the individual or simultaneous estimation of **resveratrol, quercetin, and ascorbic acid** in bulk drugs, dietary supplements, herbal extracts, food matrices, and biological samples, a detailed review of the available literature indicates that **no RP-HPLC method has been reported for the simultaneous estimation of these three compounds in a combined pharmaceutical formulation**. Most published methods focus on the analysis of either individual compounds or binary combinations under different analytical conditions. Furthermore, no marketed pharmaceutical formulation containing this specific combination has been reported, highlighting the novelty of this work.

Therefore, the present study aims to develop and validate a simple, rapid, accurate, precise, and stability-indicating RP-HPLC method for the simultaneous estimation of resveratrol, quercetin, and ascorbic acid in a newly proposed combined pharmaceutical formulation. The developed method will be validated in accordance with ICH guidelines and is expected to provide a reliable analytical tool for routine quality control, formulation development, and future research involving this novel combination.

This version is suitable for the **novelty statement** or the concluding paragraph of the literature review in a thesis or research manuscript. [10-33]

MATERIAL AND METHOD

1.1 Chemicals

Resveratrol was procured from **BLD Pharma Pvt. Ltd.**, while quercetin was obtained from **Oxford Lab Fine Chem LLP**. All chemicals and reagents used in the study were of analytical or HPLC grade and were used without further purification.

1.2 Instrumentation

A **Shimadzu UV-1900i double-beam UV–Visible spectrophotometer** equipped with a photodiode detector and UV Probe version 2.7.1 software was used for spectroscopic analysis over a wavelength range of **190–1100 nm**. Accurate weighing of chemicals was carried out using a **Mettler Toledo analytical balance** with a sensitivity of **0.1 mg**. Chromatographic analysis was performed using a **Shimadzu binary gradient HPLC system (Model: SPD-10 AVP)** equipped with a **UV–Visible detector**, **deuterium lamp** as the light source, and **LC Solution** software. The detector operated over a wavelength range of **190–800 nm**. Functional group characterization and compatibility studies were conducted using a **Shimadzu IR Spirit Fourier Transform Infrared (FTIR) spectrometer** operating in **Attenuated Total Reflectance (ATR)** mode over a scanning range of **600–4000 cm^{-1}**, with **LabSolutions** software and a resolution of **0.01 cm^{-1}**.

1.3 Chromatographic Condition

Chromatographic separation was performed in isocratic mode using a mobile phase consisting of a mixture of Methanol: Water (50:50 v/v). The mobile phase was delivered at a constant flow rate of 0.8mL/min through an ODS C18 stationary phase column (250 *4.6 mm, 5 µm particle size). The injection volume for all samples and standards was 20 µL. A standard stock solution containing a mixture of Resveratrol, Quercetin and Ascorbic Acid, was prepared in methanol The solutions were scanned individually in the UV region between 200-800nm using a UV-Visible spectrophotometer and the observed spectra were overlain. A suitable common wavelength 254nm was selected as the detection wavelength for HPLC analysis because, it is a common wavelength at which all three compound exhibit adequate absorbance, providing good sensitivity and simultaneous detection during chromatographic separation.

1.4 Preparation of Stock Solution

Resveratrol Standard Stock Solution

The standard stock solution of Resveratrol was prepared by accurately weighing 50 mg of Resveratrol reference standard and transferring it into a 100 ml volumetric flask. The drug was dissolved and the volume was made up to the mark with methanol to obtain a master stock solution with a concentration of 500 µg/ml. From this master stock, an aliquot of 0.1 ml was transferred to a 10 ml volumetric flask and diluted to volume with methanol to achieve a working standard solution of 5 µg/ml.

Quercetin Standard Stock Solution

The standard stock solution of Quercetin was prepared by accurately weighing 10 mg of Quercetin reference standard and transferring it into a 100 ml volumetric flask. The analyte was dissolved and diluted to volume with methanol to yield a master stock concentration of 100 µg/ml. A working standard solution containing 1 µg/ml was subsequently prepared by pipetting 0.1 ml of the master stock solution into a 100 ml volumetric flask and making up the volume with methanol.

Ascorbic acid Standard Stock Solution:

The standard stock solution of Ascorbic acid was prepared by accurately weighing 60 mg of reference standard into a 100 ml volumetric flask, dissolving it in methanol, and bringing the total volume to the mark to attain a master stock solution of 600 µg/ml. To prepare the working standard solution at a concentration of 6 µg/ml, a 0.1 ml aliquot of the master stock solution was transferred into a 100 ml volumetric flask and diluted to the mark with methanol.

Preparation of standard stock solution of mixture

Accurately weighed amounts of 50 mg Resveratrol, 10 mg quercetin, and 60 mg Ascorbic acid were transferred into a single 100 ml volumetric flask and dissolved in methanol, resulting in a stock mixture consisting of 500 µg/ml Resveratrol, 100 µg/ml quercetin, and 600 µg/ml Ascorbic acid. A 0.1 ml aliquot of this mixed solution was further diluted to 100 ml with methanol to obtain a working standard mixture with final concentrations of 5 µg/ml Resveratrol, 1 µg/ml quercetin, and 6 µg/ml Ascorbic acid.

1.5 Validation

The method was validated as per ICH Q2 (R2) guideline for parameter like linearity, precision, LOD, LOQ, accuracy, robustness and system suitability Parameters.

1.5.1 System Suitability Parameters

A system suitability test was an integral part of the method development to verify that the system is adequate for the analysis of Resveratrol, Quercetin and Ascorbic acid to be performed. The system suitability test of the chromatography system was performed with Six replicate injections of Standard solution of both drug in mixture. Retention time, tailing factor, resolution factor, and theoretical plates were determined.

1.5.2 Linearity

The linearity of an analytical method was carried out to check its ability to give test results within given range. Different concentration of Resveratrol (5-25 µg/ml), Quercetin (1-5 µg/ml) and Ascorbic acid (6 -30 µg/ml) were prepared in to 10 ml volumetric flask with mobile phase as a diluent and inject each concentration in mixture form in HPLC system and chromatogram were recorded. A calibration graph was plotted as concentration (µg/ml) verses chromatographic peak area (mV).

1.5.3 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

1.5.4 Robustness

The robustness of the method was confirmed by making small deliberate changes in the flow rate, mobile phase, pH, temperature and wavelength. The effect of these changes was recorded and % relative standard deviation was calculated.

1.5.5 LOD and LOQ

The LOD is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels and LOQ of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy.

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

1.5.6 Accuracy

Accuracy of the analytical method has been performed by spiking of blank with the standard. Spiking of the sample was performed at 50, 100 and 150 % of the target concentration 5,15,25µg/ml for Resveratrol, 1,3,5 µg/ml for Quercetin and 6,18,30 µg/ml for Ascorbic acid. The solution was injected and peak area was recorded.

2. RESULTS AND DISCUSSION

2.1 Selection of Wavelength

As better intensity was observed at 254 nm for all the components, it was selected as analytical wavelength.

2.2 Optimization of Mobile Phase

The mobile phase consisting of Methanol: Water (50:50 v/v) was found to be optimal for method development, yielding well-resolved, symmetric peaks for all three analytes. Under these optimized chromatographic conditions, Resveratrol, Quercetin, and Ascorbic acid were successfully separated with excellent resolution. The retention times for Resveratrol, Quercetin, and Ascorbic acid were found to be 3.446 min, 6.058 min, and 2.177 min, respectively, with all system suitability parameters falling within acceptable limits.

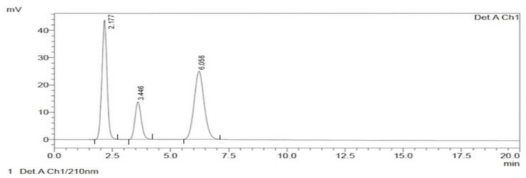

Figure 2 Optimization of chromatographic condition.

2.3 VALIDATION

2.3.1 System Suitability Parameters Result

Sr No.	Parameters	Results		
		RESVERATROL	QUERCETIN	ASCORBIC ACID
1	Tailing factor	1.679±0.03	0.922±0.01	0.988±0.02
2	Resolution	3.897±0.08	4.078±0.07	-
3	Theoretical plates	4198.52±104.4802	4404.734±91.17967	2513.401±46.91449
4	Retention time	3.454±0.05	6.191±0.09	2.011±0.03

Table 1 System Suitability Parameters Result

The system suitability test was evaluated to verify the performance and reproducibility of the chromatographic system, and the results are summarized in Table. The tailing factors for Resveratrol, Quercetin and Ascorbic acid were found to be 1.679±0.03, 0.922±0.01, and 0.988 ±0.02, respectively, indicating excellent peak symmetry. High chromatographic efficiency was observed with high theoretical plate counts, particularly for Resveratrol (4198.52±104.48) and Quercetin (4404.73±91.179). Furthermore, the system exhibited robust separation with a resolution of 4.078±0.07 between the adjacent peaks and highly reproducible retention times for all three analytes. These outcomes confirm that the developed method satisfies all regulatory system suitability requirements for simultaneous estimation.

2.3.2 Linearity

The linearity of the proposed analytical method was evaluated by constructing calibration curves over a specific concentration range for each analyte. The method exhibited an excellent linear

response, with calibration curves found to be linear in the range of 5-25 µg/mL for resveratrol, 1-5 µg/mL for quercetin, and 6-30 µg/mL for ascorbic acid. The corresponding correlation coefficients (R^2) were found to be 0.999, 0.999, and 0.999, respectively, confirming a highly reliable relationship between peak area response and concentration across all evaluated ranges.

Sr. no	Resveratrol		Quercetin		Ascorbic acid	
	Concentration	Area	Concentration	Area	Concentration	Area
1	5	88022± 1560.17	1	180945± 2604.05	6	243928± 3612.09
2	10	178712± 2404.33	2	367374± 4372.2	12	495244± 5027
3	15	266734± 2935.86	3	548320± 10849.2	18	739170± 11324.5
4	20	360091± 3898.88	4	740232± 9712.92	24	997880± 14331.5
5	25	440111± 5615.32	5	904728± 15282.19	30	1219631± 22314.91
Regression Equation	y = 17711x + 1066.9		y = 182042x + 2192.6		y = 40901x + 2958	
R^2	R^2 = 0.999492		R^2 = 0.999492		R^2 = 0.999492	

Table 2 Concentration and Area for linearity

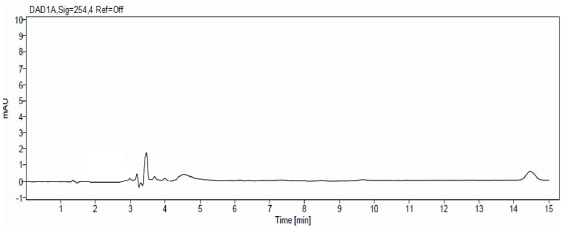

Figure 3 Linearity chromatogram (Blank)

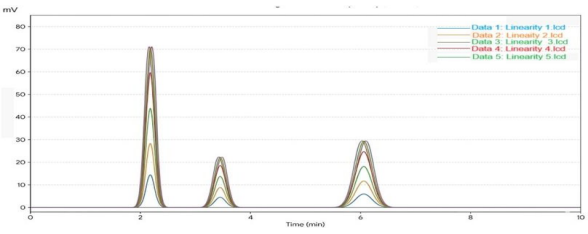

Figure 4 Linearity chromatogram Resveratrol, Quercetin and Ascorbic Acid

2.3.3 Precision

2.3.3.1 Repeatability

Table 3 Result Table for Repeatability

Concentration	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL
Resveratrol (Mean±SD)	88422± 1673.95	178712 ± 2823.26	252533.6± 2974.63	498851.6± 8505.46	747219.4± 14866.49
	1 µg/mL	2 µg/mL	3 µg/mL	4 µg/mL	5 µg/mL
Quercetin (Mean±SD)	181365 ± 3266.20	366974 ± 4058.64	548320 ± 10528.43	740234 ± 9777.57	906928 ± 15523.73
	6 µg/mL	12 µg/mL	18 µg/mL	24 µg/mL	30 µg/mL
Ascorbic acid (Mean±SD)	480850.8± 9346.017	975008.8± 9710.704	142116 7± 24841.96	191751 1± 25568.51	238672 9± 37326.91

The repeatability (injection precision) of the method was evaluated by analyzing six replicates (n = 6) of sample solutions across five different concentration levels for each analyte. The relative standard deviation (%RSD) values were found to be between 1.18%–1.99% for Resveratrol, 1.10%–1.92% for Quercetin, and 0.99%–1.94% for Ascorbic acid. All the calculated %RSD values were well below the stringent regulatory threshold of 2.0%, demonstrating an exceptionally high degree

of instrument precision and measurement repeatability.

2.3.3.2 Intraday and Interday Precision

The precision of the developed method was verified by determining intraday and interday variations at three different concentration levels for each analyte. The relative standard deviation (%RSD) values for intraday precision ranged from 0.93 % to 1.87 % for resveratrol, 1.06% to 1.61% for quercetin, and 1.96% to 1.99% for ascorbic acid. For interday precision, the %RSD values were found between 0.96%–1.72%, 1.17%–1.70%, and 1.69%–1.90% for resveratrol, quercetin, and ascorbic acid, respectively. All obtained %RSD values were well within the acceptable regulatory limit of less than 2.0%, confirming the excellent reproducibility and ruggedness of the method.

2.3.4 Robustness

Following parameters were altered one by one for determination of robustness of the method and their effect was observed by comparing with the standard preparation. Optimized flow rate was 0.8 mL/min. Mobile phase composition(±2mL), in optimized ratio. Determinations of Res+ Que +Asc+= 15+3+18 µg/mL for each alteration were carried out and RSD was measured.

Table 4 Result table for robustness

Parameters	Level of Change	Observation		
		Resveratrol		
		Peak Area	SD	%RSD
Flow Rate	0.7 mL/min	758502	1443.93	0.19
	0.8 mL/min	761219		
	0.9 mL/min	759013		
Composition of Mobile Phase	48:52	748502	5456.03	0.72
	50:50	751219		
	52:48	759013		

Parameters	Level of Change	Observation		
		Quercetin		
		Peak Area	SD	%RSD
Flow Rate	0.7 mL/min	239290	928.97	0.39
	0.8 mL/min	240986		
	0.9 mL/min	239481		
Composition of Mobile Phase	48:52	243290	1696.46	0.70
	50:50	240986		
	52:48	239981		

Parameters	Level of Change	Observation		
		Ascorbic Acid		
		Peak Area	SD	%RSD
Flow Rate	0.7 mL/min	517568	1496.77	0.29
	0.8 mL/min	514703		
	0.9 mL/min	516887		
Composition of Mobile Phase	48:52	512968	4458.41	0.86
	50:50	521703		
	52:48	518887		

2.3.5 LOD and LOQ

The sensitivity of the developed method was established by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) based on the standard deviation of the intercept (sigma) and the mean slope (S) of the calibration curves. The LOD values were found to be 1.59 µg/ml for resveratrol, 0.32 µg/ml for quercetin, and 1.91 µg/ml for ascorbic acid. The corresponding LOQ values were determined as 4.83 µg/ml 0.97 µg/ml, and 5.97 µg/ml for resveratrol, quercetin, and ascorbic acid, respectively. These low detection and quantification limits demonstrate that the proposed RP-HPLC method possesses high sensitivity for the simultaneous tracking of all three compounds.

2.3.6 Accuracy

The accuracy of the method was determined by calculating % recovery. Known amount of standard spike at three different level (50%, 100%, 150 %) to a pre quantified sample solution and percentage recovery was found within range at 98%-102 %. Calculated the percentage recovery.

Table 5 Result table for Accuracy

Drug	Level of spiking	Amount of drug added (µg/mL)	Amount of drug recovered (µg/mL)	% Mean recovery
Resveratrol	Un-spiked	-	-	-
	Spiked 50 %	5	4.89± 0.02	97.94±0.05
	Spiked 100 %	15	14.84±0.05	98.90±0.36
	Spiked 150 %	25	24.28±0.35	97.14±1.42
Quercetin	Un-spiked	-	-	-
	Spiked 50 %	1	0.97±0.02	97.50±1.71
	Spiked 100 %	3	2.96±0.01	98.77±0.37
	Spiked 150 %	5	4.90±0.07	98.05±1.32
Ascorbic Acid	Un-spiked	-	-	-
	Spiked 50 %	6	5.74±0.05	95.73±0.89
	Spiked 100 %	18	17.62±0.15	97.88±0.82
	Spiked 150 %	30	29.05±0.54	96.83±1.80
(n=3 determinations at each level)				

3. CONCLUSION

From this study, it can be concluded that a simple, rapid, accurate, and precise RP-HPLC method was successfully developed for the simultaneous estimation of resveratrol, quercetin and ascorbic acid. The statistical parameters, system suitability, and assay results confirm the high efficiency and reproducibility of the method. The proposed method was fully validated in accordance with the ICH Q2(R2) guidelines, with all evaluated parameters falling well within the established acceptance criteria. Consequently, this method proves to be highly reliable and well-suited for the routine quality control analysis of these three active components in pharmaceutical and herbal formulation.

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1. LIST OF FIGURES

2. LIST OF TABLES