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ORIGINAL ARTICLE

Design Approach Assisted RP-HPLC Method Development and Validation of Phytomarker Andrographolide in Polyherbal Formulation

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ABSTRACT

In the present work, a reliable, sensitive and reproducible RP-HPLC method for determination of AG was developed and validated by means of AQbD concept. Flow rate, composition of the mobile phase and injector volume of samples taken were chosen as the independent variables, whereas the retention time, the number of theoretical plates and the tailing factor as responses. The chromatographic separation was performed on a Chromatopak C18 (150 mm x 4.6 mm, 5 µm) column at 40 C° using mobile phase included acetonitrile and 0.1% orthophosphoric acid (OPA) with the ratio of 33:67 (v/v) with the flow rate of 1 mL/min in an isocratic mode. Detection was performed with a PDA detector at 231 nm. The retention time of AG was about 5.5 min. The method was found to be linear, sensitive with LOD and LOQ of 0.810 and 2.455 µg/mL. Method validation was carried out in compliance with ICH Q2(R1) that confirmed the accuracy, precision and robustness of the method. The developed method is applicable for the routine analysis of AG in the herbal formulation such as Nilavembu Kudineer.

Keywords: Andrographolide, RP-HPLC, AQbD, Box-Behnken Design, Method Validation, Nilavembu Kudineer

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INTRODUCTION

A natural substance called andrographolide is present in the plant Andrographis paniculata, also referred to as "Kalmegh" or "King of Bitters." [1] The plant's bitter flavor and therapeutic qualities are primarily due to its labdane diterpenoid. There are numerous biological effects of andrographolide, a diterpene lactone, including hepatoprotective, immunomodulatory, cytotoxic, neuroprotective, antibacterial, and antioxidant properties. [2] A naturally occurring substance having the chemical formula $C_{20}H_{30}O_5$ is

andrographolide. It can also have an impact on the cardiovascular system, improving cardioprotection and reducing cholesterol levels. [4] (3*E*,4*S*)-3-[2-[(1*R*,4*aS*,5*R*,6*R*,8*aS*)-6-hydroxy-5-(hydroxymethyl)-5,8*a*-dimethyl-2-methylidene-3,4,4*a*,6,7,8-hexahydro-1*H*-naphthalen-1-yl] ethylidene]-4-hydroxyoxolan-2-one. These qualities make it a popular treatment for infections, fevers, liver issues, and other ailments in traditional medical systems like Ayurveda and Traditional Chinese Medicine. [5] *Nilavembu Kudineer* is a classical polyherbal Siddha formulation, traditionally prescribed for the treatment of fever and viral infections, including dengue and chikungunya. [6] It contains *Andrographis paniculata* as a key ingredient, among other herbs. Due to its widespread use during epidemic outbreaks, there is a growing need for standardized analytical methods to ensure consistent quality, safety, and efficacy of formulations containing Andrographolide. [7] HPLC is an efficient and popular approach for separating, identifying, and quantifying active ingredients in pharmaceutical and herbal research. [8] However, a methodical and scientific approach must be used to develop and verify HPLC procedures in order to guarantee dependability and reproducibility. [9] Critical method variables are identified and controlled by Analytical Quality by Design (AQbD), which provides an organized framework for method development. [10] In order to create a reliable and well-understood method, this technique places a strong emphasis on risk assessment, design of experiments (DoE), and method operable design region (MODR). In addition to guaranteeing regulatory compliance, AQbD makes method transfer and lifecycle management easier. [11] In this context, the present study aims to develop and validate a reverse-phase HPLC method for the quantification of Andrographolide in herbal extracts and Nilavembu Kudineer, following AQbD principles and ICH Q2(R1) guidelines. The method is designed to be accurate, precise, and suitable for routine quality control applications. [12]

MATERIAL AND METHODS

Andrographolide was procured from Yucca Enterprises Mumbai, India, all the solvents Acetonitrile and Ortho phosphoric acid HPLC Grade were procured from Merck

Instruments used

The chromatographic analysis was performed on the Shimadzu HPLC-ultravoilet (UV) system. The instrument consisted of a quaternary pump gradient LC 20AD, the injector used was an autosampler SIL 20AC, the column oven consisted of CTO 10 AS, the column is a C18, 100 A°, (5 µm, 4.6 × 150mm) and the detector was a UV detector SPD M 20 A (Shimadzu, Japan).

Preparation of Standard and sample solution

Andrographolide 1 mg was precisely measured and taken in a VF of 10 ml and the contents were solubilized in mobile phase consisting of ACN and OPA. The volume was increased with diluent solution after sonification for 5 mins to bring the conc. of 100 µg/ml.

Fractionation of Phytoconstituents Andrographolide from Nilavembu Kudineer (NK)

A separating funnel was filled with a 10 mL aliquot of NK. After adding 30 mL of hexane, the mixture was gently shaken for five minutes and left to stand for thirty minutes, allowing two separate layers to form. After being separated, the topmost layer of hexane was gathered in a beaker, designated as the hexane fraction, and placed aside. Following a similar procedure, the remaining aqueous layer was extracted using chloroform, and the chloroform layer was subsequently collected independently. The residue fraction was then removed by adding 10 mL of mobile phase to the remaining layer and extracting it. A rotary evaporator was employed to evaporate this residual fraction, which was then used to prepare the sample.

Preparation of sample solution

Sample solution was established by taking 1 mg of residual fraction and mobile phase ACN and OPA.

System suitability

The chromatographic system's performance was confirmed through system suitability testing before sample analysis. After being prepared at a known concentration, a standard solution of andrographolide was injected six times in a row. The following parameters were noted: theoretical plates (N), peak area, retention period, resolution, and tailing factor. Acceptance criteria were as follows: theoretical plates > 2000, tailing factor < 2.0, and RSD of peak area ≤ 2%. The system was only deemed appropriate if every parameter satisfied the established standards.[13]

Linearity

By preparing a number of standard solutions of andrographolide at various concentrations—typically between 50 and 150 µg/ml of the target concentration—the linearity of the procedure was assessed. The HPLC system was filled with three injections of each concentration, and the peak regions were noted. The correlation coefficient (R^2) was computed by plotting a calibration curve between peak area and concentration. A strong linear association was shown by a value of R2 ≥ 0.999.[14]

Precision

Repeatability (Intra-day precision): A standard solution of andrographolide at 100% concentration was prepared and injected six times within the same day under identical conditions. The relative standard deviation (RSD) of peak areas was calculated. [15]

Accuracy

A recovery study was used to assess the method's accuracy. Three concentration levels of the andrographolide standard—50%, 100%, and 150% of the target level—were added to a pre-analyzed sample matrix. Every level was examined three times. The recovery % was computed. Recovery rates between 98 and 102% on average were deemed satisfactory. [16]

Robustness

To determine robustness, essential chromatographic parameters were purposefully changed within a narrow range to gauge the method's dependability under slight modifications. The following parameters were changed: column temperature: ±2°C, detection wavelength: ±2 nm, mobile phase composition: ±2%, and flow rate: ±0.1 mL/min. Every adjusted condition was examined using a standard solution of andrographolide, and the parameters pertaining to system appropriateness were tracked. If there were no appreciable changes to the retention duration, resolution, or peak symmetry, the approach was deemed robust. [17]

LOD & LOQ

Using the slope (S) of the calibration curve and the response standard deviation (σ), LOD and LOQ were calculated. From the y-intercepts of the regression lines of several calibration curves, the standard deviation was calculated. By injecting solutions at the appropriate concentrations and making sure that the signal-to-noise ratios were roughly 3:1 and 10:1, respectively, the computed LOD and LOQ values were confirmed. [18]

RESULT

AQBD method development

The table lists 17 runs with different combinations of A, B, and C, showing how these factors affect the responses. Retention time varied from ~5.47 min to 7.82 min depending on conditions. Theoretical plates ranged ~15,210 to 21,755, showing efficiency changes. Tailing factor stayed mostly close to 1.1–1.2, suggesting reasonable symmetry. This design helps identify which factors significantly impact method performance. Design Expert software (Design-Expert 13, Stat Ease) was used. Table 1summarizes Factors and responses selected in BBD design for Andrographolide. The significant components B (flow rate) of retention time have a strong influence (F value = 35.47, p = 0.0006), whereas C (injection volume) has a significant borderline (p = 0.0503), and B & C have a significant interaction (p = 0.0091). Quadratic terms A, AB, and AC, as well as A^2, B, and C^2, are not significant. Retention duration is greatly influenced by the flow rate and the BC interaction, and the lack of fit p value of 0.1517 indicates that the model does not fit the data well. [19] Table 2 represents Response 1 Retention time**.** Figure 1 Displays the 3D Plot, Counter Plot and Perturbation plot of Andrographolide on (Retention Time). Key elements of theoretical pates the quadratic terms A, C, and interactions are mostly non-significant, but B (flow rate) is highly significant ($p < 0.0001$), as is B^2. poor fit; the model is sufficient because the p value of 0.3675 is not significant. The primary factor governing efficiency is the flow rate (including linear and quadratic impacts). [20] The summary of Response 2 Theoretical Plate is shown in Table 3. The 3D Plot Counter Plot Theoretical Plate and Perturbation plot of Andrographolide are represented in Figure 2. Important aspects related to the tailing factor B stands for flow rate. All the interactions A (linear) and C are non-significant, with the p values of 0.0172, A^2, and B^2 being 0.0153 and 0.0332, respectively. Since the p-value for lack of fit (0.2208) is not significant, the model fits well. Flow rate and curvature effects (A^2, B^2) influence peak symmetry. [21] Table 4 shows Response 3 Tailing Factor. Figure 3 shows the 3D plot, Counter Plot Tailing Factor and Perturbation plot of Andrographolide. Figure 4 shows the desirability Plot of Andrographolide and Figure 5 displays the overlay plot of Andrographolide.

Validation

The validation for andrographolide was evaluated by determining system suitability, precision, robustness study, linearity, LOD & LOQ and accuracy. Figure 6 represents standard Chromatogram of Andrographolide and Figure 7 represents sample Chromatogram of Andrographolide.

Precision

The % RSD of 0.12% from six replicate injections confirms excellent precision. All peak areas are consistent, indicating minimal instrumental or procedural variation. [22] Precision results for andrographolide are summarized in Table 5.

Robustness

Evaluated under deliberate changes in column temperature and flow rate. All % RSD values were below 0.5%, showing high method robustness and resilience to small variations. [23] The robustness results for andrographolide are summarized in table 6.

Linearity

Andrographolide showed linear response in the 50–150 µg/mL range. The Correlation coefficient (R^2 = 0.9954) confirms strong linearity. The Slope and intercept values support a reliable calibration curve. [24] The linearity results for Andrographolide are displayed in Table 7.

LOD & LOQ

LOD = 0.810 µg/mL; LOQ = 2.455 µg/mL. Indicates the method's high sensitivity and suitability for detecting low levels of Andrographolide. [25] LOD & LOQ for Andrographolide results are summarized in Table 8.

Accuracy

Recovery studies at 50%, 100%, and 150% levels yielded a mean recovery of 100.04%. This confirms that the method is accurate and unbiased across different concentration levels. [26] Table 9 represents accuracy results for Andrographolide.

DISCUSSION

The AQbD-driven approach of the RP-HPLC for Andrographolide demonstrated robust and optimized chromatographic performance. The design of experiments (DoE) facilitated a comprehensive understanding of the influence of critical factors include flow rate, composition of mobile phase and injection volume on key analytical responses such as retention time, theoretical plate count, and peak symmetry. [27,28] Retention Time model is Significant (p = 0.0064). Flow rate and injection volume significantly influence RT (p < 0.005). Quadratic terms and interaction terms (especially AC) show moderate effects. The Theoretical Plates Model is statistically significant (p = 0.0375). Flow rate and the interaction BC (Flow Rate × Injection Volume) are significant. Indicates that column efficiency is strongly influenced by these two parameters. The Tailing Factor Model is significant (p = 0.0223). Injection volume (p = 0.0008) is the most critical factor affecting tailing. Interaction AB (Mobile Phase × Flow Rate) also significantly affects symmetry [29] The statistical analysis revealed that injection volume significantly influenced the tailing factor, while flow rate and its interaction with injection volume had notable impacts on column efficiency. The optimized method showed excellent system precision (% RSD = 0.12), linearity (R^2 = 0.9954), and accuracy (mean recovery = 100.04%). [30] Furthermore, the method was proven robust against minor changes in flow rate and column temperature, which is crucial for its application in quality control settings. Low LOD and LOQ values also confirmed the method's high sensitivity, making it suitable for detecting trace levels of Andrographolide in herbal extracts and formulations such as Nilavembu Kudineer.

Table 1: Factors and responses selected in BBD design for Andrographolide

Std	Run	Factor 1 Flow rate	Factor 2 Mobile phase ratio	Factor3 Inj Vol	Response1 Retention time	Res 2 Theoretical Plate	Res3 Tailing Factor
7	1	0.8	67	12	6.992	23250	1.248
2	2	1.2	64	10	6.642	49226	1.201
6	3	1.2	67	8	6.067	71881	1.198
1	4	0.8	64	10	6.833	44342	1.205
9	5	1	64	8	4.6	26393	1.293
3	6	0.8	70	10	5.725	24147	1.283
4	7	1.2	70	10	3.858	17349	1.283
17	8	1	67	10	6.006	10820	1.211
11	9	1	64	12	6.125	58706	1.207
13	10	1	67	10	6.006	10820	1.211
14	11	1	67	10	6.006	10820	1.211
8	12	1.2	67	12	3.842	16122	1.303
5	13	0.8	67	8	6.986	35582	1.265
16	14	1	67	10	6.006	10820	1.211
12	15	1	70	12	5.808	13736	1.282
10	16	1	70	8	7.217	36213	1.251
15	17	1	67	10	6.006	10820	1.211

Table 2: Response 1 Retention time

	Sum of Squares	df	Mean Square	F-value	p-value	
Model	14.36	9	1.60	7.82	0.0064	significant
A-Mobile phase	1.15	1	1.15	5.65	0.0491	
B-Flow rate	3.72	1	3.72	18.24	0.0037	
C-Injection volume	3.76	1	3.76	18.46	0.0036	
AB	0.2611	1	0.2611	1.28	0.2951	
AC	1.59	1	1.59	7.78	0.0269	
BC	0.8921	1	0.8921	4.37	0.0748	
A^2	0.0206	1	0.0206	0.1012	0.7597	
B^2	0.1504	1	0.1504	0.7375	0.4189	
C^2	2.69	1	2.69	13.19	0.0084	
Residual	1.43	7	0.2039			
Lack of Fit	0.3503	3	0.1168	0.4336	0.7408	not significant
Pure Error	1.08	4	0.2693			
Cor Total	15.79	16				

Table 3: Response 2 Theoretical Plate

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.871E+09	6	6.452E+08	3.56	0.0375	significant
A-Mobile phase	3.656E+08	1	3.656E+08	2.02	0.1859	
B-Flow rate	1.270E+09	1	1.270E+09	7.01	0.0244	
C-Injection volume	6.371E+08	1	6.371E+08	3.52	0.0903	
AB	1.385E+07	1	1.385E+07	0.0764	0.7878	
AC	5.390E+08	1	5.390E+08	2.97	0.1153	
BC	1.046E+09	1	1.046E+09	5.77	0.0372	
Residual	1.812E+09	10	1.812E+08			
Lack of Fit	1.991E+08	6	3.318E+07	0.0823	0.9951	not significant
Pure Error	1.613E+09	4	4.033E+08			
Cor Total	5.683E+09	16				

Table 4: Response 3 Tailing Factor

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0198	9	0.0022	5.04	0.0223	significant
A-Mobile phase	0.0003	1	0.0003	0.6057	0.4619	
B-Flow rate	0.0005	1	0.0005	1.10	0.3290	
C-Injection volume	0.0136	1	0.0136	31.17	0.0008	
AB	0.0041	1	0.0041	9.38	0.0183	
AC	0.0002	1	0.0002	0.5153	0.4961	
BC	0.0000	1	0.0000	0.0824	0.7823	
A^2	0.0003	1	0.0003	0.7467	0.4161	
B^2	0.0000	1	0.0000	0.0756	0.7913	
C^2	0.0006	1	0.0006	1.46	0.2663	
Residual	0.0031	7	0.0004			
Lack of Fit	0.0003	3	0.0001	0.1289	0.9380	not significant
Pure Error	0.0028	4	0.0007			
Cor Total	0.0229	16				

Table 5: Precision results for andrographolide

Injection	Area of Andrographolide
Injection 1	2100524
Injection 2	2097275
Injection 3	2100662
Injection 4	2104356
Injection 5	2101286
Injection 6	2103341
Average	2101241
Standard deviation	2477.65
Relative standard deviation	0.12

Table 6: Robustness results for andrographolide

Parameters	Deliberate Changes	Andrographolide	Parameters	Deliberate Changes	Andrographolide
Column Temperature (-)	39⁰ C	2118766	Column Flow Rate (-)	0.9 ml	2354962
		2119159			2355873
		2121539			2354196
Average		2119821	Average		2355010
Standard deviation		1500.465	Standard deviation		839.5441
%RSD		0.07	%RSD		0.04
Column Temperature (+)	41⁰ C	2095858	Column Flow Rate (+)	1.1ml	1930479
		2094149			1935580
		2081529			1933591
Average		2090512	Average		1933217
Standard deviation		7826.295	Standard deviation		2571.02
%RSD		0.37	%RSD		0.13

Table 7: Linearity results for andrographolide

Andrographolide		
S.No	**Concentration (µg/ml)**	**Area**
1	50	1654231
2	75	1854612
3	100	2100524
4	125	2369548
5	150	2658462
	Slope (m)	10093.59
	Std	2477.65
	Intercept	1118116
	Correlation coefficient (R2)	0.9954

Table 8: LOD & LOQ

Parameters	Values
LOD	0.810043
LOQ	2.454676

Table 9: Accuracy results for andrographolide

% Concentration	Area	%Recovery	Mean Recovery
50%	1654231	100.28	100.04
100%	2100524	100.04	
150%	2658462	99.45	

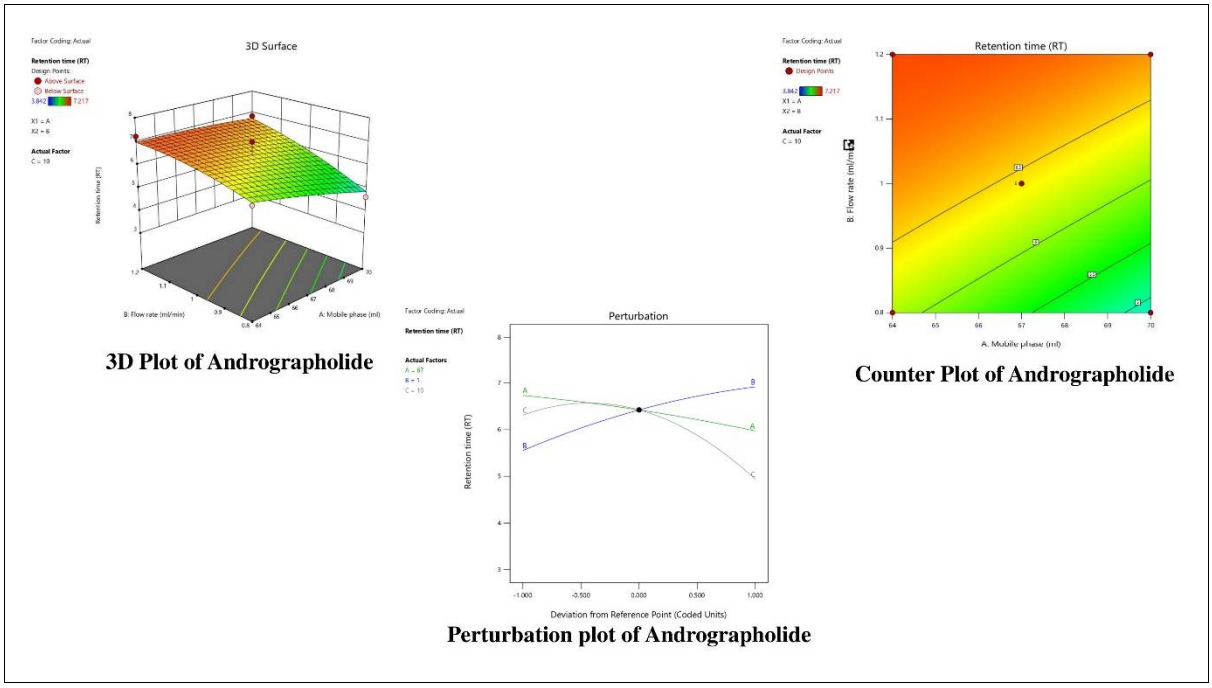


Figure 1: 3D Plot, Counter Plot and Perturbation plot of Andrographolide (Retention Time)

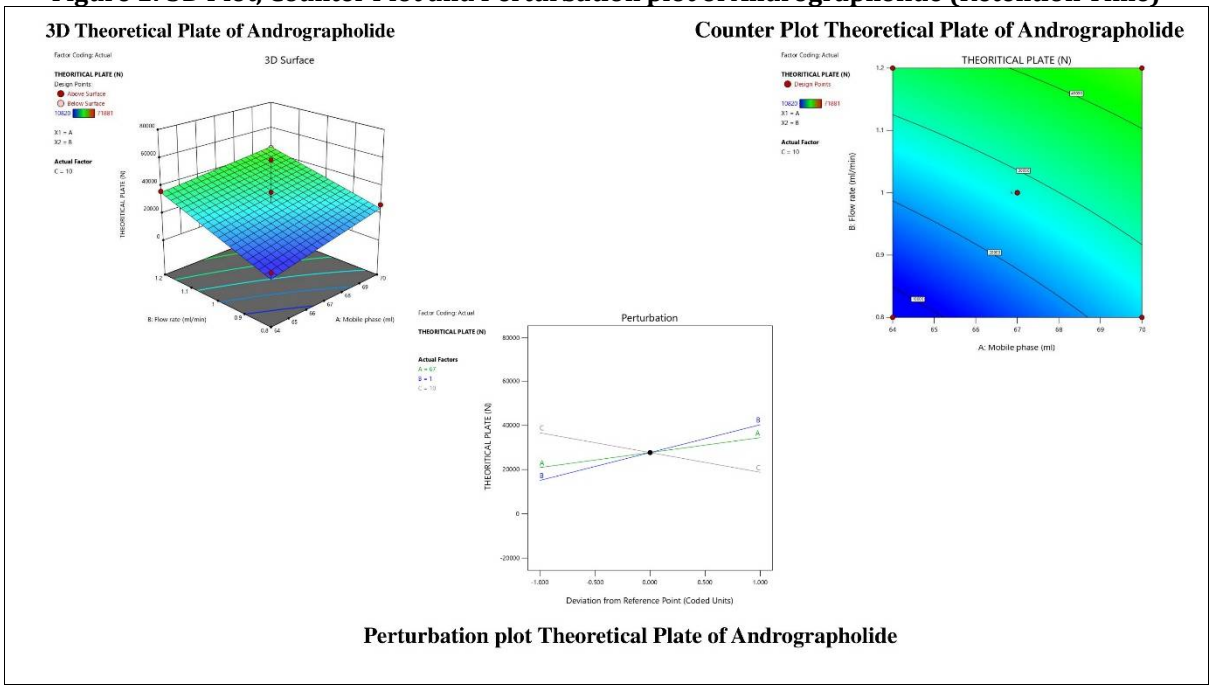
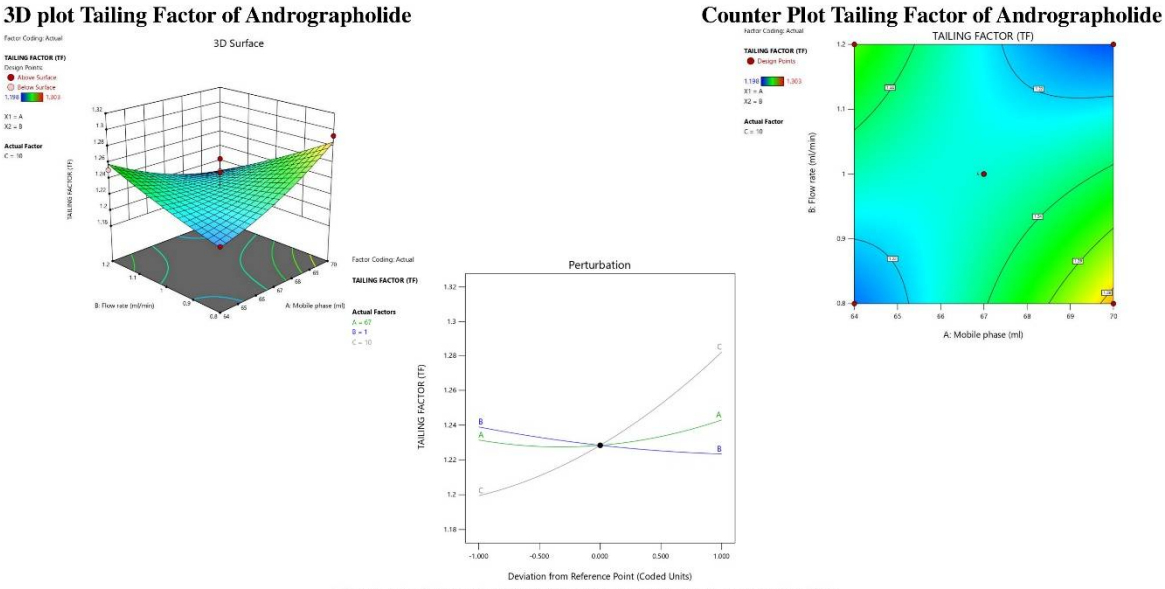


Figure 2: 3D Plot Counter Plot Theoretical Plate and Perturbation plot of Andrographolide



Perturbation plot Tailing Factor of Andrographolide

Figure 3: 3D plot, Counter Plot Tailing Factor and Perturbation plot of Andrographolide

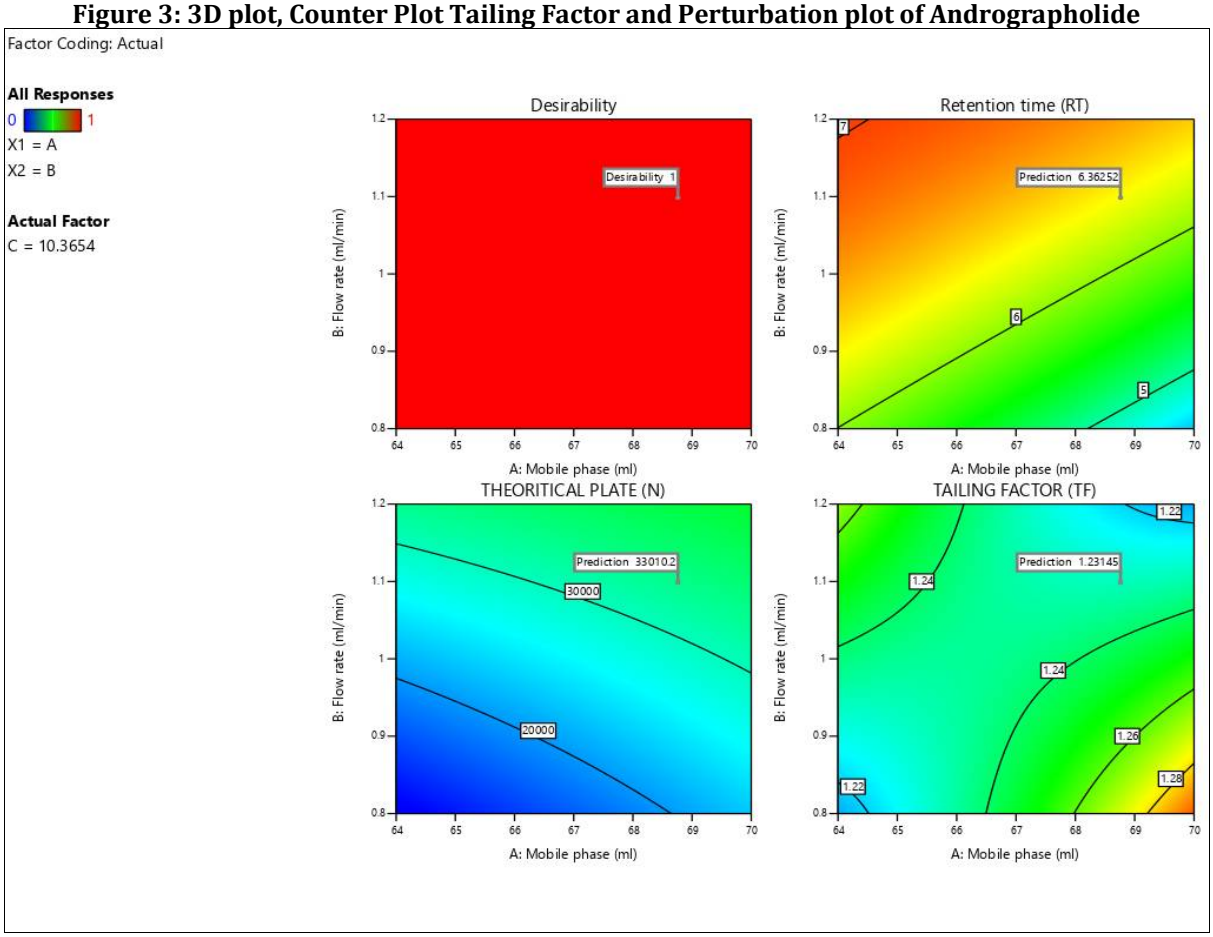


Figure 4: Desirability Plot of Andrographolide

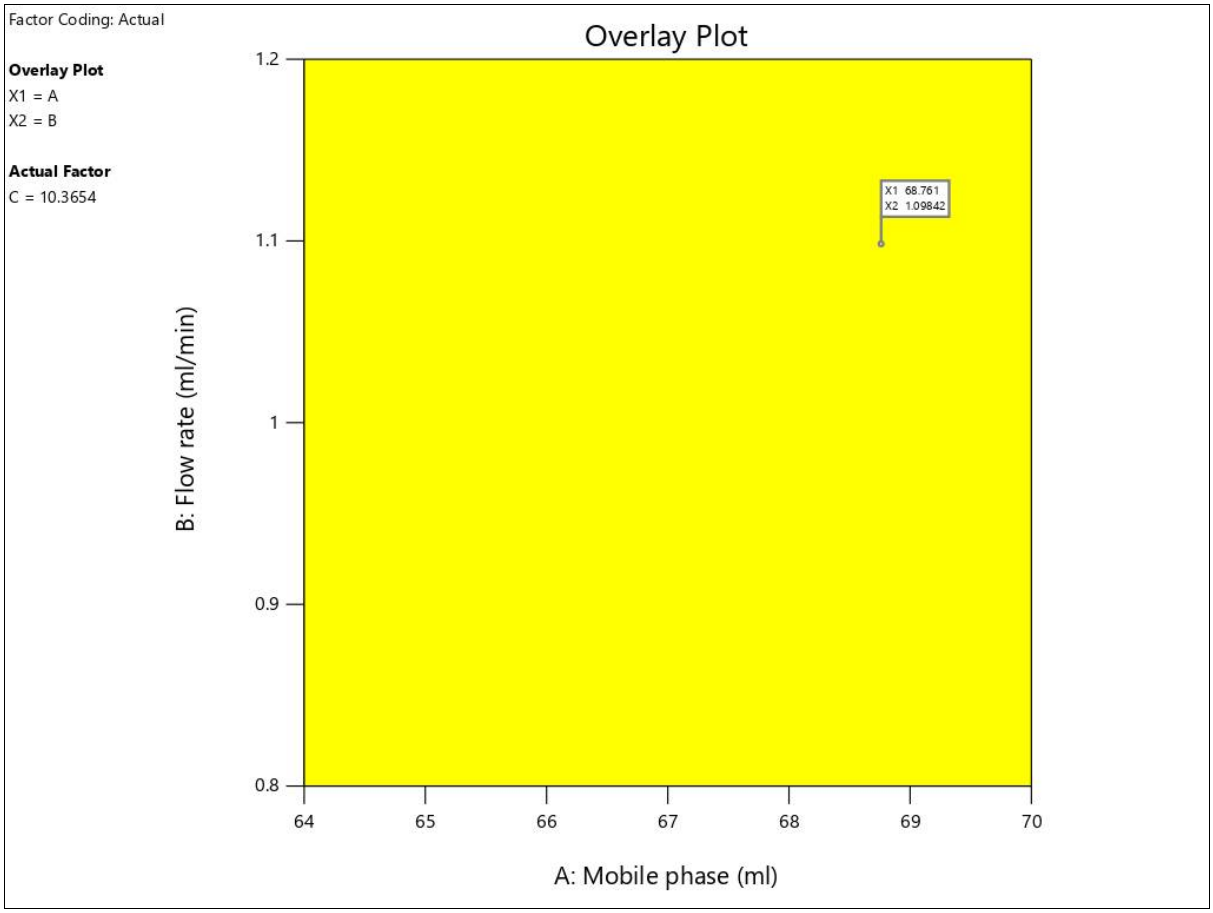


Figure 5: Overlay plot of Andrographolide

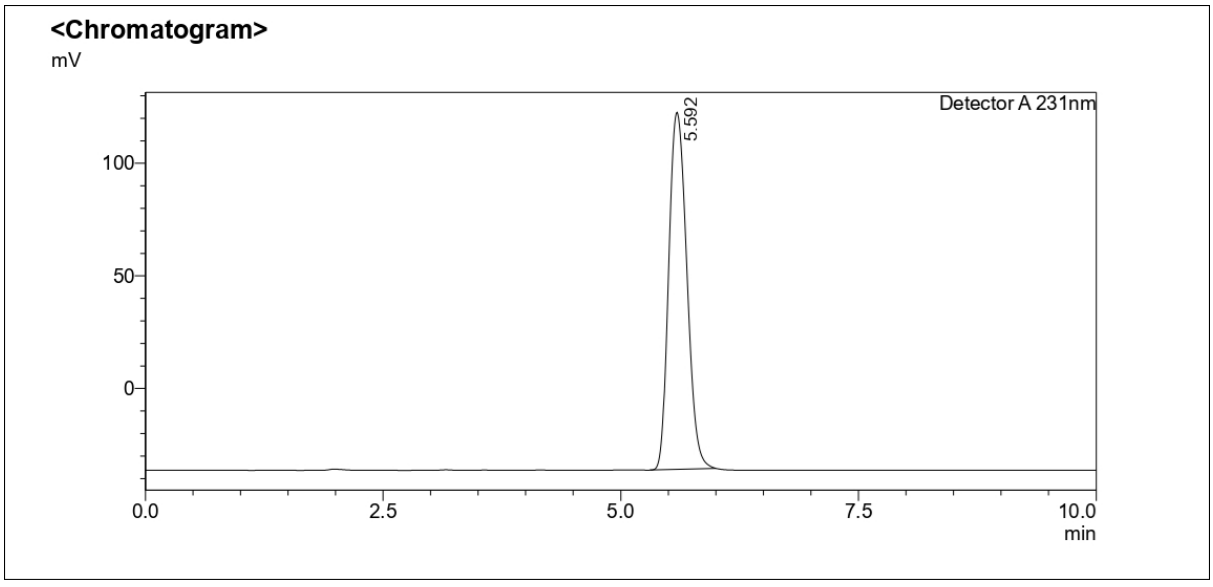


Figure 6: Standard Chromatogram of Andrographolide

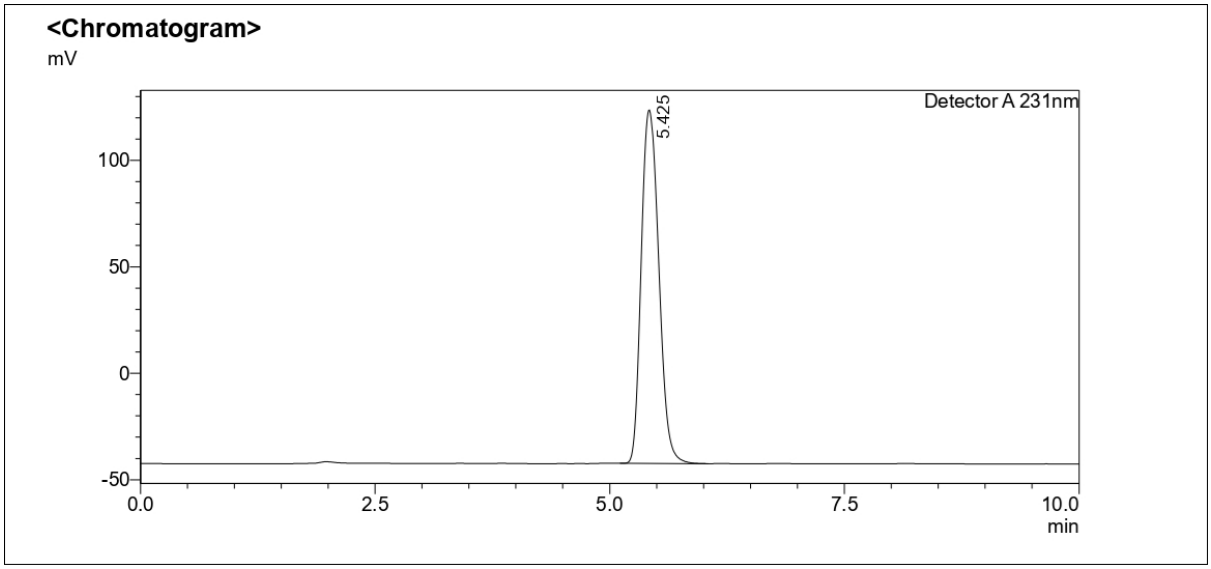


Figure 7: Sample Chromatogram of Andrographolide

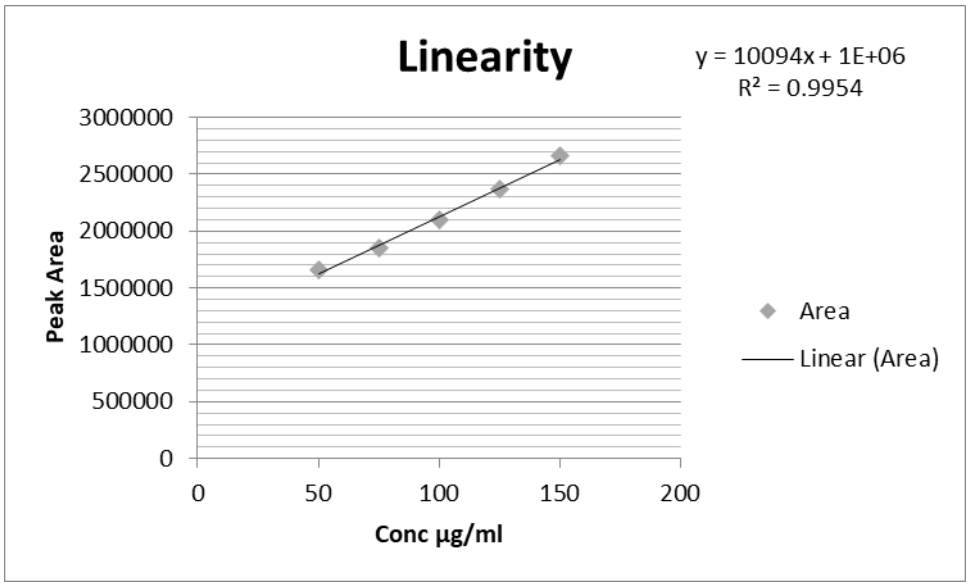


Figure 8: Linearity plot of Andrographolide

CONCLUSION

A robust and sensitive RP-HPLC method for the quantification of Andrographolide was successfully developed and validated using an Analytical Quality by Design (AQbD) framework. The method demonstrated excellent linearity, precision, accuracy, robustness and sensitivity in accordance with ICH Q2(R1) guidelines. Its effectiveness in quantifying Andrographolide from herbal matrices like Nilavembu Kudineer supports its application in routine quality control and standardization of herbal formulations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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