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RESEARCH ARTICLE

HPTLC Method Development and Validation for the Simultaneous Estimation of Embelin, Gallic Acid and Piperine in an *Ayurvedic* Formulation: A DOE Approach

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Abstract

Background: India has a longstanding legacy in *Ayurveda*, a holistic system of medicine that relies profoundly on herbal formulations. As worldwide interest in plant-based therapeutics grows, the need for scientific validation and quality control of *Ayurvedic* formulation has become increasingly important. Standardization through analytical methods ensures therapeutic consistency, safety, and efficacy. Embelin (from *Embelia ribes*), Gallic acid (from *Emblica officinalis* and other sources), and Piperine (from *Piper nigrum*) are well-documented phytoconstituents known for their anti-inflammatory, anti-oxidant, and bio-enhancing activities. These phytoconstituents are often present together in polyherbal formulations used in treating metabolic and inflammatory disorders. However, simultaneous estimation of these markers is rarely reported using a validated, robust analytical method. **Aim:** This study aimed to develop and validate a novel High-Performance Thin-Layer Chromatography (HPTLC) method for the simultaneous estimation of embelin, gallic acid, and piperine in an Ayurvedic formulation, using a Design of Experiments (DoE) approach for method optimization. **Method:** Chromatographic separation was achieved on silica gel 60 F254 plates using a mobile phase of toluene: ethyl acetate: petroleum ether: formic acid (7:1:1:1.5 v/v/v/v). Detection wavelengths were optimized to 284 nm (embelin), 278 nm (gallic acid), and 333 nm (piperine). Method validation was carried out per ICH Q2 (R2) guidelines. Robustness was assessed using a 2^{4-1} fractional factorial design. **Results:** The method showed linearity (1000–5000 ng/band) with r^2 values of 0.9982, 0.9984, and 0.9986 for embelin, gallic acid, and piperine, respectively. LOD/LOQ values were within acceptable limits. Accuracy ranged from 99.90–101.02%, and precision showed %RSD <2%. The robustness study identified petroleum ether as a critical factor influencing gallic acid and piperine estimation. **Conclusion:** The validated HPTLC method is accurate, precise, robust, and suitable for routine quality control of Ayurvedic formulations containing these phytoconstituents.

Major Findings: A validated HPTLC method was developed for the simultaneous estimation of embelin, gallic acid, and piperine with excellent linearity, accuracy, and precision as per ICH guidelines. DoE-based robustness testing identified petroleum ether as a critical factor affecting the resolution of gallic acid and piperine.

Keywords: *Ayurvedic* Formulation, Embelin, Factorial Design, Gallic Acid, HPTLC Piperine

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1. Introduction

Ayurvedic concepts can be applied more widely worldwide if current analytical methods are combined with a broader perspective. One crucial aspect is the accurate identification and quantification of the active compounds present in herbal formulations, which can have a direct impact on their efficacy and safety[1,2]. The development of an HPTLC method for the simultaneous detection of three key compounds found in various ayurvedic preparations: embelin, gallic acid, and piperine.

The selected Ayurvedic formulation for the present study is *Vidanga vati*, which is useful for the treatment of infections in children, particularly due to its antifungal and anthelmintic properties. *Vidanga vati* is produced as per the *Kusth Chikitsa Sthana* chapter of the *Yogratnakar Samhita*, an ancient Ayurvedic classic that serves as an Ayurvedic textbook[3,4]. The key ingredients of this Ayurvedic Formulation include *Vidanga* (*E. ribes* Burm F.), *Triphala* (a combination of *E. officinalis*, *Terminalia belerica*, and *Terminalia chebula Linn*), and *Pippali* (*Piper longum Linn*). These three phytomarkers contain embelin, a benzoquinone derivative, which is known for its anti-inflammatory, analgesic, and antioxidant properties, gallic acid, a tannins derivative reported to possess strong antioxidant and carminative properties isolated from the fruit pulp of *T. chebula* Linn belongs to the *Combretaceae* family and contains piperine, piperidine alkaloids which belong to the *P. longum* Linn family and have antibacterial and antiulcer activities[5,6].

The design of experiments is a powerful methodology for developing and validating analytical techniques, notably HPTLC. DoE employs statistical designs, such as factorial designs, to systematically investigate the impact of multiple factors on analytical responses. By constructing mathematical models and visualizing factor-response relationships, DoE enables the identification and optimization of critical chromatographic conditions. This study employed a two-level fractional factorial design to assess the robustness of a novel HPTLC method for quantifying embelin, gallic acid, and piperine, ensuring its reliability and accuracy[7-10].

2. Materials and Methods

2.1 Standards and Reagents

The standard biomarkers embelin, gallic acid, and piperine were procured from Yucca Enterprises, Maharashtra, India. All analytical grade (AR-grade) solvents from HPTLC were purchased from Sulab (Vadodara, Gujarat, India) and Oxford Lab (Maharashtra, India).

2.2 Marketed Formulation

Vidanga vati were procured from the department of *Kaumarabhritya*, the Ayurved Department, Parul University, Waghodia, Vadodara, Gujarat, India.

2.3 Preparation of Reference Standard Solution

Each standard (1 mg) was accurately weighed, transferred to a 10 mL volumetric flask, and diluted with methanol to obtain a 100 µg/mL solution.

2.4 Preparation of Sample Solution

Tablets containing 500 mg of active content were precisely collected and crushed with a mortar and pestle. After that, 10 g of powder was refluxed in methanol for 30 minutes. The extract was filtered, dried, and stored in a desiccator for further use. The extract was diluted in methanol to achieve a 1000 µg/ml concentration in the sample solution.

2.5 Instrumentation

The CAMAG HPTLC system (Switzerland), equipped with a Linomat 5 automatic sample applicator and a TLC Scanner-4, operated using Vision CATS software (version 3.0), was utilized for scanning and analysis. UV chamber, twin trough chamber (20 × 10 cm), precoated silica gel G60 F254 aluminium plate (20 × 20cm, 100µm thickness), was utilized. Design Expert Version 13 was used for both experimental design and optimization analysis.

2.6 Optimization of Chromatographic Conditions

Based on a literature survey and numerous experiments, the mobile phase was optimized such that the ratio of toluene: ethyl acetate: petroleum ether: formic acid (7:1:1:1.5, v/v/v/v). Before each run, the chamber underwent a 20-minute presaturation period with the mobile phase.

2.7 Method Validation

The method was thoroughly validated as per the ICH guidelines Q2 (R2), encompassing various parameters

such as system suitability, specificity, linearity, precision, accuracy, LOD, LOQ, and robustness[11].

2.7.1 System Suitability

System suitability testing confirmed the method's appropriateness. A standard solution of embelin, gallic acid, and piperine was spotted five times at 4000 ng/band, and the retardation factor (R_f) and peak area were accurately measured and analyzed under the standardized chromatographic conditions.

2.7.2 Specificity

In the analysis of an Ayurvedic formulation containing multiple markers, it is vital to compare the selected standards with the other biomarkers, like ellagic acid, lupeol, berberine, and quercetin were tentatively identified through comparison of R_f values with literature-reported standards and their known occurrence in ayurvedic formulations. Each reference spot was carefully validated by correlating its distinct peak R_f values to ensure accurate identification and comparison.

2.7.3 Linearity and Range

A mixed standard solution containing 1000 each of embelin, gallic acid, and piperine was prepared in methanol. Linearity was assessed over the range of 1000- 5000 ng/band by applying spot volumes of 2, 4, 6, 8 and 10 µL. Calibration curves were obtained by plotting peak area against concentration, and high regression coefficients confirmed excellent linearity for all three compounds.

2.7.4 Precision

Precision was rigorously assessed through repeatability, intraday, and interday measurements to ensure method reliability. Repeatability was tested by performing six measurements of the same concentration (2000 ng/band) within a single day, followed by determining the mean standard deviation and % RSD. Intraday precision was evaluated by taking three measurements of three different concentrations (1000, 2000, 3000 ng/band) at three separate time intervals within the same day, with the mean standard deviation and % RSD calculated for each. Inter-day precision was determined by measuring the same three concentrations over three consecutive days, with the mean standard deviation and % RSD also calculated. These assessments confirmed the method's robustness and consistency across various conditions.

2.7.5 Accuracy

Accuracy was assessed through triplicate analyses at 80%, 100%, and 120% concentrations. Known standards of embelin, gallic acid, and piperine were added to the formulation in unknown quantities, and the recovery percentages and % RSD were analyzed to evaluate the results.

2.7.6 Limit of Detection and Limit of Quantification

The calibration curve approach was used to determine the LODs and LOQs. The standard deviation is determined by the standard deviation of the regression lines' y-intercepts. These values indicate the minimum concentrations that can be reliably quantified and calculated using the standard deviation and slope method. It was determined via ICH guidelines.

2.7.7 Robustness Study Using Full Factorial Design[12]

In this study, a 2^{4-1} Fractional Factorial Design (FFD) was utilized to evaluate the robustness of the system. The FFD approach focused on four key factors: toluene (A), petroleum ether (B), chamber saturation time (C), and solvent front (D). Variations in these parameters were intentionally introduced to assess their influence on the retardation factor (R_f) values of all biomarkers. By deliberately altering these parameters from their optimal conditions, the extent of deviation in the R_f response was examined. To minimize bias, the experimental runs were randomized. A total of eight experimental trials were conducted using coded factor-level values in the FFD framework.

2.7.8 Quantification of the Formulation[13]

Accurate quantification of the standards within the formulation was a key aspect of the study and was achieved through detailed analysis of the formulation areas combined with the application of regression equations for precise measurements.

3. Results and Discussion

The simultaneous evaluation of three compounds was performed using a mobile phase consisting of toluene: ethyl acetate: petroleum ether: formic acid in a ratio of

7:1:1:1.5 (v/v/v/v). Embelin, gallic acid, and piperine were detected at wavelengths of 278 nm, 284 nm, and 333 nm, respectively, with R_f values of 0.497, 0.144, and 0.608. The optimized chromatographic conditions are detailed in Table 1. All three individual standards and a mixture of all three standards chromatographs are reported in Figure 1.

Table 1. Optimized chromatographic conditions

Parameters	Specifications
Stationary phase	Aluminium plates pre-coated with Silica G 60 F_{254} (Merck)
Mobile phase	Toluene: Ethyl acetate: Petroleum ether: Formic acid (7:1:1:1.5, v/v/v/v)
Solvent	Methanol
Application mode	Band
Chamber	Twin trough chamber (10 × 20 cm) with stainless steel lid
Saturation time	20 min
Separation technique	Ascending
Scanning Wavelength	Embelin: 278 nm Gallic acid: 284 nm Piperine: 333 nm
Visualization in UV	UV chamber used for Embelin, Gallic acid, and Piperine at 254 nm

3.1 Validation of the Developed HPTLC Method

3.1.1 System Suitability

The system suitability parameters, including the retardation factor and peak area, are comprehensively detailed in Table 2.

3.1.2 Specificity

The peak profiles (R_f values) of all standards and formulations are compared and shown in Figure 2.

Table 2. System suitability of embelin, gallic acid, and piperine

Conc. (ng/band)	Embelin		Gallic acid		Piperine	
	R_f	Area	R_f	Area	R_f	Area
4000	0.499	0.00129	0.145	0.02033	0.608	0.07824
	0.494	0.00131	0.143	0.02021	0.606	0.07833
	0.480	0.0013	0.141	0.02019	0.600	0.07816
	0.493	0.00133	0.150	0.02018	0.559	0.07809
	0.476	0.00131	0.144	0.02042	0.555	0.07842
Mean	0.001308		0.020266		0.078248	
SD	0.0000148324		0.000105		0.000131	
% RSD	1.13		0.51		0.16	

*n=6, SD= standard deviation, % RSD < 2
The method's suitability was validated by achieving a % RSD of less than 2%, confirming its reliability.

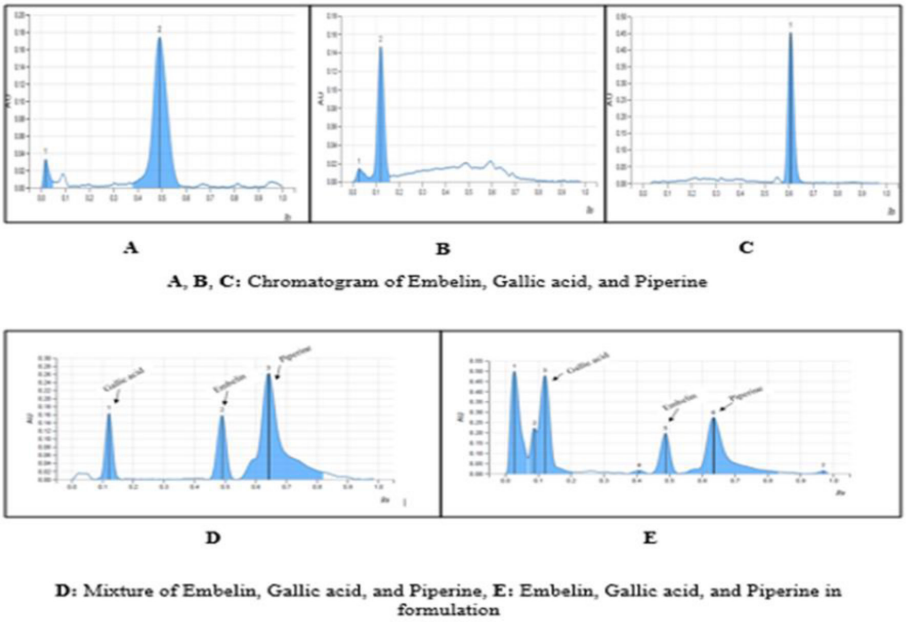


Figure 1. Chromatograms of embelin (A), gallic acid (B), and piperine (C) in standards mixtures (D), and formulations (E).

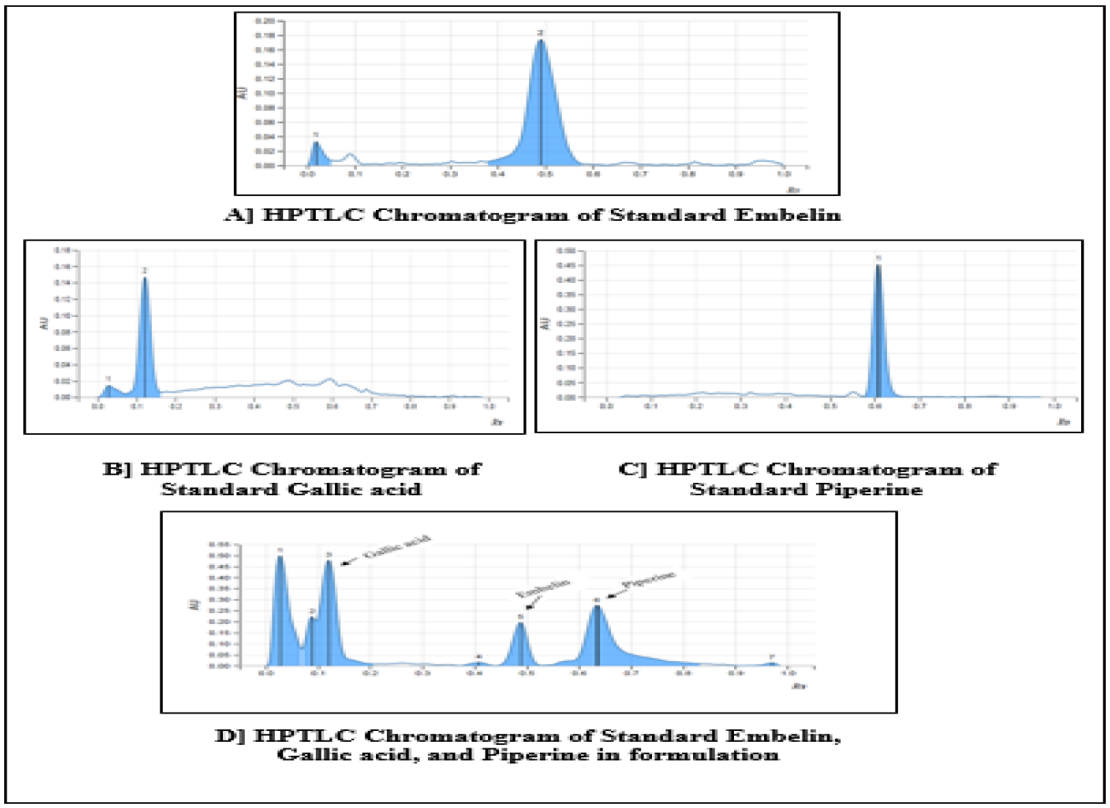


Figure 2. Chromatograms of standard embelin (A), gallic acid (B), and piperine (C); chromatogram of embelin, gallic acid, and piperine in formulation (D).

Table 3. Specificity values of embelin, gallic acid, and piperine

Markers	R_f value of standard	R_f value of formulation
Embelin	0.497	0.575
Gallic acid	0.144	0.147
Piperine	0.608	0.613

Method Specificity was verified as no interference was detected in the peaks of embelin, gallic acid, and piperine. Each standard spot in the sample was confirmed by comparing its R_f values with those of the individual standard peaks in Table 3.

3.1.3 Linearity

Linearity was carefully evaluated across a range of five concentrations, from 1000 to 5000 ng/band, for the standards of embelin, gallic acid, and piperine. This analysis aimed to establish the method's accuracy and consistency, ensuring a direct correlation between concentration and response within the specified range. Calibration curves were plotted for peak area versus concentration, and individual calibration curves were generated in Figure 3 (A, B, and C). Embelin, gallic acid, and piperine exhibited linear responses in the range of 1000-5000 ng/band in HPTLC, with correlation values of 0.9982, 0.9984, and 0.9986,

Table 4. Linearity parameters

Parameters	Embelin	Gallic acid	Piperine
Linearity range (ng/band)	1000-5000 ng/band		
Regression equation	0.0000003x + 0.000009	0.000003x + 0.0082	0.000008x + 0.0473
R^2	0.9982	0.9984	0.9986
Slope	0.0000003	0.000003	0.0000008667
Intercept	0.000010333	0.0001	0.0000833333
LOD (ng/band)	104.54	110	109.91
LOQ (ng/band)	316.81	333.33	333.08

*n=3

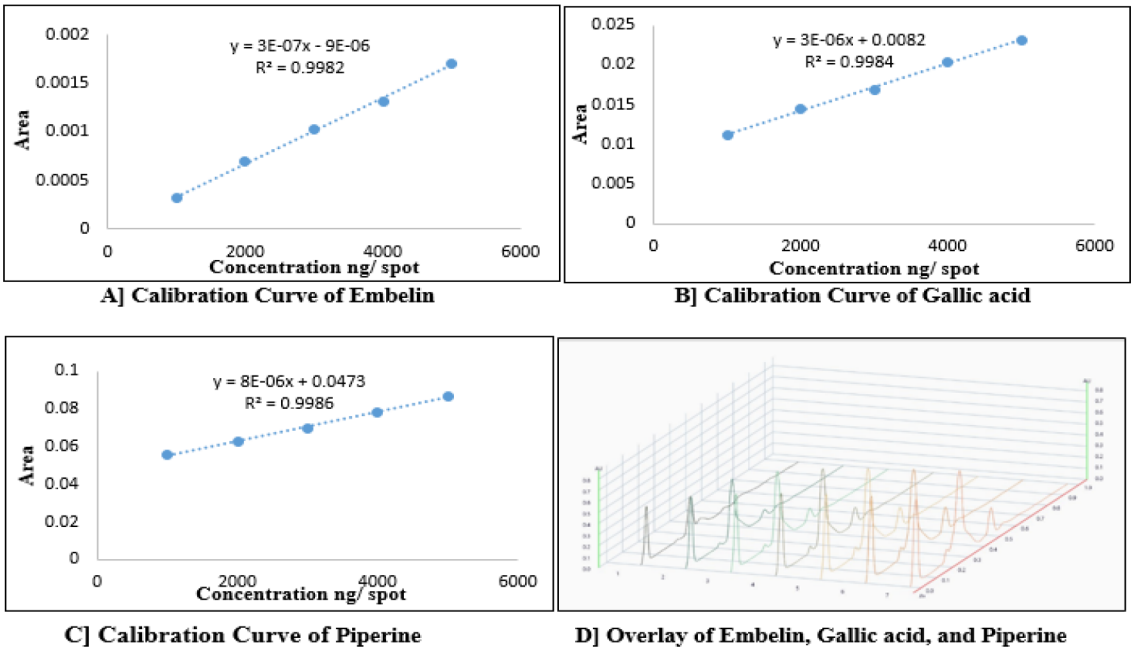


Figure 3. Calibration curves of embelin (A), gallic acid (B), and piperine (C), Overlay curves of embelin, gallic acid, and piperine (D).

Table 5. Accuracy study data for embelin, gallic acid, and piperine

Standard	% spiked	Amount present (ng/band)	Amount added (ng/band)	Amount recovered (ng/band)	% Recovery	% RSD
Embelin	80	1830	1464	3296	99.90	0.23
	100	1830	1830	3674	100.13	0.21
	120	1830	2196	4019	100.02	0.17
Gallic acid	80	2000	1600	3600	101.02	0.81
	100	2000	2000	4000	100.01	0.09
	120	2000	2400	4400	100.3	0.07
Piperine	80	2231	1784.8	4019.8	100.02	0.07
	100	2231	2231	4471	99.98	0.20
	120	2231	2677.2	4904.2	100.12	0.22

*n=3, % RSD< 2

respectively. Moreover, Figure 3 (D) displays an overlay of embelin, gallic acid, and piperine. Linearity parameters are listed in Table 4.

3.1.4 Accuracy

The accuracy of the standards was assessed via the standard recovery method at three different concentrations (1400ng/band at 80% level, 2000ng/band at 100% level, and 2400ng/band at120% level). The accuracy data are detailed in Table 5.

All three compounds achieved recovery percentages between 98% - 102%, with % RSD values below 2%, meeting the accuracy standards set by the ICH guidelines.

3.1.5 Precision

Six determinations were performed to assess repeatability at a concentration of 2000 ng/band (Table 6). Intraday and interday (Tables 7 and 8) assessments were performed with three determinations at

Table 6. Repeatability of embelin, gallic acid, and piperine

Conc. (ng/ spot)	Conc. (ng/ band)	Mean	SD	%RSD
Embelin	2000	0.012263	0.000139	1.13
Gallic acid		0.0144	0.000154	1.06
Piperine		0.06273833	0.0000699762	0.11

*n=6, % RSD < 2

Table 7. Intraday precision of embelin, gallic acid, and piperine

Mar kers	Conc. (ng/ band)	Mean peak area	SD	% RSD
Em belin	1000	0.01229	0.00004358	0.35
	2000	0.010567	0.00015695	1.48
	3000	0.055337	0.000130512	0.23
Gallic acid	1000	0.011173	0.000120139	1.05
	2000	0.01432	0.000101489	0.70
	3000	0.01678	0.000134536	0.80
Pip erine	1000	0.055373	0.00012897	0.23
	2000	0.062607	0.000162891	0.26
	3000	0.069853	0.000100167	0.14

*n=3

Table 8. Interday precision of embelin, gallic acid, and piperine

Markers	Conc. (ng/ band)	Mean peak area	SD	% RSD
Embelin	1000	0.013297	0.0000802081	0.60
	2000	0.014363	0.000219393	1.52
	3000	0.01238	0.0000556776	0.44
Gallic acid	1000	0.011367	0.000188768	1.66
	2000	0.014523	0.000217332	1.49
	3000	0.069877	0.000120544	0.17
Piperine	1000	0.05545	0.00007	0.12
	2000	0.06259	0.00019975	0.31
	3000	0.069807	0.000120554	0.17

*n=3

The repeatability and both intraday and interday precision % RSD values were all below 2%, reflecting a high degree of precision.

Table 9. LOD and LOQ values of embelin, gallic acid, and piperine

Biomarkers	LOD (ng/band)	LOQ (ng/band)
Embelin	104.54	316.81
Gallic acid	110	333.33
Piperine	109.91	333.08

concentrations of 1000, 2000, and 3000 ng/band, with the results and percentage RSD determined.

3.1.6 LOD and LOQ

The detection and quantification limits were calculated using the response standard deviation and calibration curve slope. LOD and LOQ of embelin, gallic acid and piperine results are reported in Table 9.

The proposed method was capable of detecting and quantifying small numbers of markers with precision. Consequently, the method was found to be highly sensitive.

3.1.7 Robustness Study Employing Experimental Design

The experimental results were analyzed using Design Expert Trial Version 13, which adjusts the method factors simultaneously through a design matrix. Robustness was evaluated using Fractional Factorial Design (2^{4-1}). Each run was independent and conducted within the design domain described in Table 10. This approach helps identify significant factors influencing the separation of embelin, gallic acid, and piperine, highlighting potential problems arising during their application. The chosen factor intervals represent expected variations during method transfer, with responses including the retardation factors embelin, gallic acid, and piperine (Table 11).

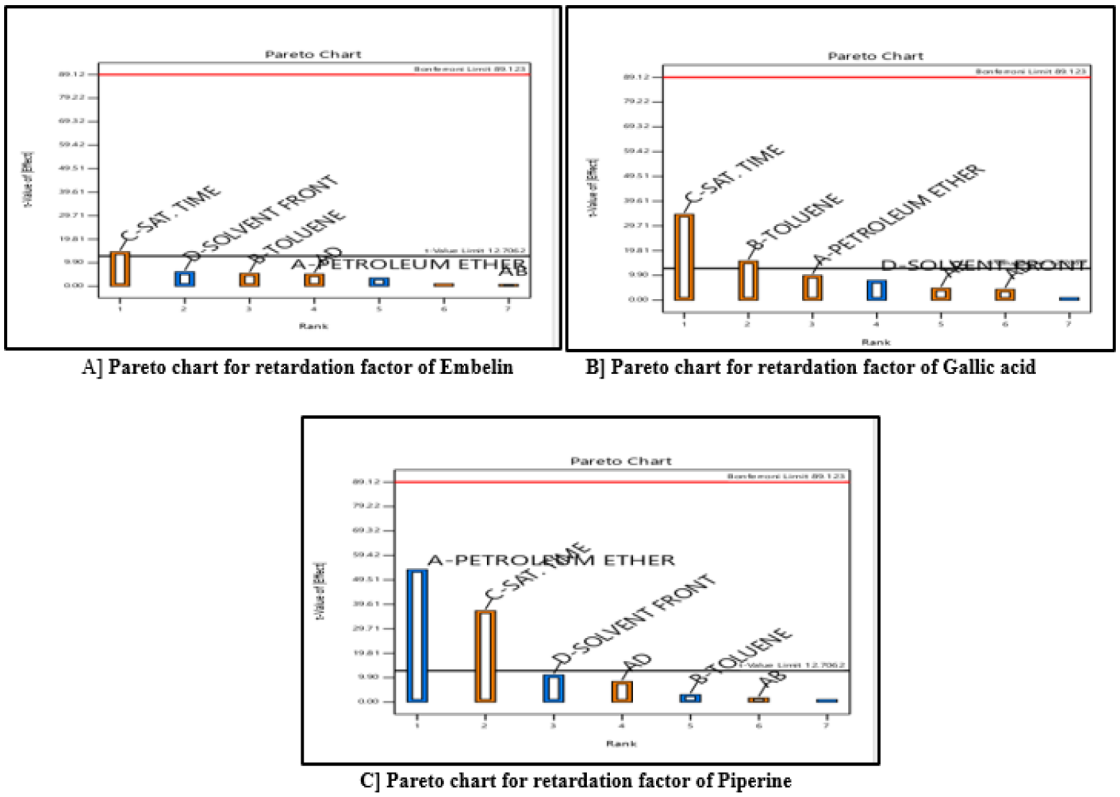
As shown in Figure 4 (A), the Pareto chart, response, and retardation factor of embelin were not significantly impacted by variations in any factors, ensuring the robustness of the analytical method. The Pareto chart for the retardation factor of gallic acid (B) shows that, in the Pareto chart, saturation time appeared to have a significant effect on robustness compared with the other parameters; thus, it was required to be carefully monitored. As shown in Figure 4 (C), in the Pareto plot, petroleum ether appeared to have a significant effect on robustness, compared with the other factors; hence, it was important to control this factor carefully.

Table 10. Factor design obtained responses

A: Volume of Toluene (ml)	B: Volume of Petroleum ether (ml)	C: Saturation Time (min)	D: Solvent Front (mm)	R_f of Embelin	R_f of Gallic acid	R_f of Piperine
7.2	0.8	22	78	0.417	0.158	0.599
7.2	1.2	18	78	0.496	0.146	0.608
7.2	1.2	22	82	0.486	0.140	0.603
6.8	1.2	22	78	0.432	0.156	0.608
7.2	0.8	18	82	0.422	0.161	0.553
6.8	1.2	18	82	0.456	0.156	0.568
6.8	0.8	22	82	0.472	0.149	0.597
6.8	0.8	18	78	0.491	0.154	0.605

Table 11. Predicted response model and statistical parameters

Response	Polynomial equation	Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adequate precision
R_f of embelin	0.4481-0.0091A+0.0141B+0.0379C-0.0159D+0.0019AB+0.0134AD	0.0074	0.4481	1.66	0.9968	0.9777	0.7957	22.17
R_f of gallic acid	0.1984+0.0111A+0.0176B+0.0386C-0.0089D+0.0054AB+0.0049AD	0.0032	0.1984	1.60	0.9994	0.9957	0.9607	41.4081
R_f of Piperine	0.6093-0.0402A-0.0023B+0.0277C-0.0082D+0.0012AB+0.0062AD	0.0021	0.6093	0.3482	0.9998	0.9984	0.9856	74.5850



A] Pareto chart for retardation factor of Embelin

B] Pareto chart for retardation factor of Gallic acid

C] Pareto chart for retardation factor of Piperine

Figure 4. Pareto chart for the retardation factors of embelin (A), gallic acid (B), and piperine (C).

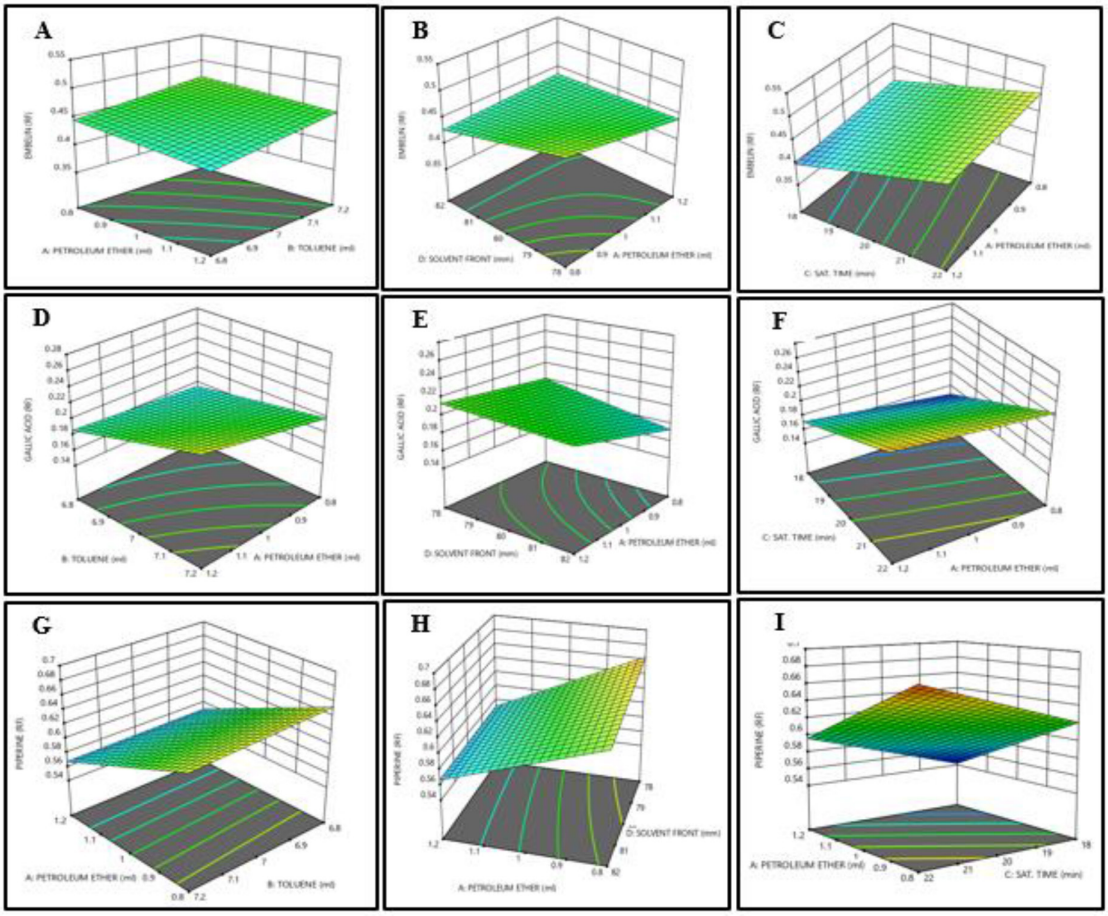


Figure 5. 3D response plots of embelin, gallic acid, and piperine.

All standards interactions reported in 3D response plots in Figure 5. In plots, A, B, and C represent embelin interactions (AB, AD, AC); D, E, and F represent gallic acid interactions (AB, AD, AC); G, H, and I represent piperine interactions (AB, AD, AC).

A^{24-1} fractional factorial design was applied to study the chromatographic behavior of embelin, gallic acid, and piperine under varying experimental conditions.

Embelin (AB, AD, AC interactions): R_f values remained consistent across all conditions, showing no significant sensitivity to changes in solvent composition or saturation time.

Gallic acid (AB, AD, AC interactions): R_f values increased with petroleum ether volume, indicating enhanced mobility. However, longer saturation times reduced R_f, suggesting delayed migration.

Piperine (AB, AD, AC interactions): R_f increased with embelin volume, possibly due to co-solvent effects, but decreased with increased petroleum ether and saturation time.

Table 12. Quantification data of embelin, gallic acid, and piperine

Standards	Concentration in sample (ng/spot)
Embelin	1830
Gallic acid	2000
Piperine	2231

3.1.8 Quantification of formulation

Table 12 shows the results of quantifying all three marker compounds with a standard calibration curve calculation.

4. Conclusion

This study successfully developed and validated analytical techniques for evaluating ayurvedic medicines, demonstrating that the method is innovative, rapid, specific, and reliable for simultaneously estimating embelin, gallic acid, and piperine. The R_f values were

0.497 for embelin, 0.144 for gallic acid, and 0.608 for piperine. The precision and accuracy were confirmed across a concentration range of 1000-5000 ng/band, with high correlation coefficients of 0.9982 for embelin, 0.9984 for gallic acid, and 0.9986 for piperine. The recovery rates were impressive, ranging from 99.90% to 100.13% for embelin, 100.01% to 101.02% for gallic acid, and 99.98% to 100.12% for piperine. The LODs and LOQs were (104.54 and 316.81 ng/band) for embelin, (110 and 333.33 ng/band) for gallic acid, and (109.91 and 333.08 ng/band) for piperine, respectively. Additionally, precision studies indicated a %RSD of less than 2%. These findings confirm the robustness and suitability of the method for accurately assessing embelin, gallic acid, and piperine in an Ayurveda formulation. The fractional factorial design (2^{4-1}) revealed that the Embelin retention factors did not significantly change with altering factors; however, the petroleum ether volume had a considerable effect on gallic acid and piperine robustness, which was carefully regulated. This method is efficient for simultaneously quantifying standards in various plant, herbal, and ayurvedic compositions.

5. Acknowledgments

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6. Author Contribution

Sakshi Srivastava was involved in the design and execution of experiments, data acquisition, statistical analysis, and drafting of the manuscript. Dr. Jaya Patel guided the research design, supervised the experimental procedures, interpreted the data, and critically reviewed and edited the manuscript for scholarly content and accuracy. Dr. Akanksha Patel and Anamika Singh helped with the DoE experiment data interpretation and design in software. All authors have read and agreed to the published version of the manuscript.

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