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RESEARCH ARTICLE

Simultaneous Quantification of Embelin, Piperine and Gallic Acid in *Vidanaga Vati* by High-Performance Liquid Chromatography

Pranali Jadav[1], Jaya Patel[2*], Akanksha Patel[3], Swapnil Raskar[4] and Nikita Vasava[4]

[1]Department of Pharmaceutical Analysis, Parul Institute of Pharmacy and Research, Parul University, Vadodara – 391760, Gujarat, India
[2]Department of Pharmacognosy, Parul Institute of Pharmacy and Research, Parul University, Vadodara – 391760, Gujarat, India; jaya1985@gmail.com
[3]Department of Pharmaceutics, Parul Institute of Pharmacy, Parul University, Vadodara – 391760, Gujarat, India
[4]Department of Kaumarbhritya, Parul Institute of Ayurveda, Parul University, Vadodara – 391760, Gujarat, India

Abstract

Background: *Vidanga vati* is a polyherbal formulation extensively used in *Ayurveda* as a remedy to treat fungal infections and warm infections. It mainly contains fruit parts of *Embelia ribes*, *Piper nigrum*, *Phyllanthus emblica*, *Terminalia bellerica* and *Terminalia chebula.* **Aim:** The present study aimed at developing a validated and reliable High-Performance Liquid Chromatography (HPLC) method for the simultaneous analysis of Embelin, Piperine and Gallic acid from *Vidanga vati*. **Method:** The method employed BDS Hypersil C_{18} column (250mm x 4.6mm, 5µm) as the stationary phase and Methanol: Phosphate buffer pH 2.4 (98:2 v/v) as the mobile phase to identify and quantify Embelin, Piperine and Gallic acid using HPLC. **Results:** Linear regression data for the calibration curves of standard Embelin, Piperine and Gallic acid showed a good linear relationship over a concentration range of 2-10µg/mL, 100-500µg/mL and 1000-5000µg/mL respectively. The percentage recoveries for Embelin, Piperine and Gallic acid were found to be in the range of 99.26-99.87%, 98.25-99.25% and 99.31-99.92% respectively. The method was assessed for accuracy, precision, selectivity and robustness. **Conclusion:** It was concluded that the developed HPLC method was efficient, simple, accurate and valid in the quantitative determination of Embelin, Piperine and Gallic acid.

Keywords: Anthelmintic Activity, Antifungal, *Embelia ribes*, HPLC, Polyherbal

1. Introduction

Herbs and herbal formulations are in high demand since they have fewer negative effects than synthetic medications. More than 80% of the world's population still relies on herbs and herbal remedies for medical purposes[1]. Preference has been seen for plant and plant-based formulations as a preventive measure and a cure to help persons suffering from chronic illnesses such as gastrointestinal problems, chronic arthritis, respiratory distress, diabetes mellitus, chronic skin inflammation and xerotic conditions[2]. Plants provide essential compounds with a wide spectrum of bioactive capabilities. Demand for economically relevant natural goods has increased in recent years in both domestic and international markets[3].

Extensive research has been conducted in the plant kingdom to uncover valuable chemicals in response to the high demand for plant-based products in medicine and industry. *E. ribes* are commonly used in traditional herbal medicine in India. The fruits of *E. ribes* are considered to be high in benzoquinone compounds as shown in Figure 1.

Embelia ribes include an alkaloid, christembine, a resinoid, tannins and trace amounts of volatile oil as well as embelin, quercitol and fatty components. Since ancient times, the dried fruit has been used as an anthelmintic in India. *Embelia ribes* have been

*Author for correspondence

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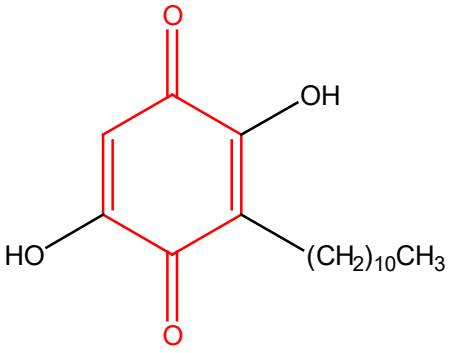

Figure 1. Structure of Embelin.

demonstrated to have astringent[4-6], carminative, stimulant, antioxidant[7], anti-spermetogenic[8-12], anti-bacterial[13] and anticancer properties[14].

Piperine (1-piperoylpiperidine) structure shown in Figure 2 is an alkaloid present in the fruits of black pepper (*Piper nigrum*) as well as other piper species[15].

The fruits include Central Nervous System (CNS) depressants, antipyretic, analgesic, hepatoprotective, bioavailability enhancers[16-17], antioxidant[18] and anti-inflammatory activities. Piplartin, Piperlogumine, Piperidine, Starch, Resin and pungent alkaloids are all found in the fruits of *P.nigrum* and *P.longum*. Piperine is the primary pharmacologically active ingredient of this plant[19-21].

Gallic Acid (GA) is a 3,4,5-trihydroxy benzoic acid polyphenolic molecule found largely in *Triphala churna* Figure 3.

Triphala is a plant product used in India for many years. *Triphala churna* is normally composed in a 1:1:1 ratio of *P. emblica*, *T. bellerica and T. Chebula*[22]. When ingested regularly *Triphala churna* is said to enhance health and longevity. The major ingredients of *Triphala* products contain gallic acid, ellagic acid, chebulinic acid, ascorbic acid, syringic acid, tannic acid and other phenolic chemicals[23-25]. *Triphala churna* is believed to have anti-cancer, anti-inflammatory, anti-diabetic, muscle-relaxing, anti-microbial, anti-depression, immune-boosting and ulcer-healing qualities[26-27].

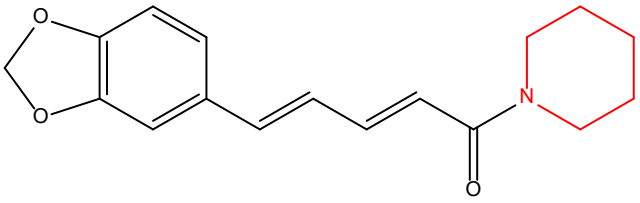

Figure 2. Structure of Piperine.

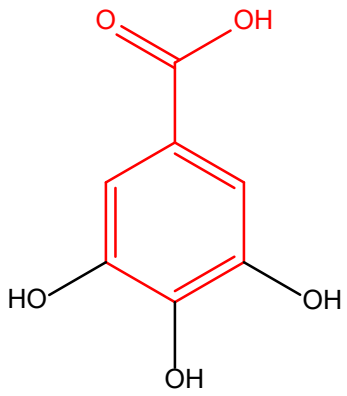

Figure 3. Structure of Gallic acid.

The current research uses HPLC to estimate Embelin, Piperine and Gallic acid from polyherbal formulations. The fruit powders of *Vidanga, Triphala* (*Amla, Baheda* and *Harde*) and *Pippali* are used to cure fungal infections, parasitic worms (helminths) and other internal parasites in the body. According to the literature, several analytical techniques have been used to estimate Embelin, Piperine and Gallic acid independently and in combination with other phytomarkers. However, no HPLC approach was presented for the simultaneous estimation of Embelin, Gallic acid and Piperine.

2. Materials and Methods

2.1 Materials

Polyherbal formulation which is traditionally prepared as per the ayurvedic formulary '*yogratnakar*' was procured from Parul Institute of Ayurveda. Authentic reference standards of Embelin, Piperine and Gallic acid were purchased from Yucca Enterprises, Maharashtra, India. HPLC chromatographic optimisation was accomplished on the Shimadzu (LC 2030) model using Lab Solution software. Separation was achieved on BDS Hypersil C18 (250 x 4.6mm) 5µm column. Wavelength was selected on Shimadzu UV-1780 Ultraviolet (UV)-visible spectrometer. All solvents used were of LC grade.

2.2 Methods

2.2.1 Preparation of 0.02 M Phosphate Buffer (pH2.3)

After precisely weighing 2.72gm of potassium dihydrogen phosphate buffer, 950ml of water was added to the mixture. Ortho-phosphoric acid was used

to get the pH level to 2.3, a volumetric flask was filled to a capacity of 1000 ml and the solution was filtered.

2.2.2 Standard Stock Solution Preparation

2.2.2.1 Embelin Standard Stock Solution

Accurately weighed 1mg Embelin was filled in a 10ml volumetric flask (100µg/ml) and volume was made up to mark with methanol.

2.2.2.2 Piperine Standard Stock Solution

Accurately weighed 20mg Piperine was filled in a 20ml volumetric flask (1000µg/ml) and volume was made up to mark with methanol.

2.2.2.3 Gallic Acid Standard Stock Solution

Accurately weighed 200mg Embelin was filled in a 20ml volumetric flask (10000 µg/ml) and volume was made up to mark with methanol.

2.2.3 Sample Preparation

Five tablets were powdered and 100mg of the resulting powder was precisely weighed before being collected for extraction. The powder was extracted with methanol for 30 minutes with gentle heating. Using methanol, an extract volume of 100ml was created. The mixture was then filtered through Whatman filter paper. After the appropriate dilutions, the solution was injected.

2.2.4 Selection of Wavelength in UV Spectroscopy

To determine a suitable wavelength, different concentrations of standard Embelin, Piperine and Gallic acid solutions were prepared and scanned with a UV Spectrophotometer spectrum. Embelin, Piperine and Gallic acid overlay spectra were obtained by applying a detection range of 200-400nm.

2.2.5 Final Optimisation of Chromatographical Condition

Working standard solution Embelin (4µg/ml), Gallic acid (2000µg/ml), Piperine (200µg/ml) and standard mixtures were prepared and chromatograms were recorded.

2.2.6 Validation of HPLC Method

2.2.6.1 Specificity

The specificity of the method was investigated by injecting blank samples of the mobile phase to demonstrate the absence of interference in standard samples.

2.2.6.2 Linearity

In five concentration ranges, the linearity of the calibration curve for Embelin, Piperine and Gallic acid was established. Each of the Embelin, Piperine and Gallic acids was placed in a volumetric flask and diluted to the mark with methanol to create the linearity stock solution. Embelin in the ranges of 2, 4, 6, 8, and 10 µg/ml, Gallic acid in the ranges of 1000, 2000, 3000, 4000, and 5000 µg/ml and Piperine in the ranges of 100, 200, 300, 400, and 500 µg/ml prepared from the stock solution were used to determine the linearity. The calibration curve was plotted against the area of the peak vs the standard concentration. The calibration curve was constructed by plotting area vs. concentration and the regression coefficient equation was calculated.

2.2.6.3 Limit of Detection and Limit of Quantification (LOD and LOQ)

The calibration curve method was used for LOD. It is recommended to examine a particular calibration curve using a sample that contains an analyte that is within the Detection Limit's (DL) range. The y-intercept standard deviation of the regression line is used as the standard deviation. It was calculated using the equation in the ICH Guideline (Q2R1), as shown below.

$$DL = 3.3 \times \sigma / S.$$

LOQ was carried out based on the calibration curve method. A specific calibration curve should be studied using a sample containing an analyte in the range of Quantification Limits (QL). The standard deviation of y-intercepts of regression lines was used as the standard deviation. It was calculated as per the ICH Guideline (Q2R1) equation as shown below $QL = 10 \times \sigma / S$.

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

2.2.6.4 Accuracy

The accuracy of the method was confined by recovery study from tablet sample solution at 3 levels of standard spiking 80%, 100% and 120% of the targeted solution. By spiking a standard and a sample with a known quantity, accuracy was studied. The percentage recoveries of Embelin, Piperine and Gallic acid were found at three different concentration levels of 80%, 100% and 120%

and then each concentration was injected in triplicate into HPLC and the chromatograms were recorded. The % recoveries achieved from each level for Embelin, Piperine and Gallic acid were calculated.

2.2.6.5 Precision

Repeatability was determined by analysing Embelin test solutions having a concentration of 4 µg/ml, Gallic acid having a concentration of 2000µg/ml and Piperine test solutions having a concentration of 200µg/ml. Chromatograms were measured 6 times and % RSD was calculated. Intraday precision was determined by analysing standard solutions on the same day by the proposed method. The chromatogram was statistically analysed and mean, standard deviation and % RSD were calculated. (Results are given in section 5.12). Interday precision was determined by analysing the standard solution of Embelin (4µg/ml), Gallic acid (2000µg/ml) and Piperine (200µg/ml) on three different days by the proposed method. The chromatogram was statistically analysed and mean, standard deviation and % RSD were calculated.

2.2.6.6 Robustness

A robustness study was conducted by changing two parameters of HPLC chromatographic conditions, to show the effect of minor changes. One was the flow rate from 1ml/min to 0.8ml/min and 1.2ml/min and the second was the wavelength from 283nm to 285nm and 287nm to evaluate the robustness of this method[28].

3. Results and Discussions

3.1 Selection of Detection Wavelength

Different concentrations of a standard solution of Embelin, Piperine and Gallic acid were prepared and scanned using a UV-visible spectrophotometer separately. The detection range was 200-800nm. The overlay spectra of all three phytomarkers are shown in Figure 4.

The detection wavelength was selected as 285nm because at that wavelength all three phytomarkers showed appreciable absorption.

3.2 Optimisation of Chromatographical Condition

The table of optimised chromatographic conditions is shown in Table 1 and Figures 5 to 8.

3.3 Validation of Developed HPLC Method

3.3.1 Specificity

There were no additional peaks eluting at the Rt of Embelin, Piperine and Gallic acid. Hence, the method was specific.

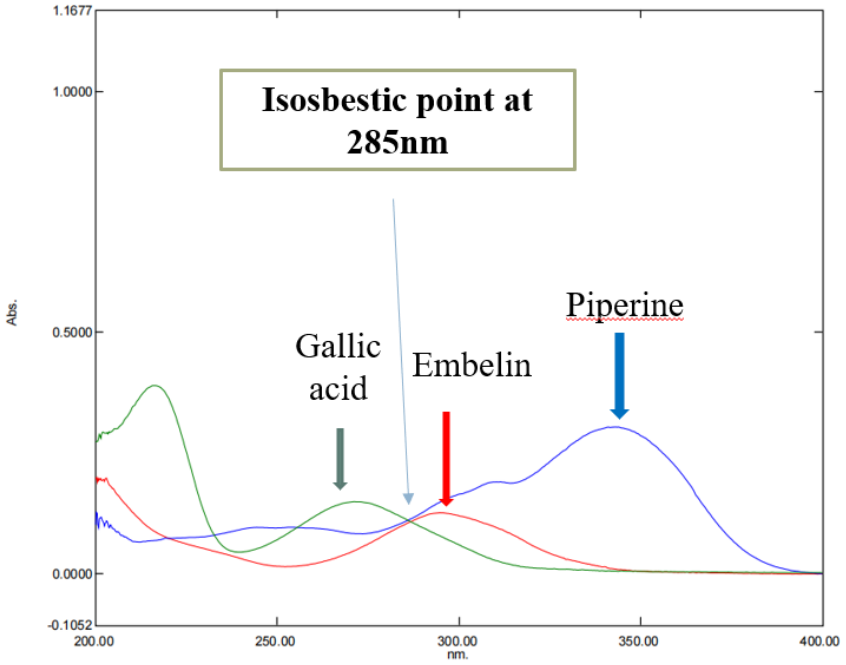

Figure 4. Overlay spectra of 2µg/ml of Embelin, Piperine and Gallic acid.

Table 1. Optimisation of HPLC chromatographic conditions

Parameter	Condition
Stationary Phase	BDS Hypersil C_{18} column (250mm x 4.6mm, 5µm)
Mobile Phase	Methanol: Phosphate buffer pH 2.4 (98:2 v/v)
Flow Rate	1ml/min
Column Oven Temperature	Ambient
Detection Limit	285nm
Run Time	5min
Retention Time	Embelin: 4.3min Piperine: 3.2min Gallic acid: 2.3min

3.3.2 Linearity

The result table of linearity is shown in Table 2 and the calibration curve of all three phytomarkers is shown in Figures 9 to 11.

The calibration curve of Embelin, Piperine and Gallic acid was found to be linear in the concentration ranges from 2-10 µg/mL, 1000-5000 µg/mL and 100-500 µg/mL respectively. The regression equation was found at 285nm, where y = 238227x - 74467 for Embelin and the correlation coefficient was found to be 0.9996, for Gallic acid y = 5277.4x - 4E+06 and the correlation coefficient was found to be 0.9998 and for Piperine y = 18230x + 66226 and the correlation coefficient was found to be 0.995. Linear regression data for calibration plots of Embelin, Piperine and Gallic acid are shown in Table 3.

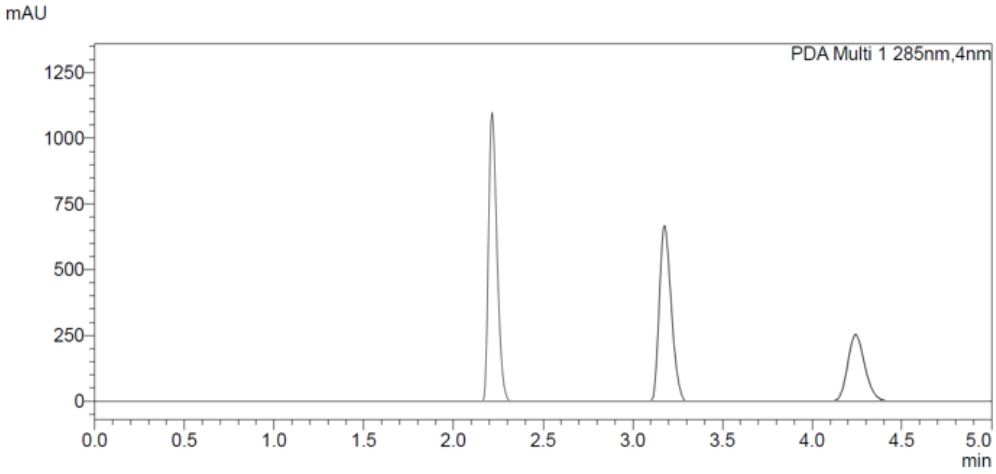

Figure 5. Chromatogram of the standard mixture.

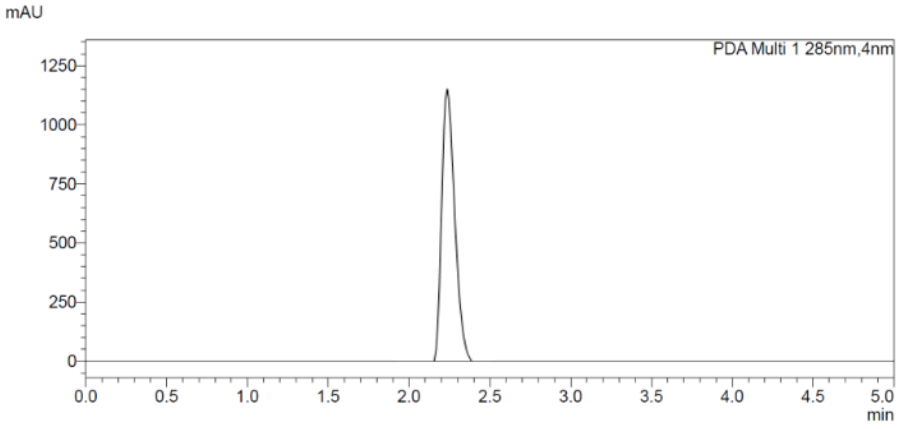

Figure 6. Chromatogram of Gallic acid.

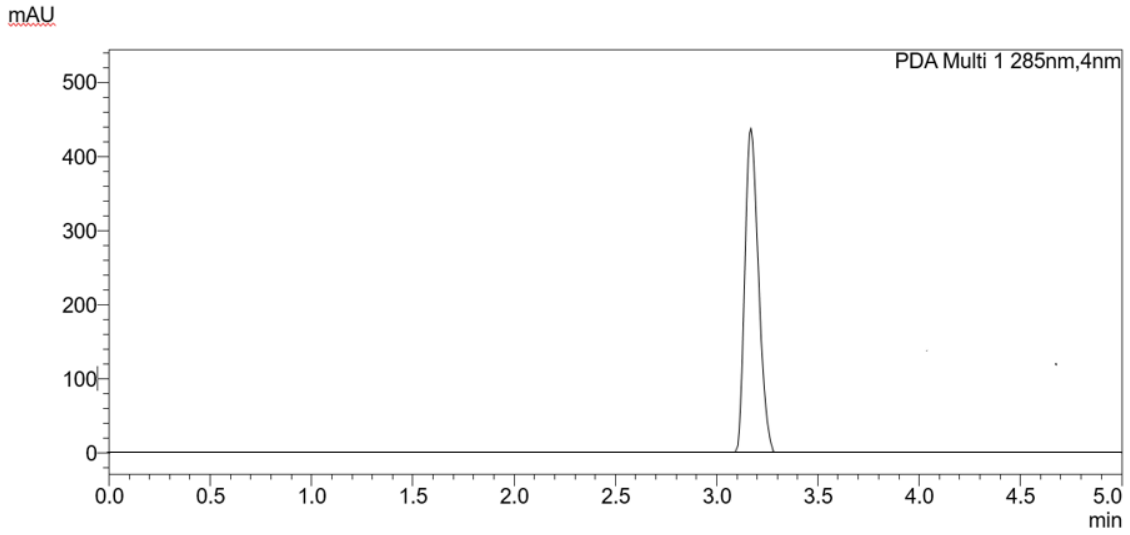

Figure 7. Chromatogram of Piperine.

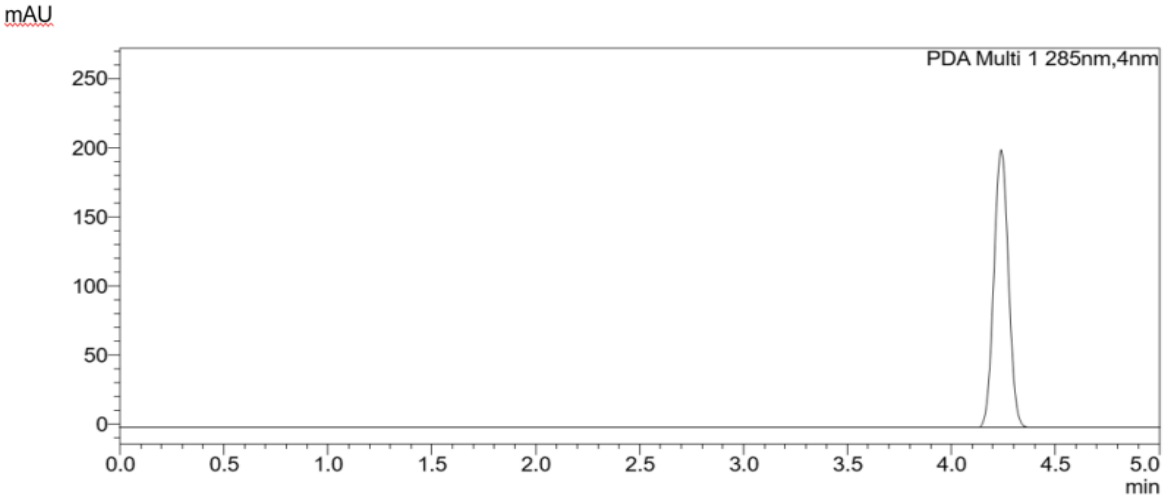

Figure 8. Chromatogram of Embelin.

Table 2. Linearity concentrations and mean peak areas

Concentration of Embelin (µg/ml)	Mean Peak Area of Embelin±SD	Concentration of Piperine (µg/ml)	Mean Peak Area of Piperine±SD	Concentration of Gallic Acid (µg/ml)	Mean Peak Area of Gallic Acid±SD
2	415756±2241.35	100	2117190.8±2559.90	1000	2416973.5±2894.97
4	866435±1147.30	200	3588256.3±1794.79	2000	6555266.2±2605.42
6	1337801±2262.55	300	5298868.5±2724.70	3000	11484497±2004.90
8	1846491±2106.90	400	7290013.5±2649.196	4000	17365346±4322.12
10	2308002±1283.54	500	9381069.2±1052.08	5000	22599056±1627.30

* SD: Standard Deviation

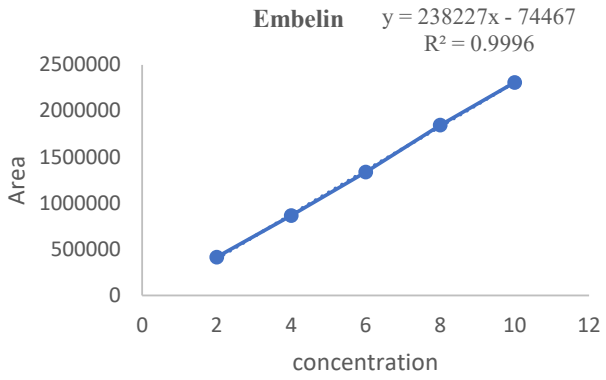

Figure 9. Calibration curve of Embelin.

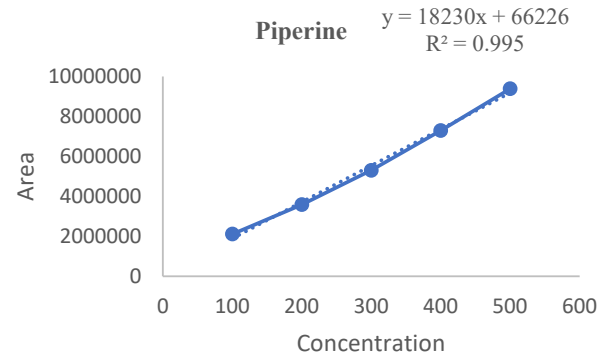

Figure 10. Calibration curve of Piperine.

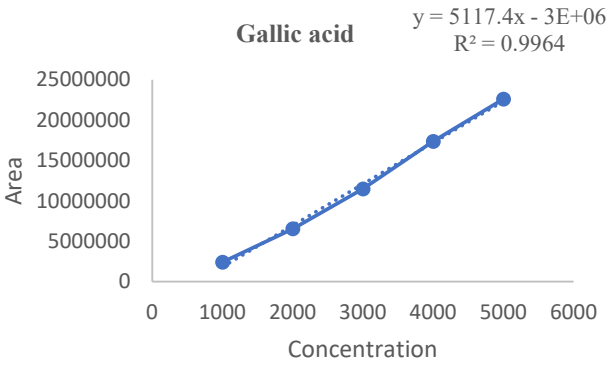

Figure 11. Calibration curve of Gallic acid.

3.3.3 *Limit of Detection and Limit of Quantitation*

The limit of detection and quantification was determined by the standard calibration curve method as per the ICH Guideline (Q2R1), results as shown in Table 4.

3.3.4 *Accuracy*

The accuracy was determined by the standard recovery method. The result % recovery of Embelin, Piperine and Gallic acid are shown in Table 5.

The % recovery of all the three phytomarkers was found to be in the range of 98-102%. According to the ICH Guideline (Q2R1), the method was found to be accurate as it is within the acceptable limits.

3.3.5 *Precision*

% RSD of repeatability, intraday and interday precision of all the standards were found to be < 2 respectively. So, it can be concluded that the proposed method for estimation of Embelin, Piperine and Gallic acid is precise. The results of intraday and interday precision are shown in Tables 6 to 8 respectively.

3.3.6 *Robustness Study*

A robustness study was done by making the deliberate change in wavelength and flow rate.

The results of the robustness study are reported in Tables 9 and 10.

Table 4. LOD and LOQ of phytomarkers

Phytomarkers	LOD (µg/ml)	LOQ (µg/ml)
Embelin	0.10	0.33
Piperine	20.04	60.74
Gallic acid	42.93	130.09

Table 3. Linear regression data for calibration lot of Embelin, Piperine and Gallic acid

Parameters	Embelin	Piperine	Gallic Acid
Linearity Range (µg/ml)	2-10µg/ml	100-500µg/ml	1000-5000µg/mL
Regression Equation	y = 238227x – 74467	y = 18230x + 66226	y = 5277.4x - 4E+06
Correlation Coefficient (r^2)	0.996	0.995	0.999
Slope	238227	18230	5277.4
Intercept	-74467	248039.4	-3808045.53

Table 5. Accuracy of Embelin, Piperine and Gallic acid

Compound	% Level	Area Found	Concentration Found	% Recovery	RSD%
Embelin	80	1180917	1.74	99.59	0.11
	100	1362091	2.25	99.87	0.98
	120	1534925	2.73	99.26	0.29
Piperine	80	3652604	198.58	99.25	0.83
	100	4354971	223.26	99.00	0.40
	120	5024661	246.78	98.25	0.47
Gallic Acid	80	20126871	4527.85	99.31	0.48
	100	21342124	4746.84	99.86	0.40
	120	22536819	4962.12	99.92	0.14

* RSD: Relative Standard Deviation

Table 6. Results of intraday precision of the developed method

Phyto markers	Interval1		Interval2		Interval3	
	Mean Area ±SD	%RSD	Mean Area±SD	%RSD	Mean Area ±SD	%RSD
Embelin	897387±1024.12	1.14	896480±3368.2	0.37	897057±3417.1	0.38
Gallic Acid	5956431±18474.36	0.31	5956121±16634.11	0.27	5956277±26250.47	0.44
Piperine	3562133±21275.02	0.59	3568168±14530.57	0.40	3562169±14415.78	0.49

* SD: Standard Deviation
* RSD: Relative Standard Deviation

Table 7. Results of interday precision of the developed method

Phyto-markers	Day1		Day2		Day3	
	Mean Area±SD	%RSD	Mean Area±SD	%RSD	Mean Area±SD	%RSD
Embelin	897129±2895.4	0.34	897129±2890.1	0.32	897172±2506.5	0.27
Gallic Acid	595643±21275.24	0.56	5956431±21271.24	0.35	3562197±33179.14	0.93
Piperine	356210±20294.78	0.37	3562103±20291.35	0.56	5956138±11972.22	0.20

* SD: Standard Deviation
* RSD: Relative Standard Deviation

Table 8. Results of reproducibility of developed method

S. No.	Area of Embelin	Area of Piperine	Area of Gallic Acid
1	897284	3562101	5955808
2	896848	3559052	5946071
3	897527	3562219	5938503
4	896351	3548907	5936047
5	894515	3562258	5948645
6	895921	3557894	5939516
Mean± SD	896407.6±1003.07	3558738.5± 4713.04	5944098.3± 6816.76
%RSD	0.11	0.13	0.11

Table 9. Robustness results from wavelength change

Phytomarkers	Wavelength	Mean Area±SD	%RSD
Embelin	**283nm**	888483±2347.11	0.26
	285nm	881320.3±11632.61	1.31
	287nm	890026.3±1914.63	0.21
Gallic Acid	**283nm**	5954594±22239.4	0.37
	285nm	5955808±18572.18	0.31
	287nm	5952772±94870.5	1.59
Piperine	**283nm**	3555434± 5194.19	0.14
	285nm	3562134±18476.8	0.51
	287nm	3557442±23667.3	0.66

* SD: Standard deviation
* RSD: Relative standard deviation

Table 10. Robustness study data for Flow rate change

Phytomarkers	Flowrate (mL/min)	Mean Area±SD	%RSD
Embelin	0.8	888344.3±5763.07	0.64
	1	890026.3±1914.63	0.21
	1.2	880177±12087.24	1.37
Piperine	0.8	3555971±5899.155	0.16
	1	3560800±18852.43	0.52
	1.2	3555464±18740.7	0.53
Gallic acid	0.8	5953261±34783.3	0.58
	1	5955436±16239.9	0.27
	1.2	5952775±10117.3	0.16

* SD: Standard Deviation
* RSD: Relative Standard Deviation

3.3.7 Quantification of Phytomarkers

The markers were quantified through HPLC analysis of sample solutions as shown in Figure 12.

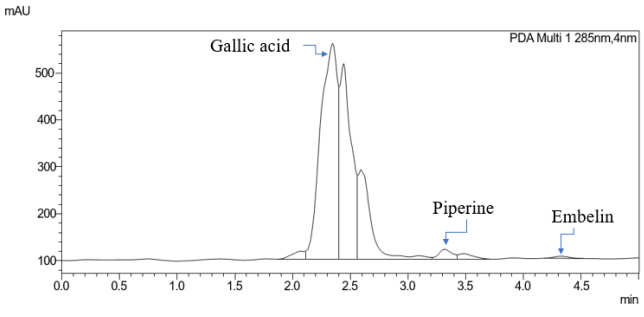

Figure 12. Chromatogram of formulation.

In *Vidanga vati* formulation, Embelin, Piperine and Gallic acid were found to be 0.04mg, 0.16mg and 2.24mg respectively.

4. Conclusion

The current study presents a novel rapid HPLC method for quantifying Embelin, Piperine and Gallic acid in polyherbal formulations. The developed HPLC method was validated by the ICH Guideline (Q2R1) and the results were found to be accurate, linear, repeatable, sensitive and precise for detection and quantification. As a result, the proposed method was shown to be adequate and suitable for routine qualitative and quantitative evaluation of Embelin, Piperine, and Gallic acid in polyherbal formulations including these markers.

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