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Research Article

RP-HPLC Method Development and Validation for the Marker Compound Embelin in *Palashabijadi Churna*

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Abstract

Aim and Objectives: The present study aimed to prepare *Palashabijadi Churna* under laboratory conditions and to develop and validate a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantitative estimation determination of embelin as, a marker compound. Methods: Palashabijadi Churna was prepared in accordance with the standards prescribed in the Ayurvedic Pharmacopoeia. RP-HPLC was then used to analyse the prepared formulation for the quantitative determination of embelin. Several preliminary chromatographic trials were performed to optimize the analytical conditions. The optimised mobile phase consisted of methanol and 0.1% orthophosphoric acid in a ratio of 90:10 (v/v), using a C-18 column. The analysis was carried out at 290 nm, with the column temperature maintained at 40°C. Before HPLC analysis, embelin was preliminarily identified by thin-layer chromatography (TLC) using a standard reference compound. Results: Embelin was detected in the laboratory-prepared formulation at an Rf of 0.32 by TLC using toluene: acetone: acetic acid (4.5:0.5:0.25, v/v/v) as the mobile phase and silica gel GF254 as the stationary phase. The λmax of embelin was found at 290 nm**.** In the RP-HPLC analysis, the standard embelin showed a retention time of 8.13 minutes, whereas the formulation sample exhibited a retention time of 8.07 minutes, thereby confirming its presence. Using the optimised mobile phase consisting of methanol and 0.1% orthophosphoric acid (90:10, v/v), the embelin content in the prepared formulation was quantified as 0.004966. The developed RP-HPLC method was validated according to standard analytical validation parameters and demonstrated precision, accuracy, and reliability. Conclusion: The laboratory-prepared Palashabijadi Churna was systematically standardized through marker-based evaluation. The validated RP-HPLC method was found suitable for routine quantitative analysis of embelin, thereby supporting the quality control and standardization of the formulation.

Keywords: *Palashabijadi Churna, Embelin, RP-HPLC Development and Validation.*

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Introduction

Palashabijadi Churna is a classical Ayurvedic polyherbal formulation described in the Ayurvedic Pharmacopoeia of India (1). The formulation is also referenced in Rasoddara Tantra (also spelled Rasodhara Tantra), a classical text of Rasa shastra. The ingredients of *Palashabijadi Churna (PC) include Palasha bija (Butea monosperma L.), Indrayava (Holarrhena pubescens L.), Vidanga (Embelia ribes L.), Nimba bija (Azadirachta indica A. Juss.), and Kiratatikta (Swertia chirata Buch.-Ham.).* According to the Ayurvedic Pharmacopoeia of India, the formulation is indicated for the management of worm infestation (Krimi Roga) (1).

A review of the literature revealed limited data regarding the standardization of *Palashabijadi Churna.* A TLC Identification and Spectrophotometric estimation of Embelin in *Embelia ribes* was carried out by Sk Chauhan etal (2). Although previous studies have reported its antimicrobial activity (3) and its high-performance thin-layer chromatography (HPTLC) evaluation (4), no clear journal report has been found on the quantitative estimation of embelin as a marker compound using reverse-phase high-performance liquid chromatography (RP-HPLC) in *Palashabijadi churna*. Pharmacognostic Standardization of the Ayurvedic formulation *Palashabijadi Churna* was reported by K.N. Anuradha et al. (5). Development and validation of an RP-HPLC method for the simultaneous determination of Embelin, Rottlerin and Ellagic acid in *Vidangadi churna* was reported (6). Therefore, the present study aimed to prepare *Palashabijadi Churna* under laboratory conditions and to carry out marker-based

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standardization by quantifying embelin using a validated RP-HPLC method.

Materials and methods

Preparation of *Palashabijadi churna*

Palashabijadi Churna consists of 5 ingredients: *Palashabijadi churna* (PC) include *palash bija* (*Butea monosperma* L.), *Indra yava* (*Holarrhena pubescens* L.), *Vidanga* (*Embelia ribes* L.), *Nimba bija* (*Azadirachta indica*.), and *Kiratatikta* (*Swertia chirata*).

All raw materials were procured from authorized local Ayurvedic drug suppliers. The botanical identity of Palasha Bija (*Butea monosperma* L.) was established based on the macroscopic and microscopic characteristics described in the *Ayurvedic Pharmacopoeia of India* (Part I, Vol. V) (7). Indrayava (*Holarrhena pubescens* L.) was identified in accordance with the standards specified in the *Ayurvedic Pharmacopoeia of India* (Part I, Vol. I) (9). Vidanga (*Embelia ribes* L.) was authenticated as per the official monograph given in the *Ayurvedic Pharmacopoeia of India* (Part I, Vol. 1) (11). The macroscopic and microscopic features documented in the *Ayurvedic Pharmacopoeia of India* (Part I, Vol. V) (10) were used to authenticate Nimba Bija (*Azadirachta indica* A. Juss.). Authentication of Kiratatikta (*Swertia chirata* Buch.-Ham.) was carried out in accordance with the standards prescribed in the *Ayurvedic Pharmacopoeia of India* (Part I, Vol. I) (8). Voucher specimens of all authenticated drugs were deposited in the Department of Pharmacognosy for future reference.

The crude drugs were cleaned to remove extraneous matter and, if required, dried. separately pulverized Each ingredient to obtain a fine powder and passed through sieve No. 80 to ensure uniform particle size. Weighed accurately equal proportions (1 part each) of *Butea monosperma*, *Holarrhena pubescens*, *Embelia ribes*, *Azadirachta indica*, and *Swertia chirata* then blended thoroughly to obtain a homogeneous formulation. The composition of the lab formulation is shown in Table 1.

Table 1: Ingredients of laboratory formulation of *Palashabijadi churna*

S. No	Drug	Lab Formulation	Part	Quantity
1	*Palash Bija*	*Butea monosperma* L	Seed	1 part
2	*Indra yava*	*Holarrhena pubescens*	Seed	1 part
3	*Vidang*	*Embelia ribes*	Fruits	1 part
4	*Nimba bij*	*Azadirachta indica*	Seed	1 part
5	*Kiratatikta*	*Swertia chirata*	Arial parts	1 part

Identification of Embelin in *Palashabijadi churna* by TLC (12)

Since embelin is the principal bioactive constituent of *Embelia ribes*, one of the ingredients of *Palashabijadi Churna*, the formulation was subjected to thin-layer chromatographic (TLC) analysis for its identification.

For sample preparation, 5 g of the laboratory-prepared formulation was accurately weighed and extracted with 25 mL of chloroform under reflux for 30 minutes. The extraction procedure was repeated twice to ensure complete recovery of the analyte. The combined extracts were cooled and filtered, and the filtrate was concentrated under reduced pressure using a rotary evaporator. The solvent was removed entirely to obtain a residue, which was reconstituted in 2 mL of methanol and used as the test solution. Prepared the standard solution by accurately weighing embelin and dissolving it in methanol to obtain a concentration of 1 mg/mL.

Carried out TLC analysis on silica gel GF_{254} precoated aluminium plates. The mobile phase used by the Soni etal. 2024 (12), with modification, was used and consisted of toluene: acetone: acetic acid (4.5:0.5:0.25, v/v/v). The standard and sample solutions were spotted on the plate using a capillary tube. Following chromatographic development, the plates were air-dried and observed under UV light at 254 nm. The occurrence of embelin in the formulation was verified by matching the Rf values and spot characteristics of the sample with those of the reference standard.

Estimation of Embelin content in formulation by HPLC

An established, validated reverse-phase high-performance liquid chromatography (RP-HPLC) method was used to quantify the marker compound embelin in laboratory-prepared *Palashabijadi Churna.*

Preparation of Mobile Phase

The mobile phase comprised methanol and 0.1% orthophosphoric acid in a ratio of 90:10 (v/v). This composition was finalized after multiple preliminary experiments to obtain acceptable resolution, good peak symmetry, and suitable retention behavior.

Preparation of Sample Solution

Triplicate portions (5 g each) of the laboratory-prepared formulation was carefully weighed and refluxed with 25 mL of chloroform for 30 minutes. The residual marc after filtration was extracted again with a further 25 mL of chloroform under the same conditions to ensure exhaustive recovery of the analyte. The pooled extracts were then filtered and concentrated under reduced pressure using a rotary evaporator. The residue dried was dissolved in 10 mL of the mobile phase, passed through an appropriate membrane filter, and subsequently analyzed by RP-HPLC.

Preparation of Standard Solution of Embelin

Prepared a standard stock solution of embelin by accurately weighing 10 mg of embelin and dissolving it in 10 mL of the mobile phase to obtain a concentration of 1 mg/mL. Sonicated the solution to ensure complete dissolution.

Determination of Analytical Wavelength

To determine the maximum absorption wavelength (λmax), 1 mL of the standard embelin stock solution (1 mg/mL) was diluted to 10 mL with the mobile phase to obtain a concentration of 100 µg/mL. Scanned the resulting solution in a UV–Visible spectrophotometer over the wavelength range of 200–400 nm. The λmax was determined from the absorption spectrum. A standard stock solution of embelin was prepared by accurately weighing 10 mg of embelin and dissolving it in 10 mL of the mobile phase to obtain a concentration of 1 mg/mL and the solution was sonicated to ensure complete dissolution.

System Suitability Study

Assessed the system suitability by preparing six replicate injections of the standard solution at a concentration of 30 µg/mL. The solutions were filtered through an appropriate membrane filter prior to injection. Retention time, peak area, tailing factor, and number of theoretical plates were recorded and evaluated to ensure compliance with established acceptance criteria.

Linearity

The linearity of the method was assessed by analysing standard solutions of embelin within the concentration range of 10–50 µg/mL. Appropriately diluted aliquots from the stock solution were with the mobile phase to prepare working standard solutions at concentrations of 10, 20, 30, 40, and 50 µg/mL for calibration purposes.

Each concentration level was prepared separately and injected into the HPLC system in triplicate. The peak areas corresponding to each concentration were recorded. A calibration curve was generated by plotting concentration (µg/mL) on the X-axis against peak area on the Y-axis. Linearity was evaluated through linear regression analysis, and the correlation coefficient (R^2) was calculated.

Precision

Intra-day Precision

Assessed Intra-day precision by injecting the optimised concentration of 30 µg/mL six times within the same day at different time intervals. The peak areas were recorded, and the results were expressed as percentage relative standard deviation (%RSD).

Inter-day Precision

Inter-day precision was assessed by analysing the optimized concentration of 30 µg/mL over three successive days. On each day, six replicate injections were carried out and the corresponding peak responses were noted. The reproducibility of the method across different days was evaluated by calculating the % RSD of the results.

Accuracy

Accuracy of the developed method was evaluated using the standard addition technique. Previously analysed sample solutions were fortified with known quantities of standard embelin at three levels—50%, 100%, and 150% of the nominal concentration. Each level was prepared in triplicate and injected into the HPLC system under the optimized conditions. The method's accuracy was evaluated by calculating the percentage recovery of embelin.

Limit of detection & Quantification

The solutions for a limit of detection & quantification were prepared based on the signal-to-noise ratio (S/N) obtained from standard deviation and slope, filtered, and injected.

LOD= 3.3 x standard deviation/ slope

LOQ= 10 x standard deviation/ slope

Robustness

Evaluated the robustness of the proposed RP-HPLC method by analysing a 30 µg/mL embelin standard while deliberately introducing minor variations in chromatographic conditions. Examined parameters such as flow rate (±0.1 mL/min from the optimized value), detection wavelength (±5 nm around the selected wavelength, e.g., 290 nm), and column temperature (±5°C from the optimized temperature, e.g., 40°C). Under each modified condition, the standard solution was injected and system suitability metrics including retention time, peak area, tailing factor, and theoretical plate count, were recorded to determine the method's tolerance to small operational changes.

Assay of Embelin

Triplicate samples (5 g each) of the laboratory-prepared formulation were accurately weighed and refluxed with 25 mL of chloroform for 30 minutes. The mixture was filtered, and the remaining marc was extracted once more with a further 25 mL of chloroform under the same conditions to ensure exhaustive extraction. The pooled extracts were filtered and dried under reduced pressure in a rotary evaporator to obtain the residue.

Dissolved the residue in 10 mL of the mobile phase consisting of methanol: 0.1% orthophosphoric acid (90:10, v/v) to obtain the stock solution. From this solution, 0.1 mL was transferred into a 10 mL volumetric flask and diluted to volume with the mobile phase to obtain the working sample solution. Injected a volume of 20 µL of the prepared solution into the HPLC system and recorded the chromatogram. Performed the extraction and analysis in triplicate. Measured the peak areas and calculated the content of embelin in the formulation using the calibration curve.

Results and Discussion

The formulation was prepared in the laboratory as per Ayurvedic Pharmacopoeia of India as mentioned in Table 1.

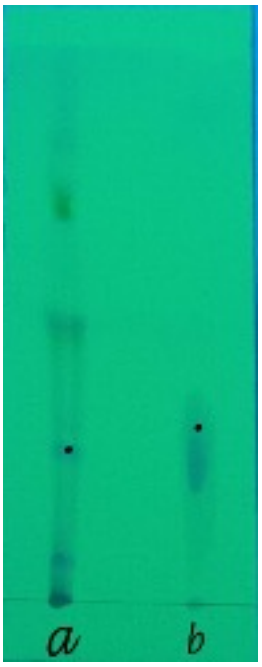
Identification of Embelin by Thin Layer Chromatography

The TLC analysis of the laboratory-prepared *Palashabijadi Churna* confirmed the presence of embelin. The standard embelin solution produced a distinct, well-defined spot under UV light at 254 nm and exhibited a characteristic purple colour upon visual observation. The test sample extract showed a prominent spot at the same Rf as the standard and exhibited colour characteristics similar to those of the standard, thereby confirming the presence of embelin in the formulation.

The Rf of standard embelin was approximately 0.33, while the formulation extract showed a spot with a comparable Rf (0.32), indicating the presence of embelin in the prepared formulation. The colour and fluorescence characteristics of the test sample spot were consistent with those of the standard compound when observed under UV illumination. The TLC chromatogram is presented in Figure 1.

The results demonstrate that embelin was successfully identified in the laboratory-prepared *Palashabijadi Churna,* thereby confirming the presence of the selected marker compound.

Figure 1: Thin layer chromatogram of embelin in the *Palashabijadi churna*



a: Extract of *Palashabijadi churna,* b: Standard Embelin.

Estimation of Embelin content in formulation by HPLC

The standard embelin solution exhibited maximum absorbance at 290 nm in the UV region, which was selected as the detection wavelength for HPLC analysis. Based on several preliminary chromatographic trials, the optimised mobile phase was determined to be methanol: 0.1% orthophosphoric acid in the ratio

of 90:10 (v/v), providing satisfactory resolution and peak characteristics.

The UV absorption spectrum of embelin is presented in Figure 2. The representative HPLC chromatogram of standard embelin under optimized conditions is shown in Figure 3. The optimized chromatographic parameters employed for the analysis are summarized in Table 2.

As part of the analytical method validation, system suitability, linearity, precision, robustness, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were carried out.

Figure 2: λ max of the Embelin in UV Spectroscopy

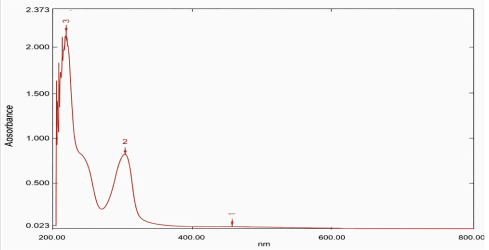


Figure 3: Optimized HPLC chromatogram of standard Embelin (10 µg/ml)

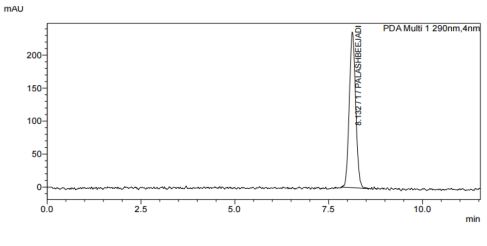


Figure 4: Calibration curve of Embelin.

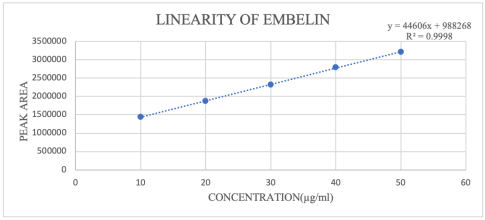


Table 2: HPLC optimized conditions for Embelin Estimation

S. No	Parameters	Conclusion
1	Mobile Phase	Methanol: 0.1% Ortho Phosphoric acid
2	Column	C-18
3	Wavelength	290nm
4	Column temperature	40^0 C
5	Run time	10 min
6	Retention time	8.15
7	Flow rate	1.2 mL/min
8	Injection volume	20 µl
9	Concentration	10 µg/ml
10	Theoretical plates	9668

System suitability

System suitability was carried out by injecting 6 replicates of 30 µg/ml solutions and the results were represented in Table 3.

Table 3: Data representing system suitability

S. No	Peak area	Ret. time	Plate count	Peak height	Tailing factor
1	2336974	8.123	9557	193456	1.054
2	2345624	8.112	9964	194330	1.012
3	2358077	8.134	9982	191162	1.003
4	2347227	8.111	9798	198656	1.052
5	2328140	8.103	9706	195340	1.056
6	2315451	8.1	9889	196633	1.038
Average	2338582.17	8.11	9816.00	194929.50	1.26
SD	15172.64	0.01	163.89	2595.45	0.02
% RSD	0.65	0.16	1.67	1.33	1.83

Limits: Theoretical plates, >2000; Tailing factor, ≤ 2; % RSD, <2

Linearity

Standard solutions in the concentration range of 10 - 50 µg/ml, in triplicate, were injected into the HPLC system. Peak responses were noted, and the calibration curve was plotted using concentration on the X-axis and peak area on the Y-axis. The calibration curve of Embelin was represented in Figure 4.

Precision

Data representing Intra-day precision within a day at different time intervals. and Inter-day precision on different days was represented in Table 4 and 5.

Table 4: Data representing Intra-day precision

S. No	Peak area		
	9.00 am	1.00 pm	5.00 pm
1	2333902	2347089	2356698
2	2331770	2322225	2302271
3	2334420	2354511	2354395
4	2318575	2340674	2345180
5	2328762	2326075	2315536
6	2333387	2349092	2359920
Average	2330136.00	2339944.33	2339000.00
SD	6021.94	13067.36	24189.43
% RSD	0.26	0.56	1.03
Limits	% RSD < 2.0	% RSD < 2.0	% RSD < 2.0

Table 5: Data representing Inter-day precision

S. No	Peak area		
	Day 1	Day 2	Day 3
1	2359011	2349043	2363071
2	2340315	2341309	2349975
3	2354361	2323423	2399626
4	2344287	2310814	2341668
5	2367842	2348687	2371311
6	2354365	2390973	2319171
Average	2353363.50	2344041.50	2357470.33
SD	9961.58	27516.60	27469.13
% RSD	0.42	1.17	1.17
Limits	% RSD < 2.0	% RSD < 2.0	% RSD < 2.0

Accuracy

Accuracy of the developed RP-HPLC method was evaluated by the standard addition method. A pre-analyzed solution of embelin at a concentration of 10 µg/mL was spiked in triplicate with known quantities of standard embelin corresponding to 50%, 100%, and 150% of the initial concentration. The spiked samples

were analyzed under optimized chromatographic conditions, and the percentage recoveries were calculated.

The mean percentage recoveries for the 50%, 100%, and 150% levels were found to be 98.46%, 101.83%, and 101.90%, respectively, indicating good accuracy of the method.

Limit of Detection & limit of Quantification (LOD & LOQ):

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of the calibration curve. The LOD and LOQ were 1.122 µg/mL and 3.401 µg/mL, respectively, indicating adequate sensitivity of the developed RP-HPLC method for the estimation of embelin.

Robustness

Assessed the robustness of the developed RP-HPLC method was by deliberately varying chromatographic parameters such as detection wavelength, flow rate, and column temperature from the optimised conditions. The results obtained under these modified conditions are presented in Table 6, demonstrating the method's reliability against small, intentional variations.

Table 6: Data representing wavelength variation ± 5 nm, Flow rate variation ± 0.1ml and Temperature variation ± 5 °C

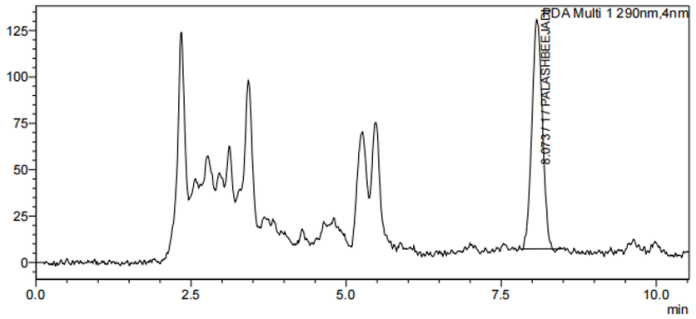
S. No	Parameter	Condition	Peak area	Retention time	Standard deviation	% RSD
1	Wave length	285nm	2370410	8.033	3311.27	0.139
2		290nm	2358077	8.134	3875.57	0.165
3		295nm	2049901	8.048	5159.94	0.252
1	Flow rate	1.1 ml	2567935	8.717	5143.70	0.20
2		1.2 ml	2345624	8.112	25243.32	1.07
3		1.3 ml	2233851	7.389	4459.28	0.199
1	Tempe-rature	35°C	2368764	8.215	28086.46	1.19
2		40°C	2347227	8.111	3619.69	0.154
3		45°C	2371977	7.924	11770.55	0.499

Assay of Embelin in *Palashabijadi churna*

The methanolic extracts of laboratory-prepared formulation was analysed by HPLC, and the corresponding peak areas were recorded. The concentration of embelin in the samples was determined using the calibration curve derived from the linear regression equation. The embelin content in the laboratory-prepared formulation was found to be 0.04966 mg/g, which corresponds to 0.004966% w/w.

The HPLC chromatograms of the methanolic extract of laboratory formulations was represented in Figure 5.

Figure 5: HPLC chromatogram of the laboratory formulation of Palashabijadi churna



Discussion

A selective and dependable RP-HPLC method was developed for quantifying embelin in the laboratory-prepared *Palasabijadi Churna*. The chromatographic conditions yielded a sharp and well-resolved embelin peak with no detectable interference from other formulation components, demonstrating the method's specificity for this polyherbal matrix. All system suitability criteria were found to be within acceptable limits, confirming satisfactory chromatographic performance.

Method validation carried out in accordance with ICH guidelines showed excellent linearity with a high correlation coefficient. The procedure exhibited good precision, with %RSD values within permissible limits, and the recovery studies confirmed the accuracy of the method. The calculated LOD and LOQ values indicated sufficient sensitivity for routine quality control, and the robustness study established that minor deliberate variations in analytical conditions did not significantly affect method performance.

The quantified embelin content in the prepared formulation was consistent, supporting uniformity of the laboratory batch. Although embelin estimation has been reported in plant materials (2) and other formulations (6), and HPTLC methods (4) exist for *Palashabijadi Churna*, this study provides a validated RP-HPLC approach for this formulation. The method is therefore suitable for routine quality control and contributes to the scientific standardization of Ayurvedic formulations.

Conclusion

The formulation was quantitatively analyzed for embelin, the chosen marker compound, using the validated RP-HPLC method under controlled laboratory conditions. The analytical results support the establishment of a reliable method for standardizing this formulation. The developed procedure proved to be rapid, straightforward, cost-effective, and sufficiently sensitive for the estimation of embelin in the samples. Owing to its satisfactory validation characteristics, the method can be applied confidently for routine analysis in pharmaceutical industries, academic settings, and quality control laboratories. Overall, the study provides useful analytical evidence to support the formulation's preparation, quality standardization, and therapeutic relevance within the traditional system of medicine.

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