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RESEARCH ARTICLE

Reversed Phase - HPLC Method Development and Validation for Simultaneous Estimation of Berberine Hydrochloride, Plumbagin, Conessine in *Ayurvedic* Formulation

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Abstract

Background: The objective of this research was to create a dependable RP-HPLC analytical technique for concurrently determining the levels of conessine, berberine HCl, and plumbagin in an ayurvedic formulation. **Aim:** The main goals were to develop a straightforward, precise, and consistent method for the pharmaceutical analysis of these compounds. **Methods:** The analytical method was developed using a BDS hypersil C18 tertiary mode column with a mobile phase incorporating 1% perchloric acid in water: methanol: acetonitrile (10:40:65% v/v/v). The analysis utilized a 1 ml/min flow rate, and detection was performed at 212 nm utilizing a PDA detector, SPD-20 A. The retention times for conessine, berberine HCl, and plumbagin were determined to be 3.12 minutes, 6.60 minutes, and 13.64 minutes, respectively. Linearity assessments were conducted in the concentration range of 25–75 ppm for all three phytomarkers. The correlation coefficients for conessine, berberine HCl, and plumbagin were found to be 0.999, 0.999, and 0.997, respectively. **Results:** The developed RP-HPLC method exhibited excellent linearity for all three compounds. Limit of detection and limit of quantification values were determined, with conessine at 1.36 µg/ml and 1.78 µg/ml, berberine HCl at 3.52 µg/ml and 4.12 µg/ml, and plumbagin at 5.40 µg/ml and 10.67 µg/ml, respectively. Precision studies, including inter-day and intra-day, demonstrated RSD values less than 2.00%. Accuracy was assessed through standard recovery methods, revealing satisfactory recovery values for conessine (100.0%–99.8%), berberine HCl (101.0%–100.9%), and plumbagin (101.4%–99.4%). **Conclusion:** The developed RP-HPLC method offers a robust and precise tool for the simultaneous estimation of conessine, berberine HCl, and plumbagin in ayurvedic formulations. The method's accuracy and reproducibility make it suitable for use as a standardization tool in the evaluation of formulations containing these marker compounds. This study contributes to the analytical methods available for quality control in the pharmaceutical industry, particularly in the context of ayurvedic formulations.

Keywords: *Ayurvedic* Formulation, Berberine, Conessine, Plumbagin, Validation

1. Introduction

Ayurvedic formulations use effective biomarkers present in herbal plants to provide healthcare for patients. Several chromatographic and spectroscopic techniques are used to evaluate the dynamic nature of biomarkers, ensuring the purity and uniformity of marketed pharmaceutical product[1]. HPLC plays a vital role in pharmaceutical analysis because of its simplicity, precision, accuracy, and reproducibility of results. This study used the RP-HPLC method for analysis of related substances[2]. In this mode, the phase that is stationary

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is nonpolar while the mobile phase is polar. In this, the polar component eluted first, and the nonpolar compound was retained for a longer period[3,4].

Vaidyaratnam Shaddaranam Gulika tablet is an *Ayurvedic* formulation used for skin disorders. It contains six herbs and helps remove ama or toxins from the body, and treats gastric diseases caused by *vata*. *Citra* is the Sanskrit name for *Berberis vulgaris*, which belongs to the Berberidaceae family. The dried stem of *B. vulgaris* contains berberine, which is an alkaloid[5-7]. *Plumbago zeylanica*, also known as *chitraka*, belongs to the Plumbaginaceae family. The dried root of *Plumbago zeylanica* contains Plumbagin, which is a Napthoquinone[8]. *Vatsaka* is made from the dried stem bark of *Holarrhena antidysenterica*, which belongs to the Apocynaceae family. The dried stem bark of *Holarrhena antidysenterica* contains Conessine, which is a steroid alkaloid[9,10].

2. Materials and Methods

2.1 Standards and Chemicals

Merck Specialties Pvt. Ltd. (Mumbai) supplied the analytical range of the organic solvents, while conessine, plumbagin, and berberine HCl were obtained from Yucca Chemicals in Mumbai as identifying markers.

2.2 Marketed Formulation

Marketed formulation of brand *Shaddaranam Gulika* name *Shaddaranam Gulika* tablet manufactured by *Vaidyaratnam Aushadhasala* was procured from local market of Vadodara, Gujarat, India.

2.3 Preparation of Standard Reference Solution (Berberine HCl, Plumbaginand Conessine)

Weigh accurately about 2.5 mg of the Conessine, Plumbaginand Berberine into a 25 ml volumetric flask containing 10 ml Acetonitrile. Combine thoroughly, sonicate to dissolve, and fill up to the mark using acetonitrile.

2.4 Preparation of Sample Solution

Approximately 10 tablets were crushed and exactly weighed 30.75 mg of powdered tablet was transferred to 150 ml volumetric flask. Then, add around 100 ml of acetonitrile was added and sonicate and stirred well to make sure all the ingredients were completely dissolved. After adding acetonitrile to adjust the volume, the mixture was filtered through a 0.2µ nylon syringe filter.

2.5 Chromatographic Condition

Shimadzu LC-205 C auto sampler series system was used for analysis. The stationary phase employed was a BDS hypersil column (5µm, 250×4.6 mm) at room temperature. Detection was performed at a wavelength of 212 nm. The mobile phase consisted of 1% perchloric acid in water: methanol: acetonitrile (10:40:65% v/v/v), with a flow rate set at 1 ml/min.

2.6 Preparation of Perchloric Acid (1.0%)

Measure accurately 1.0 ml HPLC grade Perchloric Acid and add in the 1000 ml of the HPLC grade water and filter through 0.45µ nylon filter.

3. Validation of HPLC Method

3.1 Linearity

The calibration curve's linearity for berberine HCl, plumbagin, and conessine was established within the concentration range of 25 to 75 µg/ml. To determine linearity, 12.5 mg of each standard was taken in a 50 ml volumetric flask and diluted with acetonitrile up to the mark to create the linearity stock solution (250 µg/ml). The linearity stock solution was diluted in series to produce concentrations from 25 to 75 µg/ml. The calibration curve was developed by graphing the area of the peak against the amount contained of the standard.

3.2 Precision

Precision was assessed through intra-day and inter-day evaluations. Intra-day precision involved measuring the same concentration (1000 µg/ml) five times within a single day. The mean standard deviation and % RSD were calculated based on these measurements. For inter-day precision, the same concentration (1000 µg/ml) was measured five times over three different days, and the mean standard deviation and % RSD were computed from the obtained data.

3.3 Accuracy

To assess accuracy, standard spiking was conducted on both the placebo and the sample using a known

quantity. The method's accuracy was determined through the measurement of relative recovery. The percentage recoveries of all three phytomarkers were found at three different concentration levels of 80%, 100% and 120%.

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

LOD was calculated using the calibration curve approach. A calibration curve should be studied with a sample that contains an analyte within the Detection Limit (DL). The standard deviation is calculated using the y-intercepts of regression lines. It was estimated using the ICH guidelines equation, as shown below.

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

The LOQ was carried out using the calibration curve approach. A calibration curve should be examined using a sample containing an analyte within the range of Quantification Limits (QL). The standard deviation is calculated using the y-intercepts of regression lines. It was estimated using the ICH guidelines equation, as shown below.

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

where, σ places the standard deviation of y-intercepts of regression lines and S place for the slop of the calibration curve.

3.5 Robustness

To assess the impact of minor modifications, the effectiveness of the developed method was tested by intentionally altering two parameters. The first variation involved adjusting the flowrate, specifically from 1 ml/min to both 0.8 ml/min and 1.2 ml/min. The second change pertained to the wavelength, shifting it from 198 nm to 202 nm. This deliberate manipulation aimed to evaluate the robustness of the methodology. The accuracy of the method is tested on two occasions at a same concentration of the standard drug. The mean and percent relative standard deviation values were calculated.

4. Result and Discussion

4.1 Selection of Detection Wavelength

The overall spectra of all three markers are shown in Figure 1 and different concentrations of standard solutions of berberine HCl, plumbagin, and conessine was prepared and scanned separately using a UV spectrophotometer with the detection range kept between 200-400 nm. The detection wavelength was chosen as 212 nm because at that wavelength all three markers showed appreciable absorption.

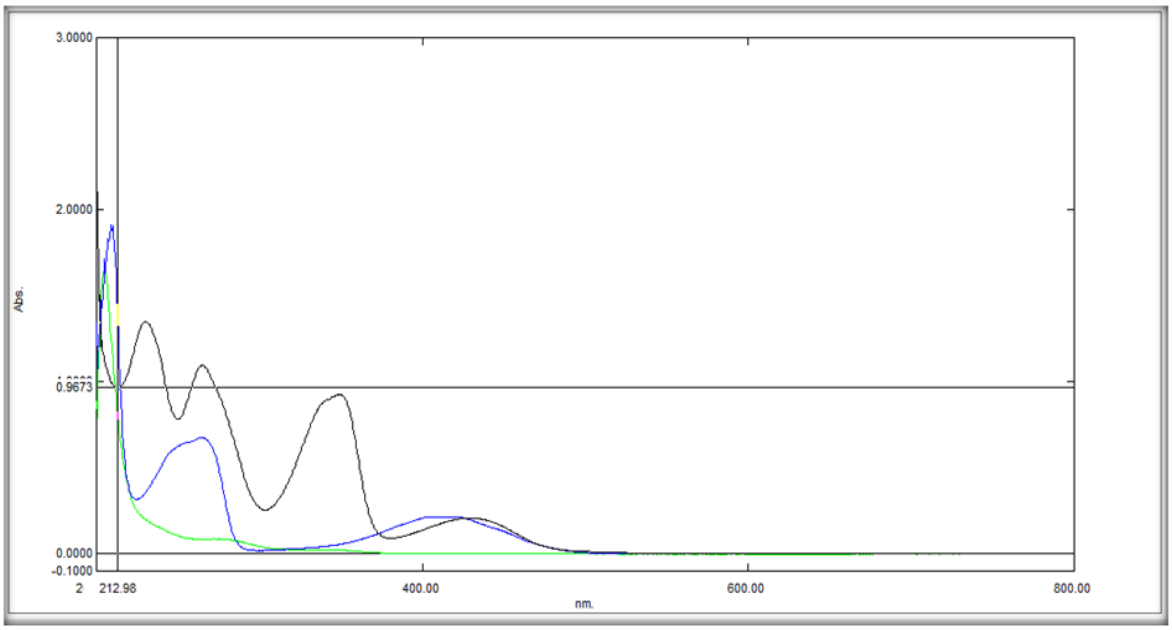

Figure 1. Overlay spectra of Berberine HCl, Plumbagin, Conessine (1µg/ml).

4.2 HPLC Method Development

The solvent's chromatogram was blank, showing that it has no effect on the ability to identify the chosen marker compound (Figure 2). The retention time for the conessine was determined to be 3.61 min as shown by the chromatogram in Figure 3. Berberine HCl chromatogram, shown in Figure 4, has a retention time of 4.59 minutes, and plumbagin chromatogram, shown in Figure 5, has a retention time of 8.73 minutes. Figure 6 displays the chromatograms of all three markers combined in mixture. Figure 7 displays the chromatogram of formulation and Figure 8 chromatogram of formulation with standard markers. The optimized chromatographic conditions for the developed HPLC method are described in Table 1.

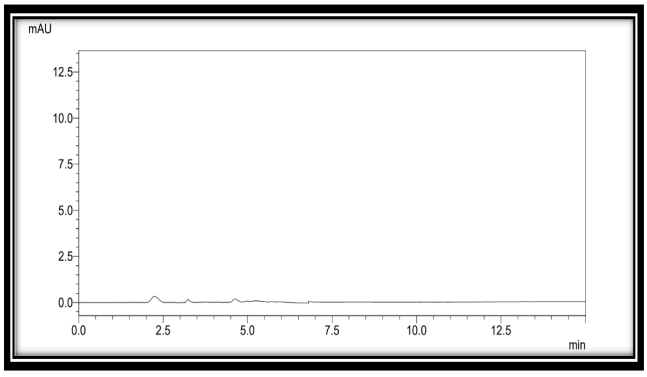

Figure 2. Blank chromatogram.

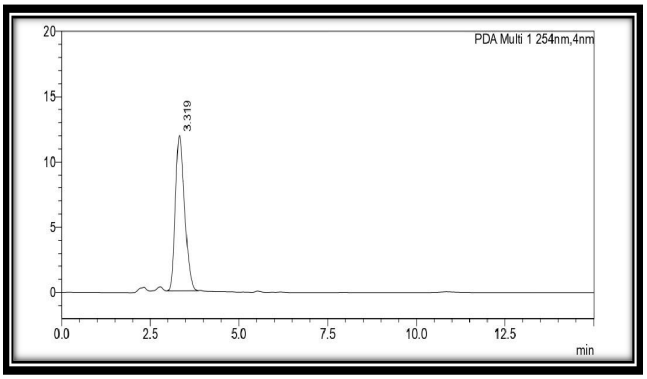

Figure 3. Chromatogram of conessine.

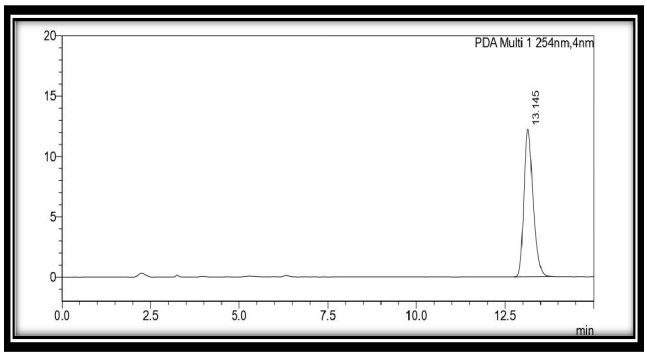

Figure 5. Chromatogram of Plumbagin.

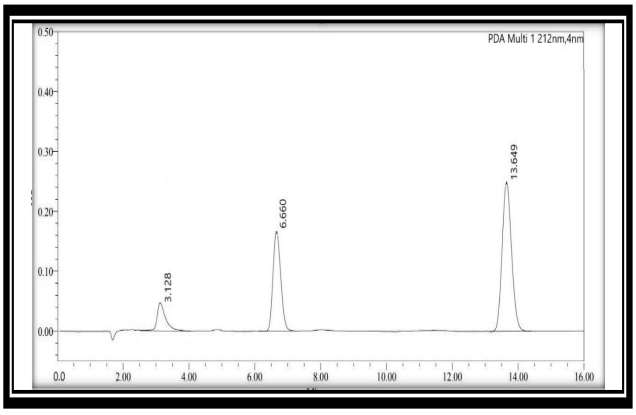

Figure 6. Chromatogram of Conessine, Berberine HCl and Plumbagin.

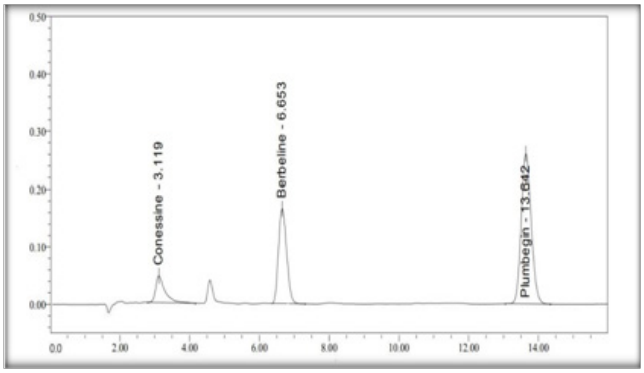

Figure 7. Chromatogram of formulation.

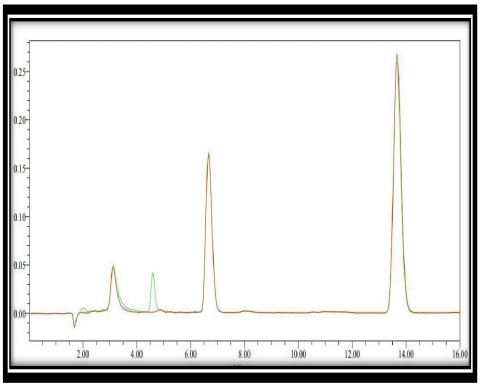

Figure 8. Chromatogram of formulation with standard markers.

Table 1. Optimized chromatographic condition for RP-HPLC

Sr. No.	Parameter	Condition
1	Column	C18 Column (250×4.6 mm (5µm))
2	Mobile phase	1% Perchloric acid in water: methanol: acetonitrile (10:40:65% v/v/v)
3	Flow rate	1 ml/min
4	Injection Volume	20 Ml
5	Wavelength	200 nm
6	Column temperature	Ambient
7	Mode of separation	Isocratic
8	Runtime	15 minutes
9	Retention time	Conessine- 3.319 Berberine HCl- 6.301 Plumbagin - 13.145

4.3 Validation of the Developed HPLC method

4.3.1 System Suitability

The system suitability parameters like height, retention time, area, resolution, theoretical plate and tailing factor was reported in Table 2 and the chromatogram is shown in Figure 9.

4.3.2 Linearity

The assessment of the proposed method's linearity involved the examination of diverse concentrations, and a calibration curve depicting the relationship between concentration and area was constructed[7]. Table 3 presents various linearity parameters of the method validation. Calibration curves of concentration versus area were individually generated for conessine (Figure 10), berberine HCl (Figure 11), and plumbagin (Figure 12). Furthermore, the linearity across the measured wavelength was evidenced in the 3D chromatogram of berberine, ellagic acid, and ferulic acid (Figure 13).

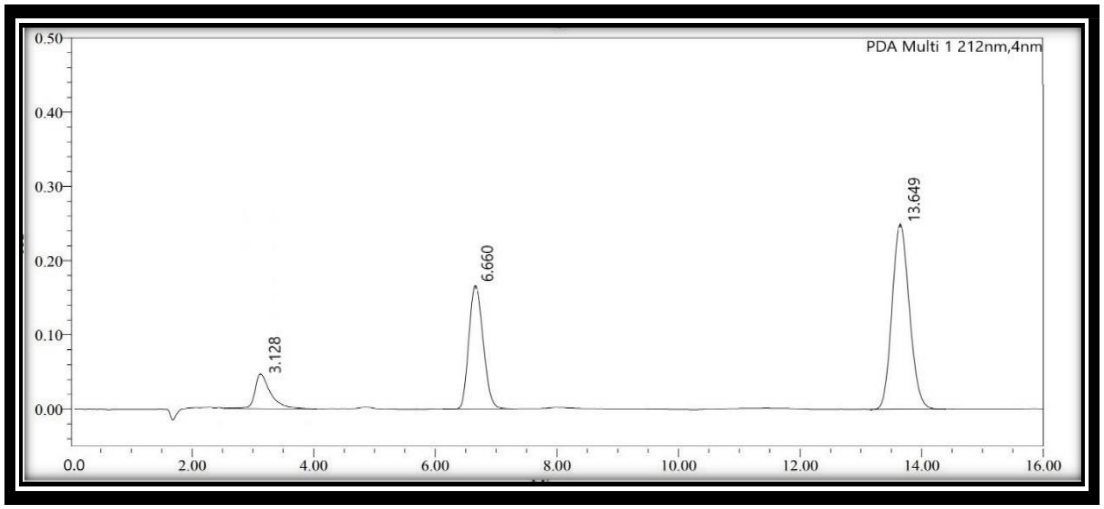

Figure 9. Chromatogram for system suitability parameter.

Table 2. System suitability parameters of the developed method

Marker compound	Retention Time	Area	Theoretical Plate	Tailing Factor
Conessine	3.12	790096	927	1.47
Berberine HCl	6.66	2668065	3675	1.17
Plumbagin	13.64	5334941	10598	1.13

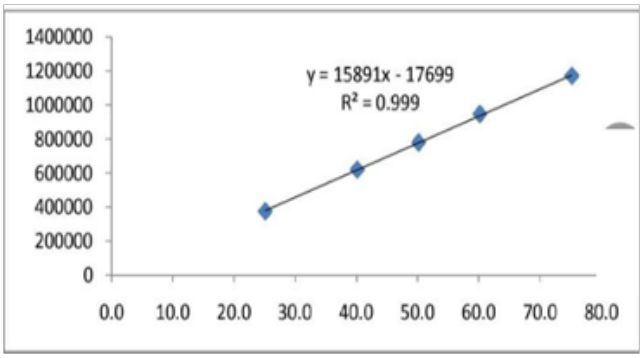

Figure 10. Calibration curves of Conessin HCl.

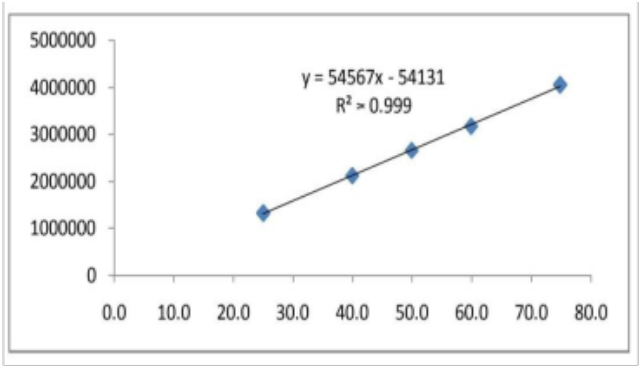

Figure 11. Calibration curves of Berberine.

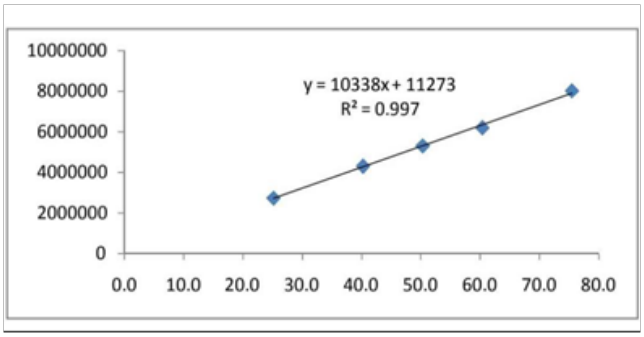

Figure 12. Calibration curves of Plumbagin.

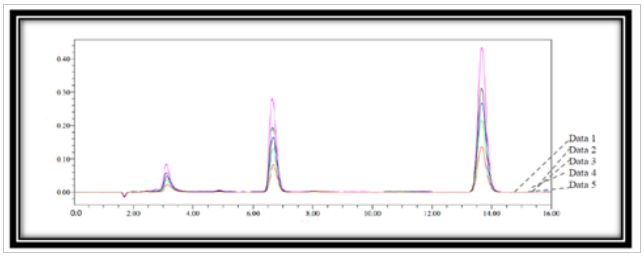

Figure 13. Linearity chromatogram of Conessine, Berberine HCl and Plumbagin.

Table 3. Linearity data of method validation

Parameters	Conessine	Berberine HCl	Plumbagin
Linearity range (µg/ml)	25 to 75µg/ml	25 to 75µg/ml	25 to 75µg/ml
Regression equation	y = 15891x - 17699	y = 54567x - 54131	y = 10338x + 11273
R^2	0.999	0.999	0.997
Standard deviation of slop	15890.699	54566.933	103381.403
LOD (µg/ml)	1.36	1.78	5.40
LODQ (µg/ml)	4.12	5.40	10.67

4.3.3 Accuracy

The method's accuracy was proven by a recovery study from a marketable formulation at three levels (80%, 100%, and 120% standard addition). The accuracy was determined by standard recovery method. The result % recovery of conessine, berberine HCl and plumbagin are shown in Table 4. The % recovery of all the three compounds was found to be in the range of 98-102 %. According to ICH guidelines the method was found to be accurate as it is with in the acceptable limits[11].

4.3.4 Precision

The developed method's precision was determined by completing repeatability, inter and intraday precision tests[10-12].

4.3.4.1 Repeatability

Repeatability performed by taking the 80 µg/ml concentration for each standard. The SD and % RSD for repeatability of all three standards were found to be < 2, respectively. So, it can be concluded that proposed

Table 4. Accuracy data of conessine, berberine HCl and plumbagin

Standards	Level of recovery	Amount of Std Spiked in mg	Area Found	Amount Recovered in mg	% recovery	% mean recovery + SD
Conessine	80	4.01	1438264	4.01	100.2	100.43±0.58
	100	5.01	1595571	5.01	100	
	120	6.01	1750989	6.08	101.1	
Berberine HCl	80	3.99	4826279	4.04	101	99.6±1.57
	100	4.99	5335511	4.89	97.9	
	120	5.99	583775	6.0	99.9	
Plumbagin	80	4.03	9275160	4.0	100.6	99.53±1.22
	100	5.03	10191503	4.94	98.2	
	120	6.04	11213458	6.02	99.8	

SD = Standard Deviation

method for estimation of conessine berberine HCl and plumbagin is precise[13,14]. The result of repeatability was shown in Table 5.

Table 5. Repeatability data of conessine, berberine HCl and plumbagin

Sr. No.	Standards	Mean Area	SD	% RSD
1	Conessine	819768	8292.18	1.011528
2	Berberine HCl	2729423	19368.38	0709615
3	Plumbagin	4983383	8710.658	0.174794

4.3.4.2 Inter and Intraday Precision

So, it can be concluded that proposed method for estimation of conessine, berberine HCl and plumbagin is precise. The results of interday and intraday precision are shown in Tables 6 and 7, respectively.

4.3.5 Robustness Study

Robustness study was done by making the deliberate change in wavelength and flow rate. The results of the robustness study are reported in Tables 8 and 9. The results show % RSD < 2 indicating that the proposed method to be robust[15].

Table 6. Interday precision data of conessine, berberine HCl and plumbagin

Standards	Mean Area			SD			%RSD		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Conessine	819684.3	819851.7	828700	10971.05	7177.45	3048.327	1.338448	0.875457	0.367844
Berberine HCl	2712972	2745874	2764192	7894.68	7977.245	7231.713	0.290997	0.290518	0.261621
Plumbagin	4981467	4985300	4977891	10675.85	8043.401	3824.323	0.214311	0161342	0.076826

Table 7. Intraday precision data of conessine, berberine HCl and plumbagin

	Conessine			Berberine HCl			Plumbagin		
Hrs	**1 hour**	**2 hours**	**3 hours**	**1 hour**	**2 hours**	**3 hours**	**1 hour**	**2 hours**	**3 hours**
Mean	376317.3	780146.3	1170272	1324885	2657980	4059171	2736338	5312030	8016070
SD	1939.525	10019.21	1181	7453.262	8803.455	6297.179	1652.365	19971.22	10287.53
%RSD	0.515396	1.284273	0.100917	0.562559	0.331208	0.155135	0.060386	0.375962	0.128336

4.3.6.1 Change in Wavelength

Table 8. Robustness study data for wavelength change

Wavelength	Standards	1	2	3	Mean	SD	%RSD
198	Conessine	977030	963226	971601	970619	6954	0.72
	Berberine HCl	2813407	2817377	2834828	2821871	11396	0.40
	Plumbagin	4414723	4395339	94408990	4406351	9958	0.23
202	Conessine	643438	634081	619944	632488	11828	1.87
	Berberine HCl	2759869	2768661	2782643	2770391	11486	0.41
	Plumbagin	6055283	6049652	6049652	6049652	9041	0.15

4.3.6.2 Change in Flow Rate

Table 9. Robustness study data for flow rate change

Flow rate	Standards	1	2	3	Mean	SD	%RSD
0.8	Conessine	1131353	991354	987297	1036668	82025	7.91
	Berberine HCl	3515845	3504123	3529843	3516604	12877	0.37
	Plumbagin	6386947	6384871	6379639	6383819	3766	0.06
1.2	Conessine	639055	640353	645907	641772	3640	0.57
	Berberine HCl	2334223	2299641	2295941	2309935	21115	0.91
	Plumbagin	3939883	3960069	3941712	3947221	11164	0.28

4.3.7 Quantification of Markers

The all three marker compounds are quantified by using standard calibration curve equation and the results are shown in the Table 10.

Table 10. Content of markers in formulation

Sr. No.	Phytomarkers	Amount present in Formulation
1	Conessine	102 mg
2	Berberine HCl	97 mg
3	Plumbagin	95 mg

5. Conclusion

Given the complexity in ayurvedic and polyherbal formulation, its crucial to guarantee that the completed product is of high quality and is produced using a trustworthy scientific process. In the current study, analytical methods for assessing the efficacy of ayurvedic formulations are being developed and validated. By using phytomarkers that are biologically active, the designed and verified RP-HPLC technique was standardised with a polyherbal formulation. The retention times for conessine, berberine HCl, and plumbagin were determined as 3.12 minutes, 6.60 minutes, and 13.64 minutes, respectively, using the developed RP-HPLC method. The 80-150 ppm range was found to be the linearity range for all three phytomarkers. Plumbagin, conessine, and berberine HCl all had correlation coefficients of 0.999, 0.999, and 0.997, respectively. The Levels of Detection (LOD) and Limits of Quantification (LOQ) for conessine, berberine HCl, and plumbagin were found to be 1.36 µg/ml and 1.78 µg/ml, 3.52 µg/ml and 4.12 µg/ml, 5.40 ug/ml, and 10.67 µg/ml, respectively. Utilising intra-day and inter-day intervals, precision studies were conducted that revealed a relative standard deviation (%RSD) value under 2.00%. Using the traditional recovery approach, the accuracy test was evaluated. The conessine recovery values of 100.0% and 99.8%, berberine HCl of 101.0% and 100.9%, and plumbagin of 101.4% and 99.4% show the accuracy of the procedure.

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