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ORIGINAL ARTICLE

Quality Control of *Bacopa monnieri* by High-Performance Liquid Chromatography Fingerprinting Combined with Chemometric Methods

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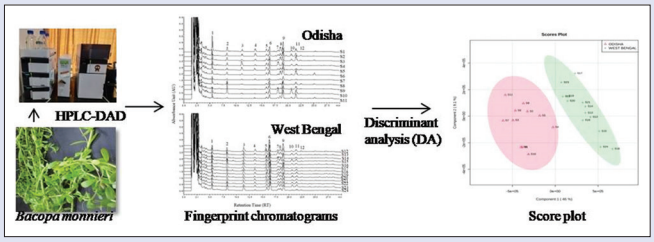
ABSTRACT

Background: *Bacopa monnieri* globally known for its tremendous memory enhancing and vitality potential which is mainly attributed to its major bioactive compound, i.e., bacoside A and bacopaside I. But differences in harvesting time, maturity, and geographical locations might result in batch-to-batch variation in active constituents which leads to quality inconsistency among samples. **Objectives:** The present work was carried out to develop the chemical fingerprint and quality consistency of *B. monnieri*. **Materials and Methods:** Twenty-four accessions of *B. monnieri* were collected from two provinces of Eastern India (Odisha and West Bengal) and were evaluated by high-performance liquid chromatography (HPLC) technique by selecting twelve common peaks. In addition, the method was validated in terms of precision, stability, repeatability, and recovery test. Moreover, chemometric analyses such as hierarchical cluster analysis, principal component analysis, and discriminant analysis were constructed from chemical fingerprint data to classify sample of different geographical origins. **Results:** The similarities analysis values were within the range of 0.81%–0.98%. In quantitative analysis, eight analytes displayed acceptable regression (R^2 > 0.989%) within the test range. The results of chemometrics were in agreement and grouped *B. monnieri* samples into two groups in accordance with the geographical origin. Bacopaside II, bacopaside I, and bacoside A3 are the influencing variable responsible for differentiating *B. monnieri* samples from different regions. **Conclusion:** The results suggested that HPLC fingerprint in combination with chemometric techniques and quantitative analysis could be used for differentiation and assessing the quality consistency of *B. monnieri*.

Key words: *Bacopa monnieri,* discriminant analysis, hierarchical cluster analysis, high-performance liquid chromatography fingerprint, principal component analysis, quality control

SUMMARY

- The objective of the present study was to develop a simple and reliable high-performance liquid chromatography (HPLC) fingerprint in combination with the chemometric method for assessing the quality of *Bacopa monnieri* collected from different geographical origins of Eastern India. The result revealed the presence of two different chemotype viz. bacopasaponin C rich and bacopaside II rich chemotype in Odisha and West Bengal respectively. HPLC fingerprint of *B. monnieri* revealed the presence of twelve characteristic peaks among twenty-four accessions of *B. monnieri* and the similarities of all the accessions were above 0.814%. Significant quantitative variations of eight components in twenty-four accessions of *B. monnieri* were observed. Chemometric approaches such as hierarchical cluster analysis, principal component analysis, and partial least square discriminant analysis could distinguish and classify the *B. monnieri* sample in accordance with the geographical origin.



Abbreviations used: DA: Discriminant analysis; HCA: Hierarchical cluster analysis; PCA: Principal component analysis; HPLC: High performance liquid chromatography; HPTLC: High performance thin layer chromatography; GC: Gas chromatography; CE: Capillary electrophoresis; SFDA: State food and drug administration; LOD: Limits of detection; LOQ: Limits of quantification; ICH: International conference on harmonization; PLS-DA: Partial least square discriminant analysis; HUVEC: Human umbilical vein endothelial cells; RRT: Relative retention time; RPA: Relative peak area; RSD: Relative standard deviation

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INTRODUCTION

Bacopa monnieri (L.) (*Scrophulariaceae*) commonly known as water hyssop is an important herb known for its medicinal properties. It is distributed in the warmer and marshy wetland regions of India, East Asia, Australia, and the United States.[1] It is used in the traditional system of Indian medicine such as Ayurveda, Siddha, and Unani for almost 3000 years as Medhya Rasayana.[2] It is gaining attention worldwide due to the inherent potential of enhancing memory and vitality.[3] Further, *B. monnieri* possesses significant pharmacological properties

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such as anti-inflammatory,[1-5] hepatoprotective,[6] anti-cancer,[7] immunostimulatory,[8] and anti-depressant activity.[9] It is considered useful in treating skin disorders, epilepsy, depression, anemia, diabetes, inflammation, and hyperpyrexia.[10]

The pharmacological activity of *B. monnieri* can be attributed due to the presence of several chemical classes of compounds such as alkaloids (brahmine, nicotine, and herpestine), steroids (stigmasterol, stigmastanol, and β-sitosterol), flavonoids (apigenin, luteolin), saponins (hersaponin, monnierin, bacopaside I, bacoside A, and bacoside B).[11-14] It can be modified as presence of several chemical classes of compounds such as alkaloids, steroids, flavonoids and saponins.[11-14] Since most of the herbal extracts exert therapeutic effects depending on the combined effects of their multiple components and multiple functions, it is inadequate to find out hardly one or two bioactive markers constituents in the complex preparations. Previous studies have reported the presence of only two bioactive markers i.e., Bacoside A and Bacopaside I for assessment of quality control in *B. monnieri.*[15] Moreover, differences in harvesting time, maturity, and geographical locations might result in batch-to-batch variation in active constituents which might lead to quality inconsistency among samples.[16,17]

Among the various chemical fingerprinting methods such as high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), gas chromatography, and capillary electrophoresis, HPLC based fingerprint is emerging as the preferred chromatographic fingerprint method.[18,19] The quality assessment of botanical extracts by qualitative and quantitative analysis of chemical constituents using chemical fingerprint has been acknowledged by various agencies such as World Health Organization,[20] European Medicine Agency,[21] U. S Food and Drug Administration.[22] Chemical fingerprint has the potential to characterize both the marker and obscure components in the plant sample due to better resolution and separation. Fingerprint pattern is a complex dataset due to the complex matrix of herbs. Therefore, the chemical patterns are processed using a chemometric technique such as similarity analysis, hierarchical cluster analysis (HCA), principal component analysis (PCA), and discriminant analysis (DA) to distinguish the accessions based on taxonomy, geographical locations, and processing methods.[23-25]

Previously, bacoside A and bacopaside I have been used as marker constituents to monitor the quality of *B. monnieri* samples using several analytical approaches such as HPLC,[26,27] HPTLC.[15,16] However, it would be difficult to describe the pharmacological efficacy of *B. monnieri* using this two compounds. None of the reports have carried out multicomponents determination of chemical fingerprint of So far no literatures have been reported regarding simultaneous separation and determination of multiple bioactive components of *B. monnieri.* There is a great need to evaluate the similarity and differences among chemical fingerprint using pattern recognition methods. Thus, the present work was accomplished to develop a simple and reliable multiple marker-based HPLC fingerprint method for quality consistency evaluation of *B. monnieri* in combination with chemometric methods. Unsupervised and supervised techniques such as HCA, PCA, and DA, respectively, were successfully used to classify and differentiate *B. monnieri* samples of different geographical locations of Eastern India.

MATERIALS AND METHODS

Plant materials

Twenty-four accessions of fresh *B. monnieri* were collected from the different geographical locations of two provinces (Odisha and West Bengal) of Eastern India in the month of July to October 2019 at their flowering stage. The identification and authentication of plant samples were carried out by Principal Scientist, Taxonomy and Conservation Division, Regional Plant Resource Centre, and voucher specimens were deposited in the herbarium of the institute.

Chemicals and reagents

Water, methanol, and acetonitrile of HPLC grade were procured from Sigma Aldrich Co. (St. Louis, Missouri, USA). Orthophosphoric acid and potassium dihydrogen phosphate were procured from Merck India Ltd., Mumbai, India. Reference standards such as luteolin, apigenin, bacopaside I, bacopaside II, bacoside A3, jujubogenin isomer of bacopasaponin C, bacopasaponin C, and bacopaside V of purity >99% were purchased from Natural Remedies Private Limited (Bangalore, Karnataka, India). The structure of these reference compounds is shown in Table 1.

Preparation of sample and standard solution

The sample solutions were prepared by taking 0.5 g of dried powder sample in a 20 mL conical flask and 20 ml of methanol was added to it. The mixture solution was sonicated in a water bath at 60°C for 20 min. The extracted solution was filtrated through a 0.22 μm membrane filter before HPLC analysis. The stock solutions of eight standards were prepared by accurately weighing these eight standards and dissolving with methanol. The calibration curves were made by serially diluting the stock solutions. All the solutions were stored at 4°C until further analysis.

Instrumentation and chromatographic conditions

HPLC analysis was accomplished using a modular Shimadzu HPLC instrument (Shimadzu, Kyoto, Japan) assembled with a binary LC-20 AD pump, a Rheodyne 8125 injector, an SPD-20 A diode array detector, and a CTO-20AC column oven. The separation was carried out in Restek C18 reverse-phase column (Shimadzu, Kyoto, Japan, 250 mm × 4.6 mm, 5 μm) with a binary gradient mode composed of 0.001M potassium dihydrogen phosphate buffer (pH adjusted to 2.4 with orthophosphoric acid) as solvent A and acetonitrile as solvent B. The gradient program was set as follows 0–0.01 min, 0%–30% B; 0.01–25 min, 30%–40% B; 25–26 min, 40%–30% B; 26–30 min, 30%–30% B. Column temperature was set at 27°C. The flow rate and injection volume of each sample and standard solution were set to 1.5 mL/min and 20 μL, respectively. The detector wavelength was set at 205 nm for acquiring the chromatograms. Before HPLC analysis, the freshly prepared HPLC mobile phase was passed through a 0.45 μm membrane filter and degassed using a sonicator.

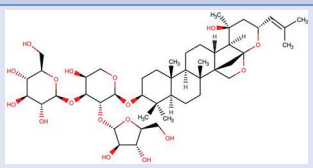
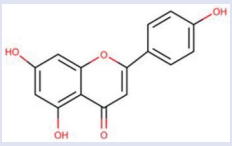
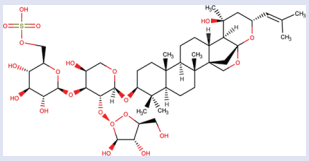
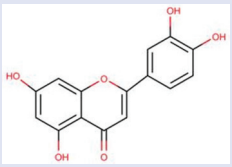
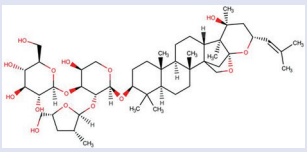
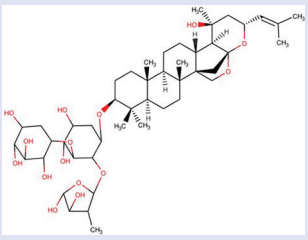
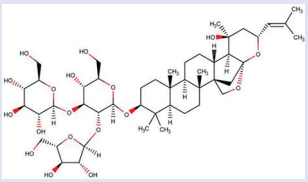
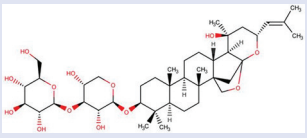
Method validation of high-performance liquid chromatography fingerprint and quantitative analysis

The developed HPLC fingerprint method was validated by assessing parameters viz. precision, stability, and repeatability of sample solution (sample 1) as per the guideline of SFDA.[28] The method validation of quantitative analysis was carried out for linearity, limits of detection (LOD), limits of quantification (LOQ), precision, recovery, repeatability, and stability as per the guidelines set by the International Conference on Harmonization.[29]

High-performance liquid chromatography fingerprint and chemometric analysis

HPLC chromatograms were analyzed for similarities using professional software named Computer-Aided Similarity Evaluation System developed by the Chinese Pharmacopoeia Committee

Table 1: Retention time, relative retention time, peak area, and relative peak area of twelve characteristic peaks of *Bacopa monnieri*

Peak	Component	Chemical structures	RT	RRT		PA	RPA	
				Average	RSD (%)		Average	RSD (%)
1	Luteolin		5.42	0.31	0.31	340784	0.40	28.26
2	Apigenin		8.31	0.48	0.31	320720	0.36	27.59
3	Bacopaside I		11.43	0.66	0.34	255946	0.28	32.50
4	Unknown		13.51	0.78	0.34	139671	0.16	51.99
5	Bacoside A3		15.76	0.90	0.31	317312	0.35	29.95
6	Bacopaside II		16.36	0.94	0.31	754313	0.82	12.28
7	Unknown		17.97	1.03	0.31	257251	0.30	32.40
8	Jujubogenin Isomer of Bacopasaponin C		18.42	1.06	0.31	204204	0.23	36.88
9	Bacopasaponin C		18.94	1.09	0.31	760589	0.87	17.00
10	Unknown		20.60	1.18	0.31	143643	0.17	40.26
11	Unknown		21.50	1.23	0.31	266412	0.30	29.19
12	Bacopaside V		22.45	1.29	0.31	51895	0.06	37.81

RT: Retention time, RRT: Relative RT, RSD: Relative standard deviation, PA: Peak area, RPA: Relative PA

(Version 2004A, Chinese Pharmacopoeia Committee, Beijing, China). The correlation coefficient of all the batches was calculated and the simulated mean chromatograms, as well as common fingerprint peaks were generated. Relative peak areas (RPA) of common peaks were normalized, scaled, and filtered before statistical analysis, then the data are mean-centered with unit variance scaling for log-transformed statistical analysis. HCA and PCA were performed using metaboanalyst 4.0, a comprehensive web-based metabolomics analysis tool (https://www.metaboanalyst.ca/). HCA with Euclidian distance similarity measure and average linkage method as

agglomeration rule was used to explore the clustering pattern among the sample. PCA constructed two major plot; score plot and loading plot by which, the sample can be grouped based on similar behavior and the impact of variables on clustering could be investigated.[30,31] Partial least square (PLS)-DA model was validated based on multiple correlation coefficients (R^2) in cross-validation and permutation test by applying 1000 interactions. The significance of metabolites was ranked using variable importance in projection score (VIP >1) from the PLS-DA model.

RESULTS AND DISCUSSION

Optimization of sample extraction and chromatographic conditions

An efficient extraction technique is necessary for obtaining optimal quantitative extraction and getting well-separated fingerprint profiles with the low background signal from the matrices. Various extraction methods (heat reflux and ultrasonication) and extraction conditions such as solvent (methanol, ethanol, and water), solvent volume (10, 20, 40 mL), extraction temperature (20°C, 40°C, and 60°C) and extraction time (10, 15 and 20 min) were tested based on single-factor experiments to maximize the extraction efficiency of target constituents. The effective extraction results was found using ultrasonication with methanol (20 mL) at 60°C for 20 min.

To achieve the peak separation in the fingerprint chromatograms of *B. monnieri* sample, optimization of the column, detection wavelength, and the mobile phase composition was also carried out. Three reverse phase column was investigated namely, Pinnacle DB C_{18} reverse-phase column (Restek Corporation, 250 mm ID × 4.6 mm ID, 5 µm), Shimpack GWS C_{18} column (Shimadzu, 250 mm × 4.6 mm, ID 5 µm), Luna C_{18} column (Phenomenex, 250 mm ID × 4.6 mm ID, 5 µm). The Restek Pinnacle DB C_{18} reverse phase column was found to be more applicable as minimized the peak tailing of Bacopaside I and also gave good peak separation with stable baselines for other compounds. An investigation of the mobile phase on chromatographic separation was carried out by taking orthophosphate buffer/methanol and orthophosphate buffer/acetonitrile. Finally, the mobile phase consist of orthophosphate buffer with pH 2.4 and acetonitrile, and sample run time of 30 min were preferred for the establishment of large number of peaks in the chromatograms of the sample solution. It was also observed that separation was better when column temperature was kept at 27°C. The detection wavelength was important for developing a reliable fingerprint and for precise quantitative analysis of target constituents, hence the chromatogram was scanned in the entire UV range. The maximum absorbance intensity of eight target constituents in samples and reference standards was observed at 205 nm, hence characteristic fingerprints of *B. monnieri* samples was taken at a detection wavelength of 205 nm.

Establishment of high performance liquid chromatography fingerprint of *Bacopa monnieri* sample and similarity analysis

The developments of chromatographic fingerprint of twenty-four batches of *B. monnieri* samples from different geographical locations of Eastern India were carried out by optimized HPLC method and matched by professional software *Similarity Evaluation System for chromatographic fingerprint of traditional Chinese medicines* (version 2004A). The profiles of HPLC fingerprints of *B. monnieri* accession collected from different geographical locations of Odisha and West Bengal are shown in Figure 1. The reference chromatogram of each province was generated as shown in Figure 2. Peaks which excelled in the entire sample with relatively high intensity and good resolution were assigned as common peaks. A total of 12 peaks (Peak 1–12) found in *B. monnieri* samples were assigned as "common peaks" based on the principle of fingerprinting, out of which eight peaks were identified as luteolin (Peak 1), apigenin (Peak 2), bacopaside I (Peak 3), bacoside A3 (Peak 5), bacopaside II (Peak 6), jujubogenin isomer bacopasaponin C (Peak 7), bacopasaponin C (peak 9), and bacopaside V (Peak 12) by comparing each peak retention time and UV absorption spectrum with the standard compounds. Peak 9 (bacopasaponin C) and 6 (bacopaside II) were taken as reference peak for Odisha and West Bengal sample respectively as it has higher peak area and better peak shape among all fingerprint peaks. The reference peak was selected to calculate the relative retention time (RRT) and RPA for twelve common peaks of all the *B. monnieri* extract which were evaluated and shown in Table 1. Thus, from the RRT and RPA data of common peaks, it could be possible to speculate the quantitative data of the HPLC fingerprint of *B. monnieri* samples. Among the twelve characteristic peaks only two peaks (peak 6 and peak 9) RSD value of RPA was below 20% and these two peaks were the predominant constituent of Odisha and West Bengal, respectively. Remaining ten peaks RSD value of RPA were more than 25% in twenty-four accessions of *B. monnieri*. There is evident batch-to-batch variation in the sample as they were collected from geographical origin. Further, it was noted that the RSD peak area of none of the twelve constituents was below 5%. Thereby we may conclude that it was not possible to monitor the RPA value, however, the RSD of RRT of twelve peak was below 1% which meet the national standard criteria of fingerprint stated by the State food drug administration. Chromatographic fingerprints should be assessed by their similarities, which should be resulted from the calculation of the correlative coefficient of the samples.[32] Therefore, fingerprint similarities analysis of *B. monnieri* samples was carried out by comparing the correlation coefficient of each chromatogram their reference chromatographic fingerprint. A correlation coefficient near to 1 suggests a high similarity value between samples. The correlation coefficient values of all the samples from different regions were more than 0.815% as listed in Table 2. These results indicated that the chromatographic patterns were usually consistent, although some peaks showed different absorption intensities. The common pattern of Odisha and West Bengal sample obtained in the present study could be used as a reference HPLC fingerprint to distinguish as well as to evaluate the *B. monnieri* sample.

Method validation of the high performance liquid chromatography fingerprint

The method validation of HPLC fingerprint analysis was validated in terms of precision, repeatability, and stability. The precision test was determined by analyzing three replicate sample solutions of sample 1 within 24 h and expressing the result as relative standard deviation (RSD) of RRT and RPA of 12 common peaks with respect to reference peak 9. The stability of the sample solution was performed by analyzing the same sample solution (sample 1) at 0, 3, 6, 12, 24, and 48 h. The repeatability of the sample was determined by testing three independently prepared same sample solutions (sample 1). The RSD values of stability, precision, and repeatability tests are listed in Table 3. The overall RSD value of RRT of precision, stability, and repeatability was 0.12%–0.56%, 0.13%–0.98%, and 0.05%–0.16%, respectively. Similarly, the overall RSD value of RPA of precision, stability, and repeatability was 0.09%–0.97%, 0.23%–2.78%, and 0.01%–1.57%, respectively. From the above test, it was observed that RSD of RRT and RPA of common peaks were <1% and 3%, respectively. The proposed HPLC fingerprint method met the national standard of the fingerprint.[28]

Table 2: Content of components 1-8 (%) and similarity value in *Bacopa monnieri* (S1-S24) samples by high-performance liquid chromatography

Sample code	Contents (%)								Similarity (%)
	Luteolin	Apigenin	Bacopaside I	Bacopaside A3	Bacopaside II	Jujubogenin Isomer of Bacopasaponin C	Bacopasaponin C	Bacopaside V	
S1	0.026	0.078	0.142	0.253	0.545	0.109	0.782	0.017	0.857
S2	0.009	0.053	0.167	0.221	0.566	0.164	0.644	0.016	0.965
S3	0.015	0.054	0.086	0.238	0.524	0.114	0.802	0.027	0.958
S4	0.010	0.041	0.347	0.260	0.759	0.395	0.545	0.028	0.821
S5	0.009	0.034	0.242	0.315	0.601	0.119	0.413	0.013	0.833
S6	0.006	0.056	0.087	0.226	0.584	0.076	0.938	0.056	0.941
S7	0.004	0.017	0.046	0.062	0.166	0.018	0.267	0.007	0.905
S8	0.010	0.064	0.023	0.123	0.530	0.022	0.349	0.073	0.857
S9	0.003	0.009	0.012	0.254	0.614	0.136	0.616	0.032	0.923
S10	0.009	0.187	0.040	0.209	0.550	0.516	0.669	0.047	0.815
S11	0.008	0.013	0.015	0.173	0.445	0.082	0.688	0.027	0.870
S12	0.010	0.070	0.343	0.509	1.333	0.183	0.712	0.037	0.982
S13	0.007	0.065	0.36	0.431	1.285	0.1383	0.706	0.041	0.971
S14	0.014	0.080	0.309	0.693	1.097	0.407	0.558	0.044	0.962
S15	0.007	0.097	0.367	0.378	1.378	0.200	0.437	0.035	0.962
S16	0.006	0.052	0.299	0.637	1.001	0.470	0.635	0.036	0.949
S17	0.012	0.092	0.309	0.589	1.032	0.389	0.428	0.041	0.915
S18	0.011	0.137	0.366	0.450	1.533	0.202	0.413	0.038	0.927
S19	0.019	0.220	0.092	0.386	0.692	0.618	0.628	0.016	0.939
S20	0.009	0.065	0.28	0.555	0.811	0.408	0.386	0.036	0.935
S21	0.007	0.055	0.307	0.463	1.224	0.194	0.652	0.036	0.981
S22	0.007	0.052	0.262	0.459	0.865	0.290	0.487	0.035	0.979
S23	0.004	0.035	0.22	0.398	0.851	0.377	0.423	0.034	0.906
S24	0.007	0.055	0.313	0.422	1.507	0.230	0.496	0.043	0.946

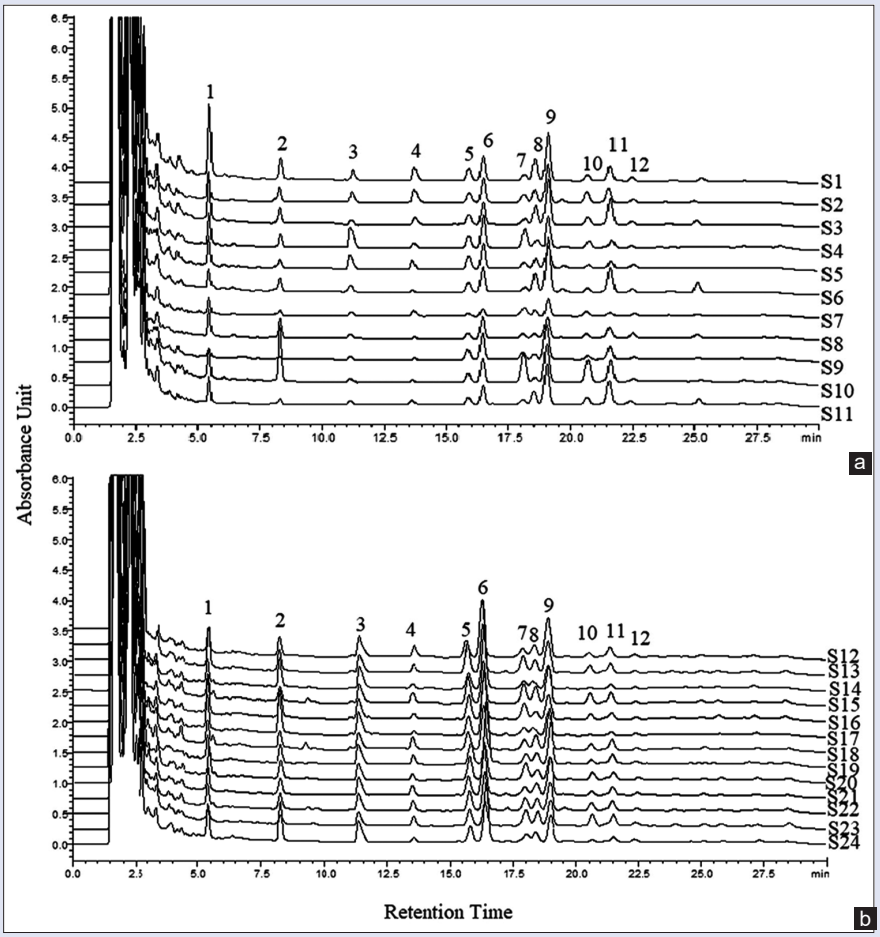


Figure 1: High-performance liquid chromatography chromatographic fingerprints of *Bacopa monnieri* accession collected from different geographical locations of Odisha (a) and West Bengal (b). The peaks marked with 1–12 in the chromatogram represent the twelve common peaks

Table 3: Summary results of precision, stability, and repeatability of 12 characteristic peaks in *Bacopa monnieri* sample (sample 1)

Peak number	RSD of RRT (%)			RSD of RPA (%)		
	Precision	Stability	Repeatability	Precision	Stability	Repeatability
1	0.33	0.24	0.16	0.56	1.32	1.51
2	0.55	0.49	0.10	0.43	1.65	0.34
3	0.53	0.23	0.05	0.90	2.78	1.18
4	0.48	0.26	0.09	0.97	0.23	1.57
5	0.31	0.39	0.08	0.68	1.89	0.50
6	0.21	0.32	0.03	0.35	1.33	0.21
7	0.30	0.18	0.02	0.83	1.55	0.71
8	0.56	0.13	0.24	0.71	1.35	1.40
9(R)	0.00	0.00	0.00	0.00	0.00	0.00
10	0.41	0.98	0.08	0.53	1.65	0.01
11	0.18	0.62	0.10	0.25	1.70	0.19
12	0.12	0.16	0.16	0.09	0.25	0.51

RT: Retention time, RRT: Relative RT, RSD: Relative standard deviation, PA: Peak area, RPA: Relative PA

Table 4: Linear range, regression equation, R^2, limits of detection, and limits of quantification of eight components

Component	Linear range (mg/L)	Regression equation	R^2	LOD (mg/L)	LOQ (mg/L)
Luteolin	6.5-32.5	y=898.9x+1.75	0.995	0.40	1.20
Apigenin	20-100	y=191.0x+0.46	0.992	3.70	11.20
Bacopaside I	35-140	y=138.4x+0.12	0.995	11.20	34.00
Bacoside A3	28-135	y=33.8x+0.03	0.999	8.90	27.05
Bacopaside II	25-125	y=34.4x+0.01	0.993	7.40	22.30
JI Bacopasaponin C	22-110	y=41.8x−0.01	0.998	6.70	20.45
Bacopasaponin C	21-105	y=53.2x+0.04	0.989	7.20	21.80
Bacopaside V	33-132	y=6110.x−26.7	0.999	10.70	32.50

Y is the peak area; X refers to the concentration of compound, R^2 refer to the regression value. LOD: Limits of detection, LOQ: Limits of quantification

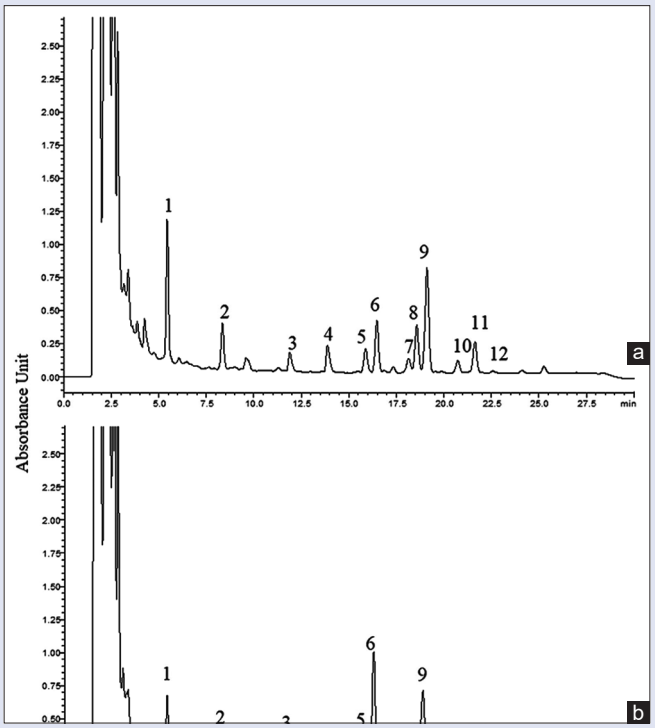


Figure 2: Reference chromatographic fingerprint of *Bacopa monnieri* samples of Odisha (a) and West Bengal (b)

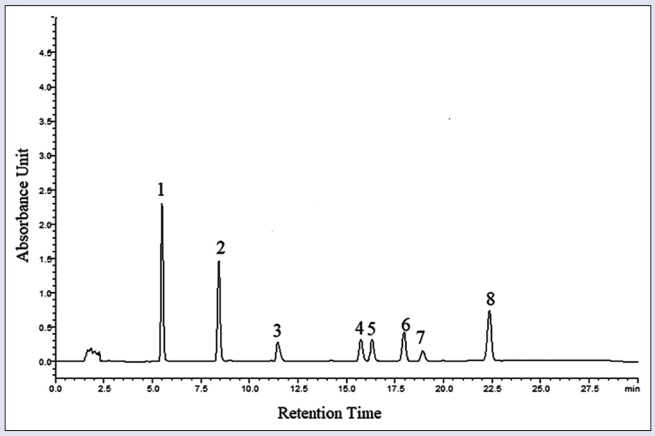


Figure 3: Typical high performance liquid chromatography chromatographic profile of eight reference compounds. 1: Luteolin, 2: Apigenin, 3: Bacopaside I, 4: Bacoside A3, 5: Bacopaside II 6: Jujubogenin Isomer of Bacopasaponin C 7: Bacopasaponin C, 8: Bacopaside V

Method validation of the quantitative data

The calibration curves were generated by plotting the peak area (y) against concentration (x, mg/l) of eight compounds. On the basis of chromatographic conditions established above, the calibration curve showed good linearity ($R2 > 0.989$) for all the analytes within the tested concentration range [Table 4]. The representative HPLC chromatogram of the eight mixed standards is shown in Figure 3. The LOD and LOQ of eight compounds were calculated as the least concentration for which the signal-to-noise ratios (S/N) were three and ten times, respectively. The LOD and LOQ of eight components were 0.40-11.20 mg/L and 1.20-34 mg/L, respectively. The values found were sufficiently low enough to allow the determination of these analytes in *B. monnieri* samples. The precision was determined by injecting the replicate solution of each standard for three times within a day. The RSD values of precision for all the investigated analytes were <2% [Table 5]. The stability test of the

Table 5: Precision, stability, and repeatability of eight components

Component	Precision RSD (*n*=3) (%)	Stability RSD (*n*=4) (%)	Repeatability RSD (*n*=3) (%)
Luteolin	1.67	1.51	0.26
Apigenin	1.13	1.83	1.11
Bacopaside I	0.87	0.77	0.78
Bacoside A3	0.75	0.61	1.37
Bacopaside II	1.39	1.59	1.03
Jujubogenin Isomer of Bacopasaponin C	1.84	1.92	0.59
Bacopasaponin C	1.17	0.98	0.87
Bacopaside V	0.99	1.26	1.08

RSD: Relative standard deviation

Table 6: Recovery rates of the eight components in *Bacopa monnieri*

Component	Original (mg/L)	Added (mg/L)	Found (mg/L)	Recovery rate (%)	Average recovery (%)	RSD (%)
Luteolin	4.75	1.00	5.59	97.20	98.26	0.82
		1.50	6.20	99.20		
		2.00	6.64	98.37		
Apigenin	32.75	4.00	35.74	97.25	98.13	0.78
		8.00	39.93	97.98		
		12.00	44.37	99.15		
Bacopaside I	63.50	5.00	67.58	98.65	99.00	0.25
		10.00	72.93	99.22		
		15.00	77.81	99.12		
Bacoside A3	69.50	10.00	78.45	98.67	99.10	0.34
		15.00	84.09	99.51		
		20.00	88.72	99.12		
Bacopaside II	150.28	5.00	150.45	96.89	98.46	1.11
		10.00	159.27	99.37		
		15.00	163.83	99.12		
JIB C	27.99	3.00	30.41	98.13	98.73	0.71
		6.00	33.43	98.35		
		9.00	36.89	99.73		
Bacopasaponin C	82.41	5.00	87.04	99.58	99.56	0.22
		10.00	91.75	99.29		
		15.00	97.23	99.82		
Bacopaside V	7.63	2.00	9.39	97.51	97.76	0.96
		4.00	11.25	96.73		
		6.00	13.50	99.04		

RSD: Relative standard deviation, JIB: Jujubogenin isomer of bacopasaponin C

sample was determined by injecting the same solution of each standard for three consecutive days (0, 24, 48, and 72 h). It observed that the solution was stable with RSD of compounds <2% [Table 5]. Repeatability of each compound was determined by independently preparing three replicates of each reference standard solutions. The RSD values of the compounds were in the range of 0.26%–1.37% [Table 5], indicating that the method repeatability was suitable. The recovery test was determined by the method of standard addition, which was confirmed by spiking known amount of standard reference compounds to the analyzed sample. Afterward, the analysis was carried out and the result of recovery for all the eight compounds was in the range of 98.13-99.56% and their RSD value were <2% given in Table 6.

Quantitative estimation of eight components in *Bacopa monnieri* sample

In this study by applying the proposed HPLC method the contents of apigenin, bacopaside I, bacoside A3, bacopaside II, jujubogenin isomer of bacopasaponin C, bacopasaponin C, and bacopaside V in twenty-four accessions of *B. monnieri* sample was estimated. The identification of marker compounds in the sample chromatogram was carried out by matching with the retention times and the UV absorption spectra of reference standards. Quantification of eight marker compounds was performed based on the external standard method using the linear range fitted calibration curve. The quantification results of eight compounds in twenty-four batches of *B. monnieri* extract are shown in Table 2. The content greatly varied in all the samples of *B. monnieri* collected from different geographical regions. The content ranges were 0.003%–0.026% (luteolin), 0.013%–0.220% (apigenin), 0.086%–0.367% (bacopaside I), 0.062%–0.693% (bacopaside A3), 0.166%–1.533% (bacopaside II), 0.018%–0.618% (Jujubogenin isomer of bacaposaponin C), 0.386%–0.938% (bacaposaponin C), and 0.013%–0.073% (bacopaside V), respectively. Interestingly, the average content of bacopaside II from West Bengal province was 1.124%. It was higher than those samples from Odisha whose average content was 0.535%. Similarly, the average content of bacopasaponin C was higher in Odisha (0.683%) than in West Bengal (0.459%). The results obtained, revealed the presence of two different types of chemotype namely, one is bacopasaponin C rich and bacopaside II rich in Odisha and West Bengal, respectively.

This study coincided with the results previously determined by HPLC and HPTLC approaches in *B. monnieri* accessions collected from different regions of India.[26] The concentration range of bacopaside II and bacopasaponin C in samples of *B. monnieri* were 0.12%–0.69% and 0.05%–0.44%, respectively. Viewed chemically, bacopasaponin C and bacopaside II are bioactive components of Bacoside A. Therefore, the quality of *B. monnieri* sample could be determined on the basis of Bacoside A contents. This difference in content could be attributed to the variations of climate, soil, and other environmental factors.

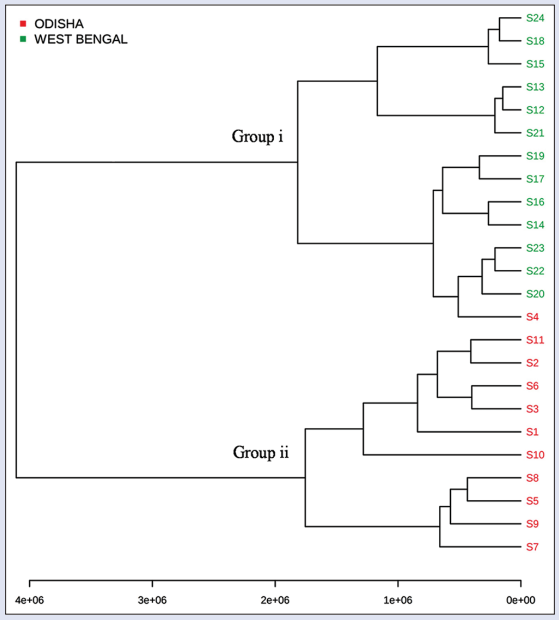


Figure 4: Dendrogram obtained by hierarchical cluster analysis using ward linkage method and Euclidian distance matrix for *Bacopa monnieri* accessions collected from different geographical locations of Odisha and West Bengal

Chemometric analysis

Hierarchical cluster analysis

HCA is an ideal unsupervised multivariate analysis technique used to cluster the sample in two-dimensional spaces on the basis of resemblance and difference among the sample.[33] A 12 × 24 matrix was formed by the RPA of common peaks in 24 accessions of *B. monnieri* from different geographical origins. Dendrogram was created using Euclidian matrix and wards linkage method as amalgamation rule. From Figure 4, it was evident that the samples were divided into two groups: Group I (S1-S11) from Odisha and Group II (S12-S24) from West Bengal provinces, respectively. The quality of sample from West Bengal province had distant relationships with those from Odisha province. Samples from Odisha had high contents of bacopasaponin C rich whereas that from West Bengal had high contents of bacopaside II, respectively. This is also in agreement with the visual comparison of their HPLC chromatograms. This is likely related to different climate conditions, geographical environments, soil nutrients between these two geographical provinces.

Principal component analysis

PCA is an unsupervised mathematical tool widely used to reduce the dimension of original data set without losing enough information by transforming large no of variables into orthogonal ones known as principal components.[34] In this study, PCA was performed by taking RPA of twelve common peaks (twelve variables) of twenty-four accessions of *B. monnieri* samples of Odisha and West Bengal province. The result of

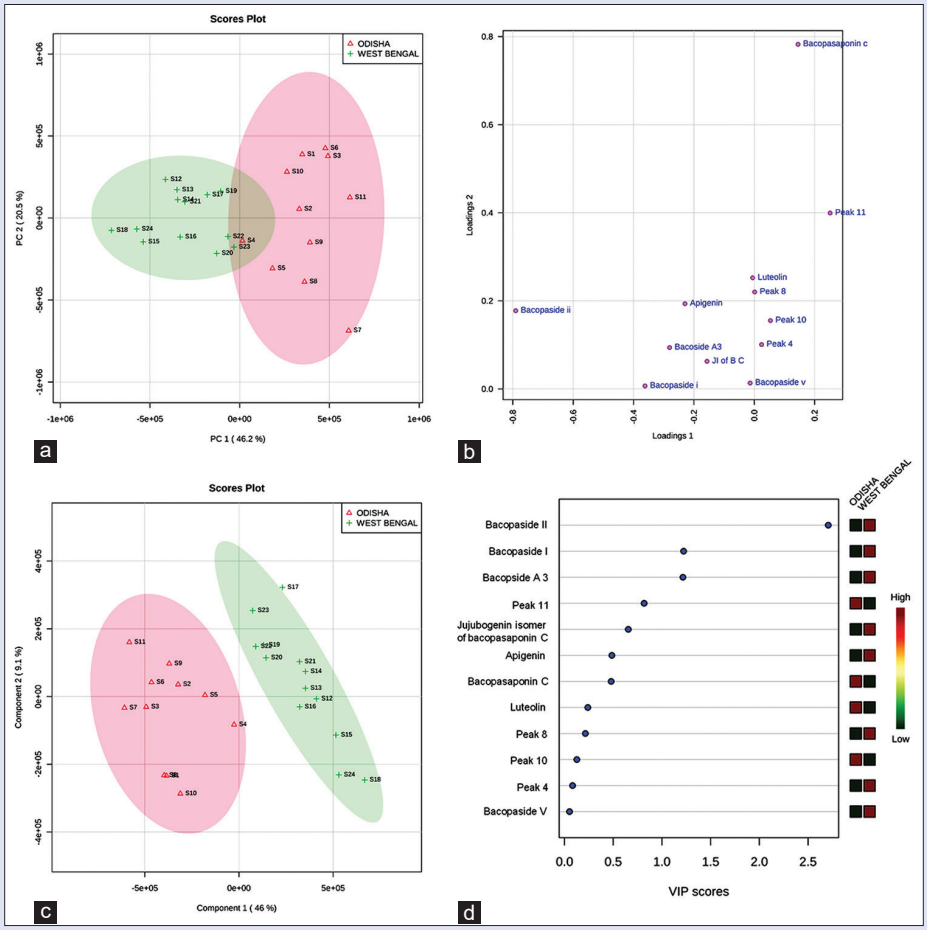


Figure 5: Chemometric analysis of twenty-four accessions of *Bacopa monnieri*. Principal component analysis score plot (a), principal component analysis loading plot (b), partial least square discriminant analysis score plot (c), and VIP score plot (d)

the PCA score plot explained 98.8% of the total variance of dataset. The first two principal components PC 1 and PC2 accounted for maximum variance namely, 46.2% and 20.5% respectively [Figure 5a]. It can be interpreted in the loading plot that bacopasaponin C and bacopaside II have more influence on the ability to discriminate between samples from the two provinces than other peaks [Figure 5b]. In addition, it is clearly visible that the sample was grouped into two groups in accordance with HCA results. There was a slight overlap between samples from Odisha and West Bengal province, therefore PLS-DA was carried out.

Partial least square discriminant analysis

PLS-DA is a supervised recognition tool used to exploit the variation among the groups and to find out the chemical constituents responsible for discrimination among diverse class of samples.[35,36] PLS-DA was carried out to enhance the group separation between the sample of Odisha and West Bengal province. The separation of the axis was shown in the score plot. The obtained score plot showed better and distinct separation of samples into two groups as compared to PCA. The first two components described 55.1% of the total variation [Figure 5c]. The predictive performance of the model was evaluated on the basis of model parameters R^2Y and Q^2Y. The values obtained for R^2Y and Q^2Y were 0.91 and 0.78 computed using cross-validation which indicates that the developed PLS-DA model has high goodness of fit and prediction value. The variable importance plot was generated to measure the influence of every chemical constituent on sample discrimination. Based on the variable important plot score >1, it can be concluded that peak 6 (bacopaside II), peak 3 (bacopaside I) and peak 5 (bacoside A3) might be the influencing variable responsible for distinguishing *B. monnieri* sample from different geographical provinces [Figure 5d]. Bacopaside II, bacopaside I, and bacoside A3 have neuroprotective and anticancer activity. Bacopaside II is a potential anti-angiogenic agent and can be used in cancer therapy as it inhibited endothelial cell migration and tube formation and induced apoptosis in mouse endothelial cell lines (2H11 and 3B11) and human umbilical vein endothelial cells cell lines.[37] Another report have revealed the neuro-protective effect of bacopaside I by reducing neurological defects, cerebral infarct volume, and edema against injury caused by cerebral ischemia.[38] Similarly, another study has shown the neuro-protective effect of bacopaside I by increasing the level of antioxidant enzymes and reducing lipid peroxidation levels, thereby protecting from oxidative stress.[39] Thus, these three bioactive constituents which are present in high concentration have numerous biological efficacies and can be used as marker constituents for assessing the quality of *B. monnieri* samples.

CONCLUSION

A simple and stable HPLC fingerprint in combination with the chemometric method was developed to assess the quality of *B. monnieri* of the various geographical origin of Eastern India. The result revealed the presence of two different types of chemotype viz. one is bacopasaponin C rich and bacopaside II rich in Odisha and West Bengal respectively. Additionally, for the first time, the HPLC fingerprint of *B. monnieri* was developed. A total of twelve characteristic peaks were selected to evaluate the similarities among twenty-four accessions of *B. monnieri* samples and they showed good similarities of above 0.814%. Furthermore, for quantitative determination, eight components in twenty-four accession of *B. monnieri* were successfully separated and determined. The method validation showed satisfactory results for all the critical parameters studied. Additionally, chemometric methods like HCA, PCA, and PLS-DA could distinguish and classify the *B. monnieri* sample in accordance with their geographical origin. The outcome showed that the developed HPLC method combined with the chemometric method could be suitable for the quality assessment and authentic identification of the *B. monnieri* sample. However, in future, analytical methods with shorter analysis time and reduced quantity of solvent consumption can be established for quality control of *B. monnieri*.

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Conflicts of interest

There are no conflicts of interest.

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