

Determination of Bergenin in Different Parts of *Bergenia ciliata* using a Validated RP-HPLC Method

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Abstract – *Bergenia ciliata* (Family: Saxifragaceae) is a folklore remedy for the treatment of various ailments in Asian countries. Bergenin (**1**) has been isolated as an active constituent in many studies, however, the amount of bergenin has not been determined in all parts of the plant. A simple RP-HPLC method was developed to determine the amount of bergenin in methanol extracts of leaves, rhizomes and roots of the plant. Separation was achieved on an Agilent Eclipse XDB-C18 column maintained at 25 °C using isocratic solvent system (water: methanol: acetic acid; 62.5:37:0.5 v/v/v) adjusted at pH 2.0 at a flow rate of 1.0 mL/min. and detected at 275 nm. Correlation coefficient (0.9952) showed linearity of concentration (5-200 µg/mL) and response. The values of LOD (0.00947 µg/mL) and LOQ (0.02869 µg/mL) indicated that method was sensitive. The recovery of bergenin was 99.99-100% indicating accuracy of method. The methanol extract of rhizomes contained higher amount of bergenin (19.4%) than roots (9.2%) and leaves (6.9%). It is concluded that methanol extract of rhizomes is a better source of bergenin than other parts of the plant. The findings are useful for standardization of bergenin containing extracts and herbal preparations.

Keywords – *Bergenia ciliata*, Saxifragaceae, bergenin, RP-HPLC

Introduction

Bergenia ciliata is a medicinal plant indigenous to the temperate Himalayan regions of Pakistan, India and Nepal. The plant consists of densely ciliated aerial leaves and barrel shaped underground rhizomes with associated small roots.¹ The plant is used traditionally for the treatment of various diseases including kidney and gall bladder stones, menorrhagia, skin, gastro-intestinal, respiratory, rheumatic and cardiac diseases.^{2,3}

The plant has been investigated for different pharmacological activities such as antioxidant, antibacterial, antitussive, antifungal, antiurolithiatic, anti-inflammatory antidiabetic, antimalarial, immunostimulant, anticancer and antiulcer activities.⁴⁻⁹ The scientific reports have linked the diverse pharmacological properties of the plant to the presence of phytochemicals such as arbutin, bergenin,

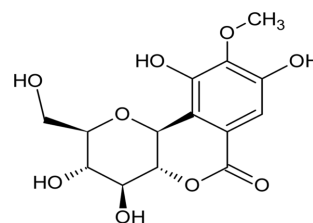


Fig. 1. Structure of bergenin (**1**).

catechin, afzelechin, proto catechuic acid and their glycosides.¹⁰

Bergenin (Fig. 1) is a polyphenolic compound possessing antiulcerogenic, antifungal, hepatoprotective, antiarrhythmic, neuroprotective, anti-inflammatory, immunomodulatory, antinociceptive, antidiabetic, antioxidant, antimicrobial, antirheumatic, antiurease and anti-*H. pylori* activities.¹¹⁻¹⁵ Bergenin has been isolated and quantified in a number of medicinal plants including *Ardisia creanta*, *Bergenia crassifolia*, *Bergenia ligulata*, *Bergenia ciliata*, *Dipterocarpus grandifloru*, and *Mallotus japonicas*.¹⁶

The huge medicinal potential of bergenin has led the

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researchers to develop different methods for quantification of bergenin in the plants. Various analytical techniques used for determination of bergenin included electro-analytical, capillary electrophoresis and chromatographic techniques.¹⁷⁻¹⁹ Generally, TLC and HPTLC methods are less sensitive, whereas HPLC studies have used complex mobile phases and gradient elution which make them complicated methods. The results of these reports revealed that *Bergenia ciliata* is a rich source of bergenin. From the literature review, it was found that the amount of bergenin in rhizomes and leaves of the plant have been determined using different HPLC methods, as a result the data regarding the quantity of bergenin in rhizomes and leaves of the plant is conflicting and unreliable. Moreover, there is no such report regarding bergenin contents of roots of the plant. As leaves, rhizomes and roots of *Bergenia ciliata* are used in traditional medicine, it is likely that bergenin may be present in all parts of the plant. Therefore, there is a dire need of development of a simple HPLC method and using a single method for the quantification of bergenin in different parts of the plant.

The aim of present study was to develop a simple validated RP-HPLC method for bergenin and to determine the amount of bergenin in methanol extracts of leaves, rhizomes and roots of the plant. The findings might be useful for standardization of bergenin containing extracts to maintain the quality of different batches and for dose calculations of bergenin containing herbal preparations.

Experimental

General experimental procedures – HPLC grade methanol (Duksan Pure Chemicals, Korea), acetonitrile, tetrahydrofuran (Tedia Company, Fairfield, USA) and acetic acid. Polytetrafluoroethylene membrane syringe filters (0.45 μm , Minisart SRP15, Sartorius Stedim Biotech, Germany), rotary evaporator (Heidolph 4002) and nylon-H filters (0.45 μm , Micropore) were procured from the local market. Bergenin was isolated from methanol extract of rhizomes of *Bergenia ciliata* by column chromatography and crystallization in the laboratory. The in-house prepared ultrapure water filtered through 0.45 μm nylon membrane was used throughout the study.

The plant was collected from Ayubia National Park (geographic coordinates; 33°51'54.83"N 73°8'19.57"E), Abbottabad, Pakistan, identified by a Taxonomist and a voucher specimen was deposited at herbarium of Government College University, Lahore, Pakistan, vide GC. Herb. Bot. 2427. The leaves, rhizomes and roots

were separated, dried under shade at room temperature and ground to a fine powder. The powders of different parts were separately subjected to extraction by maceration with methanol using powder and solvent in 1:3 (w/v). The maceration was carried out in closed flask for two days with occasional shaking. The solvent of the extract was evaporated at 40 °C under reduced pressure using rotary evaporator (Heidolph 4002). The extracts were preserved in refrigerator at 2-8 °C.

Preparation of standard solutions – A stock solution of bergenin (1.0 mg/mL) was prepared using HPLC grade methanol. Then standard solutions of different concentrations (5, 10, 20, 35, 50, 100 and 200 $\mu\text{g/mL}$) were prepared by dilution of stock solution with mobile phase.

HPLC apparatus and chromatographic conditions – HPLC system; 1200 series, Agilent Technologies, Waldron, Germany, equipped with degasser (G1379 A), isocratic pump (G1310 A), auto-sampler (G1329 A), column thermostat (G1316 A), fluorescent light detector (G 1321 A) and ChemStation LC/LCMS for Windows, Rev. B.01.03. Other instruments included UV lamp (UVGL-58, USA), ultrasonicator (DSA50-SK1-1.8L, Germany), oven (U10 Memmert, Germany) and pH Meter (Hanna Instruments, Romania).

Method development – A volume (10 μL) was eluted through column-Eclipse X DB-C18 (5 μm , 4.6 \times 250 mm) maintained at 25 °C and a flow rate of 1.0 mL/min, using different isocratic mobile phases consisting of water, methanol, acetonitrile, tetrahydrofuran and acetic acid. The detection was carried out using diode array detector (DAD) operated at 275 nm.

System suitability – The system suitability parameters such as capacity factor (k'), tailing factor, number of theoretical plates (N), height equivalent to theoretical plate (HETP), reduced plate height and peak repeatability were calculated from the chromatogram of standard solution of bergenin.

Method validation – The method validation was carried out as per standard guidelines ICH.²⁰⁻²²

Linearity, Beer's range, limit of detection and quantification – The working solutions (5-200 $\mu\text{g/mL}$) were analyzed in triplicate and independent repetitions using the chromatographic conditions mentioned earlier. The linearity was found by visual observation of chromatogram and validated using the linear regression and correlation coefficient (R^2) and range was derived from the linearity studies. The sensitivity parameters including limit of detection (LOD) at a signal to noise (S/N) ratio 3:1 and limit of quantification (LOQ) at S/N ratio 10:1, were determined statistically from standard deviation

(SD) of the intercepts and mean of slope (S) of the standard curves.

Recovery – The recovery of the method was estimated at lower, middle and high concentration levels (5, 50 and 200 $\mu\text{g/mL}$), taken as unknown and quantifying them from the calibration curve. The amounts thus calculated were compared to the respective true values to determine percent recovery.

Intra-day and inter-day accuracy and precision – Accuracy and precision (repeatability, reproducibility and ruggedness) of the method was determined at the same concentration levels as taken in recovery studies. Repeatability and reproducibility (intra- and inter-day accuracy and precision) were assessed by analyzing the solutions 5 times in a single day and once daily for five consecutive days, respectively. Ruggedness (intermediate accuracy and precision) was determined by performing the analyses on the same instrument by two different analysts. Percent recovery and relative standard deviation (RSD) were taken as the accuracy and precision measures, respectively.

Robustness – Robustness of the method was determined by deliberately changing the sample preparation, column temperature, detection wavelengths, mobile phase composition and pH, and observing their effect on the percent recovery of the compound.

Quantification of bergenin in the methanol extracts of *Bergenia ciliata* – The methanol extracts of leaves, rhizomes and roots of the plant were dissolved in HPLC grade methanol (500 $\mu\text{g/mL}$) and analyzed using the RP-HPLC method. The amount of bergenin was calculated using linear regression equation obtained for standard solutions of bergenin.

Data analysis – The samples and standards were

analyzed in triplicate and the results were presented as mean \pm SD.

Results and Discussion

The development of RP-HPLC method for bergenin involved testing of different chromatographic conditions including composition and pH of mobile phase, column temperature and wavelengths of UV detector to achieve the optimum separation. The mobile phase consisting of water: methanol: acetic acid (62.5:37:0.5 v/v/v) adjusted at pH 2 produced the best separation. The optimum column temperature was found to be 25 $^{\circ}\text{C}$ and wavelength with maximum absorbance was 275 nm.

HPLC chromatogram of the standard bergenin (Fig. 2) showed a single symmetrical peak of bergenin T_R of 4.177 min. indicating the purity of the standard. The system suitability parameters were evaluated using the chromatogram of the standard and are presented in Table 1. The results showed that the parameters were within reference values recommended by center for drug evaluation and research (CDER). The reference values for height equivalent to theoretical plate (HETP) and reduced plate height are not available (NA) but generally lower the values of these parameters higher will be the efficiency of chromatographic separation.

The method validation parameters are presented in Table 2. A high value up to (0.9952) of correlation coefficient (R^2) indicated linearity of concentration and area under the curve (AUC). The mean slope of curve was 4.24. LOD and LOQ were found to be 0.00947 $\mu\text{g/mL}$ and 0.02869 $\mu\text{g/mL}$, respectively. From these findings it was clear that the method was linear over the range of 5-200 $\mu\text{g/mL}$ and was sensitive to detect and quantify

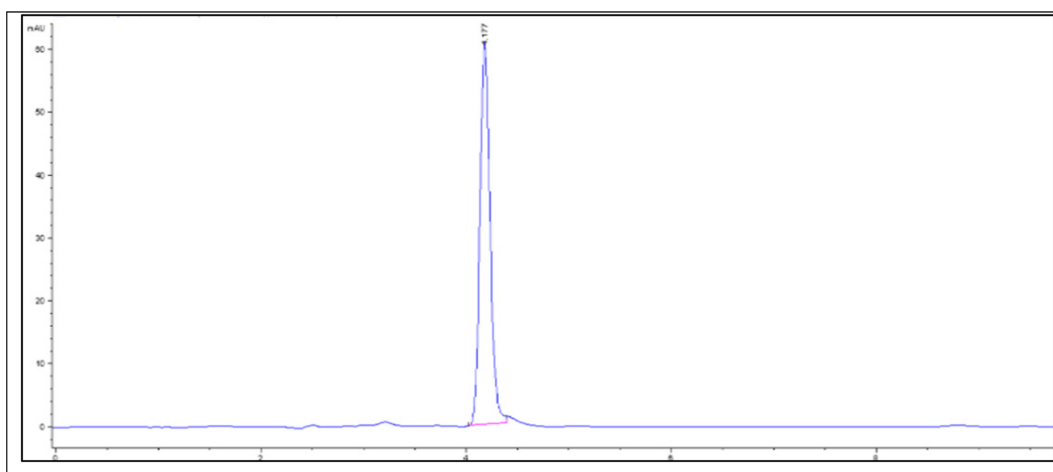


Fig. 2. HPLC chromatogram of bergenin (100 $\mu\text{g/mL}$).

Table 1. System suitability parameters calculated from a chromatogram of standard solution of bergenin (100 µg/mL)

System suitability parameters	Values	Reference values
Retention time (T_R)	4.177	>4
Capacity factor (k')	3.177	>2
Peak repeatability response	0.282	≤1%
Tailing factor (T)	1.08	≤2
Number of theoretical plates (N)	25760	>2000
Height equivalent to theoretical plate (HETP)	10 µm	NA
Reduced plate height	5 µm	NA

Table 2. Results of calibration, limit of detection (LOD), limit of quantification (LOQ) and correlation coefficient of bergenin

Sr. no.	Concentration (µg/mL)	Linear regression equation	Slope	Intercept	Correlation coefficient (R^2)
1	5-200	$y = 4.2401x + 14.017$	4.2401	14.017	0.9945
2	5-200	$y = 4.231x + 13.896$	4.231	13.896	0.9951
3	5-200	$y = 4.2569x + 11.526$	4.2569	11.526	0.9950
4	5-200	$y = 4.2312x + 12.852$	4.2312	12.852	0.9952
Mean slope (S)			4.24		
Standard deviation (SD)			0.0122		
LOD = 3.3*SD/S			0.00947		
LOQ = 10*SD/S			0.02869		

Table 3. Recovery, intra- and inter-day and intermediate accuracy and precision of HPLC method for determination of bergenin

Concentration (µg/mL)	% Recovery ± SD (n=3)	Intra-day precision %RSD (n=6)	Inter-day precision %RSD (n=6)	Intermediate precision %RSD (n=6)
5	99.99 ± 0.025	16.89 ± 0.55	16.83 ± 1.23	17.28 ± 0.60
10	100 ± 0.000	43.56 ± 0.42	45.64 ± 0.55	45.53 ± 0.63
20	100 ± 0.0400	96.90 ± 0.38	98.48 ± 0.22	98.77 ± 0.14
35	100 ± 0.000	176.64 ± 0.37	176.96 ± 0.43	179.07 ± 0.09
50	100 ± 0.000	262.26 ± 0.34	256.89 ± 1.08	262.59 ± 0.23
100	99.89 ± 0.100	412.31 ± 0.05	413.37 ± 0.10	412.48 ± 0.15
200	99.89 ± 0.088	863.46 ± 0.05	861.20 ± 0.04	862.36 ± 0.02

even small amount of bergenin in the sample. The recovery, inter- and intra-day and intermediate precisions are given in Table 3. The recovery of bergenin at lower, middle and higher concentrations were ranging from 99.99-100% which showed that method was accurate. The relative standard deviation (%RSD) values for intra, inter-day and intermediate precision were less than 1 except with concentrations 5 and 50 µg/mL which indicated that the method was repeatable and reproducible on different days and under different conditions. The method was found to be robust as small deliberate changes in sample, solvent, mobile phase composition, pH, and column temperature did not change the recovery of analyte.

The HPLC methods reported earlier for the determination of bergenin used complex mobile phase (component A (water-phosphoric acid 99.7:0.3) and component

B (acetonitrile-water-phosphoric acid 79.7:20:0.3)) and gradient elution (0-5 min, 88-85% A; 5-10 min, 85-75% A; 10-20 min, 75-70%). Moreover, the method was linear over a short range (5-50 µg/mL) and was less sensitive (LOD; 1.16 µg/mL and LOQ; 3.9 µg/mL).²³ In another study, HPLC method was developed using chromatographic conditions including reverse phase C18 column, gradient mobile phase prepared from water: phosphoric acid 99.6:0.3 (component A) and acetonitrile: water: phosphoric acid 79.6:20:0.3 (component B) and wavelength of 280 nm. The gradient was run up to 0-5 min at 88-85% A, 5-15 min at 85-75% A, 15-25 min at 75-70% A, 25-35 min at 70-88% A, 35-50 min 88% A with a flow rate 1 mL/min. The method validation parameters were found to be; retention time T_R (8.8 min), linearity range (15-20 µg/mL), LOD (2960.3 µg/mL) and LOQ (8970.8 µg/

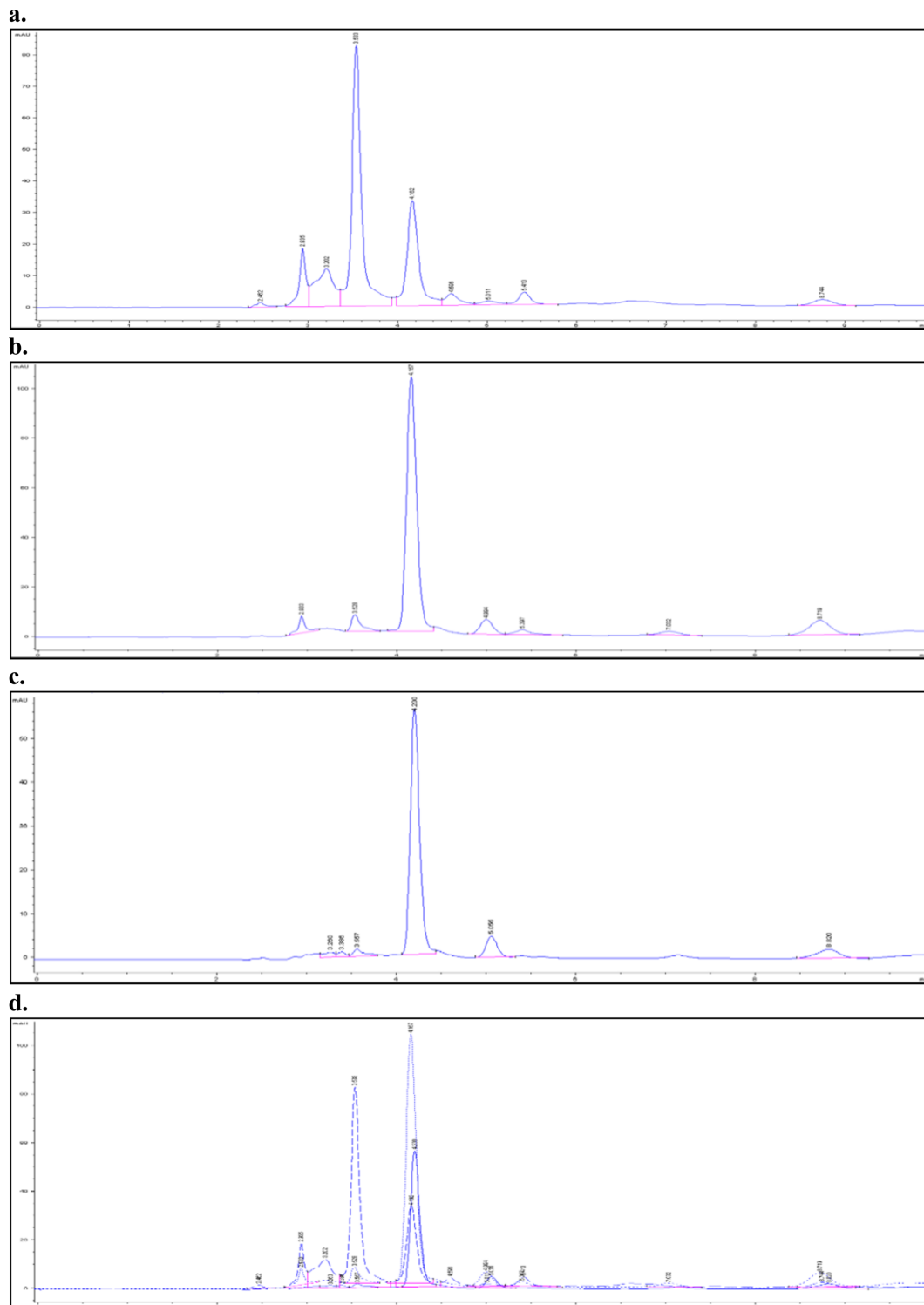


Fig. 3. HPLC chromatogram of methanol extract of leaves (a.), rhizome (b.) and roots (c.) of *Bergenia ciliata* and overlay report of the chromatograms (d.).

mL).²⁴ A comparison of findings of present study with the previous ones shows that we used a simple mobile phase and used isocratic elution and our method was linear over

wide range and was more sensitive.

The newly developed RP-HPLC method was used for the quantitative analysis of bergenin in methanol extracts

Table 4. Contents of bergenin in methanol extracts of leaves, rhizomes and roots of *Bergenia ciliata*

Extracts	Peak area (m AU*s)	Amount	
		mg/g	(%)
Leaves	310.70	68.74 ± 1.412	6.9
Rhizomes	815.59	194.01 ± 4.296	19.4
Roots	401.51	92.00 ± 0.276	9.2

of leaves, rhizomes and roots of *Bergenia ciliata* (Fig. 3) and the results are shown in Table 4. The chromatograms of methanol extracts of leaves, rhizomes and roots showed well resolved peaks and the peak corresponding to bergenin was found in all three chromatograms indicating the presence of bergenin in methanol extracts of all three parts of the plant. The peak areas of chromatograms of methanol extract of leaves (Fig. 3a.), rhizomes (Fig. 3b.) and roots (Fig. 3c.) were 310.70, 815.59 and 401.51 m AU*s, respectively. The overlay of three chromatograms (Fig. 3d) showed that the peaks corresponding to bergenin were perfectly overlapped with a clear indication of difference in the area of peaks. The results of quantitative analysis of bergenin showed that bergenin was present in the highest concentration (19.4%) in methanol extract of rhizomes followed by roots (9.2%) and leaves (6.9%). Previous studies have reported that rhizomes of *Bergenia ciliata* contained bergenin (3.3%)²³ and contents were maximum (1.326 µg/mg) in the plant grown at altitude of 7000 feet.²⁴

The results of the present study indicated that newly developed RP-HPLC method was simple, sensitive, reliable, accurate and precise. The results of the quantitative analysis indicated that bergenin is found in all parts of the plant. However, higher concentration is found in the rhizomes, followed by roots and leaves, hence, rhizomes are better source of bergenin than other parts of the plant.

Acknowledgments

We are thankful to the technical staff of Punjab University College of Pharmacy, University of the Punjab, Lahore for supporting the project.

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Received February 15, 2021

Revised March 15, 2021

Accepted March 19, 2021