Simultaneous Quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid; and quantification of β -Sitosterol using HPTLC from *Bergenia ciliata* (Haw.) Sternb. Forma *ligulata* Yeo (*Pasanbheda*)

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Abstract

In the present paper we report our work on development and validation of TLC densitometric method for simultaneous quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid, and quantification of β-Sitosterol using HPTLC.To the best of our knowledge, this is the first report of simultaneous quantification of these four bioactive compounds viz. Bergenin, (+)-Catechin, Gallicin and Gallic acid using HPTLC from this plant. Simultaneous quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid was carried out from the methanolic and hydrolysed extract (2N HCl) using the Solvent System of Toluene: Ethyl acetate: Formic acid (6: 6: 1, v/v/v). β-Sitosterol was quantified from petroleum ether extract using the Solvent System of Toluene : Methanol (9: 1, v/v). The methods were validated using ICH guidelines in terms of precision, repeatability and accuracy. The thin layer chromatography densitometric methods were found to be precise with RSD for intra-day in the range of 2.62-4.26, 0.35-2.5, 0.61-1.83, 1.14-1.57 and 0.15-0.52 and for interday in the range of 1.90-4.27, 0.85-3.05, 0.97-1.45, 0.58-1.27 and 0.26-0.61 for different concentrations of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β-Sitosterol respectively. The linearity ranges for Bergenin, (+)-Catechin, Gallicin and Gallic acid were found to be the same (160-720 ng/spot) with correlation coefficients (r-values) of 0.999, 0.997, 0.998 and 0.996 respectively. The linearity range for β-Sitosterol was 80-480 ng/spot with correlation coefficient (r-values) of 0.995. Instrumental precision was 3.39, 3.36, 3.05, 1.20 and 0.85 (% RSD) for Bergenin, (+)-Catechin, Gallicin, Gallic acid and β-Sitosterol respectively. Accuracy of the method was checked by conducting recovery studies at three different levels for both the compounds and the average percentage recoveries obtained were 100.38, 101.45, 102.38, 99.9 and 99.92% respectively for Bergenin, (+)-Catechin, Gallicin, Gallic acid and -Sitosterol. The amount of Bergenin, (+)-Catechin and Gallic acid was 0.22, 0.063 and 0.25 % w/w respectively whereas Gallicin was not in detectable amount. Developed method permitted simultaneous quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid, and showed good resolution and separation from other constituents of extract and was found to be simple, precise, specific, sensitive and accurate. It can be adopted for routine quality control of herbal material and formulations containing Bergenia ciliata.

Keywords: Bergenia ciliata; Bergenin; (+)-Catechin; Gallicin; Gallic acid; β-Sitosterol; HPTLC.

Introduction

Bergenia ciliata (Haw.) Sternb. forma ligulata Yeo. (syn. Saxifraga ligulata Wall., B. ligulata (Wall) Engl.); Fam. Saxifragaceae is a medicinal plant distributed in South East Asia [1]. The plant is found throughout the temperate Himalayas from Kashmir to Bhutan, between 7000-10,000 ft. and in the Khasia hills at 400 ft. [2]. It is a reputed drug of Ayurveda, commonly known as Pasanbheda. This plant has been recognized for its action on the urinary tract since ancient times, the name Pasanbheda being descriptive of its litholytic property [3]. The rhizomes have been used for centuries in Avurvedic formulations for various ailments [4]. It has been used as a poultice, for treating boils, for curing diarrhea and vomiting, for treatment of fever, cough, menorrhagia, excessive uterine hemorrhage and pulmonary infections [5, 6]. The rhizome is bruised and applied to boils, in ophthalmia, in eyesore and also used as anthelmintic [5, 7]. The marketed composite herbal formulations, Cystone (Himalaya Drug Company, India), Calcuri (Charak Pharmaceuticals, Bombay, India) and Chandraprabha Vati (Baidyanath, India) have been widely used clinically to dissolve urinary calculi in the kidney and urinary bladder [8]. Alcoholic extracts of plant exhibited significant anti-inflammatory, analgesic and diuretic properties [9] and antiurolithiatic activity [8]. Methanolic extract of rhizome showed significant inhibition of cough reflex [10] and exhibited a broad spectrum of antibacterial activity [11]. The major chemical constituents reported from *B. ciliata* are 11-O-galloyl Bergenin [12], Bergenin (1) and its glycosides, (+)-Catechin (2), Methyl Gallate (Gallicin) (3), Gallic acid (4) and leucocyanidin [13], (+)-Catechin-3-gallate [14], (+)-Catechin-7-O- β –D-glucopyranoside [12] and Paashaanolactone [15]. β -Sitosterol (5), β -Sitosterol-*D*-glucoside [16], (+)-Afzelechin [17].

Nowadays, HPTLC has become a routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness [18]. It has proved a very useful technique because of its low operating cost, high samplethroughput and need for minimum sample clean-up. The major advantage of HPTLC is in reducing analysis time and cost per analysis.

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Unlike HPLC, for which substantial amounts of mobile phase and long analysis times are required for quantification of multiple samples, HPTLC has the advantage that several samples can be estimated simultaneously using a small quantity of mobile phase. HPTLC also has the advantage of providing visualization of the separated constituents of the sample. It also provides on line identification of the analyte by in-situ spectrum scanning and post chromatographic derivatization, along with Rf comparison with the standard. It requires very little sample clean up since the layer is disposable. Several samples can be run simultaneously using a small quantity of mobile phase, thus reducing the time and cost per analysis. Due to low consumption of solvent the methodology is eco friendly. Another advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase unlike HPLC. This reduces the time and cost of analysis and possibilities of pollution of the environment. HPTLC also facilitates repeated detection (scanning) of the chromatogram with same or different parameters. Simultaneous assay of several components in a multicomponent formulation is also possible. Due to several advantages, such as the rapidity, the fewer amounts of sample, and an extremely limited solvent waste, HPTLC has gained widespread interest as a favorable technique for the determination of pharmacologically interesting compounds in biological matrices, such as plants, leaves, and flowers and herbal formulations [19–24]. A number of high performance liquid chromatography (HPLC) which need sample cleanup to remove the interfering constituents in the plant extracts, making the procedure more tedious and unsuitable for screening large number of samples. Recently, high performance thin laver chromatography (HPTLC) has been widely employed for the quantification of secondary metabolites [25-30].

Some of the analytical methods reported for the qualitative and quantitative analysis of Bergenin, Gallic acid and Catechin from B. ligulata are discussed herewith. Chauhan et al. [31] and Srivastava et al. [32] reported a method for determination of Bergenin and Gallic acid from *B. ligulata* and in different *Bergenia* species using HPTLC, but there are no report of simultaneous quantification of bergenin, gallic acid, catechin and gallicin. Yu et al. [33] also repoted a method for quantitation of bergenin in human plasma by LC-MS/MS. Reddy et al. [34] reported the determination of Bergenin and (+) -Afzelechin from different parts of Paashaanbhed (Bergenia ligulata Yeo) by HPLC using C18 column. Dhalwal et al. [35] repoted the Simultaneous quantification of bergenin, catechin and gallic acid from Bergenia ciliata and Bergenia ligulata by using TLC but there is no report of simultaneous quantification of the four major biomarkers present in this plant i.e. bergenin, gallic acid, catechin and gallicin, further the quantification of β -Sitosterol is also reported for the first time from this plant. Shi et al. [36] and Wang et al. [37] developed and reported the methods for determination and pharmacokinetic study of bergenin in biological fluid i.e. rat plasma by RP-HPLC method and Nunomura et al. [38] also reported the determination of bergenin by RP-HPLC method. HPLC determination of bergenin in different Bergenia species was also done by Singh et al. [39].

In the present paper, we report our work on quantification of five marker compounds viz., Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol by HPTLC (structures. Figure 1) at the same wavelength (λ max 280 nm) from the rhizomes of *B. ligulata*. Gallicin was quantified densitometrically from the methanolic extract but Bergenin, (+)-Catechin and Gallic acid as present in bound form were quantified from the hydrolysed extract (5N methanolic H₂SO₄). We developed TLC densitometric methods for simultaneous quantification of Bergenin, (+)-Catechin, Gallicin, Gallic acid and



Figure 1: TLC fingerprint profile of *Bergenia ciliata* Rhizome under UV 254 nm (Sample Solution A and B, Solvent System 1).



quantification of β -Sitosterol. To the best of our knowledge this is the first report of simultaneous quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid from *B. ligulata*. Furthermore, no TLC densitometric methods have been reported for the quantification of β -Sitosterol from *B. ligulata*. These five compounds have been shown to have significant pharmacological activities. Bergenin is reported to have hepatoprotective [40], and immunomodulatory [41] activity. (+)-Catechin has been shown to have antioxidant [42], glucosidase-II [43] and matrix metalloproteinase inhibitory activity [44] and renoprotective effect [45]. It has also been reported to induce cancer preventive activity mediated through a chaperone

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like property [46]. Gallicin is reported to have antioxidant [47], antitumor [48] antiplatelet [49], antimicrobial [50], anti-inflammatory and cyclooxygenase-2/5-lipoxygenase inhibitory [51] activity. Gallic acid has been shown to have antioxidant [52,53], anti-inflammatory, cytotoxic and bactericidal activity [53], gastric protective action [53] and antiangiogenic activity [54]. β -Sitosterol is reported to show antioxidant [55], anti-inflammatory [56], analgesic and anthelmintic [57] activity. It is also found to be effective in the treatment of benign prostatic hyperplasia [58].

We developed a simple TLC densitometric method for the simultaneous quantification of Bergenin, Gallic acid, Gallicin and (+)-Catechin and also report the quantification of β -Sitosterol from *B. ligulata.* For quantification of Bergenin and (+)-Catechin, we developed the method, whereas for the quantification of Gallic acid [59-63], Gallicin [59] and β -Sitosterol [59] we adopted the methods reported earlier from our laboratory with some modifications to suit the sample. The present work describes a simple yet sensitive, specific, and reproducible HPTLC method for the quantification of Bergenin, Gallic acid, Gallicin and (+)-Catechin and also for β -Sitosterol in *B. ligulata* rhizomes.

Materials and Methods

Plant material

Rhizome of *B. ciliata* was procured from the market in Jamnagar Dist. Gujarat, India. It was authenticated by our taxonomist and a voucher specimen (NIPER-A/NP/0408/02) was preserved in the Dept. of Natural Products. The plant material was dried in a hot air oven at < 50 C, stored in airtight glass bottle at 30°C and powdered to 40 mesh whenever required.

Chemicals

All the solvents of analytical grade were purchased from Qualigens Fine Chemicals, Mumbai, India. Bergenin (Purity: 98% w/w), (+)-Catechin (Purity: 98% w/w) and β -Sitosterol (Purity: 97% w/w) were purchased from Natural Remedies Pvt. Ltd, Bangalore, India. Gallicin (Purity: 98% w/w) was purchased from ICN Biomedicals, USA; Gallic acid (Purity: 99% w/w) was a gift sample from Tetrahedron Ltd, India.

TLC conditions

The TLC plates were 20 × 10 cm, precoated with silica gel 60 F_{254} TLC plates (E. Merck) (0.2 mm thickness); spotting device was Camag Linomat V Automatic Sample Spotter, Camag (Muttenz, Switzerland); syringe was a 100 μ L (Hamilton); developing chamber was a CAMAG glass twin trough chamber (20 × 10 cm); densitometer a Camag TLC Scanner 3 linked to winCATS software. Experimental conditions: Temperature 25 ± 2°C, relative humidity 40%.

TLC fingerprinting profile

Sample solutions: Preparation of Sample Solution was optimized to achieve good fingerprinting and also to extract the marker compounds efficiently. Of these, the preparations of selected sample solutions were:

A. Methanolic extract: Since all the five marker compounds were soluble in methanol, we prepared methanolic extract. Accurately weighed 1 g of the powdered drug was extracted with methanol ($25 \text{ mL} \times 4$) under reflux on a water bath. The methanolic extract was filtered through Whatman 1 filter paper, filtrates were combined, concentrated under vacuum and the volume was made upto 25 mL in a volumetric flask (Sample Solution A). This extract

was used for TLC fingerprinting and co-chromatography with marker compounds.

B. Hydrolyzed extract: Bergenin, (+)-Catechin, Gallic acid and β -Sitosterol are reported to be present in bound form in the drug. Hence we subjected the drug to hydrolysis by the following procedure: Accurately weighed 2 g of the powdered drug was hydrolyzed with 2N aqueous hydrochloric acid (50 mL) under reflux on a water bath for 2 hours at 100°C. The extract was filtered through Whatman I filter paper and the marc was washed with minimum amount of double distilled water (~ 5 to 6 mL) and filtered. The combined filtrates were transferred to a separating funnel and further extracted with ethyl acetate (25 mL × 4), dried over sodium sulphate, pooled, concentrated and the volume was made upto 25 mL with ethyl acetate (Sample Solution B). This extract was used for TLC fingerprinting and co-chromatography with Bergenin, (+)-Catechin and Gallic acid.

Standard solutions of bergenin, (+)-catechin, gallicin, gallic acid and β -sitosterol

2 mg each of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol were dissolved separately in methanol and the volume was made upto 25 mL with methanol in volumetric flasks individually.

Solvent systems

Two Solvent Systems were used for chromatographic separation.

Solvent System I– Toluene: Ethyl acetate: Formic acid (6: 6: 1, v/v/v) for co-chromatography with Bergenin, (+)-Catechin, Gallicin and Gallic acid.

Solvent System II– Toluene: Methanol (9: 1, v/v) for cochromatography with β -Sitosterol.

Procedure

For co-chromatography with Bergenin, (+)-Catechin, Gallicin and, Gallic acid, 10 μ L each of Sample Solution A and B along with the standards were applied on a TLC plate and the plate was developed in Solvent System I to a distance of 8 cm. The plates were observed under UV 254 nm and UV 366 nm. The R_r values of resolved bands were noted. The identity of the bands of Bergenin, (+)-Catechin, Gallicin and Gallic acid in the sample track were confirmed by overlaying their UV absorption spectra with those of the respective reference standards using a Camag TLC Scanner 3 with winCATS software. The purity of each of these bands in the sample extract track was checked by comparing the absorption spectra recorded at start, middle and end positions of each of the band.

For co-chromatography with β -Sitosterol, 10 μ L each of Sample Solution A along with the standard was applied on a TLC plate and the plate was developed in Solvent System II to a distance of 8 cm. The plates were dried at room temperature in air and derivatized with anisaldehyde-sulphuric acid reagent and heated at 105° for 5 min. The R_r values and colour of the resolved bands were noted.

TLC densitometric quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid using HPTLC

Sample solution-1 (methanolic extract): Sample Solution-A described under the previous section was used for simultaneous quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid.

Sample solution-2 (prepared by hydrolysis of drug): Sample Solution-B described under the previous section was used for quantification of Bergenin, (+)-Catechin and Gallic acid.

Preparation of standard solutions of bergenin, (+)-catechin, gallicin and gallic acid: Stock solution of 80 µg/mL, each of Bergenin, (+)-Catechin, Gallicin and Gallic acid were prepared by dissolving 4 mg each of accurately weighed Bergenin, (+)-Catechin, Gallicin and Gallic acid separately in methanol and making up the volume of the solutions to 50 mL with methanol in volumetric flasks. The aliquots (2 to 9 mL) of stock solutions were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol, to obtain standard solutions containing 16 µg/mL, 24 µg/mL, 32 µg/mL, 40 µg/mL, 48 µg/mL, 56 µg/mL, 64 µg/mL and 72 µg/mL of Bergenin, (+)-Catechin, Gallicin and Gallic acid respectively.

Preparation of calibration curve of Bergenin (+)-Catechin, Gallicin and Gallic acid: 10 μ L each of the

standard solutions of Bergenin, (+)-Catechin, Gallicin and Gallic acid (160 to 720 ng/spot) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicates on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chamber (20×10 cm) upto a distance of 8 cm using a Solvent System of Toluene : Ethyl acetate : Formic acid (6: 6 : 1, v/v/v) (13 mL) at 25 ± 2°C temperature and 40% relative humidity. The plates were dried at room temperature and scanned at 280 nm in absorbance mode using deuterium lamp. The areas of the resolved peaks were recorded. Calibration curve of Bergenin, (+)-Catechin, Gallicin and Gallic acid was obtained by plotting peak areas *vs* applied concentrations of Bergenin, (+)-Catechin, Gallicin and Gallic acid respectively.

Simultaneous quantification of bergenin, (+)-catechin, gallicin and gallic acid in the samples: The solvent system was optimized to Toluene: Ethyl acetate: Formic acid (6: 6 : 1 v/v/v). 10 μ L each of suitably diluted Sample Solution–1 and –2 along with the marker compounds were applied in triplicate on a TLC plate. The plate was developed in the given Solvent system and scanned as mentioned above. The peak areas and absorption spectra were recorded and the amount of Bergenin, (+)-Catechin, Gallicin and Gallic acid were calculated using their respective calibration curves.

Quantification of β-sitosterol using HPTLC

For the quantification of β -Sitosterol, we adopted the method reported by us earlier [59].

Sample solution-3 (petroleum ether extract): In order to reduce the interference of polar compounds, we prepared petroleum ether extract for quantification of β -Sitosterol. 1gm of powdered drug was extracted with petroleum ether (25 mL × 4) under reflux. The extract was filtered, pooled, concentrated and the volume was made upto 25 mL with petroleum ether.

Preparation of standard solutions of β -sitosterol: A stock solution of β -Sitosterol (50 µg/mL) was prepared by dissolving 5 mg of accurately weighed β -Sitosterol in methanol and making up the volume of the solution to 100 mL with methanol in a volumetric flask. The aliquots (1.6 to 9.6 mL) of the stock solution were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol to obtain standard solutions containing 8 µg/mL, 16 µg/mL, 24 µg/mL, 32 µg/mL, 40 µg/mL and 48 µg/mL of β -Sitosterol respectively.

Preparation of calibration curve of β -sitosterol: 10 µL each of the standard solutions of β -Sitosterol (80 to 480 ng/ spot) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicates on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chamber (20 × 10 cm) upto

a distance of 8 cm using a Solvent System of Toluene: Methanol (10 mL) (9: 1, v/v) at 25 \pm 2°C temperature and 40% relative humidity. After development, the plates were dried at room temperature in air, derivatized with anisaldehyde-sulphuric acid reagent, heated at 105° for 5 min. and scanned densitometrically at 525 nm in absorbance mode using tungsten lamp. The area of the resolved peaks was recorded. Calibration curve of β -Sitosterol was obtained by plotting peak areas vs concentrations of β -Sitosterol applied.

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Quantification of β -sitosterol in the sample: 15 μ l of suitably diluted Sample Solution-3 was applied in triplicates on a TLC plate. The plate was developed and scanned as mentioned above. The peak areas were recorded and the amount of β -Sitosterol was calculated using the calibration curve

Validation of the methods

ICH guidelines were followed for the validation of the analytical methods developed (CPMP/ICH/281/95 and CPMP/ICH/381/95) for precision, repeatability and accuracy.

Instrumental precision: Instrumental precision was checked by repeated scanning (n = 7) of the same spot of Bergenin (400ng/spot), (+)-Catechin (400ng/spot), Gallicin (480 ng/spot), Gallic acid (480 ng/spot) and β -Sitosterol (160 ng/spot) and expressed as relative standard deviation (% R.S.D.).

Repeatability

The repeatability of the method was affirmed by analyzing 400ng/ spot of Bergenin, 400ng/spot of (+)-Catechin, 480 ng/spot of Gallicin,





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480 ng/spot of Gallic acid and 160 ng/spot of β -Sitosterol individually on TLC plate (n = 5) and expressed as% R.S.D.

Inter-day and intra-day variation

Variability of the method was studied by analyzing aliquots of standard solution containing 400, 480, 560 ng/spot of Bergenin, 400, 480, 560 ng/spot of (+)-Catechin, 320, 480, 640 ng/spot of Gallicin, 320, 480, 640 ng/spot of Gallic acid and 80, 160, 240 ng/spot of β -Sitosterol on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as% R.S.D.

Limit of detection and limit of quantification

For the evaluation of limit of detection and limit of quantification different concentrations of the standard solutions of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol were applied along with methanol as blank and determined on the basis of signal to noise ratio.

Recovery

The accuracy of the method was assessed by performing recovery study at three different levels (50%, 100% and 125% addition of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol). The percent recoveries and the average percent recoveries were calculated for each.

Specificity

Specificity of the method was carried out as mentioned by Bhandari et al. [64]. Specificity was ascertained by analyzing standard compounds and samples. The bands for glycyrrhizin, glycyrrhetinic acid, apigenin, kaempferol and quercetin from sample solutions were confirmed by comparing the Rf and spectra of the bands to those of the standards. The peak purity of all the compounds (Figure 2, Figure 3 and Figure 4) was analysed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

Results and Discussion

B. ciliata is an important plant of Indian System of Medicine. There is no report of simultaneous quantification of Bergenin, (+)-Catechin, Gallicin and Gallic acid. Hence we developed a simple and precise method for quantification of these four marker compounds.

TLC fingerprint and co-chromatography

Quality control of herbal medicines is a tedious and difficult job. Herbal medicines differ from that of the conventional drugs and so some innovative methods are coming into being for the sake of quality assessment of herbal drugs. Quality control and quality assurance of herbal drugs remains a challenge as they contain a myriad of compounds in complex matrices in which no single active constituent is responsible for the overall efficacy [65].





R, values			
Sample Solution-A	Sample Solution-B		
0.08	0.09		
0.13 (Bergenin standard)	0.13		
0.19	0.22		
0.38	0.39		
0.49 (+)-Catechin standard)	0.49		
0.56 (Gallic acid standard)	0.56		
0.64 (Gallicin standard)	0.64		
0.71	0.74		
0.78	0.78		

 Table 1: TLC fingerprinting profile Bergenia ciliata rhizomes (Sample Solution–A and Sample Solution–B, Solvent System–1) under UV 254nm.



band in the sample extract and standard; **B**: Overlay UV absorption spectra of Gallic acid in the sample track at start, middle and end positions.

Fingerprint analysis approach using chromatography has become the most potent tools for quality control of herbal medicines because of its simplicity and reliability. It can serve as a tool for identification, authentication and quality control of herbal drugs. The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines [66]. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of the herbal medicines and therefore be used for authentication and identification of the herbal products. Based on the concept of phytoequivalence, the chromatographic fingerprints of herbal medicines could be utilized for addressing the problem of quality control of herbal medicines [67].

Hence a systematic consideration of all its phytoconstituents is as important as the quantification of the active constituents present in it. TLC fingerprint profile of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality and ensuring the consistency and stability of herbal drugs and their products. Literature survey revealed that some work using HPTLC has been reported on this plant. The method reported by Chauhan et al. [31] is less sensitive as the range was in micrograms, while the methods reported by Dhalwal et al. [35] and Srivastava et al. [32] were not able to simultaneously quantify all the four biomarkers. Other methods reported were more tedious and time consuming either done by LC-MS/MS, HPLC or by HPLC MS/MS. In the present study, we developed TLC fingerprint profile

Sr. No.	Parameter	Bergenin	(+)-Catechin	Gallicin	Gallic acid	β -Sitosterol
1	Instrumental precision (% CV, n = 7)	3.29	3.36	3.05	1.20	0.85
2	Repeatability (% CV, n = 5)	1.95	1.83	1.15	1.12	1.51
4	Accuracy (average % recovery)	100.38	101.45	102.38	99.9	99.92
5	Limit of detection (ng)	60	60	48	64	20
6	Limit of quantification (ng)	160	160	112	112	60
7	Specificity	Specific	Specific	Specific	Specific	Specific
8	Linearity (Correlation coefficient)	0.999	0.997	0.998	0.996	0.995
9	Range (ng/ spot)	160-720	160-720	160-720	160- 720	80-480

Table 2: Method validation parameters for the quantification of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol by the proposed TLC densitometric methods.

Marker	Concentration (ng/ spot)	Intra-day precision*	Inter-day precision*	
	400	3.12	3.15	
Bergenin	480	2.62	1.90	
	560	4.26	4.27	
	400	1.04	0.85	
(+)-Catechin	480	2.5	3.05	
	560	0.35	2.43	
Gallicin	320	1.83	0.97	
	480	1.20	1.25	
	640	0.61	1.45	
Gallic acid	320	1.56	0.64	
	480	1.57	1.27	
	640	1.14	0.58	
β-Sitosterol	80	0.15	0.26	
	160	0.52	0.43	
	240	0.46	0.61	

* % R.S.D.; Mean (n=3)

Table 3: Intra-day and Inter-day precision of Bergenin, (+)-Catechin, Gallicin, Gallic acid, and $\beta\text{-Sitosterol}.$

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for *B. ciliata* and carried out co-chromatography with five marker compounds *viz*. Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol. The developed methods were further validated and used for the quantification of these compounds. Different sample solutions and solvent systems were tried in order to resolve the marker compounds.

Bergenin, (+)-Catechin, Gallicin and Gallic acid resolved well at R_r 0.13, 049, 0.56 and 0.64 (Table 1, Figure 1) from sample Solution A and B when the plate was developed in Solvent System–I. The identity of the band for Bergenin, (+)-Catechin, Gallicin and Gallic acid in the sample extract was confirmed by overlaying their UV absorption spectra with those of respective reference standards using CAMAG TLC scanner 3 with WINCATS software (Figure 2A, Figure 3A, Figure 4A and Figure 5A). The purity of each of these bands in the sample extract was confirmed by comparing the absorption spectra recorded at start, middle and end positions of the band (Figure 2B, Figure 3B, Figure 4B and Figure 5B). β -Sitosterol resolved at R_f 0.50 along with one more compound from Sample Solution A when the plate was developed in Solvent System–II and derivatized as mentioned.

Preliminary TLC and co-TLC indicated the possible presence of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol in *B. ligulata.* Hence, we quantified these five compounds from *B. ligulata.*

TLC densitometric quantification of bergenin, (+)-catechin, gallicin, gallic acid and β -Sitosterol using HPTLC

The simplicity of the sample preparation, and the possibility of analyzing several sample of herbal products simultaneously in a short time, make HPTLC the method of choice. In the present work five compounds *viz*. Bergenin, (+)-Catechin, Gallicin, Gallic acid and

Marker	Amount of marker present (µg)	Amount of marker added (µg)	Amount of marker found (µg)	Recovery* (%)	Average Recovery (%)	
	110	55	160.25 ± 3.50	97.12 ± 0.73		
Bergenin	110	110	222.5 ± 7.23	101.13 ± 1.18	100.38	
	110	140	255.23 ± 4.71	102.9 ± 1.02		
	60	30	92.37 ± 4.58	102.63 ± 0.96	101.45	
(+)-Catechin	60	60	118.91 ± 7.25	99.09 ± 1.20		
	60	75	138.59 ± 2.17	102.65 ± 0.64		
	40	20	60.89 ± 1.21	101.48 ± 0.52		
Gallicin	40	40	83.05 ± 4.15	103.81 ± 0.87	102.38	
	40	50	91.68 ± 3.17	101.86 ± 0.69		
	252	125	376.54 ± 3.21	99.87 ± 0.73		
Gallic acid	252	252	504.72 ± 2.31	100.14 ± 0.58	99.9	
	252	316	566.25 ± 3.37	99.69 ± 0.76		
β-Sitosterol	45	22	67.54 ± 4.71	100.81 ± 1.05		
	45	45	89.75 ± 7.14	99.72 ± 1.19	99.92	
	45	54	98.23 ± 3.13	99.22 ± 0.87	1	

*Mean ± SD (n=3)

Table 4: Recovery studies of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol at 50 %, 100 % and 125 % addition by the proposed TLC densitometric method.

Sample Solution	Bergenin (% w/w)*	(+)-Catechin (% w/w)*	Gallicin (% w/w)*	Gallic acid (% w/w)*	β-Sitosterol (% w/w)*
A	0.11 ± 0.0026	0.048 ± 0.007	0.036 ± 0.003	0.022 ± 0.002	NQ
В	0.22 ± 0.0026	0.063 ± 0.004	NQ	0.25 ± 0.003	NQ
С	NQ	NQ	NQ	NQ	0.044 ± 0.031
*Mean ± S.D. (n = 3): NQ = Not quantified					

Table 5: Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol content estimated in *Bergenia ciliata* by proposed TLC densitometric method.





-Sitosterol were quantified from *B. ciliata* by TLC densitometric methods using HPTLC. For the quantification of Bergenin and (+)-Catechin, TLC densitometric method was established and described. For the quantification of Gallicin, Gallic acid [59-63] and β -Sitosterol [63], methods reported by us earlier were adopted with modifications to suit the sample.

The TLC densitometric methods were validated in terms of precision, repeatability, and accuracy (Table 2, Table 3 and Table 4). The linearity ranges for Bergenin, (+)-Catechin, Gallicin and Gallic acid were found to be the same (160-720 ng/spot) with correlation coefficients (r-values) of 0.999, 0.997, 0.998 and 0.996 respectively (Table 2). The linearity range for β -Sitosterol was 80-480 ng/spot with correlation coefficient (r-values) of 0.995. The TLC densitometric methods were found to be precise with R.S.Ds for intraday in the range of 2.62-4.26, 0.35-2.5, 0.61-1.83, 1.14-1.57 and 0.15-0.52 and for interday in the range of 1.90-4.27, 0.85-3.05, 0.97-1.45, 0.58-1.27 and 0.26-0.61 for different concentrations of Bergenin, (+)-Catechin, Gallicin, Gallic acid and -Sitosterol respectively (Table 3). This indicates that the proposed methods were precise and reproducible. The limit of detection (LOD) values for Bergenin, (+)-Catechin, Gallicin, Gallic acid and β-Sitosterol were found to be 60, 60, 48, 64 and 20 ng, respectively, and limit of quantification (LOQ) values were 160, 160, 160, 160 and 80 ng, respectively (Table 2). The average percent recoveries at 3 different levels of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol were found to be 100.38, 101.45, 102.38, 99.9 and 99.92%, respectively (Table 4).

Bergenin, (+)-Catechin, Gallicin and Gallic acid were simultaneously quantified from Sample Solution–1 and –2. The amount of Bergenin, (+)-Catechin, Gallicin and Gallic acid were found to be 0.11, 0.048, 0.036 and 0.022% w/w respectively in Sample Solution-1(Table 5, Figure 6). Bergenin is reported to be present in glycoside form in *B. cilita* [12, 13]. Gallic acid is known to be present in ester form in plant materials (polygalloyl ester chains of gallotannins) [68]. (+)-Catechin is also present in bound form as (+)-Catechin-3-gallate

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[16] and (+)-Catechin-7-O- β –D-glucopyranoside [12]. Hence we hydrolyzed the sample (Sample Solution–2) and quantified bergenin, (+)-Catechin and Gallic acid from the same. After hydrolysis, the amount of Bergenin, (+)-Catechin and Gallic acid was found to be 0.22, 0.063 and 0.25% w/w respectively (Table 5, Figure 6). Gallicin was present in Sample Solution-2 but not in detectable amount.

For quantification of β -Sitosterol petroleum ether extract was prepared to minimize the interference by other compounds. The content of β -Sitosterol quantified using TLC densitometric method was found to be 0.044% w/w (Table 5, Figure 7).

Conclusion

We established TLC densitometric method for the simultaneous quantification of four bioactive compounds viz. Bergenin, (+)-Catechin, Gallicin and Gallic acid and quantification of β -Sitosterol from rhizomes of *B. ciliata* using HPTLC. The method was found to be simple, precise, specific sensitive and accurate and can also be used for the quantification of Bergenin, (+)-Catechin, Gallicin, Gallic acid and β -Sitosterol in the herbal raw materials. It can also be used in routine quality control of herbal materials as well as formulations containing any or all of these compounds.

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