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Phytochemical evaluation and free radical scavenging properties of rhizome of *Bergenia ciliata* (Haw.) Sternb. forma *ligulata* Yeo

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Abstract

Objective : To carry out phytochemical analysis of *Bergenia ciliata* forma *ligulata* rhizome and to evaluate its free radical scavenging properties. Materials and methods : Preliminary phytochemical analysis, estimation of total phenolic content, development of fingerprint profiles and estimation of gallic acid by HPTLC method were carried out as a part of phytochemical evaluation of *Bergenia ciliata* forma *ligulata* rhizome. Further, the free radical scavenging activity was evaluated using three *in vitro* models. Results : Preliminary phytochemical screening showed the presence of phenols, tannins, flavonoids and sterols. Total phenolic content was found to be 5.78% (w/w). The solvent system developed for fingerprint profile and gallic acid estimation gave good separation of gallic acid from the other components of the extract. The HPTLC method for gallic acid estimation was validated in terms of instrumental precision, accuracy and repeatability. The amount of gallic acid in the rhizome of *B. ciliata* forma *ligulata* was found to be 1.54% (w/w, dwb). The methanolic extract was found to be a good scavenger of DPPH radical with an EC_{50} of 36.24 µg/ml. The extract scavenged superoxide radical in a dose dependent manner with EC_{50} of 106.48 µg/ml. Conclusion : Phytochemical evaluation was carried out for *Bergenia ciliata* forma *ligulata*, and a sensitive HPTLC method was developed for the estimation of gallic acid as a marker compound. This developed method can be adopted for the estimation of gallic acid from various herbal drugs and formulations. Further, the methanolic extract of *B. ciliata* forma *ligulata* rhizome was found to scavenge DPPH and superoxide radicals in the models tested.

Key words : *Bergenia ciliata* forma *ligulata*, Gallic acid, HPTLC, Antioxidant activity.

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1. Introduction

With the phenomenal increase in the demand for herbal medicine in the last two decades, a need has been felt for ensuring the quality, safety and efficacy of the herbal drugs. Phytochemical evaluation is one of the tools for quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques.

In the last one decade HPTLC emerged as an important tool for qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers [1, 2, 3, 4].

Bergenia ciliata forma *ligulata* Wall (Syn. *Saxifraga ligulata* Wall) Fam. Saxifragaceae, commonly known as *pashanbheda*, is widely used in Ayurveda as astringent, tonic, antiscorbutic and laxative and is recommended in ulcers, dysuria, spleen enlargement, cough and fever [5]. Alcoholic extract of the plant exhibited significant anti-inflammatory, analgesic and diuretic activity [6].

Some chemical constituents of the rhizome include bergenin (~0.9%), afzelechin, leucocyanidine, gallic acid, methyl gallate, (+)-catechin, (+)-catechin-7-*O* -β-D-glucopyranoside, 11-*O*-galloyl bergenin, paashanolactone, β-sitosterol, β-sitosterol-D-glucoside and sitoindoside I [7].

In the present study, we report our work on the phytochemical evaluation of rhizome of *Bergenia ciliata* forma *ligulata*. From the chemical constituents reported and our findings of phytochemical analysis, it was predicted to have free radical scavenging activity. Hence it was evaluated for its free radical scavenging property using different *in vitro* models.

2. Materials and methods

2.1 Plant Material

B. ciliata forma *ligulata* rhizome was procured from Haldwani, Himachal Pradesh, India, authenticated in Pharmacognosy and Phytochemistry Department and a voucher specimen was preserved. Sample was dried, powdered to 40 mesh and stored in an air-tight container at 25°C.

2.2 Chemicals

1,1-diphenyl-2-picryl hydrazyl (DPPH) was purchased from Sigma Ltd. Ethylene diamine tetra acetate (EDTA), sodium nitroprusside, naphthalene diamine dihydrochloride and Folin ciocalteu reagent were purchased from SD Fine Chemicals, India. Riboflavin, nitro blue tetrazolium (NBT) and pyrogallol were purchased from HiMedia Ltd. Sulphanilamide and Ascorbic acid were obtained as gift samples from Cadila Pharmaceuticals Ltd., India. Gallic acid was a gift sample from Tetrahedron Ltd., India. All the other chemicals used for the experiments were of analytical grade.

2.3 Preparation of methanolic extract of B. ciliata forma ligulata rhizome

B. ciliata forma *ligulata* rhizome powder (10 gm) was extracted with methanol (3 X 50 ml) and filtered, and the solvent was evaporated under reduced pressure (extract obtained = 2.65 gm).

2.4 Phytochemical evaluation of the methanolic extract of rhizome of B. ciliata forma ligulata

A stock solution was prepared by dissolving 500 mg of methanolic extract in 20 ml of methanol, and it was subjected to preliminary phytochemical testing for the detection of major chemical groups. [8, 9]

2.5 Estimation of total phenolics

The total phenolic content of the methanolic extract was estimated according to the method

described by Singleton & Rossi [10,11]. Briefly, the method is as follows : A stock solution (1mg/ml) of the extract was prepared in methanol. From the stock solution, suitable quantity of the extract was taken into a 25 ml volumetric flask and 10 ml of water and 1.5 ml of Folin Ciocalteau reagent were added to it. The mixture was kept for 5 min, and then 4 ml of 20% sodium carbonate solution was added and made up to 25 ml with double distilled water.

The mixture was kept for 30 min and absorbance was recorded at 765 nm. Percentage of total phenolics was calculated from calibration curve of gallic acid plotted by using the above procedure, and total phenolics were expressed as % gallic acid.

2.6 Development of TLC fingerprint profile of rhizome of B. ciliata forma ligulata

TLC fingerprint profile of methanolic extract was established using HPTLC. Suitably diluted stock solution of methanolic extract (section 2.4) was spotted on a pre-coated Silica gel 60 F254 TLC plate (E.Merck) using CAMAG Linomat IV Automatic Sample Spotter and the plate was developed in the solvent system of Toluene : Ethyl acetate : Formic acid : Methanol (3 : 3 : 0.8 : 0.2).

The plate was dried at room temperature and scanned using CAMAG TLC Scanner 3 at UV 254 nm and R_f values, spectra, λ_{max} and peak area of the resolved bands were recorded. Relative percentage area of each band was calculated from peak areas. The TLC plate was derivatised by spraying with 5% methanolic ferric chloride solution for the detection of phenolic compounds.

2.7 Estimation of gallic acid in the rhizome of B. ciliata forma ligulata using HPTLC method

2.7.1 Extraction of B. ciliata forma ligulata rhizome

500 mg of the rhizome powder was taken in a conical flask and extracted with methanol (4 X 25 ml), filtered, the filtrates were pooled and concentrated to 25 ml.

2.7.2 Calibration curve for gallic acid

10 mg of gallic acid standard was dissolved in 100 ml of methanol in a volumetric flask. From this stock solution standard solutions of 15 - 75 µg/ml were prepared by transferring aliquots (1.5 to 7.5 ml) of stock solution to 10 ml volumetric flasks and adjusting the volume to 10 ml with methanol. 10 µl of each of the standard solutions were applied on precoated silica gel 60 F254 TLC plate.

Plate was developed in the solvent system of toluene : ethyl acetate : formic acid : methanol (3 : 3 : 0.8 : 0.2) up to a distance of 8 cm. Plate was dried in air and scanned at 280 nm. Peak area was recorded and calibration curve was prepared by plotting peak area *vs* concentration of gallic acid applied.

2.7.3 Procedure

10 µl of the sample solution was applied on a precoated silica gel 60 F254 TLC plate (E. MERCK). The plate was developed in the solvent system and chromatogram was recorded as described above for the calibration curve. Amount of gallic acid present in the sample was calculated from the calibration curve of gallic acid.

2.8 Validation

The method was validated for instrumental precision, repeatability and accuracy. Precision of the instrument was checked by repeated scanning of the same spot of gallic acid (450 ng) seven times and the coefficient of variation (% CV) was calculated. The repeatability of the method was tested by analyzing 450 ng/spot of standard solution of gallic acid after application on a TLC plate (n = 5) and calculating % CV. Accuracy of the method was tested by

Table 1.
Method validation parameters for the estimation of gallic acid in methanolic extract of rhizome of *B. ciliata* forma *ligulata* by HPTLC method

Sl. No.	Parameters	Result
1	Instrumental precision (% CV) (n=7)	0.091
2	Repeatability (% CV) (n=5)	1.05
3	Limit of detection (ng/spot)	50 ng
4	Limit of quantification (ng/spot)	150 ng
5	Specificity	Specific
6	Linearity (correlation coefficient)	0.997
7	Range	150-750 ng/spot
8	Recovery (50% addition) (77 ng of gallic acid standard was added)	101.67% recovery (234.9 ng)

performing the recovery study at single level by addition of approximately 50% of gallic acid to one of the sample powders and the percentage recovery was calculated.

2.9 Free radical scavenging activity

2.9.1 Assay for antiradical activity

Antiradical activity was measured by a decrease in the absorbance at 516 nm of methanolic solution of coloured DPPH brought about by the sample [8,11]. A stock solution of DPPH (1.3 mg/ml methanol) was prepared such that 75 µl of it in 3 ml methanol gave an initial absorbance of 0.9.

This stock solution was used to measure the antiradical activity. Decrease in the absorbance in the presence of methanolic extract of *B. ciliata* forma *ligulata* rhizome at different concentrations was noted after 15 min. EC_{50} was calculated from % inhibition. Pyrogallol was used as positive control.

2.9.2 Assay for superoxide radical scavenging activity

The assay was based on the capacity of the extract to inhibit formazon formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system [11,12]. The reaction mixture contains 50 mM phosphate buffer pH 7.6, 20 µg riboflavin, 12 mM EDTA, NBT 0.1mg/3ml, added in that sequence. Reaction was started by illuminating the reaction mixture containing different concentrations of sample extract for 90 seconds and the absorbance was measured immediately at 590 nm. Ascorbic acid was used as positive control.

2.9.3 Assay for nitric oxide scavenging activity

The procedure is based on the principle that, sodium nitroprusside solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavenger of nitric oxide competes with oxygen, leading to reduced production of nitrite ions.

For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of the methanolic extract dissolved in methanol and incubated at room temperature for 150 min. The same reaction without the methanolic extract of sample but equivalent amount of methanol served as control.

Table 2.
Antiradical activity of methanolic extract of *B. ciliata* forma *ligulata* rhizome

Sample	Concentration (µg/ml)	% inhibition[a]	EC_{50} (µg/ml)
Methanolic extract	25.0	36.47 ± 2.59	
	37.5	51.52 ± 4.55	
	50.0	58.44 ± 0.81	36.24
	67.5	72.40 ± 4.47	
	75.0	84.09 ± 1.27	
Pyrogallol			4.50

n = 3, [a]Mean ± SD

After the incubation period, 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Curcumin was used as positive control [11,13].

3. Results and discussion

Bergenia ciliata forma *ligulata* is one of the widely used drugs in various classical and herbal formulations. In the present study, preliminary phytochemical testing showed the presence of high amount of phenolics and tannins along with flavonoids. The amount of total phenolics was found to be 5.78% (w/w). TLC of methanolic extract showed 6 bands in UV 254 nm. After spraying with 5% methanolic ferric chloride solution, 5 bluish coloured bands were observed out of which three bands at R_f 0.18, 0.42, 0.51 were found to be major.

In the preliminary TLC experiments, gallic acid was found to be one of the major compounds in the rhizome of *B. ciliata* forma *ligulata*. Considering the importance of gallic acid [14,15], in the present work, a simple, sensitive HPTLC method was developed for the estimation of gallic acid as a marker compound. Of the several solvent systems tried, the solvent system of toluene : ethyl acetate : formic acid : methanol (3 : 3 : 0.8 : 0.2 v/v) gave good separation of gallic acid (R_f = 0.49) from the other components present in the sample extract.

The band of gallic acid from the samples was confirmed by comparing the UV absorption spectrum of the standard with the corresponding band in the sample.

3.1 Validation

The HPTLC method was validated in terms of instrumental precision, accuracy and repeatability (Table 1). The method is specific as it well resolved gallic acid in the presence of other components in the samples. A linear relationship was obtained within the

Table 3.
Superoxide anion scavenging activity of methanolic extract of *B. ciliata* forma *ligulata* rhizome

Sample	Concentration (µg/ml)	% inhibition[a]	EC_{50} (µg/ml)
Methanolic	50.0	22.70 ± 2.68	
extract	75.0	37.70 ± 0.73	
	100.0	44.16 ± 5.69	106.48
	150.0	84.09 ± 11.49	
Ascorbic acid			43.19

n = 3, [a]Mean ± SD

concentration range of 150-750 ng/spot for gallic acid with a correlation coefficient of 0.997. The instrumental precision was studied by repeated scanning of the same spot seven times (% CV = 0.091). Repeatability of the method was tested by analyzing the standard solution (450 ng/spot) five times (% CV=1.05).

Accuracy of the method was determined by analysis at single level (50% addition) by adding a known amount of gallic acid to the powder. The recovery was found to be 101.67%. The validated method was applied for the estimation of gallic acid from *B. ciliata* forma *ligulata* rhizome. The content of gallic acid was found to be 1.54% (w/w).

3.2 Free radical scavenging activity

Various disease conditions are associated with free radical oxidative stress [16]. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial biomolecules. If they are not effectively scavenged by cellular constituents, they lead to disease condition [17,18]. Herbal drugs containing free radical scavengers are well known for their therapeutic activity. Certain plants exhibit antioxidant properties due to the phenolic constituents, especially compounds like tannins and flavonoids.

In the present study methanolic extract of *B. ciliata* forma *ligulata* rhizome was found to be a good scavenger of DPPH radical with an EC_{50} of 36.24 µg/ml (Table 2). The extract also scavenged superoxide radical in a dose dependent manner with EC_{50} of 106.48 µg/ml (Table 3).

The NO scavenging property of the extract could not be assessed as the coloured components of the extract interfered with the measurement of the chromophore formed in the reaction mixture.

In summary, phytochemical evaluation of *B. ciliata* forma *ligulata* rhizome revealed the presence of steroids, flavonoids and large quantities of phenol and tannins. The simple HPTLC method developed for the estimation of the biomarker gallic acid has the applicability in the quantification of gallic acid from the herbal drugs.

As the chemical constituents like phenolics including flavanoids, tannins and gallic acid were present, free radical scavenging property of *B. ciliata* forma *ligulata* rhizome was tested in a few *in vitro* models and it was found to scavenge DPPH and superoxide radicals.

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