

Pharmacological and phytochemical analysis of *Bergenia ciliata* leaf and rhizome extracts

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Abstract: Antibacterial, antifungal, antioxidant, cytotoxic, and anti-haemolytic activity of various rhizome extracts of *Bergenia ciliata* were evaluated in this study. The results showed inhibition of the growth of all selected bacterial and fungal strains in comparison with standard antibiotics. The antioxidant activities of *Bergenia ciliata* extracts were evaluated against DPPH, H₂O₂, ABTS, total antioxidant capacity and reducing power assays. The order of antioxidant activity of various extracts were methanol> ethanol>n-hexane> aqueous>chloroform. The cytotoxicity (brine shrimp assay) and anti-haemolytic activities of plant extracts were also promising and varies in dose depended manner. The phytochemical analysis of rhizome extracts of *Bergenia ciliata* revealed presence of various secondary metabolites which might be responsible for the antimicrobial, antioxidant, cytotoxic and anti-haemolytic activities.

Keywords: *Bergenia ciliata*, secondary metabolites, antimicrobial activity, antioxidant activity.

INTRODUCTION

Drug resistance towards human pathogenic organisms is an important global health issue (Nguessan *et al.*, 2007). The random use of synthetic antimicrobial compounds over prolonged periods is one of the key factor for the bacterial resistance to these specific compounds (Andremont, 2001). Therefore, interest to the use of natural products of plant origin as safe alternative source is increasing. The phytochemicals from medicinal plants are well known to possess antimicrobial effects and thus could be of major importance for therapeutic applications. According to the modern theory of free radical biology and medicines, free radicals are known for major causes of many chronic, infectious and degenerative disorders (Halliwell, 1995; Qureshi *et al.*, 2016). Oxidative stress resulting from free radicals has been associated with pathogenic diseases like atherosclerosis, neural diseases (Alzheimer and Parkinsons), diabetes mellitus, inflammatory disorders (Nazif, 2007).

Natural antioxidants can be classified into a lipophilic group, tocopherols, and a hydrophilic group, including simple phenolics, phenolic acids, anthocyanins, flavonoids and tannins. The phenolic compounds from plants are recognized in conferring stability against oxidative stress. Studies have shown that natural antioxidant components could lower the risk of cardiovascular diseases and several cancers (Valko *et al.*, 2006; Ul-Haq *et al.*, 2012). According to World Health Organization 80% of the population from African

and Asian countries are utilizing herbal medicine as chief health concerning source (WHO, 2003).

Bergenia ciliata (elephant's ears) plant extracts has been used in folk medicines against number of human ailments like liver diseases, kidney and gall bladder stones, healing of wounds, cough, inflammation, pathogenic microbes, oxidative stress and various types of ulcers (Kakub and Gulfraz, 2007; Sertié *et al.*, 2000). Therefore current study was conducted to assess phytochemicals from rhizome extracts of *B. ciliata* and to evaluate their antimicrobial and antioxidants activity as well as *in vitro* assessment of anti-cytotoxic and anti-haemolytic activities.

MATERIALS AND METHODS

Collection and preparation of plant samples

Samples (rhizomes) of *Bergenia ciliata* were collected from the hilly areas of Neelum valley, Azad Jammu and Kashmir (AJK), Pakistan. The plant materials were stored in fine plastic bags, properly labelled with date of samples collection. The samples were identified by taxonomic characterization and registered as voucher specimen for future references (No. 5562). Before analysis, all samples were thoroughly washed with distilled water and dried at room temperature for a week and then sun dried followed by oven drying for overnight at 60°C. The dried samples were ground into powder form and passed through 80 mesh sieve. The sieved samples were stored in normal room temperature in plastic bags till further use.

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Preparation of extracts

The aqueous, methanolic, ethanolic, chloroform and *n*-hexane extracts were prepared using 50g sample. Extracts were prepared by using Soxhlet and rotary evaporator followed by shaking over-night and filtration. The percentage yield of every extract was calculated. Later the extracts were dried and stored in air tight vials for further analyses.

Qualitative estimation of phytonutrients

Qualitative estimation of flavonoids, alkaloids, phenols, tannins, gallic acids, saponins glycosides, terpenoids and steroids from rhizome of *B. ciliata* was carried out using the specific methods (Harborne, 1998; Sazada *et al.*, 2009).

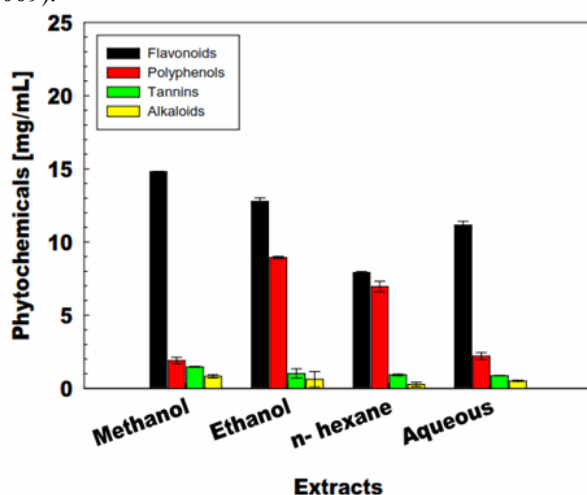


Fig. 1: Total flavonoids, polyphenols, tannins and alkaloids in different extracts of *B. ciliata* rhizome, flavonoids were extracted as quercetin equivalents and polyphenols were quantified as gallic acid equivalent.

Quantitative estimation of phytochemicals

Samples of *B. ciliata* were analysed for total phenolic, flavonoid, tannins and saponin contents. Total phenolic and tannins components were investigated by using the Folin-Ciocalteu's phenol reagent as described by Kim *et al.* (2003). Whereas, total flavonoid contents were estimated using quercetin as standard (Hussain *et al.*, 2013). For HPLC quantification 1% (w/w) *B. ciliata* extract was prepared in methanol. The samples were sonicated for 10-12min followed by filtration and the final volume was made up to 200mL with methanol. A 5mL aliquot was filtered through C-18 column and eluted with 4mL of methanol and total volume made up-to 10mL followed by filtration. A 20μL aliquot was injected into HPLC column with isocratic flow rate adjusted to 1 mL/min, retention time 6 mins and absorbance measured at 200 nm. Acetonitrile and 0.1% phosphoric acid (36: 64 (w/w)) were used as mobile phase.

Estimation of antioxidant activity

The antioxidant activities of plants extracts were determined by using various methods. The capacity of

the plant extracts to reduce Fe^{+3} ions into Fe^{+2} ions was assessed by the method reported by Yildirim *et al.* (2001). Phosphomolybdate assay method, reported by Dillard and German (2000) was applied to assess total antioxidant capacity of the plant samples. The scavenging ability of plant extract was assessed using 1,1 diphenyl 1-2 -picryl-hydrazyl (DPPH) assay by Moon and Shibamoto *et al.* (2009). Freer radical scavenging assay was carried out by method reported by Ashafa *et al.* (2010) using 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS). The capacity of plant extracts to scavenge H_2O_2 was determined by method reported by Ruch *et al.* (1989).

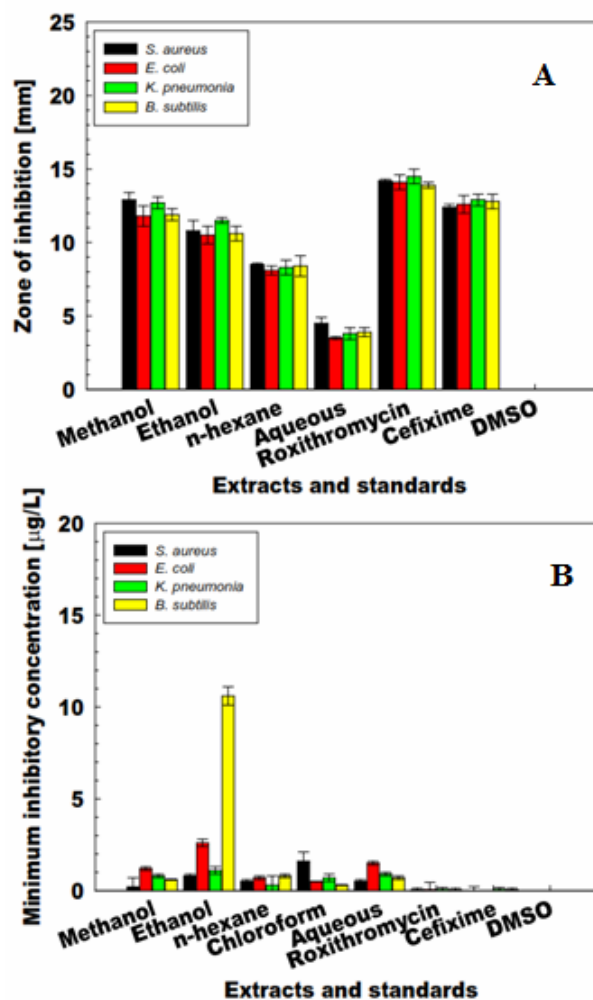


Fig. 2: (a) Antibacterial potential of various rhizome extracts of *B. ciliata* (b) Minimum inhibitory concentrations ($\mu\text{g/mL}$) of *B. ciliata* rhizome extracts tested against bacteria. Roxithromycin, cefixime and DMSO were used as control.

Estimation of antimicrobial and antifungal activities

Rhizome extracts of *B. ciliata* were screened for antibacterial potential using agar well diffusion assay (Boyed, 1980) against four bacterial strains, *Staphylococcus aureus* (ATCC 6538), *Escherichia Coli*

(ATCC15224), *Klebsiella pneumonia* (MTCC618) and *Bacillus subtilis* (ATCC6633). Standard antibiotics include, cefixime and roxithromycin. The minimum inhibitory concentration (MIC) was estimated as minimum concentration of extracts that restrict the bacterial growth after 24 h of incubation period (Ettebong & Nwafor, 2009).

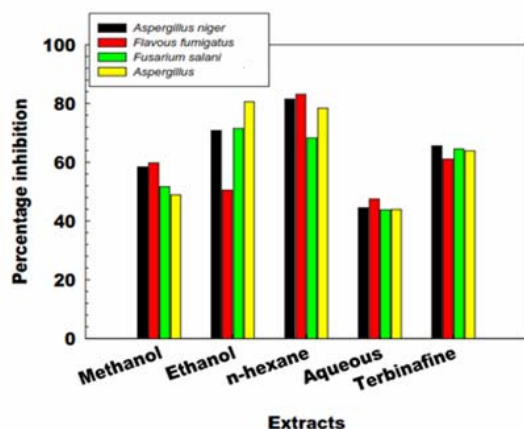


Fig. 3: Antifungal potential of various rhizome extracts of *B. ciliata*. The percentage zone of inhibition against tested fungal strains.

The antifungal ability of plant extracts was estimated by the agar tube dilution method against four fungal strains; *Aspergillus niger* 0198; *Aspergillus flavus* 0064; *Aspergillus fumigatus* 66 and *Fusarium solani* 0291. Fungal growth was evaluated by measuring linear expansion in millimetres while growth inhibition was determined with respect to negative control as;

$$\% \text{ age inhibition} = \frac{100 - \text{Linear growth test (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

Determination of cytotoxic and haemolytic activity

Brine shrimps cytotoxicity assay was carried out to evaluate the cytotoxic effect extracts of *B. ciliata* extract using method reported by Meyer *et al* (2000) with minor modifications. The percentage mortality and log concentrations were determined to calculate lethal dose (LD_{50}) of the samples. Anti-haemolytic activity of plant extract was determined based on standard method reported by Meyer *et al.* (2000).

STATISTICAL ANALYSIS

The data obtained from various analyses were statistically analysed using the analysis of variance (ANOVA). The means were compared through least significance difference (LSD) at 95% probability level according to the method described by Steel and Torrie (1997).

RESULTS

Results of phytochemicals obtained after analysis of plant samples were presented in table 1. Phytochemicals

are non-nutritive plant based chemical compounds and have the protective or preventive features against the different diseases.

The results of total flavonoids contents from various extracts of *B. ciliata* rhizome were presented in fig. 1. The methanol extract of rhizome contained higher amount of flavonoid contents ($14.81 \pm 0.021 \text{ mg/mL}$) followed by ethanol and aqueous extracts while *n*-hexane fraction had lower amounts of flavonoids ($7.91 \pm 0.07 \text{ mg/mL}$). Results indicate that total tannin contents (fig.1) were variable in different plant extracts. Higher tannin contents were found in methanolic extract of rhizome followed by ethanolic extracts while *n*-hexane and aqueous extracts had lower contents. The results from present study indicated that both methanolic and ethanolic extracts of rhizome possess higher amount of alkaloids in comparison to *n*-hexane and aqueous fractions (fig.1). Over 3000 different types of alkaloids are reported in literature like nicotine, caffeine and morphine etc. The compounds are known to possess a variety of bioactivities and interact with enzymes, proteins and nucleic acids (Devasagayam *et al.*, 2004).

Antimicrobial activity of *Bergenia ciliata*

All plant extracts showed antibacterial activity against selected Grams positive and Gram negative strains (fig. 2a). Among all extracts, methanolic fraction of rhizome showed highest inhibitory potential (12.9 ± 0.5 to $11.8 \pm 0.7 \text{ mm}$) against all tested bacterial strains; *S. aureus*, *E.coli*, *K. pneumonia* and *B. subtilis* in comparison to ethanolic and *n*-hexane extracts. Whereas, lowest antibacterial inhibitory potential (4.5 ± 0.3 to $3.5 \pm 0.1 \text{ mm}$) was found with aqueous plants extract. Our results are in parallel with previous findings, indicating presence of active antimicrobial phytochemicals like flavonoids in the extract of *B. ciliata* rhizome (Ettebong and Nwafor, 2009). The minimum inhibitory concentrations (MIC) of *B. ciliata* rhizome extracts, along with standard antibiotics, showing significant antimicrobial potential against tested bacterial strains were presented in fig. 2a. The results show variability in the inhibitory concentrations produced by the lowest amount of each tested extract and bacteria strain. The lowest MIC (fig. 2b) was observed in case of methanol extracts against *S. aureus* ($0.2 \pm 0.5 \text{ mm}$) perhaps due to its purity or solubility of plant materials in this solvent.

The antifungal activity of plant extracts (fig. 3), follows the order; *n*-hexane extracts followed by ethanol, methanol extracts and as well as terbinafine, whereas lowest activity was shown by aqueous extracts. Extracts prepared in organic solvents showed activity against fungal strains and these results are supported by Fawole *et al.* (2009). Parekh and Chanda (2007) found that water extracts showed no/poor fungi toxicity than organic solvents.

Table 1: Qualitative estimation of phytochemicals from plant extracts

Phytochemicals	Methanolic extracts	Ethanol extracts
	Rhizome	Rhizome
Alkaloids	+	+
Steroids	+	+
Phenols	+	+
Saponins	+	+
Tannins	+	+
Gallic acid	+	+
Terpenoides	—	+
Glycosides	+	+

+sign indicates presence of a phytochemicals while - sign is for absence.

Table 2: Antioxidant potential of various rhizome extracts of *B. ciliata* at different concentration. The radical scavenging values were presented in $\mu\text{g/mL}$.

Plant extracts	DPPH	Phosphate Molybdate	H ₂ O ₂	ABTS	Reducing power assay
Methanol	41.6 $\pm$ 0.3	31.5 $\pm$ 1.7	61.3 $\pm$ 0.5	132.3 $\pm$ 0.8	1.2 $\pm$ 0.7
Ethanol	47.2 $\pm$ 0.9	37.6 $\pm$ 0.6	66.5 $\pm$ 0.2	156.7 $\pm$ 0.7	1.9 $\pm$ 0.4
<i>n</i> -hexane	58.2 $\pm$ 0.8	41.5 $\pm$ 1.4	74.5 $\pm$ 2.6	225.2 $\pm$ 0.7	1.5 $\pm$ 0.5
Chloroform	86.5 $\pm$ 1.5	81.6 $\pm$ 1.9	71.9 $\pm$ 1.7	76.5 $\pm$ 0.5	0.9 $\pm$ 0.1
Aqueous	73.8 $\pm$ 0.7	87.5 $\pm$ 0.8	81.3 $\pm$ 0.6	91.3 $\pm$ 0.6	29.7 $\pm$ 0.8
Ascorbic acid	15.1 $\pm$ 0.3	22.3 $\pm$ 0.9	19.1 $\pm$ 0.8	17.2 $\pm$ 0.5	1.5 $\pm$ 0.9
Rutin	33.4 $\pm$ 1.7	-	37.6 $\pm$ 1.8	-	-

250 $\mu\text{g/mL}$ extract was used to measuring the scavenging activities. The power reducing assay was quantified at 700 nm. Ascorbic acid and rutin were used as standards.

Table 3: Anti-haemolytic activity of plant extracts at different concentrations. The values and concentrations were presented in $\mu\text{g/mL}$.

Extracts	Positive control	Negative control	10	50	100	200	300
Methanol	0.6 $\pm$ 0.01	1.1 $\pm$ 0.02	1.03 $\pm$ 0.2	1.0 $\pm$ 0.5	0.9 $\pm$ 0.2	0.8 $\pm$ 0.6	0.7 $\pm$ 0.3
Ethanol	0.6 $\pm$ 0.01	1.1 $\pm$ 0.02	1.02 $\pm$ 0.4	1.0 $\pm$ 0.5	0.9 $\pm$ 0.4	0.9 $\pm$ 0.3	0.7 $\pm$ 0.4
<i>n</i> -hexane	0.6 $\pm$ 0.01	1.1 $\pm$ 0.02	1.01 $\pm$ 0.7	1.0 $\pm$ 0.3	0.7 $\pm$ 0.3	0.8 $\pm$ 0.4	0.7 $\pm$ 0.5
Chloroform	0.6 $\pm$ 0.01	1.1 $\pm$ 0.02	0.95 $\pm$ 0.3	0.8 $\pm$ 0.4	0.7 $\pm$ 0.5	0.7 $\pm$ 0.5	0.6 $\pm$ 0.3
Aqueous	0.6 $\pm$ 0.01	1.1 $\pm$ 0.02	0.86 $\pm$ 0.4	0.8 $\pm$ 0.8	0.7 $\pm$ 0.6	0.6 $\pm$ 0.2	0.6 $\pm$ 0.6

Water was used as positive control and provided 100% haemolysis rate. Phosphate buffer were used as negative control and provided 0% haemolysis rate. The lower values were related with greater cell lysis.

Table 4: Cytotoxicity screening of various concentration ($\mu\text{g/mL}$) of rhizome extracts of *B. ciliate*

Extracts	10	100	1000	LD ₅₀
Methanol	27.2 $\pm$ 0.7	38.5 $\pm$ 0.5	46.5 $\pm$ 0.8	>1000
Ethanol	31.5 $\pm$ 0.5	34.6 $\pm$ 0.5	44.8 $\pm$ 0.5	650
<i>n</i> -hexane	29.1 $\pm$ 0.7	36.1 $\pm$ 0.6	55.3 $\pm$ 0.6	64
Chloroform	34.4 $\pm$ 0.5	53.8 $\pm$ 0.8	65.5 $\pm$ 0.1	100
Aqueous	36.6 $\pm$ 0.9	56.9 $\pm$ 0.6	77.8 $\pm$ 0.4	278
Positive control	53.8 $\pm$ 0.1	76.6 $\pm$ 0.5	91.4 $\pm$ 0.5	<10

Saline sea water was used as positive control.

***In vitro* antioxidant assay**

To check the antioxidant potential of rhizome extracts five different assays i.e. DPPH, H₂O₂, ABTS phosphomolybdenum and reducing power assays were performed with ascorbic acid as positive control. All solvents extracts (methanol, ethanol, *n*-hexane,

chloroform and water) showed a remarkable quantity of antioxidant activity (table 2), although the values were quite less than the positive control (ascorbic acid) and standard rutin. However, order of antioxidant activity of various extracts was methanol > ethanol > *n*-hexane > aqueous > chloroform) as shown in table 2.

***In vitro* anti haemolytic activity of plant extracts**

Results regarding anti-haemolytic activity of plant extracts are presented in table 3, which revealed an importance of this plant extracts in local medicines required for human health.

Brine shrimps lethality assay

Five different dilutions of rhizome extracts (10,100 and 1000µg/mL) were made to check brine shrimps cytotoxicity assay. The results (table 4) exposed the better brine shrimps larvicidal potential and lethality was maximum at higher concentration of plant extracts and was concentration dependent. It was assumed that plant extracts might be composed of antitumor components in the form essential phytonutrients. The plant extracts whose value i.e. $LC_{50} < 1000\mu\text{g/mL}$ was biologically active while $LC_{50} > 1000\mu\text{g/mL}$ was biologically inactive (non-toxic). The highest mortality was found in *n*-hexane and chloroform extracts of *B. ciliata*.

DISCUSSION

Plants are limitless source of secondary metabolites those due to their therapeutic properties are usually rectified after their phytochemicals screening and pharmacological testing. Bagul *et al.* (2003) reported phytochemicals from rhizome of *B. ciliata*, however, amounts of these parameters are lower as compared to those found in current study. Large quantities of phyto-constituents in rhizome of *B. ciliata* clearly indicates high therapeutic properties of these plant extracts like antioxidant, cytotoxic, antimicrobial and antifungal (Cetin *et al.*, 2011). A group of naturally occurring benzo-g-pyrone derivatives are known as flavonoids. They possess many biological and chemical properties including hepato protective, anti-thrombotic, antiviral, and anti-inflammatory (Dillard and German, 2000; Okwu *et al.*, 2006). Majority of the above mentioned activities might be related to the antioxidant and free-radical-scavenging activity of the phyto chemicals especially flavonoids present in the plant extracts (fig. 1). The ethanolic rhizome extracts showed higher amount of phenolic compounds ($8.94 \pm 0.09\text{mg/mL}$) followed by *n*-hexane. In comparison, lowest quantities of phenolic contents were found in aqueous ($2.21 \pm 0.237\text{mg/mL}$) and methanolic ($1.91 \pm 0.22\text{mg/mL}$) extracts (fig. 1). Phenolic compounds are known for their anticancer activity particularly due to their radical scavenging properties (Ashafa *et al.*, 2010; Bagul *et al.*, 2003).

Antimicrobial resistance (AMR) is considered as a major health threat globally (Tambekar *et al.*, 2010). The herbal practitioners commonly used different medicinal plants as remedies for the treatment of infectious diseases in all over the world and are potential candidates as a effective treatments against some antibiotic-resistant infections (Baydar *et al.*, 2004; Fawole *et al.*, 2009).

It was reported elsewhere that majority of the antioxidant activities of plant extracts are due to the flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins. A group of dietary flavanoids belongs to natural antioxidants, are considered to be having a strong antioxidant activity and received increasing attention as potential protectors against a variety of human infectious diseases, in particular cardiovascular disease (CVD) and also cancer. Due to the presence of higher total phenolic contents, the plant extracts had shown potential anti-haemolytic activity (table 3) and the level of the activity was in dose depend manner. Where as cytotoxicity assay also revealed importance of this plant extracts for its utilization for the development of drugs in future that might be useful against human disorders.

CONCLUSION

The study revealed that phytochemicals present in rhizome extracts of *B. ciliata* exhibit biological properties. The various extracts of rhizome showed antibacterial, antifungal, antioxidants, cytotoxic and anti-haemolytic activities. The obtained results could provide a good basis for selection of this medicinal plant for further phytochemicals screening in the potential discovery of new bioactive compounds might be useful for preparation of drugs in future.

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