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Studies on fungal endophytes and antibacterial activity of medicinal plant *Bergenia ciliata* (haw.) Sternb

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Abstract
In the present study, the endophytes and antibacterial activity of *Bergenia ciliata* (Haw.) Sternb. were investigated. Studies on endophytes revealed the presence of eighteen fungal species belonging to ten genera (*Alternaria, Aspergillus, Cunninghamella, Cephalosporium, Epicoccum, Fusarium, Penicillium, Rhizopus, Sporotrichium* and *Trichoderma)* from the leaf, stem and rhizome of *B. ciliata*. *Aspergillus* was the most abundant genus among the endophytes and maximum number of fungi was observed during rainy (14 spp.) season followed by summer (11 spp.) and winter (8spp.). The antibacterial activity of the methanol, ethanol and acetone leaf and rhizome extracts of *B. ciliata* was determined *in-vitro* against medically important pathogenic bacteria such as *Escherichia coli*, *Yersinia pestis* and *Staphylococcus aureus* following agar-well diffusion method using different concentrations (25%, 50%, 75% and 100%). Methanol leaf and rhizome extracts were found to be more effective against selected pathogenic bacterial spp. as compared to ethanol and acetone leaf and rhizome extract. Further the leaf and rhizome extracts of plant inhibited gram- positive bacteria, *S. aureus* more efficiently than gram- negative bacteria *E. coli* and *Y. pestis.* Therefore, the leaf and rhizome extracts of this plant can be selected for further investigation to check their therapeutic potential.

Keywords: Endophytes, *Bergenia ciliata*, antibacterial activity, leaf extract, rhizome extract, agar-well diffusion

Introduction
Earth is a home for various microorganisms. Of all the microorganisms, fungi constitute a very large group which are found on earth. About 1.5 million species of fungi are estimated to be present all over the world [1]. They are ubiquitous and occur in almost every habitat where organic matter is available. Fungi play an important role in the decomposition of organic matter. They have been used as a direct source of food, in the form of mushroom and truffles, and as a leavening agent for bread [2]. Not all the fungi which show association with higher plants are detrimental to them. There are some beneficial fungi which are widely distributed in soil. Some fungi live within the plant tissues and are called as endophytes, enabling the plant to grow under harsh conditions which they could not do otherwise [3].
The endophytes are microfungi that colonize living tissues of plants without producing any symptoms or negative effects [4]. Endophytes include specific group of microorganisms such as bacteria, fungi and actinomycetes that inhabit some period of their life in the interior of a host plant. It is now confirmed that in nature, each single plant is the host to one or more type of endophyte. Endophytic fungi live internally, moreover intercellularly or intracellularly. They usually occur in above-ground plant tissues, as well as in below ground plant tissues. Endophytes play vital role in nature for promoting plant growth, stress tolerance, adaptation and development.
The use of plants for treating various diseases is as old as the human civilization. Plants are traditionally being used for medicinal treatment of numerous human disorders including infectious diseases caused by microorganisms [5]. A major part of population in developing countries still uses traditional folk medicines which are obtained from plant resources [6-7]. Medicinal plants are used in the Ayurvedic, Unani and other traditional systems of medicine and in plant-based pharmaceutical industries. About 80% of individuals from developed countries are using traditional medicines which have bioactive compounds derived from

medicinal plants hence such plants should be investigated further for better understanding of their properties, safety, efficacy and efficiency. The present investigation is aimed to focus on the antibacterial activity of a valuable medicinal plant, *Bergenia ciliata.*

Materials and Methods

Selection of plant for isolation of endophytes and antibacterial activity.

Bergenia ciliata (Haw.) Sternb. was selected for the isolation of endophytes and antibacterial activity.

Sampling area and collection of plant material

Banjar valley of Distt. Kullu (H.P.) was selected for the collection of study material (leaf, stem, and rhizome) of *B. ciliata*. The collections were made during summer, rainy and winter seasons. The samples were brought to the laboratory for further use.

Methodology for isolation of Endophytes

(a) Hot water treatment

The endophytes were isolated from small pieces of leaf, stem and rhizome of *Bergenia ciliata* These were washed with hot water (60 °C) for 15 min in a test tube. Then three pieces of each sample were inoculated on Petri plates containing PDA medium supplemented with penicillin or streptomycin (150 mgL^{-1}). These Petri plates were incubated at 25±2 °C in an incubator for one week. After the fungal growth, sub-culturing was done on PDA slants and slants were preserved in refrigerator.

(b) Three step method

The samples were washed with sterilized distilled water. Then these were surface sterilized with 25% methanol for 5 minutes, followed by 50% methanol for 3 min, again followed by 75% methanol for 2 min. Finally these samples were washed in sterilized water for 5 minutes. Then three pieces of each sample were inoculated on Petri plates containing PDA medium supplemented with penicillin or streptomycin (150 mg^{-1}). The Petri plates were incubated at 25±2 °C for few days. The growing fungal colonies were then transferred on PDA slants.

Slide preparation

For identification, temporary mount of fungi were made in 0.1% cotton blue and lactophenol. An adequate high power microscope was used for observing the slides. Fungi were identified following Nagmani *et al.* (2005) [8].

Maintenance and Preservation of culture

Pure culture of different fungal genera was maintained on PDA which was preserved in refrigerator. Sub culturing was done at regular intervals in order to maintain cultures. Each fungal species was transferred from parent source to a fresh slant in order to maintain and preserve the culture.

Materials and methods for Antibacterial Activity

Procurement of Bacteria

Different strains of bacteria (*Staphylococcus aureus, Escherichia coli, Yersinia pestis*) have been procured from IGMC Shimla, IMTECH Chandigarh and Department of Biotechnology, HPU Shimla for screening antibacterial properties of plant extracts.

Revival of Pathogen

The collected pathogens were revived in nutrient broth and stored in nutrient agar slants at 4 °C.

Extract Preparation

Extracts (acetone, ethanol, methanol) of medicinal plant *Bergenia ciliata* have been prepared to check antimicrobial activity. 5 gm dried plant material was taken in a separate Erlenmeyer flasks to which 50mL of required solvents i.e., methanol, acetone, ethanol were added. The flasks were covered with aluminium foil and allowed to stand for 3-5 days for extraction. These extracts were filtered through Whatman filter paper no. 1 and evaporated at 40 °C using rotary evaporator. The extracts were collected and weighed. Finally, stock solution of conc. 50 mg/mL was prepared.

In vitro testing of extracts for antimicrobial activity

Agar-well Diffusion Method

Testing of plant extracts (methanol, acetone, ethanol) of *B. ciliata* was done using agar-well diffusion method. Nutrient agar medium (Beef extract 1 g, Yeast extract 2 g, Sodium Chloride 1 g, Peptone 5 g, Agar 20 g, Distilled Water 1000 mL) was used throughout the investigations. The medium was autoclaved at 121.6 °C for 30 minutes and poured into Petri plates. Bacteria were grown in nutrient broth for 24 hours. A 100µL of bacterial suspension was spread on each nutrient agar plate. Agar wells of 8 mm diameter were prepared with the help of sterilized stainless steel cork borer in each Petri plate. The wells in each plate were loaded with 25%, 50%, 75% and 100% concentration of prepared plant extracts. The Petri plate kept as a control contained pure solvent only. The plates were incubated at 37±2°C for 24 hours in the incubation chamber. The zone of growth inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in perpendicular direction in all the three replicates and the average values were tabulated. Percent inhibition of bacterial species was calculated after subtracting control from the values of inhibition diameter using control as standard [9].

Percentage of growth inhibition = (Control-Test/Control) x100

Control = average diameter of bacterial colony in control.

Test = average diameter of bacterial colony in treatment sets [10].

Results and Discussion

Eighteen species of fungi were isolated from different parts of *B. ciliata* plant (leaf, rhizome and stem) in different seasons which fall into 10 genera as shown in Table-1. These genera were *Alternaria, Aspergillus, Cunninghamella, Cephalosporium, Epicoccum, Fusarium, Penicillium, Rhizopus, Sporotrichum* and *Trichoderma.* The genus *Aspergillus* was represented by five species i.e (*A. nigar, A. wentii, A. versicolor, A. glaucus* and *A. ustus)*. The genus *Rhizopus* was represented by two species (*R. nigricans* and *R. oryzae)*. The genus *Penicillium* was represented by two species (*P. chrysogenum* and *P. funiculosum)*. The genus *Trichoderma* was represented by two species (*T. viride* and *T. harzianum)*. The genus *Cunninghamella* was represented by two species (*C. elegans* and *C. echinulata).* The genus *Alternaria, Fusarium, Cephalosporium, Epicoccum, Sporotrichium* were all represented by one species each i.e (*Alternaria alternata,*

Fusarium oxysporum, Cephalosporium acremonium, Epicoccum nigrum, Sporotrichum sp.*).*

A comparison of seasonal distribution of these isolates revealed that maximum number of fungi were recorded in rainy season (14 spp.) followed by summer season (11 spp.) and winter season (8 spp.) respectively as shown in Table-2 of fig-1. Most predominant genus reported was *Aspergillus* (5 spp.). These fungal isolates from plant parts (viz. leaf, rhizome and stem) of *B. ciliata* were further grouped into Zygomycota, Ascomycota and Deuteromycota. The maximum representatives isolated were of Ascomycota as shown in Table-3 of Fig-2).

Table 1: List of endophytic fungi isolated from various plant parts (viz. stem, leaf and rhizome) of Bergenia ciliata (Haw.) Sternb.

Sr. No.	Isolated Endophytic Fungi
1.	*Alternaria alternata*
2.	*Aspergillus glaucus*
3.	*Aspergillus niger*
4.	*Aspergillus ustus*
5.	*Aspergillus versicolor*
6.	*Aspergillus wentii*
7.	*Cephalosporium acremonium*
8.	*Cunninghamella elegans*
9.	*Cunninghamella echinulata*
10.	*Epicoccum nigrum*
11.	*Fusarium oxysporum*
12.	*Penicillium chrysogenum*
13.	*Penicillium funiculosum*
14.	*Rhizopus nigricans*
15.	*Rhizopus oryzae.*
16.	*Sporotrichium sp.*
17.	*Trichoderma harzianum*
18.	*Trichoderma viride*

Table 2: Comparison of occurrence of endophytic fungi isolated from various plant parts (viz. stem, leaves and rhizome) of *B. ciliata* during different Seasons

Sr. No.	Isolated Endophytic Fungi	Summer	Rainy	Winter
1.	*Alternaria alternata*	-	+	-
2.	*Aspergillus glaucus*	+	+	+
3.	*Aspergillus niger*	+	+	+
4.	*Aspergillus ustus*	+	+	+
5.	*Aspergillus versicolor*	+	-	+
6.	*Aspergillus wentii*	+	-	+
7.	*Cephalosporium acremonium*	-	+	-
8.	*Cunninghamella elegans*	+	+	+
9.	*Cunninghamella echinulata*	-	+	-
10.	*Epicoccum nigrum*	+	+	-
11.	*Fusarium oxysporum*	+	+	-
12.	*Penicillium chrysogenum*	-	-	+
13.	*Penicillium funiculosum*	+	+	-
14.	*Rhizopus nigricans*	+	+	+
15.	*Rhizopus oryzae*	+	+	+
16.	*Sporotrichium sp.*	-	+	-
17.	*Trichoderma harzianum*	-	+	-
18.	*Trichoderma viride*	+	-	-

Table 3: Categorization of endophytic fungi isolated from various plant parts (viz. leaf, stem and rhizome) of *B. ciliata* into different fungal division

Sr. No.	Division	Genus
1.	Zygomycota	*Rhizopus, Cunninghamella*
2.	Ascomycota	*Alternaria, Aspergillus, Epicoccum, Penicillium, Cephalosporium, Sporotrichium*
3.	Deuteromycota	*Fusarium, Trichoderma*

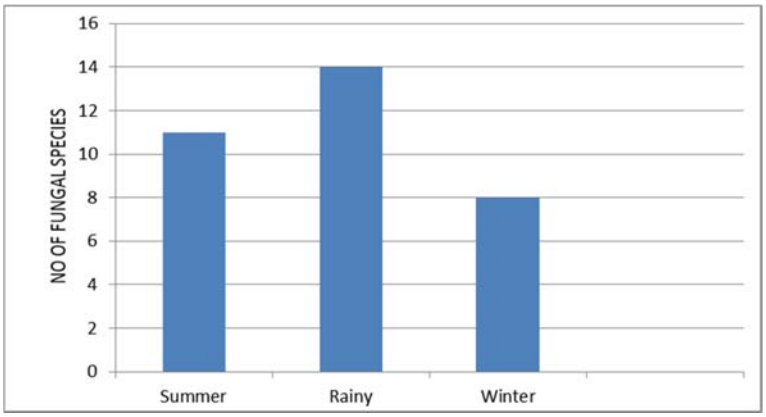

Fig 1: Histogram showing the distribution of endophytic fungal species (in different seasons) isolated from various plant parts (viz. leaf, stem and rhizome) of *B. ciliata*

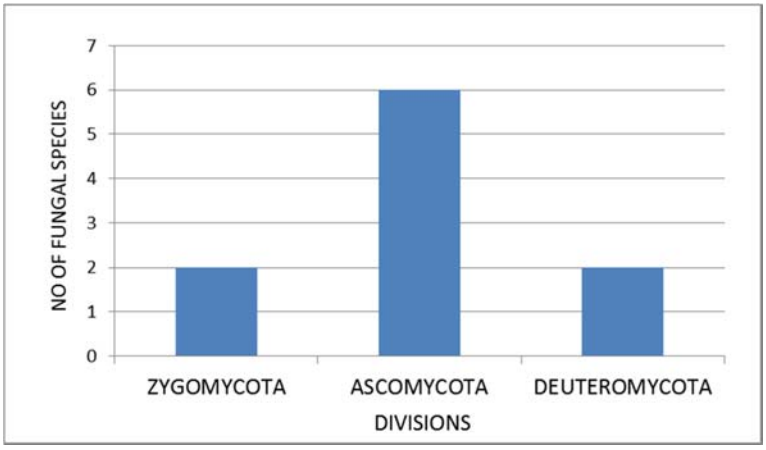

Fig 2: Histogram showing the distribution of endophytic fungal species (in different fungal divisions) isolated from various plant parts (viz. leaf, stem and rhizome) of *B. ciliata*

Antibacterial screening of leaf extracts (methanol, ethanol and acetone) of *B. ciliata* against *Staphylococcus aureus, Escherichia coli* and *Yersinia pestis* bacteria.

The results are presented in Table-4

It is clear from Table-4 that methanol leaf extract of *B. ciliata* showed a maximum zone of inhibition for *S.aureus* (24.04mm at 100%) and minimum zone of inhibition for *E.coli* (20.87mm at 100%). For ethanol leaf extract *Y. pestis* showed a maximum zone of inhibition (22.19mm at 100%) and minimum zone of inhibition (20.29mm at 100%) by *E.coli* For acetone leaf extract a maximum zone of inhibition was given by *S.aureus* (24.35mm at 100%) whereas *E.coli* showed a minimum zone of inhibition (19.22mm at 100%).

Table 4: Percent inhibition of growth of pathogenic bacterial spp. at different concentrations of methanol, acetone and ethanol leaf extracts of *B. ciliate*

Extract	Concentrations (In %)	Percent inhibition zone of test bacteria (mm±S.E)		
		S. aureus	***E. coli.***	***Y. pestis***
Methanol Extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	14.21±0.36	12.77±0.27	15.07±0.20
	50	16.79±0.37	15.78±0.38	16.57±0.28
	75	20.08±0.35	18.84±0.0.23	19.69±0.21
	100	24.04±0.06	20.87±0.44	22.60±0.28
Ethanol extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	13.00±0.24	12.72±0.38	12.54±0.32
	50	16.56±0.37	15.22±0.22	17.71±0.40
	75	18.98±0.24	16.47±0.68	20.12±0.36
	100	21.79±0.26	20.29±0.70	22.19±0.44
Acetone extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	13.74±0.20	11.60±0.22	12.69±0.31
	50	16.22±0.26	13.40±0.36	16.29±0.51
	75	19.15±0.65	16.54±0.76	18.50±0.30
	100	23.07±0.86	19.22±0.29	22.06±0.25

Each data represents mean of three replicates ± S.E.

Antibacterial screening of root extracts (methanol, ethanol and acetone) of *B. ciliata* against *S. aureus, E. coli* and *Y. pestis.*

The results are presented in Table-5.

Table 5: Percent inhibition of growth of pathogenic bacterial spp. at different concentrations of methanol, acetone and ethanol root extracts of *B. ciliata*

Extract	Concentrations (In %)	Percent inhibition of growth of test bacteria (mm±S.E)		
		S. aureus	***E. coli.***	***Y. pestis***
Methanol extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	12.89±0.27	13.09±0.53	12.42±0.69
	50	15.43±0.78	15.16±0.23	13.49±0.42
	75	17.64±0.27	16.83±0.22	15.83±0.89
	100	22.42±0.48	19.04±0.34	17.08±0.44
Ethanol extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	11.85±0.42	10.85±0.32	10.11±0.04
	50	14.00±0.40	12.57±0.34	11.70±0.33
	75	15.60±0.30	15.09±0.18	12.69±0.22
	100	17.47±0.40	19.07±0.33	14.79±0.16
Acetone Extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	50	12.96±0.44	12.99±0.39	10.89±0.41
	50	15.50±0.31	15.34±0.66	13.13±0.23
	75	17.11±0.21	18.51±0.61	14.85±0.43
	100	20.84±0.49	21.62±0.36	18.16±0.54

Each data represents mean of three replicates ± S.E.

Table-5 is showing data pertaining to antimicrobial activity of methanol root extract of *B. ciliata* showed a maximum zone of inhibition for *S.aureus* (21.42mm at 100%) and a minimum for *Y. pestis* (17.08mm at 100%) for ethanol root extract *E.coli* showed a maximum zone of inhibition (19.07mm at 100%) and *Y. pestis* showed a minimum zone of inhibition (14.79mm at 100%) for acetone root extract a maximum zone of inhibition was shown by *E.coli* (22.52mm at 100%) and *Y. pestis* showed a minimum zone of inhibition (18.16mm at 100%).

Discussion

Bergenia ciliata (Saxifragaceae) is a plant of great economic and medicinal importance and present worldwide. The plant is widely used for the treatment of various diseases. In the present investigation, work has been conducted on the endophytes and antibacterial activity of *B. ciliata*. Eighteen species belonging to ten genera were isolated as endophytes of this plant. Maximum endophytes were isolated in rainy season followed by summer and winter.

Various workers have reported similar fungal endophytes from different plants. Bharti *et al.* (2016) [11] have reported 12 species of endophytic fungi belonging to different genera (viz. *Aspergillus, Cladosporium, Fusarium, Penicillium, Rhizoctonia, Rhizopus, Stachybotrys* and *Trichoderma)* from the leaf, bark, stem and root of *Rhododendron arboreum*. Maximum endophytic fungi were isolated during rainy season followed by winter season and summer season. Most abundant genus was *Aspergillus.* Sagar and Kaur (2010) [12] isolated the rhizosphere fungi of *Aesculus indica* and found that maximum number of fungi were recorded during rainy season (8 spp.) followed by spring (7 spp.), winter (6 spp.) and summer (5 spp.) Anitha *et al*. (2013) [13] investigated endemic medicinal plants of Tirumala hills under the Eastern Ghats of India for the presence of endophytes and *Aspergillus* was found to be the dominant fungus.

Antibacterial activity of *Bergenia ciliata* was tested for three pathogenic bacteria (viz. *S. aureus. E. coli and Y. pestis*) using leaf and rhizome extracts (methanol, ethanol and acetone) The screening revealed that Methanol leaf and root extracts were most effective and showed greater activity against *S.aureus* as compared to *Y. pestis* and *E. coli*. Further it was shown that rhizome and leaf extract were found to be more effective against gram positive bacteria as compared to gram negative bacteria.

There are reports of work done on antibacterial activities *of Bergenia* sp. Jahan *et al.* (2011) [14] studied the antimicrobial activity of ethanol leaf extracts of 5 traditionally used medicinal plants, *Syzygium cumini, Lawsonia inermis*, *Ficus religiosa, Ocimum sanctum and Zizyphus mauritiana* against *S. aureus* strains by agar well method. The study revealed that all the medicinal plants showed a remarkable antimicrobial activity and can be used in the treatment of various diseases. Chauhan *et al*. (2012) [15] have reported antibacterial activity of *B. ciliata*. The roots and leaves extracts were used to test the antibacterial activity. *B. ciliata* rhizome extract was found to inhibit the growth of gram positive bacteria as compared to gram negative strain.

Findings of the present study confirm that the plant extract of *B. ciliata* can be used as a potential antimicrobial agent against various pathogenic bacteria.

Conclusion

In the present work, maximum number of endophytes were observed during rainy season followed by summer and winter. It can be attributed to the fact that variation in individual fungal species distribution depend upon the type of soil, moisture content, depth, season of the year and concentration of organic matter. For antibacterial activity, it was concluded from the results that methanol leaf and root extract of *B. ciliata* were found to be quite effective in inhibiting the growth of *Staphylococcus*. Further the leaf and rhizome extract of plant showed more inhibitory effects in gram positive-bacteria than in gram-negative bacteria. Possible reasons for this antibacterial activity of *B. ciliata* are presence of bioactive compounds like alkaloids, phenolics and flavonoids which are present in leaf stem and rhizome of this plant and need further investigation.

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Conflict of interest
The authors declare that there is no conflict of interest.

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