Isolation and antimicrobial efficacy tests of *Bergenia ciliate* using *in vitro* models

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Antimicrobial activities of five compounds isolated from ethyl acetate fractions of *Bergenia ciliate* were investigated. All compounds exhibited antimicrobial efficacy on the tested microorganisms, that included two human Gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*) and four Gram-negative ones (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Klebsiella Pneumonia*). Compound 1 exhibited the highest antimicrobial activity compared to the other compounds. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of compound 1 was carried out by agar dilution method and viable cell count method, respectively. These constituents were isolated first time from *B. ciliate* which is credible as standard antimicrobial drugs. MICs ranged from 0.156 to >10 µg/ml and MBCs from 1.26 to 15 µg/ml. Compound 1 showed antifungal activity 7 (±0.3) mm while compounds 2 to 5 have moderate activity 5 (±0.12) ~ 2 (±0.1) mm.

**Key words:** *Bergenia ciliate*, isolation, antimicrobial activity, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC).

INTRODUCTION

The species of genus *Bergenia* (Saxifragaceae), popularly known in the folk medicine as Paashaanbhed, grow at Himalayas, usually on rocky, moist and shady places. Many plants of this genus have been used for long in the folk medicine. The rhizomes of *Bergenia* have been used for treatment of diarrhea, vomiting, fever, cough, pulmonary infections, menorrhagia, excessive uterine hemorrhage, kidney stones and ulcer of large intestines (Ahmed et al., 2004; Sinha et al., 2001a; Uniyal et al., 2006). They have also been used externally for healing wounds, eye sores and boils. Their alcoholic extracts have significant analgesic, anti-inflammatory, diuretic and antibacterial activities (Sinha et al., 2001a,b; Uniyal et al., 2006). Bergenin (compound 1), a C-glycoside of 4-O-methyl gallic acid, is the main constituent of rhizomes of these species. Other compounds isolated from *Bergenia* species include polyphenols, galloylarbutin, afzelechin, sitosterol, paashaanolactone and bergenan (Bahl et al., 1974; Umashankar et al., 1998; Popov et al., 2005).

Bergenin is reported to have anti-inflammatory (Swarnalakshmi et al., 1984), hypolipidaemic (Jahromi et
Table 1. Zone of Inhibitions of reference antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Ec</th>
<th>Kp</th>
<th>Ps</th>
<th>Ent</th>
<th>MI</th>
<th>Sta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicilline</td>
<td>15.1(±0.02)</td>
<td>14(±0.01)</td>
<td>11.9(±0.01)</td>
<td>13(±0)</td>
<td>10(±0.11)</td>
<td>9.06(±0.05)</td>
</tr>
<tr>
<td>Ofloxacine</td>
<td>14.1(±0.05)</td>
<td>12.3(±0.5)</td>
<td>11(±0)</td>
<td>14(±0.11)</td>
<td>13(±0.05)</td>
<td>14(±0.05)</td>
</tr>
</tbody>
</table>

E.c: Escherichia coli; K.p: Klebsiella pneumoniae; p.s: Pseudomonas aeruginosa; Ent: Enterobacter cloacae; M.I: Micrococcus luteous; Sta: Staphylococcus aureus (methicillin resistant); mm: milli metre.

MATERIALS AND METHODS

General experimental procedure

Aluminum thin layer chromatography (TLC) plates (20 x 20, 0.5 mm thick) was carried out to check the purity of the compounds pre-coated with silica gel 60 F254 (20 x 20 cm, 0.2 mm thick; E. Merck, Darmstadt, Germany). Column chromatography (CC) was done using silica gel of 230-400 mesh (E. Merck, Darmstadt, Germany). Ceric sulphate and potassium permanganate solutions were used as spray reagents. The UV spectra (λmax nm) were recorded on Shimadzu UV-2700 spectrophotometer (Shimadzu, Japan) in EtOH. Mass Spectra was recorded on Bruker TOF Mass spectrometers (Billerica, USA) using electrospray ionization (ESI). The $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker DPX-400 NMR spectrometer (Billerica, USA) (400 MHz for $^1$H and 100 MHz for $^{13}$C-NMR), using CDCl$_3$ as solvents.

Plant

The plant was collected from Bara Gali Hazara Division, NWFP, Pakistan. The plant was identified by Prof. Dr. Manzoor Ahmad, Botany Department, Government Post Graduate College, Abbottabad.

Extraction and isolation

Shade dried whole plant of B. ciliate (24 kg) was ground and extracted with methanol at 25°C (3 x 25 L). The whole methanolic extract was evaporated under reduced pressure to obtain a thick blackish gummy material (crude). It was fractionated with n-hexane (116 g), chloroform (165 g), ethyl acetate (171 g), and n-butanol (180 g) soluble fractions, respectively. The ethyl acetate soluble fraction was subjected to column chromatography over silica gel (65-225 mesh) eluting with n-hexane (100%), n-hexane:EtOAc (0.4:9.6 to 9.6:0.4), EtOAc (100%), EtOAc:EtOH (1:9 to 9:1), EtOH (100%), in increasing order of polarity to obtain 11 fractions A to K. A series of silica gel column chromatography was carried out of fraction C eluting with n-hexane, n-hexane-EtOAc and EtOAc in increasing order of polarity to get compound 1 and to a preparative TLC using n-hexane:EtOAc (1:5:3.5) as solvent system to get compounds 2 and 3. Fraction D (5 g) was again introduced to column chromatography over silica gel and fractionized with n-hexane, n-hexane-EtOAc and EtOAc in increasing order of polarity. Which was followed by preparative TLC washed with n-hexane: CH$_2$Cl$_2$ (1:1.5) and got compounds 4 and 5.

Microorganisms

Six bacterial cultures, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603, Pseudomonas aeruginosa (clinical strain/PIMS), Enterobacter cloacae (clinical strain/PIMS), Staphylococcus aureus (MRSA, clinical strain/PIMS), Enterobacter cloacae (clinical strain/PIMS) were used in antibacterial assay. Cultures were obtained from School of Life Science and Technology, Beijing University of Chemical Technology (BUCT) China, where they were identified and characterized. These strains were maintained on agar slants at 4°C for antimicrobial tests. Microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.2. The reference antibiotics were used as Ofloxacine (10 µg) and Ampicilline (10 µg) (Oxoid) (Table 1).

Antimicrobial screening

Screening for antibacterial activity

The agar well diffusion method (Hadacek and Greger, 2000) was used to check the antibacterial activity. All bacterial cultures were first grown in nutrient broth at 37°C for 20 to 24 h incubated till turbidity became correspondent to McFarland 0.5 turbidity standard was obtained. The inocula of the respective bacteria were splashed on to the Mueller Hinton agar (Oxoid) plates using a sterile swab in order to make sure a uniform thick lawn of growth following incubation. With the help of sterile cork borer, wells of 6 mm in diameter were formed on to nutrient agar plates. The test agents (100 µl) were put to the wells and the plates were then allowed to stay for 1 h at room temperature. At last, the plates were incubated at 37°C for 20 to 24 h and the resulting diameters of zones of inhibition were measured.

Determination of minimum inhibitory concentration (MIC)

MIC of the compound 1 was carried out by agar dilution method (EUCAST Definitive Document, 2000; Mukherjee, 2002;
Table 2. Inhibition zones of compounds 1-5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>E. coli (mm)</th>
<th>K. pneumoniae (mm)</th>
<th>E. cloacae (mm)</th>
<th>P. aeruginosa (mm)</th>
<th>M. luteus (mm)</th>
<th>S. aureus (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15(±0.5)</td>
<td>14(±0.4)</td>
<td>14(±0.1)</td>
<td>15(±0.01)</td>
<td>16(±0.6)</td>
<td>14(±0.5)</td>
</tr>
<tr>
<td>2</td>
<td>12(±0.3)</td>
<td>9(±0.4)</td>
<td>10(±0.1)</td>
<td>8(±0.6)</td>
<td>8(±0.1)</td>
<td>5(±0.1)</td>
</tr>
<tr>
<td>3</td>
<td>7(±0.2)</td>
<td>7(±0.4)</td>
<td>8(±0.3)</td>
<td>5(±0.1)</td>
<td>5(±0.5)</td>
<td>4(±0.7)</td>
</tr>
<tr>
<td>4</td>
<td>4(±0.2)</td>
<td>5(±0.01)</td>
<td>6(±0.6)</td>
<td>5(±0.4)</td>
<td>5(±0.1)</td>
<td>3(±0.4)</td>
</tr>
<tr>
<td>5</td>
<td>5(±0.5)</td>
<td>3(±0.4)</td>
<td>5(±0.3)</td>
<td>5(±0.3)</td>
<td>4(±0.2)</td>
<td>3(±0.2)</td>
</tr>
</tbody>
</table>

E. c: *Escherichia coli*; K. p: *Klebsiella pneumoniae*; p. s: *Pseudomonas aeruginosa*; Ent: *Enterobacter cloacae*; M. l: *Micrococcus luteus*; Sta: *Staphylococcus aureus* (methycillin resistant); mm: milli metre

Table 3. MIC and MBC *B. ciliata* of compound 1.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>&gt;10</td>
<td>N.d</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>&gt;10</td>
<td>N.d</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>&gt;10</td>
<td>N.d</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>M. luteus</td>
<td>0.625</td>
<td>1.877</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.156</td>
<td>1.260</td>
</tr>
</tbody>
</table>

Anonymous, 2000). The sterilized Muller Hinton Agar (oxoid) was allowed to cool to 50°C and about 19 ml of this was introduced to test tubes which contained 1 ml of different concentration of compounds. This mixture was thoroughly mixed and poured into pre-labeled sterile Petri dishes. Petri dishes having only growth media were prepared in the same way so as to serve for comparison with petri plate containing compound. The concentrations of the compound used in this test ranged from 2000 to 0.156 μg/ml. The suspensions of the respective microorganisms having density adjusted to 0.5 McFarland turbidity standards were inoculated onto the series of agar plates using standard loop. The plates were then incubated at 37°C for 24 h. The lowest concentration which inhibited the growth of the respective organisms was considered as MIC. All tests were carried out in triplicate.

Determination of minimum bactericidal concentration (MBC)

MBC of the compound 1 was done by the viable cell count method (Toda et al., 1989; Anonymous, 2003). The results were expressed as the number of viable cells as a percentage of the control.

Screening for antifungal activity

The required amount of each fungal strain was introduced in 2 ml of Sabauraud Dextrose broth. This suspension was homogeneously spread on Petri plates containing Sabauraud Dextrose agar media using sterile swabs. Samples were applied into wells using same technique for bacteria and incubated at 25°C for 72 h. The plates were then checked for the presence of zones of inhibition and the results were recorded. Itraconazole was used as a standard.

RESULTS

Almost all fractions of *B. ciliata* presented activity against both Gram positive and Gram negative bacterial pathogens.

Compounds 1 and 2 show high antibacterial activity while the other three compounds have moderate antibacterial activity as shown in Table 2 and Figure 2.

Compound 1 was subjected for the determination of MIC and MBC, respectively. The MIC values ranged from 0.156 to >10 mg/ml for all tested strains while the MBC values reported were higher than MIC (Table 3 and Figure 3).

Likewise nearly similar pattern of susceptibility was reported against fungal strain *Aspergillus niger*. The widest zones of inhibition (maximum antifungal activity) were presented by Compounds 1 and 2. Compounds 3, 4 and 5 have reasonable zone of inhibition (Table 4 and Figure 4).

DISCUSSION

All the compounds, namely, Afzelechin (Compound 1), Asarone (Compound 2), Terpinen-4-ol (Compound 3), Parasorbic acid (Compound 4) and Damascenone (Compound 5) were isolated from ethyl acetate fraction of *B. ciliata* having antibacterial activity. Compound 1 have the highest antibacterial activity. The MIC of compound 1 was taken as 0.156 mg/ml. The MBC value of compound 1 is many times higher than MIC. The MBC values for *E. coli*, *K. pneumonia*, and *P. aeruginosa* were not detected. All the five compounds isolated from the ethyl acetate fraction of *B. ciliata* are polar and were primarily tested as antimicrobial reagents.
This investigation is probably the first to explore the antimicrobial activities of compounds of *B. ciliata*, as a comprehensive literature review; to the best of our knowledge, there is no information about the antimicrobial activities of these isolated compounds (1 to 5) from *B. ciliata*.

**Conclusion**

The isolated five antimicrobial compounds from ethyl acetate fraction showed that the plant *B. ciliata* has potential antimicrobial activities. Almost all the compounds isolated from *B. ciliata* were primarily...
Figure 3. MIC and MBC *Bergenia ciliata* of compound 1.

Figure 4. Antifungal activities of compounds 1 to 5.

Table 4. Antifungal activities of compounds 1-5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Zone of inhibition (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7(±0.3)</td>
</tr>
<tr>
<td>2</td>
<td>5(±0.12)</td>
</tr>
<tr>
<td>3</td>
<td>2(±0.1)</td>
</tr>
<tr>
<td>4</td>
<td>3(±0.8)</td>
</tr>
<tr>
<td>5</td>
<td>4(±0.8)</td>
</tr>
<tr>
<td>Standard</td>
<td>8(0)</td>
</tr>
</tbody>
</table>

reported as active against the *A. niger*. Compounds 1 and 2 showed the highest antifungal activities, while compound 3 showed the least antifungal activity. Therefore, the other three fractions (*n*-hexane, dichloromethane and methanol) are further recommended for investigations to isolate the compounds and expose the hidden medicinal value of *B. ciliata*. 
Conflict of Interest

The authors have not declared any conflict of interest.

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REFERENCES


