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Isolation, Antioxidant and Antifungal Activities of Two Newly Reported Compounds from *Bergenia ciliata*

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Ethyl acetate fraction of *Bergenia ciliata* was subjected to various chromatographic techniques such as silica gel CC, size exclusion CC and reverse phase CC, which resulted in isolation of two new compounds from this specie. Modern spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR (BB and DEPT), 2D NMR, ESI and ESI-HR-MS were utilized to elucidate the structures of isolated secondary metabolites. Both the compounds **A**, **B** showed a remarkable fungicidal activity against *Cladosporium cladosporioides* in TLC bio-autography method. Compound **B** showed maximum activity (90.34 ± 0.81 %) against control drug.

Keywords: *Bergenia ciliata*, Antioxidant Activity, Antibacterial activity.

INTRODUCTION

Bergenia is a genus, which has ten flowering plant species, native to central Asia and the Himalayan region. Some species of this genus is also present in Pakistan. The flowers grow on a stem similar in colour to a rhubarb stalks and most varieties have cone-shaped flowers in varying shades of pink [1]. 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 [2-4]. 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 [3-5]. Bergenin, 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 [6-8]. Bergenin is reported to have anti-inflammatory [9], hypolipidaemic [10], anti-HIV [11], antiarrhythmic [12] and hepatoprotective [13].

In this study, the isolation, separation, antioxidant and antifungal activities of the known compounds which were isolated for first time from *Bergenia ciliata*, namely kaempferol-7-methoxy-3-O-β-D-glucopyranosyl-3'-oic acid and debenzoyl-paeoniflorin (4-methylether-8-benzoyl-1-O-β-D-glucopyranoside) were reported.

EXPERIMENTAL

Un-correct melting points were measured using Stuart SMP 10 apparatus. (UV) spectra were performed on Hitachi-U-3200 and (IR) were recorded using JASCO-A-302. ^{1}H NMR and ^{13}C NMR spectra were recorded by a Varian 500 MHz. Finnigan-MAT-311 and Varian MAT 312 spectrometers were used to determine the mass spectrum at 250 °C with ionization potential of 70 eV. Jeol JMS-600H mass spectrometer was used for high resolution mass spectra (HRMS) utilizing PFK as an internal standard. Fast atom bombardment mass spectra (FAB +ve) was obtained by using JMS HX-110 double focusing mass spectrophotometer.

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

Extraction and isolation: The shade dried roots and aerial parts were separately extracted with commercial grade ethanol at room temperature and concentrated in rotary evaporator at 45 °C to thick syrup which was further concentrated in water bath at 45 °C for three days to dark brown colour solid mass. The combined extract was suspended in water and extracted with *n*-hexane, dichloromethane and ethyl acetate in order to

fractionate the complex mixture into non-polar, slightly polar and medium polar sub-fractions while highly polar compounds remained in the aqueous phase.

Compound A: (9-Kaempferol-7-methoxy-3-O-β-D-glucopyranosyl-3'-oic acid) white amorphous powder Yield: 14 mg, IR (ν_{max}): 3380, 1640, 1710, 1550 cm^{-1}; UV λ_{max} (MeOH): 355, 320 nm HRESIMS: *m/z* 507.1130 $[M+H]^+$ calcd. for $C_{23}H_{22}O_{13}$ 507.1126 ESI (+)-MS *m/z*: 507.1130, 477.1022, 463.1235, 285.0754 ^1H NMR and ^{13}C NMR and HSQC and HMBC correlation (Table-1).

TABLE-1
^1H NMR AND ^{13}C NMR CHEMICAL SHIFTS OF COMPOUND **A** IN CD_3OD

C. No.	^{13}C NMR (δ_c)[a]	Multiplicity (DEPT)[bd]	^1H NMR (δ_H) coupling constants *J*HH (Hz)[cd]
2	158.8	C	–
3	135.6	C	–
4	179.2	C	–
5	163.0	C	–
6	99.8	CH	6.28, d (*J* = 1.8)
7	132.2	C	–
8	94.7	CH	6.39, d (*J* = 1.8)
9	159.0	C	–
10	104.3	C	–
OCH_3	56.6	CH_3	3.81,s
1'	123.0	C	–
2'	117.5	CH	7.70 d (*J* = 2.0)
3'	145.9	C	–
4'	149.8	C	–
5'	116.0	CH	6.84, d (*J* = 8.6)
6'	123.2	CH	7.57, dd (*J* = 8.5, 2.0)
7'	168.3	C=O	–
1"	104.2	CH	5.25, d (*J* = 6.5)
2"	75.7	CH	3.47, t (*J* = 6.5)
3"	78.1	CH	3.42, t (*J* = 6.5)
4"	71.2	CH	3.34, t (*J* = 6.5)
5"	78.4	CH	3.21, m
6"	62.5	CH_2	3.57, dd (*J* = 6.5, 2.0) 3.70, dd (*J* = 6.5, 2.0)

[a]Broad band; [b]DEPT; [c]^1H NMR; [d]HSQC interactions

Compound B: (4-Methylether-8-benzoyl-1-O-β-D-glucopyranoside) amorphous powder Yield: 21 mg, IR (ν_{max}): 3410, 1710, 1540, 1030 cm^{-1}; UV λ_{max} (MeOH): 265, 225 nm HRESIMS: *m/z* 543.1708 $[M+H]^+$ calcd. for $C_{24}H_{30}O_{11}$ 543.1706 ^1H NMR and ^{13}C NMR and HSQC and HMBC correlation (Table-2).

Antifungal bioassay: The EtOAc extract of compounds **A** and **B** were tested for antifungal activity by direct bioautography on aluminum-backed TLC sheets [14] against the wheat pathogenic fungus. *Cladosporium cladosprioides* was assessed on glass-backed TLC plates in agar overlay assay [15]. After elution, the chromatograms were thoroughly dried to remove any solvent residues before being sprayed with the suspension of the fungus. Nystatine (Sigma) and amphotericin B (Sigma) were used as controls.

Test against *Cladosporium cladosprioides* strains: Inoculated sabouraud maltose liquid medium with *Cladosporium cladosprioides* was sprayed on TLC. Clear inhibition zone were observed against a blue-raddish background after 48 h incubation at room temperature in humid atmosphere. Conidial suspension of *Cladosporium cladosprioides* was supplemented with a solution of thiazolium (0.25 % MTT) before being sprayed on TLC. The activity of the extract and compound appeared as clear inhibition zone (Table-3) against reddish background 48 h post incubation.

TABLE-2
^1H NMR AND ^{13}C NMR CHEMICAL SHIFTS OF COMPOUND **B** IN CD_3OD

C/H. No.	^{13}C NMR (δ_c)[a]	Multiplicity (DEPT)[bd]	^1H NMR (δ_H) coupling constants *J*HH (Hz)[cd]
1	89.1	C	–
2	86.0	C	–
3	44.5	CH_2	1.85, d (*J* = 12.5) 2.19, d (*J* = 12.5)
4	106.0	C	-
5	44.0	CH	2.57, t (*J* = 6.5)
6	71.7	C	–
7	23.4	CH_2	1.96, dd (*J* = 10.7, 1.5) 2.50, dd (*J* = 10.7, 1.5)
8	60.8	CH	4.81, s
9	100.2	CH	5.41, s
10	19.6	CH_3	1.35, s
11	22.2	CH_2	4.74, s
1'	102.3	CH	5.41, d (*J* = 7.6)
2'	75.0	CH	4.08, t (*J* = 7.6)
3'	77.9	CH	4.33, t (*J* = 7.6)
4'	72.2	CH	5.79, t (*J* = 7.6)
5'	78.1	CH	3.99, m
6'	62.9	CH_2	4.10, dd (*J* = 7.6, 1.5) 4.21, dd (*J* = 7.6, 1.5)
1"	130.7	C	–
2", 6"	130.0	CH	8.03 dd (*J* = 7.5, 2)
3", 5"	129.6	CH	7.48, t (*J* = 7.5)
4"	134.4	CH	7.60, t (*J* = 7.5)
7"	168.4	C	–

[a]Broad band; [b]DEPT; [c]^1H NMR; [d]HSQC interactions

TABLE-3
ANTIFUNGAL ACTIVITY OF COMPOUNDS **A** and **B**

S. No.	Test sample	Zone of inhibition (mm)*
1	Compound **A**	6 (± 0.30)
2	Compound **B**	4 (± 0.12)
3	Standard	8 (0)

DPPH antioxidant assay: Both the isolated compounds from *B. ciliata* were evaluated for their radical scavenging potential and it was found out that these compounds are the most potent antioxidant with IC_{50} value of 14.6 ± 1.25 μM. Moreover, it was found that the isolated from *B. ciliata* indicating that the OH groups attached to benzene ring have key function in scavenging of the free radicals (Table-4).

TABLE-4
DPPH ANTIOXIDANT ACTIVITY AND IC_{50} VALUES OF COMPOUND **A** AND COMPOUND **B** FROM *B. ciliata*

S. No.	Test sample[a]	IC_{50} (μg/mL)	% DPPH inhibition ± SEMC
1	Compound **A**	4.70	83.63 ± 1.50
2	Compound **B**	3.41	90.42 ± 0.81
3	Gallic acid[b]	3.08	96.34 ± 0.26
4	Ascorbic acid[b]	3.15	96.33 ± 0.56

[a]Test samples were TLC DPPH scavangers at amount of 1 μg/spot.
[b]Positive control used in assay.
[c]Standard error of mean of three assays.

Statistical analysis: The data is presented as ± standard deviation for the three determinations. The data of DPPH was analyzed for statistical significance using analysis of variance with SPSS 12 software.

RESULTS AND DISCUSSION

Compound **1** was isolated as white amorphous powder. Molecular formula ($C_{23}H_{22}O_{13}$) was established by ESI-HR-MS at *m/z* 507.1130 a.m.u. (calcd. 506.1127 a.m.u.). The IR indicated the presence of carboxylic –OH group (3451 cm^{-1}), phenolic OH group 3364 cm^{-1}, carbonyl group 1641 cm^{-1} and 1711 cm^{-1} for α,β-unsaturated ketone. The UV spectrum is also consistent with the presence of benzene ring and a carbonyl group showing λ_{max} at 356 nm. The ^{1}H NMR spectrum further displayed adouble doublet at δ_H 7.56 (1H, *J* = 8.6, 2 Hz) which was assigned to H-6' due to the presence of a *meta* proton (H-5') in the vicinity of C-6' where H-5' resonated as a doublet at δ_H 6.84 (*J* = 8.6).

The glucose protons signals are observed in ^{1}H NMR spectrum resonated at δ_H 5.28(1H, d, *J* = 6.4 Hz, H-1'), 3.43 (1H, t, *J* = 6.5 Hz, H-2'), 3.41 (1H, t, *J* = 6.5 Hz, H-3'),3.39 (1H, t, *J* = 6.3 Hz, H-4'), 3.22(1H, m, H-5'), 3.54 (1H, dd, *J* = 6.4, 2.1 Hz, H-6a') and δ_H 3.68 (1H, dd, *J* = 6.6, 2.0 Hz, H-6b') and the structure of compound A (Fig. 1) was confirmed.

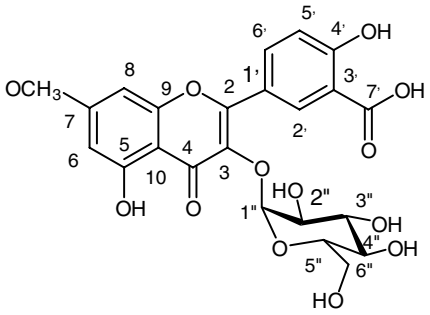

Fig. 1. Structure of compound **A**

The ^{13}C NMR spectrum displayed signal at δ_C 168.3 for carboxyl carbon (C-7') and a signal at δ_C 145.9 was assigned to C-3', slightly downfield due to attachment of carboxyl carbon at C-3 which was confirmed by HMBC experiment.

The coupling of protons was confirmed by ^{1}H-^{1}H COSY experiment which showed coupling of H-6/H-8, H-4'/H-5' and H-5'/H-1'.

The relative position of various groups with respect to each other was confirmed by the HMBC experiment *i.e.* H-6 and H-8 both showed correlation with OCH_3 carbon indicating that OCH_3 carbon is in neighboring of both of these protons suggesting the attachment of a methoxy group at C-7. In addition, H-6 and H-8 are showing correlation with C-10. H-2' showed correlation with C=O carboxylic and also with C-2 while H-5' is showed correlation with C-1' and also with C-3'. The anomeric carbon is showed correlation with C-3 indicating the attachment of glucose with the C-3.

The ESIMS spectrum of compound **16** was analyzed and the presence of M^+-45 peak at *m/z* 463.1234 a.m.u. indicated presence of carboxyl function, in addition the peak at *m/z* 477.1023 a.m.u is due to loss of OCH_3 (Fig. 2).

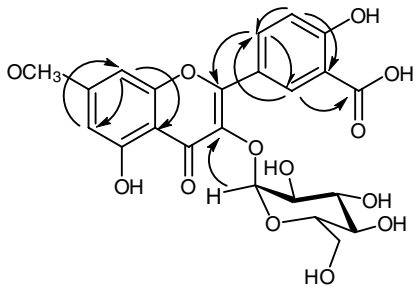

Fig. 2. Selected key COSY (H----H) and HMBC (H----C) interactions of compound **A**

Compound **2** was isolated as amorphous powder. The IR spectrum is consistent with the presence of benzene ring (1541 cm^{-1}), ester linkage (1711 cm^{-1}), glycoside linkage (1031 cm^{-1}) and hydroxyl groups (3411 cm^{-1}). UV spectrum also indicated presence of highly conjugated system (λ_{max} 266 nm). Molecular formula was established by ESI-HR-MS *m/z* 543.1709 a.m.u. calcd. 543.1707 a.m.u. for $C_{24}H_{30}O_{11}$ (Fig. 3).

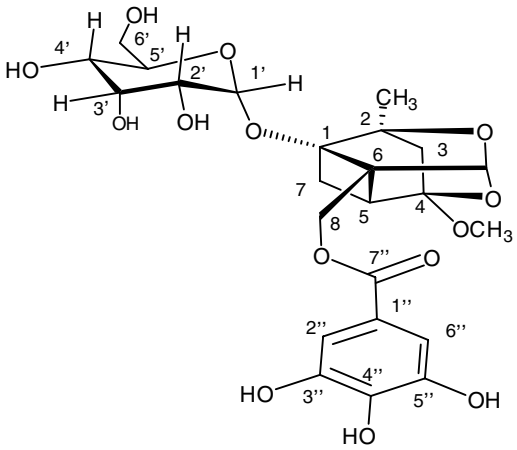

Fig. 3. Structure of compound **B**

The ^{1}H NMR showed signals at δ_H 1.79 (1H, d, *J* = 11.5 Hz, H-3a), 2.25 (1H, d, *J* = 11.6 Hz, H-3b), 2.69 (1H, t, *J* = 6.51 Hz, H-5), 2.05 (1H, dd, *J* = 10.4, 2 Hz, H-7a), 2.62 (1H, dd, *J* = 10.6, 2 Hz, H-7b), 4.82 (2H, s, H-8), 5.52 (1H, s, H-9), 1.48 (1H, s, H-10) and OCH_3 appeared at δ_H 3.98 as a singlet. The glucose moiety showed signals in ^{1}H NMR spectrum at δ_H 5.63 (1H, d,

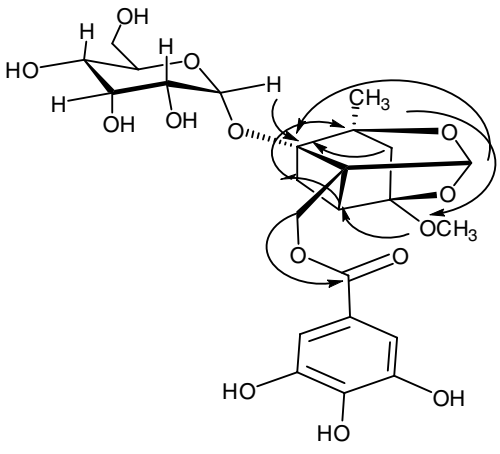

Fig. 4. Selected key COSY (H----H) and HMBC (H----C) interactions of compound **B**

J = 7.6 Hz, H-1'), 4.22 (1H, t, *J* = 7.5 Hz, H-2'), 4.53 (1H,t, *J* = 7.5 Hz, H-3'), 5.69 (1H, t, *J* = 7.5 Hz, H-4'), 3.85 (1H, m, H-5'), 4.09 (1H, dd, *J* = 7.6Hz, 1.5 Hz, H-6a') and δ_H 4.38 (1H, dd, *J* = 7.50, 1.5 Hz, H-6b') while the two aromatic protons appeared at δ_H 7.69 as a singlet [16,17] (Fig. 4).

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