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Article

Cashmirins A and B, new antifungal and urease inhibitory prenylated coumarins from *Sorbus cashmiriana*

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Cashmirins A (**1**) and B (**2**), new prenylated coumarins, have been isolated from the EtOAc-soluble fraction of the whole plant of *Sorbus cashmiriana* Hedlung, Monog. along with seselin (**3**), scopoletin (**4**), 3-hydroxyxanthyletin (**5**) and luteolin (**6**), reported for the first time from this species. Their structures were elucidated by spectroscopic techniques including MS, 1D and 2D NMR spectroscopy. Both new compounds **1** and **2** were investigated for biological activities and showed significant antifungal and urease inhibitory activities. Compounds **1** and **2** exhibited significant activity against *Aspergillus flavus*, *Macrophomina phaseolina*, *Trichophyton simii*, *Trichophyton schoenleinii*, and *Pseudallescheria boydri*. Both compounds also exhibited significant inhibitory activity against Jack bean urease with IC_{50} values of 28.2±0.12 µM and 30.3±0.18 µM, respectively compared to thiourea used as positive control.

Keywords: *Sorbus cashmiriana*. Prenylated coumarins. Cashmirins. Antifungal activity. Urease inhibition.

INTRODUCTION

The genus *Sorbus* (Rosaceae) comprises of 200 species which are commonly grown in Asia, Africa and South America. Plants of the genus *Sorbus* are found in customary and local medicines that are used as anti-diarrhea, diuretic, anti-inflammatory, anti-diabetic, vaso- protective, broncho- and vasorelaxant, along with potent antioxidative qualities. In Pakistan, it is represented by seven species. One of these is *Sorbus cashmiriana* Hedlung, Monog, which is a tree of two seasons, one in the spring with lovely pink-tinted flowers and one in the autumn when the leaves are gone, and glorious white fruits shine out. It is distributed in Kashmir and the western Himalayas. A tea made from its bark is used to treat nausea. The bark preparation is also used to treat heart diseases. Berries are used to cure scurvy (Bhattacharjee, 2003; Perry, Metzger, 1980; Krishna, 1972; Jayaweera, 1982; Krachmal, 1980). Previously, six triterpenes (Kazmi *et al*., 2007; Kazmi *et al*., 2009; Kazmi *et al*., 2011) and two coumarins (Khan *et al*., 2015) have been reported by us from this species. The ethnopharmacologic and chemotaxonomic importance of the genus *Sorbus* prompted us to carry out further phytochemical studies on *S. cashmiriana*. Herein we report the isolation and structural elucidation of two new prenylated coumarins named as cashmirins A (**1**) and B (**2**) (Figure 1) along with seselin (**3**) (Cazal *et al*., 2009), scopoletin (**4**) (Darmawan *et al*., 2012), 3-hydroxyxanthyletin (**5**) (Chen *et al*., 2010) and luteolin (**6**) (Nissler, Gebhardt, Berger, 2004), reported for the first time from this species.

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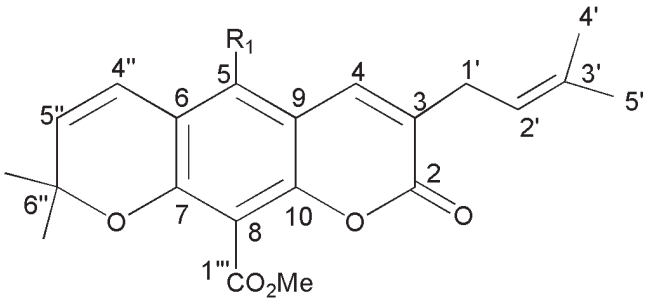

1: R_1 = H

2: R_1 = OMe

FIGURE 1 - Structures of cashmirins A (**1**) and B (**2**).

The plant kingdom has provided a number of therapeutic compounds, such as antifungal, antibacterial, antiparasitic, antihistamine, analgesics, anti-inflammatory, asthma medications and many more (Ficker *et al*., 2003; Islam *et al*., 2001; Jones *et al*., 2000; Omar *et al*., 2000). Some of the traditionally used plants may lead to the development of new antifungal agents that are in increasing demand due to their resistance to traditional medicines (White *et al*., 1998).

Urease (EC.3.5.1.5) has been identified as a key virulence determinant in the pathogenesis of a variety of clinical conditions, with implications for human and animal health, as well as agriculture. It leads to the pathogenesis of urolithiasus, pyelonephritis, hepatic coma, and urinary catheter encrustation by being specifically involved in the formation of infection stones (Mobley *et al*., 1989; Mobley *et al*., 1995).

In the current study, we have described the antifungal and urease inhibitory activity of the new prenylated coumarins named as cashmirins A (**1**) and B (**2**).

MATERIAL AND METHODS

General experimental procedure

The UV and IR spectra were recorded on a Hitachi UV-3200 and JASCO 320-A spectrometers, respectively. NMR data were recorded on a Bruker AV-500MHz spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard. The chemical shift values are reported in ppm (**δ**) and the coupling constant (*J*) is in Hz. EI- and HREI-MS were recorded on Finnigan MAT 12 and MAT 312 spectrometers in *m/z* and %. Thin layer chromatography (TLC) was executed on precoated with silica gel F_{254} plates (E. Merck, Darmstadt, Germany): detection at 254 nm and by spraying with ceric sulfate in 10% H_2SO_4 solution. Silica gel (230–400 mesh, E. Merck, Darmstadt, Germany) was used for column chromatography.

Plant material

The whole plant of *Sorbus cashmiriana* Hedlung, Monog was collected from Kashmir and identified by Sher Wali Khan, Department of Biological Science, Karakoram International University Gilgit. A voucher specimen (No. KUH 73/67 760) has been deposited with the Herbarium of the Department of Botany, University of Karachi, Pakistan.

Extraction and isolation

The whole plant *S. cashmiriana* (16 kg) was shade dried ground and extracted with MeOH (3 × 40 L). The MeOH extract was evaporated under reduced pressure to yield a residue (300 g), which was divided into n-hexane (70 g), $CHCl_3$ (60 g), EtOAc (78 g), and H_2O (80 g) soluble sub-fractions. The EtOAc sub-fraction was subjected to column chromatography over silica gel eluting with n-hexane/$CHCl_3$ in increasing order of polarity to obtain five fractions F_1-F_5. The fraction F_4 which eluted with n-hexane/$CHCl_3$ (30:70) was further purified through preparative TLC using n-hexane/$CHCl_3$ (45:55) to afford compound **6** (18 mg). The fraction F_3 which eluted with n-hexane/$CHCl_3$ (40:60) was further chromatographed, eluting with n-hexane/$CHCl_3$ (55:45) to obtain compounds **4** (35 mg) and **5** (30 mg) from the top and the tail fractions, respectively. The fraction F_2 which eluted with n-hexane/$CHCl_3$ (50:50) was further chromatographed and successively eluted with n-hexane/$CHCl_3$ (60:40) and (55:45) to obtain compounds **1** (5 mg) and **2** (5.5 mg), respectively. The fraction F_1 which eluted with n-hexane/$CHCl_3$ (60:40) furnished compound **3** (25 mg).

Cashmirin A (1)

White amorphous solid; m.p. 107-108 °C; UV λ_{max} (MeOH) 339, 264, 235, 220 nm; IR υ_{max} (KBr) 1730, 1720, 1635, 1602, 1518 cm^{-1}; EIMS *m/z* (rel. int.) 354 (10), 339 (32), 300 (21), 296 (100), 285 (45), 162 (72); HR-EIMS (*m/z*) 354.1463 $[M]^+$ (calcd for $C_{21}H_{22}O_5$, 354.1467); ^1H and ^{13}C NMR data, see Table I.

CashmirinB (2)

White amorphous solid; m.p. 107-108 °C; UV λ_{max} (MeOH) 338, 264, 233, 222 nm; IR υ_{max} (KBr) 1728, 1722, 1632, 1602, 1522 cm^{-1}; EIMS *m/z* (rel. int.) 384 (9), 369 (28), 330 (18), 316 (20), 302 (32), 296 (100), 285 (52), 244 (40), 162 (75); HR-EIMS (*m/z*) 384.1576 $[M]^+$ (calcd for $C_{22}H_{24}O_6$, 354.1572); ^1H and ^{13}C NMR data, see Table I.

Antifungal activity assay

The antifungal assay was performed on human, animals and plants pathogens by agar tube dilution method (Ahmad *et al*., 2007) using ten pathogenic fungi which are illustrated in Table II. The crude extracts, compounds **1**, **2** and the standard drugs (each 200 µg/ml of Sabourd Dextose Agar) were subjected to antifungal activity against *Aspergillus flavus* ATCC 32611, *Aspergillus niger* ATCC 1015, *Macrophomina phaseolina* ATCC 53789, *Trichophyton simii* ATCC 25923, *Microsporum canis* ATCC 36299, *Trichophyton schoenleinii* ATCC 22775, *Fusarium solani* ATCC 36031, *Pseudallescheria boydri* ATCC 44330, *Candida glabrata* ATCC 90030, and *Rhizoctonia solani* ATCC 76131.

Urease inhibitory activity

Reaction mixtures comprising 25 µL of enzyme (Jack bean urease) solution and 55 µL of buffers containing 100 mM urea were incubated with 5 µL of test compounds at 30 °C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method (Weatherburn, 1967). 45 µL each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). In a final volume of 200 µL, all reactions were performed in triplicate. The results were processed by using Soft Max Pro software (Molecular Device, USA). Assays were performed at pH 8.2 potassium phosphate buffer (0.01 M $K_2HPO_4.3H_2O$, 1 mM EDTA and 0.01 M LiCl). Percentage inhibitions were calculated from the formula $100 - (OD_{test\ well}/OD_{control}) \times 100$). Thiourea was used as the standard inhibitor of urease.

RESULTS AND DISCUSSION

Cashmirin A (**1**) was obtained as a white amorphous solid. The molecular formula was established as $C_{21}H_{22}O_5$ through HR-EIMS showing an $[M]^+$ peak at *m/z* 354.1463 (calcd 354.1467). The IR spectrum showed the presence of an α, β-unsaturated lactone (1720 cm^{-1}), ester carbonyl (1730 cm^{-1}), aromatic C=C (1602 and 1518 cm^{-1}) and olefinic (1635 cm^{-1}) functionalities. The UV spectrum showed absorption maxima at λ_{max} 220, 235, 264, and 339 nm, characteristic of 6-substituted-7-oxygenated-pyranocoumarins (Seiji *et al*., 1989).

The ^{13}C NMR (BB and DEPT) spectra of **1** showed 21 carbon signals, including five methyl, five methine, one methylene and ten quaternary carbons (Table I). The downfield signal at δ_C 164.0 and 161.4 ppm were assigned to the carbonyl carbons of the ester and α, β-unsaturated lactone moieties, respectively. The olefinic carbons of the coumarin nucleus were observed at δ_C 139.2 and 128.2 ppm. The oxygenated aromatic carbons appeared at δ_C 166.7 and 155 ppm. The signals of dimethylchromene moiety were observed at δ_C 129.4, 123.0 and 28.0 ppm (2 × Me), respectively. The signals of tri-substituted olefinic carbons at δ_C 133.2 and 121.7 ppm, methyl groups at δ_C 25.0 and 18.9 ppm together with methylene carbon at δ_C 30.2 ppm could be attributed to γ,γ-dimethylallyl moiety. In the EIMS, the γ,γ-dimethylallyl moiety was further confirmed by a base peak at *m/z* 285 $[M\text{-}C_5H_9]^+$. The signal at δ_C 56.4 ppm could be assigned to the methoxyl carbon of the methyl ester.

The ^1H NMR spectrum (Table I) showed the signals of **γ,γ**-dimethylallyl moiety at $\boldsymbol{\delta}_H$ 1.75 and 1.62 (3H each,

br. s, Me), 3.23 (d, J = 7.0 Hz, 2H) and 5.25 ppm (m, CH). The notable up-field shift of the H-4 signal to δ_H 7.29 ppm instead of 7.65–8.03 ppm and the lack of its coupling with H-3, allowed us to assign the γ,γ-dimethylallyl moiety to C-3 (*Cordova*, Garelli, 1974). It further showed characteristic signals of dimethylchromene ring [δ_H 1.38 ppm (6''-Me × 2) and olefinic protons at δ_H 5.49 and 6.41 ppm (d, J = 9.5 Hz)]. The spectrum further showed an aromatic proton as singlet at δ_H 7.02 ppm and carbomethoxy protons at δ_H 3.78 ppm. One of the major fragment at *m/z* 296 [M-59]$^+$ in EIMS resulted from the loss of methyl carboxylate group. The collective data showed close resemblance to those of previously reported 3-prenylxanthyletin (*Cordova*, Garelli, 1974), the only notable difference being the presence of methyl carboxylate group at C-8. The location of various substituents was finally confirmed by HMBC correlations (Figure 2). The presence of γ,γ-dimethylallyl moiety at C-3 could be confirmed by ^3J correlations of H-1' with C-4 (δ_C 139.2 ppm), C-2 (δ_C 161.4 ppm) and C-3' (δ_C 133.2 ppm) as well as ^2J correlations with C-3 (δ_C 128.2 ppm) and C-2' (δ_C 121.7 ppm). The attachment of 2, 2-dimethylchromene ring at C-6 and C-7 positions of the aromatic ring could be inferred by ^2J correlation of H-4'' with C-6 (δ_C 116.7 ppm) and ^3J correlations with C-7 (δ_C 158.7 ppm) and C-5 (δ_C 120.7 ppm). The presence of the methyl ester group was evident by the ^3J correlation of methoxyl protons (δ_C 3.78 ppm) with ester carbonyl (δ_C 164.0 ppm). Its presence at C-8 was authenticated by aromatic proton H-5 which showed ^3J correlations with C-4 (δ_C 139.2 ppm), C-7 (δ_C 158.7 ppm), C-10 (δ_C 155.0 ppm) and C-4'' (δ_C 123.0 ppm), revealing substitution at C-8. All these data were in complete agreement with the assigned structure of cashmirin A (**1**) as 3-(γ,γ-dimethylallyl)-6'',6''-dimethylpyrano-8-(methylcarboxylate)-8*H*-pyrano[2'',3'',7,8]-chromen-2-one.

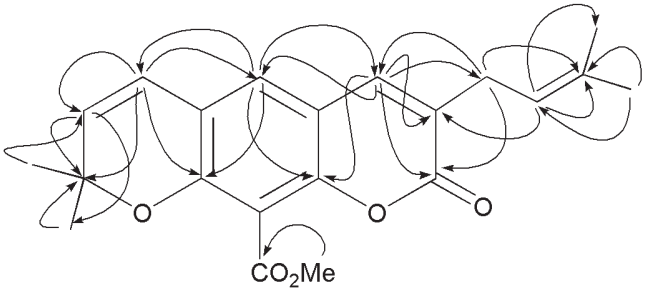

FIGURE 2 - Important HMBC correlations of cashmirin A (**1**).

Cashmirin B (**2**) was obtained as a white amorphous solid. The molecular formula was established as $C_{22}H_{24}O_6$ through HR-EIMS, showing an [M]$^+$ peak at *m/z* 384.1576 (calcd 384.1572). The IR and UV spectra were similar to that of **1**. The ^{1}H and ^{13}C NMR spectra (Table I) were also similar to those of **1** except for an additional signal due to a methoxyl group at δ_H 3.89 ppm and δ_C 62.2 ppm. The absence of aromatic signal and the downfield shift of C-5 allowed us to assign it to C-5. It was confirmed by HMBC and NOESY spectra. The methoxyl protons at δ_H 3.89 ppm showed ^3J correlation with C-5 (δ_C 151.8 ppm) as well as NOESY interactions of 5-OCH_3 with both H-4 and H-4'', respectively. The structure of cashmirin B (**2**) could thus be assigned as 5-methoxy-3-(γ,γ-dimethylallyl)-6'',6''-dimethylpyrano-8-(methylcarboxylate)-8*H*-pyrano[2'',3'',7,8]-chromen-2-one.

TABLE I - ^{1}H and ^{13}C NMR data (500 and 125 MHz) for **1** and **2** ($CDCl_3$, δ in ppm, J in Hz)

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
2	-	161.4	-	160.8
3	-	128.2	-	128.5
4	7.29 s	139.2	7.64 s	140.0
5	7.09 s	120.7	-	151.8
6	-	116.7	-	110.2
7	-	166.7	-	169.1

TABLE I - ^{1}H and ^{13}C NMR data (500 and 125 MHz) for **1** and **2** ($CDCl_3$, δ in ppm, *J* in Hz)

Position	1		2	
8	-	111.5	-	100.8
9	-	109.3	-	101.2
10	-	155.0	-	156.5
1’	3.23 d (7.0)	30.2	3.21 d (7.0)	30.4
2’	5.25 m	121.7	5.26 m	121.5
3’	-	133.2	-	132.9
4’	1.62 s	18.9	1.61 s	18.9
5’	1.75 s	25.0	1.76 s	25.5
4’’	6.41 d (9.5)	123.0	6.53 d (9.5)	118.2
5’’	5.49 d (9.5)	129.4	5.67 d (9.5)	129.7
6’’	-	75.2	-	75.2
6’’-2Me	1.38 s	28.0	1.39 s	28.0
1’’’	-	164.0	-	164.0
1’’’-OMe	3.78 s	56.4	3.79 s	56.2
5-OMe	-	-	3.89 s	62.2

The antifungal activity of both **1** and **2** was determined by the agar tube dilution method using ten pathogenic fungi. Both of these showed significant activity against *A. flavus*, *M. phaseolina*, *T. simii*, *T. schoenleinii*, and *P. boydri*; moderate activity against *A. niger*, *F. solani*, and *C. glabrata* and weak activity against *M. canis*, and *R. solani* (Table II). It is important to note that compound **2** showed slightly more potency than **1** which is probably is due to the presence of additional methoxyl group at C-5.

TABLE II - *In vitro* fungicidal bioassay of crude extract and cashmirins A (**1**) and B (**2**)

Organisms	Inhibition (%) of crude extract	Inhibition (%)		Standard drugs	Inhibition (%) of standard drugs
		1	2		
A. flavus	68	78	80	Amphotericin-B	100
A. niger	50	54	62	Miconazole	90
M. phaseolina	58	69	72	Miconazole	100
T. simii	67	80	82	Ketoconazole	100
M. canis	35	16	25	Benlate	90
T. schoenleinii	62	75	78	Ketoconazole	100
F. solani	48	57	60	Benlate	100
P. boydri	60	74	78	Nystain	90
C. glabrata	58	60	65	Miconazole	100
R. solani	24	11	17	Ketoconazole	100

The inhibitory activity of compounds **1** and **2** against Jack bean urease was determined by the method described in the experimental section. The IC_{50} values of compounds **1** and **2** were found to be 28.2±0.12 **µM** and 30.3±0.18 µM, respectively, as against IC_{50} value of 22.4±0.15 µM, which was observed for thiourea that was used as a positive control (Table III).

TABLE III - *In vitro* inhibition of urease of cashmirins A (**1**) and B (**2**)

Compound	IC_{50} ± S.E.M (µM)
1	28.2 ± 0.12
2	30.3 ± 0.18
Thiourea	22.4 ± 0.15

S.E.M.: standard error of the mean (n = 3-5).

CONCLUSION

In conclusion, two new prenylated coumarins, named as cashmirins A (**1**) and B (**2**) have been isolated from *Sorbus cashmiriana* Hedlung, Monog. along with four known compounds. Their structures were elucidated by spectroscopic techniques including MS, 1D and 2D NMR spectroscopy. Both new compounds showed significant antifungal activity against *A. flavus*, *M. phaseolina*, *T. simii*, *T. schoenleinii*, and *P. boydri*. The results showed compound **2** was slightly more antifungal than **1**. Both of these compounds also exhibited significant inhibitory activity against the enzyme urease.

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