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FLAVONOIDAL *C*-GLYCOSIDES FROM *ABUTILON PAKISTANICUM*

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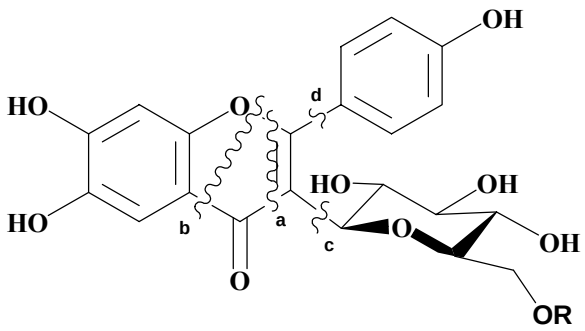
Absract - Two new flavonoidal *C*-glycosides provisionally named as pakistoside A (**1**) and pakistoside B (**2**) have been isolated from the aerial parts of *Abutilon pakistanicum.* Their structures have been assigned through detailed spectral analyses including 2D-NMR techniques. In addition kaempferol, kaempferol-3-*O*-α-L-rhamnopyranoside and luteolin are isolated for the first time from this plant.

INTRODUCTION

The genus *Abutilon* belongs to the family *Malvaceae* and it comprises of about 150 species distributed in tropical and sub-tropical regions of Asia and other parts of the world.[1,2] The species of genus *Abutilon* contain considerable amounts of mucilage due to which these find use in indigenous medicine for the treatment of rheumatism and as demulcents, emollients and diuretics.[3] The multifold uses of these species have created wide interest in their phytochemistry. From this genus the presence of sesquiterpenes, triterpenes, steroids, flavonoid aglycones and flavonoid glycosides have been reported while only triterpenes and steroids are reported from *Abutilon pakistanicum.*[4,5] Herein, we report the isolation and structure elucidation of two new flavonoidal *C*-glycosides, named as pakistoside A (**1**) and pakistoside B (**2**) along with kaempferol, kaempferol-3-*O*-α-L-rhamnopyranoside and luteolin which are isolated for the first time from this species. This is the first report of the occurrence of flavones and their glycosides from *Abutilon pakistanicum*.

RESULTS AND DISCUSSION



1 R= H

2 R= β-D-GlcP

Figure 1

The ethyl acetate soluble fraction of the methanolic extract of the aerial parts of *A. pakistanicum* was subjected to flash chromatography eluting with different mobile phases in order of increasing polarity. This resulted in the isolation of pakistoside A (**1**) and pakistoside B (**2**) along wih kaempferol, kaempferol-3-*O*-α-L-rhamnopyranoside and luteolin. The structures of compounds **1** and **2** were established by spectroscopic methods while the known compounds were identified by comparing their data with those reported in literature.[6-8]

The 5-deoxygenated flavones and isoflavones have been encountered in several plants[9-12] and the 5-deoxy compounds of kaempferol, quercetin and myricetin are also very common for example 7,4′-dihydroxyflavonol, fisetin and robinetin.[13] However, the 5-deoxy-6,7-dihydroxylated flavonols are very rare and the first member of this series, rhynchosin, was isolated from the leaves of *Rhynchosia beddomei* in 1980 by Adinarayana *et al.*[9] Herein we report two new members **1** and **2** of this class of compounds.

The compound **(1)** was isolated as a pale yellow powder. The HR FABMS (positive ion mode) provided $[M+H]^+$ at *m/z* 433.3938 indicating a molecular mass of 432 and the molecular formula $C_{21}H_{20}O_{10}$ which suggested 12 degrees of unsaturation. The UV spectrum exhibited absorptions at 350, 315, 267, 247, and 206 nm. The IR spectrum showed strong absorption bands for hydroxyl group at 3461-3261 cm^{-1}, a carbonyl group at 1678 cm^{-1} and aromatic ring at 1607, 1589 and 1501 cm^{-1}.

The EI-MS fragmentation pattern of **1** was typical of flavonoids. The cleavage "**a**" from aglycone gave fragments "i" and "ii" with additional one hydrogen atoms at *m/z* 153.0174 ($C_7H_5O_4$) and 118.0411 (C_8H_6O), respectively.[14,16] On the other hand the cleavage "**b**" yielded fragments "iii" at *m/z* 124.0146 ($C_6H_4O_3$) and fragment "iv" with additional two hydrogen atoms at *m/z* 310.1034 ($C_{15}H_{18}O_7$).[15,16] The formation of these fragments confirmed the presence of two hydroxyl groups in ring A, one hydroxyl

group in ring B and a hexose moiety in ring C. The cleavage "**c**" yielded a prominent ion "v" at *m/z* 270.0519 $[M-162]^+$ resulted from the loss of a hexose moiety.[17] The cleavage "**d**" provided fragments "vi" and "vii" at m/z 339.0520 ($C_{15}H_{15}O_9$) and 93.0334 (C_6H_5O), respectively,[16] which ultimately confirm the attachment of hexose moiety at C-3 in ring C.

Table 1. NMR data of compounds **1** and **2** in CD_3OD

Carbon Position	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
2	162.9	-	163.1	-
3	111.8	-	111.9	
4	178.7	-	178.2	
5	109.7	7.23 (1H, s)	109.9	7.19 (1H, s)
6	144.5	-	144.1	-
7	153.3	-	153.7	-
8	102.4	7.10 (1H, s)	102.9	7.12 (1H, s)
9	152.8	-	152.6	-
10	119.7	-	120.6	-
1′	121.6	-	121.3	-
2′, 6′	129.2	7.31 (2H, d, 8.5)	129.2	7.33 (2H, d, 8.1)
3′, 5′	115.7	6.91 (2H, d, 8.5)	115.9	6.95 (2H, d, 8.5)
4′	157.7	-	157.4	-
1″	75.6	4.30 (1H, d, 9.8)	75.1	4.32 (1H, d, 10.2)
2″	70.6	3.41-3.35 (4H, m)	70.2	3.45-3.31 (4H, m)
3″	78.1		79.1	
4″	71.3		70.6	
5″	78.9		80.1	
6″	61.8	4.16 (1H, d, 11.8); 3.45 (1H,	66.7	4.01 (1H, d, 11.2); 3.71 (1H,

		dd, 11.8, 4.7)		dd, 11.2, 3.7)
1‴	-	-	104.1	5.13 (1H, d, 9.5)
2‴	-	-	73.6	3.45-3.31 (4H, m)
3‴	-	-	78.9	
4‴	-	-	71.7	
5‴	-	-	78.7	
6‴	-	-	61.6	3.95 (1H, d, 11.5); 3.87 (1H, dd, 11.5, 3.7)

The NMR data of compound **1** represented a typical flavonoidal skeleton. The ^{13}C-NMR spectrum showed presence of 21 carbon atoms out of which six corresponded to a hexose moiety and the remaining 15 carbons were due to aglycone. The DEPT experiments also revealed 6 methine and 9 quaternary carbons in aglycone out of which 6 were oxygenated. The methine and methylene signals at δ_C 75.6 and 61.8 were assigned to C-1″ and C-6″ of the hexose moiety. The H-1″ proton of the hexose moiety resonated as a doublet at δ 4.30. The coupling constant J=9.8 Hz indicated axial-axial relatonship of the anomeric proton with the adjacent proton, H-2, and is in agreement with the reported values for β-glucopyranose.[17,18] On the other hand the upfield shifts of C-1″ and H-1″ as compared to those of aromatic *O*-glucopyranosides indicated that compound **1** is a *C*-glucopyranoside which was also depicted by its resistance to acid hydrolysis.[19] The H-1″ showed ^{2}J correlations with carbons at δ_C 111.8 (C-3) and 70.6 (C-2″) and ^{3}J correlations with carbons at δ_C 162.9 (C-2), 178.7 (C-4) and 78.1 (C-3″) in HMBC experiment of **1** which confirmed that **1** is the 3-*C*-glucopyranosyl flavone.[19,20]

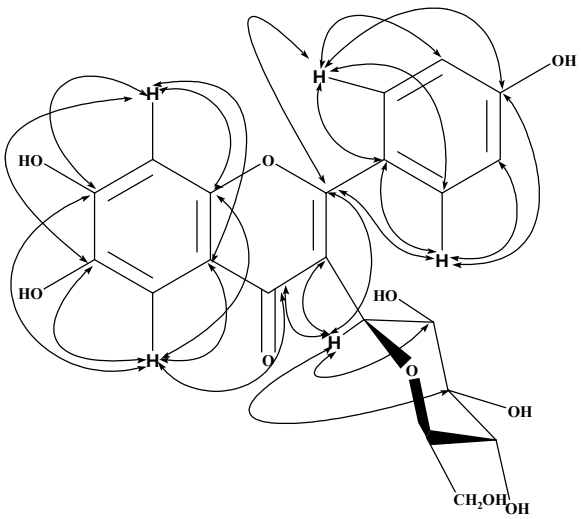


Figure 2: Important HMBC correlations of compound **1**

In the ^{1}H-NMR spectrum of **1** the signals at δ_H 7.31 (2H, d, *J*=8.5 Hz) and δ_H 6.91 (2H, d, *J*=8.5 Hz) were assigned to the flavonoidal protons H-2′,6′ and H-3′,5′, respectively, and inferred *p*-hydroxyl group in ring B. The remaining problem was the placement of two hydroxyl groups in ring A. The protons resonating at δ_H 7.23 (1H, s) and δ_H 7.10 (1H, s) were assigned to H-5 and H-8, respectively which confirmed hydroxylations at positions C-6 and C-7.[9,10] This was further confirmed by the HMBC experiments in which H-5 showed ^2J correlations with carbons at δ_C 144.5 (C-6) and 119.7 (C-10) and ^3J correlations with carbons at δ_C 153.3 (C-7), 178.7 (C-4) and 152.8 (C-9). On the other hand H-8 showed ^2J correlations with carbons at δ_C 153.3 (C-7) and 152.8 (C-9) and ^3J correlations with carbons at δ_C 144.5 (C-6) and 119.7 (C-10) only. All these evidences suggested that the structure of **1** is 2-(4′-hydroxyphenyl)-6,7-dihydroxy-3-*C*-β-glucopyranosyl-4*H*-1-benzopyran-4-one.

The compound **(2)** was isolated as a pale yellow powder. The HR FABMS (positive ion mode) provided $[M+H]^+$ at *m/z* 595.5253 indicating a molecular mass of 594 and the molecular formula $C_{27}H_{30}O_{15}$. This also suggested the presence of 13 degrees of unsaturation. The ion at *m/z* 431.0943 indicated the rapid loss of a hexose unit from the molecular ion peak. The remaining portion of mass spectrum was identical to that of compound **1** indicating that compound **2** differs from **1** only in having one additional hexose moiety. The UV spectrum exhibited absorptions at 355, 315, 277, 247 and 206 nm. The IR spectrum showed strong absorption for hydroxyl group at 3460-3250 cm^{-1}, a carbonyl group at 1684 cm^{-1} and aromatic system at 1610, 1590 and 1510 cm^{-1}. The ^{1}H-NMR spectrum of **2** showed flavonoidal protons H-2′, 6′, H-3′-5′, H-5 and H-8 at δ_H 7.33 (2H, d, *J*=8.1 Hz), 6.95 (2H, d, *J*=8.5 Hz), 7.19 (1H, s) and 7.12 (1H, s), respectively. The H-1″ appeared at δ 4.32 (1H, d, *J*=10.2 Hz) and H-1‴ appeared at δ 5.13 (1H, d, *J*=9.5 Hz) while the H_a-6″ and H_b-6″ protons resonated at δ 4.01 (1H, d, *J*=11.2 Hz, H_a-6″) and δ 3.71 (1H, dd, *J*=11.2 and 3.7 Hz, H_b-6″). On the other hand the H_a-6‴ and H_b-6‴ protons appeared at δ 3.95 (1H, d, *J*=11.5 Hz, H_b-6‴) and δ 3.87 (1H, dd, *J*=11.5 and 3.7 Hz, H_a-6‴)**.** The ^{13}C-NMR data of compound **2** was also identical to that of **1** except an additional glucose moiety in **2**. The downfield shift in the carbons C-6″ and C-1‴ which appeared at δ 66.7 and 104.1, respectively, indicated that the two glucose units are linked through (6→1) linkage. This was confirmed by the acid hydrolysis of **2** which yielded compound **1**, authenticated by its R_f value, HR EIMS and ^{1}H-NMR spectral data, and one glucose unit which was identified through coTLC with the standard and sign of its optical rotation. All these evidences concluded the structure of compound **2** as 2-(4′-hydroxyphenyl)-6,7-dihydroxy-3-*C*-[β-glucopyranosyl-(6→1)-β-glucopyranosyl]-4*H*-1-benzopyran-4-one.

For the inhibitory activity experiments both the xanthine oxidase and superoxide anion radical scavenging assays were performed for compounds **1** and **2** and the results have been summarized in Table 2.

EXPERIMENTAL

General Flash silica (230-400 mesh) was used for column chromatography. For TLC aluminum sheets precoated with silica gel 60 F_{254} (20 x 20 cm, 0.2 mm thick; E-Merck) were used. Optical rotations were measured on a JASCO DIP-360 polarimeter. IR spectra were recorded on a 460 Shimadzu spectrometer. FDMS, EIMS and HRFABMS were recorded on JMS-HX-110 with a data system on JMS-DA 500 mass spectrometers. ^{1}H and ^{13}C-NMR, DEPT and HMBC spectra were recorded on Bruker spectrometers operating at 300 MHz and 500 MHz using Me_4Si as internal standard; chemical shifts δ in ppm and coupling constants *J* in Hz.

Material The plant *Abutilon pakistanicum* was collected from the Karachi District (Sindh), Pakistan and was identified by Prof. Dr. Surraiya Khatoon, the Plant Taxonomist, Department of Botany, University of Karachi, Karachi where a voucher specimen was deposited in the Herbarium.

Extraction and Isolation The aerial parts of the freshly collected plant material (60 kg) were dried in the shade, chopped and then extracted with MeOH (3 x 50 L). The combined methanolic extract was evaporated *in vacuo* to give the residue (2.4 kg) which was partitioned with water and hexane followed by CH_2Cl_2 and EtOAc. The EtOAc layer was evaporated to give 40 g residue. The 20 g of this residue was subjected to flash chromatography and eluted with increasing polarity of solvents from hexane, CH_2Cl_2, EtOAc and EtOH. The fraction eluted with CH_2Cl_2 / EtOAc (7:3) yielded a mixture of two compounds which were resolved by TLC using the same solvent system and identified as kaempferol (45 mg) and luteolin (37 mg).[6,8] The elution with CH_2Cl_2 / EtOAc (1:1) yielded kaempferol-3-*O*-α-L-rhamnopyranoside[7] (30 mg). The elution with CH_2Cl_2 / MeOH (8:2) yielded a yellow residue which was filtered and subjected to further CC using the solvent system CH_2Cl_2 / MeOH (9:1) to give compounds **1** (20 mg) and **2** (17 mg) as yellow powders.

Pakistoside A [2-(4′-hydroxyphenyl)-6,7-dihydroxy-3-*C*-β-glucopyranosyl-4*H*-1-benzopyran-4-one] (1) Pale yellow powder. $[\alpha]^{26}_{D}$-17.3 (c 0.15, MeOH). UV λ_{max}^{MeOH} nm: 350, 315, 267, 247, and 206. IR ν_{max}^{KBr} cm^{-1}: 3461-3261, 1678, 1607 , 1589, 1501. ^{1}H-NMR (300 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) *see Table 1.*

FDMS 432. HRFAB-MS *m/z* 433.3938 $[M+H]^+$, ($C_{21}H_{21}O_{10}$) (Calcd for $C_{21}H_{21}O_{10}$:433.3846). HR EIMS 432.3614 (M^+) (Calcd for $C_{21}H_{20}O_{10}$:432.3767), 404.0882 (M^+-CO), 403.0822 (M^+-CHO), 388.0664 (M^+-CO_2), 339.0520 (M^+-C_6H_5O), 310.1034 (M^+-$C_6H_2O_3$), 270.0519 (M^+-$C_6H_{10}O_5$), 269.0439 (M^+-$C_6H_{11}O_5$), 163.0387 (M^+- $C_{15}H_9O_5$), 153.0174 (M^+-$C_{14}H_{15}O_6$), 124.0146 (M^+-$C_{15}H_{16}O_7$), 118.0411 (M^+-$C_{13}H_{14}O_9$), 93.0334 (M^+- $C_{15}H_{15}O_9$).

Pakistoside B [2-(4′-hydroxyphenyl)-6,7-dihydroxy-3-*C*-β-glucopyranosyl-(6→1)-β-glucopyranosyl -4*H*-1-benzopyran-4-one] (2) Pale yellow powder. $[\alpha]^{23}_{D}$ -11.5 (c || 0.19, MeOH). UV λ_{max}^{MeOH} nm: 355, 315, 277, 247, 206. IR ν_{max}^{KBr} cm^{-1}: 3460-3250, 1684, 1610, 1590, 1510. ^{1}H-NMR (300 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) *see Table 1*. FDMS 594. HRFAB-MS *m/z* 595.5253 $[M+H]^+$ ($C_{27}H_{31}O_{15}$) (Calcd for $C_{27}H_{31}O_{15}$:595.5148), 431.0943 (M^+-$C_6H_{11}O_5$). HR EIMS 431.0781 (M^+-$C_6H_{11}O_5$), 402.0713 (M^+- $C_7H_{12}O_6$), 387.0803 (M^+- $C_7H_{11}O_7$), 269.0435 (M^+-$C_{12}H_{21}O_{10}$), 153.0203 (M^+-$C_{20}H_{25}O_{11}$), 124.0139 (M^+-$C_{21}H_{26}O_{12}$),118.0395 (M^+-$C_{19}H_{24}O_{14}$), 93.0347 (M^+- $C_{21}H_{25}O_{14}$).

Hydrolysis of Pakistoside B (2) and Detection of the Sugar Moiety. The compound **2** (7 mg) was refluxed with 18 % HCl (5 ml) for 2.5 h. The reaction mixture afforded glucose and compound **I** in 1:1 ratio.

Xanthine oxidase inhibition assay The xanthine oxidase (XO) inhibition activity was assayed in phosphate buffer (0.1 M, pH 7.5). XO (0.003 unit / well), 20 μL and various concentrations of test samples in DMSO (10 μL) were mixed in 96-well microplate and pre-incubated for 10 min at rt. The reaction was initiated by adding 20 μL of 0.1 mM of xanthine and uric acid formation was measured spectrophotometrically at 295 nm by using Molecular Devices, Spectramax 384.[21]

Superoxide anion radical scavenging assay The reaction mixture contained 280 μM β nicotinamide adenine dinucleotide reduced form (NADH), 80 μM nitro blue tetrazolium (NBT), 8 μM phenazine methosulphate (PMS) and test samples in 200 μL of 0.1 M phosphate buffer (pH 7.5). The NBT, NADH and PMS were prepared in the same buffer. Test samples were dissolved in DMSO. The reaction was performed in 96-well microtitre plates (Molecular Devices, Spectramax 340) at rt and absorbance was measured at 560 nm.[22] The percent radical scavenging activity by samples was determined in comparison with a DMSO treated control group. The % radical scavenging activity was calculated by using the formula:

% RSA = 100 – [(OD test compound / OD control) X 100]

Table 2. Antioxidant activities of compounds **1** and **2**

	Xanthine Oxidase Assay	Superoxide Scavenging Assay	
Sample	IC_{50} μ M	% Radical Scavenging Activity	IC_{50} μ M

pakistoside A	430.70	85.41	791.47
pakistoside B	129.54	45.63	-
allopurinol	7.02	-	-
propyl galate	-	90.01	106±5.0

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