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Flavonoid glycosides from *Salvia moorcroftiana* Wall.

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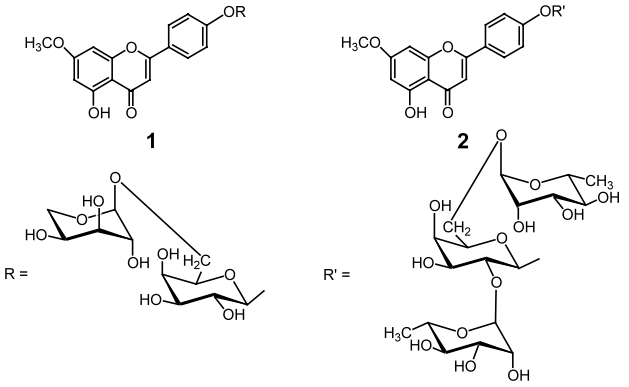
Abstract

Phytochemical analysis of the whole plant of *Salvia moorcroftiana* Wall. (Lamiaceae) resulted in the isolation of two new flavonoid glycosides, together with three known compounds. The structures of the new compounds were established as genkwanin 4′-*O*-α-L-arabinopyranosyl-(1→6)-β-D-galactopyranoside (**1**) and genkwanin 4′-*O*-{α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside} (**2**). The structures of all compounds were elucidated by spectroscopic methods, including 2D NMR techniques. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Salvia moorcroftiana*; Lamiaceae; Flavonoid glycosides; 1D and 2D NMR spectroscopy

1. Introduction

Salvia moorcroftiana Wall., commonly known as 'kallijari',[1] belongs to the family Lamiaceaea, members of which are well known for their medicinal properties. Some of their constituents show antitumor activity.[2] The seeds and roots of *S. moorcroftiana* Wall. are used as emetics, antitumor agents and also as a treatment for hemorrhoids.[3,4] The leaves serve as agents against the guinea worm and itch, and in the form of a poultice they are applied to wounds. Previous phytochemical investigations of this plant revealed the presence of diterpenoids[4–7] and flavonoids.[8] In this paper we wish to describe the isolation and characterization of two new flavanoid glycosides **1** and **2**, along with three known compounds, apigenin,[8] apigenin 7-*O*-dirhamnoside,[9] and luteolin 3′-*O*-glucoside.[10] The later two compounds have been isolated for the first time from our source.



2. Results and discussion

The BuOH-soluble fraction (see Section 3) yielded compound **1**, mp 208–211 °C, which was recognized as a flavonoid glycoside from its positive reactions with the Molish and Shinoda reagents.[11] The UV spectrum combined with the ^{1}H and ^{13}C NMR data indicated the flavone skeleton with oxy-substituents at C-7 and C-4′ and a free OH group at C-5.[12,13] Its IR spectrum (KBr) exhibited absorptions at ν_{max} 3510–3485 (OH), 2950 (CH), 2868 (C=C), 1690 (C=O), 1130–1015 (O-glycosidic linkage) and 825 cm^{-1}. The positive-ion FAB mass spectrum exhibited the protonated molecular ion

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peak at m/z 579 $[M+H]^+$. The fragment ions at m/z 447 $[(M+H)-Ara]^+$ and 285 $[(M+H)-(Gal+Ara)]^+$ confirm the presence of two sugar moieties in the molecule.

The ^{13}C NMR spectrum of **1** showed the presence of 25 signals, (two of which represented two carbons each), which were resolved through DEPT experiments as one methyl, two methylene, 16 methine and eight quaternary carbons. The ^1H NMR spectrum showed two pair of doublets at δ 8.01(2 H, J 7.5 Hz) and δ 7.20 (2 H, J 7.5 Hz), which were assigned to H-2′/H-6′ and H-3′/H-5′ due to their HMBC interactions with δ 164.1 (C-2)/161.7 (C-4′) and δ 122.1 (C-1′), respectively.[12,14] The same value coupling constant (7.5 Hz) and mutual interaction of these protons in the COSY-45° experiment confirmed their ortho position in ring B.[9] In the same spectrum, a singlet which integrated for one proton at δ 6.92, is assigned to H-3.[12]

The ^1H NMR spectrum also had a pair of doublets at δ 6.40 and 6.80 (J 2.0 Hz), each with a one-proton integration that is correlated to carbons at δ 99.3 and 94.0, respectively, in the HMQC spectrum. These chemical shifts are characteristic values for C-6 and C-8 in ring A of the flavonoids, provided C-5, C-7, and C-9 have a direct link with the oxygen atoms.[9,14] The downfield chemical shift of the C-4 carbonyl (δ 182.5) in the ^{13}C NMR spectrum indicated the presence of a chelated hydroxyl functionality at C-5, which was also in accordance to the chemical shift of C-5. A signal, which integrated for three protons at δ 3.80 and its respective carbon in the HMQC spectrum at δ 55.0, showed the presence of only one methoxyl group in the molecule, which is attached to a quaternary carbon at δ 163.5. This value is assigned to C-7 due to its HMBC interaction with the H-6 and H-8 protons.

The presence of two doublets in the ^1H NMR spectrum of **1** at δ 5.45 (d, J 7.8 Hz) and 5.20 (d, J 3.1 Hz), and their correlated carbons in the ^{13}C NMR spectrum at δ 102.2 and 106.8 indicated the presence of two sugar moieties in the compound. The 1.9 ppm upfield shift of C-4′ in the ^{13}C NMR spectrum indicated the attachment of sugar residue at C-4′ instead of a free hydroxyl group, as the presence of the methoxy group has already been confirmed at C-7.[15] This was further confirmed by the HMBC spectrum in which the anomeric proton of the galactopyranosyl (H-1″, δ 5.45) showed the connectivity with C-4′ (δ 161.7). The presence of 11 carbons in the ^{13}C NMR spectrum, together with 19 protons in the ^1H NMR spectrum, showed the presence of one pentose and one hexose sugar in the molecule, Further, it was also clearly evident by the loss of 132 amu from the molecular-ion peak, and then the loss of 162 amu from the resulting fragment in the positive-ion FAB mass spectrum, that the pentose sugar was attached to the hexose, which itself is attached to the aglycone.[16] The assignment of the various sugar protons were made by their spin-pattern analysis, COSY 45° and HMQC experiments. The two sugar moieties were indicated to be the arabinopyranose and galactopyranose by comparing their chemical shifts in the ^{13}C NMR values with the reference data.[17]

The fact that two anomeric protons signals in the ^1H NMR spectrum appeared at δ 5.45 (J 7.8 Hz) and 5.20 (J 3.1 Hz) indicated that L-arabinose is attached to D-galactose through an α linkage, and D-galactose is linked with the aglycone through a β linkage.[18–20] The absolute configurations of galactose and arabinose were assumed to be D and L, respectively, because only these forms are found in flavone glycosides.[21] The downfield shift of the methylene group in galactose by about 5 ppm indicated the attachment of rhamnose at C-6 of the hexose.[12,15] This was also confirmed by the HMBC interaction of the anomeric proton of arabinose at δ 5.20 (J 3.1 Hz) to C-6 (δ 67.6) of galactose. A literature search of the ^{13}C NMR chemical shifts of glycosides containing terminal arabinose moieties revealed that the values observed for **1** are close to those reported for α-L-arabinopyranoside,[22] but not to those published for α-L-arabinofuranoside.[23] Therefore, arabinose is present in the pyranose form.

Acid hydrolysis of compound **1** yielded arabinose and galactose and yellow needles of the aglycone **3** (mp 286 °C), and was characterized as genkawanin by comparison of its spectral data with that reported in the literature.[15,24] The sugars were further confirmed as galactose and arabinose by co-TLC with authentic samples using different solvent systems, and their D and L configuration was proved by GLC analysis of the thiazolidine derivatives.[25]

Enzymatic hydrolysis of compound **1** with takadiastase yielded L-arabinose and the proaglycone, 5,4′-dihydroxy-7-methoxyflavone 4′-*O*-β-D-galactopyranoside. Further hydrolysis with almond emulsin yielded D-galactose and the aglycone. All the assignments were confirmed by HMQC and $^3J_{H,C}$ interactions in the HMBC spectrum. These results confirmed the structure of compound **1** as genkwanin 4′-*O*-α-L-(arabinopyranosyl-(1→6)-β-D-galactopyranoside).

Compound **2** appeared as a dark purple spot under UV light, which was unchanged after exposure with ammonia fumes. Its FAB mass spectrum shown to have a molecular ion at m/z 739 $[M+H]$, corresponding to the molecular formula $C_{34}H_{42}O_{18}$ by ^{13}C NMR with DEPT.

The UV spectrum of **2** is characteristic for the flavones having substituted C-7/C-4′ and a free hydroxyl group at C-5.[12,13] In the ^1H and ^{13}C NMR spectral data of **2**, the signals due to aglycone are similar to that of compound **1**, although the signals due to the sugar moiety were not identical. Acid hydrolysis of **2** yielded genkawanin as the aglycone,[15,22] and galactose and rhamnose as the carbohydrate components.

The sugars were confirmed by co-TLC with authentic samples using different solvent systems and by GLC analysis of the thiazolidine derivatives, which proved the absolute configurations of D-galactose and L-rhamnose,[25] which is also exclusively found in flavone glycosides.[21]

The diagnostic resonances for the anomeric protons and carbons at δ 5.56 (J 8.0 Hz), 5.45 (J 3.5 Hz), 5.42 (J 3.0 Hz), and their correlated carbons at δ 99.5, 100.7 and 100.3 in the ^{1}H and ^{13}C NMR spectra, combined with other resonances for sugar protons and carbons, inferred the presence of branched triglycoside moieties in **2**, including two 6-deoxy sugars. The β and α orientation of the anomeric center of D-galactose and L-rhamnose was supported by relatively large and small J values of anomeric protons (7.5 for galactose, 3.5 and 3.0 for rhamnose), whereas the α orientation of the L-rhamnopyranosyl residues was further supported by their ^{13}C NMR shifts of C-3 and C-5.[18,19]

The fragments in the positive FABMS at m/z 593 $[(M+H)-Rha]^+$ 447 $[(M+H)-(2\times Rha)]^+$ and 285 $[(M+H)-(2\times Rha+Gal)]^+$ indicated the attachment of two rhamnoses to galactose and the attachment of galactose to the aglycone. The downfield chemical shifts of C-2″ and C-6″ and slight upfield shift of C-1′ and C-5′ of galactopyranosyl confirmed the site of attachment of the rhamnoses to the galactose.[15] In the HMBC spectrum, correlation peaks were observed δ 5.45 (H-1‴ of rhamnosyl) to δ 75.6 (C-2″ of substituted galactosyl) and δ 5.42 (H-1⁗ of rhamnosyl) to δ 66.4 (C-6″ of substituted galactosyl). Thus the rhamnosyl-(1→2)-[rhamnosyl-(1→6)]-galactosyl structure was revealed. The position of attachment of branched triglycoside moiety with the aglycone was confirmed by the ^3J interactions of δ 5.56 (H-1″ of substituted galactosyl) to δ 161.9 (C-4′ of the aglycone), which was further supported by an upfield shift of 1.7 ppm for the C-4′, which is analogous to that reported when a 4′-hydroxy group is glycosylated.[12,15] All these cumulative results confirmed the structure of compound **2** as genkwanin 4′-*O*-{α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside}.

3. Experimental

General.—Fast-atom bombardment mass spectra (FABMS) were recorded on a double-focusing Varian MAT-312 spectrometer in a positive-ion mode using lactic acid as the matrix. The IR and UV spectra were recorded on a Shimadzu IR-46 and Shimadzu UV-240, respectively. TLC was carried out on E. Merck silica gel plates using the indicated solvents: BAW = 12:3:5 butanol–AcOH–water; BEW = 12:3:5 butanol–EtOH–water. Purities of the compounds were checked by HPLC on a Zorbax ODS C_{18} (25 × 4.6 mm) column at flow rate of 1.7 mL min^{-1}, with UV detection at 280 nm, using an isocratic system of 20:4:1 MeOH–water–HOAc. The ^{1}H and ^{13}C NMR spectra were recorded in Me_2SO-d_6 for compound **1** and **2** and in CD_3OD for compound **3** at 500 and 125 MHz, respectively, using a Bruker AM 500, spectrometer. GLC was carried out on a GC-18A equipped with FID.

Collection, identification and extraction.—The plant material (whole parts, 6 kg) was collected from Manshera, Peshawar, Pakistan in June 2000 and identified by Dr Abdd-ur-Rashid (Department of Botany, University of Peshawar, Peshawar, Pakistan). A voucher specimen (no. 854) was deposited in the herbarium of the same department. The shade-dried plant material was extracted repeatedly with MeOH at rt. The combined methanolic extracts were evaporated in vacuo to afford a gummy residue (345 g). This residue was partitioned between water and hexane. The aqueous layer was then extracted 3× each with EtOAc (118 g) and then with BuOH (76.8 g).

Isolation, purification and characterization.—The BuOH extract was subjected to column chromatography (CC) on silica gel using a gradient of MeOH in $CHCl_3$. The fractions eluted with 20–25% $CHCl_3$ in MeOH that contained compound **1** were combined, applied to a Sephadex LH-20 column, and eluted with 70% MeOH and allowed to stand at 5 °C for 2 days. Finally compound **1** was obtained as yellowish powder (10.5 mg).

Compound **1**: mp 208–211 °C; R_f 0.70 (BAW), 0.59 (BEW), 0.41 (15:85 HOAc–water); UV λ_{max} (MeOH): 269, and 320; +NaOMe 287, and 360; +$AlCl_3$·HCl 279, 297, 333, and 380; +NaOAc 270, and 314; +NaOAc–H_3BO_3, 269, and 320 nm; IR ν_{max} (KBr): 3510–3485 (OH), 2950 (CH), 2868 (C=C), 1690 (C=O), 1130–1015 (O-glycosidic linkage), and 825 cm^{-1}; FABMS: m/z 579 $[M+H]^+$, 447 $[(M+H)-Ara]^+$, and 285 $[(M+H)-(Gal+Ara)]^+$; HRMS: m/z 578.5191 (Calcd m/z 578.5198 for $C_{27}H_{30}O_{14}$); ^{1}H NMR (Me_2SO-d_6, 500 MHz): δ 6.92 (s, 1 H, H-3), 6.40 (d, 1 H, J 2 Hz, H-6), 6.80 (d, 1 H, J 2 Hz, H-8), 8.01 (d, 2 H, J 7.5 Hz, H-2′,6′), 7.20 (d, 2 H, J 7.5 Hz, H-3′,5′), 5.45 (d, 1 H, J 7.8 Hz, H-1″), 5.20 (d, 1 H, J 3.1 Hz, H-1‴), 3.80 (s, 3 H, OMe); for ^{13}C NMR data, see Table 1.

The fraction containing compound **2** was eluted from the main column with BuOH with 30% MeOH in $CHCl_3$. This fraction was further subjected to a Sephadex LH-20 column and eluted with 65% MeOH. Finally, compound **2** was purified using preparative TLC plates, and a purple band under UV was scratched to yield compound **2** (9.32 mg).

Compound **2**: mp 228–230 °C; R_f 0.54 (BAW), 0.41 (BEW), 0.36 (15:85 HOAc–water); UV λ_{max} (MeOH): 270, and 320; +NaOMe 285, and 358; +$AlCl_3$·HCl 275, 299, 336, and 380; +NaOAc 271, and 316;

+ NaOAc–H_3BO_3, 270, and 320 nm; IR ν_{max} (KBr): 3510–3470 (OH), 2955 (CH), 2875 (C=C), 1695 (C=O), 1130–1010 (O-glycosidic linkage), and 825 cm^{-1}; FABMS: m/z 739 $[M+H]^+$, 593 $[(M+H)-Rha]^+$ 447 $[(M+H)-2\times Rha]^+$, and 285 $[(M+H)-(2\times Rha+Gal)]^+$; HRMS: m/z 738.6870 (Calcd m/z: 738.6878 for $C_{34}H_{42}O_{18}$); ^1H NMR (Me_2SO-d_6, 500 MHz): δ 6.93 (1 H, s, H-3), 6.45 (d, 1 H, J 2 Hz, H-6), 6.85 (d, 1 H, J 2.2 Hz, H-8), 8.04 (d, 2 H, J 7.9 Hz, H-2′,6′), 7.18 (d, 2 H, J 7.9 Hz, H-3′,5′), 5.56 (d, 1 H, J 8.0 Hz, H-1″), 5.45 (d, 1 H, J 3.5 Hz, H-1‴), 5.42 (d, 1 H, J 3.0 Hz, H-1⁗), 1.05 (d, 3 H, J 5.8 Hz, Rha-Me‴), 0.89 (d, 3 H, J 5.5 Hz, Rha-Me⁗), 3.85 (s, 3 H, OMe); for ^{13}C NMR data, see Table 1.

Table 1
^{13}C NMR spectral data for compounds **1**–**3** [a]

Carbon no.	DEPT	δ C (compound **1**)	δ C (compound **2**)	δ C (compound **3**)
2	C	164.1	164.0	164.3
3	CH	103.9	103.5	103.4
4	C	182.5	182.6	182.3
5	C	161.2	161.5	159.7
6	CH	99.3	99.9	99.0
7	C	163.5	163.8	164.0
8	CH	94.0	94.5	93.9
9	C	157.2	157.9	158.2
10	C	104.9	105.1	105.0
1′	C	122.1	122.5	121.8
2′	CH	128.6	128.1	128.5
3′	CH	115.1	115.6	116.1
4′	C	161.7	161.9	163.6
5′	CH	115.1	115.6	116.1
6′	CH	128.6	128.1	128.5
	OMe	55.0	56.2	55.6
Gal				
1″	CH	102.2	99.5	
2″	CH	71.5	75.6	
3″	CH	73.6	73.7	
4″	CH	68.4	68.3	
5″	CH	73.9	74.1	
6″	CH_2	67.6	66.4	
Ara				
1‴	CH	106.8		
2‴	CH	72.3		
3‴	CH	74.4		
4‴	CH	69.1		
5‴	CH_2	66.5		
Rha-1				
1‴	CH		100.7	
2‴	CH		70.9	
3‴	CH		70.8	
4‴	CH		72.3	
5‴	CH		68.8	
6‴	Me		17.5	
Rha-2				
1⁗	CH		100.3	
2⁗	CH		70.9	
3⁗	CH		70.5	
4⁗	CH		72.3	
5⁗	CH		68.3	
6⁗	Me		17.7	

[a] δ units in ppm downfield from internal Me_4Si in Me_2SO-d_6.

Compound **3**, genkwanin (5,4′-dihydroxy-7-methoxyflavone): yellow needles; mp 285–286 °C; R_f 0.24 (3:7 HOAc–water), 0.98 (4:1:2 EtOAc–HOAc–water, organic phase), HPLC: R_t 39.3 spot appearance, dark (UV), yellow–green (UV/NH_3), UV λ_{max} (MeOH): 267, 300 sh, and 333; + NaOMe 273, 300 sh, and 385; + $AlCl_3$, 275, 301, 342, and 382 sh; + NaOAc 267, 299 sh, 369, and 380; + H_3BO_3, 267, 299 sh, and 337 nm; EIMS (70 eV): m/z 284 $[M]^+$, 256, 25, 166, 121, 118; HRMS: m/z 284.2663 (Calcd m/z: 284.2658 for $C_{16}H_{12}O_5$); ^{1}H NMR (Me_2SO-d_6, 500 MHz): δ 6.82 (s, 1 H, H-3), 6.40 (d, 1 H, J 2 Hz, H-6), 6.81 (d, 1 H, J 2.0 Hz, H-8), 7.99 (d, 2 H, J 8.0 Hz, H-2′,6′), 7.05 (d, 2 H, J 8.0 Hz, H-3′,5′). 3.85 (s, 3 H, 7-OMe).

Acid hydrolysis of compounds **1** *and* **2**.—Compounds **1** and **2** (5 mg each) were refluxed with 10% aq HCl for 3 h at 100 °C. On cooling, the aglycone was recrystallized from $CHCl_3$ and identified as genkwanin by comparison of its spectral data with that of an authentic specimen.[15,22] The aqueous hydrolyzate was neutralized with silver carbonate and concentrated; the sugars were found to be galactose, arabinose, and rhamnose by co-TLC with authentic sugars using different solvent systems. The concentrated residue was further treated with L-cysteine methyl ester hydrochloride (1 mg) in pyridine (0.125 mL) at 60 °C for 1 h. The solution was then treated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.05 mL) at 60 °C for 1 h. The supernatant was applied to GLC; GLC conditions: column, Supelco SPB™-1, 30 m × 0.25 mm; column temperature, 230 °C; N_2 flow rate, 0.8 mL min^{-1}; t_R of derivatives, D-galactose 11.09 min (L-galactose 11.89 min), L-rhamnose 9.41 min (D-rhamnose 9.72 min), L-arabinose 7.79 min (D-arabinose 8.40 min).

Enzymatic hydrolysis of compounds **1** *and* **2**.—Compounds **1** and **2** on enzymatic hydrolysis with takadiastase yielded a proaglycone plus arabinose and rhamnose, indicating that the arabinose (for compound **1**) and rhamnose (for compound **2**) were linked to galactose through α linkages. The proaglycone on further hydrolysis with enzyme almond emulsin yielded galactose and the aglycone.

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