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The active constituents from Gualou-xiebai-baijiu-tang part I: Active saponins

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THE ACTIVE CONSTITUENTS FROM *GUALOU-XIEBAI-BAIJIU-TANG* PART I: ACTIVE SAPONINS

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Two new steroidal saponins and a new triterpenoidal saponin, together with nine known steroidal saponins, were isolated from "Gualou-xiebai-baijiu-tang" consisting of *Fructus trichosanthis* and *Bulbus allii macrostemi*. The structures of the three new compounds were determined as 3-*O*-β-D-galactopyranosyl-hederagenin 28-*O*-β-D-xylopyranosyl (1 → 6)-β-D-galactopyranosyl ester (**1**), spirost 25(27)-ene-2β,3β-diol-3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-galactopyranoside (**2**) and 26-*O*-β-D-glucopyranosyl-22α-hydroxy-5β-furost-25(27)-ene-1β,3β,6β,26-tetraol-3-*O*-β-D-galactopyranoside (**3**), respectively, by means of chemical evidences and spectral analysis.

Keywords: Gualou-xiebai-baijiu-tang; *Trichosanthes kirilowii* Maxim; *Allium macrostemon* Bge; Triterpenoidal saponin; Steroidal saponins

INTRODUCTION

"Gualou-xiebai-baijiu-tang" is a well-known classic recipe in Traditional Chinese Medicinal Sciences. This prescription consists of *Fructus trichosanthis* and *Bulbus allii macrostemi* extracted with wine. It has been used for the treatment of coronary heart disease and angina pectoris for several hundreds years and got very eminent effects in modern clinic [1]. So far research about the prescription was focused on studying its pharmacological activities and the chemical constituents of *Trichosanthes kirilowii* Maxim. and *Allium macrostemon* Bge. [2,3]. There were no reports on the constituents of the whole prescription yet. Our research was focused on elucidating the effective principles and mechanism of actions of the prescription. A new triterpenoidal saponin (**1**) and two new steroidal saponins (**2–3**), together with nine known steroidal saponins (**4–12**), were isolated by bioactivity-guided isolation from the prescription. The present paper describes the isolation, structure determination of three novel compounds.

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TABLE I The assignment of carbon and proton signals of compound **1**

No.	^{13}C	^{1}H	No.	^{13}C	^{1}H
1	38.9	1.57(H,m) 1.04(H,m)	C-3 Gal-1	106.6	4.98(H, d, $J = 7.2$ Hz)
2	26.1	2.25(H,m) 2.02(H,m)	2	71.0	4.27(H, m)
3	82.0	4.23(H,m)	3	74.8	4.06(H, m)
4	42.2		4	69.5	4.21(H, m)
5	47.7	1.67(H,m)	5	78.0	4.10(H, m)
6	18.2	1.66(2H,m)	6	62.7	4.47(H, dd, $J = 11.4, 2.4$ Hz) 4.32(H,m)
7	34.0	1.30(H, m) 1.11(H, m)	C-28 Gal-1	95.7	6.25(H, d, $J = 7.8$ Hz)
8	40.0		2	71.6	4.20(H,m)
9	48.2	1.77(H,m)	3	73.9	4.11(H,m)
10	37.0		4	69.6	4.33(H,m)
11	23.4	1.90(2H,m)	5	78.7	4.21(H,m)
12	122.9	5.42(H. t, $J = 3.0$ Hz)	6	69.6	4.25(2H,m)
13	144.2		Xyl-1	105.3	5.03(H, d, $J = 7.8$ Hz)
14	43.5		2	75.2	4.02(H, t, $J = 8.4$ Hz)
15	28.3	2.30(H, m) 1.08 (H,m)	3	78.4	3.88(H,m)
16	23.7	1.98(2H,m)	4	71.0	4.28(H,m)
17	47.0		5	67.0	4.27(H,m) 3.73(H, d, $J = 10.8$ Hz)
18	41.7	3.19(H, dd, $J = 13.8, 4.2$ Hz)			
19	46.2	1.71(H,m) 1.22(H, dd, $J = 13.8$ MHz)			
20	30.7				
21	32.9	1.61(H, m) 1.30(H, m)			
22	32.6	1.88(H, m)1.75(H, m)			
23	64.6	4.21(H, m) 3.70(H, m)			
24	13.6	0.94(3H, s)			
25	16.3	0.99(3H, s)			
26	17.6	1.13(3H, s)			
27	26.1	1.18(3H, s)			
28	176.6				
29	23.1	0.88(3H, s)			
30	23.9	0.87(3H, s)			

All spectra were recorded on INOVA 600 MHz NMR spectrometer; The signals of carbon and proton were unambiguously assigned through HMQC, DQCOSY, TOCSY and HMBC.

RESULTS AND DISCUSSION

The prescription was extracted with 60% EtOH and the extract was sequentially partitioned with EtOAc and *n*-BuOH. The saponins were isolated through repeated chromatography.

Compound **1**, a colorless amorphous powder, m.p. 276–277°C (uncorrected), showed a quasimolecular ion peak at *m/z* 951 $[M + Na]^+$ in the positive FAB-MS. A molecular formula of $C_{47}H_{76}O_{18}$ can be settled by FAB-MS as well as ^{13}C and ^{1}HNMR data. The IR spectrum showed the presence of an ester carbonyl group at 1724.0 cm^{-1} and an olefinic group at 1629.6 cm^{-1}.

The ^{1}HNMR spectrum revealed signals due to six tertiary methyl groups (δ 0.87, 0.88, 0.94, 0.99, 1.13, 1.18), one trisubstituted olefinic proton (δ 5.42, t, $J = 3.0$ Hz) and three anomeric protons (δ 4.98, 5.03 and 6.25). The ^{13}CNMR spectrum showed signals of a pair of olefinic carbons at δ 122.9 and 144.2, three anomeric carbons at δ 95.7, 105.3 and 106.6, and a carbonyl carbon at δ 176.6. All these data suggested that **1** was a triterpenoidal saponin related to oleanolic acid. Comparison of the signals from the aglycone of **1** in the ^{13}CNMR spectrum with the literature [4,5] showed that the aglycone of **1** was hederagenin.

On acid hydrolysis, xylose and galactose were identified by co-chromatography on HPTLC with authentic samples. According to FAB-MS fragments, it can be concluded that the ratio of galactose and xylose in the molecule of **1** was 2:1. Hydrolysis of **1** with ammonia at room temperature for 10 h, xylose and galactose were examined on HPTLC, suggesting there were xylose and galactose linked at C-28 as a sugar linkage. The β-form anomeric configurations for the galactoses and xylose were judged from their $^{3}J_{H1,H2}$ coupling constants ($J = 7.2$, 7.8 and 7.8 Hz, respectively). The spin systems for sugars were assigned

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TABLE II The assignment of carbon signals of compound **2** and **3**

No.	**2**	Samogenin	**3**	No.	**2**	**3**
1	40.2	39.2	72.2	C3 Gal-1	103.4	104.7
2	67.2	70.2	28.3	2	81.8	71.5
3	82.0	67.5	74.9	3	75.2	74.9
4	31.8	33.5	27.6	4	69.8	71.5
5	36.6	36.1	34.1	5	77.0	75.2
6	26.3	26.3	66.8	6	62.0	63.7
7	26.8	26.8	43.1	Glc-1	106.3	
8	35.6	35.7	35.0	2	75.2	
9	40.6	41.5	41.6	3	78.6	
10	37.1	37.0	41.0	4	71.7	
11	21.3	21.3	21.2	5	78.0	
12	40.8	40.4	40.0	6	62.8	
13	41.4	40.9	43.1			
14	56.3	56.5	55.9	26-Glc-1		103.7
15	32.1	32.1	32.3	2		73.7
16	81.6	81.1	81.0	3		78.3
17	63.1	63.1	64.5	4		71.5
18	16.6	16.6	16.6	5		78.3
19	23.9	24.1	19.8	6		62.5
20	41.9	42.0	41.6			
21	15.0	14.9	16.2			
22	109.4	109.1	110.2			
23	29.0	31.8	28.3			
24	33.3	29.2	35.5			
25	144.5	30.6	147.1			
26	65.0	66.8	72.0			
27	108.7	17.3	110.7			

All spectra were recorded on 500 MHz NMR in pyridine-d_5; The signals of carbon were unambiguously assigned through DEPT, HMQC, COSY, and HMBC.

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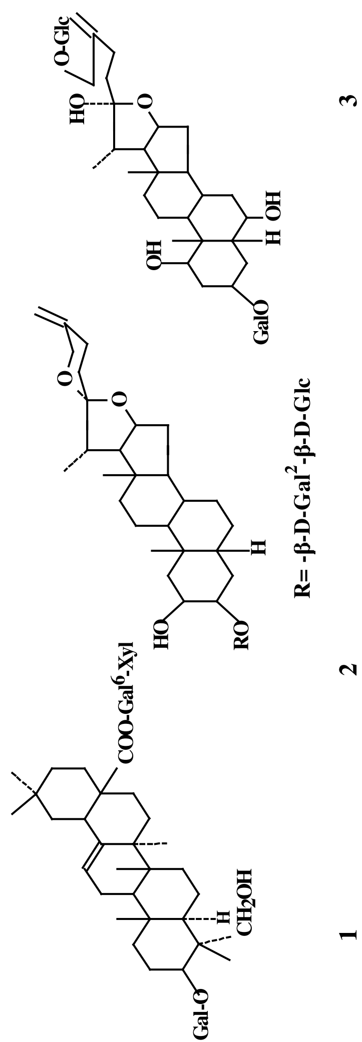

FIGURE 1 The structures of Compound **1**, **2** and **3**.

on the basis of spectroscopic evidences obtained by DQCOSY, HMQC and TOCSY experiments. The sugar linkages were determined on the basis of HMBC spectrum. Namely, a cross peak of long-range correlations was observed between a proton signal at δ 6.25 (Gal-H-1) and a carbonyl carbon signal at 176.6 (C-28), whereas a proton signal at δ 5.03 (Xyl-H-1′) correlated with carbon signal at 69.6 (Gal-C-6). A proton signal at δ 4.98 (Gal-H-1″) had a cross peak with carbon signal at 82.0 (C-3) in HMBC spectrum. All these evidences indicated that the two sugar moieties of **1** were linked at both C-3 and C-28 of oleanolic acid. The relative configuration of **1** was confirmed by NOESY spectrum. Furthermore, the characteristic ion peaks at *m/z* 929$[M + H]^+$, 796$[\text{M-pentose}]^+$, 766$[\text{M-hexose}]^+$, 617$[\text{M-pentose-hexose-}H_2O + H]^+$, 472$[\text{aglycone}]^+$ and 437$[\text{aglycone-}2{\cdot}H_2O + H]^+$ in the positive ion FAB-MS confirmed the sugar linkages mentioned above.

From the above evidences, the structure of **1** was concluded to be 3-*O*-β-D-galactopyranosyl-hederagenin 28-*O*-β-D-xylopyranosyl (1 → 6)-β-D-galactopyranosyl ester.

Compound **2**, obtained as an amorphous powder, m.p. 265–266°C (uncorrected), which was shown to have the molecular formula $C_{39}H_{62}O_{14}$ by HR-FAB-MS (negative mode) ($m/z = 753.4084[M-H]^-$, calcd. 753.4061). The IR spectrum showed a strong absorption at 3425.4 cm^{-1} due to hydroxyl groups and an olefinic group at 1629.0 cm^{-1}, but lacked the characteristic bands of the spirostanol rings just like tupichigenin B [6]. It was positive to anisaldehyde and Molish reaction, but negative to Ehrlish reagent.

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The ^{1}HNMR spectrum of **2** in pyridine-d_5 displayed signals for two tertiary methyl groups at δ 0.78 (3H, s, Me-18) and 0.92 (3H, s, Me-19), a secondary methyl group at δ 1.06 (3H, d, $J = 8.0$ Hz, Me-21), and two anomeric proton signals at δ 4.98 (1H, d, $J = 7.8$ Hz) and 5.28(1H, d, $J = 7.6$ Hz), suggesting that **2** was a spirostanol diglycoside. The carbonyl signal located at δ 109.4 in the ^{13}CNMR spectrum was assigned to C-22 of spirostanol skeleton. Two signals at δ 144.5 ($=C<$) and 108.7 ($=CH_2$) were assigned to the C-25 and C-27, respectively [7]. Apart from some signals due to F-ring and C-3, most of the ^{13}CNMR data of the aglycone part of **2** are almost at the same positions with samogenin [2]. All these facts showed that the aglycone of **2** was 25(27)-ene samogenin. On acid hydrolysis of **2** with 1 M HCl in dioxane/H_2O (1:1), glucose and galactose were identified by co-chromatography on HPTLC with authentic samples. According to FAB-MS fragments, it can be concluded that the ratio of glucose and galactose was 1:1. The β-form anomeric configurations of the galactose and glucose were judged from their coupling constants of anomeric protons ($J = 7.8$ and 7.6 Hz, respectively).

The sugar linkages were determined by comparison with the known compound, samogenin 3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-galactopyranoside [8], which was isolated from bulbs of *Allium macrostemon*. The ^{13}CNMR spectral data of two compounds at sugar moieties were almost identical. Thus combining with the results of acid hydrolysis and spectroscopic evidences, the sugar moiety of **2** could be determined. By comparison of the ^{13}CNMR spectral data of **2** with those of samogenin, the signal due to C-3 shifted to downfield by approximately $+14.5$ ppm, the signals due to C-2 and C-4 shifted to higher field by -3.0 and -1.7 ppm, respectively. According to the glycosylation shift value of 1,2-diol-type saponin proposed by Tanaka [9], the absolute stereochemistry of C-3 was deduced to be *R*-configuration. All these indicated that the sugar moiety of **2** was linked at C-3 position of samogenin. Furthermore, the characteristic ion peaks in the positive ion FAB-MS was also confirmed to the sugar linkages. Thus, the structure of **2** was elucidated to be spirost 25(27)-ene-2β, 3β-diol-3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-galactopyranoside.

Compound **3** was obtained as an amorphous powder, m.p. 175–176°C (uncorrected), which was shown to have the molecular formula $C_{39}H_{64}O_{16}$ by negative FAB-MS (*m/z* 787 $[M\text{-}H]^-$) in conjunction with the ^{13}CNMR data (39 carbon signals). The IR spectrum showed

a strong absorption at 3414.8 cm^{-1} due to hydroxyl groups and an olefinic group at 1631.9 cm^{-1}. It was positive to Molish, anisaldehyde and Ehrlish reactions. These facts suggested that **3** was a furostanol saponin.

The ^{1}HNMR spectrum of **3** showed two methyl signals at δ 0.92 (3H, s, Me-18) and 1.05 (3H, s, Me-19), one three-proton doublet at δ 1.60 (3H,d, $J = 6.9$ Hz, Me-21), and two anomeric proton signals at δ 4.76 (1H, d, $J = 7.5$ Hz, Gal-H-1) and 4.84 (1H, d, $J = 7.8$ Hz, Glc-H-1). In the ^{13}CNMR spectrum, the carbonyl signal at δ 110.2 was assigned to C-22 of furostanol skeleton, which suggested that a hydroxyl group instead of a methoxyl group linked at C-22 according to the literature [7]. The signals at δ 147.1 (= C <) and 110.7 (= CH_2) were a pair of olefinic carbons due to C-25 and C-27 positions. Comparing the ^{13}CNMR spectral data of aglycone part with the known compound, which was isolated from *Allium tuberosum* [10], it displayed similarity except for some variation at A rings and C-22–C-27 positions. Combining with the molecular formula of **3**, suggested that the aglycone of **3** had an additional hydroxyl group as compared with β-chologenin [11]. The signals due to C-2 and C-19 shifted to downfield by approximately +1.6 and +2.2 ppm, respectively, while the signal due to C-3 shifted to a higher field by −5.2 ppm. In HMBC spectrum, 19-CH_3 (1.05 ppm) has long-range correlation with C-1 (72.2 ppm) and C-5 (34.1 ppm). So the free hydroxyl group can be assigned to the C-1 rather than to C-2 position. Thus **3** was suggested to be a (5β)-25(27)-ene-1β, 3β, 6β-trihydroxyl furostanol saponin.

The spin systems for sugars were assigned on the basis of COSY and HMQC spectra, while the sugar linkages of **3** were determined on the basis of HMBC spectrum. Namely, long-range couplings ($^{3}J_{H,C}$) were observed between a proton signal at δ 4.84 (Glc-H-1) and a carbon signal at δ 72.0 (C-26), whereas a proton signal at δ 4.76 (Gal-H-1′) had a cross peak with carbon signal at δ 74.9 (C-3) in the HMBC spectrum. All these indicated that the two sugar moieties of **3** were linked at C-3 and C-26 of the aglycone, respectively. From the above evidence, the structure of **3** was established to be 26-*O*-β-D-glucopyranosyl-22α-hydroxy-5β-furost-25(27)-ene-1β,3β,6β,26-tetraol-3-*O*-β-D-galactopyranoside.

The structures of known compounds **4**–**12** were determined by means of chemical and spectral ways. Their chemical names were: Tigogenin 3-*O*-β-D-glucopyranosyl (1 → 2)[β-D-glucopyranosyl (1 → 3)]-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (**4**), Laxogenin 3-*O*-α-arabinopyranosyl (1 → 6)-β-D-glucopyranoside (**5**), Laxogenin 3-*O*-β-xylopyranosyl (1 → 4)[α-arabinopyranosyl (1 → 6)]-β-D-glucopyranoside (**6**), Samogenin 3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-galactopyranoside (**7**), (25S)Samogenin 3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-galactopyranoside (**8**), (25R)-26-*O*-β-D-glucopyranosyl-5α-furost-20(22)-ene-3β,26-diol-3-*O*-β-D-glucopyranosyl (1 → 2) [β-D-glucopyranosyl (1 → 3)]-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (**9**), (25R)-26-*O*-β-D-glucopyranosyl-5α-furost-3β,26-diol-3-*O*-β-D-glucopyranosyl (1 → 2)[β-D-glucopyranosyl (1 → 3)-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (**10**), 26-*O*-β-D-glucopyranosyl-22-hydroxy-5β-furost-25(27)-ene-3β,12β,26-triol-3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-galactopyranoside (**11**) and 26-*O*-β-D-glucopyranosyl-22-methoxy-5β-furost-25(27)-ene-3β,12β,26-triol-3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-galactopyranoside (**12**), respectively.

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points were measured on Yanaco Micro melting point apparatus (uncorrected). NMR spectra were recorded on INOVA at 600 or 500 MHz for ^{1}H and 150 or 125 MHz for

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^{13}CNMR in C_5D_5N at room temperature, using TMS as internal standard. IR spectra were determined with a Bruker infrared spectrometer. FABMS and HR-FABMS were measured on VG Autospec 300 Mass Spectrometer. Preparative HPLC was carried out on LC-10 (Japan Analytical Industry Co., Ltd), using Econosphere C_{18} column (22 × 250 mm, Alltech, USA); mobile phase: MeOH–H_2O; Flow rate: 6.0 ml/min.

Plant Material

Trichosanthes kirilowii Maxim. and *Allium macrostemon* Bge. were purchased from Liaoning provincial station of medicinal materials, Shenyang, China, and were identified by Prof. Qishi Sun, Department of Pharmacognosy, Shenyang Pharmaceutical University. The voucher specimens are deposited in the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

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Extraction and Isolation

The prescription (25 kg, *Trichosanthes kirilowii* Maxim. 15 kg and *Allium macrostemon* Bge. 10 kg) was extracted with 60% EtOH at reflux temperature for three times. The extracts were evaporated under reduced pressure and partitioned with EtOAc and *n*-BuOH successfully. The *n*-BuOH layer (448 g) was subjected to Diaion (D101) chromatography and eluted with H_2O, 10, 30 and 50% and EtOH gradually. The part of 30% EtOH elute (40.13 g) and EtOH elute (50.34 g) were subjected to chromatography on silica gel using a $CHCl_3$–MeOH system. Further separation using Diaion HP-20, repeated ODS opening column and HPLC (Rp18) with H_2O–MeOH system, were performed to obtain **3–11** from 30% EtOH part and **1–2**, **12** from ethanol part, respectively.

Compound **1**, $C_{47}H_{76}O_{18}$, colorless amorphous powder, m.p. 276–277°C (uncorrected), IR (KBr)cm^{-1}: 3425.2, 2927.8, 1724.0, 1629.6, 1385.6, 1076.9, 567.7. ^{1}HNMR and ^{13}CNMR data see Table I. Positive ion FAB-MS *m/z*: 929[M + H]$^+$, 796[M-pentose]$^+$, 766[M-hexose]$^+$, 617[M-pentose-hexose-H_2O + H]$^+$, 472 [aglycone]$^+$ and 437[aglycone-2·H_2O + H]$^+$.

Compound **2**, $C_{39}H_{62}O_{14}$, colorless amorphous powder, m.p.265–266°C (uncorrected). IR (KBr) cm^{-1}: 3425.4,2927.3,1629.0,1384.1,1075.5. ^{1}HNMR (500 MHz, ppm, in C_5D_5N): 0.78 (3H, s), 0.92 (3H, s), 1.06(3H, d, $J = 8.0$ Hz), 4.78 (H, s), 4.82 (H, s), 4.98 (H, d, $J = 7.8$ Hz), 5.28 (H, d, $J = 7.6$ Hz). ^{13}CNMR (125 MHz, ppm, in C_5D_5N) data are shown in Table II. FAB-MS (pos.) *m/z*: 755[M + H]$^+$, 593[M + H-Glc]$^+$, 431[M + H-Glc-Gal]$^+$ and 413[Aglycone + H-H_2O]$^+$.

Compound **3** $C_{39}H_{64}O_{16}$ m.p. 175–176°C (uncorrected), colorless amorphous powder. IR (KBr) cm^{-1}: 3414.8,2931.1,1631.9,1452.3,1076.6, 609.6. ^{1}HNMR (500 MHz, ppm, in C_5D_5N): 0.92 (3H, s), 1.05 (3H, s), 1.60 (3H, d, $J = 6.9$ Hz), 3.98 (H-3, m), 4.18 (H-1, m), 4.40 (H-6, m), 4.76 (1H, d, $J = 7.5$ Hz), 4.84 (1H, d, $J = 7.8$ Hz). ^{13}CNMR (125 MHz, ppm, in C_5D_5N) data are shown in Table II. FAB-MS (neg.) *m/z*: 787[M-H]$^-$, 626[M-H-Glc]$^-$, and 463 [Aglycone-H]$^-$ (Fig. 1).

Acid Hydrolysis of Saponins

Compound **1** (2 mg) was heated with 7% sulfuric acid at 80°C for 12 h. The reaction mixture was neutralized with 1 M NaOH and filtered. The filtrate was extracted with $CHCl_3$ and H_2O. The water layer was concentrated and galactose and xylose were identified by HPTLC with authentic samples.

Compound **2** and **3** (2 mg, respectively) was heated with 1 M HCl in dioxane–H_2O at rt. for 8 h. The coming procedures were the same with the acid hydrolysis of compound **1** galactose and glucose were identified by HPTLC with authentic samples.

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