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Five new polyhydroxylated furostanol saponins from the rhizomes of *Tupistra chinensis*

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[ABSTRACT] Five new polyhydroxylated furostanol saponins were isolated from the roots and rhizomes of *Tupistra chinensis*, and their structures were determined as tupistrosides J–N (**1**–**5**), together with four known furostanol saponins (**6**–**9**), on the basis of physico-chemical properties and spectral analysis. Among them, compounds **3** and **5** showed cytotoxicity against human cancer cell lines SW620 with IC_{50} values of 72.5 ± 2.4 and 77.3 ± 2.5 μmol·L^{-1}, respectively. Compound **4** showed cytotoxicity against human cancer cell line HepG2 with IC_{50} value of 88.6 ± 2.1 μmol·L^{-1}.

[KEY WORDS] *Tupistra chinensis*; Liliaceae; Furostanol saponins; Cytotoxicity

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Introduction

Tupistra chinensis Baker., a species in the genus *Tupistra* of the family Liliaceae, is used as an endemic herbal medicine, known as "Kai-Kou-Jian", in the Qinba Mountains of Shaanxi Province in China [1]. The roots and rhizomes of *T. chinensis* are commonly used as Chinese traditional medicine for the treatment of throat irritation, rheumatic diseases and snake-bites [2-3]. Previous phytochemical investigations on *T. chinensis* have resulted in the isolation of steroidal sapogenins and their glycosides [4-7], cardenolides [3], a pregnane genin and its glycoside [8-9] and flavonoids [10]. Steroidal saponins were the most abundant active constituents in this plant, which mainly included spirostanol and furostanol saponins. As part of our research project to find more diverse bioactive leading compounds from the medicinal herbs of the Qinba Mountains [11-14], the chemical constituents and pharmacological studies of *T. chinensis* were investigated, and five new furostanol saponins tupistrosides J–N (**1**–**5**), together with four known furostanol saponins 5*β*-furost-25(27)-en-1*β*, 2*β*, 3*β*, 4*β*, 5*β*, 6*β*, 7*α*, 22*α*, 26-nonaol-26-*O*-*β*-D-glucopyranoside (**6**) [15], (25*R*)-26-*O*-*β*-D-glucopyranosyl-furost-1*β*, 3*β*, 26-trihydroxy-5(6), 20(22)-dien-3-*O*-*β*-D-glucopyranoside (**7**) [3], tupistroside E (**8**) [16] and (25*S*)-26-*O*-*β*-D-glucopyranosyl-5*β*- furost-1*β*, 3*β*, 22*α*, 26-tetraol-3-*O*-*β*-D-glucopyranoside (**9**) [4] were obtained (Fig. 1). In this article, we reported the isolation and structure elucidation of five new compounds, and their cytotoxic evaluation against A549, SW620 and HepG 2 tumor cell lines.

Results and Discussion

Compound **1** was obtained as a white amorphous powder, which showed positive reactions in the Liebermann-Burchard, Ehrlich and Molisch reactions, suggesting that **1** was a furostanol glycoside. Its molecular formula was determined as $C_{33}H_{54}O_{12}$ from the HR-ESI-MS peak at *m/z* 641.3528 [M −

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H]$^-$. The ^{1}H NMR spectrum showed three methyl protons at δ_H 0.94 (3H, s, Me-18), 1.62 (3H, s, Me-19) and 1.35 (3H, d, J = 6.9 Hz, Me-21), two exo-methylene protons [δ_H 5.36 (1H, brs) and 5.07 (1H, brs)], as well as signal for one anomeric proton at (δ_H 4.92 (d, J = 7.8 Hz). The ^{13}C NMR spectrum displayed 33 carbon signals, 27 of which belonged to the aglycone carbons, while the remaining signals were assignable to one glucosyl moiety (δ_C 104.4, 75.7, 79.1, 71.7, 79.0, 63.3). Among carbon signals of the aglycone, δ_C 147.7 and 111.2 were due to an olefinic bond group, δ_C 17.2, 14.4 and 16.9 were due to three methyl groups. The structure of **1** was finally determined by analysis of its 2D NMR data (Fig. 2). The HSQC experiment allowed for the assignments of the proton and protonated carbon resonances in the NMR spectra of **1**. HSQC correlations of [δ_H 5.36 (H-27a) and 5.07 (H-27b)] to δ_C 111.2, showed the appearance of a terminal olefinic bond at C-27. HMBC correlations of H-3/C-1, C-2 and C-5, H-6/C-4, C-5, C-8 and C-10, H-19/C-1, C-5, C-9 and C-10, indicated the existence of 1-OH, 3-OH, 4-OH and 5-OH. HMBC correlations of H-27/C-24, C-25 and C-26, H-24/C-22, C-23, C-25 and C-26, H-26/C-24, C-25 and C-27, indicated that the appearance of an isopentene group, linked at C-22 of the tetrahydrofuran ring of the furostanol saponin. Moreover, HMBC correlations of H-18/C-12, C-13, C-14 and C-17, H-16/C-13, C-17, C-20 and C-22, H-21/C-17, C-20 and C-22, were assigned. Therefore, the aglycone of **1** was identified as 1, 3, 4, 5, 22, 26-hexanol-furost-25(27)-en. In addition, the HMBC correlation signal of H-1′ (δ_H 4.92) of Glc moiety and C-26 (δ_C 72.6) of aglycone, indicated that sugar group was connected as (Glc-1-*O*-C-26). The glucosyl moiety was identified as D-glucose by acid hydrolysis of **1**, followed by TLC comparison with a reference compound and optical rotation determination [17], and judged to be in a *β*-configuration [18] from the coupling constant of the anomeric proton (7.8 Hz). In the NOESY spectrum of **1**, the NOE correlations of H-9/H-2a/H-1, H-9/H-2a/H-3/H-4/H-7a and H-1/Me-19, indicated *α*-axial configurations of H-1, H-2a, H-4, H-3 H-7a and H-9 and *β*-orientations of 1-OH, 3-OH, 4-OH and 5-OH [5], which supported the A/B *cis* ring junction pattern; the NOE correlations of Me-19/H-8, H-8/Me-18 and H-14/H-9, H-16 and H-17, supported the B/C and C/D *trans* ring junction pattern; and the NOE correlations of Me-18/H-15b, H-15a/H-16 and H-17, and H-17/Me-21, suggested an *α*-orientation of Me-21 (Fig. 2). Therefore, tupistroside J (**1**) was identified as 1*β*, 3*β*, 4*β*, 5*β*, 22*α*, 26-hexahydroxy-furost-25(27)-en-26-*O*-*β*-D-glucopyranoside.

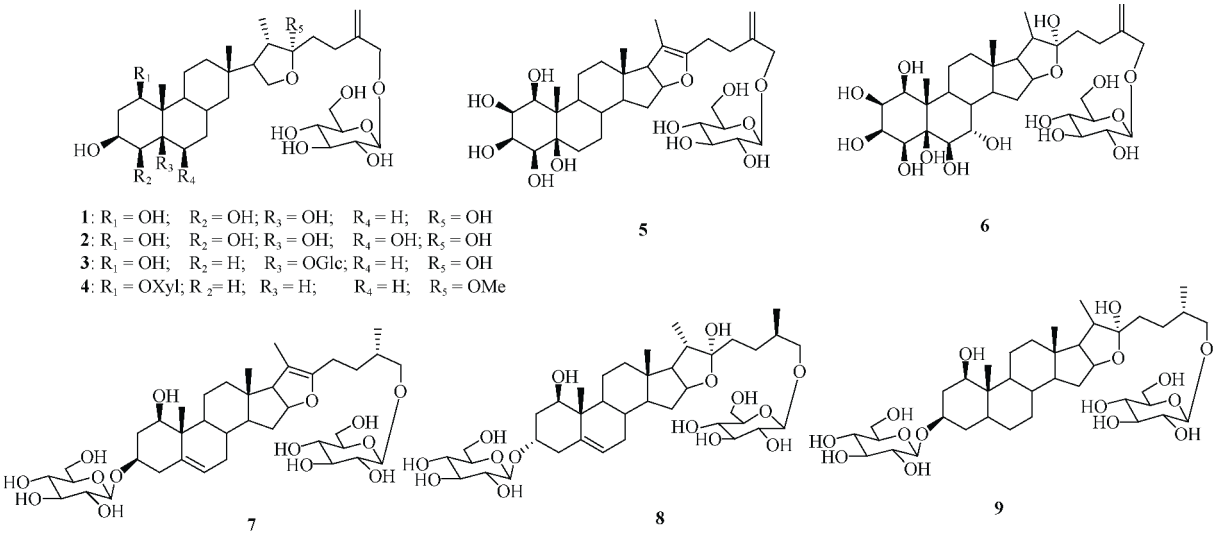

Fig. 1 Structures of compounds 1–9

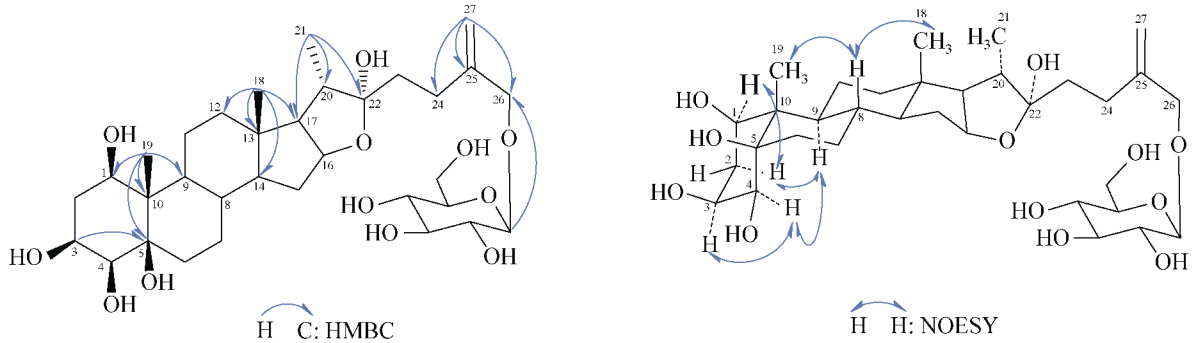

Fig. 2 Key HMBC and NOESY correlations of the compound 1

Compound **2** was obtained as a white amorphous powder, which showed positive reactions in the Liebermann-Burchard, Ehrlich and Molisch reactions, suggesting that **2** was a furostanol glycoside. Its molecular formula was determined as $C_{33}H_{54}O_{13}$ from the HR-ESI-MS peak at *m/z* 681.3494 $[M + Na]^+$. Comparison of the NMR, HR-ESI-MS data of **2** and **1**, compound **2** exhibited spectroscopic features similar to those of **1**, except an increase of 6-OH. The proton and carbon NMR signals of [δ_H 1.73 (1H, *ca.*, H-6a), 2.5 (1H, *ca.*, H-6b) and δ_C 30.9 (C-6)] and [δ_H 1.16 (1H, *ca.*, H-7a), 1.53 (1H, *ca.*, H-7b) and δ_C 29.0 (C-7)] in **1**, were replaced by [δ_H 4.88 (1H, brs, H-6), δ_C 70.0 (C-6)] and [δ_H 1.56 (1H, *ca.*, H-7a), 2.07 (1H, *ca.*, H-7b) and δ_C 35.9 (C-7)] in **2**, which was supported by HSQC, HMBC and NOESY spectrums. HMBC correlations of H-3/C-1, C-2 and C-5, H-6/C-4, C-5, C-8 and C-10, indicated the existence of 1-OH, 3-OH, 4-OH, 5-OH and 6-OH. HMBC correlation signal of H-1′ (δ_H 4.91) of Glc moiety and C-26 (δ_C 72.6) of aglycone indicated the glucosyl group was linked to C-26. Similarly as compound **1**, the results of the acid hydrolysis procedure and analysis of detail NOESY spectra data showed the structure of tupistroside K (**2**) was identified as 1*β*, 3*β*, 4*β*, 5*β*, 6*β*, 22*α*, 26-heptolfurost-25(27)-en-26-*O*-*β*-D-glucopyranoside.

Compound **3** was obtained as a white amorphous powder, which showed positive reactions in the Liebermann-Burchard, Ehrlich and Molisch reactions, suggesting that **3** was a furostanol glycoside. Its molecular formula was determined as $C_{39}H_{64}O_{16}$ from the HR-ESI-MS peak at *m/z* 789.4147 $[M + H]^+$. Comparison of the NMR, HR-ESI-MS data of **3** and **1**, compound **3** exhibited spectroscopic features similar to those of **1**, except an absence of 4-OH and an increase of 5-*O*-*β*-D-glucopyranoside, which was confirmed by the HMBC correlations of H-19/C-1, C-5, C-9 and C-10, and H-6/C-4 and C-5. In addition, the glucosyl moiety was identified as *β*-D-glucose by the acid hydrolysis procedure and the coupling constant analysis of the anomeric protons (7.7, 7.8 Hz), according to the same protocol as that described for **1**. Thus, the planar structure of **3** was deduced as 1, 3, 5, 22, 26-pentanol-furost-25(27)-en-5, 26-*O*-*β*-D-glucopyranoside. In addition, in the NOESY spectrum, the NOE correlations of H-9/H-2a/H-1, H-9/H-2a/H-3/H-4a/H-7a, and H-1/Me-19, indicated *α*-axial configurations of H-1, H-2a, H-3, H-7a and H-9 and *β*-orientations of 1-OH, 3-OH and 5-OH. Therefore, the structure of tupistroside L (**3**) was identified as 1*β*, 3*β*, 5*β*, 22*α*, 26-pentaol-furost-25(27)-en-5, 26-*O*-*β*-D-glucopyran oside.

Compound **4** was obtained as a white amorphous powder, which showed positive reactions in the Liebermann-Burchard, Ehrlich and Molisch reactions, suggesting that **4** was a furostanol glycoside. Its molecular formula was determined as $C_{39}H_{64}O_{14}$ from the HR-ESI-MS peak at *m/z* 779.4188 $[M + Na]^+$. The ^{1}H NMR spectrum showed three methyl protons at δ_H 0.82 (3H, s, Me-18), 1.35 (3H, s, Me-19) and 1.18 (3H, d, J = 6.9 Hz, Me-21), two exo-methylene protons (δ_H 5.38 (1H, brs) and 5.09 (1H, brs)), as well as signals for two anomeric protons at [δ_H 4.96 (d, J = 7.8 Hz) and 4.90 (1H, d, J = 7.6 Hz)]. Comparison of the ^{13}C NMR data of **4** and a reference compound 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-5*β*-furost-25(27)-en-1*β*, 3*β*, 22*α*, 26-tetraol-26-*O*-*β*-D-glucopyranoside [19], compound **4** showed identical carbon signals in the B–F rings, except for C-1-C-4 due to the different link of the sugar. Thus, the aglycone of compound **4** was determined as 25(27)-en-1*β*, 3*β*, 22*α*, 26-tetra-furostanol. The appearance of a methoxyl signal at δ_C 47.9 in the ^{13}C NMR spectrum and the downfield shift at δ_C 112.9 (C-22) suggested that the 22-OH was methylated. The above inference was supported by HSQC and HMBC spectrums. In addition, the HMBC correlation signals of H-1′ (δ_H 4.90) of Xyl moiety and C-1 (δ_C 80.0) of aglycone and H-1′ (δ_H 4.96) of Glc moiety and C-26 (δ_C 72.6) of aglycone, indicated that sugar groups were connected as (Glc-1-*O*-C-26) and (Xyl-1-*O*-C-1). In the NOESY spectrum, the NOE correlations of H-9/H-2a/H-1, H-9/H-2a/H-4/H-3 and Me-19/H-8 were observed, indicated *α*-axial configurations of H-1 and H-3, and *β*-orientation of 1-OH and 3-OH (Fig. 3). The 5*β*-H configuration was revealed by a group of carbon signals due to C-4, C-5, C-6, C-7, C-8, C-9 and C-19 at δ_C 35.0, 32.0, 26.7, 26.9, 36.1, 42.2 and 20.2 [20]. Therefore, tupistroside M (**4**) was identified as 22-methoxy-26-*O*-*β*-D-glucopyranosyl-5*β*-furostan-25(27)-en-1*β*, 3*β*, 22*α*, 26-tetraol-1-*O*-*β*-D-xylose.

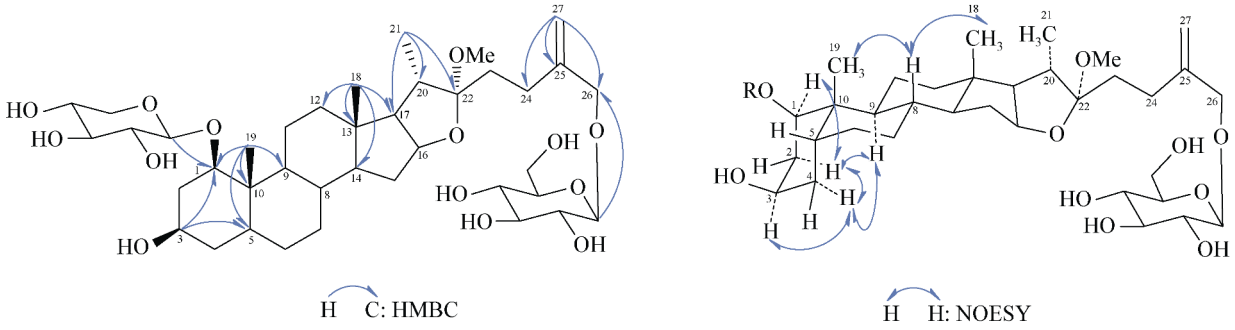

Fig. 3 Key HMBC and NOESY correlations of the compound 4

Compound **5** was obtained as a white amorphous powder, which showed positive reactions in the Liebermann-Burchard, Ehrlich and Molisch reactions, suggesting that **5** was a furostanol glycoside. Its molecular formula was determined as

$C_{33}H_{52}O_{12}$ from the HR-ESI-MS peak at *m/z* 663.3374 [M + Na]$^+$. Comparison of the NMR, HR-ESI-MS data of **5** and the know compound 1*β*, 2*β*, 3*β*, 4*β*, 5*β*, 26-hexahydroxy furost-20(22), 25(27)-dien-5, 26-*O*-*β*-D-glucopyranoside [7], compound **5** exhibited spectroscopic features similar to those of it, except an absence of 5-*O*-*β*-D-glucopyranoside, which was confirmed by the HMBC correlations of H-19/C-1, C-5, C-9 and C-10, and H-6/C-4 and C-5. In the NOESY spectrum, the NOE correlations of Me-19/H-8, H-9/H-4, H-4/H-3 and H-2, and H-2/H-1 were observed (Fig. 4), indicated *α*-axial configurations of H-1, H-2, H-3 and H-4, and *β*-orientation of Me-19, 1-OH, 2-OH, 3-OH, 4-OH and 5-OH. Therefore, tupistroside N (**5**) was identified as 1*β*, 2*β*, 3*β*, 4*β*, 5*β*, 26-hexahydroxyfurost-20(22), 25(27)-dien-26-*O*-*β*-D-glucopyranoside.

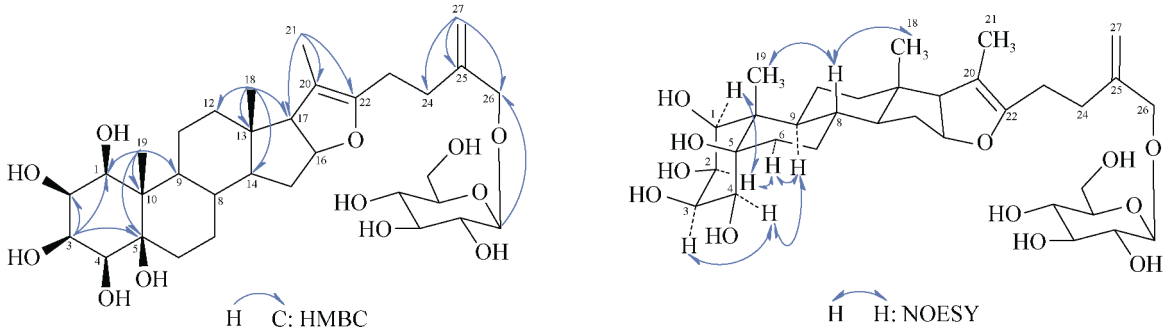

Fig. 4 Key HMBC and NOESY correlations of the compound 5

The known furostanol saponins were identified as 5*β*-furost-25(27)-en-1*β*, 2*β*, 3*β*, 4*β*, 5*β*, 6*β*, 7*α*, 22*α*, 26-nonaol-26-*O*-*β*-D-glucopyranoside (**6**) [15], (25*R*)-26-*O*-*β*-D-glucopyranosyl-furost-1*β*, 3*β*, 26-trihydroxy-5(6), 20(22)-dien-3-*O*-*β*-D-glucopyranoside (**7**) [3], tupistroside E (**8**) [16] and (25*S*)-26-*O*-*β*-D-glucopyranosyl-5*β*-furost-1*β*, 3*β*, 22*α*, 26-tetraol-3-*O*-*β*-D-glucopyranoside (**9**) [4] by comparison of spectral data with those reported in the literature.

Compounds **1**–**9** were evaluated for their cytotoxic activity against SW620, A549 and HepG2 tumor cell lines. Among them, compounds **3** and **5** showed cytotoxicity against human cancer cell lines SW620 with IC_{50} values of 72.5 ± 2.4 and 77.3 ± 2.5 μmol·L^{-1}, respectively. Compound **4** showed cytotoxicity against human cancer cell line HepG2 with IC_{50} value of 88.6 ± 2.1 μmol·L^{-1}. For the limitation in the number of the isolated furostanol glycosides from *T. chinensis*, more extensive studies are needed before a clear structure-activity relationship can be reached.

Experimental

General procedures

Optical rotation indices were determined in methanol on a Rudolph Autopol II digital polarimeter (Rudolph, Hackettstown, NJ, USA). UV spectra were recorded on a Shimadzu-2201 (Kyoto, Japan). The IR spectra were recorded on a Bruker TENSOR-27 instrument (Bruker, Rheinstetten, Germany). ESI-MS was performed on Waters Quattro Premier instrument (Waters, Milford, MA, USA). The HR-ESI-MS spectra was taken on an Agilent Technologies 6550 Q-TOF (Santa Clara, CA, USA). 1D and 2D NMR spectra were recorded on a Bruker-AVANCE400 instrument (Bruker, Rheinstetten, Germany) with TMS (Meryer Chemical Technology Co., Ltd., Shanghai, China, purity ≥ 99%, batch number: 20180915) as an internal standard. The analytical HPLC was performed on a Waters 2695 Separations Module coupled with a 2996 Photodiode Array Detector (Waters, Milford, MA, USA) and a Accurasil C_{18} column (4.6 mm × 250 mm, 5 mm particles, Ameritech, Chicago, IL, USA). Semipreparative HPLC was performed on a system comprising a Shimadzu LC-6AD pump equipped with a SPD-20A UV detector (Shimadzu, Kyoto, Japan) and a Ultimate XB-C_{18} (10 mm × 250 mm, 5 mm particles, Welch, Shanghai, China) or YMC-Pack-ODS-A (10 mm × 250 mm, 5 mm particles, YMC, Kyoto, Japan). D101 was from Sunresin New Materials Co., Ltd. (Xi'an, China). Silica gel was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

Plant materials

The roots and rhizomes of *T. chinensis* Baker were collected from Taibai region of Qinba mountains in Shaanxi Province on August in 2010, and identified by Senior experimentalist WANG Ji-Tao, the School of Pharmacy, Shaanxi University of Chinese Medicine (Shaanxi, China). A voucher specimen (herbarium No. 20100816) has been deposited in the Medicinal Plants Herbarium (MPH), Shaanxi University of Chinese Medicine, Xianyang, China.

Extraction and isolation

The air-dried and powdered underground parts of *T. chinensis* (1.5 kg) were extracted with 65% EtOH three times at 80 °C. The combined EtOH extracts were evaporated to 6 L, and applied to a resin D101 column, eluting with H_2O, 20% EtOH, 60% EtOH, and 95% EtOH to give four fractions (Frs.1–4). Fr. 3 (72 g) were separated by silica gel column chromatography in elution with gradient solvent system ($CHCl_3$–MeOH–H_2O, from 100 : 0 : 0 to 0 : 50 : 50, *V/V/V*) producing ten fractions (Frs. 3-1–3-10). Fr. 3-6 (5.5g) was subjected to reversed-phase ODS, using MeOH–H_2O (from 0 : 100 to 70 : 30, *V/V*) as the eluent to afford six fractions (Frs. 3-6-1–3-6-6). Fr. 3-6-3 (0.9 g) was purified by HPLC (Ultimate XB-C_{18}, 10 mm × 250 mm, 5 mm particles, flow rate: 1.0 mL·min^{-1}) with MeCN–H_2O (28 : 72, *V/V*) as mobile phase

to afford compound **1** (8 mg, t_R = 29.3 min), **2** (9 mg, t_R = 42.5 min) and **3** (8 mg, t_R = 52.1 min). Fr. 3-6-1 was purified by HPLC with MeCN–H_2O (24 : 76, *V/V*) as mobile phase to afford compound **4** (12 mg, t_R = 35.5 min), **5** (9 mg, t_R = 39.2 min) and **9** (11 mg, t_R = 54.3 min). Fr. 3-5 was purified by HPLC with MeCN–H_2O (26 : 74, *V/V*) as mobile phase to afford compound **6** (10 mg, t_R = 45.3 min), **7** (11 mg, t_R = 50.2 min) and **8** (6 mg, t_R = 63.4 min).

Table 1 ^{13}C NMR spectral data of compounds 1–5 (100 MHz in pyridine-d_5)

No.	1	2	3	4	5
1	74.3	75.5	72.6	80.0	78.7
2	34.0	33.6	25.7	29.8	68.0
3	72.2	71.6	68.1	67.1	76.2
4	68.6	70.0	28.5	35.0	68.8
5	78.9	79.7	87.8	32.0	78.5
6	30.9	70.0	25.4	26.7	29.0
7	29.0	35.9	28.9	26.9	30.9
8	35.5	30.7	34.9	36.1	34.9
9	46.3	46.2	44.6	42.2	45.8
10	45.9	46.0	43.7	39.9	45.6
11	22.0	21.8	22.1	21.8	22.3
12	40.7	40.7	40.7	40.6	40.3
13	41.5	41.5	41.6	41.6	44.0
14	56.7	56.5	56.7	56.8	55.0
15	32.9	33.0	32.8	32.1	31.5
16	81.7	81.7	81.8	81.9	84.9
17	64.4	64.3	64.3	64.9	65.0
18	17.2	17.2	17.2	17.1	14.9
19	14.4	16.8	18.1	20.2	14.3
20	41.2	41.1	41.2	40.9	104.4
21	16.9	16.9	16.9	16.8	12.3
22	110.9	111.2	110.9	112.9	152.3
23	38.5	38.4	38.5	32.6	35.0
24	28.9	28.9	29.0	28.6	25.1
25	147.7	147.7	147.7	147.3	146.7
26	72.6	72.6	72.6	72.6	72.2
27	111.2	110.9	111.2	111.6	112.2
-OMe				47.9	
Glc-1′	104.4	104.4	104.4	104.4	104.3
2′	75.7	75.7	75.7	75.7	75.7
3′	79.1	79.1	79.1	79.1	79.1
4′	71.7	72.1	72.2	71.7	72.2
5′	79.0	79.1	79.0	79.1	79.1
6′	63.3	63.2	63.2	63.3	63.3
Glc-1″			97.6		
2″			76.3		
3″			79.5		
4″			72.4		
5″			79.1		
6″			63.3		
Xyl-1				102.8	
2				75.6	
3				79.3	
4				72.2	
5				68.1	

Continued

Table 2 ^{1}H NMR spectral data of compounds 1–5 (400 MHz in pyridine-d_5)

No.	1	2	3	4	5
1	4.23 (*ca.*)	4.18 (*ca.*)	4.37 (*ca.*)	4.09 (*ca.*)	4.40 (*ca.*)
2	2.12 (*ca.*)	2.54 (*ca.*)	1.34 (*ca.*)	1.68 (d, 14.3)	4.25 (*ca.*)
	2.55 (*ca.*)	2.14 (*ca.*)	2.26 (*ca.*)	2.35 (d, 15.5)	
3	4.24 (*ca.*)	4.58 (brs)	4.17 (*ca.*)	4.38 (*ca.*)	4.87 (*ca.*)
4	4.27 (*ca.*)	4.22 (*ca.*)	1.72 (*ca.*)	2.11 (*ca.*)	4.42 (*ca.*)
			1.84 (*ca.*)	1.66 (*ca.*)	
5	-	-	-	2.43 (*ca.*)	-
6	1.73 (*ca.*)	4.88 (brs)	1.82 (*ca.*)	1.33 (*ca.*)	1.55 (*ca.*)
	2.5 (*ca.*)		2.72 (d, 12.4)	1.07 (*ca.*)	1.17 (*ca.*)
7	1.16 (*ca.*)	1.56 (*ca.*)	2.28 (*ca.*)	1.81 (*ca.*)	2.53 (*ca.*)
	1.53 (*ca.*)	2.07 (*ca.*)	2.74 (*ca.*)	1.21 (*ca.*)	1.75 (*ca.*)
8	1.75 (*ca.*)	2.38 (*ca.*)	1.63 (*ca.*)	1.6 (*ca.*)	2.1 (*ca.*)
9	1.25 (*ca.*)	1.25 (*ca.*)	1.31 (*ca.*)	1.27 (*ca.*)	1.25 (*ca.*)
10	-	-	-	-	-
11	1.42 (2H, *ca.*)	1.51 (*ca.*) 1.39 (*ca.*)	1.42 (2H, *ca.*)	1.28 (2H, *ca.*)	1.46 (*ca.*) 1.51 (*ca.*)
12	1.12 (*ca.*)	1.13 (*ca.*)	1.16 (*ca.*)	1.72 (*ca.*)	1.68 (*ca.*)
	1.77 (*ca.*)	1.77 (*ca.*)	1.77 (*ca.*)	1.10 (*ca.*)	1.1 (*ca.*)
13	-	-	-	-	-
14	1.12 (*ca.*)	1.14 (*ca.*)	1.08 (*ca.*)	1.13 (*ca.*)	0.85 (*ca.*)
15	1.48 (*ca.*)	2.12 (*ca.*)	1.47 (*ca.*)	2.18 (*ca.*)	2.43 (*ca.*)
	2.05 (*ca.*)	1.47 (*ca.*)	2.02 (*ca.*)	2.03 (*ca.*)	2.52 (*ca.*)
16	4.99 (*ca.*)	4.97 (*ca.*)	4.97 (*ca.*)	4.53 (dd, 7.3, 7.7)	4.82 (*ca.*)
17	2.01 (*ca.*)	2.02 (*ca.*)	2.04 (*ca.*)	1.84 (*ca.*)	2.48 (*ca.*)
18	0.94 (s.)	0.93 (s)	0.92 (s)	0.82 (s)	0.73 (s)
19	1.62 (s.)	1.90 (s)	1.38 (s.)	1.35 (s)	1.66 (s)
20	2.33 (*ca.*)	2.26 (*ca.*)	2.3 (*ca.*)	2.26 (t, 6.4)	-
21	1.35 (d, 6.9)	1.34 (d, 6.9)	1.34 (d, 6.8)	1.18 (d, 6.9)	1.63 (s)

Continued

No.	1	2	3	4	5
22	-	-	-	-	-
23	2.29 (*ca.*)	2.23 (*ca.*)	2.27 (*ca.*)	2.46 (*ca.*)	1.50 (*ca.*)
	2.26 (*ca.*)	2.25 (*ca.*)	2.28 (*ca.*)	2.45 (*ca.*)	1.74 (*ca.*)
24	2.74 (*ca.*)	2.69 (*ca.*)	1.56 (*ca.*)	2.01 (*ca.*)	2.45 (*ca.*)
	2.76 (*ca.*)	2.72 (*ca.*)	1.13 (*ca.*)	1.41 (dd, 6.1, 13.3)	2.41 (*ca.*)
25	-	-	-	-	-
26	4.37 (d, 13.0)	4.36 (d, 12.9)	4.37 (d, 12.9)	4.65 (d, 12.9)	4.38 (*ca.*)
	4.64 (d, 12.7)	4.63 (d, 13.0)	4.64 (d, 12.9)	4.37 (*ca.*)	4.62 (*ca.*)
27	5.07 (brs)	5.36 (brs)	5.36 (brs)	5.38 (s)	5.09 (br.s)
	5.36 (brs)	5.07 (brs)	5.07 (brs)	5.09 (s)	5.4 (br.s)
-OMe				3.28 (s)	
Glc-1′	4.92 (d, 7.8)	4.91 (d, 7.8)	4.92 (d, 7.7)	4.96 (d, 7.8)	4.95 (d, 7.8)
2′	4.08 (*ca.*)	4.08 (*ca.*)	4.07 (*ca.*)	4.05 (*ca.*)	4.11 (*ca.*)
3′	4.25 (*ca.*)	4.25 (*ca.*)	4.27 (*ca.*)	4.28 (*ca.*)	4.29 (*ca.*)
4′	4.63 (*ca.*)	4.26 (*ca.*)	4.09 (*ca.*)	4.24 (*ca.*)	4.27 (*ca.*)
5′	3.95 (*ca.*)	3.95 (*ca.*)	3.95 (*ca.*)	4.03 (*ca.*)	3.98 (*ca.*)
6′	4.42 (dd, 5.9, 11.8)	4.40 (*ca.*)	4.42 (*ca.*)	4.6 (d, 12.9)	4.59 (dd, 2.5, 12.0)
	4.57 (*ca.*)	4.55 (*ca.*)	4.57 (*ca.*)	4.44 (*ca.*)	4.43 (dd, 5.3, 11.3)
Glc-1″			5.24 (d, 7.8)		
2″			4.01 (*ca.*)		
3″			4.08 (*ca.*)		
4″			4.23 (*ca.*)		
5″			4.25 (*ca.*)		
6″			4.24 (*ca.*)		
			4.57 (*ca.*)		
Xyl-1				4.90 (d, 7.6)	
2				4.05 (*ca.*)	
3				4.29 (*ca.*)	
4				4.42 (*ca.*)	
5				3.77 (t, 10.6)	

Note: *Ca.* means multiplet.

Identification of compounds

Tupistroside J (**1**): a white amorphous powder; $[\alpha]_D^{16.6}$ –100 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (logε): 193 (4.03) nm; IR (KBr) ν_{max}: 3456, 2925, 1695, 1448, 1026, 907, 804, 772 cm^{-1}; negative HR-ESI-MS *m/z* 641.3528 $[M - H]^-$ (Calcd. for $C_{33}H_{53}O_{12}$, 641.3537). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2.

Tupistroside K (**2**): a white amorphous powder; $[\alpha]_D^{15.8}$ –73.0 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (logε): 194 (3.91) nm; IR (KBr) ν_{max}: 3455, 2975, 1670, 1025, 897, 806, 773 cm^{-1}; positive HR-ESI-MS *m/z* 681.3494 $[M + Na]^+$ (Calcd. for $C_{33}H_{54}O_{13}Na$, 681.3462). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2.

Tupistroside L (**3**): a white amorphous powder; $[\alpha]_D^{15.3}$ –96.0 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (logε): 194 (3.89) nm; IR (KBr) ν_{max}: 3450, 2930, 1715, 1450, 1025, 918, 805 cm^{-1}; positive HR-ESI-MS *m/z* 789.4147 $[M + H]^+$ (Calcd. for $C_{39}H_{65}O_{16}$, 789.4173). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2.

Tupistroside M (**4**): a white amorphous powder; $[\alpha]_D^{14.8}$ –116 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (logε): 193 (4.05) nm; IR (KBr) ν_{max}: 3445, 2941, 1647, 1452, 1030, 899 cm^{-1}; positive HR-ESI-MS *m/z* 779.4188 $[M + Na]^+$ (Calcd. for $C_{39}H_{64}O_{14}Na$, 779.4194). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2.

Tupistroside N (**5**): a white amorphous powder; $[\alpha]_D^{16.2}$ –32 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (logε): 203 (3.61) nm; IR (KBr) ν_{max}: 3465, 2945, 1650, 1445, 1025, 913, 802, 771 cm^{-1}; positive HR-ESI-MS *m/z* 663.3374 $[M + Na]^+$ (Calcd. for $C_{33}H_{52}O_{12}Na$, 663.3356). ^1H and ^{13}C NMR spectral data are shown in Tables 1 and 2.

*Acidic hydrolysis of compounds **1–5***

The solutions of compounds **1** (4 mg), **2** (5 mg), **3** (6 mg), **4** (5 mg) and **5** (4 mg) were hydrolyzed with 2 $mol \cdot L^{-1}$ HCl (5 mL) for 5 h at 80 °C, respectively. The reaction mixtures were concentrated and dried by N_2, and then water (5 mL) was added and the mixtures were extracted with EtOAc (3 × 5 mL). The aqueous layers of **1**–**3** and **5** were subjected to CC over silica gel eluted with MeCN–H_2O (8 : 1, *V/V*) to yield D-glucose, which was determined by TLC comparison (MeCN–H_2O, 6 : 1, *V/V*) with the authentic sugar and the optical rotation determination $[\alpha]_D^{20}$ +49.2 (*c* 0.16, H_2O). The aqueous layer of **4** was subjected to CC over silica gel eluted with MeCN–H_2O (from 8 : 1 to 15 : 1, *V/V*) to yield D-glucose and D-xylose, which was identified by TLC comparison with the authentic sugar and the optical rotation determination ($[\alpha]_D^{20}$ +49.2 (*c* 0.16, H_2O), $[\alpha]_D^{20}$ +17.9 (*c* 0.14, H_2O)).

Table 3 Cytotoxicities of compounds 1–9 against human cancer cell lines

Compounds	IC_{50} ($\mu mol \cdot L^{-1}$)		
	A549	SW620	HepG2
1	> 100	> 100	> 100
2	> 100	> 100	> 100
3	> 100	72.5 ± 2.4	> 100
4	> 100	> 100	88.6 ± 2.1
5	> 100	77.3 ± 2.5	> 100
6	> 100	90.2 ± 2.3	89.4 ± 2.4
7	> 100	> 100	> 100
8	> 100	> 100	> 100
9	> 100	> 100	> 100
5-FU	40.6 ± 1.6	23.6 ± 1.4	55.1 ± 1.8

Cytotoxicity assay

The cytotoxic activity assay toward the A549, SW620 and HepG 2 tumor cell lines were measured by the MTT method *in vitro*, using 5-fluorouracil as positive control. Briefly, 1×10^4 mL^{-1} cells were seeded into 96-well plates and allowed to adhere for 24 h. Compounds **1**–**9** were dissolved in DMSO and diluted with complete medium to six degrees of concentration (from 0.001 to 0.4 $mmol \cdot L^{-1}$) for inhibition rate determination. After incubation at 37 °C for 4 h, the supernatant fraction was removed before adding DMSO (100 μL) to each well. The inhibition rate (IR) and IC_{50} were calculated (see Table 3). Values are mean ± SD, $n = 3$.

References

[1] Song XM, Liu HJ. *Research and Application of "Qi- Medicines" in Taibai Mountains* [M]. Beijing: People's Medical Publishing House, 2011.

[2] Wu XF, Fan JJ, Ouyang ZJ, *et al*. *Tupistra chinensis* extract attenuates murine fulminant hepatitis with multiple targets against activated T lymphocytes [J]. *J Pharm Pharmacol*, 2014, **66**(3): 453-465.

[3] Pan ZH, Li Y, Liu JL, *et al*. A cytotoxic cardenolide and a saponin from the rhizomes of *Tupistra chinensis* [J]. *Fitoterapia*, 2012, **83**(8): 1489-1493.

[4] Xiang LM, Wang YH, Yi XM, *et al*. Antiproliferative and anti-inflammatory furostanol saponins from the rhizomes of *Tupistra chinensis* [J]. *Steroids*, 2016, **116**: 28-37.

[5] Xiang LM, Wang YH, Yi XM, *et al*. Bioactive spirostanol saponins from the rhizome of *Tupistra chinensis* [J]. *Steroids*, 2016, **108**: 39-46.

[6] Liu CX, Guo ZY, Xue YH, *et al*. Five new furostanol saponins from the rhizomes of *Tupistra chinensis* [J]. *Fitoterapia*, 2012, **83**(2): 323-328.

[7] Li YZ, Wang X, He H, *et al*. Steroidal saponins from the roots and rhizomes of *Tupistra chinensis* [J]. *Molecules*, 2015, **20**(8): 13659-13669.

[8] Pan WB, Wei LM, Wei LL, *et al*. Chemical constituents of *Tupistra chinensis* rhizomes [J]. *Chem Pharm Bull*, 2006, **54**(7): 954-958.

[9] Pan WB, Chang FR, Wei LM, *et al*. New flavans, spirostanol sapogenins, and a pregnane genin from *Tupistra chinensis* and their cytotoxicity [J]. *J Nat Prod*, 2003, **66**(2): 161-168.

[10] Xiao YH, Yin HL, Chen L, *et al*. Three spirostanol saponins and a flavane-*O*-glucoside from the fresh rhizomes of *Tupistra chinensis* [J]. *Fitoterapia*, 2015, **102**: 102-108.

[11] Song XM, Li YZ, Zhang DD, *et al*. Two new spirostanol saponins from the the roots and rhizomes of *Tupistra chinensis* [J]. *Phytochem Lett*, 2015, **13**: 6-10.

[12] Song XM, Zhang DD, He H, *et al*. Steroidal glycosides from *Reineckia carnea* [J]. *Fitoterapia*, 2015, **105**: 240-245.

[13] Zhang DD, Wang W, Li YZ, *et al*. Two new pregnane glycosides from *Reineckia carnea* [J]. *Phytochem Lett*, 2016, **15**: 142-146.

[14] Cui YW, Yang XJ, Zhang DD, *et al*. Steroidal constituents from roots and rhizomes of *Smilacina japonica* [J]. *Molecules*, 2018, **23**(4): 798-787.

[15] Xu LL, Zou K, Wang JZ, *et al*. New polyhydroxylated furostanol saponins with inhibitory action against NO production from *Tupistra chinensis* rhizomes [J]. *Molecules*, 2007, **12**(8): 2029-2037.

[16] Yang QX, Zhang YJ, Li HZ, *et al*. Polyhydroxylated steroidal constituents from the fresh rhizomes of *Tupistra yunnanensis* [J]. *Steroids*, 2005, **70**(10): 732-737.

[17] Hudson CS, Dale JK. Studies on the forms of D-glucose and their mutarotation [J]. *J Am Chem Soc*, 1917, **39**(2): 320-328.

[18] Zhang XD, Chen CX, Yang JY, *et al*. New minor spirostane glycosides from *Ypsilandra thibetica* [J]. *Helv Chim Acta*, 2012, **95**(7): 1087-1093.

[19] Liu CX, Guo ZY, Deng ZS, *et al*. New furostanol saponins from the rhizomes of *Tupistra chinensis* [J]. *Nat Prod Res*, 2013, **27**(2): 123-129.

[20] Agrawal PK, Jain DC, Pathak AK. NMR spectroscopy of steroidal sapogenins and steroidal saponins: an update [J]. *Magn Reson Chem*, 2010, **33**(12): 923-953.

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