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Article

Steroidal Saponins from the Roots and Rhizomes of *Tupistra chinensis*

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Abstract: Two new furostanol saponins **1**–**2** and a new spirostanol saponin **3** were isolated together with two known furostanol saponins **4**–**5** from the roots and rhizomes of *Tupistra chinensis*. Their structures were characterized as 1β,2β,3β,4β,5β,26-hexahydroxyfurost-20(22), 25(27)-dien-5,26-*O*-β-D-glucopyranoside (**1**), 1β,2β,3β,4β,5β,6β,7α,23ξ,26-nona-hydroxyfurost-20(22),25(27)-dien-26-*O*-β-D-glucopyranoside (**2**), (20*S*,22*R*)-spirost-25 (27)-en-1β,3β,5β-trihydroxy-1-*O*-β-D-xyloside (**3**), tupisteroide B (**4**) and 5β-furost-$\Delta^{25(27)}$-en-1β,2β,3β,4β,5β,7α,22ξ,26-octahydroxy-6-one-26-*O*-β-D-glucopyranoside (**5**), respectively, by extensive use of spectroscopic techniques and chemical evidence. Additionally, the *in vitro* cytotoxic activity of **1**–**4** was evaluated on human A549 and H1299 tumor cell lines, and compound **3** exhibited cytotoxicity against A549 cells (IC_{50} 86.63 ± 2.33 μmol·L^{-1}) and H1299 cells (IC_{50} 88.21 ± 1.34 μmol·L^{-1}).

Keywords: *Tupistra chinensis*; steroidal saponins; structure identification; cytotoxic activity

1. Introduction

Tupistra chinensis Baker., a species in the *Tupistra* genus of the Liliaceae family, 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.* c*hinensis* are commonly used as folk medicine to treat throat irritation, rheumatic diseases and snake-bites [2,3]. Modern pharmacological experiments have showed that the extracts of this species possess significant antitumor activities [4,5], moreover, two main kinds of components—cardenolides and saponins—were isolated from *T. chinensis* [3,6,7]. As part of our research project to find more diverse bioactive leading compounds from the medicinal herbs of the Qinba Mountains [8–11], the chemical constituents and pharmacological studies of *T. chinensis* were investigated, and two new furostanol saponins, 1β,2β,3β,4β,5β,26-hexahydroxyfurost-20(22),25(27)-dien-5,26-*O*-β-D-glucopyranoside (**1**), 1β,2β,3β,4β,5β,6β,7α,23ξ,26-nonahydroxyfurost-20(22),25(27)-dien-26-*O*-β-D-glucopyranoside (**2**), and a new spirostanol saponin (20*S*,22*R*)-spirost-25(27)-en-1β,3β,5β-trihydroxy-1-*O*-β-D-xyloside (**3**) were obtained from the roots and rhizomes of *T. chinensis* together with the two known compounds tupisteroide B (**4**) and 5β-furost-$\Delta^{25(27)}$-en-1β,2β,3β,4β,5β,7α,22ξ,26-octahydroxy-6-one-26-*O*-β-D-glucopyranoside (**5**) (Figure 1). The cytotoxic activity of **1**–**4** was evaluated on human A549 and H1299 tumor cells.

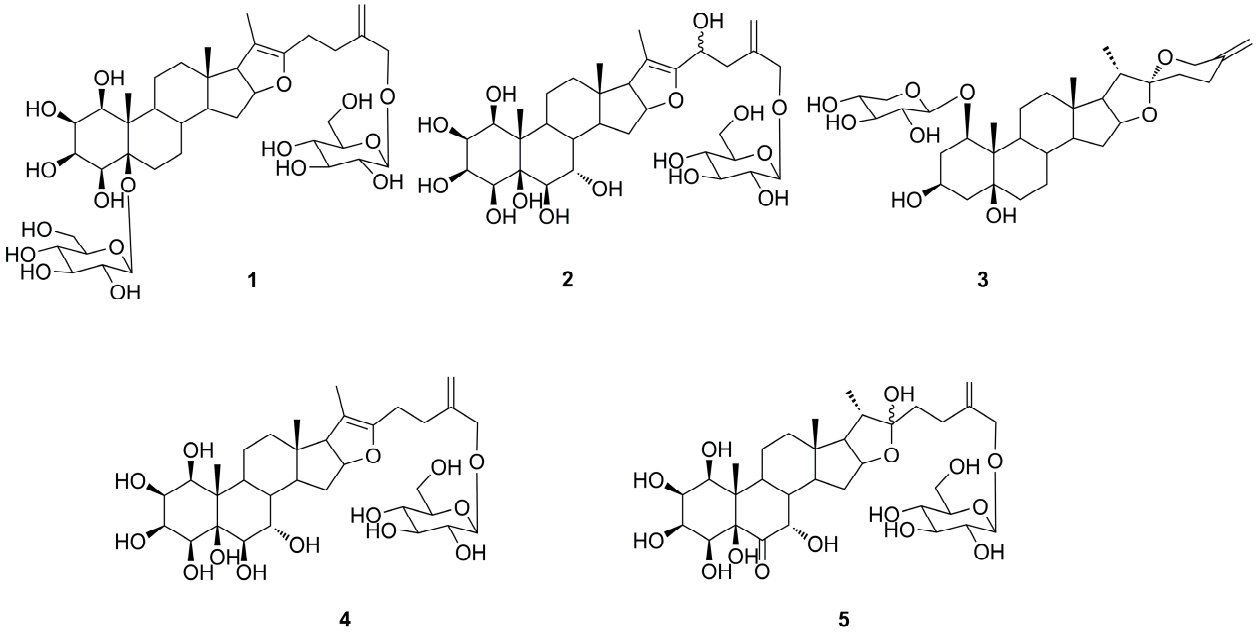

Figure 1. Structures of compounds **1**–**5**.

2. 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 $C_{39}H_{62}O_{17}$ from the HR-ESI-MS peak at *m*/*z* 801.3855 $[M - H]^-$.

The ^{1}H-NMR spectrum showed three methyl protons at δ_H 0.67 (3H, s), 1.70 (3H, s) and 1.58 (3H, s), two *exo*-methylene protons (δ_H 5.35 (1H, brs) and 5.04 (1H, brs)), as well as signals for two anomeric protons at (δ_H 5.28 (d, J = 7.8 Hz) and 4.89 (1H, d, J = 7.7 Hz)). The ^{13}C-NMR spectrum displayed 39 carbon signals, 27 of which belonged to the aglycone carbons, while the remaining signals were assignable to two glucosyl moieties (δ_C 103.8, 75.8, 78.5, 71.7, 78.6 and 62.6, and δ_C 97.4, 76.2, 78.6, 71.9, 78.8 and 62.8). Among carbon signals of the aglycone, δ_C 146.2 and 111.6 were due to an olefinic bond group, δ_C 14.3, 13.7 and 11.7 were due to three methyl groups, and δ_C 77.8, 68.1, 75.2, 67.6, 87.4, 84.4, 64.5 and 71.7 were due to eight oxygenated carbon groups, which indicated that **1** was a furostanol saponin with multiple hydroxyl groups. The structure of **1** was finally determined by analysis of its 2D NMR data (see Figure 2). The HMQC experiment allowed for the assignments of the proton and protonated carbon resonances in the NMR spectra of **1**. HMQC correlations of (δ_H 5.35 (H-27a) and 5.04 (H-27b)) to δ_C 111.6, showed the appearance of a terminal olefinic bond at C-27. Then, 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-19/C-1, C-5, C-9 and C-10, H-3/C-1, C-2 and C-5, and H-6/C-4 and C-5, indicated that all hydroxyl groups were linked at C-1–C-5 of the A ring of the furostanol saponin (see Figure 2). Furthermore, the remaining 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 (see Figure 2). Therefore, the aglycone of **1** was identified as 1, 2, 3, 4, 5, 26-hexanol-furost-20 (22),25(27)-dien. In addition, the HMBC correlation signals of H-Glc-1′/C-5 and H-Glc-1″/C-26, indicated that glucosyl groups were connected as (Glc-1″-*O*-C-26) and (Glc-1′-*O*-C-5) (see Figure 2). The two glucosyl moieties were identified as D-glucose by acid hydrolysis of **1**, followed by TLC comparison with a reference compound and optical rotation determination [12], and judged to be in a β-configuration [13] from the coupling constants of the anomeric protons (7.8 Hz and 7.7 Hz, respectively). In the NOESY spectrum of **1**, 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 (see Figure 2), 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, 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 (see Figure 2). Therefore, compound **1** was identified as 1β,2β,3β,4β,5β,26-hexahydroxyfurost-20(22),25(27)-dien-5,26-*O*-β-D-glucopyranoside.

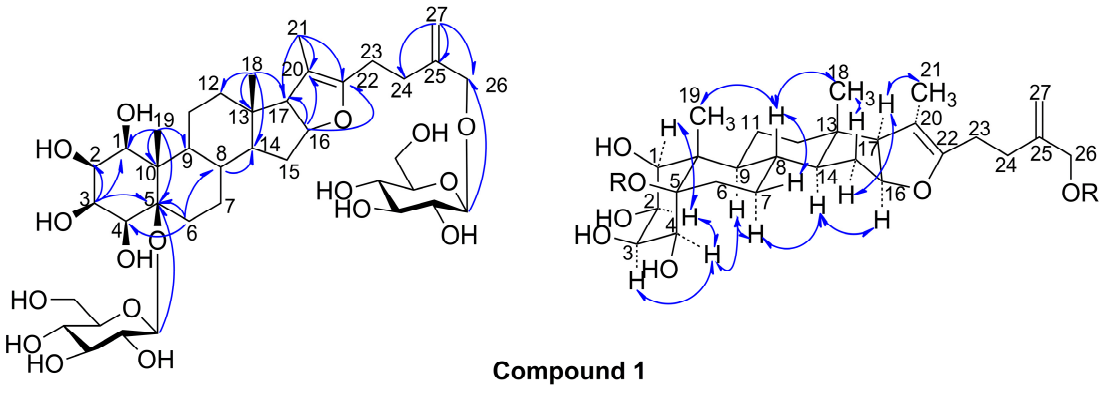

Figure 2. Key HMBC and NOESY correlations of the compound **1**.

Compound **2** was obtained as a white amorphous powder, which showed positive reactions in the Liebermann-Burchard, Ehrlich, and Molisch tests, suggesting that **2** was a furostanol glycoside. The molecular formula $C_{33}H_{52}O_{15}$ was deduced from the HR-ESI-MS peak at *m/z* 711.3198 $[M + Na]^+$. Comparison of the HR-ESI-MS and NMR data of **2** and **1**, indicated almost similar NMR spectroscopic features, except for the number of oxygenated methine groups. In the ^{13}C-NMR spectrum of **2**, only one glucosyl moiety (δ_C 104.2, 75.6, 80.0, 72.1, 79.0, 63.2) was recognized, however, nine oxygenated carbon groups of the aglycone at δ_C 79.1, 67.7, 76.1, 70.2, 78.6, 74.0, 72.5, 64.8 and 72.7 were identified. Meanwhile, the spectroscopic features of **2** were similar to those of tupisteroide B (4), indicating that seven hydroxyl groups were linked at C-1–C-7 of the furostanol saponin, which was confirmed by the ^{1}H-^{1}H COSY correlation of H-1/H-2/H-3/H-4 and H-6/H-7 and the HMBC correlation of H-19/C-1, C-5, C-9 and C-10, and H-6/C-4 and C-5 (see Figure 3). The 26-OH was connected with the glucosyl moiety from the correlation signals of H-Glc-1′/C-26 in the HMBC spectra (see Figure 3). The remaining hydroxyl group was deduced to be linked at C-23, from one oxygen-bearing methine signal occurring at δ_C 64.8 in **2**, instead of a methylene carbon (C-23) at δ_C 34.3 in **4**, which was correlated with a proton signal at δ_H 5.13 (dd, *J* = 6.0, 8.0 Hz, H-23) in the HMQC spectrum, and the correlation signals of H-23/H-24 in the ^{1}H-^{1}H COSY spectrum, the correlation signals of H-23/C-20, C-22, C-24 and C-25, H-24/C-22, C-23, C-25, C-26 and C-27, and H-27/C-24, C-25 and C-26 in the HMBC spectrum (see Figure 3). In addition, the glucosyl moiety was identified as β-D-glucose by the acid hydrolysis procedure and the coupling constant analysis of the anomeric proton (*J* = 7.8 Hz), according to the same protocol as that described for **1**. Thus, the planar structure of **2** was deduced as 1,2,3,4,5,6,7,23,26-nonanolfurost-20(22),25(27)-dien-26-*O*-β-D-glucose. In the NOESY spectrum of **2**, the NOE correlations of Me-19/H-8, H-4/H-2, H-3 and H-9, and H-2/H-1 were observed, indicating 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, which supported the A/B *cis* ring junction pattern (see Figure 3). Besides, NOE correlation of H-7/H-8 was observed and no correlation signals was occurred between Me-19/H-6, which indicated α-axial configuration of 7-OH and β-orientation of 6-OH (see Figure 3). Finally, the NOE correlations of H-8/Me-19 and Me-18, and H-14/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 the α-orientation of Me-21 (see Figure 3). Therefore, compound **2** was identified as 1β,2β,3β,4β,5β,6β,7α,23ξ,26-nonahydroxyfurost-20(22),25(27)-dien-26-*O*-β-D-glucopyranoside.

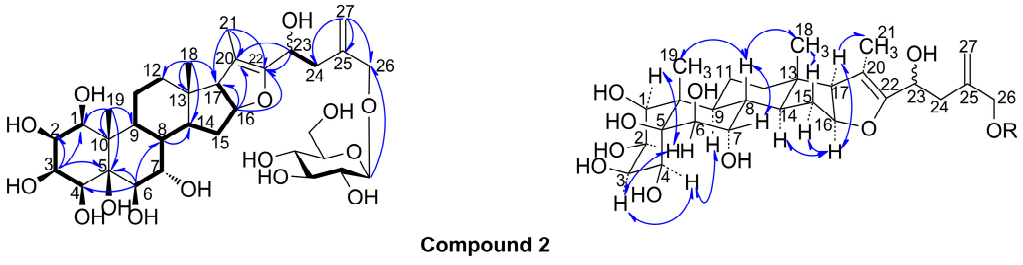

Figure 3. Key HMBC, ^{1}H-^{1}H COSY and NOESY correlations of the compound **2**.

Compound **3** was obtained as a white amorphous powder, and the molecular formula of $C_{32}H_{50}O_9$ was established by the HR-ESI-MS signal at *m/z* 579.3590 $[M + H]^+$. The ^{13}C-NMR spectrum exhibited 32 carbon signals, 27 of which were attributed to the aglycone carbons, while the remaining signals were

assignable to a characteristic of a xylosyl moiety (δC 104.1, 75.8, 78.9, 71.5 and 68.1), which was identified as β-D-xylose by the coupling constant analysis of the anomeric proton (*J* = 7.2 Hz), the acid hydrolysis procedure, TLC comparison, and the optical rotation determination. Among the aglycone carbon signals, the quaternary carbon signal at δC 109.9 (see, Table 1), was identified as an acetal carbon (C-22), a characteristic signal of spirostanol or norspirostanol saponin [14]. In HMBC spectrum, the anomeric proton [4.81 (1H, d, *J* = 7.2 Hz)] of the xylose was correlated with δC 82.5, which was confirmed as C-1 for the HMQC correlation of δH 4.26 (H-1)/δC 82.5 (C-1), ^{1}H-^{1}H COSY correlations of H-1/H-2/H-3/H-4, and HMBC correlations of H-19/C-1, C-5, C-9 and C-10 (see, Figure 4). Moreover, HMBC correlations of H-18/C-12, C-13, C-14 and C-17, H-21/C-17, C-20 and C-22, H-23/C-22, and H-27/C-24, C-25 and C-26, were observed (see, Figure 4).The above data indicated the planar structure of **3** as spirost-25(27)-en-1,3,5-trihydroxy-1-*O*-β-D-xyloside. In the NOESY spectrum of **3** (see, Figure 4), the NOE correlations of Me-19/H-8, H-3/H-2a and H-4, H-2a/H-1, and H-4a/H-7a and H-9, indicated α-axial configurations of H-1 and H-3, and β-orientation of Me-19, 1-OH, 3-OH and 5-OH, which supported the A/B *cis* ring junction pattern; the NOE correlations of H-8/Me-19 and Me-18, and H-14/H-9 and H-7a, supported the B/C and C/D *trans* ring junction pattern; the NOE correlations of Me-18/H-15b and H-20, H-15a/H-16 and H-17, and H-17/Me-21, suggested α-orientation of Me-21. These spectra data was almost similar to those of (20*S*,22*R*)-*1*β,3β,5β-trihydroxyspirost-25(27)-en-5-*O*-β-D-glucopyranoside [8], expect for the site of glycosylation. Therefore, compound **3** was elucidated as (20*S*, 22*R*)-spirost-25(27)-en-1β,3β,5β-trihydroxy-1-*O*-β-D-xyloside.

Table 1. ^{1}H-NMR and ^{13}C-NMR spectral data of compounds **1**–**3**.

Position	1		2		3	
	δc [a]	**δH [a] (*J* in Hz)**	**δc [b]**	**δH [b] (*J* in Hz)**	**δc [c]**	**δH [c] (*J* in Hz)**
1	77.8	4.25 (brs)	79.1	4.29 (brs)	82.5	4.26 (brs)
2	68.1	4.38 (brs)	67.7	4.33 (brs)	30.4	2.53 (H-2a, *ca.*) 1.85 (H-2b, *ca.*)
3	75.2	4.70 (brs)	76.1	4.77 (brs)	67.8	4.59 (brs)
4	67.6	4.08 (brs)	70.2	5.33 (brs)	40.0	2.40 (H-4a, *ca.*) 2.04 (H-4b, *ca.*)
5	87.4	-	78.6	-	74.7	-
6	24.9	1.93 (*ca.*), 2.80 (*ca.*)	74.0	5.03 (brs)	36.3	1.54 (*ca.*), 1.90 (*ca.*)
7	28.5	1.1 (*ca.*), 1.51 (*ca.*)	72.5	4.49 (brs)	29.2	0.98 (H-7a, *ca.*) 1.51 (H-7b, *ca.*)
8	34.4	1.59 (*ca.*)	34.8	2.62 (*ca.*)	35.4	1.67 (*ca.*)
9	46.6	1.19 (*ca.*)	37.8	2.05 (*ca.*)	46.3	1.15 (*ca.*)
10	46.2	-	46.3	-	44.9	-
11	21.9	1.41 (*ca.*), 1.44 (*ca.*)	21.9	1.61 (*ca.*), 1.67 (*ca.*)	22.3	1.14(*ca.*),1.38 (*ca.*)
12	39.7	1.62 (d, 12.0), 1.15 (*ca.*)	40.0	1.70 (d, 12.0), 1.24 (*ca.*)	40.5	1.73 (d, 12.5), 1.13 (*ca.*)
13	43.3	-	43.8	-	41.2	-
14	54.3	0.76 (*ca.*)	48.9	1.96 (*ca.*)	56.7	1.12 (*ca.*)
15	31.0	2.48 (H-15a, *ca.*) 2.38 (H-15b, *ca.*)	34.7	2.58 (H-15a, *ca.*) 1.65 (H-15b, *ca.*)	32.7	2.07 (H-15a, *ca.*) 1.48 (H-15b, *ca.*)
16	84.4	4.77 (q, 7.5)	85.1	4.87 (*ca.*)	81.9	4.62 (q, 7.2)

Table 1. *Cont.*

Position	1		2		3	
	δc [a]	δ_H [a] (*J* in Hz)	δc [b]	δ_H [b] (*J* in Hz)	δc [c]	δ_H [c] (*J* in Hz)
17	64.5	2.42 (*ca.*)	65.3	2.57 (*ca.*)	63.5	1.88 (*ca.*)
18	14.3	0.67 (s)	14.6	0.81 (s)	17.0	0.87 (s)
19	13.7	1.70 (s)	16.0	1.99 (s)	14.4	1.59 (s)
20	103.9	-	105.9	-	42.4	2.00(*ca.*)
21	11.7	1.58 (s)	12.1	1.74 (s)	15.5	1.10 (d, 8.0)
22	151.8	-	154.2	-	109.9	-
23	34.3	1.45 (*ca.*), 2.04 (*ca.*)	64.8	5.13 (dd, 6.0, 8.0)	33.7	1.81 (*ca.*)
24	24.6	2.37 (*ca.*), 2.47 (*ca.*)	40.3	2.88 (H-24a, dd, 6.0, 14.3), 3.10 (H-24b, dd, 8.0, 14.3)	29.4	2.26 (*ca.*) 2.74 (*ca.*)
25	146.2	-	144.4	-	144.9	-
26	71.7	4.58 (d, 13.0) 4.34 (d, 13.0)	72.7	4.75 (d, 13.0) 4.61 (d, 13.0)	65.5	4.50 (d, 12.1) 4.07 (d, 12.1)
27	111.6	5.35 (H-27a, s) 5.04 (H-27b, s)	114.6	5.47 (H-27a, s) 5.28 (H-27b, s)	109.2	4.81(H-27a, s) 4.84 (H-27b, s)
1′	97.4	5.28 (d, 7.8)	104.2	5.0 (d, 7.8)	104.1	4.81 (d, 7.2)
2′	76.2	3.95 (*ca.*)	75.6	4.12 (*ca.*)	75.8	3.99 (*ca.*)
3′	78.6	4.01 (*ca.*)	80.0	4.36 (*ca.*)	78.9	4.21 (*ca.*)
4′	71.9	4.02 (*ca.*)	72.1	4.27 (*ca.*)	71.5	4.23 (*ca.*)
5′	78.8	4.22 (*ca.*)	79.0	3.96 (*ca.*)	68.1	3.78 (t, 10.5), 4.42 (dd, 4.5, 11.5)
6′	62.8	4.52 (*ca.*), 4.21 (*ca.*)	63.2	4.58 (dd, 2.0, 11.8), 4.41 (dd, 5.5, 11.8)	-	-
1″	103.8	4.89 (d, 7.7)	-	-	-	-
2″	75.8	4.03 (*ca.*)	-	-	-	-
3″	78.5	4.22 (*ca.*)	-	-	-	-
4″	71.7	4.19 (*ca.*)	-	-	-	-
5″	78.6	3.92 (*ca.*)	-	-	-	-
6″	62.6	4.52 (*ca.*), 4.35 (*ca.*)	-	-	-	-

[a] δ in pyridine-d_5, in ppm from TMS; coupling constants (*J*) in Hz; ^{1}H-NMR at 500 MHz and ^{13}C-NMR at 125 MHz; [b] δ in pyridine-d_5, ^{1}H-NMR at 600 MHz and ^{13}C-NMR at 150 MHz; [c] δ in pyridine-d_5, ^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz.

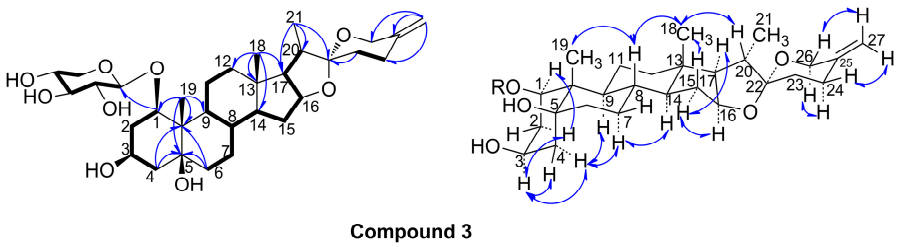

Figure 4. Key HMBC, ^{1}H-^{1}H COSY and NOESY correlations of the compound **3**.

Additionally, the known furostanol saponins were identified by comparison of their spectroscopic data with those reported in the literature as tupisteroide B (**4**) [15] and 5β-furost-$\Delta^{25(27)}$-en-1β,2β,3β,4β,5β,7α,22ξ,26-octaol-6-one-26-*O*-β-D-glucopyranoside (**5**) [16].

The cytotoxic activity of **1**–**4** towards the A549 and H1299 tumor cell lines was measured by the MTT method. Compound **3** exhibited cytotoxicity against A549 cells (IC_{50} 86.63 ± 2.33 μmol·L^{-1}) and H1299 cells (IC_{50} 88.21 ± 1.34 μmol·L^{-1}, see Tables 2 and 3). Considering **3** is a spirostanol saponin, our results showed the cytotoxic activity of this type of steroidal saponin as mentioned in the literature [8,17–19].

Table 2. Activities of compounds **1**–**4** on proliferation of the H1299 cells.

Comp.	1 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} μM
1	1.93 ± 0.95 **	13.50 ± 1.81 **	14.69 ± 1.41 **	16.53 ± 1.26 **	16.90 ± 0.69 **	>100
2	3.95 ± 2.09 **	5.75 ± 1.48 **	11.50 ± 3.22 **	16.17 ± 1.50 **	20.04 ± 1.36 **	>100
3	4.55 ± 1.10 **	8.04 ± 1.94 **	13.47 ± 0.61 **	17.39 ± 0.73 **	55.74 ± 0.87 **	88.21 ± 1.34
4	4.01 ± 0.86 **	9.26 ± 0.44 **	11.46 ± 2.91 **	13.47 ± 1.49 **	26.07 ± 0.99 **	>100
5-FU	3.07 ± 0.52	5.21 ± 0.28	17.39 ± 1.11	47.88 ± 1.38	71.96 ± 2.49	38.65 ± 1.59

The data are expressed as mean ± SD of three independent experiments (** $p < 0.01$ *vs.* control).

Table 3. Activities of compounds **1**–**4** on proliferation of the A549 cells.

Comp.	1 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} μM
1	3.75 ± 1.24 **	11.62 ± 1.88 **	12.83 ± 2.02 **	14.35 ± 0.77 **	20.19 ± 3.63 **	>100
2	4.17 ± 1.30 **	7.68 ± 1.27 **	11.07 ± 1.57 **	13.80 ± 2.05 **	23.11 ± 0.74 **	>100
3	3.95 ± 0.95 **	7.90 ± 1.67 **	13.05 ± 1.75 **	20.60 ± 2.40 **	56.17 ± 1.98 **	86.63 ± 2.33
4	2.93 ± 1.18 **	6.65 ± 0.94 **	7.01 ± 2.47 **	13.21 ± 1.40 **	24.75 ± 1.62 **	>100
5-FU	6.97 ± 0.82	9.03 ± 1.21	23.76 ± 1.22	42.18 ± 1.22	69.24 ± 2.05	42.78 ± 1.63

The data are expressed as mean ± SD of three independent experiments (** $p < 0.01$ *vs.* control).

3. Experimental Section

3.1. General Information

The IR spectra were recorded on a TENSOR-27 instrument (Bruker, Rheinstetten, Germany). ESI-MS was performed on a Quattro Premier instrument (Waters, Milford, MA, USA). The HR-ESI-MS spectra were recorded on an Agilent Technologies 6550 Q-TOF (Santa Clara, CA, USA). 1D and 2D NMR spectra were recorded on Bruker-AVANCE 400, Bruker-AVANCE 500 and Bruker-AVANCE 600 instrument (Bruker, Rheinstetten, Germany) with TMS as an internal standard. The analytical HPLC was performed on a Waters 2695 Separations Module coupled with a 2996 Photodiode Array Detector and a Accurasil C18 column (4.6 mm × 250 mm, 5 mm particles, Ameritech, Chicago, IL, USA). Semipreparative HPLC was performed on a system comprising an LC-6AD pump (Shimadzu, Kyoto, Japan) equipped with a SPD-20A UV detector and a Ultimate XB-C18 (10 mm × 250 mm, 5 mm particles) or YMC-Pack-ODS-A (10 mm × 250 mm, 5 mm particles). D101 was from Sunresin New Materials Co. Ltd. (Xi'an, China). Silica gel was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

3.2. Plant Material

The roots and rhizomes of *T. chinensis* Baker were collected from the Taibai region of Qinba Mountains in Shaanxi Province, China, in August 2010, and identified by senior experimentalist Jitao

Wang. A voucher specimen (herbarium No. 20100816) has been deposited in the Medicinal Plants Herbarium (MPH), Shaanxi University of Chinese Medicine, Xianyang, China.

3.3. Extraction and Isolation

The air-dried and powdered underground parts of *T. chinensis* (1.5 kg) were extracted with 65% EtOH (15 L) 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 (Fr.1–Fr.4). Fr.3 (75 g) was subjected to column chromatography (CC) on silica gel, eluting with gradient solvent system ($CHCl_3$–MeOH–H_2O, 100:0:0–0:50:50) to yield nine fractions (Fr.3-1–Fr.3-9). Fr.3-6 (5 g) was separated over silica gel using $CHCl_3$–MeOH (100:1–50:50) as eluent to obtain eight fractions (Fr.3-6-1–Fr.3-6-8). Fr.3-6-5 (150 mg) and Fr.3-6-7 (370 mg) were purified by HPLC (YMC-Pack-ODS-A, 10 mm × 250 mm, 5 μm particles, flow rate: 1.0 mL·min^{-1}) with CH_3OH–H_2O (45:55) as mobile phase to afford **1** (23 mg; t_R = 35 min), **2** (15 mg; t_R = 27 min), **3** (20 mg; t_R = 43 min), **4** (27 mg; t_R = 47 min) and **5** (1.8 mg; t_R = 65 min).

3.4. 1β,2β,3β,4β,5β,26-Hexahydroxyfurost-20(22),25(27)-dien-5,26-O-β-D-glucopyranoside (**1**)

A white amorphous powder, IR (KBr) ν_{max}: 3450, 2980, 1694, 1025, 907, 804, 772 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (125 MHz, pyridine-d_5) spectral data, see Table 1; *m*/*z* 801.3855 $[M - H]^-$ (calcd. for $C_{39}H_{61}O_{17}$, 801.3909).

3.5. 1β,2β,3β,4β,5β,6β,7α,23ξ,26-Nonahydroxyfurost-20(22),25(27)-dien-26-O-β-D-glucopyranoside (**2**)

A white amorphous powder, IR (KBr) ν_{max}: 3475, 2980, 1742, 1062, 904, 804 cm^{-1}. ^{1}H-NMR (600 MHz, pyridine-d_5) and ^{13}C-NMR (150 MHz, pyridine-d_5) spectral data, see Table 1; *m*/*z* 711.3198 $[M + Na]^+$ (calcd. for $C_{33}H_{52}O_{15}Na$, 711.3204).

3.6. (20S,22R)-Spirost-25(27)-en-1β,3β,5β-trihydroxy-1-O-β-D-xyloside (**3**)

A white amorphous powder; IR (KBr) ν_{max}: 3306, 2922, 1650, 1042, 989, 917, 892, 876 cm^{-1}; ^{1}H-NMR (400 MHz, pyridine-d_5) and ^{13}C-NMR (100 MHz, pyridine-d_5) spectral data, see Table 1; *m*/*z* 579.3590 $[M + H]^+$ (calcd. for $C_{32}H_{51}O_9$, 579.3633).

3.7. Acid Hydrolysis of Compounds **1**, **2**, **3** *and Absolute Sugar Configuration Determination*

The solutions of compounds **1** (3 mg), **2** (3 mg) and **3** (5 mg) were hydrolyzed with 2*N* 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** and **2** were subjected to CC over silica gel eluted with MeCN–H_2O (8:1) to yield D-glucose, which was determined by TLC comparison (MeCN–H_2O, 6:1) with the authentic sugar and the optical rotation determination $[\alpha]_D^{20}$ +49.2 (*c* 0.16, H_2O). The aqueous layer of **3** was subjected to CC over silica gel eluted with MeCN–H_2O (8:1–15:1) to yield D-xylose, which was identified by TLC comparison with the authentic sugar and the optical rotation determination $[\alpha]_D^{20}$ +17.9 (*c* 0.14, H_2O).

3.8. Cytotoxicity Assay

The cytotoxic activity assays towards the A549 and H1299 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**–**4** were dissolved in DMSO and diluted with complete medium to five concentration levels (from 0.001 $mmol \cdot L^{-1}$ to 0.1 $mmol \cdot L^{-1}$) for inhibition rate determination. After incubation at 37 °C for 4 h, the supernatant was removed before adding DMSO (100 μL) to each well. 5-Fluorouracil (5-Fu) was used as positive control. The inhibition rate (IR) and IC_{50} were calculated. Values are mean ± SD, $n = 3$, ** $p < 0.01$ *vs.* DMEM control. Compound **3** exhibited cytotoxicity against A549 cells (IC_{50} 86.63 ± 2.33 $\mu mol \cdot L^{-1}$) and H1299 cells (IC_{50} 88.21 ± 1.34 $\mu mol \cdot L^{-1}$), while the positive control of 5-Fu exhibited cytotoxicity against A549 cells (IC_{50} 42.78 ± 1.63 $\mu mol \cdot L^{-1}$) and H1299 cells (IC_{50} 38.65 ± 1.59 $\mu mol \cdot L^{-1}$), respectively, (see Tables 2 and 3).

Supplementary Materials

IR, HR-ESI-MS, ^{1}H-NMR ^{13}C-NMR and 2D NMR spectra for compounds **1**–**3** can be accessed at: http://www.mdpi.com/1420-3049/20/08/13659/s1.

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Author Contributions

Every author has participated in the research and did his or her individual contribution to the article: Y.L. and X.W. conducted the experiments and collected the data; D.Z. and Y.J. planned and coordinated the experiments; H.H. and F.W. carried out the cytotoxicity biology experiments; X.Y. and Z.T. analysed the data; X.S. designed the study, and Z.Y. planned and oversaw the research project and drafted the paper. Finally, All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **3**–**5** are available from the authors.