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Steroidal saponins from *Tribulus terrestris*

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ABSTRACT

Sixteen steroidal saponins, including seven previously unreported compounds, were isolated from *Tribulus terrestris*. The structures of the saponins were established using 1D and 2D NMR spectroscopy, mass spectrometry, and chemical methods. They were identified as: 26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-4-en-2*α*,3*β*,22*α*,26-tetrol-12-one (terrestrinin C), 26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-4-en-22*α*,26-diol-3,12-dione (terrestrinin D), 26-*O*-*β*-D-glucopyranosyl-(25*S*)-furost-4-en-22*α*,26-diol-3,6,12-trione (terrestrinin E), 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furostan-3*β*,22*α*,26-triol-12-one (terrestrinin F), 26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-4-en-12*β*,22*α*,26-triol-3-one (terrestrinin G), 26-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl-(25*R*)-furost-4-en-22*α*,26-diol-3,12-dione (terrestrinin H), and 24-*O*-*β*-D-glucopyranosyl-(25*S*)-5*α*-spirostan-3*β*,24*β*-diol-12-one-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (terrestrinin I). The isolated compounds were evaluated for their platelet aggregation activities. Three of the known saponins exhibited strong effects on the induction of platelet aggregation.

1. Introduction

Tribulus terrestris L. is an annual creeping herb native to the Mediterranean region, but it now grows worldwide, especially in subtropical areas. The plant is used in folk medicine in China, India, Bulgaria and other countries. The fruits of *T. terrestris*, a Chinese traditional medicine termed "Ji Li", are used to treat eye inflammation, skin irritation, high blood pressure, abdominal distention, and cardiovascular diseases. A wide range of compounds have been extracted from this plant: saponins (Tomova et al., 1974; Wang et al., 1996, 1997, 2009; Cai et al., 2001; Huang et al., 2003; Kostova and Dinchev, 2005; Su et al., 2009; Xu et al., 2009, 2010a, 2010b; Liu et al., 2010; Chen et al., 2012, 2013), flavonoids (Bhutani et al., 1969; Saleh et al., 1982), and alkaloids (Wo et al., 1999). Among the compounds, several studies have demonstrated that saponins are responsible for the biological activities of *T. terrestris* extracts (Tomova et al., 1981; Bedir et al., 2002; Zhang et al., 2010). To identify additional active steroidal saponins from *T. terrestris*, five known compounds (**8**, **9**, **11**, **13**, and **14**) were recently reported (Wu et al., 2012). Further phytochemical analysis of the whole plant extract led to seven new steroidal saponins being isolated namely six furostanol saponins (**1**–**6**) and one spirostanol saponin (**7**), along with four known steroidal saponins (**10**, **12**, **15**, and **16**) (Fig. 1). Among the identified compounds, the aglycones of compounds **1**, **3**, **5**, and **7** were observed for the first time. In the present paper, the isolation and structural elucidation of the seven unreported steroidal saponins are described. Since the saponin fraction of Chinese *T. terrestris* shows a significant effect on cardiovascular diseases (Kostova and Dinchev, 2005; Yin et al., 2005) and the effect of steroidal saponins on platelet aggregation was previously reported (Fu et al., 2008; Cong et al., 2010, 2012; Kang et al., 2012), the activities of isolated saponins (**1**, **2**, and **4**–**16**) on platelet aggregation were also evaluated in this paper.

2. Results and discussion

The whole plant of *T. terrestris* was extracted using 70% aq. EtOH. The extract was subjected to macroporous resin SP825 column to afford two saponin-rich fractions. The two fractions were further separated chromatographically using a series of silica-gel columns, ODS silica-gel columns, and semi-preparative HPLC to afford sixteen steroidal saponins. The structures of the seven previously unreported compounds, termed terrestrinins C–I (**1**–**7**), were elucidated by analysis using HRESIMS and 1D- and 2D-NMR, including COSY, TOCSY, HSQC, HMBC, and ROESY

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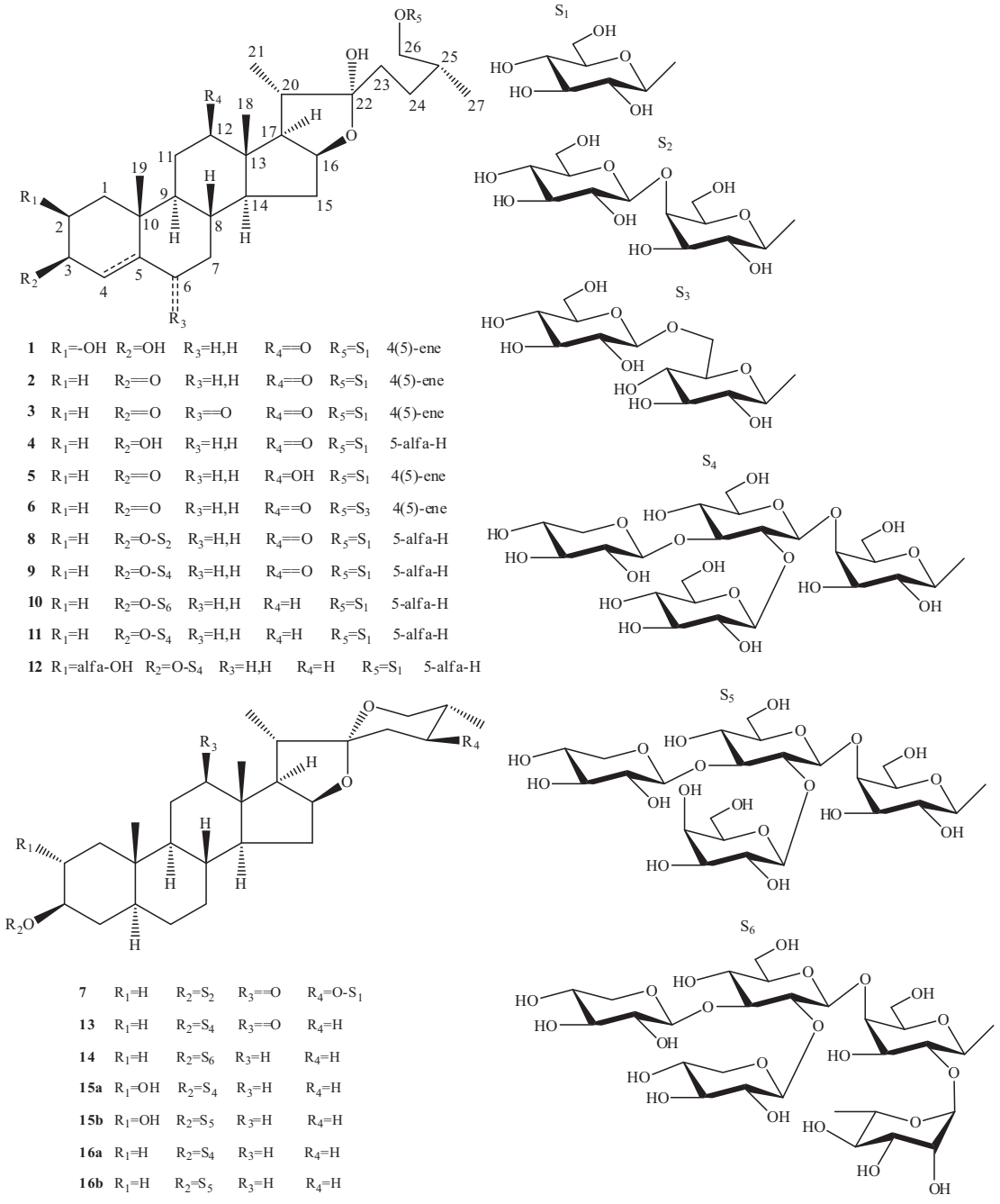

1	R_1=-OH	R_2=OH	R_3=H,H	R_4=O	R_5=S_1	4(5)-ene
2	R_1=H	R_2=O	R_3=H,H	R_4=O	R_5=S_1	4(5)-ene
3	R_1=H	R_2=O	R_3=O	R_4=O	R_5=S_1	4(5)-ene
4	R_1=H	R_2=OH	R_3=H,H	R_4=O	R_5=S_1	5-alfa-H
5	R_1=H	R_2=O	R_3=H,H	R_4=OH	R_5=S_1	4(5)-ene
6	R_1=H	R_2=O	R_3=H,H	R_4=O	R_5=S_3	4(5)-ene
8	R_1=H	R_2=O-S_2	R_3=H,H	R_4=O	R_5=S_1	5-alfa-H
9	R_1=H	R_2=O-S_4	R_3=H,H	R_4=O	R_5=S_1	5-alfa-H
10	R_1=H	R_2=O-S_6	R_3=H,H	R_4=H	R_5=S_1	5-alfa-H
11	R_1=H	R_2=O-S_4	R_3=H,H	R_4=H	R_5=S_1	5-alfa-H
12	R_1=alfa-OH	R_2=O-S_4	R_3=H,H	R_4=H	R_5=S_1	5-alfa-H

7	R_1=H	R_2=S_2	R_3=O	R_4=O-S_1
13	R_1=H	R_2=S_4	R_3=O	R_4=H
14	R_1=H	R_2=S_6	R_3=H	R_4=H
15a	R_1=OH	R_2=S_4	R_3=H	R_4=H
15b	R_1=OH	R_2=S_5	R_3=H	R_4=H
16a	R_1=H	R_2=S_4	R_3=H	R_4=H
16b	R_1=H	R_2=S_5	R_3=H	R_4=H

Fig. 1. Structures of compounds **1**–**16**.

experiments. The four known compounds were identified as tribuluside A (**10**) (Xu et al., 2007), purpureagitosid (**12**) (Tschesche et al., 1974; Kang et al., 2006), a mixture of F-gitonin (**15a**) and desglucolanatigonin II (**15b**) (Inoue et al., 1995; Wang et al., 1996), and a mixture of desgalactotigonin (**16a**) and gitonin (**16b**) (Wang et al., 1996) by comparison of their NMR and MS data with those previously reported, and the five previously isolated compounds (Wu et al., 2012) were identified as 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furostan-3*β*,22*α*,26-triol-12-one-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (**8**) (Chen et al., 2012; Wu et al., 2012), polianthoside D (**9**) (Jin et al., 2004; Wu et al., 2012), uttroside B (**11**) (Zhou et al., 2006; Wu et al., 2012), hecogenin-3-*O*-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (**13**) (Huang et al., 2003; Wu et al., 2012), 25*R*-tribulosin (**14**) (Mahato et al., 1981; Wang et al., 1996; Wu et al., 2012).

Compound **1** was isolated as a white amorphous powder. The negative HRESIMS showed an $[M-H]^-$ ion peak at *m/z* 623.3433, corresponding to the molecular formula $C_{33}H_{52}O_{11}$. The ^{1}H NMR spectrum of **1** showed two methyl singlets at δ 1.17 (s, CH_3-18) and 1.19 (s, CH_3-19); two methyl doublets at δ 1.54 (d, J = 7.2 Hz, CH_3-21) and 0.99 (d, J = 7.2 Hz, CH_3-27); three methine protons indicative of secondary alcoholic functions at δ 4.20 (m, H-2), 4.52 (br d, J = 4.8 Hz, H-3), and 4.87 (m, H-16); and one olefinic proton at δ 5.71 (br s, H-4). The ^{13}C NMR spectrum showed 33 carbons, including two olefinic carbons at δ 123.2 (C-4) and 145.7 (C-5) and one ketone group δ 212.3 (C-12). The ^{1}H and ^{13}CNMR spectroscopic assignments of the aglycone moiety of **1**

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Table 1
^{1}H and ^{13}C NMR chemical shifts assignments of the aglycone moiety of saponins **1–7** in pyridine-d_5.

Position	**1**		**2**		**3**		**4**		**5**		**6**		**7**	
	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H/ (Hz)
1	40.5	1.71 dd (10.2, 13.8) 2.19 dd (3.0, 13.8)	35.4	1.42 td (5.1, 13.8) 1.65 o*	35.0	1.68 m 1.80 m	36.9	0.85 m 1.45 m	35.8	1.48 dt (4.5, 13.8) 1.81 o	35.4	1.43 m 1.66 m	36.6	0.71 m 1.30 m
2	70.6	4.20 m	34.1	2.36 m 2.33 m	34.1	2.43 m 2.54 m	32.2	1.63 m 1.98 m	34.3	2.29 o, 2.36 m	34.2	2.37 m, 2.33 m	29.8	1.53 m 1.97 m
3	73.1	4.52br d (4.8)	197.9	–	198.8	–	70.3	3.79 m	198.2	–	197.9		76.9	3.86 m
4	123.2	5.71 br s	124.7	5.84 s	126.5	6.47 s	39.1	1.54 m 1.79 m	124.2	5.84 br s	124.7	5.83 br s	34.6	1.30 m 1.80 br d (12.0)
5	145.7	–	168.6	–	159.1	–	45.0	1.03 m	170.1	–	168.6	–	44.4	0.83 m
6	31.7	2.03 o 2.29 br t (13.2)	32.4	2.11 m 2.28 m	200.5	–	28.7	1.23 m 1.25 m	32.8	2.07 o 2.23 dt (4.8, 14.0)	32.4	2.10 m 2.29 m	28.6	1.12 o
7	33.4	1.64 m, 0.75 m	31.5	0.88 qd (13.0, 4.0) 1.67 o	46.0	2.15 dd (8.8, 16.5) 2.80 dd (4.5, 16.5)	31.7	0.79 m, 1.59 m	32.1	0.82 br q (13.0) 1.65 o	31.5	1.66 m, 0.83 qd (4.2, 13.2)	31.7	1.51 m 0.74 m
8	34.9	2.00 m	34.3	1.96 m	32.5	2.47 m	34.4	1.81 m	34.3	1.63 o	34.2	1.96 m	34.3	1.67 m
9	53.1	1.50 m	54.7	1.17 o	51.5	1.79 m	55.7	0.96 m	53.1	0.91 br t (10.4)	54.7	1.18 m	55.3	0.87 m
10	39.9	–	38.9	–	39.4	–	36.4	–	38.7	–	38.9	–	36.3	–
11	38.7	2.48 dd (4.8, 13.8) 2.55 dd (13.2, 13.8)	37.4	2.25 m 2.53 t (13.8)	37.0	2.42 dd (5.5, 14.6) 2.67 t (14.4)	38.1	2.31 dd (5.0, 13.8) 2.45 t (13.8)	31.2	1.64 o, 1.81 m	37.3	2.25 m, 2.53 t (13.8)	37.9	2.20 dd (4.9, 14.0) 2.33 t (13.4)
12	212.3	–	211.9	–	211.2	–	213.1	–	78.9	3.57 dd (4.8, 10.8)	212.0	–	212.7	–
13	55.8	–	55.5	–	55.4	–	55.7	–	46.7	–	55.5	–	55.3	–
14	55.4	1.28 m	55.0	1.40 m	55.4	1.67 m	55.8	1.41 m	54.4	1.08 m	55.0	1.40 m	55.5	1.33 m
15	31.7	1.61 m 2.04 o	31.7	1.64 o 2.09 m	31.4	1.65 m 2.12 m	31.8	1.61 m 2.08 m	32.1	1.65 o 2.07 m	31.7	1.64 o 2.08 m	31.3	1.49 m 2.03 m
16	79.7	4.87 m	79.6	4.87 q (7.1)	79.4	4.89 q (7.0)	79.7	4.88 m	81.1	5.01 q (7.2)	79.5	4.87 q (8.4)	80.1	4.44 q (7.0)
17	55.2	2.86 qd (7.2, 8.4)	55.1	2.88 qd (6.6, 8.6)	55.0	2.93 dd (6.7, 8.5)	55.1	2.91 dd (6.6, 8.4)	63.7	2.31 o	55.1	2.88 dd (6.0 8.6)	53.9	2.69 dd (6.6 8.4)
18	16.3	1.17 s	16.2	1.19 s	16.3	1.24 s	16.3	1.15 s	11.3	1.19 s	16.2	1.20 s	16.1	0.98 s
19	21.7	1.19 s	16.5	1.08 s	16.8	1.15 s	11.9	0.82 s	17.1	1.04 s	16.5	1.08 s	11.7	0.65 s
20	41.2	2.21 m	41.3	2.22 m	41.3	2.24 m	41.3	2.22 qd (6.6, 6.0)	41.7	2.50 p (7.8)	41.2	2.23 m	42.8	1.87 m
21	15.3	1.54 d (7.2)	15.2	1.54 d *J* = 7.2	15.2	1.57 d (6.9)	15.3	1.56 d (6.6)	15.7	1.61 d *J* = 6.6	15.3	1.55 d (6.6)	13.8	1.26 d (7.2)
22	110.7	–	110.8	–	110.8	–	110.8	–	110.9	–	110.8	–	111.7	–
23	37.1	2.00 o 2.06 o	37.1	2.02 o 2.05 o	37.1	2.06 o	37.1	2.02 o	37.3	2.08 o	37.1	2.05 o	40.9	1.95 t (12.6) 2.67 dd (4.8, 13.1)
24	28.4	1.66 o 2.03 o	28.4	1.66 o 2.04 o	28.4	1.68 m 2.06 o	28.4	1.65 m 2.04 o	28.5	1.70 m 2.04 m	28.5	1.68 m 2.05 o	81.5	4.01 m
25	34.3	1.92 m	34.3	1.93 m	34.5	1.96 m	34.3	1.94 m	34.3	1.92 m	34.2	1.93 m	38.1	1.89 m
26	75.3	3.61 dd (6.0, 9.0) 3.94 m	75.3	3.61 dd (6.0, 8.4) 3.94 m	75.3	3.63 t (5.9, 9.3) 3.97 m	75.3	3.61 dd (8.4, 6.6) 3.94 m	75.3	3.63 dd (6.0, 9.0) 3.92 m	75.3	3.57 dd (6.0, 9.6) 4.03 m	65.2	3.55 t (11.5) 3.63 dd (5.0, 11.5)
27	17.4	0.99 d (7.2)	17.4	0.98 d (6.6)	17.4	0.99 d (6.7)	17.4	0.98 d (6.6)	17.5	0.98 d (6.6)	17.4	0.99 d (6.6)	13.5	1.13 d (6.6)

(Table 1) were established from the analysis of the ^{1}H–^{1}H COSY, HSQC and HMBC experiments. The $^{3}J_{H-H}$ COSY correlations for δ 5.71 (H-4)/δ 4.52 (H-3)/δ 4.20 (H-2)/δ 2.19 and 1.71 (H_2-1) indicated two hydroxyl groups at the C-2 and C-3 positions of the aglycone of **1**. The HMBC correlations between the proton signal at δ 5.71 (H-4) and the carbon resonances at δ 70.6 (C-2), 39.9 (C-10), and 31.7 (C-6), and between δ 1.19 (H_3-19) and δ 39.9 (C-10), 40.5 (C-1), 53.1 (C-9), and 145.7 (C-5) also supported the presence of the OH-2 and OH-3 in the aglycone. The β orientation of the oxygen atom at C-2 and C-3 positions was confirmed by the spin-coupling constants between the proton signals of H-2 (δ 4.20) and H_2-1 ($^{3}J_{H\text{-}1ax\text{–}H\text{-}2ax}$ = 10.2 Hz and $^{3}J_{H\text{-}1eq\text{–}H\text{-}2ax}$ = 3.0 Hz), and between the proton resonances of H-3 (δ 4.52) and H-2 ($^{3}J_{H\text{-}2ax\text{–}H\text{-}3eq}$ = 4.8 - Hz), and NOE correlations between H-2 (δ 4.20) and H-9 (δ 1.50), whereas H-3 at δ 4.52 possessed a NOE correlation with H-2. The C-25 configuration was deduced to be (*R*) based on the difference of the chemical shifts of the geminal protons CH_2-26 (Δab = 0.33 ppm). The Δab was established to be <0.48 ppm in 25*R*-furostane steroids (Agrawal, 2005). NOE correlations were observed between H_3-19/H-11, H-8/H_3-18, H_3-18/H-20, H-9/H-14, H-16/H-17, and H-17/H-21, suggesting the usual *trans* junction for the B/C and C/D rings and a *cis* ring fusion for the D/E rings. The α orientation of OH-22 in the aglycone moiety was deduced from the semiketal carbon signal at δ 110.7 (Fattorusso et al., 2002), and was further confirmed by the NOE correlation between H-20 (δ 2.21) and H-23 (δ 2.06). Thus, the aglycone moiety of **1** was deduced to be (25*R*)-furost-4-en-2β,3β,22α,26-tetrol-12-one, a sapogenin previously unreported. The anomeric regions in the ^{1}H and ^{13}C NMR spectra of **1** showed one anomeric proton at δ 4.81 (d, *J* = 7.8 Hz), corresponding to the anomeric carbon signal at δ 105.0. Glucose was detected after acidic hydrolysis of **1** and its absolute configuration was identified as D by GC analysis. The large coupling constants ($^{3}J_{1,2}$ > 7 Hz) were consistent with the β configuration of the sugar (Agrawal, 1992). The long-range correlation between δ 4.81 (H-1-Glc) and δ 75.3 (C-26) in the HMBC spectrum and the NOE correlation between δ 4.81 (H-1-Glc) and δ 3.94 (H-26) revealed the sugar linkage site to be the aglycone moiety. Thus, the structure of **1** was elucidated as 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-4-en-2β,3β,22α,26-tetrol-12-one, named terrestrinin C.

Compound **2** was isolated as a white amorphous powder with a molecular formula of $C_{33}H_{50}O_{10}$, as determined by HRESIMS (*m*/*z*: 605.3347 $[M-H]^-$). A comparison of the NMR and MS data of **2** with those of **1** (Tables 1 and 2) indicated that while they have similar structures, they exhibited significant differences in the A ring of the aglycone. Because the ^{13}C NMR resonances of C-2–C-5 were markedly shifted [δ 34.1 (C-2), 197.9 (C-3), 124.7 (C-4), and 168.6 (C-5) instead of δ 70.6 (C-2), 73.1 (C-3), 123.2 (C-4), and 145.7 (C-5) in compound **1**], the lack of two hydroxyl groups at C-2 and C-3 positions and a ketone group as the substituent at the C-3 position of the aglycone in **2** could be deduced. This was confirmed by the HMBC correlation between the C-3 (δ 197.9) and H_2-2 (δ 2.36 and 2.33) and H_2-1 (δ 1.42 and 1.65). Thus, the aglycone of saponin **2** was identified as (25*R*)-furost-4-en-22α, 26-diol-3,12-dione (Xu et al., 1998). The sugar unit and its linkage site to the aglycone were identified as in **1**. Thus, **2** was determined to be 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-4-en-22α,26-diol-3,12-dione, named terrestrinin D.

Compound **3** showed an $[M-H]^-$ ion peak at *m*/*z* 619.3146 in the negative HRESIMS, corresponding to the empirical molecular formula $C_{33}H_{48}O_{11}$, suggesting that saponin **3** was an oxo derivative of saponin **2**. A detailed comparison of the NMR spectroscopic

Table 2
^{1}H and ^{13}C NMR chemical shifts assignments of the glycosides part of saponins **1**–**7** in pyridine-d_5.

Position	**1**		**2**		**3**		**4**		**5**		**6**		**7**	
	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)
	Glc		Glc		Glc		Glc		Glc		Glc′		Gal′	
1′	105.0	4.81 d (7.8)	105.0	4.81 d (7.8)	105.0	4.83 d (7.8)	105.0	4.81 d (8.4)	105.0	4.81 d (7.8)	104.9	4.74 d (7.8)	102.5	4.86 d (7.8)
2′	75.2	4.02 dd (7.8, 8.4)	75.2	4.01 m	75.3	4.04 t (8.5)	75.2	4.01 t (8.4)	75.2	4.02 dd (7.8, 9.0)	75.1	3.96 t (8.2)	73.5	4.38 dd (7.8, 9.6)
3′	78.7	4.22 o	78.7	4.22 o	78.7	4.25 o	78.7	4.22 o	78.7	4.23 o	78.6	4.17 dd (8.7, 8.8)	75.3	4.24 o
4′	71.8	4.21 o	71.8	4.21 0	71.7	4.24 o	71.8	4.22 o	71.7	4.24 o	71.7	4.15 dd (8.7, 9.8)	80.1	4.70 br d (3.0)
5′′	78.5	3.93 m	78.5	3.93 m	78.5	3.96 m	78.5	3.94 m	78.5	3.93 m	77.3	4.05 m	75.5	4.08 m
6′	62.9	4.37 dd (5.4, 12.0), 4.54 dd (1.8, 12.0)	62.9	4.38 m, 4.54 brd (11.7)	62.9	4.40 dd (5.0, 11.7) 4.56 dd (2.2, 11.7)	62.9	4.38 dd (5.4, 12.0) 4.54 dd (1.8, 12.0)	62.9	4.39 dd (4.8, 11.4) 4.55 br d (11.4)	70.2	4.33 dd (6.0, 11.4) 4.83 dd (1.8, 11.4)	61.1	4.27 dd (5.4, 10.7) 4.66 dd (8.5, 10.7)
											Glc′′		Glc′′	
1′′											105.5	5.11 (7.8)	107.2	5.28 d (7.8)
2′′											75.3	4.04 m	76.0	4.13 dd (7.8, 9.0)
3′′											78.5	4.21 o	78.7	4.22 o
4′′											71.7	4.22 o	71.8	4.24 o
5′′											78.5	3.92 m	78.1	3.87 m
6′′											62.8	4.37 dd (5.0, 11.8) 4.51 br d (10.0)	62.9	4.35 dd (5.3, 11.7) 4.51 dd (2.4, 11.7)
													Glc′′′	
1′′′													106.5	4.92 d (7.8)
2′′′													75.7	4.04 o
3′′′													78.6	4.20 o
4′′′													72.3	4.06 o
5′′′													78.5	4.01 o
6′′′													63.2	4.20 o 4.59 dd (2.2, 11.6)

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data of **3** with those of **2** indicated that an additional carbonyl was at δ 200.5 (Table 1), this being attached to the A or B rings of the aglycone. In the HMBC spectrum, long range correlations were observed between C-6 (δ 200.5) and H-4 (δ 6.47) and H_2-7 (δ 2.15 and 2.80), and between H_3-19 (δ 1.15) and C-1 (δ 35.0), C-10 (δ 39.4), C-9 (δ 51.5), and C-5 (δ 159.1), confirming the 6-one structure. The difference in the chemical shifts of the geminal H_2-26 protons ($\Delta\delta_H$ = 0.34 ppm) proved the configuration of C-25 to be *R*. The analysis of the 1D- and 2D-NMR experiments allowed the aglycone of **3** to be identified as (25*R*)-furost-4-en-22α,26-diol-3,6,12-trione, an aglycone reported for the first time. The sugar unit and its linkage site were identified as in compound **1** (Table 2). Thus, the structure of **3** was established as 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-4-en-22α,26-diol-3,6,12-trione, termed terrestrinin E.

Compound **4** displayed an $[M-H]^-$ ion peak at *m*/*z* 607.3497 in the negative HRESIMS, indicating a molecular formula of $C_{33}H_{54}O_{10}$. A detailed comparison of the NMR spectroscopic data of **4** with those of **1** indicated they shared similar aglycone and sugar moieties, except at the A and B rings of the aglycone moiety. The lack of one hydroxy group at position C-2 and a double bond at position C-4(5) of the aglycone in compound **4** was deduced from the chemical shift of its C-1–C6 (δ 36.9, 32.2, 70.3, 39.1, 45.0, and 28.7 instead of δ 40.5, 70.6, 73.1, 123.2, 145.7, and 31.7 in compound **1**) (Table 1). The chemical shift of C-19 at δ 11.9 indicated an α orientation for H-5 (Agrawal et al., 1995). Through analysis of the 1D- and 2D-NMR spectroscopic data of **4**, its structure was defined as 26-*O*-β-D-glucopyranosyl-(25*R*)-5α-furostan-3β,22α,26-triol-12-one, named terrestrinin F.

Compound **5** showed an $[M-H]^-$ ion peak at *m*/*z* 609.3638 in the negative HRESIMS, corresponding to a molecular formula of $C_{33}H_{54}O_{10}$. A detailed comparison of the NMR spectroscopic data of **5** with those of **2** established that they shared similar aglycone and sugar moieties, except that the C and D rings of the aglycone lack a ketone group but possess an additional hydroxy group at position C-12. The long range correlations between the proton at δ 3.57 (H-12) and the carbon signals at δ 11.3 (C-18), 46.7 (C-13), 54.4 (C-14), and 63.7 (C-17) in the HMBC spectrum and the cross-peak correlations between δ 3.57 (H-12) and δ 1.64 and 1.81 (H_2-11) in the ^1H–^1H COSY spectrum indicated that the hydroxy group was located at the C-12 position of the aglycone. The β orientation of OH-12 was confirmed by the spin-coupling constant between the proton resonances of 12-H and 11-H_2 ($^3J_{H\text{-}12ax\text{–}H\text{-}11ax}$ = 10.8 Hz and $^3J_{H\text{-}12ax\text{–}H\text{-}11eq}$ = 4.8 Hz) and the NOE correlations between H-12/H-9/H-14/H-11/H-17 in the ROESY spectrum. Therefore, the aglycone of **5** was elucidated as (25*R*)-furost-4-en-12β,22α,26-triol-3-one (Hamed et al., 2004; Mimaki et al., 1998). Through analysis of the 1D- and 2D-NMR spectroscopic data of **5**, its structure was defined as 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-4-en-12β,22α,26-triol-3-one, named terrestrinin G.

Compound **6** was isolated as a white amorphous powder. Its negative HRESIMS showed an $[M-H]^-$ ion peak at *m*/*z* 767.3849, corresponding to a molecular formula of $C_{39}H_{60}O_{15}$. Comparing the NMR and MS data with those of **2**, compound **6** was determined to have the same aglycone as **2** but with an additional sugar residue. The ^1H NMR spectrum showed two anomeric protons at δ 4.74 (d, *J* = 7.8 Hz) and 5.11 (d, *J* = 7.8 Hz), corresponding to two anomeric carbon signals at δ 104.9 and 105.5 in the ^{13}C NMR spectrum. The ^1H and ^{13}C NMR assignments of the sugar moiety of **6** (Table 2) were established by analysis of the ^1H–^1H COSY, HSQC and HMBC experiments. The HMBC cross peaks of δ 4.74 (H'-1-Glc) with δ 75.3 (C-26), and δ 5.11 (H''-1-Glc) with δ 70.2 (C-6′) permitted deduction of the sequence of the sugars and their linkage site to the aglycone moiety. Thus, the structure of **6** was elucidated as 26-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(25*R*)-furost-4-en-22α,26-diol-3,12-dione, named terrestrinin H.

Compound **7** was isolated as a white amorphous powder. It showed an $[M-H]^-$ ion peak at *m*/*z* 931.4561 in the negative HRESIMS, corresponding to a molecular formula of $C_{45}H_{72}O_{20}$. Its ^1H NMR spectrum showed four methyl signals at δ 0.65 (s, CH_3-19), 0.98 (s, CH_3-18), 1.13 (d, *J* = 6.6 Hz, CH_3-27), and 1.26 (d, *J* = 7.2 Hz, CH_3-21); two methylene proton resonances of CH_2OR at δ 3.55 (t, *J* = 11.5 Hz, Hax-26) and 3.63 (dd, *J* = 5.0, 11.5 Hz, Heq-26); and three methine proton signals of CHOR at δ 3.86 (m, H-3), 4.01 (m, H-24), and 4.44 (q, *J* = 7.0 Hz, H-16). The hydroxy group at the C-24 position was confirmed by the HMBC corrections between the proton resonances at δ 1.95 and 2.67 (H_2-24), 1.89 (H-25), 3.55 and 3.63 (H_2-26), and 1.13 (H_3-27) and the carbon signal at δ 81.5 (C-24). The C-24*S* and C-25*S* configurations were deduced from the proton multiplicities of H-23 and H-26, with *J* values of 12.6 Hz (H-24/H-23ax), 4.8 Hz (H-24/H-23 eq), 11.5 Hz (H-25/H-26ax), and 5.0 Hz (H-25/H-26 eq). The NOE correlations from H-23ax to H-20 and H-24 and from H-26ax to H-16 and H-24 in the ROESY spectrum were consistent with the C-22*R*, C-24*S*, and C-25*S* configurations (Su et al., 2009). The α orientation of H-5 was identified by the chemical shift of C-19 at δ 11.7 (Agrawal et al., 1995) and the NOE correlations from H-5 to H-3 and H-9. Therefore, the aglycone of **7** was identified as (25*S*)-5α-spirostan-3β,24β-diol-12-one. The nature of the monosaccharides was identified as D-galactose and D-glucose from acid hydrolysis and GC analysis. The ^1H and ^{13}C NMR spectra showed three anomeric protons at δ 4.86 (d, *J* = 7.8 Hz), 5.28 (d, *J* = 7.8 Hz), and 4.92 (d, *J* = 7.8 Hz), corresponding to the anomeric carbon signal at δ 102.5, 107.2, and 105.0 (Table 2). Complete assignment of the sugar protons and carbons by COSY, HSQC and HMBC experiments showed the presence of two β-D-glucopyranosyl units and one β-D-galactopyranosyl unit. The HMBC corrections between H-1′ (δ 4.86) and C-3 (76.9), H-1″ (δ 5.28) and C-4′ (δ 80.1), and H-1‴ (δ 4.92) and C-24 (δ 81.5) allowed deduction of the sugar sequencing and the linkage sites. Therefore, the structure of **7** was assigned as 24-*O*-β-D-glucopyranosyl-(25*S*)-5α-spirostan-3β,24β-diol-12-one-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside, named terrestrinin I.

In a previous study, pennogenin glycosides with spirostanol structures were reported as strong platelet agonists (Fu et al., 2008; Cong et al., 2010, 2012), and it was also found that some saponins with sarsasapogenin have strong antiplatelet activities (Zhang et al., 1999; Kang et al., 2012). Structure–activity relationship analysis suggested that steroidal saponins exhibited either agonistic or inhibitory activities on platelet aggregation based on the difference in their structures. So, the activities of the isolated saponins on platelet aggregation were evaluated. Using U46619 (a TxA2 analog) -induced rat platelet aggregation as positive control, the inductive activity of isolated saponins on platelet aggregation was evaluated. The effect of compounds **1**, **2**, and **4**–**16** on platelet was further investigated. The results showed that compounds isolated from *T. terrestris* exhibited diverse platelet activities (Table 3). The screening concentration of compounds was 250 μmol/L. Compounds **13**–**16** exhibited agonistic activity on platelet aggregation, the induction rate were 73%, 72%, and 74%, respectively. With the concentration decreased to 25 μM, compounds **13**, **15**, and **16** also exhibited significant inductive effects on platelet aggregation. The compounds **1**, **2**, and **4**–**12** exhibited weak (or no) inhibitory effects on U46619-induced platelet aggregation. Then the inhibitory activity was evaluated on U46619-induced platelet aggregation. Here, some steroidal saponins extracted from Chinese *T. terrestris* exhibited stronger agonistic activities on rat platelet aggregation, which indicates they may have a role on hemostasis.

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3. Experimental

3.1. General methods

The NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C-NMR, Karlsruhe, Germany) and a Varian ^{UNITY}INOVA 600 spectrometer (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C-NMR, Palo Alto, USA) in pyridine-d_5, and 2D NMR experiments were performed using standard microprograms. HRESIMS was recorded on a Synapt MS (Waters Corporation, Milford, MA, USA). The optical rotations were measured with a Perkin-Elmer 343 polarimeter (PerkinElmer, Waltham, MA, USA). HPLC was performed on a Waters 2695 series; XBP C_{18} and XBP C_{18}-2 columns (5 μm, 4.6 × 250 mm, Agela, Tianjin, China); Alltech 2000 ELSD detector (Alltech Corporation, Deerfield, USA). TLC was performed on silica gel GF254 plates (Qingdao marine Chemical, China). Column chromatography (CC) was performed on macroporous resin SP825 (Mitsubishi Chemicals, Japan), silica gel (Qingdao Haiyang Chemical Co., Ltd., China), and ODS-A silica gel (120 Å, 50 μm, YMC, Japan). U46619 (⩾98%) and PGE1 (⩾98%) was purchased from Cayman. The male Wistar rats were housed separately and acclimatized in a temperature controlled (25 ± 2 °C) and illumination-controlled (12-h light/dark cycle) room for at least 5 days prior to the experiments. The animals were fed with standard animal food and water.

3.2. Plant material

Fresh whole *T. terrestris* L. plants were collected from Beijing, China in May 2010. The plant was identified by Prof. Li-juan Zhang (Tianjin University of Traditional Chinese Medicine). A voucher specimen (No. 100501) is deposited in the Herbarium of the Beijing Institute of Radiation Medicine, Beijing.

3.3. Extraction and isolation

The whole plant material of *T. terrestris* L. (20 kg) was extracted using EtOH–H_2O (7:3, 40 L × 2, each for 1 h), under conditions of reflux, this being continued for. The combined extract was evaporated under reduced pressure, and applied to a macroporous resin SP825 column which was eluted with a gradient mixture of EtOH-H_2O (5:95, 60:40, and 90:10; 10 L of each solvent) to yield three factions (A–C). Fr. B (60 g) was subjected to silica-gel CC with a gradient mixture of $CHCl_3$–MeOH–H_2O (15:1:0.01, 65:25:4, and 2:1:0.01) as the eluent, and four fractions were obtained (B_1–B_4). Fr. B_1 was purified repeatedly by silica gel CC with a gradient mixture of $CHCl_3$–MeOH–H_2O (10:1:0.01, and 3:1:0.01) as the eluent, and a total of 280 tubes were collected. Tubes 48–57 were purified by semi-preparative HPLC with MeCN–H_2O (25:75) to yield **4** (6.9 mg) and **8** (12.9 mg). Tubes 63–88 were subjected to ODS silica-gel CC eluted with MeCN–H_2O (24:76) and semi-preparative HPLC with MeCN–H_2O (26:74) to yield **5** (4.8 mg). Tubes 121–164 were purified by semi-preparative HPLC with MeCN–H_2O (25:75) to yield **1** (7.8 mg) and **3** (3.1 mg). Fr. B_2 was subjected to an ODS silica-gel CC eluted with MeCN–H_2O (23:77) and 112 tubes were collected. Tubes 56–63 were purified by semi-preparative HPLC with MeCN–H_2O (26:74) to yield **6** (6.4 mg). Tubes 89–92 were purified by semi-preparative HPLC with MeCN–H_2O (26:74) to yield **2** (59.0 mg) and **10** (180.4 mg). Fr. B_3 was subjected to ODS silica-gel CC eluted with MeCN–H_2O (23:77) and 40 tubes were collected. Tubes 6–8 were purified by semi-preparative HPLC with MeCN–H_2O (23:77) to yield **12** (9.0 mg). Tubes 10–12 were purified by semi-preparative HPLC with MeCN–H_2O (26:74) to yield **7** (7.7 mg). Fr. B_4 was separated chromatographically on a silica-gel column with $CHCl_3$–MeOH–H_2O (3:1:0.01) to give 150 tubes. Tubes 68–119 were purified by semi-preparative HPLC with MeCN–H_2O (28:72) to yield **9** (9.5 mg) and **11** (11.4 mg). Fr. C (30 g) was subjected to CC on a silica-gel column with a gradient mixture of $CHCl_3$–MeOH–H_2O (9:1:0.01, 5:1:0.01, and 2:1:0.01) as the eluent, and 287 tubes were obtained. Tubes 81–113 was purified by CC on ODS silica gel with MeCN–H_2O (48:52) to give 50 tubes. Tubes 10–21 were purified by semi-preparative HPLC with MeCN–H_2O (53:47) to yield **13** (196.3 mg) and tubes 30–35 were purified by semi-preparative HPLC with MeCN–H_2O (50:50) to yield **16** (38.1 mg). Tubes 114–142 were purified by CC on ODS silica gel with MeCN–H_2O (40:60) to give 100 tubes, and tubes 73–86 were purified by recrystallization to yield **15** (31.5 mg). Tubes 231–250 were purified by recrystallization to yield **14** (172.4 mg).

3.4. Compound *1*

White amorphous power; $[\alpha]_D^{20}$ + 10.0 (c 0.05, pyridine); For ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz) spectroscopic data, see Tables 1 and 2; ESIMS (negative) 659.3 $[M+Cl]^-$, 623.3 $[M-H]^-$, 605.3 $[M-H-H_2O]^-$, 443.3 $[M-H-H_2O-Glc]^-$, 425.3 $[M-H-3\times H_2O-Glc]^-$, and 407.3 $[M-H-3\times H_2O-Glc]^-$; HRESIMS (negative) *m/z*: 623.3433 $[M-H]^-$ (calc. for $C_{33}H_{51}O_{11}$, 623.3431).

3.5. Compound *2*

White amorphous power; $[\alpha]_D^{20}$ + 48.0 (*c* 0.05, pyridine); For ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz) spectroscopic data, see Tables 1 and 2; ESIMS (negative) 641.3 $[M+Cl]^-$, 605.3 $[M-H]^-$, 587.3 $[M-H-H_2O]^-$, and 425.3 $[M-H-H_2O-Glc]^-$; HRESIMS (negative) *m/z*: 605.3347 $[M-H]^-$ (calc. for $C_{33}H_{49}O_{10}$, 605.3326).

3.6. Compound *3*

White amorphous power; $[\alpha]_D^{20}$ + 40.5 (*c* 0.04, pyridine); For ^{1}H-NMR (pyridine-d_5, 500 MHz) and ^{13}C-NMR (pyridine-d_5, 125 MHz) spectroscopic data, see Tables 1 and 2; ESIMS (negative) 665.3 $[M-HCOO]^-$, 655.3 $[M+Cl]^-$, 619.3 $[M-H]^-$, 601.3 $[M-H-H_2O]^-$ and 439.3 $[M-H-Glc]^-$; HRESIMS (negative) *m/z* 619.3146 $[M-H]^-$ (calc. for $C_{33}H_{47}O_{11}$, 619.3118).

3.7. Compound *4*

White amorphous power; $[\alpha]_D^{20}$ + 30.0 (c 0.04, pyridine); For ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz)

Table 3
The effect of compounds **1**, **2**, and **4–16** on rat platelet activities.

Compounds	Dose	Max. inhibition rate (%)	Max. induction rate (%)
PGE1	50 μg/mL	70.4	–
U46619	1.25 μM	–	83
1	250 μM	16.8	–
2	250 μM	7.2	–
4	250 μM	9.6	–
5	250 μM	18.1	–
6	250 μM	7.2	–
7	250 μM	9.6	–
8	250 μM	14.5	–
9	250 μM	10.8	–
10	250 μM	13.2	–
11	250 μM	15.6	–
12	250 μM	-2.9	–
13	250 μM	–	73
14	250 μM	–	38
15	250 μM	–	72
16	250 μM	–	74

spectroscopic data, see Tables 1 and 2; ESIMS (negative) 609.4 $[M-H]^-$, 591.4 $[M-H-H_2O]^-$, and 429.3 $[M-H-H_2O-Glc]^-$; HRESIMS (negative) *m/z* 609.3638 $[M-H]^-$ (calc. for $C_{33}H_{53}O_{10}$, 609.3639).

3.8. Compound **5**

White amorphous power; $[\alpha]_D^{20}$ + 25.7 (*c* 0.04, pyridine); For ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz) spectroscopic data, see Tables 1 and 2; ESIMS (negative) 607.4 $[M-H]^-$, 445.3 $[M-H-Glc]^-$, and 427.3 $[M-H-Glc-H_2O]^-$; HRESIMS (negative) *m/z* 607.3497 $[M-H]^-$ (calc. for $C_{33}H_{11}O_{10}$, 607.3482).

3.9. Compound **6**

White amorphous power; $[\alpha]_D^{20}$ + 32.4 (*c* 0.09, pyridine); For ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz) spectroscopic data, see Tables 1 and 2; ESIMS (negative) 767.4 $[M-H]^-$, 749.4 $[M-H-H_2O]^-$, 587.3 $[M-H-H_2O-Glc]^-$, and 425.3 $[M-H-H_2O-2\times Glc]^-$; HRESIMS (negative) *m/z* 767.3849 $[M-H]^-$ (calc. for $C_{39}H_{59}O_{15}$, 767.3854).

3.10. Compound **7**

White amorphous power; $[\alpha]_D^{20}$ −22.5 (*c* 0.05, pyridine); For ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz) spectroscopic data, see Tables 1 and 2; ESIMS (negative) 931.5 $[M-H]^-$, 769.4 $[M-H-Glc]^-$, and 607.4 $[M-H-2\times Glc]^-$; HRESIMS (negative) *m/z* 931.4531 $[M-H]^-$ (calc. for $C_{45}H_{71}O_{20}$, 931.4539).

3.11. Acid hydrolysis

Compounds **1**–**7** (1.5 mg each) were individually treated in 2 N $CF_3COOH-H_2O$ (2 mL) at 95 °C for 4 h. Each reaction mixture was extracted with CH_2Cl_2 (2 mL) three times, with the aqueous layer repeatedly evaporated to dryness until neutral. Then, in the monosaccharide mixture, glucose and galactose were detected by TLC analysis on a cellulose plate using *n*-BuOH–EtOAc–C_5H_5N–H_2O (6:1:5:4) as the developing solution and aniline-*o*-phthalic acid as the detection solution and were then compared with the control samples: glucose (R_f 0.46) and galactose (R_f 0.69). The sugar residue in pyridine (1 mL) was added to L-cysteine methyl ester hydrochloride (3.0 mg), and the mixture was kept at 60 °C for 1 h. Then, HMDS-TMCS (hexamethyldisilazane–trimethylchlorosilane) (0.6 mL) was added to the reaction mixture, which was then kept at 60 °C for 0.5 h. The supernatant (1.0 mL) was analyzed by GC under the following conditions: Agilent Technologies 6890 gas chromatograph was the equipment, carrying a 5973 mass spectrograph detector, and a HP-5 capillary column (30 m × 0.25 mm × 0.25 μm) was used. The conditions were as follow: column temperature: 180 °C/250 °C; programmed increase, 15 °C/min; carrier gas: N_2 (1 mL/min); injection and detector temperature: 250 °C; injection volume: 4.0 μL; and split ratio: 1/50. The absolute configurations of the monosaccharides were confirmed by comparison of the retention times of the monosaccharide derivatives with those of standard samples: D-glucose (17.95 min) and D-galactose (18.57 min). The D-glucose was detected for compounds **1**–**6**, and D-glucose and D-galactose were detected for compound **7**.

3.12. Platelet aggregation assay

Blood samples were obtained by cardiac puncture from male Wistar rats weighing 200–250 g, and the blood was anti-coagulated with a one-ninth volume of 200 U (v/v) heparin. The blood samples were immediately centrifuged at 2300*g* for 1 min at room temperature, and the resulting supernatant platelet-rich plasma (PRP) fraction was obtained. The remaining blood was further centrifuged at 1500*g* for 10 min, and the resulting supernatant used as the platelet-poor plasma (PPP) fraction. Platelet aggregation was measured using a Lumi aggregometer (Chrono-Log, Havertown, PA). The PRP was equilibrated at 37 °C without stirring for 3 min prior to the initiation of each experiment, and compounds **1**, **2**, and **4–12** (250 μmol L^{-1}) or PGE1 (50 μg/mL) or dimethyl sulfoxide (DMSO) was added 2 min prior to the addition of U46619 (1.25 μmol L^{-1}) with continuous stirring at 37 °C. Compounds **13–16** were added without the addition of U46619 (1.25 μmol L^{-1}) with continuous stirring at 37 °C. The baseline was determined from the PRP, and the maximum possible increase in light transmission (platelet aggregation rate: 100%) was established with PPP. H_1 was the height of maximum aggregation by given each compound with U46619 gathered away from PPP baseline, and H_0 was the height of maximum aggregation by giving U46619 without other compounds gathered away from the PPP baseline. Then platelet max inhibition rate (MIR_1) was obtained by the following formula System rate: $MIR_1 = (1-H_1/H_0)\times 100\%$ and the maximum induction rate (MIR_2) was calculated based on the following formula System rate: $MIR_2 = H_1/100 \times 100\%$. U46619-inducing rat platelet aggregation was used as positive control in the biological test.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.08.003.

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