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Dammarane-type saponins from *Ziziphus jujube* and their inhibitory effects against TNF-α release in LPS-induced RAW 246.7 macrophages

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ABSTRACT

Four new dammarane-type saponins jujubosides F–J, together with six known compounds were isolated from the seeds of *Ziziphus jujube*. Their structures were elucidated on the basis of chemical and spectroscopic evidences. Compounds **1**–**10** showed moderate inhibitory effects against pro-inflammatory cytokine TNF-α release in LPS-induced RAW 246.7 macrophages.

1. Introduction

Ziziphus jujube Mill. is a thorny rhamnaceous plant which is widely distributed in northern China. Its seeds have been used as a traditional Chinese medicine, which is prescribed for tonic and sedative purposes and treatment of insomnia (Yoshikawa et al., 1997). Up to now, many chemical components have been isolated from this plant, including triterpene saponins (Yoshikawa et al., 1997; Matsuda et al., 1999), flavonoids (Cheng et al., 2000), and alkaloids (Tripathia et al., 2001). In previous phytochemical investigation, a series of dammarane saponins with a sugar chain linked to C-3 of aglycone have been isolated. Some of them showed inhibitory activities against histamine release and lipoxygenase (Yu et al., 2013; Yoshikawa et al., 1997), and 3-glycoside moiety is important to show immunological adjuvant activity (Matsuda et al., 1999). In the course of our studies on the anti-inflammation saponins of natural medicines, we have isolated twenty four triterpene saponins from *Clematis chinensis* (Ranunculaceae) and three dimeric monoterpene glycosides from *Paeonia lactiflora* (Ranunculaceae); these compouns were found to show inhibitory activities against COX-1/COX-2 enzymes and nitric oxide release in LPS-induced RAW 246.7 macrophages (Fu et al., 2010, 2015). As a continuing part of our screening for anti-inflammatory saponins of natural medicines, four new saponins jujubosides F–J (Fig. 1) and six known saponins were isolated from the seeds of *Z. jujube*. Herein, we report the isolation and structure elucidation, and anti-inflammatory activity of these compounds.

2. Results and discussion

The EtOH extract of dried seeds of *Z. jujube* was subjected to silica gel column chromatography and prep-HPLC to obtain four new compounds (**1**–**4**), together with six known structures, (**5**) jujuboside I (Wang et al., 2013), (**6**) jujuboside A_1 (Yoshikawa et al., 1997), (**7**) jujuboside A (Renault et al., 1997), (**8**) jujuboside B (Okamura et al., 1981), (**9**) jujuboside C (Yoshikawa et al., 1997), (**10**) jujubasaponin IV (Yoshikawa et al., 1992),

Their structures were elucidated on the basis of spectroscopic evidence and hydrolysis products. The inhibitory effects against pro-inflammatory cytokine TNF-α release in LPS-induced RAW 246.7 macrophages of all compounds were also evaluated.

Compound **1** was isolated as white, amorphous powder. The HRESIMS (positive-ion mode) experiment reveled a pseudo-molecular ion peak $[M+Na]^+$ at *m/z* 1141.5765, in agreement with the molecular formula $C_{55}H_{90}O_{23}$. The aglycone of **1** was identified as jujubogenin by comparison of the ^1H and ^{13}C NMR data obtained in 2D NMR experiments with literature values (Wang et al., 2013). The ROESY correlations between H-17, Me-21, and H-22b and between H-22a and H-23 and the magnitude of the coupling

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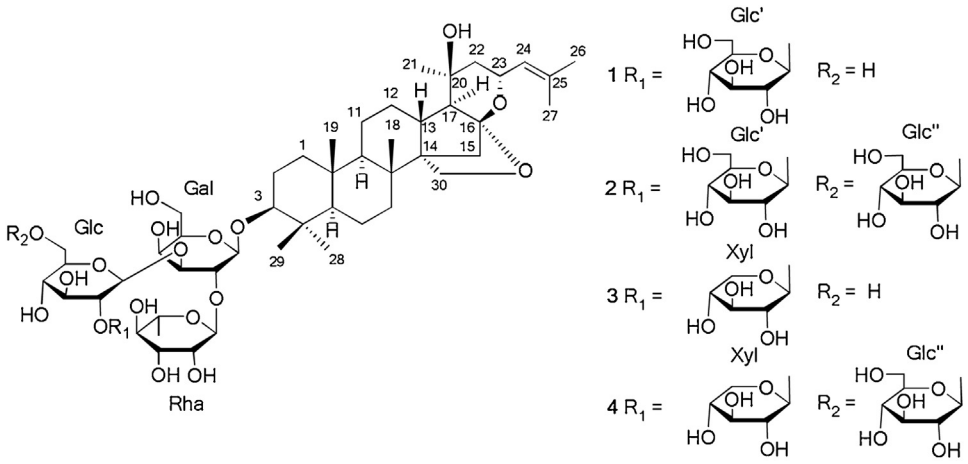

Fig. 1. The structures of compounds **1**–**4**.

constant between H-13 and H-17 (J = 7.0 Hz) are in agreement with the configuration of rings D-F of jujubogenin (Wang et al., 2013; Yu et al., 2013). The downfield shifts of C-3 at δ_C 88.6 suggested that **1** was a monodesmosidic saponin.

The ^{1}H NMR spectrum of **1** exhibited 4 anomeric proton resonances at δ 6.40 (1H, brs), 5.29 (1H, d, J = 7.5 Hz), 5.16 (1H, d, J = 7.5 Hz), and 4.81 (1H, d, J = 7.0 Hz). Acid hydrolysis of **1** yielded galactose, glucose, and rhamnose, which were detected by TLC comparison with authentic samples. The absolute configuration of the monosaccharides was determined to be D for glucose and galactose and L for rhamnose by GC analysis of chiral derivatives of the monosaccharides in the hydrolysate of each compound (see experimental section). The relatively large coupling constants (7.0–7.5 Hz) for the anomeric protons in the ^{1}H NMR spectra (see

Table 1
^{1}H NMR and ^{13}C NMR spectral data for the aglycone moieties of compounds **1**–**4.** (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR, in Pyridine-d_5).

	1		2		3		4	
No.	δ_H	δ_c	δ_H	δ_c	δ_H	δ_c	δ_H	δ_c
1	1.52 m	39.2	1.53 m	39.0	1.52 m	39.2	1.51 m	39.1
	0.75 m		0.75 m		0.76 m		0.74 m	
2	2.12 m	27.2	2.12 m	27.1	2.12 m	27.2	2.13 m	27.2
	1.86 m		1.88 m		1.86 m		1.87 m	
3	3.23 dd (12.0, 4.0)	88.6	3.22 dd (12.0, 4.5)	88.6	3.23 dd (12.0, 4.0)	88.6	3.22 dd (12.0, 4.5)	88.5
4		40.1		40.0		40.1		40.0
5	0.64 d (11.0)	56.8	0.66 d (10.5)	56.7	0.64 d (10.5)	56.7	0.65 d (11.0)	56.7
6	1.44 m	18.9	1.44 m	18.8	1.43 m	18.8	1.45 m	18.8
	1.34 m		1.33 m		1.36 m		1.36 m	
7	1.49 m	36.4	1.48 m	36.4	1.48 m	36.4	1.48 m	36.3
	1.38 m		1.38 m		1.38 m		1.39 m	
8		38.1		38.0		38.0		37.9
9	0.82 dd (12.0, 2.0)	53.3	0.81 dd (12.0, 2.0)	53.2	0.81 dd (12.0, 2.0)	53.3	0.82 dd (12.0, 2.0)	53.2
10		37.7		37.7		37.7		37.7
11	1.52 m	21.9	1.53 m	21.8	1.52 m	21.8	1.54 m	21.8
	1.31 m		1.32 m		1.33 m		1.33 m	
12	1.90 m	28.9	1.90 m	28.9	1.91 m	28.8	1.92 m	28.8
	1.79 m		1.79 m		1.80 m		1.81 m	
13	2.81 m	37.8	2.82 m	37.8	2.81 m	37.8	2.82 m	37.8
14		54.2		54.1		54.2		54.2
15	2.48 d (8.0)	37.5	2.48 d (8.0)	37.4	2.47 d (8.0)	37.4	2.48 d (8.0)	37.4
	1.52 m		1.53 m		1.50 m		1.52 m	
16		111.2		111.1		111.2		111.1
17	1.41 d 7.0	54.4	1.41 d 7.0	54.4	1.42 d 7.0	54.4	1.42 d 7.0	54.3
18	1.07 s	19.5	1.07 s	19.4	1.06 s	19.4	1.06 s	19.4
19	0.75 s	17.0	0.76 s	17.0	0.75 s	17.1	0.74 s	17.0
20		69.1		69.0		69.2		69.1
21	1.38 s	30.5	1.39 s	30.5	1.38 s	30.5	1.38 s	30.5
22	1.75 d (12.0)	46.1	1.74 d (12.0)	46.2	1.75 d (12.0)	46.2	1.76 d (11.5)	46.1
	1.66 m		1.67 m		1.67 m		1.68 m	
23	5.20 m	69.0	5.20 m	69.1	5.21 m	69.0	5.22 m	69.0
24	5.52 br d (7.5)	127.5	5.53 br d (7.5)	127.4	5.53 br d (7.0)	127.4	5.53 br d (7.5)	127.4
25		134.6		134.5		134.5		134.4
26	1.67 s	26.1	1.66 s	26.1	1.67 s	26.2	1.67 s	26.1
27	1.69 s	18.8	1.69 s	18.8	1.68 s	18.8	1.69 s	18.7
28	1.23 s	28.4	1.24 s	28.4	1.24 s	28.4	1.24 s	28.3
29	1.15 s	17.4	1.14 s	17.3	1.16 s	17.4	1.16 s	17.3
30	4.27 m	66.2	4.26 m	66.1	4.28 m	66.1	4.28 m	66.1
	4.20 m		4.21 m		4.21 m		4.21 m	

Experimental Section) of **1** suggested that the galactopyranosyl and glucopyranosyl moieties have a β-configuration. The α-configuration of the rhamnopyranosyl moiety was determined from the broad singlet observed for the anomeric proton.

The spin–spin coupling system of individual monosaccharide units was identified by analysis of 1D TOCSY and 2D NMR spectra. ^{1}H NMR spectral data of individual monosaccharide units were obtained by selective irradiation of the anomeric protons or methyl groups of rhamnopyranosyl units in a series of 1D TOCSY experiments. Analysis of the ^{1}H–^{1}H COSY spectrum resulted in sequential assignment of all proton resonances of the 4 monosaccharide units, including identification of most of their multiple splitting patterns and coupling constants, as shown in Table 2. In the HSQC experiment, proton resonances were correlated with those of the corresponding carbons, and associated anomeric protons were correlated with their respective carbon atoms from HSQC-TOCSY data, leading to unambiguous assignments of the carbons in each monosaccharide unit (see Table 2). Taking into account the known effects of *O*-glycosylation, **1** contains one D-galactopyranosyl unit (Gal), one L- rhamnopyranosyl unit (Rha), and two D-glucopyranosyl units (Glc).

In the HMBC spectrum, the anomeric proton signals at δ_H 4.81 (Gal-1), 6.40 (Rha-1), 5.16 (Glc-1), and 5.29 (Glc′-2) showed cross-peaks with the carbon signals at δ_C 88.6 (Aglycone-C-3), 74.9 (Gal-C-2), 85.8 (Gal-C-3), and 85.1 (Glc-C-2), respectively. These signals provide evidence to determine the linkages between the sugars, and the sugar and the aglycone. These linkages were also confirmed by NOESY correlations between Aglycone-H-3/Gal-H-1, Gal-H-2/Rha-H-1, Gal-H-3/Glc-H-1, and Glc-H-2/Glc′-H-1 (see Tables 1 and 2). From the above evidence, the structure of **1** was established as 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 3)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranosyl jujubogenin, which has been named jujuboside F.

Compound **2** was isolated as white, amorphous powder. The HRESIMS (positive-ion mode) experiment revealed a pseudomolecular ion peak $[M+Na]^+$ at *m*/*z* 1303.6303, in agreement with the molecular formula $C_{61}H_{100}O_{28}$. The spectroscopic properties of **2** were closely related to those of **1**, except for the appearance of signals due to Glc″ connected to Glc-C-6 in **2**. This observation was supported by a relative downfield shift of Glc-C-6 of **2** at δc 70.2 (Glc-C-6 of **1** at δc 62.8), and confirmed by HMBC correlation between Glc″-H-1 and Glc-C-6. Thus, the structure of **2** was determined as 3-*O*-β-D-glucopyranosyl-(1 → 6)-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranosyl-(1 → 3)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranosyl jujubogenin, which has been named jujuboside G.

Table 2
^{1}H NMR and ^{13}C NMR spectral data for the sugar moieties of compounds **1**–**4**. (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR, in Pyridine-d_5).

	1		2		3		4	
No.	δ_H	δ_c	δ_H	δ_c	δ_H	δ_c	δ_H	δ_c
	Gal		Gal		Gal		Gal	
1	4.81 d (7.0)	105.9	4.80 d (7.0)	105.8	4.81 d (7.0)	105.8	4.82 d (7.0)	105.8
2	4.65 dd (9.5, 7.0)	74.9	4.63 dd (9.0, 7.0)	74.9	4.65 dd (9.5, 7.0)	74.8	4.64 dd (9.0, 7.0)	74.8
3	4.25 m	85.8	4.26 m	85.8	4.25 m	85.8	4.24 m	85.7
4	4.82 m	70.5	4.82 m	70.5	4.82 m	70.5	4.81 m	70.4
5	4.10 m	76.7	4.12 m	76.6	4.10 m	76.6	4.11 m	76.5
6	4.30 dd (12.0, 6.0)	62.6	4.33 dd (12.0, 6.0)	62.5	4.30 dd (12.0, 6.0)	62.5	4.32 dd (12.0, 6.0)	62.6
	4.41 dd (12.0, 2.0)		4.42 dd (12.0, 2.0)		4.41 dd (12.0, 2.0)		4.43 dd (12.0, 2.0)	
	Rha		Rha	Rha		Rha		
1	6.40 br s	101.7	6.41 br s	101.7	6.40 br s	101.6	6.42 br s	101.6
2	4.71 br s	73.0	4.72 br s	73.0	4.71 br s	73.0	4.71 br s	73.1
3	4.58 dd (9.0, 3.0)	73.2	4.58 dd (9.0, 2.5)	73.2	4.58 dd (9.0, 3.0)	73.2	4.60 dd (9.0, 2.5)	73.3
4	4.28 m	74.4	4.29 m	74.4	4.28 m	74.4	4.28 m	74.4
5	4.72 m	70.4	4.73 m	70.4	4.72 m	70.4	4.72 m	70.3
6	1.69 d (6.5)	18.8	1.70 d (6.0)	18.8	1.69 d (6.5)	18.8	1.70 d (6.0)	18.8
	Glc		Glc		Glc		Glc	
1	5.16 d (7.5)	103.5	5.07 d (7.5)	103.8	5.18 d (7.5)	103.7	5.05 d (7.0)	103.9
2	4.01 dd (9.0, 7.5)	85.1	3.98 dd (9.0, 7.5)	84.9	4.04 dd (9.0, 7.5)	83.2	4.02 dd (9.0, 7.5)	83.5
3	4.28 m	78.8	4.20 m	78.7	4.27 m	78.6	4.21 m	78.6
4	4.12 m	71.2	3.88 m	71.3	4.14 m	71.2	3.89 m	71.3
5	3.90 m	78.7	3.98 m	76.2	3.91 m	78.6	3.98 m	76.2
6	4.49 dd (12.0, 2.0)	62.8	4.88 dd (12.0, 2.0)	70.2	4.47 dd (12.0, 2.0)	62.7	4.89 dd (12.0, 2.0)	70.e
	4.32 dd (12.0, 6.0)		3.92 dd (12.0,6.0)		4.34 dd (12.0, 6.0)		3.90 dd (12.0,6.0)	
	Glc′		Glc′		Xyl		Xyl	
1	5.29 d (7.5)	106.8	5.28 d (7.0)	106.8	5.35 d (7.0)	106.8	5.34 d (7.0)	106.8
2	4.24 dd (9.0, 7.5)	76.4	4.24 dd (9.0, 7.0)	76.4	4.19 dd (9.0, 7.0)	76.4	4.20 dd (9.0, 7.0)	76.3
3	4.17 t (9.0)	78.6	4.16 t (9.0)	78.5	4.16 m	78.5	4.17 m	78.4
4	4.32 m	70.8	4.30 m	70.8	4.22 m	71.2	4.21 m	71.2
5	3.82 m	79.2	3.81 m	79.1	4.50 m	68.2	4.50 m	68.3
6	4.41 dd (12.0, 2.0)	62.4	4.40 dd (12.0, 1.5)	62.3	3.76 t (11.0)		3.78 t (11.0)	
	4.34 dd (12.0, 6.0)		4.32 dd (12.0, 6.0)					
			Glc″				Glc′	
1			4.88 d (7.5)	105.3			4.87 d (7.5)	105.2
2			4.04 d (9.0, 7.5)	75.8			4.03 d (9.0, 7.5)	75.7
3			4.14 m	78.3			4.13 m	78.2
4			4.22 m	71.4			4.21 m	71.3
5			3.86 m	78.6			3.84 m	78.7
6			4.48 dd (12.0, 1.5)	62.8			4.47 dd (12.0, 2.0)	62.8
			4.34 dd (12.0, 6.0)				4.32 dd (12.0, 6.0)	

Compound **3** was isolated as white, amorphous powder. The HRESIMS (positive-ion mode) experiment revealed a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 1111.5668, in agreement with the molecular formula $C_{54}H_{88}O_{22}$. Acid hydrolysis of **3** yielded galactose, glucose, xylose, and rhamnose, which were detected by TLC comparison with authentic samples. The absolute configuration of the xylose was determined to be D by GC analysis of chiral derivatives. The coupling constants (7.0 Hz) for the anomeric protons of xylopyranosyl (Xyl) suggested that the Xyl moiety has a β-configuration. A comparison of the ^{13}C NMR spectra of **3** with that of **1** revealed the similar aglycone. The only difference was that the signals for Glc′ in **1** were replaced by those for Xyl in **3**. The correlation observed between Xyl-H-1 and the Glc-C-2 in the HMBC spectrum indicated that the Xyl is linked to the C-2 of Glc. From the above evidence, the structure of **3** was determined as 3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 3)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D galactopyranosyl jujubogenin, which has been named jujuboside H.

The molecular formula of **4** was determined as $C_{60}H_{98}O_{27}$ by HRESIMS, which showed a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 1273.6188. The spectroscopic properties of **4** were closely related to those of **3**, except for the appearance of signals due to Glc′ connected to Glc-C-6 in **4**. This observation was supported by a relative downfield shift of Glc-C-6 of **4** at δc 70.3 (Glc-C-6 of **3** at δc 62.7), and confirmed by HMBC correlation between Glc′-H-1 and Glc-C-6. Thus, the structure of **4** was determined as 3-*O*-β-D-glucopyranosyl-(1 → 6)-[β-D-xylopyranosyl −(1 → 2)]-β-D-glucopyranosyl-(1 → 3)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranosyl jujubogenin, which has been named jujuboside J.

All isolates were evaluated for inhibitory activity against LPS-induced TNF-α production in RAW 246.7 macrophages (see Experimental). Curcumin was used as positive control (72.3% inhibition rate, 10 μM). Those compounds showed moderate inhibitory effects against TNF-α production with the inhibition ratios of 33.3%, 29.1%, 27.2%, 18.8%, 42.6%, 17.4%, 18.5%, 26.9%, 29.1% and 19.5% at 50 μM, respectively. No cytotoxicity was observed in compounds **1**–**10** treated cells (cell viability >90%).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 digital polarimeter (Jasco, Tokyo, Japan). Spots were visualized by spraying 10% H_2SO_4–EtOH followed by heating. IR spectra were obtained on a Bruker IFS-55 plus spectrometer (Bruker, Ettlingen, German). NMR spectra were recorded on an Inova 500 spectrometer with TMS as an internal standard, operating at 500 MHz for $^1H$and 125 MHz for ^{13}C (Bruker, Waltham, MA, USA). HR-ESI–MS data were obtained on a Bruker-Daltonics APES-III 7.0 TESLA FTMS spectrometer (Bruker, Billerica, MA, USA). GC was obtained on a SHIMADZU GC-14D. Precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co., Qingdao, China) were employed for thin layer chromatography. Column chromatography was performed with silica gel (Merck, Darmstadt, Germany) and C18 silica gel (150–200 mesh, Merck). High performance liquid chromatography (HPLC) separation was carried out on an octadecylsilanized column (YMC-pack ODS-A, 250 × 10 mm, i.d. 5 μm, YMC, Kyoto, Japan) with an Alltech ES 2000 evaporative light scattering detector (Grace, Crutis Bay, MD, USA).

3.2. Plant material

The seeds of *Z. jujube* were collected in May 2015 in Chengdu city, Sichuan Province of China, and identified by author (Qiang Fu). A voucher specimen (ZJ 201505) is maintained in the herbarium of School of Pharmacy and Bioengineering, Chengdu University.

3.3. Extraction and isolation

The dried seeds (9.6 kg) of *Z. jujube* were refluxed two times with EtOH, each for 2 h. After concentrated *in vacuo*, the residue (1332 g) was suspended in water, and partitioned with ethyl acetate and *n*-butanol successively. The *n*-butanol-soluble fraction (337 g) was further chromatographed over a silica gel column chromatography using $CHCl_3$-MeOH (100:1 → 10:90) as an eluent to give fractions 1–7(8.7, 22.3, 151.7, 38.4, 23.2, 18.8 and 14.9 g, respectively). Fraction 2 (10.5 g) was subjected to ODS open column chromatography (MeOH—H_2O, 10:90 → 95:5) to afford sub-fractions 1–5 (2.2, 1.5, 1.8, 2.4 and 1.6 g, respectively). Sub-fraction 3 (1.8 g) was separated by prep-HPLC (MeOH—H_2O, 38:62, 2.0 ml/min, tube temperature 120 °C, gas flow 2.5 l/min) to yield compound **10** (42 mg, t_R 18.1 min). Fraction 3 (11.2 g) was subjected to C_{18} silica gel chromatography (MeOH—H_2O, 25:75 → 60:40) to afford sub-fractions 1–8 (0.9, 1.5, 1.3, 0.6, 0.4, 1.8, 2.4 and 1.5 g, respectively). Sub-fraction 2 (1.5 g) was separated by prep-HPLC (ACN—H_2O, 32:68, 2.0 ml/min, tube temperature 120 °C, gas flow 2.5 l/min), affording compound **5** (51 mg, t_R 19.4 min) and **8** (37 mg, t_R 23.2 min). Sub-fraction 3 (1.3 g) was separated by prep-HPLC (ACN—H_2O, 30:70, 2.0 ml/min, tube temperature 120 °C, gas flow 2.5 l/min), affording compound **6** (71 mg, t_R 22.7 min), **7** (69 mg, t_R 24.6 min), **3** (21 mg, t_R 27.3 min), and **1** (19 mg, t_R 33.2 min). Sub-fraction 4 (0.6 g) was separated by prep-HPLC (ACN—H_2O, 28:72, 2.0 ml/min, tube temperature 120 °C, gas flow 2.5 l/min) to yield compound **9** (73 mg, t_R 18.7 min), **4** (23 mg, t_R 21.7 min), and **2** (28 mg, t_R 24.2 min).

3.3.1. Jujuboside F (*1*)

White amorphous power, $[\alpha]^{20}_D$ −42, (*c* 0.5, MeOH); IR(KBr) ν_{max} (cm^{-1}): 3430, 1647, 1046; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HRESIMS: *m/z* 1141.5765 $[M+Na]^+$ (calcd for $C_{55}H_{90}O_{23}Na$, 1141.5771).

3.3.2. Jujuboside G (*2*)

White amorphous power, $[\alpha]^{20}_D$ −56 (*c* 0.5, MeOH); IR(KBr) ν_{max} (cm^{-1}): 3422, 1645, 1036; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HRESIMS: *m/z* 1303.6303 $[M+Na]^+$ (calcd for $C_{61}H_{100}O_{28}Na$, 1303.6299).

3.3.3. Jujuboside H (*3*)

White amorphous power, $[\alpha]^{20}_D$ −44, (*c* 0.5, MeOH); IR(KBr) ν_{max} (cm^{-1}): 3418, 1636, 1072; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HRESIMS: *m/z* 1111.5668 $[M+Na]^+$ (calcd for $C_{54}H_{88}O_{22}Na$, 1111.5665).

3.3.4. Jujuboside J (*4*)

White amorphous power, $[\alpha]^{20}_D$ −59 (*c* 0.5, MeOH); IR(KBr) ν_{max} (cm^{-1}): 3424, 1729, 1049; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HRESIMS: *m/z* 1273.6188 $[M+Na]^+$ (calcd for $C_{60}H_{98}O_{27}Na$, 1273.6193).

3.4. Acid hydrolysis

Each compound (5 mg) was heated in 0.5 ml of 2 M HCl at 95 °C for 10 h in a sealed tube. After filtration of the reaction mixture, the filtrate was evaporated under vacuum. After addition of H_2O, the acidic solution was evaporated again to remove HCl. This procedure was repeated until a neutral solution was obtained, which was finally evaporated and dried in vacuo to furnish a monosaccharide residue. The sugar components obtained after acid hydrolysis of **1**–**10** were analyzed by GC analysis of the methyl

sugar peracetates. The monosaccharide residue was dissolved in anhydrous pyridine (100 ml), 0.1 M L-cysteinemethyl ester hydrochloride (200 ml) was added, and the resultant was warmed at 60 °C for 2 h. The trimethylsilylation reagent HMDS–TMCS (hexamethyldisilazane–trimethylchlorosilane–pyridine, 2:1:10; Acros Organics, Geel, Belgium) was added and warmed at 60 °C for 30 min. To the mixture, the thiazolidine derivatives were analyzed by GC for sugar identification. Separations were carried out on a DB-5 column (30 m × 0.25 mm × 0.25 mm). Highly pure N_2 was employed as carrier gas (1.0 ml/min flow rate), and the Flame Ionization Detector detector operated at 280 °C (column initial temperature was maintained at 100 °C for 2 min and then raised to 280 °C at the rate of 10 °C/min). The retention times of L-rhamnose (9.50 min), D-xylose (11.31 min), D-glactose (13.49) and D-glucose (15.68 min) were confirmed by comparison with those of authentic standards.

3.5. Assay for inhibitory activity against LPS-induced TNF-α production

Tested compounds were separately dissolved in DMSO, and diluted with phosphate buffered saline (PBS) to a final concentration of 10 mM. Such solution was then diluted to various test concentrations. RAW 264.7 macrophages were seeded in 96 well plates (1×10^5 cells/well). Cells were co-incubated with tested samples and lipopolysaccharide (LPS, 1 μg/mL) for 24 h. TNF-α production levels were determined using a commercially available TNF-α ELISA kit (BioLegend, Inc., CA) according to the protocol provided by the manufacturers.

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