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•Research article•

New triterpenoid saponins from the leaves of *Ilex chinensis* and their hepatoprotective activity

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[ABSTRACT] Seven new triterpenoid saponins, including five ursane-type saponins, ilexchinenosides R–V (**1**–**5**), and two oleanane-type saponins, ilexchinenosides W–X (**6**–**7**), with four known triterpenoid saponins (**8**–**11**) were isolated from the leaves of *Ilex chinensis*. Their structures were elucidated by comprehensive spectroscopic 1D and 2D NMR and HR-ESI-MS data. Their sugar moieties were determined by HPLC analysis compared with standards after hydrolysis and derivatization. Compounds **1**, **2**, **4**, **9** and **10** exhibited potential hepatoprotective activity against *N*-acetyl-*p*-aminophenol (APAP)-induced HepG2 cell injury *in vitro*.

[KEY WORDS] *Ilex chinensis*; Ursane and oleanane; Triterpenoid saponins; Hepatoprotective activity
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Introduction

Ilex chinensis is a kind of arbors from the Aquifoliaceae family, which is widely distributed in the south area of China [1]. Its leaves have been used as Traditional Chinese Medicine (TCM) named “Si-ji-qing” over a thousand of years for the treatment of cough, pharyngitis, dysentery, stranguria and ulceration [2]. Phytochemical investigations reported a large number of compounds isolated from the genus *Ilex*, among which triterpenoids were regarded as the major constituents [3,4]. Pharmacological studies elucidated that the triterpenoids from *Ilex* species possess various bioactivities [5], especially the hepatoprotective [6] and the anti-inflammatory activity [7], which have attracted extensive interests of researchers. Our previous study has identified lots of triterpenoids from the leaves of *I. chinensis* and their hepatoprotective and anti-inflammatory activity were evaluated, too [8,9]. As a continuing investigation to find more undescribed and bioactive triterpenoids, seven new triterpenoid saponins, including five ursane-type triterpenoid saponins, ilexchinenosides R–V (**1**–**5**), and two oleanane-type triterpenoid saponins, ilexchinenosides W-X (**6**–**7**) (Fig. 1), with four known triterpenoid saponins (**8**–**11**) were isolated from the leaves of *I. chinensis*. Meanwhile, the hepatoprotective activities of these compounds were evaluated *in vitro* assay.

Results and Discussion

Ilexchinenoside R (**1**) was obtained as a white amorphous powder. Its molecular formula of $C_{41}H_{66}O_{13}$ was determined by the HR-ESI-MS *quasi* ion peak at *m*/*z* 789.4394 $[M + Na]^+$ (Calcd. for $C_{41}H_{66}NaO_{13}$, 789.4396). The ^{1}H NMR data of **1** (Table 1) exhibited five methyl singlets at δ_H 0.90, 1.00, 1.18, 1.25, and 1.31, and one methyl doublet at δ_H 1.11 (d, J = 6.0 Hz). Meanwhile, one olefinic proton at δ_H 5.50 (t, J = 3.0 Hz), one oxy-methine proton at δ_H 3.36 (dd, J = 12.0, 4.2 Hz), two oxy-methylene protons at δ_H 3.91 (2H, m), and several overlapped methylene and methane signals between δ_H 0.81–2.66. The characteristic ^{1}H NMR data implied that it was a triterpenoid. In addition, two anomeric protons at δ_H 4.85 (d, J = 7.8 Hz) and 6.32 (d, J = 8.4 Hz), along with 11 protons between δ_H 3.80–4.48 revealed that a β-xylose and β-glucose existed in **1**. The ^{13}C NMR data of **1** (Table 1) showed 41 carbons, consisting of 30 signals from the triterpenoid skeleton and 11 signals for the sugars. The characteristic olefinic bond carbon signals at δ_C 126.3 (C-12) and 138.4 (C-13) indicated it was an ursane-type triterpenoid [10]. Analysis the 1D NMR data of **1** suggested that it was very similar with that of monepaloside F [10]. The only difference between them is that the OH-19 of monepaloside F is instead by OH-30 in **1**. It is confirmed by the HMBC correlations of H-30 with C-19, C-20 and C-21 (Fig. 2). The HMBC correla-

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These authors have no conflict of interest to declare.

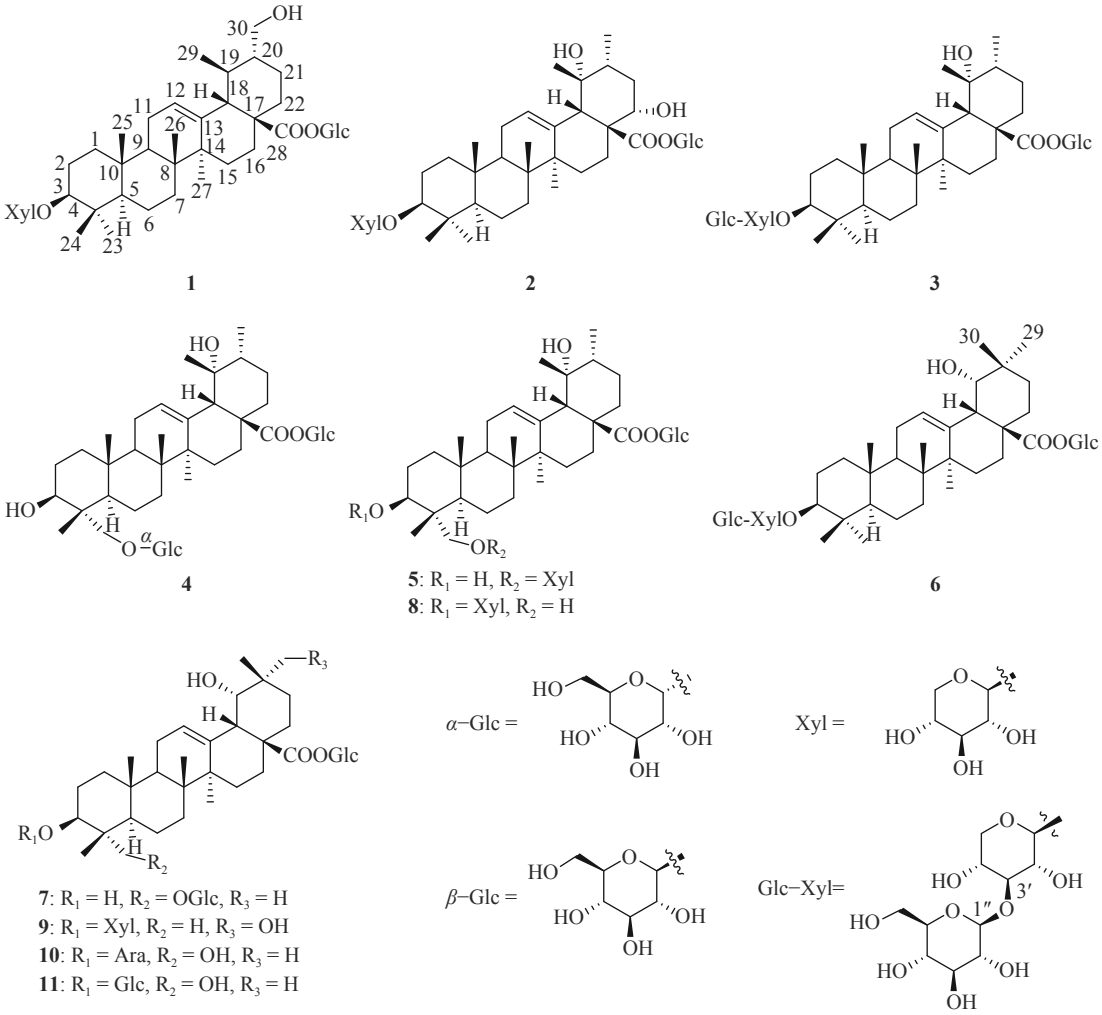

Fig. 1 Structures identified from the leaves of *I. chinensis*

tions of H-1′ with C-3 and H-1″ with C-28 revealed that the pentose and hexose were connected at C-3 and C-28, respectively.

The NOESY correlations between H-3 and H-5, and between H-19 and H_2-30 revealed that H-3, H-5, and H_2-30 were *α*-oriented, while the cross-peak between H-18 and H_3-29 suggested they were *β*-oriented (Fig. 3). The sugars were determined to be D-xylose and D-glucose by HPLC analysis after hydrolysis and derivatization. Thus, the structure of **1** was established as 3-*O*-*β*-D-xylopyranosyl-3*β*, 30-dihydroxyurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside in Fig. 1.

Ilexchinenoside S (**2**) has a molecular formula of $C_{41}H_{66}O_{14}$ determined by HR-ESI-MS data. Its ^{1}H NMR data (Table 1) showed two anomeric protons at δ_H 4.84 (H-1′, d, J = 7.5 Hz) and 6.36 (H-1″, d, J = 7.5 Hz), together with 11 protons between δ_H 3.79–4.47, which revealed the presence of a *β*-xylose and *β*-glucose. The sugars were identified to be D-xylose and D-glucose by HPLC analysis after hydrolysis and derivatization. Analysis of the ^{1}H and ^{13}C NMR data of **2** revealed that they were very similar with those of monepaloside F except for the value of C-22 shifted from 37.0 to 74.4 ppm [10]. It was speculated that a hydroxyl group was connected on C-22. This was confirmed by the HMBC correlations of H-22 with C-20 and C-28 (Fig. 2). Additionally, the NOESY correlations of H-22 with H-18 and H-20 indicated that H-22 was *α*-oriented. Therefore, the structure of **2** was named as 3-*O*-*β*-D-xylopyranosyl-3*β*, 19*α*, 22*α*-trihydroxyurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside in Fig. 1.

Ilexchinenoside T (**3**) was isolated as a white amorphous powder. The molecular formula was identified to be $C_{47}H_{76}O_{18}$ by the HR-ESI-MS *quasi* ion peak. Comparing with monepaloside F [10], the ^{1}H NMR data of **3** showed an extra anomeric proton at δ_H 5.32 (H-1″, d, J = 8.0 Hz) and six protons between δ_H 4.03−4.54 suggested the presence of another *β*-glucose. The ^{13}C NMR data of **3** displayed six corresponding carbon signals at δ_C 105.8 (C-1″), 75.6 (C-2″), 78.3 (C-3″), 71.7 (C-4″), 78.7 (C-5″), and 62.5 (C-6″). All the data above suggested that the structure of **3** had one more *β*-glucose than that of monepaloside F. The sugars of **3** were identified to be D-xylose and D-glucose by HPLC analysis after hydrolysis and derivatization. In addition, the HMBC correlation of H-1″ with C-3′ indicated the extra glucose was con-

Table 1 ^{1}H and ^{13}C NMR spectroscopic data of compounds 1–5 in C_5D_5N

No.	**1**[a]		**2**[b]		**3**[b]		**4**[b]		**5**[a]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1*α*	0.93, m	39.0	0.96, m	38.9	0.94, m	38.9	0.85, m	38.7	0.90, m	38.5
1*β*	1.56, m		1.56, m		1.55, m		1.50, m			1.52, m
2*α*	2.18, m	26.8	2.17, m	26.8	2.11, m	26.7	1.76, m	27.6	1.80, m	27.6
2*β*	1.92, m		1.91, m		2.00, m		1.87, m			1.89, m
3	3.36, dd, 12.0, 4.2	88.7	3.36, dd, 11.5, 4.5	88.7	3.32, dd, 11.5, 4.5	88.9	3.99, m	72.1	4.20, dd, 11.4, 4.2	72.5
4		39.6		39.6		39.6		42.8		42.8
5	0.81, br d, 12.0	55.9	0.86, br d, 11.5	55.9	0.85, br d, 11.5	55.9	1.49, m	48.4	1.52, m	48.8
6*α*	1.47, m	18.5	1.49, m	18.7	1.50, m	18.7	1.54, m	18.9	1.70, m	18.8
6*β*	1.29, m		1.34, m		1.34, m		1.37, m		1.42, m	
7*α*	1.49, m	33.5	1.63, m	33.5	1.62, m	33.5	1.46, m	33.2	1.47, m	33.2
7*β*	1.36, m		1.46, m		1.46, m		1.70, m		1.77, m	
8		40.2		40.7		40.6		40.5		40.5
9	1.60, dd, 10.8, 6.6	48.1	1.81, m	47.7	1.81, m	47.8	1.81, m	47.9	1.84, m	47.7
10		37.0		37.0		37.0		37.0		37.2
11	1.96, m	23.7	2.05, m	24.1	2.05, m	24.1	2.04, m	24.0	2.05, m	24.0
12	5.50, t, 3.0	126.3	5.56, br s	128.8	5.57,t, 3.5	128.4	5.58, t, 3.0	128.4	5.56, t, 3.0	128.4
13		138.4		138.9		139.3		139.3		139.4
14		42.5		42.5		42.2		42.1		42.1
15*α*	1.18, m	28.7	1.34, m	28.6	1.26, m	29.3	1.20, m	29.1	1.21, m	29.2
15*β*	2.49, td, 13.8, 4.2		2.59, td, 13.5, 5.0		2.50, td, 13.5, 3.5		2.47, td, 14.0, 4.5		2.47, td, 13.8, 4.2	
16*α*	2.23, td, 13.8, 4.2	24.7	3.00, td, 13.5, 5.0	19.2	3.13, td, 13.5, 3.5	26.1	3.08, td, 14.0, 4.5	26.2	3.09, td, 13.8, 4.2	26.2
16*β*	2.04, m		2.77, br d, 13.0		2.05, m		2.05, m		2.00, m	
17		48.4		55.3		48.7		48.7		48.6
18	2.66, br d, 11.4	53.4	3.00, s	55.1	2.95, s	54.5	2.95, s	54.5	2.95, s	54.4
19	2.04, m	33.7		72.4		72.7		72.7		72.7
OH-19			5.38, s		5.14, s		4.96, s		5.09, s	
20	1.17, m	47.1	1.51, m	40.4	1.38, m	42.1	1.37, m	42.1	1.37, m	42.1
21*α*	1.86, m	25.5	2.39, q, 12.0	35.9	1.88, m	26.7	1.99, m	26.7	2.00, m	26.7
21*β*			1.79, m		1.24, m		1.22, m		1.22, m	
22*α*	2.07, m	36.8	4.40, m	74.4	2.08, m	37.8	2.08, m	37.7	2.06, m	37.7
22*β*	1.83, m				1.86, m		1.88, m		1.86, m	
23	1.31, s	28.3	1.31, s	28.2	1.29, s	28.1	3.35, d, 9.5 4.32, d, 9.5	71.5	3.99, d, 9.6 4.04, d, 9.6	75.2
24	1.00, s	17.0	1.01, s	17.0	1.01, s	17.0	0.93, s	13.1	1.06, s	13.2
25	0.90, s	15.8	0.95, s	15.7	0.92, s	15.7	0.98, s	16.1	1.00, s	16.1
26	1.18, s	17.7	1.24, s	17.4	1.21, s	17.4	1.22, s	17.5	1.22, s	17.5
27	1.25, s	23.8	1.76, s	24.9	1.72, s	24.6	1.60, s	24.4	1.64, s	24.6

Continued

No.	**1**[a]		**2**[b]		**3**[b]		**4**[b]		**5**[a]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
28		176.3		175.9		177.0		177.0		177.0
29	1.11, d, 6.0	17.2	1.37, s	26.8	1.42, s	27.1	1.42, s	27.1	1.41, s	27.1
30	3.91, m	65.0	1.12, d, 6.5	16.6	1.08, d, 6.5	16.7	1.07, d, 6.5	16.7	1.07, d, 6.6	16.7
	Xyl		Xyl		Xyl		Glc		Xyl	
1′	4.85, d, 7.8	107.7	4.84, d, 7.5	107.8	4.77, d, 7.5	107.2	5.37, br s	102.0	4.81, d, 7.2	105.6
2′	4.04, m	75.6	4.03, m	75.6	4.04, m	74.2	4.56, m	73.5	4.02, m	74.7
3′	4.18, t, 9.0	78.7	4.17, t, 9.0	78.7	4.13, m	88.5	4.44, m	75.6	4.14, t, 8.4	77.9
4′	4.24, m	71.3	4.24, m	71.3	4.10, m	69.6	4.66, t, 9.5	69.2	4.17, m	71.0
5′	3.80, t, 10.8 4.39, m	67.2	3.79, t, 10.0 4.39, m	67.2	3.69, t, 10.0 4.32, m	66.4	4.58, m	72.4	3.70, dd, 11.4, 9.0 4.36, m	66.8
6′							4.38, m 4.56, m	63.1		
	Glc		Glc		Glc		Glc		Glc	
1″	6.32, d, 8.4	95.7	6.36, d, 7.5	96.2	5.32, d, 8.0	105.8	6.32, d, 8.0	95.9	6.32, d, 8.4	95.9
2″	4.24, m	74.1	4.28, m	74.2	4.08, m	75.6	4.25, m	74.1	4.25, t, 9.0	74.1
3″	4.31, t, 9.0	78.9	4.30, m	78.8	4.25, m	78.3	4.33, m	79.0	4.33, t, 9.0	79.0
4″	4.39, m	71.2	4.33, m	71.3	4.22, m	71.7	4.38, m	71.3	4.39, t, 9.0	71.3
5″	4.05, m	79.3	4.05, m	79.1	4.03, m	78.7	4.08, m	79.3	4.08, m	79.3
6″	4.42, dd, 12.0, 10.2 4.48, dd, 12.0, 1.8	62.3	4.40, m 4.47, dd, 11.5, 2.5	62.4	4.31, m 4.54, br d, 14.5	62.5	4.43, m 4.50, m	62.3	4.43, dd, 12.0, 4.2 4.50, d, 12.0	62.4
					Glc					
1‴					6.33, d, 8.0	95.9				
2‴					4.25, m	74.1				
3‴					4.32, m	79.0				
4‴					4.38, m	71.2				
5‴					4.06, m	79.3				
6‴					4.42, m 4.50, m	62.3				

[a] ^{1}H NMR data recorded at 600 MHz and ^{13}C NMR data at 150 MHz;
[b] ^{1}H NMR data recorded at 500 MHz and ^{13}C NMR data at 125 MHz.

nected on C-3′ of the xylose. Consequently, the structure of **3** was established as 3-*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*β*-D-xylopyranosyl-3*β*, 19*α*-dihydroxyurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside in Fig. 1.

Ilexchinenoside U (**4**) was obtained as a white amorphous powder. The molecular formula was determined to be $C_{42}H_{68}O_{15}$ by the HR-ESI-MS. Its ^{1}H and ^{13}C NMR data (Table 1) showed an aglycone very similar with that of compound **3** except for an extra hydroxyl group. It was located at C-23 confirmed by the HMBC correlations of H-23 with C-3, C-5 and C-24 (Fig. 2). Additionally, the anomeric proton at δ_H 5.37 (H-1′, br s) and the corresponding anomeric carbon at δ_C 102.0 (C-1′) revealed that a *α*-glucose existed in **4**. While, the anomeric proton at δ_H 6.32 (H-1″, d, J = 8.0 Hz) indicated the presence of *β*-glucose. The sugars were determined to be D-glucose by HPLC analysis after hydrolysis and derivatization. They were located at C-23 and C-28, respectively, which confirmed by the HMBC correlations of H-1′ with C-23 and H-1″ with C-28. Thus, the structure of compound **4** was determined and named as 23-*O*-*α*-D-glucopyranosyl-3*β*, 19*α*, 23-trihydroxyurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside in Fig. 1.

Ilexchinenoside V (**5**) has the molecular of $C_{41}H_{66}O_{14}$ elucidated by the HR-ESI-MS *quasi* ion peak at *m*/*z*

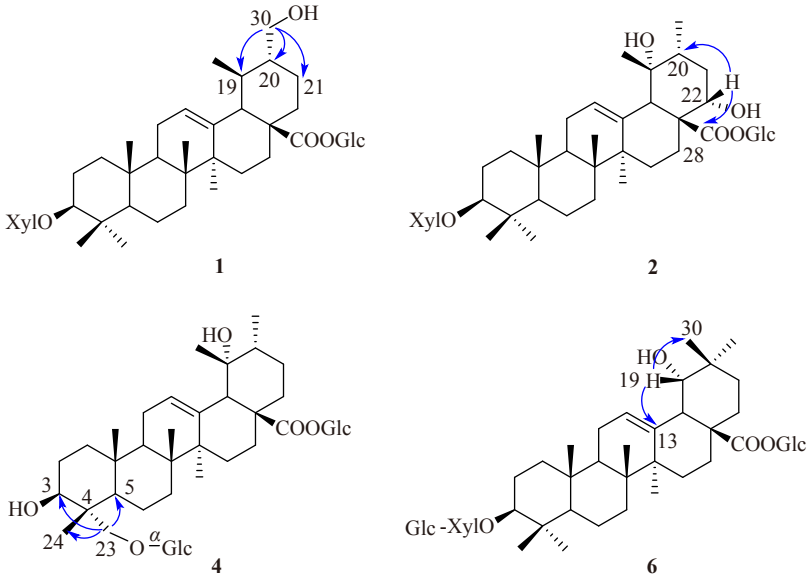

Fig. 2 The key HMBC correlations of compounds 1, 2, 4 and 6

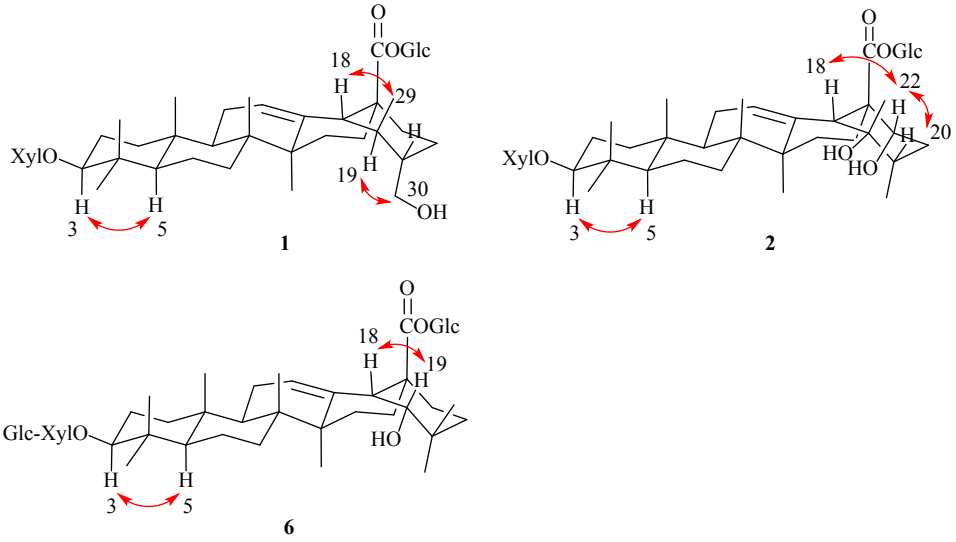

Fig. 3 The key NOESY correlations of compounds 1, 2 and 6

805.4403, the same with that of compound **2**. Its ^{1}H and ^{13}C NMR data (Table 1) indicated an ursane-type triterpenoid, a *β*-xylose [δ_H 4.81 (H-1′, d, J = 7.2 Hz)] and *β*-glucose [δ_H 6.32 (H-1″, d, J = 8.4 Hz)], which were also the same with those of compound **2**. The sugars were confirmed to be D-glucose and D-xylose by HPLC analysis after hydrolysis and derivatization. Furthermore, the xylose and glucose of **5** were located at C-23 and C-28 confirmed by its HMBC cross-peaks of H-1′ with C-23 and H-1″ with C-28. As a result, the structure of **5** was established as 23-*O*-*β*-D-xylopyranosyl-3*β*, 19*α*, 23-trihydroxyurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside in Fig. 1.

Ilexchinenoside W (**6**) was yielded as a white amorphous powder with the molecular formula of $C_{47}H_{76}O_{18}$ determined by the HR-ESI-MS positive ion peak at *m*/*z* 951.4952 (calcd for $C_{47}H_{76}NaO_{18}$, 951.4924). The ^{1}H NMR data of **6** (Table 2) showed seven methyl singlets at δ_H 0.91, 1.00, 1.01, 1.17, 1.17, 1.30 and 1.66, one olefinic proton at δ_H 5.53 (t, J = 3.0 Hz), two oxy-methine protons at δ_H 3.32 (H-3, dd, J = 11.5, 4.0 Hz) and 3.60 (H-19, m), and many methylene and methane signals between δ_H 0.84–2.87. The typical ^{1}H NMR data indicated that it was a triterpenoid. Additionally, three anomeric protons at δ_H 4.79 (d, J = 7.5 Hz), 5.33 (d, J = 8.0 Hz), and 6.41 (d, J = 8.0 Hz), along with 17 signals between δ_H 3.71–4.55 revealed the presence of two *β*-glucoses and one *β*-xylose. Moreover, HPLC analysis confirmed that they were D-xylose and D-glucose after acid hydrolysis and derivatization. The ^{13}C NMR data of **6** (Table 2) showed 47 carbons, consisting of 30 carbons from the triterpenoid skeleton and 17 signals for the sugars. The characteristic olefinic bond carbon signals at δ_C 123.7 (C-12) and 144.3 (C-13) indicated that it was an oleanane-type triterpenoid [11]. Analysis of the

Table 2 ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compounds 6 and 7 in C_5D_5N

No.	6		7	
	δ_H	δ_C	δ_H	δ_C
1*α*	0.93, m	38.6	0.89, m	38.4
1*β*	1.50, m		0.89, m	
2*α*	2.10, m	26.7	1.81, m	27.5
2*β*	1.87, m		1.89, m	
3	3.32, dd, 11.5, 4.0	88.8	4.23, m	72.3
4		39.6		42.9
5	0.84, br d, 11.5	55.9	1.49, m	48.8
6*α*	1.50, m	18.7	1.39, m	18.8
6*β*	1.34, m		1.68, m	
7*α*	1.56, m	33.2	1.43, m	32.9
7*β*	1.47, m		1.66, m	
8		40.2		40.2
9	1.83, m	48.3	1.86, m	48.3
10		37.2		37.4
11	1.98, m	24.2	1.99, m	24.2
12	5.53, t, 3.0	123.7	5.56, t, 3.0	123.6
13		144.3		144.5
14		42.1		42.1
15*α*	1.28, m	29.1	1.24, m	29.0
15*β*	2.40, td, 13.5, 3.0		2.37, td, 14.0, 3.0	
16*α*	2.16, m	28.0	2.83, td, 14.0, 3.0	28.0
16*β*	2.87, td, 13.5, 3.0		2.11, m	
17		46.5		46.4
18	3.56, br s	44.7	3.54, br s	44.5
19	3.60, m	81.0	3.57, m	81.1
OH-19	6.05, d, 6.0		5.98, d, 6.0	
20		35.6		35.6
21*α*	1.06, m	29.0	1.04, m	28.9
21*β*	2.08, m		2.06, m	
22*α*	1.98, m	33.1	1.95, m	33.1
22*β*	2.08, m		2.06, m	
23	1.30, s	28.1	4.01, d, 10.04.08, d, 10.0	75.2
24	1.01, s	16.9	1.02, s	13.2
25	0.91, s	15.5	0.98, s	16.0
26	1.17, s	17.6	1.17, s	17.7
27	1.66, s	24.9	1.56, s	25.1
28		177.3		177.3
29	1.17, s	28.8	1.14, s	28.8
30	1.00, s	24.7	0.98, s	24.6
	Xyl		Glc	
1′	4.79, d, 7.5	107.2	4.92, d, 7.5	105.5
2′	4.04, m	74.2	4.04, m	75.2

Continued

No.	**6**		**7**	
	δ_H	δ_C	δ_H	δ_C
3′	4.14, m	88.5	4.19, m	78.7
4′	4.11, m	69.6	4.18, m	71.7
5′	3.71, t, 10.04.34, m	66.5	3.93, m	78.5
6′			4.38, m 4.54, br d, 11.5	62.9
	Glc		Glc	
1″	5.33, d, 8.0	105.8	6.39, d, 8.5	95.9
2″	4.09, m	75.6	4.23, m	74.1
3″	4.26, m	78.3	4.31, t, 9.0	79.0
4″	4.21, m	71.7	4.39, m	71.1
5″	4.04, m	78.7	4.04, m	79.3
6″	4.32, m 4.55, dd, 11.5, 1.5	62.6	4.42, m 4.47, br d, 12.5	62.2
	Glc			
1‴	6.41, d, 8.0	95.9		
2‴	4.24, m	74.2		
3‴	4.31, m	79.0		
4‴	4.40, m	71.1		
5‴	4.05, m	79.4		
6‴	4.42, m 4.47, br d, 10.5	62.2		

1D NMR data of **6** suggested that it was similar with those of oblonganoside K [11]. The difference between them was that compound **6** had one more glucose moiety. The HMBC correlations of H-19 with C-13 and C-30 indicated the hydroxyl group was located at C-19 (Fig. 2). The extra glucose was connected on the C-3′ of the xylose determined by HMBC correlation of H-1″ with C-3′. In addition, the NOESY correlations between H-3 and H-5, and between H-18 and H-19 revealed that H-3 and H-5 were *α*-oriented, while the H-18 and H-19 were *β*-oriented (Fig. 3). Thus, the structure of **6** was determined as 3-*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*β*-D-xylopyranosyl-3*β*, 19*α*-dihydroxy olean-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside in Fig. 1.

Ilexchinenoside X (**7**) has a molecular formula of $C_{42}H_{68}O_{15}$ determined by the HR-ESI-MS data. Its ^{1}H NMR data (Table 2) exhibited two anomeric protons at δ_H 4.92 (H-1′, d, J = 7.5 Hz), and 6.39 (H-1″, d, J = 8.5 Hz), and 12 protons between δ_H 3.93 and 4.47, which indicated the presence of two *β*-glucoses. The HPLC analysis confirmed that the sugars were D-glucose after acid hydrolysis and derivatization. Comparison of the NMR data of **7** with those of **6** suggested that they were very similar except for the lack of xylose and the position of glucose. The HMBC correlations of H-1′ with C-23 indicated that the changed glucose was located at C-23. Therefore, the structure of **7** was identified and named as 23-*O*-*β*-D-glucopyranosyl-3*β*, 19*α*, 23-trihydroxy-olean-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside in Fig. 1.

Additionally, four known triterpenoid saponins, oblonganoside I [12], oblonganoside L [13], randiasaponin VI [14], and randiasaponin VII [14] were isolated from the leaves of *I. chinensis*. Their structures were elucidated by comparison of the NMR and ESI data with those reported in literatures.

All isolated compounds **1–11** were evaluated for their hepatoprotective activities against APAP-induced HepG2 cell injury *in vitro*. The results showed that compounds **1**, **2**, **4**, **9**, and **10** exhibited potential activity with the survival rates higher than the bicyclol (positive control, 46.82%), among which compound **10** possessed the highest survival rate of 61.35% at 10 μmol·L^{-1} (Table 3). According to the results, both the ursane-type and oleanane-type triterpenoids showed potential hepatoprotective effects, moreover, the effect of oleanane-type triterpenoid with an arabinose at C-3 was stronger.

Experimental

General experimental procedures

UV spectra were taken on a JASCO J-650 spectrophotometer (JASCO, Easton, MD, USA). IR spectra were carried out on a Nicolet 5700 spectrometer *via* FT-IR microscope transmission (Thermo Scientific, Waltham, MA, USA). Optical rotations were measured at a JASCO J-815 spectrometer (JASCO, Easton, MD, USA). The 1D and 2D NMR spectra were measured at Inova-500 and VNS-600 (Varian, USA) spectrometer with C_5D_5N as the deuterated solvents. HR-ESI-MS experiments were performed on an Agilent 6520 Accurate-Mass Q-Tof LC/MS ion trap mass spectrometer (Agilent Technologies, Waldbronn, Germany). HPLC experiments were conducted by a Shimadzu instrument LC-6AD equipped

Table 3 Hepatoprotective activities of compounds 1–11 against APAP-induced HepG2 cell damage [a]

Group	Optical density (*OD*)	Cell survival rate (%)
Control	1.184 ± 0.120	100.00
APAP (8 mmol·L^{-1})	0.343 ± 0.058***	30.76
Bicyclol [b]	0.522 ± 0.027$^{\#\#}$	46.82
1	0.584 ± 0.070$^{\#\#}$	**52.38**
2	0.583 ± 0.089$^{\#\#}$	**52.26**
3	0.379 ± 0.036	33.99
4	0.567 ± 0.036$^{\#\#}$	**50.88**
5	0.468 ± 0.042	42.00
6	0.415 ± 0.025	37.25
7	0.351 ± 0.013	31.45
8	0.359 ± 0.023	23.12
9	0.573 ± 0.053$^{\#\#}$	**51.39**
10	0.684 ± 0.015$^{\#\#}$	**61.35**
11	0.476 ± 0.051$^{\#}$	42.72

The compounds were tested at 10 μmol·L^{-1}. $^{***}P < 0.001$ *vs* control group; $^{\#\#\#}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ *vs* model group. [a] Results are expressed as the means ± SD (n = 3). [b] Bicyclol as the positive control

with an SPD-20A UV detector using an YMC-Pack ODS-A column (250 mm × 10 mm, 5 μm; YMC Corp., Kyoto, Japan). Column chromatography was performed on an Isco CombiFlash Rf2000 chromatograph or atmospheric pressure chromatograph using silica gel (200–300 mesh, Qingdao Marine Chemical Co. Ltd.), RP-18 (45–75 μm, Alltech Bulk Higt Capacity C_{18}), and Sephadex LH-20 (GE HealthcareBio-Science AB, Uppsala).

Plant material

The leaves of *I. chinensis* were collected from Jiujiang City of Jiangxi Province, People's Republic of China in March 2012. They were identified by Professor Tan Ce-ming of the Herbarium of Jiujiang Forestry Institute. A voucher specimen (ID-S-2597) has been deposited at the Herbarium of Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences.

Extraction and isolation

The leaves of *I. chinensis* (20 kg) were shattered and extracted with 70% C_2H_5OH−H_2O (50 L) under reflux for three times, 2 h for each time to produce the crude extract. Then, the extract was suspended in water (3 L) and partitioned with ethyl acetate (3 × 3 L). The aqueous fraction was carried out on a macroporous adsorption resin (HP-20) column with a mixture gradient of CH_3OH−H_2O (0%, 20%, 50%, 95%) to produce four fractions.

The 50% fraction (300 g) was subjected to another HP-20 column with CH_3OH−H_2O (0%, 40%, 70%, 95%) to obtain four fractions (Frs. WC1−WC4). Fr. WC3 (30 g) was subjected to a silica gel column with a mixture of CH_2Cl_2−CH_3OH−H_2O (10 : 1 : 0.1−5 : 1 : 0.1) to yield 10 fractions (Frs. WC3A−WC3J). Fr. WC3E (2.2 g) was separated by an ODS-AQ-packed column using a gradient elution of CH_3OH−H_2O (50%−100%) to produce four fractions (Fr. WC3E1−Fr. WC3E4). Then, Fr. WC3E3 (728 mg) was purified by the semipreparative HPLC using CH_3OH−H_2O (58 : 42) to yield compounds **9** (35 mg, 26 min), **8** (120 mg, 38 min) and five other subfractions (Fr. WC3E3B−Fr. WC3E3F). Fr. WC3E3B was further separated by the semipreparative HPLC using CH_3CN−H_2O (50 : 50) to yield compounds **1** (3 mg, 10 min) and **2** (3 mg, 11 min). Fr. WC3E3C was further purified by the semipreparative HPLC with CH_3CN−H_2O (50 : 50) to yield compound **10** (2 mg, 16 min). Fr. WC3E3D was purified by the semipreparative HPLC with CH_3CN−H_2O (52 : 48) to yield compound **5** (3 mg, 11 min).

Fr. WC3G (1.5 g) was subjected to an ODS-AQ-packed column with CH_3OH−H_2O (50%−100%) to produce three fractions (Fr. WC3G1−Fr. WC3G3). Fr. WC3G2 (710 mg) was purified by the semipreparative HPLC with CH_3CN−H_2O (40 : 60) to yield compound **11** (47 mg, 12 min) and two subfractions (Fr. WC3G2D and Fr. WC3G2E). Fr. WC3G2D was further purified by the semipreparative HPLC with CH_3OH−H_2O (60 : 40) to yield compound **4** (4 mg, 27 min). Fr. WC3G2E was further purified by the semipreparative HPLC with CH_3OH−H_2O (66 : 34) to yield compound **7** (20 mg, 13 min). Fr. WC3G3 (523 mg) was separated by the semipreparative HPLC with CH_3CN−H_2O (43 : 57) to yield compounds **3** (35 mg, 24 min) and **6** (35 mg, 26 min).

Ilexchinenoside R (**1**): white amorphous powder; mp 201−202 °C; $[\alpha]_D^{20}$ +13.1 (*c* 0.14, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 211 (3.50), 257 (2.70); IR ν_{max} (Microscope) 3402, 2929, 2879, 1731, 1458, 1375, 1165, 1073, 1048 cm^{-1}; HR-ESI-MS *m/z* 789.4394 $[M + Na]^+$ ($C_{41}H_{66}NaO_{13}$, Calcd. 789.4396); ^{1}H NMR and ^{13}C NMR data see Table 1.

Ilexchinenoside S (**2**): white amorphous powder; mp 203−205 °C; $[\alpha]_D^{20}$ +9.4 (*c* 0.13, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 213 (2.83); IR ν_{max} (Microscope) 3397, 2942, 1742, 1649, 1459, 1388, 1363, 1165, 1072 cm^{-1}; HR-ESI-MS *m/z* 805.4296 $[M + Na]^+$ ($C_{41}H_{66}NaO_{14}$, Calcd. 805.4345); ^{1}H NMR and ^{13}C NMR data see Table 1.

Ilexchinenoside T (**3**): white amorphous powder; mp 235−236 °C; $[\alpha]_D^{20}$ −3.4 (*c* 0.21, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 211 (3.31); IR ν_{max} (Microscope) 3376, 2926, 2877, 1732, 1646, 1452, 1368, 1166, 1072, 1030 cm^{-1}; HR-ESI-MS *m/z* 951.4902 $[M + Na]^+$ ($C_{47}H_{76}NaO_{18}$, Calcd. 951.4924); ^{1}H NMR and ^{13}C NMR data see Table 1.

Ilexchinenoside U (**4**): white amorphous powder; mp 201−203 °C; $[\alpha]_D^{20}$ +25.8 (*c* 0.09, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 219 (3.35), 258 (2.84); IR ν_{max} (Microscope) 3403, 2928, 2889, 1735, 1451, 1386, 1133, 1072, 1030 cm^{-1}; HR-ESI-MS *m/z* 835.4466 $[M + Na]^+$ ($C_{42}H_{68}NaO_{15}$, Calcd. 835.4450); ^{1}H NMR and ^{13}C NMR data see Table 1.

Ilexchinenoside V (**5**): white amorphous powder; mp 202−203 °C; $[\alpha]_D^{20}$ −2.6 (*c* 0.12, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 216 (3.25), 258 (2.74); IR ν_{max} (Microscope) 3386, 2928, 1735, 1452, 1372, 1164, 1072, 1046 cm^{-1}; HR-ESI-MS *m/z* 805.4403 $[M + Na]^+$ ($C_{41}H_{66}NaO_{14}$, Calcd. 805.4345); ^1H NMR and ^{13}C NMR data see Table 1.

Ilexchinenoside W (**6**): white amorphous powder; mp 235−236 °C; $[\alpha]_D^{20}$ −1.8 (*c* 0.17, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 211 (3.16); IR ν_{max} (Microscope) 3399, 2942, 2881, 1730, 1457, 1388, 1165, 1071, 1029 cm^{-1}; HR-ESI-MS *m/z* 951.4952 $[M + Na]^+$ ($C_{47}H_{76}NaO_{18}$, Calcd. 951.4924), ^1H NMR and ^{13}C NMR data see Table 2.

Ilexchinenoside X (**7**): white amorphous powder; mp 199−200 °C; $[\alpha]_D^{20}$ +4.3 (*c* 0.17, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 211 (3.48); IR ν_{max} (Microscope) 3373, 2831, 2877, 1731, 1454, 1388, 1073, 1032 cm^{-1}; HR-ESI-MS 835.4507 *m/z* $[M + Na]^+$ ($C_{42}H_{58}NaO_{15}$, Calcd. 835.4450); ^1H NMR and ^{13}C NMR data see Table 2.

Determination the absolute configuration of sugars [15]

Compound **1** (1 mg) was dissolved in 3 mL CH_3OH−H_2O (2 : 1), 1 mol·L^{-1} HCl (1 mL) was then added and reacted at 70 °C for 4 h in oil-bath. The mixture reaction was evaporated under reduced pressure to yield a residue. The residue was solved in 2 mL H_2O and extracted by ethyl acetate for four times (each time 5 mL). The aqueous layer was evaporated by freeze drier to obtain sugars. The sugars were confirmed to be glucose and xylose by comparison of their R_f values with those of standardized glucose and xylose. The sugars were solved in 1 mL pyridine and reacted with L-cysteine methyl ester hydrochloride (2 mg) under 60 °C for 1 h. Then, the *N*-trimethylsilylimidazole (2 mg) was reacted with the mixture under 60 °C for 1 h. The reaction mixture was analyzed by the HPLC with a Cosmosil-Packed 5C18-AR-II column using CH_3CN−H_2O (25 : 75) in 50 mmol·L^{-1} H_3PO_4 at the flow rate of 1 mL·min^{-1}. Compounds **2**–**7** were treated as well as **1**. The D-glucose (t_R = 12.36 min) and D-xylose (t_R = 14.47 min) were determined via comparison with those retention times of the standards after derivatization (Supplementary material, Fig. S1).

Hepatoprotective activity assay

The hepatoprotective activities of compounds **1**–**11** were evaluated by the MTT method [16]. HepG2 cells were cultivated in DMEM medium with 10% fetal bovine serum and penicillin (100 U·mL^{-1})-streptomycin (100 μg·mL^{-1}) at 37 °C in a 5% CO_2 atmosphere. The cells were put in 96-well plates and cultivated for 24 h. Then, they were treated with APAP (end with 8 mmol·L^{-1}) and the tested compounds (10 μmol·L^{-1}) and cultivated for another 48 h. The bicyclol was set as the positive control. Then, 100 μL MTT (0.5 mg·mL^{-1}) was added in each well after removing the DMEM and subsequently cultivated for 4 h. Finally, 150 μL DMSO was added in each well after removing the MTT. The optical density values were detected at 570 nm using a microplate reader.

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