Ilexpublesnins C–M, Eleven New Triterpene Saponins from the Roots of *Ilex pubescens*

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Key words
- *Ilex pubescens*
- Aquifoliaceae
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- 24-aldehyde group
- cytotoxicity

Abstract

Eleven new triterpene saponins, ilexpublesnins C–M (**1**–**11**), along with ten known analogues were isolated from the roots of *Ilex pubescens*. Their structures were elucidated on the basis of extensive spectroscopic analysis, including 1D and 2D NMR experiments. Compounds **1, 2, 10**, and **11** contain a 24-aldehyde, which is rare for triterpene saponins from *Ilex*. These compounds were evaluated *in vitro* for their cytotoxic effects on human cancer cell lines HepG2, HLE, BEL7402, BEL7403, BEL7405, MCF-7, and HeLa. Among them, only compounds **6** and **19** showed cytotoxicity against the MCF-7 cell line [inhibition (%): 33.14 and 34.03, respectively].

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Ilex pubescens Hook. et Arn., known under the Chinese name 'Mao-dong-qing', is an evergreen shrub belonging to the Aquifoliaceae family. Its roots are commonly used as Chinese herbal medicine for treatment of cardiovascular disease and hypercholestemia in south China [1]. Previous phytochemical investigation on its roots and leaves led to the isolation of triterpene saponins [2], lignan glycosides [3], phenylethanols [4,5], and other minor compounds [6–8]. Pharmacological evaluation proved that *Ilex pubescens* extracts exhibited a variety of bioactivities, i.e., to enlarge blood vessels, improve microcirculation, ease blood pressure, inhibit platelet aggregation, prevent thrombosis, and reduce cardiac ischemia, as well as to decrease the excitation of the cardiac conduction system and other effects [9].

As part of a systematic research on seeking the bioactive constituents from *Ilex* species [10–18], a phytochemical investigation on the roots of *I. pubescens* was undertaken, which led to the isolation of eleven new triterpene saponins, namely ilexpublesnins C–M (**1**–**11**) (**Fig. 1**), and ten known ones (**12**–**21**). This paper describes the isolation and structural elucidation of these compounds. In addition, the cytotoxic evaluation for these compounds against the human cancer cell lines HepG2, HLE, BEL-7402, BEL-7403, BEL-7405, MCF-7, and HeLa is included.

Materials and Methods

Instruments

HRESIMS were measured on a Bruker APEX IV FT_MS (7.0 T) spectrometer in the positive ion mode. Optical rotations were measured on an Autopol-IV polarimeter (RudoPH Research analytical). IR spectra were obtained using a NEXUS-470 FTIR (Nicolet) spectrometer. 1D and 2D NMR spectra were recorded on Bruker Avance DRX-400 and Vnmrs-500 spectrometers. Semipreparative HPLC was performed on a Waters model 2487 (Agilent ODS column, 250 × 10 mm i.d., 5 µm) with an Alltech evaporative light scattering detector (ELSD). GC analysis was carried out on an Agilent 6890 N gas chromatograph, with an HP-5 capillary column (28 m × 0.32 mm) and an FID detector operated at 260 °C (column temp. 180 °C), and 1.0 mL/min N_2 as the carrier gas. Macroporous resin (HPD100) was purchased from Hebei Bao-En Biotech Ltd. Thin-layer and column chromatography was performed using silica gel (Qingdao Haiyang Chemical Co. Ltd., GF_{254} or 200–300 mesh), Sephadex LH-20 (Pharmacia Biotech Ltd.), and ODS (Merck & Co., Inc.). All the solvents were

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of analytical grade and purchased from Beijing Chemical Company Ltd.

Plant material

The roots of *Ilex pubescens* were purchased from Guilin San-Jin Pharmaceutical Co. Ltd and originally collected from Guangxi province, China. The plants were identified by Prof. Peng-Fei Tu (one of the authors in this paper). A voucher specimen (No. 091005) is deposited in the Herbarium of Modern Research Center for Traditional Chinese Medicine (TCM), Peking University, Beijing.

Extraction and isolation

Dry crude materials (18 kg) were grinded and extracted with 70% EtOH at the temperature of 70 °C. After the retrieval of the ethanol, the residue suspended in water (50 L) was subjected to column chromatograph (CC) on macroporous resin with an EtOH-H_2O gradient (30 : 70, 70 : 30, 90 : 10) to yield three fractions (Frs.1–3). Fr.2 (160 g) was chromatographed on silica gel (2.5 kg, 100 × 10 cm) with a gradient of $CHCl_3$-MeOH (40 : 1–1 : 1) elution to yield thirteen fractions (Frs. A–M). Then these fractions were applied to CC on silica gel, Sephadex LH-20, ODS, and semipreparative HPLC to afford compounds **1** (30 mg), **2** (5 mg), **3** (80 mg), **4** (200 mg), **5** (15 mg), **6** (15 mg), **7** (50 mg), **8** (9 mg), **9** (12 mg), **10** (28 mg), **11** (10 mg), **12** (9 mg), **13** (120 mg), **14** (160 mg), **15** (250 mg), **16** (10 mg), **17** (8 mg), **18** (25 mg), **19** (24 mg), **20** (22 mg), and **21** (500 mg). For detailed isolation and purification protocol, see Supporting Information.

Ilexpublesnin C (**1**): white, amorphous powder; $[\alpha]_D^{20}$ + 9.47 (*c* 0.19, MeOH); IR (KBr) ν_{max} 3423, 2933, 1719, 1611, 1072 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 500/125 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 842.45015 $[M + NH_4]^+$ (calcd. for $C_{42}H_{64}O_{16}NH_4$, 842.45326).

Ilexpublesnin D (**2**): white, amorphous powder; $[\alpha]_D^{20}$ + 9.34 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3433, 2932, 1692, 1631, 1068 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 500/125 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 680.40011 $[M + NH_4]^+$ (calcd. for $C_{36}H_{54}O_{11}NH_4$, 680.40044).

Ilexpublesnin E (**3**): white, amorphous powder; $[\alpha]_D^{20}$ – 56.0 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3420, 2933, 1750, 1642, 1073 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 400/100 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 1092.59670 $[M + NH_4]^+$ (calcd. for $C_{53}H_{86}O_{22}NH_4$, 1092.59490).

Ilexpublesnin F (**4**): white, amorphous powder; $[\alpha]_D^{20}$ + 52.6 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3433, 2932, 1695, 1078 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 400/100 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 766.47571 $[M + NH_4]^+$ (calcd. for $C_{41}H_{64}O_{12}NH_4$, 766.47360).

Ilexpublesnin G (**5**): white, amorphous powder; $[\alpha]_D^{20}$ + 3.3 (*c* 0.12, MeOH); IR (KBr) ν_{max} 3420, 2936, 1731, 1072 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 500/125 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 1565.89765 $[2M + H]^+$ (calcd. for $C_{82}H_{133}O_{28}$, 1565.89779).

Ilexpublesnin H (**6**): white, amorphous powder; $[\alpha]_D^{20}$ + 8.33 (*c* 0.12, MeOH); IR (KBr) ν_{max} 3434, 2943, 2879, 1732, 1639, 1073 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 400/100 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 845.39863 $[M - H]^-$ (calcd. for $C_{41}H_{66}O_{16}S$, 845.39988).

Ilexpublesnin I (**7**): white, amorphous powder; $[\alpha]_D^{20}$ + 2.33 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3453, 2940, 2875, 1738, 1637, 1063 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 400/100 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 869.39648 $[M + Na]^+$ (calcd. for $C_{41}H_{66}O_{16}SNa$, 869.39638).

Ilexpublesnin J (**8**): white, amorphous powder; $[\alpha]_D^{20}$ + 2.0 (*c* 0.08, MeOH); IR (KBr) ν_{max} 3428, 2930, 2879, 1730, 1076 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 400/100 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 1108.58861 $[M + NH_4]^+$ (calcd. for $C_{53}H_{86}O_{23}NH_4$, 1108.58981).

Ilexpublesnin K (**9**): white, amorphous powder; $[\alpha]_D^{20}$ – 4.1 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3421, 2929 2875, 1732, 1075 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 400/100 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 1078.58298 $[M + NH_4]^+$ (calcd. for $C_{53}H_{86}O_{23}NH_4$, 1078.57925).

Ilexpublesnin L (**10**): white, amorphous powder; $[\alpha]_D^{20}$ + 12.05 (*c* 0.19, MeOH); IR (KBr) ν_{max} 3427, 2941, 1718, 1615, 1071 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 500/125 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 842.45024 $[M + NH_4]^+$ (calcd. for $C_{42}H_{64}O_{16}NH_4$, 842.45326).

Ilexpublesnin M (**11**): white, amorphous powder; $[\alpha]_D^{20}$ + 8.57 (*c* 0.14, MeOH); IR (KBr) ν_{max} 3433, 2944, 1689, 1073 cm^{-1}; ^1H and ^{13}C NMR data (pyridine-d_5, 500/125 MHz), see **Tables 1** and **2**; HRESIMS *m/z* 680.39823 $[M + NH_4]^+$ (calcd. for $C_{36}H_{54}O_{11}NH_4$, 680.40044).

Acid hydrolysis

Compounds **1, 3**–**11** (4 mg), and **2** (2 mg) were heated in 3 mL of 10% HCl-dioxane (1 : 1) at 90 °C for 4 h in a sealed amp. After the dioxane was removed, the solution was extracted with EtOAc (3 mL × 3) to yield the aglycon and the sugar, respectively. The sugar components in the aqueous layer left after acid hydrolysis of **1**–**11** were analyzed by silica gel TLC by comparison with standard sugars. The solvent system was *n*-BuOH-TEA-H_2O (60 : 0.7 : 30), and spots were visualized by spraying with phenylamine-diphenylamine, and then heated at 200 °C for 1 min. For the sugars of **1**–**11**, the R_f of glucuronic acid, glucose, arabinose, xylose, and rhamnose by TLC was 0.44, 0.64, 0.65, 0.71, 0.77, respectively. The results were confirmed by GC analysis of the methyl sugar peracetates. The aqueous layer was evaporated and dissolved in anhydrous pyridine (100 µL); 0.1 mL cysteine methyl ester hydrochloride (200 µL) was added, and the mixture was warmed at 60 °C for 1 h. The trimethysilylation reagent HMDS-TMCS (hexamethyldisilazane-trimethylchlorosilanepyridine, 2 : 1 : 10) (Acros Organics) was added and warmed at 60 °C for 30 min. The thiazolidine derivatives were analyzed by GC for sugar identification. The retention times of L-arabinose (t_R, 5.19 min), L-rhamnose (t_R, 5.34 min), D-xylose (t_R, 5.46 min), D-glucose (t_R, 11.63 min), and D-glucuronic acid (t_R, 23.56 min) were confirmed by comparison with those of authentic standards [10].

Cytotoxicity assay

The method described in the literature [19] was used for the measurement of cytotoxic activities against human cancer cell lines. For detailed protocols, see Supporting Information.

Supporting information

The detailed isolation and purification protocol of **1**–**11**, cytotoxicity assay and MTT data of **1**–**21**, structures of **12**–**21**, and the HRESIMS, IR, 1D and 2D NMR spectra of **1**–**11** are available as Supporting Information.

Table 1 ^{1}H NMR data (in pyridine-d_5) of **1, 2, 5, 10, 11** (500 MHz), **3, 4**, and **6**–**9** (400 MHz), J in Hz and δ in ppm.

Pos.	1	2	3	4	5	6	7	8	9	10	11
1	0.85 m; 1.50 m	0.87 m; 1.45 m	0.94 m; 1.52 m	0.84 m; 1.54 m	0.89 m; 1.53 m	0.87 m; 1.52 m	0.89 m; 1.52 m	0.88 m; 1.52 m	0.89 m; 1.53 m	0.81 m; 1.44 m	0.82 m; 1.43 m
2	2.00 m; 2.46 m	2.02 m; 2.44 m	1.86 m; 2.08 m	1.87 m; 2.06 m	1.99 m; 2.23 m	1.82 m; 2.02 m	1.90 m; 2.07	1.78 m; 2.06 m	1.77 m; 2.08 m	2.09 m; 2.48 m	2.10 m; 2.44 m
3	3.56 m	3.59 m	3.24 m	3.27 m	3.52 m	3.29 m	3.24 m	3.21 m	3.24 m	3.55 m	3.57 m
5	1.05 m	1.06 m	0.83 d (11.6)	0.72 d (11.6)	0.93 m	0.76 d (11.6)	0.79 m	0.76 d (11.6)	0.79 d (11.6)	1.00 m	1.04 m
6	1.37 m; 1.74 m	1.29 m; 1.48 m	1.31 m; 1.45 m	1.23 m; 1.45 m	1.33 m; 1.62 m	1.27 m; 1.46 m	1.26 m; 1.45 m	1.28 m; 1.45 m	1.27 m; 1.45 m	1.41 m; 1.75 m	1.42 m; 1.80 m
7	1.38 m; 1.47 m	1.32 m; 1.48 m	1.41 m; 1.59 m	1.52 m; 2.41 m	1.35 m; 1.50 m	1.30 m; 1.58 m	1.38 m; 1.52 m	1.38 m; 1.45 m	1.38; 1.45 m	1.33 m; 1.48 m	1.28 m; 1.43 m
9	1.72 m	1.79 m	1.78 m	1.46 m	1.78 m	1.80 m	1.76 m	1.78 m	1.78 m	1.74 m	1.81 m
11	1.97 m; 2.05 m	1.86 m; 2.04 m	1.68 m; 2.00 m	1.61 m; 1.91 m	1.62 m; 1.98 m	1.68 m; 2.07 m	1.62 m; 2.00 m	1.61 m; 2.01 m	1.62 m; 2.00 m	1.85 m; 2.06 m	1.88 m; 1.96 m
12	5.49 br s	5.56 br s	5.52 br s	5.60 t (4.0)	5.48 br s	5.52 br s	5.48 br s	5.49 br s	5.48 br s	5.44 br s	5.51 br s
15	1.16 m; 2.37 m	1.25 m; 1.99 m	1.13 m; 2.42 m	2.13 m; 2.30 m	1.26 m; 1.93 m	1.25 m; 2.48 m	1.25 m; 2.45 m	1.22 m; 2.44 m	1.22 m; 2.48 m	1.21 m; 2.25 m	1.21 m; 2.09 m
16	1.73 m; 1.96 m	1.24 m; 2.25 m	1.86 m; 2.06 m	2.30 m; 2.43 m	2.04 m; 2.11 m	1.82 m; 2.07 m	1.87 m; 2.07 m	1.93 m; 2.06 m	1.91 m; 2.09 m	1.17 m; 2.02 m	1.16 m; 2.07 m
18	2.89 s	3.02 s	2.91 s		3.17 s	3.18 br s	3.16 s	3.17 s	3.16 s	3.46 br s	3.59 br s
19										3.55 br s	3.59 br s
20	1.32 m	1.36 m	1.33 m	2.11 m	2.00 m	1.95 m	1.93 m	1.93 m	1.91 m		
21	1.96 m; 2.50 m	1.44 m; 2.44 m	1.85 m; 2.04 m	1.55 m; 1.66 m	1.93 m; 2.48 m	1.68 m; 2.00 m	1.69 m; 2.07 m	1.69 m; 1.99 m	1.69 m; 1.98 m	1.23 m; 1.38 m	1.24 m; 1.40 m
22	1.79 m; 2.01 m	1.79 m; 2.08 m	1.81 m; 2.06 m	2.29; 2.41 m	1.92 m; 2.20 m	1.87 m; 2.07 m	1.87 m; 2.07 m	1.88 m; 2.09 m	1.87 m; 2.09 m	1.91 m; 2.00 m	2.00 m; 2.05 m
23	1.54 s	1.58 s	1.31 s	1.25 s	1.51 s	1.21 s	1.37 m	1.20 s	1.37 m	1.52 s	1.59 s
24	10.32 s	10.32 s	1.06 s	1.05 s	3.61 d (11.5); 4.36 d (11.5)`	0.96 s	1.15 m	1.06 s	1.15 m	10.30 s	10.32 s
25	0.72 s	0.69 s	0.86 s	0.83 s	0.84 s	0.89 s	0.86 m	0.89 s	0.89 m	0.69 s	0.69 s
26	1.06 s	0.98 s	1.16 s	0.97 s	1.16 s	1.18 s	1.14 s	1.18 s	1.18 m	1.12 s	0.93 s
27	1.65 s	1.72 s	1.67 s	1.07 s	1.71 s	1.75 s	1.69 s	1.69 s	1.69 m	1.58 s	1.65 s
29	1.38 s	1.43 s	1.36 s	1.82 s	1.37 s	1.40 s	1.36 s	1.38 s	1.38 m	1.20 s	1.18 s
30	1.04 d (6.5)	1.10 d (6.5)	1.05 d (6.8)	1.08 d (6.0)	0.97 d (7.0)	0.98 d (7.6)	0.97 d (6.8)	0.98 d (6.8)	0.98 d (7.2)	0.95 s	1.10 s
3-*O*-	**GlcA**	**GlcA**	**Xyl**	**Xyl**	**Xyl**	**Ara**	**Xyl**	**Xyl**	**Xyl**	**GlcA**	**GlcA**
1	4.89 d (8.0)	4.98 d (5.5)	4.88 d (6.8)	4.80 d (6.4)	4.90 d (8.0)	5.07 d (7.2)	4.95 d (7.2)	4.96 d (6.0)	4.87 d (6.8)	4.82 d (6.0)	5.00 d (7.0)
2	4.01 m	4.05 m	4.05 m	4.19 m	3.97 m	4.20 m	5.03 m	4.09 m	4.08 m	3.97 m	4.03 m
3	4.27 m	4.23 m	3.84 m	4.26 m	4.12 m	5.55 t (8.4)	4.41 m	3.85 m	3.84 m	4.30 m	4.27 m
4	4.18 m	4.17 m	4.32 m	4.21 m	4.03 m	4.18 m	4.15 m	4.19 m	4.23 m	4.20 m	4.22 m
5	4.46 m	4.52 m	3.68 m; 4.28 m	3.65 t (11.6); 4.29 dd (11.6, 5.2)	3.78 t (10.5); 4.37 d (10.5)	3.99 m; 4.18 m	3.68 m; 4.30 m	3.74 m; 4.32 m	3.71 m; 4.32 m	4.44 m	4.47 m
Intermediate sugar			**Glc**					**Glc**	**Glc**		
1			5.80 d (7.2)					5.44 d (7.6)	5.49 d (8.0)		
2			4.38 m					4.14 m	4.17 m		
3			4.42 m					3.85 m	4.28 m		
4			4.73 m					4.11 m	4.37 m		
5			4.08 m					4.03 m	4.25 m		
6			4.29 m; 4.45 m					3.72 m; 4.54 m	4.39 m; 4.43 m		
Terminal			**Rha**	**Glc**				**Glc**	**Ara**		
1			6.38 br s	5.33 d (7.6)				5.38 d (7.6)	5.50 d (8.0)		
2			4.69 m	4.09 m				4.19 m	4.20 m[b]		
3			4.09 m	3.90 m				3.94 m	4.10 m		

cont.

Table 1 *Continued*

Pos.	1	2	3	4	5	6	7	8	9	10	11
4			4.26 m	4.14 m				4.19 m	4.12 m[c]		
5			5.00 m	4.26 m				4.05 m[a]	3.73 m; 4.32 m		
6			1.76 d (6.0)	3.65 t (11.6); 4.29 dd (11.6, 5.2)				4.42 m, 4.56 m			
28-*O*-	**Glc**		**Glc**		**Glc**	**Glc**	**Glc**	**Glc**	**Glc**	**Glc**	
1	6.21 d (8.5)		6.29 d (8.0)		6.31 d (8.0)	6.32 d (8.0)	6.32 d (8.0)	6.35 d (8.0)	6.35 d (8.0)	6.33 d (8.0)	
2	4.20 m		4.24 m		4.22 m	4.09 m	4.27 m	4.20 m	4.20 m[b]	4.19 m	
3	4.02 m		4.39 m		4.04 m	4.06 m	4.05 m	4.39 m	4.38 m	4.21 m	
4	4.37 m		4.32 m		4.19 m	4.32 m	4.38 m	4.23 m	4.12 m[c]	4.37 m	
5	4.26 m		4.06 m		4.29 m	4.28 m	4.29 m	4.05 m[a]	4.03 m	4.27 m	
6	4.36 m; 4.44 m		4.37 m; 4.45 m		4.37 m; 4.42 m	4.37 m; 4.44 m	4.38 m; 4.42 m	4.34 m; 4.42 m	4.39 m; 4.43 m	4.37 m; 4.42 m	

[a, b, c] The signals under the same superscript were overlapped

Results and Discussion

Ilexpublesnin C (**1**) was assigned the molecular formula $C_{42}H_{64}O_{16}$, determined on the basis of its positive HRESIMS [M + NH_4]$^+$ ion peak at *m*/*z* 842.45015 (calcd. 842.45326). The IR spectrum showed absorption bands for hydroxyl (3423 cm^{-1}), methyl (2933 cm^{-1}), carbonyl (1719 cm^{-1}), and olefinic (1611 cm^{-1}) groups. Acid hydrolysis of **1** afforded sugar components as D-glucose and D-glucuronic acid identified by TLC and GC analyses, which was also verified by comparing ^{13}C NMR data of chikusetusaponin IVa: 107.2, 75.5, 78.1, 73.4, 77.8, 172.8 [20]. The ^1H and ^{13}C NMR data (**Tables 1** and **2**) assigned by HSQC and HMBC experiments revealed five singlets for a tertiary methyl at δ_H 0.72, 1.06, 1.38, 1.54, 1.65, one doublet at δ_H 1.04 (d, *J* = 6.5 Hz), one olefinic linkage (δ_H 5.49, δ_C 128.2, C-12; δ_C 139.3, C-13), two carboxyls (δ_C 177.0, COOR-28; 175.1, COOH-GlcA-6), one low-field signal for an aldehyde group (δ_H 10.32, δ_C 206.2, CHO-24), and two sugar components (δ_H 4.89, δ_C 106.7, CH-GlcA-1; δ_H 6.21, δ_C 95.7, CH-Glc-1) in **1**.

Analysis of the above data suggested **1** to be a hydroxylated ursane triterpene saponin with an aldehyde substitution. Detailed HMBC correlations, including from H-12 to C-11, C-14, and C-18, from CH_3-24 to aldehyde carbon (δ_C 206.2), C-3, and C-5, from CH_3-29 to C-18 and C-20, and from CH_3-30 to C-19 and C-20, supported this assignment, and this analysis further indicated the structure of **1** to closely resemble ilexpublesnin A [21] except for the main difference at the sugar moiety. HMBC correlations (**Fig. 2**) from GlcA–H-1 (δ_H 4.89, d, *J* = 8.0 Hz) to C-3 (δ_C 86.9) and from Glc-H-1 (δ_H 6.21, d, *J* = 8.5 Hz) to C-28 (δ_C 177.0) suggested the linkages of a 3-*O*-, 28-*O*-diglycosylation. After the construction of its planar structure, the stereochemistry was revealed by analysis of NOESY correlations (**Fig. 2**). In the NOESY spectrum, cross-peaks of CH_3-29/CH_3-26, CH_3-27/CH_3-30 suggested an *α* configuration of 19-OH and CH_3-30, and correlations between H-24 (δ_H 10.32, s) and CH_3-25 (δ_H 0.72, s) showed that they were on the same face, confirming the *β* configuration of the 24-aldehyde. Based on the above analysis, compound **1** was elucidated as 3-*O*-*β*-D-glucuronide-3*β*,19*α*-dihydroxyurs-24-oxo-12-en-28-oic-*O*-*β*-D-glucopyranosyl ester.

Ilexpublesnin D (**2**) was assigned the molecular formula $C_{36}H_{54}O_{11}$ determined by the HRESIMS data. The IR, ^1H and ^{13}C NMR data (**Tables 1** and **2**) indicated that compound **2** possessed a similar structure to **1,** except for the disappearance of a glucosylation site at C-28. The preliminary deduction was supported by comparing its ^1H and ^{13}C data with those of **1** and confirmed by HMBC correlations. Therefore, compound **2** was elucidated as 3-*O*-*β*-D-glucuronide-3*β*,19*α*-dihydroxyurs-24-oxo-12-en-28-oic acid.

Analysis of the HRESIMS data ([M + NH_4]$^+$ at *m*/*z* 1092.59670, calcd. 1092.59490) of ilexpublesnin E (**3**) determined its molecular formula to be $C_{53}H_{86}O_{22}$. Its IR spectrum showed absorption bands for hydroxyl, methyl, carbonyl, and olefinic functional groups. Acid hydrolysis of **3** afforded sugar components of D-glucose, D-xylose, and L-rhamnose identified by TLC and GC analyses. The ^1H and ^{13}C NMR data (**Tables 1** and **2**) revealed **3** to be a hydroxylated ursane triterpene saponin, as **1** and **2**. A comprehensive analysis and comparison of its ^1H and ^{13}C NMR data with those of ilexoside II [22] implied that they were highly similar in structure, with the exception of an additional sugar unit in **3**. HMBC correlations from the *inner*-xyl-H-1 (δ_H 4.88, d, *J* = 6.8 Hz) to C-3 (δ_C 89.6), and from Glc-H-1 (δ_H 6.29, d, *J* = 8.0 Hz) to C-28 (δ_C 176.9) determined its glycosylation sites at 3-*O*- and *28-O*-. Furthermore, HMBC correlations from the intermediate-glc-H-1 (δ_H 5.80, d, *J* = 7.2 Hz) to inner-xyl-C-2 (δ_C 79.4) and from terminal-rha-H-1 (δ_H 6.38, br s) to intermediate-glc-C-2 (δ_C 79.3) established all the linkages of the sugar moieties. Eventually, **3** was elucidated as 3-*O*-[*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-xylopyranosyl]-3*β*,19*α*-dihydroxyurs-12-en-28-oic-*O*-*β*-D-glucopyranosyl ester.

Ilexpublesnin F (**4**) was obtained as white amorphous powder with a molecular formula of $C_{41}H_{64}O_{12}$ determined by the HRESIMS data. Overview of its IR, ^1H and ^{13}C NMR data (**Tables 1** and **2**) implied it to be a hydroxylated ursane triterpene saponin. A careful comparison of its ^1H and ^{13}C NMR data with those of pubescenoside C [23] revealed their structural similarity, except for the difference of no sugar moiety being present at the C-28 carboxyl in **4**. All chemical and spectroscopic methods, including acid hydrolysis and HMBC experiments, established the glycosylation sites and all the linkages between sugars and aglycon. Therefore, **4** was elucidated as 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)]-*β*-D-xylopyranosyl-3*β*-hydroxyurs-12,18-dien-28-oic acid.

Table 2 ^{13}C NMR data (in pyridine-d_5) of **1, 2, 5, 10, 11** (125 MHz), **3, 4**, and **6–9** (100 MHz), δ in ppm.

Position	1	2	3	4	5	6	7	8	9	10	11
1	37.9	37.9	38.8	39.2	38.6	38.7	38.8	38.8	38.9	37.6	37.6
2	27.0	27.0	26.6	26.7	26.9	26.8	26.6	26.8	26.8	27.1	27.0
3	86.9	87.0	89.6	88.8	88.9	88.4	89.6	88.9	88.7	87.0	87.0
4	53.6	53.6	39.6	39.5	44.3	39.4	39.6	39.6	39.6	53.5	53.6
5	57.6	57.6	56.0	56.0	56.3	55.7	55.9	55.9	55.9	57.5	57.5
6	19.3	19.2	18.7	18.3	19.0	18.6	18.6	18.7	18.7	19.3	19.2
7	33.4	33.4	33.5	35.6	33.7	33.4	33.5	33.5	33.5	33.0	33.1
8	40.2	40.1	40.5	39.2	40.4	40.4	40.4	40.4	40.4	39.8	39.7
9	46.4	46.4	47.7	48.1	47.6	47.7	47.7	47.7	47.8	47.0	47.0
10	36.8	36.8	37.0	36.7	36.7	37.0	37.0	37.0	37.0	37.0	37.0
11	24.6	24.4	24.3	23.4	24.7	24.7	24.7	24.7	24.7	24.6	24.5
12	128.2	127.8	128.3	126.3	127.6	127.7	127.6	127.6	127.7	123.5	123.5
13	139.3	140.0	139.2	139.5	138.8	138.8	138.8	138.8	138.2	144.2	144.9
14	42.1	42.1	42.1	44.7	42.1	42.2	42.1	42.1	42.1	42.0	42.1
15	29.2	29.2	29.2	28.8	29.2	29.2	29.2	29.2	29.2	28.4	28.3
16	24.8	24.7	26.6	34.8	26.8	26.6	26.6	26.8	26.6	28.9	29.1
17	48.6	48.2	48.6	50.2	48.3	47.9	48.3	48.3	48.3	46.4	46.0
18	54.4	54.6	54.4	135.0	27.2	47.2	47.2	47.2	47.2	44.6	44.8
19	72.6	72.7	72.6	135.9	73.4	73.4	73.4	73.4	73.4	81.0	81.2
20	42.1	42.4	42.1	37.4	42.8	42.8	42.8	42.9	42.8	35.5	35.7
21	26.0	27.1	27.0	29.2	24.2	24.1	24.0	24.0	24.0	28.9	29.2
22	37.6	38.5	36.9	34.8	31.8	31.9	31.8	31.9	31.9	33.1	33.6
23	21.4	21.5	28.4	28.1	23.4	28.3	28.3	28.2	28.2	21.3	21.5
24	206.2	206.2	16.7	16.7	63.3	16.0	17.4	16.8	16.8	206.4	205.9
25	16.2	16.1	15.6	16.1	15.4	15.6	15.6	15.7	15.7	15.9	15.9
26	17.2	17.0	17.4	18.0	17.4	17.5	16.9	17.5	17.5	17.4	17.2
27	24.5	24.6	24.0	22.2	24.2	24.4	24.3	24.3	24.3	24.8	24.8
28	177.0	180.6	176.9	178.9	177.0	177.1	177.1	177.0	177.0	177.2	180.8
29	26.7	26.9	26.8	20.2	29.7	29.7	29.7	29.7	29.7	28.8	28.8
30	16.7	16.8	16.6	20.5	16.0	17.0	16.0	16.0	16.0	24.6	24.7
3-*O*-	**GlcA**	**GlcA**	**Xyl**	**Xyl**	**Xyl**	**Ara**	**Xyl**	**Xyl**	**Xyl**	**GlcA**	**GlcA**
1	106.7	107.4	105.8	105.7	106.7	106.3	104.8	105.0	105.2	106.5	107.3
2	75.1	75.2	79.4	82.7	75.3	74.1	80.2	82.1	82.6	75.0	75.2
3	78.2	78.0	77.7	77.8	78.5	84.0	77.4	77.8	77.8	76.8	78.0
4	73.4	73.4	71.2	71.5	71.2[c]	69.9	70.8	71.1	71.6	73.4	73.4
5	76.8	76.8	66.6	66.6	67.2	66.2	65.9	65.9	66.5	76.8	77.9
6	175.1	–[a]								176.0	–[a]
Intermediate			**Glc**					**Glc**	**Glc**		
1			102.3					103.2	103.2		
2			79.3					85.4	84.4		
3			79.2					77.8	77.9[f]		
4			72.6					70.1	70.7		
5			79.0[b]					77.8	78.0[f]		
6			63.2					62.7	62.7		
Terminal			**Rha**	**Glc**				**Glc**	**Ara**		
1			102.0	105.6				106.4	106.5		
2			72.3	76.8				76.6	71.1		
3			72.6	77.9				79.1[e]	76.0		
4			74.3	70.8				71.0	70.5		
5			69.5	78.3				79.3	67.4		
6			19.0	62.6				62.5			
28-*O*-	**Glc**		**Glc**		**Glc**	**Glc**	**Glc**	**Glc**	**Glc**	**Glc**	
1	95.7		95.8		95.8	95.8	95.8	95.8	95.8	95.8	
2	74.0		74.1		74.1	73.9	74.1	74.1	74.1	74.1	
3	78.8		78.4		79.0	78.9	79.0[d]	79.0[e]	79.0	78.9	
4	71.2		71.2		71.1[c]	71.1	71.1	71.1	70.7	71.0	
5	79.2		78.9[b]		79.2	79.2	79.2[d]	79.3	79.3	79.3	
6	62.2		62.3		62.2	62.2	62.2	62.2	62.2	62.0	

[a] Signals were absent; [b, c, d, e, f] The signals under the same superscript may be interchanged

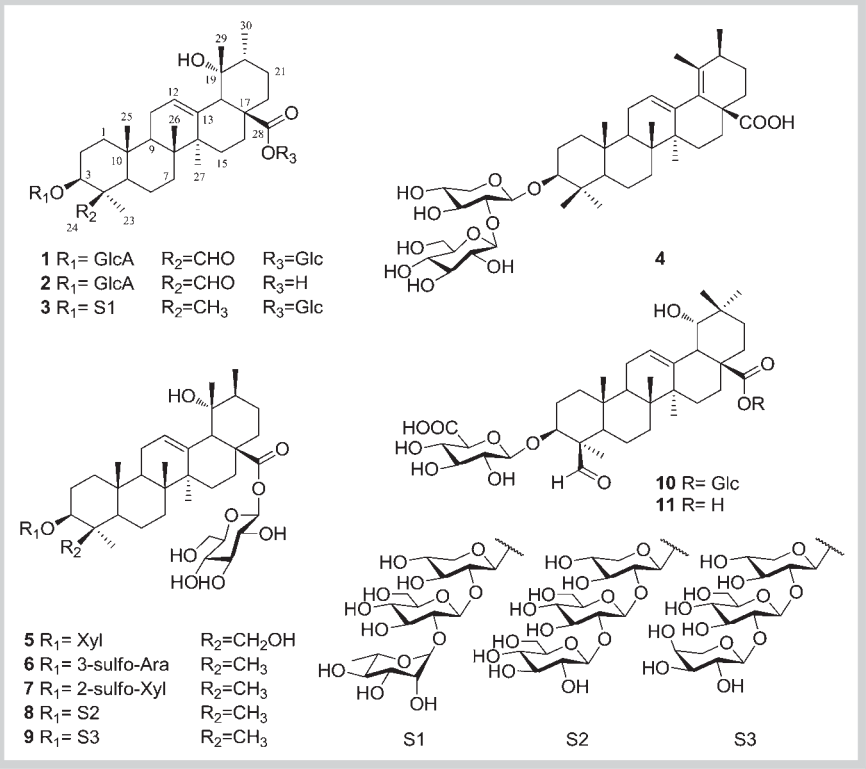

Fig. 1 Structures of compounds **1**–**11**.

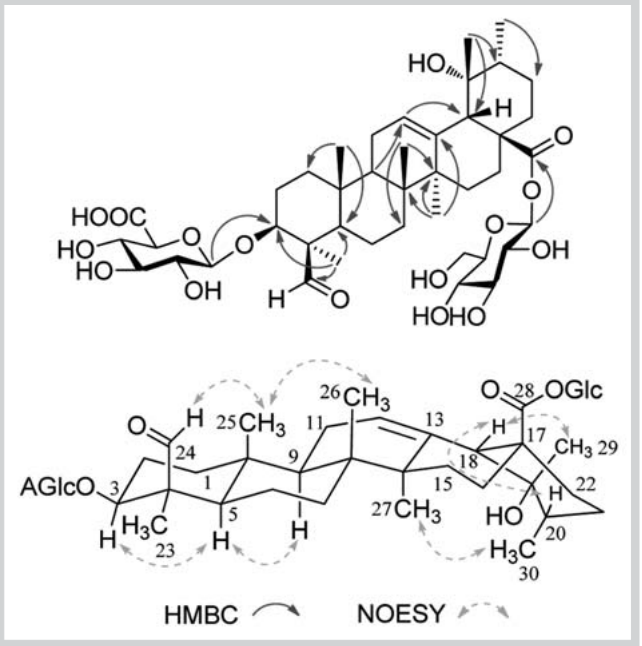

Fig. 2 Key HMBC and NOESY correlations of **1**.

Ilexpublesnin G (**5**) was given the molecular formula $C_{41}H_{66}O_{14}$ by HRESIMS data ($[2M + H]^+$ at *m/z* 1565.89765, calcd. 1565.89779). The IR spectrum showed absorption bands for hydroxyl, methyl, carbonyl, and olefinics. Acid hydrolysis of **5** afforded D-glucose and D-xylose, identified by TLC and GC analyses. The signals characteristic for an ursane triterpene saponin were observed from its ^1H and ^{13}C NMR data (**Tables 1** and **2**), including five singlets for tertiary methyls, an olefinic proton at δ_H 5.48 (br s), and two anomeric carbons as well. Detailed interpretation of its ^1H and ^{13}C NMR data suggested **5** to be closely related to ilexoside A [2], except for the oxidization of 24-CH_3 leading to 24-hydroxymethine, which was consistent with the chemical shifts of C-5 (δ_C 56.3) and C-23 (δ_C 23.4), respectively, compared to chemical shifts of C-5 (δ_C 48.2) and C-24 (δ_C 14.3) when hydroxylation happened at C-23 [24,25]. This was further confirmed by a NOESY correlation between H-24a (δ_H 4.36) and CH_3-25. Finally, **5** was elucidated as 3-*O*-*β*-D-xylpyranosyl-3*β*,19*α*,24*β*-trihydroxyurs-12-en-28-oic-*O*-*β*-D-glucopyranosyl ester.

The molecular formula $C_{41}H_{66}O_{16}S$ was determined for ilexpublesnin H (**6**) on the basis of its negative HRESIMS ($[M - H]^-$ at *m/z* 845.39863, calcd. 845.39988). Overall appearance of its IR spectrum and ^1H, ^{13}C NMR data (**Tables 1** and **2**) displayed a high resemblance with those of ilexoside A [2]. Interpretation of the spectroscopic data of **6** revealed the only difference between both compounds to be a sugar moiety. Acid hydrolysis of **6** yielded ilexgenin B [26], D-glucose, and L-arabinose identified by TLC and GC analyses. Comparison of the ^{13}C NMR data with those of ziyu-glycoside I [27] revealed sulfonylation on the hydroxyl attaching to Ara-C-3, which was supported by HMBC correlations from Ara-H-3 (δ_H 5.50) to Ara-C-2 (δ_C 74.1) and Ara-C-4 (δ_C 69.9). The same analysis of the HMBC correlations from Ara-H-1 (δ_H 5.07) to C-3 (δ_C 88.4), and from Glc-H-1 (δ_H 6.32) to C-28 (δ_C 177.1) suggested **6** to be a 3-*O*-, 28-*O*-diglycoside of ilexgenin B. Subsequently, **6** was elucidated as 3-*O*-*α*-L-(3′-sulfonyl)-arabinpyranosyl ilexgenin B 28-*O*-*β*-D-glucopyranosyl ester.

The molecular formula $C_{41}H_{66}O_{16}S$ for ilexpublesnin I (**7**) was given by HRESIMS data. The IR, ^1H and ^{13}C NMR data (**Tables 1** and **2**) exhibited a high similarity with those of **6**, suggesting related structures. Acid hydrolysis of **7** gave ilexgenin B, D-glucose, and D-xylose after TLC and GC analyses. Comparison of the ^{13}C NMR data with those of ilexoside A [2] revealed sulfonylation on

the hydroxyl attaching to Xyl-C-2 compared to **7**. HMBC correlations from Xyl-H-2 (δ_H 5.03, m) to Xyl-C-1 (δ_C 104.8) and Xyl-C-3 (δ_C 77.4) confirmed these assignments. Eventually, an extensive analysis of the HMBC correlations of **7** was applied to establish its structure as 3-*O*-*β*-D-(2′-sulfonyl)-xylpyranosyl ilexgenin B 28-*O*-*β*-D-glucopyranosyl ester.

Ilexpublesnin J (**8**) was deduced to have the molecular formula $C_{53}H_{86}O_{23}$ by HRESIMS ($[M + NH_4]^+$ at *m/z* 1108.58661, calcd. 1108.58981). Acid hydrolysis of **8** afforded ilexgenin B, D-glucose, and D-xylose after identification by TLC and GC analyses. The ^{1}H and ^{13}C NMR data (**Tables 1** and **2**) assigned by various NMR experiments exhibited similarity with those of ilexsaponin B_3 [28], indicating their similar structures. Comparison of their ^{1}H and ^{13}C NMR data revealed that **8** had one additional sugar unit, supported by HMBC correlations from *inner*-xyl-H-1 (δ_H 4.96) to C-3 (δ_C 88.9) and from Glc-H-1 (δ_H 6.35) to C-28 (δ_C 177.0). This determined for **8** a 3-*O*-, *28*-*O*-disaccharide structure. The positions and sequence of the rest of the sugar moieties were defined by HMBC correlations from intermediate-glc-H-1 (δ_H 5.44) to inner-xyl-C-2 (δ_C 82.1) and from terminal-glc-H-1 (δ_H 5.38) to intermediate-glc-C-2 (δ_C 85.4). Thus, **8** was unambiguously elucidated as 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-(1 → 2)]-*β*-D-xylopyranosyl ilexgenin B 28-*O*-*β*-D-glucopyranosyl ester.

Ilexpublesnin K (**9**) has the molecular formula $C_{52}H_{84}O_{22}$ determined by its HRESIMS data. Comparison of its ^{1}H and ^{13}C NMR data (**Tables 1** and **2**) with those of **8** suggested that they were closely related in structures, except for a difference in the sugar moiety. Acid hydrolysis of **9** afforded D-glucose, D-xylose, and L-arabinose which were identified by TLC and GC analyses. The structure assignments and the linkages of the sugar moiety were confirmed by HMBC correlations. Accordingly, **9** was elucidated as 3-*O*-[α-L-arabinpyranosyl-(1 → 2)-*β*-D-glucopyranosyl-(1 → 2)]-*β*-D-xylopyranosyl ilexgenin B 28-*O*-*β*-D-glucopyranosyl ester.

Ilexpublesnin L (**10**) and ilexpublesnin M (**11**) were obtained as white amorphous powders. Their molecular formulas were determined as $C_{42}H_{64}O_{16}$ and $C_{36}H_{54}O_{11}$, respectively, by the HRESIMS data. The IR spectra of both compounds showed absorption bands for hydroxyl, methyl, carbonyl, and olefinics. The ^{1}H and ^{13}C NMR data (**Tables 1** and **2**) displayed signals for six methyls, an olefinic, an aldehyde, and a sugar moiety, including glucose and glucuronic acid for **10** and a glucuronic acid for **11**, respectively. These assignments were confirmed by acid hydrolysis and/or ^{1}H and ^{13}C NMR comparisons with reported data. Analysis of ^{13}C NMR data of their aglycones indicated that both **10** and **11** closely resembled ilexoside XXXIII [29], with an aldehyde on C-24 in **10** and **11**, differently from ilexoside XXXIII with the aldehyde substitution on C-23. The determination was further supported by the observation of NOESY correlations between H-24 with CH_3-25, suggesting the *β* configuration of the aldehyde.

The complete structures, including all the linkages of sugars and aglycones, were established by extensive HMBC correlation analyses. Therefore, **10** and **11** were elucidated as 3-*O*-*β*-D-glucuronide-3*β*, 19α-dihydroxyolea-24-oxo-12-en-28-oic-*O*-*β*-D-glucopyranosyl ester and 3-*O*-*β*-D-glucuronide-3*β*, 19α-dihydroxyolea-24-oxo-12-en-28-oic acid, respectively.

Additionally, ten known analogues (**12**–**21**) (see Supporting Information) were also isolated. By comparing their ^{1}H and ^{13}C NMR as well as MS data with reported values, their structures were identified as ilexoside II (**12**) [22], ilexsaponin B_1 (**13**) [28], ilexsaponin B_3 (**14**) [28], ilexoside O (**15**) [30], ilexsaponin B_4 (**16**) [31], ilexoside XV (**17**) [32], ilexsaponin C (**18**) [33], chikusetusaponin IVa (**19**) [22], oleanolic acid 28-*O*-*β*-D-glucopyranol ester (**20**) [34], and pubescenoside C (**21**) [4].

Compounds **1, 2, 10**, and **11** are considerably special, as they contain the rare 24-aldehyde group, compared to the common 23-aldehyde- [21] or 24-COOH-substituted triterpene saponins from *Ilex* species.

Compounds **1**–**21** were evaluated for their cytotoxic activities *in vitro* against the human hepatoma cell lines HepG2, HLE, BEL-7402, BEL-7403, BEL-7405, human breast cancer cell line MCF-7, and human cervical cancer cell line HeLa, using the MTT assay [20]. Unfortunately, no significant cytotoxic results [inhibition (%) < 50] were observed against all seven cancer cell lines at the concentration of 40 μM. Among these isolates, only **6** and **19** showed cytotoxicity against the MCF-7 cell line [inhibition (%) = 33.14, 34.03, respectively], and **20** exhibited a weak cytotoxic activity on the HeLa cell line [inhibition (%) = 36.86]. The cytotoxic effects of **19** and **20** may be due to their aglycon oleanic acid.

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Conflict of Interest

The authors of the present manuscript have declared that no competing interests exist.

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