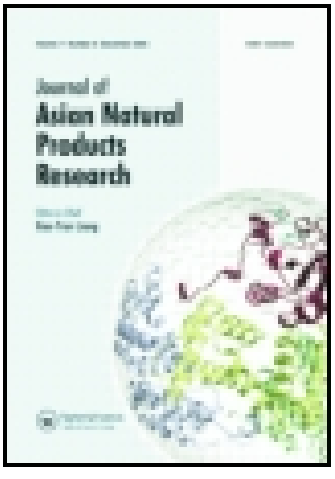

Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ganp20

Cytotoxic triterpenesaponins from Ilex pubescens

Ling Li[a], Li-Shi Feng[a] & Yu-Xin He[a]

[a] Bioengineering College, Xihua University, Chengdu 610039, China

Published online: 01 Aug 2014.

To cite this article: Ling Li, Li-Shi Feng & Yu-Xin He (2014): Cytotoxic triterpenesaponins from Ilex pubescens, Journal of Asian Natural Products Research, DOI: 10.1080/10286020.2014.920012

To link to this article: http://dx.doi.org/10.1080/10286020.2014.920012

Journal of Asian Natural Products Research, 2014
http://dx.doi.org/10.1080/10286020.2014.920012

Cytotoxic triterpenesaponins from *Ilex pubescens*

Ling Li, Li-Shi Feng and Yu-Xin He*

Bioengineering College, Xihua University, Chengdu 610039, China

(*Received 14 April 2014; final version received 28 April 2014*)

Two new triterpenesaponins, ilexsaponins G and H (**1** and **2**), together with six known triterpenesaponins (**3**–**8**), were isolated from the roots of *Ilex pubescens*. Their structures were elucidated on the basis of spectroscopic evidence and hydrolysis products. Those compounds showed inhibitory activities against two human colorectal cancer cell lines HCT 116 and HT-29.

Keywords: *Ilex pubescens*; triterpenesaponins; ilexsaponin G; ilexsaponin H; cytotoxicity

1. Introduction

The roots of *Ilex pubescens* Hook et Arn. are a traditional Chinese medicine commonly used in South China for the treatment of cardiovascular disease and hypercholesterolemia [1]. Phytochemical investigation of its roots and leaves led to the isolation of glaberides, triterpenesaponins, and phenolics, specifically vomifoliol [2–8]. In our previous study, a series of saponins were isolated from this plant [9]; however, their bioactivity has not been investigated. As a continuation of study on this plant, we present in this report the isolation and structural elucidation of two new triterpenesaponins (**1** and **2**) (Figure 1) and six known compounds from the roots of *I. pubescens*. All of these compounds were screened for their cytotoxicity.

2. Results and discussion

The 70% EtOH extract of dried roots of *I. pubescens* was subjected to silica gel column chromatography and prep-HPLC to obtain two new compounds (**1** and **2**), together with six known structures, 3β,21α,23-trihydroxy-urs-12-en-28-oic acid 21-*O*-β-D-glucopyranoside (**3**) [8], 3-*O*-β-D-glucopyranosyl-3β,21α,23-trihydroxy-urs-12-en-28-oic acid 21-*O*-β-D-glucopyranoside (**4**) [8], ilexsaponin D (**5**) [9], ilexsaponin E (**6**) [9], ilexsaponin F (**7**) [9], and ilexoside XXX (**8**) [10]. Their structures were elucidated on the basis of spectroscopic evidence and hydrolysis products. The cytotoxicity of all compounds against two colorectal human cancer cell lines HCT 116 and HT-29 was also evaluated.

Compound **1** was isolated as a white amorphous powder. The HR-ESI-MS experiment revealed a pseudomolecular ion peak at *m/z* 939.4592 $[M - H]^-$, in agreement with the molecular formula $C_{47}H_{72}O_{19}$. The monosaccharides obtained after aqueous acid hydrolysis of **1** were identified as glucose and arabinose by thin layer chromotography (TLC) comparison with authentic samples. The absolute configuration of the monosaccharides was determined to be D for glucose and L for arabinose by GC analysis of chiral derivatives of the monosaccharides in the hydrolysate of each compound (see Section 3). The relatively large coupling constants (5.0–8.0 Hz) for the anomeric protons in the ^{1}H NMR spectrum

*Corresponding author. Email: heyuxin66@tom.com

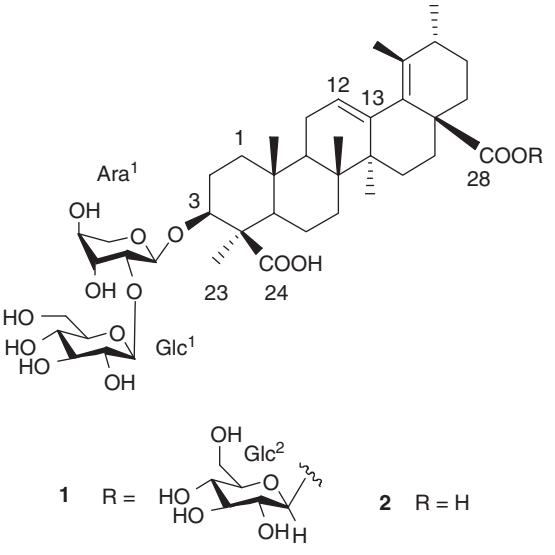

Figure 1. The structures of compounds **1** and **2**.

(see Section 3) of **1** suggested that the arabinopyranosyl moiety has an α-configuration and the glucopyranosyl moiety has a β-configuration. The ^{1}H NMR spectrum showed six methyl resonances at δ 1.70, 1.16, 1.20, 1.09, 1.73, (s, 3H each), and 1.06 (3H, d, $J = 7.0$ Hz) and an olefinic proton at δ 5.72. There were two carboxyl carbons at δ 174.6 and 180.5 and two pairs of olefinic carbon signals at δ 126.4, 138.7, 133.6, and 136.0 in the ^{13}C NMR spectrum. Above information indicated the presence of an ursane-type triterpene skeleton [9]. The aglycone of **1** was identified as 3β-hydroxyurs-12,18-dien-24,28-dioic acid by comparison of its ^{1}H and ^{13}C NMR spectral data obtained in 2D NMR experiments with values reported in the literature (see Table 1) [8]. The ^{1}H NMR spectrum of **1** exhibited three anomeric proton resonances at δ 5.12 (1H, d, $J = 6.0$ Hz), 5.15 (1H, d, $J = 8.5$ Hz), and 6.28 (1H, d, $J = 8.5$ Hz), respectively. The spin–spin coupling system of individual monosaccharide units was identified by the 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 in

Table 1. ^{13}C NMR spectral data for the aglycone moieties of **1** and **2** (125 MHz, in C_5D_5N).

Position	**1**	**2**	Position	**1**	**2**	Position	**1**	**2**
1	39.1	39.2	11	23.9	23.8	21	26.3	26.2
2	28.1	28.4	12	126.4	126.3	22	31.0	31.1
3	88.7	88.6	13	138.7	138.8	23	24.7	24.7
4	48.3	48.3	14	45.1	45.0	24	180.5	180.4
5	57.1	57.2	15	28.9	28.9	25	14.2	14.2
6	20.5	20.5	16	35.2	35.1	26	18.6	18.8
7	35.3	35.4	17	49.6	49.7	27	22.1	22.1
8	39.6	39.5	18	133.6	133.6	28	174.6	180.5
9	47.2	47.2	19	136.0	136.0	29	19.3	19.2
10	37.4	37.6	20	34.4	34.4	30	18.8	18.9

a series of 1D TOCSY experiments. Analysis of the $^{1}H-^{1}H$ COSY spectrum resulted in sequential assignment of all proton resonances of three monosaccharide units, 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). In the HMBC spectrum, the anomeric proton signals at δ_H 5.12 (Ara-H-1), 5.15 (Glc^1-H-1), and 6.28 (Glc^2-H-1) showed cross-peaks with the carbon signals at δ_C 88.7 (Agly-C-3), 81.0 (Ara^1-C-2), and 174.6 (Agly-C-28), respectively. These signals provide ample evidence to determine the linkages between the sugars, and between the sugar and the aglycone. From the above evidence, the structure of **1** was established as 3-*O*-β-D-glucopyranosyl (1 → 2)-α-L-arabinopyranosyl urs-12,18-diene-24,28-dioic acid 28-*O*-β-D-glucopyranosyl ester, named ilexsaponin G.

Compound **2** was isolated as a white amorphous powder. The HR-ESI-MS experiment revealed a pseudomolecular ion peak at *m/z* 777.4066 $[M - H]^-$, in agreement with the molecular formula $C_{41}H_{62}O_{14}$. Comparison of the NMR spectral data for **2** with those of **1** revealed that both of compounds are similar except that the signals due to the D-glucopyranosyl linked to C-28 in **1** were absent in **2**. This observation was supported by a relative downfield shift of C-28 (δ 180.5) of **2** in the ^{13}C NMR spectrum. Thus, the structure of **2** was established as 3-*O*-β-D-glucopyranosyl(1 → 2)-α-L-arabinopyranosyl urs-12,18-diene-24,28-dioic acid, named ilexsaponin H.

Table 2. ^{1}H NMR and ^{13}C NMR spectral data for the sugar moieties of compounds **1** and **2** (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR, in C_5D_5N).

	1		**2**	
Position	δ_H	δ_C	δ_H	δ_C
Ara				
1	5.12 d (6.0)	103.6	5.11 d (6.0)	103.5
2	5.52 dd (8.0, 6.0)	81.0	5.52 dd (8.0, 6.0)	81.1
3	4.22 dd (8.0, 4.5)	73.4	4.22–4.24 m	73.3
4	4.27–4.28 m	68.0	4.27–4.29 m	68.0
5a	3.58d (9.0)	64.7	3.58d (9.0)	64.8
5b	4.21d (9.0)		4.21d (9.0)	
Glc^1				
1	5.15 d (8.5)	105.7	5.14 d (8.0)	105.8
2	4.03 dd (8.5, 9.0)	76.2	4.01 dd (8.0, 9.0)	76.3
3	4.12 dd (9.0, 9.0)	78.1	4.11 dd (9.0, 9.0)	78.0
4	4.23–4.25 m	71.2	4.23 dd (9.0, 9.0)	71.2
5	3.67–3.69 m	78.2	3.67–3.69 m	78.1
6a	4.33 dd (12.0, 7.5)	62.4	4.33 dd (12.0, 7.0)	62.3
6b	4.42 dd (12.0, 2.0)		4.42 dd (12.0, 2.0)	
Glc^2				
1	6.28 d (8.5)	95.7		
2	4.11 dd (8.5, 9.0)	74.3		
3	4.22 dd (9.0, 9.0)	78.7		
4	4.30–4.32 m	71.2		
5	4.02–4.04 m	79.1		
6a	4.34 dd (12.0, 7.0)	62.1		
6b	4.47 dd (12.0, 2.0)			

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a JASCO P-1020 digital polarimeter (JASCO Corporation, Tokyo, Japan). UV spectra were obtained on a TU-1901 spectrometer (Beijing Purkinje General Instrument Co., Ltd, Beijing, China). IR spectra were obtained on a Perkin-Elmer 577 spectrometer (PerkinElmer, Waltham, MA, USA). NMR spectra were recorded on an Inova 500 spectrometer (Varian, Palo Alto, CA, USA), operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. HR-ESI-MS data were obtained on a G1969A TOF-MS instrument (Agilent Technologies Inc., Santa Clara, CA, USA). Precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co., Qingdao, China) were used for TLC. Spots were visualized by spraying 10% H_2SO_4–EtOH followed by heating. Column chromatography was carried out on silica gel (Qingdao Haiyang Chemical Co.) and macroporous resin D101 (pore size B 13–14 nm, 26–60 mesh; Bohong resin technology Co., Tianjin, China). High-performance liquid chromatography (HPLC) separation was carried out on a Waters 1525 EF system (Waters, Millford, MA, USA; YMC-pack ODS-A, 250 mm × 10 mm, i.d. 5 μm, YMC, Kyoto, Japan). GC analysis was carried out on an Agilent 6890N gas chromatograph (Agilent, Waldbronn, Germany) using a HP-5 capillary column with an FID detector.

3.2 Plant material

The roots of *I. pubescens* were collected in Guangzhou, Guangdong Province, China, in June 2010, and identified by Prof. Y.-X. He, Xihua University. A voucher specimen (No. 20100810) has been deposited in the herbarium of Bioengineering College of Xihua University.

3.3 Extraction and isolation

The dried roots (5 kg) of *I. pubescens* were extracted with 70% EtOH. After removing the solvent, the residue (854 g) was suspended in H_2O and extracted with *n*-BuOH. The *n*-BuOH extract (114 g) was further chromatographed over a macroporous resin D101 column eluted initially with water, then with 30%, 50%, and 70% EtOH to give fractions A–C (fraction A, 22 g; fraction C, 17 g). Fraction B (34 g) was subjected to a silica gel column using $CHCl_3$–MeOH–H_2O (90:10:0.1 to 70:30:5) as an eluent to give fractions B_1–B_9. Fraction B_3 (2.9 g) was subjected to ODS open column chromatography (MeOH–H_2O 40:60 to 80:20) to afford fractions $B_{3\text{-}1}$–$B_{3\text{-}4}$. Fraction $B_{3\text{-}1}$ (600 mg) was subjected to prep-HPLC (MeOH–H_2O, 40:60, UV detection at 210 nm), affording **5** (18 mg, 16.7 min), **6** (12 mg, 20.7 min), and **7** (5 mg, 18.5 min), respectively. Fraction B_4 (1.7 g) was subjected to ODS open column chromatography (MeOH–H_2O; 40:60 to 80:20) to afford fractions $B_{4\text{-}1}$–$B_{4\text{-}5}$. Fraction $B_{4\text{-}2}$ (310 mg) was subjected to prep-HPLC (ACN–H_2O, 26:74; UV detection at 210 nm), affording **1** (26 mg, 14.3 min) and **2** (29 mg, 18.9 min), respectively. Fraction $B_{4\text{-}5}$ (95 mg) was subjected to prep-HPLC (CH_3CN–H_2O, 29:71, UV detection at 210 nm, affording **8** (17 mg, 12.4 min) and **3** (14 mg, 14.4 min), respectively.

3.3.1 Ilexsaponin G (1)

White amorphous solid, $[\alpha]_D^{22} + 36$ ($c = 0.20$, MeOH); UV (MeOH) λ_{max} 225 nm; IR (KBr): ν_{max} (cm^{-1}) 3422, 1720, 1682; ^{1}H NMR (C_5D_5N, 500 MHz): δ 1.73 (3H, s, Me-29), 1.70 (3H, s, Me-23), 1.20 (3H, s, Me-26), 1.16 (3H, s, Me-25), 1.09 (3H, s, Me-27), 1.06 (3H, d, $J = 7.0$ Hz, Me-30), 3.32 (1H, dd, $J = 12.5$, 4.0 Hz, H-3), 5.72 (1H, br t, $J = 3.0$ Hz, H-12). For ^{13}C NMR spectral data, see Tables 1 and 2. HR-ESI-MS: *m/z* 939.4592 $[M - H]^-$ (calcd for $C_{47}H_{71}O_{19}$, 939.4590).

Table 3. Inhibitory effects of compounds **1**–**8** against two colorectal human cancer cell lines HCT 116 and HT-29.

	IC_{50} value (μM)	
Compound	HCT 116	HT-29
1	12.4 ± 3.2	10.8 ± 2.1
2	3.2 ± 0.3	3.7 ± 0.4
3	2.7 ± 0.5	2.2 ± 0.4
4	5.2 ± 1.2	4.9 ± 0.7
5	13.3 ± 2.2	15.9 ± 3.5
6	6.6 ± 1.5	7.2 ± 0.8
7	13.1 ± 3.5	11.9 ± 2.7
8	8.3 ± 1.9	7.1 ± 1.0
Paclitaxel	0.0038 ± 0.0003	0.0040 ± 0.0004

3.3.2 *Ilexsaponin H (2)*

White amorphous solid, $[\alpha]_D^{22} + 38$ ($c = 0.20$, MeOH); UV (MeOH) λ_{max} 225 nm; IR(KBr) ν_{max} (cm^{-1}): 3424,1721, 1683; ^1H NMR (C_5D_5N, 500 MHz): δ 1.72 (3H, s, Me-29), 1.71 (3H, s, Me-23), 1.20 (3H, s, Me-26), 1.15 (3H, s, Me-25), 1.08 (3H, s, Me-27), 1.06 (3H, d, $J = 7.0$ Hz, Me-30), 3.34 (1H, dd, $J = 12.0$, 3.5 Hz, H-3), 5.71 (1H, brt, $J = 3.5$ Hz, H-12). For ^{13}C NMR spectral data, see Tables 1 and 2. HR-ESI-MS: *m/z* 777.4066 $[M - H]^-$ (calcd for $C_{41}H_{61}O_{14}$, 777.4061).

3.4 *Acid hydrolysis*

A solution of compounds **1** or **2** (5.0 mg each) in 2 M trifluoroacetic acid (1 ml) was heated at 110°C for 2 h, and then dried by N_2 gas. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 resin (OH^- form) and the resin was filtered. After removal of the solvent under pressure from the filtrate, the residue was passed through a Sep-Pak C_{18} cartridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (2.0 mg) in pyridine (1.0 ml) at 60°C for 2 h. After dried by N_2 gas, the residue was treated with *N*-(trimethylsilyl) imidazol (0.2 ml) at 60°C for 1 h. The reaction was ended by adding water (1.0 ml), and then extracted with cyclohexane (1.0 ml, three times). The cyclohexane layer was collected and concentrated to 1.0 ml for GC analysis. Separations were carried out on an HP-5 column (28 m × 0.32 mm). Highly pure He was used as carrier gas (1.0 ml/min flow rate), and the FID detector was operated at 260°C (column temp. 180°C). The retention times of the monosaccharide derivatives were as follows: L-Ara, 5.45 min, and D-Glc, 12.45 min.

3.5 *MTT cytotoxicity assay*

The inhibitory activities against two colorectal human cancer cell lines HCT 116 and HT-29 were carried out according to a previously described method [11]. Paclitaxel was used as a positive control (Table 3). The experiments were carried out for three times.

Acknowledgments

This work was financially supported by the National Natural Science Fund of China (Grant No. 81202897); the Chunhui Project of Ministry of Education (Z2010102); the Chun hui Project of Ministry of Education (Z2010103); the Open Research Fund of Key Laboratory of Food Biotechnology, Xihua University (SZJJ2014-008); and the Innovation Fund of Postgraduate, Xihua University (YCJJ2014106).

References

[1] Jiangsu New Medical College, editors, *Directory of Chinese Materia Medica (Zhong Yao Da Ci)* (Shanghai Scientific and Technological Press, Shanghai, 1977), p. 441.

[2] C.X. Zhang, C.Z. Lin, T.Q. Xiong, C.C. Zhu, J.Y. Yang, and Z.X. Zhao, *Fitoterapia* **81**, 788 (2010).

[3] K. Hidaka, M. Ito, Y. Matsuda, H. Kohda, K. Yamasaki, and J. Yamahara, *Phytochemistry* **26**, 2023 (1987).

[4] K. Hidaka, M. Ito, Y. Matsuda, H. Kohda, K. Yamasaki, J. Yamahara, T. Chisaka, Y. Kawakami, T. Sato, and K. Kagei, *Chem. Pharm. Bull.* **35**, 524 (1987).

[5] L.P. Lin, W. Qu, and J.Y. Liang, *Chin. Chem. Lett.* **22**, 697 (2011).

[6] F. Feng, M.X. Zhu, N. Xie, W.Y. Liu, D.J. Chen, and Q.D. You, *J. Asian Nat. Prod. Res.* **10**, 71 (2008).

[7] Y.B. Zhou, J.H. Wang, X.M. Li, X.C. Fu, Z. Yan, Y.M. Zeng, and X. Li, *J. Asian Nat. Prod. Res.* **10**, 827 (2008).

[8] G.B. Xie, S.X. Zhou, Y.N. Lu, L.D. Lei, and P.F. Tu, *Chem. Pharm. Bull.* **57**, 520 (2009).

[9] L. Li, Y.X. He, M.L. Gou, and C. Dai, *J. Asian Nat. Prod. Res.* **14**, 1169 (2012).

[10] K. Amimoto, K. Yoshikawa, and S. Arihara, *Chem. Pharm. Bull.* **40**, 3138 (1992).

[11] J. Carmichael, W.G. de Graff, A.F. Gazdar, J.D. Minna, and J.B. Mitchell, *Cancer Res.* **47**, 936 (1987).