Phytochemistry xxx (2016) 1–11

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Triterpenoid saponins with anti-inflammatory activities from *Ilex pubescens* roots

Peng Wu [a], Hui Gao [a], Jian-Xin Liu [b], Liang Liu [b], Hua Zhou [b, **], Zhong-Qiu Liu [a, b, *]

[a] International Institute for Translational Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, 510006, People's Republic of China
[b] State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Taipa, Macau, People's Republic of China

ARTICLE INFO

Article history:
Received 17 February 2016
Received in revised form
21 November 2016
Accepted 22 November 2016
Available online xxx

Keywords:
Ilex pubescens
Aquifoliaceae
Triterpenoid saponins
RAW 264.7 macrophages
Anti-inflammatory

ABSTRACT:

Seven triterpenoid saponins, named ilexsaponin I–O, along with twelve known ones, were isolated from the roots of *Ilex pubescens*. The structures of all compounds were elucidated by use of extensive spectroscopic methods (IR, HR-ESI-MS, and 1D and 2D NMR). Sugar residues obtained after acid hydrolysis were identified by TLC and HPLC. The *in vitro* anti-inflammatory effects of the triterpenoid saponins were also evaluated in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Among the isolated saponins, seven compounds were shown to inhibit LPS-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by suppressing the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively, in LPS-stimulated RAW 264.7 cells. Ilexsaponin I and β-D-glucopyranosyl 3-β-[β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyloxy]-olea-12-en-28-oate exerted more potent anti-inflammatory effects than the other compounds tested.

1. Introduction

Ilex pubescens Hook et Arn., also known under the Chinese name "Mao Dong Qing," is an evergreen bush that belongs to the Aquifoliaceae family. The roots have been widely used in traditional Chinese medicine for treatment of cardiocerebral and cardiovascular diseases, such as stroke, coronary arterial disease, and peripheral vascular diseases (Jiangsu New Medical College, editors, 1997). Previous phytochemical investigations established the presence of triterpenoid saponins (Hidaka et al., 1987b; Feng et al., 2008; Zhang et al., 2010a, 2010b, 2011; Lin et al., 2011; Li et al., 2012, 2014a; Zhou et al., 2013a, 2013b, 2014; Wu et al., 2015), lignan glucosides (Zhou et al., 2008a, 2008b), iridoids (Yang et al., 2007), phenolics (Yang et al., 2006), flavonoids (Xing et al., 2012), and hemiterpene glycosides (Jiang et al., 2005; Wu et al., 2012). Pharmacological evaluation further established that *I. pubescens* extracts exhibit several bioactivities, such as alleviation of inflammation, inhibition of virus and platelet aggregation, enlargement of blood vessels, and improvement of microcirculation and blood pressure; these extracts can also prevent thrombosis, reduce cardiac ischemia, and decrease the excitation of the cardiac conduction system (Yang and Pang, 1986; Han et al., 1993; Wu et al., 2007a; Wang et al., 2008a,b).

Inflammation is a complex response mediated by immune cells, including monocytes and macrophages (Munhoz et al., 2008). However, an excessive inflammatory response can result in many inflammatory diseases, such as rheumatoid arthritis, atherosclerosis, and cancer (Epstein et al., 2001; Barton et al., 2007; Grivennikov et al., 2010). Macrophages play a critical role in the initiation, maintenance, and resolution of the inflammatory response. Soluble stimuli such as lipopolysaccharide (LPS) can activate macrophages to produce pro-inflammatory mediators, including nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Song et al., 2014). NO and PGE_2 are the most prominent inflammatory mediators. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are responsible for increased levels of NO and PGE_2, respectively (Lee et al., 2012).

In a previous study, a purified saponin fraction (PSF) derived from the root of *I. pubescens* was identified as a bioactive fraction exhibiting anti-inflammatory and analgesic activity. Dominant constituents in PSF were triterpenoid saponins of ursane and

* Corresponding author. International Institute for Translational Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, 510006, People's Republic of China.
** Corresponding author. State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Taipa, Macau, People's Republic of China
E-mail addresses: hzhou@must.edu.mo (H. Zhou), liuzq@gzucm.edu.cn (Z.-Q. Liu).

http://dx.doi.org/10.1016/j.phytochem.2016.11.012
0031-9422/

oleanane types (Wang et al., 2008a,b). A phytochemical study on the roots of *I. pubescens* established the structure of eight triterpenoid saponins (**20**–**27**) (see Supporting Information) (Wu et al., 2015). In an ongoing program toward the discovery of additional bioactive constituents, seven new triterpenoid saponins, namely, ilexsaponin I-O (**1**–**7**), and twelve triterpenoid saponins (**8**–**19**) were isolated. Therefore, the anti-inflammatory activity of all triterpenoid saponins (**1**–**27**) isolated from this plant was investigated by detecting the iNOS and COX-2 protein expression levels in LPS-stimulated RAW264.7 cells.

2. Results and discussion

2.1. Characterization of the compounds

Ilexsaponin I (**1**), a white amorphous powder, had a molecular formula of $C_{47}H_{74}O_{17}$, as determined from the ^{13}C NMR data and a quasi-molecular ion peak $[M+Na]^+$ at *m/z* 933.4830 (calcd. 933.4824) in the positive-ion HR-ESI-MS. Its IR spectrum showed absorption bands for hydroxyl (3429 cm^{-1}), alkyl (2960 cm^{-1}), carbonyl (1705 cm^{-1}), and double bond (1632 cm^{-1}) groups. The sugar component of acid-hydrolyzed **1** included D-glucose and D-xylose as identified by TLC and HPLC analyses. According to the ^{13}C NMR spectroscopic data in the literature (Zhou et al., 2013a), the hexose moieties of **1** were identified as glucopyranoside and xylopyranoside. The ^1H and ^{13}C NMR data (Tables 1 and 2) obtained using HSQC and HMBC (Fig. 2) experiments displayed: six singlets for tertiary methyls at δ_H 0.87, 1.05, 1.06, 1.08, 1.24, and 1.25; one terminal double bond (δ_C 153.5, C-20; δ_H 5.05, δ_C 113.0, C-30); one trisubstituted double bond (δ_H 5.46, δ_C 127.8, C-12; δ_C 137.7, C-13); one carboxyl (δ_C 176.4, COOR-28); and three anomeric signals (δ_H 4.81, δ_C 106.0, CH-Xyl-1; δ_H 5.34, δ_C 106.1, CH-Glc-1; δ_H 6.28, δ_C 96.1, CH-Glc-1). All NMR spectroscopic data in **1** indicated an ursane type of aglycone. Comparison of ^1H and ^{13}C NMR data with those of oblonganoside F (Wu et al., 2007b) established their structural similarities, except for the absence of a hydroxyl group at C-19 and a difference in the sugar moiety. The HMBC analysis supported the assignment as follow from: H-12 to C-11, C-14, and C-18; CH_3-23 to C-24, C-3, and C-5; CH_3-25 to C-5 and C-9; CH_3-26 to C-7 and C-14; CH_3-27 to C-8 and C-13; CH_3-29 to C-18 and C-20; and CH_2-30 to C-19 and C-21. The HMBC from inner-Xyl-H-1 (δ_H 4.81, d, $J = 5.6$ Hz) to C-3 (δ_C 89.1) and from Glc-H-1 (δ_H 6.28, d, $J = 8.0$ Hz) to C-28 (δ_C 176.4) determined its glycosylation sites at the 3-*O*- and 28-*O*-positions. Furthermore, the HMBC from the terminal-Glc-H-1 (δ_H 5.34, d, $J = 7.2$ Hz) to the inner-Xyl-C-2 (δ_C 83.2) established the linkages of the sugar moieties. After the overall structure was constructed, the relative configuration was established by NOESY correlations. In the NOESY (Fig. 2) spectrum, correlations between H-3 and H-5, H-5 and H-9, H-9 and CH_3-27, as well as CH_3-27 and H-19, indicated that the methyl groups 23-CH_3 and 27-CH_3 were in an α configuration. The cross-peaks of H-24/CH_3-25, CH_3-25/CH_3-26, and H-18/CH_3-29 indicated that the sugar moiety of the C-3, 24-CH_3, 25-CH_3, 26-CH_3, and 29-CH_3 methyl groups had a β orientation. Thus, compound **1** was elucidated as β-D-glucopyranosyl 3β-[β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyloxy]-urs-12,20(30)-dien-28-oate (Fig. 1).

Ilexsaponin J (**2**), a white amorphous powder, had a molecular formula of $C_{47}H_{76}O_{17}$ as determined from the ^{13}C NMR data and a quasi-molecular ion peak $[M+Na]^+$ at *m/z* 935.4974 (calcd. 935.4980) in the positive-ion HR-ESI-MS. Its IR spectrum also had absorption bands for hydroxyl, alkyl, carbonyl, and double bond groups. The sugar component of acid-hydrolyzed **2** included D-glucose and D-xylose as identified by TLC and HPLC analyses. The signals characteristic for an ursane triterpene saponin were observed from its ^1H and ^{13}C NMR data (Tables 1 and 2), including six singlets for tertiary methyls and one methyl doublet, one trisubstituted double bond, one carboxyl and three anomeric carbons. The ^1H and ^{13}C NMR data of **2** were similar to those of **1**; however, the double bond at C-12 in **1** (δ_H 5.46, δ_C 127.8, C-12; δ_C 137.7, C-13) was hydrogenated (δ_C 33.6, C-12; δ_C 37.9, C-13). This was confirmed by HMBC cross-peaks between H-12 and C-11, C-14, and C-18. The exo-methylene group in **1** (δ_C 153.5, C-20; δ_H 5.05, δ_C 113.0, C-30) was shifted to C-20 and C-21 (δ_C 143.4, C-20; δ_C 117.7, C-21) in **2**. The location of the double bond was further confirmed by the HMBC from H-21 to C-17 and C-30. Therefore, compound **2** was elucidated as β-D-glucopyranosyl 3β-[β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyloxy]-urs-20-en-28-oate (Fig. 1).

The HR-ESI-MS data ([M+Na]+ at *m/z* 933.4830, calcd. 933.4824) indicated that the molecular formula of ilexsaponin K (**3**) was $C_{47}H_{74}O_{17}$. Its IR spectrum also had absorption bands for hydroxyl, alkyl, carbonyl, and double bond groups. The sugar component of acid-hydrolyzed **3** included D-glucose and D-xylose as identified by TLC and HPLC analyses. The ^1H and ^{13}C NMR data (Tables 1 and 2) assigned using HSQC and HMBC experiments established: seven singlets for tertiary methyls at δ_H 0.89, 1.09, 1.11 (6H), 1.27, 1.60, and 1.65; one trisubstituted double bond (δ_H 5.66, δ_C 128.1, C-12; δ_C 138.1, C-13); one tetrasubstituted double bond (δ_C 129.0, C-19; δ_C 124.0, C-20); one carboxyl (δ_C 176.6, COOR-28); and three anomeric signals (δ_H 4.83, δ_C 106.1, CH-Xyl-1; δ_H 5.37, δ_C 106.3, CH-Glc-1; δ_H 6.34, δ_C 96.1, CH-Glc-1). Comparison of the ^1H and ^{13}C data with those of oblonganoside B (Wu et al., 2007b) indicated structural similarity; the main difference was presence of additional signals of a glucose moiety. The location of the double bond at C-19 and C-20 was further confirmed by the HMBC analysis from CH_3-29 and CH_3-30 to C-19 and C-20. Comparison of the ^1H and ^{13}C NMR data with those of the aglycone of oblonganoside B (Wu et al., 2007b) confirmed these data. Eventually, compound **3** was elucidated as β-D-glucopyranosyl 3β-[β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyloxy]-urs-12,19-dien-28-oate (Fig. 1).

The HR-ESI-MS data ($[M+Na]^+$ at *m/z* 933.4821, calcd. 933.4824) indicated that the molecular formula of ilexsaponin L (**4**) was $C_{47}H_{74}O_{17}$. Its IR spectrum also presented absorption bands for hydroxyl, alkyl, carbonyl, and double bond groups. The sugar component of acid-hydrolyzed **4** included D-glucose and D-xylose as identified by TLC and HPLC analyses. The ^1H and ^{13}C NMR data (Tables 1 and 2), assigned using HSQC and HMBC experiments, had five singlets for tertiary methyls and two methyl doublets, two trisubstituted double bonds, one carboxyl and three anomeric carbons. Comparison of the NMR data of **3** and **4** showed the same sugar moiety and linkages; the main differences were in their methyl signals and in the presence of two trisubstituted double bonds (δ_H 6.63, δ_C 134.8, C-9; δ_C 128.8, C-11; δ_H 5.66, δ_C 125.9, C-12; δ_C 138.8, C-13). In the ^1H NMR spectrum of **4**, the two vinylic protons at δ_H 6.63 (d, $J = 10.0$ Hz) and δ_H 5.66 (d, $J = 10.0$ Hz), were correlated in the COSY spectrum indicating that the two double bonds were adjacent. The locations of the double bonds at C-9, C-11 and C-12, C-13 were further confirmed by the HMBC between H-11 and C-8, C-9; H-12 and C-9, C-14, C-18, respectively. Thus, compound **4** was elucidated as β-D-glucopyranosyl 3β-[β-D-glucopyranosyl-(1 → 2)-β-D-xylopyranosyloxy]-urs-9(11),12-dien-28-oate (Fig. 1).

Ilexsaponin M (**5**) was obtained as a white amorphous powder. Its HR-ESI-MS analysis showed a pseudomolecular ion at *m/z* 911.4951 $[M-H]^-$ (calcd. for $C_{47}H_{75}O_{17}$, 911.5004), corresponding to the molecular formula of $C_{47}H_{76}O_{17}$. Its IR spectrum showed absorption bands for hydroxyl, alkyl, carbonyl, and olefinic functional groups. The sugar components of acid-hydrolyzed **5** included D-glucose, L-rhamnose, and D-xylose, as identified by TLC and HPLC analyses. The ^1H and ^{13}C NMR data (Tables 1 and 2), assigned using HSQC and HMBC experiments, showed: six singlets for tertiary

Please cite this article in press as: Wu, P., et al., Triterpenoid saponins with anti-inflammatory activities from *Ilex pubescens* roots, Phytochemistry (2016), http://dx.doi.org/10.1016/j.phytochem.2016.11.012