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Triterpenoid saponins from *Aesculus sylvatica* W. Bartram

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ABSTRACT

16 triterpenoid saponins including two new compounds were isolated from the seeds of A *esculus sylvatica* W. Bartram. The two new saponins were assigned as 3-*O*-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinofuranosyl-(1 → 3)-β-D-glucuronopyranosyl-21,22-*O*-ditigloyl-3β,16α,21β,22α,24,28 hexahydroxyolean-12-ene (aesculioside S1, **1**) and 3-*O*-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinofuranosyl-(1 → 3)-β-D-glucuronopyranosyl-21-*O*-tigloyl-22-*O*-angeloyl 3β,16α,21β,22α,24,28-hexahydroxyolean-12-ene (aesculioside S2, **2**). Aesculioside S1 and S2 displayed moderate cytotoxicity against human non-small cell lung cancer cells (A549) and prostate cancer cells (PC3) (GI_{50} ranged from 8.7 to 18.2 μM). The structural analysis of the saponins isolated from *Aesculus* supports the taxonomic placement of *A. sylvatica* under the section *Pavia* of *Aesculus* genus.

1. Introduction

Aesculus L. is a genus of Sapindaceae with 12 shrub or small deciduous tree species in the northern hemisphere (Zhang et al., 2010). As a remarkable example of an intercontinental disjunction genus, the taxonomy, biogeography, and evolution of *Aesculus* have attracted great attention from botanists (Harris and Xiang, 2009; Harris et al., 2009; Modliszewski et al., 2006; Xiang et al., 1998). The phylogenetic and biogeographic analysis of *Aesculus* was made using molecular and morphological data with some controversial results (Harris et al., 2009; Xiang et al., 1998). Since 2004, we have conducted extensive phytochemical investigations of North America *Aesculus* species to identify bioactive saponins against human cancer (Yuan et al., 2012, 2013; Zhang and Li, 2007; Zhang et al., 2010, 2006). 61 polyhydroxyoleanene saponins including 56 previously unknown compounds were reported from *A. pavia* L. and *A. glabra* Willd. distributed in eastern North America and *A. californica* (Spach) Nutt. distributed in western North America. The phytochemical data of these *Aesculus* species formed a basis for medical uses of *Aesculus* and its triterpenoid saponins and provided new evidences for understanding systematics and evolutionary pattern of the genus. In the present study, we report 16 triterpenoid saponins isolated from *Aesculus sylvatica* W. Bartram (commonly known as painted buckeye), another species of *Aesculus* in the southeastern USA.

2. Results and discussion

From 400 g *A. sylvatica* seeds, two new oleanane saponins (**1–2**) together with 14 known saponins (**3–16**) were isolated (Fig. 1). By analysis of their NMR spectral data, the known saponins were identified as aesculioside IIe (**3**) (Zhang and Li, 2007), aesculioside IIf (**4**) (Zhang and Li, 2007), aesculioside IIg (**5**) (Zhang and Li, 2007), aesculioside IIh (**6**) (Zhang and Li, 2007), aesculioside IIk (**7**) (Zhang and Li, 2007), aesculioside IIj (**8**) (Zhang and Li, 2007), aesculioside IIId (**9**) (Zhang and Li, 2007), aesculioside IIIe (**10**) (Zhang and Li, 2007), aesculioside IIIf (**11**) (Zhang and Li, 2007), aesculioside G6 (**12**) (Yuan et al., 2012), xanifolia-Y8 (**13**) (Chan et al., 2008; Voutquenne et al., 2005), aesculioside G9 (**14**) (Yuan et al., 2012), aesculioside G10 (**15**) (Yuan et al., 2012), and xanifolia-Y10 (**16**) (Voutquenne et al., 2005). Known saponins **3–11** were previously reported in *A. pavia* (Zhang et al., 2006). **12**, **14** and **15** were reported in *A. glabra* (Yuan et al., 2012). **13** was reported from *A. pavia*, *A. glabra*, *Xanthoceras sorbifolia*, and *Harpullia austro-caledonica* (Chan et al., 2008; Voutquenne et al., 2005; Yuan et al., 2012; Zhang et al., 2006). **16** was reported from *A. pavia*, *X. sorbifolia*, *and H. austro-caledonica* (Chan et al., 2008; Voutquenne et al., 2005; Yuan et al., 2012; Zhang et al., 2006). The structures of the two new saponins (**1-2**) were elucidated by extensive analysis of their spectroscopic data as well as chemical analysis.

The molecular formula of **1** was determined to be $C_{57}H_{87}O_{24}$ from its HR-ESI-MS data ($[M\text{-}H]^-$ *m/z*, 1155.5589) and ^{13}C NMR spectrum (Table 1). Similar to the saponins previously reported from the genus *Aesculus*, the NMR data of **1** displayed signals of a

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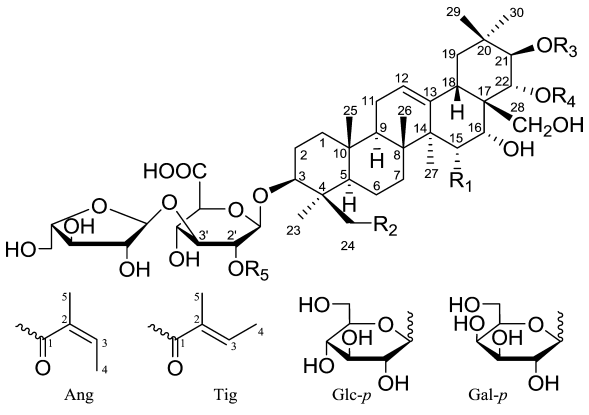

	R_1	R_2	R_3	R_4	R_5
1	H	OH	Tig	Tig	Glc-*p*
2	H	OH	Tig	Ang	Glc-*p*
3	OH	OH	Tig	Ac	Glc-*p*
4	OH	H	Tig	Ac	Gal-*p*
5	OH	H	Ang	Ac	Gal-*p*
6	H	OH	Tig	Ac	Glc-*p*
7	H	H	Ang	Ac	Gal-*p*
8	H	H	Tig	Ac	Gal-*p*
9	OH	OH	Tig	Tig	Glc-*p*
10	OH	OH	Tig	Ang	Glc-*p*
11	OH	H	Tig	Tig	Gal-*p*
12	OH	H	Tig	Ang	Gal-*p*
13	H	OH	Ang	Ang	Glc-*p*
14	H	H	Tig	Ang	Gal-*p*
15	H	H	Ang	Tig	Gal-*p*
16	H	H	Ang	Ang	Gal-*p*

Fig. 1. The structures of triterpenoid saponins **1–16** from *Aesculus sylvatica*.

30-carbon olean-12-ene type aglycone, two five carbon side chains and a trisaccharide chain. Following detailed analyses of the NMR spectroscopic data (COSY, HSQC, HMBC and NOESY) and comparison with literature data, the triterpenoid skeleton was identified as protoaescigenin or 3β,16α,21β,22α,24,28-hexahydroxyolean-12-ene as in compounds **6** and **13** (Voutquenne et al., 2005; Zhang and Li, 2007). The relatively high field shift of the H-23 methyl signal at δ_H 0.66 and downfield shift of C-3 at δ_C 91.2 indicated a C-24 hydroxylation. The relatively high field shift of H-7 (δ_H, 1.27, 1.55) and the relatively low field shift observed for methyl in position 27 (δ_H 1.85 and δ_C 27.6, respectively) revealed no hydroxyl substitution at C-15 but oxygen bearing at C-16. There were two sets of tigloyl group signals in ^{1}H-NMR spectra of **1** (δ_H 7.07 (1H, *q*, *J* = 7.1), 1.63 (3H, *d*, *J* = 7.1), and 1.93 (3H, *s*) and δ_H 6.95 (1H, *q*, *J* = 7.1), 1.45 (3H, *d*, *J* = 7.1), and 1.84 (3H, *s*), respectively). By analysis of COSY, HSQC, and HMBC spectra, the signals of the two tigloyl groups were assigned unambiguously at C-21 and C-22, respectively. The presence of trisaccharide residues were indicated by three anomeric proton signals at δ_H 4.94 (1H, *d*, *J* = 7.2 Hz), 5.52 (1H, *d*, *J* = 7.0 Hz), 6.08 (1H, *br s*) and the corresponding anomeric carbons at δ_C 104.2, 103.3, and 110.7. Acid hydrolysis of **1** afforded D-glucuronic acid, D-glucose and L-arabinose as analyzed following aldose derivatization and HPLC analysis (Tanaka et al., 2007; Yuan et al., 2013). By HSQC, DQF-COSY, HMBC and 2D-TOCSY, it was possible to characterize one β-glucuronopyranosyl, one β-glucopyranosyl and one α-arabinofuranosyl moieties. The glucuronic acid was determined to be in pyranose form from the ^{13}C NMR spectroscopic data. The β-anomeric configuration of glucuronic acid was determined according to the 3*J*H1, H2 coupling constants (1H, *d*, *J* = 7.2). The D-glucopyranosyl was determined to be in β-pyranose form according to its ^{13}C-NMR spectroscopic data and 3*J*H1, H2 coupling constant (1H, *d*, *J* = 7.0). The ^{1}H-NMR and ^{13}C-NMR signal assignments of α-arabinofuranosyl were in accordance with those of previous reported *Aesculus* saponins. The HMBC correlation of glucuronic acid anomeric protons at δ_H 4.94 (1H, *d*, *J* = 7.2 Hz) with the downfield shift signal of C-3 at δ_C 91.2 established the linkage of glucuronic acid to C-3 of the aglycone. The attachment of D-glucopyranosyl and L-arabinofuranosyl to C-2′ and C-3′ of D-glucuronopyranosyl acid were determined by their HMBC correlations, H-1 of Glc-*p* and Ara-*f* (δ_H 5.52 and 6.08) to C-2′ (δ_C 78.0) and C-3′ (δ_C 86.3) of GlcA, respectively. Therefore, the structure of **1** was assigned as 3-*O*-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinofuranosyl-(1 → 3)-β-D-glucuronopyranosyl-21,22-*O*-ditigloyl-3β,16α,21β,22α,24,28-hexahydroxyolean-12-ene (aesculioside S1).

Compound **2** was assigned a molecular formula of $C_{57}H_{87}O_{24}$ from its negative HR-ESI-MS ion peak at *m/z* 1155.5583 $[M-H]^-$, same as aesculioside S1 (**1**). By detailed analysis and comparison of ^{1}H NMR, ^{13}C and HSQC spectra, the signals of **2** were almost superimposable to those of **1** in terms of the triterpenoid aglycone and oligosaccharide side chain. The trisaccharide groups of **2** were also confirmed as D-glucuronic acid, D-glucose and L-arabinose by acid hydrolysis and HPLC analysis. Compared with **1**, one of the tigloyl groups apparently disappeared in **2**. The ^{1}H-NMR spectra of **2** showed a set of characteristic angeloyl signals at δ_H 5.87 (1H, *q*, *J* = 7.1 Hz), 2.03 (3H, *d*, *J* = 7.1 Hz), and 1.89 (3H, *s*). The angeloyl groups were established as C-22 substitution by HMBC correlations of angeloyl carboxyl carbons at δ_C 168.5 with the corresponding H-22 at δ_H 6.40 (3H, *d*, *J* = 10.2 Hz). The structure of **2** was then elucidated as 3-*O*-[β-D-glucopyranosyl-(1→2)]-α-L-arabinofuranosyl-(1 → 3)-β-D-glucuronopyranosyl-21-*O*-tigloyl-22-*O*-angeloyl-3β,16α,21β,22α,24,28-hexahydroxyolean-12-ene (aesculioside S2).

Aesculioside S1 (**1**) and S2 (**2**) were evaluated for their cytotoxicity against human lung adenocarcinoma epithelial cell (A549) and human prostate cancer cells (PC3). Aesculioside S1 (**1**) exhibited cytotoxic activity against A549 and PC3 with GI_{50} values of 18.2 ± 4.3 and 13.6 ± 2.1 μM, respectively (doxorubicin as positive control, GI_{50} against A549 and PC3 for 0.58±0.04 and 0.81 ± 0.06 μM, respectively). The cytotoxicity of Aesculioside S2 (**2**) against A549 and PC3 was evaluated at GI_{50} values of 11.7 ± 1.3 and 8.7 ± 2.5 μM, respectively. The cytotoxicity data of these polyhydroxyoleanene saponins are consistent with the similar compounds isolated from *A. pavia* and *A. glabra* (Yuan et al., 2012; Zhang and Li, 2007,2006).

Our previous investigations found that the structures of triterpenoid saponins isolated from *A. pavia* and *A. glabra* of the section *Pavia* in eastern North America can be easily distinguished from those from other sections of *Aesculus* in Asia, Europe and western North America and all saponins in these two species are characterized by trisaccharide chains with an arabinofuranosyl unit fixed to C-3 of the glucuronopyranosyl unit (Yuan et al., 2012; Zhang et al., 2010). *A. sylvatica* is usually placed in the section *Pavia* with *A. pavia*, *A. glabra*, and *A. flava* distributed in the same region. All 16 saponins isolated from *A. sylvatica* have an arabinofuranosyl unit affixed to C-3 of the glucuronopyranosyl unit in the trisaccharide chain. This result supports the placement of *A. pavia*, *A. glabra*, and *A. sylvatica* under the same *Pavia* section.

As reported before, all the saponins in *A. glabra* have no hydroxyl group at C-24 (Yuan et al., 2012). However, saponins in both *A. sylvatica* and *A. pavia* have either hydroxyl or no hydroxyl group at C-24. Based on analysis of the saponin structures, *A. sylvatica* may be positioned more closely to *A. pavia* than to *A. glabra* within the *Pavia* section (Fig. 2). This phylogenetic relationship among *A. pavia*, *A. glabra*, and *A. sylvatica* drawn from the saponin structure similarity agrees with the phylogeny derived from combined analysis of internal transcribed spacer (ITS)

Table 1
^{1}H NMR and ^{13}C NMR spectroscopic data for compounds **1–2** (400 MHz in pyridine-d_5).

	1		2			1		2	
	δ_H	δ_C	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C
1	0.84 m, 1.36 *m*	38.8	0.80 m, 1.37 m	38.8	C_{21}	Tig		Tig	
2	1.96 m, 2.31 *m*	26.6	1.91 m, 2.30 m	26.9	1	–	168.4	–	168.2
3	3.45 (*dd*, 11.5, 3.0)	91.2	3.44 (*dd*, 11.0, 3.0)	91.8	2	–	129.9	–	129.8
4	–	43.7	–	43.7	3	7.07 (*q*, 7.1)	137.1	7.09 (*q*, 7.0)	137.1
5	0.89 *m*	55.7	0.88	56.3	4	1.63 (*d*, 7.1)	14.5	1.64 (*d*, 7.0)	14.1
6	1.55 *m*	18.3	1.53	18.9	5	1.93 s	12.7	1.94 *s*	12.9
7	1.27 *m*, 1.55 *m*	33.2	1.27, 1.55	33.5	C_{22}	Tig		Ang	
8	–	40.6	–	40.4	1	–	168.8	–	168.5
9	1.69 *m*	46.3	1.67	47.2	2	–	129.4	–	129.5
10	-	37.2	–	36.7	3	6.95 (*q*, 7.1)	137.5	5.87 (*q*, 7.1)	136.6
11	1.76 *m*, 1.86 *m*	23.9	1.74 m, 1.84 m	24.3	4	1.45 (*d*, 7.1)	14.0	2.03 (*d*, 7.1)	16.2
12	5.44	123.3	5.42 *br s*	123.9	5	1.84 *s*	12.7	1.89 *s*	21.3
13	–*brs*	143.0	–	143.6	C_3	GlcA-*p*		GlcA-*p*	
14	–	41.5	–	41.8	1	4.94 (*d*, 7.2)	104.2	4.92 (*d*, 7.4)	105.1
15	1.64 *m*, 1.88 (1H, *d*, 10.0)	35.1	1.61 *m*, 1.82 (1H, *d*, 11.0)	35.2	2	4.27 (*dd*, 8.9, 7.2)	78.0	4.27 (*dd*, 8.2, 7.4)	78.5
16	4.51 *m*	68.7	4.51 *m*	68.9	3	4.26 (*t*, 8.9)	86.3	4.27 (*t*, 8.2)	87.0
17	–	48.6	–	48.4	4	4.50 (*t*, 8.9)	71.5	4.51 (*t*, 8.2)	72.0
18	3.13 (*dd*, 13.1, 2.9)	40.0	3.13 (*dd*, 13.6, 3.2)	40.6	5	4.57 (*d*, 8.9)	77.1	4.58 (*d*, 8.2)	77.9
19	1.46 m, 3.16 (*t*, 13.1)	47.2	1.14 *m*, 3.31 (*t*, 13.6)	47.8	6		172.0		172.1
20	–	37.1	–	36.9	$C_{2'}$	Glc-*p*		Glc-*p*	
21	6.74 (d, 10.0)	79.0	6.69 (*d*, 10.2)	79.5	1	5.52 (*d*, 7.0)	103.3	5.52 (*d*, 7.0)	104.0
22	6.35 (d, 10.0)	73.5	6.40 (*d*, 10.2)	73.8	2	4.08 (*dd*, 9.0, 7.0)	75.2	4.08 (*dd*, 9.1, 7.0)	75.6
23	1.37 *s*	22.1	1.37 *s*	22.6	3	4.27 (*t*, 9.0)	78.0	4.27 (*t*, 9.1)	78.5
24	3.31, 4.31 (*d*, 11.0)	63.1	3.32, 4.31 (*d*, 10.5)	63.5	4	4.59 (*dd*, 9.0, 8.7)	69.2	4.58 (*dd*, 9.1, 8.6)	69.7
25	0.66 *s*	15.4	0.65 *s*	15.9	5	3.66 *m*	78.0	3.66 *m*	78.6
26	0.81 *s*	16.9	0.81 *s*	17.1	6	4.36 *m*, 4.48 (*dd*, 10.9, 7.3)	61.2	4.35 *m*, 4.47(dd, 11.2, 7.3)	61.9
27	1.85 *s*	27.6	1.85 *s*	28.0	$C_{3'}$	Ara-*f*		Ara-*f*	
28	3.42, 3.66 (*d*, 11.0)	63.1	3.44, 3.67 (d, 11.0)	64.1	1	6.08 (*br s*)	110.7	6.08 (*br s*)	111.8
29	1.14 *s*	29.5	1.13 *s*	29.9	2	5.00 *m*	83.5	5.00 *m*	84.0
30	1.37 *s*	20.1	1.35 *s*	20.7	3	4.81 *br d*, 5.8	77.1	4.82 *br d*, 5.6	78.0
					4	4.89 (*td*, 5.8, 3.4)	85.4	4.89 (*td*, 5.6, 3.4)	85.8
					5	4.21 (dd, 11.1, 3.4), 4.36 *m*	62.2	4.18 (*dd*, 10.5, 3.4), 4.34 *m*	62.9

sequence and morphological dataset (Xiang et al., 1998). In a more recent reconstructed phylogeny inferred from a combination of DNA sequence (*LFY*, *trnHK*, *rps16*, *matK* and ITS), morphology and fossil information, *A. pavia*, *A. glabra*, and *A. sylvatica* were still placed in the same eastern North America section *Pavia*. However, within the section *Pavia*, *A. pavia* was placed as the sister of the group of *A. sylvatica* and *A. glabra* (Harris et al., 2009). More new emerging data contribution seemed to make the taxonomy of *Aesculus* genus more complicated and controversial at this point. Further investigations are needed to resolve the long time taxonomy controversy.

Saponins from *A. californica* have more diversified structures which share more similar features to eastern Asian species than to eastern North American species (Yuan et al., 2013). This chemical evidence agrees to its closer relationship to the Asian clade in phylogeny derived from DNA and morphology data (Harris et al., 2009). According to analysis of the saponins isolated from *Aesculus* to date, we expect that *A. flava* in the section *Pavia* have similar saponins with the other species in the same section. Due to their distinctive morphological features, other two American *Aesculus* species, *A. parviflora* Walter and *A. parryi* A. Gray are usually placed into two different monotypic sections *Macrothyrsus* and *Parryana*, respectively. The chemical investigation of these two species will provide important clues to understand the systematics and evolutionary pattern of the genus *Aesculus*.

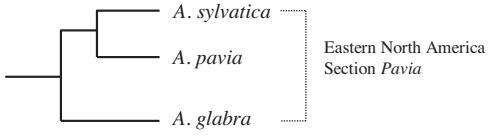

Fig. 2. The phylogenic relationship among three eastern North America *Aesculus* species *A. pavia*,*A. glabra*, and *A. sylvatica* derived from their saponin structure data.

3. Experimental

3.1. General experimental procedures

NMR experiments were performed on a JEOL ECS 400 spectrometer, with spectroscopic data referenced to the solvent used. HR-mass spectra were acquired using a Waters Q-Tof Premier mass spectrometer. HPLC analysis was performed on an Agilent 1260HPLC system using Agilent ODS columns (Column A: Poroshell 120 EC-C18, 2.7 μm). Preparative HPLC was performed with a Lab-Alliance Series III Isocratic HPLC System using an Alltima C18 column (Column B: 250 × 22 mm, 10 μm, Grace).

3.2. Plant material

Seeds of *A. sylvatica* were collected from Georgia, USA in 2012. The plant sample was identified by Dr. Shiyou Li and the voucher specimen (Ga-Fayetteville-NNN-10122012-AS-Fr) was deposited at the National Center for Pharmaceutical Crops at Stephen F. Austin State University, USA.

3.3. Extraction and isolation

The seeds of *A. sylvatica* (400 g, dry weight) were ground to a coarse powder using a Thomas Model 4 Wiley® Mill. The powder was percolated with 4L methanol using a 75 × 600 mm glass

column. After evaporated under reduced pressure, the methanol extracts were pooled, concentrated and partitioned with *n*-hexane to remove compounds of low polarity (*n*-hexane phase, 7 g). The lower layer extracts were dried to give 19 g powder as the raw saponin fraction. The raw saponin was ground and mixed with 30 g octadecyl-functionalized silica gel (C_{18}) and loaded onto a pre-equilibrated open ODS column (75 × 200 mm). The column was eluted with 45%, 65%, 80% and 100% methanol in water (each for 1 L). The eluents were collected with a Spectra/Chrom CF-2 Fraction Collector (Houston, TX, USA) (50 mL for each fraction) and analyzed with Agilent 1260HPLC (Column A: 45% acetonitrile in 0.5% HOAc water, 0.5 mL/min, 210 nm). Sub-fractions were subjected for further preparative HPLC separation according to the analysis results. The sub-fractions 7-9 were combined and separated by preparative HPLC system (column B, 40% acetonitrile in 0.5% HOAc water, 3 mL/min, 210 nm) to yield **3** (54.3 min, 3.7 mg) and **4** (65.0 min, 6.3 mg). The sub-fractions 21–25 furnished **5** (54.3 min, 2.8 mg) and **6** (65.0 min, 7.1 mg) (column B, 46% acetonitrile in 0.5% HOAc water, 3 mL/min, 210 nm). **7** (54.3 min, 11.4 mg) and **8** (65.0 min, 7.1 mg) were isolated from the sub-fractions 28 and 29 by preparative HPLC system (column B, 46% acetonitrile in 0.5% HOAc water, 3 mL/min, 210 nm). The sub-fractions 32–33 were combined and separated by a preparative HPLC system (column B, 50% acetonitrile in 0.5% HOAc water, 3 mL/min, 210 nm) to afford **9** (50.8 min, 7.4 mg), **10** (53.4 min, 8.6 mg), **11** (57.3 min, 9.6 mg) and **12** (60.5 min, 7.5 mg). **1** (55.1 min, 10.4 mg), **2** (57.8 min, 11.7 mg), and **13** (60.7 min, 8.0 mg) were prepared from sub-fraction 35 by preparative HPLC system (column B, 53% acetonitrile in 0.5% HOAc water, 3 mL/min, 210 nm). The sub-fraction 39 give **14** (56.3 min, 6.3 mg), **15** (59.8 min, 12.8 mg), and **16** (64.9 min, 4.6 mg) by using preparative HPLC (column B, 55% acetonitrile in 0.5% HOAc water, 3 mL/min, 210 nm).

*3.4. 3-O-[β-D-glucopyranosyl-(1→2)]-α-L-arabinofuranosyl-(1→3)-β-D-glucuronopyranosyl-21,22-O-ditigloyl-3β,16α,21β,22α,24,28-hexahydroxyolean-12-ene (aesculioside S1, **1**)*

White amorphous powder; $[\alpha]^{20}_{D}$ +17.2 (c 0.12, MeOH); For ^{1}H NMR and ^{13}C spectroscopic data, see Table 1; HRESIMS: *m/z* 1155.5589 $[M-H]^-$ (calcd. for $C_{57}H_{87}O_{24}$, 1155.5587).

*3.5. 3-O-[β-D-glucopyranosyl-(1→2)]-α-L-arabinofuranosyl-(1→3)-β-D-glucuronopyranosyl-21-O-tigloyl-22-O-angeloyl-3β,16α,21β,22α,24,28-hexahydroxyolean-12-ene (aesculioside S2, **2**)*

White amorphous powder; $[\alpha]^{20}_{D}$ + 11.7 (c 0.13, MeOH); For ^{1}H NMR and ^{13}C spectroscopic data, see Table 1; HRESIMS: *m/z* 1155.5583 $[M-H]^-$ (calcd. for $C_{57}H_{87}O_{24}$, 1155.5587).

*3.6. Acid hydrolysis of new saponins aesculioside S1 (**1**) and S2 (**2**) and determination of absolute configuration of sugars*

Acid hydrolysis of aesculioside S1 (**1**) and S2 (**2**) and sugar determination followed a method described before (Tanaka et al., 2007; Yuan et al., 2013). By retention time comparison and co-HPLC, the absolute configuration of sugars in each hydrolysis was identified as D-glucose, L-Arabinose and D-Glucuronic acid (Rt observed at 15.1, 16.3 and 17.9 min, respectively).

3.7. Cytotoxicity assays

The cytotoxicity assays were performed using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) as previously described (Wang et al., 2010). Cytotoxicity was reported as GI_{50} ($GI = [1-(T_D-T_0/T_C-T_0)] \times 100$. T_0 was the initial reading before the treatment. T_C or T_D represents the readings of the untreated control or with the tested compounds. Doxorubicin was used as the positive cytotoxicity control (GI_{50} against A549 and PC3 for 0.58 ± 0.04 and 0.81 ± 0.06 μM, respectively).

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