Phytochemistry 70 (2009) 1195–1202

Contents lists available at ScienceDirect

Phytochemistry

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

Cytotoxic 16-β-[(D-xylopyranosyl)oxy]oxohexadecanyl triterpene glycosides from a Malagasy plant, *Physena sessiliflora*

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ARTICLE INFO

Article history:
Received 27 February 2009
Received in revised form 21 May 2009
Available online 13 July 2009

Keywords:
Physena sessiliflora
Capparidaceae
Physenaceae
Physenoside
Triterpene glycoside
Cytotoxicity
16-Hydroxy-oxohexadecanoic acid

ABSTRACT

Brine shrimp lethality assay-guided separation of the MeOH extract of leaves of *Physena sessiliflora*, which is endemic to Madagascar, afforded eight triterpene glycosides, Physenoside S1–4 and 16-β-[(D-xylopyranosyl)oxy]oxohexadecanyl homologues, Physenoside S5–8. Structural elucidation of these compounds was based on both spectroscopic analyses and chemical properties. Physenoside S7 and S8 have significant cytotoxic activities in the brine shrimp lethality assay.

1. Introduction

A bushy tree, *Physena sessiliflora* Tul. is endemic to Madagascar and only two species, *P. sessiliflora* and *Physena madagascariensis*, belong to this genus *Physena*. This genus generally classifies into Capparidaceae, but some botanists classify it into the Physenaceae (Dickison and Miller, 1993), which is an endemic family in Madagascar and contains only this genus. In Madagascar, the decoction of these leaves is used for rites by witch doctor (Samyn, 1999). Triterpenes (Deng et al., 1999) and prenyl biflavones (Deng et al., 2000; Cao et al., 2006) were isolated from *P. madagascariensis*, but no phytochemical and biological investigations on *P. sessiliflora* have been reported. As a part of our studies on the phytochemical constituents of Malagasy plants, we have undertaken chemical investigation of the leaves of this plant. Besides, the MeOH extract of these leaves showed cytotoxic activity in the brine shrimp lethality assay (Meyer et al., 1982; Anderson et al., 1991; Solis et al., 1993; Carballo et al., 2002). Herein, we describe isolation and structure elucidation of eight novel triterpene glycosides, Physenoside S1–4, and 16-β-[(D-xylosyl)oxy]oxohexadecanyl homologues, Physenoside S5–8. In general, keto-fatty acids such as oxohexadecanoic acid are rarely found as plant lipid constituents (Deas et al., 1974).

2. Results and discussion

The methanolic extract of the leaves of *P. sessiliflora* showed cytotoxic activity using the brine shrimp lethality assay at 100 μg/mL. This extract was partitioned into *n*-hexane, EtOAc, *n*-BuOH, and water layers, successively. The *n*-BuOH extract exhibited the strongest activity and its activity-guided separation extract afforded eight triterpene glycosides, Physenoside S1–8(**1**–**8**), whose structures are shown in Fig. 1. An alkaline hydrolysis product **9** was obtained from **7** and **8**. The various NMR spectroscopic analyses such as ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC, in conjunction with FABMS spectroscopy, established that the compounds **1**–**9** (Fig. 1) were olean-12-en-28-oic acid type triterpene glycosides with oxygenated carbons (C-2, C-3, C-23, and/or C-16).

Acid hydrolysis of **1**–**6** afforded the same aglycone, bayogenin (2β,3β,23-trihydroxyolean-12-en-28-oic acid) **1a** (see Fig. 2), which was identified on the basis of NMR spectroscopic data when compared with literature data (Jurenitsch et al., 1986). On the other hand, alkaline hydrolysis of **1**–**6** gave a glucoside **1b** (Fig. 2), which was identified as 3-*O*-β-D-glucopyranoside of **1a** based on the comparison of its spectroscopic data with reference data (Kasai et al., 1986; Marston et al., 1988).

Compound **1**, named Physenoside S1, was obtained as a white amorphous powder. Its negative FABMS exhibited a quasi-molecular ion peak at *m/z* 1191 $[M-H]^-$, indicating a molecular weight of 1192. The molecular formula was established as $C_{57}H_{92}O_{26}$ by a

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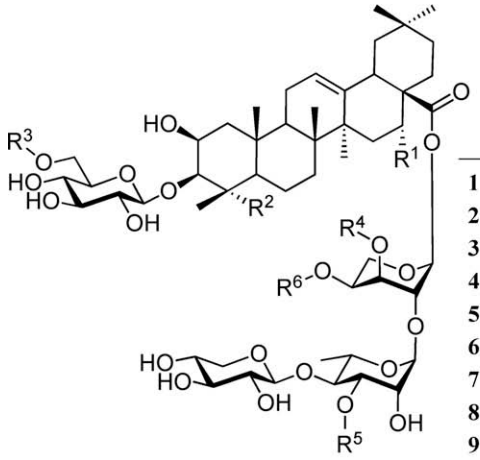
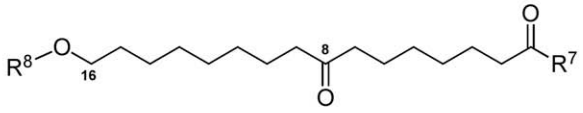
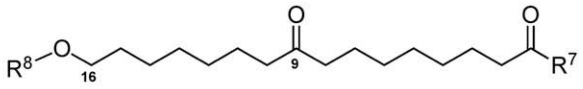
doi:10.1016/j.phytochem.2009.06.002



	R1	R2	R3	R4	R5	R6
1	H	CH_2OH	H	α-L-Ara	H	H
2	H	CH_2OH	H	β-D-Api	H	H
3	H	CH_2OH	H	α-L-Ara	α-L-Rha	H
4	H	CH_2OH	β-D-Glc	α-L-Ara	α-L-Rha	H
5	H	CH_2OH	H	β-D-Api	H	**Acyl A**
6	H	CH_2OH	H	β-D-Api	H	**Acyl B**
7	OH	COOH	H	α-L-Ara	H	**Acyl A**
8	OH	COOH	H	α-L-Ara	H	**Acyl B**
9	OH	COOH	H	α-L-Ara	H	H

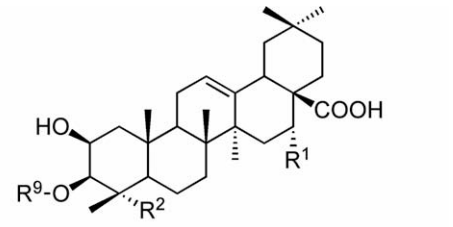




	R7	R8
Acyl A		β-D-Xyl
10	OH	β-D-Xyl
11	OH	H
12	OCH_3	$Si(CH_3)_3$
Acyl B		β-D-Xyl
13	OH	β-D-Xyl
14	OH	H
15	OCH_3	$Si(CH_3)_3$

Fig. 1. Structures of **1**–**15,** Acyl A, and Acyl B.

positive-ion mode HRFABMS with a pseudo-molecular ion peak at *m/z* 1215.5790 $[M+Na]^+$ (calcd for 1215.5775 $C_{57}H_{92}O_{26}Na$). Acid hydrolysis of **1** gave D-glucose (Glc), L-arabinose (Ara), L-rhamnose (Rha), and D-xylose (Xyl) as sugar components. The ^1H and ^{13}C NMR spectra of **1** displayed five anomeric protons at δ 6.48 (*d*, *J* = 3.0 Hz), 5.80 (*brs*), 5.12 (*d*, *J* = 7.7 Hz), 5.11 (*d*, *J* = 6.2 Hz), and 5.08 (*d*, *J* = 7.3 Hz), and five anomeric carbons at *δ* 107.1, 105.5, 103.4, 101.7, and 92.7 (see Tables 1 and 2). Each proton and carbon signal of **1** was assigned to individual functional groups using 1D-TOCSY, COSY, and HMQC spectra. An HMBC experiment was carried out for determination of the glycosyl linkages. The HMBC spectrum showed cross-peaks between each anomeric proton and the corresponding carbon resonance; δ_H 5.12 (H-1 of Glc) and δ_C 83.1 (C-3 of aglycone), δ_H 6.48 (H-1 of inner Ara) and δ_C 176.2 (C-28 of aglycone), δ_H 5.80 (H-1 of Rha) and δ_C 74.1 (C-2 of inner Ara), δ_H 5.08 (H-1 of Xyl) and δ_C 84.0 (C-4 of Rha), and δ_H 5.11 (H-1 of outer Ara) and δ_C 74.9 (C-3 of inner Ara). Based on the ^{13}C chemical shifts and coupling constants of proton signals of inner arabinosyl residue, the conformation of ester-linked arabinosyl residue of **1** was $^1C_4$ form, which is congruent with the data reported by Nagao et al. (1989). The configuration of the rhamnosyl residue was determined by the same method as reported



	R1	R2	R9
1a	H	CH_2OH	H
1b	H	CH_2OH	β-D-Glc
4b	H	CH_2OH	β-D-Glc-(1-6)-β-D-Glc
9a	OH	COOH	H
9b	OH	COOH	β-D-Glc

Fig. 2. Structures of the hydrolysis products **1a**, **1b**, **4b**, **9a**, and **9b**.

Table 1
Selected ^1H NMR spectroscopic data (500 MHz, in pyridine-d_5) of the aglycone and anomeric protons of Physenoside S1 (**1**), S7 (**7**), and S8 (**8**).

		1	**7**	**8**
Aglycone	16	5.50 (*m*)	5.14 (*brs*)	5.14 (*brs*)
	18	3.20 (*dd*, 13.7, 4.3)		
	23	3.68 (*d*, 10.6)		
	24	1.30 (*s*)	1.85 (*s*)	1.86 (*s*)
	25	1.52 (*s*)	1.46 (*s*)	1.46 (*s*)
	26	1.12 (*s*)	1.03 (*s*)	1.03 (*s*)
	27	1.23 (*s*)	1.67 (*s*)	1.66 (*s*)
	29	0.88 (*s*)	0.88 (*s*)	0.88 (*s*)
	30	0.95 (*s*)	1.00 (*s*)	1.00 (*s*)
C-3				
Glc-	1	5.11 (*d*, 6.2)	4.99 (*d*, 7.8)	4.99 (*d*, 7.8)
C-28				
inner-Ara-	1	6.48 (*d*, 3.0)	6.17 (*d*, 4.6)	6.17 (*d*, 3.7)
outer-Ara-	1	5.12 (*d*, 7.7)	4.92 (*d*, 6.4)	4.91 (*d*, 6.4)
Rha-	1	5.80 (*brs*)	5.90 (*brs*)	5.90 (*brs*)
Xyl-	1	5.08 (*d*, 7.3)	5.04 (*d*, 7.6)	5.04 (*d*, 7.6)
Acyl group				
Xyl-	1		4.64 (*d*, 7.6)	4.64 (*d*, 7.6)

Table 2
^{13}C NMR spectroscopic data (125 MHz, in pyridine-d_5) of the glycosidic parts of **1**–**9**.

		1	2	3	4	5	6	7	8	9
C-3										
Glc-	1	105.5	105.2	105.6	105.0	105.6	105.6	104.7	104.7	105.7
	2	75.5	75.2	75.5	75.2	75.6	75.6	74.7	74.7	75.5
	3	78.6	78.2	78.6	78.5	78.6	78.6	77.9	77.9	78.6
	4	71.6	71.3	71.7	71.6	71.6	71.6	71.0	71.0	71.7
	5	78.2	78.0	78.3	76.6	78.2	78.2	77.8	77.8	78.3
	6	62.6	62.3	62.7	70.1	62.7	62.7	62.1	62.1	62.7
Glc-	1				104.9					
	2				75.3					
	3				78.2					
	4				71.7					
	5				78.4					
	6				62.7					
C-28										
inner-Ara-	1	92.7	92.9	92.4	92.5	93.2	93.2	93.8	93.8	92.7
	2	74.1	73.3	74.1	74.1	73.4	73.4	73.5	73.5	74.1
	3	74.9	77.0	73.2	74.2	73.3	73.3	73.3	73.3	74.9
	4	65.3	65.5	64.7	64.7	69.9	69.9	69.9	69.9	65.3
	5	63.5	63.6	63.0	63.0	62.6	62.6	62.2	62.3	63.5
outer-Ara-	1	103.4		103.0	103.0			104.9	104.9	103.4
	2	71.1		71.1	70.8			71.6	71.6	71.1
	3	74.1		74.2	74.2			73.7	73.7	74.1
	4	68.4		68.4	68.3			68.2	68.2	68.4
	5	66.2		66.3	66.2			66.0	66.0	66.2
Api-	1		110.0			110.3	110.3			
	2		77.9			78.2	78.2			
	3		80.1			80.5	80.5			
	4		75.2			75.5	75.5			
	5		64.9			65.8	65.8			
Rha-	1	101.7	101.5	101.6	101.5	101.9	101.9	101.3	101.3	101.6
	2	71.9	71.5	72.3	71.6	71.7	71.7	71.4	71.4	71.9
	3	72.5	72.2	80.7	80.7	72.5	72.5	71.9	71.9	72.5
	4	84.0	83.5	77.8	77.8	84.1	84.1	82.8	82.8	84.0
	5	68.7	68.7	69.4	69.3	68.9	68.9	68.5	68.6	68.7
	6	18.4	18.3	18.7	18.6	18.6	18.4	18.2	18.2	18.4
Rha-	1			103.9	104.0					
	2			71.7	72.2					
	3			72.7	72.7					
	4			74.4	74.1					
	5			70.1	70.0					
	6			18.7	18.6					
Xyl-	1	107.1	106.7	105.5	105.4	107.2	107.2	106.2	106.2	107.1
	2	76.0	75.7	75.5	75.2	76.0	76.0	75.5	75.5	76.0
	3	78.6	78.2	78.3	78.2	78.6	78.6	77.8	77.8	78.6
	4	70.9	70.7	70.9	71.1	71.0	71.0	70.7	70.7	70.9
	5	67.4	67.1	67.1	67.1	67.5	67.5	66.9	66.9	67.4
Acyl group										
Xyl-	1					105.3	105.3	104.8	104.8	
	2					75.0	75.0	74.4	74.5	
	3					78.4	78.4	77.6	77.6	
	4					71.2	71.2	70.5	70.5	
	5					67.2	67.2	66.7	66.7	

previously (Kasai et al., 1979). From these experimental facts, we confirmed the structure of compound **1** to be β-D-xylopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-[α-L-arabinopyranosyl-(1-3)]-α-L-arabinopyranosyl 3-*O*-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate.

Physenoside S2 (**2**) was obtained a white powder, and its positive HRFABMS spectrum exhibited a quasi-molecular ion peak at *m/z* 1215.5801 $[M+Na]^+$ (calcd. for 1215.5775 $C_{57}H_{92}O_{26}Na$) as for as **1**. The ^{13}C NMR spectrum of **2** was similar to that of **1** except for a terminal L-arabinosyl residue. On acid hydrolysis of **2**, D-glucose, L-arabinose, L-rhamnose, D-xylose, and D-apiose (Api) were detected. HMBC spectrum coupled with 1D-TOCSY and HMQC suggested that compound **2** had a structure in which the terminal α-L-arabinose of **1** was replaced by β-D-apio-D-furanose. The anomeric center of the apio-D-furanose was determined to be β-configuration by comparing the ^{13}C NMR spectroscopic data with the reported values for α- and β-D-apiofranosides (Kitagawa et al., 1993). Consequently, the structure of **2** was assigned to β-D-xylopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-[β-D-apio-D-furanosyl-(1-3)]-α-L-arabinopyranosyl 3-*O*-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate.

The FABMS spectrum of Physenoside S3 (**3**) showed a quasi-molecular ion peak at 1337 $[M-H]^-$, which is 146 mass units greater than **1**. Its positive HRFABMS spectrum also exhibited a quasi-molecular ion peak at *m/z* 1361.6371 $[M+Na]^+$ (calcd for 1361.6354 $C_{63}H_{102}O_{30}Na$). Acid hydrolysis of **3** gave D-glucose, L-arabinose, L-rhamnose, and D-xylose. Two methyl proton signals at δ 1.74 (*d*, *J* = 6.7 Hz) and 1.64 (*d*, *J* = 6.7 Hz) indicated the presence of two rhamnosyl residues, and fragment ion peak at *m/z* 1191 $[M-146]^-$ suggesting that one of rhamnosyl residues is

the terminal residue. A comparison of the ^{13}C NMR spectrum of **3** with that of **1** showed glycosylation shifts for C-3 of Rha ($\Delta\delta$ +8.2 ppm) on going from **1** to **3**. The HMBC spectrum of **3,** followed by assignments of protons and carbons of sugar moieties, showed a cross-peak between δ_H 5.78 (H-1 of terminal Rha) and δ_C 80.7 (C-3 of inner Rha). These data of **3** were consistent with the structure of β-D-xylopyranosyl-(1-4)-[α-L-rhamnopyranosyl-(1-3)]-α-L-rhamnopyranosyl-(1-2)-[α-L-arabinopyranosyl-(1-3)]-α-L-arabinopyranosyl 3-*O*-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate.

Physenoside S4 (**4**), $C_{69}H_{112}O_{35}$, was isolated as a white powder having a molecular weight of 1500. Acid hydrolysis of **4** gave D-glucose, L-arabinose, L-rhamnose, and D-xylose. On the other hand, alkaline hydrolysis of **4** yielded compound **4b**. Two anomeric protons at δ 4.89 (*d*, *J* = 7.8 Hz) and 4.98 (*d*, *J* = 7.8 Hz) in ^1H NMR, and two anomeric carbons at δ 104.9 and 105.0 in ^{13}C NMR were observed. The carbon signals indicated the presence of gentiobiosyl unit in **4b**. These experimental results established that compound **4b** is 3-*O*-β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl-bayogenin. The ^1H and ^{13}C NMR spectra of **4** showed seven anomeric signals. In the ^{13}C NMR spectrum of **4,** carbon resonances of sugar chain at C-28 were essentially the same as those of **3**. These NMR spectroscopic data suggested that compound **4** is β-D-xylopyranosyl-(1-4)-[α-L-rhamnopyranosyl-(1-3)]-α-L-rhamnopyranosyl-(1-2)-[α-L-arabinopyranosyl-(1-3)]-α-L-arabinopyranosyl 3-O-β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate. This formulation was confirmed by HMBC experiment coupled with COSY, 1D-TOCSY, and HMQC.

Physenoside S5 (**5**) showed a quasi-molecular ion peak $[M+Na]^+$ at *m/z* 1615.8251 in the positive HRFABMS, in accordance with an empirical molecular formula of $C_{78}H_{128}O_{33}Na$. The ^1H and ^{13}C NMR spectra of **5** displayed six anomeric protons at δ 6.33 (*brs*), 5.78 (*brs*), 5.76 (*d*, *J* = 1.6 Hz), 5.12 (*d*, *J* = 7.8 Hz), 5.07 (*d*, *J* = 7.3 Hz), and 4.71 (*d*, *J* = 7.6 Hz), and six anomeric carbons at δ 110.3, 107.2, 105.6, 105.3, 101.9, and 93.2. Acid hydrolysis of **5** afforded D-glucose, L-arabinose, D-apiose, L-rhamnose, and D-xylose as sugar components. Alkaline hydrolysis of **5** gave compound **10** as well as **1a**. Alkaline treatment of **5** with 2% KOH*aq* also yielded **10** in addition to compound **1**. These results indicated that compound **5** is ester of **1** and **10**. In the ^{13}C NMR spectrum of **10**, one carbonyl (δ_C 210.3), one carboxyl (δ_C 172.9), 14 methylene carbon signals together with signals assigned as xylosyl residue were observed. The HRFABMS of **10** showed a quassi-molecular ion peak at *m/z* 441.2450 $[M+Na]^+$, suggesting the molecular formula $C_{21}H_{38}O_8$ (calcd. 441.2464 for $C_{21}H_{38}O_8Na$). Acid hydrolysis of **10** gave D-xylose and compound **11**, and the HMBC spectrum displayed a cross-peak between δ_H 4.71 (*d*, *J* = 7.6 Hz, H-1 of Xyl) and δ_C 69.7 (CH_2). These results showed that compound **10** is 16-β-[(D-xylopyranosyl)oxy]-χ-oxohexadecanoic acid. To determine the position of the carbonyl group, compound **11** was converted to **12** by methylation followed by trimethylsilylation. The ELMS of **12** showed a typical fragment ion peak at *m/z* 171 (Fig. 3), indicating that the carbonyl group exist at C-8 position. This result was accorded with the data in the literature (Deas et al., 1974; Ray et al., 1995). Thus, compound **10** was elucidated as 8-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoic acid. The ester substituent in **5** was placed at C-4 of ester-linked arabinosyl residue as a result of downfield shifts observed for H-4 and C-4 of arabinosyl unit in the ^1H and ^{13}C NMR spectra, respectively, compared to those of **1**; This was also confirmed by observation of correlations between δ_H 5.62 (*ddd*, *J* = 3.2, 3.2, 8.0 Hz, H-4 of inner Ara) and δ_C 173.3 (C-1 of **Acyl A**) in the HMBC spectrum of compound **5**. Consequently, the structure of **5** was determined to be β-D-xylopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-{[β-D-apio-D-furanosyl-(1-3)]-4-*O*-(8-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoyl)}-α-L-arabinopyranosyl 3-*O*-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate.

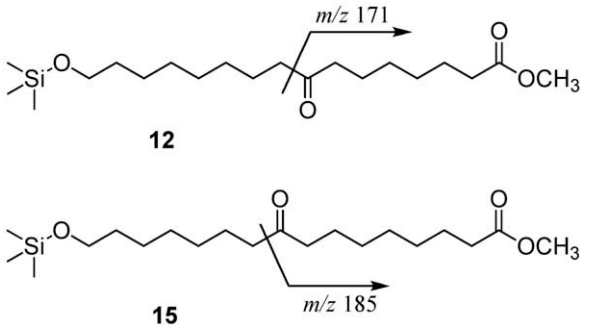


Fig. 3. Fragmentation points observed in EIMS of **12** and **15**.

The chromatographic behavior of Physenoside S6 (**6**) was almost the same as **5**. The ^1H and ^{13}C NMR spectroscopic data of **6** were also quite similar to those of **5**. Alkaline hydrolysis of **6** with 2% KOH*aq* at room temperature yielded **1** and **13**. The NMR spectroscopic data suggested that compound **13** is the isomer of **10**, and that the difference between **10** and **13** is the position of the carbonyl group. In the EIMS of the trimethylsililated methyl ester **15**, a critical fragment ion at *m/z* 185 (Fig. 3) was observed (Deas et al., 1974; Ray et al., 1995). Therefore, the structure of **13** was assigned to 9-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoic acid. In the similar manner, the position of ester linkage between **1** and **13** in **6** was determined as *O*-4 of 28-*O*-linked arabinose. The above evidence showed that compound **6** is β-D-xylopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-{[β-D-apio-D-furanosyl-(1-3)]-4-*O*-(9-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoyl)}-α-L-arabinopyranosyl 3-*O*-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate.

Alkaline treatment of Physenoside S7 (**7**) and S8 (**8**) with 2.5% NaOH*aq* gave the same compound **9**. On the other hand, acid hydrolysis of physenoside S7 and S8 afforded the same aglycone, zanhic acid (Dimbi et al., 1984) (2β,3β,16α-trihydroxyolean-12-en-23,28-dioic acid) **9a**, which was identified on the basis of the NMR spectroscopic data compared with the reported values (Lavaud et al., 1998; Sakai et al., 1999). Furthermore, alkaline hydrolysis of **7** and **8** yielded the same compound (**9b**), which was identified as 3-*O*-β-D-glucopyranoside of **9a**.

The ^1H and ^{13}C NMR spectroscopic data of the sugar moieties including the β-[(D-xylopyranosyl)oxy]acyl group of compounds **7** and **8** were essentially identical to those of corresponding compounds **1** and each acyl component (Tables 1 and 2). Therefore, the structures of **7** and **8** were identified as 28-*O*-β-D-xylopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-{[α-L-arabinopyranosyl-(1-3)]-4-*O*-(8-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoyl)}-α-L-arabinopyranosyl 3-*O*-β-D-glucopyranosyl-2β,3β,16α-trihydroxyolean-12-en-23,28-dioic acid and 28- *O*-β-D-xylopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-{[α-L-arabinopyranosyl-(1-3)]-4-*O*-(9-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoyl)}-α-L-arabinopyranosyl 3-*O*-β-D-glucopyranosyl-2β,3β,16α-trihydroxyolean-12-en-23,28-dioic acid, respectively.

Finally, we conducted the brine shrimp lethality assay for determination of the toxicity of the isolated compounds **1**–**8**. The assay is proven to be an effective and rapid screening method to determine cytotoxicity of water-soluble compounds (Meyer et al., 1982; Anderson et al., 1991; Solis et al., 1993; Carballo et al., 2002). Table 3 shows the assay results as LD_{50} values with comparison of a positive control, Etoposide phosphate. Among the subfractions prepared from the MeOH extract by solvent partition, only *n*-BuOH extract exhibited the lethality at 32 μg/mL. This result indicated that toxic compounds of this plant are less-lipophilic substances. Compounds **7** (8.5 μg/mL) and **8** (22 μg/mL) exhibited high degrees of toxicity in isolated compounds.

Table 3
Brine shrimp lethality assay results of the extracts of *P. sessiliflora*, the isolated compounds **1**–**9**, and Etoposide phosphate (a positive control).

	Brine shrimp lethality (LD_{50} in μg/mL)
MeOH extract	50.4
MeOH → *n*-hexane	>500.0
MeOH → EtOAc	>500.0
MeOH → *n*-BuOH	32.0
1	>500.0
2	>500.0
3	>500.0
4	>500.0
5	394.0
6	>500.0
7	8.5
8	22.1
9	420.0
Etoposide phosphate	3.5

3. Conclusions

In this study, we isolated eight new triterpene glycosides (Pysenoside S1–8) in the methanolic extract of the leaves of *P. sessiliflora*. For four of the eight compounds, novel acyl moieties were found as 16-β-[(D-xylopyranosyl)oxy]-8-oxohexadecanyl and 16-β-[(D-xylopyranosyl)oxy]-9-oxohexadecanyl groups. Pysenoside S7 and S8 gave significant lethal activities for the brine shrimp, suggesting that the β-[(D-xylopyranosyl)oxy]acyl moiety and the aglycone having 23-carboxylic and 16α-hydroxyl groups play important role for the cytotoxicity.

4. Experimental

4.1. General procedure

Optical rotations were measured on a JASCO DIP-1030 automatic polarimeter in MeOH at 30 °C. NMR spectra were recorded on a JEOL JNM ECP-500 with a field gradient unit (500 MHz for ^{1}H and 125 MHz for ^{13}C) in pyridine-d_5, unless otherwise stated, using TMS as an internal standard at 35.0 ± 0.1 °C. FABMS in glycerol matrix in the negative-ion mode and EIMS were recorded on a JEOL SX-102A instrument. FL-100D (Fuji silysia, Tokyo, Japan) and ODS-A-120 (YMC, Kyoto, Japan) were used for column chromatography. HPLC were carried out using a D-ODS-5 (20 mm i.d. × 15 cm, YMC) column or Polyamine-II (20 mm i.d. × 15 cm, YMC) with two JASCO PU-1500 pumps, and a JASCO UV-1500 UV detector, and/or a JASCO RI-1500 differential refractometer as a detector. TLC was performed using a Merck Art. 5554 (silica-gel) TLC plate. All reagents and solvents were of the highest commercial quality and were used without further purification.

4.2. Plant material

The leaves of *P. sessiliflora* were collected in the Berenty Reserve in March 2000. The identity of the plant was confirmed by Dr. Armand Rakotozafy from the Institut Malgache de Recherches Appliquées. A voucher specimen (PHYS200012) is on deposit at ESSD, Antananarivo, Madagascar.

4.3. Extraction and isolation

Air-dried leaves (200 g) were extracted with MeOH (2 L × 3) under conditions where the suspension was heated until reflux began, thus being maintained for 3 h. The suspensions were filtered with the solvent removed from the filtrate *in vacuo* to yield the crude residue (55 g). The MeOH extract was partitioned between equal portions of *n*-hexane/MeOH–H_2O (7:3), then the solvent was removed from the *n*-hexane layer to yield the *n*-hexane extract (3.2 g). After removing MeOH from the aqueous layer, H_2O then added until one liter, the latter was partitioned with EtOAc (1 L) and then *n*-BuOH (1 L) successively. From each layer, the solvents were removed *in vacuo* to afford residues of 9.1 g (EtOAc), 21.4 g (*n*-BuOH), and 21.5 g (H_2O), respectively. The active *n*-BuOH soluble fraction was subjected to silica-gel column chromatography (CC) with a step gradient solvent system of CH_2Cl_2–MeOH–H_2O (30:10:1, 20:10:1, 10:5:1, and finally 7:5:1), to give six fractions (Fr. I to VI). Active fraction IV was applied to a silica-gel eluted with CH_2Cl_2–MeOH–H_2O (30:10:1) to give 5 fractions (Fr. IVa to IVe). Fraction IVb was further separated by HPLC on an ODS column with MeCN–H_2O (3:7) to give compound **1**–**3** (176, 32, and 128 mg, respectively). Compound **7** (15 mg) and **8** (22 mg) was purified by HPLC on an ODS column with MeCN–H_2O (37:63) and further with MeOH/0.05% TFA*aq* (65:35) from Fr. IVc. Fr. III was subjected to an ODS CC using a MeOH–H_2O (4:6 to 9:1) gradient solvent system give four fractions and then Fr. IIIc was purified by HPLC on a Polyamine-II column with MeCN–H_2O 82.5: 17.5) to afford compounds **5** (14 mg) and **6** (16 mg), respectively. Fr. V was separated by MPLC on an ODS using a MeOH–H_2O gradient (4:6 to 100:0) to give eight fractions. Fr. Vc was purified by HPLC on a Polyamine-II column with MeCN–H_2O (77.5:22.5) afforded compound **4** (18 mg). All separations was guided by brine shrimp lethality testing.

4.4. Identification of the component monosaccharides

Compounds (2 mg) were heated with 1 mol/L H_2SO_4 (1 mL) at 80 °C for 2 h with reaction mixtures washed with Et_2O, and aqueous layers neutralized with amberlite MB-3 resin and dried. Each residue dissolved in H_2O (100 μL) and subjected to HPLC analysis equipped with an optical rotation detector. HPLC conditions: column, Polyamine-II (4.6 mm i.d. × 15 cm, YMC); column temp., 40 °C; mobile phase, MeCN–H_2O (4:1); flow rate, 1.0 mL/min; detection, OR-2090 detector (JASCO). Retention time and a symbol of optical rotation: D-Glc, 12.5 min, +; L-Ara, 8.3 min, +; L-Rha, 6.0 min, −; D-Api, 5.7 min, −; D-Xyl, 9.1 min, +.

4.5. Physenoside S1 (*1*)

White powder; $[\alpha]_D$ +17.8° (*c* 1.0, MeOH); for ^{1}H NMR spectrum (pyridine-d_5) assignments, see Table 1; and for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts, see Table 2; ^{13}C NMR data of aglycone δ 15.0 (C-24), 17.3 (C-25), 17.6 (C-26), 18.1 (C-6), 23.2 (C-16), 23.7 (C-30), 24.0 (C-11), 26.1 (C-27), 28.3 (C-15), 30.8 (C-20), 32.7 (C-22), 33.1 (C-7), 33.1 (C-29), 34.1 (C-21), 37.0 (C-10), 40.0 (C-8), 41.9 (C-18), 42.4 (C-14), 42.7 (C-4), 44.1 (C-1), 46.2 (C-19), 47.3 (C-17), 47.8 (C-9), 48.5 (C-5), 65.6 (C-23), 70.4 (C-2), 83.1 (C-3), 122.9 (C-12), 144.1 (C-13), 176.2 (C-28); FABMS (negative ion mode) *m/z* 1191 $[M-H]^-$, 1059 $[M-132-H]^-$, 1029 $[M-162-H]^-$, 913 $[M-132-146-H]^-$, 649 $[M-132\times3-146-H]^-$; HRFABMS (positive-ion mode) *m/z* 1215.5790 $[M+Na]^+$ (calcd. for 1215.5775 $C_{57}H_{92}O_{26}Na$).

4.6. Acid hydrolysis of *1*

Compound **1** (10 mg) was dissolved in 1 mol/L H_2SO_4 (2 mL) and heated at 80 °C for 2 h. The reaction mixture was extracted with Et_2O, and the organic layer washed with saturated $NaHCO_3$, and solvent then removed. The residue was crystallized from MeOH to give **1a** as colorless needles. Bayogenin (**1a**): $[\alpha]_D$ +17.8° (c 1.0, MeOH); ^{1}H NMR (pyridine-d_5) δ 5.49 (1H, *t*, *J* = 3.6 Hz, H-12), 4.80 (1H, *ddd*, *J* = 3.9, 3.6, 3.6 Hz, H-2), 4.35, 3.68

(2H, each *d*, *J* = 10.8 Hz, H-23), 4.31 (1H, *d*, *J* = 3.9 Hz, H-3), 3.29 (1H, *dd*, *J* = 4.4, 13.4 Hz, H-18), 1.54 (3H, *s*, H-25), 1.33 (3H, *s*, H-24), 1.27 (3H, *s*, H-26), 1.07 (3H, *s*, H-27), 1.00 (3H, *s*, H-30), 0.93 (3H, *s*, H-29); ^{13}C NMR (pyridine-d_5) δ 15.0 (C-24), 17.2 (C-25), 17.5 (C-26), 18.4 (C-6), 23.7 (C-16), 23.8 (C-30), 24.0 (C-11), 26.3 (C-27), 28.3 (C-15), 31.0 (C-20), 33.0 (C-22), 32.8 (C-7), 33.3 (C-29), 34.3 (C-21), 37.2 (C-10), 49.9 (C-8), 42.1 (C-18), 42.4 (C-14), 42.2 (C-4), 44.1 (C-1), 46.5 (C-19), 46.7 (C-17), 48.3 (C-9), 48.5 (C-5), 65.6 (C-23), 71.6 (C-2), 73.3 (C-3), 123.0 (C-12), 144.2 (C-13), 180.2 (C-28).

4.7. Alkaline hydrolysis of **1**

Compound **1** (20 mg) was treated with 1 mol/L NaOH*aq* at 80 °C for 30 min. The reaction mixture was acidified with 2 mol/L HCl, and then extracted with *n*-BuOH. The *n*-BuOH layer was washed with saturated $NaHCO_3$ and evaporated to dryness. The residue was chromatographed on a silica-gel with CH_2Cl_2–MeOH–H_2O (30:10:1) to give **1b** (10 mg). 3-*O*-β-D-glucopyranosyl bayogenin (**1b**): ^{1}H NMR (pyridine-d_5) δ 5.49 (1H, *t*, *J* = 3.6 Hz, H-12), 4.80 (1H, *ddd*, *J* = 3.9, 3.6, 3.6 Hz, H-2), 4.35, 3.68 (2H, each *d*, *J* = 10.8 Hz, H-23), 4.31 (1H, *d*, *J* = 3.9 Hz, H-3), 3.29 (1H, *dd*, *J* = 4.4, 13.4 Hz, H-18), 1.54 (3H, *s*, H-25), 1.33 (3H, *s*, H-24), 1.27 (3H, *s*, H-26), 1.07 (3H, *s*, H-27), 1.00 (3H, *s*, H-30), 0.93 (3H, *s*, H-29), 5.15 (1H, *d*, *J* = 7.8 Hz, H-1 of Glc), 4.17 (1H, *dd*, *J* = 7.8, 8.2 Hz, H-2 of Glc), 4.20 (1H, *dd*, *J* = 8.2, 8.7 Hz, H-3 of Glc), 4.16 (1H, *dd*, *J* = 8.7, 8.9 Hz, H-4 of Glc), 3.90 (1H, *ddd*, *J* = 8.9, 2.7, 5.0 Hz, H-5 of Glc), 4.45 (1H, *dd*, *J* = 2.7, 11.8 Hz, H-6a of Glc), 4.31 (1H, *dd*, *J* = 5.0, 11.8 Hz, H-6b of Glc); ^{13}C NMR (pyridine-d_5) δ 15.0 (C-24), 17.2 (C-25), 17.5 (C-26),18.0 (C-6), 23.7 (C-16), 23.8 (C-30), 24.0 (C-11), 26.3 (C-27), 28.3 (C-15), 31.0 (C-20), 33.0 (C-22), 33.3 (C-7), 33.3 (C-29), 34.3 (C-21), 37.0 (C-10), 40.0 (C-8), 42.1 (C-18), 42.4 (C-14), 42.8 (C-4), 44.1 (C-1), 46.5 (C-19), 46.7 (C-17), 47.8 (C-9), 48.6 (C-5), 62.7 (Glc-1), 65.6 (C-23), 70.4 (C-2), 71.7 (Glc-4), 75.5 (Glc-2), 78.3 (Glc-5), 78.6 (Glc-3), 83.1 (C-3), 105.7 (Glc-1), 123.3 (C-12), 144.9 (C-13), 180.2 (C-28).

4.8. Physenoside S2 (**2**)

White powder; $[\alpha]_D$ +18.8° (*c* 0.6, MeOH); for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts: see Table 2. ^{13}C NMR data of the aglycone were almost the same (within ±0.2 ppm) as those for compound **1**; FABMS (negative ion mode) *m/z* 1191 $[M-H]^-$, 1159 $[M-132-H]^-$, 1029 $[M-162-H]^-$, 913 $[M-132-146-H]^-$, 649 $[M-132\times3-146-H]^-$; HRFABMS (positive-ion mode) *m/z* 1215.5801 $[M+Na]^+$ (calcd. for 1215.5775 $C_{57}H_{92}O_{26}Na$); Acid hydrolysis in the same manner as described above gave **1a**. Alkaline hydrolysis gave **1b**.

4.9. Physenoside S3 (**3**)

White powder; $[\alpha]_D$ +12.7° (*c* 0.9, MeOH); for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts: see Table 2. ^{13}C NMR data of the aglycone were almost the same (within ±0.2 ppm) as those for compound **1**; FABMS (negative ion mode) *m/z* 1337 $[M-H]^-$, 1205 $[M-132-H]^-$, 1191 $[M-146-H]^-$, 1175 $[M-162-H]^-$, 649 $[M-132\times3-146\times12-H]^-$; HRFABMS (positive-ion mode) *m/z* 1361.6371 $[M+Na]^+$ (calcd. for 1361.6354 $C_{63}H_{102}O_{30}Na$); Acid hydrolysis in the same manner as described above gave **1a**. Alkaline hydrolysis gave **1b**.

4.10. Physenoside S4 (**4**)

White powder; $[\alpha]_D$ +16.6° (*c* 0.5, MeOH); for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts: see Table 2. ^{13}C NMR data of the aglycone were almost the same (within ±0.2 ppm) as those for compound **1**; FABMS (negative ion mode) *m/z* 1499 $[M-H]^-$, 1367 $[M-132-H]^-$, 1353 $[M-146-H]^-$, 1337 $[M-162-H]^-$, 1175 $[M-162\times2-H]^-$, 811 $[M-132\times3-146-H]^-$; HRFABMS (positive-ion mode) *m/z* 1523.6901 $[M+Na]^+$ (calcd. for 1523.6882 $C_{69}H_{112}O_{35}Na$); Acid hydrolysis in the same manner as described above gave **1a**. Alkaline hydrolysis gave **1b**.

4.11. Physenoside S5 (**5**)

White powder; $[\alpha]_D$ +1.3° (*c* 1.0, MeOH); for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts: see Table 2. ^{13}C NMR data of the aglycone were almost the same (within ±0.2 ppm) as those for compound **1**; FABMS (negative ion mode) m/z 1621 $[M-H]^-$, 1489 $[M-132-H]^-$, 1459 $[M-162-H]^-$, 1343 $[M-146-132-H]^-$, 649 $[M-132\times3-146-430-H]^-$, 441 $[Acyl]^-$; HRFABMS (positive-ion mode) *m/z* 1615.8252 $[M+Na]^+$ (calcd. for 1615.8235 $C_{78}H_{128}O_{33}Na$); Acid hydrolysis in the same manner as described above gave **1a**. Alkaline hydrolysis gave **1b**.

4.12. Physenoside S6 (**6**)

White powder; $[\alpha]_D$ +10.2° (*c* 1.0, MeOH); for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts: see Table 2. ^{13}C NMR data of the aglycone were almost the same (within ±0.2 ppm) as those for compound **1**; FABMS (negative ion mode) *m/z* 1621 $[M-H]^-$, 1489 $[M-132-H]^-$, 1459 $[M-162-H]^-$, 1343 $[M-146-132-H]^-$, 649 $[M-132\times3-146-430-H]^-$, 441 $[Acyl]^-$; HRFABMS (positive-ion mode) *m/z* 1615.8239 $[M+Na]^+$ (calcd. for 1615.8235 $C_{78}H_{128}O_{33}Na$); Acid hydrolysis in the same manner as described above gave **1a**. Alkaline hydrolysis gave **1b**.

4.13. Mild alkaline hydrolysis of **5** and **6**

Each of the saponins (10 mg) was dissolved into 0.5 (w/v)% KOH*aq* and left at 20 °C for 15 min. The reaction mixture was acidified by 1 mol/L HCl and extracted with EtOAc, and then the organic layer was washed with saturated $NaHCO_3$. The solvent was removed *in vacuo* from the EtOAc layer to afford corresponding 8/9-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoic acid (**10** and **13**, respectively). The aqueous layer was extracted *n*-BuOH to give the corresponding saponin.

4.14. 8-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoic acid (**10**)

White powder; $[\alpha]_D$ +52.1° (*c* 0.3, MeOH); ^{1}H NMR (pyridine-d_5) δ 1.26–1.41 (12H, *m*, H-4, H-5, H-11, H-12, H-13 and H-14), 1.58 (8H, *m*, H-3, H-6, H-10 and H-15), 2.15 (2H, *t*, *J* = 7.6 Hz, H-2), 2.44 (4H, *m*, H-7 and H-9), 3.15 (1H, *dd*, *J* = 7.5, 9.2 Hz, H-2 of Xyl), 3.18 (1H, *dd*, *J* = 10.3, 11.4 Hz, H-5a of Xyl), 3.31 (1H, *dd*, *J* = 8.7, 9.2 Hz, H-3 of Xyl), 3.47 (1H, *d*, *J* = 5.3, 8.7, 10.3 Hz, H-4 of Xyl), 3.53 (1H, *dt*, *J* = 9.6, 6.7 Hz, H-16a), 3.79 (1H, *dt*, *J* = 9.6, 6.7 Hz, H-16b), 3.84 (1H, *dd*, *J* = 5.2, 11.4 Hz, H-5b of Xyl), 4.18 (1H, *d*, *J* = 7.5 Hz, H-1 of Xyl); ^{13}C NMR (pyridine-d_5) δ 24.5 (C-6), 24.8 (C-10), 25.8 (C-3), 26.9 (C-14), 29.7 (C-4), 30.2 (C-5), 30.4 (C-11, C-12 and C-13), 30.7 (C-15), 34.0 (C-2), 43.3 (C-7), 43.5 (C-9), 66.9 (Xyl-5), 70.9 (C-16), 71.2(Xyl-4), 74.9(Xyl-2), 77.9 (Xyl-3), 104.8(Xyl-1), 178.4 (C-1), 214.3 (C-8); HRFABMS (positive-ion mode) *m/z* 441.2450 $[M+Na]^+$ (calcd. 441.2464 for $C_{21}H_{38}O_8Na$); Compound **10** (10 mg) was dissolved in 1 mol/L H_2SO_4 (2 ml) and heated at 80 °C for 2 h. The reaction mixture was extracted with Et_2O, and the organic layer washed with saturated $NaHCO_3$, and the solvent was removed to give **11** (5 mg).

4.15. 9-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoic acid (**13**)

White powder; $[\alpha]_D$ +48.5° (*c* 0.3, MeOH); ^{1}H NMR (pyridine-d_5) δ 1.26–1.41 (12H, *m*, H-4, H-5, H-6, H-12, H-13 and H-14), 1.58 (8H, *m*, H-3, H-7, H-11 and H-15), 2.15 (2H, *t*, *J* = 7.6 Hz, H-2), 2.43 (4H, *m*, H-8 and H-10), 3.15 (1H, *dd*, *J* = 7.5, 9.2 Hz, H-2 of Xyl), 3.18 (1H, *dd*, *J* = 10.3, 11.4 Hz, H-5a of Xyl), 3.31 (1H, *dd*, *J* = 8.7, 9.2 Hz, H-3 of Xyl), 3.48 (1H, *d*, *J* = 5.3, 8.7, 10.3 Hz, H-4 of Xyl), 3.51 (1H, *dt*, *J* = 9.6, 6.7 Hz, H-16a), 3.80 (1H, *dt*, *J* = 9.6, 6.7 Hz, H-16b), 3.84 (1H, *dd*, *J* = 5.2, 11.4 Hz, H-5b of Xyl), 4.19 (1H, *d*, *J* = 7.5 Hz, H-1 of Xyl); ^{13}C NMR (pyridine-d_5) δ 24.8 (C-7), 24.9 (C-11), 25.9 (C-3), 29.8 (C-4), 30.1 (C-5), 30.2 (C-6, C-12 and C-13), 30.7 (C-14 and C-15), 34.0 (C-2), 43.3 (C-8 and C-10), 66.9 (Xyl-5), 70.9 (C-16), 71.2(Xyl-4), 74.9(Xyl-2), 77.9 (Xyl-3), 105.1 (Xyl-1), 178.4 (C-1), 214.5 (C-9); HRFABMS (positive-ion mode) *m/z* 441.2488 $[M+Na]^+$ (calcd. 441.2464 for $C_{21}H_{38}O_8Na$); Acid hydrolysis of **13** (10 mg) gave **14** (5 mg).

4.16. Preparation of methyl 16-trimethylsilyloxy-8/9-oxohexadecanoate for EIMS

Trimethylsilyldiazomethane (20 μL) was added to an EtOH solution (100 μL) of compounds **11** or **14** (1 mg). After 1 h, the solvent was removed from the reaction mixture, and then added TMSI-H (100 μL) to the residue. After 15 min, H_2O (1 drop) was added to stop the reaction, and the reaction mixture was extracted with *n*-hexane. The organic layer furnished methyl 16-trimethylsilyloxy-8/9-oxohexadecanoate (**12** and **15**, respectively).

4.17. Compound **9** (alkaline treatment product of Physenoside S7 (**7**) and S8 (**8**) with 2.5% NaOHaq)

White powder; $[\alpha]_D$ +6.9° (*c* 1.0, MeOH); for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts, see Table 2. ^{13}C NMR (pyridine-d_5) data of the aglycone: δ 13.9 (C-24), 16.8 (C-25), 17.2 (C-26), 20.9 (C-6), 23.7 (C-11), 24.4 (C-30), 26.8 (C-27), 30.5 (C-20), 31.6 (C-22), 32.8 (C-29), 33.0 (C-7), 35.6 (C-15), 35.7 (C-21), 36.5 (C-10), 40.2 (C-8), 41.3 (C-18), 42.0 (C-14), 44.1 (C-1), 47.0 (C-19), 47.5 (C-9), 49.3 (C-17), 52.3 (C-5), 52.6 (C-4), 69.8 (C-2), 73.4 (C-16), 85.6 (C-3), 122.3 (C-12), 144.2 (C-13), 175.7 (C-28), 180.9 (C-23); FABMS (negative ion mode) *m/z* 1221 $[M-H]^-$, 1089 $[M-132-H]^-$, 1059 $[M-162-H]^-$, 943 $[M-132-146-H]^-$, 649 $[M-132\times3-146-H]^-$; HRFABMS (positive-ion mode) m/z 1245.5562 $[M+Na]^+$ (calcd. for 1245.5516 $C_{57}H_{90}O_{28}Na$).

4.18. Physenoside S7 (**7**)

White powder; $[\alpha]_D$ +5.2° (*c* 1.0, MeOH); for ^{1}H NMR spectroscopic (pyridine-d_5) assignments, see Table 1, and for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts: see Table 2; ^{13}C NMR data of the aglycone were almost the same (within ±0.2 ppm) as those for compound **9**; FABMS (negative ion mode) *m/z* 1621 $[M-H]^-$, 1489 $[M-132-H]^-$, 1459 $[M-162-H]^-$, 1343 $[M-132-146-H]^-$; Acid hydrolysis in the same manner as described above gave **9a**. Alkaline hydrolysis of **7** gave **9b**. Mild alkaline hydrolysis with 0.5 (w/v)% KOH*aq* also gave 8-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoic acid (**10**).

4.19. Physenoside S8 (**8**)

White powder. $[\alpha]_D$ +1.2° (*c* 1.5, MeOH); for ^{1}H NMR spectroscopic (pyridine-d_5) assignments, see Table 1, and for ^{13}C NMR (pyridine-d_5) assignments of the glycosidic parts, see Table 2. ^{13}C NMR data of the aglycone were almost the same (within ±0.2 ppm) as those for compound **9**; FABMS (negative ion mode) *m/z* 1621 $[M-H]^-$, 1489 $[M-132-H]^-$, 1459 $[M-162-H]^-$, 1343 $[M-132-146-H]^-$; Acid hydrolysis in the same manner as described above gave **9a**. Alkaline hydrolysis of **8** gave **9b**. Mild alkaline hydrolysis with 0.5 (w/v)% KOH*aq* also gave 9-oxo-16-β-[(D-xylopyranosyl)oxy]hexadecanoic acid (**13**).

4.20. Brine shrimp lethality assay

The bioassay was performed in the similar way described in reference (Meyer et al., 1982; Anderson et al., 1991; Solis et al., 1993; Carballo et al., 2002). Brine shrimp eggs (Japan Pet Drugs Co., Tokyo, Japan) were hatched in artificial sea water prepared with commercial salt mixture (Senju Phamaceutical Co., Osaka, Japan) and oxygenated with an aquarium pump. After 48 h incubation at 28 °C, nauplii were collected with pasteur pipette after attracting the organisms to one side of vessel with a light source. Ten shrimps were transferred to each sample vial, and artificial sea water was added to make 2.5 mL. Samples for testing were made up to 1 mg/mL in artificial sea water (2.5 mL) except for water insoluble samples which were dissolved in 50 μL DMSO prior to adding sea water. Sample solutions (2.5 mL) were added to each test vial (finally, total 5 mL). The vials were maintained under illumination. Survivors were counted after 2 and 24 h, and the percent deaths at each dose and control were determined. DMSO in this concentration did not affect this bioassay. The LC_{50} values were determined from 24 h counts using the probit analysis. The results are summarized in Table 3.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (B) (14380289 and 15390013) from the Japan Society for the Promotion of Science.

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