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Acetylated glucuronide triterpene bidesmosidic saponins from *Symplocos glomerata*

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Abstract

Nine new bidesmosidic 3-*O*-glucuronide oleanane triterpenoid saponins were isolated from the stem bark of *Symplocos glomerata* King along with two known saponins, salsoloside C and copteroside E, and two major lignans, (−)-pinoresinol and (−)-pinoresinol-4′-*O*-β-D-glucopyranoside. The structures of the new saponins were established using one- and two-dimensional NMR spectroscopy and mass spectrometry as, 3-*O*-[β-D-xylopyranosyl(1→4)-[2-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, 3-*O*-[β-D-xylopyranosyl(1→4)-[3-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, 3-*O*-[β-D-xylopyranosyl (1→4)-[2,3-*O*-diacetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, 3-*O*-[α-L-arabinopyranosyl(1→4)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, 3-*O*-[α-L-arabinopyranosyl (1→4)-[2-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, 3-*O*-{[β-D-xylopyranosyl (1→2)]-[β-D-xylopyranosyl (1→4)]-[3-*O*-acetyl]-β-D-glucuronopyranosyl}-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, 3-*O*-{[β-D-glucopyranosyl (1→2)]-[β-D-xylopyranosyl (1→4)]-[3-*O*-acetyl]-β-D-glucuronopyranosyl}-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, 3-*O*-{[β-D-glucopyranosyl (1→2)]-[α-L-arabinofuranosyl (1→4)]-[3-*O*-acetyl]-β-D-glucuronopyranosyl}-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, and 3β-*O*-[β-D-xylopyranosyl(1→4)-[2-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-morolic acid. The EtOH and EtOAc extracts of the stem bark showed no cytotoxic activity. At a concentration of 370 μg/ml, the saponin mixture showed haemolytic activity and caused 50% haemolysis of a 10% suspension of sheep erythrocytes.

Keywords: *Symplocos glomerata*; Symplocaceae; Triterpenoid saponins; Oleanolic acid; Morolic acid; Haemolysis

1. Introduction

The genus *Symplocos* of the Symplocaceae family consists of 300–500 species, and is most common in tropical and subtropical Asia, Malaysia and America (Hegnauer, 1973). Previously, flavonoids (Tschesche et al., 1980; Tanaka et al., 1980, 1982; Lin et al., 1996), iridoids (Iida et al., 1990), lignans (Ishida et al., 2001), steroids (Frotan et al., 1983) and triterpenoids (Ali et al., 1990) have been found as constituents of this genus. Previous phytochemical studies revealed the presence of triterpenoid saponins in *Symplocos spicata* (Higuchi et al., 1982). *Symplocos* species are used for antimicrobial activity (Khan et al., 2001), and for the treatment of diarrhoea, dysentery, eyes diseases, hemorrhagic gingivitic, menorrhagia and uterine disorders (Ali et al., 1990), female diseases (Ahmad et al., 2003), bowel complaints and ulcers (Dhaon et al., 1989).

The stem bark of *S. glomerata* King was collected in Pà Co, Mai Chàu (Hoà Binh) in Vietnam. The chemical constituents and biological activity of this plant have not been previously investigated. This species was selected in a screening program for potential cytotoxic compounds from plants growing in Vietnam. The ethanolic and ethyl acetate extracts of the stem bark were tested in vitro against KB cells, but showed no cytotoxic activity. Primary chemical studies on the ethanolic and ethyl

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acetate extracts of leaves, stem bark and fruits, showed the presence of saponins. The present paper reports the isolation and the structural elucidation of one bidesmosidic morolic acid saponin and eight bidesmosidic saponins possessing oleanolic acid as aglycone, along with two known saponins, salsoloside C and copteroside E.

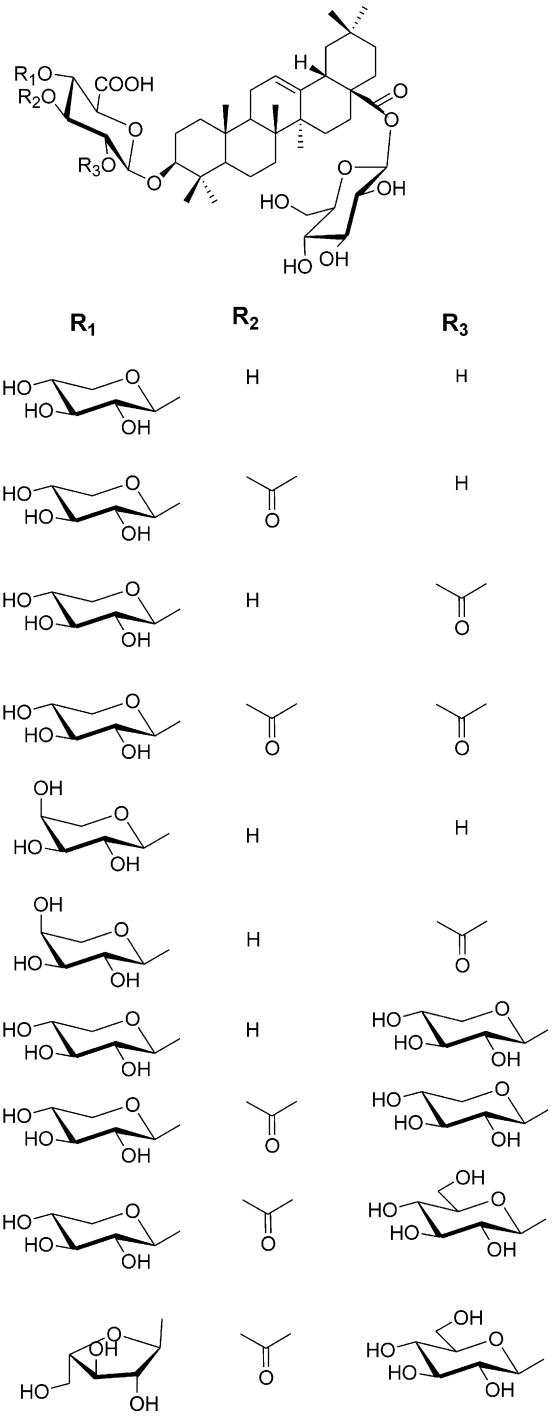
2. Results and discussion

The dried and powdered stem bark of *S. glomerata* was extracted with boiling 80% methanol. The aqueous methanolic extract was concentrated and precipitated into acetone to give saponin mixture A. The filtrate was concentrated and again precipitated in diethyl ether to give saponin mixture B. Each crude saponin precipitate was dialysed. Saponin mixture A and B showed the same contents by TLC analysis, and saponin mixture B, was chromatographed on a silica gel column. Purification by reversed phase C-18 column chromatography and semi-preparative HPLC over C-18 reversed phase afforded eleven saponins (**1**–**11**). In addition to saponins, two lignans, (−)-pinoresinol and (−)-pinoresinol-4′-*O*-β-D-glucopyranoside were also isolated from saponin mixture B. The spectroscopic data of these compounds were found to be identical with those reported in the literature (Cuenca and Catalan, 1991; Nishibe et al., 1984; Casabuono and Pomilio, 1994).

Acid hydrolysis of saponin mixture A gave only oleanolic acid, purified by silica gel column chromatography. The sugars were identified as D-glucose, D-glucuronic acid, L-arabinose, and D-xylose by TLC and their absolute configurations were determined by the measurement of optical rotation after separation by prep. TLC.

Oleanolic acid was identified as the aglycone of compounds **1**–**10** on the basis of its ^{1}H-NMR, ^{13}C-NMR, COSY, ROESY, HSQC, and HMBC spectra. The ^{13}C chemical shifts for C-28 between δ 176.2 and 176.6, and for C-3 between δ 89.7 and 91 suggested that sugars were connected to these points (Tables 1 and 2). Moreover, the ^{13}C-NMR shifts of the aglycone part of compounds **1**–**10** were in good agreement with those reported for bidesmosidic saponins of oleanolic acid (Tan et al., 1999).

Saponin **1**, molecular formula $C_{47}H_{74}O_{18}$ (ESI-MS^{-}: m/z 925 $[M-H]^{-}$) and saponin **7**, molecular formula $C_{52}H_{82}O_{22}$ (ESI-MS^{-}: m/z 1057 $[M-H]^{-}$), were identified as salsoloside C, 3-*O*-[β-D-xylopyranosyl-(1→4)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, and copteroside E, 3-*O*-{[β-D-xylopyranosyl (1→2)]-[β-D-xylopyranosyl (1→4)]-β-D-glucuronopyranosyl}-28-*O*-[β-D-glucopyranosyl]-oleanolic acid, respectively, on the basis of their spectral data. Compound **1** was previously isolated from *Salsola micranthera* (Annaev et al., 1983a), *Aralia cordata* (Kawai et al., 1989), *Melanthera scandens* (Penders and Delaude, 1994) and *Aralia continentalis* (Kim and Kang, 1998), while compound 7 was isolated from *Climacoptera transoxana* (Annaev et al., 1983b) and *Verbesina suncho* (Cerda-Garcia-Rojas et al., 2000).



The positive ESI-MS of compound **2** gave a quasi-molecular ion peak at m/z 991 $[M+Na]^{+}$ while, in the negative mode, a quasi-molecular ion was detected at m/z 967 $[M-H]^{-}$ indicating a M_r of 968 amu in agreement with a molecular formula of $C_{49}H_{76}O_{19}$ and

Table 1
^{1}H and ^{13}C NMR data of saponins **2**, **3**, **4**, **5** and **11** in CD_3OD

Genin	**2**		**3**		**4**		**5**		**11**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
3	3.22 *dd* (11.5, 4.5)	89.9	3.17 *dd* (11.7, 4.2)	89.9	3.20 *dd* (11.8, 3.7)	89.8	3.19 *dd* (11.4, 4.2)	89.8	3.16 *dd* (12, 5)	89.7
12 ax	5.27 *t* (3.7)	122.3	5.27 *t* (3.4)	122.3	5.27 *t* (3.6)	122.3	5.27 *t* (3.4)	122.3	1.6 *m*	25.7
12 eq	—		—		—		—		1.26 *m*	
13	—	143.4	—	143.4	—	143.4	—	143.4	2.29 *dd* (11.4, 2.5)	40.8
18	2.87 *dd* (13.7, 4)	41.1	2.87 *dd* (13.5, 3.4)	41.1	2.87 *dd* (13.6, 4.5)	41.1	2.87 *dd* (13.7, 4.4)	41.1	—	137
19 ax	1.73 *t* (13.5)	45.8	1.73 *t* (13.5)	45.8	1.73 *t* (13.5)	45.8	1.73 *t* (13.5)	45.8	5.16 *s*	132.5
19 eq	1.17 *t* (13.5)		1.17 *brd* (13.5)		1.17 *brd* (13.5)		1.17 *brd* (13.5)			
23	1.06 *s*	27	0.97 *s*	27	0.97 *s*	26.9	1.07 *s*	27	0.95 *s*	26.7
24	0.86 *s*	15.5	0.75 *s*	15.5	0.75 *s*	15.4	0.87 *s*	15.5	0.73 *s*	15.3
25	0.97 *s*	14.5	0.95 *s*	14.5	0.96 *s*	14.5	0.97 *s*	14.5	0.91 *s*	15.7
26	0.82 *s*	16.3	0.81 *s*	16.3	0.81 *s*	16.2	0.82 *s*	16.2	1.03 s	15.1
27	1.18 *s*	24.8	1.17 *s*	24.9	1.17 *s*	24.8	1.18 *s*	24.8	0.83 *s*	14.1
28	—	176.6	-	176.6	-	176.6	-	176.6	-	175.4
29	0.93 *s*	32	0.93 *s*	32	0.93 *s*	32	0.93 *s*	32	1.01 *s*	29.4
30	0.95 *s*	22.5	0.96 *s*	22.5	0.95 *s*	22.5	0.95 *s*	22.5	0.99 *s*	27.9
3-β-D-glucuronic acid										
1′	4.51 *d* (7.8)	105.2	4.63 *d* (8)	102.9	4.74 *d* (8.1)	102.6	4.44 *d* (7.8)	105.4	4.6 *d* (7.9)	102.9
2′	3.41 *dd* (9.4, 7.8)	71.9	4.83 *dd* (9.5, 8.1)	73.4	4.9 *t* (9.8)	71.7	3.31 *dd* (7.9, 9.3)	73.5	4.83 *t* (8.6)	73.4
3′	5.03 *t* (9.4)	75.1	3.71 *t* (9.4)	72.4	5.19 *t* (9.5)	73.1	3.54 *t* (9)	74.5	3.69 *t* (8.4)	72.5
4′	3.86 *t* (9.4)	77.2	3.82 *t* (9.5)	79.6	3.94 *brt* (9.6)	78	3.72 *t* (9)	79.7	3.82 *dd* (9.3, 8.4)	79.8
5′	4.03 *brd* (9.2)	73.4	4.03 *brd* (9.6)	73.4	4.05 *brs*	nd	3.98 *brd* (9.2)	73.5	nd	nd
6′	—	171.1	—	170	—	171.2	—	nd	—	170
28-β-D-glucose										
1″	5.4 *d* (8.1)	94.2	5.4 *d* (8.2)	94.2	5.4 *d* (8.1)	94.2	5.4 *d* (8.2)	94.2	5.53 *d* (8.2)	94.3
2″	3.34 *t* (8.6)	72.4	3.33 *m*	72.7	3.33 *dd* (9.1, 8.1)	72.4	3.34 *dd* (9, 8)	72.5	3.32 *t* (8.7)	72.6
3″	3.43 *t* (8.4)	76.8	3.43 *t* (9)	76.8	3.42 *t* (9.1)	76.8	3.41 *t* (9)	76.8	3.44 *t* (8.9)	77
4″	3.37 *m*	69.6	3.39 *t* (9.5)	69.6	3.37 *t* (9.1)	69.6	3.38 *m*	69.6	3.41 *m*	69.6
5″	3.37 *m*	77.3	3.37 *m*	77.2	3.36 *m*	77.2	3.37 *m*	77.2	3.40 *m*	77.3
6a″	3.7 *dd-like* (12.1, 4.5)	60.9	3.70 *dd* (12, 4.2)	60.9	3.7 *dd* (12.2, 4.6)	60.9	3.7 *dd-like* (12.1, 4.3)	60.9	3.72 *dd* (11.9, 3.9)	61
6b″	3.84 *dd* (12.1, 1.4)		3.83 *dd* (12, 1.4)		3.84 *dd* (12.2, 1.7)		3.84 *dd* (12.1, 1.6)		3.85 *dd* (11.9, 1.7)	
	4′-β-D-xylose		4′-β-D-xylose		4′-β-D-xylose		4′-α-L-arabinopyranose		4′-β-D-xylose	
1‴	4.28 *d* (7.4)	103.8	4.4 *d* (7.5)	103.4	4.31 *brd* (5.2)	104.1	4.35 *d* (6.7)	103.4	4.41 *brd* (7.5)	103.5
2‴	3.06 *dd* (9, 7.4)	73.4	3.22 *dd* (9, 7.5)	73.1	3.1 *brt* (8)	73.5	3.57 *dd* (9, 6.7)	70.9	3.23 *brdd* (9.5, 7.5)	73.2
3‴	3.27 *t* (9)	76.1	3.33 *t* 9.4	75.9	3.28 *dd* (9.5, 8.5)	76.1	3.54 *dd* (9.2, 3.3)	72.5	3.33 *t* (9.8)	76
4‴	3.45 *ddd* (10, 9, 5.3)	69.6	3.51 *ddd* (9.9, 8.9, 5.3)	69.4	3.45 *ddd* (9.9, 9, 5)	69.6	3.83 *brs*	68.1	3.52 *ddd* (11.4, 10.4, 5.3)	69.4
5ax‴	3.17 *dd* (11.5, 10)	65.6	3.26 *dd* (11.5, 10)	65.5	3.17 *t* (11)	65.7	3.62 *dd* (12.5, 1.3)	65.9	3.26 *dd* (11.4, 10.4)	65.5
5eq‴	3.83 *dd* (11.5, 5.2)		3.93 *dd* (5.3, 11.5)		3.83 *dd* (11.4, 5)		3.95 *dd* (12.5, 2.8)		3.93 *dd* (11.7, 5.3)	
2′-acetate										
CH_3			2.09 *s*	19.7	2.02 *s*	19.6			2.08 *s*	19.7
C=O				170		169.6				170
3′-acetate										
CH_3	2.08 *s*	19.8			2.01 *s*	19.4				
C=O		171.1				170.6				

nd = not determinated.

suggesting the presence of an acetyl group compared to saponin **1**. The ^{1}H and ^{13}C NMR spectra of **2** showed signals for a single acetyl ester at δ_H 2.08 and δ_C 19.8 (CH_3) and 171.1 (CO). The MS^2 experiment of the $[M+Na]^+$ ion, gave positive fragments at m/z 859 $[M+Na-132]^+$, 829 $[M+Na-162]^+$, 697 $[M+Na-132-162]^+$ and 623 $[M+Na-132-176-CH_3CO_2H]^+$, attributed to the loss of a terminal pentose, a terminal hexose, both terminal sugars and a diglycoside chain consisting of a pentose and an acetylated uronic acid, respectively. These results suggested that the saponin **2** contained three sugars units, one of which is an acetylated uronic acid.

Table 2
^{1}H and ^{13}C NMR data of saponins **6**, **8**, **9** and **10** in CD_3OD

Genin	**6**		**8**		**9**[a]		**10**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
3	3.14 *dd* (11.7, 4.2)	89.8	3.17 *dd* (11.7, 4.2)	90.5	3.11 *dd* (12.3, 4.3)	89.7	3.24 *dd* (12, 4.4)	91
12	5.23 *t* (3.4)	122.3	5.27 *t* (3.6)	122.3	5.38 *t* (3.5)	123.6	5.27 *t* (4.6)	122.3
13	—	143.4	—	143.4	—	143.8	—	143.4
18	2.84 *dd* (13.4, 3.8)	41.1	2.87 *dd* (13.6, 4.1)	41.1	3.6 *dd* (13.6, 4)	41.4	2.88 *dd* (13.5, 4)	41.1
23	0.94 *s*	26.9	1.06 *s*	26.8	1.09 *s*	27.5	1.07 *s*	26.8
24	0.72 *s*	15.4	0.86 *s*	15.1	1.04 *s*	16.3	0.88 *s*	15.4
25	0.93 *s*	14.5	0.97 *s*	14.6	0.74 *s*	15.1	0.98 *s*	14.5
26	0.78 *s*	16.2	0.81 *s*	16.3	1.04 *s*	17.1	0.82 *s*	16.3
27	1.14 *s*	24.8	1.18 *s*	24.9	1.23 *s*	25.8	1.18 *s*	24.9
28	—	176.6	—	176.6	—	176.2	—	176.6
29	0.9 *s*	32	0.93 *s*	32.1	0.88 *s*	32.8	0.93 *s*	32
30	0.92 *s*	22.5	0.96 *s*	22.5	0.85 *s*	23.3	0.95 *s*	22.5
3-β-D-glucuronic acid								
1′	4.61 *d* (8)	102.9	4.65 *d* (7.3)	103.8	4.85 *d* (7.5)	104.7	4.69 *d* (7.4)	103.7
2′	4.8 *dd* (9.4, 8.1)	73.3	3.71 *dd* (9, 7.3)	77.2	4.42 *dd* (9.2, 7.3)	77.2	3.86 *dd* (9.2, 7.6)	75.9
3′	3.7 *t* (9.3)	72.4	5.2 *t* (9)	74.5	5.86 *t* (9)	74.9	5.27 *t* (9.2)	74.6
4′	3.78 *t* (9.3)	79.7	3.94 *t* (9.3)	77.1	4.54 *m*	78.9	3.9 *t* (9.2)	77.2
5′	4.01 *brd* (9.2)	na	4.09 *d* (9.3)	73.6	4.55 *m*	75.7	4.08 *brd* (9)	nd
6′	—	170	—	171.1	—	170.1	—	171.1
28-β-D-glucose								
1″	5.37 *d* (8.2)	94.2	5.4 *d* (8.2)	94.2	6.31 *d* (8.1)	95.5	5.41 *d* (8.1)	94.2
2″	3.30 *t* (8.4)	72.4	3.33 *t* (8.4)	72.4	4.21 *t* (8.8)	73.8	3.34 *t* (8.4)	72.5
3″	3.40 *t* (8.8)	76.8	3.42 *t* (9.2)	76.8	4.29 *t* (8.7)	78.6	3.44 *t* (8.9)	76.8
4″	3.36 *t* (8.8)	69.6	3.38 *m*	69.7	4.36 *t* (8.8)	70.7	3.38 *m*	69.6
5″	3.33 *m*	77.2	3.37 *m*	77.2	4.03 *m*	79.1	3.37 *m*	77.2
6a″	3.67 *dd* (11.9, 4.3)	60.9	3.7 *dd* (12.2, 4.1)	60.9	4.4 *dd* (12.1, 4.3)	61.9	3.7 *dd* (11.9, 5.3)	60.9
6b″	3.8 *dd* (11.9, 1.4)	60.9	3.84 *dd* (12.2, 1.9)		4.47 *dd* (12.1, 2.3)		3.84 *dd* (11.9, 1.9)	
			2′-β-D-xylose		2′-β-D-glucose		2′-β-D-glucose	
1‴			4.37 *d* (7.6)	104	5.13 *d* (7.6)	104.3	4.46 *d* (7.7)	102.8
2‴			3.13 *dd* (9.1, 7.4)	73.6	3.96 *dd* (8.6, 7.6)	75.1	3.14 *dd* (9.2, 7.7)	73.7
3‴			3.28 *t* (9)	76.4	4.22 *t* (9)	78.1	3.33 *t* (9.2)	76.4
4‴			3.44 *m*	69.6	4.12 *t* (8.9)	72	3.11 *t* (9.3)	70.7
5ax‴			3.13 *t* (11)	65.6	4.03 *m*	78.2	3.27 *m*	77.1
5eq‴/6a‴			3.82 *dd* (11.4, 5.4)		4.37 *dd* (11.5, 5.5)	63.2	3.58 *dd* (12, 7.5)	62.1
6b‴					4.62 *dd* (11.5, 2.5)		3.86 *dd* (12, 1.9)	
	4′-α-L-arabinopyranose		4′-β-D-xylose		4′-β-D-xylose		4′-α-L-arabinofuranose	
1⁗	4.34 *d* (6.6)	103.3	4.29 *d* (7.5)	103.8	5.03 *d* (7.5)	105.8	4.96 *brs*	109.4
2⁗	3.53 *brt* (9)	70.9	3.08 *dd* (9, 7.5)	73.4	3.92 *dd* (8.3, 7.5)	74.7	3.9 *m*	81.7
3⁗	3.51 *dd* (9.1, 3.4)	72.5	3.27 *t* (9)	76.1	4.07 *t* (8.8)	77.9	3.83 *dd* (5.6, 3.4)	76.8
4⁗	3.79 *brs*	68	3.44 *m*	69.6	4.15 *ddd* (10.9, 8.8, 5.6)	70.6	3.9 *m*	85
5ax⁗	3.58 *dd* (12.6, 1.3)	65.8	3.17 *dd* (13, 11.2)	65.4	3.67 *t* (10.8)	67.1	3.63 *dd* (12, 4.8)	61.4
5eq⁗	3.91 *dd* (12.6, 2.9)	65.8	3.84 *dd* (11.4, 5.1)	65.6	4.31 *dd* (10.8, 5)		3.71 *dd* (12, 3.6)	
	2′-acetate		3′-acetate		3′-acetate		3′-acetate	
CH_3	2.06 *s*	19.7	2.09 *s*	20.1	2.2 *s*	21.3	2.18 *s*	20.4
C=O		170		171.1		170.1		171.1

na: not assigned; signal was too weak.
[a] Measured in C_5D_5N.

The three anomeric carbons were detected at 94.2, 103.8 and 105.2 in the ^{13}C-NMR spectrum, attached to doublets at δ 5.4, 4.28 and 4.51, respectively in the HSQC experiment. Complete assignments of each glycosidic proton system were achieved by analysis of COSY and TOCSY experiments. The units with anomeric protons at δ 5.4 ($J=8.1$) and 4.28 ($J=7.4$) corresponded to an hexose and a pentose with their methylene carbons at δ 60.9 and 65.6, respectively, and were identified as β-D-glucose and β-D-xylose (Table 1). The third glycosidic unit with anomeric proton at δ 4.51 ($J=7.8$) was identified as a β-D-glucuronic acid which possessed a deshielded H-3′ at δ 5.03 indicating the position of acetylation. The deshielding of C-4′ (δ 77.2) of glucuronic acid suggested the point of linkage of the xylose (Table 1).

Sequencing of the glycosidic chains in saponin **2** was achieved by analysis of HMBC and ROESY experiments. The HMBC spectrum showed cross peaks between C-28 (δ 176.6) of oleanolic acid and H-1‴ of the glucose ester (δ 5.40), between C-3 (δ 89.9) of oleanolic acid and H-1′ of esterified glucuronic acid (δ 4.51), and between C-4′ (δ 77.2) of this glucuronic acid and H-1⁗ of the terminal xylose unit (δ 4.28). Thus, saponin **2** is 3-*O*-[β-D-xylopyranosyl-(1→4)-[3-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid or 3′-*O*-acetylsalsoloside C.

The ESI-MS of compound **3** gave quasi-molecular ion peaks at *m/z* 991 $[M+Na]^+$ (positive mode) and *m/z* 967 $[M-H]^-$ (negative mode) indicating a M_r of 968 amu in agreement with a molecular formula of $C_{49}H_{76}O_{19}$ isomeric with saponin **2**. The MS^2 experiment of the $[M+Na]^+$ ion, gave the same positive fragments described for **2**. The MS^4 experiment of the ion at *m/z* 641 gave an ion fragment at *m/z* 479 $[genin+Na]^+$ due to the loss of the glycosidic chains. The presence of three sugar residues was confirmed from the observation of signals for three anomeric positions at δ_C 94.2, 102.9 and 103.4 and δ_H 5.4 ($J=8.2$ Hz), 4.63 ($J=8$ Hz) and 4.4 ($J=7.5$ Hz). Inspection of the spectroscopic data indicated that saponin **3** had the same glycosidic part as saponin **2**: a β-D-glucopyranosyl ester (δ_H 5.4), a terminal β-D-xylopyranosyl (δ_H 4.40) and a 4-substituted β-D-glucuronopyranosyl (δ_H 4.63). These results suggested that compound **3** is a regioisomer of **2** with the acetate attached to C-2 rather than to C-3 (see Table 1). Thus, saponin **3** is 3-*O*-[β-D-xylopyranosyl-(1→4)-[2-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid or 2′-*O*-acetylsalsoloside C.

The negative ESI-MS of saponin **4** gave a quasi-molecular ion at *m/z* 1009 $[M-H]^-$, indicating a molecular formula of $C_{51}H_{78}O_{20}$ (M_r 1010). The MS/MS fragmentation indicated a supplementary acetyl unit compared to saponins **2** and **3**. The ^{1}H and ^{13}C NMR data of compound **4** were closely comparable to those of **2** and **3** except for the signals of glucuronic acid and the presence of two acetate methyls (δ_H 2.01 and 2.02) (Table 1). The β-D-glucuronic acid possessed two deshielded protons H-2′ and H-3′ at δ 4.9 and 5.19, respectively, indicating the position of the acetates. Thus saponin **4** is 3-*O*-[β-D-xylopyranosyl-(1→4)-[2,3-*O*-diacetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid or 2′,3′-*O*-diacetylsalsoloside C.

The positive ESI-MS of compound **5** gave a quasi-molecular ion peak at *m/z* 971 $[M+2Na-H]^+$ while, in the negative mode, a quasi-molecular ion was detected at *m/z* 925 $[M-H]^-$ indicating a M_r of 926 amu in agreement with a molecular formula of $C_{47}H_{74}O_{18}$. The MS^2 experiment of the $[M+2Na-H]^+$ ion, gave positive fragments at *m/z* 839 $[M+Na-H-132]^+$ and 809 $[M+2Na-H-162]^+$, attributed to the loss of a terminal pentose and a terminal hexose. The MS^3 fragmentation of the ion at *m/z* 809 gave an ion fragment at *m/z* 677 $[M+2Na-H-162-132]^+$ corresponding to the loss of both hexose and pentose. The MS^4 fragmentation of the ion at *m/z* 677 gave a product ion at *m/z* 501 $[genin+2Na-H]^+$ after the loss of an uronic acid unit. This analysis suggested that saponin **5** contained three sugars units and was isomeric of saponin **1**.

Three anomeric carbons were detected at δ 94.2, 103.4 and 105.4 in the ^{13}C-NMR spectrum, attached to doublets at δ 5.4, 4.35 and 4.44, respectively in HSQC experiment (Table 1). Analysis of 2D experiments (COSY, TOCSY and HSQC) revealed a β-D-glucopyranosyl ester (δ_H 5.4, δ 94.2) attached to C-28 of the genin, a 4-substituted β-D-glucuronic acid, and of a terminal α-L-arabinose. The cross peaks observed in the HMBC experiment between C-28 (δ 176.6) of oleanolic acid and H-1″ of glucose (δ 5.4), between C-3 (δ 89.8) of the genin and H-1′ of glucuronic acid (δ 4.44) and between C-4′ (δ 79.7) of this glucuronic acid and H-1⁗ (δ 4.35) of the α-L-arabinose unit, showed that compound **5** is 3-*O*-[α-L-arabinopyranosyl-(1→4)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid.

Saponin **6** exhibited an intense quasi-molecular ion peak at *m/z* 1013 $[M+2Na-H]^+$ while, in the negative mode, a quasi-molecular ion was detected at *m/z* 967 $[M-H]^-$ indicating a M_r of 968 ($C_{49}H_{76}O_{19}$). This result suggested an additional acetyl group compared with saponin **5**. The MS^2 experiment of the $[M+2Na-H]^+$ ion gave positive fragments at *m/z* 953 $[M+2Na-H-60]^+$, 881 $[M+2Na-H-132]^+$ and 851 $[M+2Na-H-162]^+$, attributed to the loss of acetic acid, a terminal pentose and a terminal hexose, respectively. The 2D experiments (COSY, TOCSY and HSQC) permitted the assignment of all the ^{1}H and ^{13}C signals of the sugars, identified as a terminal β-D-glucopyranosyl (δ_H 5.37, δ_C 94.2), a terminal α-L-arabinopyranosyl (δ_H 4.34, δ_C 103.3) and a 4-substituted β-D-glucuronic acid (δ_H 4.61, δ_C 102.9). Relative to **5**, the

identical value observed for C-4′ of β-D-glucuronic acid at δ 79.7 ppm confirmed that the α-L-arabinopyranosyl was attached at this position and the deshielded value of H-2′ (δ 4.8) suggested that the glucuronic acid was acetylated at position 2. HMBC correlations showed that the sugars were attached in the same way in both saponins **5** and **6**. Consequently, the structure of saponin **6** is 3-*O*-[α-L-arabinopyranosyl-(1→4)-[2-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-oleanolic acid.

The ESI-MS of saponin **8** gave a quasi-molecular ion peaks at *m/z* 1145 $[M+2Na-H]^+$ (positive mode) and at *m/z* 1099 $[M-H]^-$ (negative mode) indicating a M_r of 1100 amu ($C_{54}H_{84}O_{23}$), and suggesting a supplementary pentose unit relative to saponin **2**. Saponin **8** was shown to contain four glycosidic residues and one acetate group from the HSQC spectrum. The anomeric proton signals at δ 5.4, 4.65, 4.37 and 4.29 had correlations with carbon signals at δ 94.2, 103.8, 104 and 103.8, respectively. Analysis of 2D experiments permitted the assignment of the four sugars: a β-D-glucopyranosyl ester (δ_H 5.4, δ 94.2) attached to C-28 of oleanolic acid, two terminal β-D-xyloses and one 2,4-disubstituted β-D-glucuronic acid. The cross peaks observed in the ROESY experiment between H-3 (δ 3.17) of the genin and H-1′ of glucuronic acid (δ 4.65) and between H-2′ (δ 3.71) and H-4′ (δ 3.94) of this glucuronic acid and H-1‴ of the first β-D-xylose (δ 4.37) and H-1⁗ of the second β-D-xylose (δ 4.29), respectively, showed that the saponin **8** was an acetylated copteroside E **7**. The acetyl group was readily assigned to C-3 of the glucuronic acid (see Table 2). Thus, saponin **8** is 3-*O*-{[β-D-xylopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→4)]-[3-*O*-acetyl]-β-D-glucuronopyranosyl}-28-*O*-[β-D-glucopyranosyl]-oleanolic acid. This saponin corresponded to the 3′-*O*-acetyl derivative of the known copteroside E (Annaev et al., 1983b).

The negative ESI-MS of saponin **9** gave a quasi-molecular ion peak at *m/z* 1129 $[M-H]^-$ indicating a M_r of 1130 amu ($C_{55}H_{86}O_{24}$), and suggesting a supplementary hexose unit compared to saponin **2**. The analysis of MS/MS fragmentation showed that this supplementary hexose unit replaced the pentose unit found in saponin **8**.

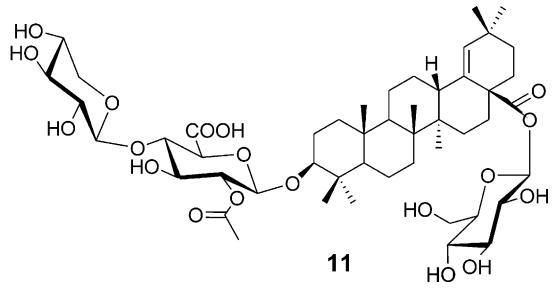
Analysis of 2D experiments (COSY, TOCSY, HSQC and HMBC) allowed the identification of the glycosidic part of saponin **8**: a β-D-glucopyranosyl ester (δ_H 5.4, δ 94.2) attached to C-28 of oleanolic acid, a terminal β-D-xylose, and a 2,4-disubstituted β-D-glucuronic acid acetylated in position 3 (deshielded H-3′ at δ 5.86). The anomeric proton of the fourth sugar appeared at δ 5.13 ($J=7.6$ Hz). This was identified as a second β-D-glucopyranosyl unit with its hydroxymethyl group at δ_C 63.2 (Table 2). The HMBC experiment showed correlations between C-3 (δ 89.7) of the genin and H-1′ of glucuronic acid (δ 4.85) and between C-2′ (δ 77.2) and C-4′ (δ 78.9) of glucuronic acid and H-1‴ of the second β-D-glucose (δ 5.13) and H-1⁗ of β-D-xylose (δ 5.03), respectively. Thus, compound **9** is 3-*O*-{[β-D-glucopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→4)]-[3-*O*-acetyl]-β-D-glucuronopyranosyl}-28-*O*-[β-D-glucopyranosyl]-oleanolic acid. This saponin corresponded to the 3′-*O*-acetyl derivative of the known salsoloside E (Annaev et al., 1984).

The positive ESI-MS of compound **10** gave a quasi-molecular ion peak at *m/z* 1153 $[M+Na]^+$ while, in the negative mode, a quasi-molecular ion was detected at *m/z* 1129 $[M-H]^-$ indicating a M_r of 1130 amu ($C_{55}H_{86}O_{24}$) as for saponin **9**. The MS^2 experiment of the $[M+Na]^+$ ion, gave positive fragments at *m/z* 1021 $[M+Na-132]^+$ and 991 $[M+Na-162]^+$, attributed to the loss of a terminal pentose and the loss of a terminal hexose, and at *m/z* 535 corresponding to the loss of the triglycosidic chain at position 3 of oleanolic acid. Comparison of the NMR spectra of compound **10** and saponin **9** indicated that **10** possessed an α-L-arabinofuranose instead of a β-D-xylose (Table 2). This α-L-arabinofuranose was characterized by the chemical shifts of its anomeric proton (δ_H 4.96) and carbon (δ_C 109.4).

Sequencing of the sugar chains in saponin **10** was achieved by analysis of HMBC experiments, which showed cross peaks between C-28 (δ 176.6) of oleanolic acid and H-1″ of the glucose ester (δ 5.41), and between H-1′ (δ 4.69), H-2′ (δ 3.86), H-3′ (δ 5.27) and H-4′ (δ 3.9) of the trisubstituted glucuronic acid with C-3 (δ 91) of oleanolic acid, C-1‴ (δ 102.8) of glucose, the carbonyl (δ 171.1) of the acetyl unit and C-1⁗ (δ 109.4) of arabinose, respectively. Thus, saponin **10** was concluded to be 3-*O*-{[β-D-glucopyranosyl (1→2)] [α-L-arabinofuranosyl-(1→4)]-[3-*O*-acetyl]-β-D-glucuronopyranosyl}-28-*O*-[β-D-glucopyranosyl]-oleanolic acid.

The negative ESI-MS of compound **11** gave a quasi-molecular ion at *m/z* 967 $[M-H]^-$ indicating a molecular formula of $C_{49}H_{76}O_{19}$ (M_r 968) isomeric with saponins **2**, **3** and **6**. The MS^2 experiment of the positive $[M+2Na-H]^+$ molecular ion at *m/z* 1013, gave positive fragments at *m/z* 953 $[M+2Na-H-60]^+$ and 851 $[M+2Na-H-162]^+$, attributed to the loss of acetic acid and a terminal hexose. The MS^3 experiment of the $[M+2Na-H-162]^+$ ion, gave positive fragments at *m/z* 791 $[M+2Na-H-162-60]^+$ and 719 $[M+2Na-H-162-132]^+$, attributed to the loss of acetic acid, and a terminal pentose. The analysis of 1D- and 2D-NMR experiments (^1H, ^{13}C, COSY, TOCSY, HSQC and HMBC) of saponin **11** demonstrated that the glycosidic chains of compounds **3** and **11** were identical (Table 1). The ^1H NMR spectrum of **11** showed a trisubstituted olefinic proton at δ 5.16 (H-19) correlated in the HMBC spectrum with C-13 (δ 40.8), C-18 (δ 137), C-20 (δ 31.5), C-29 (δ 29.3) and C-30 (δ 27.9), indicating that the double bond is between C-18 and C-19 instead of C-12 and C13. The ^{13}C NMR chemical shifts of the signals

due to the aglycone part of saponin **11** were in close agreement with those reported for morolic acid (Yoshikawa et al., 1997). In the ROESY spectrum, the Overhauser interaction of the axial proton H-13 at δ 2.29 (*dd*, $J = 11.4$, 2.5) with the methyl group at δ 1.03 (CH_3-26), assigned from HMBC correlations, indicated that H-13 is in a β-axial position. Thus, the structure of saponin **11** is 3β-*O*-[β-D-xylopyranosyl(1→4)-[2-*O*-acetyl]-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl]-morolic acid.



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The haemolytic activity of the purified saponin mixture and of pure saponins **1**, **2**, **3**, **8**, **9** and **10** was assessed on sheep erythrocytes using the method previously described (Voutquenne et al., 2002). The isolated quantities of saponins **4**, **5**, **6**, **7** and **11** were not sufficient to allow evaluation of biological activity. Saponin mixture B was active and 50% haemolysis was obtained at 370 μg/ml. Amongst the tested compounds, only saponin **2** exhibited haemolytic activity with an HD_{50} at 216 μM (209.1 μg/ml).

3. Experimental

3.1. General experimental procedures

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 (^1H at 500 MHz and ^{13}C at 125 MHz). 2D experiments were performed using standard Bruker microprograms. ESI-MS and MS-MS experiments were recorded on a Finningan LCQ deca quadripole ion trap mass spectrometer (Finnigan MAT, San Jose, USA). The samples were introduced by direct infusion in a solution of MeOH at a rate of 5 μl min^{-1}. Optical rotations were determined in MeOH or pyridine with a Perkin-Elmer 241 polarimeter. TLC was performed on pre-coated silica gel 60 F_{254} Merck and detection was achieved by H_2SO_4 50%. CC were carried out on Kieselgel 60 (63–200 mesh) Merck or LiChroprep RP-18 (40–63 μm) Merck. HPLC was performed on a DIONEX apparatus equipped with an ASI-100 autosampler, a P580 pump, a diode array detector UVD 340S and a Chromeleon® software. C18 reversed phase column (201SP, 250×10 mm, 5 μm, Dionex, Jouyensosap, France) was used for semi-preparative HPLC with a binary gradient eluent (solvent A, H_2O (pH 2.4 with TFA); solvent B, MeCN) and a flow rate 5 ml/min; the chromatogram was monitored at 205 nm.

3.2. Plant material

Stem bark of *S. glomerata* King. was collected in PàCo, Mai chau (Hoa Binh), Vietnam in April 1996. The specimen of the plant VN076 was deposited in the herbarium of CNST of Hanoi (Vietnam). The identification was confirmed by Dr. A. Hladik, Botanist at MNHN Paris.

3.3. Extraction and isolation

Dried and finely powdered stem bark of the plant (1 kg) was macerated in 20% aq. MeOH (15 l) for 2 h and then boiled for 3 h. The hydromethanolic extract was filtered, and then evaporated to yield a residue (125 g). This residue was suspended in MeOH (300 ml), then poured dropwise into 1.5 l of Me_2CO. The resulting ppt. was filtered and dried over KOH in vacuo (42.8 g). The filtrate was evaporated, dried, suspended in MeOH and precipitated into Et_2O and the ppt. was filtered and dried over KOH in vacuo (62.5 g). The two dried ppt. (62.5 g) were dissolved in H_2O and dialysed against H_2O in seamless cellulose tubing with agitation during 48 h. The contents of the tubes were freeze-dried to afford 10.5 g of saponin mixt. A (from ppt. Me_2CO) and 10.7 g of saponin mixt. B (from ppt. Et_2O).

An aliquot of saponin mixt. B (10 g) was fractionated on silica gel CC, using a gradient of $CHCl_3$/MeOH (100/0 to 0/100). Frs. [9–16] = I eluted with $CHCl_3$/MeOH (98/2) was purified by preparative TLC in $CHCl_3$/MeOH (9/1) to give (−)-pinoresinol (20 mg). Frs [46-62] = II eluted with $CHCl_3$/MeOH (95/5) yielded (−)-pinoresinol-4′-*O*-β-D-glucopyranoside (1.5 g). Frs [131–134] = III eluted with $CHCl_3$/MeOH (7/3) and containing saponins **1–4** and **11** was purified on a reversed-phase RP18 CC using a gradient of MeOH/H_2O (6/4 to 8/2): Frs. [6–18] eluted with MeOH/H_2O (6/4) was purified by semi-prep. HPLC with the elution prog.: 30–50% B (0–20 min) and 50% B (20–25 min) to give saponin 1 (rt = 14 min, 11 mg) and saponin **2** (rt = 16 min, 10 mg); Frs [22–46] eluted with MeOH/H_2O (6/4) was purified by semi-prep. HPLC 45% B (0–15 min) to give saponins **3** (rt = 8.78 min, 14 mg) and 4 (rt = 10 min, 5 mg); saponin **11** (rt = 9.32 min, 5 mg) was purified from Frs. [53–54] eluted with MeOH/H_2O (7/3) by semi-prep. HPLC using the same prog. as above. Frs. [135–139] = IV eluted with $CHCl_3$/MeOH (7/3) and containing saponins **8**, **9** and **10** was chromatographed on a reversed-phase RP18 CC using a gradient of MeOH/H_2O (5/5 to 7/3): Frs. [35–57] eluted with MeOH/H_2O (6/4) was purified by semi-prep. HPLC, using a linear gradient 30–35% B in 40 min to yield saponins **9** (rt = 10.90 min, 23 mg), 10 (rt = 12.58 min, 14

mg) and 8 (rt = 13.52 min, 18 mg). Frs. [145–157] = V eluted with $CHCl_3$/MeOH (7/3) was purified on a RP18 CC using a gradient of MeOH/H_2O (5/5 to 7/3) to give saponins **5**, **6** and **7**: Frs. [42–45] eluted with MeOH/H_2O (6/4) was purified by semi-prep. HPLC, using a linear gradient 30–50% B in 30 min to give saponins **5** (rt = 8.33 min, 6 mg) and **7** (rt = 9.61 min, 8 mg). Saponin **6** (rt = 14.19 min, 9 mg) eluted with MeOH/H_2O (7/3) from Frs. [58–61] was finally purified by semi-prep. HPLC using the same prog. as above.

3.4. Saponin 2

White powder $[\alpha]_D^{21}$ +9 (MeOH, *c* 0.5); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 1; ^{13}C NMR (CD_3OD) of the aglycone δ 14.5 (C-25), 15.5 (C-24), 16.2 (C-26), 17.8 (C-6), 22.5 (C-30), 22.5 (C-16), 23.1 (C-11), 24.8 (C-27), 25.5 (C-2), 27 (C-23), 27.4 (C-15), 30.1 (C-20), 31.7 (C-22), 32 (C-29), 32.5 (C-7), 33.4 (C-21), 36.4 (C-10), 38.3 (C-1), 38.7 (C-4), 39.2 (C-8), 41.1 (C-18), 41.6 (C-14), 45.8 (C-19), 46.5 (C-17), 47.7 (C-9), 55.5 (C-5), 89.9 (C-3), 122.3 (C-12), 143.4 (C-13), 176.6 (C-28); ESI-MS (negative ion mode) *m/z* 967 $[M-H]^-$; ESI-MS-MS: MS^2 (967) *m/z* 817 $[(M-H)-132-H_2O]^-$, 805 $[(M-H)-162]^-$, 757 $[(M-H)-132-H_2O-CH_3COOH]^-$, 611 $[(M-H)-132-162-CH_3CO-H_2O]^-$, MS^3 (757) *m/z* 595 $[(M-H)-132-H_2O-CH_3CO_2H-162]^-$; ESI-MS (positive ion mode) *m/z* 991 $[M+Na]^+$; ESI-MS-MS: MS^2 (991) *m/z* 859 $[(M+Na)-132]^+$, 829 $[(M+Na)-162]^+$, 697 $[(M+Na)-132-162]^+$, 641 $[(M+Na)-132-176-CH_3CO_2H]^+$, 623 $[(M+Na)-132-176-CH_3CO_2H-H_2O]^+$, MS^4 (623) *m/z* 461 $[(genin+Na)\text{-}H_2O]^+$.

3.5. Saponin 3

White powder $[\alpha]_D^{21}$ 0 (MeOH, *c* 0.46); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 1, ^3C NMR chemical shift values of aglycone are identical to those described for saponin **2**; ESI-MS (negative ion mode) *m/z* 967 $[M-H]^-$; ESI-MS-MS: MS^2 (967) *m/z* 817 $[(M-H)-132-H_2O]^-$, 655 $[(M-H)-132-162-H_2O]^-$, MS^3 (817) *m/z* 655 $[(M-H)-132-162-H_2O]^-$; ESI-MS (positive ion mode) *m/z* 991 $[M+Na]^+$; ESI-MS-MS: MS^2 (991) *m/z* 859 $[(M+Na)-132]^+$, 829 $[(M+Na)-162]^+$, 641 $[(M+Na)-132-176-CH_3CO_2H]^+$, MS^3 (859) *m/z* 623 $[(M+Na)-132-176-CH_3CO_2H-H_2O]^+$, MS^3 (641) *m/z* 479 $[genin+Na]^+$.

3.6. Saponin 4

White powder $[\alpha]_D^{21}$ +1.5 (MeOH, *c* 0.2); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 1, ^{13}C NMR chemical shift values of the aglycone are identical to those described for saponin **2** to ±0.2 ppm; ESI-MS (negative ion mode) *m/z* 1009 $[M-H]^-$; ESI-MS-MS: MS^2 (1009) *m/z* 859 $[(M-H)-132-H_2O]^-$, 799 $[(M-H)-132-H_2O-CH_3CO_2H]^-$, 653 $[(M-H)-132-162-CO_2-H_2O]^-$, 637 $[(M-H)-132-162-H_2O-CH_3CO_2H]^-$, 593 $[(M-H)-132-162-CO_2-H_2O-CH_3CO_2H]^-$, 577 $[(M-H)-132-162-H_2O-2CH_3CO_2H]^-$; ESI-MS (positive ion mode) *m/z* 1055 $[M+2Na-H]^+$, 1033 $[M+Na]^+$; ESI-MS-MS: MS^2 (1055) *m/z* 893 $[(M+2Na-H)-162]^+$, MS^3 (893) *m/z* 875 $[(M+2Na-H)-162-H_2O]^+$, 833 $[(M+2Na-H)-162-CH_3CO_2H]^+$, 639 $[(M+2Na-H)-132-162-CH_3CO_2H-CO_2]^+$.

3.7. Saponin 5

White powder $[\alpha]_D^{21}$ +4.7 (MeOH, *c* 0.38); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 1, ^{13}C NMR chemical shift values of the aglycone are identical to those described for saponin **2**; ESI-MS (negative ion mode) *m/z* 925 $[M-H]^-$; ESI-MS-MS: MS^2 (925) *m/z* 775 $[(M-H)-132-H_2O]^-$, 613 $[(M-H)-132-162-H_2O]^-$, MS^4 (613) *m/z* 455 $[genin-H]^-$; ESI-MS (positive ion mode) *m/z* 971 $[M+2Na-H]^+$; ESI-MS-MS : MS^2 (971) *m/z* 839 $[(M+2Na-H)-132]^+$, 809 $[(M+2Na-H)-162]^+$,MS^3 (809) *m/z* 791 $[(M+2Na-H)-162-H_2O]^+$, 677 $[(M+2Na-H)-162-132]^+$, MS^4 (677) *m/z* 659 $[(M+2Na-H)-162-132-H_2O]^+$, 501 $[genin+2Na-H]^+$.

3.8. Saponin 6

White powder $[\alpha]_D^{21}$ +8 (MeOH, *c* 0.55); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 2, ^{13}C NMR chemical shift values of the aglycone are identical to those described for saponin **2** to ±0.3 ppm; ESI-MS (negative ion mode) *m/z* 967 $[M-H]^-$; ESI-MS-MS: MS^2 (967) *m/z* 817 $[(M-H)-132-H_2O]^-$, 655 $[(M-H)-132-162-H_2O]^-$, 611 $[(M-H)-132-162-CO_2]^-$, MS^4 (655) *m/z* 595 $[(M-H)-132-H_2O-CH_3CO_2H]^-$, 455 $[genin\text{-}H]^-$; ESI-MS (positive ion mode) *m/z* 1013 $[M+2Na-H]^+$; ESI-MS-MS: MS^2 (1013) *m/z* 953 $[(M+2Na-H)-CH_3CO_2H]^+$, 881 $[(M+2Na-H)-132]^+$ 851 $[(M+2Na-H)-162]^+$, MS^3 (851) *m/z* 791 $[(M+2Na-H)-162-CH_3CO_2H]^+$, 719 $[(M+2Na-H)-132-162]^+$, MS^4 (791) *m/z* 773 $[(M+2Na-H)-162-CH_3CO_2H-H_2O]^+$, 659 $[(M+2Na-H)-162-132-CH_3CO_2H]^+$, 501 $[genin+2Na-H]^+$.

3.9. Saponin 8

White powder $[\alpha]_D^{21}$ +1.4 (pyridine, *c* 0.416); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 2, ^{13}C NMR chemical shift values of the aglycone are identical to those described for saponin **2** to ±0.3 ppm; ESI-MS (negative ion mode) *m/z* 1099 $[M-H]^-$, 743

$[(M-H)-132-162-CH_3CO_2H-2H]^-$; ESI-MS (positive ion mode) *m/z* 1145 $[M+2Na-H]^+$; ESI-MS-MS: MS^2 (1145) *m/z* 995 $[(M+2Na-H)-132-H_2O]^+$, 983 $[(M+2Na-H)-162]^+$, 935 $[(M+2Na-H)-132-H_2O-CH_3CO_2H]^+$, 869 $[(M+2Na-H)-132-H_2O-CO_2Na-CH_3CO_2H+H]^+$, 983 $[(M+2Na-H)-132-162-H_2O-CO_2Na+H]^+$, 729 $[(M+2Na-H)-132-162-H_2O-CO_2Na-CH_3CO_2H+H]^+$, MS^3 (869) *m/z* 737 $[(M+2Na)-132-162-H_2O-CO_2Na-CH_3CO_2H+H]^+$.

3.10. Saponin **9**

White powder $[\alpha]_D^{21}$ +7.7 (pyridine, *c* 0.21); ^1H and ^{13}C NMR (C_5D_5N) of the glycosidic part: see Table 2; ^{13}C NMR chemical shift values of the aglycone are identical to those described in the literature (Tan et al., 1999); ESI-MS (negative ion mode) *m/z* 1129 $[M-H]^-$; ESI-MS-MS: MS^2 (1129) *m/z* 979 $[(M-H)-132-H_2O]^-$, 919 $[(M-H)-132-H_2O- CH_3CO_2H]^-$, 773 $[(M-H)-132-162-CO_2-H_2O]^-$, 713 $[(M-H)-132-162-CO_2-H_2O-CH_3CO_2H]^-$, MS^3 (919) *m/z* 757 $[(M-H)-132-H_2O-CH_3CO_2H-162]^-$; ESI-MS (positive ion mode) *m/z* 1175 $[M+2Na-H]^+$, 1153 $[M+Na]^+$; ESI-MS-MS: MS^2 (1153) *m/z* 1021 $[(M+Na)-132]^+$, 991 $[(M+Na)-162]^+$, 965 $[(M+2Na-H)-132-H_2O-CH_3CO_2H]^+$, 899 $[(M+2Na-H)-132-H_2O-CH_3CO_2H-CO_2Na+H]^+$ MS^3 (899) *m/z* 737 $[(M+2Na-H)-132-162-H_2O-CH_3\ CO_2H-CO_2Na+H]^+$, MS^4 (737) *m/z* 575 $[(M+2Na-H)-132-162-162-H_2O-CH_3CO_2H-CO_2Na+H]^+$.

3.11. Saponin **10**

White powder $[\alpha]_D^{21}$ −10.8 (MeOH, *c* 0.61); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 2, ^{13}C NMR chemical shift values of the aglycone are identical to those described for saponin **2** to ±0.3 ppm; ESI-MS (negative ion mode) *m/z* 1129 $[M-H]^-$, 773 $[(M-H)-132-162-CO_2-H_2O]^-$; ESI-MS (positive ion mode) *m/z* 1153 $[M+Na]^+$; ESI-MS-MS : MS^2 (1153) *m/z* 1021 $[(M+Na)-132]^+$, 991 $[(M+Na)-162]^+$, 535 [triglycosidic chain + CH_3CO_2H + Na]$^+$, MS^3 (535) *m/z* 475 [triglycosidic chain + Na]$^+$.

3.12. Saponin **11**

$[\alpha]_D^{21}$ −8.1 (MeOH, *c* 0.28); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part: see Table 1, ^{13}C NMR (CD_3OD) of the aglycone δ 14.1 (C-27), 15.1 (C-26), 15.3 (C-24), 15.7 (C-25), 17.7 (C-6), 19.7 (C-11), 25.6 (C-2), 25.7 (C-12), 26.7 (C-23), 27.9 (C-30), 29 (C-15), 29.4 (C-29), 31.5 (C-20), 32.6 (C-16), 33 (C-21), 33 (C-22), 34.3 (C-7), 36.6 (C-10), 38.5 (C-1), 38.6 (C-4), 40.5 (C-8), 40.8 (C-13), 42.2 (C-14), 48.4 (C-17), 51.1 (C-9), 55.6 (C-5), 89.7 (C-3), 132.5 (C-19), 137 (C-18), 175.4 (C-28); ESI-MS (negative ion mode) *m/z* 967 $[M-H]^-$; ESI-MS-MS: MS^2 (967) *m/z* 817 $[(M-H)-132-H_2O]^-$, 655 $[(M-H)-132-162-H_2O]^-$; ESI-MS (positive ion mode) *m/z* 1013 $[M+2Na-H]^+$; ESI-MS-MS : MS^2 (1013) *m/z* 953 $[(M+2Na-H)-CH_3CO_2H]^+$, 851 $[(M+2Na-H)-162]^+$, MS^3 (851) *m/z* 833 $[(M+2Na-H)-162-H_2O]^+$, 807 $[(M+2Na-H)-162-CO_2]^+$, 791 $[(M+2Na-H)-162-CH_3CO_2H]^+$, 773 $[(M+2Na-H)-162-CH_3CO_2H-H_2O]^+$, 719 $[(M+2Na-H)-132-162]^+$, MS^4 (791) *m/z* 659 $[(M+2Na-H)-162-132-CH_3CO_2H]^+$, 640 $[(M+2Na-H)-162-132-CH_3CO_2H-H_2O]^+$.

3.13. Acid hydrolysis of saponins

The crude saponin mixt. A (1 g) was refluxed with 30 ml of 2N HCl for 4 h 30 min. The sapogenin mixture was extracted with EtOAc (3×15 ml), washed with H_2O, dried over Na_2SO_4 and evapd to dryness. Oleanolic acid was identified from the sapogenin residue with an authentic sample by TLC in $CHCl_3$/MeOH (98/2). The acid aq. layer was neutralised with 0.5 M KOH and freeze-dried. Three sugars were identified with authentic samples by TLC in MeCOEt/iso-PrOH/Me_2CO/H_2O (20/10/7/6) as glucuronic acid, glucose, xylose and arabinose. After prep. TLC of the sugar mixt. in this solvent, the optical rotation of each purified sugar was measured.

3.14. Haemolytic activity

This assay was performed as described previously (Voutquenne et al., 2002). Sheep erythrocyte suspension (10%) was obtained by dilution of a commercial 50% suspension from Biomerieux® Lyon with phosphate buffer saline (PBS). Saponin mixt. was dissolved in PBS. 25 µl of erythrocyte suspension were added to 1 ml of the sample and rapidly stirred. Absorbance of the supernatant was measured at 540 nm after 60 min of incubation and centrifuged at 3000 rpm for 5 min. HD_{50} and HD_{100} were the concentrations of sample which cause 50% and 100% of haemolysis, respectively.

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