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Oleanane-*type* Saponins from *Glochidion hirsutum* and Their Cytotoxic Activities

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Five new oleanane-*type* saponins, hirsutosides A – E, were isolated from the leaves of *Glochidion hirsutum* (Roxb.) Voigt. Their structures were elucidated as 21β-benzoyloxy-3β,16β,23,28-tetrahydroxyolean-12-ene 3-*O*-β-D-glucopyranoside (**1**), 21β-benzoyloxy-3β,16β,23,28-tetrahydroxyolean-12-ene 3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranoside (**2**), 21β-benzoyloxy-3β,16β,23,28-tetrahydroxyolean-12-ene 3-*O*-6-acetyl-[β-D-glucopyranosyl-(1 → 3)]-β-D-glucopyranoside (**3**), 21β-benzoyloxy-3β,16β,23,28-tetrahydroxyolean-12-ene 3-*O*-β-D-glucopyranosyl-(1 → 3)-⟨-L-arabinopyranoside (**4**), and 21β-benzoyloxy-3β,16β,23-trihydroxyolean-12-ene-28-al 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranoside (**5**). All isolated compounds were evaluated for cytotoxic activities on four human cancer cell lines, HepG-2, A-549, MCF-7, and SW-626 using the SRB assay. Compounds **1**, **2**, **4**, and **5** showed significant cytotoxic activities against all human cancer cell lines with IC_{50} values ranging from 3.4 to 10.2 μM. Compound **3** containing acetyl group at glc C(6″) exhibited weak cytotoxic activity with IC_{50} values ranging from 47.0 to 54.4 μM.

Keywords: *Glochidion hirsutum*, Euphorbiaceae, Hirsutosides A – E, Oleanane-*type* saponins, Cytotoxic activities.

Introduction

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. Accordingly, the development of new and efficient anticancer drugs has been interested from the scientists around the world. Natural products are potential sources of novel drugs with a broad range of biological and pharmacological activities, including anticancer activities.[1] There are more than 60% of currently anticancer drugs from natural sources.[2] Moreover, many oleanane triterpene-*type* saponins from various plants such as *Bolbostemma paniculatum*,[3] *Platycodon grandiflorum*,[4] *Glochidion eriocarpum*[5] exhibited cytotoxic activities.

Glochidion is a large genus of the Euphorbiaceae family, comprising more than 250 species in the world. *Glochidion hirsutum* (Roxb.) Voigt is a shrub or small tree distributed throughout Southeast Asia. The leaves of *G. hirsutum* have been used in folk medicine to treat toothaches; the roots are used as medicine for rheumatism and pneumonia.[6] Phytochemical studies of *G. hirsutum* have shown the presence of flavonols.[7][8] Previous our investigation program on cytotoxic constituents of *Glochidion* genus identified cytotoxic oleanane saponins from *G. eriocarpum*[5] and *G. glomerulatum*.[9][10] Herein, we reported the isolation, structural elucidation of oleanane-*type* saponins from the leaves of *G. hirsutum*, and their cytotoxic activity against four human cancer cell lines, HepG-2, A-549, MCF-7, and SW-626.

Results and Discussion

Structure Elucidation

The methanol extract of the *G. hirsutum* leaves was suspended in water and then partitioned with CH_2Cl_2 and AcOEt to obtain three layers. The AcOEt layer was separated using a combination of silica gel and *RP-18* column chromatographic steps to afford five new oleanane-*type* saponins (*Fig. 1*). Their structures were elucidated by extensive spectroscopic methods including 1D- and 2D-NMR experiments, as well as by HR-ESI-MS analysis.

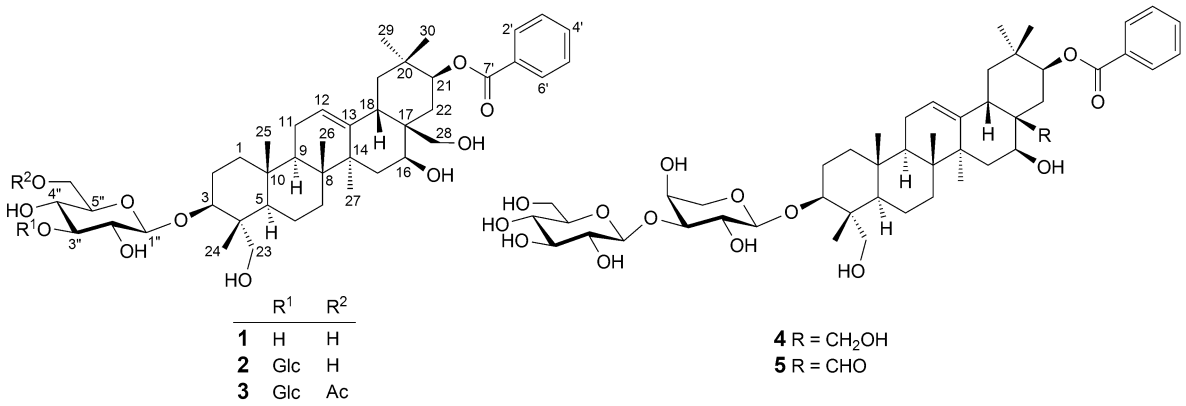

Figure 1. Chemical structures of compounds **1** – **5** from *Glochidion hirsutum*.

Compound **1** was obtained as a white amorphous powder and its molecular formula was determined as $C_{43}H_{64}O_{11}$ by HR-ESI-MS ion at *m/z* 779.4370 $[M + Na]^+$ (calc. for $C_{43}H_{64}NaO_{11}^+$, 779.4346). The ^{1}H-NMR spectrum of **1** showed the signals of six Me groups at δ(H) 0.75, 0.96, 1.04, 1.06, 1.17, and 1.34 (each, 3 H, *s*) and one olefinic H-atom at δ(H) 5.37 (1 H, *t*, *J* = 3.0), which indicated an oleanane aglycone. In addition to these, H-atoms of a benzoyloxy were observed at δ(H) 7.51 (2 H, *t*, *J* = 8.0), 7.62 (1 H, *t*, *J* = 8.0), and 8.04 (2 H, *d*, *J* = 8.0). One anomeric H-atom at δ(H) 4.43 (1 H, *d*, *J* = 8.0) showed the presence of a sugar moiety. The ^{13}C-NMR and DEPT spectra of **1** showed the presence of 43 C-atoms, including one CO group, eight quaternary C-atoms, 17 CH groups,eleven CH_2 groups, and six Me C-atoms (*Table 1*). The analysis of ^{1}H- and ^{13}C-NMR spectroscopic data indicated that the aglycone of **1** was similar to those of 21β-(benzoyloxy)olean-12-ene-3β,16β,23,28-tetraol, an oleanane-type triterpene isolated from *Glochidion assamicum*.[11] The HMBCs between H–C(18) (δ(H) 2.51) and C(13) (δ(C) 143.0) /C(16) (δ(C) 67.9)/C(17) (δ(C) 44.7)/C(28) (δ(C) 66.6) as well as the COSY correlations between H–C(15) (δ(H) 1.40 – 1.46 and 1.80 – 1.84) and H–C(16) (δ(H) 4.36) confirmed the positions of two OH groups at C(16) and C(28) (*Fig. 2*). The β configuration (*equatorial* orientation) of the OH group at C(16) was confirmed by NOESY correlations between H–C(16) (δ(H) 4.36) and H–C(27) (δ(H) 1.34)/H$_\alpha$–C(19) (δ(H) 2.10) as well as by the coupling constant of H–C(15) and H–C(16), $J_{eq\text{-}ax}$ = 5.0 and $J_{ax\text{-}ax}$ = 12.0. The location of a O-bearing group at C(21) was assigned based on the HMBCs between H–C(29) (δ(H) 0.96)/H–C(30) (δ(H) 1.17) and C(19) (δ(C) 48.0)/C(20) (δ(C) 36.6)/C(21) (δ(C) 78.2). Furthermore, the esterification location of benzoic acid at C(21) was confirmed by a HMBC between H–C(21) (δ(H) 5.16) and Bz C(7′) (δ(C) 167.9). The configuration of the benzoyloxy group was determined as β by the NOESY observations between H–C(21) (δ(H) 5.16) and H$_\alpha$-C(19) (δ(H) 2.08 – 2.12)/H–C(29) (δ(H) 0.96). The HMBCs from H–C(3) (δ(H) 3.67) to C(4) (δ(C) 43.9)/C(5) (δ(C) 48.1)/C(23) (δ(C) 64.8)/C(24) (δ(C) 13.4), from H–C(23) (δ(H) 3.31 and 3.67)/H–C(24) (δ(H) 0.75) to C(3) (δ(C) 83.3)/C(4) (δ(C) 43.9)/C(5) (δ(C) 48.1) suggested the location of the O-bearing and OH groups at C(3) and C(23), respectively. The α-orientations of H–C(3) and the hydroxylmethylene group at C(4) were determined by the NOESY observation of H–C(3) (δ(H) 3.67) and H–C(5) (δ(H) 1.63-1.69)/H–C(23) (δ(H) 3.31 and 3.67) and of H–C(24) (δ(H) 0.75) and H–C(25) (δ(H) 1.04). Acid hydrolysis of **1** afforded D-glucose as sugar component (identified as TMS derivatives by GC). Also, the HMBC from glc H–C(1″) (δ(H) 4.43) to C(3) (δ(C) 83.3) confirmed the location of glucopyranosyl moiety at C(3). Consequently, the structure of **1** was elucidated to be 21β-benzoyloxy-3β,16β,23,28-tetrahydroxy-olean-12-ene 3-*O*-β-D-glucopyranoside and named hirsutoside A.

The molecular formula of **2** was determined as $C_{49}H_{74}O_{16}$ by the HR-ESI-MS ion at *m/z* 941.4896 $[M + Na]^+$ (calc. for $C_{49}H_{74}NaO_{16}^+$, 941.4875). The ^{1}H- and ^{13}C-NMR spectra exhibited the presence of one oleanane aglycone, one benzoyloxy, and two sugar moieties (*Table 1*). The NMR data of **2** were similar to those of hirsutoside A (**1**), except for the addition of a sugar moiety at glc C(3″). The aglycone was recognized to be 21β-(benzoyloxy)olean-12-ene-3β,16β,23,28-tetraol.[11] The configurations of functional groups for aglycone **2** were similar to those of **1**, confirmed by NOESY experiments. Acid hydrolysis of **2** gave D-glucose (identified as TMS derivatives by GC). The HMBCs between glc H–C(1‴) (δ(H) 4.57) and glc C(3″) (δ(C) 88.3); glc H–C(3″) (δ(H) 3.55) and glc C(1‴) (δ(C) 105.3) confirmed the sequence of sugar linkages as 3-*O*-β-D-glucopyranosyl (1 → 3)-β-D-

Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data for Compounds **1** – **3**

C	**1**		**2**		**3**	
	δ(H) (mult., *J*, in Hz)	δ(C)	δ(H) (mult., *J*, in Hz)	δ(C)	δ(H) (mult., *J*, in Hz)	δ(C)
Aglycone						
1	0.98 – 1.02 (*m*)	39.6	1.01 – 1.05 (*m*)	39.6	0.99 – 1.03 (*m*)	39.7
	1.64 – 1.68 (*m*)		1.64 – 1.70 (*m*)		1.64 – 1.68 (*m*)	
2	1.70 – 1.76 (*m*)	26.3	1.75 – 1.79 (*m*)	26.3	1.74 – 1.80 (*m*)	26.4
	1.95 – 2.01 (*m*)		1.95 – 2.01 (*m*)		1.97 – 2.03 (*m*)	
3	3.67 (*dd*, *J* = 3.5, 13.0)	83.3	3.68 (*m*)	83.4	3.65 (*dd*, *J* = 4.5, 12.0)	83.3
4	–	43.9	–	43.9	–	44.1
5	1.63 – 1.69 (*m*)	48.1	1.64 – 1.70 (*m*)	48.2	1.63 – 1.70 (*m*)	47.8
6	1.42 – 1.46 (*m*)	18.8	1.45 – 1.49 (*m*)	18.9	1.42 – 1.46 (*m*)	18.9
	1.55 – 1.59 (*m*)		1.55 – 1.59 (*m*)		1.52 – 1.56 (*m*)	
7	1.33 – 1.39 (*m*)	33.3	1.35 – 1.41 (*m*)	33.3	1.35 – 1.41 (*m*)	33.3
	1.72 – 1.76 (*m*)		1.73 – 1.79 (*m*)		1.72 – 1.79 (*m*)	
8	–	41.1	–	41.1	–	41.1
9	1.25 – 1.30 (*m*)	48.1	1.25 – 1.30 (*m*)	48.5	1.25 – 1.30 (*m*)	48.2
10	–	37.5	–	37.5	–	37.5
11	1.94 – 2.00 (*m*)	24.7	1.94 – 2.00 (*m*)	24.7	1.94 – 2.00 (*m*)	24.7
12	5.37 (*t*, *J* = 3.0)	124.9	5.37 (br. *s*)	124.9	5.37 (*t*, *J* = 3.0)	125.0
13	–	143.0	–	143.0	–	143.0
14	–	44.6	–	44.6	–	44.6
15	1.40 – 1.46 (*m*)	36.5	1.40 – 1.46 (*m*)	36.5	1.40 – 1.46 (*m*)	36.5
	1.80 – 1.84 (*m*)		1.81 – 1.85 (*m*)		1.80 – 1.84 (*m*)	
16	4.36 (*dd*, *J* = 5.0, 12.0)	67.9	4.36 (*dd*, *J* = 5.0, 12.0)	67.9	4.36 (*dd*, *J* = 5.0, 12.0)	67.9
17	–	44.7	–	44.8	–	44.8
18	2.51 (*dd*, *J* = 4.5, 14.0)	43.6	2.52 (*dd*, *J* = 4.5, 13.5)	43.6	2.52 (*dd*, *J* = 4.5, 13.0)	43.7
19	1.30 – 1.36 (*m*)	48.0	1.30 – 1.35 (*m*)	48.0	1.30 – 1.35 (*m*)	48.0
	2.08 – 2.12 (*m*)		2.00 – 2.06 (*m*)		2.00 – 2.06 (*m*)	
20	–	36.6	–	36.6	–	36.6
21	5.16 (*dd*, *J* = 5.0, 12.0)	78.2	5.16 (*dd*, *J* = 4.5, 12.0)	78.2	5.16 (*dd*, *J* = 5.0, 12.5)	78.2
22	1.73 (*dd*, *J* = 12.0, 13.5)	30.2	1.73 (*dd*, *J* = 12.0, 13.5)	30.2	1.73 (*dd*, *J* = 12.5, 13.5)	30.2
	2.39 (*dd*, *J* = 5.0, 13.5)		2.38 (*dd*, *J* = 4.5, 13.5)		2.38 (*dd*, *J* = 4.5, 13.5)	
23	3.31 (*d*, *J* = 13.0)	64.8	3.32 (*d*, *J* = 13.0)	65.0	3.23 (*d*, *J* = 11.0)	64.3
	3.67 (*d*, *J* = 13.0)		3.68 (*d*, *J* = 13.0)		3.83 (*d*, *J* = 11.0)	
24	0.75 (*s*)	13.4	0.75 (*s*)	13.4	0.71 (*s*)	13.5
25	1.04 (*s*)	16.6	1.05 (*s*)	16.6	1.04 (*s*)	16.6
26	1.06 (*s*)	17.5	1.07 (*s*)	17.5	1.06 (*s*)	17.5
27	1.34 (*s*)	27.4	1.32 (*s*)	27.4	1.32 (*s*)	27.4
28	3.42 (*d*, *J* = 11.0)	66.6	3.42 (*d*, *J* = 10.5)	66.6	3.41 (*d*, *J* = 11.0)	66.6
	3.73 (*d*, *J* = 11.0)		3.73 (*d*, *J* = 10.5)		3.72 (*d*, *J* = 11.0)	
29	0.96 (*s*)	29.4	0.96 (*s*)	29.4	0.96 (*s*)	29.4
30	1.17 (*s*)	18.9	1.17 (*s*)	18.9	1.17 (*s*)	18.9
21-*O*-Bz						
1′	–	134.2	–	134.2	–	134.2
2′, 6′	8.04 (*d*, *J* = 8.0)	130.4	8.05 (*d*, *J* = 8.0)	130.4	8.05 (*d*, *J* = 8.0)	130.4
3′, 5′	7.51 (*t*, *J* = 8.0)	129.6	7.51 (*t*, *J* = 8.0)	129.6	7.51 (*t*, *J* = 8.0)	129.6
4′	7.62 (*t*, *J* = 8.0)	131.9	7.63 (*t*, *J* = 8.0)	131.9	7.63 (*t*, *J* = 8.0)	131.9
7′	–	167.9	–	167.8	–	167.8
MeCO					–	172.8
*Me*CO					2.08 (*s*)	20.9
3-*O*-Glc						
1″	4.43 (*d*, *J* = 8.0)	105.7	4.49 (*d*, *J* = 8.0)	105.3	4.68 (*d*, *J* = 7.5)	105.3
2″	3.20 (*t*, *J* = 8.0)	75.6	3.38 – 3.42 (*m*)	74.9	3.26 – 3.30 (*m*)	76.2
3″	3.34 – 3.38 (*m*)	77.7	3.53 – 3.57 (*m*)	88.3	3.43 – 3.49 (*m*)	83.4
4″	3.29 – 3.33 (*m*)	71.6	3.40 – 3.46 (*m*)	70.0	3.30 – 3.36 (*m*)	71.3
5″	3.28 – 3.32 (*m*)	78.3	3.30 – 3.34 (*m*)	77.4	3.26 – 3.32 (*m*)	77.6

Table 1. (cont.)

C	**1**		**2**		**3**	
	δ(H) (mult., *J*, in Hz)	δ(C)	δ(H) (mult., *J*, in Hz)	δ(C)	δ(H) (mult., *J*, in Hz)	δ(C)
6″	3.69 (*dd*, *J* = 4.5, 12.0) 3.86 (*dd*, *J* = 2.0, 12.0)	62.7	3.71 (*dd*, *J* = 6.0, 11.5) 3.90 (*dd*, *J* = 2.0, 11.5)	62.4	4.18 (*dd*, *J* = 5.0, 12.0) 4.36 (*dd*, *J* = 2.0, 12.0)	64.9
3″-*O*-Glc						
1‴			4.57 (*d*, *J* = 8.0)	105.3	4.53 (*d*, *J* = 8.0)	104.4
2‴			3.29 – 3.33 (*m*)	75.5	3.42 – 3.48 (*m*)	75.4
3‴			3.38 – 3.42 (*m*)	77.8	3.37 – 3.41 (*m*)	77.8
4‴			3.37 – 3.36 (*m*)	71.6	3.31 – 3.35 (*m*)	71.3
5‴			3.32 – 3.38 (*m*)	78.2	3.56 – 3.60 (*m*)	78.6
6‴			3.64 (*dd*, *J* = 6.0, 11.5) 3.88 (*dd*, *J* = 2.0, 11.5)	62.4	3.67 (*dd*, *J* = 5.0, 12.0) 3.86 (*dd*, *J* = 2.0, 12.0)	62.7

Assignments were done by HSQC, HMBC, COSY, and NOESY experiments. Ara, arabinopyranosyl; Bz, benzoyl; Glc, glucopyranosyl.

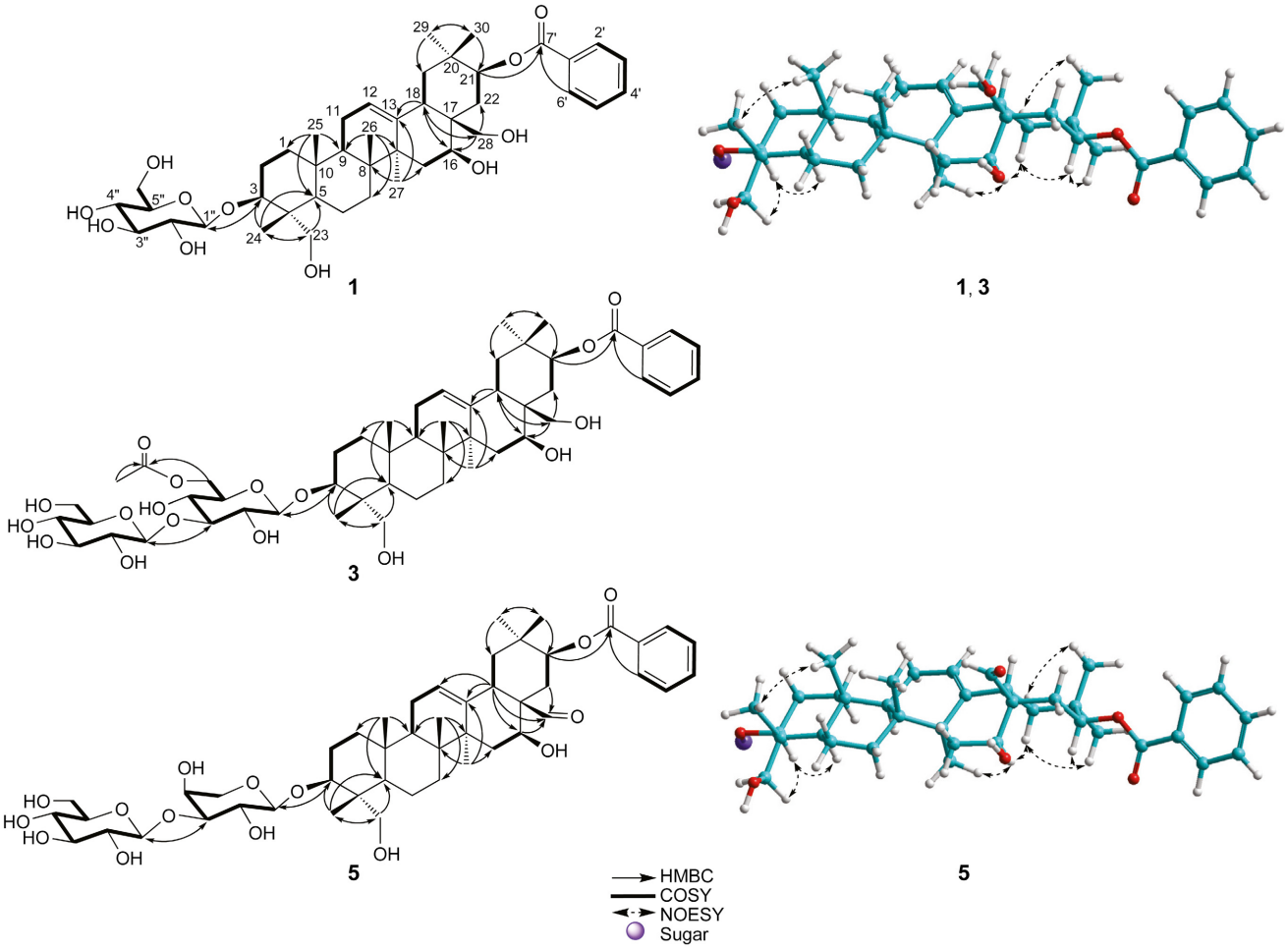

Figure 2. The key HMBC, ^{1}H,^{1}H-COSY, and NOESY correlations of **1**, **3**, and **5**.

glucopyranoside. The sequence of sugar linkages at C (3) of aglycone was proved by HMBCs between glc H–C(1″) (δ(H) 4.49) and C(3) (δ(C) 83.4). Consequently, the structure of **2** was determined as 21β-benzoyloxy-3β,16β,23,28-tetrahydroxyolean-12-ene 3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranoside and named hirsutoside B.

The molecular formula of **3** was deduced as $C_{51}H_{76}O_{17}$ by the HR-ESI-MS ion at *m/z* 983.4965 $[M + Na]^+$ (calc. for $C_{51}H_{76}NaO_{17}^+$, 983.4980). Analysis

of the NMR data of **3** indicated that the structure of aglycone was similar to those of **1** and **2**. The HMBCs between glc H–C(1‴) (δ(H) 4.53) and glc C(3″) (δ(C) 83.4); between glc H–C(6″) (δ(H) 4.18 and 4.36) and acetyl group (δ(C) 172.8) confirmed the sugar linkages to be 3-*O*-6-acetyl-[β-D-glucopyranosyl-(1 → 3)]-β-D-glucopyranoside. Moreover, the sequence of sugar linkages was located at C(3) of aglycone by HMBC between glc H–C(1″) (δ(H) 4.68) and C(3) (δ(C) 83.3). Based on the above evidence, compound **3** was defined as 21β-benzoyloxy-3β,16β,23,28-tetrahydroxyolean-12-ene 3-*O*-6-acetyl-[β-D-glucopyranosyl-(1 → 3)]-β-D-glucopyranoside and named hirsutoside C.

Compound **4** was also obtained as a white amorphous powder. The molecular formula was determined as $C_{48}H_{72}O_{15}$ by the HR-ESI-MS ion peak at *m/z* 911.4779 $[M + Na]^+$ (calc. for $C_{48}H_{72}NaO_{15}^+$, 911.4769). The ^{1}H-, ^{13}C-NMR, and DEPT spectra of compound **4** showed one olean-12-ene triterpene aglycone, one benzoyloxy, and two sugar moieties (*Table 2*). The aglycone of **4** was found to be similar to those of hirsutoside A (**1**). Acid hydrolysis and GC analysis of **4** confirmed the presence of D-glucose and L-arabinose. In addition, the coupling constants of ara H–C(1″) and ara H–C(2″), J = 7.5 Hz; glc H–C(1‴) and glc H–C(2‴), J = 8.0 Hz, confirmed the configurations of the *O*-glycoside bonds as β-D-glucopyranosyl and α-L-arabinopyranosyl. The sequence of sugar linkages was as 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranoside confirming by the HMBC from glc H–C(1‴) (δ(H) 4.57) to ara C(3″) (δ(C) 84.2). Furthermore, the location of sugar at C(3) of aglycone was confirmed by the HMBC between ara H–C(1″) (δ(H) 4.38) and C(3) (δ(C) 83.3). Consequently, compound **4** was defined as 21β-benzoyloxy-3β,16β,23,28-tetrahydroxyolean-12-ene 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranoside and named hirsutoside D.

The molecular formula of compound **5** was determined as $C_{48}H_{70}O_{15}$ by the HR-ESI-MS ion at *m/z* 909.4602 $[M + Na]^+$ (calc. for $C_{48}H_{70}NaO_{15}^+$, 909.4612). Analysis of the NMR data of **5** indicated that the structure of **5** was similar to those of **4** except for the presence of aldehydic group instead of hydroxylmethylene at C(17). The HMBCs from H–C(16) (δ(H) 4.45)/H–C(22) (δ(H) 1.45 and 2.46) to C(28) (δ(C) 207.4); from H–C(18) (δ(H) 2.92) to C(12) (δ(C) 125.2)/C(13) (δ(C) 142.1)/C(16) (δ(C) 66.1)/C(17) (δ(C) 42.7) confirmed the positions of OH and aldehyde groups at C(16) and C(17), respectively. In addition, the β configuration of the OH group at C(16) was confirmed by NOESY correlations between H–C(16) (δ(H) 4.45) and H–C(27) (δ(H) 1.32). The location of a benzoyloxy group at C(21) was proved by the HMBCs between H–C(29) (δ(H) 0.99)/H–C(30) (δ(H) 1.17) and C(19) (δ(C) 47.8)/C(20) (δ(C) 36.5)/C(21) (δ(C) 77.1); between H–C(21) (δ(H) 5.15) and Bz C(7′) (δ(C) 167.7). The configuration of this benzoyloxy group was determined as β by the NOESY observations between H–C(21) (δ(H) 5.15) and H–C(29) (δ(H) 0.99). The orientation of remaining functional groups of aglycone were based on NOESY experiments and coupling constant analysis. The position and sequence of sugar linkages were similar to those of compound **4**. Consequently, the structure of **5** was elucidated to be 21β-benzoyloxy-3β,16β,23-trihydroxyolean-12-ene-28-al 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranoside, and named hirsutoside E.

Biological Studies

All compounds were evaluated against four human cancer cell lines, HepG-2 (human liver hepatocellular carcinoma), A-549 (human lung carcinoma), MCF-7 (human breast carcinoma), and SW-626 (human ovarian carcinoma) using the SRB assay. Ellipticine, an anticancer agent, was used as a positive control with IC_{50} values ranging from 1.4 to 2.1 μM for all the human cancer cell lines (*Table 3*).

Comparing to ellipticine, compounds **1**, **2**, **4**, and **5** showed significant cytotoxic activities against all human cancer cells with IC_{50} values ranging from 3.4 to 10.2 μM. Compound **3** containing acetyl group at glc C(6″) exhibited weak cytotoxic activity with IC_{50} values ranging from 47.0 to 54.4 μM. In the structure–activity relationship of isolated compounds **1** – **3**: with an additional sugar moiety at glc C(3″) (**2**), the cytotoxic activity exhibited stronger, however, when an AcO group was placed at glc C(6″) (**3**), the cytotoxic activity decreased. The current study demonstrated that the cytotoxic activity of **2** on all tested human cancer cell lines comparable to those of ellipticine. This work has thus provided a further example of the importance of oleanane-type saponins contain a benzoyloxy group at C(21) as potential anticancer agents.

Experimental Section

General

Optical rotations were determined on a *Jasco DIP-370* automatic polarimeter. The NMR spectra were recorded using a *Bruker DRX 500* spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz). The HR-ESI-MS were obtained using an *Agilent 6550 iFunnel Q-TOF* LC/MS system. Column chromatography was performed using silicagel (*Kieselgel 60*, 70 – 230 mesh and 230 – 400 mesh, *Merck*) or *RP-18* resins (30 – 50 μm, *Fujisilisa Chemical*

Table 2. ^{1}H- and ^{13}C-NMR Spectroscopic Data for Compounds **4** and **5**

C	**4**		**5**	
	δ(H) (mult., *J*, in Hz)	δ(C)	δ(H) (mult., *J*, in Hz)	δ(C)
Aglycone				
1	0.98 – 1.02 (*m*)	39.6	0.98 – 1.02 (*m*)	39.5
	1.64 – 1.68 (*m*)		1.64 – 1.68 (*m*)	
2	1.70 – 1.76 (*m*)	26.3	1.73 – 2.00 (*m*)	26.3
	1.96 – 2.00 (*m*)		1.88 – 1.92 (*m*)	
3	3.67 (*dd*, *J* = 3.5, 13.0)	83.3	3.61 – 3.67 (*m*)	83.4
4	–	43.9	–	43.9
5	1.63 – 1.66 (*m*)	48.1	1.62 – 1.68 (*m*)	48.1
6	1.44 – 1.48 (*m*)	18.8	1.42 – 1.46 (*m*)	18.8
	1.52 – 1.56 (*m*)		1.52 – 1.56 (*m*)	
7	1.35 – 1.41 (*m*)	33.3	1.35 – 1.41 (*m*)	33.6
	1.72 – 1.78 (*m*)		1.69 – 1.75 (*m*)	
8	–	41.1	–	40.9
9	1.25 – 1.29 (*m*)	48.1	1.25 – 1.29 (*m*)	48.3
10	–	37.5	–	37.6
11	1.94 – 1.98 (*m*)	24.7	1.95 – 1.99 (*m*)	24.6
12	5.37 (br. *s*)	124.9	5.42 (*t*, *J* = 3.0)	125.2
13	–	143.0	–	142.1
14	–	44.6	–	44.9
15	1.41 – 1.45 (*m*)	36.5	1.53 – 1.60 (*m*)	38.1
	1.80 – 1.84 (*m*)		1.81 – 1.85 (*m*)	
16	4.36 (*dd*, *J* = 5.0, 12.0)	67.9	4.45 (*dd*, *J* = 5.0, 12.0)	66.1
17	–	43.9	–	42.7
18	2.51 (*dd*, *J* = 4.5, 14.0)	43.6	2.92 (*dd*, *J* = 4.5, 14.0)	43.9
19	1.30 – 1.36 (*m*)	48.0	1.37 – 1.43 (*m*)	47.8
	2.09 – 2.11 (*m*)		2.09 – 2.11 (*m*)	
20	–	36.6	–	36.5
21	5.16 (*dd*, *J* = 5.0, 12.0)	78.2	5.15 (*dd*, *J* = 4.5, 12.0)	77.1
22	1.73 (*dd*, *J* = 12.0, 13.5)	30.2	1.45 (*dd*, *J* = 12.0, 13.0)	28.3
	2.39 (*dd*, *J* = 4.5, 13.5)		2.46 (*dd*, *J* = 4.5, 13.0)	
23	3.31 (*d*, *J* = 13.0)	64.8	3.33 (*d*, *J* = 13.0)	65.1
	3.67 (*d*, *J* = 13.0)		3.67 (*d*, *J* = 13.0)	
24	0.75 (*s*)	13.4	0.75 (*s*)	13.4
25	1.04 (*s*)	16.6	1.03 (*s*)	16.5
26	1.06 (*s*)	17.5	0.87 (*s*)	17.7
27	1.34 (*s*)	27.4	1.32 (*s*)	26.9
28	3.42 (*d*, *J* = 11.0)	66.6	9.78 (*s*)	207.4
	3.73 (*d*, *J* = 11.0)			
29	0.96 (*s*)	29.4	0.99 (*s*)	29.2
30	1.17 (*s*)	18.9	1.17 (*s*)	18.8
21-*O*-Bz				
1′	–	134.2	–	134.4
2′, 6′	8.04 (*d*, *J* = 8.0)	130.4	8.04 (*d*, *J* = 8.0)	130.5
3′, 5′	7.51 (*t*, *J* = 8.0)	129.6	7.50 (*t*, *J* = 8.0)	129.6
4′	7.63 (*t*, *J* = 8.0)	131.8	7.64 (*t*, *J* = 8.0)	131.6
7′	–	167.8	–	167.7
3-*O*-Ara				
1″	4.38 (*d*, *J* = 7.5)	106.1	4.37 (*d*, *J* = 7.5)	106.1
2″	3.70 – 3.74 (*m*)	72.1	3.69 – 3.73 (*m*)	72.1
3″	3.65 – 3.71 (*m*)	84.2	3.63 – 3.69 (*m*)	84.3
4″	4.06 (br. *s*)	69.5	4.06 (br. *s*)	69.6
5″	3.60 (br. *d*, *J* = 12.0)	66.9	3.60 (br. *d*, *J* = 14.0)	66.9
	3.88 (br. *d*, *J* = 12.0)		3.88 (*dd*, *J* = 2.0, 14.0)	
3″-*O*-Glc				

Table 2. (cont.)

C	4		5	
	δ(H) (mult., *J*, in Hz)	δ(C)	δ(H) (mult., *J*, in Hz)	δ(C)
1‴	4.57 (*d*, *J* = 8.0)	105.5	4.56 (*d*, *J* = 8.0)	105.5
2‴	3.30 – 3.34 (*m*)	75.3	3.30 – 3.34 (*m*)	75.3
3‴	3.37 – 3.43 (*m*)	77.6	3.37 – 3.43 (*m*)	77.7
4‴	3.34 – 3.40 (*m*)	71.1	3.31 – 3.37 (*m*)	71.2
5‴	3.29 – 3.33 (*m*)	77.9	3.29 – 3.33 (*m*)	78.0
6‴	3.71 (*dd*, *J* = 5.0, 12.0) 3.87 (br. *d*, *J* = 12.0)	62.3	3.71 (*dd*, *J* = 5.0, 12.0) 3.85 (*dd*, *J* = 2.0, 12.0)	62.4

Assignments were done by HSQC, HMBC, COSY, and NOESY experiments. Ara, arabinopyranosyl, Bz, benzoyl, Glc, glucopyranosyl.

Table 3. Effects of **1** – **5** from *Glochidion hirsutum* on the Growth of Human Cancer Cells

Compound	IC_{50} [μM]			
	HepG-2	A-549	MCF-7	SW-626
1	8.2 ± 1.3	9.3 ± 0.3	9.2 ± 0.5	8.5 ± 1.3
2	3.4 ± 0.3	4.4 ± 0.7	4.7 ± 0.6	6.6 ± 1.0
3	47.0 ± 5.6	49.3 ± 4.1	51.9 ± 3.7	54.4 ± 1.5
4	7.6 ± 0.8	8.0 ± 2.2	8.8 ± 1.3	9.1 ± 1.1
5	9.9 ± 3.1	8.6 ± 1.3	10.2 ± 2.4	10.1 ± 1.9
Ellipticine	1.4 ± 0.2	1.8 ± 0.3	2.0 ± 0.3	2.1 ± 0.3

Ellipticine was used as a positive control. Data are presented as mean ± SD of experiments performed in triplicate.

Ltd.), and thin layer chromatography was performed using a pre-coated silica-gel *60* F_{254} (0.25 mm, *Merck*) and *RP-18* $F_{254}S$ plates (0.25 mm, *Merck*).

Plant Material

The leaves of *G. hirsutum* (Roxb.) Voigt were collected in Sondong, Bacgiang, Vietnam in December 2012 and identified by Dr. *Nguyen The Cuong*, Institute of Ecology and Biological Resources, VAST. A voucher specimen (GH1212) was deposited with the Herbarium of the Institute of Marine Biochemistry, Hanoi, Vietnam.

Extraction and Isolation

Dried leaves of *G. hirsutum* (4.0 kg) were sonicated in MeOH (7 l × 3 times) for 15 h to yield a MeOH extract (355 g) after evaporating under reduced pressure. The MeOH extract was suspended in H_2O and successively partitioned with CH_2Cl_2 and AcOEt to obtain CH_2Cl_2 (*GH1*, 120.0 g), AcOEt (*GH2*, 50.0 g), and H_2O (*GH3*, 180.0 g) layers after removal of the solvents in *vacuo*. The *GH2* layer was applied to a silica gel CC eluted with a gradient elution of CH_2Cl_2/MeOH (100:1, 30:1, 10:1, 5:1, 1:1, 0:1, *v/v*) to give six smaller fractions, *GH2A* (4.0 g), *GH2B* (3.1 g), *GH2C* (3.6 g), *GH2D* (8.5 g), *GH2E* (4.3 g), and *GH2F* (7.0 g). The *GH2C* fraction was applied to a silica gel CC eluting with CH_2Cl_2/MeOH (8:1, *v/v*) to give two fractions, *GH2C1* and *GH2C2*. The *GH2C1* fraction was further purified by silica gel CC eluting with AcOEt/MeOH/H_2O (12:1:0.01, *v/v/v*) to yield **1** (12.0 mg). The *GH2D* fraction was subjected to a silica gel CC eluting with CH_2Cl_2/MeOH/H_2O (5:1:0.1, *v/v/v*) to give three smaller fractions, *GH2D1* – *GH2D3*. The *GH2D1* fraction was applied to a silica gel CC eluting with AcOEt/MeOH/H_2O (6:1:0.01, *v/v/v*) to yield compound **4** (18.0 mg). The *GH2D2* fraction was subjected to an *RP-18* CC eluting with MeOH/H_2O (3.5:1, *v/v*) to yield compound **5** (32.0 mg). The *GH2D3* fraction was applied to a silica gel CC, eluted with CH_2Cl_2/MeOH/H_2O (6:1:0.05, *v/v/v*) to yield compound **3** (21.0 mg). Compound **2** (9.0 mg) was obtained from *GH2E* fraction, using a silica gel CC eluting with CH_2Cl_2/MeOH/H_2O (6:1:0.05, *v/v/v*) then an *RP-18* CC eluent of MeOH/H_2O (3:1, *v/v*). The purity of the compounds was assessed by HPLC-DAD at 210 nm as > 95%.

(3*β*,16*β*,21*β*)-3-(*β*-D-Glucopyranosyloxy)-16,23,28-trihydroxyolean-12-en-21-yl Benzoate (**1**). White amorphous powder. $[\alpha]_D^{25}$ = +30.0 (*c* = 0.1, MeOH). ^{1}H-(CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125 MHz): see *Table 1*. HR-ESI-MS: 779.4370 ($[M + Na]^+$, $C_{43}H_{64}NaO_{11}^+$; calc. 779.4346).

(3*β*,16*β*,21*β*)-3-{[3-*O*-(*β*-D-Glucopyranosyl)-*β*-D-glucopyranosyl]oxy}-16,23,28-trihydroxyolean-12-en-21-yl Benzoate (**2**). White amorphous powder. $[\alpha]_D^{25}$ = +50.0 (*c* = 0.1, MeOH). ^{1}H- (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125 MHz): see *Table 1*. HR-ESI-MS: 941.4896 ($[M + Na]^+$, $C_{49}H_{74}NaO_{16}^+$; calc. 941.4875).

(3*β*,16*β*,21*β*)-3-{[6-*O*-Acetyl-3-*O*-(*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl]oxy}-16,23,28-trihydroxyolean-12-en-21-yl Benzoate (**3**). White amorphous powder.

$[\alpha]_D^{25} = -20.0$ (c = 0.1, MeOH). ^{1}H- (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125 MHz): see *Table 1*. HR-ESI-MS: 983.4965 ($[M + Na]^+$, $C_{51}H_{76}NaO_{17}^+$; calc. 983.4980).

(3β,16β,21β)-3-{[3-*O*-(β-D-Glucopyranosyl)-α-L-arabinopyranosyl]oxy}-16,23,28-trihydroxyolean-12-en-21-yl Benzoate (**4**). White amorphous powder. $[\alpha]_D^{25} = +41.0$ (c = 0.1, MeOH). ^{1}H- (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125 MHz): see *Table 2*. HR-ESI-MS: 911.4779 ($[M + Na]^+$, $C_{48}H_{72}NaO_{15}^+$; calc. 911.4769).

(3β,16β,21β)-3-{[3-*O*-(β-D-Glucopyranosyl)-α-L-arabinopyranosyl]oxy}-16,23-dihydroxy-28-oxoolean-12-en-21-yl Benzoate (**5**). White amorphous powder. $[\alpha]_D^{25} = -9.0$ (c = 0.1, MeOH). ^{1}H- (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125 MHz): see *Table 2*. HR-ESI-MS: 909.4602 ($[M + Na]^+$, $C_{48}H_{70}NaO_{15}^+$; calc. 909.4612).

Acid Hydrolysis

Each compound (**1** – **5**, 2.0 mg) was separately dissolved in 1.0N HCl (dioxane/H_2O, 1:1, *v/v*, 1.0 ml) and heated to 80 °C in a water bath for 3 h. The solvent in acidic solution was removed under an N_2 stream. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2. The residue was dissolved in dry pyridine (0.1 ml), followed by addition of L-cysteine methyl ester hydrochloride in pyridine (0.06M, 0.1 ml). The mixture was heated at 6 °C for 2 h. Trimethylsilylimidazole solution (0.1 ml) was then added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with hexane and H_2O (0.1 ml each), and the organic layer was analyzed by gas chromatography (GC): column *DB-5* (0.32 mm ID × 30 m length), detector FID, column temp. 210 °C, injector temp. 270 °C, detector temp. 300 °C, carrier gas He (2 ml/min). Under these conditions, the standard sugars gave peaks at t_R (min) 14.11 and 14.26 for D- and L-glucose, 9.82 and 15.24 for D- and L-arabinose, resp. Peaks at t_R (min) 14.11 of D-glucose for **1** – **3**; 14.11 and 15.24 of D-glucose and L-arabinose for **4** and **5**, were observed.

Cytotoxic Assay

Tumour cells were cultivated in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. Cell viability was examined by SRB method for the determination of cell density, based on the measurement of cellular protein content. Viable cells were seeded in the growth medium (180 μl) into 96-well microwell plates (4×10^4 cells per well) and allowed to attach overnight. Test samples were added carefully into wells of 96-well plates and the cultivation was continued under the same conditions for another 48 h. Thereafter, the medium was removed and the remaining cell monolayers are fixed with the cold 20% (*w/v*) trichloroacetic acid for 1 h at 4 °C and stained by *1X SRB* staining solution at r.t. for 30 min, after which the unbound dye was removed by washing repeatedly with 1% (*v/v*) acetic acid. The proteinbound dye is dissolved in 10 mM *Tris* base solution for OD determination at 515 nm on an *ELISA Plate Reader* (*Bio-Rad*). DMSO 10% was used as blank sample while ellipticine was used as positive control. The cytotoxicity was measured at doses of 100.0, 20.0, 4.0, and 0.8 μM and estimated as a half maximal inhibitory concentration (IC_{50}), which was calculated by the program TableCurve Version 4.0. All experiments were prepared in triplicates. The inhibition rate (*IR*) of cells was calculated by the following formula $IR\% = [100 - (\text{absorbance}_t - \text{absorbance}_0)/(\text{absorbance}_c - \text{absorbance}_0)] \times 100\%$, where *IR* indicates inhibition rate of cell growth, absorbancet indicates average optical density value at day 2; absorbance_0 indicates average optical density value at time-zero and absorbancec indicates average optical density value of the blank DMSO control sample.

Conflict of Interest

The authors declare no competing financial interest.

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org//10.1002/cbdv.201600445.

References

[1] A. Vickers, 'Botanical Medicines for the Treatment of Cancer: Rationale, Overview of Current Data, and Methodological Considerations for Phase I and II Trials', *Cancer Invest.* **2002**, *20*, 1069 – 1079.

[2] D. J. Newman, G. M. Cragg, 'Natural Products as Sources of New Drugs over the Last 25 Years', *J. Nat. Prod.* **2007**, *70*, 461 – 477.

[3] L. Yu, R. Ma, Y. Wang, H. Nishino, J. Takayasu, W. He, M. Chang, J. Zhen, W. Liu, S. Fan, 'Potent Anti-tumorigenic Effect of Tubeimoside 1 Isolated from the Bulb of *Bolbostemma paniculatum* (Maxim) Franquet', *Int. J. Cancer* **1992**, *50*, 635 – 638.

[4] K. J. Lee, S. J. Hwang, J. H. Choi, H. G. Jeong, 'Saponins Derived from the Roots of *Platycodon grandiflorum* Inhibit HT-1080 Cell Invasion and MMPs Activities: Regulation of NF-κB Activation via ROS Signal Pathway', *Cancer Lett.* **2008**, *268*, 233 – 243.

[5] N. X. Nhiem, V. K. Thu, P. V. Kiem, C. V. Minh, B. H. Tai, T. H. Quang, N. X. Cuong, P. H. Yen, H.-J. Boo, H.-K. Kang, Y.

H. Kim, 'Cytotoxic Oleane-type Triterpene Saponins from *Glochidion eriocarpum*', *Arch. Pharm. Res.* **2012**, *35*, 19 – 26.
[6] V. V. Chi, 'The Dictionary of Medicinal Plants in Vietnam', Vol. 2, Medical Publishing House, Hanoi, 2012.
[7] J. Yang, X. D. Yang, S. Yang, J. F. Zhao, L. Li, 'Study on Flavanols from *Glochidion hirsutum*', *China J. Chin. Mater. Med.* **2007**, *32*, 593 – 596.
[8] J. Yang, X.-D. Yang, H.-Y. Wu, Q.-L. Zhao, J.-F. Zhao, L. Li, 'Chemical Study on *Glochidion hirsutum* (II)', *Nat. Prod. Res. Dev.* **2007**, *986 – 988*, 1012.
[9] V. K. Thu, N. V. Thang, N. X. Nhiem, B. H. Tai, N. H. Nam, P. V. Kiem, C. V. Minh, H. L. T. Anh, N. Kim, S. Park, S. H. Kim, 'Oleanane-type Saponins from *Glochidion glomerulatum* and Their Cytotoxic Activities', *Phytochemistry* **2015**, *116*, 213 – 220.
[10] V. K. Thu, N. V. Thang, N. X. Nhiem, H. L. T. Anh, P. H. Yen, C. V. Minh, P. V. Kiem, N. Y. Kim, S. J. Park, S. H. Kim, 'Oleanane-type Triterpene Saponins from *Glochidion glomerulatum*', *Nat. Prod. Commun.* **2015**, *10*, 875 – 876.
[11] X.-Q. Liu, H.-L. Huang, M.-J. Yao, G.-T. Han, N. Liu, J.-C. Yuan, C.-S. Yuan, 'Oleanane-Type Triterpenoids from *Glochidion assamicum*', *Helv. Chim. Acta* **2011**, *94*, 2264 – 2271.

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