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ScienceDirect
Phytochemistry 69 (2008) 1609–1616
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Triterpenoid saponins from the fruits and galls of *Sapindus mukorossi*

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Received 1 December 2006; received in revised form 31 August 2007
Available online 10 March 2008

Abstract

Six saponins, sapinmusaponin K (**1**) [hederagenin-3-*O*-(3-*O*-acetyl-α-L-arabinopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside], sapinmusaponin L (**2**) [hederagenin-3-*O*-(4-*O*-acetyl-α-L-arabinopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabino-pyranoside], sapinmusaponin M (**3**) [hederagenin-3-*O*-(2,3-*O*-diacetyl-β-D-xylopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside], sapinmusaponin N (**4**) [hederagenin-3-*O*-(2,4-*O*-diacetyl-β-D-xylopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside], sapinmusaponin O (**5**) [3,7,20(*S*)-trihydroxydammar-24-ene-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside], and sapinmusaponin P (**6**) [3,7,20(*R*)-trihydroxydammar-24-ene-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside], along with seven known saponins (**7**–**13**), were isolated from fruits and the galls of *Sapindus mukorossi*. Their structures were elucidated by 1D and 2D NMR spectroscopic techniques and acid hydrolysis. Biological evaluation indicated that saponins **1**–**4** and **7**–**13** showed moderate cytotoxicity against several human tumor cell lines.

Keywords: *Sapindus mukorossi*; Sapindaceae; Oleanane-type saponins; Dammarane-type saponins; Cytotoxic activity

1. Introduction

The fruit of *Sapindus mukorossi* Gaertn. (Sapindaceae), better known as soapnuts in tropical and sub-tropical regions of Asia (Nakayama et al., 1986), is generally used a commercial cleanser and has been shown to have medical applications based on its usages including antidermatophytic, antitussive, and antihelmintic activities (Waller and Yamasaki, 1996). In our work on the development of naturally-occurring bioactive agents, we have recently reported the isolation of several dammarane-type (sapinmusaponins A–E) (Kuo et al., 2005) and tirucallane-type (sapinmusaponins F–J) (Huang et al., 2006; Ni et al., 2006) saponins from the galls of *S. mukorossi*. A biological evaluation indicated that dammarane-type saponins had moderate cytotoxicity and that tirucallane-type saponins showed potent anti-platelet aggregation activity. The pericarps of *S. mukorossi* were also studied and several oleanane-type saponins that exhibited molluscicidal effects against *Pomacea canaliculata* and moderate cytotoxicity against human tumor cells (Huang et al., 2003) were isolated. Upon further investigation of the other parts of *S. mukorossi*, we have isolated and characterized four new oleanane-type saponins, sapinmusaponins K–N (**1**–**4**), and seven known saponins (**7**–**13**) from the EtOH extract of fruits of the title plant. In addition, further isolation of other active fractions from the previously collected galls of the title plant led to two dammarane-type saponins, sapinmusaponin O (**5**) and sapinmusaponin P (**6**). The structures of all newly isolated saponins (**1**–**6**) were

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doi:10.1016/j.phytochem.2007.10.033

established by spectroscopic analyses, mainly 2D NMR techniques, and chemical methods. Moreover, all of the isolated triterpenoid saponins (**1**–**13**), together with previously isolated **14** and **15** (Kuo et al., 2005), were evaluated for cytotoxicity against several human tumor cell lines.

2. Results and discussion

The EtOH extract of the fruit of *S. mukorossi* was extracted successively with *n*-hexane, $CHCl_3$ and *n*-BuOH. After evaporation of the $CHCl_3$ solvent, the residue was successively subjected to column chromatography on silica gel, Diaion HP-20 and Sephadex LH-20, and then separated by HPLC to give **1**–**4** and **7**–**13**. Compounds **5** and **6** were obtained as described in Section 3 by repeated chromatography on silica gel, Sephadex LH-20, and HPLC of the EtOH extract of the galls of the title plant.

The molecular formula of **1** was determined to be $C_{48}H_{76}O_{17}$ by HR-FAB-MS, which exhibited a quasi-molecular ion peak at *m/z* 947.4974 $[M+Na]^+$. The IR spectrum showed absorptions at 3398 (OH) and 1692 (C=O of COOH), 1458 (C=C), 1085 (C–O–C) cm^{-1}. The ^1H, ^{13}C NMR and DEPT spectra displayed six singlet methyls (δ_H 0.91, 0.93, 0.99, 1.01, 1.11 and 1.22; δ_C 14.1, 16.0, 17.4, 23.6, 26.1, and 33.2), an olefinic (δ_H 5.45; δ_C 122.5 and 144.7), and three anomeric signals (δ_H 5.00, 5.24 and 6.27; δ_C 101.1, 104.5 and 107.3), suggesting that **1** possessed a oleanane-type triterpene along with three sugar moieties (Table 1) (Abdulmagid Alabdul et al., 2006). Other resonances included an oxygenated methylene (δ_H 3.90, 4.26; δ_C 64.0), a carboxyl carbon (δ_C 180.2), and an acetate group (δ_C 21.0, δ_C 170.6; δ_H 2.01). Acid hydrolysis of **1** with 1 N HCl gave L-arabinose and L-rhamnose (2:1) as the component sugars, which were further treated with 1-(trimethylsilyl)imidazole and identified by comparison with authentic samples in a GC analysis. These findings indicated that saponin **1** possessed a hederagenin saponin with two L-arabinose units and an L-rhamnose unit, as well as an acetate group (Kanchanapoom et al., 2001). In the TOCSY spectrum of **1** (Fig. 1), the anomeric proton that was ascribed to L-arabinose [δ_H 5.00 (Ara-1′)] showed connectivity with three methines [δ_H 4.53 (Ara-2′), 3.95 (Ara-3′), and 4.09 (Ara-4′)] and two methylene protons [δ_H 4.20 (Ara-5a′), and 3.62 (Ara-5b′)]. The TOCSY spectrum also showed correlations between each glycosidic H-atom for the L-rhamnose and terminal L-arabinose. Moreover, the α-anomeric configurations of the L-arabinose ($J = 6.0$, 7.5 Hz) and L-rhamnose ($J = br\ s$) units were confirmed by their coupling constants (Lavaud et al., 2001). As to

Table 1
^{13}C and ^1H NMR spectroscopic data of the sugar moieties of sapinmusaponins K (**1**), L (**2**), M (**3**), and N (**4**)[a]

	1[b]		**2**[c]			**3**[d]		**4**[d]	
	^{13}C	^1H	^{13}C	^1H		^{13}C		^{13}C	^1H
Ara-					Ara-				
1′	104.5	5.00 *d* (6.0)	104.5	5.05 *d* (6.4)	1′	104.5	4.53 *d* (6.0)	104.0	4.52 *d* (6.0)
2′	75.1	4.53 *t* (7.5)	75.1	4.58 *t* (7.2)	2′	76.5	3.73 *t* (6.0)	76.3	3.69[e]
3′	75.1	3.95[e]	75.1	4.01 *dd* (8.4, 3.2)	3′	73.8	3.71[e]	73.8	3.68 *dd* (8.4, 3.6)
4′	69.5	4.09 *br s*	69.5	4.10 *br s*	4′	69.2	3.77 *br s*	69.3	3.74 *br s*
5′	66.2	4.20 *dd* (11.5, 2.5)	66.2	4.25[e]	5′	65.1	3.84 *br d* (12.4)	65.1	3.84 *br d* (12.0)
		3.62 *br d* (11.5)		3.68 *br d* (12.4)			3.52 *dd* (12.0, 2.0)		3.51 *dd* (11.6, 2.4)
Rha-					Rha-				
1″	101.0	6.27 *br s*	101.3	6.34 *br s*	1″	101.4	5.17 *d* (1.6)	101.3	5.16 *d* (1.6)
2″	71.9	4.84 *br s* ($W_{1/2}$ 3.0)	71.9	4.90 *br* ($W_{1/2}$ 2.8)	2″	71.6	4.06 *dd* (2.8, 2.0)	71.6	4.04 *dd* (3.6, 2.0)
3″	82.5	4.73 *dd* (9.0, 3.0)	82.6	4.78 *dd* (9.2, 2.8)	3″	81.5	3.83 *dd* (9.6, 2.8)	81.2	3.79 *dd* (9.6, 3.6)
4″	72.3	4.42 *t* (9.0)	72.3	4.43 *t* (9.2)	4″	72.5	3.45 *t* (9.6)	72.7	3.46 *t* (9.6)
5″	69.6	4.69 *dd* (9.0, 6.0)	69.5	4.73 *dd* (9.2, 6.0)	5″	70.5	3.86 *dd* (9.6, 6.0)	70.5	3.85 *dd* (9.6, 6.4)
6″	18.3	1.49 *d* (6.0)	18.3	1.55 *d* (6.0)	6″	17.9	1.23 *d* (6.0)	18.8	1.24 *d* (6.4)
Ara-					Xyl-				
1‴	107.3	5.24 *d* (7.5)	107.2	5.32 *d* (7.6)	1‴	104.0	4.77 *d* (7.6)	104.5	4.73 *d* (7.2)
2‴	70.0	4.69[e]	73.2	4.43 *t* (7.2)	2‴	73.5	4.81 *t* (9.2)	76.9	4.90 *t* (8.8)
3‴	77.1	5.30[e]	73.0	4.24 *dd* (7.2, 2.8)	3‴	76.9	4.99 *t* (9.2)	72.5	3.76 *t* (8.8)
4‴	67.1	5.46 *br s*	73.2	5.52 *br s*	4‴	69.2	3.79 *m*	72.4	4.74 *m*
5‴	66.9	4.13 *dd* (11.5, 2.5)	64.5	4.14 *dd* (12.4, 2.4)	5‴	66.7	3.96 *dd* (11.6, 6.0)	63.1	4.06 *dd* (12.4, 6.6)
		3.73 *br d* (11.5)		3.77 *br d* (12.4)			3.33[e]		3.33[e]
CH_3CO	170.6	2.01 *s*	170.6	2.02 *s*	CH_3CO	172.2	2.01 *s*, 2.02 *s*	172.3	2.07 *s*, 2.11 *s*
	21.0		21.0			172.1		172.0	
						20.8		20.7	
						20.8		21.0	

[a] *J* values (Hz) in parentheses.
[b] 125 MHz for ^{13}C in C_5D_5N.
[c] 100 MHz for ^{13}C in C_5D_5N.
[d] 100 MHz for ^{13}C in CD_3OD.
[e] Overlapping signals.

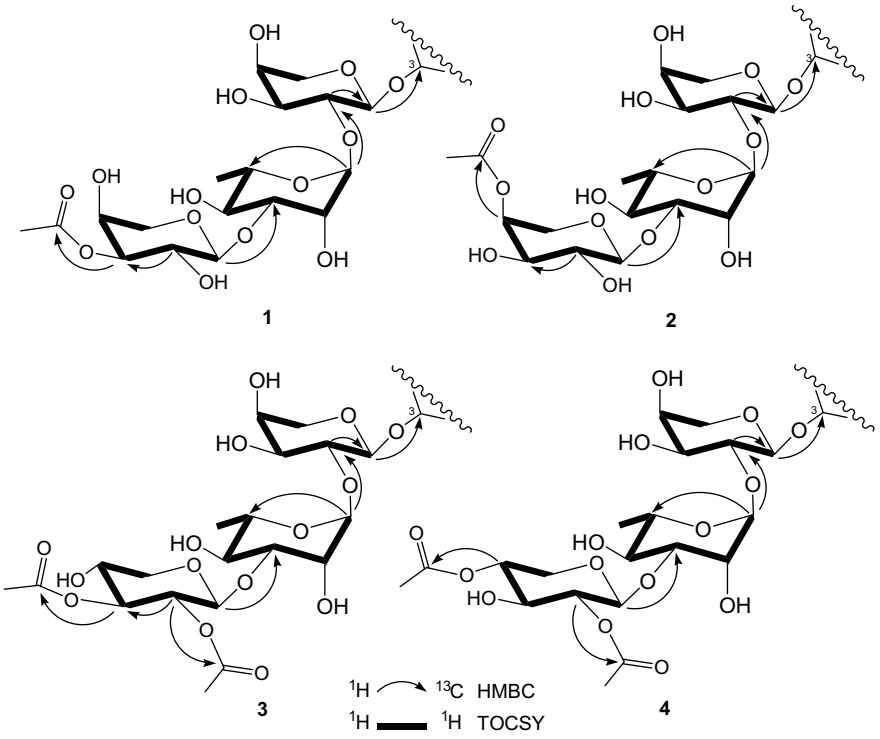


Fig. 1. TOCSY and HMBC correlations of sugar moieties of **1**–**4**.

the interglycosidic linkage positions, they were determined from the following HMBC correlations (Fig. 1): δ_H 5.00 (H-1′ of Ara)/δ_C 81.1 (C-3 of the aglycone); δ_H 6.27 (H-1″ of Rha)/δ_C 75.1 (C-2′ of Ara), and 5.24 (H-1‴ of the terminal Ara)/δ_C 82.5 (C-3″ of the inner Rha), and therefore the sugar units were deduced to be linked by Ara-(1 → 3)-Rha-(1 → 2)-Ara-(1 → 3)-aglycone. Due to the long-range correlation between δ_H 5.30 (Ara-3‴) and δ_C 170.6 (acetyl C═O), the location of the acetate group was assigned at C-3‴ of Ara (Fig. 1). Based on these findings, the structure of **1** was elucidated to be hederagenin-3-*O*-(3-*O*-acetyl-α-L-arabinopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside, and was named sapinmusaponin K.

Sapinmusaponin L (**2**) was determined to have an elemental composition of $C_{48}H_{76}O_{17}$, based on the results of HR-FAB-MS (*m*/*z* 947.4970 $[M+Na]^+$). The IR spectrum showed absorptions at 3424 (OH) and 1693 (C═O of COOH), 1455 (C═C), 1052 (C–O–C) cm^{-1}. As in **1**, the sugars L-arabinose and L-rhamnose (2:1) in **2** were verified by GC analysis after acid hydrolysis. The ^{1}H and ^{13}C NMR spectra of **2** showed signal patterns similar to those of **1**, indicating that **2** possessed a hederagenin with two α-L-arabinopyranosyl units [anomeric signals at δ_H 5.05 (*d*, *J* = 6.4 Hz) and 5.32 (*d*, *J* = 7.6 Hz); δ_C 104.5 and 107.2] and one α-L-rhamnopyranosyl unit [anomeric signals at δ_H 6.34 (*br s*); δ_C 101.3]. A detailed comparison of the ^{13}C NMR spectra of **1** and **2**, the downfield shifts for C-2‴ of Ara (Δδ + 3.2 ppm) and C-4‴ of Ara (Δδ +6.1 ppm) and upfield shifts for C-3‴ of Ara (Δδ −4.1 ppm) and C-5‴ of Ara (Δδ −2.4 ppm) in **2**, suggested that the acetate group in **2** was located at C-4‴ of Ara, instead of at C-3‴ as in **1**. In the HMBC spectrum, the evidence of correlation between δ_H 5.52 (Ara-4‴) and δ_C 170.6 also supported the assignment of the acetate group as mentioned (Fig. 1) above. Consequently, the structure of **2** was determined to be hederagenin-3-*O*-(4-*O*-acetyl-α-L-arabinopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside.

HR-FAB-MS of sapinmusaponin M (**3**) showed a quasi-molecular ion at 989.5073 $[M+Na]^+$, consistent with a formula of $C_{50}H_{78}O_{18}$. The IR spectrum showed absorptions at 3418 (OH) and 1693 (C═O of COOH), 1461 (C═C), 1051 (C–O–C) cm^{-1}. After acid hydrolysis of **3** with 1 N HCl and then treated with 1-(trimethylsilyl)imidazole, the sugar moieties in **3**, including L-arabinose, D-xylose and L-rhamnose (1:1:1), were identified by GC analysis. Based on a comparison of the NMR spectroscopic data of **3** and **1**, the former was suggested to have the same triterpenoid skeleton as **1**, except that Ara in **1** was replaced by Xyl in **3**, and there were two acetate groups [δ_H 2.01, 2.02 (3H × 2, s); δ_C 172.2, 172.1 (C═O × 2), 20.8 (CH_3 × 2)] in **3** rather than one in **1**. The HMBC spectrum of **3** showed correlations between δ_H 4.53 (Ara-1′) and δ_C 82.4 (C-3), between δ_H 5.17 (Rha-1″) and δ_C 76.5 (Ara-2′), and between δ_H 4.77 (Xyl-1‴) and δ_C 81.5 (Rha-3″), which suggested the linkage of Ara-C-1′ to C-3 of the aglycone, Rha-C-1″ to Ara-C-2′, and Xyl-C-1‴ to Rha-C-3″, respectively. Moreover, long-range correlations between δ_H 4.81 (Xyl-2‴) and δ_C 172.2, and between δ_H 4.99 (Xyl-3‴) and δ_C 172.1 permitted the assignment of two acetate groups at C-2‴ and C-3‴ of Xyl, respectively (Fig. 1). Thus, the

structure of **3** was elucidated to be hederagenin-3-*O*-(2,3-*O*-diacetyl-β-D-xylopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside.

Sapinmusaponin N (**4**) has a formula of $C_{50}H_{78}O_{18}$, due to a pseudomolecular ion at 989.5069 $[M+Na]^+$ in the HR-FAB-MS spectrum. The IR spectrum showed absorptions at 3433 (OH) and 1696 (C=O of COOH), 1464 (C=C), 1053 (C–O–C) cm^{-1}. Compound **4** was hydrolyzed and identified to have L-arabinose, D-xylose and L-rhamnose (1:1:1) as sugar moieties. The ^1H and ^{13}C NMR spectra of **4** showed signal patterns similar to those of **3**, except for the upfield shifts for C-3‴ of Xyl ($\Delta\delta$ −4.4 ppm) and C-5‴ of Xyl ($\Delta\delta$ −3.6 ppm), and the downfield shifts for C-2‴ of Xyl ($\Delta\delta$ +1.4 ppm) and C-4‴ of Xyl ($\Delta\delta$ +3.2 ppm) in **4**. The evidence suggested that the acetate group was located at C-4‴ of Xyl in **4**, instead of at C-3‴ in **3**. Moreover, correlations between δ_H 4.80 (Xyl-2‴) and δ_C 172.3, and between δ_H 4.74 (Xyl-4‴) and δ_C 172.0 in the HMBC spectrum further confirmed the locations of two acetate units at C-2‴ and 4‴ of Xyl, respectively (Fig. 1). Accordingly, **4** was unambiguously assigned to be hederagenin-3-*O*-(2,4-*O*-diacetyl-β-D-xylopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside, and named as sapinmusaponin N.

Sapinmusaponin O (**5**) and sapinmusaponin P (**6**) have the same molecular formula $C_{42}H_{72}O_{12}$, and pseudomolecular ions at 791.4930 $[M+Na]^+$ and 791.4908 $[M+Na]^+$, respectively, as deduced from HR-FAB-MS. Both the ^{13}C NMR (Table 2) and DEPT spectra of **5** and **6** showed 42 similar signals, which could be assigned to a triterpene and two sugar moieties. The characteristic signals for aglycone in **5**, including six tertiary methyls (δ_H 1.08, 1.04, 0.96, 0.93, 0.85 × 2), two allylic methyls (δH 1.66, 1.60), one olefinic proton [δ_H 5.09 (*t*, $J = 6.8$ Hz)], and two oxygenated methines [δ_H 3.17 (*dd*, $J = 10.8$, 4.0 Hz); δ_H 3.68 (*dd*, $J = 10.8$, 4.8 Hz), as well as similar to those resonances in **6** were found in the ^1H NMR spectrum. Additionally, two anomeric signals [δ_H 5.35 (*br s*), δC 101.8; δ_H 4.38 (*d*, $J = 7.2$ Hz), δ_C 105.5 in **5** and δ_H 5.34 (*br s*), δC 101.8; δ_H 4.40 (*d*, $J = 7.2$ Hz), δ_C 105.5 in **6**] were further determined to be L-rhamnose and D-glucose, respectively, by GC analysis of an acid hydrolysate treated as described above. These findings indicated that **5** and **6** have structures similar to previously isolated 3,7,20,22-tetrahydroxydammar-24-ene saponin, sapinmusaponin A (Kuo et al., 2005; Zou et al., 2002), except for the lack of a secondary hydroxyl group at C-22 of the side-chain in **5** and **6**.

Based on NOESY spectroscopic analysis, the correlations between CH_3-21 and H-17 in **5** suggested the assignment of an *S* configuration for C-20 in **5**. The steric difference at C-20 may be explained, due to the obvious difference in the chemical shifts at C-21 [δ_C 25.3 (CD_3OD), δ_C 27.9 (C_5D_5N) in **5;** δ_C 23.4 (CD_3OD), δ_C 23.3 (C_5D_5N) in **6**], C-22 [δ_C 42.2 (CD_3OD), δ_C 36.8 (C_5D_5N) in **5**; δ_C 43.1 (CD_3OD), δ_C 43.5 (C_5D_5N) in **6**] and C-17 [δ_C 49.7 (CD_3OD), δ_C 51.7 (C_5D_5N) in **5**; δ_C 49.7 (CD_3OD), δ_C 49.6 (C_5D_5N) in **6**] between **5** and **6,** especially measured in C_5D_5N. These findings suggest that **6** was an *R* configuration at C-20, which were consistent with the ^{13}C NMR spectroscopic data in the literature (Duc et al., 1994; Jiang et al., 1999; Teng et al., 2002). Accordingly, the structures of **5** and **6** were established to be 3,7,20(*S*)-trihydroxydammar-24-ene-3-*O*-α-L-rhamnopyrnosyl-(1 → 2)-β-D-glucopyranoside (**5**), and 3,7,20(*R*)-trihydroxydammar-24-ene-3-*O*-α-L-rhamnopyrnosyl-(1 → 2)-β-D-glucopyranoside (**6**).

Compounds **7**–**13** were identified to be the known mukurozi-saponin G (**7**) (Huang et al., 2003), hederagenin-3-*O*-(2-*O*-acetyl-β-D-xylopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside (**8**) (Nakayama et al., 1986), hederagenin-3-*O*-(3-*O*-acetyl-β-D-xylopyranosyl)-(1 → 3)-α-L-rhamnopyranosyl-(1→2)-α-L-rabinopyranoside (**9**) (Huang et al., 2003), mukurozi-saponin E1 (**10**) (Waller and Yamasaki, 1996; Huang et al., 2003), hederagenin-3-*O*-α-L-arabinopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside (**11**) (Waller and Yamasaki, 1996), sapindoside B (**12**) (Nakayama et al., 1986), and sapindoside A (**13**) (Waller and Yamasaki, 1996). All of the isolated triterpenoid saponins (**1**–**13**) were evaluated for cytotoxicity against several human tumor cell lines [Hela (epitheloid carcinoma), WiDr (colon adenocarcinoma), KB (oral epidermoid carcinoma), Daoy (medulloblastoma), and Hepa59T/VGH (hepatoma)]. The bioassay data (Table 3) exhibited that while most of the oleanane-type triterpene saponins have weak cytotoxicity, the dammarane-type triterpene saponins (**5** and **6**) were inactive (IC50 > 20 μM) in the tumor cell lines tested.

2.1. Concluding remarks

The present results with oleanane-type triterpene saponins are consistent those in the literature, even in different tumor cell lines. These findings suggest that the sugars and the number of acetates in sugar moieties of oleanane may not be the major factors in their cytotoxicity. Moreover, in a comparison of the cytotoxic effects of isomers **5** and **6** with those of similar previously identified saponins (**14** and **15**) (Kuo et al., 2005), **14**, which had two hydroxyl groups in its side chain, showed moderate cytotoxic effects compared to those of **5**, **6** and **15** (see Table 3) which had one or three hydroxyl groups, implying that the number of hydroxyl groups in the side-chain of dammarane-type triterpene saponins may play a crucial role in their cytotoxicity.

3. Experimental

3.1. General

Infrared (IR) spectra were measured on a Mattson Genesis II spectrophotometer using a KBr matrix, whereas optical rotations were acquired on a JASCO P-1020 polarimeter equipped with a sodium lamp (589 nm). ^1H and ^{13}C NMR spectra were obtained on Bruker NMR (Avance

Table 2
^{13}C and ^{1}H NMR spectroscopic data of sapinmusaponins O (**5**), and P (**6**)[a]

	5			**6**		
	^{13}C[b]	^{13}C[c]	^{1}H[b]	^{13}C[b]	^{13}C[c]	^{1}H[b]
1	40.2	39.5	1.63[d], 0.95 *m*	40.2	39.5	1.63[d], 0.95 *m*
2	27.4	27.9	1.95 *m*, 1.64[d]	27.4	27.9	1.95 *m*, 1.64[d]
3	90.0	88.6	3.17 *dd* (10.8, 4.0)	90.0	88.9	3.17 *dd* (11.6, 4.0)
4	40.3	29.5		40.3	39.5	
5	55.4	54.2	0.81 *m*	55.4	54.1	0.81 *m*
6	29.0	29.1	1.63[d], 1.55 *m*	29.0	29.5	1.63[d], 1.55 *m*
7	75.8	76.7	3.68 *dd* (10.8, 4.8)	75.9	76.7	3.70 *dd* (10.4, 4.4)
8	47.2	46.6	–	47.3	46.5	–
9	51.8	46.0	1.25 *m*	51.8	46.6	1.24 *m*
10	37.8	36.8	–	37.8	36.9	–
11	22.6	22.0	1.52[d], 1.31 *m*	22.5	21.9	1.52 *m*, 1.34 *m*
12	26.1	27.0	1.53[d], 1.33[d]	26.6	27.0	1.69[d], 1.35[d]
13	44.3	42.8	1.63[d]	44.2	42.9	1.70[d]
14	50.9	50.2	–	50.6	50.3	–
15	35.6	35.5	1.68[d], 1.35[d]	35.4	35.8	1.69[d], 1.33[d]
16	28.7	25.2	1.85 *m*, 1.22 *m*	28.6	25.8	1.94 *m*, 1.24 *m*
17	49.7	51.7	1.70 *m*	49.7	49.6	1.70[d]
18	10.4	10.5	0.96 *s*	10.4	10.4	0.96 *s*
19	16.9	16.6	0.85 *s*	16.9	16.6	0.85 *s*
20	76.0	74.7	–	76.3	74.6	–
21	25.3	27.9	1.11 *s*	23.4	23.3	1.08 *s*
22	42.2	36.8	1.44 *m*, 1.42 *m*	43.1	43.5	1.44 *m*, 1.39 *m*
23	23.5	23.3	2.03 *m*, 2.01 *m*	23.2	23.3	2.04 *m*, 2.02 *m*
24	125.9	126.2	5.09 *t* (6.8)	125.8	126.1	5.08 *t* (7.2)
25	131.8	130.7	–	131.9	130.8	–
26	25.9	25.7	1.66 *s*	25.9	26.0	1.66 *s*
27	17.7	17.7	1.60 *s*	17.7	17.7	1.58 *s*
28	28.3	27.9	1.04 *s*	28.3	27.9	1.04 *s*
29	17.0	17.1	0.85 *s*	17.1	17.1	0.85 *s*
30	16.7	18.6	0.93 *s*	16.6	18.6	0.93 *s*
Glc-						
1′	105.5	105.4	4.38 *d* (7.2)	105.5	105.4	4.40 *d* (7.2)
2′	78.9	79.9	3.39[d]	78.9	79.8	3.39[d]
3′	79.4	78.2	3.45 *t* (8.8)	79.4	78.2	3.45 *t* (8.8)
4′	72.1	72.1	3.28 *t* (8.4)	72.1	72.4	3.28 *t* (8.4)
5′	77.5	77.7	3.23[d]	77.5	77.7	3.23[d]
6′	62.7	62.9	3.84 *dd* (11.6, 1.2) 3.64 *dd* (11.6, 5.2)	62.7	62.9	3.82 *dd* (10.4, 1.2) 3.64 *dd* (11.6, 5.2)
Rha-						
1″	101.8	101.7	5.35 *br s*	101.8	101.7	5.34 *br s*
2″	71.9	72.5	3.94[d]	72.0	72.4	3.95[d]
3″	71.9	72.4	3.74 *dd* (9.2, 2.4)	72.0	72.4	3.75 *dd* (9.6, 2.4)
4″	73.9	74.1	3.35 *t* (9.6)	73.9	74.2	3.35 *t* (9.6)
5″	69.9	69.6	3.97[d]	69.9	69.6	3.98[d]
6″	17.9	17.1	1.21 *d* (6.0)	17.9	17.1	1.21 *d* (6.0)

[a] *J* values (Hz) in parentheses.
[b] 100 MHz for ^{13}C NMR in CD_3OD.
[c] 125 MHz for ^{13}C NMR in C_5D_5N.
[d] Overlapping signals.

400 MHz) and Varian NMR spectrometers (Unity Plus 500 MHz) using CD_3OD and C_5D_5N as solvent for measurement. FABMS data were performed on a Jeol SX-102A instrument. High-resolution FABMS were measured on a Finnigan/Thermo Quest MAT mass spectrometer. Diaion HP-20, Sephadex LH-20 and silica gel (Merck 70–230 mesh and 230–400 mesh) were used for cc, and precoated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected by spraying with 5% H_2SO_4 and then by heating at 100 °C. Preparative HPLC was performed using a reversed phase column (Cosmosil $5C_{18}$-AR-II column or Hypersil ODS column, 250 × 20 mm i.d.) on a Shimadzu LC-6AD series apparatus with a RID-10A Refractive Index. GC was performed on a Agilent Technologies 6890N Network GC System.

3.2. Plant material

Fruits of *S. mukorossi* were collected from Tainan County, Taiwan in 2004 and the galls of this plant were

Table 3
Cytotoxicity data of **1**–**13** against human tumor cells

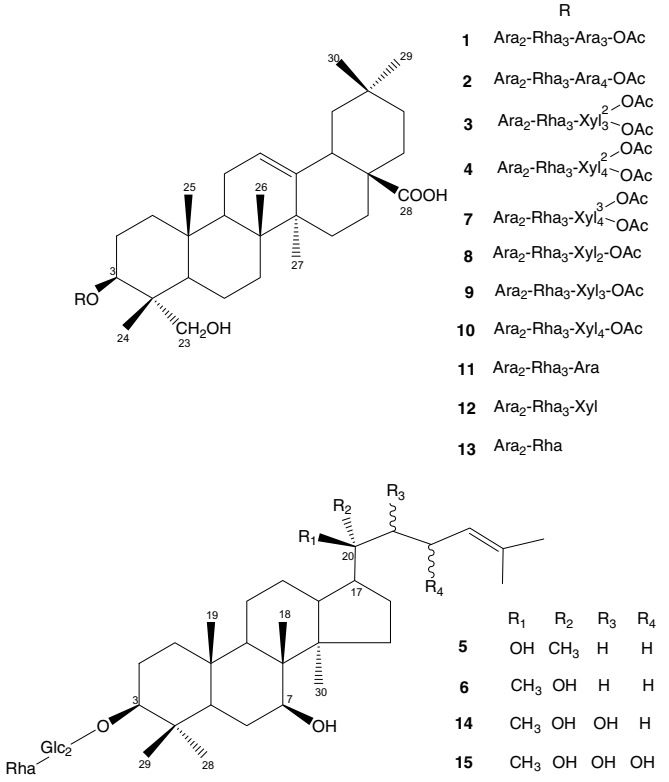
Compounds	ED_{50} (μM)				
	Hela	WiDr	KB	Daoy	Hepa59T/VGH
1	18.10	19.31	5.49	7.09	11.07
2	(–)[a]	13.21	13.33	9.00	19.49
3	11.60	9.68	13.71	17.85	16.78
4	11.36	[b]	5.67	[b]	13.27
5	[a]	[a]	[b]	[a]	[a]
6	[a]	[a]	[b]	[a]	[a]
7	[b]	10.59	14.82	[b]	[b]
8	15.98	12.69	4.53	5.24	15.26
9	19.61	15.26	19.31	[b]	[b]
10	15.98	12.69	12.52	10.04	[b]
11	17.80	6.92	12.93	7.51	[a]
12	[b]	[a]	19.14	19.54	[b]
13	16.16	7.01	[b]	[b]	9.41
Mitomycin-C	0.52	0.38	0.70	0.38	0.35

[a] (–): Inactive, $ED_{50} > 20$ μM.
[b] No test.

collected in October 2001 in the northern mountains of Taiwan, Taipei County. The plants and galls were identified by Professor Muh-Tsuen Kao, National Institute of Chinese Medicine. Voucher specimens (No. NRICM20041117A1 for fruits and NRICM20011007A2 for galls) have been deposited at herbarium for 20 °C in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

3.3. Extraction and isolation

The dried fruits of *S. mukorossi* (5.5 kg) were extracted with EtOH (20 L × 3). The ethanol extracts was partitioned between H_2O and *n*-hexane, $CHCl_3$, and *n*-BuOH, successively. The $CHCl_3$ layer was subjected to a silica gel cc, eluting with $CHCl_3$/MeOH (containing 5% H_2O) in a gradient manner to give 13 fractions. Fraction 7 ($CHCl_3$:MeOH, 1:1) was applied to a 60 × 5.7 cm Diaion HP-20 porous polymer resin column, eluting with 30%, 50%, 70%, 80%, 90%, and 100% aq. MeOH, successively, to yield six fractions (fr. 7.1–7.6). Fr. 7.2 was further separated by chromatography on a Sephadex LH-20 column (30 × 2.5 cm) with MeOH to yield five fractions (fr. 7.2.1–7.2.5). Fraction 7.2.4 was further purified by HPLC on a Hypersil ODS column (250 × 20.0 mm i.d., flow rate: 5 mL/min) with MeOH–H_2O (3:1, v/v) to afford **2** (R_t = 68.6 min, 7.9 mg), and **4** (R_t = 78.7 min, 6.0 mg). Compound **1** (R_t = 50.1 min, 4.6 mg), **3** (R_t = 68.8 min, 8.5 mg), **7** (R_t = 66.0 min, 20 mg) and **8** (R_t = 48.1 min, 20.3 mg) were obtained from fraction 7.2.5 by HPLC with 80% MeOH. Fr. 7.5 was separated by a Sephadex LH-20 cc (35 × 2.5 cm) with MeOH to yield 4 fractions (fr. 7.5.1–7.5.4). Using the same column as that of fraction 7.5.3 on HPLC with MeOH–H_2O (3:2, v/v), **7** (R_t = 85.1 min, 18.5 mg), **9** (R_t = 70.3 min, 20 mg), and **11** (R_t = 72.5 min, 18 mg) were isolated, and **10** (R_t = 34.0 min, 38.3 mg) was yielded from fraction 7.5.4 eluting with MeOH–H_2O (3:2, v/v).



For the treatment of dried galls of *S. mukorossi* (8.5 kg) to give 13 fractions (fr. 1–13), see the previous paper (Kuo et al., 2005). When fraction 11 was further purified by chromatography on a Sephadex LH-20 column (35 × 2.5 cm) with MeOH (1.5 L), four fractions (fr. 11.1–11.4) were obtained. Fraction 11.4 was further purified by HPLC on a Cosmosil 5C_{18}-AR-II column (250 × 20.0 mm i.d., flow rate: 5 min/mL) with 75% MeOH to give **5** (R_t = 93.5 min, 23.0 mg) and **6** (R_t = 104.8 min, 21.0 mg).

3.4. Acid hydrolysis of saponins

Sapinmusaponins K–P (**1**–**6**) (1.5 mg for **1**; each 2.0 mg for **2**–**6**) were refluxed with 2 mL 1 N HCl (1,4-dioxane–H_2O, v/v, 1:1) at 80 °C under reflux conditions for 3 h, respectively. Each mixture was extracted with CH_2Cl_2 to afford the aglycone part, and the aqueous layer was neutralized with Na_2CO_3 and filtered. To the evaporated filtrates were added 1-(trimethylsilyl)imidazole and pyridine (0.2 ml), with the whole stirred at 60 °C for 5 min. After the reaction mixture was dried under a steam of N_2, each residue was partitioned between $CHCl_3$ and H_2O. Each $CHCl_3$ layer was analyzed by GC experiment [conditions: column, CP-Chirasil-L-Val column (25 m × 0.25 mm); injection temperature: 200 °C; column temperature: 100–200 °C; rate: 4 °C/min; and retention times: L-arabinose (12.09), L-rhamnose (12.24 min), D-xylose (15.55 min) and D-glucose (21.24 min)]. Peaks of hydrolysates were detected by comparison with retention times of authentic samples

(L-arabinose and L-rhamnose for **1** and **2**; L-arabinose, L-rhamnose and D-xylose for **3** and **4**; L-rhamnose and D-glucose for **5** and **6**) treating with 1-(trimethylsilyl)imidazole.

3.5. Sapinmusaponin K (*1*)

White powder; $[\alpha]_D^{24}$ +18.9 (MeOH; *c* 0.10); IR (KBr) ν_{max}: 3398, 1692, 1458, 1085 cm^{-1}; for ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) of the sugar moieties, see Table 1; ^1H NMR of the aglycone moiety: δ 0.91, 0.93, 0.99, 1.01, 1.11, 1.22 (each 3H, *s*, CH_3 of C-29, C-25, C-30, C-26, C-24, and C-27) and 3.27 (1H, *dd*, $J = 14.0$, 3.0 Hz, H-18), 3.90, 4.26 (2H, *m*, H-23), 4.26 (1H, *dd*, $J = 12.0$, 5.6 Hz, H-3), 5.45 (1H, *br s*, H-12); ^{13}C NMR of the aglycone moiety: δ 14.1 (C-24), 16.0 (C-25), 17.4 (C-26), 18.1 (C-6), 23.6 (C-30), 23.7 (C-16), 23.7 (C-11), 26.0 (C-27), 26.4 (C-2), 28.2 (C-15), 30.8 (C-20), 32.8 (C-7), 33.1 (C-22), 33.1 (C-29), 34.1 (C-21), 36.8 (C-10), 38.9 (C-1), 39.7 (C-8), 41.9 (C-18), 42.1 (C-14), 43.5 (C-4), 46.3 (C-19),46.5 (C-17), 47.5 (C-5), 48.1 (C-9), 63.9 (C-23), 81.1 (C-3), 122.5 (C-12), 144.7 (C-13), 180.1 (C-28); HR-FAB-MS: *m/z* 947.4974 $[M+Na]^+$ (calcd for $C_{48}H_{76}O_{17}Na$, 947.4980).

3.6. Sapinmusaponin L (*2*)

White powder; $[\alpha]_D^{24}$ +23.1 (MeOH; *c* 0.17); IR (KBr) ν_{max}: 3424, 1693, 1455, 1052 cm^{-1}; for ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) of the sugar moieties, see Table 1; HR-FAB-MS *m/z*: 947.4970 $[M+Na]^+$ (calcd for $C_{48}H_{76}O_{17}Na$, 947.4980).

3.7. Sapinmusaponin M (*3*)

White powder; $[\alpha]_D^{24}$ +2.01 (MeOH; *c* 0.10); IR (KBr) ν_{max}: 3418, 1693, 1461, 1051 cm^{-1}; for ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) of the sugar moieties, see Table 1; HR-FAB-MS *m/z*: 989.5073 $[M+Na]^+$ (calcd for $C_{50}H_{78}O_{18}Na$, 989.5086).

3.8. Sapinmusaponin N (*4*)

White powder; $[\alpha]_D^{24}$ +28.7 (MeOH, *c* 0.12); IR (KBr) ν_{max}: 3433, 1720, 1696, 1464, 1375, 1249, 1053 cm^{-1}; for ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) of the sugar moieties, see Table 1; HR-FAB-MS *m/z*: 989.5069 $[M+Na]^+$ (calcd for $C_{50}H_{78}O_{18}Na$, 989.5086).

3.9. Sapinmusaponin O (*5*)

White powder; $[\alpha]_D^{24}$ −38.4 (MeOH, *c* 0.20); IR (KBr) ν_{max}: 3404, 2938, 1645, 1378, 1047 cm^{-1}; for ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectra, see Table 2; HR-FAB-MS *m/z*: 791.4930 $[M+Na]^+$ (calcd for $C_{42}H_{72}O_{12}Na$, 791.4921).

3.10. Sapinmusaponin P (*6*)

White powder; $[\alpha]_D^{24}$ −18.3 (MeOH, *c* 0.14); IR (KBr) ν_{max}: 3417, 2932, 1646, 1375, 1047 cm^{-1}, for ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectra, see Table 2; HR-FAB-MS *m/z*: 791.4908 $[M+Na]^+$ (calcd for $C_{42}H_{72}O_{12}Na$, 791.4921).

3.11. Cytotoxicity assay

The assay using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-di-phenyltetrazolium bromide] against Hela, WiDr, KB, Daoy, and Hepa59T/VGH human tumor cells was based on the reported methods (Kuo et al., 2001). In brief, the cells were cultured in RPMI-1640 medium supplemented with serum in 5% CO_2 incubated at 37 °C. Test samples and control drug standard were prepared at concentrations of 1, 10, 20 and 40 μg/mL. After seeding 2880 cells/well in a 96-well microplate for 4 h, 20 μL of sample or standard agent was placed in each well and incubated at 37 °C for 3 days, and then 20 μL MTT was added for 5 h. After removing the medium and adding DMSO (200 μL/well) into the microplate with shaking for 10 min, the formazan crystals (the product of MTT reacting with dehydrogenase existing in mitochondria) were redissolved and their absorbance was measured on a model MR 7000 microtiter plate reader (Dynatech International Corporation, Edgewood, New York) at a wavelength of 550 nm. The ED_{50} was defined by comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance.

Acknowledgements

Financial support of this work by the National Science Council (NSC94-2313-B-077-002) and the National Research Institute of Chinese Medicine (NRICM-95-DHM-02) of the Republic of China to Y. H. Kuo is gratefully acknowledged.

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