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Acylation with diangeloyl groups at C21–22 positions in triterpenoid saponins is essential for cytotoxcity towards tumor cells

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ABSTRACT

Saponins are natural surfactants, found in many plants. Certain saponins are bioactive compounds with anticancer, antivirus and hemolytic activities. The mechanism is unknown. A saponin with antitumor activity was identified in *Xanthoceras sorbifolia Bunge* (*Sapindaceae*) and purified. This saponin is a triterpenoid saponin with a trisaccharide chain attached at C3 of the aglycone and two angeloyl groups acylated at C21 and C22. It inhibits the growth of tumor cells with IC50 = 2 μg/ml in OVCAR3 cells. To study the structure–activity relationship, the diangeloyl group or the carbohydrates of Xanifolia-Y were removed and tested for activity. It was found that removal of both angeloyl groups in C21 and C22 positions completely abolished its activity (IC50 > 120 μg/ml). However, when carbohydrates were remove, its activity was reduced but was not abolished (IC50 = 10 μg/ml). These results suggest that a presence of diangeloyl group in the triterpene structure play an essential role for activity. By comparison, compounds with a similar structure as Xanifolia-Y but have only one angeloyl group at C22: Xanifolia-X (IC50 = 6 μg/ml) or at C21: β-escin (IC50 = 10 μg/ml), have less activity. Results suggest that diangeloyl group in both C21 and C22 positions are important contributing activity. Similar results were observed in hemolytic activity. It is concluded that acylation with angeloyl group at C21 and C22 positions of triterpenoid saponin is essential for its activity.

1. Introduction

Saponins are natural surfactants, found in many plants. Saponins are also identified in medicinal plants such as ginseng. Chemically, saponins are glycosides with a distinctive foaming characteristic. They consist of a polycyclic aglycone that is either a steroid or triterpenoid attached at C3 with a sugar side chain.

Certain saponins are bioactive compounds and have medicinal uses. It has been reported that saponins have properties such as anticancer, hypocholesterolemic, immunomodulatory, antiviral, hypoglycemic and anti-oxidant activity [1,2]. The diverse activity of saponins could be explained by its complexity of chemical natures. Structurally, saponin has a general backbone structure, a polycyclic aglycone of either steroid or triterpenoid in nature. However, a complexity of carbohydrate and various acylations attached on the aglycone makes saponin a very heterogenous compound. A specific activity may be derived from a distinct structure. Currently, the drug mechanism of saponin toward a specific action is not well defined. Insufficient information of the structure–activity relationship of saponin hindrances the progress of drug development with saponin. While most of studies focus on the carbohydrate constituents and structures,

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information of the acylated side chains on the aglycone towards functional activity is not known. In this report, a family of compound, Xanifolia-Y from *Xanthoceras sorbifolia* was purified and showed that it has anti-tumor activity. These compounds also have potent hemolytic activity. It was found that acylation at C21 and C22 with angeloyl groups in the triterpene is essential for its activity.

2. Materials and methods

2.1. Preparation of plant extract

The dried husks of *Xanthoceras sorbifolia* was extracted three times with 80% ethanol at 80 °C. The extract was filtered, dried and used for further purification.

2.2. HPLC analysis

The extract of *Xanthoceras sorbifolia* (plant extract), dissolved in 10% acetonitrile–0.005% trifluoro-acetic acid (TFA), was applied into a C18 reverse phase column (μbondapak, 3.9 mm × 300 mm). Bound materials were eluted (flow rate 0.5 ml/min) with an acetonitrile gradient from 10% to 80% in 70 min, and then remains at 80% for 10 min. Fractions were monitored at 207 nm.

2.3. Fractionation of plant extract by FPLC

An Octadecyl functionalized silica column (2 cm × 28 cm) was used. Column was equilibrated with 10% acetonitrile–0.005% TFA before application of samples. The sample was eluted with a gradient of 10–80% acetonitrile. Samples were monitored at 254 nm and collected.

2.4. Isolation and fractionation of individual active components (Xanifolia-Ys)

The active fractions from FPLC were further separated with HPLC with a C18 column (Waters Delta Pak C18-300A). Samples were eluted (isocratic) with 45% acetonitrile–0.005% TFA. Selected fractions (Y1–Y10) were collected and lyophilized.

2.5. Cells

Human cancer cell lines (derived from different tissue types) were obtained from American Type Culture Collection (ATCC). HTB-9 (bladder), HeLa-S3 (cervix), DU145 (prostate), H460 (lung), MCF-7 (breast), K562 (leukocytes), HCT116 (colon), HepG2 (liver), U2OS (bone), SK-Mel-5 (skin), T98G (brain) and OVCAR3 (ovary) were used. Cells were grown in culture medium (HeLa-S3, DU145, MCF-7, HepG2, SK-Mel-5 and T98G in MEN (Earle's salts); HTB-9, H460, K562, OVCAR3 in RPMI-1640; HCT116, U2OS in McCoy-5A) supplemented with 10% fetal calf serum, glutamine and antibiotics in a 5% CO_2 humidified incubator at 37 °C.

2.6. MTT assay

Cell growth activity was determined by MTT assay [3]. Cells were seeded overnight in a 96-wells plate and were exposed to plant extract for 48–72 h. Lyophilized plant extract was weighted and dissolved in DMSO (0.1 g/ml) as stock solution. Appropriate amounts of extract in stock solution were added to culture medium. After the drug-treatment, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (0.5 mg/ml) was added to cultures for an hour. The formation of formazan was dissolved with DMSO and the OD at 490 nm was measured by an ELISA reader. The MTT level of cells before drug-treatment was also measured (T0). The % cell-growth (%*G*) [4] is calculated as

$$\%G = \frac{TD - T0}{TC - T0} \times 100 \quad (1)$$

where TC or TD represent OD readings of control or drug-treated cells.

When T0 > TD, the negative growth is expressed as—% of the control and is calculated as

$$\%G = \frac{TD - T0}{T0} \times 100 \quad (2)$$

The optical density of each drug concentration was determined from the average readings of nine wells of the micro-titer plate. The percentage of cell growth (%*G*) was then plotted against drug concentrations. An *X*–*Y* scatter plot with data points connected by smooth lines (growth curves) was used (Microsoft Excel). The drug concentration at which the growth curve crosses 50% growth is defined as the IC50 value. Experiments were repeated two to three times to obtain an average IC50 (±S.D.) value for a particular cell line (Table 1).

2.7. Determination of hemolytic activity

The hemolysis test is same as described by Voutquenne et al. [5] except that human erythrocytes instead of sheep erythrocytes were used. Erythrocytes (RBC) were isolated from human blood (EDTA whole blood, collected randomly from Texas Gulf Coast Regional Blood Center) through Ficoll-Paque gradient centrifugation. RBC were washed three times with PBS before used. Fifty microliters of the 10% RBC suspension was added to

Table 1 – IC50 of *Xanthoceras sorbifolia* plant extract in different cancer cell lines determined by the MTT assay

Human cancer cell lines (tissue)	Average IC50 values ± S.D. (μg/ml)[a]
OVCAR3 (ovary)	14.5 ± 1
HTB-9 (bladder)	48.3 ± 3
U2OS (bone)	46.7 ± 8
DU145 (prostate)	41.7 ± 8
K562 (leukocyte)	44.3 ± 6
HepG2 (liver)	57 ± 11
MCF-7 (breast)	65 ± 0
T98G (brain)	77.5 ± 11
HCT116 (colon)	103.3 ± 3
H460 (lung)	112.5 ± 4
SK-MEL-5 (skin)	115 ± 7
HeLa-S3 (cervix)	130 ± 14

[a] Results were obtained from two to three independent experiments for each cell lines.

2 ml of saponin solutions (with various concentrations) in PBS. The mixture was vortexed briefly and sat for 60 min at room temperature. The mixture was spun at 3 K for 10 min (table-top centrifuge) and the lysed hemoglobin in the supernatant was measured at 540 nm.

2.8. NMR analysis of Xanifolia-Ys

The pure compound was dissolved in pyridine-D5 with 0.05% (v/v) TMS (tetramethylsilane). NMR spectra (^{1}H, ^{13}C, HMQC, HMBC, NOESY and HOHAHA) were acquired with a Bruker Advance 600 MHz NMR spectrometer with a QXI probe (1H/13C/15N/31P) at 298 K in The Keck/IMD NMR Center, The University of Houston.

2.9. Mass analysis

High resolution mass spectra (HR-MS) of samples was obtained on Applied Biosystems MALDI-TOF Voyager DE-STR Mass Spectrometer. ESI-MS Mass spectra were recorded with LCQ DECA XP plus machine made by Thermo Finnigan.

2.10. Preparation and purification of aglycone and prosapogenin of Xanifolia-Y

Xanifolia-Y was hydrolyzed with 1N HCl in 50% methanol at 80 °C for 5 h. The solution was then neutralized with 1N NaOH. The aglycone was then extracted with ethyl acetate. Purification of aglycone was achieved by chromatography (HPLC) in a C18 reverse phase column eluted with 80% acetonitrile–0.005% TFA. Alkaline hydrolysis was performed at 80 °C in 1 M NaOH for 4 h. The prosapogenin was extracted with 1-butanol, and finally purified with HPLC (C18 reverse column eluted with 25% acetonitrile–0.005% TFA).

3. Results

3.1. Anti-tumor activity of the plant extract of Xanthoceras sorbifolia

The plant extract of *Xanthoceras sorbifolia* (plant extract) was tested for cell growth activity in human tumor cell lines with the MTT assay. Human cells lines derived from 12 tissue types: HTB-9 (bladder), HeLa-S3 (cervix), DU145 (prostate), H460 (lung), MCF-7 (breast), K562 (leukocytes), HCT116 (colon), HepG2 (liver), U2OS (bone), T98G (brain), SK-MEL-5 (skin) and OVCAR3 (ovary) were examined. It was found that the sensitivity among these cells toward cell growth inhibition is heterogeneous. Some cells (e.g. OVCAR3) are sensitive while others (e.g. HeLa-S3) are minimally affected by the plant extract. The IC50 values of plant extract in these cell lines are listed in Table 1. Among these cell lines studied, OVCAR3 cells which derived from ovary are most sensitive toward inhibition of cell growth.

3.2. Fractionation of plant extract and identification of active ingredient

To study the active component of *Xanthoceras sorbifolia*, the plant extract was analyzed with HPLC. Over 30 peaks can be accounted for as shown in the elution profile (Fig. 1A). Four of them are major peaks and the rest are small fractions. These peaks are labeled "A" to "Z" in the elution profile following the increased concentration of acetonitrile.

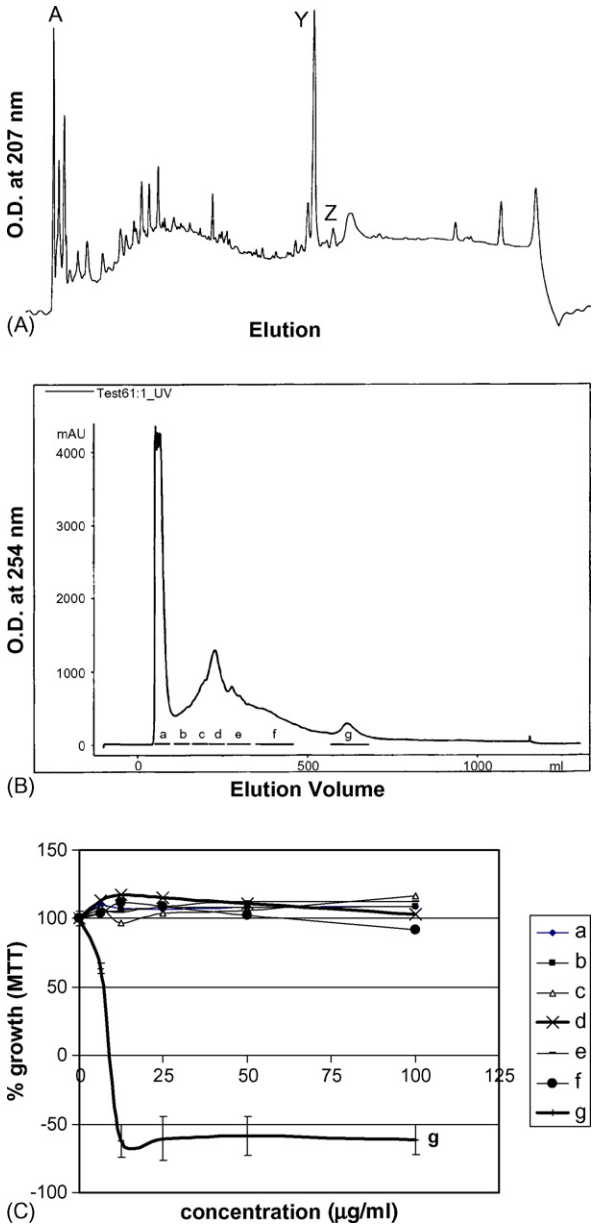

Fig. 1 – Isolation and identification of the active ingredient of *Xanthoceras sorbifolia*. (A) HPLC profile of plant extract. The extract of *Xanthoceras sorbifolia* was applied into a C18 reverse phase column (μbondapak). Bound materials were eluted with acetonitrile (a gradient from 10% to 80%). Over 30 peaks (labeled "A" to "Z") were observed. (B) FPLC profile of plant extract. The plant extract was fractionated with a preparative C18 column. The sample was eluted with a gradient of 10–80% acetonitrile. The FPLC fractions (grouped into seven pools, labeled a to g) were collected for analysis. (C) Inhibition activity of FPLC fractions. The FPLC fractions were analyzed for cell growth inhibition activity in HTB-9 cells (human bladder cancer) with the MTT assay. Only the pool "g" contains inhibition activity.

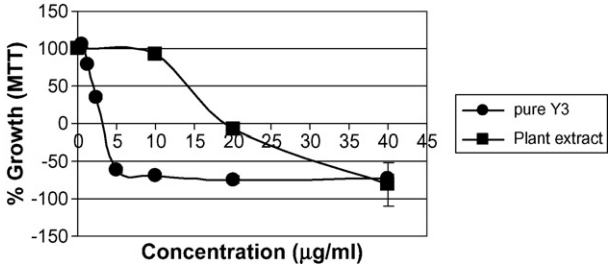

Fig. 2 – Growth curves of cells exposed to Xanifolia-Y or plant extract. Human ovarian cancer cells (OVCAR3) were exposed to Xanifolia-Y or plant extract for 2 days. Inhibition of cells growth was determined by MTT assay. The IC50 for Xanifolia-Y and plant extract are 2.5 and 15 μg/ml, respectively.

The plant extract was then fractionated with a preparative C18 column (Section 2.4) in order to obtain a sufficient quantity for analysis. The elution profile is shown in Fig. 1B. FPLC fractions are grouped into seven pools (labeled a to g in Fig. 1B) and were analyzed for cell growth inhibition activity in HTB-9 cells. Only the pool "g" contains inhibition activity (Fig. 1C). The yield (g) is approximately 6%. Fraction "g" contains components comprising fraction Y in the HPLC profile (Fig. 1A).

Further purification of the active component (from the FPLC fraction g) was achieved with HPLC in a C18 column employing 45% acetonitrile isocratic elution (Section 2.4). Ten compounds (Y1–Y10) were identified in which Y3 is a major component. The major component Y3 was tested and showed inhibition activity as determined by MTT assay with OVCAR3 cells. Fig. 2 shows the growth curves of OVCAR3 cells after exposed to Xanifolia-Y3. The IC50 values is 2.5 ± 0.5 μg/ml which is approximately five to six times more potent than the crude plant extract (IC50 = 15 μg/ml).

3.3. *Chemical analysis*

The chemical structure of the major active compound, Y3 (designated as Xanifolia-Y) was determined with NMR (^{1}H, ^{13}C, HMQC, HMBC, NOESY, HOHAHA) and MS (MALDI-TOF) spectroscopy analysis (Sections 2.8 and 2.9). The NMR spectral data were shown in Table 2. The mass spectral fragments data was shown in Table 3.

Table 2 – ^{13}C and ^{1}H NMR data for Xanifolia-Ys and Xanifolia-X (in Pyridine-d_5)[a]

Position	Compound							
	Xanifolia-Y		AKOH-Y		ACH-Y		Xanifolia-X	
	C	H	C	H	C	H	C	H
1	38.7	0.83, 1.40	38.7	0.79, 1.39	39.1	1.12, 1.64	38.5	1.00, 1.64
2	26.4	1.81, 2.14	26.4	1.82, 2.11	28.1	1.87, 1.90	25.7	1.70, 1.85
3	89.6	3.25 (1H, dd, 12.0/4.0 Hz)	89.7	3.22 (1H, dd, 11.4 Hz/3.6 Hz)	77.8	3.49 (1H, dd, *J* = 10.8 Hz/4.8 Hz)	90.9	3.18 (1H, dd, *J* = 11.4/3.6 Hz)
4	39.4	–	39.5	–	39.5	–	39.1	–
5	55.3	0.78	55.5	.075	55.4	0.99	55.6	0.78 (1H, d, *J* = 11.4 Hz)
6	18.5	1.55, 1.59	18.6	1.35, 1.54	18.9	1.48, 1.65	17.9	1.44, 1.58
7	36.5	2.00, 2.10	36.5	2.00, 2.08	36.6	2.14, 2.21	33.5	1.40, 1.69
8	41.2	–	40.1	–	40.1	–	39.8	–
9	47.0	3.06	47.1	1.67	47.1	1.80	46.4	1.67
10	37.2	–	36.9	–	36.9	–	36.4	–
11	23.7	1.74, 1.89	23.8	1.75, 1.88	23.8	1.89, 2.03	23.3	1.89, 1.93
12	125.2	5.49 (1H, br s)	123.4	5.43 (1H, br s)	125.3	5.59 (1H, br s)	123.9	5.38 (1H, br s)
13	143.4	–	143.1	–	143.2	–	143.7	–
14	47.5	–	47.2	–	47.2	–	40.9	–
15	67.3	4.21	67.1	4.35	67.4	4.28 (1H, d, *J* = 3.0 Hz)	32.5	1.38, 1.60
16	73.6	4.45	71.6	4.88	73.3	4.47 (1H, d, *J* = 3.0 Hz)	68.8	3.99
17	48.3	–	48.2	–	48.2	–	48.5	–
18	40.8	3.07	41.7	2.73 (1H, m)	41.7	3.11 (1H, m)	39.6	2.64 (1H, m)
19	46.8	1.41, 1.69	47.6	1.38, 2.97 (1H, m)	47.0	1.49, 3.14 (1H, m)	46.6	1.20, 2.68 (1H, m)
20	36.2	–	36.3	–	36.3	–	35.8	–
21	79.3	6.71 (1H, d, 10 Hz)	78.2	4.78 (1H, d, *J* = 9.0 Hz)	78.3	6.73 (1H, d, *J* = 10.2 Hz)	80.6	6.00 (1H, d, *J* = 10.2 Hz)
22	73.5	6.32 (1H, d, 10 Hz)	76.4	4.58 (1H, d, *J* = 9.0 Hz)	73.4	6.35 (1H, d, *J* = 10.2 Hz)	72.4	5.88 (1H, d, *J* = 10.2 Hz)
23	27.7	1.26 (3H, s)	27.7	1.25 (3H, s)	28.4	1.26 (3H, s)	27.0	1.08 (3H, s)
24	16.5	1.16, 3H, s	16.5	1.15 (3H, s)	16.5	1.08 (3H, s)	15.0	0.98 (3H, s)
25	16.0	0.81 (3H, s)	15.6	0.82 (3H, s)	16.4	1.00 (3H, s)	15.4	0.88 (3H, s)
26	17.3	0.99 (3H, s)	17.3	1.00 (3H, s)	17.4	1.12 (3H, s)	15.9	0.94 (3H, s)
27	21.0	1.85 (3H, s)	20.9	1.82 (3H, s)	20.1	1.75 (3H, s)	26.3	1.49 (3H, s)

Table 2 (*Continued*)

Position	Compound							
	Xanifolia-Y		AKOH-Y		ACH-Y		Xanifolia-X	
	C	H	C	H	C	H	C	H
28	62.9	3.50, 3.76 (each, 1H, d, *J* = 11.0 Hz)	67.1	3.74, 4.04 (each, 1H, d, *J* = 9.6 Hz)	63.0	3.54, 3.81 (each, 1H, d, *J* = 10.8 Hz)	63.0	2.91, 3.25 (each, 1H, d, *J* = 11.4 Hz)
29	29.2	1.09 (3H, s)	30.3	1.36 (3H, s)	29.3	1.14 (3H, s)	28.4	0.90 (3H, s)
30	20.0	1.32 (3H, s)	19.9	1.32 (3H, s)	20.0	1.37 (3H, s)	18.8	1.11 (3H, s)
3-O-GlcA-p								
1′	104.9	4.89 (1H, d, 7.8 Hz)	104.8	4.87 (1H, d, *J* = 7.2 Hz)			104.1	4.55 (1H, d, *J* = 7.8 Hz)
2′	79.1	4.38	78.2	4.34			77.3	3.74
3′	86.1	4.20	86.1	4.17			85.0	3.68
4′	71.5	4.42	71.6	4.38			70.7	3.62
5′	78.0	4.52	76.4	4.46			75.0	3.86
6′	171.9	–	171.9	–			170.3	–
Butyl-1							59.8	3.02 (2H, m)
Butyl-2							29.3	1.05
Butyl-3							18.4	1.51
Buty-4							12.5	1.16
3-O-Gal-p								
1″	104.6	5.32 (1H, d, 7.7 Hz)	104.5	5.31 (1H, d, *J* = 7.8 Hz)			103.1	4.65 (1H, d, *J* = 7.8 Hz)
2″	73.6	4.42	73.3	4.41			72.0	3.54
3″	74.9	4.10	74.9	4.07			73.6	3.50
4″	69.5	4.56	69.6	4.52			69.1	3.82
5″	76.4	3.94	76.1	3.90			75.8	3.46
6″	61.6	4.43, 4.52	61.7	4.41, 4.47			61.3	3.59, 3.70
Ara-f								
1‴	110.6	6.03 (1H, br s)	110.9	6.04 (1H, br s)			109.4	5.24 (1H, d, *J* = 1.8 Hz)
2‴	83.4	4.94	83.4	4.91			82.0	4.10
3‴	78.3	4.78	77.5	4.72			76.4	3.87
4‴	85.2	4.82	85.0	4.80			83.9	4.06
5‴	62.2	4.12, 4.28	62.2	4.09, 4.29			61.5	3.59, 3.70
21-O-Ang								
1⁗	167.7	–			168.1	–		
2⁗	129.6	–			129.8	–		
3⁗	137.2	5.96 (1H, dq, 7.0/1.5 Hz)			137.2	5.98 (1H, q, 6.0 Hz)		
4⁗	15.5	2.10 (3H, dq, 7.0/1.5 Hz)			15.7	2.11 (1H, dd, 6.0/1.2 Hz)		
5⁗	20.8	2.00 (3H, s)			20.9	2.04 (1H, s)		
21-O-acetyl								
1							169.9	–
2							21.6	2.14 (3H, s)
22-O-Ang								
1⁗	167.9	–			167.9	–	167.8	–
2⁗	129.8	–			129.9	–	127.5	–
3⁗	136.3	5.78 (1H, dq, 7.0/1.5 Hz)			136.2	5.80 (1H, q, 6.0 Hz)	140.0	6.17 (1H, q, *J* = 7.2 Hz)
4⁗	15.5	1.93 (3H, dq, 7.0/1.5 Hz)			15.6	1.98 (1H, dd, 6.0/1.2 Hz)	14.7	2.02 (1H, d, *J* = 7.2 Hz)
5⁗	20.5	1.74 (3H, s)			20.9	1.88 (1H, s)	19.6	1.85 (3H, s)

[a] The data were assigned based on HMQC and HMBC correlations.

The MALDI MS spectrum of Xanifolia-Y (Table 3) exhibited three quasi-molecular ion peaks at *m*/*z* 1163 $[M + Na]^+$, 1185 $[M + 2Na–H]^+$, and 1201 $[M + Na + K–H]^+$, in agreement with a molecular formula of C_{57} H_{88} O_{23}. The ^{13}C NMR data were identical with those of 21, 22-di-O-angeloyl R1-barrigenol (3β, 15α, 16α, 21β, 22α, 28-hexahydroxyolean-12-ene) [6,7] except for C3 and the sugar portion, leading to the assignment of a glycoside of 21, 22-di-O-angeloyl R1-barrigenol. The presence

Table 3 – Mass spectral fragments of Xanifolia-Ys (*m/z*)

Compounds	Xanifolia-Y	AKOH-Y	ACH-Y	Xanifolia-X
Exact mass [M]	1140.5716	976.4879	670.4445	1140.6080
Fragments $[M + Na]^+$	1163.5673	999.4777	693.4377	1163.8761
Fragments $[M + 2Na–H]^+$	1185.5389	1021.4843		1185.8761
Fragments $[M + Na + K–H]^+$	1201.5109	1037.4521		1201.8579

of two angeloyl ester groups in Xanifolia-Y was suggested by the characteristic ^1H and ^{13}C NMR data (Table 2) and their locations at C21 and C22 positions were determined on the basis of long range proton–carbon correlations observed between δH 6.71 (1H, d, J = 10 Hz, H-21) and δC 167.7 (21-*O*-Ang-1‴), as well as between δH 6.32 (1H, d, J = 10 Hz, H-22) and δC 167.9 (22-*O*-Ang-1‴). The carbohydrates of Xanifolia-Y consists of three monosaccharide residues, with anomeric carbon signals observed at δC 104.9, 104.6 and 110.6. These carbons are attached to protons at δH 4.89 (d, J = 7.8 Hz), 5.32 (d,

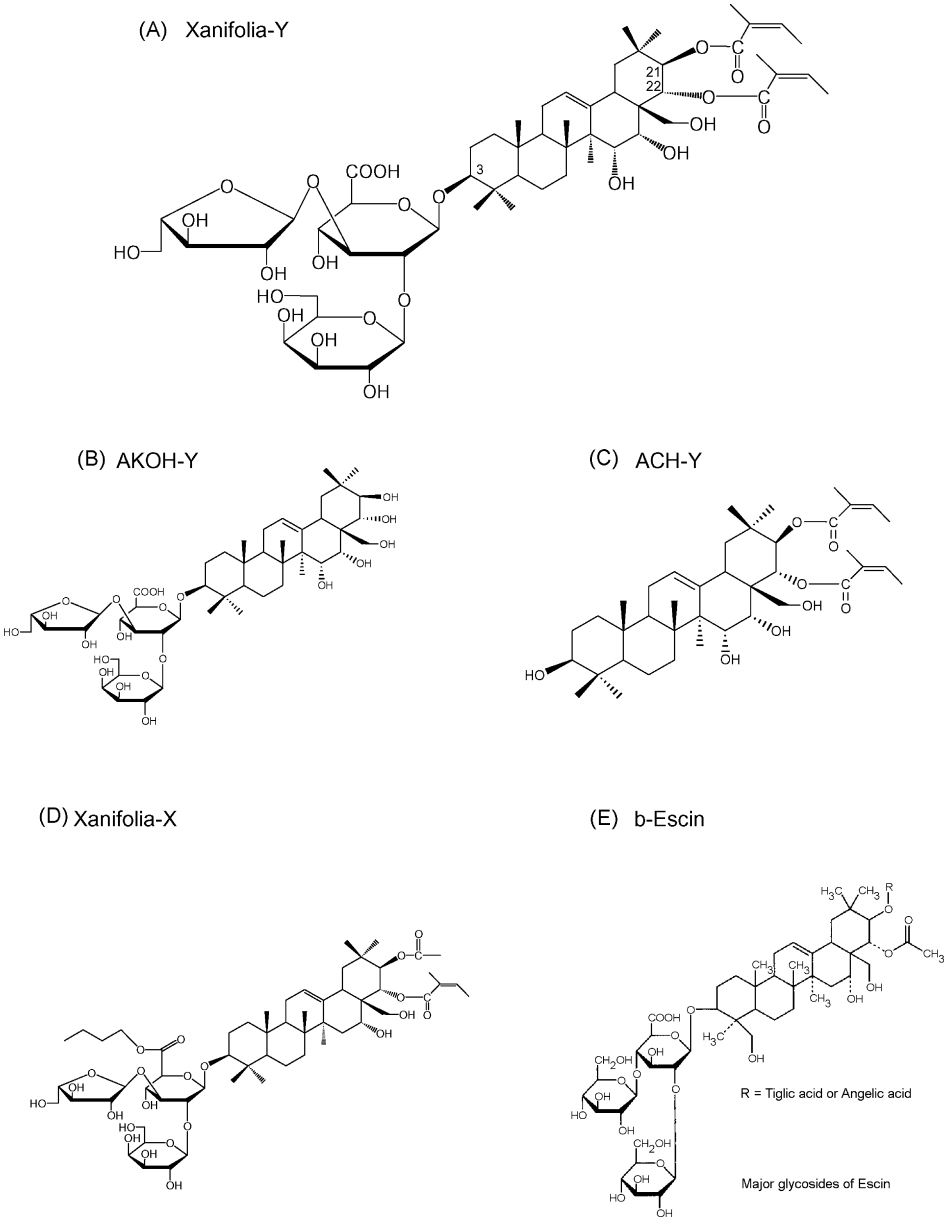

Fig. 3 – Chemical structures of Xanifolia-Y and its derivatives. (A) Xanifolia-Y; (B) AKOH-Y is prosapogenin (without diangeloyl group) of Xanifolia-Y; (C) ACH-Y is sapogenin (without carbohydrates) of Xanifolia-Y; (D) Xanifolia-X, eluted in the HPLC chromatography prior to Xanifolia-Ys, has a similar structure to Xanifolia-Y but has only one angeloyl group at C22. All these compounds were purified and structurally verified before tested for activity. (E) β-Escin (purchased from SIGMA, the picture was obtained from Merck index [11072-93-8]).

J = 7.7 Hz) and 6.03 (br s), respectively (HMQC). The sugar residues were identified to be β-D-glucuronic acid, β-D-galactose [8] and α-L-arabinofuranose [9] on the basis of their NMR data (Table 2). The sequence of sugar residues and the linkage to the aglycone were established by analysis of the HMBC results. Thus, HMBC correlations between δH 4.89 (Glc A-1′) and δC 89.6 (C3), between δH 5.32 (Gal-1″) and δC 79.1 (Glc A-2′), as well as between δH 6.03 (Ara-f-1‴) and δC 86.1 (Glc A-3′) suggested a trisaccharide structure of [β-D-galactopyranosyl-(1 → 2)]-α-L-arabinofuranosyl-(1 → 3)-β-D-glucuronopyranosyl, which is attached to the C3 position of 21, 22-di-O-angeloyl R1-barrigenol through a glycosidic linkage. The NOESY data revealed the relative stereochemistry of the triterpene structure, in particular αH-3, βH-15, βH-16, αH-21 and βH-22. Thus, the following NOESY correlations were observed: H-3 and αCH3-23; H-15 and βCH3-26, βCH3-28; H-16 and βCH3-26, βCH3-28; H-21 and αCH3-27, αCH3-29; H-22 and βCH3-28, βCH3-30.

Based on these NMR data, Xanifolia-Y was determined to be 3-O-[β-D-galactopyranosyl-(1 → 2)]-α-L-arabinofuranosyl-(1→3)-β-D-glucuronopyranosyl-21, 22-O-diangeloyl-3β, 15α, 16α, 21β, 22α, 28-hexahydroxyolean-12-ene. It is a new triterpenoid glycoside. It has a trisaccharide chain attached at C3 of the aglycone and two angeloyl groups are acylated at C21 and C22. Fig. 3A shows the chemical structure of Xanifolia-Y.

3.4. *Studies of structure–activity relationship of Xanifolia-Y: importance of the diangeloyl groups for activity*

A characteristic feature of Xanifolia-Y is the presence of diangeloyl group at C21–22 and a trisaccharide (-[β-D-galactopyranosyl-(1 → 2)]-α-L-arabinofuranosyl-(1 → 3)-β-D-glucuronopyranosyl) at C3. To study the contribution of the diangeloyl group or the carbohydrates of Xanifolia-Y towards its inhibition activity, these groups were separately removed. Removal of the diangeloyl group or carbohydrate, was achieved by alkaline or acid hydrolysis, respectively (Section 2.10). The prosapogenin (AKOH-Y, without diangeloyl group) and the aglycone (ACH-Y, without carbohydrates) of Xanifolia-Y, were purified with HPLC (Section 2.10) and their structures were verified by NMR and MS spectral analysis (Tables 2 and 3 and Fig. 3B and C). For comparison purposes, two compounds: Xanifolia-X and β-escin were also used. Xanifolia-X was eluted in the HPLC chromatography prior to Xanifolia-Ys, and has a similar structure to Xanifolia-Y but has only one angeloyl group at C22 (Tables 2 and 3; Fig. 3D). β-Escin (SIGMA), a triterpenoidal saponin from horse chestnut which has one angeloyl group at C21 position (Merck index [11072-93-8]) (Fig. 3E).

Fig. 4 shows the cell growth inhibition activity with these compounds in OVCAR3 cells as determined by MTT assay. It was found that removal of both angeloyl groups from C21 and C22 positions (AKOH-Y) completely abolishes its activity (no activity observed at120 μg/ml). However, Xanifolia-X, which has one angeloyl group at C22 position and an acetyl group in C21 position, retains 50% of activity (IC50 = 6 μg/ml) as compare to Xanifolia-Y (IC50 = 3 μg/ml). β-Escin which has one angeloyl at C21 position and a acetyl group at C22 has a lower activity (IC50 = 10 μg/ml). Results indicate that compounds with one angeloyl group (at either C21 or C22) have

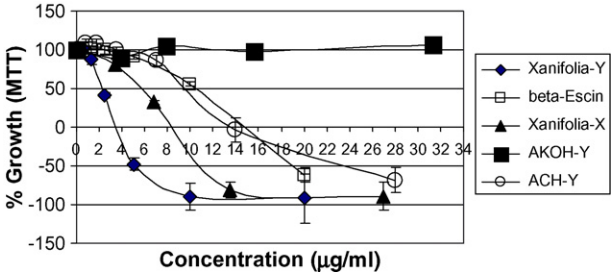

Fig. 4 – Growth curves of cells exposed to Xanifolia-Y and its derivatives. Human ovarian cancer cells (OVCAR3) were exposed to Xanifolia-Y and derivatives for 2 days. Inhibition of cells growth was determined by MTT assay. The IC50 values for these compounds are: Xanifolia-Y, 2.5 μg/ml; Xanifolia-X, 6 μg/ml; ACH-Y, 9.5 μg/ml; β-escin, 10 μg/ml, and AKOH-Y >120 μg/ml.

reduced activities as compared with compound that have two angeloyl groups at C21 and C22 positions. Accordingly, acylation of two angeloyl groups in C21 and C22 positions is more potent for anti-tumor activity.

Fig. 4 also shows the activity of ACH-Y (Xanifolia-Y with carbohydrates removed). It was found that when carbohydrates were remove, it also lost the inhibition activity but the activity was not completely abolished (IC50 = 9.5 μg/ml), suggesting that a presence of diangeloyl group in the triterpenoid structure play an essential role for activity.

Hemolytic activity is generally observed in saponins [5]. It was found that Xanifolia-Y is potent in hemolytic activity. To compare activities among these compounds in respect to the presence of angeloyl group, human blood as hemolytic targets was used. As shown in Fig. 5 and Table 4, the hemolytic activity of Xanifolia-Y is most potent (IC50 = 0.5 μg/ml) among these compounds, next are β-escin (IC50 = 1.8 μg/ml) and Xanifolia-X (IC50 = 2.5 μg/ml). The least active is ACH-Y in which

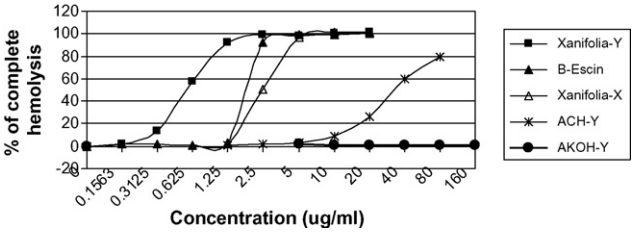

Fig. 5 – Hemolytic activity induced by Xanifolia-Y and derivatives. Human erythrocytes (RBC) were isolated from human blood through Ficoll-Paque gradient centrifugation. RBC were washed three times with PBS before exposed to PBS containing different amounts of saponin. The lysed hemoglobin was measured at 540 nm. Maximum lysis is regarded as complete lysis. Xanifolia-Y is most potent (IC50 = 0.5 μg/ml) among these compounds, next are β-escin (IC50 = 1.8 μg/ml) and Xanifolia-X (IC50 = 2.5). The least active is ACH-Y in which carbohydrate was removed (IC50 = 30). However, AKOH-Y which does not have the diangeloyl groups but retains carbohydrates has no activity (up to 160 μg/ml).

Table 4 – Comparison of cytotoxic and hemolytic activities of Xanifolia-Y

Compounds	Cytotoxic activity against OVCAR3 cells (IC50, μg/ml)	Hemolytic activity against human red blood cells (IC50, μg/ml)
Xanifolia-Y	3	0.5
AKOH-Y	>120	>160
ACH-Y	9.5	30
Xanifolia-X	6	2.5
β-Escin	10	1.8

carbohydrate was removed (IC50 = 30 μg/ml). However, AKOH-Y which does not have the diangeloyl group but retains carbohydrates has no activity (up to 160 μg/ml). These results corroborate with the tumor growth inhibition activity (Fig. 4) that presence of diangeloyl group is essential for the functional activity of Xanifolia-Y.

4. Conclusion

Based on these studies, it is concluded that: (1) Xanifolia-Y isolated from *Xanthoceras sorbifolia* have anti-tumor and hemolytic activities; (2) removal of the carbohydrates from Xanifolia-Y (at C3 position) reduce the cell growth inhibition activity and hemolytic activity. However, removal of both angeloyl groups from C21 and C22 positions abolish both activities. These results indicate that acylation of angeloyl group at C21 and C22 positions of triterpenoid saponin are essential for its activity.

5. Discussion

Saponins with acylations at C21 or C22 positions were reported in literatures. Yet their functional significance is not known. Some of these saponins showed anti-tumor or anti-viral activity. For examples, Jiang et al. [10] purified six triterpenoid saponins from *Maesa Laxiflora* which have one angeloyl group at C22 and pentasaccharide moiety linked to C3 of the aglycon. The plant extract exhibited moderate cytotoxic activity against A549 cells and inhibited microtubules. However, no information about the structure–activity relationship was reported. Apers et al. [11] reported a new series of acylated triterpenoid saponins from *Maesa lanceolata*. These acylated saponins consist of mono-, di- and tri-esters in which C21 is always substituted with an angeloyloxy group and C22 with a variable acyloxy groups. These compounds have antiviral and hemolytic activity. There is no structure–activity relationship reported. Yang et al. [12] studied the anti-HIV activity of several triterpenoid saponins from seeds of *Aesculus chinensis* and compared them with escin. They found that there is no major difference in activity among compounds with in angeloyl or tigloyl group in either C21 or C22 positions. Lu et al. [13] reported triterpenoid saponins from roots of tea plant (*Camellia sinensis var. assamica*). These compounds have diangeloyl group at C21, 22. However, the function of the compound is not known. Seo et al. [14] isolated triterpenoidal saponin from *Pittosporum viridiflorum*, with angeloyl and senecioyl groups in C21, 22 which have weak anti-tumor activity. There is no study on the effect of either these groups or carbohydrates. In all these reports, there is very little study on the structure–activity relationship on these compounds.

Hemolytic activity is a well known property of certain saponins. The structure–activity relationship of hemolytic saponins was extensively reviewed by Voutquenne et al. [5]. In this review, 59 saponins from different sources were compared. It was concluded that hemolytic activity is dependent on the number of sugar unites in the chain, as well as on the presence of an osidic chain in position 3 or of functional groups on the genin such as carboxylic or 16α-OH. Monodesmosidic saponins were generally more active than bidesmodsidic saponins. However, the effect of acylation of C21 and C22 related to activity was not elaborated.

Recently, Voutquenne et al. [15] isolated 8 new acylated triterpenoid saponins from *Harpullia austro-caledonica*. All of them have diangeloyl group in C21–22 position. Interestingly, two of the compounds reported, 3 and 7, have the same structures as Y10 and Y8 reported in our study which was isolated from *Xanthoceras sorbifolia*. Recently, same two compounds were also identified in *Aesculus pavia* [16]. These compounds have potent hemolytic activity against sheep erythrocytes. As reported by Voutquenne et al. [15], a mixture of these compounds as well as two pure compounds (2 and 3) were used and show their hemolytic activities are much higher than commercial saponins (SIGMA). Structure–activity studies were investigated regarding to the carbohydrate's contribution to hemolytic activity and found that arabinofuranosyl instead of rhamnosyl at position 3 of glucuronic acid is more effective.

While structure–activity relationship studies for hemolytic activity have been described in these reviews, structure–activity relationship for anti-tumor activity is yet to be explored. Whether there is correlation between anti-tumor and hemolytic activity of saponin is not known. In Voutquenne's studies, the ethanol extract of the plant exhibited cytotoxic activity against KB cells. In this study, 12 cells lines derived from different human tumor tissues were employed and found that the cells' responses with plant extract is heterogeneous, with certain cell types are more sensitive. Whether other components in the plant extract contribute to the heterogeneous effect remains to be investigated. With the pure compound, its efficacy is much higher. In regards of structure–activity relationship, it was found that triterpenoid saponins (Xanifolia-Y) with two angeloyl groups at C21 and C22 positions has much higher activity than compounds with one angeloyl group on either C21 or C22 position. However, if both angeloyl groups are removed, it completely eliminates activity. In comparing acylation with acetyl or angeloyl group, it seems that angeloyl group is more potent. Same results were

observed in hemolytic activity (Table 4). Accordingly, these results suggest that there is a correlation between anti-tumor and hemolytic activity.

Saponins as a group have multiple biological and pharmacological properties including hemolytic, antiviral, anti-tumor, molluscicidal, anti-inflammatory, antifungal, anti-microbial and anti-parasitic activity [1,2]. The drug mechanism for saponins remains largely unknown and is investigated only recently [17–25]. Weakening membrane integrity and channels forming in membranes after triterpenoid saponin-treatments were recently reported [17,18]. Whether there is a unified or general mechanism for saponin remains to be proven. The diverse and heterogeneous effect of saponin suggests that each individual saponin may have a distinct drug-effect outcome depending on cell types and physiological conditions. Saponin is a group/class of compound which has a diverse structure. The current studies focus on the acylation with angeloyl groups at C21 and C22 position of triterpenoid saponin and show its importance for anti-tumor and hemolytic activity. The fact that loosing two angeloyl groups at C21 and C22 position abolish activity indicates there is an involvement of binding target(s) in cells. It was found that certain cell lines are sensitive while others are not (Table 1), leading to a possibility that sensitive cells may have targets that bind to this drug tighter than those of non-sensitive cells. Future research on this direction will be fruitful.

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