Korean J. Food Preserv.
Vol. 17, No. 3. pp. 311-319, June 2010

사단법인 한국식품저장유통학회
THE KOREAN SOCIETY OF FOOD PRESERVATION

Cytotoxic and Apoptotic Effects of Saponins from *Akebia quinata* on HepG2 Hepatocarcinoma Cells

Hye-Sook Kang, Jae-Seon Kang[1] and Woo-Sik Jeong[†]

School of Food and Life Sciences, Food Science Institute, Inje University, Gimhae 621-749, Korea
[1]Department of Pharmacology, Kyungsung University, Busan 608-736, Korea

으름유래 사포닌의 HepG2 간암세포에 대한 세포독성 및 세포자살유도 효과

강혜숙·강재선[1]·정우식[†]

인제대학교 식품생명과학부, [1]경성대학교 약학과

Abstract

Four saponins (1~4) were isolated from *Akebia quinata* pericarp through bioassay-guided fractionation. Pericarps of *A. quinata* were extracted with ethanol and sequentially fractionated with dichloromethane, ethyl acetate, butanol and water. Compounds 1~4 from the butanol fraction were identified as 3-O-α-L-arabinopyranosyl hederagenin (δ-hederin), 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranoly oleanolic acid (β-hederin), 3-O-β-D-xylopyranosyl (1→3) α-L-arabinopyranosyl hederagenin (saponin C), and 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranosyl hederagenin (α-hederin) based on the spectroscopic evidences, respectively. Oleanolic acid and hederagenin were identified as the corresponding sapogenins by acid-hydrolysis. These compounds exhibited strong cytotoxic activity in MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt] assay on HepG2 cells. β-Hederin obviously attenuated the expression of bcl-2, an anti-apoptotic protein. All of the compounds also induced the activity of caspase-3, an apoptotic enzyme, while α-hederin was the most potent activator of the enzyme. Our data demonstrate for the first time the apoptosis-inducing activity of *A. quinata*. These results suggest that *A. quinata* could be used as a potential source of natural cancer chemopreventive agents.

Key words : *Akebia quinata,* apoptosis, cytotoxicity, saponin, α-hederin, HepG2

Introduction

Apoptosis is the stereotypical program of cellular suicide and an inadequate apoptotic regulation can result in several physiological disorders such as cancer, rheumatoid arthritis and neurodegenerative diseases (1). Bcl-2 families, which either reside or congregate to mitochondrial surface during apoptosis, play a central regulatory role by interacting pro- and anti- apoptotic members (2). Anti-apoptotic proteins such as Bcl-2 inhibit the release of cytochrome c that binds to apoptotic protease-activating factor 1 (Apaf-1) in the cytoplasm and trigger a cascade of caspase activation (3). Caspases, a family of cysteinyl aspartate-specific proteases, are considered responsible for activating other caspases in a cascade or cleaving key cellular proteins such as cytoskeletal proteins, and these processes lead to apoptosis (4). Several natural chemopreventive compounds including catechins, isothiocyanates, anthocyanins, gingerols, curcuminoids and so on, have been reported to induce apoptosis (5-9).

Akebia quinata Dence (Lardizabalaceae), also called 'Moktong', has been used as an antiphlogistic, diuretic and analgesic traditional medicine in Korea, which is a creeping vine widely distributed around East Asia (10,11). Few data have been reported to demonstrate the biological activity of the fruits of *A. quinata.*

[†]Corresponding author. E-mail : jeongws@inje.ac.kr
Phone : 82-55-320-3238, Fax: 82-55-321-0691

In this study, we evaluated bioactivities of the ethanol extract from *A. quinata* fruits and the structural components including whole fruit, pericarp, flesh, and seed. Based on the activity-guided fraction, the most potent pericarp extract was further fractionated and four saponins were identified. Apoptosis-inducing effects of these compounds on HepG2 hepatocarcinoma cells were evaluated.

Materials and Methods

Materials and reagents

Fruits of *Akebia quinata* were collected around the Mt. Jiri, Hamyang Kyoungnam, Korea, in October 2007 and the whole fruits (W) were separated into several part including pericarp (P), flesh (F) and seed (S). They were sliced, dried with hot air (80℃) and powdered. An antibody against Bcl-2 was obtained from Cell signaling Technology, Inc. (Beverly, MA, USA) and β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tris-HCl (pH 7.4), β-glycerophosphate, $MgCl_2$, EDTA, phenylmethylsulfonyl fluoride (PMSF), dithiothrietol (DTT), sucrose, HEPES, CHAPS and digitonin were purchased from Sigma-Aldrich chemicals Co. (St Louis, MO, USA). Colorimetric peptide substrate of caspase-3 (Ac-DEVD-*p*NA) was obtained from Biomol International Inc. (Plymouth Meeting, PA, USA). All the other reagents were of analytical or molecular grade from commercial sources.

Extraction and Isolation

The pericarp powder (3.3 kg) was extracted with 10 L of 95% ethanol (EtOH) at room temperature for 24 hours. The powders of whole fruits, flesh and seed were extracted as the same way. The pericarp EtOH extract (839 g) was suspended in water and partitioned with dichloromethane (DCM), ethyl acetate (EtOAc), n-butanol (BuOH), and water fractions (Fig. 1). BuOH fr. (441.2 g) was subjected to 70-230 mesh silica gel (Merck, Darmstadt, Germany) column chromatography by eluting with lower layer of DCM-MeOH-water (65:35:10) to obtain seven fractions (B1~B7). The B3 (218.4 g) was further fractionated by the silica gel column chromatography with DCM-MeOH-water (100:10:1) as eluting solvent to give 19 sub-fractions (B3-1~B3-19) and yield compound 1 (1.23 g), 2 (0.99 g), and 4 (71 g). The B3-9 fraction (7.9 g) was re-chromatographed on the silica gel with DCM-MeOH-water (80:10:1) to obtain compound 3 (25 mg). Thin layer chromatography (TLC) was also performed on a pre-coated Merck Kisselgel 60 F_{254} plates (0.25 mm) and 50% H_2SO_4 was used as a color detecting reagent.

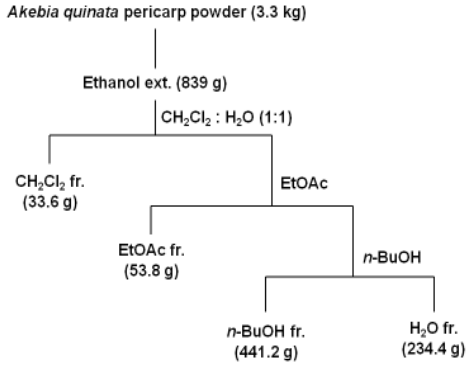


Fig. 1. Extract and fractionation procedure of pericarp of *A. quinata* fruits.

Compound 1 (δ-hederin; 3-O-α-L-arabinopyranosyl hederagenin)

Amorphous white powder, negative LC-MS m/z 604.2 $[M]^-$, positive LC-MS m/z 588.0 $[M+2H-H_2O]^+$, 456.0 $[M+2H-ara-H_2O]^+$, 438.0 $[M+2H-ara-2H_2O]^+$, $C_{35}H_{56}O_8$, ^{1}H-NMR (400MHz, pyridine-d_5) δ: 5.49(1H, brs, H-12), 5.01(1H, d, J=7.03, ara H-1), 3.30(1H, d, J=13.62, H-18), 1.25(3H, s, H-27), 1.04(3H, s, H-26), 1.01(3H, s, H-30), 0.95(3H, s, H-25), 0.94(6H, s, H-24, 29). ^{13}C-NMR (100MHz, pyridine-d_5) data are listed in Table 1.

Compound 2 (β-hederin; 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranoly oleanolic acid)

Amorphous white powder, negative LC-MS m/z 734.2 $[M]^-$, positive LC-MS m/z 440.2 $[M+2H-rhm0ara-H_2O]^+$, $C_{41}H_{66}O_{11}$, ^{1}H-NMR (400MHz, pyridine-d_5) δ: 6.21(1H, s, rhm H-1), 5.49(1H, brs, H-12), 4.92(1H, d, J=5.49 Hz, ara H-1), 3.32(1H, dd, J=3.66, 13.91 Hz, H-18), 1.65(3H, d, J=6.22 Hz, rhm H-6), 1.31(3H, s, H-27), 1.20(3H, s, H-23), 1.09(3H, s, H-24), 1.02(3H, s, H-30), 1.01(3H, s, H-26), 0.97(3H, s, H-29), 0.85(3H, s, H-25). ^{13}C-NMR (100MHz, pyridine-d_5) data are listed in Table 1.

Compound 3 (3-O-β-D-xylopyranosyl (1→3) α-L-arabinopyranosyl hederagenin)

Amorphous white powder, negative LC-MS m/z 736.0$[M]^-$, 472.0 $[M-xyl-ara]^-$, positive LC-MS m/z 738.0 $[M+2H]^+$, 588.0 $[M+2H-xyl-H_2O]^+$, 456.0 $[M+2H-xyl-ara-H_2O]^+$, 438.0 $[M+2H-xyl-ara-2H_2O]^+$, $C_{40}H_{64}O_{12}$, ^{1}H-NMR (400MHz, pyridine-d_5) δ: 5.49(1H, brs, H-12), 5.22(1H, dd, J=2.56, 6.96

Table 1. ^{13}C-NMR spectra of compounds from pericarps of *A. quinata* fruits in pyridine-d_5.

No of carbon	1	2	3	4	Hederagenin	Oleanolic acid [1)]
1	39.26	39.32	39.29	39.45	39.28	38.40
2	26.61	27.01	26.62	26.71	28.16	27.18
3	82.33	89.20	82.23	81.50	73.90	79.03
4	43.99	39.97	44.03	43.99	42.67	38.76
5	50.16	56.38	48.65	50.16	49.10	55.21
6	18.63	18.98	18.61	18.61	19.08	18.30
7	33.36	33.61	33.37	33.32	33.46	32.64
8	40.25	40.19	40.26	40.21	40.25	39.26
9	48.64	48.49	48.04	48.19	46.94	47.61
10	37.43	37.49	37.41	37.36	37.72	37.07
11	24.14	24.24	24.35	24.13	24.34	23.40
12	123.07	123.00	123.08	123.08	123.08	122.66
13	145.28	145.27	145.28	145.26	145.32	143.56
14	42.62	42.62	42.44	42.60	43.37	41.64
15	28.81	28.77	28.81	28.82	28.83	27.68
16	24.35	24.14	24.16	24.31	24.19	22.97
17	47.12	47.12	47.12	47.10	47.15	46.48
18	42.44	42.44	42.63	42.42	42.50	41.05
19	48.07	46.91	46.88	48.63	48.65	45.87
20	31.42	31.44	31.42	31.42	31.44	30.67
21	46.86	34.68	34.68	46.85	34.70	33.79
22	34.67	33.67	33.73	34.66	33.69	32.42
23	64.93	28.53	64.69	64.41	68.40	28.10
24	14.10	17.49	14.14	14.49	13.61	15.54
25	16.58	15.99	16.57	16.54	16.46	15.32
26	17.94	17.83	17.94	17.91	17.97	17.09
27	26.61	26.64	26.62	26.63	26.64	25.90
28	180.66	180.66	180.66	180.67	180.67	181.90
29	33.73	33.76	33.73	33.73	33.73	33.06
30	24.24	24.24	24.24	24.24	24.25	23.56
ara-1	107.22	105.43	107.15	104.95		
2	73.61	76.36	73.33	76.25		
3	75.22	74.47	83.78	75.37		
4	70.14	69.30	69.78	69.94		
5	67.49	65.36	67.29	66.33		
rhm-1		102.25		102.17		
2		72.92		72.88		
3		73.07		73.04		
4		74.56		74.62		
5		70.36		70.17		
6		19.08		19.05		
xyl-1			107.08			
2			74.92			
3			78.75			
4			72.39			
5			67.55			

[1)]Data were determined in $CDCl_3$.

Hz, xyl H-1), 5.02(1H, d, J=7.32 Hz, ara H-1), 3.31(1H, dd, J=4, 13.92 Hz, H-18), 1.26(3H, s, H-27), 1.04(3H, s, H-26), 1.02(3H, s, H-30), 0.94(9H, s, H-24, 25, 29). ^{13}C-NMR (100MHz, pyridine-d_5) data are listed in Table 1.

Compound 4 (α-hederin; 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranosyl hederagenin)

Amorphous white powder, negative LC-MS m/z 750.3 $[M]^-$, positive LC-MS m/z 752 $[M+2H]^+$, 602.0 $[M+2H\text{-rhm}]^+$, 456.0 $[M+2H\text{-rhm-ara-}H_2O]^+$, 438.0 $[M+2H\text{-rhm-ara-}2H_2O]^+$, $C_{41}H_{66}O_{12}$, ^{1}H-NMR (400MHz, pyridine-d_5) δ:6.31(1H, s, rhm H-1), 5.48(1H, brs, H-12), 5.13(1H, d, J=6.64 Hz, ara H-1), 4.18(1H, brs, H-23a), 3.76(1H, d, J=10.37 Hz, H-23b), 3.30(1H, dd, J=3.32, 13.69 Hz, H-18), 1.24(3H, s, H-27), 1.09(3H, s, H-24), 1.03(3H, s, H-30), 1.01(3H, s, H-26), 0.95(3H, s, H-25), 0.94(3H, s, H-29). ^{13}C-NMR (100MHz, pyridine-d_5) data are listed in Table 1.

Hederagenin (230hydroxy-oleanolic acid)

^{1}H-NMR (400MHz, pyridine-d_5) δ: 5.51(1H, brs, H-12), 4.22(1H, dd, J=5.84, 11.36 Hz, H-3), 4.20(1H, d, J=10.6, H-23a), 3.74(1H, d, J=10.24, H-23b), 3.32(1H, dd, J=4, 13.56 Hz, H-18), 1.26(3H, s, H-29), 1.07(6H, s, H-27, 30), 1.02(3H, s, H-26), 0.99(3H, s, H-25), 0.95(3H, s, H-24). ^{13}C-NMR (100MHz, pyridine-d_5) data are listed in Table 1.

Oleanolic acid

^{1}H-NMR (400MHz, $CDCl_3$) δ: 5.29(1H, brs, H-12), 3.12(1H, dd, J=4.1, 10.5 Hz, H-3), 2.82(1H, dd, J=4.1, 13.7 Hz, H-18), 1.14(3H, s, H-27), 0.99(3H, s, H-23), 0.93(3H, s, H-24), 0.91(3H, s, H-30), 0.90(3H, s, H-26), 0.77(3H, s, H-29), 0.76(3H, s, H-25). ^{13}C-NMR (100MHz, $CDCl_3$) data are listed in Table 1.

Acid-hydrolysis of saponin

The acid hydrolysis of saponin (50 mg) was carried out by refluxing with 5% H_2SO_4 (20 mL) at 90℃ for 3 hours. The reaction mixture was partitioned with EtOAc and water, and the upper layer was washed 3 times with water for neutralization. The EtOAc fr. was concentrated and purified on a silica gel column chromatography with EtOAC-Hexane (3:7) to produce sapogenin, which were identified as hederagenin and oleanolic acid by TLC spectroscopic with authentic compounds.

Cell culture and treatments

HepG2 human hepatocarcinoma cells were obtained from

American Type Culture Collections (Manassas, VA, USA). Cells were maintained in F-12 media supplemented with 10% FBS, 1.17 g/L sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, 1% essential amino acids, and 0.1% insulin, in a humidified atmosphere of 95%, 5% CO_2 at 37℃. Cells were seeded in 6-well plates and allowed to grow for 24 hours (about 80% to 90% confluency). Then, the cells were starved overnight with serum-free media prior to further treatments with either vehicle (DMSO, 0.1%) or samples from *A. quinata*.

MTS assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed with CellTiter 96 Aqueous nonradioactive cell proliferation assay kit (Promega Corp., Madison, WI, USA) by the manufacturer's instructions. Briefly, the cells were plated on 96-well plates and incubate for 24 hours. The cells were starved overnight with serum-free media prior to treatments with different doses of each sample for 24 hours. After removing the media, culture media containing MTS and phenazine methosulfate solution were added and incubated for 1-2 hours. The absorbance was measured at 490 nm with ❍Quant ELISA reader (BIO-TEK Instrument, Inc., Winooski, VT, USA).

Western blot analysis

For the evaluation of anti-apoptotic protein expression, cells were treated with different concentrations of samples for various time periods. After treatments, cells were washed with ice-cold PBS (pH 7.4) and harvested with 200 μL of a whole cell lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton-X 100. After homogenizing and standing on ice for 30 minutes, cell lysates were centrifuged at 13,000 × g for 15 minutes at 4℃. The supernatants were collected and equal amounts of total protein of each sample, as determined by BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA), were mixed with 4 × loading buffer, and heated at 95℃ for 5 minutes. The samples were loaded and electrophoresed in a 10% SDS-polyacrylamide gel at 150 V and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) for 1.5 hour using a semi-dry transfer system (Bio-rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in 1 × PBST buffer (0.1% Tween 20 in PBS) for 1 hour at room temperature and incubated with anti-Bcl-2 antibodies in 3% nonfat dry milk (1:500 dilution) overnight at 4℃. After hybridization with primary antibody, membranes were washed three times with PBST, and then incubated with anti-rabbit (for Bcl-2) or anti-goat (for β-actin) antibodies with horseradish peroxidase (Santa Cruz Biotechnology) for 1 hr at room temperature and washed with PBST three times. Final detection was performed with enhanced chemiluminescence (ECL^{TM}) Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Caspase assay

After treatment with isolated compounds for various times (2, 6, 12, 24, 36 and 48 hours) at the concentration of each IC_{50} value observed in MTS assay, cells were harvested in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothrietol (DTT) and 150 μg/mL digitonin. The lysate was passed through 26-G needle 5~6 times in order to homogenize, and centrifuged at 13,000 × g for 10 minutes at 4℃, after leaving for 30 minutes on ice. The supernatants were analyzed for their protein concentrations. The caspase-3 activity was determined in a reaction mixture containing of 10 μg protein and 200 μM Ac-DEVD-pNA as a substrate of caspase-3 in assay buffer (100 mM HEPES, 10% sucrose, 10 mM DTT, and 0.1% CHAPS). After 2~4 hours incubation of the reaction mixture at 37℃, the absorbance was measured at 405 nm using a microplate reader.

Instrumental analyses

The isolated compounds and solvent fractions were analyzed on an Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE, USA). Mobile phase was consisted with HPLC grade water (A) and acetonitrile (B): 0 minute, 15% B; 10 minutes, 30% B; 17 minutes, 65% B; 30 minutes, 90% B; 40 minutes, 90% B; 41 minutes, 15% B; 45 minutes, 15% B. Detection was set at 203 nm at 40℃ with a flow rate of 1 mL/minute. APCI-MS was measured on Agilent-1100 HPLC/MS (G2708DA, Agilent Technologies) using Alltima HP C18 AQ (5μm, 250 × 4.6 mm, Alltech, IL, USA) with HPLC grade water and acetonitrile (Duksan, Korea). ^{1}H-, ^{13}C-NMR and HMQC, HMBC spectra were

measured by JNM ECP-400 spectrometer (Tokyo, Japan).

Results and Discussion

Cytotoxicicity of structural components from *A. quinata* fruit on HepG2 cells

Ethanol extracts of whole fruits of *A. quanta* as well as the structural components including pericarps, flesh, and seeds were evaluated for the inhibitory activity on cell viability of HepG2 cells by a colorimetric MTS assay. The activity was expressed as percent cell viability against that of control (DMSO, 0.1%) (Fig. 2). The concentration of each treatment ranged 2.5 to 100 μg/mL. Pericarp extract displayed the most potent inhibitory activity (IC_{50} 4.70 μg/mL) followed by whole fruits (IC_{50} 8.33 μg/mL) and seeds (IC_{50} 23.5 μg/mL) while the flesh part was not effective even at 100 μg/mL. The most active pericarp extract was divided into five solvent fractions by liquid-liquid partition using hexane, DCM, EtOAc, BuOH and water as shown in Fig.1. Inhibition of each solvent fraction on the cell viability was also determined by MTS assay and the results are in Fig. 3. BuOH fraction exhibited almost complete inhibition even at the concentrations as low as 5 μg/mL and EtOAc fraction also displayed similar results. Based on the activity and the extraction yield, we decided to further fractionate the BuOH fraction to isolate the active compounds.

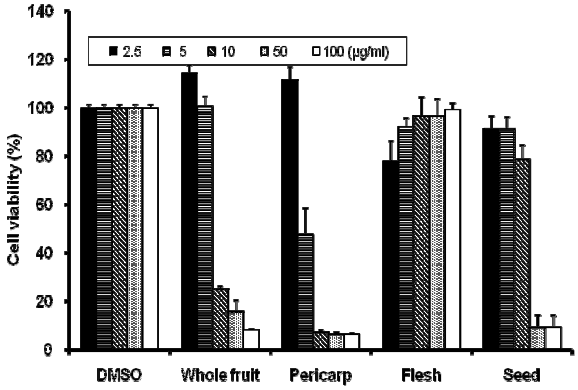


Fig. 2. Effects of ethanol extracts *A. quinata* fruits and its structural components on cell viability of HepG2 cells.

HepG2 cells were treated with vehicle (DMSO, 0.1%) or each EtOH extract for 24 hours and the cell viability was measured by MTS assay. Vertical bars show the mean of 3 replicates and standard deviation.

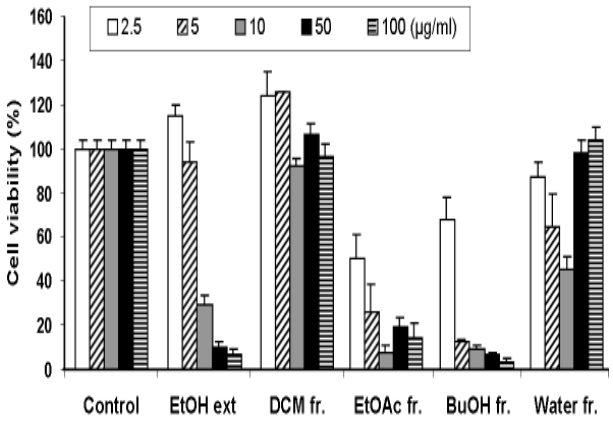


Fig. 3. Effects of solvent fractions from pericarps of *A. quinata* fruits on cell viability of HepG2 cells.

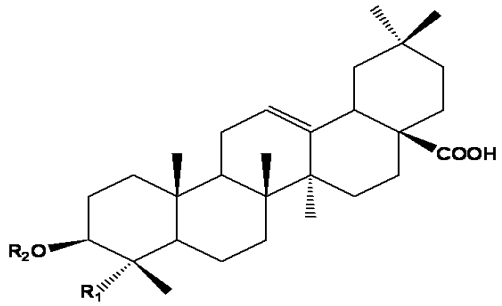
HepG2 cells were treated with vehicle (DMSO, 0.1%) or each solvent fraction for 24 hours and the cell viability was measured by MTS assay. Vertical bars show the mean of 3 replicates and standard deviation.

Purification and Identification of active compounds

Four saponins were isolated from BuOH fraction as described in the materials and method section. Compounds 1~4 were obtained as white amorphous powder and APCI mass spectra of these compounds revealed that they had one or two molecules of sugar moieties (see the materials and methods). The ^{1}H-NMR spectrum of compound 1 showed six tertiary methyl singlets at δ 1.25, 1.04, 1.01, 0.95, 0.94 and 0.94, one broad singlet of olefinic proton at δ 5.49 and one doublet of anomeric proton at δ 5.01 (J=7.03 Hz). The ^{13}C-NMR spectrum of compound 1 revealed the presence of 35 carbon atoms in the molecule. Five carbon signals were assigned to a sugar moiety and the anomeric carbon was shown at δ 107.22. The olefinic carbon signals were at δ 123.07 and 145.28. Based on these results, compound 1 was considered as olean-12-en type triterpenoid glycoside. The HMBC data also showed the location of glycosidic bond at C-3, and finally the compound 1 was identified as 3-O-α-L-arabinopyranosyl hederagenin (δ-hederin). The data of compound 1 were well agreed to the previously published data (12).

The ^{1}H-^{13}-C NMR data of compounds 3 and 4 were very similar to that of compound 1 except the additional sugar moiety of xylose or rhamnose, respectively. Each glycosidic bond was determined by the HMQC and HMBC analyses. Compounds 3 and 4 were identified as 3-O-β-D-xylopyranosyl (1→3) α-L-arabinopyranosyl hederagenin (saponin C) and 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranosyl hederagenin (α-hederin). The spectral data of compound 2 were almost same as that of compound 4 except the methyl moiety of C-23 and it was identified as 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranoly oleanolic acid (β-hederin). The NMR data of those

compounds were consistent with references (12-14). Importantly, it is the first report on original NMR data of saponins from *A. quinata* pericarp, because previous article by Higuchi and Kawasaki (15) reported incomplete ^{1}H- and ^{13}C-NMR spectra of intrinsic compounds.



	R1	R2
OA	CH_3	H
Hgn	CH_2OH	H
1	CH_2OH	Ara
2	CH_3	Rham (1-2) Ara
3	CH_2OH	Xyl (1-3) Ara
4	CH_2OH	Rham (1-2) Ara

Fig. 4. Structures of compounds identified from pericarps of *A. quinata* fruits.

OA, oleanolic acid; Hgn, hederagenin; 1, compound 1; 2, compound 2; 3, compound 3; 4, compound 4

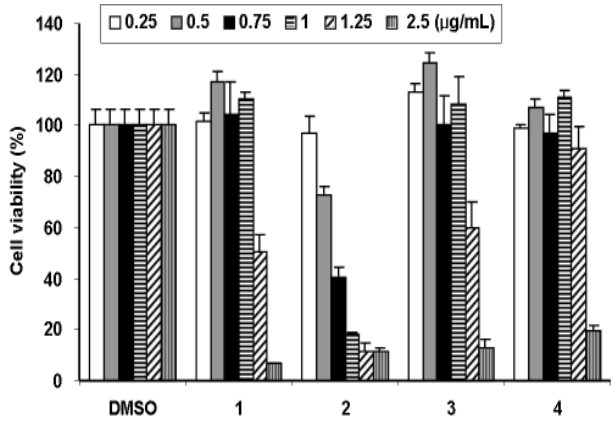


Fig. 5. Effects of saponins isolated from pericarps of *A. quinata* fruits on cell viability of HepG2 cells.

HepG2 cells were treated with vehicle (DMSO, 0.1%) or each compound for 24 hours and the cell viability was measured by MTS assay. 1, compound 1; 2, compound 2; 3, compound 3; 4, compound 4. Vertical bars show the mean of 3 replicates and standard deviation.

Effects of saponins and the corresponding aglycones from *A. quinata* pericarps on the viability of HepG2 cells

The effects of compounds from *A. quinata* pericarp were determined by MTS assay after 24 hours treatment. Compounds 1~4 showed extremely strong cytotoxicity with IC_{50} values of 1.25, 0.68, 1.57 and 1.96 μg/mL, respectively (Fig. 5), suggesting that the cytotoxicity of this fruit on HepG2 cells came from the activity of these isolated compounds. Compound containing oleanolic acid skeleton displayed more potent inhibitory activity than that of hederagenin glycosides. It has been suggested that the type of aglycone, number of sugar and the sequence considerably affect the bioactivities of saponins. Chwalek *et al.* have reported that α-L-rhamnopyranosyl (1→2) α-L-arabinopyranosidic chain in α-hederin is critical for its cytotoxicity in KB cells (16). In this study, β-hederin was more cytotoxic to the cells than α-hederin. Our results indicate that the C-23 methyl group of oleanolic acid is crucial in the cytotoxicity on HepG2 cells. Chwalek *et al*. also suggested the role of methyl group, explaining that the saponin methyl esters were more active than the free carboxylic acid saponins (17). Taken together, the structure of sapogenin, the aglycone, seems obviously more effective in inhibition of HepG2 hepatocarcinoma cells than the corresponding glycosides. In fact, oleanolic acid itself showed stronger activity than hederagenin. The IC_{50} values of oleanolic acid and hederagenin were 4.32 and 12.28 μg/mL, respectively (Fig. 6).

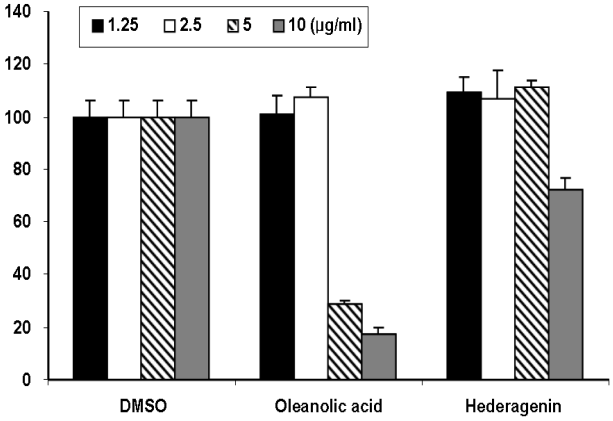


Fig. 6. Effects of sapogenins from pericarps of *A. quinata* fruits on cell viability of HepG2 cells.

HepG2 cells were treated with vehicle (DMSO, 0.1%) or the sapogenins for 24 hours and the cell viability was measured by MTS assay. Vertical bars show the mean of 3 replicates and standard deviation.

Induction of apoptosis by the saponins and sapogenins from *A. quinata* pericarp

In order to analyze the cytotoxic principle of isolated compounds, we determined the effects of these compounds on the apoptosis-related proteins including Bcl-2 and caspase. Cells were treated with compounds from *A. quinata* pericarp for 6, 12 and 24 hours and results are shown in Fig. 7. After 12 hours of treatments, the compound 2 (β

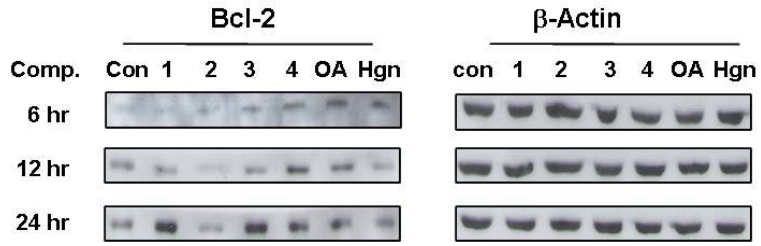


Fig. 7. Bcl-2 protein expression by saponin and sapogenins from pericarps of *A. quinata* fruits in HepG2 cells.

HepG2 cells were treated with vehicle (DMSO, 0.1%) or each compound for various time periods and equal amount of proteins from whole cell lysates were analyzed for Bcl-2 and β-actin by Western blotting. The data shown are representative of three independent experiments with similar results. Con, control; OA, oleanolic acid; Hgn, hederagenin; 1, compound 1; 2, compound 2; 3, compound 3; 4, compound 4

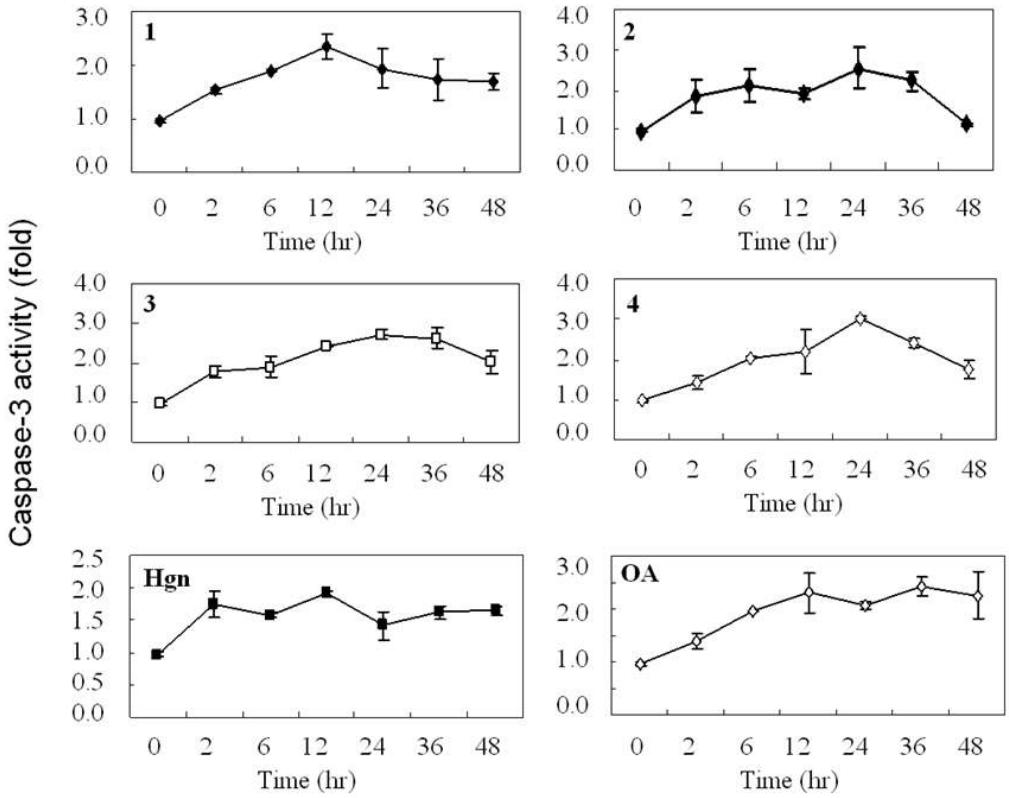


Fig. 8. Caspase-3 actvities of saponins and sapogenins from pericarps of *A. quinata* fruits in HepG2 cells.

HepG2 cells were treated with vehicle (DMSO, 0.1%) or each compound for various time periods at the concentrations of IC_{50} values observed in MTS assay and the casapse-3 activity was measured by a microplate reader as described in the Materials and Methods. OA, oleanolic acid; Hgn, hederagenin; 1, compound 1; 2, compound 2; 3, compound 3; 4, compound 4. Vertical bars show the mean of 3 replicates and standard deviation.

-hederin) clearly attenuated the expression of anti-apoptotic Bcl-2 protein while other compounds did not inhibit the expression of Bcl-2. In addition to the Bcl-2 inhibitory activity, we evaluated the effects of the isolated compounds on the enzyme activity of an apoptotic protein caspase-3 and the results are shown in Fig. 8. After exposure of each compound to HepG2 cells for various time periods (0 to 48 hours), the caspase-3 activity was measured using a colorimetric method described above and the enzyme activity was expressed as the fold induction compared to the activity of the control (0 hour). The treatment concentration of each compound was decided according to the IC50 values in MTS assay. All of the compounds stimulated the enzyme activity of caspase-3 about 2- to 3-fold. Taken together, the cancer cell death by β-Hederin could come not only from its activity to down-regulate the expression of anti-apoptotic protein Bcl-2 but also from its ability to induce the enzyme activity of apoptotic protein caspase-3. Although other compounds

did not inhibit the expression of Bcl-2 protein, they seemed to induce apoptosis of HepG2 cells via activation of capase-3.

In conclusion, the inhibitory activity of *A. quinata* fruits on HepG cells seems to be at least in part originated from the pericarp with active compounds of saponins. Our current results imply that the fruits of *A. quinata* as well as its active compounds can be a potential source of natural chemopreventive and/or chemotherapeutic agents. Further studies on the upstream signaling pathway and biological consequences in vivo should be followed.

요 약

생리활성에 따른 용매분획을 통해 으름(Akebia quinata) 과피로부터 4종의 사포닌을 분리하였다. 으름 과피를 에탄올로 추출한 후 디클로로메탄, 에틸아세테이트, 부탄올 및 물 층으로 순차분획하였으며 분광학적 분석을 통해 부탄올 분획으로부터 3-O-α-L-arabinopyranosyl hederagenin (δ-hederin), 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranoly oleanolic acid (β-hederin), 3-O-β-D-xylopyranosyl (1→3) α-L-arabinopyranosyl hederagenin (saponin C), 및 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranosyl hederagenin (α-hederin)을 구조동정하였다. 또한, 산분해 분석을 통해 oleanolic acid 및 hederagenin을 해당 sapogenin으로 확인하였다. 이들 화합물들은 HepG2 간암세포에서 강력한 세포독성을 나타내었으며 β-hederin의 경우 항세포사멸단백질인 bcl-2의 발현을 억제하는 것으로 나타났다. 분리한 모든 화합물은 세포사멸유도효소인 caspase-3의 효소활성을 촉진하였으며 이중 α-hederin의 활성이 가장 우수한 것으로 확인되었다. 본 연구를 통해 으름의 세포자살유도활성을 최초로 보고하는 바이며 이러한 결과는 으름이 향후 천연항암제로 사용될 수 있는 가능성을 제시하고 있다.

Acknowledgement

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-355-F00019) and also supported in part by a grant (grant No. 0009201000100 to W.S. Jeong) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

References

1. Adams, J.M. and Cory, S. (2001) Life-or-death decisions by the bcl-2 protein family. Trends Biochem. Sci., 26, 61-66
2. Gross, A., McDonnell. J.M. and Korsmeyer, S.J. (1999) Bcl-2 family members and the mitochondria in apoptosis. Genes Dev., 13, 1899-1911.
3. Adams, J.M. and Cory, S. (1998) The Bcl-2 protein family: arbiters of cell survival. Science, 281, 1322-1326
4. Khan, N., Afaq, F. and Mukhtar, H. (2007). Apoptosis by dietary factors: the suicide solution for delaying cancer growth. Carcinogenesis, 28, 233-239
5. Gao, X., Deeb, D., Jiang, H., Liu, YB., Dulchavsky, S.A. and Gautam, S.C. (2005) Curcumin differentially sensitizes malignant glioma cells to TRAIL/Apo2L-mediated apoptosis through activation of procaspases and release of cytochrome c from mitochondria. J. Exp. Ther. Oncol., 5, 39-48.
6. Baliga, M.S., Meleth, S. and Katiyar, S.K. (2005) Growth inhibitory and antimetastatic effect of green tea polyphenols on metastasis-specific mouse mammary carcinoma 4T1 cells *in vitro* and *in vivo* systems. Clin. Cancer Res., 11, 1918-27
7. Larrosa, M., Tomás-Barberán, F.A. and Espín, J.C. (2006) The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J. Nutr. Biochem., 17, 611-625
8. Miyoshi, N., Nakamura, Y., Ueda, Y., Abe, M., Ozawa, Y., Uchida, K. and Osawa T. (2003) Dietary ginger constituent s, galanals A and B, are potent apoptosis inducers in human T lymphoma Jurkat cells. Cancer Lett., 199, 113-119
9. Yang, Y.M., Conaway, C.C., Chiao, J.W., Wang, C.X., Amin, S., Whysner, J., Dai, W., Reinhardt, J. and Chung, F.L. (2002) Inhibition of benzo(a)pyrene-induced lung tumorigenesis in A/J mice by dietary N-acetylcysteine conjugates of benzyl and phenethyl isothiocyanates during the postinitiation phase is associated with activation of mitogen-activated protein kinases and p53 activity and induction of apoptosis. Cancer Res., 62, 2-7
10. Ikuta, A. (1995) Saponins and triterpenes from callus tissues of Akebia trifoliate and comparision with the constituents of other Lardizabalaceous callus tissues. J. Nat. Prod., 58, 1378-1383
11. Park, J.H. (2004) Medicinal plants of Korea. Shinilbooks, Seoul, Korea, p 524-525.
12. Jayasinghe, L., Shimada, H., Hara, N. and Fujimoto, Y. (1995) Hederagenin glycosides from *Pometia eximia*. Phytochemistry, 40, 891-897

13. Park, M.J. and Hahn, D.R. 1991. Saponins from the stem bark of *Kalopanax pictum* var. magnificum (I). Arch. Pharm. Res., 14, 7-11
14. Cheng, M.S., Yang, M.C., Liu, Y., Zheng, L.G. and Liu, J. (2006) Synthesis of β-hederin and hederacolchiside A1: triterpenoid saponins bearing a unique cytotoxicity-inducing disaccharide moiety. Carbohydr. Res., 341, 60-67
15. Higuchi, R. and Kawasaki, T. (1976) Pericarp saponins of *Akebia quinata* Dence. I. Glycosides of hederagenin and oleanolic acid. Chem. Pharm. Bull., 24, 1021-1032
16. Chwalek, M., Lalun, N., Bobichon, H., Plé, K. and Voutquenne-Nazabadioko, L. (2006) Structure-activity relationships of some hederagenin diglycosides: hemolysis, cytotoxicity and apoptosis induction. Biochim. Biophys. Acta, 1760, 1418-1427
17. Chwalek, M., Plé, K. and Voutquenne-Nazabadioko, L. (2004) Synthesis and hemolytic activity of some hederagenin diglycosides. Chem. Pharm. Bull., 52, 965-971

(접수 2010년 2월 3일, 수정 2010년 6월 1일, 채택 2010년 6월 4일)