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ORIGINAL ARTICLE

Chemical constituents of the *Annona glabra* fruit and their cytotoxic activity

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Abstract

Context: Traditional Chinese medicines have attracted increasing interest as potential sources of novel drugs with a wide range of biological and pharmacological activities. *Annona glabra* Linn (Annonaceae) is used in traditional medicine as an anticancer drug. Phytochemical investigation of this plant led to the isolation of acetogenins, *ent*-kauranes, peptides, and alkaloids. In addition, compounds exhibited anticancer, anti-HIV-reserve, and antimalaria.

Objective: Isolation, structure determination, and cytotoxic activity evaluation of compounds from the methanol extract from *A. glabra* fruits.

Materials and methods: Using chromatographic methods to isolate compounds from the *A. glabra* methanol extract. The cytotoxic activity of compounds was evaluated by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, compounds which showed significant cytotoxic activity were chosen for further study apoptosis characteristics.

Results: One new, (2*E*,4*E*,1′*R*,3′*S*,5′*R*,6′*S*)-dihydrophaseic acid 1,3′-di-*O*-β-D-glucopyranoside, and eight known compounds, (2*E*,4*E*,1′*R*,3′*S*,5′*R*,6′*S*)-dihydrophaseic acid 3′-*O*-β-D-glucopyranoside (**2**), icariside D_2 (**3**), icariside D_2 6′-*O*-β-D-xylopyranoside (**4**), 3,4-dimethoxyphenyl *O*-β-D-glucopyranoside (**5**), 3,4-dihydroxybenzoic acid (**6**), blumenol A (**7**), cucumegastigmane I (**8**), and icariside B_1 (**9**), were isolated from the fruits of *A. glabra*. Icariside D_2 (**3**) was found to show significant cytotoxic activity on the HL-60 cell line with the IC_{50} value of 9.0 ± 1.0 μM and did not show cytotoxic activity on the Hel-299 normal cell line. The further test indicated that compound **3** induced apoptosis via alteration of expression of apoptosis-related proteins and decreased phosphorylation of AKT in HL-60 cells.

Discussion and conclusion: The results suggested that the constituents from *A. glabra* may contain effective compounds which can be used as anticancer agents.

Keywords

Annonaceae, apoptosis, HL-60

History

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Introduction

Traditional Chinese medicines have attracted increasing interest as potential sources of novel drugs with a wide range of biological and pharmacological activities, including anticancer and other activities (Vickers, 2002). In 2014, there will be an estimated 1 665 540 new cancer cases diagnosed and 585 720 cancer deaths in the United States. Cancer remains the second most common cause of death in the US, accounting for nearly one of every four death. More than half of currently available drugs are natural compounds or are related to them, and in the case of cancer, this proportion surpasses 60% (Newman & Cragg, 2007). The discovery and the identification of new antitumor drugs with low side effects on the immune system have become an essential goal in many studies of immunopharmacology (Altmann & Gertsch, 2007). To find new medications, many attentions have been focused on natural compounds in plants, marine organisms, and microorganisms.

Annona glabra L. (Annonaceae) is a tropical tree growing widely in the America and Asia. The whole plant is used in traditional medicine as an anticancer drug (Cochrane et al., 2008). Phytochemical investigation of this plant led to the isolation of acetogenins (Liu et al., 1999), *ent*-kauranes (Chen et al., 2000, 2004), peptides (Li et al., 1999), and alkaloids (Likhitwitayawuid et al., 1993; Tsai & Lee, 2010). In addition, they exhibited anticancer (Chen et al., 2004), anti-HIV-reserve (Chang et al., 1998), and antimalaria activities (Likhitwitayawuid et al., 1993). As part of our continuing efforts to find anticancer compounds, one new megastigmane glycoside and eight known compounds were isolated from the fruit of *A. glabra*.

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Materials and methods

General experiment

The NMR spectra were recorded on a Bruker DRX 500 spectrometer (Bruker, Rheinstetten, Germany) using TMS as an internal standard. The HR-ESI-MS were obtained using an AGILENT 6550 iFunnel Q-TOF LC/MS system (Agilent Technologies Inc., Santa Clara, CA). GC spectra were recorded on a Shimadzu-2010 spectrometer (Shimadzu Corporation, Kyoto, Japan). Circular dichroism spectra were determined on a Chirascan™ CD spectrometer (Applied Photophysics Ltd., Surrey, UK). Optical rotations were determined on a Jasco DIP-370 automatic polarimeter (Jasco, Tokyo, Japan).

Plant material

The fruit of *A. glabra* was collected in Ho Chi Minh City, Vietnam, during May 2013, and identified by Dr. Bui Van Thanh, Institute of Ecology and Biological Resources, VAST. A voucher specimen (AG1605) was deposited at the Herbarium of Institute of Marine Biochemistry, VAST.

Extraction and isolation

The dried fruit of *A. glabra* (4.0 kg) was extracted with MeOH (3×5 L, 50 °C) under sonication for 1 h to yield 300.0 g extract after evaporation of the solvent. This extract was suspended in H_2O (2.0 L) and successively partitioned with *n*-hexane, $CHCl_3$, and ethyl acetate (EtOAc) to obtain the *n*-hexane (AG1, 51.0 g), $CHCl_3$ (AG2, 190.5 g), EtOAc (AG3, 3.5 g), and H_2O (AG4, 54.0 g) extracts after removal of the solvents *in vacuo*. The AG2 extract was chromatographed on a silica gel column and eluted with *n*-hexane–EtOAc gradient (100:1→1:1, v/v) to obtain four fractions AG2A–AG2D. The AG2D fraction was chromatographed on a silica gel column eluting with *n*-hexane–acetone (2:1, v/v) to obtain three fractions AG2D1–AG2D3. The AG2D2 fraction was chromatographed on an YMC RP-18 column eluting with acetone–water (2.5:1, v/v) to yield compound **7** (7.0 mg). The water soluble fraction AG4 was chromatographed on a Diaion HP-20 column (Mitsubishi Chem. Ind. Co., Tokyo, Japan) eluting with water containing increasing concentrations of MeOH (0, 25, 50, 75, and 100% MeOH) to give four fractions AG4A–AG4D. The AG4A fraction was chromatographed on an YMC RP-18 column eluting with acetone–H_2O (1:2, v/v) to give three fractions AG4A1–AG4A3. The AG4A1 fraction was chromatographed on a silica gel column eluting with CH_2Cl_2–MeOH (5:1, v/v) to give two fractions AG4A1A–AG4A1B. The AG4A1B fraction was chromatographed on a silica gel column eluting with *n*-hexane–EtOAc (2:1, v/v) to yield compound **1** (5.0 mg). The AG4A2 fraction was chromatographed on a silica gel column eluting with CH_2Cl_2–MeOH (10:1, v/v) to give two fractions AG4A2A–AG4A2B. The AG4A2B fraction was chromatographed on a silica gel column eluting with *n*-hexane–EtOAc (3:1, v/v) to yield compounds **4** (6.0 mg) and **6** (5.0 mg). The AG4A3 fraction was chromatographed on an YMC RP-18 column eluting with acetone–H_2O (3:1, v/v) to yield compounds **2** (8.0 mg), **8** (6.0 mg), and **9** (7.0 mg). The AG4C fraction was chromatographed on a silica gel column eluting with CH_2Cl_2–MeOH (6:1, v/v) to give three fractions AG4C1–AG4C3. The AG4C2 fraction was chromatographed on a silica gel column eluting with CH_2Cl_2–EtOAc (10:1, v/v) to give three fractions AG4C2A–AG4C2C. The AG4C2B was chromatographed on an YMC RP-18 column eluting with MeOH–H_2O (1:1, v/v) to yield compounds **3** (5.0 mg) and **5** (5.0 mg).

Table 1. ^{1}H- and ^{13}C-NMR data for compound **1**.

Pos.	δ_C[a,b]	δ_H[a,c] (mult., *J* in Hz)
1	166.0	–
2	117.8	5.86 (s)
3	154.0	–
4	131.8	8.05 (d, 16.0)
5	136.4	6.62 (d, 16.0)
6	21.3	2.14 (s)
1′	48.8	–
2′	42.8	1.82 (m)
		2.02 (m)
3′	73.9	4.28 (m)
4′	42.9	1.82 (m)
		2.21 (m)
5′	87.6	–
6′	83.3	–
7′	77.1	3.78 (d, 7.0)
		3.83 (d, 7.0)
8′	16.3	0.96 (s)
9′	19.7	1.19 (s)
1-*O*-Glc		
1″	95.4	5.54 (d, 8.0)
2″	75.1	3.17 (t, 8.0)
3″	78.0	3.30 (m)
4″	71.7	3.30 (m)
5″	78.8	3.42
6″	62.8	3.70 (m)
		3.87 (m)
3′-*O*-Glc		
1‴	103.0	4.38 (d, 8.0)
2‴	74.0	3.40 (m)
3‴	78.0	3.46 (t, 8.0)
4‴	71.1	3.39 (m)
5‴	78.1	3.39
6‴	62.4	3.70 (m)
		3.87 (m)

[a]Measured in CD_3OD.
[b]125 MHz.
[c]500 MHz. Assignments were done by HMQC, HMBC, and NOESY experiments.

(2*E*,4*E*,1′*R*,3′*S*,5′*R*,6′*S*)-*dihydrophaseic acid* 1,3′-di-*O*-β-D-*glucopyranoside* (**1**): A white amorphous powder $[\alpha]_D^{25}$: −25.0 ($c = 0.1$, MeOH), $C_{27}H_{42}O_{15}$, HR-ESI-MS found *m/z* 629.2431 $[M + Na]^+$ (Calcd $C_{27}H_{42}O_{15}Na$ for 629.2416), CD ($c = 1.5 \times 10^{-5}$, MeOH), $[\theta]$ (λ_{max}, nm) −52481 (237), ^{1}H- and ^{13}C-NMR (Table 1).

Acid hydrolysis

Compound **1** (0.5 mg) was dissolved in 1N HCl (dioxane–H_2O, 1:1, 1 mL) and heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with Ag_2CO_3 and the solvent evaporated under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 mL of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solutions. The mixture was heated at 60 °C for 2 h, and 0.1 mL of TMS was added, followed by heating at 60 °C for 1.5 h. The dried

products were partitioned with *n*-hexane and H_2O (0.1 mL each), and the organic layer was analyzed by GC: column SPB-1 (0.25 mm × 30 m); detector FID, column temp. 210 °C, injector temp. 270 °C, detector temp. 300 °C, and carrier gas He (2 mL/min). A peak was found at the retention time of 14.11 min. When standard solutions were prepared by the same reaction from the standard glucoses, the retention times of persilylated D-glucose and L-glucose were 14.11 and 14.26, respectively. By comparing of retention times, the sugar in compound **1** was determined to be D-glucose.

Cytotoxic assay

Effects of compounds **1–9** on the growth of human cancer cells, HL-60 (leukemia cancer), were determined by measuring the cytotoxic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The HL-60 and HEL-299 cell lines were grown in the RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 g/mL, respectively) at 37 °C in a humidified 5% CO_2 atmosphere. The exponentially growing cells were used throughout the experiments. The MTT assays were performed as follows: human cancer cells (3×10^5 cells/mL) were treated for 3 d with 0.01, 0.1, 1, 10, 50, and 100 μM of compounds and as mitoxantrone. After incubation, 0.1 mg (50 μL of a 2 mg/mL solution) MTT (Sigma, St. Louis, MO) was added to each well and the cells were then incubated at 37 °C for 4 h. The plates were centrifuged at 1000 rpm for 5 min at room temperature and the media were then carefully aspirated. Dimethylsulfoxide (150 μL) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech, Piscataway, NJ). All the experiments were performed three times and the mean absorbance values were calculated. The results are expressed as the percentage of inhibition that produced a reduction in the absorbance by the treatment of samples compared with the untreated controls. A dose–response curve was generated and the inhibitory concentration of 50% (IC_{50}) was determined for each compound.

Morphological analysis of apoptosis by Hoechst 33342 staining

For detection of apoptosis, cells were seeded at 3×10^5 cells/mL (HL-60) in 1 mL on 24-well microplates. After 24 h of incubation to allow cell attachment, cells were treated for 24 and 48 h with the IC_{50} concentration of compound **3**. The cells were incubated in a Hoechst 33342 (Hoechst 33342 was diluted in culture medium at a final concentration of 10 μg/mL) staining solution at 37 °C for 20 min. The stained cells were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (Olympus Imaging America Inc., Center Valley, PA) (magnification × 200).

Western blot analyses

HL-60 cell line (3×10^5 cells/mL) was treated with the IC_{50} concentration of compound **3** for 24 h and 48 h. After treatment, the cells were harvested and washed two times with cold PBS. The cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 25 μg/mL aprotinin, 25 μg/mL leupeptin, and 1% Nonidet P-40) and kept on ice for 30 min. The lysates were centrifuged at 15 000 rpm at 4 °C for 15 min. The supernatants were stored at −20 °C until use. The protein content was determined by the Bradford assay. The same amount of lysates was separated on 8–10% SDS-PAGE gels and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) by glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], and 20% MeOH [v/v]) at 100 V for 2 h. After blocking with 5% non-fat dried milk, the membrane was incubated with primary antibody against PARP (1:1000), cleaved caspase-3 (1:1000), Bcl-2 (1:500), Bax (1:1000), P-AKT (1:1000), AKT (1:1000), C-myc (1:1000), and β-actin antibodies and incubated with a secondary HRP antibody (1:5000; Vector Laboratories, Burlingame, VT) at room temperature. The membrane was exposed on X-ray films (AGFA, Mortsel, Belgium), and protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON., Gyeonggi-do, Korea).

Statistical analysis

Data are presented as the means ± SD. Statistical significance was set at $p < 0.05$ and $p < 0.01$. Statistically significant differences were determined by the analysis of variance using SPSS statistical software (SPSS Inc., Chicago, IL).

Results and discussion

Compound **1** was obtained as a white amorphous powder and its molecular formula was determined to be $C_{27}H_{42}O_{15}$ by HR-ESI-MS at *m/z* 629.2431 (Calcd $[C_{27}H_{42}O_{15}Na]^+$ for 629.2416). The ^{1}H-NMR spectrum of compound **1** showed the signals for three tertiary methyl at δ_H 0.96, 1.19, and 2.14 (each 3H, s), three olefinic protons at δ_H 5.86 (1H, s), 6.62 (1H, d, $J = 16.0$ Hz), 8.05 (1H, d, $J = 16.0$ Hz), two anomeric protons at δ_H 4.38 (1H, d, $J = 8.0$ Hz), and δ_H 5.54 (1H, d, $J = 8.0$ Hz), indicating the presence of one megastigmane aglycone and two sugar moieties as listed in Table 1. The ^{13}C-NMR and DEPT spectra of compound **1** revealed the signals for 27 carbons: one carbonyl, four nonprotonated carbons, 14 methine (including eleven oxygenated methines), five methylene, and three methyl carbons. The ^{1}H and ^{13}C-NMR data of compound **1** were similar to those of (2*E*,4*E*,1′*R*,3′*S*,5′*R*,6′*S*)-dihydrophaseic acid 3′-*O*-β-D-glucopyranoside (**2**) (Ngan et al., 2012), except for an addition of one sugar moiety at C-1 of the aglycone. The HMBC cross peaks from H-5 (δ_H 6.62) to C-3 (δ_C 154.0), C-1′ (δ_C 48.8), C-5′ (δ_C 87.6), and C-6′ (δ_C 83.3); from H-6 (δ_H 2.14) to C-2 (δ_C 117.8), C-3 (δ_C 154.0), and C-4 (δ_C 131.8); and from H-2 (δ_H 5.86) to C-1 (δ_C 166.0), C-3 (δ_C 154.0), and C-6 (δ_C 21.3) confirmed that two double bonds were at C-2/C-3 and C-4/C-5 (see Figure 1). In addition, the coupling constant between H-4 and H-5, $J = 16.0$ Hz, confirmed the *E* configuration at C-4/C-5. The observation of NOESY correlation between H-5 (δ_H 6.62) and H-6 (δ_H 2.14) as well as no NOESY correlation between H-2 (δ_H 5.86) and H-6 (δ_H 2.14) proved the

E configuration at C-2/C-3. The position of oxygen bridge between C-5′ and C-7′ was based on the HMBC cross peaks from H-7′ (δ_H 3.78 and 3.83) to C-1′ (δ_C 48.8), C-2′ (δ_C 42.8), C-5′ (δ_C 87.6), C-6′ (δ_C 83.3), and C-8′ (δ_C 16.3). Moreover, the CD spectrum of compound **1** showed one negative Cotton effect at 237 nm, similar to those of (2*E*,4*E*,1′*R*,3′*S*,5′*R*,6′*S*)-dihydrophaseic acid 3′-*O*-β-D-glucopyranoside (**2**) (Ngan et al., 2012), suggested that the configuration at C-6′ was *S*. The H-3′ (δ_H 4.28) showed the NOESY correlation with H_a-7′ (δ_H 3.83), indicating an α-orientation of H-3′. Acid hydrolysis of compound **1** revealed D-glucose (identified as a TMS derivative by a gas chromatography method). Furthermore, the HMBC correlations between H-1″ (δ_H 5.54) and C-1 (δ_C 166.0); H-1‴ (δ_H 4.38) and C-3 (δ_C 73.9) suggested that two glucopyranosyl moieties were at C-1 and C-3′ of the aglycone. Based on the above evidence, the structure of compound **1** was determined to be (2*E*,4*E*,1′*R*,3′*S*,5′*R*,6′*S*)-dihydrophaseic acid 1,3′-di-*O*-β-D-glucopyranoside.

The remaining compounds were identified as (2*E*,4*E*,1′*R*,3′*S*,5′*R*,6′*S*)-dihydrophaseic acid 3′-*O*-β-D-glucopyranoside (**2**) (Ngan et al., 2012), icariside D_2 (**3**) (Miyase et al., 1989), icariside D_2 6′-*O*-β-D-xylopyranoside (**4**) (Zhao et al., 2001), 3,4-dimethoxyphenyl *O*-β-D-glucopyranoside (**5**) (Pan & Lundgren, 1995), 3,4-dihydroxybenzoic acid (**6**) (Scott, 1972), blumenol A (**7**) (González et al., 1994), cucumegastigmane I (**8**) (Kai et al., 2007), and icariside B_1 (**9**) (Hisamoto et al., 2004) (see Figure 2). Their structures were established on the basis of spectral and chemical evidence, which were in agreement with those reported in the literature.

All compounds were evaluated for cytotoxic activity on HL-60 cancer cell line. Mitoxantrone was used as a positive control with the IC_{50} value of 6.8 ± 0.9 μM (Table 2). As the results, compound **3** showed significant activity on HL-60 with the IC_{50} value of 9.0 ± 1.0 μM. Compounds **4–6** exhibited moderate activity with the IC_{50} values ranging from 32.2 to 64.6 μM. The remaining compounds did not show cytotoxic activity. Compound **3** was chosen for evaluating on the Hel-299 normal cell line and this compound did not show cytotoxic activity (IC_{50} value >100 μM). Due to the significant cytotoxic activity of compound **3** on the HL-60 cell line, we investigated whether the inhibitory effect of compound **3** might arise from the induction of apoptosis. Apoptotic cell death has typical characteristics, such as chromatin condensation, membrane blebbing, cell shrinkage,

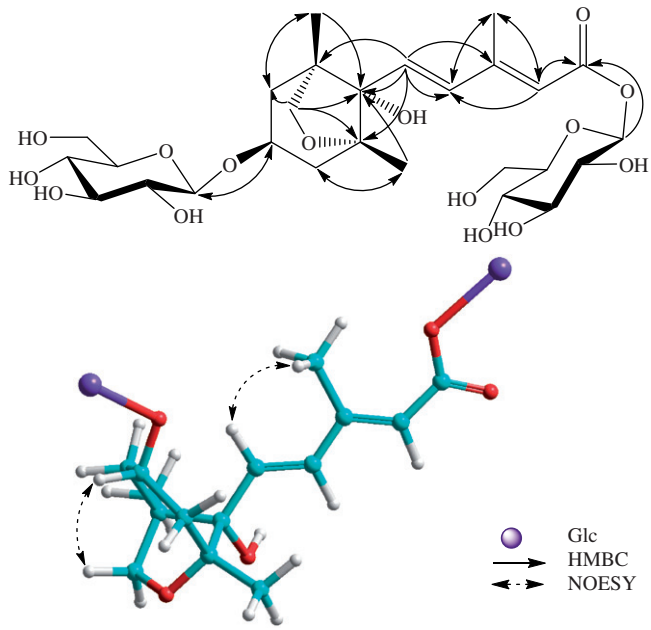

Figure 2. The important HMBC and NOESY correlations of compound **1**.

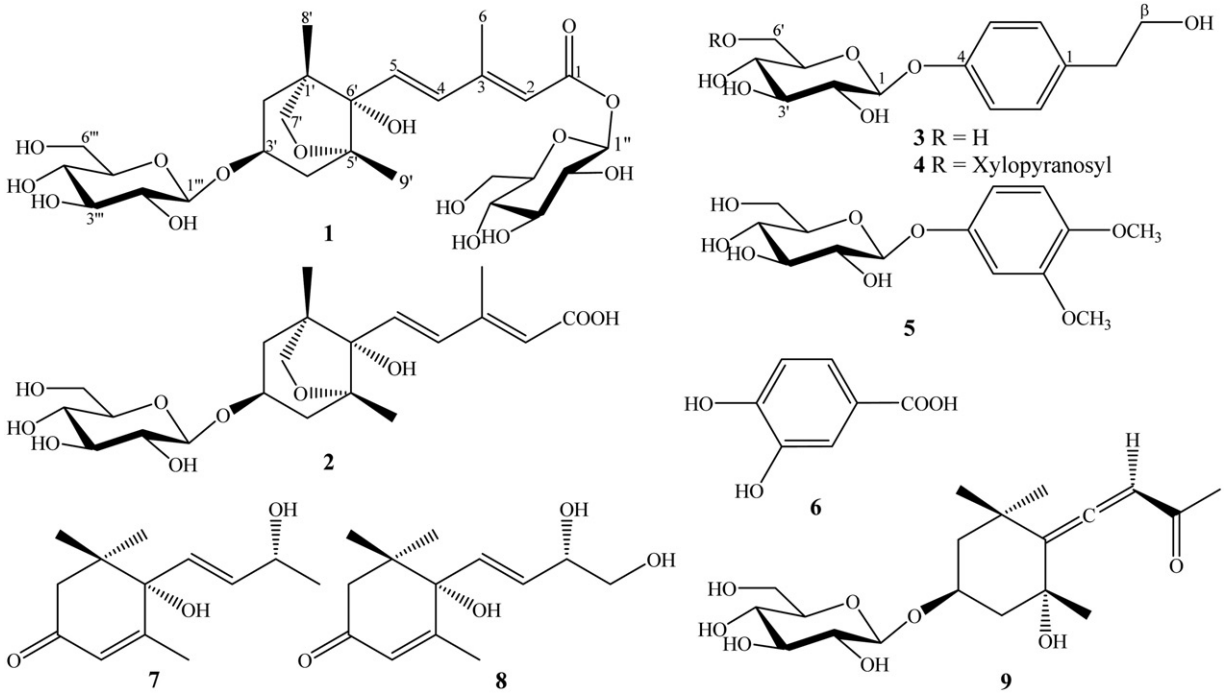

Figure 1. Structures of compounds **1–9** from the fruit of *A. glabra*.

and an increased population of sub-G1 hypodiploid cells (Yang et al., 2007). When treated with compound **3** for 24 and 48 h, apoptotic characteristics were observed such as increasing apoptotic bodies (Figure 3). In addition, treatment of compound **3** increased the sub-G1 hypodiploid cell population in a time-dependent manner. The Bcl-2 family is separated into anti-apoptotic proteins, such as Bcl-2, and pro-apoptotic proteins, such as Bax. The Bax induces apoptosis by the releasing of cytochrome *c* from mitochondria. In contrast, Bcl-2 inhibits the releasing of cytochrome *c*. During apoptosis, released cytochrome *c* induces the cleavage of caspase-9, which is followed by the cleavage of caspase-3 and cleavage of poly (ADP-ribose) polymerase (PARP) (Zimmermann & Green, 2001). Therefore, to investigate the possible mechanism underlying the induction of apoptosis, we examined the levels of apoptosis-related proteins. When treated with compound **3** (9.0 μM) for 24 and 48 h, we could observe the alteration of expression of apoptosis-related proteins such as up-regulation of Bax, down-regulation of Bcl-2, cleaves of caspase-3, and cleaves of PARP (Figure 4). These results indicated that compound **3** induced apoptosis via alteration of expression of apoptosis-related proteins in HL-60 cells.

Table 2. The effects of compounds **1–9** on the growth of HL-60 leukemia and Hel-299 normal cells.

	IC_{50} (μM)	
Compound	HL-60	Hel-299
1	>100	NT
2	>100	NT
3	9.0 ± 1.0	>100
4	49.1 ± 4.9	>100
5	32.2 ± 2.1	>100
6	64.6 ± 3.6	>100
7	>100	NT
8	>100	NT
9	>100	NT
Mitoxantrone	6.8 ± 0.9	>100

Mitoxantrone was used as a positive control. NT: not tested.

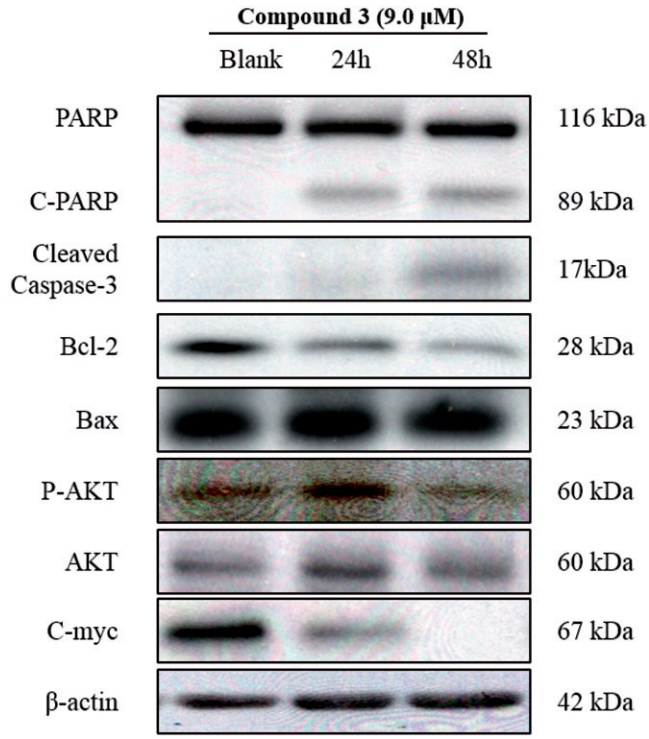

Figure 4. The effects of **3** on the expressions of apoptosis-related proteins, p-AKT and c-myc. The HL-60 cells (3×10^5 cells/mL) were treated with compound **3** (9.0 μM) for 24 h and 48 h.

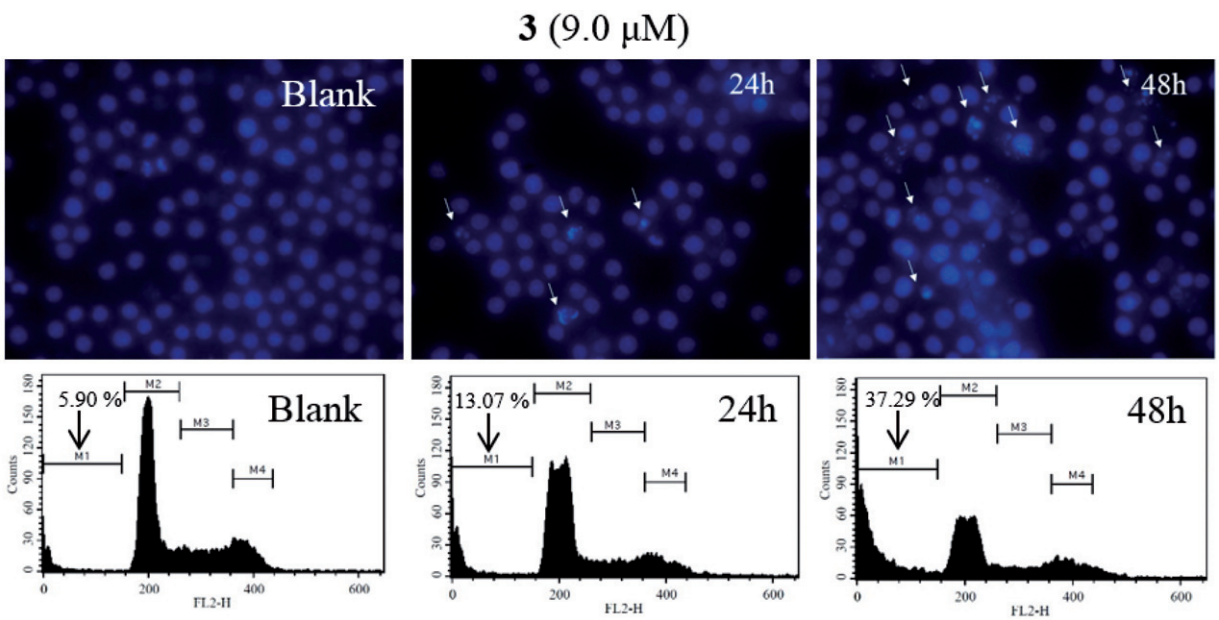

Figure 3. The apoptosis degree of **3** as represented as the fluorescent image of nuclei in HL-60 cells by Hoechst 33342 staining and DNA content measured by flow cytometric analysis in HL-60 cells. The HL-60 cells were treated with **3** (9.0 μM) for 24 h and 48 h. Apoptotic bodies were pointed by arrows.

The PI3K/AKT signaling pathways regulate cell survival, cell growth, and apoptosis. Especially, activated AKT contributes the survival and the growth of cancer cell via c-myc (Kim & Chung, 2002). The c-myc is frequently over-expressed in various types of tumors. In order to investigate intracellular signaling compound **3** induced, we analyzed the phosphorylation of AKT and the level of c-myc by Western blotting. As the results, the treatment of compound **3** decreased phosphorylation of AKT in conditions that could induce apoptosis in HL-60 cells. Furthermore, that was also accompanied by down-regulation of c-myc. These findings provide evidence demonstrating that the apoptosis-inducing effects of compound **3** are mediated by down-regulation of p-AKT and c-myc.

Declaration of interest

The authors report that they have no conflicts of interest. This research was supported by Vietnam Academy of Science and Technology (VAST 04.04/13-14) and by a grant from the Priority Research Center Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815), Republic of Korea.

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