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C_{29} sterols with a cyclopropane ring at C-25 and 26 from the Vietnamese marine sponge *Ianthella* sp. and their anticancer properties

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ABSTRACT

Two new C_{29} sterols with a cyclopropane ring at C-25 and C-26, petrosterol-3,6-dione (**1**) and 5α,6α-epoxy-petrosterol (**2**), along with petrosterol (**3**), were isolated from the Vietnamese marine sponge *Ianthella* sp. The structures of the new compounds were elucidated by comprehensive spectroscopic analyses. Compounds **1**–**3** showed cytotoxic activities on A549, HL-60, MCF-7, SK-OV-3, and U937 cancer cell lines with IC_{50} in the range of 8.4–22.6 μM, whereas compounds **1**–**3** exhibited only weak cytotoxic activities on HT-29 cell. After HL-60 cells were treated with the compounds, several apoptosis events like chromatin condensation and the increase of the population of sub-G1 hypodiploid cells were observed. These data supported that the compounds might have potential for leukemia treatment.

Marine sponges are sources of numerous novel sterols, particularly in terms of unique side chain structures such as those with high degree of alkylation and unusual function groups.[1] In which, steroids with a cyclopropane ring at C-25 and C-26 are relatively rare and first reported in 1978 from sponges, the *Petrosia ficiformis*[2] and *Halichondria* sp.[3] Some of these analogs have been reported to show biological activities including anti-inflammation and cytotoxicity.[4–6] The sponges of the genus *Ianthella* (order Verongida, family Ianthellidae) have been reported to contain several bromotyrosine-derived metabolites, such as the ianthesines, purealin, and others, which were mainly isolated from their polar extracts.[7–9]

In our research on secondary metabolites from Vietnamese marine organisms, chromatographic fractionation and isolation led to results of three sterols (**1**–**3**)[10] including two new sterols, namely petrosterol-3,6-dione (**1**) and 5α,6α-epoxy-petrosterol (**2**), with a cyclopropane ring at C-25 and C-26 and a known one, petrosterol (**3**),[4] from the MeOH extract of the sponge *Ianthella* sp.

The marine sponge *Ianthella* sp. (2.4 kg, wet weight) were collected at Namyet island, Khanh Hoa province in March, 2007, and was kept frozen (−20 °C) until used; it was identified by Dr. Do Cong Thung, Institute of Marine Resources and Environment, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam. The specimen vouchers of the *Ianthella* sp. (No. 20070306) have been deposited at Institute of Natural Products Chemistry and Institute of Marine Resources and Environment, VAST, Vietnam. The frozen sample *Ianthella* sp. was ground and exhaustively extracted by the ultrasound-assisted manner with methanol (5 L × 3, each one day) at room temperature, and combined MeOH extract was concentrated in vacuo to give a black gum residue (70 g). The residue was suspended in H_2O (0.5 L), and partitioned with EtOAc (0.5 L × 3). Next, the EtOAc-soluble portion (9.3 g) was subjected to silica gel column eluted with a gradient of MeOH in $CHCl_3$ (30:1–1:1, v/v) to give four fractions (1a–d). The fraction 1a was repeatedly chromatographed using silica gel column with an eluent of $CHCl_3$–MeOH (30:1, v/v) to afford **1** (20 mg). Thereafter, fraction 1b was further chromatographed using reversed-phase C-18 YMC column with an eluent of MeOH–acetone–H_2O (5:1:3, v/v/v), followed by silica gel column chromatography (*n*-hexane–EtOAc, 3:1, v/v) to afford **2** (7.5 mg). Finally, repeated column chromatography of the fraction 1c (1.8 g) using silica gel (*n*-hexane–EtOAc, 5:1, v/v) and reversed-phase C-18 YMC (MeOH–H_2O–acetone, 5:1:3, v/v/v) resulted in the isolation of **3** (12.5 mg).

The structure of compounds **1**–**3** (Fig. 1) were elucidated by using NMR, UV, IR, and MS spectra. The IR (1685 cm^{-1}) and UV (250 nm) spectra of **1** verified the presence of carbonyl groups

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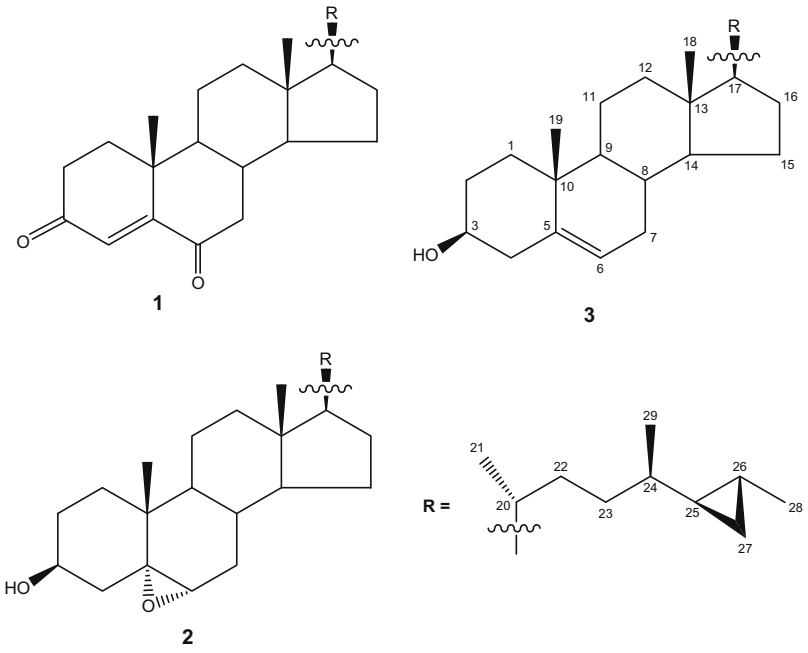

Figure 1. Structures of **1**–**3**.

Table 1
^{1}H and ^{13}C NMR data for **1** and **2**

C no.	1		2	
	δ_C[a,b]	δ_H[a,c] (*J* in Hz)	δ_C[a,b]	δ_H[a,d] (*J* in Hz)
1	35.8	2.18, m	32.4	1.70, m
		1.90, m		1.36, m
2	34.2	2.54, m	31.1	1.92, m
		2.30, t (7.6)		1.62, m
3	199.7		68.8	3.92, m
4	125.7	6.18, s	39.8	2.08, m
				1.31, m
5	161.3		65.7	
6	202.6		59.3	2.92, d (4.5)
7	47.0	2.68, dd (16.0, 4.0)	28.8	1.93, m
		2.06, m		1.50, m
8	34.4	1.90, m	29.9	1.38, m
9	51.2	1.40, m	42.6	1.26, m
10	40.0		34.9	
11	21.1	1.65, m	20.6	1.37, m
		1.51, m		1.25, m
12	39.4	2.11, m	39.8	1.95, m
		1.28, m		1.10, m
13	42.8		42.3	
14	56.2	1.20, m	55.8	1.17, m
15	24.2	1.63, m	24.0	1.55, m
		1.18, m		1.13, m
16	28.2	1.90, m	28.1	1.82, m
		1.33, m		1.26, m
17	56.8	1.20, m	56.9	1.20, m
18	12.1	0.73, s	11.9	0.61, s
19	17.7	1.17, s	15.9	1.07, s
20	36.1	1.38, m	35.9	1.33, m
21	18.9	0.94, d (6.8)	18.6	0.90, d (6.5)
22	33.6	1.50, m	33.4	1.46, m
		1.22, m		1.19, m
23	34.1	1.36, m	33.9	1.34, m
		1.25, m		1.23, m
24	38.9	0.61, m	39.4	0.59, m
25	27.6	0.15, m	27.4	0.14, m
26	13.0	0.46, m	12.8	0.45, m
27	11.8	0.14, m	11.6	0.14, m
		0.07, m		0.08, m
28	19.4	1.02, d (6.4)	19.1	1.00, d (6.0)
29	20.1	0.90, d (6.4)	19.9	0.88, d (6.5)

[a]Measured in $CDCl_3$, [b]at 100 MHz, [c]at 400 MHz, [d]at 500 MHz. Assignment were confirmed by COSY, HMQC, HMBC, and NOESY.

and a conjugated double bond system, Δ^4-3,6 dione. The quasimolecular peak was appeared at *m/z* 425 $[M+H]^+$ in FABMS. The molecular formula of **1**, $C_{29}H_{44}O_2$, was deduced by HRFABMS (detected peak at *m/z* $[M+H]^+$ 425.3397, calcd for $C_{29}H_{45}O_2$, 425.3375).

The IR spectrum of **2** displayed absorptions at 3443 cm^{-1} (OH group) and 1260 cm^{-1} (epoxy group). The molecular formula of

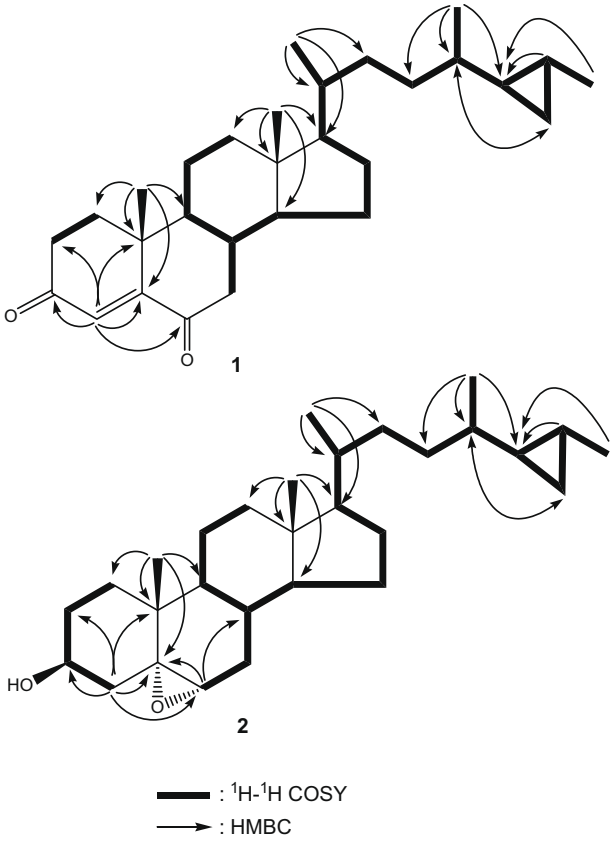

Figure 2. ^{1}H–^{1}H COSY and selected HMBC correlations for **1** and **2**.

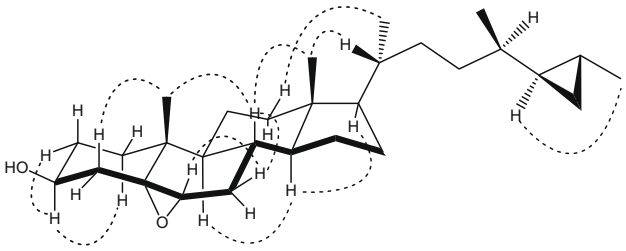

Figure 3. Key NOESY correlations of **2**.

2, $C_{29}H_{48}O_2$, was established based on HREIMS (detected peak at *m/z* $[M]^+$ 428.3656, calcd for $C_{29}H_{48}O_2$ 428.3654) and FABMS (observed peak at *m/z* 429 $[M+H]^+$).

The ^1H and ^{13}C NMR data of **1** and **2** are summarized in Table 1. The assignments were confirmed by HMQC, HMBC, COSY, and NOESY spectra. The NMR data suggested all three compounds belonging to C_{29} steroids with C_{10} side chain containing a cyclopropane ring at C-25 and C-26. The presence of the cyclopropane ring was defined due to upfield resonance signals, typically, in ^1H NMR (H-25, H-26, and H-27) and ^{13}C NMR (C-26 and C-27) of **1**–**3**.[2,3,5,6]

Table 2
Cytotoxicity data of compounds **1**–**3**

Cell line	Compound[a]			
	1	**2**	**3**	Mitoxantrone[b]
A549 (lung)	8.4	9.8	11.5	8.0
HL-60 (leukemia)	19.9	21.3	21.5	6.3
HT-29 (colon)	48.2	69.9	46.5	8.7
MCF-7 (breast)	17.8	19.4	16.4	7.1
SK-OV-3 (ovary)	16.2	22.6	19.8	9.8
U937 (leukemia)	22.1	19.9	18.7	6.2

[a] Results are expressed as IC_{50} values (μM), and values <100 μM are considered to be active.
[b] Positive control.

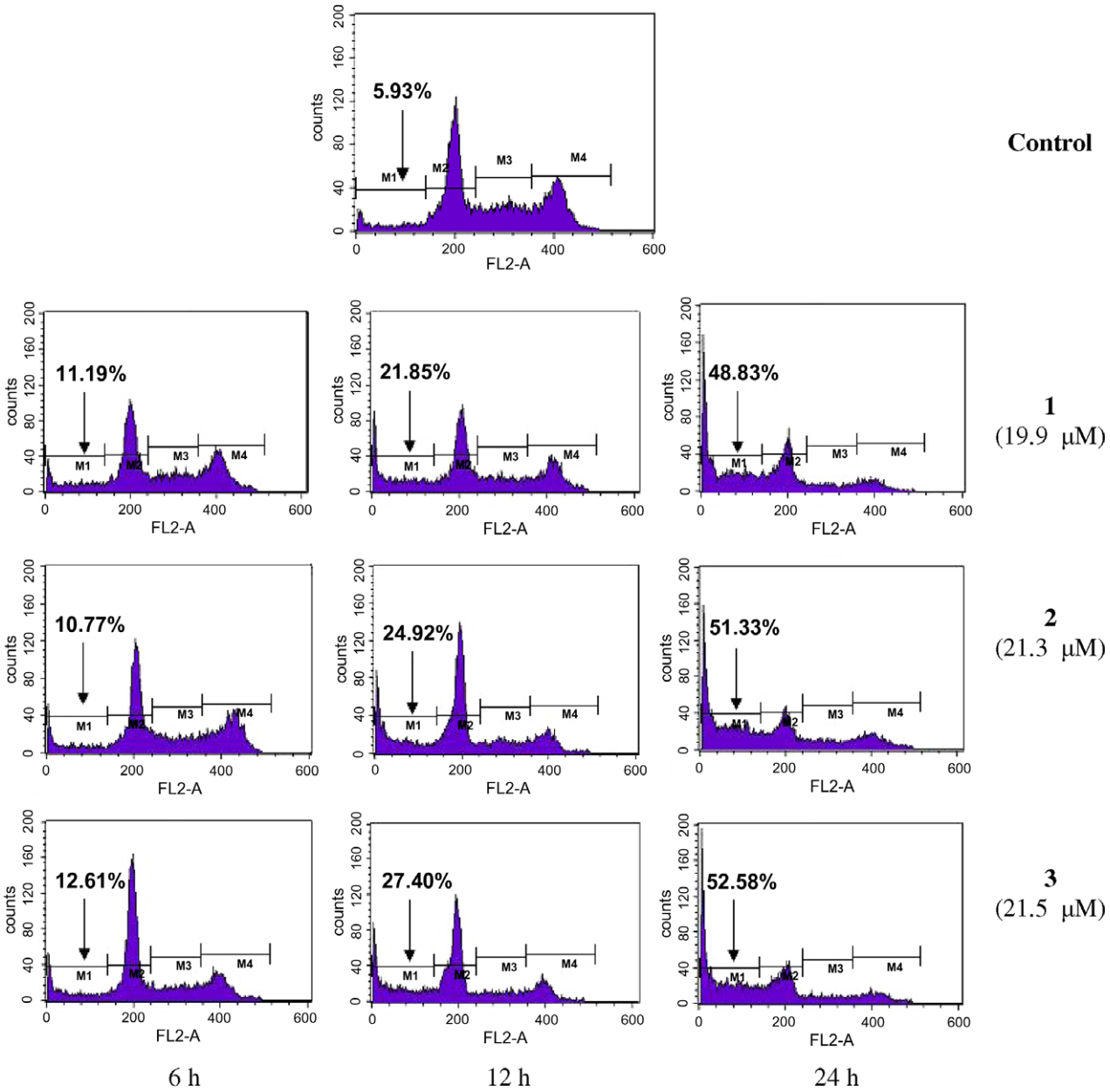

Figure 4. The degree of apoptosis represented as the DNA content measured by flow cytometric analysis in HL-60 cells.

In addition, five methyl groups were observed in the ^{1}H NMR spectra of **1**–**3** including two singlets (H-18 and H-19) and three doublets (H-21, H-28, and H-29) which were integrated relatively for 3H, respectively.

In compound **1**, a steroid with Δ^4-3,6 dione containing nucleus was proposed based on UV, IR properties (see above) and NMR spectra. The ^{13}C NMR spectrum showed the presence of a carbon–carbon double bond (δ 125.7, C-4; δ 161.3, C-5), and indicated the presence of two conjugated carbonyls (δ 199.7, C-3; δ 202.6, C-6).[11] In addition, the ^{1}H NMR spectrum had a singlet olefinic proton (δ 6.18, H-4). The position of Δ^4-3,6 dione was assured by a HMBC experiment, which disclosed cross correlations of the H-4/C-3, H-4/C-2, H-4/C-5, and H-4/C-6. Furthermore, as shown in Figure 2, the double quantum filter correlation spectroscopy (DQF COSY) experiment on **1** indicated the presence of partial structures written in bold lines.

The ^{13}C NMR spectrum of **2** was in difference from those of petrosterol (**3**) by the appearance of two carbon signals (δ 65.7 and 59.3) instead of two olefinic carbons at δ 141.8 (C-5) and δ 122.3 (C-6) in petrosterol (**3**) together with a proton signal at δ 2.92 (d, J = 4.5 Hz, H-6) in ^{1}H NMR spectrum, which indicated the existence of a trisubstituted epoxide group at C-5 and C-6 position. Additionally, in the HMBC spectrum of **2,** cross correlations of H-4/C-5, H-4/C-6, H-6/C-5, and H-6/C-8 further confirmed this indicating, respectively (Fig. 2). α-Orientation of epoxy group was proposed based on NMR agreement with the steroids containing similar sterol nucleus with 5α,6α-epoxy group. Accordingly, there are differences in ^{1}H and ^{13}C NMR between α and β forms.[12,13] The downfield-shifted resonance of the methine proton H-3 at δ 3.92 is alternatively due to deshield effect by the 5α,6α-epoxy form. Furthermore, the β-orientation of H-6 was deduced from its NOESY correlation with H_β-7 at δ 1.93, which in turn showed a NOESY correlation with the H_β-8 at δ 1.38, respectively (Fig. 3).[12,13]

Relative stereochemistry of the side chain of all compounds **1**–**3** was established from careful comparison of their ^{1}H and ^{13}C spectra with literature data and NOESY experiments.[4,5,14] Consequently, the 17β-orientation of the side chain was disclosed by NOESY cross-peaks of H-12/H-21, H-14/H-17, and H-18/H-20. The geometry of the cyclopropane ring was deduced to be *E* by NOESY the cross-peak of H-25/H-28 (Fig. 3).

The cytotoxic activities of compounds **1**–**3** were tested against a panel of human tumor cell lines including lung (A549), colon (HT-29), breast (MCF-7), ovary (SK-OV-3), and two types of leukemia (HL-60 and U937) human cancer cell lines using 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[15] Compounds **1**–**3** showed cytotoxicity against A549, HT-29, HL-60, MCF-7, SK-OV-3, and U937 with IC_{50} values from 8.4 to 69.9 μM. Compound **1** showed growth-inhibitory effects with IC_{50} values of 8.4, 19.9, 17.8, 16.2, and 22.1 μM against A549, HL-60, MCF-7, SK-OV-3, and U937 cell lines, respectively. Compound **2** showed cytotoxic effects with IC_{50} values of 9.8, 21.3, 19.4, 22.6, and 19.9 μM. Case of compound **3** showed growth-inhibitory activities with 11.5, 21.5, 16.4, 19.8, and 18.7 μM, respectively. However, compounds **1**–**3** showed less cytotoxic effects against HT-29 cells (Table 2). Compounds **1**–**3** might show effects on diverse cellular pathways associated with cell survival and apoptosis such as extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathway as well as phosphoinositide 3 (PI3) kinase/Akt pathway.[16–19] In order to explain the potency difference between cell lines, the cellular action mechanisms of the compounds should be studied in detail.

We investigated whether the inhibitory effects of compounds **1**–**3** on the growth of cancer cells might arise from the induction of apoptosis.[20] The apoptotic characteristics were examined after the HL-60 were treated with IC_{50} of compounds **1**–**3** for 6, 12, and 24 h. The percentage of sub-G1 hypodiploid cells by the treatment of compound **1** increased from 11.2% to 48.8% in a time-dependent manner. In the treatment of compounds **2** and **3**, Sub-G1 hypodiploid cells increased from 10.8% to 51.3% and from 12.6% to 52.6% in time-dependent manners (Fig. 4). These data

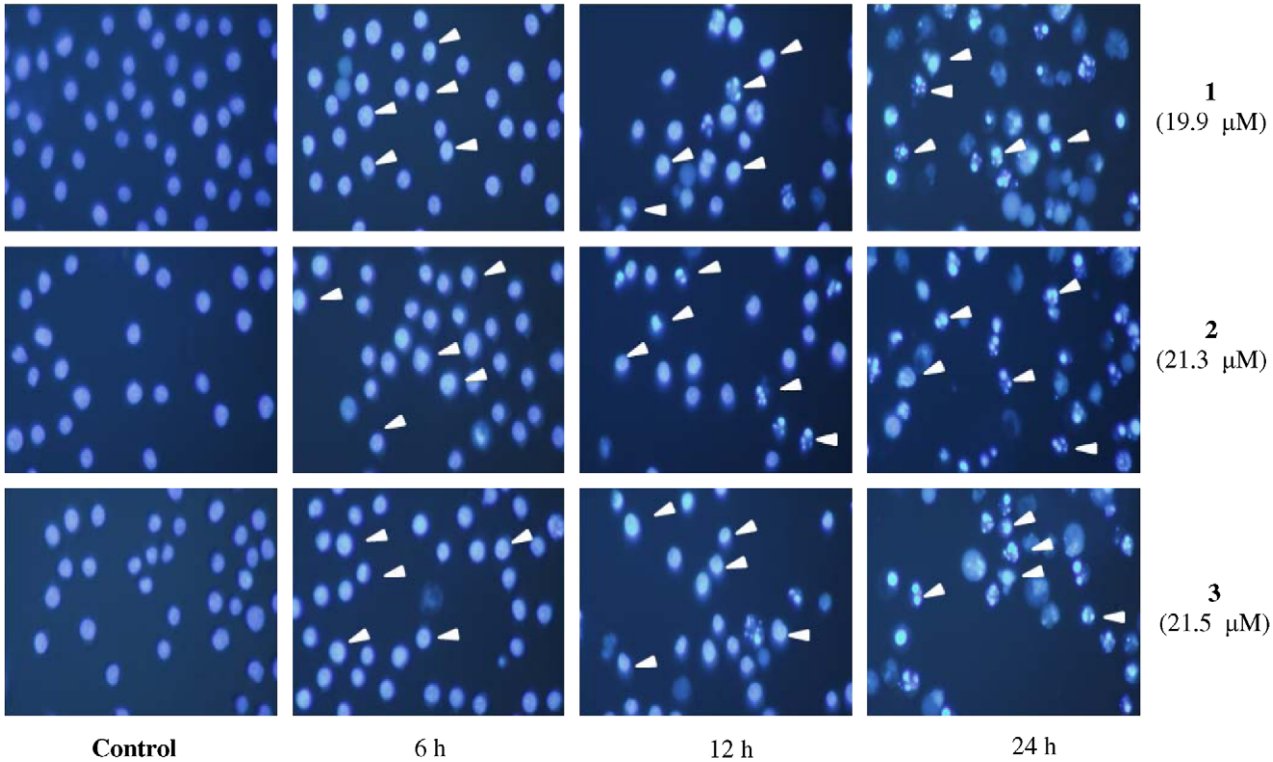

Figure 5. The degree of apoptosis represented as the fluorescent image of nuclei in cells by fluorescent microscope.

show that compounds induce the apoptosis of HL-60 cells, and are supported by the increase in the number of apoptotic bodies which were easily found by H33342 staining in compounds-treated cells after over 6, 12, and 24 h-incubation (Fig. 5). This study observed that compounds **1**–**3** markedly inhibited the growth of various cancer cells suggesting that the compounds induced necrosis or apoptosis. Epigallocatechin gallate (EGCG), one of green tea catechins, has been reported to induce the apoptosis of HL-60, acute promyelocytic leukemia cells, while EGCG was demonstrated to cause caspase-independent necrosis-like cell death in chronic myelogenous leukemia such as K562 and C2F8.[21,22] As the compounds **1**–**3** induced the apoptosis of HL-60 leukemia cells, the compounds are expected to be able to induce the apoptosis in the other tested cancer cells. However, in further study, we need to evaluate the mode of cell death by compounds **1**–**3** based on cancer cell types.

In summary, this study demonstrated that compounds **1**–**3** inhibited the growth of the HL-60 cells by the induction of apoptosis.

Acknowledgements

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10. Petrosterol-3,6-dione (**1**): yellow amorphous powder; $[\alpha]_D^{20}$ −2.8 (*c* 0.06, $CHCl_3$); UV: λ_{max}^{MeOH} (nm) (log ε) 204.0 (1.04), 250.0 (1.49); IR (KBr) ν_{max} 2945, 1685, 1242 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz): see Table 1; positive FABMS *m/z* 425 $[M+H]^+$; positive HRFABMS *m/z* $[M+H]^+$ 425.3397 (Calcd for $C_{29}H_{45}O_2$ 425.3375).
5α,6α-Epoxy-petrosterol (**2**): white solid; $[\alpha]_D^{20}$ −1.9 (*c* 0.07, $CHCl_3$); UV λ_{max}^{MeOH} (nm) (log ε) 223.0 (2.29); IR (KBr) ν_{max} 3443, 2951, 2924, 1260, 1041 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz): see Table 1; FABMS *m/z* 429 $[M+H]^+$; HREIMS *m/z* $[M]^+$ 428.3656 (Calcd for $C_{29}H_{48}O_2$ 428.3654).
Petrosterol (**3**): white powder; mp 121−122 °C; $[\alpha]_D^{20}$ −16 (*c* 0.8, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ 5.30 (1H, br d, *J* = 5.5 Hz, H-6), 3.48 (1H, tt, *J* = 11.5, 5.5 Hz, H-3), 1.04 (3H, s, H-19), 1.03 (3H, d, *J* = 6.0 Hz, H-28), 0.92 (3H, d, *J* = 6.5, H-21), 0.88 (3H, d, *J* = 6.5 Hz, H-29), 0.68 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 38.2 (C-1), 31.9 (C-2), 72.1 (C-3), 42.7 (C-4), 141.8 (C-5), 122.3 (C-6), 32.8 (C-7), 32.7 (C-8), 51.2 (C-9), 37.4 (C-10), 21.9 (C-11), 40.7 (C-12), 43.2 (C-13), 57.7 (C-14), 25.1 (C-15), 29.1 (C-16), 57.1 (C-17), 12.4 (C-18), 19.6 (C-19), 36.9 (C-20), 19.3 (C-21), 34.4 (C-22), 34.8 (C-23), 39.7 (C-24), 28.2 (C-25), 13.6 (C-26), 12.2 (C-27), 19.9 (C-28), 20.4 (C-29); FABMS *m/z* 413 $[M+H]^+$.
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20. *Flow cytometric analysis:* HL-60 cells (3×10^5 cells/mL) were treated with IC_{50} of compounds for 6, 12, and 24 h. For the flow cytometric analysis to determine cell cycle phase distribution, the treated cells were washed twice with PBS and fixed in 70% ethanol for 30 min at 4 °C. The cells were then rinsed with PBS and incubated in 50 μg/mL propidium iodide solution (PI; Sigma) and 50 μg/mL RNase A in the dark for 30 min at 37 °C. Flow cytometry analysis was performed using an flow cytometer (Becton Dickinson FACS Caliber, BD Biosciences, USA). The DNA histograms obtained were analyzed to measure the proportion of sub-G1 hypodiploid cells.
Morphology analysis: HL-60 cells (3×10^5 cells/mL) were treated with IC_{50} of compounds for 6, 12, and 24 h. Cells were washed twice with PBS before being stained with 1 mg/mL Hoechst 33342 for 30 min at 37 °C. Apoptotic bodies, with condensed and fragmented nuclei, were observed with a fluorescence microscope (400×, BX-50, Olympus, Japan).
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