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Apoptotic-inducing epidioxysterols identified in hard clam (*Meretrix lusoria*)

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Abstract

Hard clams (HC), *Meretrix lusoria* are a popular seafood and traditionally used as a Chinese remedy for liver disease and chronic hepatitis. The goal of this study was to identify compounds from hard clam, which are responsible for inducing apoptosis using bioassay-directed isolation. The effects of ethyl acetate extract of HC (HC-EA) on cell viability in human cancer cells were investigated. The ethyl acetate fraction was then subjected to separation and purification using silica gel column chromatography. The compounds showing strong apoptosis inducing activity were identified by spectral methods as epidioxysterols (EDS). The molecular mechanisms of epidioxysterolds-induced apoptosis as determined by annexin V apoptosis assay, DNA condensation, and sub-G1 DNA were investigated. The results suggest that induction of apoptosis by epidioxysterols may provide a pivotal mechanism for its cancer chemoprevention.

Keywords: Hard clam; *Meretrix lusoria*; Epidioxysterols; Apoptosis

1. Introduction

Human cancer development requires 20–40 years or more, and the scope of chemoprevention encompasses cohorts at all phases of this process (Kelloff et al., 1996). Epidemiological studies provide convincing evidence that dietary factors can modify the processes of carcinogenesis, including initiation, promotion and progression of several types of human cancer (Kelloff et al., 1994). Because of their expected safety and because they are not perceived as "medicine", food derived products may find widespread, long term use among the populations at normal risk; thus they are highly interesting for development as chemopreventive agents.

Apoptosis is a defined type of cell death and differs from traditional cell death, necrosis. Many recent studies have indicated that anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent tumor promotion, progression, and the occurrence of cellular inflammatory responses other than necrosis (Jacks & Weinberg, 2002; Nicholson, 2000). Apoptosis is also a gene-directed form of cell death with well-characterized morphological and biochemical features (Boise et al., 1993). Initiation of apoptosis appears to be a common mechanism of many cytotoxic agents used in chemotherapy (Pan, Lin-Shiau, & Lin, 2000b; Pan, Chang, Lin-Shiau, Ho, & Lin, 2001).

Hard clams, *Meretrix lusoria* are a popular seafood and traditionally used as a Chinese remedy for liver disease and chronic hepatitis. Research indicates that the extracts of hard clams exhibited anti-tumor activity and enhanced IgM secretion (Kong, Chiang, Fang, Shinohara, & Pan, 1997). However, the specific compounds responsible for

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDS, epidioxysterols.

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the induced apoptosis activity of hard clams remained unknown. This study was designed to identify compounds from hard clam and elucidate the mechanisms of apoptosis by constituents in hard clam. Many recent studies indicate that anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent tumor promotion, progression and the occurrence of cellular inflammatory responses other than necrosis (Jacks & Weinberg, 2002; Nicholson, 2000).

In the current study, we first examined the apoptotic effects of HC-EA on human cancer cells. Our results clearly demonstrate that HC-EA can inhibit cell proliferation in a dose dependent manner in HL-60 cells. Two compounds, 5α,8α-epidioxy-24(*S*)-methylcholest-6-en-3β-ol and 5α,8α-epidioxy-24(*R*)-methylcholest-6-en-3β-ol showing strong apoptosis inducing activity were isolated and identified for the first time from *M. lusoria*. We further evaluated the molecular mechanisms of apoptotic effects induced by epidioxysterols.

2. Materials and methods

2.1. Cell culture and regents

Human promyelocytic leukemia (HL-60) cells obtained from American Type Culture Collection (Rockville, MD), COLO 205 cells, HT-29 cells, THP-1 cells and K562 cells were grown in RPMI 1640; SK-Hep 1 cells were grown in DMEM medium and 10% fetal bovine serum (Gibco BRL, Grand Island, NY), supplemented with 2 mM glutamine (Gibco BRL) and 1% penicillin/streptomycin (10 000 units of penicillin/ml and 10 mg/ml streptomycin). Human polymorphonuclear cells (PMNs) were obtained from healthy male donors and were separated by Ficoll–Hypaque density gradient. Human PMNs were washed twice in 0.9% NaCl and resuspended in RPMI-1640 medium. The human AGS gastric carcinoma cells (CCRC 60102) were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium/Nutrient mixture F-12 containing 10% heat inactivated fetal bovine serum (Gibco BRL, Grand Island, NY), 100 units/ml of penicillin, (100 μg/ml of streptomycin), and 2 mM L-glutamine (Gibco BRL) and were kept at 37 °C in a humidified 5% CO_2 incubator.

RAW 264.7 cells, which were derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/ml penicillin, and 100 μg/ml streptomycin. When the cells reached a density of $2–3 \times 10^6$ cells/ml, they were activated by incubation in medium containing *Escherichia coli* LPS (100 ng/ml). Various concentrations of test compounds dissolved in dimethylsulfoxide were added together with LPS. Lipopolysaccharide (LPS) (*E. coli* 0127: E8), sulfanilamide, naphthylethylenediamine dihydrochloride, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of HC-EA from hard clam

Hard clams were purchased from Budai Aquaculture Farm (Chaiyi, Taiwan) in December, 2004. They were cut into small piece and homogenized into a mixture in a blender and extracted with ethyl acetate. This procedure was repeated three times. The resulting supernatant extract was filtered and concentrated by rotary evaporator working under vacuum and then freeze-dried (HC-EA). The resulting deep blue lyophilized powder was dissolved directly in DMSO for further bioassay.

2.3. Cell survival assay

Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, HL-60 cells and selected cells were plated at a density of 1×10^5 cells/ml into 24-well plates. After overnight growth, cells were pretreated with a series of concentrations of HC-EA for 6 h. The final concentration of dimethyl sulfoxide in the culture medium was <0.1%. At the end of treatment, 30 μl of MTT was added, and cells were incubated for a further 4 h. Cell viability was determined by scanning with an enzyme-linked immunosorbent assay reader with a 570 nm filter. On the other hand, Cells were plated at a density of 1×10^5 cells/100 μl/well into 96-well plates. After overnight growth, cells were treated with 25 and 50 μg/ml HC-EA and epidioxysterols, respectively. The final concentrations of dimethyl sulfoxide (DMSO) in the culture medium were less than 0.1%. Following 24 h of incubation with HC-EA and epidioxysterols, the cell viability was assayed with Luminescent ATP Detection Assay Kit (Packard BioScience B. V.). Briefly, 50 μl of cell lysate was used to assay luminescent ATP. Luminescence was measured on a Luminescence Counter (LMax II; Molecular Devices, CA) in single photon counting mode for 0.1 min/well, following 2 min adaptation in the dark (Pan et al., 2000a).

2.4. General procedures

Optical rotations were measured using a JASCO DIP-180 digital spectropolarimeter. The IR spectra were recorded on a Nicolet 510 P FT-IR spectrometer. The NMR spectra were recorded in $CDCl_3$ at room temperature on a Varian Mercury plus 400 NMR spectrometer, and the solvent resonance was used as internal shift reference (TMS as standard). EI-MS was recorded on a Finnigan TSQ-700 and a JEOL SX-102A spectrometer. TLC was performed using silica gel 60 F_{254} plates (200 μm, Merck). High performance liquid chromatography (HPLC) was performed with a L7100 instrument (Hitachi,

Japan) and a differential refractive index detector RI 8120 (Bischoff, Germany). A 10 × 250 mm, 5 μm, Lichrosorb Si 60 column (Merck, Germany) was used for analysis.

2.5. Extraction and isolation

The scheme for the column chromatography of the apoptosis-inducing substance from *M. lusoria* is shown in Fig. 1. The fresh pieces of the 4500 g of hard clam were extracted three times with ethyl acetate (4.5 l) at room temperature (2 days each time). The EtOAc extract was evaporated *in vacuo* to yield a black residue. The EtOAc fraction (8 g) was chromatographed on Si gel using *n*-hexane and EtOAc of increasing polarity as eluent to obtain 19 fractions. Fraction 13 was further chromatographed on a Silica gel column (3 × 45 cm, Merck 230–400 mesh) eluted with *n*-hexane/acetone (10/1) to obtain 8 fractions, fraction 13A–fraction 13H. HPLC of fraction 13E on a Merck Lichrosorb Si 60 column (5 μm, 250 × 10 mm) with *n*-hexane/ EtOAc (8/1) as an eluent, afforded a mixture of 5α,8α-epidioxy-24(*S*)-methylcholest-6-en-3β-ol and 5α,8α-epidioxy-24(*R*)-methylcholest-6-en-3β-ol (32 mg). The purity of epidioxysterols (EDS) is above 95% detected by the 1H NMR spectrum.

2.6. Structural determination of isolated compound

5α,8α-epidioxy-24(*R*)-methylcholest-6-en-3β-ol (**1**) and 5α,8α-epidioxy-24(*S*)-methylcholest-6-en-3β-ol (**2**): white needles: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.78 (3H, s, H-18), 0.82 (3H, d, $J = 6.8$ Hz, H-26), 0.83 (each 3H, d, $J = 6.8$ Hz, H-27), 0.86 (3H, s, H-19), 0.89 (3H, d, $J = 6.8$ Hz, H-28), 1.00 (3H, d, $J = 7.0$ Hz, H-21), 3.95 (1H, m, H-3), 6.22 (each 1H, d, $J = 8.5$ Hz, H-6), 6.48 (each 1H, d, $J = 8.5$ Hz, H-7); EIMS m/z (%) 430 ($M^{+\cdot}$, 7), 412 (17), 396 (38), 379 (5), 365 (18), 339 (8), 253 (7) [identical with the literature values (Gauvin, Smadja, Aknin, Faure, & Gaydou, 2000)].

2.7. Acridine orange staining assay

Cells (5×10^5) were seeded into 60 mm Petri dishes and incubated at 37 °C for 24 h. The cells were harvested after treatment for 24 h, and 5 μl of cell suspension was mixed on a slide with an equal volume of acridine orange solution (10 μg/ml in PBS). Green fluorescence was detected between 500 and 525 nm using an Olympus microscope (Olympus America, Inc., Lake Success, NY). Bright-staining condensed chromatin was detected in apoptotic cells.

2.8. Annexin V flow cytometric assay

Perturbations in the cellular membrane occur during the early stages of apoptosis and lead to a redistribution of phosphatidylserine to the external side of the cell membrane. Annexin V selectively binds to phosphatidylserine, and this has enabled the use of fluorescein-labeled annexin V for identification of cells undergoing apoptosis. FITC-labeled annexin V Apoptosis Detection Kit (Pharmingen, Becton Dickinson Company, San Diego, CA). Ten thousand events were collected with a FACScan flow cytometry (Becton Dickinson, San Jose, CA).

2.9. Flow cytometry

HL-60 cells (2×10^5) were cultured in 60-mm Petri dishes and incubated for 24 h. The cells were then harvested, washed with PBS, resuspended in 200 μl of

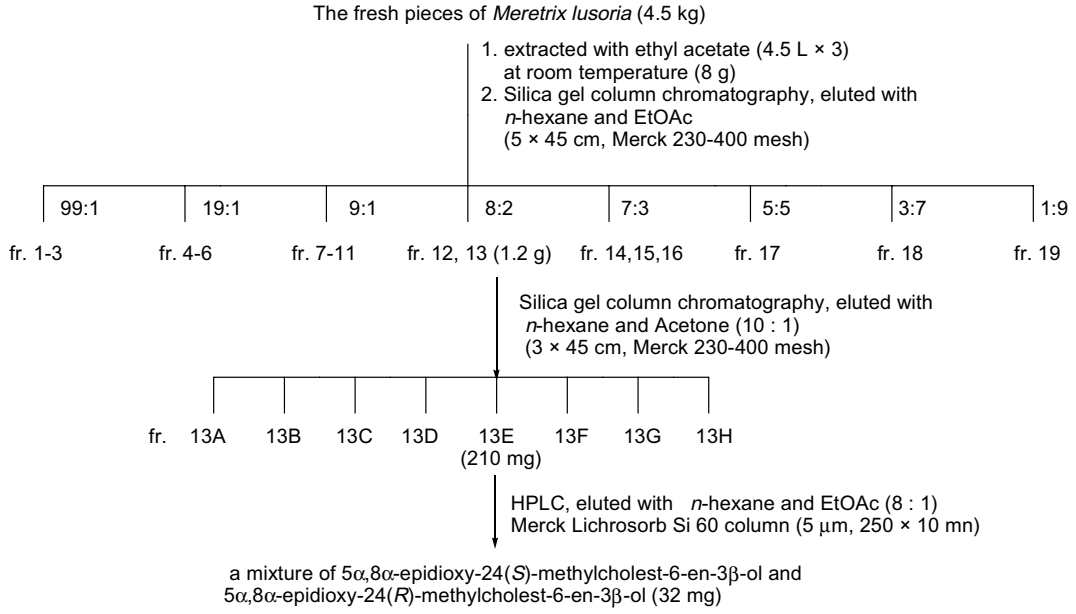

Fig. 1. Separation scheme of the apoptosis-inducing substance from *Meretrix lusoria*.

PBS and fixed in 800 μl of iced 100% ethanol at −20 °C. After they were left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase), and incubated at 37 °C for 30 min. Next, 1 ml of propidium iodide solution (50 μg/ml) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

2.10. DPPH free radicals scavenge activity

α,α-Diphenyl-β-picrylhydrazyl (DPPH) was purchased from Sigma (St. Louis, MO). DPPH (150 μM in 60% absolute alcohol) was only/or mixed with different concentrations of epidioxysterols. Absorption at 517 nm was measured using an UV-visible spectrophotometer (Hitachi, U2000). The decrease in absorbance was shown when tested compounds possessed free radicals scavenge activity.

2.11. Nitrite assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction (Pan et al., 2000). One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was determined with an enzyme-linked immunosorbent assay plate reader (Thermo Labsystems; Multiskan Ascent, Finland).

3. Results and discussion

To determine whether HC-EA has a growth inhibition effect on human cancer cells we performed cell viability assays using seven human cancer cell lines. HC-EA significantly decreased the cell viability of all seven of human cancer lines tested in a dose dependent manner (Fig. 2). Assuming an IC_{50} value of ~198, and ~275 μg/ml concentration, HC-EA markedly blocked cancer cell growth in HL-60 and AGS cells. The anti-proliferation activity of HC-EA was observed in cancer cell lines, but not in pri-

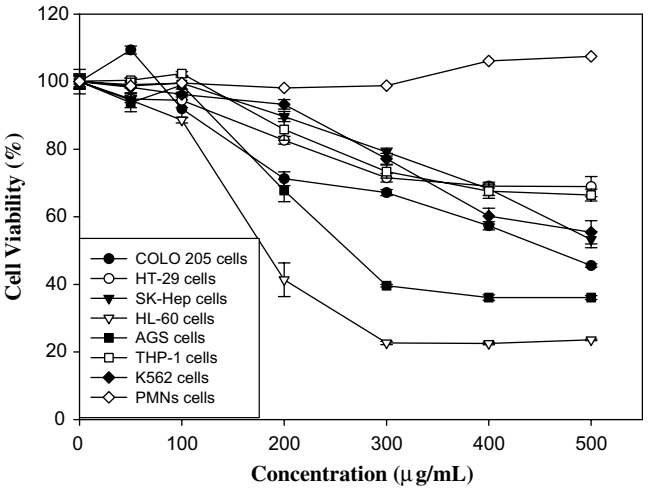

Fig. 2. Analysis of cell viability of HC-EA-treated human cancer cells as determined by MTT assay. Cells were treated with different concentrations of HC-EA for 6 h. Viability of the cells was determined by MTT assay. Cells were treated with 0.1% DMSO as vehicle control. Data are represented as means ± SE for three determinations.

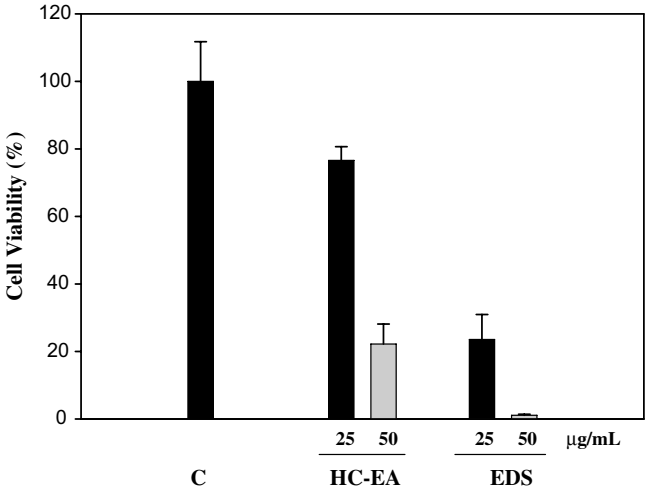

Fig. 4. Effects of HC-EA and epidioxysterols (EDS) on cell viability. HL-60 cells were either treated with 0.05% DMSO as vehicle control (C) or treated with HC-EA and (or epidioxysterols) for 24 h. Cell viability then was determined by Luminescent ATP Detection Assay Kit, as described in Section 2. Data are represented as means ± SE for three determinations.

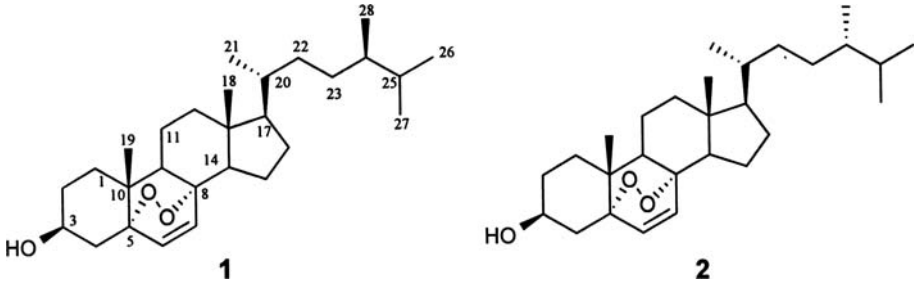

Fig. 3. Structure of epidioxysterols (EDS) isolated from *Meretrix lusoria*.

mary human PMNs, as analyzed by MTT assays. These results suggest the specific suppression of growth activity by HC-EA in human leukemia HL-60 cells.

Carcinogens usually cause genomic damage in exposed cells. As a consequence, the damaged cells may be triggered either to undergo apoptosis or to proliferate with genomic damage, leading to the formation of cancerous cells that usually exhibit cell cycle abnormalities and which are more susceptible to various apoptosis-inducing agents (Steller, 1995; Thompson, 1995). Therefore, identifying active compounds from food with apoptosis-inducing activity against cell lines is considered to be an important search for the chemoprevention of cancer. The apoptosis-inducing fraction 13 was further fractionated to obtained the most active compounds epidioxysterols in yield of 32 mg. Epidioxysterols was identified as a mixture of 5α,8α-epidioxy-24(*R*)-methylcholest-6-en-3β-ol (**1**) and 5α,8α-epidioxy-24(*S*)-methylcholest-6-en-3β-ol (**2**). The structures as shown in Fig. 3, were determined by NMR and confirmed by the literature data (Gauvin et al., 2000). We first tested the effect of HC-EA and epidioxysterols on cell viability. Human leukemia HL-60 cells were treated with 25 μg/ml and 50 μg/ml of HC-EA and epidioxysterols. After 24 h of treatment, a Luminescent ATP cell proliferation assay kit as described in Section 2 determined the live cells. As shown in Fig. 4, epidioxysterols appeared to be a more potent inhibitor of cell viability.

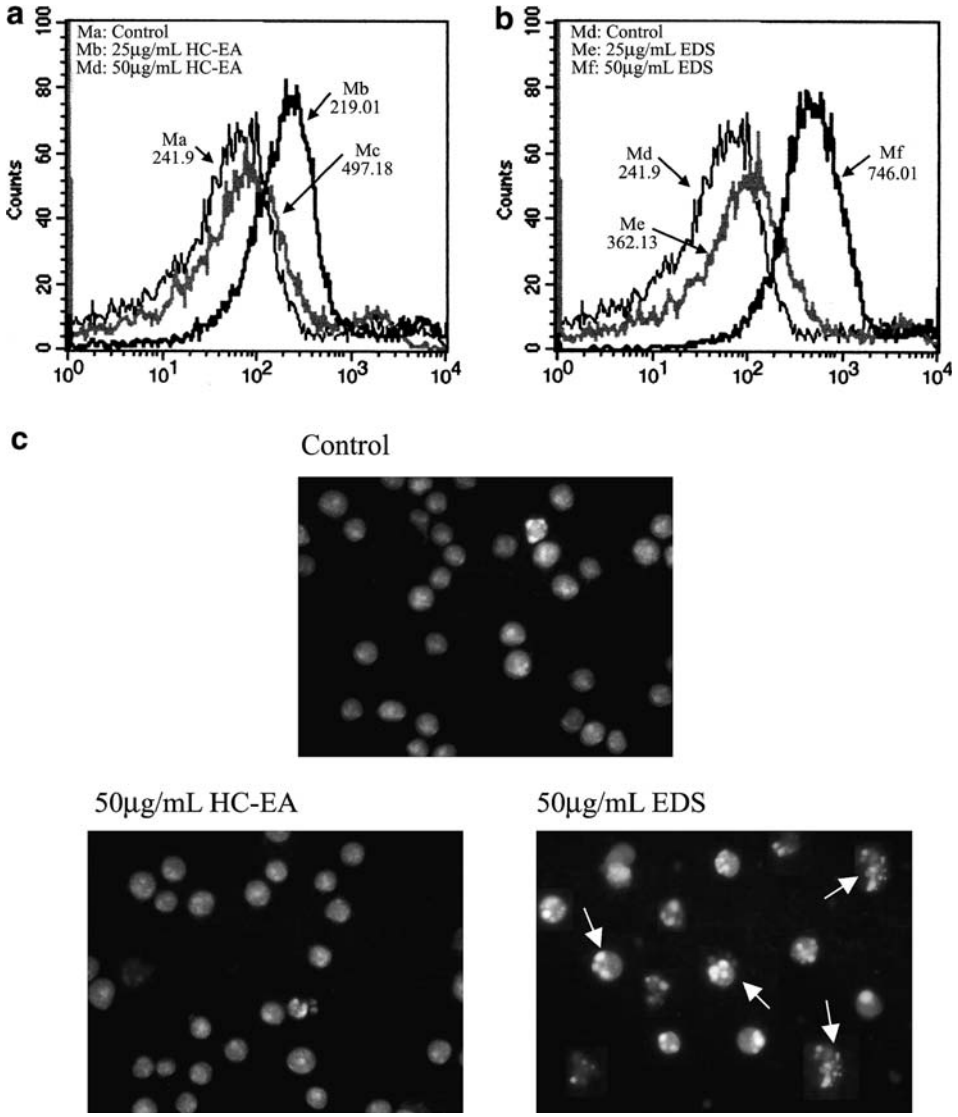

Fig. 5. Induction of apoptosis by EDS in HL-60 cells. (a) Determination of apoptosis in HC-EA and EDS treated HL-60 cells by annexin V-FITC staining was quantitated by flow cytometry. HL-60 cells were treated with 25 μg/ml and 50 μg/ml HC-EA and EDS (b), respectively, or treated with 0.05% DMSO as vehicle control for 15 min. The method of flow used is described in Section 2. Data are presented as log fluorescence intensity. (c) HL-60 cells were treated with 0.05% DMSO as vehicle control or treated with 50 μg/ml HC-EA and EDS, respectively, for 24 h, and cells were harvested and washed with PBS followed by staining with acridine orange. Nuclear staining was examined by fluorescence microscopy. The data presented are representative of three independent experiments.

Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosome degradation of DNA, and apoptotic body formation (Pan, Lin, Lin-Shiau, & Lin, 1999). To determine whether the inhibition of cell growth by epidioxysterols resulted from the induction of apoptosis, the early translocation of phosphatidyserine (PS) from the internal to external leaflet, chromatin condensation, and sub-G1 cell population, a characteristics of apoptosis, were demonstrated by incubating HL-60 cells with epidioxysterols. The means of FITC fluorescence clearly exhibited a significant increase from 241.9 to 497.2, as detected by annex V-FITC, during 50 μg/ml HC-EA-treatment of HL-60 cells (Fig. 5a). However, the

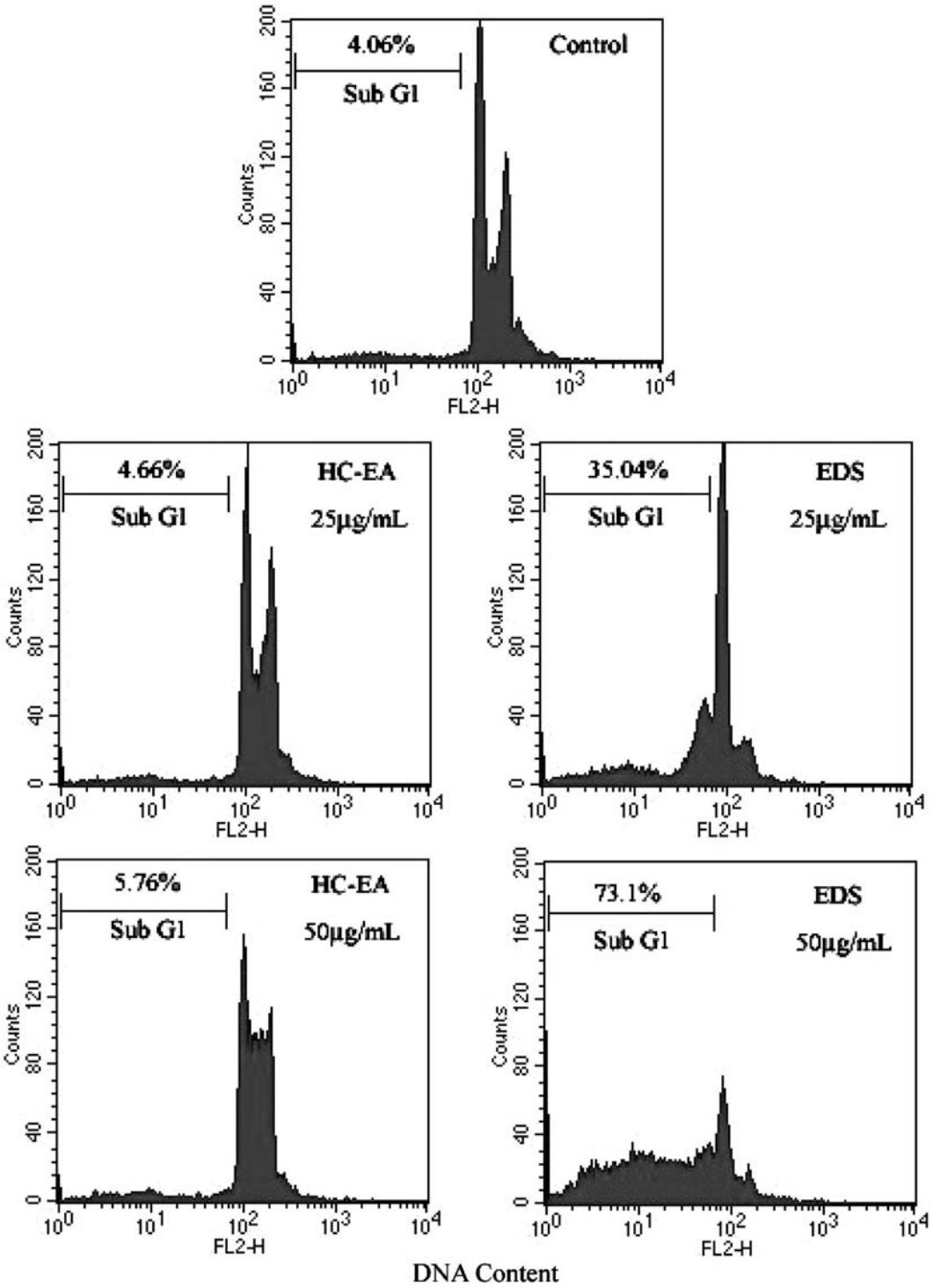

Fig. 6. Determination of sub-G1 cells in HC-EA- and EDS-treated HL-60 cells. HL-60 cells were treated with DMSO only as control or treated with HC-EA and EDS with 25 μg/ml and 50 μg/ml for 24 h, respectively. AP (apoptotic peak) represents apoptotic cells with a lower DNA content.

means of FITC fluorescence marked increase from 249.1 to 361.2 and 746.0 during 25 μg/ml and 50 μg/ml epidioxysterols-treatment in HL-60 cells (Fig. 5b). To characterize the cell death induced by epidioxysterols, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding agent, acridine orange. Within 24 h of treatment with 50 μg/ml epidioxysterols, cells clearly exhibited significant morphological changes and chromosomal condensation, which is indicative of apoptotic cell death (Fig. 5c). Such results imply that the cytotoxic action of epidioxysterols was due to its ability to induce apoptosis.

A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA, a hallmark of apoptosis, was used to guide the fractionation and identification of the apoptosis-inducing components in *M. lusoria*. As shown in Fig. 6, the percentages of apoptotic HL-60 cells were 35.0% and 73.1% with 25 μg/ml and 50 μg/ml compounds, respectively. In contrast, there was no changed in the percentages of apoptosis in HL-60 cells treated with 25 μg/ml or 50 μg/ml of HC-EA.

Next, we determined the effects of HC-EA and EDS on free radicals scavenger and anti-inflammatory activity. The antioxidant activity of HC-EA and EDS was assayed with DPPH free radical. HC-EA and EDS showed less antioxidant activity. To investigate the anti-inflammatory effects of HC-EA and EDS shown in Fig. 7, they were tested for their ability to inhibit NO generation in LPS-activated macrophages. Of the compounds tested, EDS strongly inhibited LPS-stimulated NO generation in a concentration-dependent manner. At the concentrations of 5, 10, 25, and 50 μg/ml, EDS inhibited NO generation by 24%, 27%, 46%, and 76%, respectively. Inhibition of NO production was not due to cytotoxicity, as determined with the trypan blue exclusion assay.

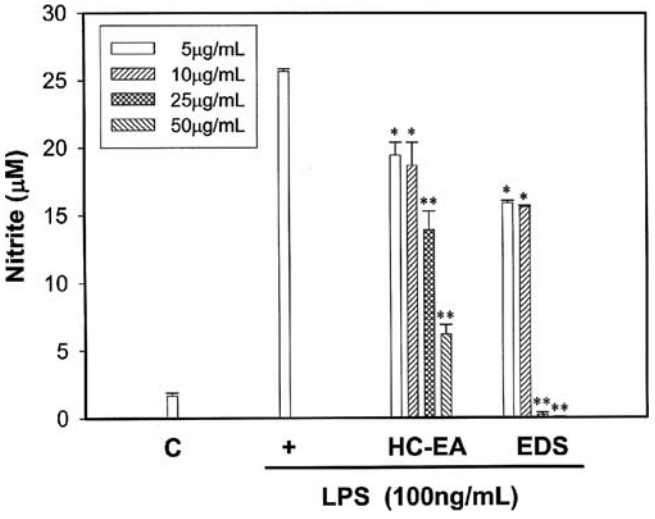

Fig. 7. Effect of HC-EA and EDS on LPS-induced nitrite production in RAW 264.7 cells. The cells were treated with or without LPS (100 ng/ml) and different concentrations of compounds or DMSO (0.05%) for 24 h. Nitrite were determined by Griess reaction as described in Section 2. The values are expressed as means ± SE of triplicate testes. $^{*}P < 0.05$; $^{**}P < 0.001$ versus LPS treatment.

This lipid extract of the *M. lusoria* has proven to be an extraordinarily rich source of a wide variety of unusual steroids belonging to the 5α, 8α-epidioxy sterol family. The epidioxysterols identified showed apoptosis-inducing activity against the human leukemia HL-60 cells. Recently, this sterol peroxide family from the marine sponge *Luffariella* cf. *variabilis* has been reported to possess inhibitory activity against the human T-cell leukemia/lymphotropic virus type I (HTLV-I) and also displayed cytotoxic activity against the human breast cancer cell line (Gauvin et al., 2000). The cytotoxic 5α,8α-epidioxysterol from a soft coral *Sinularia* species has been found to exhibit significant cytotoxicity against P-388 (mouse lymphocytic leukemia), KB (human nasopharyngeal carcinoma) cells, A549, and HT-29 cells (Sheu, Chang, & Duh, 2000). In addition, the sterol, ergosterol peroxide, isolated from edible mushrooms *Sarcodon aspratus* has been shown to inhibit the growth of HL-60 cells by inducing apoptosis (Takei, Yoshida, Ohnishi-Kameyama, & Kobori, 2005). Taken together, these results suggest that steroids containing 5α,8α-epidioxy functional group warrant further antitumor studies. Therefore, we speculate that the induction of apoptosis observed in this study may provide a distinct mechanism for the chemopreventive function of *M. lusoria*.

Acknowledgements

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