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Eudesmols Induce Apoptosis through Release of Cytochrome *c* in HL-60 Cells

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Abstract – We verified that the apoptosis activities were examined by DNA fragmentation, flow cytometric analysis with annexin V staining, activation of caspase-3, and cytochrome c release. In the result, α- and β-eudesmol induced DNA fragmentation in HL-60 cells at a concentration of 80 μM, respectively. Additionally, pro-apoptotic cells sorted by flow cytometry analysis were detected in HL-60 cells to 31.77 and 29.67% with á- and β-eudesmol of 80 μM. Thus, both α- and β-eudesmol exerted caspase-3 activation and cytochrome c release at 80 μM in HL-60 cells. These results are firstly reported that the sesquiterpenes, α- and β-eudesmol are apoptosis inducers through mitochondria-dependent caspase cascade in HL-60 cells.
Keywords – *Magnolia obovata*; sesquiterpene; eudesmols; apoptosis inducer; phytochemical

Introduction

The stem bark of *Magnolia obovata* (Magnoliaceae) has been used as traditional medicine for the treatment of gastrointestinal disorders, anxiety, and allergic diseases including bronchial asthma in Korea, Japan, and China. Previously, phytochemicals isolated from *Magnolia obovata* studied have been revealed a variety of neolignans, sesquiterpenes, sesquiterpene-neolignan, phenylpropanoids, and alkaloids. These compounds have reported to exert antibacterial (Bae *et al*., 1998), neurotrophic, antialergic (Hamasaki *et al*., 1999), vasorelation (Teng *et al*., 1990), muscle relaxation, central depressant and anti-gastric ulcer activities. Additionally, a lot of biological activities of sesquiterpenes isolated from natural sources have been reported with antitumor, antifungal, antibacterial, antioxidant, and antivenom activities (Denyer *et al*., 1994). Therefore, we have commenced a screening program to identify and develop apoptosis inducers from medicinal plants. Accordingly, we had the identification of two apoptosis inducers, α-eudesmol (**1**) and β-eudesmol (**2**) isolated from *Magnolia obovata*. Both α-eudesmol and β-eudesmol, eudesmane-type phytochemicals, are belonging to sesquiterpene family, and also have wide biological activities, such as antifreedant (Vera *et al*., 2008), antibacterial (Al-Dabbas *et al*., 2006) and glucose consumption activities (Sun *et al*., 2004). Especially, β-eudesmol is known to have various unique effects on the nervous system. β-Eudesmol acts as a channel blocker for nicotinic acetylcholine receptors at the neuromuscular junction (Kimura *et al*., 1991), antimicrobial (Denyer *et al*., 1994), antiangiogenic activity (Ikeda *et al*., 2002). However, there is not reported that whether β-eudesmol has activity of inducing apoptosis in tumor cells. We whence thought that it is desirable that for new biological function to investigate the apoptotic effects of α- and β-eudesmol in tumor cells.

Experimental

Plant material – The stem barks of *Magnolia obovata* were purchased from Il-sin Oriental market in Daejeon, Korea. The botanical sample was identified by Professor KiHwan Bae, College of Pharmacy, Chungnam National University, Daejeon, Korea. A voucher specimen has been deposited in the Chemical Biology Research Center, Korea Institute of Bioscience and Biotechnology, Daejeon, Korea.

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Extraction and isolation – The dried stems barks (3 kg) of *M. obovata* were extracted three times with MeOH for 10 days at room temperature. The combined extract was suspended in H_2O, and sequentially partitioned with *n*-hexane, EtOAc, and BuOH. The hexane-solution fraction was subjected to a silica gel column and α- and β-eudesmol were isolated by using semi-preparative HPLC. The purity of α- and β-eudesmol was higher than 98% with the analysis of HPLC and NMR measurement.

α-Eudesmol (1): Colorless needle, mp 80 - 82 °C, $[\alpha]^{24}_D$ + 3.12 ° (*c* 0.16, CH_2Cl_2); UV λ_{max} (CH_2Cl_2) 228 nm (log ε 3.25); IR ν_{max} (KBr) 3490 (OH), 3377 (CH, aliphatic), 1051, 888 cm^{-1}; EIMS *m/z* 222.3 $[M]^+$; ^1H and ^{13}C NMR data were in accordance with previous reported data (Raharivelomanana *et al*., 1998).

β-Eudesmol (2): Colorless needle, mp 80 - 82 °C, $[\alpha]^{24}_D$ + 3.12 ° (*c* 0.16, CH_2Cl_2); UV λ_{max} (CH_2Cl_2) 228 nm (log ε 3.25); IR ν_{max} (KBr) 3490 (OH), 3377 (CH, aliphatic), 1051, 888 cm^{-1}; EIMS *m/z* 222.3 $[M]^+$; ^1H and ^{13}C NMR data were in accordance with previous reported data (Raharivelomanana *et al*., 1998).

Cell culture and cell viability assay – Human leukemia HL-60 cells were maintained in RPMI-1640 supplemented with 10% FBS. The culture was incubated at 37 °C in a 5% CO_2 atmosphere. Various concentrations of α- and β-eudesmol were added to 1×10^4 human leukemia HL60 cells suspended in the culture medium supplemented with 10% FBS, in 9 cm dish. The culture was incubated at 37 °C for 4 hrs in a 5% CO_2 atmosphere. Number of viable cells was counted by trypan-blue dye exclusion method.

Analysis of DNA fragmentation – Cells (1×10^6 cells/well) were collected and washed by cold phosphate-buffered saline (PBS) after they were treated with various concentration of α- and β-eudesmol for 4 hrs. At appropriate times, cells were transferred to 1.5 ml tubes, centrifuged for 30 sec at 12,000 rpm, and resuspended in 20 µl of lysis buffer (0.8% sodium lauryl sarcosine, 20 mM EDTA, 100 mM Tris pH 8.0). The cell extracts were treated with 10 µl of RNase A (1 mg/ml) for 30 min at 37 °C, and further incubated for 1.5 hrs at 50 °C in the presence of 10 µl of proteinase K (20 mg/ml). After mixing with loading dye, the lysates were subjected to a 2% agarose gel electrophoresis at 100 V for 1.5 hrs.

Flow cytometric analysis of apoptosis – Cells were seeded into 6-well plates at a density of 2×10^6 cells/ml and added stimulating antibody. After treatment of α- and β-eudesmol, cells were harvested and washed twice with cold PBS. Apoptosis assessment was performed using FITC-conjugated annexin V antibody (BD PharMingen, San Diego, CA) and propidium iodide (Sigma), according to the manufacturer's recommendations. The proportion of apoptotic cells in the prepared cells was determined by flow cytometric analysis using FACS Calibur (Becton Dickinson, Mountain View, CA), and data were analyzed using CellQuest software (Becton Dickinson).

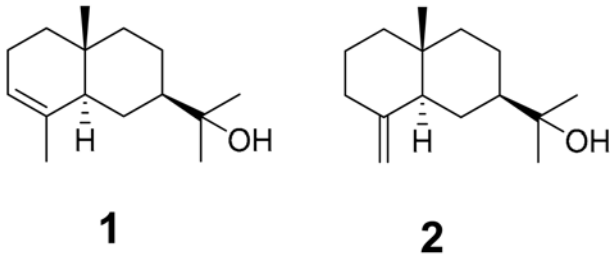

Fig. 1. Chemical structures of α- and β-eudesmol.

Immunoblotting analysis – HL-60 cells (1×10^6 cell/well) were treated with α- and β-eudesmol for 4 hrs, then collected and washed with PBS. The cells were lysed [25 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM ethylene glycol bis-(2-aminoethylether) *N, N, N', N*-tetraacetic acid (EGTA), containing 1 mM phenylmethylsunfonyl fluoride (PMSF) and 5 mM dithiothreitol (DTT)] and centrifuged at 15,000 rpm for 30 min at 4 °C. The protein concentration was determined with Bio-Rad protein assay reagent. Equal amounts of cytoplasmic or nuclear extract were subsequently fractionated by 10~15% SDS-PAGE. The fractionated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% skim milk diluted in PBS (or TBS) containing 0.1% Tween-20 for 1 hr and incubated with the primary antibodies, The blots were incubated with monoclonal anti-cytochrome *c*, anti-caspase-3, GAPDH antibodies followed by enhance chemiluminescence (ECL)-based detection (Amersham Pharmacia Biotech). The secondary antibodies were used at a 1 : 1000 dilution.

Results and Discussion

α- and β-Eudesmol induced the cell viability of leukemia HL-60 cells – To confirm the effect of α- and β-eudesmol on HL-60 cells, we first examined the cell survival of HL-60 cells treated with various concentrations of α- and β-eudesmol. Treatment of HL-60 cells with α- and β-eudesmol at 40 µM did not significantly decreased cell survival for 4 hrs. In contrast treatment of HL-60 cells with α- and β-eudesmol at 80 µM, cell viability was decreased with 44.6% and 51.6%, respectively (Fig. 2) for 4hrs. Additionally, investigating

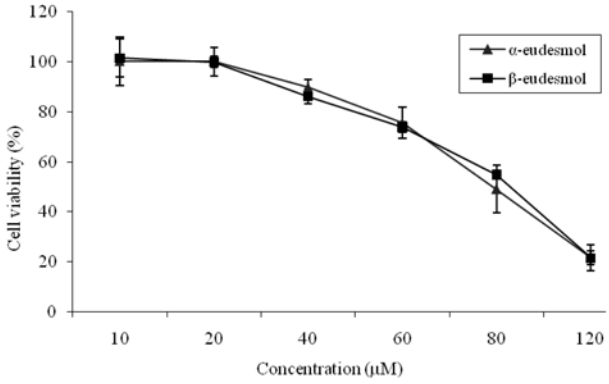

Fig. 2. α- and β-Eudesmol inhibit HL-60 cell viability in dose dependent manner.
Cultured HL-60 cells in 96-well plate treated with α-eudesmol (close triangle) and β-eudesmol (close square). Test compounds were dissolved in DMSO at appropriate concentrations treated for 4 hrs, and the cell viability was measured by trypan-blue dye exclusion method as described in material method. All the points show means ± S.E. from a representative triplicate experiment.

whether the inhibitory effects on HL-60 cells are caused by apoptosis, the morphology of HL-60 cells was observed under a microscope by treating with α- and β-eudesmol. The morphology of HL-60 cells observed under a microscope by treating with α- and β-eudesmol at 40 μM and 80 μM showed typical morphological characteristics of apoptosis, such as swelling-shape (data not show).

Induction of apoptosis by α- and β-eudesmol in leukemia HL-60 cells – To determine whether α- and β-eudesmol could induce apoptosis in HL-60 cells, we performed DNA laddering experiments following α- and β-eudesmol treatment. As shown in Fig. 3A, the DNA laddering was observed after incubation with α- and β-eudesmol at concentrations of 80 μM for 4 hrs as well as CPT (Campothecin, 10 μM) treatment. Particularly, investigating α- and β-eudesmol acts as apoptosis, flow cytometric analysis for apoptosis quantification was carried out. The analysis data did not show the apoptosis

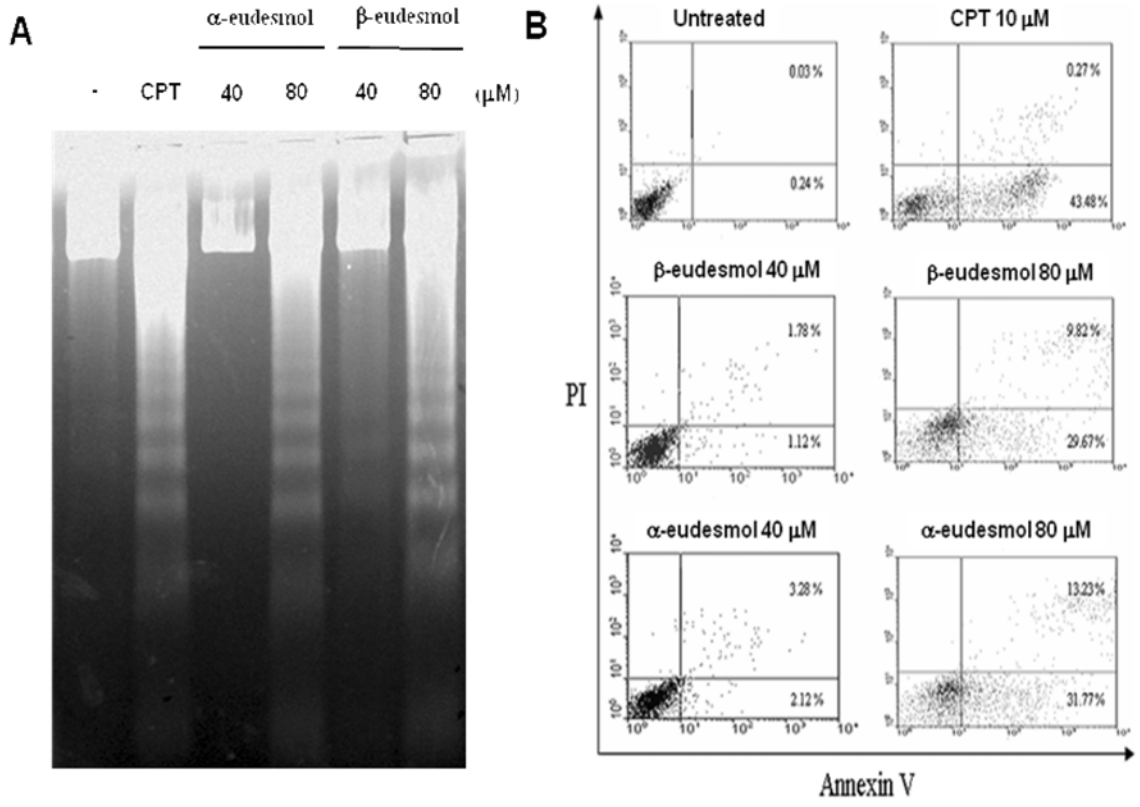

Fig. 3. α- and β-Eudesmol induce apoptosis in HL-60 cells.
(A) DNA fragmnetation of leukemia HL60 cells treated with α- and β-eudesmol. Cell cultured in a 12-well plate exposed to both compounds with indicated concentration for 4 hrs, the agarose gel electrophoresis as described in Materials and methods. Campothecin (CPT) used as a positive control (10 μM). (B) Flow cytometric analysis of apoptosis of HL-60 cells treated with α- and β-eudesmol. Evaluation of apoptosis by annexin V positivity after α- and β-eudesmol treatment for 4 hrs. HL-60 cells were exposed with 40 μM and 80 μM α- and β-eudesmol and examined by flow cytometry after annexin V and propidium iodium (PI) staining. Evidence of apoptotic cells was provided by the binding of annexin V and the retention of PI (low right quadrant) and double-positive cells underwent secondary necrosis (upper right quadrant). Data are representative of three independent experiments.

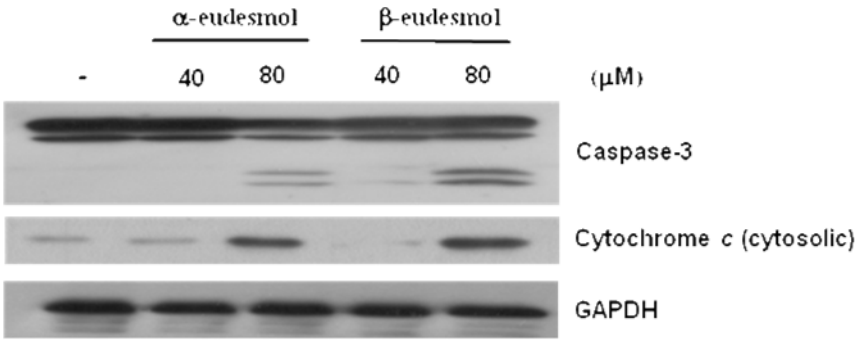

Fig. 4. α-eudesmol and β-eudesmol activates caspase-3 and release cytochrome *c* to cytoplasm from mitochondria. Cells were treated with both compounds with indicated concentration for 4hrs. Equal amounts (30 μg) of the cytoplasmic fractions obtained as described in Materials and methods. Samples were applied to western blot analysis with a caspase-3 and cytochrome *c* specific antibodies.

cells at 40 μM. In contrast, at 80 μM of α- and β-eudesmol, the apoptosis were induced with 29.6% and 31.7%, respectively (Fig. 3B). These results indicated that α- and β-eudesmol reduced cell viability by inducing of apoptosis in HL-60 cells.

Activation of caspase-3 with α- and β-eudesmol induced apoptosis in HL-60 cells – Principally, proteolytic cleavage of caspases from the precursor to the active form is necessary to activate the caspase-cascade and downstream proteolytic effectors function for apoptosis. In order to determine the involvement of caspase- cascade with α- and β-eudesmol induced apoptosis, we examined caspase-3 activity with α- and β-eudesmol treated cells by measurement of the cleavage of the caspase-3 using western blot experiments. The activation of caspase-3 was observed at 80 μM after α- and β-eudesmol treatment for 4 hrs (Fig. 4). These results suggested that caspase-3 was functionally activated during α- and β-eudesmol-induced apoptosis in HL-60 cells.

α- and β-Eudesmol induced the release of cytochrome c into the cytoplasm – Mitochondrial permeabilization has been shown to activate caspase-9 through the release of cytochrome *c*. We verified that whether α- and β-eudesmol induce the apoptosis via an mitochondria-dependent caspase-cascade. Predictably, α- and β-eudesmol induced the release to cytochrome *c* into the cytoplasm from mitochondria in at 80 M for 4 hrs (Fig. 4). These results revealed that the release of cytochrome *c* occurred during the process of α- and β-eudesmol induced apoptosis. These data suggested that α- and β-eudesmol induced apoptosis is through the pathway of caspase-3 via caspase-9 activation by cytochrome *c*. In conclusion, we revealed that effects of α- and β-eudesmol on caspase-3 activation will be correlated with its apoptosis-inducing activities in HL-60 cells. Moreover, the precise molecular target, containing caspases-related molecules, associated with the apoptosis-inducing activities of α- and β-eudesmol may provide a new drug target for the development of novel apoptosis-inducing compounds.

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