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The Cytotoxicity of Eutigosides from *Eurya emarginata* Against HL-60 Promyelocytic Leukemia Cells

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Two phenolic glucosides, eutigoside B and eutigoside C were isolated from the fresh leaves of *Eurya emarginata*. These two phenolic glucosides exerted a significant inhibitory effect on the growth of HL-60 promyelocytic leukemia cells. Furthermore, when the HL-60 cells were treated with eutigoside C, several apoptotic characteristics such as DNA fragmentation, morphologic changes, and increase of the population of sub-G1 hypodiploid cells were observed. In order to understand the mechanism of apoptosis induction by eutigoside C, we examined the changes of Bcl-2 and Bax expression levels. The eutigoside C reduced Bcl-2 protein and mRNA levels, but slightly increased Bax protein and mRNA levels in a time-dependent manner. When we examined the activation of caspase-3, an effector of apoptosis, the eutigoside C increased the expression of active form (19-kDa) of caspase-3 and the increase of their activities was demonstrated by the cleavage of poly (ADP-ribose) polymerase, a substrate of caspase-3, to 85-kDa. The results suggest that the inhibitory effect of eutigoside C from *E. emarginata* on the growth of HL-60 appears to arise from the induction of apoptosis *via* the down-regulation of Bcl-2 and the activation of caspase.

Key words: *Eurya emarginata*, HL-60, Eutigosides B, Eutigoside C, Apoptosis. Bcl-2, Bax, Caspase-3

INTRODUCTION

Recently, a large number of new anticancer agents, which target one or more of the extracellular, transmembrane, or intracellular (but extranuclear) processes involved in the malignant transformation of cells or carcinogenesis, have been developed from natural resources (Greenwald *et al.*, 1990; Pezzuto, 1997; Sporn, 1994; Wattenberg, 1992). In the continuing search for antitumor compounds from plants growing in Jeju Island, the crude extract of the leaves from *Eurya emarginata* markedly inhibited the growth of leukemia cells and lymphoma cells such as HL-60, KG-1, U937 and Jurkat cells (Park and Kang, 2000; Park *et al.*, 2004). Moreover, the ethylacetate fraction of *E. emarginata* induced the apoptosis of HL-60 promyelocytic leukemia cells (Park *et al.*, 2004). *Eurya emarginata* (Thunb.) Makino (Theaceae) is a dioecious and insect-pollinated tree, which combines both sexual reproduction and clonal spread. It is narrowly distributed in coastal areas ranging from southern China, along southern Korea, and extending to central and southern Japan (Chung and Epperson, 2000). The leaves of *Eurya* are used in the traditional medicine of the coastal areas of Jeju Island with the aim of diuresis or to treat ulcers. Nevertheless, there are few reports on the biological activity and constituents of *E. emarginata*. Therefore, this report dealt with the isolation and the structure elucidation of eutigosides from leaves of *E. emarginata*, as well as their anti-cancer activity and their action mechanism against human leukemia HL-60 cells.

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MATERIALS AND METHODS

Plant materials

The leaves of *Eurya emarginata* (Thumb.) Makino were collected in May 2000 at Cheju National University, Jeju Island, Korea and dried at room temperature.

Characterization experimental procedures

^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JNM-LA 400 (FT NMR system, Nuclear Magnetic Resonance, Japan) spectrometer. TLC (Thin layer chromatography) was carried out on Merck precoated silica gel 60 F_{254} plates and silica gel for normal-phase column chromatography was Kieselgel 60 (230-400 mesh ASTM, Merck, Germany). Reverse-phase column chromatography was carried out with silica gel 100 C_{18}-reversed phase (3 × 15, silica gel 100 C_{18}-reversed phase, Merck). C_{18} reverse phase HPLC column (μBondapak C_{18}, 7.8 × 300 mm column, Merck; Waters 2487, Waters) was used for HPLC analysis.

Isolation of eutigosides B (1) and C (2)

The dried leaves (150 g) were extracted using 80% aqueous methanol (1 L × 3) after standing a week at room temperature. The liquid layer was obtained by filtration, and the solvent was evaporated under reduced pressure to give the crude extract (48.8 g). After water (1 L) was added to the extract, the aqueous layer was successively partitioned into hexane, ethyl acetate (EtOAc) and *n*-butanol. A part (1.5 g) of the ethyl acetate fraction (7.7 g) was purified through reversed phase SiO_2 column using gradient elution of aqueous methanol (20% to 100%) to give five fractions (A–E). The polar fraction A (600 mg) was subjected to sephadex LH-20 column chromatography using CH_2Cl_2/acetone/methanol (3/2/0 to 1/4/0 to 2/7/1) to give 32 fractions. The fraction 26 (13 mg) was further purified by HPLC to give eutigoside C (5.3 mg). The fraction 30 (15 mg) was also purified by HPLC to give the eutigoside B (3.8 mg).

Eutigoside B (1)

^{1}H-NMR (400 MHz, methanol-d_4) δ 7.63 (1H, d, 16.0, H7"), 7.47 (2H, d, 8.6, H2", H6"), 6.97 (2H, m, H2, H6), 6.81 (2H, br d, 8.6, H3", H5"), 6.35 (1H, d, 16.0, H8"), 6.07 (2H, d, 10.0, H3, H5), 4.48 (1H, dd, 12.0, 2.2, H6'), 4.29 (1H, dd, 12.0, 6.0, H6'), 4.24 (1H, d, 7.8, H1'), 3.92 (1H, dt, 11.9, 6.1, H8), 3.64 (1H, dt, 11.9, 6.1, H8), 3.49 (1H, dd, 9.0, 6.0, H5'), 3.31-3.39 (2H, m, H3', H4'), 3.16 (1H, dd, 9.0, 7.8, H2'), 2.04 (2H, t, 6.1, H7); ^{13}C-NMR (methanol-d_4) δ 187.8 (C4), 169.1 (C9"), 161.4 (C4"), 154.4 (C2), 154.3 (C6), 146.8 (C7"), 131.2 (C2", C6"), 128.0 (C3), 127.9 (C5), 116.9 (C3", C5"), 115.0 (C8"), 104.4 (C1'), 77.9 (C3'), 75.5 (C5'), 75.0 (C2'), 71.8 (C4'), 69.2 (C1), 65.9 (C8), 74.6 (C6'), 41.0 (C7); Consistent with $C_{23}H_{26}O_{10}$

Eutigoside C (2)

^{1}H-NMR (400 MHz, methanol-d_4) δ 7.71 (1H, d, 16.0, H8"), 7.62 (2H, m, H2" and H6"), 7.40 (3H, m, H3", H4", H5"), 6.97 (2H, m, H2, H6), 6.56 (1H, d, 16.0, H8"), 6.06 (2H, br d, 10.4, H3, H5), 4.50 (1H, dd, 11.8, 2.0, H6'), 4.32 (1H, dd, 11.8, 2.0, H6'), 4.25 (1H, d, 7.8, H1'), 3.92 (1H, dt, 10.2, 6.5, H8), 3.65 (1H, dt, 10.2, 6.5, H8), 3.51 (1H, dd, 9.0, 6.0, H5'), 3.34 (2H, m, H3', H4'), 3.16 (1H, dd, 9.0, 7.8, H2'), 2.04 (2H, t, 6.5); ^{13}C-NMR (methanol-d_4) δ 187.8 (C4), 168.5 (C9"), 154.4 (C2), 154.3 (C6), 146.5 (C7"), 135.7 (C1"), 131.6 (C4"), 130.1 (C2", C6"), 129.3 (C3", C5"), 128.0 (C3), 127.9 (C5), 118.7 (C8"), 104.4 (C1'), 77.9 (C3'), 75.4 (C2'), 75.0 (C5'), 71.7 (C4'), 69.2 (C1), 65.9 (C8), 64.8 (C6"), 41.0 (C7); Consistent with $C_{23}H_{26}O_9$

Cell cultures

The HL-60 acute promyelocytic leukemia cells were obtained from the KCLB (Korean Cell Line Bank). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; GIBCO). The exponentially growing cells were used throughout the experiments.

Cytotoxicity tests

Effects of testing materials on the growth of promyelocytic leukemia HL-60 cells were determined by the measurement of metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Carmichael *et al.*, 1987). MTT assays were performed as follows. HL-60 cells (2.5 × 10^5/mL) were treated for 4 days with 100 μg/mL of testing materials in 96-microwell plates. After incubation, 0.1 mg (50 μL of a 2 mg/mL solution) of MTT was added to each well and cells were then incubated at 37°C for 4 h. Plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. 150 μL of dimethylsulfoxide was then added to each well to solubilize the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech). All experiments were performed three times and the mean absorbance values were calculated. Results are expressed as the percentage of inhibition that produced reduction of absorbance in material-treated cells as compare with untreated controls.

Assays for apoptosis

The HL-60 cells (2.5 × 10^5/mL) were treated with 100 μg/mL of testing materials for 24 h. For the DNA fragmentation assay, the cells were collected by centrifugation and DNA was extracted with Wizard Genomic DNA purification kit (Promega). The DNA fragmentation pattern was analyzed by electrophoresis on a 1.5% agarose gel containing 0.1 μg/mL ethidium bromide for 1 h at 100 V (Oberhammer *et al.*, 1993). For the flow cytometric analysis to determine cell cycle phase distribution, the treated cells were washed twice with phosphate-buffered saline (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 of a propidium iodide (PI) solution and 50 μg/mL of RNase A in the dark for 30 min at 37°C. Flow cytometry analysis was performed using an EPICS-XL FACScan flow cytometer (Coulter). The DNA histograms obtained were analyzed to determine the distribution of cells in the cell cycle phase and to measure the proportion of sub-G1 hypodiploid cells (Sherwood *et al.*, 1994). In order to examine the morphological changes, the treated cells were harvested, fixed with 4% paraformaldehyde and incubated in 1 mL of a staining solution containing 2.5 μg/mL of 4.6-diamidino-2-phenyl-indole (DAPI) in the dark place for 30 min. After washing the cells with PBS, 10 mL of the stained cells were mounted. The nuclei were visualized using a BX-50 fluorescence microscope (Olympus) (Wyllie, 1992).

RNA isolation and RT-PCR analysis

HL-60 cells (2.5×10^5/mL) were treated with 100 μg/mL of eutigoside C and then harvested at 3, 6, 12, and 24 h. The cells were washed with PBS at 4°C and homogenized. Total cellular RNA was extracted by the Tri-reagent (MRC) according to the manufacturer's instructions. The RNA was quantified by reading the absorbance at 260 nm according to the methods described by Sambrook *et al.* (Sambrook *et al.*, 1989). The reverse transcription of 1 μg RNA was carried out with M-MuLV reverse transcriptase (MBI). The generated cDNA was amplified by using primers. The primers were for Bcl-2 (Agarwal and Mehta, 1997): TGC ACC TGA CGC CCT TCA C (S) and AGA CAG CCA GGA GAA ATC AAA CAG (A); for Bax (Agarwal and Mehta, 1997): ACC AAG AAG CTG AGC GAG TGT C (S) and ACA AAG ATG GTC ACG GTC TGC C (A); and for β-Actin: ATG GGT CAG AAG GAT TCC TAT G (S) and CAG CTC GTA GCT CTT CTC CA (A). The (S) and (A) denote forward primer and backward primer, respectively. Polymerase chain reaction (PCR) was performed with a particular primer pair for 4 min at 94°C, followed by 35 cycles for 30 sec at 94°C (denaturing), for 30 sec at 55-60°C (annealing) and for 30 sec at 72°C (primer extension). PCR products were detected on 1.5% agarose gel electrophoresis.

Western blot analysis

To examine the expressions of Bcl-2 family proteins, HL-60 cells (2.5×10^5/mL) were treated with 100 μg/mL of eutigoside C and then harvested at 3, 6, 12, and 24 h. To observe the activation of caspase-3 and cleavage of poly (ADP-ribose) polymerase (PARP), HL-60 cells (2.5×10^5/mL) were treated with different concentrations of eutigoside C for 24 h. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 mg/mL aprotinin, 25 μg/mL leupeptin] and kept on ice for 30 min. The cell lysates were centrifuged at $12,000 \times g$ at 4°C for 15 min and the supernatants were then stored at -70°C until used. The protein concentration was measured using the Bradford method (Bradford, 1976). Aliquots of the lysates (30-50 μg of protein) were resolved on 10% SDS-polyacrylamide gels for the detection of PARP and 12% SDS-polyacrylamide gels for the detections of caspase-3 and Bcl-2 family proteins, and transferred to a polyvinylidene fluoride (PVDF) membrane (BIO-RAD) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 5% nonfat dried milk, the membrane was then incubated with specific mouse monoclonal anti-human Bcl-2 Ab (1:1000, Santa-Cruz), rabbit polyclonal anti-human Bax Ab (1:1000, Santa-Cruz), rabbit polyclonal anti-human caspase-3 (1:1000, Cell Signaling) and rabbit polyclonal anti-human PARP Ab (1:1000, Santa-Cruz) as primary antibodies at 4°C overnight. The membrane was further incubated for 30 min with a secondary peroxidase-conjugated IgG (1:5000, Santa-Cruz) to mouse or rabbit. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (Amersharm-pharmacia Biotech.).

RESULTS AND DISCUSSION

In present study, we isolated two cytotoxic compounds, eutigoside B and C from the fresh leaves of *E. emarginata* and investigated the mechanism by which eutigoside C inhibited the proliferation of HL-60 leukemia cells. Two phenolic glucosides, eutigoside B and C were reported only from *Eurya tigang* (Theaceae) (Khan *et al.*, 1992) and the biological functions of these compounds were not defined. To the best of our knowledge, this is the first report to isolate eutigoside B and eutigoside C from *E. emarginata* as well as to show that eutigoside C induces the apoptosis of HL-60 leukemia cells.

Previously, we reported that the EtOAc fraction of the *E. emarginata* leaves markedly inhibited the growth of HL-60 leukemia cells and the inhibitory effect of *E. emarginata* on the growth of HL-60 appeared to arise from the induction of apoptosis (Park *et al.*, 2004). So, we isolated eutigoside B and eutigoside C, the cytotoxic compounds from the EtOAc-soluble fraction of *E. emarginata* (Fig. 1). Cornoside and quercitrin were also isolated from the EtOAc-soluble fraction, but they did not show cytotoxicity (not shown). The effects of eutigosides on the growth of HL-60 cells were assessed using a MTT assay. The MTT assay showed (Fig. 2) that eutigoside C than eutigoside B much more inhibited the growth of HL-60 cells. IC_{50} values

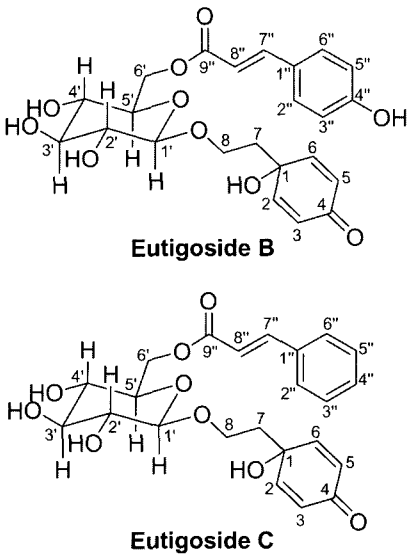

Fig. 1. The structures of eutigoside B and eutigoside C

of eutigoside B and eutigoside C were found to be 139.20 and 70.66 μg/mL in HL-60 acute promyelocytic leukemia cells, respectively.

The apoptosis induction of HL-60 cells by eutigoside C was assessed by observing the chromosomal DNA fragmentation. When the HL-60 cells were treated with 100 μg/mL of eutigoside C for 24 h, the internucleosomal cleavage of the genomic DNA resulting in the DNA ladder was observed (Fig. 3). When the HL-60 cells were stained with the DNA-specific fluorochrome, propidium iodide, eutigoside C was found to increase the proportion of sub-G1 hypodiploid cells (Fig. 4A). These results show that eutigoside C induces the apoptosis of HL-60 cells, and is supported by the increase in the number of morphologic

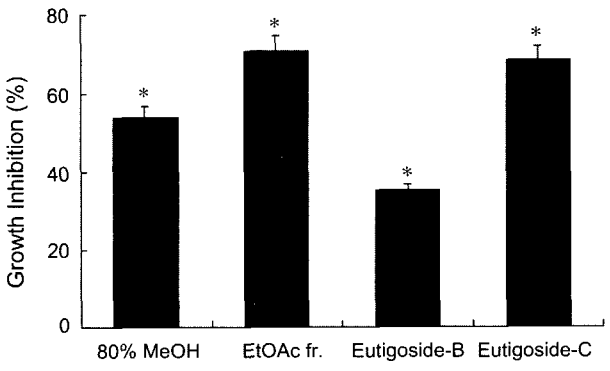

Fig. 2. Inhibitory effects of MeOH extract, EtOAc fraction, eutigoside B, or eutigoside C from *E. emarginata* on the growth of HL-60 cells. HL-60 cells (2.5×10^5/mL) were treated with 100 μg/mL of MeOH extract, EtOAc fraction, eutigoside B, or eutigoside C from *E. emarginata* for 4 days and measured for viability by MTT assay. All experiments were performed in triplicate. Data are presented as a mean ± S.D. *$p < 0.05$ compared with the control.

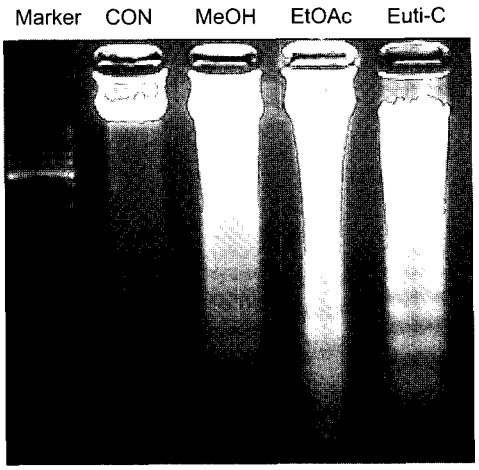

Fig. 3. DNA fragmentation by MeOH extract, EtOAc fraction, or eutigoside C from *E. emarginata* in HL-60 cells. HL-60 cells (2.5×10^5/mL) were treated with 100 μg/mL of MeOH extract, EtOAc fraction, or eutigoside C from *E. emarginata* for 24 h. The DNA was isolated and electrophoresed on a 1.5 % agarose gel containing 0.1 mg/ml ethidium bromide for 1 h at 100 V, and visualized under ultraviolet light.

changes (cell shrinking, the condensation of chromatin, and the membranous apoptotic bodies), which are another characteristics of apoptosis (Fig. 4B) (Sherwood *et al.*, 1994). Apoptosis (programmed cell death) is known as an important biologic mechanism that contributes to the maintenance of the integrity of multicellular organisms. It is induced by a wide variety of cellular stresses such as DNA damage, UV radiation, ionizing radiation and

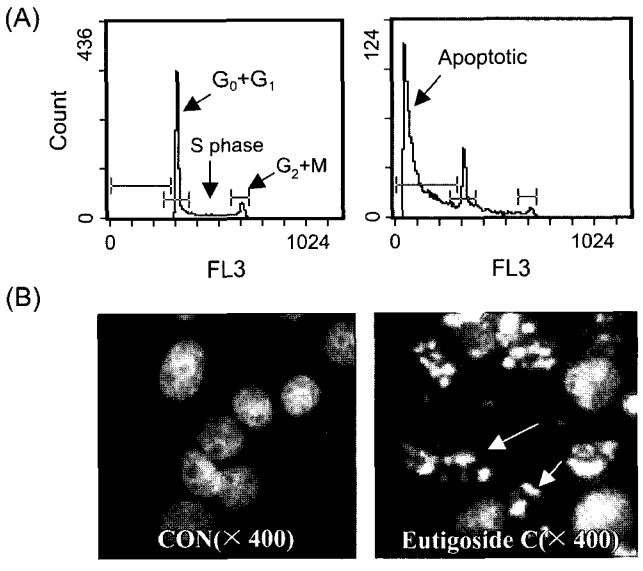

Fig. 4. The degree of apoptosis is represented as the DNA content measured by flow cytometric analysis and the fluorescence micrographs of cells with highly condensed nuclei stained with DAPI. Cells (2.5×10^5/mL) were treated without or with eutigoside C from *E. emarginata* for 24 h for the measurement of the sub-G1 hypodiploid cells (A) or for the examination of the morphological changes (B).

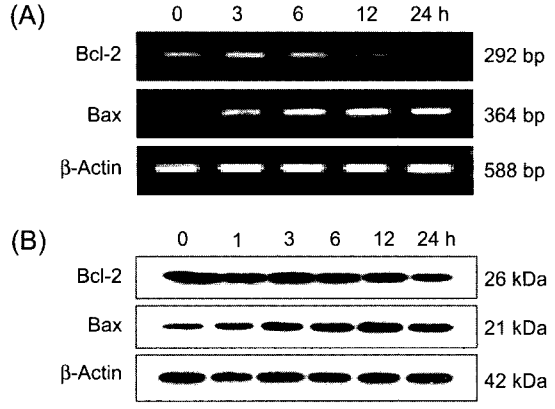

Fig. 5. Expressions of Bcl-2 and Bax in HL-60 cells by a treatment with the eutigoside C. HL-60 cells (2.5×10^5 /mL) were treated with eutigoside C (100 μg/mL) from *E. emarginata* for the indicated hours. (A) Reverse transcription polymerase chain reaction (RT-PCR) analyses of Bcl-2 and Bax were performed after synthesizing the cDNA as described in the "materials and methods". (B) Western blot analyses of Bcl-2 and Bax using the antibodies against Bcl-2 recognized a protein at 26 kDa and a 21 kDa protein for Bax.

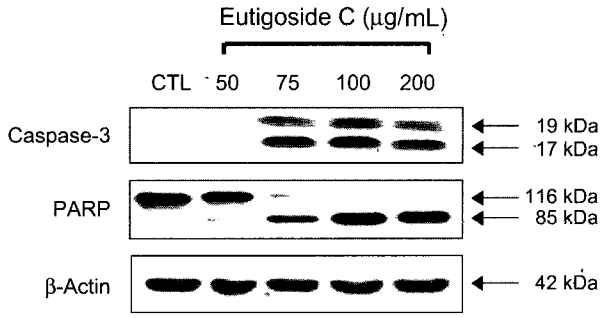

Fig. 6. Immunoblot analyses of caspase-3 and PARP. HL-60 cells (2.5×10^5/mL) were treated with various concentrations (25, 50, 75 and 100 μg/mL) of eutigoside C from *E. emarginata* for 24 h, and the activation of caspase-3 and PARP cleavage were analyzed with specific antibodies.

oxidative stress (Nagata, 1997). Antitumor agents also induce apoptosis in some cancer cells both *in vitro* and *in vivo*, indicating that apoptosis plays a very important role in cancer chemotherapy (Kaufmann, 1989; Meyn *et al.*, 1995). As a result of treatment with eutigoside C from *E. emarginata* leaves, HL-60 cells exhibited the apoptotic characteristic patterns of DNA fragment ladders, the increase of sub-G1 hypodiploid cells, cell shrinkage, and chromatin condensation.

Bcl-2 is known to suppress apoptosis by controlling the membrane potential of the mitochondria and/or preventing calcium depletion of the endoplasmic reticulum (Distelhorst *et al.*, 1996; Shimizu and Pommier, 1996). The anti-apoptotic activity of Bcl-2 correlates with its intracellular ratio to Bax, a pro-apoptotic factor. High expression levels of Bax have been shown to favor apoptosis in the cells subjected to growth factor deprivation, whereas high levels of Bcl-2 prolong cell survival under certain conditions (Baff *et al.*, 1993). Since Bcl-2 functions by forming a hetero-dimer with its pro-apoptotic partner, Bax, the Bcl-2 : Bax ratio is proportional to the relative sensitivity or resistance of the cells to various apoptotic stimuli (Oltvai and Korsmeyer, 1994). A number of studies have shown that the constitutive expression of the Bcl-2 proto-oncogene in a variety of cells results in a heightened resistance to chemotherapeutic agents that function by inducing apoptosis (Campos *et al.*, 1993; Hanada *et al.*, 1993; Miyashita and Reed, 1993). The central component of apoptosis is a proteolytic system involving a family of cysteine proteases referred to as caspases. All caspases are expressed as proenzymes, and their activation involves proteolytic processing (Kohler *et al.*, 2002; Nicholson and Thomberry, 1997). In response to various apoptotic stimuli, caspase cascades are activated leading to an irreversible commitment to cell death. This process serves as positive feedback to amplify the apoptotic signal in the caspase activation pathway (Zou *et al.*, 1997). Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins such as PARP, a nuclear enzyme that is involved in DNA repair in response to environmental stress (Fernandes-Alnemri *et al.*, 1994).

In order to elucidate the induction mechanism of apoptosis by eutigoside C from *E. emarginata*, we examined expressions of Bcl-2 and Bax using RT-PCR analysis and western blot. As shown in Fig. 5, eutigoside C reduced Bcl-2 protein and mRNA levels, whereas increased expressions of Bax protein and mRNA in a time-dependent manner. We then examined the activation of caspase-3, an effector of apoptosis. As shown in Fig. 6, the active form (19 kDa) of caspase-3 was increased in a dose-dependent manner. The activation of caspase-3 was demonstrate by the proteolytic cleavage of PARP (116 kDa) to 85 kDa cleavage products. PARP cleavage was markedly increased at concentration higher than 75 μg/mL of eutigoside C, paralleled with an increase of caspase-3 activity. These results suggest the involvement of caspase-3 in the onset of apoptosis by the eutigoside C.

Our present results indicate that eutigoside C from *E. emarginata* leaves induces the apoptosis of HL-60 cells via the down-regulation of Bcl-2 and the activation of caspase. This study supports the possibility that eutigoside C from *E. emarginata* might play a therapeutic role for treating human leukemia.

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