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Apoptosis-Inducing Costunolide and a Novel Acyclic Monoterpene from the Stem Bark of *Magnolia sieboldii*

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In a course of obtaining more amount of bioactive costunolide and successive phytochemical isolation from *Magnolia sieboldii* (Magnoliaceae), a novel acyclic monoterpene **1** named deoxygeraniol {2,6(*E*)-dimethyl-2,6-octadiene} was isolated along with β-sitosterol 3-O-linoleate (**2**), trilinolein (**3**) and high amount of costunolide (**4**) in the pure state. The structure of compound **1** was determined on the basis of spectroscopic data. Costunolide was found to induce apoptotic cell death in a dose-dependent manner by nucleosomal DNA ladder and flow cytometric analysis. Immunoblot analysis showed that the level of the anti-apoptotic protein, Bcl-2, was decreased, whereas the cleavage of poly-(ADP-ribose) polymerase was activated. Furthermore, the N-acetyl-L-cysteine antioxidant effectively prevented costunolide-induced cytotoxicity. These results suggest that costunolide-induced cell death is mediated by reactive oxygen species

Key words: *Magnolia sieboldii*, Magnoliaceae, Costunolide, Deoxygeraniol, Apoptosis, PARP, Bcl-2

INTRODUCTION

Most plants belonging to Magnoliaceae family, such as *Magnolia officinalis* (Baek *et al.*, 1992), *Magnolia obovata* (Kawai *et al.*, 1994) and *Magnolia fargesii* (Chen *et al.*, 1988) have been used as oriental herbal medicines. Many important biologically active substances such as magnolol, honokiol, magnoshinin, and magnosalin (Namba *et al.*, 1982; Kimura *et al.*, 1985) have been also isolated from the Magnoliaceae. However, *Magnolia sieboldii* is very little used for medicinal therapeutics in Korean folkloric medicine. From this plant, we have reported potent nitric oxide (NO) inhibitory activity of costunolide in the endotoxin-induced murine macrophage, J774.1, and the isolation of other substances {costunolide, syringin-4-O-glucoside, (-)-germacresne, (+)-elemene, echinacoside, β-sitosterol, and β-sitosterol β-glucoside} (Park *et al.*, 1996). Tada *et al.* (1982) isolated the 15-acetocycostunolide for the first time from *M. sieboldii*. Cho *et al.* (1988) and Lee *et al.* (1999) reported the TNF-α inhibitory effect of sesquiterpene lactones in macrophage cells. In particular, it is also known that sulfhydryl compounds attenuate TNF-a inhibitory effect of sesquiterpene lactones (Cho *et al.*, 1988).

Apoptosis is cellular suicide or programmed cell death which is mediated by the activation of an evolutionary conserved intracellular pathway. Recently, the relation of apoptosis and cancer has been emphasized and increasing evidence suggests that the processes of neoplastic transformation, progression and metastasis involve alteration of normal apoptotic pathway (Bold *et al.*, 1997). Apoptosis also gives some clues about effective anticancer therapy, and many chemotherapeutic agents were reported to exert their anti-tumor effects by inducing apoptosis of cancer cells (Kamesaki *et al.*, 1988).

In the present study, we have demonstrated the phytochemical isolation and costunlide-induced apoptosis in HL-60 cells. The mechanism of costunolide-induced apoptosis can be mediated through the suppression of

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the Bcl-2 expression and activation of caspase-3 which cleavage of poly-(ADP-ribose) polymerase (PARP). We also explored the reactive oxygen species (ROS)-mediated cytotoxicity.

MATERIALS AND METHODS

Plant material

The stem bark of *Magnolia sieboldii* was collected in August 2000, in Pyongchang, Kangwondo, South Korea, and the plant was identified by Dr. Gap-Tae Kim (Division of Applied Plant Sciences, Sang-ji University, Wonju, Korea). A voucher specimen (GT-000819) is deposited in the herbarium of Division of Applied Plant Sciences, Sang-ji University. This plant was air-dried and pulverized for the experiment.

General experimental procedures

IR spectra were recorded on a Hitachi 260-01 spectrometer in KBr cells (neat). EIMS (ionization voltage, 70 eV) were measured with a Finnigan Mat TSQ-700 mass spectrometer. ^{1}H-, ^{13}C-NMR, and NOESY spectra were taken on a Bruker DRX-300 spectrometer with TMS as the internal standard.

Extraction, fractionation and isolation

The dried stem bark (2.5 kg) was extracted three times with hot MeOH under reflux. The MeOH extract was then filtered and evaporated on a rotatory evaporator under reduced pressure to obtain a viscous mass (160 g) of MeOH extract. This material was suspended with H_2O and partitioned with n-hexane to give a n-hexane fraction (48 g). A part (20 g) of n-hexane fraction was subjected to column chromatography on a silica gel (700 g, 7 × 80 cm, Merck, Art No. 7734, Germany). After the column was eluted with n-hexane by 540 ml retention volume, it was successively eluted with n-hexane-EtOAc (12:1) with collecting 60 ml each. The fractions were combined to six fractions by TLC check using vanillin-sulfuric acid reagent. The volume of each combined fraction is as follows: fraction 1 (fr. 180 ml), fr. 2 (120 ml), fr. 3 (180 ml), fr. 4 (120 ml), fr. 5 (180 ml), and fr. 6 (240 ml). Repeated chromatography of fr. 2, fr. 4, fr. 5 and fr. 6 yielded compound **1**-**4**, respectively. Compounds **1**-**3** were colorless oils but compound **4** was crystalline (needle).

Compound **1**: Colorless oil, IR (KBr): ν_{max}= 3068 (=C-H), 2962 (C-H), 1645 cm^{-1} (C=C); ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 1.61 (3H×2, each s, H-9, 10), 1.69 (3H, s, H-1), 2.02 (3H, d, *J*=6.2 Hz), 1.99-2.08 (4H, overlapped, H-4, 5), 5.11-5.16 (1H × 2, m, H-3, 7); ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ=16.0 (C-9), 17.6 (C-10), 25.7 (C-1), 26.7 (C-8), 28.3 (C-4), 39.7 (C-5), 124.3 (C-3), 124.4 (C-7), 131.2 (C-2), 135.1 (C-6); EI-MS (70 eV) m/z (%): m/z 138.1 M^+ (64), 69.1 $[C_5H_{11}]^+$ (84), 55.1 $[C_4H_7]^+$ (100).

Compound **2**: Colorless oil, IR ν (KBr) cm^{-1}: no OH band, 1732 (ester); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.68-1.05 (6 × CH_3), 5.33 (1H, t-like, H-5), 2.36 (2H, t, J=7.4 Hz), 2.78 (2.36, t, J=7.4 Hz, H-2), 2.78 (2H, t, J=6.2 Hz); ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ: 34.2 (C-2), 65.8 (C-3), 122.6 (H-6), 127.9, 128.0 (C-9, 13), 130.0, 130.2 (C-10, 12), 139.7 (C-5); EI-MS (70 eV) m/z (%): no M^+, 397.4 [M-linoleic acid+H]$^+$.

Alkaline hydrolysis of compound 2

Compound **2** (35 mg) was hydrolyzed by 1 M KOH aqueous MeOH solution under reflux for 2 h. After cooling, the reactant was neutralized with HCl and followed by being partitioned between H_2O and $CHCl_3$. The lower phase $CHCl_3$ fraction was dried *in vacuo* and subjected to silica gel column chromatography with eluting solvent of benzene-EtOAc (20:1) to afford sterol and fatty acid fraction. Sterol fraction was dried *in vacuo* and this was applied for mass spectrometer. And dried fatty acid fraction was methylated in MeOH (10 ml) under H_2SO_4 catalyst (1.5 ml) by heating. The product was partitioned between H_2O and $CHCl_3$ and further $CHCl_3$ fraction was washed with H_2O. $CHCl_3$ fraction was dried *in vacuo*. The resulting oily substance was applied for mass spectrometer and showed the molecular ion m/z 276.

Compound **3**: Colorless oil, IR ν (KBr) cm^{-1}: no OH band, 1730 (ester); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.90 (3H × 3, t, *J*=6.6 Hz, H-1′, 1″, 1‴), 2.36 (2H × 3, t, *J*=7.4 Hz, H-2′, 2″, 2‴), 2.78 (2H × 3, t, *J*=6.2 Hz, H-11′, 11″, 11‴), 4.15 (1H × 2, dd, *J*=6.2, 12.2 Hz, H_a-1, 3), 4.30 (1H × 2, dd, *J*=4.0, 6.2 Hz, H_b-1, 3), 5.27 (1H, dd, *J*=4.0, 6.2 Hz), 5.4-5.5 (8H in total, m, H-9′, 9″, 9‴, 10′, 10″, 10‴, 12′, 12″, 12‴, 13′, 13″, 13‴); ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ: 14.1 (C-1, 1, 1), 34.05 (C-2, 2, 2), 62.1 (C-1, 3), 68.8 (C-2), 127.9, 128.0 (C-9, 9, 9, 10, 10, 10), 130.0, 130.2 (C-10, 10, 10, 12, 12, 12), 173 (C-1, 1, 1)

Identification fatty acid

By the same way as compound **2**, linoleic acid was found as the only fatty acid moiety of compound **3**.

Compound **4**: Colorless needles, mp 105-106°C; $[\alpha]_D$ 76.7 (*c* 0.3, MeOH). ^{1}H- and ^{13}C-NMR ($CDCl_3$) δ: literature (6).

Cell lines and culture media

Tumor cell lines such as Colon 26 (mouse colon adenocarcinoma), 3LL Lewis (mouse lung carcinoma), J82 (human bladder carcinoma), T24 (human bladder carcinoma), and HL-60 cells (human acute promyelocytic leukemia) were obtained from the American Tissue Culture Collec-

tion (ATCC, Rocksville, MD, USA). Cisplatin, N-acetyl-L cysteine (NAC) and 3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.).

Cytotoxicity assay

Aliquots of 2×10^5 cells were seeded on 96-well flat microtiter plates in RPMI medium supplemented with 10 % FBS, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml). Compounds dissolved in DMSO at various concentrations was added to the culture and adjusted to a final concentration of 0.1% (v/v). Above cells were grown in a humidified atmosphere of 95% air and 5% CO_2, and the cells were seeded in plates after three passages. The cell viability was determined in the presence or absence of a compound or cisplatin, as a positive control, using standard MTT assay (Lee *et al.*, 2000). Briefly, twenty four hours after seeding, 100 new media or a test compound were added and the plates were incubated for 48 h. Cells were washed once before adding 50 FBS-free medium containing 5 mg/ml MTT. After incubation at 37 for 4 h, the medium was discarded and formazan blue formed in the cells was dissolved with 50 μl DMSO. Optical density was measured at 570 nm. The concentration required to reduce absorbance by 50% (ED_{50}) in comparison to control cells was determined.

DNA fragment assay

For assays of fragmentation of chromosomal DNA, 1×10^6 HL-60 cells were collected by centrifugation and washed two times with PBS. The cells were fixed in 70% ethanol and stained overnight with propidium iodide (100 μg/ml) in PBS containing RNase A (500 μg/ml) at 4°C. The hypodiploid DNA was determined by flow cytometry using Cell Quest program (Becton Dickinson, San Jose, CA, U.S.A.). A portion of cells after incubation was suspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% sodium dodecyl sulfate solution). The lysate was centrifuged at $13000 \times g$ for 20 min to obtain the supernatant containing fragmented DNA. An aliquot (200 μl) of the supernatant and the pellet were used for the assay of DNA content. The other half was incubated with 1 μg/ml RNase A (Sigma, St. Louis, MO) at 37°C for 60min, followed by treatment with 100 μg/ml proteinase K (Wako Pure Chemical Industries, Osaka, Japan) for an additional 45 min at 50°C. DNAs were pre-incubated by adding 50 μl of 5 M NaCl and 250 μl of isopropyl alcohol at -20°C overnight. DNA samples were resolved by eletrophoresis on 2% agarose gel. After electrophoresis for 90 min at 50 V, the gel was stained with ethidium bromide, and DNA was visualized by a UV transilluminator.

SDS-PAGE and Western blot analysis

Cellular proteins were extracted from control and costunolide treated HL-60 cells. The washed cell pellets were resuspended in ELB buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated at 4°C for 15 min. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein was determined by Bio-Rad (Hercules, CA, U.S.A.) protein assay reagent as described by the manufacturer. 200 μg of cellular proteins from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% non-fat milk in 0.02 % Tween 20 containing PBS) at 4°C, followed incubated for 4 h with a 1:500 dilution of monoclonal anti-Bcl-2 antibody (Santacruz, CA, U.S.A.) or 1:1000 of PARP polyclonal antibody. Blots were washed 2 times with 0.02% Tween 20 containing PBS and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santacruz) for 1 h at room temperature. Blots were again washed three times in 0.02% Tween 20 containing PBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

Statistics

The results were expressed as the mean ± standard deviation. The significance of the difference between the control and the drug treated groups was analyzed by Student's t-test.

RESULTS AND DISCUSSIONS

Successive phytochemical studies on the isolation of n-hexane fraction from *M. sieboldii* afforded a new monoterpene (**1**, $C_{10}H_{18}$) named deoxygeraniol, β-sitosterol 3-O-linoleate, trilinolein (glycerol tri-9,12-octadecadienoate) and high amount of costunolide. Both isolated fatty acid esters are composed of the only linoleoyl moiety, though most fatty acid esters are occurred with analogue complex from a natural source. Spectroscopic analysis including 2D-NMR experiments on compound **1** determined the structure of deoxygeraniol as 2,6(*E*)-dimethyl-2,6-octadiene. Numbering was inevitably given as shown in Fig. 1, not according to that of general monoterpene but according to IUPAC nomenclature method. In ^{1}H-NMR spectrum of compound **1**, four methyl peaks including three singlets (two at d 1.61 and one at δ 1.69, each 3Hs)

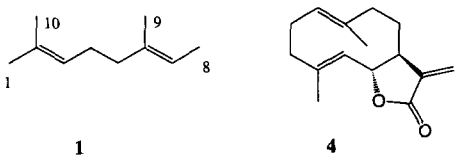

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Fig. 1. Structure of compound **1** (deoxygeraniol) and **4** (costunolide) isolated from *Magnolia sieboldii*

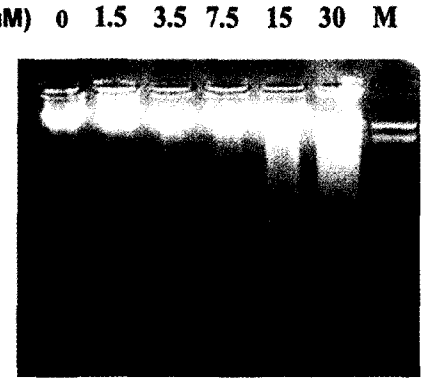

Fig. 2. Detection of DNA fragmentation in HL-60 cells treated with costunolide by agarose gel electrophoresis. Cells were treated without (control) or with various samples for 4 d, then detergent soluble cleaved DNA was subjected to electrophoresis on 1.5% agarose gel elctrophoresis, as described in Material and Methods. Lane 1, DNA from untreated HL-60 cells; Lanes 2, 3, 4, 5 and 6 are treated with 1.5, 3.5 7.5, 15 or 30 μM costunolide; M. marker.

and a doublet {δ 2.02 (3H, d, *J*=6.2 Hz)} were shown and two olefinic protons were shown being overlapped at d 5.11-5.16. These results indicate that compound **1** is a monoterpene, but it showed no functions of OH or CHO in any of NMR and IR spectra. Therefore, compound **1** is a monoterpene with a structure of hydrocarbon (alkadiene). The ^{13}C-NMR spectral data was examined comparing with those of a related monoterepene, geraniol (Atta-ur-Rahman and Ahmad 1992). Although the data was similar to those of geraniol, the peaks of C_5-C_8 and 6-Me were significantly shifted from the known geraniol data. An NOE correlation of C-8 with 6-Me established stereochemistry of compound 1 as 6(*E*)-configuration. The structure could be confirmed by prominent molecular ion at *m/z* 138.1 (rel. int., 64%) and its prominent fragment ions at *m/z* 69 (rel. int., 84%) and *m/z* 55 (rel. int., 100%). This has not been reported from a natural source, though compound 1 has been chemically known (Matsushita and Neigishi, 1982). This compound was not an artifact and further it could not be formed biosynthetically from the only dehydration process of related monoterpene alcohols, i.e., geraniol, nerol and (-)-linalool but could be formed by reduction of citral. The only natural compound with a chemical formula $C_{10}H_{18}$ as an acyclic monoterpene is citronellene but this bears an exomethylene. Therefore, a name of geraniane could be given for a basic acyclic monoterpene as farnesane in a basic structure of sesquiterpene. It is of interest that colorless oils of deoxygeraniol (**1**), β-sitosterol 3-O-linoleate (**2**) and trilinolein (**3**) were obtained, respectively, together with very high amount of costunolide (**4**). The structures (**2**-**4**) were also established by chemical and physicochemical data as described in experimental section. The function of exomethylene in costunolide may be labile to the groups of OH, NH, and especially SH with nucleophilicities, based on the backgrounds of organic chemistry.

Costunolide exhibited a remarkable cytotoxic effect on various cancer cells, including Colon 26 (colon adenocarcinoma), 3LL Lewis (lung carcinoma), T24 (bladder carcinoma), HepG2 (hepatoma) and HL-60 (leukemia) (Table I). However, other compounds did not show any significant cytotoxicty up to 100 M. Among them, HL-60 cells seemed to be the strong susceptibility to costunolide treatment, with an EC_{50} of 10.6 μM. To further determine the mode of cell death induced by costunolide, HL-60 cells were treated with various concentrations of costunolide and apoptosis was subsequently examined. Agarose gel electrophoresis of costunolide-treated chromosomal DNA was demonstrated by incubations with different concentrations of costunolide for 24 h (Fig. 2). A ladder-like pattern of DNA fragments became apparent from 7.5 M. To further determine the degree of apoptosis, we employed flow cytometry to quantify the sub-G1 peak (apoptotic peak). As shown in Fig. 3, when HL-60 cells were incubated with costunolide at concentration of 3.5 μM, 7 μM and 15 μM for 24 h, approximately 33.8%, 55.4% and 79.8% of HL-60 cells became positive in

Table I. Cytotoxic activity of costunolide on cancer cell growth *in vitro*

Cell line	Origin		ED_{50}[a] (μM)	
			Costunolide	Cisplatin
Colon 26	Colon adenocarcinoma	Mouse	17.2 ± 1.4	12.3 ± 0.9
3LL Lewis	Lung carcinoma	Mouse	19.3 ± 1.9	15.5 ± 1.1
J82	Bladder carcinoma	Human	16.4 ± 2.5	16.1 ± 2.9
T24	Bladder carcinoma	Human	23.9 ± 3.5	21.9 ± 3.0
HL-60	Leukemia	Human	9.5 ± 0.8	10.6 ± 1.6

[a]ED_{50} is defined as the concentration which resulted in a 50% decrease in cell number.
[b]The values represent the mean ± S.D of three independent experiments.

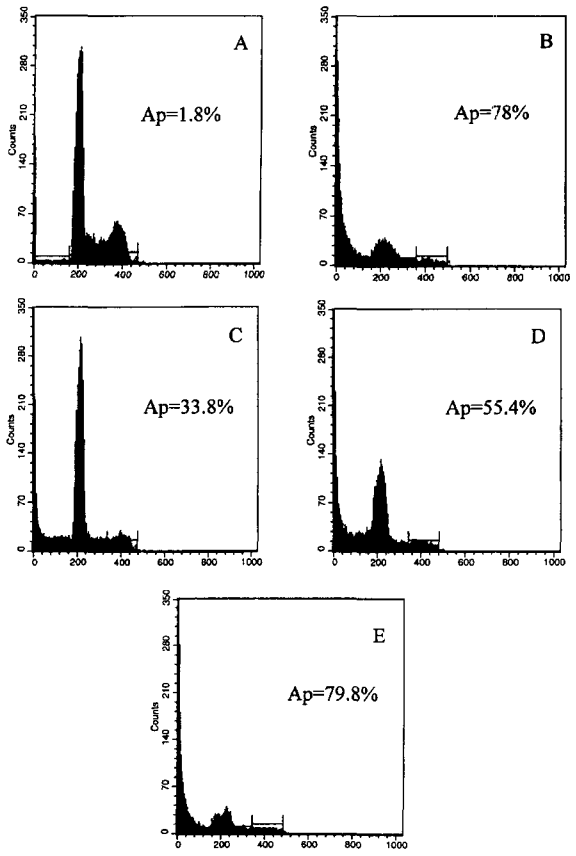

Fig. 3. DNA fluorescence histograms of PI-stained HL-60 cells treated with costunolide for 24 h.DNA from the cells treated with various samples for 4 d was analyzed by flow cytometry as described in Materials and Methods. (A) control, (B) 2.5 μM cisplatin, (C) 3.5 μM costunolide (D) 7.5 μM costunolide (E) 15 mM costunolide

apoptosis, respectively.

In an attempt to explore the role of Bcl-2 protein and caspase-3 activity in costunolide-induced apoptosis, we examined the level of Bcl-2 protein in HL-60 cells after treatment with costunolide. Immunoblot analysis of Bcl-2 showed that the anti-apoptotic protein was decreased in a dose-dependent manner (Fig. 4). This result suggests that Bcl-2 inhibition is involved in the apoptosis of costunolide. Bcl-2, apoptosis-inhibiting factor, is known to inhibit the signal transduction to apoptosis being involved in the activation process of caspase-3 (Fadeel *et al.*, 1999). The substrate for caspase-3, PARP, can be cleaved into 85 kDa peptide with carboxy terminal and 25 kDa peptide with amino terminal from 116 kDa (Zhivotovsky *et al.*, 1999). Treatment with costunolide in HL-60 cells for 24 h caused a dose-dependent proteolytic cleavage of PARP, with a accumulation of the 85 KDa fragments during apoptosis (Fig. 5). This result supported the increase of caspase-3 protease activity in HL-60 and was consistent with DNA fragmentation. To determine the events involved in costunolide-induced apoptosis, we examined whether the NAC of known antioxidant for their capacity interferes with the cell death process. The results showed here that costunolide-induced cell death can be suppressed by NAC, a well-known scavenger of reactive oxygen intermediate (ROI) (Fig. 6). This strongly implied that oxidative injury may play a pivotal role in costunolide-elicited apoptotic cell death.

Several observations indicate that oxidative stress is an important mediator of apoptosis. For instance, ionizing and ultraviolet, low concentration of H_2O_2, and certain

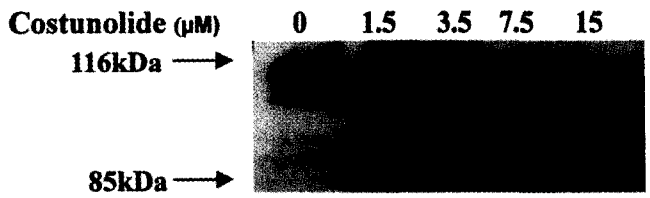

Fig. 5. Cleavage of PARP during costunolide-induced apoptosis in HL-60 cells.The cleavage of the caspase substrate PARP was examined in apoptotic HL-60 cells. HL-60 cells were treated with indicated costunolide concentrations and subjected to western blotting as described in materials and methods. The characteristic 85 kDa PARP fragment is arrowed.

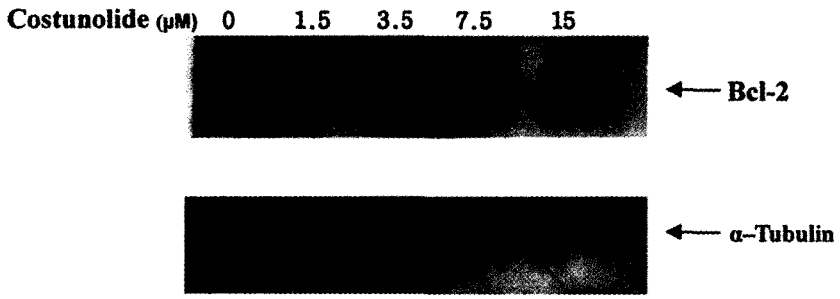

Fig. 4. Effect of cosrunolide on Bcl-2 protein expression in HL-60 cells as determined by western blot analysis. Cells were incubated at 37°C with costunolide for 4 d. Total protein (100 μg/lane) after lysis were resolved on 10% SDS-PAGE gel and the human Bcl-2 Mab was used to detect human Bcl-2 protein, which migrates at Mr 26,000. Equaling loading of the protein sample was confirmed by Coomassie blue staining of gel strips cut from the gels prior to Western blotting (data not shown). The blots are representative of three similar experiments.

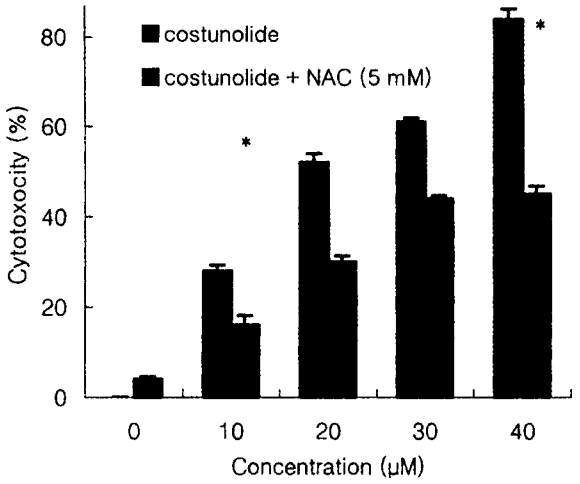

Fig. 6. Effect of N-aetyl-L-cysteine on the costunolide-induced cytotoxicity on HL-60 cells. Each column represents the mean ± S.D. (n=3).

cytokines stimulate apoptosis and generation ROI (Kiyoshi, 2000). On the other hand, antioxidants, such as gallic acid and curcumin, also induced apoptosis in different cell lines by generation of oxidative stress (Inoue *et al.*, 1994; Kuo *et al.*, 1996). The experiments on cytotoxicity and apoptosis of costunolide suggested that the electrophilicity of costunolide induces a strong apoptosis in HL-60 cells together with a notion that cellular sulfhydryl may be mediated. This electrophilic damage to HL-60 cells can induce apoptosis through Bcl-2 inhibition and subsequent caspase-3 protease activation.

In conclusion, a new acyclic monoterpene, deoxygeraniol and apoptosis-inducing costunolide were isolated together with β-sitosterol 3-O-linoleate and trilinolein from the stem bark of *M. sieboldii*. Many other sesquiterpene lactones may follow this biological feature of costunolide by their exomethylene moieties. In addition, the usage of *M. sieboldii* was considerably restricted in the folkloric societies of Korea and the reason may be attributed to the strong electrophilic toxicity of costunolide, though many other *Magnolia* species were preferred for medicinal usages. However, costunolide and this plant could be evaluated as a promising candidate for cancer therapy. Together with this, it seems likely that the occurrence of compounds **1-3** with no the hydroxy group may be closely associated with the existence of high content of costunolide that is responsible for performing of phytoalexin functions in plant kingdom.

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