A Cytotoxic and Apoptosis-Inducing Sesquiterpenoid Isolated from the Aerial Parts of *Artemisia princeps* Pampanini (Sajabalssuk)

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Repeated silica gel and octadecyl silica gel (ODS) column chromatography of the aerial parts of *Artemisia princeps* Pampanini (Sajabalssuk) led to the isolation of a new sesquiterpenoid, 3-((*S*)-2-methylbutyryloxy)-costu-1(10),4(5)-dien-12,6α-olide (2), along with two previously reported sesquiterpenoids: 8α-angeloyloxy-3β,4β-epoxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (1, carlaolide B) and 3β,4β-epoxy-8α-isobutyryloxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (3, carlaolide A). The structure of compound 2 was elucidated by spectroscopic data analysis, including one dimensional (1D) and two dimensional (2D) nuclear magnetic resonance (NMR) experiments. Of the isolates, compound 2 exhibited potent cytotoxicity against human cervix adenocarcinoma cells and induced apoptosis.

Key words *Artemisia princeps*; carlaolide A; carlaolide B; 3-((*S*)-2-methylbutyryloxy)-costu-1(10),4(5)-dien-12,6α-olide; cytotoxicity; apoptosis

More than 200 *Artemisia* species have been described worldwide, with 38 identified in Korea; many species have yet to be undescribed.[1)] Knowledge of these undescribed species is important, not only to enable their conservation, but also for the discovery of novel compounds which may be effective in treating diseases. *Artemisia* has traditionally been used in medicine as an antiphlogistic, an anodyne and a cardiotonic drug.[2)] The chemical constituents of the genus *Artemisia* have been studied by a number of researchers who have isolated terpenoids, sesquiterpenoids, lignans, phenylpropanoids.[3—5)] As of yet, however, There has been very little research focusing on *Artemisia princeps* Pampanini (Sajabalssuk).

"Sajabalssuk" is a variety of *Artemisia princeps*, an annual herb that grows in the wild in Ganghwa Island, Korea. Sajabalssuk has been used as an antiphlogistic, a cardiotonic drug, and to reduce bleeding, regulate menses and cure menstrual disorders, and alleviate pain. The constituents of Sajabalssuk include flavonoids, such as eupatilin, jaceosidin, apigenin and eupafolin[6)] and steroids, such as β-sitosterol, ergosterol peroxide, daucosterol and stigmasterol.[7)] This paper details the isolation and identification of two previously known and one new sesquiterpenoids from the aerial parts of *Artemisia princeps* Pampanini (Sajabalssuk), and evaluates their cytotoxic and apoptosis-inducing activities. The ethyl acetate (EtOAc) layer of the 80% methanol extract was developed using silica gel thin layer chromatography (TLC). After spraying a 10% H_2SO_4 solution and heating the TLC plate, the spots showed purple coloration, indicating the presence of sesquiterpenoids in the fraction. Repeated silica gel and octadecyl silica gel (ODS) column chromatography of the EtOAc layer yielded three sesquiterpenoids, compounds **1**—**3**.

Compounds **1** and **3** were identified as 8α-angeloyloxy-3β,4β-epoxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (carlaolide B) and 3β,4β-epoxy-8α-isobutyryloxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (carlaolide A), respectively, through the comparison of their spectroscopic data with those in the existing literature[8,9)] (Fig. 1).

Compound **2**, which was obtained as a yellowish, amorphous powder from chloroform ($CHCl_3$), exhibited absorbance bands of ester (1763 cm^{-1}) and olefine (1670 cm^{-1}) in the infra red spectrometer (IR) spectrum. The molecular ion peak $[M]^+$ at *m*/*z* 332 in the electron ionization mass spectrometer (EI-MS) spectrum and *m*/*z* 332.2006 in the high resolution (HR) EI-MS spectrum as a mass number of $C_{20}H_{28}O_4$ (calcd mass number=332.1987) were obtained. The proton-NMR (^{1}H-NMR) spectrum showed two exomethylene signals [δ_H 6.25 (H-13a) and δ_H 5.51 (H-13b)], two olefine methine signals [δ_H 4.89 (H-1) and δ_H 4.86 (H-5)], two oxygenated methine signals [δ_H 5.18 (H-3) and δ_H 4.57 (H-6)], two methine signals [δ_H 2.57 (H-7) and δ_H 2.39 (H-2′)], three methylene signals exhibiting germinal couplings (J_2) [δ_H 2.48 and δ_H 2.25 (H-2); δ_H 2.10 and δ_H 1.62 (H-8); δ_H 2.42 and δ_H 2.41 (H-9)], one methylene signal [δ_H 1.65

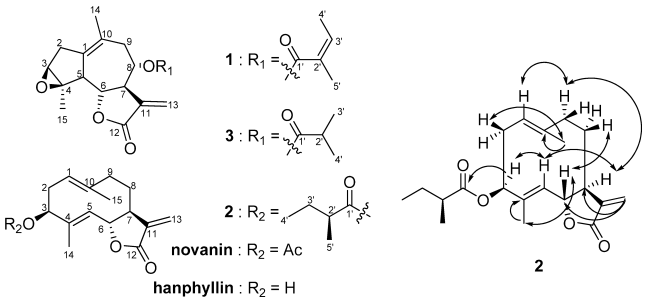

Fig. 1. Chemical Structures of Sesquiterpenoids Isolated from the Aerial Parts of *Artemisia princeps* Pampanini (Sajabalssuk)

The two-way arrows indicate the correlations between proton and proton signals in the NOESY spectrum, and one-way arrows indicate the long-range correlations between proton and carbon signals in the gHMBC spectrum.

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(H-3′)], and two allylic singlet methyl signals [δ_H 1.69 (H-15) and δ_H 1.44 (H-14)], a doublet methyl signal at δ_H 1.14 (H-5′), and a triplet methyl signal at δ_H 0.90 (H-4′). In the ^{13}C-NMR spectrum, a total of twenty signals were observed. In the sp^2 carbon region we observed two carbonyl carbon signals [δ_C 175.4 (C-1′), δ_C 170.0 (C-12)], three olefine quaternary carbon signals [δ_C 139.4 (C-4), δ_C 138.8 (C-10), and δ_C 138.6 (C-11)], two olefine methine carbon signals [δ_C 125.5 (C-5) and δ_C 124.2 (C-1)], and one exomethylene carbon signal [δ_C 120.0 (C-13)]. In the sp^3 carbon region, signals were observed for two oxygenated-methine carbons [δ_C 81.0 (C-6) and δ_C 78.6 (C-3)], two methine carbons [δ_C 50.0 (C-7) and δ_C 41.3 (C-2′)], four methylene carbons [δ_C 41.0 (C-8), δ_C 32.2 (C-2), δ_C 28.3 (C-9), and δ_C 26.8 (C-3′)] and four methyl carbons [δ_C 16.7 (C-5′), δ_C 16.4 (C-14), δ_C 12.7 (C-15), and δ_C 11.7 (C-4′)]. We concluded, therefore, that compound **2** is a germacrane-type sesquiterpenoid with two olefins—one between C-1 and C-10 and another between C-4 and C-5—an exomethylene on C-1, and a 2-methylbutyryloxy group on C-3. Determination of the final structure of compound **2**, including the location of the functional group, was accomplished using gradient heteronuclear multiple-bond connectivity (gHMBC). The exomethylene signals at δ_H 6.25 (H-13a) and δ_H 5.51 (H-13b) were correlated with methine carbons at δ_C 50.0 (C-7) and δ_C 81.0 (C-6). Two methyl signals, H-15 (δ_H 1.69) and H-14 (δ_H 1.44), correlated with two olefine quaternary carbons, C-4 (δ_C 139.4) and C-10 (δ_C 138.8). These observations revealed the costunolide skeleton. The oxygenated methine proton signal at δ_H 5.18 (H-3) correlated with the carbonyl carbon of 2-methylbutyryloxy at δ_C 175.5 (C-1′), indicating that the 2-methylbutyryloxy group attaches to C-3 through an ester bond. The relative stereochemistry for chiral carbons such as C-3, C-6 and C-7, and the configurations of double bonds were determined using a NOE experiment. The H-3 proton signal at δ_H 5.18 showed a NOE effect with an olefin proton signal at δ_H 4.86 (H-5) and H-5 with a methine proton signal at δ_H 2.57 (H-7); this indicates that the stereostructure of the double bond between C-4 and C-5 was *E*. Both H-5 and H-7 showed the same configuration. The NOESY spectrum exhibited correlations between H-6 (δ_H 4.57) and an equatorial proton signal at δ_H 1.62 (H-8β), and between H-7 and an axial proton signal at δ_H 2.11 (H-9α), which indicates they have identical configurations. The cross peaks between H-9α and an olefin proton signal at δ_H 4.89 (H-1), and between an allylic methyl proton signal at δ_H 1.69 (H-15) and an axial proton signal at δ_H 2.48 (H-2β) verify the stereochemistry of a double bond between C-1 and C-10 as *E*. The absolute stereochemistry of compound **2** was confirmed by a comparison of specific rotation ([α]$_D$ +133.2) and the chemical shifts and the coupling patterns of C-3 (δ_H 5.18, dd, J=10.4, 5.2 Hz; δ_C 78.6), C-6 (δ_H 4.57, dd, J=9.2, 9.6 Hz; δ_C 81.0), and C-7 (δ_H 2.57, m; δ_C 50.0) in the NMR spectra with those of 3*S*,6*S*,7*R*-3β-hydroxygermacra-1(10)*E*,4*E*,11(13)-trien-12,6α-olide [3β-hydroxycostunolide, hanphyllin; [α]$_D$ +155.2[10]; +103.8[11]] and other relevant components.[12—15] The configuration of C-2′ was subsequently determined using GC analysis with a chiral column.[16] The retention times of (*S*)-2-methylbutanoic acid methyl ester and (*R*)-2-methylbutanoic acid methyl ester appeared at 8.66 min and 9.27 min, respectively, while that of 2-methylbutanoic acid methyl ester from compound **2** was 8.59 min. The stereochemistry of chiral carbon (C-2′) for 2-methylbutanoic acid from compound **2** was therefore determined to be an *S*-configuration. Finally, the structure of compound **2** was concluded to be 3-((*S*)-2-methylbutyryloxy)-costu-1(10),4(5)-dien-12,6α-olide (Fig. 1). This is the first report addressing the isolation of compound **2** from a natural source.

Guaianolide sesquiterpenoids have been reported to have cytotoxic effects on various cancer cell lines.[17,18] We previously reported that costunolide sesquiterpenoids are potent cytotoxins[19] and induce apoptosis in human cancer cells *via* depletion of intracellular thiols.[20] Accordingly, in this study the three isolated sesquiterpenoids were evaluated for their cytotoxic and apoptosis-inducing abilities.

The cytotoxic activity of the three isolated sesquiterpenoids in human cervical adenocarcinoma (HeLa), human leukemia (U937) and human lung adenocarcinoma (A549) cell lines was examined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each compound showed significant cytotoxicity against all cancer cells (Table 1). Cytotoxicity most likely occurs at level of replication as opposed to an effect on the biosynthesis of DNA precursors.[21] The active chemicals may penetrate the cell membrane to the DNA and react with nucleophiles, especially the cystein sulfhydryl groups of enzymes, through a Michael-type addition.[22] Variance in cytotoxicity may therefore largely be explained by differences in electronics, lipophilicity and molecular geometry.[23] The cytotoxicity of compounds **1**—**3** was chemically mediated by an α,β-unsaturated carbonyl structure—*e.g.* an α-methylene-γ-lactone—and organic acid moieties—angeloyl, methylbutyroyl and isobutyroyl—which increase the lipophilicity of the molecule. Compared to other compounds, compound **2** has more double bonds in the ring structure and showed relatively higher activity. The level of activity exhibited by compound **2** is almost identical to the well known anti-cancer compound, cisplatin. The double bonds increase the electron cloud of the molecules and can, in addition, influence the molecular form with conformations. The presence of an epoxide group in compounds **1** and **3** will also increase the electron cloud; however, these structures appeared to have only a minor effect on activity.[23] Therefore, we conclude that the double bond has more of an influence on activity level than the epoxide group.

Because apoptosis-inducing compounds are potential anti-tumor agents, we tested the ability of compound **2** to induce apoptosis in HeLa cells. Apoptotic cells have several typical

Table 1. The Cytotoxicity of Sesquiterpenoids Isolated from Aerial Parts of *Artemisia princeps* Pampanini (Sajabalssuk) against Human Cancer Cell Lines

Compound	IC_{50} (μg/ml)		
	HeLa	U937	A549
1	22.8	17.2	43.5
2	15.5	16.0	22.1
3	22.9	22.6	40.5
Cisplatin	15.7	12.5	24.9

IC_{50} is defined as the concentration that results in a 50% decrease in the number of cells compared to that of the control cultures in the absence of compound. The data are representative examples for tests performed in triplicate.

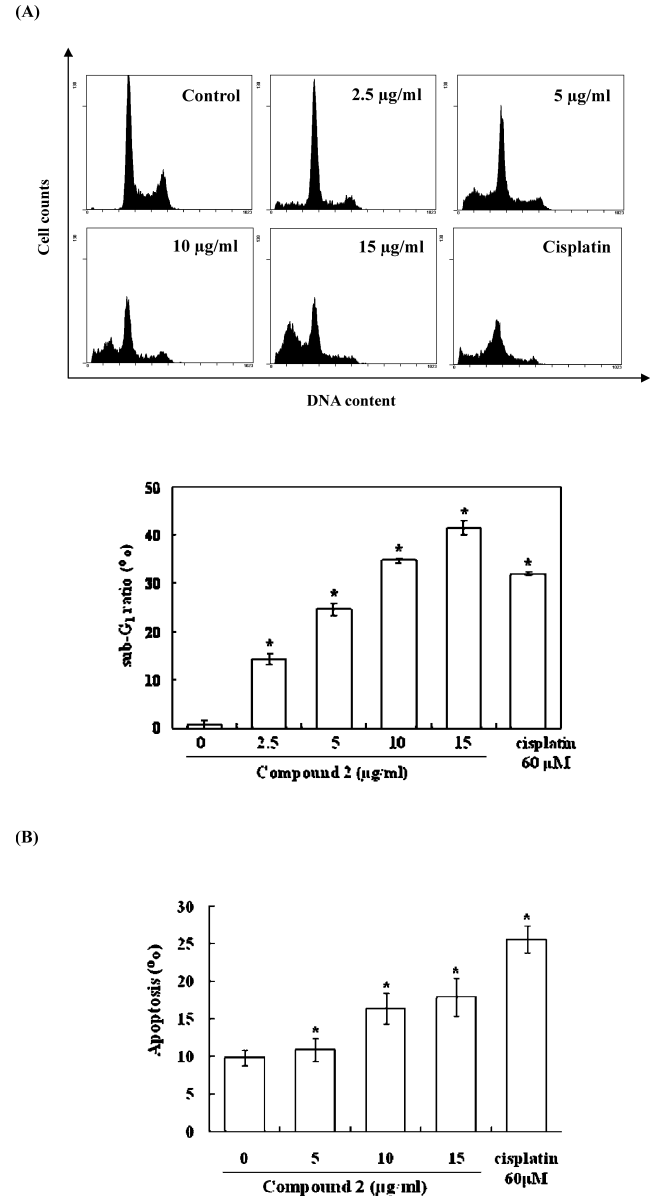

Fig. 2. Accumulation of Cells with Sub-G_1 DNA Content (A) and DNA Fragmentation (B) in Compound **2**-Treated HeLa Cells

HeLa cells were treated with compound **2** at the indicated concentrations for 24 h, and then induction of apoptosis was estimated. (A) Accumulation of cells with sub-G_1 DNA content. Cell distribution according to DNA content was measured by PI incorporation and apoptotic DNA contents (sub-G_1 phase) analyzed by flow cytometry. (B) The extent (%) of DNA fragmentation was determined by a fluorometric method using DAPI. Data presented are the means±S.D. of results from three independent experiments. Cisplatin was compared as a positive control. $*p<0.05$ *vs.* control group; significance between treated groups were determined using the Student–Newman–Keuls method.

features, such as accumulation of cells with sub-G_1 DNA content, DNA fragmentation into nucleosomal fragments and the appearance of nuclear condensation.[24,25] The number of HeLa cells in the sub-G_1 fraction increased in a concentration-dependent manner (Fig. 2A). Compared to the control cells (0.64%), treatment with compound **2** (15 μg/ml) for 24 h resulted in 41.44% sub-G_1 ratio (Fig. 2A). In addition, compound **2** also induced DNA fragmentation in a concentration-dependent manner in the HeLa cells (Fig. 2B). Because it has been suggested that cytosolic aspartate-specific proteases, called caspases, are responsible for the intentional disassembly of a cell into apoptotic bodies,[26] we examined

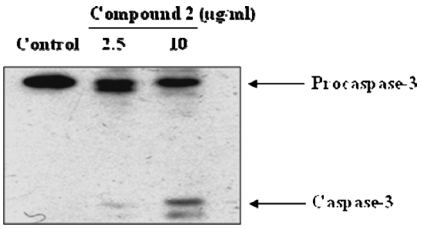

Fig. 3. Activation of Caspase-3 during Compound **2**-Induced Apoptosis

After treatment with 2.5 and 15 μg/ml compound **2**, the cleavage of procaspase-3 was examined by Western blot analysis.

the involvement of caspase activation in compound **2**-induced apoptosis in HeLa cells. Western blotting was performed to analyze caspase activation. Treatment of cells with compound **2** (2.5, 10 μg/ml) for 24 h increased the cleavage form of procaspase-3 (Fig. 3). These results indicate that compound **2** is a potent inducer of apoptosis in HeLa cells *via* caspase-3 activation. Although further structure–activity relationship studies are necessary, a sesquiterpenoid such as compound **2**, has the potential to be a powerful anti-tumor agent. Moreover, *Artemisia princeps* Pampanini (Sajabalssuk), which is frequently ingested in Korea in both traditional medication and as an ingredient when cooking, might prove useful for the development of anti-cancer functional foods or medicine.

Experimental

General Experimental Procedures Melting points were determined on a Fisher–John apparatus and uncorrected. Optical rotation was measured on a JASCO P-1010 digital polarimeter (Jasco, Tokyo, Japan). The IR spectrum was run on a Perkin Elmer spectrum One FT-IR spectrometer (Perkin Elmer, Norwalk, U.S.A.). EI-MS was recorded on a JEOL JMS 700 (JEOL, Tokyo, Japan). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were taken on a Varian Unity Inova AS 400 FT-NMR spectrometer (Varian, California, U.S.A.). Isotopic analysis was carried out using a liquid scintillation counter (1450 Microbeta Trilux Wallac Cy, Turku, Finland).

Plant Materials *Artemisia princeps* Pampanini (Sajabalssuk) was harvested at Ganghwa in 2003, and was provided by Ganghwa Agricultural R&D Center, Incheon, Korea, and identified by Prof. Jae-Ho Pyee of Dankook University, Seoul, Korea. The Sajabalssuk was stored in the shade for two years and then used for the experiments. A voucher specimen (KHU-NPCL-051020) was lodged at the Laboratory of Natural Products Chemistry, Kyung Hee University, Suwon, Korea.

Chemicals Methanol-d_4 and $CDCl_3$ as the internal standard was purchased from Sigma (St.Louis, MO, U.S.A.). RPMI Medium 1640, Dulbecco's Modified Eagle Medium (GIBCO BRL, Life Technologies Inc., NY, U.S.A.) and Penicillin-Streptomycin were purchased from GIBCO (Grandisland, NY, U.S.A.). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, U.S.A.). MTT, RNase, leupeptin, aprotinin, phenylmethylsulfonylfluoride (PMSF), 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Antibodies for caspase-3 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Extraction and Isolation The dried aerial parts of *Artemisia princeps* Pampanini (Sajabalssuk) (4 kg) were extracted at room temperature with 80% methanol (MeOH, 22 l). The extract was partitioned with water (4 l), EtOAc (4 l×2) and normal butanol (*n*-BuOH, 4 l×2), successively, and concentrated *in vacuo* to obtain the EtOAc (47 g, SJBE), *n*-BuOH (107 g, SJBB), and H_2O extracts (45 g, SJBW). The EtOAc extract was applied to a silica gel (70—230 mesh) column (8×25 cm) for chromatography (c.c), and eluted with *n*-hexane : EtOAc (7 : 1→5 : 1→3 : 1→1 : 1, each 5 l) with monitoring by TLC to produce 20 fractions (SJBE-1 to SJBE-20). SJBE-11 (Ve/Vt=0.55, elution volume/total volume, 664 mg) was applied to the silica gel c.c. (4×5 cm) and eluted with *n*-hexane : EtOAc (5 : 1, 3 l), which resulted in 19 fractions (SJBE-11-1 to SJBE-11-19). The SJBE-11-5 (Ve/Vt= 0.26, 190 mg) fraction was subjected to ODS c.c. (2×5 cm) and eluted with MeOH : H_2O (2 : 1, 1 l) to produce purified compound **1** (SJBE-11-5-5,

Ve/Vt=0.71, ODS TLC *Rf*=0.5 in MeOH : H_2O=5 : 1, 19 mg). The SJBE-12 (Ve/Vt=0.6, 1.26 g) fraction was applied to a silica gel c.c. (4×10 cm) and eluted with *n*-hexane : EtOAc (5 : 1, 3 l) to give 10 fractions (SJBE-12-1 to SJBE-12-10), and the SJBE-12-4 (Ve/Vt=0.4, 401 mg) fraction was applied to an ODS c.c. (2×4 cm) and eluted with MeOH : H_2O (2 : 1, 1.5 l) to yield compound **2** (SJBE-12-4-10, Ve/Vt=0.9, ODS TLC *Rf*=0.4 in MeOH : H_2O=7 : 1, 17 mg). The SJBE-12-7 (Ve/Vt=0.7, 100 mg) fraction was eluted with MeOH : H_2O (5 : 1, 2 l) from an ODS c.c. (2×4 cm) to produce compound **3** (SJBE-12-7-7, Ve/Vt=0.7, Silica gel TLC *Rf*=0.5 in *n*-hexane : EtOAc=3 : 1, 38 mg).

8α-Angeloyloxy-3β,4β-epoxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (**1**, Carlaolide B): Yellowish powder (MeOH); mp 180—181 °C; $[\alpha]_D$ −11.3 (*c*=0.52, $CHCl_3$); EI-MS *m/z*: 344 $[M]^+$; IR ν (KBr) cm^{-1}: 1765, 1735, 1668, 1625.

3-((*S*)-2-Methylbutyryloxy)-costu-1(10),4(5)-dien-12,6α-olide (**2**): Yellowish powder (MeOH); mp 103—105 °C; $[\alpha]_D$ +133.2 (*c*=1.03, MeOH); EI-MS *m/z*: 332 $[M]^+$, 217, 189, 175, 150, 123, 109, 53; HR-EI-MS *m/z* 332.1996 $[M]^+$ (Calcd for $C_{20}H_{28}O_4$=332.1987); IR ν (KBr) cm^{-1}: 1763, 1670, 1450, 1140; ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 6.25 (1H, d, *J*=3.2 Hz, H-13a), 5.51 (1H, d, *J*=3.2 Hz, H-13b), 5.18 (1H, dd, *J*=10.4, 5.2 Hz, H-3), 4.89 (1H, br d, *J*=9.6 Hz, H-1), 4.86 (1H, d, *J*=9.6 Hz, H-5), 4.57 (1H, dd, *J*=9.2, 9.6 Hz, H-6), 2.57 (1H, m, H-7), 2.48 (1H, m, H-2α), 2.42 (1H, m, H-9β), 2.39 (1H, qt, *J*=7.2, 6.4 Hz, H-2′), 2.25 (1H, m, H-2β), 2.11 (1H, m, H-9α), 2.10 (1H, m, H-8α), 1.69 (3H, br s, H-15), 1.65 (2H, qd, *J*=7.2, 6.4 Hz, H-3′), 1.62 (1H, m, H-8β), 1.44 (3H, br s, H-14), 1.14 (3H, d, *J*=7.2 Hz, H-5′), 0.90 (3H, d, *J*=7.2 Hz, H-4′); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 175.5 (C-1′), 170.0 (C-12), 139.4 (C-4), 138.8 (C-10), 138.6 (C-11), 125.5 (C-5), 124.2 (C-1), 120.0 (C-13), 81.0 (C-6), 78.6 (C-3), 50.0 (C-7), 41.3 (C-2′), 41.0 (C-9), 32.2 (C-2), 28.3 (C-8), 26.8 (C-3′), 16.7 (C-5′), 16.4 (C-14), 12.7 (C-15), 11.7 (C-4′).

3β,4β-Epoxy-8α-isobutyryloxy-6βH,7αH,8βH-guaia-1(10),11(13)-dien-12,6α-olide (**3**, Carlaolide): White powder (MeOH); mp 183—191 °C; $[\alpha]_D$ −16.5 (*c*=0.1, $CHCl_3$); EI-MS *m/z*: 332 $[M]^+$, 314; IR ν (KBr) cm^{-1}: 1760, 1735, 1670, 1625.

Alkaline-Hydrolysis of Compound 2 Followed by Methylesterification and GC Analysis Compound **2** (3 mg) was dissolved in a 10% KOH solution (3 ml, H_2O–MeOH=1 : 10) and refluxed for 2 h, at which time TLC ($CHCl_3$–MeOH=7 : 1) indicated that the reaction was complete. After the reaction mixture was neutralized with cation exchange resin (DOWEX 50WX4-400, Aldrich Inc., Milwaukee, WI, U.S.A.) and filtrated, the solution was concentrated under reduced pressure to dryness. The concentrate was dissolved in MeOH (1 ml) and esterified by adding diazomethane in ether (*ca.* 1 ml). Gas chromatography was used to analyze the solution. A Shimadzu gas chromatograph Model GC-14B (Japan), equipped with an on-column injection system and FID was used. The column used was an Alltech Chiraldex G-Ta capillary column (30 m×0.25 mm i.d., film thickness 0.125 μm; Alltech Associates Inc., Deerfield, IL, U.S.A.). The operating conditions were as follows: carrier gas flow (N_2), 1.5 ml/min with split; H_2, 60 kPa; air, 50 kPa; make-up gas (N_2), 29 ml/min; injector, 200 °C; detector, 220 °C. The oven temperature was maintained at 40 °C for 4 min, then increased to 80 °C at the rate of 2 °C/min, and subsequently maintained at 80 °C for 4 min. 0.5 μl of sample was injected directly into the inject port. Both the (*S*)-(+)-2-methylbutanoic acid (2 mg, Sigma-Aldrich Korea, Yongin, Korea) and (±)-2-methylbutanoic acid (2 mg, Sigma-Aldrich Korea, Yongin, Korea) were esterified and analyzed using the same procedure as described previously. The retention times of (*S*)-2-methylbutanoic acid methyl ester and (*R*)-2-methylbutanoic acid methyl ester were at 8.66 min and 9.27 min, respectively.

Cell Culture Human cervical adenocarcinoma HeLa, human histocytic lymphoma U937 and human lung adenocarcinoma A549 cell lines were purchased from the Korean cell line bank (KCLB, Seoul, Korea) and cultured in RPMI 1640 media supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). Cells were maintained at 37 °C in an atmosphere of 5% CO_2 in air.

Cell Viability Assay Cytotoxicity was assessed by the MTT assay.[27] Briefly, cells were seeded at 1×10^5/ml in each well containing 100 μl of the RPMI medium supplemented with 10% FBS in a 96-well plate. After 12 h, various concentrations of tested samples were added. After 48 h, 50 μl of MTT (5 mg/ml stock solution, in PBS) was added per well and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which formed in the cells, was dissolved with 100 μl DMSO. The optical density was measured at 540 nm using an Enzyme-Linked Immuno Sorbent Assay (ELISA) system version 2 (Amersham Pharmacia Biotech).

Flow-Cytometric Cell Analysis by PI Staining The cell cycle distribution has been previously described.[28] The cells were collected by centrifugation at 2000 rpm for 4 min. The cell pellets were then resuspended in 1 ml of phosphate-buffered saline (PBS), fixed in 70% ice-cold ethanol, and kept in a freezer overnight. The fixed cells were centrifuged, washed once in PBS and resuspended in PI staining solution containing 50 mg/ml of propidium iodide and 100 μg/ml of RNase A. The cell suspension, which was shielded from light, was incubated for 30 min at 37 °C and analyzed using the FACS (Becton-Dickinson, Heidelberg, Germany). A total of 10000 events were acquired for analysis using CellQuest software.

DAPI Assay DNA fragmentation was quantitated, as previously reported.[26] In brief, cells were lysed in a solution containing 5 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 0.5% (w/v) Triton X-100 for 20 min on ice. The lysate and supernatant obtained after centrifugation at 27000×***g*** for 20 min were sonicated for 40 s, and the level of DNA in each fraction was measured with a fluorometric method using DAPI. The amount of fragmented DNA was calculated as the ratio of the amount of DNA in the supernatant to that in the lysate.

Western Blot Analysis HeLa cells (2.5×10^7) were harvested and washed twice with ice-cold PBS. Cell pellets were then lysed in an ice-cold cell extraction buffer [50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 0.1 mM PMSF, 0.5 mM dithiothreitol (DTT), 5 mM NaF, 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated for 30 min at 4 °C. Cell debris was removed by microcentrifugation (10000***g***, 5 min), followed by quick freezing of the supernatants. Protein concentration was determined by a Bio-Rad protein assay reagent. Cellular proteins (50 μg) were electroblotted onto nitrocellulose membrane following separation on a 10—15% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk in TBS containing 0.1% Tween 20) at 4 °C, and then incubated for 4 h with a 1 : 1000 dilution of anti-caspase-3 antibody. Blots were washed three times with TTBS (Tris-buffered saline, 0.1% Tween 20), and then incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed again three times with TTBS, and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

Statistical Analysis Data presented are the means±S.D. from three independent experiments. The Student–Newman–Keuls method was used to compare treated groups and controls. $*p<0.05$ indicates statistical significance.

Acknowledgements This study was supported by the Ganghwa R&D Center of 'Evaluation of Biological activity and Pharmacological efficacy for plants indigenous to Ganghaw (Incheon)' and by a grant from the Korean Science & Technology Foundation through the Plant Metabolism Research Center, Kyung Hee University.

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