Increase of Caspase-3 Activity by Lignans from *Machilus thunbergii* in HL-60 Cells

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Nine lignans and two butanolides were isolated from the stem bark of *Machilus thunbergii* and their structures were identified as machilin A (1), licarin B (2), zuonin B (3), macelignan (4), secoisolancifolide (5), isolancifolide (6), oleiferin C (7), *meso*-dihydroguaiaretic acid (8), licarin A (9), machilin F (10), and nectandrin B (11) by spectroscopic means. These compounds were assessed for their abilities to activate a caspase-3 activity in human promyeloid leukemic HL-60 cells. The intracellular caspase-3 activity of macelignan (4), oleiferin C (7), *meso*-dihydroguaiaretic acid (8), and licarin A (9) increased approximately 3.04, 6.16, 2.10, and 3.10-fold at 100 μM over that of untreated control. In addition, compounds 4, 7, 8, and 9 induced internucleosomal DNA fragmentation in HL-60 cells.

Key words *Machilus thunbergii*; lignan; caspase-3; DNA fragmentation; apoptosis

Apoptosis is a form of cell death that occurs during several pathologic situations in organisms and contributes to cell replacement, tissue remodeling, and removal of damaged cells under normal conditions.[1] However, inappropriate regulation of apoptosis may cause many serious disorders, such as neural generation, AIDS, autoimmune disease, and cancers. In numerous studies it has been documented that the process of apoptosis is regulated by the expression of several proteins. Of these, the caspases make up a family of cysteine proteases that cleave substrates after aspartic residue. These proteins are considered to play a central role in the apoptotic process and to trigger a cascade of proteolytic cleavage in mammalian cells. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or wholly for the proteolytic cleavage of various proteins.[2,3]

The stem bark of *Machilus thunbergii* Sieb. *et* Zucc. (Lauraceae) is one of the well-known medicinal herbs used to treat a wide variety of diseases including edema, abdominal pain, and abdominal distension.[4] Lignans, lignan glycosides, alkaloids, flavonoids, and essential oils have been reported from this plant.[5] Some lignans from *M. thunbergii* have been shown to have potent antioxidant activity.[6] In addition, Kim and Ryu reported that butanolides from *M. thunbergii* have an inhibitory effect on nitric oxide synthesis in activated macrophages.[7] During the course of our search for new caspase-3 activators from natural sources, we found that a MeOH extract of the stem bark of *M. thunbergii* showed activity. In this study we describe the increase of caspase-3 activity and internucleosomal DNA fragmentation of compounds from this source.

MATERIALS AND METHODS

Plant Material The stem bark of *M. thunbergii* was collected during September 2001 at Ulleung-Do, Kyungbook, Korea, and dried at room temperature. A voucher specimen (PB-2910) is deposited at the Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea.

Chemicals RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco Laboratories. Ac-Asp-Glu-Val-Asp 7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) was obtained from Enzyme Systems Products (Livermore, CA, U.S.A.). Camptothecin, penicillin, streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), propidium iodide (PI), and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Extraction and Isolation Stem bark of *M. thunbergii* (1.8 kg) was extracted with MeOH at room temperature to give a dark-brown extract (290 g). The MeOH extract was suspended in H_2O and extracted with hexane to give a hexane-soluble fraction (35.3 g). The resulting H_2O layer was extracted with $CHCl_3$ and EtOAc to yield a $CHCl_3$-soluble fraction (2.7 g) and an EtOAc-soluble fraction (68.1 g), respectively. The hexane-soluble fraction was chromatographed on a column of silica gel (500 g). The column was eluted using a gradient of hexane and acetone to give four fractions. Repeated column chromatography of Fr. 2 (10.9 g) on silica gel (hexane/acetone; 7 : 3 and benzene/EtOAc; 20 : 1) and ODS column (MeOH, 10% aq. MeOH) afforded compounds **1** (146 mg), **2** (26 mg), **3** (75 mg), **4** (147 mg), **5** (26 mg), **6** (32 mg), and **7** (7 mg). Repeated column chromatography of Fr. 3 on silica gel (hexane/EtOAc; 5 : 3, benzene/acetone; 30 : 1, and $CHCl_3$/EtOAc; 5 : 1) and ODS column (15% aq. MeOH) furnished compounds **8** (147 mg), **9** (27 mg), **10** (25 mg), and **11** (62 mg).

Machilin A (**1**): Colorless needles; mp 66—67 °C; $[\alpha]_D$ 0° (c=0.53, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 264 (2.46); FAB-MS m/z: 325 $[M-H]^+$.

Licarin B (**2**): White amorphous powder; $[\alpha]_D$ −50.8° (c=0.25, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 273 (2.56); FAB-MS m/z: 324 $[M]^+$.

Zuonin B (**3**): Colorless needles; mp 49—51 °C; $[\alpha]_D$ 0° (c=0.33, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 294 (2.45); FAB-MS m/z: 363 $[M+Na]^+$.

Macelignan (**4**): Yellow oil; $[\alpha]_D$ +4.8° (c=0.1, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 302 (2.62), 274 (2.60); FAB-MS m/z: 327 $[M-H]^+$.

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Secoisolancifolide (**5**): Colorless oil; $[\alpha]_D$ +75.0° (c=0.2, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 259 (2.35); FAB-MS m/z: 283 $[M-H]^+$.

Isolancifolide (**6**): Colorless oil; $[\alpha]_D$ −69.0° (c=0.13, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 258 (2.41); FAB-MS m/z: 251 $[M-H]^+$.

Oleiferin C (**7**): Colorless oil; $[\alpha]_D$ +40.0° (c=0.5, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 251 (3.01), 285 (3.09); FAB-MS m/z: 341 $[M-H]^+$.

meso-Dihydroguaiaretic Acid (**8**): White amorphous powder; $[\alpha]_D$ 0° (c=0.47, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 250 (2.89), 276 (3.00); FAB-MS m/z: 330 $[M]^+$.

Licarin A (**9**): White amorphous powder; $[\alpha]_D$ −26° (c=0.34, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 277 (2.80); FAB-MS m/z: 326 $[M]^+$.

Machilin F (**10**): Yellow oil; $[\alpha]_D$ +2.3° (c=0.2, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 296 (2.64); FAB-MS m/z: 342 $[M]^+$.

Nectandrin B (**11**): Yellow oil; $[\alpha]_D$ 0° (c=0.05, $CHCl_3$); UV λ_{max} ($CHCl_3$) nm (log ε): 285 (2.54); FAB-MS m/z: 344 $[M]^+$.

Cell Culture Human promyelocytic leukemia HL-60 cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in IMDM medium supplemented with 20% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell culture was maintained at 37 °C in a 5% CO_2 humidified incubator.

Cell Viability Cell viability was assessed by the standard MTT technique. Cells were seeded to a concentration of 5×10^5 cells/ml, then compounds **1**—**11** were added to the suspension. After 24 h incubation (37 °C), 10 μl MTT (5 mg/ml) was added to each well. After 4 h incubation (37 °C) and 5 min centrifugation (3000 rpm), the resulting formazan precipitate was dissolved with 100 μl DMSO and the absorption was measured at 570 nm on a microplate reader (BIO-RAD, U.S.A.).[8] The growth inhibition was determined using;

growth inhibition=(control's OD−sample's OD)/control's OD

Assay of Caspase-3 Activity Caspase-3 enzyme activity was measured by proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC by counting on a microplate fluorometer (Perkin-Elmer, LS50B, U.S.A.). After incubation with 100 μM compounds **1**—**11** for 16 h, cells were harvested and washed once with cold PBS. The pellets were lysed using 15 μl of lysis buffer containing 10 mM EDTA, 0.5% Triton X-100, and 10 mM Tris–HCl (pH 8.0) at room temperature for 10 min, and then 100 μl of assay buffer (100 mM HEPES; pH 7.5, 10 mM dithiothreitol, 10% sucrose, 0.1% CHAPS, 0.1% BSA) and 10 μl of substrate solution (10 μl of 20 mM substrate+1000 μl assay buffer) were added on ice. Fluoroscene at 400 nm (excitation) and 505 nm (emission) was measured after incubation at 37 °C for 1 h.[9]

DNA Fragmentation After incubation with compounds **4**, **7**—**9** (at 100 μM) for 16 h, HL-60 cells were harvested and washed with PBS. Apoptotic DNA was purified using an Apoptotic DNA Ladder Kit (Roche, Mannleim, Germany). Then cell pellets were lysed in 200 μl of lysis buffer (10 mM Tris–HCl; pH 8.0, 10 mM Urea, 6 M guanidine-HCl, and 20% Triton X-100; pH 4.4) for 10 min. The lysate flowed to the filter and the filter was washed with 4 mM NaCl, 0.4 mM Tris–HCl (pH 7.5) and 80% EtOH. The DNA bound at the filter was eluted with 200 μl elution buffer (10 nM Tris–HCl, pH 8.5). The isolated DNA fragments were separated by 1% agarose gel containing 0.5 μg/ml of ethidium bromide and analyzed under an ultraviolet illuminator.[10]

RESULTS AND DISCUSSION

As part of the ongoing program of research in our laboratory to detect natural products by the increase of caspase-3 activation, the activated properties of compounds from the stem bark of *M. thunbergii* were investigated. Repeated column chromatography of the hexane-soluble fraction of the MeOH extract of *M. thunbergii* on silica gel and ODS led to the isolation of nine lignans (**1**—**4**, **7**—**11**) and two butanolides (**5**, **6**). These compounds were identified as machilin A (**1**), licarin B (**2**), zuonin B (**3**), macelignan (**4**), secoisolancifolide (**5**), isolancifolide (**6**), oleiferin C (**7**), *meso*-dihydroguaiaretic acid (**8**), licarin A (**9**), machilin F (**10**), and nectandrin B (**11**) by comparing their spectral data to those previously reported (Fig. 1).[11—16]

The compounds **1**—**11** isolated were tested for their increasing activity of caspase-3 enzyme in human promyelocytic leukemia HL-60 cells (doubling time; about 24 h) for 16 h. After 16 h of exposure of compounds (**1**—**11**) from *M. thunbergii* at 100 μM, macelignan (**4**), oleiferin C (**7**), *meso*-dihydroguaiaretic acid (**8**), and licarin A (**9**) were significantly increased to a level approximately 3.04, 6.16, 2.10, and 3.10-fold that of the non-treated control (Fig. 2). HL-60 cells exhibited typical morphological changes of apoptosis after treatment with compounds **4**, **7**—**9**. On the other hand, compounds **1**—**3**, **5**, **6**, **10**, and **11** showed cytotoxic activity at 100 μM and were inactive for caspase-3 activation. In the structure–activity relationship of lignans from *M. thunbergii*, the dibenzylbutanedimethyl lignans (**4**, **7**, **8**) showed more potent increasing activity of caspase-3 than neolignans (**2**, **9**)

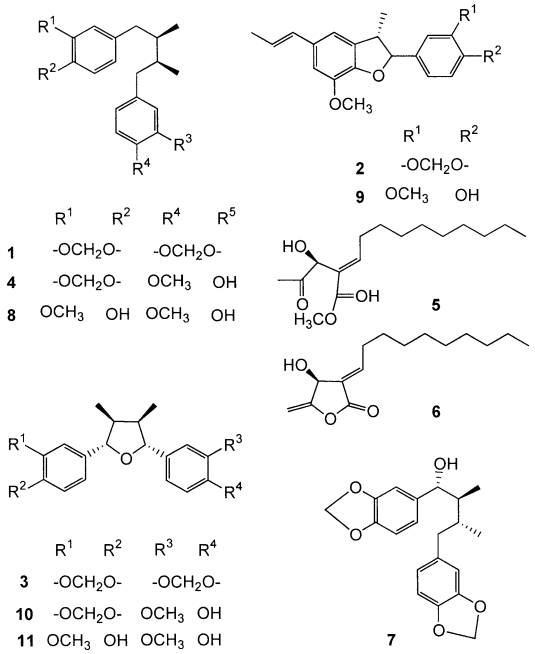

Fig. 1. Chemical Structures of Compounds **1**—**11** Isolated from *M. thunbergii*

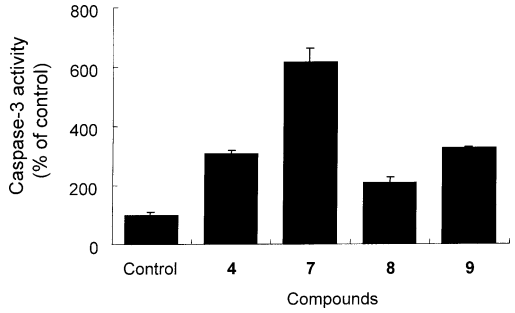

Fig. 2. Effect of Macelignan (**4**), Oleiferin C (**7**), *meso*-Dihydroguaiaretic Acid (**8**), and Licarin A (**9**) on Caspase-3 Activation

HL-60 cells were treated with compounds **4**, **7**—**9** at 100 μM for 16 h, then collected and washed with PBS. Cell lysate was incubated at 37 °C with Ac-DEVD-AFC for 1 h, after which the fluroscence intensity was measured.

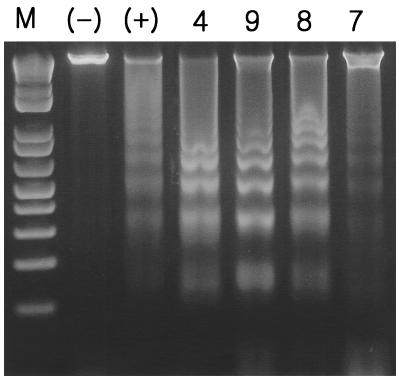

Fig. 3. DNA Fragmentation Induced by Macelignan (**4**), Oleiferin C (**7**), *meso*-Dihydroguaiaretic Acid (**8**), and Licarin A (**9**)

HL-60 cells were treated with compounds **4**, **7**—**9** for 16 h and then collected. DNA fragmentation of treated cells was detected by 1% agarose gel electrophoresis. M is a DNA size marker.

or tetrahydrofuran lignans (**3**, **10**, **11**). Of the compounds tested, oleiferin C (**7**) was found to have the greatest increase of caspase-3 activation. Our results suggest that a hydroxyl group on butane moiety of dibenzylbutanedimethyl seems to significantly enhance caspase-3 activity.

Apoptosis involves the activation of endonucleases and this activation results in the cleavage of genomic DNA into a characteristic ladder pattern.[17] The proteolytic activity was due to caspase-3.[18] To provide evidence supporting the involvement of apoptosis, a DNA fragmentation assay was performed. HL-60 cells treated with compounds **4**, **7**—**9** at 100 μM for 16 h showed the distinctive ladder pattern characteristic of apoptosis (Fig. 3).

In summary, the results of the present study demonstrated that macelignan (**4**), oleiferin C (**7**), *meso*-dihydroguaiaretic acid (**8**), and licarin A (**9**) induce an apoptotic effect in HL-60 cells in a caspase-3 activation manner. It is possible that these compounds may be valuable as cancer chemopreventive agents.

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