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NOTE

4-Hydroxyderricin from *Angelica keiskei* Roots Induces Caspase-dependent Apoptotic Cell Death in HL60 Human Leukemia Cells

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Abstract: The ethyl acetate (EtOAc)-soluble fraction of a methanol extract of *Angelica keiskei* roots exhibited cytotoxic activity against 4 human tumor cell lines, HL60 (leukemia), CRL1579 (melanoma), A549 (lung), and AZ521 (stomach). Nine chalcones (1–9), 5 coumarins (10–14), and 4 flavanones (15–18), isolated from the EtOAc-soluble fraction, were examined for their cytotoxic activities in the 4 human tumor cell lines. Among the compounds tested, 4-hydroxyderricin (2), a major chalcone constituent, exhibited potent cytotoxic activities in all 4 tumor cell lines with IC_{50} values of 5.5 μM (HL60), 4.8 μM (CRL1579), 10.2 μM (A549), and 4.2 μM (AZ521). 4-Hydroxyderricin induced early apoptosis in HL60 cells, observed as membrane phospholipid exposure in flow cytometry. Western blot analysis showed that 4-hydroxyderricin markedly reduced the levels of procaspases-3, -8, and -9, while increasing the levels of cleaved caspases-3, -8, and -9. In addition, 4-hydroxyderricin exhibited potent inhibitory activity on human DNA topoisomerase (Topo) II (IC_{50} 21.9 μM). These results suggested that 4-hydroxyderricin induces apoptotic cell death in HL60 *via* both the death receptor-mediated pathway and the mitochondrial pathway by, at least in part, Topo II inhibition. 4-Hydroxyderricin may therefore hold promise as an effective antitumor agent.

Key words: *Angelica keiskei*, 4-hydroxyderricin, chalcone, leukemia, cytotoxic activity, caspase-dependent apoptosis

1 INTRODUCTION

Angelica keiskei Koidzumi (Japanese name "Ashitaba", Umbelliferae), a hardy perennial herb growing mainly along the Pacific coast of Japan, has been used in traditional medicine, food, and beverages[1]. The herb has been reported to contain bioactive chalcones, flavanones, and coumarins, exhibiting antiallergic[2], antibacterial[1], antidiabetic[3], antimetastatic[4], antioxidant[5,6], antitumor[4,7–10], antiulcer[1], cancer chemopreventive[11–13], hypotensive[14], lipid regulatory[14], and nuclear factor-κB inhibitory[15] effects. In the course of our search for potential antitumor agents from natural sources[7–9], we have found that the ethyl acetate (EtOAc)-soluble fraction of a methanol (MeOH) extract of *A. keiskei* roots exhibited potent cytotoxic activities against 4 human tumor cell lines, HL60 (leukemia), CRL1579 (melanoma), A549 (lung), and AZ521 (stomach). We now report the isolation and identification of 9 chalcones (**1-9**), 5 coumarins (**10-14**), and 4 flavanones (**15-18**) from the EtOAc-soluble fraction of *A. keiskei* root extract and the evaluation of their cytotoxic activities against 4 human tumor cell lines. We also report on the apoptosis-inducing activity of 4-hydroxyderricin (**2**), a major chalcone constituent, in HL60 cells; 4-hydroxyderricin also exhibited potent cytotoxic activities against all 4 cancer cell lines.

2 EXPERIMENTAL PROCEDURES

2.1 General experimental procedures

The ^{1}H NMR spectra were recorded with a JEOL ECX-400 (400 MHz) in C_5D_5N with tetramethylsilane as an

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internal standard. ESIMS were recorded in positive-ion mode on an Agilent 1100 LC/MSD TOF (time-of-flight) system. Silica gel (Silica gel 60, 220－400 mesh, Merck & Co. Inc.) and octadecyl silica (ODS) (Chromatorex-ODS, 100－200 mesh; Fuji Silysia Chemical, Ltd., Aichi) were used for open column chromatography. Reversed-phase preparative HPLC (with a refractive index detector) was carried out on an ODS column (Pegasil ODS-II 5 μm column, 25 cm × 10 mm i.d.; Senshu Scientific Co., Ltd., Tokyo) with MeOH: H_2O:AcOH as the eluting solvent at a ratio of 85:15:1 (flow rate of the eluent: 3.0 mL/min; system Ⅰ), 75:25:1 (3.0 mL/min; system Ⅱ), 60:40:1 (2.0 mL/min; system Ⅲ), 80:20:0 (2.0 mL/min; system Ⅳ), 60:40:0 (3.0 mL/min; system Ⅴ), or 55:45:0 (2.0 mL/min; system Ⅵ).

2.2 Materials and chemicals

The dried roots of *Angelica keiskei* Koidzumi (Umbelliferae) were purchased from Ashitaba Fabrication Plant Co., Ltd. (Hachijojima, Tokyo) in June, 2006. The plant material was authenticated by Mr. Yukinari Yamada (Ashitaba Fabrication Plant Co., Ltd.), and a voucher specimen (No. SB-1291) has been deposited in the authors' laboratory (Coll. Sci. Technol., Nihon Univ.). Fifteen compounds, 4-hydroxyderricin (**2**), xanthoangelol (**3**), xanthoangelol F (**4**), xanthoangelol H (**6**), selinidin (**10**), laserpitin (**11**), isolaserpitin (**12**), 3'-senecioyl khellactone (**13**), 4'-senecioyl khellactone (**14**), prostratol F (**16**), 4'-*O*-geranyl naringenin (**18**)[12], isobavachalcone (**1**), deoxyxanthoangelol H (**5**), xanthoangelol I (**7**), and isobavachin (**15**)[13], were used as reference compounds. Chemicals and reagents were purchased as follows: Fetal bovine serum (FBS), RPMI 1640 medium, penicillin-streptomycin, and non essential amino acid (NEAA) from Invitrogen Co. (Auckland, New Zealand), Dulbecco's modified eagle's medium (D-MEM), Eagle's minimal essential medium (MEM), and thiazoyl blue tetrazolium bromide (MTT) from Sigma Aldrich Japan Co. (Tokyo), camptothecin and etoposide from EMD Biosciences, Inc. (La Jolla, CA, U.S.A.), human DNA topoisomerases (Topo) I and II from TopoGen (Columbus, OH, U.S.A.), supercoiled pBR 322 plasmid DNA from Takara Bio Inc. (Ohtsu), and rh Annexin V/FITC kit (Bender MedSystems®) from Cosmo Bio Co. Ltd. (Tokyo). All other chemicals and reagents were of analytical grade.

2.3 Cell lines and culture conditions

HL60 (human leukemia), CRL1579 (human melanoma), A549 (human lung), and AZ521 (human stomach) cell lines were obtained from Riken Cell Bank (Tsukuba, Ibaraki). HL60 and CRL1579 were grown in RPMI 1640 medium, while A549 and AZ521 were grown in D-MEM and in 90% D-MEM + 10% MEM + 0.1 mM NEAA, respectively. The medium was supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). The cells were cultured in a 5% CO_2 humidified incubator at 37℃.

2.4 Extraction, isolation, and identification

The dried and pulverized roots of *A. keiskei* (2970 g) were extracted three times with MeOH under reflux (3 h) to yield MeOH extract (477 g). The MeOH extract was partitioned with *n*-hexane-MeOH-H_2O (19:19:2) giving *n*-hexane- (28 g) and MeOH-H_2O- (364 g) soluble fractions. The latter, suspended in H_2O, was partitioned successively with EtOAc and *n*-butanol (*n*-BuOH) to sequentially yield EtOAc- (41 g), *n*-BuOH- (31 g), and H_2O- (262 g) soluble fractions. A portion (36 g) of the yellow-colored EtOAc fraction was chromatographed on a silica gel (1 kg) column, which was eluted successively with solvents of increasing polarity (*n*-hexane-EtOAc, 9:1→0:1; EtOAc-MeOH, 1:0→1:4) to yield 11 fractions, A-K. Fraction C (972 mg) eluted with *n*-hexane-EtOAc (7:3) was subjected to further chromatography on silica gel and yielded 9 fractions, C1-C9. Fraction C1 (316 mg), upon chromatography on an ODS column (eluting solvent: MeOH-H_2O, 3:2→9:1), yielded **10** (251 mg). Fraction C2 (142 mg), upon preparative HPLC (system I), yielded **8** (2.5 mg) and **18** (61.4 mg). Fraction E (3564 mg), eluted with *n*-hexane-EtOAc (7:3), was chromatographed on an ODS column (eluting solvent: MeOH-H_2O, 1:1→9:1) to yield **4** (377 mg). Fraction F (1717 mg), eluted with *n*-hexane-EtOAc (7:3), upon successive column chromatography on silica gel and ODS, yielded **3** (67 mg). Fraction G (13.0 g) was crystallized from MeOH, yielding crystallized (Gc; 2567 mg) and mother liquor (Gm; 9398 mg) fractions. Chromatography of fraction Gc on an ODS column (eluting solvent: MeOH-H_2O, 1:1→1:0) yielded **2** (216 mg; fraction Gc3) and **4** (130 mg; fraction Gc6), along with 4 other fractions, Gc1, Gc2, Gc4, and Gc5. A portion (600 mg) of fraction Gc4 (1317 mg) was subjected to preparative HPLC (system Ⅳ) which yielded **2** (136 mg), **5** (2.0 mg), and **17** (9.0 mg). Fraction Gm was chromatographed on a silica gel column to yield 10 fractions, Gm1-Gm10. Further ODS column chromatography of fraction Gm4 (3960 mg) yielded **3** (1219 mg; fraction Gm4-6) and **11** (163 mg; fraction Gm4-3), and 5 other fractions, Gm4-1, Gm4-2, Gm4-4, Gm4-5, and Gm4-7. Preparative HPLC (system Ⅲ) of fraction Gm4-2 yielded **11** (73.2 mg) and **12** (5.0 mg). Chromatography of fraction J on an ODS column yielded 6 fractions, J1-J6. A portion (250 mg) of fraction J2 (283 mg) was further chromatographed on a silica gel column to yield 6 fractions, J2-1-J2-6. Preparative HPLC of fractions J2-1 (29 mg; system VI) and J2-2 (90 mg; system V) yielded **13** (1.3 mg) and **14** (2.2 mg), and **1** (1.1 mg), **6** (0.6 mg), **15** (2.5 mg), and **16** (2.2 mg), respectively. Furthermore, preparative HPLC of fractions J3 (44 mg; system Ⅳ) and J4 (22 mg; system Ⅱ) yielded **9** (9.8 mg) and **7** (1.2 mg), respectively. Identification of 15 compounds, **1**-**7**, **10**-**16**, and **18** (Fig. 1), was performed by NMR and MS comparison with reference compounds. Compounds **8**, **9**, and **17** were iden-

tified as lespeol[16], 3',4'-dihydro-3'-hydroxylespeol[17], and 7-*O*-methyl prostratol F[18], respectively, by comparison of NMR and MS data with the corresponding compounds in the literature (Fig. 1). The percentage purity of compounds **1-18** was more than 95%.

2.5 Cytotoxicity assay

The cytotoxicity assay was performed according to a previously described method[7, 19].

2.6 Annexin V-propidium iodide double staining

Apoptosis was detected as described in our recent paper using a recombinant human (rh) Annexin V/FITC kit[19].

2.7 Western blot analysis

Western blot analysis was performed according to a previously described method[7, 19].

2.8 DNA Topoisomerase inhibition assay

DNA Topo I and II inhibition assays were performed according to the previous method[19, 20].

3 RESULTS AND DISCUSSION

The MeOH extract of *A. keiskei* roots and its *n*-hexane-, EtOAc-, *n*-BuOH-, and H_2O-soluble fractions were assayed for cytotoxic activity against 4 human tumor cell lines, HL60 (leukemia), CRL1579 (melanoma), A549 (lung), and AZ521 (stomach). As shown in **Table 1**, the EtOAc-soluble and *n*-hexane-soluble fractions exhibited potent activities against all 4 tumor cell lines. The EtOAc-soluble fraction was further investigated for the active constituents in this study. Nine chalcones (**1-9**), 5 coumarins (**10-14**), and 6 flavanones (**15-18**), were isolated and identified from the EtOAc-soluble fraction. Among the compounds identified, 2 chalcones, **3** followed by **2**, were detected as the most abundant constituents . The predominance of these 2 chalcones in the EtOAc-soluble fraction of the *A. keiskei* root extract is consistent with observations in the stem extract of *A. keiskei*[12]. Whereas several flavanones have recently been identified in the stem extract of *A. keiskei*[12, 13], this is the first report of the isolation and identification of these compounds, *i.e.*, **15-18**, in the root extract of *A. keiskei*.

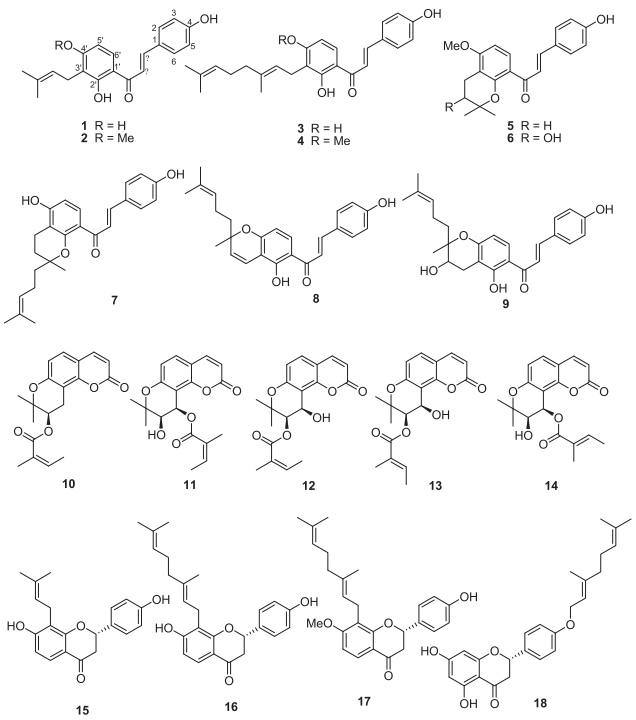

Fig. 1 Structures of compounds **1-18** isolated from *Angelica keiskei* root extract.

Eighteen compounds, **1-18**, were assayed for cytotoxic activity against HL60, CRL1579, A549, and AZ521 cell lines. Six compounds, **2**, **3**, **5**, **8**, **16**, and **18**, exhibited potent cytotoxicity against one or more tumor cell line (**Table 2**). Compound **2** against HL60 (IC_{50} 5.5 μM), CRL1579 (IC_{50} 4.8 μM), A549 (IC_{50} 10.2 μM), and AZ521 (IC_{50} 4.2 μM) cells, **3** and **16** against HL60 and CRL1579 cells, **5** and **8** against HL60 cells, and **18** against CRL1579 cells, exhibited potent cytotoxicities with IC_{50} values of 3.4-10.2 μM. The cytotoxicities of these compounds against CRL 1579, A549, and AZ521 cell lines were superior to those of the reference compounds, cisplatin and 5-fluorourasil. Cyclization of the prenyl chain at C-3' of compound **2** did not influence activity (compound **5**); demethylation of the methoxy group at C-4' (compound **1**) and substitution of the prenyl chain with the geranyl chain (compounds **3** and **4**) reduced the cytotoxic activity of compound **2** in HL60. All of 5 coumarins, **10-14**, showed the least cytotoxicity.

Compound **2**, the most potent cytotoxic compound against all 4 tumor cell lines, was then evaluated for its induction of early apoptosis in HL60 cells. Exposure of the membrane phospholipid, phosphatidylserine, to the external cellular environment is one of the earliest markers of apoptotic cell death[21]. Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine expressed on the cell surface. Propidium iodide (PI) does not enter whole cells with intact membranes and was used to differentiate between early apoptotic (Annexin V positive, PI negative), late apoptotic (Annexin V, PI double positive), or necrotic cell death (Annexin V negative, PI positive). In the early phases of apoptotic cell death, PS is translocated to the outer layer of the membrane, *i.e.*, the external surface of the cell, during which process the cell membrane remains intact. Late apoptotic cells lose their membrane integrity as the PI is incorporated into the cells[22]. The proportion of early apoptotic HL60 cells (lower right) was significantly increased after 8 h of incubation with **2** (40 μM) (29.4% vs. 4.9%) (**Fig. 2**). After

Table 1 Cycotoxicities in Four Human Tumor Cells of the Extracts of *Angelika keiskei* Roots

Extract and fraction	Cytotoxicity (IC_{50}; μg/mL)			
	HL60 (leukemia)	CRL1579 (melanoma)	A549 (lung)	AZ521 (stomach)
MeOH extract	72.2	>100	93.5	89.2
n-Hexane-soluble fraction	14.1	53.1	27.4	40.8
EtOAc-soluble fraction	10.6	14.9	8.5	13.3
n-BuOH-soluble fraction	>100	>100	>100	25.2
H_2O-soluble frtion	>100	>100	>100	>100
Cisplatin[a)]	0.6	6.3	7.5	4.1

[a)] Reference compound.

Table 2 Cycotoxicities in Four Human Tumor Cells and Inhibitory Activities of Human DNA Topoisomerases I and II of the Compounds from *Angelika keiskei* Root Extract

Compound		Cytotoxicity (IC_{50}; μM)[a,b)]				Inhibitory Activity on Human Topoisomerases (IC_{50}; μM)[b)]	
		HL60 (leukemia)	CRL1579 (melanoma)	A549 (lung)	AZ521 (stomach)	Topo I	Topo II
Chalcone							
1	Isobavachalcone	10.0	20.9	>100	>100	>100	>100
2	4-Hydroxyderrincin	5.5	4.8	10.2	4.2	>100	21.9
3	Xanthoangelol	9.9	3.4	>100	13.9	>100	>100
4	Xanthoangelol F	10.5	61.0	33.2	79.5	>100	>100
5	2''-Deoxyxanthoangelol H	4.1	16.7	96.2	85.5	>100	>100
6	Xanthoangelol H	14.5	40.7	>100	72.5	>100	>100
7	Xanthoangelol I	12.6	21.7	>100	>100	>100	>100
8	Lespeol	9.4	49.3	34.0	28.4	>100	>100
9	6'-Dihydro-7'-hydroxylespeol	7.7	61.1	20.5	18.3	>100	>100
Coumarin							
10	Selinidin	12.1	29.6	74.8	82.5	>100	>100
11	Laserpitin	81.5	>100	>100	>100	>100	>100
12	Isolaserpitin	14.0	15.6	64.4	22.8	>100	>100
13	3'-Senecioyl khellactone	75.0	>100	>100	>100	>100	>100
14	4'-Senecioyl khellactone	76.4	87.5	>100	>100	>100	>100
Flavanone							
15	Isobavachin	54.2	15.6	28.0	28.3	>100	>100
16	Prostratol F	7.2	9.4	23.4	25.3	>100	>100
17	7-*O*-Methyl prostratol F	13.0	17.1	>100	16.6	>100	>100
18	4'-*O*-Geranyl naringenin	13.3	7.2	76.3	>100	>100	>100
Reference compound							
	Cisplatin	1.9	21.1	24.9	5.1		
	5-Fluorouracil	9.5	>100	>100	11.3		
	Camptothecin					5.7	
	Etoposide						34.5

a) Cells were treated with compounds (1×10^{-4} - 1×10^{-6} M) for 48 h, and cell viabilitiy was analyzed by the MTT assay.
b) IC_{50} based on triplicate three points.

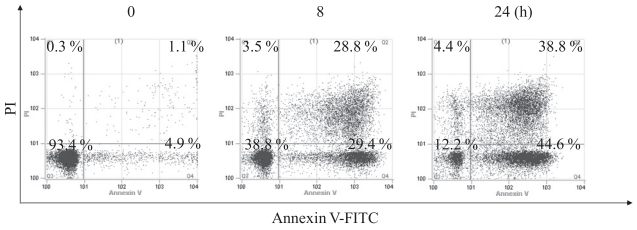

Fig. 2 Detection of early apoptotic cells by annexin V-PI double staining.
HL60 cells were cultured with 40 μM 4-hydroxyderricin (**2**) for 8 h and 24 h. Results shown are representative of 3 independent experiments with similar results.

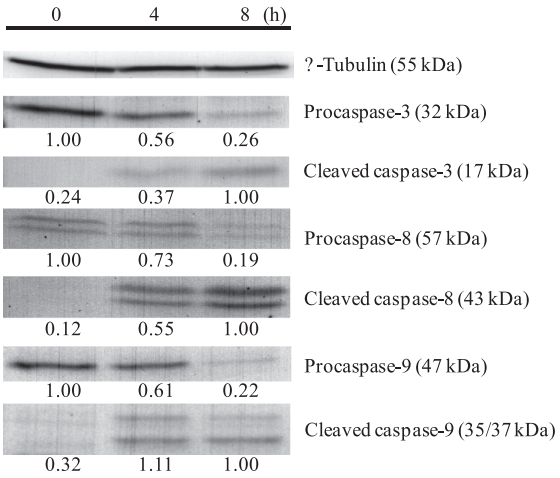

Fig. 3. Western blot analysis of caspases-3, -8, and -9 in HL60 cells treated with 4-hydroxyderricin (**2**).
Relative amount of expressed protein is shown at each immunoblotting band. Results shown are representative of 3 independent experiments with similar results.

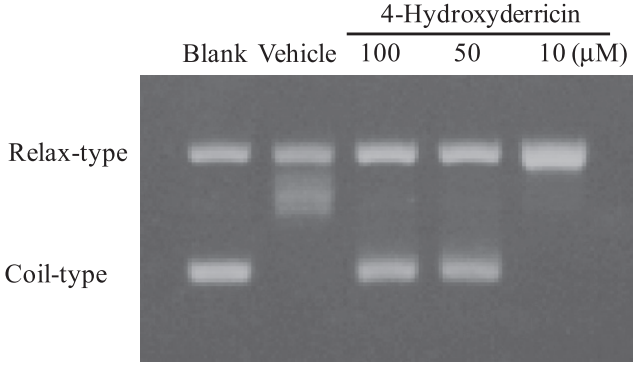

Fig. 4. DNA Topoisomerase II inhibitory activity of 4-hydroxyderricin (**2**).
Results shown are representative of 3 independent experiments with similar results.

treatment with **2** for 24 h, the proportion of early apoptotic cells further increased (44.6% vs. 29.4% for 8 h) and was accompanied by the accumulation of late apoptotic cells (upper right; 38.8% vs. 28.8% at 8 h vs. 1.1% at 0 h). These results suggested that most of the cytotoxicity of **2** is due to the induction of apoptotic cell death in HL60.

Apoptosis is induced mainly by triggering of the death receptors (extrinsic pathway) and/or mitochondria (intrinsic pathway), leading to the activation of caspases[23, 24]. In the death receptor-mediated apoptosis pathway, tumor necrosis factor activates upstream caspase-8. Activation of caspase-8 can in turn directly activate caspase-3. The mitochondrial-mediated apoptosis pathway activates procaspase-9 by releasing cytochrome *c*. Caspase-9 forms an active holoenzyme that processes and activates downstream caspase-3. Inhibitors of DNA Topo Ⅱ are known to stimulate apoptosis by activating the mitochondrial pathway[24].

To characterize the mechanism by which 4-hydroxyderricin (**2**) induces apoptotic cell death, activation of caspases-3, -8, and -9 was evaluated by Western blot analysis. After treatment of HL60 cells with **2** (40 μM), the level of procaspases-3, -8, and -9 gradually diminished in a time-dependent manner, and cleaved caspases-3, -8, and -9 were detected (Fig. 3). These results suggest that **2**-induced cell death occurs through activation of caspases-3, -8, and -9. Upon investigation of Topo I and II inhibition by compounds **1**-**18**, only compound **2** exhibited potent Topo Ⅱ-inhibitory activity (IC_{50} 21.9 μM) (Table 2, Fig. 4), almost comparable with that of etoposide (IC_{50} 34.5 μM), a well known Topo Ⅱ poison[25]. Compound **2** exhibited, however, almost no inhibitory effect on Topo Ⅰ (IC_{50} > 100 μM) (Table 2) whereas camptothecin, a positive control[26], showed potent activity (IC_{50} 1.9 μM). These suggested that the mitochondrial signal transduction pathway involving Topo II inhibition by **2** is one of the triggers of apoptosis in HL60 cells.

We have shown that compound **2** induces apoptotic cell death *via* both the death receptor-mediated and the mitochondrial pathways in HL60 cell lines. Whereas the induction of apoptosis by **2** in human stomach cancer KATO III cells has recently been reported[10], this is the first report of caspase-dependent apoptosis induction by **2** in HL60 cells. Xanthoangelol (**3**), the most abundant chalcone constituent of the EtOAc-soluble fraction of *A. keiskei* root extract, has recently been demonstrated to possess apoptosis-inducing activities in human neuroblastoma (IMR-32), leukemia (Jurkat)[7], and stomach cancer (KATO Ⅲ) cells[10].

In summary, we have shown that the EtOAc-soluble fraction of a MeOH extract of *A. keiskei* roots exhibits cytotoxic activity in 4 human tumor cell lines, HL60 (leukemia), CRL1579 (melanoma), A549 (lung), and AZ521 (stomach). Eighteen phenolic compounds (**1**-**18**) isolated from the

EtOAc-soluble fraction were examined for their cytotoxic activities against the 4 human tumor cell lines. Among the compounds tested, 4-Hydroxyderricin (**2**), a major chalcone constituent, exhibited potent cytotoxic activities in all 4 tumor cell lines. 4-Hydroxyderricin induces apoptotic cell death in HL60 cells *via* both the death receptor-mediated pathway and the mitochondrial pathway by, at least in part, Topo II inhibition. It appears that 4-hydroxyderricin as well as xanthoangelol (**3**)[7, 10] may hold promise as effective antitumor agents.

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