ANTICANCER RESEARCH *25*: 2055-2064 (2005)

Tumor-specificity and Apoptosis-inducing Activity of Stilbenes and Flavonoids

SHAHEAD ALI CHOWDHURY[1], KAORI KISHINO[2], RIE SATOH[2], KEN HASHIMOTO[2], HIROTAKA KIKUCHI[3], HIROFUMI NISHIKAWA[3], YOSHIAKI SHIRATAKI[4] and HIROSHI SAKAGAMI[2]

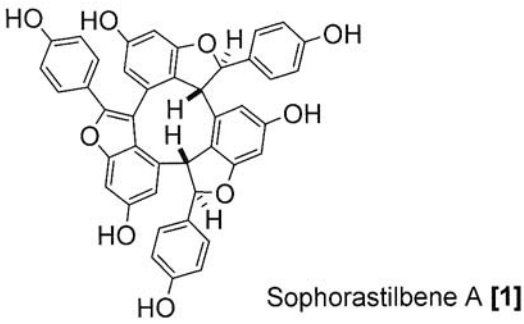
[1]Meikai Pharmaco-Medical Laboratory (MPL), [2]Department of Dental Pharmacology and [3]Department of Endodontics, Meikai University School of Dentistry, Sakado, Saitama; [4]Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan

Abstract. *A total of eleven stilbenes **[1-6]** and flavonoids **[7-11]** were investigated for their tumor- specific cytotoxicity and apoptosis-inducing activity, using four human tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG and promyelocytic leukemia HL-60) and three normal human oral cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF). All of the compounds, especially sophorastilbene A **[1]**, (+)-α-viniferin **[2]**, piceatannol **[5]**, quercetin **[9]** and isoliquiritigenin **[10]**, showed higher cytotoxicity against the tumor cell lines than normal cells, yielding tumor-specific indices of 3.6, 4.7, >3.5, >3.3 and 4.0, respectively. Among the seven cell lines, HSC-2 and HL-60 cells were the most sensitive to the cytotoxic action of these compounds. Sophorastilbene A **[1]**, piceatannol **[5]**, quercetin **[9]** and isoliquiritigenin **[10]** induced internucleosomal DNA fragmentation and activation of caspases -3, -8 and -9 dose-dependently in HL-60 cells. (+)-α-Viniferin **[2]** showed similar activity, but only at higher concentrations. All the compounds failed to induce DNA fragmentation and activated caspases to much lesser extents in HSC-2 cells. Western blot analysis showed that sophorastilbene A **[1]**, piceatannol **[5]** and quercetin **[9]** did not induce any consistent changes in the expression of pro-apoptotic proteins (Bax, Bad) and anti-apoptotic protein (Bcl-2) in HL-60 and HSC-2 cells. An undetectable expression of Bcl-2 protein in control and drug-treated HSC-2 cells may explain the relatively higher sensitivity of this cell line to stilbenes and flavonoids.*

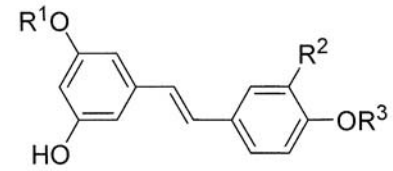
Correspondence to: Prof. Hiroshi Sakagami, Department of Dental Pharmacology, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: (+81) 49-285-5511, ex 336, 429, 690, Fax: (+81) 49-285-5171, e-mail: sakagami@dent.meikai.ac.jp

Key Words: Stilbenes, flavonoids, oral tumor cells, DNA fragmentation, caspase, Bcl-2, Bax, Bad.

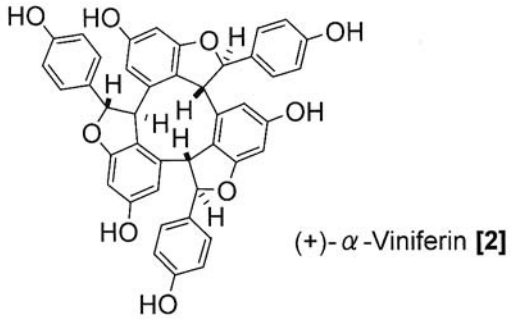
We have previously reported the tumor-specific cytotoxicity of flavonoids and analogous phenols. Most pryranoflavones and their derivatives and prenylated or geranylated flavones were cytotoxic, but showed weak tumor-specificity (TS=0.3-2.3), suggesting that the presence of both hydrophobic and hydrophilic groups within the molecule are necessary for the cytotoxic activity (1-3). Licochalcone B, a chalcone derivative without the isoprenoid group, showed the highest tumor-specificity (TS=31.7). Isoprenoid-substituted chalcone showed higher cytotoxicity, and prenylation(s) on an isoflavone, genistein, also increased the cytotoxicity, but is not necessary for tumor-specificity (4). Among flavonoids and 2-arylbenzofurans with isoprenoid substituents, sanggenol M, sanggenon C and sanggenon B showed some tumor-specific cytotoxicity (TS= 2.5, 2.7 and 2.3, respectively). These compounds are Diels-Alder-type adducts with a chalcone and a 6-dehydrogeranyl(prenyl)flavanone and its derivative. Seven other flavanones showed similar tumor-specificity (TS=1.6-3.0), whereas the more hydrophobic 2-arylbenzofurans showed much weaker cytotoxicity and tumor-specificity (TS=1.0-1.5) (5). Benzophenones, compounds with two isoprenoid groups, showed higher cytotoxicity than the monoprenylated compound, but they showed weak tumor-specific cytotoxicity (TS=1.2-1.3). Fourteen xanthones showed marginal tumor-specific cytotoxicity (TS=1.1->2.0) (6). Thirteen anthraquinones showed relatively higher tumor-specific cytotoxicity. Among them, emodin and aloe-emodin, without glycosylation, were the most potent (TS=8.5 and >18.6, respectively), whereas other anthraquinone glycosides (TS=1.0->3.4), phenylbutane glucosides (TS=1.5-3.3) and naphthalene glucosides (TS=1.1->1.4) were less active. These data suggest that the glycosyl moiety is not necessary for the tumor-specificity of anthraquinones (7). Studies with eleven isoflavones and isoflavanones from *Sophora* species suggest that: (i) compounds with two isoprenyl groups (one in the A-ring and the other in the B-ring) or the α,α-dimethylallyl group at C-5' of the B-ring should have



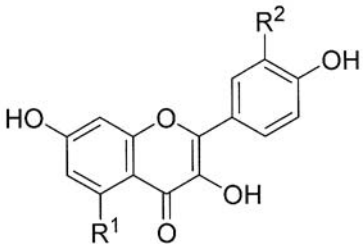
Sophorastilbene A **[1]**



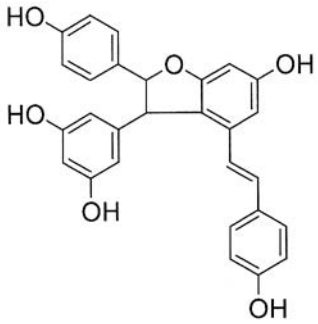
	R^1	R^2	R^3
Resveratrol **[4]**	H	H	H
Piceatannol **[5]**	H	OH	H
Rhaponticin **[6]**	Glc	OH	CH_3



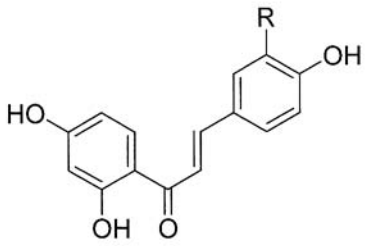
(+)-α-Viniferin **[2]**



	R^1	R^2
Kaempferol **[7]**	OH	H
Fisetin **[8]**	H	OH
Quercetin **[9]**	OH	OH



(−)-ε-Viniferin **[3]**



	R
Isoliquiritigenin **[10]**	H
Butein **[11]**	OH

Figure 1. *Structure of stilbenes **[1-6]** and flavonoids **[7-11]** used in this study.*

relatively higher cytotoxic activity; (ii) their cytotoxic activity reached the maximum level when the log P was around 4; and (iii) tumor-specificity (TS= <2.8) was not directly coupled with the cytotoxic activity (8).

Our recent studies with twenty-six α,β-unsaturated ketones (9), eight hydoxyketones (10) and twenty-three β-diketones (11) demonstrated that apoptosis-inducers did not always show tumor-specific cytotoxicity, and that compounds with higher tumor-specific cytotoxicity did not always induce apoptosis in tumor cell lines. Recently, stilbenes (resveratrol [**4**], piceatannol [**5**]) and flavonoids (fisetin [**8**], quercetin [**9**], isoliquiritigenin [**10**], butein [**11**])

have been shown to prolong the lifespan of *Saccharomycess cerevisiae* through the activation of NAD-dependent protein deacetylate (12). We investigated, here, whether eleven related compounds including six stilbenes (sophorastilbene A [**1**], (+)-α-viniferin [**2**], (-)-ε-viniferin [**3**], resveratrol [**4**], piceatannol [**5**], rhaponticin [**6**]) and flavonoids (kaempferol [**7**], fisetin [**8**], quercetin [**9**], isoliquiritigenin [**10**], butein [**11**]) (Figure 1) induce tumor-specific cytotoxicity and apoptotic cell death, using four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG, promyelocytic leukemia HL-60) and three normal human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF). We used internucleosomal DNA fragmentation, caspase activation and expression of apoptosis-related proteins as markers for apoptosis.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RPMI 1640 medium, DMEM (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Ind., St. Louis, MO, USA), dimethyl sulfoxide (DMSO), (Wako Pure Chem Ind., Osaka, Japan). Sophorastilbene A [**1**] (MW676.7), (+)-α-viniferin [**2**] (MW678.7), (-)-ε-viniferin [**3**] (MW454.5) and isoliquiritigenin [**10**] (MW256.3) were prepared as previously described (13, 14). Resveratrol [**4**] (MW22.8), piceatannol [**5**] (MW244.2), rhaponticin [**6**] (MW420.4), kaempherol [**7**] (MW286.2), fesetin [**8**] (MW286.2), quercetin [**9**] (MW302.2) and butein [**11**] (MW272.3) were purchased from Tokyo Kasei Kogyo Co., Ltd., Japan.

Cell culture. Normal human cells, such as gingival fibroblast (HGF), pulp cell (HPC) and periodontal ligament fibroblast (HPLF), were obtained from human periodontal tissue after informed consent, according to the guidelines of Meikai University Ethics Committee, Japan (No. 0206). Since normal cells have a limited lifespan (15), cells at 6-8 population doubling level (PDL) were used for the present study. The human oral squamous cell carcinoma cell lines (HSC-2, HSC-3) were supplied by Prof. Nagumo, Showa University, and Dr. Fukuda, Meikai University, Japan, respectively. The human submandibular gland carcinoma cell line (HSG) was supplied by Drs. Atsumi and Kurihara, Meikai University School of Dentistry, Japan. The human promyelocytic leukemia cell line (HL-60) was supplied by Prof. Nakaya, Showa University, Japan.

HL-60 cells were maintained at 37˚C in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. The other cells were cultured as monolayer cultures at 37˚C in DMEM supplemented with 10% heat-inactivated FBS, in a humidified 5% CO_2 atmosphere, and subcultured by trypsinization.

Cytotoxic activity. The relative viable cell number of adherent cells was determined by MTT methods, while that of non-adherent cells (HL-60 cells) was determined by trypan blue dye exclusion. For the MTT assay, the cells in 96-microwell plate (Falcon, flat bottom, Becton Dickinson, Franklin Lakes, NJ, USA) were treated for 24 hours without (control) or with various concentrations of the test samples. The cells were washed once with phosphate-buffered saline without Mg^{2+} or Ca^{2+} [PBS(-)], and further incubated for 4 hours with 0.2 mg/mL MTT in culture medium. After removal of the medium, the cells were lysed with 0.1 mL of DMSO. The absorbance at 540 nm of the solubilized formazan pellet (which reflects the relative viable cell number) was then determined by microplate reader (Biochromatic Labsystem, Helsinki, Finland). For the trypan blue dye exclusion assay, the number of viable cells which did not incorporate the trypan blue dye was calculated by hemocytometer. From the dose-response curve, the 50% cytotoxic concentration (CC_{50}) was determined. Tumor-specific cytotoxicity (Sl value) was determined by the following equation:

$$Sl=\{[CC_{50}\,(HGF) + CC_{50}\,(HPC) + CC_{50}\,(HPLF)] / [CC_{50}\,(HSC\text{-}2) + CC_{50}\,(HSC\text{-}3) + CC_{50}\,(HSG) + CC_{50}\,(HL\text{-}60)]\} \times (4/3)$$

Assay for DNA fragmentation. The cells were lysed with 50 μL lysate buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate), and incubated sequentially with 1 mg/mL RNase A (Boehringer Mannheim, Germany) for 1 hour at 50˚C, and with 1 mg/mL proteinase K (Boehringer) for 1 hour at 50˚C. DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with ethanol. The DNA was dissolved with DNA loading buffer, and then applied to 2% agarose gel electrophoresis. After staining with ethidium bromide, the DNA was visualized by UV irradiation and photographed by CCD camera (Bio Doc Inc., UVP).

Assay for caspase activity. The cells were lysed with 200 μL of lysis solution (MBL, Nagoya, Japan). After standing on ice for 10 minutes and centrifugation at 10,000 xg and 4˚C, for 5 minutes, the supernatant was collected. To 50 μL sample (equivalent to 0.2 mg protein), 50 μL of 2 x reaction buffer (MBL) containing the substrate for caspase-3 (DEVD-*p*NA(*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA) was added. After incubation at 37˚C for 4 hours, the absorbance at 405 nm of *p*NA produced by the cleavage of the substrates was measured by microplate reader, according to the manufacturer's instruction (MBL).

Western blotting. The cell pellets were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 minutes in ice-water, and then incubated for 50 minutes at 4˚C with RT-5 ROTATOR (Titec, Saitama, Japan). The cell lysates were centrifuged at 16,000 xg for 20 minutes at 4˚C to remove the insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of protein from the cell lysates (10 μg) was mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-mercaptoethanol), boiled for 10 minutes and applied to the SDS-7% polyacrylamide gel electrophoresis, before being transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in PBS(-) plus 0.05% Tween 20 for 90 minutes and incubated with anti-Bcl-2 antibody (1:1,000), anti-Bax antibody (1:1,000), anti-Bad antibody (1:1,000) (Santa Cruz Biotechnology, Delaware, CA, USA) or anti-actin antibody (1:1,000) (Sigma) for 90 minutes at room temperature. After incubation for 60 minutes at room temperature with HRP-conjugated anti-IgG (1:2,000,Santa

Table I. *Tumor-specific cytotoxic activity of flavonoids and stilbenes.*

		CC_{50} (μM)							
		Normal cells			Tumor cells				
	MW	HGF	HPC	HPLF	HSC-2	HSC-3	HSG	HL-60	TS
Stilbenes									
1	677	196	210	207	27	61	108	28	3.6
2	679	464	573	553	36	60	349	6	4.7
3	455	111	146	94	42	84	110	31	1.8
4	228	500	676	582	155	288	316	45	2.9
5	244	367	414	>1000	63	232	373	11	>3.5
6	420	>1000	979	>1000	500	588	>1000	810	><1.4
Flavonoids									
7	286	>1000	>1000	>1000	743	>1000	>1000	29	><1.4
8	286	608	170	641	39	188	375	16	3.1
9	302	750	909	>1000	35	250	745	31	>3.3
10	256	311	319	336	79	97	125	22	4
11	272	274	180	234	26	69	188	5	3.2
	A_{540}	0.389	0.433	0.448	2.146	1.572	0.462	-	

$TS=\{\Sigma CC_{50}(\text{normal cells})/\ \Sigma CC_{50}(\text{tumor cell lines})\} \times (4/3)$

Cruz Biotech), immunoblots were detected by Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA).

Results

Tumor-specific cytotoxicity. We found that four tumor cell lines (HSC-2, HSC-3, HSG, HL-60) were more sensitive to all the stilbenes and flavonoids investigated than the three normal cells (HGF, HPC, HPLF), yielding a tumor-specificity index (Table I). However, the tumor cell lines showed considerable variation in sensitivity. HL-60 cells were the most sensitive, followed by HSC-2, HSC-3 and HSG. On the other hand, the normal cells showed comparable sensitivity with each other. There was no apparent difference in the cytotoxicity between stilbenes [**1-6**] and flavonoids [**7-11**]. Especially, sophorastilbene A [**1**], (+)-α-viniferin [**2**], piceatannol [**5**], quercetin [**9**] and isoliquiritigenin [**10**] showed higher cytotoxicity against the tumor cell lines than normal cells, yielding tumor-specific indices of 3.6, 4.7, >3.5, >3.3 and 4.0, respectively. There was no clear-cut relationship between the cytoxicity and molecular weight, or between the tumor-specificity and molecular weight (Table I).

Induction of apoptosis. Sophorastilbene A [**1**] (at >50 μM), piceatannol [**5**] (at >10 mM), quercetin [**9**] (at >40 μM) and isoliquiritigenin [**10**] (at >10 μM) induced internucleosomal DNA fragmentation and activation of caspases -3, -8 and -9 dose-dependently in HL-60 cells (left two columns in Figure 2). (+)-α-Viniferin [**2**] induced these apoptosis markers only at much higher concentrations (>200 μM). All these compounds (up to 320 μM) failed to induce internucleosomal DNA fragmentation and activated caspases -3, -8 and -9 to much lesser extents in HSC-2 cells (right two columns in Figure 2).

Western blot analysis shows that sophorastilbene A [**1**], piceatannol [**5**] and quercetin [**9**] did not induce any consistent changes in the expression of pro-apoptotic proteins (Bax, Bad) and anti-apoptotic protein (Bcl-2) in HL-60 (upper panel in Figure 3) and HSC-2 cells (lower panel in Figure 3). No expression of Bcl-2 protein was detectable in HSC-2 cells, without or with treatment with any compounds (lower panel in Figure 3).

Discussion

The present study demonstrated that stilbenes and flavonoids induced tumor-specific cytotoxicity to various extents (Table I). Among these eleven compounds, (+)-α-viniferin [**2**], a cyclic trimer of resveratrol [**4**] (16), showed the highest tumor-specificity (TS=4.7), but this compound induced apoptosis markers to a much lesser extent than that attained by the other compounds investigated. This further supports the fact that tumor-specificity and apoptosis induction do not always correlate with each other. The tumor-specificity of (-)-ε-viniferin [**3**] (TS=1.9), a dimer of resveratrol [**4**], was lower than that of resveratrol [**4**] (TS=2.9). It should be noted that another trimer, sophorastilbene A [**1**] (13) also showed higher tumor-specificity (TS=3.6). This suggests that the higher tumor-specificity of trimers [**1, 2**] might be due to a higher order conformation. Piceatannol [**5**], a hydroxylated analog of

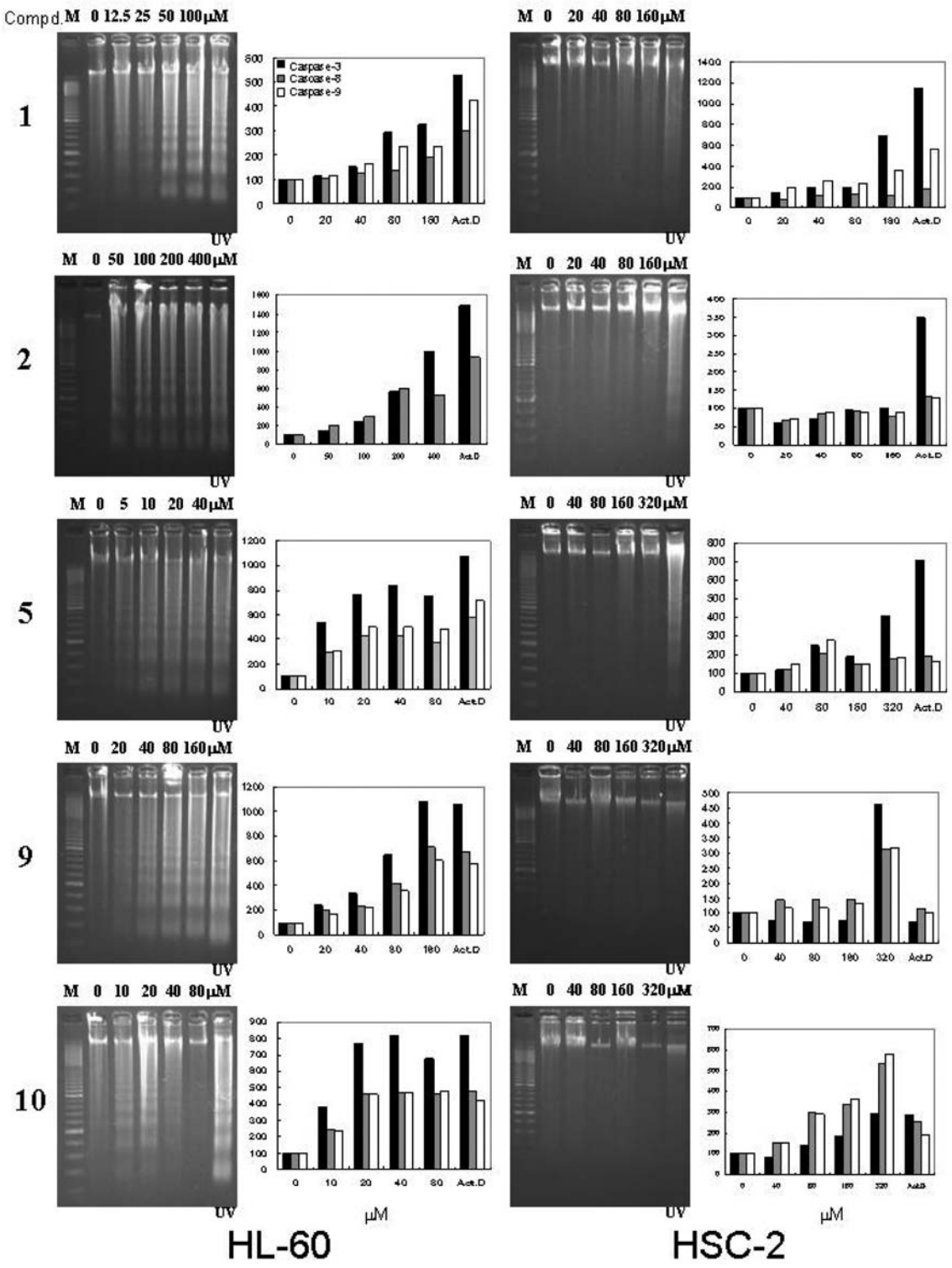


Figure 2. *Induction of internucleosomal DNA fragmentation and caspase activation by flavonoids and stilbenes. HL-60 cells (1 x 10^6/mL) (left two columns) or near confluent HSC-2 cells (right two columns) were incubated for 6 or 4 hours with the indicated concentrations of compounds* ***[1, 2, 5, 9, 10]****. DNA fragmentation and activation of caspases -3, -8 and -9 were then assayed by agarose gel electrophoresis and substrate cleavage assay. The data of capase-9 in* ***[2]****-treated HL-60 cells were omitted due to the fact that the expression of this protein in the control was too low to calculate the relative expression. UV, DNA from the apoptotic HL-60 cells induced by UV irradiation. Act.D., 1 μg/mL actinomycin D*

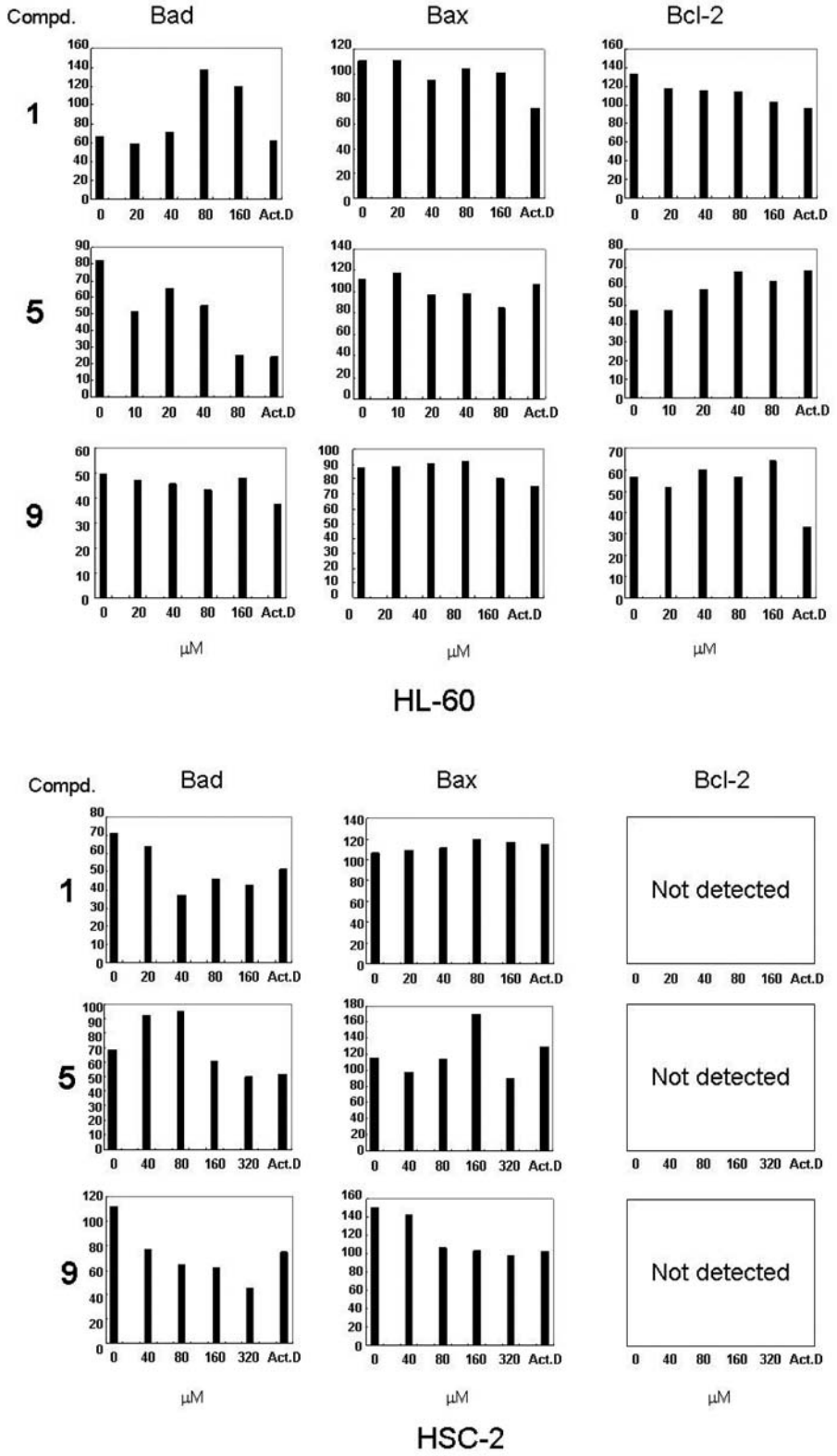


Figure 3. *Effect of compounds **[1, 5, 9]** on the expression of apoptosis-related proteins in HL-60 and HSC-2 cells. HL-60 cells (1 x 10^6/mL) (upper panel) or near confluent HSC-2 cells (lower panel) were incubated for 4 hours with the indicated concentrations of compounds **[1, 5, 9]**, and the expression of Bad, Bax and Bcl-2 proteins was then monitored by Western blot analysis. The expression of each protein was normalized to that of actin and expressed as an arbitrary value. Act.D., 1 μg/mL actinomycin D*

resveratrol [**4**], showed higher tumor-specificity (TS= >3.5) than [**4**]. Rhaponticin [**6**], which has a glucose and methyl group in two different benzene rings, had much lower cytotoxicity (Table I). Resveratrol [**4**] has been reported to induce apoptosis in many tumor cell lines including HL-60 cells (17-19), human acute lymphoma cells (BJAB)(20), human monocytic leukemia THP-1 (21), human colon carcinoma cells (Caco-2, HTC116) (22-24), human breast, colon and prostate cancer cells (25), gastric adenocarcinoma (26), human medulloblastoma cells (Med-3, UW228-1, -2 and -3) (27) and melanoma cells (28). The mechanism of apoptosis induction by resveratrol [**4**] includes the increase of cells in the S-phase of the cell cycle and the decrease of cells in G2/M-phase (25), the inhibition of polyamine synthesis and increased polyamine catabolism (22), the decline of Bcl-2 (18), the involvement of Bax (23), the depolarizing mitochondrial membranes (20), the dependent (19) and independent (21, 24, 27) on CD95-signaling, the phosphorylation of ERK1/2 (28) and the inhibition of protein kinase C activity (26). Piceatannol [**5**]- induced apoptosis in BJAB cells has been reported to be independent of the CD95/Fas signaling pathway (29). Stilbene oligomers such as (-)-ε-viniferin [**3**], gnetin H, suffruticosol B and vaticanol C also induced apoptosis of various tumor cells lines (30-32).

In addition, the present study demonstrated that five flavonoids, kaempferol [**7**], fisetin [**8**], quercetin [**9**], isoliquiritigenin [**10**] and butein [**11**], showed similar tumor-specific cytotoxicity to that of stilbenes [**1-6**] (Table I), suggesting that the stilbene structure is not an important factor in determining tumor-specificity and apoptosis. The tumor-specificity index of kaempferol [**7**] could not be accurately calculated due to much lower cytotoxicity against both normal and tumor cells. Another four compounds [**8-11**] showed comparable cytotoxicity and tumor-specificity (Table I). Quercetin [**9**], a widely distributed natural flavonoid, has shown a variety of biological functions. It [**9**] induced an antiproliferative effect or apoptosis in transformed hepatic cell lines (33), melanoma (34, 35), prostate cancer (36, 37) and breast cancer cells (37), by a mechanism including growth arrest at the S-phase of the cell cycle (36), loss of mitochondrial membrane potential, decline of Bcl-2, inhibition of PKC-alpha expression, translocation of PKC-delta (35) and inhibition of fatty acid synthase activity (37). Quercetin [**9**] showed selective growth inhibition and apoptosis in hepatic tumor cells, but not in normal cells (33), in agreement with its higher tumor-specificity found in this study (TS=>3.3). Quercetin [**9**] enhanced the sensitivity of the K562/ADM and HL-60/ADM cell lines to daunorubicin by restoring the subcellular distribution of daunorubicin (38). Quercetin [**9**] is also a well known antioxidant, scavenging radicals (39), decreasing the production of leukotriene and reactive oxygen species (ROS) (40), inhibiting ROS and reactive nitrogen species (RNS)-caused tissue damage (41) and protecting cells from oxidative stress-induced cell death (42, 43). Fisetin [**8**] and quercetin [**9**] induced apoptosis in human leukemia U937 cells through the activation of caspases -3 and -8 (for [**9**]) and caspases and calpains (for [**8**]), respectively, and sensitized U937 cells to apoptosis induced by tumor necrosis factor (44). The structure-activity relationship showed that at least two hydroxylations in positions 3, 5 or 7 of the A-ring were needed to induce apoptosis, whereas hydroxylation in 3' and/or 4' of the B-ring enhanced pro-apoptotic activity (44). Isoliquiritigenin [**10**] is a natural pigment with the simple chalcone structure 4, 2', 4'-trihydroxychalcone. It [**10**] has been reported to induce apoptosis in various tumor cell lines including human non-small cell lung cancer cells (45, 46), leukemia, melanoma (47, 48), prostate cancer (49) and gastric cancer (50), by mechanisms involving growth arrest at the S- and G2/M-phase (46, 49), expression of GADD153 mRNA and protein associated with cell cycle arrest (49), increase of the intracellular concentration of free calcium (50) and collapse of the mitochondrial transmembrane potential (47, 50), depletion of gluthathione (GSH) and increase of oxidized GSH (47), p53 and the Fas/FasL system (45) and p21 (CIP1/WAF1) (46), down-regulation of Bcl-2 and promotion of Bax expression (48). Isoliquiritigenin [**10**] also inhibited carcinogenesis (51) and pulmonary metastasis of renal cell carcinoma (52) and showed cytoprotective effects against cadmium-induced toxicity (53). However, most of these studies have focused on the mechanism of cell death, without paying attention to their tumor-specificity. Our data demonstrated that the stilbenes [**1-6**] and flavonoids [**7-11**] investigated showed some, though not potent, tumor-specificity (TS=1.8-4.7). Based upon these findings, the optimum concentration of these compounds for each cell must be set so as to minimize the cytotoxicity against normal cells.

HSC-2 cells were found not to express Bcl-2 protein, without or with induction of apoptosis by stilbenes or flavonoids. The relatively higher sensitivity of HSC-2 cells to stilbenes and flavonoids (Table I) may be due to the lower level of expression of Bcl-2. The expression of Bcl-2 is regulated by interaction with the 70-kDa heat-shock protein (54). The lower level of Bcl-2 protein expression in HSC-2 cells may be compensated for by Bcl-X(L) protein expression (55).

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Culture, Education, Science, Sports and Culture of Japan (Sakagami, No.14370607).

References

1 Sakagami H, Jiang Y, Kusama K, Atsumi T, Ueha T, Toguchi M, Iwakura I, Satoh K, Fukai T and Nomura T: Induction of apoptosis by flavones, flavonols (3-hydroxylflavones) and isoprenoid-substituted flavonoids in human oral tumor cell lines. Anticancer Res *20*: 271-278, 2000.

2 Satoh K, Ida Y, Sakagami H, Tanaka T and Fujisawa S: Effect of antioxidants on radical intensity and cytotoxic activity of eugenol. Anticancer Res *18*: 1549-1552, 1998.

3 Nonuma T: *In:* Progress in the Chemistry of Organic Natural Products 53 (Herz W, Gresebach H, Kirby GW, Tamm C, eds.), Springer, pp. 87-201, 1988.

4 Fukai T, Sakagami H, Toguchi M, Takayama F, Iwakura I, Atsumi T, Ueha T, Nakashima H and Nomura T: Cytotoxic activity of low molecular weight polyphenols against human oral tumor cell lines. Anticancer Res *20*: 2525-2536, 2000.

5 Shi Y-Q, Fukai T, Sakagami H, Chang W-J, Yang P-Q, Wang F-P and Nomura T: Cytotoxic flavonoids with isoprenoid groups from *Morus mongolica*. J Nat Prod *64*: 181-188, 2001.

6 Hou A-J, Fukai T, Shimazaki M, Sakagami H, Sun H-D and Nomura T: Benzophenones and xanthones with isoprenoid groups from *Cudrania cochinchinensis*. J Nat Prod *64*: 65-70, 2001.

7 Shi Y-Q, Fukai T, Sakagami H, Kuroda J, Miyaoka R, Tamura M, Yoshida N and Nomura T: Cytotoxic and DNA damage-inducing activities of low molecular weight phenols from rhubarb. Anticancer Res *21*: 2847-2854, 2001.

8 Shirataki Y, Wakae M, Yamamoto Y, Hashimoto K, Satoh K, Ishihara M, Kikuchi H, Nishikawa H, Minagawa K, Motohashi N and Sakagami H: Cytotoxicity and radical modulating activity of isoflavones and isoflavones from *Sophora* species. Anticancer Res *24*: 1481-1488, 2004.

9 Nakayachi T, Yasumoto E, Nakano K, Morshed SRM, Hashimoto K, Kikuchi H, Nishikawa H, Kawase M and Sakagami H: Structure-activity relationships of α, β-unsaturated ketones as assessed by their cytotoxicity against oral tumor cells. Anticancer Res *24*: 737-742, 2004.

10 Yasumoto E, Nakano K, Nakayachi T, Morshed SRM, Hashimoto K, Kikuchi H, Nishikawa H, Kawase M and Sakagami H: Cytotoxic activity of deferiprone, maltol and related hydroxyketones against human tumor cell lines. Anticancer Res *24*: 755-762, 2004.

11 Nakano K, Nakayachi T, Yasumoto E, Morshed SRM, Hashimoto K, Kikuchi H, Nishikawa H, Sugiyama K, Amano O, Kawase M and Sakagami H: Induction of apoptosis by β-diketones in human tumor cells. Anticancer Res *24*: 711-718, 2004.

12 Houritz KT, Bitterman KJ, Cohen HY, Lamming BW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Schera B and Sinclain DA: Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature *425*: 191-196, 2003.

13 Shirataki Y, Tanaka T, Ohyama M, Toda S and Iinuma M: Stilbene oligomers in roots of *Sophora moorcroftiana*. Natural Med *56*: 139-142, 2002.

14 Komatsu M, Yokoe I and Shirataki Y: Studies on the constituents of *Sophora* species. XII. Constituents of the aerial part of *Sophora tomentosa* L. (1). Chem Pharm Bull *26*: 1274-1278, 1978.

15 Heyflick L: The limited *in vitro* lifetime of human diploid cell strains. Exp Cell Res *37*: 614-636, 1965.

16 Kitanaka S, Ikezawa T, Yasukawa K, Yamanouchi S, Takido M, Sung HK and Kim IH: (+)-α-Viniferin, an anti-inflammatory compound from *Caragana chamlagu* root. Chem Pharm Bull *38*: 432-435, 1990.

17 Gautam SC, Xu YX, Dumaguin M, Janakiraman N and Chapman RA: Resveratrol selectively inhibits leukemia cells: a prospective agent for *ex vivo* marrow purging. Bone Marrow Transplant *25*: 639-645, 2000.

18 Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G and Lee SJ: Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Cancer Lett *140*: 1-10, 1999.

19 Clement MV, Hirpara JL, Chawdhury SH and Pervaiz S: Chemopreventive agent, resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. Blood *92*: 996-1002, 1998.

20 Dorris J, Gerauer H, Wachter Y and Zunino SJ: Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. Cancer Res *61*: 4731-4739, 2001.

21 Tsan MF, White JE, Maheshwari JG, Bremner TA and Sacco J: Resveratrol induces Fas signaling-independent apoptosis in THP-1 human monocytic leukaemia cells. Br J Haematol *109*: 405-412, 2000.

22 Wolter F, Turchanowa L and Stein J: Resveratrol-induced modification of polyamine metabolism is accompanied by induction of c-Fos. Carcinogenesis *24*: 469-474, 2003.

23 Mahyar-Roemer M, Kohler H and Roemer K: Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells. BMC Cancer *17*: 27, 2002.

24 Delmac D, Rebe C, Lacour S, Filomenko R, Athias A, Gambert P, Cherkaoui-Malki M, Jannin B, Dubrez-Daloz L, Latruffe N and Solary N: Resveratrol-induced apotosis is associated with Fas redistribution in the rafts and the formation of a death-inducing complex. J Biol Chem *278*: 41482-41490, 2003.

25 Sgambato A, Ardito R, Faraglia B, Boninsegna A, Wolf FI and Cittadini A: Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. Mutat Res *20*: 171-180, 2001.

26 Atten MJ, Attar BM, Milson T and Holian O: Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism. Biochem Pharmacol *62*: 1423-1432, 2001.

27 Wang Q, Li H, Wang XW, Wu DG, Chen XY and Liu J: Resveratrol promotes differentiation and induces Fas-independent apoptosis of human medulloblastoma cells. Neurosci Lett *351*: 83-86, 2003.

28 Niles RM, McFarland M, Weimer MB, Redkar A, Fu YM and Meadows GG: Resveratrol is a potent inducer of apoptosis in human melanoma cells. Cancer Lett *190*: 157-163, 2003.

29 Wieder T, Prokop A, Bagci B, Essmann F, Bernicke D, Schulze-Osthoff K, Dorken B, Schmalz HG, Daniel PT and Henze G: Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. Leukemia *15*: 1735-1742, 2001.

30 Kang JH, Park YH, Choi SW, Yang EK and Lee WJ: Resveratrol derivatives potently induce apoptosis in human promyelocytic leukemia cells. Exp Mol Med *35*: 467-474, 2003.
31 Ito T, Akao Y, Yi H, Ohguchi K, Matsumoto K, Tanaka T, Iinuma M and Nozawa Y: Antitumor effect of resveratrol oligomers against human cancer cell lines and the molecular mechanism of apoptosis induced by vaticanol. Carcinogenesis *24*: 1489-1497, 2003.
32 Kim HJ, Chang EJ, Bae SJ, Shim SM, Park HD, Rhee CH, Park JH and Choi SW: Cytotoxic and antimutagenic stibenes from seeds of *Paeonia lactiflora*. Arch Pharm Res *25*: 293-299, 2002.
33 Son YO, Lee KY, Kook SH, Lee JC, Kim JG, Jeon YM and Jang YS: Selective effects of quercetin on the cell growth and antioxidant defense system in normal *versus* transformed mouse hepatic cell lines. Eur J Pharmacol *502*: 195-204, 2004.
34 Yanez J, Vicente V, Alcaraz M, Castillo J, Benavente-Garcia O, Canteras M and Teruel JA: Cytotoxicity and antiproliferative activities of several phenolic compounds against three melanocytes cell lines: relationship between structure and activity. Nutr Cancer *49*: 191-199, 2004.
35 Zhang XM, Chen J, Xia YG and Xu Q: Apoptosis of murine melanoma B16-BL6 cells induced by quercetin targeting mitochondria, inhibiting expression of PKC-alpha and translocating PKC-delta. Cancer Chemother Pharmacol *55*: 251-262, 2005.
36 Shenouda NS, Zhou C, Browning JD, Ansell PJ, Sakla MS, Lubahn DB and Macdonald RS: Phytoestrogens in common herbs regulate prostate cancer cell growth *in vitro*. Nutr Cancer *49*: 200-208, 2004.
37 Brusselmans K, Vrolix R, Verhoeven G and Swinnen JV: Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem *18*: 5636-5645, 2005.
38 Cai X, Chen FY, Han JY, Gu CH, Zhong H and Quyang RR: Restorative effect of quercetin on subcellular distribution of daunorubicin in multidrug resistant leukemia cell lines K562/ADM (in Chinese). Ai Zheng *23*: 1611-1615, 2004.
39 Rajendron M, Manisankar P, Gandhidasan R and Murugesan R: Free radical scavenging efficiency of a few naturally occurring flavonoids: a comparative study. J Agric Food Chem *52*: 7389-7394, 2004.
40 Young JF, Hansen-Moller J and Oksbjerg N: Effect of flavonoids on stress response in myotube culture. J Agric Food Chem *52*: 7158-7163, 2004.
41 Zhao Y, Gao Z, Li H and Xu H: Hemin/nitric/H_2O_2 induces brain homogenate oxidation and nitration: effects of some flavonoids. Biochim Biophys Acta *1675*: 105-112, 2004.
42 Echeverry C, Blasina F, Arredondo F, Ferrira M, Abin-Carriquiry JA, Vasquez L, Aspillaga AA, Diez MS, Leighton F and Dajas F: Cytoprotection by neutral fraction of tannat red wine against oxidative stress-induced cell death. J Agric Food Chem *52*: 7395-7399, 2004.
43 Kabakov AE, Budagova KR, Malyutina YU, Latchman DS and Csermely P: Pharmacological attenuation of apoptosis in reoxygenated endothelial cells. Cell Mol Life Sci *61*: 3076-3086, 2004.
44 Monasterio A, Urdaci MC, Pinchuk IV, Lopez-Moratalla N and Martinez-Irujo JJ: Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase-calpain-dependent pathways. Nutr Cancer *50*: 90-100, 2004.
45 Hsu YL, Kuo PL, Chiang LC and Lin CC: Isoliquiritigenin inhibits the proliferation and induces the apoptosis of human non-small cell lung cancer A549 cells. Clin Exp Pharmacol Physiol *31*: 414-418, 2004.
46 Li T, Satome Y, Katoh D, Shimada J, Baba M, Okuyama T, Nishino H and Kitamura N: Induction of cell cycle arrest and p21 (CIP1/WAF1) expression in human lung cancer cells by isoliquiritigenin. Cancer Lett *207*: 27-35, 2004.
47 Sabzevari O, Galati G, Moridani MY, Siraki A and O'Brien PJ: Molecular cytotoxic mechanisms of anticancer hydroxychalcones. Chem Bio Interact *148*: 57-67, 2004.
48 Iwashita K, Kobori M, Yamaki K and Tsushida T: Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. Biosci Biotechnol Biochem *64*: 1813-1820, 2000.
49 Kanazawa M, Satomi Y, Mizutani Y, Ukimura O, Kawauchi A, Sakai T, Baba M, Okuyama T, Nishio H and Miki T: Isoliquiritigenin inhibits the growth of prostate cancer. Eur Urol *43*: 580-586, 2003.
50 Ma J, Fu NY, Pang DB, Wu WY and Xu AL: Apoptosis induced by isoliquiritigenin in human gastric cancer MGC-803 cells. Planta Med *67*: 754-757, 2001.
51 Kinghorn AD, Su BN, Jang DS, Chang LC, Lee D, Gu JQ, Carcache-Blanco EJ, Pawlus AD, Lee SK, Park EJ, Cuendet M, Gills JJ, Bhat K, Park HS, Mata-Greenwood E, Song LL, Jang M and Pezzuto JM: Natural inhibitors of carcinogenesis. Planta Med *70*: 691-705, 2004.
52 Yamazaki S, Morita T, Endo H, Hamamoto T, Baba M, Joichi Y, Kaneko S, Okada Y, Okuyama T, Nishino H and Tokue A: Isoliquiritigenin suppresses pulmonary metastasis of mouse renal cell carcinoma. Cancer Lett *83*: 23-30, 2002.
53 Kim SC, Byun SH, Yang CH, Kim CY, Kim JM and Kim SG: Cytoprotective effects of *Glycyrrhizae radix* extract and its active component isoliquiritigenin against cadmium-induced toxicity (effects on bad translocation and cytochrome c-mediated PARP cleavage). Toxicology *197*: 239-251, 2004.
54 Kaur J, Kaur J and Ralhan R: Induction of apoptosis by abrogation of HSP70 expression in human oral cancer cells. Int J Cancer *85*: 1-5, 2000.
55 Otani Y, Tsutsumi K, Kuwahara D, Oyake D, Ohta T, Nishikawa H and Koizuka I: Sensitization of head and neck squamous cell carcinoma cells to Fas-mediated apoptosis by the inhibition of Bcl-X(L) expression. Airos Nasus Larynx *30(suppl)*: S79-84, 2003.

Received January 17, 2005
Accepted April 6, 2005