Relationship between Cytotoxic Activity and Radical Intensity of Isoflavones from *Sophora* Species

YOSHIAKI SHIRATAKI[1], SATORU TANI[1], HIROSHI SAKAGAMI[2], KAZUE SATOH[3], HIDEKI NAKASHIMA[4], KAZUYO GOTOH[4] and NOBORU MOTOHASHI[5]

[1]*Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan;* [2]*Department of Dental Pharmacology, Meikai University School of Dentistry, Saitama, Japan;* [3]*Analysis Center, School of Pharmaceutical Sciences, Showa University, Tokyo, Japan;* [4]*Department of Microbiology and Immunology, Kagoshima University Dental School, Kagoshima-shi, Kagoshima, Japan;* [5]*Meiji Pharmaceutical University, Kiyose, Tokyo, Japan*

Abstract. *Among 11 isoflavones tested, genistein [**YS13**] produced higher cytotoxic activity against human oral tumor cell lines (HSC-2, HSG) than against normal cells (human gingival fibroblast, HGF), suggesting its tumor-specific action. Electron spin resonance (ESR) spectroscopy showed that **YS13** did not produce radical, nor scavenged $O_2^{\bullet -}$, generated by hypoxanthine-xanthine oxidase reaction system, suggesting that radical-mediated oxidation mechanism is not be involved in the **YS13**-induced cytotoxicity. Addition of one prenyl group produced **YS18** and **YS19** with higher anti-Helicobacter pylori activity. Addition of two prenyl groups produced **YS21** with the highest cytotoxic activity but lower tumor-specificity. Since **YS21** produced the highest amount of radical and most efficiently scavenged $O_2^{\bullet -}$, this compound may induce cytotoxicity by radical-mediated oxidation mechanism. All isoflavones failed to induce anti-human immunodeficiency virus (HIV) activity. These data suggest the medicinal efficacy of isoflavones.*

We have suggested the usefulness of polyphenols (tannins, flavonoids, lignins) for prevention of oral diseases (1). Millimolar concentrations of tannin-related compounds (epigallocatechin gallate (EGCG), gallic acid, hydrolyzable tannins) induced apoptotic cell death (characterized by DNA fragmentation and caspase activation) in human leukemic (2) and oral tumor cell lines (3, 4). We have recently found that flavonoids induced apoptosis in human oral tumor cell lines at slightly lower concentrations (5, 6). On the other hand, lignins showed much lower cytotoxic activity, but significantly enhanced the cytotoxic activity of ascorbate and vitamin K_3 (7, 8).

The *Sophora* species contains different type of flavonoids including isoflavones (9). Flavonoids are medicinally important, since they have shown diverse biological activities such as antioxidant (10-12), anticarcinogenic (13), antimutagenic (14), anti-inflammatory (15-17), antiviral (11), immunosuppressing (11, 16), nitric oxide-producing (18), differentiation-inducing (19) and anticancer (17, 20) activity.

We have recently found that the antioxidative activity of isoflavones, one of the flavonoids, is superior or similar to those of butyl hydroxyltoluenes (21). The antioxidant activities of isoflavones depend on the relation between their chemical structures and reactive oxygen species (22). The prenylflavanones had antitumor, antimicrobial and anti-human immunodeficiency virus (HIV) activity and we found the relationship between radical generation and radical scavenging activity in prenylflavanones (23). In continuation with previous studies, we further investigated the relationship between isoflavones and their bioactivity.

Correspondence to: Dr. Yoshiaki Shirataki, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan. Tel & Fax: (+)-81-49-271-7053; e-mail: <shiratak@josai.ac.jp>

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Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RPMI1640 medium, Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chem Ind., Ltd., Osaka, Japan); 3'-azido-2',3'-dideoxythymidine (AZT), dideoxycytidine (ddC) (Sigma); dextran sulfate (8 kD) (Kowa, Tokyo); diethylenetriaminepentaacetic acid (DETAPAC), gallic acid (Sigma Chem. Co., St. Louis, MO, USA); 3,4-dihydro-2,2-dimethyl-2*H*-pyrrole-1-oxide (DMPO) (a spin trap agent) (Aldrich Chemical Co. In, U.S.A.); superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan); Daidzein [**YS11**] (Tokyo Kasei Organic Chemicals, Tokyo, Japan). Formononetin [**YS12**] (24), genistein [**YS13**] (25), biochanin A [**YS14**] (26), irisolidone [**YS15**] (26), calycosin [YS16] (27), 7,4'-dihydroxy-3'-methoxyisoflavone [**YS17**] (28), licoisoflavone A [**YS18**] (29), sophoraisoflavone A [**YS19**] (27), licoisoflavone B [**YS20**] (27) and 6,8-diprenylgenistein [**YS21**] (30) were purified and characterized as described (Figure 1). A strain of *Helicobacter pylori* (ATCC43504) was purchased from the American Type Culture Collection (Rockville, MD, USA).

Assay for cytotoxic activity. Human squamous cell carcinoma (HSC-2), human salivary gland tumor (HSG) and human gingival fibroblasts (HGF) (5-7 population doubling levels) were cultured in DMEM

Table I. *Diverse biological activity of isoflavones [YS11-YS21].*

Compd	Cytotoxic actlvity[1] (CC_{50} μg/ml)				Anti--HIV activity			Anti-*H.pylori* actlvity	Radical generation (at pH10.5)	O_2^- scavenging activity[2]
	Human oral tumor cell line		Human gingival fibroblast (HGF)	SI (HGF/HSC-2)	CC_{50} (μg/mL)	EC_{50} (μg/mL)	SI (CC_{50}/ EC_{50})	MIC_{50} (μg/mL)[3]		SOD (U/1.5 mg)
	HSC-2	HSG								
YS11	114	177	625	5.5	>200	>200	><1	>100	<0-03	0-13
YS12	596	584	726	1.2	>200	>200	><1	>100	<0.03	0.51
YS13	31	54	401	12.9	9	>40	<1	>100	<0.03	0.75
YS14	28	125	165	5.9	24	>40	<1	>100	<0.03	0.78
YS15	199	233	529	2.7	>200	>200	><1	>100	<0.03	0.61
YS16	179	142	206	1.2	104	>200	<1	>100	0.13	7.10
YS17	500	524	583	1.2	>200	>2.00	><1	>100	<0.03	1.10
YS18	25	31	38	1.5	39	>40	<1	33	<0.03	1.30
YS19	26	26	41	1.6	41	>200	<1	55	<0.03	2.60
YS20	11	19	20	1.8	5	8	<1	>100	<0.03	1.50
YS2l	10	13	15	1.5	14	>40	<1	>100	0.30	14.60
Dextran sulfate					>1000	4.14	>242			
Curdlan sulfate					>1000	0.72	>1396			
AZT (μM)					276	0.03	8291			
ddC (μM)					475	0.16	3012			
Clarithromycin								10		

1) Near confluent HSC-2 HSG and HGF cells were incubated for 24 hours with various concentrations of each compound and the relative viable cell number (A_{540}) was determined by MTT method. Each value represents mean from duplicate determinations. Control (A_{540}) values of HSC-2 HSG and HGF cells were 0.97, 0.54 and 0.31, respectively. 2) ESR condition:see in text. 3) The MIC value was determined for each compound by a broth microdilution method using *H. pylori* (ATCC43504) by the following literatures (32 33).

medium supplemented with 10% heat-inactivated FBS. These cells were incubated for 24 hours with the indicated concentrations of test samples. The relative viable cell number (absorbance at 540 nm (A_{540})) was then determined by MTT assay. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (Table I) (4).

Assay for anti-HIV activity (31). Human T cell leukemia virus 1 (HTLV1)-bearing CD4 positive human T cell lines, MT-4 cells, were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or MOCK-infected MT-4 cells (1.5 x 10^5/mL, 200 μL) were placed into 96-well microtiter plates and incubated in the presence of various concentrations of the fractions in RPMI1640 medium supplemented with 10% FBS. After incubation for 5 days at 37°C in a 5% CO_2 incubator, cell viability was quantified by a colorimetric assay (at 540 nm and 690 nm), monitoring the ability of viable cells to reduce MTT to a blue formazan product. All data represent the mean values of triplicate measurements. CC_{50} was determined with MOCK-infected cells, whereas EC_{50} was determined with HIV-infected cells. The selectivity index (SI) was defined as follows: $SI=CC_{50}/EC_{50}$ (Table I) (31).

Anti-Helicobacter pylori activity. A strain of *Helicobacter pylori* (ATCC43504) was purchased from the American Type Culture Collection (Rockville, MD, USA). Mueller-Hilton broth containing 5% FBS was used as the medium and was cultured in a jar conditioned with Campylo Pack (Dia Iatron) for 48 hours. Briefly, *H. pylori* strains were inoculated on a *Brucella* agar plate containing 10% horse serum, and cultured at 37°C for 48 hours. The bacterial colonies collected were diluted to 10^7 colony forming units (CFU)/mL with 0.9% saline. The extracts were dissolved in DMSO and then diluted with Mueller-Hilton broth. To the solution of the extracts, each bacterial suspension was added to a density of 10^6 colony forming units (CFU)/100 mL/well. The mixture was incubated at 37°C for 48 hours. The minimum inhibitory concentration (MIC) of each fraction was calculated from the dose-response curve (32, 33) (Table I).

Assay for radical intensity. Radical intensity was determined at 25°C (RT) using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 335.6 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630 time constant, 0.1 seconds; scanning time, 2 minutes. Radical intensity was determined in 0.1M $NaHCO_3/Na_2CO_3$ buffer (pH 10.5) containing 50% DMSO. The final concentration of compound was 3 mg/mL and the radical intensity was defined as the ratio of peak heights of these radicals to that of MnO (Figure 2) (34).

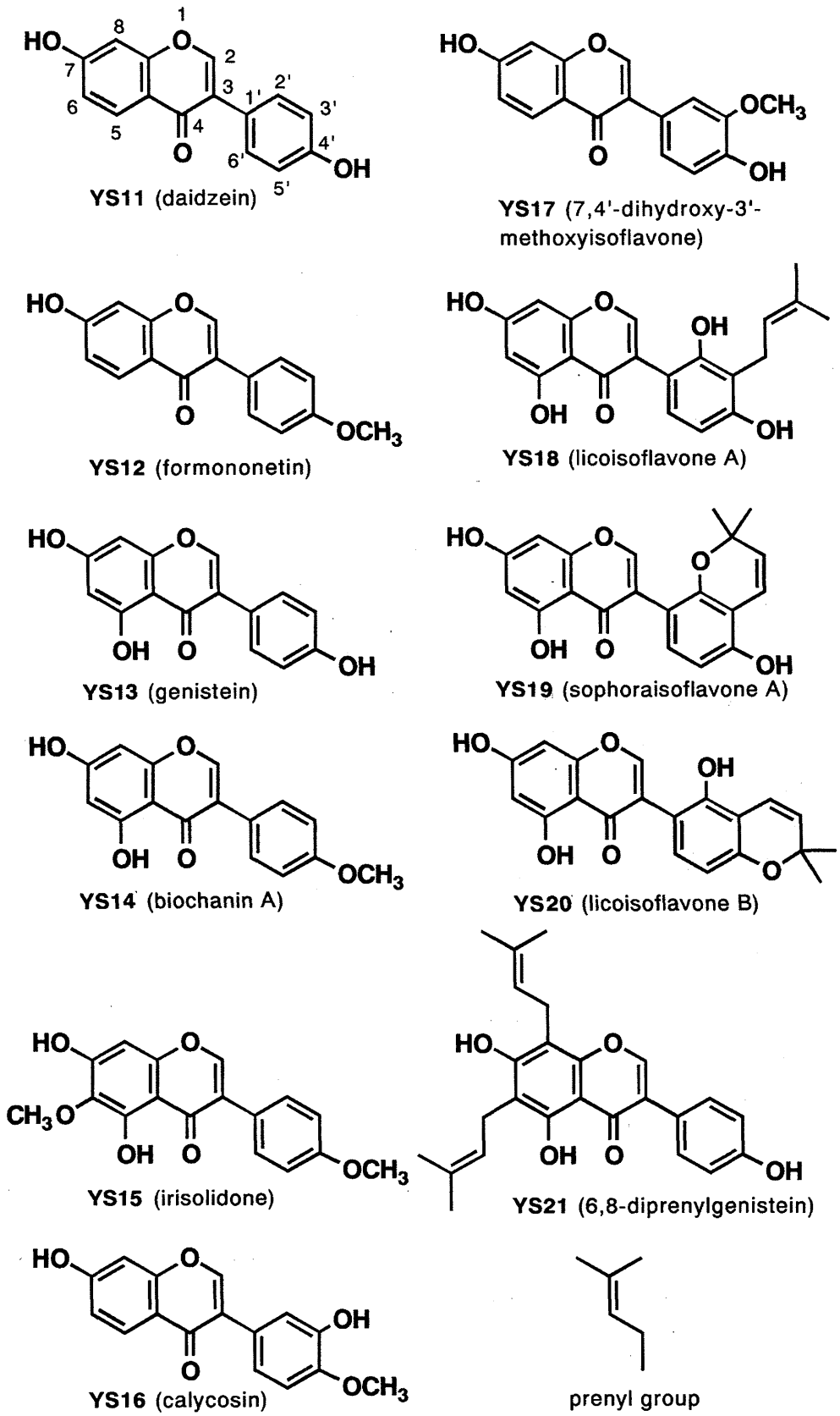

Figure 1. *Structures of isoflavones [YS11-YS21].*

Superoxide anion ($O_2\bullet^-$) scavenging activity. A superoxide anion radical ($O_2\bullet^-$) was generated by hypoxanthine (HX) and xanthine oxidase (XOD) system (200 μL) [2 mM HX (in 0.1 M phosphate buffer, pH 7.8) (PB) 50 μL, 0.5 μM DETAPAC 20 μL, DMPO 10 μL, sample (in DMSO) 50 μL, H_2O or SOD 30 μL, SOD (0.5 U/mL in 0.1M PB) 40 μL]. After 1 minutes, the measurement was begun. The gain was changed to 250. $O_2\bullet^-$ scavenging activity was expressed as SOD unit/1.5 mg sample (Table I) (35).

Results

Structure-activity relationship. We first compared the cytotoxic activity and tumor specificity (as measured by CC_{50} against HGF/CC_{50} against HSC-2 (SI)) of 11 isoflavones (Table I). Daidzein [**YS11**] and formononetin [**YS12**] (OH at C-4′ position were replaced with OCH_3 in **YS11**) showed weak cytotoxicity against human oral tumor cell lines (HSC-2, HSG) (CC_{50}=114, 117; 596, 584 μg/mL), but much weaker cytotoxicity against normal cells (HGF) (CC_{50}=625, 726 μg/mL), suggesting their tumor-specific action (SI=5.5, 1.1) (Table I). Addition of the OH group to C-5 of **YS11** or **YS12** produced genistein [**YS13**] and biochanin A [**YS14**], which showed higher cytotoxic activity (CC_{50}=31, 54; 26, 125 μg/mL) and tumor specificity (SI=12.9, 5.9). Further addition of OCH_3 group to C-6 of **YS14** produced irisolidone [**YS15**], which showed reduced cytotoxic activity (CC_{50}=199, 233 μg/mL). On the other hand, the addition of OH or OCH_3 groups to C-3′ of **YS12** or **YS11** produced calycosin [**YS16**] (CC_{50}=179, 142 μg/mL) and 7,4′-dihydroxy-3′-methoxyisoflavone [**YS17**] (CC_{50}=500, 524 μg/mL), which showed comparable activity with **YS11** or **YS12**, respectively. These data suggest that **YS13** or **YS14** might be the minimum structure required for production of higher cytotoxicity and tumor-specificity. Further addition of one or two prenyl groups produced licoisoflavone A [**YS18**] and 6,8-diprenylgenistein [**YS21**], which have slightly higher cytotoxicity (CC_{50}=25, 31; 10, 13 μg/mL), but reduced tumor specificity (SI=1.5, 1.5). An addition of 2,2-dimethyl-chromene ring to 5,7,4′ or 2′-trihydroxyisoflavone produced sophoraisoflavone A [**YS19**] or licoisoflavone B [**YS20**], which had also higher cytotoxicity (CC_{50}=26, 26; 11, 19 μg/mL), but reduced tumor specificity (SI=1.6, 1.8) (Table I).

Anti-human immunodeficiency virus (HIV) activity. All isoflavones [**YS11-YS21**] showed no apparent anti-HIV activity (SI<1), in contrast to popular anti-HIV agents, such as dextran sulfate (SI=154), curdlan sulfate (SI>1396), AZT (SI=8291) and ddC (SI=3012) (Table I).

Anti-Helicobacter pyroli activity. **YS18** and **YS19** showed slightly weaker anti-*H. pylori* activity (CC_{50}=33 and 55 μg/mL, respectively) than clarithromycin (CC_{50}=10 μg/mL). On the other hand, other isoflavones were inactive (Table I).

Radical generation and $O_2\bullet^-$ scavenging activity. ESR spectroscopy demonstrates that **YS16** and **YS21** produced radical(s) under alkaline condition (pH 10.5), whereas other isoflavones produced no detectable radical (Figure 2).

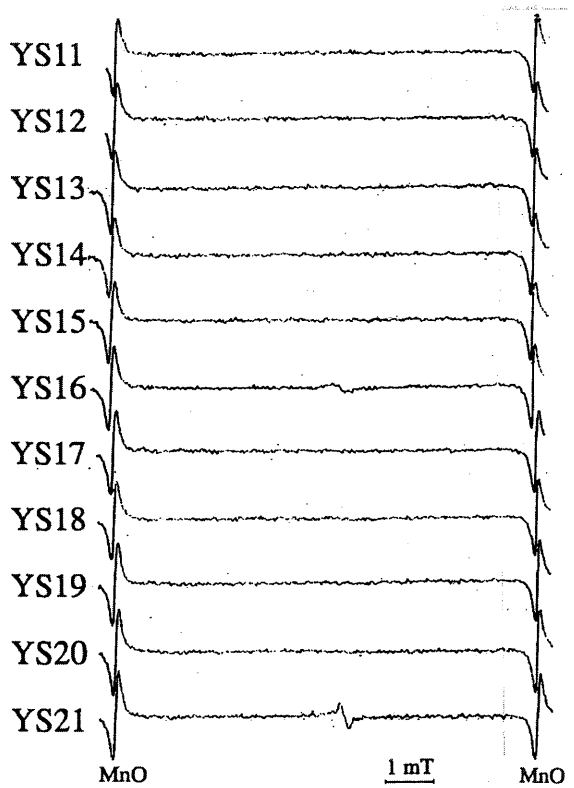

Figure 2. *ESR spectra of 11 isoflavones (3 mg/mL) in 0.1M $NaHCO_3/Na_2CO_3$ (pH10.5).*

YS21 scavenged most efficiently $O_2\bullet^-$, produced hypoxanthine-xanthine oxidase reaction system. Titration with superoxide dismutase (SOD) standard demonstrated that **YS21** had the highest SOD activity (146 SOD unit/1.5 mg), followed by **YS16** (7.1 SOD unit/1.5 mg) (Table I). Other isoflavones had much reduced SOD activity (<2.5 SOD unit/1.5 mg). When radical intensity was plotted as a function of SOD activity, a positive relationship was found between these two parameters (Figure 3).

Discussion

The present study demonstrated that among 11 isoflavones, genistein [**YS13**] showed the highest tumor-specific cytotoxic activity (SI=12.9). Since this genistein [**YS13**] did not show detectable radical production nor $O_2\bullet^-$ scavenging activity, **YS13** might induce cytotoxicity by non-oxidation mechanism. On the other hand, the addition of two isoprenyl groups to **YS13** produced 6,8-diprenylgenistein [**YS21**], which showed much higher cytotoxic activity than **YS13**, but lesser tumor-specificity. Of note, **YS21** produced the highest amount of radical, suggesting that **YS21** might produce cytotoxicity by a

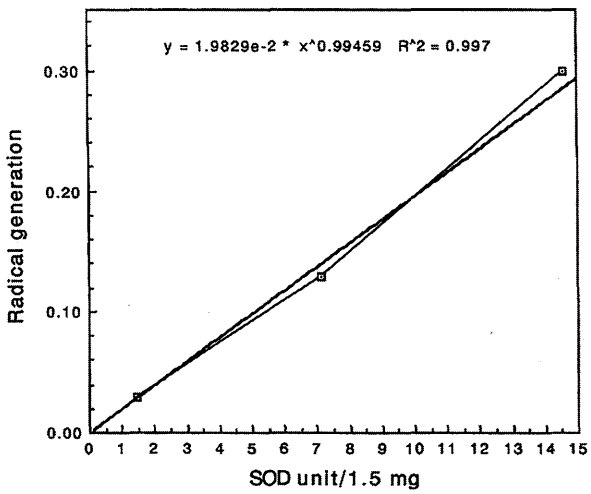

Figure 3. *Relationship between radical generation (at pH 10.5) and* $O_2^{\bullet -}$ *scavenging (SOD) activity (at pH 7.8).*

radical-mediated oxidation mechanism. Therefore, combination with other compounds which show tumor-specificity, such as ascorbate, benzaldehyde, epigallocatechin gallate, macrocyclic ellagitannins, curcumin, dopamin, flavonoids, steroidal saponins (4, 5, 36, 37) might furhter potentiate the cytotoxic activity of **YS21**.

We also found that **YS21** not only produced the highest amount of radicals, but showed the highest $O_2^{\bullet -}$ scavenging activity. This suggests that **YS21** acts as both an antioxidant and a prooxidants, depending on the condition where **YS21** is present. We have previously reported that sodium ascorbate (38) and vitamin K_3 (39) displayed similar bimodal action. The mechanism by which these isoflavones switch antioxidant action to prooxidant action and vice versa remains to be investigated.

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