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Cytotoxicty and Radical Modulating Activity of Isoflavones and Isoflavanones from *Sophora* Species

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Abstract. *We investigated 2 isoflavones and 9 isoflavanones from Sophora species for their cytotoxic activity against 3 normal human cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast) and 2 human tumor cell lines (squamous cell carcinoma HSC-2, submandibular gland carcinoma HSG). Compounds with 2 isoprenyl groups (one in A-ring and the other in B-ring) such as tetrapterol G [YS31] and isosophoranone [YS24], and those with α, α-dimethylallyl group at C-5' of B-ring [YS26 (secundifloran), YS27 (secundiflorol A), YS28 (secundiflorol D), YS29 (secundiflorol E)] showed relatively higher cytotoxic activity. When hydrophobicity was assessed by octanol-water partition coefficient (log P), the maximum cytotoxic activity was observed at a log P value around 4. Compounds with intermediate cytotoxic activity [YS27, genistein, YS28, YS29, YS30 (secundiflorol F)] showed relatively higher tumor specificity. All isoflavones and isoflavanones did not stimulate the nitric oxide (NO) production by mouse macrophage-like Raw 264.7 cells, but almost completely inhibited the NO production by lipopolysaccharide (LPS)-activated Raw 264.7 cells. ESR spectroscopy showed that YS26 and YS28, which are the most inhibitory for NO production, efficiently scavenged superoxide anion and NO radicals. These data suggest that the inhibition of macrophage NO production by these isoflavanones may, at least in part, be explained by their radical scavenging or reduction activity.*

Natural polyphenols such as lignins, tannins and flavonoids have displayed diverse biological activities (1, 2). Lignins with higher molecular weight showed lower cytotoxicity, but synergistically enhanced the cytotoxic activity of vitamin C (3) and vitamin K (4). On the other hand, lower molecular components such as hydrolyzable tannins (5) and prenylflavonoids (6-8) directly displayed higher cytotoxicity and induced apoptotic cell death characterized by DNA fragmentation and caspase activation, in cultured human oral tumor cell lines when compared with lignins. We have recently found that lignin stimulated the production of nitric oxide (NO) by mouse macrophage-like cells Raw 264.7 (9), whereas flavanones and prenylflavanones apparently inhibited the NO production by lipopolysaccharide (LPS)-activated Raw 264.7 cells (10, 11), suggesting that these three distinct groups of polyphenols might modify the function of macrophages in different fashions. To clarify the generality of our findings, we investigated here the cytotoxic activity of 2 isoflavones (daidzein, genistein) and 9 isoflavanones [YS23 (sophoronol), YS24 (isosophoranone), YS25 (sophoraisoflavanone A), YS26 (secundifloran), YS27 (secundiflorol A), YS28 (secundiflorol D), YS29 (secundiflorol E), YS30 (secundiflorol F), YS31 (tetrapterol G) (12-19)] from *Sophora* species against 3 normal human cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] and 2 human tumor cell lines (squamous cell carcinoma HSC-2, submandibular gland carcinoma HSG). We also investigated the relationship between their cytotoxic activity and lipophilicity assessed by octanol-water partition coefficient (log P), and the possible radical scavenging activity, which may reduce the extracellular concentration of NO, with ESR spectroscopy.

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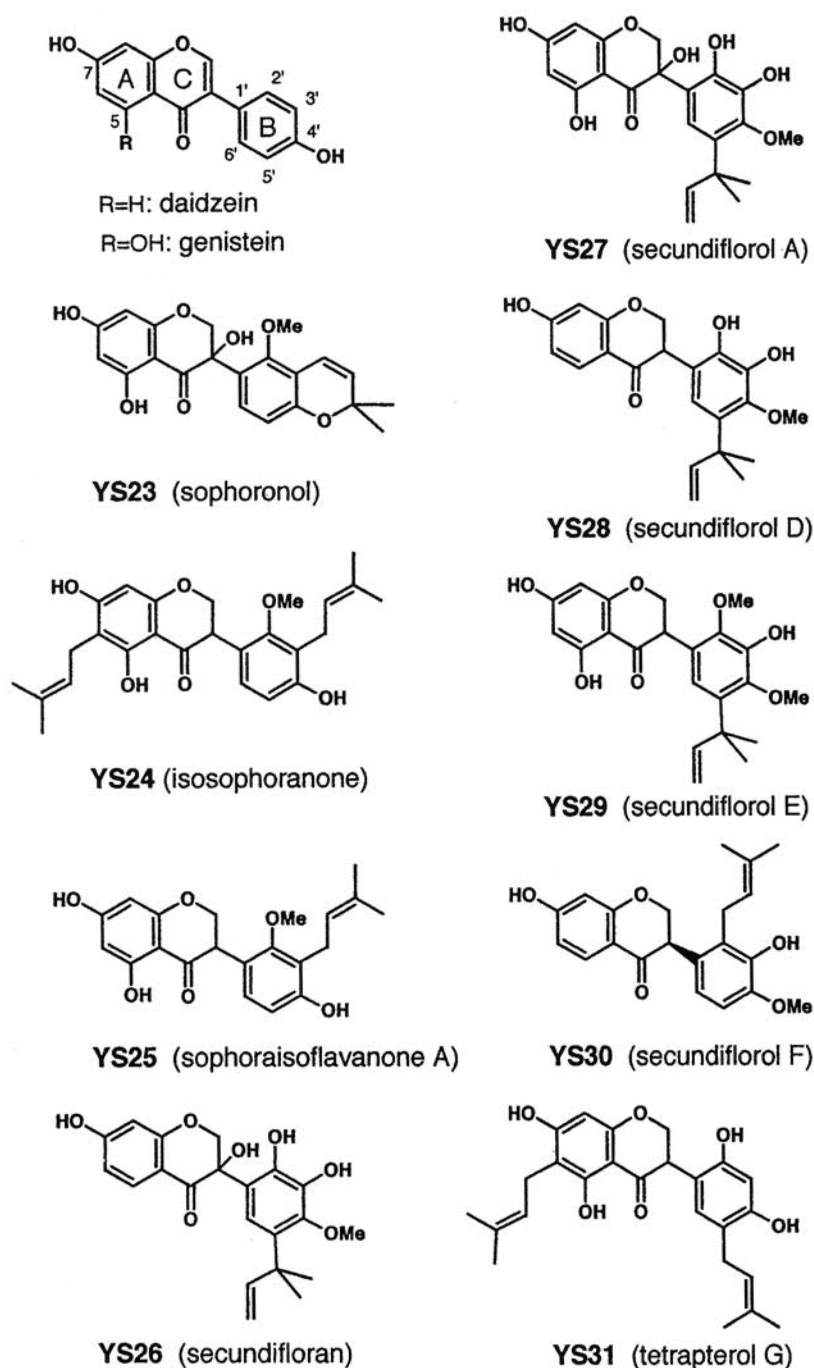

Figure 1. *Structure of isoflavones and isoflavanones.*

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), phenol red-free DMEM (Gibco BRL, NY, USA); fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), LPS from *E. coli* (Serotype 0111: B4), daidzein ($C_{15}H_{10}O_4$=254.23), genistein ($C_{15}H_{10}O_5$=270.23) (Sigma Chem. Co., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind. Ltd., Osaka, Japan); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) (NO generator) (Dojin, Kumamoto, Japan).

Preparation of isoflavanones. YS23 ($C_{21}H_{20}O_7$=384.39) (12-14), YS24 ($C_{26}H_{30}O_6$=438.52) (13, 15), YS25 ($C_{21}H_{22}O_6$=370.40) (13), YS26 ($C_{21}H_{22}O_7$=386.40) (16, 17), YS27 ($C_{21}H_{22}O_8$=402.40) (17), YS28 ($C_{21}H_{22}O_6$=370.40) (18), YS29 ($C_{22}H_{24}O_7$=400.43) (18), YS30 ($C_{21}H_{22}O_5$=354.40) (18) and YS31 ($C_{25}H_{28}O_6$=424.49) (19) were prepared, as described in the literature cited above, and their structures are shown in Figure 1. Purifications of these compounds (YS23-31) was performed by TLC (Merck: Silica gel 60F254; solvent: benzene : EtOAc=1:1) and HPLC apparatus (Waters: 600-MI-UV system; column: symmetry C_{18} 5 μm 4.6 x 150 mm; Mobile phase: MeOH:H_2O=4:1; flow rate: 0.4 mL/min; pressure: 600 psi; detector: UV254 nm; temperature: 23˚C). All isoflavones and isoflavanones were preserved at 4˚C.

Cell culture. Mouse macrophage-like Raw 264.7 cells (20), human tumor cell lines (HSC-2, HSG) and human normal cells (HGF, HPC, HPLF) (5-7 population doubling level (PDL)) were cultured in DMEM supplemented with 10% heat-inactivated FBS, under a humidified 5% CO_2 atmosphere. These HGF, HPC and HPLF cells were isolated from the gingival biopsies from a 10-year-old girl, according to the Guideline of Meikai University Ethics Committee, after obtaining the informed consent of the patient.

Determination of cytotoxic activity. Near confluent Raw 264.7 cells were treated for 24 hours with various concentrations of test compounds. Cells were washed once and incubated with 0.1 mL of fresh medium containing 0.2 mg/mL MTT. The cells were incubated for 4 hours at 37˚C. After removal of the medium, the cells were lysed with 0.1 mL of DMSO. The absorbance at 540 nm (A_{540}) of the lysate, which reflects the relative viable cell number, was then determined and the 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (5-7).

Determination of NO concentration. Near confluent Raw 264.7 cells were incubated for 24 hours with each test compound in phenol red-free DMEM supplemented with 10% FBS and the extracellular concentration of NO produced by Raw 264.7 cells was quantified with Griess reagent (Molecular Probes Inc., Netherlands), using the standard curve of NO_2^- (21).

Assay for radical intensity. The radical intensity of the test sample was determined at 25˚C in 0.1 M Tris-HCl buffer (pH 7.4, 8.0), 0.1 M $NaHCO_3$/Na_2CO_3 buffer (pH 9, 10) or in 0.1 M KOH (pH 12.5), using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.03 seconds; scanning time, 2 minutes. The radical intensity was defined as the ratio of peak height of these radicals to that of MnO (21).

To determine the concentration of O_2^-, produced by HX-XOD reaction (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, sample (in DMSO) 40 μL, SOD or H_2O 30 μL, XOD (0.5 U/mL in PB) 30 μL], the gain, time constant and scanning time were changed to 320, 0.1 seconds and 2 minutes, respectively. The radical intensity was determined 1 minute after mixing. The O_2^- scavenging activity was expressed as SOD unit/mg sample, by calibration with the standard curve of SOD (21).

For the determination of NO radical scavenging activity, sample was added to the reaction mixture of 20 μM carboxy-PTIO and 50 μM NOC-7 (NO generator) in 0.12 M phosphate buffer, pH 7.4. Three minutes later, the radical intensity of the first peak of carboxy-PTI (second peak indicated by symbols in Figure 6: the first peak is not derived from carboxy-PTI), which was produced by the reaction of NO (derived from NOC-7) and C-PTIO (21), was determined (microwave power: 5 mW; gain: 125; scanning time: 2 minutes). The production of carboxy-PTI from carboxy-PTIO and NO proceeds with incubation time and requires 10 minutes for completion (22). If the added sample has NO scavenging activity, the intensity of the first peak of carboxy-PTI should be decreased. To determine the general reduction activity, the sample was added to the carboxy-PTI solution (which was produced by preincubation for 10 minutes with carboxy-PTIO and NOC-7) and 3 minutes later the radical intensity was determined.

Computational details. Theoretical calculations were carried out, using the restricted Hartree-Fock level (HPL) PM3 semiempirical method, as implemented in the MOPAC program on a Tektronix CAChe work system (version 3.8). Log P was calculated in the presence of water, according to the COSMO method. Geometries were optimized in internal coordinates and were terminated when the Herberts test was satisfied in the eigenvector following EP method (23).

Results and Discussion

Cytotoxic activity. Among 11 compounds, YS31 (mean of CC_{50} of a total of 3 normal and 2 tumor cells = 28 μM) showed the highest cytotoxic activity, followed by YS24 (42 μM) > YS29 (106 μM) > YS25 (120 μM) > YS28 (134 μM) > YS26 (139 μM) > YS27 (230 μM) > YS23 (296 μM) > YS30 (302 μM) > daidzein (324 μM) > genistein (375 μM) (Table I). These data suggest that compounds with 2 isoprenyl groups (one in A-ring and the other in B-ring) [YS24, YS31] or α, α-dimethylallyl group at C-5' of B-ring [YS26, YS27, YS28, YS29] showed relatively higher cytotoxic activity.

YS27 showed the highest tumor-specific cytotoxic action (tumor specificity index (TS)=2.8), followed by genistein (TS=2.4) > YS28 (TS=1.9), YS29 (TS=1.9), YS30 (TS=1.9) > YS25 (TS=1.8) > YS23 (TS=1.7), YS26 (TS=1.7) > YS31 (TS=1.6) > YS24 (TS=1.5) > daidzein (TS=1.5) (Table I). Tumor specificity was not directly coupled with the cytotoxic activity. Compounds with intermediate magnitude of cytotoxic activity [YS27, genistein, YS28, YS29, YS30] showed relatively higher tumor specificity (Table I).

Computational analysis. The relationship between cytotoxicity and hydrophobicity of 11 compounds was further investigated, using the log P value as the marker of hydrophobicity. When the log P value for each compound was plotted against the cytotoxic activity (expressed as CC_{50}), a parabola curve was described, except for daizein, genistein, YS23 and YS30, which were slightly separated away from the curve (Figure 2A). The cytotoxic activity of these compounds against HSC-2 cells reached the maximum level when the log P value was around 4.

Table I. *Cytotoxic activity of isoflavones and isoflavanones from Sophora species.*

Compound	CC_{50} (μM)						TS[2]
	Tumor cell		Normal cell				
	HSC-2	HSG	HGF	HPC	HPLF	mean[1]	
Daidzein	91	508	524	150	346	324	1.1
Genistein	156	259	533	444	481	375	2.4
YS23	171	250	378	313	367	296	1.7
YS24	25	41	62	39	43	42	1.5
YS25	65	100	200	111	122	120	1.8
YS26	34	166	194	166	135	139	1.7
YS27	15	204	286	258	388	230	2.8
YS28	30	143	203	141	154	134	1.9
YS29	53	88	120	110	160	106	1.9
YS30	133	266	508	226	376	302	1.9
YS31	17	26	42	26	31	28	1.6

[1] $$\text{mean} = \frac{CC_{50}\,(\text{HSC-2}) + CC_{50}\,(\text{HSG}) + CC_{50}\,(\text{HGF}) + CC_{50}\,(\text{HPC}) + CC_{50}\,(\text{HPLF})}{5}$$

[2] $$\text{TS} = \frac{CC_{50}\,(\text{HGF}) + CC_{50}\,(\text{HPC}) + CC_{50}\,(\text{HPLF})}{CC_{50}\,(\text{HSC-2}) + CC_{50}\,(\text{HSG})} \times \frac{2}{3}$$

When the cytotoxic activity (CC_{50}) was plotted against the stabilization of hydration, a straight line was produced, except for daizein, genistein, YS23 and YS30, which were slightly separated away from the line (Figure 2B).

NO production. Raw 264.7 cells spontaneously produced a low level of NO_2^- (0 ~ 0.1 μM) (Table II). When Raw 264.7 cells were treated with 100 ng/mL LPS, the production of NO was increased up to 20 μM. YS23~YS31 alone did not significantly stimulate the NO production by Raw 264.7 cells (0 ~ 0.5 μM), but rather inhibited the LPS-stimulated NO production (Figure 3, Table II). YS28 was the most inhibitory (SI=20.8), followed by YS26 (SI=11.3) > YS27 (SI=6.7) > YS25 (SI=6.0) > YS29 (SI=4.9) >YS23 (SI=4.0) > YS30 (SI=3.7) > YS31 (SI=1.9) > YS24 (SI=1.5). These data indicate that compounds with intermediate magnitude of cytotoxic activity [YS25, YS26, YS27, YS28, YS29] or those with an α, α-dimethylallyl group at C-5' of the B-ring [YS26, YS27, YS28, YS29] showed relatively higher inhibitory effect on the LPS-stimulated NO production by Raw 264.7 cells.

Radical scavenging activity. ESR spectroscopy shows that YS26 (A) and YS28 (B) produced radicals under alkaline conditions (Figure 4). At pH 7.4, a lower concentration (0.1 mg/mL) of these compounds did not produce radical. However, the radical became detectable when the concentration of these compounds was increased from 0.1 to 1 mg/mL (indicated by symbols, Figure 4). At pH 9 or 10, the radical intensity was significantly augmented even at lower concentration, reaching the maximum level within 2 minutes, accompanied by the rapid radical decay.

Figure 5 shows that both YS26 and YS28 scavenged the O_2^- (detected as a DMPO adduct, DMPO-OOH) produced by the HX-XOD reaction. At 10 μM of YS26 or YS28, the DMPO-OOH radical completely disappeared and was replaced by the compound's own radical. From the dose-response curve, the concentration which reduced the DMPO-OOH radical by 50% (IC_{50}) was calculated to be 0.53 and 0.59 μM, respectively.

We next investigated the NO scavenging activity. We utilized the reaction system of carboxy-PTIO and NOC-7 (NO generator), which produces carboxy-PTI. If a sample with NO scavenging activity is added to this reaction system, the production of carboxy-PTI should be decreased. Figure 6 shows that both YS26 and YS28 dose-dependently reduced the radical intensity of the 1st peak of carboxy-PTI (IC_{50} = 27 and 41 μM, respectively). However, there was a possibility that this apparent NO scavenging activity might simply be due to their general reduction activity. To test this possibility, a sample was added to the carboxy-PTI solution (which was prepared by 10-minute preincubation with carboxy-PTIO and NOC-7). YS26 and YS28 again reduced the radical intensity of carboxy-PTI to similar extents (Table III), suggesting that the major part of the NO radical scavenging activity of YS26 and YS28 derives from their reduction activity.

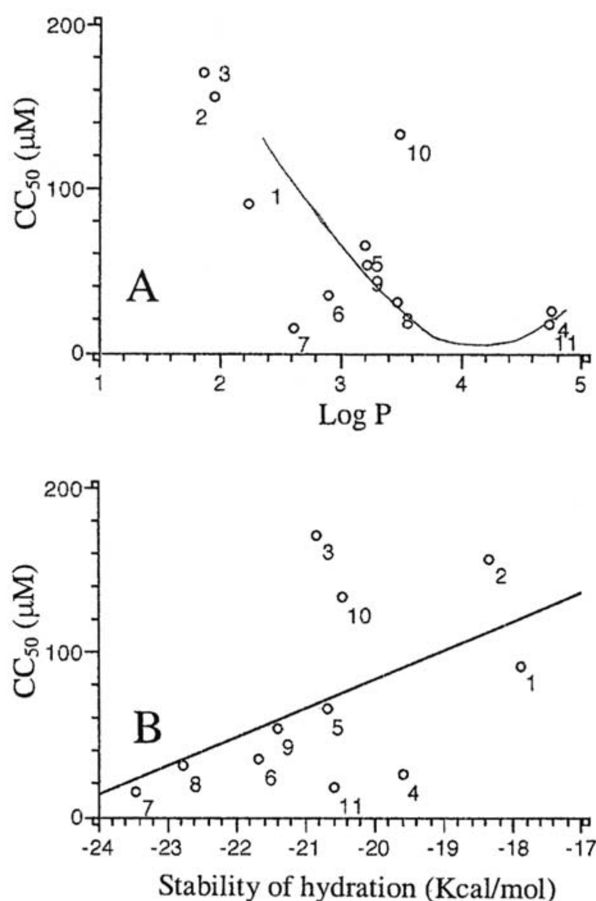

Figure 2. *(A) Relationship between hydrophobicity (log P) (A) or stabilization of hydration (B) and their cytotoxic activity (expressed as CC_{50} (μM)) against HSG cells. 1) daidzein; 2) genistein; 3) YS23; 4) YS24; 5) YS25; 6) YS26; 7) YS27; 8) YS28; 9) YS29; 10) YS30; 11) YS31.*

Table II. *Effect of isoflavanones on NO production by Raw 264.7 cells.*

Compound	CC_{50} (μM)		IC_{50} (μM)	SI = CC_{50}/IC_{50}
	LPS (-)	LPS (+)	LPS(+)	
YS23	129	126	32	4.0
YS24	25	25	17	1.5
YS25	58	51	8.4	6.0
YS26	99	66	5.8	11.3
YS27	76	87	13	6.7
YS28	66	64	3.1	20.8
YS29	63	83	17	4.9
YS30	117	131	35	3.7
YS31	28	24	12	1.9

Raw 264.7 cells were incubated for 24 hours with various concentrations of each isoflavone in the absence or presence of LPS (10 ng/mL).

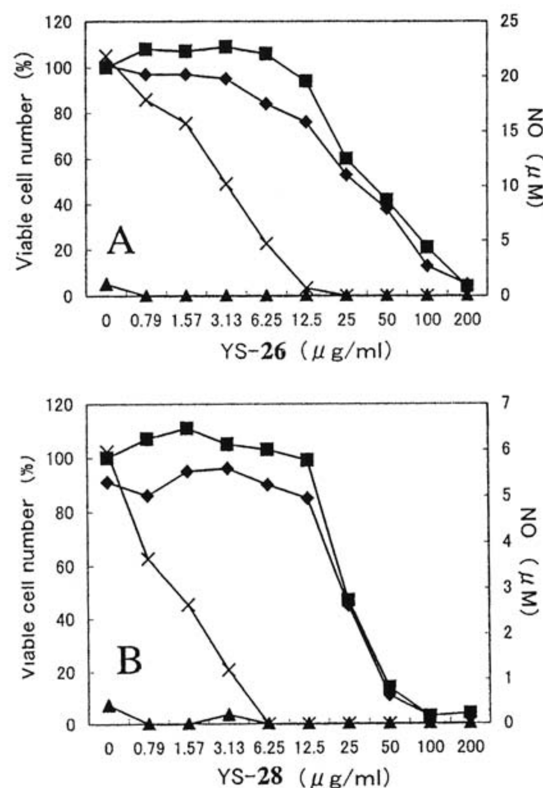

Figure 3. *Effect of YS26 and YS28 on the viable cell number and NO production by unstimulated or LPS-stimulated Raw 264.7 cells. Raw 264.7 cells (30 x 10^4/mL) were seeded onto 96-microwell plate and allowed to attach for 24 hours. After replacement with 0.1 mL of fresh medium containing the indicated concentrations of YS26 (A) or YS28 (B) in the presence (◆, ×) or absence (■, ▲) of 100 ng/mL LPS, the absorbance at 540 nm of the MTT-stained cell lysate (which reflects the relative viable cell number) (■, ◆) and extracellular NO production (×, ▲) were determined. Each symbol represents the mean of 4 determinations.*

The present study demonstrates that isoflavanones especially with an α, α-dimethylallyl group at C-5' of the B-ring [YS26, YS28] showed some tumor-specific cytotoxic activity and inhibited the NO production by LPS-activated macrophages. The apparent inhibition of NO production might at least in part be explained by their radical scavenging activity or general reduction activity. Flavonoids have been reported to act as scavengers of nitric oxide radical (24). However, it is important to measure both NO scavenging activity and reduction activity in each flavonoid sample, since these activities may differ from sample to sample (Satoh *et al.* unpublished data).

We have recently found that epigallocatechin gallate (EGCG), a main component of green tea polyphenol, induced apoptosis in Raw 264.7 cells (as judged by the expression of TUNEL-positive cells) and inhibited the LPS-stimulated NO and TNF production, possibly by its

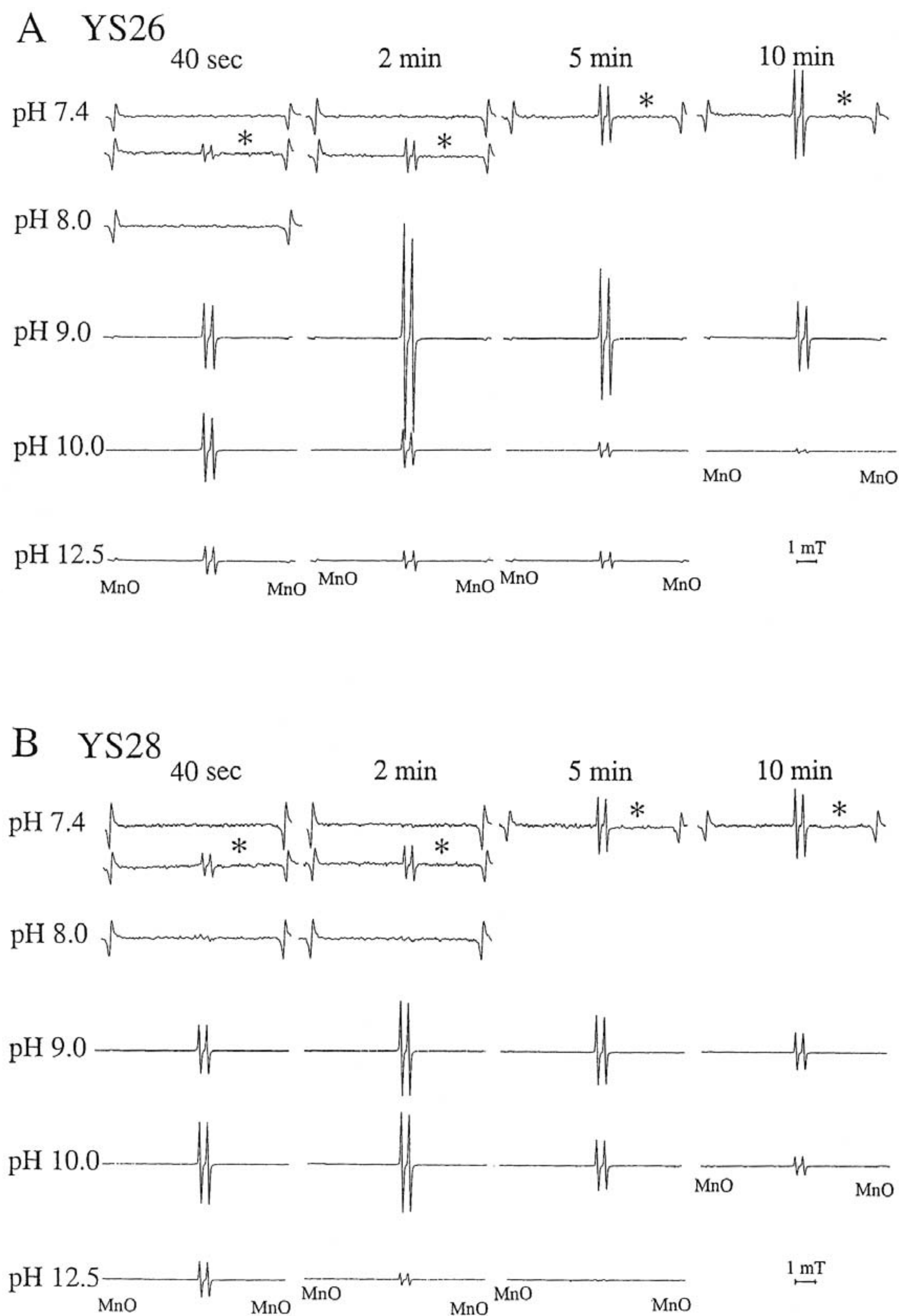

Figure 4. *Time course of the radical generation by YS26 (A) and YS28 (B) at the indicated pH. The concentration of compounds was 100 µg/mL except the sample indicated by * (1 mg/mL).*

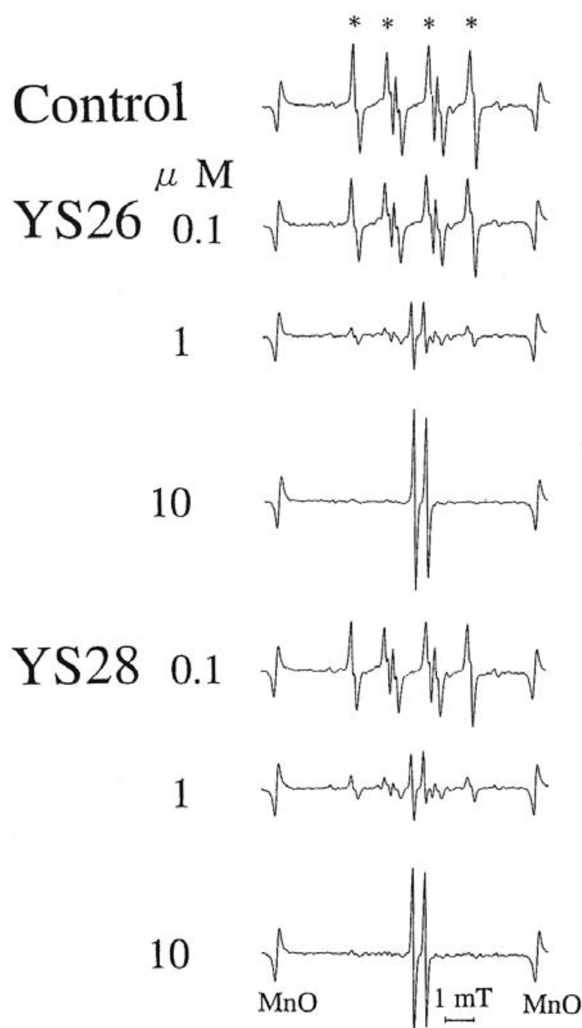

Figure 5. *Effect of YS26 and YS28 on DMPO-OOH radical intensity. * indicates DMPO-OOH adduct produced by HX-XOD reaction.*

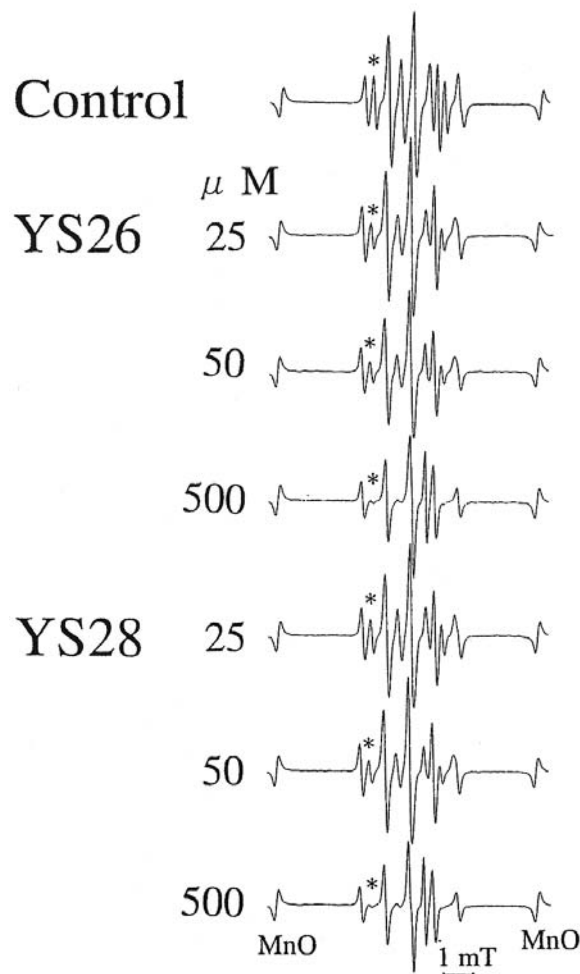

Figure 6. *NO radical scavenging activity of YS26 and YS28. The reaction mixture containing 20 μM carboxy-PTIO + 50 μM NOC-7 without (control) or with the indicated concentrations of YS26 or YS28. The mixture was subjected to ESR spectroscopy 3 minutes later.*

apoptosis-inducing activity (Jiang *et al.*, in preparation). Therefore, it remains to be investigated whether YS26 or YS28 directly induces the apoptosis in Raw 264.7 cells and thereby inhibits the NO production.

We found that the cytotoxic activity of isoflavanones was significantly increased when the log P value reached around 4. This is consistent with our previous finding that the optimal log P value of eugenol-related compounds (25), vitamin K compounds (26) and prenylflavanones (10), for induction of maximum cytotoxicity, was around 3-4. The semiempirical method may be applicable not only to estimate the cytotoxic activity of the related compounds, but also to design more active derivatives.

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Table III. *NO scavenging activity of YS26 and YS28.*

Compound	Radical intensity	
	Assay for NO scavenging activity [a]	Assay for reduction activity [b]
Control	100	100
YS26 (25 μM)	51	53
YS28 (40 μM)	51	51

Representative data are shown in Figure 6.
[a] Carboxy-PTIO, NOC-7 and sample were mixed. Three minutes later, the radical intensity of the first peak of carboxy-PTI was determined by ESR spectroscopy and expressed as % of control.
[b] Carboxy-PTIO and NOC-7 were incubated for 10 minutes to achieve the complete conversion to carboxy-PTI. Sample was then added and further incubated for 3 minutes to determine the radical intensity, which was expressed as % of control.

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