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 cells Raw 264.7, 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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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_15H_10O_4=254.23), genistein (C_15H_10O_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).

Figure 1. Structure of isoflavones and isoflavanones.
Preparation of isoflavanones. YS23 (C_{21}H_{20}O_{7}=384.39) (12-14), YS24 (C_{22}H_{22}O_{7}=438.52) (13, 15), YS25 (C_{21}H_{22}O_{7}=370.40) (13), YS26 (C_{22}H_{22}O_{7}=386.40) (16, 17), YS27 (C_{22}H_{22}O_{7}=402.40) (17), YS28 (C_{22}H_{22}O_{7}=370.40) (18), YS29 (C_{22}H_{22}O_{7}=400.43) (18), YS30 (C_{22}H_{22}O_{7}=354.40) (18) and YS31 (C_{22}H_{22}O_{7}=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 then determined and the 50% cytotoxic concentration (CC50) was performed by TLC (Merck: Silica gel 60F254; structures are shown in Figure 1. Purifications of these compounds were prepared, as described in the literature cited above, and their structures are shown in Figure 1. Purifications of these compounds (YS23-31) was then determined and the 50% cytotoxic concentration (CC50) was performed by TLC (Merck: Silica gel 60F254; 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 production of carboxy-PTI from carboxy-PTIO (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 CC50 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) > YS32 (296 μM) > YS30 (302 μM) > daidzein (324 μM) > genistein (375 μM) > germane (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 CC50), a parabola curve was described, except for daidzein, 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.
When the cytotoxic activity (CC50) 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 NO2- (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 O2- (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% (IC50) 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 (IC50 = 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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC50 (μM)</th>
<th>TS</th>
<th>Tumor cell</th>
<th>Normal cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSC-2</td>
<td>HSG</td>
<td>HGF</td>
<td>HPC</td>
</tr>
<tr>
<td>Daidzein</td>
<td>91</td>
<td>508</td>
<td>524</td>
<td>150</td>
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<tr>
<td>Genistein</td>
<td>156</td>
<td>259</td>
<td>533</td>
<td>444</td>
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<td>YS23</td>
<td>171</td>
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<td>YS30</td>
<td>133</td>
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<td>508</td>
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<tr>
<td>YS31</td>
<td>17</td>
<td>26</td>
<td>42</td>
<td>26</td>
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</table>

1) \( \text{mean} = \frac{\text{CC50 (HSC-2) + CC50 (HSG) + CC50 (HGF) + CC50 (HPC) + CC50 (HPLF)}}{5} \)

2) \( \text{TS} = \frac{\text{CC50 (HGF) + CC50 (HPC) + CC50 (HPLF)}}{\text{CC50 (HSC-2) + CC50 (HSG)}} \times \frac{2}{3} \)
The present study demonstrates that isoflavanones, especially with a $\alpha$, $\alpha$-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).
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 isoflavones 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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References

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.

Table III. NO scavenging activity of YS26 and YS28.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radical intensity</th>
<th>Assay for NO scavenging activity</th>
<th>Assay for reduction activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>YS26 (25 μM)</td>
<td>51</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>YS28 (40 μM)</td>
<td>51</td>
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