Polyphenols from *Eriobotrya japonica* and Their Cytotoxicity against Human Oral Tumor Cell Lines

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Received December 8, 1999; accepted January 29, 2000

Three new flavonoid glycosides, together with 15 known flavonoids, have been isolated from the leaves of *Eriobotrya japonica*, and characterized as (2*S*)- and (2*R*)-naringenin 8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosides, and cinchonain Id 7-*O*-β-D-glucopyranoside, respectively, based on spectral analyses including two dimensional (2D) NMR techniques. Higher proanthocyanidin fraction in the water-soluble portion of the extract was characterized as a procyanidin oligomer mixture mainly composed of undecameric procyanidin. These polyphenols have also been assessed for cytotoxic activity against two human oral tumor (human squamous cell carcinoma and human salivary gland tumor) cell lines. Selective cytotoxicity of the procyanidin oligomer between tumor and normal gingival fibroblast cells, and its possible mechanism, were also described.

Key words *Eriobotrya japonica*; Rosaceae; cinchonain Id 7-*O*-β-D-glucopyranoside; naringenin 8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside; undecameric procyanidin; human oral tumor cell line

Eriobotrya japonica Lindl. (Rosaceae) is a well-known medicinal plant in Japan and China. Its astringent leaves have been used a long time to treat chronic bronchitis, coughs, phlegm, high fever and gastroenteric disorders. A decoction of the leaves has been known to be a cooling beverage preventing sunstroke and thirst, and has also been applied locally to wounds, ulcers and cancers.[1)] The occurrence of triterpenoids with anti-inflammatory[2)] and antiviral[3)] effects, megastigmane glycoside[4)] and a small amount of amygdalin[2)] in the leaves has been reported. Although many of the rosaceous plants are known to be rich in flavonoids and tannins, only several polyphenolic constituents of *E. japonica* have been reported.[5)] In addition, the anti-tumorgenic effect has not been extensively examined for any ingredients with a defined structure.

This paper describes the isolation and characterization of 18 polyphenolic compounds from the leaves of this plant, three of which are new flavonoid glycosides. Furthermore, a higher oligomeric proanthocyanidin fraction in the water-soluble portion of the extract was characterized to be composed mainly of procyanidin undecamer with 4β→8 interflavanyl linkages. In order to clarify which constituents of this plant contribute to its anti-tumorgenic effect, we also investigated here the cytotoxic effect of these polyphenols against human oral tumor cell lines [human squamous cell carcinoma (HSC-2), human salivary gland tumor (HSG)], in comparison with the effect on human normal gingival fibroblasts (HGF), and found that the procyanidin oligomer among the isolated polyphenols exhibited potent selective cytotoxicity to tumor cell lines. Apoptosis-inducing activity of the oligomer against tumor cell lines, as revealed by agarose gel electrophoresis which detects DNA fragments,[6)] and immunocytochemical staining with M30 monoclonal antibody, which reacts with the degradation product of cytokeratin 18 by activated caspases,[7)] is also demonstrated.

Results and Discussion

A 70% aqueous acetone homogenate of the leaves of *E. japonica* was extracted successively with ether, AcOEt and 1-butanol. Repeated chromatographies of the AcOEt and 1-butanol extracts over polystyrene and polyvinyl gels gave 19 polyphenols, including three new compounds (**1**, **2**, **3**).

Known polyphenols were identified as chlorogenic acid,[8)] 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 4-*O*-*p*-coumaroylquinic acid, quercetin,[9)] isoquercitrin,[10)] quercetin 3-*O*-sophoroside,[11)] kaempferol 3-*O*-sophoroside,[11)] kaempferol 3-*O*-α-rhamnopyranosyl-(1→2)-β-D-glucopyranoside,[12)] (−)-epicatechin (**5**), procyanidin B-2 (**6**),[13)] procyanidin C-1 (**7**),[14)] 1-*O*-feruloyl-β-D-glucopyranose,[15)] and 4-*O*-β-glucopyranosyl-*cis*-*p*-coumaric acid.[16)] From the water-soluble fraction remaining after 1-butanol extraction, cinchonain Ia (**4**)[17,18)] and procyanidin oligomer (**8**) were obtained.

Structures of Flavonoid Glycosides Compound **1** was isolated as a pale yellow amorphous powder. The molecular formula of **1** was determined to be $C_{27}H_{32}O_{14}$ based on high-resolution electrospray ionization mass spectrum (HR ESI-MS) [*m*/*z* 581.1815 $(M+H)^+$ (Calcd for $C_{27}H_{32}O_{14}+H$, 581.1870)]. The UV spectrum showed an absorption maximum at 290 nm with a shoulder at 325 nm, characteristic of a flavanone skeleton.[19)] The ^{1}H-NMR spectrum (acetone-d_6+D_2O) of **1** showed mutually coupled methine and methylene proton signals [δ 5.40 (dd, *J*=2, 14 Hz), δ 3.36 (dd, *J*=14, 17 Hz), δ 2.59 (dd, *J*=2, 17 Hz)], and AA′BB′-type signals [δ 7.45 (2H, d, *J*=8.5 Hz), δ 6.90 (2H, d, *J*=8.5 Hz)] and a 1H-singlet at δ 5.96 in the aromatic proton region. These spectral futures, together with a hydrogen-bonded hydroxyl proton signal at δ 12.34 (s), which disappeared upon the addition of D_2O, indicated the presence of a naringenin skeleton with a C_6- or C_8-substituted A-ring in the molecule of **1**. The spectrum also exhibited signals ascribable to a sugar moiety in the aliphatic proton region. Although the presence

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of rhamnose residue was suggested by an anomeric proton signal [δ 5.29 (br s)] and a methyl signal [δ 0.76 (d, J=6 Hz)], full assignment of the other signals was difficult due to partial overlapping and/or broadening. Measurement of the spectrum in methanol-d_4 gave, however, well-resolved signals for all of the sugar protons, and their full assignment was made with the aid of ^{1}H–^{1}H shift correlation spectroscopy (COSY) (see Experimental). The presence of rhamnose and glucose residues in **1** was thus revealed by coupling patterns characteristic of the respective sugars. The ^{13}C resonances of the glucose moiety, which were assigned by heteronuclear multiple quantum coherence (HMQC) spectrum (in methanol-d_4), were δ 74.4 (C-1), 78.2 (C-2), 81.1 (C-3), 71.4 (C-4), 82.2 (C-5) and 62.4 (C-6). These data, along with the appearance of a glucose H-1 signal at a higher field (δ 4.85) than that expected for *O*-glucoside, were indicative of a *C*-glucopyranosyl moiety. The *C*-glucosyl and *O*-rhamnosyl structure was supported by acid hydrolysis of **1**, which liberated only rhamnose. The ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) spectrum ($^{2,3}J_{CH}$=6 Hz) in acetone-d_6+D_2O revealed that the anomeric proton signal at δ 5.29 (rhamnose H-1‴) exhibited three-bond coupling with the glucose C-2″ signal (δ 77.3), indicating the presence of a rhamnopyranosyl (1→2) glucopyranosyl moiety as the disaccharide sugar chain. The HMBC spectrum also showed two and three bond correlations of the anomeric proton signal (δ 4.83) of the glucose core with C-7 (δ 166.0), C-8 (δ 104.9) and C-9 (δ 161.3) of the aglycone, establishing that the glucose residue is attached to C-8 of the aglycone through a C–C bond. The absolute configuration at C-2 of the aglycone was assigned an (*S*)-orientation, based on a negative Cotton effect at 290 nm[20] in the CD spectrum of **1**. The coupling constants of H-1″ (δ 4.83, J=10 Hz) and H-1‴ (δ 5.29, br s) were consistent with β- and α-glycosidic linkages for glucopyranosyl and rhamnopyranosyl residues, respectively. Based on these data, taking into consideration the D- and L-series for almost all of glucose and rhamnose in higher plants, compound **1** was determined to be (2*S*)-naringenin 8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.

Compound **2**, a pale yellow amorphous powder, exhibited a pseudomolecular ion peak at *m/z* 581.1858 $(M+H)^+$ (Calcd for $C_{27}H_{32}O_{14}$+H, 581.1870) in an HR ESI-MS spectrum, establishing its molecular formula as $C_{27}H_{32}O_{14}$, the same as that of **1**. The spectral data (UV, MS, ^{1}H-NMR) of **2** were almost superimposable on those of compound **1**, except for downfield shifts for H-2 ($\Delta\delta$ 0.20 ppm) and one of the C-3 methylene proton signals ($\Delta\delta$ 0.18 ppm) as compared with the corresponding signals of **1** in the NMR spectra. A sign of the Cotton effect at 291 nm ($\Delta\varepsilon$ +5.1) in the CD spectrum of **2**, which is associated with the absolute configuration at C-2 of flavanols, was opposite to that of **1**. Compound **2** was thus assigned to be a C-2 epimeric isomer of **1** [(2*R*)-naringenin 8-*C*-α-rhamnopyranosyl-(1→2)-β-glucopyranoside], although it lacks further evidence because an insufficient amount was available. It is not a rare case that (2*R*)- and (2*S*)-isomers of flavanone glycosides coexist in a plant, as exemplified by previous reports for those in the Zizyphi fructus[21] and in licorice.[22]

The structure of compound **3**, a pale yellow amorphous powder, was determined as follows. The molecular formula was established to be $C_{30}H_{30}O_{14}$ based on HR ESI-MS that showed an $(M+NH_4)^+$ ion peak at *m/z* 632.1990 (Calcd for $C_{30}H_{30}O_{14}$+NH_4, 632.1979). Its ^{1}H-NMR spectrum revealed the signals of an ABX system [δ 7.07 (d, J=2 Hz), δ 6.84 (dd, J=2, 8 Hz), δ 6.79 (d, J=8 Hz), B-ring], a 1H-aromatic singlet (δ 6.56, A-ring), two methine signals [δ 4.95 (br s), δ 4.28 (m)] and a methylene signal [δ 3.02 (br dd, J=3, 17 Hz), δ 2.83 (dd, J=3, 17 Hz), C-ring], suggesting the presence of an epicatechin moiety with a monosubstituted A-ring. In addition to those signals, mutually coupled methine [δ 4.72 (dd, J=2, 7 Hz)] and methylene [δ 3.02 (dd, J=7, 16 Hz), δ 2.88 (dd, J=2, 16 Hz), D-ring] proton signals, and ABX-type proton signals [δ 6.72 (d, J=2.5 Hz), δ 6.70 (d, J=8.5 Hz), δ 6.51 (dd, J=2.5, 8.5 Hz), E-ring] were observed. These data were similar to those of cinchonain-type flavanols. A sequentially coupled seven-spin system characteristic of glucopyranose residue was also observed in the ^{1}H-NMR spectrum. Compound **3** was thus assumed to be a glucoside of either of cinchonains Ia (**4**), Ib (**9**), Ic (**10**) or Id (**11**)[17,18] The HMBC spectrum of **3** exhibited three-bond correlations of one of the H-4 signals (δ 3.02) with oxygen-bearing carbon resonances at δ 155.9 (C-9) and δ 151.5 (C-5), which in turn were associated with the A-ring proton (H-8, δ 6.56) and H-7″ (δ 4.72), respectively, through two- and three-bond couplings. Both of these proton signals (H-8, H-7″) were also correlated with an oxygen-bearing carbon resonance at δ 154.3 (C-7), which showed three-bond coupling with an anomeric proton (H-1‴) signal at δ 4.94 (Fig. 1). It was thus concluded that the location of the glucose residue is at O-7, and the aglycone should be either cinchonains Ic (**10**) or Id (**11**).[17,18] These diastereomers, **10** and **11**, were reported to be distinguished from each other by the CD spectrum.[17,18] The CD spectrum of **3** showed a negative Cotton effect at 286 nm ($\Delta\varepsilon$ −2.2) and a positive one at 246 nm ($\Delta\varepsilon$ +5.5) similar to those of **11**, establishing a (7″*S*)-configuration. Glucose was determined to be D-series by a positive reaction of acid hydrolysate from **3** to D-glucose oxidase.[23] The β-glucosidic linkage was indicated by a large coupling constant (J=7 Hz)

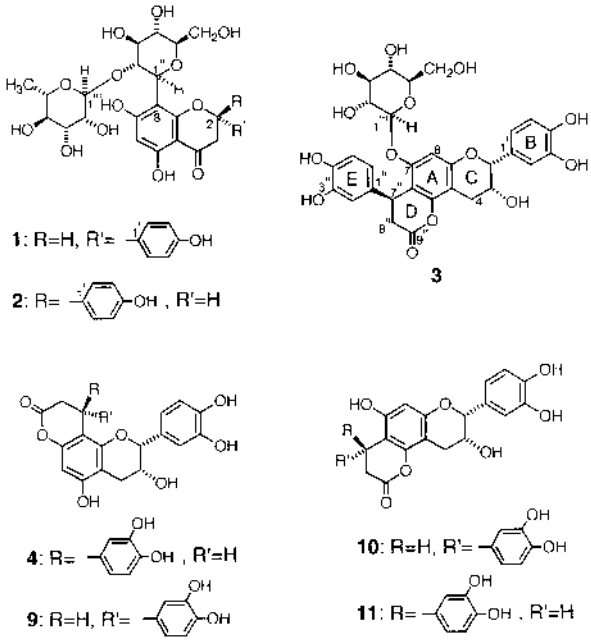
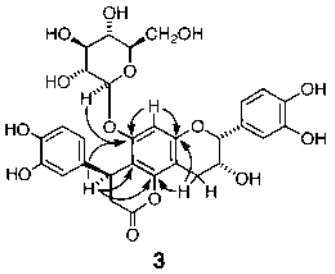


Chart 1

of the H-1‴ signal. Consequently, the structure of **7** was determined to be cinchonain Id 7-*O*-β-D-glucopyranoside. Although cinchonains were previously isolated from *Cinchona succirubra* (Rubiaceae),[17] *Phyllocladus trichomonoides* (Podocarpaceae)[24,25] and *Apocynum venetum* (Apocynaceae),[26] compound **3** is the first example of a chinchonain glycoside.

Compound **8** was shown to be procyanidin by the formation of cyanidin (TLC, HPLC) upon heating with *n*-BuOH–HCl.[27] Its polymeric nature was apparent from a very broad peak both on normal- and reversed-phase HPLC. Acid-catalysed cleavage of **8** in the presence of excess phloroglucinol was attempted in order to establish the identity of the constituent units and the nature of the interflavonoid linkage in the oligomer.[28] HPLC of the reaction mixture revealed formations of epicatechin (**5**) and epicatechin-(4β→2)-phloroglucinol (**12**) as major products, along with small amounts of **6** and **7**. The phloroglucinol addition product (**12**) was produced by the reaction between nucleophilic phloroglucinol and C-4 carbocation generated by cleavage of the interflavonoid bond in an acidic medium, while **5** was produced from the terminal unit of **8** (Chart 2). These products were identified by HPLC comparison with authentic samples obtained by similar reactions of **6** or **7**. The formation of **6** and **7** from **8** provided conclusive evidence that **8** was a procyanidin oligomer in which epicatechin units link with each other predominantly through 4β→8 interflavanyl linkages. Although it was difficult to estimate quantitatively the products in the reaction, an average degree of polymerization was ascertained by the ^{13}C-NMR spectrum. The ^{13}C-NMR spectrum of **8**, which showed broad and unresolved signals reflecting a large molecular weight, was closely similar to that reported for epicatechin-(4β→8)-epicatechin oligomers.[29,30] It is reported that a ratio of peak areas of the C-3 or C-4 signal of a terminal unit to that of extension units is diagnostic for estimating the polymerization degree.[29] The peak ratio of the C-3 resonances due to terminal and extension units at δ 66.8 and 72.7 ppm, respectively, in the ^{13}C-NMR spectrum of **8** was estimated as *ca.* 1 : 10, suggesting that **8** is an undecameric procyanidin. This was in agreement with the molecular weight estimated from gel permeation chromatography (GPC)[31] of an acetyl derivative of this higher oligomeric mixture, which showed that number average molecular weight (M_n) of the acetate was 5400 [weight average molecular weight (M_w): 14800], corresponding to an undecamer. The predominant (4β→8) interflavanyl linkage in the oligomer was supported by a strong positive couplet Cotton effect centered at 211 nm.[32] The procyanidin oligomer fraction was thus assigned to involve an undecamer (on the average) composed of an epicatechin-(4β→8)-epicatechin unit.



3

Fig. 1. Selective HMBC Correlations of **3**

Cytotoxic Activity of Polyphenols We recently found that epigallocatechin gallate (EGCG), a main polyphenol of green tea, and some ellagitannin monomers and oligomers from medicinal plants, exhibit potent cytotoxic activity against human oral tumor cell lines (HSG, HSC-2), and those anti-tumorgenic substances show negligible or weak toxicity to normal cells (HGF).[33] In a survey of possible anti-tumor ingredients in *E. japonica*, polyphenolics isolated in the present study, *i.e.* procyanidins B-2 (**6**), C-1 (**7**) and oligomer (**8**), flavan 3-ols related with EGCG and flavonoids, were then assessed for cytotoxic activity against those tumor cell lines.

As shown in Table 1, the cytotoxic activity of procyanidins and related polyphenols against HSC-2 cells was increased as molecular weight increased: epicatechin (**5**) (CC_{50}> 1.72 mM)<procyanidin B-2 (**6**) (CC_{50}=0.39 mM)<procyani-

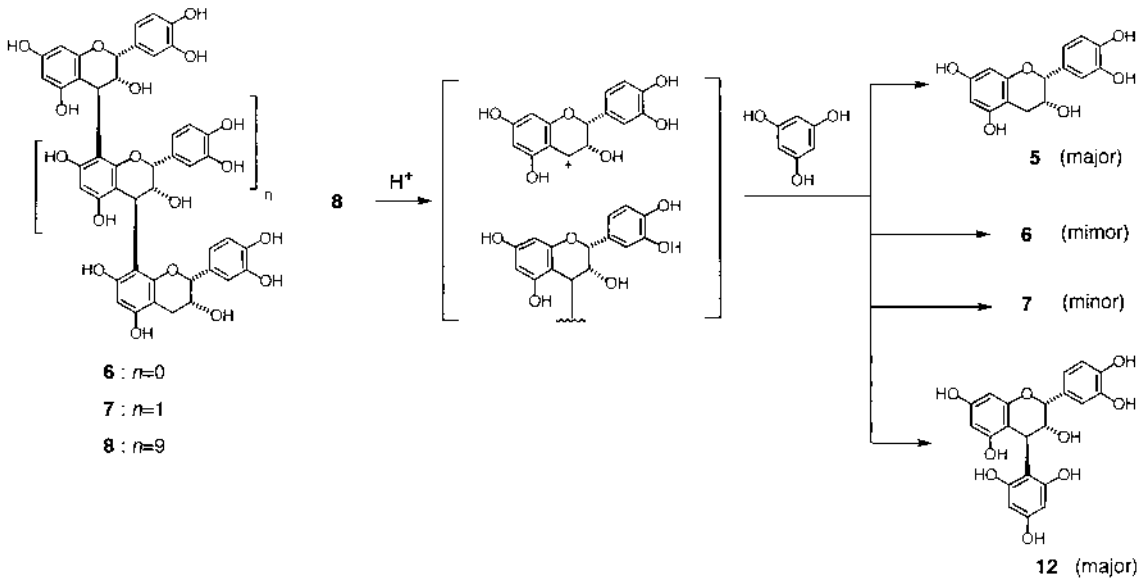


Chart 2

Table 1. Cytotoxicity (CC_{50}) of Polyphenols from *E. japonica*

	MW	CC_{50} (μg/ml)(mM)		
		HSC-2	HSG	HGF
Procyanidins and related flavan 3-ols				
Catechin	290	>500 (>1.72)	>500 (>1.72)	>500 (>1.72)
(−)-Epicatechin (**5**)	290	>500 (>1.72)	>500 (>1.72)	>2000 (>6.90)
Procyanidin B-2 (**6**)	578	225 (0.39)	415 (0.72)	1850 (3.20)
Procyanidin C-1 (**7**)	866	205 (0.24)	395 (0.46)	>2000 (>2.31)
Procyanidin oligomer (**8**)	3170	189 (0.060)	353 (0.11)	>2000 (>0.63)
(−)-Epigallocatechin gallate	458	206 (0.45)	216 (0.47)	870 (1.90)
Flavonoids				
Quercitrin	448	337 (0.75)	>500 (>1.12)	>500 (>1.12)
Quercetin 3-*O*-sophoroside	626	>500 (>0.80)	>500 (>0.80)	>500 (>0.80)
Kaempferol 3-*O*-sophoroside	610	>500 (>0.82)	>500 (>0.82)	>500 (>0.82)
Naringenin-8-*C*-rhamnoglucoside (**1**)	580	>500 (>0.86)	>500 (>0.86)	>500 (>0.86)
Others				
Gallic acid	170	77 (0.45)	80 (0.47)	90 (0.53)
Ellagic acid	334	>500 (>1.50)	>500 (>1.50)	>500 (>1.50)

Relative viable cell number (A_{540}) was determined by MTT method. The A_{540} of control HSC-2, HSG and HGF cells was 1.17, 0.59 and 0.29, respectively. Each value represents mean from triplicate assays. S.D.<10%.

din C-1 (**7**) (CC_{50}=0.24 mM)<procyanidin oligomer (**8**) (CC_{50}=0.060 mM). The cytotoxic activity of **8** in a molar basis exceeded that of gallic acid (CC_{50}=0.45 mM), ellagic acid (CC_{50}>1.5 mM) or EGCG (CC_{50}=0.45 mM) (Table 1). The cytotoxic activity of quercitrin, quercetin 3-*O*-sophoroside, kaempferol 3-*O*-sophoroside and naringenin-8-*C*-rhamnoglucoside was much less (CC_{50}>0.75 mM). As compared with the HSC-2 cell line, the human salivary gland tumor cell line HSG was slightly resistant to all of these polyphenols, whereas normal human gingival fibroblast HGF was very resistant, suggesting that these polyphenols display selective cytotoxic activity against cancer cell lines relative to normal cells.

Possible Mechanism of Cytotoxicity We have recently established that the prooxidant action of polyphenols can be detected by ESR spectroscopy, using sodium ascorbate as a radical generator.[33] Figure 2A shows that the radical intensity of sodium ascorbate was slightly reduced by a lower concentration (<3 μM) of **8**, but enhanced by higher concentrations (>10 μM). This suggests that the cytotoxic activity of procyanidin oligomer observed at higher concentrations is due to their prooxidant action. This was confirmed by radical generation by **8**, under an alkaline condition (Fig. 2B). It was, however, unexpected that catalase, which decomposes extracellular H_2O_2, did not significantly affect the cytotoxic activity of the procyanidin oligomer (B/A ratio=1.0–1.4, see Table 2), whereas the cytotoxic activity of EGCG (B/A=1.4–1.8) and gallic acid (B/A=18–29) was significantly reduced. (Table 2) Procyanidin oligomer (**8**) also induced two apoptosis-associated characteristics: DNA fragmentation (as demonstrated by agarose gel electrophoresis) (Fig. 3A) and degradation of cytokeratin 18 by activated caspase(s) (as demonstrated by cytoplasmic staining with an M30 monoclonal antibody) (Fig. 3B).

EGCG, as well as tea extracts,[34] which are known as antitumor agents,[35—38] has been reported to induce apoptosis in various human tumor cell lines, and to inhibit neovasculization in the tumor.[39] In the present study, procyanidin oligomer **8** showed more potent cytotoxic activity against oral tumor cell lines than EGCG. However, procyanidin

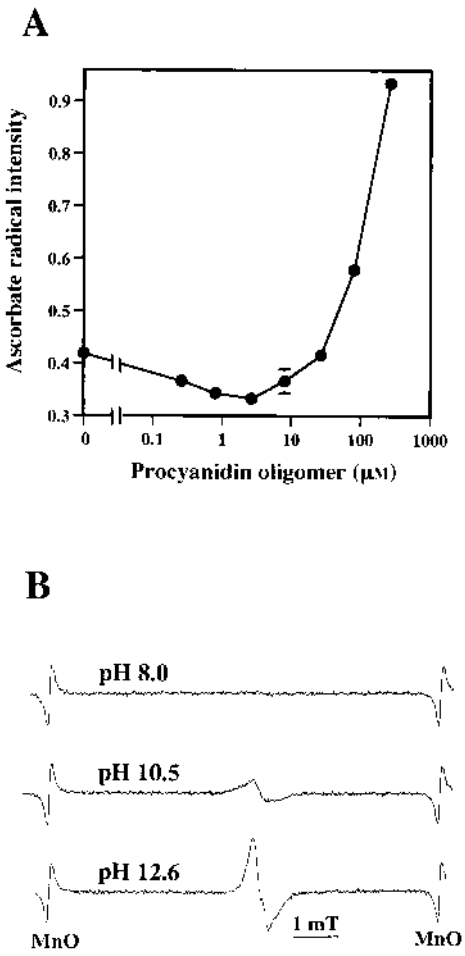


Fig. 2. Effect of Procyanidin Oligomer (**8**) on Ascorbate Radical Intensity (A) and Radical Generation by **8** (B)

(A): Sodium ascorbate (3 mM) was mixed with an equal volume of various concentrations of procyanidin oligomer in 50% DMSO, 0.08 M Tris–HCl, pH 8.0, and the radical intensity of sodium ascorbate was measured 1 min thereafter. The gain was 5×100. Each value represents the mean from triplicate assays. S.D.<5%. (B): Procyanidin oligomer (3 mg/ml in DMSO) was mixed with an equal volume of 0.2 M Tris–HCl (pH 8.0), 0.2 M $NaHCO_3/Na_2CO_3$, pH 10.5, or 0.2 N KOH (pH 12.6), and the mixture was applied to ESR spectroscopy. The gain was 6.3×100.

Table 2. Failure of Catalase to Inhibit the Cytotoxic Activity of Procyanidin Oligomer

Compounds	CC_{50} (mM)								
	HSC-2			HSG			HGF		
	(A)	(B) +Cat	B/A	(A)	(B) +Cat	B/A	(A)	(B) +Cat	B/A
Procyanidin oligomer (**8**)	0.046	0.065	1.4	0.10	0.10	1.0	>0.13	>0.13	1.0
EGCG	0.47	0.79	1.7	0.37	0.53	1.4	0.87	1.60	1.8
Gallic acid	0.56	9.78	17.5	0.48	13.9	29.0	0.69	>16.0	>23.2

Cells were incubated for 24 h with various concentrations of the indicated test compounds without (A) or with (B) 3000 U/ml catalase, and the relative viable cell number (A_{540}) was determined by MTT method. The A_{540} of control HSC-2, HSG and HGF cells was 1.20, 0.57 and 0.17, respectively. Each value represents mean of triplicate assays. S.D.<10%. The ability of catalase to inhibit the cytotoxic activity of each compound was determined by the ratio of B to A.

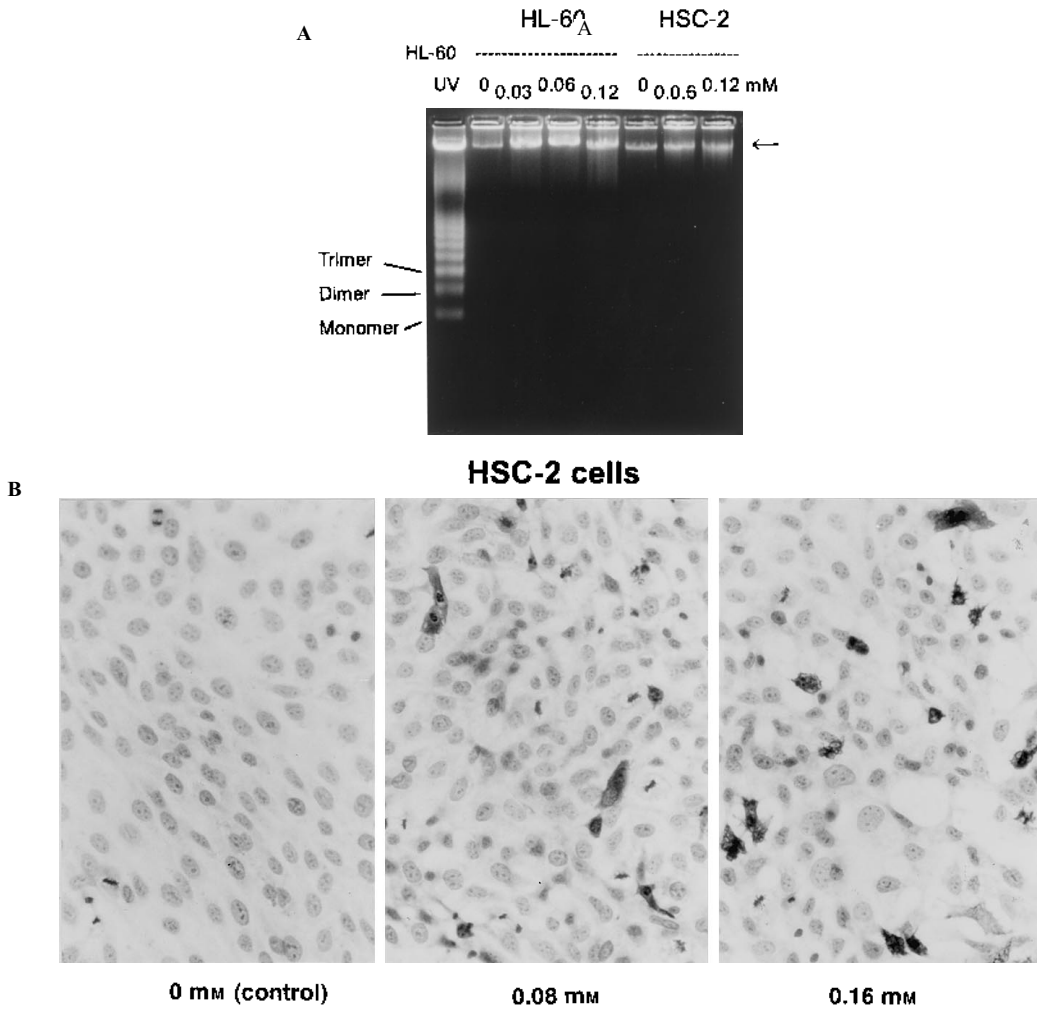


Fig. 3. Induction of DNA Fragmentation by Procyanidin Oligomer (**8**) in HSC-2 Cells (A) and Activation of Caspase by **8** (B)

(A): Near confluent HSC-2 cells and HL-60 cells (1×10^6/ml) were treated for 6 h with the indicated concentrations of **8**. The cells were lysed, treated with RNase A and proteinase K, and DNA was isolated and applied to 1.8% agarose gel electrophoresis. Lane 1 is the DNA from UV-induced apoptotic HL-60 cells. (B): Near confluent HSC-2 cells were treated for 6 h without (control) or with 0.08 or 0.16 mM procyanidin oligomer (**8**). The cells were fixed in 95% ethanol and 5% acetic acid, then treated with M30 monoclonal antibody. Original magnification, ×33. Note the cytoplasmic staining of the cytokeratin 18 degradation products in the cells treated with procyanidin oligomer.

oligomer (Fig. 2A), but not EGCG, enhanced the radical intensity of sodium ascorbate. These data suggest that these two compounds have different sites of action.

Although further details regarding the effect of procyanidin oligomer against these tumor cells remain to be investigated, the present results suggest that the extract of *E. japonica* would be beneficial in the chemoprevention of oral cancer.

Experimental

Optical rotations were recorded on a JASCO DIP-1000 polarimeter. ^{1}H- and ^{13}C-NMR spectra were measured in acetone-d_6+D_2O on Varian VXR-500 (500 MHz for ^{1}H-NMR and 125.7 and 50 MHz for ^{13}C-NMR) instruments. Chemical shifts are given in δ (ppm) values relative to that of the solvent acetone-d_6 (δ_H 2.04; δ_C 29.8) and methanol-d_4 (δ_C 49.0) on a tetramethylsilane scale. ESI-MS were carried out using a Micromass Auto Spec OA-Tof mass spectrometer (solvent: 50%MeOH+0.1%$AcONH_4$, flow rate: 20 μl/min). CD spectra were measured on a JASCO J-720W spectrometer. Normal-phase HPLC was conducted on a YMC-Pack SIL A-003 (YMC Co.,

Ltd.) column (4.6 mm i.d.×250 mm) developed with *n*-hexane–MeOH–tetrahydrofuran (THF)–formic acid (55 : 33 : 11 : 1) containing oxalic acid (450 mg/1 l) (flow rate, 1.5 ml/min; detection 280 nm) at room temperature. Reversed-phase HPLC was performed on a YMC-Pack ODS-A A-302 (YMC Co., Ltd.) column (4.6×150 mm) developed with 10 mM H_3PO_4–10 mM KH_2PO_4–EtOH–AcOEt (42.5 : 42.5 : 10 : 5) (flow rate, 1 ml/min; detection 280 nm) at 40 °C. Detection was made with a Shimadzu SPD-6A spectrophotometric detector at 280 nm. Column chromatography was performed with Diaion HP-20 and MCI gel CHP-20P (75—150 μm) (Mitsubishi), and Toyopearl HW-40 (coarse grade) (Tosoh). Solvents were evaporated under reduced pressure below 40 °C.

Plant Materials The fresh leaves of *E. japonica* cultivated in the herbal garden of our Faculty were collected in April, 1996. A voucher specimen is deposited in the Herbarium, Faculty of Pharmaceutical Sciences, Okayama University.

Extraction and Isolation The fresh leaves (1.0 kg) of *E. japonica* were homogenized in 70% acetone (10 l), and the concentrated solution (1 l) was extracted with ether (1 l×5), AcOEt (1 l×6) and 1-butanol saturated with water (1 l×4), successively. A part (2.8 g) of the AcOEt extract (4.0 g) was chromatographed over Diaion HP-20 (3.3 cm i.d.×40 cm) with H_2O→aqueous MeOH (10%→30%→50% MeOH)→MeOH→70% acetone–H_2O. The 10% MeOH (109 mg) and 30% MeOH (230 mg) eluates were separately purified by rechromatography over MCI-gel CHP-20P (1.1 cm i.d.×40 cm) with aqueous MeOH to give commonly chlorogenic acid (25 mg in total), 3-*O*-caffeoylquinic acid (2 mg), (−)-epicatechin (**5**) (14 mg) and procyanidin B-2 (**6**) (15 mg). The eluate from 50% MeOH (1.3 g) was subjected to column chromatography over Toyopearl HW-40 (coarse grade, 2.2 cm i.d.×60 cm) with aqueous MeOH yielding procyanidin C-1 (**7**) (15 mg) and isoquercitrin (4 mg). The MeOH eluate (727 mg) was similarly submitted to column chromatography over Toyopearl HW-40 (coarse grade, 2.2 cm i.d.×60 cm) with aqueous MeOH to give quercetin (18 mg). The 1-butanol extract (65.8 g) was chromatographed over Diaion HP-20 (2.2 cm i.d.×60 cm) with H_2O containing increasing amounts of MeOH in a stepwise gradient mode. The 50% MeOH eluate (4.4 g) was similarly purified by a combination of column chromatographies over Toyopearl HW-40 (coarse grade) and MCI-CHP-20P to afford 4-*O*-caffeoylquinic acid (15 mg), 4-*O*-*p*-coumaroylquinic acid (11 mg), quercetin 3-*O*-sophoroside (14 mg), kaempferol 3-*O*-sophoroside (28 mg), kaempferol 3-*O*-α-rhamnopyranosyl-(1→2)-β-glucopyranoside (10 mg), 1-*O*-feruloyl β-glucopyranose (10 mg), 4-*O*-β-glucopyranosyl-*cis*-*p*-coumaric acid (1 mg), (2*S*)-naringenin 8-*C*-α-rhamnopyranosyl-(1→2)-β-glucopyranoside (**1**) (40 mg), (2*R*)-naringenin 8-*C*-α-rhamnopyranosyl-(1→2)-β-glucopyranoside (**2**) (3 mg) and cinchonain Id 7-*O*-β-glucopyranoside (**3**) (6 mg). The aqueous extract (48.6 g) was subjected to column chromatography over Diaion HP-20 (5.0 cm i.d.×47 cm) and developed with H_2O→10% MeOH→20%→30%→40%→60%→MeOH→70% acetone–H_2O. A part (2.0 g) of the 60% MeOH eluate (5.0 g) was rechromatographed over Toyopearl HW-40 (coarse grade, 2.2 cm i.d.×34 cm) with 40% MeOH→50%→60%→70%→MeOH acetone–H_2O (7 : 1 : 2→7 : 2 : 1)→70% aqueous acetone. The 60% MeOH eluate (23 mg) was further purified by a combination of column chromatographies over Toyopearl HW-40 (coarse grade) and MCI-CHP-20P to yield cinchonain Ia (**4**) (1 mg). The eluate from 70% aqueous acetone gave a procyanidin oligomer (**8**) (662 mg). The known compounds were identified by direct comparison with authentic specimens or by comparisons of their physicochemical data with those reported in the literature.

(2*S*)-Naringenin 8-*C*-α-Rhamnopyranosyl-(1→2)-β-glucopyranoside (1): A pale yellow amorphous powder, $[\alpha]_D$ +25.3° (*c*=1.0, MeOH). ESI-MS *m/z*: 598 $(M+NH_4)^+$, 581 $(M+H)^+$. HR ESI-MS *m/z* 581.1815 $(M+H)^+$, Calcd for $C_{27}H_{32}O_{14}$+H, 581.1870. UV λ_{max} (MeOH) nm (log ε): 214 (4.39), 225 (sh 4.36), 290 (4.12), 325 (sh 3.64). CD (MeOH) $\Delta\varepsilon$ (nm): +10.5 (218), +1.5 (253), −9.0 (290), +2.3 (329). ^{1}H-NMR (methanol-d_4) δ: 7.42 (2H, d, *J*=8.5 Hz, H-2′, 6′), 6.90 (2H, d, *J*=8.5 Hz, H-3′, 5′), 6.02 (1H, s, H-6), 5.34 (1H, dd, *J*=2, 14 Hz, H-2), 5.27 [1H, br s, rhamnose (rha) H-1‴], 4.85 [1H, d, *J*=9 Hz, glucose (glc) H-1″], 4.07 (1H, t, *J*=9 Hz, glc H-2″), 3.94 (1H, br s, rha H-2‴), 3.82 (1H, br d, glc H-6″), 3.74 (1H, dd, *J*=5, 12 Hz, glc H-6″), 3.68 (1H, dd, *J*=3, 9 Hz, rha H-3‴), 3.54 (1H, t, *J*=9 Hz, glc H-3″), 3.48 (1H, t, *J*=9 Hz, glc H-4″), 3.34 (1H, m, glc H-5″), 3.32 (1H, dd, *J*=14, 17 Hz, H-3), 3.25 (1H, t, *J*=9 Hz, rha H-4‴), 2.79 (1H, dd, *J*=6, 9 Hz, rha H-5‴), 2.64 (1H, dd, *J*=2, 17 Hz, H-3), 0.85 (3H, d, *J*=6 Hz, rha H-6‴). ^{1}H-NMR (acetone-d_6+D_2O) δ: 7.45 (2H, d, *J*=8.5 Hz, H-2′, 6′), 6.90 (2H, d, *J*=8.5 Hz, H-3′, 5′), 5.96 (1H, s, H-6), 5.40 (1H, dd, *J*=2, 14 Hz, H-2), 5.29 [1H, br s, rha H-1‴], 4.83 (1H, d, *J*=10 Hz, glc H-1″), 3.36 (1H, dd, *J*=14, 17 Hz, H-3), 2.59 (1H, dd, *J*=2, 17 Hz, H-3), 0.76 (3H, d, *J*=6 Hz, rha H-6‴). ^{13}C-NMR (acetone-d_6+D_2O) δ: 198.1 (C-4), 166.0 (C-7), 164.6 (C-5), 161.3 (C-9), 158.6 (C-4′), 130.5 (C-1′), 129.1 (2C) (C-2′, 6′), 116.2 (2C) (C-3′, 5′), 104.9 (C-8), 103.2 (C-10), 101.2 (rha C-1‴), 98.0 (C-6), 81.4 (glc C-5″), 80.3 (C-2), 80.1 (glc C-3″), 77.3 (glc C-2″), 74.2 (glc C-1″), 73.1 (rha C-4‴), 72.0 (rha C-2‴), 71.6 (rha C-3‴), 70.3 (glc C-4″), 69.0 (rha C-5‴), 61.1 (glc C-6″), 43.6 (C-3), 17.9 (rha C-6‴).

Acid Hydrolysis of 1 A solution of **1** (1 mg) in 2.5% H_2SO_4 (0.5 ml) was heated in a boiling-water bath for 2 h. After cooling, the reaction mixture was extracted with AcOEt. The aqueous layer was neutralized with Amberlite IR-120 (OH form), and evaporated to dryness. The residue after trimethylsilylation was analyzed by GLC (2% OV-17, column temperature: 170 °C), in which the retention time of the sugar unit was identical with that of the standard L-rhamnose.

(2*R*)-Naringenin 8-*C*-α-Rhamnopyranosyl-(1→2)-β-glucopyranoside (2): A pale yellow amorphous powder, $[\alpha]_D$ +5.3° (*c*=1.0, MeOH). ESI-MS *m/z*: 598 $(M+NH_4)^+$, 581 $(M+H)^+$. HR ESI-MS *m/z* 581.1858 $(M+H)^+$, Calcd for $C_{27}H_{32}O_{14}$+H, 581.1870. UV λ_{max} (MeOH) nm (log ε): 218 (4.43), 290 (4.20), 328 (sh 3.63). CD (MeOH) $\Delta\varepsilon$ (nm): −4.45 (219), −0.6 (254), +5.1 (291), −1.3 (327). ^{1}H-NMR (acetone-d_6+D_2O) δ: 7.39 (2H, d, *J*=8.5 Hz, H-2′, 6′), 6.87 (2H, d, *J*=8.5 Hz, H-3′, 5′), 5.96 (1H, s, H-6), 5.62 (1H, dd, *J*=2, 14 Hz, H-2), 5.27 (1H, br s, rha H-1‴), 4.83 (1H, d, *J*=8 Hz, glc H-1″), 3.10—3.95 (H-3, glc H-2″—H-6″ and rha H-2‴—H-4‴), 2.77 (2H, m, H-3, rha H-5‴), 0.82 (3H, d, *J*=6 Hz, rha H-6‴).

Cinchonain Id 7-*O*-β-Glucopyranoside (3): A pale yellow amorphous powder, $[\alpha]_D$ −37.5° (*c*=1.0, MeOH). ESI-MS *m/z*: 632 $(M+NH_4)^+$, 615 $(M+H)^+$. HR ESI-MS *m/z* 632.1990 $(M+NH_4)^+$, Calcd for $C_{30}H_{30}O_{14}$+NH_4, 632.1979. UV λ_{max} (MeOH) nm (log ε): 284 (3.97), 328 (3.38). CD (MeOH) $\Delta\varepsilon$ (nm): +11.4 (210), −11.2 (228), +5.5 (246), −2.2 (286). ^{1}H-NMR (acetone-d_6+D_2O) δ: 7.07 (1H, d, *J*=2 Hz, H-2′), 6.84 (1H, dd, *J*=2, 8 Hz, H-6′), 6.79 (1H, d, *J*=8 Hz, H-5′), 6.72 (1H, d, *J*=2.5 Hz, H-2″), 6.70 (1H, d, *J*=8.5 Hz, H-5″), 6.56 (1H, s, H-8), 6.51 (1H, dd, *J*=2.5, 8.5 Hz, H-6″), 4.95 (1H, s, H-2), 4.94 (1H, d, *J*=7 Hz, glc H-1‴), 4.72 (1H, br dd, *J*=2, 7 Hz, H-7″), 4.28 (1H, m, H-3), 3.81 (1H, dd, *J*=2.5, 12 Hz, glc H-6‴), 3.64 (1H, dd, *J*=5, 12 Hz, glc H-6‴), 3.37—3.53 (4H, m, glc H-2‴—5‴), 3.02 (1H, br dd, *J*=3, 17 Hz, H-4), 3.02 (1H, dd, *J*=7, 16 Hz, H-8″), 2.88 (1H, dd, *J*=2, 16 Hz, H-8″), 2.83 (1H, dd, *J*=3, 17 Hz, H-4). ^{13}C-NMR (126 MHz, in acetone-d_6+D_2O): δ 168.6 (C-9″), 155.9 (C-9), 154.3 (C-7), 151.5 (C-5), 145.8 (C-3″), 145.4 (2C) (C-3′, 4′), 144.7 (C-4″), 134.6 (C-1″), 131.4 (C-1′), 119.2 (C-6′), 118.5 (C-6″), 115.9 (C-5″), 115.4 (C-5′), 115.2 (2C) (C-2′, 2″), 108.9 (C-6), 103.6 (C-10), 102.6 (glc C-1‴), 100.1 (C-8), 79.7 (C-2), 77.5 (glc C-3‴, 5‴), 74.9 (glc C-2‴), 70.7 (glc C-4‴), 65.9 (C-3), 62.1 (glc C-6‴), 37.3 (C-8″), 34.1 (C-7″), 29.0 (C-4).

Acid Hydrolysis of 3 A solution of **3** (0.5 mg) in 1% H_2SO_4 (0.5 ml) was heated in a boiling-water bath for 2 h. After cooling, the reaction mixture was subjected to a Bond Elut C_{18} cartridge column with H_2O and MeOH. Addition of the H_2O eluate to a solution of Glucose CII-test Wako (Wako Pure Chemicals Industries, Ltd.) produced a reddish color, indicating that a sugar unit of **3** was D-glucose.[23]

Procyanidin Oligomer (8): CD (MeOH) [θ] (nm): -4.7×10^5 (204), $+4.7\times10^5$ (225). ^{13}C-NMR (50 MHz, in methanol-d_4, 45 °C): δ 154.9—157.1 (C-5, 7, 8a), 145.1—145.8 (C-3′, 4′), 132.6 (C-1′), 119.2 (C-6′), 116.1 (C-5′), 115.3 (C-2′), 107.7 (C-8), 102.4 (C-4a), 97.8 (C-6), 79.8 (external C-2), 77.1 (C-2), 72.7 (C-3), 66.8 (terminal C-3), 37.6 (C-4), 30.0 (terminal C-4).

Degradation of 8 with Phloroglucinol A mixture of procyanidin polymer fraction (5 mg) and phloroglucinol (5 mg) in 1% HCl–EtOH (1 ml) was left standing overnight at room temperature. The reaction mixture was analyzed by reversed-phase HPLC to show the peaks due to (−)-epicatechin (**5**) and (−)-epicatechin-(4β→2)-phloroglucinol (**12**) as the main products. Their identity was confirmed by HPLC comparison with authentic samples obtained by similar treatment of **6** and **7**. Small peaks due to **6** and **7** were also detected in the HPLC.

GPC Analysis The fraction of the procyanidin oligomer was acetylated with acetic anhydride and pyridine. After the reaction mixture was poured into water, the acetate deposited was collected and washed with water. The acetate was then analyzed by GPC [column, TSK gel G4000H_{XL} (7.8 mm i.d.×300 mm) (Tosoh); solvent, THF; flow rate, 0.8 ml/min; column temperature, 40 °C]. Molecular weight was calibrated by the standard peracetates of (−)-epicatechin (**5**), procyanidins B-2 (**6**) (procyanidin dimer), C-1 (**7**) (procyanidin trimer), and cinnamtannin A_2 (procyanidin tetramer). An average molecular weight as the acetate (M_n: 5400, M_w: 14800) of the procyanidin oligomer was defined by the equation ($\log MW = -0.67\times t_R + 10.89$).

Assay for Cytotoxic Activity HSC-2 and HSG cell lines were main-

tained as monolayer cultures at 37 °C in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, U.S.A.) in a humidified 5% CO_2 atmosphere. HGF cells were isolated from healthy gingival biopsies from a 10-year-old female, a patient undergoing periodontal surgery, as described previously.[40] Cells between the fifth and seventh passages were used.

The relative viable cell number was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, U.S.A.) method. Near confluent cells grown in a 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson) were incubated for 24 h with or without test compounds. All tannin samples were dissolved in dimethylsulfoxide (DMSO) or 25% DMSO at 50 mg/ml. The final concentration of DMSO in the medium was below 1%. The cells were washed once with phosphate-buffered saline (PBS) and incubated for 4 h with 0.2 mg/ml MTT in DMEM medium supplemented with 10% FBS. After removing the medium, cells were lysed with 100 μl of DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate with Labsystems Multiskan® (Biochromatic) with a Star/DOT Matrix printer JL-10. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Assay for Radical Intensity The radical intensity of test samples was determined at 25 °C in the indicated buffer solution 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, 5 or 6.3×100; time constant, 0.1 s; scanning time, 4 min. Radical intensity was defined as the ratio of peak heights of these radicals to that of MnO.[41,42]

Assay for DNA Fragmentation Cells were lysed with 20 μl lysis buffer [50 mM Tris–HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate]. The solutions were incubated sequentially with 0.5 mg/ml RNase A for 60 min at 50 °C, and 0.5 mg/ml proteinase K for 60 min at 50 °C. DNA was extracted and precipitated by 70% ethanol, then dissolved in TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, pH 8.0). DNA, equivalent to 5×10^6 cells, was applied to 1.8% agarose gel electrophoresis. The DNA fragmentation pattern was examined in photographs taken under UV illumination.[43]

Immunocytochemistry Cultured cells on chamber slides were fixed with 95% ethanol and 5% acetic acid for 10 min at 4 °C. After washing in PBS, each slide was incubated in 2% bovine serum albumin (BSA)–PBS to block a nonspecific reaction for 15 min at room temperature. An appropriately diluted M30 monoclonal antibody (Boehringer Mannheim) was applied to each slide as a primary antibody and incubated for 60 min at room temperature. After washing in PBS, the slides were incubated with biotinylated horse anti-mouse IgG (H and L chains) antibody (1 : 200, Vector) for 30 min at room temperature. The slides were subsequently incubated with streptavidin-peroxidase and colored as described above. To block endogenous peroxidase activity, a 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.05% NaN_3 was used.

Acknowledgements The authors thank the SC-NMR Laboratory of Okayama University for NMR experiments using their Varian VXR-500 instrument.

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