Effects of the Interaction of Tannins with Co-existing Substances. VI.[1] Effects of Tannins and Related Polyphenols on Superoxide Anion Radical, and on 1,1-Diphenyl-2-picrylhydrazyl Radical

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The scavenging effects of twenty-five tannins including low-molecular polyphenols on the superoxide anion radical ($O_2^{\cdot-}$) generated in the hypoxanthine–xanthine oxidase system were estimated by electron spin resonance (ESR) measurements of the adducts formed by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and the radical. The scavenging effects of tannins and related polyphenols having *ortho*-trihydroxyl (pyrogallol) structure [galloyl, hexahydroxydiphenoyl (HHDP) groups in hydrolyzable tannins, galloyl group in acylated proanthocyanidins, and the B-ring of some flavan-3-ols] were stronger than the effects of unacylated proanthocyanidins. The effects of tannins and related polyphenols on the superoxide anion radical were also compared with those on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Each tannin in an ethanol solution of DPPH radical reduced the intensity of the signal of the DPPH radical, and gave a weak signal assignable to a radical derived from that tannin, in a similar way to the appearance of the signal of *dl*-α-tocopherol radical, accompanied with reduction of the signal of DPPH radical, in a mixture of *dl*-α-tocopherol and the DPPH radical. In contrast to the case of the superoxide anion radical, the effects of unacylated proanthocyanidins on DPPH radical were comparable with those of the other types of tannins. The scavenging effects of all of the tannins and related polyphenols tested in the experiments on DPPH radical were stronger than that of *dl*-α-tocopherol.

Keywords tannin; hydrolyzable tannin; condensed tannin; proanthocyanidin; polyphenol; radical scavenger; superoxide anion; xanthine oxidase; 1,1-diphenyl-2-picrylhydrazyl radical; α-tocopherol

Investigations on the mechanism of the inhibitory effects of various tannins and related polyphenols on the lipid peroxidation in rat liver mitochondria and liver microsomes[2,3] have revealed that the scavenging action of tannins on the peroxy radicals participates in the inhibition.[4–6] Recently, tannins and related polyphenols also have been shown to inhibit ocular lens peroxidation induced by the xanthine–xanthine oxidase system,[7] which has frequently been used to generate the superoxide anion radical ($O_2^{\cdot-}$). Other experiments also showed that the polyphenols involving flavonoids[8] and some condensed tannins (galloylated proanthocyanidins)[9] scavenge the superoxide anion radical, formed in the xanthine (or hypoxanthine)–xanthine oxidase system. As the effects of tannins on co-existing substances, including the inhibitory effects on the lipid peroxidation in mitochondria and in microsomes, varied depending on the chemical structures of the tannins,[2–5,7,10] we have now investigated the radical scavenging effects of various types of tannins (*i.e.*, monomeric and dimeric hydrolyzable tannins, and acylated and unacylated condensed tannins) and related polyphenols of low molecular weight.

The scavenging effects of several tannins and related polyphenols on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and the correlation of these effects of some of them with the inhibitory effects on lipid peroxidation in rat liver mitochondria, and in liver microsomes, have been reported.[4,11] On the other hand, the scavenging effects of galloylated proanthocyanidins on the DPPH radical were also reported based on an electron spin resonance (ESR) study.[9] However, the reported scavenging effects of these compounds relative to that of α-tocopherol were much stronger than the effects of the other types of tannins, which were estimated by colorimetry.[4,11] Thus, we re-examined the scavenging effects of various types of tannins and related polyphenols on the DPPH radical, and compared the effects of tannins with that of α-tocopherol.

Experimental

The ESR spectra were measured on a JEOL JES-FE1XG spectrometer

TABLE I. Sources of Tannins and Related Polyphenols

Tannin or related polyphenol	Plant	Reference
1,2,6-Tri-*O*-galloyl-β-D-glucose (1,2,6-trigalloylglucose) (**1**)	*Rosa rugosa*	12*a*
1,2,3,6-Tetra-*O*-galloyl-β-D-glucose (1,2,3,6-tetragalloylglucose) (**2**)	*Cornus officinalis*	12*b*
Pedunculagin (**4**)	*Casuarina stricta*	12*c*
Casuarictin (**5**)	*Casuarina stricta*	12*c*
Tellimagrandin II (**6**)	*Cornus officinalis*	12*d*
Rugosin A (**7**)	*Rosa rugosa*	12*a*, *e*
Furosinin (**8**)	*Geranium thunbergii*	12*f*
Geraniin (**9**)	*Geranium thunbergii*	12*g*
Isoterchebin (**10**)	*Cornus officinalis*	12*d*
Cornusiin A (**11**)	*Cornus officinalis*	12*b*, *h*
Rugosin D (**12**)	*Rosa rugosa*	12*i*
Coriariin A (**13**)	*Coriaria japonica*	12*j*
(−)-Epigallocatechin (**17**)	*Thea sinensis*	12*k*
(−)-Epigallocatechin gallate (**18**)	*Thea sinensis*	12*k*
Procyanidin B-2 (**20**)	*Chaenomeles sinensis*	12*l*
Procyanidin C-1 (**21**)	*Chaenomeles sinensis*	12*l*
(−)-Epicatechin gallate (ECG) (=3-*O*-galloylepicatechin) (**22**)	*Thea sinensis*	12*k*
3-*O*-Galloylepicatechin-(4β→8)-3-*O*-galloylepicatechin (ECG dimer) (**23**)	*Saxifraga stolonifera*	12*l*
3-*O*-Galloylepicatechin-(4β→8)-3-*O*-galloylepicatechin-(4β→8)-3-*O*-galloylepicatechin (ECG trimer) (**24**)	*Saxifraga stolonifera*	12*l*
3-*O*-Galloylepicatechin-(4β→8)-3-*O*-galloylepicatechin-(4β→8)-3-*O*-galloylepicatechin-(4β→8)-3-*O*-galloylepicatechin (ECG tetramer) (**25**)	*Saxifraga stolonifera*	12*m*

(X-band; field modulation, 100 kHz) at room temperature, and the *g*-factor of each radical was estimated from the signals of external manganese dioxide at $g=1.981$ and $g=2.034$. Microwave power and sweep time for the ESR measurements were set at 0.8 mW and 2 min, respectively. A Shimadzu UV-180 spectrophotometer was used for colorimetry.

Materials Tannins and related compounds **1**, **2**, **4**—**13**, **17**, **18**, **20**—**25** (Chart 1) were isolated from medicinal plants as in the previous experi-

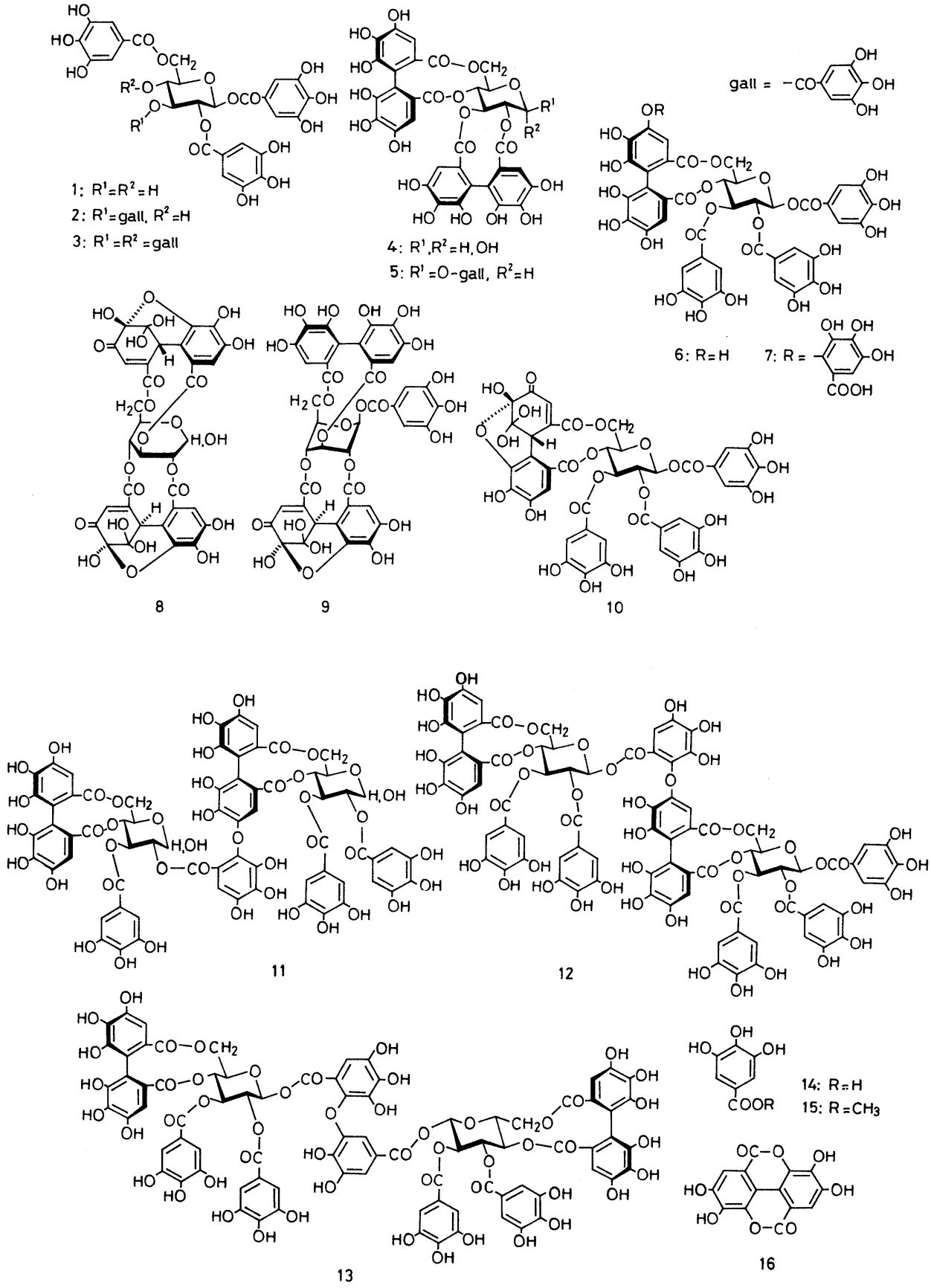

Chart 1

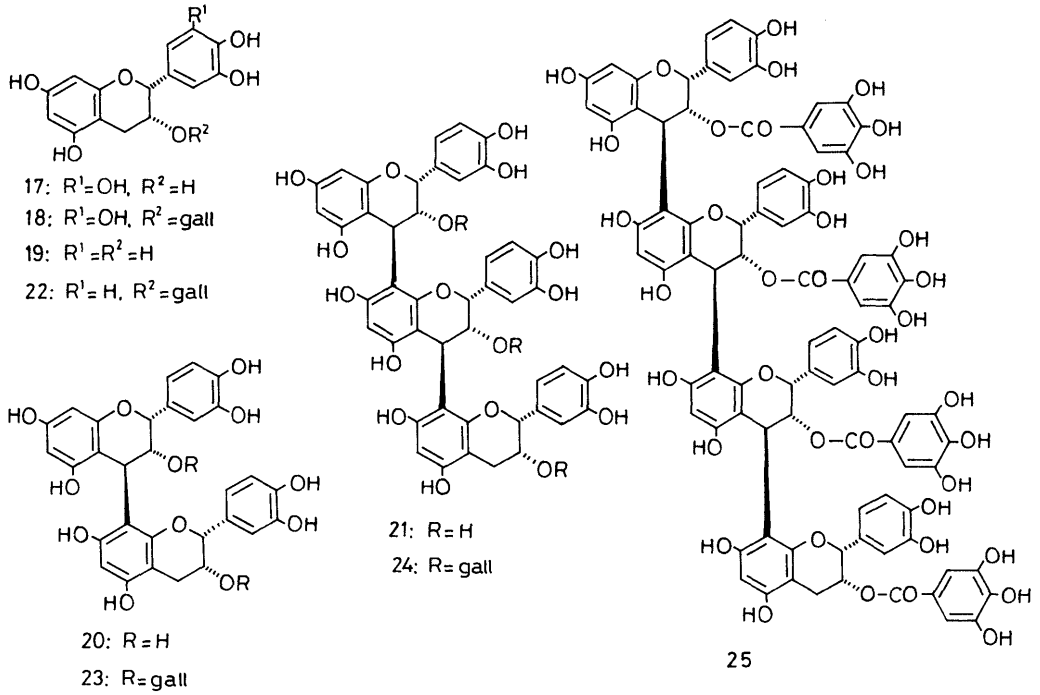

Chart 1 (continued)

ments (Table I).[12] Penta-*O*-galloyl-*β*-D-glucose (**3**) and methyl gallate (**15**) were prepared by methanolysis[13] of tannic acid (Dainippon Pharmaceutical Co.). Other reagents were purchased from the following companies: ellagic acid (**16**), (−)-epicatechin (**19**), DPPH, hypoxanthine and diethylenetriaminepentaacetic acid (DETAPAC), from Sigma Chemical Co.; xanthine oxidase (from cow's milk), from Boehringer Mannheim Co.; gallic acid (**14**) from Tokyo Kasei Co.; 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Daiichi Pure Chemicals Co.; *dl*-*α*-tocopherol from Wako Pure Chemical Industries.

Effects of Tannins and Related Compounds on Superoxide Anion Radical[9,14] A solution of xanthine oxidase [0.3 unit/ml in 0.1 M sodium phosphate buffer (pH 7.8), 50 *μ*l] was added to a mixture of 2 mM hypoxanthine solution (in the same buffer, 50 *μ*l), 11 mM DETAPAC solution (in the same buffer, 35 *μ*l), a solution of each tannin (or related compound) (in the same buffer, 100 *μ*l), and DMPO (15 *μ*l). After mixing for 2 s on a vortex mixer, the mixed solution was placed in a flat cell for the ESR measurement. The sweeping of the spectrometer was started 60 s after the addition of the xanthine oxidase solution. The scavenging effect of the tannin was expressed in terms of EC_{50}, the concentration required to give a 50% decrease in the intensity of the signal of the DMPO adduct of the superoxide anion radical. The coefficient of variation (C.V.) of EC_{50} values for a tested compound was within *ca.* 10%.

Effects of Tannins and Related Compounds on DPPH Radical[9,15] A solution of each tannin or related compound in ethanol (200 *μ*l) was added to an ethanol solution of DPPH radical (30 *μ*M, 200 *μ*l). After mixing for 10 s (unless otherwise stated) on a vortex mixer, the resulting solution was placed in a flat cell. The sweeping for the ESR spectrum was started 4 min after the addition of the tannin solution. The scavenging activity of the tannin was also expressed in terms of EC_{50}, the concentration of the tannin required to give a 50% decrease of the intensity of the signal of the DPPH radical. The coefficient of variation of EC_{50} values for a tested compound was within *ca.* 10%.

Colorimetric Determination of the Scavenging Effect of *dl*-*α*-Tocopherol on DPPH Radical[4,11] To 400 *μ*l of 30 *μ*M DPPH solution in ethanol, 400 *μ*l of an ethanol solution of *dl*-*α*-tocopherol was added, and the mixture was agitated on a vortex mixer for 10 s. The absorbance of the mixed solution at 520 nm was recorded 5 min after the addition of the tocopherol solution.

Results and Discussion

Effects of Tannins on the Superoxide Anion Radical The superoxide anion radical generated by hypoxanthine and xanthine oxidase was detected as the DMPO adduct by ESR spectroscopy. All the tannins and related compounds tested in the present experiments inhibited the appearance of the signal of the DMPO adduct, in a dose-dependent manner, as represented in Fig. 1, which shows the inhibition by penta-*O*-galloyl-*β*-D-glucose (**3**). Figure 1 also shows the appearance of a signal ($g=2.006$, $A_N=15.5$ G, $a_\beta^H=23$ G) which resembles the reported signal[16] of the DMPO adduct

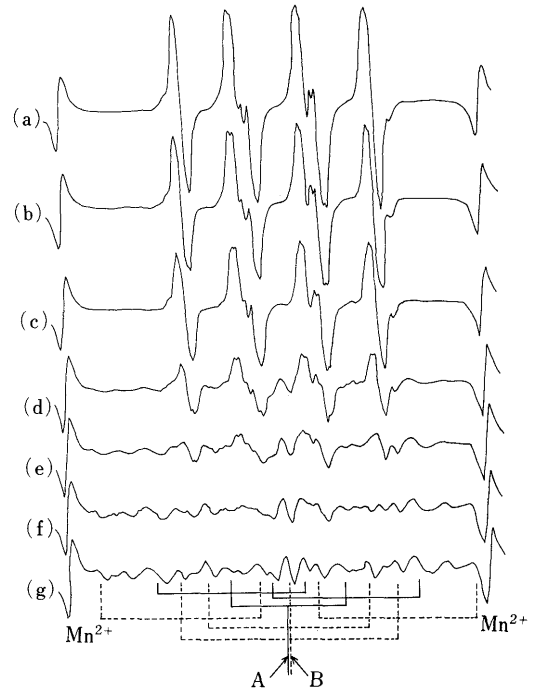

Fig. 1. Effects of Penta-*O*-galloyl-*β*-D-glucose (**3**) on the ESR Spectrum of the DMPO Adduct of Superoxide Anion Radical

The spectra were recorded in the absence [(a)] and in the presence [(b) 1.0×10^{-6} M, (c) 2.5×10^{-6} M, (d) 5.1×10^{-6} M, (e) 1.0×10^{-5} M, (f) 2.5×10^{-5} M, (g) 5.1×10^{-5} M] of penta-*O*-galloyl-*β*-D-glucose. The solid (A) and broken (B) lines below the spectrum (g) indicate the assignments of the hyperfine splitting patterns for the DMPO adduct of a C-centered radical and the hydrogen adduct of DMPO (DMPO-H), respectively.

of C-centered radical, together with the signal of a hydrogen adduct of DMPO (DMPO-H) ($g=2.006$, $A_N=16.6$ G, $a_\beta^H=22.5$ G), and a decrease in the intensity of the signal of the DMPO adduct of the superoxide anion radical. The appearance of the signal assignable to the adduct of the C-centered radical suggested that scavenging of the superoxide anion radical by tannins occurred through the formation of phenoxy radicals from the tannins.

Table II shows that the scavenging effects of hydrolyzable tannins and related compounds on the superoxide anion radical increase with increase of the molecular weight (and hence with increase of the number of phenolic hydroxyl groups in the molecule). In particular, dimeric hydrolyzable tannins having twenty or more phenolic hydroxyl groups strongly inhibit the appearance of the superoxide anion radical. However, ellagic acid (**16**) having four phenolic hydroxyl groups shows activity weaker than that of gallic acid (**14**) [and methyl gallate (**15**)] having three phenolic hydroxyl groups.[17] Therefore, the *ortho*-trihydroxyl (pyrogallol) structures in the hydrolyzable tannins and related polyphenols may be contributing to the strong activities of these compounds.

Table II. Effects of Hydrolyzable Tannins and Related Polyphenols on Superoxide Anion Radical ($O_2^{\cdot-}$) Generated by Hypoxanthine and Xanthine Oxidase

Compound	Molecular weight	Number of phenolic hydroxyl groups	EC_{50} [a] (μM)
Monomeric hydrolyzable tannins			
Galloylglucoses			
1,2,6-Trigalloylglucose (**1**)	636.5	9	4.1
1,2,3,6-Tetragalloylglucose (**2**)	788.6	12	2.8
Penta-*O*-galloyl-β-D-glucose (**3**)	940.7	15	3.4
Ellagitannins			
Pedunculagin (**4**)	784.6	12	2.8
Casuarictin (**5**)	936.7	15	2.8
Tellimagrandin II (**6**)	938.7	15	3.1
Rugosin A (**7**)	1106.8	17	2.7
Dehydroellagitannins			
Furosinin (**8**)	816.6	4	7.5
Geraniin (**9**)	952.7	11	3.0
Isoterchebin (**10**)	954.7	11	3.1
Dimeric hydrolyzable tannins			
Cornusiin A (**11**)	1571.1	23	1.6
Rugosin D (**12**)	1875.3	29	1.7
Coriariin A (**13**)	1875.3	29	1.3
Related polyphenols and others			
Gallic acid (**14**)	170.1	3	6.8
Methyl gallate (**15**)	184.1	3	6.1
Ellagic acid (**16**)	302.2	4	19
Ascorbic acid	176.1	—	32

a) Concentration giving a 50% decrease of $O_2^{\cdot-}$. The values are the means of triplicate experiments.

Table III. Effects of Condensed Tannins and Related Polyphenols on Superoxide Anion Radical ($O_2^{\cdot-}$) Generated by Hypoxanthine and Xanthine Oxidase

Compound	Molecular weight	Number of phenolic hydroxyl groups	EC_{50} [a] (μM)
Flavanols having three hydroxyl groups on the B-ring			
(−)-Epigallocatechin (**17**)	306.3	5	1.6
(−)-Epigallocatechin gallate (**18**)	458.4	8	1.8
Flavanols having two hydroxyl groups on the B-ring and their oligomers			
Epicatechin and its oligomers			
(−)-Epicatechin (**19**)	290.3	4	23
Procyanidin B-2 (**20**)	578.5	8	14
Procyanidin C-1 (**21**)	866.8	12	8.8
Epicatechin gallate and its oligomers			
ECG (**22**)	442.4	5	4.8
ECG dimer (**23**)	882.7	10	5.0
ECG trimer (**24**)	1323.1	15	2.6
ECG tetramer (**25**)	1763.5	20	2.1

a) Concentration giving a 50% decrease of $O_2^{\cdot-}$. The values are the means of triplicate experiments.

Table III shows the scavenging effects of condensed tannins and related monomeric flavan-3-ols on the superoxide anion radical. Although the scavenging activity increases with increase of the molecular weight [epicatechin (**19**) < procyanidin B-2 (**20**) < procyanidin C-1 (**21**); ECG (**22**) < ECG dimer (**23**) < ECG trimer (**24**) < ECG tetramer (**25**)], the activities of galloylated proanthocyanidins [having *ortho*-trihydroxyl groups] were much stronger than those of the unacylated proanthocyanidins having *ortho*-dihydroxyl groups alone. (−)-Epigallocatechin (**17**) having an *ortho*-trihydroxyl group on the B-ring of the flavan skeleton also showed strong inhibition, in spite of its low molecular weight, while the activity of (−)-epicatechin (**19**) having an *ortho*-dihydroxyl group was weak.

Such strong activities of these compounds having the *ortho*-trihydroxyl groups are probably due to the stability of the phenoxy radicals formed in the scavenging reactions.

Effects of Tannins and Related Compounds on the DPPH Radical Prior to the analyses of the effects of tannins and related compounds on the DPPH radical, the scavenging effect of *dl*-α-tocopherol on this radical was re-examined. Upon the addition of *dl*-α-tocopherol solution to a DPPH radical solution, the intensity of the ESR signal of the DPPH radical decreased dose-dependently, and the appearance of a signal due to the formation of another radical

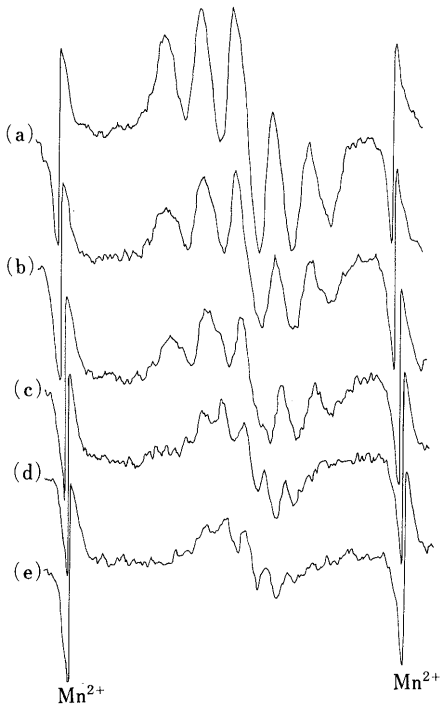

Fig. 2. Effects of *dl*-α-Tocopherol on the ESR Spectrum of DPPH

The spectra were recorded in the absence [(a)] and in the presence [(b) 5.9×10^{-6} M, (c) 7.9×10^{-6} M, (d) 4.7×10^{-5} M, (e) 1.2×10^{-4} M] of *dl*-α-tocopherol.

($g=2.005$) was observed (Fig. 2). This newly appeared signal, as shown in Fig. 2(d) and Fig. 2(e), forms a splitting pattern attributable to the phenoxy radical derived from *dl*-α-tocopherol.[18] The appearance of this signal, in spite of the lability of the tocopherol radical, can be regarded as a consequence of the consumption of oxygen in the closed system (flat cell) by a part of the produced tocopherol radicals.

Although the estimation of the intensity of the signal of the DPPH radical was hampered by the overlapping signal of the tocopherol radical, careful examination led us to find that the concentration of α-tocopherol required to give a 50% decrease of the intensity of the signal of the DPPH radical (EC_{50}) was 8.4 μM [about 6 times of the value for (−)-epicatechin gallate (**22**)], and the scavenging activity of α-tocopherol was therefore not as weak as has been reported.[9] The EC_{50} value of *dl*-α-tocopherol estimated by colorimetry was also 8.4 μM, identical with the value obtained in the ESR experiment.

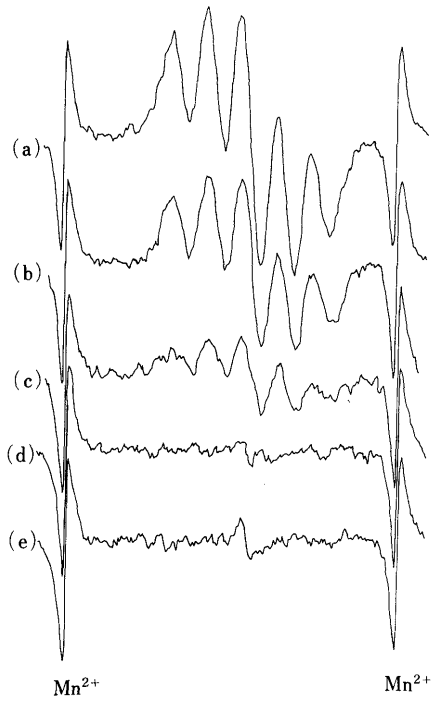

Fig. 3. Effects of Geraniin (**9**) on the ESR spectrum of DPPH

The spectra were recorded in the absence [(a)] and in the presence [(b) 5.0×10^{-7} M, (c) 1.0×10^{-6} M, (d) 2.5×10^{-6} M, (e) 5.0×10^{-6} M] of geraniin.

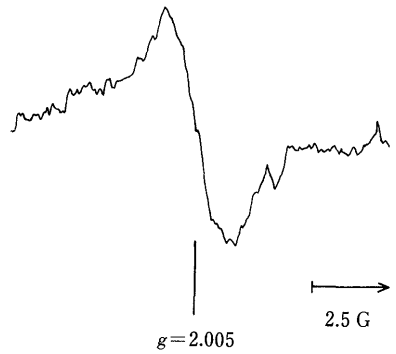

Fig. 4. The ESR Signal Obtained by the Mixing of 15 μM DPPH and 5.8×10^{-5} M Geraniin (**3**)

Final concentrations are given. After mixing for 5 s on a vortex mixer, the resulting solution was placed in a flat cell. The spectral sweep was started 4.5 min after the addition of the geraniin solution.

The change in the ESR spectrum upon the addition of geraniin (**9**) at various concentrations to the DPPH radical is shown in Fig. 3. The intensity of the signal of the DPPH radical decreased with increase of the concentration of geraniin. When the concentration of geraniin was high enough to induce the almost complete disappearance of the signal of the DPPH radical, a new signal of $g=2.005$ was observed [Fig. 3(e) and Fig. 4]. This g=factor suggested that a phenoxy radical was formed in a similar way to the tocopherol radical, although further experiments would be needed for the confirmation of the assignment, since the experimental conditions employed in the present study did not cause the hyperfine splitting of the signal.

The scavenging effects of various types of tannins and related compounds tested in the present investigation are

Table IV. Effects of Hydrolyzable Tannins and Related Polyphenols on Diphenylpicrylhydrazyl Radical

Compound	EC_{50}[a] (μM)
Monomeric hydrolyzable tannins	
Galloylglucoses	
1,2,6-Trigalloylglucose (**1**)	1.0
1,2,3,6-Tetragalloylglucose (**2**)	0.95
Penta-*O*-galloyl-β-D-glucose (**3**)	0.66
Ellagitannins	
Pedunculagin (**4**)	1.0
Casuarictin (**5**)	0.72
Tellimagrandin II (**6**)	0.72
Rugosin A (**7**)	0.87
Dehydroellagitannins	
Furosinin (**8**)	1.4
Geraniin (**9**)	0.82
Isoterchebin (**10**)	0.76
Dimeric hydrolyzable tannins	
Cornusiin A (**11**)	0.71
Rugosin D (**12**)	0.64
Coriariin A (**13**)	0.81
Related polyphenols and others	
Gallic acid (**14**)	2.0
Methyl gallate (**15**)	2.0
Ellagic acid (**16**)	1.7
Ascorbic acid	4.1
dl-α-Tocopherol	8.3

a) Concentration giving a 50% decrease of DPPH radical. The values are the means of duplicate experiments.

Table V. Effects of Condensed Tannins and Related Polyphenols on Diphenylpicrylhydrazyl Radical

Compound	EC_{50}[a] (μM)
Flavanols having three hydroxyl groups on the B-ring	
(−)-Epigallocatechin (**17**)	2.5
(−)-Epigallocatechin gallate (**18**)	1.2
Flavanols having two hydroxyl groups on the B-ring and their oligomers	
Epicatechin and its oligomers	
(−)-Epicatechin (**19**)	3.9
Procyanidin B-2 (**20**)	1.6
Procyanidin C-1 (**21**)	0.85
Epicatechin gallate and its oligomers	
ECG (**22**)	1.5
ECG dimer (**23**)	1.2
ECG trimer (**24**)	0.86
ECG tetramer (**25**)	0.60

a) Concentration giving a 50% decrease of DPPH radical. The values are the means of duplicate experiments.

shown in Tables IV and V. The indicated values confirmed the previous observation[9,11] that tannins scavenged the DPPH radical at lower concentrations than *dl*-α-tocopherol. The present investigation clearly indicates that the increase of molecular weight of tannins and related phenolics, with the accompanying increase of number of phenolic hydroxyl groups, resulted in an increase of the scavenging effects on the DPPH radical. In contrast to the scavenging effects of these compounds on the superoxide anion radical, the compounds having *ortho*-dihydroxyl groups also showed scavenging effects on the DPPH radical, comparable to those of compounds having *ortho*-trihydroxyl groups.

These results suggest that the radical scavenging effects of tannins and related compounds vary depending on the character of the radicals to be scavenged. This variation may have to be taken into consideration in connection with the pharmacological actions of tannin-rich medicinal plants.

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