Effects of Catechins on Superoxide and Hydroxyl Radical

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Superoxide ($O_2^-\cdot$) was reduced by the addition of superoxide dismutase (SOD: $O_2^-\cdot$ scavenger) and catechins. In competitive reactions utilizing different concentrations of spin-trap agent, the IC_{50} values of each sample were changed. With regard to the Cu^{2+}/H_2O_2 and Fe^{2+}/H_2O_2 reaction systems, metal chelater, hydroxyl radical (·OH) scavenger and catechins eliminated the levels of ·OH. For the Cu^{2+}/H_2O_2 reaction systems, the IC_{50} for ·OH scavenger changed, but that for metal chelater and catechins did not. However, for the Fe^{2+}/H_2O_2 reaction system, the IC_{50} for ·OH scavenger and catechins changed, whereas that for metal chelater did not. The ESR signal for free Cu^{2+} was changed by addition of metal chelater and catechins. In the spectrophotometer experiments, it was confirmed that the $CuCl_2$ spectrum was changed by addition of metal chelater and catechins but not by ·OH scavenger. Conversely, the $FeSO_4$ spectrum was not changed by addition of ·OH scavenger or catechins, but was altered by metal chelater. Lipid peroxidation was inhibited by catechins in a concentration-dependent manner.

Therefore, it was suggested that the catechins did not scavenge directly the generated ·OH from the Cu^{2+}/H_2O_2 reaction system, but inhibited the generation of ·OH by acting on the Cu^{2+}/H_2O_2 reaction system. On the other hand, with respect to the ·OH generated from the Fe^{2+}/H_2O_2 reaction system, it was suggested that the catechins had a direct scavenging capacity of the ·OH, but had little chelating activity of iron.

It was confirmed that catechins have the ability to scavenge for $O_2^-\cdot$ as well as ·OH and to inhibit the generation of ·OH by chelation with metal ions.

Key words catechin; hydroxyl radical; superoxide; metal chelation; scavenger

In recent years, among various medical, pharmacological, dental and nutritional substances, Japanese green-tea catechins have been focused upon with great concern. It has been found that catechins have many biological functions such as inhibition of cancer,[1,2] antiviral activity,[3] antioxidative activity[4] and antibacterial activity.[5] Catechin molecules which have iron chelating activity[6] possess many hydroxide elements, and flavonoids to inhibit lipid peroxidation. With regard to active oxygen species, it has been reported that catechins can eliminate active oxygen species such as superoxide ($O_2^-\cdot$) and hydroxyl radical (·OH) which induce lipid peroxidation of cell walls and cause various diseases.[4,7—9] However, previous report did not demonstrate the real scavenging ability of catechins; they did not present the detailed mechanisms underlying the scavenging and/or inhibition of the generation of $O_2^-\cdot$ and ·OH Using one concentration spin-trapping agent for scavenging experiments, as in the previous reports, it should be hard to understand the elimination effect of catechins on active oxygen species. Since the extraction of hydrogen from lipids by ·OH is considered to be the first step of lipid peroxidation and the action of catechins on anti-lipid peroxidation, the reactivity of ·OH must be evaluated in detail.

In the study described here, the properties of catechins as inhibitors and/or scavengers of $O_2^-\cdot$ and ·OH were investigated using different concentration of spin-trapping agents, spectrophotometer and anti-lipid peroxidation experiments.

Experimental

Materials The catechins, theaflan-30 and theaflan-90S were obtained from the Itoen Central Research Institute; their composition is given in Table 1 and Fig. 1. The concentrations of catechins were calculated from percentage of their weight. The chemical species used to generate ·OH were $CuCl_2$, $FeSO_4$ and H_2O_2, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DETAPAC), and 2Na-EDTA was used for metal chelation, and dimethyl sulfoxide (DMSO) was used as an agent that scavenges ·OH; all of these were obtained from Wako Pure Chemicals (Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), which is a trap agent for radicals, was obtained from Dojin Chemicals (Japan). Hypoxanthine (HPX) and xanthine oxidase (XOD) were obtained from Sigma Chemicals (U.S.A.) and Boehringer Mannheim (Germany), respectively. Human superoxide dismutase (SOD), a scavenger of $O_2^-\cdot$ was obtained from Sigma Chemicals.

Human peripheral blood was obtained from healthy volunteers, and the 2-thiobarbituric acid (TBA) used for the analysis of lipid peroxidation was obtained from Wako Pure Chemicals. All other chemicals used were obtained from local sources and were of the highest grade available.

Effects of Catechins on ·OH Elimination The amounts of ·OH generated from the $CuCl_2+H_2O_2$ (Cu^{2+}/H_2O_2) and $FeSO_4+H_2O_2$ (Fe^{2+}/H_2O_2) reaction systems were measured using the ESR spin-trapping method.[10—12] The ·OH-eliminating potency of catechins was analyzed by adding catechins and an ·OH scavenging agent to these reaction systems and making react them competitively with DMPO, the spin-trapping agent. The final concentration of DMPO in 200 μl of the Cu^{2+}/H_2O_2 (1 mM $CuCl_2$/100 mM H_2O_2) reaction mixture was adjusted to either 89.0 or 8.9 mM for this experiment. Catechins and DMSO were added to the reaction mixture. In the Fe^{2+}/H_2O_2 reaction system, the reaction mixture of 1.0 mM $FeSO_4$ solution and H_2O_2 with 0.8 mM DETAPAC was used. The reaction was started by adding H_2O_2, and the generated ·OH was measured with ESR after 50 s. The quantity of ·OH was normalized relative to the standard signal intensity of the manganese oxide marker as:

·OH relative intensity
= ·OH signal intensity (first peak)/Mn^{2+} marker intensity[12]

The ESR spectra were measured using a JEOL JES FR-80 Radical Bio Sensor. The measurement conditions were as follows: microwave power, 8 mW; magnetic field, 335.4±5 mT; sweep time, 2 min; modulation frequency, 100 kHz; and time constant, 0.3 s.

Measurement of the ESR Signal for Cu^{2+} Since the ESR signals of EDTA-chelated Cu^{2+} and free Cu^{2+} are known to be different, in this experiment the influence of catechins on Cu^{2+} was studied using ESR at a temperature of 77 K. For measurement of the free Cu^{2+} ESR signal, the final concentration of $CuCl_2$ was adjusted to 1 mM. Several concentrations of EDTA and/or catechin solutions were mixed with $CuCl_2$, and then their ESR signals were measured.

The measurement conditions were as follows: microwave power, 1.0 mW; magnetic field, 300.0±100 mT; sweep time, 2 or 4 min; modulation frequency, 100 kHz; and time constant, 0.1 s. The coordinate bond of Cu^{2+} and the catechin was assessed from the ESR parameters obtained from the ESR

Table 1. Components of Theaflans

	Theaflan-30 (Lot. 950113)	Theaflan-90S [Lot. 960625(-3)
Epigallocatechin (EGC)	12.30%	2.00%
Epigallocatechin gallate (EGCg)	6.84%	66.26%
Epicatechin (EC)	1.64%	—
Epicatechin gallate (ECg)	1.05%	15.29%
Total concentration of catechins	21.83%	83.55%

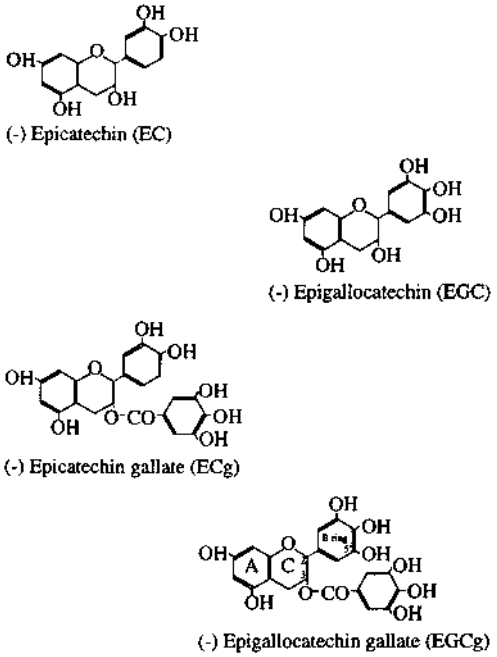

Fig. 1. Chemical Structures of Catechins

signal. Fe^{2+} could not be measured as the diamagnetic.

Measurement of the Absorption Spectra of $CuCl_2$ and $FeSO_4$ The influence of catechins, EDTA and DMSO on the absorption spectra of $CuCl_2$ and $FeSO_4$ were studied. The final concentration of $CuCl_2$ or $FeSO_4$ was 1 mM in the total amount of 1200 μl. Alterations to the absorption spectrum were observed using a Shimadzu spectrophotometer UV-2200 over a 50 s period. The absorption range of Cu and Fe (from $CuCl_2$ and $FeSO_4$) was 202.4—327.4 nm and 208.4—511.0 nm, respectively. The influences of catechins, chelating agents and radical scavenging agents (changes in $CuCl_2$ and $FeSO_4$ orientation) were determined in terms of the changes in instinct absorbance.

Examination of the Effect of Catechins on $O_2^-\cdot$ Elimination $O_2^-\cdot$, generated from a HPX/XOD reaction system, was trapped with DMPO, forming a DMPO–O_2^- adduct, the levels of which were measured with the aid of ESR. Catechins or SOD were added to the reaction system and their effects on $O_2^-\cdot$ generation were studied.[13,14] When measuring the $O_2^-\cdot$ generated from the HPX/XOD reaction system 100 μM HPX, 0.1 U/ml XOD and 667.5 or 66.8 mM DMPO were used. DETAPAC (962 μM), a chelating agent, SOD and/or catechins of various concentrations were added to the reaction mixture. The reaction was started by adding XOD, and measurement by ESR was started after 50 s. All of the $O_2^-\cdot$ signals observed were measured with reference to the first peak. The quantitative change in DMPO–O_2^- adduct was determined by the same method used to quantitate changes in DMPO–OH.

Influence of Catechins on Lipid Peroxidation The effect of catechins on Cu^{2+}/H_2O_2-dependent lipid peroxidation was studied by the TBA-method.[15]

Preparation of Erythrocyte Membranes Human peripheral blood was donated by healthy 20 to 40 years old volunteers (with their consent). Hemoglobin-free erythrocyte membranes were prepared according to the method of Dodge *et al*.[16] Briefly, erythrocytes were hemolyzed and washed with an excess of 10 mM Tris–HCl buffer (pH 7.4), to thoroughly eliminate contamination with hemoglobin. The final precipitate, thus obtained, was suspended in the same buffer. All of these procedures were performed below 4 °C, except where noted.

Formation and Determination of the TBA-Reactive Substance Lipid peroxidation was determined by measuring the amount of TBA-reactive substance formed. The standard reaction mixture for the formation of TBA-reactive substance contained 2.6 mM Tris–HCl buffer (pH 7.4), 100 μM $CuCl_2$, 20 mM H_2O_2 and 300 μl of erythrocyte membrane solution in a final volume of 500 μl. The reaction was initiated by the addition of the membrane fraction. After incubation at 37 °C for the indicated periods of time, the reaction was terminated by the addition of 500 μl of 10% trichloroacetic acid solution. The TBA-reactive substance was then determined at 530 nm by the method of Wilbur *et al*.,[17] using a Hitachi 624 spectrophotometer.

Protein Determination Protein concentration was determined by the method of Bradford[18] with bovine serum albumin as a standard.

Results

Examination of the Effects of Catechins on ·OH Elimination Potency of Eliminating the ·OH Generated from the Cu^{2+}/H_2O_2 Reaction System: When DMPO was added as a spin-trapping agent to the reaction mixture of 1 mM $CuCl_2$/100 mM H_2O_2, the ESR spectrum of the spin adduct which was developed by reacting DMPO with ·OH (DMPO–OH) was detected. This adduct was confirmed to be ·OH because the hyperfine coupling constant was consistent with that given in a previous report ($a_N = a_{H\beta} = 1.48$ mT).[14] A reduction in the signal was observed (data not shown), and this reduction was dependent upon the increase in concentration of DMSO, working as a ·OH scavenger. As a result of adding theaflan-30, theaflan-90S or EDTA to this reaction system, there was a reduction in DMPO–OH adducts that was dependent upon the concentration of the substance added [Fig. 2A)—D)].

An alteration in DMPO–OH results in a change in DMPO concentration, showing that they are involved in a competitive reaction. When different concentrations of DMPO were used, the sigmoid curve was shifted laterally with the addition of DMSO, but not with the addition of other agents. The concentration required to eliminate IC_{50} was determined from the regression line obtained by the least-squares method shown in Fig. 2 (Table 2). As a result, no changes in IC_{50} of theaflan-30 [Fig. 2C)], theaflan-90S [Fig. 2D)] or EDTA [Fig. 2B)] were observed with addition of different concentrations of DMPO; the IC_{50} values of theaflan-30, theaflan-90S and EDTA were: 7.4×10^{-3}, 4.8×10^{-4} and 5.7×10^{-5} M, respectively. However, the IC_{50} of DMSO [Fig. 2A)] was changed by adding different concentrations of DMPO. The IC_{50} values of DMSO at 89.0 and 8.9 mM DMPO were 9.0×10^{-2} and 4.6×10^{-3} M, respectively.

Elimination of the ·OH Generated from the Fe^{2+}/H_2O_2 Reaction System: When DMSO, theaflan-30, theaflan-90S or EDTA was added to the Fe^{2+}/H_2O_2 reaction system, the levels of DMPO–OH adducts were reduced in a concentration-dependent manner. It was also noted that the sigmoid curve shifted laterally with addition of DMSO, theaflan-30 or theaflan-90S, depending upon the concentration of DMPO used, however, a similar shift was not observed with EDTA (data not shown). The IC_{50} values at 89.0 mM DMPO; DMSO, theaflan-30 and theaflan-90S were 9.0×10^{-3}, 1.2×10^{-2} and 1.2×10^{-2} M, respectively. And the IC_{50} values at 8.9 mM DMPO; DMSO, theaflan-30 and theaflan-90S were 1.3×10^{-4}, 7.3×10^{-3} and 2.4×10^{-3} M, respectively. The IC_{50} of EDTA was 1.2×10^{-3} M regardless of DMPO concentration (Table 3).

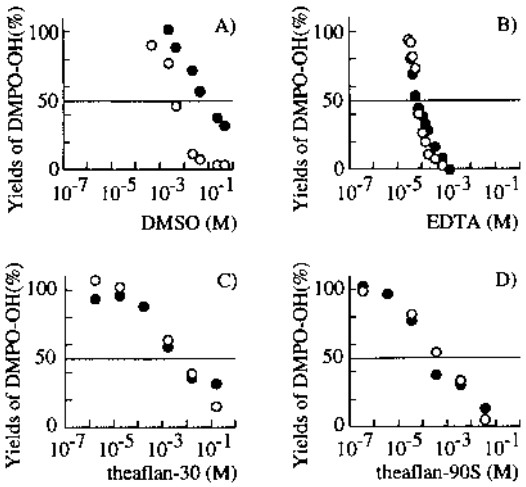

Fig. 2. Elimination Modes of Hydroxyl Radicals by Adding Samples to the Cu^{2+}/H_2O_2 Reaction System

A) DMSO, B) EDTA, C) theaflan-30, D) theaflan-90S, ●: 89.0 mM DMPO, ○: 8.9 mM DMPO.

Table 2. IC_{50} of Samples on Cu^{2+}/H_2O_2 Reaction System

	IC_{50} (M) (DMPO 89.0 mM)	IC_{50} (M) (DMPO 8.9 mM)
DMSO	9.0×10^{-2}	4.6×10^{-3}
EDTA	5.7×10^{-5}	5.7×10^{-5}
Theaflan-30	7.4×10^{-3}	7.4×10^{-3}
Theaflan-90S	4.8×10^{-4}	4.8×10^{-4}

Table 3. IC_{50} of Samples on Fe^{2+}/H_2O_2 Reaction System

	IC_{50} (M) (DMPO 89.0 mM)	IC_{50} (M) (DMPO 8.9 mM)
DMSO	9.0×10^{-3}	1.3×10^{-4}
EDTA	1.2×10^{-3}	1.2×10^{-3}
Theaflan-30	1.2×10^{-2}	7.3×10^{-3}
Theaflan-90S	1.2×10^{-2}	2.4×10^{-3}

Interaction between Cu^{2+} or Fe^{2+} and Catechins Changes in the ESR signals for Cu^{2+} and in the absorption spectra of Cu^{2+} and Fe^{2+} were examined to confirm whether catechins acted on the ·OH generation system or on generated ·OH.

The alteration of the ESR signal of Cu^{2+} is shown in Fig. 3. The ESR signal obtained from the condition with EDTA [Fig. 3B)] shifted to the right as compared with the control [Fig. 3A)], indicating chelation with Cu^{2+}. When activity developed in theaflan-30 and theaflan-90S experiments, the ESR signal for Cu^{2+} changed as the activity caused chelation with Cu^{2+} [Fig. 3C), D)].

The effects of EDTA, DMSO, theaflan-30 and theaflan-90S on the $CuCl_2$ and $FeSO_4$ absorption spectra are shown in Fig. 4. Regarding EDTA, the absorption spectra obtained by reacting $CuCl_2$ and $FeSO_4$ with EDTA shifted more to the right compared with the spectrum of EDTA alone, and moved to the high wavelength side. When DMSO was added, no changes were seen in the $CuCl_2$ or $FeSO_4$ absorption spectra. When theaflan-30 or theaflan-90S was added to $CuCl_2$, the absorption spectrum moved a little, but changes were not

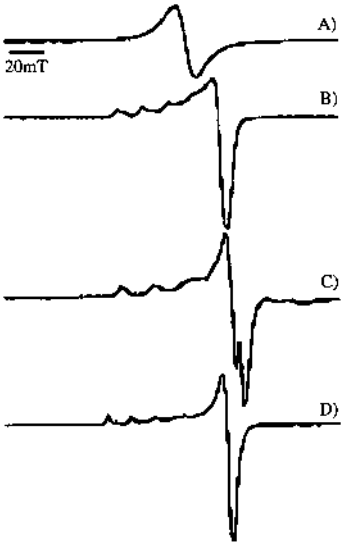

Fig. 3. ESR Signals from 1 mM $CuCl_2$ with/without Each Solution

A) 1 mM $CuCl_2$; B) 1 mM $CuCl_2$+7.5 mM EDTA; C) 1 mM $CuCl_2$+127.5 mM theaflan-30; D) 1 mM $CuCl_2$+26.5 mM theaflan-90S.

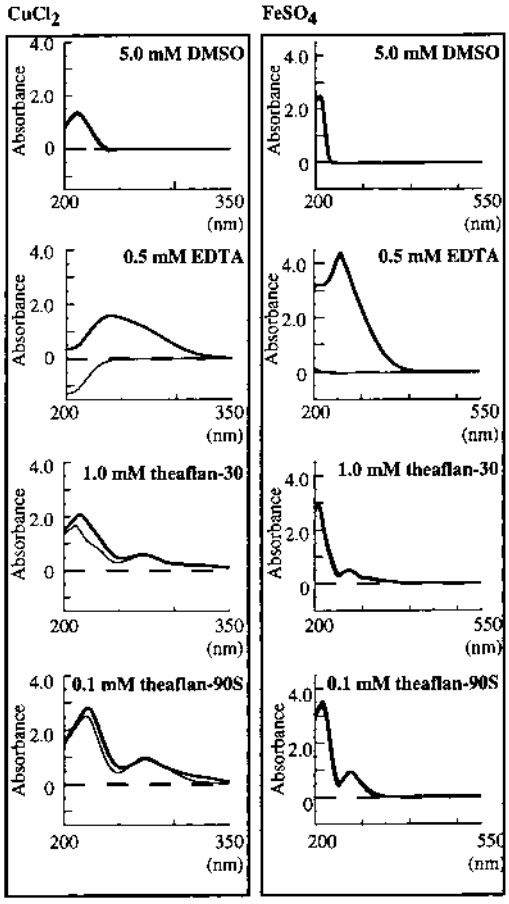

Fig. 4. The Influence of Catechins, EDTA and DMSO on the Absorption Spectra of $CuCl_2$ and $FeSO_4$

– – –, 1 mM $CuCl_2$ or 1 mM $FeSO_4$; ——, sample only; ▬, sample+1 mM $CuCl_2$ or 1 mM $FeSO_4$.

observed with the addition of $FeSO_4$.

Effects of Catechins on O_2^-· Elimination Catechins eliminated the O_2^-· which is generated from the HPX/XOD reaction system in a concentration-dependent manner (data

Table 4. IC_{50} of Samples on HPX/XOD Reaction System

	IC_{50} (M) (DMPO 667.5 mM)	IC_{50} (M) (DMPO 66.8 mM)
SOD	1.2×10^{-9}	1.8×10^{-10}
Theaflan-30	7.4×10^{-5}	8.9×10^{-6}
Theaflan-90S	2.2×10^{-6}	2.5×10^{-7}

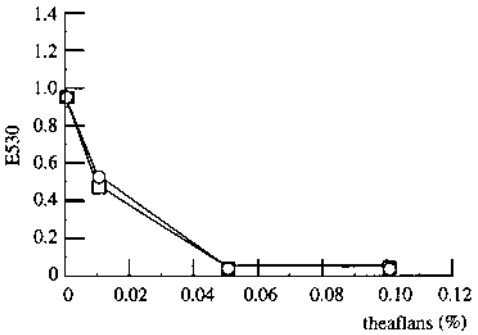

Fig. 5. Concentration-Dependent Effects of Theaflan-30 and Theaflan-90S on Cu^{2+}/H_2O_2-Dependent Lipid Peroxidation

The reaction mixture contained 2.6 mM Tris–HCl buffer, pH 7.4, 100 μM $CuCl_2$, 20 mM H_2O_2 and erythrocyte membrane (31.5 μg protein/100 μl) in a final volume of 500 μl. The reaction was carried out at 37 °C for 120 min with different concentrations of either theaflan-30 or theaflan-90S. –□– theaflan-30, –○– theaflan-90S.

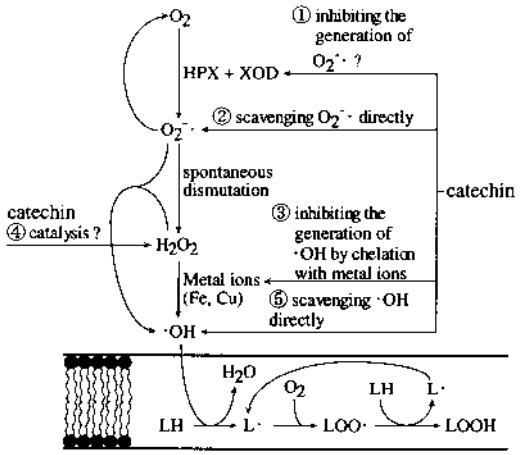

Fig. 6. Possible Course of Inhibition of Lipid Peroxidation by Catechins

not shown). When a competitive reaction was caused after changing the trapping speed by altering the concentration of DMPO, the sigmoid curves for the catechins shifted in a similar manner to that of SOD, a scavenging reagent for $O_2^-\cdot$. At a concentration of 667.5 mM DMPO, the IC_{50} of SOD was 1.2×10^{-9} M in this reaction system, while that of the catechins was 7.4×10^{-5} M for theaflan-30 and 2.2×10^{-6} M for theaflan-90S. At a DMPO concentration of 66.8 mM, the IC_{50} values for SOD, theaflan-30 and theaflan-90S were 1.8×10^{-10}, 8.9×10^{-6} and 2.5×10^{-7} M, respectively (Table 4).

Influence of Catechins on Lipid Peroxidation Lipid peroxidation, which is dependent upon the active oxygen species generated as a result of the Cu^{2+}/H_2O_2 reaction system, was measured by Nagashima's method.[15] Catechins were added to this system to investigate the potency of the anti-lipid peroxidation activity of the catechins (Fig. 5). The degree of lipid peroxidation increased as the concentrations of theaflan-30 and theaflan-90S decreased.

Discussion

The mechanism of the lipid peroxidation reaction induced by active oxygen species, hydroperoxide (LOOH) is considered by the following reactions. Firstly, ·OH extracts H from the lipid (LH), then lipid radical (·L) becomes peroxy radical (·LOO) under the presence of O_2, then ·LOO and extracts H from LH become LOOH. Such reactions continue for lipid peroxidation.[19] It is generally considered that the antioxidative activity of catechins is the result of a total of the five activities of ① to ⑤ shown in Fig. 6.[19,20] For example, it has been reported that catechins have a radical-scavenging ability for ·OH and $O_2^-\cdot$, as shown in Fig. 6 ②, ⑤.

Regarding the ·OH generated from the Cu^{2+}/H_2O_2 reaction system in the present experiment, no change was observed in IC_{50} values when theaflan-30, theaflan-90S or EDTA was added, however, the IC_{50} was changed when DMSO was added as a ·OH scavenger, even though the trapping speed was changed by different concentrations of DMPO. Therefore, it is presumed that the ·OH that was generated from the Cu^{2+}/H_2O_2 reaction system was not scavenged directly by the catechins, as shown in Fig 6 ⑤, but that the catechins inhibited the generation of ·OH, as shown in ③, by acting on the Cu^{2+}/H_2O_2 reaction system.

Furthermore, the results of the ESR analysis, the change in the free Cu^{2+} signal and the change in the $CuCl_2$ spectrum suggest that catechins inhibit the generation of ·OH by chelating the Cu^{2+} (Figs. 3, 4). The reduction in Cu^{2+}/H_2O_2-dependent lipid peroxidation observed following the addition of catechins suggests that they inhibit the ·OH generating system (Fig. 5).

The direct radical scavenging was observed as described in a previous report[8] with respect to the ·OH generated from the Fe^{2+}/H_2O_2 reaction system and the $O_2^-\cdot$ generated from the HPX/XOD reaction system. Since the sigmoid curve shifted and IC_{50} value changed (Tables 3, 4) when the trapping speed was altered by adding different concentrations of DMPO, it is suggested that catechins scavenge directly the ·OH generated from the Fe^{2+}/H_2O_2 reaction system in addition to the $O_2^-\cdot$ generated from the HPX/XOD reaction system (Fig. 6 ②, ⑤). Catechins have no inhibiting effect on the generation of $O_2^-\cdot$ (Fig. 6 ①), furthermore, they have scavenging capacity rather than iron chelating activity.

The catalysis effect (Fig. 6 ④) of catechins to H_2O_2 was not determined in this experiment, and further studies are required on this.

In summary, it is presumed that catechins induce chelation by reacting with metal ions selectively. This possibility is supported by the findings of Morel *et al.*[6] who investigated that the establishment of a relationship between the antioxidant activity of flavonoids and their iron chelating capacity is of major importance in explaining their mechanism of action. This is the first report to demonstrate that catechins have two actions, namely, scavenging ·OH directly and inhibiting the generation of ·OH by chelation with metal ions. These functions also depend on the characterization of metal ions.

As shown in Table 2, the IC_{50} of the ·OH generated from the Cu^{2+}/H_2O_2 reaction system was 7.4×10^{-3} and 4.8×10^{-4} M in the presence of theaflan-30 and theaflan-90S, respectively. Thus theaflan-90S was the more effective inhibitor of the two. Correlating this result with the component ratio of the catechins shown in Table l, theaflan-90S contains about

10—15 times as much epicatechin gallate (ECg) and epigallocatechin gallate (EGCg) as theaflan-30, hence EGCg and ECg are thought to participate strongly in the chelation that occurs in the Cu^{2+}/H_2O_2 reaction system. Therefore, theaflan-30 and theaflan-90S may have different modes of action since they react with metal ions and radicals depending upon their component balance.

Further studies are required to elucidate the properties of individual pure catechins themselves. However, the data presented here clearly show that catechins possess the ability to eliminate $O_2^- \cdot$ and $\cdot OH$, which are responsible for various diseases.

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