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Electron spin resonance spectroscopic evaluation of scavenging activity of tea catechins on superoxide radicals generated by a phenazine methosulfate and NADH system

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Abstract

The scavenging effects of tea catechins on superoxide radicals ($\cdot O_2^-$) generated non-enzymatically by a phenazine methosulfate (PMS) and reduced β-nicotinamide adenine dinucleotide (NADH) system, was studied using an electron spin resonance (ESR) spectrometer along with a spin-trapping agent, 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The presence of 3′, 4′, 5′-trihydroxyl groups attached to the B-ring of the flavan skeleton enhanced the radical scavenging efficiency displayed by the catechin family in comparison to those with 3′, 4′-dihydroxyl groups, and the insertion of a galloyl moiety into three positions of the C-ring exerted a synergistic impact on $\cdot O_2^-$ scavenging activity. Catechin constituents accounted for 86% of the total $\cdot O_2^-$ scavenging capacity of green tea extract, with a particularly high contribution ascribed to (−)-epigallocatechin gallate [(−)-EGCG] (48%) and (−)-epigallocatechin [(−)-EGC] (26%).

Keywords: Catechins; Superoxide radical; Phenazine methosulfate; NADH; ESR; (−)-Epigallocatechin gallate; Green tea

1. Introduction

Reactive oxygen species have attracted a great deal of attention due to their role in the progression of major human degenerative diseases and conditions (Ames, Shigenaga, & Hagen, 1993). Various lines of evidence have shown that superoxide radicals $\cdot O_2^-$ are generated in living cells by one electron reduction of oxygen under physiological conditions, and that they play a harmful role as a precursor of more reactive oxygen species, thus contributing to the pathological process of many diseases (McCord, 1985). It is generally considered, therefore, that the elimination of $\cdot O_2^-$, the origin of various types of active oxygen in the body, is absolutely crucial.

Green and black teas prepared from the leaves of the plant *Camellia sinensis* are the major fluids ingested by many people around the world. There has been renewed interest in the antioxidant potential of green tea, largely because of its ability to eliminate reactive oxygen species (Wiseman, Balentine, & Frei, 1997). Green tea is characterized by its remarkable content of polyphenolic antioxidants such as (−)-epigallocatechin gallete [(−)-EGCG], (−)-epigallocatechin [(−)-EGC], (−)-epicatechin gallate [(−)-ECG] and (−)-epicatechin [(−)-EC] (Fig. 1), whose combined content in green tea leaves accounts for 10–16% of the mass on a dry weight basis (Goto, Yoshida, Kiso, & Nagashima, 1996). In addition, heat treatment of a green tea infusion results in the conversion of the four dominant types of tea catechins into their corresponding epimers, namely (−)-gallocatechin gallate [(−)-GCG], (−)-gallocatechin [(−)-GC], (−)-catechin gallate [(−)-CG] and (−)-catechin [(−)-C], respectively (Seto, Nakamura, Nanjo, & Hara, 1997; Wang & Helliwell, 2000).

Tea catechins have been scientifically investigated to show the excellent $\cdot O_2^-$ scavenging activity (Guo, Zhao, Shen, Hou, Hu, & Xin, 1999; Hatano et al., 1989; Jovanovic, Hara, Steenken, & Simic, 1995; Kashima, 1999; Nanjo, Mori, Goto, & Hara, 1999; Uchida et al., 1987). Our previous work also showed the structure–activity relationship of tea catechins and their epimers

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by a hypoxanthine (HPX) and xanthine oxidase (XOD) system (Unno, Sugimoto, & Kakuda, 2000). This system is frequently used as a generator of $\cdot O_2^-$. However, when assessing the scavenging power of test compounds by this enzymatic system, a possible inhibitory action should be noted on the primary function of the enzyme to oxidize xanthine or HPX to uric acid. There are reports documenting that some kinds of polyphenols including (−)-EGCG, acted, not only as $\cdot O_2^-$ scavengers, but also as xanthine oxidase inhibitors (Aucamp, Gasper, Hara, & Apostolides, 1997; Cos et al., 1998; Lin, Chen, Ho, & Lin-Shiau, 2000; Nagao, Seki, & Kobayashi, 1999). From this viewpoint, one may safely state that the use of a non-enzymatic generation system of $\cdot O_2^-$ can be a better way to assess the $\cdot O_2^-$ scavenging activity of the catechin family. To date, several non-enzymatic methods employing other $\cdot O_2^-$ generating systems have been identified, for example a hydrogen peroxide degradation system in alkaline dimethylsulfoxide (Hyland, Voisin, Banoun, & Auclair, 1983; Yoshimura, Inomata, Nakazawa, Kubo, Yamaguchi, & Ariga, 1999), a riboflavin irradiation system (Guo et al., 1999; Zhao, Li, He, Cheng, & Wenjuan, 1989), and a β nicotinamide adenine methosulfate (NADH) oxidation system by phenazine dinucleotide (PMS; Robak & Gryglewski, 1988; Van Noorden, & Butcher, 1989). Here, the PMS–NADH system has virtues as a convenient non-enzymatic source of $\cdot O_2^-$ under aerobic conditions, aqueous media and physiological pH (Fried, 1975). Electron spin resonance (ESR) studies are often used for a wide variety of free-radical research, and their results have provided much valuable information, offering attractive possibilities of estimating antioxidant potency. Hence, an ESR spectroscopic technique was employed to assess the scavenging activity of tea catechins and their corresponding epimers on $\cdot O_2^-$ generated by the PMS–NADH system, as being different, in an in vitro experimental model, from the HPX–XOD system. The aim was to gain a deeper insight into the $\cdot O_2^-$ scavenging potentials.

2. Materials and methods

2.1. Chemicals

Tea catechins [(−)-EGCG, (−)-EGC, (−)-ECG and (−)-EC], their corresponding epimers [(−)-GCG, (−)-GC, (−)-CG and (−)-C], (+)-epicatechin [(+)-EC] and (+)-catechin [(+)-C] were purchased from Funakoshi, Ltd. (Tokyo, Japan). Gallic acid (GA) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). L-ascorbic acid, PMS and NADH were from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Superoxide dismutase (SOD) from bovine milk, DMPO as the spin-trapping agent of high purity, and diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic

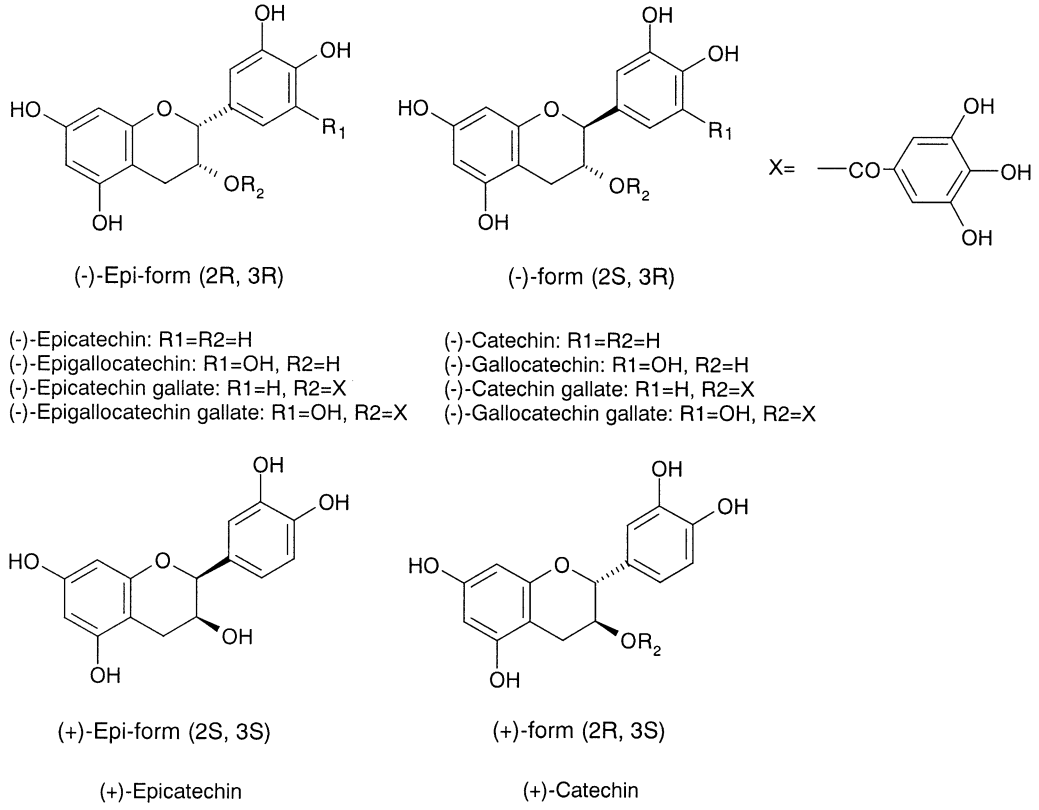

Fig. 1. Chemical structures of the catechin family.

acid (DTPA) as the metal-chelating agent were purchased from Toyobo, Co. Ltd. (Tokyo, Japan), Labotec, Ltd. (Tokyo, Japan), and Dojindo Laboratory (Kumamoto, Japan), respectively. All other chemicals used were of reagent grade.

2.2. Experimental design

In the first experiment, using commercial standards of tea catechins, the possible involvement of structural features of the catechin family in $\cdot O_2^-$ scavenging activity was studied by the PMS–NADH system with the ESR technique. Values for the $\cdot O_2^-$ scavenging activity of the catechin family were converted to values representing the equivalent activity of known concentrations of SOD standard.

Green tea contains other polyphenolic antioxidants, such as quercetin, kaempferol and myricetin (Hertog, Hollman, & van de Putte, 1993), and non-polyphenolic antioxidants such as L-ascorbic acid (Liang, Liu, Xu, & Hu, 1990) and pheophytin (Higashi-Okai, Taniguchi, & Okai, 2000). Accordingly, the second experiment was carried out to estimate the contribution of the catechin family to the total $\cdot O_2^-$ scavenging activity of green tea extract, calculated by a summation of their individual contributions based on the proportion of catechin content in green tea extract.

2.3. Measurement of $\cdot O_2^-$ scavenging activity

The $\cdot O_2^-$ was essentially generated by the PMS–NADH system, according to the method of Robak and Gryglewski (1988) with a slight modification, and was detected by ESR spectrometry in conjunction with DMPO. All the chemicals, except samples, were dissolved in 0.1 M sodium phosphate buffer (pH 7.4). The solution of PMS was cooled in ice avoiding light. Fifteen microlitres of 8.9 M DMPO, 35 μl of 5.5 mM DTPA, 50 μl of 0.6 mM NADH, and 50 μl of sample solution dissolved in distilled water, were put into a test tube. To this mixed solution, 50 μl of 0.12 mM PMS was added. After vortex stirring, the mixture was transferred into a flat cell. The recording of ESR spectra started exactly 40 s after the addition of PMS solution. The signal intensities were evaluated by comparing the peak height of the first DMPO-O_2^- signal relative with the peak height of the Mn^{2+} signal as an internal standard.

2.4. ESR spectrometer

ESR spectra were recorded on a computerized JES-FR30 spectrometer (JEOL Ltd., Tokyo, Japan). The ESR spectroscopic conditions were set as follows: magnetic field, 335.6±5 mT; power, 4 mW; sweep time, 2 min; modulation, 79 μT; amplitude, 200; time constant, 0.1 s.

3. Results and discussion

3.1. ESR spectrum

When DMPO was added to the reaction mixture, $\cdot O_2^-$ was detected as DMPO–O_2^- spin adducts in the ESR spectrum. With regard to the consumption of $\cdot O_2^-$, the antioxidant competes with a spin-trapping agent, DMPO; thus a decrement in spin adducts reflects its scavenging ability. Fig. 2A shows a typical ESR spectrum of DMPO–O_2^- spin adducts obtained under controlled conditions conducted by the PMS–NADH system. The pattern of ESR signals detected by this reaction system was almost the same as that by the HPX–XOD system. The addition of (−)−EGCG to the reaction mixture, at various concentrations, caused attenuation of the DMPO–O_2^- signal intensities in a dose-dependent fashion (Fig. 2B to 2C).

3.2. $\cdot O_2^-$ scavenging activity of SOD

According to the report by Sekine, Masumizu, Maitani, and Nagai (1998), the $\cdot O_2^-$ scavenging activity of tested materials was estimated from the concentrations required to reduce the relative peak height of DMPO–O_2^- by 50% (IC_{50}). Calculation of IC_{50} was performed by the following equation:

$$V = (I_{\text{control}}/I_{\text{test}}) - 1 \quad (1)$$

where I_{control} and I_{test} are the spin adduct signal intensities observed in the absence and presence of the tested

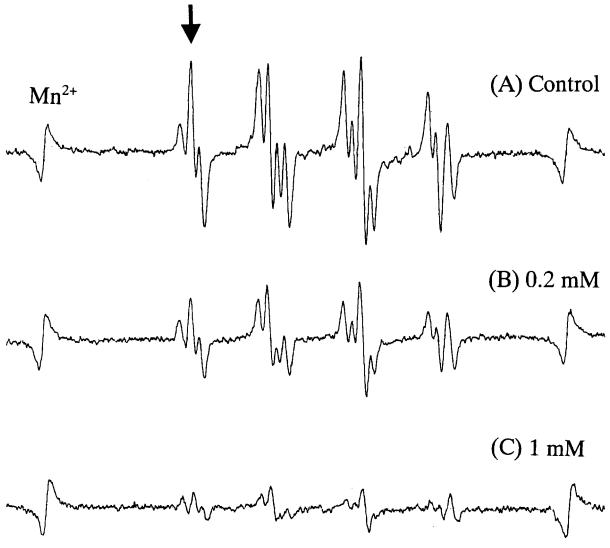

Fig. 2. Electron spin resonance (ESR) spectra of $\cdot O_2^-$ with 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO-O_2^-) observed upon the addition of EGCG solution to the phenazine methosulfate and reduced β-nicotinamide adenine dinucleotide (PMS–NADH) system. The arrow shows the first peak of DMPO–O_2^- spin adduct.

antioxidant, respectively. After V values were plotted using the least-squares method, IC_{50} values of the tested substance were calculated at $V=1$ in triplicate measurements. The relationship between the added concentration of SOD and the V values on the formation of DMPO–O_2^- is shown in Fig. 3. From the linear regression line for the O_2^- scavenging activity of SOD, the IC_{50} value of SOD was computed to be 77 ± 5 unit/ml ($n=3$).

3.3. O_2^- scavenging effect of catechin family

The O_2^- scavenging activity of tested samples was expressed in terms of SOD-equivalent activity (unit/μmol) by the following equation:

$$\text{SOD-equivalent activity (unit/}\mu\text{mol)} = 77\ \text{(unit/ml)}/IC_{50}\ \text{of tested sample (}\mu\text{mol/ml)} \quad (2)$$

Fig. 4 illustrates the linear curves relating to the correlation between the concentrations of each catechin and V values in the PMS–NADH system. The addition of catechin solution to the reaction system increased the V value in a dose-dependent fashion. The calculated IC_{50} values of tested samples (the concentration needed to give $V=1$) are summarized in Table 1, and O_2^- scavenging power is expressed as SOD-equivalent activity. Among the samples tested, L-ascorbic acid had the most effective O_2^- scavenging power. The O_2^- scavenging activity of the four dominant catechins was in the order: (−)-EGCG > (−)-EGC > (−)-ECG > (−)-EC. As regards the structural relationship of tea catechins to O_2^- scavenging activity, (−)-EGCG, (−)-EGC, (−)-GCG and (−)-GC, which have 3′, 4′, 5′-trihydroxyl groups (pyrogallol type) in the B-ring of the flavan skeleton, were more potent radical scavengers than (−)-ECG, (−)-EC, (−)-CG and (−)-C, which have 3′, 4′-dihydroxyl groups (catechol type) in the B-ring. An investigation of the structural importance of the 3′, 4′, 5′-trihydroxyl groups in the B-ring for O_2^- scavenging activity, in the PMS–NADH system, reinforces our previous results in the HPX–XOD system (Unno, Sugimoto, & Kakuda, 2000). The O_2^- scavenging activity responds broadly to the tenet that the structures with more hydroxyl groups in the B-ring exert the greater scavenging activity. Additionally, the fact that the scavenging activity of (−)-EGCG, (−)-GCG, (−)-ECG and (−)-CG on O_2^- generated by the PMS–NADH system was strong, and that of (−)-EGC, (−)-GC, (−)-EC and (−)-C was relatively weak, may be due to the structural modification by ester linkage via the 3-hydroxyl group to GA. The SOD-equivalent values of the four dominant catechins [(−)-EGCG, (−)-EGC, (−)-ECG and (−)-EC] were almost the same as those of their corresponding epimers [(−)-GCG, (−)-GC, (−)-CG and (−)-C]. Table 1 also shows that there was not much difference in SOD-equivalent value among (−)-EC, (−)-C, (+)-EC and (+)-C. These results led us to the conclusion that the stereochemical configuration of flavan-3-ol will not function as an advantageous structure for the O_2^- scavenging activity. Moreover, the SOD-equivalent activities of (−)-EGCG (216 ± 3 unit/μmol) and (−)-ECG (88 ± 10 unit/μmol) were not equal to the sum of (−)-EGC (103 ± 11 unit/μmol) and GA (60 ± 6 unit/μmol) and the sum of (−)-EC (14 ± 2 unit/

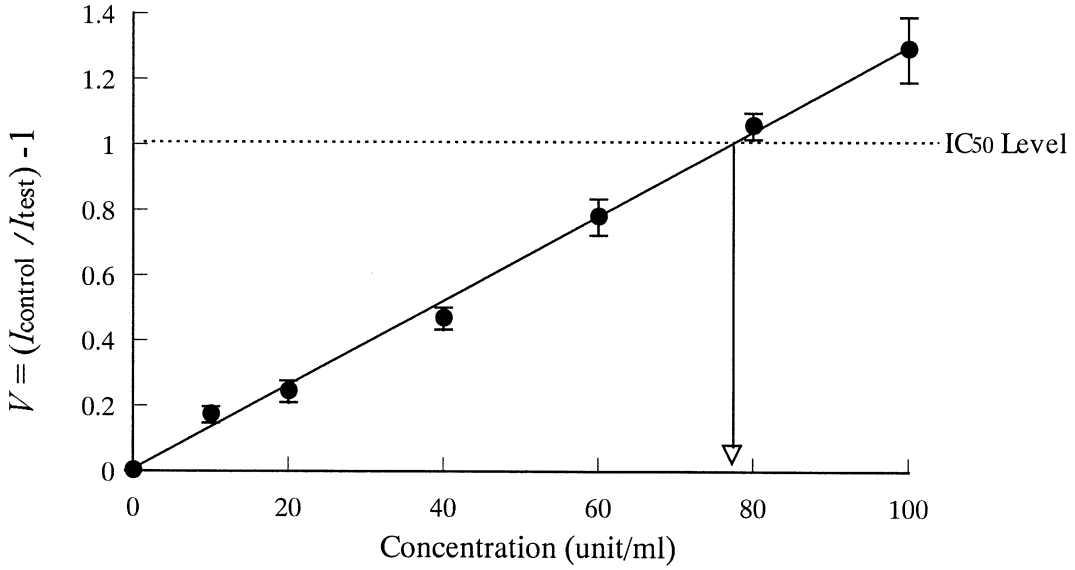

Fig. 3. The relationship between the added concentration of superoxide dismutase (SOD) and V value on the O_2^- scavenging activity in the phenazine methosulfate and reduced β-nicofinamide adenine dinucleotide (PMS–NADH) system, as measured by the electron spin resonance (ESR) spin-trapping technique. Each point represents the mean±S.D. from triplicate measurements.

μmol) and GA (60±6 unit/μmol), respectively. On the basis of these outcomes, the most persuasive explanation is that the $\cdot O_2^-$ scavenging reaction of (−)-EGCG or (−)-ECG is not due to the respectively independent actions of their B-ring and galloyl moiety, and that, preferably, the presence of a galloyl moiety in catechin molecules may synergistically enhance the elimination power against $\cdot O_2^-$.

3.4. Total $\cdot O_2^-$ scavenging activity of green tea extract

As already described, catechins are particularly important candidates for understanding the $\cdot O_2^-$ scavenging properties of green tea. The $\cdot O_2^-$ scavenging capacities of tea catechins in relation to their concentrations in green tea extract are used to calculate their contribution to the $\cdot O_2^-$ scavenging potential of that extract. A commercial product of spray-dried green tea extract (Itoen, Ltd., Tokyo, Japan), contained 29.0% catechins on a dry weight basis, composed of (−)-EGCG, 12.5%; (−)-EGC, 9.5%; (−)-ECG, 2.5%; (−)-EC, 1.7%; (−)-GCG, 0.4%; (−)-GC, 1.9%; (−)-CG, not detected (ND); and C, 0.5% (Table 2). The measurement of the total $\cdot O_2^-$ scavenging activity of green tea extract gives an SOD-equivalent value of 123±9 unit/mg of dry matter ($n=3$). The $\cdot O_2^-$ scavenging capacity of each catechin in relation to their concentrations in green tea extract is used to calculate their

Table 1
Assessment of $\cdot^-O_2^-$ scavenging activity of tested samples in the PMS and NADH system[a]

Test materials	Configuration	IC_{50} value(mM)	SOD-equivalent activity(unit/μmol)
(−)-Epigallocatechin gallate	2R, 3R	0.36±0.01	216±3
(−)-Epigallocatechin	2R, 3R	0.75±0.08	103±11
(−)-Epicatechin gallate	2R, 3R	0.89±0.09	88±10
(−)-Epicatechin	2R, 3R	5.75±0.75	14±2
(−)-Gallocatechin gallate	2S, 3R	0.39±0.07	205±43
(−)-Gallocatechin	2S, 3R	0.71±0.06	109±9
(−)-Catechin gallate	2S, 3R	1.05±0.11	74±7
(−)-Catechin	2S, 3R	4.82±0.28	16±1
(+)-Epicatechin	2S, 3S	6.06±0.62	13±1
(+)-Catechin	2R, 3S	6.08±0.50	13±1
Gallic acid		1.30±0.13	60±6
L-Ascorbic acid		0.33±0.02	237±15
Superoxide dismutase		77±5[b]	

[a] Results are represented as mean±S.D. from triplicate measurements.
[b] unit/ml.

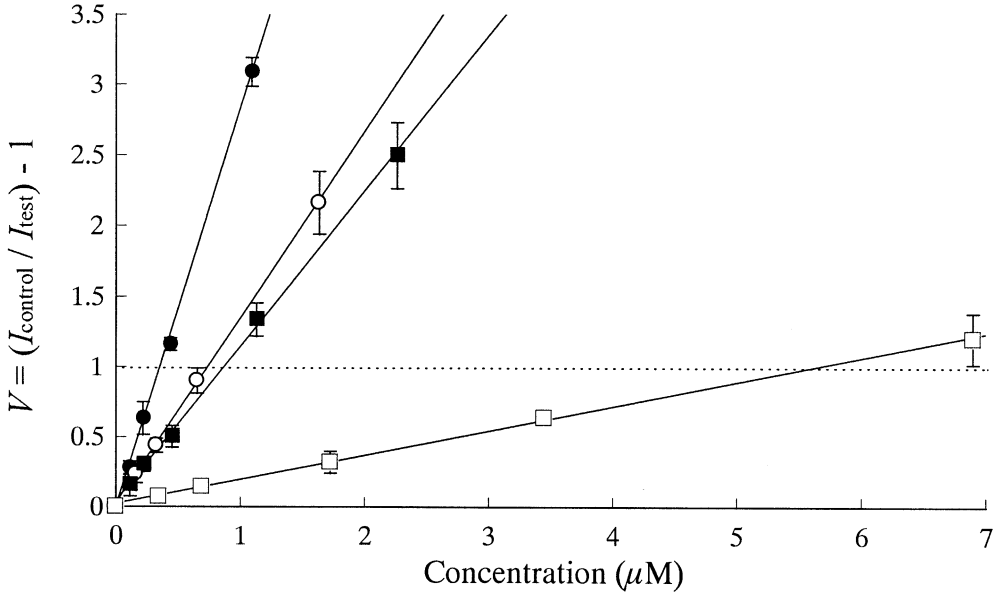

Fig. 4. Scavenging effect of four dominant catechins contained in green tea on $\cdot O_2^-$. Each point represents the mean±S.D. from triplicate measurements. (●) EGCG; (○) EGC; (■) ECG; (□) EC.

Table 2
Contribution of catechins to the $\cdot O_2^-$ scavenging activity of green tea extract

Catechins	Composition of green tea extract[a] (% of dry matter)	Measured $\cdot O_2^-$ scavenging activity[b] (unit/μmol)	(unit/mg)	Calculated contribution to scavenging activity[c] (unit of relative proportion)	Actual contribution to scavenging activity of green tea extract[d] (%)
(−)-Epigallocatechin gallate	12.5	216	472	59	48
(−)-Epigallocatechin	9.5	103	337	32	26
(−)-Epicatechin gallate	2.5	88	199	5	4
(−)-Epicatechin	1.7	14	48	1	1
(−)-Gallocatechin gallate	0.4	205	448	2	1
(−)-Gallocatechin	1.9	109	356	7	6
(−)-Gatechin gallate	N.D.	74	167	–	–
(−)-Catechin	0.5	16	55	0	0
	=29.0			=106	=86

The contribution of each catechin to the $\cdot O_2^-$ scavenging activity was calculated as follows: Column [a] depicts the composition of catechins in green tea extracts as percent dry weight; columns [b] shows the measured $\cdot O_2^-$ scavenging activity of individual catechins as units/mg and units/μmol (from Table 1); column [c] gives the calculated contribution of each catechin to the $\cdot O_2^-$ scavenging activity in green tea extracts, derived from the relative proportions in column [a] ; column [d] indicates the calculated percentage contribution of each catechin to the totally measured $\cdot O_2^-$ scavenging activity in green tea extract (123±9 unit/mg).

predicted contributions to the total $\cdot O_2^-$ scavenging potential of the extract, and the computed result of SOD-equivalence is 106 unit/mg of dry matter as shown in Table 2. From the measured results of green tea extract, 86% of the $\cdot O_2^-$ scavenging activity of such extract can be accounted for by the catechin constituents from the calculated data. Especially, (−)-EGCG (48%) and (−)-EGC (26%) had great impacts on the $\cdot O_2^-$ scavenging effect of the extract. A similar observation seems to apply to different types of radical species. Gardner, McPhail, and Duthie (1998) reported that 77% and 82% of the total scavenging activity of green tea was due to catechin constituents against Fremy's salt radical and galvinoxyl radical, respectively, and Salah, Miller, Paganga, Tijburg, Bolwell, and Rice-Evans (1995) also found 78% against 2, 2′-azinobis- (3-ethylbenzthiazoline-6-sulfonic acid) radical cation. Thus, such a high contribution of catechin components to the total antioxidant activity of green tea extract is now widely accepted. In this respect, the scavenging effect of green tea extract against a variety of radical species, as well as $\cdot O_2^-$, can be explained quite naturally as due to the catechin family.

4. Conclusions

In summary, this study elucidates the scavenging activity of tea catechins on $\cdot O_2^-$ generated non-enzymatically by the PMS–NADH system using the ESR spectroscopic spin-trapping technique. Provided that the polyphenolic compounds has an inhibitory effect on XOD, the results obtained in this PMS–NADH system are more informative for an accurate assessment of $\cdot O_2^-$ scavenging activity.

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