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Superoxide Scavenging Activities of Sixty Chinese Medicines Determined by an ESR Spin-Trapping Method Using Electrogenerated Superoxide

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Superoxide-scavenging activities of 60 kinds of Chinese herbal medicines were determined accurately by an electron spin resonance (ESR) spin-trapping technique using 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) as a spin-trapping reagent. As a source of superoxide in this method, superoxide generated by one-electron reduction of the oxygen molecule in dimethyl sulfoxide solution was used. As a result of these studies, very powerful scavenging activity was found in Chinese medicines for inflammation, diseases of blood circulation and for tumors.

Key words——Chinese medicines; superoxide; scavenging activity; ESR; electrolysis of oxygen

INTRODUCTION

It is well known that reactive oxygen species are closely involved with various pathological events such as aging,[1,2] cancer,[3] inflammation,[4] and certain diseases on blood circulation.[5] Many researchers have therefore shown that the intake of natural antioxidants results in a reduction in the risk from these diseases.[6] From this viewpoint, traditional Chinese medicines have attracted special interest as a natural antioxidant source.[7]

Superoxide (O_2^-) is the first reactive oxygen radical produced by one-electron reduction of molecular oxygen in metabolism processes and the source of other radicals. Therefore, it is very important to examine the O_2^- scavenging ability of antioxidants. Recently, hypoxanthine-xanthin oxidase (HPX-XOD) has widely been used as a O_2^- source for evaluating O_2^- scavenging abilities of antioxidants by using the ESR technique.[8] Ohsugi et al. examined the O_2^- scavenging activities of 70 kinds of traditional Chinese herbal medicines by this method and reported very important results.[7] However, this HPX-XOD method has the following problem. When the test sample contains an XOD inhibitor, in the HPX-XOD system the ESR signal intensity of the spin adduct (DMPO-O_2^- or DMPO-OOH) is decreased not only by the reaction of the sample of interest with O_2^- but also by inhibition of XOD. As a result, the O_2^- scavenging ability of a sample containing an XOD inhibitor is overestimated.[9,10] Some researchers have reported the XOD inhibition by some flavonoids, which are often contained in plants.[11] Therefore, XOD inhibition is a factor that can not be ignored in evaluating the O_2^- scavenging activities of Chinese herbal medicines. In fact, our preliminary experiments showed XOD inhibition for the major Chinese herbal medicines.

As described in the literature,[12] electrochemical reaction of molecular oxygen gives the most pure O_2^- solution in DMSO solvent. In this study, we used the ESR spin-trapping technique combining the electrolytically generated superoxide as a source of O_2^- and 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) as a spin-trapping reagent. In this case the competition reaction of O_2^- is between the sample and DMPO without any O_2^--generating inhibition. Therefore, it is thought that the true values of O_2^- scavenging abilities can be obtained by this electrolytically generated O_2^- method. Using this method, we investigated the O_2^- scavenging activities of 60 kinds of Chinese medicines, which have already been shown to be effective for anti-aging, diminishing inflammation, and promoting blood circulation and have anti-tumor properties, according to pharmacology and the traditional clinical applications.[13]

EXPERIMENTS

Materials Dried Chinese medicines examined in this study were identified and supplied by Zhejiang Drug Administration (China). The Latin names of these Chinese medicines and their original plants are

listed in Table 1. Phosphate buffer solution (PBS) and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Industries, Ltd. Japan. Copper-zinc superoxide dismutase (SOD, 3000 units, 0.8 mg from human erythrocytes) was obtained from Sigma Chemical Co. USA. Spin trapping reagent, 5, 5′-Dimethyl-1-pyrroline 1-oxide (DMPO) was obtained from Labotec Co., Ltd. Japan. Tetra-n-butylammonium tetrafluoroborate (Bu_4NBF_4) was obtained from NACALAI TESQUE, INC, Japan. The reagents except for DMSO were used without further purification.

Preparation of samples and superoxide solution A dried sample (0.2 g) was broken to small pieces and steeped in 8 ml PBS for 30 min, then homogenized at 13000 rpm for 4 min with Nissei Biomixer (Nihon Seiki Kaisha, Ltd., Japan). These conditions were determined by a series of trial experiments for the extractability (unpublished data). The suspended solutions of Chinese medicines were separated at 6000 rpm for 6 min with KUBOTA 1025 mini centrifuge (Sigma, Laborzentrifugen, Germany). Each supernatant was diluted with PBS to suitable concentrations for ESR analysis. Three milliliters of original supernatant of each medicine was evaporated under reduced pressure below 50℃. The obtained solid was weighed to calculate the extract yield of each sample. All treatments, except for the experiments for extract yields, were performed at room temperature (about 25℃).

Refined DMSO was prepared by drying with Molecular Sieves 4A for one day and distillation at 60 ℃ and 7 mmHg. Supporting electrolyte solution was prepared with 0.05 M Bu_4NBF_4 and the newly refined DMSO in an electrolysis cell of 8 ml volume. When oxygen gas was bubbled into the DMSO solution, O_2^- could be generated by electrode reaction using three electrodes. Silver wire, platinum wire, and glassy carbon (GC10) disc electrodes were used as the reference electrode, counter electrode, and working electrode, respectively. In order to get quantitative results, controlled potential electrolysis was carried out at −1.2 V *vs.* silver electrode for about 10 min with potentiostat/galvanostat HA-211 (Hokuto Electron-

Table 1. Superoxide scavenging activities of Chinese Medicines determined by ESR spin-trapping[a)]

No.	Scientific name	Original plant	Function[b)]	Extract yield /%	SOD-like activity /units g^{-1}
1	*Rhei rhizoma exsiccata*	Rheum palmatum L.	III	36.6	1.2×10^5
2	*Salviae miltiorrhizae radix*	Salviae miltiorrhiza Bunge.	I, IV	29.2	1.1×10^5
3	*Rabdosiae macrocalyx radix*	Rabdosia macrocalyx (Dunn) Hara	IV	9.6	8.6×10^4
4	*Rhei rhizoma conquitum*	Rheum palmatum L.	II	46.6	7.1×10^4
5	*Rabdosiae amethystoidis radix*	Rabdosia amethystoides (Benth.) Hara	IV	7.0	5.7×10^4
6	*Rehmanniae radix conquita*	Rehmannia glutinosa (Gaertn.) Libosch	I	71.8	5.4×10^4
7	*Epimedii radix*	Epimedium sagittatum (Sieb. et Zucc.) Maxim	I	23.6	5.2×10^4
8	*Nandinae fructus*	Nandina domestica Thunb.	II, IV	16.0	4.2×10^4
9	*Epimedii herba*	Epimedium sagittatum (Sieb. et Zucc.) Maxim	I	16.0	3.7×10^4
10	*Polygoni multiflori radix* (processed)	Polygonum multiflorum Thunb.	I	29.8	3.0×10^4
11	*Rehmanniae radix exsiccata*	Rehmannia glutinosa (Gaertn.) Libosch	III	67.4	2.7×10^4
12	*Lysionoti herba*	Lysionotus pauciflora Maxim.	IV	21.2	2.6×10^4
13	*Astragali complanati semen*	Astragalus complanatus R. Br.	I, III	45.2	2.1×10^4
14	*Polygoni multiflori radix exsiccata*	Polygonum multiflorum Thunb.	III	10.6	1.8×10^4
15	*Cistanches herba*	Cistanche deserticola Y. C. Ma	I, III	52.8	1.6×10^4
16	*Rhodiola rhizoma*	Rhizoma sachalinesis A. Bor	I, III	31.4	1.5×10^4
17	*Rabdosiae amethystoidis herba*	Rabdosia amethystoides (Benth.) Hara	IV	7.6	1.5×10^4
18	*Fagopyri rhizoma*	Fagopyrum dibotrys (D. Don) Hara	IV	6.6	1.4×10^4
19	*Ilicis chinensis semen*	Ilex chinensis Sims	I	10.2	9.9×10^3

Table 1. (continued)

No.	Scientific name	Original plant	Function[b]	Extract yield /%	SOD-like activity /units g^{-1}
20	*Ginkgo folium*	Ginkgo biloba L.	III	29.6	9.4×10^3
21	*Rabdosiae macrocalyx herba*	Rabdosia macrocalyx (Dunn) Hara	IV	12.4	7.7×10^3
22	*Ligustri fructus*	Ligustrum lucidum Ait.	I	14.4	5.7×10^3
23	*Schisandrae fructus*	Schisandra chinensis (Turcz.) Baill.	I	42.0	5.5×10^3
24	*Cuscutae semen*	Cuscuta chinensis Lam.	I	35.8	4.5×10^3
25	*Tetrastigmatis radix*	Tetrastigma hemsleyanum Diels et Gilg	III	4.8	4.4×10^3
26	*Scrophulariae radix*	Scrophularia ningpoensis Hemsl.	III	64.8	4.2×10^3
27	*Morindae officinalis radix*	Morinda officinalis How.	I	63.0	4.0×10^3
28	*Actinidiae radix*	Actinidia chinensis planch	IV	4.8	3.1×10^3
29	*Phytolaccae radix*	Phytolacca acinosa Roxb.	III	36.0	3.0×10^3
30	*Fici pumilae sterile syconium*	Ficus pumila L.	II, III	8.8	2.6×10^3
31	*Cnidii rhizoma*	Ligusticum chuanxiong Hort.	I, II	39.8	2.0×10^3
32	*Leonuri herba*	Leonurus heterophyllus Sweet	II	10.8	1.8×10^3
33	*Angelicae sinensis radix*	Angelica sinensis (Oliv.) Diels	I, III	60.8	1.6×10^3
34	*Carthami flos*	Carthamus tinctorius L.	II	36.8	1.5×10^3
35	*Apiotis radix*	Apios fortunei Maxim	III	8.4	1.5×10^3
36	*Ginseng radix*	Panax ginseng C. A. Mey.	I	30.0	1.0×10^3
37	*Sojae nigrum semen*	Glycine max (L.) Merr.	III	35.6	1.0×10^3
38	*Dioscoreae rhizoma*	Dioscorea opposita Thunb	I	2.0	9.9×10^2
39	*Lycii fructus*	Lycium chinense Mill.	I	56.8	8.5×10^2
40	*Polygonati rhizoma*	Polygonatum sibiricum Delar. ex Redoute	I	71.6	6.3×10^2
41	*Ginseng radix rubra*	Panax ginseng C. A. Mey.	I	49.0	5.5×10^2
42	*Curcumae rhizoma*	Curcuma aromatica Salisb.	II	17.4	5.5×10^2
43	*Polygonati odorati rhizoma*	Polygonatum odoratum (Mill.) Druce	I	63.2	5.4×10^2
44	*Pseudostellariae radix*	Pseudostellaria heterophylla (Miq.) Pax ex Pax et Hoffm.	I	44.8	4.7×10^2
45	*Zizyphi spinosi semen*	Zizyphus jujuba Mill.	I	17.2	4.4×10^2
46	*Glycyrrhizae radix*	Glycyrrhiza uralensis Fisch.	I, III	20.2	3.7×10^2
47	*Hoelen*	Pachyma hoelen Rumph	I	ND[c]	3.6×10^2
48	*Broussonetiae fructus*	Broussonetia papyrifera (L.) Vent	I, III	7.6	3.4×10^2
49	*Ophiopogonis tuber*	Ophiopogon japonicus Ker.-Gawl.	I, IV	76.8	3.3×10^2
50	*Codonopsitis lanceolatae radix*	Codonopsis lanceolate Benth. et Hook.	I, III	39.6	3.0×10^2
51	*Glehniae radix cum rhizoma*	Glehnia littoralis Fr. Schmidt ex Miq.	I	28.4	1.7×10^2
52	*Astragali radix*	Astragalus mongholicus Bunge	I, III	26.6	1.2×10^2
53	*Codonopsitis radix*	Codonopsis pilosula (Franch.) Nannf.	I	56.8	1.1×10^2
54	*Ganoderma*	Ganoderma lucidum (Leyss. ex Franch.) karst	I	27.6	9.6×10^1
55	*Changii Praeparata radix*	Changium smyrnioides Wolff	I, III	28.6	8.6×10^1
56	*Achyranthis radix*	Achyranthes bidentata Blume	II	56.6	8.0×10^1
57	*Persicae semen*	Prunus persica var. davidiana Maxim	II	37.0	7.6×10^1
58	*Actinidiae valvatae radix*	Actinidia valvata Dunn	III	12.2	6.6×10^1
59	*Ledebouriellae radix*	Ledebouriella seseloides Wolff.	III	20.2	6.3×10^1
60	*Margaria*	Pteria martensii Dunker	I	ND[c]	1.5×10^1

a) The activities are given as the average of triplicate measurements.
b) According to the traditional Chinese medicinal dictionary[13], functions of these medicines can be indicated as follows: I : anti-aging, II : diminishing inflammation, III : promoting blood circulation, IV: anti-tumor.
c) Not detected.

ics Industry Co., Japan), which was controlled accurately by monitoring the amount of electricity. The concentration of superoxide in DMSO solution amounted to 0.14 mM, which was obtained from nitro blue tetrazolium (NBT) test[14] by measuring the absorption at 520 nm. The generated O_2^- solution was used within 30 minutes.

ESR measurements In a test tube, 200 μl of SOD solution or extract solution of Chinese medicine and 20 μl of 9.2 M DMPO were mixed, then 200 μl of the mixed solution was mixed with 40 μl of the DMSO solution of superoxide using a home-made mixing apparatus, which made it possible to inject two kinds of solution into a test tube simultaneously. The mixture was transferred to an ESR analyzing capillary, which was then inserted into the cavity of a JES-FR30 free radical monitor (X-band ESR spectrometer, JEOL Co. Ltd., Japan). Thirty five seconds after mixing, ESR measurement of the DMPO-OOH signal at the lowest field was started and this signal was recorded at 15 s intervals for a total of five times at 25℃. The microwave power was 4 mW, the magnetic field 336.4 $\pm$5 mT, the sweep time 2 min, the modulation amplitude 0.1 mT (100 kHz), and the time constant 0.1 s. After recording the ESR spectra, the signal intensity of DMPO-OOH at the lowest magnetic field was normalized as a relative height against the internal standard signal (g=2.033) of Mn(II) doped in MgO.

RESULTS AND DISCUSSION

ESR spin-trapping In the ESR spin trapping, the generated O_2^- was trapped by DMPO to form the spin adduct, DMPO-O_2^- or DMPO-OOH, which showed the ESR signal in Fig. 1a. When the extract solution of Chinese medicine or SOD solution was added to the system, the signal intensity of DMPO-OOH decreased (shown in Fig. 1b for 0.032 mg/ml *Salviae miltiorrhizae radix* as an example) with an increase in the extract or SOD concentration, because of the competition reaction between DMPO and the scavengers. The competition reaction is illustrated in Fig. 2. In general, Eq. (1) is valid for SOD and also other scavengers.[15]

$$I_0/I-1=(k_{scav}[\text{Scavenger}])/(k_{DMPO}[\text{DMPO}]) \quad (1)$$

I_0 and I indicate DMPO-OOH signal intensities in the absence and the presence of SOD or scavenger, respectively, and k_{scav} and k_{DMPO} represent the second order rate constant for the reaction of scavenger and DMPO with superoxide, respectively.

It was found that the signal intensity of DMPO-OOH itself decreased according to the rate equation of a first order reaction, expressed by $I_{t=t}=I_{t=0}\exp(-kt)$, where $I_{t=t}$ is the relative intensity of the signal for DMPO-OOH against the intensity of the Mn(II) marker at time $(t)=t$, $I_{t=0}$ the relative intensity of

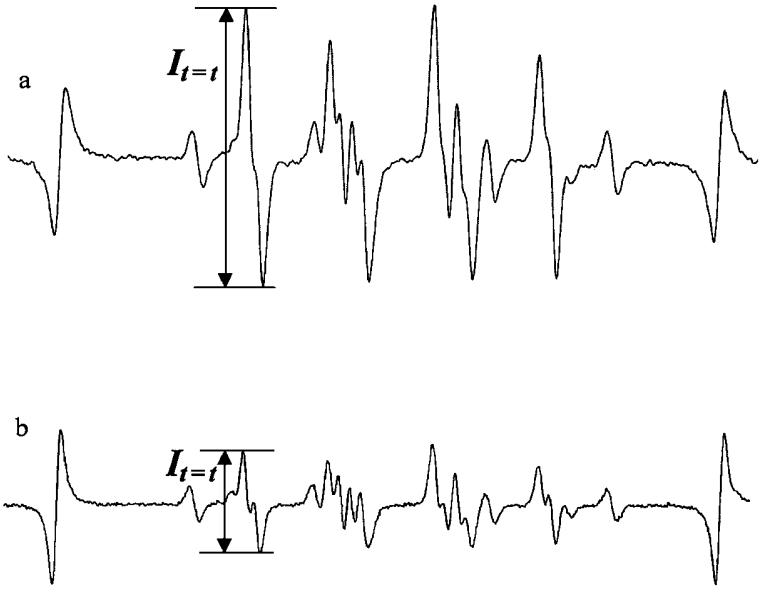

Fig. 1. Typical ESR Spectra of DMPO Adduct Trapping Superoxide (a) without and (b) with 0.032 mg/ml *Salviae miltiorrhizae radix* at 30 s after mixing the test solution

From each time course of $I_{t=t}$, the signal intensities of I_0 and I at time zero were calculated. The spectra were recorded at room temperature. The magnetic field increases from left to right. The two signals on both sides of the spectrum are the third and fourth manganese (II) signals used as markers.

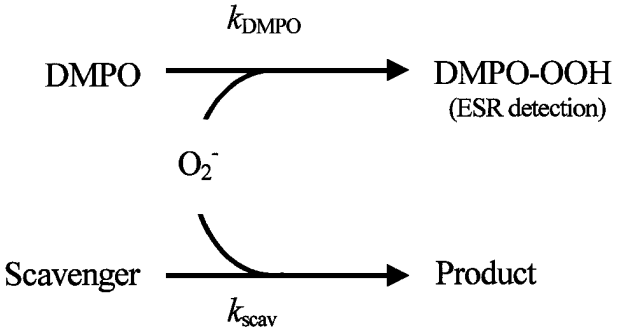

Fig. 2. Scheme of the Competitive Reaction of Superoxide to DMPO and Scavenger

DMPO-OOH at time $(t)=0$, k the first order rate constant. In general, the decrease of DMPO-OOH is due to the instability of DMPO-OOH itself. The value of k was found to be about 1.2×10^{-2} s^{-1} in the control solution. In addition, this decrease is accelerated if the test solution contains a reducing substance such as ascorbic acid. Therefore, in order to avoid this disturbance, $I_{t=0}$ was calculated as the comparable signal intensities of control buffer solution (I_0) or scavenger samples (I). The relationship between the $I_0/I-1$ value and the sample concentration could be expressed by the linear relationship curve as given in Eq. (1).

In this study, we express the O_2^- scavenging activities of Chinese medicines with SOD-like activity.[15] The linear calibration curve of SOD was obtained with a correlation coefficient of 0.9975 as shown in Eq. (2).

$$I_0/I-1=0.663\,[\mathrm{SOD}] \qquad (2)$$

In this equation, [SOD] is SOD final concentration with units/ml dimension.

It has been shown that SOD does not inhibit XOD in the HPX-XOD system.[15] To compare the two superoxide-generating systems, the calibration curve of SOD was also established in the HPX-XOD system containing DMSO. As expected, the rate constant of SOD obtained in the two systems remained constant. This fact confirmed the reliability of this electrolytically generated superoxide system.

Superoxide scavenging activity of Chinese medicines The SOD-like activity of the sample can be determined by substituting the $I_0/I-1$ value of the sample into Eq. (2) SOD calibration curve. The results are indicated in Table 1, where the values of SOD-like activity against one gram weight of dried original medicine are presented and are arranged in order value of activity.

In Table 1, extract yields are also given. Since Chinese medicines showed quite different yields of extracts, there is the question of whether or not the extract amounts played a crucial role in O_2^- scavenging. The answer can be read easily from Table 1, that is, the scavenging activity did not always increase with an increase in extract yields. Therefore, it is thought that the crucial factor deciding the O_2^- scavenging activity is more based on the components in Chinese medicines rather than the extract amount.

The effect of DMSO on the O_2^- scavenging activities of Chinese medicines was examined. At double the concentration of DMSO the obtained values of O_2^- scavenging activities per gram of the sample selected at random was the same as the data in Table 1. From this result, it is thought that the O_2^- scavenging ability obtained using the electrolytically generated superoxide system represents the real value.

The Chinese medicines tested here were classified into four types from clinical effects; medicines for aging, inflammation, diseases of blood circulation, and tumors as given in Table 1. From the SOD-like activities, we found that among the 60 kinds of medicines, the one with the most powerful superoxide scavenging activity is *Rhei rhizoma exsiccata*, which is a medicine promoting blood circulation. The anti-tumor medicines, *Salviae miltiorrhizae radix* and *Rabdosiae macrocalyx radix*, occupy the second and third positions in terms of order of superoxide scavenging activities, respectively. Among the ten medicines with the most powerful superoxide scavenging activity, anti-tumor medicines are represented four times. Furthermore, the medicines for aging are also represented four times, but their general effectiveness is not stronger than the former tumor medicines. These results showed that there might be a relation between the superoxide scavenging activity of Chinese medicine and its function. As reported in recent literature, the assimilation and metabolism of natural substances such as food,[16] vitamin E,[17] quercetin[18] and flavonoids,[19,20] play a key role in the actual physiological events. Therefore, in order to confirm the relationship between the superoxide scavenging activity of Chinese medicine and its function, it is necessary to investigate the assimilation and metabolism of the components of Chinese medicines, and this is a subject of future research.

Finally, this research provides accurate data on

the O_2^- scavenging activity of traditional Chinese medicines. We expect that traditional Chinese medicines, especially those for inflammation, diseases of blood circulation and tumors, will become a potential source of excellent antioxidants.

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要旨

スピントラップ試薬として5,5-ジメチル-1-ピロリン1-オキシド（DMPO）を用いる電子スピン共鳴（ESR）スピントラップ法により，60種類の植物由来漢方薬のスーパーオキシド（O_2^-）消去能が正確に決定された．このESR法におけるO_2^-源として，ジメチルスルホキシド中で酸素分子の一電子還元によって生成させたO_2^-を使用した．測定の結果，炎症，血管疾病，および，腫瘍に効果のある生薬の中に，極めて強いO_2^-消去活性を示すものを見出した．