Chem. Pharm. Bull. 40(2) 304—307 (1992)   Vol. 40, No. 2

Scavenging Effects of Dihydric and Polyhydric Phenols on Superoxide Anion Radicals, Studied by Electron Spin Resonance Spectrometry

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We investigated the scavenging effects of dihydric, trihydric phenols and related polyphenols on superoxide anion radicals (O_2^-) generated from the hypoxanthine–xanthine oxidase system by ESR (electron spin resonance) spin trapping technique using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a trapping reagent. The O_2^- scavenging activities of non-substituted dihydric phenols were in the order of pyrocatechol $\gg$ hydroquinone $\gg$ resorcinol. Trihydric phenols, pyrogallol and gallic acid, which have phenolic OH groups at *ortho* positions, had stronger activities than pyrocatechol; phloroglucinol, on the other hand, which has phenolic OH groups at *meta* positions, had much weaker effect. Polyhydric phenols like quercetin and hematoxylin, which have catechol groups, had significant O_2^- scavenging activities. In order to clarify the physico-chemical property of these phenolic compounds which determines their O_2^- scavenging activities, we investigated the effects of six 4-substituents of pyrocatechol on O_2^- scavenging effects. The effects of these substituents were well correlated with their redox potentials, indicating that O_2^- scavenging activities of catechol derivatives are controlled by their electron donor activities.

We also observed the scavenging effects of these phenolic compounds on O_2^- from a different source: potassium superoxide (KO_2) solubilized in dimethyl sulfoxide by 18-crown-6. ESR analysis in this system revealed that one-electron oxidized products of these compounds were concomitantly formed accompanied by the reduction of O_2^-. The results revealed that phenolic compounds directly react with O_2^- to form stable radicals, which account for the O_2^- scavenging activities of the compounds.

Keywords phenolic compound; superoxide anion radical; ESR spin trapping; radical scavenger; redox potential

It has been revealed that various phenolic antioxidants like flavonoids, tannins and α-tocopherol scavenge active oxygen species such as superoxide anion radicals[1–3] and lipid peroxy radicals[4–6] and thus prevent cell damage.[7,8] They are viewed as promising therapeutic drugs for free radical pathologies such as ischemia, anemia and arthritis.[4] Although there have been many studies on their free radical scavenging reactions, the relationship between their chemical structure and their scavenging activities is still unknown. In particular, little work has been done to reveal the physico-chemical properties which determine the activities of phenolic antioxidants, except on tocopherol derivatives.[9,10] Therefore, in this study we investigated by ESR (electron spin resonance) spectrometry the effects of dihydric and trihydric phenols as well as some polyhydric phenols, which have the dihydric phenol moieties as their basic structures, on superoxide anion radicals generated in the hypoxanthine (HPX)–xanthine oxidase (XOD) system in order to clarify their activities toward superoxide anion radicals (O_2^-). Through this work we attempted to clarify the relationship of O_2^- scavenging activities of these phenolic compounds and their chemical structures and physico-chemical properties. Moreover, since the scavenging mechanism of these phenols is unknown, by ESR spectrometry we also studied the scavenging mechanism of these phenols against O_2^- prepared from potassium superoxide (KO_2) solubilized with 18-crown-6.[1,11]

Experimental

Materials All phenolic compounds, KO_2 and 18-crown-6 were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Nakarai Tesque, Inc. (Kyoto, Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was from Labotec, Inc. (Tokyo, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) was from Dojindo Laboratories (Kumamoto, Japan). HPX and XOD were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Effects of Phenolic Compounds on Superoxide Anion Radicals Generated in HPX–XOD System The O_2^- scavenging activities of the compounds were evaluated by ESR spectrometry according to the method of Mitsuta *et al.*[12] A 0.12 unit/ml XOD was added to 0.4 mM HPX in 80 mM sodium phosphate buffer (pH 7.0) which contains 1.1 mM DTPA and various concentrations of phenolic compound. Almost simultaneously DMPO was added to the solution to a final amount of 100 mM and mixed on a vortex mixer. 1 min after the mixing, ESR spectra were measured with a JEOL JES-FE1XG (X-band) spectrometer with 100 kHz field modulation frequency and 1 G modulation amplitude at an output power of 8 mW unless otherwise indicated. Mn(II) in MnO was used as a standard. All experiments were done at room temperature (21 °C).

Reaction of Phenolic Compounds with O_2^- from KO_2 in Dimethyl Sulfoxide A 0.5 mM of KO_2 was dissolved in dimethyl sulfoxide (DMSO) in the presence of 5 mM 18-crown-6. O_2^- scavenging activities of phenolic compounds, which were also added to the DMSO solution, were evaluated from ESR spectra measured at 77 K after 1 min incubation of the compounds with O_2^- at 21 °C. Identification of radicals of phenolic compounds generated in the reaction was done from ESR spectra measured at 21 °C.

Results and Discussion

Effects of Phenolic Compounds on Superoxide Anion Radicals Generated in HPX–XOD System We first investigated the O_2^- scavenging effects of phenolic compounds shown in Fig. 1; as shown in Fig. 2 for pyrocatechol, they scavenged O_2^-. Since phenolic compounds were found to scavenge O_2^- dose-dependently, the concentrations of phenolic compounds causing 50% decrease of O_2^- (IC_{50} values) were determined as shown in Fig. 3 for pyrocatechol and are listed in Table I. Phenol itself did not exhibit O_2^- scavenging activity even at 20 mM. Among dihydric phenols, the activity of pyrocatechol, which has phenolic OH at *ortho* position, was strongest; the activity of hydroquinone was next and that of resorcinol was weakest.

Among trihydric phenols, pyrogallol and gallic acid, which have phenolic OH groups at neighboring positions, have more prominent O_2^- scavenging activities than the

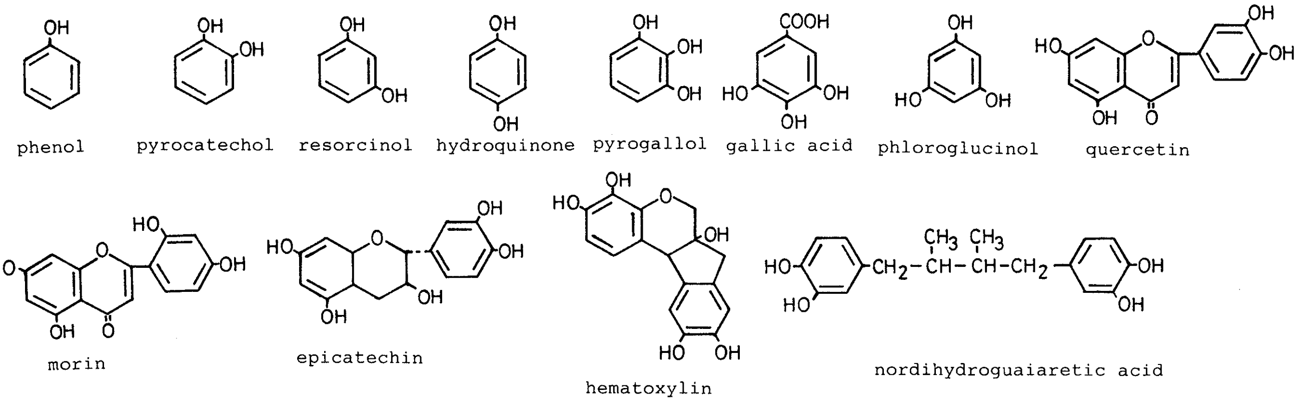

Fig. 1. Phenolic Compounds Tested in This Study

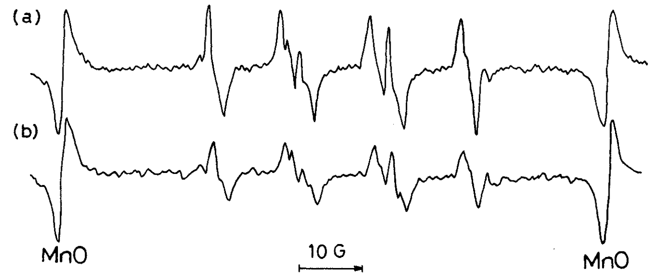

Fig. 2. Effect of Pyrocatechol on Superoxide Anion (O_2^-) Generated in Hypoxanthine (HPX)–Xanthine Oxidase (XOD) System: (a) Control, (b) with 10 μM Pyrocatechol

ESR spectra were measured after 1 min incubation at 21 °C.

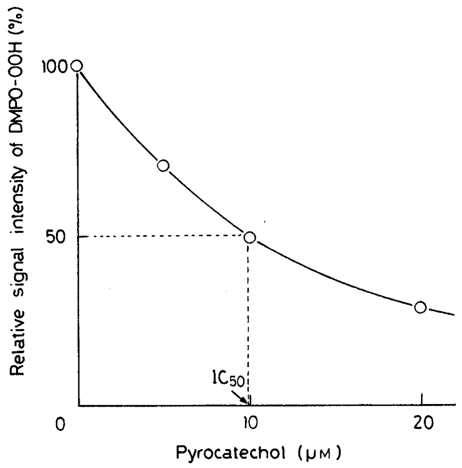

Fig. 3. Example of Dose-Dependent O_2^- Scavenging Effect of Pyrocatechol

Concentration causing 50% decrease of O_2^- (IC_{50} value) was defined as that required to give a 50% decrease of the intensity of the signal of DMPO-OOH, DMPO adduct of O_2^-.

TABLE I. Concentrations of Monohydric and Dihydric Phenols Causing 50% Decrease of O_2^-, IC_{50} Values in HPX–XOD System

Compound	IC_{50} (μM)[a]
Phenol	>20000
Pyrocatechol	10.9± 1.7 (5)
Resorcinol	18800±2600 (3)
Hydroquinone	280± 69 (3)
Pyrogallol	1.45± 0.13 (4)
Gallic acid	1.57± 0.11 (3)
Phloroglucinol	7280± 920 (3)
Quercetin	2.75± 0.31 (3)
Morin	210± 23 (3)
Epicatechin	9.32± 1.22 (3)
Hematoxylin	2.22± 0.12 (3)
Nordihydroguaiaretic acid	8.73± 2.14 (3)

a) Numbers represent means±S.D. of results from 3—5 (numbers in parentheses) different preparations.

dihydric phenols mentioned above. On the other hand, the activity of phloroglucinol, which has three phenolic OH groups at *meta* positions, was much weaker than those of pyrogallol and gallic acid.

Among the polyhydric phenols tested, quercetin, which has a catechol group and long conjugated double bonds and has been reported to have significant antioxidant activity,[1,2,4] had a strong O_2^- scavenging activity. Its IC_{50} value was about 1/4 that of pyrocatechol. The activity of morin, in contrast, which has a resorcinol group instead of a catechol group in quercetin, was much weaker. These results suggest that the catechol group is important for scavenging O_2^-. These estimations are supported by the findings that epicatechin, hematoxylin and nordihydroguaiaretic acid, which also have one or two catechol groups in the molecule, have prominent O_2^- scavenging activities as shown in this work. These results are also consistent with the recent report on the significant antioxidant activities of catechol compounds.[13]

Relationship between O_2^- Scavenging Effects of Catechol Derivatives and Their Redox Potentials In order to clarify the physico-chemical properties which may determine the O_2^- scavenging activity of the phenolic compounds, especially that of catechol compounds which exhibited strong activities, we next investigated the relationship between redox potentials[14] and O_2^- scavenging effects for 4-substituents of pyrocatechol. Correlation between their redox potentials, E_o (V), and their O_2^- scavenging activities expressed as logarithm values of $1/IC_{50}$ was examined following Naito *et al.*[15] As shown in Fig. 4 for six 4-substituents of pyrocatechol, the activity of *tert*-butyl substituent was strongest and that of carboxyl substituent (protocatechuic acid) was weakest. Moreover, a good linear correlation was found between the redox potentials and scavenging activities of these catechol derivatives as shown in Eq. 1.

$$\log(1/IC_{50})(\mu M^{-1}) = -4.61E_o + 2.63 \qquad (r=0.991) \qquad (1)$$

Here, r is the correlation coefficient. Equation 1 revealed

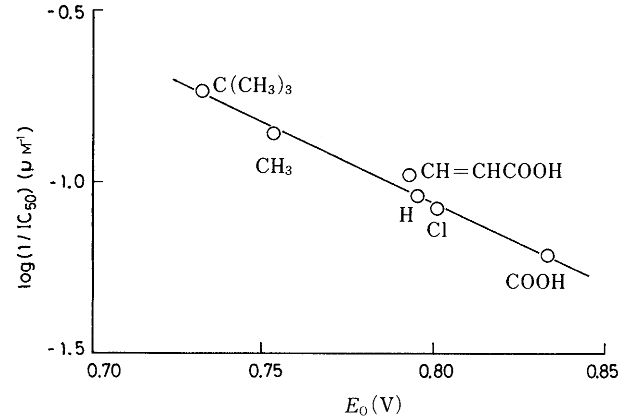

Fig. 4. Relation between Redox Potentials, E_0, of 4-Substituents of Pyrocatechol and Their O_2^- Scavenging Effects, Expressed as $1/IC_{50}$ Values

The values of E_0 (V) are cited from Ref. 14.

that catechol derivatives with lower redox potentials have stronger scavenging effects on O_2^-. These results indicated that O_2^- scavenging activities of catechol derivatives are controlled by their electron donor activities. This conclusion is consistent with the results on the scavenging effects of tocopherol derivatives against active oxygen species.[9,10] However, the results revealed here were different from those on the inhibitory effects of the same catechol derivatives on arachidonic acid metabolism in blood platelets,[16] in which radical chain reactions are involved[17] and which seems to be carried out under hydrophobic circumstances.

O_2^- Scavenging Activities of Phenolic Compounds against O_2^- Originated from KO_2 in DMSO and Identification of the Generated Radicals Reaction sites of these phenolic compounds with O_2^- under physiological conditions seem most likely to be hydrophobic surroundings like cellular lipid bilayer. In fact, the inhibitory effects of 4-substituents of pyrocatechol on the arachidonic acid metabolism mentioned above depended on their hydrophobicities in addition to their redox potentials.[16] Therefore, we next investigated the O_2^- scavenging activities of these phenolic compounds against O_2^- in an organic solvent like DMSO by using KO_2 which was solubilized by 18-crown-6 as the source of O_2^-. This system is also useful for another reason, because the high concentration of O_2^- is expected to make it possible to identify the radicals of phenolic compounds generated in the reaction process with O_2^-.

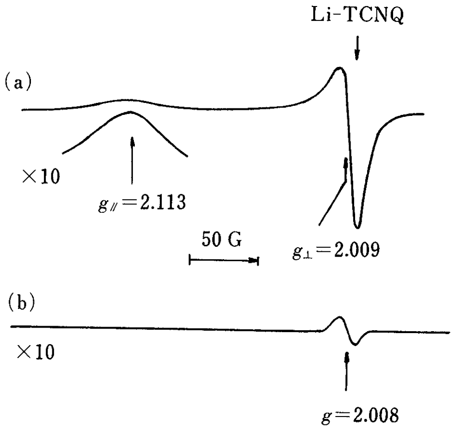

Fig. 5. ESR Spectrum of O_2^- Originated from 0.5 mM KO_2 in DMSO at 77 K (a) and That in the Presence of 1 mM Pyrocatechol (b)

Spectra were measured after 1 min incubation at 21 °C with 6.3 G modulation amplitude at the recording magnitudes of 1 (a) and 10 (b).

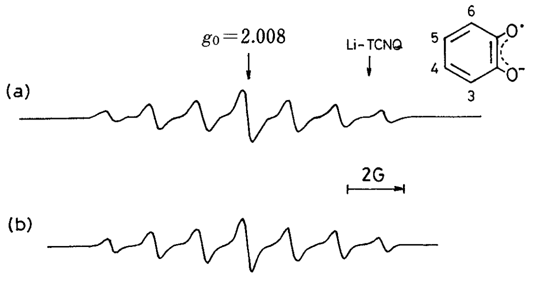

Fig. 6. ESR Spectrum Obtained by the Reaction of 1 mM Pyrocatechol with O_2^- Originated from 0.5 mM KO_2 in DMSO at 21 °C (a) and Its Simulated Spectrum (b)

Hyperfine splitting constants, $a^H_{3,6}$ and $a^H_{4,5}$, are 1.64 G (2H) and 3.45 G (2H), respectively. ESR spectrum was measured with 0.1 G modulation amplitude after 4 min incubation at 21 °C.

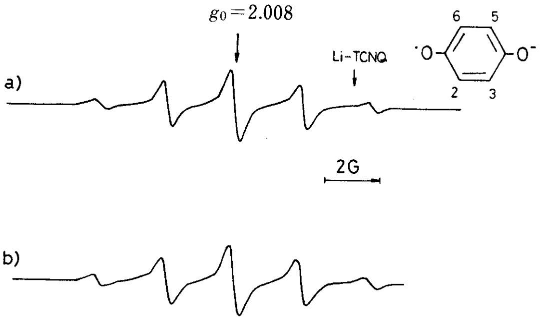

Fig. 7. ESR Spectrum Obtained by the Reaction of 1 mM Hydroquinone with O_2^- Originated from 0.5 mM KO_2 in DMSO at 21 °C (a) and Its Simulated Spectrum (b)

Hyperfine splitting constant, $a^H_{2,3,5,6}$, is 2.42 G (4H). ESR spectrum was measured with 0.1 G modulation amplitude after 4 min incubation at 21 °C.

Figure 5 shows the ESR spectra of O_2^- originated from 0.5 mM KO_2 observed at 77 K. In the presence of 1 mM pyrocatechol, the signal of O_2^- disappeared and a new ESR signal developed. In order to identify this new ESR signal, ESR spectra were recorded at 21 °C. As a result, a signal with seven lines ($g_0 = 2.008$, $a^H_{3,6} = 1.64$ G (2H) and $a^H_{4,5} = 3.45$ G (2H)) was observed as shown in Fig. 6. Computer simulation was then carried out according to the previous report,[18] and it was revealed that this signal is attributable to pyrocatechol radical. As reported for the radicals of tocopherol derivatives which are generated following the reaction with O_2^-,[19,20] the pyrocatechol radical observed here is thought to be formed by deprotonation of the unstable pyrocatechol cation radical which was produced due to one-electron oxidation of pyrocatechol by O_2^-. This prediction is consistent with the results that O_2^- scavenging activities of catechol derivatives are controlled by their electron donor activities. The pyrocatechol radical observed here was stable and did not deteriorate during the 20 min after the beginning of the reaction. Similar productions of radicals following one-electron oxidation were also observed for other phenolic compounds. As an example, the ESR signal for hydroquinone is shown in Fig. 7. Computer simulation revealed the generation of hydroquinone radicals.

There have been a number of studies on the free radical scavenging activities of various antioxidants including phenolic compounds. However, the mechanisms of their free radical scavenging as well as the physico-chemical properties which determine the activities of these compounds are scarcely known. The results obtained here for O_2^- scavenging effects of phenolic compounds indicate that these compounds directly react with O_2^-, induce one electron reduction of O_2^- due to their electron donor activities and form stable radicals of the compounds themselves. This formation of stable radicals seems to be the cause of the O_2^- scavenging activities of phenolic compounds. However, there is still one problem to be solved. Although good correlation was found between redox potentials of 4-substituents of pyrocatechol and their scavenging effects against O_2^- generated in the HPX–XOD system, certain other phenolic compounds with different structures showed variations from the correlation. For example, the O_2^- scavenging activity of hydroquinone was much weaker than that of pyrocatechol, although redox potential of the former (0.700)[14] is smaller than that of the latter (0.795).[14] Therefore, other physico-chemical factors may also govern the O_2^- scavenging activities of phenolic compounds. Binding of the compounds to the enzyme may also have to be considered.

Acknowledgment This work was partially supported by a grant from the Ministry of Education, Science and Culture of Japan (03354020).

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