Relationship between Effects of Phenolic Compounds on the Generation of Free Radicals from Lactoperoxidase-Catalyzed Oxidation of NAD(P)H or GSH and their DPPH Scavenging Ability

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The influence of various phenolic compounds on the lactoperoxidase (LPO)/hydrogen peroxide (H_2O_2)-catalyzed oxidation of biochemical reductants such as reduced β-nicotinamide adenine dinucleotide (NADH), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) or reduced glutathione (GSH) was investigated by electron spin resonance (ESR) spectroscopy. Micromolar quantities of phenolic compounds such as 17β-estradiol, phenol, and *p*-chlorophenol enhanced the LPO/H_2O_2-catalyzed oxidation of NAD(P)H or GSH to generate a large amount of superoxide radical ($O_2^{\cdot-}$) or glutathione thiyl radical ($GS^{\cdot}$), while, phenolic compounds such as quercetin and Trolox C greatly suppressed the generation of $O_2^{\cdot-}$ and $GS^{\cdot}$. In order to elucidate the effects of phenolic compounds on the generation of $O_2^{\cdot-}$ and $GS^{\cdot}$, their quenching activities for a stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), were investigated by ESR spectroscopy. 17β-Estradiol, phenol, and *p*-chlorophenol showed very weak scavenging activities for DPPH, but quercetin and Trolox C showed strong activities. This suggests that the ability of phenolic compounds to enhance LPO/H_2O_2-catalyzed oxidation of NAD(P)H or GSH relates inversely to their ability to quench DPPH. That is, phenolic compounds having weak quenching activity against DPPH may enhance the LPO/H_2O_2-catalyzed oxidation of NAD(P)H or GSH to generate a large amount of $O_2^{\cdot-}$or $GS^{\cdot}$.

Key words phenolic compound; superoxide radical; oxidation; electron spin resonance

Reactive oxygen species such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals, and singlet oxygen have been implicated both in the aging process and in degenerative disease.[1—4)]

Phenolic compounds are known to act as poisons under certain conditions. The mechanism of toxicity of phenolic compounds such as phenol[5)] and 17β-estradiol[6)] is suggested to be the generation of $O_2^{\cdot-}$ during the reaction of the compounds with oxidative enzymes (peroxidases, tyrosinase, prostaglandin synthase, *etc*.) in the presence of biological reductants such as reduced β-nicotinamide adenine dinucleotide (NADH), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) or glutathione (GSH). On the other hand, phenolic compounds that scavenge $O_2^{\cdot-}$ may be of potential therapeutic use as antioxidants.[7,8)]

In consideration of these points, we are interested in elucidating how phenolic compounds can enhance the generation of $O_2^{\cdot-}$ from the reaction of lactoperoxidase (LPO) with NAD(P)H and GSH, and whether there is a relationship between the enhancing activity of the phenolic compounds and their electrochemical properties. LPO is a mammalian hemoprotein found in saliva, tears, and milk, and forms part of the antimicrobial defense system. As the electrochemical data for phenolic compounds have been obtained under a wide range of experimental conditions, however, it is difficult to make direct comparisons. Thus, the ability of a phenolic compound to scavenge a stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), was used in this study to assess the electrochemical properties of the compounds. 1,1-Diphenyl-2-picrylhydrazyl is a radical widely used for the rough estimation of the antioxidant activity of phenolic compounds. The ability of a phenolic compound to scavenge DPPH is recently suggested to be related to the redox potential.[9)] The chemical structure of the nineteen phenolic compounds used in this study are depicted in Fig. 1. The compounds are 17β-estradiol, phenol, *p*-chlorophenol, *p*-eugenol, isoeugenol, 3,4-dimethylphenol, quercetin, Trolox C, diethylstilbestrol, bisphenol A, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, acetaminophen, guaiacol, *p*-methoxyphenol, curcumin, 2-hydroxyestradiol, gallic acid, and 3(2)-*tert*-butyl-4-hydroxyanisole. Phenolic dental medicaments including phenol, *p*-chlorophenol, *p*-eugenol, isoeugenol, and guaiacol have been used for disinfection and sedative treatment for pulpitis in dental practice.[10)] We report here that the ability of phenolic compounds to enhance the LPO/H_2O_2-catalyzed oxidation of NAD(P)H or GSH is in inverse proportion to their quenching activity against the DPPH.

Materials and Methods

Materials 17β-Estradiol was purchased from Steraloids Inc. (U.S.A.). *p*-Chlorophenol, diethylstilbestrol (DES), reduced β-nicotinamide adenine dinucleotide (NADH), lactoperoxidase (LPO), catalase, superoxide dismutase (SOD), and reduced glutathione (GSH) were purchased from Sigma Chemical Co. (U.S.A.). Trolox® (abbreviated as Trolox C hereinafter) was obtained from Aldrich Chemical Co. (U.S.A.). Phenol, guaiacol, *p*-eugenol, isoeugenol, 3,4-dimethylphenol, quercetin, bisphenol A, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, acetaminophen, *p*-methoxyphenol, 2-hydroxyestradiol, gallic acid, 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), ethyl alcohol, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and hydrogen peroxide (30%) (H_2O_2) were obtained from Wako Pure Chemical Co. (Japan). Curcumin was bought from Cayman Chemical Co. (U.S.A.). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from LABOTECK (Japan) and used without further purification. Chelex 100 resin (sodium form) was obtained from Bio-Rad Co. (U.S.A.).

ESR Measurements for Detection of $O_2^{\cdot-}$ or Glutathione Thiyl Radical Adducts with DMPO The reaction mixture contained 4 mM NAD(P)H or GSH, 10 μM phenol or other phenolic compounds, LPO (5 U/ml) and 100 mM DMPO in 0.1 M phosphate buffer at pH 7.4. In the case of GSH, 54 μM H_2O_2 was further added to the reaction mixture. ESR spectra were recorded at 1 min after mixing unless otherwise stated. The experiment was performed in air-saturated solutions. ESR measurements were carried out on a JES-RE1X spectrometer (X-band) (JEOL, Japan) with 100 kHz field modulation. ESR spectra were recorded at room temperature in a JEOL flat quartz cell. ESR parameters were calibrated by comparison with a standard

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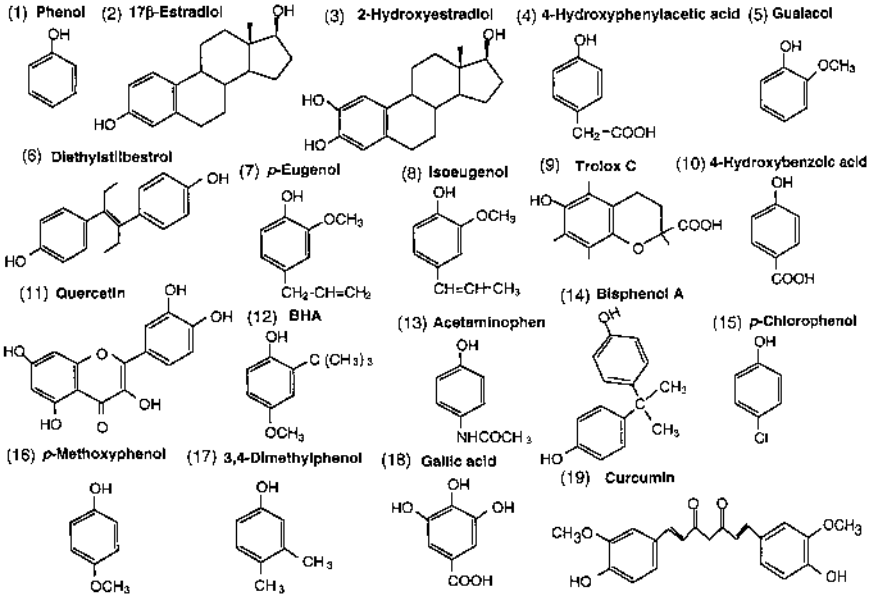

Fig. 1. The Structures of Phenolic Compounds Used Here

Mn^{2+}/MgO marker.

Reactivities of Antioxidants against DPPH An ethanol solution of each sample (200 μl) was added to 200 μl of 210 μM DPPH in ethanol. After mixing for 10 s vigorously, the solution was transferred into a cell. Exactly 1 min later, the ESR spectrum of DPPH was measured.[9)]

Statistical Analysis Linear regression analysis was performed using StatView-4.5J (Abacus Concepts, Berkeley, CA, U.S.A.) and used to determine the relation between the signal intensities of DMPO adducts and IC_{50}, which is the concentration of phenolic compounds required to reduce by 50% the signal intensity of DPPH.

Results and Discussion

Reaction of Phenolic Compounds in the Presence of NADH The characteristic twelve-line ESR spectrum was obtained from a reaction mixture containing 4 mM NADH, 10 μM phenol, LPO (5 U/ml) and 100 mM DMPO (Fig. 2A). The solution also contained 2% ethanol, because phenol is solubilized by ethanol. The hyperfine coupling constants (hfsc) (a^N=1.42, a_β^H=1.15 and a_γ^H=0.12 mT) of this ESR spectrum are identical with those previously assigned to the DMPO adduct of $O_2^{\cdot-}$.[11)] Furthermore, the addition of SOD to the reaction mixture resulted in the disappearance of the ESR signal as shown in Fig. 2F. Thus, this radical species is assigned to $O_2^{\cdot-}$. When phenol was omitted from the reaction mixture, the ESR signal was greatly decreased (Fig. 2B). Thus, the addition of phenol leads to enhancement of the generation of $O_2^{\cdot-}$. No signal was observed in the absence of NADH or LPO as shown in Figs. 2C and D, respectively. The partial inhibition by catalase demonstrates that H_2O_2 is necessary for the reaction to proceed (Fig. 2E). The presence of H_2O_2 is probably a result of the auto-oxidation of NADH.[6)] The generation of $O_2^{\cdot-}$ during aerobic oxidation of NADH by the LPO/H_2O_2/phenol system can be rationalized by Eqs. 1—5.[12,13)]

$$LPO + H_2O_2 \rightarrow LPO\text{-}I + H_2O \quad (1)$$

$$LPO\text{-}I + PhOH \rightarrow LPO\text{-}II + PhO^{\cdot} \quad (2)$$

$$LPO\text{-}II + PhOH \rightarrow LPO + PhO^{\cdot} + H_2O \quad (3)$$

$$2PhO^{\cdot} + 2NADH \rightleftarrows 2PhOH + 2NAD^{\cdot} \quad (4)$$

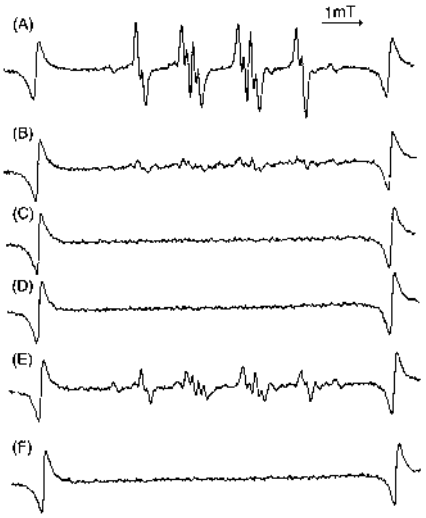

Fig. 2. ESR Spectrum of the DMPO-Superoxide Adduct Detected in a Reaction Mixture Containing NADH, Phenol, and Lactoperoxidase

(A) Complete system containing 10 μM phenol, 4 mM NADH, 5 units/ml lactoperoxidase and 100 mM DMPO in 100 mM phosphate buffer, pH 7.4. The sample also contained 2% ethanol from the phenol stock solution. (B) as in (A), but without addition of phenol. (C) as in (A), but without NADH. (D) as in (A), but without lactoperoxidase. (E) as in (A), but with 1641 units/ml catalase. (F) as in (A), but with 126 units/ml superoxide dismutase. Conditions: microwave power, 10 mW; modulation amplitude, 0.079 mT; scan range, 10 mT; receiver gain, 200; time constants, 0.03 s; scan time, 2 min.

$$2NAD^{\cdot} + 2O_2 \rightarrow 2\,NAD^{+} + 2O_2^{\cdot-} \quad (5)$$

where LPO, LPO-I, and LPO-II represent lactoperoxidase, lactoperoxidase compound I, and lactoperoxidase compound II, respectively.

Oxidation by peroxidases such as LPO is usually explained by a cycle of electron-transfer reactions in which the first step is the formation of an electron-deficient LPO-I, the product of two-electron oxidation of the enzyme by H_2O_2 (Eq. 1). In subsequent steps, LPO-I and then LPO-II reacts with phenol. One-electron oxidation of phenolic compounds by oxidative enzymes (peroxidases, tyrosinase, prostaglandin synthase) gives phenoxyl radicals. For example, Thompson

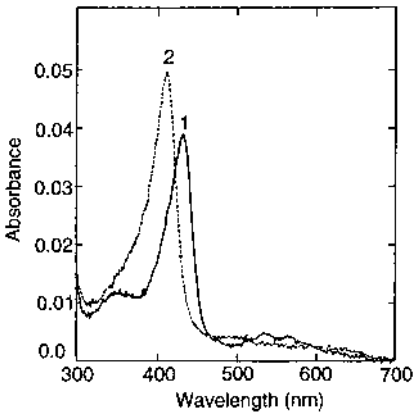

Fig. 3. Spectral Evidence for the Oxidation of Phenol by Lactoperoxidase Compound II

(A) Lactoperoxidase (5 units/ml) and 1.4 μM H_2O_2 (compound II, trace 1) plus 10 μM phenol (trace 2) in 100 mM phosphate buffer, pH 7.4.

et al. suggested that oxidation of *p*-eugenol by horseradish peroxidase occurs *via* a one-electron pathway yielding a phenoxyl radical.[14] Similar observations were made for other phenolic compounds, such as phenol,[5] BHA,[15] acetaminophen,[16] quercetin[17] and 17β-estradiol.[6] Next, we investigated how phenolic compounds affect Eq. 3. Figure 3 indicates that the addition of phenol (10 μM) to LPO-II, which was alternatively prepared from the reaction of LPO (44 μg/ml) with H_2O_2 (1.5 μM), immediately caused a change from LPO-II (λ_{max}=430 nm) to LPO (λ_{max}=412 nm),[18] suggesting that the lifetime of LPO-II is shortened by the addition of phenol and this decay of LPO-II is consistent with the change to its native, ferric form. Similar results were observed in all phenolic compounds except 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, and Trolox C.[19] These three compounds slowly changed LPO-II to LPO as shown in Fig. 4. 4-Hydroxybenzoic acid has the carboxyl group (COO^-) being strongly hydrophilic and thus, it is interfered with its accessibility to the active site of LPO-II, slowing the reaction.[13] Furthermore, the bigger chromane ring of Trolox C compared to the single benzene ring of 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid may result in some steric hindrance for Trolox C to reach the heme active site in LPO. The phenoxyl radical generated during the oxidation of phenol by LPO-II abstracts hydrogen from NADH to generate the $NAD^{\cdot}$ radical. The difference in the redox potentials between the phenoxyl radical ($PhO^{\cdot}$)/phenol (PhOH) (970 mV[20]) and $NAD^{\cdot}$/NADH (282 mV[21]) couples at pH 7 makes this reaction (Eq. 4) thermodynamically feasible. The $NAD^{\cdot}$ radical is a powerful reducing species ($E(NAD^+/NAD^{\cdot})=-930$ mV[21] (pH 7)) and readily reduces molecular oxygen to $O_2^{\cdot-}$ ($E(O_2/O_2^{\cdot-})=-330$ mV[22]), which is then spin-trapped by DMPO and subsequently detected by ESR spectroscopy. The signal intensities of the DMPO/$O_2^{\cdot-}$ adduct obtained during the reaction of LPO with NADH in the presence of phenol together with other phenolic compounds are listed in Table 1. Phenolic compounds yielding a large amount of $O_2^{\cdot-}$ included phenol, *p*-chlorophenol, 17β-estradiol, bisphenol A and 3,4-dimethylphenol. In contrast, the addition of quercetin or *p*-methoxyphenol to the mixture of LPO and NADH resulted in the disappearance of the DMPO/$O_2^{\cdot-}$ adduct, suggesting that these compounds either

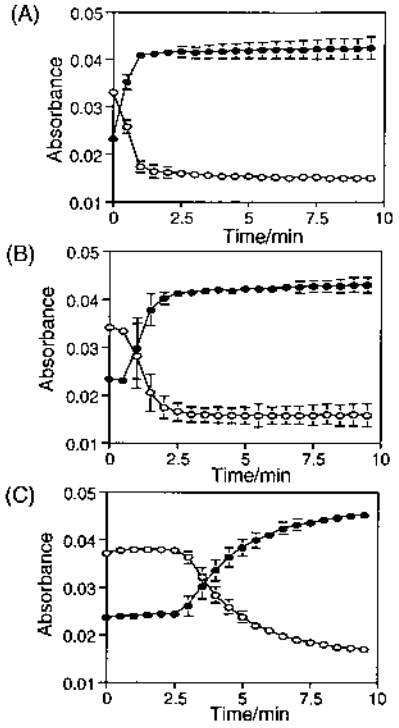

Fig. 4. Time Course of Absorbance at 430 nm (○) (λ_{max} of LPO-II) and 412 nm (●) (λ_{max} of LPO) during the Oxidation of 4-Hydroxybenzoic Acid (A), 4-Hydroxyphenylacetic Acid (B), and Trolox C (C) by LPO-II

scavenge $O_2^{\cdot-}$ generated from the reaction of LPO with NADH[8] or quench $NAD^{\cdot}$ radical to suppress the formation of $O_2^{\cdot-}$. Phenolic compounds with low reduction potential are easily oxidized and efficiently accelerate steps (2) and (3), but they shift step (4) towards the left, and hence they may quench the $NAD^{\cdot}$ radical.

Reaction of Phenolic Compounds in the Presence of NADPH Using NADPH in place of NADH, the same characteristic twelve-line ESR spectrum of the DMPO adduct of $O_2^{\cdot-}$ as that of NADH was observed as shown in Fig. 5A. This assignment was supported by the disappearance of the ESR signal on the addition of SOD as shown in Fig. 5B. The signal intensities of the DMPO/$O_2^{\cdot-}$ adduct obtained in the reaction of LPO with NADPH in the presence of phenol together with other phenolic compounds are summarized in Table 1. When NADH was replaced by NADPH, similar ESR spectra to those in Fig. 2 were obtained, indicating that the electron transport in NADPH undergoes the same operational processes as in NADH.

Reaction of Phenolic Compounds in the Presence of Glutathione and Hydrogen Peroxide Glutathione thiyl free radical ($GS^{\cdot}$) generated by the lactoperoxidase-catalyzed reaction of phenol in the presence of glutathione (GSH) was demonstrated by spin trapping with DMPO. The four-line ESR spectrum was detected in a reaction medium containing 10 μM phenol, 4 mM GSH, LPO (5 U/ml), 54 μM H_2O_2 and 100 mM DMPO at pH 7.4 (Fig. 6A). The hyperfine coupling constants (a^N=1.51 and a_β^H=1.62 mT) of this spectrum are very similar to those of the DMPO-glutathione thiyl radical adduct.[11] When phenol was omitted from the reaction mixture, the signal became very weak, indicating direct oxidation of GSH by LPO/H_2O_2 (Fig. 6B). No signal was observed

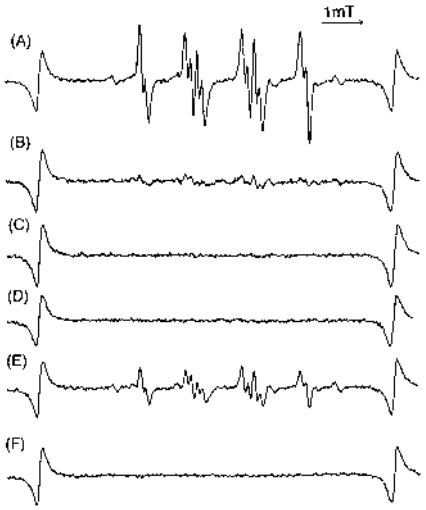

Fig. 5. ESR Spectrum of the DMPO-Superoxide Adduct Detected in a Reaction Mixture Containing NADPH, Phenol, and Lactoperoxidase

(A) Complete system containing 10 μM phenol, 4 mM NADPH, 5 units/ml lactoperoxidase and 100 mM DMPO in 100 mM phosphate buffer, pH 7.4. The sample also contained 2% ethanol from the phenol stock solution. (B) as in (A), but without addition of phenol. (C) as in (A), but without NADPH. (D) as in (A), but without lactoperoxidase. (E) as in (A), but with 1641 units/ml catalase. (F) as in (A), but with 126 units/ml superoxide dismutase. The conditions were as described for Fig. 2.

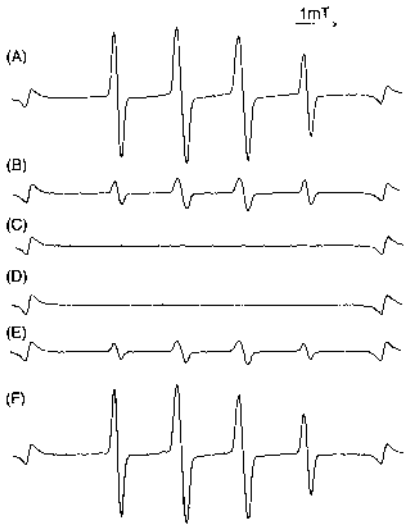

Fig. 6. ESR Spectrum of the DMPO-Glutathione Thiyl Radical Adduct Detected in a Reaction Mixture Containing GSH, Phenol, and Lactoperoxidase

(A) Complete system containing 10 μM phenol, 4 mM GSH, 54 mM H_2O_2, 5 units/ml lactoperoxidase and 100 mM DMPO in 100 mM phosphate buffer, pH 7.4. The sample also contained 2% ethanol from the phenol stock solution. (B) as in (A), but without addition of phenol. (C) as in (A), but without glutathione. (D) as in (A), but without lactoperoxidase. (E) as in (A), but with 1641 units/ml catalase. (F) as in (A), but with 126 units/ml superoxide dismutase. The instrumental conditions except receiver gain were as described for Fig. 2. Receiver gain was 63.

in the absence of GSH or LPO (Figs. 6C, D). Furthermore, the partial inhibition by catalase demonstrates that H_2O_2 was necessary for the reaction to proceed (Fig. 6E). The addition of SOD to the reaction mixture did not affect the ESR signal as shown in Fig. 6F, suggesting that this DMPO adduct was not derived from $O_2^{\cdot-}$. These results indicate that the phenoxyl radical probably generated during the metabolism of phenol by LPO and H_2O_2 abstracts hydrogen from GSH to give GS$^{\cdot}$ (Eq. 6), which is trapped by DMPO.

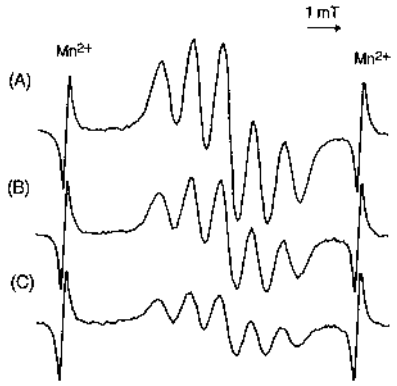

Fig. 7. ESR Spectra of the DPPH Radical after the Addition of Trolox C at Different Concentrations

The spectra were recorded without (A) and in the presence [(B) 10 μM, (C) 20 μM] of Trolox C. Conditions: microwave power, 7 mW; modulation amplitude, 0.25 mT; scan range, 20 mT; time constants, 0.1 s; scan time, 2 min.

$$2PhO^{\cdot}+2GSH \rightleftarrows 2PhOH+2GS^{\cdot} \quad (6)$$

The signal intensities of the DMPO/GS$^{\cdot}$ adduct obtained in the reaction of LPO with GSH in the presence of phenol together with other phenolic compounds are listed in Table 2. Phenolic compounds generating a large amount of GS$^{\cdot}$ included 17β-estradiol, phenol, *p*-chlorophenol, bisphenol A, 3,4-dimethylphenol, acetaminophen, and DES. In contrast, the addition of Trolox C to the mixture of LPO and GSH resulted in the disappearance of the DMPO/GS$^{\cdot}$ adduct.

Scavenging Effects of Phenolic Compounds on DPPH To elucidate the electrochemical properties of the phenolic compounds used here, we have investigated the quenching effects of phenolic compounds for DPPH.[23—25] The reaction of phenolic compounds with stable radical, DPPH, is essentially the reverse reaction for Eq. 4 or 6. Therefore, the results obtained here may reflect the reactivity of the phenoxyl radical against NADH, NADPH, or GSH. Figure 7A shows a typical ESR spectrum for DPPH. In the presence of Trolox C at different concentrations in ethanol, the signal intensities of DPPH decreased as shown in Figs. 7B and C. The scavenging effects of phenolic compounds are expressed as the IC_{50}, which is the concentration required to reduce by 50% the signal intensity of DPPH. IC_{50} values of phenolic compounds for DPPH are presented in Table 1. Seven compounds, that is, 17β-estradiol, *p*-chlorophenol, phenol, DES, bisphenol A, 4-hydroxyphenylacetic acid, and 4-hydroxybenzoic acid, did not give an IC_{50} under the experimental conditions used here, suggesting that these phenolic compounds have no or very weak DPPH quenching abilities. Gallic acid, quercetin, and Trolox C showed the strongest quenching ability against DPPH among the phenolic compounds used here. Nanjo *et al*. have described that the strong scavenging activity of tea catechins for DPPH is due to their low redox potential.[9] In fact, the redox potentials of hydroxybenzoic acid,[20] phenol[20] and *p*-chlorophenol[20] at pH 7, showing no quenching activity for the DPPH, are 1040, 970 and 940 mV, and those of quercetin[8] and Trolox C[8] showing the highest scavenging activity for the DPPH were 600 and 480 mV, respectively. This suggests that the strong scavenging activity of phenolic compounds for DPPH is due to their low redox potentials.

Relationship between the Generation of Superoxide or Glutathione Thiyl Radical and Scavenging Effects on

Table 1. Relationship between DPPH Scavenging Ability and Signal Intensities of DMPO/$O_2^{\cdot-}$ Adducts[a)]

Compounds	IC_{50} (μM)[b)]	DMPO/$O_2^{\cdot-}$ (a.u.)	
		NADH	NADPH
None		0.29 (1.0)[c)]	0.22 (1.0)[c)]
p-Chlorophenol	>2500	2.88 (9.9)	2.79 (12.7)
17β-Estradiol	>2500	2.61 (9.0)	2.20 (10.0)
3,4-Dimethylphenol	>2500	2.20 (7.6)	1.48 (6.7)
Phenol	>2500	1.26 (4.3)	1.08 (4.9)
Bisphenol A	>2500	1.06 (3.7)	0.65 (3.0)
4-Hydroxyphenylacetic acid	>2500	0.45 (1.6)	0.41 (1.9)
4-Hydroxybenzoic acid	>2500	0.29 (1.0)	0.35 (1.6)
Acetaminophen	1940	0.65 (2.2)	0.41 (1.9)
Diethylstilbestrol	1460	0.47 (1.6)	0.43 (2.0)
Guaiacol	880	0.21 (0.7)	0.10 (0.5)
p-Methoxyphenol	265	0.11 (0.4)	0.00 (0)
p-Eugenol	263	0.50 (1.7)	0.41 (1.9)
3(2)-*tert*-Butyl-4-hydroxyanisole	204	0.30 (1.0)	0.19 (0.9)
Curcumin	45	0.41 (1.4)	0.27 (1.2)
Isoeugenol	32	0.28 (1.0)	0.17 (0.8)
Trolox C	17	0.27 (0.9)	0.16 (0.7)
2-Hydroxyestradiol	17	0.22 (0.8)	0.14 (0.6)
Quercetin	16	0.00 (0)	0.00 (0)
Gallic acid	10	0.16 (0.6)	0.14 (0.6)

a) Values are expressed as the mean (n=3). *b*) [DPPH]=105 μM. *c*) Relative signal intensity.

Table 2. Relationship between DPPH Scavenging Ability and Signal Intensities of DMPO/GS$^{\cdot}$ Adducts[a)]

Compounds	IC_{50} (μM)[b)]	DMPO/GS$^{\cdot}$ (a.u.)
None		1.28 (1.0)[c)]
p-Chlorophenol	>2500	9.46 (7.4)
Phenol	>2500	8.19 (6.4)
3,4-Dimethylphenol	>2500	6.40 (5.0)
Bisphenol A	>2500	5.74 (4.5)
17β-Estradiol	>2500	4.83 (3.8)
4-Hydroxybenzoic acid	>2500	2.16 (1.7)
4-Hydroxyphenylacetic acid	>2500	1.94 (1.5)
Acetaminophen	1940	5.01 (3.9)
Diethylstilbestrol	1460	4.28 (3.3)
Guaiacol	880	1.80 (1.4)
p-Methoxyphenol	265	1.95 (1.5)
p-Eugenol	263	1.98 (1.6)
3(2)-*tert*-Butyl-4-hydroxyanisole	204	0.93 (0.7)
Curcumin	45	1.89 (1.5)
Isoeugenol	32	1.85 (1.5)
Trolox C	17	0.41 (0.3)
2-Hydroxyestradiol	17	0.99 (0.8)
Quercetin	16	1.45 (1.1)
Gallic acid	10	0.91 (0.7)

a) Values are expressed as the mean (n=3). *b*) [DPPH]=105 μM. *c*) Relative signal intensity.

DPPH In the absence of phenolic compounds, LPO/H_2O_2-induced oxidation of NAD(P)H or GSH gives a weak ESR signal of DMPO/$O_2^{\cdot-}$ or DMPO/GS$^{\cdot}$ adducts. LPO/H_2O_2-catalyzed oxidation of some phenolic compounds, which had very weak scavenging activity towards DPPH, like 17β-estradiol, phenol, and *p*-chlorophenol (but not 4-hydroxyphenylacetic acid or 4-hydroxybenzoic acid) in the presence of either NAD(P)H or GSH caused a strong ESR signal of DMPO/$O_2^{\cdot-}$ or DMPO/GS$^{\cdot}$ adducts as shown in Tables 1 and 2. Furthermore, in the case of an IC_{50} value below 2500, linear regression analysis shows a correlation between IC_{50} values and signal intensities of DMPO/$O_2^{\cdot-}$ (NADH), DMPO/$O_2^{\cdot-}$ (NADPH) and DMPO/GS$^{\cdot}$ adducts as shown in Fig. 8. The correlation coefficients are 0.666, 0.601, and 0.914, respectively. The low correlation coefficient for $O_2^{\cdot-}$ may be caused by scavenging abilities of some phenolic compounds against $O_2^{\cdot-}$ generated from the reaction of LPO with NAD(P)H. In particular, there was a significant correlation between IC_{50} values and signal intensities of DMPO/GS$^{\cdot}$ adducts.

Phenoxyl radicals of phenolic compounds generated by LPO and H_2O_2 enhanced the formation of NAD(P)$^{\cdot}$ radicals and glutathione thiyl radicals, respectively,[10] because having a higher redox potential they can oxidize NADPH[26] or GSH directly. NAD(P)$^{\cdot}$ radicals reacted with oxygen to generate $O_2^{\cdot-}$. On the other hand, the thiyl radical may react with another GSH molecule to form the glutathione disulfide radical anion, which reacts rapidly with oxygen to produce $O_2^{\cdot-}$. Superoxide thus formed may cause intracellular damage.[27,28] Phenolic compounds having strong quenching activity for DPPH can scavenge $O_2^{\cdot-}$. These results suggest that the reaction of the phenoxyl radical with NADH, NADPH, or GSH is the limiting step, and the amount of $O_2^{\cdot-}$ or GS$^{\cdot}$ formed determines whether a particular phenolic compound favors the right or left reaction for Eq. 4 or 6. In conclusion, by knowing the quenching abilities of unknown or synthetic phenolic compounds against DPPH, one can predict whether they would enhance the LPO/H_2O_2-catalyzed oxidation of NAD(P)H or GSH to generate $O_2^{\cdot-}$ or scavenge $O_2^{\cdot-}$ itself.

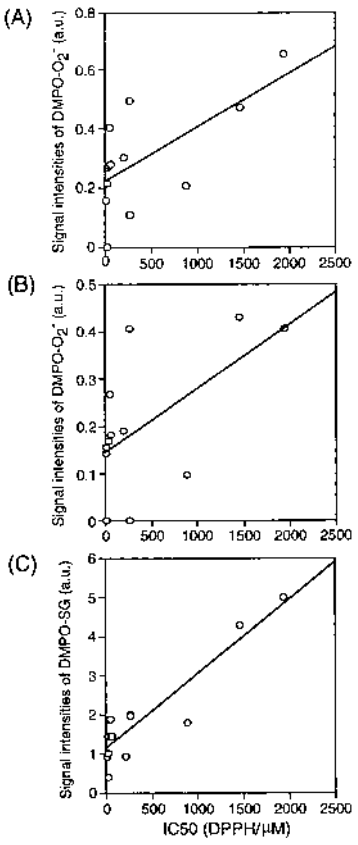

Fig. 8. Correlation between Signal Intensities of DMPO Adducts and IC_{50}
(A) DMPO/$O_2^{\cdot-}$ (NADH), (B) DMPO/$O_2^{\cdot-}$ (NADPH), (C) DMPO/GS$^{\cdot}$ adducts.

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