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ESSENTIAL OIL PHENYL PROPANOIDS. USEFUL AS ·OH SCAVENGERS?

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In order to search for radical scavengers which could be used as raw materials for cosmetics, phenyl propanoids (eugenol, isoeugenol, dehydrodieugenol, dehydrodieugenol B and coniferyl aldehyde) were examined for their hydroxyl radical (·OH) scavenging ability. A Fenton system was used to produce ·OH. In order to see scavenging by these phenyl propanoids, competition reactions between a spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and these phenyl propanoids for ·OH were studied. The relative yield of the spin adduct of ·OH (DMPO–OH) was measured by electron spin resonance spectroscopy. The approximate rate constants of the reactions between these phenyl propanoids and ·OH estimated by measuring the reduced height of the ESR signals of DMPO–OH were found to be at least in the order of $10^9\,M^{-1}s^{-1}$ (diffusion-controlled). Also, using the TBA tests, the reactions between ·OH and several compounds reactive with ·OH were investigated in the presence of the phenyl propanoids and it was found that the phenyl propanoids compete with such reactive compounds for ·OH. These results indicate that these phenyl propanoids can be used as antioxidants for skin damage perhaps caused by ·OH generated by UV-light.

KEY WORDS: DMPO, TBA test, Phenyl Propanoids, Radical scavenging, ·OH

INTRODUCTION

It has been reported that spontaneous free radical reactions occurring in living bodies, such as lipid peroxidation, are directly related to a variety of diseases.[1–4] Lipid peroxidation leads to fatal damage to cellular organelle[5] as well as to deterioration of food.[6–9] Active oxygen species such as hydroxyl radicals, superoxide anion radicals, and singlet oxygen are reported to take part in the induction of lipid peroxidation.[5,10–12] Therefore, in order to prevent such undesirable damage caused by the products, which may arise from lipid peroxidation, great effort has been placed on finding effective scavengers for these initial species.

Skin is also a target organ of environmental oxidative damage. One of the active oxygen species yielding severe skin damage could be the hydroxyl radical, which may be induced by UV light-irradiation. If effective scavengers for ·OH can be used as raw materials for cosmetics, such skin damage could be prevented. In the present study, we have examined phenyl propanoids, which are components of essential oils commonly used as raw materials for cosmetics, to determine their ·OH scavenging ability.

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MATERIALS AND METHODS

Materials

In the present study, five phenyl propanoids were examined for ·OH scavenging ability. Eugenol and isoeugenol were purchased from Takasago Perfumery Co., Ltd. (Tokyo). Dehydrodieugenol B, dehydrodieugenol, and coniferyl aldehyde were prepared from clove bud oil in our laboratory by the following procedure. Clove bud oil, which was prepared by steam distillation of extract of clove bud cultivated in Indonesia in 1987, was obtained from Takasago Perfumery Co., Ltd. Volatile components of clove bud oil (300 g) were removed by distillation at 120°C at 0.5 mmHg and 60 g of the residual fraction was obtained. This redisue (40 g) was dissolved in 10 mL of hexane and loaded on a silica gel column (300 g). The column was successively eluted with hexane (1.5 L), chloroform (1.0 L), ethyl acetate (1.0 L) and methanol (0.8 L). Components (5 g) obtained in the ethyl acetate fraction were dissolved in 2 mL of hexane/acetone (8/1 in v/v) and loaded on a silica gel column (200 g) and eluted with benzene/acetone (8/1 in v/v). The collected fractions were grouped into 8 fractions by monitoring the components using silica gel thin layer chromatography (TLC) coupled with benzene/acetone (6/1 in v/v). From the three fractions, phenyl propanoids could be obtained, namely Compound I (R_f = 0.84), II (R_f = 0.61), and III (R_f = 0.48), as semi-crystal, white crystal, and yellow powder, respectively. These crude products were further recrystallized from ethyl acetate/hexane and analyzed as follows.

Melting points of these three compounds were measured on a Yanagimoto Melting Point apparatus (Yanagimoto, Kyoto, Japan): 60–62.5°C, 106.5–108°C, 73.5–75°C for Compounds I, II, and III, respectively. Proton and carbon nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were obtained on JEOL GX-270 FT (JEOL, Tokyo). The solvent used was $CDCl_3$. The ^{1}H-chemical shifts in ppm obtained are as follows: 3.2 (d, 2H), 3.3 (d, 2H), 3.8 (s 3H), 3.8 (s, 3H), 4.9–5.1 (m, 4H), 5.9–6.0 (m, 2H), 5.9 (s, 1H), and 6.5–6.95 (m, 5H) for Compound I; 3.3 (d, 4H), 3.9 (s, 6H), 5.0–5.1 (m, 4H), 5.9–6.0 (m, 2H), 6.0 (s, 2H), and 6.7 (m, 4H) for Compound II; 3.9 (s, 3H), 6.4 (s, 1H), 6.5 (dd, 1H), 6.8–7.2 (m, 3H), 7.4 (d, 1H), and 9.6 (d, 1H) for Compound III. The values (in ppm) of ^{13}C-NMR obtained are as follows: 39.9, 39.9, 56.0, 56.2, 107.3, 111.9, 113.0, 115.7, 116.0, 119.5, 120.8, 131.1, 135.2, 136.4, 137.2, 137.4, 144.3, 144.5, 147.9, and 150.4 for Compound I; 39.9, 56.1, 110.7, 115.7, 123.1, 124.4, 131.9, 137.7, 141.0, and 147.2 for Compound II; 56.0, 109.7, 115.1, 124.1, 126.3, 126.6, 147.1, 149.1, 153.3, and 193.8 for Compound III. Mass spectra were measured on a JOEL JMS-DX303 mass spectrometer (JEOL, Tokyo). The mass numbers obtained by the electron impact method are 326, 326, and 178 for Compounds I, II, and III, respectively. Based on these data, Compounds I, II, and III were identified as dehydrodieugenol B, dehydrodieugenol and coniferyl aldehyde, respectively. The chemical structures of the phenyl propanoids tested here are illustrated in Figure 1.

Thiobarbituric acid (TBA) was purchased from Merck Co. (Darmstadt, Germany). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) with high purity was obtained from Mitsui Toatsu Co. (Tokyo). Other chemicals of reagent grade were purchased from Wako Pure Chemicals, Ltd. (Osaka, Japan). Water was purified sequentially by Milli R/Q and Milli QII (Millipore, MA, USA).

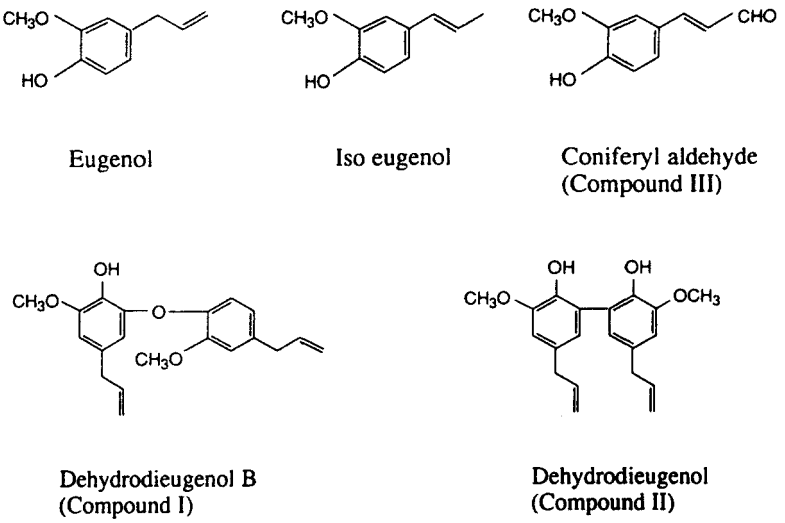

FIGURE 1 Structures of phenyl propanoids tested.

ESR Measurements

ESR measurements were carried out on a JEOL PE-3X spectrometer (X-band and 100 kHz field modulation, JEOL). The markers used for the measurement of the hyperfine splitting constants and g-values were Mn^{2+} in MgO.

Assay with Fenton System

The ·OH radical was generated with a Fenton system, and ESR intensities were calculated by double integration. Since phenyl propanoids are poorly soluble in water, samples were prepared by the following procedure: To aqueous solutions (0.1 M, 100 mL), phenyl propanoids dissolved in ethanol (20 mM, 1 mL) were added, and 1 mL of these prepared aqueous solutions were mixed with 1 mL of KH_2PO_4–Na_2HPO_2 buffer (pH 7.4, 100 mM) containing $FeSO_4 \cdot 7H_2O$ (1 mM), diethylene-triamine-N,N,N′,N″,N″-pentaacetic acid (DETAPAC) (50 mM), and DMPO (100 mM). Then, the total volume was adjusted to 9 mL by adding water. Immediately prior to the ESR measurements, 1 mL of H_2O_2 (145 mM) was added to the finally prepared sample solutions to start the Fenton reaction. The ESR spectra were recorded 2 min after the addition of H_2O_2 at room temperature. The final solution contained $FeSO_4 \cdot 7H_2O$ (0.1 mM), H_2O_2 (14.5 mM), DETAPAC (5 mM), DMPO (10 mM), ethanol (32 mM), and phenyl propanoids (0.02 mM). As a reference, a KH_2PO_4–Na_2HPO_4 buffer (pH 7.4, 10 mM) containing the same amount of $FeSO_4 \cdot 7H_2O$, H_2O_2, DETAPAC and DMPO was measured.

Microsomal Lipid Peroxidation Assay

Liver microsomes were separated from 6 weeks old male SD rats as follows: Animals were sacrificed and livers were quickly removed and perfused in physiological salt solutions. Livers were then minced and homogenized in 0.25 M sucrose using a Tefron-grass homogenizer. The homogenate was centrifuged at 10,000 rpm for

20 min. The supernatant was centrifuged at 40,000 rpm for 60 min. These prepared pellets (precipitation) were subsequently centrifuged at 40,000 rpm for 20 min in 0.15 M KCl.[13] These treated pellets were used as samples (microsomal samples) for the following measurements. To obtain reliable data, content of protein in the microsomal samples was measured by the Lowry method, and the samples showing equivalent absorbance at 750 nm were used. Phenyl propanoids were dissolved in ethanol (20 mM) and 0.15 mL of this ethanol solution was added to 0.15 mL of the prepared microsomes (containing 26.3 mg protein/1 mL) in 0.15 mL of KH_2PO_4–Na_2HPO_4 buffer (100 mM, pH 7.4) containing KCl (1.5 M), adenosine diphosphate (20 mM), and $FeSO_4 \cdot 7H_2O$ (1 mM). Subsequently water was added to the mixture to make the total volume 1.35 mL: the final concentration of phenyl propanoids was 2 mM in the sample (that of the reference was 0 mM). In this system, lipid peroxidation was initiated by a Fenton reaction: 0.15 mL of H_2O_2 (145 mM) was added to the sample solutions immediately prior to the reactions. Samples containing various phenyl propanoids were incubated at 37°C for 1 hr. Aliquots (0.2 mL) were sampled and to these, a mixture of 0.2 mL sodium dodecylsulfate (8%), 1.5 mL acetate buffer (3 M, pH 3.5), and 1.5 mL TBA (0.5%) was added and subsequently boiled. TBA-malonaldehyde (MDA) reaction products formed in the aliquots were extracted with *n*-butanol (5 mL) and the extracts measured for the absorbance (A_1) at 532 nm.[14] The reference values (A_2 and A_3) were obtained from the samples: One free of phenyl propanoids (mentioned earlier) and the other composed of only microsomes (the same amount as that in the samples) and KH_2PO_4–Na_2HPO_4 buffer (10 mM, pH 7.4).

Assay with Deoxyribose Degradation

Samples were prepared as follows: phenyl propanoids were dissolved in ethanol (2 mM) and 1 mL of this solution was added to a mixture (1 mL) consisting of KH_2PO_4–KOH buffer (200 mM, pH 7.4) containing deoxyribose (28 mM), ethylenediamine tetraacetic acid disodium salt (1 mM), ascorbate (1 mM), and $FeCl_3$ (1 mM). To these sample solution, water was added to make the total volume 9 mL. Immediately prior to the reaction, 1 mL of H_2O_2 (1 mM) was added and these prepared samples were incubated at 37°C for 1 hr. Subsequently TBA testing reagent was added.[15] TBA–MDA products were extracted with *n*-butanol and measured for the absorbance at 532 nm.[14]

RESULTS AND DISCUSSION

In order to see how effectively the phenyl propanoids scavenge ·OH, the method of DMPO spin trapping was used. The ·OH radical was generated with a Fenton system.

The ESR spectrum obtained from the reference is represented in Figure 2(a), where strong ESR lines appear because of the absence of scavengers such as ethanol and phenyl propanoids. Although it has been pointed out that Fe^{2+}-DETAPAC chelate oxidizes DMPO,[16,17] no ESR line was produced from DMPO in the absence of H_2O_2. In the spectrum, only a 1:2:2:1 quartet due to a DMPO spin adduct of ·OH (DMPO–OH: $g = 2.0059$, $a(N) = 1.49$ mT, and $a(\beta H) = 1.49$ mT) appears, indicating the generation of ·OH in these reference solutions.

Also, sample solutions containing ethanol as another standard, which is a good scavenger for ·OH ($k_{ethanol+\cdot OH} = 1 \times 10^9 M^{-1} s^{-1}$), [18] were studied. The ESR spectrum obtained from this standard is depicted in Figure 2(b). As is seen in Figures. 2(a)

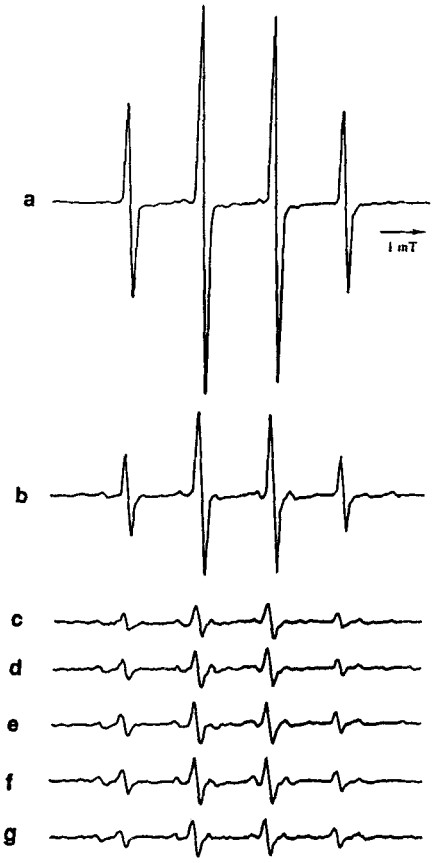

FIGURE 2 ESR spectra obtained from KH_2PO_4–Na_2HPO_4 buffer solutions (10 mM) containing $FeSO_4$ (0.1 mM), H_2O_2 (14.5 mM), DETAPAC (5 mM), DMPO (10 mM), and (a) none, (b) ethanol (100 mM), and (c–g) ethanol (32 mM) and 0.02 mM phenyl propanoids ((c) eugenol, (d) isoeugenol, (e) dehydrodieugenol, (f) dehydrodieugenol B, and (g) coniferyl aldehyde). ESR settings are as follows: microwave power, 5 mW; modulation amplitude, 0.05 mT; time constant, 0.1 sec; gain, 160; sweep time, 2 min.

and (b), the ESR intensity of a quartet decreased by ca. 42% by the addition of ethanol (100 mM). This result implies that in the sample solutions containing smaller amounts of ethanol (32 mM), the ESR intensity is sufficiently large for the estimation of the scavenging ability of phenyl propanoids.

When phenyl propanoids (0.02 mM) were present, the ESR intensities of DMPO–OH decreased significantly, consistent with the scavenging of ·OH by the phenyl propanoids. Shown in Figures 2(c–g) are ESR spectra obtained from the samples containing 0.02 mM eugenol, isoeugenol, dehydrodieugenol, dehydrodieugenol B, and coniferyl aldehyde, respectively. In the spectra, ca. 92 and 58% of the ESR intensities of a 1 : 2 : 2 : 1 quartet due to DMPO–OH was decreased compared to the signal in Figure 2(a) and (b), respectively: The difference of 34% decrease is due to the scavenging by ethanol. This result implies that the phenyl propanoids are much

TABLE I
Inhibition of microsomal lipid peroxidation and deoxyribose degradation by phenyl propanoids. Experimental details are shown in the text

Phenyl propanoid	*I*Hm (%)			*I*Hd (%)		
	Ex.1	Ex.2	Average	Ex.1	Ex.2	Average
Eugenol	45	50	47	73	75	74
Isoeugenol	61	56	59	72	77	75
Dehydrodieugenol	67	61	64	75	73	74
Dehydrodieugenol B	67	67	67	73	75	74
Coniferyl aldehyde	39	39	39	73	70	72

more efficient scavengers for ·OH than ethanol (the concentrations of phenyl propanoids and ethanol were 0.02 mM and 32 mM, respectively.). Using the rate constant of the reaction between ·OH and DMPO ($k = 3.4 \times 10^9\,M^{-1}s^{-1}$),[19] the rate constants for the reactions of all the phenyl propanoids tested here with ·OH ($k_{s+\cdot OH}$) were calculated by the Eq. (1),

$$k_{s+\cdot OH} = k_{DMPO+\cdot OH} \cdot F \cdot [DMPO]/1\text{-}F] \cdot [S] \quad (1)$$

where F is the percent decrease in the ESR intensity by the phenyl propanoids and [S] and [DMPO] are the concentrations of phenyl propanoids and DMPO present, respectively: F was obtained by the Eq. (2),

$$F = (I_0 - I)/I_0 \times 100 \quad (2)$$

where I_0 is the intensity of the low field line in Figure 2(b) and I is that in Figure 2(c–g). Apparent rate constants of phenyl propanoids were roughly estimated to be of the order of $10^{11}\,M^{-1}s^{-1}$: Considering that diffusion controls these reactions, the rate constants will be 10^9 to $10^{10}\,M^{-1}s^{-1}$. This overestimation of the rate constants may arise from the reduction of DMPO–OH by phenyl propanoid radicals. These values are equivalent to or higher than the rate constant of the reaction between ·OH and phenol (reported value, $k = 10^{10}\,M^{-1}s^{-1}$).[20] The rate constant estimated for ethanol using Eq. (1), in which [S] is the concentration of ethanol (100 mM), was roughly $10^9\,M^{-1}s^{-1}$. This value is consistent with the value reported previously.[18].

In order to ascertain the strong scavenging ability of the phenyl propanoids, another measurement for free radical inhibition by phenyl propanoids was performed for microsomal lipid peroxidation. Liver microsomes were separated from 6 weeks old male SD rats. Inhibition (*I*Hm) by phenyl propanoids against lipid peroxidation occuring in microsomes was obtained by the Eq. (3),

$$I\text{Hm}\ (\%) = \{1 - (A_2 - A_3)/(A_1 - A_3)\} \times 100 \quad (3)$$

The absorbance at 532 nm (A_1) was obtained from the samples by the TBA test. The A_2 values indicate ther amount of lipid peroxidation products formed by the Fenton reaction in the absence of phenyl propanoid and A_3 gives the amount of those products generated by the microsomal systems without the aid of the Fenton reaction. The *I*Hm values obtained for all the phenyl propanoids tested are summarized in Table I. The *I*Hm values range between 39 to 67%. This result indicates that the phenyl propanoids have effective antioxidant activity in a biological system. Therefore, it can be expected that these phenyl propanoids are also effective in living systems.

The ·OH scavenging ability of the phenyl propanoids was also investigated by

measuring the amount of products generated by ·OH oxidation of deoxyribose by the TBA test.[14,15] Inhibition (*I*Hd) for degradation of deoxyribose by phenyl propanoids was obtained by the following Eq. (4),

$$I\text{Hd}\ (\%) = \{1 - (B_2/B_1)\} \times 100 \quad (4)$$

where B_1 and B_2 are the absorbansce at 532 nm obtained in the presence and the absence of phenyl propanoids, respectively. In Table I, the *I*Hd values obtained for the phenyl propanoids are listed. The inhibition was found to be more than 70% at 0.2 mM phenyl propanoid concentration although 220 mM ethanol coexisted in the samples. This result is consistent with that obtained using microsomes and implies that these phenyl propanoids have strong antioxidant activity for ·OH.

It has been reported that metallothionein has a rate constant of $10^{12}\,M^{-1}s^{-1}$ for ·OH. In aqueous solutions, these values should be 10^9 to $10^{10}\,M^{-1}s^{-1}$ in aqueous solutions because diffusion controls the reaction rate. The metallothionein behaves as a sacrificial target for ·OH-mediated cellular damage.[21] In the present study, we have found that phenyl propanoids tested here also have effective antioxidant ability for ·OH (Figure 2 and Table I.). The reaction rate between these compounds and ·OH is nearly of the same order as that of metallothionein. We therefore suggest that the phenyl propanoids may be effective inhibitors for ·OH-mediated cellular damages when this radical is the pro-active agent. The use of phenyl propanoids in cosmetics may provide the needed protection against cellular damage caused by ·OH radicals.

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