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PEROXYL RADICAL SCAVENGING ACTIVITIES OF HAMAMELITANNIN IN CHEMICAL AND BIOLOGICAL SYSTEMS

HITOSHI MASAKI$*, TAKAMASA ATSUMI$ and HIROMU SAKURAI¶*

$Shiga Central Laboratory, Noevir Co., LTD., 112-1, Okada-cho, Youkaichi 527, Japan
¶Department of Analytical Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan

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The antioxidative activities of hamamelitannin (2′,5-di-*O*-galloyl-hamamelose), gallic acid and *dl*-α-tocopherol against lipid peroxyl radicals were evaluated in chemical and biological systems. The peroxyl radical scavenging activity was evaluated by electron spin resonance (ESR) method in which both spin-trapping and direct reaction methods were used. In the spin-trapping method, as evaluated by 50% inhibition concentration (IC_{50}) of peroxyl radicals generated in a t-butylhydroperoxide-methemoglobin system, hamamelitannin (IC_{50} = 95.3 ± 2.7 μM) showed the highest activity, followed by gallic acid (IC_{50} = 152.8 ± 14.6 μM) and *dl*-α-tocopherol (IC_{50} = 221.5 ± 4.6 μM) as a positive control. When estimating by the direct method, IC_{50} values of hamamelitannin, gallic acid and *dl*-α-tocopherol were 93.5 ± 2.1 μM, 141.6 ± 2.0 μM and 1590.0 ± 330.0 μM, respectively.

On peroxidation of lipid bilayers induced by 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) in terms of inhibition period (tinh), hamamelitannin showed the longest tinh time (1107.0 ± 38.18 sec), followed by those of *dl*-α-tocopherol (877.5 ± 31.8 sec) and gallic acid (771.0 ± 4.2 sec). The kinetic chain length, defined as the propagation numbers of a lipid peroxyl radical, and calculated from the ratio (Rp/Ri) of the rate of lipid peroxidation to that of inhibition by *dl*-α-tocopherol, hamamelitannin and gallic acid were 27.23, 7.86 and 7.09, respectively. The effects of hamamelitannin, gallic acid and *dl*-α-tocopherol were evaluated on murine fibroblasts exposed to t-butylhydroperoxide (BHP) in terms of the cell survivals. In the protection, hamamelitannin induced the highest survival of 27.6 ± 0.6 % at 50 μM, while both gallic acid and *dl*-α-tocopherol were less active at the same concentrations. On the basis of the results, hamamelitannin was concluded to have a high protective activity on cell damage induced by peroxides.

KEY WORDS: hamamelitannin, gallic acid, t-butylhydroperoxide, antioxidant, ESR, fibroblast.

INTRODUCTION

Skin-aging induced by chronic ultraviolet light (UV) exposure is defined as 'photoaging' and characterized by deep wrinkles and pigmentation. Chronic UV exposure on the skin (photoaging skin) causes denaturations of connective tissue components,[1] such as decrease of collagen, increased staining for glycosaminoglycans[2] and accumulation of elastin.[3] These photochemical reactions in the skin with UV light may produce active oxygen species such as superoxide anion radicals, hydroxyl radi-

*Correspondence to: Hitoshi Masaki, Shiga Central Laboratory, Noevir Co., LTD., 112-1, Okada-cho, Youkaichi 527, Japan. Tel 0748-23-6810, Fax 0748-23-1479
Hiromu Sakurai, Department of Analytical Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan. Tel 075-595-4629, Fax 075-595-4753

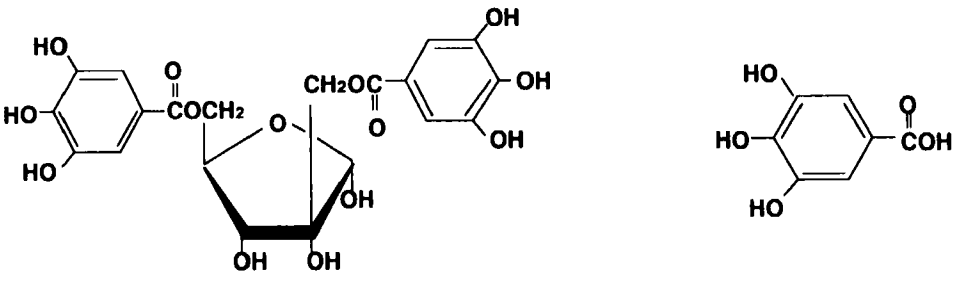
cals, hydrogen peroxides, singlet oxygens and lipid peroxides. The relationship between such dermal denaturations and these active oxygens has been reported.[4] For example, collagen is degraded by exposure of active oxygens such as superoxide anions, hydroxyl radicals[5] and singlet oxygens.[6] There are several lines of experiment to suggest the contribution of active oxygen species on tissue or cell damage under UV irradiation. ESR spin-trapping method suggested the possibility of superoxide anion radical generation,[7] when homogenates of epidermis were exposed to UV light. In organ culture study, the addition of SOD and catalase prevent the formations of sunburn cells.[8] Furthermore, in *in vivo* study, formation of the sunburn cells was found to be accelerated by depletion of glutathione.[9] These observations suggest that connective tissues and skin cells, keratinocytes, melanocytes and fibroblasts are damaged by active oxygens generated by UV irradiation in the skin.

In the previous study, we reported that hamamelitannin which contains two galloyl groups and hamamelose (Figure 1) has a strong scavenging activity against superoxide anion radicals.[10] We investigated further the scavenging activity of hamamelitannin against lipid peroxyl radicals in chemical and biological systems. We report here the results on the lipid peroxyl radical scavenging activity of hamamelitannin as well as the protective activity on murine dermal fibroblast damage induced by t-butylhydroperoxide (BHP).

EXPERIMENTAL

Chemicals

Hamamelitannin, 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), t-butylhydroperoxide (BHP), tetraethoxypropane, gallic acid, Dulbecco's modified eagle medium (Nissui®) (DMEM), *l*-glutamine and 5% fetal bovine serum (FBS) were obtained from Nacalai Tesque. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was from LABOTEC Co. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and *dl*-α-tocopherol were obtained from Tokyo Kasei Kogyo Co.. Methemoglobin (MetHb) and phosphatidylcholine from soybean were obtained from Sigma Co. Ltd.



Hamamelitannin
(2',5-di-*O*-galloyl hamamelose)

Gallic acid

FIGURE 1. Chemical structures of hamamelitannin (2′,5-di-*O*-galloyl-hamamelose) and gallic acid.

Evaluation of Lipid Peroxyl Radical Scavenging Activity by ESR-Spin Trapping Method

Lipid peroxyl radical scavenging activities of the compounds were estimated by ESR spin-trapping technique according to the method of Akaike *et al.*[11] A 100 mM t-butylhydroperoxide was added to metHb (50 μg/ml) in 5 mM sodium phosphate buffer (pH 7.4) which contains 0.7 mM DTPA and various concentrations of the test compounds. Hamamelitannin or *dl*-α-tocopherol was added in the system as the ethanolic solution. Almost simultaneously DMPO was added to the solution to a final concentration of 10 mM. After mixing the solution on a vortex mixer for 1 min, ESR spectra were recorded with a JEOL RE1XG (X-band) spectrometer at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; scanning field, 336.7 ± 5 mT; receiver gain, 1000; response time 0.03 s; sweep time, 2 min and output power, 5 mW. Mn(II) doped in manganese oxide (MnO) was used as a standard. A quantitative analysis of spin adducts of alkyl peroxyl radicals was performed as described below. After recording the ESR spectra, the signal intensity of DMPO-OOR was normalized as a relative signal height against the standard signal of MnO marker. The scavenging activity was expressed as IC_{50}, 50 % inhibition concentration of t-butylperoxyl radicals generated by BHP-MetHb system. Furthermore, to validate the peroxyl radical scavenging activity by the spin trapping technique, the scavenging activities were measured using two different concentrations (10 mM and 30 mM) of DMPO.[12] In the presence of a scavenger, peroxyl radicals react with DMPO and a scavenger competitively as the following equations:[13]

$$\text{DMPO} + \text{ROO}^{\cdot} \xrightarrow{k_d} \text{DMPO–OOR} \qquad [1]$$

$$\text{Scavenger} + \text{ROO}^{\cdot} \xrightarrow{k_s} \text{Product} \qquad [2]$$

where k*d* and k*s* are second-order rate constants of DMPO and the scavenger, respectively. If the concentrations of DMPO and scavenger are much higher than peroxyl radicals, the following equation is derived from equations (1) and (2):

$$Io/I = 1 + k_s[S]/k_d[D] \qquad [3]$$

where Io and I are the ESR signal intensities of DMPO-OOR without and with the scavenger, respectively, and [S] and [D] are the initial concentrations of the scavenger and DMPO, respectively. If the scavenger does not directly react with DMPO, the linear relationship between Io/I-1 and [S]/[D] at various concentrations of DMPO is recognized.

Evaluation of Lipid Peroxyl Radical Scavenging Activity by ESR-Direct Method

Lipid peroxyl radical scavenging activities of the compounds were estimated by the ESR direct method. A 100 mM t-butylhydroperoxide was added to metHb (50μg/ml) in 5 mM sodium phosphate buffer (pH 7.4) which contains 0.7 mM DTPA and various concentrations of the test compounds. Hamamelitannin or *dl*-α-tocopherol was added in the system as the ethanolic solution. After mixing the solution on a vortex mixer for 1 min, ESR spectra recording and quantitative analysis of radicals were performed as described above. The relative signal height was saturated in the presence of the excess

scavenger. The scavenging of peroxyl radicals was calculated as percentages of the saturated value, and its activity was expressed as IC_{50}, 50 % inhibition concentration of the radicals generated in the reaction of the scavenger and t-butylperoxyl radical generating system.

Estimation of Antioxidative Activity on Peroxidation of SPC-Liposome

a) Liposome Preparation Liposome was prepared with a phosphatidylcholine from soybean (SPC). SPC-liposome was prepared according to the method of Watt *et al.*[14] as follows: A dried thin film, which contains SPC (47.5 μmol) and the test compounds, was swollen with phosphate buffered saline (pH 7.4) containing 0.1 mM EDTA and was sonicated with a bath-type sonicator (Bransoni 2200, Yamato Co., Ltd) for 1 min under N_2 gas atmosphere. The resulting suspension under N_2 gas was sonicated with a probe-type sonicator (Handy Sonic UR-20P, Tomy Seiko Co., Ltd) for 10 min in an ice-bath.

b) Measurement of Oxygen Consumption[15] A 2 nM liposome solution with or without the test compounds was transferred into a measuring cell at 37°C. After addition of 10 mM AAPH, oxygen consumption profiles were recorded with a Clark type electrode with high sensitivity membranes combined to a YSI model 53 oxygen monitor (Yellow Springs Instruments, Yellow Springs, OH). The inductive period of peroxidation was measured from each profile of oxygen consumption.

c) Stoichiometric Factors of Antioxidants The stoichiometric factors (n) that represent the number of peroxyl radicals scavenged by each antioxidant were measured by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as an organic radical model.[16] A 0.5 mM DPPH in 50 % EtOH–0.1 M acetate buffer (pH 5.5) and various concentrations of the test compounds were mixed and stood for 30 min at room temperature. Changes of the absorbance at 517 nm were monitored with a Hitachi UV spectrometer U-2000.

Protective Activity on Cell Damage Induced by BHP

a) Cells and Culture Conditions Dermal fibroblasts were primary-cultured from ICR mouse (4-days old). Cells were maintained with Dulbecco's modified eagle medium (Nissui®) (DMEM) supplemented with 0.1 mM L-glutamine and 5% fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37°C with 5% CO_2.

b) BHP Exposing to Murine Fibroblasts and Assay of the Survivals Fibroblasts were placed in a 96-well microplate at a cell density of 3×10^4 cells per well. After 1 day cultivation, the cells were exposed to Hank's buffer solution containing 1.26 mM $CaCl_2$ and 0.81 mM $MgSO_4$ (HBS) supplemented with 50 mM BHP and the compounds tested. After incubation for 5 h at 37°C, the cells were washed with HBS, and the viabilities of the cells were estimated by MTT test. MTT test, which is a rapid colorimetric test to quantify the cell growth and survival, was used to estimate the cell viability.[17] When a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is reduced by NADH in mitochondria of living cells, the blue formazan is formed. The cultured cells were incubated in DMEM supplemented with 5% FBS and 1 mM MTT for 3 h at 37°C. After incubation, the blue formazan was extracted with 100μl of 2-propanol. The absorbances at 560 nm and 650 nm were measured and the formazan formation was estimated by the difference between both absorbances.

TABLE 1
t-Butylperoxyl radical scavenging activities of hamamelitannin, gallic acid and *dl*-α-tocopherol as estimated by ESR spectrometry

	$IC_{50}(\mu M)^{a)}$	$IC_{50}(\mu M)^{b)}$
Hamamelitannin	95.3 ± 2.7	93.8 ± 2.1
Gallic acid	152.8 ± 14.6	141.6 ± 2.0
dl-α-Tocopherol	221.5 ± 4.6	1590.0 ± 330.0

a): IC_{50} values were estimated by spin trapping method. b): IC_{50} values were estimated by the direct reaction method without DMPO as described experimental section. Data are expressed as the mean values ± standard deviations of 3 experiments.

RESULTS

Lipid Peroxyl Radical Scavenging Activities of Hamamelitannin

Scavenging activities of hamamelitannin and its active moiety, gallic acid, against t-butylperoxyl radicals were evaluated by both ESR spin trapping and direct reaction method, and the results are summarized in Table 1. Both hamamelitannin (IC_{50} = 95.3 ± 2.7 μM) and gallic acid (152.8 ± 14.7 μM) were active much more than *dl*-α-tocopherol (221.5 ± 4.6 μM), as evaluated by spin trapping method. We examined the scavenging activities of hamamelitannin using two different concentrations of DMPO (10 mM and 30 mM). The relationship between Io/I-1 and [S]/[D] is shown in Figure 2. The slopes of regression line at 10 mM and 30 mM DMPO were found to be different each other, suggesting that equations (1) and (2) should not be used to analyze this system.

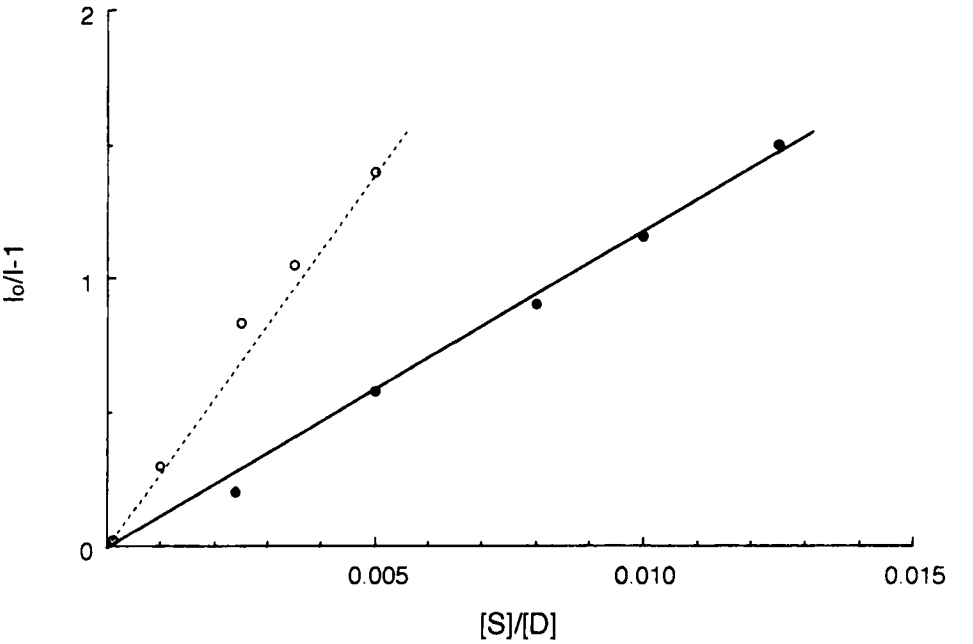


FIGURE 2. Peroxyl radical scavenging activities of hamamelitannin at two different concentrations of DMPO (10 mM and 30 mM) estimated by ESR spin trapping method.
The relationships between Io/I-1 and [S]/[D] were calculated by using eq [3] as described in experimental section. ●: 10 mM DMPO, ○: 30 mM DMPO

Addition of the compounds decreased the radical intensities due to DMPO-t-butylperoxyl radical adducts (g = 2.006, a_H^γ = 0.14 mT, a_H^β = 1.00 mT, a_N = 1.45 mT) with appearance of a new signal due to another types of radicals (Figure 3). The hyperfine splitting constants of the DMPO-t-butylperoxyl radical adducts was consistent with the results of Akaike *et al.*[11] When DMPO was lacked from the system in the ESR spin trapping, the new radicals were disappeared and another type of signals were observed in the presence of hamamelitannin and gallic acid (Figure 4). To examine the mechanism of the radical formation in the system, we measured ESR spectra by omitting t-BHP or metHb from the t-butylperoxyl radical generating system. Since no ESR signals were found under the conditions, the radicals in Figure 4 were concluded to be derived from the direct reaction of t-butylperoxyl radicals with hamamelitannin or gallic acid. The intensities of the new radicals increased with the amount of the compounds in a dose dependent manner (Figure 5). Furthermore, we examined the peroxyl radical scavenging activities of the compounds using the radicals derived from the compounds and t-butylperoxyl radicals. The IC_{50} values for hamamelitannin, gallic acid and *dl*-α-tocopherol were 93.5 ± 2.1 μM, 141.6 ± 2.0 μM and 1590.0 ± 330.0 μM, respectively (Table 1).

Antioxidative Activity of Hamamelitannin

Lipid peroxidation is characterized by radical chain reactions which are expressed by the following equations.[15,18]

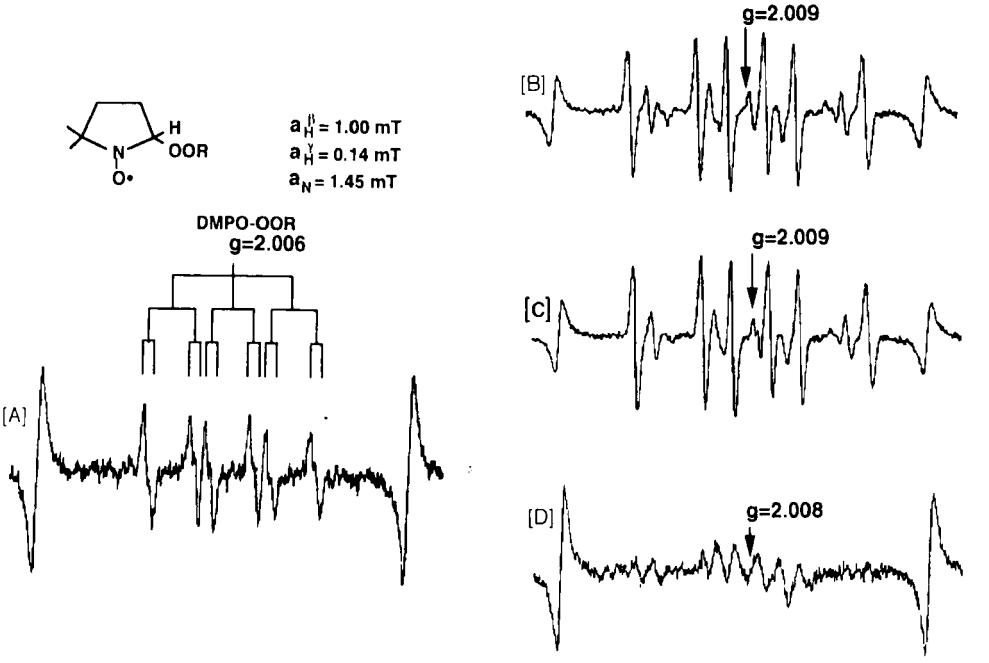


FIGURE 3. ESR spectra of DMPO spin adducts in the reaction systems of t-butylhydroperoxide (t-BHP) plus methemoglobin (MetHb) in the presence of hamamelitannin, gallic acid and *dl*-α-tocopherol.

The reaction system contained 100 mM t-BHP, 50 μg/ml MetHb, 0.7 mM DTPA and 10 mM DMPO in 5 mM sodium phosphate buffer (pH 7.4), [A]: spectrum for the reaction system without scavengers, [B]: spectrum for the reaction system in the presence of 500 mM hamamelitannin, [C]: spectrum for the reaction system in the presence of 500 mM gallic acid, [D]: spectrum for the reaction system in the presence of 500 mM *dl*-α-tocopherol.

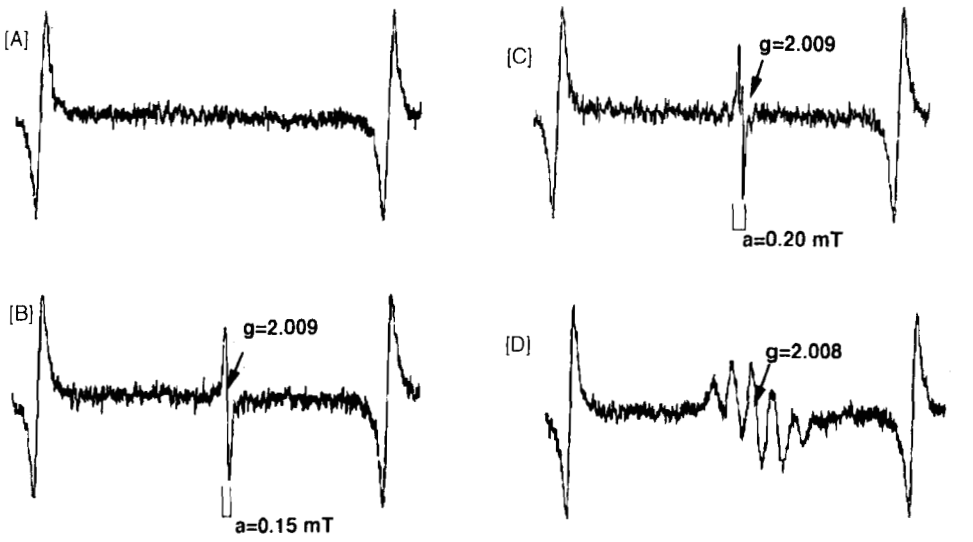


FIGURE 4. ESR spectra of radicals generated by the reaction with t-butylhydroperoxide (t-BHP) plus methemoglobin (MetHb) system and hamamelitannin, gallic acid and *dl*-α-tocopherol.

The reaction system contained 100 mM t-BHP, 50 μg/ml MetHb, and 0.7 mM DTPA in 5 mM sodium phosphate buffer (pH 7.4) [A]: spectrum for the reaction system without scavengers, [B]: spectrum for the reaction system in the presence of 500 mM hamamelitannin, [C]: spectrum for the reaction system in the presence of 500 mM gallic acid, [D]: spectrum for the reaction system in the presence of 500 mM *dl*-α-tocopherol.

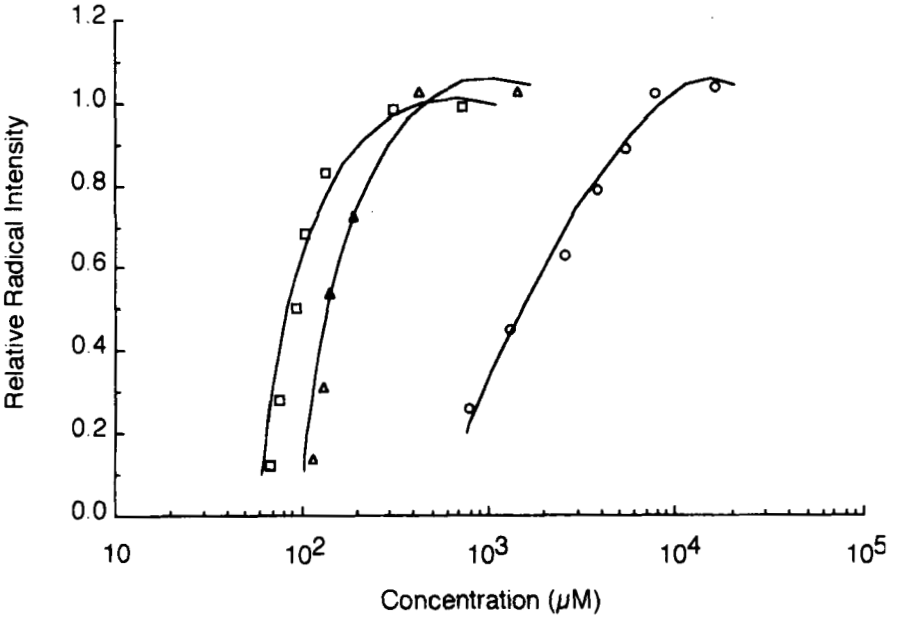


FIGURE 5. Dose dependent t-butylperoxyl radical scavenging profiles of hamamelitannin, gallic acid and *dl*-α-tocopherol estimated by ESR direct method.

Radicals generated by reactions with compounds and t-butylperoxyl radicals were detected by ESR spectrometer. After recording the ESR spectra, the signal intensity was normalized as a relative signal height against the standard signal of MnO marker. □ Hamamelitannin, △ Gallic acid, ○ *dl*-α-Tocopherol

Initiation

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad [4]$$

$$ROO^{\cdot} + LH \rightarrow ROOH + L^{\cdot} \quad [5]$$

Propagation

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad [6]$$

$$LOO^{\cdot} + LH \overset{kd}{\leftrightarrows} LOOH + L^{\cdot} \quad [7]$$

Termination

$$2LOO^{\cdot} \overset{kt}{\rightarrow} \text{nonradical products} \quad [8]$$

where $R^{\cdot}$ is the initiator radical, LH represents the lipid molecule, $L^{\cdot}$, $LOO^{\cdot}$ and LOOH are the alkyl and alkylperoxyl radicals and hydroperoxide respectively, and *k*p and *k*t are the rate constants for propagation and termination of the radical chain reactions, respectively.

The rates of lipid oxidation (Rp) and inhibition period (Rinh) can be determined quantitatively by the rate of oxygen consumption. The initiation rate (Ri) is calculated by the inhibition period (tinh) produced by a known amount of an antioxidant as follows,

$$Ri = n[IH]/tinh \quad [9]$$

where [IH] is the concentration of an antioxidant and 'n' corresponds to the stoichiometric factor that represents the number of peroxyl radicals scavenged by each molecule of antioxidant. Furthermore, the inhibition rate constant (*k*inh) is calculated by tinh and Rinh as follows,

$$kinh/kp = [LH]/Rinh \cdot tinh \quad [10]$$

where [LH] is the concentration of the lipid molecule.

Antioxidative activities of hamamelitannin and gallic acid on peroxidations of lipid bilayers induced by AAPH, which is a water soluble radical initiator, were evaluated by both oxygen consumption profiles and lipid peroxides. In these systems, *dl*-α-tocopherol was used as a standard antioxidant. Obtained parameters for the antioxidant activities of the compounds are summarized in Table 2. The tinh values of *dl*-α-tocopherol and gallic were 877.5 ± 31.8 sec and 771.0 ± 4.2 sec, respectively. On the other hand, hamamelitannin gave the longest tinh (1107.0 ± 38.18 sec) among the compounds tested. The stoichiometric factors (n) of *dl*-α-tocopherol, hamamelitannin and gallic acid were found to be 2.2 ± 0.1, 9.4 ± 0.8 and 8.8 ± 1.2, respectively.

The kinetic chain length, obtained from the ratio Rp/Ri, for *dl*-α-tocopherol, hamamelitannin and gallic acid were 27.23, 7.86 and 7.09, respectively. The ratio of *k*inh/*k*p of *dl*-α-tocopherol, hamamelitannin and gallic acid were 108.96, 79.35 and

TABLE 2
Parameters for the antioxidant activities of hamamelitannin, gallic acid and *dl*-α-tocopherol

	tinh s	Rinh $\times 10^8$ M/s	Rp $\times 10^7$ M/s	n	Ri $\times 10^9$ M/s	Rp/Ri	kinh/kp $M^{-1}s^{-1}$
Hamamelitannin	1107.00	2.45	1.47	9.40	18.69	7.86	79.35
Gallic acid	771.00	3.15	1.55	8.80	21.32	7.09	76.05
dl-α-Tocopherol	877.50	2.50	1.54	2.20	5.66	27.23	108.96

Rp (rate of lipid oxidation), Rinh (rate of inhibition period) and tinh (inhibition period) were estimated by the rate of oxygen consumption. The values of n (the stoichiometric factors of antioxidant) and Ri (initiation rate) was calculated by the Eq [9] in the text. Rp/Ri (kinetic chain length), *k*inh (inhibition rate constant), *k*p (rate constants for propagation) and *k*inh/*k*p were obtained by the Eq [10] in the text.

76.05, respectively. The results indicate that hamamelitannin and gallic acid had the higher abilities to stop the propagation of lipid peroxidation than *dl*-α-tocopherol, in contrast, both hamamelitannin and gallic acid were inferior to *dl*-α-tocopherol in terms of the inhibition rate constant.

Protective Activity of Hamamelitannin on Cell Damage Induced by BHP

Effects of hamamelitannin, gallic acid and *dl*-α-tocopherol were evaluated on murine fibroblasts exposed to BHP in terms of the cell survival. In the protection, hamamelitannin showed higher survivals at the low concentration of 50 μM (27.6 ± 0.6 %), in contrast to that of control (14.1 ± 0.8%). Gallic acid was not effective at all concentrations tested. The protective activity of *dl*-α-tocopherol was observed at 100 μM (21.3 ± 1.7 %) (control survival: 13.0 ± 1.2 %). (Figure 6) From these observations, hamamelitannin was concluded to have a higher protective activity on cell damages induced by BHP, compared with those of gallic acid and *dl*-α-tocopherol.

DISCUSSION

Since hamamelitannin contains two galloyl groups in the chemical structure (Figure 1), gallic acid was also used to discuss a role of a galloyl group on the scavenging activity of hamamelitannin for active oxygens. The results on the scavenging activities of these compounds against t-butylperoxyl radicals as estimated by ESR spin-trapping method indicated that hamamelitannin has a potent activity (Table 1). Further, we examined whether hamamelitannin reacts with DMPO or peroxyl radicals. Since the ratio (k*s*/k*d*) of second-order rate constant in the spin trapping reaction of hamamelitannin at different concentrations of DMPO gave the different values under the conditions, the interaction of hamamelitannin with DMPO was suggested (Figure 2). No changes of absorbance at 227 nm due to DMPO were found in the presence of hamamelitannin, suggesting that the compounds did not react with DMPO. Hamamelitannin, gallic acid and *dl*-α-tocopherol gave the ESR signals in the reaction with peroxyl radicals without DMPO (Figure 4). Furthermore, in the presence of DMPO, both hamamelitannin and gallic acid developed the new signals in the process of the scavenging of t-butylperoxyl radicals (Figure 3). These signals are due to DMPO adducts of the reaction products of t-butylperoxyl radicals with hamamelitannin or gallic acid (Figure 4). From these results, the reactions in the ESR spin trapping were speculated as follows:

$$\text{DMPO} + \text{ROO}^{\cdot} \overset{k_{d1}}{\rightleftharpoons} \text{DMPO–OOR} \qquad [11]$$

$$\text{Scavenger} + \text{ROO}^{\cdot} \overset{k_s}{\rightleftharpoons} \text{Radical (I)} \qquad [12]$$

$$\text{Radical (I)} + \text{DMPO} \overset{k_{d2}}{\rightleftharpoons} \text{Radical (II)} \qquad [13]$$

During the spin trapping of t-butylperoxyl radicals (ROO$^{\cdot}$) with DMPO, hamamelitannin or gallic acid reacts with ROO$^{\cdot}$ and gives radical (I) (equation 12). Radical (I) is trapped by DMPO, and is converted to radical (II) (equation 13). Since ROO$^{\cdot}$ and radical (I) derived from the reaction with t-butylperoxyl radicals and hamamelitannin or gallic acid, are trapped by DMPO competitively, equations (1) and (2) should not be used to the spin trapping on the scavenging of t-butylperoxyl radicals by hamamelitannin and gallic acid. Then, we estimated IC_{50} values of the peroxyl radical scavenging using the direct reaction of t-butylperoxyl radicals and the compounds (Table 1). The IC_{50} values of the direct reaction method were consistent with those of the spin trapping method in hamamelitannin and gallic acid, however, the IC_{50} of *dl*-α-tocopherol by the direct reaction method showed much higher than that by the spin trapping method. From the results of the kinetic study on the antioxidants, the spin trapping method was found to be adequate in comparison with the direct reaction method. Furthermore, the appearance of the direct radicals indicates that hamamelitannin reacts with peroxyl radicals generated in the BHP-metHb system by scavenging the peroxyl radicals.

We performed the kinetic study on the antioxidants in terms of lipid peroxidation. Parameters for antioxidant activities of hamamelitannin were similar to those of gallic acid (Table 2), indicating that the active site of hamamelitannin in scavenging peroxyl radicals is due to the galloyl moiety. By analyzing antioxidant parameters, the reactivity of hamamelitannin against peroxyl radicals was suggested to be lower than that of

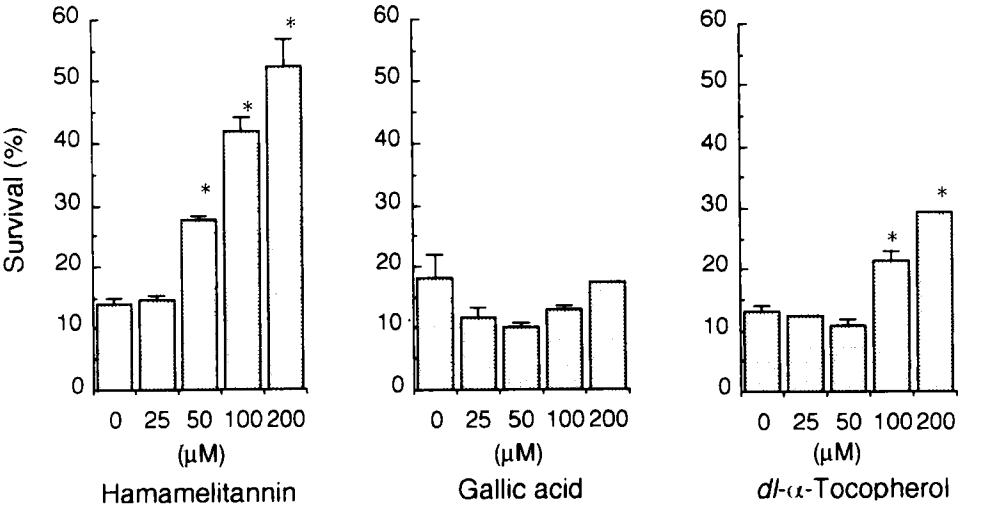


FIGURE 6. Protective activities of hamamelitannin, gallic acid and *dl*-α-tocopherol against cell damage induced by t-butylhydroperoxide.

Data are expressed as the mean values ± standard deviations of 4 experiments

Significance *: $P < 0.01$.

dl-α-tocopherol in terms of *k*inh (inhibition rate constant). However, the parameters such as tinh, n, and Rp/Ri for hamamelitannin were superior to those of *dl*-α-tocopherol (Table 2).

In protection the BHP-exposed cells, hamamelitannin was revealed to have the highest activity among the compounds tested. However, gallic acid did not protect the cells under the same conditions (Figure 6). In the processes of cell damage induced by BHP, it may be incorporated into the cell membranes and converted to the corresponding peroxyl radicals by biometal ions such as Fe^{3+} and Cu^{2+}. Thus the peroxyl radicals will propagate the peroxidation of the membrane molecules, and lead the cell death. Hamamelitannin is the more lipophilic than gallic acid, suggesting that hamamelitannin will be able to migrate in the lipid regions of cell membrane due to its lipophilicity.

In conclusion, hamamelitannin was revealed to have the high peroxyl radical scavenging activity as well as the high superoxide anion scavenging activity, in which the galloyl group is responsible for the activity. Furthermore, hamamelitannin was found to have the protective activity on cell damage exposed peroxyl radicals, supporting the results on chemical systems.

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