

Protective Activity of Hamamelitannin on Cell Damage Induced by Superoxide Anion Radicals in Murine Dermal Fibroblasts

Hitoshi MASAKI,*^a Takamasa ATSUMI,^a and Hiromu SAKURAI^b

Shiga Central Laboratory, Noevir Co., Ltd.,^a 112-1, Okada-cho, Youkaichi 527, Japan and Department of Analytical Chemistry, Kyoto Pharmaceutical University,^b Yamashina-ku, Kyoto 607, Japan.

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Previously we demonstrated that hamamelitannin (2',5-di-*O*-galloyl hamamelose) in *Hamamelis virginiana* L. exhibits potent superoxide-anion scavenging activity. We then examined the physiological and pharmacological activities of hamamelitannin as well as its functional homologues, gallic acid and syringic acid. The following results were obtained: (1) Hamamelitannin has a higher protective activity against cell damages induced by superoxide anions than gallic acid which is the functional moiety of hamamelitannin. The protective activity of hamamelitannin on murine fibroblast-damage induced by superoxide anions was found at a minimum concentration of 50 μM , while the corresponding figure for gallic acid was 100 μM . (2) Pre-treatment of fibroblasts with hamamelitannin enhances cell survival. (3) The superoxide-anion scavenging activity of the compound in terms of its IC_{50} value (50% inhibition concentration of superoxide anion radicals generated) was evaluated by ESR spin-trapping. Both hamamelitannin ($\text{IC}_{50} = 1.31 \pm 0.06 \mu\text{M}$) and gallic acid ($\text{IC}_{50} = 1.01 \pm 0.03 \mu\text{M}$) exhibited high superoxide-anion scavenging activity followed by syringic acid ($\text{IC}_{50} = 13.90 \pm 2.38 \mu\text{M}$). (4) When hamamelitannin was treated with superoxide anions generated by a KO_2 -crown ether system, HPLC analysis showed the disappearance of hamamelitannin and the concomitant formation of hamamelitannin-derived radicals ($g = 2.005$, $\Delta H_1 = 2.16 \text{ G}$, $\Delta H_2 = 4.69 \text{ G}$) was detected by ESR spectrometry. From these observations, we concluded that (i) a compound with polyphenolic hydroxyl groups, especially the galloyl group (3,4,5-trihydroxy-), has potent scavenging activity against superoxide anions, and (ii) hamamelitannin is superior to gallic acid in protecting against cell damage induced by superoxide anions, suggesting that the high affinity of hamamelitannin for cells or membranes may be an important factor for protecting cells against active oxygen species.

Keywords hamamelitannin; superoxide anion radical; fibroblast; ESR-spin trapping method; cytotoxicity

Recently, the relationship between skin aging and solar irradiation has interested many researchers.^{1–3} Such aging has been defined as cutaneous photoaging and is characterized by the formation of deep wrinkles. Ultraviolet (UV) rays from the sun play a significant role in the development of skin aging.⁴ Chronic UV irradiation of the skin causes denaturation of connective tissue components, such as collagen, increased staining of glycosaminoglycans⁵ and accumulation of elastin.⁶ The relationship between this denaturation and active oxygen *in vitro* has been reported.⁷ For example, collagen is degraded by exposure to active-oxygen species such as superoxide anions, hydroxyl radicals⁸ and singlet oxygens.⁹ Previously, we reported that hyaluronic acid, the major component of glycosaminoglycans, is decomposed by the superoxide anion–Fe–EDTA system.¹⁰ The formation of “sunburn cells” in the epidermis was observed following exposure to UV light.¹¹ However, this was prevented by active oxygen-scavengers such as superoxide dismutase, catalase, xanthine or *D*-mannitol.¹² The depletion of glutathione in hairless mice enhanced sunburn cell formation produced by exposure to UVB light.¹³ Furthermore, it has been reported that the homogenized epidermis, when exposed to UV light, generates lipid-derived radicals, detected by ESR spin-trapping, and these signals disappeared with the addition of superoxide dismutase.¹⁴ From these observations, several types of active oxygens are suggested to be produced in the skin by UV irradiation. Such active oxygens play a significant role in the development of damage to connective tissues and skin cells, keratinocytes,

fibroblasts and melanocytes.

During our investigations on antioxidative compounds in plants, we found that the extract of *Hamamelis virginiana* L. among 65 plant extracts exhibits good superoxide anion scavenging activity, estimated by neotetrazolium method¹⁵ and hamamelitannin, 2',5-di-*O*-galloyl hamamelose, which is a major component of the plant bark (Fig. 1), exhibits potent superoxide-anion scavenging activity, as estimated by ESR-spin trapping.¹⁰ The object of this study was to evaluate the protective activity of hamamelitannin on cell damage induced by superoxide anions compared with that of polyphenol compounds and to clarify the mechanism of its scavenging activity as far as reactions with superoxide anion radicals are considered.

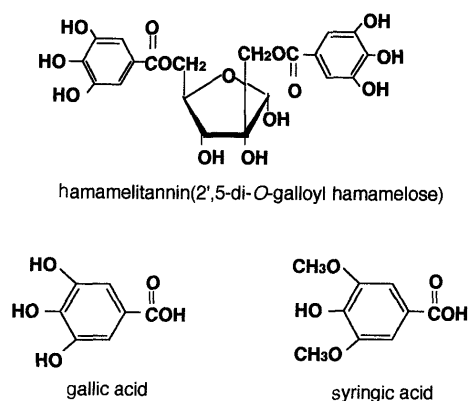


Fig. 1. Chemical Structures of Hamamelitannin (2',5-Di-*O*-galloyl Hamamelose), Gallic Acid and Syringic Acid

MATERIALS AND METHODS

Chemicals Hamamelitannin (2',5-di-*O*-galloyl hamamelose) was purchased from Funakoshi Co., Ltd. Gallic acid, syringic acid and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Tokyo Kasei Co., Ltd. Hypoxanthine (HPX), xanthine oxidase (XOD) and catalase were obtained from Nacalai Tesque. Diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid (DTPA) was obtained from Dojindo Laboratories. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Labotec Co. Potassium superoxide (KO_2) and 18-crown-6 ether (18C6) were obtained from Aldrich Chemical Co., Ltd. Dimethylsulfoxide (DMSO) was distilled *in vacuo* and used after drying with a molecular sieve (Wako Pure Chemical Co., mesh size of 3 Å) followed by filtration.

Cells and Culture Conditions Dermal fibroblasts were primary-cultured from ICR mice (4-d 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 .

Protective Activity on Cell Damage Induced by Superoxide Anion Radicals The protective activity on cell damage induced by superoxide anion radicals was evaluated by the method reported by Richard *et al.*¹⁶⁾ Fibroblasts were placed in a 96-well microplate at a cell density of 3×10^4 cells per well. After 1 d of cultivation, cells were exposed to the superoxide-anions generating system in the presence of the compounds to be tested. After 2.5 h incubation at 37°C, the cells were washed with Hank's buffer containing 1.26 mM CaCl_2 and 0.81 mM MgSO_4 (HBS), and the viabilities of the cells were estimated by the MTT test described below. The superoxide-anion generating system, consisting of 1.5 mM HPX, 500 unit/ml catalase and 40 munit/ml XOD, was dissolved in 100 μl HBS and placed on the cells in the presence of the test compounds. Survival was expressed as the percentage of control value exhibited by the system containing HPX, catalase, and the test compound.

Pre-treatment Effect on Cell Damage Induced by Superoxide Anion Radicals Fibroblasts were placed in a 96-well microplate at a cell density of 3×10^4 cells per well. After 1 d cultivation, the cells were cultivated with DMEM supplemented with 5% FBS and the test compounds for 24 h at 37°C. The cells were washed with Hank's buffer to remove the compounds. The superoxide-anion generating system, consisting of 1.5 mM HPX, 500 unit/ml catalase and 100 munit/ml XOD, was dissolved in 100 μl HBS, and placed on the cells. After 2.5 h incubation at 37°C, the cells were washed with HBS and the viability of the cells was estimated by the MTT test. The survivals were expressed as the percentage of the control value exhibited by the system containing HPX, catalase, and the test compound.

Estimation of Cell Viability The MTT test, which is a rapid colorimetric test to quantify cell growth and survival, was used to estimate cell viability.¹⁷⁾ When MTT is reduced by NADH in the mitochondria of living cells, blue formazan is formed. The cultured cells were incubated

in DMEM supplemented with 5% FBS and 1 mM MTT for 2 h at 37°C. After incubation, the blue formazan was extracted with 100 μl 2-propanol. The absorbances at 560 and 650 nm were measured and the formazan formed was estimated from the difference between both the two.

Superoxide-Anion Scavenging Activity Estimated by ESR Spin-Trapping The superoxide-anion scavenging activity of the compounds was estimated by ESR spin-trapping according to the method of Kitagawa *et al.*¹⁸⁾ XOD, 0.12 unit/ml, was added to 0.4 mM HPX in 100 mM sodium phosphate buffer (pH 7.4) which contains 0.7 mM DTPA and various concentrations of the test compounds. Almost simultaneously, DMPO was added to the solution to give a final concentration of 90 mM and then mixed on a vortex mixer. After mixing for 1 min, ESR spectra were recorded with a JEOL RE1XG (X-band) spectrometer at a field modulation frequency of 100 kHz and a modulation amplitude of 1 G at an output power of 5 mW. Mn(II) doped in MgO was used as a standard. All experiments were carried out at room temperature (21°C). A quantitative analysis of the spin adducts of the superoxide anion radicals was performed as described below. After recording the ESR spectra, the signal intensity due to DMPO-OOH was normalized as a relative height against the standard signal of the Mn(II) marker. The scavenging activity is expressed as the IC_{50} , the 50% inhibition concentration of the superoxide anion radicals generated by the HPX-XOD system.

Reaction of Hamamelitannin with Superoxide Anion Radicals The reaction of hamamelitannin with superoxide anion radicals was estimated by monitoring the decomposition of hamamelitannin by HPLC analysis. A KO_2 -18C6 mixture was used as the superoxide-anion generating system.¹⁹⁾ KO_2 (0.015 mmol) and 18C6 (0.075 mmol) were transferred to a test-tube and dimethyl sulfoxide (DMSO) (5 ml) was added. The mixture was purged with argon gas for 30 s and sealed with a septum, followed by sonication for 60 s. The supernatant was used for the reaction with hamamelitannin. The concentration of superoxide anions was estimated from the molecular extinction coefficient of reduced cytochrome c, $\epsilon = 21000/\text{M}/\text{cm}$.²⁰⁾ The superoxide anion solution (150 μl) and 0.4 mM hamamelitannin solution (150 μl) were mixed. After allowing the solution to stand for 30 min at room temperature, hamamelitannin was determined by HPLC (Shimadzu SLC-10A and SPD-10A), using a Capcell Pack C18 column (4.6 \times 150 mm). The elution solvent was 12% CH_3CN and 88% 0.05% H_3PO_4 , and the detector was operated at 280 nm.

Detection of Radicals Derived from Hamamelitannin and Superoxide Anion Radicals The KO_2 -18C6 system¹⁹⁾ was used as a superoxide anion source in an organic solvent. The superoxide anion solution (200 μl) and 0.4 mM hamamelitannin solution (100 μl) in DMSO were mixed. The mixed solution was immediately transferred to a glass capillary tube (Microcaps, Drummond Scientific Co.) and sealed with clay. Two capillary tubes transferred in an ESR quartz were set in the ESR cavity and the spectrum was recorded after 60 s of mixing.

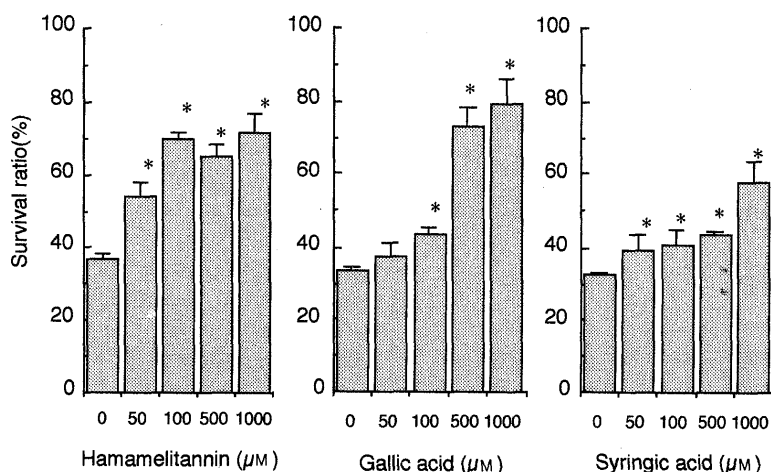


Fig. 2. Protective Activities of Hamamelitannin and Its Homologues on Cell Damage Induced by Superoxide Anion Radicals

Data are expressed as the mean values \pm standard deviation of 4 experiments, significance *, $p < 0.01$.

RESULTS

Protective Activities of Hamamelitannin and Its Homologues on Cell Damage to Murine Fibroblasts Induced by Superoxide Anion Radicals The protective activity of hamamelitannin and its homologues, such as gallic acid and syringic acid, on the cell damage induced by superoxide anion radicals was evaluated. Superoxide anion radicals were generated by the HPX-XOD system. To evaluate the effect of superoxide anion radicals on the damage, catalase was added to the generating system to minimize the effect of hydrogen peroxide. The results are summarized in Fig. 2. In contrast to the survival of the control ($36.9 \pm 1.5\%$), the addition of $50 \mu\text{M}$ hamamelitannin enhanced the survival of fibroblasts to $52.4 \pm 3.7\%$. Gallic acid had little effect on the survival at $50 \mu\text{M}$, but significant enhancement of survival was observed at a concentration of $100 \mu\text{M}$ and over ($43.5 \pm 2.3\%$ at $100 \mu\text{M}$, $73.5 \pm 5.3\%$ at $500 \mu\text{M}$ and $79.7 \pm 6.8\%$ at 1 mM). Syringic acid produced survivals of $40.9 \pm 4.0\%$ at $100 \mu\text{M}$, $43.4 \pm 1.2\%$ at $500 \mu\text{M}$ and $58.1 \pm 6.0\%$ at 1 mM , indicating that its protective activity was inferior to that of gallic acid.

Effects of Pre-treatment by Hamamelitannin or Gallic Acid on Cell Damage to Murine Fibroblasts Fibroblasts were treated with hamamelitannin or gallic acid for 24 h at 37°C . After washing to remove hamamelitannin or gallic acid, fibroblasts were placed in contact with superoxide anions generated by the HPX-XOD system. The survival of the control and fibroblasts pre-treated with hamamelitannin ($200 \mu\text{M}$) were $19.0 \pm 3.9\%$ and $63.8 \pm 4.4\%$, respectively (Fig. 3). On the other hand, the survival of the fibroblasts pre-treated with $200 \mu\text{M}$ gallic acid was $25.4 \pm 0.7\%$, similar to that of the control, $17.7 \pm 0.5\%$. These results confirmed that fibroblasts which have been pre-treated with hamamelitannin are highly resistant to damage induced by superoxide anion.

Superoxide-Anion Radical-Scavenging Activity of Hamamelitannin and Its Homologues Estimated by ESR Spin-Trapping The superoxide anion radical scavenging activity of hamamelitannin and its homologues was evaluated by ESR spin-trapping using DMPO as a spin-trap reagent. The superoxide-anion scavenging

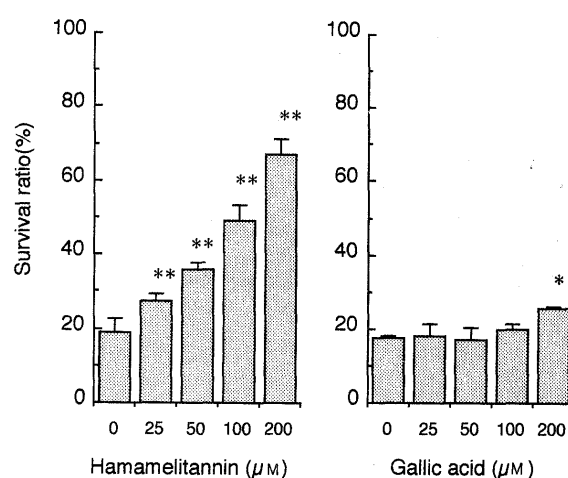


Fig. 3. Pre-treatment Effects of Hamamelitannin and Gallic Acid on Cell Damage Induced by Superoxide Anion Radicals

Data are expressed as the mean values \pm standard deviation of 4 experiments, significance *, $p < 0.05$; **, $p < 0.01$.

TABLE I. Superoxide Anion Scavenging Activities of Hamamelitannin and Its Homologues Estimated by ESR Spin-Trapping

Compound	IC ₅₀ (μM)
Hamamelitannin	1.31 \pm 0.06
Gallic acid	1.01 \pm 0.03*
Syringic acid	13.90 \pm 2.38*

Data are expressed as the mean values \pm standard deviation of 3 experiments. Significance *, $p < 0.01$.

activity was expressed in terms of the concentration producing 50% inhibition of superoxide anion radical (IC_{50}). The IC_{50} s of hamamelitannin, gallic acid and syringic acid were found to be 1.31 ± 0.06 , 1.01 ± 0.03 , and $13.90 \pm 2.38 \mu\text{M}$, respectively (Table I). Although hamamelitannin contains two galloyl groups, its scavenging activity was lower than that of gallic acid.

Reaction of Hamamelitannin with Superoxide Anion Radicals Hamamelitannin was treated with superoxide anion radicals generated in the KO_2 -18C6 system and the amount of hamamelitannin was monitored by HPLC at

room temperature (Fig. 4). The reaction rate was too fast to detect the conversion of hamamelitannin, however, when low concentrations of superoxide anion radicals were used, the rapid disappearance of hamamelitannin was observed to take place in a concentration-dependent manner (Fig. 5). These results confirm that the disappearance of hamamelitannin is due to the addition of

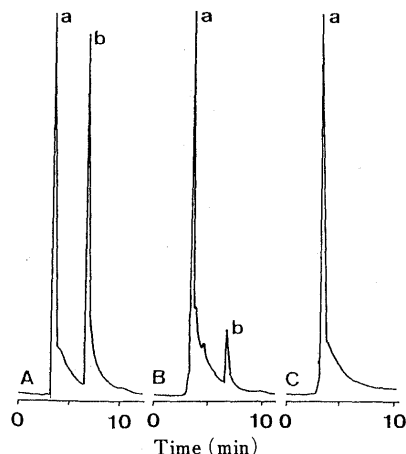


Fig. 4. HPLC Profiles of the Reactions between Hamamelitannin and Superoxide Anion Radicals

HPLC conditions: column, capcell pack C_{18} ; solvent, CH_3CN : 0.05% H_3PO_4 = 12:88; flow rate, 0.5 mm/min; temp. 40°C; a, peak due to solvent; b, peak due to hamamelitannin; A, before the reaction; B, after reaction with 10 nmol superoxide anions; C, after reaction with 20 nmol superoxide anions.

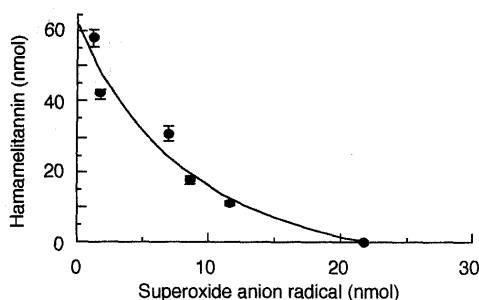


Fig. 5. Conversion of Hamamelitannin during the Reaction with Superoxide Anion Radicals

The determination was carried out by HPLC, as described in Materials and Methods.

superoxide anion radicals, suggesting a direct reaction between the two.

Detection of Hamamelitannin-Derived Radicals by ESR Spectrometry When superoxide anion radicals and hamamelitannin were mixed at room temperature, an ESR spectrum ($g=2.005$, $\Delta H_1=2.16$ G, $\Delta H_2=4.69$ G) due to hamamelitannin-derived radicals was recorded, being stable for 2 h at room temperature (Fig. 6). It was tentatively presumed that these radicals are hamamelitannin-derived. We attempted detection of the radicals due to gallic acid under the same conditions, but no stable radicals were detected.

DISCUSSION

UV damage by active oxygen species has been studied by many investigators.¹⁻³⁾ We speculate that UV-induced skin damage, including wrinkle formation, may be prevented by scavenging active oxygens. To examine this hypothesis, we studied the scavenging activity on active oxygen species exhibited by plant products and found that hamamelitannin (Fig. 1) which consists of two galloyl groups and hamamelose, has potent superoxide anion radical scavenging activity.¹⁰⁾

In the present study, we characterized the protective activity of hamamelitannin on cell damage induced by superoxide anion radicals, compared with that of homologues such as gallic acid and syringic acid; hamamelitannin was found to have the highest activity. Hamamelitannin protected against cell damage at lower concentrations than gallic acid (Fig. 2). To support these observations, we estimated the scavenging activities with respect to superoxide anion radicals, using ESR spin-trapping. Both hamamelitannin and gallic acid exhibited high scavenging activity, with gallic acid being slightly more active than hamamelitannin, while syringic acid was less active. These results suggest that the scavenging activity of hamamelitannin is due to the galloyl groups (Table I).

From the results in Fig. 2 and Table I, hamamelitannin and gallic acid seen to have different features in terms of their protective activity on cell damage induced by superoxide anion radicals. We examined the pre-treatment effects of both compounds on cell damage induced by

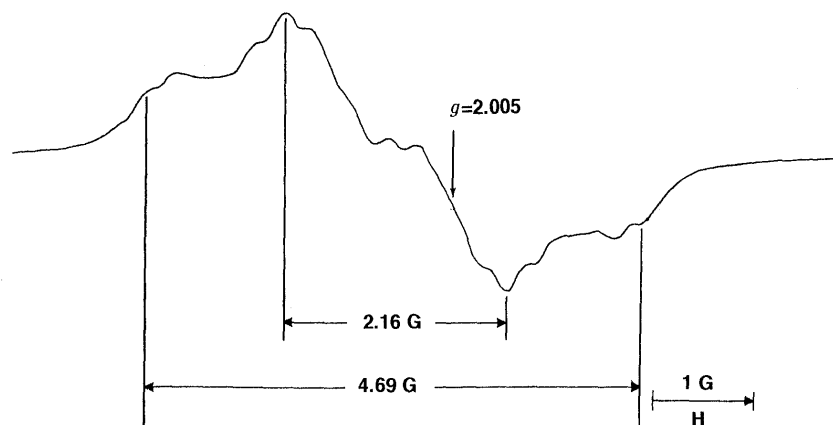


Fig. 6. ESR Spectrum of Hamamelitannin-Derived Radicals Generated by Reaction with Superoxide Anion Radicals

ESR spectrum was recorded with a JEOL RE1XG (X-band) spectrometer at room temperature (22°C) at a field modulation frequency of 100 kHz and a modulation amplitude of 1 G at an output power of 5 mW.

superoxide anion radicals. The cells pre-treated with hamamelitannin showed a higher survival ratio (%) than those treated with gallic acid (Fig. 3). These results indicate that hamamelitannin has a higher affinity for cells or cell membranes than gallic acid, under the same conditions, suggesting an interaction between the cell membranes and hamamelitannin, probably through the hamamelose moiety.

To understand the scavenging mechanism of hamamelitannin with respect to superoxide anion radicals, the reaction was examined by HPLC. Hamamelitannin was found to be destroyed by the addition of superoxide anion radicals (Figs. 4 and 5), with a concomitant formation of hamamelitannin-derived radicals as detected by ESR spectrometry (Fig. 6). The hamamelitannin-derived radicals, whose electronic structure was not yet been determined, were stable for 2 h. The reactivity of hamamelitannin with superoxide anion radicals may be involved in the enhanced survival of cells exposed to superoxide anion radicals, when cells are pre-treated with this compound. On the other hand, no ESR signals, due to gallic acid-derived radicals were observed in the reaction of superoxide anion radicals, probably due to the short half-life of the gallic acid-derived radicals, which may promote radical chain reactions. Further physico-chemical investigation on the properties of hamamelitannin-derived radicals is needed.

In this study, we propose a reaction mechanism for hamamelitannin, which protects against cell damage induced by superoxide anion radicals, related to its structure: (1) the galloyl group in the molecule is essential for the scavenging effect on superoxide anion radicals and (2) the high affinity of hamamelitannin for cells or cell membranes may be due to the hamamelose moiety of compound.

In conclusion, hamamelitannin was found to protect against cell damage induced by superoxide anion radicals. Recently, we found that hamamelis extract has a relatively

high hydroxyl-radical scavenging activity¹⁵⁾ and we have plans to study at a cellular level the interactions of this plant as well as its active components with several active oxygen species, including hydroxyl radicals.

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