Free Rad. Res. Comms., Vol. 19, No. 5, pp. 333-340
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EVALUATION OF SUPEROXIDE SCAVENGING ACTIVITIES OF HAMAMELIS EXTRACT AND HAMAMELITANNIN

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(Received February 25, 1993; in final form August 12, 1993)

Hamamelitannin, which is a component of bark extract of hamamelis (*Hamamelis virginior L.*), was found to be a potent scavenger of superoxide anion radicals. Superoxide anion scavenging activity of the compound was evaluated by ESR-spin trap method using DMPO (5,5'-dimethyl-1-pyrroline-N-oxide) as a spin trapping agent. The IC_{50} value (the concentration producing 50% inhibition of superoxide anion radicals) of hamamelitannin was found to be 1.38 ± 0.06 μM much lower than that of ascorbic acid (23.31 ± 2.23 μM). Supporting the superoxide scavenging activity of hamamelitannin, the compound showed both suppresive ability against depolymelization of hyaluronic acid and protective ability against cytotoxicity induced by superoxide anion radicals. Hamamelitannin increased the survival rate of fibroblast to 85.5 ± 3.3%, compared with that of control (27.2 ± 4.3%).

KEY WORDS: Hamamelis extract, Hamamelitannin, superoxide anion scavenging activity, ESR, fibroblast, cytotoxicity.

INTRODUCTION

It has been reported that skin-aging is accelerated by ultra-violet (UV) irradiation. Such a skin-aging is called as photo-aging[1], which is characterized by deep wrinkles. The UV-irradiation for the skin presumably causes the formation of the active oxygens such as superoxide anion radicals, hydrogen peroxides and hydroxy radicals in the skin.[2] When the UVB (ultra-violet light of wavelength at 290 nm–320 nm) was irradiated to the homogenized skin of hairless mice, the generation of superoxide anions has been detected by the electron spin resonance (ESR) spectrometry.[3] Therefore, it is assumed that the photo-aging, and thus the wrinkle formation is prevented by the scavengers for superoxide anion radicals. We measured the superoxide anion scavenging (SAS) activities of 16 kinds of plant extracts and found that hamamelis (*Hamamelis virginiana L.*) bark extract has the highest SAS activity among the plant extracts tested. The active component in the extracts was identified as hamamelitannin (Figure 1) by HPLC and TLC.[4]

In this communication, we report the biochemical activities of hamamelitannin

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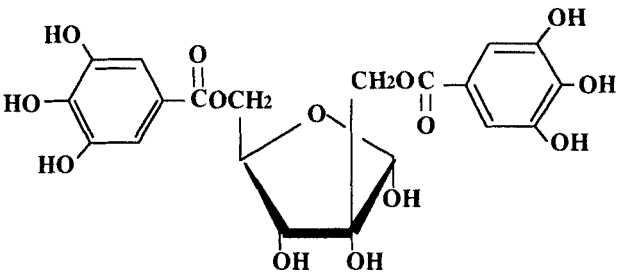

FIGURE 1 Chemical structure of hamamelitannin (2',5-di-O-galloyl-hamamelose).

on the follows points: 1. SAS activity of hamamelitannin estimated by ESR-spin trap method using 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trapping agent. 2. Inhibitory effect of hamamelitannin against hyarulonic acid depolymerization. 3. Protective ability of hamamelitannin against cell damages induced by superoxide anion radicals.

EXPERIMENTAL

Reagent

Hamamelis virginiana L. was used during the experiments. Hamamelis extract was obtained from the hamamelis bark with 95% EtOH. Hamamelitannin (2',5-di-O-galloyl-hamamelose) was purchased from Funakoshi Co. Ltd. L-Ascorbic acid, neutral red, xanthine, hypoxanthine (HPX), xanthine oxidase (XOD) and superoxide dismutase (SOD) were purchased from Nakalai Tesque. Diethylenetriamine-N,N,N',N",N"-pentaacetic acid (DTPA) and ethylendiamine tetraacetic acid (EDTA) were from Dojindo Laboratries. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from LABOTEC Co.

Superoxide Anion Scavenging (SAS) Activity

SAS activities of the compounds were estimated by ESR-spin trap method according to the method of Kitagawa *et al.*[5] A 0.12 unit/ml XOD 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 sample. Almost simultaneously DMPO was added to the solution to a final concentration of 90 mM and mixed on a vortex mixer. After the mixing for 1 min, ESR spectra were recorded with a JEOL JES-FE1XG (X-band) spectrometer with 100 kHz field modulation frequency and 0.1 mT modulation amplitude at output power of 5 mW. Mn(II) in MnO was used as a standard. All experiments were carried out at room temperature (21°C).

Hyaluronic Acid Depolymerization

Hyaluronic acid depolymerization was estimated by measuring the viscosity of hyaluronic acid solution.[6] The measuring solution contained 0.9 mg/ml hyaluronic acid, 0.1 mM xanthine and 0.15 μM $FeCl_3$, 1.5 mM EDTA (to promote the

formation of hydroxy radicals)[7,8] in a total volume of 9 ml in the presence of various concentrations of SOD, L-ascorbic acid or hamamelitannin. The solution was placed into a cannon fenske viscometer (Top LABO-WARE). After an equilibrium period of 5 min, the depolymerization was started by the addition of XOD. The flow time was measured for 30 min at 5 min intervals.

Cell Culture

Human dermal fibroblasts were used and cultured with Dulbecco's modified eagle medium (DMEM) (Nissui®) supplimented with 0.1 mM L-glutamine and 5% fetal bovine serum (FBS), and maintained in a 5% CO_2 atmosphere at 37°C.

Application of Superoxide Anions against the Cultured Fibroblasts

Fibroblasts were placed in 96-well microplate at a cell density of 3×10^4 cells per well. After 1 day cultivation, cells were washed with Hank's buffer solution containing 1.26 mM $CaCl_2$ and 0.81 mM $MgSO_4$ (HBS). The superoxide anion generating system consisting 1.5 mM hypoxanthine and XOD was dissolved with 100 μl of HBS, and was placed on the cells with the test compound in the presence of 500 units/ml catalase. After 2.5 h incubation at 37°C, the cells were washed with HBS and the viabilities of the cells were estimated by the neutral red test as follows.

Estimation of the cell viability[9]

Neutral red test is a rapid colorimetric test to quantify the cell growth and survival. The culture cells were incubated in Dulbecco's modified egle medium supplimented with 5% FBS and 0.005% natural red for 3 h at 37°C. After incubation, the neutral red in cells was extracted with 30% EtOH in 0.1 M HCl, and the absorbance at 545 nm was measured.

RESULTS

Superoxide Anion Scavenging (SAS) Activities of Hamamelis Extract and Hamamelitannin Estimated by ESR-Spin Trapping Method

We measured SAS activities for 16 kinds of plant extracts evaluated by neotetrazolium method[10] and found that hamamelis extract has the highest SAS activity among them (unpublished data). Thus, the SAS activities of hamamelis extract and hamamelitannin, which has been found to be a main component of hamamelis extract[4], were estimated by ESR-spin trapping technique using DMPO as a spin trapping agent. The ESR spectrum of a superoxide generating solution which contains HPX, XOD and DMPO was identified as DMPO-superoxide (DMPO-OOH) adduct by their hyperfine splitting constants ($a_N = 1.43$ mT, $a_{H\beta} = 1.15$ mT and $a_{H\beta} = 0.13$ mT).[5] The various concentrations of hamamelis extract and hamamelitannin were added to the superoxide generating solution to evaluate the superoxide scavenging ability. Figures 2 and 3 show the dose-dependent superoxide scavenging rate. The IC_{50} value of hamamelis extract in this system was estimated to be 0.72 ± 0.06 μg (dry material)/ml. The SAS activity of hamamelitannin was thus compared with that of ascorbic acid. The IC_{50} values of

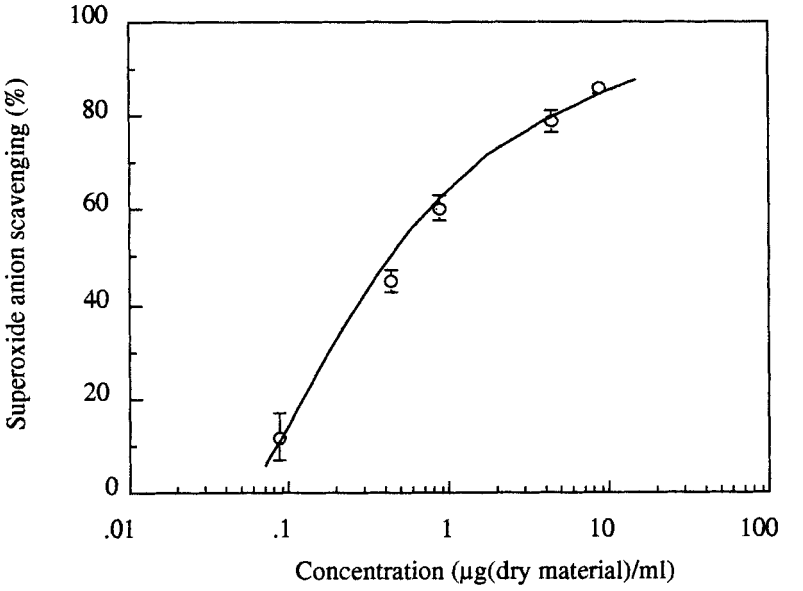

FIGURE 2 Dose dependent superoxide anion scavenging activity of hamamelis extract estimated by ESR spin trap method.

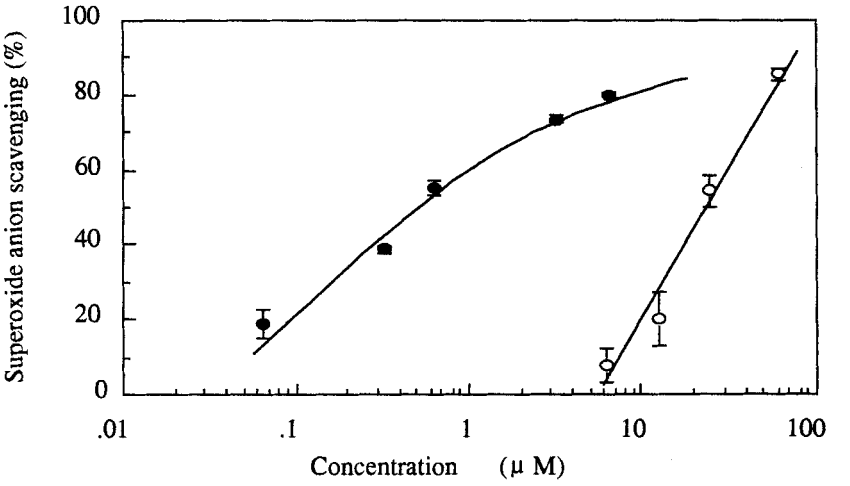

FIGURE 3 Dosedependent superoxide anion scavenging activities of hamamelitannin and ascorbic acid estimated by ESR spin trap method. The HPX-XOD system was used as the superoxide anion radical generating system. ESR spectra were recorded with the following conditions: center field 336.5 ± 5 mT, modulation amplitude 0.1 mT, output power 5 mW. ○: Ascorbic acid ●: Hamamelitannin.

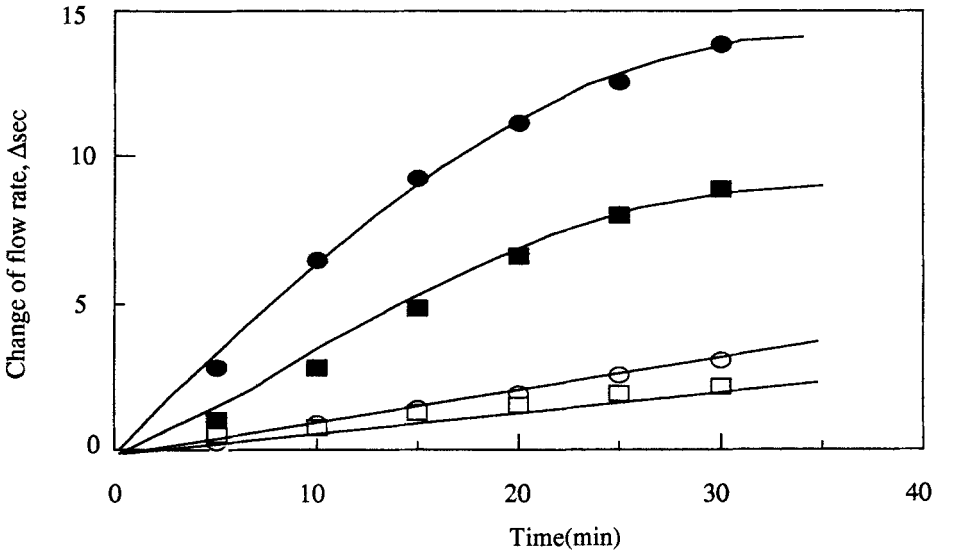

FIGURE 4 Inhibitory effects of hamamelitannin and ascorbic acid on hyaluronic acid depolymerization. Inhibitory effects of compounds on hyaluronic acid depolymerization were evaluated by using the change in the viscosity, which was measured with a cannon fenske viscometer. The viscosities were expressed by the change of the flow rate as follows. △sec = Tt-T0; T0 = Inital flow time(sec); Tt = Flow time at each incubation time(sec); ●: Control; □: 100 units/ml SOD; ○: 1mM Hamamelitannin; ■: 1mM Ascorbic acid.

hamamelitannin and ascorbic acid in this system were estimated to be 1.38 ± 0.06 μM and 23.31 ± 2.23 μM, respectively. Hamamelitannin showed much higher SAS activity, compared with that of ascorbic acid.

Inhibititory Effect of Hamamelitannin and Ascorbic Acid on Hyaluronic Acid Depolymerization

Inhibitory effects of hamamelitannin, ascorbic acid and SOD on hyaluronic acid depolymerization were estimated by the viscocity measurement of hyaluronic acid solution (Figure 4). The inhibitory rates, which are expressed as percentage of the depolymerization rate of hyaluronic acid, of ascorbic acid, hamamelitannin and SOD were found to be 24.67%, 73.85%, and 84.43%, respectively. Thus, it was found that the depolymerization was highly suppressed by SOD. The results indicate that the superoxide anion is an important reactant on the hyaluronic acid depolymerization, being in good agreement with those of ESR-spin trapping method described before.

Protective Effect of Hamamelitannin against the Cell Damages Induced by Superoxide Anion Radicals

Protective effect of hamamelitannin against the cell damages induced by superoxide anion radicals was evaluated. First, in order to clarify an influence of hydrogen peroxide by-produced by superoxide anion radicals generating system, the effect of

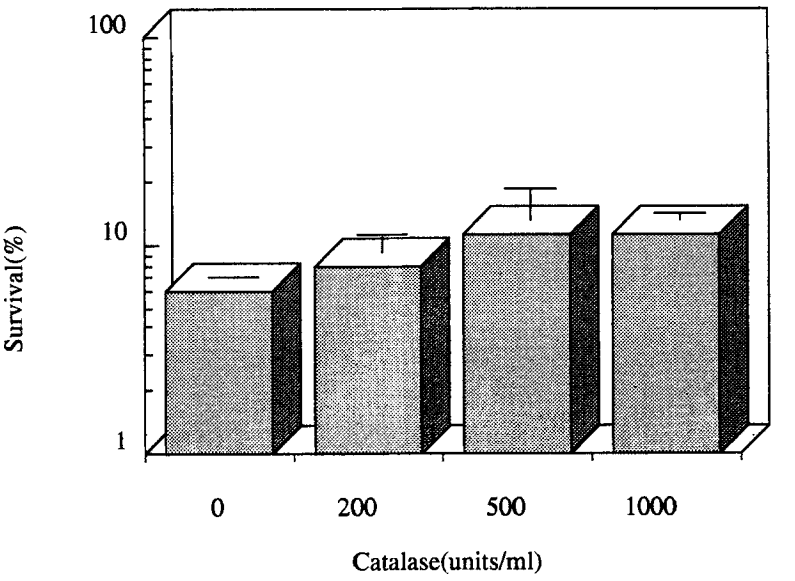

FIGURE 5 Inflience of catalase against the cell damages induced by superoxide anion generating system.

catalase was examined. In the system, hydrogen peroxide slightly injuried fibroblasts, however, the potent cytotoxic oxygens were indicated due to the superoxide anion radicals (Figure 5). The level of superoxide anion radicals were regulated by the unit numbers of XOD added. The survival rate of fibroblast decreased with increase of the additional XOD units, indicating that the decrease of survival rate of fibroblast depends on the levels of superoxide anion radicals. Figure 6 shows the protective effect of this compound on the cell damages in a dose-dependent manner. The survival rate of the fibroblast was found to be 85.5 ± 3.3% in the presence of 100 μM hamamelitannin, while that was 27.2 ± 4.3% without the compound, suggesting that hamamelitannin is a good protective agent against the cell damages induced by superoxide anion radicals.

DISCUSSION

It has been reported that chronic UV irradiation accelerates the wrinkle formation.[11] The alterations of dermal connective tissue may be responsible for the generation of wrinkles. On the other hand, the increases of the level of collagen and elastin, which are the major components of the dermis, have been observed by UV irradiation.[2] Histologic studies on actinically damaged skin have shown that the connective tissue components of the dermis were alternated.[13,14] Involvement of active oxygen species in the skin may accelerate the effects of UV irradiation and result in harmful effects on the surrounding tissues.[2] Thus, the interactions between the active oxygens and collagen have been investigated.[15] When the soluble collagen was exposed with superoxide anion radicals and hydroxy radicals, the rapid degradations of the collagen were observed. Furthermore, hyaluronic acid, which

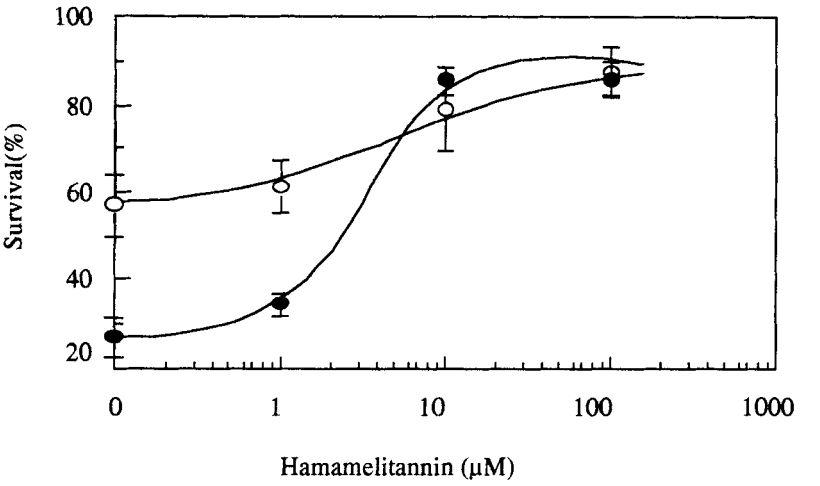

FIGURE 6 Protective effect of hamamelitannin against the cell damages induced by superoxide anion radicals. The protective activity of hamamelitannin was evaluated using a human fibroblasts culture system, where the hypoxanthine-XOD system was used as the superoxide anion generating system. The levels of superoxide anion radicals were regulated by the amount of XOD added. ○: XOD munits/ml; ●: XOD 100 munits/ml.

is the major component of glycosaminoglycans in the dermis was reported to be depolymerized by hydroxy radicals. From these observations, the use of radical scavenger against active oxygen species is thought to be very effective to suppress the harmful alteration of dermis. Therefore, we tested the effects of the anti-aging agent, especially the anti-wrinkle agent, of plant extracts in terms of superoxide anion scavenging activity, and found that hamamelis and thus hamamelitannin which is one of the major components of the hamamelis extract, had the highest activity among them tested. The IC_{50} value for the superoxide anion scavenging activity of hamamelitannin was found to be $1.38 \pm 0.06\,\mu M$ (Figure 2), which is much higher than the IC_{50} that for ascorbic acid. In order to evaluate the availability of hamamelitannin as the anti-wrinkle agent, hyaluronic acid and the dermis fibroblast which play the important roles in wrinkle formations, were used as the evaluation systems. The mechanism of wrinkle formation has yet been unknown. However, it has been thought that the wrinkle formation results from the denaturation of dermis matrix. Hyaluronic acid is synthesized in fibroblast, and excreted into the dermis matrix. In the dermis, hyaluronic acid acts as a water reservoir and supplies water for the epidermis. Thus, the denaturation of hyaluronic acid will be responsible for the dry skin and wrinkle formation. Therefore, the inhibitory effect of hamamelitannin on hyaluronic acid depolymerization was evaluated. The depolymerization of hyaluronic acid was caused by hydroxy radicals which were generated by superoxide anion radicals and Fe^{3+}-EDTA complex. The inhibitory rate of hamamelitannin on the depolymerization was found to be 73.85% (Figure 4), confirming that hamamelitannin suppresses the denaturation of the dermis component induced by hydroxy radicals. Hyaluronic acid should be the target molecule of the active oxygen species in the skin. Then, we tested the efficacy of hamamelitannin using a cell culture system. In the examination, hypoxantine-XOD system

was used as superoxide anion radicals source. It has been known that the system generates hydrogen peroxide as by-product. It was found that hydrogen peroxide did not influence the survival rate and superoxide anion radicals were the potent reactants in the system (Figure 5). Hamamelitannin enhanced the survival of the cells which were exposed to superoxide anion radicals generated by hypoxanthine-XOD system. It was found that hamamelitannin protected fibroblasts from the damage induced by superoxide anion radicals (Figure 6).

Based on these results, hamamelitannin, which has a high superoxide anion scavenging activity, was concluded to act as a potent anti-aging and anti-wrinkle agent.

Acknowledgement

The authors wish to thank Mr. Sakon, the managing director of Noevir Co., LTD Shiga Central Research Laboratory, for his encouragement and discussions during this work.

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Accepted by Professor T. Yoshikawa