Inhibitory Effects of *Citrus hassaku* Extract and Its Flavanone Glycosides on Melanogenesis

Kimihisa Itoh,[a] Noriko Hirata,[a] Megumi Masuda,[a] Shunsuke Naruto,[a] Kazuya Murata,[a] Keitaro Wakabayashi,[b] and Hideaki Matsuda*,[a]

[a] *School of Pharmacy, Kinki University; 3–4–1 Kowakae, Higashiosaka, Osaka 577–8502, Japan:* [b] *Department of Product Development, Wellco Co., Ltd.; 370 Fukudome-machi, Hakusan, Ishikawa 924–0051, Japan.*
Received September 4, 2008; accepted October 30, 2008; published online December 26, 2008

The 50% ethanolic extract (CH-ext) obtained from the unripe fruit of *Citrus hassaku* exhibited significant tyrosinase inhibitory activity. The CH-ext showed antioxidant activity, such as superoxide dismutase (SOD)-like activity and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity. Activity-guided fractionation of the CH-ext indicated that flavanone glycoside-rich fractions showed potent tyrosinase inhibitory activity. Further examination revealed that the tyrosinase inhibitory activity and antioxidant activity of the CH-ext were attributable to naringin and neohesperidin, respectively. The CH-ext showed inhibition of melanogenesis without any effects on cell proliferation in cultured murine B16 melanoma cells after glucosamine exposure. The topical application of the CH-ext to the dorsal skin of brownish guinea pigs showed *in vivo* preventive effects against UVB-induced pigmentation.

Key words *Citrus hassaku*; flavanone glycoside; tyrosinase; melanogenesis

Melanogenesis stimulated by UV irradiation occurs in plants, microorganisms, and mammalian cells by an enzymatic oxidation process starting with L-tyrosine. Various ingredients for skin-whitening cosmetics are developing to reduce melanogenesis. Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA), followed by the oxidation of L-DOPA to dopaquinone, and oxidative polymerization of several dopaquinone derivatives produces melanin. Thus the tyrosinase inhibitor is one of the candidates for reduction of melanogenesis.[1)] On the other hand, it has been reported that superoxide dismutase (SOD) is one of the key factors that reduce melanin production caused by UV irradiation.[2)] Therefore tyrosinase inhibitors with SOD-like activity and/or antioxidant activity may be useful ingredients in the field of skin-whitening cosmetics. During our screening program to find a potential tyrosinase inhibitor from natural resources, we reported several crude drugs, such as *Glebnia littoralis* F. Schmidt, *Prunus zippeliana* M., *Myrica rubra* S. *et* Zucc., and *Arctostaphylos uva-ursi* L. Sprengel, some of which have been applied to cosmetic beauty preparations.[1,3—5)]

Recently, the tyrosinase inhibitory activities of the peel of *Citrus* fruit (*Citrus unshiu* Markovich) and its flavonoids, such as nobiletin, have been reported.[6,7)] As a part of our continuous studies on the biological activities of *Citrus* species,[8—12)] we found that a 50% ethanolic extract (CH-ext) obtained from the unripe fruit of *Citrus hassaku* Hort ex T. Tanaka, which was collected by thinning out in July, exhibited potent mushroom tyrosinase inhibitory activity. Thinning out the unripe fruit of *C. hassaku* in July is important for a rich harvest of ripe fruit in December. Thus this study was undertaken to examine whether the unripe fruit of *C. hassaku* collected in July by thinning can be utilized as a plant resource for skin-whitening cosmetic agents, because, to the best of our knowledge, there is no report on the tyrosinase inhibitory activity of *C. hassaku*. First, to identify the active component, we carried out activity-guided fractionation of the CH-ext using tyrosinase inhibitory assay. For antioxidant activity, SOD-like and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of the CH-ext and its flavanone glycosides were also studied. Second, according to the method of Imokawa,[13)] we examined the effects of the CH-ext on melanogenesis using cultured murine B16 melanoma cells after exposure to glucosamine. Third, we examined the *in vivo* preventive effects of the CH-ext against UVB-induced pigmentation of dorsal skin in brownish guinea pigs.[14)]

MATERIALS AND METHODS

Reagents Hesperidin, naringin, and neohesperidin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Narirutin was isolated from fruit of *C. unshiu*.[10)] Other chemical and biochemical reagents were of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and/or Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise noted.

Preparation and Fractionation of CH-ext Unripe fruit of *C. hassaku* were collected in Wakayama prefecture, Japan in July, 2004, air-dried at 50 °C for 48 h in an automatic air-drying apparatus (Vianove Inc., Tokyo, Japan), and powdered. The powder (10 g) was extracted with 50% ethanol (EtOH) (100 ml) for 2 h under reflux. The extract was evaporated under reduced pressure and then lyophilized to give the 50% EtOH extract (CH-ext) in 25.7% yield.

Animals Female Wiser-Maple brownish guinea pigs (4 weeks of age) were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan). They were maintained in an air-conditioned room with lighting from 07:00 to 19:00. The room temperature (about 23 °C) and humidity (about 60%) were controlled automatically. Laboratory pellet chow (RC4, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were freely available. All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the

* To whom correspondence should be addressed. e-mail: matsuda@phar.kindai.ac.jp

US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Tyrosinase Inhibitory Activity Tyrosinase activity was measured according to the method of Mason and Peterson,[15] as described in previous papers.[1,3—5] The test sample was dissolved with dimethyl sulfoxide (DMSO) and diluted with 1/15 M phosphate-buffered saline (PBS, pH 6.8) to a final DMSO concentration of 5% v/v. After incubation of 0.5 ml of the test solution at 25 °C for 10 min, 0.5 ml of mushroom tyrosinase (135 U/ml, Sigma-Aldrich Japan, Tokyo, Japan) and 0.5 ml of 0.03% DOPA solution were added. The mixture was incubated at 25 °C for 5 min. The amount of dopachrome in the mixture was determined based on the optical density (OD) at 475 nm using a Hitachi 200-10 spectrophotometer. Arbutin (Tokyo Chemical Industry, Tokyo, Japan) and kojic acid were used as standard agents. The inhibitory percentage of tyrosinase was calculated as follows:

$$\% \text{ inhibition} = [(A-B)-(C-D)]/(A-B)\times 100$$

where A is the OD at 475 nm with enzyme, but without test substance; B the OD at 475 nm without test substance and enzyme; C the OD at 475 nm with test substance and enzyme; and D the OD at 475 nm with test substance, but without enzyme.

Fractionation of CH-ext A suspension of the CH-ext (10 g) in water (100 ml) was extracted with hexane (200 ml×3) followed by ethyl acetate (200 ml×3). Evaporation of the solvent gave a hexane-soluble fraction (0.38 g), an ethyl acetate-soluble fraction (1.8 g), a water-soluble fraction (6.6 g), and an ethyl acetate-water-insoluble intermediate fraction (0.7 g) which was obtained as an intermediate layer during the process of ethyl acetate extraction. The tyrosinase inhibition percentage in each fraction was evaluated. The results were: hexane-soluble fraction, inhibition percent 0% at a concentration of 1 mg/ml; ethyl acetate-soluble fraction, 24% at 0.5 mg/ml and 43% at 1 mg/ml; water-soluble fraction, 2% at 0.5 mg/ml and 7% at 1 mg/ml; and ethyl acetate-water-insoluble intermediate fraction, 30% at 0.5 mg/ml and 45% at 1 mg/ml.

Determination of Flavanone Glycosides in CH-ext and Its Fractions Using HPLC The flavanone glycoside content in each sample was determined using the HPLC method.[11] The CH-ext (300 mg) was accurately weighed and extracted with MeOH (50 ml×3) for 30 min under reflux. After filtration, the combined methanolic extracts were collected in a volumetric flask (200 ml). An appropriate volume of MeOH was added in the volumetric flask to 200 ml to give a sample solution. After filtration with a membrane filter (0.45 μm, GL Sciences Inc. Tokyo, Japan), 5 μl of the sample solution was injected into the HPLC system. On the other hand, each fraction (4 mg) obtained from the CH-ext was accurately weighed and dissolved with MeOH in a volumetric flask (20 ml) under ultrasonic radiation. After filtration with a membrane filter (0.45 μm), 5 to 20 μl of each sample solution was injected into the HPLC system. The HPLC system consisted of a Shimadzu SCL-10A*vp* (Shimadzu, Kyoto, Japan) with a Shimadzu UV–Vis detector SPD-10A*vp* and Shimadzu Chromatopack C-R8A. The TSK gel ODS-120T (5 μm, 250×4.6 mm i.d.) column (Tosoh Co., Tokyo, Japan) was used at 37 °C. The mobile phase was a gradient system of a solution A [0.1% H_3PO_4 in distilled water : CH_3CN (9 : 1, v/v)] and solution B [0.1% H_3PO_4 in distilled water : CH_3CN (1 : 4, v/v)] in the following ratio 0 min, solution A: solution B 10 : 0; for 25 min, 7 : 3; for 35 min, and 0 : 10 v/v. The flow rate was 0.8 ml/min; detection was at UV 280 nm; and the t_R for narirutin was 27.6 min, for naringin 28.7 min, for hesperidin 29.4 min, and for neohesperidin 30.5 min. The peak area ratios *versus* concentrations of naringin (r=0.9987) or neohesperidin (r=0.9985) yielded straight-line relationships in the range of 3—200 μg/ml with the above correlation coefficients. In the range of 6—200 μg/ml, hesperidin and narirutin showed similar straight-line relationships with correlation coefficients of 0.9998 and 0.9999, respectively.

SOD-Like Activity SOD-like activity was measured according to the method of Oyanagui[16] with minor modification. The test sample was dissolved with DMSO and diluted with 0.5 mM disodium dihydrogen ethylenediamine tetraacetate (EDTA)-PBS buffer (pH 8.2) to a final DMSO concentration of 1% v/v. SOD, as a reference, originating from cow's milk (Roche Co., Tokyo, Japan) was dissolved with 0.5 mM EDTA-PBS buffer (pH 8.2). A mixture of 0.5 mM EDTA-PBS buffer (pH 8.2) (0.2 ml), 0.5 mM hypoxanthine in EDTA-PBS buffer (pH 8.2) (0.2 ml), reagent A solution (10 mM hydroxylamine hydrochloride and 1 mg/ml hydroxylamine-*o*-sulfonic acid in water) (0.1 ml), water (0.2 ml) and the sample solution (0.1 ml) was preincubated at 37 °C for 10 min. Five mU/ml xanthine oxidase (Roche Co., Tokyo, Japan) solution in 0.5 mM EDTA-PBS buffer (pH 8.2) (0.2 ml) was added to the above solution, and the mixture was incubated at 37 °C for 30 min. Reagent B (30 μM *N*-1-naphthylethylenediamine·2HCl, 3 mM sulfanilic acid, and 25% acetic acid in water) (2 ml) was added to the reaction mixture. The resulting mixture was allowed to stand for 30 min at room temperature, and then OD was measured at 550 nm with a Hitachi 200-10 spectrophotometer. The SOD-like activity of each sample was expressed as percentage of the decrease in OD compared with that of control A or B solution. The IC_{50} value represents the concentration of sample required to scavenge 50% of the superoxide anions produced by the hypoxanthine-xanthine oxidase system.

Radical-Scavenging Activity Radical-scavenging activity was measured according to the method of Blois[17] with minor modification. The test sample was dissolved with DMSO and diluted with 0.5 M acetate buffer (pH 5.5) to a final DMSO concentration of 5% v/v. A mixture of test sample solution (2 ml), EtOH (1.6 ml), 0.5 M acetate buffer (pH 5.5) (0.4 ml), and 0.5 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH)/EtOH solution (1.0 ml) was allowed to stand for 30 min at room temperature. The OD of the resulting mixture at 520 nm was determined with a Hitachi 200-10 spectrophotometer. L-Ascorbic acid was used as a reference agent. The scavenging activity of each sample was expressed as percentage of the decrease in OD against compared with that of control DPPH solution. The IC_{50} value represents the concentration of sample required to scavenge 50% of DPPH free radicals.

Cell Culture A cultured murine B16 melanoma cell line (B16F1) was purchased from Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan) in May 2005. Following to the method of Imokawa[13] with minor modification, the murine B16 melanoma cells were precultured in Dulbecco's modified Eagle's medium (D-MEM, Invitrogen Corp., Carls-

bad, CA, U.S.A.) supplemented with 10% fetal bovine serum (ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.), 0.1% glucosamine and 1% antibiotic-antimycotic solution (a mixture of 10000 U/ml penicillin, 10000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B, Invitrogen Corp.) at 37 °C in a humidified incubator in 5% CO_2–95% air (CO_2 incubator).

Measurement of Melanin in Cultured B16 Melanoma Cells The amount of melanin in cultured murine B16 melanoma cells (intracellular melanin) was measured according to the method of Hill *et al.*,[18] as described in our previous paper.[19] Briefly, test samples were dissolved in DMSO/Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS (CMF-D-PBS, Invitrogen Corp.) (1 : 1, v/v) and then diluted with D-MEM to an appropriate concentration. The final concentration of DMSO was 0.1%. In the control group, DMSO/CMF-D-PBS (1 : 1, v/v) solution diluted with D-MEM to 0.1% of the final DMSO concentration was used instead of the sample solution.

Assay of Cell Proliferation The cell proliferation of murine B16 melanoma cells was assessed using the MTT method described in our previous paper.[19] Cell proliferation in the treated group was compared with that in the control group.

Brownish Guinea Pig Skin Pigmentation Induced by UVB Irradiation According to the method described by Imokawa *et al.*,[14] with minor modification, skin pigmentation was induced by UVB irradiation of the dorsal skin of brownish guinea pigs. After 1 week for adaptation, the dorsal hair of 5 female brownish guinea pigs (4 weeks of age) was shaved with an electric hair clipper and then treated with hair remover cream (Kanebo, Tokyo, Japan) to remove the hair completely. Five or six separate rectangular (1.5 cm×1.5 cm) areas of the flank of each guinea pig anesthetized with pentobarbital (35 mg/kg, i.p., Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) and exposed to UVB light (305 nm; FL20S·E-30/DMR, Toshiba, Tokyo, Japan) for 5 min/d for 3 successive days (total energy, 450 mJ/cm^2/d) from the next day (Day 1) after shaving. Vehicle areas did not receive UVB irradiation. Test samples were dissolved with a mixture of EtOH and propyleneglycol (1 : 9, v/v). From the next day (Day 4) after the last UVB irradiation, each sample solution was applied topically with a pipette to each separate shaved area once a day for 21 successive days. Vehicle areas received a topical mixture of EtOH and propyleneglycol (1 : 9, v/v). Control areas received UVB irradiation and a topical mixture of EtOH and propyleneglycol (1 : 9, v/v).

On the following day, immediately before the first UVB irradiation (Day 1), and on Day 4, Day 7, Day10, Day 13, Day 17, Day 21, and Day 24, skin pigmentation levels of the shaved dorsal skin were visually assessed according to the following scale (0—8): 0, no pigmentation; 2, little pigmentation; 4, slight pigmentation; 6, moderate pigmentation; and 8, intense deep pigmentation. Photographs of dorsal skin were taken on the same days.

Six hours after the last application of the sample on Day 24, a skin specimen (1 cm×1 cm) was removed from the treated flank of the guinea pigs. The skin specimens were rinsed twice with 0.1 M phosphate buffer (pH 6.8), and incubated in 1 M sodium bromide dissolved with 0.1 M phosphate buffer (pH 6.8) at 37 °C for 5 h. The epidermal sheets separated from the specimens were fixed in 10% cold neutral formalin for 30 min, washed twice with 0.1 M phosphate buffer (pH 6.8), and incubated in 0.1% DOPA dissolved with 0.1 M phosphate buffer (pH 6.8) at 37 °C for 5 h. The number of DOPA-stained melanocytes (per square millimeter) was counted using an Olympus-BHA microscope with a micrometer (Olympus, HWK10X) at a magnification of ×200. In each specimen, the number of melanocytes was calculated by averaging the numbers found in 100 fields.

Statistical Analysis The experimental data were evaluated for statistical significance using Bonferroni/Dunn's multiple-range test.

RESULTS AND DISCUSSION

Unripe *Citrus* fruit has been used in traditional Chinese medicine and contain several types of biologically active compounds such as limonoids, alkaloids, and flavonoids.[7] In the previous study on seasonal variation in several *Citrus* fruit extracts in the relationship between anti-allergic activity and the content of flavanone glycosides, we reported that unripe *C. unshiu* fruit extract showed more potent activity than that of ripe fruit, and that the flavanone glycoside content in unripe fruit extract was richer than that in the ripe extract.[10] During the course of our preliminary comparative screening for mushroom tyrosinase inhibitory activity of 50% ethanolic extracts obtained from *C. hassaku* fruit collected monthly in July, August, September, October, and November, we found that the extract obtained from unripe fruit collected by thinning out in July showed the most potent activity (data not shown) as in the case of the anti-allergic activity of *C. unshiu* fruit extract.[10] Moreover, thinning out the unripe fruit of *C. hassaku* in July is important for a rich harvest of superior ripe fruit in December. Thus we focused on the tyrosinase inhibitory activity of the CH-ext of unripe fruit of *C. hassaku* collected in July when thinning. The CH-ext showed concentration-dependent inhibition of tyrosinase, and the IC_{50} value of the extract was 4.7 mg/ml (Table 1). Arbutin showed weak inhibition (IC_{50} >10 mM). Kojic acid inhibited the enzyme at IC_{50} value of 0.02 mM. HPLC analysis[11] revealed that the flavanone glycoside content of the CH-ext was: naringin, 206.8; neohesperidin 94.7; narirutin, 28.5; and hesperidin, 8.9 mg/g of the extract. To identify the active component, the CH-ext was fractionated by solvent extraction to give a hexane-solu-

Table 1. Tyrosinase Inhibitory Activities of CH-ext, Arbutin, and Kojic Acid

Samples	Concentration	OD (×1000)[a] at 475 nm	% Inhibition	IC_{50} value
Control		749±7	0	
CH-ext	2 mg/ml	566±3**	24	
	5 mg/ml	358±4**	52	4.7 mg/ml
	10 mg/ml	219±8**	71	
Arbutin	1 mM	844±7**	−13	
	5 mM	809±8**	−8	>10 mM
	10 mM	791±8**	−6	
Kojic acid	0.01 mM	488±9**	35	
	0.05 mM	229±12**	69	0.02 mM
	0.1 mM	139±2**	81	

a) OD: optical density. Each value represents mean±S.E. of 3 experiments. Significantly different from control group, ** $p<0.01$.

ble fraction, an ethyl acetate-soluble fraction, a water-soluble fraction and a water-ethyl acetate-insoluble fraction. Among them, the ethyl acetate-soluble fraction and water-ethyl acetate-insoluble intermediate fraction showed significant tyrosinase inhibitory activities. On HPLC analysis, the flavanone glycoside content of the ethyl acetate-soluble fraction was: naringin, 345.9; neohesperidin, 189.4; narirutin, 56.3; and hesperidin, 41.6 mg/g. That of the water-ethyl acetate-insoluble intermediate fraction was: naringin, 674.2; neohesperidin, 81.1 mg/g; narirutin, not detected; and hesperidin, not detected. Thus it was found that these two fractions were flavanone glycoside-rich fractions in which naringin and neohesperidin were major flavanones, and narirutin as well as hesperidin were minor flavanones. These results are in accordance with the reports[6,7] that some flavanone glycosides exhibit tyrosinase inhibitory activities. The tyrosinase inhibitory activities of naringin, neohesperidin, narirutin, and hesperidin are shown in Table 2. Naringin showed the most potent activity.

Since SOD is one of key factors that reduce the production of melanin caused by UV irradiation,[2] tyrosinase inhibitors with SOD-like activity and/or antioxidant activity may be useful ingredients in the field of skin-whitening cosmetics. Thus SOD-like activities and DPPH radical-scavenging activities of the CH-ext and its four flavanone glycosides were examined.

SOD-like activity was evaluated using SOD (IC_{50} 0.2 U/ml) as a positive agent. In this experiment, the IC_{50} value represents the concentration of sample required to scavenge 50% of the superoxide anions produced by the hypoxanthine–xanthine oxidase system. As shown in Table 3, the CH-ext exhibited significant SOD-like activity (IC_{50} 0.5 mg/ml). The IC_{50} values of neohesperidin and hesperidin were 26 μM and 268 μM, respectively, as shown in Table 4, whereas naringin and narirutin showed weak activities. The antioxidant activity was evaluated using a DPPH radical-scavenging method that has been widely used to measure the radical-scavenging ability of plant extracts and their constituents, and the IC_{50} value represents the concentration of sample required to scavenge 50% of DPPH free radicals.[20,21] The CH-ext exhibited potent radical-scavenging activity (IC_{50} 0.2 mg/ml) as shown in Table 4. As shown in Table 2, the radical-scavenging activity of neohesperidin (IC_{50} 0.6 mM) was more potent than that of hesperidin (IC_{50} 3.2 mM), whereas naringin (IC_{50} >4 mM) and narirutin (IC_{50} >4 mM) were inactive. L-Ascorbic acid, a reference agent, showed potent activity (IC_{50} 0.03 mM).

According to the method of Imokawa,[13] the inhibitory effects of the CH-ext on melanogenesis were evaluated using cultured murine B16 melanoma cells after exposure to glucosamine. Two tyrosinase inhibitors, arbutin and kojic acid, and an antioxidant, L-ascorbic acid, were used as reference compounds. The inhibitory effects of the CH-ext, arbutin, kojic acid, and L-ascorbic acid on melanogenesis were expressed as the amount of intracellular melanin in B16 cells and cell proliferation. As shown in Table 5, the CH-ext showed significant inhibitory activity in a concentration-dependent manner without any significant effects on cell proliferation at a concentration of 100—500 μg/ml. Arbutin with weak tyrosinase inhibitory activity showed significant inhibitory effects in a concentration-dependent manner without any significant effects on cell proliferation at a concentration of 50—500 μM. Kojic acid slightly decreased the amount of melanin, whereas L-ascorbic acid slightly increased the amount of melanin. These results suggest that down-regulation of melanogenesis can not be explained by tyrosinase inhibitory activity or antioxidant activity alone.

Using UVB-induced skin pigmentation in brownish guinea pigs,[14] we examined *in vivo* inhibitory effects of the CH-ext on melanogenesis. The CH-ext was topically applied to the flank skin for 21 d after UVB irradiation. The efficacy of samples was evaluated by comparison of the visible degree of pigmentation of the treated skin, as shown in Fig. 1. Topical application of 1% and 5% CH-ext significantly decreased

Table 2. IC_{50} Values of Tyrosinase Inhibitory, SOD-Like, and Radical-Scavenging Activities of Naringin, Neohesperidin, Narirutin, Hesperidin, Arbutin, Kojic Acid, SOD, and L-Ascorbic Acid

Samples	Tyrosinase inhibitory activity (mM)	SOD-like activity (μM or U/ml)	Radical scavenging activity (mM)
Naringin	1.9	>2000 μM	>4
Neohesperidin	>5	26 μM	0.6
Narirutin	2	>2000 μM	>4
Hesperidin	>5	268 μM	3.2
Arbutin	>10	N.D.	N.D.
Kojic acid	0.02	N.D.	N.D.
SOD	N.D.	0.2 U/ml	N.D.
L-Ascorbic acid	N.D.	N.D.	0.03

N.D.: not determined.

Table 3. SOD-Like Activities of CH-ext and SOD

Samples	Concentration	OD (×1000)[a] at 550 nm	% Inhibition	IC_{50} value[b]
Control A[c]		123±1	0	
CH-ext	0.2 mg/ml	88±0**	29	
	0.5 mg/ml	63±1**	49	0.5 mg/ml
	1.0 mg/ml	40±2**	67	
Control B[c]		132±1	0	
SOD	0.02 U/ml	118±5##	11	
	0.1 U/ml	88±5##	33	0.2 U/ml
	0.5 U/ml	40±2##	69	

a) OD: optical density. Each value represents mean±S.E. of 3 experiments. Significantly different from control A (DMSO/buffer) group, $^{**}p<0.01$. Significantly different from control B (buffer) group, $^{\#\#}p<0.01$. *b*) IC_{50} value represents the concentration required to scavenge 50% of superoxide anions. *c*) Control A is a DMSO/buffer solution. Control B is a buffer solution.

Table 4. Radical-Scavenging Activities of CH-ext and L-Ascorbic Acid

Samples	Concentration	OD (×1000)[a] at 520 nm	% Inhibition	IC_{50} value[b]
Control		891±3	0	
CH-ext	0.1 mg/ml	668±4**	25	
	0.2 mg/ml	526±4**	41	0.2 mg/ml
	0.5 mg/ml	155±13**	83	
L-Ascorbic acid	0.01 mM	714±4**	20	
	0.02 mM	488±3**	45	0.03 mM
	0.05 mM	77±3**	91	

a) OD: optical density. Each value represents mean±S.E. of 3 experiments. Significantly different from control group, $^{**}p<0.01$. *b*) IC_{50} value represents the concentration required to scavenge 50% of DPPH free radicals.

pigmentation from Day 21 to Day 24. Representative photographs of skin pigmentation taken on the last day (Day 24) are depicted in Fig. 2. As shown in Fig. 2, UVB exposure control areas (Fig. 2-c and 2-d) showed pigmentation, although the vehicle areas (Fig. 2-a and 2-b) of the same animal did not show any pigmentation. After UVB irradiation, none of the test animals showed any abnormalities throughout the experiments, and the UVB-exposed sites were without skin inflammation, such as erythema and desquamation. As shown in Fig. 2, there was a visible reduction in pigmentation in the areas treated with 5% CH-ext for 3 weeks (Fig. 2-h and 2-i) in comparison with control areas (Fig. 2-e). The efficacy of samples was evaluated based on the number of DOPA-positive melanocytes in the removed skin sheet. The effects of the CH-ext and kojic acid are shown in Table 6. The number of DOPA-positive melanocytes was much higher in the irradiated skin (control area) than in the non irradiated skin (vehicle area). In both 1% and 5% CH-ext treated areas, the increase in DOPA-positive melanocytes after UVB irradiation was lower than that in the control area. These results indicate that the CH-ext might have prevented the increase in UVB-induced pigmentation.

In conclusion, the CH-ext exhibited tyrosinase inhibitory, SOD-like, and antioxidant activities. It was revealed that part of the tyrosinase inhibitory activity of the CH-ext was attributable to naringin, and the antioxidant activity of the extract was due to neohesperidin. To the best of our knowledge, this is the first report of the melanogenesis inhibitory effect of *C. hassaku*. The CH-ext showed *in vitro* inhibitory effects on melanogenesis in B16 cells and *in vivo* prevention against UVB-induced pigmentation of dorsal skin in brownish guinea pigs. These results suggest that the CH-ext may be an useful ingredient for skin-whitening cosmetics.

Table 5. Effects of CH-ext, Arbutin, Kojic Acid, and L-Ascorbic Acid on Melanin Content in Cultured B16 Murine Melanoma Cells

Samples	Concentration	Melanin content (μg/well)	Cell proliferation (%)
Control		13.3±0.6	100.0±5.9
CH-ext	100 μg/ml	11.2±0.6**	113.3±5.7
	250 μg/ml	9.9±0.5**	122.2±6.4
	500 μg/ml	7.0±0.2**	101.8±13.1
Arbutin	50 μM	11.5±0.1*	84.6±5.9
	100 μM	11.6±0.1*	91.9±7.6
	250 μM	8.5±0.2**	103.3±12.0
	500 μM	6.5±0.3**	100.2±8.8
Kojic acid	50 μM	13.6±1.0	100.9±6.6
	100 μM	13.2±0.2	101.7±11.1
	250 μM	12.5±0.6	116.4±13.2
	500 μM	10.6±0.2**	129.7±10.1
L-Ascorbic acid	50 μM	13.0±0.4	120.7±10.1
	100 μM	15.4±0.8**	136.4±15.2*
	250 μM	13.9±0.8	134.1±13.2
	500 μM	14.9±0.4*	118.9±15.1

B16 murine melanoma cells (passage number 6) were cultured in D-MEM (final DMSO conc. 0.5%) for 4 d, and the amount of intracellular melanin was assayed. Each value in melanin content represents mean±S.E. of 3 experiments. Significantly different from control group, $*p<0.05$, $**p<0.01$. Each value in cell proliferation represents mean±S.E. of 3 experiments. Significantly different from control group, $*p<0.05$.

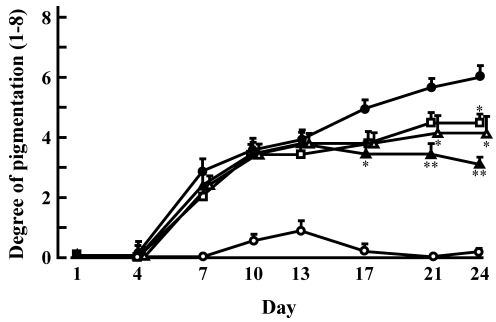

Fig. 1. Effects of CH-ext and Kojic Acid on UVB-Induced Skin Pigmentation in Brownish Guinea Pigs

Each value represents mean±S.E. ($n=5$). Significantly different from control area, $*p<0.05$, $**p<0.01$. ○; vehicle, ●; control, □; 1% kojic acid, △; 1% CH-ext, ▲; 5% CH-ext.

Table 6. Effects of CH-ext and Kojic Acid on Number of Melanocytes after UVB Irradiation

Area	UV irradiation (mJ/cm^2/d)	Treatment	Number of melanocytes[a] (Number/mm^2, mean±S.E.)
Vehicle	0	Solvent[b]	56±6
Control	450	Solvent	1133±26##
1% CH-ext	450	1% CH-ext in solvent	737±26**
5% CH-ext	450	5% CH-ext in solvent	405±19**
1% Kojic acid	450	1% Kojic acid in solvent	773±25**

a) Each value represents mean±S.E. ($n=5$). Significantly different from vehicle area, $\#\#p<0.01$. Significantly different from control area, $**p<0.01$. *b*) Solvent: a mixture of EtOH and propyleneglycol (1 : 9, v/v).

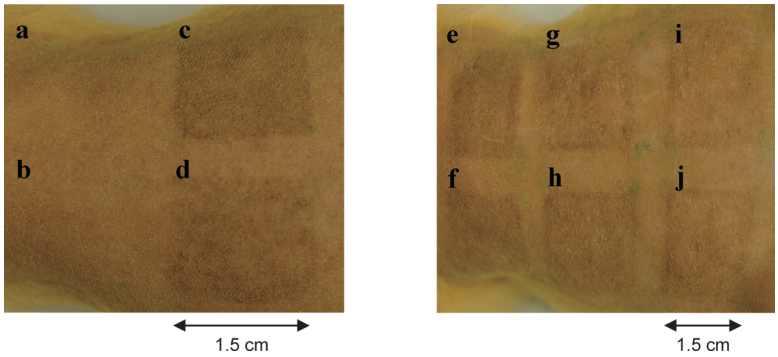

Fig. 2. Effects of CH-ext and Kojic Acid on UVB-Induced Skin Pigmentation in Brownish Guinea Pigs

a, b) Vehicle area: no UVB irradiation, c, d) control area: UVB irradiation 450 mJ/cm^2. The photographs (a, b, c, and d) of a guinea pig (no. 1) were taken on the last day (Day 24, see text). e) Control area, f, j) 1% CH-ext treated area, g) 1% Kojic acid treated area, h, i) 5% CH-ext-treated area. The photographs (e, f, g, h, i, and j) of a guinea pig (no. 4) were taken on the last day (Day 24, see text).

Acknowledgments We appreciate all staff of Yuasa Experimental Farm of Kinki University for the collection of unripe fruit of *C. hassaku*. This work was financially supported by the "High-Tech Research Center" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology of Japan), 2007—2011.

REFERENCES

1) Matsuda H., Higashino M., Nakai Y., Iinuma M., Kubo M., Frank A. L., *Biol. Pharm. Bull.*, **19**, 153—156 (1996).
2) Tobin D., Thody A. J., *Exp. Dermatol.*, **3**, 99—105 (1994).
3) Masamoto Y., Iida S., Kubo M., *Planta Med.*, **40**, 361—365 (1980).
4) Matsuda H., Nakamura S., Kubo M., *Biol. Pharm. Bull.*, **17**, 1417—1420 (1994).
5) Matsuda H., Higashino M., Chen W., Tosa H., Iinuma M., Kubo M., *Biol. Pharm. Bull.*, **18**, 1148—1150 (1995).
6) Sasaki K., Yoshizaki F., *Biol. Pharm. Bull.*, **25**, 806—808 (2002).
7) Zhang C., Lu Y., Tao L., Tao X., Su X., Wei D., *J. Enzyme Inhib. Med. Chem.*, **22**, 83—90 (2007).
8) Kubo M., Yano M., Matsuda H., *Yakugaku Zasshi*, **109**, 835—842 (1989).
9) Matsuda H., Yano M., Kubo M., Iinuma M., Oyama M., Mizuno M., *Yakugaku Zasshi*, **111**, 193—198 (1991).
10) Kubo M., Fujita T., Nishimura S., Tokunaga M., Matsuda H., Gato T., Tomohiro N., Sasaki K., Utsunomiya N., *Nat. Med.*, **58**, 284—294 (2004).
11) Fujita T., Kawase A., Nishijima N., Masuda M., Matsuda H., Iwaki M., *Shoyakugaku Zasshi*, **62**, 8—14 (2008).
12) Fujita T., Kawase A., Niwa T., Tomohiro N., Masuda M., Matsuda H., Iwaki M., *Biol. Pharm. Bull.*, **31**, 925—930 (2008).
13) Imokawa G., *J. Invest. Dermatol.*, **95**, 39—49 (1990).
14) Imokawa G., Kawai M., Mishima Y., Motegi I., *Arch. Dermatol. Res.*, **278**, 352—362 (1986).
15) Mason H. S., Peterson E. W., *Biochim. Biophys. Acta*, **111**, 134—146 (1965).
16) Oyanagui Y., *Anal. Biochem.*, **142**, 290—296 (1984).
17) Blois M. S., *Nature* (London), **181**, 1199—1200 (1958).
18) Hill S. E., Buffey J., Thody A. J., Oliver I., Bleehen S. S., Neil S. M., *Pigment Cell Res.*, **2**, 161—166 (1989).
19) Matsuda H., Hirata N., Kawaguchi Y., Naruto S., Takata T., Oyama M., Iinuma M., Kubo M., *Biol. Pharm. Bull.*, **29**, 834—837 (2006).
20) Hou W. C., Lin R. D., Chen K. T., Hung Y. T., Cho C. H., Chen C. H., Hwang S. Y., Lee M. H., *Phytomedicine*, **10**, 170—175 (2003).
21) Cho E. J., Yokozawa T., Rhyu D. Y., Kim S. C., Shibahara N., Park J. C., *Phytomedicine*, **10**, 544—551 (2003).