J Nat Med (2009) 63:443–450
DOI 10.1007/s11418-009-0349-1

ORIGINAL PAPER

Antiallergic activity of unripe *Citrus hassaku* fruits extract and its flavanone glycosides on chemical substance-induced dermatitis in mice

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Received: 24 April 2009 / Accepted: 18 June 2009 / Published online: 15 July 2009

Abstract Oral administration of a 50% ethanolic extract (CH-ext) obtained from unripe *Citrus hassaku* fruits collected in July exhibited a potent dose-dependent inhibition of IgE (immunoglobulin E)-mediated triphasic cutaneous reaction at 1 h [immediate phase response (IPR)], 24 h [late phase response (LPR)] and 8 days [very late phase response (vLPR)] after dinitrofluorobenzene challenge in mice. Naringin, a major flavanone glycoside component of CH-ext, showed a potent dose-dependent inhibition against IPR, LPR and vLPR. Neohesperidin, another major glycoside component of CH-ext, showed an inhibition against vLPR. The effect of CH-ext on type IV allergic reaction was examined by determining inhibitory activity against ear swelling in mice by using the picryl chloride-induced contact dermatitis (PC-CD) model. Oral administration (p.o.) of CH-ext and subcutaneous administration (s.c.) of prednisolone inhibited ear swelling during the induction phase of PC-CD. The inhibitory activities of combinations of CH-ext (p.o.) and prednisolone (s.c.) against PC-CD in mice were more potent than those of CH-ext alone and prednisolone alone, without enhancing the adverse effects. Other combinations of prednisolone (s.c.) and flavanone glycoside (p.o.) components of CH-ext, i.e. naringin and neohesperidin, exerted similar synergistic effects.

Keywords *Citrus hassaku* · Naringin · Neohesperidin · Prednisolone · Antiallergic effect

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Introduction

During the course of our ongoing search for antiallergic agents from natural resources, we found that a 50% ethanolic extract of unripe *Citrus* fruits showed antiallergic activities against type I, II and IV allergic reactions in several pharmacological assays [1]. Unripe *Citrus* fruit has been used in traditional Chinese medicine and contains several types of biologically active compounds such as limonoids, alkaloids and flavonoids [2]. In a previous study on seasonal variation in several *Citrus* fruit extracts in the relationship between antiallergic activity and the content of flavanone glycosides [3], it was found that 50% ethanolic extracts obtained from unripe fruits of some *Citrus* species, e.g. *C. unshiu* Markovich, *C. reticulata* Blanco, *C. sudachi* Hort et Shirai, and *C. hassaku* Hort ex T. Tanaka, showed in vitro inhibitory effect on compound 48/80-induced histamine release from rat peritoneal mast cells. Among them, the extract of unripe fruit of *C. unshiu* collected in July (CU-ext) exhibited antiallergic activities against type I, II and IV allergic reactions in several in vivo assays [1], and hesperidin, a major flavanone glycoside component of CU-ext, was shown to be an active component against type I and type IV allergies [4]. Furthermore, we reported that the efficacy of prednisolone was enhanced by administration of a combination of prednisolone [subcutaneous administration (s.c.)] and CU-ext [oral administration (p.o.)] without enhancing the adverse effects that accompany steroidal agents [5].

Ripe fruits of *C. hassaku* have been used for foods in Japan; the thinning out of unripe fruits in July is important for a rich harvest of superior ripe fruit in December, and any collected unripe fruit, deemed unworthy, is usually discarded. We previously reported that CH-ext obtained from unripe *C. hassaku* fruits collected in July exhibited in

vitro inhibitory activity on compound 48/80-induced histamine release [3]. However, there is no report on in vivo antiallergic effects of *C. hassaku*. Thus, since the unripe fruit of *C. hassaku* collected by thinning out can be considered a plant resource, we examined in vivo antiallergic effects of CH-ext and its two major flavanone glycosides, naringin and neohesperidin, against type I and type IV allergy models in mice. 2,4-Dinitrofluorobenzene (DNFB) has been shown to induce triphasic increase in ear thickness in mice after a passive sensitization with monoclonal anti-dinitrophenyl (DNP) IgE antibody [6, 7]. This method serves as an animal model for type I allergic reaction [6, 7]. As type IV allergic reaction model, we used picryl chloride-induced contact dermatitis (PC-CD) in mice [8], as applied by others [9]. Moreover, we examined whether a combination of CH-ext and prednisolone exerts a synergistic effect on PC-CD in mice, as in the case for CU-ext [5]. In addition, anti-type IV allergic effects of other combinations of prednisolone and major flavanone glycoside components of *C. hassaku* were studied in the PC-CD model.

Materials and methods

Preparation of CH-ext

Fruits of *C. hassaku* were collected monthly in the Wakayama Prefecture, Japan, from July to November, 2004, air-dried at 50°C for 48 h in automatic air-drier apparatus (Vianove Inc., Japan) and powdered. A 50% ethanolic extract of *C. hassaku* fruits was obtained according to the preceding paper [10] in yields of 26% (collected in July; this extract is abbreviated as CH-ext throughout this paper), 22% (August), 26% (September), 30% (October) and 30% (November).

Reagents

DNFB, PC, prednisolone and carboxymethylcellulose sodium (CMC-Na) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Monoclonal anti-DNP antibody, naringin and neohesperidin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). 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.

Fractionation of CH-ext

A suspension of 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 ethyl acetate extraction. The percentage inhibition of histamine release was evaluated for each fraction at 200 μg/ml and the results were: hexane-soluble fraction, 20% inhibition; ethyl acetate-soluble fraction, 27%; water-soluble fraction, 9%; and ethyl acetate–water-insoluble intermediate fraction, 24%.

Animals

Female BALB/c strain mice (14–19 g) and female ICR strain mice (30–32 g) were provided by SLC (Japan SLC, Hamamatsu, Japan). Mice 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 (Labo MR Stock, Nihon Nosan Kogyo 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 US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Compound 48/80-induced histamine release from rat peritoneal mast cells

Mast cells were prepared from the peritoneal cavity fluid of male Wistar strain rats by a slight modification of the method described by Uvnäs and Thon [6]. The cells were suspended in Hanks' solution containing heparin (10 U/ml), then layered on 40% Ficoll in a test tube for 30 min. After centrifugation at 150 *g* and 4°C for 10 min, the layer containing mast cells was pipetted out. The cells were washed three times with 5 ml of phosphate-buffered saline (PBS, pH 7.0) and suspended in the same medium at 2.9×10^6 cells/ml. The test sample was dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS (pH 7.0) to a final DMSO concentration of 0.5% v/v, and then the mixture was incubated at 37°C. After 10 min, 0.1 ml of compound 48/80 solution (0.2 mg/ml) was added, and the mixture was incubated at 37°C for 10 min in a final volume of 2 ml. The reaction was quenched by cooling the mixture on ice. The mixture was centrifuged at 150 *g* and 5°C for 5 min, then histamine in the supernatant fluid was assayed fluorometrically according to the method described in the previous report [7]. The effect of the test substance on histamine release from mast cells induced by compound 48/80 was expressed as histamine release percentage. PBS

(pH 7.0) was used as control. SCG was used as a reference drug.

IgE-mediated triphasic cutaneous reaction in mice

IgE-mediated triphasic cutaneous reaction was induced according to the methods of Yamaguchi et al. [11] and Tahara et al. [12] with minor modifications. Female BALB/c strain mice weighing 14–19 g ($n = 10$–11 per group) were passively sensitized with intravenous injection of 10 μg of monoclonal anti-DNP IgE antibody dissolved in 0.5 ml of saline. Twenty-four hours after the sensitization, the mice were challenged by painting 25 μl of 0.15% DNFB solution in acetone/olive oil (3:1 v/v) to each side of the right and left ears. The control mice received intravenous injection of saline (0.5 ml) instead of IgE antibody and were painted with 25 μl of 0.15% DNFB solution. The vehicle control mice received intravenous injection of IgE antibody and were painted with 25 μl of 0.15% DNFB solution. Prednisolone was suspended in 0.2% CMC-Na solution. An appropriate amount of test sample was suspended in 0.2% CMC-Na solution. The suspension of test sample was administered orally (0.2 ml/10 g body weight of mouse per day) at 1 h before DNFB challenge, 23 h after DNFB challenge, and every day for 7 days. The control and vehicle control mice were administered 0.2% CMC-Na solution (0.2 ml/10 g body weight of mouse per day). The thickness of the right ear was measured by using a dial thickness gauge (Mitutoyo Co., Kawasaki, Tokyo) immediately before and at 1 h [immediate phase response (IPR)], 24 h [late phase response (LPR)] and 8 days [very late phase response (vLPR)] after the DNFB challenge. The ear swelling (cutaneous reaction) was expressed as the difference in the ear thickness between immediately before the DNFB challenge and those at 1 h (IPR), 24 h (LPR) or 8 days (vLPR) after the DNFB challenge, respectively. The experimental results were expressed as the average of increase in ear thickness ± standard error (SE) ($n = 7$–11 per group).

Picryl chloride-induced contact dermatitis (PC-CD) in mice

Following the method described by Asherson and Ptak [8] with slight modification, female ICR strain mice weighing 30–32 g were sensitized by topical application of 0.1 ml of 7% PC solution in EtOH to the shaved abdomen (the first sensitization). After the first sensitization, test sample was suspended in 0.2% CMC-Na solution and administered orally (0.2 ml/10 g body weight of mouse per day) from day −1 to day 5 for 7 days to the CH-ext group and the flavanone glycoside group, respectively. Prednisolone suspended in saline was administered subcutaneously (0.1 ml/10 g body weight of mouse per day) from day 0 to day 5 for 6 days to the prednisolone group. The combination group was treated with prednisolone (s.c., for 6 days) and CH-ext or flavanone glycoside (p.o., for 7 days) in a given portion. The control group was treated with 0.2% CMC-Na solution (p.o., for 7 days) and saline (s.c., for 6 days). Six days after the first sensitization, the treated mice were challenged by painting the inside of the ears with 0.02 ml of 1% PC solution in olive oil to induce PC-CD (the first PC challenge). The ear swelling was expressed as the difference in the ear thickness between immediately before and 24 h after the first PC challenge. The ear thickness was measured by using a dial thickness gauge. The experimental results were expressed as the average of increase in ear thickness ± SE. To evaluate the effect of test sample on the induction phase of PC-CD, the difference of increase in ear thickness between the test group and the control group was calculated and expressed as the percentage inhibition value compared with the control group. After the last measurement of ear thickness (24 h after the first PC challenge), the mice were killed by cervical vertebrae dislocation, and three organs (thymus, spleen and adrenal gland) were isolated. The weights of the organs were measured and expressed as a ratio of organ weight to 10 g body weight of mouse.

Statistical analysis

The experimental data were evaluated for statistical significance using the Tukey–Kramer post hoc test (computer program was StatView for Windows, Ver. 5.0, SAS Institute Inc., 1998).

Results and discussion

Histamine release inhibitory activity

Our preliminary comparative screening for compound 48/80-induced histamine release inhibitory activity of 50% ethanolic extracts obtained from the fruit of *C. hassaku* collected monthly in July, August, September, October and November gave the following results: the percentage inhibition values of an extract (CH-ext) obtained from unripe fruits collected in July were 15% at a concentration of 200 μg/ml and 43% at a concentration of 500 μg/ml, the corresponding values of the extract of August were 13% at a concentration of 200 μg/ml and 16% at a concentration of 500 μg/ml, but other extracts obtained from fruits collected in September, October and November showed no significant effect at a concentration of 500 μg/ml. These results indicated that CH-ext exhibited the most potent inhibitory activity.

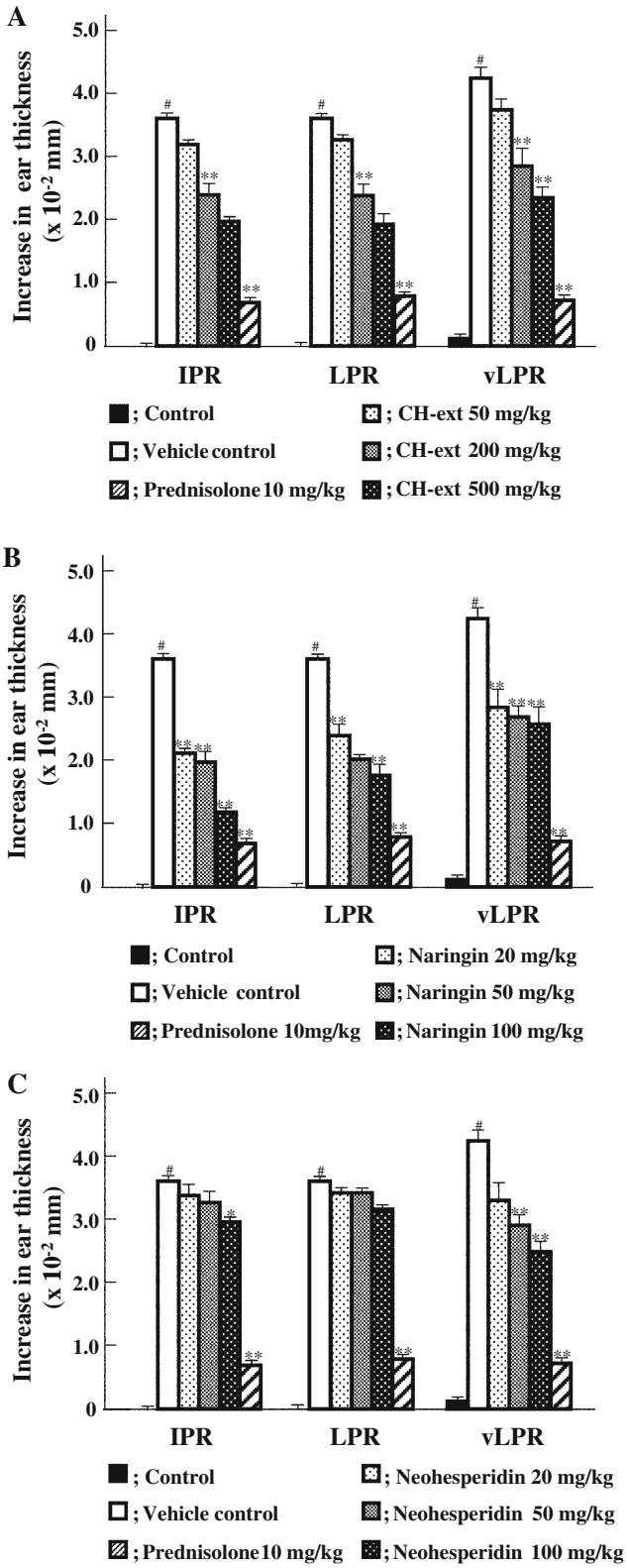

Fig. 1 Effects of CH-ext, naringin, neohesperidin and prednisolone on IgE-mediated triphasic cutaneous reaction in passively sensitized mice. **a** CH-ext and prednisolone, **b** naringin and prednisolone, **c** neohesperidin and prednisolone. Mice were passively sensitized by anti-DNP IgE antibody before the DNFB challenge. Each test substance was orally administered. Ear thickness was measured at immediately before the DNFB challenge, and 1 h (IPR), 24 h (LPR) and 8 days (vLPR) after the DNFB challenge. Each value represents the mean ± SE of 7–11 mice. $^{\#}P < 0.01$, significantly different from the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the vehicle control group

For the purpose of identification of active constituents of unripe *C. hassaku* fruits, CH-ext was fractionated by solvent extraction to give a hexane-soluble fraction, an ethyl acetate-soluble fraction, a water-soluble fraction and a water–ethyl acetate-insoluble intermediate fraction. Among them, the ethyl acetate-soluble fraction and the water–ethyl acetate-insoluble intermediate fraction showed more potent histamine release inhibitory activities than the hexane-soluble fraction as described above in Fractionation of CH-ext, whereas the water-soluble fraction was inactive. On HPLC analysis [10], the flavanone glycoside content (in milligrams per gram) of CH-ext was: naringin, 206.8; neohesperidin, 94.7; narirutin, 28.5; and hesperidin, 8.9. That of the ethyl acetate-soluble fraction was: naringin, 345.9; neohesperidin, 189.4; narirutin, 56.3; and hesperidin, 41.6. That of the water–ethyl acetate-insoluble intermediate fraction was: naringin, 674.2; neohesperidin, 81.1; narirutin, not detected; and hesperidin, not detected. Thus the two fractions were flavanone glycoside-rich fractions in which naringin and neohesperidin were major flavanones, and narirutin as well as hesperidin were minor flavanones. In the previous paper [3], we reported that four flavanone glycosides of *Citrus* fruits, namely hesperidin, narirutin, naringin and neohesperidin, showed potent histamine release inhibitory activity. Percentage inhibition values at 1 mM of naringin, neohesperidin, narirutin, hesperidin and sodium cromoglycate (SCG, a reference compound) were 43.7, 26.7, 47.3, 49.8 and 36.5, respectively. On the basis of these finding, we reasonably assumed that the histamine release inhibitory effect of CH-ext is attributable to naringin and neohesperidin.

Effects of CH-ext, naringin, neohesperidin and prednisolone on IgE-mediated triphasic cutaneous reaction in mice

The antiallergic effect of oral administration of test samples on DNFB-induced triphasic cutaneous reaction was examined by measuring ear swelling in mice passively sensitized with anti-DNP IgE antibody. As shown in Fig. 1, in comparison with the control and the vehicle control groups, DNFB induced triphasic cutaneous reaction (ear swelling) at 1 h (IPR) after, 24 h (LPR) after and 8 days (vLPR) after DNFB challenge. The efficacy of the test substances against ear swelling was evaluated by

measuring ear thickness at 1 h (IPR) after, 24 h (LPR) after and 8 days (vLPR) after DNFB challenge as shown in Fig. 1. Among these responses, IPR is considered to be a type I allergy model. Prednisolone (10 mg/kg, p.o.), a positive reference drug, had a potent inhibitory effect on IPR, LPR and vLPR (Fig. 1). As shown in Fig. 1a, CH-ext (50, 200 and 500 mg/kg, p.o.) dose-dependently inhibited ear swelling of IPR, LPR and vLPR. Naringin (20, 50 and 100 mg/kg, p.o.) dose-dependently inhibited IPR, LPR and vLPR as illustrated in Fig. 1b. Neohesperidin (200 and 500 mg/kg, p.o.) was almost inactive at IPR and LPR, but showed inhibitory effects on ear swelling of vLPR (Fig. 1c).

Effect of CH-ext, prednisolone and combinations of CH-ext and prednisolone on induction phase of PC-CD

Antiallergic activity of CH-ext, prednisolone and combinations of CH-ext and prednisolone against the type IV allergic reaction during induction phase of PC-CD in mice was examined by successive oral administration after sensitization. Prednisolone, a reference drug, was subcutaneously administered as in the experiments with combinations of CU-ext and prednisolone [5]. Activity of test samples was evaluated by the increase of ear thickness during induction phase of PC-CD in mice. Percentage inhibition values in Tables 1 and 3 are the calculated values. Adverse effect of test substance was checked by the weight of three organs (thymus, spleen and adrenal gland) isolated from the mice after the last measurement of ear thickness, because the weight of these organs may reflect the adverse effects of drugs especially adrenocortical hormone agents.

As shown in Table 1, successive administrations of CH-ext (50 and 200 mg/kg for 7 days, p.o.) and prednisolone (0.2 and 1 mg/kg for 6 days, s.c.) dose-dependently inhibited the ear swelling during the induction phase of PC-CD in mice. Successive administration of combinations of CH-ext (p.o.) and prednisolone (s.c.) in a given portion showed inhibitory effects against ear swelling. The inhibitory activities of combination of CH-ext and prednisolone were more potent than those of CH-ext (10 and 50 mg/kg for 7 days, p.o.) alone and prednisolone (0.05 and 0.2 mg/kg for 6 days, s.c.) alone. Results of organ weight measurement are shown in Table 2. Slight decrements of the weight ratios in thymus and spleen were observed in the highest dose group of prednisolone (0.2 mg/kg for 6 days, s.c.). These results indicated the combination of CH-ext and prednisolone exerted a synergistic effect without enhancement of the adverse reaction of prednisolone.

Table 1 Effects of CH-ext, prednisolone and combinations of CH-ext and prednisolone on ear swelling during the induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Number of mice	Increase in ear thickness ($\times 10^{-2}$ mm)	Inhibition (%)
Vehicle control	–		10	11.5 ± 0.1	–
CH-ext	10	p.o.	10	11.3 ± 0.2	1.7
	50	p.o.	10	8.6 ± 0.1**	25.2
	200	p.o.	10	6.5 ± 0.2**	43.8
Prednisolone	0.05	s.c.	10	10.8 ± 0.4	6.1
	0.2	s.c.	10	8.8 ± 0.1**	23.5
	1	s.c.	10	6.5 ± 0.3**	43.5
CH-ext + prednisolone	10 + 0.05	p.o. + s.c.	11	7.9 ± 0.2**[a,c]	31.3
	10 + 0.2	p.o. + s.c.	11	7.4 ± 0.2**[a,d]	35.7
	50 + 0.05	p.o. + s.c.	11	7.0 ± 0.1**[b,c]	39.1
	50 + 0.2	p.o. + s.c.	11	5.5 ± 0.2**[b,d]	52.3

Ear thickness was measured immediately before and 24 h after the first PC challenge. Each value represents the mean ± SE

p.o. oral administration, *s.c.* subcutaneous administration

** $P < 0.01$, significantly different from the vehicle control group

[a] $P < 0.01$, significantly different from 10 mg/kg of CH-ext group

[b] $P < 0.01$, significantly different from 50 mg/kg of CH-ext group

[c] $P < 0.01$ significantly different from 0.05 mg/kg of prednisolone group

[d] $P < 0.01$ significantly different from 0.2 mg/kg of prednisolone group

Table 2 Effects of CH-ext, prednisolone and combinations of CH-ext and prednisolone on the weight of adrenal gland, thymus and spleen during the induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Number of mice	Organ weight (mg/10 g body weight)		
				Adrenal gland	Thymus	Spleen
Vehicle control	–		10	1.5 ± 0.1	19.8 ± 1.2	49.7 ± 1.3
CH-ext	10	p.o.	10	1.4 ± 0.1	25.4 ± 1.7	52.9 ± 3.2
	50	p.o.	10	1.5 ± 0.1	20.8 ± 1.2	50.9 ± 3.6
	200	p.o.	10	1.8 ± 0.1	21.0 ± 1.3	48.3 ± 2.6
Prednisolone	0.05	s.c.	10	1.6 ± 0.1	21.4 ± 1.5	42.8 ± 2.2
	0.2	s.c.	10	1.5 ± 0.1	19.2 ± 1.5	46.5 ± 3.8
	1	s.c.	10	1.7 ± 0.1	12.7 ± 0.9**	31.9 ± 1.2**
CH-ext + prednisolone	10 + 0.05	p.o. + s.c.	11	1.8 ± 0.1	19.7 ± 1.8	48.1 ± 3.6
	10 + 0.2	p.o. + s.c.	11	1.5 ± 0.1	19.2 ± 1.2	44.9 ± 2.5
	50 + 0.05	p.o. + s.c.	11	1.4 ± 0.1	19.6 ± 1.3	45.8 ± 3.4
	50 + 0.2	p.o. + s.c.	11	1.7 ± 0.1	18.5 ± 0.8	45.2 ± 1.9

The organs were isolated after the last measurement (24 h after the first PC challenge) of ear thickness. Each value represents the mean ± SE

p.o. oral administration, *s.c.* subcutaneous administration

** $P < 0.01$, significantly different from the vehicle control group

Table 3 Effects of naringin, neohesperidin, prednisolone and combinations of naringin or neohesperidin and prednisolone on ear swelling during the induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Number of mice	Increase in ear thickness ($\times 10^{-2}$ mm)	Inhibition (%)
Vehicle control	–		10	12.5 ± 0.3	–
Naringin	5	p.o.	7	10.9 ± 0.3	12.8
	20	p.o.	7	9.1 ± 0.3**	27.2
	50	p.o.	7	6.8 ± 0.2**	45.6
Neohesperidin	5	p.o.	7	12.5 ± 0.2	0
	20	p.o.	7	10.4 ± 0.5	16.8
	50	p.o.	8	9.2 ± 0.4**	26.4
Prednisolone	0.05	s.c.	7	12.1 ± 0.4	3.2
	0.2	s.c.	7	9.7 ± 0.3**	22.4
	1	s.c.	7	7.3 ± 0.2**	41.6
Naringin + prednisolone	5 + 0.05	p.o. + s.c.	9	7.2 ± 0.5**,a,d	42.4
	5 + 0.2	p.o. + s.c.	9	5.5 ± 0.7**,a,e	56.0
	20 + 0.05	p.o. + s.c.	9	6.4 ± 0.8**,b,d	48.8
	20 + 0.2	p.o. + s.c.	10	3.9 ± 0.6**,b,e	68.8
Neohesperidin + prednisolone	5 + 0.05	p.o. + s.c.	9	10.7 ± 0.3	14.4
	5 + 0.2	p.o. + s.c.	9	8.8 ± 0.3**,f	29.6
	20 + 0.05	p.o. + s.c.	9	9.6 ± 0.3**,c	23.2
	20 + 0.2	p.o. + s.c.	10	7.2 ± 0.2**,e,g	42.4

Ear thickness was measured immediately before and 24 h after the first PC challenge. Each value represents the mean ± SE

p.o. oral administration, *s.c.* subcutaneous administration

** $P < 0.01$, significantly different from the vehicle control group

[a] $P < 0.01$, significantly different from 5 mg/kg of naringin group

[b] $P < 0.01$, significantly different from 20 mg/kg of naringin group

[c] $P < 0.05$, [d] $P < 0.01$ significantly different from 0.05 mg/kg of prednisolone group

[e] $P < 0.01$ significantly different from 0.2 mg/kg of prednisolone group

[f] $P < 0.01$ significantly different from 5 mg/kg of neohesperidin group

[g] $P < 0.01$ significantly different from 20 mg/kg of neohesperidin group

Table 4 Effects of naringin, neohesperidin, prednisolone and combinations of naringin or neohesperidin and prednisolone on the weight of adrenal gland, thymus and spleen during the induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Number of mice	Organ weight (mg/10 g body weight)		
				Adrenal gland	Thymus	Spleen
Vehicle control	–		10	1.4 ± 0.1	24.0 ± 2.0	47.4 ± 1.3
Naringin	5	p.o.	7	1.5 ± 0.1	18.8 ± 0.9	36.8 ± 0.8*
	20	p.o.	7	1.5 ± 0.1	21.2 ± 1.4	44.3 ± 2.5
	50	p.o.	7	1.6 ± 0.1	22.1 ± 1.5	41.2 ± 1.7
Neohesperidin	5	p.o.	7	1.6 ± 0.1	21.8 ± 2.2	37.7 ± 5.7*
	20	p.o.	7	1.5 ± 0.1	19.0 ± 0.3	48.2 ± 1.6
	50	p.o.	8	1.4 ± 0.1	20.7 ± 0.5	43.8 ± 1.0
Prednisolone	0.05	s.c.	7	1.5 ± 0.1	21.9 ± 1.5	44.5 ± 1.6
	0.2	s.c.	7	1.3 ± 0.1	18.2 ± 0.9	37.5 ± 1.5*
	1	s.c.	7	1.3 ± 0.1	13.1 ± 0.4**	37.5 ± 1.3*
Naringin + prednisolone	5 + 0.05	p.o. + s.c.	9	1.5 ± 0.1	21.1 ± 1.4	41.5 ± 1.2
	5 + 0.2	p.o. + s.c.	9	1.2 ± 0.1	21.9 ± 1.1	42.4 ± 2.3
	20 + 0.05	p.o. + s.c.	9	1.2 ± 0.2	19.8 ± 0.8	40.7 ± 1.9
	20 + 0.2	p.o. + s.c.	10	1.4 ± 0.1	18.3 ± 0.4*	44.7 ± 0.9
Neohesperidin + prednisolone	5 + 0.05	p.o. + s.c.	9	1.4 ± 0.1	21.9 ± 1.3	46.4 ± 1.6
	5 + 0.2	p.o. + s.c.	9	1.3 ± 0.1	19.9 ± 1.0	38.1 ± 2.1*
	20 + 0.05	p.o. + s.c.	9	1.3 ± 0.1	22.0 ± 0.9	44.3 ± 1.1
	20 + 0.2	p.o. + s.c.	10	1.6 ± 0.1	18.8 ± 1.4	39.4 ± 1.5

The organs were isolated after the last measurement (24 h after the first PC challenge) of ear thickness. Each value represents the mean ± SE

p.o. oral administration, *s.c.* subcutaneous administration

* $P < 0.05$, ** $P < 0.01$, significantly different from the vehicle control group

Effect of naringin, neohesperidin, prednisolone, combinations of naringin and prednisolone and combinations of neohesperidin and prednisolone on induction phase of PC-CD

As shown in Table 3, successive oral administration of naringin (20 and 50 mg/kg for 7 days, p.o.) as well as successive subcutaneous administration of prednisolone (0.2 and 1 mg/kg for 6 days, s.c.) dose-dependently inhibited the ear swelling during the induction phase of PC-CD in mice. Neohesperidin showed inhibitory effects at the highest dose (50 mg/kg for 7 days, p.o.). Successive administration of a combination of naringin (p.o.) and prednisolone (s.c.) in a given portion inhibited the ear swelling. The inhibitory activities of combinations of flavanone glycoside and prednisolone were more potent than each flavanone glycoside (5 and 20 mg/kg for 7 days, p.o.) alone and prednisolone (0.05 and 0.2 mg/kg for 6 days, s.c.) alone. These results indicated a synergistic effect of the combination of each flavanone glycoside and prednisolone as well as the combination of CH-ext and prednisolone. Results of the organ weight measurement are shown in Table 4. Significant decrement of spleen weight ratio was observed in the groups of naringin (5 mg/kg, p.o.) and neohesperidin (5 mg/kg, p.o.), but there was no dose-dependency. Considering the contents of naringin and neohesperidin in CH-ext, the inhibitory activity of CH-ext against the type IV allergic reaction is not fully explained by the presence of these two flavanone glycosides.

In conclusion, CH-ext and naringin have antiallergic effects against both type I and type IV allergies. The combination of CH-ext and prednisolone as well as the combination of various kinds of flavanone glycoside alone and prednisolone exerted synergistic effect without enhancement of adverse reaction of steroidal agents as in the case of the combination of CU-ext and prednisolone as well as the combination of hesperidin and prednisolone [5]. The synergistic effect of combinations of *Citrus* fruit extract and its flavanone glycosides in combination with prednisolone on ear swelling in PC-CD in mice is a tractable phenomenon. However further investigations are required to reveal the mechanisms involved.

Acknowledgments We are grateful to all staff at Yuasa Experimental Farm, Kinki University, for the collection of unripe fruit of

C. hassaku. This work was financially supported by "Antiaging Center Project" for Private Universities from Ministry of Education, Culture, Sports, Science and Technology, 2008–2012.

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