

Anti-allergic effect of a combination of *Citrus unshiu* unripe fruits extract and prednisolone on picryl chloride-induced contact dermatitis in mice

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Abstract Effect of 50% ethanolic extract of unripe fruits of *Citrus unshiu* (CU-ext) on type IV allergic reaction was examined by inhibitory activity of ear swelling of picryl chloride-induced contact dermatitis (PC-CD) in mice. Oral administration of CU-ext and subcutaneous administration of prednisolone showed inhibition of ear swelling during both induction and effector phases of PC-CD. The inhibitory activities of combinations of CU-ext (p.o.) and prednisolone (s.c.) during induction phase of PC-CD were more potent than those of CU-ext alone and prednisolone alone. Successive oral administration of hesperidin, a major flavanone glycoside of CU-ext, inhibited ear swelling during induction phase of PC-CD. The inhibitory activities of combinations of hesperidin (p.o.) and prednisolone (s.c.) were more potent than those of hesperidin alone and prednisolone alone. These results indicated that the combinations of prednisolone and CU-ext or hesperidin exerted a synergistic effect.

Keywords *Citrus unshiu* · Hesperidin · Prednisolone · Anti-allergic effect

Introduction

Fruits of *Citrus unshiu* Markovich have been used for food as well as a traditional medicine for treating gastrointestinal and inflammatory diseases in China and Japan. During the course of our studies on the anti-allergic effect of citrus fruits [1–3], it was found that 50% ethanolic extract of unripe fruits of *C. unshiu* harvested in July (CU-ext) showed anti-allergic activities against type I, II and IV allergic reactions in several pharmacological assays [1]. Furthermore, we previously identified hesperidin, a major flavanone glycoside of unripe fruits of *C. unshiu*, as an active component that exhibited anti-allergic activity against type I allergy [2]. However, no active constituent of the *C. unshiu* unripe fruits has been known to be a compound having anti-allergic activity against type IV allergic reaction. At the same time, we reported that the efficacy of prednisolone was enhanced by administration of a combination of prednisolone [subcutaneous administration (s.c.)] and an extract of bearberry leaf [leaves of *Arctostaphylos uva-ursi* (L.) Spreng] [oral administration (p.o.)] without enhancing the side effects that accompany steroidal agents [4]. Therefore, it was assumed that a combination of prednisolone and CU-ext may have a synergistic effect and could reduce the adverse effect of steroidal agents. In order to confirm this assumption, firstly we examined the anti-type IV allergic effect of CU-ext during either induction or effector phase of picryl chloride-induced contact dermatitis (PC-CD) in mice. Secondly we examined whether a combination of prednisolone and CU-ext exerts a synergistic effect. In addition, the anti-type IV allergic effect of another combination of prednisolone and hesperidin was examined.

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Materials and methods

Plant material

Unripe fruits of *C. unshiu* were harvested in Arita-gun, Wakayama, Japan, on July 2004, air-dried, and characterized by one of the authors (K. Sasaki). A voucher specimen (CU-04-07) is deposited at Kinki University.

Preparation of 50% ethanol extract from unripe fruits of *C. unshiu*

The crushed fruits (300 g) were extracted once with 50% EtOH (3 l) under reflux for 2 h. The filtrates were concentrated under reduced pressure and lyophilized to give brown powder (CU-ext) (yield: 73.5 g, 24.5%).

Chemicals

Picryl chloride (PC), prednisolone, and carboxymethyl-cellulose sodium (CMC-Na) were purchased from Nacalai Tesque, Kyoto, Japan. Hesperidin (purity: 92%) purchased from Wako Pure Chemical Industries (Osaka, Japan) was recrystallized from MeOH to give purified hesperidin (purity: 96% determined by HPLC [3]), which was used for this experiment.

Animals

Female ICR strain mice (27–29 g) were provided by SLC (Japan SLC, Hamamatsu, Japan). They were maintained in an air-conditioned room with lighting from 7 AM to 7 PM. The room temperature (about 23°C) and humidity (about 60%) were controlled automatically. Laboratory pellet chows (Labo MR Stock, Nihon Nosan Kogyo K.K., Yokohama, Japan) and water were freely available.

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

Anti-allergic effect on the induction phase

Following the method described by Asherson and Ptak [5] with modification, 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, CU-ext and hesperidin were suspended in 0.2% CMC-Na and administered orally (0.2 ml/10 g body weight of mouse/day) from day -1 to day 5 for 7 days

to the CU-ext group and the hesperidin group, respectively. Prednisolone suspended in saline was administered subcutaneously (0.1 ml/10 g body weight of mouse/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 CU-ext or hesperidin (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). To evaluate the effect on the induction phase of PC-CD, the ear thickness was measured by using a dial thickness gauge (Mitsutoyo, Tokyo, Japan) immediately before and 24 h after the first PC challenge, and the difference in the thickness was calculated and expressed in percent value compared to 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 in a ratio of organ weight to 10 g body weight of mouse.

Anti-allergic effect on the effector phase

Mice weighing 30–32 g were sensitized by 7% PC solution as described above (the first sensitization). Six days after the first sensitization, the mice were challenged by 1% PC solution as described above (the first PC challenge). Ear thickness was measured immediately before and 24 h after the first PC challenge. Mice with a certain percentage (over 25%) of ear swelling after the first PC challenge were chosen. Three days thereafter, the selected mice ($n = 8–11$ per group) were sensitized again by application of 0.1 ml of 7% PC solution in EtOH to the shaved abdomen (the second sensitization). Six days later, the mice were challenged again by painting the inside of ears with 0.02 ml of 1% PC solution in olive oil (the second PC challenge).

CU-ext suspended in 0.2% CMC-Na was administered orally twice immediately before and 16 h after the second PC challenge. Prednisolone suspended in saline was administered subcutaneously 16 h after the second PC challenge. The control group was treated with 0.2% CMC-Na solution (p.o., twice) or saline (s.c., once). To evaluate the effect on the effector phase, ear thickness was measured immediately before and 24 h after the second PC challenge. After the last measurement (24 h after the second PC challenge) of ear thickness, the weight of three organs (thymus, spleen, and adrenal gland) was measured as described above.

Statistical analysis

The experimental data were tested for statistically significant differences by means of Bonferroni/Dunn's method (multiple-range tests).

Results and discussion

Anti-allergic activity of test samples against type IV allergic reaction during the induction phase of PC-CD in mice was examined by successive oral administration after sensitization, whereas the activity during the effector phase of PC-CD was examined by two oral administrations of test samples immediately before and 16 h after the second PC challenge. In order to avoid an interaction during gastrointestinal absorption, prednisolone, a reference drug, was subcutaneously administered. In the study on the effector phase of PC-CD, prednisolone was administered only at 16 h after the second PC challenge.

Activity of test samples was evaluated as percentage of the ear swelling of PC-CD in mice. Inhibition percentages in tables are calculated values. Adverse effects of test samples were checked by measuring the weight of three organs (thymus, spleen, and adrenal gland) isolated from the mice after the last measurement of ear swelling.

Effect of CU-ext and prednisolone on effector phase in PC-CD mice

As shown in Table 1, twice oral administration of CU-ext (200 and 500 mg/kg) exhibited an inhibitory effect on the effector phase of PC-CD in mice. These results were in accordance with the previous report [1]. Single

Table 1 Effect of CU-ext and prednisolone on ear swelling during the effector phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Swelling (%)	Inhibition (%)
Control	–	<i>p.o.</i>	59.4 ± 5.1	–
CU-ext	50	<i>p.o.</i>	47.3 ± 4.8	20.4
	200	<i>p.o.</i>	45.4 ± 5.8*	23.6
	500	<i>p.o.</i>	40.6 ± 3.0**	31.6
Control	–	<i>s.c.</i>	64.4 ± 9.5	–
Prednisolone	1	<i>s.c.</i>	40.8 ± 4.4 ^{##}	36.6

Ear thickness was measured immediately before and 24 h after the second PC challenge. Each value represents the mean ± SE of 8–11 mice

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

P* < 0.05, *P* < 0.01, significantly different from the control (*p.o.*) group; ^{##}*P* < 0.01, significantly different from the control (*s.c.*) group

administration of prednisolone (1 mg/kg, *s.c.*) showed the inhibition of ear swelling. CU-ext and prednisolone did not affect weights of three organs (thymus, spleen and adrenal gland) (data not shown).

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

As shown in Table 2, successive administrations of CU-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 CU-ext (*p.o.*) and prednisolone (*s.c.*) in a given portion showed the inhibitory effects on the ear swelling. The inhibitory activities of a combination of CU-ext and prednisolone were more potent than those of CU-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 3. A slight decrease in thymus weight ratio was observed in the combination group, but there was no significant difference between the treated group and control group. These results indicated that the combination of CU-ext and prednisolone exerted a synergistic effect without enhancement of the adverse reactions of prednisolone.

Effect of hesperidin, prednisolone, and combinations of hesperidin and prednisolone on the induction phase of PC-CD

As shown in Table 4, successive oral administration of hesperidin (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) exhibited dose-dependent inhibitory effects on the ear swelling during the induction phase of PC-CD in mice. Successive administration of a combination of hesperidin (*p.o.*) and prednisolone (*s.c.*) in a given portion inhibited the ear swelling. The inhibitory activities of combinations of hesperidin and prednisolone were more potent than those of hesperidin (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 hesperidin and prednisolone as well as the combination of CU-ext and prednisolone. Results of organ weight measurement are shown in Table 5. The decrease in thymus weight was observed in the group that was treated with a combination of hesperidin (5 mg/kg, *p.o.*) and prednisolone (0.2 mg/kg, *s.c.*). Considering the

Table 2 Effect of CU-ext, prednisolone, and combinations of CU-ext and prednisolone on ear swelling during the induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Swelling (%)	Inhibition (%)
Control	–		71.6 ± 1.0	–
CU-ext	10	<i>p.o.</i>	67.0 ± 1.6	6.4
	50	<i>p.o.</i>	48.4 ± 2.2**	32.4
	200	<i>p.o.</i>	34.5 ± 1.9**	51.8
Prednisolone	0.05	<i>s.c.</i>	68.0 ± 1.1	5.0
	0.2	<i>s.c.</i>	50.8 ± 1.3**	29.1
	1	<i>s.c.</i>	32.8 ± 2.4**	54.2
CU-ext + prednisolone	10 + 0.05	<i>p.o.</i> + <i>s.c.</i>	48.8 ± 1.8**,\$\$	31.8
	10 + 0.2	<i>p.o.</i> + <i>s.c.</i>	39.0 ± 1.7**,\$\$	45.5
	50 + 0.05	<i>p.o.</i> + <i>s.c.</i>	30.5 ± 1.8**,\$\$	57.4
	50 + 0.2	<i>p.o.</i> + <i>s.c.</i>	15.8 ± 1.8**,\$\$	77.9

Ear thickness was measured immediately before and 24 h after the first PC challenge. Each value represents the mean ± SE of 11 mice

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

***P* < 0.01, significantly different from the control group; ***P* < 0.01, significantly different from CU-ext group; \$\$*P* < 0.01, significantly different from prednisolone group

Table 3 Effect of CU-ext, prednisolone, and combinations of CU-ext and prednisolone on the weight of adrenal gland, thymus, and spleen during induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Organ weight (mg/10 g body weight)		
			Adrenal gland	Thymus	Spleen
Control	–		1.4 ± 0.1	26.5 ± 1.9	42.0 ± 2.4
CU-ext	10	<i>p.o.</i>	1.4 ± 0.1	28.2 ± 1.4	46.9 ± 1.9
	50	<i>p.o.</i>	1.4 ± 0.1	26.4 ± 1.7	43.7 ± 3.4
	200	<i>p.o.</i>	1.4 ± 0.1	26.0 ± 3.3	40.8 ± 2.5
Prednisolone	0.05	<i>s.c.</i>	1.6 ± 0.1	25.1 ± 2.1	38.9 ± 2.0
	0.2	<i>s.c.</i>	1.4 ± 0.1	24.9 ± 1.2	41.4 ± 1.7
	1	<i>s.c.</i>	1.4 ± 0.1	20.8 ± 1.9**	33.5 ± 1.7**
CU-ext + prednisolone	10 + 0.05	<i>p.o.</i> + <i>s.c.</i>	1.4 ± 0.1	23.1 ± 1.5	42.0 ± 2.1
	10 + 0.2	<i>p.o.</i> + <i>s.c.</i>	1.6 ± 0.1	23.0 ± 1.5	43.4 ± 1.9
	50 + 0.05	<i>p.o.</i> + <i>s.c.</i>	1.3 ± 0.1	24.8 ± 2.0	43.4 ± 2.5
	50 + 0.2	<i>p.o.</i> + <i>s.c.</i>	1.3 ± 0.1	23.4 ± 1.6	41.7 ± 1.6

The organs were isolated after the last measurement (24 h after the first PC challenge) of ear thickness. Each value represents the mean ± SE of 11 mice

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

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

content of hesperidin (3.7% determined by HPLC [3]) in CU-ext, the inhibitory activity of CU-ext against a type IV allergic reaction is not fully explained by hesperidin alone.

In conclusion, it was found that CU-ext exhibited inhibitory effects on the ear swelling during both the induction and effector phases of PC-CD in mice and that

hesperidin showed inhibitory effect on the ear swelling during the induction phase of PC-CD. It was interesting that the combination of CU-ext and prednisolone as well as the combination of hesperidin and prednisolone exerted a synergistic effect without enhancement of the adverse reactions of steroidal agents. However, further investigations are required to understand the mechanisms involved.

Table 4 Effect of hesperidin, prednisolone, and combinations of hesperidin and prednisolone on ear swelling during induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Swelling (%)	Inhibition (%)
Control	–		69.7 ± 0.9	–
Hesperidin	5	<i>p.o.</i>	66.6 ± 0.8	4.4
	20	<i>p.o.</i>	52.5 ± 1.1**	24.7
	50	<i>p.o.</i>	40.2 ± 1.4**	42.3
Prednisolone	0.05	<i>s.c.</i>	68.8 ± 1.5	1.3
	0.2	<i>s.c.</i>	56.2 ± 1.3**	19.4
	1	<i>s.c.</i>	37.5 ± 1.8**	46.2
Hesperidin + prednisolone	5 + 0.05	<i>p.o.</i> + <i>s.c.</i>	37.7 ± 1.8**,\$\$	45.9
	5 + 0.2	<i>p.o.</i> + <i>s.c.</i>	33.4 ± 1.2**,\$\$	52.1
	20 + 0.05	<i>p.o.</i> + <i>s.c.</i>	33.7 ± 0.8**,\$\$	51.6
	20 + 0.2	<i>p.o.</i> + <i>s.c.</i>	19.0 ± 2.8**,\$\$	72.7

Ear thickness was measured immediately before and 24 h after the first PC challenge. Each value represents the mean ± S.E. of 7–10 mice

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

***P* < 0.01, significantly different from the control group; ##*P* < 0.01, significantly different from CU-ext group; \$\$*P* < 0.01, significantly different from prednisolone group

Table 5 Effect of hesperidin, prednisolone, and combinations of hesperidin and prednisolone on the weight of adrenal gland, thymus, and spleen during induction phase of PC-CD in mice

Treatment	Dose (mg/kg)	Route	Organ weight (mg/10 g body weight)		
			Adrenal gland	Thymus	Spleen
Control	–		1.5 ± 0.1	23.5 ± 2.1	46.0 ± 1.6
Hesperidin	5	<i>p.o.</i>	1.5 ± 0.1	19.7 ± 1.3	42.9 ± 2.5
	20	<i>p.o.</i>	1.5 ± 0.1	19.9 ± 3.7	50.0 ± 3.9
	50	<i>p.o.</i>	1.5 ± 0.1	23.5 ± 1.8	50.4 ± 3.8
Prednisolone	0.05	<i>s.c.</i>	1.5 ± 0.1	20.5 ± 1.7	44.0 ± 1.6
	0.2	<i>s.c.</i>	1.4 ± 0.1	17.7 ± 1.6*	41.4 ± 2.5
	1	<i>s.c.</i>	1.7 ± 0.2	13.0 ± 1.3**	44.1 ± 2.7
Hesperidin + prednisolone	5 + 0.05	<i>p.o.</i> + <i>s.c.</i>	1.5 ± 0.1	23.0 ± 1.3	50.1 ± 2.1
	5 + 0.2	<i>p.o.</i> + <i>s.c.</i>	1.5 ± 0.1	18.1 ± 1.8*	45.1 ± 3.1
	20 + 0.05	<i>p.o.</i> + <i>s.c.</i>	1.6 ± 0.1	19.2 ± 2.0	46.6 ± 2.1
	20 + 0.2	<i>p.o.</i> + <i>s.c.</i>	1.7 ± 0.1	17.9 ± 2.8*	46.2 ± 3.5

The organs were isolated after the last measurement (24 h after the first PC challenge) of ear thickness. Each value represents the mean ± S.E. of 7–10 mice

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

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

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