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Anti-allergic Components from the Peels of *Citrus unshiu*

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In a bioassay-guided search for anti-allergic compounds from higher plants of Korea, polymethoxyflavones, 3',4',5,6,7,8-hexamethoxyflavone (I), 5-hydroxy-3',4',6,7,8-pentamethoxyflavone (II) and 3',4',5,7,8,-pentamethoxyflavone (III) have been isolated from the immature peels of *Citrus unshiu*. Structures of these compounds were elucidated on the basis of spectroscopic techniques. Compounds **I** and **II** inhibited dose-dependently histamine release from the rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE.

Key words: Anti-allergic effect, *Citrus unshiu*, 3',4',5,6,7,8-Hexamethoxyflavone, 5-Hydroxy-3',4',6,7,8-pentamethoxyflavone, Compound 48/80, Anti-DNP IgE

INTRODUCTION

Citrus unshiu Markovich (Rutaceae) is widely cultivated in Cheju, Korea. The dried peels of this immature fruit have been used to soothe liver and to remove retained food (Namba, 1993). Iinuma *et al.* reported the isolation of several polymethoxyflavones (Iinuma *et al.*, 1980 a, b), and Chun, *et al.*, reported nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone, and tangeretin as anti-allergic principles from Aurantii Fructus Immaturus (Chun and Sankawa, 1989).

In a search for anti-allergic compounds derived from higher plants, the water extract of the peels of *C.unshiu* was found to inhibit histamine release from rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE. And the anti-allergic effect was mainly concentrated in the CH_2Cl_2-soluble fraction.

In this paper, we report the isolation and structure elucidation of three polymethoxyflavones (**I-III**) as well as their anti-allergic activities.

MATERIALS AND METHODS

General experimental procedures

Melting points were obtained on Gallenkamp melting point apparatus (uncorr.). IR spectra were recorded on a Nicolet model 205 FT-IR spectrophotometer. UV spectra were obtained on a Shimadzu UV_{240} UV-Visible recording spectrometer. ^{1}H- and ^{13}C-NMR spectra were determined on a Bruker AMX 400 spectrophotometer. MS was recorded on a VG70-VSEQ instrument. Analytical TLC was carried out on Merck aluminium plates precoated with Si gel 60 F_{254}. Chromatography was performed on Merck Si gel 60 (230-400 mesh) and Sephadex LH 20. LPLC was carried out on Duramat 80 equipped with a Merck Lichroprep Si 60 (240 × 10 mm) column.

Plant material

The fresh immature peels of *Citrus unshiu* were collected at Chejudo, Korea in 1998. A voucher specimen is deposited in the herbarium of College of Pharmacy, Woosuk University (WSU-98-004).

Reagents

Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), O-phthaldialdehyde (OPA), evans blue, α-minimum essential medium (α-MEM) and metrizamide were purchased from Sigma Chemical Co.

Animals

The original stock of Wistar rats were purchased from the Dae Han Experimental Animal Center (Taejeon, Korea) and the rats were maintained at the College of Pharmacy, Woosuk University. The rats were housed five to ten per cage in a laminar air flow room maintained at a temperature of 22 ± 1°C and relative humidity of

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55±10% through out the study.

Preparation of RPMC and histamine determination

RPMC were isolated as previously described (Kanemoto *et al.*,1993). In brief, rats were anesthetized by ether and injected with 20 ml of Tyrode buffer B (NaCl, glucose, $NaHCO_3$, KCl, NaH_2PO_4) containing 0.1% gelatin into the peritoneal cavity and the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened and the fluid containing peritoneal cells was aspirated by a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150×g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e. macrophages and small lymphocytes, according to the method described by Yurt *et al.* (1977). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B were layered on 2 ml of metrizamide (22.5% W/V) and centrifuged at room temperature for 15 min at 400×g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A containing calcium. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Histamine content was measured by the OPA spectroflurometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer (Shimadzu, RF-5301, Japan).

Inhibition of histamine release

Purified mast cells were resuspended in Tyrode buffer A containing calcium for the treatment of compound 48/80. Mast cell suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37 before the addition the compound 48/80 or the challenge with DNP-HSA. The cells were preincubated with the compounds **I**, **II** and **III** preparations and then incubated (10 min) with compound 48/80 or DNP-HSA. The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400×g for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400×g for 5 min at 4°C.

Assay of histamine release

The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{A-B}{A} \times 100$$

A: Histamine release without compounds **I**, **II** and **III**
B: Histamine release with compounds **I**, **II** and **III**

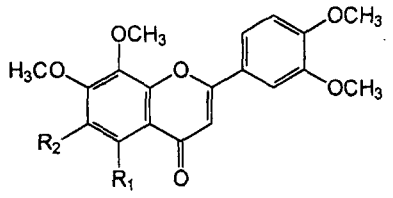
Statistical analysis

The results obtained were expressed as means S.E. The Student's *t*-test was used to make a statistical comparison between the groups. Results with $p<0.05$ were considered statistically significant.

Extraction and isolation

The air-dried plant material (300g) was finely ground and extracted at room temperature with H_2O (2L×3) for 2 weeks. The resulting H_2O extract (45 g) was subjected to successive solvent partitioning to give n-hexane (9 g), CH_2Cl_2 (10 g), n-BuOH (13 g) and water soluble fractions. The anti-allergic CH_2Cl_2 fraction was applied over silica gel using gradient solvent system of EtOAc : MeOH (1:0 → 0:1) as an eluent to give six fractions A-G. Fraction C (180 mg) was applied over silica gel eluted with n-hexane : $CHCl_3$: MeOH (10:10:1) and purified with Sephadex LH-20 column (MeOH) to yield **I** (35 mg). Faction A (100 mg) was chromatographed on silica gel eluted with n-hexane : $CHCl_3$: MeOH (15 : 10 : 1) and purified with Sephadex LH-20 column chromatography (MeOH) to yield **II** (14mg). Fraction G (80 mg) was rechromatographed on LPLC (Lobar A, n-hexane : EtOAc : MeOH, 10 : 10 : 1) and purified with Sephadex LH-20 column (MeOH) to yield **III** (15 mg).

Compound I (3',4',5,6,7,8-hexamethoxyflavone)
colorless needles in MeOH, mp. 136-137°C, UV λ_{max}^{MeOH} nm 245, 266, 330, EIMS : m/z(rel. int.) ; 402 (M^+, 38), 387 (100), 240 (3), 225 (10), 197 (15), 162 (10), ^{1}H-NMR ($CDCl_3$, δ) : 7.57 (1H, dd, *J*=8.3, 2.0 Hz, H-6'), 7.42 (1H, d, *J*=2.0 Hz, H-2'), 7.00 (1H, d, *J*=8.3 Hz, H-5'), 6.63 (1H, s, H-3), 4.12, 4.04, 3.99, 3.97, 3.96, 3.96 (18H, each s, 6×OCH_3), ^{13}C-NMR ($CDCl_3$, δ) : 177.2 (C-4), 160.9 (C-2), 151.8 (C-4'), 151.3 (C-7), 149.1 (C-



	R_1	R_2
I	OCH_3	OCH_3
II	OH	OCH_3
III	OCH_3	H

Fig. 1. Structures of compound **I**, **II** and **III**

3'), 148.3 (C-9), 143.9 (C-5), 137.9 (C-6, 8), 123.8 (C-6'), 119.5 (C-1'), 114.5

$\underline{C}H_3$)

Compound II (5-hydrox,y-3',4',6,7,8-pentamethoxyflavone)

colorless needles in MeOH, mp. 141-143°C, Mg-HCl, Zn-HCl test, $FeCl_3$ positive, EIMS : *m/z* (rel. int.) ; 388 (M^+, 28), 373 (100), 226 (3), 211 (10), 183 (7), ^{1}H-NMR ($CDCl_3$, δ) : 12.55 (1H, s, 5-OH), 7.60 (1H, dd, *J*=8.8, 2.0 Hz, H-6'), 7.43 (1H, d, *J*=2.0, H-2'), 7.01 (1H, d, *J*=8.8, Hz, H-5'), 6.62 (1H, s, H-3), 4.12, 3.99, 3.98, 3.97, 3.95 (15H, each s, $5OCH_3$), ^{13}C-NMR ($CDCl_3$, δ) : 183.0 (C-4), 163.9 (C-2), 153.0 (C-7), 152.5 (C-4'), 149.6 (C-3'), 149.4 (C-9), 145.8 (C-5), 136.6 (C-6), 132.9 (C-8), 123.7 (C-6'), 120.2 (C-1'), 111.3 (C-5'), 108.7 (C-2'), 107.0 (C-10), 104.0 (C-3), 62.1, 61.7, 61.2, 56.1, 56.0 (each $O\underline{C}H_3$)

Compound III (3',4',5,7,8,-pentamethoxyflavone)

colorless needles in MeOH, mp. 196-197°C, UV λ_{max}^{MeOH} (MeOH) 240, 275, 335, EIMS : *m/z* (rel. int.) ; 372 (M^+, 70), 357 (100), 210 (5), 195 (8), 167 (22), ^{1}H-NMR (400 MHz, $CDCl_3$) δ : 7.59 (1H, dd, *J*=8.3, 2.0Hz, H-6'), 7.42 (1H, d, *J*=2.0Hz, H-2'), 6.99 (1H, d, *J*= 8.3Hz, H-5'), 6.62 (1H, s, H-3), 6.44 (1H, s, H-6), 4.01, 3.99, 3.98 (each 3H, s, OCH_3), 3.96 (6H, s, $OCH_3 \times 2$); ^{13}C-NMR(100 MHz, $CDCl_3$) δ : 177.9 (C-4), 160.5 (C-2), 156.5 (C-7), 156.4 (C-9), 152.0 (C-5), 151.8 (C-4'), 149.3 (C-3'), 130.7 (C-8), 124.1 (C-1'), 119.6. (C-6'), 111.2 (C-5'), 109.0 (C-10), 108.6 (C-2'), 107.2 (C-3), 92.5 (C-6), 61.5, 56.6, 56.3, 56.1, 56.0 (each $O\underline{C}H_3$)

RESULTS AND DISCUSSION

The H_2O extract of the peels of *Citrus unshiu* yielded two active compounds, which are traced according to the inhibition of histamine release from the rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE.

Compounds **I**, **II**, and **III** had very similar patterns in their UV and NMR spectra. Compound **I**, mp 136-137°C was obtained as colorless needles. The ^{1}H-NMR spectrum showed four aromatic protons attributable to H-3, H-2', H-5', and H-6' of a flavone skeleton at δ 6.63 (s), 7.42 (d), 7.00 (d) and 7.57 (dd) (Mizuno *et al.*, 1987). In the ^{13}C-NMR spectrum, the six signals of six methoxyl carbons were observed at low magnetic field, suggesting the presence of six methoxyls at both ortho positions (Machida and Osawa, 1989), and these signals were assigned to the methoxyls on the C-3', C-4', C-5, C-6, C-7, and C-8 carbons. Based on these results and values reported in the literature, compound **I** was identified as 3',4',5,6,7,8-hexamethoxyflavone (Iinuma *et al.*, 1980 a, b; Machida and Osawa, 1989).

Compound **II**, mp 143-144°C, was obtained as colorless needles. The ^{1}H- and ^{13}C-NMR spectra of **II** was very similar to those of **I**, suggesting the same skeleton. The main difference was the absence of a methoxyl signal. The ^{1}H-NMR spectrum showed the presence of five methoxyls and one proton singlet at δ 6.62. In the aromatic region of the spectrum, the signals of the remaining three proton signals coincided well with those of **I**, and these were assigned to the B-ring protons at 2',5', and 6' positions, respectively. In the ^{13}C-NMR spectrum, the five signals of five methoxyl carbons were observed at low magnetic field. On the basis of these results, together with comparison of its data with those reported in the literature (Iinuma *et al.*, 1980a, b) compound **II** was well consistent with 5-hydroxy-3',4',6,7,8-pentamethoxyflavone.

Compound **III**, mp 196-197°C was obtained as colorless needles. The ^{1}H-NMR spectrum showed the presence of five methoxyls and two isolated proton singlets at δ 6.44 and 6.62. The B-ring proton signals coincided well with those of **I**. In the ^{13}C-NMR spectrum, five methoxyl carbons were observed at low magnetic field, and the C-6 carbon was observed at δ 92.5 (Iinuma *et al.*, 1980 a, b). Based on these finding, one proton singlet at δ 6.44 was assigned to H-6. Compound **III**, therefore, determined to be 3',4',5,7,8-pentamethoxyflavone (Machida and Osawa, 1989).

We examined the effect of compounds **I**, **II**, and **III** on compound 48/80 or anti-DNP IgE-induced histamine release from RPMC. The inhibitory effects of isolated compounds **I**, **II**, and **III** on compound 48/80-induced or IgE-mediated histamine release from RPMC are shown in Fig. 2 and Fig. 3. Compounds **I** and **II** inhibited concentration-dependently compound 48/80-induced or anti-

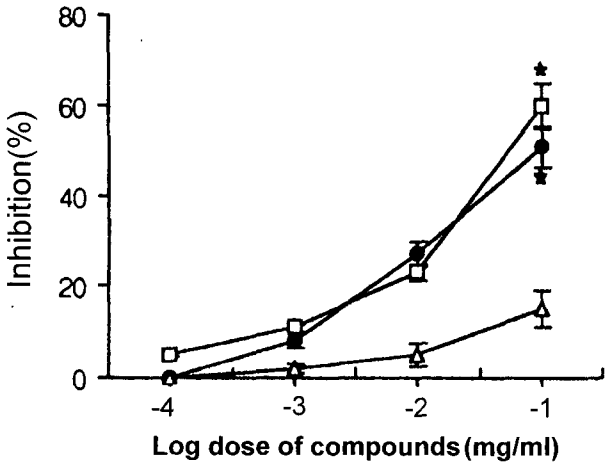


Fig. 2. Effect of compound I (○), II (□) and III (▲) on compound 48/80-induced histamine release from RPMC. The cells (2×10^5 cells/ml) were preincubated with drug at 37°C for 10 min prior to incubation with compound 48/80. *P<0.05; significantly different from the saline value.

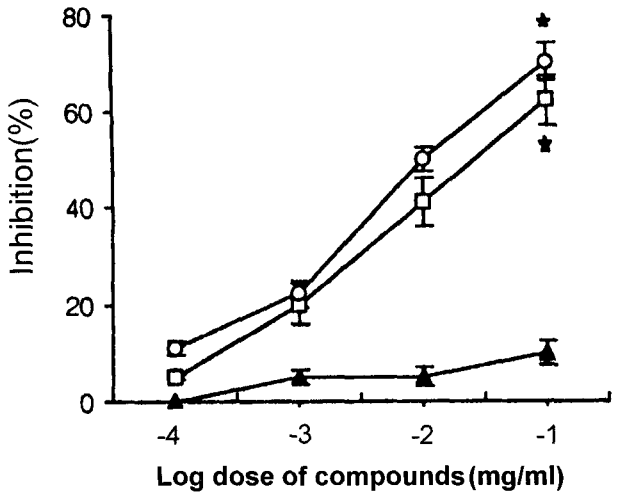


Fig. 3. Effect of compound I (○), II (□) and III (▲) on anti-DNP IgE-mediated histamine release from RPMC. The cells (2×10^5 cells/ml) were preincubated with drug at 37°C for 10 min prior to incubation with each stimulator for 10 min. *P<0.05;significantly different from the saline value.

DNP IgE-mediated histamine release at concentrations of 10^{-4}-10^{-1} mg/ml. The inhibitory effect of compounds **I** and **II** on histamine release was more significant in the case of anti-DNP IgE treatment. Therefore, we simply speculate that this result indicate that anaphylactic degranulation of mast cells is inhibited by compound **I** and **II**. There is no doubt that stimulation of mast cells with compound 48/80 initiates the activation of a signal-transduction pathway that leads to histamine release. Some recent studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G proteins (Mousli *et al.*, 1990a, b). The evidence indicates that the protein is Gi-like and that the activation is inhibited by benzalkonium chloride (Bueb *et al.*, 1990)

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