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Inhibitory Effect of Fulvic Acid Extracted from Canadian Sphagnum Peat on Chemical Mediator Release by RBL-2H3 and KU812 Cells

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Fulvic acid (FA) was extracted and purified from Canadian *Sphagnum* peat (CP-FA) and characterized by using an element analysis meter, Fourier transform infrared (FT-IR) spectroscopy, electron spin resonance (ESR) spectroscopy, and ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectroscopy. To investigate the antiallergic effect of CP-FA, we incubated rat basophilic leukemia (RBL-2H3) cells with 0.001–10.0 μg/ml of CP-FA and determined the β-hexosaminidase release inhibition at different response stages. The intracellular calcium $[Ca^{2+}]_i$ level was also determined by using Fluo 3-AM, a calcium-specific fluorescent probe, and the cytotoxicity of CP-FA was determined by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The results revealed that RBL-2H3 cells incubated for 48 h with 0.001–10.0 μg/ml of CP-FA did not show any decreased viability. CP-FA inhibited the β-hexosaminidase release by IgE-sensitized, antigen-stimulated RBL-2H3 cells at the antigen-antibody binding stage and the antibody-receptor binding stage. CP-FA also inhibited histamine release from A23187 plus PMA- or compound 48/80-stimulated KU812 cells. Furthermore, there was a decrease in the intracellular $[Ca^{2+}]_i$ level in IgE-sensitized cells incubated with CP-FA and stimulated with antigen. Our results show that CP-FA may be useful for the treatment or prevention of allergic diseases.

Key words: antiallergy; β-hexosaminidase; fulvic acid; peat; rat basophilic leukemia (RBL-2H3) cells

Type I allergy is induced by certain types of antigens such as foods, dust, mites, medicines, cosmetics, mold spores, and pollen. This class of antigens induces the production of antigen-specific IgE antibodies that bind to receptors on mast cells or basophils. The early phase reaction in type I allergy occurs within minutes and then mediators such as histamine and serotonin are released from the cell. These mediators induce vasodilation, mucous secretion, and bronchoconstriction. Histamine, which is released from mast cells stimulated by an antigen or degranulation inducers, is usually determined by using a degranulation marker in experiments on the immediate allergic reaction *in vitro*.

RBL-2H3 cells, a tumor analog of mast cells, display characteristics of mucosal-type mast cells and express several hundred thousand IgE receptors on the membrane surface. After sensitization with IgE, the cells respond to the antigen and release histamine. β-Hexosaminidase, which is stored in the secretory granules of mast cells, is released concomitantly with histamine when mast cells are immunologically activated.[1,2] Thus, the β-hexosaminidase activity in the medium is used as a marker of mast cell degranulation.[3] RBL-2H3 cells are therefore considered as a good tool for studying the effect of unknown compounds on histamine release and β-hexosaminidase release activity.

Medically important humic substances such as humic acid, fulvic acid and humin are found abundantly in peat, weathered coal, and other humified sources.[4,5] Humic substances are used in medicine as antimicrobial, anti-inflammatory, and antitumor agents, as liver stimulants, as remedies for gastric ulcers to stop bleeding, and for the treatment of skin burns.[6] Natural humification products have been used to develop pharmacologic agents with diverse applications *in vivo*.[7,8]

Humic substances consist of a mixture of closely

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Abbreviations: FA, fulvic acid; IHSS, International Humic Substances Society; CP, Canadian *Sphagnum* peat; FT-IR, fourier transform infrared; ESR, electron spin resonance; NMR, nuclear magnetic resonance; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; RBL-2H3, rat basophilic leukemia; HA, humic acid; DNP, dinitrophenol; WC, weathered coal; WC-SFA, FA extracted with hot compressed water from weathered-coal-extracted HA; ROS, reactive oxygen species

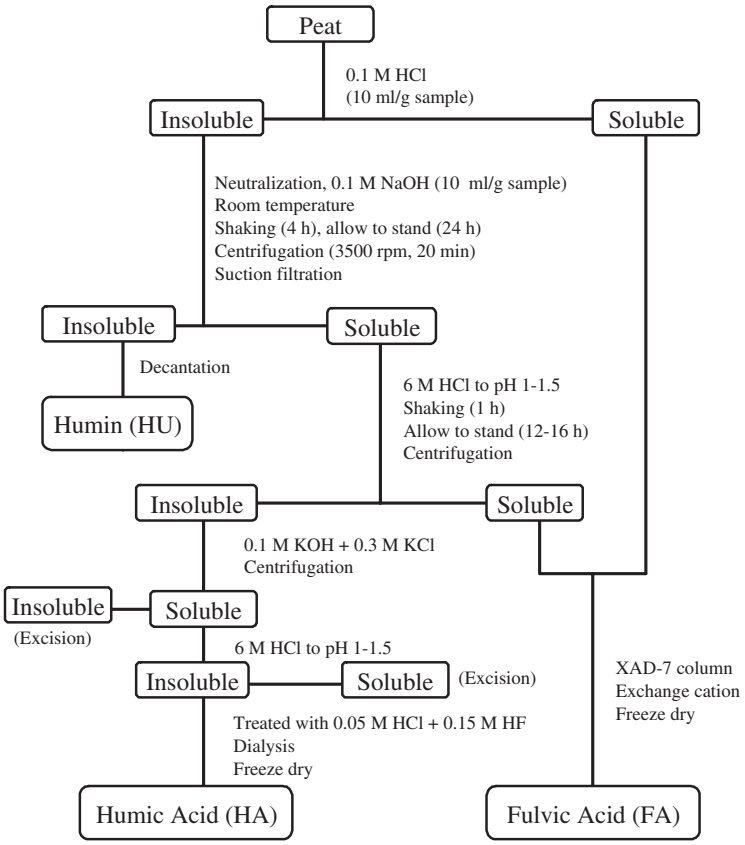

Fig. 1. Method Used to Extract Humic Acid (HA) and Fulvic Acid (FA) from Canadian *Sphagnum* Peat.

related complex aromatic polymers; the exact composition of humic acid (HA) and FA varies according to geographic location. Chemical and spectroscopic analyses have revealed the presence of aromatic rings, and phenolic hydroxyl, ketone carbonyl, quinone carbonyl, carboxyl and alkoxyl groups in HA and FA.[9] The biological effects of humic substances can be different, depending on their chemical structure and physicochemical properties. The chemical composition, structure and functional groups can vary markedly depending on the origin and age of the humic substances and on the conditions of the humification process (humidity, aeration, temperature, mineral microenvironment, *etc.*).[10,11] Furthermore, the characteristics and functional properties of humic substances depend on the extraction method.[12] The physicochemical properties of humic substances isolated from soil, peat, weathered coal and aquatic reservoirs have been investigated.[13–16]

Peat and various peat preparations have been successfully used in the balneological practice of clinical medicine.[17] It is known that FA extracted from peat has antibacterial and anti-inflammatory properties and can boost the immune system.[8] However, the antiallergic effect of FA and its mechanism of action remain undefined. In June 2005, the Ministry of Health and Welfare of Japan designated fulvic acid as a food, and thus, we focused on the effect of CP-FA on type I allergy and investigated its *in vitro* antiallergic activity by using RBL-2H3 cells.

Materials and Methods

Extraction. Sphagnum (moss) peat was commercially available from Fisons Horticulture, Canada. FA was extracted and purified from Canadian *Sphagnum* peat (CP) by the standard method of the International Humic Substances Society (IHSS).[18] Briefly, 1 g of dry peat was stirred for 4 h in 10 ml of 0.1 M NaOH and then allowed to stand for 24 h at room temperature. The residue (humin and other insoluble compounds) was separated from the supernatant by centrifugation (3500 rpm for 20 min). The supernatant was acidified with 6 M HCl to pH 1.0, and after centrifugation, the soluble fraction was passed through a column of XAD-7 resin (Organo Co.). The fraction retained by the XAD-7 resin column was collected with 0.1 M NaOH and then freeze-dried, after desalination by ion-exchange chromatography using AG MP-50 cation exchange resin (Bio-Rad) (Fig. 1). The CP-FA extraction efficiency was calculated by comparing the dry weights of the freeze-dried extract with the raw material.

Elemental analysis. The carbon, hydrogen and nitrogen contents of each sample were determined by using CHN Corder MT-5 element analysis meter (Yanaco

Co.). The oxygen content was calculated by subtracting the C, H, and N contents from 100% and is expressed on a moisture- and ash-free basis (d.a.f.). The ash content of CP-FA was also determined by using the element analysis meter. The percentage purity of CP-FA was calculated from the weight of the ash divided by the weight of the original sample multiplied by 100.

Carboxyl group content. The carboxyl group content was determined by the calcium acetate method.[19] Dried CP-FA (500 mg) was placed in 50 ml of a 0.5 M aqueous calcium acetate solution after a pretreatment with 50 ml of methanol. After 24 h, the filtrate was treated with 0.1 M NaOH at pH 9.8, with phenolphthalein used as an indicator. The carboxyl group content was calculated by subtracting from the result of the blank test.

Phenolic hydroxyl group content. The phenolic hydroxyl group content was determined by using the Folin-Ciocalteu reagent method (Schnitzer and Khan, 1972).[6] Dried CP-FA (2 mg) was dissolved in 10 ml of 0.1 M NaOH and diluted to 100 ml with demineralized water. A mixture of 10 ml of this solution and 10 ml of 20% Na_2CO_3 and 2 ml of the Folin-Ciocalteu reagent was diluted to 25 ml. After allowing the solution to stand for 1 h, its absorbance was measured at 760 nm with a UV-1200 spectrometer (Shimadzu Co.). The phenolic hydroxyl group content was calculated from the difference between the absorbance of the initial solution and that of a standard phenol solution.

Molecular weight. The molecular weight distribution of CP-FA was determined by high-performance size-exclusion chromatography (HPSEC).[20] An HPLC system (Waters 600, Millipore Co.) fitted with UV (280 nm) and IR (400 nm) detectors and a TSK gel G 2000 SW_{XL} + G 3000 SW_{XL} column (30 × 2.0 cm, 7.5 mm, Tohsoh) for this purpose was used. The elution rate was 0.5 ml/min. The gel mobile phases were a 0.05 M phosphate buffer (pH 7.5) plus 0.03 M NaCl plus 30% acetonitrile. Polyethylene glycol (Nacalai Tesque Co.) was used as the molecular weight marker.

FT-IR spectroscopy. A dry sample (1 mg) was mixed with 100 mg of dry potassium bromide (KBr) and pressed into a disk. The infrared spectrum (400–4000 cm^{-1}) was recorded with an FT-IR-3 spectrophotometer (Jasco Co).

ESR. ESR measurements were carried out by a Jeol RE-1X spectrometer (X-band) with 100-kHz field modulation. The ESR spectrum was recorded at room temperature in a Jeol flat quartz cell.

CP/MAS ^{13}C-NMR. Solid-state measurement was performed with an AMX-400 spectrophotometer (Bruker Co.), using the CP/MAS technique at 100.0 MHz. The measurement conditions were as follows: rotor frequency, 3.0 KHz; contact time established, 5 s; multiplication operation frequency, 10,000 times.

Reagents. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo (Japan); dinitrophenylated bovine serum albumin (DNP-BSA) was purchased from Cosmo Biotechnology Co. (Japan); anti-DNP-IgE, compound 48/80, ketotifen, and L-glutamine were purchased from Sigma (Japan). Fetal bovine serum (FBS) was purchased from Hyclone Co. (Japan), and Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co. (Japan). Calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Japan (Tokyo) and dissolved in DMSO at a concentration of 10 mM.

Cells and cell culture. RBL-2H3 cells were purchased from Riken Cell Bank, Japan. The cells were maintained in MEM supplemented with 10% FBS and 2 mM L-glutamine, and incubated at 37 °C in a 5% CO_2 incubator, while KU812 cells were maintained in an RPMI 1640 medium supplemented with 10% FBS at 37 °C in a 5% CO_2 incubator.

MTT assay. The MTT assay is a sensitive and quantitative colorimetric assay that is used to determine cell viability and proliferation.[21] RBL-2H3 cells were harvested at approximately 60–80% confluence and seeded on to 96-well plates at 5.0×10^4 cells per well in 100 µl of medium. After an overnight incubation, 10 µl of a sample was added to obtain final dilutions of 0.001, 0.01, 0.1, 1.0, 10.0, and 100.0 µg/ml with PBS (−). The cells were then incubated for 48 h, before 10 µl of 5 mg/ml of MTT was added. After 24 h of incubation, 100 µl of 10% sodium dodecyl sulfate (SDS) was added, this being followed by another 24 h of incubation to completely dissolve the formazan produced by the cells. The absorbance was then spectrophotometrically determined at 570 nm with a multidetection microplate reader (Power Scan HT, Dainippon Pharmaceutical Co.). Blanks were prepared at the same time to correct for the absorbance caused by sample color and by the inherent ability of a sample to reduce MTT in the absence of cells. The optical density of the formazan produced by the untreated control cells was considered as representing 100% viability.

β-Hexosaminidase inhibition assay at the antigen-antibody binding stage. The β-hexosaminidase release inhibition assay with RBL-2H3 cells was performed according to the method described by Kawasaki[22] with some modifications. A test sample was added at two stages, the IgE sensitization stage and antigen stimulation stage. For the β-hexosaminidase inhibition assay at the antigen-antibody binding stage, RBL-2H3 cells were seeded onto 96-well plates (Falcon Co.) at 5.0×10^4 cells/well in 100 µl of medium. The cells were incu-

bated and sensitized for 24 h at 37 °C with 0.3 μg/ml anti-DNP-IgE. The cells were then washed twice with PBS (−) to eliminate free IgE. After incubating the cells at 37 °C for 10 min in 60 μl per well of a releasing mixture (116.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.6 mM glucose, 25 mM HEPES, 2.0 mM $CaCl_2$ and 1 mg/ml BSA at pH 7.7) containing 5 μl of a test sample (0, 0.001, 0.01, 0.1, 1.0, and 10.0 μg/ml CP-FA), the cells were exposed to 5 μl per well of 4 μg/ml DNP-BSA in PBS (−), before being incubated at 37 °C for 1 h. The plate was put on ice for 10 min, before 20 μl of the supernatant was transferred to another plate; 80 μl of a substrate solution (5 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide in a 50 mM $C_6H_8O_7$ buffer at pH 4.5) was then added to the supernatant with subsequent incubation at 37 °C for 30 min. After adding 200 μl/well of a stop buffer (0.1 M $NaHCO_3$ at pH 10.0), the absorbance at 405 nm was obtained with the Power Scan HT microplate reader. The percentage inhibition of β-hexosaminidase release from RBL-2H3 cells by the test sample was calculated using the following equation:

$$\text{Inhibition } (\%) = \{1 - (To - Bl)/(Co - Bl)\} \times 100$$

Control (*Co*): cell (+), DNP-BSA (+), test sample (−); Test (T*o*): cell (+), DNP-BSA (+), test sample (+); Blank (*Bl*): cell (−), DNP-BSA (+), test sample (+).

β-Hexosaminidase inhibition assay at the antibody-receptor binding stage. To determine the effect of the sample at the antibody-receptor binding stage, the test sample was added to the cells before IgE sensitization. RBL-2H3 cells were seeded on to 96-well plates at 1.0×10^5 cells/well in 50 μl of the medium, and 5 μl/well of a test sample (0.01, 0.1, 1.0, 5.0, and 10.0 μg/ml of CP-FA) was then added, before being incubated at 37 °C for 30 min. Then, 50 μl of the medium with 0.6 μg/ml anti-DNP-IgE was added to each well, before being incubated at 37 °C for 24 h. The cells were washed twice with PBS (−) to eliminate free IgE. After incubating the cells at 37 °C for 10 min in 60 μl/well of the releasing mixture, the cells were exposed to 5 μl/well of 4 μg/ml DNP-BSA in PBS (−), before being incubated at 37 °C for 1 h. As positive and negative controls, 3 mM ketotifen and PBS (−) were used respectively. The plate was put on ice for 10 min, and then 20 μl of the supernatant was transferred to another plate; 80 μl of the substrate solution was then added to the supernatant, before being incubated at 37 °C for 30 min. After adding 200 μl per well of the stop buffer, the absorbance at 405 nm was obtained with the microplate reader. The percentage inhibition of β-hexosaminidase release from RBL-2H3 cells was calculated as described in the previous section.

Determination of the intracellular calcium [Ca^{2+}]i level. [Ca^{2+}]*i* measurement was performed by the method of Aase and Arna[23] with some modifications. Cells (1.5×10^4 cells per well in 100 μl of the medium) were precultured at 37 °C for 24 h in 96-well plates, the medium containing 10% FBS and anti-DNP IgE. The final concentration of IgE was 0.3 μg/ml. The cells were washed twice with 200 μl of PBS (−) to eliminate free IgE. The cells were next incubated with 100 μl per well of a loading buffer containing Fluo3-AM (Calcium Kit-Fluo3, Dojindo Co.) at 37 °C for 1 h. The cells were then washed twice with 200 μl of PBS (−) to eliminate free Fluo3-AM, before being incubated with 100 μl per well of a recording medium (Calcium Kit-Fluo3, Dojindo Co.) at 37 °C for 1 h in a 5.0% CO_2 incubator with the sample (0.001, 0.01, 0.1, 1.0, and 10.0 μg/ml CP-FA). For positive and negative controls, 3 mM ketotifen and sterile water were used respectively. The fluorescence intensity (FI) was determined 30 s after adding 4 μl/ml of the DNP-BSA antigen. FI was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm with the microplate reader.

Histamine release assay. The histamine release inhibition assay using KU812 cells was performed according to the method described by Shin *et al.*[24] with some modifications. KU812 cells were resuspended at 2.0×10^5 cells/well in 200 μl of Tyrode buffer A (30 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.03% bovine serum albumin) for treatment with CP-FA. The cells were next incubated without or with 25 μl/well of various concentrations of a test sample (0.1, 1.0, and 10.0 μg/ml of CP-FA) for 15 min, and then stimulated with A23187 (1 μM) plus PMA (20 nM) or compound 48/80 (10 μg/ml) for 30 min at 37 °C. The reaction was terminated by incubating in ice for 15 min. The resulting cell suspension was centrifuged at 400 × *g* for 5 min at 4 °C. The supernatant (50 μl) was transferred to a 96-well ELISA plate, and the histamine concentration was measured by ELISA according to the manufacturer's instructions. A Histamine EIA kit (Oxford Biomedical Research, USA) was used to detect the histamine released. The absorbance at 650 nm was determined with the microplate reader. The percentage of histamine released from A23187 plus PMA- or compound 48/80-stimulated KU812 cells by the test sample was calculated by using the following equation:

$$\text{Histamine release } (\%) = \{(T - N)/(C - N)\} \times 100$$

Control (C): A23187+PMA (+), test sample (−); Test (T): A23187+PMA (+), test sample (+); Normal (N): A23187+PAM (−), test sample (−).

Statistical analysis. Each result is expressed as the mean ± standard deviation. Statistical comparisons were carried out by Student's *t*-test for paired values.

Results

Extraction and characteristics of CP-FA

Dry Canadian *Sphagnum* peat contained 12.8% HA and 4.8% FA. The main characteristics of CP-FA are shown in Table 1. The elemental composition of CP-FA

Table 1. Main Characteristics of FA Extracted from *Sphagnum* Peat

	Elemental composition (% d.a.f.)
C	47.8
H	4.6
N	0.3
O+S (diff)	47.3
Ash (%)	0.2
Molecular weight	780.0
COOH (mmol/g)	3.98
Ph-OH (mmol/g)	0.75
ESR (spins/g)	7.27×10^{15}

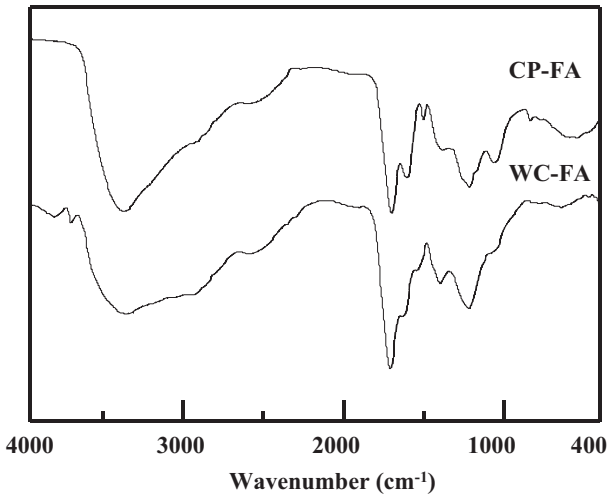

Fig. 2. FT-IR Spectra of Fulvic Acid Extracted from *Sphagnum* Peat and Weathered Coal.

CP-FA, fulvic acid extracted from *Sphagnum* peat; WC-FA, fulvic acid extracted from weathered coal.

was determined to be C, 47.8; H, 4.6; N, 0.3; O, 47.3, and its molecular weight was 780. The purity of CP-FA was calculated to be 99.8%, and the structural formula of CP-FA was calculated to be $C_{31}H_{36}O_{22}N$ from the obtained molecular weight. The carboxyl group content was 3.98 mmol/g, the phenolic hydroxyl group content was 0.75 mmol/g, and ESR was 7.27×10^{15} spins/g.

The infrared spectrum of CP-FA is shown in Fig. 2. The spectrum of FA extracted from weathered coal (WC-FA) is shown for comparison. The spectrum shows strong absorbance at 3400, 2920, 1720, 1620, and 1400–1200 cm^{-1}. These bands were interpreted according to the study by Stevenson and Goh.[25] The 3400 cm^{-1} band is attributed to H-bonded OH groups and was relatively strong and broad for all FA. The 2920 cm^{-1} band was relatively weak and is attributed to aliphatic C–H stretching vibrations, indicating the presence of CH_3 and CH_2 groups. The 1720 cm^{-1} band in FA was strong, and is attributed to C=O stretching vibrations, which is associated with COOH groups. CP-FA and WC-FA had a strong band in this region. The band in the 1660–1600 cm^{-1} region is attributed to aromatic C=C vibrations, strongly H-bonded C=O of quinines, and/or H-bonded and conjugated ketones. CP-FA and WC-FA both showed strong absorption in this region, but the absorption of the latter was stronger than that of the former. The absorption near 1400 cm^{-1} was probably due to C–H deformation of the CH_3 and CH_2 groups, or to OH deformation and C–O stretching of phenolic OH groups. This characteristic band was apparent in the spectra of both CP-FA and WC-FA, and it is suggested that aliphatic C–H of CP-FA was higher than that of WC-FA. The band near 1200 cm^{-1} is attributed to C–O stretching and OH deformation of COOH groups, corresponding to the C=O band near 1720 cm^{-1}. It is suggested that CP-FA and WC-FA may contain aromatic rings, phenolic hydroxyl, ketone carbonyl, quinone carbonyl, carboxyl, and alkoxyl groups, and that they have the same basic chemical structure, although their content is different in each FA.

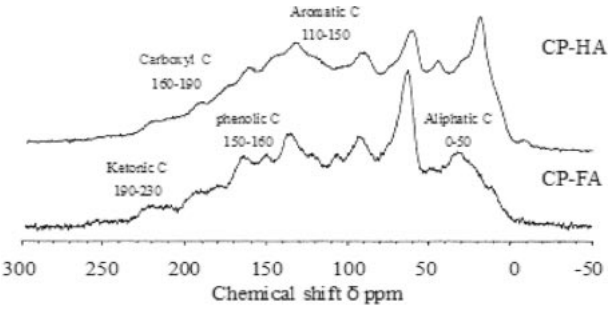

Fig. 3. CP/MAS ^{13}C-NMR Spectra of Humic Acids (HA) and Fulvic Acids (FA).

CP-HA, humic acid extracted from *Sphagnum* peat; CP-FA, fulvic acid extracted from *Sphagnum* peat.

The CP/MAS ^{13}C-NMR spectra of CP-HA and CP-FA are shown in Fig. 3. In some studies, the first two chemical shift zones (0–60 ppm) are combined and labeled as alkyl C; in other cases, the 50–60 ppm zone is assigned exclusively to methoxyl groups. The regions corresponding to 60–110 ppm (C–O resonance) are normally attributed to carbohydrates, but other compounds may also contribute to resonance in this region. The chemical shift zones (110–150 ppm) have been labeled as aromatic C, phenolic C (150–160 ppm), carboxyl C (160–190 ppm), and ketone C=O (190–230 ppm).[26,27] In Fig. 3, the spectrum of the CP-FA fraction is compared with that of CP-HA. The resonance of CP-FA was stronger than that of CP-HA in the 60–90 ppm region; it is suggested that CP-FA contained larger amounts of methoxyl C (6%), carbohydrates (27%) and aromatic C (16%), and lower aliphatic C (29%) and carboxyl C (11%) than CP-HA, but the difference between the two was not significant (Table 2) in the other regions.

Cytotoxicity effect of CP-FA on RBL-2H3 cells

We used the MTT assay to assess the cytotoxicity of CP-FA on RBL-2H3 cells which were treated for 48 h at a final concentration of 0.001, 0.01, 0.1, 1.0, 10.0 and

Table 2. Carbon Distribution Calculated from ^{13}C-NMR Spectra of HA and FA Extracted from *Sphagnum* Peat

	Aliphatic C (0–50 ppm)	N-Alkyl+Methoxy C (60–50 ppm)	Aliphatic C–O (60–110 ppm)	Aromatic C (110–150 ppm) (%)	Phenolic C (150–160 ppm)	Carboxylic C (160–190 ppm)	Ketonic C=O (190–230 ppm)
CP-HA	37	4	18	14	5	14	7
CP-FA	29	6	27	16	5	11	6
WC-FA[13]	23	1	11	20	8	26	12

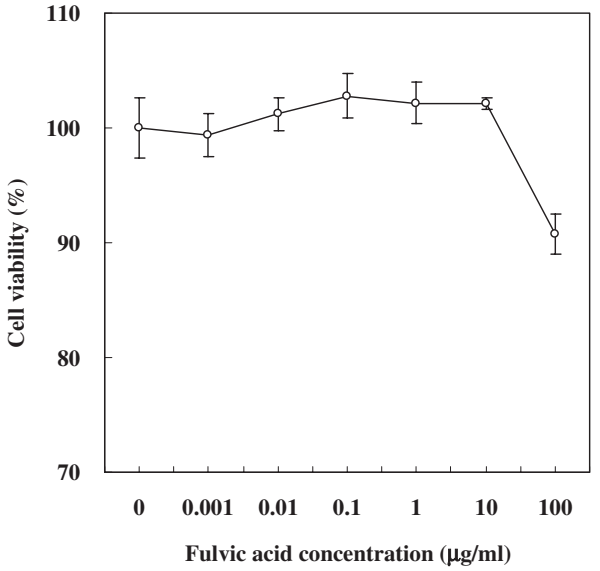

Fig. 4. Cytotoxicity Effect of FA on RBL-2H3 Cells by MTT Assay. The percent cell viability was calculated relative to the untreated control. The cells (5.0×10^4 cells/well) were incubated with CP-FA at 37 °C for 48 h in 5% CO_2.

100.0 μg/ml. The results show that CP-FA in the range 0.001–10.0 μg/ml did not cause any cytotoxicity after 48 h (Fig. 4). The viability of cells treated with 100.0 μg/ml of CP-FA was 90%.

Effect of CP-FA on β-hexosaminidase release at the antigen-antibody binding stage

The β-hexosaminidase release inhibition effect of CP-FA on RBL-2H3 cells is shown in Fig. 5. At 0.001–10.0 μg/ml, CP-FA inhibited β-hexosaminidase release from RBL-2H3 cells, the effect being significant at 0.01–10.0 μg/ml ($p < 0.05$ *vs.* the negative control).

Effect of CP-FA on β-hexosaminidase release at the antibody-receptor binding stage

CP-FA at 0.01–10.0 μg/ml showed a β-hexosaminidase release inhibition effect at the antibody-receptor binding stage (Fig. 6), and at 0.01–5.0 μg/ml, the effect was significant ($p < 0.05$ *vs.* the negative control). We compared the effect of CP-FA with the clinically available antiallergic drug, ketotifen fumarate (Keto.), which is known as a mast cell stabilizer, H1-receptor antagonist and eosinophil inhibitor.[28,29] The results show that the inhibitory effect was lower than that of 3 mM ketotifen (final 214 μM, IC_{50} = 200–300 μM).

Effect of CP-FA on the intracellular $[Ca^{2+}]_i$ level

The intracellular $[Ca^{2+}]_i$ level is increased by an antigen, thereby causing histamine and β-hexosaminidase to be released.[30] To examine the relationship between the β-hexosaminidase release and $[Ca^{2+}]_i$ levels in RBL-2H3 cells, we determined the change in the $[Ca^{2+}]_i$ levels using Fluo3-AM, a calcium-specific fluorescent probe. The results reveal that 30 s after DNP-BSA stimulation, the RBL-2H3 cells showed a decrease in $[Ca^{2+}]_i$ level at each CP-FA concentration (Fig. 7).

Nicola *et al.*[31] have pointed out that FA has a fluorescent absorptive quality and that the fluorescent absorption spectra of various FAs are also different. Therefore, we used a blank for each concentration to correct for the fluorescence absorption spectrum from CP-FA. At 0.001–10.0 μg/ml, no difference in fluorescence from that of the control was apparent.

Inhibitory effect of CP-FA on histamine release from KU812 cells

We used A23187 plus PMA- or compound 48/80-stimulated KU812 cells to confirm the inhibitory effect on the histamine release of CP-FA from human cells. The inhibitory effects of CP-FA on A23187 plus PMA- or compound 48/80-mediated histamine release from KU812 cells are shown in Fig. 8. CP-FA dose-dependently inhibited A23187 plus PMA- or compound 48/80-mediated histamine release in a concentration range of 1.0–10.0 μg/ml, but enhanced histamine release at a concentration of 0.1 μg/ml after stimulation with compound 48/80.

Discussion

Sphagnum peat is a very valuable raw material for the extraction and purification of humic substances because of its high content of such substances.[15,32] The results of this study demonstrated that 0.001–10.0 μg/ml CP-FA is not cytotoxic to RBL-2H3 cells, unlike humic acid which is growth inhibitory to endothelial and fibroblast cells.[33,34] This difference may be attributable to their physicochemical properties and chemical structure. The molecular weights of FAs are lower, and their solubilities are higher than those of HAs. The elemental composition shows, indeed, that CP-FA has relatively higher oxygen and lower nitrogen contents than CP-HA.[14] CP-FA is typically more hydrophilic than CP-HA because it has a higher density of more aliphatic functional groups (Fig. 3 and Table 2). HA can enhance

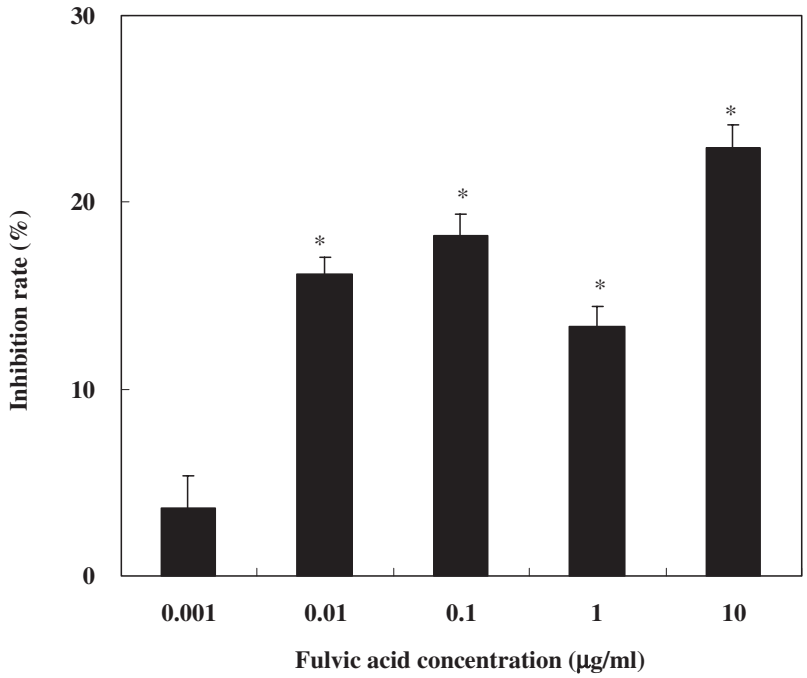

Fig. 5. Inhibitory Effect of Fulvic Acid Extracted from *Sphagnum* Peat (CP-FA) on the β-Hexosaminidase Release from RBL-2H3 Cells at the Antigen-Antibody Binding Stage.

The cells (5.0×10^4 cells/well) were preincubated with CP-FA at 37 °C for 10 min prior to their incubation with DNP-BSA. Results represent one trial (n = 6–12). Two additional trials show similar results. *Significantly different from the negative control (PBS (−)) ($P < 0.05$, Student's *t*-test).

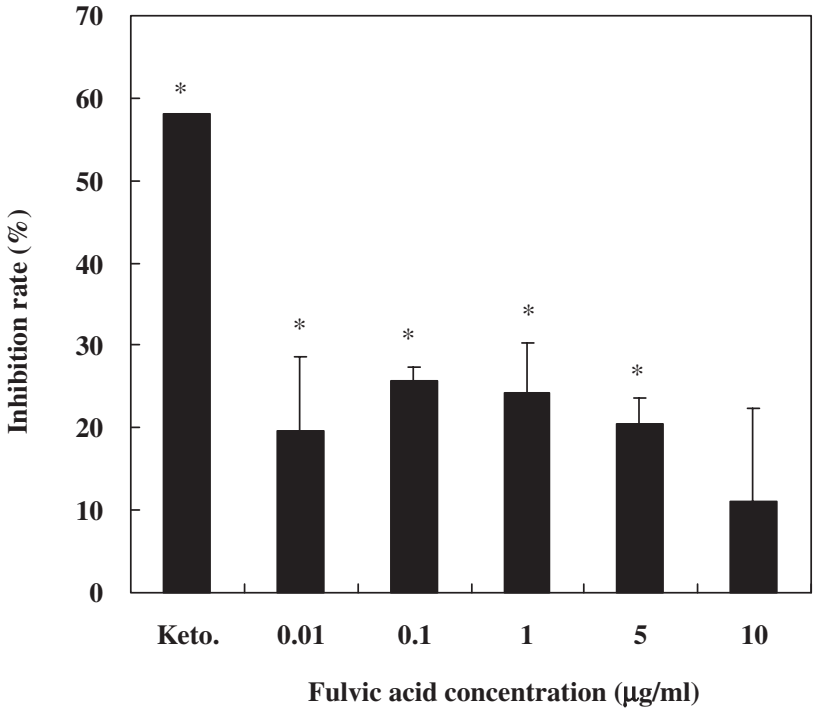

Fig. 6. Inhibitory Effect of Fulvic Acid Extracted from *Spaghnum* Peat (CP-FA) on the β-Hexosaminidase Release from RBL-2H3 Cells at the Antibody-Receptor Binding Stage.

The cells (1.0×10^5 cell/well) were preincubated with CP-FA at 37 °C for 1 h prior to their IgE sensitization, and then preincubated with CP-FA at 37 °C for 24 h prior to their DNP-BSA stimulation. Results represent one trial (n = 6–12). Two additional trials show similar results. *Significantly different from the negative control (PBS (−)) ($P < 0.05$, Student's *t*-test).

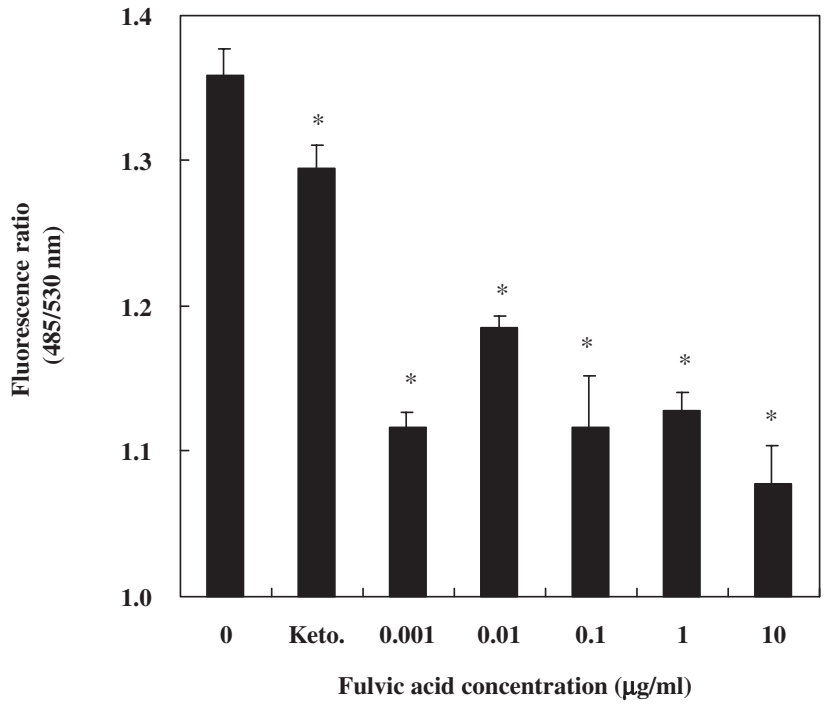

Fig. 7. Effect of Fulvic Acid Extracted from *Sphagnum* Peat (CP-FA) on Intracellular $[Ca^{+2}]_i$ Levels 30 s after Stimulation with the DNP-BSA Antigen.

The cells (1.5×10^4 cells/well) were preincubated with CP-FA at 37 °C for 1 h prior to their incubation with DNP-BSA. Results represent three trials (n = 10–16). Four additional trials show similar results. *Significantly different from the negative control (PBS (−)) ($P < 0.05$, Student's *t*-test).

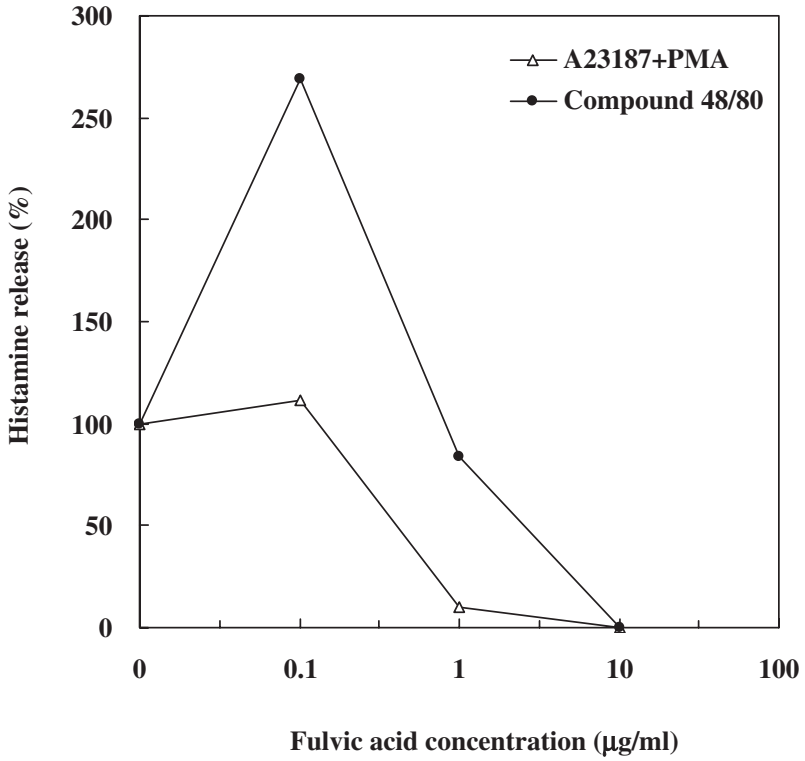

Fig. 8. Effect of Fulvic Acid Extracted from *Sphagnum* Peat (CP-FA) on the A23187+PMA- and Compound 48/80-Induced Histamine Release from KU812 Cells.

The cells (2.0×10^5 cells/well) were preincubated with CP-FA at 37 °C for 15 min prior to their incubation with A23187+PMA or compound 48/80. Results represent two trials.

the cell surface expression of tissue factor activity by permeabilizing the cell membrane to extracellular Ca^{+2}, leading to an elevation of [Ca2+]i that functions as a second messenger to activate protein kinase C (PKC), finally leading to enhanced cell surface tissue factor (TF) expression by cultured endothelial cells.[35] However, it has also been reported that FA adsorption to algal surfaces is strongly dependent on pH; FA has a lower ability to adsorb on cell membranes at $pH > 7$.[36]

Mast cells play a crucial role in inflammatory and immediate allergic responses. Type I immediate hypersensitivity reactions are mediated through the effects of antigen-specific IgE. IgE binds to high-affinity IgE receptors (FcεRI) on human or mouse mast cells and, when cross-linked by an antigen, causes the release of preformed mediators, the release of newly formed lipid-derived mediators, and later production of proinflammatory cytokines. They express FcεRI, a high-affinity IgE receptor, on the cell surface and incorporate cytoplasmic granules containing various potent inflammatory mediators such as histamine, proteases and chemotactic factors.[37,38]

CP-FA was found to have antiallergic properties as shown by its ability to inhibit IgE-mediated local allergic reactions. CP-FA inhibited the β-hexosaminidase release from IgE-sensitized, antigen-stimulated RBL-2H3 cells by blocking antigen-antibody binding and antibody-receptor binding. Moreover, CP-FA decreased the intracellular $[Ca^{2+}]_i$ level after antigen stimulation. Furthermore, CP-FA inhibited the A23187 plus PMA- or compound 48/80-mediated histamine release from KU812 cells at higher concentrations.

Flavonoids are widely distributed in the plant kingdom, and have been recognized to have various biological activities including antiallergic actions. Various *in vitro* and *in vivo* studies on the antiallergy effects of flavonoids have been reported. Matsuda *et al.* have reported that several flavonoids, such as apigenin, luteolin, diosmetin and fisetin, show potent inhibitory activity toward the β-hexosaminidase release from RBL-2H3 cells, and that the 2–3 double bond of flavones and flavonols is essential for this activity. As the hydroxyl groups at the 3′, 4′, 5, 6, and 7 positions increased in number, the inhibitory activity became stronger. Methylation of flavonols at the 3 position reduced the activity.[3] Furthermore, concerning the structure-activity relationship of curcuminoids on antiallergy activity, it can be concluded that the conjugated olefins at the 1–7 positions and the 4′ and 4″ hydroxyl groups of curcuminoids are essential for this activity, whereas the 3′ and 3″ methoxyl groups only enhanced the activity.[39] Matsuda *et al.* have further demonstrated that the conjugated acetoxyl groups at the 4 and 1″ positions of phenylpropanoid are essential for the activity, and that the 2′–3′ double bond of phenylpropanoid enhances the activity.[39]

The physiological effect of CP-FA is considered to be attributable to its chemical composition. FT-IR and ^{13}C-NMR spectra have shown that CP-FA contains aromatic rings, and phenolic hydroxyl, ketone carbonyl, quinone carbonyl, carboxyl, and methoxyl groups. However, CP-FA had higher methoxyl, lower aromatic, and lower carboxyl contents than WC-FA (Table 2).[10] These differences may be attributable to the humification process conditions such as humidity, aeration, temperature, mineral microenvironment, and other factors. Their content and positions in the FA structure may play important roles in the antiallergy activity, including the different mechanisms behind the degranulation inhibition of RBL-2H3 cells. In a similar antiallergy test using WC-SFA, the result showed that the β-hexosaminidase release inhibition effect of WC-SFA on RBL-2H3 cells was lower than that of CP-FA (data not shown). WC-SFA is FA extracted with hot compressed water from weathered-coal-extracted HA. The carboxyl group (6.65 mmol/g) and phenolic OH group (1.26 mmol/g) contents of WC-SFA are higher, and the free radical content (4.37×10^{15} spins/g) is lower than that of CP-FA. Our previous studies on FA extracted from CP and WC have indicated that the methoxy group content of WC-SFA is lower than that of CP-FA.[10,40] These differences appeared to have an influence on their antiallergy effects. This is because CP-FA contains more methoxy carbon and free radicals than WC-SFA; furthermore, the methoxy carbon position of CP-FA may be different from that of WC-SFA. This suggests that quinone groups, hydroxyl groups, and methoxyl groups and their positions in FA play important roles in the antiallergy activity.

The degranulation of mast cells is closely related to $[Ca^{2+}]_i$. An increase in cytosolic calcium level $[Ca^{2+}]_i$ is considered to be an essential and ubiquitous mechanism in the process of mast cell degranulation.[41–43] Our results indicate that the intracellular $[Ca^{2+}]_i$ level was lower in the CP-FA-treated, DNP-BSA-stimulated RBL-2H3 cells (Fig. 7), which is consistent with other reports,[44,45] and these results agree with those of Fig. 5. We consider from these observations that the decrease in intracellular Ca^{2+} is involved in the inhibitory effect of CP-FA on β-hexosaminidase release. Suzuki *et al.*[46] have demonstrated that the IgE-specific receptor signaling of mast cells activates the intracellular oxidative burst involved in the regulation of calcium signals and degranulation. The sustained elevation of cytosolic calcium through store-operated calcium entry was totally abolished when the reactive oxygen species (ROS) production was blocked. We have previously found that FA had scavenging activity.[47] Furthermore, Schnitzer and Khan[6] have reported that FA has a chelating effect on metal ions. It is suggested that the CP-FA-inhibited intracellular calcium release is related to the free radical scavenging activity and calcium ion chelating activity of CP-FA.

To determine whether the CP-FA inhibited the early step of FcεRI signaling, its inhibitory activities against A23187 plus PMA- or compound 48/80-mediated his-

tamine release from KU812 cells were measured. CP-FA showed strong inhibitory activity against A23187 plus PMA- or compound 48/80-mediated histamine release at higher concentrations (Fig. 8). On the other hand, as shown in Fig. 8, the histamine release was enhanced after stimulation by compound 48/80 for treatment with 0.1 μg/ml CP-FA, as has been reported by Senyshyn *et al.*,[48] who have shown that basophilic cells acquire sensitivity to compound 48/80 after a pretreatment with quercetin. However, Fowler *et al.*[49] have demonstrated that a preincubation of quercetin-treated cells with EDTA followed by washing the cells with PBS considerably reduced the effect of compound 48/80 on chemical mediator release. Flavonoids such as quercetin, luteolin, apigenin and kaempferol, which are normally regarded as anti-inflammatory agents, reversibly inhibit the stimulatory signals and release of inflammatory mediators in basophilic cells.[3,50] Moreover, rutin, quercetin, luteolin and apigenin isolated and purified from the olive extract have been shown to have antioxidant activities.[51] Compound 48/80-induced secretion is associated with a transient increase in cytosolic calcium ions. This secretion is blocked by the calcium chelator and PKC inhibitor.[48] Agullo *et al.*[52] have reported that quercetin, luteolin, apigenin, and kaempferol inhibit more or less phosphatidylinositol 3-kinase, tyrosine kinase, and PKC *in vitro* at varying amounts. Our results clearly indicate that CP-FA decreased the intracellular $[Ca^{2+}]_i$ level after stimulation by the anti-IgE antigen, and that CP-FA inhibited the A23187 plus PMA-mediated histamine release by basophilic cells (Fig. 8). It is suggested that the CP-FA derivatives inhibited an earlier and later phase than the intracellular $[Ca^{2+}]_i$ level and PKC activation. This result supports our hypothesis that the CP-FA inhibitory effect on a chemical mediator by basophilic cells is similar to that of flavonoids such as the antiallergy compounds quercetin, luteolin, and apigenin. In addition, CP-FA may have different activities toward the cells at different concentrations; this would be because the macromolecular conformation of FA varies according to such properties as the concentration, composition and pH of the solution.[53] These observations suggest that several stimulation mechanisms are operating simultaneously and intracellularly in response to CP-FA. The inhibitory effect of CP-FA on the chemical mediator release and intracellular [Ca2+]i level may involve FA macromolecular conformations. It will be necessary to clarify the complete mechanism in the future.

The results of our studies suggest that FA extracted and purified from *Sphagnum* peat inhibited the β-hexosaminidase release and intracellular $[Ca^{2+}]_i$ influx by IgE-sensitized, antigen-stimulated RBL-2H3 cells. CP-FA also inhibited the histamine release from A23187 plus PMA- or compound 48/80-stimulated KU812 cells. The detailed mechanism behind the antiallergy affect of CP-FA is the subject of a future study.

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