Anti-allergic activity of the *Morinda citrifolia* extract and its constituents

Kazuya Murata, Yumi Abe[1], Kaito Shinohara, Megumi Futamura-Masuda, Akemi Uwaya[1], Fumiyuki Isami[1], Hideaki Matsuda

Faculty of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502, [1]Research and Development, Morinda Worldwide, Inc., 3-2-2 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

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ABSTRACT

Background: *Morinda citrifolia* (Rubiaceae), commonly known as noni is distributed throughout tropical and sub-tropical regions of the world. Anti-allergic effects of noni have not been reported despite the clinical usage as an anti-allergic agent. **Materials and Methods:** To investigate the anti-allergic effects of the 50% ethanolic extract of *M. citrifolia* fruits and leaves (MCF-ext and MCL-ext), dinitrofluorobenzene (DNFB)-induced triphasic cutaneous reaction and picryl chloride-induced contact dermatitis (PC-CD) tests were performed. **Results:** In DNFB-induced triphasic cutaneous reaction, oral administration of MCF-ext and MCL-ext exhibited dose-dependent inhibition of cutaneous reaction at 1 h (immediate phase response) after the DNFB challenge. MCF-ext also inhibited ear swelling at 24 h (late phase response) and 8 days (very late phase response) after the DNFB challenge. The effect of MCL-ext on the immediate phase response was attributed to the anti-degranulation from RBL-2H3 cells, while MCF-ext had no significant effect on degranulation. The active components of anti-degranulation activity in MCL-ext were determined to be ursolic acid, rutin and kaempferol-3-*O*-α-L-rhamnopyranosyl-(1$\rightarrow$6)-β-D-glucopyranoside. In the PC-CD test, both MCF-ext and MCL-ext showed an anti-swelling effect but the potency of MCF-ext was stronger than MCL-ext. **Conclusion:** These data suggest that noni fruits and leaves can be a daily consumable material for the prevention of allergic symptoms.

Key words: Allergy, contact dermatitis, degranulation, IgE-mediated triphasic cutaneous reaction, *Morinda citrifolia*

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INTRODUCTION

Morinda citrifolia (Rubiaceae), commonly known as noni, is distributed throughout the tropical and sub-tropical regions of the world such as French Polynesia and Hawaii. It has been used to treat various symptoms in these regions[1,2] and there is much interest in its various physiological effects. These effects include potential anti-hypertensive,[3] hypoglycemic[4] and anti-dementia[5] effects. Furthermore, noni was traditionally used to relieve allergic symptoms such as bronchitis, asthma and pruritus.[2]

The number of individuals with allergies including atopic dermatitis, allergic rhinitis, asthma and so on is rapidly increasing worldwide.[6-8] Definitive treatment has not been established, leaving only symptomatic therapy to ameliorate symptoms.[7,8] Safe, effective and convenient supplements or functional foods are in high demand given that allergy medications may cause side effects.[7,8]

Allergies are disorder of the immune system. Most allergies are result of a dramatic reaction against pollens, house dust and other chemical substances that act as antigens.[9,10] An antigen induces the production of specific immunoglobulin E (IgE) antibodies, which interact with receptors on the mast cell membrane surface.[8,10] Once mast cells are activated by the binding of IgE, degranulation occurs, which leads to the release of inflammatory mediators. This reaction is known as "immediate-type hypersensitivity (ITH)".[11,12] Another common allergic reaction is "delayed-type hypersensitivity (DTH)," which is mediated by the response of monocytes and lymphocytes to antigens.[12] By blocking both pathways, allergic reactions could be significantly suppressed.

Address for correspondence:
Prof. Hideaki Matsuda, Faculty of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502, Japan.
E-mail: matsuda@phar.kindai.ac.jp

From our investigation of the traditional use of noni, we have encountered that noni had been used to cure itchiness and inflammation which may relate to some allergic symptoms. This prompted us to investigate the anti-allergic activities of noni. Here we investigated the anti-allergic effects of noni in two pathways: ITH by the dinitrofluorobenzene (DNFB)-induced triphasic cutaneous reaction test and DTH by utilizing the picryl chloride-induced contact dermatitis (PC-CD) test. Degranulation inhibitory activities were also examined by secretion of β-hexosaminidase from RBL-2H3 cells.

MATERIALS AND METHODS

Plant materials

Fruits and leaves of *M. citrifolia* were collected in French Polynesia during 2004 to 2006 and identified by Tropical Resources, Inc. (Provo, UT). The fruits were separated into flesh and seeds by hand. The flesh was then freeze-dried and the leaves were air-dried. Voucher specimens of dried fruit flesh (Noni: Lot Code. 2555900), leaves (Noni leaf: Lot Code. 8281M5/G13) were deposited at Kinki University.

Reagents

Ursolic acid (1), rutin (2) and kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (3) were isolated from MCL-ext according to the method described previously.[13] Dinitrophenyl (DNP)-labeled human serum albumin (HSA), monoclonal anti-DNP (mouse IgE isotype, IgE anti-DNP) and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were purchased from Sigma-Aldrich (St. Louis, MO). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Nacalai Tesque, Inc. (Kyoto, Japan), unless otherwise noted.

Preparation of 50% ethanolic extracts and fractionation

Preparation of the 50% ethanolic extracts of fruit flesh (MCF-ext, yield 53%) and leaves (MCL-ext, yield 36%) were performed according to the method described previously.[14] The MCL-ext (100 g) was suspended in water (300 ml) and was extracted successively with *n*-hexane (200 ml × 2), ethyl acetate (EtOAc, 200 ml × 8) and *n*-butanol (BuOH, 200 ml × 8). Evaporation of the solvent gave a hexane-soluble fraction (2.0 g), an EtOAc-soluble fraction (8.6 g), a BuOH-soluble fraction (41.6 g) and a water-soluble fraction (42.8 g).[13]

Cell culture

The rat basophilic leukemia RBL-2H3 cells were purchased from Japan Health Sciences Foundation (Osaka, Japan) in November 2012. The RBL-2H3 cells were cultured in Eagle's minimum essential medium (E-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Nichirei Biosciences Inc., Tokyo, Japan) and 1% Pen Strep (a mixture of 10,000 U/ml penicillin and 10,000 μg/ml streptomycin; Life Technologies Japan Ltd., Tokyo, Japan) at 37°C in a humidified, CO_2-controlled (5%) incubator.

Animals

Female BALB/c mice (5 weeks of age; 14 to 19 g) and female ICR mice (6 weeks of age; 25 to 27g) were purchased from Shimizu Laboratory Supply (Kyoto, Japan). They were maintained in an air-conditioned room with lighting from 07:00 to 19:00. The room temperature (about 23°C) and humidity (about 60%) were controlled automatically. Laboratory pellet chow (Labo MR Stock, Nihon Nosan Kogyo Co. Ltd., Tokyo, Japan) and water were freely available. The body weights were measured every 2 days and the body weight changes were confirmed during the experiment. All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University (Protocol No. KAPS-24-006).

IgE-mediated triphasic cutaneous reaction test

IgE-mediated triphasic cutaneous reaction by DNFB was induced according to the methods of Yamaguchi *et al.*,[15] and Tahara *et al.*,[16] with minor modifications. Female BALB/c mice (7-9 mice/group) were passively sensitized with intravenous injection of 10 μg of IgE anti-DNP dissolved in saline (0.9% w/v of NaCl and water) (day 1). 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) to each side of the right and left ears. The test sample and prednisolone (reference drug) were suspended in 0.2% carboxymethylcellulose sodium (CMC-Na) solution and administered orally (0.2 ml/10 g body weight of mouse/day) 1 h before DNFB challenge (day 2), 23 h after DNFB challenge (day 3) and days 4 to 10 (for 7 days). Control mice were administered 0.2% CMC-Na solution (0.2 ml/10 g body weight of mouse/day). Ear thickness 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. Ear swelling (cutaneous reaction) was expressed as the difference in 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 (S.E.).

The inhibition (%) of increase in ear thickness value was calculated by the following formula: Inhibition (%) = (control group - sample group/control group) ×100. After

the last measurement of ear thickness (8 days after the challenge), the mice were sacrificed by cervical vertebrae dislocation and the adrenal glands, thymuses and spleens were harvested. The weights of the organs were measured and expressed as a ratio of organ weight to 10 g body weight of mice.

β-Hexosaminidase release assay

Inhibitory effect on the release of β-hexosaminidase from RBL-2H3 cells was evaluated according to the methods of Murata *et al*.[17] Briefly, RBL-2H3 cells were plated at a density of 2×10^5 cells/well in 24-well plates. After 24 h of incubation at 37°C, 5% CO_2, 100 μl of IgE anti-DNP antibody (final concentration: 0.45 μg/ml) was added for sensitization and incubated for 24 h at 37°C, 5% CO_2. The cells were then washed twice with 500 μl of siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH, pH 7.2) followed by180 μl of siraganian (+) buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH, 5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA, pH 7.2). Test samples (20 μl) and the reference drug baicalein dissolved in dimethyl sulfoxide (DMSO): Siraganian (+) buffer (1:100) were then added to each well. After incubation at 37°C, 5% CO_2 for 30 min, 50 μl of DNP-labeled HSA (final concentration: 0.01 μg/ml in siraganian (+) buffer) was added to induce degranulation and incubated for 30 min at 37°C, 5% CO_2. A portion of the supernatant (50 μl) was transferred to a 96-well plate and 50 μl of β-hexosaminidase substrate, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (1 mM) dissolved in citrate buffer (100 mM citric acid, pH 4.6 to 5.0) was added. After incubation at 37°C, 5% CO_2 for 1 h, the reaction was stopped by adding 100 μl of stop buffer (50 mM $NaHCO_3$, 50 mM Na_2CO_3, pH 10.0). Finally, the absorbance at 405 nm was measured using a microplate reader (Rainbow, Tecan, Männedorf, Switzerland). The inhibition of release of β-hexosaminidase was calculated by the following formula: Inhibition (%) = $[1-(T-B_T)/(C-B_C)] \times 100$. Control (C): cell (+), 0.1% DMSO (+); test (T): cell (+), test sample in 0.1% DMSO (+); control blank (B_C): cell (−), 0.1% DMSO (+); test blank (B_T): cell (−), test sample in 0.1% DMSO (+).

Cell viability assay

Cell viability was determined by the cleavage of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfpphenyl)-2*H*-tetrazolium salt (WST-8) using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Test samples were dissolved in DMSO then diluted with E-MEM to an appropriate concentration. In the control group, 1% (v/v) DMSO/E-MEM solution was used instead of sample solution. The final concentration of DMSO was 0.1% (v/v). 2×10^3 cells were placed into a 96-well plate. After 48 h of incubation at 37°C, 5% CO_2, several concentrations of samples were added. After incubation for 1 h, the medium was replaced with a WST solution (WST: culture medium (1:10, 100 μl)). After incubation for 1 h, the resulting formazan was photometrically determined from absorption at 450 nm with a microplate reader. Cell viability was expressed as a percentage of the control group.

Picryl chloride-induced contact dermatitis test

The test was performed according to the method described by Asherson and Ptak[18] with modifications. Female ICR mice (7-9 mice/group) were sensitized by topical application of 0.1 ml of 7% picryl chloride (PC) solution in EtOH to the shaved abdomen (day 1). After the sensitization, test sample was suspended in 0.2% CMC-Na solution and administered orally (0.2 ml/10 g body weight of mouse/day) from days 1 to 7. The control group was treated with 0.2% CMC-Na solution. The reference drug prednisolone was suspended in saline and administered subcutaneously (0.2 ml/10 g body weight of mouse/day) from days 2 to 7. On the next day (day 8), the 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 ear swelling was expressed as the difference in the ear thickness between immediately before and 24 h after the 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 ± S.E. The inhibition of increase in ear thickness value was calculated by the following formula: Inhibition (%) = (control group − sample group/control group) ×100.

After the last measurement of ear thickness (24 h after the PC challenge), the mice were sacrificed by cervical vertebrae dislocation and their adrenal glands, thymuses and spleens were harvested. The weights of the organs were measured and expressed in a ratio of organ weight to 10 g body weight of mice.

Statistical analysis

Intergroup differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's or Steel's multiple comparison test with Statcel3 software (OMS Publishing Inc., Saitama, Japan, 2011).

RESULTS AND DISCUSSION

Effect of noni-ext on IgE-mediated triphasic cutaneous reaction induced by DNFB

The effect of oral administration of MCF-ext or MCL-ext on DNFB-induced triphasic cutaneous reaction was examined by ear swelling in mice passively sensitized with

IgE anti-DNP. Ear thickness was measured immediately before DNFB challenge and at 1 h, 24 h, and 8 days after DNFB challenge. One hour after the challenge (IPR), MCL-ext at 200, 500, and 1000 mg/kg (p.o.) inhibited ear swelling by 31, 48, and 63% respectively. In the same case, MCF-ext at 500 and 1000 mg/kg (p. o.) showed 21 and 34% inhibition, respectively [Figure 1a]. While administration of MCF-ext at 500 and 1000 mg/kg (p. o.) inhibited ear swelling by 36 and 48%, respectively at 24 h after challenge (LPR), MCL-ext at 1000 mg/kg (p. o.) demonstrated weak inhibition of 28% [Figure 1b]. At 8 days after challenge (vLPR), MCF-ext at 500 and 100 mg/kg (p. o.) showed 17 and 28% inhibition, respectively while no effect was observed in the MCL-ext administered group [Figure 1c].

Triphasic cutaneous reactions (IPR, LPR and vLRP) were observed in mice sensitized with IgE anti-DNP after DNFB challenge.[19] IPR is caused by inflammatory mediators secreted upon mast cell degranulation.[19] LPR is said to be maximized at 24 h after challenge and is attributed to inflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). This reaction is independent of mast cells.[19] vLPR is maximized 8 days after the challenge and is characterized by an eosinophil infiltration.[19] Eosinophil infiltration is common among most atopic dermatitis patients which is correlated with the development of a rash.[20] Therefore, vLPR was studied as an atopic dermatitis model.[16]

Administration of MCF-ext and MCL-ext inhibited ear swelling at IPR. These results suggested that MCF-ext and MCL-ext might inhibit mast cell activation or histamine release or other inflammatory substances. In addition, mice fed with MCF-ext showed inhibition not only in IPR but also in LPR and vLPR. It raised the possibility that noni would be an anti-allergic agent against DTH or atopic dermatitis. Their safety was confirmed since MCF-ext and MCL-ext treated mice maintained healthy body and organ weights (data not shown). Our data suggested that MCF-ext and MCL-ext have anti-allergic properties with few side effects.

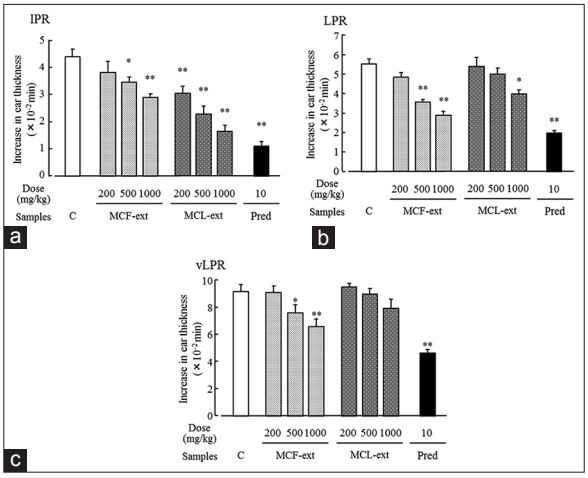

Figure 1: Effects of MCF-ext, MCL-ext and prednisolone on IgE-mediated triphasic cutaneous reaction induced by DNFB. Mice were passively sensitized by IgE anti-DNP before the DNFB challenge and each test sample was administered. Ear thickness was measured immediately before the DNFB challenge and (a) 1 h (IPR), (b) 24 h (LPR) and (c) 8 days (vLPR) after the DNFB challenge. Each value represents the mean ± S.E. of 7-9 mice. Significantly different from the control group: **P*< 0.05, ***P*< 0.01. Pred = prednisolone

Inhibitory effect of noni-ext on degranulation of RBL-2H3 cells

Inhibition of degranulation by MCF-ext and MCL-ext was examined by a β-hexosaminidase release assay with RBL-2H3 cells. MCL-ext at 200 and 500 μg/ml showed 50 and 62% inhibitory activity, respectively without cytotoxicity. MCF-ext at 500 μg/ml, on the other hand, showed weak inhibitory activity of 18% [Table 1]. These results suggested that MCL-ext inhibited IPR through inactivation of degranulation. The inhibitory activity of MCF-ext against IPR was not due to degranulation inhibition but possibly through inhibition of prostaglandin production (another type of inflammatory mediator) as previously reported.[21,22]

Inhibitory effects of ursolic acid (1), rutin (2) and kaempferol-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (3) on degranulation of RBL-2H3 cells

We next performed purification of the active compounds from MCL-ext since MCL-ext was more potent than MCF-ext. Initially, MCL-ext was divided into four fractions, including a hexane-soluble fraction (yield from MCL-ext; 2%), an EtOAc-soluble fraction (9%), a BuOH-soluble fraction (42%) and a water-soluble fraction (43%). The EtOAc-soluble and BuOH-soluble fractions (both at 50 μg/ml) showed 63 and 53% inhibition without cytotoxicity [Table 2]. In our previous report,[13] we identified 1 (isolation yield from MCL-ext;

Table 1: Effects of MCF-ext, MCL-ext and baicalein on degranulation and cell proliferation in RBL-2H3 cells

Samples	Concentration (μg/ml)	Absorbance	Inhibition (%)	Cell proliferation (%)
Control	-	0.632±0.021	-	100.0±1.5
MCF-ext	50	0.547±0.050	13	105.9±2.5
	200	0.538±0.041	15	102.0±1.5
	500	0.520±0.041*	18	106.8±2.0
MCL-ext	50	0.643±0.011	−2	105.1±3.3
	200	0.318±0.017**	50	104.4±0.7
	500	0.243±0.006**	62	103.8±1.4
Baicalein	25 (μM)	0.180±0.005**	72	114.9±4.1**
	50	0.135±0.003**	79	104.1±1.8

Each value represents the mean±S.E. of 6 experiments. Significantly different from the control group: **P*<0.05, ***P*<0.01; MCF: *M. citrifolia* fruits; MCL: *M. citrifolia* leaves

Table 2: Effects of hexane-, EtOAc-, BuOH- and water-soluble fraction from MCL-ext and baicalein on degranulation and cell proliferation in RBL-2H3 cells

Samples	Concentration (μg/ml)	Absorbance	Inhibition (%)	Cell proliferation (%)
Control	-	0.530±0.074	-	100.0±2.8
Hexane fr.	50	0.310±0.015**	42	100.8±3.4
EtOAc fr.	50	0.199±0.002**	63	104.1±2.4
BuOH fr.	50	0.250±0.012**	53	99.3±2.2
Water fr.	50	0.284±0.015**	47	101.1±3.0
Baicalein	25 (μM)	0.266±0.004**	50	100.1±4.5
	50	0.183±0.007**	65	119.0±3.5**

Each value represents the mean±S.E. of 6 experiments. Significantly different from the control group: **P<0.01, MCL: *M. citrifolia* leaves; EtOAc: Ethyl acetate; BUOH: Butanol

0.4%, isolation yield from dried leaf; 0.15%) from the EtOAc-soluble fraction and 2 (0.27%, 0.1%) and 3 (1.24%, 0.45%) from the BuOH-soluble fraction. We examined the inhibitory activity of these three compounds. As seen in Table 3, 1 at 5 and 10 μM showed 14 and 30% inhibition, respectively. Compound 2 at 0.3, 0.5 and 1 μM showed 39, 45 and 49% inhibition, respectively. Compound 3 at 2.5, 5 and 10 μM showed 44, 47 and 48% inhibition, respectively. This is the first report that compound 3 has anti-degranulation effects while the anti-degranulation activities of the other two compounds have already been reported.[23-25] In spite of former reports on anti-degranulation, the anti-allergic effects of 1 and 2 have not been reported as well as 3. In light of their potency and contents, these three compounds can be regarded as the main contributors of the anti-degranulation effects of MCL-ext.

Effect of noni-ext on PC-CD

We next investigated the anti-DTH activity of both MCF-ext and MCL-ext using the PC-CD model. As shown in Figure 2a, administration of MCF-ext at 500 and 1000 mg/kg, for 7 days (p. o.) inhibited ear swelling at a rate of 23 and 54%, respectively, during the induction phase of PC-CD in mice. MCL-ext at 500 and 1000 mg/kg had weaker activities but still inhibited ear swelling by 16 and 29%, respectively [Figure 2b]. No significant changes were observed in organ and body weights (data not shown). These results demonstrated that MCL-ext and MCF-ext have anti-allergic properties in DTH and can be potent material which alleviates allergic symptoms.

CONCLUSION

Using the DNFB-induced triphasic cutaneous reaction method, we demonstrated that MCL-ext was effective

Table 3: Effects of compounds 1, 2, 3 and baicalein on degranulation and cell proliferation in RBL-2H3 cells

Samples	Concentration (μM)	Absorbance	Inhibition (%)	Cell proliferation (%)
Run 1				
Control	-	0.534±0.015	-	100.0±2.6
1	2.5	0.503±0.011	6	97.6±3.4
	5.0	0.461±0.016**	14	96.2±3.6
	10.0	0.372±0.013**	30	107.1±2.6
Baicalein	25.0	0.259±0.017**	52	101.5±1.2
	50.0	0.131±0.005**	76	101.9±1.4
Run 2				
Control	-	0.500±0.015	-	100.0±2.6
2	0.3	0.303±0.012**	39	106.3±2.8
	0.5	0.275±0.013**	45	106.7±1.8
	1.0	0.254±0.005**	49	103.6±0.9
Baicalein	25.0	0.193±0.005**	61	101.5±1.2
	50.0	0.075±0.005**	85	101.9±1.4
Run 3				
Control	-	0.525±0.042	-	100.0±2.6
3	2.5	0.292±0.024**	44	106.8±2.4
	5.0	0.278±0.025**	47	98.1±2.2
	10.0	0.271±0.031**	48	100.9±1.5
Baicalein	25.0	0.229±0.018**	56	101.5±1.2
	50.0	0.156±0.008**	70	101.9±1.4

Each value represents the mean±S.E. of 6 experiments. Significantly different from the control group: **P<0.01

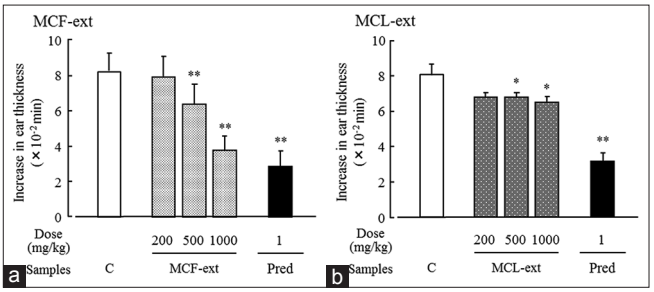

Figure 2: Effects of MCF-ext, MCL-ext and prednisolone on PC-CD test. Ear thickness was measured immediately before and 24 h after the PC challenge along with treatment with (a) MCF-ext or (b) MCL-ext. Each value represents the mean ± S.E. of 7-9 mice. Significantly different from the control group: *P< 0.05, **P< 0.01. Pred = prednisolone

in ITH. Using the β-hexosaminidase release assay, we also showed that inhibiting degranulation was the main mechanism of action. On the other hand, MCF-ext had no impact on degranulation *in vitro*. However, *in vivo* MCF-ext inhibited ear swelling in the ITH and DTH models, similar to atopic dermatitis. Oral administration of MCF-ext and MCL-ext did not cause any negative side effects, including changes in body and organ weights.

Taken together, anti-allergic properties were observed in extracts from noni fruits and leaves. The efficacy was not as strong as pharmaceutical products, but noni fruits and leaves are a more practical approach for treatment because they are already traditionally consumed as food.

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