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ORIGINAL ARTICLE

Anti-inflammatory effects of flavonoids in *Citrus jabara* fruit peels

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ABSTRACT

Currently, about half of people in Japan suffer from allergic diseases. Thus, *Citrus jabara* fruits have been paid attention as one of quite effective anti-allergic functional foods. *C. jabara* is an endemic species originally grown only in Kitayama village, Wakayama prefecture in Japan. Although genetic characterization and diversity of various *Citrus* fruits including *C. jabara* were researched, but there is room for the study on flavonoids characteristics in *C. jabara* fruit. For the alleviation of allergic symptom, anti-inflammatory effects are also important. In this study, characteristics of flavonoids in *C. jabara* fruit peels, and the anti-inflammatory effects of these purified flavonoids were investigated. Our results revealed that *C. jabara* is a unique *Citrus* that almost all of flavonoids in fruit peels was narirutin. There was no *Citrus* species with a flavanone glycosides content ratio like *C. jabara*. Although anti-inflammatory effects of narirutin was weak, but its aglycone naringenin exhibited following inhibitory effects: nitric oxide synthesis (IC_{50} = 105 μM), nitric oxide synthase induction, Interleukin-6 synthesis (IC_{50} = 65 μM), and inducible soluble epoxide hydrolase activity (IC_{50} = 267 μM). Since narirutin is deglycosylated to naringenin that is then absorbed by colonocytes, it is considered that narirutin exists like a prodrug and its aglycone naringenin works as an active form of anti-inflammatory effect in a living body at oral ingestion of *C. jabara* fruit peels.

Keywords: *Citrus jabara; anti-inflammatory effects; flavonoids; narirutin; naringenin*

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Introduction

Currently, about half of people in Japan suffer from allergic diseases such as pollen allergy, perennial rhinitis, and atopic dermatitis. So, it is figuratively said that allergic diseases are the current national disease in Japan. Under such condition, the functional foods to alleviate allergic symptoms, such as *Citrus unshiu* unripe fruit, were focused[1,2].

In recent years, *Citrus jabara* fruits has also been paid attention as one of quite effective anti-allergic functional foods. *C. jabara* is an endemic species originally grown only in Kitayama village, Wakayama prefecture in Japan, and is estimated as a natural hybrid species of *Citrus junos*, *Citrus reticulata*, and *Citrus kinokuni*[3]. *C. jabara* was recognized as a new variety of citrus by Dr. Tanaka in 1971, and then registered as a new nursery plant in 1979 by Mr. Fukuda (Japan the plant variety protection NO. 10). Concerning with anti-allergic activities of *C. jabara* fruits, the anti-degranulation activity[3], the improving effect of its juice on symptoms and quality of life in patients with Japanese cedar pollinosis[4], and the suppressive effect of its extracts on mucin production in human lung epithelial cells[5] were reported. Although genetic characterization and diversity of various citrus fruits including *C. jabara* were researched [6–8], but there is room for the study on flavonoids characteristics in *C. jabara* fruit. For the alleviation of allergic symptom, both anti-allergic effect and anti-inflammatory effects are important. Thus, in this study, we investigated the component analysis of flavonoids in *C. jabara* fruit peels and the anti-inflammatory effects of these isolated flavonoids.

Materials and Methods

Extract and isolation

C. jabara fruits were harvested in Kitayama village, Wakayama prefecture in Japan and on 3 November 2010. The residue of *C. jabara* fruits by commercial roller juice squeezer was used as *C. jabara* fruit peels. After drying, these peels were chopped and sealed in aluminum puck until use. Extract and isolation were carried out in 2010.

The dried of *C. jabara* fruit peels (1 Kg) were chopped into small pieces and extracted with *n*-hexane (3 L x 5), EtOAc (3 L x 3) and acetone (3 L x 3) using a ultrasonic extraction. The combined *n*-hexane, EtOAc and acetone extracts were concentrated to dryness in vacuo, respectively. The *n*-hexane residue (11.7 g) was subjected to column chromatography on silica gel and ODS, and eluted successively with a *n*-hexane – EtOAc and MeOH – H_2O solvent system to afford fourteen compounds, eight flavones: 3, 3', 4', 5, 6, 7, 8-heptamethoxyflavone (**FLV1**, 265.8 mg), natsudaidain (3-hydroxy-3', 4', 5, 6, 7, 8-hexamethoxyflavone) (**FLV2**, 130.3 mg), 5-hydroxy-3, 3', 4', 6, 7, 8-hexamethoxyflavone (**FLV3**, 29.3 mg), 3, 3', 4', 5, 6, 7-hexamethoxyflavone (**FLV4**, 14.6 mg), 8-hydroxy-3, 4', 5, 6, 7-pentamethoxyflavone (**FLV5**, 7.1 mg), 4', 5-dihydroxy-3, 3', 6, 7, 8-pentamethoxyflavone (**FLV6**, 2.9 mg), retusin (5-hydroxy-3, 3', 4', 7-tetramethoxyflavone) (**FLV7**, 1.9 mg), nobiletin (3', 4', 5, 6, 7, 8-hexamethoxyflavone) (**FLV8**, 1.9 mg), three flavanones: 3', 4', 5, 6, 7, 8-hexamethoxyflavanone (**FNN2**, 32.1 mg), 3', 4', 5, 6, 7-pentamethoxyflavanone (**FNN3**, 6.7 mg), 4', 5, 6, 7-tetramethoxyflavanone (**FNN4**, 1.4 mg), three chalcones: 2'-hydroxy-3, 3', 4, 4', 5', 6'-hexamethoxychalcone (**CHL1**, 31.7 mg), 2'-hydroxy-3, 4, 4', 5', 6'-pentamethoxychalcone (**CHL2**, 8.2 mg), 2'-hydroxy-4, 4', 5', 6'-tetramethoxychalcone (**CHL3**, 7.8 mg). The EtOAc and acetone residues were filtered to obtain an amorphous powder (narirutin: **FNN1**, 17.7 g). These compounds were determined by comparison of physical data and NMR spectral data with articles[9–13].

Chemical structures of isolated compounds were shown in **Figure 1**, and their names and amounts were listed in **Table 1**. Among these compounds, **FLV3, 5, 6, 7** and **CHL1-3** are the compounds which were isolated for the first time from *C. jabara* fruit peels[14]. Seven compounds that were obtained more than 10 mg (**FLV1 - 4**, **FNN1 - 2**, and **CHL1**) were evaluated in cell-based assays.

Materials

Naringenin was purchased from LKT Laboratory (St. Paul, MN, USA). Hesperidin, neohesperidin, and naringin were purchased from Nacalai Tesque

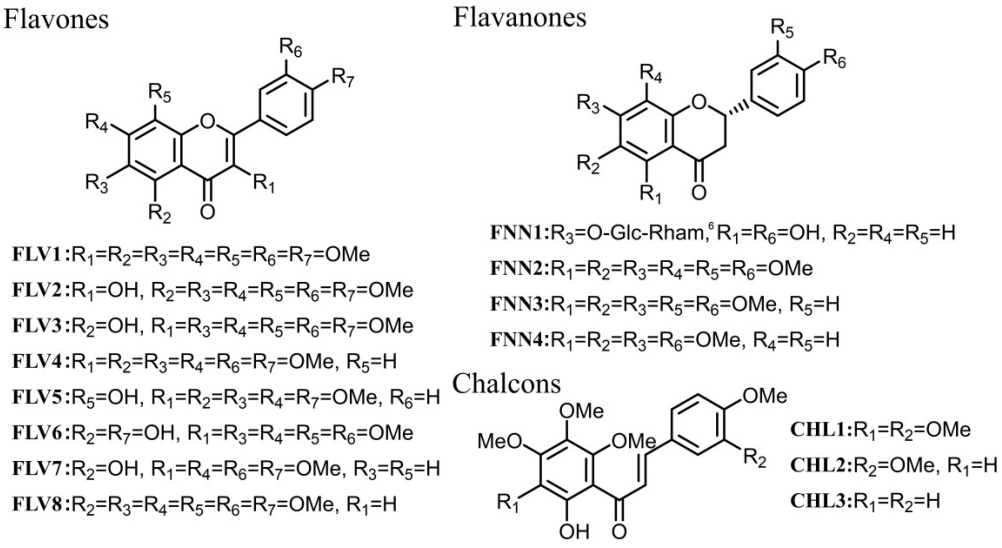

Figure 1. Structures of isolated flavonoids from dried *C. jabara* fruit peels

Table 1. Flavonoids isolated from dried *C. jabara* fruit peels (1 kg).

	Compound	Amount (mg)
Flavones		
FLV1	3, 3', 4', 5, 6, 7, 8-heptamethoxyflavone	265.8
FLV2	natsudaidain (3-hydroxy-3', 4', 5, 6, 7, 8-hexamethoxyflavone)	130.3
FLV3	5-hydroxy-3, 3', 4', 6, 7, 8-hexamethoxyflavone	29.3
FLV4	3, 3', 4', 5, 6, 7-hexamethoxyflavone	14.6
FLV5	8-hydroxy-3, 4', 5, 6, 7-pentamethoxyflavone	7.1
FLV6	4',5-dihydroxy-3, 3', 6, 7, 8-pentamethoxyflavone	2.9
FLV7	retusin (5-hydroxy-3, 3', 4', 7-tetramethoxyflavone)	1.9
FLV8	nobiletin (3', 4', 5, 6, 7, 8-hexamethoxyflavone)	1.9
Flavanones		
FNN1	narirutin	17,700.0
FNN2	3', 4', 5, 6, 7, 8-hexamethoxyflavanone	32.1
FNN3	3', 4', 5, 6, 7-pentamethoxyflavanone	6.7
FNN4	4', 5, 6, 7-tetramethoxyflavanone	1.4
Chalcones		
CHL1	2'-hydroxy-3, 3', 4, 4', 5', 6'-hexamethoxychalcone	31.7
CHL2	2'-hydroxy-3, 4, 4', 5', 6'-pentamethoxychalcone	8.2
CHL3	2'-hydroxy-4, 4', 5', 6'-tetramethoxychalcone	7.8

(Kyoto, Japan). Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin mixed solution (stabilized) were from Nacalai Tesque. Fetal bovine serum was obtained from Biowest (Nuaillé, France). Lipopolysaccharide (LPS) from *Escherichia coli* O55 was obtained from Wako Pure Chemical Industries (Osaka, Japan). Griess reagent system was obtained from Promega (Fitchburg, WI, USA). Cell Counting Kit-8 was from Dojindo Laboratories (Kumamoto, Japan). RIPA buffer and protease inhibitor cocktail were purchased from Wako Pure Chemical. BCA Protein Assay Kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Polyvinyliden fluoride (PVDF) membrane was obtained from Bio-Rad (Hercules, CA, USA). Blocking one, a blocking solution for blot membrane, was obtained from Nacalai Tesque. Rabbit antibody to inducible nitric oxide synthase (iNOS) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and anti-rabbit IgG HRP-linked whole antibody was from GE Healthcare (Buckinghamshire, UK). Monoclonal antibody to β-actin HRP conjugate was obtained from Wako Pure Chemical Industries. Amersham ECL Prime Western Blotting Detection Reagent, chemiluminescent reaction reagent, was obtained from GE Healthcare. Enzyme linked immunosorbent assay (ELISA) kits for Interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) ware obtained from BioLegend (San Diego, CA, USA). Human recombinant soluble epoxide hydrolase (sEH) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and its substrate, *(2S,3S)-trans*-3-phenyl-2-oxiranylmethyl-4-nitrophenyl carbonate (S-NEPC), was from Sigma-Aldrich. Human recombinant chymase was obtained from Sigma-Aldrich, and its substrate, *N*-Succinyl-Ala-His-Pro-Phe-*p*-nitroanilide, was from Peptide Institute (Osaka, Japan). Hyaluronidase from bovine testes and Compound 48/80 were obtained from Sigma-Aldrich. Hyaluronic acid sodium salt from Rooster Comb was obtained from Wako Pure Chemical Industries.

HPLC analysis for quantification of hesperidin, neohesperidin, naringin, and naringenin

Prior to the sample preparation, dried *C. jabara* fruit peels were powdered. Amounts of hesperidin, neohesperidin, naringin, and naringenin were quantified as reported by Tosa *et al.*[15] with some modifications. Briefly, 50 mg of *C. jabara* powder were taken into a 10 mL vial and extracted with 50% methanol for 20 min using an ultrasonic extraction. Then, extract was passed through a 0.45 μm syringe

filter into a glass vial, and 5.0 µL was injected into the HPLC. Chromatographic separation was performed on a COSMOSIL 5C18-AR-II column (φ 4.6 x 250 mm) with a following condition: temperature, 40 °C; mobile phase, 20% acetonitrile containing 0.8% acetic acid; flow rate, 1.0 mL/min. Quantitative analysis was performed with purchased standard samples of hesperidin, neohesperidin, naringin, and naringenin. Peaks were detected at 280 nm. The limits of detection of each flavanone were follows: hesperidin, 40 ng/mL; neohesperidin, 92 ng/mL; naringin, 65 ng/mL; naringenin, 10 ng/ml, respectively. The quantification limits of each flavanone were follows: hesperidin, 120 ng/mL; neohesperidin, 280 ng/mL; naringin, 200 ng/mL; naringenin, 30 ng/mL, respectively.

Cell culture

The murine macrophage-like cell line, RAW 264 (RCB0535), was provided from the RIKEN BioResource Center (Ibaraki, Japan). RAW 264 cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 µg/ mL streptomycin. Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere.

Cell viability assay

The cytotoxicity of flavonoids on RAW 264 cells were measured by Cell Counting Kit-8. Briefly, 1.0 x 10_5 cells/well of RAW 264 cells were seeded on a 96 well plate in DMEM with 10% FBS and antibiotics with various flavonoids at 37 °C for 24 h. Then, cells were washed, and added new 200 µL of DMEM with 10% FBS and 10 µL of WST-8 reagent. After further incubation for 2 h, cell viability was determined by measuring the absorbance at a wavelength of 450 nm. The cell viability ratio was calculated by the following formula: Cell viability (%) = (average of treated group/average of control group) x 100.

Measurement of nitric oxide (NO), IL-6, and TNF-α production

RAW 264 cells were seeded in a 96 well plate at a density of 1.0 x 10^5 cells/well in DMEM with 10% FBS and antibiotics at 37 °C for 2 h. Next, cells were cultured with or without flavonoids for 2 h, then, stimulated with 1 µg/mL of LPS for an additional 24 h. After treatment, culture supernatants were collected and stored -20 °C until assayed. The NO concentration was measured by using Griess reagent system. Average of LPS-induced NO production was 26.5 ± 1.4 µM. The concentration of IL-6 and TNF-α was measured by using mouse ELISA kits. Average of LPS-induced IL-6 protein was 1.034 ± 0.121 nM, and that of TNF-α protein was 5.030 ± 0.774 nM, respectively. The inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = (1-average of treated group/ average of control group) x 100.

Western blot analysis

RAW 264 cells were seeded in φ 60 mm dishes at a density of 6.0 x 10^6 cells/dish in DMEM with 10% FBS at 37 °C for 2 h. Next, cells were cultured with or without flavonoids for 2 h, then, stimulated with 1 µg/mL of LPS for an additional 24 h. After treatment, cells were washed and lysed with RIPA buffer and centrifuged at 20,600 xg for 30 min at 4 °C. Protein concentrations of supernatants were estimated by BCA protein assay kit, and stored at -80˚C until analysis. Equal amounts of protein (2.5 µg) of the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were pre-incubated in blocking solution at room temperature for 1 h. For iNOS protein detection, a membrane was incubated with a 1: 2,000 dilution of a primary antibody against iNOS at room temperature for 1 h. The blot was washed with T-PBS (-) and incubated with a 1:4,000 dilution of HRP-conjugated IgG secondary antibody. In case of β-actin, the transferred membrane was incubated with a 1:8,000 dilution of a HRP-conjugated antibody against β-actin at room temperature for 1 h. The target proteins on the membranes were detected using a chemiluminescent reaction and LAS-3000 Imaging System (Fuji film, Tokyo, Japan).

Enzyme inhibitory assay

sEH activity was measured as reported by Bahl *et al*.[16] with some modifications. Briefly, samples were incubated at 37 °C for 30 min in a 100 µL volume containing 0.58 U/mL sEH and 0.4 mM s-NEPC. Hyaluronidase activity was measured by the Morgan-Elson reaction as reported by Ratnasooriya *et al*.[17] Chymase activity was measured as reported by Tani *et al*.[18] with some modification. Briefly, samples were incubated at 37 °C for 60 min in a 100 µL volume containing 8.8 µg/mL chymase and 0.8 mM *N*-Succinyl-Ala-His-Pro-Phe-*p*-nitroanilide. These assays were carried out in 96-well plates and the optical density was measured at 405 nm in case of sEH and chymase, and at 585 nm in case of hyaluronidase, respectively. The inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = (1-average of increase in absorbance of sample/ average of increase in absorbance of control) x 100.

Results

Flavonoids in *C. jabara* fruit peels

Amounts of flavonoids in *C. jabara* fruit peels were quantified to clarify the distribution characteristics in *C. jabara* fruit peels. Eight flavones, 4 flavanones, and 3 chalcones were detected in *C. jabara* fruit peels, but more than 99% of flavonoids were narirutin (**Table 1**). Typical flavanone glycosides of *Citrus* fruit peels, such as hesperidin, neohesperidin, nor naringin, were not detected during the isolation and quantification process of *C. jabara* fruit peels. Furthermore, naringenin, an aglycone of narirutin, was also not detected.

For confirmation, amounts of hesperidin, naringin, neohesperidin, and naringenin in *C. jabara* fruit peels were quantified by HPLC analysis. Amounts of these flavonoids were below detection limit.

Toxicities of flavonoids in *C. jabara* fruit peels

Prior to investigate the anti-inflammatory effects, toxicities of purified flavonoids in *C. jabara* fruit peels were examined. Cell viabilities of RAW 264 cells cultivated with 100 and 200 μM flavonoids for 24 h were measured with Cell Counting Kit-8. In case of **FLV4**, toxicity of 200 μM was not evaluated because of its low solubility. At 100μM, no toxicity was observed on **FLV4**, narirutin, and its aglycone naringenin (**Figure 2**). Mild toxicity was observed on **FLV1-3** and **FNN2**. Strong toxicity was observed on **CHL1**. Toxicities of narirutin and naringenin were examined until 500 μM. Narirutin up to 500 μM and naringenin up to 400 μM exhibit no toxicity. At 500 μM naringenin, cell viability was 85.8 ± 6.0 %.

Flavonoids in *C. jabara* fruit peels inhibit LPS-induced NO production and iNOS protein generation in RAW 264 cells

In order to investigate the suppressive effects on NO synthesis and iNOS inhibitory activities, RAW 264 cells were cultivated with 1 μg/mL of LPS and 50, 100, and 200 μM of flavonoids for 24 h. NO concentrations in the culture supernatants were quantified by the Griess reaction. In case of **FLV4** at 200 μM, NO was not evaluated because of its low solubility. Expression levels of iNOS protein at 100 μM flavonoids were visualized by immunoblot analysis. All tested flavonoids, except for narirutin, exhibited inhibitory effects equally or more than that of L-NAME (**Figure 3A**). With a few exceptions, dose-dependently inhibitory effects were observed on each tested flavonoid. These flavonoids, except for narirutin, significantly reduced LPS-induced iNOS protein expression, but did not change β-actin expression (**Figure 3B**).

Flavonoids in *C. jabara* fruit peels inhibit LPS-induced IL-6 and TNF-α secretion in RAW 264 cells

In order to investigate the suppressive effects on IL-6 and TNF-α secretion, RAW 264 cells were cultivated with 1 μg/mL of LPS and 50, 100, and 200 μM of flavonoids for 24 h. IL-6 and TNF-α concentrations in the culture supernatants were quantified by the ELISA kits. In case of **FLV4** at 200

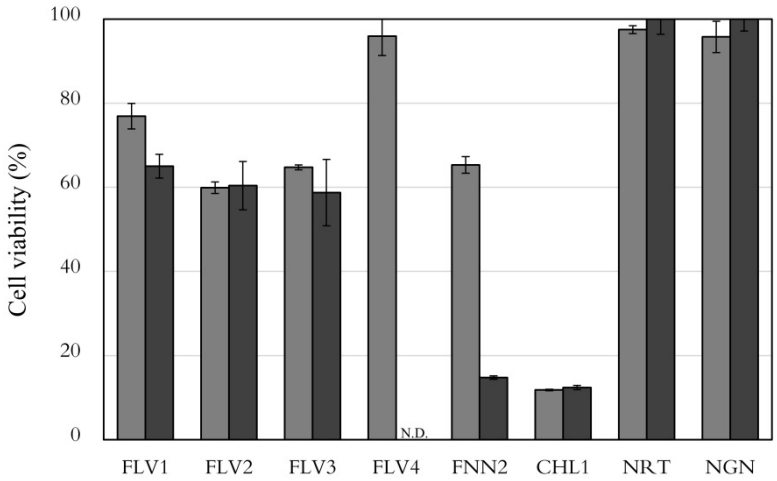

Figure 2. Toxicities of flavonoids in *C. jabara* fruit peels on RAW 264 cells. Flavonoids were tested at concentrations of 100 μM(■) and 200 μM(■). The values express the means ± S.D. of three experiments. NRT, narirutin; NGN, naringenin. Other abbreviations of flavonoids are listed in **Table 1**. N.D., no data.

μM, IL-6 and TNF-α were not evaluated because of its low solubility. Dose-dependently inhibitory effects were observed on each tested flavonoid. Among these flavonoids, **FNN2**, naringenin, and **FLV1** inhibited IL-6 secretion strongly, but narirutin and **FLV3** did weakly (**Figure 4A**). In case of TNF-α, only **FNN2** exhibited strong inhibitory activity (**Figure 4B**).

Inhibitory effects of narirutin and naringenin on inflammatory related factors

Inhibitory effects of narirutin and its aglycone naringenin on following inflammatory related factors were examined; secretions of NO, IL-6 and TNF-α in LPS-stimulated RAW 264 cells, and inflammation related enzymes, such as soluble epoxide hydrolase (sEH), hyaluronidase and chymase. Dose-dependent inhibitory effects were observed other than chymase (**Figure 5**). The inhibitory concentration 50% (IC_{50}) values of naringenin on inflammatory related factors were follows; NO production was 105 μM, IL-6 secretion was 65 μM, sEH was 267 μM, and hyaruronidase was 1,470 μM, respectively. On the other hand, narirutin did not exhibit significant inhibitory effects in the range of tested concentrations.

Discussion

In this study, we clarified the characteristics of flavonoids in *C. jabara* fruit peels, and investigated the anti-inflammatory effects of these purified flavonoids. Furthermore, we estimated the most

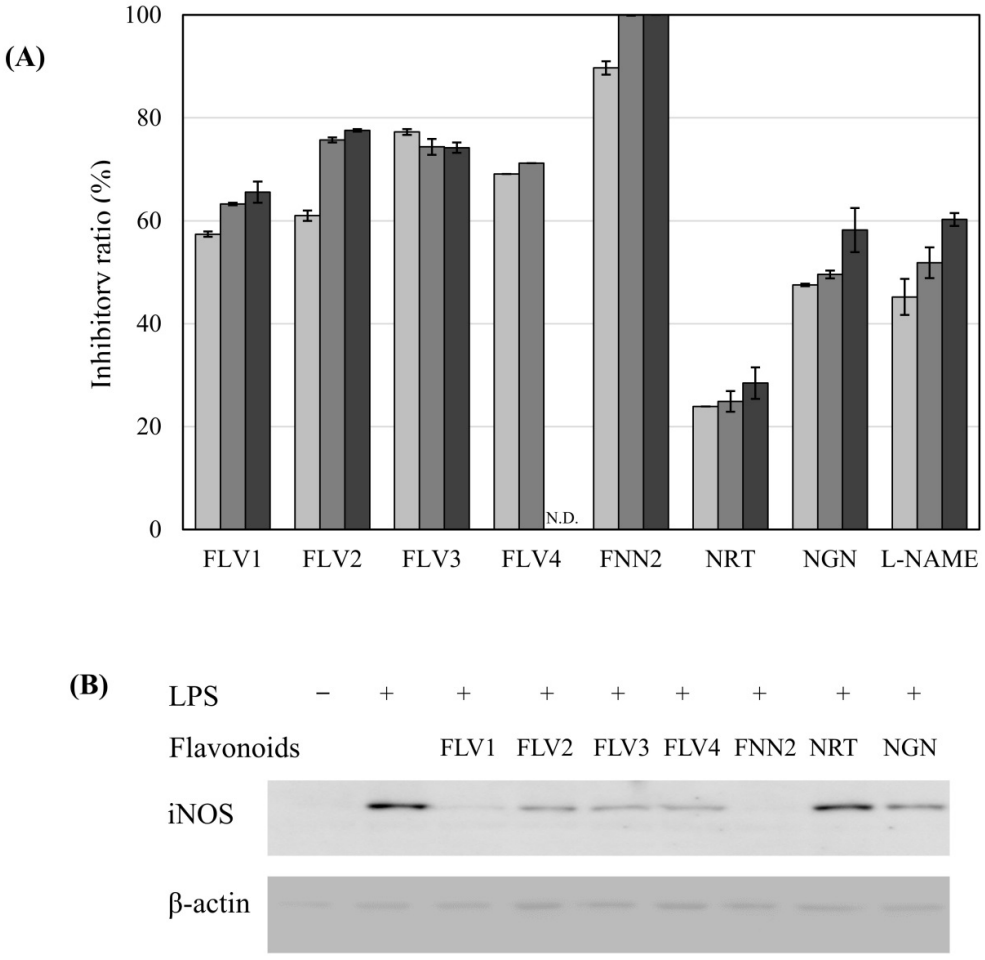

Figure 3. Flavonoids in *C. jabara* fruit peels inhibit LPS-induced NO production (**A**) and iNOS generation (**B**) in RAW 246 cells.

For NO production, flavonoids were tested at concentrations of 50 μM(■), 100 μM(■), and 200 μM(■). For iNOS generation, flavonoids were tested at concentration of 100.The values express the means ± S.D. of three experiments. NRT, narirutin; NGN, naringenin; L-NAME, N-Nitro-L-arginine methyl ester hydrochloride (positive control). Other abbreviations of flavonoids are listed in **Table 1**. N.D., no data.

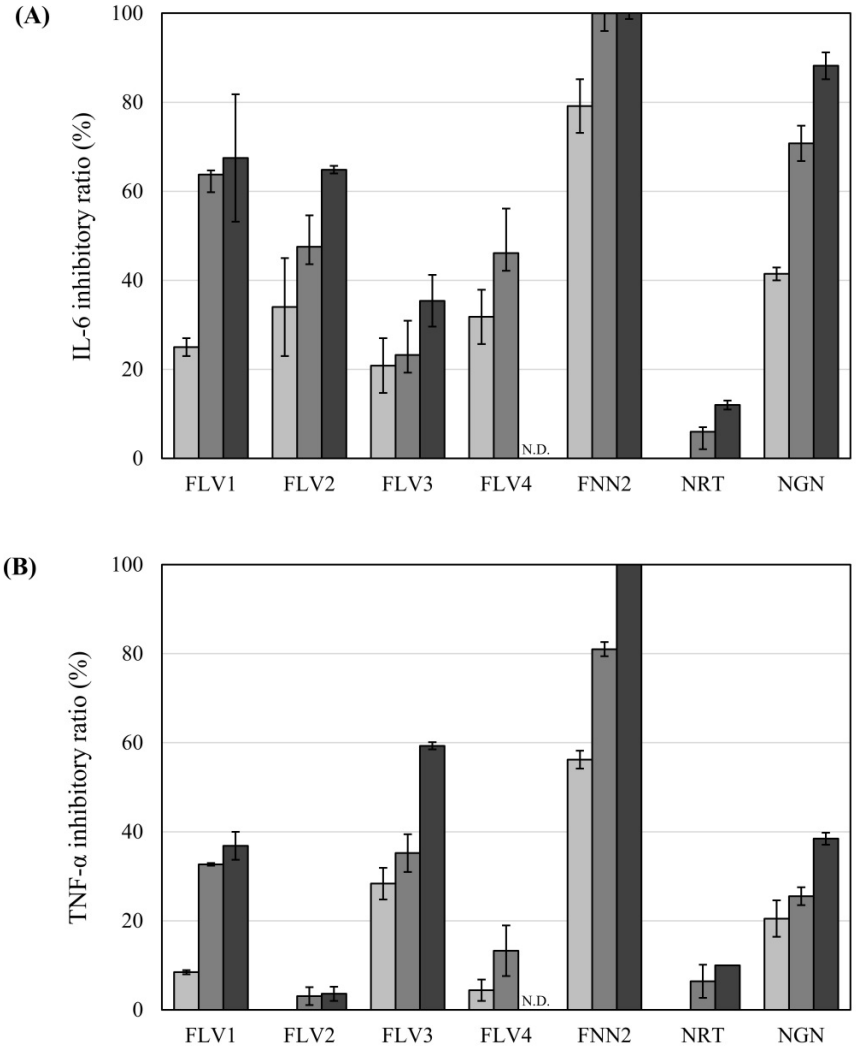

Figure 4. Flavonoids in *C. jabara* fruit peels inhibit LPS-induced IL-6 (A) and TNF-α (B) in RAW 246 cells. Flavonoids were tested at concentrations of 50 μM(■), 100 μM(■), and 200 μM(■). The values express the means ± S.D. of three experiments. NRT, narirutin; NGN, naringenin; L-NAME. Other abbreviations of flavonoids are listed in **Table 1**. N.D., no data.

effective anti-inflammatory compound in a living body.

Citrus could be classified by the content ratio of following 4 flavanone glycosides, such as narirutin, hesperidin, naringin, and neohesperidin, in its fruits[19]. Principal component analysis of *Citrus* fruits flavonoids indicated that distributions of *Citrus* species belonging to different classes were largely in accordance with Tanaka's classification system[20]. *C. jabara* is recognized as a closely-related species to *C. sphaerocarpa*, *C. sudachi*, *C. junos*, and *C. reticulate* by genetic characterization and diversity research[6–8]. As shown in **Table 1**, almost all of flavonoids in *C. jabara* fruit peels was narirutin, but hesperidin, naringin, and neohesperidin were below detection limit. There was no *Citrus* species with a flavanone glycosides content ratio like *C. jabara*[19,20]. This result indicates that *C. jabara* is a unique citrus.

Flavonoids in *C. jabara* fruit peels did not exhibit strong toxicity except for **CHL1** (**Figure 2**). Especially, narirutin and its aglycone naringenin were nontoxic. Thus, *C. jabara* fruit peel is considered as a nontoxic material, because almost all of flavonoids are narirutin in *C. jabara* fruit peels.

Anti-inflammatory effects of flavonoids were observed on inflammatory mediators in LPS-stimulated macrophage-like cells (**Figures 3 and 4**). Flavonoids are known as suppressors of nuclear factor κB (NF-κB) pathway[21,22]. Among

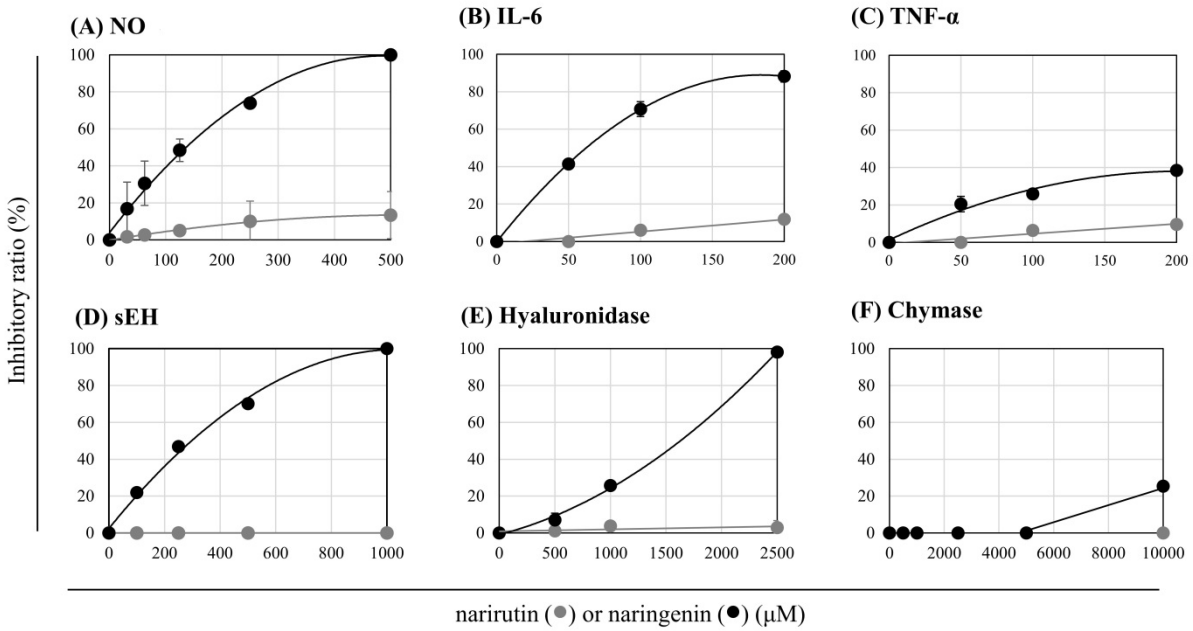

Figure 5. Inhibitory effects of narirutin and naringenin on inflammatory factors. (A)NO, (B)IL-6, (C)TNF-α, (D)sEH, (F)hyaluronidase, and (F)chymase were examined. The values express the means S.D. of three experiments.

tested flavonoids, **FNN2** was most effective on NO synthesis, iNOS induction, IL-6 synthesis, and TNF-α synthesis (**Figures 3** and **4**). These results may be effected by the reduction of cell viability, thus, toxicities of each flavonoid should also be taken into consideration. Although anti-inflammatory effects of narirutin was weak, but, its aglycone naringenin exhibited enough inhibitory effects (**Figures 3–5**). The difference in the inhibitory activity of narirutin and naringenin, in other word, glycosides and aglycone, may be partially explained by the difference in cell membrane permeability[23,24]. In this study, other structure-activity relations of inhibitory effects of tested flavonoids were not clear. For example, Takano-Ishikawa *et al*. reported that C2-C3 double-bond and 4-oxo functional group of the C-ring in flavonoids are important factors for the high inhibition activities of prostaglandin E_2 (PGE_2) induction by LPS in rat peritoneal macrophages[23]. In this study, all tested flavonoids have 4-oxo functional group in the C-ring. The C2-C3 double bond of C-ring, in short, flavone (**FLV**) skeletal structure, was not relevant to the inhibition activities in our results (**Figures 2–4**). **FNN2**, the most effective flavonoids, is one of most hydrophobic flavonoids in this study. But, the relationship between hydrophobicity of flavonoids and inhibition activities is considered not so high, because the inhibition activities of other most hydrophobic flavonoids, such as **FLV1** and **FLV4**, were not so high compare to other tested flavonoids.

Thinking about the abundance ratio of flavonoids, narirutin is considered to contribute to anti-inflammatory effect at oral ingestion of *C. jabara* fruit peels. Funaguchi *et al*. also reported that oral administration of narirutin inhibited airway inflammation in allergic model mouse[25]. On the other hand, *in vitro* cell assay, narirutin did not exhibited enough anti-inflammatory effects (**Figures 2–5**) nor anti-degranulating effects[26]. These conflicting results between *in vivo* and *in vitro* examinations may be explained by the metabolism of narirutin in a living body. Pharmacokinetics of narirutin after ingestion of orange juice in healthy humans were reported by Silveria *et al*.[27]. The majority of oral intake-narirutin is considered to reach the colon, and colonic microbiota deglycosylate narirutin to naringenin, that is then absorbed by colonocytes[28]. Thus, it is considered that narirutin exists like a prodrug, and its aglycone naringenin works as an active form within the body.

Anti-inflammatory effects of naringenin were also observed on inflammation related enzymes, such as sEH and hyaluronidase (**Figure 5D–5F**). sEH is known as a pro-inflammatory factor by metabolizing anti-inflammatory epoxyeicosatrienoic acids (EETs) to less potent dihydroxyeicosatrienoic acids (DHETs)[29]. Relationship of inflammation to hyaluronidase is also reported[30]. Therefore, naringenin is thought to work as an enzyme inhibitor, too. The wide difference of effective concentration of naringenin

was observed on each inflammatory related parameter (**Figure 5**), but this phenomenon is also observed in case of other flavonoids[31].

In this study, we clarified the large difference between narirutin and its aglycone naringenin on anti-inflammatory effects, and suggested the importance of deglycosylation of narirutin to naringenin at oral ingestion of *C. jabara* fruit peels. In fact, when patients with allergic disease intake *C. jabara* fruit peels, there are individual differences on anti-allergic and anti-inflammatory effects. Our findings may contribute to dissolve this problem. As shown in **Figure 1**, narirutin is a flavanone that contains rutinose group (Glc-Rham). Although hydrolysis of rhamnosides was not observed in human small intestinal cell extract[32], but rhamnosyl moiety of flavonoids could be hydrolyzed by strains of intestinal *Bacteroides* from humans[33]. Therefore, intestinal bacterial flora is considered as an important element for *C. jabara* fruit peels to exhibit anti-inflammatory effects. Now, we are trying to explore most suitable intestinal bacteria for deglycosylation of narirutin in *C. jabara* fruit peels in order to be more effective in more patients with allergic diseases.

Conclusion

C. jabara was a unique citrus that almost all of flavonoids in fruit peels were narirutin. Although anti-inflammatory effects of narirutin was weak, but, its aglycone naringenin exhibited following inhibitory effects: NO synthesis, iNOS induction, IL-6 synthesis, and sEH activity. Since narirutin is deglycosylated to naringenin that is then absorbed by colonocytes, it is considered that narirutin exists like a prodrug and its aglycone naringenin works as an active form of anti-inflammatory effect in a living body at oral ingestion of *C. jabara* fruit peels.

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Conflict of interest

The authors declare no potential conflicts of interest.

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