Yuzu (*Citrus junos* Tanaka) Peel Attenuates Dextran Sulfate Sodium-induced Murine Experimental Colitis

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Abstract: Ulcerative colitis is a well-known inflammatory bowel disease. Although there are drugs that are effective against this disease, the prevention and attenuation of ulcerative colitis by food rich in functional ingredients without side effects is desired because some drugs have side effects. In this study, we investigated the effects of yuzu (*Citrus junos* Tanaka), a citrus fruit native to northeast Asia, on a mouse dextran sulfate sodium (DSS)-induced colitis model. Mice given drinking water containing DSS showed significant weight loss, colon shortening, diarrhea, and visible fecal blood. In contrast, mice fed a diet containing 5% yuzu peel for 14 d before receiving DSS showed significant attenuation of these phenotypes. To clarify the mechanism underlying the attenuation, we investigated the anti-inflammatory and antioxidant effects of yuzu peel. We found that yuzu peel extract suppressed tumor necrosis factor- α (TNF- α) production in lipopolysaccharide (LPS)-stimulated mice and murine macrophage cell line through suppression of nuclear factor- κ B (NF- κ B) activation. In addition, we confirmed that yuzu peel extract had a moderate antioxidant effect. These results suggest that yuzu peel attenuates the pathologies of DSS-induced colitis by coordinately suppressing inflammation and oxidative stress against lipids *in vivo*.

Key words: yuzu, experimental colitis, inflammation, oxidative stress, antioxidant against lipids, citrus flavonoid

1 Introduction

Ulcerative colitis is an idiopathic inflammatory bowel disease characterized by intestinal inflammation, and the number of patients is increasing in developed countries¹⁾. It has been reported that patients with active ulcerative colitis status have high activated macrophage, monocyte, and neutrophil counts in peripheral blood or inflamed gut^{2,3)}. The activated macrophages and monocytes generate proinflammatory cytokines, including tumor necrosis factor- α (TNF- α)^{4,5)}, which induce inflammation in the intestines⁶⁾. Reactive oxygen species generated by the activated macrophages and neutrophils also contribute to intestinal oxidative injury in ulcerative colitis patients⁷⁾. Thus, it has been believed that both inflammation and oxidative stress are involved in the development and progress of ulcerative

colitis⁸⁾.

Such drugs as aminosalicylates and corticosteroids are currently used for the treatment of this disease. However, they have serious side effects, including allergic reaction^{9,10)}. It is therefore ideal that the pathogenesis of ulcerative colitis be prevented or attenuated by the ingestion of food without side effects.

Yuzu (*Citrus junos* Tanaka) is a popular citrus fruit native to northeast Asia, including Japan, China, and Korea¹¹⁾. Yuzu has been used in traditional Chinese medi-

Abbreviations: DSS; dextran sulfate sodium, HPLC; high performance liquid chromatography, IL; interleukin, LPS; lipopolysaccharide, MS; mass spectrometry, NF- κ B; nuclear factor- κ B, PBS; phosphate-buffered saline, TNF- α ; tumor necrosis factor- α

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cine¹²⁾, beverages, cuisines, and cosmetics^{13, 14)}. It has been reported that yuzu contains many bioactive compounds that show anti-inflammatory and/or antioxidant activities, including ascorbic acid, β -carotene, flavonoids, and limonoids¹⁵⁾. The extract of yuzu inhibits platelet aggregation, prevents myocardial infarction-induced ventricular dysfunction and structural remodeling of myocardium, and exerts an antidiabetic effect^{11, 16, 17)}. Although yuzu has many health benefits, there is no report of its beneficial effects on ulcerative colitis.

In this study, we examined the effect of yuzu on ulcerative colitis induced by dextran sulfate sodium (DSS) in mouse. We found that yuzu peel significantly attenuated DSS-induced colitis symptoms due to the coordination between its anti-inflammatory and antioxidant effects.

2 Experimental

2.1 Extraction of yuzu peel and preparation of samples

Yuzu peel paste YP-01, which consists of yuzu peel after squeezing out the juice, was obtained from Shikoku Research Institute Inc. (Kagawa, Japan). The yuzu peel paste was stored at -80°C . For the extraction of the aqueous fraction, 10 g of frozen yuzu peel paste and 10 mL of cold serum-free RPMI 1640 medium (Sigma-Aldrich, MO) supplemented with 2 mM L-glutamine or cold phosphate-buffered saline (PBS) were transferred to a 50 mL conical centrifuge tube. The mixture was vortexed vigorously on the highest setting for 30 seconds to produce a suspension of yuzu peel paste. The suspension was further mixed in a rotator (RT-5; TAITEC Corporation., Saitama, Japan) at 2.5 r/min for 15 min at 5°C , and then centrifuged at $1100 \times g$ for 5 min at 5°C . The resulting supernatant was used as 50% yuzu peel extract.

2.2 Quantitative analysis of flavonoids and ascorbic acid

The 50% yuzu peel extract extracted with RPMI 1640 medium (hereinafter, RPMI 1640 extract of yuzu peel) was diluted 5-fold with DMSO/MeOH (1:1 v/v) and then measured in a UFLC system (Prominence UFLC; Shimadzu Corporation, Kyoto, Japan) equipped with a Shim-pack XR-ODS column (Shimadzu Corporation) maintained at 30°C at the flow rate of 0.6 mL/min. The absorbance at 280 nm was monitored. Formic acid (0.1% (v/v); A) and 99.9% acetonitrile containing 0.1% formic acid (B) were used as the gradient elution system. The mobile phase was 95% A, which was decreased to 50% over a period of 18.7 min. It was further decreased to 5% A in 0.5 min and maintained at that concentration for another 1.5 min. Hesperidin (Wako Pure Industries, Ltd., Osaka, Japan) and naringin (Sigma-Aldrich) were dissolved in DMSO/MeOH (1:1 v/v) and diluted to prepare standard solutions (8 μM to 324 μM) for the creation of a calibration curve for each chemical. Each

flavonoid was quantified on the basis of the calibration curve of hesperidin or naringin.

Ascorbic acid content in the yuzu peel extract extracted with PBS (hereinafter, PBS extract of yuzu peel) was determined with an Ascorbic Acid Colorimetric/Fluorometric Assay Kit (BioVision, CA) in accordance with the manufacturer's instructions.

2.3 Animals

Male BALB/c mice were purchased from Clea Japan Inc. (Tokyo, Japan). The animals were fed a standard laboratory diet (CE-2; Clea Japan Inc.) and water ad libitum. The animal room was maintained at $23 \pm 2^{\circ}\text{C}$ and $50 \pm 10\%$ humidity under a 12 h light (8:00–20:00) and dark (20:00–8:00) cycle. The animals were acclimated to the laboratory environment for at least one week before the experiment. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology.

2.4 Mouse DSS-colitis model

Mice were randomly divided into four experimental groups: two groups (5 animals each) were fed a diet containing 50 g/kg (w/w) yuzu peel paste daily for 14 d. The remaining two groups were fed a standard diet. One group fed yuzu peel paste was given drinking water containing 4% (w/v) DSS for 5 d to induce colitis while yuzu peel paste feeding was continued. One group fed the standard diet was also given drinking water containing 4% (w/v) DSS for 5 d. To determine the severity of clinical colitis, body weight, stool consistency, and visible fecal blood were evaluated daily. Stool consistency and visible fecal blood were scored as described by Melgar *et al.*¹⁸⁾ with slight modifications, as shown in Table 1.

2.5 LPS-induced inflammation *in vivo* and measurement of plasma cytokines

Male BALB/c mice (6 weeks old, 18 to 22 g) without anesthesia were intraperitoneally administered 13 μg of lipopolysaccharide (LPS) with or without PBS extract of yuzu peel at various concentrations (200 $\mu\text{L}/\text{cavity}$). A mixture of LPS and yuzu peel extract for intraperitoneal administration was prepared by mixing in PBS. A 26-gauge, 1/2-inch injection needle (Terumo Co., Tokyo, Japan) and a 1 mL syringe (Terumo Co., Tokyo, Japan) were used for each

Table 1 Scores of diarrhea and visible fecal blood.

Score	Diarrhea Score	Visible Fecal Blood
0	Normal pellets	Normal
1	Slightly loose feces	Slightly bloody
2	Loose feces	Adherence of blood around anus
3	Watery diarrhea	Bleeding through the anus

mouse. Peripheral blood of individual mice was collected from the retro-orbital sinus. ELISA kits were used for measuring plasma cytokines, namely, TNF- α (Biosource Europe S. A., Nivelles, Belgium), interleukin (IL)-12(total), and interferon- γ (IFN- γ) (Thermo Fisher Scientific Inc., MA) in accordance with the manufacturer's instructions.

2.6 Cells and cell culture

The murine macrophage-like cell line RAW 264.7 (DS Pharma Biomedical Co., Osaka, Japan) and RAW 264.7 cells expressing secreted embryonic alkaline phosphatase (SEAP) (Raw-Blue cells, InvivoGen, CA) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin, and were maintained at 37°C in a humidified atmosphere of 5% CO₂.

2.7 Measurement of TNF- α produced by RAW 264.7

RAW 264.7 cells (1×10^5 cells/well) were plated into 96-well plates for 4 h. The cells were treated with RPMI 1640 extract of yuzu peel at the concentration of 0 to 1% with or without LPS (250 ng/mL), and incubation was carried out for 24 h.

2.8 SEAP reporter assay

Raw-Blue cells (1×10^5 cells/well) were plated into 96-well plates and treated with RPMI 1640 extract of yuzu peel at the concentration of 0 to 1% with LPS (50 ng/mL). Then, the cells were incubated for 24 h. SEAP activity in the supernatants was measured using Quanti-Blue (InvivoGen).

2.9 Indirect immunofluorescence assays and confocal microscopy

RAW 264.7 cells (2.5×10^4 cells/well) were cultured in an 8-well cell culture chamber slide (Thermo Fisher Scientific). The cells were treated with 1% RPMI 1640 extract of yuzu peel for 24 h at 37°C, and then treated with LPS (50 ng/mL) for 30 min at 37°C. Subcellular localization of nuclear factor- κ B (NF- κ B) p65 was examined by indirect immunofluorescence assay as reporter previously¹⁹ using a rabbit polyclonal antibody against p65 (1:500, Cell Signaling Technology Inc., MA) and an Alexa Fluor 546-conjugated goat anti-rabbit antibody (1:500, Thermo Fisher Scientific).

Nuclear counterstaining was performed with TO-PRO-3 (1:10000, Thermo Fisher Scientific). The cells were imaged under a confocal laser scanning microscope (LSM 700, Carl Zeiss, Oberkochen, Germany). Fluorescence intensity in the cytoplasm was determined from the p65 fluorescence intensity in the TO-PRO-3-negative region divided by the p65 fluorescence intensity in the entire region including the TO-PRO-3-positive region.

2.10 Measurement of lipid peroxidation

The antioxidant activity of PBS extract of yuzu peel was determined by measuring oxygen uptake as described previously²⁰. Briefly, micelles were prepared by vigorously vortexing methyl linoleate (0.2% (v/v)) in PBS containing 0.05 M cholic acid. Free-radical-mediated oxidation of methyl linoleate generates conjugated diene hydroperoxides together with the consumption of oxygen via a straightforward mechanism. The oxidation of methyl linoleate and its inhibition by antioxidants were monitored by oxygen uptake measurement using an oxygen monitor equipped with a Clark-type oxygen electrode (YSI model 5300, OH) at 37°C. Hydrophilic 2,2'-azobis(2-amidinopropane) dihydrochloride was used as an azo initiator.

2.11 Statistical analysis

Data are expressed as means $\pm$ standard error (SE). Statistical analyses were performed using Prism version 5.0c (GraphPad Software, CA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) and Dunnett's test as the post hoc test. *p* Values < 0.05 were considered statistically significant.

3 Results and discussion

3.1 Characterization of bioactive components in yuzu peel

First, we analyzed the bioactive components in yuzu peel used in this study. RPMI 1640 extract of yuzu peel was subjected to HPLC and HPLC-MS/MS. The results showed that narirutin (774 ± 12 μ M), hesperetin 7-(2,6-dirhamnosylglucoside) (356 ± 5 μ M), naringin (513 ± 10 μ M), hesperidin (1118 ± 15 μ M), neohesperidin (635 ± 9 μ M), and rhamnetin 3-*o*-rutinoside (concentration not determined) were the major compounds (Table 2, Fig. S1). The bioactive components and their concentration in PBS extract of yuzu peel were almost the same as those in the RPMI 1640 extract of yuzu peel (data not shown). We also identified ascorbic acid (602 ± 68 μ M) in the PBS extract of yuzu peel. These results suggest the possibility of yuzu peel having an attenuating effect on ulcerative colitis because the bioactive components identified in yuzu peel exhibit both anti-

Table 2 Flavonoid concentrations in yuzu peel extract.

Compound	Concentration (μ M)
Narirutin	773.9 $\pm$ 11.8
Hesperetin 7-(2,6-dirhamnosylglucoside)	355.8 $\pm$ 4.5
Naringin	512.6 $\pm$ 10.4
Hesperidin	1117.9 $\pm$ 14.9
Neohesperidin	635.2 $\pm$ 9.2

Means and standard deviations of concentrations of four replicates.

inflammatory and antioxidant effects.

3.2 Attenuating effect of yuzu peel in mouse DSS-induced colitis model

Next, we examined whether yuzu peel attenuates DSS-induced colitis in a mouse model. Mice were fed powder diet containing 50 g/kg yuzu peel paste for 14 d and then given drinking water containing 4% (w/v) DSS for 5 d to induce colitis while yuzu peel paste feeding was continued. Mice given DSS showed typical colitis symptoms, including

weight loss (Fig. 1A, white circles), diarrhea (Fig. 1B, white circles), rectal bleeding (Fig. 1C, white circles), and colon shortening caused by edematous inflammation of the colon²¹⁾ (Fig. 1D, yuzu - and DSS +). Diarrhea (Fig. 1B, black circles) and visible fecal blood (Fig. 1C, black circles) were significantly attenuated in DSS-treated mice fed yuzu peel. In addition, colon shortening was also significantly suppressed (Fig. 1D, yuzu + and DSS +). We noted that TNF- α and IL-12 levels in the culture supernatants from a section of colon in DSS-treated mice fed yuzu peel tended

Fig. 1 Attenuating effect of yuzu peel on DSS-induced colitis. Mice were fed powder diet containing 50 g/kg yuzu peel paste for 14 d and then given drinking water containing 4% (w/v) DSS for 5 d to induce colitis while yuzu peel paste feeding was continued. The attenuating effect of yuzu peel was evaluated in terms of (A) body weight, (B) diarrhea score, (C) visible fecal blood score, and (D) colon length. Data are expressed as means $\pm$ SE of five animals in each group. * $p < 0.05$, ** $p < 0.01$ for comparison of DSS-treated mice fed the standard diet (DSS) with DSS-treated mice fed diet containing yuzu peel (DSS + yuzu). (A) Body weight change was calculated using body weight on day 0 as the initial body weight. (D) Relative colon length was compared with that of non-treated mice fed the standard diet (yuzu- and DSS-).

to be lower than those in the DSS-treated mice fed the standard diet, although these differences were not statistically significant (Fig. S2). Furthermore, we examined the plasma levels of totally assessed hydroxyeicosatetraenoic acids (tHETEs), which are the lipid peroxidation products of the free and ester forms of arachidonic acids. The plasma levels of tHETEs (5-, 12-, and 15-HETEs) tended to be suppressed in mice fed yuzu peel compared to those fed the control diet with and without DSS, although the differences were not statistically significant²²⁾ (Fig. S3). These results suggested that yuzu peel partially suppressed inflammation of the colon and oxidative stress. On the other hand, the phenotypes of non-treated mice fed diet containing yuzu peel were not significantly different from those of non-treated mice fed the standard diet (Fig. 1). Together, these results demonstrate that yuzu peel had an attenuating effect on DSS-induced colitis.

3.3 Anti-inflammatory effect of yuzu peel

To elucidate the mechanism underlying the attenuating effect of yuzu peel on DSS-induced colitis, we investigated the anti-inflammatory effect of yuzu peel *in vivo* and *in vitro*. In the *in vivo* experiment, 13 µg of LPS and PBS extract of yuzu peel of different concentrations were simultaneously administered intraperitoneally into the mice. Plasma was collected at 1, 3, and 6 h for measurements of TNF- α , IL-12, and IFN- γ , respectively, based on our previous finding of the peak times of the respective proinflammatory cytokines in plasma of LPS-treated mice²³⁾. As shown in Fig. 2A, TNF- α level in plasma of mice injected with 0.5% yuzu peel extract was significantly reduced compared with that of mice treated with LPS alone. The levels of IL-12 and IFN- γ also tended to decrease, but the decrease was not statistically significant (Fig. 2A). Suppression of TNF- α production was also observed in RAW264.7 cells (Fig. 2B) without exhibiting cytotoxicity (Fig. S4). These results demonstrated that yuzu peel has anti-inflammatory effect, as reported previously^{12, 16)}.

It has been reported that the activation of the NF- κ B pathway, a representative proinflammatory signaling pathway²⁴⁾, following production of proinflammatory cytokines, including TNF- α , is deeply involved in the pathogenesis of DSS-induced colitis²⁵⁾. We therefore examined whether yuzu peel suppresses the activation of the NF- κ B pathway by using SEAP-expressing RAW 264.7 cells (Raw-Blue cells) in which SEAP reporter is expressed under the control of NF- κ B response elements²⁶⁾. RAW 264.7 cells were simultaneously treated with 50 ng/mL of LPS and different concentrations of RPMI 1640 extract of yuzu peel, and SEAP protein secreted into the culture medium was measured after incubation for 24 h. As shown in Fig. 3A, yuzu peel extract significantly suppressed LPS-induced SEAP production, demonstrating that the extract potentially suppressed the activation of the NF- κ B pathway.

To further confirm whether yuzu peel extract suppresses endogenous NF- κ B pathway activation, we performed immunohistochemical analysis of p65 protein, a major subunit of NF- κ B. NF- κ B exists in an inactive form in the cytoplasm owing to its interaction with I κ B α , an inhibitor of NF- κ B. After activation of I κ B kinase by stimulation, including LPS and TNF- α , I κ B α is phosphorylated, ubiquitinated, and degraded by proteasomes. The dissociated NF- κ B becomes free to translocate to the nucleus where it activates specific target genes through selective binding to the NF- κ B response element. Through such mechanisms, NF- κ B regulates the expression of a wide variety of genes, including TNF- α . Thus, the activation of the NF- κ B pathway is characterized by the translocation of p65 from the cytoplasm to the nucleus.

To examine the subcellular localization of p65, RAW 264.7 cells were pretreated with RPMI 1640 extract of yuzu peel for 24 h, and then treated with LPS for 30 min. As shown in Fig. 3B, whereas p65 was localized in the cytosol in unstimulated cells (upper panel), it was translocated to the nucleus by LPS stimulation (middle panel). As expected, p65 partially remained in the cytoplasm by the pretreatment with yuzu peel extract (Fig. 3B, bottom panel). Quantitative analysis demonstrated that the LPS-induced translocation of p65 from the cytoplasm to the nucleus was suppressed by yuzu peel extract (Fig. 3C), indicating that yuzu peel extract suppresses the activation of the endogenous NF- κ B pathway by inhibiting upstream of I κ B α phosphorylation.

Hesperidin, which is present in yuzu peel extract in a large amount, has been reported to exert an inhibitory effect on NF- κ B-mediated inflammatory responses²⁷⁾. Naringin also has been reported to inhibit LPS-induced endotoxin shock in mice by suppressing NF- κ B pathway activation²⁸⁾. In addition, neohesperidin has been found to inhibit receptor activator of NF- κ B ligand (RANKL)-induced activation²⁹⁾. Thus, although we cannot identify all the yuzu peel components involved in NF- κ B suppression, it may be reasonable to assume that flavonoids, including hesperidin and naringin, play an important role in the suppression of NF- κ B pathway activation.

3.4 Antioxidant effect of yuzu peel against lipid

Finally, we investigated the antioxidant effect of yuzu peel based on its radical scavenging activity. Antioxidant effect was examined by using the method for monitoring methyl linoleate oxidation, as reported previously³⁰⁾. The same concentrations of ascorbic acid and hesperidin in PBS extract of yuzu peel were tested as controls, because we have identified the large amounts of hesperidin and ascorbic acid in yuzu peel extract. As shown in Fig. 4, ascorbic acid showed radical scavenging activity at the early stage of oxidation, whereas hesperidin exhibited radical scavenging activity at the later stage. The reason

A

B

Fig. 2 Effects of yuzu peel extract on proinflammatory cytokine production in LPS-stimulated mice and RAW 264.7 cells. (A) Mice were intraperitoneally injected with 13 μ g of LPS alone or 0.25% or 0.5% PBS extract of yuzu peel and 13 μ g of LPS. Proinflammatory cytokine levels in plasma were assessed by ELISA. Data are expressed as means $\pm$ SE of five animals in each group. * $p < 0.05$ for comparison of 0% with each concentration of yuzu peel extract. (B) RAW264.7 cells were cultured with RPMI 1640 extract of yuzu peel and 250 ng/mL of LPS for 24 h. Experiments were performed in triplicate and data are expressed as means $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ for comparison of 0% with each concentration of yuzu peel extract.

Fig. 3 Effects of yuzu peel extract on NF- κ B activation in RAW 264.7 cells. (A) SEAP reporter assay using RAW 264.7 cells. Cells (Raw-Blue cells) were cultured with 50 ng/mL of LPS in the presence or absence of RPMI 1640 extract of yuzu peel for 24 h, and then SEAP activity in culture medium was measured. Experiments were performed in four replicates, and data are expressed as means $\pm$ SE. ** $p < 0.01$ for comparison of 0% with each concentration of yuzu peel extract. SEAP activity in the absence of yuzu peel extract was set at 100%. (B) Representative fluorescence images of intracellular localization of p65 protein. p65 localization was examined under a confocal laser scanning microscope with indirect immunofluorescence using NF- κ B p65 rabbit polyclonal antibody. Cells were stained with TO-PRO-3 to visualize nuclei. (C) Relative p65 fluorescence intensity in cytoplasm. Data are expressed as means $\pm$ SE. * $p < 0.05$ for comparison of LPS-alone-treated group with LPS + yuzu group. p65 fluorescence intensity in cytoplasm of unstimulated cells was set at 100%.

for this is unknown; nevertheless, we speculate that the oxidation products of hesperidin generated by radicals may be also effective against the next-coming radicals formed at the later stage. It is interesting that yuzu peel extract, whose hesperidin and ascorbic acid concentrations are the same as those of hesperidin and ascorbic acid positive controls, inhibited the oxidation much more efficiently than those controls. This may be attributed to the fact that antioxidative compounds other than ascorbic acid and hesperidin are present in the extract and flavonoids other than hesperidin contributed synergistically to the total antioxidant effect³⁰⁾. It is therefore concluded that yuzu peel extract possesses moderate antioxidative effect against radicals and its effect is stronger than that of ascorbic acid and hesperidin at the same concentration.

4 Conclusion

We demonstrated that yuzu peel extract has an attenuating effect on DSS-induced symptoms, including weight loss, colon shortening, diarrhea, and visible fecal blood. We confirmed that yuzu peel extract suppressed LPS-stimulated TNF- α production (Fig. 2) and showed radical scavenging activity (Fig. 4), demonstrating that yuzu peel possesses both anti-inflammatory and antioxidant effects. Thus, it may be reasonable to assume that yuzu peel attenuated DSS-induced colitis symptoms due to the coordination

between anti-inflammatory effect and antioxidant effect against lipids *in vivo*.

Conflicts of Interest

A part of this study was funded by Shikoku Research Institute Inc. for about three years from 2010 to 2012. Mika Ishioka was an employee of Shikoku Research Institute Inc.

Supporting information

The material is available free of charge via the Internet at <http://dx.doi.org/10.5650/jos.ess17184>

Fig. S1: HPLC chart of yuzu peel extract (A) and (B-G) MS and MS/MS spectra for peaks 1 (B), 2 (C), 3 (D), 4 (E), 5 (F), and 6 (G) in A. Fig. S2: Cytokine levels in supernatant of colon tissue culture. Fig. S3: The plasma levels of tHETE in DSS-treated mice fed yuzu peel or the standard diet. Fig. S4: Effects of yuzu peel extract on viability.

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Fig. 4 Antioxidant activity of yuzu peel extract. PBS extract of yuzu peel was used at 250-fold dilution with PBS. As positive control, hesperidin and ascorbic acid were dissolved in DMSO and PBS, respectively, to make the same concentrations as those of hesperidin and ascorbic acid in the extract.

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