Inhibitory Effects of Hana-yuzu (*Citrus hanaju*) on Mouse Stress-induced Gastritis and its Antioxidative Activities

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(Received February 10, 2010; Accepted March 5, 2010)

Abstract

The inhibitory effects of the oral administration of Hana-yuzu (*Citrus hanaju*) and New Summer Orange (*Citrus tamurana*) on mouse gastritis induced by cold-restraint stress and the relationship with their antioxidative activities were investigated. Whole pastes of Hana-yuzu and New Summer Orange significantly suppressed mouse gastritis at doses of 100 and 200 mg/kg body weight. The suppressive effect of Hana-yuzu tended to be higher than that of New Summer Orange. Among the parts of Hana-yuzu, the paste of the albedo at a dose of 200 mg/kg body weight and the aqueous extract of the flavedo at a dose of 200 μL/kg body weight significantly suppressed mouse gastritis. These samples also significantly suppressed the increases in lipid peroxide levels in the stomachs of mice with gastritis. In the *in vitro* experiments, the aqueous extracts of the flavedo and albedo showed strong activities as substrates for peroxidase and radical scavengers. Considerable amounts of some antioxidative components, β-cryptoxanthin, β-carotene, and auraptene, were determined in the pastes of Hana-yuzu flavedo and albedo. The sum of the flavedo and albedo weight accounted for ca. 82% of the whole Hana-yuzu. These results suggest that Hana-yuzu is an available food material for the prevention of gastritis and the antioxidative activities of its flavedo and albedo appear to contribute to the antiinflammatory effects.

Keywords: Hana-yuzu, Stress-induced gastritis, Antioxidant, Mouse

1. Introduction

Recent increases in the incidence rates of various inflammatory diseases, such as digestive tract ulcers, due to lifestyle changes, including dietary habits, have become serious social problems[1]. Hydrochloric acid and digestive enzymes, such as pepsin, in gastric juice and *Helicobacter pylori* in the gastric mucosa, are direct causative factors of human gastric ulcers, chronic gastritis, and/or gastric cancer[2-4]. Injury of the cells in the mucous membranes may provoke production of active oxygen species, such as nitrogen monoxide and superoxide anion radicals, by macrophages and neutrophils permeated into them. Active oxygen species injure the surrounding cells and
extracellular matrix, and produce lipid peroxides and metabolites of arachidonic acid. In general, active oxygen species are thought to promote inflammation through these processes[5,6]. With regard to gastric inflammation, it was found that the levels of lipid peroxides were elevated in rats exposed to water immersion restraint stress[7]. We reported previously that antioxidative food materials, such as oregano and acidic xylooligosaccharide, could suppress stress-induced gastric inflammation in mice[8,9]. It is well known that citrus fruits contain many antioxidative components[10] including α-tocopherol in the flavedo, β-cryptoxanthin and β-carotene as carotenoids in the flavedo and/or pulp, and auraptene[11] as an antiinflammatory coumarin in the flavedo and/or albedo, limonin[12] as an antioxidative limonoid in the flavedo and/or albedo, limonene[13,14] as an antiinflammatory monoterpenoid in the flavedo, nobiletin and hesperidin[15] as antioxidative flavonoids in the albedo, and ascorbic acid in the flavedo and/or pulp. The ingestion of citrus fruits is expected to prevent gastric inflammation. Some antioxidative constituents among these compounds are present in the pericarp of citrus fruits, the flavedo and albedo. Hana-yuzu (Citrus hanaju) and New Summer Orange (Citrus tamurana) are cultivated on the east coast of Izu area in Shizuoka Prefecture and their whole fruits are habitually eaten in Japan. In the present study, the inhibitory effects of these citrus fruits on mouse stress-induced gastritis and their antioxidative activities were investigated.

2. Materials and Methods

2.1 Materials

Hana-yuzu and New Summer Orange were cultivated at Shizuoka Prefectural Research Institute of Agriculture and Forestry. Whole pastes of these fruits and the pastes of flavedo, albedo, and pulp of Hana-yuzu were prepared by homogenization with 4 volumes of distilled water. The homogenates of the various parts of Hana-yuzu were centrifuged at 5000 rpm for 15 min and the supernatants were used as the aqueous extracts. Thiobarbituric acid (TBA) and horseradish peroxidase (POD) were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Other chemicals used were of reagent grade.

2.2 Mouse model of stress-induced gastric inflammation

Four-week-old male ddY mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The effects of various citrus samples on stress-induced gastric inflammation in mice were examined by a modification of the method of Chen et al.[16]. Briefly, mice were starved for 23 h but given tap water ad libitum. Whole pastes of Hana-yuzu and New Summer Orange were dissolved in 0.5 mL of 0.5% (w/v) tragacanth gum solution and administered orally to mice with a gastric sonde at doses of 50, 100, or 200 mg/kg body weight. The pastes and aqueous extracts of flavedo, albedo, and pulp of Hana-yuzu were administered orally to mice at doses of 200 mg/kg body weight and 200 μL/kg body weight. Mice were immobilized in stress cages and left at 4°C for 90 min under close observation to prevent death. Hydrocortisone, a well-known steroid-type antiinflammatory agent, was administered orally to mice as a positive control at the same doses as the samples. A control experiment was performed with only tragacanth gum solution. After stress loading, mice were immediately sacrificed under anesthesia with diethyl ether and the stomach was dissected out. The stomachs were incised in line with the greater curvature and the contents were washed out with
chilled saline. The number of bleeding points in the inner mucosa of the mouse stomach was counted. Each group consisted of 8 mice. Throughout the experiment, the animals were handled in accordance with “The Guide for Animal Experiments in the Numazu National College of Technology.”

2.3 Determination of lipid peroxide levels in the stomachs of mice administered Hana-yuzu samples

The stomachs of mice administered Hana-yuzu samples were homogenized in 9 volumes of 40 mM phosphate buffer (pH 7.4). Lipid peroxide levels were determined as TBA reactive substances in the mouse stomach according to the method of Yagi[17]. The contents were expressed in terms of malondialdehyde.

2.4 Evaluation of the activities of Hana-yuzu samples as substrates for peroxidase

The activities of the Hana-yuzu flavedo, albedo, and pulp aqueous extracts as substrates for peroxidase were determined by the method reported previously[18]. Briefly, reaction mixtures consisted of 5% (v/v) samples, 0.35 mM hydrogen peroxide, 0.25 mM 4-aminoantipyrine with or without 0.1 U/mL POD in 84 mM phosphate buffer (pH 7.0). The total volume was 1.0 mL. Reaction mixtures were incubated at 37°C for 10 min and 100 mL of 1 N hydrochloric acid was added to stop the reaction. The absorbance at 500 nm was determined immediately. As a negative control, phosphate buffer was added instead of samples. Scavenging rates of hydrogen peroxide were calculated.

2.5 Evaluation of the 1,1-diphenyl-2-picrylhydrazyl-scavenging activities of Hana-yuzu samples

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of the Hana-yuzu flavedo, albedo, and pulp aqueous extracts were determined by the method reported previously with slight modifications[19]. Reaction mixtures consisted of 6.7% (v/v) samples and 32 μg/mL DPPH in a total volume of 5.0 mL. These mixtures were incubated at 37°C for 10 min and chilled on ice water to stop the reaction. The absorbance at 525 nm was determined immediately. As a negative control, distilled water was added instead of samples. Scavenging rates of DPPH were calculated.

2.6 Determination of antioxidants in Hana-yuzu samples

Samples of 10 g of the Hana-yuzu flavedo, albedo, and pulp pastes were added to ethyl acetate-saturated sodium chloride solution (5:3) in a total volume of 40 mL. The mixtures were homogenized and centrifuged at 3000 rpm for 10 min. The upper layers were collected and evaporated in vacuo. Analytical sample solutions were prepared by dissolving the residues with 1.0 mL of acetone-ethanol (1:1). The contents of β-cryptoxanthin, β-carotene, and auraptene in the analytical sample solutions were determined by high performance liquid chromatography (HPLC) (model LC-10AD; Shimadzu Co., Kyoto, Japan). The following analytical conditions were used: column, Wakosil-II 5C18HG (5 µm, 150 × Φ 4.6 mm) (Wako Pure Chem. Ind.); column temperature, room temperature; elution solvent, 80% methanol; flow rate, 1.0 mL/min; detection, UVD (325 nm) (model SPD-10A; Shimadzu Co.). Identification of the compound was performed by comparison of the retention times of the samples and standard. Under these analytical conditions, β-cryptoxanthin and β-carotene were not isolated sufficiently and the contents of these compounds taken together were calculated as the contents of β-carotene. The chemical structures of β-cryptoxanthin, β-carotene, and auraptene are shown in Figure 1.
2.7 Statistical Analysis

Statistical analyses were performed with a non-parametric method, the Mann-Whitney U-test, to determine the significance of differences between the appropriate experimental groups. In all analyses, \( P < 0.05 \) was considered statistically significant.

3. Results and Discussion

3.1 Inhibitory effect of Hana-yuzu and New Summer Orange on stress-induced gastritis in mice

The inhibitory effects of whole pastes of Hana-yuzu and New Summer Orange on mouse gastritis are shown in Figure 2 and 3. In this study, cold-restraint stress was used to promote mouse gastritis, because it did not require induction with agents, such as ethanol and hydrochloric acid. So any direct interactions between the samples and inflammation-inducible agents were negligible. As indicated by the arrows in Figure 2, many bleeding points were observed in the gastric mucosa of mice subjected to cold-restraint stress. Oral administration of whole Hana-yuzu and New Summer Orange pastes and hydrocortisone at a dose of 200 mg/kg body weight reduced the number of bleeding points in the gastric mucosa. As shown in Figure 3, oral administration of whole Hana-yuzu and New Summer Orange pastes dose-dependently reduced the number of bleeding points in the gastric mucosa of mice subjected to cold-restraint stress, and the effects were significant at doses of 100 and 200 mg/kg body weight. Hydrocortisone also showed significant suppressive effects on mouse gastric inflammation at doses of 50, 100, and 200 mg/kg body weight. The activities of Hana-yuzu were significantly higher than those of New Summer Orange at doses of 100 and 200 mg/kg body weight (\( P < 0.05 \)).

Next, Hana-yuzu fruits were divided into three parts, i.e., the flavedo, albedo, and pulp, and pastes and aqueous extracts were prepared from each part. The yields of flavedo, albedo, and pulp from fruits of Hana-yuzu were 16.6, 17.1, and 7.3 g, respectively. The concentrations of nonvolatile components in the aqueous extracts of the flavedo, albedo, and pulp were 15.0, 21.4, and 18.0 mg/mL, respectively. The inhibitory effects of the pastes and aqueous extracts of the Hana-yuzu flavedo, albedo, and pulp on mouse gastritis are shown in Figure 4. Oral administration of

![Chemical structures of the antioxidative components of citrus fruits.](http://bigjohn.fukui-nct.ac.jp/journal/)

![Figure 2](http://bigjohn.fukui-nct.ac.jp/journal/) Inhibitory effects of oral administration of whole pastes of Hana-yuzu and New Summer Orange on mouse gastritis induced by cold-restraint stress. Dose, 200 mg/kg body weight. Arrows show the bleeding points.
the paste from the albedo at a dose of 200 mg/kg body weight and the aqueous extract of the flavedo at a dose of 200 μL/kg body weight significantly reduced the number of bleeding points in the gastric mucosa of mice subjected to cold-restraint stress. These results suggest that the flavedo and albedo, which account for ca. 82% of the whole Hana-yuzu, contribute to the inhibitory effects on mouse gastritis.

3.2 Lipid peroxide levels in stomach of mice subjected to cold-restraint stress and the effects of Hana-yuzu samples

The effects of the pastes and aqueous extracts of the Hana-yuzu flavedo, albedo, and pulp on lipid peroxide levels in the stomachs of mice affected with gastritis are shown in Figure 5. Lipid peroxide levels in the stomachs of mice with gastritis were significantly higher than those of normal mice (P < 0.05). This observation indicated a close relationship between the generation of stress-induced gastritis and the promotion of oxidative stress in mice. Oral administration of the Hana-yuzu flavedo, albedo, and pulp pastes at a dose of 200 mg/kg body weight and their aqueous extracts at a dose of 200 μL/kg body weight significantly reduced the lipid peroxide levels in

![Figure 3](image-url)  Inhibitory effects of oral administration of whole pastes of Hana-yuzu and New Summer Orange on mouse gastritis induced by cold-restraint stress. Means ± SE. n = 8. Dose: 50, 100, and 200 mg of paste/kg body weight. * P < 0.01, Significantly different from control value.

![Figure 4](image-url)  Inhibitory effects of oral administration of pastes and aqueous extracts of flavedo, albedo, and pulp of Hana-yuzu on mouse gastritis induced by cold-restraint stress. Means ± SE. n = 8. Dose: 200 mg of paste and 200 μL of solution/kg body weight. * P < 0.05, ** P < 0.01, Significantly different from control value.
mouse stomach, except for the aqueous pulp extract. Especially, oral administration of the albedo paste and the flavedo aqueous extract tended to be more effective than the other samples. The preventive effects of Hana-yuzu samples against oxidative stress in the stomach appeared to be closely related to the suppression of gastritis in mice.

3.3 Antioxidative activities of the Hana-yuzu flavedo, albedo, and pulp aqueous extracts in vitro

The activities of the Hana-yuzu flavedo, albedo, and pulp aqueous extracts as substrates for POD are shown in Figure 6(A). All of the sample solutions acted as substrates for POD, and the order of the activities was flavedo > albedo > pulp. The digestive tracts of animals contain salivary peroxidase (s-POD) and microperoxidase (m-POD). The usual substrates for these enzymes are thought to be SCN⁻ for s-POD and m-POD in the mouth, and Cl⁻ for s-POD and m-POD in the stomach. The flavedo and albedo of Hana-yuzu are expected to include some components that act as good electron donors for s-POD and m-POD and scavenge various peroxides.

DPHH radical scavenging activities of the aqueous extracts of the flavedo, albedo, and pulp of Hana-yuzu are shown in Figure 6(B). All of the sample solutions scavenged DPPH radicals and the order of the activities was the flavedo > albedo > pulp, the same as their activities as substrates for POD. The parts of Hana-yuzu with high antioxidative activities showed strong preventive effects on mouse stress-induced gastritis. Thus, the antioxidant activities of Hana-yuzu...
samples were thought to contribute, at least in part, to the preventive effects on mouse gastric inflammation.

3.4 Contents of β-cryptoxanthin, β-carotene, and auraptene in the pastes of Hana-yuzu flavedo, albedo, and pulp

The contents of β-cryptoxanthin, β-carotene, and auraptene in the pastes of Hana-yuzu flavedo, albedo, and pulp are shown in Figure 7. As described in the Methods section, the sum of the β-cryptoxanthin and β-carotene contents is presented in this study. In general, the content of β-cryptoxanthin is high in some citrus fruits with vermilion color, such as Unshu-mikan (Citrus unshiu). The order of the contents was flavedo > albedo > pulp for both β-cryptoxanthin + β-carotene and auraptene. The order was the same as that of antioxidative activities associated with these parts. The contents of β-cryptoxanthin + β-carotene and auraptene in the flavedo and albedo were much higher than those in the pulp. Therefore, β-cryptoxanthin, β-carotene, and auraptene would be related to the antioxidative activities of Hana-yuzu.

The results of the present study indicated that Hana-yuzu and New Summer Orange may be useful food materials for the prevention of gastritis, and this function is mainly related to the flavedo and albedo with high antioxidative activities. However, antioxidative activities were highest in the flavedo, whereas the preventive effects on mouse gastritis were strongest in the albedo. The dietary fiber pectin in the albedo may show simple protective effects against gastric juice by coating the surface of the mouse gastric mucosa. Further investigations are necessary to clarify the active components of Hana-yuzu involved in its preventive effects on gastritis and their mechanisms of action.

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花ユズ（Citrus hanaju）のストレス誘発マウス胃炎抑制作用とその抗酸化作用

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要旨
花ユズ（Citrus hanaju）とニューサーオレンジ（Citrus tamurana）の経口投与による、寒冷拘束ストレス誘発マウス胃炎の抑制とその抗酸化作用について検討を行った。花ユズとニューサーオレンジの果実全体をペーストにしたものは、100および200 mg/kg体重の投与量においてマウスの胃炎を有意に抑制した。花ユズの効果は、ニューサマーオレンジに比較して高い傾向が見られた。花ユズの様々な部位の中では、中果皮のペーストが200 mg/kg体重の投与量で、また外果皮の水抽出液が200μL/kg体重の投与量で、それぞれ有意な抗胃炎作用を示した。これらの試料は、胃炎を発症したマウスの胃中の過酸化脂質レベルの上昇も有意に抑制した。in vitroの実験において、外果皮と中果皮の水抽出物は、ペルオキシダーゼに対する基質として、あるいはラジカル捕捉剤としての強い活性を示した。さらに、外果皮と中果皮のペースト中に、抗酸化成分としてβ-クリプトキサンチン、β-カロテンおよびオーラプテンが検出された。外果皮と中果皮の重量の合計は、花ユズ全体の約82%を占めていた。これらの結果は、花ユズが胃炎の予防に有効な食品素材であり、この作用に外果皮と中果皮の抗酸化作用が寄与していることを示唆している。

キーワード：花ユズ、ストレス性胃炎、抗酸化作用、マウス