We investigated whether Hyuganatsu orange (Citrus tamurana Hort. ex Tanaka) contains water and acetic-acid soluble substances that increase bone mineral density (BMD) in ovariectomized rats. In in vivo study, femoral BMD can significantly increased. In in vitro study, tartrate-resistant acid phosphatase (TRAP) positive cells significantly decreased. We speculate that Hyuganatsu orange contains biologically active substances other than hesperidin that increase BMD.

Key words: Citrus tamurana; osteoporosis; bone mineral density; rat-preosteoclast cells; tartrate-resistant acid phosphatase positive cells

Osteoporosis compromises bone strength predisposing a person to increased risk of bone fracture and impaired quality of life. Since it is more prevalent in older women than in older men, exploring methods of preventing osteoporosis is important for gynecologists dealing with postmenopausal women. It has been reported that various citrus fruits contain biologically active substances¹⁻³ that prevent osteoporosis. These substances include, for example, β-cryptoxanthin, isoflavone, and hesperidin, which are extracted with hexane and methanol.¹⁻³ However, to our knowledge, there is no report on water-soluble substances in citrus fruits. The hyuganatsu orange (Citrus tamurana Hort. ex Tanaka) originated in Miyazaki Prefecture Japan, and is unique because the albedo part is edible, making the fruit sweeter, suggesting that it contains biologically active substances. In ancient China, the peel of the unshu orange used as an herbal medicine. This means that the ancient Chinese knew that orange peel contains biological active substance. In Chinese herbal medicine, water extraction is a common method of preparing drugs. Since, it is very difficult to determine the structure of the water-soluble substances, extraction with an organic solvent such as ethyl acetate is a common method in modern pharmacology. To our knowledge, no organic solvent soluble substance from orange, one that suppresses osteoclast formation, has been isolated. Hence, we decided to investigate the effects of the hyuganatsu orange on ovariectomized rats. In an unpublished pilot study, administration of homogenate of hyuganatsu orange at about 5 g/kg increased BMD in ovariectomized rats, but the hexane and methanol extracts did not contain biological active substances strengthening bones. Hence, we hypothesized that a water extract or acetic acid extract of hyuganatsu orange increases bone mineral density in ovariectomized rats, an animal model of osteoporosis, and affects bone loss.

Dried, powdered residue of hyuganatsu orange-juice was provided by Nokyo-Kaju Co., Ltd., (Miyazaki, Japan). Flow chart of isolation procedures is shown in Fig. 1. The isolation procedure was based on a review,⁴ with modifications. Briefly, the hyuganatsu powders were first mixed well and extracted using hexane over 6 h, and filtrated. The hexane extract was evaporated in vacuo. The residues were then mixed and extracted using 10% methanol over 2 h. The methanol extract was isolated by filtration and evaporated in vacuo. These two procedures removed β-cryptoxanthin, isoflavone, and hesperidin. The residues were further extracted using water at 60 °C over 2 h (water extract), followed by using 1 M acetic acid for 2 h (acetic acid extract). Both the water and the 1-M acetic acid extracts were evaporated and dried for later use. For the experiments, they were dissolved in phosphate buffer saline and sterilized with a 0.2-micron filter.

In vivo study: The animal experiments were approved by the Animal Care and Use, Committee of the Faculty of Medicine of the University of Miyazaki. In total, 24 10-week-old female Wistar rats were ovariectomized (n = 19) or sham-operated (n = 5) under ether anesthesia. In the pilot study, 5 g wet weight/kg/d of hyuganatsu orange homogenate increased bone mineral density. We assumed about that 0.5 g/kg/d was adequate amount in an water extract. Nineteen ovariectomized rats were divided into 3 groups. Six rats were ovariectomy only (OVX control). Six were treated with 0.25 g/kg/d of the hyuganatsu water extract. The
remaining seven were treated with 0.7 g/kg/d of the water extracts. The water extract was given once daily with a gastric tube. In a sham operation and the OVX control group, water was given with a gastric tube. All the rats were maintained at the Animal Center of University of Miyazaki with water and food ad libitum. After 8 weeks, they were sacrificed and the femoral bones were collected. BMD was measured by dual energy X-ray absorptiometry at 25 mm from the proximal edge (2 mm width) at a scan speed of 25 ms (DEXA; DCS-600EX-IIIR, Aloka, Tokyo). Bone tissue specimens were prepared by Kureha Special Laboratory (Iwaki, Japan). Osteoblast cell surfaces, the surface of bone resorption and the osteoclast cell numbers were analyzed by histomorphometry as a parameter of bone formation,19 described as Ob.S/BS in the lower panel of Fig. 2, increased. At the same time, the parameter of bone absorption, surfaces of bone resorption (N.Oc/B.Pm/100 mm), and osteoclast cell numbers (Oc.S/BS) were decreased by the water extracts. However, due to the small number of histomorphological data, the osteoblast cell surfaces, a parameter of bone formation and bone absorption.

**In vitro** study: Rat pre-osteoclast cells (Primary Cell, Ishikari, Japan) were cultured and water extract or acetic acid extract was added to the culture medium in a dose-response manner (0.05, 0.2, and 0.5 mg/mL final concentration) following the manufacturer’s instruction manual.33 The cells were maintained in culture medium (α-MEM medium containing 10% fetal calf serum, 10 units/mL, penicillin, and 10 μg/mL, streptomycin) in a 5% CO₂ incubator. After 4 h of pre-incubation, osteoblast cells were treated with the water extract or the acetic acid extract at concentrations of 0.05 mg/mL, 0.2 mg/mL, and 0.5 mg/mL. Since the hyuganatsu treated cells became confluent within 3 d, their log proliferation phase was arbitrarily set at 24 h of culture. After 24 h of incubation, 10 μL of Tetracolor One™ (Seikagaku, Tokyo) was added to each well. The optical density was measured at 450 nm (Micro Titer Plate Reader, ImmunoMini J2300, System Instruments, Tokyo).

Statistical evaluation: Mann-Whitney test was used to analyze differences in bone mineral density. One-way ANOVA followed by the Bonferroni/Dunn test were used to analyze osteoclast and osteoblast cell culture data. The data were expressed as mean ± SD, and p < 0.05 was considered statistically significant.

Aging induces osteoporosis with accompanying decreases in BMD. Osteoporosis is now widely recognized as a major public health problem. The symptomatic bone loss results from decreased bone formation and increased bone resorption. Recently, many plants have been reported to be effective in preventing osteoporosis, e.g., herbal extracts,6–8 soy bean,9–11 black cohosh,12,13 garlic,14 onion,15 and grape seed.16 Tylavsky et al.17 have reported that fruit and vegetable intake is an independent predictor of bone size in early pubescent children. The albedo of the Hyuganatsu orange itself is not sweet, and the fruit is very sour. However, if one eats the fruit with the albedo, it tastes sweet. Citrus species contain biologically active factors. For example, Hosseimimehr and Karami18 reported that citrus extract modulates the genotoxicity induced by cyclophosphamide in mouse bone-marrow cells. Deyhim et al.2 reported that citrus prevents osteoporosis of orchidectomized rats by enhancing serum antioxidant status. Chiba et al.23 reported that hesperidin, a citrus flavonoid, inhibits bone loss in ovariectomized mice. Yamaguchi and Uchiyama19 reported that β-cryptoxanthin is the main substance of Citrus unshiu that inhibits osteoclast formation. On the basis of these results, we previously used hexane and methanol extracts, which did not increase bone mineral density. Hence, we assumed that hyuganatsu contains water-soluble substances that can suppress bone loss. As Fig. 2 indicates ovariectomy significantly decreased femoral bone BMD as compared with sham-operated ones. The water extract of hyuganatsu significantly increased BMD at 0.25 g/kg/d and 0.7 g/kg/d as compared with the ovariectomized controls, but not with the sham-operated ones. In histomorphological data, the osteoblast cell surfaces, a parameter of bone formation,29 described as Ob.S/BS in the lower panel of Fig. 2, increased. At the same time, the parameter of bone absorption, surfaces of bone resorption (N.Oc/B.Pm/100 mm), and osteoclast cell numbers (Oc.S/BS) were decreased by the water extracts. However, due to the small number of histomorphological analyse, further investigation is necessary. The effects of the water and acetic acid extracts on TRAP

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**Fig. 1.** Extraction Scheme of the Water Fraction and the Acetic Acid Fractions of Hyuganatsu Orange

β-Cryptoxanthin is extracted in the hexane fraction (fraction 1) and hesperidin is extracted in the methanol fraction (fraction 2).
positive osteoclast cells are shown in the upper panel of Fig. 3. Both the water and the acetic acid extract significantly decreased the number of TRAP-positive cells in a dose-dependent manner. The mechanism of action of the biologically active substances is not fully understood. Since free radicals suppress osteoblast formation and increase osteoclast formation,20 one possible mechanism is that the water extract suppressed free radical formation.

Some plants decrease osteoclast formation by modulation of the receptor activator of nuclear factor kappa-B ligand (RANKL)12,21,22 or core binding factor alpha1. Especially, RANKL plays a key role in osteoclast formation. Flavonoids,6,22 isoflavones and phytoestrogens10,23 contained in plants might decrease osteoclast formation by a RANKL-related mechanism. There is a report that hesperidin affects bone anabolism.24–25 Orange peel is known to contain high levels of hesperidin.26 Our results might be explained by herperidin. However, hesperidin and β-cryptoxanthin are lipophilic and alcohol soluble substances, respectively.13 By our procedure, most of the flavonoids were removed. The water and acetic acid fraction contained only 0.1% low molecular weight substances, such as flavonoids. Hence, we concluded, our result cannot be explained by hesperidin.

The effects of the water and acetic acid extracts on osteoblasts are shown in lower panel of Fig. 3. The water extracts significantly increased the optical density of 450 nm in a dose-dependent manner, suggesting the osteoblast cell numbers increased. However, the acetic acid extracts did not show any significant effects on osteoblast activity. We speculate that the biologically active substance in the water fraction is different from the active substance in acetic acid fraction. In other words, hyuganatsu orange may contain at least two biologically active substances.

In conclusion, the hyuganatsu orange contains biologically active water-soluble substances, possibly other than hesperidin, which inhibit bone loss.

References


