ORYZA OIL & FAT CHEMICAL CO., LTD.

CITRUS UNSHIU EXTRACT

Health Ingredient for prevention of osteoporosis

Health Ingredient for whitening and aesthetic

Ingredient for cosmetics

- **CITRUS UNSHIU EXTRACT-P**

  (Powder, Food Grade)

- **CITRUS UNSHIU EXTRACT-L**

  (Liquid, Food Grade)

- **CITRUS UNSHIU EXTRACT-PC**

  (Powder, Cosmetic Grade)

- **CITRUS UNSHIU EXTRACT-LC**

  (Liquid, Cosmetic Grade)

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Ver.4.1 YF

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1. Introduction

Advancement in science & technology and higher standard of living have significantly increased life expectancy in human. Japan is the nation with the highest longevity in the world. However, incidence of osteoporosis increases with increasing life expectancy among the aged population. Osteoporosis is a major and growing public health concern especially for older women and men. Incidence of post-menopausal osteoporosis is high in women aged 50 and above and is classified as primary osteoporosis. More than 90% post-menopausal women are diagnosed with osteoporosis. Risk for dementia increases as complications of osteoporosis related bed-ridden condition

Osteoporosis, or porous bone, is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures of the hip, spine and wrist. Men as well as women suffer from osteoporosis, a disease that can be prevented and treated. Osteoporosis is a multifactorial disease and is influenced by factors like nutritions, hormone and physical activity. The process of bone resorption and reformation is continuous and osteoclasts are important in the regulation of bone metabolism. Osteoblasts decrease with age while growth, maintenance and constant repair of bone tissue depend on the balance between rates of bone resorption and bone formation. Osteoporosis occurs when rate of bone resorption is greater than bone formation leading to loss of bone mass.

Adequate calcium intake, timely use of estrogen replacement therapy, activated vitamin D3, ipriflavone, vitamin K2 and bisphosphonate-related compound have been identified to prevent osteoporosis and constrain bone loss. However, therapeutic treatment remains a formidable challenge, e.g., poor absorption of calcium, risk of cancer development with long term administration of estrogen replacement therapy. Hence, development of preventive / therapeutic agents or supplements that prevent osteoporosis is essential.

In addition, desire to remain youthful and healthy has been increasing in recent years. UV radiation, sedentary lifestyle, diet and usage of medicines in the modern society are major risk factors of aging resulting in the appearance of fine lines and wrinkles. Whitening products has

captured a major share in the cosmeceutical industry in Asia. Fair, silky skin has emerged as the concept of beauty.

Studies conducted in Oryza Oil & Fat Chemical Co., Ltd. confirmed the beneficial effects of Citrus Unshiu Extract on prevention against osteoporosis and aesthetic enhancement among ladies. Citrus Unshiu Extract is highly recommended as functional ingredient for nutraceutical and cosmeceutical industries.

2. What is Citrus Unshiu ?

Unshiu mikan or ***Citrus Unshiu Marc*** is commonly known as ***Satsuma, Satsuma Mandarin*** or ***Satsuma Orange*** in the Western society. Unshiu mikan is originated from Kagoshima (Satsuma).

There are approximately 900 species of citrus in the world. Japanese oranges are unique to Japan and appeared 1,200 years ago. It was regarded as fruit for perpetual youth and longevity as described in Kojiki and Nihon Shoki.

Japanese seedless orange was generated by mutation 400 years ago. The name Unshiu mikan originated from Unshiu area in China where it is famous for production of oranges. There are various type of Citrus Unshiu, e.g. "Kumamoto mikan", "Ehime mikan", "Arita mikan" and "Sizuoka mikan".

Citrus Unshiu is listed in the Jouhon in Shinnouhonzoukyou as follows:

[Origin] Mature pericarp of citrus unshiu or related plants of the rue family

[Pharmacological action] CNS depression, anticonvulsant, anti-inflammatory & anti-allergic

[Indications] Anorexia, diarrhea, vomiting, pain, cough (as expectorant and anti-tussive)

Chinpi, mandarin orange peel, and mandarin chinpi are dried citrus unshiu peels. They are also referred to as mandarin.

Citrus unshiu (fruit)

Citrus unshiu (peel)

3. Functional Components of Citrus Unshiu Extract

Citrus unshiu is rich in carotenoids, (β-cryptoxanthin, β-carotene), coumarins (auraptene, etc), limonoids (limonins, etc), and flavonoids (tangeretin, nobiletin, hesperidin).

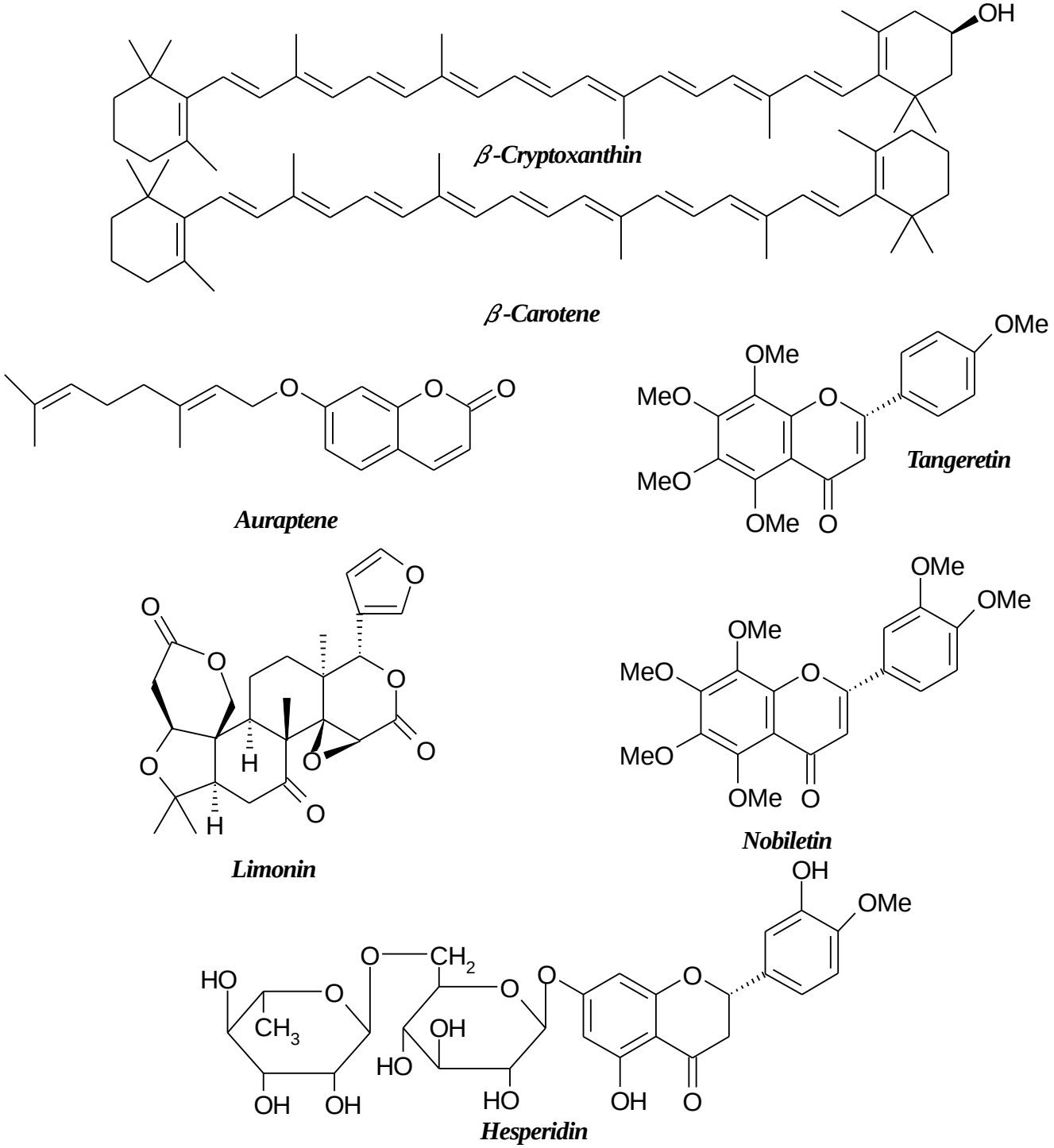


Fig. 1. Functional Components of CITRUS UNSHIU EXTRACT

4. Pathophysiology
(A) Pathophysiology of Osteoporosis
1) Bone Remodeling

Growth and development of endochondral bone are driven by a process called modeling. Once new bone is laid down, it is subject to a continuous process of breakdown and renewal called remodeling that continues throughout life. New bone is formed every 3-5 months and 30% of the entire bone is remodeled every year. There are 2 types of bone cells, osteoclasts and osteoblasts that are responsible for bone resorption and bone formation respectively. When old bone is remodeled, osteoclasts will dissolve the bone initiating the process of bone resorption. Next, new matrix is secreted by osteoblasts with the initiation of calcification followed by mineralization of the new matrix leading to formation of new bone. The combination of simultaneous resorption and deposition creates continual remodeling of bone while excess osteoclasts activity leads to an imbalance and a loss of bone density, causing osteoporosis.

2) Osteoporosis and Menopause

Osteoporosis is a condition of low bone mass and the microstructure disruption that results in fractures with minimal trauma. [Diagnostic criteria for primary osteoporosis (2,000 revised version), Osteoporosis Japan 9 (1): 9-14, 2001]

Hunchback and bent hips are now considered as a phenomenon of osteoporosis. “Porosis” is defined as coarse, sparse and pine needle-like transparent condition. Osteoporosis occur when the inner region of bone, substantia spongiosa become porous. Bone resorption reduces bone mass of both vertical trabecula and the non-weight bearing horizontal trabecula. This is visible as large holes under X-ray examination. Thus, bone is porous, fragile and susceptible to fractures.

Primary Osteoporosis was composed of two separate entities: one related to menopausal estrogen loss, and the other due to aging. The latter occurs when the rate of bone resoprtion is greater than bone formation and commonly found in individuals of both sexes aged 75 years and above. Postmenopausal osteoporosis occurs in women upon menopause when estrogen production is ceased. However, estrogen related osteoporosis also occur in young women with low estrogen level due to malfunction of the ovaries, stress and sedentary lifestyle. Nutrition imbalance and reduction in female hormones, estrogen, may cause a rapid loss of bone mass.

What are the effects of estrogen? In the mid-follicular phase of female maturation stage, 1ml of blood contains 100-200pcg(pg, 1/trillion g) of estrogen (E2). This level decreases to 30-50pcg during menopause and remained at 10pcg or lower after menopause. Bone mineral density changes with the amount of estrogen in the body. Estrogen secretion decreases when ovarian

function stops after menopause. This results in a reduction of activated vitamin D in the body. Activated vitamin D is essential for calcium absorption in the intestine. As a result, intestinal calcium absorption markedly reduced causing a reduction in blood calcium concentration. Thus, parathyroid hormone (PTH), which is responsible for maintenance of a constant blood calcium level, is secreted. Osteoclasts are activated to initiate bone resorption for the release of calcium into the circulation. Estrogen, which inhibits calcium resorption is not secreted in a similar way. Bone resorption continues to progress causing loss of bone mass, hence osteoporosis.

Development and advances in modern medicine and improvement of quality of life successfully increased the mean life expectancy of women. However, estrogen related menopausal symptoms and osteoporosis are major health concerns.

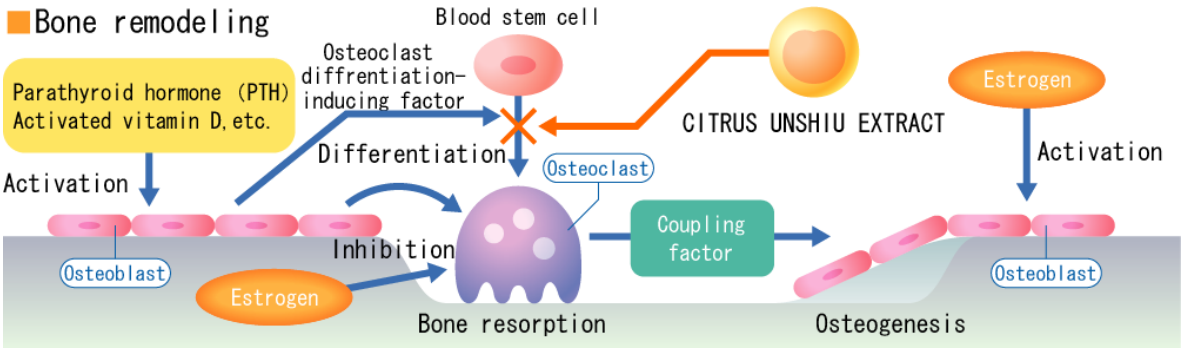


Fig. 2. Pathophysiology of Osteoporosis (Bone Remodeling)

(B) Melanin Production

Melanin - pigment that gives the skin color and protects the underlying skin against damage by ultraviolet light; produced by melanocytes in the inner layer of the epidermis. However, aging, hormonal change and over exposure to ultraviolet (UV) light increases the pigmentation levels resulting in the appearance of fine lines, freckles and age spots.

Upon exposure to UV light, series of signal transduction occur where enzyme tyrosinase is activated to convert tyrosine to dopa, and later to dopa quinone. Dopa quinone is further oxidized to melanin. In addition, Stem Cell Factor (SCF) has been found to play an important role in skin hyperpigmentation. Upon exposure to UV light, SCF and endothelin activate chromocytes that stimulate the production of melanin pigment as a result of natural defense mechanism to protect skin from harmful effect of UV radiation. Turn over of skin tissues occur in a 28-day cycle when the melanin and old cells are removed and replaced by new skin cells. However, metabolic rate declines with age resulting in an accumulation of melanin pigment over time, hence hyperpigmentation.

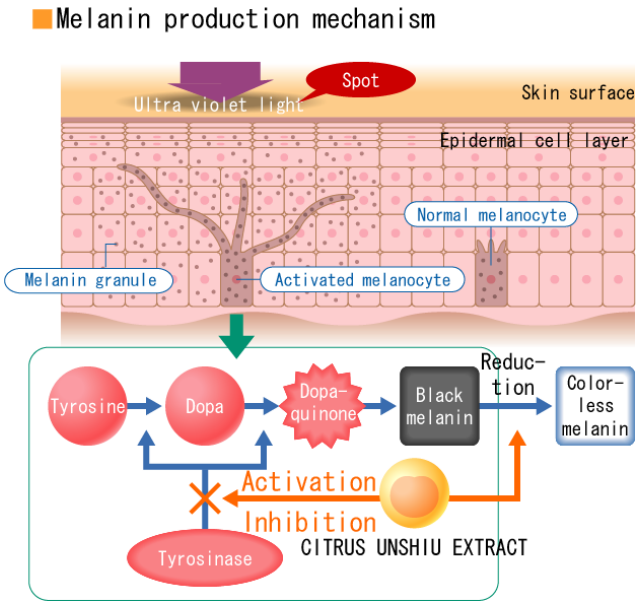


Fig. 3. Pathophysiology of Melanin Production

5. Beneficial Effects of Citrus Unshiu Extract

(A) Prevention of Osteoporosis

1) Inhibition of Osteoclasts Production

Osteoclasts are multinucleated cells associated with bone resorption process. Histologically, osteoclasts are tartaric acid-resistant, acidic phosphatase-positive multinucleated cells. Osteoclasts usually present in large quantities in porous bone surface. Bone density and bone mass is maintained through a balance of continuous process of bone resoprtion by osteoclasts and bone formation by osteoblasts. Recent studies suggested that estrogen receptor α is present in osteoclasts and effectively inhibits bone resorption. Production of estrogen decreases in menopause causing an imbalance in bone metabolism. Osteoporosis arises as rate of bone resorption increases.

Theoretically, inhibiting production of osteoclasts may prevent activity of bone resorption, hence prevention against osteoporosis. Study was conducted to examine the effect of Citrus Unshiu Extract on osteoclasts.

Osteoclast cells culture kit (by Hokudo Co.) was used. Study shown that **β-cryptoxanthin** of Citrus Unshiu Extract inhibited the production of osteoclasts at concentration 0.05µg/ml and 0.5µg/ml (as illustrated in Figure. 4). Citrus Unshiu Extract is beneficial in the prevention of osteoporosis with its inhibitory effect on osteoclasts production.

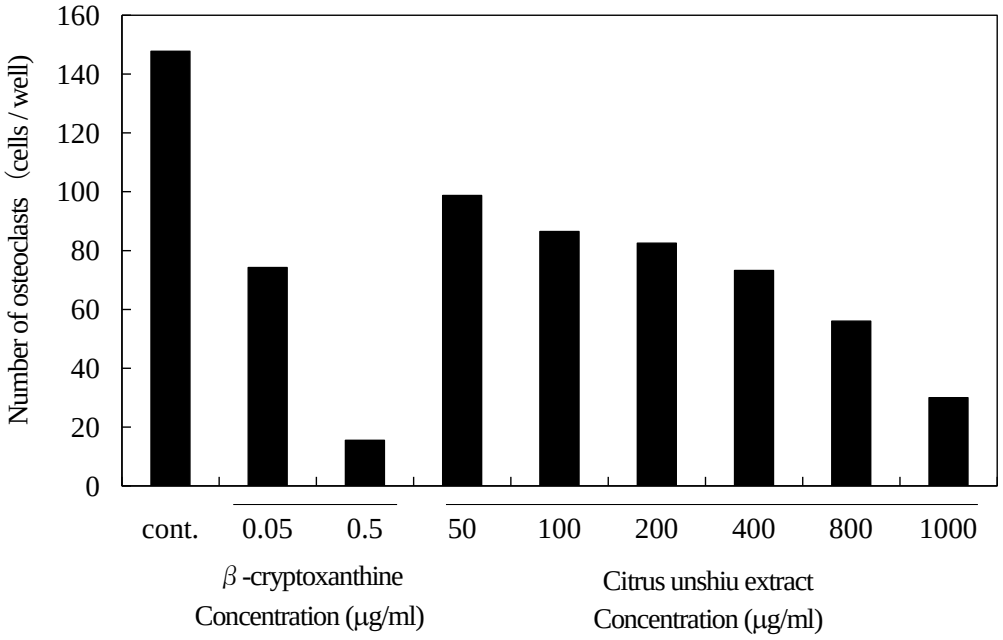


Fig. 4. Inhibition of Osteoclasts Production by Citrus Unshiu Extract

2) Prevention of Osteoporosis (*in vivo*)

With reference to the above finding, further investigation was prompted using an osteoporosis model in mouse whose ovary has been removed.

Method:

Ovariectomized (OVX) mice (ICR, female, 5 weeks old) were prepared by removing the bilateral ovaries under anesthesia. Meanwhile, mice with intact ovaries, sham-operated mice (Sham) were used as control. Powdered feed (AIN-93G) ad libitum was given to mice for 7 days. The average intake of food is 10g/day/mice in both OVX and Sham model. The mice were divided into 4 groups with different diet composition as tabulated below:

	Diet
Control	50% (5g/day)
MX-1	50% diet + 1% citrus unshiu extract
MX-2	50% diet + 2% citrus unshiu extract
Sham	AIN-96 ad libitum

· Diet composition was prepared according to the literatures below:

※ Man S. L., Tamaki H., Ohta Y., Katsuyama N., Chinen I. Effect of exercise on osteopenia caused by restricted food intake in rats. *J. Jpn. Soc. Nutr. Food Sci.*, **56**, 237-242 (2003).

※ Man S. L., Ohta Y., Katsuyama N., Tamaki H., Oku H., Chinen I. Effect of estrogen on osteoporosis caused by restricted food intake in rats. *J. Jpn. Soc. Nutr. Food Sci.*, **55**, 149-155 (2002).

Urine deoxypyridinoline concentration, bone mineral density and bone strength in mice were measured in week 2 and week 4. Results are shown in Table 1, 2 & 3.

i) Urine Deoxypyridinoline Concentration

Bone resorption is usually determined using urine deoxypyridinoline concentration as parameter. Concentration of urine deoxypyridinoline is usually high in osteoporotic and menopausal individuals. Concentration of urine deoxypyridinoline is significantly reduced in MX-1 & MX-2 groups (as shown in Table 1) in which Citrus Unshiu Extract were fed.

Table 1. Effect of Citrus Unshiu Extract on Urinary Deoxypyridinoline Concentration in Ovariectomized Mice

	2 weeks	4 weeks
Control	38.4±4.4 (ng/mL)	22.7±12.5
MX-1	**29.2±7.0**	**16.5±7.5**
MX-2	**33.6±11.3**	23.9±13.4
Sham	24.9±0.5	19.0±4.6

MX-1 : Citrus unshiu extract 1% Mean±SD, n=4
MX-2 : Citrus unshiu extract 2%
Sham : Sham-operated group

ii) Bone Mineral Density

Bone mineral density was measured using pQCT method. The metaphysis and disphysis of mice femoral bone were measured. The bone mineral density in MX-2 group fed with 2% Citrus Unshiu Extract was significantly higher (as shown in Table 2).

Table 2. Effect of Citrus Unshiu Extract on Bone Mineral Density in Ovariectomized Mice

	Cancellous bone	Cortical bone
Control	297±13 (mg/cm^3)	978±38
MX-1	281±46	987±49
MX-2	**302±28**	**994±29**
Sham	521±67	1117±26

MX-1 : Citrus unshiu extract 1% Mean±SD, n=4
MX-2 : Citrus unshiu extract 2%
Sham : Sham-operated group

iii) Bone Strength

Similarly, bone strength of the metaphysis and disphysis of mice femoral bone was measured using a 3-point bending test. Table 3 shown that MX-2 group fed with 2% Citrus Unshiu Extract has a significantly higher fracture displacement value.

Table 3. Effect of Citrus Unshiu Extract on Bone Strength in Ovariectomized Mice

	Bone fracture force	Fracture displacement
Control	54.7±12.5 ($\times 10^{-5}$N・m)	0.78±0.31 (mm)
MX-1	44.2±10.3	0.70±0.20
MX-2	**71.6±26.2**	**1.07±0.49**
Sham	82.3±15.4	0.60±0.07

MX-1：Citrus unshiu extract 1%
MX-2：Citrus unshiu extract 2%
Sham：Sham-operated group
Mean±SD, n=4

(B) Skin Whitening and Aesthetic Effect

1) Inhibition of Tyrosinase

As mentioned above, tyrosinase is the enzyme responsible for skin hyperpigmentation. As shown in Figure. 5, Citrus Unshiu Extract effectively inhibited the activity of tyrosinase, thus prevents against skin hyperpigmentation.

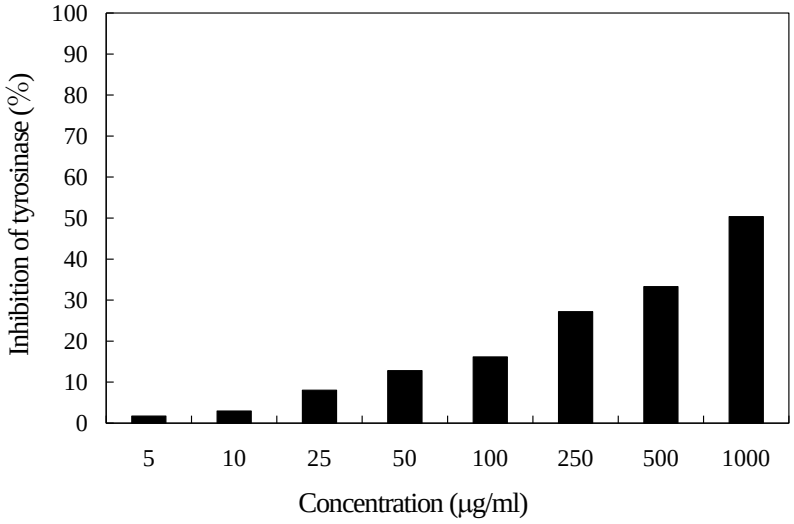


Fig. 5. Inhibition of Tyrosinase by Citrus Unshiu Extract

2) Inhibition of Melanin Production in B16 Melanoma Cells

The effect of Citrus Unshiu Extract on melanin production was examined using B16 melanoma cells. B16 melanoma cells are melanin pigment producing cells. Production of melanin pigment was significantly inhibited in culture cells supplemented with Citrus Unshiu Extract (as illustrated in Figure.6)

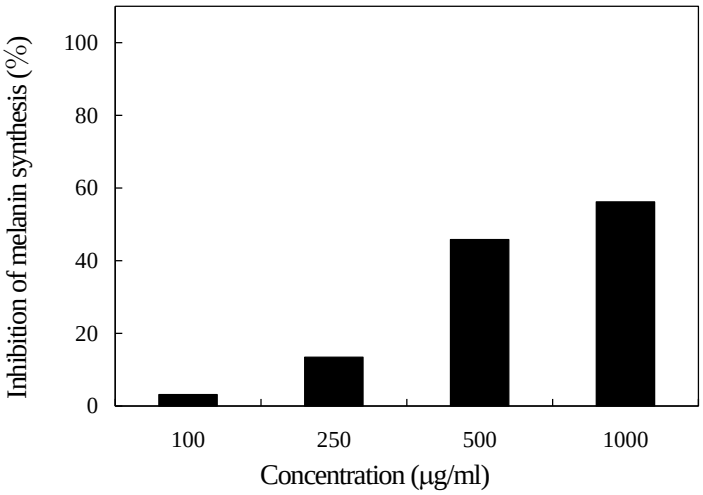
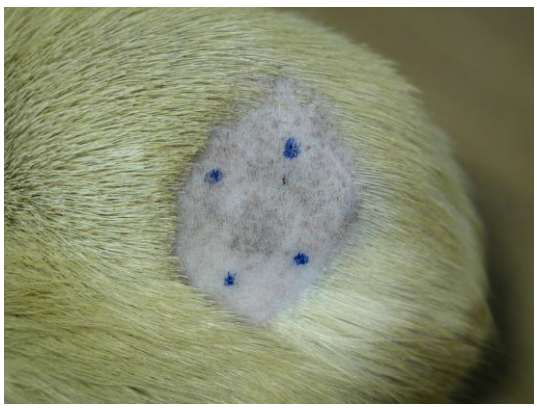


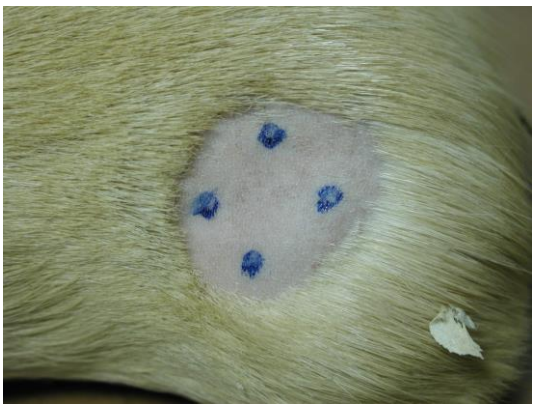
Fig. 6. Inhibition of Melanin Production by Citrus Unshiu Extract in B16 Melanoma Cells

3) Inhibition of Pigmentation

The whitening effect of Citrus Unshiu Extract was further investigated using UV induced hyperpigmented skin. Oral Citrus Unshiu Extract was given to guinea pigs for 8 days prior to UV irradiation. UV-B was irradiated at 1,000mJ/cm2 for 3 days, the brightness (L* value) was measured on day 0, 4, 6 and 10 using spectrophotometer. As illustrated in the photos bellow and Figure. 7, the hyperpigmented area faded significantly in group fed with Citrus Unshiu Extract 800mg/kg.



Control



800 mg/kg

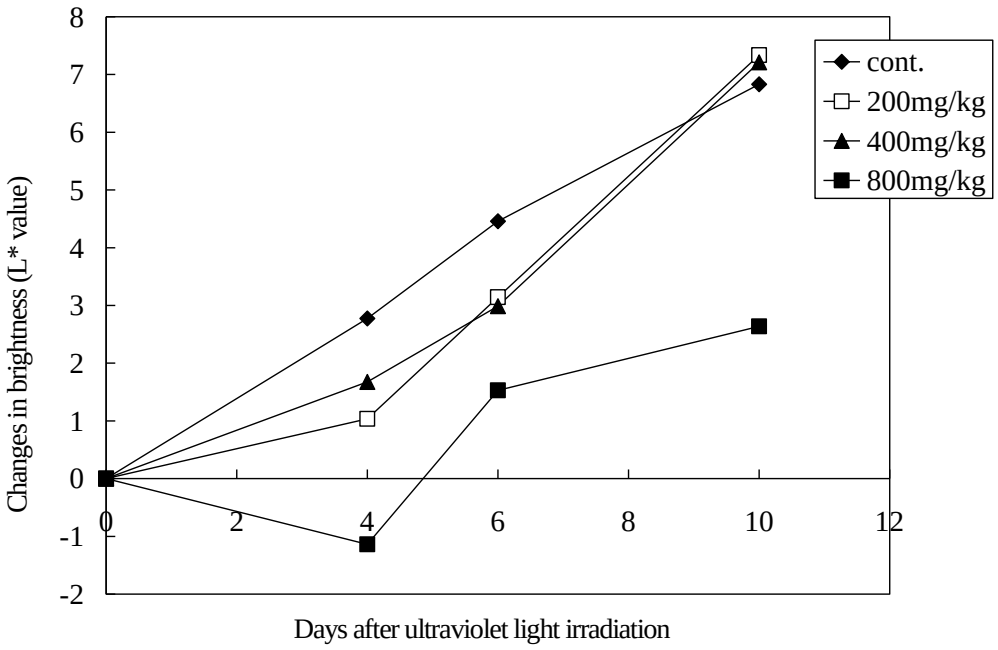


Fig. 7. Inhibition of Pigmentation by Citrus Unshiu Extract

4) Inhibition of Skin mRNA Expression Related to Pigmentation in UV-irradiated Mice

We evaluated the effect of Citrus Unshiu Extract containing 0.5% beta-cryptoxanthin on skin pigmentation in UV-irradiated mice by topical and oral application. As shown in Fig. 8, topical application of the extract reduced pigmentation.

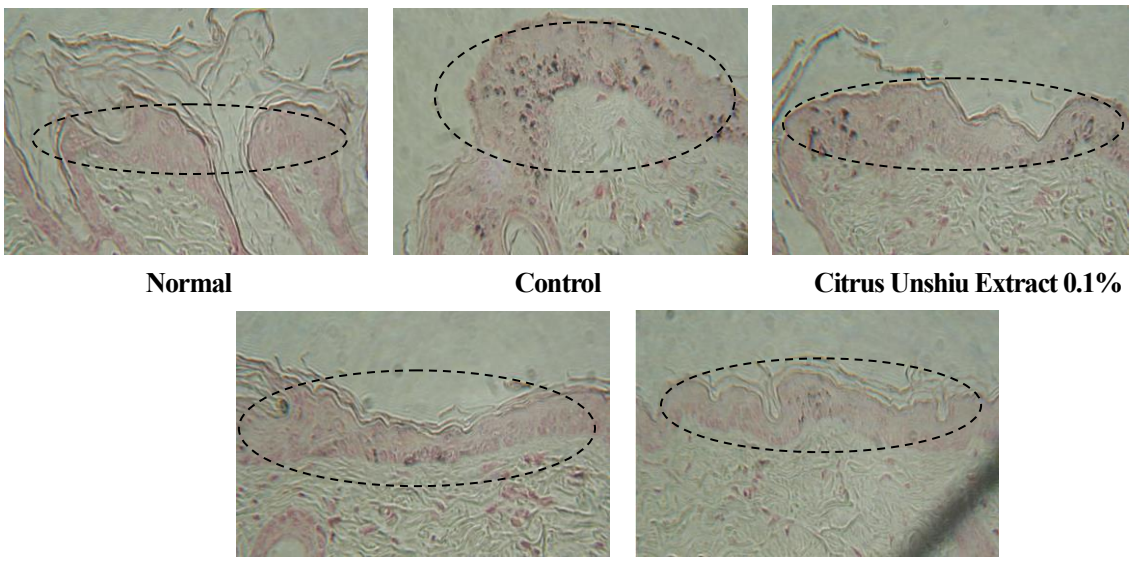


Fig. 8. Microscopic image of mouse epidermis topically treated with Citrus Unshiu Extract (x 200, Fontana-Masson staining)

In the mouse skin treated with UV, mRNA expression related to pigmentation was enhanced (Table 4). Citrus Unshiu Extract significantly suppressed mRNA expression of tyrosinase, tyrosinase related protein, melanocortin receptor 1, COX-2 and endothelin A receptor.

Table 4. Effect of topical treatment of Citru unshiu extract on skin mRNA expression related to pigmentation in UV-irradiated mice.

	Normal	Control	Citrus unshiu extract		Arbutin
			0.1%	1%	1%
Tyrosinase	0.26±0.06	1.00±0.14	1.25±0.23	**0.40±0.02** ↓	**0.27±0.07** ↓
Tyrosinase related protein	0.28±0.08	1.00±0.16	1.15±0.26	**0.39±0.03** ↓	**0.32±0.10** ↓
Melanocortin receptor 1	0.16±0.04	1.00±0.23	0.91±0.15	**0.23±0.01** ↓	**0.25±0.07** ↓
COX-2	0.51±0.16	1.00±0.18	**0.72±0.08** ↓	**0.21±0.01** ↓	**0.23±0.05** ↓
Endothelin A receptor	0.23±0.04	1.00±0.21	**0.68±0.08** ↓	**0.23±0.01** ↓	**0.23±0.01** ↓

Similar result was obtained by oral administration of Citrus unshiu extract (Table 5).

Table 5. Effect of oral treatment of Citru unshiu extract on skin mRNA expression related to pigmentation in UV-irradiated mice.

	Normal	Control	Citrus unshiu extract		Arbutin (mg/kg)
			10 (mg/kg)	100	10
Tyrosinase	0.92±0.04	1.00±0.03	1.06±0.01	1.11±0.09	0.94±0.07
Tyrosinase related protein	0.89±0.01	1.00±0.04	1.09±0.03	0.95±0.09	**0.84±0.01** ↓
Melanocortin receptor 1	0.84±0.05	1.00±0.07	**0.75±0.02** ↓	**0.61±0.03** ↓	**0.45±0.02** ↓
COX-2	1.88±0.07	1.00±0.03	0.92±0.01	0.95±0.04	**0.87±0.02** ↓
Endothelin A receptor	0.92±0.02	1.00±0.03	**0.82±0.01** ↓	**0.73±0.01** ↓	0.95±0.02

5) Aniti-melanogenic Activity of β-CPX

We investigated the anti-melanogenic action and its mechanism of β-CPX stimulated by prostaglandin (PG) E_2, melanocyte-stimulating hormone (MSH) and endothelin (ET-) 1. β-CPX (10 μg/mL) suppressed melanogenesis induced by PGE_2, MSH and ET-1 (Fig. 9-11). In the PGE_2-stimulated melanocytes, mRNA expressions of EP-1, Tyr and Tyrp1 and phosphorylation of CREB protein were suppressed. In the ET-1-stimulated cells, only expression of CREB protein was suppressed. In the MSH-induced cells, mRNA expression of MC1R and Tyrp1 and protein expression of CREB were suppressed. In conclusion, suppression of melanogenic enzymes, receptors of melanogenic stimulators, expression and phosphorylation of CREB is thought to be involved in the mechanism.

Shimoda H., Shan S.H., Tanaka J., Maoka T., β-Cryptoxantin suppressed UVB-induced melanogenesis in mouse: involvement of the inhibition of prostaglandin E_2 and melanocyte-stimulating hormone pathway. *J. Pharmacy Pharmacol*. 64, 1165-76 (2012).

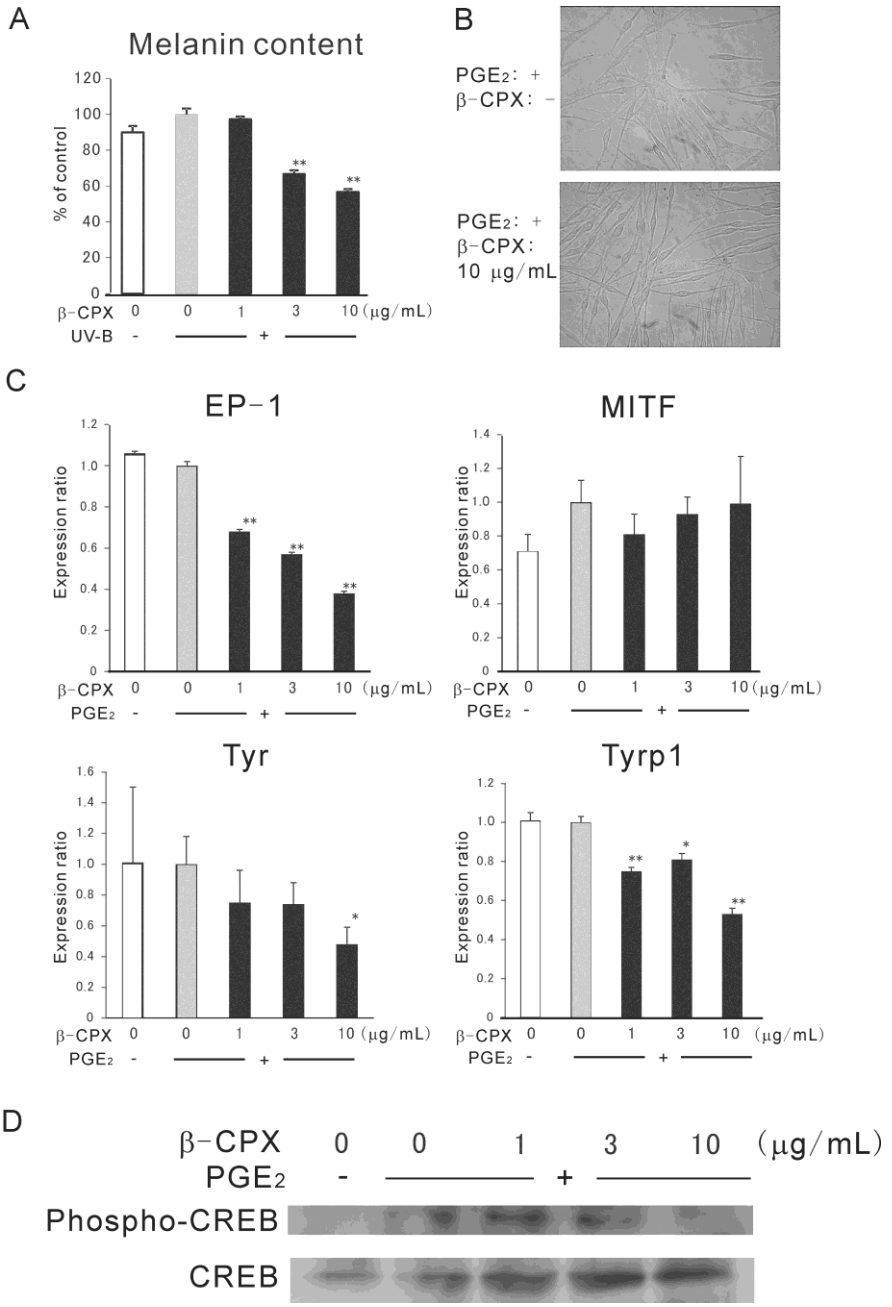


Fig. 9. Suppression by β-CPX of melanogenesis, dendricity, melanogenic mRNA expression and phosphorylation of CREB in PGE_2-stimulated melanocytes.
The melanocytes were cultured with β-CPX (1-10 μg/mL) and PGE_2 (0.3 μM) for 6 days. A) Cellular melanin contents. B) Comparison of PGE_2-induced dendrite formation in melanocyte treated with β-CPX (10 μg/mL, lower) or without β-CPX (upper). C) mRNA expression of melanogenic molecules. D) Expression of CREB and phosphorylated CREB. Each column represents mean with the S.E. of 4-5 experiments. Asterisks denote significant differences from the control group at *: $p<0.05$, **: $p<0.01$.

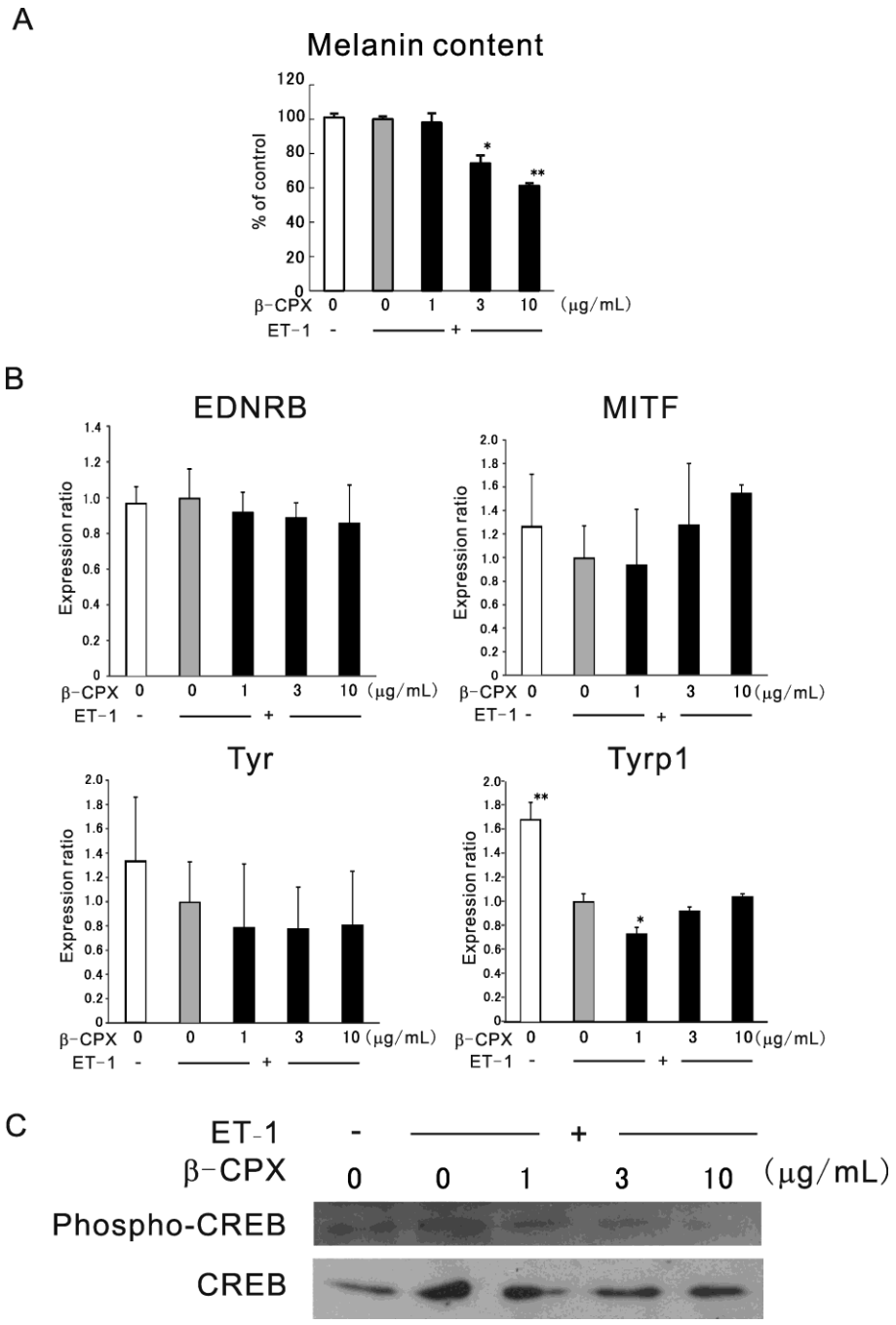


Fig. 10. Suppression by β-CPX of melanogenesis, melanogenic mRNA expression and phosphorylation of CREB in ET-1-stimulated melanocytes.

The melanocytes were cultured with β-CPX (1-10 μg/mL) and ET-1 (10 nM) for 10 days with one change of the medium. A) Cellular melanin contents. B) mRNA expression of melanogenic molecules. C) Expression of CREB and phosphorylated CREB. Each column represents mean with the S.E. of 4-5 experiments. Asterisks denote significant differences from the control group at *: $p<0.05$, **: $p<0.01$.

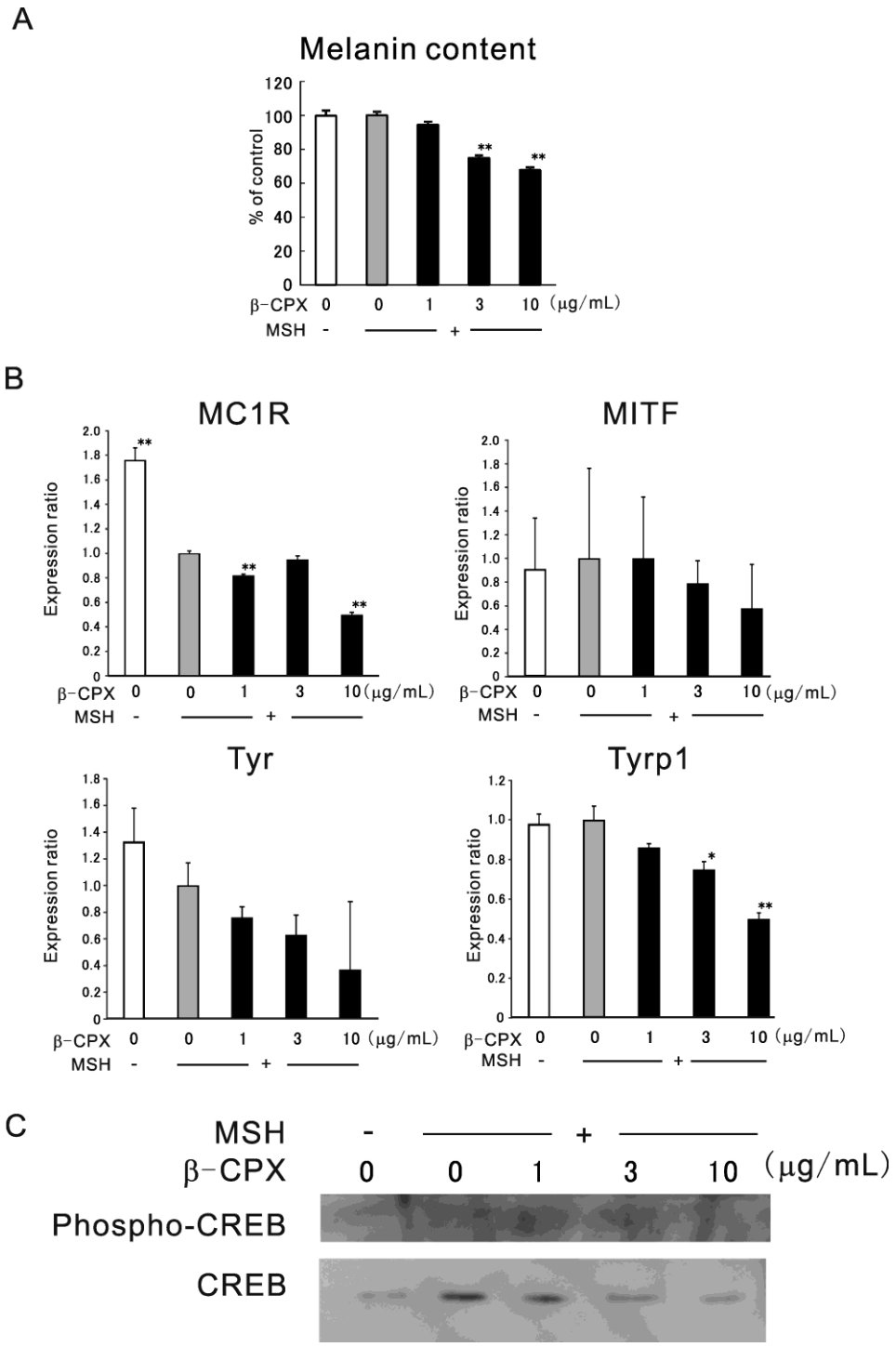


Fig. 11. Suppression by β-CPX of melanogenesis, melanogenic mRNA expression and phosphorylation of CREB in MSH-stimulated melanocytes.

The melanocytes were cultured with β-CPX (1-10 μg/mL) and MSH (0.1 μM) for 10 days with one change of the medium. A) Cellular melanin contents. B) mRNA expression of melanogenic molecules. C) Expression of CREB and phosphorylated CREB. Each column represents mean with the S.E. of 4-5 experiments. Asterisks denote significant differences from the control group at*: $p<0.05$, **: $p<0.01$.

6) Cell Activation

Neonatal dermal fibroblasts (NG1RGB by RIKEN, Japan) was used and cultured in medium containing 1% FBS (serum) and Citrus Unshiu Extract. Figure 12 shown that Citrus Unshiu Extract effectively promotes the proliferation of dermal fibroblast cells suggesting its effect on skin rejuvenation.

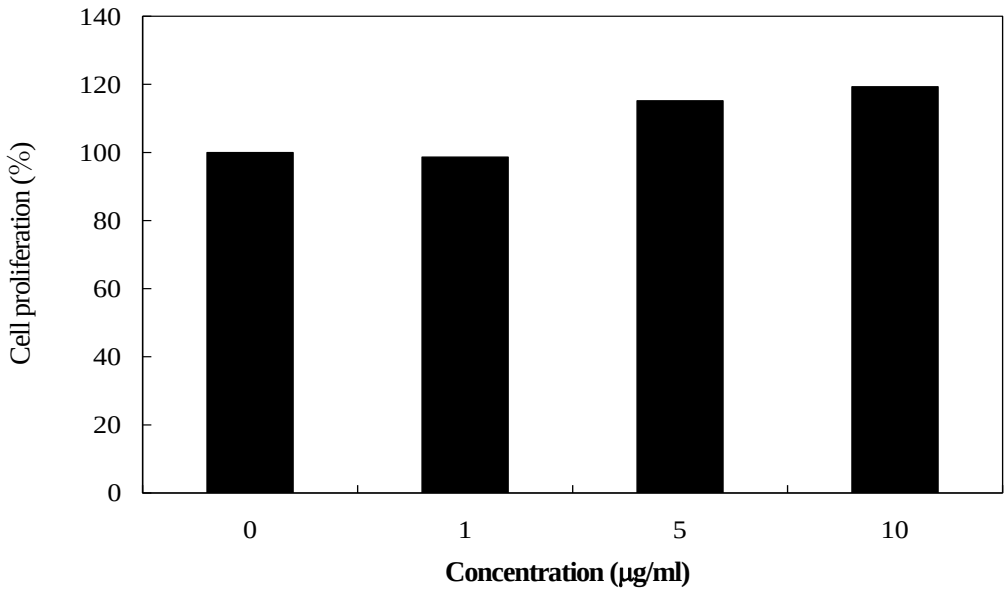


Fig. 12. Cell Activation by Citrus Unshiu Extract

(C) Antioxidative Action

Free radicals such as reactive oxygen species & hydroxyradicals are unstable molecules that cause various degenerative diseases in human (e.g., cancers, freckles, and wrinkles)

1) DPPH Radical Scavenger Activity

The DPPH radical scavenging effect of Citrus Unshiu Extract was measured. Citrus Unshiu Extract demonstrated excellent anti-oxidative effect as shown in Figure 13.

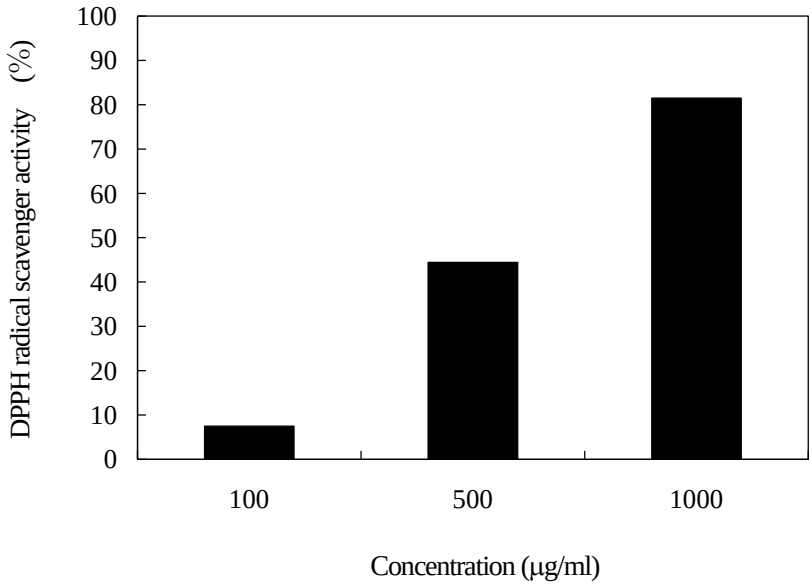


Fig. 13. DPPH Radical Scavenger Activity of Citrus Unshiu Extract

2) SOD-like Activity

Citrus Unshiu Extract demonstrated superior anti-oxidative effect similar to SOD mechanism

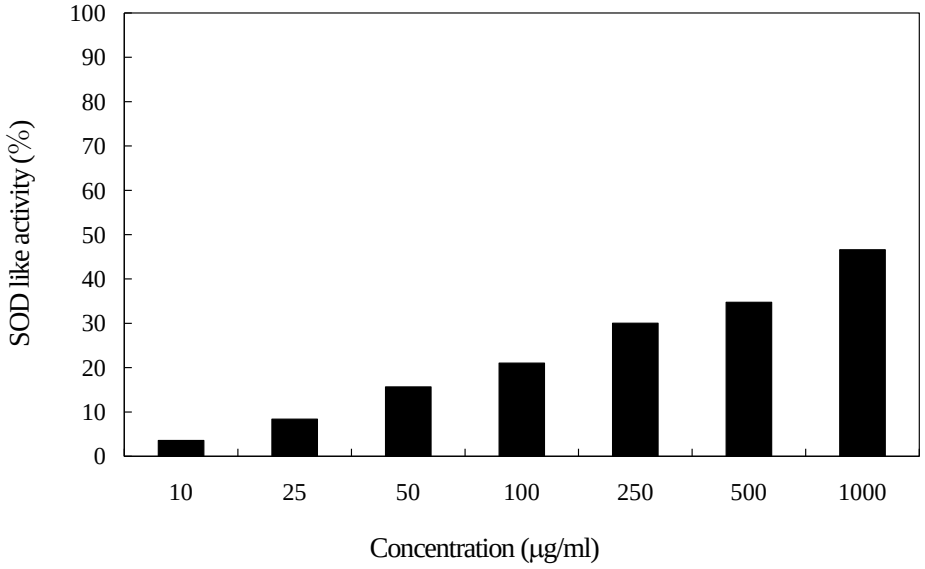


Fig. 14. SOD-like Activity of Citrus Unshiu Extract

(D) Citrus Unshiu Extract - Moisturizing Effect (topical use)

The moisturizing effect of Citrus Unshiu Extract product [Citrus Unshiu Extract – LC, which contains 99% 1, 3 - butylene glycol (BG) and 1% Citrus Unshiu Extract; Lot No. Z – 419] upon topical usage was evaluated and compared with vehicle (BG). Fig. 15 A and B revealed that the product Citrus Unshiu Extract – LC and its twice-diluted sample increased and maintained skin moisture up to 120 minutes while maintenance of 99% and 49.5% BG aqueous solutions lasted only for 80 minutes.

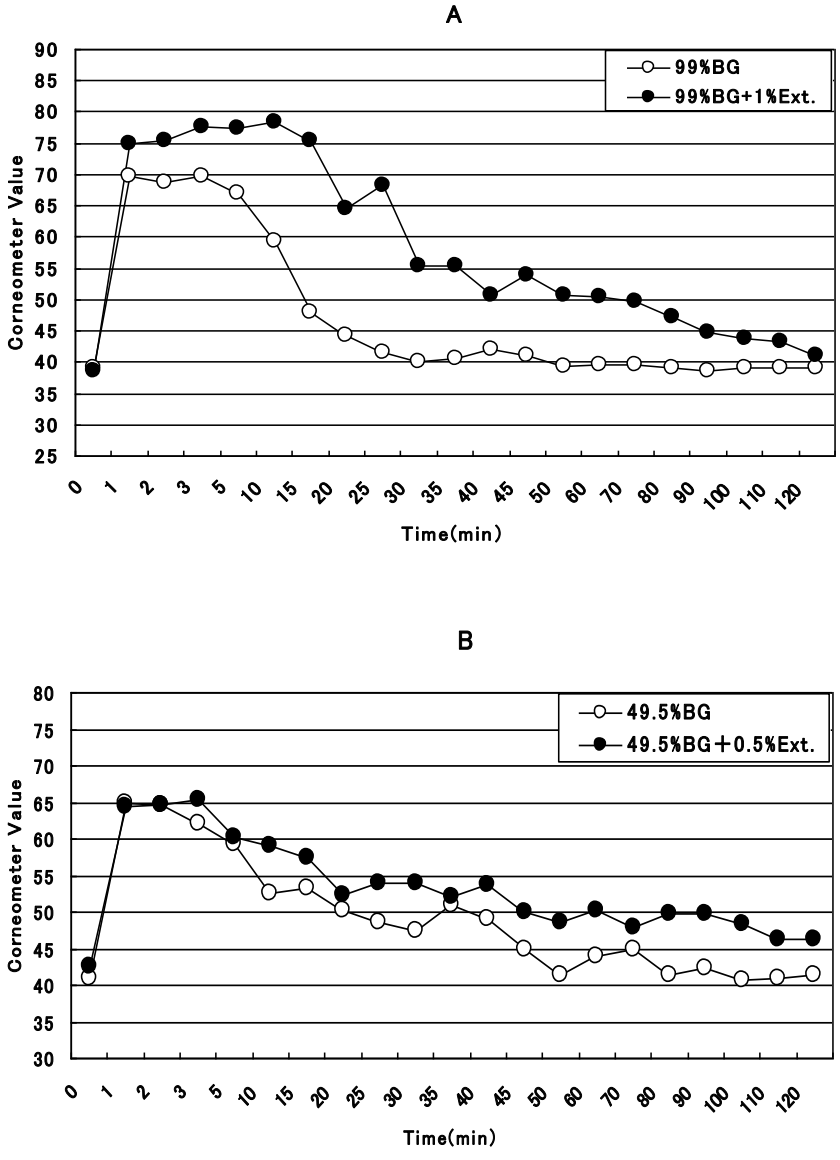


Fig. 15. Moisturizing Effect (topical use) of Citrus Unshiu Extract

6. Stability of Citrus Unshiu Extract

(1) Heat Stability

After continuous heating at 100°C for 30minutes, levels of ***β-cryptoxanthin*** slowly deteriorate (as shown in Fig. 16). Caution is required for application in beverage during sterilization.

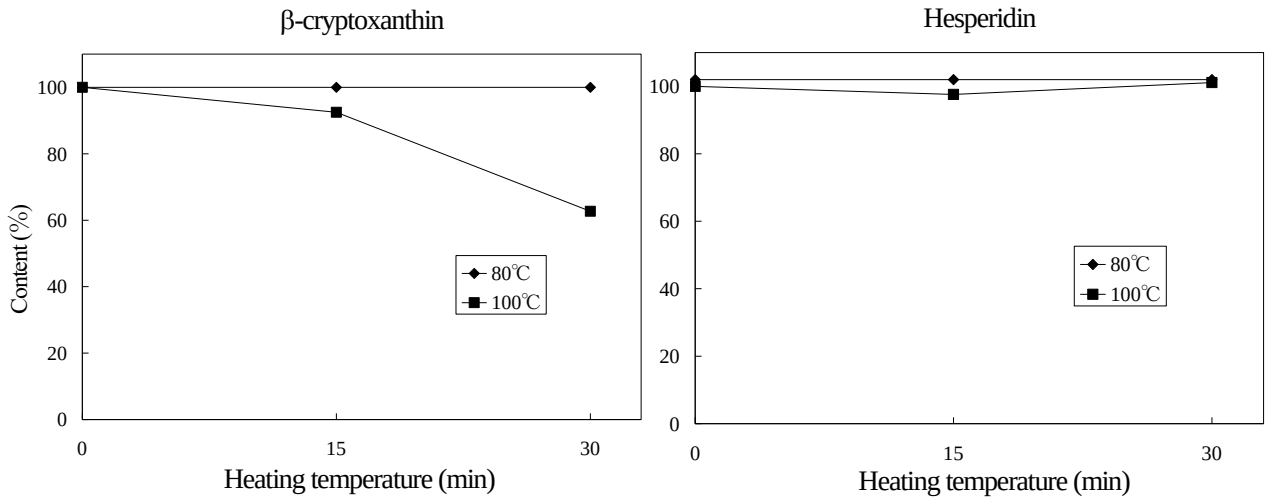


Fig. 16. Heat Stability of Citrus Unshiu Extract

* The initial value before heating was designated as 100%.

(2) pH Stability

β-cryptoxanthin and ***hesperidin*** in Citrus Unshiu Extract remained stable within range of pH 3 to 9. It is suitable for application in food preparation at normal pH range.

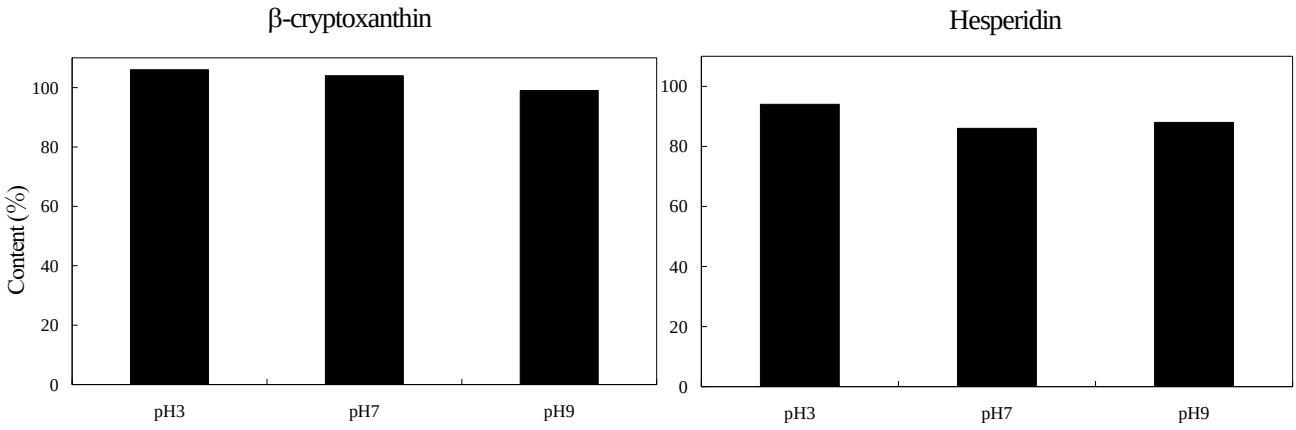


Fig. 17. pH Stability of Citrus Unshiu Extract

* The initial value before pH adjustment was designated as 100%.

7. Recommended Daily Dosage

Recommended daily dosage of Citrus Unshiu Extract: 200mg-500mg/day

※CITRUS UNSHIU EXTRACT is approved for food applications by Ministry of Health, Labor and Welfare in Japan. Please refer to section 9 for product safety profile.

8. Nutritional Information

	Results	Method
Moisture	5.3g/100g	Heat drying method under low pressure
Protein	2.6g/100g 1)	Kieldahl method
Fat	7.2g/100g	Acid fat dissolution method
Ash	0.3g/100g	Direct ashing method
Carbohydrate	84.6g/100g 2)	
Energy	414kcal/100g 3)	Modified Atwater method
Dietary fiber	0.0g/100g	Prosky method
Sodium	10mg/100g	Atomic absorption spectrophotometory

1) *N*=6.25

2) 100-(moisture + protein + fat + ash + dietary fiber)。

3) According to the Japanese Nutrition Composition Standard: No. 146, 1996 from Ministry of Health.

Factors for calculating the energy value: protein,4; fat, 9; carbohydrate,4

Test trustee: SRL, Inc.

Date of issue of the test result report: September 2, 2004

Research result issue number: No. 200408200016

9. Safety Profile

(A) Residual Agricultural Chemicals

Citrus Unshiu Extract complianced with the 61 requirements specified for citrus fruits by Food Sanitation Law for residual agricultural chemicals.

Test trustee: Kyusai Analytical Research Laboratory Co. Ltd.

Date of issue of the test result report: September 13, 2004

Research result issue number: No. 2004902-3

(B) Acute Toxicity (LD_{50})

No toxic effect observed at 5000mg/kg body weight in mice (ICR, male, 5 weeks old). It is deduced that the LD_{50} (in rat) is >5000mg/kg body weight.

10. Applications

Preparations	Examples
Food supplements	Soft gel capsule, tablet, hard capsule, *etc.*
Foods / Functional Food	Candy, gum, chewing gum, tablet, cookies, chocolate, jelly, drinks (beverage, juice, *etc.*) *etc.*
Cosmetics	Soap, facial soap, shampoo, hair lotion, lotion, foundation, lipstick, lip cream, tooth paste, *etc.*

11. Packaging

CITRUS UNSHIU EXTRACT-P（Powder, food）
CITRUS UNSHIU EXTRACT-PC（Powder, cosmetic）

5kg Interior packaging: aluminium-coated plastic bag
Exterior packaging: cardboard box

CITRUS UNSHIU EXTRACT -L（Liquid, food）
CITRUS UNSHIU EXTRACT-LC（Liquid, cosmetic）

5kg Interior packaging: cubic polyethylene container
Exterior packaging: cardboard box

12. Storage

Store in cool, dry place. Avoid humidity.

13. Expression

CITRUS UNSHIU EXTRACT-P, CITRUS UNSHIU EXTRACT-L
Expression : Citrus unshiu extract
CITRUS UNSHIU EXTRACT-PC
INCI name : Citrus Unshiu Pericarp Extract
CITRUS UNSHIU EXTRACT-LC
INCI name : Butylene Glycol, Citrus Unshiu Pericarp Extract

Test Method

Fig. 4. Inhibition of osteoclast production by Citrus Unshiu Extract

The test was performed using an osteoclast culture kit (Hokudo Co.). Rat-derived osteoclasts, 4×10^6 cells (2 vials), were suspended in 10 ml of specific culture medium containing 10 ng/ml M-CSF and 10 ng/ml RANKL, and distributed to 96 wells (2x48-well plates), 100 μl per well, and cultured at 37°C in 5% CO_2. 24 hours later, β-cryptoxanthin (0.05 and 0.5 μg/ml), Citrus Unshiu Extract (0, 5, 10, 50, 100, 200, 400, 600, 800, and 1,000 μg/ml) were added. After continuous culturing for 4 days, osteoclasts were stained using the TRAP staining reagents attached to the kit, and the cells were counted.

Table 1. Effect of citrus unshiu extract on urinary deoxypyridinoline concentration in ovariectomized mice

Table 2. Effect of citrus unshiu extract on bone mineral density in ovariectomized mice

Table 3. Effect of citrus unshiu extract on bone strength in ovariectomized mice

OVX mice (ICR, female, 5 weeks old) were prepared by removing the bilateral ovaries under anesthesia. Meanwhile, mice with intact ovaries, sham-operated mice (Sham) were used as control. Powdered feed (AIN-93G) ad libitum were given to mice for 7 days. The average intake of food is 10g/day/mice in both OVX and Sham model. The mice were divided into 4 groups with different diet composition as tabulated below:

	Diet
Control	50% (5g/day)
MX-1	50% diet + 1% citrus unshiu extract
MX-2	50% diet + 2% citrus unshiu extract
Sham	AIN-96 ad libitum

· Diet composition was prepared according to the literatures below:

※ Man S. L., Tamaki H., Ohta Y., Katsuyama N., Chinen I. Effect of exercise on osteopenia caused by restricted food intake in rats. *J. Jpn. Soc. Nutr. Food Sci.*, **56**, 237-242 (2003).

※ Man S. L., Ohta Y., Katsuyama N., Tamaki H., Oku H., Chinen I. Effect of estrogen on osteoporosis caused by restricted food intake in rats. *J. Jpn. Soc. Nutr. Food Sci.*, **55**, 149-155 (2002).

After 2 weeks, 4 animals were randomly selected from each group, urine sample was collected for 24 hours with feeding and giving drinking water, urinary deoxypyridinoline content was measured with ELISA method. Similarly, urine deoxypyridinoline was measured on week 4 for analysis.

Next, the mice were anesthetized with ether, and blood was collected from the abdominal aorta. Serum was separated by centrifugation, and blood tartaric acid-resistant acidic phosphatase was measured with ELISA method (Mouse TRAP assay, Suomen Bioanalytikka

Oy SBA Sciences, Finland). The femoral bone was excised, stored at –80°C, and the bone mineral density of metaphysis (cancellous bone) and disphysis (cortical bone) were measured using the pQCT method. The bone strength was measured using the 3-point bending test.

Fig. 5. Inhibition of tyrosinase by Citrus Unshiu Extract

Citrus Unshiu Extract was dissolved in distilled water and tested. Tyrosinase solution (derived from mushroom) was added, and the reaction of L-tyrosine conversion to dopa-quinone was measured. Absorbance of dopa-quinone was measured.

Fig. 6. Inhibition of melanin production in B16 melanoma cells by Citrus Unshiu Extract

B16 melanoma cells were precultured in 0.1% glucosamine-supplemented medium. After whitening was confirmed, 2 ml of cell suspension adjusted to 3×10^5 cells/ml with 2mM theophylline-supplemented medium was added to a 6-well plate, and the sample of Citrus Unshiu Extract was added at 100 μg/ml. The cells were recovered after culturing for 3 days, samples were mixed with 1 N NaOH containing 0.1% Triton × 100, and solubilized at 100°C for 30 minutes. The absorbance at wavelength 415 nm was measured using a microplate reader.

Fig. 7. Inhibition of pigmentation by Citrus Unshiu Extract

The sample was administered orally to guinea pigs for 8 days before UV irradiation. Using an ultraviolet light irradiator (solar simulator), ultraviolet light (UV-B) was irradiated at 1,000 mJ/cm^2 for 3 days, and the brightness (L* value) was measured on days 0, 4, 6, and 10 using a spectrophotometer.

Fig. 8, Table 4, 5

Citus unshiu extract contrining 0.5% beta-cryptoxanthin was mixed in white vaserin to prepare 0.1 and 1% ointments. UV-B (160 mJ/cm^2) was irradiated to hairless mice (Hos; HRM2, 7 weeks old) and then ointment (0.1 mL) was applied same area. In a case of oral administration, UV-irradiation was performed 2 hr later of administration. This procedure was repeated for 7 days and power of UV-B was increased to 320 mJ/cm^2 and irradiated another 8 days.

Fig. 9-11

Melanocytes (5×10^4 cells/ml) suspended in the medium(2, 0.5 and 0.2 ml) attached to the kit were seeded onto 6-, 24- and 48-well culture plates, respectively. After 4 h of culture, β-CPX (final concentration: 1–10 mg/ml) and melanogenic stimulants (final concentration of PGE2: 0.3 mm, ET-1: 10 nm and MSH: 0.1 mm) were added. b-CPX was suspended in dimethyl sulfoxide (DMSO) and dispersed in the medium (final concentration of DMSO: 1%). The cells treated

with PGE_2 were cultured for 6 days and the dendricity was observed under a microscope (×400). The cells treated with ET-1 and MSH were cultured for 6 days and the culture was continued for an additional 4 days after exchange of the medium, β-CPX and the stimulants. After culturing, cellular melanin, mRNA expression of melanogenic molecules, protein expression and phosphorylation of CREBwere evaluated as follows. For determination of melanin contents, the medium was removed from the 48-well culture plate and 1N NaOH (100 ml) was added. After extraction of the melanin, the absorbance of the solution was measured at 400 nm. The cells in the 24-well culture plate were collected with the lysis solution (350 ml) attached to the RNAeasy TM Protect Mini Kit. The collected lysate was stored at -20°C for RT-PCR. ForWestern blotting of CREB protein, the medium was removed from the 6-well plate and the cell surface was washed with warmed PBS. The cell lysate was harvested with ice-cold RIPA buffer (100 ml, pH: 7.2) containing protein inhibitor cocktail and stored at -20°C.

Fig. 12. Cell activation by Citrus unshiu extract

NG1RGB cells suspended with 1% FBS-αMEM and distributed into 96 well-plates at a cell density of 2×10^4 cells/well.

After 24 hours, the medium was exchanged with 1% FBS-α–MEM containing the sample at the specified concentration. 48 hours later, cell proliferation was evaluated by MTT assay.

Fig. 13. DPPH radical scavenger activity of Citrus Unshiu Extract

Citrus Unshiu Extract was dissolved in 70% ethanol, and tested. Citrus Unshiu Extract was added to 1,1-diphenyl-2-picrylhydraxyl (DPPH) solution, and color changes of the DPPH solution was measured using spectrophotometer.

Fig. 14. SOD-like activity of Citrus Unshiu Extract

Citrus Unshiu Extract was dissolved in distilled water and tested, using an SOD test Wako Kit.

Fig. 15. Moisturizing Effect (topical use) of Citrus Unshiu Extract

The moisture content of the skin was measured prior to the evaluation. Citrus Unshiu Extract product samples or vehicle were applied on a 2x2cm area on the inside left forearm of test subjects. Moisture content of the applied areas of the skin was measured again after absorption of the samples in approximately 1 minute. Corneometer SM825 (Courage-Khazaka Electronics GmbH) was used for the above measurement at 26°C with 42% humidity.

PRODUCT STANDARD

PRODUCT NAME

CITRUS UNSHIU EXTRACT-P

(FOOD)

This product is extracted from Satsuma mandarin, the fruits of *Citrus unshiu* (Rutaceae), with ethanol. It contains minimum of 1000μg/100g *β*-cryptoxanthin and minimum of 0.3 % hesperidin.

Appearance	Light yellowish powder with slightly unique smell
β-Cryptoxanthin	Min. 1000 μg/100g (HPLC)
Hesperidin	Min. 0.3 % (HPLC)
Loss on Drying	Max. 10.0 % (Analysis for Hygienic Chemists, 1g, 105℃, 2 h)
Purity Test	
(1) Heavy Metals (as Pb)	Max. 10 ppm (Sodium Sulfide Colorimetric Method)
(2) Arsenic (as As_2O_3)	Max. 1 ppm (Standard Methods of Analysis in Food Safety Regulation, The Third Method, Apparatus B)
Standard Plate Counts	Max. 1×10^3 cfu/g (Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/g (Analysis for Hygienic Chemists)
Coliforms	Negative (Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Citrus Unshiu Extract	30%
Cyclodextrin	70%
Total	100%

PRODUCT STANDARD

PRODUCT NAME

CITRUS UNSHIU EXTRACT-L

(FOOD)

This product is emulsifying liquid of constituents extracted from Satsuma mandarin, the fruits of *Citrus Unshiu* (Rutaceae) with ethanol. It contains minimum of 330μg/100g β-cryptoxanthin and minimum of 0.10 % hesperidin.

Appearance	Yellow to dark brown liquid with slight unique aroma
β-Cryptoxanthin	Min. 330μg/100g (HPLC)
Hesperidin	Min. 0.10 % (HPLC)
Purity Test	
(1) Heavy Metals (as Pb)	Max. 10 ppm (Sodium Sulfide Colorimetric Method)
(2) Arsenic (as As_2O_3)	Max. 1 ppm (Standard Methods of Analysis in Food Safety Regulation, The Third Method , Apparatus B)
Standard Plate Counts	Max. 1×10^3 cfu/g (Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/g (Analysis for Hygienic Chemists)
Coliforms	Negative (Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Hydrogenated Glucose Syrup	58.0%
Purified Water	20.0%
Citrus Unshiu Extract	10.0%
Glycerin	10.0%
Enzymatic Lysolecithin (Soy Bean)	1.5%
Sucrose Fatty Acid Ester	0.5%
Total	100.0%

PRODUCT STANDARD

PRODUCT NAME

CITRUS UNSHIU EXTRACT-PC

(COSMETIC)

This product is extracted with ethanol from peels of Satsuma mandarin, the fruits of *Citrus unshiu* (Rutaceae). It contains minimum of 1000μg/100g β-cryptoxanthin and minimum of 0.3 % hesperidin.

Appearance	Yellowish powder with slightly unique smell	
β-Cryptoxanthin	Min. 1000 μ g/100g (HPLC)	
Hesperidin	Min. 0.3 %	(HPLC)
Loss on Drying	Max. 10.0 %	(1g, 105℃,2 h)
Purity Test		
(1) Heavy Metals (as Pb)	Max. 10 ppm	(The Second Method of The Japanese Standards of Cosmetic Ingredients)
(2) Arsenic (as As_2O_3)	Max. 1 ppm	(The Third Method of The Japanese Standards of Cosmetic Ingredients)
Standard Plate Counts	Max. 1 × 10^2 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1 × 10^2 cfu/g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Cyclodextrin	70%
Citrus Unshiu Peel Extract	30%
Total	100%

PRODUCT STANDARD

PRODUCT NAME

CITRUS UNSHIU EXTRACT-LC

(COSMETIC)

This product is extracted with 1,3-butylene glycol from peels of Satsuma mandarin, the fruits of *Citrus unshiu* (Rutaceae).

Appearance	Yellowish liquid with slightly unique smell	
Certification Test (Polyphenols)	Mix this product (0.5 ml) with water (2.0 ml), and Folin-Denis reagent (0.2 ml) and saturated Na_2CO_3 solution (0.4 ml) are added. The solution reveals blue color.	
Purity Test		
(1)Heavy Metals (as Pb)	Max. 10 ppm	(The Second Method of The Japanese Standards of Cosmetic Ingredients)
(2)Arsenic (as As_2O_3)	Max. 1 ppm	(The Third Method of The Japanese Standards of Cosmetic Ingredients.)
Standard Plate Counts	Max. 1 × 10^2 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1 × 10^2 cfu/g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Butylene Glycol	99%
Citrus Unshiu Peel Extract	1%
Total	100%

ORYZA OIL & FAT CHEMICAL CO., LTD. striving for the development of the new functional food materials to promote health and general well-being.

From product planning to OEM - For any additional information or assistance, please contact :

ORYZA OIL & FAT CHEMICAL CO., LTD.

No.1, Numata Kitagata-cho, Ichinomiya-city, Aichi-pref.,
493-8001 JAPAN
TEL : +81 (0) 586 86 5141
FAX : +81 (0) 586 86 6191
URL/http : //www.oryza.co.jp/
E-mail : info@oryza.co.jp

Tokyo sales office:
5F Diamant-building 1-5 Kanda-suda-cho
Chiyoda-ku, Tokyo, 101-0041 JAPAN
TEL +81-3-5209-9150 FAX +81-3-5209-9151
E-mail: tokyo@oryza.co.jp

適正製造規範 GMP JIHFS 一般社団法人 日本健康食品規格協会

ISO 22000 BUREAU VERITAS Certification

ISO 22716 BUREAU VERITAS Certification

*The contents of this catalogue may be changed without prior notice.

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