ORYZA OIL & FAT CHEMICAL CO., LTD.

PhytoRetinol™

Psoralea corylifolia Fruit Extract

Improvement Effect of wrinkle and Sagging

Beauty Food Material

■**PhytoRetinol™-3**
(Oil, Food Grade)

■**PhytoRetinol™-P1**
(Powder, Food Grade)

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ver. 1.0 KM

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Improvement Effect of **Wrinkle and Sagging**
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1. Introduction

Every woman wants to look 'younger than her age'. Recently, there has been a trend for men to use the same skincare and make-up products as women. As a result, the number of people interested in beauty is increasing worldwide, regardless of gender. However, as people get older, their 'apparent age' (based on the impression given by their appearance) increases more. A major factor in apparent age is facial changes such as wrinkles and sagging skin, which increase with age (Fig 1). Large pores, acne and rough skin are the most common skin problems in younger people. As people get older, these problems change to sagging and wrinkles, which become more common. Japan has one of the highest life expectancies in the world and there are many anti-wrinkle creams (medicines) with retinol as the main ingredient. However, retinol has safety and stability issues. Recently, **bakuchiol**, a plant ingredient, has been used in cosmetics as an alternative anti-wrinkle ingredient. In addition, there have been few ingredients that could be expected to have wrinkle-improving effects when taken orally.]

Figure 1. Aging changes in facial expression (purchased image).

Based on this information, Oryza Oil & Fat Chemical Co. Ltd. has launched PhytoRetinol™, standardised bakuchiol with retinoid-like action, for use as a wrinkle-improving supplement.

PhytoRetinol™ is produced from the fruits of *Psoralea corylifolia* and the main ingredient is bakuchiol. *Psoralea corylifolia* fruit is used as a tonic in Ayurveda and Chinese medicine, and is also used in OTC medicines and dietary supplements in Japan. We have identified PhytoRetinol™ functions such as inhibiting fibroblast skeletal atrophy caused by UV irradiation, improving collagen gel contraction by increasing integrin a1 in a 3D dermis model, increasing elastin-related genes, increasing laminin 5 which improves adhesion between epidermis and dermis, and hyaluronidase inhibition. In addition, in an in-house clinical trial, we evaluated the use of PhytoRetinol™ for anti-wrinkle effect according to guidelines. The results of PhytoRetinol™ use showed an improvement in maximum wrinkle depth and maximum wrinkle width in subjects with particularly deep wrinkles at the corners of the eyes.

This evidence suggests that PhytoRetinol™ can also be used in foods and cosmetics for the prevention of wrinkles and sagging.

2. Vitamin A (Retinol, Retinoic acid)

Vitamins are a generic term for organic compounds other than the three macronutrients (carbohydrates, proteins and fats) that are essential for human life. Together with minerals, vitamins, even in minute quantities, play a vital role in helping the human body to metabolize the three macronutrients. If the three macronutrients were the 'fuel' in a car, vitamins would be the 'grease' in the engine's gears. However, most vitamins cannot be produced by the body, so we have to take them from food. Vitamins are broadly divided into fat-soluble and water-soluble vitamins, of which there are more than ten. Among the vitamins, vitamins A and C are the best known for beauty. Vitamin C is a kind of water-soluble, so characteristically it is inefficiently absorbed in the body and easily (safely) excreted from the body. Therefore, a lot of research has been done by pharmaceutical and cosmetic companies to improve the absorption of vitamin C in the body.

We have focused on vitamin A, which is a fat-soluble vitamin with cosmetic properties. **Vitamin A** is known to be involved in maintaining functions such as vision, hearing and reproduction, promoting growth and normal epithelial tissues such as skin and mucous membranes. Vitamin A is therefore essential in a wide range of tissues. The biological potency of vitamin A is expressed in 'retinol activity equivalents' (μg **RAE**). This index is the total amount of retinol in foods of animal origin and the equivalent amount of carotenoids, such as β-carotene. These carotenoids come mainly from plant foods and act as vitamin A in the body. In terms of the RAE, 1 μg of retinol is equivalent to 24 μg of α-carotene, 12 μg of β-carotene and 24 μg of β-cryptoxanthin (Table 1). However, some of us have food preferences as well as allergies and vegetarianism (vegan). Vitamin A can also be obtained from carotenoids, which are abundant in plants, but as the retinol equivalent is one 24th of vitamin A. So, it must be consumed in higher quantities.

We have taken the Sustainable Development Goals (SDGs) into consideration and been investigating active ingredients from natural plants that are relevant to people's health. In this study, we have developed an extract with an action similar to that of vitamin A.

Table 1. Vitamin and provitamin A content of animal foods (in μg/100 g).

Food name	Retinol	α-carotene	β-carotene	cryptoxanthin	β-carotene equivalent	Retinol equivalent
Raw chicken liver	14,000	-	-	-	30	14,000
Pork raw liver	13,000	-	-	-	Trace	13,000
Beef liver raw	1,100	-	-	-	40	1,100
Raw lamprey eel	8,200	0	0	0	0	8,200
Boiled Firefly Squid	1,900	-	-	-	Trace	1,900
Grilled Eel	1,500	0	0	0	0	1,500
Lampreys raw	1,100	0	0	0	0	1,100
Raw conger eel	500	0	0	0	0	500
Grilled Saury	13	0	0	0	0	13
Boiled whole hen egg	130	0	3	26	16	140
Processed cheese	240	-	-	-	230	260
Raw milk	38	0	6	0	6	38
Baby carrots	0	2,400	7,500	0	8,600	720
Spinach	0	0	5,400	45	5,400	450
Chrysanthemum garland	0	0	5,300	0	5,300	440
Carrot Juice	0	1,300	3,800	0	4,500	370
Western Squash	0	18	3,900	90	4,000	330
Japanese mustard spinach	0	0	3,100	28	3,100	260
Broccoli Flower	0	0	770	5	770	64
Tomatoes	0	4	540	0	540	45
Sweet Corn	0	7	22	53	49	4

3. What is *Psoralea corylifolia* ?

A) Plant

Figure 2. The fruits (Left side) and flowers (Right side) of Babchi (Purchased photo)

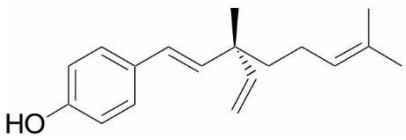
Psoralea corylifolia Linn. is a 60-120 cm tall annual plant of the Fabaceae family (Fig. 2). It is native to all of **India** and is widespread in semi-arid areas (e.g. Rajasthan, eastern Punjab and Uttar Pradesh) and in tropical and subtropical regions (**China**)[1,2]. Its flowers are small, light purple in colour and have a distinctive, pleasant fragrance. The fruits are also brownish-black, kidney-shaped, 2-4 mm long, 2-3 mm wide and pleasantly fragrant (Fig. 2)[3, 4].

B) Application

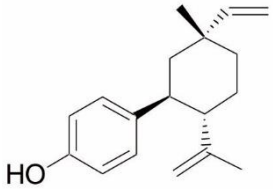
The fruit of *Psoralea corylifolia* originated in Ayurveda and is mentioned in the Chinese Pharmacopoeia. In India it is known as 'Babchi' and is used as a folk medicine for psoriasis, leprosy, vitiligo and other skin conditions[5]. In China, it was first introduced as an herbal medicine in the Kai Pao Hon Zao, published in 974 during the Song Dynasty 974, and is described as 'curing five labours, seven wounds, wind deficiency and cold, bone marrow injury and loss, cold sperm flow in the kidneys, female blood qi and abortion'.

C) Constituents

Phytochemical studies have been carried out on the fruits and have shown that *Psoralea corylifolia* fruits contain **bakuchiol** (major constituent), coumarins such as psoralen and isopsoralen, flavonoids, benzofuran derivatives and stigmasterol[6-11]. We have focused on bakuchiol, a characteristic and major constituent of *Psoralea corylifolia* fruit. However, psoralen and isopsoralen have been reported to cause phototoxicity to the skin. The following chapter describes the hazards of phototoxic constituents and the safety assurance data for our extracts.



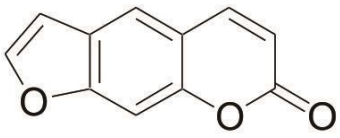
Bakuchiol



Cyclobakuchiol B

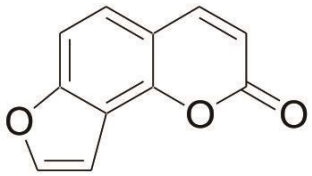
3. Phototoxicity of furanocoumarins

Furanocoumarins are a class of organic compounds produced by various plants. The chemical structure of furanocoumarins is a fused furan ring and coumarin. When the furan ring is fused in different ways, several isomers are produced. The most common parent structures are **psolarene** and **isopsolarene**. Derivatives of these two parent structures are called linear and angular furanocoumarins[12]. Many of the furanocoumarins are toxic and are known to be produced by plants to protect themselves against various types of predators, such as insects and mammals[13]. In humans, they are also abundant in citrus fruits such as grapefruit, and the furanocoumarins bergamotin and dihydroxybergamotin are known to cause drug interactions[14]. In addition, these furanocoumarins are often present in the essential oils of citrus peel and are the cause of **photosensitivity**. Therefore, it is important to check that the furanocoumarins have been removed when using them externally. Our product, **PhytoRetinol™**, has been developed with safety in mind and **remove the psolarene** while retaining the active ingredient bakuchiol, which improves skin wrinkles. Phototoxicity tests have also been carried out on PhytoRetinol™ with the psolarene removed.



Psolarene

(Linear Furanocoumarins)



Isopsolarene

(Angular Furanocoumarins)

4. Phototoxicity test of *Psoralea corylifolia* extract

We conducted the phototoxicity test for *Psoralea corylifolia* Extract (PCE, removed psolarene) and its major component, bakuchiol. Cells and animals were used in the confirmation test.

A) Phototoxicity test using mouse fibroblast cells (*in vitro*)

As shown in Table 2, the cytotoxicity (phototoxicity) induced by UVA irradiation of PCE and bakuchiol was not observed, and the PIF (Photo Irritancy Factor) was less than 2. On the other hand, phototoxicity was clearly observed in cells treated with psolarene or chlorpromazine (positive control), with a PIF greater than 5. Based on these results, it can be concluded that PCE and bakuchiol are not phototoxic.

Table 2. IC_{50} of Cytotoxicity (phototoxicity) and PIF

Sample	PCE	Bakuchiol	Psolarene	chlorpromazine
IC50 (-UVA)	100.4	72.4	10000	76.31
IC50 (+UVA)	120.1	87.2	1.75	0.20
PIF	**0.84**	**0.83**	**5714.29**	**381.55**

PIF < 2 : No Phototoxicity　　2 ≦ PIF < 5 : possible Phototoxicity

PIF ≧ 5 : Phototoxicity

[Method]

The assay was performed according to OECD Guideline No. 432 [15]. Mouse fibroblasts (BALB/3T3) were seeded (10^4 cells/100 µL) in two 96-well plates. After 1 day of culture, removed the medium and replaced with PBS (-) containing the test samples. After 1 hour of incubation, one plate was uncovered and irradiated with UVA for approximately 35 minutes (total dose 5 J) using a CL-1000 UV crosslinker (Analytik Jena AG, Germany). During this time, the other plate was left at room temperature under a light shield. After irradiation, PBS (-) was removed from the plates and washed with PBS (-). The plates were then incubated with medium for 20 hours. Finally, cell viability was calculated in neutral red (NR) and the concentration at which cell viability was 50% for each compound (IC_{50} value) was calculated by non-linear regression. Psolarene and chlorpromazine were used as positive controls.

The photo-irritation factor (PIF), a value to determine the presence or absence of phototoxicity, was calculated using the following formula

$$\mathrm{PIF} = \frac{IC_{50}\ \text{of Non UVA Irradiation}}{IC_{50}\ \text{of UVA Irradiation}}$$

B) Phototoxicity test using rat (*in vivo*)

As shown in Figure 3, treated with 8-methoxypsoralen (8-MOP) group, UVA irradiation of the rat auricle (the part of the ear that protrudes outward) resulted in edema and inflammation. On the other hand, control (with UVA irradiation), normal (without UVA irradiation), or psolarene-removed *Psoralea corylifolia* Extract (PCE) treatment groups were observed no edema or inflammation. These results indicate that PCE, PhytoRetinol™ is not also phototoxic in vivo.

[Method]

The test was performed according to Morimura et al.[16]. This test was designed to evaluate the phototoxicity of chemicals with melanin affinity. For this reason, coloured animals with melanin granules distributed in the skin and eyes are often used in a phototoxicity test. Therefore, Long-Evans rat, which has both coloured and albino parts of the skin, were used. 500 mg/kg PCE, 10 mg/kg 8-MOP (the positive control) were dissolved in corn oil and administered orally to non-fasted rats. The control and normal groups received corn oil alone orally. After oral administration of each sample 1 h, UVA irradiation was performed using a Dermaray 200 (Muranaka) (50 min, total irradiation dose 10 J). Auricular thickness and inflammation intensity were then assessed at 24, 48, 72 and 96 hours. Auricular thickness was measured with a thickness gauge and inflammation intensity was scored on a 5-point scale (0: no reaction, 1: slightly red, 2: mild inflammation, 3: obvious redness and severe inflammation, 4: dark red and very intense inflammation).

Auricular thickness was measured with a thickness gauge

From Chapter 6, we describe the anti-wrinkle action mechanism of bakuchiol and its efficacy in humans by ingestion.

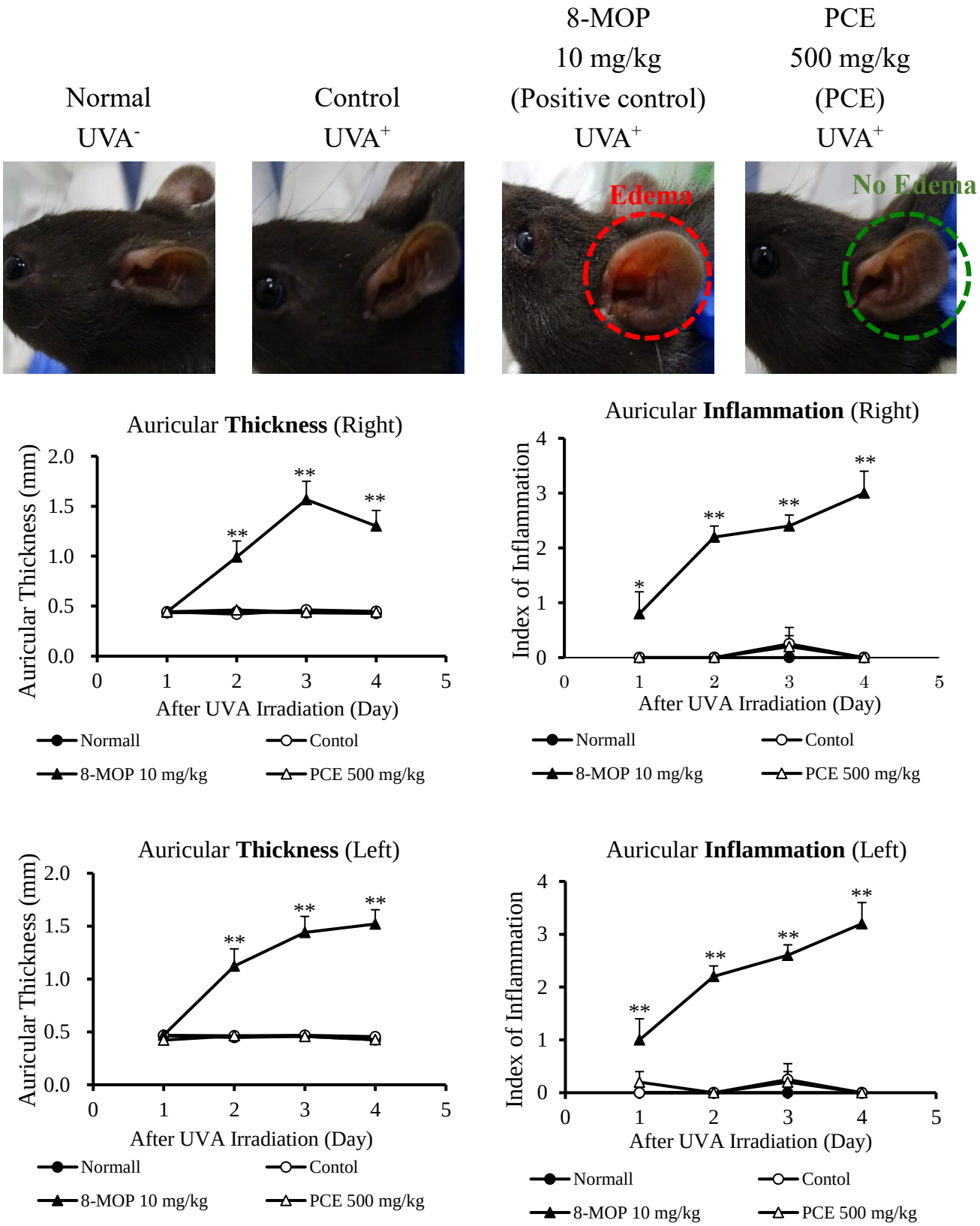


Figure 3. Phototoxicity Test of *Psoralea corylifolia* Extract (PCE) on Long-Evans rat

5. Improvement effects of PhytoRetinol™ on wrinkle (*in vitro*)

A) Abstract

PhytoRetinol™ was found to have a variety of effects on wrinkles (Fig. 4). Wrinkles are broadly divided into two categories. The first are those caused by physiological ageing and the second are those caused by photoaging, which occurs when the epidermis and dermis are damaged by UV. PhytoRetinol™ has been shown to prevent and inhibit the ageing process. This chapter provides detailed data on the six functionalities.

i. Inhibition of UVA-induced Cell Atrophy in Human Skin Fibroblast Cells
ii. Enhancement of Collagen Gel Contraction with increasing integrin α1 in a 3D model of the dermis.
iii. Promotion of Elastin-related Genes (Fibrin) in Human Older Fibroblasts Cells
iv. Promotion of Laminin 5 in Human Epidermal Cells
v. Inhibition of Hyaluronidase
vi. Anti-Inflammatory Activity (Reported by Paper)

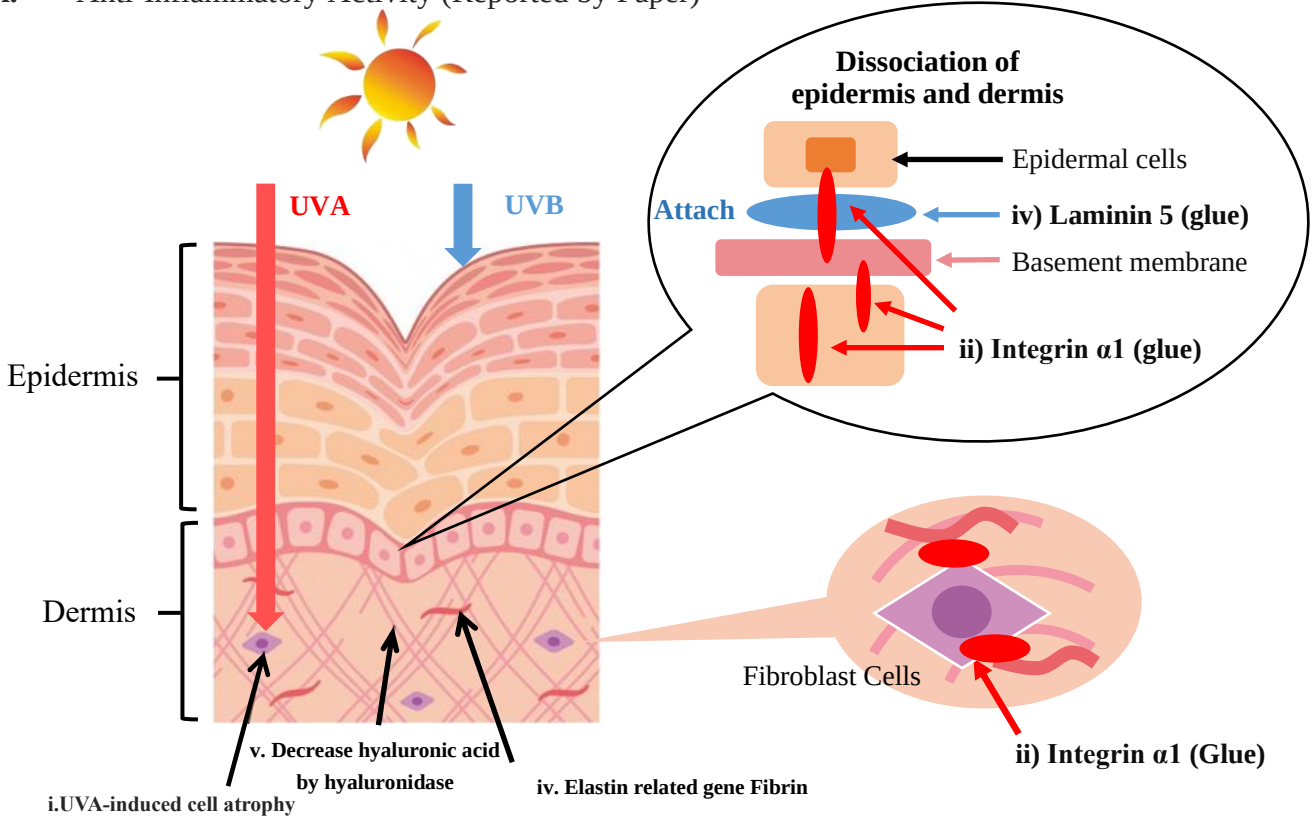


Figure 4. Evaluation of targets

i. Inhibition of UVA-induced cell atrophy in human skin fibroblast cells

The dermis is mainly composed of extracellular matrix (collagen, hyaluronic acid, elastin, etc.) and which is produced by fibroblasts. Fibroblasts in the dermis are tightly attached to collagen fibers and have an elongated shape. When fibroblasts are normally cultured on a monolayer, they expand their skeleton as shown on the left side of Figure 5. The reason for this is to form a network between the cells. However, after exposure to UVA, the cytoskeleton atrophies, as shown on the right side of Figure 5. This phenomenon occurs because the cells themselves reduce the area exposed to UV radiation in order to minimize damage. However, it has been reported that atrophied fibroblasts dissociate from collagen fibers, leading to a weakening of the collagen fiber structure and a decrease in cellular activity such as decrease in collagen production and increase in matrix metalloproteinase-1 (MMP1)[17]. Therefore, in this experiment we decided to evaluate the inhibitory effect of UVA irradiation on skeletal atrophy in fibroblasts.

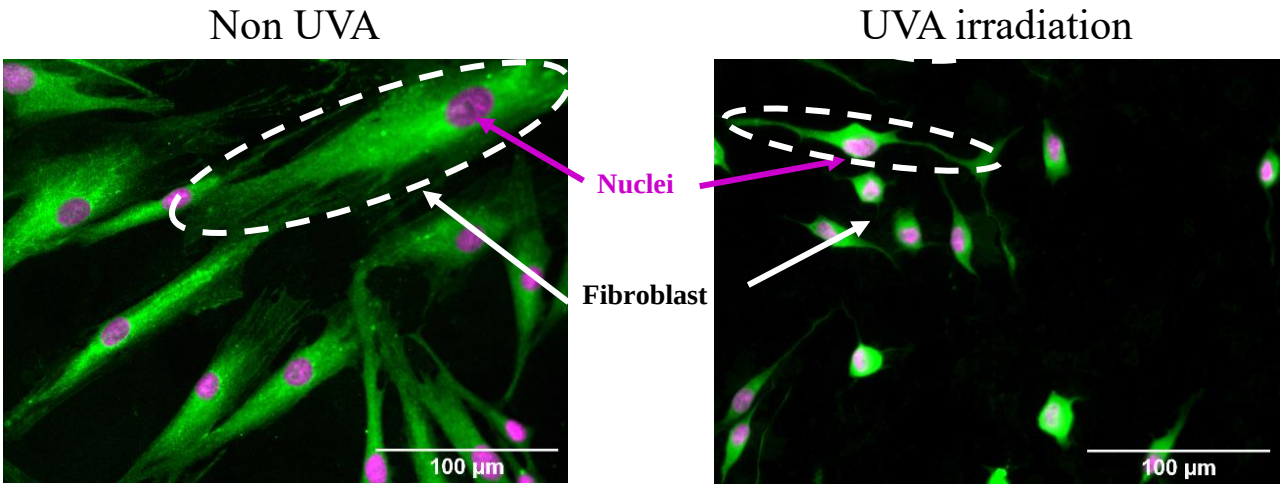


Figure 5. Morphological changes in UVA-irradiated/non-irradiated fibroblasts.

[Result]

As shown in Figure 6, 7, *Psoralea corylifolia* extract (PCE) and bakuchiol were effective against cytoskeletal atrophy induced by UVA irradiation in a concentration-dependent manner. From Figure 7, **bakuchiol** maintained a cytoskeleton similar to that of non-UVA irradiated cells. Its effect was stronger than that of the positive control, retinol acetate (RA), a vitamin A derivative. These results suggest that PCE and bakuchiol can be expected to inhibit MMP1, maintain the structure of the dermal layer and inhibit UVA-induced skin inflammation.

[Method]

Human neonatal fibroblast cell line, NB1RGB cells were seeded on culture plates and cultured with the test substance. The culture medium was then replaced with PBS (-) and irradiated with UVA (10 J/cm^2). After irradiation, the medium was changed and incubation for 3 hours. After incubation, fluorescence immunostaining for actin was performed according to the standard method. Fluorescence intensity and cell number (the number of nucleus) were measured using Image J. RA was used as a positive control.

UVA irradiation resulted in a decrease in cell death (absolute number) and atrophy of the cytoskeleton (control group in the figure). The graph shows the result of dividing the stained area of the cytoskeleton (actin) in the photograph by the number of nuclei and correcting the result to calculate the actin luminescence intensity per cell.

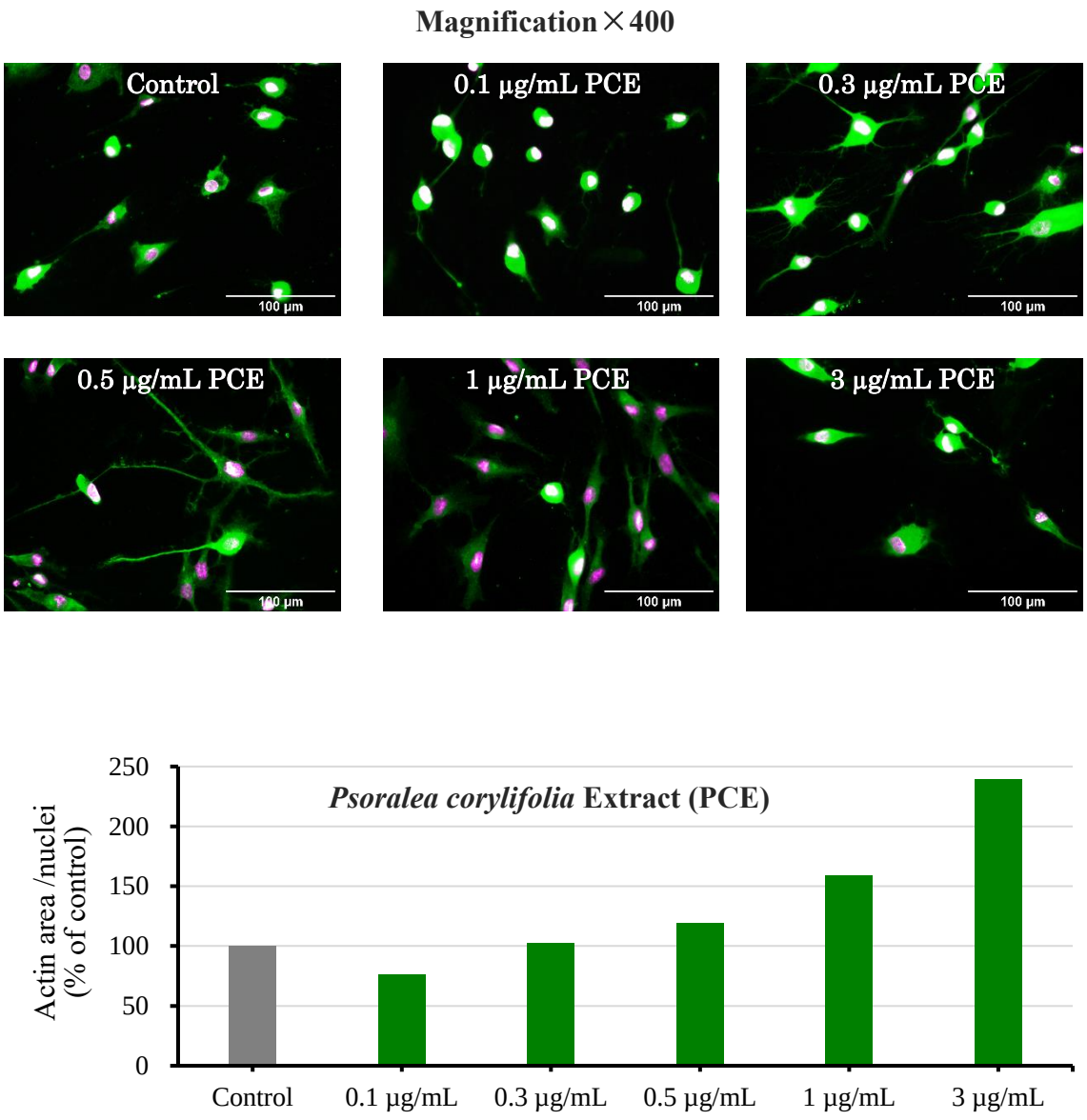


Figure 6. Inhibition of cytoskeletal atrophy by UVA irradiation of PCE.

As shown in Figure 6, PCE inhibited UVA-induced cytoskeletal atrophy on a concentration-dependent. However, cytotoxicity was also observed at concentrations above 3 μg/ml. Therefore, the results at 3 μg/mL did not use atrophy inhibition as reference.

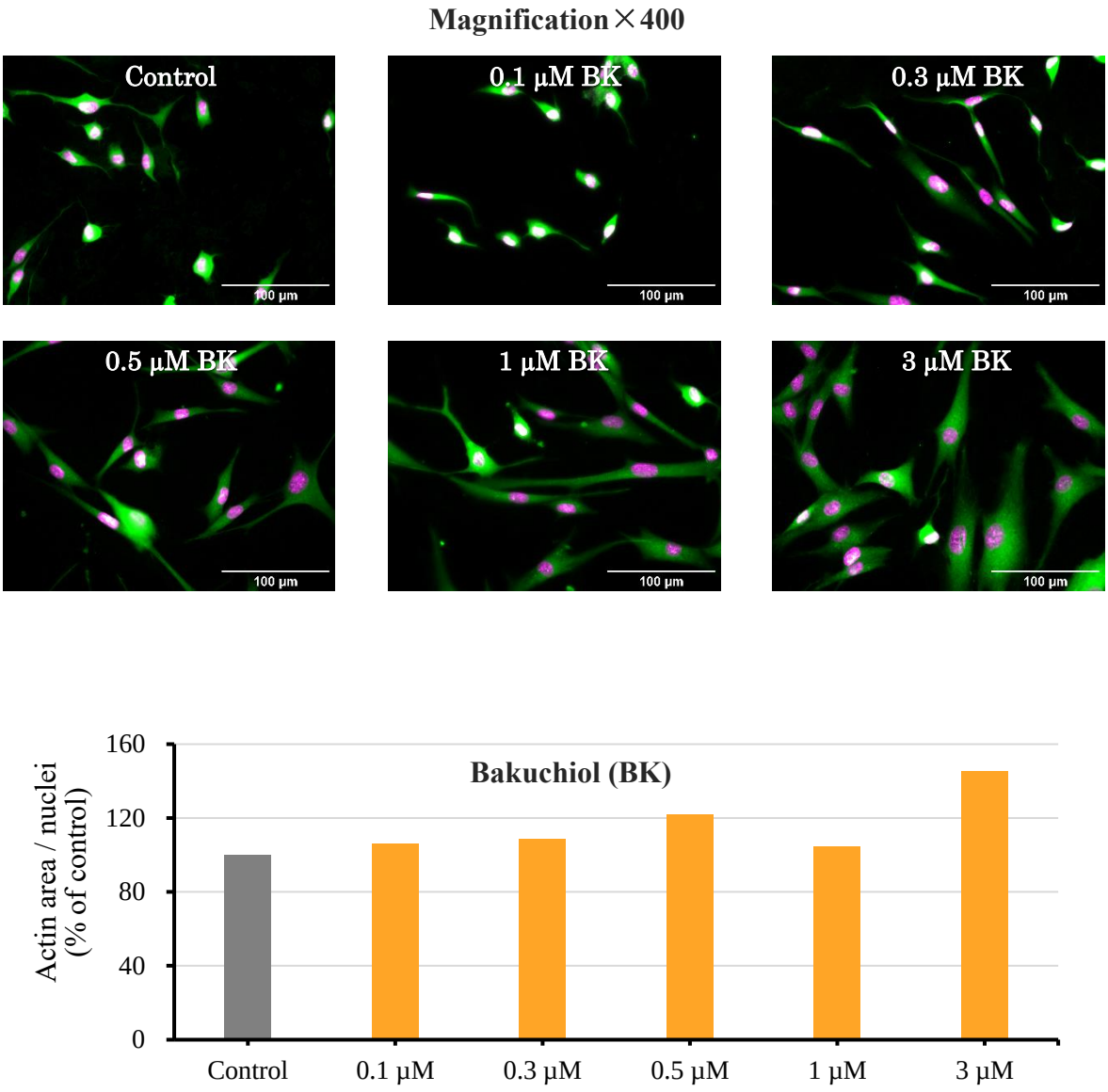


Figure 7. Inhibition of cytoskeletal atrophy by UVA irradiation of bakuchiol.

As shown in Figure 7, bakuchiol dramatically inhibited UVA-induced cytoskeletal atrophy on a concentration-dependent.

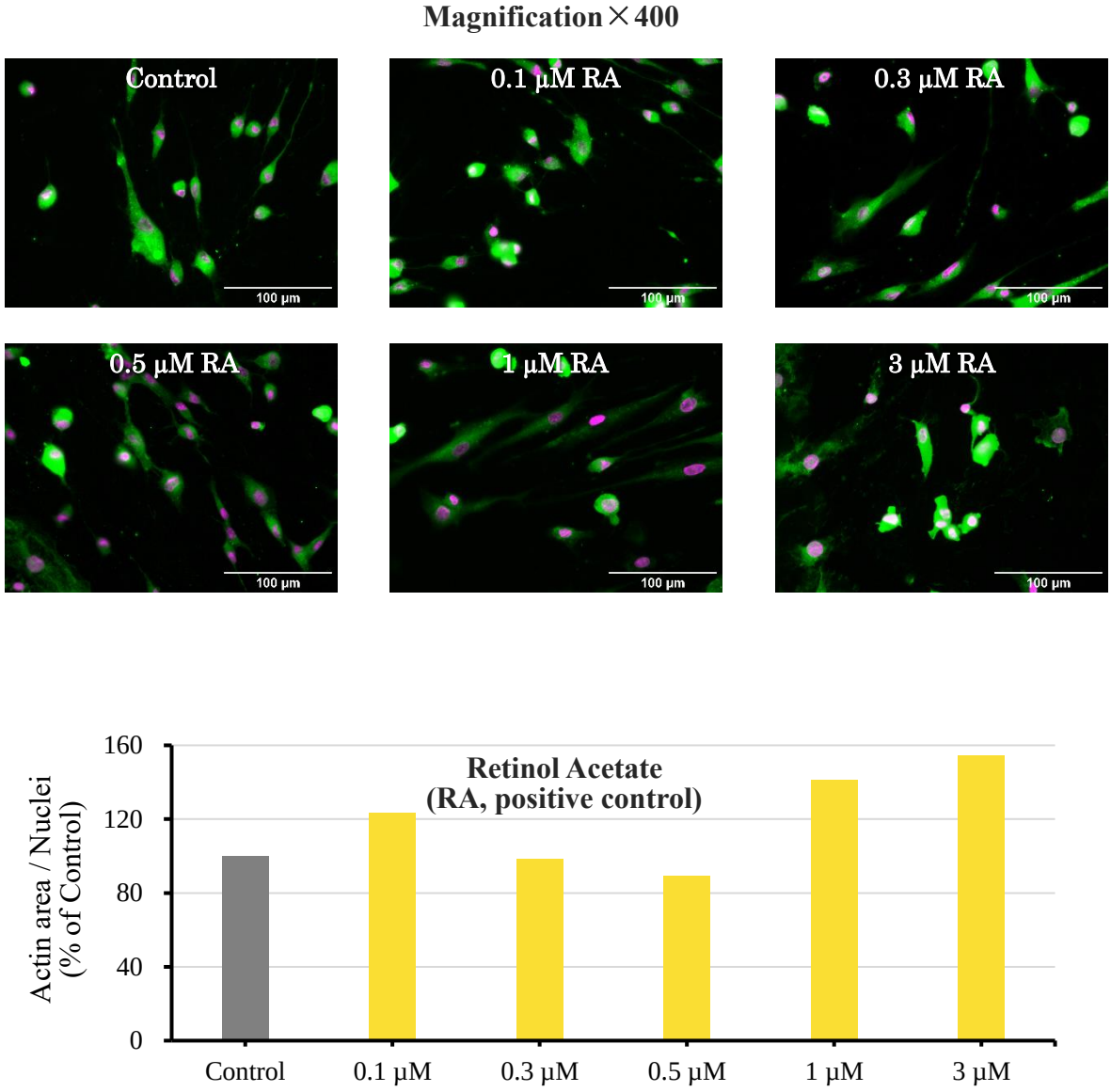


Figure 8. Inhibition of Cytoskeletal Atrophy by UVA Irradiation of Retinol Acetate.

ii. Enhancement of Collagen Gel Contraction with Increasing Integrin α1 in a 3D Model of the Dermis.

The cause of the age-related wrinkle in collagen production by fibroblasts is a reduction in the adhesive force between cells and collagen fibers. Fibroblasts exist in the dermis in a state of adhesion and elongation to collagen fibers at numerous points. However, in photo-aged skin, fibroblasts dissociate from the collagen fibers that serve as their vital scaffold and their normal functions, such as extracellular matrix production and cell division, are reduced (Fig. 9). **Integrins**, proteins found on the surface of the cell membrane, act as adhesins between cells and collagen. Integrins adhere not only to the extracellular matrix but also to laminin 5(discussed below) and maintain the structure of the dermis. However, this integrin has been reported to decrease after UVA irradiation, weakening the bond between cells and extracellular matrices such as collagen[18]. This has been shown to cause cell shape atrophy due to a reduction in the number of attachment points to collagen fibers, leading to sagging and wrinkling of the skin.

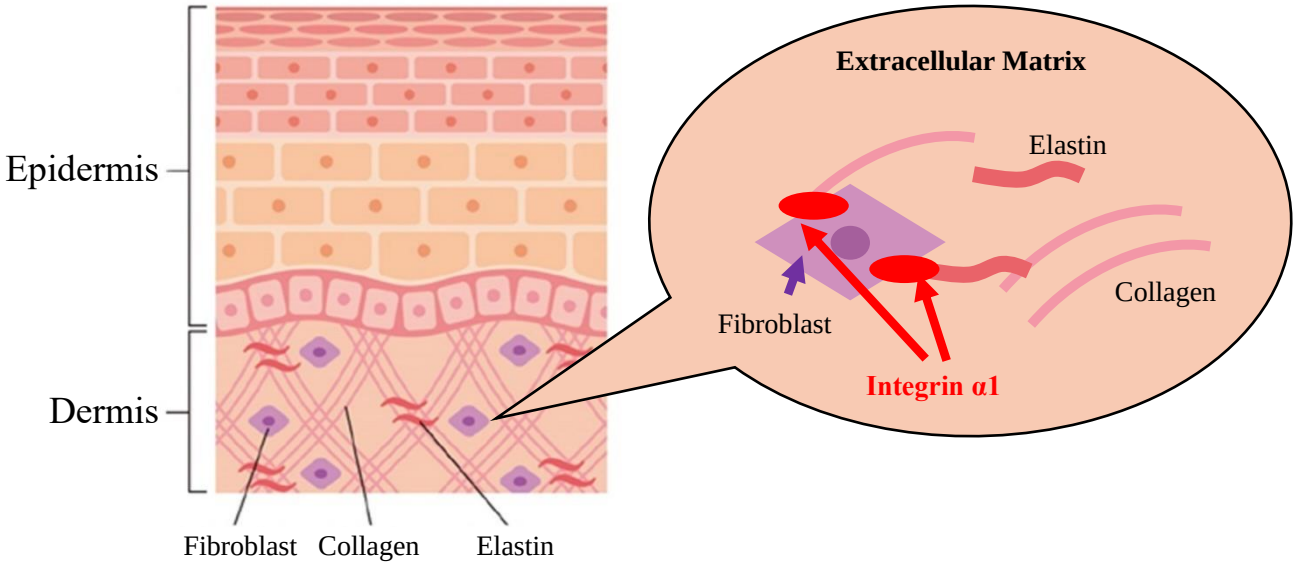


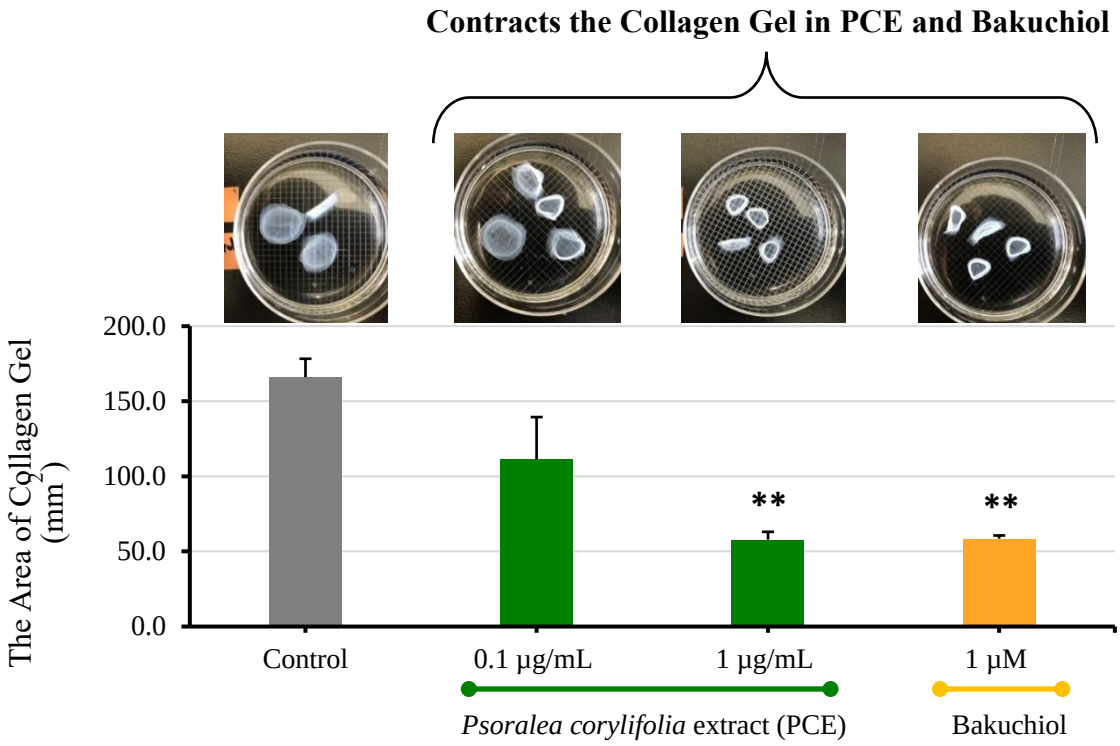
Figure 9. Relationship with Fibroblasts, Extracellular Matrix and Integrin α1

We examined *Psoralea corylifolia* extract (PCE) and bakuchiol for collagen gel contraction to evaluate the adhesion of fibroblasts to collagen fibers. When fibroblasts are embedded in collagen gel and cultured, the cells adhere to the surrounding collagen fibers and the gel shrinks because of cell-cell interaction. The stronger the cell adhesion to the collagen fibers, the stronger the contraction. Furthermore, it has also been reported that contraction strength decreases with oxidative stress and UV. after checking the contraction of the collagen gel, integrin α1 was detected by immunofluorescence.

[Result]

Fibroblasts were cultured in collagen gel to create a 3D model close to the dermis so that collagen gel contraction shows strengthening of the dermal structure because of promoting cell-collagen adhesion.

PCE and bakuchiol significantly promoted collagen gel contraction on 3D model of the dermis (Fig. 10). Integrin α1 of the collagen gel was tested by immunofluorescence. From the result, PCE and bakuchiol drastically increased integrin α1 (Fig. 11).



Each bar represents the means±S.E.M (N=4). Asterisks denote significant differences from the control group, **, p<0.01.

Figure 10. Enhancement Effect of *Psoralea corylifolia* extract (PCE) and Bakuchiol on the contraction of Collagen Gel in 3D Dermis Model

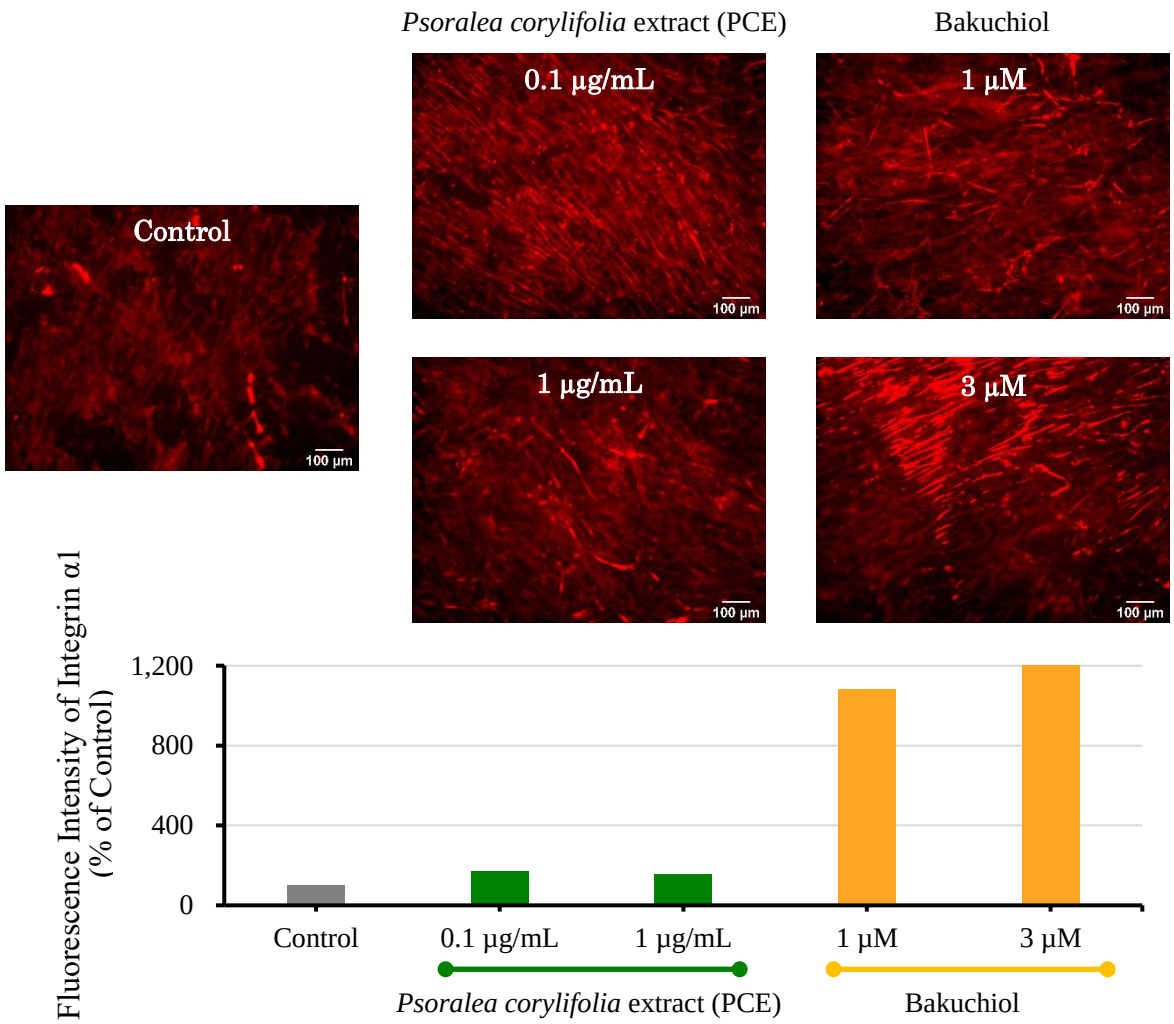


Figure 11. Enhancement Effect of *Psoralea corylifolia* extract (PCE) and Bakuchiol on Integrin α1 expression in 3D Dermis Model

PCE and bakuchiol increased integrin α1 expression. Therefore, these results suggested that PCE and bakuchiol contribute to the suppression of skin sagging, maintenance of skin firmness and skin tightening by firming the dermal connective tissue through an increase in integrin α1.

[Method]

Dermis 3D model was created by culturing NB1RGB cells in type I collagen gel and evaluated *in vitro*. Briefly, a suspension of NB1RGB cells was mixed with collagen solution (pH 7.4) and seeded onto culture plates. After gel solidification, the test substance was added and the cells were incubated for a total of 7 days. Subsequently, area measurements of the collagen gel. immunofluorescence for integrin α1 was performed and fluorescence intensity measured by image J.

iii. Promotion of Elastin-related Genes (Fibrin) in Human Older Fibroblasts Cells

Wrinkles and sagging of the skin are caused by the reduction and degeneration of the extracellular matrix of the dermal layer due to ageing. **Elastin**, the elastic fiber in the skin, is formed by the polymerization of fibrillin1, tropoelastin and **fibrin** 4, 5 produced by fibroblasts (Fig. 12). In the present study, we investigated the effect of **fibrin 4**, which acts as a 'stake' by holding fibrillin and tropoelastin together in the formation of elastic fibers, on gene expression levels.

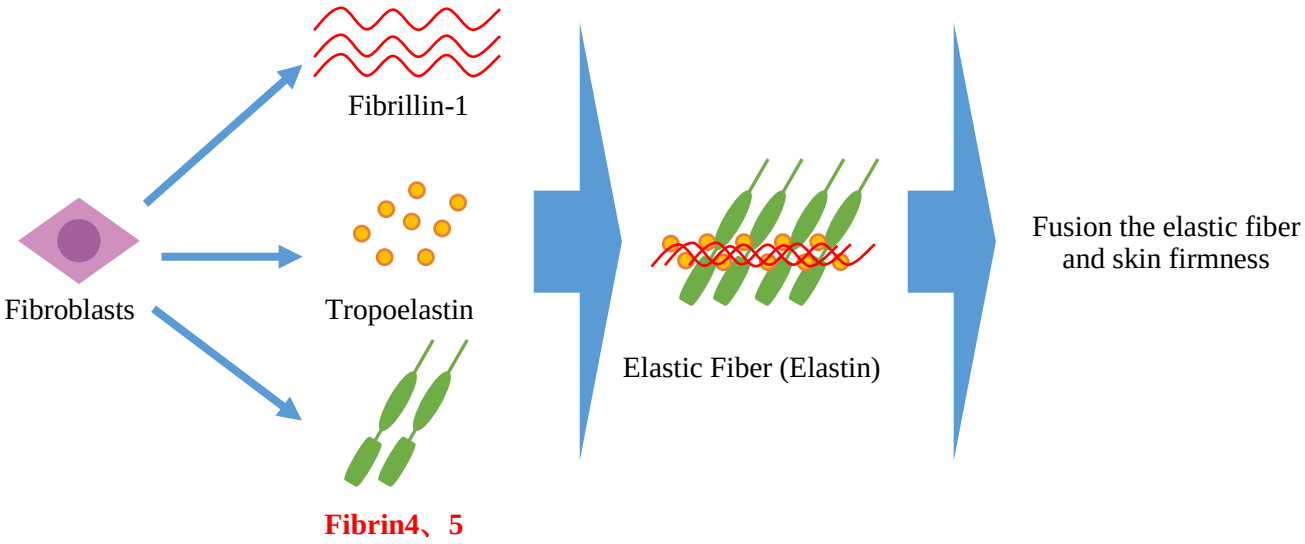


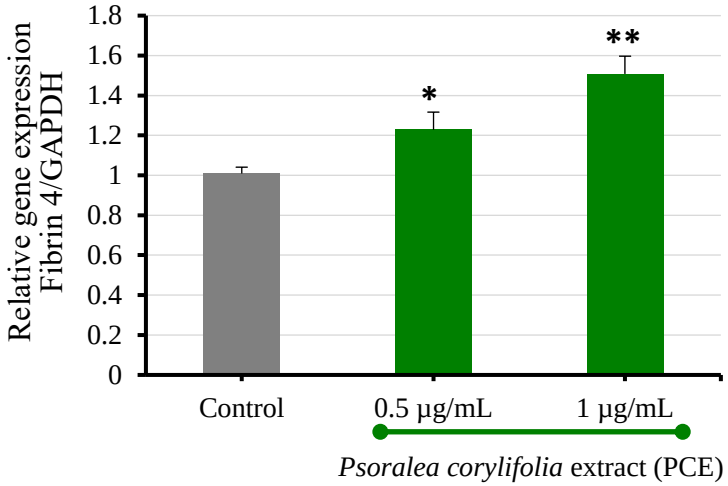
Figure 12. The Mechanism of Production of Elastin

[Result]

PCE increased the gene expression of fibrin 4 (elastin-related gene) on older fibroblasts (Tig 103) (Fig. 13).

[Method]

TIG 103 cells (human female normal diploid fibroblasts derived from a 69-year-old woman) were seeded on culture plates and cultured with the test substance. RNA was then extracted and RT-PCR was performed.



Each bar represents the means±S.E.M (N=4). Asterisks denote significant differences from the control group,*p<0.05,**, p<0.01.

Figure 13. Promotion Effect of PCE on Elastin related Gene of Expression in Tig 103.

iv. Promotion of Laminin 5 in Human Epidermal Cells

The skin is divided into three main structures: epidermis, dermis and subcutaneous tissue. The epidermis and dermis are in close contact through the epidermal basement membrane. The epidermal basement membrane functions is "grew" between the epidermis and dermis to prevent from skin rubbed[19]. When this basement membrane structure is weakened, the adhesion between the epidermis and dermis is weakened, leading to sagging skin and deep wrinkles. The protein that acts as this adhesive is **laminin 5** (Fig. 14).

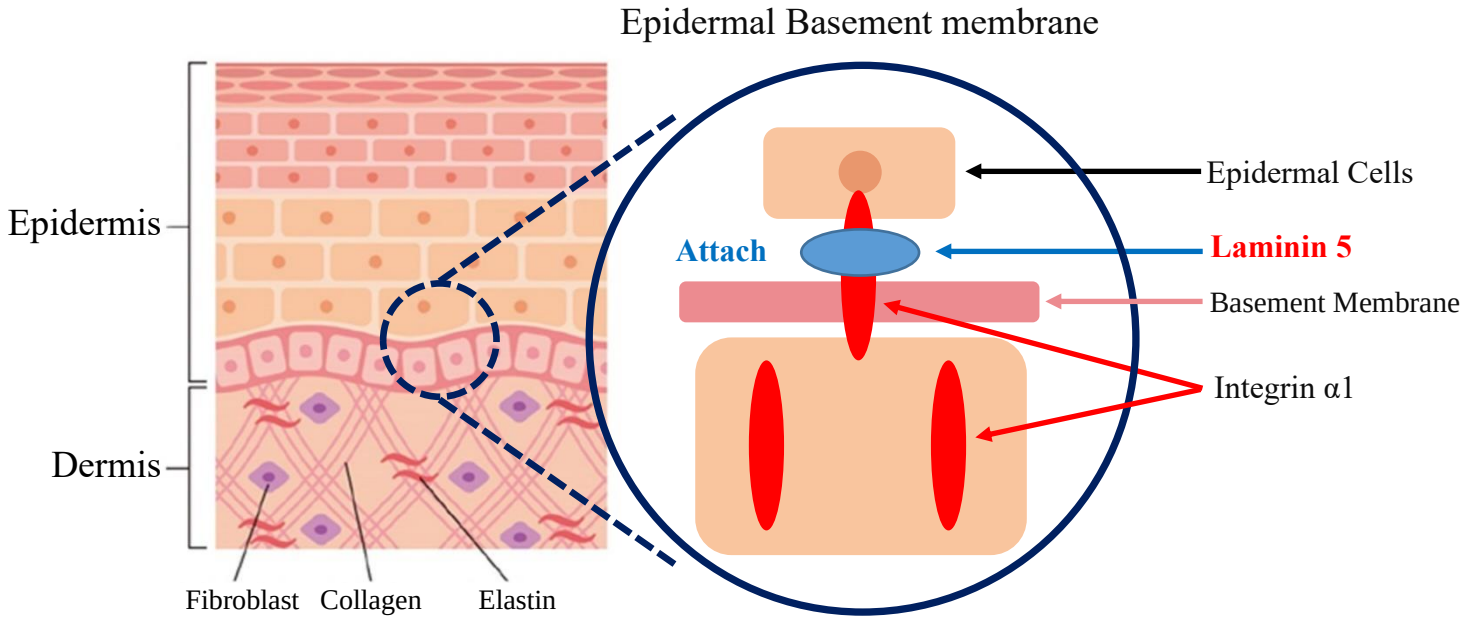


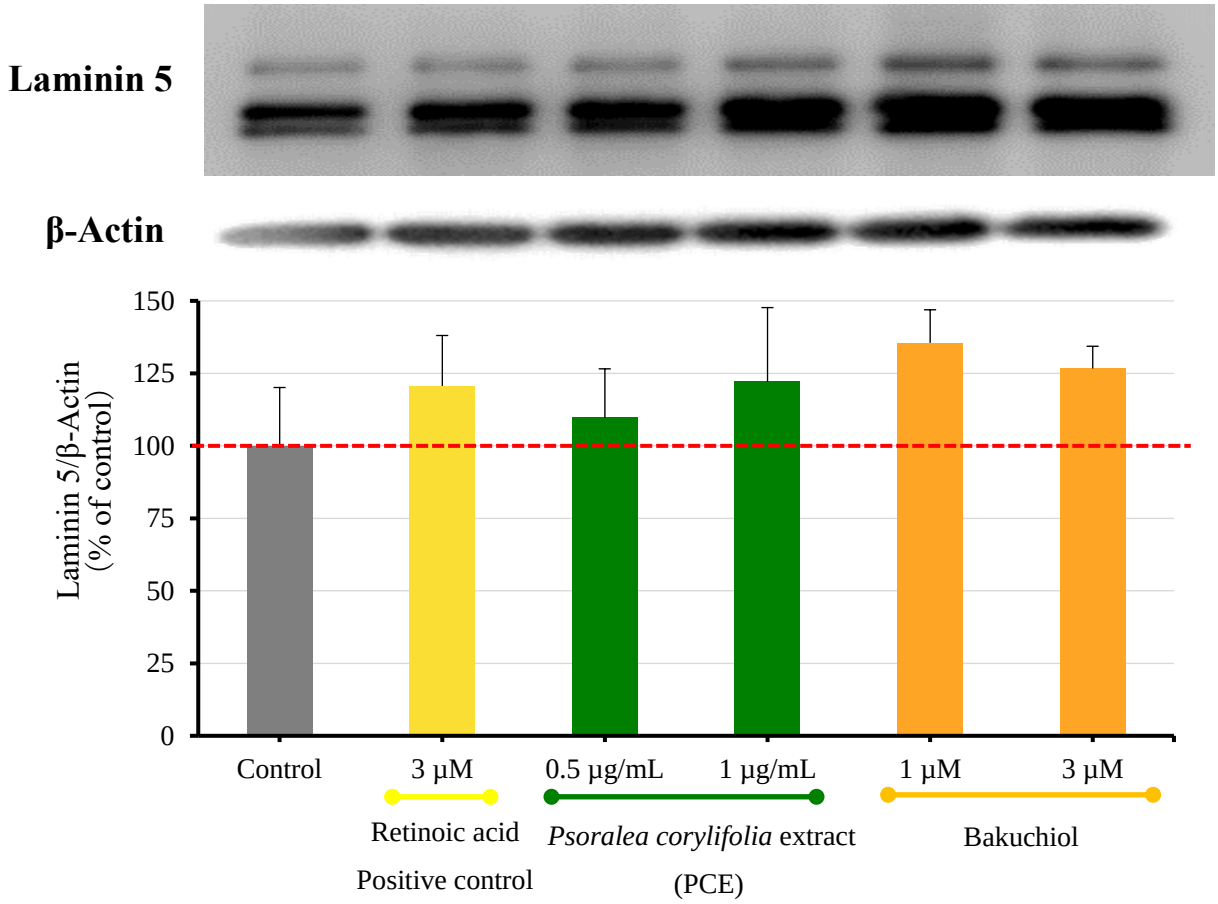
Figure 14. The Role of Laminin 5 in Skin.

Focusing on **laminin 5**, which is present in the basement membrane of the epidermis, the expression levels of laminin 5 protein were measured in *Psoralea corylifolia* extract (PCE) and Bakuchiol. Retinoic acid (vitamin A) was used as a positive control.

[Result]

PCE and bakuchiol increased laminin-5 expression as well as positive controls (Fig. 15). For bakuchiol, the effect was found to be comparable to that of the positive control.

These results suggest that PCE and bakuchiol enhance the adhesion between the epidermis and dermis and have anti-sagging and anti-wrinkle effects.



Each bar represents the means±S.E.M (N=4). Asterisks denote significant differences from the control group, **, p<0.01.

Figure 15. Effect of PCE and Bakuchiol on laminin-5 protein expression in human epidermal keratinocytes.

[Method]

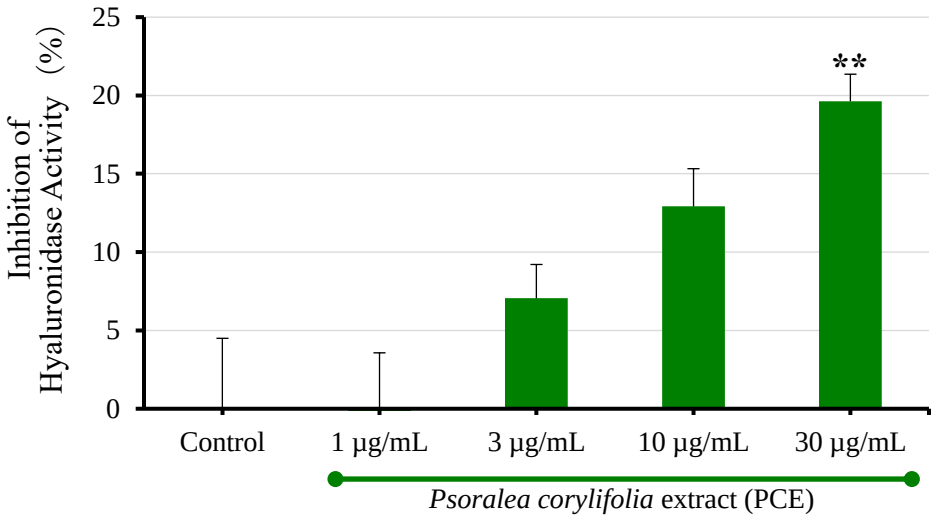
Normal human epidermal keratinocytes (NHEKs) were seeded on culture plates (collagen-coated). After 1 day of culture, the medium was replaced with a specific human epidermis medium (HuMedia-KG2) containing the test sample and cultured for 2 days. The cells were then subjected to protein extraction and western blotting using the established method targeting laminin 5.

v. Inhibition of Hyaluronidase

Hyaluronic acid is a component of the extracellular matrix and is responsible for retaining water in the dermis. The theoretical water retention capacity of hyaluronic acid is 2-6 L/g and it plays a role in maintaining tissue flexibility. However, as the skin ages, the amount of hyaluronic acid in the skin decreases due to a decrease in fibroblast production capacity and increased expression of degradative enzymes such as hyaluronidase. This leads to wrinkling, sagging and loss of skin firmness. As mentioned above, hyaluronidases have been reported to be involved in allergic and inflammatory reactions. Therefore, in this experiment, the **inhibitory effect of hyaluronidase**, an enzyme that degrades hyaluronic acid, was evaluated.

[Result]

As a result, a concentration-dependent hyaluronidase inhibitory effect was observed in *Psoralea corylifolia* extract (PCE) (Fig. 16).



Each bar represents the means±S.E.M (N=6). Asterisks denote significant differences from the control group, **, $p<0.01$.

Figure 16. Inhibition Effect of PCE on Hyaluronidase.

[Method]

The evaluation tests were carried out according to the previously reported method of Morikawa et al.[20].

vi. Anti-Inflammatory Activity (Reported by Paper)

The main cause of skin inflammation is the invasion of allergens such as UV irradiation and pollen into the skin. A review of the paper confirmed that *Psoralea corylifolia* Extract (PCE) and its constituents have anti-inflammatory activity (Fig. 17).

A) Inhibition of Inflammation Induced by UV

Continuous exposure to UV irradiation causes the generation of reactive oxygen species in the epidermis and the production of the inflammatory chemokine IL-8 in fibroblasts in the dermis. In the skin, IL-8 receptors are expressed on neutrophils, mast cells and epidermal cells. Recent studies have shown an increase in IL-8 receptors on epidermal cells in inflammatory diseases such as psoriasis and atopic dermatitis, and IL-8 is an important target for the treatment of skin diseases[21]. In addition, IL-8 is produced at higher levels in the elderly than in the young and is recognised as a mediator of ageing.

Baequeville et al.[22] reported that bakuchiol inhibits IL-8 production in UVA-irradiated fibroblasts and inhibits the production of the "p16 protein", which has the power to arrest the G1 phase of the cell cycle, the first phase in which mRNA and proteins are synthesised.

B) Inhibition of Inflammation by Allergens (Pollen, Dust)

When allergens such as pollen and dust enter the skin, they bind to the surface of mast cells and release inflammatory cytokines (TNF-α, histamine and β-hexosaminidase) as a type I allergic reaction (degranulation). These inflammatory cytokines not only cause redness, itching and other inflammation, but also cause an influx of calcium ions into the cells of the dermis, leading to the activation of collagenases and hyaluronidases. These cause degradation of the extracellular matrix, leading to wrinkles, sagging, etc.

Morikawa et al.[10] found degranulation inhibition of mast cells (RBL-2H3 cells) in PCE. In addition, Morikawa et al. isolated and identified various components of the PCE and elucidated the active contributing components.

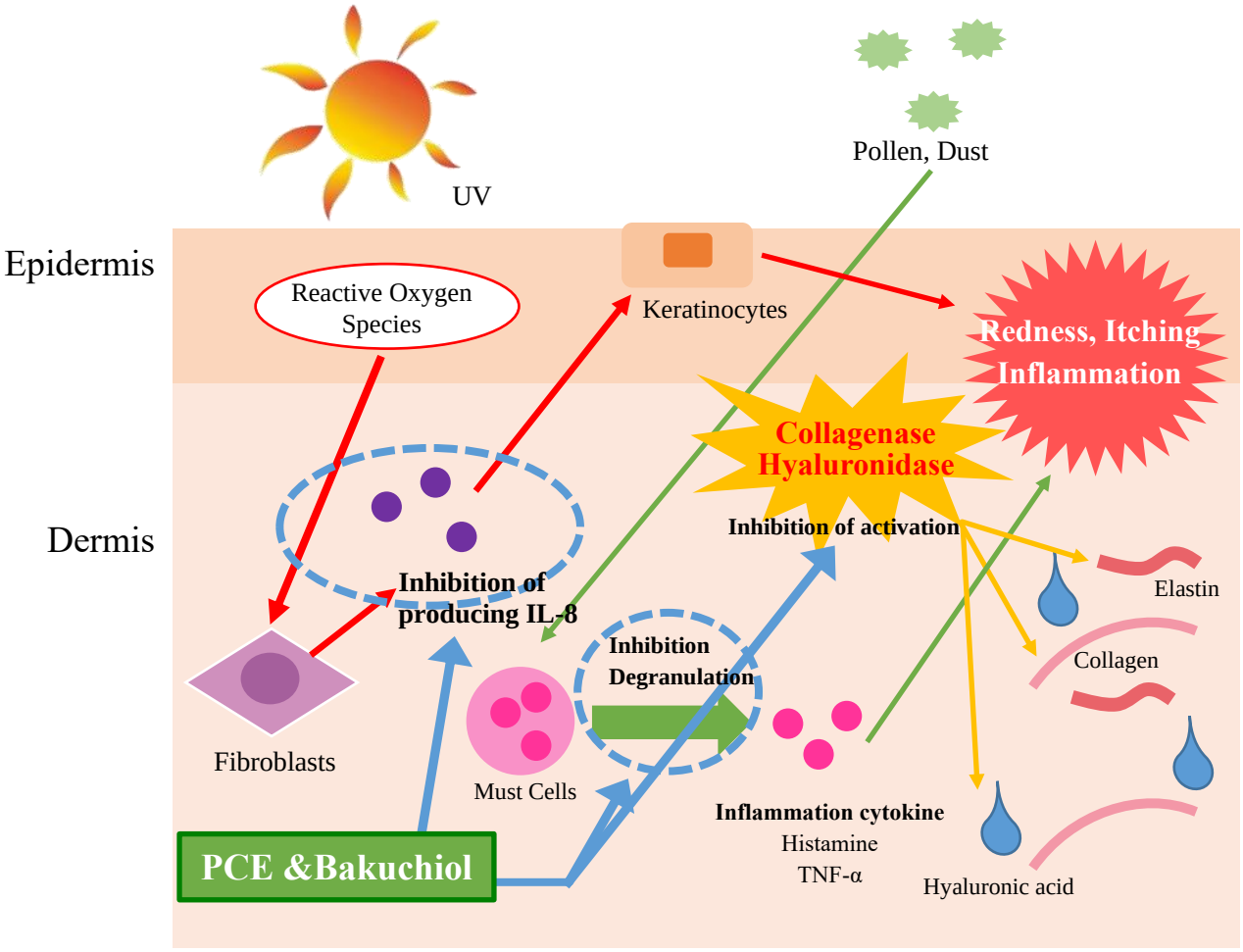


Figure 17. Mechanism of Skin inflammation and Anti-Inflammatory Effect of *Psoralea corylifolia* Extract (PCE) and Bakuchiol

These papers and our results suggest that PCE and bakuchiol prevent the destruction of intercellular networks and dermal structure by inhibiting UVA-induced cytoskeletal atrophy in fibroblasts in the dermis. In addition, PCE and bakuchiol are thought to have anti-inflammatory effects in the skin by suppressing the production of inflammatory cytokine (IL-8) released by UVA-irradiated fibroblasts and inhibiting degranulation in mast cells. Furthermore, suppressing inflammation, it also suppresses the production of p16 protein, which is a cause of ageing, and is thought to have anti-ageing effects.

6. Human Clinical Trial in-House (Oral Intake)

Psoralea corylifolia Extract (PCE), a component of PhytoRetinol™ (product specification on page 37) was monitored for wrinkles by 19 internal volunteers who took it for 4 weeks. This clinical trial was examined according to the Guidelines for the Evaluation of Cosmetic Functions (Guidelines for the Evaluation of Anti-Wrinkle Products). We evaluated improvement effect of PhytoRetinol™ in maximum wrinkle depth and width at the corners of the eyes.

The main component of PCE, bakuchiol, has a vitamin A-like effect. So, the intake of bakuchiol was adjusted at 1 mg/day. So as not to exceed the standard intake of vitamin A, in accordance with the Dietary Intake Standards for Japanese People published by the Ministry of Health, Labour and Welfare.

[Method]

i. Study design

Open-trial and intake period is 4 weeks. We examined photographing the wrinkles at the corners of the eyes before and 4 weeks after taking the product and by analysing the wrinkles using a wrinkle replica at the corners of the eyes.

ii. Test Sample

Hard capsule containing 35 mg Phytoretinol™-3 (1 mg as bakuchiol) and 150 mg excipients.

iii. Subjects.

Subjects were recruited from Oryza Oil & Fat Chemical Co. Ltd. (employees working at the head office). After an explanation of the study, subjects were asked to agree to participate in the study. Subjects were healthy adult men and women with the following exclusion criteria: (1) those undergoing hormone therapy, (2) those who were pregnant or lactating, (3) those with allergic reactions to the test products, and (4) those with a history of cosmetic treatment affecting the test area. As a result, 19 healthy male and female subjects were chosen.

iv. Measurements.

Photography and 3D analysis of the collected wrinkle replicas.

v. Statistical analysis

Measurements for each item was presented as mean±standard deviation. Significant difference tests were performed after analysis of variance and comparison of pre-test to post-test (paired t-test).

[Result]

Analysis of the wrinkle replicas of all subjects (19 subjects) showed a trend towards improvement in wrinkles in terms of total volume rate, wrinkle area rate and wrinkle volume rate (Fig. 19). When analysed by wrinkle grade*, a significant improvement in the number of wrinkles was observed in subjects with wrinkle grades 2-3 (Fig. 20). Subjects with wrinkle grade 4 and above showed a trend towards improvement in total volume ratio, wrinkle area ratio, wrinkle volume ratio and maximum wrinkle width (Fig. 21). Examples of two subjects who showed a significant effect on wrinkles at the corners of the eyes are shown in Figures 22 and 23.

[What is wrinkle grade?]

Wrinkle grade refers to the evaluation criteria for the degree of wrinkling set out in the Guidelines for the Evaluation of Anti-Wrinkle Products (Fig. 18). The testers determined the subject's wrinkle grade.

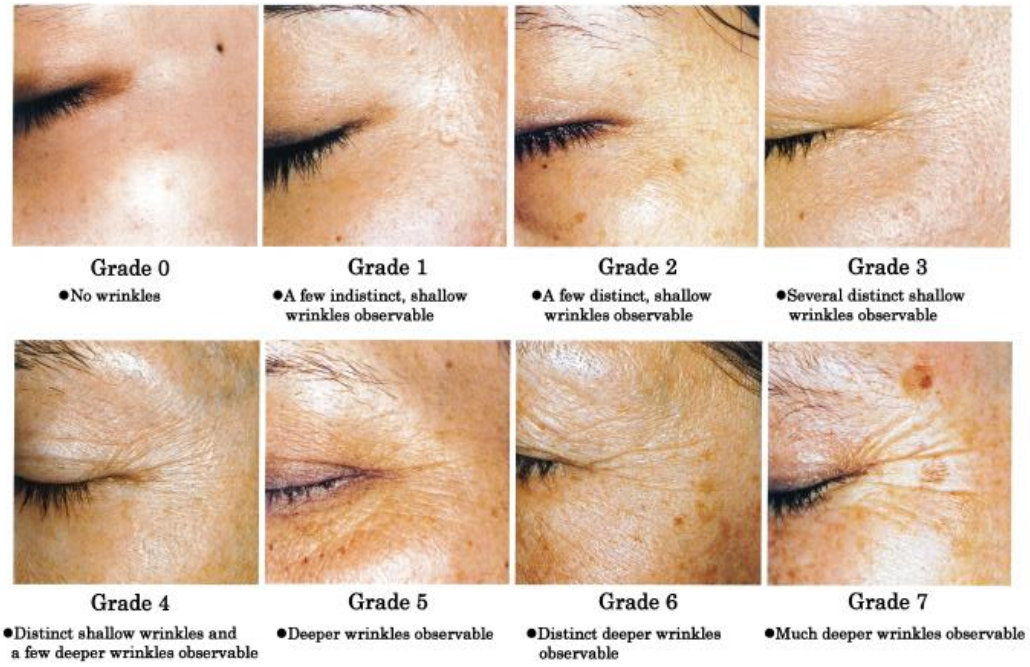


Figure 18. Wrinkle Grade

Film scenes

Replica analysis system for reflection (ASA-03RXD)

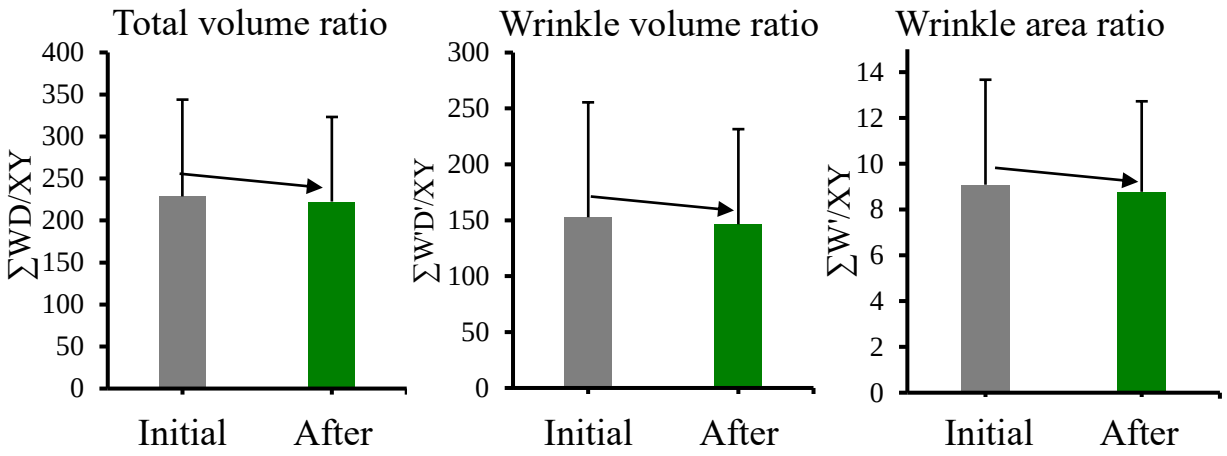


Figure 19. Total volume, wrinkle volume and are ratio for all subjects (n=19)

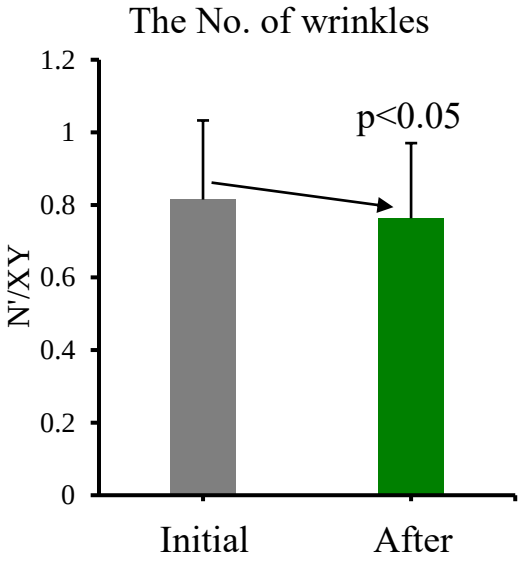


Figure 20. The number of wrinkles in grades 2-3 (n=13)

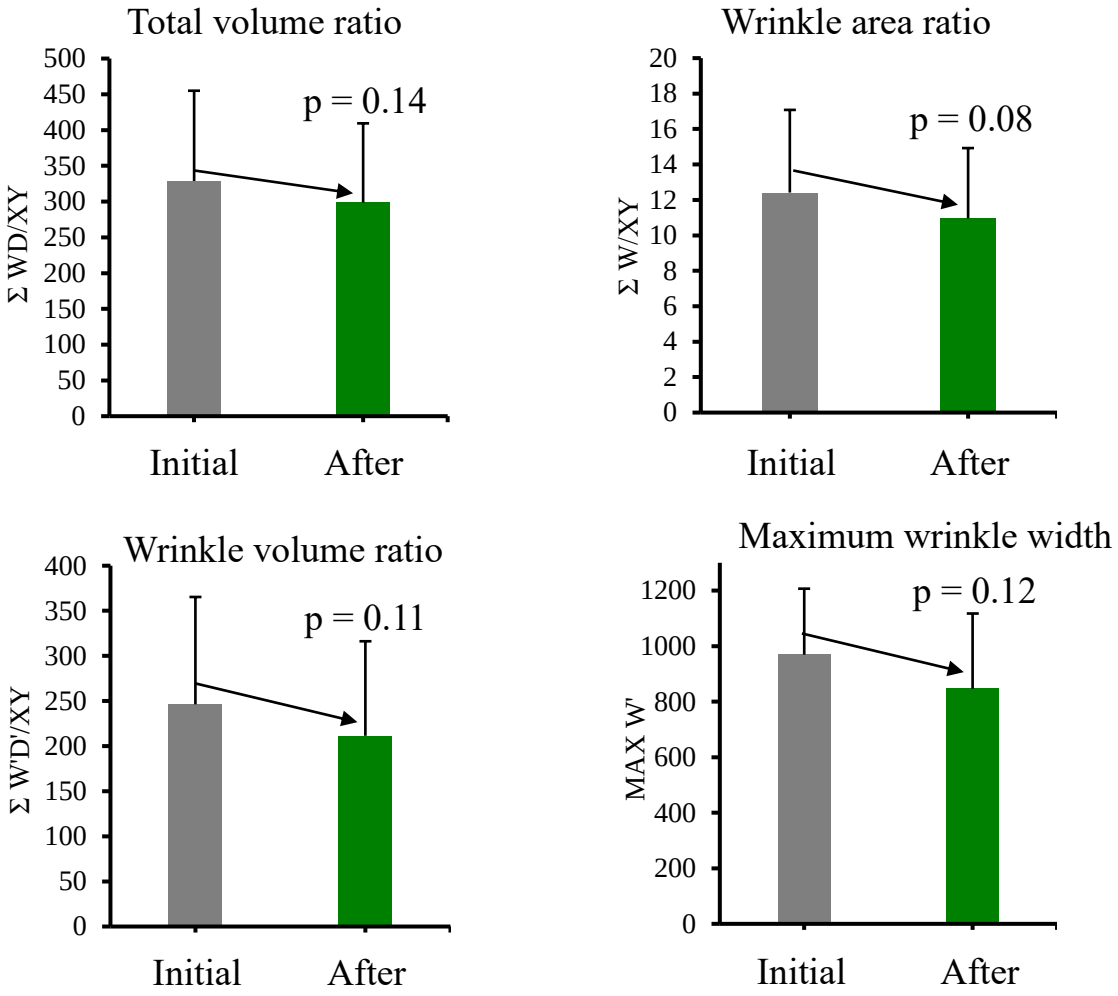


Figure 21. Total volume ratio, wrinkle area and volume ratio and maximum wrinkle width in grade 4 and above (n=6)

*Units for each analysis point.

Total volume ratio

(W: width in μm, D: depth in μm, X: width of rectangle in mm, Y: number of lines).

Wrinkle area ratio

(W: width of the wrinkle in μm, X: width of the rectangle in mm, Y: number of lines)

Wrinkle volume ratio

(W: Wrinkle width in μm, D: Wrinkle depth in μm, X: Width of rectangle in mm, Y: Number of lines)

Maximum wrinkle depth

(maximum depth detected as wrinkle, D: wrinkle depth in μm).

Number of wrinkles (the number of wrinkles counted as wrinkles in the quadrangle is converted by image analysis per point per mm, N: number of wrinkles, X: width of the quadrangle in mm, Y: number of lines).

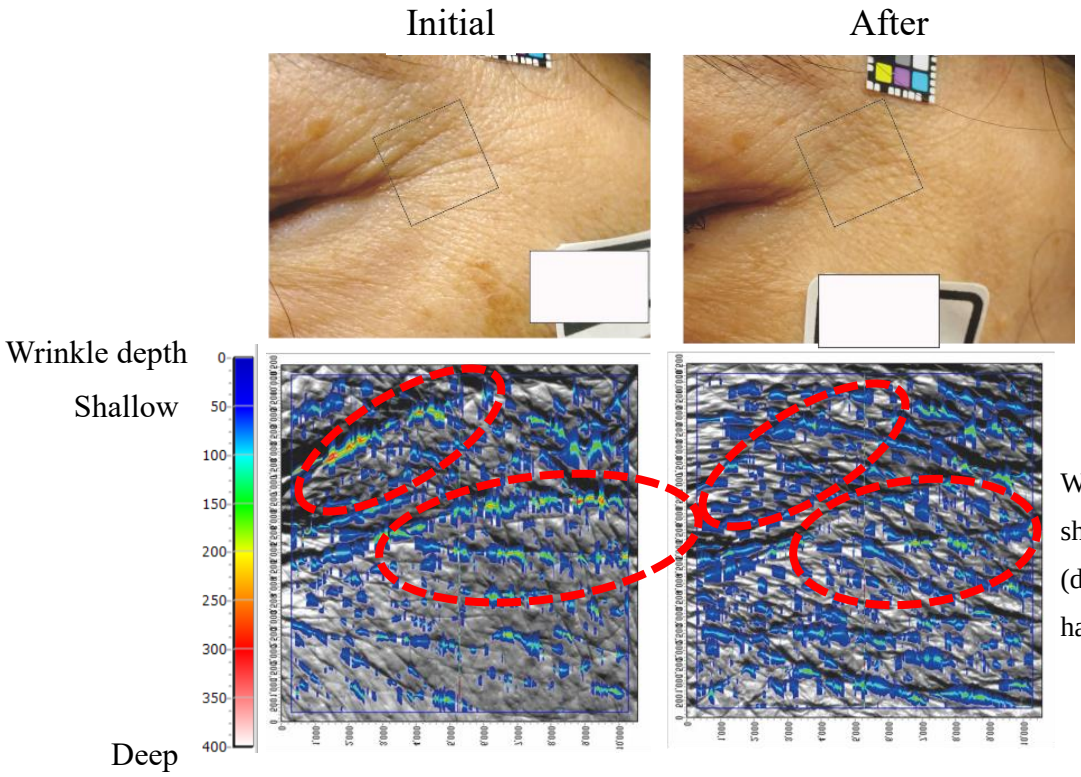


Figure 22. Comparison of initial and after ingestionon subject A

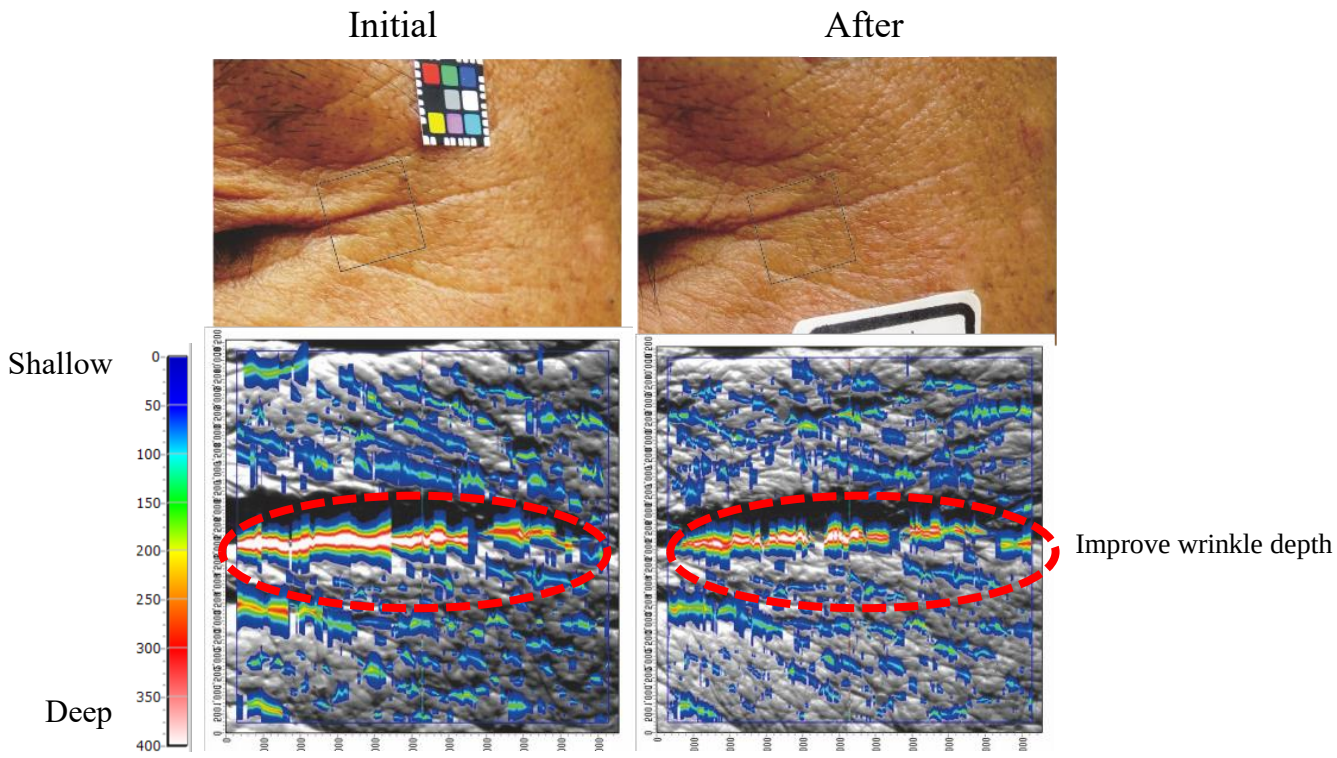


Figure 23. Comparison of initial and after ingestion on subject B

The results show that after 4 weeks of continuous intake of PhytoRetinol™-3 (35 mg/day) in 19 healthy male and female subjects aged 36-61 years, **the number of wrinkles was significantly improved** in the group of subjects with wrinkle grades 2-3 compared to before and after intake. In subjects with wrinkle grade 4 and above, there was also a trend towards wrinkle improvement in photographs and analysed images, although the difference was not significant. This suggests that **PhytoRetinol™ may not only improve shallow wrinkles, but also deeper wrinkles** of grade 4 and above (Figure 24).

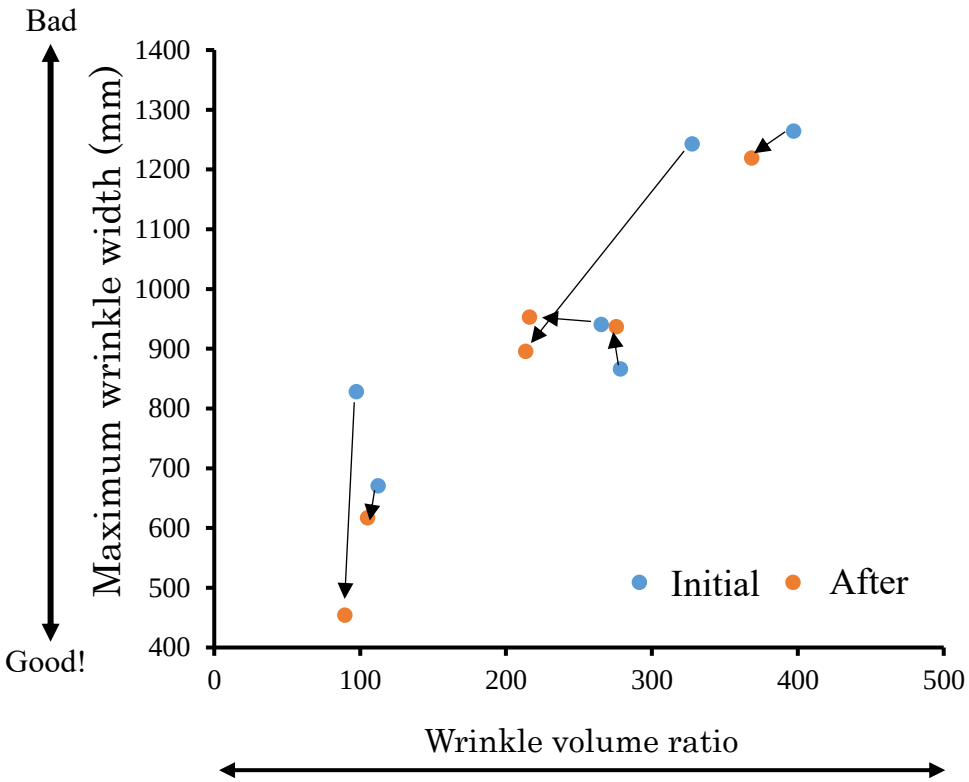


Figure 24. The changes of wrinkle statuses in above grade 4 subject.

7. Product Stability of PhytoRetinol™

i. Heat stability

Heat stability of PhytoRetinol™-3, P1 were examined by heating at 100℃ and 120℃ continuously for 1hour. As shown in Fig. 25, bakuchiol contents was not decreased after heating. Therefore, PhytoRetinol™-3, P1 are highly stable upon heating at normal food processing temperature.

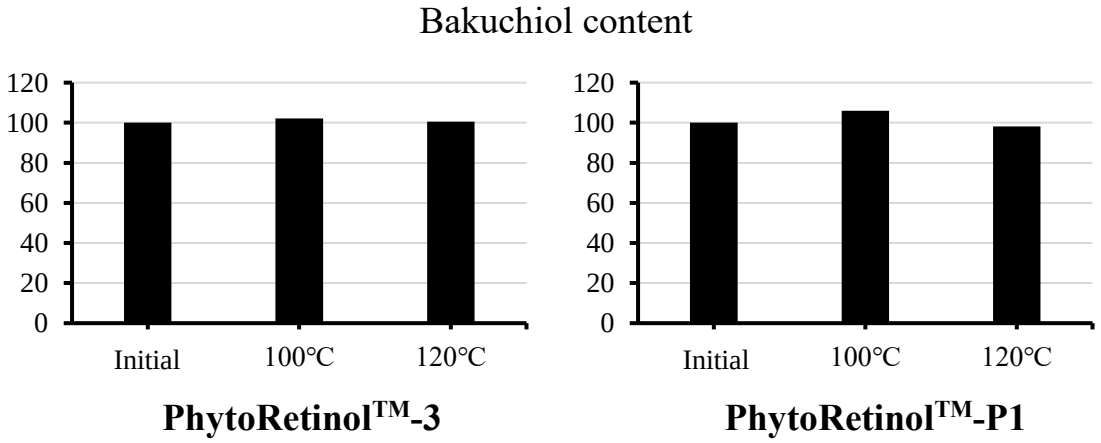


Fig. 25. Heat Stability of Phytoretinol™-P Heating for 1 hours at each temperature

ii. pH stability

Bakuchiol is unstable in water especially low pH.

8. Nutrition Profiles

Analyzed item (/100g)	PhytoRetinol™ -3	PhytoRetinol™ -P1	Method
Water (g)	<0.1	5.8	Heating drying method under normal pressure
Protein (g)	<0.1	0.2	Kjeldahl method, nitrogen protein conversion factor: 6.25
Fat (g)	100	3.6	Acid decomposition method
Ash (g)	<0.1	0.2	Direct incineration method
Carbohydrate (g) －Sugar (g) －Fiber (g)	0 0 0	90.2 83.4 6.8	Refer note 1 Prosky's method
Energy (kcal)	900	380	Refer note 2
Sodium (mg)	0.4	13.3	Atomic absorption spectrophotometory
Sodium chloride equivalent (g)	<0.01	0.03	Refer note 3

The nutritional information of Phytoretinol™ was analyzed according to the standard in nutrition labeling (March 30, 2015; No 139 Eishin)

Note 1: Calculation: 100-(water + protein + fat + ash)

Note 2: Energy conversion factor: Protein 4, fat 9, sugar 4, dietary fiber 2

Note 3: In terms of sodium

Test trustee: SUNATECH/ Dte of analysis: October 20, 2020

Test No: PhytoRetinol™-3: 201009631-002-01

PhytoRetinol™-3: 201009631-001-01

9. Safety Profiles

i. Residual agricultural chemicals

Psoralea corylifolia Extract was screened and analyzed for residual agricultural chemicals (504 items) stipulated under the Food Sanitation Act and Pesticides Control Act, presence of the test items was lower than the allowed limits.
Test trustee: SANATECH Co., Ltd.
Date: October 26, 2020
Report No: 201009631-003-01

ii. Acute Toxicity (LD_{50})

Acute Toxicity test was conducted according to the Guidelines for Single-Dose Toxicity Tests for Pharmaceutical Products. Tomato Seed Extract 2000 mg/kg was orally given to fasted ICR mice (6 weeks old). After 14 days, no abnormalities and fatal event were observed at 2000 mg/kg. No abnormalities were observed under macroscopic examination upon autopsy. Thus, LD_{50} of *Psoralea corylifolia* Extract is deduced to be > 2000 mg/kg.

iii. Mutagenicity (Ames test)

Ames test was conducted to evaluate the mutagenicity of *Psoralea corylifolia* Extract using Salmonella typhimurium TA98, TA100, TA1535, TA1537 and E. coil WP2 at concentration 19.5-5,000 μg/plate. No mutagenicity was observed.

iv. Phototoxicity Test

Phototoxicity safety tests conducted on cells and animals for *Psoralea corylifolia* Extract (psolarene-removed product) and bakuchiol both showed no phototoxicity.

10. Recommended Dosage

We recommend 35 mg/day of Phytoretinol™-3 or 100 mg/day Phytoretinol™-P1 based on the result of human clinical trial.

11. Application

	Applications	Indication	Examples
Food	Nutritional supplement, Beauty food	Improvement Effect of Wrinkle and Sugging	Beverages, Hard and soft capsules, tablets, Candies, Chewing gums, Gummies, Cookies, Chocolates, Wafers, Jellies etc.

12. Packing

PhytoRetinol™-3

1 kg, 5kg — Interior packing: Coated Can
Exterior packing: Cardboard box

PhytoRetinol™-P1

1 kg, 5kg — Interior packing: Aluminium bag
Exterior packing: Cardboard box

13. Storage

Store in a dry, ventilated location. Keep away from high temperature and sun light, store in the closed containers.

14. Expression

<Food>

PhytoRetinol™-3

Expression : Edible Oil, Psoralea corylifolia Fruit Oil/Antioxodant (Rosemary extract)

PhytoRetinol™-P1

Expression : Psoralea corylifolia Fruit Oil/cyclodextrin, Arabic gum

15. Reference

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PRODUCT STANDARD

PRODUCT NAME : **PhytoRetinol™-3** (FOOD)

(Psoralea corylifolia fruit oil)

This oil is extracted and diluted from the fruits of *Psoralea corylifolia* Linne (*Legminosae*).

It contains minimum of 3.0% bakuchiol.

Appearance	Brown to orange brown oil with characteristic odor.	
Bakuchiol	Min. 3.0%	(HPLC)
Acid Value	Max. 10	
Purity Test		
(1) Heavy Metals (as Pb)	Max. 20 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	Negative	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredient	Content (Values are just a guide)
Triglyceride	90.0%
Psoralea corylifolia fruits oil	9.9%
Rosemary extract	0.1%
Total	100.0%

Expiry date	2 years from date of manufacturing.
Storage	Store in a dry, ventilated location. Keep away from high temperature and sunlight.

PRODUCT STANDARD

PRODUCT NAME : **PhytoRetinol™-P1** (FOOD)

(Psoralea corylifolia fruit oil)

This product is powder of oil extracted from the fruits of *Psoralea corylifolia* Linne (*Legminosae*). It contains a minimum of 1.0% bakuchiol.

Appearance	Yellowish white to pale yellowish brown powder with slightly characteristic odor.	
Bakuchiol	Min. 1.0 %	(HPLC)
Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105ºC, 2 hr)
Purity Test		
(1) Heavy Metals (as Pb)	Max. 20 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

Ingredient	Content (Values are just a guide)
Cyclodextrin	90 %
Acacia Gum	7 %
Psoralea corylifolia fruits oil	3 %
Total	100 %

Expiry date	2 years from date of manufacturing.
Storage	Store in a dry, ventilated location. Keep away from high temperature and sunlight.

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 :

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Established Date : July 31, 2024
Revised Date: July 31, 2024

ORYZA OIL & FAT CHEMICAL CO., LTD.