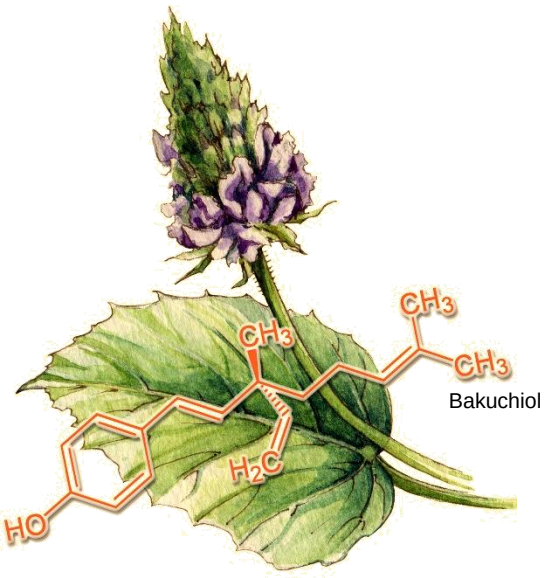
ORYZA OIL & FAT CHEMICAL CO., LTD.

Improvement Effect of Fine Wrinkle by Drying

PhytoRetinol™

Psoralea Corylifolia Fruit Extract



Bakuchiol

Psoralea corylifolia Linnè *(Leguminosae)*

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1. Introduction

"You look much younger than you actually are. I can't believe you're XX years old!"

This is a classic compliment given to women, but has anyone ever told you this? The latest research has revealed that in these cases, not only do these people look young, but their body is really younger than their actual age. Surprisingly, it is said that people do not age equally.

An international team of researchers examined the process and effects of aging on 1,000 men and women born in New Zealand between 1972 and 1973. In 2011, when researchers examined the degree of aging of the internal organs of examinees aged 38 years old, they found that the age of their internal environment differs from 30 at the youngest to 60 at the oldest. This was the result of examining every element including organs, immune system, health condition of the heart, as well as chromosomes. This shocking result was published in the scientific journal “The Proceedings of the National Academy of Sciences (PNAS)”.

Daniel Belsky, a professor of medicine at Duke University, says "There's a correlation between how old a person looks and how fast their internal environment ages.” But why is there such a huge individual difference? There was a time when researchers could not answer the reason as to why some people aged faster than others. However, Belsky reports that "genetics, stress and eating habits" are key factors in the aging process. Currently, research is being conducted on how to slow down the aging process by taking these factors into account.

Even by global standards, Japan has long been described as a super-aging society, where the proportion of elderly people in the population is very high. Everyone has the goal of staying young and healthy both mentally and physically. A major part of everyone's desire to look young forever is strongly related to their concern about their appearance. Especially, reducing expression wrinkles is a key factor in looking younger than our actual age. Currently in Japan, retinol is used in many wrinkle-reducing creams (quasi-drugs). Although it has a high wrinkle-reducing effect, its use requires an understanding as it breaks down easily when exposed to light or heat.

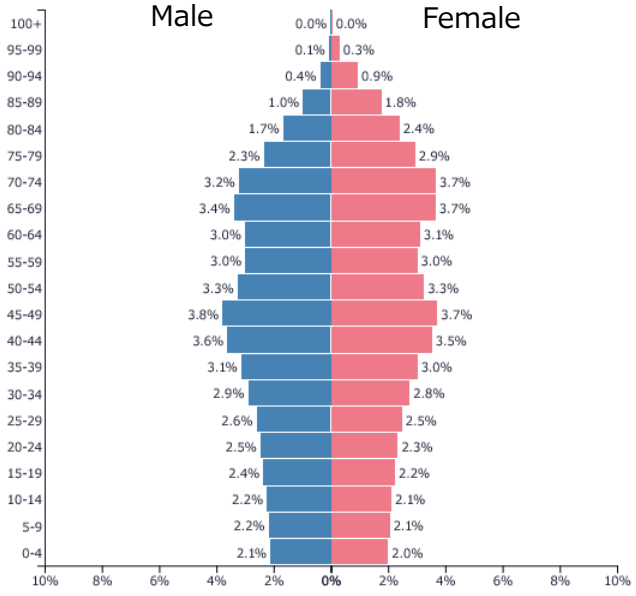


Fig.1: Japanese Population Pyramid 2018

From the statistics of
the Ministry of Internal Affairs and Communications

We have screened plant extracts that have an effect similar to retinol, and have developed PhytoRetinol™, which reduces wrinkles especially ones caused by drying.

As shown in Figure 1, the percentage of women in their teens to 24 years old, during which they start to use cosmetics, is 4.5% of the total Japanese population. The percentage of women aged 25 to 49 is 15.5% and the percentage of women aged 50 or older is 25.1%.

The percentage for men is 4.9%, 16.0%, and 21.4% respectively, for the same age groups. Notably, men and women aged 65 to 74 account for 14% of the total population of Japan, creating a generational pyramid that represents Japan's aging society.

Referring to "World Health Statistics 2019" released by the World Health Organization (WHO) in April 2019, we summarized the average life expectancy in 2016 for Japan, the United States, China, Russia, as well as Lesotho, a country surrounded by South Africa and regarded to be the poorest in the world and whose life expectancy is the lowest in the world.

	World	Japan	U.S.A.	China	Russia	Lesotho
Male	69.8s	81.1s	76.1s	75.0s	66.4s	51.0s
Female	74.2s	87.1s	81.1s	77.9s	77.2s	54.6s
Total	72.0s	84.2s	78.6s	76.4s	72.0s	52.9s
World Ranking		1st	34th	50th	103th	183th

Table 1: Comparison of life expectancy in the world and each country
(Excerpt from WHO announcement in April 2019, World Health Statistics 2019)

From this table, you can see that Japan has one of the longest life expectancies in the world, and there is a strong need for anti-aging ingredients, especially anti-wrinkle ingredients, that are in demand among the elderly. Therefore, health food and cosmetic ingredient manufacturers have been expected to develop products specifically designed with anti-wrinkle effects.

To maintain youthfulness, it is recommended to combine high moisture retaining ingredients with properties to reduce fine lines as well as ingredients that can be expected to improve wrinkles.

2. Physiological aging and Photo-aging[1), 2)]

Biologically, aging is a process of changing that occurs in an individual organism over time, and in particular, it can be said that it is a functional decline or process that occurs before an organism dies. Particularly, as the skin is exposed to UV-rays which are from the external environment, photo-aging, which is an environmental factor, inevitably occurs simultaneously, in addition to physiological aging.

Physiological aging is an aging process of the skin caused by gaining age, and is visually characterized by fine wrinkles and dryness. The skin becomes thinner, metabolism decreases, and the skin turnover rate slows. This is due to the reduction in collagen in the dermis and the decline of elastin's elasticity, which is an elastic fiber. Proteins such as collagen and elastin bind to active sugar and cause the Maillard reaction (glycation reaction), which reduces water retention capacity and skin elasticity. The epidermal barrier function also declines due to a decrease in sweat as well as natural moisturizing factors. This reduces the moisture content of horny cells and leads to dryness.

On the other hand, photo-aging is mainly a change in the skin caused by UV-rays, and is characterized by deeper wrinkles compared to that of physiological aging. This is due to the changes in the epidermal cells themselves and the degeneration of elastic fibers. Furthermore, hyperpigmentation, or blotchiness as it is commonly called, is also mainly caused by melanin pigments taking roots in the epidermis due to UV-rays. As dryness and wrinkles are more notable on the face, which is exposed to the external environment as compared to other body parts covered by clothing, 80% of the aging process is photo-aging.

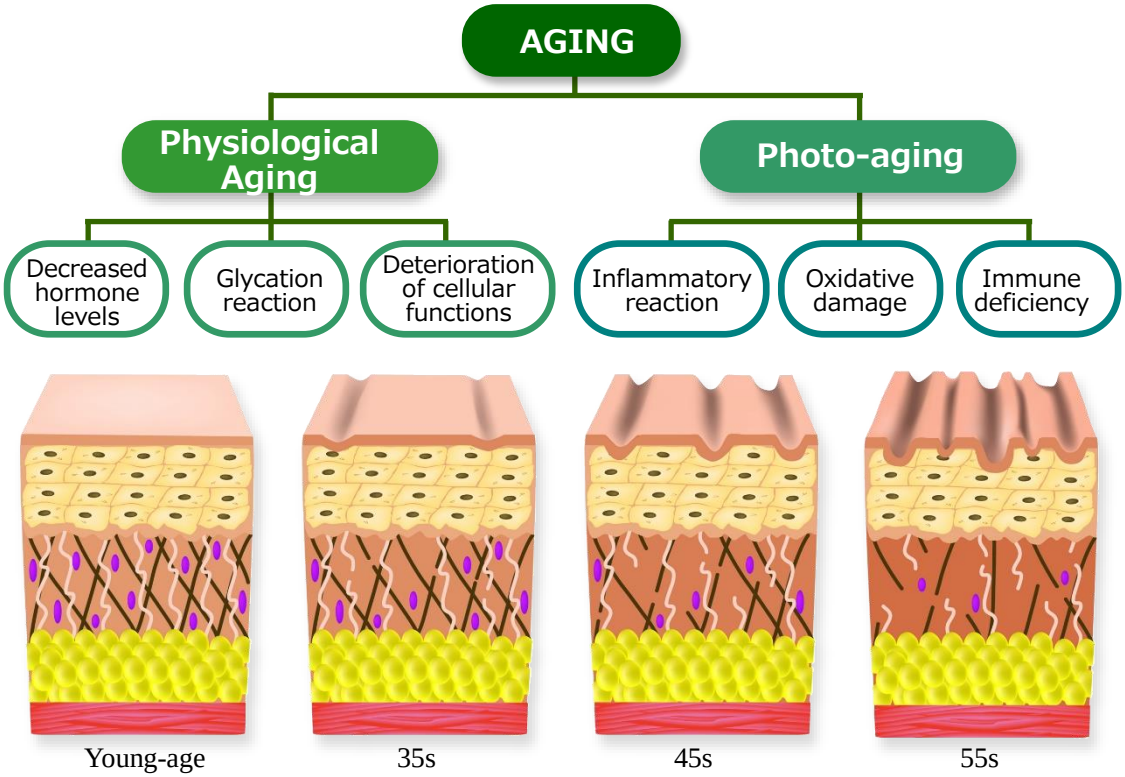


Fig.2: Types of aging and wrinkle formation process

3. Active Ingredient for Improving Wrinkles: Retinol[3)]

Retinol is a vitamin in the vitamin A family, and in the broad sense, the word retinol is used interchangeably with retinoid (vitamin A analogues). Also, it occasionally includes not only retinol ($C_{20}H_{30}O$), but also retinal ($C_{20}H_{28}O$) and retinoic acid ($C_{20}H_{28}O_2$).
It has long been known that retinol is involved in cell differentiation and proliferation. When applied to the skin, it has the effect of creating new cells and rejuvenating the skin.

As the table below shows, retinoic acid is a medication that is prescribed under the supervision of a dermatologist, but as a side effect, the following symptoms are known to be negative aspects of using retinol:

1) Skin becomes more susceptible to UV damage.
2) Dryness
3) Tingling feeling
4) Redness and rashes

There are examples of its use in Japan through parallel importation since its use as a cosmetic product is approved in other countries and its price is relatively reasonable. However, it is necessary to note that if any of the side effects described above are noticed, its use should be discontinued immediately and medical attention from a dermatologist will be required.

	Retinol (Vitamin A)	Retinoic acid (Tretinoin)
Category	Quasi-drug/ Cosmetics	Drug
Biological activity	1	50 to 100
Effects on the skin	Small	Large
Side-effect	Small	Large
Stability	Bad	Bad

Table 2: Retinol and Retinoic acid

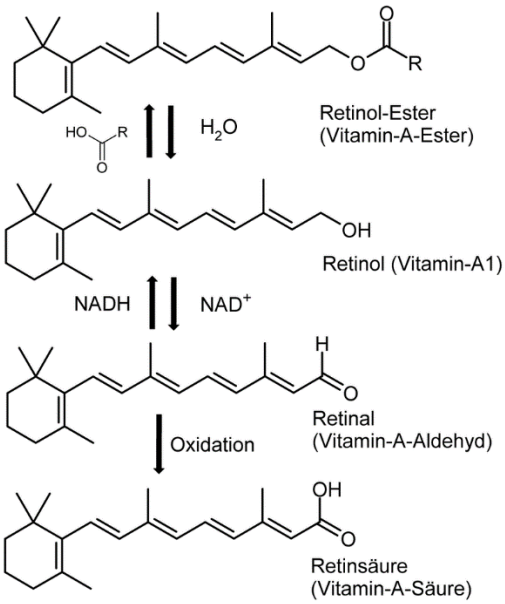


Fig.3: Retinoid Compounds

【Problems with Retinoid】

Tretinoin (vitamin A acid) had once gained prominence in the United States as a wrinkle removing ingredient. However, in Japan, it is a pharmaceutical product only prescribed under the supervision of a dermatologist. Although retinol has milder effects and less stimulation compared to tretinoin, both compounds promote inflammation and suntan on the skin and have poor formulation stability. For this reason, a process such as encapsulating retinol in capsules of liposomes is required for before use.

4. What is Vitamin A[4)]?

Vitamin A is a fat-soluble vitamin, and is the generic name for all substances that exhibit the biological effects of vitamin A. For humans, vitamin A is believed to play an important role in maintaining vision, audibility, reproduction ability, etc.; in promoting growth, in maintaining normal conditions of epithelial tissues such as skin and mucous membranes; as well as in gene expression-mediated anticancer activity and protein synthesis.[4)] Vitamin A includes retinol (vitamin A1), 3-dehydro-retinol (vitamin A2), and derivatives of these vitamins. Vitamin A is extremely unstable chemically and can be isomerized, decomposed, or polymerized by acid, air, light, and heat.

Provitamin A (the precursor to vitamin A), which is converted to vitamin A in the body, belongs to a group of carotenoid pigments that has about 50 different types including major ones such as β-carotene, α-carotene, and cryptoxanthin. In particular, β-carotene is converted into retinol efficiently compared to other carotenoids.

The term "retinol equivalents (RE)" is used to describe its biological potency as vitamin A. 1 μg of retinol is equivalent to 24 μg of α-carotene, 12 μg of β-carotene, and 24 μg of β-cryptoxanthin.

Food Name	Retinol	α-Carotene	β-Caroten	Cryptxanthin	β-Carotene Equivalent	Retinol Equivalent
Raw chicken liver	14,000	-	-	-	30	14,000
Raw pork liver	13,000	-	-	-	Trace	13,000
Raw beef liver	1,100	-	-	-	40	1,100
Raw lamprey	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
Raw Silverfish	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 chicken egg	130	0	3	26	16	140
Processed cheese	240	-	-	-	230	260
Normal milk	38	0	6	0	6	38
Carrot	0	2,400	7,500	0	8,600	720
Spinach	0	0	5,400	45	5,400	450
Crown daisy	0	0	5,300	0	5,300	440
Carrot juice	0	1,300	3,800	0	4,500	370
Western pumpkin	0	18	3,900	90	4,000	330
Turnip leaf	0	0	3,100	28	3,100	260
Broccoli	0	0	770	5	770	64
Tomato	0	4	540	0	540	45
Sweet corn	0	7	22	53	49	4

Excerpt from STANDARD TABLES OF FOOD COMPOSITION IN JAPAN 5th Revised and Enlarged Edition

Table 3: Animal and vegetable foods containing vitamin A and provitamin A (unit: μg / 100g)

5. Retinoid derivative and similar functional components from plants [5), 6)]

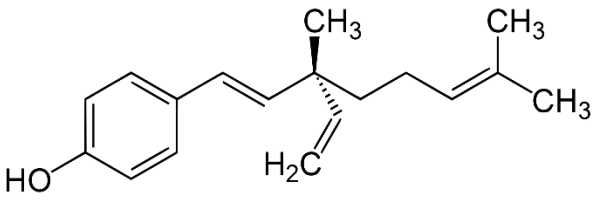
At the International Dermatology Research Conference in 2018, Estee Lauder, a leading cosmetics company in the U.S, explained that although retinol is well known for its anti-aging effects for the skin, its dermal irritancy and optical instability are the drawbacks for use in cosmetics. As a new compound that is more stable and less irritating, they introduced hydroxypinacolone retinoate, a retinoic acid ester. They announced that this compound does not have skin irritancy like tretinoin, and unlike retinol, which metabolizes to the bioactive retinoic acid, hydroxypinacolone retinoate can bind directly to retinoic acid receptors.

Also, the results of a randomized trial that demonstrated the efficacy of bakuchiol, which has been drawing attention as an anti-aging phytochemical, was published online in the "British Journal of Dermatology" on June 27, 2018.

Bakuchiol is a natural organic compound isolated from the legume plant. So far, in addition to anti-aging effects, anti-influenza and other activities have been confirmed. Also, the induction of gene expression similar to a standard treatment retinoid used on skin for anti-aging has been identified, showing that it would lead to amelioration of optical damage to the skin. Bakuchiol was expected to have similar effects as retinoids. However, this was the first time an in vivo study comparing both efficacy and side effects was published.

According to the report, 44 study participants were randomly assigned to two groups, one group applying a cream with 0.5% bakuchiol to the face once a day, and the other group doing the same with a cream with 0.5% retinol. Evaluations were made by comparing high-resolution photos of faces taken at weeks 0, 4, 8, and 12. Side effects were considered through an acceptability questionnaire and board-certified dermatologist's evaluation of hyperpigmentation and skin redness. As a result, significant reductions in wrinkle surface area and hyperpigmentation was confirmed in both groups. Although there was no statistical difference between the two groups, the retinol group had more frequent reports of facial skin desquamation and irritation. Therefore, we have developed and studied plant extracts containing bakuchiol as an ingredient for health food and cosmetics.

(a) Bakuchiol



(b) Retinol

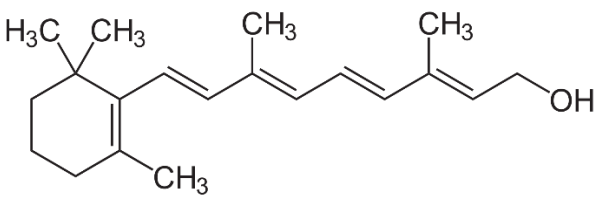
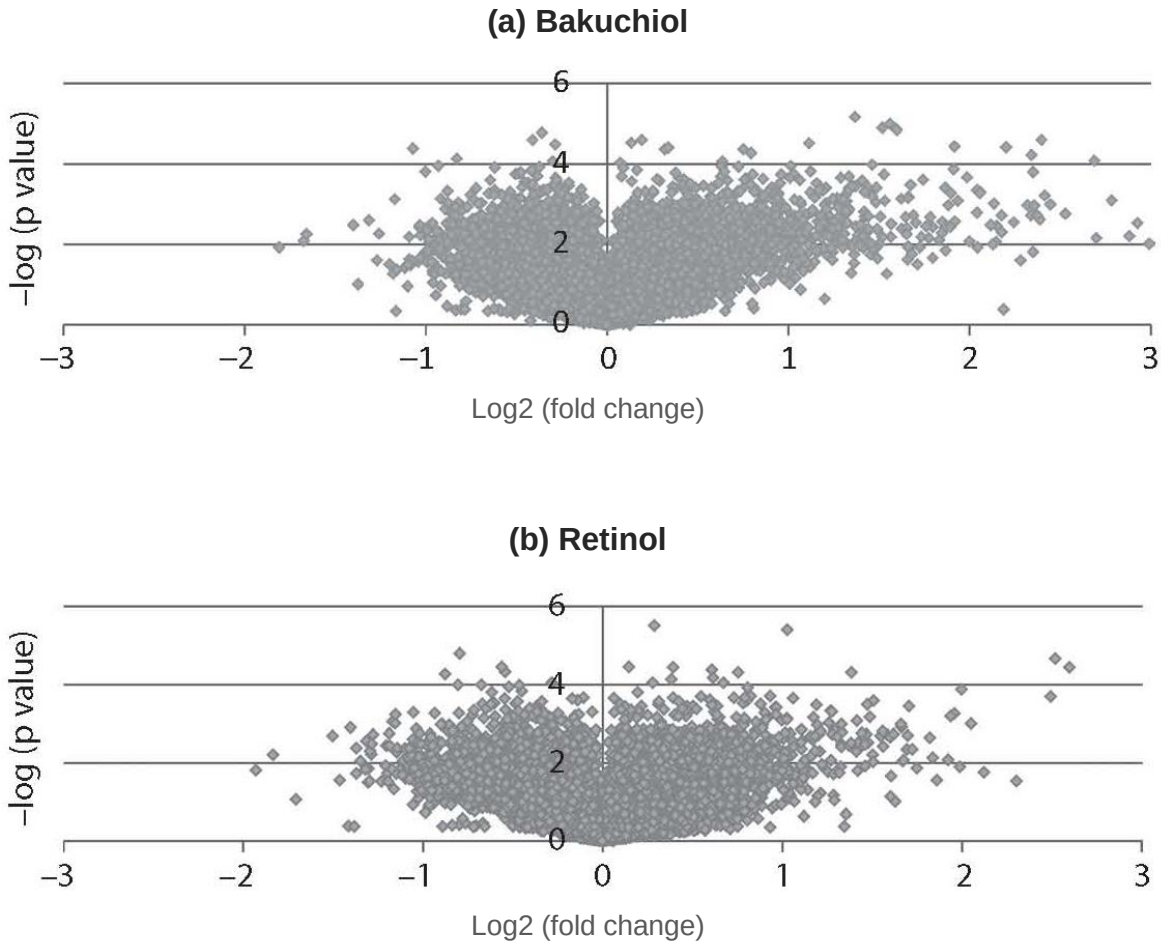


Table 4: Comparison between Bakuchiol and Retinol

Compounds	Bakuchiol	Retinol
IUPAC Name	4-[(1E,3S)-3-ethenyl-3,7-dimethylocta-1,6-dienyl]phenol	2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraen-1-ol
Formula	$C_{18}H_{24}O$	$C_{20}H_{30}O$
Molecular weight	256.4	286.45

A DNA microarray is DNA fragments (called DNA probes) from a large number of known genes (from thousands to tens of thousands of different types) arranged on a solid surface. This technology enables comprehensive analysis of gene expression in cells.



(a) Volcanic plot of DNA microarray data – Bakuchiol. (b) Volcanic plot of DNA array data – Retinol. From Chaudhuri RK, Bojanowski K. Int'l J. Cosmet Sci 2014: 36(3): 221-30

Fig.4: DNA microarray analysis of Bakuchiol and Retinol

6. Bakuchiol

- **[Bakuchiol] Excerpt from Wikipedia**

Bakuchiol (INCI) is a type of meroterpene, a terpene phenol compound. It is a substance naturally produced in *Psoralea corylifolia* and *Otholobium pubescens*. It is **functional analog** of retinol. Since it induces retinol-like gene expression and reduces wrinkles and pigmentation without causing peeling of the skin which retinol would, it is drawing attention as a naturally-derived wrinkle-reducing ingredient.

- **[Reference: VOGUE]**

"A Complete Guide of the Accelerating 'Clean Beauty' Movement." VOGUE. March 28, 2019

- **The latest alternative ingredient to look out for**

Bakuchiol, a plant-derived retinol substitute found in the seeds of the psoralea corylifolia, should not be overlooked. Bakuchiol is classified in the vitamin A group, and is said to promote the growth of collagen and elastin. Belinsky has high expectations for this alternative ingredient. “Since it acts similarly, bakuchiol is a great alternative to retinol. Unlike retinol, which can irritate the skin and cause hyperpigmentation, bakuchiol has no side effects. It is a revolutionary natural ingredient that makes you forget that you are getting old. While brightening the skin, it works on fine lines, wrinkles, pores, and blemishes.” he says.

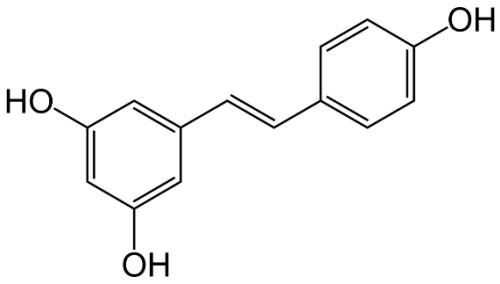


Fig.5-1: Bakuchiol

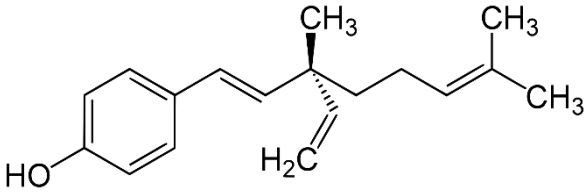


Fig.5-2: Resveratrol

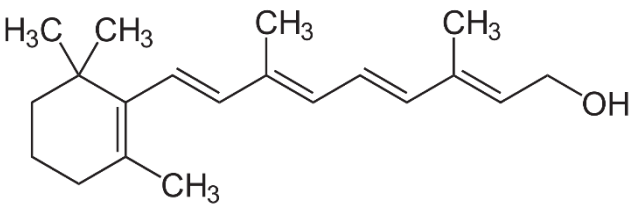


Fig.5-3: Retinol

7. Phototoxicity of Furanocoumarin[7)]

The furanocoumarins, or furocoumarins, are a class of organic chemical compounds produced by a variety of plants. The chemical structure of furanocoumarins consists of a furan ring fused with a coumarin. The furan ring may be fused in various ways producing several different isomers. The most common mother nucleus structures are psoralen and angelicin. Derivatives of these two mother nucleus structures are referred to respectively as linear and angular furanocoumarins.

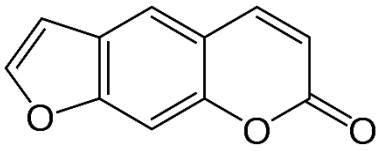


Fig.6-1: Psoralen

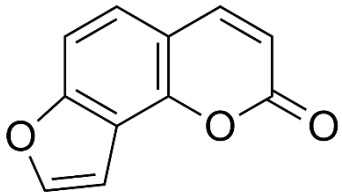


Fig.6-2: Angelicin (Isopsoralen)

Many furanocoumarin compounds are toxic. It is known that plants produce furanocoumarins as a defense mechanism against many types of predators such as insects and mammals. For humans, furanocoumarins are abundant in citrus fruits such as grapefruit, and the furanocoumarins bergamottin and dihydroxybergamottin have become well known as a cause of drug interactions. These furanocoumarins are also known for often being present in the essential oils of citrus fruit peels and causing photosensitivity. Thus, when applying them topically, it is essential to confirm that the furanocoumarins have been removed.

We have developed PhytoRetinol™ focusing on safety. By establishing a process that specifically removes phototoxic furanocoumarins typified by psoralen, while retaining the Bakuchiol, which has a wrinkle-reducing effect on the skin as an active ingredient, we have enabled the start of development of cosmetic and food ingredients using the fruit of *Psoralea corylifolia*, which was considered to be challenging to use.

8. Psoralea Corylifolia / Crude Drug Name: Bu-gu-zhi (補骨脂)

Psoralea corylifolia is a dicotyledonous annual upright plant with a strong odor. It is a species in the genes *Psoralea* in the *Leguminosae* family. Approximately, more than 100 species of psoralea are distributed in the tropics and subtropics. [8)]

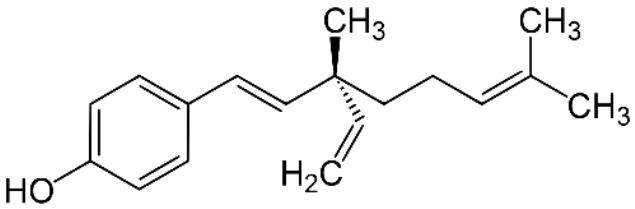
In China, *Psoralea corylifolia* Linnè (*Leguminosae*) was first introduced as a crude drug in the book on medicine called〝開宝本草 (kai-bao-ben-cao)", which was published in 974 during the Song dynasty. It was described that it "cures five strains, seven impairments, wind coldness, bone marrow injury, cold essence of kidney, female hysteria, and abortion". In the "開宝本草", the plant was also called "破故紙 (po-gu-zhi)". Besides, in other medicinal essays such as "薬性論 (yao-xing-lun) " and "日華子 (ri-hua-zi)", it was described as "婆固脂 (po-gu-zhi)" and "胡韮子 (hu-jiu-zi)" respectively, but all of these names indicate the fruits of *Psoralea corylifolia*.[9)]

It is an annual legume distributed from India to Southeast Asia and China (mainly Sichuan, Henan, Shaanxi and Anhui provinces). In Chinese medicine, foreign products were considered better than those produced in China. In Asia, it has been used as a crude drug since ancient times. It is called BAKUCI in the folklore Ayurveda in India, and its seeds are used in a tonic. In Indonesia, seeds are used to cure skin diseases. In China, it is called Buguzhi, and it's a Chinese medicine used for tonicity and coldness.

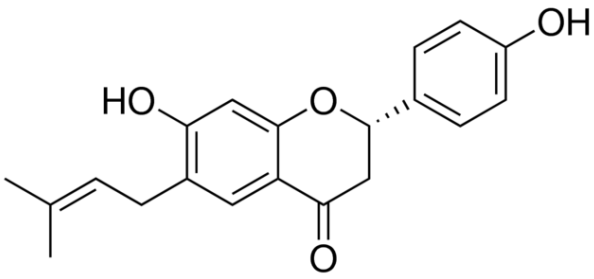
Photo1: *Psoralea corylifolia* in flowering period

9. Components of the fruits of *Psoralea corylifolia*[9)]

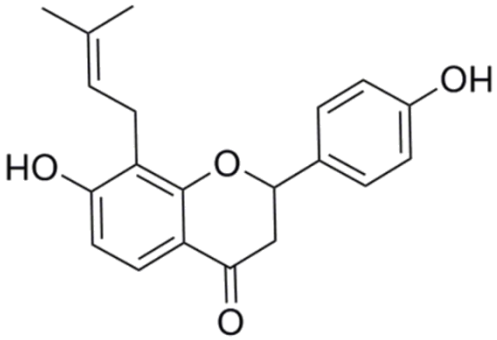
The seeds of the psoralea corylifolia contain a large amount of resin and essential oil components, most of which being Bakuchiol, whose name came from BAKUCI, a herb used in Ayurveda, an Indian folklore. As for the flavones, it is known to contain compounds such as bavachin, bavachinin, and isobavachin. The essential oil also contains psoralen, which is a type of furanocoumarin that can cause phototoxicity on the skin, and its use once required extreme caution. However, to ensure safety, which is the main feature of our unique product, PhytoRetinol™, we have established a method of removing the furanocoumarins during the manufacturing process, and provide a certificate for every lot proving that psoralen was not found in the purity test.



Bakuchiol



Bavachin



Isobavachin

Fig.7: Components in *Psoralea corylifolia*

10. Effectiveness Evaluation

10-1 *in vitro* Evaluation Test

PhytoRetinol™ has been confirmed to be effective in improving wrinkles. Broadly, wrinkles have two factors: first, physiological aging, and second, photo-aging, which is caused when epidermis or dermis is damaged by UV-rays. Regarding wrinkles, photo-aging is the underlying cause for most of them. According to a report by Yamada et al.[10], when exposed to UV-rays, fibroblasts atrophy their cytoskeleton compared to when not, and they take a contractionary form, allowing the cells themselves to minimize the damage from UV-rays. However, it is reported that as a detriment, the production capacity of extracellular matrix (ECM) such as collagen is significantly reduced in atrophied fibroblasts. We found that adding PhytoRetinol™, which we developed, as well as retinol acetate, which was used as a positive control, during medium replacement suppressed morphological atrophy of fibroblasts. This enables fibroblasts to maintain their collagen production capacity, and thus they can contribute to the reduction of wrinkles.

① Inhibitory Effect of the Skeletal Atrophy of Fibroblasts caused by UVA irradiation
② Tightening Effect of Collagen gel with an Increase of Integrin-α1
③ Increase Effect of Elastin-related genes in fibroblasts from elderly people
④ Increase Effect of Laminin 5 in keratinocyte
⑤ Inhibitory Effect of Hyaluronidase
⑥ Monitor Test Results

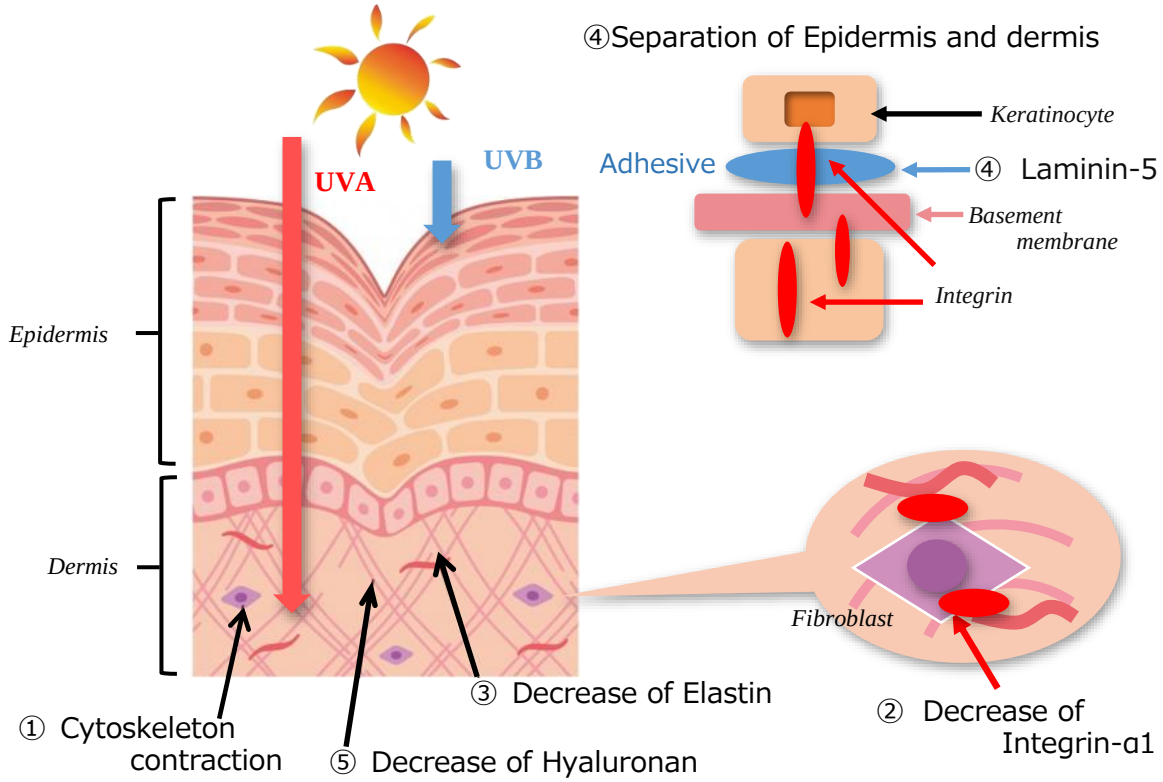


Fig.8: Evaluation items and evaluation sites

10-1-1 Inhibitory Effect of the skeletal atrophy of fibroblasts caused by UVA irradiation

The dermis is mainly composed of the extracellular matrix (collagen, hyaluronic acid, elastin, etc.) and fibroblasts. Fibroblasts are important mother cells that synthesize the major extracellular matrix components. Fibroblasts in the dermis firmly adhere to collagen fibers at numerous points and exhibit an elongated shape. However, fibroblasts atrophied in their skeletal shape have been observed in photo-aged skin. It can be easily assumed that this leads to a decrease in collagen production capacity in fibroblasts as well as their dissociation from collagen fibers. It has also been reported that cells expand their skeleton in order to build a network between cells. In fact, it is reported that atrophy of cells causes a reduction of the collagen fibril structure and cell inactivation (decreased collagen production and increased MMP-1, a collagen-degrading enzyme).[8)]

Therefore, in this experiment, we decided to evaluate the action to suppress the skeletal atrophy of fibroblasts caused by UV irradiation.

Normally, when fibroblasts are cultured on a monolayer, they expand the skeleton as shown on the left side of the figure. However, it has been confirmed that the skeleton atrophies when fibroblasts are exposed to UV-rays as shown in the picture on the right side.

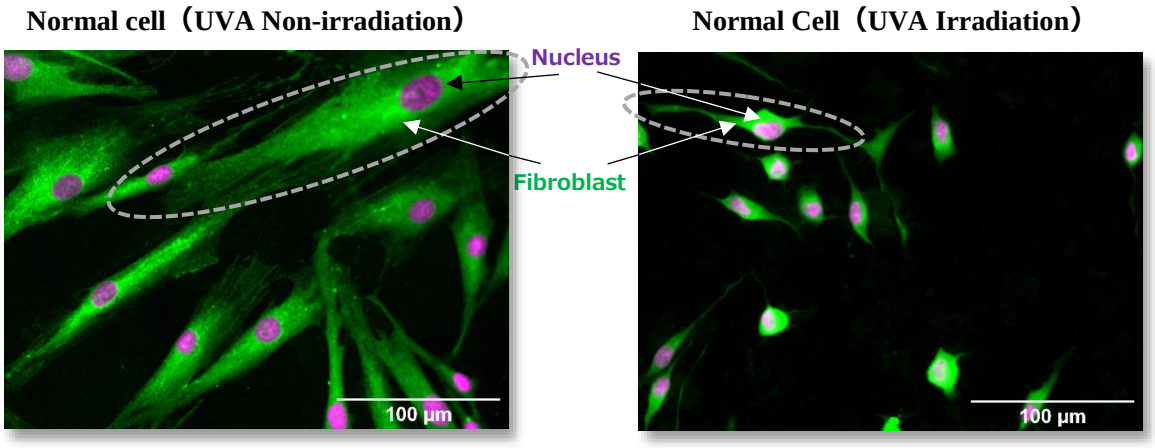


Photo 2: Morphological changes in fibroblasts by UVA-Irradiation / Non-irradiation

【Preparation of test solutions】

Psoralea corylifolia fruit extract and bakuchiol were adjusted in DMSO and a medium to achieve the final concentration and were used in the test. Psoralea corylifolia fruit extract concentrations 1 μg/mL and 3 μg/mL are equivalent to 0.001% and 0.003% of PhytoRetinol™-3C respectively.

We adjusted the DMSO final concentration used in all in vitro tests described in this catalog to 0.5% (v/v) and we confirmed that this DMSO concentration does not influence evaluation tests.

【Test Method】

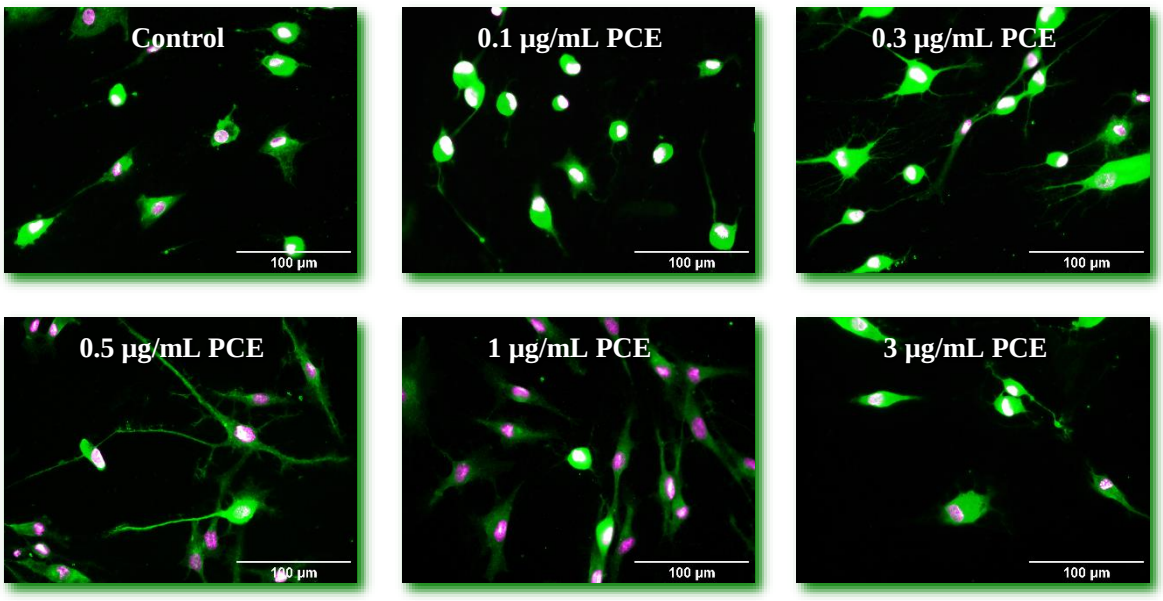
・Cell culturing method

We cultured fibroblast lines from newborn human NB1RGB (RCB 0222) purchased from RIKEN to use in the experiments. For medium, Dulbecco's Modified Eagles Medium (containing 1000 mg/L glucose, DMEM, Sigma-Aldrich) was used with 10 vol.% FBS (Fetal bovine serum, Biosera) with 100 units/mL penicillin G, 100 mg/mL streptomycin (Wako Pure Chemical) added. Cells were cultured in 75 cm^2 cultivation flasks (Sumitomo Bakelite) in the presence of 5% CO_2 at 37℃. Regarding subculturing, the cultured cells were washed with PBS (-) (Wako Pure Chemical) twice and removed from the flask using 0.25 w/v% Trypsin-1 mM EDTA-4Na solution containing Phenol Red (Wako Pure Chemical) to be used in the experiments.

・Skeletal atrophy suppression test on fibroblasts with UVA irradiation

NB1RGB cells were inseminated in an ibidi 8 well plate (Nippon Genetics) (9000 cells/well), they were cultured for one day, the medium was replaced with non serum-containing DMEM with the test substance, and then cultured for one day. After cultivation, the medium was replaced with PBS (-), the cover of the plate was removed, and UVA was irradiated using the CL-1000 UV Crosslinker (Analytik Jena AG, Germany) (10 J/cm^2). After irradiation, the medium was replaced with non serum-containing DMEM and the sample was cultured for a further 3 hours. Then fluorescent immunostaining was performed in accordance with the specified method. After fluorescent immunostaining, the fluorescence intensity and the number of cells (nuclear number) were analyzed using Image J. Retinol acetate (Wako Pure Chemical), which is a vitamin A derivative, was used as a positive control.

※Note: It was observed that irradiation of UVA reduced cellular death (absolute number) in addition to the reduction in atrophy of cell skeleton (control group in the figure). The actin emission intensity per cell is calculated as the fluorescence intensity of cell skeleton (actin) in the photo divided by the number of cell nuclei and the corrected results are applied to the graph.



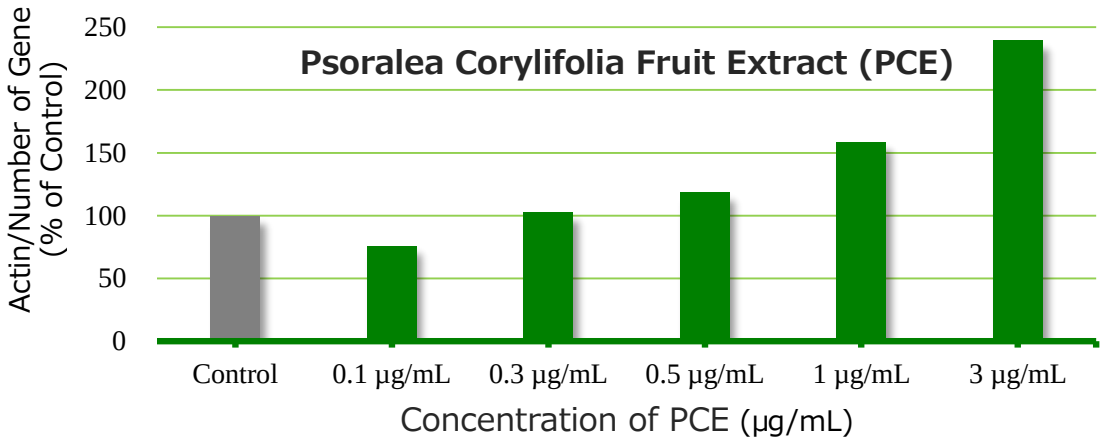


Fig.9: Inhibitory Effect of the skeletal atrophy of fibroblasts caused by UVA irradiation of PCE

【Results: PCE】

As shown in Figure 9, it has been confirmed that adding Psoralea Corylifolia Fruit Extract (PCE) prior to UVA irradiation and culturing the cells provides a concentration-dependent effect to suppress the atrophy of cell skeleton caused by UVA irradiation. However, cell toxicity was also observed with the concentration of 3 µg/mL.

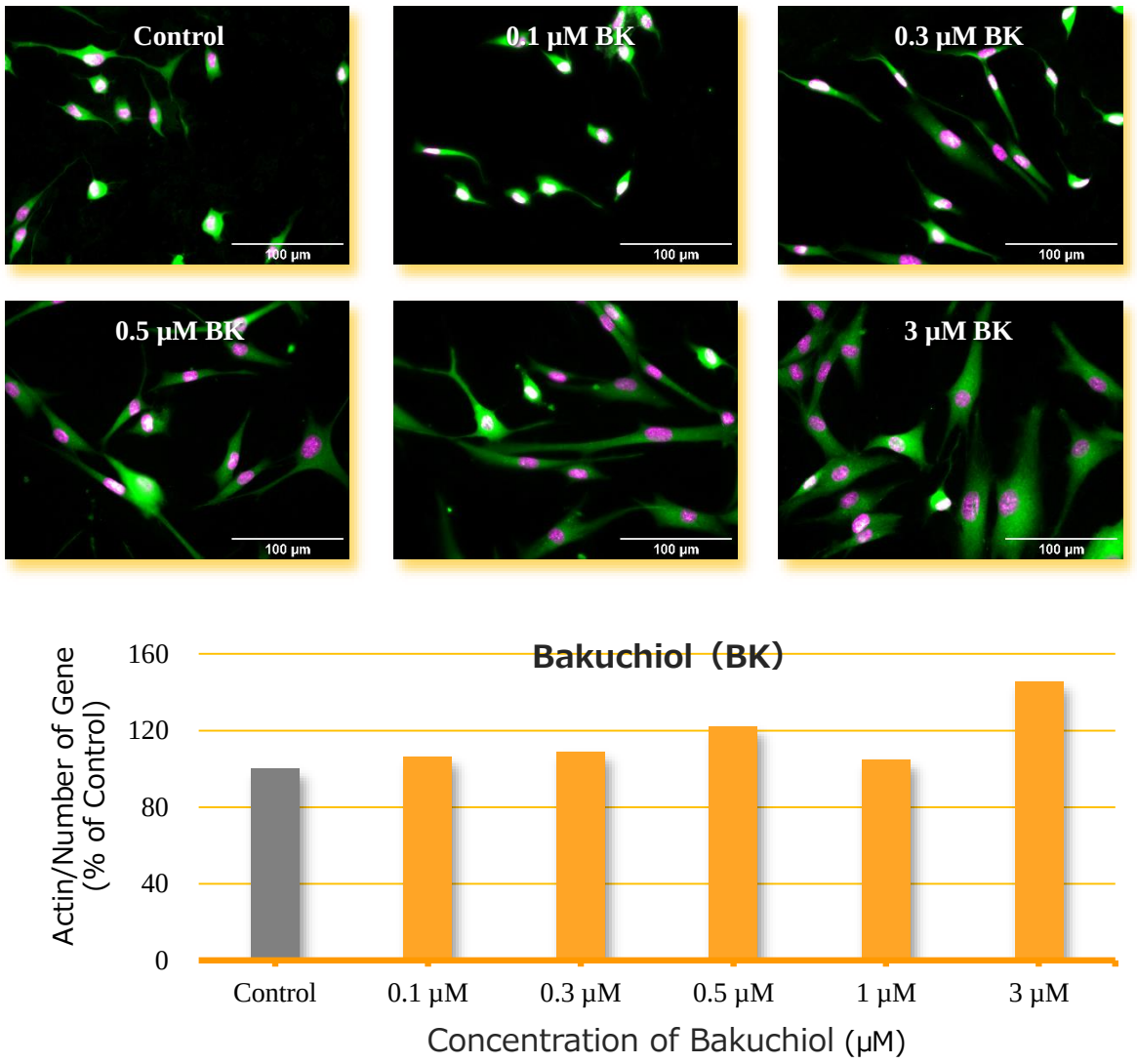


Fig.10: Inhibitory Effect of the skeletal atrophy of fibroblasts caused by UVA irradiation of BK

【Results: BK】

It has been confirmed that adding bakuchiol prior to UVA irradiation and culturing the cells provides a concentration-dependent effect to suppress the atrophy of cell skeleton caused by UVA irradiation (Figure 10). Furthermore, as shown in the photos of the group in which bakuchiol was added, it was observed that adding bakuchiol helps to maintain the skeleton that is close to that of normal cells.

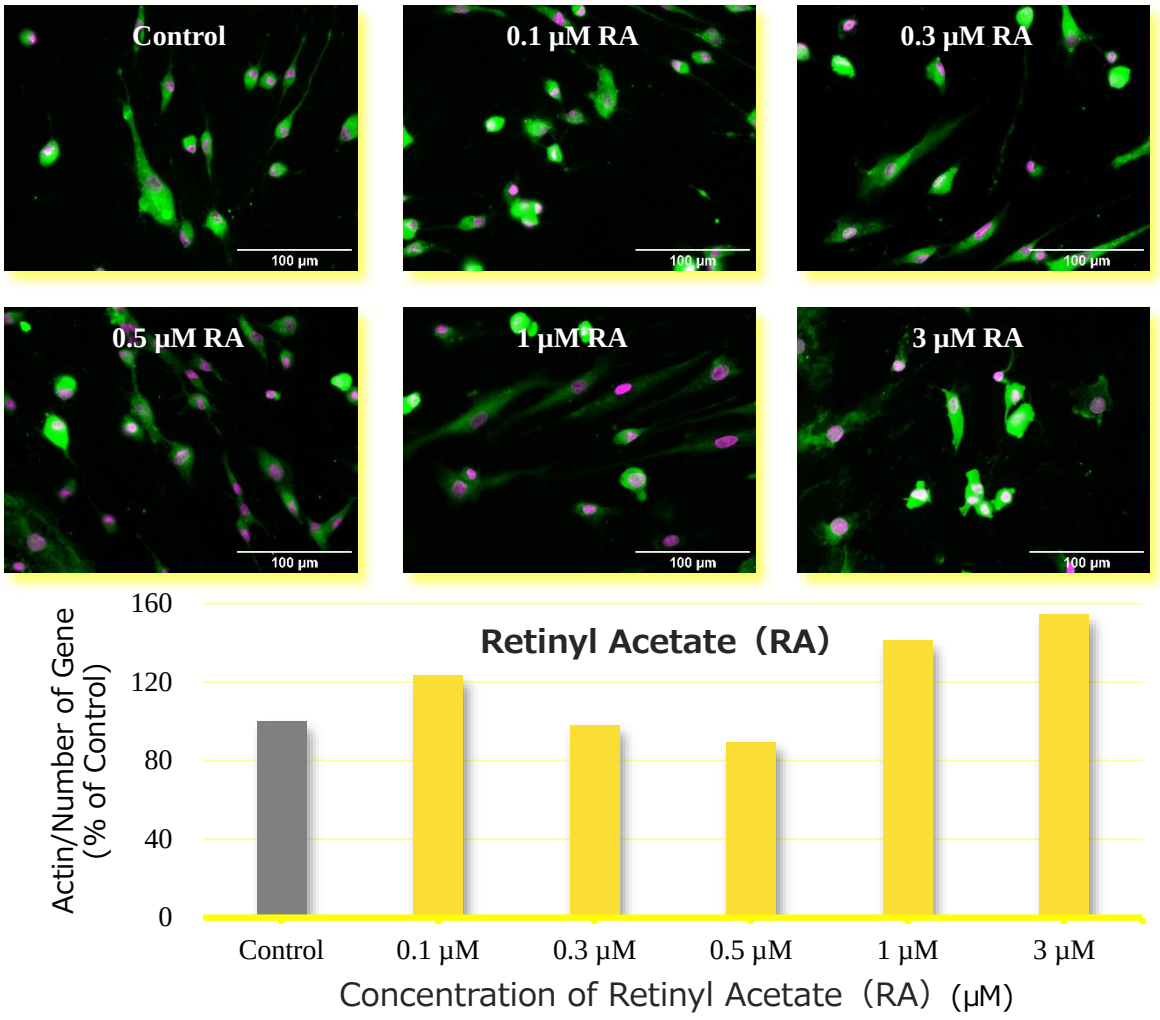


Fig.11: Inhibitory Effect of the skeletal atrophy of fibroblasts caused by UVA irradiation of RA

【Result: RA】

It has been confirmed that adding Retinyl Acetate (RA) prior to UVA irradiation and culturing the cells provides a concentration-dependent effect to suppresses the atrophy of cell skeleton caused by UVA irradiation (Figure 11). Furthermore, as shown in the photos of the group in which RA was added, it was observed that adding RA helps to maintain the skeleton that is close to that of normal cells.

【Consideration】

A comparison test was conducted using the Psoralea Corylifolia Fruit Extract, bakuchiol, and positive control Retinyl Acetate, which is mainly used as an ingredient for anti-wrinkle cosmetics. All samples were confirmed to have the action of suppressing the atrophy of cells induced by UVA. Therefore, they are expected to be effective in suppressing the reduction of collagen generation due to the deactivation of fibroblasts seen under UVA irradiation and activation of MMP-1, maintaining the structure of the dermis, and suppressing skin inflammation. In the test, Bakuchiol demonstrated a stronger action in suppressing the atrophy of cells than Retinyl Acetate when compared with the same concentration.

10-1-2 Tightening Effect of Collagen gel with an Increase of Integrin-α1

Collagen production in fibroblasts decreases as you age because the bond of cells and collagen fibers decays in addition to a decline in the production capacity. Fibroblasts adhere to collagen fibers at many points and they exist in the dermis in an extended state. However, in photo-aged skin, fibroblasts are dissociated from collagen fibers that are a critical foothold for them. In this condition, normal functions such as production of the extracellular matrix and cell division become impaired. A protein called integrin is present in the surface of the cell membrane and functions as a bond between cells and collagen. Integrin adheres not only to the extracellular matrix but also to laminin 5 described later in order to maintain the structure of the dermis. However, it has been reported that irradiation of UVA reduces integrin, weakening the bond of cells and extracellular matrix such as collagen.[11)] Because of this, the number of attachment points with collagen fibers decreases and the atrophy of cells occurs, leading to sagging skin and wrinkles.

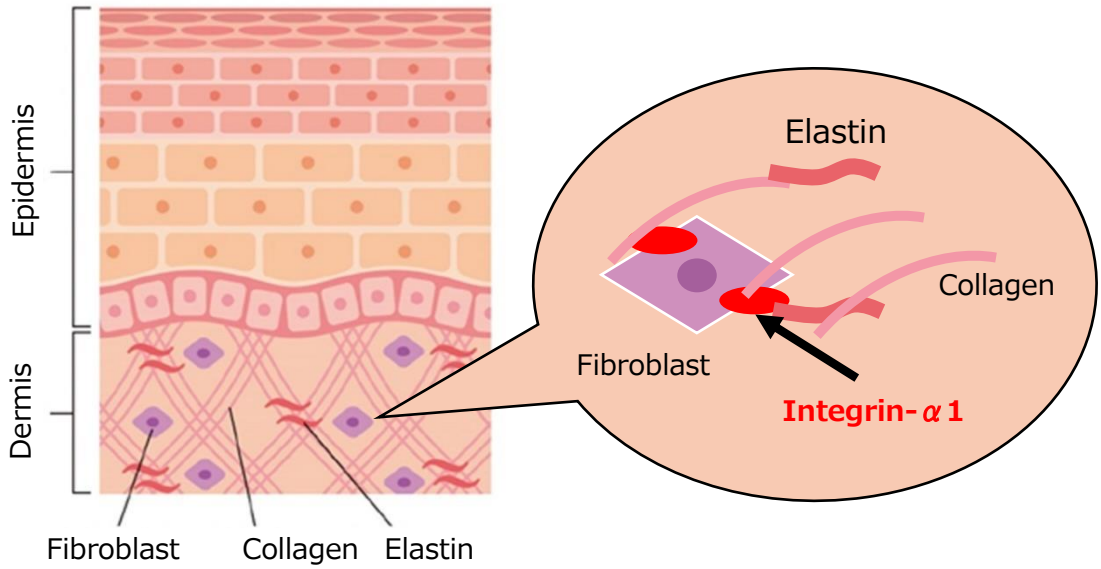


Fig.12: Role of Integrin-α1 in skin

So, we carried out a collagen gel contraction experiment on the Psoralea Corylifolia Fruit Extract and Bakuchiol as a system to evaluate the adhesion of fibroblasts and collagen fibers. When fibroblasts are embedded in collagen gel and cultured, cells adhere to any collagen fibers around them and gel contracts over time due to interactions of cells. It has been reported that the tighter the cells adhere to collagen fibers, the higher the contraction level and the level lowers due to oxidative stress, etc. Furthermore, after checking the collagen gel contraction level, fluorescent immunostaining was implemented on the surface of the cell membrane to detect integrin-α.

【Test Method】

NB1RGB cells were cultured in type I collagen gel (PureCol®, Advanced Bio Matrix, U.S.) in order to create 3D models of the dermis and the results were evaluated *in vitro*. Namely, a collagen solution (pH 7.4) with Hanks buffer solution (Sigma-Aldrich) added was mixed with a suspension of NB1RGB cells, and the sample was inseminated in a 24 well plate (1.2 x 10^5 cells/well). After that, DMEM containing 10% FBS was added to the gel (500 µL/well) and the sample was cultured for one day. The medium was then replaced with DMEM containing 10% FBS and the sample was cultured for two days. The medium was replaced with DMEM containing 10% FBS and the sample was cultured for five days in total (the medium was replaced every two days). Then, the collagen gel area value was measured, fluorescent immunostaining was implemented on integrin alpha-1, and the fluorescence intensity was measured. (Calculations were performed using Image J, open-source image processing software in the public domain.)

【Results】

In the experiment, 3D models similar to the dermis were created by culturing fibroblasts in collagen gel. Contraction of the collagen gel can be considered as a phenomenon where adhesion of the cells and collagen have become stronger and the structure of the dermis has been enhanced.

When the Psoralea Corylifolia Fruit Extract and Bakuchiol were added to the 3D dermis models, an action to significantly increase the contractile capacity on collagen gel was observed (Fig. 13).

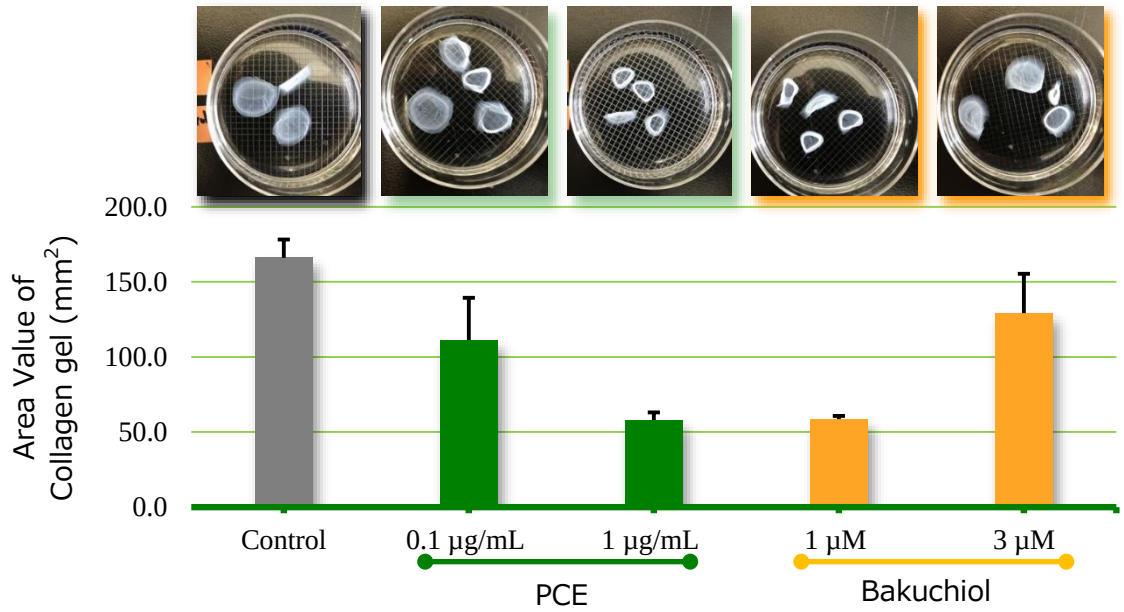


Fig.13: Tightening Effect of Collagen gel using 3D-Skin model

Each bar represents the means±S.E.M (N=3). Asterisks denote significant differences from the control group,*p<0.05,**, p<0.01.

Hint! "Concentration"

0.1 µg/mL of PCE = 0.0001 % of PhytoRetinol™-3C
1.0 µg/mL of PCE = 0.001 % of PhytoRetinol™-3C
1 µM of Bakuchiol = 0.00085％ of PhytoRetinol™-3C
3 µM of Bakuchiol = 0.0255％ of PhytoRetinol™-3C

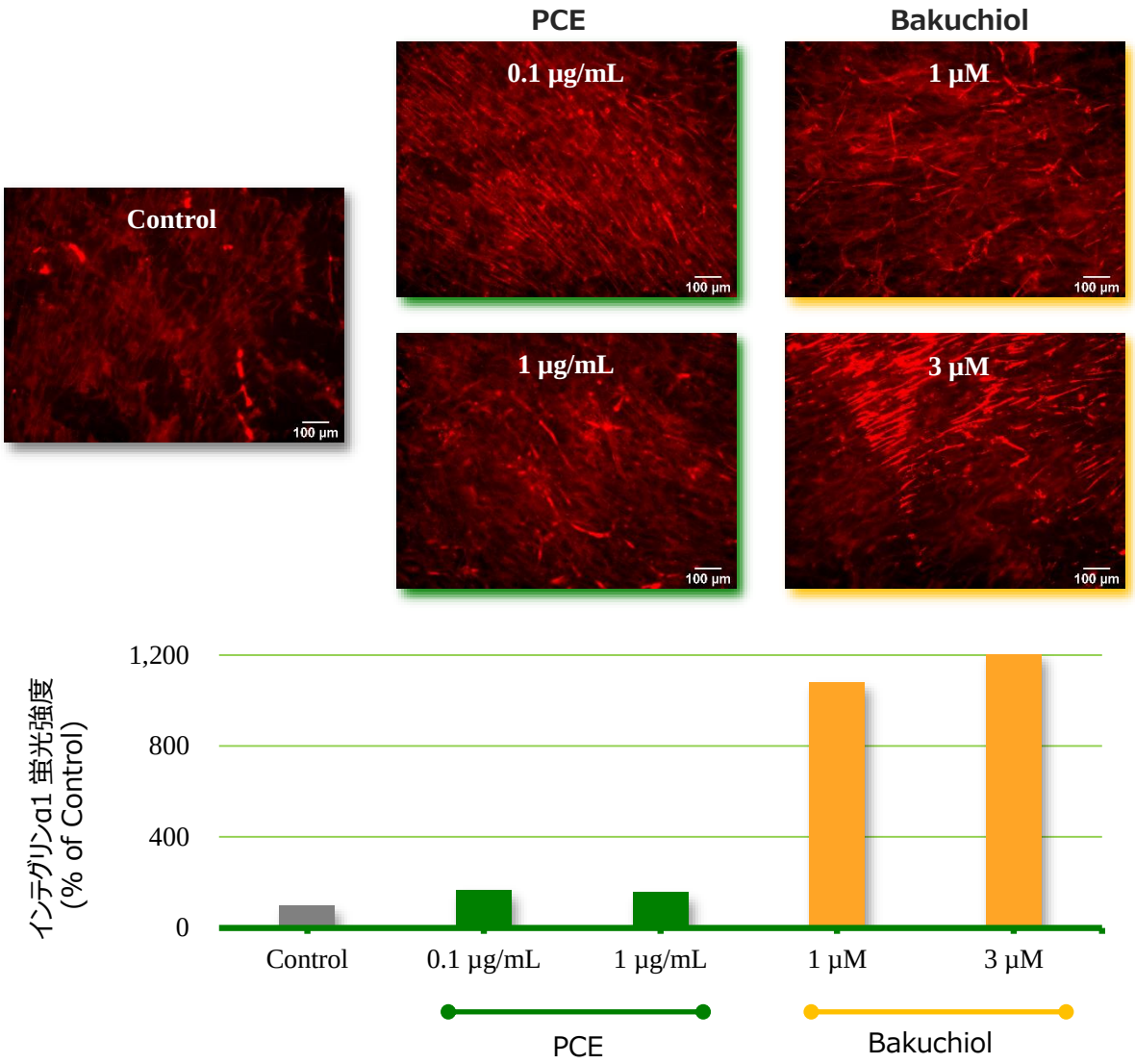


Fig.14: Integrin-α1 Increasing Effect during Collagen gel Formation

【Results】

When fluorescent immunostaining was implemented on the surface of the cell membrane and integrin alpha-1 was detected using the collagen gel shown in Figure 13 on the previous page, the Psoralea Corylifolia Fruit Extract and Bakuchiol demonstrated a strong action to increase integrin alpha-1 (Fig. 14).

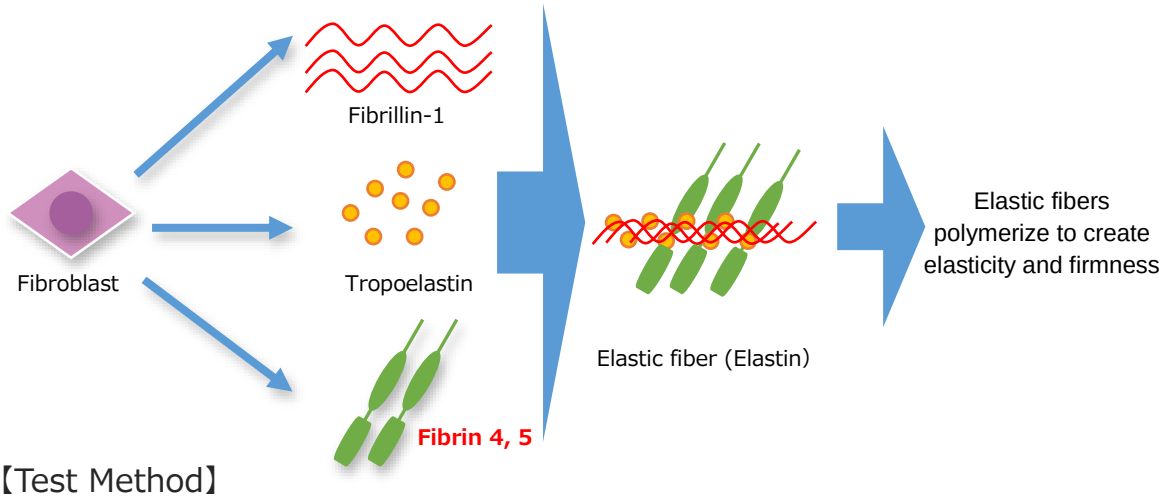
【Consideration】

The results suggest that the Psoralea Corylifolia Fruit Extract and Bakuchiol have an action to enhance the structure of the dermis via increasing integrin alpha-1.

As the Psoralea Corylifolia Fruit Extract and Bakuchiol make connective tissues of the dermis contract more and tighten them while increasing the integrin alpha-1 protein amount, it was suggested that they are effective in increasing the strength of the dermis, improving the elasticity of the skin when it has dropped due to aging, etc., suppressing sagging of the skin, recovering from it, maintaining or recovering skin's firmness, and tightening the skin.

10-1-3 Increase Effect of Elastin-related genes in fibroblasts from elderly people

Wrinkles and sagging skin occur due to a decrease or degeneration of the extracellular matrix in the dermis due to aging. Elastin is an elastic fiber in the skin and is formed when fibrillin 1, tropoelastin, fibrin 4 or 5, etc. generated from fibroblasts polymerize. We studied the influence on the gene expression of fibrin 4 which serves as a “pile” to connect fibrillin and tropoelastin while forming elastic fibers.



【Test Method】

TIG 103 (JCRB0528) cells purchased from JCRB Cell Bank (human normal diploid fibroblast collected from 69 year old person’s skin and made into cell strains) were inseminated in a 12 well plate (1.0 x 105 cells/well/1 mL) and cultured for one day. Then, the medium was replaced with a medium containing 10 % FBS with the test substance and cultured for 48 hours. Then, RNAs were extracted and RT-PCR was performed.

【Results】

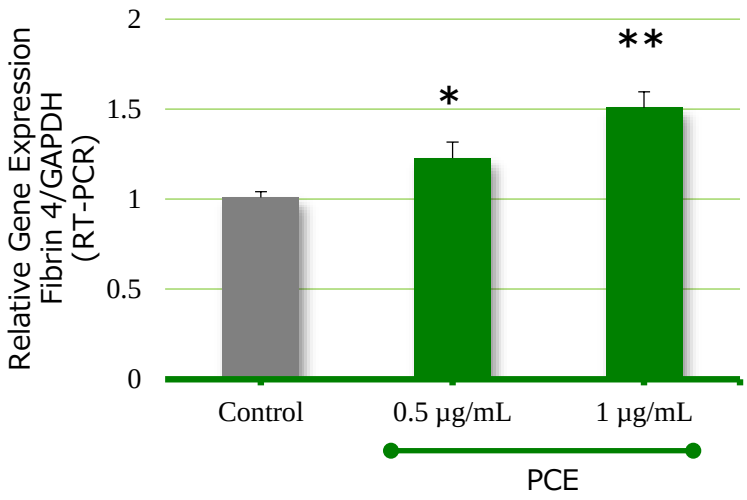


Fig.15: Increase Effect of Fibrin-4 Gene Expression in PCE

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

【Consideration】

As adding the psoralea corylifolia fruit extract increased the expression of fibrin 4 in a concentration-dependent manner, the extract is believed to be involved with the reduction of wrinkles by recovering elasticity.

10-1-4 Increase Effect of Laminin 5 in keratinocyte

Starting from the outer most layer, skin roughly consists of three structures, epidermis, dermis, and subcutaneous tissue. The epidermis and dermis closely contact with each other via the epidermal basement membrane structure. The epidermis and dermis do not separate even when friction is applied to the skin as the epidermal basement membrane normally functions[12]. When this basement membrane structure wanes, the adhesiveness of the epidermis and dermis lowers, leading to sagging skin and deep wrinkles. Laminin 5 is a protein that serves as a bond between them. For this reason, we evaluated the Psoralea Corylifolia Fruit Extract and Bakuchiol focusing on laminin 5 that is present in the epidermal basement membrane. We used Retinoic acid (Vitamin A acid) as the positive control.

【Test Method】

Normal human adult epidermal keratinocytes (NHEK) were inseminated in a 6 well plate (collagen coating) (2.0 x 10^5 cells/well/1 mL) and cultured for one day. Then, the medium was replaced with the medium dedicated for human epidermis (HuMedia-KG2) containing the test substance and was cultured for two days. After protein extraction, Western Blotting was performed in accordance with its specifications.

【Results】

Positive control retinoic acid as well as test substances psoralea corylifolia fruit extract and bakuchiol showed a tendency to increase the expression of laminin 5 (Fig. 16). Bakuchiol was confirmed to have an action equivalent to the positive control.

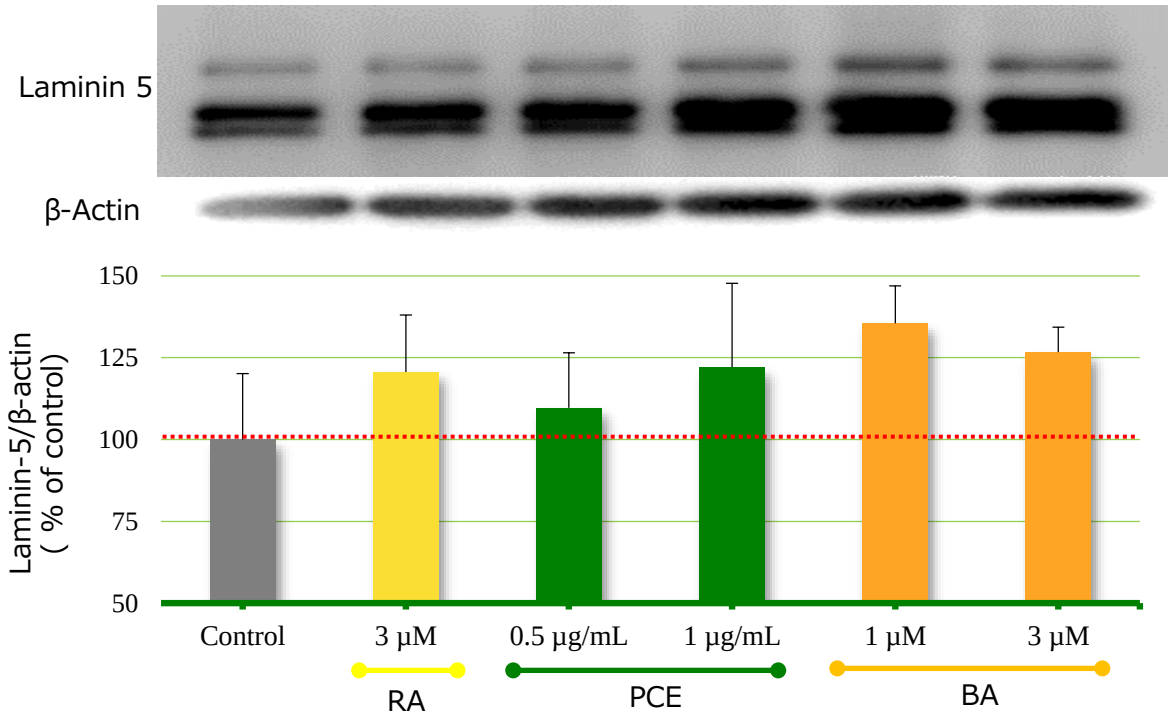


Fig. 16: Lamin-5 Increase Effect of PCE

【Consideration】

The results suggest that the Psoralea Corylifolia Fruit Extract and Bakuchiol have an action to improve the adhesiveness of the epidermis and dermis, anti-sagging action, as well as anti-wrinkle action.

10-1-5 Inhibitory Effect of Hyaluronidase

Hyaluronic acid is a component of the extracellular matrix and has a role to retain the moisture in the dermis. It is believed to have a capacity to maintain 2 to 6 L/g of moisture theoretically and it maintains the flexibility of tissue. However, as you age, hyaluronic acid in the skin decreases due to a decline in the fibroblasts' capacity to produce it as well as degrading enzymes. This leads to wrinkles, sagging skin, as well as a drop in firmness. For this reason, we evaluated the action to inhibit hyaluronidase which is an enzyme that decomposes hyaluronic acid in this experiment.

【Test Method】

The test was carried out in a 0.1 M acetic acid buffer solution (pH 4.0). Scaling was implemented in a 96 well plate. Psoralea Corylifolia Fruit Extract at various concentrations (20 µL/well), 4000 units/mL of bovine testicle-derived hyaluronidase (Sigma Aldrich) solution (10 µL/well), 800 µg/mL hyaluronic acid (Sigma Aldrich) solution which is the ground substance, as well as 500 µg/mL Compound 48/80 (Sigma Aldrich) solution (20 µL/well) were added and an enzyme reaction (37 ℃, 40 min.) was performed. Then, 0.4N NaOH solution (20 µL/well) was added and the sample was promptly left on the ice to stop the reaction. Next, 0.8 M boric acid (Wako Pure Chemical) solution (pH 9.1, 20 µL/well) was added and the entire 96 well plate was boiled (90℃, 3 min.). Immediately after boiling, the sample was left on the ice and the reaction solution was moved to a new 96 well plate (50 µL/well). Then, *p*-dimethylaminobenzaldehyde reagent (200 µL/well) was added and the sample was incubated at 37℃ for 20 minutes. The absorbance of the obtained reaction solution at 585 nm was measured.

【Results】

Psoralea Corylifolia Fruit Extract was found to have a concentration-dependent hyaluronidase inhibitory action.

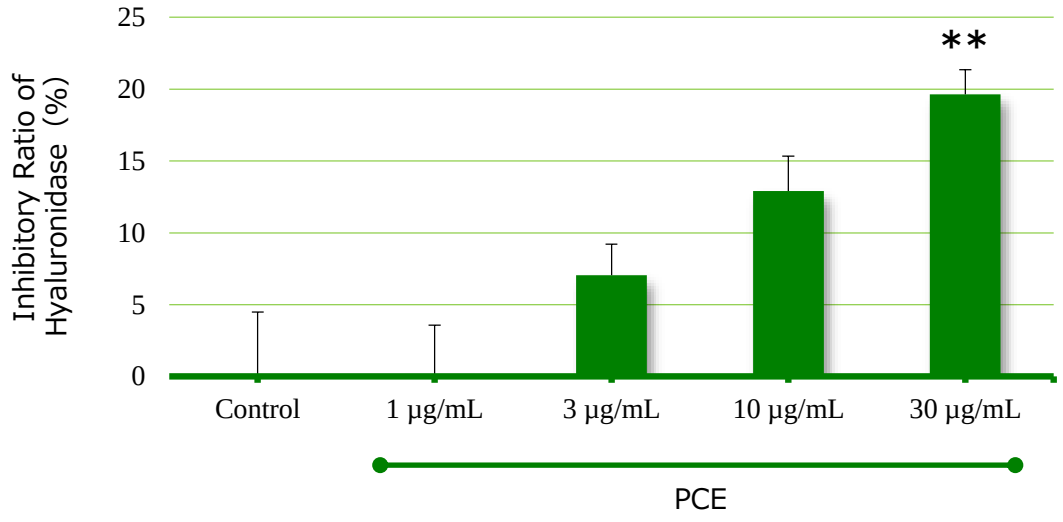


Fig.17: Inhibitory Effect of Hyaluronidase

Each bar represents the means±S.E.M (N=6). Asterisks denote significant differences from the control group,*p<0.05,, p<0.01.**

10-1-6 Summary of *in vitro* evaluation tests

As a result of carrying out various *in vitro* tests using PhytoRetinol™, its actions were confirmed via the five types of tests shown below.

1. Inhibitory Effect of the skeletal atrophy of fibroblasts caused by UVA irradiation
2. Tightening Effect of Collagen gel with an Increase of Integrin-α1
3. Increase Effect of Elastin-related genes in fibroblasts from elderly people
4. Increase Effect of Laminin 5 in keratinocyte
5. Inhibitory Effect of Hyaluronidase

The content is summarized below.

1) Fibroblast

Fibroblasts are important mother cells that synthesize major extracellular matrix components. Their actions cannot be performed sufficiently under irradiation of UV-rays as they contract. Our test confirmed that adding PhytoRetinol™ suppresses the contraction of fibroblasts.

2) Integrin alpha

Skin can be classified into the epidermis on the outer surface and the dermis in deeper parts. There is a membrane called the basement membrane at the border of them. A factor called integrin alpha connects the epidermis and the basement membrane as well as the dermis and basement membrane like a stapler. Wrinkles are formed when these 'staples' fall out. It was observed that adding PhytoRetinol™ increased integrin alpha.

3) Elastin

Elastin is a protein in the extracellular matrix. For example, when you pinch the skin on the back of your hand and pull it, the skin is stretched. The ability to stretch the skin is provided by collagen and the ability to recover the skin's original state after being pulled is provided by elastin. As we age, the ability to recover declines as elastin decreases so wrinkles are formed. PhytoRetinol™ has been confirmed to increase a material called fibrin 4 that forms the protein elastin.

4) Laminin 5

As described in 2) above, integrin alpha connects the epidermis and basement membrane as well as the dermis and basement membrane like a stapler. Furthermore, Laminin 5, which is a bond, is present between the epidermis and basement membrane, making the epidermis and basement membrane hard to separate with a stapler effect. However, when the generation of these factors slows down due to aging, wrinkles are formed. PhytoRetinol™ has been confirmed to increase laminin 5 in a test.

5) Hyaluronidase

Hyaluronic acid is a factor that moisturizes living organisms and can hold 6 L of water per 1 g. It is decomposed by the enzyme hyaluronidase. PhytoRetinol™ has been confirmed to inhibit the activation of hyaluronidase. It prevents fine wrinkles caused by dryness through the prevention of hyaluronic acid decomposition.

10-1-7 Anti-inflammatory Effect of Psoralea Corylifolia Fruit Extract

[quoted from literature] [13)]

Skin inflammations are mainly caused by allergens such as UV-rays and pollens entering the skin. As a result of studying literatures, we confirmed that the psoralea corylifolia fruit extract and its components have an anti-inflammatory action (Fig. 18).

A) Inhibitory Effect of UV-induced Inflammation

When continuously exposed to UV-rays, reactive oxygen species are generated in the epidermis, and IL-8, a proinflammator chemokine present in the dermis, is produced in fibroblast. In the skin, IL-8 receptors are expressed in neutrophils, mast cells and epidermal cells. Since an increase in IL-8 receptors in epidermal cells has been observed in psoriasis, atopic dermatitis, and other inflammatory diseases in recent studies, it is said to be an important target for the treatment of skin diseases[13)]. Also, as IL-8 is produced more frequently in elderly people than in young people, it is recognized as a mediator of aging.

According to the report by Baequeville et al.[13)], not only has Bakuchiol been confirmed to inhibit the production of IL-8 in UVA-irradiated fibroblasts, it is also confirmed to inhibit the production of "p16 protein" which has an effect to stop the G1 phase of the cell cycle.

B) Inhibitory Effect of Inflammation caused by Allergens (Pollen, Dust)

When allergens such as pollen or dust enter the skin, they bind to the surface of mast cells, and pro-inflammatory cytokines (TNF-alpha, histamine and beta-hexosaminidase) are released (degranulation) as a type I allergic reaction. Not only do these pro-inflammatory cytokines cause inflammation such as redness and itching, but they also activate the collagenase and hyaluronidase as calcium ions flow into the cells in the dermis. These lead to the degradation of extracellular matrix, causing wrinkles and sagging.

Matsuda et al.[14)] have found that the psoralea corylifolia fruit extract inhibits the degranulation of mast cells (RBL-2H3 cells). In addition, they have identified various components of psoralea corylifolia fruit extract by isolating them and have also identified components that contribute to this activity.

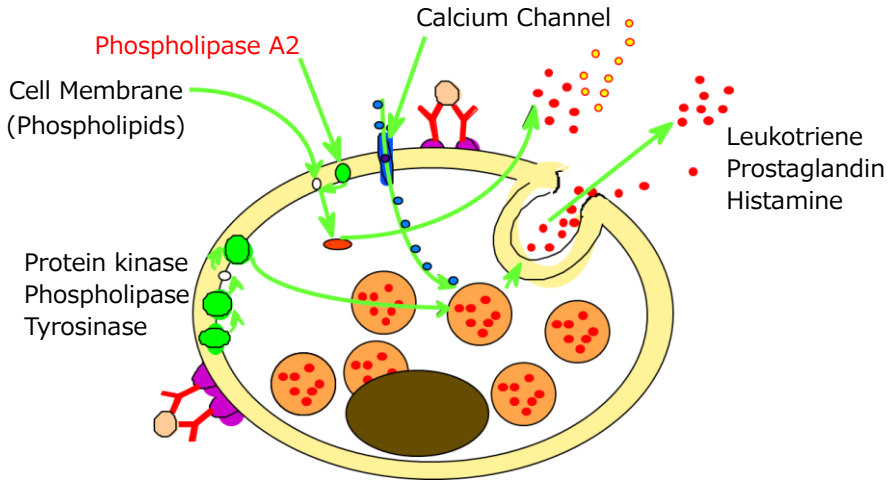


Fig18: Chemical Mediator Release from Mast Cell

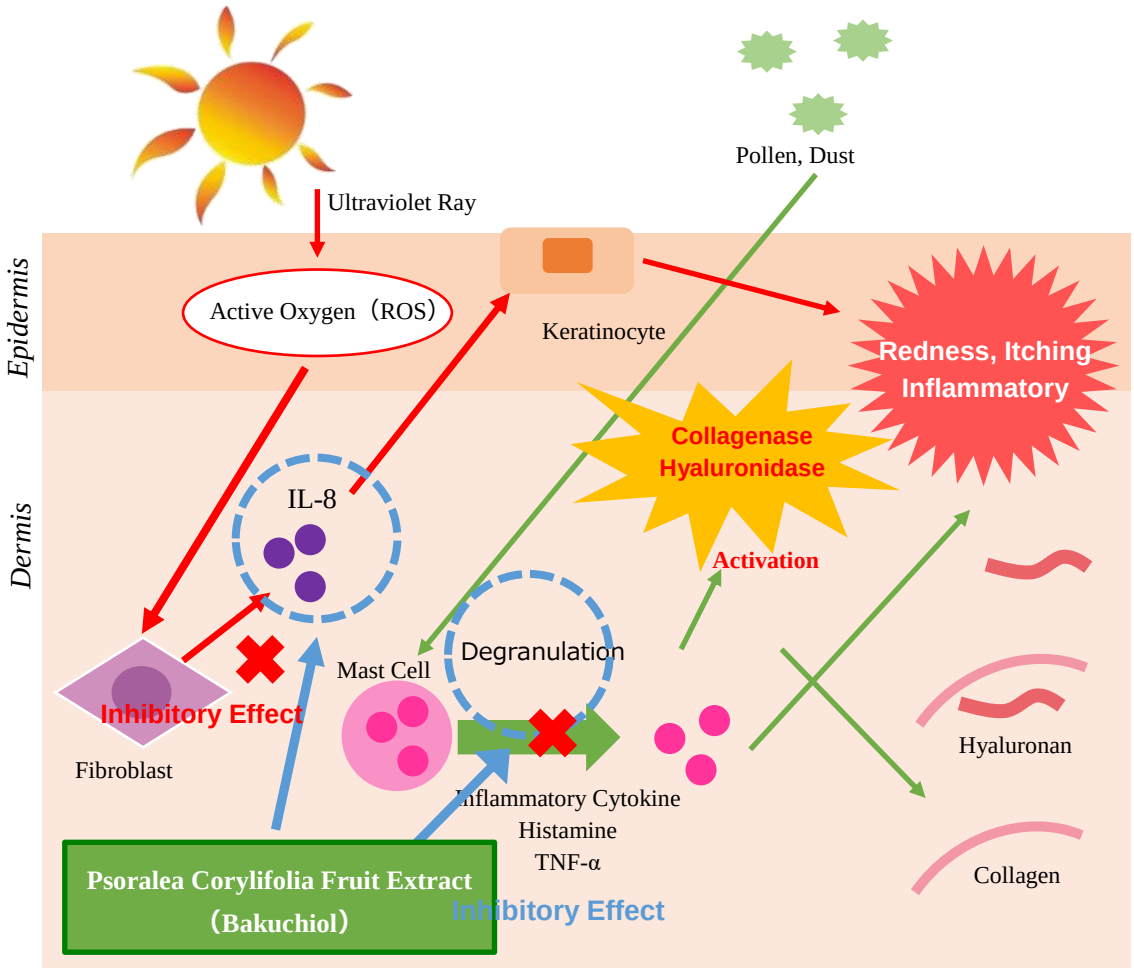


Fig.19: Inflammatory Mechanism of Skin and
Anti-inflammatory effect of Psolarea Corylifolia Fruit Extract

The results reported in these papers as well as our results suggest that both Psoralea Corylifolia Fruit Extract and Bakuchiol prevent the structural breakdown of intercellular networks and dermis by inhibiting UV induced cytoskeletal atrophy in fibroblasts in dermis. In addition, since Bakuchiol and Psoralea Corylifolia Fruit Extract inhibit the production of pro-inflammatory cytokines (IL-8) released from UV irradiated fibroblasts, and suppress the degranulation in mast cells, it can be considered to have an anti-inflammatory effect in the skin. Also, as it not only reduces inflammation, but also inhibits the production of p16 protein, which is a cause of aging, an anti-aging effect can be expected.

10-2 *in vivo* Evaluation Test

10-2-1 Clinical Test

Based on the results of *in vitro* evaluation tests described in 10-1, we prepared a cream containing 2% PhytoRetinol™-3C. We then had test subjects apply the cream and placebo cream on their left and right eye corners in order to study the effect to improve wrinkles.

【Test Design】

A test was carried out for comparing effects in a group of subjects that applied the product and a group of subjects that did not apply the product according to the "Guidelines for the Evaluation of Anti-Wrinkle Products for Acquiring New Efficacy" in "Guidelines on Methods to Evaluate Cosmetic Products' Functions". The test was carried out on 20 healthy men and women as an open test. Test subjects applied cream containing 2% PhytoRetinol™-3C and a placebo cream on their left and right eye corners twice a day (after a facial wash in the morning and before bed at night) continuously for four weeks. Photos of their eye corners were taken and replicas of their eye corners were created before the test and four weeks later in order to analyze their wrinkles.

【Test Samples】

A cream containing PhytoRetinol™-3C and placebo cream

	Trade Name	Maker	Sample	Placebo	INCI Name
1	PhytoRetinol™-3	A	2.0	—	Caprylic/Capric Triglyceride, Psoralea Corylifolia Fruit Extract, Rosmarinus Officinalis (Rosemary) Leaf Extract
1'	TCG-M	B	—	2.0	Caprylic/Capric Triglyceride
2	NIKKOL LecinolS-10	C	0.5	0.5	Hydrogenated Lecithin
3	NIKKOL Decaglyn 1-M	C	3.0	3.0	Polyglyceryl-10 Myristate
4	NIKKOL Selachyl Alcohol	C	2.0	2.0	Oleyl Glyceryl Ether
5	NIKKOL N-SPV	C	4.0	4.0	Cetyl Palmitate
6	ORYZASQUALANE®	A	10.0	10.0	Squalane
7	T.I.O	D	8.0	8.0	Triethylhexanoin
8	STEARYL ALCOHOL NX	B	6.0	6.0	PEG-75
			35.5	35.5	
9	GLYCERIN, conc.	E	1.5	1.5	Glycerin
10	1,3-BG <UK grade>	F	1.5	1.5	Butylene Glycol
11	KELTROL CG-T (2% aq)	G	10.0	10.0	Water, Xanthan Gum
12	MEKKINS™-M	H	0.1	0.1	Methylparaben
13	PURIFIED WATER	I	51.4	51.4	Water
			64.5	64.5	
			100.0	100.0	

【Manufacturer】

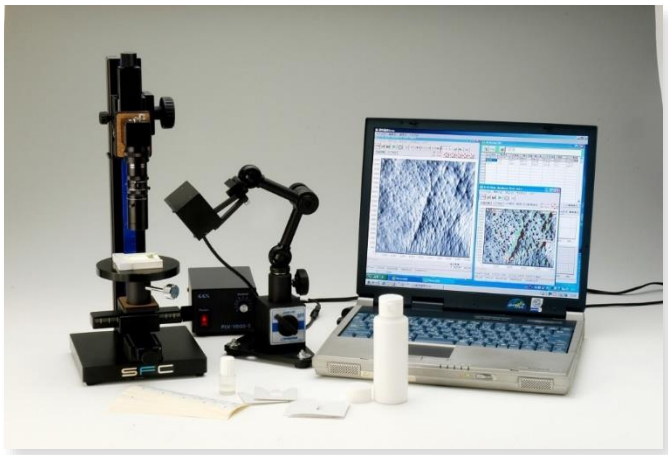
A: ORYZA Oil & Fat Chemical Co., Ltd. B: KOKYU Alcohol Industry Co., Ltd.
C: NIKKO Chemical Co., Ltd. D: NISSHIN OilliO Group Ltd.
E: KAO Corporation F: DICEL Corporation G: SANSHO Co., Ltd.
H: UENO Fine Chemicals Co., Ltd. I: TOKAI Pharmaceuticals Co., Ltd.

【Test Subjects】

We recruited 20 test subjects from employees working in the head office of Oryza Oil and Fat Chemical Co., Ltd. We provided them with thorough explanation about ethical principles in accordance with the Declaration of Helsinki and had them participate in the test with their consent. The criteria for the test subjects were healthy adult men and women except for those who (1) are taking hormonal treatment, (2) are pregnant or breast-feeding, (3) show allergic reaction against the test product, (4) have experienced cosmetic medical treatment that may influence the test areas.

【Measurement Items】

Photos were taken, replicas of wrinkles were created, and three-dimensional analysis was performed before and after the test using the ASA-03RXD reflection replica analysis system made by ASCH JAPAN Co., LTD.



Measurements were performed twice, on September 24, 2020 when the test started and on October 22, 2020 at the end of the test. At the start, photos of the left and right eye corners of the test subjects were taken and the replicas of their skin were created without applying the sample. The second measurement was performed after four weeks of continuous application of the sample. The test subjects removed their makeup from their face 15 minutes before entering the measurement room and they stayed in the room at a temperature of 25 ± 3℃ and humidity of 60 ± 5% for 15 minutes before measurement.

No test subjects complained about redness, itching, or any abnormal skin conditions during the test period. Although 20 test subjects completed the test, analysis was performed on data of 19 test subjects as a position gap occurred on the photos of a test subject's eye corners before and after application.

【Results and Considerations】

Test subjects were classified into a fine line group (wrinkle grade 4 or lower) and a deep wrinkle group (wrinkle grade 5 or higher) and the average value of their wrinkles was analyzed. As a result, a tendency to improve fine lines was observed in all seven wrinkle evaluation indexes in the group of wrinkle grade 4 or lower. Regarding relatively deep wrinkles of grade 5 or higher, an improvement tendency was observed in two items out of seven wrinkle evaluation indexes.

As the results of the test that we carried out in accordance with the "Guidelines for the Evaluation of Anti-Wrinkle Products for Acquiring New Efficacy" in "Guidelines on Methods to Evaluate Cosmetic Products' Functions" also show an improvement tendency on fine lines, Psoralea Corylifolia Fruit Extract containing a large amount of Bakuchiol is expected to reduce fine lines safely and effectively as a cosmetic ingredient.

【Test Results】

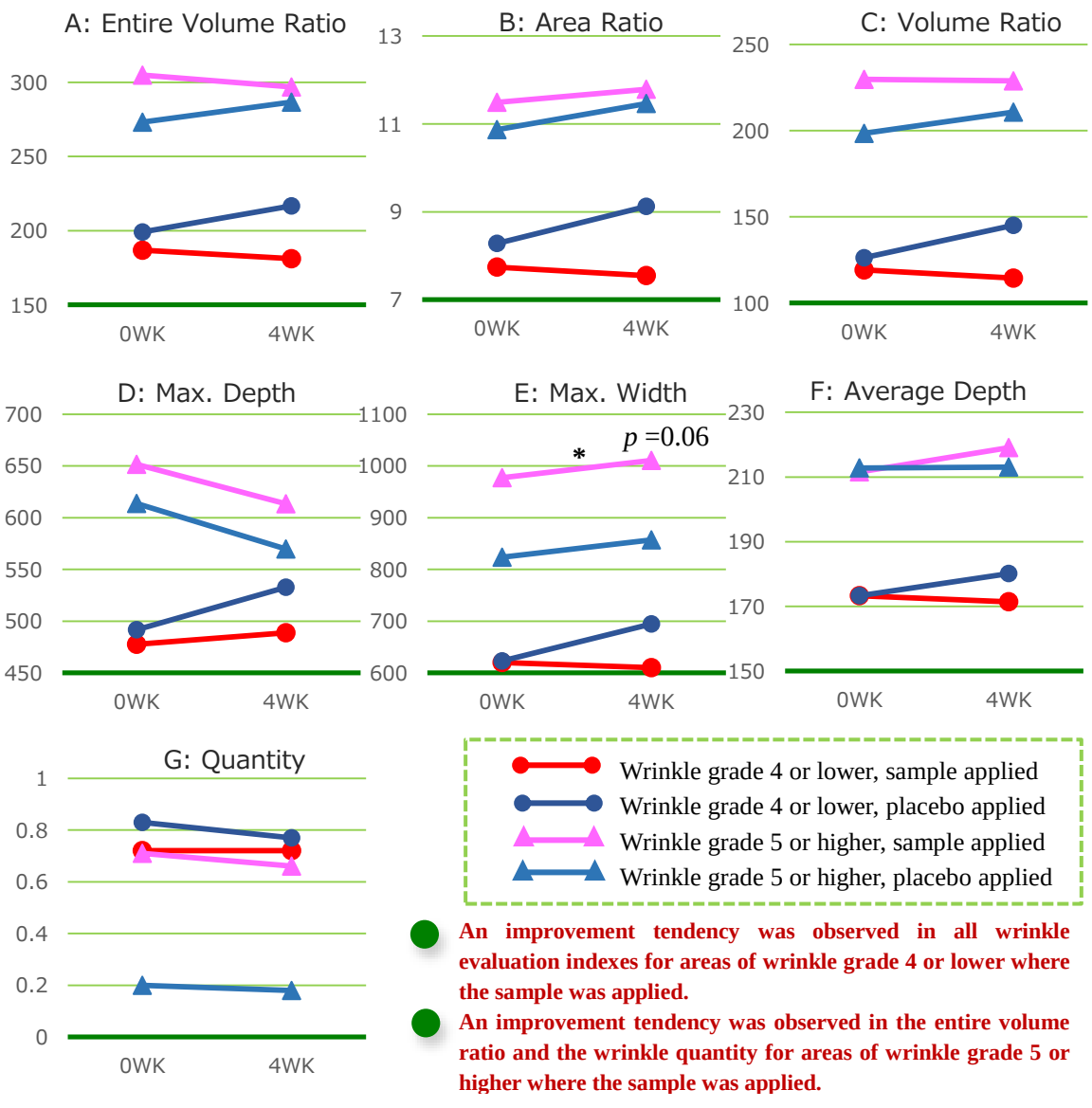
The table below shows the rate of change in indexes regarding wrinkles before applying the sample cream (applied on the left eye corner) and the placebo cream (applied on the right eye corner) and after applying them for four weeks.

No	Subjects	Gender	Age	Wrinkle Grade	Sample	Change Ratio (4 values at 4th week minus values before application)						
						A	B	C	D	E	F	G
1	JS	M	41	3	SAMPLE	22.3	0.98	15.0	36.27	39.66	1.32	0.04
					PLACEBO	-29.0	-1.03	-23.3	131.2	-178.3	-11.23	-0.03
2	KS	F	44	4	SAMPLE	4.8	0.62	13.6	-16.44	45.06	17.28	-0.11
					PLACEBO	-60.6	-2.14	-24.6	-82.8	11.86	21.6	-0.43
3	MT	F	45	3	SAMPLE	17.8	0.71	4.9	-92.03	107.63	-3.23	0.17
					PLACEBO	-21.7	-0.16	-2.7	-29.8	31.51	9.34	-0.26
4	KS	M	44	3	SAMPLE	-49.5	-1.96	-41.4	14.21	-14.76	-26.1	0.04
					PLACEBO	25.1	1.14	20.8	87.22	98.98	-5.55	0.08
5	KH	M	44	3	SAMPLE	28.9	1.55	18.2	21.05	-58.41	-5.29	0.05
					PLACEBO	37.8	1.83	35.6	103.9	-97.4	13.22	0.04
6	EN	F	48	2	SAMPLE	11.3	0.13	0.4	13.19	-129.3	-6.62	0.07
					PLACEBO	-22.3	-0.70	-6.8	61.93	-3.3	11.43	-0.22
7	DY	M	48	3	SAMPLE	-32.1	-1.30	-29.4	-51.76	-91.3	-12.7	-0.01
					PLACEBO	-15.9	-0.53	-20.0	13.63	-96.3	-12.4	0.02
8	TK	M	49	3	SAMPLE	解析中止						
					PLACEBO							
9	YT	M	50	3	SAMPLE	-11.3	-0.13	3.1	-6.61	-46.02	18.4	-0.15
					PLACEBO	32.3	1.69	20.4	-9.03	24.69	-1.23	0.09
10	MO	M	50	4	SAMPLE	15.8	0.73	15.7	221.488	121.5	11.2	0
					PLACEBO	186.8	7.18	144.6	84.56	790.0	21.7	0.2
11	KK	F	51	5	SAMPLE	-27.0	-0.44	-18.0	-146.2	-138.8	-5.01	-0.06
					PLACEBO	53.3	2.06	26.8	-41.6	39.12	-16.9	0.25
12	MH	M	52	3	SAMPLE	-58.8	-3.00	-47.5	3.78	-79.76	-1.56	-0.09
					PLACEBO	4.5	-0.21	8.5	-0.88	-41.2	13.6	-0.1
13	HS	M	52	4	SAMPLE	47.9	1.74	22.8	-61.32	19.6	-20.2	0.26
					PLACEBO	86.9	3.33	76.7	135.0	357.6	15.56	-0.05
14	KA	M	52	6	SAMPLE	-90.5	-1.54	-70.1	-125.5	-189.2	10.17	-0.14
					PLACEBO	-34.0	-1.26	-21.3	26.63	58.87	-0.05	-0.07
15	TH	M	53	6	SAMPLE	47.1	1.77	45.3	139.704	-23.95	13.06	0
					PLACEBO	7.5	0.45	8.5	44.67	67.48	0.98	-0.01
16	TS	F	56	4	SAMPLE	-66.7	-2.41	-32.4	51.14	-34.94	4.87	-0.26
					PLACEBO	-12.2	-0.25	-3.2	-1.91	-33.35	5.69	-0.07
17	SK	M	58	7	SAMPLE	-66.5	-1.64	-48.9	-37.4	-0.65	2.62	-0.14
					PLACEBO	106.1	3.49	118.1	-3.21	217.4	47.7	-0.09
18	ST	M	59	5	SAMPLE	36.6	1.93	32.1	-29.5	271.8	-6.38	0.08
					PLACEBO	-46.3	-2.25	-50.2	-83.03	-33.6	-16.7	-0.01
19	CM	F	59	5	SAMPLE	24.5	1.47	17.5	-118.9	-45.6	0.69	0.02
					PLACEBO	-7.8	-0.83	-16.0	55.00	54.9	-10.8	0.03
20	MG	M	61	7	SAMPLE	19.8	0.52	34.7	50.2	361.0	37.5	-0.14
					PLACEBO	15.7	2.46	19.9	-312.4	-169.0	-2.04	0.01
AVERAGE					SAMPLE	-6.61	-0.01	-3.39	-7.09	5.97	1.58	-0.02
					PLACEBO	16.12	0.71	16.41	9.58	57.39	4.41	-0.03

A: Entire volume ratio, B: Area ratio, C: Volume ratio, D: Maximum depth
E: Maximum width, F: Average depth, G: Quantity

Among indexes related to wrinkles described on the previous page, ones with a negative (-) rate of change are the items that an improvement on wrinkles was observed. The table below shows the averages of wrinkle evaluation indexes before and after applying the sample and placebo creams while roughly classifying wrinkle grades.

Wrinkle grade	Sample	Replica	Averages of Wrinkle Evaluation Indexes with Wrinkle grade						
			A	B	C	D	E	F	G
2, 3, 4	Sample	Start	186.88	7.74	119.17	477.64	620.08	173.27	0.72
		4weeks	181.08	7.55	114.42	488.72	609.99	171.38	0.72
	Placebo	Start	199.03	8.28	126.33	491.58	623.06	173.29	0.83
		4weeks	216.68	9.12	145.17	532.65	694.34	180.09	0.77
5, 6, 7	Sample	Start	305.00	11.49	229.84	651.52	977.26	211.54	0.71
		4weeks	297.00	11.79	228.79	613.28	1010.76	219.06	0.66
	placebo	Start	273.21	10.87	198.41	613.69	823.40	212.76	0.20
		4weeks	286.71	11.46	210.67	569.26	856.99	213.08	0.18

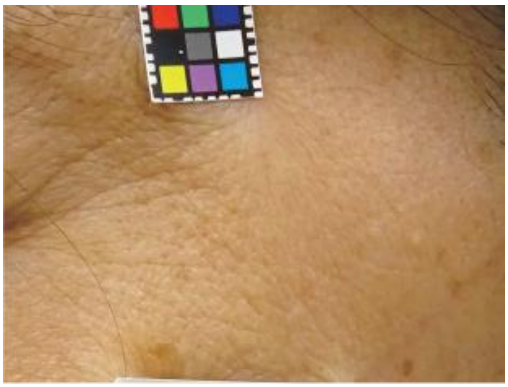


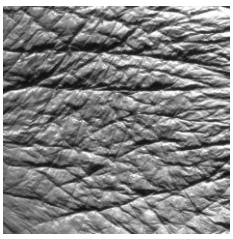
- **An improvement tendency was observed in all wrinkle evaluation indexes for areas of wrinkle grade 4 or lower where the sample was applied.**
- **An improvement tendency was observed in the entire volume ratio and the wrinkle quantity for areas of wrinkle grade 5 or higher where the sample was applied.**

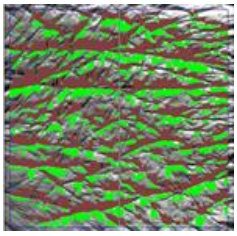
Anti-wrinkle Evaluation Test (During a continuous 4week Application)

TS Wrinkle grade 4, 56s, F

0W







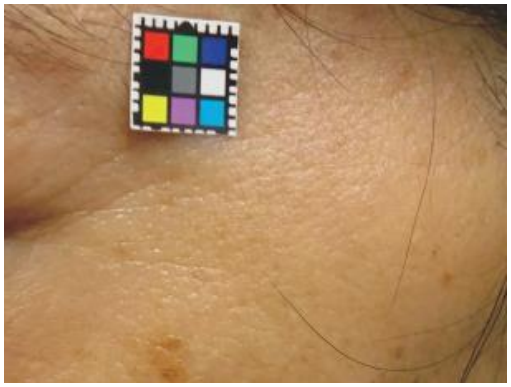
Replica Image

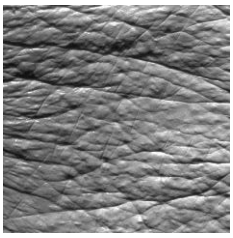
Analysis Image

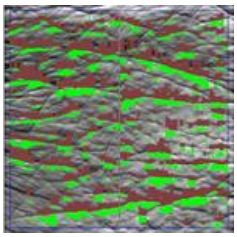
Entire volume ratio: 218.9
Volume ratio : 121.0

Area ratio : 8.60
Max. width : 555.95

4W







Replica Image

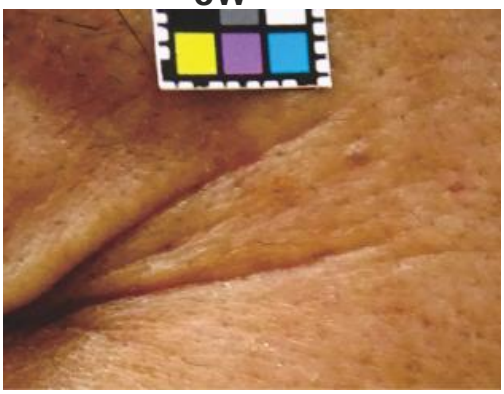
Analysis Image

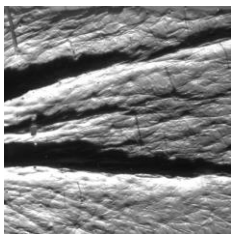
Entire volume ratio: 152.2
Volume ratio : 88.6

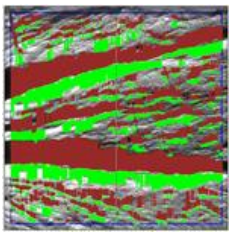
Area ratio : 6.15
Max. width : 512.01

KA Wrinkle grade 6, 52s, M

0W







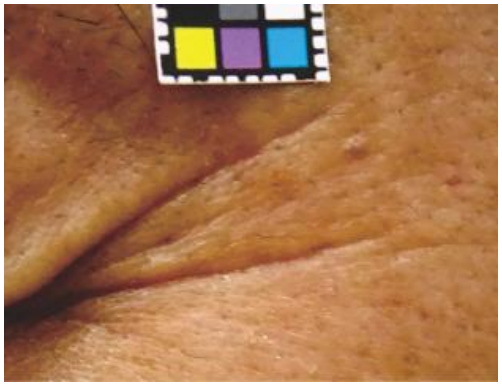
Replica Image

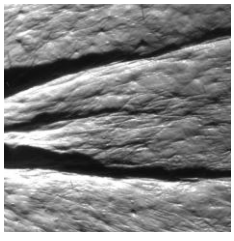
Analysis Image

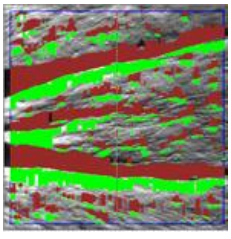
Entire volume ratio: 460.0
Volume ratio : 376.2

Area ratio : 15.74
Max. width : 1275.88

4W







Replica Image

Analysis Image

Entire volume ratio: 369.5
Volume ratio : 306.1

Area ratio : 14.20
Max. width : 1086.68

11. Stability Test

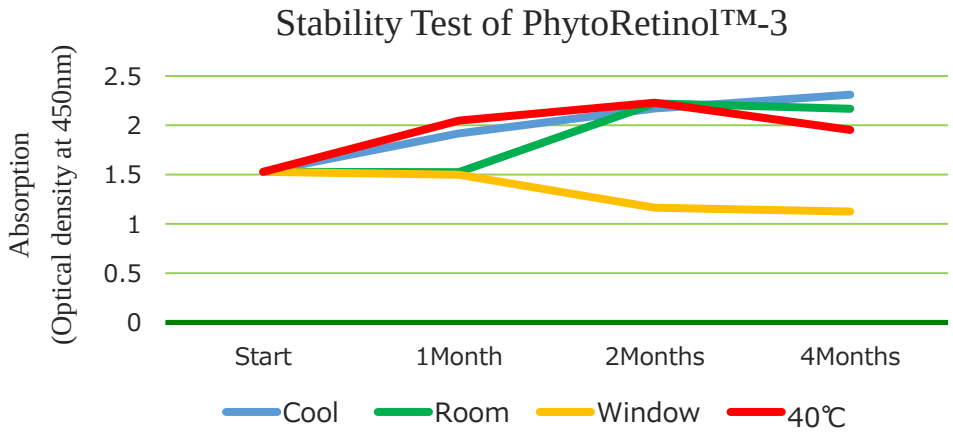
11-1 Long Term Stability Test

Store PhytoRetinol™-3C as it was, in a cool dark place at 4° C, room temperature, window side and at 40° C, observed for 4 months, and determined optical density at 450nm and observe color of liquid, change of smell, and presence of precipitates.

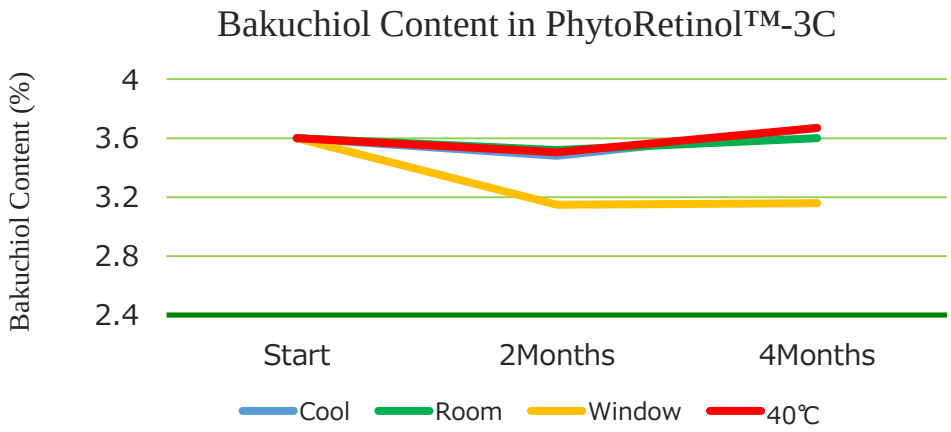
<Test Sample>

PhytoRetinol™-3C, Lot Number N-006, undiluted

<Test Result>



11-2 Long Term Stability of Bakuchiol Content



<Considerations>

Throughout the four (4) months, color tone increased for up to two months in cold, room temperature, and at 40 ° C. On the contrary, a phenomenon of color drop (color loss) was observed near the window. The color tone of the product changed over time, but the bakuchiol content in the product did not drop significantly except for the amount stored by the window.

12. Compatibility Test

（○： Clear, △： Turbid, ×：Separation / Precipitation）

	(%)	Trade Name / Manufacturer	Labelling Name	Result 1hr	Result 24hr
Cation	3.0	QUARTAMIN 86W Kao Corporation	Steartrimonium Chloride / Water	×	×
Anion	10.0	SOYPON SLE Kawaken Fine Chemical Co., Ltd.	Sodium Lauroyl Sarcosinate	▲	×
	10.0	EMAL 20C Kao Corporation	Sodium Laureth Sulfate / Water	▲	▲
	10.0	AMISOFT CT-12S Ajinomoto Co., Inc.	Water / TEA-Cocoyl Glutamate	▲	×
Nonion	10.0	PYROTER GPI-25 Nihon Emulsion Co., Ltd.	Glycereth-25 PCA Isostearate	×	×
	10.0	SALACOS PG-218 Nisshin Oilio Group Co., Ltd.	Polyglyceryl-10 Dioleate / Tocopherol	○	△
	10.0	RHEODOL 460V Kao Corporation	Sorbeth-60 Tetraoleate	△	△
	10.0	RHEODOL TW-0120V Kao Corporation	Polysorbate 80	△	△
Amphor-teric	5.0	AMPHITOL 20AB Kao Corporation	Lauramidopropyl Betaine	▲	×
	10.0	SOFTAZOLINE LSB 29% aq. Kawaken Fine Chemical Co., Ltd.	Lauramidopropyl Hydroxysulfate / Water	×	×
Silicones	10.0	KF-96A-10CS Shin-Etsu Chemical Co., Ltd.	Dimethicone	○	○
	10.0	KF-96A-300CS Shin-Etsu Chemical Co., Ltd.	Dimethicone	×	×
	10.0	KF-995 Shin-Etsu Chemical Co., Ltd.	Cyclopentasiloxane	○	○
	10.0	Silwet L-7604 Momentive Performance Materials	PEG-8 Dimethicone	△	△
	10.0	Silwet L-7622 Momentive Performance Materials	PEG-8 Dimethicone	○	○
Ester	1:1	AMIER MA-HD Nihon Emulsion Co., Ltd.	Hexyldecyl Myristoyl Methylaminopropionate	○	○
	1:1	EMALEX INTD-139 Nihon Emulsion Co., Ltd.	Isotridecyl Isononanoate	○	○
	1:1	T.I.O Nisshin Oillio Group Co., Ltd.	Triethylhexanoin	○	○
Hydrocarbon	1:1	MARCASOL R Maruzen Petroceutical Co., Ltd.	Isododecane	○	○
	1:1	PARAFOL 12-97 SASOL Ltd.	Dodecane	○	○
	1:1	NIKKOL SUGAR SQUALANE NIKKO Chemicals Co., Ltd.	Squalane	○	○
Polymer	1.0	Bio-HA 1%水溶液（MP-PE）N SHISEIDO Co., Ltd.	Water / Sodium Hyaluronate / Methylparaben	×	×

Each cosmetic ingredient was placed in a test tube, and PhytoRetinol ™ -3 was added and mixed so as to have a predetermined blending ratio. The properties of the mixed solution after 1 hour and 24 hours were observed. The compatibility with the emollient and solvent was mixed at a volume ratio of 1: 1 and the properties were confirmed.

13. Toxicological Safety Study

Trade Name	PhytoRetinol™ -3C	
INCI Name	Caprylic/Capric Triglyceride Psoralea Corylifolia Fruit Extract Rosmarinus Officinalis (Rosemary) Leaf Extract	
Safety Test Item	**Result**	**Test Method**
Acute Oral Toxicity	Not Performed	-
Primary Skin Irritation	No Category	LabCyte EPI / OECD TG 439 PhytoRetinol™-3C, undiluted
Accumulated Skin Irritancy	No Irritation	RIPT (53 volunteers) PhytoRetinol™-3C, undiluted
Sensitization	No Irritation	RIPT (53 volunteers) PhytoRetinol™-3C, undiluted
Photo Toxicity	Negative	*in vitro* 3T3 NRU / OECD TG 432 Psoralea Corylifolia Fruit Extract excluding psoralen
Photo Sensitization	Not Performed	-
Eye Irritation	No Category	EpiOcular™ / OECD TG 492 PhytoRetinol™-3C, undiluted
Mutagenicity	Negative	Ames test (5 strains) Psoralea Corylifolia Fruit Extract
Human Patch	(-) 53 volunteers	RIPT (53 volunteers) PhytoRetinol™-3C, undiluted

14. Recommended Plans and Guide Formulation

Cooperated by: NIHON Emulsion Co., Ltd.
NIKKO Chemicals Co., Ltd.
KOKYU Alcohol Industry Co., Ltd.

- Eye Cream
- Lotion
- Milky Lotion
- Anti-wrinkle Cream
- Face Mist
- Emollient Cream

14-1 Formulation 1 EYE CREAM / Modified No.142 by NIKKO Chemicals

No.	TRADE NAME	MANUFACTURER	%	INCI NAME
1	**PhytoRetinol™**	**ORYZA Oil & Fat Chemical**	2.00	Caprylic/Capric Triglyceride, Psoralea Corylifolia Fruit Extract, Rosmarinus Officinalis (Rosemary) Leaf Extract
2	**NIKKOL Lecinol S-10**	**NIKKO Chemicals Co., Ltd.**	0.50	Hydrogenated Lecithin
3	**NIKKOL Decaglyn 1-M**	**NIKKO Chemicals Co., Ltd.**	3.00	Polyglyceryl-10 Myristate
4	**NIKKOL Selachyl Alcohol**	**NIKKO Chemicals Co., Ltd.**	2.00	Oleyl Glyceryl Ether
5	**NIKKOL N-SPV**	**NIKKO Chemicals Co., Ltd.**	4.00	Cetyl Palmitate
6	**ORYZA SQUALANE™**	**ORYZA Oil & Fat Chemical**	10.00	Squalane
7	T.I.O	The NISSHIN OilliO Group	8.00	Triethylhexanoin
8	STEARYL ALCOHOL NX	**KOKYU Alcohol Industry**	6.00	Stearyl Alcohol
9	GLYCERINE, Conc.	KAO Corporation	3.00	Glycerine
10	KELTROL CG-T（2% aq）	SANSHO Co., Ltd.	10.00	Xanthan Gum, Water
11	MEKKINS™-M	UENO Fine Chemical Industry	0.10	Methylparaben
12	PURIFIED WATER	TOKAI Pharmaceutical	51.80	Water
			100.00	

Preparation Method

1) Mix and dissolve Ingredients No. 1 to 8 at 80℃.
2) Mix and dissolve Ingredients No. 9 to 12 at 80℃.
3) While stirring 1) by homogenizer, add 2) and emulsifier.
4) While stirring and cool as the product.

Note: Thoroughly study and examine the temporal stability, safety including skin irritation, and restrictions such as the patent law for this formulation before use.

14-2 Formulation 2 LOTION / Modified AP-1067-1A by NIHON Emulsion

No.	TRADE NAME	MANUFACTURER	%	INCI Name
1	**AMITER MA-HD**	**NIHON Emulsion**	4.20	Hexyldecyl Myristoyl Methylaminopropionate
2	Eldew PS-306	AJINOMOTO Co., Ltd.	1.00	Phytosteryl/Behenyl/Octyldodecyl Lauroyl Glutamate
3	**PhytoRetinol™**	**ORYZA Oil & Fat Chemical**	0.10	Caprylic/Capric Triglyceride, Psoralea Corylifolia Fruit Extract, Rosmarinus Officinalis (Rosemary) Leaf Extract
4	**Astaxanthin-20C**	**ORYZA Oil & Fat Chemical**	0.10	Haematococcus Pluvialis Extract
5	**EMALEX PEIS-6EX**	**NIHON Emulsion**	2.40	PEG-6 Isostearate
6	**EMALEX HC-20**	**NIHON Emulsion**	4.80	PEG-20 Hydrogenated Castor Oil
7	GLYCERINE, Conc.	KAO Corporation	10.00	Glycerin
8	1,3-BG, UK grade	DICEL CORPORATION	10.00	Butylene Glycol
9	MEKKINS™-M	UENO Fine Chemicals	0.10	Methylparaben
10	ETHANOL（99%）		1.00	Alcohol
11	PURIFIED WATER	TOKAI Pharmaceutical	65.30	Water
12	First stage emulsification		10.00	First stage emulsification
13	CITRIC ACID	KOMATSUYA Corporation	0.10	Citric acid
14	SODIUM CITRATE	KOMATSUYA Corporation	0.50	Sodium Citrate
15	ETHANOL（99%）		10.00	Alcohol
16	PURIFIED WATER	TOKAI Pharmaceutical	79.40	Water
			100.00	

Preparation Method

1) Mix and dissolve Ingredients No. 1 to 8 at 60℃.
2) Mix and dissolve Ingredients No. 9 to 11 at room temperature.
3) While stirring 1) by homogenizer, add 2) and emulsifier as First stage emulsification.
4) Mix and dissolve Ingredients No. 12 to 16 at room temperature.
5) While stirring [3] / First stage emulsification by stirrer, and add [4] as the product.

Note: Thoroughly study and examine the temporal stability, safety including skin irritation, and restrictions such as the patent law for this formulation before use.

15. Product Specification

15-1 PhytoRetinol™-3C

Test Item		Specification	Remarks
Product Name	:	PhytoRetinol™-3C	
Appearance			
・ Color	:	Brown to Orange brown oil	
・ Odor	:	Characteristic odor	
Identification Test (IR Spectrum)			
1) Bakuchiol	:	3350cm^{-1},,1650cm^{-1}, 980cm^{-1} , 922 cm^{-1}	
2) Caprylic/Capric Triglyceride	:	2930cm^{-1}, 1745cm^{-1}, 1455 cm^{-1}, 1160cm^{-1}	
Specific Gravity d_{25}^{25}	:	0.940 ～ 0.960	Method1. C
Refractive Index n_D^{25}	:	1.440 ～ 1.4765	
Purity Test			
1) Heavy Metals	:	10 ppm max.	
2) Arsenic	:	1 ppm max.	
Residue on Ignition (1g, 550℃)	:	0.5% max.	Method 2
Assay			
・ Bakuchiol	:	3.0 % min.	
Microbiological Examination			
1) Bacterial Count	:	1 × 10^2/g max.	Hygiene Exam.
2) Yeast･Mold Count	:	1 × 10^2/g max.	Hygiene Exam.
3) Coliform	:	Negative	Hygiene Exam.

These standards and test method are referred to General Notices and General Tests, Processes and Apparatus of The Japanese Standards of Quasi-drug Ingredients (JSQI), unless otherwise specified.

15-2 PhytoRetinol™-PC1

Test Item		Specification	Remarks
Product Name	:	PhytoRetinol™-PC1	
Appearance			
・ Color	:	Pale yellowish white to slightly white powder	
・ Odor	:	Slightly characteristic odor	
Purity Test			
1) Heavy Metals	:	20 ppm max.	
2) Arsenic	:	1 ppm max.	
Loss on Drying (1g, 105℃)	:	10.0% max.	Method 2, 2hrs
Assay			
・ Bakuchiol	:	1.0 % min.	
Microbiological Examination			
1) Bacterial Count	:	1×10^2/g max.	Hygiene Exam.
2) Yeast・Mold Count	:	1×10^2/g max.	Hygiene Exam.
3) Coliform	:	Negative	Hygiene Exam.

These standards and test method are referred to General Notices and General Tests, Processes and Apparatus of The Japanese Standards of Quasi-drug Ingredients (JSQI), unless otherwise specified.

16. Cosmetic Regulations and Certifications

16-1 PhytoRetinol™-3C

Japanese Labelling Name	:	トリ(カプリル酸/カプリン酸)グリセリル オランダビユ果実エキス ローズマリー葉エキス
Japanese Quasi-drug	:	Not Applicable
INCI Name	:	Caprylic/Capric Triglyceride Psoralea Corylifolia Fruit Extract Rosmarinus Officinalis (Rosemary) Leaf Extract
IECIC	:	None
CAS Number	:	73398-61-5 None 84604-14-8
EC Number	:	277-452-2 922-709-2 283-291-9

ISO16128	:	Natural Index	1
		Natural Origin Index	1
		Organic Index	0
		Organic Origin Index	0

16-2 PhytoRetinol™-PC1

Japanese Labelling Name	:	シクロデキストリン アラビアガム オランダビユ果実エキス
Japanese Quasi-drug	:	Not Applicable
INCI Name	:	Cyclodextrin Acacia Senegal Gum Psoralea Corylifolia Fruit Extract
IECIC	:	None
CAS Number	:	17465-86-0 9000-01-5 None
EC Number	:	241-482-4 232-519-5 922-709-2

ISO16128	:	Natural Index	0
		Natural Origin Index	1
		Organic Index	0
		Organic Origin Index	0

17. Others

17-1 Packaging Style

1 kg Tin-can, 5 kg Tin-can Outer: Carton box / PhytoRetinol™-3C

1 kg Alumi-bag, 5 kg Alumi-bag Outer: Carton box / PhytoRetinol™-PC1

17-2 Shelf Life & Storage Conditions

2years after manufacturing date

Please store room temperature avoiding high temperature and humid.

18. References

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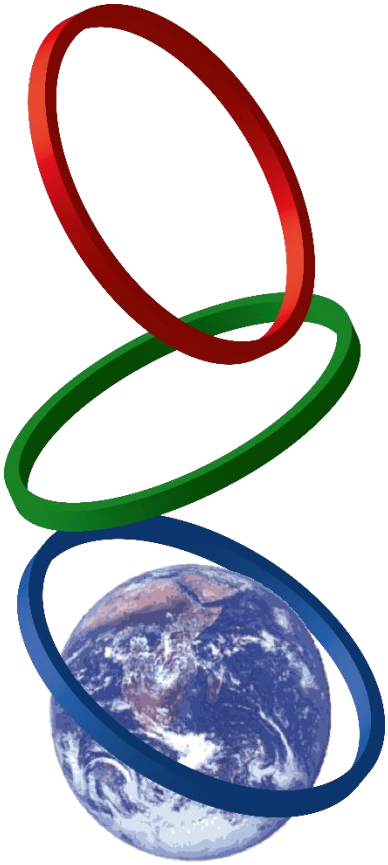
BUREAU VERITAS
Certification

Factory in Ichinomiya

Issued on December 9, 2020
K-009SO



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