Natural Cosmetic Ingredient

CureBerry

(Vaccinium Myrtillus Leaf Extract)

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Published on April 30, 2006

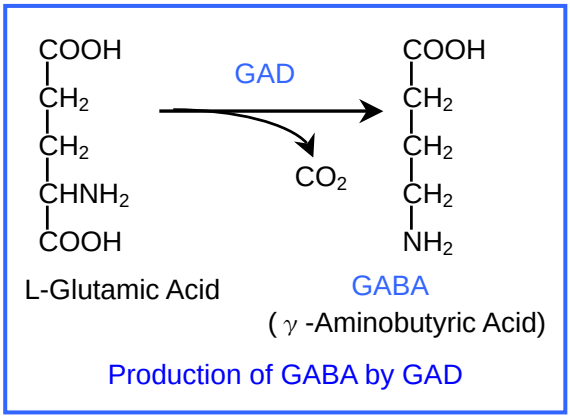
Revised on September 4, 2007

CONTENTS

Therapeutic amino acid GABA and CUREBERRY [1) to 4)]

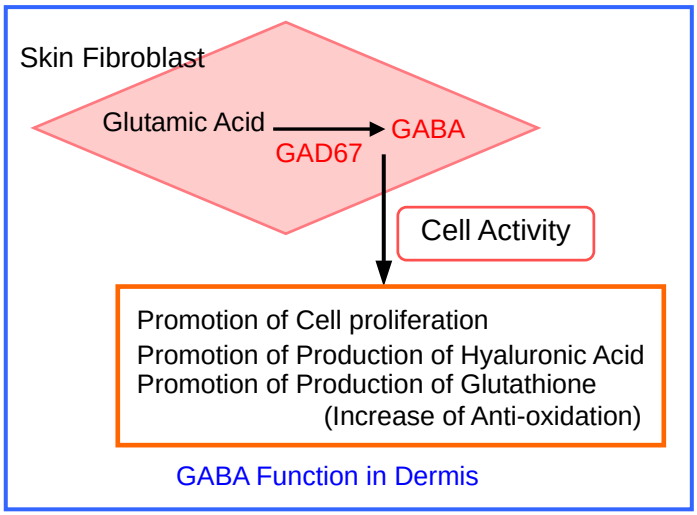
Recently, a substance called GABA has been attracting attention as a functional food component, because it has the relaxing and therapeutic actions. The formal name of GABA is γ (gamma)-amino butyric acid. The name GABA is derived from the first letters of Gamma-Amino Butyric Acid. GABA is contained in various foods such as fermented foods (pickled vegetables etc.) vegetables, fruits as well as grains. Therefore, GABA can be considered as an amino acid ingested by eating an ordinary daily diet.

A large amount of GABA exists in the brain of mammals including humans, and GABA works as an inhibitory neurotransmitter that inhibits nervous excitation in the central nervous system. Since GABA has a repressive action on cerebral excitation, the relaxing effect and the anti-stress effect after oral intake of GABA has been studied and it has been reported that Gabaron tea and cacao containing plenty of GABA have an anti-stress effect. In further use of GABA as a functional food component, it has become clear that it possess various physiological actions such as hypotensive action, diuretic action, activation of renal function, improvement of liver function, obesity prevention and stimulation of alcohol metabolism. Therefore, it has been attracting more and more attention.



Production of GABA by GAD

In the body, GABA is produced from glutamic acid by the action of Glutamic Acid Decarboxylase (GAD). GAD requires pyridoxal-5'-phosphoric acid as the coenzyme for the reaction and it has been known that GAD consists of two different structural molecules, GAD65 and GAD67. It has been reported that GAD exists in the pancreas, kidney, small intestine, tongue, testis and oviduct as well as the brain, but the function of GAD and GABA in tissues other than nerve tissue is hardly known at all.



Ichimaru Pharcos Co., Ltd. reported for the first time that GAD67, GABA synthesizing enzyme, exists in the dermal fibroblasts, suggesting that GABA is being produced in the skin.

Furthermore, we found that GABA enhanced fibroblast functions such as production of hyaluronic acid and the biological antioxidant glutathione, as well as cell growth. **(Annual Meeting of Japanese Biochemical Society, 2003).**

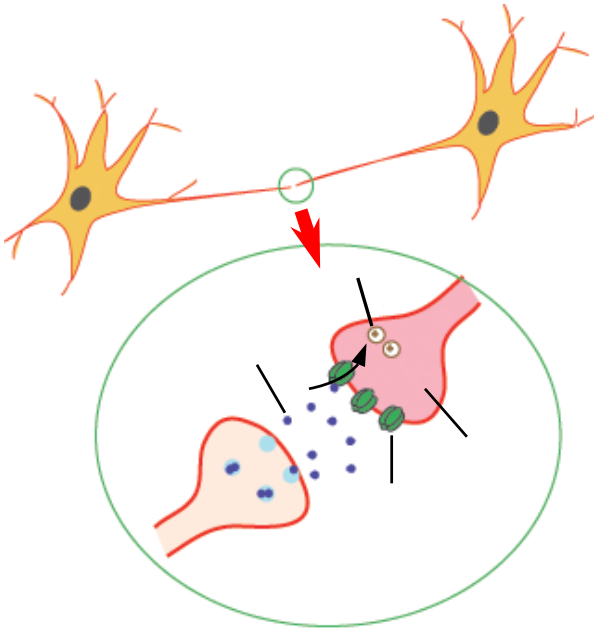
It has been frequently said that "Love makes a person beautiful." Recently, the relationship between emotion and skin has been investigated. It has been said that mental stress induces worsening of the skin conditions such as fine wrinkles and flabby skin. It is considered that the inhibitory neurotransmitters with anti-stressing action such as GABA are one of the factors that make the skin more beautiful by activating skin cell function.

Cure (Therapy) + Bilberry (Leaf) = CUREBERRY

Our recent study revealed that the amount of GAD67 decreased in the aging skin. It has been known that resistance to stress and adaptability to changes decrease with aging and the same thing has been suggested also in the skin. It is expected that decrease in GABA production due to reduction of GAD67 is one of the causes for the age-dependent decrease of stress resistance in the skin. We examined whether the production of GABA with beneficial effect on the skin could be induced in the skin instead of externally applying, and found that bilberry leaf extract had the strong ability to increase GAD67 in dermal fibroblasts. Thus we developed "CUREBERRY" this name being a combination of "Cure" and "Bilberry."

Neurotransmitters and skin [1) 5)]

Various neurotransmitters play an important role in the central nervous system. Amino acids such as glycine and GABA, monoamines such as serotonin and dopamine, and various peptides play a role in the information transfer from one cell to another cell in the nerve network. The receptors of these neurotransmitters exist at nerve cell communication sites called synapses. It is the receptors with ion channels that play the most important role in the nervous system. Ions usually cannot pass through the cell membrane. However, ion channels, pores in receptors, pass a specific ion into the cell. The binding of a neurotransmitter to the ion channel receptor opens the pore of cell membrane. When an ion channel for positive ions such as sodium and calcium is opened and a positive ion flows into the synapse, the cell membrane potential becomes positive. In the nerve cells, this is called “excitation” of cells. Conversely, when an ion channel for negative ions such as chlorine is opened and a negative ion flows into the synapse, the excitation is “repressed.” When this potential change exceeds a certain threshold, the potential difference is changed to an electric signal to transfer information through the nerve fiber. Stimulatory neurotransmitters, the key opening a positive ion channel, include acetylcholine, while inhibitory neurotransmitters, the key opening a negative ion channel, include glycine and GABA.



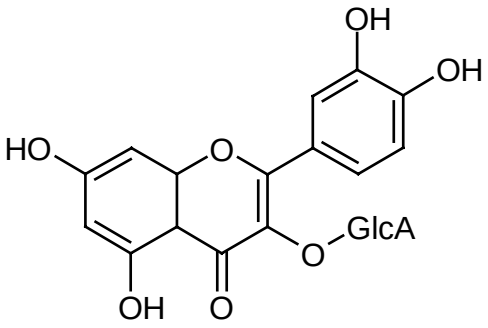
Recently, it became known that there was a receptor for a neurotransmitter even at skin epidermal cells. It has been reported that a stimulatory transmitter delayed the restoration of the damaged horny layer’s barrier function and that conversely, an inhibitory neurotransmitter stimulated the restoration of the damaged barrier function. It is considered that a neurotransmitter which acts in the nervous system is probably involved in the information transfer also in the skin.

CUREBERRY [6) to 11)]

CUREBERRY is an extract obtained from leaf of bilberry *Vaccinium myrtillus* L. (*Ericaceae*) belonging to the *Ericaceae* family, genus *Vaccinium*. Bilberry naturally grows widely from Scandinavia to northern Europe lowlands and the alpine shrub zone in Asia. The height is 20 to 60 cm, the leaf has a saw-like blade edge, and the flower is red in color and has the cup-like shape. The size of the fruits is 6 to 8 mm and the color is dark blue to purple blue. The presence of a large amount of anthocyanin is characteristic of the fruit. The fruit and leaf of Bilberry have been utilized for thousands of years, for dyestuffs and coloration of alcohol beverages as well as for foods such as raw fruit, dried fruit, juice, jam and tea.

In the genus *Vaccinium*, to which bilberry belongs, is a shrub that has bunches of indigo black to light blue color fruit has been called generally “Blueberry.” The fruit of bilberry is marketed as blueberry sometimes, but bilberry contains a very large amount of anthocyanin compared with other fruits called blueberry. With reports that anthocyanin is good for the eyes, the amount of the fruit harvested for pigment extraction and as the raw material of food supplements with high content of anthocyanin has drastically increased.

It has been said that this effect of bilberry was pointed out for the first time by a Great Britain air force pilot. Scientists in Italy and France were interested in the report that this pilot could see a target clearly from the cockpit even under the dim light by ingesting a large amount of bilberry jam. Thereafter, the beneficial effect of bilberry fruits, especially on the eyes, is much talked about in Europe. The bilberry leaf has been frequently utilized for tea, and bilberry leaf has been traditionally applied as a folk medicine mainly for diabetes. It has been reported that bilberry leaf contains polyphenols such as tannin and quercetin-3-glucuronide.



Quercetin-3-glucuronide

Introduction

Property

This product is an extract obtained by extracting the leaf of *Vaccinium myrtillus* L. (*Ericaceae*) with 1,3-butylene glycol solution. Leaf of CUREBERYY is organic plant; which is classified under Council Regulation（EEC）2092/91 in EU regulation.

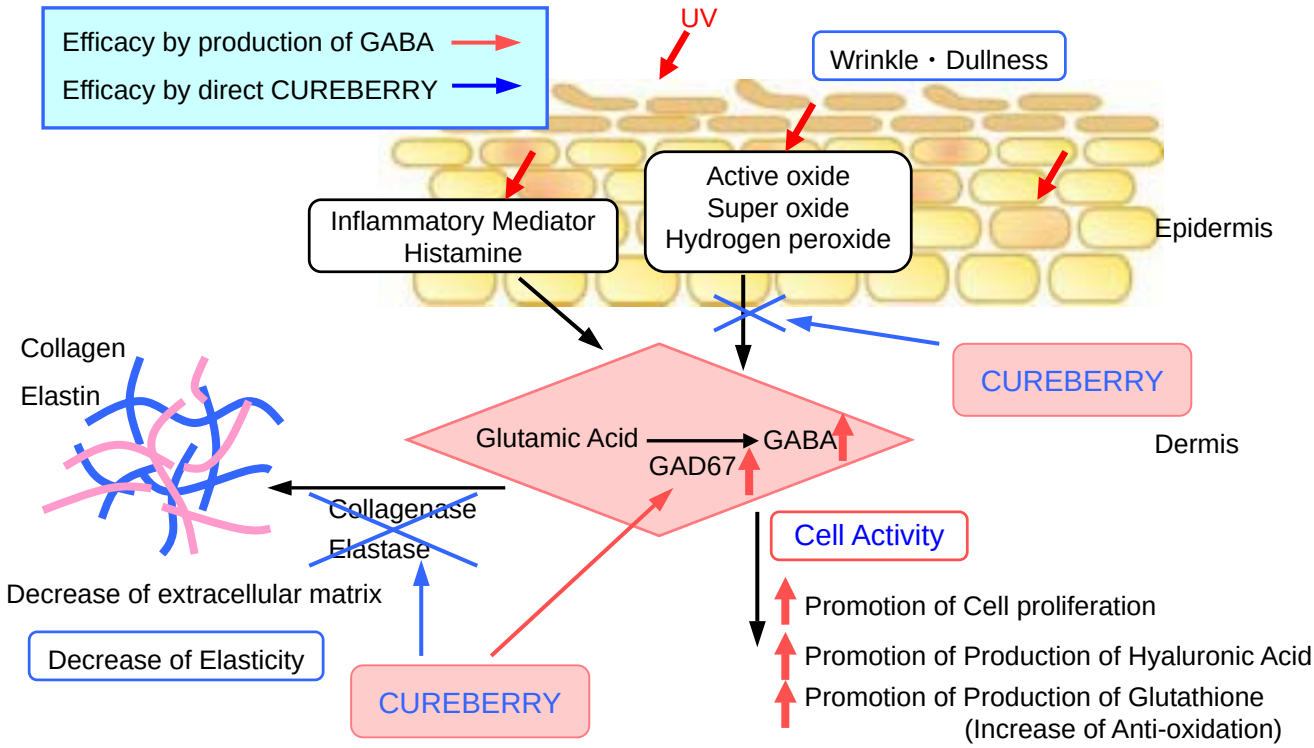
Efficacy

CUREBERRY **increased GABA synthesizing enzyme <GAD>** and below mentioned efficacies are assumed to be related to activity of GABA in the skin.

- Fibroblast Proliferation Activity
- Promotion Effect of Hyaluronic acid Production
- Promotion Effect of Glutathione Production

Also, below mentioned efficacies are observed by CUREBERRY.

- SOD like Activity
- Inhibitory Effect of Collagenase Activity
- Inhibitory Effect of Elastase Activity
- Inhibitory Effect of Lipase Activity
- Inhibitory Effect of Production of B16 Melanoma Melanin
- Inhibitory Effect of Histamine Release



Increase of GABA Synthesizing Enzyme <GAD>

Ichimaru Pharcos Co., Ltd. confirmed that GAD (glutamic acid decarboxylase) exists in dermal fibroblasts and found that therapeutic amino acid, GABA (γ-amino butyric acid), which is attracting attention for its healing and relaxing effects, is produced in the dermis. It has been reported that GABA had various actions in the skin such as cell proliferation, production of antioxidants and stimulation of the restoration of barrier function. Therefore, it can be expected that such effects of GABA on the skin are increased by increasing GAD production in the skin.

Thus the effect of CUREBERRY on the production of GAD in human skin fibroblasts was examined.

Test Sample

CUREBERRY was applied 20 μg/mL as final concentration as test sample. (Product concentration is 1%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

Human dermal fibroblasts (Kurabo) were pre-incubated until reaching to a confluent state in the presence of 5% fetal bovine serum (FBS) and a test sample was added to the culture medium. After 24-hour incubation, the medium was removed and the cells were washed with PBS. Then the cells were dissolved with a solution for protein extraction (50 mM Tris-HCl × pH 7.5, 1% Nonident P-40, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid and 1 mM PMSF). The solution was centrifuged at 15000g for 15 minutes at 4°C, and protein in the supernatant was measured by the Bradford method. After adjusting the amount of protein to 20 μg, the supernatant was separated by SDS-PAGE and the amount of GAD was determined by Western blot analysis using anti-GAD67 antibody (CHEMICON).

Result and Discussion

The result of Western blot showed that the significant increase of GAD67 in dermal fibroblast (Fig.1). GAD67 expression was enhanced by applying CUREBERRY.

According to the result, CUREBERRY can induce GAD expression which promotes production of GABA in dermis and have a role in anti-aging process.

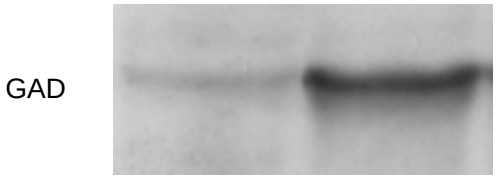


Fig.1 Increase of enzyme of GABA production <GAD>

Fibroblast Proliferation Activity

Fibroblasts exist in the dermis to produce extracellular matrices of collagen, hyaluronic acid, etc., the main dermal constituents. It has been said that the amount of fibroblasts is decreased with aging and it has been considered that the decrease in fibroblasts leads to a decrease in extracellular matrices and further to various aging phenomena such as wrinkles and flabby skin. It has been confirmed that GABA has the proliferating action on fibroblasts.

Test Sample

CUREBERRY is adjusted to 5 μ g/mL and 10 μ g/mL solid matter by 50% 1,3-Butylene Glycol. (Product concentration is 0.25% and 0.5%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

Using 24-well plates and DMEM containing 5% FBS, human dermal fibroblasts were seeded at a density of 5 x 10^4 cell/well. After cultivation under condition of 5% CO^2 and 37℃ for 3 days, culture medium was replaced by DMEM which was not contained serum. After cultivation, the numbers of cells are analyzed by WST method (Cell Counting Kit-8 DOJINDO Laboratories).

Result and Discussion

Increase fibroblast proliferation by CUREBERRY was shown in Fig.2. Concentration dependent cell growth was observed.

According to result, CUREBERRY is expected to increase fibroblast by promotion of production of GABA and improve skin aging.

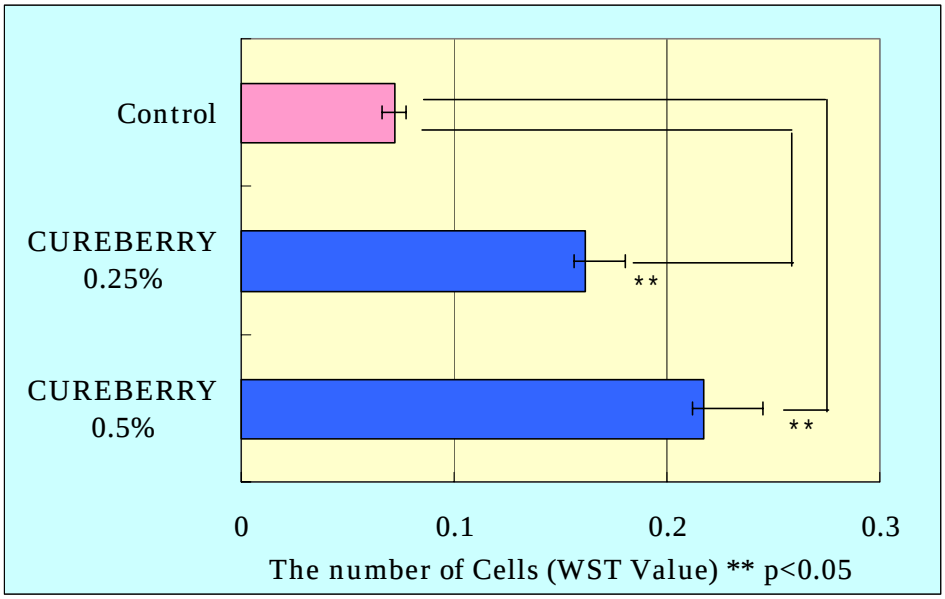


Fig.2 Fibroblast Proliferation Activity

Promotion Effect of Hyaluronic acid Production

Hyaluronic acid holds a large amount of water in human dermis and plays an important role in dermal plasticity (flexibility) by filling the gaps in the collagen and elastin matrix. The degradation of hyaluronic acid is rapid. Therefore, when the production of hyaluronic acid is decreased with aging etc., the amount of hyaluronic acid in the dermis is decreased rapidly to result in a decrease in skin flexibility. Thus it can be expected that a substance stimulating hyaluronic acid production will increase skin flexibility. It has been confirmed that GABA has a stimulating effect on hyaluronic acid production from dermal fibroblast

Test Sample

CUREBERRY is adjusted to 5 μ g/mL and 10 μ g/mL solid matter by 50% 1,3-Butylene Glycol. (Product concentration is 0.25% and 0.5%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

Using 24-well plates and DMEM containing 5% FBS, normal human dermal fibroblasts were seeded so that a pattern of 5 x 10^4 (cell/well) was formed. After cultivation under condition of 5% CO^2 and 37℃ for 3 days, their culture medium was replaced by DMEM; which was not contained serum. After cultivation, amount of hyaluronic acid in cell culture medium was analyzed by measurement kit of hyaluronic acid (Seikagaku Corp.). Number of cells were measured by Cell Counting Kit-8 and the amount of hyaluronic acid per cell was calculated. Promotion of production of hyaluronic acid was analayzed by the relative value where 100 is the control.

Result and Discussion

Promotion of production of hyaluronic acid was shown in Fig.3. CUREBERRY was observed to have promotion of production of hyaluronic acid.

According to result, CUREBERRY is expected to increase hyaluronic acid in dermis by promotion of production of GABA and increase skin flexibility.

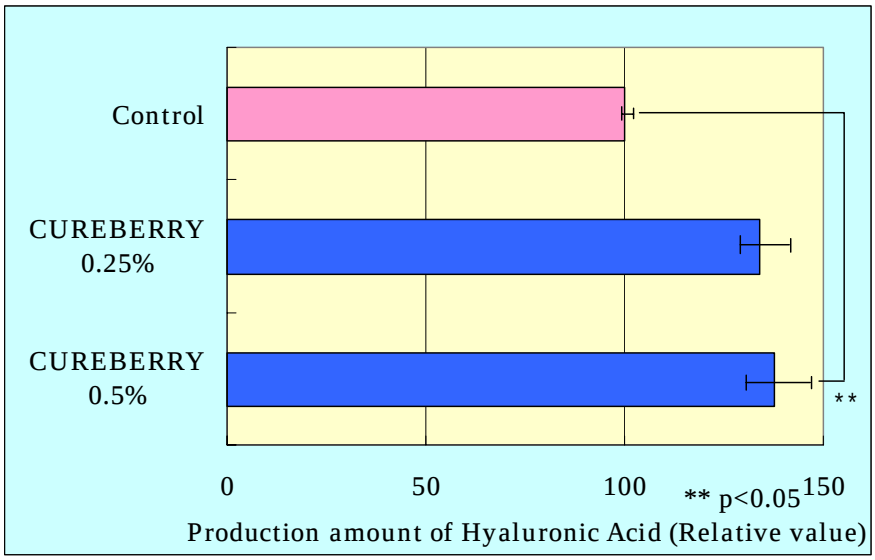


Fig.3 Promotion Effect of Hyaluronic acid Production

Promotion Effect of Glutathione Production

Glutathione is a biological antioxidant that protects cells from the oxidative damage. Glutathione reduces hydrogen peroxide, which damages cells, converting it to water. Recently, it has been reported that glutathione prevents lowering of immunity and protects against stress, and it became recognized that glutathione is an all round biological protective substance. It has been confirmed that GABA has a stimulatory effect on glutathione production from dermal fibroblast.

Test Sample

CUREBERRY is adjusted to 10 μg/mL and 20 μg/mL solid matter by 50% 1,3-Butylene Glycol. (Product concentration is 0.5% and 1.0%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

Human dermal fibroblasts were cultured until reaching a confluent state in DMEM containing 5% FBS under conditions of 5% CO_2 at 37°C. Thereafter, the medium was changed to DMEM without serum, and a test sample was added. After the incubation for 24 hrs, the medium was removed, and the cells were washed with PBS and collected. After adding 10 mM HCl solution to the collected cells, the cells were disrupted by repeated cycles of freezing and thawing, and centrifuged at 8000 g to obtain the cell extract. After adding 5% SSA (5-sulfosalicylic acid) to the extract, centrifugation was performed at 8000 g and the supernatant was used for determination of total amount of glutathione by a Total Glutathione Assay Kit (Dojin Chemical Co., Ltd.). The amount of protein in the cell extract before adding 5% SSA was determined by the Bradford method and the amount of glutathione within the total protein content was obtained to calculate the relative value where 100 is the control.

Result and Discussion

Promotion of production of glutathione was shown in Fig.4. CUREBERRY was observed to have promotion of production of glutathione by concentration dependence.

According to result, CUREBERRY is expected to increase glutathione in dermal fibroblast by promotion of production of GABA and protect skin aging.

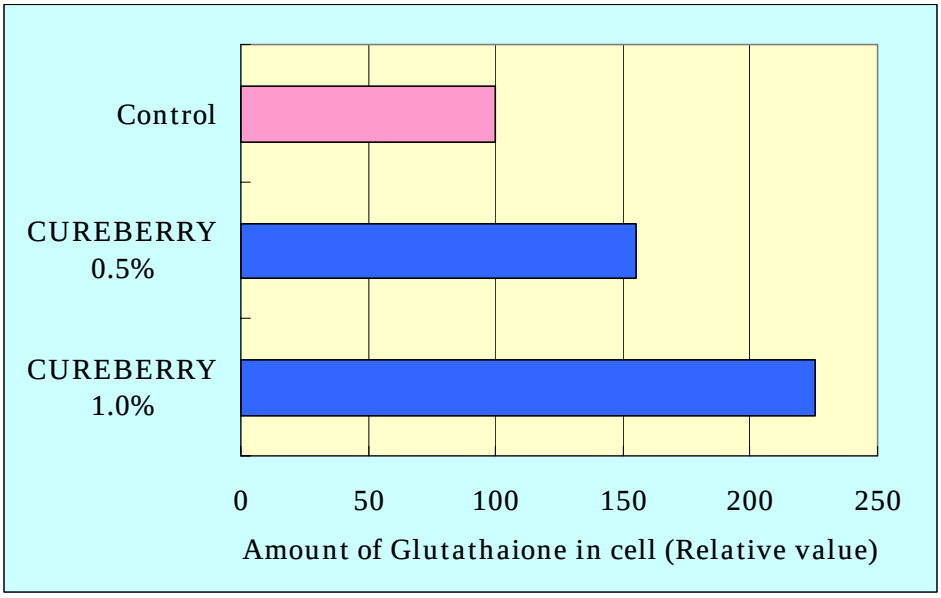


Fig.4 Promotion Effect of Glutathione Production

SOD like Activity

There are many reports that reactive oxygen species are involved in skin aging. Especially, superoxide dismutase (SOD) that eliminates superoxide (O_2^-), particularly active oxygen, has been given attention for its aging prevention action, and a substance with superoxide-eliminating activity like SOD (SOD-like activity) has been vigorously searched for.

Test Sample

CUREBERRY is adjusted to 0.1 μ g/mL and 1 μ g/mL solid matter by 50% 1,3-Butylene Glycol. (Product concentration is 0.005% and 0.05%.) As control, 50% 1,3-Butylene Glycol was applied. As positive control, OOLONG Liquid E is applied. (Product concentration is 0.004%.)

Test Method

SOD like effect was measured by SOD Assay kit-WST (DOJINDO Laboratories). This kit is measurement of SOD like activity; which super oxide (O^{2-}) let WST test-drug reduce and change color by reaction of xanthine-xanthine oxidase.

Result and Discussion

SOD like effect was shown in Fig.5. CUREBERRY was observed to have strong SOD like effect as same as OOLONG Liquid E; which has already reported strong anti-oxidation effect.

According to result, CUREBERRY is observed to have SOD like effect and expected to have strong inhibition for oxidation stress.

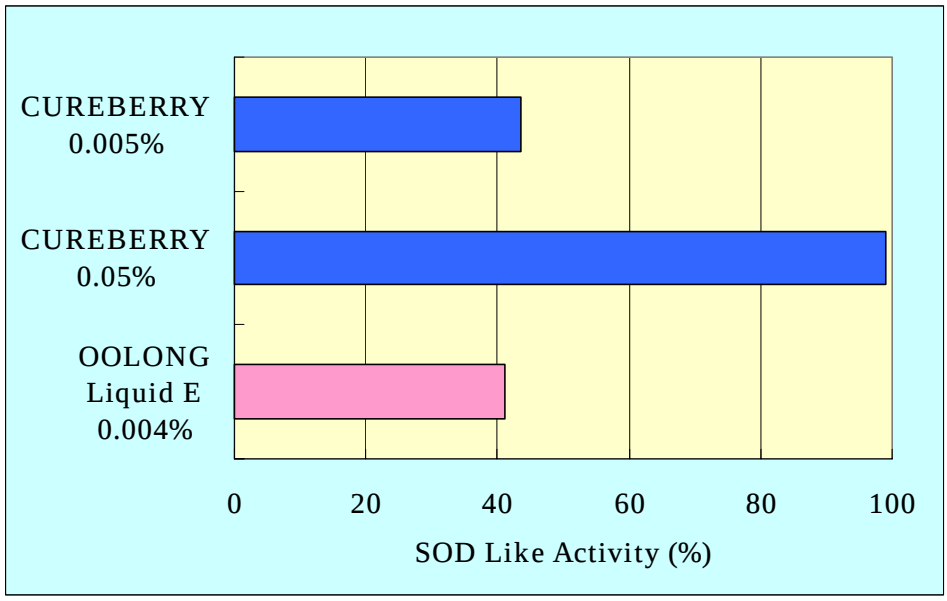


Fig.5 SOD like Activity

Inhibitory Effect of Collagenase Activity

Collagen is a major component of the matrix of human dermis, comprising 70% of the dermis, and plays an important role in the maintenance of the skin structure. Therefore, the fragmentation and denaturation of collagen by the actions of reactive oxygen species and collagen-degrading enzyme (collagenase) decrease skin elasticity to produce wrinkles and flabby skin. In other words, it can be expected that a substance inhibiting the action of collagenase has an inhibitory effect on the occurrence of wrinkles and flabby skin.

Test Sample

CUREBERRY is adjusted to 25 μg/mL, 50 μg/mL and 110 μg/mL solid matter in test reaction by 50% 1,3-Butylene Glycol. (Product concentration is 1.25%, 2.5% and 5.5%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

Collagenase was induced by IL-1α from cultured dermal fibroblast cells. Trypsin solution was added to the supernatant of this culture medium, and the mixture was left for 5 minutes at 37°C to activate collagenase in the supernatant. Thereafter, trypsin inhibitor was added to the mixture to stop trypsin reaction to prepare crude collagenase solution. Fifty microliters of ice-cold FITC labeled Type-I collagen solution (Sigma), 90 μL of Tris-HCl buffer solution (pH 7.5), 10 μL of a test sample and 50 μL of crude collagenase solution were mixed to incubate for 2 hours at 37°C. Then the reaction was terminated by adding 200 μL of ice-cold *o*-phenanthroline solution. After leaving for 30 minutes at room temperature, the reaction mixture was centrifuged at 6000 g for 10 minutes to determine the fluorescent intensity (excitation: 490 nm, fluorescence: 530 nm) of the supernatant. The blank was prepared in each test sample by adding Tris-HCl buffer solution in place of crude collagen solution and the collagenase inhibition rate in each test sample was calculated using the following equation.

Result and Discussion

Inhibition effect of Collagenase Activity was shown in Fig.6. CUREBERRY was observed to have strong inhibition effect of collagenase activity by concentration dependence.

According to result, CUREBERRY protects dermal collagen and is expected to inhibit wrinkle and slack.

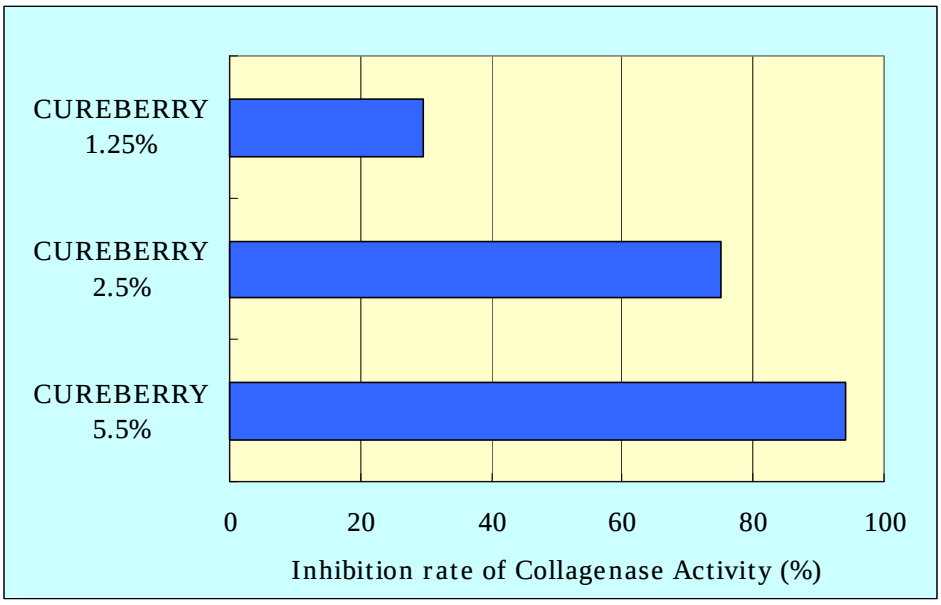


Fig.6 Inhibitory Effect of Collagenase Activity

Inhibitory Effect of Elastase Activity

Elastin is one of the major components of dermal matrices, and plays an important role in the maintenance of the skin elasticity (power to push back against external force like a spring). The fragmentation and denaturation of elastin by the actions of reactive oxygen species and an elastin degrading enzyme (elastase) lead to the decreased skin elasticity, wrinkles and flabby skin. In other words, it can be expected that a substance inhibiting the action of elastase have an inhibitory effect on the occurrence of wrinkles and flabby skin.

Test Sample

CUREBERRY is adjusted to 12.5㎍/mL, 25㎍/mL and 125㎍/mL solid matter by 50% 1,3-Butylene Glycol. (Product concentration is 0.625%, 1.25% and 6.25%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

This test is evaluated by using pig's pancreas-derived elastase and synthetic substrate N-succinyl-Ala-Ala-Ala-*p*-nitroanilide. As the buffer solution, 0.05 M Tris-HCl buffer (pH 8.8) was used. With this buffer, the enzyme and the substrate solutions were prepared to have a concentration of 0.05 unit/mL and 0.1 M by dimethyl sulfoxide, respectively. First, 50 μL of the test sample, 50 μL of the enzyme solution and 100 μL of the substrate solution were mixed together and the resulting solution was kept at 37°C for 30 minutes to promote its reaction. It was measured at 405 nm of wavelength. The inhibition rate was calculated by the following formula. Here, the extract and for the test sample was used as the reference solution. Setting up a blind test in which the substrate was added to the solution whose reaction had been stopped, the inhibition rate was calculated from the difference in absorbance between the actual test and the blind test according to the following formula.

$$\text{Inhibition Rate (\%)} = \left(1 - \frac{\text{Absorption Rate of Actual Test of Sample} - \text{Absorption Rate of Blind Test of Sample}}{\text{Absorption Rate of Actual Test of Control} - \text{Absorption Rate of Blind Test of Control}}\right) \text{X } 100$$

Result and Discussion

Inhibition effect of Elastase Activity was shown in Fig.7. CUREBERRY was observed to have strong concentration dependent elastase inhibitory effect.

According to result, CUREBERRY protects dermal elastin and is expected to inhibit wrinkle and slack.

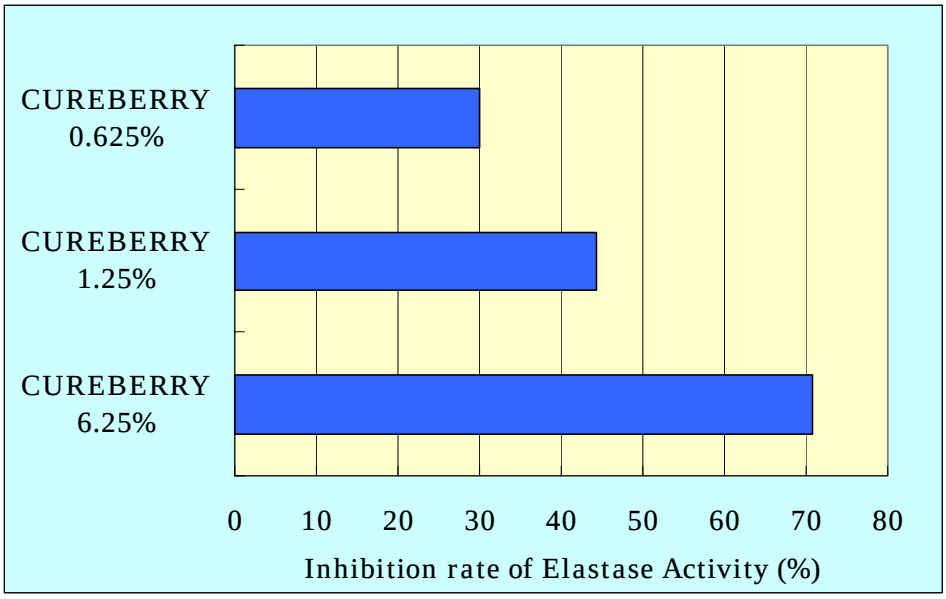


Fig.7 Inhibitory Effect of Elastase Activity

Inhibitory Effect of Lipase Activity

One of the major causes for acne inflammation is considered to be free fatty acids produced by lipase (an enzyme degrading lipid into free fatty acids and glycerol) produced from *Propionibacterium acnes* present mainly in follicles of sebaceous glands. Therefore, it can be expected that a substance inhibiting the action of lipase has an inhibitory effect on acne inflammation.

Test Sample

CUREBERRY is adjusted to 2.8g/mL, 28 μg/mL and 61.6 μg/mL solid matter by 50% 1,3-Butylene Glycol. (Product concentration is 0.14%, 1.4% and 3.08%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

Lipase activity is measured with using reagent of quantitative of human blood lipase (lipase kit S made by Dainippon Pharmaceutical Co., Ltd.). Sample (Test Sample : 0.01% of lipase of pig pancreas : purified water = 4 : 5 : 11) is mixed as a measurement sample. 8 μL of measurement sample, 76 μL of coloring solution of reagent and 8 μL of esterase inhibition of reagent is mixed and heated at 30℃ for 5 minutes. After heating, add 8 μL of substrate solution and incubate at 30℃ for 30 minutes. After incubating, add 152 μL of reaction stop solution of reagent, after stop reacting, measure absorption at 405nm. (Abs. Rate A) For the control, solvent is used. Also, after stop reacting, make blank absorption rate (Abs. Rate B), according to the balance between measurement absorption and blank absorption (Abs. Rate A - Abs. Rate B), lipase activity of CUREBERRY Liquid is measured with using below mentioned calculation.

$$\text{Inhibition Rate (\%)} = \left(1 - \frac{\text{Absorption Rate of Actual Test of Sample} - \text{Absorption Rate of Blind Test of Sample}}{\text{Absorption Rate of Actual Test of Control} - \text{Absorption Rate of Blind Test of Control}}\right) \times 100$$

Result and Discussion

Inhibition effect of Lipase Activity was shown in Fig.8. CUREBERRY inhibits lipase activity by concentration dependence compared with control.

According to result, CUREBERRY is expected to inhibit lipase activity and protect inflammation and deterioration of acne.

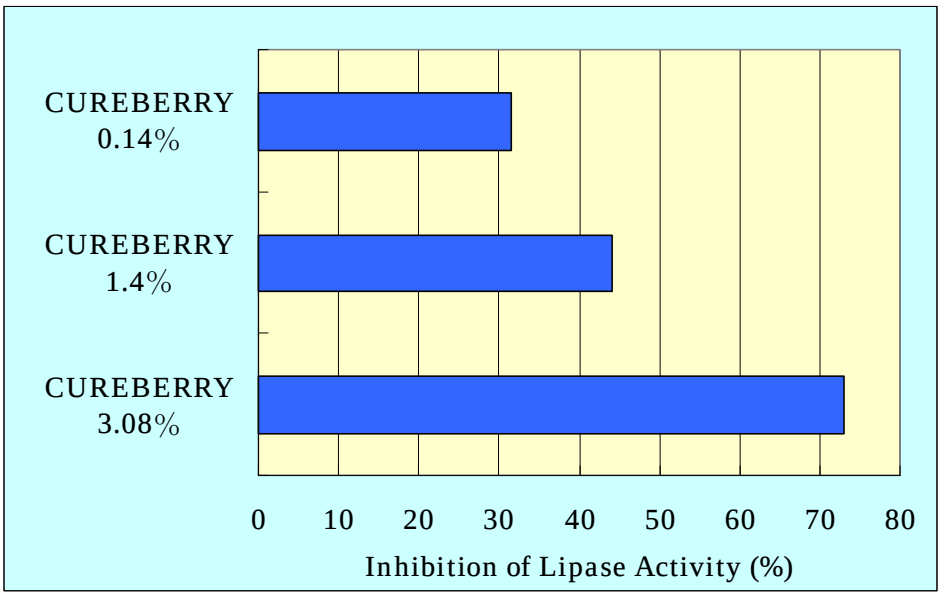


Fig.8 Inhibitory Effect of Lipase Activity

Inhibitory Effect of B16 Melanoma Cell

We investigated melanin production inhibition test on B16 melanoma cells for CUREBERRY.

Test Sample

CUREBERRY is adjusted to 25㎍/mL solid matter in test reaction by 50% 1,3-Butylene Glycol. (Product concentration is 1.25%.) As control, 50% 1,3-Butylene Glycol was applied. As positive control, Arbutin is adjusted to 50㎍/mL solid matter by 50% 1,3-Butylene Glycol.

Test Method

2 x 10^5 B16 melanoma cells were planted in a 60 mm plastic culture dish and were pre-cultured 24 hours. Then they were transferred to a fresh culture medium and test materials were added to be 25 μg/mL concentration to culture medium. The cells were collected by processing with trypsin after culturing for three days. Cells were dissolved in 1N NaOH and 10% DMSO and then absorbance was measured at 420nm. At the same time the viability of the cells which were supplemented with various materials was measured by the MTT reduction method.

Result and Discussion

Amount of melanin in medium was shown in Fig. 9. CUREBERRY was observed to have significant inhibition effect of melanin production. Cell toxicity on CUREBERRY was not observed on MTT test.

According to results, CREBERRY is expected to have strong whitening effect.

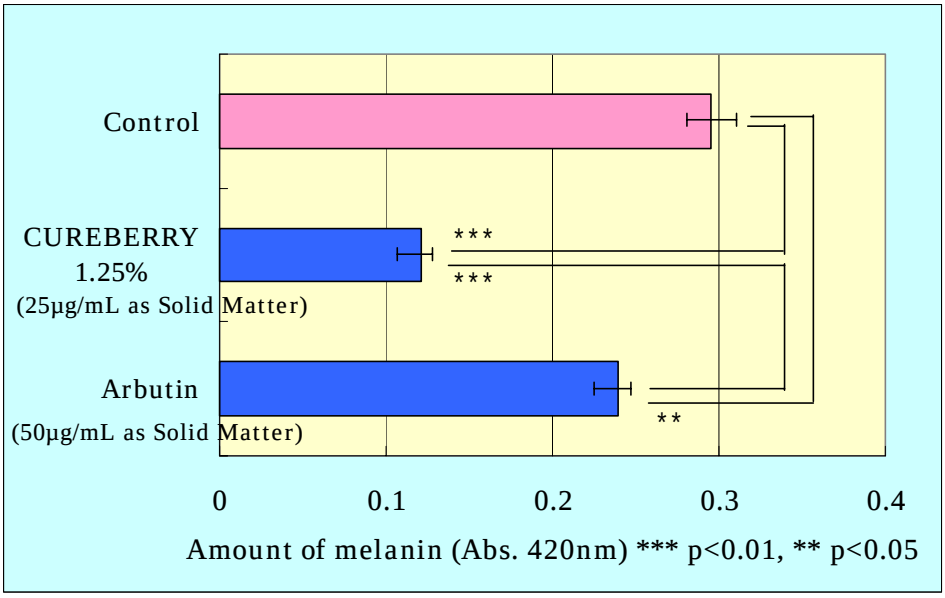


Fig.9 Inhibitory Effect of Melanin Production

Inhibitory Effect of Histamine Release

Histamine is released from mast cells and can cause allergic diseases such as eczema, asthma and itching. Therefore, substances which inhibit histamine release from mast cells are expected to show Anti-inflamatory effect and Anti-allergic effect.

We investigated the inhibition effect of histamine release on CREBERRY.

Test Sample

CUREBERRY is adjusted to 67.5 μ g/mL and 108 μ g/mL solid matter by 50% 1,3-Butylene Glycol. (Product concentration is 3.38% and 5.4%.) As control, 50% 1,3-Butylene Glycol was applied.

Test Method

Mast cells thus obtained were suspended in 2% FT solution to make about 1.0×10^5 cells/mL. After adding test substance into the cell suspension and keeping at stand for 10 min at 37°C, add histamine-releasing agent compound 48/80 (Sigma) (final concentration: 1 μg/mL) and keep at stand for 15 min at 37°C. The reaction was stopped by cooling on ice, and the reaction mixture was centrifuged at 100 ×g for 10 min. at 4°C to determine histamine in the supernatant. Briefly, purified water, 1 mol/mL of NaOH solution and 1% *o*-phthaldialdehyde-methanol solution were added to the supernatant. After keeping at stand for 5 min, the reaction was stopped by adding 3mol/L of HCl solution. At 10 min after terminating the reaction, the reaction mixture was centrifuged at 1,900 rpm for 25 min at 5°C to obtain the supernatant and sediment. Histamine in the supernatant was determined on the calibration curve of histamine using the fluorescence values at 360 nm of excitation wavelength and 450 nm of emission wavelength. Furthermore, histamine remained in the mast cells was determined by the same way as that described above in the ultrasonically treated sediment in 2% FT solution after 1-day storage at freezing. Then histamine-release ratio and the inhibition rate of histamine-release were obtained.

$$\text{Histamine Release Ratio} = \frac{\text{Histamine amount released from cell}}{\text{Total histamine amount in cell}}$$

Inhibition rate of Histamine Release (%) = [1-(A-C / B-C)] X 100

A: Histamine release ratio; which histamine release agent is added in what mast cell is added in test sample.

B: Histamine release ratio; which histamine release agent is added in mast cell.

C: Histamine release ratio; which is naturally released from the mast cell.

Result and Discussion

Inhibition rate of histamine release is shown in Fig.10. CUREBERRY was observed to have strong inhibition effect of histamine release by concentration dependence.

According to result, CUREBERRY is expected to have anti-inflammation effect and anti-allergic effect.

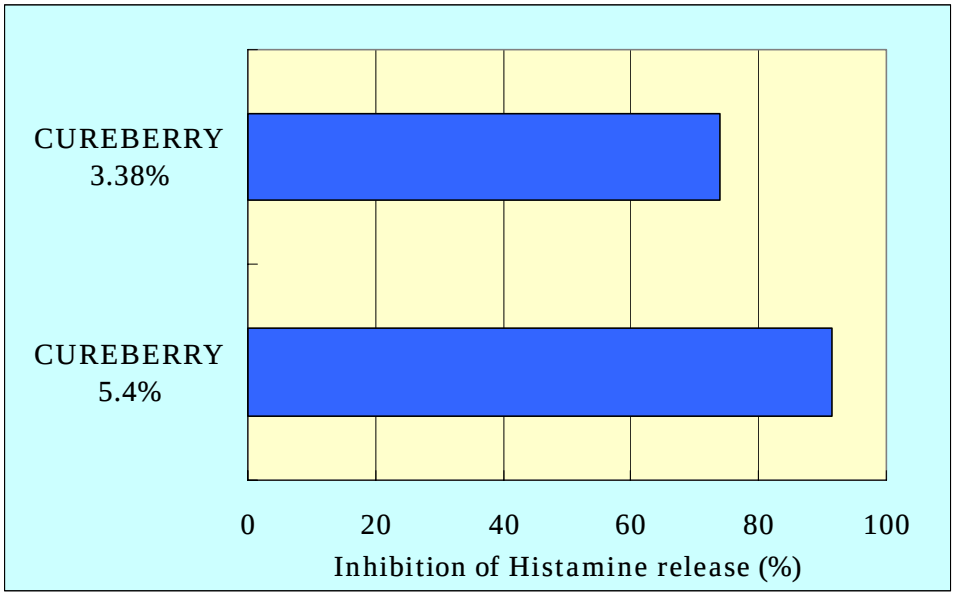


Fig.10 Inhibitory Effect of Histamine release

Recovering of Wrinkle on the Human Skin

Test Sample

CUREBERRY was diluted by purified water to adjust 5 % as product concentration. As control solution, 50% of 1,3-Butylene Glycol solution was diluted in the same manner and used in the test.

Test Method

Eight health male and female volunteers at age 30s to 50s that gave us written informed consent were enrolled in this study, and the samples were applied around their eyes two times a day for 12 weeks. The study was conducted from August 1, 2006 to October 26, 2006.

Before the treatment and 12 weeks after the treatment, a test was carried out according to the procedures for examining dermal shape by replica (SILFLO,AMIC Group). After washing face, volunteers stay in a thermo-hygrostat room (at 20℃, humidity 50%) for 20 minutes. Light was irradiated to replica from constant angle (tops and bottoms angle 25) and its shade was calculated with the use of an image data processing and picture analyzing software (WinROOF, Mitani), and area of wrinkle and depth of wrinkle were compared with the data before the application as 100 by calculating relative scale of the area of wrinkle per analyzing area and maximum depth of wrinkle.

Result and Discussion

The comparative results of the wrinkle area between that before the application and that at 12 weeks after the commencement of the test in each subject and the representative shadow images are shown in Fig 11 and Fig. 12, respectively. The decrease of the wrinkle area and maximum depth was observed at the CUREBERRY application. On the shadow images that CUREBERRY was used for 12 weeks, asperity of the skin was recovered

According to this result, CUREBERRY is expected to have recovering effect of wrinkle.

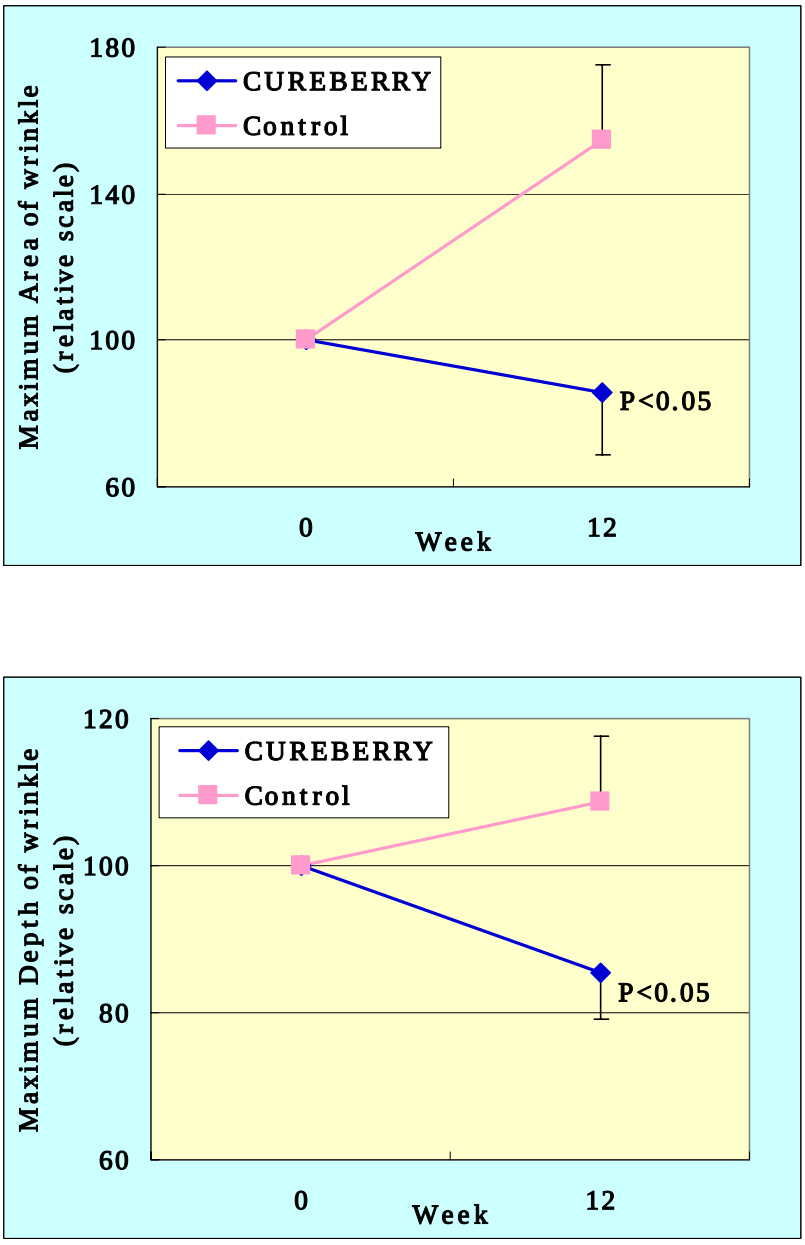


Fig. 11. Recovering of Wrinkle on the Human Skin

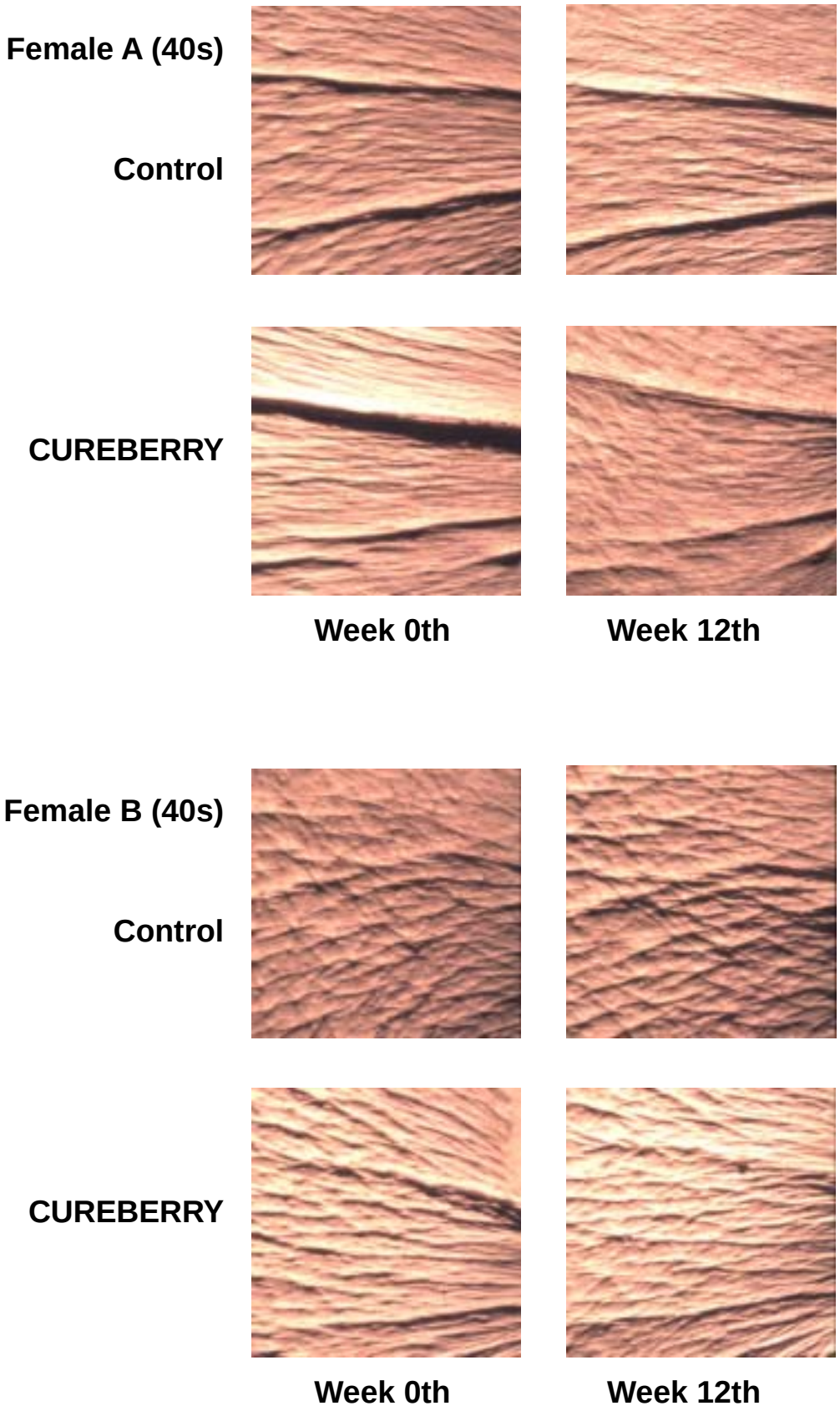


Fig. 12. Recovering of Wrinkle on the Human Skin

Whitening effect on the human skin

Test Sample

CUREBERRY was diluted by purified water to adjust 5 % as product concentration. As control solution, 50% of 1,3-Butylene Glycol solution was diluted in the same manner and used in the test.

Test Method

Eight health male and female volunteers at age 30s to 50s that gave us written informed consent were enrolled in this study, and the samples were applied around their eyes two times a day for 12 weeks. The study was conducted from August 1, 2006 to October 26, 2006.

Melanin index on the skin before the application and at week 12th by MEXAMETER MX18 (courage+Khazaka electronic GmbH), melanin index ratio, which regards the melanin index before application as 100, was determined

Result and Discussion

Whitening effect on each application was showed in Fig. 13. The melanin index relative scale on the area CUREBERRY applied was decreased; that is, whiteness of the skin tends to be increased.

According to this result, CUREBERRY is expected to have whitening effect.

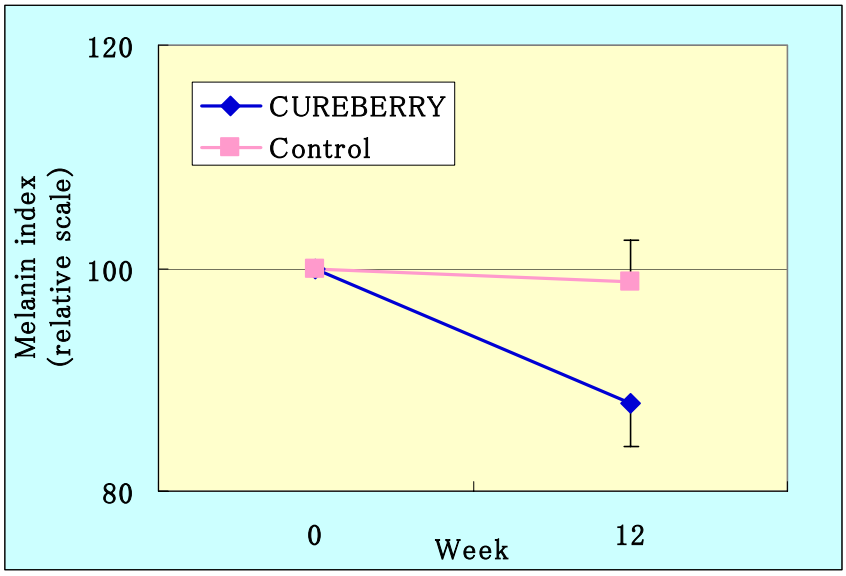


Fig. 13. Whitening effect on the human skin

Penetration into skin

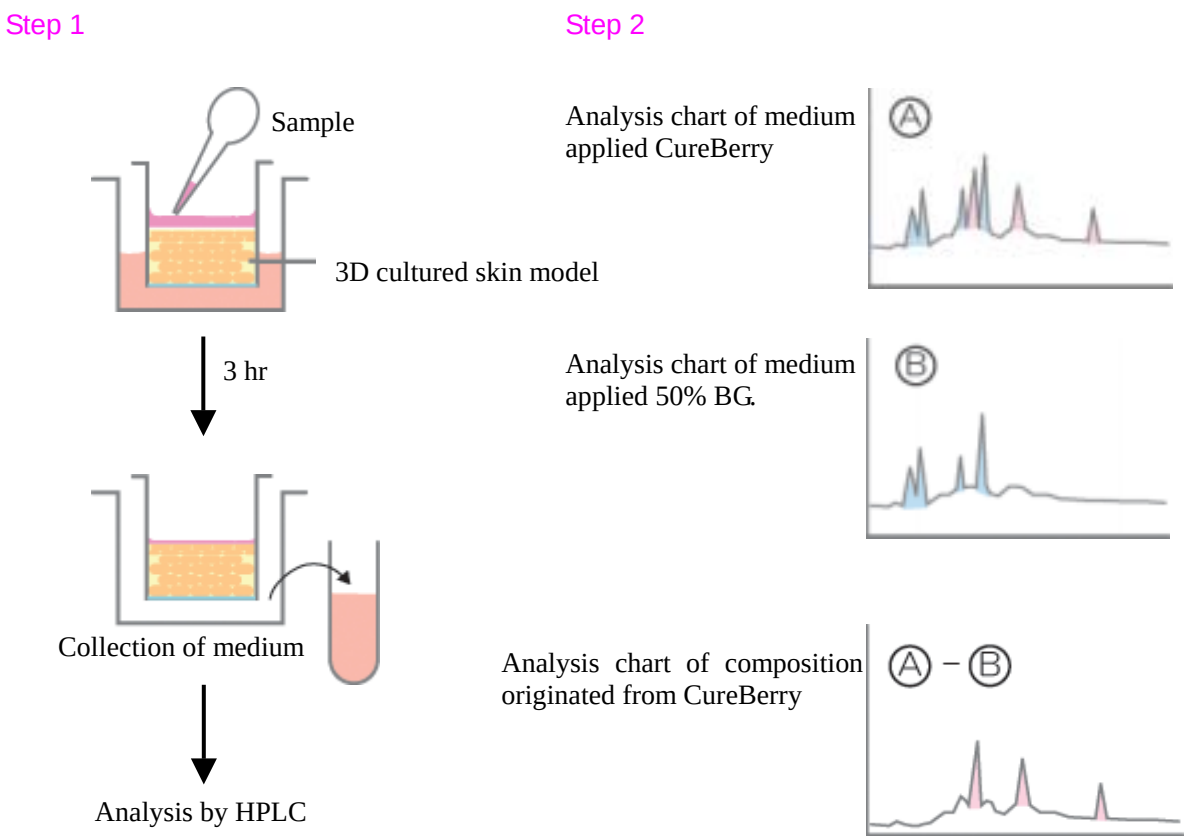
We investigate penetration into skin by using cultured 3D skin model.

Test Sample

Princess Care (0.3 mL) was placed on the surface of the three-dimensional cultured skin model (Kurabo), a human epidermal model. After 4-hr incubation, a lower layer of the culture medium equivalent to the inside of the skin was collectd to subject to HPLC analysis. As the reference, 50% BG solution was used.

Result and Discussion

According to the result of analysis of lower layer of the culture medium equivalent, peak; which is proper for CureBerry was observed and it is confirmed for CureBerry to penetrate into skin. According to this result, when CureBerry is applied in skin, CureBerry is expected to penetrate into epidermis and reach to basement membrane.



We can detect an ingredient of CureBerry origin by deducting peaks of butylene glycol from CureBerry additional group.

Abstract of penetration test method into skin

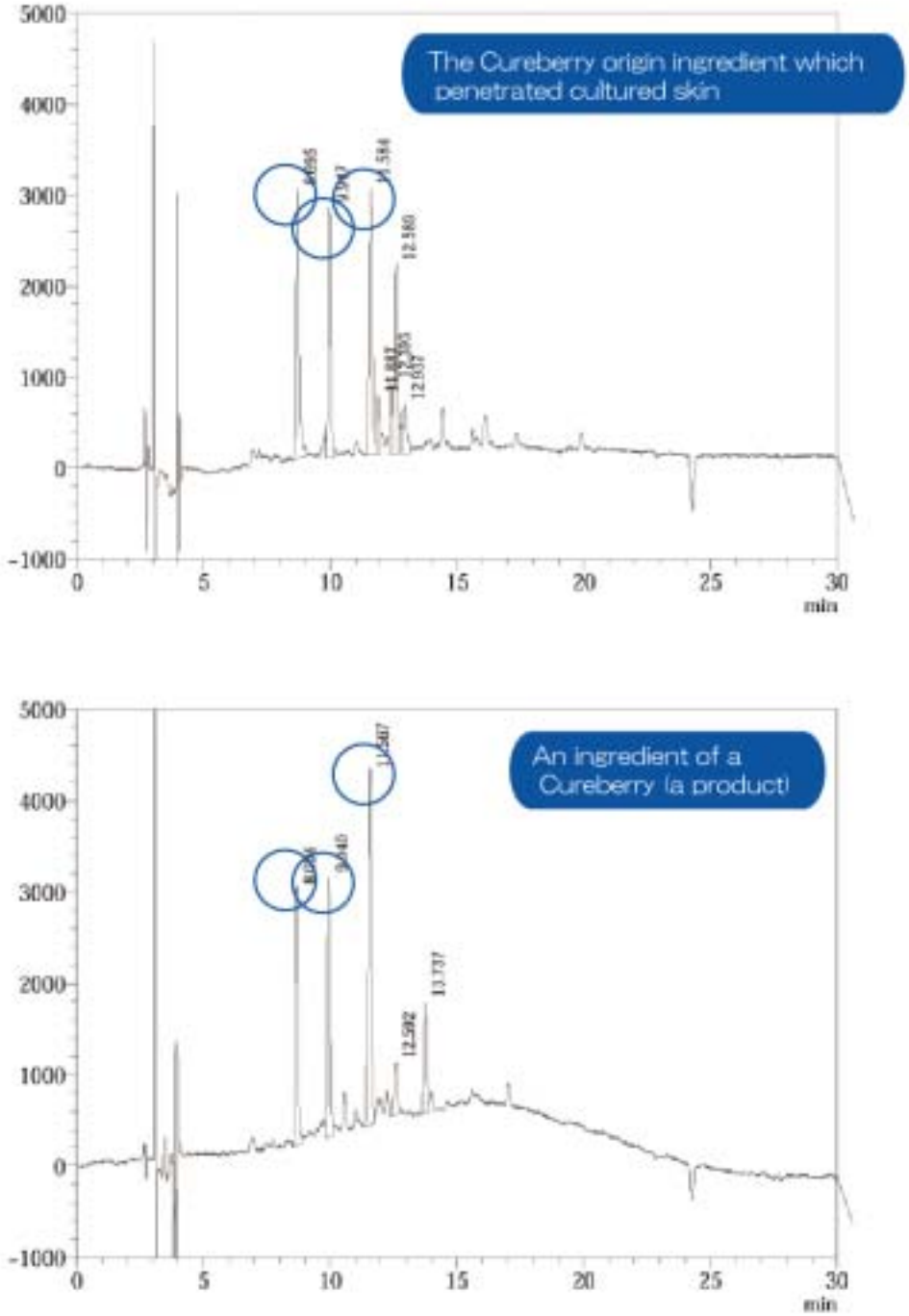
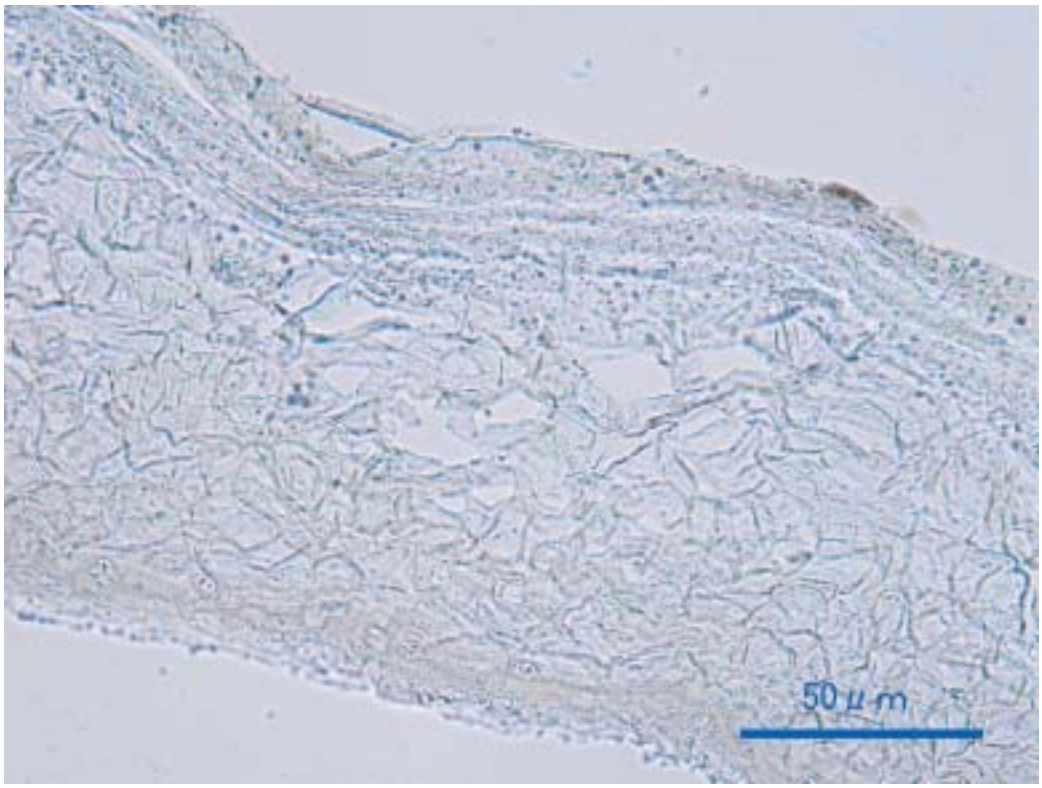
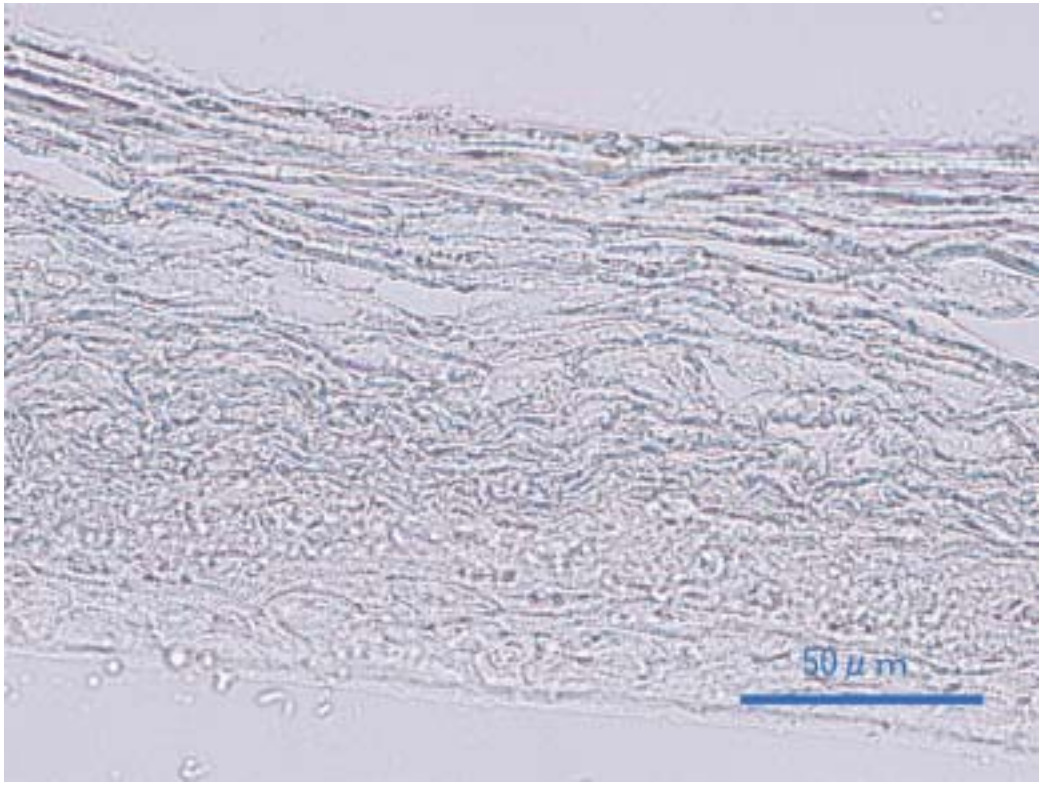


Fig. 14, The result of HPLC of CureBerry



Applied CureBerry



Applied Butylene Glycol

Stability Test

Stability of CUREBERRY was investigated.

1.Long term Stability

Store CUREBERRY in a cool dark place(4℃), room temperature, window side and at 50℃. Visual observation and absorbance value (1 → 10) at 470nm were determined.

Result and Discussion

Change of Absorbance value is shown in Fig. 15. The color turned dark at 50℃ with time, but color was almost not changed in a cool dark place.

According to result, CUREBERRY is recommended to store in cool dark place.

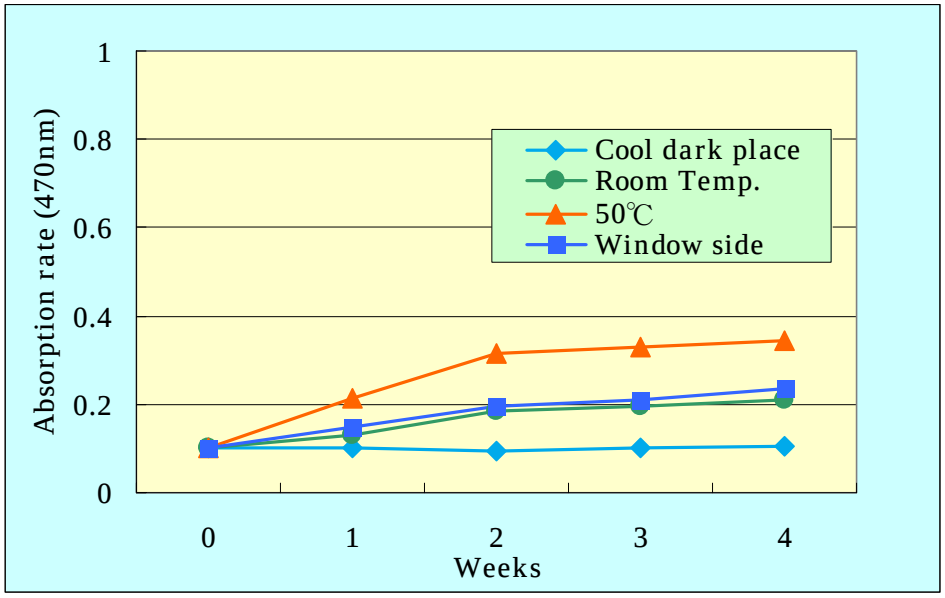


Fig.15 Long term stability

2.pH Stability

pH of CUREBERRY is adjusted from 2 to 10 by HCl and NaOH. Visual observation and absorbance value (1 → 10) at 470nm were determined.

Result and Discussion

Absorbance value of CUEBERRY is shown in Fig.16. pH 2 to 8, it was almost stable on color, but over pH8 the color was darkened. Precipitation was not observed at any pH range.

According to the result, CUEBERRY is not recommended to use under alkali conditions.

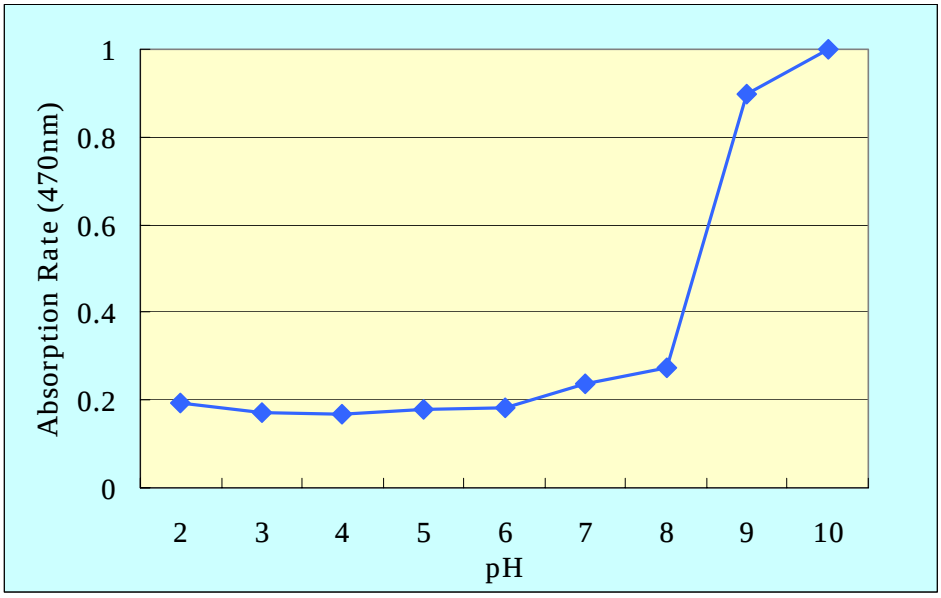


Fig.16 pH stability

3. Thermal stability

CUREBERRY was heated in a water bath at 90℃. After cooling down, it is diluted 10 times by purified water and absorbance value was measured at 470 nm.

Result and discussion

Thermal stability of CUREBERRY is shown in Fig. 17. The increase of absorbance of CUREBERRY and precipitate were not observed. According to the result, please take notice to avoid heating CUREBERRY for a long time.

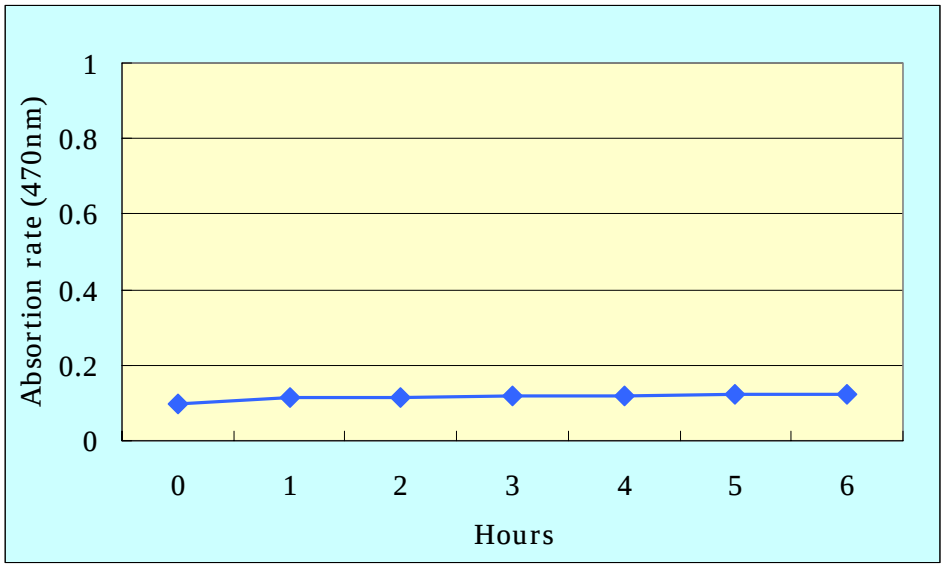


Fig.17, Thermal Stability

Compatibility Test

Compatibility of CUREBERRY with various ingredients was evaluated.

Test Method

CUREBERRY was diluted to 10 %, and the test samples were adjusted by purified water as shown on the each table. After 2 month, mixed solution was evaluated.

1.Compatibility of 10% of CUREBERRY with Surfactant

	%	Ingredients	Result
Cation	2.8	Stearyl Trimethyl Ammonium Chloride	○
	3.0	Cetyltrimehylammonium Chloride	○
	2.7	Lauryltrimethylammonium Chloride	○
Anion	10.0	Triethanolamine Lauryl Sulfate	○
	25.0	Sodium Laureth Sulfate	×
	25.0	Triethanolamine Laureth Sulfate	○
	6.25	Laureth-6 Carboxylic Acid	○
	10.0	Sodium N-Cocoyl-N-methyl Taurate	×
	10.0	Potassium N-Cocoyl Glycinate	○
	10.0	Sodium N-Cocoyl-L-glutamate	○
	7.5	Sodium Lauroyl Methylaminopropionate	×
	25.0	Sodium Tetradecenesulfonate	○
Nonion	10.0	Polyethylene Glycol (50) Oleyl Ether	○
	10.0	Coconut Tatty Acid Diethanolamide	○
	10.0	Sorbeth-60 Tetraoleate	○
	10.0	Polyoxyethylene Sorbitan Monooleate (20E.O.)	○
	10.0	Polyoxyethylene Hydrogenated Castor Oil (60E.O.)	○
Silicone	10.0	Polyoxyethylene・Methylpolysiloxane Copolymer	○
Ampholytic	3.5	Lauryl Dimethylaminoacetic Acid Betaine	×
	4.0	Sodium N-Cocoyl-N-Carboxymethyl-N-Hydroxyethyl Ethylenediamide	○
	2.9	Lauroyl Amide Propylhydroxysulfobetaine	○

○: Good, △: Slight Turbidity, ×: Precipitate

2.Compatibility of 10% of CUREBERRY with other ingredients

	%	Ingredients	Result
Solvent	50	Glycerin	○
	50	1,3-Butylene Glycol	○
	50	Propylene Glycol	○
	50	Isopropyl Alcohol	○
	50	Ethanol	○
Synthetic polymer	0.1	Carboxyvinyl polymer	○
	1	Polyvinylpyrrolidone	○
	1	Polyvinyl Alcohol	○
	1	Polyethylene glycol (6000)	○
Natural polymer	1	Sodium alginate	○
	1	Carboxymethyl cellulose	○
	1	Cationic cellulose	○
	1	Hydroxypropyl cellulose	○
Phospholipid	1	Lipidure-PMB	○
Vitamin-C derivative	2	Ascorbyl Glucoside	○
	2	Pacificos VAP	×

○: Good, △: Slight Turbidity, ×: Precipitate

3.Compatibility of 10% CUREBERRY with other our products.

%	Product name	INCI Name	Result
10	FM Extract LA-B	Lactobacillus / Milk Ferment Filtrate	×
10	OUGON Liquid SE	Scutellaria Baicalensis Root Extract	○
10	CHITIN Liquid (N)	Carboxymethyl Chitin	○
10	HPCH Liquid	Hydroxypropyl Chitosan	○
10	Clairju	Hydrolyzed Prunus Domestica	○
10	KOTHALAHIMBUTU Liquid B	Salacia Reticulata Wood Extract	○
10	SAKURA Extract B	Prunus Yedoensis Leaf Extract	○
10	MARINWORT IPC-14 SBW	Algae Extract	○
10	SILKGEN G Soluble	Hydrolyzed Silk	×
10	SILKGEN G Soluble-S	Hydrolyzed Silk	○
10	TREHALOSE 30	Trehalose	○
10	NEEM Leaf Liquid B	Melia Azadirachta Leaf Extract	○
10	Bio-PGA Solution HB	Polyglutamic Acid	○
10	Bio-PGA Solution LB	Polyglutamic Acid	○
10	PEACH Leaf Liquid B	Prunus Persica (Peach) Leaf Extract	○
10	Biocellact ALOE VERA B	Aloe Barbadensis Leaf Extract	○
10	Fermentage Chardonnary B	Lactobacillus/Grape Juice Ferment	○
10	Fermentage Pear B	Lactobacillus/Pyrus Communis (Pear) Fruit Juice Ferment	×
10	Pharconix CTP-F (BG)	Hydrolyzed Collagen	×
10	JIOU Liquid	Rehmannia Chinensis Root Extract	×
10	SOUHAKUHI Liquid (BG)	Morus Alba Root Extract	○
10	HIOUGI Liquid	Belamcanda Chinensis Root Extract	○
10	BOTANPI Liquid E	Paeonia Suffruticosa Root Extract	○
10	LEMONGRASS Liquid B	Cymbopogon Schoenanthus Extract	○
10	Phyto COLLAGE (N)	Natto Gum	○
10	Phyto HYALURON B	Hibiscus Esculentus Fruit Extract	○
10	FLAVOSTERONE SB	Glycine Soja (Soybean) Protein	○
10	YUZU Ceramide B	Citrus Junos Fruit Extract	○
10	LACTOSACCHARIDES B	Yogurt Filtrate	×
10	RYOKUCHA Liquid	Camellia Sinensis Leaf Extract	○
10	LUNAWHITE B	Oenothera Biennis (Evening Primrose) Seed Extract	○

○: Good, △: Slight Turbidity, ×: Precipitate

Specification

Subject	Specification
Appearance	Brown liquid, having a characteristic odor.
Identification Tannin Sugar	 Positive Positive
Purity Heavy metals Arsenic	 20 ppm max. 2 ppm max.
Residue on Evaporation	0.1 to 0.5 w/v%
INCI Name	Water Butylene Glycol Vaccinium Myrtillus Leaf Extract
CAS Number	84082-34-8
EINECS Number	281-983-5

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