NEEM Leaf Liquid B

(Melia Azadirachta Leaf Extract)

ICHIMARU PHARCOS CO., LTD.

318-1 Asagi, Motosu-shi, Gifu 501-0475 JAPAN

Phone : (81) 58 320－1032

Fax : (81) 58 320－1039

http://www.ichimaru.co.jp

Published on September 15, 2004

Revised on December 21, 2005

CONTENTS

Introduction

Neem has been used cosmetically and medicinally for thousands of years and is considered to be an equal to, or even superior to aloe in its healing properties. The Neem tree (Melia azadirachta) has been revered as the "village pharmacy" in India and researchers are saying that Neem could be called "a wonder tree" as every part of this sacred tree is used in some form on a daily basis; the twigs as a toothbrush, the oil for soap, and the leaves to maintain beautiful and healthy skin.

The Ayurvedic literature is full of references of Neem and it is one of the most powerful blood purifiers and detoxifiers in ayurvedic usage. Traditionally Neem has been used to heal heat rash, boils, wounds, jaundice, leprosy, diabetis, skin disorders, stomach ulcers, hay-fever, chicken pox and as a cosmetic to remove skin blemishes.

Neem trees have many unique compounds that have been identified and others that have not yet been identified. The most analyzed compounds are nimbin, nimbidin, nimbidol, gedunin, queceretin, salanin and azadirachtin. (The leaves contain nimbin, nimbinine, nimbandiol, nimbolide and quercetin)

The sanskrit name *nimba* means "to give good health" and ancient religious texts referred to Neem as *sarva roga nivarini* (the curer of all ailments).

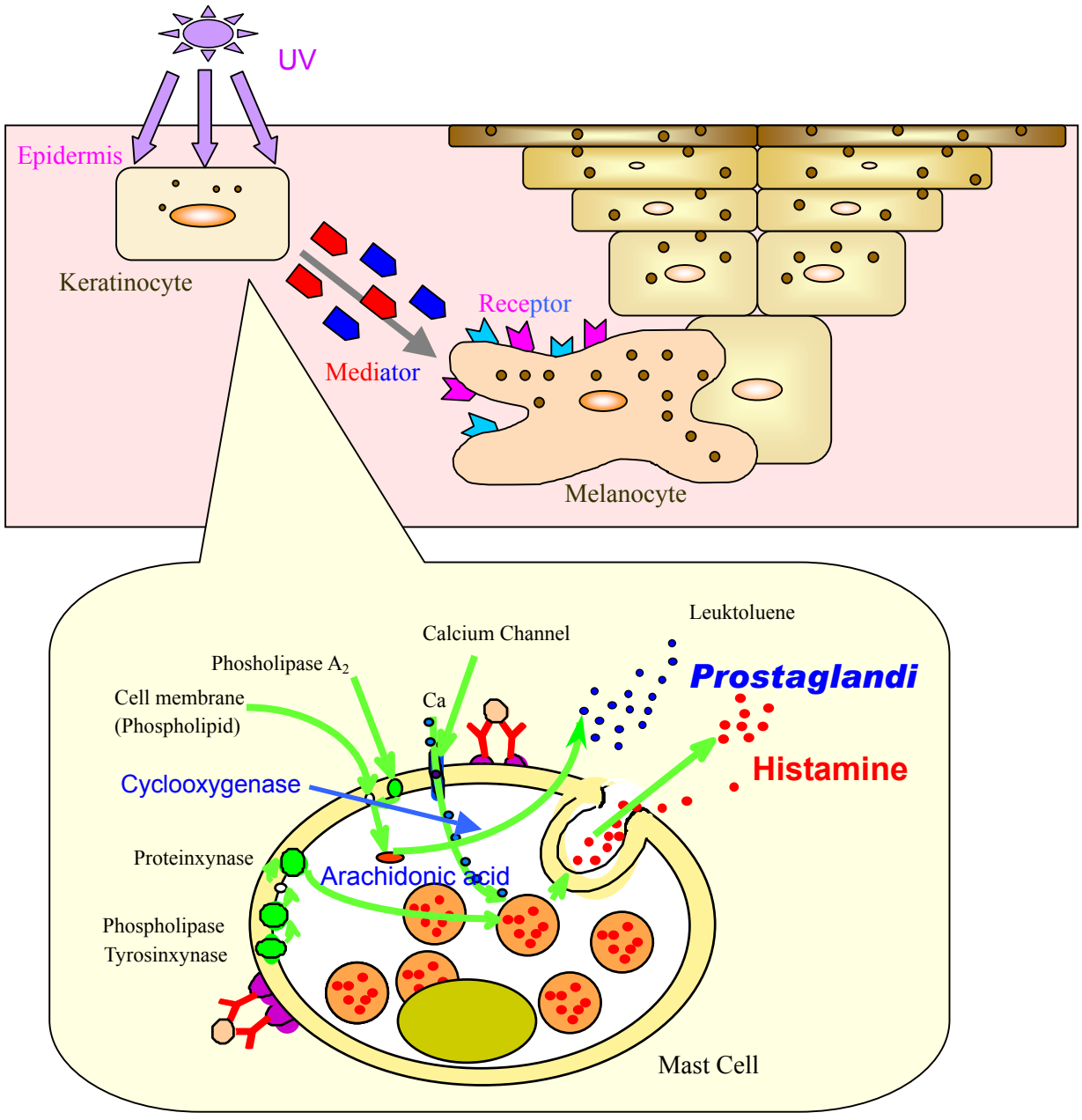
Native to India and Burma, it grows in much of South-east Asia and west Africa.

Efficacy

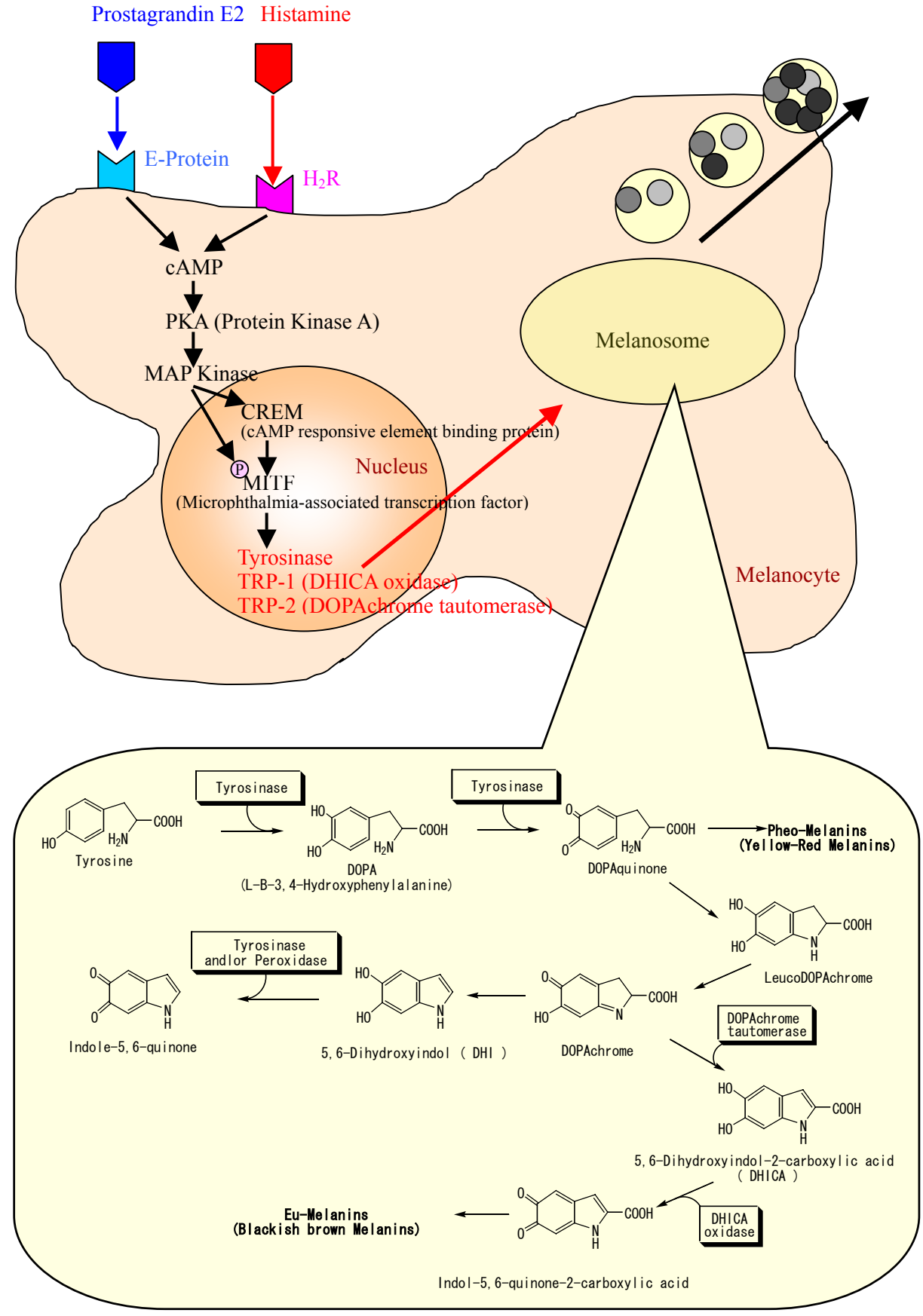
Whitening

- *B16 Melanoma cell Inhibition Effect*
- *Inhibition Effect of Melanin at 3D Skin Model*
- *Inhibition Effect of Histamine Release*
- *Inhibition Effect of Cyclooxygenase Activity*
  (Inhibition Effect of Prostagrandin Production)

Whitening Mechanism



Process of Melanin Production in Melanocyte



The melanocytes are dendritic cells, and normal melanocytes produce transparent melanosomes.

The melanosomes contain an amino acid, tyrosine and an enzyme, tyrosinase.

Tyrosinase plays a catalytic role in the conversion of tyrosine to premelanins such as dopa and dopaquinone by the oxidative reaction and the oxidative reaction is repeated to produce melanin granules.

The exposure of the skin to the strong ultraviolet rays produces inflammation, and at this time, a transmitter with the message, "The skin was exposed to ultraviolet rays," is released from keratinocytes. The transmitter triggers melanin synthesis at the melanocytes in the basal layer of the epidermis. Tyrosinase is activated in the melanocytes receiving the message of the transmitter to produce a large amount of melanin, which is a principal causative factor of chloasma and freckles.

It has been said that prostaglandins, histamine etc. are the transmitters for the activation of tyrosinase.

Prostaglandins

Prostaglandins are the transmitters for activating the melanocytes by exposure to ultraviolet B rays. Prostaglandins, metabolites of arachidonic acid, have melanocyte-stimulating actions such as an increase in the number of dendrite with the enlargement of the cell size as well as an increase in the activity of tyrosinase in the cultured melanocytes. The continuous application of prostaglandins and arachidonic acid, a precursor of prostaglandins can produce pigmentation *in vitro,* and the external application of indomethacin (a representative drug that inhibits the production of prostaglandins from arachidonic acid) inhibits pigmentation in the skin by sunburn. These facts suggest that the metabolites of arachidonic acid such as prostaglandins that have been confirmed to be increased in the skin by exposure to ultraviolet B rays are probably one of the key factors for the enhancement of pigmentation by exposure to ultraviolet B rays.

Thus the inhibition of the production of the metabolites of arachidonic acid such as prostaglandins is considered one of the most effective ways to prevent the occurrence of chloasma and freckles due to sunburn.

The production of prostaglandins is catalyzed by cyclooxygenase (COX). There are two types of COX, COX-1 and COX-2. COX-1 is produced to such extent that homeostasis in the body is maintained, while COX-2 is excessively produced at inflammation.

Histamine

Histamine is a substance that plays a role in the excessive production of melanin by stimulating the melanocytes. It is considered important to inhibit the release of histamine and not to stimulate the melanocytes for the maintenance of beauty whitening of the skin. The following were also elucidated: (1) the number of melanocytes that was considered almost constant in the whole area of the skin is increased at the site of chloasma, (2) the number of mast cells that release histamine is increased at the dermis and (3) histamine increases the number of melanocytes themselves as well as stimulates melanin synthesis. Furthermore, it has been reported that (4) histamine activates the melanocytes by binding to a receptor on the melanocytes.

It is considered that the inhibition of the release of histamine from the mast cells and blocking of binding of histamine to the receptor on the melanocytes are effective ways to increase beauty whitening of skin.

NEEM Leaf Liquid B is expected whitening effect because of inhibiting these transmitters.

B16 Melanoma cell Inhibition Effect

We investigated melanin production inhibition test on B16 melanoma cells of NEEM Leaf Liquid B.

Test Method [1)]

a. Cells and the culture condition

B16 mouse melanoma cells were used and were cultured in an MEM culture medium including 5% Fetal Bovine Serum (made by GIBCO BRL). The culture conditions were 37 ° C, 5% CO_2.

b. Test Sample

NEEM Leaf Liquid B is adjusted 50 ppm as final concentration. Arbutin is used as positive control.

c. Measurement

Plant B16 melanoma cell, 2 x 10^5 /60mm culture dish
↓
Culture at 5% FBS.MEM medium culture at 37 ° C for 24 hours
↓
Transfer to a fresh culture medium, add to each test sample (finally 50 ppm concentration to culture medium
↓
After culturing for 24 hours, remove the culture medium, process with trypsin
↓
Dissolve with 1N NaOH, 10% DMSO solution with heat
↓
Determine the absorbance at 420nm

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 50 ppm concentration to culture medium. The cells were collected by processing with trypsin after culturing for three days. After the cell were removed non-melanin matter by 5% TCA, Ether/Ethanol mixture and Ether Dehydrated, they were dissolved in 1N NaOH and 10% DMSO and then absorbance was measured at 420nm. At the same time the activity of the cells which were supplemented with various materials was measured by the MTT reduction method.

Results

Amount of melanin in medium added NEEM Leaf Liquid B and MTT activity are shown in Fig. 1 and 2. As MTT activity is relative to the number of cells, melanin production rate; which melanin amount is compensated by MT activity shown in Fig. 3.

According to results, NEM Leaf Liquid has a strong inhibition effect of melanin production.

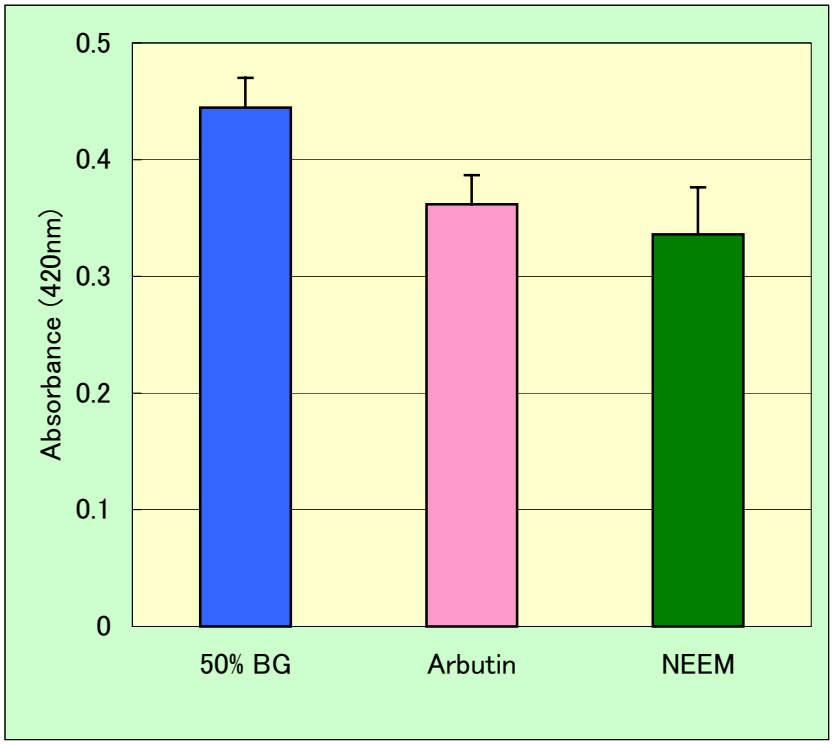


Fig. 1, Amount of Melanin

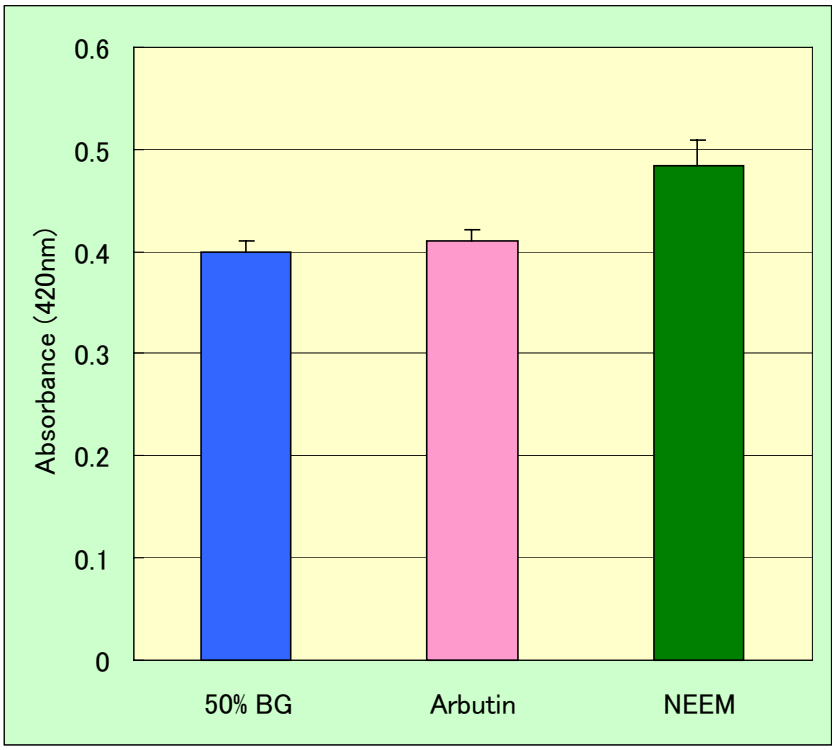


Fig. 2, MTT Activity

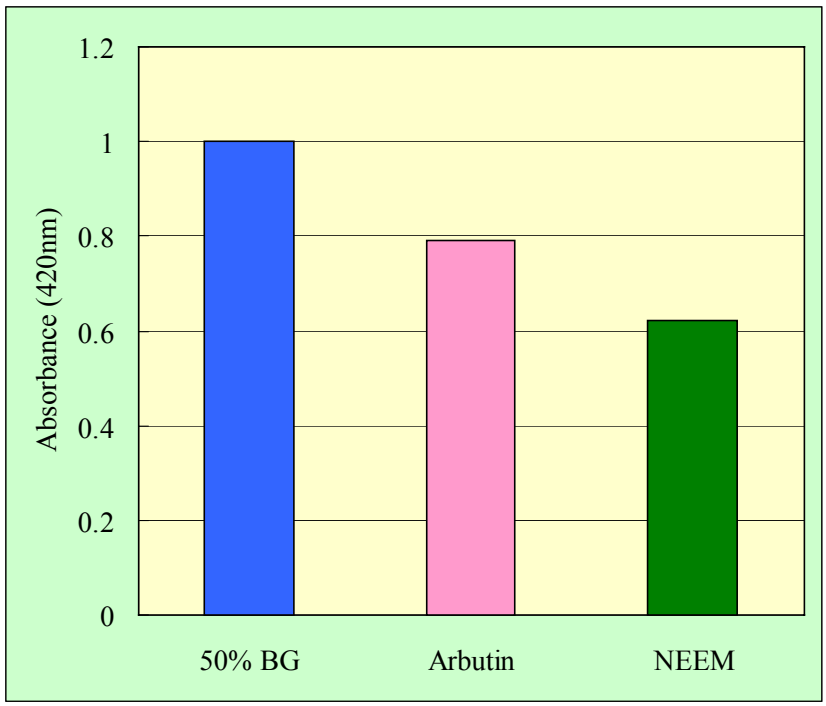


Fig. 3, Rate of Melanin Production

Inhibition Effect of Melanin Production at 3D Skin Model

We investigated inhibition effect of Melanin by using 3D skin models, which contain melanocyte.

Test Sample

NEEM Leaf Liquid B was adjusted 0.5% as final solid concentration by 50% 1,3-Buthylene Glycol solution. Same concentration of Arbutin was used as positive control.

Test Method

Using 3D skin model containing melanocyte (MEL-300B kit, Kurabo industry). Apply plate-insert containing medium into 6 well-plate, using EPI-100 LLMM of medium of 3D skin kit, cultivate at 5% CO_2 and 37℃. After washing both inner and outer by PBS (Phosphate Buffered Saline), put into 8 well-plate; which is for exposing, expose 30mJ by UV-B lump (FL-20SE, TOSHIBA). After exposing UV-B, each 50 μ L of sample is applied on horny layer. Exposing UV and applying sample took place every other day for 14 days.

After cultivating, the number of cells was measured by MTT method. After washing tissue by PBS, cut and homogenized. Homogenate of the tissue was centrifugalized at 2,000 rpm, dissolve its precipitate by 1mol/L NaOH and 10% DMS solution, and measure melanin by 420nm.

Results and discussion

Amount of melanin determined from each models was showed in Fig.4. The photograph taken from the side of horny cell layer at the 3D skin model was shown inFig.5. NEEM Leaf Liquid B is observed to have inhibition of Melanin almost equals to Arbutin. According to this result, Neem Leaf Liquid B is expected to have strong whiting effect.

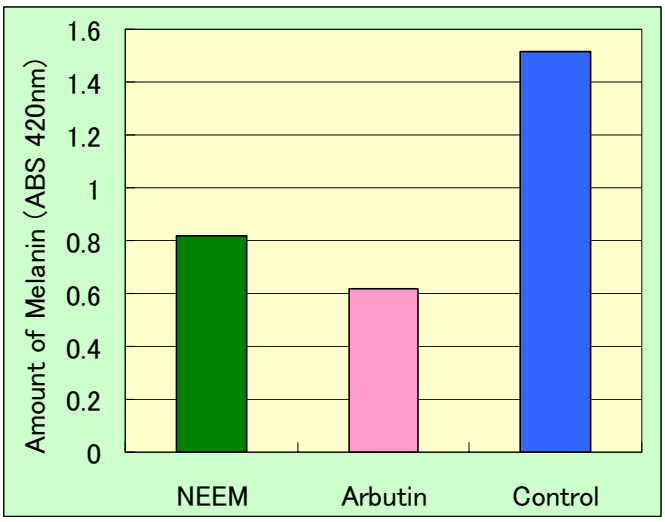


Fig.4, Amount of Melanin at 3D Skin Model

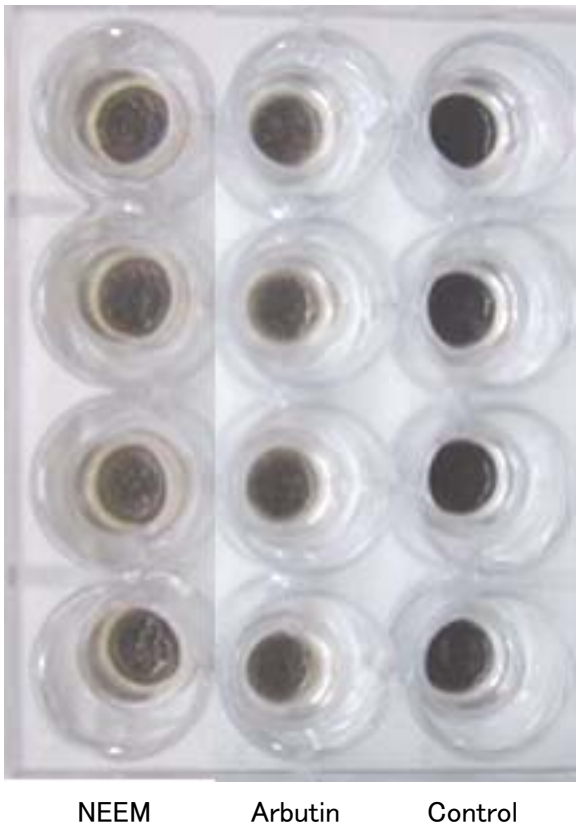


Fig. 5, 3D Skin Model at 14th day

Inhibition Effect of Histamine Release

We investigated inhibition effect of histamine release of NEEM Leaf Liquid B.

Test Sample

NEEM Leaf Liquid is adjusted to 0.1 and 0.05% solid matter by purified water. (Final concentration in test process : 137 and 67.5 μ g/ml.)

Test Method [2) 3)]

Rats were exsanguinated to death under ether anesthesia and the abdominal skin was resected to make a small hole at the median abdominal wall. Peritoneal exudates cells suspension was collected by injecting about 15 mL of Tyrode solution (2% FT solution) containing 2% FCS (fetal calf serum, GIBCO) into the peritoneal cavity and by slightly massaging the abdominal wall.

The peritoneal exudates cells suspension thus obtained was centrifuged at 500 rpm for 5 min at 4°C to obtain the sediment cells. The sediment cells were slightly stirred in 2 mL of 2% FT solution to obtain the cell suspension, which was further centrifuged at 500 rpm for 5 min at 4°C. This operation was repeated twice. Mast cells thus obtained were suspended in 2% FT solution to make about 1.0×10^5 cells/mL. After adding 25 μL of a test substance to 120 μL of the cell suspension and keeping at stand for 10 min at 37°C, 40 μL of histamine-releasing agent compound 48/80 (Sigma) (final concentration: 1 μg/mL) prewarmed at 37°C was added to the reaction mixture. After keeping 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 according to Shore's method. Briefly, 100 μL of purified water, 50 μL of 1N NaOH solution and 25 μL of 1% o-phthaldialdehyde-methanol solution were added to 25 μL of the supernatant. After keeping at stand for 5 min, the reaction was stopped by adding 25 μL of 3 N 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 fluorescence wavelength. Furthermore, histamine remained in mast cells was determined by the same way as that described above in the ultrasonically treated sediment in 125 μL of 2% FT solution after 1-day storage at frozen. 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 mast cell.

Result

Inhibition rate of histamine release of 0.1% and 0.05% NEEM Leaf Liquid B is observed strong inhibition effect as 69.7% and 31.6%. As a reference, inhibition rate of histamine release of 200 μg/mL (Final concentration in test) sodium cromoglycate; which is known as inhibition agent of degranulation is 9.9%. (This was not performed in this test. This is only reference.)

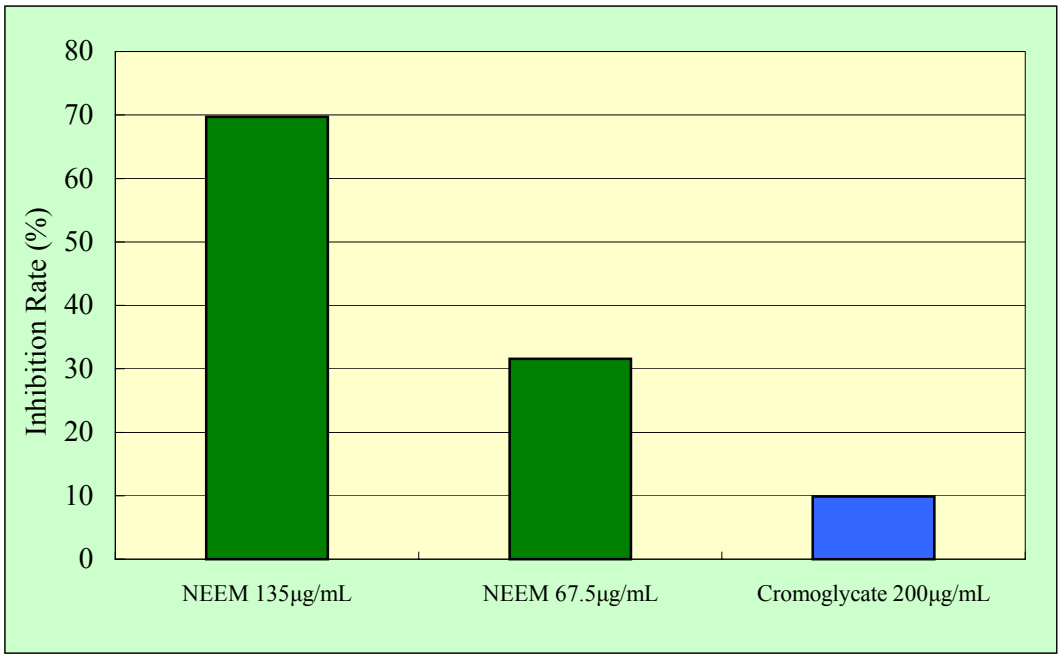


Fig. 6, Inhibition rate of Histamine Release

Inhibition Effect of Contact Dermatitis

The problem is, cosmetics can, when in direct contact with skin, caused contact dermatitis. We investigated how much NEEM Leaf Liquid B is able to inhibit contact dermatitis caused by P-1,4-phenylendiamine.

Test Sample

Hydrophilic Vaseline containing 10% of NEEM Leaf Liquid B was adjusted to use for the test, and the control sample was adjusted using same amount of purified water instead of NEEM Leaf Liquid B.

Test Method [4)]

Abdominal hair of female mice(BALB/c, 8 weeks) were shaved. In order to sensitized, 2.5% P-1,4-phenylendiamine /acetone : olive oil = 4 : 1 (PPD) applied every day for three days at the abdominal area of the mice. 5 days later, test sample applied three times on one side of ear. After 1 hour it applied last time, test sample was wiped enough off and PPD was applied, and 17 hours later test sample was applied two times again. 24 hours later both ears were cut off by punch of a certain amount of area, and they were measured. Regarding the judgment, measure the different weight between the ears applied with test sample and the other ear non-applied, and calculated the inhibition rate by means of comparing with control. 8 mice were used for each group.

$$\text{Swelling Rate (\%)} = \frac{\text{Weight of ears applied with test sample} - \text{Weight of ears non-applied}}{\text{Weight of ears non-applied}} \times 100$$

Result

Swelling rate of contact dermatitis of NEEM Leaf Liquid B and control were shown Fig. 6. NEEM Leaf Liquid B inhibited swelling by sensitization of PPD solution ($p<0.05$). According to this result, it is expected that NEEM Leaf Liquid B might inhibit contact dermatitis.

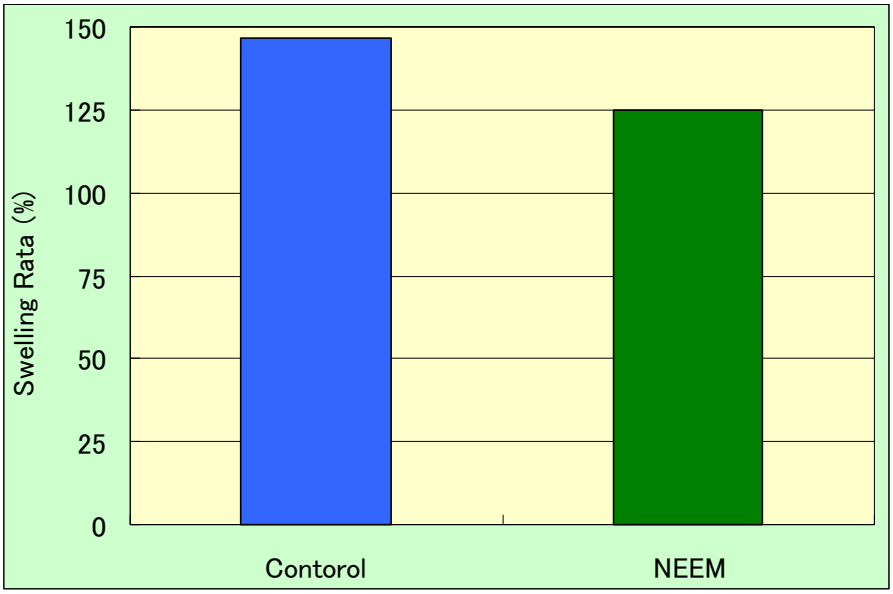


Fig. 6 Inhibition Effect of contact dermatitis of NEEM Leaf Liquid B

Inhibition Effect of Cyclooxygenase Activity

Prostaglandins produced at inflammation in a living body are known as cytokines and induce melanin synthesis etc. Prostaglandins are synthesized from arachidonic acid by the action of cyclooxygenase (COX). The compounds with inhibitory action on COX are considered to be useful for antiinflammation and for beauty whitening of the skin. It is well known that there are two kinds of COX: COX1 and COX2, and it has been said that the inhibition of COX2 activity induced at inflammation is desirable.

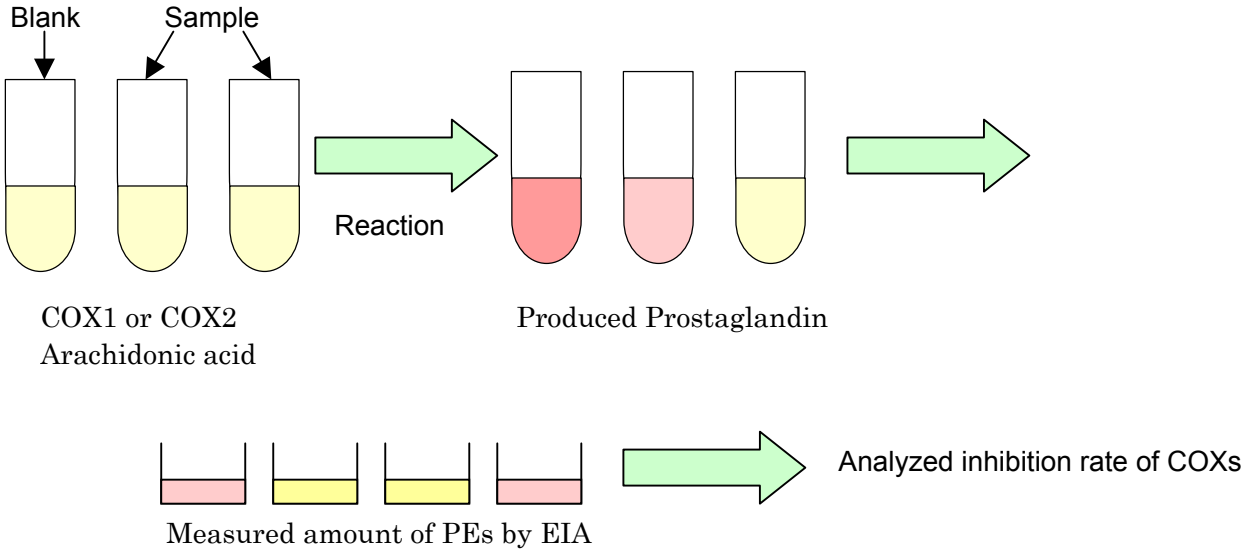
Test sample

A sample was prepared by diluting NEEM Leaf Liquid in 50% BG to make the final concentration 0.1 to 5%.

Method

The inhibitory activity of the sample in the reaction with COX was determined by using COX Inhibitor Screening Assay (Cayman Chemical Inc. catalog No. 560131).

The reaction of prostaglandin production was performed by adding arachidonic acid (substrate) to COX (enzyme) solution. At this time, the inhibitory effect on COX activity was examined by determining prostaglandin produced at the addition of each sample according to the testing method. The quantity of prostaglandin produced by the COX reaction was determined by an EIA method to calculate the inhibitory rate of the prostaglandin production.



Results and Discussion

The result of the inhibitory rate is shown below, which indicates that NEEM Leaf Liquid has an inhibitory effect on COX activity. The strong inhibitory effect was observed in the reaction with COX2, while almost no inhibitory effect was shown in the reaction with COX1. Thus it can be expected that NEEM Leaf Liquid inhibits COX2 when activity induced at inflammation specifically.

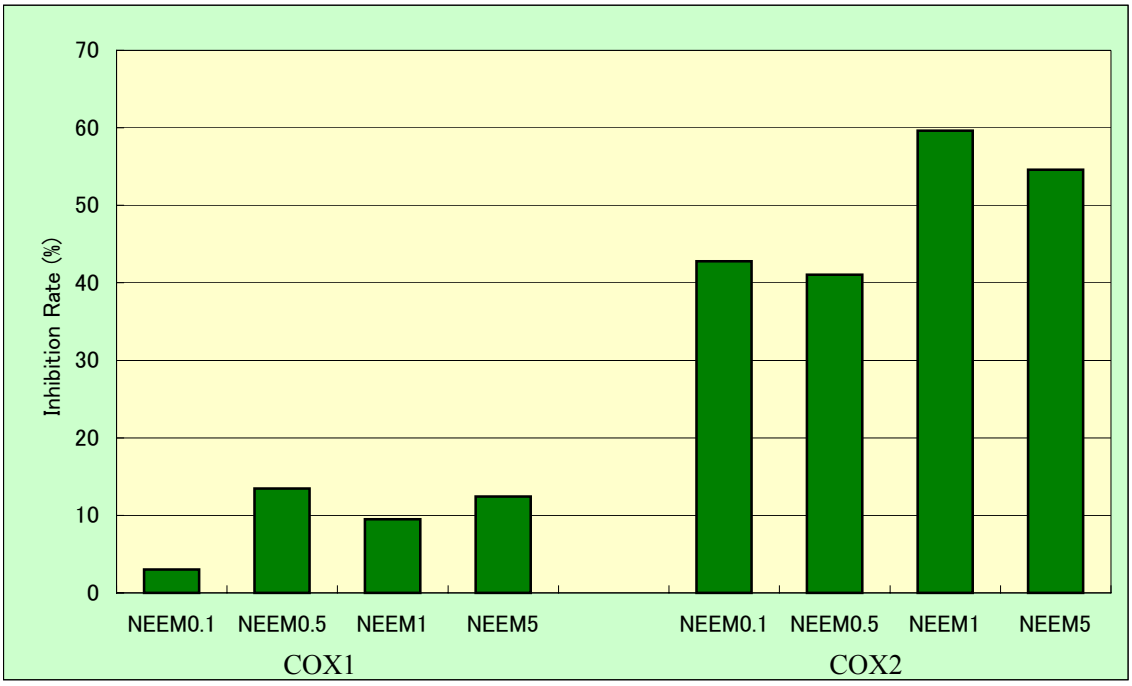


Fig. 8, Inhibition Effect of Cyclooxygenase Activity

Stability

1.Long Term stability

Sample is placed at 4℃, room temperature, 50℃, and window side, and then ABS and pH were measured.

Result and discussion

Change of Absorbance value is shown in Fig. 8. The tone color of NEEM Leaf Liquid B changed in dark place except preserving in a cold place. Color loss was observed at window side after 2 months. Precipitate was observed at 50℃ and window side, and a small amount of precipitate was observed at room temperature. According to this result, NEEM Leaf Liquid B is preserved in a place preventing from high temperature and window side.

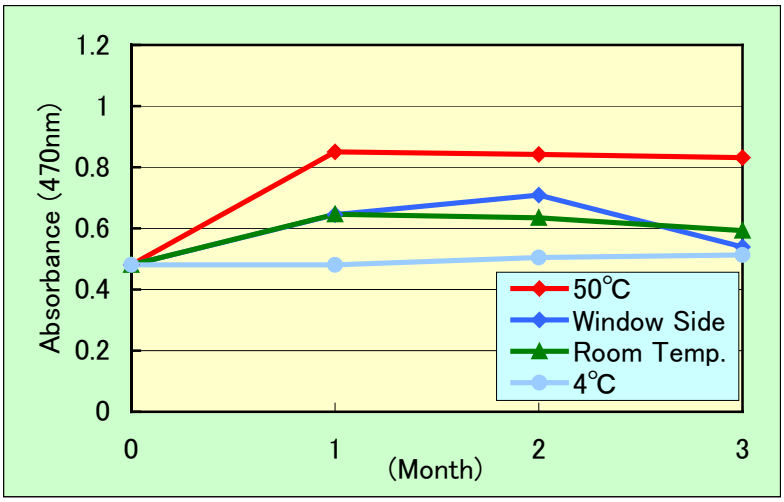


Fig. 8, Long Term stability (ABS)

Change of pH is shown in Fig. 9. pH did not virtually change at any condition.

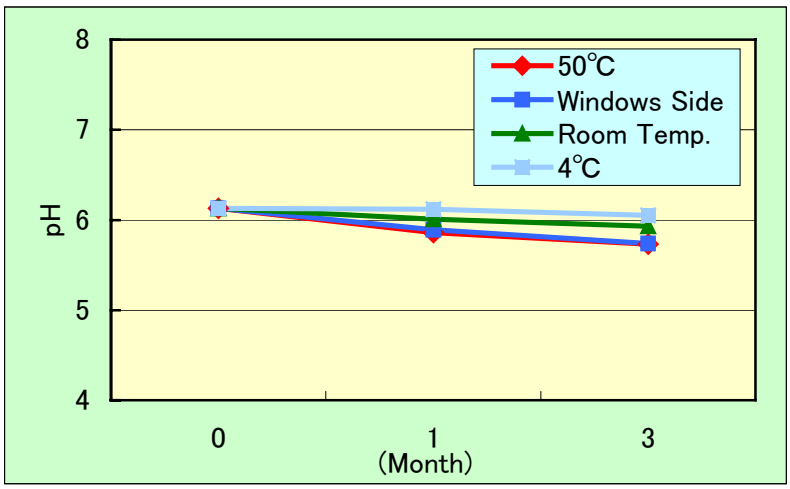


Fig. 9, Long Term stability (pH)

2.pH Stability

pH of Neem Leaf Liquid B is adjusted from 2 to 12 by 1 mol/L HCl and 2mol/L NaOH, and ABS is measured.

Result and discussion

Absorbance value of Neem Leaf Liquid B is shown in Fig.10. Absorbance value is suddenly up at more than pH 10, and precipitate was observed more than pH 8.

According to this result, it is necessary to take notice over pH 8.

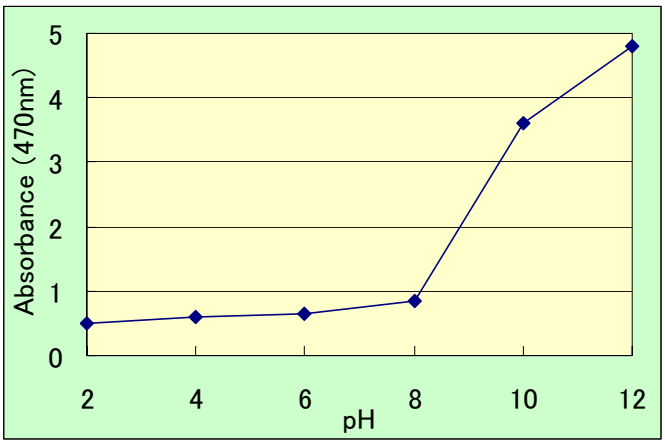


Fig. 10, pH stability

3.Thermal stability

After sample was heated in a water bath at 80℃ for 1 to 10 hours, Absorbance value was measured at 470 nm.

Result and discussion

Change of Absorbance value is shown in Fig. 11.

Although absorbance value was increased, precipitate and color tone change was not almost observed. According to this result, NEEM Leaf Liquid B is expected to be stable against heating.

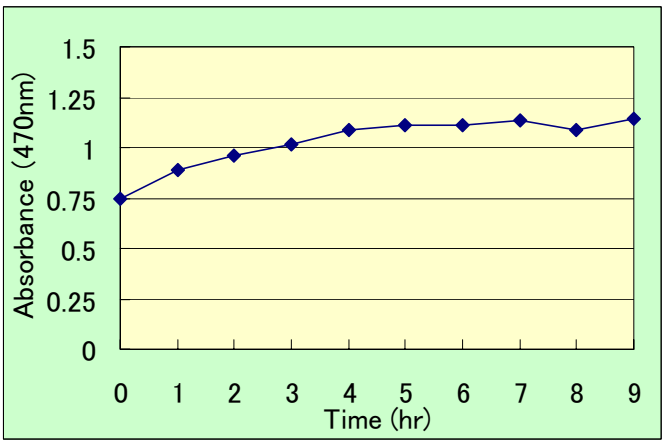


Fig.11, Thermal stability

Compatibility

1.Compatibility of 10% NEEM Leaf Liquid B with Surfactant

	%	Ingredients	10%	1%
Cation	2.8	Stearyl Trimethyl Ammonium Chloride	×	○
	3.0	Cetrimonium Chloride	○	N.P.
	2.7	Lauryltrimonium Chloride	○	N.P.
Anion	10	TEA-Lauryl Sulfate	○	N.P.
	25	Sodium Laureth Sulfate	○	N.P.
	25	TEA-Laureth Sulfate	○	N.P.
	6.25	Sodium Laureth-3 Carboxylate	○	N.P.
	6.75	Sodium Methyl Cocoyl Taurate	○	N.P.
	10	Potassium Cocoyl Glycinate	○	N.P.
	7.5	Sodium Lauroyl Methylaminopropionate	○	N.P.
Nonion	10	Oleth-50	○	N.P.
	10	Cocamide DEA		N.P.
	10	Sorbeth-60 Tetraoleate	○	N.P.
	10	Polysorbate 80	○	N.P.
Silicone	10	Dimethicone Copolyor	○	N.P.
Ampholytic	3.5	Lauryl Betaine	○	N.P.
	4.0	Sodium Cocoamphopropionate	○	N.P.
	2.9	Lauramidopropyl Hydroxysultaine (E)	○	N.P.

○: Good, △: Slight Turbidity, ×: Precipitate, N.P. : Not Performed

2.Compatibility of 10% NEEM Leaf Liquid B with other ingredients

%	Ingredients	10%	1%
0.1	Carboxyvinyl polymer	△	○
1	Carboxyvinyl alcohol	×	○
1	Polyethylene glycol (6000)	○	N.P.
1	Cationic cellulose	○	N.P.
1	Carboxymethyl cellulose	○	N.P.
1	Hydroxypropyl cellulose	○	N.P.
1	Sodium alginate	×	○
50	Glycerin	○	N.P.
50	Propylene Glycol	○	N.P.
50	Ethanol	○	N.P.
20	1,3-Butylene Glycol	×	○
50	Isopropyl Alcohol	×	○
2	Magnesium ascorbyl-2-phosphate	○	N.P.
2	Ascorbyl Glucoside	×	○

○: Good, △: Slight Turbidity, ×: Precipitate, N.P. : Not Performed

3.Compatibility of 10% NEEM Leaf Liquid B with other our products.

%	Product name	INCI Name	10%	1%
0.1	Bio Sodium Hyaluronate	Sodium Hyaluronate	○	N.P.
0.5	LEXSOD-P	Tannic Acid	○	N.P.
10	YEAST Liquid ZB	Yeast Extrcat	○	N.P.
10	Phyto COLLAGE (N)	Natto Gum	○	N.P.
10	SUPER Hair Coat	Hydroxypropyl Chitosan	○	N.P.
10	AQUACRUSTAR	Hydroxyethyl Chitosan	○	N.P.
10	Bio antiage B	Pueraria Lobata Root Extract Chlorella Vulgaris Extract Aloe Barbadensis Leaf Extract	○	N.P.
10	MARINWORT IPC-14 SBW	Algae Extract	○	N.P.
10	LUNAWHITE-B	Oenothera Biennis (Evening Primrose) Seed Extract	○	N.P.
10	MARRINNIER Liquid B	Aesculus Hippocastanum (Horse Chestnut) Seed Extract	○	N.P.
10	KOUBOKU Liquid B	Magnolia Obovata Bark Extract	○	N.P.
10	FM Extract LA-B	Lactobacillus / Milk Ferment Filtrate	×	○
10	Bio-PGA Solution HB	Polyglutamic Acid	×	○
10	Bio-PGA Solution LB	Polyglutamic Acid	×	○
10	TREHALOSE 30	Trehalose	×	○
10	SILKGEN G Soluble	Hydrolyzed Silk	×	○
10	SILKGEN G Soluble-S	Hydrolyzed Silk	×	○
10	Pharconix CTP-F (BG)	Hydrolyzed Collagen	×	○
10	FLAVOSTERONE SB	Glycine Soja (Soybean) Protein	×	○
10	Phyto HYALURON B	Hibiscus Esculentus Fruit Extract	×	○
10	Biocellact ALOE VERA B	Aloe Barbadensis Leaf Extract	×	○
10	ALPROTECTOR	Paeonia Suffruticosa Root Extract Tilia Cordata Flower Extract Althaea Officinalis Root Extract Arnica Montana Flower Extract	×	○
10	HIOUGI Liquid	Belamcanda Chinensis Root Extract	×	○
10	BOTANPI Liquid E	Paeonia Suffruticosa Root Extract	×	○
10	ROOIBOS Liquid B(N)	Aspalathus Linearis Extract	×	○
10	LEMONGRASS Liquid B	Cymbopogon Schoenanthus Extract	×	○

○: Good, △: Slight Turbidity, ×: Precipitate, N.P. : Not Performed

Specification

Subject	Specification
Appearance	Yellowish brown to brown liquid, having specific odor
Identification Tannin	 Positive
Purity Heavy metals Arsenic	 20 ppm max. 2 ppm max.
Residue on Evaporation	0.5 to 1.5 w/v%
INCI Name	Water Butylene Glycol Melia Azadirachta Leaf Extract
CAS Number	84696-25-3
EINECS Number	283-644-7

Reference

1) Satoru AKIU, *Journal of Japanese Cosmetic Science Society,* 15, (3) (1991)

2) Toshihiko TSUJI et al,*SCCJ.*, 2) 1992

3) Michinori KUBO et al, *YAKUGAKU ZASSHI*, 110, (1) 1990

4) Ken WATANABE et al, *Japanese Journal of Dermatology* 105, 440 (1995)