# BIOLOGICAL ACTIVITES OF PLANT LEAF EXTRACTS; AVAILABILITY OF STAR FRUIT LEAF EXTRACT AGAINST SKIN AGING

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## Summary

We evaluated activities of various plant leaf extracts and found the availability against skin aging in the leaf extract of star fruit (*Averrhoa carambola* L), and developed Star Fruit Leaf Extract BG30 as an ingredient of cosmetics. Star Fruit Leaf Extract BG30 was found to show scavenging activities of reactive oxygen species and an inhibitory effect on the activity of matrix metalloproteinase-1. It showed increasing activity of type I collagen and recovery effect from damage of UV-B irradiation in human fibroblast. We performed the separation of the active principal from Star Fruit Leaf Extract BG30 to give isofurcatin 2"-*O*-α-L-rhamnopyranoside, which showed increasing activity of type I collagen. To examine the anti-wrinkle effect of Star Fruit Leaf Extract BG30, seven volunteers applied a Star Fruit Leaf Extract BG30 1% cream in double blind manner to one-side of the corner of their eye and the placebo cream to the opposite side. Clinical evaluation of wrinkling was performed every week for 5 weeks using a silicone rubber replica. A statistically significant improvement of Star Fruit Leaf Extract BG30-treated site was seen in decreased wrinkles. Star Fruit Leaf Extract BG30 results in clinically visible improvement in wrinkling when used topically for 5 weeks.

## Introduction

Plants get energy from photosynthesis on its leaves by use of sunlight. On the other hand, sunlight is containing UV-rays, which produce reactive oxygen species (ROS, singlet oxygen ($^1O_2$), superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($^{\cdot}OH$)) on epidermis and mesophyll of leaf, and ROS damage to the cell. However, a lot of original components such as polyphenol and carotenoid exist in the leaf as a defense mechanism against ROS, and the leaf keeps to ability of photosynthesis and protects from aging by the use of this defense mechanism.

Human skin is directly exposed to environmental factors such as UV-rays and oxygen, and UV is trigger the generation of ROS from the cells and then these ROS cause damage. Especially UVB (UVB: 290nm-320nm) causes critical damage to the skin in the short term. However, the skin has intrinsic scavengers, such as superoxide dismutase (SOD) and catalase, as a defense mechanism against ROS, and protects form aging.

Thus, plant leaf and human skin have similar defense mechanism against UV-rays and ROS. In normal human skin, extracellular matrix (ECM) is kept balance between the biosynthesis and degradation, and kept elasticity, moisture and so on. However, decrease of defense ability against ROS by intrinsic ageing and long years of UV expose induce disruption of EMC balance and appear as photoaging such as dryness, wrinkles, looseness and coarseness. As a photoaging mechanism, it is well known that matrix metalloproteinase-1 (MMP-1) is stimulated by UV irradiation and singlet-oxygen, and then degrades type I collagen (1- 4). Therefore, topical application of preventive and radical-scavenging antioxidant, MMP-1 inhibitor and type I collagen promoter may be an effective way to prevent photoaging by UV expose (5).

In this study, we evaluated activities of various plant leaf extracts and found the availability against skin aging in the leaf extract of star fruit (*Averrhoa carambola* L) and separated active principal from this extract, and developed Star Fruit Leaf Extract BG30 as a ingredient for cosmetics. Topical treatment with the Star Fruit Leaf Extract BG30 improved in wrinkling when used for 5 weeks.

## Materials and Methods

### Chemicals

***Preparation of the leaves extract of star fruit (A. carambola L)***

The leaves (300 g) of *A. carambola* was chopped by a commercial mixer and homogenized with the addition of 3 liters of 50%-ethanol. The mixture was kept at 80-90°C for 2 hours and then filtered. This operation was repeated 2 times and collected filtrate was concentrated to 600 ml under a reduced pressure. The solution was freeze-dried, giving 109 g of powder.

***Isolation of isofurcatin 2''-O-α-L-rhamnopyranoside from the leaves of A. carambola***

The 50% ethanol extract was subjected to HP-20 ($H_2O$→ 40% MeOH→ 80% MeOH→ MeOH), silica gel ($CHCl_3$-MeOH- $H_2O$ (10:5:1)), ODS (40%-acetonitrile) column chromatography and finally HPLC (JAIGEL GS-310, MeOH) to give isofurcatin 2''-*O*-α-L-rhamnopyranoside (Fig. 1).

Isofurcatin 2''-*O*-α-L-rhamnopyranoside, a yellow powder, mp 217-219°C. $^1$H-NMR(DMSO-$d_6$) δ: 0.53 (3H, d), 1.13 (3H, d), 4.99 (1H, s), 6.53 (1H, s), 6.71 (1H, s), 6.92 (2H, d), 7.89 (2H, d). $^{13}$C-NMR (DMSO-$d_6$) δ: 163.6 (C-2), 103.0 (C-3), 181.8 (C-4), 161.1 (C-5), 109.0 (C-6), 162.7 (C-7), 94.9 (C-8), 156.7 (C-9), 103.7 (C-10), 121.3 (C-1'), 128.6 (C-2' and C-6'), 116.1 (C-3' and C-5'), 158.9 (C-4'), 75.9 (Rha-1), 74.0 (Rha-2 and Rha-3), 71.4 (Rha-4 and Rha-5), 17.9 (Rha-6), 100.7 (Rha-1'), 70.6 (Rha-2'), 72.2 (Rha-3'), 70.4 (Rha-4'), 68.4 (Rha-5'), 17.2 (Rha-6'). MS (positive mode) m/z: 563 $[M+H]^+$.

**Bioassay Methods**

***Assay for inhibitory activity of superoxide anion by xanthine oxidase***

The superoxide anion scavenging activity was determined by a modification the method of Toda et al. (6). The standard reaction mixture containing 0.1mM xanthine, 0.1mM EDTA, bovine serum albumin (50μg of protein/ml), 40mM sodium carbonate (pH10.4), 25mM nitroblue tetrazolium, sample solution and 7 x $10^{-9}$ U XOD (xanthine oxidase), with a final volume of 3ml, was incubated at 25 °C for 20 min. The reaction was terminated after incubation by the addition of 0.1ml of 6mM $CuCl_2$ solution. The absorbance of the formazan produced was determined at 560nm. Inhibitory effect of sample on superoxide anion was estimated by the equation.

Inhibition (%) = [O.D. (control) – O.D. (sample)] / O.D. (control) x 100

***Assay for radical scavenging activity on DPPH***

The radical scavenging activity was determined by a previous report of Haraguchi et al. (7). The reaction mixture consisted of 3ml of 150μM diphenyl-*p*-picrylhydrazyl (DPPH) ethanol solution and 3ml of sample solution. After allowing the mixture to stand at room temperature for 30min, the absorbance of remaining DPPH was determined colorimetrically at 520nm. The scavenging activity of sample was expressed as percentage of a decrease in absorbance of DPPH against that of a control DPPH solution.

***Assay for hemolysis***

The inhibitory effect of hemolysis induced by singlet-oxygen was determined by a modification the methods of Haraguchi et al. (7). The photosensitized hemolysis of rabbit erythrocytes was inhibited by antioxidants. Blood from rabbit was collected in heparinized tubes. Erythrocytes were separated by centrifugation from plasma and buffy coat, and washed three times with phosphate buffered saline (PBS). In a vial, 1% suspension of erythrocytes, sample solution and 10μM hematoporphyrin, with a final volume of 10ml, was irradiated with halogen beam on the tern table at room temperature for 20min. After irradiate, two samples (1ml each) were taken out from the mixture; one sample was diluted with 2ml of PBS and other with 2ml of distilled water to yield complete hemolysis. Both samples were centrifuged at 3000rpm for 5min. The absorbance of the supernatants was determined at 540nm. The singlet-oxygen scavenging activity of sample was expressed as percentage of hemolysis against that of a complete hemolysis.

***Assay for inhibitory effect of MMP-1 activity***

The inhibitory effect of MMP-1 activity was determined by a modification the method of Mineo et al. (9). In a screw-capped test tube, 0.45ml of 0.1M Tris-HCl (pH 7.1), containing 20mM $CaCl_2$, 100μg/ml MMP-1 (from *Cl-histolyticum*, SIGMA Chemical Co., St. Louis, Mo), 0.4M Pz-peptide (BACHEM Feinchemikalien AG, Switzerland) and 0.05ml of sample solution, was incubated at 37 °C for 30 min. The reaction was terminated after incubation by the addition of 1ml of 25mM citric acid

solution, and 5ml of methylene chloride was added to the reaction mixture to dissolve the residues of substance by shaking vigorously. The absorbance of the upper layer, obtained by centrifuge at 3,000r.p.m for 10 min, was determined at 320nm. The inhibitory activity is expected as the percentage inhibition of the MMP-1 in the above assay system, *i.e.* as [(A – B) / A] x 100, where *A* is the optical density of the methylene chloride phase without test sample and *B* is that of the phase with test sample.

***Promoting activity of type I collagen synthesis on human skin fibroblasts***

For the determination of promoting activity of type I collagen synthesis, CCL-110, which is normal human skin fibroblast, provided by ATCC (American Type Culture Collection, MD) were maintained in minimum essential medium (MEM, ICN Pharmaceuticals Inc., OH) supplemented with 10% fetal bovine serum (FBS, Irvine Scientific, CA), 1% non-essential amino acid and 1mM sodium pyrvate in a humidified atmosphere of 5% $CO_2$ and 95% air. After the confluent, cells were collected by trypsinization, and resuspended in MEM. The cells were plated onto a 96-well plate ($2 \times 10^4$ cells/well) in 100μl MEM. After overnight cultivation, the medium was replaced with 120μl MEM supplement with 0.5% FBS containing test sample, and the cell cultures were continued. After 72h, the culture medium or 0-2000ng/ml of human type I collagen, for the standard curve, (0.09ml) was put into each well of ELISA plate with 1mM EDTA, 1mM NEN and 0.1mM PMSF, in a total volume of 0.1ml. After gentle mixing, the plate was incubated at 4°C for overnight. The plate was washed with phosphate buffered saline, pH7.4, containing 0.05% Tween20, then incubated with PBS, pH7.4, containing 1% BSA at 37 °C for 3h. After the plate was washed, probed with the rabbit anti-human collagen type I antibody (Chemicon International, CA) at 37 °C for 90min. The plate was washed, and then probed with the horseradish peroxidase conjugated goat anti-rabbit IgG (Nichirei, Tokyo) at 37 °C for 90min. After the plate was washed, 0.15ml of 0.3mg/ml ABTS in 0.1M phosphate-citrate buffer, pH4.0, containing 0.03% $H_2O_2$ was added to the each well. After 20 to 30min, the absorbance of each well was measured at 405nm after mixing. Promoting activity of type I collagen synthesis was evaluated by using standard curve.

***Recovery effect against UVB induced cell damage on human skin fibroblasts***

RCB0222, which is normal human skin fibroblast, provided by Riken Cell Bank (Tsukuba) were maintained in MEM-alpha (GIBCO, N.Y.) supplemented with 10% FBS (Bio Whittaker, MD), penicillin (50U/ml) and streptomycin (50μg/ml) in a humidified atmosphere of 5% $CO_2$ and 95% air. After the confluent, cells were collected by trypsinization, and resuspended in MEM-alpha. The cells were plated onto 48-well-plate ($4.0 \times 10^4$ cells/well). After overnight cultivation, cells were exposed UVB at 5J/cm$^2$. Immediately, culture medium was replaced to the test sample dissolved into MEM-alpha, and cells were cultivated for 24h. After the medium removed, 200μl of tetrazolium compound MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-corboxyMethoxy-phenyl)-2-(4-sulfo-phenyl)-2H- tetrazolium; inner salt) (0.4mg/ml in PBS (-)) solution was added to each well. The plate was incubated for 2h at 37°C in a $CO_2$ incubator. After incubation MTT solution was removed from each well and 200μl of

iso-propanol was added. A570-A650 value was measured with micro plate reader, and recovery rate was determined by following formula.

Recovering rate (%) = [(Nt – C) – (Nt – Sa)] / (Nt – C) x 100

Nt: A570-A650 value on untreated group

C: A570-A650 value on UVB irradiation group

Sa: A570-A650 value on UVB irradiation and test samples group

**Clinical study**

To examine the anti-wrinkle effect of Star Fruit Leaf Extract BG30, seven volunteers (mean age: 32 +/- 7year) applied a newly formulated 1% of this extract in an o/w cream in double blind manner to one-side of the corner of their eye and the inactive o/w cream to the opposite side. Clinical evaluation of wrinkling was performed every week for 5 weeks using a silicone rubber replica and photo imaged by Photoshop®. Result of clinical study was analyzed the histogram of between before and after treatment, and efficacy of Star Fruit Leaf Extract BG30 1% cream was assessed as described below.

No effective: >5

Effective: 5 - 10

Very effective: 10<

## Results

**The antiperoxidative activity**

The antiperoxidative activities of Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside were evaluated using the different assay system; (1) radical scavenging activity on DPPH; (2) inhibitory activity on superoxide anion generation by xanthine-xanthine oxidase system; and (3) protective activity of erythrocyte from singlet oxygen-induced hemolysis.

***Inhibitory activity of superoxide anion by xanthine oxidase***

Xanthine oxidase is one of the major oxidative enzymes producing superoxide anion. Star Fruit Leaf Extract BG30 inhibited the generation of superoxide anion by the xanthine-xanthine oxidase system in a concentration dependent manner, whereas no inhibitory effect of Isofurcatin 2"-*O*-α-L-rhamnopyranoside as shown in Figure 2. The $IC_{50}$ value of Star Fruit Leaf Extract BG30 was observed at 25.7μg/ml.

***Radical scavenging activity on DPPH***

Radical scavenging activity of Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside were evaluated by decrease in absorbance of DPPH. Star Fruit Leaf Extract BG30 showed a radical scavenging activity in a concentration dependent manner, whereas no activity of Isofurcatin 2"-*O*-α-L-rhamnopyranoside as shown in Figure 3. The $EC_{50}$ value of Star

Fruit Leaf Extract BG30 was observed at 24.1μg/ml.

***Assay for hemolysis***

Singlet-oxygen scavenging activities of Star Fruit Leaf Extract BG30 and Isofurcatin 2''-O-α-L-rhamnopyranoside were evaluated by inhibitory effect of hemolysis. Erythrocyte has a large amount of lipid membrane compared to other tissues, and hemolysis can be induced easily by singlet-oxygen as a result of membrane damage via lipid peroxidation. Inhibitory effects of Star Fruit Leaf Extract BG30 and Isofurcatin 2''-O-α-L-rhamnopyranoside on hemolysis were tested. As shown in Figure 4, singlet-oxygen induced erythrocyte hemolysis was prevented effectively by Star Fruit Leaf Extract BG30 and Isofurcatin 2''-*O*-α-L-rhamnopyranoside, and $IC_{50}$ value of Star Fruit Leaf Extract BG30 was observed at 95.4μg/ml.

**Inhibitory effect of MMP-1 activity**

Inhibitory effects of Star Fruit Leaf Extract BG30 and Isofurcatin 2''-O-α-L-rhamnopyranoside on MMP-1 activity were tested by the using bacterial collagenase and synthesized substrate. As shown in Figure 5, Star Fruit Leaf Extract BG30 and Isofurcatin 2''-*O*-α-L-rhamnopyranoside has an inhibitory effect on MMP-1 in a concentration dependent manner, and $IC_{50}$ value of Star Fruit Leaf Extract BG30 was observed at 94.5μg/ml.

**Promoting activity of type I collagen synthesis in human skin fibroblast**

Promoting activities of collagen synthesis was evaluated in human skin fibroblast treated with Star Fruit Leaf Extract BG30 and Isofurcatin 2''-*O*-α-L-rhamnopyranoside by measuring type I collagen in the culture medium. As shown in Figure 6A, B, the amount of type I collagen in the culture medium were increased by treated with Star Fruit Leaf Extract BG30 and Isofurcatin 2''-*O*-α-L-rhamnopyranoside in a concentration dependent manner. The maximal increase of 160.7% ($P<0.05$) was seen in a Star Fruit Leaf Extract BG30 with a concentration of 100μg/ml.

**Recovery effect against UVB induced cell damage**

In order to study the recovery effects of Star Fruit Leaf Extract BG30 and Isofurcatin 2''-*O*-α-L-rhamnopyranoside on UVB induced cell damage, human skin fibroblasts were treated with Star Fruit Leaf Extract BG30 and Isofurcatin 2''-*O*-α-L-rhamnopyranoside followed by UVB irradiation. The viability of cells was detected by MTT assay. As shown Figure 7A, B, Star Fruit Leaf Extract BG30 was inhibited on UVB induced cell damage.

**Effect of topical application of Star Fruit Leaf Extract BG30 1% cream**

As shown in Figure 8, complete effect was seen in 2 volunteers, improved effect was seen in 1 volunteer and ineffective was seen in 4 volunteers. However, all of Star Fruit Leaf Extract BG30 1% cream-treated site was superior to placebo cream-treated site. Especially, deep, long wrinkle was changed to shallow wrinkle by the 5weeks treatment of Star Fruit Leaf Extract BG30 1% cream (Fig.

9A,B). No volunteers were found to have any evidence of inflammation.

## Conclusions and Discussion

We evaluated activities of various plant leaf extracts and found the availability against skin aging in the leaf extract of star fruit (*Averrhoa carambola* L), and the Star Fruit Leaf Extract BG30 was developed as an ingredient for cosmetics. The results presented here show that Star Fruit Leaf Extract BG30 has scavenging activity of ROS, inhibitory effect of MMP-1 activity, promoting activity of type I collagen synthesis and inhibitory effect of UVB induced cell damage. Furthermore, improved effects of wrinkle were observed in clinical study. We believe that a decrease in the ROS load by protective ingredients, such as Star Fruit Leaf Extract BG30, may prevent or at least minimize ROS-induced photoaging, as evidenced by our clinical study.

We performed the separation of the active principal from this extract to give isofurcatin 2"-*O*-α-L-rhamnopyranoside, which showed increasing activity of type I collagen protein. However, isofurcatin 2"-*O*-α-L-rhamnopyranoside had no effect on ROS scavenging activity, and it is still unknown that the active principal of ROS scavenging activities, though active principal was seemed to be polyphenol and carotenoid. We are further investigating the effect of isofurcatin 2"-*O*-α-L-rhamnopyranoside on epidermis, such as keratinocyte and melanocyte.

This study demonstrated that Star Fruit Leaf Extract BG30 was able to prevent skin aging, and useful as an ingredient for cosmetics.

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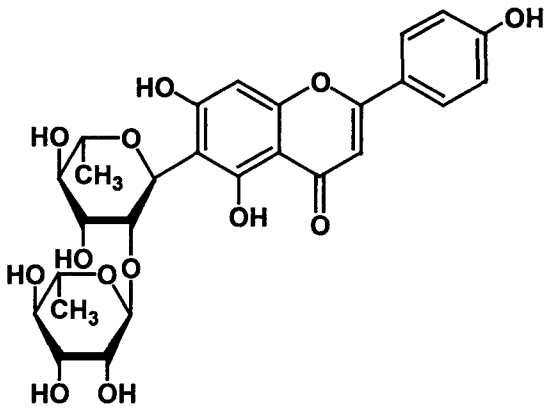


Figure 1. The structure of Isofurcatin 2"-*O*-α-L-rhamnopyranoside

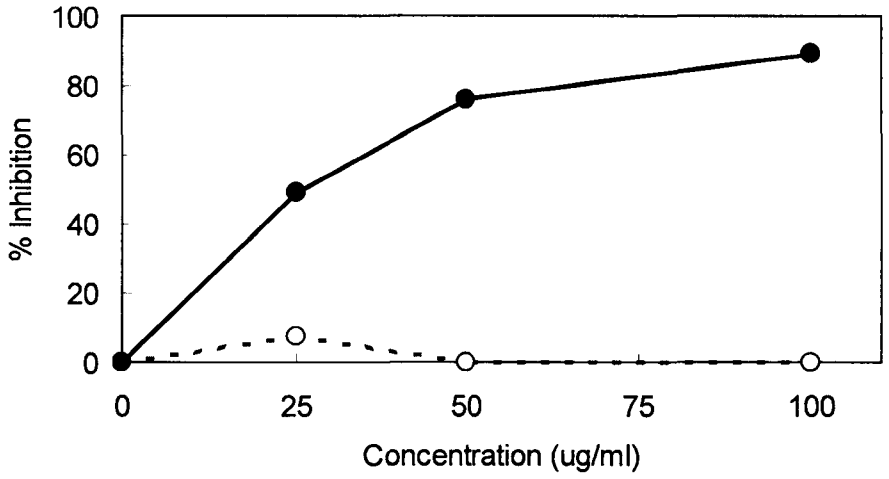


Figure 2. Inhibitory effect of Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside on the generation of superoxide anion. (•) Star Fruit Leaf Extract BG30, (○) Isofurcatin 2"-*O*-α-L-rhamnopyranoside

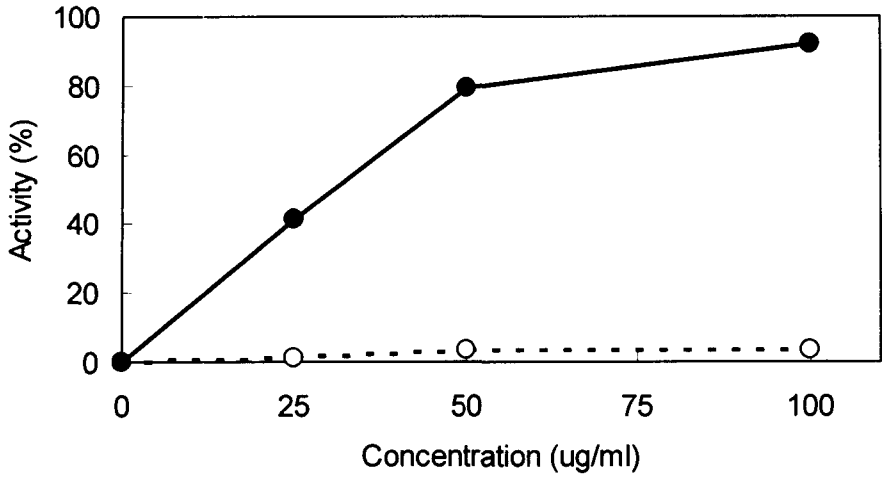


Figure 3. Scavenging activity of Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside on DPPH radical. (●) Star Fruit Leaf Extract BG30, (○) Isofurcatin 2"-*O*-α-L-rhamnopyranoside

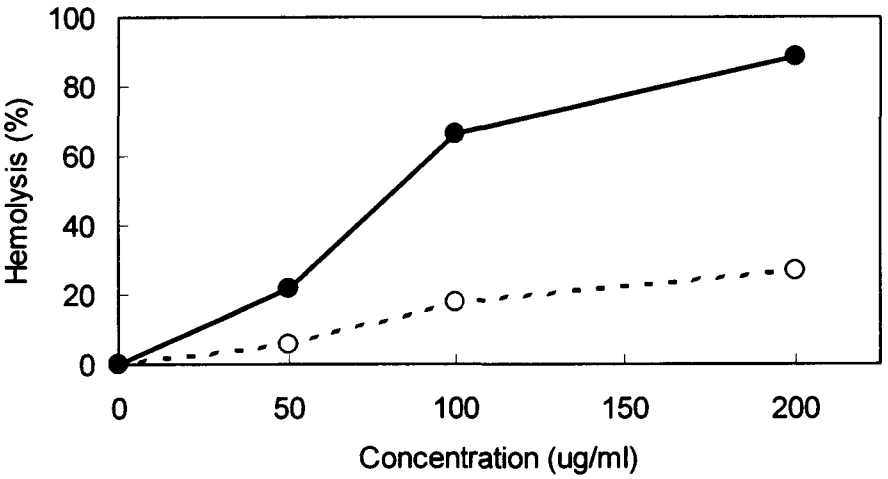


Figure 4. Inhibition of oxidative hemolysis by Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside in rabbit erythrocytes. (●) Star Fruit Leaf Extract BG30, (○) Isofurcatin 2"-*O*-α-L-rhamnopyranoside

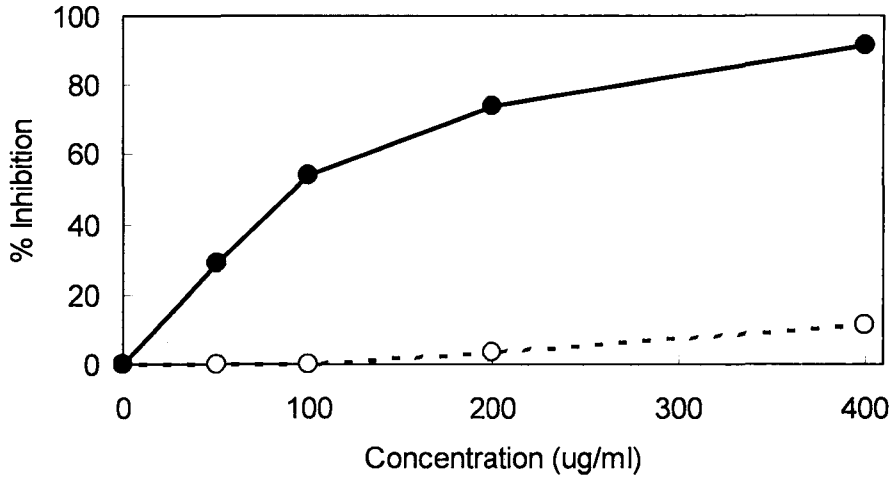


Figure 5. Inhibitory effect of Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside on MMP-1 activity. (●) Star Fruit Leaf Extract BG30, (○) Isofurcatin 2"-*O*-α-L-rhamnopyranoside

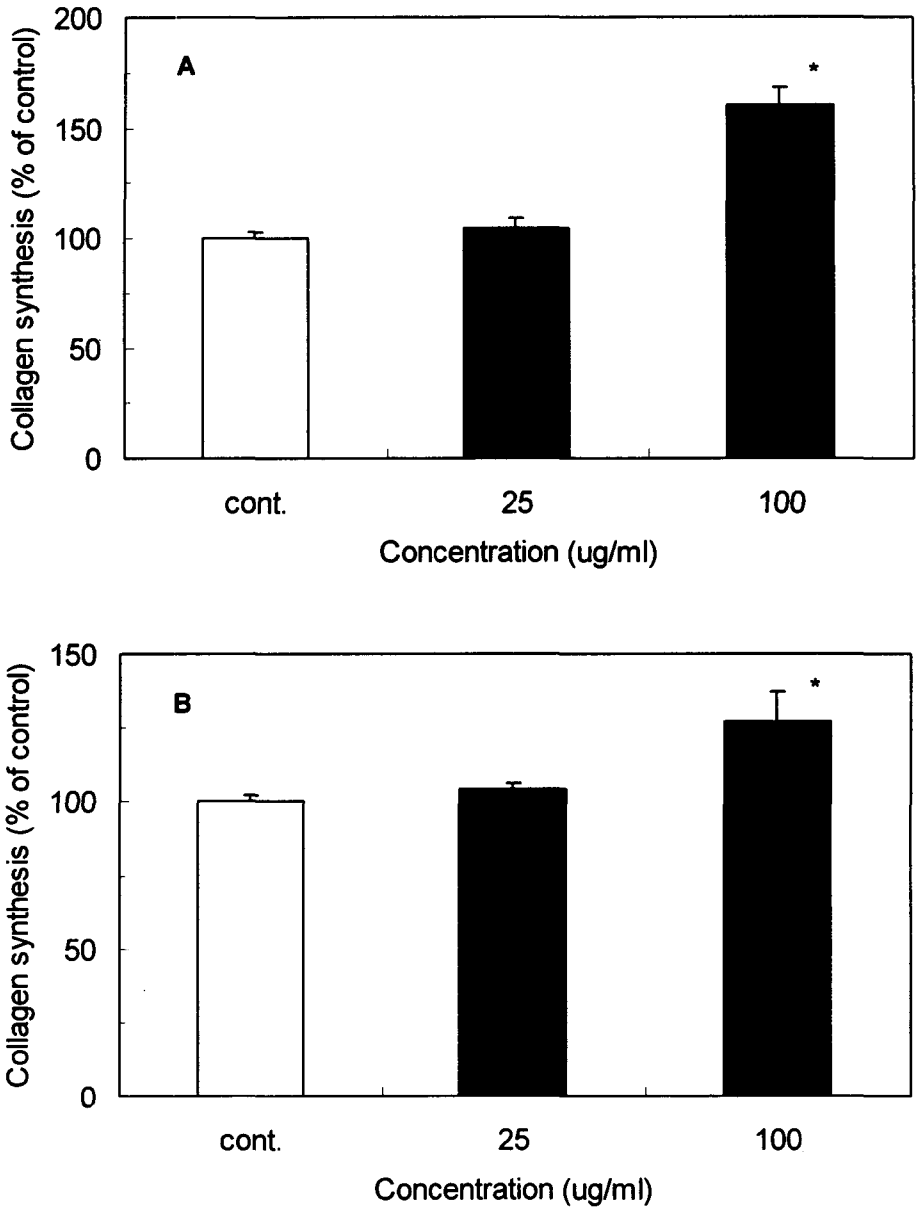


Figure 6. Promoting activity of type I collagen synthesis of Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside on human fibroblast. (**A**) Star Fruit Leaf Extract BG30, (**B**) Isofurcatin 2"-*O*-α-L-rhamnopyranoside. *; $P<0.05$ versus control group.

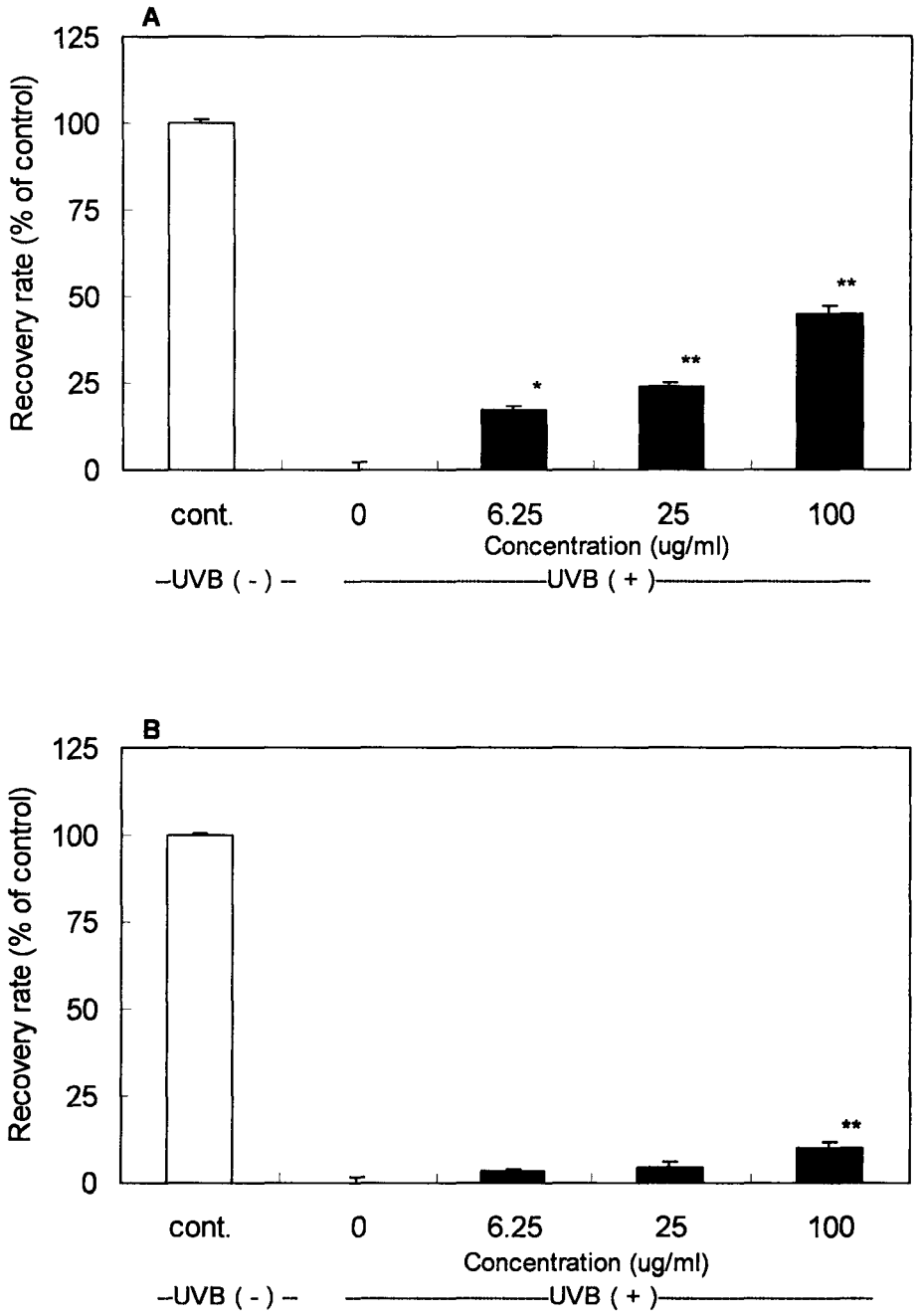


Figure 7. Recovery effect of Star Fruit Leaf Extract BG30 and Isofurcatin 2"-*O*-α-L-rhamnopyranoside on UVB induced cell damage. (**A**) Star Fruit Leaf Extract BG30, (**B**) Isofurcatin 2"-*O*-α-L-rhamnopyranoside. *; $P<0.05$, **; $P<0.01$ versus irradiated-control group.

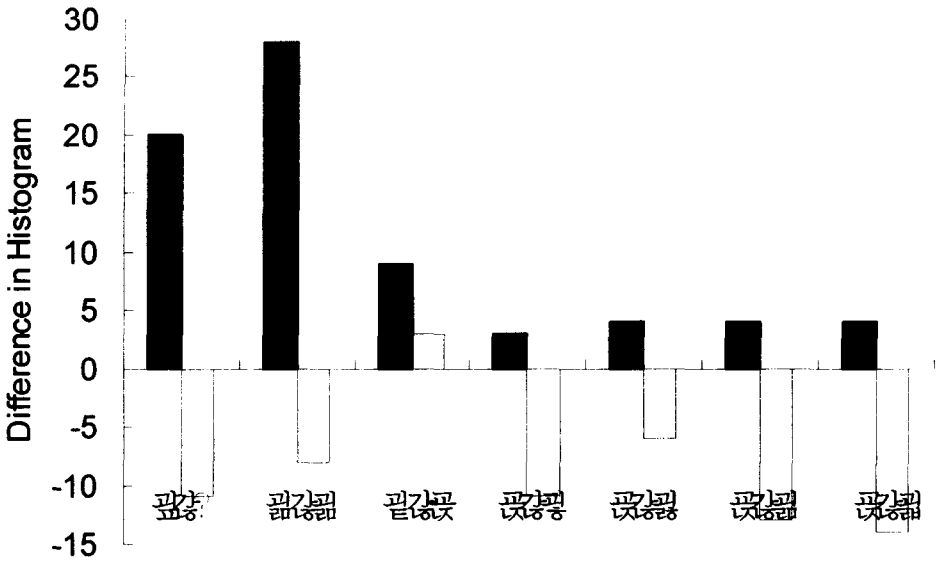


Figure 8. Effect of topical application of Star Fruit Leaf Extract BG30 1% cream. (■) application of Star Fruit Leaf Extract BG30 1% cream, (□) application of placebo cream.

A)

B)

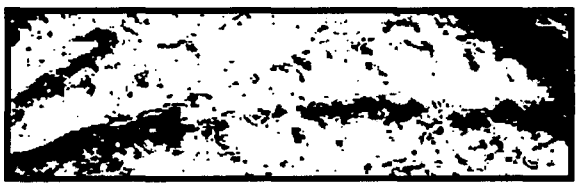

Figure 9. Typical replica of wrinkles with Star Fruit Leaf Extract BG30 1% cream. (**A**) before application, (**B**) 5 weeks after application.