# Effects and permeation property of anti-aging material from tinged autumnal leaves of Maple tree in the skin

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## Abstract

Free radicals and reactive oxygen species (ROS) caused by UV exposure or other environmental facts play critical roles in cellular damage and aging.

The extract of tinged autumnal leaves of maple tree(*Acer palmatum*) has proven to be a powerful antioxidant. The *Acer palmatum* extract is very effective on the stabilization of biological membranes(containing unsaturated fatty acid).

We studied photoprotective effect of the extract against UVB-induced cytotoxicity in human keratinocytes. The extract improved cell viability comparing to control after UVB irradiation. In the determination test of proinflammatory cytokines the extract decreased expression of interleukin 1 α and 6, which play an important role in inflammation and skin erythema caused by UV.

We also studied property of varying cosmetic formulations on the percutaneous absorption of the extract. After 24 hour *in vitro* penetration study, the content of the extract was more highly detected in skin residue part. This result showed the extract had relatively high compatibility of skin in our emulsion system.

On human skin, after appling the product containing the extract we obtained a good result of antiwrinkle effect by skin visiometer.

In conclusion, the *Acer palmatum* extract is a photoprotective and very effective in stressed and aged skin care. And we can predict the extract mainly affects on the skin cell and tissue in our emulsion system.

## 1. Introduction

Most symptoms of cutaneous aging and skin cancers are the result of exposure to solar radiation. Evidence for free radical formation in UV-radiated skin (1) provided the basis for implication of reactive oxygen species (ROS) in UV-mediated cutaneous damage including skin cancer, autoimmune disease, phototoxicity, photosensitivity and skin aging.

Thus, study concerned about UV-irradiated and chronologically aged human skin were increased and UV-induced skin aging seems to be provides help in development of new clinical strategies to impede chronological aging. It is well known that a decrease in collagen is shown with photoaging of human skin. Collagen, the predominant component of dermal connective tissue, is biosynthesized by dermal fibroblasts. UV radiation plays a major role in altering the dermis and activating a family of degradative enzymes called matrix metalloproteinase (MMPs). These enzymes target the components of the extracellular matrix (ECM) such as collagen, laminin, fibronectin and proteoglycan (2-5).

In response to UVB-irradiation keratinocytes release a variety of cytokines, including tumor necrosis factor α (TNF-α), interleukin 1 α (IL-1 α) and interleukin 6 (IL-6).

A UV-induced cytokine network consisting of IL-1 α, IL-1 β and IL-6, which via interrelated autocrine loops induce collagenase/MMP-1 and thus may contribute to the loss of interstitial collagen in cutaneous photoaging(10). And IL-1 α and –6 is key mediators of inflammation in UV-irradiated human skin (6-9).

A number of antioxidants that posses oxygen radical scavenging properties have been tested as potentially beneficial photoprotective agents from these extrinsic factors (11-12).

Intrinsic oxidation-protecting enzyme systems, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, and food-derived substances such as tocopherols, flavonoids, ascorbic acid and carotenes are known to diminish the undesired effects caused by oxidation processes in organisms (13).

Percutaneous absorption of these active ingredients via stratum corneum is very important. In order that active ingredients function properly, they must penetrate stratum corneum and reach viable epidermal or dermal layers. But barrier property of skin act as a major obstacle for the transdermal delivery, so penetration enhancer is necessary to overcome this problem. Skin delivery of an active ingredient from a cosmetic formulation depends on its release and passive diffusion through the stratum corneum. A significant number of metabolic processes take place in the skin (14) and these can only be taken into account under *in vitro* conditions if skin viability is maintained. Various animal model like mouse and human skin have been used to study the permeation of active ingredients(15-16).

In this study, we investigated the possibility of tinged autumnal leaves(*Acer palmatum*) as

an antioxidant source. The growing process of plants is varied with seasonal changes. Temperature and sunshine duration can affect the color change of leaves, which is due to the variation of pigments during the change of season. Autumnal leaves have a high concentration of secondary metabolites compared to green leaves. We screened various tinged autumnal leaves to select target materials with potent antioxidant activities.
In this work we studied the skin penetration properties of various cosmetic formulation containing *Acer Palmatum* extract and measured the efficacy of the extract.

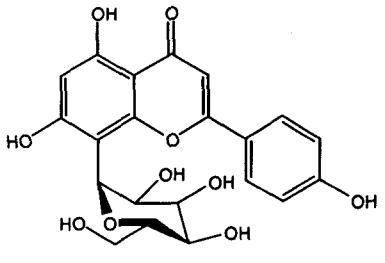
## 2. Materials and Method

### Chemicals and instruments

BHA (3-t-Butyl-4-Hydroxyanisol), Anti-MMP-1 antibody (Ab-5), Anti-Human Interleukin-1 α, Anti-Human Interleukin-6, anti-rabbit IgG conjugated with Horse radish peroxidase (HRP) and anti-mouse IgG conjugated with alkaline phosphatase (AP) were purchased from Sigma chemical Co.(St. Louis, MO, USA).

### *Plant extract preparation and Identification*

Through a series of screening works, the tinged autumnal leaves of maple tree(*Acer Palmatum* THUNBERG.(Aceraceae) was selected as our antioxidant materials. Leaves were collected between October and November in the middle part of South Korea.
The 100g of each leaf was extracted with 900g of ethanol for 10days at room temperature.
The ethanol extracts were filtered and concentrated at 40℃ in vacuum evaporator.
The major ingredient of *Acer Palmatum* extract is vitexin(up to 1%), which is a analysis target of the extract was identified by HPLC and NMR determination (17).




**Vitexin**
($C_{21}H_{20}O_{10}$ 432.38)
(Apigenin-8-C-β-D-glucopyranoside)

Figure 1. The structure of Vitexin

***Antioxidative activity***

The antioxidative activity of the extract was evaluated by nitroblue tetrazolium chloride mono hydrate (NBT) test and 1,1-diphenyl-2-picrylhydrazyl (DPPH) test with suitable dilution as follows.

First, in NBT test, 0.1ml sample, 2.4ml $Na_2CO_3$ buffer (0.05M, pH 10.2), 0.1ml xanthine(3mM), 0.1ml ethylene diamine tetraacetic acid(3mM), 0.1ml NBT solution(0.72mM) and 0.1ml bovine serum albumin solution were added in turn to each test tube and reacted at 25℃ for 10 min. To each test tube, 0.1ml xanthine oxidase(0.25unit/ml) was added and reacted at 25℃ for 20 min. The reaction was quenched with 0.1ml $CuCl_2$. Finally inhibitory effect(%) was calculated as compared with blank control after measuring absorbance at 560nm.

In DPPH test, 0.15ml of the sample and 0.15ml of DPPH alcoholic solution(0.1mM) was mixed and incubated in a test plate at 37℃ for 10 min . And the absorbance was measured at 560nm. Then, its antioxidative activity(%) was calculated with respect to blank control.

***Collagenase(MMP-1) inhibition assays***

The collagenase inhibition assay, which is based upon fluorescence measurement of collagen fragments upon cleavage by MMP-1, was performed according to the manufacturer's protocol. The enzymes were mixed with quenched fluorescent substrates (0.2ug/ml) in a final volume of 200ul reaction buffer in 96-well microplates. The enzymatic assays were optimized with 0.1 units of MMP-1. Digested products from DQ collagen substrates have absorption maxima at ~495nm and fluorescence emission maxima at ~ 515nm in a fluorescence microplate reader (Perkin Elmer, USA). For all the MMPs tested the activities under these conditions were linear for at least 15min. For each time point, correct for background fluorescence by subtracting the values derived from the no-enzyme control.

***Culture of HaCaT(Human keratinocyte)***

Human keratinocyte cell line (HaCaT) were purchased from American Type Culture Collection. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and incubated in a humidified 5% $CO_2$ incubator at 37℃.

***Protective effect of the extract against UVB-induced cytotoxicity.***

HaCaTs ($1\times10^5$/well) were seeded into 24-well plates and cultured overnight. Using the

thiazolyl blue (MTT) conversion assay as a measure of mitochondrial function, the extent of cytotoxicity induced by various doses of UVB (280-360 nm) was evaluated. The photoprotective effect of *Acer palmatum* extract on the 50% lethal damage induced by ultraviolet B (UVB), and then the culture media were replaced with DMEM containing sample. After 24 hours incubation, culture media was removed. And then 0.5% 60$\mu\ell$ MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and 500 $\mu\ell$ of fresh DMEM was added on a 24 well plate. The plate was maintained at $CO_2$ incubator for 2h to give formazan formation. The quantity of formazan produced can be regarded as an indicator of cell density or viability. After dissolving the formazan in isopropanol, the absorbance at 565nm was measured with microplate reader.

***UVB irradiation and determination of Interleukin 1α and 6 by ELISA***

The expression of IL-1α and IL-6 was assayed by enzyme-linked immunosorbent assay (ELISA). HaCaTs ($1\times10^5$/well) were seeded into 24-well plates and cultured overnight. The cells (HaCaT cell) were irradiated by 10 mJ/cm$^2$ of UVB source emitting wavelengths in the 280 ~ 360 nm range (Sankyo Denki, Japan). And then the culture media were replaced with DMEM containing sample. After 5 hours' incubation, the supernatants were transferred into a 96 well plate and incubated for 12 hours. The supernatants were removed and the coated well was washed with PBS containing 0.05% Tween 20 (PBS-T) for 3 times and followed by blocking with 3% BSA in PBS-T for 2 hours at 37℃. After washing 3 times with PBS-T, 150$\mu\ell$ of diluted primary antibody (IL-1 α ,IL-6) was added into each well and incubated for 90 min at 37℃. Washing the wells with PBS-T 3 times, 150$\mu\ell$ of diluted secondary Ab, anti-rabbit IgG conjugated with HRP (Horse radish peroxidase) in blocking buffer was added and incubated for 90 min. Washing the wells with PBS-T 5 times, 150$\mu\ell$ of OPD (o-phenylenediamine)solution was added and incubated for 40 min. After that, 50$\mu\ell$ of 2N $H_2SO_4$ was added. The optical density was measured at 490nm.

***Anti-wrinkle effect by skin visiometer***

To determine, *in vivo*, the effect of the emulsion containing *Acer palmatum* extract on skin, we performed a human use study. Volunteers consisted of 7 healthy asian females, aged between 37 and 51, selected strictly by study criteria. The sample was applied to the eye area twice daily at home for 56 days. The experimental conditions during measurements were maintained constantly under the ambient temperature of 22±2℃ and relative humidity between 40~60%.

The treated zone was compared to the placebo(the same formulation without the extract)

after twice-daily use for 56 days. We used Skin visiometer SV600(C+K, Germany) for analyzing skin condition after making skin replica(a negative of the skin).
We calculated the depth of wrinkes and skin roughness by Skin visiometer and analyzed by manufacture's protocol(18).

***Skin penetration property of the extract in O/W emulsion***

For the purpose of skin penetration study several formulations used. The composition of the emulsion formulation is given in Table 1.

**Table 1.** Composition of O/W emulsion formulation

| Ingredients | Concentration(% w/w) | | | | 비고 |
|---|---|---|---|---|---|
| Butylene glycol | 4.00 | 4.00 | 4.00 | 4.00 | Humectant |
| Glycerin | 1.00 | 1.00 | 1.00 | 1.00 | Humectant |
| TEA | 0.50 | 0.50 | 0.50 | 0.50 | pH controller |
| Tween 60 | 2.00 | 2.00 | 2.00 | 2.00 | Surfactant |
| Ar165 | 2.00 | 2.00 | 2.00 | 2.00 | Surfactant |
| IPM | 10.00 | 10.00 | 10.00 | 10.00 | Emollient |
| **Ethanol** | . | 5.00 | . | . | Solvent |
| **Ethoxydiglycol** | . | . | 5.00 | . | Emollient |
| **Urea** | . | . | . | 5.00 | Humectant |
| Water | q.s. to 100 | | | | solvent |
| others | . | | | | |

For this study the hairless mouse skin (6~8 weeks) was cut into squares of $4\times4cm^2$ pieces and placed in containers filled with PBS (Phosphate buffered saline, pH 7.4)
A flow-through system was used for conducting *in vitro* permeation experiments. The total system consisted of a receptor fluid reservoir, a variable flow rate peristatic pump (IPC 24 channel system, ISMATEC), a circulating water bath (polyscience,Hanson research), 10 flow-through diffusion cells, and Retriever IV fraction collector(ISCO Inc., US) to collect effluent fractions over the adjusted time period. Each diffusion cell had an inner diameter of 1.6mm and a surface area of $2cm^2$ exposed to the receptor fluid. The receptor fluid was pumped at a flow rate of 0.4ml/h from the reservoir to the diffusion cells placed in the holding blocks. The skin surface temperature was maintained at 37℃ by adjusting the circulating water bath temperature.

Thus, the amount of *Acer palmatum* extract was estimated in the following three locations in each *in vitro* permeation experiment : (a)receptor fluid, (b)surface washes, (c)tissues of the skin.

***HPLC Analysis(Acer palmatum* extract, *vitexin)***

For the quantitative determination of the extract, HPLC analysis was used. HPLC system consist of a Waters alliance 2695 system connected to a column(Waters Xterra RP18 3.5㎛ 3.0 ×150 mm) and a variable wavelenghth dectector(Waters 996 photodiode array detector). The analysis target of *Acer palmatum* extract was vitexin.

Vitexin was detected at 340 nm. The mobile phase for the sample was methanol : acetonitrils : water at the ratio of 30:10:60 (v/v/v). 10㎕ of each sample was injected into a column. Flow rate was 0.5 ml/min. The detection limits were 1 ㎍/ml(Vitexin) and 10 ㎍/ml(the extract). The HPLC method was validated prior to use, using USP standards. The peak areas were converted into concentrations(㎍/ml)using a standard curve.

***Statistical Analysis***

Results were presented as means ± standard error (SE). Experimental results were statistically analyzed by using Microsoft EXEL. P values <0.05 were regarded as indicating significant differences.

## 3. RESULTS AND DISCUSSION

***Antioxidative activity***

The exposure of cells to UVB radiation can induce the production of reactive oxygen species (ROS) which damage cellular components. Free radical scavengers and antioxidants can interfere with the production of ROS. Katiyar SK, et al reported that green tea polyphenol (-)-epigallocatechin-3-gallate in human skin inhibits ultraviolet radiation-induced oxidative stress. Other investigator reported the correlation of *in vitro* antioxidative and *in vivo* photoprotective effects of plant extract (19-23).

Antioxidative activity was measured by NBT test and DPPH test. Figure 1 (A) represents the results of NBT test on the extracts compared with other well-known antioxidants to measure measure Superoxide ($O_2^-$) scavensing effect. The activity of the extracts was comparatively as good as or higher than green tea extracts and BHA (3-t-Butyl-4-

Hydroxyanisol).

Figure 1(B) represents the results of DPPH test on the extracts to measure free radical scavensing effect and validate the correlation with NBT test. As a result, all the samples showed very high activities.

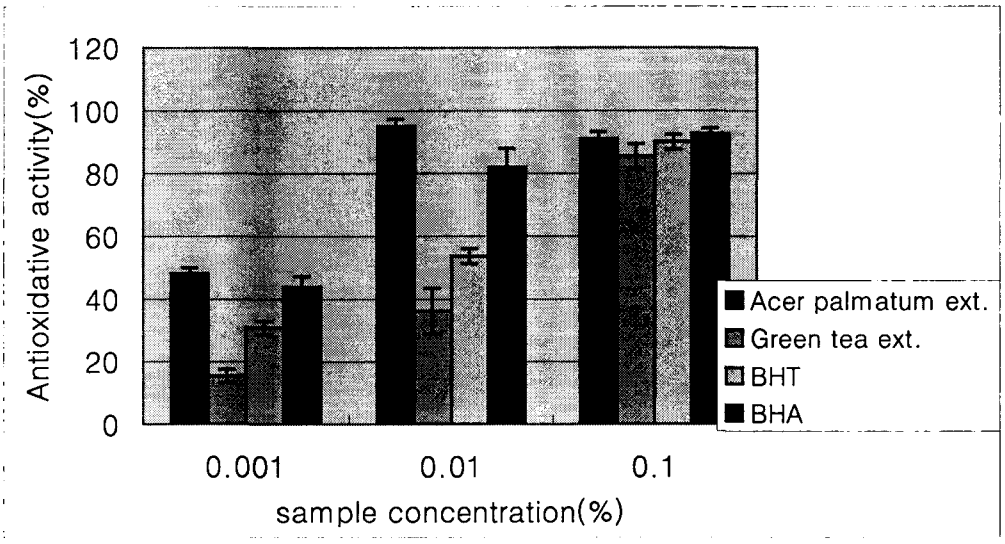



Figure 1(A). The results of antioxidative activities(NBT) on tinged autumnal leaf extracts

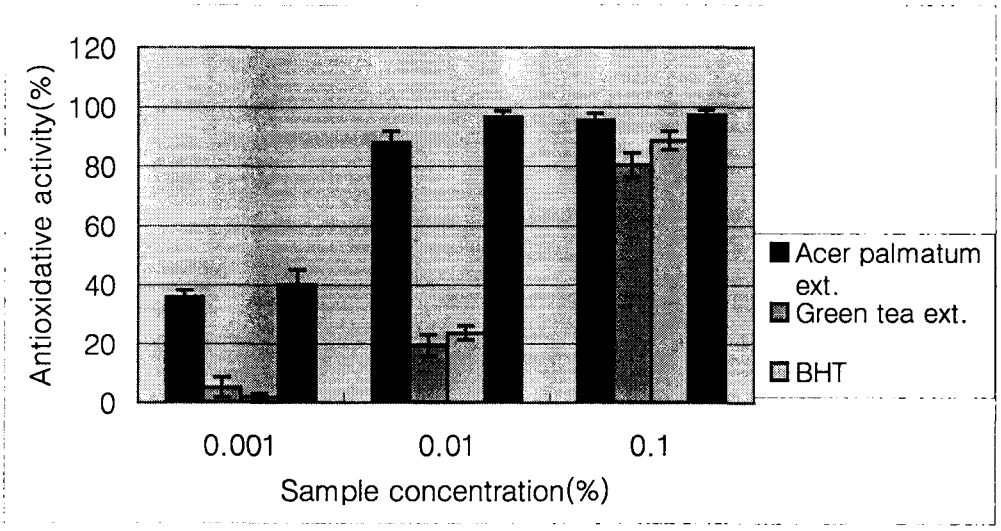



Figure 1(B). The results of free radical scavenging activities(DPPH) on *Acer palmatum* extract

### *In-vitro* Collagenase inhibition assays

Collagenase activity was measured using fluorescein-conjugated collagen as substrate and collagenase purified from *Clostridium histolyticum* was provided with the assay kit to serve as a control enzyme. We measured collagenase inhibitory of the *Acer palmatum* extract and green tea extract. Generally it is known that green tea extract have a good collagenase inhibition activity (24).

In the result of the test, the extract caused strong inhibition of collagenase activity, producing an inhibition of 90% at 0.04% (w/v)(Fig. 2). And green tea extract was shown inhibition of 50% at the same concentration.

The activities of MMP-1 were inhibited in a dose-dependent manner. The $IC_{50}$ values calculated from semi-log were 0.02%, and Green tea extract was 0.04% for MMP-1. *Acer palmatum* extract showed more effectively inhibition of MMP-1 than Green tea extract, so the data indicate that *Acer palmatum* extract is a good MMP inhibitor as a plant extract.

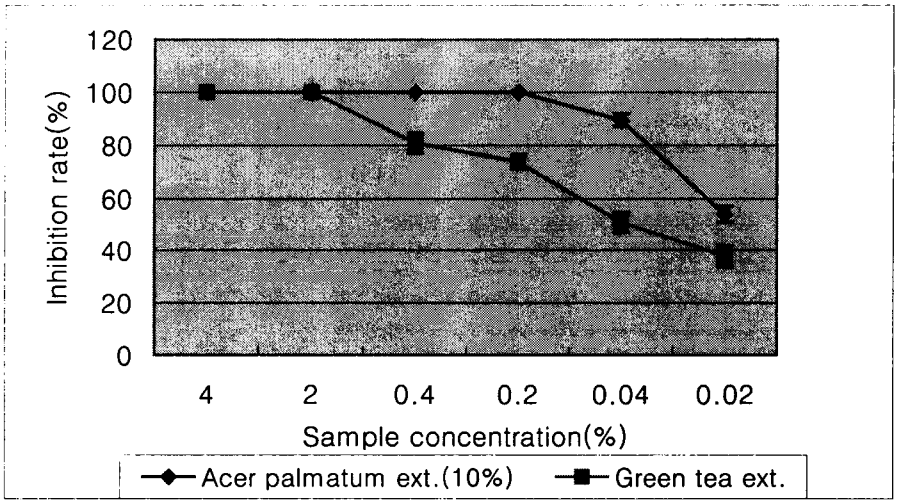



Fig. 2. The effect of *Acer palmatum* extract on MMP-1 activities. Collagenase activity were measured using a fluorometric assay in the presence of inhibitor and compared with Green tea extract. Fluorometric assays of the activities of MMP-1 was performed in the presence of increasing concentrations of *Acer palmatum* extract (◆) and Green tea extract (☒).

### *Protective effect of the extract against UVB-induced cytotoxicity*

The effect of *Acer palmatum* extract on the 50% lethal damage induced by ultraviolet B (UVB) was

studied using a human keratinocyte cell line(HaCaT). For an attempt to develop safe materials protecting solar ultraviolet (UV)-induced skin damage, plant extracts were evaluated for their inhibitory activities of free radical generation and cytotoxicity. Kim YH showed that their plant extract possessed the protective activity against UV-induced cytotoxicity of keratinocytes and fibroblasts(25). The photoprotective effect of the extract on cytotoxicity of UVB in HaCaT cells was dose dependent (0.0025-0.01 %). This data show that the *Acer palmatum* extract effectively can protect skin cells against harmful UVB irradiation.

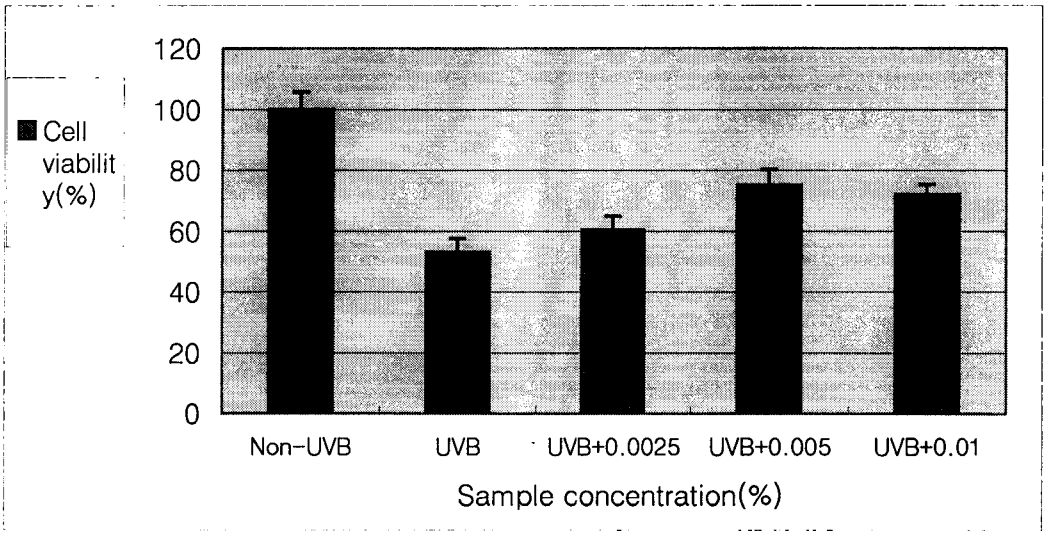



Figure 3. Protective effect of the *Acer palmatum* extract against UVB-induced cytotoxicity
After UVB irradiated, keratinocytes cultured for 24hr in the presence of increasing concentrations of *Acer palmatum* extract. MTT assay was used to measure cell viability.

***Determination of Interleukin 1α and 6 by ELISA***

The expression of IL-1α and IL-6 was assayed by enzyme-linked immunosorbent assay (ELISA) to quantify them in the culture medium of HaCaTs.

In response to UVB-irradiation keratinocytes release a variety of cytokines, including tumor necrosis factorα (TNF-α ), IL-1α and IL-6. IL-1α and IL-6 is key mediators of inflammation in UV-irradiated human skin. Robert M. et al reported that IL-1 and other cytokine were increased at 15hr and 24hr incubation after UV irradiation(6).

In order to investigate the anti-inflammatory effect of the *Acer Palmatum* extract on the UVB response in human skin, we studied the effect of the extract on UVB-modulated IL-1α and IL-6

expression in normal human keratinocytes (HaCaT). Before this test we screened the amount of interleukin production in various UVB doses and obtained the highest and appropriate test condition(UVB 10mJ). To determine whether the extract could control the production of interleukin by irradiated keratinocytes, The extract was applied for 5hr after UVB irradiation to the cells.

In these studies, we found that IL-1α and -6 production in the supernatants of HaCaTs was increased about 26, 35% over UV-untreated control (Fig. 4. , Lane 1.2).

In the concentration of the extract at 0.01%(w/v), IL-1α and –6 production were decreased 31, 22% compared with UVB irradiated cells (Lane 5). The expression rate of interleukin was inhibited in a dose-dependent manner. In conclusion, these results show that the *Acer palmatum* extract can effectively modulate UVB-induced cytokine in HaCaT.

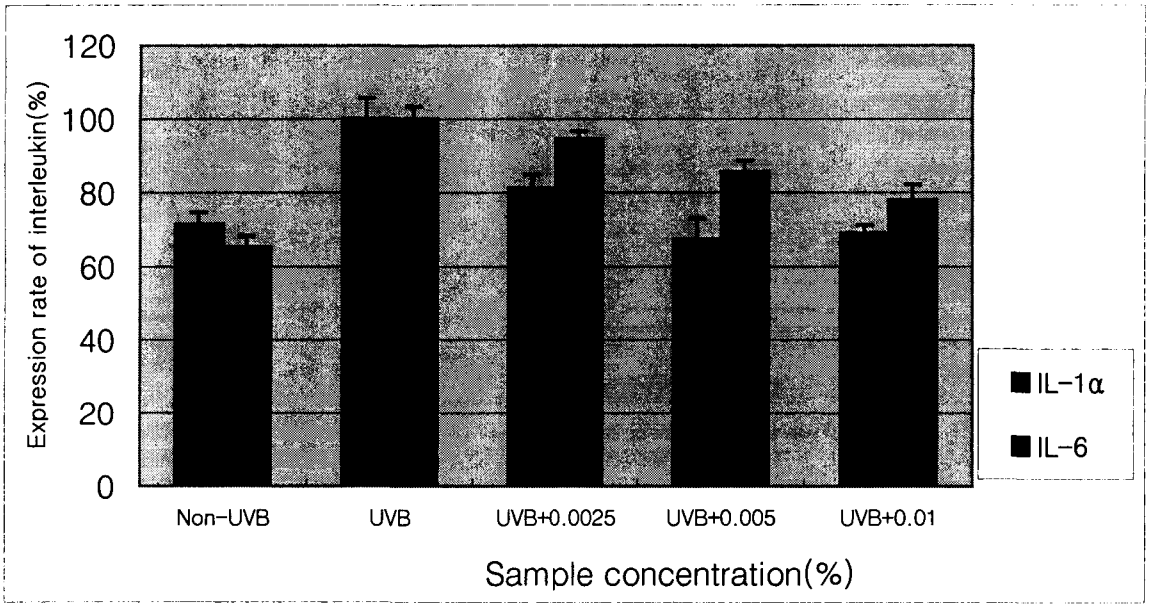



Figure 4.Expression rate of interleukin 1α and 6 after UVB irradiation with the *Acer palmatum* extract

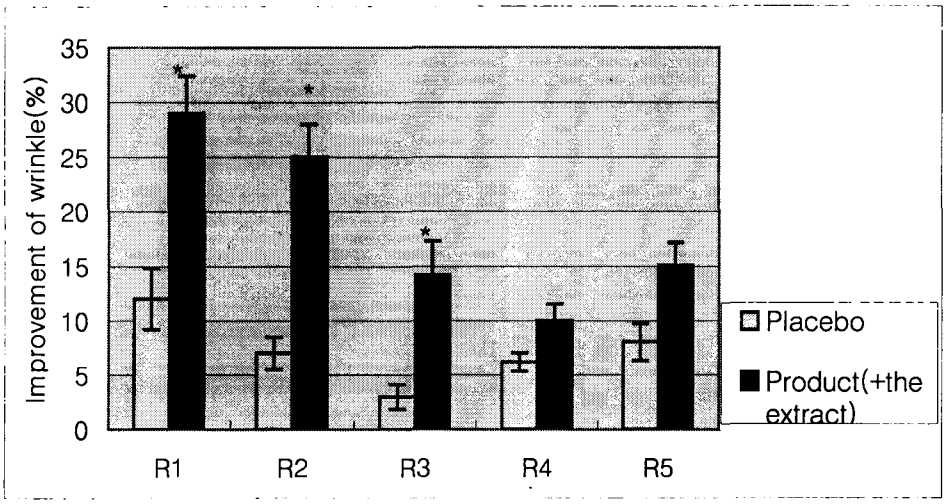
### *Anti-wrinkle effect by skin visiometer*

To estimate the anti-wrinkle effect of the *Acer palmatum* extract we used skin visiometer SV600(C+K).

After 8 weeks of application of placebo, values of R1 showed a statistically significant difference as compared with initial values ($p<0.05$). However, after 8 weeks of application the product containing the extract, values of R1, R2, R3, R4 and R5 showed a statistically

significant difference as compared with initial values ($p<0.05$) and R1, R2 and R3 showed a statistically significant difference as compared with placebo ($p<0.05$). Volunteer self-questionnaires about efficacy also showed improvement of wrinkle(data not shown).

In conclusion the emulsion containing *Acer palmatum* extract was shown anti-wrinkle effect in human clinical tests and proved to be potential cosmetic ingredient focused on anti-wrinkle effect.



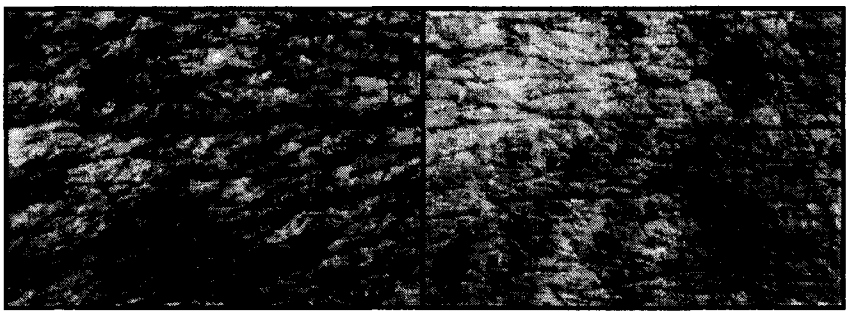

<Figure 5A> Improvement of wrinkles relative to placebo after 8weeks of application

R1 : Skin Roughness R2 : Maximum Roughness

R3 : Average Roughness R4 : Smoothness Depth

R5 : Arithmetic average roughness

* : Significally different at $p<0.05$ compared with placebo



Before after 8 weeks

<Figure 5B> Improvement of wrinkles relative to placebo after 8weeks of application

The picture of skin surface before and after application was obtained by visioscan(C+K)

***Skin penetration property of the Acer palmatum extract in O/W emulsion***

We also studied properties and effects of varying cosmetic formulations on the percutaneous absorption of the *Acer palmatum* extract . For this study, we developed the HPLC method to measured the extract concentration in the biological fluids such as skin tissue.

Figure 6 show that percutaneous penetration of the *Acer palmatum* extract from several cosmetic formulations. In this study we applied the extract 12.5 mg/0.5 ml in donor compartment. After 24 hours we measured the concentration of the extract in the skin, donor and receptor solution. Figure 6 shows the cumulative amount of penetrated extract in O/W emulsion. During 24 hours it's rarely that the extract penetrate through hairless mouse skin. But, we obtained the interesting results in the "the residue of skin" fraction of the extract. The content of the extract in skin residue is 2~3 times more highly detected in the case of the donor emulsion containng ethoxydiglycol, urea or ethanol comparing control product.

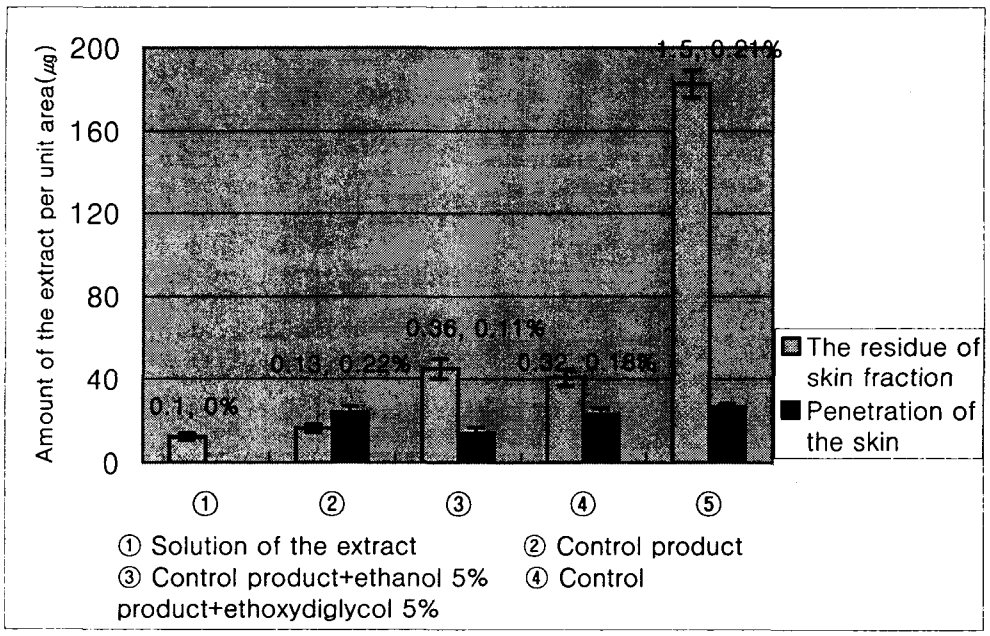



Figure 6. Cumulative amount of the *Acer palmatum* extract penetrated through hairless mouse skin over 24 hours in each emulsion

## CONCLUSIONS

In this study, *Acer palmatum* extract showed effective free radical scavenging activities and inhibition effect on MMP-1 (collagenase). In the result of the test, the extract caused strong inhibition of collagenase activity, producing an inhibition of 90% at 0.04% (w/v).

In human keratinocytes, the extract also significantly increased viability against UVB-induced cell damage and decresed the expression of IL-1 α and –6, which is key mediators of inflammation.

In skin penetration study, we showed that *Acer palmatum* extract could affect skin cells and tissue after appling our emulsion containing penetration enhancer, especially urea. The emulsion containing *Acer palmatum* extract was shown anti-wrinkle effect in human clinical tests and proved to be potential cosmetic ingredient focused on anti-wrinkle effect.

These data suggest that *Acer palmatum* extract is potent photoprotective ingredient , which prevents the inflammation and skin cell damage induced by UVB irradiation.


## ACKNOWLEDGMENT

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