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약용식물추출물의 광보호 효과와 항염증 효과 연구

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Photoprotection and Anti-inflammatory Effects of Chinese Medical Plants

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요 약: 자외선이 피부에 지속적으로 조사되면 썬번, 염증, 광노화와 같은 다양한 부작용이 생기게 된다. 따라서 광손상에 대한 보호작용은 점점 중요성이 인식되고 있으며, 천연 식물추출물에서 자외선에 대한 광보호효과가 있는 안전하고 효과적인 물질개발에 대한 연구가 진행되고 있다. 본 연구는 자외선에 의해 유도된 피부손상에 대한 보호효과가 우수한 천연식물추출물에 대한 것이다. 향나무, 능소화, 비자추출물에서 자외선에 의해 생성되는 라디칼에 대한 항산화효과, MMP 발현 및 활성 저해, 염증관련 사이토카인인 인터루킨 1 알파, 6, 프로스타글란딘 E_2 생성저해효과 등으로 피부세포보호 효과를 연구하였다. 실험결과 천연추출물 중 향나무추출물과 능소화추출물이 프리라디칼 및 슈퍼옥사이드 라디칼 소거효과가 우수하게 나타났으며, 피부 콜라겐과 같은 메트릭스를 분해하는 효소인 MMP-1의 활성 및 발현 저해효과는 섬유아세포에서 UVA 조사에 의한 실험에서 우수하게 나타났으며, 피부세포 배양액에 대해 zymography를 실시하여 활성이 감소됨을 확인하였다. 피부 각질형성세포에서 자외선에 의해 유도된 염증관련 사이토카인인 인터루킨 6의 발현량 실험에서도 무처리군에 비해 향나무추출물이 인터루킨 6을 30% 정도 저해효과가 나타났으며, 염증반응 중 cyclooxygenase (COX)에 의한 경로에서 생성되는 프로스타글란딘 E_2의 생성량도 감소시켰다. 사람피부에서 SLS (0.5%) 첩포로 유발된 자극성 피부염의 항염증 효과 평가 시 SLS에 의해 유발되는 자극정도가 피검자의 피부상태에 따라 다양하게 나타났으며, 향나무 추출물 함유 에멀젼 제품 도포 실험에서는 피부 홍반 완화 효과와 피부장벽 회복효과가 우수하게 나타났다. 이상의 결과로 향나무추출물은 자외선 조사 및 SLS에 의한 피부손상에 대한 피부세포보호작용이 우수하여 광노화에 대응하는 자극완화 소재로서의 화장품 응용 가능성을 확인하였다.

Abstract: Chronic exposure to solar radiation, particularly ultraviolet (UV) light, causes a variety of adverse reactions on human skin, such as sunburn, photoaging and photocarcinogenesis. Free radicals and reactive oxygen species (ROS) caused by UV exposure or other environmental facts play critical roles in cellular damage. And, repeated-UV irradiation activated the expression of the matrix metalloproteinase (MMP) and induced skin irritation. Therefore, the development of effective and safe photoprotectants that can reduce and improve the skin damage has been required. The purpose of this study was to investigate the photo-protective effect of several chinese medical plants (*Juniperus chinensis*) on the UV-induced skin cell damages. We tested free radical and superoxide scavenging effect *in vitro*. Fluorometric assays of the proteolytic activities of MMP-1 (collagenase) were performed using fluorescent collagen substrates. UVA induced MMP-1 synthesis and activity were analyzed by enzyme-linked immunosorbent assay (ELISA) and gelatin-based zymography in skin fibroblasts. We also examined anti-inflammatory effects by the determination test of proinflammatory cytokine, interleukin 6 in HaCaT keratinocytes. Expression of prostaglandin E_2 (PGE_2) after UVB irradiation was measured by competitive enzyme immunoassay (EIA) using PGE_2 monoclonal antibody. In the human skin we tested anti-irritation effect on the SLS-induced damage skin after appling the extract containing emulsion. We found that *Juniperus chinensis* extract had potent radical scavenging effect by 98% at 100 ug/mL. The extract of *Juniperus chinensis* showed strong inhibitory effect on MMP-1 activities by 97% at 100 ug/mL and suppressed the UVA induced expression of MMP-1 by 79% at 25 ug/mL. This extract also showed strong inhibition on MMP-2 activity in UVA irradiated fibroblast by zymography.

In the test of proinflammatory cytokines of human keratinocytes *Juniperus chinensis* extract decreased expression of interleukin 6 about 30%. The amount of PGE_2 by HaCaT keratinocytes was significantly increased at the doses of above 10 mJ/cm^2 of UVB ($p < 0.05$). At the concentrations of 3.2~25 ug/mL of this extract, the production of PGE_2 by HaCaT keratinocytes (24 h after 10 mJ/cm^2 UVB irradiation) was significantly inhibited in culture supernatants ($p < 0.05$). In

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SLS-induced skin irritation model *in vivo*, we found to reduce skin erythema and improve barrier recovery after appling *Juniperus chinensis* extract containing emulsion when compared to irritated non-treated and placebo-treated skin. Our results suggest that *Juniperus chinensis* extract can be effectively used for the prevention of UV and SLS-induced adverse skin reactions and applied as anti-aging and anti-irritation cosmetics.

Keywords: *Juniperus chinensis, skin-cell protection, anti-inflammation, prostaglandin E_2, anti-oxidation*

1. Introduction

The human skin is permanently challenged by oxidative damages generated exogenously by the environment (UV light, smoke), and endogenously by the reaction of the skin's own immune reaction: inflammatory reactions. 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 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[1].

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 metalloproteinases (MMPs). These enzymes target the components of the extracellular matrix (ECM) such as collagen, laminin, fibronectin and proteoglycan[2-5].

A UV-induced cytokine network consisting of IL-1α, IL-1β 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[8]. And IL-6 and prostaglandin E_2 (PGE_2) is key mediators of inflammation in UV-irradiated human skin[6,7].

Due to their ubiquitous use in industry, human skin is more exposed to surfactants than to any other class of chemicals. Surfantants such as sodium lauryl sulfate also destroy skin cell and induce skin inflammation[9]. So, it needs to protect skin cell from various environmental irritants such as UV and surfactants.

In order to find plant materials having protective effects on solar ultraviolet (UV) and SLS induced skin damage, various plant extracts were initially screened and the extract of *Juniperus chinensis* was selected for further study. In this work, we found the *J. chinensis* is a good antioxidative and skin protective source. We studied the inhibitory effect of proinflammatory cytokines in cultured keratinocytes and anti-irritation effect in the human skin *in vivo*.

2. Materials and Methods

2.1. Chemicals and Plant Extract Preparation

Butylated hydroxytoluene (BHT), anti-MMP-1 antibody (Ab-5), anti-human interleukin-6, sodium lauryl sulfate, 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). Collagenase inhibitory assay kit and 1,10-phenanthroline were purchased from molecular probes Co. (Eugene, OR, USA). Through a series of screening works, *Juniperus chinensis* xylem extracts was selected as photoprotective and anti-inflammatory materials. The 100 g of *Juniperus chinensis* xylem was extracted with 900 g of ethanol for 2 h at 25℃ and extracted for 4 h at 80℃. The ethanol extracts were filtered and concentrated at 40℃ in vacuum evaporator.

2.2. Antioxidative Activity

The free radical scavenging activity of the extract was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) alcoholic solution (0.1 mM) and incubated in a test plate at 37℃ for 10 min[10]. Superoxide dismutase (SOD) activity was measured using xanthine-xanthine oxidase system as a source of superoxide and nitroblue tetrazolium (NBT) as a scavenger for this radical[11].

2.3. 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 (0.2 ug/mL) in a final volume of 200 uL 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 ~495 nm and fluorescence emission maxima at ~515 nm in a fluorescence spectrophotometer (Perkin Elmer, USA).

2.4. Culture of Human Skin Cells

Human dermal fibroblasts (HDFs) from new born foreskin were acquired from Korea Cancer Center Hospital. Human HaCaT keratinocytes 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℃. HDFs from passage 6 to 10 were used in the experiments.

2.5. UV Irradiation and Determination of MMP-1 by ELISA

The expression of MMP-1 was assayed by enzyme-linked immunosorbent assay (ELISA). HDFs (8×10^3/well) were seeded into 96-well plates and cultured overnight. UVA irradiation doses were 4.2 J/cm^2 and the radiation intensity was measured using UV radiometer (EKO, JAPAN). The culture media were replaced with DMEM containing the extract. After 24 h incubation, the supernatants were transferred into a 96 well plate and incubated for 12 h. After blocking, 50 μL of diluted primary antibody, Ab-5 in PBS-T were added into each well and incubated for 40 min. Washing the wells, 50 μL of diluted secondary Ab, anti-mouse IgG conjugated with alkaline phosphatase in PBS-T was added and incubated for 40 min. After washing, 100 μL of 1 mg/mL pNPP (p-nitrophenyl phosphate) in diethanolamine buffer was added. The optical density was measured at 405 nm after 30 min.

2.6. MMP-2 Zymography

Zymography in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.15% gelatin was performed according to the method of Demeule *et al.*[12]. The samples were mixed with SDS sample buffer in the absence of reducing agent, incubated at 37℃ for 20 min, and electrophoresed on 10% polyacrylamide gels at 4℃. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h to remove SDS and incubated for 12 h at 37℃ in 50 mM Tris-HCl, pH 7.6, 0.15 NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 and then stained with 0.1% Coomassie Brilliant Blue R250.

2.7. UVB Irradiation and Determination of Proinflammatory Cytokine (Interleukin 6, Prostaglandin E_2)

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). The expression of IL-6 was assayed by enzyme-linked immunosorbent assay (ELISA)[7].

PGE_2 assay was performed by enzyme immunoassay after 24 h incubation in keratinocytes. This assay is based on the competition between PGE_2 and a PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody (enzyme immunoassay kit, Cayman chemical, Ann Arbor, MI, USA)[13].

2.8. Anti-inflammatory Effect of SLS-induced Erythema in Human Skin

The anti-inflammatory effect of the extract was evaluated in the sodium lauryl sulfate (SLS)-induced skin irritation. 10 healthy volunteers had patch tests with 0.5% SLS applied on their forearms for 24 h. After irritation of the skin all volunteers had applied an emulsion containing *J. chinensis* extract on one forearm skin twice a day for 5 days. The other forearm served as an untreated and placebo control[14]. Erythema was quantified by skin color reflectance measurements with spectrophotometer CM 2002 (Minolta, Japan). Basically, in the L*a*b*-mode a* represents the color spectrum from total green to pure red. Factor a* correlates closely with erythema values[15]. Transepidermal water loss (TEWL) is a measure for the integrity of stratum corneum (SC) barrier fuction[16,17], and it was measured with Tewameter (TM210, C+K, Germany).

2.9. 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

3.1. 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. Many investigators reported the correlation of *in vitro* antioxidative and *in vivo* photoprotective effects of plant extract[18,19].

Antioxidative activity was measured by NBT test and DPPH test. Figure 1 represents the results of DPPH test on the extracts compared with other well-known antioxidant to measure free radical scavensing effect. The activity of the extracts was comparatively as good as or higher than BHT. Figure 2 represents the results of NBT test on the extracts to measure superoxide scavensing effect and validate the correlation with DPPH test. As both of the results, the *J. chinensis* extract showed very effective radical scavenging activities.

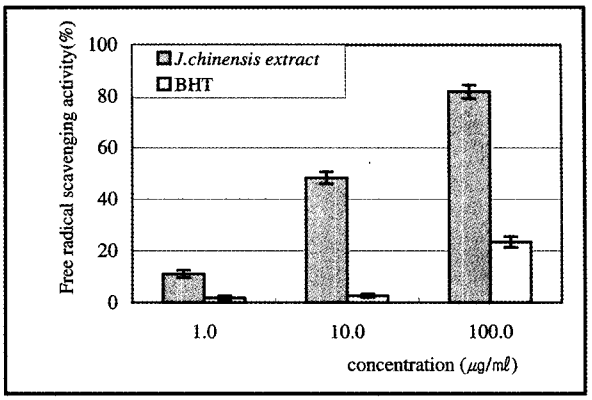

Figure 1. Free radical scavenging activity (DPPH method).

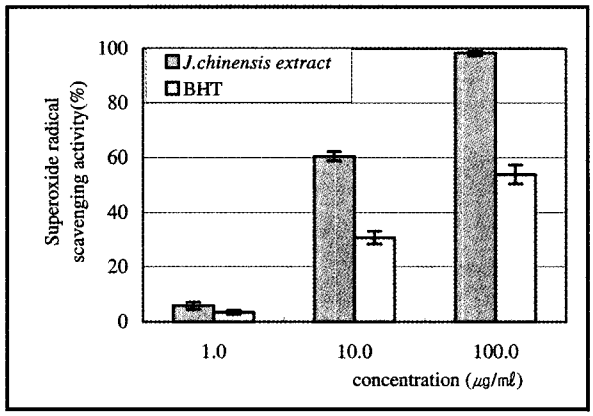

Figure 2. Superoxide scavenging activity (NBT method).

3.2. *In-vitro* Collagenase Inhibition Assays

We measured collagenase inhibitory effect of the *J. chinensis* extract. We found that 1,10-phenanthroline at 40 ug/mL effectively inhibits the activity of collagenase. In the result of the test, the extract had strong inhibition effect of collagenase activity, producing an inhibition of 97% at 100 ug/mL. The activities of MMP-1 were inhibited in a dose-dependent manner. The IC_{50} values calculated from semi-log were about 40 ug/mL. *J. chinensis* extract showed very effective inhibitory activity of MMP-1 (Figure 3).

3.3. Determination of MMP-1 by ELISA

To estimate the effect of *J. chinensis* extract on MMP expression from UVA irradiated HDFs, ELISA was used to quantify protein respectively for MMP-1 in the culture medium of HDFs. It is well documented that UVA irradiation stimulates the production of interstitial collagenase mRNA and protein in cultured HDFs[20,21]. To determine whether *J. chinensis* extract could modulate the production of MMP-1 by irradiated HDFs, *J. chinensis* extract was applied for 24 h after UVA irradiation to the cells. In the presence of added *J. chinensis* extract at 25 ug/mL, MMP-1 production was decreased about 80% compared with UVA irradiated cells, and the reduction of MMP by *J. chinensis* extract was shown in a dose dependent manner. These results indicate that *J. chinensis* extract was able to regulate MMP-1 production effectively (Figure 4).

3.4. MMP-2 Zymography

The effect of *J. chinensis* extract on MMP-2 gelatinase activities was confirmed by zymography using supernatant from UVA irradiated HDFs. We found that the gelatinase activities were proportionally increased with the UV irradiation of HDFs using gelatin zymography. In the presence of added *J. chinensis* extract at 25, 50

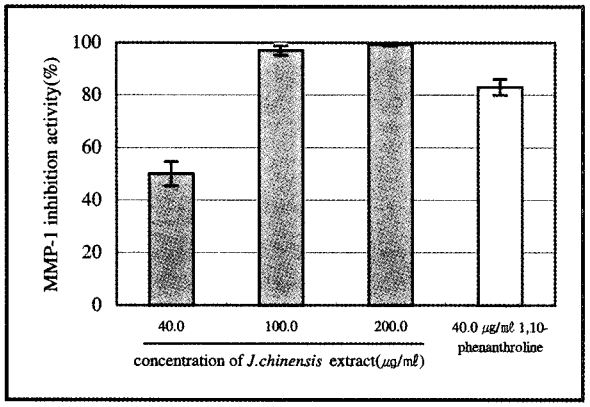

Figure 3. MMP-1 inhibition activity.

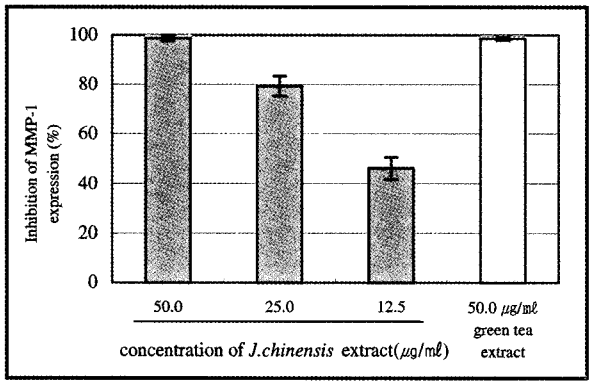

Figure 4. Inhibition of MMP-1 expression in human dermal fibroblasts.

ug/mL, MMP-2 activity were decreased compared with UVA irradiated cells, and the reduction of MMP-2 by *J. chinensis* extract was shown in a dose dependent manner (Figure 5). These data indicated that *J. chinensis* extract showed not only the inhibitory activity of MMP-1 (collagenase) but also MMP-2 (gelatinase) from UVA irradiated HDFs.

3.5. Determination of Proinflammatory Cytokine (Interleukin 6, Prostaglandin E_2)

In order to investigate the anti-inflammatory effect of the *J. chinensis* extract on the UVB response in human skin, we studied the effect of the extract on UVB-modulated IL-6 and PGE_2 expression in normal human keratinocytes (HaCaT). Before this test we screened the amount of IL-6 and PGE_2 production in various UVB doses and obtained appropriate test condition (UVB 10 mJ).

In these studies, we found that IL-6 production in

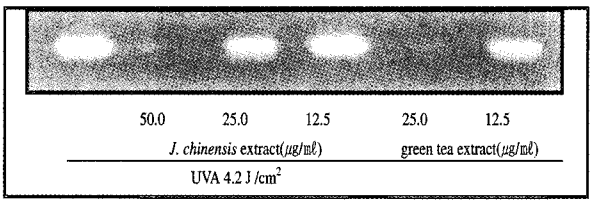

Figure 5. Inhibition of MMP-2 in human dermal fibroblast (*MMP-2 zymography*).

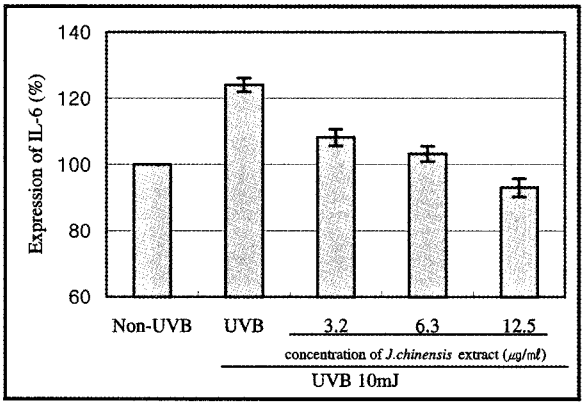

Figure 6. Inhibition of UVB-induced IL-6 expression.

the supernatants of keratinocytes was increased about 25% over UV-untreated control. In the concentration of the extract at 12.5 ug/mL, IL-6 production were decreased 32% compared with UVB irradiated cells (Figure 6). After irradiation UVB 10 mJ, the expression of PGE_2 was about 60 ng/mL, and in the concentration of the extract at 25 ug/mL, PGE_2 production was inhibited about 50% compared with UVB irradiated cells (Figure 7). The expression rate of IL-6 and PGE_2 was inhibited in a dose-dependent manner. In conclusion, these results show that the *J. chinensis* extract can effectively modulate UVB-induced proinflammatory cytokine in HaCaT keratinocytes.

3.6. Anti-inflammatory Effect of SLS-induced Erythema in Human Skin

In this short-term irritation model, skin irritant was used by 0.5% SLS. Erythema was most increased directly after removal of SLS with a slow gradual decrease thereafter. After appling *J. chinensis* extract containing emulsion for 5 days, value of a^* (erythema) was significantly reduced comparing to untreatment and placebo (Figure 8). SLS also induced a significantly high TEWL. The extract was found to accelerate skin

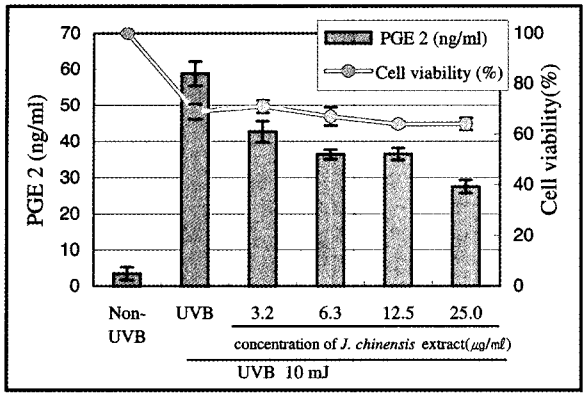

Figure 7. Inhibition of UVB-induced PGE_2 expression.

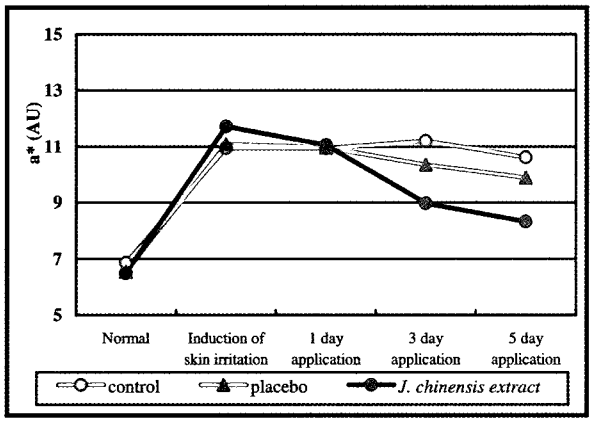

Figure 8. Changes in the a* values of spectrophotometer (Minolta, Japan) after inducing irritant contact dermatitis caused by SLS.

recovery of SLS-irritated skin after 3~5 days of treatment (Figure 9). In conclusion the *J. chinensis* extract tested in the present study was found to reduce skin erythema and improve barrier regeneration in SLS-induced dermatitis when compared to untreatment and placebo.

4. Conclusions

In this study, *J. chinensis* extract showed effective free radical and superoxide radical scavenging activities and inhibition effect on MMP-1 (collagenase). The *J. chinensis* extract had potent inhibitory effect of MMP-1 and MMP-2 expression from UVA-irradiated human dermal fibroblasts. In human keratinocytes, the extract also significantly decreased the expression of IL6 and prostaglandin E_2, which is key mediators of inflammation. *In vivo* human skin test, the extract reduced skin erythema in SLS-unduced skin irritation and improve barrier regeneration compared to untreatment and placebo.

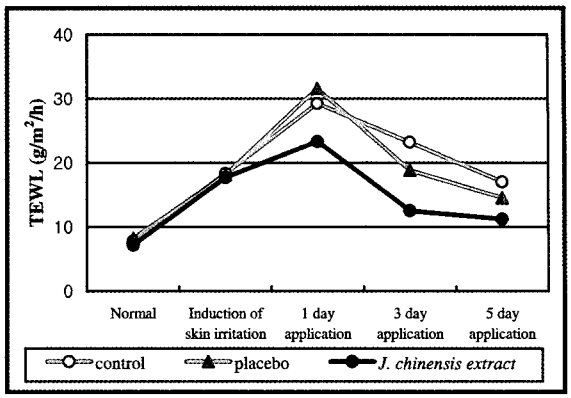

Figure 9. The effect of skin barrier recovery after appling *J. chinensis* xylem extracts on the SLS-induced irritant contact dermatitis measured by Tewameter (C+K, Germany).

These data suggest that *J. chinensis* extract is potent photoprotective and anti-inflammatory ingredient, which protect skin cells from UV and SLS induced cellular damage.

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