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화장품소재로서 유자발효물의 *In Vitro* 효능 연구

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An *In Vitro* Study on the Effect of Fermented *Citrus junos* as a Cosmetic Material

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요 약: 본 연구에서는 화장품소재로서 락토바실러스 람노서스(*Lactobacillus rhamnosus*)로 발효된 유자발효물의 항산화 활성, wound healing, 보습 및 미세먼지 차단 효능을 조사하였다. 항산화 활성을 측정한 결과, 유자발효물에서 우수한 DPPH 라디칼 소거 활성이 있음을 확인하였다. 또한, 유자발효물은 scratch-induced wound healing 실험에서 무처리군 대비 세포 이동을 증가시키는 효과를 나타내었다. 유자발효물에서 보습 인자인 필라그린의 생성량이 증가하는 것을 확인하였다. PM_{10}으로 유도된 세포 자극에 대한 미세먼지 차단 효과를 확인한 결과, 유자발효물의 세포 보호 효과를 확인하였다. 이러한 결과를 바탕으로 유자발효물의 다양한 효과가 화장품 원료 개발에 대한 과학적 근거를 제공할 수 있을 것으로 사료된다.

Abstract: In this study, we investigated the antioxidant activity, wound healing, moisturizing and anti-pollution effects of fermented *Citrus junos* (FCJ) with *Lactobacillus rhamnosus* as a cosmetic material. For the anti-oxidative activities, the FCJ showed potent DPPH radical scavenging activities. In addition, FCJ increased cell migration compared to the untreated group in scratch-induced wound healing assay. It was confirmed that the amount of filagrin (FLG), a moisturizing factor, increased in FCJ. FCJ prevented the decrease in cell viability, stimulated by PM_{10} at in vitro. Based on these results, it is believed that various effects of FCJ can provide a scientific basis for the development of cosmetic raw materials.

Keywords: *fermented Citrus junos, anti-oxidation, moisturizing, wound healing, anti-pollution*

1. Introduction

Nowadays, concerns regarding the harmful skin effect are growing as contamination of environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere[1]. Because human skin has been exposed to external stimuli such as UV radiation, microbes and particulate matter (PM). PM is a fine dust that floats for a long time in the atmosphere containing numerous pollutants along with sulfur oxides, nitrogen oxides, lead, ozone and carbon monoxide[2]. when it is deposited in the body, it acts as a toxic substance to the human body. PM produces a lot of free radicals, which are toxic substances in the human body, and these free radicals destroy collagen in

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the skin, reducing skin elasticity, causing exogenous aging[3]. The epidermis, the outermost part of the skin, provides a physical and functional barrier to prevent invasions of allergens, pathogens, and air pollutants such as PM into the human body[4-6]. However, PM skin exposure may promote the development of allergic diseases by inducing filaggrin (FLG) deficiency and skin barrier dysfunction, allowing antigen penetration through the skin[7]. Furthermore, PMs delay the wound healing of the skin by topical exposure[8]. When skin cells are exposed to air pollution factors, changes in a number of skin properties can be observed. Therefore, recently, the demand for anti-pollution cosmetics has been increasing.

Yuzu (*Citrus junos* Sieb ex TAVAKA) is a citrus fruit and plant in the family Rutaceae of east asian origin. Citron is known for containing abundant anti-oxidants such as vitamin C, flavonoids[9]. Flavonoids from plants possess various effects such as anti-inflammatory, anti-oxidant, and radical scavenging activities[10].

Fermentation is a metabolic process caused by microorganisms producing organic acids. Release of various bioactive ingredients including polyphenols and flavonoids increased during fermentation because cell wall structure and the linkages within chemical substances embedded in cell wall were broken down. So, various functional plant-based materials were often combined in order to maximize quality properties of the final products by fermentation[11].

However, the effects of fermented *Citrus junos* (FCJ) on the bioactive characteristics were not investigated. In this study, we investigated the skin improvement effects of FCJ.

2. Materials and Methods

2.1. Reagents and Instruments

Ascorbic acid and 2,2-diphenyl-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemical Co. (USA). A microplate reader (VersaMax, China) was used to measure absorbance for physiological activities search. Optical microscope (INV-100, OLYMPUS, Japan) observed to monitor the scratch wound healing.

2.2. Preparation of Fermented *Citrus junos* Extract (FCJ)

Citrus junos produced from Jeju-do (Korea) was used in this study. 1 L of water was added to 50g of *Citrus junos* powder (5% of *Citrus junos* powder [w/w]). The *Citrus junos* extract followed by heat treatment at 60 ℃ for 60 mins. The prepared *Citrus junos* extract was fermented at 32 ~ 35 ℃ for 4 days with a *Lactobacillus rhamnosus*. The fermented liquid was filtered through a membrane filter (0.45 ㎛, HYUNDAI Micro, Korea) to remove lactobacillus.

2.3. 2,2-Diphenyl-picrylhydrazyl (DPPH) Radical Scavenging Activity

The determination of the DPPH radical scavenging activities by the sample was carried out according to the method by the Blois[12]. The reaction mixture containing various concentrations of the test samples and DPPH methanol solution (0.3 mM) was incubated at room temperature at 517 nm. The scavenging activity percentage of DPPH radical was calculated according to the formula: DPPH radical scavenging capacity(%) = $[(A_0 - A)/A_0] \times 100$, Where A_0 and A are the absorbance values of the control and tested samples, respectively. Ascorbic acid were used in experiments as a positive control. The determinations were performed in triplicate.

2.4. Cell Culture

Human keratinocyte (HaCaT) were obtained from Cell Lines Service (CLS, Germany). The HaCaT Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose medium with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37 ℃ in a humidified atmosphere containing 5% CO_2. The cells were sub-cultured every 2 ~ 3 days to obtain enough quantity of cell for test.

2.5. Scratch Wound Assay

Scratch wound healing assay was used to determine the wound healing effect of FCJ was determined[13]. The HaCaT cells were seeded at 1×10^5 cells/well in 24 well plates and cultivated for 24 h. A scratch wound was made across the well, using yellow micropipette tips. The wells were washed twice with the phosphate buffer saline (PBS) and were further allowed to grow in the medium containing FCJ. The HaCaT cells were measured in the subsequent time points, including

0 h, 24 h. Cell migration was quantified as the remaining scratch wound area relative to the initial wound area using microscope.

2.6. Cell Proliferation Assay

The HaCaT cells were plated at a density of 1×10^5 cells/well in 24 well plates. After 24 h of incubation, various concentrations of FCJ (0.5, 1.0 and 2.0% in fresh medium) were used to treat the cells. The percentage viability was estimated by WST-1 assay at 450 nm after 24 h incubation, using the microplate reader.

2.7. Determination of Filaggrin Synthesis in Human Keratinocytes

The HaCaT cells were grown to high density of 1×10^5 cells/well in 24 well plates and cultivated for 24 h. Subsequently, the culture medium was changed to serum-free DMEM and cultured with or without FCJ for 24 h. At the indicated time, cell lysates were collected from each well, and the amount of FLG using an ELISA kit (Cusabio Biotech, China).

2.8. Cell Protective Effect on PM_{10}-induced Cell Damage in Human Keratinocytes

To evaluate the cell protective effect of FCJ, each concentration (0.5, 1.0, 2.0%) was pretreated 2 h before PM_{10}-induced cell damage. PM_{10} (200 μg/mL) treatment was added and incubated for 48 h. The cell viability was estimated by WST-1 assay at 450 nm after 48 h incubation, using the microplate reader[14].

2.9. Statistical Analysis

All tests were repeated three times, and the experimental results were shown as the average and standard deviation. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results and Discussion

3.1. The Anti-oxidant Activity of FCJ

Among reactive oxygen species harmful to the human body, free radicals having unpaired electrons such as hydroxyl radical (•OH) are included. In general, electrons tend to exist in pairs, so they try to react with other molecules when they are alone. Therefore, free electrons of reactive oxygen species such as •OH cause structural instability, which quickly reacts with electrons in biomolecules and damages cells. The anti-oxidant activity of the sample can be measured through a reducing power, which is the activity to scavenging radicals by providing electrons to these unpaired electrons. Therefore, the radical scavenging activity of the FCJ product was measured using DPPH existing as a relatively stable radical.

As shown in Figure 1, the *Citrus junos* extract (CJ) increased antioxidant activity by lactobacillus fermentation. FCJ showed potent DPPH radical scavenging activity in a dose dependent manner (SC_{50}: 0.6%). This result reveals that FCJ have remarkable anti-oxidant effect.

3.2. Wound Healing and Cell Proliferation of FCJ

As the largest organ of the human body, skin displays significant influence on diverse human activities and functions, including protection from pathogens, sensing of external environment, and thermoregulation. However, laying on the outermost of the human body and facing incessant conflicts of external environment, skin owing to its elastic and soft nature is susceptible to generate defects that are referred to as wounds[15]. Wound healing is the natural process of regenerating dermal and epidermal tissue in the body. During wound healing, a set of complex biochemical events take place in a

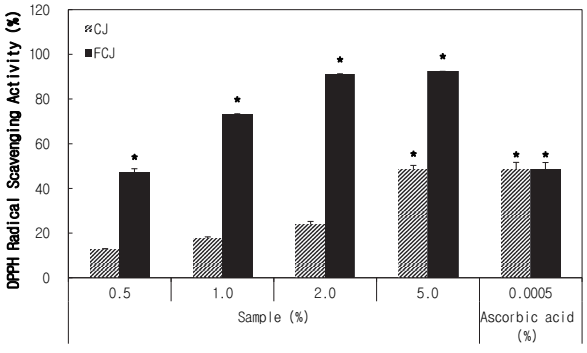

Figure 1. DPPH radical scavenging activity of FCJ. The data are expressed as a percentage of control and represent the mean ± SD of triplicate experiments. $^*p < 0.05$. CJ : *Citrus junos* extract, FCJ : fermented *Citrus junos* extract.

closely orchestrated cascade to repair the damage. These events overlap in time and may be artificially categorized into separate steps: the inflammatory, proliferative, and remodeling phases. In the inflammatory phase, bacteria and debris are phagocytized and removed, and different factors are released, which cause the migration and division of cells involved in the proliferative phase. The proliferative phase is characterized by angiogenesis, collagen deposition, epithelialization, and wound contraction[8].

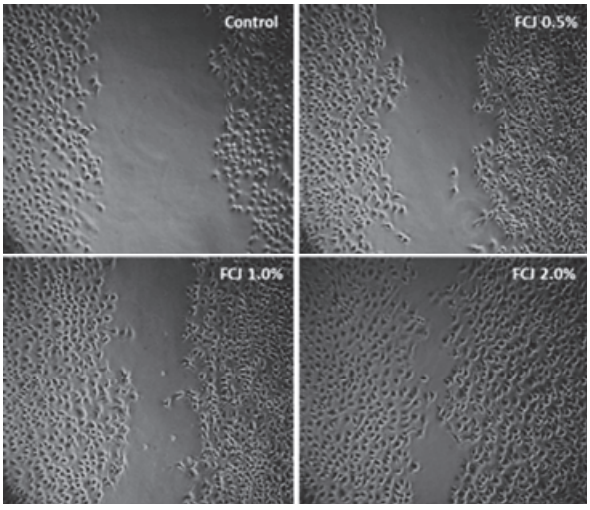

Figure 2. FCJ accelerated cell wound healing of HaCaT cells in the scratch wound assay. A scratch was produced in a monolayer of HaCaT cells and photographs were taken after 24 h of treatment with FCJ.

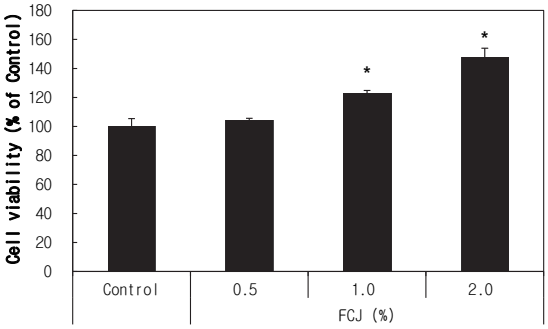

Figure 3. Effect of FCJ on cell proliferation of HaCaT cells at 24 h. An increase of cell proliferation of HaCaT cells was produced by FCJ (0.5 ~ 2.0%) after 24 h incubation, by WST-1 assay. The data represent the means ± SD of triplicate experiments. $^{*}p < 0.05$. FCJ : fermented *Citrus junos* extract.

As a step for evaluating the wound healing effect of FCJ on human keratinocytes, a scratch wound healing assay was performed on FCJ-treated HaCaT cells for 24 h. The data demonstrated that FCJ triggered HaCaT cell migration in a concentration-dependent manner at time points after 24 h of incubation compared to the control group (Figure 2). Interestingly, when FCJ was cultured for 24 h in the same culture condition, it was confirmed that cell proliferation increased in a concentration-dependent manner (Figure 3). These results suggest the possibility that FCJ can accelerate the culture of wounded monolayers of human keratinocytes by activating cell proliferation or cell migration processes.

3.3. Effect of FCJ on the Filaggrin (FLG) Synthesis in Keratinocyte

HaCaT cells were treated with CJ and FCJ at non-cytotoxic concentrations. CJ treated cells did not affect the amount of FLG production, and FCJ treated cells produced higher levels of detectable FLG than untreated cells. FCJ was increased significantly in the amount of FLG, natural moisturizing factor, in a dose dependent manner (Figure 4).

3.4. Effect of FCJ on the Anti-pollution

Cell protection effects and prevention of adsorption from particulate matter were performed to find out how FCJ affect. In HaCaT cells, cell survival was reduced by PM_{10} treatment.

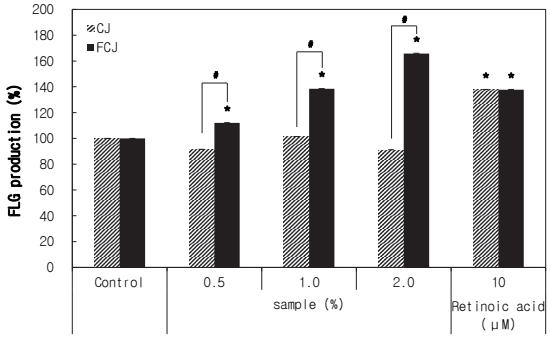

Figure 4. Effect of FCJ on filaggrin (FLG) production in HaCaT cells. Cells were treated with the indicated concentrations of FCJ (0.5 ~ 2.0%) for 24 h. The data represent the means ± SD of triplicate experiments. $^{*}p < 0.05$ compared with control group, $^{\#}p < 0.05$ compared with CJ group. CJ : *Citrus junos* extract, FCJ : fermented *Citrus junos* extract.

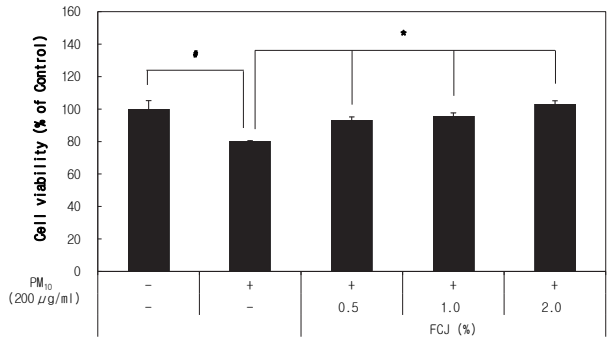

Figure 5. Effects of FCJ on the viability of HaCaT cells exposed to PM_{10}. The cells were exposed to PM_{10} (200 μg/mL) for 48 h in the absence or presence of FCJ at the indicated concentrations. The data represent the means ± SD of triplicate experiments. $^{\#}p < 0.05$ compared with control group, $^{*}p < 0.05$ compared with PM_{10}-treated group.

However, FCJ prevented the decrease in cell viability, stimulated by PM_{10}, in a dose-dependent manner (Figure 5).

4. Conclusion

In conclusion, the FCJ exhibited stronger anti-oxidant activity than CJ. In addition, FCJ showed moisturizing effect by increasing FLG, wound healing effect by cell growth and migration, and protective effect against fine dust in keratinocytes. Based on the findings in this study, it was suggested that FCJ could be potentially applicable as cosmeceutical ingredients in cosmetic industries.

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