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Research Paper

Artemisia vulgaris 발효물을 이용한 노화방지 및 주름방지 효과의 적절성

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Adequacy of the Anti-aging and Anti-wrinkle Effects of the *Artemisia vulgaris* Fermented Solvent Fraction

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Abstract: Collagen is decomposed by MMP-1, an enzyme induced by transcription factor activator protein 1 (AP-1) and matrix metalloproteinase-9 (MMP-9). Action of MMPs in inflammatory response promotes inflammatory cell movement and secretion, resulting in wrinkles on the skin. After using *Bacillus* sp. fermentation system and water, *Artemisia vulgaris* was fermented to prepare different solvent fractions using water, dichloromethane, hex ane, n-butanol, and ethyl acetate. These fractions were used to assess their effects on cell survival, wound healing, MMP-1/MMP-9 and procollagen type I C-peptide (PICP) expression, and skin turnover. MTT assay showed that cell viability of each treated group was 103% to 121%, indicating that *A. vulgaris* fractions were not toxic compared to control (cell viability: 100%). Wound healing assay revealed that wound healing ability in each treated group was 51% to 61%. This was lower than wound healing area in the control. Using RT-PCR, inhibition of MMP-1/MMP-9 gene expression was examined. As a result, each group treated with fraction showed reduced expression of both MMP-1 and MMP-9 compared to tumor necrosis factor-α (TNF-α) treatment group. Effects on collagen biosynthesis were analyzed using a PICP ELISA Kit. The group in which *Artemisia vulgaris* was treated increased collagen synthesis from 141 to 262ng/mL compared to the control group. Three-dimensional cell culture revealed that each fraction could increase skin wall formation. These results suggest that each fractions has anti-aging and anti-wrinkle effects on the skin, indicating its suitability as a functional material.

Keywords: anti-aging, anti-wrinkle, *Artemisia vulgaris* fermented solvent fraction, *MMP-1/MMP-9,* procollagen

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1. INTRODUCTION

Skin aging can be classified as intrinsic aging and photoaging. It is influenced by various environmental factors, psychological stress, and hormonal changes. [1,2] Intrinsic or endogenous aging is a constant process. However, photoaging occurs upon exposure to radiation. It can be prevented by avoiding exposure to ultraviolet (UV) rays caused by extended exposure to sunlight. [3] Intrinsic aging and photoaging share some common biological,

biochemical, and molecular mechanisms. Exposure to large amounts of UV light can reduce the amount of antioxidants and subsequently lead to generation of reactive oxygen species (ROS) in the skin. ROS such as hydroxyl, superoxide, and lipid peroxide radicals will become more reactive during this process and react with major constituents of the skin, including lipids, proteins, polysaccharides, and nucleic acids, and damage cells, causing the skin to wrinkle and ultimately age due to inadequate wound healing. [4-6] UV light is also believed to promote melanin production and produce wrinkles. Upon exposure to UV light, skin matrix proteins such as collagen and elastin fibers are damaged. Subsequently, the amount of collagen in the skin becomes insufficient and elastic fibers are denatured, thereby causing wrinkles. [7- 9]

The dermis is the second outermost layer of the skin. It primarily consists of type I collagen and some type III collagen, elastin, proteoglycan, and fibronectin. [10] Collagen imparts strength and tension to the skin, thus protecting the skin from external stimuli and stress. It accounts for 90% of the dermis. Reduction in its amount is closely associated with skin aging. [11] Metalloproteinases (MMPs) are proteins that contain zinc at the active center. They are secreted in the form of a latent zymogen *in vivo*. To become enzymatically active, structural modification, including cleavage and activation of the amino terminal region, is required. Activated MMPs are regulated by inhibitors such as α2-macroglobulin and tissue inhibitors of MMPs. The vast majority of cells, including skin keratinocytes and fibroblasts, secrete MMPs. [12,13] Among various MMPs, MMP-1 is a typical collagenase that degrades collagen type I and most of extracellular matrix and basement membrane. MMP-9 is produced by inflammatory cells such as neutrophils, macrophages, and connective tissue cells. It is known to degrade type IV and V collagens and elastin after conversion to an active form. [14,15] Thus, wrinkle formation on skin is affected by MMP factors. Suppression of wrinkle formation is mainly achieved by controlling these MMP factors. [16,17]

Artemisia vulgaris belongs to family Arduaceae. It is a perennial plant found throughout Korea with a strong breeding capacity. It has been historically used for its medicinal and antioxidant properties. It has been reported to be an effective antiaging agent. [18] *Bacillus* sp. is known to produce physiologically active substances such as peptides, antioxidants, polysaccharides such as viscous substances, and fibrin by microbial fermentation. [19, 20] It is expected that combining ***Artemisia vulgaris*** and *Bacillus* sp. can enhance the observed fermentation effects.

Thus, in this study, we will perform fermentation using two kinds of mixed microorganisms and determine their effects on skin cell regeneration. Fermentation is one common methods for developing new medicinal materials. It involves processes of fermenting natural ingredients through catalytic decomposition of microorganisms at appropriate temperatures and humidity after certain treatments. Unabsorbed glycosides in the intestine can be bioconverted into non-glycosides via fermentation, thus increasing the absorption and bioavailability of active ingredient in the body.

This study was conducted to determine the suitability of functional materials for antiaging and antiwrinkle effects by inhibiting MMP-1/MMP-9 production, cell regeneration, and collagen synthesis upon reaction with a fractionated component using N-BuOH solvent after fermentation of ***Artemisia vulgaris***.

2. MATERIALS and METHODS

2.1. *Preparation of* each fraction

Artemisia vulgaris was purchased from BS corporation (KOREA). It was grown in Korea. Hot water extraction was performed for ***Artemisia vulgaris*** fermented with two species of microorganisms: *B. methulotrophicus* and *B. subtilis*. ***Artemisia vulgaris*** and medium were mixed with glucose, yeast extract, and soytone. Fermentation was conducted at 37°C for 72 h in a fermentor (Fermentec, KOREA). After completion of fermentation, centrifugation was carried out, followed by filtration with a 0.2 μm filter to completely eliminate microorganisms. Fermented ***Artemisia vulgaris*** extract was sequentially separated into hexane, dichloromethane, ethyl acetate, n-butanol, and water fractions. Fractions were concentrated under vacuum. Each fraction was then dissolved in distilled water or Dimethyl sulfoxide (DMSO).

2.3. REAGENT

Specific primers targeting MMP-1, MMP-9, and β-actin were obtained from Bioneer (Deajeon, Chungnam, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), tumor necrosis factor-alpha (TNF-α), and cDNA synthesis kit were purchased from Sigma (St. Louis, MO, USA). Procollagen type I C-peptide (PCIP) ELISA kits were purchased from Takara Bio (Kusatsu, Shiga, Japan). DiaStar™ RT Kit and Sol-g™RNase Inhibitor were purchased from Solgent (Deajeon, Chungnam, Korea). RiboEx was purchased from GeneAll Biotechnology (Seoul, Gyeonggido, Korea).

2.4. Cell culture

HaCaT and fibroblast cells were obtained from the American Type Culture Collection (ATCC, USA). Cells were grown in Dulbecco's modified Eagle's medium (Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% antibiotics (100 mg/L streptomycin with 100 U/

mL penicillin) at 37°C with 5% CO_2. Cells were then detached using Trypsin-EDTA (Hyclone, USA) and split every 48 h to yield 1×10^6 cells/mL per plate.

2.5. MTT assay

Cells were seeded into 12-well plates at density of 1×10^4 cells/mL and cultured for 24 h. They were subsequently incubated with each fraction (100 μg/mL) for 24 h. Following incubation with test compound, cells were incubated with 40 μL MTT solution (5 mg/mL) for 1 h. After washing cells with phosphate buffered saline, 200μL of dimethyl sulfoxide (Sigma) was added to dissolve purple formazan crystals. Optical densities of solutions were quantified at 595 nm using a microplate reader (BIO-RAD Laboratories, Inc. Tokyo, Japan).

2.6. *Procollagen type I C-peptide (PCIP) ELISA*

Cells were seeded into 24-well plates at density of 1×10^5 cells/ml and cultured for 24 h. They were then incubated with each solvent fraction for 24 h. Following incubation, 100 μL of antibody-POD-conjugated solution was transferred to a 96-well plate. To each well, 20 μL sample mix was added. The 96-well plate was then sealed (e.g., with a foil) and allowed to stand at 37°C for 3 h. Subsequently, 100 μL of substrate solution was added into each well and incubated at room temperature (~20°C–30°C) for 15 min. After completion of reaction, 100 μL of stopping solution was added into each well in the same order as that for the substrate. The plate was tapped gently to allow mixture of contents. The absorbance was then measured at 450 nm using a FLUOstar Omega (BMG labtech, Germany).

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using RiboEx according to the manufacturer's instructions and cDNA was generated using a ReverseAids cDNA synthesis kit (Bioneer, Daejean, Korea) according to the manufacturer's instructions. RT-PCR was performed with the following temperature profile: pre-denaturation at 95°C for 10 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing and extension at 72°C for 30 s, and a final extension step at 72°C for 10 min. Specific primers are listed in Table 1. Cell viability, number of invasive cells, and EGFR activity were statistically analyzed using unpaired t-test (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

2.8. Wound healing assay

Cells were seeded into 6-well plates at density of 2.5×10^6/mL and cultured to reach 100% confluence. After incubation, a wound was made in the cell monolayer at the center of the well. Cells were then treated with each fraction (100 μg/mL) at indicated dose for 24 h. Wound healing was detected with a bright field microscope (Carl Zeiss, Oberkochen, Germany).

Table 1. Primer sequences target gene amplification

Gene	Primer sequence
MMP-1	F: 5'-GGTCTCTGAGGGTCAAGCAG-3' R: 5'-AGTTCATGAGCTGCAACACG-3'
MMP-9	F: 5'-GAGACCGGTGAGCTGGATAG-3' R: 5'-TACACGCGAGTGAAGGTGAG-3
GAPDH	F: 5'-TTCCTCGGTGATACCCACTC-3' R: 5'-AGGACCTTCCCGTTTCACTT-3

2.9. Organotypic 3D cell culture model

First, 6-well plates were pre-coated with type I collagen and incubated at room temperature for 10 min. Subsequently, 1×10^6 cells/mL human dermal fibroblast cells were seeded onto a 0.3 μm pore size cell culture insert plate and incubated with Matrigel and type I collagen mixture for 45 min. After the cell mixture was detached from the insert plate, 1×10^6 cells/mL of HaCaT human keratinocyte cells were placed in the mixture and incubated with complete medium for one week. After incubation, the 3D cell formation medium was placed in the bottom well and cultured for three weeks. The medium was changed every two days. An appropriate amount of *Artemisia Vulgaris* Extract (AVE) was added to the new medium and placed in the bottom well, followed by incubation for one week. 3D cell culture models were then subjected to hematoxylin and eosin (H & E) staining.

2.10. Statistical analysis

An unpaired *t*-test (SPSS, Inc., Chicago, IL, USA) was used to statistically analyze cell viability and tumor volume data. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Preparation and measurement of *Artemisia vulgaris*

Hexane, CH2Cl2, EtOAc, n-BuOH, and water were used as solvents to isolate biological active substances present in *Artemisia vulgaris*. (Fig. 1) Separated fractions were referred to as *Artemisia vulgaris* total (AVT), *Artemisia vulgaris* water (AVW), *Artemisia vulgaris* dichloromethane (AVD), *Artemisia vulgaris* hexane (AVH), *Artemisia vulgaris* n-butanol (AVB), and *Artemisia vulgaris* ethyl acetate (AVE). They were used in the experiment.

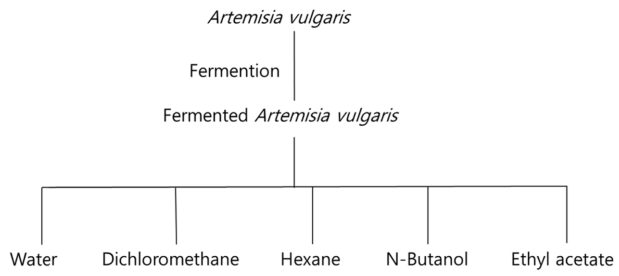

Fig. 1. Water fractionation of fermented *Artemisia vulgaris.*

3.2. Cytotoxicity assay of each fraction by MTT assay

Each fraction (100 μg/ml) was used to treat human dermal fibroblasts. MTT assay was performed to measure cell viability and cytotoxicity of each fraction to human dermal fibroblasts. Cell survival rate was observed to be > 90% at all tested concentrations. Furthermore, no fraction exhibited cytotoxicity to or inhibition on proliferation of human dermal fibroblasts (Fig. 2). Therefore, each fraction was considered suitable for further experiments on human dermal fibroblasts.

3.3. Validation of collagen synthesis activity

Effect of each fraction on collagen biosynthesis by dermal fibroblasts was analyzed (Fig. 3). Negative control produced 82.25 ng/mL of collagen. In each fraction-treated group (500 μg/mL), collagen production was increased compared to that in the negative control group. Collagen biosynthesis result was observed as follows: APT-treated group, 141.19 ng/mL; APW-treated group, 245.51 ng/mL; APD-treated group, 190.99 ng/mL, APH-treated group, 189.89 ng/mL; APB-treated group, 262.72 ng/mL and APE-treated group, 181.37 ng/mL. APB and APW exhibited excellent collagen biosynthesis. These group show

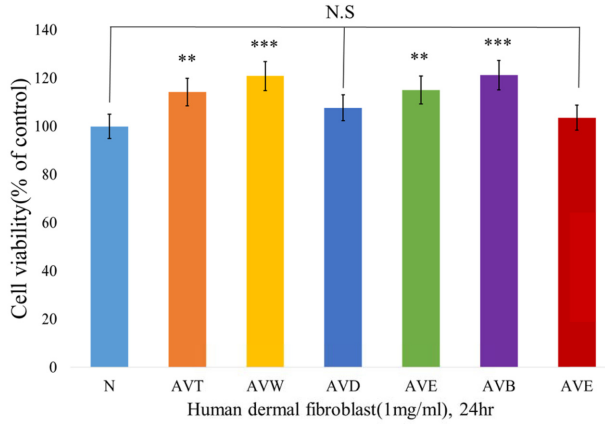

Fig. 2. Measurement of cell viability by MTT assay. Cells were treated with each fraction at 0.1% and 1% for 24 h. Statistical analysis of data was performed using an independent sample t-test. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. con (24 h). ###p < 0.001 (each experiment, n = 3). NS, not significant

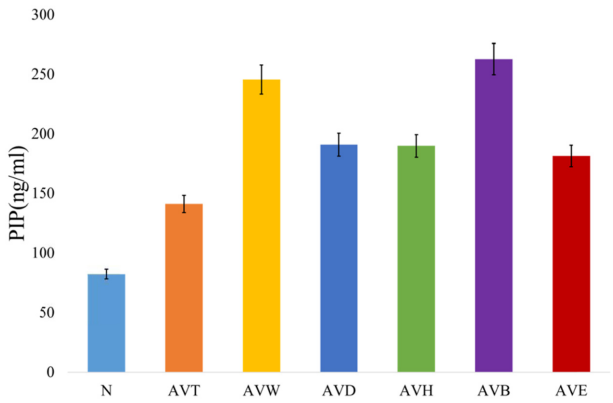

Fig. 3. Measurement of type I 5α-reductase using ELISA. Cells were treated with each fraction at 0.1% and 1% for 24 h. Type I 5α-reductase was decreased in human fibroblast cells.

approximately three times the effect of the negative control. APD, APH, and APE treatment groups showed approximately twice the effect, whereas APT treatment group showed the weakest result among all treatment groups.

3.4. Confirmation of inhibition of fraction on expression of MMP-1 and MMP-9 by RT-PCR

After treatment with TNF-α to induce inflammatory stress, cells were treated simultaneously with each fraction followed by RNA extraction and quantification. cDNA was synthesized and subjected to RT-PCR to determine expression levels of MMP-1 and MMP-9 (Fig. 4). Compared with the negative control, amounts of collagenases MMP-1 and MMP-9 were increased in the TNF-α-treated group owing to inflammatory stress. On the other hand, all fractions-treated groups showed significantly decreased levels of MMP-1 and MMP-9 compared to TNF-α-treated group.

3.5. Determination of skin regeneration effects using wound healing assay

Skin fibroblasts were wounded, treated with each fraction, and observed after 24 h to investigate effects of each fraction on

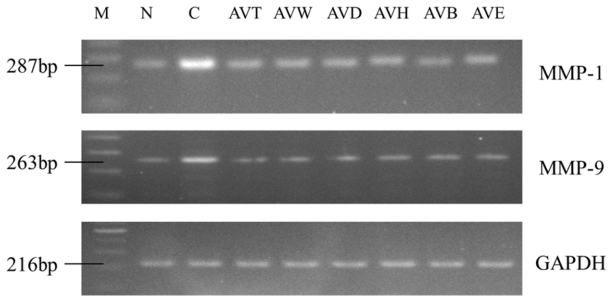

Fig. 4. MMP-1/MMP-9 pathway. Changes in GAPDH polarization protein levels and MMP-1/MMP-9 gene expression levels were examined using PCR.

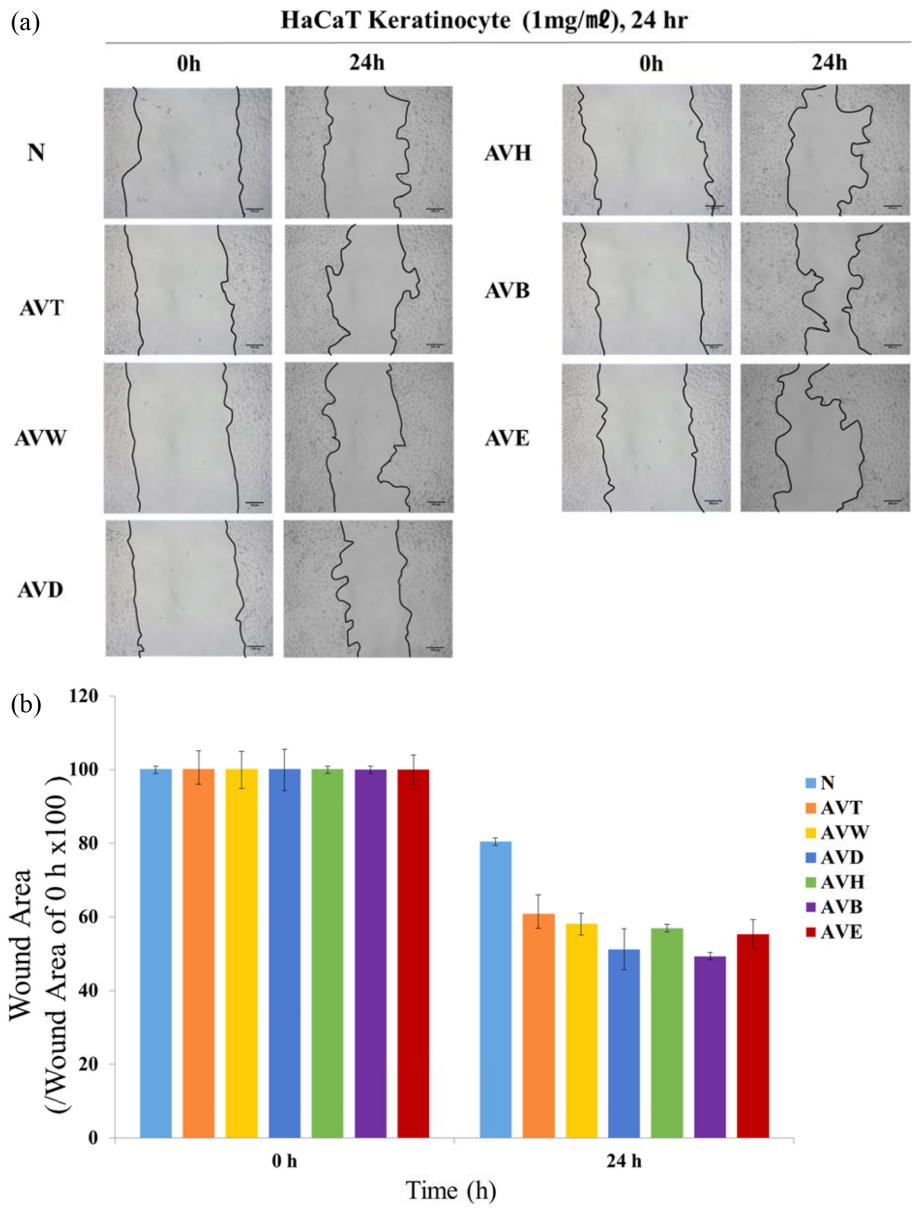

Fig. 5. Cell motility was measured using a wound healing assay. Representative images of cell migration were captured (original magnification, x10). (a) Photo taken before processing each substance in HaCaT cells (0 h). (b) Graphs of comparison before and after treating substances by graphically forming the wound area.

cell regeneration and migration. Skin regeneration using dermal fibroblasts after treatment with each fraction was confirmed (Fig. 5). In the negative control group, wound area was 80.48% while cell regeneration and migration were hardly induced after 24 h. On the other hand, in each fraction-treated group, cell regeneration rate was increased by narrowing of intercellular distance. Percentages of the wound areas were as follows: AVT-treated group, 60.85%; AVW-treated group, 58.09%, AVD-treated group, 51.24%; AVH-treated group, 56.97%; AVB-treated group, 49.35%; and AVE-treated group, 55.30%. The smaller the percentage of the wound area, the wider the cell area, indicating that cell regeneration had occurred.

3.6. Analysis of skin turnover improvement effect using 3D cell culture

To confirm the turnover effect of cells, each fraction was used for treatment with a 3D cell culture model. The 3D culture cell layer was fixed with 4% formalin. The cross-sectional layer was stained using H & E and change in thickness of cell layer was qualitatively analyzed (Fig. 6(a)). The outermost cell layer is the cell barrier. Each arrow represents the thickness of skin barrier. Compared with the control group, skin barrier formation was increased in each fraction-treated group. As a result of quantitative comparison of cell wall, cell wall thickness was 4.8 in the control, 8.8 in AVT-treated group, 8.0

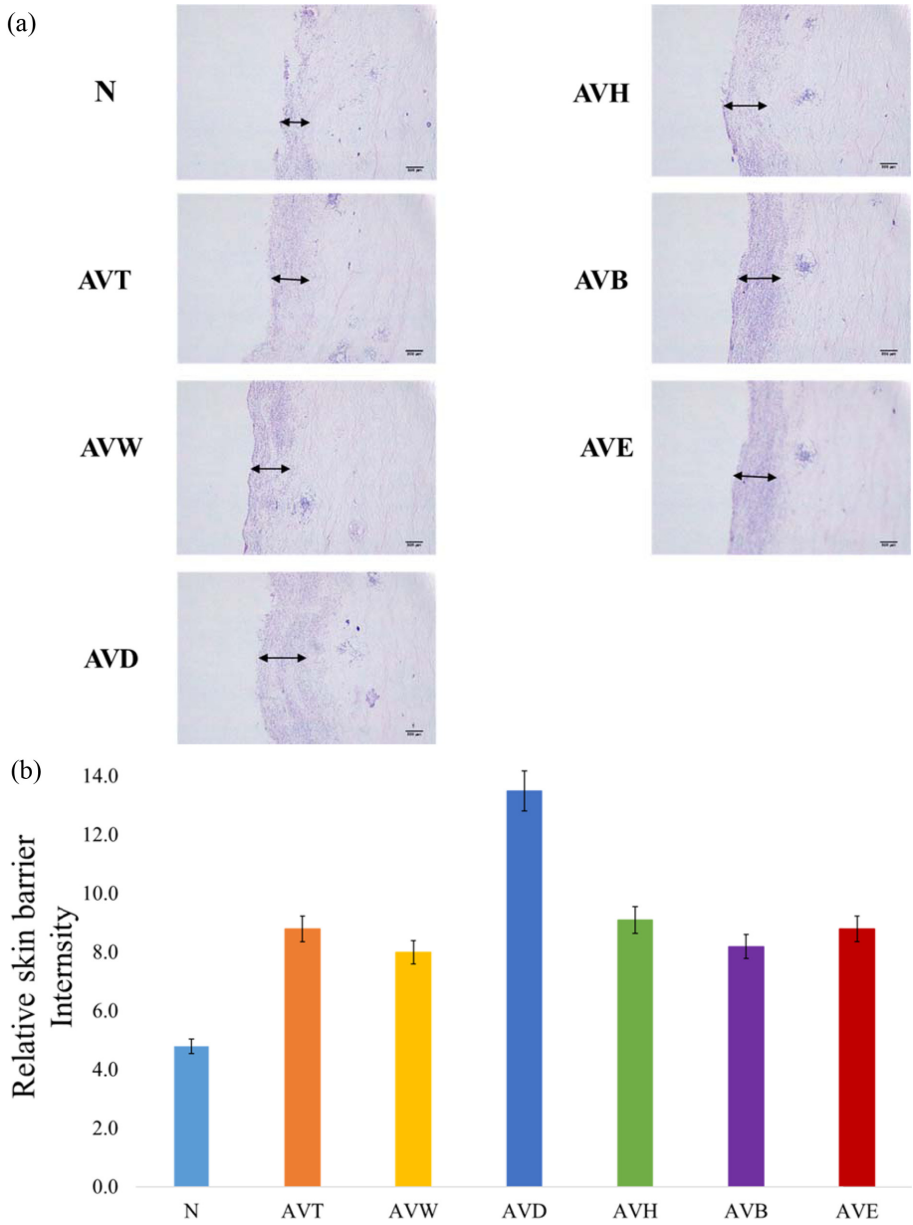

Fig. 6. Three-dimensional models of HaCaT cells and human dermal fibroblast cells were stained with hematoxylin and eosin. Sections were viewed using an optical microscope. (a) Aphotograph of a three-dimensional model after two weeks of processing of each substance. (a) (b) Graph presentation of changes in skin barrier shown in the picture.

in AVW-treated group, 13.5 in AVD-treated group, 9.1 in AVH-treated group, 8.2 in AVB-treated group, and 8.8 in AVE-treated group. Skin barrier formation is shown to increase by about two times.

4. CONCLUSION

Each fraction was not toxic. These fractions promoted collagen synthesis, MMP-1/MMP-9 inhibition, skin regeneration, and skin turnover. Different types of collagen (type I, II, III, IV, and V) are synthesized in the form of precursors called procollagen. [21,22] Procollagen comprises a peptide base sequence called propeptide at the amino and carboxy terminuses. The propeptide aids in folding procollagen molecule within endoplasmic reticulum. It cleaves itself and separates from collagen molecule after polymerization of collagen. [23,24] Therefore, the degree of collagen biosynthesis in cells can be estimated by measuring the amount of propeptides. [25] Here, we showed that more than 140 ng/mL of collagen was produced after treatment with each fraction at 1 mg/mL. Compared to controls, each fraction was found to be effective in collagen synthesis.

TNF-α is an inflammatory cytokine that increases inflammatory response and oxidative stress through production of proteolytic enzymes and ROS. [26,27] TNF-α binds to TNFR (TNF-α receptor) to activate intracellular signaling pathways and NF-κB to stimulate inflammatory response or induce cell death. [28,29] RT-PCR was performed to confirm the inhibitory effect of collagen-degrading MMP-1/MMP-9 after treatment with TNF-α. We confirmed that MMP-1/MMP-9 was increased in the induced inflammatory reaction. Compared with controls, each fraction inhibited collagen degradation by inhibiting MMP-1/MMP-9. Thus, collagen degradation factor MMP-1/MMP-9 can be removed to help maintain collagen and prevent aging. [30]

To confirm cell regeneration and migration, cells were subjected to wound healing assay. Results showed that each fraction was effective in cell regeneration and migration. These results indicate that each fraction can promote skin cell regeneration and aid skin healing and regeneration.

The epidermis is composed of several epithelial tissues, with keratinocyte as the primary constituent. In the basal layer, the lowest layer of the epidermis, cell division occurs constantly. Cell divisions rise in order of the superficial layer, granular layer, and stratum corneum. Desquamation occurs in the skin. [31,32] Cells dislodge from the basal layer in approximately 14 days. This cycle is called turnover. This division of cell layer creates a cell barrier, which acts as a defense against external factors and protects the skin. The 3D cell culture model can be used to identify turnover and cell barrier effects on skin. To confirm the effect of skin barrier formation, the 3D cell culture model was constructed and H & E staining were used to identify cell barriers. Each fraction-treated group showed effectively increased cell barrier formation compared to the control group. Thus, formation of the cell layer can protect the skin, exert an antiaging effect due to collagen synthesis, and help the skin regenerate.

Collectively, these results indicate that each fraction has a positive effect on cell barrier formation, cell regeneration, wound healing, collagen synthesis, and wrinkle improvement. Therefore, such fraction is suitable as a functional material in reducing wrinkles. As an outlook, we expect to select novel wrinkle-improving substances that are effective antiaging agents by analyzing individual component. This process is currently underway in our laboratory. Antiaging effects of new components will be reported in the future.

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