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Effects on skin of *Stichopus japonicus* viscera extracts detected with saponin including Holothurin A: Down-regulation of melanin synthesis and up-regulation of neocollagenesis mediated by ERK signaling pathway

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ABSTRACT

Ethnopharmacological relevance: *Stichopus japonicus* (sea cucumber), edible traditional food in Asia, and its extracts are renowned for their wound healing, pain relieving, and cosmetic effects in traditional medicine. Holothurins, toxins isolated from sea cucumber, are thought to be active components for their beneficial effects. However, researchers have yet to outline specific mechanisms thereof.

Aim of the study: The present study was designed to evaluate the anti-melanogenic and anti-wrinkle properties of *S. japonicus* viscera extracts (VF) on the skin via *in vitro* and *ex vivo* experiments and to assess the anti-aging effects of *S. japonicus* viscera extracts in relation to known wound healing and cosmetic processes.

Materials and methods: The viscera of live *S. japonicus* specimens were freeze dried and ground into a powder. Aqueous extracts were subsequently prepared from the concentrated powder using a water extraction method. To investigate the inhibitory effects of VF on melanogenesis, mushroom tyrosinase activity assay and melanin assay were performed on Melan-A cells. To further delineate the anti-melanogenic properties of VF, western blot analysis for tyrosinase, TRP-1, TRP-2, MITF, and ERK was conducted. Changes in collagen synthesis in human dermal fibroblast (HDF) were evaluated via CCK-8 assay and immunocytochemistry to determine the anti-wrinkle effects of VF. Finally, anti-aging properties were examined in a human skin equivalent *ex vivo* model.

Results: In Melan-A cells, VF treatment reduced melanin contents in a concentration-dependent manner. The anti-melanogenic effects of VF appeared to be due to enzymatic inhibition of tyrosinase. In CCK-8 assay, VF also significantly increased the viability of HDFs in a concentration-dependent manner. Immunoblot analysis revealed phosphorylation of ERK in HDFs treated with VF. In a human skin equivalent *ex vivo* model (Neoderm®-ED), VF treatment at a concentration of 50 μg/ml enhanced collagen type IV and Ki-67 expression and down-regulated MMP-9 expression.

Conclusion: This study demonstrated that aqueous extracts from *S. japonicus* viscera are effective whitening and anti-aging agents that stimulate ERK signaling to inhibit melanin synthesis and promote collagen synthesis.

1. Introduction

Recent trends in the search for drugs from natural sources reflect increasing interest in natural products from marine environments (Baker et al., 1995). In traditional medicine, sea cucumbers (*Stichopus japonicus*) have long been used to treat wounds and to improve skin tone: they are perceived to increase vascularity and to promote a vivid or glittering appearance (Bordbar et al., 2011; Fredalina et al., 1999; Menton and Eisen, 1973). Sea cucumbers are enriched with natural collagen, peptides, sphingosine, saponins, mucopolysaccharides,

Abbreviations: VF, *Stichopus japonicus* viscera extracts; HDF, human dermal fibroblast; MMP, Matrix metalloproteinase; ERK, extracellular-regulated kinase

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vitamins, 18 amino acids, and nutritional supplements (Bordbar et al., 2011). Also, they contain distinct substances, such as sulfated polysaccharides, chondroitin sulfates philinopside E, triterpene glycoside compounds, and glycosaminoglycan (Xue et al., 2015), that are known to have anti-microbial (Omran and Allam, 2013), anti-oxidant (Ghanbari et al., 2015), anti-angiogenic (Soltani et al., 2015), anti-inflammatory (Himaya et al., 2010), immunomodulatory (Pislyagin et al., 2014), and anti-tumoral properties (Tian et al., 2005). Interestingly, viscera specimens have been found to yield higher diversity and greater amounts of these natural compounds than body wall specimens (Bahrami et al., 2014).

Upon repeated exposure, ultraviolet (UV) radiation adversely effects the skin, inducing skin thickening, wrinkle formation, inflammation, and pigmentation (Moyal, 2004). Changes to the structure of the connective tissue that forms the skin upon UV radiation may be attributable to reduced synthesis and elevated degradation of type I collagen (Rittie and Fisher, 2002). Meanwhile, melanin, which is secreted by melanocytes and is the primary component of the pigmentation of the skin (Kollias et al., 1991), acts to protect the skin from harmful UV rays from the sun (Brenner and Hearing, 2008). However, accumulation of melanin pigments in the sun-damaged skin results in various hyperpigmentation disorders, which can be a source of great distress (Ortonne and Passeron, 2005).

In the present study, we aimed to outline the anti-melanogenic and anti-wrinkle properties of *S. japonicus* viscera extracts on the skin via *in vitro* and *ex vivo* experiments. Additionally, we attempted to determine the anti-aging effects of *S. japonicus* viscera extracts in relation to known wound healing and cosmetic processes.

2. Materials and methods

2.1. Sample preparation and extraction procedure

Live specimens of *S. japonicus* (average body weight 180 g) from the Sochong and Daechong Islands of South Korea were divided into visceral organs and body portions and rinsed in clean water. Then, the fresh viscera specimens were frozen at −80 to −90 °C. To prepare a concentrated *S. japonicus* viscera powder, the frozen viscera specimens were freeze-dried (PVTFD-100, ilShinBioBase, Gyeonggi-do, Korea) and ground in a blender. Next, 150 g of the powder were incubated in 2 L of 65% ethanol for 2 h and concentrated by a vacuum evaporator (EYELA N-12, EYEKA CA-1112, Tokyo, Japan) after filtration. Aqueous extracts were prepared from the concentrated powder of *S. japonicus* viscera using a water extraction method at Catholic Kwandong University International St. Mary's Hospital.

2.2. Culture of murine Melan-A melanocytes

Melan-A cells were obtained as a gift from Prof. Dorothy C. Bennett (St. George's Hospital, London, UK) (Bennett et al., 1987). They were cultured in RPMI 1640 medium, containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS), and 200 nM phorbol 12-myristate 13-actate, in 10% CO2 at 37 °C.

2.3. Culture of human dermal fibroblasts

Human dermal fibroblasts (HDF) were isolated from foreskin tissue. HDFs were obtained by normal skin biopsy from one male healthy donors (12 years of age) in September 2014 (Chung-Ang university hospital, Seoul, Korea) in accordance with the Ethical committee approval process of Chung-Ang University Hospital (C2015051(1509)) (Seoul, Korea). Written informed consent was obtained from the legal guardians of all donors prior to enrolment. The epidermis and dermis were separated via incubation in medium containing 0.9 U/ml dispase at 4 °C (Lee et al., 2009). After the epidermis and dermis were mechanically separated, the dermis was minced, and then plated on the surface of a tissue culture flask. The explants were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS for 1–2 weeks. Dermal fibroblasts were cultured in DMEM containing 10% FBS and 1% PS at 37 °C in a 5% CO2 incubator.

2.4. Cell viability assay

Melan-A and HDF cells were seeded in 96-well plates. After 24 h, media was removed, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and buffer was replaced with media containing *S. japonicus* viscera extracts (VF) diluted to various concentrations. After 24 h, the cells were washed with DPBS, and buffer was replaced with media containing 10% EZ-Cytox solution for 30 min. The cells were then incubated at room temperature and measured at 450 nm using a microplate reader (Molecular Devices, CA, USA).

2.5. Mushroom tyrosinase activity assay

Mushroom tyrosinase activity assay was conducted using a slightly modified protocol from that previously described (Shin et al., 2015). In brief, mushroom tyrosinase activity was assessed according to DOPA oxidase activity. A total of 190 µl of 0.1 M sodium phosphate buffer (pH 6.5) containing kojic acid and VF, as well as 30 µl of mushroom tyrosinase (1500 U/ml), were added to each well, followed by 30 µl of 5 mM L-DOPA. Absorbance was then measured at 475 nm using a microplate reader.

2.6. Melanin assay

Melan-A cells were seeded in 24-well plates. After 24 h, the plates were washed with DPBS; replenished with media containing VF, dimethyl sulfoxide (DMSO), or phenylthiourea (Roberts and Aptula, 2008); and incubated for three days. Cells were then dissolved in 120 µl of 1 N NaOH at 50 °C for 15 min. Next, the lysed cells were measured at 470 nm using a microplate reader. All results were normalized to protein concentrations of the pellet using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

2.7. Collagen measurements in cell-culture supernatants

Aliquots of cell supernatant (200 µl) were assayed for collagen levels and compared with a standard curve prepared from bovine skin using the Sircol collagen dye-binding assay, according to the manufacturer's instructions (Biocolor, Belfast, Northern Ireland).

2.8. Western blot analysis

Cells were prepared using lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate acid) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, IN, USA). Proteins were quantified using a Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA, USA), separated on 10% SDS-PAGE gel, and electrotransferred to a nitrocellulose membrane. After blocking with 5% nonfat milk, the blot was probed with antibodies against tyrosinase, tyrosinase-related protein (TRP)-1, TRP-2, microphthalmia-associated transcription factor (MITF), extracellular-regulated kinase (ERK), type I collagen, and actin (Abcam, Cambridge, UK), and then exposed to HRP-conjugated anti-mouse or anti-rabbit secondary antibodies. Protein expression was detected using an enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, NJ, USA). Protein levels were compared to a loading control, such as actin or non-phosphorylated protein.

2.9. Immunocytochemistry

Cells at 1.0 × 104 cells/500 µL per chamber were seeded in a

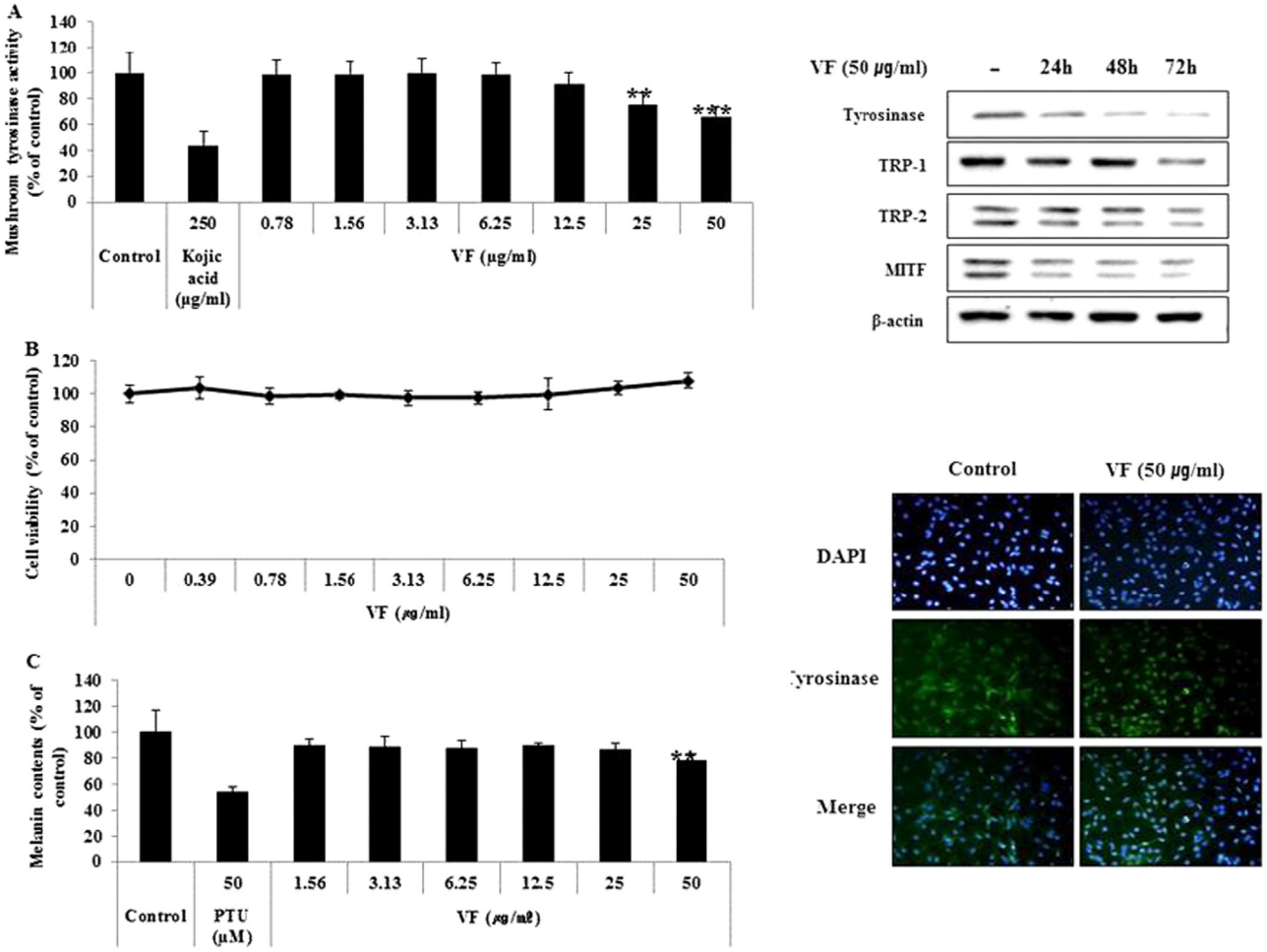

Fig. 1. Effects of *S. japonicus* viscera extract on melanogenesis in Melan-A cells. (A) *S. japonicus* viscera extracts (VF) were incubated with mushroom tyrosinase for 30 min at 37 °C. Absorbance was measured as described in the Materials and methods section. (B) Melan-A cells were treated with VF at concentrations of 0 – 50 μg/ml for 24 h. Cell viability was measured using EZ-Cytox solution. (C) The melanin contents of Melan-A cells were measured after incubation with VF or phenylthiourea (PTU; 50 μM) for three days. Each experiment was conducted in triplicate, and the data are represented as mean ± SD. $p < 0.01$ (**) and $p < 0.005$ (***), compared to control. (D) Melan-A cells were treated with VF (50 μg/ml) for three days. Cell lysates were harvested for western blot analysis using primary antibodies against tyrosinase, TRP-1, TRP-2, and MITF. Equal protein loading was confirmed using a β-actin antibody. (E) The expression of tyrosinase was examined using immunofluorescence staining and a specific tyrosinase antibody (green). Corresponding 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining and merged images are shown. Tyrosinase expression was confirmed via a fluorescence microscope. The samples were imaged using DP Controller software (x200). The results represent averages obtained from triplicate experiments.

chamber slide, serum-starved for 24 h, and then treated with VF for 48 h. After treatment with 4% paraformaldehyde for 10 min and 0.1% Triton X-100 for 5 min, the cultured cells were incubated with anti-type I collagen antibody (1:500, ab34710, Abcam Cambridge, UK) at 4 °C overnight and then with FITC-labeled goat anti-mouse IgG (1:1000, NB720-F, Novus Biologicals, CO, USA). A 4', 6-diamidino-2-phenylindole (DAPI) mounting media kit was used to counterstain the nuclei. The immunostained cells were mounted with medium containing DAPI and visualized using an Olympus FLUOVIEW FV10i confocal microscope.

2.10. Preparation of a human skin equivalent model

Neoderm®-ED, a human skin equivalent model, was purchased from TEGO Science (Seoul, Korea). Briefly, HDFs were cultured onto a collagen matrix for one day, after which keratinocytes were seeded on top of the matrix and co-cultured for four days. Next, the keratinocytes and HDF block were exposed to air. VF was then treated for 48 h: media was changed every 24 h, and the skin layer was incubated at 37 °C with 5% CO2.

2.11. Biopsy specimens and histologic measurements

Biopsied skin tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 5 μm thick sections. The skin sections were then transferred to probe-on-plus slides (Fisher Scientific, Pittsburgh, PA). Immunofluorescence staining, including that for collagen type IV and Ki-67, were performed. For immunofluorescence, sections were blocked at room temperature for 2 h in phosphate-buffered saline (PBS) containing 0.2% Triton X-100 and normal horse serum. Sections were stained using mouse monoclonal antibodies against anti-collagen type IV (1:500, ab6586, Abcam, MA, USA) and anti-Ki-67 (1:500, ab1558, Abcam, MA, USA) and incubated at 4 °C overnight. Following incubation, sections were washed three times for 5 min with 0.2% Triton X-100 in PBS. Sections were then incubated at room temperature with FITC-conjugated anti-rabbit (1:200, sc-2012, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min, and then counterstained for 5 min with DAPI.

2.12. Matrix metalloproteinase-9 inhibition assay

Matrix metalloproteinase-9 (MMP-9) activity was determined with

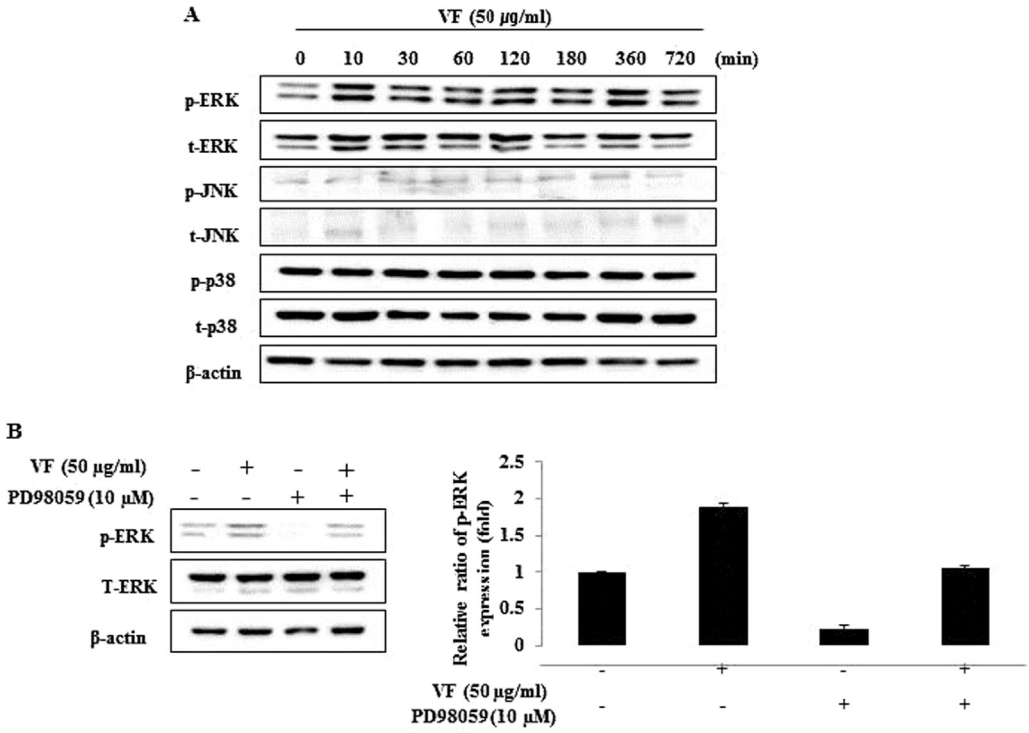

Fig. 2. Effects of *S. japonicus* viscera extract on signal transduction pathways and phosphorylation of ERK in Melan-A cells. (A) After 24 h of serum starvation, Melan-A cells were treated with viscera extracts (VF) for the indicated time period. Cell lysates were harvested for western blot analysis using primary antibodies against p-ERK, p-JNK, and p-p38. Equal amounts of protein were loaded and examined using ERK, JNK, p38, and β-actin antibodies. (B) Melan-A cells were treated with VF for 30 min in the presence or absence of PD98059 (10 μM). Cell lysates were harvested for western blot analysis using a primary antibody against p-ERK. Equal protein loading was determined using ERK and β-actin antibodies. The results represent the averages of triplicate experiments.

an MMP-9 immunoassay kit (R&D Systems, Minneapolis, MN, USA). Supernatants from the human skin equivalent model were also assayed for MMP-9 with a final volume of 200 μl per well. Samples were incubated for 2 h at room temperature and 18 h at 4 °C. Assays were performed in duplicate and completed according to the manufacturer's instructions.

2.13. High-performance liquid chromatography (HPLC) analysis

S. japonicus viscera extracts (VF) was dissolved in methanol and analyzed using an HPLC system (Agilent 1260 series, USA) equipped with a DAD detector. The extract was separated on Agilent ZORBAX SB-C18 column (5□m, 4.6 × 250 mm) at a flow rate of 0.8 ml/min. To detect Chondroitin sulfate and Holothurin A, the mobile phase was composed of 0.1% Phosphoric acid in distilled water (solution A) and 70% Acetonitrile (solution B); gradient, 0–20 min (5–5% B), 20–50 min (5–25% B), 50–50.1 (25–5% B), 50.1–55 (5–5% B) for 55 min.

2.14. Statistical analysis

Data are presented as the mean ± SD of at least three separate experiments. Statistical significance was calculated by one-way ANOVA, followed by Duncan's multiple range test. All *P*-values of $p < 0.05$*, $p < 0.01$**, and $p < 0.001$*** were considered statistically significant.

3. Results

3.1. Inhibitory effects of VF on tyrosinase activity in Melan-A cells

Melanogenesis is regulated by a tyrosinase enzymatic cascade. For this reason, various skin-whitening compounds have been developed to reduce tyrosinase activity (Smit et al., 2009). To confirm whether VF inhibits tyrosinase, we measured tyrosinase activity using mushroom tyrosinase activity assay. Indeed, VF decreased tyrosinase activity directly by 25–35% at concentrations of 25 μg/ml and 50 μg/ml (Fig. 1A). Kojic acid, a common reagent used as a tyrosinase inhibitor, was used as a positive control (Cabanes et al., 1994).

In order to confirm whether VF inhibits melanogenesis in Melan-A cells, cells were treated with the extracts. First, however, the cell viability of VF-treated Melan-A cells was confirmed using the cell counting kit-8 (CCK-8) assay. Therein, cells were treated with VF at concentrations of 0–50 μg/ml. As shown in Fig. 1B, no discernable cytotoxicity was detected at any concentration. Thus, a maximum concentration of 50 μg/ml of VF was used in all subsequent experiments. To determine whether VF regulates melanin synthesis, melanin contents were measured after VF treatment at concentrations of 1.56–50 μg/ml. At a concentration of 50 μg/ml, VF reduced melanin contents to 25% that of the DMSO-treatment control group (Fig. 1C). PTU, a well-known tyrosinase inhibitor, was used as a positive control (Oh et al., 2014).

In order to investigate whether VF affects melanogenesis signaling pathways, including the expression of tyrosinase, TRP-1, TRP-2, and MITF, Melan-A cells were treated with VF for 24–72 h. As shown Fig. 1D, tyrosinase, TRP-1, and MITF protein levels were significantly inhibited by VF treatment. Additionally, the effects of VF on tyrosinase expression in Melan-A cells were visualized using immunofluorescence (Fig. 1E). Therein, tyrosinase expression levels in Melan-A cells were inhibited following VF treatment for 72 h. All together, these results suggest that VF inhibits melanin synthesis through the inhibition of MITF and tyrosinase expression.

3.2. Effects of VF on signal transduction pathways and phosphorylation of ERK

To highlight effects of VF treatment on signaling mechanisms involved in pigmentation, we assessed changes in mitogen-activated protein kinase (MAPK) levels, including ERK, c-Jun NH2-terminal kinase (JNK), and p38, upon treatment with VF using western blot analysis. In Melan-A cells, ERK phosphorylation increased significantly at 10 min after VF treatment. However, phosphorylation of JNK and p38 remained unchanged (Fig. 2A). As the phosphorylation of ERK has been reported to inhibit tyrosinase expression, which subsequently inhibits cellular melanin synthesis (Lee et al., 2015), we attempted to confirm whether VF-induced phosphorylation of ERK signaling regulates melanin synthesis in Melan-A cells treated with VF for 30 min in the presence or absence of PD98059, a specific ERK inhibitor. Indeed, as

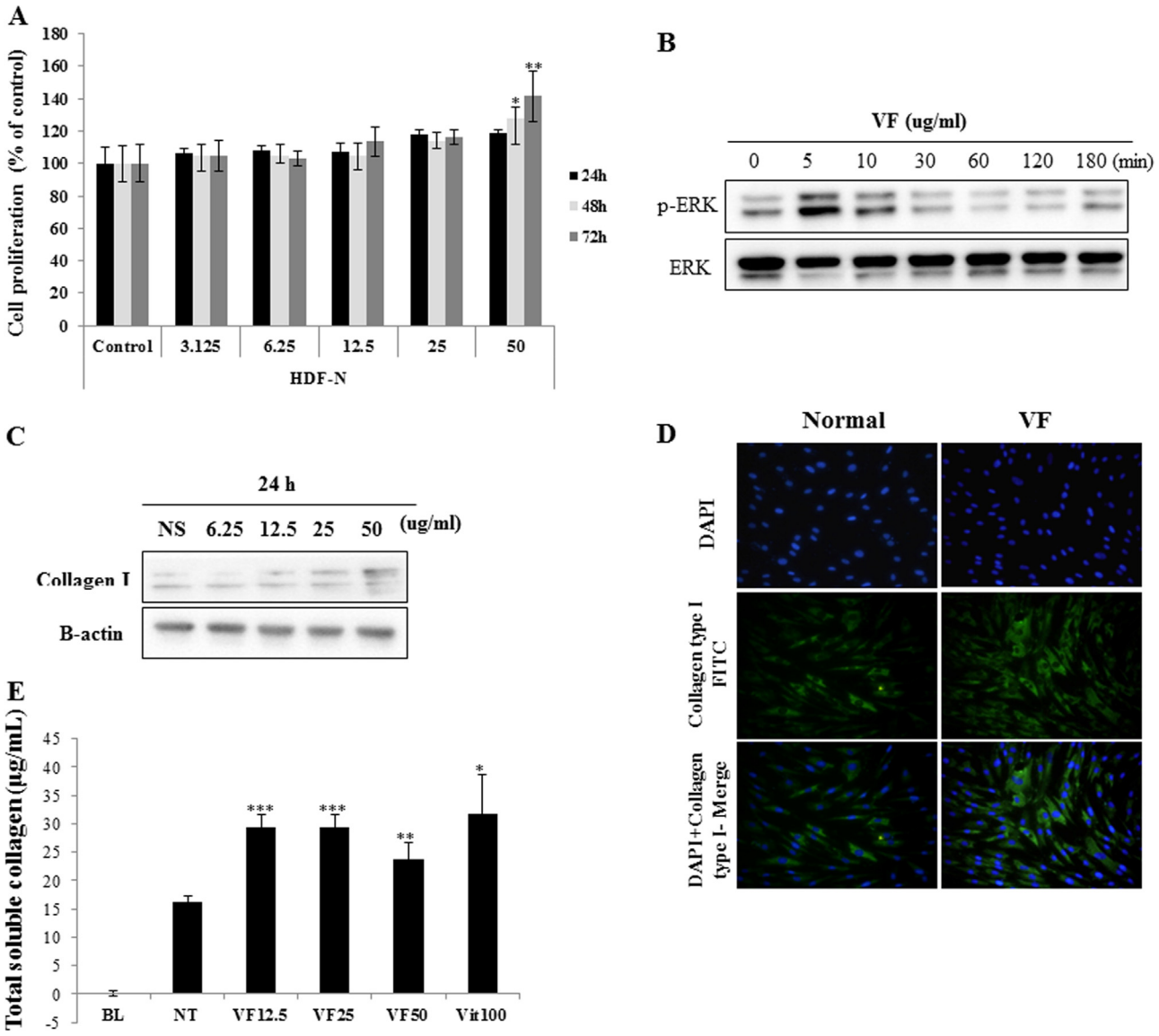

Fig. 3. Viscera extract from *S. japonicus* induces collagen production and promotes proliferation of human dermal fibroblasts. (A) Proliferative effect of VF on HDFs. Cells were treated with VF at different concentrations for 24, 48, and 72 h. The proliferation of HDFs was evaluated by cell counting kit-8 (CCK-8) assay. Values represent the mean ± SEM. (B) Cell lysates were harvested for western blot analysis using primary antibodies against p-ERK. (C) Western blot analysis for type I collagen in HDFs treated with 0 – 50 μg/ml of VF for 24 h. Equal amounts of protein were loaded and examined using ERK antibodies. (D) The expression of collagen I was examined using immunofluorescence staining and a type I collagen antibody (green). Corresponding 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining (41) and merged images are shown. (E) Histogram of extracellular secreted-collagen contents from supernatant by Sircol collagen assay at 72 h after VF treatment. Results are shown as mean ± SD. $p < 0.01$ (**) and $p < 0.005$ (***), compared to control. Each experiment was performed in triplicate.

shown in Fig. 2B, PD98059 inhibited ERK phosphorylation in VF-treated cells. These results indicate that ERK phosphorylation may be an important mechanism of VF-regulated inhibition of melanin synthesis.

3.3. VF induces collagen production and ERK phosphorylation in dermal fibroblasts

To examine the effects of sea cucumber extract on dermal fibroblasts, we first examined whether VF treatment affects fibroblast proliferation. Cells were treated for 72 h with VF at the indicated concentrations, and cell viability was measured using the CCK-8 assay. Serum deprivation for 72 h induced a significant increase in the cell viability of HDFs (Fig. 3A). To investigate the effects of VF on collagen synthesis, we measured the expression of type I collagen in HDFs. Therein, type I collagen expression in HDFs was dose-dependently increased by VF treatment (Fig. 3B).

As collagenase is primarily regulated by activation of MAPK signaling, we next examined the effect of VF on MAPK phosphorylation. Immunoblot analysis showed that exposure to VF for as little as 15 min led to phosphorylation of ERK. To verify the anti-wrinkle effects of VF on collagen production, we performed immunofluorescence staining of HDFs treated with VF. Therein, immunofluorescence staining revealed marked increases in type I collagen levels upon treatment with VF (Fig. 3C). At 72 h after VF treatment, soluble collagen concentrations in the cell culture media were determined using a Sircol collagen assay (Fig. 3E). Fig. 3D&E show that both intracellular collagen production and extracellular collagen secretion were markedly increased after 72 h of treatment with 50 μg/ml of VF. Thus, our results demonstrated that VF directly activates type I collagen production and fibroblast proliferation via the ERK 1/2 signaling pathway.

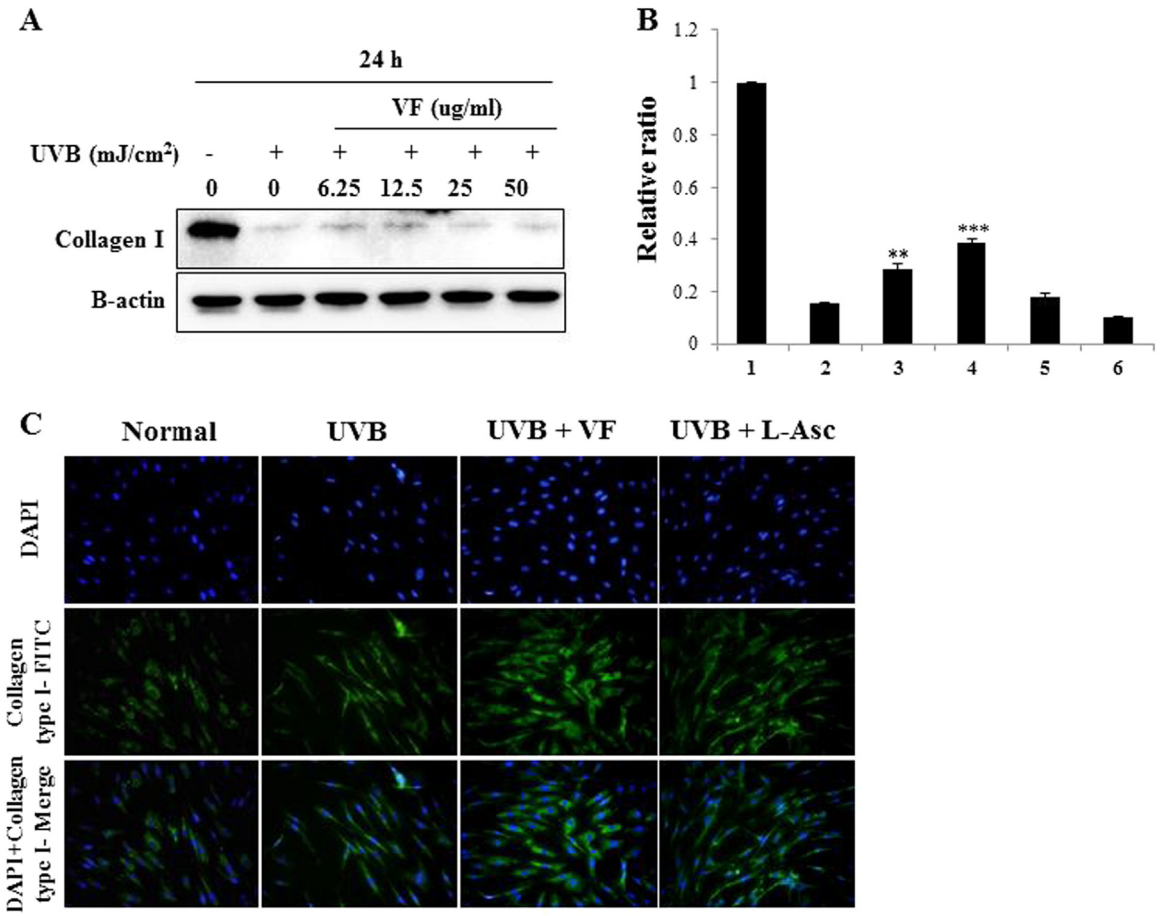

Fig. 4. Effects of *S. japonicus* viscera extract on type I collagen levels in human dermal fibroblasts upon UVB radiation. (A) Cells were treated with 0 – 50 μg/ml of VF after 30 mJ/cm2 of UVB radiation. Western blot analysis was performed to detect type I collagen protein levels in HDFs. (B) Blots were quantified by densitometry as percentages of the control. $p < 0.01$ (**) and $p < 0.005$ (***), compared to control. (C) Cells were treated with 100 μg/ml of L-Asc after 30 mJ/cm2 of UVB radiation. Collagen I expression was examined according to immunofluorescence staining and type I collagen antibody (green). Corresponding 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining (41) and merged images are shown. VF: *Stichopus japonicus* viscera extract, UVB: Ultraviolet B, L-ASC: L-ascorbic acid.

3.4. VF restores collagen synthesis and attenuates MMP-1 expression in a human skin equivalent model

To determine the effects of VF on collagen production after exposure to UV light, we examined type I collagen expression in biopsied tissue irradiated with UVB radiation. As shown in Fig. 4A&B, emission of 30 mJ/cm2 of UVB radiation suppressed type I collagen levels in HDFs, although they were subsequently restored by treatment with 12.5 μg/ml of VF. Immunofluorescence assay highlighted significant increases in type I collagen protein after treatment with VF for 72 h, compared to HDFs treated with UVB alone. Importantly, the effect of VF was stronger than that of L-Asc, which was used as a positive control (Fig. 4C). These results indicate that VF treatment could effectively restore the capacity to synthesize collagen among dermal fibroblasts.

Next, to examine the effects of VF in a skin equivalent model, collagen type IV synthesis and MMP-9 expression were examined via immunofluorescence staining and ELISA, respectively: type IV collagen is the most abundant structural basement membrane component, and MMP-2 and MMP-9, type IV collagen-specific collagenases, participate in extracellular matrix degradation. In the Neoderm®-ED skin equivalent model, we found that VF promoted collagen type IV and Ki-67 expression (Fig. 5A and B) and downregulated MMP-9 expression (Fig. 5C). At a concentration of 50 μg/ml, VF activated Ki-67-positive cells by 48.3%, compared to 12.5% for cells treated with vehicle alone. Furthermore, 50 μg/ml of VF effectively decreased MMP-1 enzyme activity in the Neoderm®-ED model. Our observation that VF effectively promotes collagen type IV and Ki-67 activity suggests that VF might stimulate extracellular matrix components to induce re-epithelialization.

3.5. The detection of chondroitin sulfate and Holothurin A from VF by HPLC analysis

To evaluate active ingredients of VF associated with skin-regeneration function, HPLC analysis was performed with VF. From HPLC profile, chondroitin sulfate was detected at 6.219 min, the area was 2025.4 mAU*s, and the area percent was 43.59%. Also, Holothurin A showed at 10.840 min, the area was 863.2 mAU*s, and the area percent was 18.57% in VF (Fig. 6).

4. Discussion

Many researchers are interested in various active molecules from sea cucumber extracts because of their natural origin, edibility, and lack of toxic effects. Whole sea cucumber has been found to exhibit antioxidant with hepatoprotective properties (Esmat et al., 2013), as well as anti-inflammatory and immunomodulatory effects (Smith, 1978). Despite the use of sea cucumber extracts for food and traditional medicinal purposes, their specific properties have not yet been examined in detail, although, recently, the active ingredients in viscera and body wall extracts were elucidated (Bahrami et al., 2014), (Dhinakaran and Lipton, 2014). Moreover, methods to isolate and purify individual bioactive

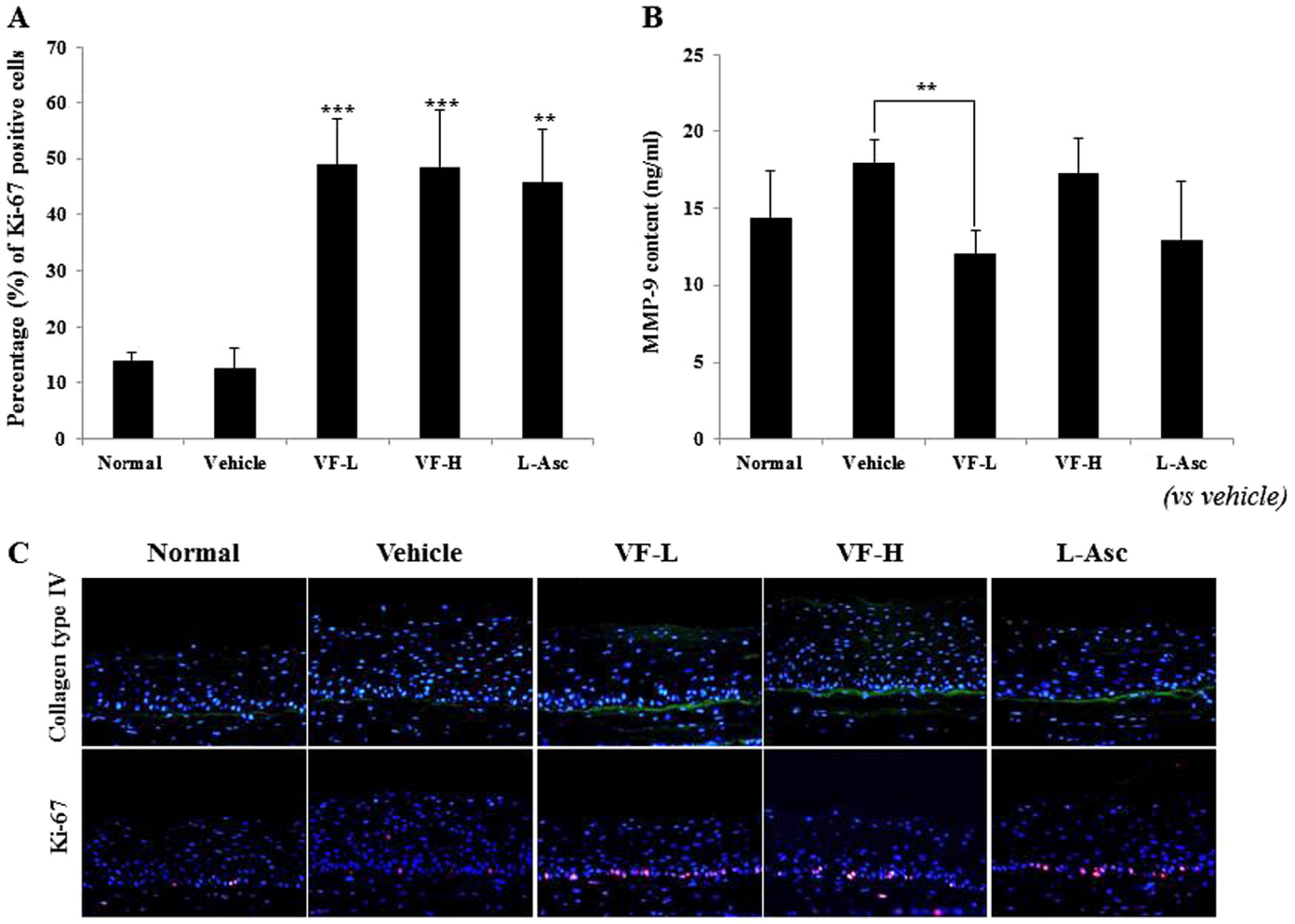

Fig. 5. Viscera extract from *S. japonicus* enhances collagen type IV and Ki-67 expression in Neoderm®-ED. (A) Neoderm®-ED was treated with various concentrations of DMSO (0.1%; Vehicle control), VF (50, 500 μg/ml), and L-Asc (100 μg/ml). Serial sections from the Neoderm®-ED were mounted onto silane-coated slides and subjected to immunofluorescence staining using anti-collagen type IV and anti-Ki-67 antibodies as described in the Materials and methods section. (B) The bar graph represents the average number of Ki-67 positive cells from immunofluorescence assays. Data (n = 4) represent the means ± SD. (C) Measurement of MMP-9 production in supernatants. $p < 0.01$ (**) and $p < 0.005$ (***), compared to vehicle control.

compounds in sea cucumber extracts need developing, and studies of the medicinal value of the natural products found in sea cucumber extracts, which could be utilized in pharmaceutical or cosmetic applications, are lacking. Lastly, we stress that the compositions and concentrations of sea cucumber extracts for use in clinical applications must be standardized.

Tyrosinase is an crucial enzyme in melanogenesis of melanin in mammalian cells (del Marmol and Beermann, 1996). Previously, researchers found that tyrosinase acts a key role in catalyzing the hydroxylation of monophenols (tyrosine) to o-diphenols and their subsequent oxidation to o-quinones (Munoz-Munoz et al., 2011). Additionally, tyrosinase activity has been linked to cancer (Jawaid et al., 2009) and neurodegenerative disease (Paun et al., 2015). Inhibitors of tyrosinase have found use in cosmetic and pharmaceutical applications. In this study, we discovered that VF down-regulates tyrosinase activity and inhibits cellular melanin synthesis in Melan-A cells. Also, VF significantly suppressed protein levels of MITF, TRP-1, and TRP-2 in Melan-A cells. In general, the down-regulation of MITF and TRP is considered an important marker of reduced melanogenesis. Thus, our results suggest that VF effectively down-regulates MITF and TRPs to inhibit the production of melanin by melanocytes.

The MAPK family of proteins, including ERK, JNK, and p38, play an important role in melanogenesis (Ng et al., 2014) and collagenogenesis via activation of MMPs (Zeldich et al., 2007), both directly and indirectly. Thus, we aimed to examine the influence of VF on the activation of ERK, JNK, and p38 MAPKs in an attempt to further understand the molecular mechanisms involved in VF treatment. In doing so, we found that VF treatment stimulates the phosphorylation of ERK in both melanocytes and fibroblasts. This finding suggests that VF exerts its anti- melanogenic and collagenogenic effects through MAPK mediated pathways.

Fibroblasts perform a crucial function in wound healing, as they produce the majority of extracellular matrix components required for re-epithelialization (Brown, 2004). In the present study, VF was shown to promote the proliferation of dermal fibroblasts and procollagen synthesis in a concentration-dependent manner. According to several reports, the transcription factor endothelin (ET)-1 is activated by ERK signaling (Ahmedat et al., 2013), whereby it plays a crucial role in tissue remodeling and fibrogenesis by induction of the synthesis of collagen I via protein kinase C (Horstmeyer et al., 2005). Interestingly, we discovered that VF stimulates the phosphorylation of ERK in HDFs. In light of these results, we suggest that VF effectively stimulates the proliferation of fibroblasts and ERK signaling to promote collagen synthesis.

Human skin equivalent models are three-dimensional cell culture systems developed to overcome the limits of two-dimensional cell culture systems (Wang et al., 2014). These models have seen frequent use in studies of skin diseases, such as ichthyosis (Rosenberger et al., 2014)

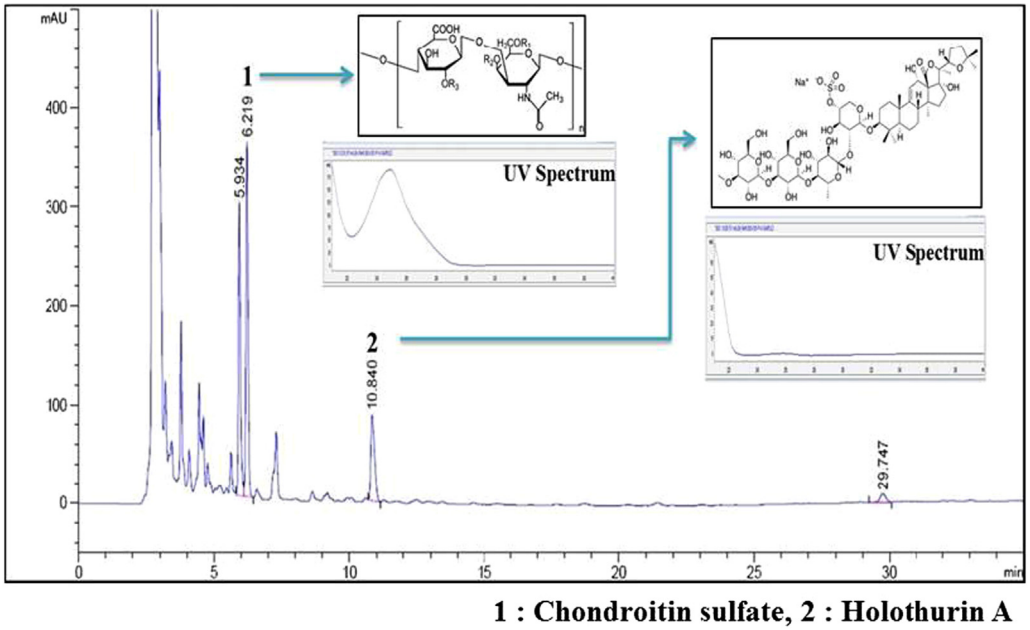

Fig. 6. HPLC profile of viscera extract from *S. japonicus* and its verified components. Chondroitin sulfate was detected at 6.219 min, the area was 2025.4 mAU*s, and the area percent was 43.59%. Also, Holothurin A showed at 10.840 min, the area was 863.2 mAU*s, and the area percent was 18.57% in VF.

and inflammation (van den Bogaard et al., 2014). In the present study, VF effectively decreased the activity of MMP-9 enzyme our human skin equivalent model, Neoderm®-ED. Also, VF treatment in the model induced the expression of collagen type IV and Ki-67, which play a role in regulating extracellular matrix components and stimulating wound healing.

In the chemical analysis of VF, chondroitin sulfate and Holothurin A were detected with HPLC. In the previous studies, chondroitin sulfate was shown as active component of anti-inflammatory, anticancer, antiviral, and anti-coagulant functions in the body of sea cucumber (Pomin, 2014). Also, Bahrami et al. (2014) reported that various novel saponins were in the viscera of Holothuria lessoni. Especially, Holothurin A among saponins, isolated from sea cucumber *Pearsonothuria graeffei*, showed anti-metastatic activity through down-regulation of MMP-9 and up-regulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) in chick embryo using the chorioallantoic membrane assay *in vivo* study (Zhao et al., 2010). Similarly, our study revealed that chondroitin sulfate and Holothurin A were found in the viscera of *S. japonicus* using HPLC. Even though our experiments were not performed with pure filtrated chondroitin sulfate and Holothurin A detected in VF of *S. japonicus*, alone. It is considered that they might decrease the expression of MMP-9 and increase collagen synthesis via ERK signaling pathway in human skin equivalent model. In the future, further experiments will be carried out with purified chondroitin sulfate and Holothurin A from VF of *S. japonicus*.

In conclusion, we suggest that VF can be used in functional cosmetics to improve signs of aging and to whiten the skin. While our results suggest that VF inhibits cellular melanogenesis and promotes collagen synthesis, further studies are needed to examine the anti-aging effects including anti-wrinkle and anti-melanogenic functions of cosmetic formulations containing these bioactive compounds on human skin *in vivo*.

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Author contributions

Tae-Rin Kwon, Chang Taek Oh, Beom Joon Kim, and Heesu Kim conceived and designed the experiments; Tae-Rin Kwon, Chang Taek Oh, Dong-Ho Bak, Jong Hwan Kim, Joon Seok¸ Jong Hoon Lee¸ Su Hwan Lim performed the experiments; Kwang Ho Yoo, Beom Joon Kim, and Heesu Kim analyzed and interpreted the data; Tae-Rin Kwon, Chang Taek Oh, and Heesu Kim wrote the paper; all authors contributed to the final approval of manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.08.007.

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