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β-Glucogallin isolated from *Fusidium coccineum* and its enhancement of skin barrier effects

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Abstract

Soil has been used for treatment of wound and skin diseases and for cosmetic purposes. *Fusidium coccineum* (FC) SA-1FC (Ascomycota) is a fungus found in nature, and its by-products are present in humid soils with plant humus. This study investigates the medium of fermented FC as a covering for all skin problems, including dryness, inflammation, and wounds. A preliminary study revealed that an alcohol extract of FC had a skin-enhancing effect, and thin-layer chromatography revealed a major component in a non-polar fraction. Here we identify a major compound isolated from a non-polar fraction as *β*-glucogallin. The mRNA levels of filaggrin and HAS3 are upregulated by FC and *β*-glucogallin treatment in keratinocytes and immortalized human keratinocytes cells. In addition, FC and *β*-glucogallin exert anti-inflammatory effects by suppressing expression of interleukin-4/poly(I:C)-induced chemokines and inflammatory cytokines. In fibroblasts, Hs68 cells, FC and *β*-glucogallin stimulate cell migration. These results suggest that FC and *β*-glucogallin can enhance skin barrier function.

Keywords: *β*-Glucogallin, Filaggrin, *Fusidium coccineum*, HAS3, Keratinocyte

Introduction

Soil, which is composed of inorganic and organic substances, has long been used for treatment of wounds and various skin diseases and for cosmetic purposes, as confirmed by Egyptian records, in particular. *Fusidium coccineum* (FC) SA-1FC is a fungus found commonly in nature, and its by-products are typically present in humid soils plant humus. A fungal species belonging to the Ascomycota family, strain SA-1FC was first isolated from soil in 2017. *Fusidium coccineum* has been widely used as a strain that produces a substance called Fusidic acid in the past, and many studies are focused on the production and analysis of fusidic acid [1–3]. However, there are no research results on the effect of *Fusidium coccineum* on the skin, and in this study, various effects and functional substances of skin cells were screened as metabolites of *Fusidium coccineum*. In addition, effective substances were found for skin cell regeneration-recovery-soothing-moisturizing, etc., and results were derived for efficacy and material properties. Although FC can produce antibiotics, such as fusidic acid for inhibiting skin infection, special processes are required to extract these products [1, 2]. In this study, we cultured FC SA-1FC under conditions that maximized its proliferation after approximately 7 days. In general, the mycelium was well formed and the growth time was 7 days [3]. Also, *β*-glucogallin was isolated from FC culture medium as a new active compound. While *β*-glucogallin has many biological activities, like anti-inflammation, anti-oxidation, anti-cancer, and photo-protection [4], the effects of FC and *β*-glucogallin on skin hydration in human epidermal

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keratinocytes have received little attention. We investigated the efficacy of FC and β-glucogallin on skin barrier development and regeneration of keratinocytes and fibroblasts.

Skin protects organisms against external environmental damage and water loss. The stratum corneum (SC), the outermost layer of the epidermis, is composed of terminally differentiated keratinocytes [5]. Corneocytes are surrounded by an insoluble protein structure, also known as the cornified envelope (CE), and high concentrations of natural moisturizing factors (NMFs) exist within corneocytes. Both the CE and NMFs play pivotal roles in skin hydration and barrier functions [6–8]. Keratinocyte differentiation is required for skin hydration and barrier functions, and CE and NMF dysfunction can lead to defective skin. Inflammatory skin diseases such as itching, erythema, and inflamed lesions are caused by impaired inflammatory and immune responses. Pro-inflammatory cytokines, such as tumor necrosis factor alpha, interleukin (IL)-6, and IL-1, are important mediators in many inflammatory diseases and are expressed at high levels in lesional skin of atopic dermatitis (AD) patients. In AD, keratinocyte-derived thymic stromal lymphopoietin (TSLP) enhances the release of cytokines, which trigger itch-evoked scratching, inflammation, and development of AD [9, 10]. As a defensive response to protect the human skin from harmful environmental materials, wound healing is important and consists of three phases: inflammation, proliferation, and remodeling. The proliferative stage refers to the period of fibroblast regeneration and can induce cell migration associated with regeneration in the wound healing process [11].

In the present study, β-glucogallin was isolated from FC as a principal compound for skin protection. We investigated the effects of FC and β-glucogallin on skin barrier development and skin regeneration. These results suggest that FC and β-glucogallin are potential ingredients to enhance skin barrier function, as showing skin effects in every aspect.

Materials and methods

Fungus materials

Samples of FC were provided from SINABT. Co., Republic of Korea. A voucher specimen (KHU2018-0036) was stored at the NPCL (Natural Products Chemistry Laboratory), Kyung Hee University, Yongin, Republic of Korea.

General experimental procedures

The materials and methods used for this study were same as those in the previous study [9].

Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz FT-NMR spectrometer (Varian Inova AS-400, Palo Alto, CA, USA). IR spectra were obtained from a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). Fast atom bombardment mass spectrometry (FAB/MS) spectra were recorded on a JEOL JMS-700 (Tokyo, Japan). Melting points were measured using a Fisher-John's melting point apparatus (Fisher Scientific, Miami, FL, USA) with a microscope.

Extraction and isolation

Preparation of methanol (MeOH) extract from FC and solvent fractions and ethyl acetate (EtOAc) (SHE, 4.5 g) and normal-butanol (*n*-BuOH) (SHB, 19.0 g) fractions (Frs) followed procedures described in a previous study [12].

SHE was subjected to silica gel column chromatography (SiO_2 CC) (CC, 5.0 × 22.0 cm, *n*-hexane–EtOAc (HE) = 10:1 → 5:1 → 3:1 → 1:1, 2000 mL of each → chloroform ($CHCl_3$)-MeOH (CM) = 10:1 → 7:1 → 5:1 → 3:1 → 2:1 → 1:1, 1500 mL of each) to produce 19 Frs (SHE-1 to SHE-19).

SHE-12 [57 mg, elution volume/total volume (EV/TV) 0.312–0.454] was applied to the SiO_2 CC (3.0 × 15.0 cm, HE = 10:1 → 7:1 → 5:1 → 3:1 → 2:1 → 1:1, 1000 mL) to yield eight Frs (SHE-12–1 to SHE-12–8). SHE-12–4 (49 mg, EV/TV 0.040–0.080) was applied to a Sephadex LH-20 CC (2.5 × 14.0 cm, 75% aqueous MeOH, 1300 mL) to yield three Frs (SHE-12–4-1 to SHE-12–4-3) and compound **1** (SHE-12–4-2, β-glucogallin, 10 mg, EV/TV 0.197–0.369, R_f 0.46 on SiO_2 TLC, $CHCl_3$-MeOH = 5:1).

β-Glucogallin (1)

Ocher amorphous powder (MeOH); positive FAB/MS *m/z* 333 $[M+H]^+$; IR (KBr, *ν*) 3400, 1660, 1605, 1500 cm^{-1}; ^{1}H-NMR (600 MHz, Methanol-d_4, δ_H, δ in ppm, *J* in Hz) 8.31 (br. s, H2), 8.18 (brs, H6), 5.97 (d, 7.2, H1′), 4.74 (dd, 7.2, 7.2, H2′), 4.46 (m, H5′), 4.32 (dd, 7.2, 7.2, H4′), 3.88 (dd, 12.0, 2.4, H6′a), 3.74 (overlapped, H6′b), 3.66 (overlapped, H3′); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 166.4 (C7), 153.6 (C3, C5), 142.2 (C4), 121.2 (C1), 103.9 (C2), 102.8 (C6), 91.4 (C1′), 79.5 (C3′), 78.2 (C5′), 74.0 (C2′), 72.7 (C4′), 63.6 (C6′).

Cell culture

Immortalized human keratinocytes (HaCaT) and human fibroblasts (Hs68) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 1% antibiotic antimycotic solution and 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37 ℃. HaCaT and Hs68 cells were maintained until 80% confluence and then cells were treated with various concentrations of FC (1%,

10%) and β-glucogallin (1 ppm, 10 ppm) in serum-free medium and incubated for 24 h.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from HaCaT cells using a TRIzol reagent according to the manufacturer's instruction (TaKaRa, Shiga, Japan). cDNA was synthesized from 1 μg of total RNA using a reverse transcription premix (Elpisbiotech, Daejeon, Korea) under the following reaction conditions: 45 °C for 45 min and 95 °C for 5 min. Gene expression signals were quantified with real-time polymerase chain reaction (PCR) amplification, and the data were analyzed using StepOne Plus software (Applied Biosystems, Foster City, CA, USA). Real-time PCR amplification reactions were performed using an SYBR Green PCR Master Mix with premixed ROX (Applied Biosystems, Foster City, CA, USA). The following primer pairs (Bioneer, Daejeon, Korea) were used in the reactions performed in an ABI 7300 following the manufacturer's protocol: β-actin (F: 5′-GGC CAT CTC TTG CTC GAA GT-3′ and R: 5′-GAC ACC TTC AAC ACC CCA GC-3′), filaggrin (F: 5′-AGT GCA CTC AGG GGG CTC ACA-3′ and R: 5′-CCG GCT TGG CCG TAA TGT GT-3′), HAS3 (F: 5′-CTT AAG GGT TGC TTG CTT GC-3′ and R: 5′-GTT CGT GGG AGA TGA AGG AA-3′), IL-1β (F: 5′-GTC ATT CGC TCC CAC ATT CT-3′ and R: 5′-ACT TCT TGC CCC CTT TGA AT-3′), and TSLP (F: 5′-GCT ATC TGG TGC CCA GGC TAT-3′ and R: 5′-CGA CGC CAC AAT CCT TGT AAT-3′). Real-time PCR was performed using an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Waltham, MA, USA). The reaction conditions were as follows: initiation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Expression of β-actin was used as an internal control.

Cell migration assay

Cell migration was defined as the actual movement of individual cells. Relative wound confluency can also be used to measure Hs68 cell migration. Wounds were made with WoundMaker in the middle of cells, and live images of Hs68 cells were captured by an IncuCyte ZOOM system (Essen BioScience, Ann Arbor, MI). The IncuCyte ZOOM system also measured the spatial cell density in the wound area relative to spatial cell confluency of the wound area at each time point.

Statistical analysis

All experiments were repeated at least three times, and each experiment was performed in triplicate. Results are presented as mean ± standard deviation. For in vitro cell assay analysis, statistical analysis was performed using SPSS statistical package version 25.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were performed with a two-tailed Student's t-test. $p < 0.05$ and $p < 0.01$ were considered statistically significant.

Results and discussion

Chemical structure of compound 1

Fusidium coccineum extracts were produced by aqueous MeOH, and the concentrated FC extracts were successively partitioned into EtOAc (SHE), *n*-BuOH (SHB), and H_2O (SHW) Frs. Evaluation of skin-enhancing effects of FC and its fractions revealed that the SHE was most effective. The major compound of the SHE showed UV absorption at 254 nm and turned light brown on a TLC plate after being sprayed with 10% H_2SO_4 and alcohol lamp heated. Based on these findings, it was predicted that this major compound had a phenolic structure. The SHE was used to isolate the major compound.

Compound **1** was obtained as an ocher amorphous powder that maroon color on a TLC plate upon spraying with 10% H_2SO_4 and alcohol lamp heating. The molecular weight was 332, based on a detected molecular ion peak *m/z* 333 $[M+H]^+$ in the positive FAB/MS spectrum. The infrared spectrum (IR, cm^{-1}) suggested the presence of hydroxyl (3402), conjugated ketone (1664), and aromatic groups (1607 and 1501). In the ^{1}H-NMR spectrum, two aromatic methines (chemical shift, coupling pattern, *J* in Hz, proton number; δ_H 8.31, br. s, H2; δ_H 8.18, br. s, H6) were due to one 1,2,3,5-tetrasubstituted benzene ring moiety. Proton signals of a hexose moiety were also detected: one hemiacetal (δ_H 5.97, d, 7.2, H1′), four oxygenated methines (δ_H 4.74, dd, 7.2, 7.2, H2′; δ_H 4.46, m, H5′; δ_H 4.32, dd, 7.2, 7.2, H4′; δ_H 3.66, overlapped, H3′), and one oxygenated methylene (δ_H 3.88, dd, 12.0, 2.4, H6′a/ 3.74, overlapped, H6′b). The ^{1}H-NMR data indicated that **1** was a glycoside composed of 1,2,3-trisubstituted phenyl and hexose moieties. A ^{13}C-NMR spectrum was observed 13 carbon aglycon signals including one ester (δ_C 166.4, C-7), three oxygenated aromatic quaternaries (δ_C 153.6, C3; δ_C 153.6, C5; δ_C 142.2, C4), one aromatic quaternary (δ_C 121.2, C1), and two aromatic methines (δ_C 103.9, C2; δ_C 102.8, C6), which was assume to be a trihydroxyphenol from chemical shifts. Also, the chemical shifts of one hemiacetal (δ_C 91.4, C1′), four oxygenated methines (δ_C 79.5, C3′; δ_C 78.2, C5′; δ_C 74.0, C2′; δ_C 72.7, C4′), and one oxygenated methylene (δ_C 63.6, C6′) revealed the sugar to be a β-glucopyranose, with its coupling constant (7.2 Hz) confirming the β configuration of the hemiacetal. In a gradient-selected heteronuclear multiple bond coherence spectrum, a hemiacetal proton signal (δ_H 5.97, H1′) showed a cross-peak with an ester carbon signal (δ_C 166.4, C7), suggesting the presence of β-D-glucopyranose at the C7 position. A

hemiacetal carbon signal (δ_C 91.4, C1′), which is usually observed at δ_C 101.8 in *β*-glucopyranose [12], was shifted upfield due to the ester-glycosylation effect. This evidence indicates that **1** was 1-(3,4,5-trihydroxybenzoate) 7-*O*-*β*- *d*-glucopyranoside, *β*-glucogallin (Fig. 1).

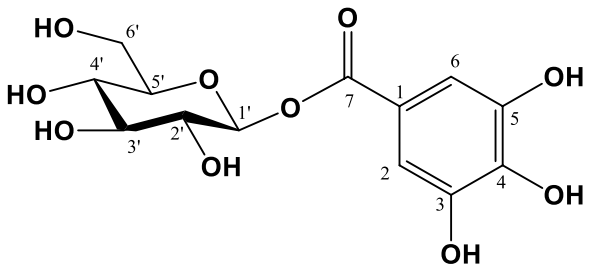

Fig. 1 Chemical structure of *β*-glucogallin isolated from *Fusidium coccineum*

Effects of FC and β-glucogallin on skin barrier development and hydration

A CE is formed during keratinocyte differentiation [5]. The mRNA level of keratinocyte differentiation marker was investigated to clarify the ability of FC and *β*-glucogallin to form a CE. Filaggrin, acting as a key factor of skin hydration, is generated by proteolysis of profilaggrin and degraded by caspase 14, resulting in NMF production [6]. The mRNA level of filaggrin was significantly increased ($p < 0.05$) by a factor of approximately 8 when treated with 10% FC and doubled when treated with 10 ppm of *β*-glucogallin compared with a non-treated control (Fig. 2a, c). Hyaluronan is a crucial extracellular glycosaminoglycan synthesized directly into the extracellular matrix by hyaluronan synthase (HAS) genes in epidermis. The HAS3 mRNA expression regulates of hyaluronan synthesis in the epidermis [13]. FC and *β*-glucogallin treatment also significantly

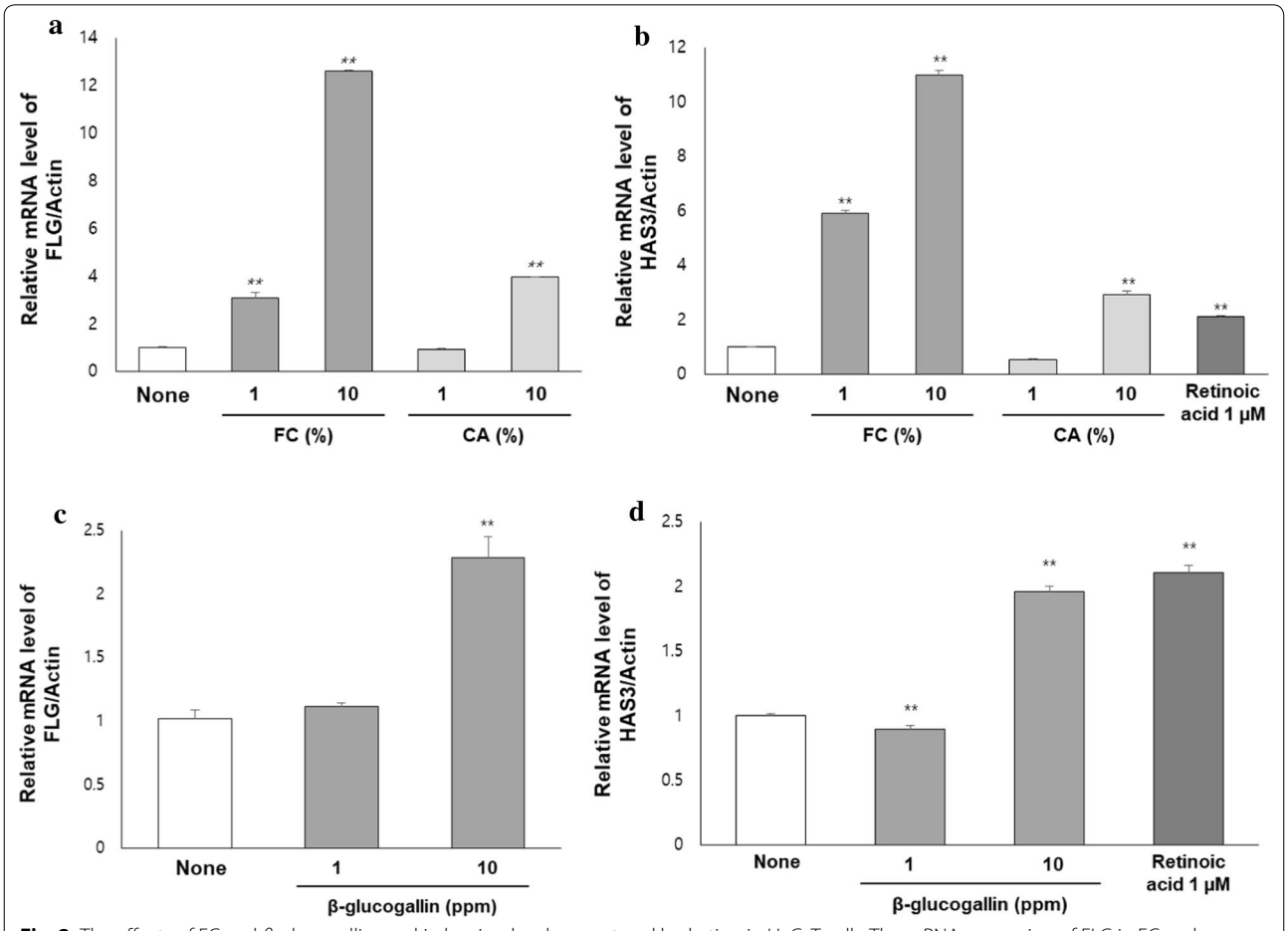

Fig. 2 The effects of FC and *β*-glucogallin on skin barrier development and hydration in HaCaT cells. The mRNA expression of FLG in FC and *β*-glucogallin treated HaCaT cells (**a**, **c**). The mRNA expression of HAS3 in FC and *β*-glucogallin treated HaCaT cells (**b**, **d**). HaCaT cells were pretreated with various concentrations of FC and *β*-glucogallin for 24 h. The mRNA expression of FLG and HAS3 was determined by RT-PCR and quantified by densitometric anlaysis. Results are expressed as mean ± SD (% control) of three independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$. FLG, Filaggrin; HAS3, Hyaluronan synthase 3; FC, *Fusidium coccineum*; CA, *Centella asiatica*

increased mRNA expression of HAS3, an enzyme that produces hyaluronic acid (Fig. 2b, d). *Centella asiatica* (CA), which has been used widely to treat skin diseases, served as a control. When evaluated under the same conditions, the regenerative and soothing effects of the FC were superior to those of CA. These results suggest that epidermal hyaluronan synthesis, through changes in the expression of HAS3, correlates with epidermal differentiation. FC and *β*-glucogallin were revealed to improve skin hydration and barrier function.

Inhibitory effects of FC and β-glucogallin on cytokine production

An IL-4 and poly(I:C) mixture was used to induce inflammation and disrupt skin barriers. Cytokine levels, including those of IL-1*α*, significantly increased after IL-4/poly(I:C) treatment but were significantly suppressed after treatment by FC and *β*-glucogallin (Fig. 3a, c). Also, itching-related chemokines, such as TSLP, were inhibited by FC and *β*-glucogallin (Fig. 3b, d). These results demonstrated that FC and *β*-glucogallin possess anti-inflammatory and anti-itching effects, and that FC and *β*-glucogallin contribute to formation of healthy skin barriers.

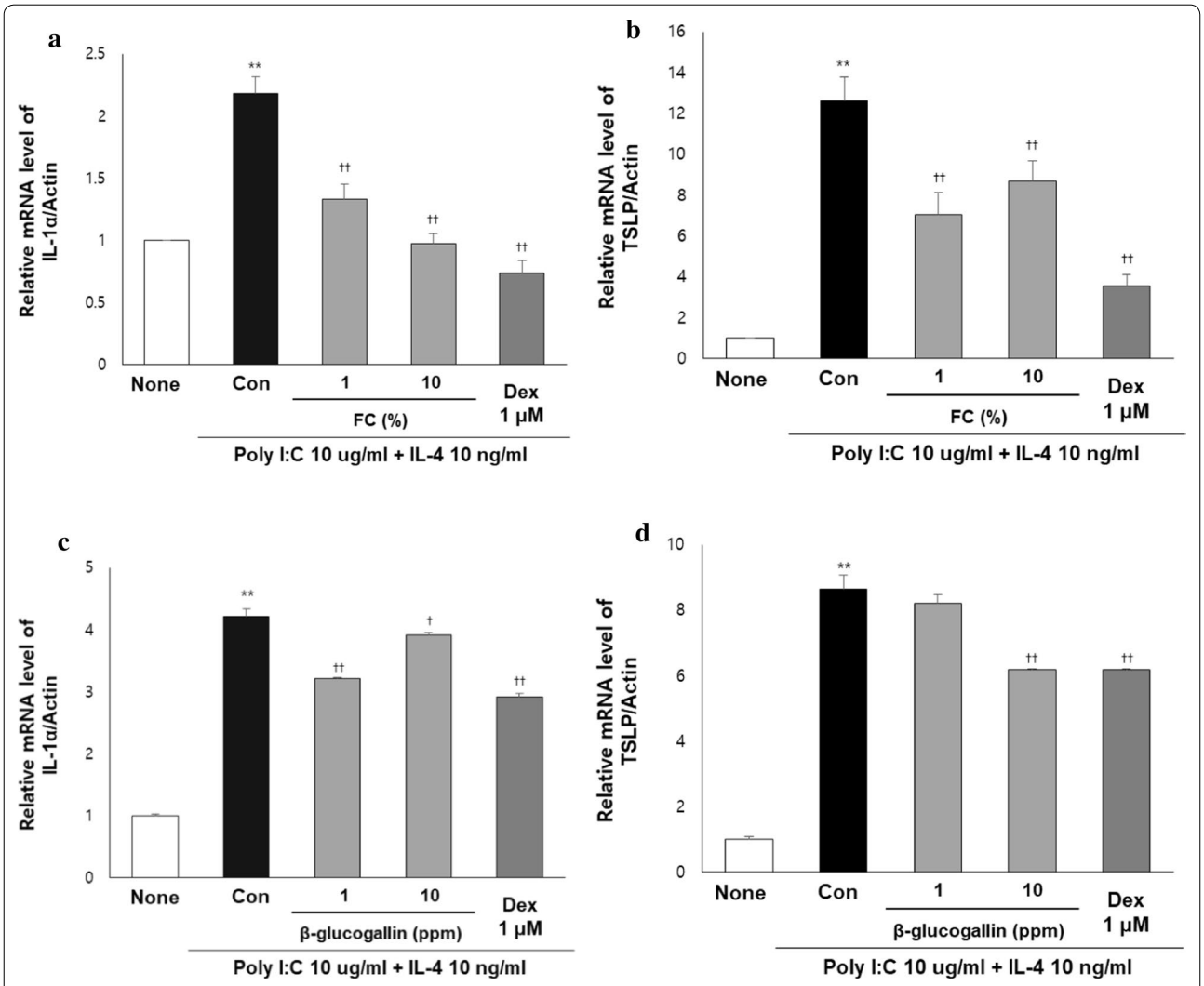

Fig. 3 The inhibitory effects of FC and β-glucogallin on cytokine production. The mRNA expression of IL-1α in FC and *β*-glucogallin treated HaCaT cells (**a**, **c**). The mRNA expression of TSLP in FC and *β*-glucogallin treated HaCaT cells (**b**, **d**). The mRNA expression of IL-1α and TSLP was determined by RT-PCR and quantified by densitometric anlaysis. Results are expressed as mean ± SD (% control) of three independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$ vs none, $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs IL-4/Poly(I:C) con. IL-1α, Interleukin 1 alpha; TSLP, Thymic stromal lymphopoietin; FC, *Fusidium coccineum*; Dex, Dexamethasone

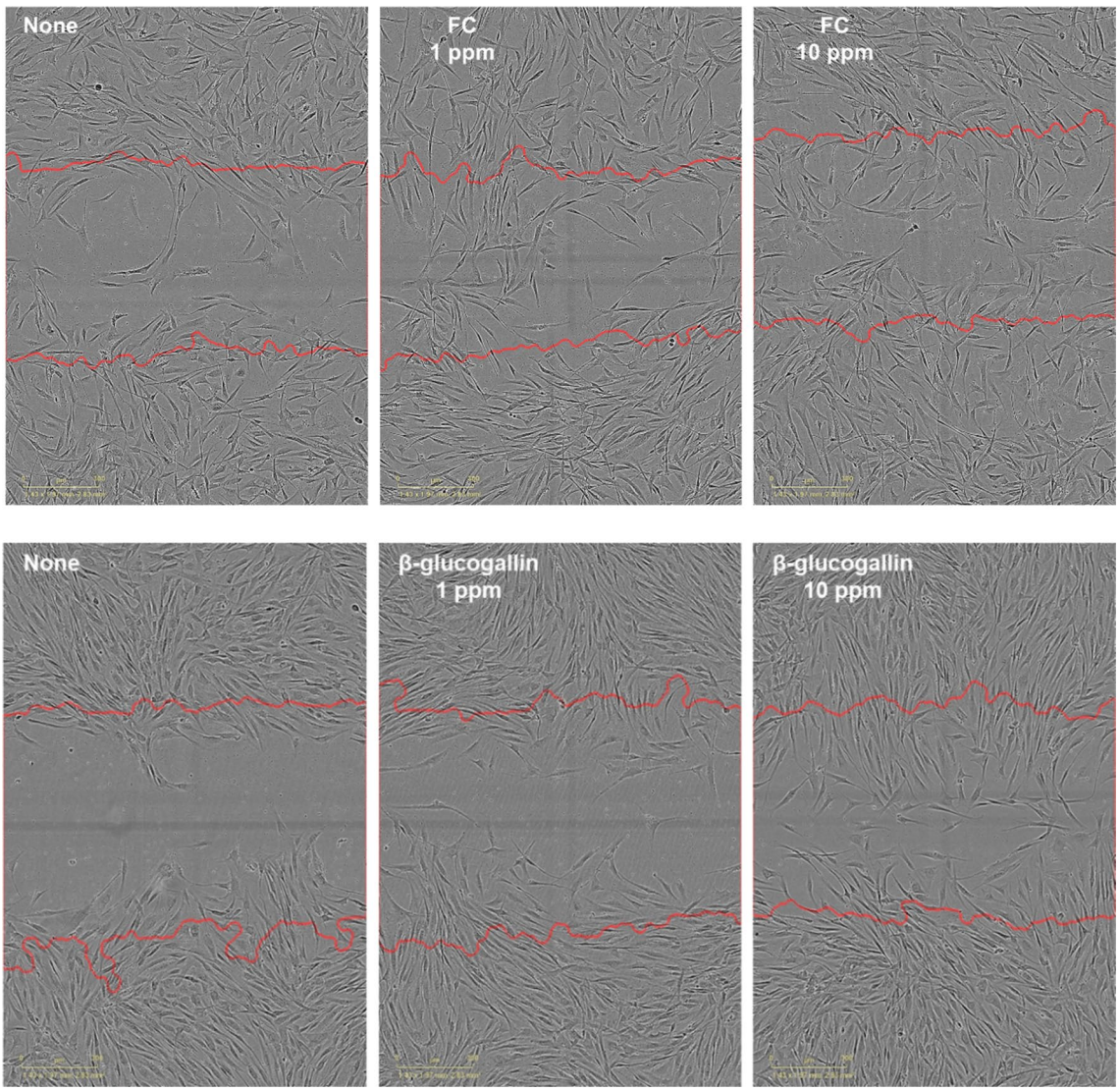

Fig. 4 Regeneration effect of FC and *β*-glucogallin in Hs68 cell migration. The Monolayers of confluent Hs68 cells were wounded with IncuCyte Woundmaker. Hs68 cells were treated with indicated concentrations of FC and *β*-glucogallin. HS68 cells were photographed at 24 h after wounding by IncuCyte ZOOM™. FC, *Fusidium coccineum*

Effects of FC and β-glucogallin on wound healing

Skin plays an important role in protecting the body from external stimuli [14]. Damage to the skin, and the dermis of which it is composed, can interrupt the continuity of the organ. The process of restoring a wounded site in multiple stages is called wound healing [15]. In general, side effects such as ointment-containing sodium fusidate, which is frequently used on wounds to induce an allergic reaction on the skin or form scars, and adherent tissues have been reported [16, 17]. Various studies are underway to develop natural products with medicinal or cosmetic properties. The wound healing effects of FC and *β*-glucogallin were evaluated for migration assays using Hs68 fibroblast cells. Both showed skin regeneration capacity through enhancement of cell migration in Hs68 cells (Fig. 4).

In conclusion, evaluation of the skin-enhancing effects of FC and its fractions revealed the EtOAc fraction to be most effective. The major compound of the EtOAc fraction from FC was isolated and identified as *β*-glucogallin based on UV, 1D, 2D-NMR, IR, and FAB/MS data. FC and *β*-glucogallin were found to upregulate mRNA levels

of filaggrin and HAS3 in keratinocytes and HaCaT cells, inhibit expression of IL-4/poly(I:C)-induced chemokines and inflammatory cytokines, and enhance wound healing by stimulating cell migration in fibroblasts and Hs68 cells. Our data indicate that FC and β-glucogallin are potential natural nutraceuticals that can enhance skin health by encouraging development of skin barriers and hydration and through anti-inflammatory and wound-healing effects. Clinical studies are necessary to elucidate the effects on skin health.

Supporting information

^{1}H–NMR and ^{13}C–NMR spectra of β-glucogallin are available in supporting information.

Authors' contributions

H-GK, KSK, D-GL, and N-IB planned this study and made in paper. H-GK, MHB, and N-IB isolated and identified β-glucogallin. KSK, MJK, SHS, and D-GL performed experiments about enhanced skin barrier. All authors read and approved the final manuscript.

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Availability of data and materials

The data and materials used in this study are available under permission from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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