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anti-aging cosmetic ingredient

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1. Introduction

Skin aging is a direct manifestation of the overall aging procedure, representing a complex and irreversible process that negatively affects most features of normal skin morphology and function. It is driven by both intrinsic and extrinsic factors. Intrinsic factors contributing to skin aging include the natural passage of time, hormonal regulation, genetic predisposition, and gradual shifts in the cellular redox environment. Extrinsic factors include environmental stresses such as prolonged sunlight exposure and various forms of pollution [1]. Aging underlies the decline of skin function and is the predominant risk factor for numerous skin problems [2]. Thus, a deeper understanding of aging could provide valuable insights into the mechanisms of skin diseases and help facilitate the development of novel anti-aging skincare treatments. The ultimate goal of cosmetic research is to prevent skin aging and restore dermal health.

As part of this effort, we identified four genes as aging markers—FSTL3, GDF15, MMP12, and CCDC80—whose expression in skin cells changes significantly during the first peak of aging that happens around the humans age of 40. MMP12, an elastase, was found to increase in amount along with skin cell aging, promoting the degradation of elastin leading to reduce skin elasticity. MMP12 is a matrix metalloprotease that degrades the ECM and elastin [3], and is involved in integrin signaling related to dermatitis. Therefore, downregulating MMP12 through a cosmetic formula could have anti-aging effects. Growth differentiation factor 15 (GDF15) is overexpressed in melanoma cells and is associated with tumor invasion and metastasis [4]. Dermal fibroblasts also express GDF15, which can be induced by reactive oxygen species (ROS) or visible light [5]. A recent study revealed that upregulation of GDF15 induces cellular senescence in human aortic endothelial cells (HAECs), as evidenced by G0/G1 cell cycle arrest, decreased cell proliferation, and increased SA-β-gal staining. GDF15 may play a crucial role in cellular senescence via a ROS-mediated p16 pathway [6]. Follistatin-like 3 (FSTL3) is a glycoprotein that binds and inhibits the action of TGF-β ligands, such as activin. The physiological role of FSTL3 in adults is not yet fully understood, but it is critical for maintaining normal adult metabolic homeostasis [7]. In the testis, FSTL3 deletion leads to increased AKT signaling and SIRT1 expression, indicating that the interaction between TGF-β ligands and AKT signaling affects cell survival and anti-aging [8]. It has been reported that Coiled-coil domain containing 80 (CCDC80) inhibits melanoma cell migration, indicating its potential of an anticancer role [9]. However, the role of CCDC80 in aging, particularly of skin, has not been well studied. In this present study of

investigating the mechanism of cellular senescence driven by aging markers, the authors clarified that CCDC80 suppresses ROS generation and inhibits skin cell aging, likely through the p16 signaling pathway.

The main objective of this study is to confirm the identification and variation of four senescence genes related to skin aging, with the goal of developing cosmetic ingredients from natural source that control the expression of these genes.

2. Materials and Methods

1.1. Preparation of botanical extracts

Punica granatum (pomegranate) flower and Hamamelis virginiana (witch hazel) were extracted using 40% 1,3-propanediol (DuPont Tate and Lyle, UK) or 40% 1,3-butylene glycol (OQ Chemical corporation, USA) at 60 ℃ for 24 hours. The extracts were filtered and used for the tests. [10].

1.2. Cell culture

To screen age-related gene expression in skin cells, keratinocytes (HaCaT) cells and human primary fibroblast cells (HDFn, Normal human dermal fibroblasts juvenile foreskin, C-12300, PromoCell) were cultured in Dulbecco's modified Eagle's medium high glucose (DMEM high glucose, SH30243.01, Hyclone) containing 10% (v/v) of fetal bovine serum (FBS, SH30084.03, Hyclone) and 1% antibiotic-antimycotic agents (Anti-anti, 15240-062, Gibco). Cell cultures were maintained at subconfluence in a 95% air, 5% CO2 humidified atmosphere at 37 °C. HDFn cells were serially passaged from passage 6 through passage 41. Recombinant human GDF15 (rh GDF15, OriGene, Rockville, Maryland, USA), recombinant human FSTL3 (rh FSTL3, OriGene, Rockville, Maryland, USA), and recombinant human CCDC80 (rh CCDC80, OriGene, Rockville, Maryland, USA) were used as aging marker proteins to evaluate their cellular senescence-inducing functions. To estimate variation of aging markers with aging inducement, passages 9 and 15 were selected in this study as representative of early and late passage cells.

1.3. Senescence induction using UVB and hydrogen peroxide

HDFn cells were plated at a density of 2 X 10^4 cells per well in a 96-well plate and cultured with 5% CO_2 at 37°C for 24 h. To induce senescence, the supernatants were removed and the cells were exposed to UVB, a total dose of 100 mJ/cm^2 (Bio-Sun system, Vilber Lourmat, Inc., Torcy, France). Following irradiation, the cells were treated with the botanical extracts for 6 hours. Alternatively, hydrogen peroxide was used to induce senescence. In this case, cells were incubated with the botanical extracts at 37 °C for 1 hour. After incubation, the medium was removed, and the cells were treated with 0.5 mM hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 4 hours.

1.4. RNA preparation and quantitative real-time PCR

To screen age-related gene expression in skin cells and assess the effect of aging markers on the mRNA expression of SASP (senescence-associated secretory phenotypes) components, RNA was extracted from the cells using an RNA extraction kit (TaKaRa MiniBEST Universal RNA Extraction Kit, 9767A, Takara Bio, Inc.) according to the manufacturer’s protocol. The isolated RNA (1 μg) was reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (RR047A, Takara Bio, Inc.). The primer sequences for RT-PCR and qRT-PCR are listed in a poster. All PCR reactions were performed in a total volume of 50 μl, containing 2 μl primers, 2 μl of cDNA, and 25 μl of Taq

polymerase 2X premix (Solgent, Korea), using a MiniAmp Plus Thermal Cycler (A37835, Thermo Fisher Scientific, Inc.). After initial denaturation at 95°C for 90 seconds, amplification was performed for 35 cycles: 30 seconds at 95°C, 30 seconds of annealing at 60°C, and 1 minute of extension at 72°C, followed by a final extension of 5 minutes at 72°C. Then, 10 µl of the RT-PCR product was subjected to electrophoresis on a 1.2% agarose gel in TAE buffer (40 mM Tris, 40 mM acetate, 1.0 mM EDTA), containing 0.5 µl/ml ethidium bromide. Gel images were captured by UV transillumination using a GelDoc apparatus (Bio-Print, Vilber-Lourmat, Inc.). For real-time PCR, the gene expression levels were quantified using the SYBR Green Realtime PCR Master Mix (Power SYBR™ Green PCR Master Mix, 4367659, Applied Biosystems™) and the QuantStudio™ 3 Real-Time PCR Instrument (A28132, Thermo Fisher Scientific, Inc.). Gene expression was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used in this study are shown in a poster. All experiments were performed in duplicate across three independent biological repeats. Melting curve analysis was conducted for each primer set.
To estimate the variation of aging markers in response to aging inducement, total RNA was extracted from dermal fibroblasts grown in 96-well plates, and cDNA was synthesized. The cells were washed once with PBS. Cell lysates were prepared, and reverse transcription was performed using a commercial kit (SuperPrep II Cell Lysis & RT Kit for qPCR; TOYOBO, Osaka, Japan). Quantitative real-time PCR was conducted on the LightCycler® 96 (Roche Diagnostics, Mannheim, Germany) using FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany), with 10 pM of each primer (see in a poster) and 100 ng of cDNA. Gene expression levels were analyzed by normalizing against β-actin expression.

1.5. RNA Melanin content assay

B16F10 melanoma cells were seeded at a density of 2×10^5 cells per well in 3 mL of medium in 6-well culture plates and incubated overnight to allow for adherence. The cells were then treated with various concentrations (50, 100, 150, and 100 ng/mL) of recombinant aging marker proteins for 48 hours. Following the treatment, the cells were washed with PBS and lysed with 800 µL of 1 N NaOH (Merck, Germany) containing 10% DMSO at 60 °C for 1 hour. Absorbance was measured at 420 nm using an EPOCH2 ELISA reader (Bio-Tek, Winooski, VT, USA).

1.6. Data analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) or Student's t-test. Dunnett's test was applied for post hoc analysis to identify differences compared to the negative control. P-values of ≤ 0.05 were considered statistically significant.

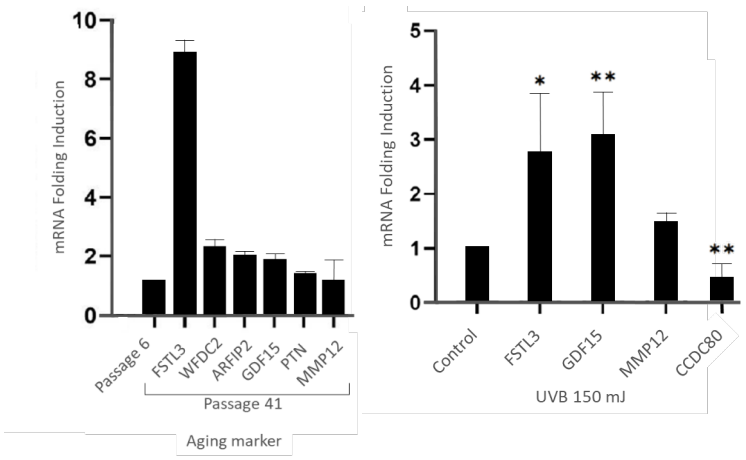
3. Results

1.1. Age-related gene expression in skin cells

The the expression of age-related genes was assessed via RT-PCR in HDFn and HaCaT cells. The genes WFDC2, FSTL3, PTN, MMP12, GDF15, and ARFIP2 were expressed in both HDFn and HaCaT cells. The genes SVEP1, CCDC80, SMOC1, PTGDS, SCARF2, and MSMP were expressed exclusively in HDFn cells, while the Sost gene was detected only in HaCaT cells. Conversely, the genes CHRD1, RSPO4, NPPB, EPHB6, SCG3, SERPINE2, OMD, CHAD, COL11A2, and RET were not expressed in either cell type.

Finally, four age-related genes expressed in senescent cells under the UVB-applied model were selected from those identified in HDFn and HaCaT cells, or exclusively in HDFn cells.

Among these, three age-related genes (FSTL3, GDF15, and MMP12) were found to be up-regulated with aging induction, while one gene (CCDC80) was downregulated with aging induction as shown in Fig.1.



(**a**) (**b**)

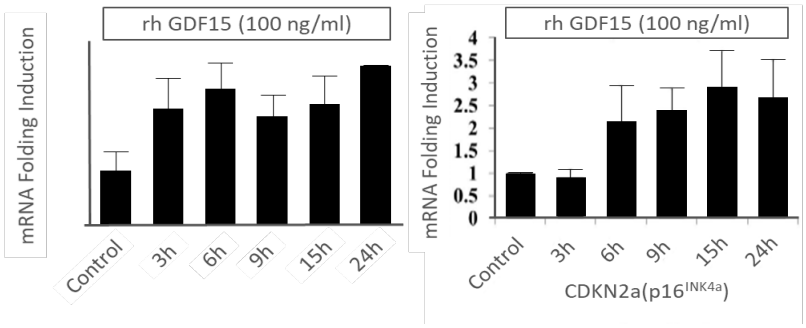
Figure 1. Changes in age-related gene expression in HDFn cells (A) passage 6 vs. passage 41 (B) gene expression in UVB-irradiated cells. Data are shown as the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control.

1.2. Cellular senescence-inducing function of aging markers

This study identified several skin aging markers that are closely associated with cellular senescence. Notably, we found that the markers GDF15 and CCDC80 promote cellular senescence acting oppositely on reactive oxygen species (ROS) generation through the p16 signaling pathway.

The rhGDF15 and rhFSTL3 promote cellular senescence, as indicated by an increase in blue staining in SA-β-gal assays shown in a poster. This suggests that the two markers increase in action during cellular aging process induced by oxidative stress. However, in the case of rhCCDC80, SA-β-gal activity was reduced, indicating that rhCCDC80 mitigates cellular aging induced by oxidative stress.

It was confirmed that rhGDF15 increases the gene expression levels of CDKN1A (p21) and CDKN2A (p16INK4a) in a time-dependent manner in HDFn cells. In contrast, treatment with rhCCDC80 appeared to attenuate the mRNA expression levels of CDKN2A (p16INK4a) and CDKN1A (p21) over time, as shown in Fig. 2.



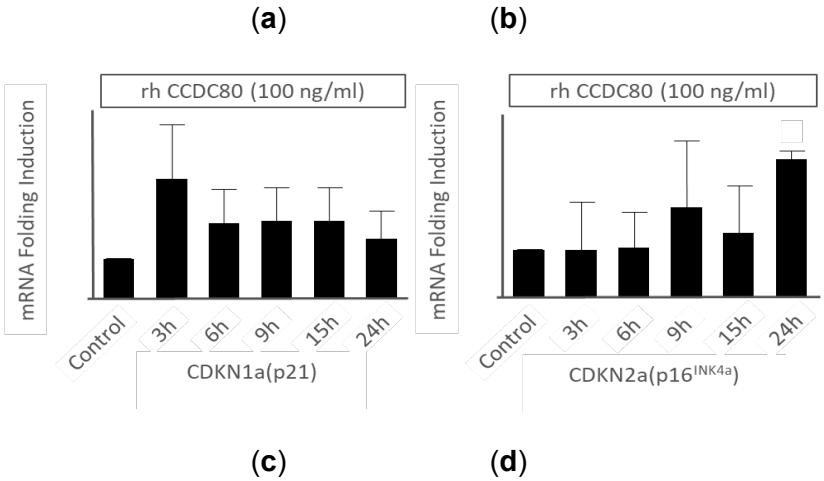
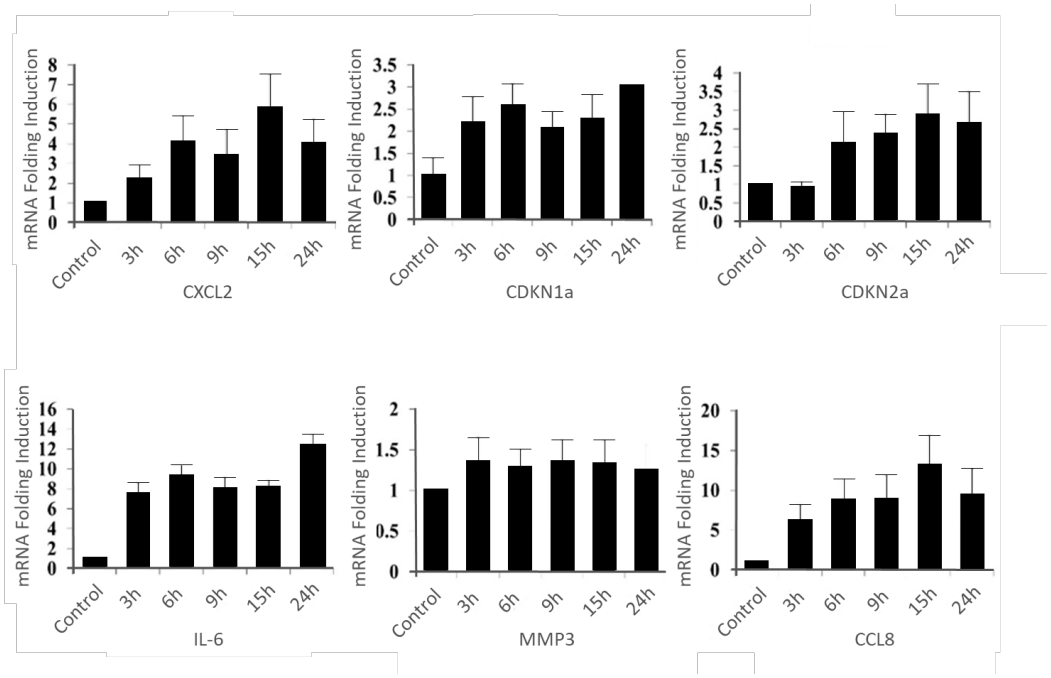


Figure 2. Effect of aging markers on p16 Signaling Pathway. (A) CDKN1a (p21) level; (B) CDKN2a (P16INK4a) level in rhGDF15 treated in HDFn cell; (C) CDKN1a (p21) and (D) CDKN2a (P16INK4a) level in rhCCDC80 treated in HDFn cell.

To investigate, rhGDF15 was applied to HDFn cells, and gene expression levels were measured at multiple time points (0 h, 3 h, 6 h, 9 h, 15 h, and 24 h). The results showed that rhGDF15 significantly increased the expression levels of CXCL2, CDKN1a, CDKN2a, IL-6, MMP3, and CCL8, as shown in Fig. 3(A). Conversely, rhCCDC80 suppressed the expression of CXCL1, MMP3, and IL-6 induced by H_2O_2 treatment, as shown in Fig. 3(B). These findings suggest that rhGDF15 promotes cellular senescence, while rhCCDC80 inhibits cellular senescence caused by oxidative stress.



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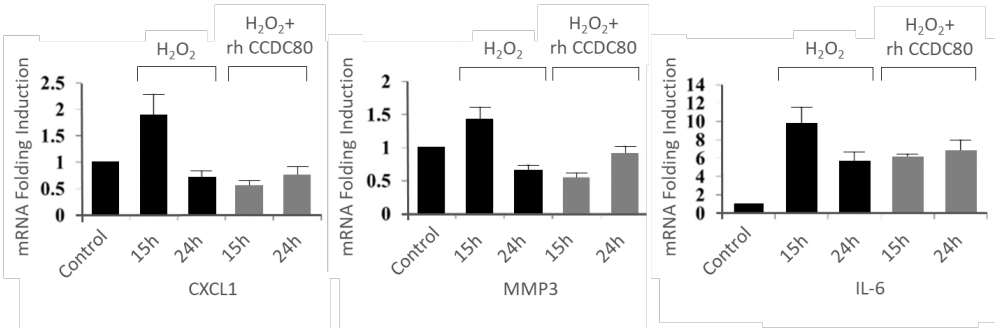

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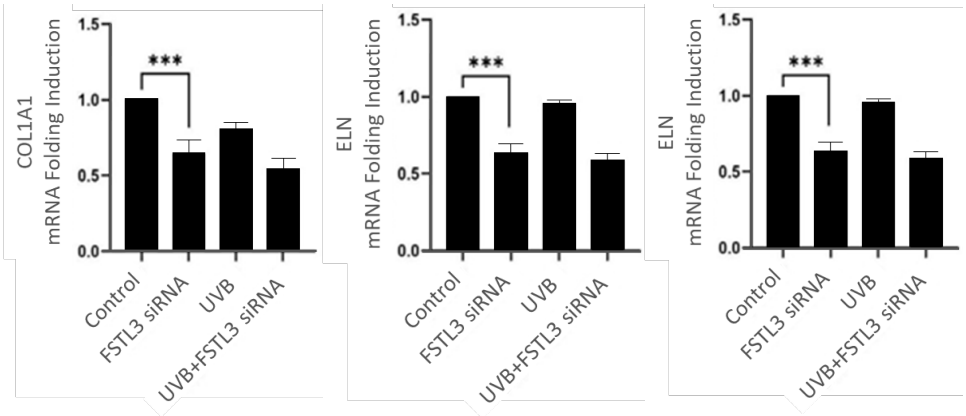
Figure 3. Effect of Aging markers on mRNA expression of SASP components (A) SASP gene expression levels in HDFn cells treated by rhGDF15 (100 ng/ml). Total RNA was extracted on the indicated time after treatment. CXCL2, CDKN1a, CDKN2a, IL-6, Mmp3 and Ccl8 mRNA levels were then determined by quantitative RT-PCR analysis.; (B) Gene expression levels in HDFn cells treated by rhCCDC 80 (100 ng/ml) for 24 hours were treated with H_2O_2 to give oxidative stress, and total RNA was extracted 15h and 24h later. CXCL1, Mmp3 and IL-6 mRNA levels were then determined by quantitative RT-PCR analysis. The levels of the indicated mRNA were normalized to that of GAPDH mRNA and shown as fold induction by 0h.

1.3. Effects of the recombinant aging marker protein on collagen and elastin expression

In this study, FSTL3 was found to be related to skin elasticity. COL1A1 and ELN gene expression levels, as well as collagen synthesis, were shown to be influenced by the FSTL3 gene, as shown in Fig. 4. As mentioned earlier, the FSTL3 gene can be upregulated in aged cells by harmful factors such as UVB and ROS. It was observed that when FSTL3 gene expression was increased by UVB irradiation, COL1A1 gene expression and collagen synthesis. Based on these findings, the FSTL3 gene could be a promising new target for skincare strategies in cosmetic formulations.



(**a**) (**b**) (**c**)

Figure 4. Effects of Fstl3 on Collagen and Elastin expression. Expression of (A) Col1A1 gene (B) Eln gene and (C) Collagen synthesis level in the Fstl3 Knockdown condition of the UVB applied model. Data are shown as the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared to the control

1.4. Effects of the recombinant aging marker protein on skin pigmentation

As the skin ages, it becomes darker and loses its radiance. Fortunately, some of the screened aging markers shown the potential to influence melanin synthesis. To investigate this, B16F10 cells were treated with recombinant aging marker proteins rhGDF15, rhFSTL3, and rhCCDC80 at various concentrations (50, 100, 150, and 200 ng/mL), and melanin synthesis was measured. The results showed that melanin synthesis was particularly increased by rhGDF15 at 50 and 100 ng/mL, as shown in Fig. 5.

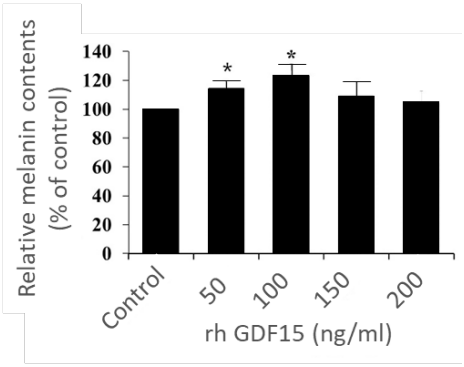
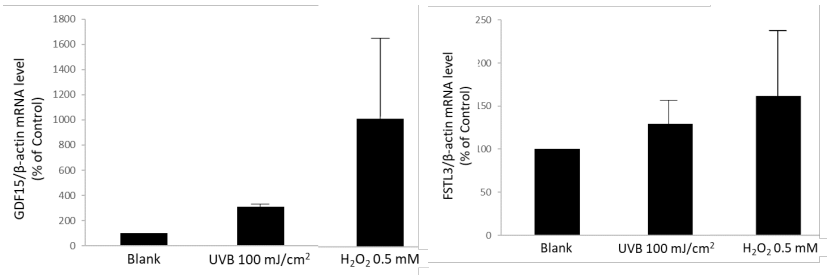


Figure 5. Effect of rh GDF15 on the regulation of pigmentation. Analysis of melanin content in B16F10 melanoma cells incubated for 48 h with rh GDF15. Data are shown as mean±S.D. of three experiments. * <0.05 compared to control.

1.5. Variation of the aging marker proteins on skin cell senescence

Fortunately, as shown in Fig. 6, the four screened senescence markers exhibit consistent changes in skin cells when exposed to aging inducers of UVB and H_2O_2. This suggests that skin aging could be delayed by either activating or suppressing the markers deliberately. In previous studies, it was found that GDF15 and CCDC80 influence cellular senescence induced by oxidative stress. Since GDF15 increases and CCDC80 decreases in skin cells exposed to aging inducers, down-regulating GDF15 and activating CCDC80 are considered the key factors in delaying skin aging. Moreover, skin elasticity can be improved by suppressing FSTL3, which is associated with COL1A1 and ELN genes. As referenced in previous studies, suppressing FSTL3 is expected to be beneficial for the reason that it inhibits AKT-mediated activation of zyxin and SIRT1, thereby reducing their anti-aging effects [11]. Skin pigmentation may also be improved by suppressing GDF15, which has been shown to increase melanin synthesis according to previous reports.



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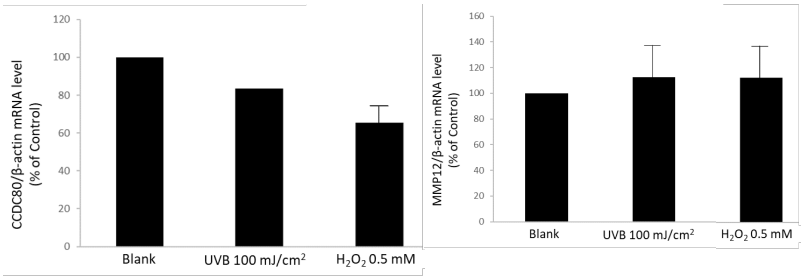

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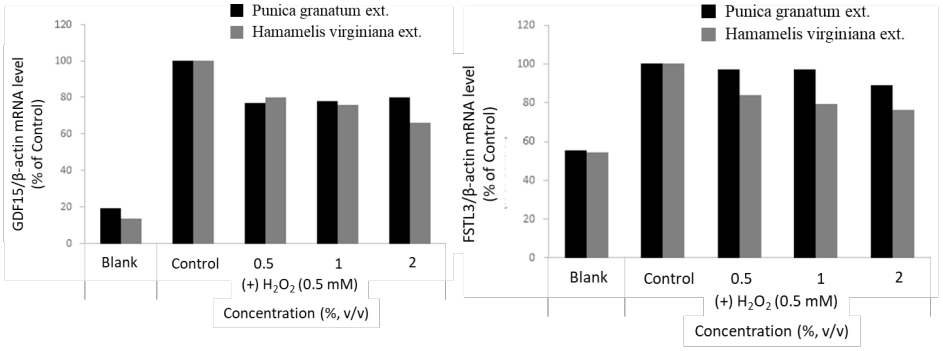
Figure 6. Variation of expression of aging markers, (A) GDF15; (B) FSTL3; (C) CCDC80 and (D) MMP12 within HDFn cell affected by aging inducers, UVB and H_2O_2.

1.6. Management of the aging marker protein by classified extracts

Eventually, we screened an anti-aging ingredient that regulates the mRNA expression of selected senescence markers—GDF15, FSTL3, MMP12, and CCDC80—which play important roles in cellular senescence.



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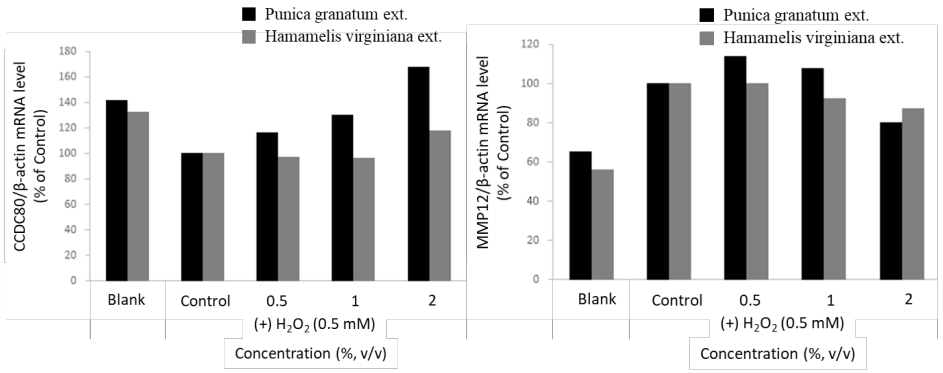

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Figure 7. Effects of the skincare ingredients on age-related gene expression (A) GDF15; (B) FSTL3; (C) CCDC80 and (D) MMP12 treated with Punica granatum extract and hamamelis virginiana extract under aging induced conditions, H_2O_2.

Punica granatum (pomegranate) flower and hamamelis virginiana (witch hazel) effectively regulated the expression of the four senescence markers, as shown in Fig. 7. Both extracts successfully inactivated GDF15, FSTL3, and MMP12, while activating CCDC80, in a concentration-dependent manner. Witch hazel has been used by Native Americans for wound healing and eye care. Witch hazel extracts have been used for decades as cosmetic ingredients in skin care products due to its anti-bacterial properties, particularly for cleansing the skin. The main compounds from the leaves or bark of witch hazel turned out to be most of polyphenols such as hydroxycinnamic acids, flavonoids, and tannin which can be specially applied to anti-pollution products through recover skin barrier function via tight junction protein expression. [15]. The increasing consumer demand for natural products in skincare is driven by their efficacy, mildness, and biodegradability. These findings highlight the potential of incorporating natural extracts into personal and skincare products, offering both beneficial properties and strong consumer appeal [12].

4. Conclusion

Anti-aging of skin is one of the most important fields in cosmetics. In an effort to develop new, scientifically grounded, and effective cosmetic products, a significant achievement in this study was the screening of four senescence markers whose expressions change notably during the first aging peak around the age of 40. These markers reflect the progress of skin aging, influenced by genetics, lifestyle, and environmental factors.

As for future works, it is crucial to study the skincare mechanisms in-depth under various harmful conditions related to senescence genes. In addition, there is a need for further exploration to improve the effectiveness of ingredients for cosmetic application. This includes advancing ingredient potency, developing effective ingredient delivery systems to meet the dermal target, conducting clinical tests to further clarify the underlying mechanisms, and more.

6. References

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