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"Skin physiological actions of Centella asiatica triterpenoids through OR2AT4 activation in human keratinocytes"

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1. Introduction

Olfactory receptors (ORs), the largest protein superfamily of G protein-coupled receptors (GPCRs), are traditionally known for their role in detecting odorant molecules in the olfactory epithelium [1]. However, recent studies have shown that these receptors are also ectopically expressed in non-olfactory tissues, including the skin, and are involved in regulating several physiological functions.

Keratinocytes, the major cell type in the epidermis, play a critical role in physiological functions such as wound healing and skin barrier restoration. These cells express a variety of olfactory receptors that enable them to sense external chemicals and environmental stimuli [2]. Olfactory receptors can be activated not only by odorants but also by various molecules, leading to the activation of G-proteins such as GNAL (guanine nucleotide-binding protein alpha L), which in turn stimulates adenylate cyclase 3 (ADCY3), converting ATP (adenosine triphosphate) into cAMP (cyclic adenosine monophosphate) [3,4]. As a key second messenger, cAMP initiates intracellular signaling cascades that contribute to skin homeostasis [5].

Among the ORs expressed in the skin, OR2AT4 (olfactory receptor family 2 subfamily AT member 4) has been the most extensively studied [2]. OR2AT4 can be activated by agonists such as sandalore, and its activation has been shown to exert several physiological benefits, including antioxidant, anti-aging, and wound-healing effects [4–6]. In particular, OR2AT4 activation in keratinocytes enhances cell proliferation and migration, which can lead to increased expression of extracellular matrix (ECM) and basement membrane proteins that contribute to the structural stability of the dermal-epidermal junction (DEJ) [6,7]. The DEJ, located at the interface between the epidermis and dermis, plays a pivotal role in maintaining skin barrier, improving elasticity, and regenerating tissue by firmly anchoring the two layers and mediating the exchange of nutrients and signaling molecules.

Centella asiatica is a traditional medicinal plant that has long been used in Asian medicine for treating skin disorders and is now widely incorporated into various skincare formulations. The primary active compounds of *C. asiatica* include madecassoside, asiaticoside, and their aglycones—madecassic acid and asiatic acid—[8], which have been reported to exhibit anti-inflammatory, antioxidant, and wound-healing effects [9].

Although these four major triterpenoids derived from *C. asiatica* (INCI: Madecassoside, Asiaticoside, Madecassic Acid, Asiatic Acid) have been extensively studied for their skin-related

effects, it remains unclear whether their effects are mediated through olfactory receptors, particularly OR2AT4.

Therefore, in this study, we aimed to investigate whether the major *C. asiatica*-derived triterpenoids (hereafter referred to as CATs) could activate OR2AT4 in human keratinocytes, and whether this activation contributes to the suppression of oxidative stress and the enhancement of DEJ structure, ultimately improving skin elasticity and exerting anti-aging effects

2. Materials and Methods

2.1. Preparation of CATs

CATs were solubilized and diluted in the media at the desired concentrations, by using a proprietary solution.

2.2. Cell culture

Human keratinocytes (HaCaT) and human dermal fibroblasts, neonatal (HDFn) were obtained from ATCC (USA). HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher, USA) and 1% penicillin-streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2.

2.3. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher, USA). Complementary DNA (cDNA) was synthesized from the RNA using the SuperPrep II Cell Lysis & RT Kit (Toyobo, Japan). Real-time PCR was performed using FastStart SYBR Green Master (Roche Diagnostics, Switzerland) on a LightCycler® 96 System (Roche Diagnostics, Switzerland). The PCR conditions were as follows: initial denaturation at 95°C for 10min, followed by 45 cycles of 95°C for 10s, 60°C for 10s, and 72°C for 20s. Gene expression levels were quantified using the ΔCt method. Primer sequences used in this study are listed in Table 1.

Table 1. Primer sequences used for RT-qPCR.

Target genes	Primer	Sequence
OR2AT4	Forward	GCCCATCCCAGCAGTAGTAAG
	Reverse	GAGGGGGTTGAGAATTGGTGT
GNAL	Forward	CAGCAAGACGACGGAAGACC
	Reverse	CGCTCTTTCTGCAACTGCTTC
ADCY3	Forward	GGAATTGGACTGGTGTTGGAC
	Reverse	GATCTGGGCGGTTATGAGCA
COL7A1	Forward	GTTGGAGAGAAAGGTGACGAG
	Reverse	TGGTCTCCCTTTTCACCCACA
COL17A1	Forward	GCTCTTGGCATTCCTAGTGGT
	Reverse	GATGTACTGCTGAATCTCCTG
β-actin	Forward	CCTCGCCTTTGCCGATCC
	Reverse	CGCGGCGATATCATCATCC

2.4. Measurement of Intracellular ROS generation

Intracellular reactive oxygen species (ROS) levels were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, USA). HaCaT cells were seeded in black 96-well plates at a density of 3 × 10^4 cells per well and incubated for 24 hours. After treatment with CATs for 1 hour, cells were washed with phosphate-buffered saline (PBS; Thermo Fisher, USA). Subsequently, 20 μM DCFH-DA and 0.5 mM H_2O_2 (Sigma-Aldrich, USA) were added in PBS and incubated in the dark for 30 minutes. Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a microplate reader (SpectraMax iD3, Molecular Devices, USA).

2.5. Wound Healing Assay

The scratch assay was performed to evaluate keratinocyte migration and wound closure. HaCaT cells were seeded in 24-well plates at a density of 1.2 × 10^5 cells per well. Once a confluent monolayer was formed, a linear scratch was made across the center of each well using a 1 mL pipette tip. Subsequently, cells were treated with various concentrations of CATs and incubated at 37 °C with 5% CO_2. The wound areas were imaged at 0, 6, and 24 hours to assess the degree of wound closure.

2.6. Senescence-Associated β-Galactosidase (SA-β-gal) Staining

Senescent cells were stained using a SA-β-gal staining kit (Abcam, UK), following the manufacturer's instructions. HaCaT cells were seeded in 24-well plates at a density of 5 × 10^4 cells per well. After overnight incubation, the cells were treated with CATs at various concentrations for 4 hours, followed by the addition of 0.1 mM H_2O_2 for 3 days. Cells were then washed with PBS and fixed with fixative solution for 15 minutes at room temperature. After fixation, cells were washed twice with PBS and incubated with SA-β-gal staining solution mix (20 mg/mL X-gal, 1× staining supplement, 1× staining solution) overnight at 37 °C without CO_2. SA-β-gal positive cells were observed under a microscope (Lionheart FX, Agilent, USA) at 20X magnification and quantified using ImageJ software (NIH, USA).

2.7. Western Blot Analysis

Cells were lysed using RIPA buffer (Sigma-Aldrich, USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, USA). Equal amounts of protein were separated on 12% SDS-PAGE gels (Thermo Fisher, USA) and transferred to PVDF membranes for Western blot analysis. The membranes were incubated with the following primary antibodies: p16 INK4A (80772S) and p21 Waf1/Cip1 (2947S) from Cell Signaling Technology (USA), each diluted 1:1000; and β-actin (A1978) from Sigma-Aldrich (USA), diluted 1:10,000 as a loading control. Protein bands were visualized using the ImageQuant LAS 4000 system (GE Healthcare, USA) and quantified with ImageJ software.

3. Results

3.1. OR2AT4 signaling was upregulated by CATs in HaCaT cells.

OR2AT4 is an ectopically expressed olfactory receptor in keratinocytes, known to regulate various skin physiological processes. Upon ligand binding, OR2AT4 activates the G-protein isoform GNAL, which subsequently stimulates adenylate cyclase 3 (ADCY3), leading to the production of cyclic AMP (cAMP) [3,4].

In this study, we investigated the effects of CATs on the expression of OR2AT4 and its downstream signaling components in HaCaT cells. To verify pathway specificity, we used sandalore (a known OR2AT4 agonist) and oxyphenylon (an antagonist) as positive and negative controls, respectively. RT-qPCR analysis revealed that CATs significantly

upregulated the mRNA expression of OR2AT4, GNAL, and ADCY3 (Figure 1A). Similar upregulation was observed upon sandalore treatment (Figure 1B), while oxyphenylon did not cause any significant changes in the expression of these genes (Figure 1C). These results suggest that CATs activate OR2AT4 signaling and modulate its downstream targets in HaCaT cells, like sandalore [5].

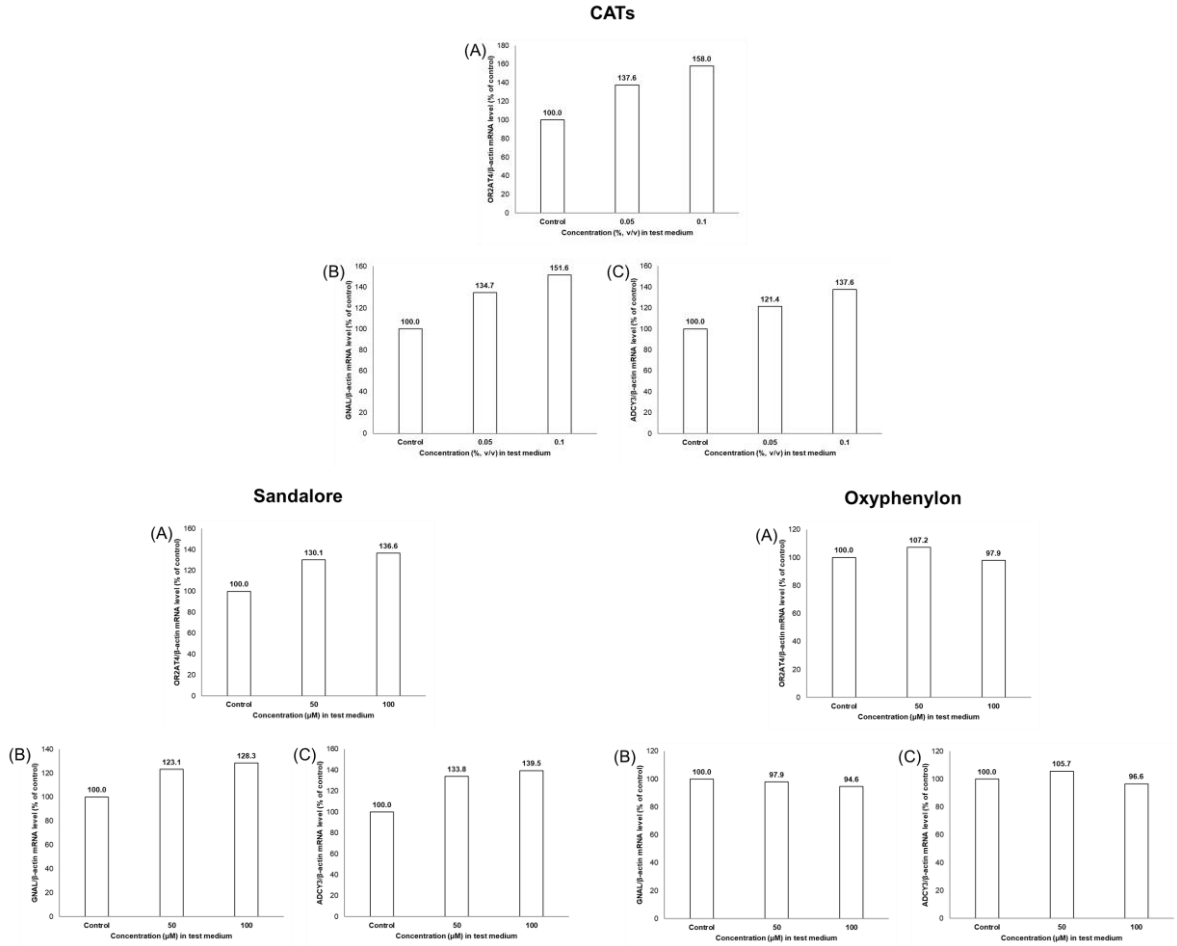

Figure 1. CATs upregulated OR2AT4 and its downstream signaling components in HaCaT cells. HaCaT cells were treated with CATs, sandalore, or oxyphenylon for 24 hours. The mRNA levels of (A) OR2AT4, (B) GNAL, and (C) ADCY3 were quantified by RT-qPCR.

3.2. CATs enhanced DEJ-related protein expression via OR2AT4 signaling.

Activation of OR2AT4 in keratinocytes has been reported to promote cell proliferation and migration, which may contribute to the upregulation of extracellular matrix (ECM) and basement membrane proteins [7], thereby reinforcing the structural stability of the dermal–epidermal junction (DEJ) and improving skin elasticity. However, the role of OR2AT4 in regulating the the exprssion of DEJ-related structural proteins remains incompletely understood. In this study, we investigated the mRNA expression of key DEJ-associated proteins following OR2AT4 activation.

Collagen type VII alpha 1 chain (COL7A1) is a major component of anchoring fibrils that connect the dermis and epidermis, while collagen type XVII alpha 1 chain (COL17A1) is a transmembrane protein essential for keratinocyte adhesion to the basement membrane [10]. RT-qPCR analysis demonstrated that CATs significantly upregulated the mRNA expression of both COL7A1 and COL17A1 in HaCaT cells (Figure 2A). Similar upregulation was

observed upon treatment with sandalore (Figure 2B), whereas oxyphenylon did not induce any significant changes (Figure 2C).

These results suggest that CATs promote the expression of DEJ-associated structural proteins through OR2AT4 signaling activation, which may contribute to the enhancement of DEJ and improve skin firmness.

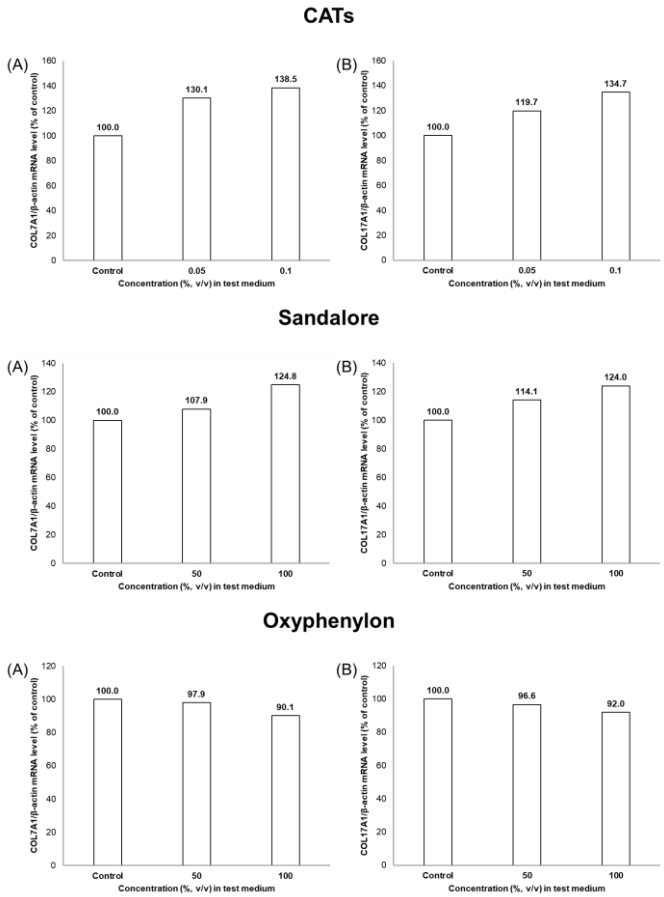

Figure 2. CATs induced DEJ-associated structural protein expression in HaCaT cells. HaCaT cells were treated with CATs, sandalore, or oxyphenylon for 24 hours, followed by RT-qPCR analysis of the mRNA expression of (A) COL7A1 and (B) COL17A1.

3.3. CATs accelerated keratinocyte migration and wound healing.

OR2AT4 is expressed in skin keratinocytes, including HaCaT cells, and its activation has been reported to promote cell proliferation and migration, contributing to the wound healing process [12]. To evaluate whether CATs, which upregulated OR2AT4 expression, exhibit similar physiological activity, a scratch assay was conducted in HaCaT cells.

Treatment with CATs for 24 hours significantly promoted cell migration and reduced the scratch width in a dose-dependent manner (Figure 3A). These findings suggest that CATs functionally activate OR2AT4 signaling, thereby enhancing both keratinocyte migration and wound healing process.

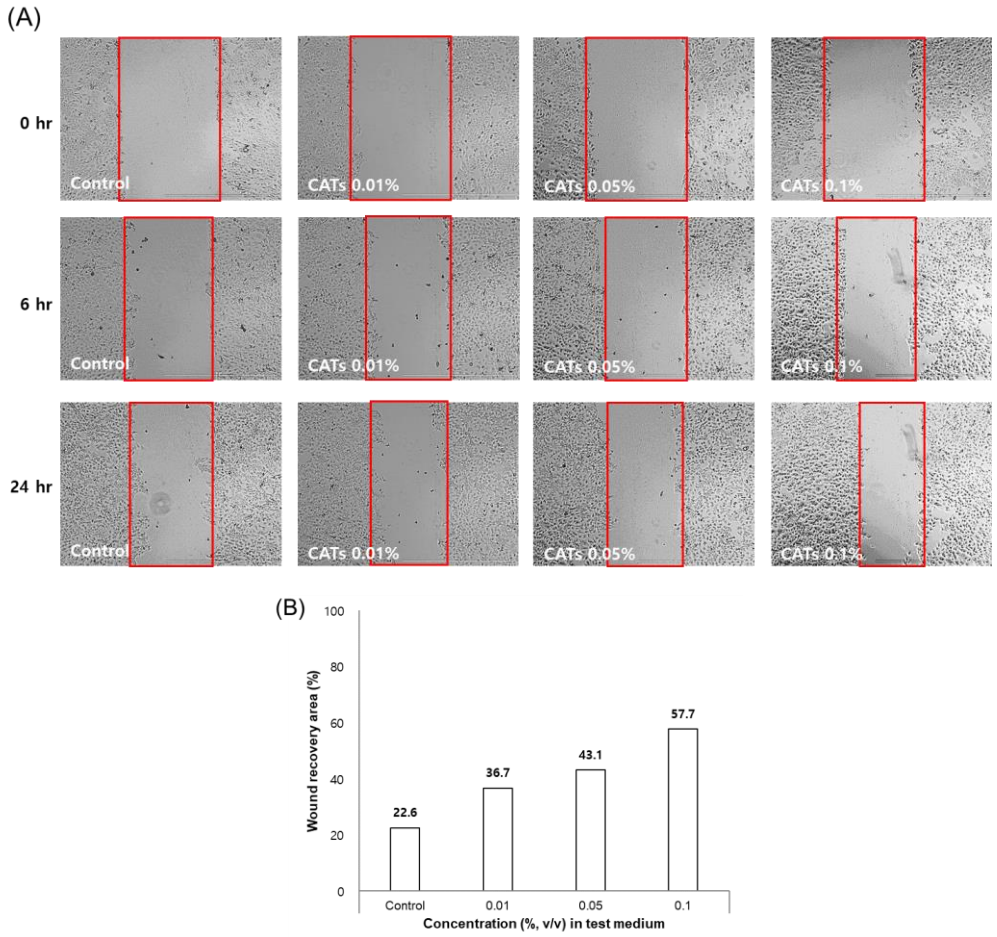

Figure 3. CATs enhanced wound healing in HaCaT cells. A scratch was induced in HaCaT cell monolayers, followed by treatment with negative control (media only) or CATs (0.01%, 0.05%, 0.1%). (A) Representative images of wound areas were captured at 0, 6, and 24 hours. (B) Wound closure was quantified using ImageJ software based on the relative wound area.

3.3. CATs inhibited H_2O_2-induced cellular senescence in HaCaT Cells.

As demonstrated in previous results, OR2AT4 promotes cell proliferation and wound healing in HaCaT keratinocytes. However, this activity is reported to decline with aging [11], and recent studies have suggested that activation of OR2AT4 can counteract cellular senescence [4]. Therefore, to investigate whether CATs can modulate H_2O_2-induced cellular senescence via OR2AT4 activation, we conducted a series of experiments using HaCaT cells.

HaCaT cells were pretreated with various concentrations of CATs for 4 hours, followed by exposure to hydrogen peroxide (H_2O_2), an inducer of oxidative stress-mediated senescence. Senescent cells were visualized using SA-β-gal staining. As shown in Figure 4A, H_2O_2 treatment significantly increased the proportion of SA-β-gal positive cells, whereas CATs treatment led to a dose-dependent reduction in cellular senescence. This effect was further confirmed by quantitative analysis using ImageJ (Figure 4B).

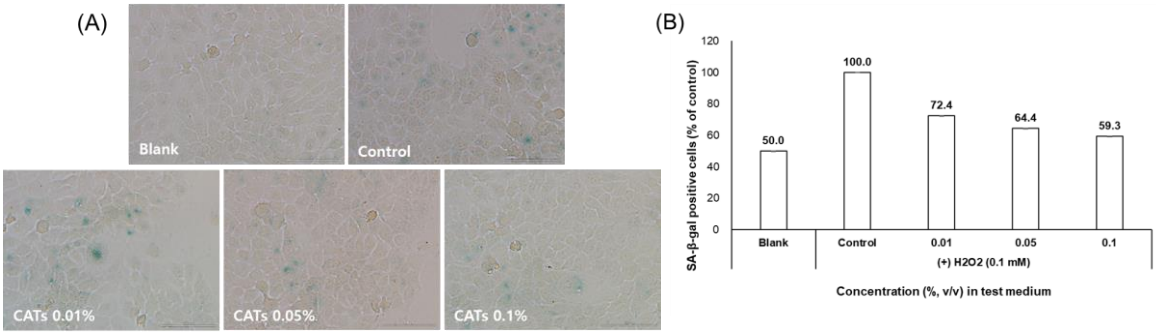

Figure 4. CATs suppressed H_2O_2-induced senescence in HaCaT cells. HaCaT cells were pretreated with CATs for 4 hours and then exposed to H_2O_2 to induce cellular senescence. (A) SA-β-gal staining was performed to identify senescent cells. (B) The percentage of SA-β-gal-positive cells was quantified using ImageJ software.

Also, we examined the expression of key senescence-associated markers—p16 INK4A (CDKN2A) and p21 Waf/CIP1 (CDKN1A) — using Western blot analysis. As shown in Figure 5, H_2O_2 treatment led to marked upregulation of both proteins, whereas CATs treatment effectively attenuated their expression levels. These results indicate that CATs can effectively suppress oxidative stress-induced cellular senescence in keratinocytes, potentially via modulation of OR2AT4 signaling pathways.

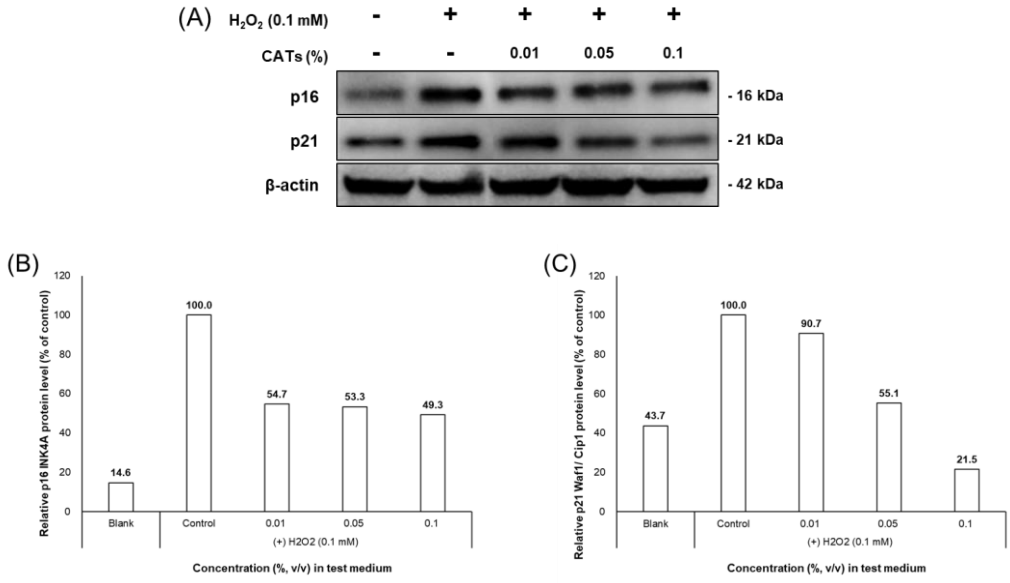

Figure 5. CATs downregulated senescence-associated proteins p16 INK4A and p21-Waf1/CIP1 in H_2O_2-treated HaCaT cells. HaCaT cells were pretreated with CATs and subsequently exposed to H_2O_2 to induce cellular senescence. (A) Protein expression levels of p16 INK4A and p21 Waf1/CIP1 were analyzed by Western blotting. Band intensity quantification of (B) p16 INK4A and (C) p21 Waf1/CIP1 was performed using ImageJ software and normalized to β-actin.

4. Discussion

With the growing interest in skin health and anti-aging, extensive research has been dedicated to naturally derived compounds that can effectively regulate cellular functions. In particular, more attention has been drawn to the roles of olfactory receptors, which have been identified in the skin, suggesting novel strategies for skincare. In this study, we propose a novel mechanism whereby CATs activate OR2AT4 and positively modulate multiple physiological functions of human keratinocytes.

Previous studies have established the role of OR2AT4 in promoting cell proliferation, migration, and wound healing. Our results extend these findings by showing that OR2AT4 activation also upregulates the expression of dermal-epidermal junction (DEJ)-related structural proteins, specifically COL7A1 and COL17A1. These proteins are essential for anchoring the epidermis to the dermis and maintaining skin elasticity, suggesting that OR2AT4 activation may contribute to skin firmness.

CATs not only enhanced OR2AT4 expression but also increased the levels of its downstream signaling molecules, GNAL and ADCY3. Furthermore, CATs treatment significantly upregulated the expression of key DEJ-associated extracellular matrix proteins. These findings indicate that CATs go beyond simple cellular stimulation and may contribute to reinforcing the DEJ structure through OR2AT4-mediated pathways.

The wound healing assay and the H_2O_2-induced senescence model further support the functional role of CATs in skin elasticity and anti-aging. CATs promoted wound closure and reduced senescence-associated markers, highlighting their potential in promoting skin repair and delaying cellular aging. Still, CATs may also act through other receptors or signaling pathways beyond OR2AT4. Therefore, future studies utilizing OR2AT4 knockdown or knockout models are warranted to better elucidate the specificity of CATs toward this receptor. This study was conducted using *in vitro* models, and further *in vivo* and clinical investigations are necessary to clarify the histological and physiological impacts of OR2AT4 activation in human skin.

5. Conclusion

This study demonstrated that CATs activate the olfactory receptor OR2AT4, resulting in enhanced expression of DEJ-associated structural proteins, accelerated wound healing, and attenuation of oxidative stress-induced cellular senescence in HaCaT cells. These findings provide novel mechanistic insights into the skin-beneficial effects of *Centella asiatica*-derived triterpenoids, and suggest OR2AT4 as a potential molecular target for skin regeration and anti-aging interventions.

By establishing the role of CATs in modulating OR2AT4-mediated skin physiology, our findings support the strategic development of OR2AT4-targeted cosmetic formulations. Overall, CATs represent a promising bioactive ingredient for maintaining and improving skin health, and this study lays the groundwork for the future development of olfactory receptor-based skincare solutions.

5. References

[1] Duroux, R., Mandeau, A., Guiraudie-Capraz, G., Quesnel, Y., & Loing, E. (2020). A rose extract protects the skin against stress mediators: a potential role of olfactory receptors. *Molecules*, *25*(20), 4743..

[2] Maßberg, D., & Hatt, H. (2018). Human olfactory receptors: novel cellular functions outside of the nose. *Physiological reviews*.

[3] Kang, W., Son, B., Park, S., Choi, D., & Park, T. (2021). UV-irradiation-and inflammation-induced skin barrier dysfunction is associated with the expression of olfactory receptor genes in human keratinocytes. *International journal of molecular sciences*, *22*(6), 2799.

[4] Kim, J. S., Lee, H. L., Jeong, J. H., Yoon, Y. E., Lee, I. R., Kim, J. M., ... & Lee, S. J. (2022). OR2AT4, an ectopic olfactory receptor, suppresses oxidative stress-induced senescence in human keratinocytes. *Antioxidants*, *11*(11), 2180.

[5] Seo, J., Choi, S., Kim, H., Park, S. H., & Lee, J. (2022). Association between olfactory receptors and skin physiology. *Annals of Dermatology*, *34*(2), 87.

[6] Busse, D., Kudella, P., Grüning, N. M., Gisselmann, G., Ständer, S., Luger, T., ... & Benecke, H. (2014). A synthetic sandalwood odorant induces wound-healing processes in human keratinocytes via the olfactory receptor OR2AT4. *Journal of investigative dermatology*, *134*(11), 2823-2832.

[7] Benny, P., Badowski, C., Lane, E. B., & Raghunath, M. (2015). Making more matrix: enhancing the deposition of dermal–epidermal junction components in vitro and accelerating organotypic skin culture development, using macromolecular crowding. *Tissue Engineering Part A*, *21*(1-2), 183-192.

[8] Park, K. S. (2021). Pharmacological effects of *Centella asiatica* on skin diseases: Evidence and possible mechanisms. *Evidence‐Based Complementary and Alternative Medicine*, *2021*(1), 5462633.

[9] Witkowska, K., Paczkowska-Walendowska, M., Garbiec, E., & Cielecka-Piontek, J. (2024). Topical Application of Centella asiatica in Wound Healing: Recent Insights into Mechanisms and Clinical Efficacy. *Pharmaceutics*, *16*(10), 1252.

[10] Lim, J. H., Bae, J. S., Lee, S. K., & Lee, D. H. (2022). Palmitoyl-RGD promotes the expression of dermal-epidermal junction components in HaCaT cells. *Molecular Medicine Reports, 26*(4), 1-6.

[11] Kita, A., Yamamoto, S., Saito, Y., & Chikenji, T. S. (2024). Cellular senescence and wound healing in aged and diabetic skin. *Frontiers in Physiology*, *15,* 1344116.

[12] Choo, J. H., Kim, D., Min, K., Lee, S. Y., & Kang, N. G. (2024). Pogostemon cablin Extract Promotes Wound Healing through OR2AT4 Activation and Exhibits Anti-Inflammatory Activity. *Current Issues in Molecular Biology*, *46*(8), 9136-9148.