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Natural Product Sciences
30(4) : 268-274 (2024)
https://doi.org/10.20307/nps.2024.30.4.268

Citronellic Acid Improves Skin Barrier Function by Activation of PPAR-α

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Abstract – Peroxisome proliferator activated receptors (PPAR)-α is a critical regulator of skin conditions of skin inflammatory disorders, hyper-proliferation, and abnormal differentiation. The new search for PPAR-α activator is required for epidermal differentiation and skin barrier improvement. Citronellic acid was selected for a new PPAR-α ligand through structure-based pharmacophore screening. Citronellic acid is an acyclic monoterpene carboxylic acid found in essential oils of lemongrass and citrus fruits. Citronellic acid enhanced the trans-activation activity of PPAR responsive element (PPRE) and cornified envelope (CE) formation, and decreased the expression of inflammatory cytokines and anti-microbial peptides. Citronellic acid also promoted the protein expression of involucrin, as a component of CE and a marker of keratinocyte differentiation, and the synthesis of hyaluronic acid (HA), a moisturizing component. These results indicate that citronellic acid may be a suitable skin treatment for improvement of epidermal barrier function.
Keywords – Citronellic acid, Peroxisome proliferator activated receptors, Hyaluronic acid, Pro-inflammatory cytokines, Anti-microbial peptides

Introduction

The skin is the largest and a complex organ consisting of multiple layers and protects a human body from chemical, physical and biological damages.[1] The epidermis is typically composed of four layers: stratum basale, stratum spinosum, stratum granulosum, and stratum corneum.[2] The stratum corneum (SC) is the outermost layer of the epidermis, which acts as the skin barrier and is composed of cornified keratinocytes.[3] The structure of stratum corneum is composed of terminally differentiated keratinocytes, namely corneocytes (bricks), are linked with the extracellular matrix of specialized lipids (mortar). As keratinocytes differentiate from the stratum basale to the stratum corneum, dramatic changes in lipid and protein compositions are induced.[4] Abnormalities of the skin barrier are known to be related to a decrease of intercellular lipid types, including ceramides, cholesterols and free fatty acids.[5] In other words, epidermal homeostasis is a crucial meaning in the dermatological treatment and cosmetics industry.

Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors belonging to the nuclear receptor steroid family.[6] There are three main isotypes of PPARs found in mammals PPAR-α, PPAR-β/δ, and PPAR-γ.[6] Among the PPAR isoforms, PPAR-α has important functions in regulating inflammatory mediators, cell proliferation and differentiation.[6,7] Therefore, PPAR-α ligands have been broadly studied for keratinocyte differentiation and epidermal barrier function, and topical treatment with selective PPAR-α agonists such as WY14643, clofibrate, and fenofibrate has been reported to restore epidermal permeability and promotes the expression of cornified envelope proteins such as loricin and involucrin.[8] Atopic dermatitis, often called eczema, is a chronic skin disease that causes inflammation, erythema, and irritation of the skin. In particular, inflammation is a type of protective response that involves a complex combination of immune cells, blood vessels, and molecular mediators. Also, inflammatory cytokines and mediators trigger or exacerbate inflammation in the immune system.[9,10] Additionally, PPAR activation has been reported to influence inflammatory responses.[11,12] Therefore, natural bioactive materials that can improve skin barrier function through PPAR-α activation and anti-inflammatory action are needed.

Citronellic acid (3,7-dimethyl-6-octenoic acid) is an acyclic monoterpene carboxylic acid, or a monounsaturated fatty acid found in the essential oils of lemongrass and citrus fruits.[13] Citronellic acid has been reported to be a component found in *Eucalyptus camaldulensis*, *Daphne*

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odora and other organisms.[14] In cosmetics industry, it is widely utilized not only as a flavor ingredient in various cosmetic products, but also as an agent that has antimicrobial effects.[15] Citronellic acid was selected as a natural PPAR-α activator by molecular docking simulation method.[16] However, the molecular mechanism of citronellic acid as a skin barrier improvement has not been investigated. In this study, citronellic acid, a plant-derived PPAR-α activator, was examined for its effectiveness in improvement of skin barrier function.

Experimental

Cell culture and materials – CV-1 cell was purchased from Korea Cell Line Bank (KCLB, Seoul, Korea) and human dermal fibroblasts HaCaT cell was purchased from American Type Culture Collection (ATTC), cultured in Dulbecco's Modified Eagle's Medium (Gibco-BRL/Life Technologies, Grand Island, NY, USA) with 1% penicillin and streptomycin, 10% fetal bovine serum (FBS)) in a humidified condition of 5% CO_2 at 37ºC.[17] Human normal keratinocytes grown to 80% confluency were maintained according to the methods of previous studies.[17] Cells were treated using citronellic acid (3,7-dimethyl-6-octenoic acid, Sigma-Aldrich, St. Louis, MO, USA) the PPAR-α activator WY14643 (Sigma-Aldrich, St. Louis, MO, USA), or $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) respectively.[17] All experiments used citronellic acid and WY14643 dissolved in dimethyl sulfoxide.

Cell cytotoxicity – Cytotoxicity was investigated by MTT assay according to previous studies.[18]

Cornified envelope formation – The rate of cornified envelope formation was measured as established in previous studies.[19–21] Human normal keratinocyte was prepared as 3×10^5 cells in 60 mm dish. After incubation for 24 h, citronellic acid and 1.2 mM $CaCl_2$ as a differentiation inducing group was added for 48 h, and cells were then washed using phosphate buffered saline (PBS) and lysed by 10 mM Tris-HCl buffer containing 20 mM dithiothreitol and 2% sodium dodecyl sulfate.[16]

DNA constructs, transfection, and PPRE transactivation assay – PPAR- transcription activity was performed using the PPAR response element (PPRE) transactivation method.[21] PPAR-α expression vector was purchased from Promega (Mannheim, Germany) and transformed into *Escherichia coli* competent cells.[17] PPAR DNA constructs were purified by a DNA preparation kit (Qiagen, Hilden, Germany). Co-transfection and transactivation assays were performed according to previous methods.[17]

Quantification of hyaluronic acid – The hyaluronan enzyme-linked immunosorbent assay was used to measure hyaluronic acid (HA) levels. Human normal keratinocytes and human dermal fibroblasts culture media with HA treatment were assayed and quantified by hyaluronic acid ELISA kit as the manufacturer's manual (Echelon Biosciences Inc., Salt Lake City, UT, USA) for 24 h.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) – HaCaT cells were prepared at a density of 2×10^4 per well and cultured as described in the Materials and methods.[17] Real-time PCR was performed according to previous methods.[17] Melting curve analysis of the PCR products was measured at the end of each assay to confirm the specificity of the amplification and absence of primer dimers (Table 1).[17]

Immunoblot analysis – Human normal keratinocytes pre-treated with citronellic acid and 1.2 mM $CaCl_2$ were cultured for 48 h.[22] Immunoblot analysis was determined

Table 1. Primers for RT-PCR used in this study

Mediator	Gene	Sense and antisense sequences
Pro-inflammatory cytokines	IL-1α	5'-GACGCACTTGTAGCCACGTA-3'
		5'-ACCGCCAATGAAATGACTCC-3'
	IL-6	5'-GAAAGCAGCAAAGAGGCAC-3'
		5'-TTTCACCAGGCAAGTCTCCT-3'
	IL-8	5'-TCTGGCAACCCTAGTCTGCT-3'
		5'-GCTTCCACATGTCCTCACAA-3'
	TNF-α	5'-ATGTTCGTCCTCCTCACAGG-3'
		5'-CTATCTGGGAGGGGTCTTCC-3'
Antimicrobial peptides	hBD-2	5'-CAGCCCATTGAAACCAACTT-3'
		5'-CTCTGGTGCCTCTCAGAACC-3'
	LL-37	5'-GACATGGGGACCATGAAGA-3'
		5'-AGGAGGCGGTAGAGGTTAG-3'
Housekeeping gene	β-actin	5'-TGAAGGTCGGAGTCAACGG-3'
		5'-CATGTGGGCCATGAGGTCCA-3'

performed according to previous methods.[17]

Statistics – All data are expressed as the mean ± standard error of the mean (S.E.M). Statistical analysis was performed with GraphPad Prism 5.0. (GraphPad Software, Inc. San Diego, CA, USA).[17]

Results and Discussion

The PPARs are the nuclear hormone receptor super family.[6] The three PPAR subtypes, α, β/δ, and γ can be activated by selective activators and regulate various gene expression as transcription factors.[23,24] Particularly, PPAR-α has been known to suppress cell proliferation, promote keratinocyte differentiation in the epidermis, improve the skin barrier function by inducing lipid metabolism, and sustain skin homeostasis by inhibiting inflammatory processes.[19] PPAR-α activation also plays an essential role in skin wound healing,[25] and PPAR-α agonists such as WY14643 and clofibrate have been shown to facilitate keratinocyte differentiation and skin barrier recovery.[26]

Therefore, the trans-activation of PPRE with citronellic acid was investigated. The dose dependency of citronellic acid significantly influenced PPAR-α ligand binding activity, and 200 μM citronellic acid treatement produced a significant increase compared to the untreated control (Fig. 1). Additionally, the treated groups with citronellic acid showed a slightly lower level of trans-activation activity than the groups treated with WY14643 at the same concentration. These results mean that citronellic acid has the potential as a novel PPAR-α activator.

To demonstrate the effects of citronellic acid on epidermal differentiation, the rate of CE formation was determined as the end product of terminal differentiation in keratinocytes (Fig. 2). Compared to the untreated

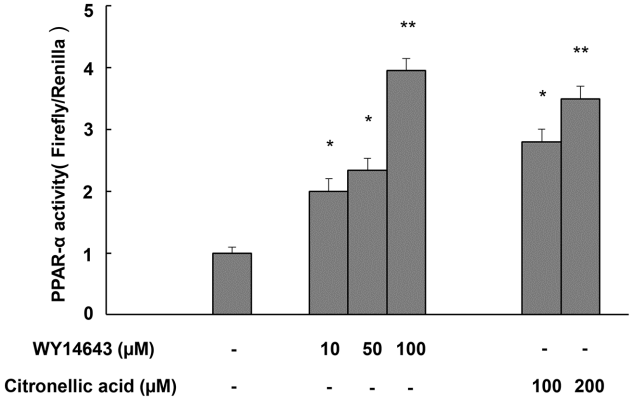

Fig. 1. Transactivation of peroxisome proliferator-activated receptor (PPAR)-responsive element (PPRE) by different concentrations of citronellic acid. CV-1 cells were transfected with PPRE luciferase. WY14643 was used as a positive control and no treatment was used as a negative control. Values are presented as means ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ compared to the untreated control group.

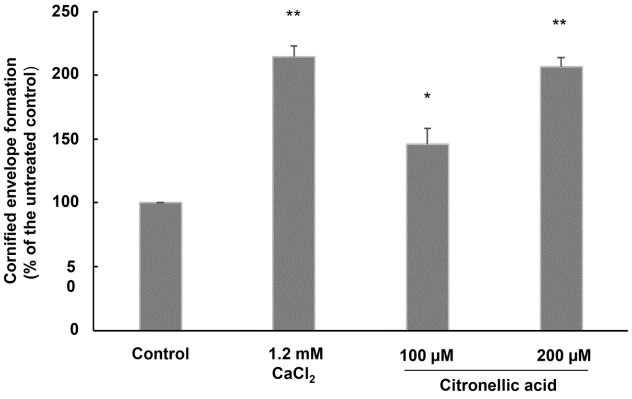

Fig. 2. Effect of citronellic acid on cornified envelope (CE) formation. CE formation composed of insoluble proteins in the cell lysate was measured at 430 nm using a spectrophotometer. $CaCl_2$ (1.2 mM) was used as a differentiation-inducing control. Values are presented as means ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ compared to the untreated group.

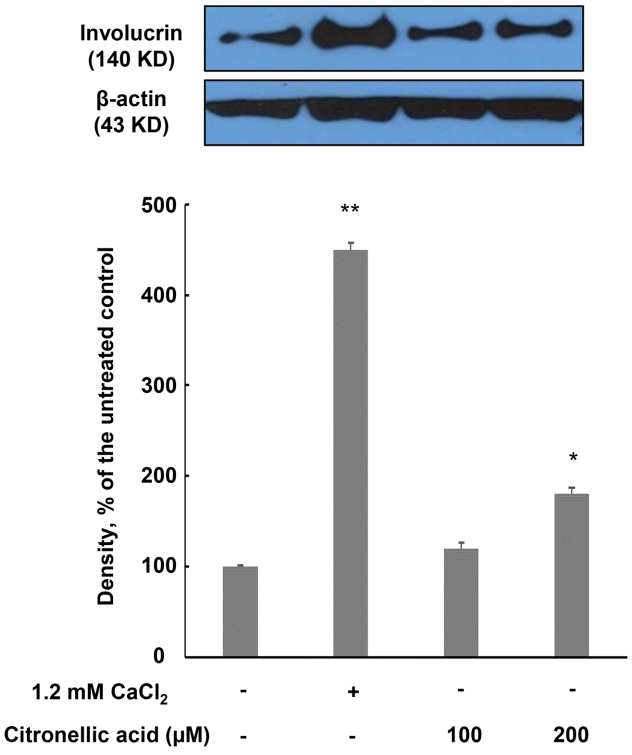

Fig. 3. Western blot analysis of involucrin. Each signal was quantified by scanning densitometry. β-actin was used as an internal standard. 1.2 mM $CaCl_2$ was used as a positive control and no treatment was used as a negative control in keratinocytes. Values are presented as means ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ compared to the untreated control group.

group, the groups treated with citronellic acid showed high CE formation at dependent on concentration. These results mean that citronellic acid has significant effects on epidermal differentiation. Involucrin contributes to the formation of cornified envelope, that protects corneocytes in the epidermis.[2] Involucrin is a major protein for the covalent linkage of ceramides to the cornified envelope. Involucrin expression is generally seen in the upper granular and spinous layers of skin.[3] The expression of involucrin was measured to confirm changes in protein expression related to CE formation. As shown in Fig. 3, in the untreated group, the protein expression level of involucrin increased depending on the concentration of citronellic acid. These results indicate that citronellic acid significantly strengthens epidermal barrier homeostasis related to CE formation. MTT assay was performed to investigate the cytotoxicity of citronellic acid in normal human keratinocyte and fibroblast. No significant differences were observed in keratinocyte and fibroblast cytotoxicity experiments, respectively, regardless of the change in citronellic acid dose (Fig. 4A, C).

Hyaluronic acid (HA) is known to be a moisturizing factor related to skin barrier function.[20] As shown in Fig. 4B, D, the levels of HA increased in the groups treated using citronellic acid more than in untreated group in keratinocyte and fibroblast. Additionally, the HA amounts in groups treated using citronellic acid was not significantly different from that of the positive groups treated using WY14643. These results showed that citronellic acid significantly increased HA as moisturizing factor related to skin hydration. HA is a non-sulfated glycosaminoglycan, anionic polymer that is a component

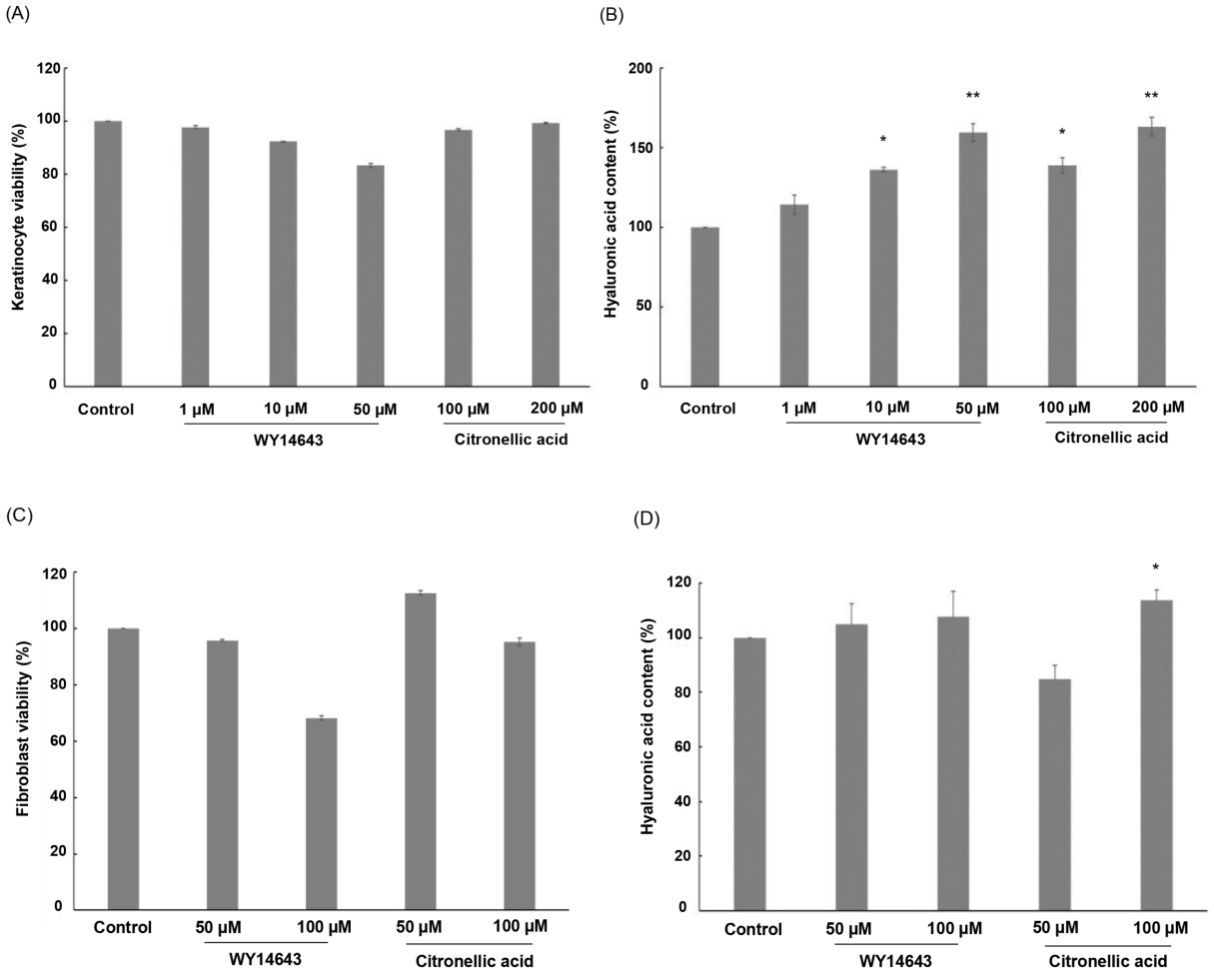

Fig. 4. Determination of cell viability and hyaluronic acid synthesis in normal human keratinocytes (A) and fibroblasts (C). Cells were treated with various concentrations (50 μM–200 μM) of citronellic acid. WY14643 was used as a positive control and no treatment was used as a negative control. Cell viabilities were determined using MTT assay. Quantification of hyaluronic acid (HA) were assayed by hyaluronic acid ELISA kit in normal human keratinocytes (B) and fibroblasts (D). Values are presented as means ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ compared to the untreated control group.

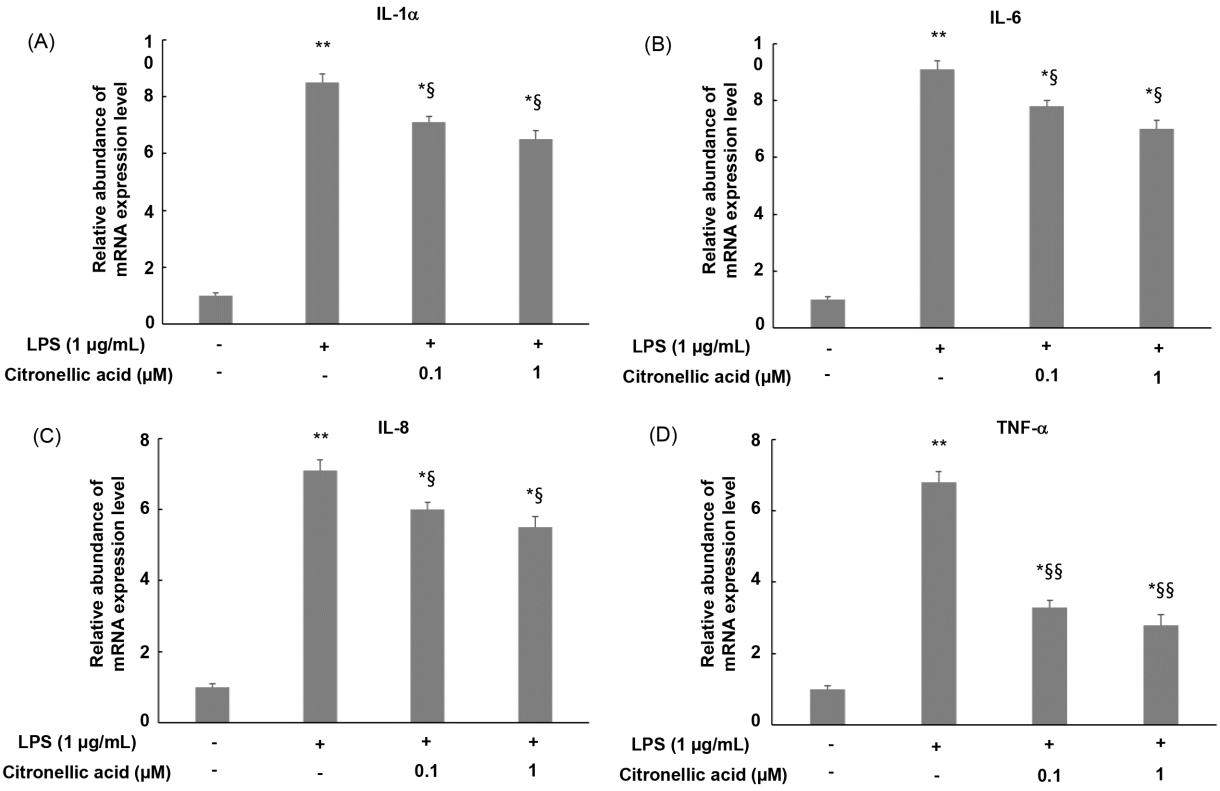

Fig. 5. Effect of citronellic acid on inflammatory cytokines. The effect of citronellic acid on the production of inflammatory cytokine was determined by quantitative qRT-PCR in HaCaT cells. Values were normalized to β-actin before calculating changes. Cells were treated with 1 μg/mL of lipopolysaccharides (LPS) alone or in combination with different concentrations of citronellic acid for 6 h. LPS was used as a positive control and no treatment was used as a negative control. Values are presented as means ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ compared to the untreated control group. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ compared to the LPS treated group.

of the extracellular matrix of the skin, and is composed of alternating D-glucuronic acid and N-acetyl-D-glucosamine.[27,28] It is known that HA synthesis can be promoted by retinoic acid[29] or esradiol treatment[30] in keratinocytes, but, L-ascorbic acid and anti-inflammatory corticoids decrease HA synthesis in dermal fibroblasts.[31] HA level decreases with skin aging, which may influence to wrinkle formation and loss of skin elasticity,[27] therefore, there is a request to develop new materials that have the effect of suppressing inflammation and reducing skin wrinkles by regulating HA production. HA is synthesized by keratinocytes and fibroblasts in the epidermis and dermis, and plays a critical function in extracellular matrix formation of collagen and elastin.[32] Furthermore, adiponectin, a hormone derived from adipocytes, is known to regulate PPAR-α and promote HA production in dermal fibroblasts.[33] These results showed that citronellic acid increased HA amount along with PPAR-α activation in keratinocytes and fibroblasts.

The inhibitory effect of citronellic acid on the production of pro-inflammatory cytokine was measured by qRT-PCR. As shown in Fig. 5, The citronellic acid repressed the gene expressions of LPS-induced inflammatory cytokine including IL-1, IL-6, IL-8, and TNF-α in HaCaT cells. These data indicate that treatment with citronellic acid resulted in the down-regulation of mRNA expression levels related to pro-inflammatory cytokines. Human beta-defensin-2 (hBD2) and LL-37, belonging to the cathelicidin family, also known as skin anti-microbial peptides are expressed in keratinocytes in skin inflammatory lesions.[34] The inhibitory effect of citronellic acid on the gene expression of skin anti-microbial peptides was measured. As shown in Fig. 6, The citronellic acid significantly suppressed the genes expression LPS-induced hBD2 and LL-37. These results indicate that citronellic acid reduces LPS-induced inflammatory responses and consequently decreases skin anti-microbial peptide synthesis. Cytokines are abundantly expressed in atopic dermatitis, and inflammatory cytokines are known to weaken ceramide synthesis throughout the epidermis which is responsible for skin barrier improvement.[35]

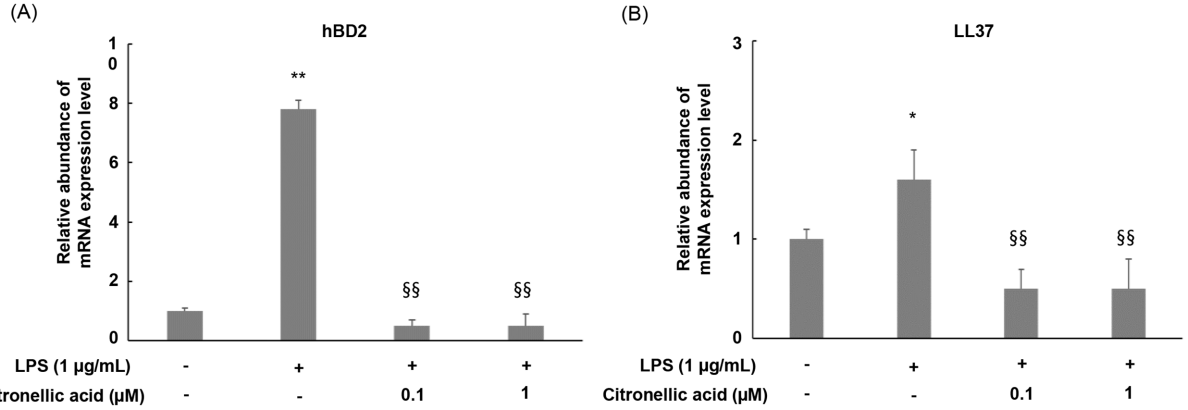

Fig. 6. Effect of citronellic acid on anti-microbial peptides. The effect of citronellic acid on the production of anti-microbial peptides was determined by quantitative RT-PCR in HaCaT cells. Values were normalized to β-actin before calculating changes. Cells were treated with 1 μg/mL of Lipopolysaccharides (LPS) alone or in combination with different concentrations of citronellic acid for 6 h. LPS was used as a positive control and no treatment was used as a negative control. Values are presented as means ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ compared to the untreated control group. $^{§§}p < 0.01$ compared to the LPS treated group.

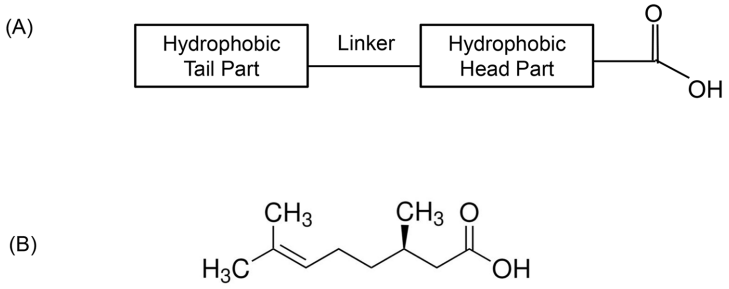

Fig. 7. Model of PPAR-α ligand structures. PPAR-α ligand structure has a carboxylic acid moiety in hydrophobic head portion and a linker between the hydrophobic tail portion and the hydrophobic head portion (A). Structure of citronellic acid (3,7-dimethyl-6-octenoic acid) (B).

Therefore, suppressing these inflammatory mediators is being considered as a new agent for skin treatment. In this study, the anti-inflammatory effects of citronellic acid were identified through inhibition of inflammatory cytokines and antimicrobial peptides. therefore, citronellic acid has been shown to be a potential agent for treating inflammatory conditions such as atopic dermatitis.

In previous study, the molecular docking simulation of PPAR-α agonists has been attempted through the natural products library of anti-inflammatory natural products from Ministry of Food and Drug Safety (MFDS) and Dictionary of Natural products (DNP) *in silico* screening to search candidates for PPAR-α agonists,[16] On the basis of the chemical structure of PPAR-α agonists such as WY14643 and clofibrate, PPAR-α ligand structure has a carboxylic acid moiety in hydrophobic head portion and a linker between the hydrophobic tail portion and the hydrophobic head portion (Fig. 7). Finally, citronellic acid was screened as the most appropriate PPAR-α ligand by simulation of structure-based pharmacophore screening[16] and has been shown to activate PPAR-α.[36] Citronellic acid enhanced the rate of keratinized epidermis formation and also increased the proteins involved in CE formation, such as involucrin. These results indicate that treatment of citronellic acid as a natural PPAR-α agonist not only increased CE formation but also increased terminal differentiation of keratinocytes, as evidenced by increased expression of involucrin.

In conclusion, these results suggest that citronellic acid can improve atopic dermatitis by activating PPAR-α to promote the production and differentiation of the stratum corneum, strengthening the skin barrier function, increasing HA, and regulating anti-inflammatory cytokines and antimicrobial peptides. Citronellic acid may be a useful candidate for epidermal barrier restoration, and may have benefits as it is a plant derived PPAR-α agonist.

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Received July 29, 2024
Revised December 1, 2024
Accepted December 2, 2024