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Harnessing Fermentation Technology: Discovery of Dual Action Bio-Transformed Compounds for Managing Chronic Pruritus in Sensitive Skin

Ka-Heng Lee[1,*], Wen-Shang Saw[1], Mei-Leng Tee[1], Thomas Chung-Lee Yee[1], Choon-Han Heh[2], Yi-Peng Ng, Ursula Rho-Wan Chong[1], Khan-Loon Ng[1], Mong-Sah Toh[1]

[1]Winnox Cosmeceutics (A R&D entity of Wipro), Malaysia;
[2]Faculty of Pharmacy, Universiti Malaya, Malaysia

1. Introduction

Pruritus or itch is defined as an unpleasant sensation that provoke the desire to scratch. It is a common symptom associated with various skin disorders such as eczema, atopic dermatitis (AD), psoriasis and xerosis cutis [1]. In cosmetic industry, itch is recognized as one of the key sensory discomforts experienced in sensitive skin, significantly affecting one's quality of life. Although numerous skincare interventions are available for sensitive skin, the global prevalence of sensitive skin symptoms continues to rise [2]. Among the discomfort symptoms associated with sensitive skin, itch remains one of the least studied, with considerable lack of in-depth mechanistic understanding.

The mechanism of itch induction is complicated that involves various cell types and signalling pathways. The perception of itch begins in the skin when endogenous or exogenous pruritogens activate specific receptors on peripheral sensory nerve fibres. These signals are then transmitted to brain, where they are interpreted as itch sensations. (Figure 1). Itch sensation can be classified into two main biological pathways: histaminergic and non-histaminergic. The histaminergic pathway is associated with acute itch that involves histamine binding to sensory neurons, typically in allergic reactions. In contrast, the non-histaminergic pathway, dominant in chronic itch conditions like eczema, dry skin and sensitive skin, involves mediators such as proteases, cytokines, and neuropeptides that activate neuron receptors [3] [4].

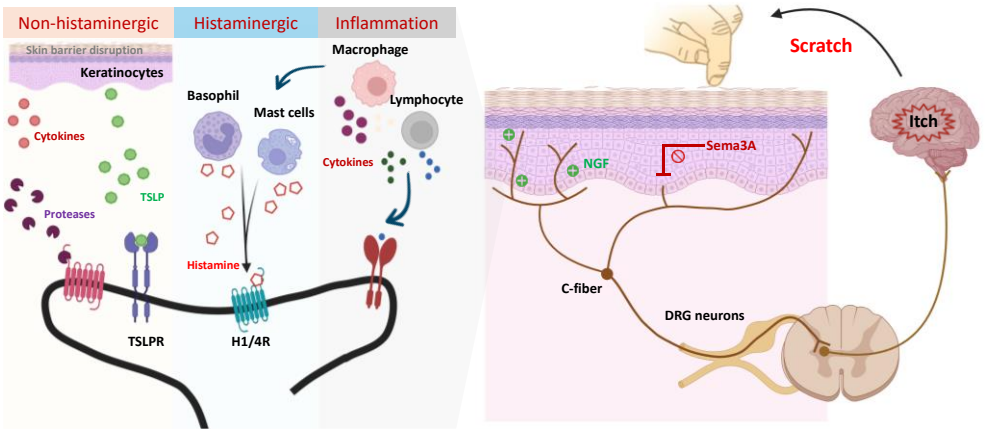


Figure 1. Overview of the itch mechanism (modified from [4] and [7])

In the basic research of itch, acetone/ether/water (AEW)-treated mouse model is a well-established method for studying mechanism of dry-skin induced itch. Notably, AEW treatment leads to the increase of trans-epidermal water loss (TEWL) and heightened scratching behaviour, highlighting the role of skin barrier dysfunction in itch induction [5]. Furthermore, intracutaneous injection of a Proteinase-Activated Receptor-2 (PAR-2) agonist, SLIGKV, has been shown to dose-dependently induce itch sensation in healthy subjects and amplify the itch intensity of patient with AD [6]. Similarly, sensitive skin is often characterized by impaired barrier function and enhanced nerve fibre density within the epidermis. Taken together, we hypothesize that barrier disruption in sensitive skin is the primary cause triggering the release of mediators via epidermal PAR-2 signalling pathway that activate cutaneous nerve fibres and elicit itch sensations.

Despite the extensive research, most studies have been conducted using animal models, which do not fully replicate human skin responses. In this study, we aim to investigate the molecular mechanisms underlying non-histaminergic pruritus using human-derived skin models, focusing on the PAR-2 signalling pathway. Our goal is to develop effective skincare solutions containing bioactive compound which potentially used to manage pruritus in sensitive skin, while leveraging fermentation technology to enhance the safety and efficacy of this bioactive compound for its full potential functionality as itch-relief actives.

2. Materials and Methods

2.1. Acetone/ether/water (AEW)-induced model of skin barrier dysfunction

The EFT-400 full-thickness reconstituted skin tissue (Mattek, USA) and NativeSkin® explant (Genoskin, France) were cultured in respective maintenance media at 37°C with 5% CO_2 for 24 hours upon receipt. Subsequently, the skin samples were treated with a 1:1 mixture of acetone and diethyl ether for 5 minutes to induce barrier disruption, followed by three rinses with deionized water. For the inhibition study, a PAR-2 antagonist peptide was topically applied to the skin post-AEW treatment. The AEW-treated tissues were then incubated under the same conditions for an additional 24 hours. Post-incubation, culture supernatants were collected for itch mediators (histamine, NGF, Semaphorin 3A, thymic stromal lymphopoietin (TSLP), IL-1α, IL-1β and IL-8) analysis using ELISA. Skin tissues were fixed in sucrose solution for histological and immunohistochemical (IHC) assessments of KLK-5 expression. Additionally, TEWL measurements were conducted using a Tewameter® (Courage+Khazaka electronic GmbH, USA) to evaluate skin barrier integrity.

2.2. PAR-2-activated skin model (non-histaminergic itch pathway)

The Human Reconstructed Epidermis (RHE from Episkin, France) and NativeSkin® (Genoskin, France) were cultured in the provided media at 37°C with 5% CO_2 for 24 hours upon receipt. The inflammatory responses of skins were induced by peptide PAR-2 agonist (SLIGKV-NH_2) or its inactive peptide (LSIGKV-NH_2) as control, followed by product treatment topically for 24 hours. For the inhibition study, PAR-2 antagonist peptide (FSSLRY) was used. Lastly, the inflammatory cytokine level at the supernatant including Interleukin-8 (IL-8) was quantified by using ELISA kit (Biolegend, USA).

2.3. Basophil co-culture skin model (histaminergic itch pathway)

Basophilic leukemia cell line KU812 (ATCC, USA) was cultured and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin antibiotics. In parallel, human full-thickness skin tissues (Mattek, USA) were equilibrated overnight in assay medium (Mattek, USA). Subsequently, the

full-thickness skins were co-cultured with KU812 cells (100,000 cells/well) in 6-well plates using serum-free medium to induce histamine release. Simultaneously, the test product was topically applied, and the co-culture system was incubated at 37°C with 5% CO_2 for 48 hours. Finally, the culture supernatant was collected for quantification of histamine levels using an ELISA kit (Abnova, Taiwan).

2.4. Fermentation of Thymoquinone

The edible strain of *Bacillus spp.*, obtained from the Korean Collection for Type Cultures (KCTC), was cultured in 500 mL of GPY medium containing 2–6% glucose, 0.1–1% peptone, and 0.1–1% yeast extract. The culture was incubated at 25 to 40°C for 24 hours. Following incubation, microbial cells were harvested by centrifugation at 3,000 to 5,000 rpm for 10 to 30 minutes. The resulting cell pellet was washed with a phosphate buffer (30–100 mM, pH 7.2) containing 1 to 4% glycerol and subsequently resuspended in 5 mL of the same buffer. Thymoquinone was then added as the substrate, and the microbial suspension was incubated at 25 to 40°C for 48 hours to facilitate biotransformation. Biotransformation yield was determined by quantifying the amount of TMQ remaining post fermentation, relative to the original amount.

2.5. Extraction and characterization of thymoquinone and its transformed compounds

TMQ and its bio-transformed derivatives present in the ferment filtrate were extracted using a liquid–liquid extraction method with methanol and water. The compounds were subsequently purified and analysed using preparative HPLC (Waters, USA) equipped with a C18 column. Elution was performed using an acetonitrile/water gradient and detection at a wavelength of 274 nm. Quantification of TMQ was achieved based on a standard calibration curve derived from peak heights of known concentrations. The chemical structures of the transformed molecules were further characterized using Fourier-transform infrared spectroscopy (FTIR, Thermo Fisher Nicolet IS10, USA) and proton nuclear magnetic resonance (H-NMR) spectroscopy, conducted on a Bruker Avance III 400 MHz system (USA).

2.6. Molecular docking of PAR-2 protein

Molecular docking of the ligand compound to the PAR-2 protein was conducted based on previously reported method with slight modification [8]. The crystal structure of a thermostabilized human PAR2 in complex with AZ8838 (PBD ID: 5NDD) was obtained from Protein Data Bank. To optimize the protein structure, water molecules and extra conformation of amino acid residues were removed using Discovery Studio Visualizer (DSV) 2025 software. The ligand structure of TMQ and DHTMQ was retrieved from PubChem. Docking simulations were performed by using AutoDock Vina 1.1.2 software (Vina). The docking results were evaluated based on the binding affinity score and binding interaction with key amino acid residues of PAR-2 binding pockets, as visualized and analyzed using DSV.

2.7. Skin Sensitization Test

The sensitizing potential of the ferment filtrate was evaluated using the in vitro skin sensitization test, which quantifies changes in CD86 cell surface marker expression on the human histiocytic lymphoma cell line U937 (OECD, 2023). Briefly, U937 cells were cultured at 37°C with 5% CO_2 and maintained in RPMI-1640 medium with phenol red, supplemented with 1 mM sodium pyruvate, 1% antibiotic-antimycotic mix, and 10% fetal bovine serum (FBS). The cells were seeded at a density of 200,000 cells per well into 6-well plates and treated with the test compound for 45 hours at 37°C under 5% CO_2. After incubation, the cells were pelleted, resuspended with APC-labelled anti-CD86 (BD Pharmingen, USA), and stained with propidium iodide (BD Pharmingen, USA). Cell analysis was performed using a BD Accuri C6 Plus Flow

Cytometer (BD Biosciences, USA). A test substance is considered positive for dendritic cell activating potential if it induces ≥150% CD86 expression with cell viability of at least 70%.

2.8. Clinical assessment of skin parameters in sensitive skin

A single blind clinical study was conducted in a group of 14 females with sensitive skin characterized with impaired skin barrier function (TEWL more than 15 g m^{-2} h^{-1}) and heightened neuro-sensitivity of Lactic Acid Stinging Test (LAST) score of more than 2, were recruited for the clinical test. All panellists were instructed to use a standard facial cleanser for 3 continuous days prior to skin evaluation as baseline measurement. Then, panellists were instructed to apply product on face twice daily for a duration of 4 weeks. Lastly, multiple skin parameters were assessed to evaluate the clinical efficacy of the product on sensitive skin. Subjective itch intensity was recorded using a standardized self-assessment scale ranging from 1 to 5. Skin barrier integrity was quantified by TEWL using a Tewameter® (Courage+Khazaka electronic GmbH, USA). Neuro-sensitivity was evaluated via the LAST, and cutaneous redness was objectively analysed using VISIA® imaging system (Canfield Scientific, USA).

3. Results

3.1. Identification of the crucial role of PAR-2 in mediating non-histaminergic induced itch mediators in human skin models

To identify epidermal-derived itch mediators, full-thickness skin and skin explants were treated with AEW. This treatment induced a consistent increasing trend in TEWL without compromising tissue morphology (Figure 2), confirming impairment of the skin barrier function. A panel of potential itch mediators, including histamine, NGF, Semaphorin 3A, TSLP, IL-1α, IL-1β, IL-8, KLK-5, and KLK-7, was subsequently analysed. Among these, IL-8 secretion was significantly elevated in both AEW-treated full-thickness skin and skin explants. Additionally, KLK-5 expression was upregulated in AEW-treated full-thickness skin, but not in skin explants (Figure 3A). These findings suggest that IL-8 may serve as a key itch mediator in barrier-compromised skin.

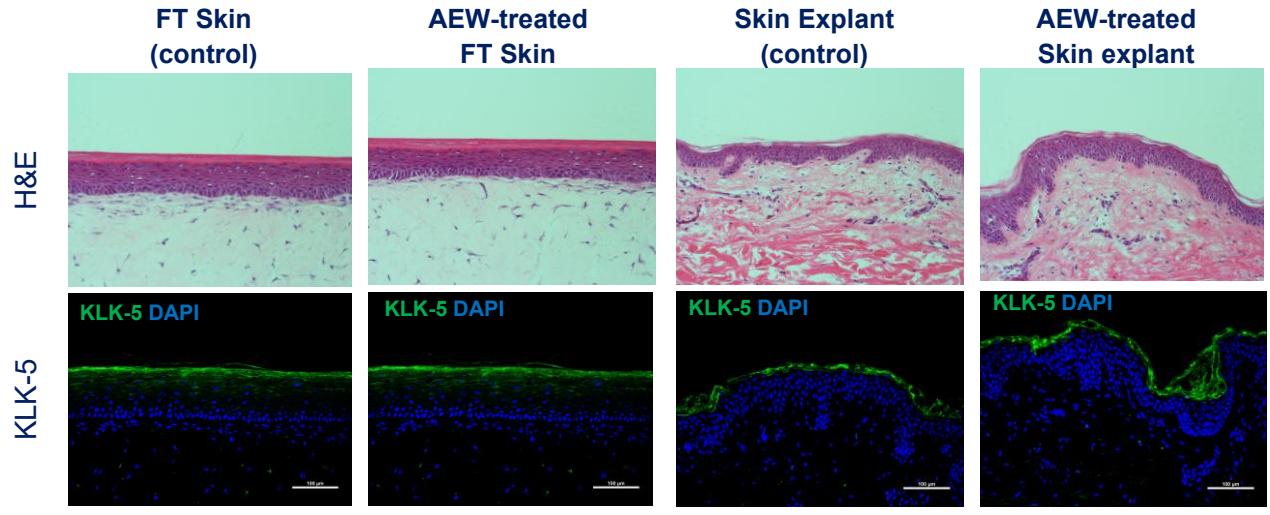


Figure 2. Effects of AEW Treatment on Skin Morphology and KLK-5 Expression in Full-Thickness (FT) Skin and Skin Explants.

To investigate the role of PAR-2 in promoting the secretion of itch mediators in skin, the AEW-treated skin explant was co-treated with PAR-2 antagonist, demonstrating inhibition of the IL-8 secretion (Figure 3b). In addition, skin explant treated with PAR-2 agonist (SLIGKV-NH_2) but not its inactive peptide (LSIGKV-NH_2), stimulated the expression of same itch mediators,

including IL-8 and KLK-5 (Figure 4a). Furthermore, the secretion of IL-8 was significantly inhibited by treatment of PAR-2 antagonist (FSSLRY) in the PAR-2-activated Reconstructed Human Epidermis (Figure 4b). Taken together, these findings suggest the crucial role of epidermal PAR-2 signalling pathway in promoting the secretion of itch mediators (IL-8 and KLK-5) via non-histaminergic route.

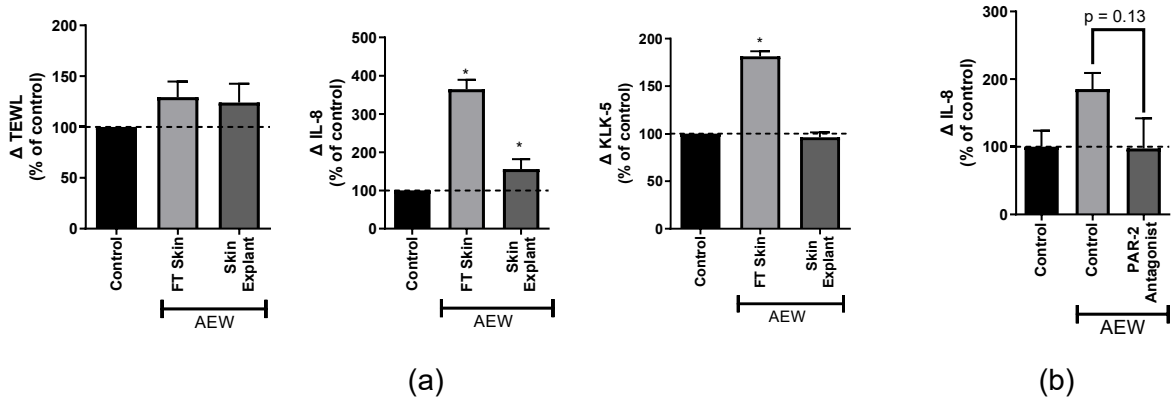


Figure 3. Effects of AEW treatment on skin barrier function and secretion of itch mediators in full-thickness (FT) skin and skin explants (a). Effects of PAR-2 inhibition on the secretion of itch mediators in AEW-treated skin explants (b). All data are presented as mean ± SEM from two independent experiments (n = 2). *p < 0.05, significantly different versus control group.

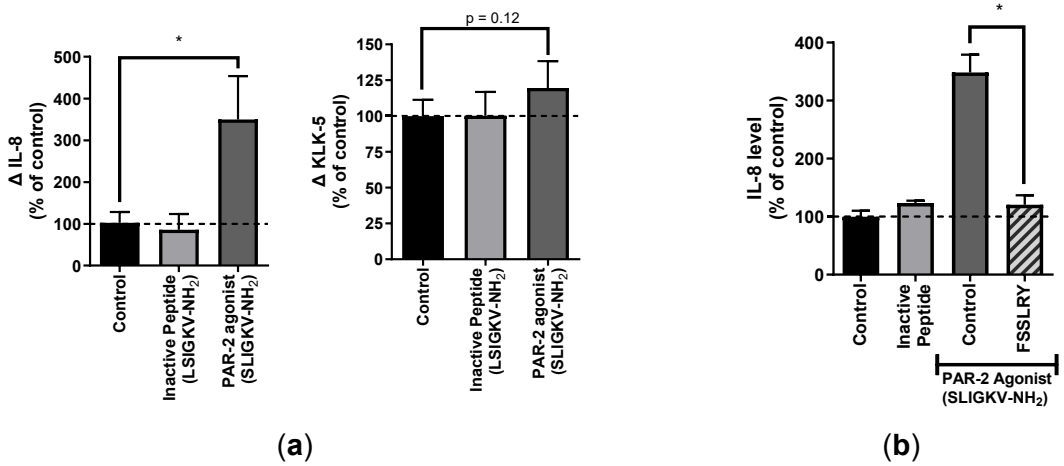
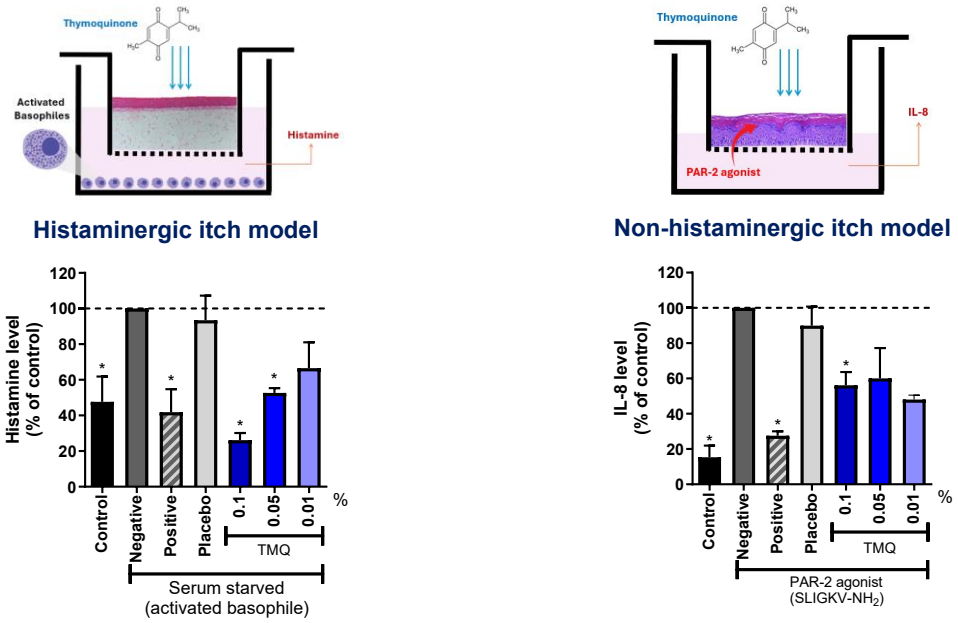


Figure 4. Effects of PAR-2 activation on the secretion of itch mediators (IL-8 and KLK-5) in skin explants (A). Effects of PAR-2 antagonist (FSSLRY) on the secretion of itch mediator (IL-8) in PAR-2-activated Reconstructed Human Epidermis (B). All data were expressed as mean ± SEM from two independent experiments (n=2). *p < 0.05, significantly different against control group.

3.2 Identification of thymoquinone as potent dual-action compound for managing skin pruritus

To identify an effective solution for managing pruritus in sensitive skin, we have developed two novel skin models for compound screening: a histaminergic and a non-histaminergic model. Through this screening, topical application of thymoquinone (TMQ), an active compound derived from *Nigella sativa* extract, demonstrated potent dual-action efficacy. Specifically, TMQ significantly attenuated histamine release dose-dependently in human full-thickness skin co-cultured with activated basophils and inhibited IL-8 secretion in a PAR-2-activated

Reconstructed Human Epidermis (Figure 5). These findings highlight the potential of TMQ in managing pruritus via both histaminergic and non-histaminergic pathways.

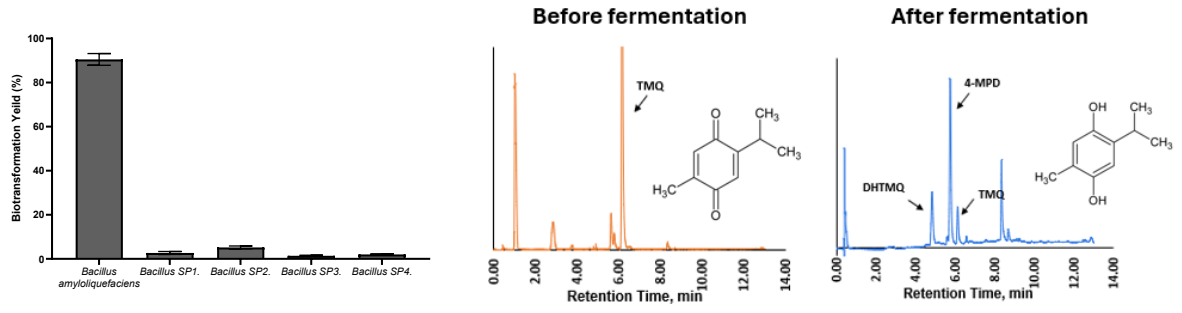


(a) (b)

Figure 5. Topical inhibitory action of Thymoquinone (TMQ) on the histamine release in FT-skin co-cultured with activated basophile cells and IL-8 secretion in PAR-2-activated Human Reconstituted Epidermal**.** All data were expressed as mean ± SEM from two independent experiments (n=2). *p < 0.05, significantly different against the control group.

3.3 Biotransformation Enhances the Safety and Efficacy of Thymoquinone

Fermentation technology is widely used to transform active molecules to enhance their bioavailability and efficacy [10]. In this study, we harnessed fermentation technology to evaluate the ability of different types of *Bacillus spp.* to bio-transform TMQ compound. As shown in Figure 6, *Bacillus amyloliquefaciens* transformed approximately 90% of the TMQ, yielding two bio-transformed compounds, dihydro-thymoquinone (DHTMQ) and 4-methylpentyldocosanoic (4-MPD).



(a) (b)

Figure 6. Biotransformation of thymoquinone via fermentation of *Bacillus spp*. (a) and HPLC profiling and characterization of transformed compounds (b).

To compare the efficacy of the transformed compounds with TMQ, we first used *in silico* molecular docking analysis to study the interaction of TMQ and DHTMQ, with the human PAR-2 protein. As shown in Figure 7a, both compounds exhibited strong binding to the PAR-2 protein, with binding energies of –7.454 kcal/mol and –6.793 kcal/mol, respectively. Notably, DHTMQ formed a hydrogen bond with aspartic acid (ASP228) at the active site of the PAR-2 protein,

indicating a higher binding affinity compared to TMQ. The anti-PAR-2 effects was validated by experimental analysis, DHTMQ exhibited significantly greater efficacy in suppressing IL-8 secretion in PAR-2-activated keratinocyte cells, with a lower IC_{50} value of 5.38 ± 0.73 µM compared to TMQ, which showed an IC_{50} of 13.04 ± 1.70 µM (Figure 7b).

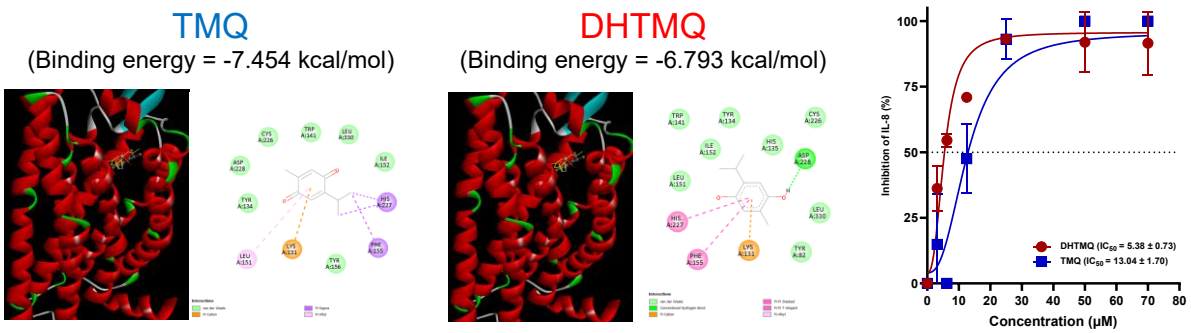


Figure 7. Comparative inhibitory action of TMQ and DHTMQ on PAR-2 protein binding and interaction via molecular docking analysis (a), and their inhibition of PAR-triggered IL-8 secretion in keratinocyte (b).

For safety evaluation, the sensitization potential of the fermented filtrate was assessed using an in vitro skin sensitization assay. In this test, a CD86 stimulatory index exceeding 150% at a concentration of 200 µg/mL indicates as potential skin sensitizer. As shown in Figure 8, both the control and fermented filtrates were classified as non-sensitizer, with CD86 stimulatory indices below 150% at 200 µg/mL. Interestingly, when challenged at higher concentration of 1000 µg/mL, the mock-control filtrate (without bacterial inoculum) exhibited a significantly elevated index, suggesting its potential allergenic property with cumulative exposure. In contrast, the fermented filtrate at 1000 µg/mL showed an improved safety profile by maintaining a low CD86 stimulatory index. Lastly, the safety of the fermented filtrate was further confirmed through a Human Repeat Insult Patch Test (HRIPT), which showed a low potential in inducing allergic skin reactions upon repeated exposure. Taken together, these findings support the successful biotransformation of TMQ via fermentation using *Bacillus amyloliquefaciens*, resulting in enhanced anti-PAR-2 efficacy and an improved safety profile.

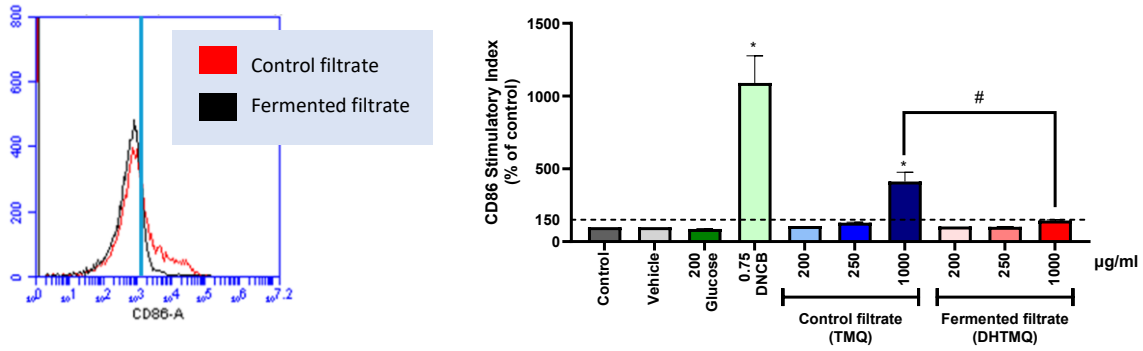


Figure 8. Effects of the ferment filtrate on the potential skin sensitization via assessment on the CD86 stimulatory index in human monocyte U937 cells by using flowcytometry. All data were expressed as mean ± SEM from two independent experiments (n=2). *p < 0.05 against untreated control group. #p < 0.05, significantly different versus control filtrate group.

3.4 Topical effects of ferment filtrate (bio-transformed compounds) in managing pruritus in sensitive skin

The developed ferment filtrate was incorporated into a topical formulation and subjected to various tests to validate its anti-itch efficacy. Using biomimetic skin models, topical application of the ferment filtrate effectively attenuated histamine release in human full-thickness skin co-cultured with activated basophils, and inhibited IL-8 secretion in a PAR-2-activated Reconstructed Human Epidermis (Figure 9). In clinical testing on subjects with sensitive skin, topical treatment with the ferment filtrate significantly reduced facial itchiness scores and improved key sensitive skin symptoms, including LAST score, TEWL, and skin redness (Figure 10). Overall, these findings highlight the dual action of the ferment filtrate in managing pruritus through both histaminergic and non-histaminergic pathways in sensitive skin.

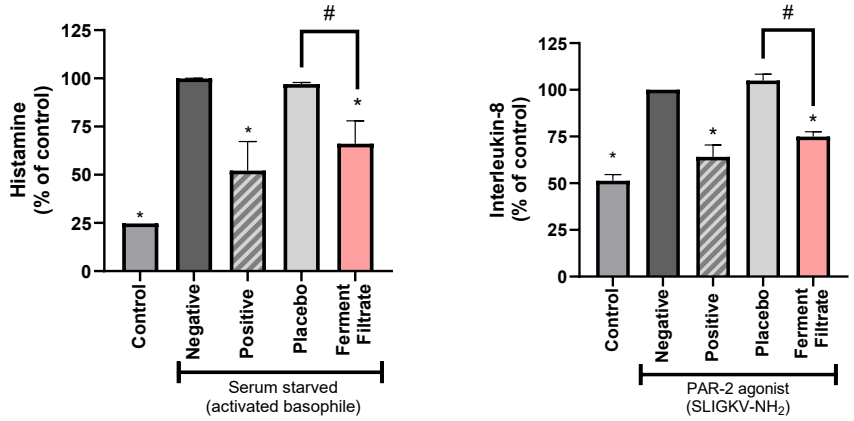


Figure 9. Topical efficacy of ferment filtrate (bio-transformed compounds) on the inhibition of itch mediators in histaminergic (a) and non-histaminergic biomimetic skin model (b). All data were expressed as mean ± SEM from two independent experiments (n=2). *p < 0.05, significantly different against control group, #p < 0.05, significantly different from placebo group.

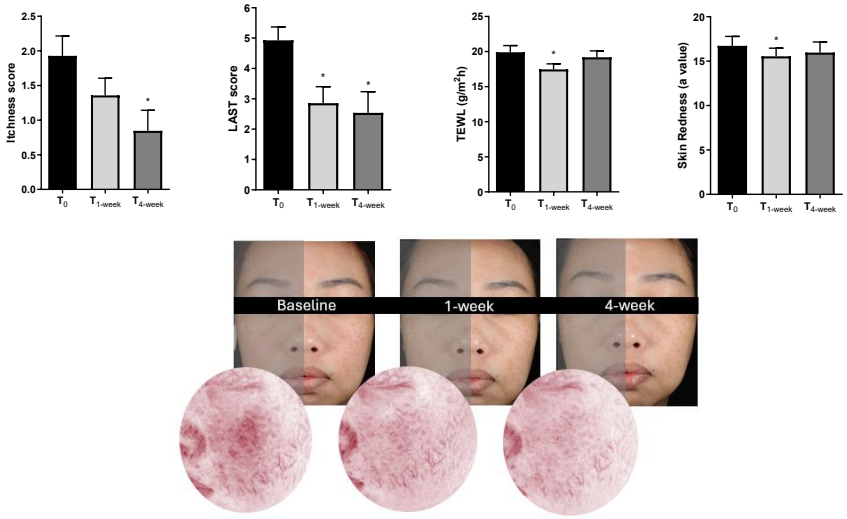


Figure 10. Topical efficacy of ferment filtrate (bio-transformed compounds) on the suppression of sensitive skin symptoms (itchiness score, LAST, TEWL and skin redness) in subjects with sensitive skin. All data were expressed as mean ± SEM from fourteen subject (n=14). *p < 0.05, significantly different against baseline.

4. Discussion

In this study, we developed a human acetone/ether/water (AEW)-induced skin barrier disruption model to investigate the role of epidermal factors in initiating itch via non-histaminergic pathways. Our findings demonstrate that barrier disruption in the AEW model enhances IL-8 and KLK5 protein expression, mediated primarily through epidermal PAR-2 signalling. Previous studies have shown that KLK5 activates PAR-2 in keratinocytes, triggering the release of proinflammatory cytokines such as TSLP, IL-8, TNF-α and IL-1β, along with epidermal barrier dysfunction and atopic dermatitis-like histopathological feature [11]. Interestingly, TSLP and inflammatory cytokines (TNF-α and IL-1β) were undetectable in our AEW-treated skin model, suggesting that IL-8 may function as the predominant itch mediator. We hypothesize that IL-8 is responsible to recruit neutrophil, which secrete interferon gamma-induced protein 10 (IP-10), activating CXCR3 receptor on sensory neurons and thereby driving itch induction [12]. Collectively, our findings highlight IL-8 secretion mediated by epidermal PAR-2 signalling in barrier-compromised skin as a promising molecular target for managing non-histaminergic pruritus in sensitive skin.

Building on these insights, TMQ was identified as a dual-action anti-pruritic agent, effectively suppressing histamine release and PAR-2-induced IL-8 secretion. Furthermore, we identified *Bacillus amyloliquefaciens* as a novel biocatalyst for transforming TMQ into DHTMQ with enhanced anti-PAR-2 activity and an improved safety profile. Although biotransformation of TMQ has been reported by using As*pergilus niger* [13], the underlying biochemical pathway remains poorly characterized. To address these gaps, we propose integrating the computational tools, such as artificial intelligence-driven pathway prediction, with experimental synthetic biology to elucidate the enzymatic steps involved and optimize production efficiency of DHTMQ.

The clinical relevance of TMQ and DHTMQ is supported by a recent meta-analysis confirming its efficacy in alleviating inflammatory skin conditions such as atopic dermatitis, eczema, psoriasis, and acne [14]. Mechanistically, bio-transformed DHTMQ interacts with epidermal PAR-2 receptors, downregulating key itch mediators. Importantly, these anti-pruritic effects were validated in a clinical test, where topical application of the bio-transformed compounds significantly improved itch symptoms and restored skin barrier function in sensitive skin subjects. Collectively, these findings highlight TMQ-derived bio-transformed compounds as promising dual-action candidates for topical treatment targeting itch in sensitive skin.

5. Conclusion

In summary, this study advances our understanding of pruritus management in sensitive skin by elucidating the interplay between skin barrier dysfunction and PAR-2 signalling in mediating non-histaminergic itch. Through microbial biotransformation of TMQ using *Bacillus amyloliquefaciens*, we successfully developed a ferment filtrate comprising DHTMQ, a dual-action anti-pruritic agent with enhanced efficacy and an improved safety profile for the treatment of pruritus in sensitive skin.

6. Acknowledgement

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