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ENRICHMENT PROGRAM REPORT

The Study of Tamanu (*Calophyllum inophyllum*) Oil Effects on Atopic Dermatitis-Related Gene Expression Levels on TNF-α and IFN-γ Induced HaCaT Cells

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Biotechnology

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RESEARCH REPORT

THE STUDY OF TAMANU (*Calophyllum inophyllum*) OIL EFFECTS ON ATOPIC DERMATITIS-RELATED GENE EXPRESSION LEVELS ON TNF-α AND IFN-γ INDUCED HaCaT CELLS

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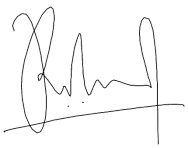

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ABSTRACT

Atopic dermatitis (AD) is a common chronic inflammatory skin disease affecting almost 2.4% of the population worldwide. Due to the complex interactions of genetic, environmental, and immunological variables, AD is still incurable as the knowledge of this complex disease is still limited. Moreover, current available treatments can only treat its symptoms and stop them from getting worse. The most widely used treatment for AD consists of applying steroid creams on the affected skin area. Prolonged use of these steroid creams carries the risks of side effects. The limited availability of safe and affordable treatments for AD makes the development of new alternative treatments for AD both crucial and urgent. Tamanu oil, which is extracted from *Calophyllum inophyllum* nuts, has been demonstrated to have anti-inflammatory and wound-healing properties. These properties make tamanu oil an ideal candidate for a natural active ingredient for treating AD as both inflammation and damaged skin barrier functions are hallmark conditions that characterize AD. In this study, HaCaT cells induced with TNF-α and IFN-γ were utilized as AD models for investigating the effects of tamanu oil on the gene expression of AD-related genes: CTACK, IL-25, IL-33, MDC, TARC, and TSLP that are related to inflammation and skin barrier genes FLG and IVL. The investigation revealed that treatments with 5 µg/mL pure tamanu oil were able to restore the expression of IL-33 gene back to normal upon TNF-α and IFN-γ induction. In contrast, 10 µg/mL was recommended for MDC, TSLP, and FLG, making these treatments the most potent concentrations to be used as AD alternative treatment candidates in further studies. However, phytochemical screening should be performed in the future to identify the specific bioactive compound responsible for tamanu oil's anti-inflammatory and wound-healing properties.

Keywords: atopic dermatitis, *Calophyllum inophyllum*, pure tamanu oil, anti-inflammatory, wound-healing, gene expression

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LIST OF ABBREVIATIONS

AD	Atopic dermatitis
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BRIN	Badan Riset dan Inovasi Nasional
CD4+	Helper T cells
CE	Cornified envelope
CO_2	Carbon dioxide
Ct	Threshold cycle
CTACK	Cutaneous T cell-attracting fchemokine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
FBS	Fetal bovine serum
FLG	Filaggrin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HaCaT	Human keratinocyte

HDF	Human dermal fibroblast
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
ILC2s	Group 2 innate lymphoid cells
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-25	Interleukin-25
IL-33	Interleukin-33
IVL	Involucrin
MDC	Macrophage-derived cytokines
MUFA	Monounsaturated acids
OX40L	Tumor necrosis factor receptor superfamily, member 4
Pen-Strep	Penicillin-Streptomycin
PUFA	Polyunsaturated acids

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SFA	Saturated fatty acids
SPF	Sun protection factor
TAE	Tris-acetate-EDTA
TARC	Thymus and activation-regulated chemokine
TCS	Topical corticosteroid
Th1	T helper 1
Th2	T helper 2
Tm	Melting temperature
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1. Background

Atopic dermatitis or AD, is a common, chronic, relapsing, inflammatory skin disorder affecting almost 2.4% of the population or around 200 million people worldwide (Urban et al., 2021). A review by Tamagawa-Mineoka & Katoh (2020) stated that sensitive, dry skin, isolated or widespread eczematous lesions on the face, flexural folds, wrists, and ankles, typically accompanied by an intense itching sensation, are common signs of AD. The first case of AD was identified in the late 1800s, but the specific causes and methods to treat the disease are still unknown. However, most researchers claimed that the disease is closely related to allergic inflammation and skin barrier dysfunction, while some also further contended that the pathogenicity of AD might be linked to environmental factors, anomalies in the skin epidermis, and immune system malfunction in addition to hereditary dysfunction (Kapur et al., 2018; Kolb & Ferrer-Bruker, 2021). AD is still a serious health concern and is still escalating in many nations, especially in developing countries such as Indonesia, due to the limited knowledge and response to available therapies (Bieber, 2021; Pribowo et al., 2021).

According to Kim et al. (2019), AD is a highly diverse illness with a complex etiology, as its risk factors are greatly affected by genetic, environmental, and immunological factors. Due to complex interactions of genetic, environmental, and immunological variables, AD is still incurable as the available treatments can only treat its symptoms and stop them from getting worse (Dębińska, 2021). Thus, AD has generally been viewed up to this point as a skin disease for which local anti-inflammatory therapy should be used as the first line of treatment when the disease flares. These include topical corticosteroids (TCSs), topical calcineurin inhibitors, phototherapy with ultraviolet (UV) light, systemic immunosuppressants, and other medications. However, these topical medications have some limitations mainly due to the risks

of side effects from long-term usage, for example skin thinning, skin peeling, stretch marks, hormonal changes, and even telangiectasias (Pribowo et al., 2021; Thomsen, 2014). On the other hand, according to Brunner et al. (2017), other alternative treatments are deemed not feasible due to their high cost and time-consuming process, showing a gap in the treatment of inflammatory skin disease, especially in Indonesia. Therefore, there is a pressing need to develop new alternative treatments for AD especially from plants, for which tamanu oil has become more and more popular due to its promising properties.

Tamanu (*Calophyllum inophyllum*) is an evergreen tree producing non-edible oil seed native to Australia, eastern Africa, Southeast Asia, southern coastal India, and South Pacific (Ansel et al., 2016). In Indonesia itself, this plant is widely known as Nyamplung and is accessible in almost every region. Oil extracted from this plant's seed is well recognized for treating various skin illnesses such as burns, eczema, acne, and many more; thus has been commercially marketed despite still lacking enough evidence from studies. In addition, due to the presence of beneficial bioactive compounds (e.g., coumarins, flavonoids, and triterpenoids) in its seed, the oil has also been demonstrated to possess anti-inflammatory, wound-healing, antibacterial, antifungal, and antioxidant properties (Raharivelomanana et al., 2018). Tamanu seed extract may be a potential and less damaging treatment to lower AD severity as most users only experience minor irritation when applying the oil onto their skin for the first time, and there has been no report of major negative effects.

In this study, anti-inflammatory and skin barrier repair activities of tamanu oil will be investigated on human keratinocyte (HaCaT) cells induced by TNF-α and IFN-γby assessing the gene expression levels of CTACK, IL-25, IL-33, MDC, TARC, and TSLP related to inflammation, as well as FLG and IVL related to skin barrier. Gene expression after treatment with tamanu oil is chosen as the main focus of this study since no work has yet evaluated it.

1.2. Objectives

This study is aimed to investigate the anti-inflammatory and skin barrier repair activities of tamanu oil as a potential treatment for atopic dermatitis by achieving the following objectives:

1. To investigate the effects of different tamanu oil concentrations on the gene expression of CTACK, IL-25, IL-33, MDC, TARC, and TSLP related to inflammation.
2. To investigate the effects of different tamanu oil concentrations on the gene expression of FLG and IVL related to skin barrier.

1.3. Scope of Work

The scope of work for this study includes the activities starting from tamanu oil preparation until statistical analysis. The detailed scope of work is as follows:

1. Obtaining tamanu oil (pure oil)
2. Preparing inducers (tumor necrosis factor-alpha/TNF-α and interferon-gamma/IFN-γ) as well as treatment solution
3. Culturing HaCaT cells, followed by induction and treatment
4. Performing RT-qPCR to investigate gene expression levels
5. Performing statistical analysis

1.4. Hypothesis

The hypotheses of this study are as follows:

1. H_0: there will be no significant effect on the gene expression of CTACK, IL-25, IL-33, MDC, TARC, and TSLP related to inflammation after treatment with different tamanu oil concentrations.

   H_1: there will be downregulation effects on the gene expression of CTACK, IL-25, IL-33, MDC, TARC, & TSLP related to inflammation after treatment with different tamanu oil concentrations.

2. H_0: there will be no significant effect on the gene expression of FLG and IVL related to skin barrier after treatment with different tamanu oil concentrations.

   H_1: there will be upregulation effects on the gene expression of FLG & IVL related to skin barrier after treatment with different tamanu oil concentrations.

CHAPTER 2

LITERATURE REVIEW

2.1. Atopic Dermatitis (AD): General Information and Risk Factors

As mentioned in the introduction, atopic dermatitis or AD is a chronic and relapsing inflammatory skin disorder typically suffered by young children (Thomsen, 2014). Silverberg et al. (2021) stated that people of various ages and ethnicities might be affected by AD, but the majority of disease cases generally begin in infancy or childhood, with a prevalence of 2.7 to 20.1%, while only around 2.1 to 4.9% prevalence in adults globally. The term atopic dermatitis comes from *atopy*, which is described as the propensity to express immunoglobulin E (IgE) in the presence of common environmental factors (e.g., pollen, house dust mites, food allergens, etc.), as well as *dermatitis*, which is derived from the Greek words "*derma*" for skin and "*itis*" for inflammation (Thomsen, 2014). Common symptoms of AD include sensitive, dry skin, isolated or widespread eczematous lesions on the face, flexural folds, wrists, and ankles, typically accompanied by an intense itching sensation (Tamagawa-Mineoka & Katoh, 2020).

There are three types of AD onset according to Kolb & Ferrer-Bruker (2022): (i) Early-onset as the most common, with 60% of cases beginning from age 1; (ii) Late-onset that starts to show symptoms after puberty; and (iii) Senile onset being the rarest subset that manifests in patients over 60. Several risk factors, including genetic, environmental, and immunological, play a part in the onset of AD; however in general, individuals with affected family members have a substantially increased risk of having AD (Nutten, 2015). For genetic factors, null mutations of filaggrin (FLG) gene result in functional deficiencies which disturb the skin barrier, thus promoting external irritants to attack underlying tissues and cause skin dehydration (Thomsen, 2014). These external irritants including allergens, climate, microorganisms, pollution, stress, and UV radiation, are the various environmental factors that have been linked to the exacerbation of AD (Altunbulakli et al., 2018). Lastly, for

immunological factors, constant production of T helper 2 (Th2) cytokines and Immunoglobulin E (IgE) due to the trigger from external irritants is believed to cause an inflammation cascade and further worsen AD condition (Irvine et al., 2011). Due to the considerable heterogeneity in this disease's natural course as affected by the aforementioned factors and the unpredictable individual trajectories (e.g., stress level, sleeping habits, sweating and scratching frequency), different patients experience different rates of disease progression (Tamagawa-Mineoka & Katoh, 2020).

2.2. Atopic Dermatitis (AD) Pathophysiology

Pathogenesis and progression of AD are believed to be influenced by a variety of factors, including genetic profile, immune system function, and environmental influences. According to Silverberg & Silverberg (2015), there are two main hypotheses of AD pathophysiology as shown in **Figure 1.** below, although it is believed that AD arises from various causal agents. The first hypothesis is known as "outside-in", where decreased skin barrier integrity is a prerequisite for immunological dysregulation to occur and cause AD. On the other hand, the second hypothesis "inside-out", is the opposite where cutaneous inflammation is thought to be the principal cause of AD as it may disrupt skin barrier integrity and even lead to the impaired skin barrier.

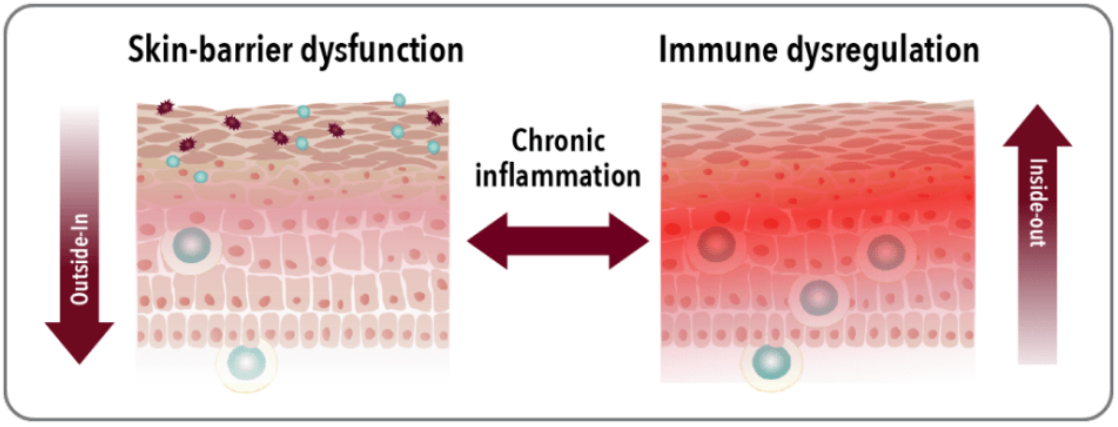


Figure 1. The "outside-in" and "inside-out" hypothesis schematic diagram.
(Boguniewicz & Leung, 2011)

2.1.1. Skin barrier dysfunction

According to Yang et al. (2020), skin's primary role is to serve as a barrier between an organism and its outside environment and offer both support as well as protection, which include reducing passive water loss, limiting environmental chemical absorption, and averting microbial infection. Filaggrin (FLG) and involucrin (IVL) are two molecules that have aberrant expressions in AD patients, and both molecules are predicted to have an impact on the homeostasis of permeability barrier (Kim et al., 2022).

FLG is a crucial structural protein which helps to keratinize, moisturize, and produce antimicrobial peptides in the skin. FLG-related genetic defects due to mutations have a well-established link to atopic dermatitis; particularly, its downregulation has been linked to the early onset of AD, higher allergies sensitivity and severity, as well as higher susceptibility to infection (Malik et al., 2017; Szegedi, 2015).

IVL is a key indicator of early epidermal differentiation which plays a role in the mature cornified envelope (CE) development encircling the terminally differentiated keratinocytes. Skin inflammation and immune response in AD have been linked to decreased IVL expression. Its expression is decreased in the skin of AD patients with lesions, whereas the Th2 cytokines (e.g., IL-4, IL-13, IL-33) expression is increased (Roesner et al., 2019; Schmidt, 2022).

2.1.2. Immune dysregulation

T helper 1 (Th1), T helper 2 (Th2), and regulatory T cell (Treg) imbalance are associated with the pathophysiology of AD, which leads to changes in Th1 and Th2-mediated immunological responses as well as IgE-mediated hypersensitivity. Various cytokines and chemokines related to immune dysregulation that causes AD include cutaneous T cell-attracting chemokine (CTACK), Interleukin-25 (IL-25),

Interleukin-33 (IL-33), macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), and thymic stromal lymphopoietin (TSLP).

The serum levels of CTACK, a chemokine generated by basal keratinocytes, are considerably increased in AD patients compared to healthy controls. Severity of AD clinical symptoms is believed to correlate with this increase (Das et al., 2022; Ferran & Santamaria-Babi, 2010).

Valizadeh et al. (2015) stated that the cytokine IL-25 contributes to the body's inflammatory pathways, particularly those that control immunological responses and immune cell migrations. Increased expression of IL-25 during AD significantly inhibits the terminal differentiation of keratinocytes, resulting in higher transepidermal water loss (TEWL), which triggers the production of a series of pro-inflammatory cytokines. FLG production is also reduced by IL-25's involvement in epidermal barrier dysfunction, which directly impairs the protective capabilities of the skin in AD patients (Basałygo et al., 2021).

In keratinocytes suffering from AD, the inflammatory cytokine IL-33 is overexpressed. Imai (2019) stated that its expression activates the immune system by causing ILC2s to rapidly grow and generate a large number of type 2 cytokines (e.g., IL-4, IL-5, and IL-13), which have been discovered to boost and eradicate AD significantly.

MDC, a chemokine expressed by macrophages and dendritic cells when activated, acts as a chemoattractant for Th2 cells. When compared to healthy controls, MDC serum levels are higher in AD patients because it causes Th2 migration, which results in inflammation (Ferran & Santamaria-Babi, 2010; Hirota et al., 2011).

Dendritic cells, endothelial cells, keratinocytes, and fibroblasts all contribute to the production of TARC expressed in the thymus. Patients with AD have high levels

of TARC serum compared to healthy controls, which is closely related to the disease severity (Umeda et al., 2020).

TSLP is a cytokine expressed by the skin, lungs, and gut's barrier surfaces epithelial cells; its high expression is believed to cause AD initiation and exacerbation. TSLP causes dendritic cells to mature and express tumor necrosis factor receptor superfamily, member 4 (OX40L), which then causes naive helper T cells (CD4+) to develop into Th2 cells to release Th2 cytokines (e.g., IL-4, IL-5, and IL-13) and secrete IgE from B cells, thus disrupting the skin barrier (Indra, 2013; Yang et al., 2020).

2.3. TNF-α and IFN-γ Induced HaCaT Cells

HaCaT cells are *in vitro* differentiable long-lived, spontaneously immortalized human keratinocyte lines. According to Colombo et al. (2017), they have been extensively employed as *in vitro* models of the proliferative epidermis and are suitable for monitoring inflammatory and repair mediators released in response to inducers.

Meanwhile, TNF-α and IFN-γ have been commonly employed as inducers to mimic the expression of AD-related cytokines in keratinocyte cell lines like HaCaT cells in order to examine the effect of tamanu oil to cure AD. TNF-α, a multifunctional cytokine expressed by activated macrophages and keratinocytes, is able to mediate inflammation, immune response, and apoptosis of cells by stimulating additional signal production (e.g., IL-1, IL-8, and others). In a healthy epidermis, keratinocytes typically release a small amount of TNF-α; however, exposure to irritants, allergens, and microbes may cause a rise in its production (Bernard et al., 2012). On the other hand, IFN-γ, a strong, well-known pro-inflammatory stimulant in a number of biological systems, has the ability to significantly increase keratinocytes' production of inflammatory cytokines and chemokines (e.g., TARC, MDC, IL-33, and others). The expression of IFN-γ is increased in chronic AD lesions when compared to healthy skin (Oh et al., 2022).

2.4. Current Treatments of AD

Since there is currently no cure to stop AD disease from relapsing permanently, palliative care is typically used as an AD treatment to lessen the disease's symptoms (Dębińska, 2021). According to Purnamawati et al. (2017), appropriate skin hydration may also assist in decreasing symptoms and stop the onset of AD illness, although avoiding allergens and irritants that may first cause the inflammatory response in the skin would be the first line of defense in prevention. Other topical medications that ease itching may also serve as AD treatments by preventing the disturbance of the skin barrier caused by scratching. When the illness causes an acute attack on the skin, topical corticosteroids (e.g., dexamethasone) can also be used. However, since the medication could have major side effects like a burning feeling on the skin, skin thinning, skin peeling, and even telangiectasias, it is only administered in emergency conditions (Lax et al., 2022). Meanwhile, patients with severe symptoms and acute illness development would receive long-term systemic therapy (e.g., oral steroids, cyclosporine, methotrexate, and many more) whose efficacies are not very high and may cause long-term adverse effects such as hormonal changes, high blood pressure, nephrotoxicity, hepatotoxicity, and many more (Brahmer et al., 2018).

2.5. Tamanu (*Calophyllum inophyllum*): General Information and Health Benefits

Calophyllum inophyllum, also known as tamanu or Nyamplung in Indonesia, is a big evergreen tree belonging to the Clusiaceae (mangosteen) family. According to Ansel et al. (2016), tamanu is native to the Indo-Pacific region, such as Australia, eastern Africa, Southeast Asia, southern coastal India, and South Pacific. When fully grown, the tree can reach heights of 8 to 20 m; it also has a dense canopy of strong, shiny leaves that are 10 to 20 cm long and 6 to 9 cm wide. During the growing period, the leaves are pale green, but when they reach maturity, they turn dark green. The tree also has fragrant white blooms that develop into large round nuts from which the oil is usually extracted, while its wood is frequently used for boat

and canoe construction, carving, and the construction of cabinets and other furniture (Vigneshwar et al., 2019).

Moreover, other various tree parts, including the leaves, roots, bark, fruit, nuts, nut oil, and resin, have been used nowadays as raw materials to make traditional medicines and cosmetics (Ginigini et al., 2019). For example, the nut oil has been used as a scar remover, a therapy to treat skin infections, and for other cosmetic purposes, while as a conjunctivitis eye wash, remedy for arthritis and joint pain, and preventative measure against infantile rash in Fiji or even as alternative oil for lamps in Polynesia (Prabakaran & Britto, 2012). Other examples, according to Ginigini et al. (2019), include its resin that can be combined with bark and leaf strips to make a remedy for irritated eyes, its green fruit as tuberculosis treatment, and many more. Studies have also revealed that the extracted oil from the nut is well recognized for treating various skin illnesses such as acne, burns, eczema, etc., and has also been demonstrated to have anti-inflammatory, antifungal, antioxidant, and wound-healing properties (Raharivelomanana et al., 2018).

2.6. Chemical Characteristics of Tamanu Oil

There are two main chemical constituents of tamanu oil, fatty acids and ethanol-soluble resinous compounds. For fatty acids, the predominant components are saturated fatty acids (SFA) (41-52%), with stearic acid as the principal constituent and palmitic acid. Meanwhile, the unsaturated fatty acids (18-22%) present are composed of monounsaturated acids (MUFA), including oleic acid and palmitoleic acid, as well as polyunsaturated acids (PUFA), including linoleic acid and alpha-linoleic acid (Léguillier et al., 2015; Raharivelomanana et al., 2018).

On the other hand, the resinous portion of tamanu oil that is soluble in ethanol primarily contains secondary metabolites made up of neoflavonoids and pyranocoumarin derivatives. This includes inophyllum C, D, E, P which contains phenyl substituents, calanolide A, B, D which contains propyl substituents, tamanolide D, P which contains sec-isobutyl

substituents, and calophyllolide which is an inophyllum derivative as the major constituent (Ansel et al., 2016; Leu et al., 2009).

2.7. Biological Activities of Tamanu Oil

Tamanu oil's impact on skin conditions including atopic dermatitis, accounts for the majority of its biological activities. As mentioned before, the benefits of the oil on anti-inflammatory, wound-healing, antibacterial, antifungal, and antioxidant properties are the subjects of various studies.

2.7.1. Anti-inflammatory properties

There have been claims that tamanu oil has anti-inflammatory properties. Calophyllolide, a resinous component isolated from *Calophyllum inophyllum*, has been demonstrated by Nguyen et al. in 2017 to have the ability to lower myeloperoxidase activity, downregulate pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α), and upregulate an anti-inflammatory cytokine called IL-10, which prevents the prolonged inflammation process.

2.7.2. Wound-healing properties

Along with the anti-inflammatory properties, Nguyen et al. (2017) also investigated how tamanu oil affects the healing of wounds. In mice models, calophyllolide was found to be able to decrease the development of fibrosis and speed up wound-healing. This is assumed to be achievable by calophyllolide's anti-inflammatory properties. Furthermore, tamanu oil has been demonstrated to stimulate wound-healing in human epidermal keratinocyte cell line (HaCaT) and human dermal fibroblast (HDF) cells in a study by Ansel et al. in 2016.

2.7.3. Antibacterial properties

It has been demonstrated that the neoflavonoid components of tamanu oil have antimicrobial properties. Yimdjo et al. (2004) reported that in comparison to the standard antibiotic, oxacillin, the neoflavonoids calopyllolide, inophyllum C, and

inophyllum E showed better antibacterial activity against *Staphylococcus aureus* which most of the time causes AD complications by infecting the skin layer. Therefore, numerous studies have been examining the effectiveness of tamanu oil against bacteria known to cause skin infections as a result of its frequent use in cases of skin diseases. Other than *S. aureus*, tamanu oil was also found to exhibit strong activity against both gram-positive (e.g., *Bacillus cereus*, *Corynebacterium minutissimum*, *Staphylococcus epidermidis*, and *Staphylococcus haemolyticus*) as well as gram-negative (*Propionibacterium acnes* and *Propionibacterium granulosum*) bacteria (Léguillier et al., 2015).

2.7.4. Antifungal properties

Other than antibacterial, tamanu oil has also been reported to have antifungal properties. In 2011, Saravanan et al. compared the antifungal activities of tamanu oil extract and the positive control fluconazole, where the extract demonstrated a higher inhibitory effect on numerous fungal strains including *Alternaria tenuissima, Aspergillus fumigatus*, *Aspergillus niger*, *Candida albicans*, and *Candida tropicalis*.

2.7.5. Antioxidant properties

Tamanu oil has been demonstrated to have the ability to significantly lower intracellular reactive oxygen species (ROS) generation, based on a study by Said et al. in 2007. Other assays were also performed in the same study: (i) UV-vis absorption assay that resulted in a maximum peak absorption at 300 nm; (ii) Sun protection factor test that resulted in a high sun protection factor (SPF) index of 18-22; (iii) Deoxyribonucleic acid (DNA) damage test that resulted in up to 85% reduction in ROS-induced damage. In light of these findings, tamanu oil may have applications as an antioxidant or sunscreen due to its high antioxidant and UV-absorption properties.

CHAPTER 3

MATERIALS AND METHODS

3.1. Tamanu Oil (Pure Oil) Preparation

Pure tamanu oil used for the study was obtained from Cahaya Naturals, a company focusing on developing natural-based skincare products, especially for AD. According to Raharivelomanana et al. (2018), the general extraction process of tamanu oil includes taking ripe fruits from the grown *Calophyllum inophyllum* tree, followed by sun-drying them for 1-2 months to promote biosynthesis and oil buildup in the nuts. The sun-dried nuts are then deshelled and put through cold, mechanical pressure to produce a viscous, yellow-greenish oil that has a strong, walnut-like aroma, known as virgin tamanu oil. After being obtained from Cahaya Naturals, the oil was put into a 50 mL falcon tube and sent to Badan Riset dan Inovasi Nasional (BRIN) Cibinong to undergo freeze drying process that was aimed to stabilize, concentrate, and/or lengthen the shelf life without altering its chemical composition (Merivaara et al., 2021).

3.2. Inducers and Treatment Solution Preparation

As mentioned above, TNF-α and IFN-γ with the concentration of 10+10 ng/mL was used to induce AD in HaCaT cells. These inducers were made by diluting the TNF-α (ab9642, abcam) and IFN-γ (285-IF-100, R&D Systems) with Type 1 water as described in the manufacturer's instructions. On the other hand, the treatment solution was prepared by mixing the extract with 0.1% dimethyl sulfoxide (DMSO) and high glucose Dulbecco's Modified Eagle Medium (DMEM) in a 15 mL falcon tube. There were four concentrations of the treatment solution used in this project: 2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 25 µg/mL. These four concentrations were chosen in accordance with the results from an unpublished preliminary study in the same laboratory. Two controls were used in this study as comparisons

for the extract: (i) Blank control, where the cells were only cultured in DMEM; and (ii) Negative control, where the cells grown in DMEM were induced without any treatment.

3.3. HaCaT Cell Culture and Induction

Immortalized human keratinocyte (HaCaT) cells were bought from i3L in the form of cryopreserved cells obtained from Prof. Ng Kee Woei at Nanyang Technological University Singapore as a gift. Cell culture and maintenance were done in a T25 or T75 flask (Biologix, Germany) with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Thermo Fisher Scientific) as well as 1% Penicillin-Streptomycin (Pen-Strep; Gibco, Thermo Fisher Scientific); incubated at 37^{o}C with 5% carbon dioxide (CO_2 incubator INCO108med, Memmert, Germany). For cell passaging, the obtained cryopreserved cells were thawed and observed under the Primovert inverted cell culture microscope (Zeiss, Germany) every day to check on their conditions. Based on the needs for which assay, the cells were passaged further and seeded.

For induction of AD, the cells were seeded into 24-well plates with a density of 5 x 10^4 or 50,000 cells per well, followed by incubation overnight. The seeded cells were observed under the microscope the next day to check for their confluency; if the confluency had not reached ~80%, the cells were incubated for another 24 hours. On the next day, the cells should have reached ~80% confluency and were induced by adding TNF-α and IFN-γ with the concentration of 10+10 ng/mL along with the treatment by adding different concentrations of tamanu oil (2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 25 µg/mL). The induced and treated cells were then incubated for 24 hours before proceeding to the following assay.

3.4. Primer Design

The majority of the primers had been designed by the students who handled the previous batch of this project. Through the use of NCBI Primer-BLAST, primers were created online. Similar melting temperatures (Tm), ranging from 59-61^{o}C, were incorporated into the

design of each primer. All primers were tested to guarantee compatibility with one another before use, and the expected size of the PCR products (amplicon) was 45-310 base pair (bp). The designed primer sequences for this study are shown in **Table 1** and were purchased from PT. Indolab Utama.

Table 1. Primer sequences that had been designed.

Abbreviation	Gene Name	Sequences (5' → 3')	Amplicon Size
CTACK	Cutaneous T cell-attracting chemokine	**F:** TAGGCTGAGCAACATGAAGGG **R:** GGAATGCTGCTGTAGGGTCT	89
IL-25	Interleukin-25	**F:** CCAGCATGTACCAGGTGGTTG **R:** TTCCCATGACCATTGCCAAGA	45
IL-33	Interleukin-33	**F:** TTATGAAGCTCCGCTCTGGC **R:** CCAAAGGCAAAGCACTCCAC	160
MDC	Macrophage-derived chemokine	**F:** ATTAGATGTCCCCTGGCCCT **R:** GCCACTTTCTGGGCTCTGAT	199
TARC	Thymus and activation-regulated chemokine	**F:** ATTCAAAACCAGGGTGTCTCC **R:** TCGCTGCCATGTCTCCAAACT	310
TSLP	Thymic stromal lymphopoietin	**F:** CTTCCTGTGGACTGGCAATG **R:** CTCTTGTTGTTGGGGTCCGA	97
FLG	Filaggrin	**F:** TCAATCTGAGGGCACTGAAAGG **R:** TAGCTGCCATGTCTCCAAACT	63
IVL	Involucrin	**F:** CTGCCCACAAAGGGAGAAGT **R:** AGCGGACCCGAAATAAGTGG	166
GAPDH (housekeeping)	Glyceraldehyde-3-phosphate dehydrogenase	**F:** ACCCACTCCTCCACCTTTGA **R:** TGAGGTCCACCACCCTGTT	115

3.5. RNA Extraction

Ribonucleic acid (RNA) extraction of the previously induced and treated cells was performed using the GENEzol™ TriRNA Pure Kit (Geneaid, Taiwan) based on the manufacturer's instructions. The steps included sample homogenization and lysis, RNA binding, washing, and elution. After the RNA extraction process, Thermo Scientific™

NanoDrop Lite Plus Spectrophotometer was used to measure the RNA concentration and purity (A260/A280 value) following the manufacturer's instructions.

3.6. Gel Electrophoresis

Gel electrophoresis was performed prior to the RT-qPCR process for checking the integrity of RNA samples to ensure reliable final results generation. To do this, agarose gel was first made by weighing agarose powder 1.5% of the total 1x Tris-acetate-EDTA (TAE) buffer in a 50 mL beaker that was wrapped with perforated cling wrap, then microwaving the mixture in a medium-high setting to dissolve the powder for around 2 minutes with occasional swirling after 30 seconds. The beaker was stored at room temperature to cool down before being poured into the gel electrophoresis casting tray. Next, the comb was put on top and left to solidify. Afterwards, 1x TAE buffer was poured into the gel chamber, followed by putting in the gel and pipetting the GeneRuler 1 kb ladder (Thermo Fisher Scientific, USA) and samples mixed with loading dye into the wells accordingly. The gel electrophoresis device was run at 100 Volt (V) and 400 milliampere (mA) for 30 minutes. Then, the gel was put into a plastic tray, covered with Diamond™ Nucleic Acid Dye (Promega, USA), and agitated for 20 minutes on Digene Hybrid Capture System Rotary Shaker (Qiagen, Germany) under no light condition. The result was checked using G: BOX Chemi XRQ (Syngene, India) in the dark room.

3.7. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR method was applied for this study to detect changes in the expression of the genes mentioned above. This was performed using SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline, USA) based on the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), whose expression is constant at different time points and various experimental manipulations according to Zainuddin et al. (2010) was used as a housekeeping gene. To start, the PCR master mix was prepared first according to the manufacturer's

instructions, followed by setting the RT-PCR Rotor-Gene Q (Qiagen, Germany) to the 3-step cycling settings shown in **Table 2**.

Table 2. Three-step cycling RT-qPCR settings.

Cycle	Temperature	Time	Notes
1	45°C	10 mins	Reverse Transcription
1	95°C	2 mins	Polymerase Activation
40	95°C	15 secs	Denaturation
	60°C	20 secs	Annealing
	72°C	45 secs	Extension

Furthermore, a post-cycling melting curve analysis was performed in order to confirm the specificity of RT-qPCR products. The settings for melting curve analysis are shown below in **Table 3**.

Table 3. Post-cycling melting curve analysis settings.

Temperature	Time	Notes
72°C	90 secs	Start
~1°C at a time until reaches 95°C	30 secs	Hold
59°C	15 secs	End

3.8. Statistical Analysis

After the whole experiment, the data obtained from the RT-qPCR machine in the form of Ct values was processed using Microsoft Excel version 16.0 (Microsoft, USA) with $2^{-\Delta\Delta Ct}$ relative quantification method, followed by statistical analysis using the GraphPad Prism 9 software (GraphPad Software, USA) to interpret the gene expression data significance by

applying a one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. The generated p-values of ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, and *$p<0.05$ were deemed significant statistically.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Tamanu Oil (Pure Oil) Preparation

Extraction process of pure tamanu oil was not performed in the study as it was provided by Cahaya Naturals, a company focusing on developing natural-based skincare products, especially for AD. The freeze-drying process yielded a more viscous deep rich greenish-colored oil compared to the pure oil before freeze-dried as its water was removed during the process (**Figure 2**).

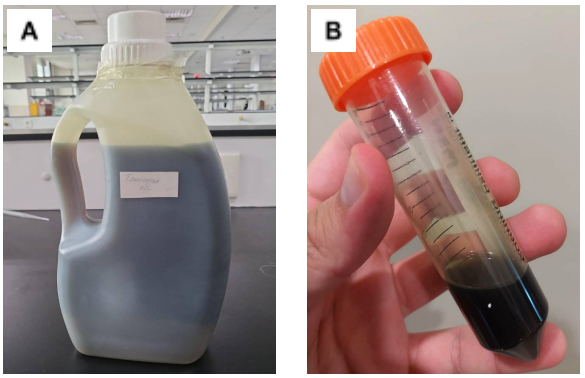


Figure 2. Pure tamanu oil (A) before and (B) after the freeze-drying process.

According to Hapsari et al. (2022), the color components of tamanu oil can be an indication of its purity where a deep rich greenish-amber color indicates a pure oil. As mentioned above, the oil used in this study has a deep rich greenish-color, thus indicating a pure tamanu oil with good quality. On the other hand, a yellowish-colored oil may indicate an oil that has been put through a chemical filtering process which might reduce its purity drastically, lightly-colored or soft green may indicate an oil that has been diluted with a carrier oil which might remove its beneficial properties, and black-colored may indicate an oil that has been made by burning the nuts that might remove its healing properties (Hapsari et al., 2022).

4.2. HaCaT Cell Culture and Induction

After seeding into 24-well plates, the HaCaT cells were induced with 10+10 ng/mL TNF-α and IFN-γ along with the tamanu oil treatment with the concentrations of 2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 25 µg/mL. Cells' morphological changes for both controls and treatments comparing before and after induction and treatment procedure are shown in **Figure 3** and **4** below.

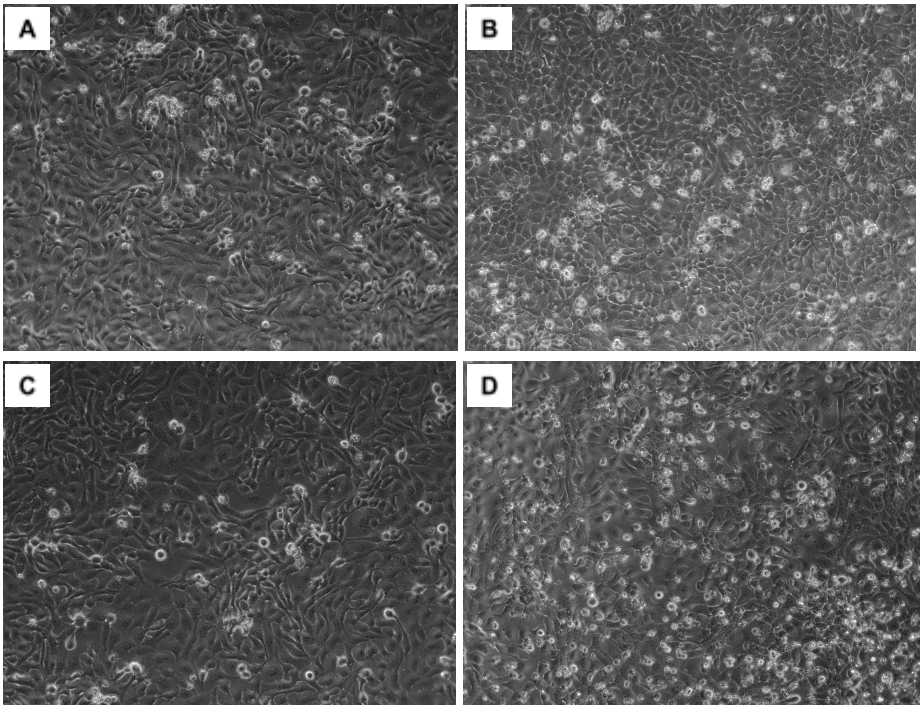
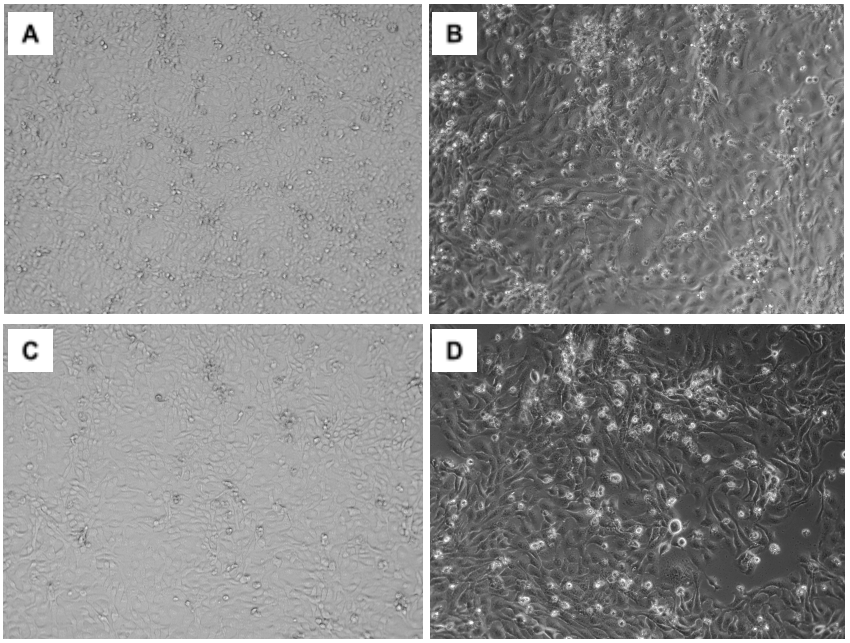


Figure 3. HaCaT cell morphology under 10X magnification - *controls*.

(A) Blank control 24 hours after seeding; (B) Blank control 48 hours after seeding; (C) Negative control 24 hours after seeding (before induction); (D) Negative control 48 hours after seeding (after 24 hours of induction).

As shown in **Figure 3A**, HaCaT cells in blank control 24 hours after seeding grew in normal conditions therefore were able to retain their spindle shapes and were closely packed to each other as to how healthy HaCaT cells should behave (Gabbott & Sun, 2018), while after 48 hours (**Figure 3B**), the cells became slightly cuboidal in shape possibly due to the formation of cell-cell tight junction (Deyrieux & Wilson, 2007). On the other hand, HaCaT cells in negative control after 24 hours of seeding grew normally (**Figure 3C**), but after being induced for 24 hours, cell shrinkage and a high number of membrane blebbing occurred (**Figure 3D**).

According to Green & Llambi (2015), both cell shrinkage and the presence of membrane blebbing are indications of cell apoptosis, a biological process of programmed cell death that occurs naturally in order for the body to continue normal functioning. Both TNF-α and IFN-γ are commonly used inducers to mimic the expression of AD-related cytokines in keratinocyte cell lines like HaCaT cells. A study by Kim et al. in 2018 revealed that a combinatorial treatment of TNF-α and IFN-γ with the concentration of 10+10 ng/mL, has important roles in inducing AD-like features in HaCaT cell through the production of cytokines and chemokines that leads to Th2 inflammation. Moreover, the effect of TNF-α and IFN-γ on HaCaT cells had also been investigated with a cell viability assay conducted in the preliminary study (**Appendix 1**) as supported by other published studies where these inducers could significantly lower the cell viability (El-Dari et al., 2017; Kim et al., 2018). Considering this basis and results from the preliminary study, the previously mentioned inducers were confirmed to successfully convert the HaCaT cells into an AD model.



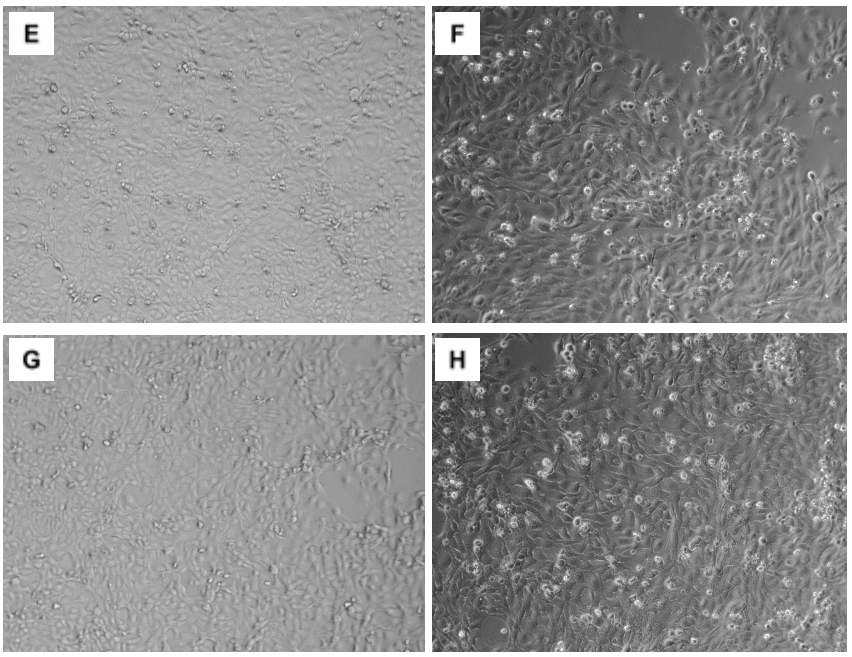


Figure 4. HaCaT cell morphology under 10X magnification - *treatments*.

(A) Before induction and treatment with 2.5 µg/mL; (B) After induction and treatment with 2.5 µg/mL; (C) Before induction and treatment with 5 µg/mL; (D) After induction and treatment with 5 µg/mL; (E) Before induction and treatment with 10 µg/mL; (F) After induction and treatment with 10 µg/mL; (G) Before induction and treatment with 25 µg/mL; (H) After induction and treatment with 25 µg/mL.

In the preliminary study, these four concentrations were chosen in accordance with the results from MTT cell viability assay in preliminary study where pure tamanu oil with the concentration of 5, 10, and 25 µg/mL were non-cytotoxic for the HaCaT cells (**Appendix 2**), and added with a lower concentration of 2.5 µg/mL. All HaCaT cells grew healthily and closely packed under normal conditions prior to induction and treatment as observed in **Figure 4A, 4C, 4E,** and **4G** above. However, upon being induced and treated for 24 hours, there were slight changes observed on their morphologies. For the 2.5 µg/mL treatment concentration (**Figure 4B**), the cells retained their spindle shapes but the number of membrane blebbing increased, while for the 5 and 10 µg/mL (**Figure 4D** and **4F** respectively), the spindle-shaped cells were less packed after 24 hours of induction and treatment as indicated by more blank spaces which might be due to decreased adherence between cells. Lastly, the cells showed little to no distinct changes on the cells with 25 µg/mL treatment solution (**Figure 4H**). Tamanu oil has been reported to possess anti-inflammatory and wound-healing properties, as well as

capable of supporting HaCaT cell proliferation (Cassien et al., 2021; Raharivelomanana et al., 2018). The results showed that the 2.5 µg/mL treatment solution was the least capable among other concentrations in reducing the cytotoxic effect elicited by the inducers, thus was the least effective in treating AD. However, before concluding the efficacies of each treatment solution, gene expression analysis needed to be performed first to observe changes in the expression of each specific gene mentioned above.

4.3. RNA Extraction and Gel Electrophoresis

The integrity of RNA extracted was checked first before proceeding to the gene expression analysis by performing Nanodrop and gel electrophoresis assays. This is crucial because although RNA is a thermodynamically stable molecule, it is quickly degraded by RNase enzymes which are present almost everywhere (throughout the prokaryotes and eukaryotes phylogenetic trees, even in viruses). Shorter RNA fragments are therefore frequently present in a sample, which may negatively impact the outcomes of a subsequent application (Schroeder et al., 2006). Concentration and purity of the extracted RNA in biological triplicates shown in **Table 4** below play a key role in determining the success of RNA extraction.

Table 4. Extracted RNA concentration and purity data.

Sample (biological triplicates)	RNA Concentration (ng/µL)			RNA Purity (A260/A280)		
Blank control	256.0	265.5	274.6	2.03	2.05	2.06
Negative control	299.7	336.2	381.0	2.05	2.05	1.97
2.5 µg/mL	335.9	402.1	350.9	2.04	2.08	2.05
5 µg/mL	368.7	401.4	389.8	2.04	2.04	2.06
10 µg/mL	262.3	379.3	368.0	2.05	2.06	2.05
25 µg/mL	279.8	459.4	379.8	2.05	2.06	2.04

For blank controls, the RNA concentration ranges between 256.0-274.6 ng/µL with A260/A280 values of 2.03-2.06, and the concentration ranges between 299.7-381 ng/µL with A260/A280 values of 1.97-2.05 were obtained for negative controls. The ranges of RNA concentration for cells treated with 2.5, 5, 10, and 25 µg/mL treatment solutions were 335.9-402.1 ng/µL, 368.7-401.4 ng/µL, 262.3-379.3 ng/µL, and 279.8-459.4 ng/µL respectively. Meanwhile, the A260/A280 values for cells treated with treatment solutions were between 2.04-2.08, indicating high RNA purity. According to Okamoto & Okabe (2000), the A260/A280 values were used to measure purity as it is based on the principle that nucleic acids, in this case RNA, absorb UV light at a specific wavelength of 260 nm. A260/A280 ratio around 2 is generally accepted as pure for RNA, while values lower than 2 may be an indication of protein, phenol, or other contaminants present in the sample that absorb UV light strongly at or near 280 nm (Okamoto & Okabe, 2000; Thermo Scientific, 2019).

Further checking of the extracted RNA integrity was done by performing gel electrophoresis, which has been used commonly due to its simple process. A study by Lee et al. in 2012 also stated that agarose gel electrophoresis is the most efficient and effective method to separate nucleic acids based on their sizes as the agarose gel may act as a molecular sieve to separate RNA fragments between 100 bp to 25,000 bp.

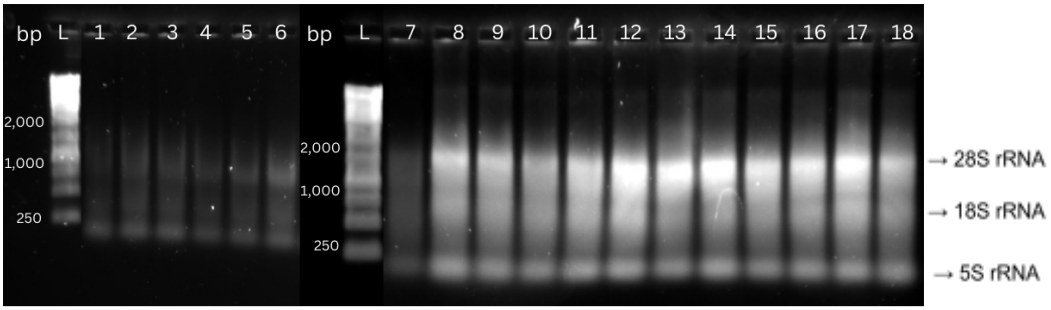


Figure 5. RNA integrity gel electrophoresis results in 1.5% agarose gel.

L: 1 kb DNA ladder; 1-3: blank control; 4-6: negative control; 7-9: 2.5 µg/mL; 10-12: 5 µg/mL; 13-15: 10 µg/mL; 16-18: 25 µg/mL

The appearance of three distinct bands indicates intact RNA when checking its integrity using gel electrophoresis; for eukaryotic RNA, the top band denotes 28S ribosomal RNA (rRNA), which is approximately twice the size of the middle band denoting 18S rRNA, and the third band denotes 5S rRNA which is usually around 120 bp (Aranda et al., 2012). According to Schroeder et al. (2006), a ratio of 28S:18S rRNA bands less than 2:1 may be an indication of low quality RNA. As shown in **Figure 5**, all three bands indicating good RNA integrity appeared after visualization with gel electrophoresis with 28S rRNA sizing ~2,000 bp, 18S rRNA sizing ~1,000 bp, and 5S rRNA sizing below 250 bp. However, the bands were not clearly defined due to smearing that might happen due to high voltage (Corthell, 2014). To avoid this from happening in the future, the researchers need to take the voltage and time into consideration which can be done by lowering the voltage while increasing the time or keeping the voltage but decreasing the time.

4.4. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

After making sure that the RNA extracted have good quality and integrity, RT-qPCR was carried out for all the controls and samples in order to detect changes in the gene expression of CTACK, IL-25, IL-33, MDC, TARC, and TSLP related to inflammation, as well as FLG and IVL related to skin barrier. The RT-qPCR method has been extensively used to measure gene expression levels mainly due to its efficiency, simplicity, and low-cost process. According to Freitas et al. (2019), by using reference genes as endogenous controls, this method can determine the relative expression of target genes. These reference genes are also known as housekeeping genes, whose expression needs to be constant throughout the experimental conditions in order to provide accurate transcriptional quantification (Kozera & Rapacz, 2013).

Results generated from the RT-qPCR machine were in the form of threshold cycle (Ct) values indicating the PCR cycle number at which the sample's fluorescence level reaches the threshold line (**Appendix 3**). The Ct values were then processed using the $2^{-\Delta\Delta Ct}$ method, a

commonly used relative quantification technique which utilizes a reference gene as normalizer to process Ct values data in determining the relative gene expression in target and reference samples, prior to being interpreted into graphs (Rao et al., 2013).

Melt curve analysis was further applied to confirm the specificity of RT-qPCR products by assessing whether or not the primers intercalated with SYBR® Green dye produced single, specific products. According to Montera et al. (2010), it is crucial to have primers that anneal specifically to only one sequence as the results' quality and reliability can be greatly impacted by them due to inefficient reverse transcription, as well as amplification.

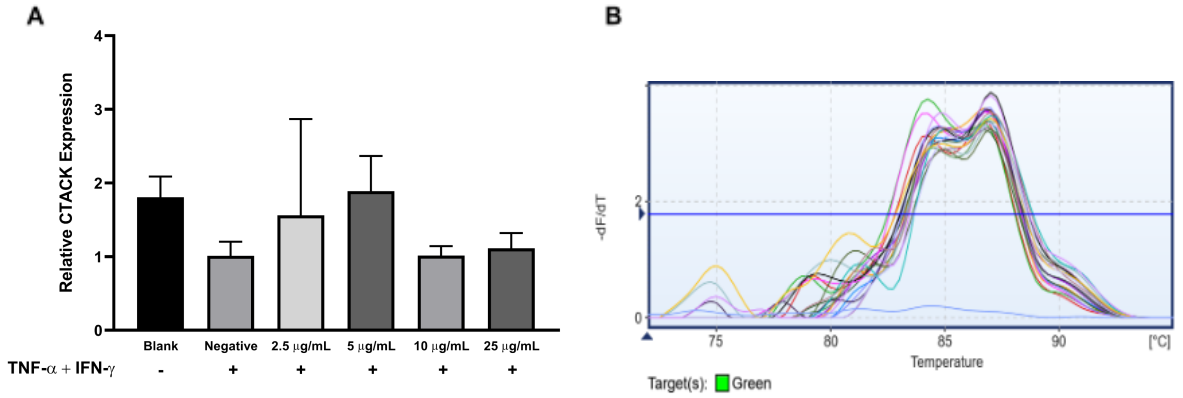


Figure 6. RT-qPCR results on CTACK gene expression.

(A) Relative mRNA expression of CTACK; (B) Melting curve of CTACK
Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment;
-dF/dT: negative derivative of fluorescence over temperature

As observed in **Figure 6A**, the relative mRNA expression of CTACK was higher in uninduced HaCaT cells (averaged $2^{-\Delta\Delta Ct}$ value of 1.81) compared to the induced cells (averaged $2^{-\Delta\Delta Ct}$ value of 1.01). This is not the expected result as CTACK, a chemokine generated by basal keratinocytes was supposed to have higher expression in the induced cells as it is important in the T-cell migration process when inflammation happens (Novak & Leung, 2010). CTACK gene expression in both 2.5 and 5 µg/mL treatment concentrations were still higher (averaged $2^{-\Delta\Delta Ct}$ values of 1.56 and 1.89 respectively) compared to the induced cells, meaning that the treatments were not strong enough to reduce the inducers' cytotoxicity or there was

something wrong with the negative control (e.g., the inducers didn't work). The 10 µg/mL treatment was able to generate equal CTACK expression with the negative control (averaged $2^{-\Delta\Delta Ct}$ value of 1.01), while the 25 µg/mL treatment had slightly higher expression (averaged $2^{-\Delta\Delta Ct}$ value of 1.11). However, these results were not able to be considered reliable as the melt curve analysis (**Figure 6B**) revealed that the primer used was not specific in the presence of multiple peaks. According to Ruiz-Villalba et al. (2017), this might happen due to non-specific amplification caused by the primer binding to multiple sites.

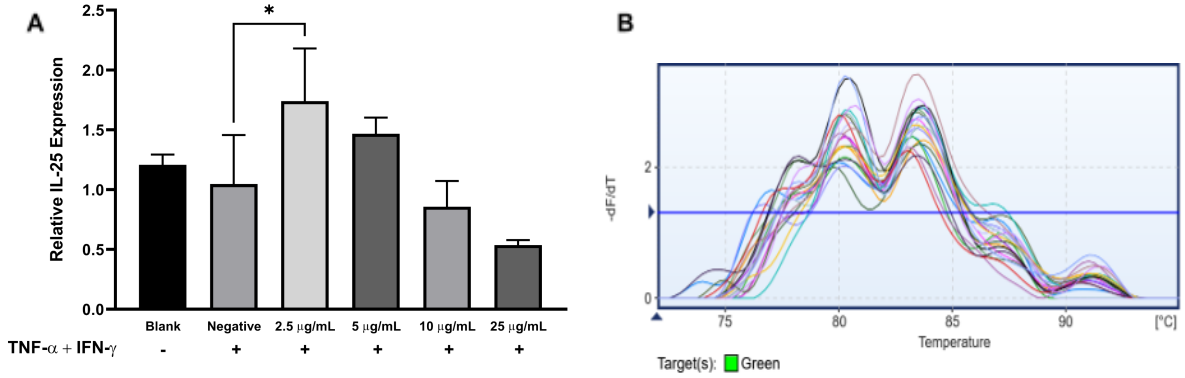


Figure 7. RT-qPCR results on IL-25 gene expression.

(A) Relative mRNA expression of IL-25; (B) Melting curve of IL-25

Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment; -dF/dT: negative derivative of fluorescence over temperature; * indicates $p<0.05$

The relative mRNA expression of IL-25 shown in **Figure 7A** was higher in uninduced HaCaT cells (averaged $2^{-\Delta\Delta Ct}$ value of 1.20) compared to the induced cells (averaged $2^{-\Delta\Delta Ct}$ value of 1.05). Just like CTACK, this is not the expected result as cytokine IL-25 contributes to the body's inflammatory pathways, particularly those that control immunological responses and immune cell migrations (Valizadeh et al., 2015). IL-25 gene expression in 2.5 µg/mL treatment was significantly higher (averaged $2^{-\Delta\Delta Ct}$ value of 1.74), while 5 µg/mL concentration was slightly higher (averaged $2^{-\Delta\Delta Ct}$ value of 1.47) compared to the induced cells, meaning that the treatments were not strong enough to reduce the inducers' cytotoxicity or there was something wrong with the negative control (e.g., the inducers didn't work). The 10 and 25

µg/mL treatments were able to generate lower IL-25 expression than the negative control (averaged $2^{-\Delta\Delta Ct}$ values of 0.86 and 0.53 respectively), but not significantly. These results were also not considered reliable as there were multiple peaks above the threshold in the melting curve indicating non-specific primer (**Figure 7B**).

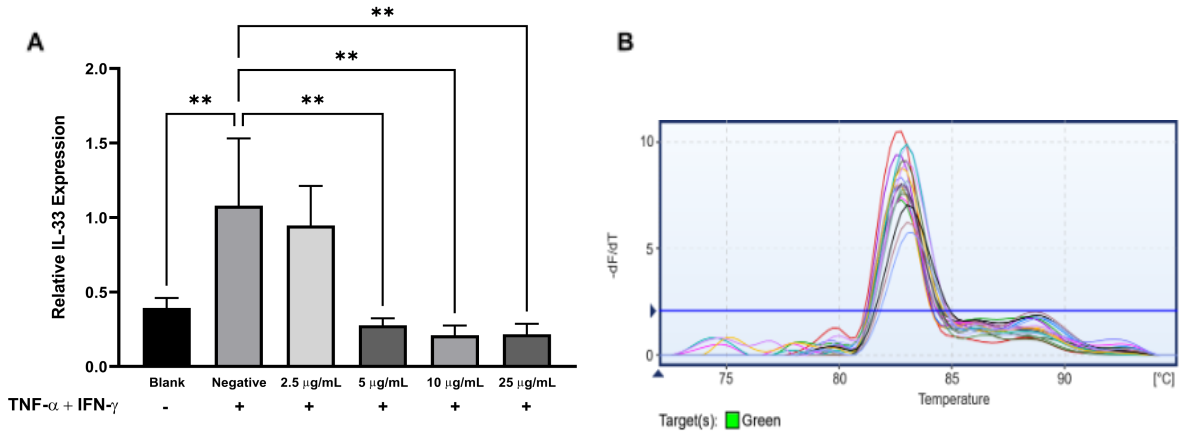


Figure 8. RT-qPCR results on IL-33 gene expression.

(A) Relative mRNA expression of IL-33; (B) Melting curve of IL-33
Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment; -dF/dT: negative derivative of fluorescence over temperature; ** indicates p<0.01

Figure 8A showing the relative mRNA expression of IL-33 revealed better results compared to the previous genes namely CTACK and IL-25. The induced cells in negative control expressed significantly higher IL-33 expression (averaged $2^{-\Delta\Delta Ct}$ value of 1.08) than the blank control (averaged $2^{-\Delta\Delta Ct}$ value of 0.39), which is as expected because of the immune system's activation by causing ILC2s to rapidly grow and generate a large number of type 2 cytokines (Imai, 2019). All treatment concentrations showing dose-dependent relationship caused the downregulation of gene expression with averaged $2^{-\Delta\Delta Ct}$ values of 0.94 for 2.5 µg/mL, 0.28 for 5 µg/mL, 0.21 for 10 µg/mL, and 0.22 for 25 µg/mL, all of which were significant except for the 2.5 µg/mL which might happen because of the low concentration that was not strong enough to downregulate IL-33 gene expression drastically. These results were also supported by the presence of a single peak above the threshold in the melting curve indicating specific primer (**Figure 8B**). On the other hand, the smaller peaks below the threshold might indicate primer

dimer formation due to slight contamination or the self-annealing of forward and reverse primer sequences (Garafutdinov et al., 2020).

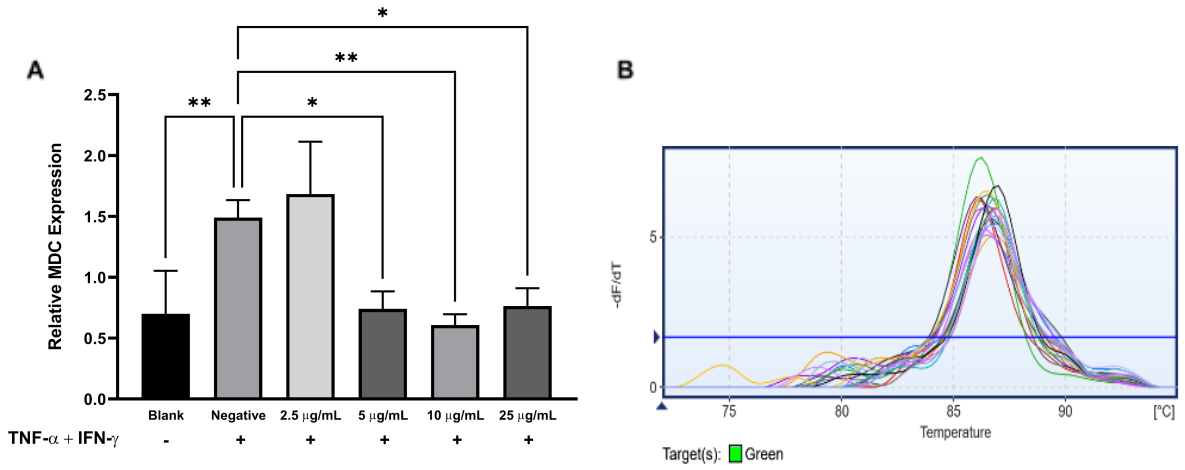


Figure 9. RT-qPCR results on MDC gene expression.

(A) Relative mRNA expression of MDC; (B) Melting curve of MDC

Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment; -dF/dT: negative derivative of fluorescence over temperature; ** indicates $p<0.01$; * indicates $p<0.05$

As shown in **Figure 9A**, the relative mRNA expression of MDC in negative control was significantly higher (averaged $2^{-\Delta\Delta Ct}$ value of 1.49) than the blank control (averaged $2^{-\Delta\Delta Ct}$ value of 0.70), which is in line as it causes Th2 migration resulting in inflammation (Ferran & Santamaria-Babi, 2010). Treatment concentrations of 5, 10, and 25 µg/mL showed promising results by downregulating MDC gene expression with averaged $2^{-\Delta\Delta Ct}$ values of 0.74, 0.61, and 0.76 respectively. However, the 2.5 µg/mL treatment (averaged $2^{-\Delta\Delta Ct}$ value of 1.68) was not able to downregulate the gene expression to be lower than the negative control as it was not strong enough. The presence of a single peak in the melting curve also supported these results, indicating the primer used was specific (**Figure 9B**). There were also smaller peaks below the threshold indicating primer dimer formation due to slight contamination or the self-annealing of forward and reverse primer sequences (Garafutdinov et al., 2020), just like IL-33.

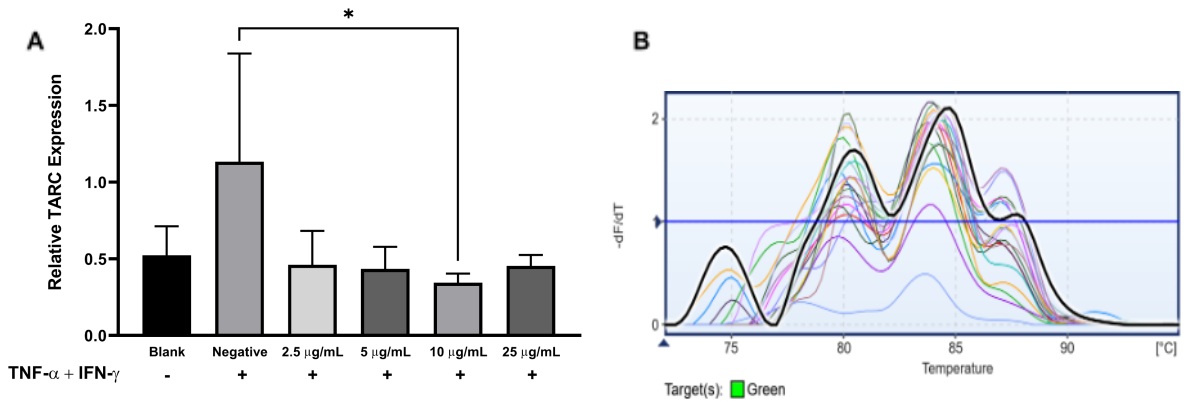


Figure 10. RT-qPCR results on TARC gene expression.

(A) Relative mRNA expression of TARC; (B) Melting curve of TARC
Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment;
-dF/dT: negative derivative of fluorescence over temperature; * indicates $p<0.05$

For TARC, the mRNA expression shown in **Figure 10A** was higher in the negative control (averaged $2^{-\Delta\Delta Ct}$ value of 1.13) than blank control (averaged $2^{-\Delta\Delta Ct}$ value of 0.53), which is the expected result as the upregulation of this chemokine plays a crucial role in activating ILC2s and Th2 cell responses when there is disruption in the epidermal skin barrier (Sun et al., 2021). All treatments were capable of downregulating TARC gene expression with the averaged $2^{-\Delta\Delta Ct}$ values of 0.46 for 2.5 μg/mL, 0.43 for 5 μg/mL, and 0.45 for 25 μg/mL, but not significantly except for the 10 μg/mL concentration (averaged $2^{-\Delta\Delta Ct}$ value of 0.35) which might be due to the non-specific primer as indicated by multiple peaks above threshold in the melting curve (**Figure 10B**). This trend might be just a coincidence that the mRNA expressed were also downregulated without any certainty proving the treatment was capable of reducing TARC gene expression as the primer used was not specific. Therefore, the RT-qPCR results on TARC gene expression were deemed not reliable enough and would need further investigation.

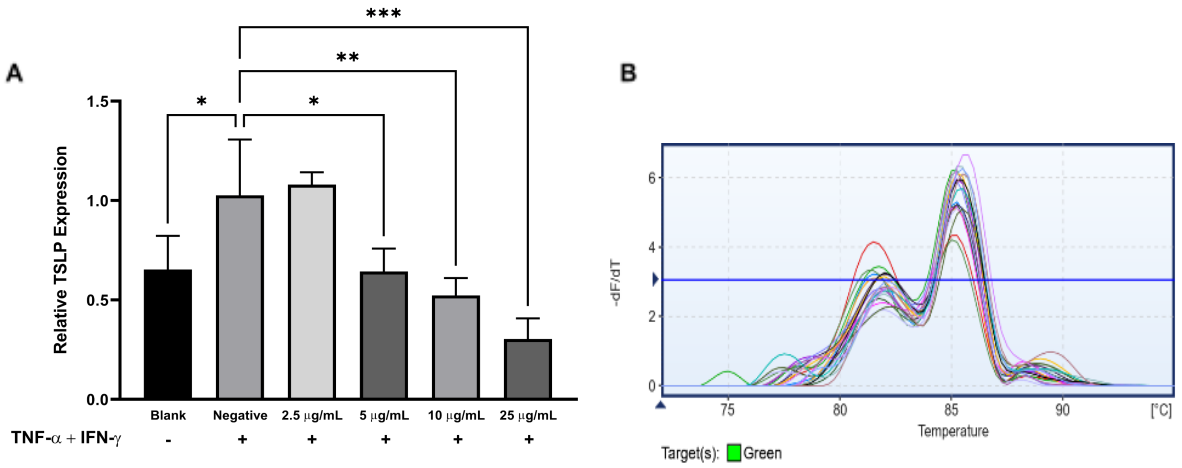


Figure 11. RT-qPCR results on TSLP gene expression.

(A) Relative mRNA expression of TSLP; (B) Melting curve of TSLP

Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment;

-dF/dT: negative derivative of fluorescence over temperature;

*** indicates p<0.001, ** indicates p<0.01, * indicates p<0.05

As shown in **Figure 11A**, the relative mRNA expression of TSLP in negative control was significantly higher (averaged $2^{-\Delta\Delta Ct}$ value of 1.03) than the blank control (averaged $2^{-\Delta\Delta Ct}$ value of 0.66), which is in line as its high expression is believed to cause AD initiation and exacerbation (Yang et al., 2020). Treatment concentrations of 5, 10, and 25 µg/mL were able to significantly downregulate TSLP gene expression with averaged $2^{-\Delta\Delta Ct}$ values of 0.64, 0.52, and 0.30 respectively. Meanwhile, the 2.5 µg/mL treatment (averaged $2^{-\Delta\Delta Ct}$ value of 1.08) was not successful in downregulating the gene expression to be lower than the negative control as it was not strong enough, indicating the treatment's dose-dependent relationship. In **Figure 11B**, however, the peaks were ambiguous whether there were multiple or only one. This ambiguity was supported by good results in the mRNA expression and the presence of a single band indicating specific RT-qPCR product in the previous batch of gel electrophoresis assay using the same primer (**Appendix 4**), suggesting a further investigation in the future. According to Bruzzone et al. (2013), multiple peaks in the melting curve does not necessarily mean there are more than one product; those extra peaks may be due to the maintenance of

double-stranded deoxyribonucleic acid (dsDNA) configuration in the stable (guanine/cytosine rich) amplicon region prior to reaching sufficient temperature to melt.

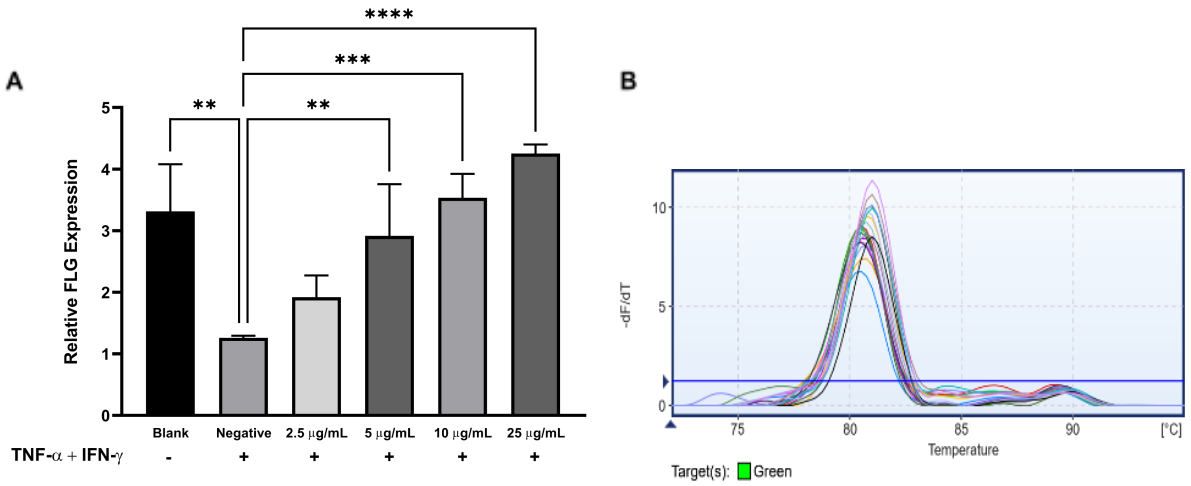


Figure 12. RT-qPCR results on FLG gene expression.

(A) Relative mRNA expression of FLG; (B) Melting curve of FLG
Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment;
-dF/dT: negative derivative of fluorescence over temperature;
**** indicates p<0.0001, *** indicates p<0.001, ** indicates p<0.01

Figure 12A showing the relative mRNA expression of FLG revealed good results just like IL-33 and MDC. However, in contrast to the genes related to inflammation, the expression in negative control was significantly lower (averaged $2^{-\Delta\Delta Ct}$ value of 1.26) than the blank control (averaged $2^{-\Delta\Delta Ct}$ value of 3.31). This is in line with the literature stating that its downregulation has been linked to the early onset of AD (Szegedi, 2015). All treatments except for the 2.5 µg/mL concentration (averaged $2^{-\Delta\Delta Ct}$ values of 1.92; still higher than the negative control but not significant) were able to significantly upregulate FLG gene expression dose-dependently compared to the negative control with averaged $2^{-\Delta\Delta Ct}$ values of 2.92 for 5 µg/mL, 3.53 for 10 µg/mL, and 4.25 for 25 µg/mL. These results were also supported by the presence of a single peak above the threshold in the melting curve indicating specific primer used (**Figure 12B**), while the smaller peaks below the threshold might indicate primer dimer formation (Garafutdinov et al., 2020).

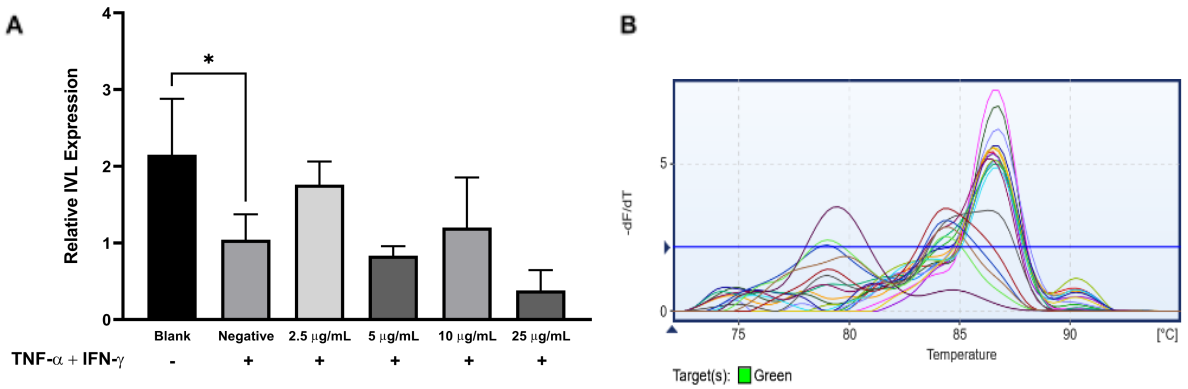


Figure 13. RT-qPCR results on IVL gene expression.

(A) Relative mRNA expression of IVL; (B) Melting curve of IVL
Blank: uninduced cells grown in DMEM; Negative: induced cells grown in DMEM without any treatment;
-dF/dT: negative derivative of fluorescence over temperature; * indicates $p<0.05$

Lastly the relative mRNA expression of IVL (**Figure 13A**), was significantly lower in the negative control (averaged $2^{-\Delta\Delta Ct}$ value of 1.04) compared to the uninduced cells (averaged $2^{-\Delta\Delta Ct}$ value of 2.15). Just like FLG, this is the expected result as the downregulation of IVL expression has been linked to skin inflammation and immune response in AD (Schmidt, 2022). Two of the treatments namely the 2.5 and 10 µg/mL (averaged $2^{-\Delta\Delta Ct}$ values of 1.76 and 1.20 respectively) were successful in upregulating the gene expression to be higher than the negative control although not significant enough, while the 5 and 25 µg/mL treatments were not able to reduce the inducers' cytotoxicity (averaged $2^{-\Delta\Delta Ct}$ values of 0.83 and 0.38). However, these results were not considered reliable as there were multiple peaks above the threshold in the melting curve indicating non-specific primer (**Figure 13B**).

The best results, meaning the recommended treatment concentrations of tamanu oil to be used for each gene are summarized in **Table 5** below. For IL-33, the recommended concentration is 5 µg/mL as it showed promising results in downregulating the inflammatory cytokine to be near the normal condition (blank control) despite its low concentration, while for MDC and TSLP, the recommended concentration that showed promising results in downregulating the inflammatory cytokines is 10 µg/mL. The recommended tamanu oil

concentrations for CTACK, IL-25, and TARC, however, can't be concluded as the results were not deemed reliable and would need further investigation in the future. On the other hand, for skin barrier-related gene FLG, 10 µg/mL showed promising results in upregulating the gene expression related to skin barrier, therefore is the recommended concentration to be used, while for IVL, no conclusion can be drawn as the results were not deemed reliable and would need further investigation.

Table 5. Summary of the recommended tamanu oil concentration for each gene.

Genes	Recommended Tamanu Oil Concentration
IL-33	5 µg/mL
MDC	10 µg/mL
TSLP	10 µg/mL
CTACK	-
IL-25	-
TARC	-
FLG	10 µg/mL
IVL	-

As seen from the table above, the gene analysis from some genes namely CTACK, IL-25, TARC, and IVL were not successful. In future study, further investigation may be done by performing gel electrophoresis after the RT-qPCR process to check the product's quantity; the appearance of a single band will indicate a single product made, showing the primer used was actually specific. By doing this, the researchers will not need to redesign and repurchase the primers, thus may save both time and money. Instead, optimization on the settings for RT-qPCR cycling and melting curve analysis can be done to generate better results.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1. Conclusion

The study in conclusion showed that treatments with 5 µg/mL pure tamanu oil was recommended for IL-33, and 10 µg/mL was recommended for MDC, TSLP, and FLG. These treatments would be the most potent concentrations to be used as AD alternative treatment candidates in further studies as both of them successfully downregulated the inflammation-related gene expression, as well as upregulated the skin-related gene expression on TNF-α and IFN-γ induced HaCaT cells. Meanwhile, further investigation and optimization would be needed for the unsuccessful gene expression analysis of CTACK, IL-25, TARC, and IVL as no conclusion can be drawn from their results.

5.2. Recommendation

Further investigation to learn particular effects of non-specific primers related to gene expression should be performed in the future to improve data reliability and accuracy. Other than that, phytochemical screening would also be a good future study direction to identify the specific bioactive compound responsible for tamanu oil's anti-inflammatory and wound-healing properties. Throughout the study, some problems were bound to occur, for example dead cells due to wrong media preparation and contamination, as well as uneven cell growth, induction, and treatment that might have affected the results. To avoid this from happening in the future, the researchers need to follow the protocol correctly, work aseptically, resuspend properly, check equipments' conditions before using, and not to over-passage the cells. According to O'Driscoll et al. (2006), P30 is generally accepted as the upper limit of passage number for certain cells in culture, as further culturing may result in substantial variation of molecular profiles or genetic abnormalities, limiting their *in vivo* applications and reproducibility.

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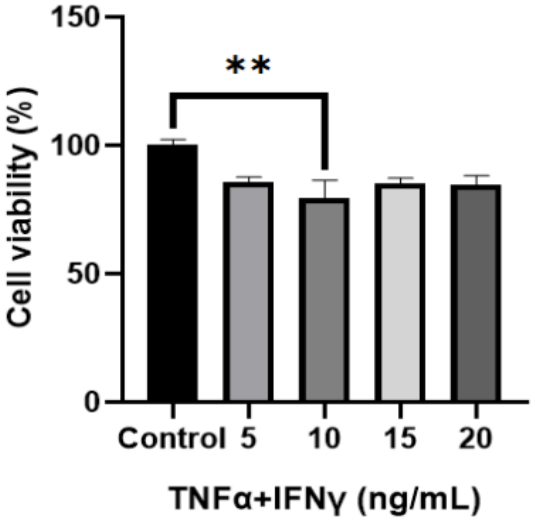
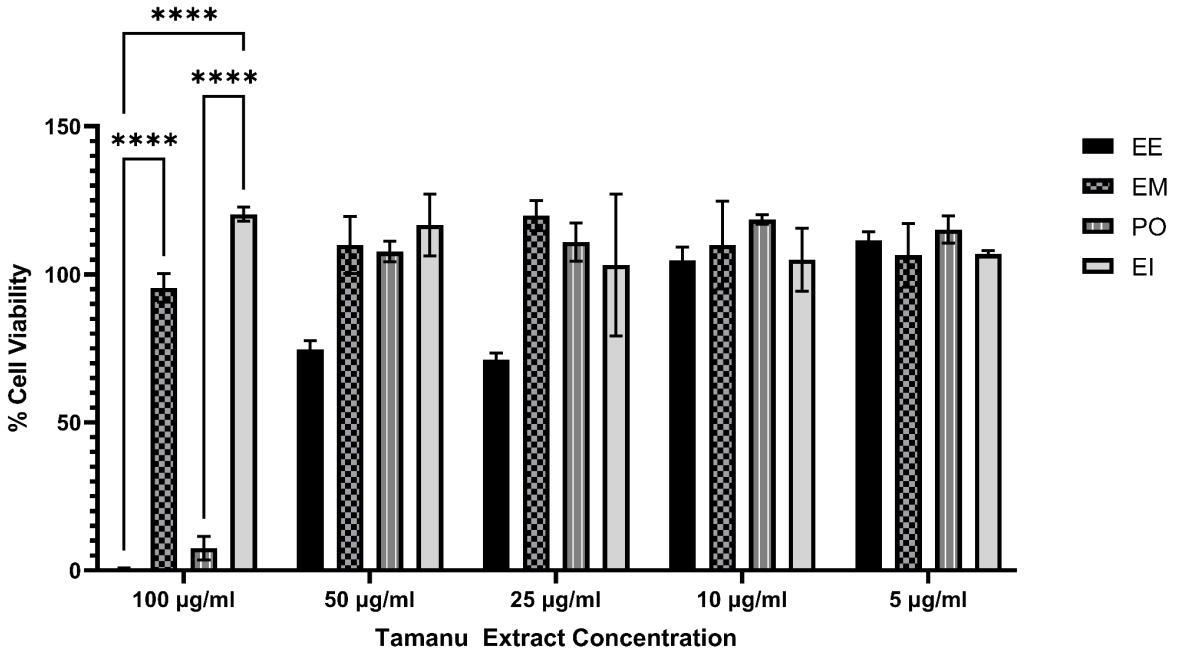
APPENDICES

Appendix 1. MTT cytotoxicity cell viability assay for AD model confirmation.
Control: untreated cells grown in DMEM media; ** indicates p<0.01



Appendix 2. MTT cytotoxicity cell viability assay for treatment solution assessment.
EE: ethanolic extract; EM: ethanol miscible; PO: pure oil; EI: ethanol immiscible



Appendix 3A. Raw data of CTACK RT-qPCR.

Sample	Target Gene Ct CTACK	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	19.95	18.163	1.58
Blank control 2	19.53		2.12
Blank control 3	19.83		1.72
Negative control 1	20.48	17.993	0.97
Negative control 2	20.16		1.22
Negative control 3	20.69		0.84
2.5 µg/mL - 1	19.72	18.527	2.39
2.5 µg/mL - 2	19.81		2.24
2.5 µg/mL - 3	25.42		0.05
5 µg/mL - 1	20.37	18.343	1.34
5 µg/mL - 2	19.71		2.12
5 µg/mL - 3	19.65		2.21
10 µg/mL - 1	20.22	17.767	1.00
10 µg/mL - 2	20.38		0.89
10 µg/mL - 3	20.02		1.15
25 µg/mL - 1	20.39	17.767	0.89
25 µg/mL - 2	19.84		1.30
25 µg/mL - 3	20.02		1.15

Appendix 3B. Raw data of IL-25 RT-qPCR.

Sample	Target Gene Ct IL-25	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	22.58	18.163	1.30
Blank control 2	22.78		1.13
Blank control 3	22.7		1.19
Negative control 1	22.93	17.993	0.90
Negative control 2	22.19		1.51
Negative control 3	23.23		0.73
2.5 µg/mL - 1	22.85	18.527	1.38
2.5 µg/mL - 2	22.16		2.23
2.5 µg/mL - 3	22.63		1.61
5 µg/mL - 1	22.74	18.343	1.31
5 µg/mL - 2	22.50		1.55
5 µg/mL - 3	22.51		1.54
10 µg/mL - 1	22.42	17.767	1.10
10 µg/mL - 2	22.92		0.78
10 µg/mL - 3	23.09		0.69
25 µg/mL - 1	23.43	17.767	0.55
25 µg/mL - 2	23.38		0.57
25 µg/mL - 3	23.58		0.49

Appendix 3C. Raw data of IL-33 RT-qPCR.

Sample	Target Gene Ct IL-33	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	20.70	18.163	0.32
Blank control 2	20.36		0.41
Blank control 3	20.52		0.45
Negative control 1	18.35	17.993	1.31
Negative control 2	19.59		0.56
Negative control 3	18.29		1.37
2.5 µg/mL - 1	19.76	18.527	0.72
2.5 µg/mL - 2	19.46		0.88
2.5 µg/mL - 3	18.97		1.24
5 µg/mL - 1	21.02	18.343	0.26
5 µg/mL - 2	21.15		0.24
5 µg/mL - 3	20.68		0.33
10 µg/mL - 1	21.21	17.767	0.15
10 µg/mL - 2	20.03		0.28
10 µg/mL - 3	20.85		0.20
25 µg/mL - 1	21.33	17.767	0.14
25 µg/mL - 2	20.35		0.28
25 µg/mL - 3	20.63		0.23

Appendix 3D. Raw data of MDC RT-qPCR.

Sample	Target Gene Ct MDC	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	21.61	18.163	0.57
Blank control 2	22.44		1.10
Blank control 3	21.73		0.43
Negative control 1	19.76	17.993	1.45
Negative control 2	19.78		1.37
Negative control 3	20.42		1.65
2.5 µg/mL - 1	20.21	18.527	1.24
2.5 µg/mL - 2	19.75		1.71
2.5 µg/mL - 3	19.45		2.10
5 µg/mL - 1	20.3	18.343	0.86
5 µg/mL - 2	20.26		0.58
5 µg/mL - 3	20.43		0.78
10 µg/mL - 1	20.56	17.767	0.69
10 µg/mL - 2	19.99		0.51
10 µg/mL - 3	19.43		0.62
25 µg/mL - 1	20.25	17.767	0.71
25 µg/mL - 2	19.87		0.93
25 µg/mL - 3	20.38		0.65

Appendix 3E. Raw data of TARC RT-qPCR.

Sample	Target Gene Ct TARC	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	24.12	18.163	0.74
Blank control 2	25.04		0.39
Blank control 3	24.86		0.44
Negative control 1	22.57	17.993	1.93
Negative control 2	24.28		0.59
Negative control 3	23.71		0.88
2.5 µg/mL - 1	24.65	18.527	0.66
2.5 µg/mL - 2	25.06		0.50
2.5 µg/mL - 3	26.25		0.22
5 µg/mL - 1	24.60	18.343	0.60
5 µg/mL - 2	25.44		0.34
5 µg/mL - 3	25.36		0.36
10 µg/mL - 1	25.11	17.767	0.28
10 µg/mL - 2	24.79		0.35
10 µg/mL - 3	24.60		0.40
25 µg/mL - 1	24.33	17.767	0.49
25 µg/mL - 2	24.72		0.37
25 µg/mL - 3	24.29		0.50

Appendix 3F. Raw data of TSLP RT-qPCR.

Sample	Target Gene Ct TSLP	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	24.58	18.163	0.51
Blank control 2	24.33		0.61
Blank control 3	23.86		0.84
Negative control 1	23.44	17.993	1.00
Negative control 2	23.05		1.32
Negative control 3	23.85		0.76
2.5 µg/mL - 1	23.90	18.527	1.06
2.5 µg/mL - 2	23.78		1.15
2.5 µg/mL - 3	23.94		1.03
5 µg/mL - 1	24.45	18.343	0.64
5 µg/mL - 2	23.43		0.53
5 µg/mL - 3	24.20		0.76
10 µg/mL - 1	23.91	17.767	0.62
10 µg/mL - 2	24.23		0.50
10 µg/mL - 3	24.36		0.45
25 µg/mL - 1	25.38	17.767	0.22
25 µg/mL - 2	24.47		0.42
25 µg/mL - 3	25.11		0.27

Appendix 3G. Raw data of FLG RT-qPCR.

Sample	Target Gene Ct FLG	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	20.00	18.163	3.88
Blank control 2	20.67		2.44
Blank control 3	20.10		3.62
Negative control 1	22.11	17.993	1.23
Negative control 2	21.67		1.25
Negative control 3	21.58		1.30
2.5 µg/mL - 1	20.10	18.527	2.32
2.5 µg/mL - 2	20.17		1.80
2.5 µg/mL - 3	20.20		1.64
5 µg/mL - 1	20.20	18.343	3.84
5 µg/mL - 2	19.75		2.20
5 µg/mL - 3	20.51		2.71
10 µg/mL - 1	19.62	17.767	3.83
10 µg/mL - 2	20.42		3.68
10 µg/mL - 3	20.12		3.09
25 µg/mL - 1	20.09	17.767	4.23
25 µg/mL - 2	20.26		4.12
25 µg/mL - 3	20.85		4.41

Appendix 3H. Raw data of IVL RT-qPCR.

Sample	Target Gene Ct IVL	Housekeeping Ct GAPDH	$2^{-\Delta\Delta Ct}$
Blank control 1	19.42	18.163	2.39
Blank control 2	20.26		1.33
Blank control 3	19.23		2.73
Negative control 1	20.27	17.993	1.18
Negative control 2	20.15		1.28
Negative control 3	21.10		0.66
2.5 µg/mL - 1	19.96	18.527	2.11
2.5 µg/mL - 2	20.37		1.59
2.5 µg/mL - 3	20.38		1.58
5 µg/mL - 1	20.91	18.343	0.96
5 µg/mL - 2	21.13		0.83
5 µg/mL - 3	21.35		0.71
10 µg/mL - 1	20.72	17.767	0.74
10 µg/mL - 2	19.32		1.95
10 µg/mL - 3	20.41		0.91
25 µg/mL - 1	23.71	17.767	0.09
25 µg/mL - 2	21.45		0.44
25 µg/mL - 3	20.99		0.61

Appendix 4. RT-qPCR product for TSLP gel electrophoresis results in 1.5% agarose gel (*previous batch*). Gel electrophoresis on TSLP gene expression revealed a single product for each sample with the same size of ~100 bp, indicating the primer used was specific.

L: 50 bp DNA ladder; 1-3: blank control; 4-6: negative control; 7-9: 2.5 μg/mL;
10-12: 5 μg/mL; 13-15: 10 μg/mL; 16-18: 25 μg/mL