Indonesia International Institute for Life Sciences

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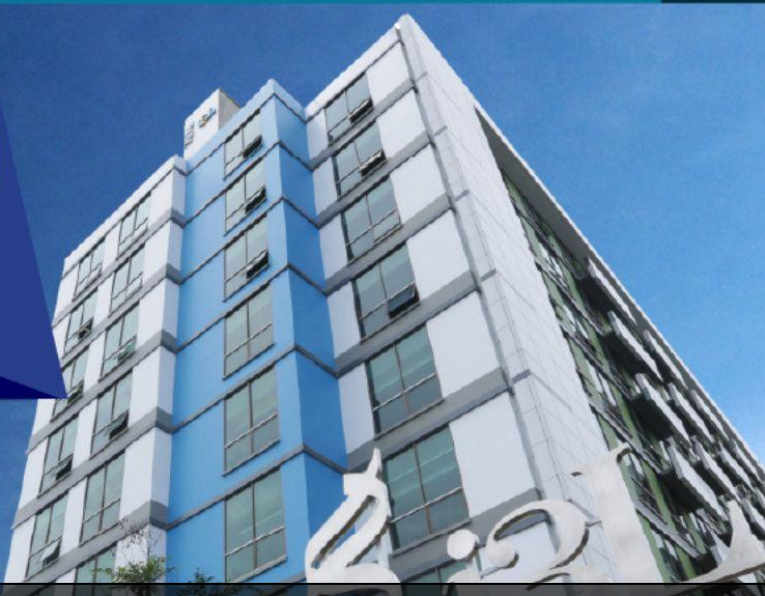

INVESTIGATING THE EFFECT OF *Calophyllum Inophyllum* ETHANOLIC EXTRACT ON THE GENE EXPRESSION IN TNF-α AND IFN-γ INDUCED HUMAN KERATINOCYTE SKIN CELLS (HaCaT)

STUDY PROGRAM
Biotechnology

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INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES (i3L)
2023

RESEARCH PROJECT PROPOSAL

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By

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Submitted to

i3L – Indonesia International Institute for Life Sciences
School of Life Sciences

in partial fulfillment of the enrichment program for the Bachelor of Science in
Biotechnology

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Jakarta, Indonesia
2022

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Thesis Committee Meeting Report

Thesis committee meetings can start without the student being present, in this case Thesis Advisor will review student's progress with committee members. At the end of the meeting, the student may opt for the Thesis Advisor to leave the room and talk alone with committee members.

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☐ Pass with revision.
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Supervisor's Approval	19 January 2023	Student's Approval
Approved		**Approved**
Amadeus Y.P. B.Sc., Ph.D.		**Agnes Maria Rosaceae**

Names and signature of other committee members present:

1	Lead Assessor	: Richard S. S.TP., Ph.D.	Approved
2	Assessor 2	: apt. Audrey A.C. M.Sc.	Approved

Note by **Amadeus Y.P. B.Sc., Ph.D.**:

- The presentation could be organized better to improve clarity and brevity

- The student could improve her understanding of the background knowledge related to her study such as the cell signaling pathways involving the genes that she investigated

Note by **Richard S. S.TP., Ph.D.**:

1. RNA extraction gel result was not in good quality. It requires more optimization in RNA extraction.

2. The melting curves of some genes are problematic. It is required to get one peak to consider qRTPCR result to be valid.

3. The discussion that led to conclusion has dicrepancies. please look at the conclusion and explain it more in the discussion.

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ABSTRACT

Atopic dermatitis is a chronic skin disease that affects 20% of children aged three to six months and 3% of adults worldwide. Despite its prevalence, the exact cause of the disease remains unclear due to its complexity of the disease. Many available drugs in the market for treating AD focus on preventing the disease from worsening, such as managing skin infection, hydration, inflammation, and itch. However, they are known to be inefficient, expensive or cause extreme side effects to the user; thus, an alternative treatment is needed for AD. The oil from *Calophyllum inophyllum* or tamanu has been used as a traditional treatment for various skin-related diseases such as psoriasis, burn, and acne. In addition, the oil extracted from tamanu seeds has been reported to have several properties, such as anti-bacterial, anti-fungal, and anti-inflammation. Since AD is related to chronic inflammation and skin barrier disruption, these properties make tamanu oil an ideal candidate for AD. Despite this potential, scientific data on the benefits of tamanu oil for AD, especially at the genetic level, remains scarce. This study investigates the effect of tamanu treatment on the expression of two skin barrier genes and six inflammatory genes in HaCaT cells induced with TNF-alpha and IFN-gamma to mimic the condition of AD patients. The investigation shows upregulation in the treated skin barrier (FLG and IVL) gene expression and downregulation in the inflammatory (IL-33, MDC, TSLP) gene expression.

Keywords: Atopic Dermatitis; RT-PCR; Gene expression; Tamanu; Ethanolic extract

ACKNOWLEDGEMENT

Throughout the whole project and writing of this report, the writer has received a lot of assistance and guidance from numerous people. Thus the writer would like to express utmost gratitude.

First and foremost, the writer would like to praise the Mighty Lord God for His grace and guidance; thus, this project and report can be completed. The writer would like to sincerely thank the family for their love, prayers, and support throughout the university journey. The writer would also like to express utmost gratitude for the support from:

1. Writer project teammates Jessica Renata Wijaya, Sarah Stefania Hutabarat, and Fanie Tansil for giving great insight as well as advice and support throughout the ups and downs of the project.
2. Mr. Amadeus Yeremia Pribowo B. Sc., Ph.D., for his guidance and input as the project supervisor throughout the project.
3. Ms. Nancy Christina Batubara, as the lab PIC, for the insight and guidance throughout the project period
4. Michael Putra Utama as the project assistant throughout the project period
5. All of the other groups at the i3L cell culture lab that is involved in this project
6. Writer friends and close ones, Stephanie Angela Yosiano and Laurentius Hardy Kurniawan, for being supportive and encouraging throughout the project
7. All the Library, cleaning, security, and building management staff for their aid during this project

This project would not be possible without their contribution and help; thus, the writer humbly expresses its utmost thanks for all the inspiration, support, and insight during this project in i3L.

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LIST OF ABBREVIATIONS

CE	Cornified envelope
CLA	Cutaneous lymphocyte antigen
CLDN1	Claudin-1
CTACK	Cutaneous T cell-Attracting Chemokine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EE	Ethanolic Extract
FBS	Fetal Bovine Serum
FLG	Filaggrin
FLG LoF	Loss of function in the filaggrin gene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HaCaT	Human Keratinocyte Cells
IFN-γ	Interferon Gamma
IL-1	Interleukin 1
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-17E	Interleukin 17E
IL-25	Interleukin 25
IL-33	Interleukin 33
ILC2s	Group 2 innate lymphoid cells
IVL	Involucrin
MDC	Macrophage Derived Cytokines
mRNA	Messenger RNA

N-3 PUFA	n-3 polyunsaturated fatty acid
RNA	Ribonucleic Acid
SC	Stratum Corneum
ST2	Serum STimulation-2
TAE	Tris-acetate-EDTA
TARC	Thymus and Activation-Regulated Chemokine
Th2	T helper 2
TNF-α	Tumor Necrosis Factor-Alpha
TRAP	Traffic-related air pollution
TSLP	Thymic Stromal Lymphopoietin

Chapter I

INTRODUCTION

1.1. BACKGROUND

Atopic dermatitis (AD) is a form of eczema, a common, chronic, long-lasting skin disease that often appears with skin rash, itchiness, fever, or even asthma. AD was first introduced in the late 1800s, however the exact cause and treatment for this disease are still unclear (Kapur et al., 2018; Kramer et al., 2017). AD affects 20% of children worldwide, and 25% will continue to be involved through adulthood, either as a relapsing disease or a continuous disease (Hadi et al., 2021). AD can occur at any age, however, people with a family history of AD and a history of gene dysfunction have a higher risk of developing AD. There are some theories related to the cause of AD, also known as the outside-in, where the concentration of responsible genes for skin barrier and moisturizing are low and inside-out hypothesis, where immune dysfunction is the main reason (Boguniewicz & Leung, 2011). AD is associated with abnormality in genes involved in skin barrier dysfunction, immune pathways, and inflammation. Over-production of various inflammatory cytokines, including IL-4R, IL-6, IL-8, IL-18, IL-32, CTACK, TARC, TSLP, etc, are often considered as the reason for AD severity (Hou et al., 2019; Kantor & Silverberg, 2017; Kanwal et al., 2021; Lee et al., 2018; J.-H. Yang et al., 2021).

Researchers also believe that AD pathology is related to environmental factors besides genetic and immunological factors. This might explain why AD prevalence differs from region to region (Kantor & Silverberg, 2017). Various environmental factors, including climate changes, stress, microorganisms, pollution, UV radiation, and allergens, combined with a genetic dysfunction, have been linked to abnormalities in the epidermis and immune system, thus contributing to the progression of AD (Kolb & Ferrer-Bruker, 2022).

Novel therapeutic approaches and preventative strategies for AD have been developed based on their facts. However, due to the complex interaction between genetic, immunological, and environmental factors and an unclear source of the disease, AD is still incurable. Furthermore, the current treatment available in the market, such as beta-Val, corticosteroid, and Kenalog-based drugs, are known to have some side effects, and the other therapy can only treat the symptoms and prevent them from getting worse (Dębińska, 2021; Nygaard et al., 2017).

Therefore, research to find alternative medication for AD has consistently been ongoing to find effective and efficient treatment without side effects.

Oil extracted from tamanu (*Calophyllum inophyllum*) has been a potential solution for AD treatment. Tamanu is a plant that originated from Polynesia, Including Asia, Africa, and the Pacific. It is known as “Nyamplung” in Indonesia, and it is widely available across Java, Kalimantan, Sulawesi, and Sumatra. Tamanu seed is known to contain 75% oil, which has been extracted and marketed commercially and used in dermal products. Tamanu oil has been recommended for treating many skin disorders, such as eczema, acne, psoriasis, and burns. It also has been reported to have antifungal, antioxidant, wound-healing, and anti-inflammatory properties (Pribowo et al., 2021).

In this study, the anti-inflammatory effect of *Calophyllum inophyllum ethanolic* extracts will be investigated using Human Keratinocyte Skin Cells (HaCaT) induced with TNF-α and IFN-γ as the AD model. This study has two hypotheses: (1) *Calophyllum inophyllum* ethanolic extracts will downregulate inflammatory gene expression in the AD model. (2) *Calophyllum inophyllum ethanolic* extracts will upregulate the gene expression of skin barrier-related genes in the AD model.

1.2. Aim and Objective

The overall aim of this study is to study the effect of Tamanu Ethanolic extract on skin barrier and inflammation genes that are expressed in people with Atopic Dermatitis by achieving the following objectives:

1. Investigate the effect of Tamanu Ethanolic Extract Treatment on the gene expression of Atopic Dermatitis-related inflammation genes (CTACK, IL-25, IL-33, MDC, TARC TSLP).

2. Investigate the effect of Tamanu Ethanolic Extract Treatment on the gene expression of Atopic Dermatitis-related skin barrier genes (IVL & FLG).

1.3. Scope of Work

This project will cover *Calophyllum inophyllum* Ethanolic Extract preparation and gene expression assays. The detailed scope of work for this project is as follows:

1. *Calophyllum inophyllum* Ethanol Extract preparation.
2. Cell Culture of human keratinocyte skin (HaCaT) cells.

3. Performing primer design for each gene through research and primer design websites.
4. RNA Extraction and Quantitative Real-Time PCR.
5. Statistical Analysis.

Chapter II

LITERATURE REVIEW

2.1. Literature review

2.1.1. Atopic Dermatitis

Atopic Dermatitis (AD) is the most common chronic inflammation in the skin that usually occurs during childhood and might last until adulthood. AD prevalence in the US was reported to be 11.3-12.7% in adults and 6.9-7.6% in children (J. Kim et al., 2019). In developing countries such as the Middle Eastern and Africa, the AD prevalence was reported to be 3-6% and 12-14%, respectively (Pribowo et al., 2021). The name atopic dermatitis came from *Atopy* and dermatitis. *Atopy* is a condition where when exposed to environmental factors (e.g. pollutants) will trigger IgE expression. Whilst Dermatitis refers to skin inflammation. Thus AD refers to a condition where when exposed to environmental factors, it will trigger skin inflammation (Papapostolou et al., 2022).

Generally, AD is a heterogenous eczematous skin disorder related to Th2 deficiency, barrier disruption, skin inflammation, and chronic pruritus. A combination of environmental, genetic, and immunological factors further complicate AD, leading to immune response dysfunction. Although the complete pathophysiology of AD has not been found, many studies believe that immune dysregulation and skin barrier dysfunction have a role in AD. Although the epidermis plays a vital role as a physical barrier, many AD cases have reported skin barrier defects. Filaggrin (*FLG*), transglutaminases, intracellular proteins, and keratins are the skin barrier's key (J. Kim et al., 2019).

2.2. Atopic Dermatitis Risk Factors

2.2.1. Environmental Risk Factors

Environmental factors hold an essential role in AD development, and many environmental risk factors that have been reported to affect AD development. However, not all factors have been scientifically proven or accepted. In industrialized countries, AD prevalence is higher, people living in the city tend to have a higher risk of getting AD than those living in the countryside. Environmental factors can be grouped into three major groups, external nonspecific (e.g. urbanization, climate, migration), external specific (e.g.

sunlight, pollution, diet, humidity, allergens, temperature), and internal (e.g. microbiome) (Ahn et al., 2020). These three groups play a significant role in AD prevalence and severity.

Climate is one of the critical factors as to why AD prevalence in different regions diversifies, climate is closely related to temperature and sunlight exposure. Some studies study the correlation between AD development and temperature, however, a future investigation is required (Bonamonte et al., 2019). Meanwhile, it has been reported that people that live in areas with less sunlight exposure have been reported to have more severe AD (Calov et al., 2020). While it is widely known that sunlight has UVB that affects Vitamin D serum production, UV light also has immunosuppressive effects that affect *FLG* production. Vitamin D has properties that aid skin protection, thus lack of Vitamin D would cause more severe AD manifestation (Vestita et al., 2015). Lifestyles such as diet and habits affect AD development to a certain degree. People who consume fruit, vegetables, and fish high in n-3 polyunsaturated fatty acid (n-3 PUFA) have been reported to have a lower risk of getting AD as n-3 PUFA has anti-inflammatory properties that can lower the risk of getting AD.

Traffic-related air pollution (TRAP) is highly related to eczema symptoms in children. Air pollution from the non-industrial and industrial processes could contain chemicals (e.g. toluene, styrene, benzene) or toxic substances (e.g. tobacco smoke). As they enter the skin, they will trigger allergic reactions by stimulating the production of Ige and Th2 cytokines. Based on a study, it was found that TRAP mainly triggers AD in children between the ages of 13 and 14. However, TRAP does not show any association with AD in the general population, thus a more profound study is required (Ahn et al., 2020). A study in South African toddlers aged between 12-36 months has shown that consuming fermented milk has reduced the risk of getting AD in urban cohorts, however, the same effect was not found in rural populations. Thus urbanization and loss of gut microbial diversity might play a role in AD development (Levin et al., 2020). However, some studies have reported that there is almost no difference in gut microbiota diversity between healthy persons and AD patients. Thus a future study that analyzes the correlation between gut microbiota diversity and AD development is required (Bonamonte et al., 2019).

2.2.2. Genetic Risk Factors

Genetic risk factors refer to particular genes related to epidermal protein and type 2 T helper lymphocytes (TH2) signalling pathway. Mutation of the filaggrin gene is the most studied genetic risk factor for AD. *FLG* has a vital role in maintaining the epidermis; mutation of the *FLG* gene has affected the *FLG* protein production and disturbed the skin barrier. *FLG*

mutations have been reported to cause loss of function in the filaggrin gene (FLG LoF), which increases the TWEL and decreases the skin's water content, increasing pH in the stratum corneum and allowing bacterial proliferation. Other than that *FLG* LoF also increases the risk of getting other allergies (e.g. asthma) (Thomsen, 2014). Other than *FLG,* many loci and genes have been identified as having a role in AD genetic risk factors. Such as inflammation related genes (e.g. CTACK, MDC, TARC), epidermal and skin barrier related genes (e.g. IVL, FLG), cytokines and chemokines (e.g. TSLP, IL-25, IL-33), protease, as well as antimicrobial genes. The many genes involved in AD development show that Many factors cause AD.

2.3. Immunology

2.3.1. CTACK

Cutaneous T cell-attracting chemokine (CTACK) or CCL27 is a chemokine that belongs to the CC chemokine family and attracts skin-homing cutaneous lymphocyte antigen (CLA). It is expressed only in the skin, thus it has been viewed as a valuable variable to evaluate AD severity; upon AD, CTACK expression in human keratinocyte cells is upregulated correlated with the severity of the disease (Blatt et al., 2017; Machura et al., 2012). It has been suggested that TARC and CTACK work together in attracting Th2 cells and migrating them into the upper layers of the skin. In addition CTACK has been reported to have a role in wound healing by attracting T cells to the skin and inducing bone marrow-derived keratinocytes, attracting Th2 cells from peripheral blood, as well as the migration, proliferation, and angiogenesis of T cells into upper layers of the skin (Karnezis et al., 2019, p. 27; Niwamoto et al., 2021).

2.3.2. *FLG*

Fliggarin (*FLG*) is an essential skin barrier protein that maintains Stratum Corneum equilibrium in the skin by producing profilaggrin protein that forms the outermost layer of skin or epidermis (Dębińska, 2021). The epidermis acts as a barrier from bacteria or foreign substances that can cause allergic reactions to the skin; lack of *FLG* will decrease keratohyalin granules, disturb barrier function, and affect the structure of the cornified envelope (CE). A deficiency of *FLG* caused by the genetics of inflammatory response allows allergens to pass through the skin barrier and migrate to lymph nodes, where it will interact with T cells and induce a Th2 immune response (Dębińska, 2021). While *FLG* is not expressed in mucosal membranes, many reported cases of people with *FLG* mutations are at higher risk of having asthma or allergic rhinitis. It is believed that *FLG* mutation promotes these allergenic

responses through sensitization of allergens that enter through a distributed skin barrier (Armengot-Carbo et al., 2015; Furue, 2020).

2.3.3. IL-25

The Interleukin 25 (IL-25), also known as Interleukin 17E (IL-17E), is a newly identified cytokine-protein from the interleukin 17 (IL-17) family that has a role in inflammation pathways that involves immune response and cell migration. IL-25 is expressed in epidermal cells and correlated to many infections, such as parasitic, viral, and bacterial infections. IL-25 is essential in regulating immune responses in two ways: as a driver for multiple allergic diseases and as an amplifier. This cytokine is known to upregulate T helper 2 (Th2) immune responses, trigger the expression of interleukin 4 (IL-4), and indirectly mediate secretion of interleukin 5 (IL-5) and interleukin 13 (IL-13) (Wu et al., 2022). During AD, IL-25 expression is upregulated and was found to lower the expression of FLG in keratinocytes upon AD, as well as directly affecting the skin barrier (Aktar et al., 2015; Deleuran et al., 2012).

2.3.4. IL-33

The Interleukin 33 (IL-33) is an inflammatory cytokine from Interleukin 1 (IL-1) family expressed in normal keratinocytes as alarmin at the protein level. However, IL-33 expression is upregulated upon cellular damage to stimulate the immune system (Cayrol & Girard, 2018). As seen in **Figure 1.** from the study of Imai (2019), overexpression of IL-33 in epidermal keratinocytes directly downregulated filaggrin and claudin-1 (CLDN1) protein which exacerbates dermatitis by activation of group 2 innate lymphoid cells (ILC2s) and basophils. IL-4 produced by basophils will then promote the activation of ILC2s, which stimulates the production of IL-5 and IL-13 and causes the accumulation of eosinophils. Eosinophils are disease-fighting white blood cells, accumulation of these cells indicates an infection or allergic reaction (Kanuru & Sapra, 2022).

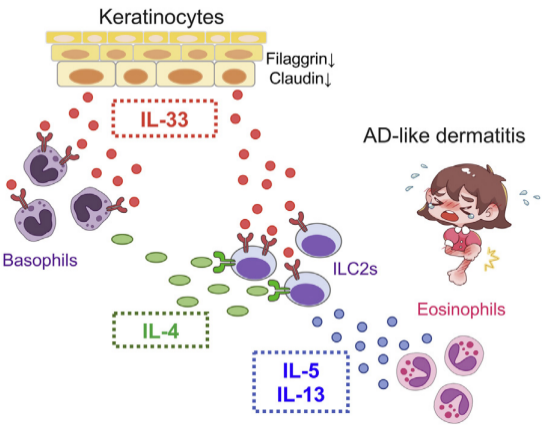

Figure 1. IL-33-induced AD-like inflammation mechanism from (Imai, 2019).

2.3.5. IVL

Involucrin (IVL) is a skin barrier protein and also a part of keratinocyte-specific differentiation protein. *IVL* is formed in the granular layer and has a specific role during the beginning of cornified envelope formation that protects the corneocytes in the skin and the base for the corneocyte-bound lipid envelope (Chiba et al., 2019). Upon AD, IVL expression is downregulated by Th2 cytokinesis and STAT-6 pathway, the absence or deficiency of this gene will lead to permeability barrier homeostasis and affect the integrity of the skin barrier (Furue, 2020).

2.3.6. MDC

Macrophage-derived chemokine (MDC) or CCL22 is one of the CCR4 ligands expressed in dendritic cells (DCs), macrophages, and thymic epithelial cells. It plays as chemoattractant for Th2 cells, NK cells, and eosinophils. MDC expression in AD is upregulated according to the severity of the disease, in AD MDC triggers Th2 migration and causes inflammation (Kakinuma et al., 2002; Richter et al., 2014).

2.3.7. TARC

Thymus and activation-regulated chemokine (TARC) are one of the essential chemokines in the skin-specific homing of T cells that are produced in keratinocytes (Umeda et al., 2020). TARC receptor is CCR4 that is expressed mainly in skin homing, TARC has been reported to play a role in inducing integrin-dependent adhesion and transendothelial migration of T-cells. Furthermore, proinflammatory cytokines such as IL-1, TNFα and IFNγ can upregulate TARC expression (Machura et al., 2012).

2.3.8. TSLP

Thymic stromal lymphopoietin (TSLP) is a cytokine from the IL-7 family that is expressed in keratinocytes and plays a role in triggering and initiating Th2 cytokine (Indra, 2013). TSLP expression is upregulated in keratinocytes with allergic dermatitis such as AD, skin barrier is disrupted during AD, making it more susceptible to allergens or irritants penetrations. Thus the keratinocytes trigger the expression of TSLP. Other than that, it has been reported that environmental factors such as microbes, viruses, chemical particles, and parasites can also trigger TSLP production. In addition TSLP will trigger the activation of STAT1, STAT3, STAT5, and STAT 6, which enhance allergic reactions, contributing to skin inflammation and tissue damage (Kang et al., 2016).

2.4. Atopic Dermatitis Model

The model used in this study is a human keratinocyte cell (HaCaT) induced by TNFα+IFNγ. This model is used based on a preliminary study from the previous students performing this project and according to previous studies by other researchers (Gil et al., 2019; H. J. Kim et al., 2018; Lee et al., 2018). TNFα+IFNγ are the most commonly used inducers to imitate the skin condition during AD. However, studies also show that this inducer induces chemokines and cytokines that trigger Th2 expression (Lee et al., 2018). Furthermore, studies by Kim et al. (2018) have found that HaCaT cells induced with TNFα+IFNγ cell morphology are more unhealthy than normal non-induced HaCaT cells. Thus the success of the inducer can be determined by observing the cell viability of the induced HaCaT cells.

2.5. Current Atopic Dermatitis Treatment

Due to the complexity and lack of study on the disease, up to date, there is no effective cure for AD. Therefore, the only available treatment is targeted to reduce the severity of the skin lesion and pruritus. AD can be prevented by ensuring skin moisture and preventing dryness using moisturizers or emollients. This will prevent itch and desire to scratch the skin, lowering the risk of skin barrier disruption and allergen infection. Depending on the severity, a water-based cream with high water content is recommended for patients with mild eczema. In contrast, thick fat-based cream is more recommended for patients with severe eczema. Topical corticosteroids (e.g. dexamethasone) are also used to treat severe eczema. However, due to the serious side effects, it is only given to patients in critical conditions. Various creams, ointments, or oral medication to treat AD are available in the market, however, those treatments have low efficiency with serious side effects when used regularly.

However, in recent years researchers have developed several medications that could block specific cytokinesis and cytokine receptors/ transcription factors, such as dupilumab and crisaborole. Dupilumab, a type of monoclonal antibody, has the ability to reduce type 2 inflammation through antagonizing IL-4 & IL-3 while increasing *FLG*, loricrin, and claudin expression (Ahn et al., 2020). Whilst crisaborole is reported to downregulate inflammatory genes (MMP12), Th2 (CCL22/MDC), Th1 (CXCL9, CXCL10), and TH17 (CXCL1 and CXCL2) while upregulating the claudin 8 (CLDN8) (G. Yang et al., 2020). Although there has been much ongoing research to find the most effective and efficient AD treatment, there have been no reports of a treatment that is free from any side effects upon long-term usage.

2.6. *Calophyllum inophyllum*

Calophyllum inophyllum is the scientific name for tamanu, a plant that belongs to the mangosteen family and is widely distributed in Southeast Asia. In Indonesia, this plant is also known as “nyamplung”. The bark of this plant has been used to make furniture and household materials, and the fruit has been used to make oil for light sources. Tamanu oil was extracted from the seeds inside the tamanu fruit. Tamanu fruit is sphere-like and has a greenish-yellow colour when ripe and brown when overripe. Tamanu seed is about 2-4 cm with a corky shell. Tamanu has been known for its biological effects: antifungal, antioxidant, anti-cancer, anti-inflammatory, UV protection, antiviral, and antibacterial activity. Other than that, tamanu also has aromatic purposes, thus, many cosmetic lines have included tamanu as one of their ingredients to promote an anti-ageing effect.

A study from 2019 by Ginigini has led to the discovery of 11 bioactive compounds in tamanu ethanolic extract, namely; calophyllolide, inophyllums (A, B, C, D, E, P), 12-oxo-calanolide, calanolide Gut 70, D, and A (Ginigini et al., 2019). The bioactive compound was reported to have anti-cancer, anti-HIV, antimicrobial, and anti-inflammatory properties, which means tamanu extract is a suitable candidate for treating AD. This is supported by a previous study by Tsai and colleagues, where tamanu extract managed to suppress and or downregulate the expression of COX-2, NF-kB, INOS, and oxide production in dose-dependent conditions (Tsai et al., 2012).

Chapter III

MATERIALS AND METHODS

3.1. *Calophyllum inophyllum* Ethanol Extract Preparation

The ethanol extraction method from *C. inophyllum* seed was performed following previous studies by previous researchers. First, fresh *C. inophyllum* seeds were de-shelled and grounded, followed by adding 100% ethanol with a 1:2 ratio to the weight of the seeds. Next the extract was covered in aluminium foil and put into a shaking incubator at room temperature for 24 hours at 100 rpm. The mixture was then filtered by the Buchner funnel and filter paper. Next, 100% ethanol was added to the solid and put back into the shaker incubator in the same condition, while the liquid was stored in the storage covered with aluminium foil. The second filtration was performed, and both extracts were combined and evaporated using a rotary evaporator. The remaining extract was placed in an evaporating dish, covered with cheese cloth to dry inside the fume hood for 1-2 weeks. The extract was then delivered to third parties (BRIN) to be freeze dried to prolong shelf life. Upon usage, extract was combined with 0.1% pure DMSO and DMEM and filtered using a syringe filter.

3.2. Cell Culture

The HaCaT cell was obtained from another project that used to work with a lecturer from Nanyang Technological University, Prof. Ng Kee Woei from the School of Materials Science and Engineering. The cells was cultured in High Glucose Dulbecco's modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin in a T25/T75 flask and was incubated in 37 ^{0}C and 5% CO_2. The cells will be monitored every 2-3 days, and the cells that will be used will be cell passage numbers 19 to 30.

3.3. Primer Design

Most of the primers have been designed by the students taking charge of this project's previous batch. Primers was designed online using the NCBI Primer-BLAST. All primers were designed to have a similar melting temperature (Tm), between 59 to 61 ^{0}C. The PCR products were set to be 45 - 310 bp, and all primers are checked to ensure compatibility from one to another. The primer for this study was brought from Pt. Indolab Utama.

3.4. RNA Extraction

The HaCaT cells were seeded into 24-well plates with $5x10^4$ cells/well for 48 hours. A 10+10 ng/ml TNFα+IFNγ with ethanolic extract of different concentrations was then added to the cells and incubated for 24 hours. The RNA extraction was performed following GENEzol^{tm} TriRNA Pure Kit manufacturer's instructions, and the RNA were measured at 260/280 nm using a Nanodrop®spectrophotometer to measure the quality and quantity. RNA was stored at -80^{0}C prior to usage.

3.5. Gel Electrophoresis

Agarose gel was weighted 1.5:100 to total 1x TAE buffer and combined in a beaker glass covered in plastic wrap with ventilation holes. The mixture was then microwaved for 1- 2 minutes in medium heat and swirled every 25 seconds to ensure that the agarose was adequately dissolved and prevent over-boiling. The mixture was cooled until there was no more steam then poured into a gel casting tray up to 6-8 mm depth, and a well-forming comb was added. After the gel had solidified, the comb was removed, and the gel was positioned into a gel tank filled with a 1x TAE buffer. A total of 7µl (1µl 6x loading dye + 6µl ladder/ samples) ladder or samples are added to each well. Then the electrophoresis tank was connected to the power supply through PowerPac Basic electrometer (Bio Rad) and run for 300 minutes at 100 V; 400 mA. The gel was then stained using Diamond Nucleic Acid Dye (Promega) that was diluted with a 1x TAE buffer for 20 minutes. Then it will be visualized in the dark room using the gel doc (GBox Chemi XRX). The ladder used in this experiment was bought from thermo scientific.

3.6. Real Time Polymerase Chain Reaction

The real-time polymerase chain reaction (RT-PCR) was performed using SensiFAST SYBR No-ROX One-Step kit (Meridian), following the manufacturer's instructions. The primer used in this study can be seen in **Table 1**. The RNA and primer will be diluted 1x to a concentration of 0.01 µg/µL and 400 nM/ 20µL, respectively, using RNAse Free Water. PCR Master Mix will be made according to the manufacturer's instructions, and the thermal cycle will be set following the manufacturer's instructions shown in **Table 2.**

Table 1. Primer Sequence for Real-Time Polymerase Chain Reaction (RT-PCR).

Symbol	Gene Name	Sequences (5' → 3')	Amplicon Size

CTACK	Cutaneous T cell-Attracting Chemokine	**Forward:** TAGGCTGAGCAACATGAAGGG	89
		Reverse: GGAATGCTGCTGTAGGGTCT	
FLG	Filaggrin	**Forward:** TCAATCTGAGGGCACTGAAAGG	63
		Reverse: TAGCTGCCATGTCTCCAAACT	
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	**Forward:** ACCCACTCCTCCACCTTTGA	115
		Reverse: TGAGGTCCACCACCCTGTT	
IL-25	Interleukin 25	**Forward:** CCAGCATGTACCAGGTGGTTG	45
		Reverse: TTCCCATGACCATTGCCAAGA	
IL-33	Interleukin 33	**Forward:** TTATGAAGCTCCGCTCTGGC	160
		Reverse: CCAAAGGCAAAGCACTCCAC	
IVL	Involucrin	**Forward:** CTGCCCACAAAGGGAGAAGT	166
		Reverse: AGCGGACCCGAAATAAGTGG	
MDC	Macrophage Derived Cytokines	**Forward:** ATTAGATGTCCCCTGGCCCT	199
		Reverse: GCCACTTTCTGGGCTCTGAT	
TARC	Thymus and Activation-Regulated Chemokine	**Forward:** ATTCAAAACCAGGGTGTCTCC	310
		Reverse: TCGCTGCCATGTCTCCAAACT	
TSLP	Thymic Stromal Lymphopoietin	**Forward:** CTTCCTGTGGACTGGCAATG	97
		Reverse: CTCTTGTTGTTGGGGTCCGA	

Table 2. Setting Condition for Real-Time Polymerase Chain Reaction (RT-PCR).

Cycle	Temperature	Time	Notes
1	45^0C	10 minutes	Reverse Transcription
1	95^0C	2 minutes	Polymerase Activation
40	95^0C	5 seconds	Denaturation
	60^0C	10 seconds	Annealing
	72^0C	5 seconds	Extension

3.7. Statistical Analysis

The data were analyzed with Microsoft Excel version 16 software, and statistical analysis were performed with GraphPad Prism 9 software. Gene expression will be analyzed with a one-way analysis of variance, and the presented data were shown as mean ± standard error of the mean (SEM). P-values of *$p<0.05$, **$p<0.01$, and ***$p<0.001$ were considered statistically significant.

Chapter IV

RESULTS AND DISCUSSION

4.1. *Calophyllum inophyllum* Seeds Ethanolic Extraction

Calophyllum inophyllum was extracted from the seeds using ethanolic extraction as suggested by the preliminary study of previous students. However, freeze-drying was added as an additional step to help stabilize the extract, as freeze-drying will help to stabilize the microparticles, solubility, and dissolution (Dixit et al., 2011). The process of freeze-drying the extract was performed by a third party (BRIN) as they are more well equipped. The ethanolic extract from the *Calophyllum inophyllum* seeds contains oil in it, thus the freeze drying products are not in the form of powder as the oil will sublimate first, resulting in a stickier consistency. As seen in **Figure 2** and **Figure 3**, there are some differences in the appearance of the oil; this could be the leftover resins from the extract, as resins will harden when exposed to air.

Figure 2. Ethanolic Extract from *Calophyllum inophyllum* Seeds Before Freeze-drying

Figure 3. *Calophyllum inophyllum* Seeds Extract After freeze-drying

4.2. Effect of *Calophyllum inophyllum* Extract on Induced HaCaT cells morphology

To better analyze the effect of TNFα+IFNγ and *Calophyllum inophyllum* treatment on the cells, a picture of the morphology of the cells before and 24 hours after treatment was taken from each triplicate. As seen in **Figure 4**, some dead cells were visible before the treatment, which can be caused by several reasons, as this picture was taken 48 h after seeding. First, the amount of nutrients in the media is already depleting. Second, as there are too many cells, there might not be enough room for them to grow, thus leading to cell death. However, HaCaT cells were still closely packed to each other and had a spindle shape, just as HaCaT cells normally behave.

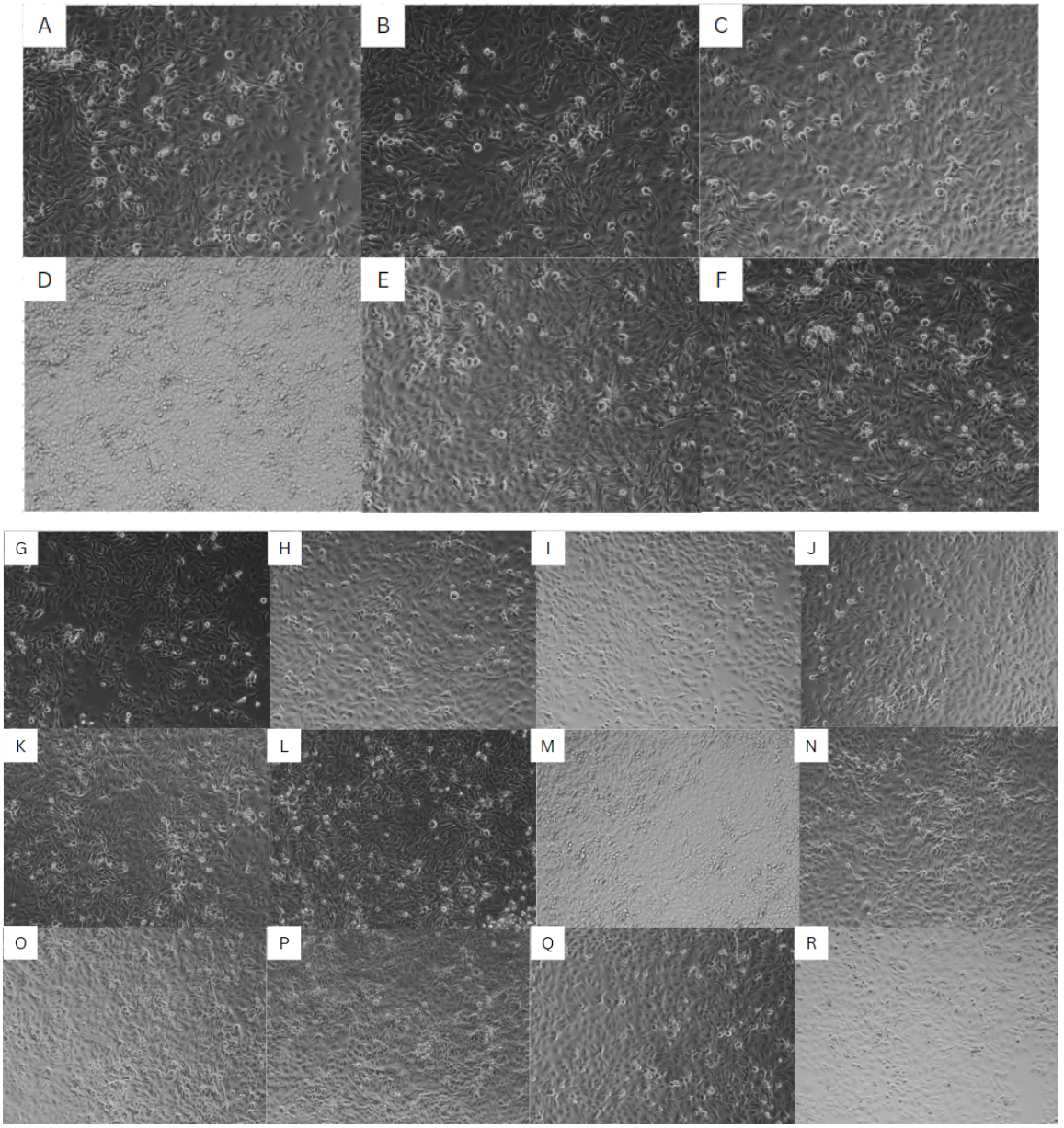

Figure 4. HaCaT Cells Morphology Before Treatment

HaCaT cell observation after 48 hour culturing, before treatment at x10 magnification (A) Negative Control 1 - Induced HaCaT cells; (B) Negative Control 2 - Induced HaCaT cells; (C) Negative Control 3 - Induced HaCaT cells; (D) Untreated HaCaT cells 1; (E) Untreated HaCaT cells 2; (F) Untreated HaCaT cells 3; (G) 25 µg/ml Ethanolic Extract 1; (H) 25 µg/ml Ethanolic Extract 2; (I) 25 µg/ml Ethanolic Extract 3; (J) 10 µg/ml Ethanolic Extract 1; (K) 10 µg/ml Ethanolic Extract 2; (L) 10 µg/ml Ethanolic Extract 3; (M) 5 µg/ml Ethanolic Extract 1; (N) 5 µg/ml Ethanolic Extract 2; (O) 5 µg/ml Ethanolic Extract 3; (P) 2.5 µg/ml Ethanolic Extract 1; (Q) 2.5 µg/ml Ethanolic Extract 2; (R) 2.5 µg/ml Ethanolic Extract 3

Cell changes were observed after 24 hours of treatment with TNFα+IFNγ and ethanol extract (**Figure 5**). The number of cell deaths is more visible after the treatment. Several factors might contribute to this. First, no nutrients are added to the media; thus, the cells fight for food. Second, the treatment concentration might be too high to the point it becomes toxic to the cell itself. Third,

when the picture was taken, it had already passed the time when the inducer was supposed to prevent cell doubling; instead, it became toxic to the cells. However, despite the amount of cell death, the HaCaT cells still show the behavior of normal HaCaT, such as being closely packed to each other and having a spindle shape. Other than that, the previous study has proven that this amount of inducer would not kill the cells upon induction for 24 hours, but it will show some difference in cell morphology (H. J. Kim et al., 2018).

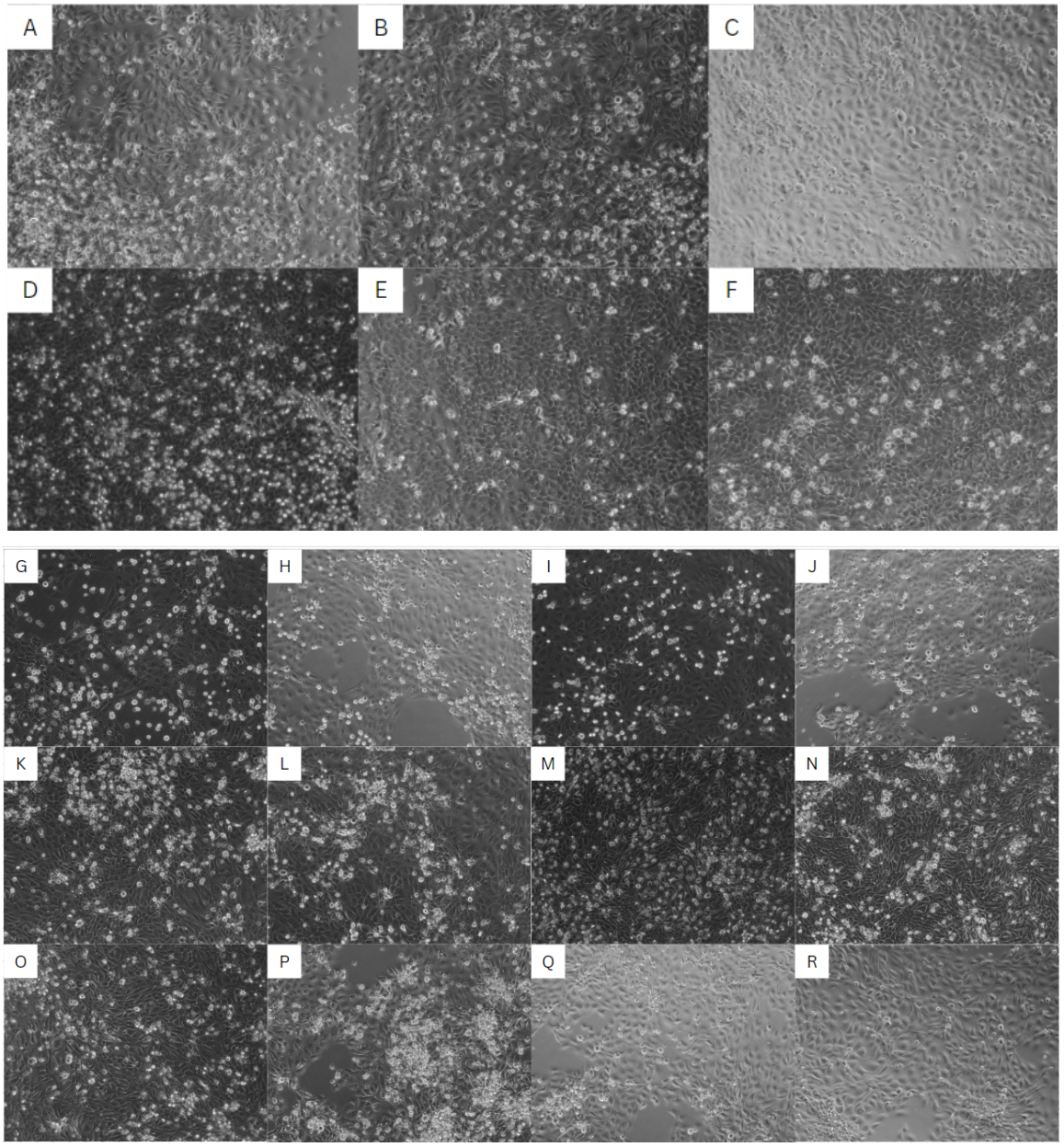

Figure 5. HaCaT Cells Morphology 24h After Treatment with *Calophyllum inophylum* and TNFα+IFNγ

HaCaT cell observation after 24 hour treatment at x10 magnification

(A) Negative Control 1 - Induced HaCaT cells; (B) Negative Control 2 - Induced HaCaT cells; (C) Negative Control 3 - Induced HaCaT cells; (D) Untreated HaCaT cells 1; (E) Untreated HaCaT cells 2; (F) Untreated HaCaT cells 3; (G) 25 µg/ml Ethanolic Extract 1; (H) 25 µg/ml Ethanolic Extract 2; (I) 25

µg/ml Ethanolic Extract 3; (J) 10 µg/ml Ethanolic Extract 1; (K) 10 µg/ml Ethanolic Extract 2; (L) 10 µg/ml Ethanolic Extract 3; (M) 5 µg/ml Ethanolic Extract 1; (N) 5 µg/ml Ethanolic Extract 2; (O) 5 µg/ml Ethanolic Extract 3; (P) 2.5 µg/ml Ethanolic Extract 1; (Q) 2.5 µg/ml Ethanolic Extract 2; (R) 2.5 µg/ml Ethanolic Extract 3

4.3. RNA Extraction

After RNA extraction, nanodrop is completed to measure each sample's quantity and purity, as shown in **Table 3**. The analysis shows different sample concentrations, varying from 256 ng/µl to 626 ng/µl according to the treatment group. The purity for each sample varies from A260/A280 of 1.97 up to 2.07, which integrity around 1.8 up to 2.1, indicating a high RNA purity (Craciun et al., 2019; Fleige & Pfaffl, 2006). RNA integrity was further analyzed by performing an agarose gel electrophoresis with a 1.5% agar concentration. As RNA is a negatively charged molecule, it will migrate to the positively charged electrode and separate according to its size when an electric current is given. However, as shown in **Figure 6**, the result from the gel is not the best, as the bands are smiley and smeared. This happens because to adjust to the new ladder, the concentration of the gel was increased from 1% to 1.5%, which required more optimization. Several matters contributing to the gel results are the voltage too high; thus, they run too fast, or the high voltage causes overheating. Other than that, there might be too many samples in each well, RNA degradation, improper washing during RNA extraction, or DNA contamination might also contribute to smearing in the agar. Some optimization that can be done to solve these problems includes replacing the TTAE buffer with TBE buffer, lowering the agar concentration, and lowering the amount of sample loaded into each well.

Table 3. RNA Concentration and Purity

Sample treatment	RNA Concentration (ng/µl)	A260/280
Negative Control 1	299.7	2.05
Negative Control 2	336.2	2.05
Negative Control 3	381.0	1.97
Untreated 1	256.0	2.03
Untreated 2	265.5	2.05
Untreated 3	274.6	2.06
25 µg/ml Ethanolic Extract 1	327.8	2.02
25 µg/ml Ethanolic Extract 2	325.7	2

25 µg/ml Ethanolic Extract 3	351.4	2.04
10 µg/ml Ethanolic Extract 1	507.6	2.07
10 µg/ml Ethanolic Extract 2	516.4	2.07
10 µg/ml Ethanolic Extract 3	501.3	2.07
5 µg/ml Ethanolic Extract 1	481.7	2.07
5 µg/ml Ethanolic Extract 2	577.0	2.03
5 µg/ml Ethanolic Extract 3	626.4	2.04
2.5 µg/ml Ethanolic Extract 1	280.8	2.03
2.5 µg/ml Ethanolic Extract 2	378.0	2.05
2.5 µg/ml Ethanolic Extract 3	559.6	2.07

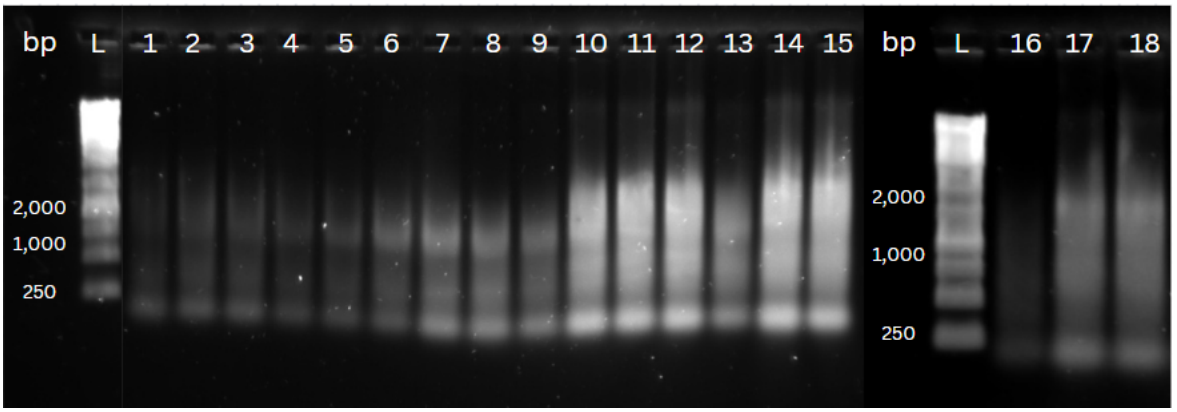

Figure 6. Gel electrophoresis of RNA in 1.5% agarose gel

4.4. RT-PCR

HaCaT cells are used as an AD model for observing both gene expression of inflammatory and skin-barrier genes. The HaCaT cells were induced with 10/10 ng/ml TNFα+IFNγ. The assay was performed by performing a Real-Time - Polymerase Chain Reaction (RT-PCR) using GAPDH as the housekeeping gene. GAPDH has been used as a housekeeping gene in the previous study as it is a gene that is equally expressed in human cells, and the expression is not distracted by external factors (Riemer et al., 2012). Eight genes were analyzed in this study with two controls: untreated (HaCaT in DMEM only) and negative control (HaCaT with TNFα+IFNγ). There are a total of 4 different concentrations of *Calophyllum inophyllum* ethanolic extract used based on the preliminary study, namely, 25 µg/ml, 10 µg/ml, 5 µg/ml, and 2.5 µg/ml. The purpose of using four different

concentrations is to investigate the effect of different concentrations towards the gene expression of each gene (CTACK, FLG, IL-25, IL-33, IVL, MDC, TARC, TSLP).

4.4.1. CTACK

CTACK, also known as CCL27, is an inflammatory gene expressed in the skin, and the expression of this gene is supposed to be upregulated upon AD and the treatment is supposed to downregulate the expression of CTACK. As seen in **Figure 7**, compared to the normal-untreated sample, the expression of the AD model is downregulated, which aligns the findings from other studies (Blatt et al., 2017; Machura et al., 2012). The figure also shows that treatment at 5 µg/ml shows a specific difference compared to the control group and the control shows a specific difference compared to the normal group. However, according to the melting curve graph shown in **Appendix 1**. Two peaks are observed, which indicate that the primer also reacts with other sequences, indicating that the primer used is not specifically targeting one gene (CTACK) or there is a DNA contamination in the sample, thus the result is deemed invalid.

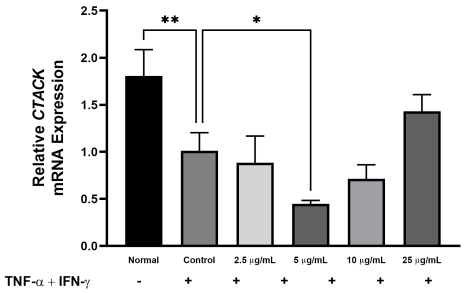

Figure 7. CTACK Gene Expression Results

4.4.2. FLG

Filaggrin is a skin barrier gene downregulated upon AD and the treatments are supposed to upregulate the expression of this gene (Dębińska, 2021). According to **Figure 8**, the expression of Fliggarin in the control (AD model) is lower compared to the normal and treated group. Which shows that the result aligns with previous study. Other than that the result at 5 µg/ml and 10 µg/ml shows a specific difference compared to the control group with 5 µg/ml being more specific. The primer specificity is also proven by the melting curve performed with the RT-PCR machine, and as seen in **Appendix 4**, the melting curve of FLG is perfect, where only one peak is visible. Thus the result for FLG can be seen as valid with 5 µg/ml and 10 µg/ml as the best candidate for skin barrier treatment.

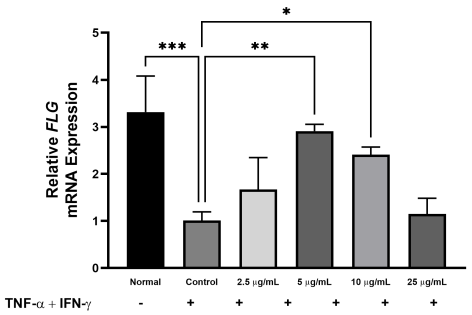

Figure 8. FLG Gene Expression Results

4.4.3. IL-25

Interleukin 25, also known as Interleukin 17E, is an inflammatory gene that is supposed to be upregulated upon AD and the treatments are expected to downregulate the expression of IL-25. As seen in **Figure 9,** the control group (AD model) has slightly lower expression compared to the normal-untreated cells but higher expression compared to the treated groups. Where 2.5 µg/ml and 5 µg/ml shows a specific difference compared to the control group. This contradicts the previous study, where the AD model is supposed to have higher gene expression than the untreated group (Aktar et al., 2015; Deleuran et al., 2012). Other than that the melting curve seen in **Appendix 7,** shows a concerning result where three peaks are visible, which might indicate three different genes targeted by this primer. Thus the results for IL-25 are deemed as invalid.

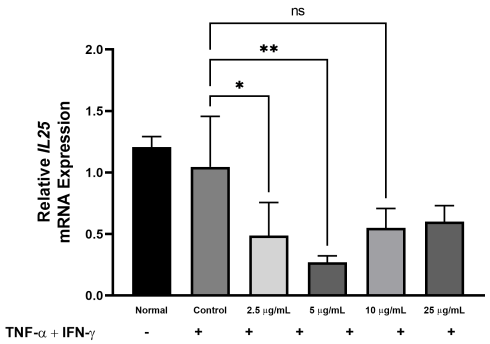

Figure 9. IL-25 Gene Expression Results

4.4.4. IL-33

Interleukin 33 is an inflammatory gene upregulated upon cellular damage such as AD and the treatments are supposed to downregulate the expression of IL-33. **Figure 10** shows that the control (AD model) gene expression is upregulated compared to the untreated group, which aligns with a previous study (Cayrol & Girard, 2018). Other than that, all treatments from 2.5 µg/ml to 25 µg/ml show lower gene expression with some degree of significance toward the control group, where 25 µg/ml is the most specific. Besides that, the

melting curve of IL-33 in **Appendix 10** shows that the primer is specific as only one peak is visible.

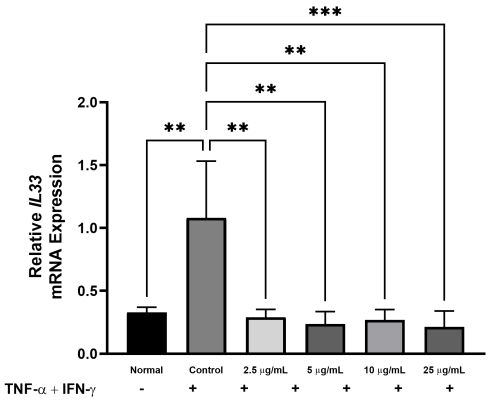

Figure 10. IL-33 Gene Expression Results

4.4.5. IVL

Involucrin (IVL) is a skin barrier gene that protects and aids in barrier homeostasis, downregulated upon AD (Furue, 2020). As seen in **Figure 11**, the control (AD model) shows a lower gene expression than the untreated cells, and the treatment causes an even lower gene expression. While lower gene expression of the control (AD model) compared to untreated samples is expected, the treatment should have increased the gene expression as the cells are being treated with the extract. However, the 2.5 μg/ml and 5 μg/ml treatments show a significant difference toward the control; there is a possibility that that treatment has some effect on the AD model. In addition, the melting curve, as seen in **Appendix 13**, shows questionable results where only one peak is visible, but not all samples have the same peak. Several explanations to this such as possible indication of unspecific amplification, contamination, or technical error that does happen with the PCR machine when running this gene (Zhang et al., 2020). Thus the result for IVL is seen as invalid.

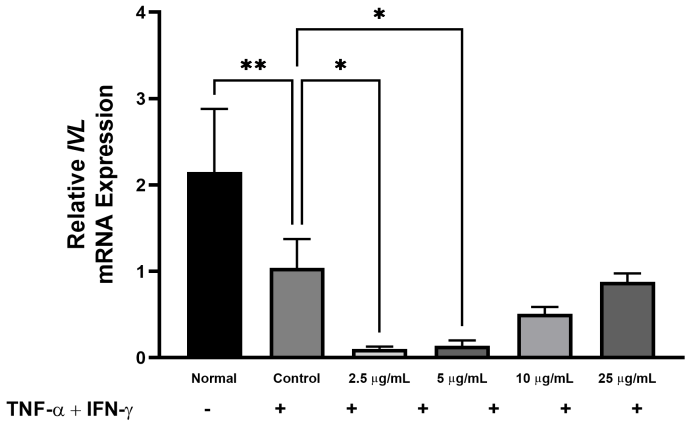

Figure 11. IVL Gene Expression Results

4.4.6. MDC

Macrophage-derived chemokine, also known as CCL22, is an inflammatory gene that is upregulated upon AD (Kakinuma et al., 2002). **Figure 12** shows the gene expression result of CCL22, where there is an upregulation in the control (AD model) group compared to the untreated group. A downregulation is also observed in all treatments with 10 µg/ml and 5 µg/ml treatments, where the 5 µg/ml are more specific compared to the 10 µg/ml treatment. However, the 25 µg/ml and 2.5 µg/ml treatments show little to no difference in gene expression compared to the control (AD model) group. When analyzed from the melting curve, shown in **Appendix 16**, the primer is a decent primer with good specificity as one peak is seen above the threshold.

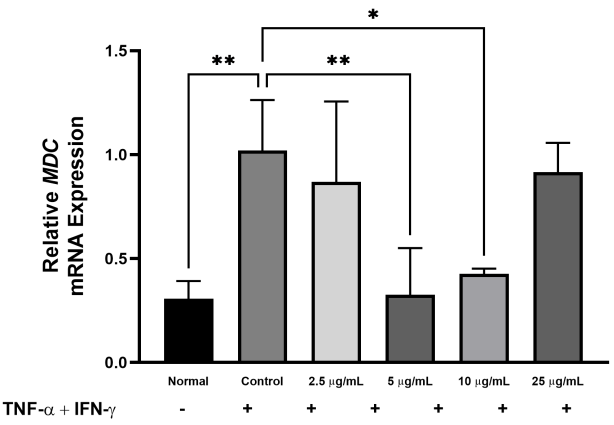

Figure 12. MDC Gene Expression Results

4.4.7. TARC

Thymus and activation-regulated chemokine is an important inflammatory gene upregulated upon AD. **Figure 13** shows an upregulation of the control (AD model) group compared to the untreated group and a downregulation in the treatment group, which aligns with the previous study about TARC (Machura et al., 2012). The 5 µg/ml treatment shows a significant comparison towards the AD model; however, upon analyzing the melting curve, the TARC primer is not specific. As seen in **Appendix 19**, there are two peaks on the melting curve, indicating that the primer targets two different genes. Therefore, while the result of the gene expression is as expected, it cannot be seen as valid as it might show a gene expression result toward other genes.

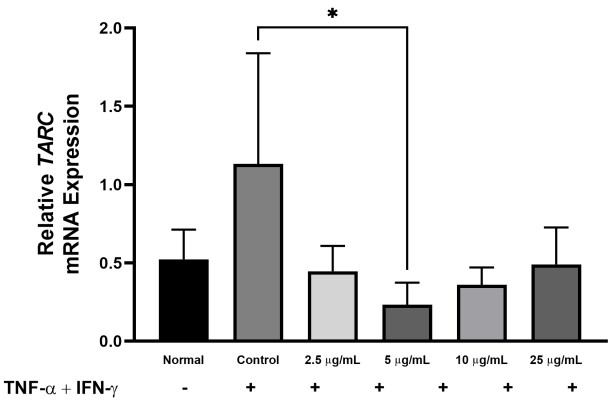

Figure 13. TARC Gene Expression Results

4.4.8. TSLP

Thymic Stromal Lymphopoietin is an inflammatory gene and is upregulated in AD (Indra, 2013). **Figure 14** shows an upregulation in the control (AD model) group compared to the untreated group. Other than that, the treatment group also showed a significant difference compared to the AD model group, with 2.5 µg/ml and 5 µg/ml being the most significant, followed by 10 µg/ml and lastly 25 µg/ml. The melting curve analysis in **Appendix 22** also shows that the TSLP primer was specific with one peak, which only indicates one target.

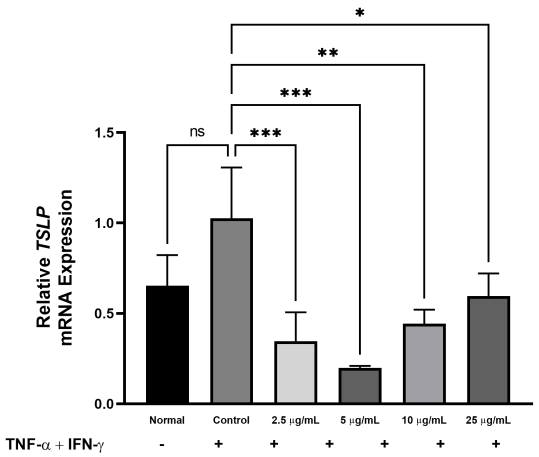

Figure 14. TSLP Gene Expression Results

The concentration of 25 µg/ml and 2.5 µg/ml in almost all primers shows a similar pattern where at those concentrations the gene expression contradicts the expected result. This could be related to the cytotoxic effect from the treatment, according to the preliminary study of cytotoxic analysis, the ethanolic extract at 25 µg/ml is quite toxic when compared with the 10 µg/ml and 5 µg/ml. Thus instead of treating the cells, the treatment at 25 µg/ml could be toxic to the cell, altering the gene expression. However this finding requires more in-deep analysis to prove the correlation of cytotoxic activity and gene expression result. Meanwhile the 2.5 µg/ml concentration might be too low thus the treatment could not work properly thus the outcome is not as expected.

Chapter V

CONCLUSION

In conclusion, this study has shown eight gene expressions resulting from inflammatory and skin barrier genes. Although half of the primer was nonspecific (CTACK, IL-25, TARC, IVL), the rest of the specific primers (IL-33, MDC, TSLP, FLG, GAPDH) shows a good and promising result that indicates ethanolic extract treatments of 5 µg/ml and 10 µg/ml concentration as the most promising potential for treating AD. Besides that, this study also shows that treatment of 2.5 µg/ml and 25 µg/ml concentration is unsuitable for treating AD. Unfortunately, further optimization on the agarose gel and primer cannot be performed due to time limitations, resulting in unsatisfactory results. Therefore, it is highly recommended to dedicate a specific time to optimizing the machine, method, and kit used for further experiments.

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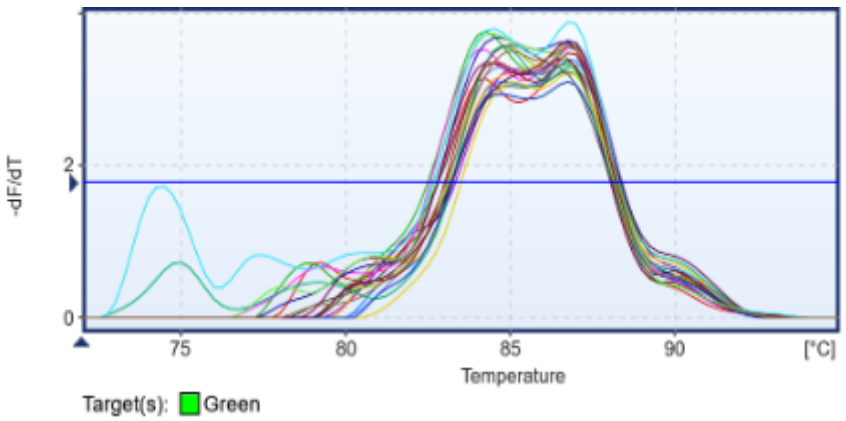
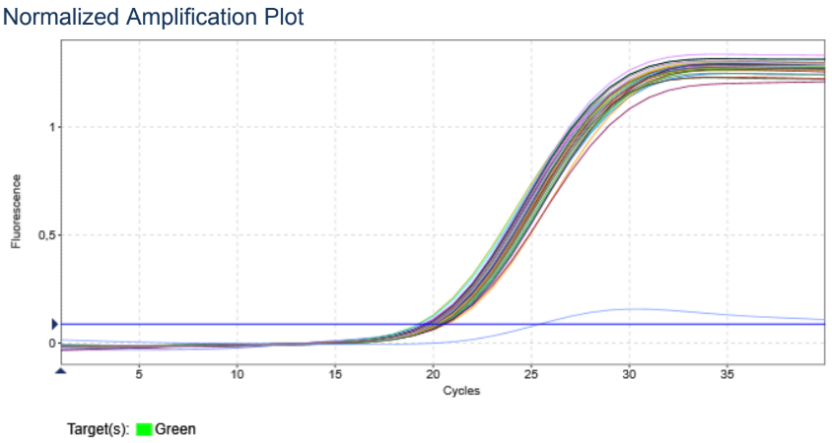
APPENDIX



Appendix 1. CTACK Melting Curve

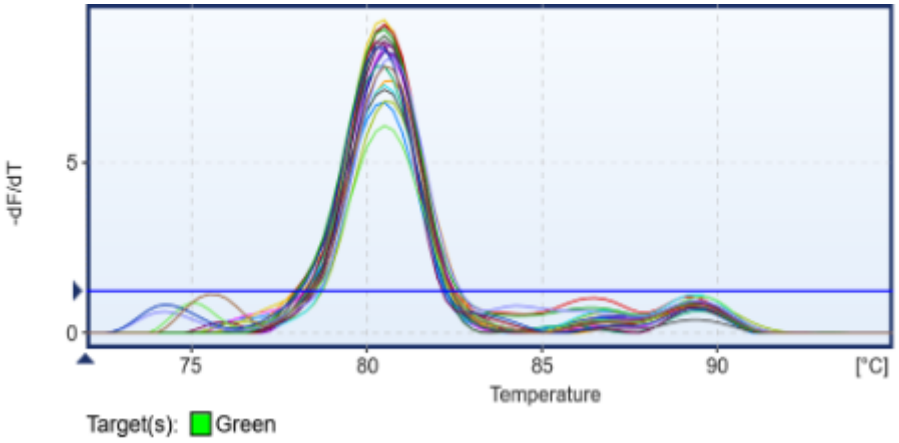


Appendix 2. CTACK Amplification Plot

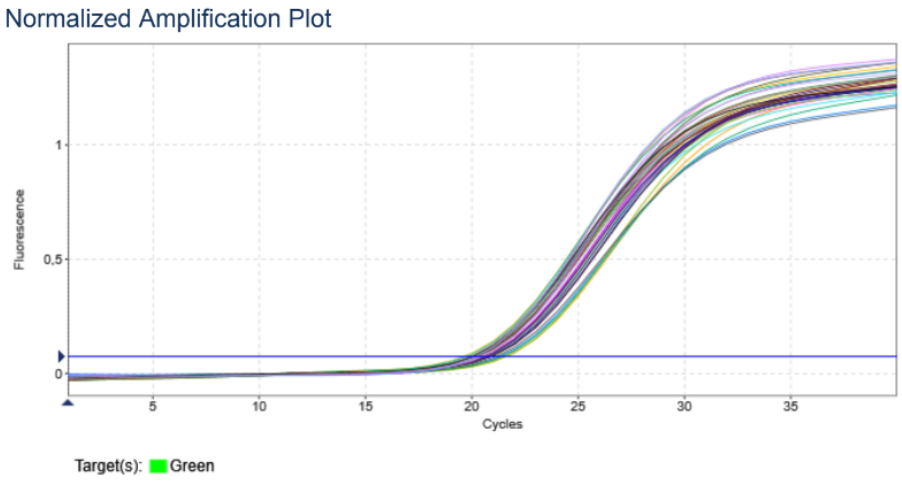
Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	CTACK	GAPDH			
Blank control 1	19.95		1.787	-0.66	1.58
Blank control 2	19.53	18.163	1.37	-1.08	2.12
Blank control 3	19.83		1.67	-0.78	1.72
Negative control 1	20.48		2.49	0.04	0.97
Negative control 2	20.16	17.993	2.17	-0.28	1.22
Negative control 3	20.69		2.70	0.25	0.84
25 - 1	19.25		1.797	-0.653	1.57
25 - 2	19.33	17.453	1.877	-0.573	1.49
25 - 3	19.6		2.147	-0.303	1.23

10 - 1	19.95		2.773	0.323	0.80
10 - 2	19.95	17.177	2.773	0.323	0.80
10 - 3	20.51		3.333	0.883	0.54
5 - 1	19.9		3.473	1.023	0.49
5 - 2	20.1	16.427	3.673	1.223	0.43
5 - 3	20.13		3.703	1.253	0.42
2.5 - 1	19.69		2.200	-0.250	1.19
2.5 - 2	20.6	17.490	3.110	0.660	0.63
2.5 - 3	20.21		2.720	0.270	0.83
Calibrator (average ΔCt control group)	2.45				

Appendix 3. CTACK Raw Data



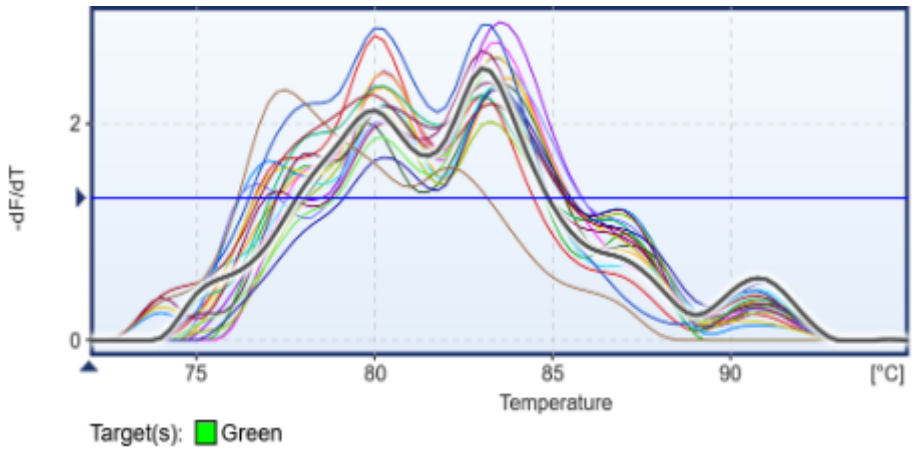
Appendix 4. FLG Melting Curve



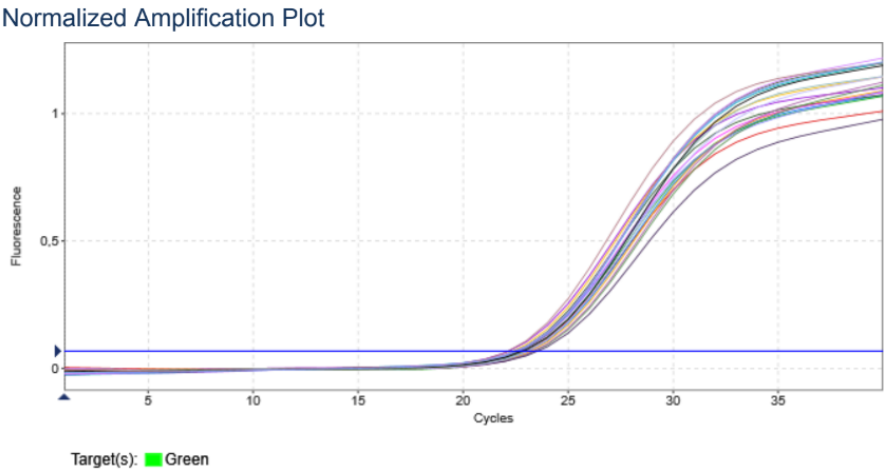
Appendix 5. FLG Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	FLG	GAPDH			
Blank control 1	20		1.837	-1.96	3.88
Blank control 2	20.67	18.163	2.51	-1.29	2.44
Blank control 3	20.1		1.94	-1.86	3.62
Negative control 1	22.11		4.12	0.32	0.80
Negative control 2	21.67	17.993	3.68	-0.12	1.08
Negative control 3	21.58		3.59	-0.21	1.15
25 - 1	21.63		4.177	0.383	0.77
25 - 2	20.76	17.453	3.307	-0.487	1.40
25 - 3	20.89		3.437	-0.357	1.28
10 - 1	19.71		2.533	-1.260	2.39
10 - 2	19.8	17.177	2.623	-1.170	2.25
10 - 3	19.6		2.423	-1.370	2.58
5 - 1	18.6		2.173	-1.620	3.07
5 - 2	18.7	16.427	2.273	-1.520	2.87
5 - 3	18.74		2.313	-1.480	2.79
2.5 - 1	20.95		3.460	-0.333	1.26
2.5 - 2	20.9	17.490	3.410	-0.383	1.30
2.5 - 3	19.99		2.500	-1.293	2.45
Calibrator (average ΔCt control group)	3.79				

Appendix 6. FLG Raw Data



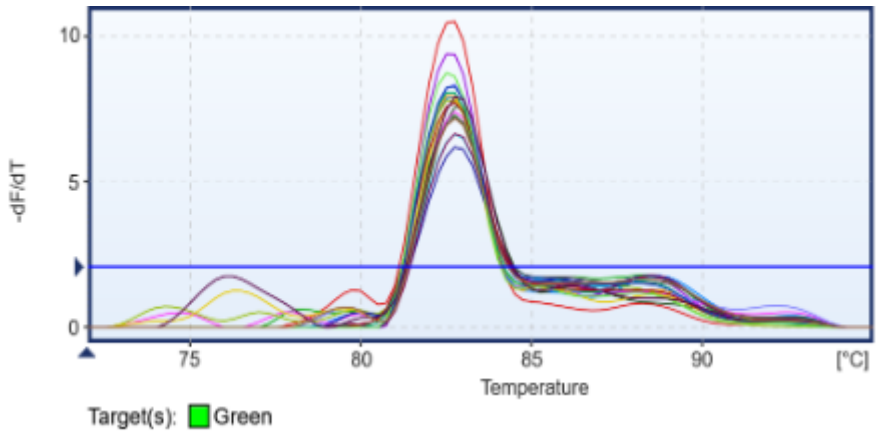
Appendix 7. IL-25 Melting Curve



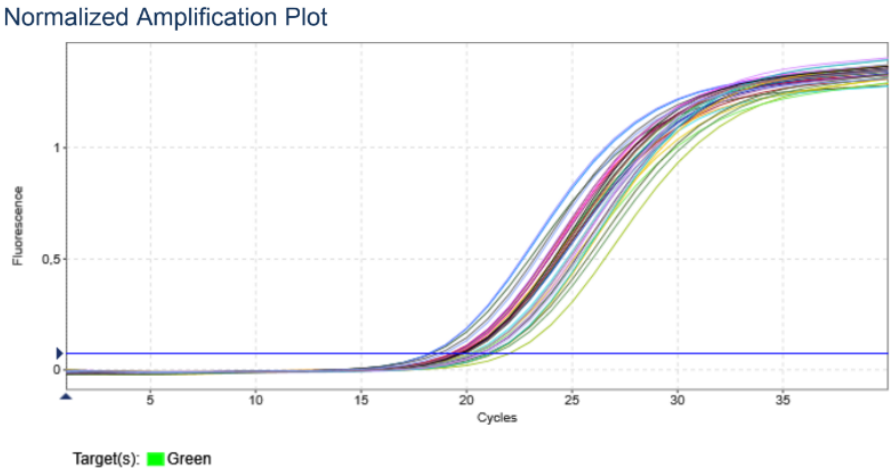
Appendix 8. IL-25 Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	IL-25	GAPDH			
Blank control 1	22.58		4.417	-0.37	1.30
Blank control 2	22.78	18.163	4.62	-0.17	1.13
Blank control 3	22.7		4.54	-0.25	1.19
Negative control 1	22.93		4.94	0.15	0.90
Negative control 2	22.19	17.993	4.20	-0.59	1.51
Negative control 3	23.23		5.24	0.45	0.73
25 - 1	23.35		5.897	1.107	0.46
25 - 2	22.94	17.453	5.487	0.697	0.62
25 - 3	22.72		5.267	0.477	0.72
10 - 1	22.9		5.723	0.933	0.52
10 - 2	23.26	17.177	6.083	1.293	0.41
10 - 3	22.44		5.263	0.473	0.72
5 - 1	22.81		6.383	1.593	0.33
5 - 2	23.33	16.427	6.903	2.113	0.23
5 - 3	23.21		6.783	1.993	0.25
2.5 - 1	22.67		5.180	0.390	0.76
2.5 - 2	23.35	17.490	5.860	1.070	0.48
2.5 - 3	24.49		7.000	2.210	0.22
Calibrator (average ΔCt control group)	4.79				

Appendix 9. IL-25 Raw Data



Appendix 10. IL-33 Melting Curve

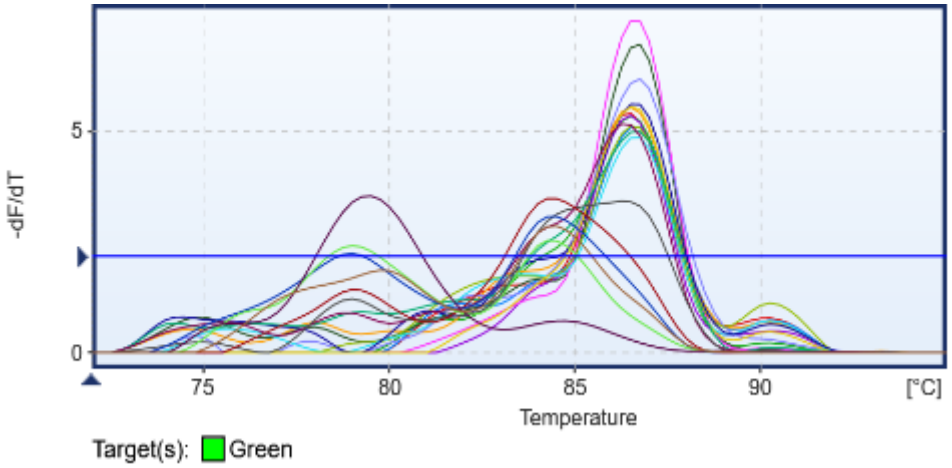


Appendix 11. IL-33 Amplification Plot

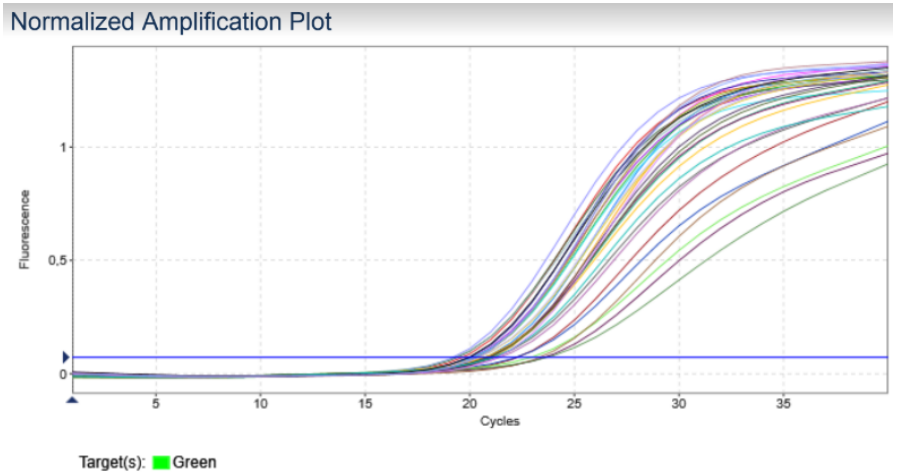
Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	IL-33	GAPDH			
Blank control 1	20.70		2.54	1.79	0.29
Blank control 2	20.36	18.163	2.20	1.45	0.37
Blank control 3	20.52		2.36	1.61	0.33
Negative control 1	18.35		0.36	-0.39	1.31
Negative control 2	19.59	17.993	1.60	0.85	0.56
Negative control 3	18.29		0.30	-0.45	1.37
25 - 1	22.04		4.587	3.837	0.07
25 - 2	20.17	17.453	2.717	1.967	0.26
25 - 3	19.89		2.437	1.687	0.31

10 - 1	20.24		3.063	2.313	0.20
10 - 2	19.9	17.177	2.723	1.973	0.25
10 - 3	19.4		2.223	1.473	0.36
5 - 1	18.69		2.263	1.513	0.35
5 - 2	19.61	16.427	3.183	2.433	0.19
5 - 3	19.76		3.333	2.583	0.17
2.5 - 1	20.41		2.920	2.170	0.22
2.5 - 2	19.79	17.490	2.300	1.550	0.34
2.5 - 3	19.93		2.440	1.690	0.31
Calibrator (average ΔCt control group)	0.75				

Appendix 12. IL-33 Raw Data



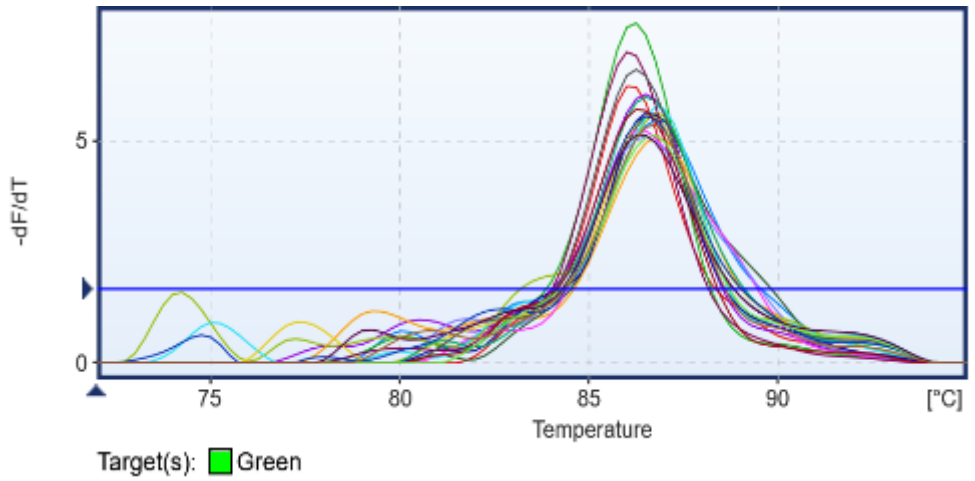
Appendix 13. IVL Melting Curve



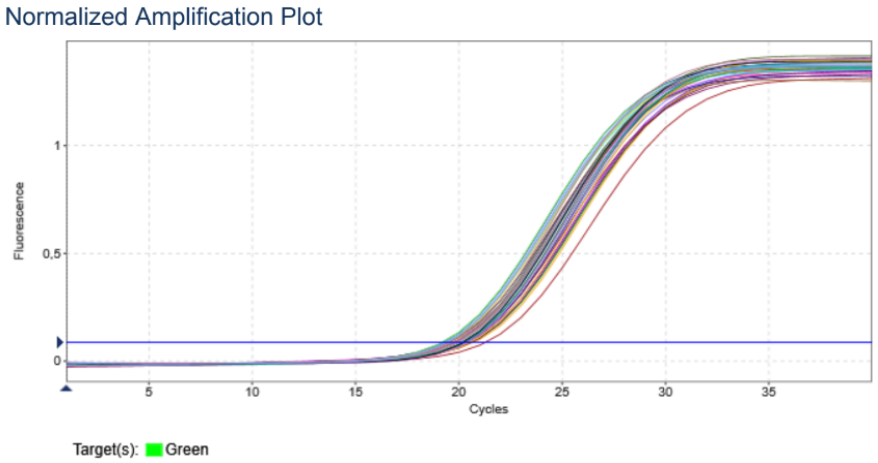
Appendix 14. IVL Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	IVL	GAPDH			
Blank control 1	19.42		1.257	-1.26	2.39
Blank control 2	20.26	18.163	2.10	-0.42	1.33
Blank control 3	19.23		1.07	-1.45	2.73
Negative control 1	20.27		2.28	-0.24	1.18
Negative control 2	20.15	17.993	2.16	-0.36	1.28
Negative control 3	21.1		3.11	0.59	0.66
25 - 1	20.33		2.877	0.363	0.78
25 - 2	20.17	17.453	2.717	0.203	0.87
25 - 3	20		2.547	0.033	0.98
10 - 1	20.42		3.243	0.730	0.60
10 - 2	20.84	17.177	3.663	1.150	0.45
10 - 3	20.77		3.593	1.080	0.47
5 - 1	21.21		4.783	2.270	0.21
5 - 2	22.29	16.427	5.863	3.350	0.10
5 - 3	22.24		5.813	3.300	0.10
2.5 - 1	22.95		5.460	2.947	0.13
2.5 - 2	23.59	17.490	6.100	3.587	0.08
2.5 - 3	23.35		5.860	3.347	0.10
Calibrator (average ΔCt control group)	2.51				

Appendix 15. IVL Raw Data



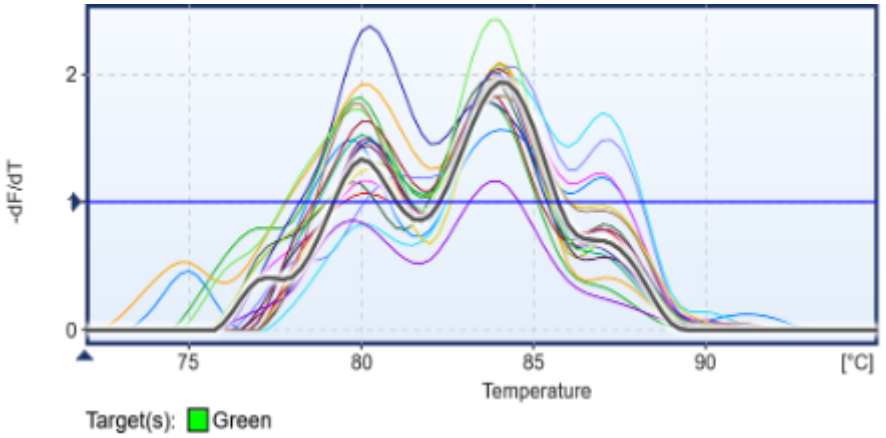
Appendix 16. MDC Melting Curve



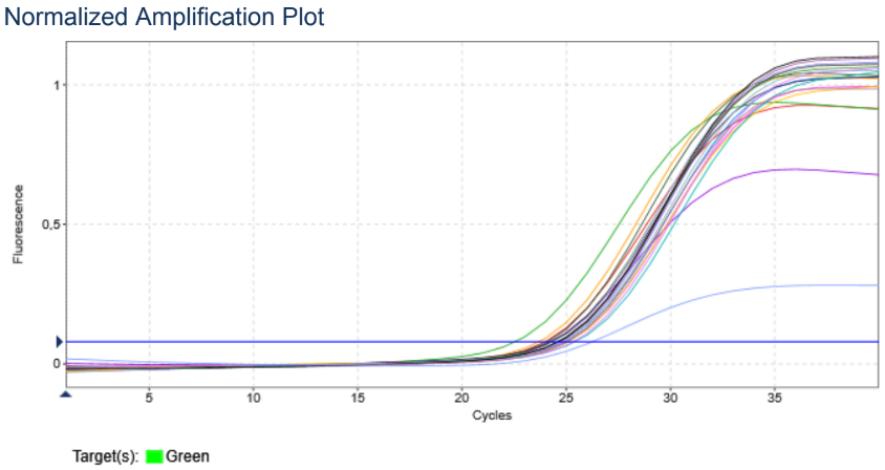
Appendix 17. MDC Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	MDC	GAPDH			
Blank control 1	21.61		3.45	1.45	0.37
Blank control 2	22.44	18.163	4.28	2.28	0.21
Blank control 3	21.73		3.57	1.57	0.34
Negative control 1	19.76		1.77	-0.23	1.17
Negative control 2	19.78	17.993	1.79	-0.21	1.15
Negative control 3	20.42		2.43	0.43	0.74
25 - 1	19.85		2.397	0.403	0.76
25 - 2	19.4	17.453	1.947	-0.047	1.03
25 - 3	19.5		2.047	0.053	0.96
10 - 1	20.5		3.323	1.330	0.40
10 - 2	20.38	17.177	3.203	1.210	0.43
10 - 3	20.33		3.153	1.160	0.45
5 - 1	20.25		3.823	1.830	0.28
5 - 2	21.31	16.427	4.883	2.890	0.13
5 - 3	19.23		2.803	0.810	0.57
2.5 - 1	19.21		1.720	-0.273	1.21
2.5 - 2	20.62	17.490	3.130	1.137	0.45
2.5 - 3	19.56		2.070	0.077	0.95
Calibrator (average ΔCt control group)	1.99				

Appendix 18. MDC Raw Data



Appendix 19. TARC Melting Curve

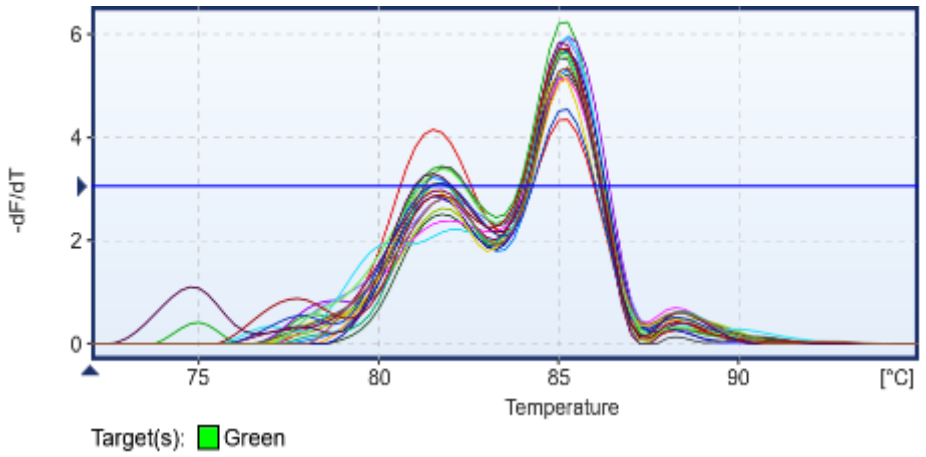


Appendix 20. TARC Amplification Plot

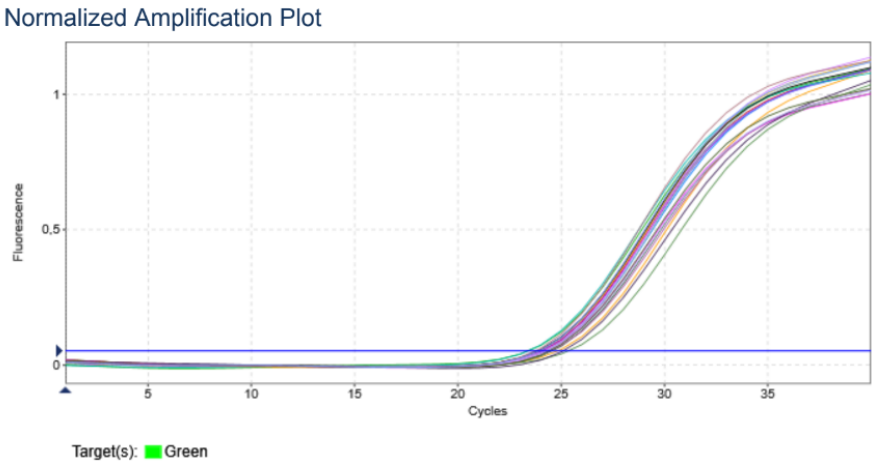
Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	TARC	GAPDH			
Blank control 1	24.12		5.957	0.43	0.74
Blank control 2	25.04	18.163	6.88	1.35	0.39
Blank control 3	24.86		6.70	1.17	0.44
Negative control 1	22.57		4.58	-0.95	1.93
Negative control 2	24.28	17.993	6.29	0.76	0.59
Negative control 3	23.71		5.72	0.19	0.88
25 - 1	23.42		5.967	0.440	0.74
25 - 2	24.87	17.453	7.417	1.890	0.27
25 - 3	24.09		6.637	1.110	0.46

10 - 1	24.79		7.613	2.087	0.24
10 - 2	23.81	17.177	6.633	1.107	0.46
10 - 3	24.11		6.933	1.407	0.38
5 - 1	25.05		8.623	3.097	0.12
5 - 2	24.37	16.427	7.943	2.417	0.19
5 - 3	23.33		6.903	1.377	0.39
2.5 - 1	23.94		6.450	0.923	0.53
2.5 - 2	23.87	17.490	6.380	0.853	0.55
2.5 - 3	24.96		7.470	1.943	0.26
Calibrator (average ΔCt control group)	5.53				

Appendix 21. TARC Raw Data



Appendix 22. TSLP Melting Curve

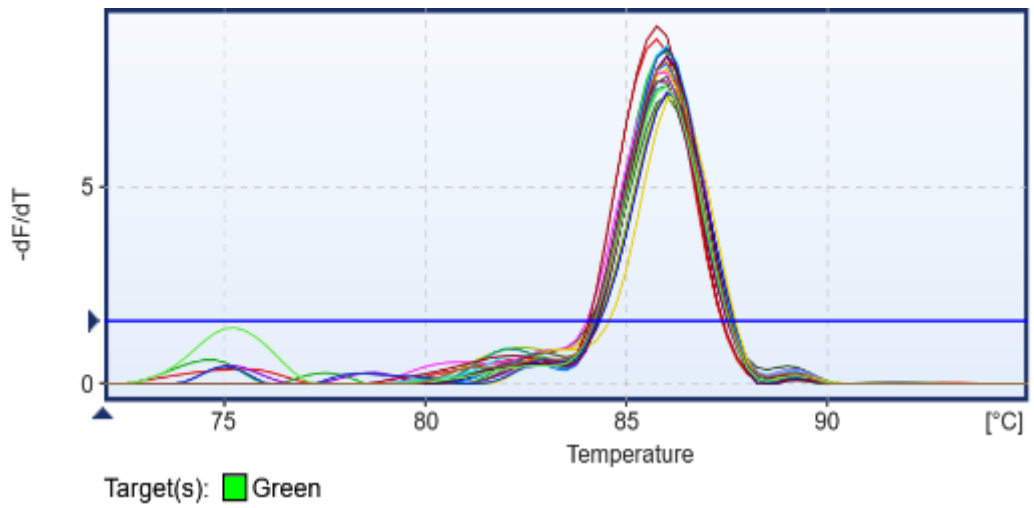


Appendix 23. TSLP Amplification Plot

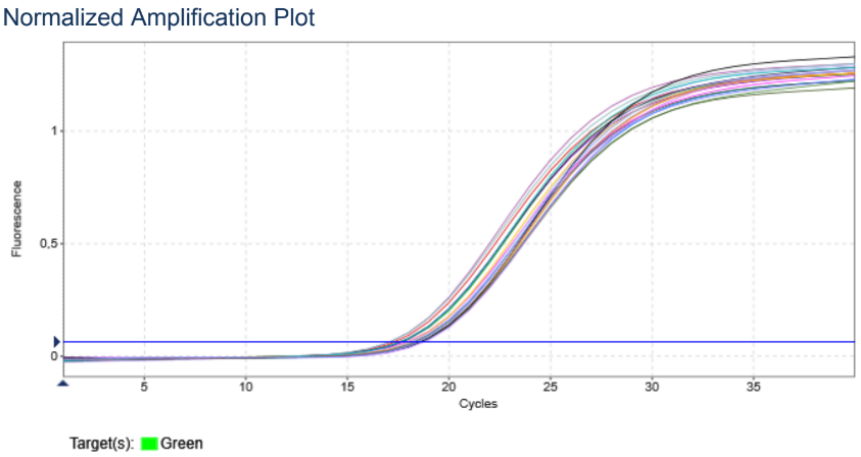
Treatment	Target	Housekeeping	ΔCt	ΔΔCt	2^-(ΔΔCt)

	Gene Ct	Ct			
	TSLP	GAPDH			
Blank control 1	24.58		6.417	0.96	0.51
Blank control 2	24.33	18.163	6.17	0.71	0.61
Blank control 3	23.86		5.70	0.24	0.84
Negative control 1	23.44		5.45	-0.01	1.00
Negative control 2	23.05	17.993	5.06	-0.40	1.32
Negative control 3	23.85		5.86	0.40	0.76
25 - 1	23.8		6.347	0.893	0.54
25 - 2	23.35	17.453	5.897	0.443	0.74
25 - 3	23.87		6.417	0.963	0.51
10 - 1	24.03		6.853	1.400	0.38
10 - 2	23.89	17.177	6.713	1.260	0.42
10 - 3	23.54		6.363	0.910	0.53
5 - 1	24.15		7.723	2.270	0.21
5 - 2	24.26	16.427	7.833	2.380	0.19
5 - 3	24.21		7.783	2.330	0.20
2.5 - 1	23.85		6.360	0.907	0.53
2.5 - 2	24.82	17.490	7.330	1.877	0.27
2.5 - 3	25		7.510	2.057	0.24
Calibrator (average ΔCt control group)	5.45				

Appendix 24. TSLP Raw Data



Appendix 25. GAPDH Melting Curve



Appendix 26. GAPDH Amplification Plot

STATEMENT OF ORIGINALITY

Submitted to

Indonesia International Institute for Life Sciences (I3L)

I, Agnes Maria Rosaceae, do hereby declare that the material contained in my report entitled:

" Investigating The Effect of *Calophyllum inophyllum* Ethanolic Extract on The Gene Expression in TNF-α and IFN-γ Induced Human Keratinocyte Skin Cells (HaCaT) "

Is an original work performed by me under the guidance and advice of my project supervisor, Amadeus Yeremia Pribowo. I have read and do understand the definition and information on use of source and citation style published by i3L. By signing this statement I unequivocally assert that the aforementioned project conforms to published information.

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☐ No

Name of student: Agnes Maria Rosaceae
Student ID: 19010004
Study Program: Biotechnology

Signature

Date: 1 January 2023

RESEARCH PROJECT PROPOSAL

INVESTIGATING THE EFFECT OF *Calophyllum Inophyllum* ETHANOLIC EXTRACT ON THE GENE EXPRESSION IN TNF-α AND IFN-γ INDUCED HUMAN KERATINOCYTE SKIN CELLS (HaCaT)

By

Agnes Maria Rosaceae
19010004

Submitted to

i3L – Indonesia International Institute for Life Sciences
School of Life Sciences

in partial fulfillment of the enrichment program for the Bachelor of Science in
Biotechnology

Research Project Supervisor: Amadeus Yeremia Pribowo, B.Sc., Ph.D.
Research Project Field Supervisor: Amadeus Yeremia Pribowo, B.Sc., Ph.D.

Jakarta, Indonesia
2022

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ABSTRACT

Atopic dermatitis is a chronic skin disease that affects 20% of children aged three to six months and 3% of adults worldwide. Despite its prevalence, the exact cause of the disease remains unclear due to its complexity of the disease. Many available drugs in the market for treating AD focus on preventing the disease from worsening, such as managing skin infection, hydration, inflammation, and itch. However, they are known to be inefficient, expensive or cause extreme side effects to the user; thus, an alternative treatment is needed for AD. The oil from *Calophyllum inophyllum* or tamanu has been used as a traditional treatment for various skin-related diseases such as psoriasis, burn, and acne. In addition, the oil extracted from tamanu seeds has been reported to have several properties, such as anti-bacterial, anti-fungal, and anti-inflammation. Since AD is related to chronic inflammation and skin barrier disruption, these properties make tamanu oil an ideal candidate for AD. Despite this potential, scientific data on the benefits of tamanu oil for AD, especially at the genetic level, remains scarce. This study investigates the effect of tamanu treatment on the expression of two skin barrier genes and six inflammatory genes in HaCaT cells induced with TNF-alpha and IFN-gamma to mimic the condition of AD patients. The investigation shows upregulation in the treated skin barrier (FLG and IVL) gene expression and downregulation in the inflammatory (IL-33, MDC, TSLP) gene expression.

Keywords: Atopic Dermatitis; RT-PCR; Gene expression; Tamanu; Ethanolic extract

ACKNOWLEDGEMENT

Throughout the whole project and writing of this report, the writer has received a lot of assistance and guidance from numerous people. Thus the writer would like to express utmost gratitude.

First and foremost, the writer would like to praise the Mighty Lord God for His grace and guidance; thus, this project and report can be completed. The writer would like to sincerely thank the family for their love, prayers, and support throughout the university journey. The writer would also like to express utmost gratitude for the support from:

1. Writer project teammates Jessica Renata Wijaya, Sarah Stefania Hutabarat, and Fanie Tansil for giving great insight as well as advice and support throughout the ups and downs of the project.
2. Mr. Amadeus Yeremia Pribowo B. Sc., Ph.D., for his guidance and input as the project supervisor throughout the project.
3. Ms. Nancy Christina Batubara, as the lab PIC, for the insight and guidance throughout the project period
4. Michael Putra Utama as the project assistant throughout the project period
5. All of the other groups at the i3L cell culture lab that is involved in this project
6. Writer friends and close ones, Stephanie Angela Yosiano and Laurentius Hardy Kurniawan, for being supportive and encouraging throughout the project
7. All the Library, cleaning, security, and building management staff for their aid during this project

This project would not be possible without their contribution and help; thus, the writer humbly expresses its utmost thanks for all the inspiration, support, and insight during this project in i3L.

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LIST OF ABBREVIATIONS

CE	Cornified envelope
CLA	Cutaneous lymphocyte antigen
CLDN1	Claudin-1
CTACK	Cutaneous T cell-Attracting Chemokine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EE	Ethanolic Extract
FBS	Fetal Bovine Serum
FLG	Filaggrin
FLG LoF	Loss of function in the filaggrin gene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HaCaT	Human Keratinocyte Cells
IFN-γ	Interferon Gamma
IL-1	Interleukin 1
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-17E	Interleukin 17E
IL-25	Interleukin 25
IL-33	Interleukin 33
ILC2s	Group 2 innate lymphoid cells
IVL	Involucrin
MDC	Macrophage Derived Cytokines
mRNA	Messenger RNA

N-3 PUFA	n-3 polyunsaturated fatty acid
RNA	Ribonucleic Acid
SC	Stratum Corneum
ST2	Serum STimulation-2
TAE	Tris-acetate-EDTA
TARC	Thymus and Activation-Regulated Chemokine
Th2	T helper 2
TNF-α	Tumor Necrosis Factor-Alpha
TRAP	Traffic-related air pollution
TSLP	Thymic Stromal Lymphopoietin

Chapter I

INTRODUCTION

1.1. BACKGROUND

Atopic dermatitis (AD) is a form of eczema, a common, chronic, long-lasting skin disease that often appears with skin rash, itchiness, fever, or even asthma. AD was first introduced in the late 1800s, however the exact cause and treatment for this disease are still unclear (Kapur et al., 2018; Kramer et al., 2017). AD affects 20% of children worldwide, and 25% will continue to be involved through adulthood, either as a relapsing disease or a continuous disease (Hadi et al., 2021). AD can occur at any age, however, people with a family history of AD and a history of gene dysfunction have a higher risk of developing AD. There are some theories related to the cause of AD, also known as the outside-in, where the concentration of responsible genes for skin barrier and moisturizing are low and inside-out hypothesis, where immune dysfunction is the main reason (Boguniewicz & Leung, 2011). AD is associated with abnormality in genes involved in skin barrier dysfunction, immune pathways, and inflammation. Over-production of various inflammatory cytokines, including IL-4R, IL-6, IL-8, IL-18, IL-32, CTACK, TARC, TSLP, etc, are often considered as the reason for AD severity (Hou et al., 2019; Kantor & Silverberg, 2017; Kanwal et al., 2021; Lee et al., 2018; J.-H. Yang et al., 2021).

Researchers also believe that AD pathology is related to environmental factors besides genetic and immunological factors. This might explain why AD prevalence differs from region to region (Kantor & Silverberg, 2017). Various environmental factors, including climate changes, stress, microorganisms, pollution, UV radiation, and allergens, combined with a genetic dysfunction, have been linked to abnormalities in the epidermis and immune system, thus contributing to the progression of AD (Kolb & Ferrer-Bruker, 2022).

Novel therapeutic approaches and preventative strategies for AD have been developed based on their facts. However, due to the complex interaction between genetic, immunological, and environmental factors and an unclear source of the disease, AD is still incurable. Furthermore, the current treatment available in the market, such as beta-Val, corticosteroid, and Kenalog-based drugs, are known to have some side effects, and the other therapy can only treat the symptoms and prevent them from getting worse (Dębińska, 2021; Nygaard et al., 2017).

Therefore, research to find alternative medication for AD has consistently been ongoing to find effective and efficient treatment without side effects.

Oil extracted from tamanu (*Calophyllum inophyllum*) has been a potential solution for AD treatment. Tamanu is a plant that originated from Polynesia, Including Asia, Africa, and the Pacific. It is known as “Nyamplung” in Indonesia, and it is widely available across Java, Kalimantan, Sulawesi, and Sumatra. Tamanu seed is known to contain 75% oil, which has been extracted and marketed commercially and used in dermal products. Tamanu oil has been recommended for treating many skin disorders, such as eczema, acne, psoriasis, and burns. It also has been reported to have antifungal, antioxidant, wound-healing, and anti-inflammatory properties (Pribowo et al., 2021).

In this study, the anti-inflammatory effect of *Calophyllum inophyllum ethanolic* extracts will be investigated using Human Keratinocyte Skin Cells (HaCaT) induced with TNF-α and IFN-γ as the AD model. This study has two hypotheses: (1) *Calophyllum inophyllum* ethanolic extracts will downregulate inflammatory gene expression in the AD model. (2) *Calophyllum inophyllum ethanolic* extracts will upregulate the gene expression of skin barrier-related genes in the AD model.

1.2. Aim and Objective

The overall aim of this study is to study the effect of Tamanu Ethanolic extract on skin barrier and inflammation genes that are expressed in people with Atopic Dermatitis by achieving the following objectives:

1. Investigate the effect of Tamanu Ethanolic Extract Treatment on the gene expression of Atopic Dermatitis-related inflammation genes (CTACK, IL-25, IL-33, MDC, TARC TSLP).

2. Investigate the effect of Tamanu Ethanolic Extract Treatment on the gene expression of Atopic Dermatitis-related skin barrier genes (IVL & FLG).

1.3. Scope of Work

This project will cover *Calophyllum inophyllum* Ethanolic Extract preparation and gene expression assays. The detailed scope of work for this project is as follows:

1. *Calophyllum inophyllum* Ethanol Extract preparation.
2. Cell Culture of human keratinocyte skin (HaCaT) cells.

3. Performing primer design for each gene through research and primer design websites.
4. RNA Extraction and Quantitative Real-Time PCR.
5. Statistical Analysis.

Chapter II

LITERATURE REVIEW

2.1. Literature review

2.1.1. Atopic Dermatitis

Atopic Dermatitis (AD) is the most common chronic inflammation in the skin that usually occurs during childhood and might last until adulthood. AD prevalence in the US was reported to be 11.3-12.7% in adults and 6.9-7.6% in children (J. Kim et al., 2019). In developing countries such as the Middle Eastern and Africa, the AD prevalence was reported to be 3-6% and 12-14%, respectively (Pribowo et al., 2021). The name atopic dermatitis came from *Atopy* and dermatitis. *Atopy* is a condition where when exposed to environmental factors (e.g. pollutants) will trigger IgE expression. Whilst Dermatitis refers to skin inflammation. Thus AD refers to a condition where when exposed to environmental factors, it will trigger skin inflammation (Papapostolou et al., 2022).

Generally, AD is a heterogenous eczematous skin disorder related to Th2 deficiency, barrier disruption, skin inflammation, and chronic pruritus. A combination of environmental, genetic, and immunological factors further complicate AD, leading to immune response dysfunction. Although the complete pathophysiology of AD has not been found, many studies believe that immune dysregulation and skin barrier dysfunction have a role in AD. Although the epidermis plays a vital role as a physical barrier, many AD cases have reported skin barrier defects. Filaggrin (*FLG*), transglutaminases, intracellular proteins, and keratins are the skin barrier's key (J. Kim et al., 2019).

2.2. Atopic Dermatitis Risk Factors

2.2.1. Environmental Risk Factors

Environmental factors hold an essential role in AD development, and many environmental risk factors that have been reported to affect AD development. However, not all factors have been scientifically proven or accepted. In industrialized countries, AD prevalence is higher, people living in the city tend to have a higher risk of getting AD than those living in the countryside. Environmental factors can be grouped into three major groups, external nonspecific (e.g. urbanization, climate, migration), external specific (e.g.

sunlight, pollution, diet, humidity, allergens, temperature), and internal (e.g. microbiome) (Ahn et al., 2020). These three groups play a significant role in AD prevalence and severity.

Climate is one of the critical factors as to why AD prevalence in different regions diversifies, climate is closely related to temperature and sunlight exposure. Some studies study the correlation between AD development and temperature, however, a future investigation is required (Bonamonte et al., 2019). Meanwhile, it has been reported that people that live in areas with less sunlight exposure have been reported to have more severe AD (Calov et al., 2020). While it is widely known that sunlight has UVB that affects Vitamin D serum production, UV light also has immunosuppressive effects that affect *FLG* production. Vitamin D has properties that aid skin protection, thus lack of Vitamin D would cause more severe AD manifestation (Vestita et al., 2015). Lifestyles such as diet and habits affect AD development to a certain degree. People who consume fruit, vegetables, and fish high in n-3 polyunsaturated fatty acid (n-3 PUFA) have been reported to have a lower risk of getting AD as n-3 PUFA has anti-inflammatory properties that can lower the risk of getting AD.

Traffic-related air pollution (TRAP) is highly related to eczema symptoms in children. Air pollution from the non-industrial and industrial processes could contain chemicals (e.g. toluene, styrene, benzene) or toxic substances (e.g. tobacco smoke). As they enter the skin, they will trigger allergic reactions by stimulating the production of Ige and Th2 cytokines. Based on a study, it was found that TRAP mainly triggers AD in children between the ages of 13 and 14. However, TRAP does not show any association with AD in the general population, thus a more profound study is required (Ahn et al., 2020). A study in South African toddlers aged between 12-36 months has shown that consuming fermented milk has reduced the risk of getting AD in urban cohorts, however, the same effect was not found in rural populations. Thus urbanization and loss of gut microbial diversity might play a role in AD development (Levin et al., 2020). However, some studies have reported that there is almost no difference in gut microbiota diversity between healthy persons and AD patients. Thus a future study that analyzes the correlation between gut microbiota diversity and AD development is required (Bonamonte et al., 2019).

2.2.2. Genetic Risk Factors

Genetic risk factors refer to particular genes related to epidermal protein and type 2 T helper lymphocytes (TH2) signalling pathway. Mutation of the filaggrin gene is the most studied genetic risk factor for AD. *FLG* has a vital role in maintaining the epidermis; mutation of the *FLG* gene has affected the *FLG* protein production and disturbed the skin barrier. *FLG*

mutations have been reported to cause loss of function in the filaggrin gene (FLG LoF), which increases the TWEL and decreases the skin's water content, increasing pH in the stratum corneum and allowing bacterial proliferation. Other than that *FLG* LoF also increases the risk of getting other allergies (e.g. asthma) (Thomsen, 2014). Other than *FLG,* many loci and genes have been identified as having a role in AD genetic risk factors. Such as inflammation related genes (e.g. CTACK, MDC, TARC), epidermal and skin barrier related genes (e.g. IVL, FLG), cytokines and chemokines (e.g. TSLP, IL-25, IL-33), protease, as well as antimicrobial genes. The many genes involved in AD development show that Many factors cause AD.

2.3. Immunology

2.3.1. CTACK

Cutaneous T cell-attracting chemokine (CTACK) or CCL27 is a chemokine that belongs to the CC chemokine family and attracts skin-homing cutaneous lymphocyte antigen (CLA). It is expressed only in the skin, thus it has been viewed as a valuable variable to evaluate AD severity; upon AD, CTACK expression in human keratinocyte cells is upregulated correlated with the severity of the disease (Blatt et al., 2017; Machura et al., 2012). It has been suggested that TARC and CTACK work together in attracting Th2 cells and migrating them into the upper layers of the skin. In addition CTACK has been reported to have a role in wound healing by attracting T cells to the skin and inducing bone marrow-derived keratinocytes, attracting Th2 cells from peripheral blood, as well as the migration, proliferation, and angiogenesis of T cells into upper layers of the skin (Karnezis et al., 2019, p. 27; Niwamoto et al., 2021).

2.3.2. *FLG*

Fliggarin (*FLG*) is an essential skin barrier protein that maintains Stratum Corneum equilibrium in the skin by producing profilaggrin protein that forms the outermost layer of skin or epidermis (Dębińska, 2021). The epidermis acts as a barrier from bacteria or foreign substances that can cause allergic reactions to the skin; lack of *FLG* will decrease keratohyalin granules, disturb barrier function, and affect the structure of the cornified envelope (CE). A deficiency of *FLG* caused by the genetics of inflammatory response allows allergens to pass through the skin barrier and migrate to lymph nodes, where it will interact with T cells and induce a Th2 immune response (Dębińska, 2021). While *FLG* is not expressed in mucosal membranes, many reported cases of people with *FLG* mutations are at higher risk of having asthma or allergic rhinitis. It is believed that *FLG* mutation promotes these allergenic

responses through sensitization of allergens that enter through a distributed skin barrier (Armengot-Carbo et al., 2015; Furue, 2020).

2.3.3. IL-25

The Interleukin 25 (IL-25), also known as Interleukin 17E (IL-17E), is a newly identified cytokine-protein from the interleukin 17 (IL-17) family that has a role in inflammation pathways that involves immune response and cell migration. IL-25 is expressed in epidermal cells and correlated to many infections, such as parasitic, viral, and bacterial infections. IL-25 is essential in regulating immune responses in two ways: as a driver for multiple allergic diseases and as an amplifier. This cytokine is known to upregulate T helper 2 (Th2) immune responses, trigger the expression of interleukin 4 (IL-4), and indirectly mediate secretion of interleukin 5 (IL-5) and interleukin 13 (IL-13) (Wu et al., 2022). During AD, IL-25 expression is upregulated and was found to lower the expression of FLG in keratinocytes upon AD, as well as directly affecting the skin barrier (Aktar et al., 2015; Deleuran et al., 2012).

2.3.4. IL-33

The Interleukin 33 (IL-33) is an inflammatory cytokine from Interleukin 1 (IL-1) family expressed in normal keratinocytes as alarmin at the protein level. However, IL-33 expression is upregulated upon cellular damage to stimulate the immune system (Cayrol & Girard, 2018). As seen in **Figure 1.** from the study of Imai (2019), overexpression of IL-33 in epidermal keratinocytes directly downregulated filaggrin and claudin-1 (CLDN1) protein which exacerbates dermatitis by activation of group 2 innate lymphoid cells (ILC2s) and basophils. IL-4 produced by basophils will then promote the activation of ILC2s, which stimulates the production of IL-5 and IL-13 and causes the accumulation of eosinophils. Eosinophils are disease-fighting white blood cells, accumulation of these cells indicates an infection or allergic reaction (Kanuru & Sapra, 2022).

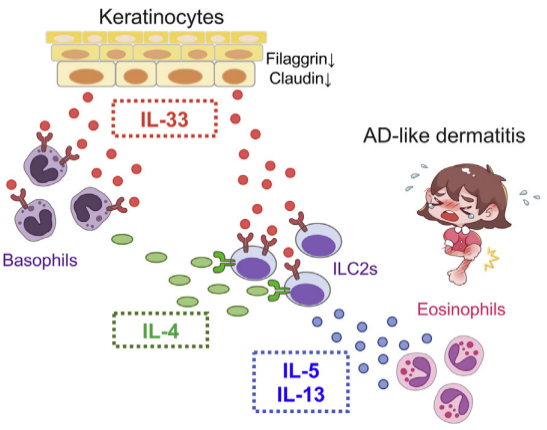

Figure 1. IL-33-induced AD-like inflammation mechanism from (Imai, 2019).

2.3.5. IVL

Involucrin (IVL) is a skin barrier protein and also a part of keratinocyte-specific differentiation protein. *IVL* is formed in the granular layer and has a specific role during the beginning of cornified envelope formation that protects the corneocytes in the skin and the base for the corneocyte-bound lipid envelope (Chiba et al., 2019). Upon AD, IVL expression is downregulated by Th2 cytokinesis and STAT-6 pathway, the absence or deficiency of this gene will lead to permeability barrier homeostasis and affect the integrity of the skin barrier (Furue, 2020).

2.3.6. MDC

Macrophage-derived chemokine (MDC) or CCL22 is one of the CCR4 ligands expressed in dendritic cells (DCs), macrophages, and thymic epithelial cells. It plays as chemoattractant for Th2 cells, NK cells, and eosinophils. MDC expression in AD is upregulated according to the severity of the disease, in AD MDC triggers Th2 migration and causes inflammation (Kakinuma et al., 2002; Richter et al., 2014).

2.3.7. TARC

Thymus and activation-regulated chemokine (TARC) are one of the essential chemokines in the skin-specific homing of T cells that are produced in keratinocytes (Umeda et al., 2020). TARC receptor is CCR4 that is expressed mainly in skin homing, TARC has been reported to play a role in inducing integrin-dependent adhesion and transendothelial migration of T-cells. Furthermore, proinflammatory cytokines such as IL-1, TNFα and IFNγ can upregulate TARC expression (Machura et al., 2012).

2.3.8. TSLP

Thymic stromal lymphopoietin (TSLP) is a cytokine from the IL-7 family that is expressed in keratinocytes and plays a role in triggering and initiating Th2 cytokine (Indra, 2013). TSLP expression is upregulated in keratinocytes with allergic dermatitis such as AD, skin barrier is disrupted during AD, making it more susceptible to allergens or irritants penetrations. Thus the keratinocytes trigger the expression of TSLP. Other than that, it has been reported that environmental factors such as microbes, viruses, chemical particles, and parasites can also trigger TSLP production. In addition TSLP will trigger the activation of STAT1, STAT3, STAT5, and STAT 6, which enhance allergic reactions, contributing to skin inflammation and tissue damage (Kang et al., 2016).

2.4. Atopic Dermatitis Model

The model used in this study is a human keratinocyte cell (HaCaT) induced by TNFα+IFNγ. This model is used based on a preliminary study from the previous students performing this project and according to previous studies by other researchers (Gil et al., 2019; H. J. Kim et al., 2018; Lee et al., 2018). TNFα+IFNγ are the most commonly used inducers to imitate the skin condition during AD. However, studies also show that this inducer induces chemokines and cytokines that trigger Th2 expression (Lee et al., 2018). Furthermore, studies by Kim et al. (2018) have found that HaCaT cells induced with TNFα+IFNγ cell morphology are more unhealthy than normal non-induced HaCaT cells. Thus the success of the inducer can be determined by observing the cell viability of the induced HaCaT cells.

2.5. Current Atopic Dermatitis Treatment

Due to the complexity and lack of study on the disease, up to date, there is no effective cure for AD. Therefore, the only available treatment is targeted to reduce the severity of the skin lesion and pruritus. AD can be prevented by ensuring skin moisture and preventing dryness using moisturizers or emollients. This will prevent itch and desire to scratch the skin, lowering the risk of skin barrier disruption and allergen infection. Depending on the severity, a water-based cream with high water content is recommended for patients with mild eczema. In contrast, thick fat-based cream is more recommended for patients with severe eczema. Topical corticosteroids (e.g. dexamethasone) are also used to treat severe eczema. However, due to the serious side effects, it is only given to patients in critical conditions. Various creams, ointments, or oral medication to treat AD are available in the market, however, those treatments have low efficiency with serious side effects when used regularly.

However, in recent years researchers have developed several medications that could block specific cytokinesis and cytokine receptors/ transcription factors, such as dupilumab and crisaborole. Dupilumab, a type of monoclonal antibody, has the ability to reduce type 2 inflammation through antagonizing IL-4 & IL-3 while increasing *FLG*, loricrin, and claudin expression (Ahn et al., 2020). Whilst crisaborole is reported to downregulate inflammatory genes (MMP12), Th2 (CCL22/MDC), Th1 (CXCL9, CXCL10), and TH17 (CXCL1 and CXCL2) while upregulating the claudin 8 (CLDN8) (G. Yang et al., 2020). Although there has been much ongoing research to find the most effective and efficient AD treatment, there have been no reports of a treatment that is free from any side effects upon long-term usage.

2.6. *Calophyllum inophyllum*

Calophyllum inophyllum is the scientific name for tamanu, a plant that belongs to the mangosteen family and is widely distributed in Southeast Asia. In Indonesia, this plant is also known as “nyamplung”. The bark of this plant has been used to make furniture and household materials, and the fruit has been used to make oil for light sources. Tamanu oil was extracted from the seeds inside the tamanu fruit. Tamanu fruit is sphere-like and has a greenish-yellow colour when ripe and brown when overripe. Tamanu seed is about 2-4 cm with a corky shell. Tamanu has been known for its biological effects: antifungal, antioxidant, anti-cancer, anti-inflammatory, UV protection, antiviral, and antibacterial activity. Other than that, tamanu also has aromatic purposes, thus, many cosmetic lines have included tamanu as one of their ingredients to promote an anti-ageing effect.

A study from 2019 by Ginigini has led to the discovery of 11 bioactive compounds in tamanu ethanolic extract, namely; calophyllolide, inophyllums (A, B, C, D, E, P), 12-oxo-calanolide, calanolide Gut 70, D, and A (Ginigini et al., 2019). The bioactive compound was reported to have anti-cancer, anti-HIV, antimicrobial, and anti-inflammatory properties, which means tamanu extract is a suitable candidate for treating AD. This is supported by a previous study by Tsai and colleagues, where tamanu extract managed to suppress and or downregulate the expression of COX-2, NF-kB, INOS, and oxide production in dose-dependent conditions (Tsai et al., 2012).

Chapter III

MATERIALS AND METHODS

3.1. *Calophyllum inophyllum* Ethanol Extract Preparation

The ethanol extraction method from *C. inophyllum* seed was performed following previous studies by previous researchers. First, fresh *C. inophyllum* seeds were de-shelled and grounded, followed by adding 100% ethanol with a 1:2 ratio to the weight of the seeds. Next the extract was covered in aluminium foil and put into a shaking incubator at room temperature for 24 hours at 100 rpm. The mixture was then filtered by the Buchner funnel and filter paper. Next, 100% ethanol was added to the solid and put back into the shaker incubator in the same condition, while the liquid was stored in the storage covered with aluminium foil. The second filtration was performed, and both extracts were combined and evaporated using a rotary evaporator. The remaining extract was placed in an evaporating dish, covered with cheese cloth to dry inside the fume hood for 1-2 weeks. The extract was then delivered to third parties (BRIN) to be freeze dried to prolong shelf life. Upon usage, extract was combined with 0.1% pure DMSO and DMEM and filtered using a syringe filter.

3.2. Cell Culture

The HaCaT cell was obtained from another project that used to work with a lecturer from Nanyang Technological University, Prof. Ng Kee Woei from the School of Materials Science and Engineering. The cells was cultured in High Glucose Dulbecco's modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin in a T25/T75 flask and was incubated in 37 ^{0}C and 5% CO_2. The cells will be monitored every 2-3 days, and the cells that will be used will be cell passage numbers 19 to 30.

3.3. Primer Design

Most of the primers have been designed by the students taking charge of this project's previous batch. Primers was designed online using the NCBI Primer-BLAST. All primers were designed to have a similar melting temperature (Tm), between 59 to 61 ^{0}C. The PCR products were set to be 45 - 310 bp, and all primers are checked to ensure compatibility from one to another. The primer for this study was brought from Pt. Indolab Utama.

3.4. RNA Extraction

The HaCaT cells were seeded into 24-well plates with $5x10^4$ cells/well for 48 hours. A 10+10 ng/ml TNFα+IFNγ with ethanolic extract of different concentrations was then added to the cells and incubated for 24 hours. The RNA extraction was performed following GENEzol^{tm} TriRNA Pure Kit manufacturer's instructions, and the RNA were measured at 260/280 nm using a Nanodrop®spectrophotometer to measure the quality and quantity. RNA was stored at -80^{0}C prior to usage.

3.5. Gel Electrophoresis

Agarose gel was weighted 1.5:100 to total 1x TAE buffer and combined in a beaker glass covered in plastic wrap with ventilation holes. The mixture was then microwaved for 1- 2 minutes in medium heat and swirled every 25 seconds to ensure that the agarose was adequately dissolved and prevent over-boiling. The mixture was cooled until there was no more steam then poured into a gel casting tray up to 6-8 mm depth, and a well-forming comb was added. After the gel had solidified, the comb was removed, and the gel was positioned into a gel tank filled with a 1x TAE buffer. A total of 7µl (1µl 6x loading dye + 6µl ladder/ samples) ladder or samples are added to each well. Then the electrophoresis tank was connected to the power supply through PowerPac Basic electrometer (Bio Rad) and run for 300 minutes at 100 V; 400 mA. The gel was then stained using Diamond Nucleic Acid Dye (Promega) that was diluted with a 1x TAE buffer for 20 minutes. Then it will be visualized in the dark room using the gel doc (GBox Chemi XRX). The ladder used in this experiment was bought from thermo scientific.

3.6. Real Time Polymerase Chain Reaction

The real-time polymerase chain reaction (RT-PCR) was performed using SensiFAST SYBR No-ROX One-Step kit (Meridian), following the manufacturer's instructions. The primer used in this study can be seen in **Table 1**. The RNA and primer will be diluted 1x to a concentration of 0.01 µg/µL and 400 nM/ 20µL, respectively, using RNAse Free Water. PCR Master Mix will be made according to the manufacturer's instructions, and the thermal cycle will be set following the manufacturer's instructions shown in **Table 2.**

Table 1. Primer Sequence for Real-Time Polymerase Chain Reaction (RT-PCR).

Symbol	Gene Name	Sequences (5' → 3')	Amplicon Size

CTACK	Cutaneous T cell-Attracting Chemokine	**Forward:** TAGGCTGAGCAACATGAAGGG	89
		Reverse: GGAATGCTGCTGTAGGGTCT	
FLG	Filaggrin	**Forward:** TCAATCTGAGGGCACTGAAAGG	63
		Reverse: TAGCTGCCATGTCTCCAAACT	
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	**Forward:** ACCCACTCCTCCACCTTTGA	115
		Reverse: TGAGGTCCACCACCCTGTT	
IL-25	Interleukin 25	**Forward:** CCAGCATGTACCAGGTGGTTG	45
		Reverse: TTCCCATGACCATTGCCAAGA	
IL-33	Interleukin 33	**Forward:** TTATGAAGCTCCGCTCTGGC	160
		Reverse: CCAAAGGCAAAGCACTCCAC	
IVL	Involucrin	**Forward:** CTGCCCACAAAGGGAGAAGT	166
		Reverse: AGCGGACCCGAAATAAGTGG	
MDC	Macrophage Derived Cytokines	**Forward:** ATTAGATGTCCCCTGGCCCT	199
		Reverse: GCCACTTTCTGGGCTCTGAT	
TARC	Thymus and Activation-Regulated Chemokine	**Forward:** ATTCAAAACCAGGGTGTCTCC	310
		Reverse: TCGCTGCCATGTCTCCAAACT	
TSLP	Thymic Stromal Lymphopoietin	**Forward:** CTTCCTGTGGACTGGCAATG	97
		Reverse: CTCTTGTTGTTGGGGTCCGA	

Table 2. Setting Condition for Real-Time Polymerase Chain Reaction (RT-PCR).

Cycle	Temperature	Time	Notes
1	45^0C	10 minutes	Reverse Transcription
1	95^0C	2 minutes	Polymerase Activation
40	95^0C	5 seconds	Denaturation
	60^0C	10 seconds	Annealing
	72^0C	5 seconds	Extension

3.7. Statistical Analysis

The data were analyzed with Microsoft Excel version 16 software, and statistical analysis were performed with GraphPad Prism 9 software. Gene expression will be analyzed with a one-way analysis of variance, and the presented data were shown as mean ± standard error of the mean (SEM). P-values of *$p<0.05$, **$p<0.01$, and ***$p<0.001$ were considered statistically significant.

Chapter IV

RESULTS AND DISCUSSION

4.1. *Calophyllum inophyllum* Seeds Ethanolic Extraction

Calophyllum inophyllum was extracted from the seeds using ethanolic extraction as suggested by the preliminary study of previous students. However, freeze-drying was added as an additional step to help stabilize the extract, as freeze-drying will help to stabilize the microparticles, solubility, and dissolution (Dixit et al., 2011). The process of freeze-drying the extract was performed by a third party (BRIN) as they are more well equipped. The ethanolic extract from the *Calophyllum inophyllum* seeds contains oil in it, thus the freeze drying products are not in the form of powder as the oil will sublimate first, resulting in a stickier consistency. As seen in **Figure 2** and **Figure 3**, there are some differences in the appearance of the oil; this could be the leftover resins from the extract, as resins will harden when exposed to air.

Figure 2. Ethanolic Extract from *Calophyllum inophyllum* Seeds Before Freeze-drying

Figure 3. *Calophyllum inophyllum* Seeds Extract After freeze-drying

4.2. Effect of *Calophyllum inophyllum* Extract on Induced HaCaT cells morphology

To better analyze the effect of TNFα+IFNγ and *Calophyllum inophyllum* treatment on the cells, a picture of the morphology of the cells before and 24 hours after treatment was taken from each triplicate. As seen in **Figure 4**, some dead cells were visible before the treatment, which can be caused by several reasons, as this picture was taken 48 h after seeding. First, the amount of nutrients in the media is already depleting. Second, as there are too many cells, there might not be enough room for them to grow, thus leading to cell death. However, HaCaT cells were still closely packed to each other and had a spindle shape, just as HaCaT cells normally behave.

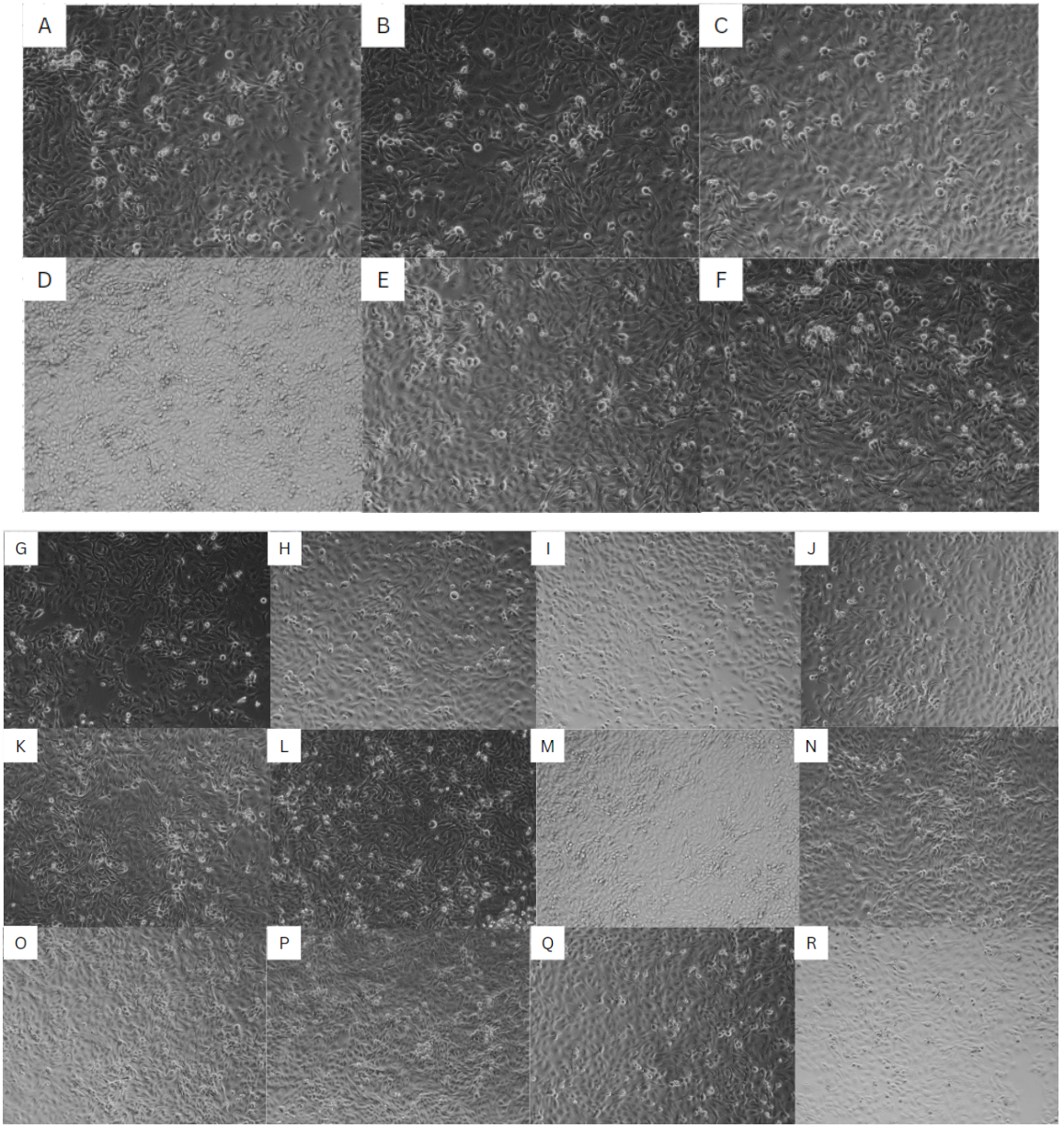

Figure 4. HaCaT Cells Morphology Before Treatment

HaCaT cell observation after 48 hour culturing, before treatment at x10 magnification (A) Negative Control 1 - Induced HaCaT cells; (B) Negative Control 2 - Induced HaCaT cells; (C) Negative Control 3 - Induced HaCaT cells; (D) Untreated HaCaT cells 1; (E) Untreated HaCaT cells 2; (F) Untreated HaCaT cells 3; (G) 25 µg/ml Ethanolic Extract 1; (H) 25 µg/ml Ethanolic Extract 2; (I) 25 µg/ml Ethanolic Extract 3; (J) 10 µg/ml Ethanolic Extract 1; (K) 10 µg/ml Ethanolic Extract 2; (L) 10 µg/ml Ethanolic Extract 3; (M) 5 µg/ml Ethanolic Extract 1; (N) 5 µg/ml Ethanolic Extract 2; (O) 5 µg/ml Ethanolic Extract 3; (P) 2.5 µg/ml Ethanolic Extract 1; (Q) 2.5 µg/ml Ethanolic Extract 2; (R) 2.5 µg/ml Ethanolic Extract 3

Cell changes were observed after 24 hours of treatment with TNFα+IFNγ and ethanol extract (**Figure 5**). The number of cell deaths is more visible after the treatment. Several factors might contribute to this. First, no nutrients are added to the media; thus, the cells fight for food. Second, the treatment concentration might be too high to the point it becomes toxic to the cell itself. Third,

when the picture was taken, it had already passed the time when the inducer was supposed to prevent cell doubling; instead, it became toxic to the cells. However, despite the amount of cell death, the HaCaT cells still show the behavior of normal HaCaT, such as being closely packed to each other and having a spindle shape. Other than that, the previous study has proven that this amount of inducer would not kill the cells upon induction for 24 hours, but it will show some difference in cell morphology (H. J. Kim et al., 2018).

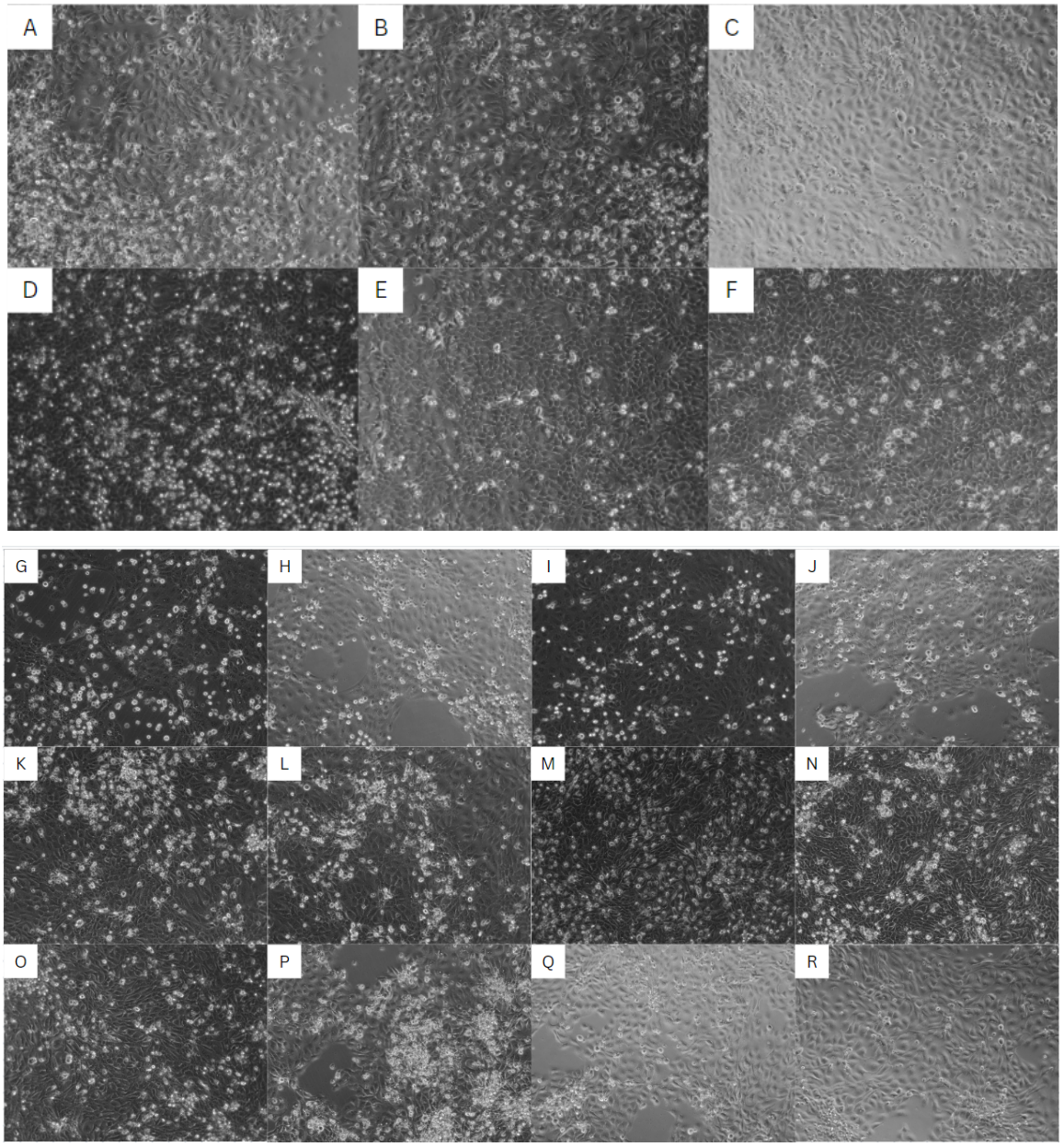

Figure 5. HaCaT Cells Morphology 24h After Treatment with *Calophyllum inophylum* and TNFα+IFNγ

HaCaT cell observation after 24 hour treatment at x10 magnification

(A) Negative Control 1 - Induced HaCaT cells; (B) Negative Control 2 - Induced HaCaT cells; (C) Negative Control 3 - Induced HaCaT cells; (D) Untreated HaCaT cells 1; (E) Untreated HaCaT cells 2; (F) Untreated HaCaT cells 3; (G) 25 µg/ml Ethanolic Extract 1; (H) 25 µg/ml Ethanolic Extract 2; (I) 25

µg/ml Ethanolic Extract 3; (J) 10 µg/ml Ethanolic Extract 1; (K) 10 µg/ml Ethanolic Extract 2; (L) 10 µg/ml Ethanolic Extract 3; (M) 5 µg/ml Ethanolic Extract 1; (N) 5 µg/ml Ethanolic Extract 2; (O) 5 µg/ml Ethanolic Extract 3; (P) 2.5 µg/ml Ethanolic Extract 1; (Q) 2.5 µg/ml Ethanolic Extract 2; (R) 2.5 µg/ml Ethanolic Extract 3

4.3. RNA Extraction

After RNA extraction, nanodrop is completed to measure each sample's quantity and purity, as shown in **Table 3**. The analysis shows different sample concentrations, varying from 256 ng/µl to 626 ng/µl according to the treatment group. The purity for each sample varies from A260/A280 of 1.97 up to 2.07, which integrity around 1.8 up to 2.1, indicating a high RNA purity (Craciun et al., 2019; Fleige & Pfaffl, 2006). RNA integrity was further analyzed by performing an agarose gel electrophoresis with a 1.5% agar concentration. As RNA is a negatively charged molecule, it will migrate to the positively charged electrode and separate according to its size when an electric current is given. However, as shown in **Figure 6**, the result from the gel is not the best, as the bands are smiley and smeared. This happens because to adjust to the new ladder, the concentration of the gel was increased from 1% to 1.5%, which required more optimization. Several matters contributing to the gel results are the voltage too high; thus, they run too fast, or the high voltage causes overheating. Other than that, there might be too many samples in each well, RNA degradation, improper washing during RNA extraction, or DNA contamination might also contribute to smearing in the agar. Some optimization that can be done to solve these problems includes replacing the TTAE buffer with TBE buffer, lowering the agar concentration, and lowering the amount of sample loaded into each well.

Table 3. RNA Concentration and Purity

Sample treatment	RNA Concentration (ng/µl)	A260/280
Negative Control 1	299.7	2.05
Negative Control 2	336.2	2.05
Negative Control 3	381.0	1.97
Untreated 1	256.0	2.03
Untreated 2	265.5	2.05
Untreated 3	274.6	2.06
25 µg/ml Ethanolic Extract 1	327.8	2.02
25 µg/ml Ethanolic Extract 2	325.7	2

25 µg/ml Ethanolic Extract 3	351.4	2.04
10 µg/ml Ethanolic Extract 1	507.6	2.07
10 µg/ml Ethanolic Extract 2	516.4	2.07
10 µg/ml Ethanolic Extract 3	501.3	2.07
5 µg/ml Ethanolic Extract 1	481.7	2.07
5 µg/ml Ethanolic Extract 2	577.0	2.03
5 µg/ml Ethanolic Extract 3	626.4	2.04
2.5 µg/ml Ethanolic Extract 1	280.8	2.03
2.5 µg/ml Ethanolic Extract 2	378.0	2.05
2.5 µg/ml Ethanolic Extract 3	559.6	2.07

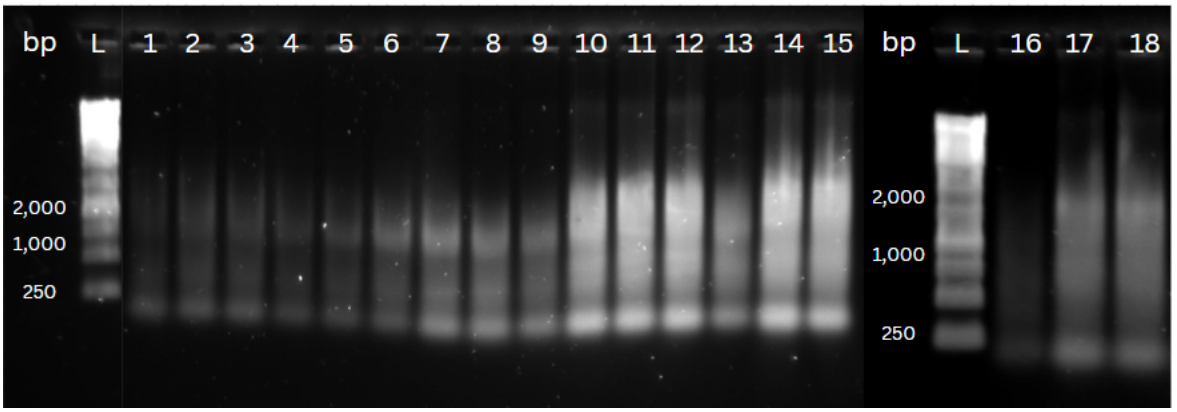

Figure 6. Gel electrophoresis of RNA in 1.5% agarose gel

4.4. RT-PCR

HaCaT cells are used as an AD model for observing both gene expression of inflammatory and skin-barrier genes. The HaCaT cells were induced with 10/10 ng/ml TNFα+IFNγ. The assay was performed by performing a Real-Time - Polymerase Chain Reaction (RT-PCR) using GAPDH as the housekeeping gene. GAPDH has been used as a housekeeping gene in the previous study as it is a gene that is equally expressed in human cells, and the expression is not distracted by external factors (Riemer et al., 2012). Eight genes were analyzed in this study with two controls: untreated (HaCaT in DMEM only) and negative control (HaCaT with TNFα+IFNγ). There are a total of 4 different concentrations of *Calophyllum inophyllum* ethanolic extract used based on the preliminary study, namely, 25 µg/ml, 10 µg/ml, 5 µg/ml, and 2.5 µg/ml. The purpose of using four different

concentrations is to investigate the effect of different concentrations towards the gene expression of each gene (CTACK, FLG, IL-25, IL-33, IVL, MDC, TARC, TSLP).

4.4.1. CTACK

CTACK, also known as CCL27, is an inflammatory gene expressed in the skin, and the expression of this gene is supposed to be upregulated upon AD and the treatment is supposed to downregulate the expression of CTACK. As seen in **Figure 7**, compared to the normal-untreated sample, the expression of the AD model is downregulated, which aligns the findings from other studies (Blatt et al., 2017; Machura et al., 2012). The figure also shows that treatment at 5 µg/ml shows a specific difference compared to the control group and the control shows a specific difference compared to the normal group. However, according to the melting curve graph shown in **Appendix 1**. Two peaks are observed, which indicate that the primer also reacts with other sequences, indicating that the primer used is not specifically targeting one gene (CTACK) or there is a DNA contamination in the sample, thus the result is deemed invalid.

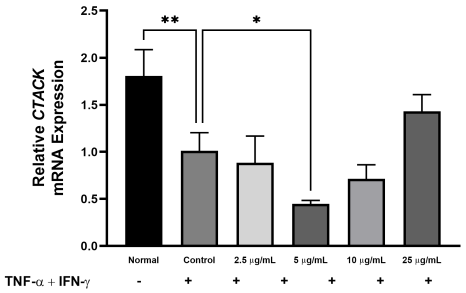

Figure 7. CTACK Gene Expression Results

4.4.2. FLG

Filaggrin is a skin barrier gene downregulated upon AD and the treatments are supposed to upregulate the expression of this gene (Dębińska, 2021). According to **Figure 8**, the expression of Fliggarin in the control (AD model) is lower compared to the normal and treated group. Which shows that the result aligns with previous study. Other than that the result at 5 µg/ml and 10 µg/ml shows a specific difference compared to the control group with 5 µg/ml being more specific. The primer specificity is also proven by the melting curve performed with the RT-PCR machine, and as seen in **Appendix 4**, the melting curve of FLG is perfect, where only one peak is visible. Thus the result for FLG can be seen as valid with 5 µg/ml and 10 µg/ml as the best candidate for skin barrier treatment.

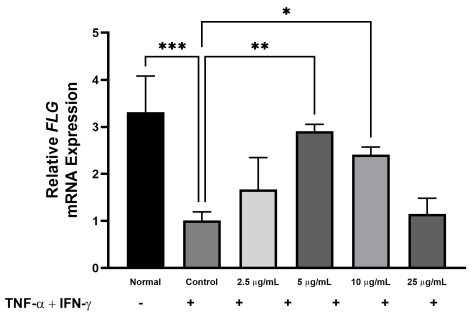

Figure 8. FLG Gene Expression Results

4.4.3. IL-25

Interleukin 25, also known as Interleukin 17E, is an inflammatory gene that is supposed to be upregulated upon AD and the treatments are expected to downregulate the expression of IL-25. As seen in **Figure 9,** the control group (AD model) has slightly lower expression compared to the normal-untreated cells but higher expression compared to the treated groups. Where 2.5 µg/ml and 5 µg/ml shows a specific difference compared to the control group. This contradicts the previous study, where the AD model is supposed to have higher gene expression than the untreated group (Aktar et al., 2015; Deleuran et al., 2012). Other than that the melting curve seen in **Appendix 7,** shows a concerning result where three peaks are visible, which might indicate three different genes targeted by this primer. Thus the results for IL-25 are deemed as invalid.

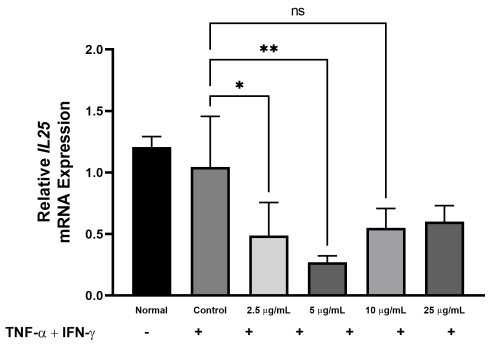

Figure 9. IL-25 Gene Expression Results

4.4.4. IL-33

Interleukin 33 is an inflammatory gene upregulated upon cellular damage such as AD and the treatments are supposed to downregulate the expression of IL-33. **Figure 10** shows that the control (AD model) gene expression is upregulated compared to the untreated group, which aligns with a previous study (Cayrol & Girard, 2018). Other than that, all treatments from 2.5 µg/ml to 25 µg/ml show lower gene expression with some degree of significance toward the control group, where 25 µg/ml is the most specific. Besides that, the

melting curve of IL-33 in **Appendix 10** shows that the primer is specific as only one peak is visible.

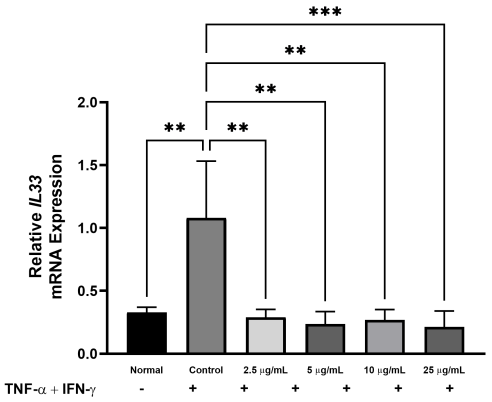

Figure 10. IL-33 Gene Expression Results

4.4.5. IVL

Involucrin (IVL) is a skin barrier gene that protects and aids in barrier homeostasis, downregulated upon AD (Furue, 2020). As seen in **Figure 11**, the control (AD model) shows a lower gene expression than the untreated cells, and the treatment causes an even lower gene expression. While lower gene expression of the control (AD model) compared to untreated samples is expected, the treatment should have increased the gene expression as the cells are being treated with the extract. However, the 2.5 μg/ml and 5 μg/ml treatments show a significant difference toward the control; there is a possibility that that treatment has some effect on the AD model. In addition, the melting curve, as seen in **Appendix 13**, shows questionable results where only one peak is visible, but not all samples have the same peak. Several explanations to this such as possible indication of unspecific amplification, contamination, or technical error that does happen with the PCR machine when running this gene (Zhang et al., 2020). Thus the result for IVL is seen as invalid.

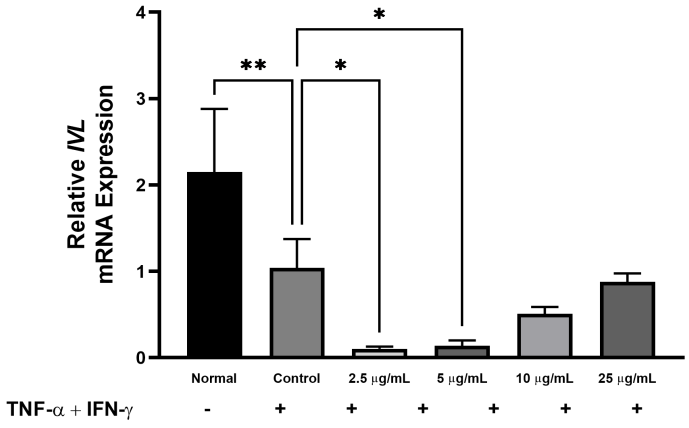

Figure 11. IVL Gene Expression Results

4.4.6. MDC

Macrophage-derived chemokine, also known as CCL22, is an inflammatory gene that is upregulated upon AD (Kakinuma et al., 2002). **Figure 12** shows the gene expression result of CCL22, where there is an upregulation in the control (AD model) group compared to the untreated group. A downregulation is also observed in all treatments with 10 µg/ml and 5 µg/ml treatments, where the 5 µg/ml are more specific compared to the 10 µg/ml treatment. However, the 25 µg/ml and 2.5 µg/ml treatments show little to no difference in gene expression compared to the control (AD model) group. When analyzed from the melting curve, shown in **Appendix 16**, the primer is a decent primer with good specificity as one peak is seen above the threshold.

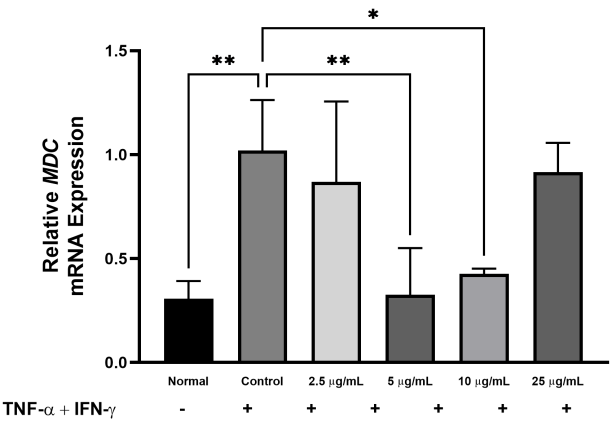

Figure 12. MDC Gene Expression Results

4.4.7. TARC

Thymus and activation-regulated chemokine is an important inflammatory gene upregulated upon AD. **Figure 13** shows an upregulation of the control (AD model) group compared to the untreated group and a downregulation in the treatment group, which aligns with the previous study about TARC (Machura et al., 2012). The 5 µg/ml treatment shows a significant comparison towards the AD model; however, upon analyzing the melting curve, the TARC primer is not specific. As seen in **Appendix 19**, there are two peaks on the melting curve, indicating that the primer targets two different genes. Therefore, while the result of the gene expression is as expected, it cannot be seen as valid as it might show a gene expression result toward other genes.

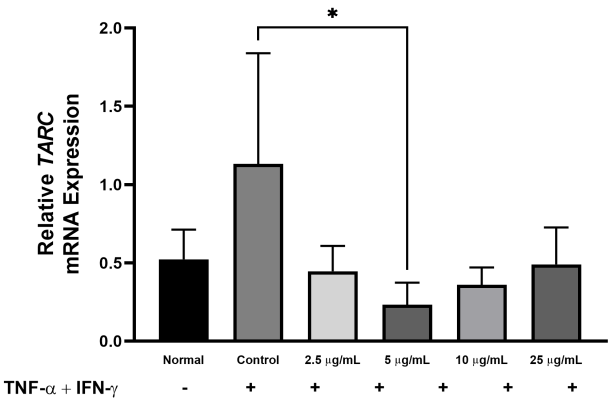

Figure 13. TARC Gene Expression Results

4.4.8. TSLP

Thymic Stromal Lymphopoietin is an inflammatory gene and is upregulated in AD (Indra, 2013). **Figure 14** shows an upregulation in the control (AD model) group compared to the untreated group. Other than that, the treatment group also showed a significant difference compared to the AD model group, with 2.5 µg/ml and 5 µg/ml being the most significant, followed by 10 µg/ml and lastly 25 µg/ml. The melting curve analysis in **Appendix 22** also shows that the TSLP primer was specific with one peak, which only indicates one target.

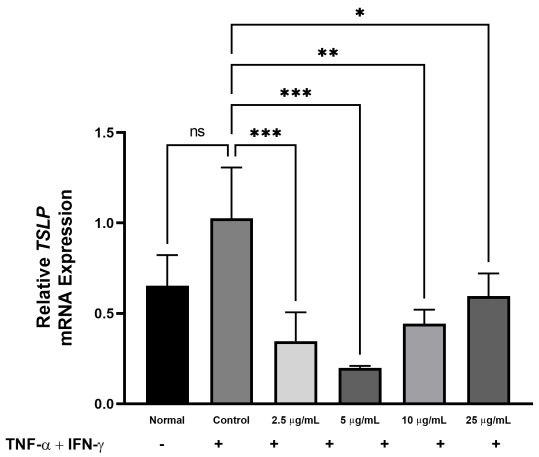

Figure 14. TSLP Gene Expression Results

The concentration of 25 µg/ml and 2.5 µg/ml in almost all primers shows a similar pattern where at those concentrations the gene expression contradicts the expected result. This could be related to the cytotoxic effect from the treatment, according to the preliminary study of cytotoxic analysis, the ethanolic extract at 25 µg/ml is quite toxic when compared with the 10 µg/ml and 5 µg/ml. Thus instead of treating the cells, the treatment at 25 µg/ml could be toxic to the cell, altering the gene expression. However this finding requires more in-deep analysis to prove the correlation of cytotoxic activity and gene expression result. Meanwhile the 2.5 µg/ml concentration might be too low thus the treatment could not work properly thus the outcome is not as expected.

Chapter V

CONCLUSION

In conclusion, this study has shown eight gene expressions resulting from inflammatory and skin barrier genes. Although half of the primer was nonspecific (CTACK, IL-25, TARC, IVL), the rest of the specific primers (IL-33, MDC, TSLP, FLG, GAPDH) shows a good and promising result that indicates ethanolic extract treatments of 5 µg/ml and 10 µg/ml concentration as the most promising potential for treating AD. Besides that, this study also shows that treatment of 2.5 µg/ml and 25 µg/ml concentration is unsuitable for treating AD. Unfortunately, further optimization on the agarose gel and primer cannot be performed due to time limitations, resulting in unsatisfactory results. Therefore, it is highly recommended to dedicate a specific time to optimizing the machine, method, and kit used for further experiments.

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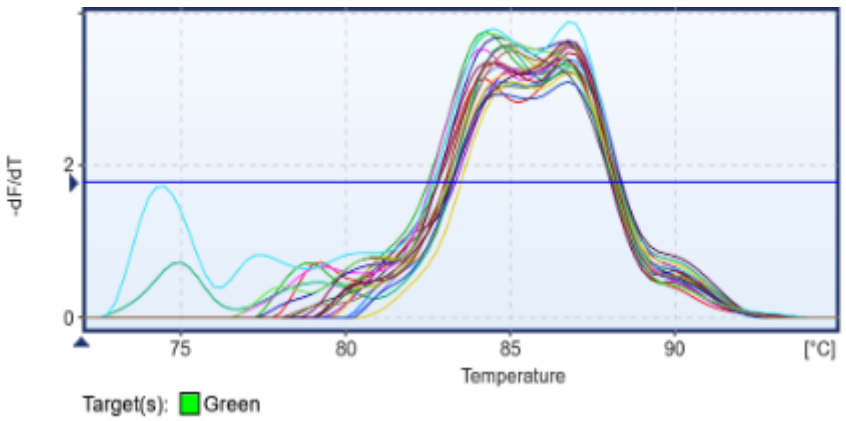
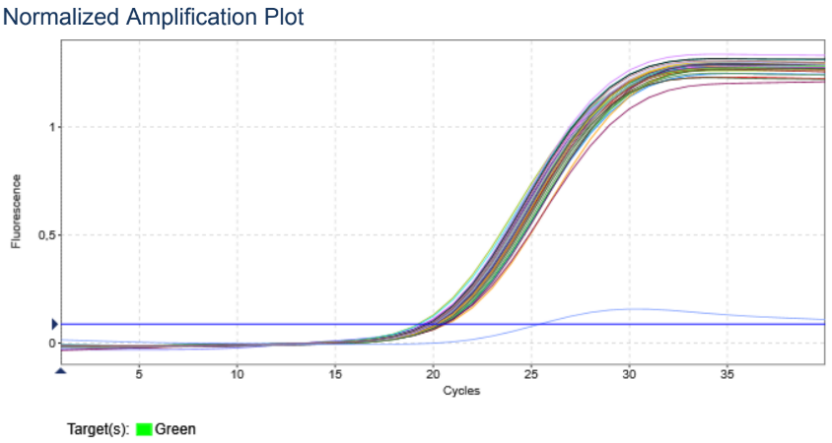
APPENDIX



Appendix 1. CTACK Melting Curve

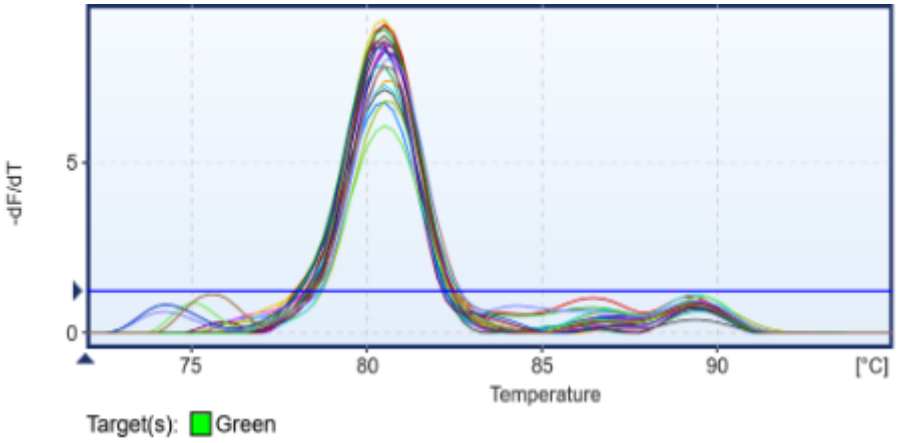


Appendix 2. CTACK Amplification Plot

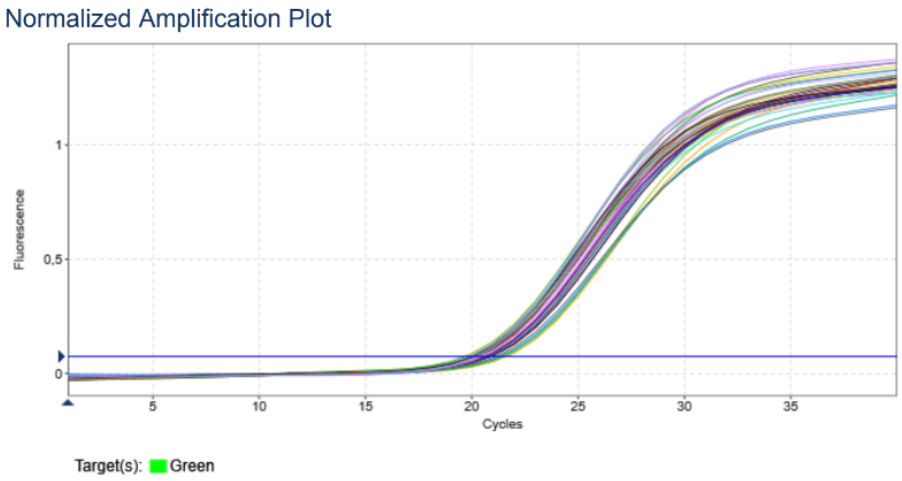
Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	CTACK	GAPDH			
Blank control 1	19.95		1.787	-0.66	1.58
Blank control 2	19.53	18.163	1.37	-1.08	2.12
Blank control 3	19.83		1.67	-0.78	1.72
Negative control 1	20.48		2.49	0.04	0.97
Negative control 2	20.16	17.993	2.17	-0.28	1.22
Negative control 3	20.69		2.70	0.25	0.84
25 - 1	19.25		1.797	-0.653	1.57
25 - 2	19.33	17.453	1.877	-0.573	1.49
25 - 3	19.6		2.147	-0.303	1.23

10 - 1	19.95		2.773	0.323	0.80
10 - 2	19.95	17.177	2.773	0.323	0.80
10 - 3	20.51		3.333	0.883	0.54
5 - 1	19.9		3.473	1.023	0.49
5 - 2	20.1	16.427	3.673	1.223	0.43
5 - 3	20.13		3.703	1.253	0.42
2.5 - 1	19.69		2.200	-0.250	1.19
2.5 - 2	20.6	17.490	3.110	0.660	0.63
2.5 - 3	20.21		2.720	0.270	0.83
Calibrator (average ΔCt control group)	2.45				

Appendix 3. CTACK Raw Data



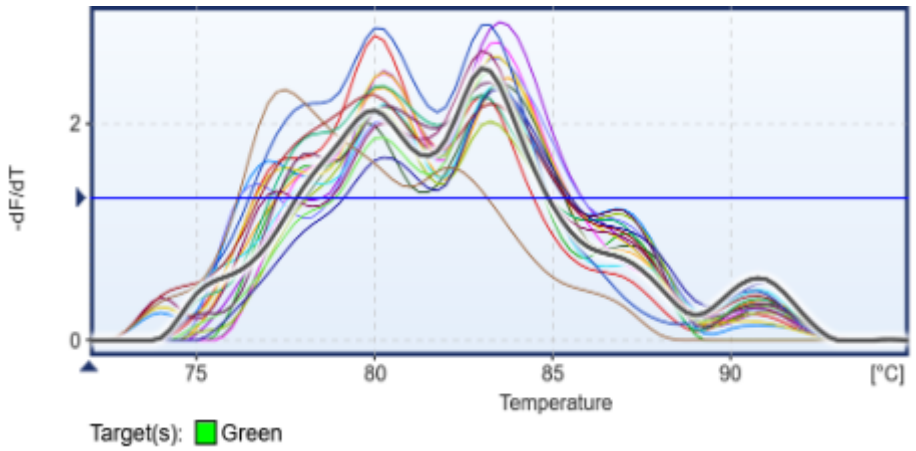
Appendix 4. FLG Melting Curve



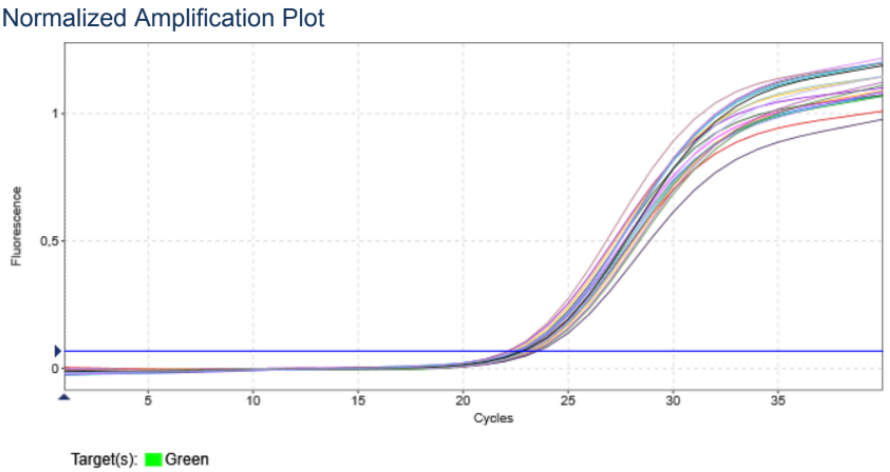
Appendix 5. FLG Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	FLG	GAPDH			
Blank control 1	20		1.837	-1.96	3.88
Blank control 2	20.67	18.163	2.51	-1.29	2.44
Blank control 3	20.1		1.94	-1.86	3.62
Negative control 1	22.11		4.12	0.32	0.80
Negative control 2	21.67	17.993	3.68	-0.12	1.08
Negative control 3	21.58		3.59	-0.21	1.15
25 - 1	21.63		4.177	0.383	0.77
25 - 2	20.76	17.453	3.307	-0.487	1.40
25 - 3	20.89		3.437	-0.357	1.28
10 - 1	19.71		2.533	-1.260	2.39
10 - 2	19.8	17.177	2.623	-1.170	2.25
10 - 3	19.6		2.423	-1.370	2.58
5 - 1	18.6		2.173	-1.620	3.07
5 - 2	18.7	16.427	2.273	-1.520	2.87
5 - 3	18.74		2.313	-1.480	2.79
2.5 - 1	20.95		3.460	-0.333	1.26
2.5 - 2	20.9	17.490	3.410	-0.383	1.30
2.5 - 3	19.99		2.500	-1.293	2.45
Calibrator (average ΔCt control group)	3.79				

Appendix 6. FLG Raw Data



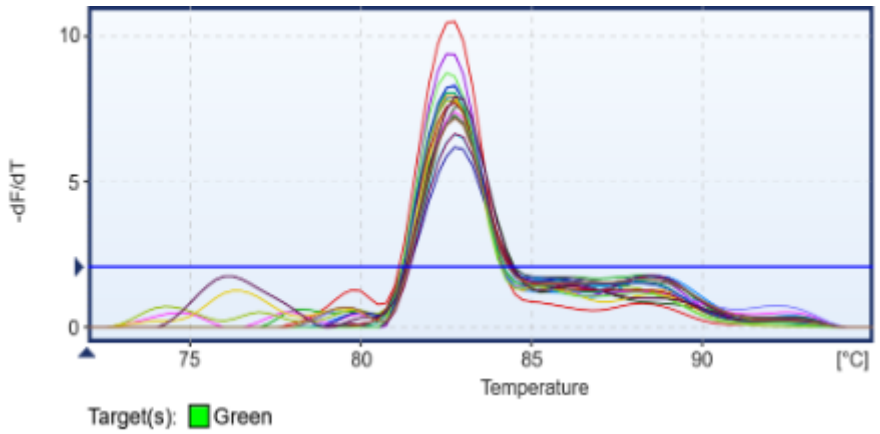
Appendix 7. IL-25 Melting Curve



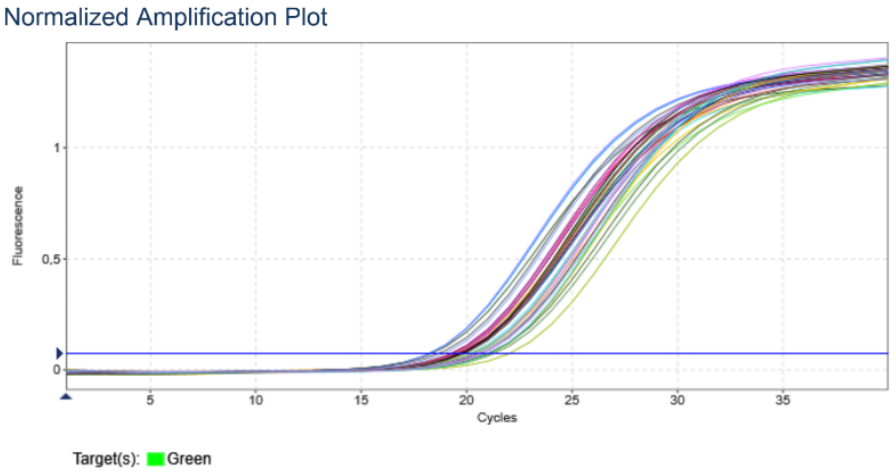
Appendix 8. IL-25 Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	IL-25	GAPDH			
Blank control 1	22.58		4.417	-0.37	1.30
Blank control 2	22.78	18.163	4.62	-0.17	1.13
Blank control 3	22.7		4.54	-0.25	1.19
Negative control 1	22.93		4.94	0.15	0.90
Negative control 2	22.19	17.993	4.20	-0.59	1.51
Negative control 3	23.23		5.24	0.45	0.73
25 - 1	23.35		5.897	1.107	0.46
25 - 2	22.94	17.453	5.487	0.697	0.62
25 - 3	22.72		5.267	0.477	0.72
10 - 1	22.9		5.723	0.933	0.52
10 - 2	23.26	17.177	6.083	1.293	0.41
10 - 3	22.44		5.263	0.473	0.72
5 - 1	22.81		6.383	1.593	0.33
5 - 2	23.33	16.427	6.903	2.113	0.23
5 - 3	23.21		6.783	1.993	0.25
2.5 - 1	22.67		5.180	0.390	0.76
2.5 - 2	23.35	17.490	5.860	1.070	0.48
2.5 - 3	24.49		7.000	2.210	0.22
Calibrator (average ΔCt control group)	4.79				

Appendix 9. IL-25 Raw Data



Appendix 10. IL-33 Melting Curve

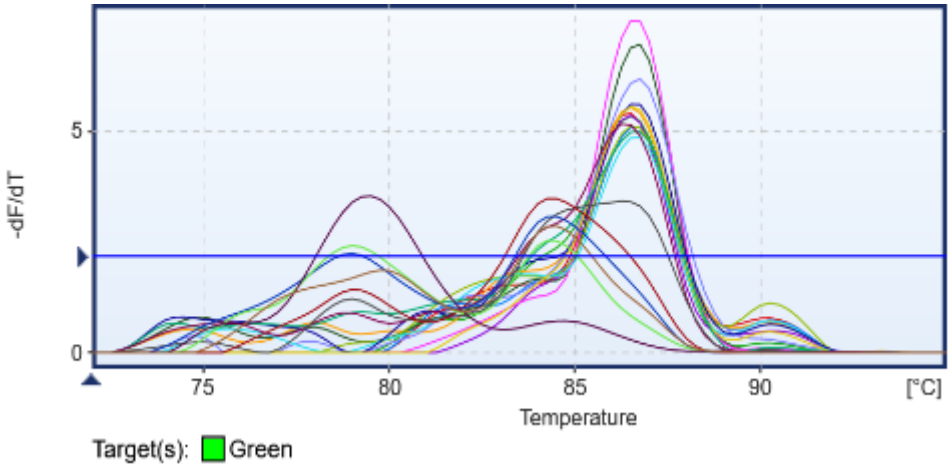


Appendix 11. IL-33 Amplification Plot

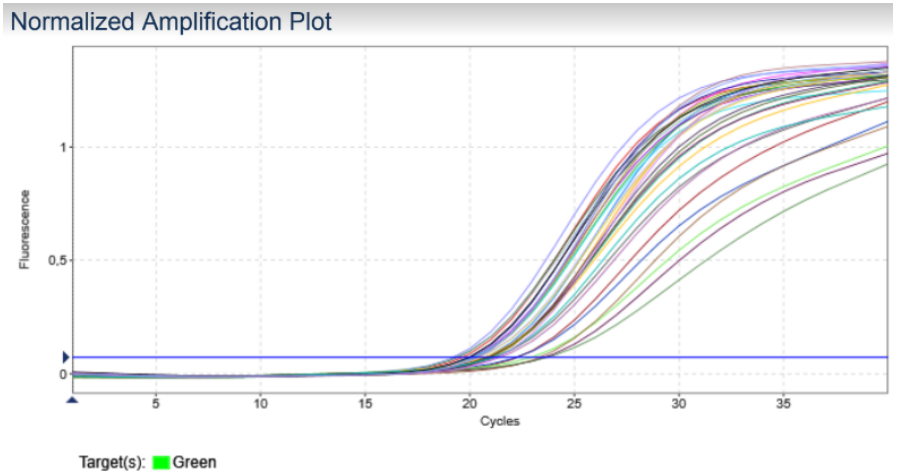
Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	IL-33	GAPDH			
Blank control 1	20.70		2.54	1.79	0.29
Blank control 2	20.36	18.163	2.20	1.45	0.37
Blank control 3	20.52		2.36	1.61	0.33
Negative control 1	18.35		0.36	-0.39	1.31
Negative control 2	19.59	17.993	1.60	0.85	0.56
Negative control 3	18.29		0.30	-0.45	1.37
25 - 1	22.04		4.587	3.837	0.07
25 - 2	20.17	17.453	2.717	1.967	0.26
25 - 3	19.89		2.437	1.687	0.31

10 - 1	20.24		3.063	2.313	0.20
10 - 2	19.9	17.177	2.723	1.973	0.25
10 - 3	19.4		2.223	1.473	0.36
5 - 1	18.69		2.263	1.513	0.35
5 - 2	19.61	16.427	3.183	2.433	0.19
5 - 3	19.76		3.333	2.583	0.17
2.5 - 1	20.41		2.920	2.170	0.22
2.5 - 2	19.79	17.490	2.300	1.550	0.34
2.5 - 3	19.93		2.440	1.690	0.31
Calibrator (average ΔCt control group)	0.75				

Appendix 12. IL-33 Raw Data



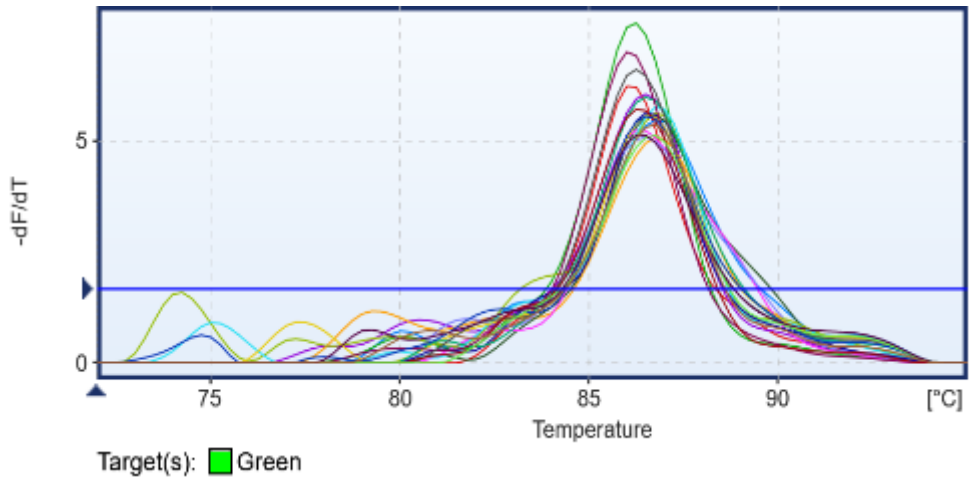
Appendix 13. IVL Melting Curve



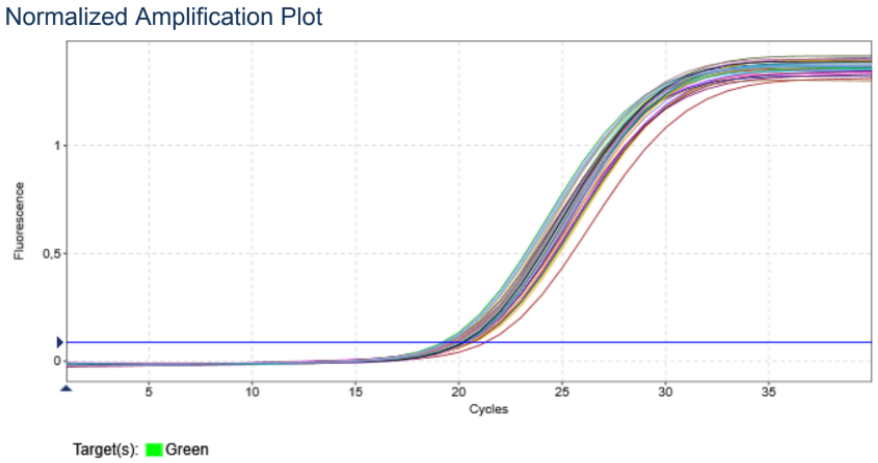
Appendix 14. IVL Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	IVL	GAPDH			
Blank control 1	19.42		1.257	-1.26	2.39
Blank control 2	20.26	18.163	2.10	-0.42	1.33
Blank control 3	19.23		1.07	-1.45	2.73
Negative control 1	20.27		2.28	-0.24	1.18
Negative control 2	20.15	17.993	2.16	-0.36	1.28
Negative control 3	21.1		3.11	0.59	0.66
25 - 1	20.33		2.877	0.363	0.78
25 - 2	20.17	17.453	2.717	0.203	0.87
25 - 3	20		2.547	0.033	0.98
10 - 1	20.42		3.243	0.730	0.60
10 - 2	20.84	17.177	3.663	1.150	0.45
10 - 3	20.77		3.593	1.080	0.47
5 - 1	21.21		4.783	2.270	0.21
5 - 2	22.29	16.427	5.863	3.350	0.10
5 - 3	22.24		5.813	3.300	0.10
2.5 - 1	22.95		5.460	2.947	0.13
2.5 - 2	23.59	17.490	6.100	3.587	0.08
2.5 - 3	23.35		5.860	3.347	0.10
Calibrator (average ΔCt control group)	2.51				

Appendix 15. IVL Raw Data



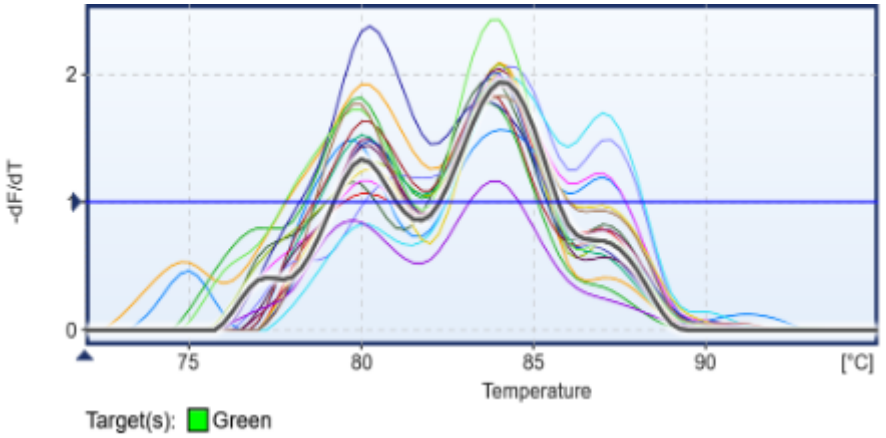
Appendix 16. MDC Melting Curve



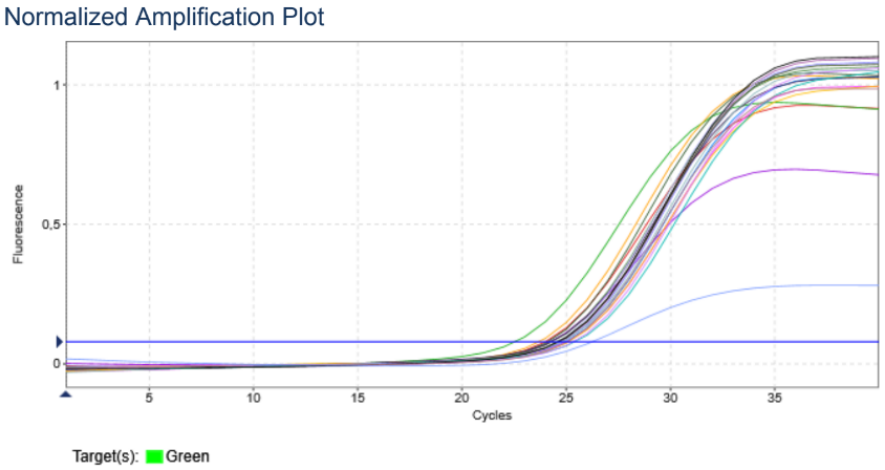
Appendix 17. MDC Amplification Plot

Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	MDC	GAPDH			
Blank control 1	21.61		3.45	1.45	0.37
Blank control 2	22.44	18.163	4.28	2.28	0.21
Blank control 3	21.73		3.57	1.57	0.34
Negative control 1	19.76		1.77	-0.23	1.17
Negative control 2	19.78	17.993	1.79	-0.21	1.15
Negative control 3	20.42		2.43	0.43	0.74
25 - 1	19.85		2.397	0.403	0.76
25 - 2	19.4	17.453	1.947	-0.047	1.03
25 - 3	19.5		2.047	0.053	0.96
10 - 1	20.5		3.323	1.330	0.40
10 - 2	20.38	17.177	3.203	1.210	0.43
10 - 3	20.33		3.153	1.160	0.45
5 - 1	20.25		3.823	1.830	0.28
5 - 2	21.31	16.427	4.883	2.890	0.13
5 - 3	19.23		2.803	0.810	0.57
2.5 - 1	19.21		1.720	-0.273	1.21
2.5 - 2	20.62	17.490	3.130	1.137	0.45
2.5 - 3	19.56		2.070	0.077	0.95
Calibrator (average ΔCt control group)	1.99				

Appendix 18. MDC Raw Data



Appendix 19. TARC Melting Curve

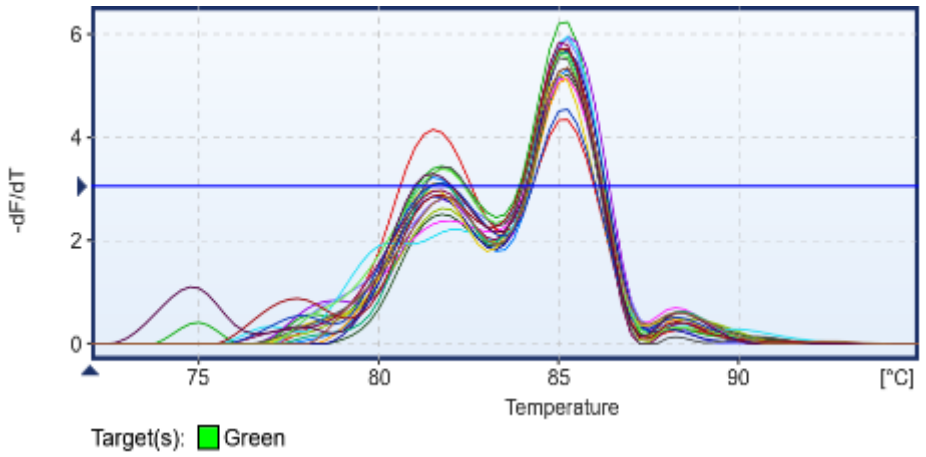


Appendix 20. TARC Amplification Plot

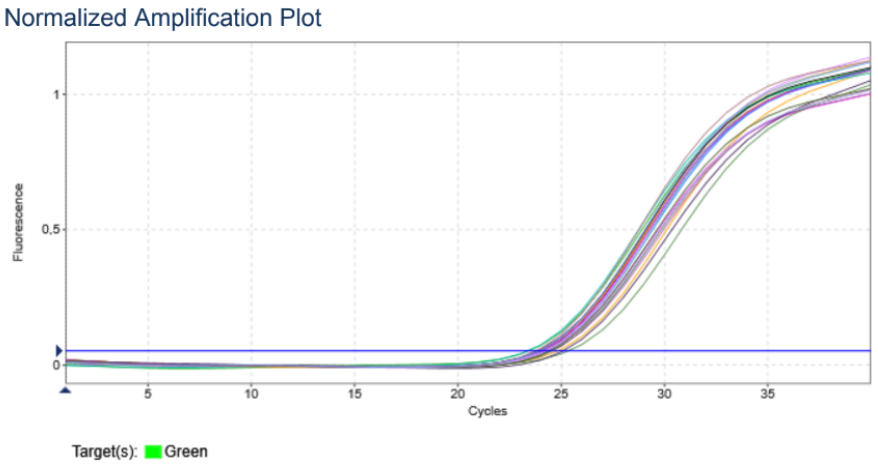
Treatment	Target Gene Ct	Housekeeping Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
	TARC	GAPDH			
Blank control 1	24.12		5.957	0.43	0.74
Blank control 2	25.04	18.163	6.88	1.35	0.39
Blank control 3	24.86		6.70	1.17	0.44
Negative control 1	22.57		4.58	-0.95	1.93
Negative control 2	24.28	17.993	6.29	0.76	0.59
Negative control 3	23.71		5.72	0.19	0.88
25 - 1	23.42		5.967	0.440	0.74
25 - 2	24.87	17.453	7.417	1.890	0.27
25 - 3	24.09		6.637	1.110	0.46

10 - 1	24.79		7.613	2.087	0.24
10 - 2	23.81	17.177	6.633	1.107	0.46
10 - 3	24.11		6.933	1.407	0.38
5 - 1	25.05		8.623	3.097	0.12
5 - 2	24.37	16.427	7.943	2.417	0.19
5 - 3	23.33		6.903	1.377	0.39
2.5 - 1	23.94		6.450	0.923	0.53
2.5 - 2	23.87	17.490	6.380	0.853	0.55
2.5 - 3	24.96		7.470	1.943	0.26
Calibrator (average ΔCt control group)	5.53				

Appendix 21. TARC Raw Data



Appendix 22. TSLP Melting Curve

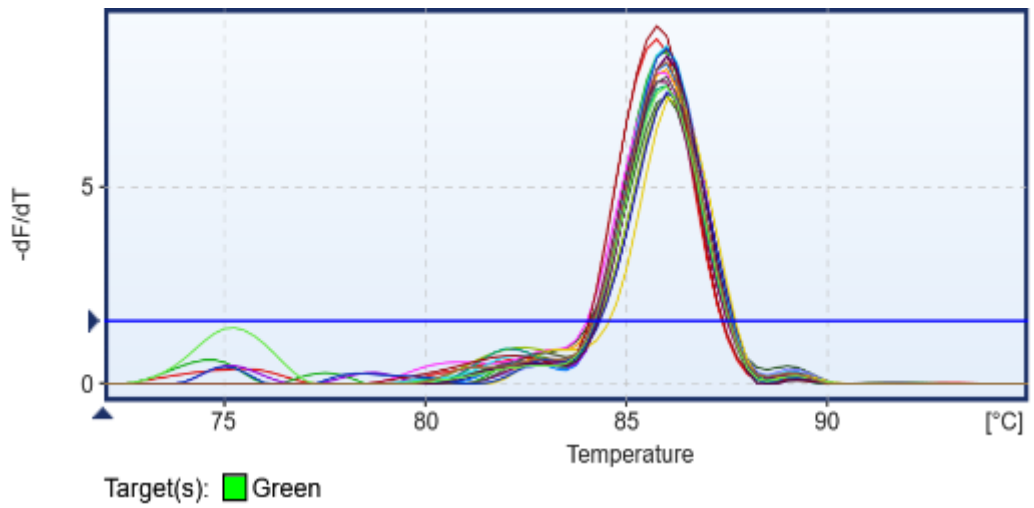


Appendix 23. TSLP Amplification Plot

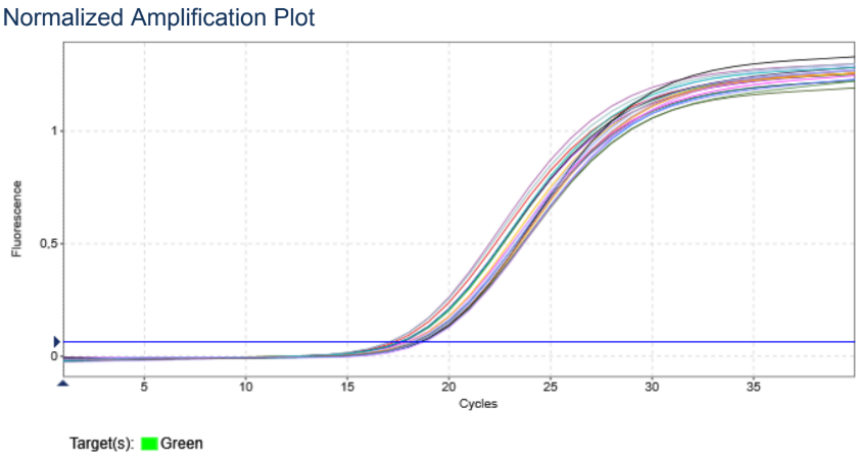
Treatment	Target	Housekeeping	ΔCt	ΔΔCt	2^-(ΔΔCt)

	Gene Ct	Ct			
	TSLP	GAPDH			
Blank control 1	24.58		6.417	0.96	0.51
Blank control 2	24.33	18.163	6.17	0.71	0.61
Blank control 3	23.86		5.70	0.24	0.84
Negative control 1	23.44		5.45	-0.01	1.00
Negative control 2	23.05	17.993	5.06	-0.40	1.32
Negative control 3	23.85		5.86	0.40	0.76
25 - 1	23.8		6.347	0.893	0.54
25 - 2	23.35	17.453	5.897	0.443	0.74
25 - 3	23.87		6.417	0.963	0.51
10 - 1	24.03		6.853	1.400	0.38
10 - 2	23.89	17.177	6.713	1.260	0.42
10 - 3	23.54		6.363	0.910	0.53
5 - 1	24.15		7.723	2.270	0.21
5 - 2	24.26	16.427	7.833	2.380	0.19
5 - 3	24.21		7.783	2.330	0.20
2.5 - 1	23.85		6.360	0.907	0.53
2.5 - 2	24.82	17.490	7.330	1.877	0.27
2.5 - 3	25		7.510	2.057	0.24
Calibrator (average ΔCt control group)	5.45				

Appendix 24. TSLP Raw Data



Appendix 25. GAPDH Melting Curve



Appendix 26. GAPDH Amplification Plot