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In-vitro Anti-Inflammatory Evaluation of Trigonelline from Fenugreek Seed (Trigonella foenum-graecum) Extract in LPS-Induced RAW264.7 Cell

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Food Science & Nutrition

KRISTOPHE GRACIANTE WIDJAJA
21010118

PROF. TAKUYA SUGAHARA, Ph.D.(FIELD SUPERVISOR)
ELEONORA VALENTINA SODE MUDA, S.T.P., M.SC (EP SUPERVISOR)

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RESEARCH REPORT

In-vitro Anti-Inflammatory Evaluation of Trigonelline from Fenugreek Seed (*Trigonella foenum-graecum*) Extract in LPS-Induced RAW264.7 Cell

By
Kristophe Graciante Widjaja
21010118

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Research Project Supervisor: Eleonora Valentia Sode Muda, S.T.P., M.Sc

Research Project Field Supervisor: Prof. Takuya Sugahara, Ph.D.

Jakarta, Indonesia
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CERTIFICATE OF APPROVAL

I hereby submit the final draft of the EP Report as a requirement to participate in EP Final Presentation.

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Student Name: Kristophe Graciante Widjaja

Student ID: 21010118

Prepared by:

Approved by,

Field Supervisor	Research Project Supervisor
	
Prof. Takuya Sugahara, Ph.D.	Eleonora Valentia Sode Muda, S.T.P., M.Sc
Date: 20 December 2024	Date: 20 December 2024

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ABSTRACT

Chronic inflammation is a significant contributor to various non-communicable diseases (NCDs), and trigonelline has shown potential in modulating inflammatory responses by affecting cytokine production and gene expression. The study investigates the anti-inflammatory effects of trigonelline, a compound derived from fenugreek seeds, on LPS-induced RAW 264.7 macrophage cells. The primary objective is to evaluate how trigonelline influences the production of pro-inflammatory cytokines such as TNF-α and IL-6, and to assess its impact on the expression of related genes using techniques like ELISA and RT-qPCR. The hypothesis posits that trigonelline can suppress inflammatory cytokines while promoting anti-inflammatory gene expression, suggesting its potential as a therapeutic agent for inflammation-related conditions. This research contributes to the understanding of natural compounds in managing inflammation and their implications for health.

Keywords: *Anti-inflammatory, cytokines, fenugreek seed, IL-6, RAW 264.7 cells, TNF-α, trigonelline.*

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LIST OF ABBREVIATIONS

WHO - World Health Organization
NCD - Non-communicable Disease
GRAS - Generally Recognized As Safe
TNF-α - Tumor Necrosis Factor-α
IL-6 - Interleukin-6
iNOS - Inducible Nitric Oxide Synthase
ELISA - Enzyme-Linked Immunosorbent Assay
IL-1β - interleukin-1β
TLRs - Toll-like receptors
IL-1R - IL-1 receptor
IL-6R - IL-6 receptor
TNFR - TNF receptor
NF-κB - Nuclear Factor Kappa-B
MAPK - Mitogen-activated protein kinase
JAK-signal - Janus Kinase

I. INTRODUCTION

1.1. Background

According to the World Health Organization (2024), the term "non-communicable disease" (NCD) refers to any illness that cannot be spread directly from one person to another. Chronic conditions often arise from a combination of factors, including lifestyle choices, biological influences, environmental factors, and genetic predispositions. Cytokines can either stimulate or inhibit inflammatory processes, making them essential for controlling inflammation. Pro-inflammatory cytokine levels are frequently shown to be high in the context of NCDs, which contributes to the pathophysiology of these conditions. For instance, low-grade chronic inflammation, which is typified by elevated production of cytokines like TNF-α and IL-6, is a feature of diseases like obesity. These cytokines increase the chance of acquiring NCDs by causing insulin resistance and other metabolic abnormalities (Hernández-Aguilera et al., 2013). It is said that non-communicable disease can be the result of inflammation inside the body which disrupts the body's immune system.

There is a strong link between the onset of NCDs and chronic inflammation, according to recent studies. The persistent production of inflammatory cytokines like TNF-α and IL-6, which can cause cellular damage and dysfunction, is a hallmark of chronic inflammation. Lifestyle factors including stress, a poor diet, and inactivity frequently cause an inflammatory state (Yu et al., 2024). Moreover, the body's immune system's intricate biochemical reaction to viruses, damaged cells, or irritants is inflammation. It acts as a protective mechanism designed to eliminate the underlying causes of cellular damage, clear away injured cells and tissues, and initiate the repair process. (Roy et al., 2016). Inflammations are divided into 2 types which are acute inflammation and chronic inflammation. Factors in which inflammation may occur includes pathogen, physical injury, chemical irritants, radiation, autoimmune disorder and chronic diseases (Chen et al., 2018). One of the substances that may have a potential to be an anti-inflammatory substance is trigonelline. Thus, trigonelline has emerged as a viable option in the numerous research that have explored the discovery of novel compounds to regulate inflammation through food sources. Coffee and fenugreek seeds contain the plant alkaloid trigonelline, which has drawn interest due to its possible anti-inflammatory qualities.

Trigonelline is a plant hormone that helps plants survive and thrive by regulating a variety of processes, including nodulation, oxidative stress, and the plant cell cycle. Numerous plants, such as fenugreek seeds and coffee beans, have large amounts of it. Trigonelline, named for its initial extraction from fenugreek seeds, is also found in various other plants, including coffee beans, garden peas, hemp seeds, and oats. Notably, coffee and fenugreek contain trigonelline in concentrations exceeding 1000 ppm (Zhou & Zhou, 2022). Research has indicated that trigonelline can help reduce diabetic auditory neuropathy and inhibit platelet aggregation. Additionally, it exhibits a range of beneficial effects, including hypoglycemic, hypolipidemic, neuroprotective, antimigraine, sedative, cognitive-enhancing, antibacterial, antiviral, and anti-cancer properties. Reactive oxygen species, β cell regeneration, insulin secretion, glucose metabolism-related enzyme activity, axonal extension, and neuron excitability are all impacted by it. However, more research into the precise mechanism and pharmacological actions of trigonelline is necessary, as is the application of this knowledge to its clinical use (Zhou et al., 2012). More specifically in the inhibition of proinflammatory cytokine and gene expression value. The FDA has classified trigonelline as GRAS (Generally Recognized as Safe). The body is thought to be better equipped to convert the new molecule back into the active, parent medicine because it is a naturally occurring metabolite found in the plants.

1.2. Objective

The main goal of this study is to investigate the anti-inflammatory properties of an aqueous trigonelline solution on LPS-stimulated RAW 264.7 macrophage cells. The evaluation will concentrate on two critical aspects: the secretion of cytokines and the expression of particular genes linked to inflammatory processes. Through this analysis, the research seeks to clarify the potential therapeutic advantages of trigonelline in reducing inflammation at the cellular level.

1.3. Hypothesis

This study hypothesizes that trigonelline has a twofold anti-inflammatory effect by inhibiting the synthesis of pro-inflammatory cytokines while enhancing the expression of anti-inflammatory genes in LPS-activated RAW 264.7 cells. This indicates that trigonelline could be a promising therapeutic agent for regulating inflammatory responses at both the cytokine and genetic levels.

1.4. Scope of Research

This study's scope includes assessing TNF-α and IL-6 protein expression using ELISA, measuring gene expression using RT-qPCR, and testing cell viability using the WST-8 reagent assay to gauge the sample's cytotoxicity. For the ELISA, it is expected that the higher the concentration of trigonelline treated to the cells, the lower the cytokine production will be and the cytokine production will increase as the depletion of the trigonelline concentration. Proinflammatory cytokines, such TNF-α and IL-6, are tiny proteins that cells make during an inflammatory response to control inflammation. The production of proinflammatory cytokines TNF-α and IL-6 was assessed during the incubation period in order to observe the sample's anti-inflammatory effects. Furthermore, β-actin, TNF-α, IL-6 and iNOS genes will be observed for its gene expression in-response towards the trigonelline treatment.

II. LITERATURE REVIEW

2.1. Inflammatory Response

An essential biological mechanism, the inflammatory response protects the body from viruses, damaged cells, and irritants, among other dangerous stimuli. This reaction is typified by a number of physiological alterations meant to identify and eradicate the harmful substance and start the healing process (Britannica, 2024). The most well known cytokines which are responsible for inflammation are IL-6 and TNF-a. Both play a crucial role in regulating the inflammation which can be caused by harmful stimuli such as pathogens, damaged cells, or irritants. It is known that the body has its own mechanism to combat the inflammation response (Pahwa, 2023). Inflammatory pathways involve various common mediators and regulatory mechanisms that influence the pathophysiology of numerous chronic diseases. Inflammatory stimuli activate intracellular signaling pathways, which subsequently lead to the production of inflammatory mediators. Key inflammatory stimuli, such as microbial products and cytokines like interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), engage with toll-like receptors (TLRs), the IL-1 receptor (IL-1R), the IL-6 receptor (IL-6R), and the TNF receptor (TNFR) to facilitate inflammation. This receptor activation triggers significant intracellular signaling pathways, including the nuclear factor kappa-B (NF-κB), mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways (Hendrayani et al., 2016).

By producing nitric oxide (NO), a signaling molecule involved in numerous physiological and pathological processes, the enzyme inducible nitric oxide synthase (iNOS) plays a crucial part in the inflammatory response. Inducible nitric oxide synthase (iNOS) is typically not expressed in resting cells but is activated during inflammatory responses due to the influence of pro-inflammatory cytokines and microbial metabolites. Nitric oxide (NO) is recognized for its antibacterial properties, contributing to the elimination of pathogens. However, excessive or sustained production of NO can lead to tissue damage and worsen chronic inflammatory conditions such as autoimmune diseases and psoriasis (Suschek et al., 2004). According to a study conducted by Zamora et al (2000), high NO levels may contribute to preventive processes, such as the control of gene expression that reduces inflammatory responses, even if they can also be harmful and encourage inflammation, according to research. Furthermore, iNOS is involved in a variety of cell types, such as macrophages and skeletal muscle cells, where it can affect both inflammatory and apoptotic processes, emphasizing its complicated role in inflammation.

Interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-alpha (TNF-α) are important mediators of the inflammatory response that have a big impact on a lot of different disorders. The pro-inflammatory cytokine TNF-α significantly impacts insulin resistance, vascular dysfunction, and chronic inflammatory conditions such as autoimmune disorders and cardiovascular diseases. Elevated levels of TNF-α are associated with negative cardiac remodeling and increased oxidative stress (Cicha et al., 2015; Patsalos et al., 2020). Interleukin-6 (IL-6) exhibits both pro-inflammatory and anti-inflammatory properties, playing a crucial role in immune regulation and metabolic processes. It serves as a biomarker for disease severity in cases like sepsis and trauma, while its influence on insulin sensitivity is complex and context-dependent. Inducible nitric oxide synthase (iNOS) produces nitric oxide (NO), which is vital for neurotransmission and vasodilation; however, excessive NO production during inflammation can lead to tissue damage. The association between heightened iNOS expression and increased pain severity

underscores its role in modulating the effects of other cytokines on vascular function and pain perception, particularly in chronic pain conditions such as diabetic neuropathy (You et al., 2021).

2.2. Trigonelline

Costa et al (2020) examines trigonelline's potential to reduce obesity-related problems by concentrating on how it affects oxidative stress, chronic inflammation, and advanced glycation end products (AGEs). Trigonelline treatment via yogurt was shown to decrease fat buildup in adipose tissue, increase insulin sensitivity and glucose tolerance, and exhibit both anti-inflammatory and antiglycation characteristics in the study, which was carried out on mice fed a high-fat diet (HFD). Notably, trigonelline-enriched yogurt increased components linked to AGE detoxification while lowering levels of AGEs and AGE receptors in the liver and kidney of HFD mice. These findings suggest trigonelline's potential as a complementary therapy for liver and kidney complications associated with obesity. Interestingly, when combined with curcumin, many of trigonelline's beneficial effects were nullified, indicating that this combination may not be effective. This study underscores trigonelline's promise as an individual treatment for obesity-related metabolic disorders, highlighting its antioxidant, anti-inflammatory, and antiglycation properties, particularly in the context of liver and kidney health.

A research carried out by Hirakawa et al (2020), by using a transgenic mouse model of Alzheimer's disease (AD), the researcher examined the possible neuroprotective effects of trigonelline. For three months, AD model mice were given trigonelline orally, and several parameters pertaining to cognitive function and AD pathology were assessed. The AD mice's spatial learning and memory were found to be considerably enhanced by trigonelline treatment. The treated group also showed enhanced expression of proteins linked to synaptic plasticity, decreased neuroinflammation, decreased oxidative stress, and decreased amyloid-β plaque deposition in the brain. These results imply that trigonelline may function as a neuroprotective drug and have a variety of positive effects on AD pathogenesis. The study shows that trigonelline can control several parts of the complicated pathophysiology of Alzheimer's disease, indicating its potential as a candidate for additional research in the prevention or therapy of the disorder.

2.3. RAW264.7 Cell

The RAW 264.7 cell line, derived from the Abelson leukemia virus-transformed cells of BALB/c mice, serves as a widely recognized model for studying monocyte/macrophage biology (Fuentes et al., 2014). This murine macrophage cell line is particularly valuable for in vitro screening of immunomodulators. Compounds that significantly reduce the expression of nitric oxide (NO) or pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells are often considered potential candidates for developing anti-inflammatory therapies in humans (Elisia et al., 2018). One key advantage of using the RAW 264.7 cell line in immunological studies is its reproducibility and stability, which facilitate the generation of reliable data across experiments. The cell line exhibits a robust response to various stimuli, including LPS, which triggers substantial inflammatory reactions. This response can be quantitatively assessed by measuring the release of cytokines and other inflammatory markers (Jannus et al., 2021). Consequently, RAW 264.7 cells provide a consistent platform for exploring immune responses and evaluating the efficacy of novel therapeutic agents.

2.4. WST-8 Cytotoxicity Assay

The WST-8 assay is a colorimetric technique widely used to evaluate cytotoxicity and cell viability. It operates on the principle that dehydrogenases in metabolically active cells reduce a tetrazolium salt, WST-8, to formazan. This reaction allows researchers to measure cell viability following exposure to various treatments or conditions, as the amount of formazan generated is directly proportional to the number of viable cells. Specifically, tetrazolium salts are reduced by NAD(P)H to produce formazan, which serves as a reliable biomarker of cell viability and metabolic activity (Eder et al., 2022). Notably, the use of WST-8 for the quantitative assessment of dehydrogenase enzyme activity has not been previously described. In our study, we documented a novel assay for quantifying dehydrogenase activity based on WST-8. This assay is performed in a microplate format, with one endpoint evaluated at 450 nm (Chamchoy et al., 2019). The process primarily occurs in the mitochondria, making it particularly effective for assessing cell viability. Since WST-8 is reduced specifically by mitochondrial dehydrogenases, any disruption in mitochondrial function—often associated with cell death—results in a decreased synthesis of formazan. Thus, mitochondrial activity serves as a robust indicator of total cell viability in the context of the WST-8 assay (Agustina et al., 2024).

2.5. ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is a widely used analytical biochemistry technique that allows for the detection and quantification of proteins, hormones, antibodies, or antigens in a sample. Among the various ELISA formats, the sandwich ELISA is particularly notable for its sensitivity and specificity. This method is especially valuable in research and clinical diagnostics for measuring biomolecules like cytokines (Alhajj, 2023). When detecting the target antigen in an unknown sample, sandwich ELISAs work incredibly well. At least two antigenic sites that can bind two distinct antibodies must be present in the target protein. A microtiter plate is coated with a capture antibody that is particular to the target antigen in a sandwich ELISA. The antibody might stick to the plate because of hydrophobic interactions. The plate is then supplemented with the target antigen. A standard curve is produced in some wells by adding a specified quantity of antigen. The plate is filled with the enzyme-conjugated detection antibody. An unlabeled detection antibody and a conjugated antibody are applied to the plate in an indirect sandwich ELISA. To avoid cross-reactivity with the conjugated antibody in indirect ELISAs, the capture antibody and the detection antibody need to belong to separate species. The substrate is added for color development after the detecting antibody has been applied. Since two antibodies detect the target antigen, sandwich ELISAs are more sensitive than direct and indirect ELISA (Anagu & Andoh, 2022). Moreover, sandwich ELISA-detected elevated cytokine levels may be a sign of pathological disorders such autoimmune illnesses or persistent immune responses. On the other hand, soluble cytokine receptors detected using this technique might be indicators for the advancement of a disease or the effectiveness of a treatment. To sum up, sandwich ELISA is an effective method for identifying and measuring antigens such as cytokines, offering vital details on immunological reactions and possible disease conditions (Chiswick et al., 2012).

2.6. RT-qPCR

The most sensitive technique for measuring mRNA is RT-qPCR, which enables the identification of uncommon transcripts and the monitoring of minute changes in gene expression. Both absolute and relative mRNA quantification are possible with RT-qPCR. A calibration curve must be created using standards of known concentration in order to use absolute quantification, which provides the target mRNAs

exact copy number. Conversely, relative quantification uses an n-fold difference with respect to a calibrator to represent the target quantity for an experimental sample. This is the preferred technique for comparing variations in mRNA expression across various samples (Ho-Pun-Cheung et al., 2009). This method uses standards of known concentration to create a calibration curve that gives the precise copy number of target mRNAs. The quality and concentration of these standards, which might be recombinant DNA or purified RT-PCR products, are critical to the precision of absolute quantification (Nolan et al., 2006). Relative quantification, on the other hand, represents the target mRNA levels as an n-fold variation from a calibrator sample. When analyzing differences in mRNA expression between various samples or experimental circumstances, this approach is quite helpful. To guarantee accurate results, it must be carefully normalized against stable housekeeping genes (Costa et al., 2013).

III. MATERIALS & METHODS

3.1. Study Design

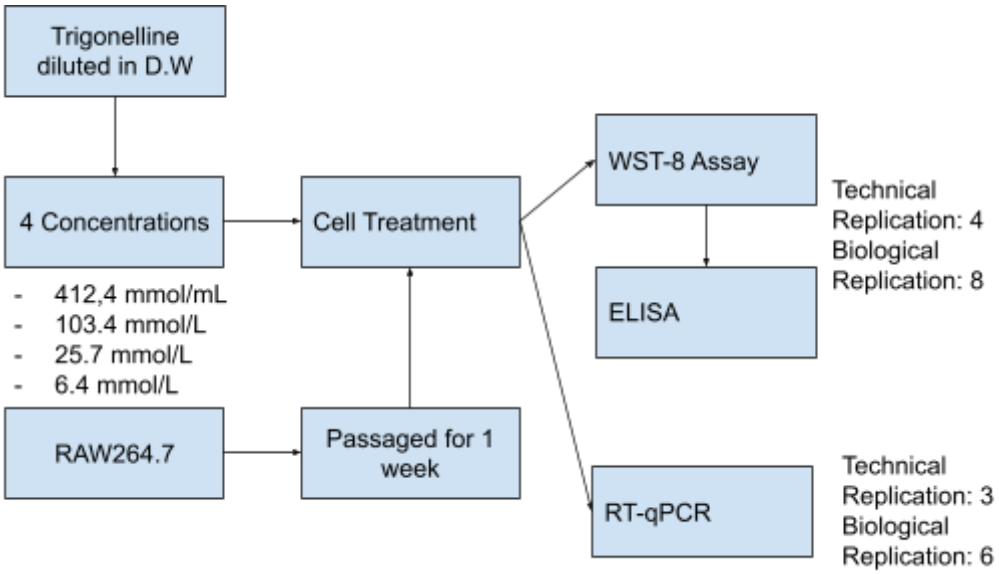

Figure 1. Experimental design of the study

3.2. Materials

In this experiment, the materials and reagents used were powdered extracted trigonelline, distilled water (D.W.), RAW 264.7 cell, DMEM media, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), Lipopolysaccharide (LPS), TNF-α ELISA kit, IL-6 ELISA kit, WST-8 reagent, Sepasol, 2-propanol, ethanol, DEPC water, 2.5mM dNTP, M-MLV RT 5x, RNase Inhibitor, M-MLV RT, MilliQ, forward primer, reverse primer, and SYBR Green mastermix.

3.3. Sample Stock Preparation

The trigonelline powder was prepared by adding 500 mg of trigonelline to a 15 mL conical tube and weighing it. Afterwards, the trigonelline was dissolved in 6.25 mL of distilled water. The pH was then measured, and adjustments were made as necessary. A total of 2.6 mL of 1M NaOH and 20 µL of 6M HCl were added to the trigonelline solution to achieve a pH in the range of 7-9. Furthermore, the sample was filtered using a syringe and a 2 µm pore filter. The final concentration of the stock solution was determined to be 56.5 mg/mL. Subsequently, the stock solution was transferred to new Falcon tubes in 1 mL volumes and stored in the refrigerator for further analysis. The dilution ratio for the stock solution was set at 1:4.

3.2. Cell Passaging and Treatment

Before being used in the experiment, the RAW 264.7 cell stock was thawed and kept for a week. 9.5 mL of warm PBS was prepared beforehand, and 500 µL of the cells were then added to the warmed PBS. The cells were then put into the cell count machine and the count was completed for approximately 2 minutes. The experiment involved seeding the cells in a 96-well plate with a cell count of 200 µl/well (cells/well) or cells/ml and a 48-well plate with a cell density of 1000 µl/well for ELISA and RT-qPCR, respectively. After

seeding, the plate was incubated at 37 degrees Celsius for 18 to 20 hours. The cell's passage number at which they were viable was 12 times of passage.

3.3. Cell Treatment

Following the incubation time, the cells were treated with four different concentrations of the sample in a 1:1 ratio of sample to 2x DMEM-20% FBS media after the old media was aspirated. To induce inflammation in the cells, 1 µg/ml of LPS was dissolved in the medium. The solution was applied at a volume of 200 µl/well. The same well was used for both the positive and negative control tests, and each sample concentration and control were repeated four times. After that, the cells were cultured for six hours. Following incubation, the surviving cells on the bottom of the plate were used for the WST-8 cytotoxicity test, and the supernatant was collected in a fresh plate for ELISA analysis.

2 x 10^5 cells/mL were thawed and placed into a 24-well plate at a volume of 1000 µl/well. The plate was incubated for 18 hours. Afterwards, 4 mL of a 20% FBS-2x DMEM solution was prepared, and 0.1% LPS was added to the solution to induce inflammation. The plate was then incubated for 6 hours. Following this, the 24-well plate was aspirated and washed with 1 mL of warm PBS, and it was aspirated again. Lastly, Sepasol was added to each well at a volume of 500 µl two times, and the mixture was transferred to PCR tubes and refrigerated at -80 degrees Celsius.

3.4. WST-8 Cytotoxicity Assay

Thirty minutes before use, the WST-8 and DMEM medium, which were the reagents used, were removed from the refrigerator to thaw. The cells were given 100 µl/well of 5% WST-8 in 2x DMEM medium. The absorbance values at 450 nm and 655 nm were then measured after the plate had been incubated for 45 minutes at 37°C.

3.5. ELISA

For the TNF-α and IL-6 analyses, two distinct ELISA plates were prepared. To obtain the standard curves, two repetitions of the standard experiment were performed on each plate. The maximum concentrations used were 500 pg/ml for IL-6 and 1000 pg/ml for TNF-α, which were diluted eight times with a dilution factor of 1. Initially, 100 µl/well of the coating solution (D.W., 1/20 coating buffer, and 1/500 capture antibody for TNF-α; coating buffer and 1/400 capture antibody for IL-6) was applied to the plates. The plates were incubated overnight. Before moving on to the next phase of the ELISA experiment, the plates were washed three times with PBS. Following the washing, 200 µl/well of the blocking solution (1% BSA-PBS for both TNF-α and IL-6) was added to each plate, and the plates were then incubated for an hour. After the incubation, the TNF-α sample supernatant was diluted 6 times for IL-6 and 9 times for TNF-α, and then added to each plate along with the standard solutions at a rate of 50 µl/well. After that, the plates were incubated for two hours. Following this, 100 µl of the second antibody solution (assay diluent and 1/500 detection antibody for TNF-α; 1% BSA-PBS and 1/400 detection antibody for IL-6) was added to each plate, and the plates were incubated for another hour. After adding 100 µl of the avidin/enzymatic solution per well, the plates were covered with aluminum foil and incubated for 30 minutes. After 30 minutes, each plate received 100 µl of the coloring solution per well, and the reaction was halted with 50 µl of H2SO4 when a noticeable color shift was observed. Finally, the absorbance value at 450 nm was measured for each plate.

3.6. RT-qPCR

The PCR tubes were thawed before use, and then chloroform was added at a ratio of 1/5 of Sepasol. The tubes were mixed vigorously by inverting them. After stabilizing for 3 minutes, the tubes were centrifuged at 11,200 rpm at 4 degrees Celsius for 15 minutes. Following the centrifugation, the supernatant was collected (450 µl) and transferred to new PCR tubes. An equal volume of 2-propanol (450 µl) was added to the collected supernatant and mixed slowly by inversion. The tubes were then left on ice for 10 minutes. Afterwards, they were centrifuged at 11,200 rpm at 4 degrees Celsius for 10 minutes. The samples were then decanted and left to air dry for approximately 10 minutes. After that, 500 µl of 75% ethanol was added twice to each PCR tube of treatments. The tubes were centrifuged again at 11,200 rpm at 4 degrees Celsius for 5 minutes. Finally, the tubes were air dried for 15 minutes, and 20 µl of MilliQ water was added before stabilizing them on ice for 15 minutes.

Then, the RNA purity was measured using the Colibri machine. The RNA concentration was adjusted to 100 µg/µL by using MilliQ water. 0.5 µL of oligo dT primer was added to each prepared tube, followed by the addition of 13.5 µL of the adjusted RNA solution to the new tube containing the oligo dT primer. Afterwards, the solution was heated in the Thermal Cycler at 70 degrees Celsius for 5 minutes. The solution was then prepared as shown in the following table:

Table 1. Reagents needed to create cDNA for RT-qPCR

2.5mM dNTP	5µL
M-MLV RT 5x	5µL
RNase Inhibitor	0.5µL
M-MLV RT	0.25µL
MilliQ	0.25µL
Total	**11.5µL**

The solution was added to the following PCR tubes after the thermal treatment and then spun down. Lastly, the PCR tubes were heated in the thermal cycler at 42 degrees Celsius for 60 minutes. Afterwards, the tubes were stored in the freezer, which contained the cDNA. The cDNA was diluted 30 times with MilliQ water and transferred to a new tube. Then, the primers were prepared and added to the well plate. The following table contained the primers and reagents used:

Table 2. Reagents and cDNA needed to prepare for RT-qPCR

cDNA	4µL
Forward Primer	0,5µL
Reverse Primer	0,5µL

SYBR Green Supermix	5µL
Total	**10µL**

Finally, the well plate was spun down for 5 minutes and inserted into the PCR Machine for 2 hours and 40 minutes.

3.7. Statistical Analysis

XLstat software was utilized to perform statistical analysis in this experiment. First, a normality test was performed on the data. The ANOVA Tukey-Kramer post-hoc test was used to determine whether there was a significant difference between each sample's data if the data was normally distributed. In the meanwhile, if the data is not normally distributed, the Kruskal Wallis nonparametric test was employed to ascertain whether each data set differed significantly.

IV. RESULTS

4.1. Correlation of Trigonelline Concentration and Cell Survivability

The study explores the effects of trigonelline on cell viability in the context of inflammation induced by lipopolysaccharide (LPS). In this experimental setup, cells are treated with LPS to simulate an inflammatory response, while also being exposed to trigonelline at four different concentrations. The experimental design includes two control groups: the negative control, which is not treated with either trigonelline or LPS, and the positive control, which is treated solely with LPS without trigonelline. To ensure the reliability of the data, all samples are prepared in quadruplicate, allowing for a more robust analysis of the results. This approach aims to elucidate trigonelline's potential therapeutic applications by assessing its impact on cell viability under inflammatory conditions.

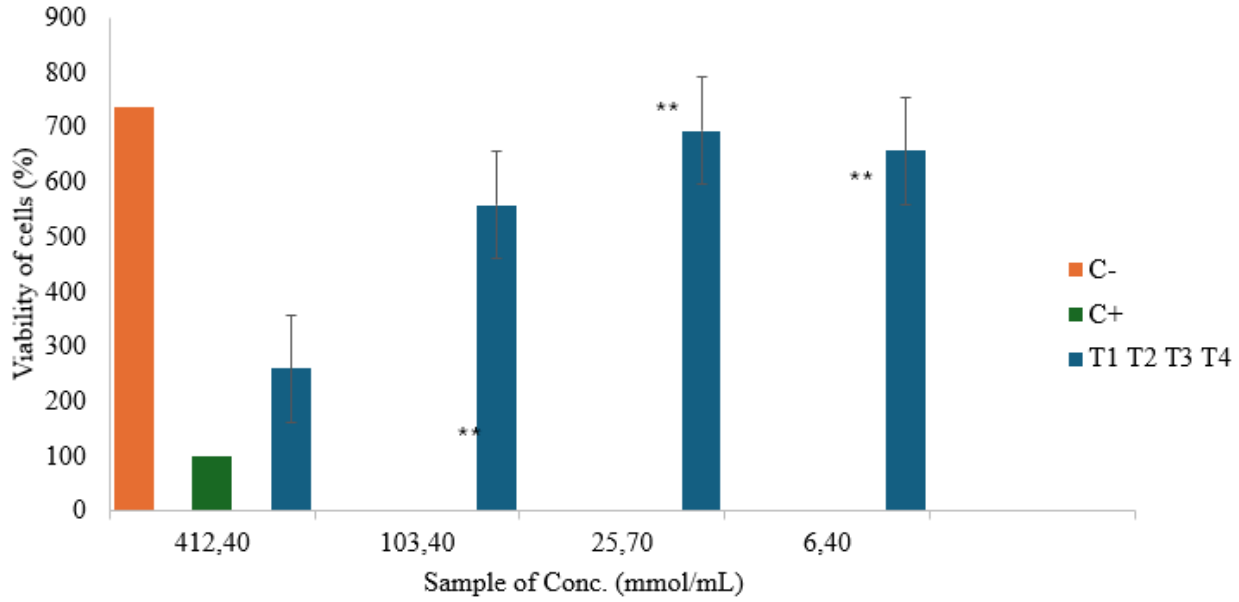

Figure 2. Effect of Trigonelline Concentration on Cell Viability. Each treatment is compared towards the control positive labeled in green. Error bars in the figure represent standard deviation (SD). Statistical significance is indicated with *p ≤ 0.05 and **p ≤ 0.01, highlighting the robustness of these findings.

The data presented in Figure 2 clearly illustrates the concentrations of trigonelline across various samples. Regarding cell viability, the negative control exhibits the highest viability at 73.8%, while the positive control shows a significantly lower viability of only 10%. Statistical analysis indicates significant differences between the concentrations of 412.4 mmol/L and 103.4 mmol/L. Additionally, both of these concentrations are significantly different from the 25.7 mmol/L concentration of trigonelline. Notably, the highest concentration (412.4 mmol/L) maintains a cell viability of 31.51%, which is acceptable for further experimentation as it exceeds the threshold of 80%. With the exception of this highest concentration, all other concentrations demonstrated cell viability levels above 80%, indicating their suitability for subsequent experiments.

4.2. Suppression of Cytokine Production

This research examines the role of trigonelline in cytokine production during inflammation induced by lipopolysaccharide (LPS). In this experimental framework, cells are treated with LPS to replicate an inflammatory response, leading to the production of cytokines such as TNF-α and IL-6. Concurrently, the cells receive trigonelline at four different concentrations. The experimental design incorporates two control groups: a negative control that is not exposed to either trigonelline or LPS, and a positive control that receives only LPS without trigonelline. To improve the reliability of the findings, all samples are prepared in quadruplicate, allowing for a more comprehensive analysis. This approach seeks to elucidate the therapeutic potential of trigonelline in modulating the inflammatory response.

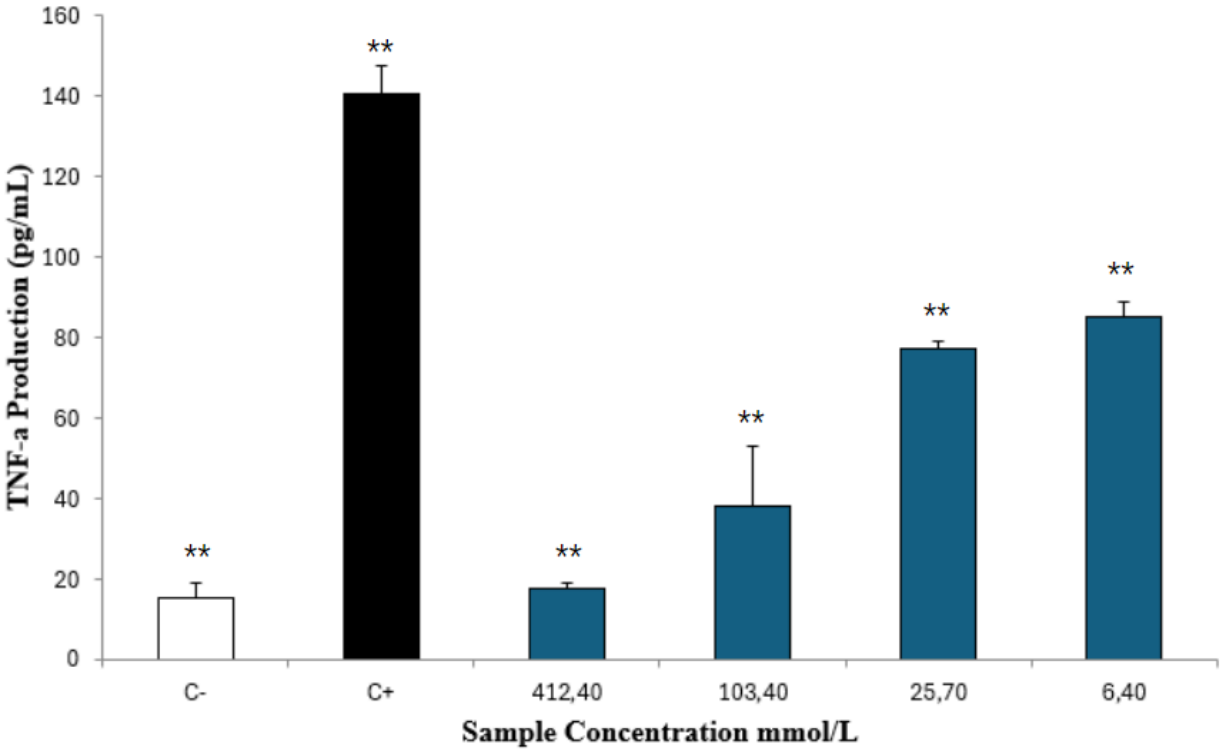

Figure 3. The Effect of Trigonelline Towards TNF-α Production. Each trigonelline concentration is evaluated relative to the positive control (black line), characterized by the highest cytokine levels, and the negative control, featuring the lowest cytokine values. Standard deviations are represented by error bars, and statistical significance is denoted by *p ≤ 0.05 and **p ≤ 0.01, confirming the reliability of our outcomes.

The results shown in Figure 3 reveal a distinct inverse relationship between trigonelline concentration and cytokine production, indicating that lower concentrations of trigonelline lead to increased cytokine levels. Statistical analysis demonstrates significant differences between the highest concentration of trigonelline (412.4 mmol/L) and the other groups, including 103.4 mmol/L, 25.7 mmol/L, and 6.4 mmol/L, as well as the negative control. These findings suggest that trigonelline effectively suppresses TNF-α production across all concentrations tested. Each concentration displayed significant differences from one another and from both the negative and positive controls. Overall, the data highlights trigonelline's potential as an anti-inflammatory agent by significantly reducing the production of pro-inflammatory cytokines.

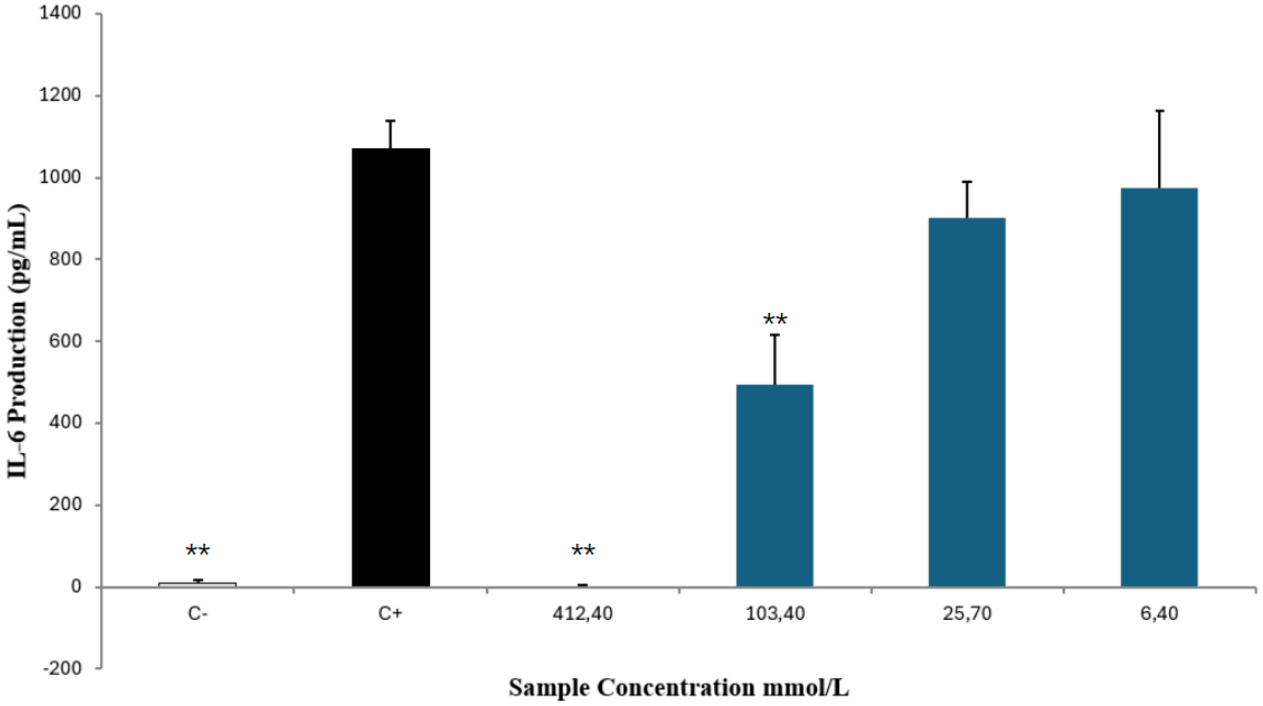

Figure 4. The Effect of Trigonelline Towards IL-6. Each trigonelline concentration is evaluated relative to the positive control (black line), characterized by the highest cytokine levels, and the negative control, featuring the lowest cytokine values. The graph's error bars denote standard deviation (SD), with statistical significance indicated by *p ≤ 0.05 and **p ≤ 0.01, underscoring the robustness of these results.

Figure 4 illustrates a clear inverse relationship between trigonelline concentration and cytokine production, indicating that as trigonelline levels decrease, cytokine levels increase. Statistical analysis reveals significant differences between the highest concentration of 412.4 mmol/L and the other groups, including 103.4 mmol/L and the negative control. These results suggest that trigonelline is effective in suppressing IL-6 production across all tested concentrations. Collectively, this data supports the idea that trigonelline may act as a potent anti-inflammatory agent by significantly reducing the production of pro-inflammatory cytokines.

4.3. Correlation of mRNA Expression with Trigonelline

This study investigates the impact of trigonelline on cytokine production during inflammation triggered by lipopolysaccharide (LPS). In this experiment, cells are treated with LPS to simulate an inflammatory response, resulting in the expression of genes encoding TNF-α, IL-6, and iNOS. Simultaneously, the cells are exposed to two different concentrations of trigonelline—412 mmol/L and 103.4 mmol/L—to assess its effects on cytokine production. The experimental design includes two control groups: a negative control that remains unexposed to both trigonelline and LPS, and a positive control that is treated with LPS alone without trigonelline. To enhance the accuracy of the results, all samples are prepared in triplicate, ensuring a more detailed analysis. This method aims to uncover the therapeutic potential of trigonelline in managing the inflammatory response by examining its ability to modulate cytokine production.

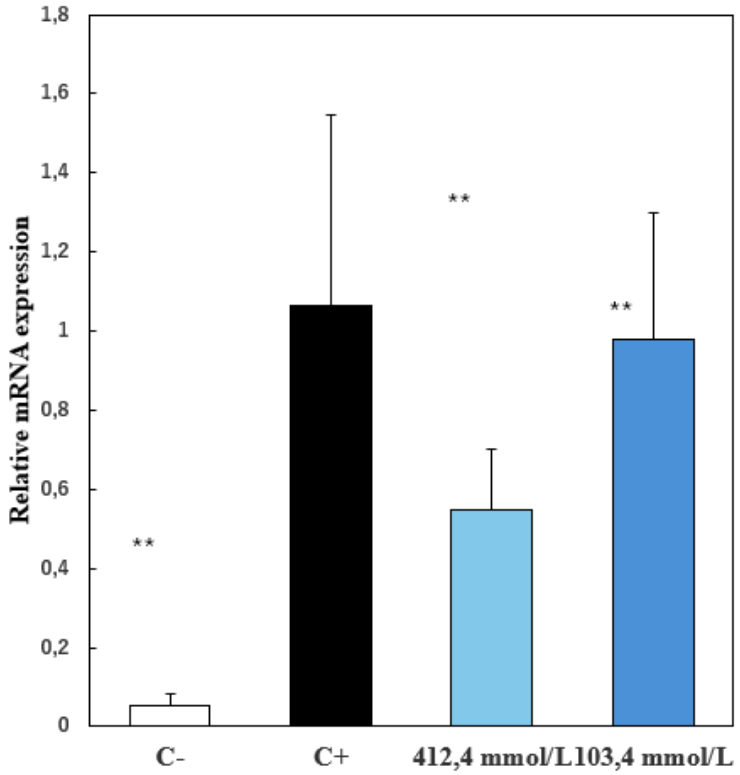

Figure 5. The Relative mRNA Expression of TNF-a Towards Trigonelline Treatment. Each concentration of samples are measured and compared towards the control positive which is the color black and control negative which is indicated by the color white. Error bars in the data represent standard deviation (SD), and statistical significance is denoted by *p ≤ 0.05 and **p ≤ 0.01.

The results indicate that the negative control exhibits the lowest mRNA expression, while the positive control shows the highest levels. Notably, the concentration of 412.4 mmol/L of trigonelline demonstrates lower mRNA expression compared to 103.4 mmol/L, with significant differences observed between these two concentrations as well as when compared to the negative control. These findings suggest that the reduction in mRNA expression may be attributed to the anti-inflammatory properties of trigonelline when applied to the RAW264.7 cell line.

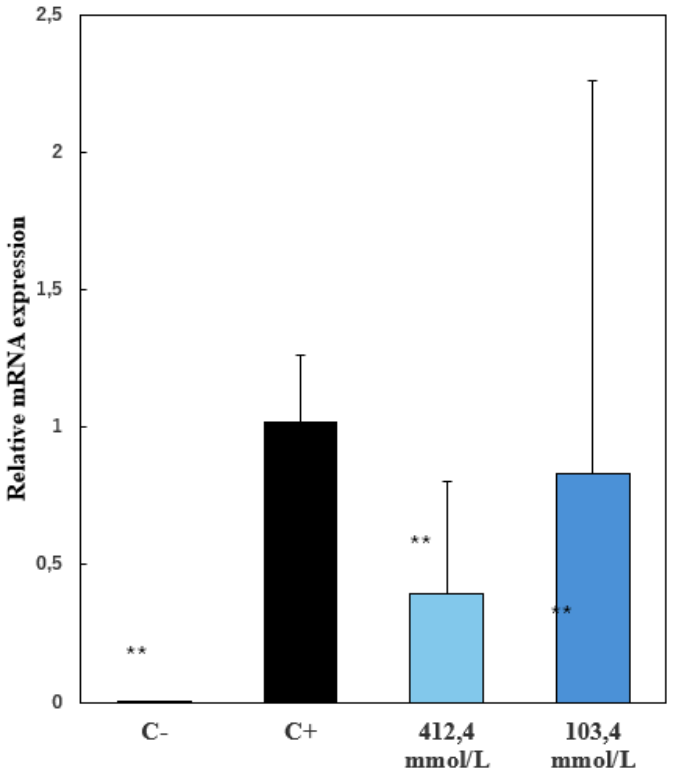

Figure 6. The Relative mRNA Expression of IL-6 Towards Trigonelline Treatment. Sample concentrations are assessed and compared against the positive control (shown in black) and the negative control (shown in white). The error bars represent standard deviation (SD), and statistical significance is indicated by *p ≤ 0.05 and **p ≤ 0.01, highlighting the strength of these results.

As depicted in Figure 5, trigonelline effectively inhibits the cytokine IL-6's mRNA expression. The treatment groups exhibit a significant decline in mRNA expression levels compared to the control positive group, which displays the highest values. Notably, there is no discernible IL-6 mRNA expression in the control sample. Conversely, the control negative is untreated with LPS, while the control positive is treated with LPS but lacks the trigonelline sample. Interestingly, there exists an inverse relationship where IL-6 mRNA synthesis increases as the treatment concentration decreases. This suggests that 412,4 mmol/L and 103,4 mmol/L of trigonelline, particularly at higher concentrations, are more effective in suppressing IL-6 expression. Significant differences exist between both trigonelline samples. These results align with previous research demonstrating the anti-inflammatory properties of trigonelline, which includes the suppression of inflammatory cytokines like TNF-α and IL-6. The mechanisms behind this action involve the modulation of signaling pathways such as NF-κB and MAPK, contributing to the overall anti-inflammatory response (Zhang et al., 2021).

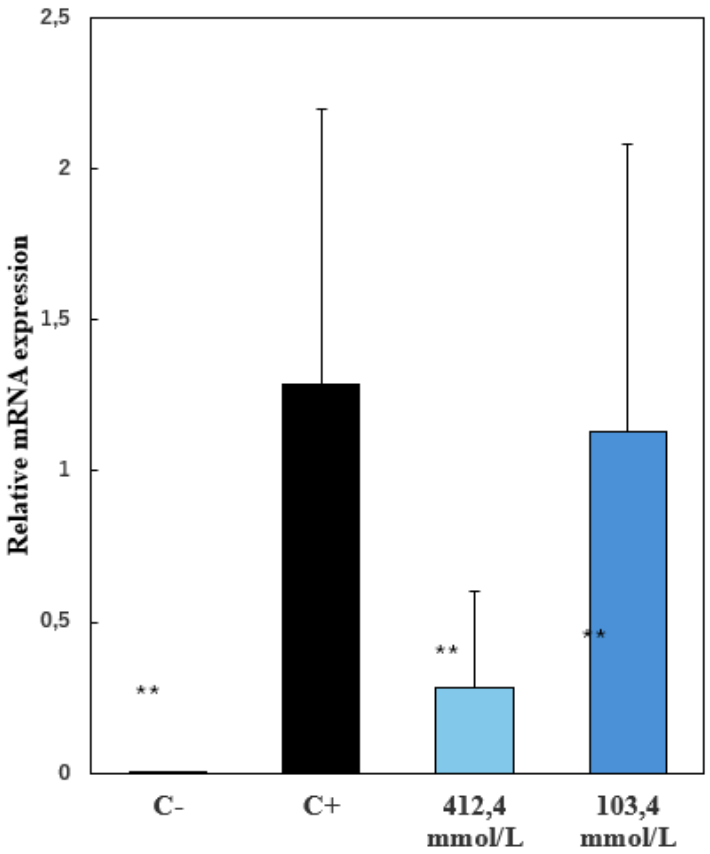

Figure 7. The Relative mRNA Expression of iNOS Towards Trigonelline Treatment. Concentrations of each sample are measured and compared to the positive control (shown in black) and the negative control (shown in white). The error bars indicate standard deviation (SD), and statistical significance is marked by *p ≤ 0.05 and **p ≤ 0.01, underscoring the reliability of these results.

Important information about the degree of inflammation in the examined samples can be gleaned from the mRNA expression of inducible nitric oxide synthase (iNOS). In contrast, the control sample is anticipated to have the highest iNOS mRNA expression since it contains lipopolysaccharide (LPS), a recognized inflammatory inducer. On the other hand, there is no discernible mRNA expression in the blank sample, which was not exposed to either trigonelline or LPS. This contrast emphasizes how trigonelline has different effects on iNOS expression and how LPS contributes to the development of inflammatory reactions.

V. DISCUSSION

5.1. Cytotoxicity Evaluation Towards Trigonelline Concentrations

Cytotoxicity assays are essential for assessing the safety and biological effects of substances on cells, as they quantify the extent to which a material can damage or kill cells (Klaunig & Xia, 2019). These assays are particularly important in evaluating medications, chemicals, and other substances. In the context of the WST-8 assay, which measures cell viability, a typical cutoff point for acceptable cell viability is approximately 70% to 80% (Huang, 2019). Although results can vary significantly across experiments, it is crucial to note that concentrations exceeding the 80% viability threshold indicate excellent cell health. Trigonelline, like many compounds, exhibits dose-dependent effects on cell viability; higher concentrations can lead to cytotoxicity. Notably, trigonelline at 50 µM (6.86 µg/mL) did not induce cytotoxicity in normal lung cells, and risk assessment studies have shown no adverse effects following acute exposure to trigonelline from coffee and its by-products (Nguyen et al., 2024). Long-term consumption of coffee products containing trigonelline has been associated with a favorable safety profile for human health.

Zhou et al. (2019) investigated the effects of trigonelline on various cell lines, including cancer cells, and found that it exhibited dose-dependent cytotoxicity, with higher doses resulting in decreased cell survival. However, in studies involving RAW264.7 cells, trigonelline demonstrated anti-inflammatory properties at concentrations ranging from 6.25 to 100 µg/mL without significant cytotoxicity within this range. To further explore its effects on inflammation, ELISA was employed to measure pro-inflammatory cytokines produced by LPS-induced RAW 264.7 cells following treatment with varying concentrations of trigonelline. Pro-inflammatory cytokines, such as TNF-α and IL-6, are small proteins that promote inflammation in response to cellular stressors like lipopolysaccharide (LPS), a component of gram-negative bacteria's outer membrane (Farhana & Khan, 2023). TNF-α plays a critical role in regulating inflammation and immune responses; it activates immune cells and stimulates the production of additional pro-inflammatory cytokines (Feng et al., 2020). The relationship between TNF-α and cell survival is complex; while it can induce apoptosis in certain cell types, it may also enhance survival in others, particularly cancer cells (Hui et al., 2023).

The results indicate that trigonelline effectively inhibits the NF-κB pathway by blocking IKKβ activation, reducing NF-κB's DNA binding activity, or preventing IκB degradation—key processes that regulate TNF-α production. Additionally, trigonelline may modulate intracellular signaling by increasing cGMP levels or activating protein kinase A, both of which contribute to TNF-α inhibition. It may also promote the synthesis of anti-inflammatory cytokines like IL-10 that inhibit TNF-α through various mechanisms. Trigonelline appears to desensitize macrophages to inflammatory stimuli such as LPS (von Bülow et al., 2007; Enomoto et al., 2002).

5.2. Cytokine Production Inhibition

Both TNF-α and IL-6 production are suppressed by trigonelline through NF-κB pathway inhibition. Furthermore, IL-6 suppression may occur via disruption of the MAPK signaling pathway. The MAPK pathway is integral to cellular signaling related to inflammatory cytokine production. Trigonelline's regulation of this pathway involves a complex series of intracellular signaling events that ultimately suppress IL-6 synthesis (Li et al., 2023). Specifically, trigonelline appears to prevent phosphorylation and activation of p38 MAPK and ERK1/2 components of the MAPK pathway, thereby reducing their ability to activate downstream transcription factors like NF-κB. This inhibition leads to decreased expression of pro-inflammatory genes such as IL-6. Additionally, trigonelline may interfere with the formation of the TLR4/MyD88 complex upstream of the MAPK pathway, further attenuating inflammatory signaling cascades. By modifying these pathways, trigonelline effectively inhibits the synthesis and release of IL-6—a key pro-inflammatory cytokine involved in various inflammatory diseases—similar to other natural compounds with known anti-inflammatory properties such as flaxseed linusorbs and fucoidan from *Sargassum autumnale* (Liyanage et al., 2023).

Trigonelline has demonstrated significant potential in suppressing pro-inflammatory responses by inhibiting key signaling pathways, particularly the NF-κB and JAK/STAT pathways. As illustrated in Figure 5, trigonelline effectively reduces the mRNA expression of pro-inflammatory cytokines, including TNF-α, through a multifaceted mechanism involving various interrelated pathways. Anti-inflammatory cytokines such as IL-10 play a crucial role in this process by inhibiting TNF-α expression at the transcriptional level. They achieve this by interfering with transcription factors that bind to the TNF-α promoter, while compounds that block the NF-κB pathway prevent its activation and translocation to the nucleus, thereby reducing TNF-α transcription (El Tahan et al., 2016). Additionally, substances like IL-27 contribute to decreased cellular sensitivity to TNF-α signaling by promoting receptor downregulation, further lowering TNF-α levels. The phosphorylation and activation of transcription factors that drive TNF-α production can also be inhibited by targeting critical signaling pathways such as JAK/STAT (Liu et al., 2000).

5.3. Suppression of mRNA Production

Post-transcriptional modifications are essential in regulating TNF-α expression. Certain microRNAs can bind to TNF-α mRNA, destabilizing it and limiting protein synthesis. Components within the 3' untranslated region (3' UTR) of TNF-α mRNA can also facilitate rapid degradation (Falvo et al., 2010). The regulation of inducible nitric oxide synthase (iNOS) mRNA expression involves similar mechanisms. Nitric oxide (NO) serves as a negative feedback regulator of iNOS production and has been shown to inhibit NF-κB, a key transcription factor that activates iNOS gene transcription in response to pro-inflammatory stimuli such as lipopolysaccharide (LPS) and cytokines like IFN-γ and IL-1β (Burke et al., 2013). NO stabilizes the NF-κB inhibitor IκBα, preventing its degradation and maintaining NF-κB's cytoplasmic retention, which significantly diminishes its nuclear translocation and subsequent iNOS transcription.

Studies indicate that NO donors can dramatically reduce iNOS mRNA levels across various cell types by inhibiting NF-κB activation (Kielbik et al., 2019). Additionally, post-transcriptional regulation of iNOS expression is influenced by RNA-binding proteins that interact with specific elements in the mRNA's 3' UTR, affecting its stability. Compounds like andrographolide have been shown to decrease iNOS activity not only

by modulating mRNA levels but also by reducing protein stability and synthesis. This highlights the importance of both transcriptional and post-transcriptional mechanisms in regulating iNOS production (Li et al., 2022). Collectively, these findings underscore trigonelline's multifaceted role in modulating inflammatory responses through intricate regulatory networks involving both transcriptional control and post-transcriptional modifications.

The decline in TNF-α, IL-6, and iNOS mRNA expression suggests a reduction in inflammation, indicating decreased synthesis of key pro-inflammatory mediators. A decrease in cytokines such as IL-6 and TNF-α is associated with diminished inflammatory activity, while reduced iNOS expression points to lower nitric oxide production, which, although essential for immune responses, can cause tissue damage when overproduced (Lee et al., 2017). The observed decrease in iNOS expression correlates with a less severe inflammatory state, collectively indicating a dampening of the overall inflammatory response. Treatments targeting these cytokines have been shown to effectively reduce inflammation; for example, anti-IL-6 antibodies in db/db mice enhanced arteriolar dilatation and decreased both TNF-α and IL-6 mRNA and protein levels. This supports the notion that suppressing these genes exerts an anti-inflammatory effect, as demonstrated by substances like saccharin, which has been shown to reduce iNOS mRNA production alongside other inflammatory markers (Park et al., 2020).

Interleukin-6 (IL-6) serves as a multifunctional cytokine that plays critical roles in various physiological and pathological processes within the body. Produced by multiple cell types including immune cells, fibroblasts, and endothelial cells, IL-6 acts as both a promoter and inhibitor of inflammation (Qin et al., 2020). It is integral to hematopoiesis, inflammation, and immune responses, functioning as a key component of the acute phase response—a systemic reaction to inflammation, injury, or infection. During this response, IL-6 stimulates the liver to produce acute phase proteins such as haptoglobin, serum amyloid A, and C-reactive protein (Okamura et al., 2023).

Trigonelline has been shown to inhibit pro-inflammatory cytokine production by modulating these pathways. Specifically, it can suppress the NF-κB pathway by blocking IKKβ activation and reducing NF-κB's DNA binding activity or limiting IκB degradation. Additionally, trigonelline may alter intracellular signaling by increasing cGMP levels or activating protein kinase A, both of which can inhibit TNF-α production. Furthermore, trigonelline may enhance the synthesis of anti-inflammatory cytokines like IL-10 that inhibit TNF-α through various mechanisms. By desensitizing macrophages to inflammatory stimuli such as lipopolysaccharide (LPS), trigonelline effectively reduces TNF-α levels (von Bülow et al., 2007; Enomoto et al., 2002).

The suppression of both TNF-α and IL-6 by trigonelline highlights its potential as an anti-inflammatory agent. In addition to inhibiting the NF-κB pathway, trigonelline may disrupt the MAPK signaling pathway, which is crucial for regulating the production of inflammatory cytokines like IL-6. This regulation involves a complex series of intracellular signaling events that ultimately lead to decreased IL-6 synthesis (Li et al., 2023). Specifically, trigonelline appears to prevent phosphorylation and activation of p38 MAPK and ERK1/2 components of the MAPK pathway, thereby reducing their ability to activate downstream transcription factors such as NF-κB. This inhibition results in decreased expression of pro-inflammatory genes like IL-6. Additionally, trigonelline may interfere with the formation of the TLR4/MyD88 complex upstream of the MAPK pathway, further attenuating inflammatory signaling cascades. By modifying these pathways, trigonelline effectively inhibits the synthesis and release of IL-6—an important pro-inflammatory cytokine

implicated in various inflammatory diseases—similar to other natural compounds known for their anti-inflammatory properties (Liyanage et al., 2023).

V. SELF REFLECTION

5.1. Self Reflection

Over the course of the three-month research internship, the author gained invaluable knowledge, experiences, and insights. Key skills developed during this period include critical thinking for problem-solving, effective time management for project formulation and trial execution, and strong communication with both the supervisor and fellow researchers. The author also learned to be adaptable and open to feedback, incorporating input and criticism from others. In terms of technical skills, the author acquired hands-on experience in various laboratory techniques such as pipetting, RT-qPCR, ELISA, and other assays. The soft skills gained from the BRIGHT sessions played a significant role in the success of the internship. For instance, the "Adulting 101" session taught the author essential skills in managing schedules, handling pressure, and taking responsibility. These soft skills were vital throughout the research internship, contributing to the author's ability to navigate challenges.

Throughout the internship, the values of i3L, including grit, integrity, and being a role model, served as guiding principles. The value of grit, which encompasses guts, resilience, initiative, and tenacity, helped the author stay focused on completing trials, data collection, and report writing on time. Integrity was applied throughout the research process to ensure the validity of the data presented in the final report. Additionally, the author received strong support and guidance from the research supervisor, which greatly contributed to the success of the internship. The knowledge gained from relevant courses and laboratory experiments, such as those from the Nutrigenetics and Nutrigenomics course, further enriched the author's understanding of genes, mRNA, cDNA, cytokines, and overall human metabolism.

VI. CONCLUSION

6.1 Conclusion

This research underscores the notable anti-inflammatory effects of trigonelline, a compound sourced from coffee and fenugreek seeds, due to its capacity to influence essential inflammatory signaling pathways. The study's results reveal that trigonelline significantly inhibits the expression and production of pro-inflammatory cytokines, including TNF-α, IL-6, and iNOS. It does so primarily by targeting the NF-κB pathway—specifically by preventing IKKβ activation and diminishing NF-κB's ability to bind to DNA—which results in lower levels of these cytokines. Notably, trigonelline shows no cytotoxicity at a concentration of 50 μM in normal lung cells, indicating its safety for prolonged consumption. Furthermore, it enhances the production of anti-inflammatory cytokines like IL-10, adding to its anti-inflammatory profile. Additionally, trigonelline interferes with the MAPK signaling pathway by inhibiting the phosphorylation and activation of crucial regulatory components involved in inflammation. This comprehensive mechanism illustrates trigonelline's promise as a therapeutic agent for treating inflammation-related disorders and suggests that incorporating it into diets could be advantageous for overall health.

6.2. Recommendations

To improve future studies on trigonelline's anti-inflammatory effects, several key recommendations can be made. First, diversifying the cell lines used in experiments will enhance the understanding of trigonelline's effects across different biological contexts; incorporating primary human macrophages or other immune cell types could provide more relevant insights. Second, conducting longitudinal studies to assess the long-term safety and efficacy of trigonelline, particularly in chronic inflammatory conditions, is essential. This should include varying concentrations to establish a comprehensive dose-response relationship while monitoring cytotoxicity. Third, employing advanced techniques such as transcriptomics and proteomics will help elucidate the broader molecular pathways influenced by trigonelline and identify novel targets for its anti-inflammatory properties. By addressing these areas, future research can significantly advance our understanding of trigonelline's therapeutic potential.

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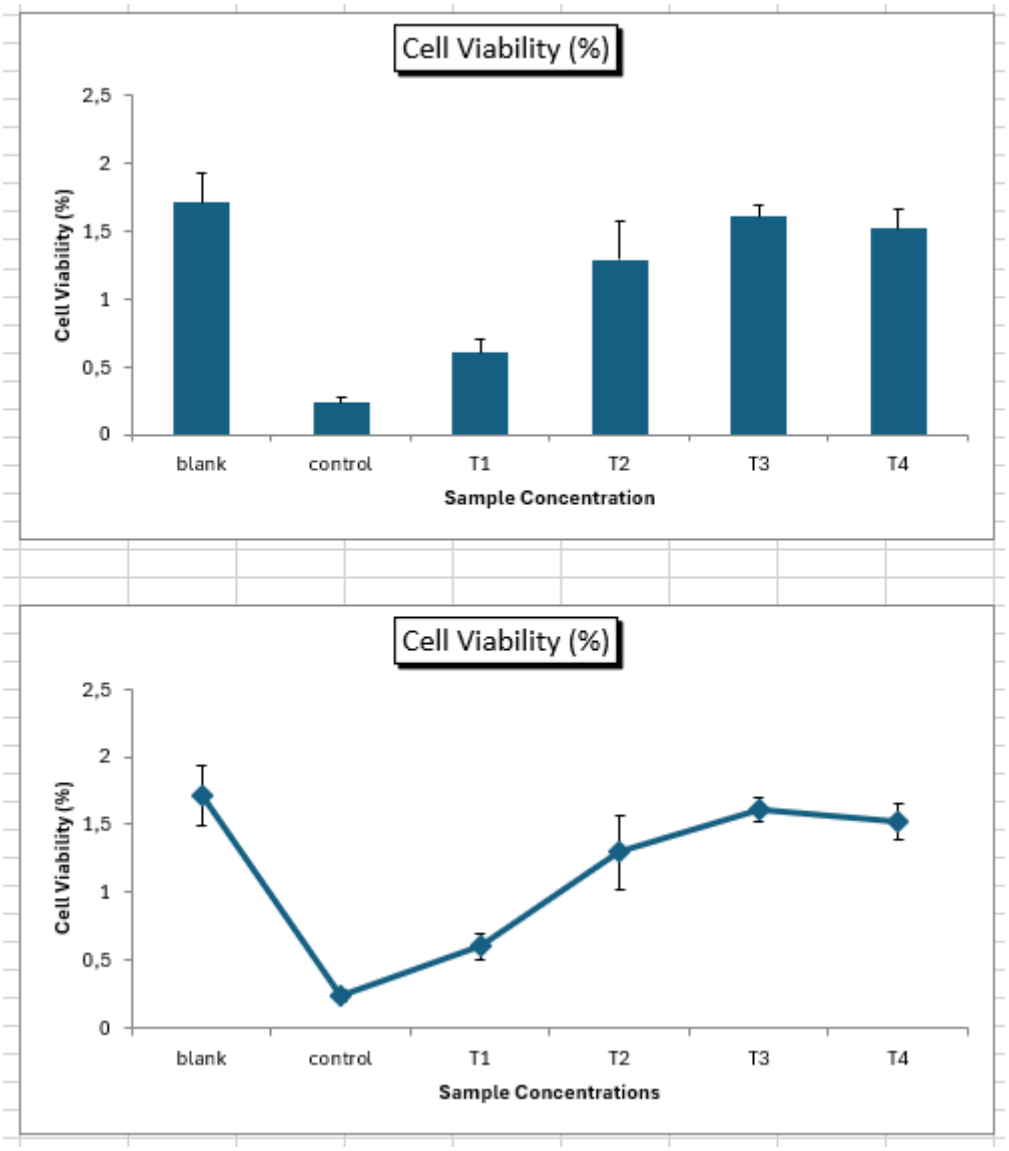
APPENDICES

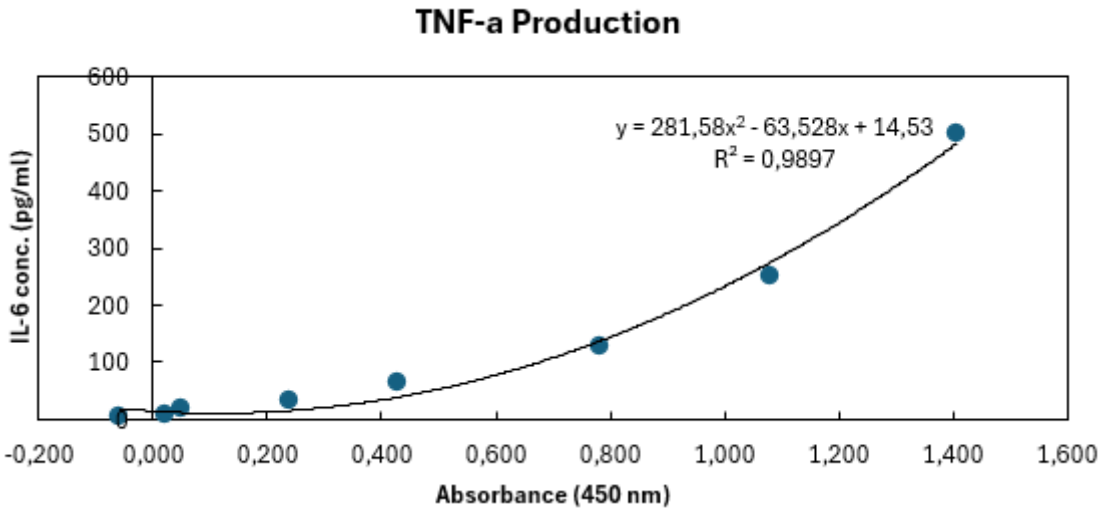
Appendix 1. Turnitin Similarity Report



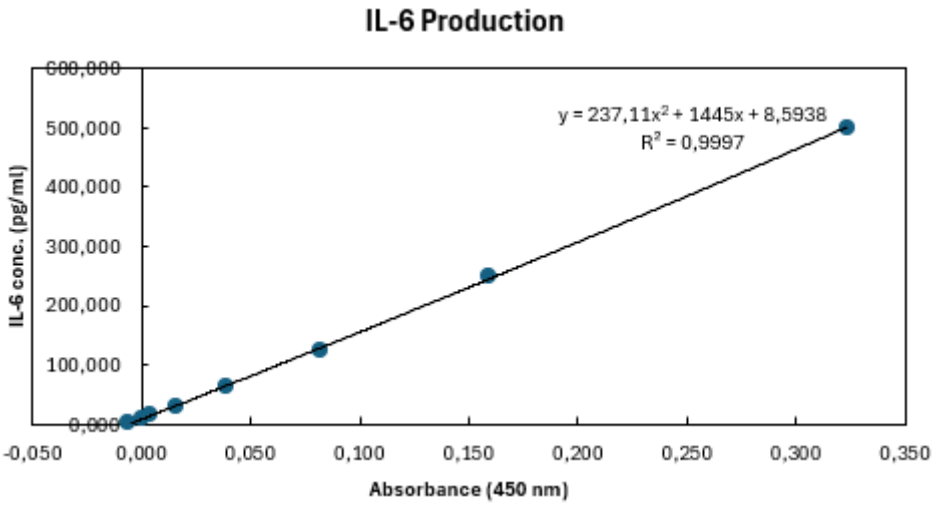
Appendix 2. The raw graph for WST-8 cytotoxicity assay

Sample conc.		#1	#2	#3	#4	No cells	#1	#2	#3	Ave.	Viability	SD
C-		2,1412	1,8102	1,7312	1,9712	0,184	1,9572	1,6262	1,5472	1,71	738	0,22
C+		0,435	0,432	0,356	0,339	0,176	0,259	0,256	0,18	0,23	100	0,04
T1	56,50	0,845	0,773	0,652	0,736	0,155	0,69	0,618	0,497	0,60	260	0,10
T2	14,13	1,1392	1,6462	1,5932	1,6702	0,167	0,9722	1,4792	1,4262	1,29	558	0,28
T3	3,53	1,8682	1,7622	1,7012	1,4002	0,17	1,6982	1,5922	1,5312	1,61	694	0,08
T4	0,88	1,5412	1,8102	1,7312	1,3712	0,173	1,3682	1,6372	1,5582	1,52	657	0,14

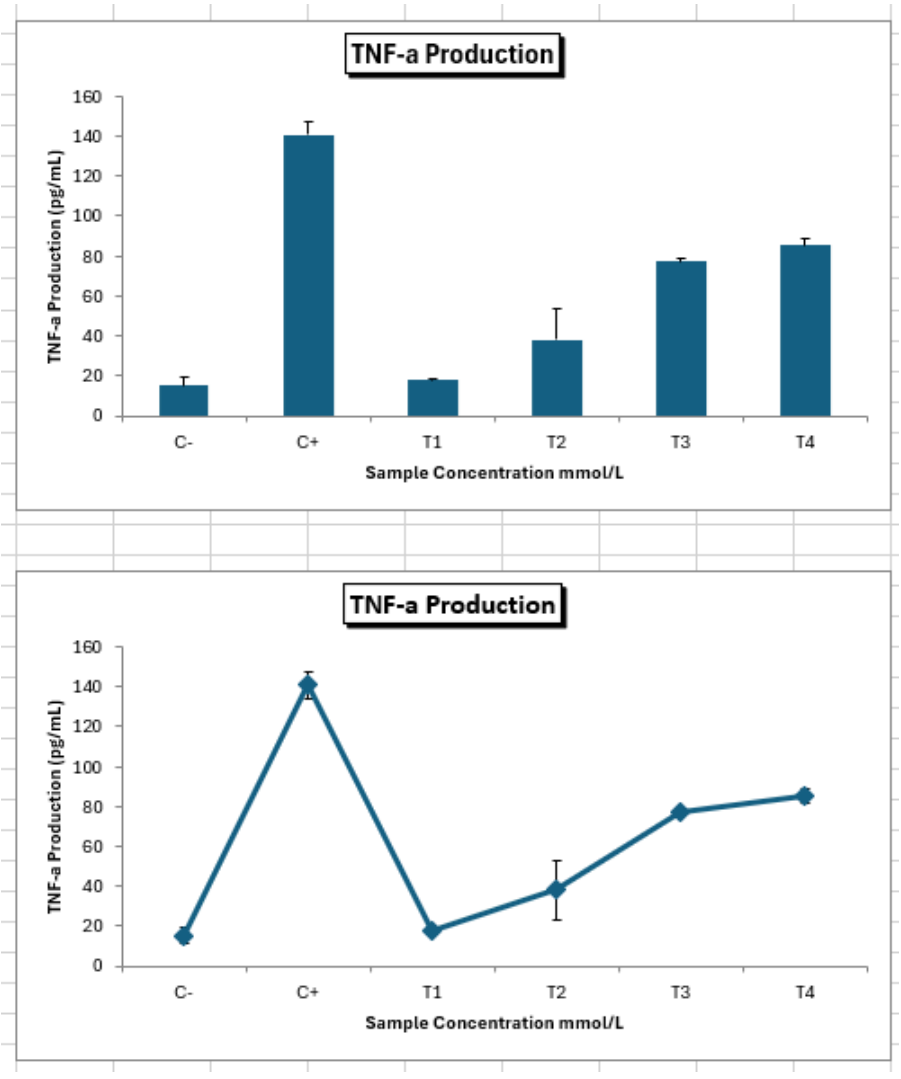
Appendix 3. The raw data for WST-8 cytotoxicity assay



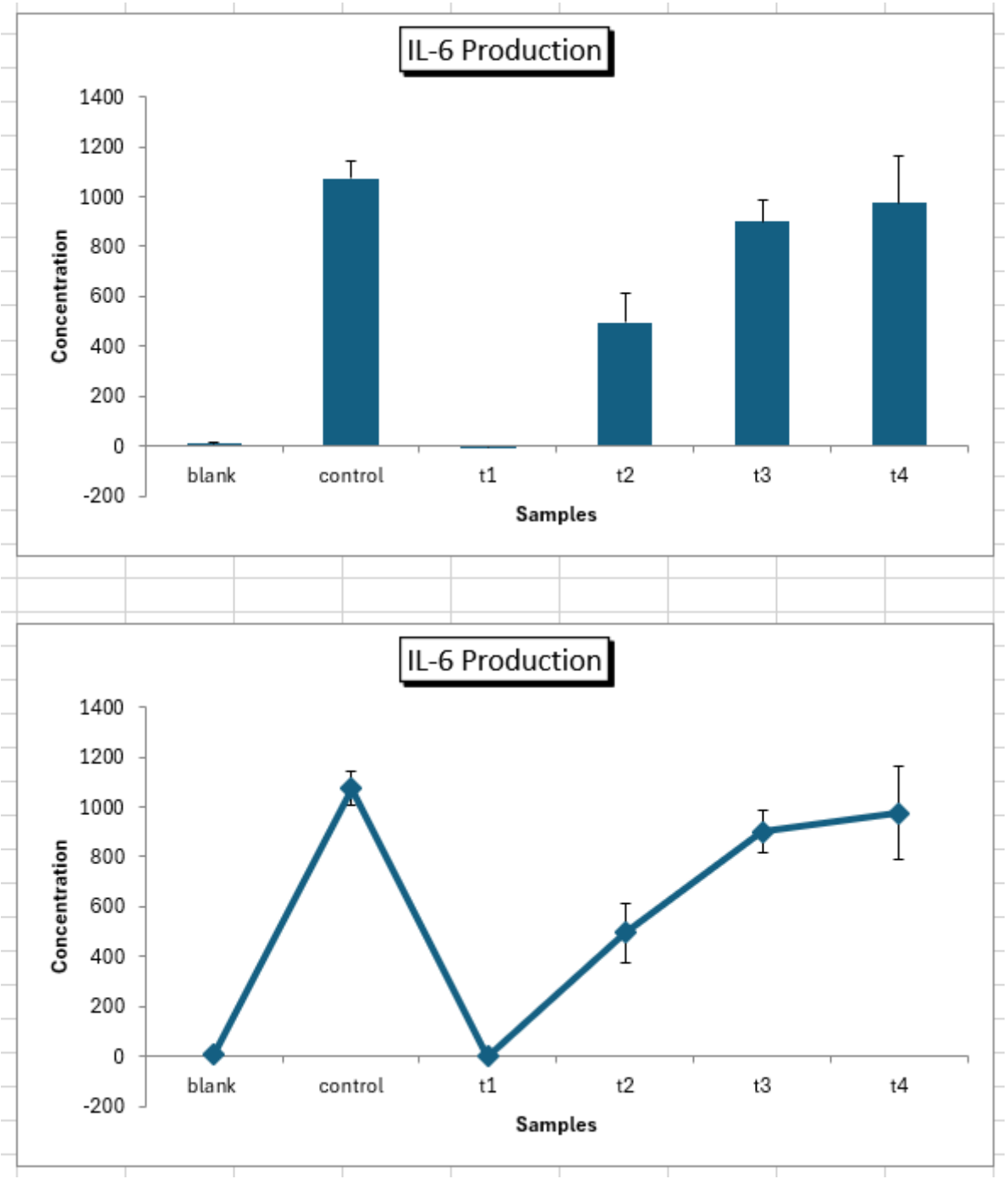
Appendix 4. Standard curve for the TNF-a



Appendix 5. Standard curve for the IL-6



Appendix 6. Raw graph for TNF-a



Appendix 7. Raw graph for IL-6

Target gene	Sample	Ct			Ct Ave.	ΔCt			ΔCt Ave.	ΔΔCt			2^(-ΔΔCt)			2^(-ΔΔCt) Ave.	S.D.	有意差
β-actin	Blank	19,9721069	19,7486248	19,5711803	19,76397069													
	Control	17,9720821	17,1508999	17,4516258	17,52486928													
	T1	19,3288784	19,9839268	19,3360462	19,54961713													
	T2	21,9158649	21,6155148	21,6951313	21,74217033													
IL-6	Blank	35,1632118	35,1632118	35,1632118	35,16321182	15,19110489	15,41458702	15,59203148	15,39924113	14,84278742	15,06626956	15,24371401	3,40311E-05	2,91475E-05	2,57742E-05	2,96509E-05	4,15141E-06	
	Control	18,2065201	17,2065201	18,2065201	17,87318675	0,234437943	0,055620193	0,754894257	0,348317464	-0,113879522	-0,292697271	0,406576792	1,082134273	1,224928271	0,754411309	1,020491284	0,241239397	
	T1	21,5668392	20,5668392	23,5668392	21,90017255	2,237960815	0,582912445	4,230792999	2,35055542	1,889643351	0,234594981	3,882475535	0,269873766	0,849923571	0,067804483	0,395867273	0,405996724	
	T2	28,7321911	29,7321911	20,7321911	26,39885775	6,816326141	8,116676331	-0,962940216	4,656687419	6,468008677	7,768358866	-1,31125768	0,011296278	0,004586601	2,481577791	0,83248689	1,428158554	
TNF-α	Blank	25,8357506	26,9725952	26,7556667	26,52133751	5,863643646	7,223970413	7,184486389	6,757366816	3,476081212	4,836407979	4,796923955	0,089865974	0,035002263	0,035973443	0,053613893	0,031398978	*
	Control	19,6737289	20,0627022	20,000864	19,91243172	1,701646805	2,911802292	2,549238205	2,387562434	-0,685915629	0,524239858	0,161675771	1,608722659	0,695325374	0,893986053	1,066011362	0,480383368	
	T1	22,8359127	22,8359127	22,8359127	22,8359127	3,507034302	2,851985931	3,499866486	3,286295573	1,119471868	0,464423498	1,112304052	0,460262284	0,724760635	0,462554719	0,549192546	0,152050745	
	T2	23,89711	24,120697	24,617548	24,211785	1,981245041	2,505182266	2,922416687	2,469614665	-0,406317393	0,117619832	0,534854253	1,325298551	0,921707035	0,690228403	0,979077996	0,321398652	
iNOS	Blank	34,2488728	33,5482121	32,6818943	33,49299304	14,27676582	13,79958725	13,11071396	13,72902234	14,28249296	13,80531438	13,11644109	5,01812E-05	6,98532E-05	0,000112605	7,75465E-05	3,19151E-05	
	Control	19,5187626	16,0337143	17,0049496	17,51914215	1,54668045	-1,117185593	-0,446676254	-0,005727132	1,552407583	-1,11145846	-0,440949122	0,340940624	2,16063962	1,357497106	1,286359117	0,911932875	
	T1	21,9739094	20,6068821	23,724411	22,10173416	2,645030975	0,622955322	4,388364792	2,55211703	2,650758108	0,628682454	4,394091924	0,159236381	0,646766807	0,047560513	0,284521234	0,31864442	
	T2	26,5886879	20,9820347	20,836998	22,80257352	4,672822952	-0,633480072	-0,858133316	1,060403188	4,678550084	-0,62775294	-0,852406184	0,039049555	1,54515647	1,805509716	1,129905247	0,953635443	

Appendix 8. Raw data for the RT-qPCR