Investigation into the Modulatory Effects of Kawakawa Extract on Macrophage Functions

by

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Abstract

Macrophage activation is essential for innate immune defence; however, excessive or prolonged activation contributes to chronic inflammation and tissue damage. Kawakawa (*Piper excelsum*), a medicinal plant traditionally used in rongoā Māori in Aotearoa New Zealand, contains bioactive compounds with reported anti-inflammatory properties, although its immunomodulatory effects on macrophages remain incompletely understood. This study investigated the concentration-, time-, stimulus-, and treatment-timing-dependent effects of a hydroethanolic kawakawa leaf extract in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS), interferon-gamma (IFN-γ), or a combination of LPS and IFN-γ. Cell viability was assessed using trypan blue exclusion and the MTT assay, while nitrite accumulation, used as an indirect indicator of nitric oxide (NO) production, was quantified via the Griess assay.

Kawakawa extract did not significantly affect macrophage viability across the concentrations tested in most conditions, indicating that the observed biological effects were generally not attributable to overt cytotoxicity. In the LPS-induced inflammation model, significant reductions in NO production were observed following pre-treatment and co-treatment with 100 μg/mL kawakawa extract ($p < 0.001$). Under the IFN-γ stimulation, the inhibitory effects of the extract were observed under both pre-treatment and co-treatment conditions after 48 hours ($p < 0.01$), suggesting a delayed anti-inflammatory response compared with the LPS model. For LPS + IFN-γ-stimulated macrophages, kawakawa extract produced the greatest inhibition under co-treatment conditions ($p < 0.001$). Across all treatment strategies, pre-treatment and co-treatment generally produced stronger inhibitory effects than post-treatment, although the magnitude and statistical significance of these responses varied according to inflammatory stimulus, incubation time, and experimental endpoint. MTT-reducing activity was not consistently affected by kawakawa treatment, with significant reductions observed under selected conditions at the highest concentration tested.

In conclusion, hydroethanolic kawakawa leaf extract modulated inflammatory nitrite accumulation in activated RAW 264.7 macrophages while generally maintaining cell viability across most experimental conditions. The findings indicate that its biological activity depends on extract concentration, inflammatory stimulus, treatment timing, and incubation period, with greater inhibitory effects observed when the extract was administered before or concurrently with inflammatory stimulation. These results provide experimental evidence supporting the immunomodulatory potential of kawakawa and establish a foundation for future studies using chemically standardised extracts to identify the active constituents, uncover the molecular mechanisms, and evaluate its potential therapeutic applications.

Contents

Abstract i

Content ii

List of Figures viii

List of Tables ix

Attestation of Authorship x

Co-Authored Works xi

Acknowledgements xii

Abbreviations xiii

1 Introduction 1

1.1 The Fundamentals of Inflammation 1

1.1.1 A Historical Overview of Inflammation 1

1.1.2 Classification of Inflammation 2

1.2 Macrophage Biology and Polarisation 3

1.2.1 The Evolution of Macrophage Theory 3

1.2.2 The Origins and Developmental Pathways of Macrophages 3

1.2.3 Central Roles of Macrophages in Immune Regulation 4

1.2.3.1 Phagocytosis and Tissue Homeostasis 4

1.2.3.2 Antigen Presentation 5

1.2.3.3 Immunomodulation and Regulation 5

1.2.4 Macrophage Polarisation: M1 vs M2 . . . 5
1.2.4.1 M1 Polarisation . . . 5
1.2.4.2 M2 Polarisation . . . 6
1.2.4.3 Balance and Pathology . . . 7
1.2.5 Regulatory Mechanisms and Natural Compounds . . . 8
1.2.5.1 JAK/STAT Signalling Pathway . . . 8
1.2.5.2 NF-κB Signalling Pathway . . . 8
1.2.5.3 PI3K/Akt Signalling Pathway . . . 9
1.2.5.4 MAPK Pathways . . . 9
1.2.5.5 Natural Compounds Influencing the Regulation of Macrophage Polarisation 10
1.3 Kawakawa (*Piper excelsum*): Traditional Use, Bioactivity, and Immunomodulatory Potential) . 10
1.3.1 Preface . . . 10
1.3.2 Abstract . . . 11
1.3.3 Connecting Traditional Knowledge and Modern Science . . . 11
1.3.4 Historical Background of Kawakawa Use . . . 12
1.3.4.1 Traditional Applications in Rongoā Māori . . . 12
1.3.4.2 Kawakawa (*Piper excelsum*) versus Kava (*Piper methysticum*): Why the Distinction Matters . . . 13
1.3.5 Advanced Pharmacological Profile . . . 13
1.3.5.1 Anti-inflammatory and Immunomodulatory Mechanisms . . . 13
1.3.5.2 Cytotoxic Activity in Cancer Cell Models . . . 14
1.3.5.3 Anthelmintic Properties and Other Reported Bioactivities . . . 15
1.3.6 Phytochemical Profile and Bioactive Compounds . . . 16
1.3.7 Interpretation of Cytokine Responses and Experimental Evidence . . . 19
1.3.7.1 Interpreting Context-Dependent IL-10 and IL-6 Responses . . . 19

1.3.7.2 Interpretive Limitations of Experimental Models 20
1.3.8 Toxicological and Safety Profile 21
1.3.9 Conclusion 21
1.4 Methodological Framework 22
1.4.1 Cell Models 22
1.4.2 Cytotoxicity Assessment 23
1.4.3 Methods for Macrophage Functional Assessment 24
1.4.3.1 Polarisation Characteristics 24
1.4.3.2 Effector Function Assessment 25
1.4.4 Controls for Experimental Integrity 26
1.4.4.1 Sterilisation Strategies for Plant Extracts 26
1.4.4.2 Solvent and Vehicle Control 27
1.4.4.3 Endotoxins in Experimental Systems 28
1.4.4.4 Types of Contamination in Cell Culture 28
1.4.4.5 Aseptic Technique and Contamination Prevention 28
1.4.4.6 Assay Controls and Quality Assurance 29
1.4.5 Summary 30
1.5 Research Aims and Objectives 30
1.5.1 Aim 30
1.5.2 Objectives 30
1.6 Significance of the Study 31

2 Materials and Methods **33**

2.1 Overview 33
2.2 Materials and Reagents 33

2.3 Preparation of Kawakawa Extract . . . 34
2.3.1 Kawakawa Extraction and Sterilisation . . . 34
2.3.2 Sterility Testing . . . 34
2.3.3 Dilution and Solvent Control . . . 35
2.4 Cell Culture Conditions . . . 35
2.4.1 RAW 264.7 Cells . . . 35
2.5 Aseptic Technique and Contamination Prevention . . . 36
2.6 Experimental Design . . . 36
2.6.1 Phase 1: Broad Concentration Screen (Co-treatment with LPS) . . . 36
2.6.1.1 Experimental Setup . . . 37
2.6.1.2 Treatment Groups . . . 37
2.6.1.3 Co-treatment Procedure . . . 38
2.6.2 Phase 2: Preventive Assessment (Kawakawa Pre-treatment before LPS Stimulation) . . . 39
2.6.2.1 Experimental Overview . . . 39
2.6.2.2 Dosing Strategy . . . 39
2.6.2.3 Time Points and Sampling . . . 40
2.6.2.4 Treatment Groups . . . 40
2.6.3 Phase 3: Mechanistic Assessment (Kawakawa Pre-treatment before IFN-γ Stimulation) 41
2.6.3.1 Experimental Overview . . . 41
2.6.3.2 Dosing Strategy . . . 42
2.6.3.3 Time Points and Treatment . . . 42
2.6.4 Phase 4: Broadening Stimuli and Intervention Timings (Co-treatment, Pre-treatment and Post-treatment) . . . 42
2.6.4.1 Detailed Procedure: . . . 42
2.6.4.2 Treatment Groups . . . 43

2.6.5 Replicates and Quality Control . . . 44
2.7 Downstream Assay Procedures . . . 44
2.7.1 Cytotoxicity: Trypan Blue Exclusion Assay . . . 45
2.7.2 Cell Viability: MTT Assay . . . 45
2.7.3 Nitric Oxide (NO) Production: Griess Assay . . . 46
2.8 Statistical Analysis . . . 47
2.8.1 Data Preparation and Preliminary Checks . . . 47
2.8.2 Phase 1: Co-Treatment Comparisons . . . 47
2.8.3 Phase 2 and 3: Time- and Dose-Dependent Effects . . . 47
2.8.4 Phase 4: Multi-Factorial Comparison . . . 48
2.8.5 Software and Significance Thresholds . . . 48

3 Context-Dependent Effects of Hydroethanolic Kawakawa (*Piper excelsum*) Leaf Extract on Nitrite Accumulation in LPS- and IFN-γ-Activated RAW 264.7 Macrophages 49

3.1 Preface . . . 49
3.2 Abstract . . . 50
3.3 Introduction . . . 50
3.4 Materials and Methods . . . 52
3.4.1 Preparation of Kawakawa Extract . . . 52
3.4.2 Cell Culture and Treatment . . . 52
3.4.3 Cell Viability (MTT) and Nitric Oxide Production (Griess) Assays . . . 52
3.4.4 Statistical Analysis . . . 53
3.5 Results . . . 53
3.5.1 Kawakawa Extract Shows No Detectable Cytotoxicity in RAW 264.7 Cells . . . 53
3.5.2 Co-treatment with Kawakawa Extract Reduces LPS-Induced Nitrite Accumulation at 100 μg/mL . . . 54

3.5.3 Pre-treatment with Kawakawa Extract Reduces LPS-Induced Nitrite Accumulation at 100 μg/mL 54

3.5.4 Higher Concentrations of Kawakawa Attenuate the Delayed Nitrite Response to IFN-γ 55

3.5.5 Timing of Kawakawa Administration Determines Its Effect on Stimulus-Induced Nitrite Accumulation 56

3.6 Discussion 57

4 General Discussion and Conclusion **65**

4.1 Overall Discussion 65

4.2 Overall Conclusion 68

References **69**

Appendices **I**

Appendix A. Representative Cell Morphology Images I

Appendix B. Statistical Analysis Details XI

Appendix C. Assay Calibration and Standard Curve Analysis XXX

List of Figures

1 Chemical structures of major bioactive compounds in kawakawa 17

2 RAW 264.7 macrophage viability is maintained following kawakawa extract treatment 60

3 Co-treatment with 100 μg/mL kawakawa extract reduces LPS-induced nitrite accumulation without decreasing MTT-reducing activity in RAW 264.7 macrophages 61

4 Kawakawa pre-treatment transiently attenuates LPS-induced nitrite accumulation without reducing MTT-reducing activity in RAW 264.7 macrophages 62

5 Kawakawa pre-treatment attenuates IFN-γ-induced nitrite accumulation at 48 h in RAW 264.7 macrophages 63

6 Co- and pre-treatment with kawakawa, but not post-treatment, attenuate stimulus-induced nitrite accumulation in RAW 264.7 macrophages 64

List of Tables

1 Macrophage polarisation subtypes 7

2 Immunomodulatory effects of kawakawa 16

3 Key bioactive compounds in kawakawa (*Piper excelsum*) 17

4 Key kawakawa bioactives and their anti-inflammatory actions 19

5 Classification and impact of contaminants 28

6 Cell culture and treatment timeline 36

7 Experimental groups (phase 1): co-treatment of macrophages with LPS and kawakawa extract 38

8 Treatment design for 24-well plate (phase 1: co-treatment of macrophages with LPS and kawakawa extract) 39

9 Experimental groups (phase 2): kawakawa pre-treatment prior to LPS-induced macrophage activation 41

10 Phase 2 experimental schedule: pre-inflammatory intervention workflow (LPS stimulation) . . 41

11 Phase 3 experimental schedule: pre-inflammatory intervention workflow (IFN-γ stimulation) . 42

12 Experimental groups (phase 4): co-, pre- and post-treatment with kawakawa under LPS and/or IFN-γ stimulation 44

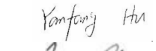
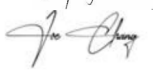
Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor used artificial intelligence tools or generative artificial intelligence tools (unless it is clearly stated, and referenced, along with the purpose of use), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Abbreviations

A-MuLV Abelson Murine Leukemia Virus

A2AR Adenosine 2A Receptor

ANOVA Analysis of Variance

AP-1 Activator Protein-1

APCs Antigen-Presenting Cells

Arg-1 Arginase-1

ASC Apoptosis-Associated Speck-Like Protein Containing a Caspase Recruitment Domain

AUTEC Auckland University of Technology Ethics Committee

BAFF B Cell Activating Factor

BCA Bicinchoninic Acid Assay

BSA Bovine Serum Albumin

BSC Biosafety Cabinet

C/EBP CCAAT/Enhancer Binding Protein

CA Cellulose Acetate

CAMs Cell Adhesion Molecules

CC50 Half Maximal Cytotoxic Concentration

CCL Chemokine (C-C motif) Ligand

CD Clusters of Differentiation

CFUs Colony-Forming Units

COX-2 Cyclooxygenase-2

CPTs Cell Preparation Tubes

CRP C-Reactive Protein

Ct Threshold cycle

CXCL Chemokine (C-X-C motif) Ligand

°C Degree Celsius

DAMPs Damage-Associated Molecular Patterns

DMEM Dulbecco's Modified Eagle Medium

DMSO Dimethyl Sulfoxide

ECL Chemiluminescent Substrate

ELISA Enzyme-Linked Immunosorbent Assay

EMMs Estimated Marginal Means

ERK1/2 Extracellular Signal-Regulated Kinase 1/2

EtOH Ethanol

FACS Fluorescence-Activated Cell Sorting

FBS Foetal Bovine Serum

FcgRI Fc-gamma Receptor I

FDR False Discovery Rate

FITC Fluorescein Isothiocyanate

Fizz1 Found in Inflammatory Zone 1 / Resistin-like Alpha

GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor

HEK Human Embryonic Kidney

HEPA High-Efficiency Particulate Air

HeLa Henrietta Lacks

HO-1 Heme Oxygenase-1

HPLC High Performance Liquid Chromatography

HRP Horseradish Peroxidase

HSCs Haematopoietic Stem Cells

HSD Honestly Significant Difference

HT29 Human Colorectal Adenocarcinoma

IC Immune Complexes

IC50 Half Maximal Inhibitory Concentration

IDO Indoleamine 2,3-Dioxygenase

IFN-γ Interferon-Gamma

IGF Insulin-like Growth Factor

IL Interleukin

IL-1β Interleukin-1 Beta

IL-1R Interleukin-1 Receptor

IL-10 Interleukin-10

IL-12 Interleukin-12

IL-13 Interleukin-13

IL-23 Interleukin-23

IL-4 Interleukin-4

IL-6 Interleukin-6

iNOS / NOS2 Inducible Nitric Oxide Synthase

IP-10 Interferon Inducible Protein-10

JAK/STAT Janus Kinase/Signal Transducer and Activator of Transcription

JAKs Janus Kinases

JNK c-Jun N-terminal Kinase

LAL Limulus Amebocyte Lysate

LC-MS/MS Liquid Chromatography-Tandem Mass Spectrometry

LDL Low-Density Lipoprotein

LIF Leukemia Inhibitory Factor

LPS Lipopolysaccharide

LTβ Lymphotoxin-β

MAPK Mitogen-Activated Protein Kinase

M-CSF Macrophage Colony-Stimulating Factor

MCP Monocyte Chemotactic Protein

MFI Mean Fluorescence Intensity

MHC Major Histocompatibility Complex

MIP-1α Macrophage Inflammatory Protein-1α

MMR Macrophage Mannose Receptor

MMP Matrix Metalloproteinase

miRNAs microRNAs

Mrc1 Mannose Receptor C-type 1

mTOR Mammalian Target of Rapamycin

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NADPH Nicotinamide Adenine Dinucleotide Phosphate

NED N-(1-naphthyl)ethylenediamine Dihydrochloride

NF-κB Nuclear Factor-κB

NFKB1 Nuclear Ractor Kappa B subunit 1

NO Nitric Oxide

NO_2^- Nitrite

NO_3^- Nitrate

NOD2 Nucleotide-Binding Oligomerisation Domain-Containing Protein 2

Nrf2/HO-1 Nuclear Factor Erythroid 2–Related Factor 2 / Heme Oxygenase-1

oxLDL Oxidized Low-Density Lipoprotein

PAMPs Pathogen-Associated Molecular Patterns

P/S Penicillin–Streptomycin

PBMCs Peripheral Blood Mononuclear Cells

PBS Phosphate-Buffered Saline

PCA Plate Count Agar

PES Polyethersulfone

PGE2 Prostaglandin E2

PI3K/Akt Phosphoinositide 3-Kinase / Protein Kinase B

PMA Phorbol 12-myristate 13-acetate

PPE Personal Protective Equipment

PSA Prostate Specific Antigen

PTFE Polytetrafluoroethylene

PTMs Post-Translational Modifications

PTPs Protein Tyrosine Phosphatases

PVDF Polyvinylidene Fluoride

qPCR Quantitative Real-time PCR

RANKL Receptor Activator of Nuclear factor κB Ligand

RIPA Radio-Immunoprecipitation Assay

RNI Reactive Nitrogen Intermediates

ROI Reactive Oxygen Intermediates

ROS Reactive Oxygen Species

RPMI Roswell Park Memorial Institute

SD Standard Deviation

SDA Sabouraud Dextrose Agar

SDS-PAGE Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SEAP Secreted Alkaline Phosphatase

SOCS/CIS Suppressor of Cytokine Signalling / Cytokine-Inducible SH2-containing protein

SPC Standard Plate Count

Srxn1 Sulfiredoxin-1

STAT Signal Transducer and Activator of Transcription

TAMs Tumour-Associated Macrophages

TBST Tris-Buffered Saline with Tween 20

TGF-β Transforming Growth Factor-Beta

THP-1 Tohoku Hospital Pediatrics-1

TLR / TLRs Toll-like Receptor(s)

Th1 T-helper 1

Th2 T-helper 2

TNF / TNF-α Tumour Necrosis Factor-Alpha

UAE Ultrasound-Assisted Extraction

VEGF Vascular Endothelial Growth Factor

VEGF-A Vascular Endothelial Growth Factor A

Ym1 Chitinase 3-Like 3

1 Introduction

This chapter provides a comprehensive overview of the fundamentals of inflammation, with a particular focus on macrophage biology. It also includes a review of the ethnobotany and phytochemistry of kawakawa (*Piper excelsum*). The methodological framework of this study is then described, covering cell models, cytotoxicity evaluation, macrophage functional assays, and strategies to maintain experimental integrity. Finally, the research aims, objectives and significance of this project are outlined in the context of current literature.

1.1 The Fundamentals of Inflammation

1.1.1 A Historical Overview of Inflammation

The understanding of inflammation, first named after the Latin word for "flame," has evolved considerably over centuries, beginning with the observations recorded by the Roman encyclopedist Aulus Cornelius Celsus (Cavaillon, 2021; Oronsky et al., 2022). He first described the four classic signs of acute inflammation: redness, swelling, warmth, and pain (Antonelli & Kushner, 2017; Korniluk et al., 2017). A fifth sign, loss of function, was added much later. This addition has been variously attributed to Thomas Sydenham and Rudolf Virchow (Cavaillon, 2021; Kołaczkowska, 2007; Oronsky et al., 2022). Although Galen of Pergamum, who advanced the humoral theory and viewed inflammation as part of the healing process, is sometimes credited with this addition, such attribution is likely anachronistic and remains debated among scholars. (Korniluk et al., 2017; Rather, 1971).

For a long time, inflammation was viewed as a purely pathological phenomenon. However this concept was further refined in the 18th and 19th centuries by John Hunter and Elie Metchnikoff, who considered it as a natural and beneficial process to sterile or infectious injury, emphasising its functional purpose in tissue repair (Cavaillon, 2021; Turk, 1994).

The introduction of microscopy into medicine in the 19th century was a landmark event, allowing for the histological diagnosis of inflammatory conditions. Researchers soon noticed that neutrophils were predominant in acute inflammation, while monocytes/macrophages and lymphocytes were associated with chronic inflammation (Antonelli & Kushner, 2017). During this time, Rudolf Virchow, often called the "father of modern pathology," significantly changed our understanding of the inflammation concept (Heidland et al., 2006). He proposed that inflammation was not a single condition but a variety of distinct processes (Ramos, 2012). Virchow categorised inflammation into four types: exudative, infiltrative, parenchymatous, and proliferative, while also reinforced the importance of the initial inflammatory stimulus (irritation) (Heidland et al., 2006).

Around the same period, William Addison and Julius Cohnheim revealed that leukocytes could migrate across the blood vessel wall into surrounding tissues, a phenomenon subsequently termed diapedesis (Cavaillon, 2021; Maulitz, 1978). Later, Wilhelm Pfeffer introduced the concept of chemotactism (now known as chemotaxis) to

describe the directed movement of cells in response to chemical stimuli (Lux & Shi, 2004).

In parallel, the phenomenon of fever was reinterpreted from a simple acceleration of circulation to a sophisticated defence mechanism against pathogens. This understanding eventually led to the identification of endogenous pyrogens, including interleukin-1β (IL-1β), tumour necrosis factor (TNF), and interleukin-6 (IL-6) (Cavaillon, 2021; Evans et al., 2015).

With the development of molecular biology in the last half-century, our comprehension of the mechanisms governing inflammation has expanded substantially. A large number of innate immune mechanisms were discovered, such as Toll-like receptors (TLRs), which can recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to trigger inflammatory responses (Akira, 2009). Additionally, C-reactive protein (CRP), an acute phase protein, has become widely applied as a biomarker of inflammation due to its elevated levels during inflammation (Gabay & Kushner, 1999). Accumulating insights in this field have prompted the continual refinement of the criteria used to identify and diagnose inflammatory conditions, thereby emphasising the importance of understanding the underlying mechanisms and diverse manifestations of inflammation.

1.1.2 Classification of Inflammation

Inflammation is a complex immune response to tissue damage or infection, and its complexity goes far beyond its initial description. The process involves a diverse range of immune cells, whose recruitment is precisely regulated by numerous extracellular signalling molecules and regulators (Punchard et al., 2004). Based on its duration, cell types, and pathogenesis, it can be categorised into two main types: acute inflammation and chronic inflammation (Abdulkhaleq et al., 2018).

Acute inflammation is a rapid and short-lived response, typically starting within minutes to hours. The defining features comprise vascular modifications, characterised by vasodilation and enhanced permeability, which result in the leakage of fluid and plasma proteins (exudation) (Ono et al., 2017; Sansbury & Spite, 2016; Wright, 1997). The process involves the rapid recruitment and infiltration of leukocytes, predominantly neutrophils, to the site of injury for the elimination of pathogens or damaged cells. This directed movement, known as extravasation, is precisely coordinated by a combination of pro-inflammatory signals, chemokines, and cell adhesion molecules (CAMs) (Kolaczkowska & Kubes, 2013). As a self-limiting process, acute inflammation usually resolves by restoring normal tissue function (Isailovic et al., 2015).

Chronic inflammation, by contrast, is a prolonged response distinguished by the infiltration of mononuclear cells, such as monocytes/macrophages and lymphocytes (Antonelli & Kushner, 2017). It is often caused by unresolved acute inflammation, persistent infections, or autoimmune reactions (Alfaro et al., 2022). This phase is marked by mononuclear cell infiltration, fibroblast proliferation, as well as the formation of collagen and connective tissue, all of which contribute to the potential development of a granuloma (Abdulkhaleq et al., 2018). Persistent inflammation contributes to the onset and development of numerous chronic diseases, including cardiovascular diseases, cancer, autoimmune disorders, metabolic syndrome and neurodegeneration (Laveti et al., 2013).

1.2 Macrophage Biology and Polarisation

1.2.1 The Evolution of Macrophage Theory

Ever since Elie Metchnikoff proposed the phagocyte theory in the late 19th century, macrophages have been recognised as a core component of host defence (Wynn et al., 2013). As a biologist with a unique background in zoology and comparative embryology, Elie Metchnikoff used microscopy and live observation—particularly with transparent marine invertebrates—to first observe phagocytic cells in starfish larvae in 1883 (Gordon, 2016). This discovery established the major involvement of phagocytes in maintaining biological homeostasis and immune defence. The concepts proposed by Metchnikoff, such as physiological inflammation and active cellular clearance, went beyond the traditional immunology and advanced understanding on the maintenance of organismal integrity (Tauber, 2003). Although his phagocytic hypothesis was initially met with strong criticism, particularly from proponents of humoral immunity, he remained steadfast in his position. His pioneering contributions eventually earned him the Nobel Prize in 1908 and the enduring title of the "Father of Innate Immunity" (Gordon, 2016).

The lasting impact of Metchnikoff's theory continues to shape modern immunology. Over the following decades, our knowledge of macrophage functions expanded far beyond simple pathogen clearance. Macrophages are now known to be integral to tissue repair, antigen presentation, homeostasis, and inflammation regulation. Their contributions extend to numerous pathological contexts, including cancer, metabolic diseases, and autoimmune disorders, demonstrating their complex and multifaceted nature (Wynn et al., 2013).

A major paradigm shift occurred in the 1960s when George B. Mackaness introduced the concept of the activated macrophage. His work in infectious models demonstrated that T-cell-derived cytokines could dramatically enhance the microbicidal capacity of macrophages, providing the first definition of a macrophage activation state and highlighting the functional connection between innate and adaptive immunity (Mackaness, 1962).

This concept was further refined in the 1990s when Stein and colleagues discovered that interleukin-4 could induce a macrophage phenotype distinct from the classical activated state, introducing the term "alternatively activated macrophage" (Stein et al., 1992). This culminated in the work of Mills et al. in 2000, who formalised the M1/M2 polarisation paradigm (Mills et al., 2000). It divides macrophage activation into two main pathways: classical activation (M1), driven by signals such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS), and alternative activation (M2), driven by interleukin-4 (IL-4) and interleukin-13 (IL-13) (Doyle et al., 1994; Mills et al., 2000; Sica & Mantovani, 2012; Stein et al., 1992). This framework has become a cornerstone of modern immunology research.

1.2.2 The Origins and Developmental Pathways of Macrophages

Macrophages are highly plastic and versatile immune cells that are essential for maintaining tissue homeostasis, initiating immune responses, resolving inflammation, and facilitating tissue repair (Locati et al., 2020; Sica & Mantovani, 2012). They are distributed throughout the body and exhibit functional specialisation depending on their microenvironment (Gordon & Taylor, 2005). The developmental origin of macrophages begins in

the bone marrow, where haematopoietic stem cells (HSCs) differentiate into monocyte precursors (Ginhoux & Guilliams, 2016). These monocytes circulate in the bloodstream and then migrate into tissues where they differentiate into specialised, tissue-resident macrophages (Ginhoux & Guilliams, 2016; Gordon & Taylor, 2005).

This process generates a heterogeneous population of macrophages that are finely tuned to the needs of their local environment (Locati et al., 2020). For example, Kupffer cells in the liver are indispensable for clearing pathogens and debris from portal blood; microglia in the central nervous system monitor neuronal health and mediate neuroinflammatory responses; alveolar macrophages in the lungs help maintain airway sterility and surfactant homeostasis; Langerhans cells in the skin perform immune surveillance by detecting and responding to foreign antigens; and osteoclasts in the bone regulate bone remodelling through the resorption of mineralised tissue. Beyond these, specialised macrophages in the kidneys and mammary glands are involved in ductal development, while those in the intestines and eyes contribute to immune surveillance and vascular remodelling, respectively (Ginhoux & Guilliams, 2016; Lendeckel et al., 2022; Pollard, 2009). In addition to these tissue-resident populations derived from circulating monocytes, some macrophages originate from embryonic precursors and can self-renew locally throughout life (Ginhoux & Guilliams, 2016).

1.2.3 Central Roles of Macrophages in Immune Regulation

Macrophages serve as the first line of host defence against disease. These multifunctional cells are pivotal for sustaining physiological balance and directing the immune response. Their functions are highly diverse and adaptable, with specific roles shaped by the stimuli they encounter and the unique environment of the tissue they inhabit (Arora et al., 2018; Wynn et al., 2013).

1.2.3.1 Phagocytosis and Tissue Homeostasis

Macrophages are professional phagocytes with the ability to engulf and eliminate harmful foreign particles and cellular waste (Lim et al., 2017). Phagocytosis, their primary defence mechanism, is essential for both early antimicrobial responses and the maintenance of healthy tissues (Rosales & Uribe-Querol, 2017). This process is enhanced by opsonisation, where pathogens are coated with antibodies or complement proteins that macrophage receptors recognise. Many of the receptors are classified as clusters of differentiation (CDs), which are used to identify different immune cell types. Once engulfed, the pathogen is enclosed in a phagolysosome, where it is destroyed using reactive oxygen species (ROS) and digestive enzymes (Lendeckel et al., 2022). The acidic conditions within lysosomes mediate proper degradation of engulfed material (Tarique et al., 2024; Yunna et al., 2020).

Beyond pathogen clearance, macrophages are principal modulators of tissue homeostasis. They remove senescent and apoptotic cells, along with other cellular debris, to preserve tissue integrity (Uribe-Querol & Rosales, 2020). They also regulate key physiological processes such as metabolism and the maintenance of neural connectivity, both vital for proper brain function (Wynn et al., 2013).

1.2.3.2 Antigen Presentation

As the major antigen-presenting cells (APCs), macrophages also bridge the interaction between innate and adaptive immunity (Lendeckel et al., 2022). Upon engulfing and lysosomally degrading pathogens, macrophages process their proteins into antigenic peptides. These peptides are then loaded onto major histocompatibility complex (MHC) class II molecules within the endocytic pathway. The resulting peptide-MHC complexes are transported to the surface of the macrophage for presentation to T helper cells (CD4+). This specific interaction activates the T helper cells, initiating a coordinated and sustained antigen-specific immune response that includes the activation of B cells for antibody production and cytotoxic T cells for eliminating infected cells (Lendeckel et al., 2022; Unanue, 1984).

1.2.3.3 Immunomodulation and Regulation

Macrophages contribute dramatically to the regulation of immune responses, /addBand are capable of adapting their function in response to environmental signals. They shift between pro-inflammatory (M1) and anti-inflammatory or tissue-repairing (M2) phenotypes, a functional plasticity that enables them to control the intensity and duration of immune responses (Lendeckel et al., 2022; Mantovani et al., 2004). Maintaining a balanced M1/M2 response supports effective pathogen elimination while minimising tissue damage.

As master regulators of inflammation, macrophages secrete a broad range of signalling molecules, including cytokines and chemokines, to coordinate immune activity (Lendeckel et al., 2022). Their influence also extends beyond immunity, as they promote angiogenesis, regulate collagen production during tissue repair, and control the proliferation of haematopoietic progenitor cells which mediate blood cell formation (Wynn et al., 2013; Yu et al., 2022).

1.2.4 Macrophage Polarisation: M1 vs M2

A defining feature of macrophages is their ability to polarise into distinct phenotypes in response to microenvironmental signals. Based on their activation state and these signals, macrophages are traditionally classified into two major phenotypic categories, although several intermediate and specialised subtypes have since been identified (Arora et al., 2018; Lampiasi, 2023; Mantovani et al., 2004).

Before activation, macrophages exist as M0 macrophages, which are unpolarised and responsible for maintaining tissue homeostasis. These resting macrophages efficiently clear senescent and apoptotic cells, as well as invading pathogens. Examples include microglia, Kupffer cells, and alveolar macrophages, all of which are specialised M0 macrophages (Lampiasi, 2023). When activated, macrophages polarise into two principal types: M1 (classically activated) macrophages and M2 (alternatively activated) macrophages (Mills et al., 2000).

1.2.4.1 M1 Polarisation

M1 macrophages, often termed "pro-inflammatory" or "killer" macrophages, are typically activated by microbial products such as LPS from Gram-negative bacteria, and by T-helper 1 (Th1) cytokines, exemplified by IFN-γ (Biswas & Mantovani, 2010; Parisi et al., 2018). This classical activation pathway leads to a ma-

jor metabolic reprogramming and the upregulation of key enzymes such as inducible nitric oxide synthase (iNOS), which produces large amounts of nitric oxide (NO), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which generates ROS (Guo et al., 2021; Lendeckel et al., 2022). Together, these molecules enable M1 macrophages to effectively eliminate pathogens and inhibit tumour growth.

In addition to their cytotoxic functions, M1 macrophages are efficient producers of pro-inflammatory cytokines, namely TNF-α, IL-1β, and IL-6, as well as interleukin-12 (IL-12) and interleukin-23 (IL-23), which enhance T-cell-mediated responses against intracellular pathogens (Biswas & Mantovani, 2010).

M1 macrophages also contribute to the activation of the adaptive immune system. They express high levels of major histocompatibility complex class II (MHC-II) molecules for antigen presentation, and co-stimulatory molecules including CD40, CD80, and CD86, which are essential for effective T-cell activation (Ambarus et al., 2012; Yamasaki & Eeden, 2018). High-affinity Fc receptors, such as CD64 (FcγRI), further enhance their ability to recognise antibodies and phagocytose opsonised pathogens (Hristodorov et al., 2015).

1.2.4.2 M2 Polarisation

Conversely, M2 macrophages, often referred to as "anti-inflammatory" or "healer" macrophages, are induced by T-helper 2 (Th2) cytokines such as IL-4 and IL-13, or by immune complexes, apoptotic cells, and certain growth factors (Bashir et al., 2016; Biswas & Mantovani, 2010; Du et al., 2024). M2 macrophages are characterised by the expression of specific markers, comprising arginase-1 (Arg1), resistin-like-α (Fizz1), mannose receptor (CD206), and scavenger receptor (CD163) (Arora et al., 2018; Martinez & Gordon, 2014). They also produce anti-inflammatory and immunosuppressive cytokines, including interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (Guo et al., 2021). Their primary roles encompass the resolution of inflammation, the efferocytosis of apoptotic cells, tissue repair and remodelling through the production of extracellular matrix components, angiogenesis, and the suppression of excessive adaptive immune responses (Arora et al., 2018).

The M2 phenotype is further divided into specialised subtypes, consisting of M2a, M2b, M2c, and M2d, each with distinct inducers and functions (Lampiasi, 2023). For example, M2a, typically activated by IL-4 and IL-13, is involved in host defence against parasites and promoting tissue repair (Arora et al., 2018). M2b macrophages are induced by immune complexes and toll-like receptor ligands, resulting in the modulation of the immune response by producing a mix of cytokines (L.-x. Wang et al., 2019). Meanwhile, M2c macrophages are activated by IL-10 and TGF-β, promoting immune regulation and inflammation resolution (Mamilos et al., 2023). Besides, M2d macrophages, often found in tumours and induced by adenosine and other factors, promote angiogenesis and facilitate immune evasion by cancer cells (Arora et al., 2018). More recently, a novel macrophage subtype, termed Mox or M4, has been identified. These cells represent intermediate phenotypes, polarised by oxidised low-density lipoprotein (oxLDL). They share characteristics of both M1 and M2 macrophages, combining pro-inflammatory ROS production with M2-like tissue repair functions (Lampiasi, 2023).

Macrophage polarisation is a dynamic and heterogeneous process that extends beyond the traditional binary M1/M2 classification. Table 1 lists the major macrophage polarisation phenotypes, detailing their inducing stimuli, characteristic markers, cytokine secretion, and biological functions.

Table 1: Macrophage polarisation subtypes

Phenotype	Inducers	Markers	Cytokines & Effectors	Functions	Reference
M1	IFN-γ, LPS, GM-CSF, TNF-α, IL-1β, IL-6; bacterial infection via TLRs	CD80, CD86, iNOS/NOS2, MHC Class II, High IL-12, Low IL-10, IL-23, IDO, CCL8, CXCL10/11, CD64 (FcγRI)	IL-1β, TNF-α, IL-6, IL-12, IL-15, IL-23; RNI/ROI, NO, COX-2	Microbial elimination, inflammation initiation, tumour suppression, phagocytosis, tissue damage	(Arora et al., 2018; Lampiasi, 2023; Martinez & Gordon, 2014)
M2a	IL-4, IL-13; mainly from mast cells, Th2 cells, and basophils	Arg1, Fizz1, Mrc1/CD206, Ym1, CD23, MHC Class II	Fibronectin, IGF, TGF-β, IL-10, CCL17/18/22	Immunoregulation, wound healing, tissue remodelling, parasite clearance	(Arora et al., 2018; Lampiasi, 2023; Martinez & Gordon, 2014)
M2b	Immune complexes + TLR ligands, IL-1R	CD86, Low IL-12, High IL-10	IL-1β, TNF-α, IL-6, IL-10, CCL1, CCL18	Immune regulation, Th2 differentiation, humoral response, LPS detoxification	(Arora et al., 2018; Lampiasi, 2023; Martinez & Gordon, 2014)
M2c	IL-10, Glucocorticoids, TGF-β	Low IL-12, High IL-10	IL-10, TGF-β, CCL16, CCL18	Inflammation suppression, repair, phagocytosis of debris	(Arora et al., 2018; Lampiasi, 2023; Martinez & Gordon, 2014)
M2d	Tumour-associated factors, A2AR	Low IL-12, High IL-10	IL-10, VEGF, TNF-α, IL-12	Tumour progression, angiogenesis, wound healing	(Arora et al., 2018; Lampiasi, 2023)
Mox / M4	Oxidised LDL (oxLDL)	HO-1, Srxn1	ROS	Mixed phenotype: inflammation + repair; linked to atherosclerosis	(Arora et al., 2018; Lampiasi, 2023)

1.2.4.3 Balance and Pathology

Proper regulation of the M1/M2 macrophage ratio supports tissue homeostasis, while imbalances often drive pathological processes (Sica & Mantovani, 2012). An M1-dominant environment tends to sustain chronic inflammation and contribute to autoimmune conditions (Martinez & Gordon, 2014). In contrast, an M2-dominant state can suppress anti-tumour immunity and promote tumour progression (N.-B. Hao et al., 2012). The dynamic transition between these two phenotypes contributes to the regulation of immune function and influences the outcome of inflammatory responses (Arora et al., 2018).

Normally, M1 macrophages are critical for killing pathogens and clearing damaged cells at the start of an infection. However, if this activation persists, it can lead to the continuous release of destructive cytokines (e.g., TNF-α and IL-1β) (Vandooren et al., 2009). A predominance of M1-polarised macrophages is frequently observed in chronic inflammatory and autoimmune diseases such as rheumatoid arthritis, lupus nephritis, atherosclerosis, and inflammatory bowel disease. In these contexts, sustained M1 activity promotes tissue damage and resolution failure (Sica & Mantovani, 2012).

In contrast, environments dominated by M2-like macrophages can suppress effective immune responses, particularly within the tumour microenvironment. These cells, often referred to as tumour-associated macrophages (TAMs), are reprogrammed by cancer-derived signals to adopt a pro-tumoural phenotype (N.-B. Hao et al., 2012). TAMs are instrumental in angiogenesis, extracellular matrix remodelling, and immune suppression, creating an environment conducive to tumour growth and metastasis. By promoting new blood vessel formation and inhibiting cytotoxic immune responses, M2-polarised TAMs enable the tumour to thrive and escape immune surveillance (Biswas & Mantovani, 2010; Sica & Mantovani, 2012).

The balance between M1 and M2 macrophages is not a static classification but a dynamic process that shifts in response to the cellular environment. In reality, rather than existing as purely M1 or M2 cells, macrophages often display a mixed phenotype, as seen in conditions like ischaemic injury (Sica & Mantovani, 2012). Targeting macrophage polarisation to favour a pro-resolving or anti-inflammatory state offers considerable potential as a precise therapy for inflammatory diseases.

1.2.5 Regulatory Mechanisms and Natural Compounds

Macrophage polarisation is tightly regulated by multiple interconnected signalling pathways, which often operate in an upstream-downstream manner. TLRs, a class of pattern recognition receptors, sense microbial components and damage-associated molecular patterns, and act as pivotal upstream sensors that initiate macrophage activation (Akira, 2009). Upon TLRs engagement, downstream signalling cascades, including NF-κB, MAPK, and PI3K/Akt pathways, are activated to coordinate inflammatory and reparative responses (Kawai & Akira, 2010). In parallel, cytokine-mediated pathways such as JAK/STAT refine macrophage polarisation, assembling signals from the microenvironment (Murray, 2017). Natural compounds can modulate these pathways through multiple regulatory mechanisms (Da Cunha et al., 2019; Gao et al., 2015; Merecz-Sadowska et al., 2020; Tian et al., 2017), thereby shaping macrophage polarisation states and influencing inflammatory responses.

1.2.5.1 JAK/STAT Signalling Pathway

The JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway is a critical cytokine-driven cascade that acts downstream of receptor engagement. While TLRs predominantly activate NF-κB and MAPK pathways, cytokines such as IFN-γ, IL-4, IL-13, and IL-10 further shape macrophage responses through JAK/STAT signalling (Hu et al., 2021). Following cytokine binding, receptor-associated JAKs (e.g., JAK1, JAK2, and JAK3) become phosphorylated and, in turn, phosphorylate specific STAT proteins (e.g., STAT1, STAT3, STAT5, and STAT6) (Hu et al., 2021; Philips et al., 2022). Activated STATs dimerise and translocate to the nucleus, where they regulate the transcription of genes involved in immune responses, inflammation, proliferation, and cell differentiation (Harrison, 2012). In macrophages, this pathway plays a central role in polarisation. IFN-γ activates the JAK1/2-STAT1 axis to promote M1 polarisation and the expression of pro-inflammatory mediators such as TNF-α and IL-1β. In contrast, after interacting with JAK family, IL-4 and IL-13 activate STAT6 to drive M2 polarisation and expression of M2 markers, such as IL-10, arginase-1 (Arg-1) and macrophage mannose receptor (MMR, also known as CD206) (Li et al., 2023). IL-10 contributes to M2-like polarisation via activating JAK1 to induce STAT3 phosphorylation (Saxton et al., 2021). The pathway is tightly regulated by negative feedback proteins such as suppressor of cytokine signalling / cytokine-inducible SH2-containing protein (SOCS/CIS) family and protein tyrosine phosphatases (PTPs), while its dysregulation is implicated in various autoimmune diseases and cancers (Hu et al., 2021).

1.2.5.2 NF-κB Signalling Pathway

As a dominant transcription factor downstream of TLRs and other pattern recognition receptors, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway is deeply involved in regulating immune-mediated inflammatory responses. Core components include NF-κB subunits (p65/RelA and p50), the inhibitory protein IκB, and the IKK complex (Anilkumar & Wright-Jin, 2024). NF-κB activation occurs

via two distinct pathways: the canonical (classical) and noncanonical (alternative) pathways, which differ in their activating stimuli and molecular mechanisms. The canonical pathway is characterised by rapid activation, typically triggered by acute inflammatory signals, for example, TNF-α, IL-1, or microbial products. This leads to activation of the IKK complex, phosphorylation and degradation of IκB, along with nuclear translocation of NF-κB dimers such as p65/p50. Once in the nucleus, NF-κB drives transcription of pro-inflammatory genes that coordinate macrophage activation and immune responses (Lawrence, 2009). Whereas, the noncanonical pathway is slower and more sustained, induced by specific TNF family members including lymphotoxin-β (LTβ), CD40 ligand (CD40L), B cell activating factor (BAFF), and receptor activator of nuclear factor κB ligand (RANKL), but not by TNF-α (Lawrence, 2009). This pathway contributes to adaptive immune functions, with roles in lymphoid organ development and B cell function (Oeckinghaus et al., 2011).

NF-κB is the principal transcription factor governing M1 macrophage polarisation, with STAT1 playing a supportive role. Although M2-like polarisation primarily depends on STAT6 activation, NF-κB signalling also contributes to this process (Mussbacher et al., 2023). During wound healing, macrophage-derived IL-1β activates NF-κB, which subsequently promotes autocrine, STAT6-dependent transcription of vascular endothelial growth factor A (VEGF-A), a pro-angiogenic factor that promotes arteriogenesis (Mantsounga et al., 2022). Impaired regulation of NF-κB signalling is associated with diseases, for instance, rheumatoid arthritis, cancer, and chronic inflammatory conditions (Oeckinghaus & Ghosh, 2009).

1.2.5.3 PI3K/Akt Signalling Pathway

Phosphoinositide 3-Kinase / Protein Kinase B (PI3K/Akt) constitutes an intracellular signalling pathway that regulates a wide range of processes, involving metabolism, growth, survival, and immune function (Vara et al., 2004). It is activated downstream of TLRs, growth factors, and cytokines through receptor tyrosine kinases or adaptor proteins, which stimulate PI3K and lead to the phosphorylation of Akt (Koyasu, 2003). Activation of the PI3K/Akt pathway regulates macrophage polarisation by promoting the transcription of main anti-inflammatory cytokines such as Arg-1 and IL-10, while simultaneously suppressing pro-inflammatory markers. In contrast, inhibition of PI3K signalling promotes M1 macrophage polarisation, emphasising the importance of this pathway in determining macrophage functional states. It also aligns the metabolic state of macrophages with the energetic demands of M2 activation, suggesting a mechanistic connection between cellular metabolism and macrophage function (Lu et al., 2017). However, this pathway also promotes the persistence and aggravation of inflammation by sustaining the continuous recruitment of immune cells through a positive feedback loop. This mechanism may be an important driver of chronic inflammatory conditions and inflammation-associated diseases (Khan et al., 2013).

1.2.5.4 MAPK Pathways

The Mitogen-Activated Protein Kinase (MAPK) pathway is a major downstream mediator of TLRs and other upstream signals. It is a complex and fundamental cellular signalling system, encompassing the three major branches: Extracellular Signal-Regulated Kinase 1/2 (ERK1/2), c-Jun N-terminal Kinase (JNK), and p38 MAPK (Pearson et al., 2001). These three pathways are activated by distinct stimuli and regulate diverse functional responses in macrophages. ERK1/2, typically triggered by growth factors, with M-CSF as a representative example, promotes macrophage proliferation and survival (Valledor et al., 1999). The JNK pathway, generally activated by stress and inflammatory signals, influences macrophage polarisation, primarily by en-

hancing the anti-inflammatory M2 phenotype in response to cytokines (e.g., IL-4) (J. Hao et al., 2017). Importantly, p38 MAPK functions as a master regulator of the acute inflammatory response, being strongly activated by stress stimuli, pro-inflammatory cytokines (e.g., IL-1 and TNF-α), and pathogen-associated molecular patterns such as LPS. Activation of this pathway drives the expression of pro-inflammatory mediators, including TNF-α and COX-2, and contributes to M1 macrophage polarisation and enhanced phagocytic activity during bacterial infection (Yang et al., 2014). Although each MAPK branch exhibits preferential activation and functional outcomes, the simultaneous activation of all three pathways by pathogens and stress reflects the complex and synergistic interaction for the full regulation of macrophage function (Rao, 2001).

1.2.5.5 Natural Compounds Influencing the Regulation of Macrophage Polarisation

Natural compounds are gaining attention as potential therapeutic agents for modulating immune responses and macrophage polarisation due to their low toxicity and multi-target effects. For example, curcumin, the primary active compound in turmeric, has been shown to influence macrophage polarisation. It mediated anti-inflammatory activity by promoting STAT6 activation and enhancing the secretion of IL-4 and/or IL-13, while downregulating pro-inflammatory markers including iNOS and IL-1β (Gao et al., 2015). This effectively shifts macrophage polarisation toward an M2 macrophage phenotype and suppresses M1-associated characteristics. Another compound, shikonin, derived from the root of *Lithospermum erythrorhizon*, presents anti-inflammatory effects by downregulating the MAPK and NF-κB pathways, thereby reducing pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Tian et al., 2017). In various inflammation and disease models, shikonin has been shown to inhibit M1 macrophage polarisation and alleviate pathological inflammation (Lianhua et al., 2022; Tian et al., 2017).

Immunomodulatory potential has also been demonstrated by several native New Zealand edible plants, specifically kawakawa, horopito, mānuka, koromiko, and pūhā (Rashidinejad, 2024). Although the exact mechanisms by which natural compounds regulate macrophage polarisation require further exploration, their therapeutic potential has been widely recognised (Earl et al., 2010; Koia & Shepherd, 2020; Mathew et al., 2020; Rashidinejad, 2024; Tautuiaki et al., 2024), providing new perspectives and strategies for immune modulation therapy.

1.3 Kawakawa (*Piper excelsum*): Traditional Use, Bioactivity, and Immunomodulatory Potential)

1.3.1 Preface

The preceding sections of this chapter have established the fundamental principles of inflammation, the critical role of macrophage polarisation in immune regulation, and how various natural compounds can modulate these pathways. Section 1.3 narrows its focus to a specific indigenous medicinal plant of Aotearoa New Zealand, *Piper excelsum* (Kawakawa).

This section examines how traditional Māori knowledge (Rongoā Māori) and modern scientific research can inform one another. It provides an overview of kawakawa's traditional uses, key phytochemical constituents,

and emerging evidence of its immunomodulatory properties. Through a synthesis of existing research, this work aims to investigate the biological mechanisms of kawakawa and its possible applications in modern medicine and nutraceutical development.

The review presented in this chapter is formatted as a manuscript prepared for submission to a peer-reviewed journal.

1.3.2 Abstract

Kawakawa (*Piper excelsum*) is a plant rooted in New Zealand's indigenous knowledge and cultural practices, and has been extensively used in Māori traditional medicine (Rongoā Māori) for its therapeutic properties. This review examines evidence concerning its traditional use, phytochemical composition, safety, and reported anti-inflammatory, antimicrobial, anthelmintic, and cytotoxic activities. Kawakawa contains diverse amides, lignans, phenylpropanoids, and flavonoids, but direct evidence for pharmacological effects of whole kawakawa preparations remains limited. Most mechanistic findings are derived from *in vitro* studies. One acute human intervention reported changes in circulating mRNA, microRNA, and cytokine measures after kawakawa tea consumption, but these results require independent replication. Available animal data support the short-term safety of aqueous leaf preparations under the conditions tested, although they do not establish long-term safety or safety across different plant parts and extraction methods. Overall, kawakawa is a promising source of bioactive compounds, but current evidence is insufficient to support therapeutic efficacy. Future work should prioritise chemically standardised preparations, dose-response studies, validated mechanistic endpoints, longer-term toxicology, and well-controlled clinical trials developed in partnership with Māori and consistent with the status of kawakawa as taonga.

Keywords: *Piper excelsum*, bioactive compounds, immunomodulation, kawakawa, macrophage defence

1.3.3 Connecting Traditional Knowledge and Modern Science

Kawakawa (*Piper excelsum*) is a native shrub of Aotearoa New Zealand, holding a central place in the traditional Māori system of medicine known as Rongoā Māori (Brooker & Cooper, 1961). The plant is a member of the *Piperaceae* family, which also includes well-known species such as black pepper (*Piper nigrum*) and kava (*Piper methysticum*) (Salehi et al., 2019). The geometrid moth *Cleora scriptaria* (S. Hodge et al., 1998) feeds on the leaves of the kawakawa plant, leaving behind distinctive holes that make the effects of herbivory more noticeable. Interestingly, Māori tradition holds a preference for kawakawa leaves with insect damage, as this damage is thought to activate defence mechanisms within the plant, which in turn increase the concentrations of bioactive compounds (Brooker & Cooper, 1961). While this explanation is biologically plausible, the effect of herbivory on therapeutically relevant metabolites in kawakawa has not been directly established and should be tested experimentally.

For centuries, the leaves, stems, and roots of the kawakawa plant have been employed by Māori healers, known as tohunga and kairongoā, in diverse therapeutic contexts (Koia & Shepherd, 2020; Ramchundar & Nlooto, 2020; Riley, 1994). This traditional use has not been limited to internal consumption but has encompassed a range of applications, including topical balms, poultices, and even steam-based treatments (Briggs, 1941;

Rashidinejad, 2024; Riley, 1994). These records provide important context and hypotheses for pharmacological research. Traditional knowledge should not, however, be framed as requiring scientific validation; rather, laboratory and clinical research can examine specific biological effects, mechanisms, dosage, and safety under defined conditions.

The numbing sensation produced by chewed kawakawa leaves is consistent with the sensory activity of pellitorine and related amides identified in the plant (Elufioye et al., 2020; Obst et al., 2017). Dopamine has also been detected in kawakawa leaf extracts (Jayaprakash et al., 2022), but its presence alone does not establish a mechanism for the traditional gastrointestinal use of kawakawa tea. The delivered concentration, stability throughout preparation and digestion, bioavailability, and peripheral pharmacological effects would all need to be demonstrated.

The primary purpose of this review is to provide a comprehensive synthesis of the available scientific literature on *Piper excelsum*, incorporating traditional Rongoā Māori knowledge with modern scientific frameworks. This review critically synthesises published research on the traditional context, phytochemistry, biological activity, safety of kawakawa, and most importantly, immunomodulatory effects. The review bridges a major knowledge gap, as the scientific community remains in the early stages of understanding the precise mechanisms of action governing the effects of kawakawa. A thorough consolidation of current findings is therefore a critical step toward its potential integration into modern therapeutic and nutraceutical applications.

1.3.4 Historical Background of Kawakawa Use

1.3.4.1 Traditional Applications in Rongoā Māori

Kawakawa has long been a key element of traditional Māori medicine, revered as one of the most important healing herbs (rongoā rākau) of the New Zealand bush (Koia & Shepherd, 2020; Riley, 1994). The plant possesses considerable therapeutic potential across diverse conditions. For internal use, kawakawa was commonly prepared as a tea or decoction to treat digestive issues such as stomach pains and indigestion, with some records indicating its use for dysentery and parasitic infections (Briggs, 1941; Riley, 1994). The leaves were also chewed to relieve toothaches, a practice attributed to their numbing properties (Obst et al., 2017). Topical applications were equally prevalent. Poultices made from the leaves were applied directly to the skin to treat abrasions, wounds, boils, stings, and various inflammatory skin conditions, including eczema and psoriasis (Brooker & Cooper, 1961). In cultural contexts, kawakawa was used to treat rheumatism and other ailments through steam baths, where heated stones and water were used to create a vapour infusion of the plant (Briggs, 1941).

Beyond its medicinal uses, the plant is considered a taonga, a highly prized cultural treasure for Māori, with its significance extending beyond its medicinal applications to its symbolic and spiritual connection. It holds an important place in rituals and ceremonies as an emblem of healing and protection, where its heart-shaped leaves are also associated with love and compassion (Aporosa & Forde, 2019; Joseph, 2022; Riley, 1994).

1.3.4.2 Kawakawa (*Piper excelsum*) versus Kava (*Piper methysticum*): Why the Distinction Matters

Kawakawa (*Piper excelsum*) and kava (*Piper methysticum*) are distinct species within the *Piperaceae* family. Kava is consumed as a psychoactive beverage in ceremonial and social contexts across the Pacific, whereas kawakawa has traditionally been used in Aotearoa New Zealand primarily as a medicinal plant (Aporosa & Forde, 2019; Butts et al., 2019). Despite their similar common names and botanical relatedness, evidence from one species should not be directly extrapolated to the other. The Māori word kawa, which also refers to marae protocol, may hint at a historical ceremonial use of kava. Although it is thought that early Polynesian settlers introduced kava to New Zealand, the plant did not survive the colder climate, and kawakawa became the culturally and therapeutically significant alternative (Aporosa & Forde, 2019).

This distinction is not merely cultural but fundamental to the safety profiles associated with kawakawa. Kava has been linked to rare but serious cases of hepatotoxicity (liver damage), which has led to its restriction or banning in several countries. These reports of liver injury have been associated with several factors, such as methods involving solvent-based extracts, low-quality cultivars containing stems and leaves, drug interactions, and possible genetic variations (Fu et al., 2008). In contrast, traditional use of kawakawa, particularly as a water-based tea, has a favourable safety record, supported by preliminary scientific findings (Butts et al., 2019).

By emphasising their different uses (medicinal versus psychoactive) and preparation methods, it becomes clear that the safety concerns associated with kava do not inherently apply to kawakawa. Differences in cultural use and botanical origin make it clear that their therapeutic profiles should be regarded independently.

1.3.5 Advanced Pharmacological Profile

1.3.5.1 Anti-inflammatory and Immunomodulatory Mechanisms

The function of kawakawa is built upon its strong immunomodulatory properties, with powerful anti-inflammatory and anticancer effects. These effects may occur through a multi-level mechanism, influencing gene expression, regulating signalling pathways, and directly modulating immune cell function. The major immunomodulatory effects of kawakawa and the supporting evidence are summarised in Table 2. The anti-inflammatory capacity constitutes its most well-studied pharmacological activity, supported by evidence from traditional use, *in vitro* studies, and human clinical trials. Its effects are not a singular action but rather a complex, multi-targeted approach to modulating the immune system (Tautuiaki et al., 2024).

Regulation of Signalling Pathways

NF-κB regulates the transcription of many inflammatory genes, including those encoding cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Anilkumar & Wright-Jin, 2024; T. Liu et al., 2017). In engineered reporter systems, secreted alkaline phosphatase (SEAP) can serve as a downstream measure of NF-κB activation (T. Liu et al., 2017). A postgraduate thesis reported that kawakawa leaf extracts reduced SEAP activity and altered IL-6 and nuclear factor kappa B subunit 1 (NFKB1) transcript abundance in human embryonic kidney (HEK)-Blue™ and human colorectal adenocarcinoma (HT-29) models (H. Xu, 2018). These results are consistent with modulation of inflammatory signalling, but they do not by themselves demonstrate

direct inhibition of NF-κB, nor have they yet been independently replicated in a peer-reviewed study. Direct measurements of pathway activation, including IκB degradation and NF-κB nuclear translocation or phosphorylation, are needed.

In addition to modulating the NF-κB pathway, clinical studies from Tūhauora Programme have shown that kawakawa intake alters the expression of microRNAs (miRNAs), small noncoding RNAs involved in post-transcriptional gene regulation (Gojer, 2023; Tautuiaki, 2023; Tautuiaki et al., 2024). These miRNA-driven changes are associated with the enrichment of key inflammation-related signalling pathways, specifically the Mitogen-Activated Protein Kinase (MAPK) and Mammalian Target of Rapamycin (mTOR) pathways. The MAPK pathway transduces extracellular inflammatory signals into intracellular responses, regulating pro-inflammatory mediators such as TNF-α, IL-12 and IL-1 (Soares-Silva et al., 2016), while the mTOR pathway integrates internal and external cellular signals to control immune cell activation, autophagy, proliferation and metabolism (Laplante & Sabatini, 2009). The influence of kawakawa on these pathways suggests that its bioactive compounds may function as multi-target modulators, mediating systems-level effects across epigenetic regulation, immune signalling, and cellular function.

Immune Cell Modulation

Macrophages coordinate antimicrobial defence, inflammation, resolution, and tissue repair (Locati et al., 2020). Kawakawa extracts have been revealed to influence the function of these cells. In a study by Newland (2016), THP-1 macrophages were found to exhibit altered expression of TNF-α and IL-6 following exposure to kawakawa extracts, with effects dependent on both extraction temperature and dose. Furthermore, the study reported that kawakawa extracts affected macrophage mitochondrial activity and cell lysis rates. Low concentrations of the extracts enhanced cell viability and function, which indicated a beneficial effect on cellular health and immune responsiveness. The effect of extraction temperature on this bioactivity further highlights the need for standardised preparation methods to ensure consistent and reproducible results in both research and product development.

Beyond the effects on macrophages, kawakawa extracts have demonstrated anti-inflammatory activity in other human cell models. A study using HEK-Blue™, nucleotide-binding oligomerisation domain-containing protein 2 (NOD2) and HT-29 cell models found that kawakawa leaf extracts presented anti-inflammatory effects by inhibiting the production of SEAP and downregulating gene expression of IL-6 and NFKB1 (H. Xu, 2018). These findings further support the broader anti-inflammatory potential of kawakawa, consistent with observations from macrophage models and indicating its potential to influence immune-related pathways across diverse cell types.

1.3.5.2 Cytotoxic Activity in Cancer Cell Models

Recent *in vitro* studies have identified promising anticancer properties in kawakawa fruit extracts and their bioactive constituents, particularly the amide compounds such as piperdardine and piperchabamide. These compounds have shown selective cytotoxicity against a variety of human cancer cell lines, including colorectal adenocarcinoma (HT-29), triple-negative breast cancer (MDA-MB-231), and prostate cancer (PC3), while exhibiting minimal or no toxicity toward non-transformed human mesenchymal stem cells (RCB2157). In some cases, low concentrations of the extracts even appeared to support the viability of normal cells, suggesting a

favourable therapeutic window and selective targeting of malignant cells (Lei et al., 2015). However, these findings are limited to *in vitro* observations. They may help identify promising compounds for further investigation, but they do not, on their own, demonstrate tumour selectivity, a meaningful therapeutic window, or anticancer efficacy *in vivo*.

Further evidence from H. Xu (2018) indicated that ethanol extracts of kawakawa leaves induced concentration-dependent cytotoxicity in HT-29 colon cancer cells, with 50% cell death observed at experimentally relevant concentrations. The half maximal inhibitory concentration (IC_{50}) values were subsequently used in gene expression assays to assess potential mechanisms of action. The results showed considerable suppression of pro-inflammatory and pro-survival genes, including IL-6 and NFKB1, both of which are implicated in tumour progression, angiogenesis, and resistance to apoptosis. These observations reveal potential anticancer effects of kawakawa, which may be mediated, at least in part, through suppression of inflammatory signalling pathways that also support tumour survival and proliferation. However, because altered viability can secondarily affect gene-expression measurements, pathway-specific conclusions require controls that separate cytotoxicity from signalling effects.

Another study using traditional Māori plant extracts, including kawakawa, puha, and watercress, explored their potential effects on prostate cancer using LnCaP and LnCaP+AKR1C3 cell lines. Kawakawa treatment modestly reduced prostate specific antigen (PSA) levels in LnCaP cells, and when combined with the carcinogen benzo(a)pyrene, PSA levels decreased more significantly, which support a possible inhibitory effect on prostate cancer progression. Kawakawa also reduced DNA damage in both LnCaP and LnCaP+AKR1C3 cells, indicating a protective effect on genomic integrity, whereas puha and watercress did not show such benefits. The extract effectively inhibited AKR1C3 enzyme activity, which is involved in androgen metabolism and associated with prostate cancer proliferation, particularly in AKR1C3-overexpressing cells (Siriwardena, 2018). This result suggests that kawakawa holds promise as a therapeutic agent for influencing molecular pathways associated with prostate cancer. These exploratory results should nevertheless be treated as preliminary since they derive from cell-line experiments and have not established efficacy, selectivity, pharmacokinetics, or safety in animals or humans. Further research, particularly in animal models, is needed to fully illustrate anticancer mechanisms of kawakawa.

1.3.5.3 Anthelmintic Properties and Other Reported Bioactivities

The biological activities of kawakawa have been investigated in relation to its traditional medicinal uses, with some studies reporting promising anthelmintic (anti-worm) properties. In particular, its potential application in controlling parasitic nematodes in livestock has attracted research interest. Gupta (2022) investigated the nematocidal activity of different kawakawa plant extracts and found that leaf extracts exhibited substantial activity against infective-stage nematode larvae. The observed activity varied depending on the extraction solvent, with the aqueous fraction achieving up to 81.7% larval mortality in one experiment. Further experiments demonstrated that combining the kawakawa aqueous fraction with ivermectin resulted in up to 92.0% larval mortality, although the effects varied between experiments and larval batches. These findings suggest that kawakawa leaves contain compounds with potential anthelmintic activity and may enhance the effectiveness of conventional antiparasitic treatments. Nevertheless, subsequent research is necessary before their potential application in veterinary or human medicine can be established.

Evidence for the antibacterial activity of kawakawa, however, is less conclusive. Although previous studies

have screened various New Zealand native plants for antimicrobial activities (Calder et al., 1986; Earl, 2010), these findings do not provide sufficient evidence to establish the antibacterial effectiveness of kawakawa. Consequently, its potential activity against bacterial pathogens remains unclear and warrants further investigation.

Table 2: Immunomodulatory effects of kawakawa

Category	Description	Relevant Sources
Immune Pathways	NF-κB signalling: Inhibition of NF-κB signalling and pro-inflammatory gene expression (e.g., IL-6, NFKB1, SEAP).	(H. Xu, 2018)
	MAPK/mTOR pathways: Modulation of these pathways via altered miRNA expression.	(Gojer, 2023; Tautuiaki et al., 2024)
	Insulin, p53, and cancer signalling: Significantly modulated by kawakawa extracts.	(Gojer, 2023; Tautuiaki et al., 2024)
Immune Cells	Macrophages (THP-1): Dose- and temperature-dependent anti-inflammatory and mitochondrial effects.	(Newland, 2016)
	HEK-Blue™, NOD2 and HT-29: Kawakawa downregulates pro-inflammatory gene expression.	(H. Xu, 2018)
	HT-29, MDA-MB-231, PC3 and RCB2157: Kawakawa-derived amides showed selective cytotoxicity against cancer cells.	(Lei et al., 2015)
	HeLa and HEPG2 cells: Evaluation of the cellular effects of kawakawa, particularly on inflammatory responses.	(Harvey, 2021)
Cytokines & Markers	IL-6, IL-8, IL-1β, TNF-α: Downregulation of these pro-inflammatory cytokines.	(Harvey, 2021; Jayaprakash, 2023; Tautuiaki, 2023)
	miRNAs (e.g., hsa-miR-17-5p, hsa-miR-144-3p): Modulation of miRNAs involved in immune response and metabolism.	(Gojer, 2023; Tautuiaki et al., 2024)

1.3.6 Phytochemical Profile and Bioactive Compounds

Kawakawa contains more than 60 bioactive compounds that are believed to contribute to its therapeutic effects (Jayaprakash et al., 2022). These phytochemicals are found in varying concentrations depending on the source and preparation method. The key compound classes identified in kawakawa through liquid chromatography–tandem mass spectrometry (LC-MS/MS) include amides, lignans, phenylpropanoids, and flavonoids (Butts et al., 2019; Jayaprakash et al., 2022). Figure 1 illustrates the chemical structures of representative bioactive compounds identified in kawakawa.

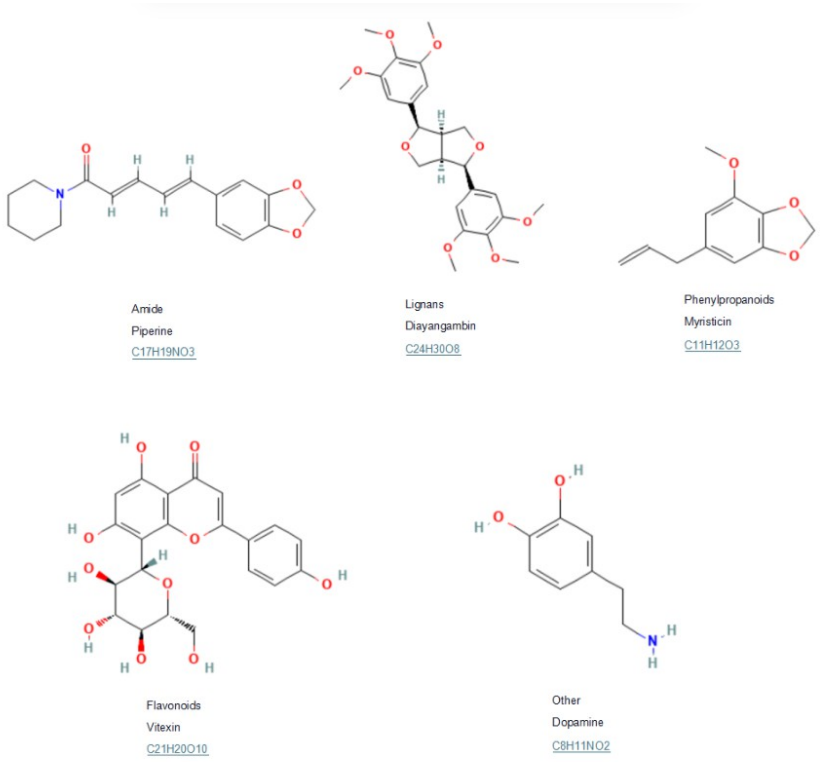


Figure 1: Chemical structures of major bioactive compounds in kawakawa. Figure adapted and compiled by the author based on PubChem reference data (PubChem, 2025).

A study found that phenylpropanoids and flavonoids were more abundant in dried leaves, whereas amides, alkaloids, and lignans predominated in freshly collected field material (Jayaprakash et al., 2022). Table 3 provides a summary of the major bioactive compounds identified in kawakawa.

Table 3: Key bioactive compounds in kawakawa (*Piper excelsum*)

Compound Class	**Specific Compounds**	**References**
Amides	Piperine, Pellitorine, Fagaramide, Piperdardine, Piperchabamide, Dihydropiperlonguminine, Dihydropipernonaline, Piperolein A	(Butts et al., 2019; Jayaprakash et al., 2022; Lei et al., 2015; Obst et al., 2017)
Lignans	Diayangambin, Excelsin	(Butts et al., 2019; Jayaprakash et al., 2022; Obst et al., 2017; Russell & Fenemore, 1973)
Phenylpropanoids	Myristicin, Elemicin	(Butts et al., 2019; Cambie, 1976; Jayaprakash et al., 2022; Obst et al., 2017)
Flavonoids	Vitexin, Isovitexin	(Butts et al., 2019; Jayaprakash et al., 2022)
Other	Dopamine	(Jayaprakash et al., 2022)

Among the amides, pellitorine is the most abundant compound found in kawakawa leaves and is specifically linked to the numbing sensation that explains its traditional use for pain relief and toothaches (Obst et al., 2017). Other amides, such as piperdardine and the rare piperchabamide A, which were isolated from kawakawa fruits, have revealed selective cytotoxic properties against cancer cells while showing a lack of toxicity in non-

transformed cell lines (Lei et al., 2015).

Piperine, a well-known amide also found in black pepper, modulates immune responses and suppresses inflammation through the regulation of molecular pathways implicated in chronic inflammatory diseases and obesity (Djaldetti, 2021). Specifically, a study on RAW264.7 cells stimulated with lipopolysaccharide (LPS) found that piperine inhibits the production of inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2) by blocking the activation of the NF-κB signalling pathway (Ying et al., 2013). Besides, its anti-inflammatory properties stem from its ability to inhibit the expression of the cyclooxygenase-2 (COX-2) gene and the subsequent production of PGE2. This is achieved by suppressing the activation and nuclear translocation of multiple signalling proteins, including NF-κB, CCAAT/enhancer binding protein (C/EBP), and activator protein-1 (AP-1) (Kim et al., 2012). Additionally, piperine analogues in kawakawa have been shown to influence the absorption of glucose and fatty acids in human epithelial cells, highlighting a role in intestinal nutrient regulation (Jayaprakash et al., 2022).

Lignans found in kawakawa also contribute to its anti-inflammatory effects. Diayangambin, in particular, possesses pronounced immunomodulatory and anti-inflammatory effects, as evidenced by its ability to inhibit human mononuclear cell proliferation, reduce PGE2 production in macrophages, and significantly decrease ear swelling and paw oedema in animal models (De León et al., 2002). The potential immunosuppressant property from lignans may explain the traditional use of kawakawa in treating autoimmune-related conditions such as psoriasis and rheumatism, where immune dysregulation leads to tissue damage.

Phenylpropanoids, particularly myristicin and elemicin, are also main contributors to the anti-inflammatory and antioxidant properties. Myristicin shows anti-inflammatory properties by inhibiting the production of inflammatory mediators, namely NO, IL-6, IL-10, interferon inducible protein-10 (IP-10), monocyte chemotactic protein (MCP)-1, MCP-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α, MIP-1β, leukaemia inhibitory factor (LIF), and intracellular calcium levels through a calcium-dependent mechanism (Lee & Park, 2011). Meanwhile, elemicin isolated from the seeds of *Myristica fragrans* displays considerable antioxidant, antibacterial, and antifungal potential (Al-Qahtani et al., 2022). Its anti-inflammatory function is closely related to this antioxidant activity, as it helps alleviate cellular damage and inflammation by scavenging free radicals and reducing oxidative stress (Hussain et al., 2016).

As a class of flavonoids, vitexin and its isomer isovitexin are recognised for their effective anti-inflammatory properties. These compounds manifest their effects by modulating major cellular signalling pathways. For instance, vitexin demonstrates its ability to alter macrophage polarisation within the inflammatory and tumour microenvironment to reduce the development of chronic colitis-related cancer (Chen et al., 2021). Additionally, isovitexin reduces inflammation, oxidative stress, and cell apoptosis both *in vitro* and *in vivo* by inhibiting MAPK and NF-κB pathways, while activating the nuclear factor erythroid 2–related factor 2 (Nrf2/HO-1) antioxidant pathway. It also suppresses LPS-induced reactive oxygen species (ROS) production, pro-inflammatory cytokines TNF-α and IL-6, as well as the expression of inducible nitric oxide synthase (iNOS) and COX-2 (Lv et al., 2016).

Altogether, reported outcomes demonstrate that multiple kawakawa bioactive compounds contribute to its anti-inflammatory activity through distinct molecular targets and signalling pathways. The available experimental evidence is summarised in Table 4.

A further area of interest is the observation that kawakawa may improve the bioavailability of other pharmacologically active compounds. A study on a functional beverage containing kawakawa and turmeric indicated that certain piperinoid compounds, for example, fagaramide, were more abundant and persisted longer in the plasma when consumed as part of the combined beverage compared to kawakawa alone (Abughazala, 2024). This suggests that kawakawa may not limit its role to intrinsic bioactivity but could extend to acting as a bioavailability enhancer in multi-ingredient formulations.

Table 4: Key kawakawa bioactives and their anti-inflammatory actions

Bioactive Compound	**Experimental Model (Cell or Animal)**	**Inflammation Outcome**	**Concentration**	**References**
Piperine	RAW264.7	↓ NO, PGE2, COX-2	10, 50 or 100 μg/ml	(Kim et al., 2012; Ying et al., 2013)
Diayangambin	RAW264.7, PBMCs, 2,4-Dinitrofluoro-benzene (DNFB)-induced Contact Hypersensitivity Model, Carrageenan-induced Paw Oedema Model	↓ PGE2, COX-2, MPO	0.5-2.8 μM, 10 μM, 40 mg/kg p.o.	(De León et al., 2002)
Myristicin	RAW264.7	↓ NO, IL-10, IL-6, IP-10, MCP-1, MCP-3, GM-CSF, MIP-1α, MIP-1β, LIF, and calcium	10, 25 or 50 μM	(Lee & Park, 2011)
Vitexin	BALB/c mice	↓ IL-1β, IL-6, TNF-α, IL-10; ↑ NO	10 mg/kg	(Chen et al., 2021)
Isovitexin	RAW264.7	↓ ROS, TNF-α, IL-6, iNOS and COX-2	25 or 50 μg/ml	(Lv et al., 2016)

1.3.7 Interpretation of Cytokine Responses and Experimental Evidence

The cytokine findings reported for purified myristicin and kawakawa tea may initially appear inconsistent, but the studies are not directly comparable. Lee and Park (2011) examined purified myristicin in polyinosinic-polycytidylic acid-stimulated RAW264.7 murine macrophages, whereas Tautuiaki et al. (2024) investigated the acute effects of a chemically complex kawakawa tea in humans. The studies differed in their interventions, species and cell sources, inflammatory stimuli, biological compartments, sampling times, and analytical endpoints. Consequently, differences between their cytokine results are more appropriately interpreted as reflecting experimental and biological context than as a direct discrepancy.

1.3.7.1 Interpreting Context-Dependent IL-10 and IL-6 Responses

After 24 h of exposure, myristicin reduced IL-10, IL-6 and several other inflammatory mediators in stimulated RAW264.7 macrophages (Lee & Park, 2011). Although IL-10 commonly contributes to the later regulatory

phase of an immune response (Carlini et al., 2023), its reduction in this model cannot be classified as either beneficial or detrimental without functional evidence. Myristicin has been shown to affect NF-κB-related signalling in other experimental systems (Badr et al., 2020; Luo et al., 2022), raising the possibility that reduced IL-10 occurred as part of broader upstream suppression rather than through selective targeting of this cytokine. However, the proposition that inhibition of the initial inflammatory response eliminated the need for IL-10-mediated feedback was not tested directly. Measurements of pathway activation, cytokine kinetics, and downstream immune function would be required to distinguish these possibilities.

In the human study, kawakawa tea consumption was associated with reduced IL-6 and IL-8 transcript abundance in peripheral blood mononuclear cells but increased plasma IL-6 concentrations at 120 min (Tautuiaki et al., 2024). Divergence between transcript and protein measurements is biologically plausible because mRNA stability, miRNA-mediated regulation, translation efficiency, secretion, tissue production, and systemic protein clearance can independently influence protein abundance (Anderson & Anderson, 2002; Buccitelli & Selbach, 2020; Vogel & Marcotte, 2012). Moreover, PBMC transcript abundance does not necessarily represent cytokine production by other tissues. Skeletal muscle, for example, can contribute substantially to circulating IL-6 during acute physiological stress, although evidence from exercise studies cannot establish the source of IL-6 following kawakawa consumption (Starkie et al., 2001). IL-6 itself can mediate both pro-inflammatory and regulatory effects depending on its signalling mode, tissue source, timing, and physiological context. Under some conditions, IL-6 induces IL-1 receptor antagonist and soluble TNF receptor p55 while suppressing IL-1 and TNF-α production (Tilg et al., 1994). Nevertheless, the observed increase in plasma IL-6 cannot be interpreted as evidence of either inflammation or a beneficial response, given that the study included only a short postprandial period and did not establish the cytokine's cellular source, signalling mode, or functional consequences.

1.3.7.2 Interpretive Limitations of Experimental Models

Comparisons between purified compounds tested *in vitro* and whole-plant preparations evaluated in humans require considerable caution. Cell-line models cannot fully reproduce the cellular interactions, feedback mechanisms, pharmacokinetics, and metabolic processes operating *in vivo* (Katt et al., 2016; Kaur & Dufour, 2012). Furthermore, myristicin represents only one of the many constituents identified in kawakawa. Within a whole-plant preparation, these compounds may interact additively, synergistically, or antagonistically, and their absorption and metabolism may further modify their biological effects. Evidence obtained with purified myristicin in stimulated murine macrophages therefore cannot be directly extrapolated to the systemic effects of kawakawa tea in humans.

The apparently divergent cytokine findings should consequently be viewed as outcomes of different experimental contexts rather than as biological contradictions. They illustrate the temporal separation between transcription and protein production, the context-dependent functions of cytokines, and the limitations of comparing isolated-compound studies with complex botanical interventions. Bridging this evidence gap will require integrated studies using chemically characterised kawakawa preparations, relevant exposure concentrations, multiple sampling times, and concordant measurements of transcript abundance, protein concentrations, pathway activation, and immune function. Such studies should progress from appropriate cell and animal models to adequately controlled human investigations.

1.3.8 Toxicological and Safety Profile

The safety of kawakawa remains a prominent area of research. While its safety profile appears promising, it is important to consider the preparation methods and the parts of the plant used. Unlike its relative kava, which has a documented association with hepatotoxicity (Fu et al., 2008), kawakawa's traditional water-based preparations and early scientific studies show a different safety profile.

A 14-day acute and 28-day subacute oral toxicity study conducted in rats provided favourable safety data for kawakawa tea. The study found no significant adverse effects on body weight or food intake, even at doses equivalent to four cups of tea per day for an adult human (Butts et al., 2019). The study concluded that the consumption of kawakawa tea could be considered safe under the specific conditions tested. This result is supported by a comprehensive phytochemical analysis, which indicated that the levels of bioactive metabolites obtained from traditional aqueous infusions are relatively lower than associated toxicity (Jayaprakash, 2023).

However, cellular toxicity studies offer a more complex perspective. *In vitro* research has shown that the biological effects of kawakawa leaf extracts can be biphasic, varying with both concentration and extraction method. Moderate concentrations may enhance mitochondrial activity and cell viability, yet a study using Tohoku Hospital Pediatrics-1 (THP-1) and Henrietta Lacks (HeLa) cell lines found increased cell lysis with room temperature extracts, while extracts prepared at 60°C did not differ significantly from controls (Newland, 2016). This implies that both extraction temperature and concentration influence cytotoxic outcomes.

Another study focusing on kawakawa fruit compounds revealed selective cytotoxicity toward cancer cells, with minimal effects on normal human mesenchymal stem cells (Lei et al., 2015). These observations indicate the therapeutic potential of specific kawakawa-derived amides, such as piperdardine and piperchabamide A, which showed considerable activity against cancer cell lines. In contrast, additional safety assessments using ethanol–water extracts from fresh and dried kawakawa plant material demonstrated no cytotoxic effects in differentiated Caco-2 intestinal cells, a model for the human intestinal barrier. Several piperamide-related compounds were tested for their impact on fatty acid and glucose uptake, and despite previous reports of cytotoxicity (Lei et al., 2015), none of the tested compounds impaired cell viability at high concentrations (Obst et al., 2017). Remarkably, compounds identified in kawakawa fruits from a previous study (Lei et al., 2015), including piperamides and related alkaloids, were not detected in the leaf samples evaluated in this study. This difference indicates that the chemical composition of kawakawa may vary across plant parts.

Overall, these results demonstrate that the safety profile of kawakawa-based products can vary widely depending on the plant parts used, extraction methods, and concentrations. This reinforces the relevance of selecting appropriate plant parts and processing techniques.

1.3.9 Conclusion

Kawakawa (*Piper excelsum*) occupies an important intersection between rongoā Māori, cultural heritage, and pharmacological research. Its diverse phytochemical composition and preliminary anti-inflammatory, cytotoxic, antimicrobial, anthelmintic, and immunomodulatory activities support continued investigation. However, the available evidence is derived predominantly from isolated compounds, cell-line studies, and postgraduate theses. Direct evidence for the efficacy of chemically characterised whole-plant preparations remains limited,

and the findings do not currently establish kawakawa as a therapeutic agent.

Future research should be Māori-led or co-governed, protect mātauranga Māori, and incorporate appropriate benefit-sharing arrangements. Experimental priorities include standardised reporting of plant provenance, plant part, harvesting conditions, extraction procedures, and chemical composition; dose-response studies with orthogonal viability controls; direct measurement of proposed signalling pathways; and pharmacokinetic and repeated-dose safety assessments. These studies should inform preregistered, adequately powered clinical trials using clinically interpretable outcomes. Such an integrated approach will help determine whether the biological activities identified in preliminary studies can be translated into safe, reproducible, and evidence-based applications while supporting the culturally appropriate and sustainable use of this taonga.

1.4 Methodological Framework

In the field of immunology, macrophages are fundamental to understand host immune responses and inflammatory processes. To investigate their functions and regulatory mechanisms, researchers rely on precise and dependable experimental methods. The following details the methodological background commonly used in macrophage function studies, including cell models, functional assessment techniques, and key controls to ensure research integrity.

1.4.1 Cell Models

For *in vitro* studies, the RAW264.7 and THP-1 cell lines are widely used tumour-derived models for simulating macrophage function. These cell lines provide valuable tools for investigating macrophage biology due to their experimental convenience and their ability to reproduce certain macrophage characteristics. In contrast, PBMCs function as a normal, non-transformed cell model, offering a more physiologically relevant perspective (Herb et al., 2024).

RAW264.7 is an immortalised cell line derived from an ascites tumour induced in BALB/c mice by the Abelson murine leukaemia virus (A-MuLV) (Raschke et al., 1978). Its primary advantages are ease of culture, rapid proliferation, and the constitutive expression of intrinsic macrophage characteristics (e.g., phagocytic ability and LPS responsiveness) without further induction (Hartley et al., 2008; Herb et al., 2024; Wu, 2025). This makes it ideal for high-throughput screening and initial mechanistic studies. However, its limitations include being of mouse origin, which may not always translate to human physiology, and its already activated state, which might not fully mimic the dynamic process of macrophage activation from a resting state. It is also prone to genetic drift, leading to genomic and epigenetic changes over successive passages that can influence cell behaviour and compromise experimental reliability (Herb et al., 2024). Moreover, the absence of critical protein such as apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) limits the suitability of this cell line for studying ASC-dependent inflammasome activation and caspase-1 activity (Pelegrin et al., 2008).

In contrast, the THP-1 cell line is derived from a human acute monocytic leukaemia (Tsuchiya et al., 1980).

While initially used for cancer research, these cells are now a common tool for immunology studies, as they can be differentiated into macrophage-like cells induced by phorbol-12-myristate-13-acetate (PMA) or macrophage colony-stimulating factor (M-CSF) (Herb et al., 2024). Given its capacity to better reflect human physiological processes, the THP-1 cell model is a valuable resource in translational medicine. It also offers the advantage of genetic uniformity during the mid-passage range (up to passage 25), with no obvious changes in cell sensitivity or activity. Besides, the cells can be stored for extended periods and recovered without noticeable effects on their characteristics or viability (Chanput et al., 2015). However, its leukaemic origin and low CD14 expression limit the application, as it is less responsive to inflammatory stimuli including LPS compared to primary cells (Herb et al., 2024). This can lead to experimental results that do not accurately reflect the responses of human monocytes.

Owing to the transformed and cancerous nature of tumour-derived cell lines, which can alter immune signalling pathways, PBMCs are often used as a more physiologically relevant model for studying normal immune cell function. PBMCs, which include monocytes, lymphocytes, and dendritic cells, are primary cells directly isolated from human peripheral blood (Kleiveland, 2015). They are routinely isolated through density gradient centrifugation methods, such as the Ficoll-Paque method, Cell Preparation Tubes (CPTs), or SepMate™ tubes in combination with Lymphoprep™ (Grievink et al., 2016). After isolation, monocytes can be differentiated into functional primary human macrophages *in vitro*, which express multiple cell surface receptors and signalling pathways. This confers a strong responsiveness to inflammatory stimuli and enables a more accurate assessment of normal cellular function, especially in studies focusing on immunomodulation, drug safety, and translational medicine. However, the isolation of PBMCs is labour-intensive, and both viability and purity can vary substantially between donors. Moreover, they exhibit a limited lifespan in culture and are prone to apoptosis following isolation, which can further restrict their use in long-term experiments and high-throughput studies (Herb et al., 2024; G. Hodge et al., 2000). These limitations make PBMCs a valuable yet complementary model, primarily used to validate fundamental findings obtained from more accessible and stable tumour-derived cell lines.

1.4.2 Cytotoxicity Assessment

Trypan blue is a dye first synthesised in 1904 by Paul Ehrlich and has since been widely used in both biology and medicine (Piccinini et al., 2017). As a cell-impermeable dye, it selectively stains only dead cells with compromised membranes, making it a reliable method for assessing cell viability based on membrane integrity. In a trypan blue assay, cells are mixed with the dye and examined under a light microscope. Viable cells repel the dye and remain unstained, while nonviable (dead) cells retain the dye and display blue due to membrane damage. By staining a cell suspension with the dye and then counting the unstained (viable) and blue-stained (non-viable) cells using a haemocytometer under a microscope, researchers can quickly estimate the percentage of living cells in a population. This provides a direct, quantifiable, and visual assessment of treatment-induced cytotoxicity in cultured cells(Strober, 2015).

Despite its simplicity and cost-effectiveness, the trypan blue assay has limitations. It is somewhat subjective, as manual counting can vary between users, and it only detects cell death based on membrane permeability (Piccinini et al., 2017). Cells in early stages of apoptosis may still exclude the dye, leading to an overestimation of viability (Henslee et al., 2016). Additionally, the timing of analysis is critical, as prolonged exposure to the dye can result in false positives (Piccinini et al., 2017).

In contrast, the MTT assay serves as a colorimetric indicator of cellular metabolic activity. This method involves the reduction of yellow tetrazolium salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into purple formazan crystals, a process primarily catalysed by NAD(P)H-dependent oxidoreductase enzymes within active mitochondria (Mosmann, 1983). Unlike the manual nature of trypan blue, the MTT assay enables high-throughput screening with microplate readers, as absorbance intensity directly correlates with mitochondrial activity and reflects cell viability (Van Meerloo et al., 2011). However, MTT is a destructive endpoint assay, and its results can be confounded by chemical reducing agents, as exogenous reducing agents or antioxidants can directly react with the tetrazolium salt to generate false-positive viability signals (Ghasemi et al., 2021). Besides, instead of measuring viable cell numbers directly, the MTT assay assesses cellular metabolic activity. Physiological shifts in mitochondrial activity may not directly indicate cell death; therefore, reduced metabolic activity should be interpreted cautiously when interpreting cell viability (Hoogstraten et al., 2022).

The selection between these assays depends on whether the primary interest is in assessing cell membrane integrity or metabolic activity. Discrepancies often arise because metabolic decline and membrane failure do not always occur simultaneously. For instance, cytotoxic agents may induce metabolic impairment (MTT negative) while membranes remain intact (trypan blue negative) (Hoogstraten et al., 2022). In contrast, early apoptotic cells may retain membrane integrity, resulting in trypan blue negative readouts despite ongoing cell death progression (Henslee et al., 2016). Therefore, cytotoxicity assessment often requires a multiparametric approach integrating structural and metabolic information to avoid misleading interpretations of cell survival.

1.4.3 Methods for Macrophage Functional Assessment

To thoroughly study macrophage function, a combination of techniques is needed to assess their polarisation status and effector functions.

1.4.3.1 Polarisation Characteristics

Macrophages can be classified into different polarisation subtypes based on their functional state, and several methods are available to assess their polarisation characteristics. A commonly used method is flow cytometry, which detects cell surface markers such as the upregulation of CD80 and CD86 on M1 macrophages, or CD206 and CD163 on M2 macrophages (Ambarus et al., 2012; Arora et al., 2018). This method employs fluorescently labelled antibodies that bind specifically to these surface proteins. As cells pass individually through a laser beam within the flow cytometer, the fluorescence emitted from each cell is detected and measured, enabling instantaneous quantification of cell populations and their phenotypes (McKinnon, 2018).

Quantitative real-time PCR (qPCR) is a molecular technique that can be used to measure macrophage gene expression. It quantifies gene expression by detecting and measuring the amplification of specific DNA sequences derived from mRNA in real time. It uses fluorescent dyes or probes that generate signals corresponding to the amplified product concentration, allowing precise analysis of target genes such as inflammatory cytokines (e.g., TNF-α, IL-6) and anti-inflammatory factors (IL-10). Gene expression levels are determined by the threshold cycle (Ct), which is inversely proportional to the initial mRNA concentration and reflects transcriptional changes during macrophage polarisation (Jozefczuk & Adjaye, 2011).

In addition, enzyme-linked immunosorbent assay (ELISA) is a commonly used antibody-based technique that detects and quantifies specific proteins, such as cytokines and chemokines, secreted into the culture medium. It relies on the specific binding between antigens and antibodies, followed by a colorimetric or fluorescent reaction that correlates with protein concentration (Shah & Maghsoudlou, 2016). ELISA thus provides a quantitative assessment of macrophage secretory activity by measuring the levels of released signalling molecules.

1.4.3.2 Effector Function Assessment

Phagocytosis and nitric oxide (NO) production are core effector functions of macrophages. Phagocytosis assays are commonly employed to assess the functional capacity of macrophages to ingest and eliminate foreign particles, including pathogens or damaged cellular components. These assays typically involve incubating macrophages with fluorescently labelled targets (e.g., beads or bacteria) (Cseresnyes et al., 2020). A common label is fluorescein isothiocyanate (FITC), which is particularly useful for this application because of its ability to covalently bind to amine groups on proteins (Lindner et al., 2020). After incubation, non-internalised particles are either washed away or quenched, and the remaining internalised fluorescence is measured. This analysis is performed using either flow cytometry, which provides fast, quantitative data on large cell populations, or fluorescence microscopy, which allows for visual confirmation of particle uptake at the single-cell level (Sharma et al., 2014). As phagocytosis is a principal function of macrophages, this assay offers direct evidence of their immune activity and pathogen-clearing ability.

For nitric oxide production assessment, the Griess reagent method offers a well-established, simple, and cost-effective colorimetric approach for indirect quantification (Tsikas, 2007). Since NO is highly unstable in biological systems, the Griess assay measures its stable breakdown products, primarily nitrite (NO_2^-), and, if a reduction step is added, total nitrate (NO_3^-) plus nitrite (NO_x^-) (Sun et al., 2003). The assay works in two steps: first, the nitrite reacts with sulfanilamide under acidic conditions to form a diazonium salt, which then couples with N-(1-naphthyl)ethylenediamine (NED) to produce a distinctive pink-purple azo dye which can be quantified by measuring absorbance at 540-550 nm (Tsikas, 2007). It is widely used in studies investigating inflammation and immune responses. Despite its application for initial screening and long-term release assessment, the Griess assay is limited by its comparatively low sensitivity (approximately 1-2 μM) and susceptibility to interference from various proteins and biological compounds (Bellavia et al., 2015).

Western blotting (immunoblotting) is an analytical technique that began in 1979 (Renart et al., 1979). It identifies a specific protein within a complex mixture by combining size-based separation and highly specific antibody detection. The process involves separating proteins electrophoretically in a complex mixture using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferring them to a solid membrane (blotting). A series of primary and labelled secondary antibodies is then used to visualise the target protein, which permits determination of its molecular weight and relative abundance (Bass et al., 2017). Even though it has faced criticism (mainly concerning antibody validation), the technique remains crucial in molecular biology due to its efficiency, lower cost, and ability to detect post-translational modifications (PTMs), with modern variations including single-cell western blots and automated capillary systems continuing to enhance its application (Sule et al., 2023).

1.4.4 Controls for Experimental Integrity

To ensure the accuracy and reproducibility of experimental results, strict control over all experimental conditions is required.

1.4.4.1 Sterilisation Strategies for Plant Extracts

Sterile filtration is an effective technique for maintaining a contamination-free environment in cell culture. This physical sterilisation method employs a 0.2 or 0.22 μm syringe filter to remove microorganisms such as bacteria and some fungi (McBurnie & Bardo, 2004; Walsh & Denyer, 2012). Acting as a fine sieve, the filter allows the passage of small bioactive molecules from the extract while effectively trapping particles larger than the hole size (Majekodunmi & Olorunsola, 2014). This approach ensures sterility without compromising the delicate chemical components of the extract. Despite that the 0.22 μm filter is the standard for sterilisation, it is not an absolute barrier, as small bacteria, mycoplasmas, and viruses are known to penetrate it (Walsh & Denyer, 2012).

The choice of filter membrane material is equally important for filtration efficiency and chemical compatibility. Hydrophilic membranes such as polyethersulfone (PES), polyvinylidene fluoride (PVDF), and cellulose acetate (CA) are regularly applied for sterile filtration of aqueous solutions. PES is a polymer with a linear backbone and amorphous morphology. It offers a low protein-binding surface, high flow rate, and broad biocompatibility, making it commonly used in medical and biotechnological applications—particularly for filtering cell culture media, serum, and biological extracts (Wasyłeczko et al., 2024). As a semi-crystalline polymer, PVDF also has low protein binding and excellent thermal and chemical resistance, suitable for both aqueous and some organic mixtures (D. Wang et al., 1999). CA is appropriate in applications where minimal protein binding is required, such as in biomedical or antimicrobial work (Abu-Zurayk et al., 2023). In contrast, polytetrafluoroethylene (PTFE) membranes are hydrophobic and primarily employed for filtering organic solvents with excellent mechanical strength, thermal stability, and resistance to chemical degradation (Zhu et al., 2013). PTFE is generally not recommended for water-based samples, unless specific pretreatment steps are taken. Proper membrane selection enhances compatibility, stable flow rate, and the preservation of sensitive compounds during filtration.

Filtration is preferred over heat-based sterilisation methods, such as autoclaving, because many plant-derived compounds, particularly phenolics and flavonoids, are heat-sensitive and susceptible to degradation or loss of activity at high temperatures (Putriani et al., 2022; G. Xu et al., 2007). By preserving the chemical stability and bioactivity of the plant extract, filtration ensures that experimental results accurately reflect its inherent properties.

Gamma irradiation is a backup sterilisation strategy. This method uses high-energy photons that create free radicals capable of breaking chemical bonds. These free radicals predominantly damage microbial DNA, inhibiting replication and leading to cell death. Sterility is thus achieved mainly through the disruption of nucleic acids and the prevention of cellular division. Well-established as a terminal sterilisation method, gamma irradiation is valued for its effectiveness and safety. It achieves a high level of microbial inactivation without exposing products to excessive heat or toxic chemicals (Hasanain et al., 2014). However, the potential for gamma irradiation to alter the chemical composition of organic compounds through free radical generation (Calucci et al., 2003) makes filtration the preferred primary method.

1.4.4.2 Solvent and Vehicle Control

Ethanol is a small, amphiphilic molecule that chiefly causes toxic effects by disrupting cell membranes and interfering with cellular metabolism, as well as directly inducing apoptosis in cell culture (Donohue et al., 2001). Its impact on cells is biphasic, with differing responses at low versus high concentrations (Tapani et al., 1996). Generally, ethanol concentrations below 0.5% (v/v) are tolerated by most cell types for short exposure periods, while higher concentrations or prolonged exposure can substantially inhibit cell growth, alter metabolism, and induce cytotoxicity (Michael et al., 2013; Nguyen et al., 2020). Because of this, when using ethanol as a solvent in biological assays, defining a safe solvent concentration that accounts for both the cell line and the specific experimental conditions is essential. Accordingly, the final ethanol concentration should be maintained at a low and consistent level to minimise any solvent-related effects on cell viability or function.

Similarly, dimethyl sulfoxide (DMSO) is a polar aprotic solvent extensively used to dissolve hydrophobic drugs and botanical extracts due to its ability to penetrate biological membranes (Brayton, 1986). However, it is known to act as a radical scavenger, modulate membrane permeability, and even induce cell differentiation or epigenetic changes at different concentrations (De Ménorval et al., 2012; Pal et al., 2012). While DMSO is a practical cryoprotectant and vehicle, its toxicity is temperature- and concentration-dependent (Pal et al., 2012). In cell-based assays, DMSO concentrations from 0.25% to 1.5% did not induce cellular toxicity (Han et al., 2023). But a lower concentration (e.g., 0.2%) as a conservative approach could be able to minimise potential effects of the solvent on inflammatory responses and signalling pathways. Thus, maintaining DMSO levels within a strictly defined range is imperative to ensure efficient compound delivery without introducing solvent-induced biological effects.

In order to maintain the safety of the solvent level and to verify that the established final concentration does not affect cell viability, a solvent tolerance test is mandatory before starting the formal experiment. This pre-experiment involves serial dilution of the solvent to determine the maximum safe final concentration that has no detrimental effect on cell viability or phenotype (Michael et al., 2013). This maximum safe concentration will define the solvent limit for all test groups (including extract and vehicle control groups).

To assess whether the observed effects are due to the extract and not the solvent, a vehicle control should be included to differentiate the impact of the active compound from that of the solvent or delivery medium. This distinction is particularly important when testing hydrophobic compounds such as plant extracts that require organic solvents to remain soluble in aqueous cell culture media (Z. Liu, 2008).

The vehicle control establishes experimental validity by confirming that observed cellular changes, including alterations in viability, gene expression, or cytokine production, result from the test agent as opposed to the solvent itself (Nguyen et al., 2020). Since solvents can be cytotoxic at higher concentrations, vehicle controls are used to validate that solvent levels stay within non-toxic or sub-toxic ranges specific to the cell line and incubation conditions. Furthermore, vehicle controls account for subtle, non-lethal effects that may arise from the solvent, for instance, changes in membrane fluidity or metabolic activity (Chin & Goldstein, 1977; Donohue et al., 2001). Failure to consider these effects may confound the interpretation of the true biological impact from the compounds. Appropriate vehicle controls therefore enhance experimental consistency, ensure accurate data interpretation, and support the validity of the findings.

1.4.4.3 Endotoxins in Experimental Systems

Endotoxin, or lipopolysaccharide (LPS), is a structural glycolipid found in the outer membrane of Gram-negative bacteria. It is released into the environment not only as bacterial death and lysis, but also continuously during the bacterial growth cycle (Zhang et al., 1998). Even minute amounts of endotoxins can trigger substantial immune responses in cell cultures, leading to inflammation and potentially interfering with experimental results (Zandieh et al., 2018). The gold standard for assessing endotoxin levels is the limulus amoebocyte lysate (LAL) assay. This test detects endotoxins by exploiting their ability to provoke a highly sensitive enzymatic cascade reaction in the amoebocytes (blood cells) of the horseshoe crab, a process that promotes clot formation (Iwanaga, 2007). During plant extraction, it is important to maintain endotoxin levels below detectable thresholds to validate that observed biological responses arise from the active compounds in the extract, rather than a toxic immune reaction—thereby minimising the risk of misinterpreting the treatment effects.

1.4.4.4 Types of Contamination in Cell Culture

Cell culture requires a highly sterile environment but faces challenges from contamination, which can greatly impact experimental outcomes. Based on their origin and nature, contaminants can be categorised into four main types (Table 5): physical, chemical, biological, and cross-contamination (Bright-Ohaeri et al., 2024). Different types of contamination pose distinct risks, each demanding careful attention to maintain the reliability of research.

Table 5: Classification and impact of contaminants

Contamination Type	Description and Source	Major Impact	References
Physical Contamination	Introduction of foreign, non-biological substances such as dust, fibers, and aerosols.	Interferes with normal cell growth and affects experimental results.	(Bright-Ohaeri et al., 2024; Dervisevic et al., 2022)
Chemical Contamination	Impurities originating from media, serum, water, plasticware, or disinfectant residues.	Affects cell viability and growth; for example, bacterial endotoxins are potently cytotoxic.	(Nims & Price, 2017)
Biological Contamination	Includes live organisms such as bacteria, molds, yeasts, viruses, and mycoplasma.	Leads to cell death or severely alters experimental results; mycoplasma and viruses are particularly difficult to detect and eliminate.	(Weiskirchen et al., 2023)
Cross-Contamination	One cell line is infiltrated and replaced by another.	Leads to cultural heterogeneity, invalid experimental results, and economic loss.	(Capes-Davis et al., 2010)

1.4.4.5 Aseptic Technique and Contamination Prevention

Aseptic technique is a set of practices designed to prevent microbial contamination. All procedures are conducted within a Class II Biosafety Cabinet (BSC), which provides a sterile workspace by filtering air through a high-efficiency particulate air (HEPA) filter, protecting both the cells and the user. Experiments are performed

under Physical Containment Level 2 (PC2) conditions, requiring lab coats, gloves, and use of containment equipment. Routine disinfection with 70% ethanol and the use of personal protective equipment (PPE), further minimise the risk of contamination (Bright-Ohaeri et al., 2024). Periodic sterility testing using Plate Count Agar (PCA) and Sabouraud Dextrose Agar (SDA) is employed to detect any microbial presence that might escape visual inspection.

Sterility testing is a quality control measure used to verify the absence of viable microorganisms in filtered extract solutions, thereby ensuring the reliability of cell culture experiments. Contaminated extracts can compromise cell viability, alter cellular behaviour, or lead to false-positive results, reinforcing the critical importance of sterility testing (Freshney, 2010). Two commonly used media for this purpose are PCA and SDA. PCA is a general-purpose medium that supports the growth of various facultative aerobic bacteria. It is typically incubated at 35 or 37°C for 48 hours, simulating body temperature and allowing most fast-growing bacteria typically associated with human and animal samples to form visible colonies for contamination detection. In contrast, SDA is selective for fungi and yeasts due to its acidic pH and high sugar concentration, which inhibit bacterial growth and promote fungal isolation. Fungal cultures are incubated on SDA at a lower temperature of 20-25°C for a longer period of seven days, reflecting the slower growth rate and room-temperature preference of many fungal species (Marsit et al., 2025; Sandle et al., 2013). After the incubation period, the total number of colony-forming units (CFUs) on a single plate is counted to calculate cell concentration in the original sample via spread plate or pour plate technique (Sanders, 2012). The absence of colony growth on both PCA and SDA plates following their respective incubation periods serves as the definitive criterion for confirming sterility.

In addition to bacterial and fungal monitoring, routine screening for mycoplasma contamination is an essential component of contamination control in mammalian cell culture. Mycoplasmas are the smallest self-replicating bacteria ($0.1\,\mu m$ to $0.2\,\mu m$), cell-wall deficient and undetectable under light microscopy. They are resistant to conventional antibiotics, and do not cause visible turbidity, making contamination hard to detect. Despite competing with host cells for nutrients, they often cause minimal observable changes in cell growth, further complicating identification (Weiskirchen et al., 2023). Stemming from their subtle yet profound effects on gene expression, metabolism, and immune responses, these factors can compromise the validity of experimental results. Detection methods include traditional culture-based assays, fluorescent DNA staining, ELISA, RNA probe hybridisation, enzymatic and flow cytometric assays, and highly sensitive PCR-based kits targeting conserved 16S rRNA regions (Volokhov et al., 2011; Weiskirchen et al., 2023). To maintain the validity of cell-based assays, mycoplasma testing should be routinely performed—preferably every month or before initiating key experiments.

1.4.4.6 Assay Controls and Quality Assurance

Beyond the solvent and aseptic controls discussed above, appropriate control groups are necessary for distinguishing true biological effects from non-specific interference, particularly when assessing the biological activity of plant extracts.

Negative controls are fundamental for establishing the baseline of an experiment and ruling out non-specific background interference. They are specifically designed to verify that unknown variables, which could unintentionally affect the outcome of the experiment, are not confounding the results. This is particularly relevant in complex biological studies, where subtle, unrecognised influences might lead to misleading conclusions, such as false positives or mistaken associations (Johnson & Besselsen, 2002). Negative controls help detect potential

experimental or biological variables, contamination, or researcher bias that could influence the results (Núñez & Matthews, 2025). For instance, in cell culture experiments, a negative control might include cells treated only with the vehicle, guaranteeing that any observed effects are not due to the solvent itself.

In contrast, positive controls are indispensable tools used to verify the efficacy and sensitivity of the experimental system. In positive control groups, a known and expected response is induced to ensure the experimental setup is functional and the reagents are working as expected (Johnson & Besselsen, 2002). The inclusion of a positive control confirms that the designed model responds appropriately to stimuli and helps validate the reliability of the entire experimental method. Failure of the positive control may indicate problems with the experimental protocol, reagent performance, or cell culture conditions, which requires further investigation and optimisation.

1.4.5 Summary

Analysis of macrophage function is a systematic process where every step from selecting the cell model and applying functional assessment techniques to strictly controlling experimental integrity is critical. Only through adherence to standardised and well-controlled procedures can researchers obtain reliable and reproducible data to accurately understand the role of macrophages in health and disease.

1.5 Research Aims and Objectives

1.5.1 Aim

The overall aim of this study is to investigate the immunomodulatory effects of kawakawa (*Piper excelsum*) extract on macrophage polarisation and function, with a specific focus on its potential anti-inflammatory properties in *in vitro* cell-based models. Given the primary role of macrophages in the initiation, maintenance, and resolution of inflammation, this study seeks to clarify whether kawakawa extract can modulate macrophage behaviour under pro-inflammatory conditions.

1.5.2 Objectives

- To determine the non-toxic concentration range of kawakawa extract in murine RAW 264.7 macrophages using viability assays.
- To assess the ability of kawakawa extract to modulate macrophage polarisation, particularly M1-type activation induced by LPS and/or IFN-γ, by measuring nitric oxide (NO) production.
- To compare the immunomodulatory effects of kawakawa extract under different treatment conditions, including co-treatment, pre-treatment, and post-treatment approaches.
- To establish and verify sterile, reproducible preparation methods for hydroethanolic kawakawa extract suitable for use in cell-based assays.

1.6 Significance of the Study

This study holds scientific, cultural, and translational importance by adopting a novel, interdisciplinary approach that bridges traditional ethnobotanical knowledge with modern pharmacology. Scientifically, it addresses a gap in biomedical understanding by investigating how kawakawa (*Piper excelsum*)—a taonga species widely used in rongoā Māori—influences macrophage function, a basic component of innate immune regulation. Kawakawa has a long history of medicinal and cultural use among Māori, and recent research has begun to investigate its phytochemical composition and potential anti-inflammatory properties (Butts et al., 2019; Tautuiaki et al., 2024). While rongoā Māori has long recognised the healing properties of kawakawa, especially for inflammatory conditions, this research does not seek to validate traditional knowledge, but instead to explore specific cellular and molecular mechanisms that may align with or complement that knowledge. This approach is grounded in respect for Mātauranga Māori as a distinct and legitimate knowledge system, and aims to contribute to a more inclusive and bicultural scientific discourse (Hudson et al., 2010).

The study will provide a comprehensive scientific characterisation of kawakawa's anti-inflammatory properties. Its integrative methodology establishes a framework that may be applied to other traditional medicinal species, enhancing pharmacognosy, ethnobotany, and translational medicine. Importantly, this study acknowledges that the plant-based component of rongoā is only one part of a multi-dimensional therapeutic tradition that encompasses whakapapa (genealogy), wairua (spirituality), and tikanga (protocols), combined with the roles of tohunga (healers) and whānau in healing processes (Mark et al., 2022; Marques et al., 2021). As such, this project acknowledges that rongoā should be understood within its broader cultural and traditional context, while investigating how kawakawa extracts influence immune function without reducing this healing tradition to its biochemical components alone. Ongoing engagement with Māori communities and knowledge holders will ensure the research remains culturally appropriate and aligned with the principles of kaitiakitanga (guardianship), manaakitanga (care), and tino rangatiratanga (self-determination) (Hudson et al., 2010).

This project is firmly grounded in the principles of Vision Mātauranga, ensuring that the research is culturally informed, ethically conducted, and of direct relevance to Māori. It upholds the principle of protection by adhering to Auckland University of Technology Ethics Committee (AUTEC) protocols and recognising Mātauranga Māori relating to kawakawa, as well as the people who hold this knowledge, as taonga. This means acknowledging the origins and cultural significance of this knowledge, while ensuring that it is represented accurately and remains connected to the communities and traditions from which it comes. By investigating the anti-inflammatory properties of kawakawa, this research seeks to contribute to Māori health aspirations while recognising its established role in rongoā Māori. Furthermore, the findings will be communicated in culturally appropriate and accessible ways, recognising the value of Māori perspectives and the cultural context of kawakawa.

The study also supports the Vision Mātauranga goals of indigenous innovation and economic development by laying a foundation for Māori-led health product development and enterprise. Its comprehensive and reproducible experimental design may serve as a methodological template for the scientific investigation of other traditional medicinal species, contributing to sustainable and respectful utilisation of native flora. In the long term, this research enhances our understanding of natural immunomodulators, strengthens the synthesis of

Māori knowledge and biomedical research, and contributes to a more equitable healthcare system in Aotearoa New Zealand.

2 Materials and Methods

2.1 Overview

This chapter outlines the methodological framework used to investigate the modulatory effects of kawakawa (*Piper excelsum*) extract on macrophage functions, including polarisation, cell viability and nitric oxide production. The study employs *in vitro* assays with murine RAW 264.7 cell lines, incorporating a staged experimental design to evaluate the extract's effects under various treatment conditions. Sterility, cytotoxicity, and consistency of treatment were prioritised to ensure data integrity.

2.2 Materials and Reagents

Cell Lines

- Murine RAW 264.7 macrophage cell line (ATCC TIB-71)

Culture Reagents

- Dulbecco's Modified Eagle Medium (DMEM) (for RAW 264.7 cells)
- Foetal Bovine Serum (FBS)
- Penicillin-Streptomycin (P/S) (1%)

Stimulants and Controls

- LPS (500 $\mathrm{ng/mL}$)
- IFN-γ (20 $\mathrm{ng/mL}$)
- Absolute Ethanol (>95% for extraction)
- 50% Ethanol (EtOH) for extraction (v/v, ethanol: DI water = 1:1)
- 70% Ethanol (EtOH) for disinfection (v/v, ethanol: DI water = 7:3)
- Dimethyl Sulfoxide (DMSO) (solvent for extract storage, cryoprotectant for cell freezing, and vehicle control; final concentration < 0.1% v/v in culture)

Kawakawa Extract

- Commercial kawakawa tea (dried leaves, OKU NZ Native Herbal Products Ltd.)
- Filter membranes: 0.45 $\mu\mathrm{m}$ PTFE pre-filters and 0.22 $\mu\mathrm{m}$ PTFE syringe filters
- Sterile solvent-resistant tubes with cap

Assay Kits & Consumables

- Trypan Blue (cell viability)
- MTT (cell viability / metabolic activity)
- Griess Reagent Kit (nitric oxide detection; inflammatory response)
- Plate Count Agar (PCA) and Sabouraud Dextrose Agar (SDA) for sterility testing

2.3 Preparation of Kawakawa Extract

2.3.1 Kawakawa Extraction and Sterilisation

Dried commercial kawakawa tea leaves (2 g) were ground under liquid nitrogen and extracted with 20 mL of 50% ethanol (1:10, w/v). The mixture underwent ultrasound-assisted extraction using an ultrasonic processor (Hielscher UP100H, Germany) operated at 50% amplitude in pulsed mode (cycle = 0.5). The extract was sonicated for 30 minutes in a water-ice bath to minimise heat-induced degradation, then allowed to stand overnight at 4°C to enhance compound release. After extraction, samples were centrifuged (10,000 × g, 15 min, 4°C) and the clarified supernatant was sequentially filtered through a 0.45 μm sterile filter. The extract was first concentrated under a nitrogen stream to remove most of the solvent, especially ethanol, and then further dried using a centrifugal vacuum concentrator (Eppendorf Concentrator Plus, Germany) at 30°C under gentle vacuum to obtain the final dried extract. The drying process required approximately 8 h (1 h nitrogen blowdown + 7 h vacuum concentration). The dried extract was reconstituted in sterile 100% DMSO and passed through a 0.22 μm sterile filter for sterilisation. The resulting stock solution was aliquoted and stored at -20°C until use. Potential DMSO-associated cytotoxicity was controlled by ensuring the final solvent concentration did not exceed 0.1% (v/v) in any treatment conditions.

Key precautions included maintaining sterility during filtration and drying, controlling sonication amplitude and duration to prevent overheating, and applying gentle concentration temperatures to preserve heat-sensitive bioactives.

2.3.2 Sterility Testing

From each filtered solution, 100 μL aliquots were aseptically plated onto Plate Count Agar (PCA) for bacterial detection, and Sabouraud Dextrose Agar (SDA) for fungal and yeast detection. PCA plates were incubated at 37°C for 48 hours, while SDA plates were incubated at 25.5°C for 7 days. Plates were checked daily for visible colony formation. The absence of colonies on both PCA and SDA plates after the incubation periods confirmed sterility. Any batch showing microbial growth was discarded immediately, and the extract was re-prepared and sterilised again before use in experiments.

2.3.3 Dilution and Solvent Control

For the dilution and solvent control protocol, the dried kawakawa extract from the previous extraction and sterilisation steps was first dissolved in DMSO to prepare a concentrated stock solution at 100 mg/mL. This stock solution was subsequently diluted in complete DMEM (RAW 264.7 culture medium) through serial dilutions to obtain the required working concentrations of up to 100 μg/mL. To minimise solvent-induced cytotoxicity, the final DMSO concentration in all treatments was maintained at or below 0.1% (v/v), with dilution volumes adjusted according to the stock concentration. A DMSO vehicle control (0.1%, v/v) without kawakawa extract was included to account for solvent-related effects on RAW 264.7 cells, ensuring that any observed cellular changes in the treatment groups could be confidently attributed to the extract itself.Finally, all prepared solutions were gently mixed by inversion and stored at 4°C until use. Repeated freeze-thaw cycles were avoided where applicable.

2.4 Cell Culture Conditions

2.4.1 RAW 264.7 Cells

RAW 264.7 cells are adherent cells that grow attached to the surface of culture flasks and are routinely maintained in DMEM supplemented with 5% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (P/S). Prior to use, culture medium and PBS were pre-warmed in a 37°C water bath for 20-30 minutes to ensure optimal handling conditions.

RAW 264.7 cells were initially grown in T25 flasks with 5 mL culture medium at 37°C in a humidified incubator containing 5% CO_2. As the cell population increased, cultures were expanded into T175 flasks when additional growth surface area was required. The culture medium was routinely replaced every 2-3 days. Before medium replacement, the cells were examined under an inverted microscope to assess cell morphology, attachment and confluency. The spent medium was aspirated, and the cell monolayer was gently washed twice with pre-warmed PBS to remove residual serum-containing medium and suspended cellular debris. For this procedure, 5 mL of PBS was used for T25 flasks and 10 mL for T175 flasks. Following washing, fresh complete medium was added at a volume of 5 mL per T25 flask and 25 mL per T175 flask, respectively.

When the cultures reached approximately 80% confluence, the medium was aspirated, and cells were washed to remove extracellular proteins and dead cells. This confluency level was selected to maintain actively growing cultures while avoiding excessive cell density and prolonged contact inhibition associated with over-confluent cultures. Cells were then detached either using a sterile 25 cm cell scraper (Costar, Corning Inc., USA) or 1-2 mL of TrypLE Express (Gibco, Thermo Fisher Scientific, USA), resuspended in fresh medium to generate a single-cell suspension, and counted using a haemocytometer with trypan blue exclusion to assess viability. Subculturing was performed at a 1:4 to 1:6 split ratio in fresh T25 or T175 flasks, depending on growth density. The passage numbers used for the experiments ranged from P5 to P20. Maintaining a consistent passage range was important since prolonged serial passaging may influence macrophage growth characteristics, metabolic activity and responses to inflammatory or cytotoxic stimuli.

For experiments, cells were diluted to working concentration using standard C1V1=C2V2 dilution principles and seeded into appropriate culture plates at the desired cell density (typically 5×10^4 cells/well for 24-well plate). Cells were then allowed to adhere overnight (18-24 h) before treatment. The overall cell culture and treatment schedule is shown in Table 6.

Table 6: Cell culture and treatment timeline

Day	Cell Line	Procedure	Reagent(s)
0	RAW 264.7	Seeding	DMEM + 5% FBS + 1% P/S
1	RAW 264.7	Treatment phase begins	LPS/IFN-γ, kawakawa extract

2.5 Aseptic Technique and Contamination Prevention

All cell culture work was conducted in a Class II biosafety cabinet under PC2 laboratory conditions. Equipment and surfaces were disinfected with 70% ethanol before and after use. PPE was worn at all times, with frequent glove changes to reduce contamination risk. Medium and reagents were handled aseptically, and cell cultures were monitored microscopically for signs of contamination. Only one cell line was handled at a time to prevent cross-contamination. Incubators were routinely cleaned and disinfected, with water trays containing a fungicidal agent. While mycoplasma testing was not included due to resource constraints, additional contamination was monitored via PCA/SDA periodically.

2.6 Experimental Design

A multi-phase, *in vitro* experimental approach was employed to investigate the effects of kawakawa extract on macrophage function across different treatment conditions and inflammatory contexts. This study focused primarily on co-treatment (simultaneous intervention) and pre-treatment (intervention before the inflammatory response was established). Post-treatment experiments were conducted subsequently using the selected target extract dose.

Prior to Phase 1 experiments, the cytotoxicity of kawakawa extract was evaluated using a trypan blue exclusion assay. Macrophages were treated with kawakawa extract at concentrations ranging from 0.01 to 100 $\mu g/mL$ to determine the non-cytotoxic working concentration range for subsequent experiments.

2.6.1 Phase 1: Broad Concentration Screen (Co-treatment with LPS)

This initial phase was designed to evaluate the immunomodulatory potential of kawakawa extract in a preventive context by co-treating macrophages with LPS and the extract simultaneously. The primary aim was to

identify effective concentration ranges for further investigation and to observe potential dose-dependent effects on macrophage activation.

2.6.1.1 Experimental Setup

RAW 264.7 cells were seeded into 24-well plates at a density of 5×10^4 cells per well in 500 μL of complete DMEM containing 5% FBS and 1% P/S. The cells were incubated overnight before treatment.

On the day of treatment, the culture medium was aspirated and replaced with 500 μL of fresh treatment medium containing the appropriate combination of reagents. LPS was added at a final concentration of 500 ng/mL, and kawakawa extract was serially diluted to final concentrations of 100, 10, 1, 0.1, and 0.01 μg/mL.

To ensure the reliability of experimental outcomes, a range of controls was included in the design. Unstimulated cells served as the negative control, representing the basal state of untreated macrophages. Cells treated with LPS and 0.1% DMSO served as the positive vehicle control, while cells treated with DMSO alone served as the solvent control to confirm that the observed effects were not due to the solvent used for extract dissolution. Treatment with LPS alone represented the primary positive inflammatory control. Kawakawa-only groups assessed any intrinsic effects of the extract in the absence of inflammatory stimuli. Finally, an additional positive control comprising LPS combined with interferon-gamma (IFN-γ) was included to induce enhanced M1-type pro-inflammatory polarisation and a stronger inflammatory response than LPS alone.

2.6.1.2 Treatment Groups

To enable direct comparison across treatment conditions, a defined set of experimental groups was established, which consisted graded concentrations of kawakawa extract alongside appropriate controls. This design allowed for the evaluation of dose-dependent responses and the specificity of kawakawa's modulatory effects on LPS-induced macrophage activation. Table 7 outlines all treatment groups used in Phase 1, which investigated the co-administration of kawakawa extract with LPS in RAW 264.7 cells.

Table 7: Experimental groups (phase 1): co-treatment of macrophages with LPS and kawakawa extract

Group Name	Kawakawa (µg/mL)	LPS (500 $\mathrm{ng/mL}$)	Other Additives
Unstimulated Control	0	No	No
Positive Vehicle Control	0	Yes	0.1% DMSO (v/v)
Vehicle Control	0	No	0.1% DMSO (v/v)
LPS Only	0	Yes	No
Kawakawa Only (High)	100	No	No
Kawakawa Only (Low)	1	No	No
Kawakawa + LPS (D1)	100	Yes	DMSO standardised $\leq 0.1\%$
Kawakawa + LPS (D2)	10	Yes	DMSO standardised $\leq 0.1\%$
Kawakawa + LPS (D3)	1	Yes	DMSO standardised $\leq 0.1\%$
Kawakawa + LPS (D4)	0.1	Yes	DMSO standardised $\leq 0.1\%$
Kawakawa + LPS (D5)	0.01	Yes	DMSO standardised $\leq 0.1\%$
Positive Control (M1)	0	Yes	IFN-γ

Note:
- Total volume per well: $500\,\mu\mathrm{L}$.
- All treatments were standardised to contain $\leq 0.1\%$ (v/v) DMSO, including the vehicle control group.

2.6.1.3 Co-treatment Procedure

1. Preparation of Working Solutions:
LPS stock solution (1 mg/mL) and IFN-γ (100 μg/mL) were diluted in culture medium to their working concentrations. Final concentrations used in stimulation were 500 ng/mL for LPS and 20 ng/mL for IFN-γ.

A working solution of the kawakawa extract was prepared from the stock in complete medium to achieve a concentration of 100 μg/mL (D1). Serial 1:10 dilutions were made by mixing 100 μL of the previous concentration with 900 μL of complete DMEM containing DMSO to achieve 10 μg/mL (D2), 1 μg/mL (D3), 0.1 μg/mL (D4), and 0.01 μg/mL (D5). Each dilution was mixed thoroughly before proceeding to the next.

The DMSO content contributed by the extract stock was taken into account during dilution preparation, and additional DMSO was added as required to ensure that all treatment groups contained a final DMSO concentration of 0.1% (v/v). This approach maintained a consistent vehicle concentration across all treatments, allowing any observed effects to be attributed to the extract rather than differences in solvent concentration.

2. Cell Seeding (Day -1):
RAW 264.7 macrophages were seeded into culture plates (24-well) one day prior to treatment, allowing them to adhere and reach approximately 80% confluence by Day 0.

3. Treatment Application (Day 0, T = 0 h):
On Day 0, treatment mixtures were freshly prepared. For co-treatment groups, cells were simultaneously exposed to kawakawa extract (at the designated concentration) and LPS. Treatments were added directly to each well using a pipette. Care was taken to avoid air bubbles, prevent cross-contamination, and gently mix for even distribution.

4. Incubation:
Cells were incubated at 37°C in 5% CO_2 for 24 hours. Plates were gently rocked every few hours to ensure

even exposure.

5. Post-treatment Analysis:
Following 24 hours of incubation, supernatants were collected and either analysed immediately or stored at -80°C for subsequent analysis. Cells were then harvested for the MTT assay.

Table 8 details the treatment layout for Phase 1 of the experiment.

Table 8: Treatment design for 24-well plate (phase 1: co-treatment of macrophages with LPS and kawakawa extract)

Plate 1

Row	1	2	3	4	5	6
A	D1-1	D2-1	D3-1	D4-1	D5-1	LPS1
B	D1-2	D2-2	D3-2	D4-2	D5-2	LPS2
C	D1-3	D2-3	D3-3	D4-3	D5-3	LPS3
D						

Plate 2

Row	1	2	3	4	5	6
A	U1	VDL1	VD1	H1	L1	P1
B	U2	VDL2	VD2	H2	L2	P2
C	U3	VDL3	VD3	H3	L3	P3
D						

Note: The experiment included eight treatments: Kawakawa + LPS combinations at descending doses (100-0.01 μg/mL, D1-D5), LPS only (LPS), unstimulated control (U), positive vehicle control (VDL), vehicle control (VD), kawakawa only at high and low doses (H: 100 μg/mL; L: 0.01 μg/mL), and enhanced positive control (P, LPS + IFN-γ). Each treatment was performed in triplicate (n = 3).

2.6.2 Phase 2: Preventive Assessment (Kawakawa Pre-treatment before LPS Stimulation)

Building on the findings from Phase 1, the same concentrations range of kawakawa extract were evaluated using a pre-treatment protocol in which cells were exposed to the extract before LPS stimulation. This design enabled direct comparisons between pre-treatment conditions and appropriate controls to evaluate the preventive potential of the extract and its ability to modulate subsequent LPS-induced macrophage activation.

2.6.2.1 Experimental Overview

RAW 264.7 macrophages were pre-treated with kawakawa extract at the designated concentrations for 2 hours before stimulation with LPS. Following pre-treatment, LPS was added to induce M1 polarisation, allowing assessment of the extract's ability to modulate the initiation of the inflammatory response. Cells were subsequently incubated for the required experimental duration depending on the endpoint being measured.

2.6.2.2 Dosing Strategy

The same concentration range used in Phase 1 (0.01-100 $\mu g/mL$) was evaluated to enable direct comparison between co-treatment and pre-treatment conditions. Extract solutions were prepared freshly by serial dilution,

and the final DMSO concentration was standardised to 0.1% (v/v) in all treatment and vehicle control wells. Control groups included a positive vehicle control (0.1% DMSO pre-treatment followed by LPS stimulation), a vehicle control (0.1% DMSO without stimulation), and kawakawa-only treatments without stimulation.

2.6.2.3 Time Points and Sampling

Three sampling schedules were designed:

A. Short-Term Kinetics
Short-term kinetics were assessed to capture early immunological events and rapid signalling changes following kawakawa pre-treatment and stimulation. Measurements were taken at 3 h and 6 h to investigate the initial cellular responses.

B. Long-Term Kinetics
Long-term kinetics were examined to assess sustained or delayed cellular responses following kawakawa pre-treatment and stimulation. Measurements were taken at 24 h and 48 h to evaluate the persistence and progression of the responses.

C. Extended Kinetic Analysis (Selected Dose)
Extended kinetic analysis was performed at the selected kawakawa dose to further investigate prolonged or delayed responses. Measurements were taken at 72 h and 120 h following kawakawa pre-treatment and stimulation.

For 3 h, 6 h, and 24 h sampling, cells were maintained in the same plate with a total volume of 1 mL per well. Supernatant samples (300 μL) were collected at 3 h and 6 h, followed by collection of 400 μL at 24 h. For 48 h, 72 h, and 120 h sampling, the total volume was maintained at 800 μL per well. The Griess assay was performed at all designated time points, whereas the MTT assay was conducted at 24 h, 48 h, 72 h, and 120 h.

2.6.2.4 Treatment Groups

To investigate the immunomodulatory effects of kawakawa extract following LPS-induced macrophage activation, the experimental groups established in Phase 2 are summarised in Table 9. This phase focused on evaluating the protective potential of the extract when administered prior to inflammatory stimulation and served as a prophylactic intervention model. RAW 264.7 macrophages were pre-treated with kawakawa extract for 2 hours prior to LPS stimulation. The group design included appropriate controls to distinguish the effects of the extract from both LPS stimulation and DMSO vehicle.

Table 9: Experimental groups (phase 2): kawakawa pre-treatment prior to LPS-induced macrophage activation

Group Name	LPS (500 ng/mL)	Kawakawa Dose (μg/mL)	0.1% DMSO (v/v)	Purpose
Positive Vehicle Control	Yes	No	Yes	Positive control
Vehicle	No	No	Yes	Solvent control
Kawakawa Alone	No	Relevant concentration	No	Extract-only effect
Kawakawa → LPS	Yes	10-fold serial dilutions	No	Pre-treatment under LPS stimulation

Note:
- A total volume of 1 mL was added to each well, with sequential sampling from the same wells at 3, 6, and 24 h. For the 48, 72, and 120 h time points, 800 μL was added to each well, with separate wells used for each sampling time point.
- All treatments were standardised to contain $\leq 0.1\%$ (v/v) DMSO, including the vehicle control group.

To evaluate both immediate and delayed responses to the kawakawa extract, samples were collected at multiple time points, covering both short-term and long-term kinetics. The full experimental timeline is detailed in Table 10.

Table 10: Phase 2 experimental schedule: pre-inflammatory intervention workflow (LPS stimulation)

Day	Time Point	Procedure
Day -1	-	Seed RAW 264.7 macrophages in appropriate plate format to reach ∼80% confluence by Day 0
Day 0	0 h	Add kawakawa extract and incubate for 2 hours
	2h	Stimulate cells with LPS (500 $\mathrm{ng/mL}$)
	3 h and 6 h post-stimulation	Collect samples for short-term kinetics
Day 1-5	24 h, 48 h, 72 h and 120 h post-stimulation	Collect samples for long-term kinetics

2.6.3 Phase 3: Mechanistic Assessment (Kawakawa Pre-treatment before IFN-γ Stimulation)

Based on the results from previous phases, the effects of kawakawa extract were further evaluated under IFN-γ stimulation conditions. This design enabled assessment of the extract's ability to modulate IFN-γ-induced macrophage activation and M1 polarisation, which provided insight into its broader immunomodulatory effects beyond LPS-driven inflammation.

2.6.3.1 Experimental Overview

RAW 264.7 macrophages were treated with kawakawa extract at the designated concentrations prior to stimulation with IFN-γ. Following extract treatment, IFN-γ was added to induce M1 polarisation, and cells were

incubated for 24 or 48 hours to evaluate time-dependent effects on macrophage responses.

2.6.3.2 Dosing Strategy

The same concentration range of kawakawa extract used in previous phases (0.01-100 $\mu g/mL$) was evaluated to maintain consistency across experimental conditions. Extract solutions were prepared freshly by serial dilution, and the final DMSO concentration was standardised to 0.1% (v/v) in all treatment and vehicle control wells. Control groups included a positive vehicle control (0.1% DMSO pre-treatment followed by IFN-γ stimulation), a vehicle control (0.1% DMSO without stimulation), and kawakawa-only treatments without stimulation.

2.6.3.3 Time Points and Treatment

All treatment conditions were identical to those in the Phase 2 LPS pre-treatment protocol, except that IFN-γ was used as the stimulus. Samples were collected at 24 h and 48 h following kawakawa pre-treatment and stimulation, with total volumes of 500 μL for 24 h and 800 μL for 48 h sampling, respectively. The experimental schedule for Phase 3 is summarised in Table 11.

Table 11: Phase 3 experimental schedule: pre-inflammatory intervention workflow (IFN-γ stimulation)

Day	Time Point	Procedure
Day -1	–	Seed RAW 264.7 macrophages in the appropriate plate format to reach ∼80% confluence by Day 0
Day 0	0 h	Add kawakawa extract for 2 hours pre-treatment
	2 h	Stimulate cells with IFN-γ
Day 1–2	24 h and 48 h post-stimulation	Collect supernatants and/or cells for subsequent analysis

2.6.4 Phase 4: Broadening Stimuli and Intervention Timings (Co-treatment, Pre-treatment and Post-treatment)

This phase expanded the investigation by including LPS, IFN-γ, and combined IFN-γ/LPS stimulation while using the selected effective kawakawa extract concentrations identified in previous phases. The aim was to compare the effects of different treatment timings, including co-treatment (simultaneous addition of kawakawa extract and inflammatory stimuli), pre-treatment (2 h kawakawa extract exposure followed by stimulation), and post-treatment (kawakawa extract addition after 4 h of initial stimulation), across different inflammatory conditions.

2.6.4.1 Detailed Procedure:

Day -1: Cell Seeding
RAW 264.7 macrophages were seeded into the appropriate culture plates one day before treatment. The seeding density was adjusted to allow the cells to reach approximately 80% confluence by Day 0, ensuring suitable cell

growth and attachment before the application of inflammatory stimuli and kawakawa extract.

Day 0 (T = 0 h): Stimulation and Treatment

Three treatment strategies were investigated to compare the effects of different intervention timings.

Co-treatment Groups

For the co-treatment groups, kawakawa extract at the selected concentration was added simultaneously with the inflammatory stimuli. The stimuli consisted of LPS at 500 ng/mL, IFN-γ at 20 ng/mL, or a combination of both. The treatment mixtures were gently mixed to ensure even distribution across the wells, and the cells were subsequently incubated under standard culture conditions.

Pre-treatment Groups

For the pre-treatment groups, RAW 264.7 macrophages were initially exposed to kawakawa extract for 2 hours before inflammatory stimulation. Following this pre-treatment period, the corresponding inflammatory stimuli (LPS, IFN-γ, or both) were added to the culture medium. The cells were then incubated for the designated experimental durations to evaluate whether prior exposure to kawakawa extract could modulate the subsequent inflammatory response.

Post-treatment Groups

For the post-treatment groups, macrophages were first stimulated with the respective inflammatory stimuli for 4 hours to establish an inflammatory response before the addition of kawakawa extract. Following this initial stimulation period, the selected extract concentration was added to each well, and incubation continued for the designated experimental durations. This treatment strategy was designed to investigate the potential effects of kawakawa extract on macrophages after inflammatory activation had commenced.

Control Groups

Appropriate control groups were included to distinguish the effects of kawakawa extract from those associated with the inflammatory stimuli and solvent. These consisted of a positive vehicle control containing 0.1% (v/v) DMSO and the respective inflammatory stimuli, a vehicle control containing 0.1% (v/v) DMSO without inflammatory stimulation, and a kawakawa-only group without inflammatory stimuli.

Incubation and Sampling Time Points

Following treatment, the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Samples were collected at 24 and 48 hours to evaluate the effects of the different treatment strategies over time. Culture supernatants were collected for subsequent nitric oxide analysis using the Griess assay, while the corresponding cell samples were used for cell viability assessment using the MTT assay, where applicable.

2.6.4.2 Treatment Groups

To further evaluate the immunomodulatory role of kawakawa extract under different inflammatory conditions, a comprehensive set of treatment groups was established in Phase 4 (Table 12). This phase compared co-treatment, pre-treatment and post-treatment strategies involving LPS and/or IFN-γ stimulation. By incorporating single and dual cytokine stimulation models, this design aimed to mimic varying degrees of macrophage

activation (e.g., M1-polarising conditions), and to determine whether kawakawa extract could modulate these states either preventively (pre-treatment and co-treatment) or therapeutically (post-treatment). Appropriate vehicle and stimulation controls were included to ensure that observed effects could be attributed to the extract itself.

Table 12: Experimental groups (phase 4): co-, pre- and post-treatment with kawakawa under LPS and/or IFN-γ stimulation

Group	Kawakawa Extract (Selected Conc.)	LPS	IFN-γ	0.1% DMSO (v/v)	Purpose
Vehicle Control	No	No	No	Yes	Negative control (baseline)
LPS Vehicle	No	Yes	No	Yes	Positive control (LPS stimulation)
IFN-γ Vehicle	No	No	Yes	Yes	Positive control (IFN-γ stimulation)
LPS + IFN-γ Vehicle	No	Yes	Yes	Yes	Positive control (LPS + IFN-γ stimulation)
Kawakawa Alone	Yes	No	No	No	Kawakawa effect without immune stimuli
Kawakawa + LPS	Yes	Yes	No	No	Co-treatment under LPS stimulation
Kawakawa + IFN-γ	Yes	No	Yes	No	Co-treatment under IFN-γ stimulation
Kawakawa + LPS + IFN-γ	Yes	Yes	Yes	No	Co-treatment under dual stimulation
Kawakawa $\rightarrow$ LPS	Yes	Yes	No	No	Pre-treatment under LPS stimulation
Kawakawa $\rightarrow$ IFN-γ	Yes	No	Yes	No	Pre-treatment under IFN-γ stimulation
Kawakawa $\rightarrow$ LPS + IFN-γ	Yes	Yes	Yes	No	Pre-treatment under dual stimulation
LPS $\rightarrow$ Kawakawa	Yes	Yes	No	No	Post-treatment under LPS stimulation
IFN-γ $\rightarrow$ Kawakawa	Yes	No	Yes	No	Post-treatment under IFN-γ stimulation
LPS + IFN-γ $\rightarrow$ Kawakawa	Yes	Yes	Yes	No	Post-treatment under dual stimulation

Note:
- Total volume per well: 24 h: $500\,\mu$L; 48 h: $800\,\mu$L.
- All treatments were standardised to contain $\leq 0.1\%$ (v/v) DMSO, including the vehicle control group.

2.6.5 Replicates and Quality Control

All treatments were performed using three independent biological replicates ($n \geq 3$) for confirmatory experiments, with technical triplicates included within each biological replicate where applicable. Cell viability was monitored using trypan blue exclusion assay, MTT assay and phase contrast microscopy prior to data analysis. Sterility was confirmed visually and by periodic culture on PCA/SDA agar plates.

2.7 Downstream Assay Procedures

Following the experimental treatments, a series of downstream assays were performed to evaluate the cellular responses and biological effects under different experimental conditions.

2.7.1 Cytotoxicity: Trypan Blue Exclusion Assay

The trypan blue exclusion assay was used as an initial assessment of cell viability following exposure to kawakawa extract. This assay was essential during the preliminary concentration screening, as it helped identify extract concentrations that could be used in subsequent functional experiments without causing substantial cytotoxicity. Maintaining an acceptable level of cell viability was critical to ensure that changes in inflammatory responses were not simply consequences of reduced cell numbers or cell death.

Following the designated treatment period, adherent RAW 264.7 macrophages were gently detached using a cell scraper or TrypLE Express. The resulting cell suspension was transferred into a microcentrifuge tube and centrifuged at 300 × g for 5 minutes. After centrifugation, the supernatant was discarded, and the cell pellet was resuspended in 1 mL of phosphate-buffered saline (PBS). An aliquot of 10 µL of cell suspension was mixed with 10 µL of 0.4% trypan blue solution at a 1:1 ratio. Subsequently, 10 µL of the mixture was loaded into a haemocytometer and examined under a light microscope. Viable cells, which excluded the dye and remained unstained, were distinguished from non-viable cells, which appeared blue. Cell counts were obtained from at least four haemocytometer quadrants.

Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100\%$$

Samples exhibiting at least 80% viability were considered suitable for subsequent functional assays. This criterion helped minimise the influence of cytotoxicity when interpreting the immunomodulatory effects of kawakawa extract.

2.7.2 Cell Viability: MTT Assay

The MTT assay was used to evaluate cellular metabolic activity after treatment with kawakawa extract under different inflammatory conditions. Unlike the trypan blue exclusion assay, which directly distinguishes viable and non-viable cells based on membrane integrity, the MTT assay measures the reduction of tetrazolium salt to coloured formazan by metabolically active cells. Therefore, it provides a complementary assessment of cellular metabolic activity and helps determine whether changes observed in nitric oxide production are associated with potential alterations in cell viability.

In this study, the MTT assay was applied to samples collected at the designated experimental time points, including 24, 48, 72 and 120 hours where applicable. Following completion of the treatment period, the culture medium was replaced with 100 µL of fresh culture medium in each well. Subsequently, 10 µL of 12 mM MTT stock solution was added to each well. For background correction, a cell-free control containing 100 µL of culture medium and 10 µL of MTT solution was included.

The plates were incubated at 37°C for 2-5 hours under standard culture conditions to allow formazan crystal formation. Following incubation, the culture medium was carefully removed, leaving approximately one-quarter of the original volume in each well. Subsequently, 50 µL of DMSO was added to dissolve the formazan crys-

tals. The contents of each well were mixed thoroughly by pipetting to ensure complete dissolution, followed by a further incubation at 37°C for 10 minutes.

The samples were mixed again before absorbance was measured at 540 nm using a microplate reader. Relative cell viability was expressed as a percentage relative to the appropriate control group, calculated using the following equation:

$$\text{Relative cell viability (\%)} = \frac{\text{Absorbance of treated group}}{\text{Absorbance of control group}} \times 100\%$$

The MTT results were interpreted alongside the Griess assay measurements to distinguish changes in nitric oxide production from potential changes in cellular metabolic activity. This helped determine whether the reduced inflammatory response observed following kawakawa treatment reflected an immunomodulatory effect or was instead associated with cytotoxicity.

2.7.3 Nitric Oxide (NO) Production: Griess Assay

The Griess assay was employed to assess nitric oxide production by measuring nitrite accumulation in the culture supernatants of RAW 264.7 macrophages. Nitric oxide is an inflammatory mediator produced by activated macrophages. Increased NO production is commonly associated with pro-inflammatory activation and the induction of inducible nitric oxide synthase (iNOS). Therefore, measuring extracellular nitrite provides an indirect indicator of NO production and enables the evaluation of the potential immunomodulatory effects of kawakawa extract.

In this study, the Griess assay was applied across the different experimental phases to investigate the effects of kawakawa extract under co-treatment, pre-treatment and post-treatment conditions. It was also used to compare macrophage responses following stimulation with LPS, IFN-γ, or their combination. Supernatants collected at the designated time points were analysed to determine whether kawakawa treatment altered nitrite accumulation relative to the corresponding inflammatory and vehicle controls.

Following the designated incubation period, culture supernatants were collected and centrifuged at 300 × g for 5 minutes to remove suspended cellular debris. The clarified supernatants were transferred into fresh tubes and either analysed immediately or stored at -80°C until analysis.

A sodium nitrite ($NaNO_2$) standard curve was prepared in complete DMEM to enable quantification of nitrite concentrations in the samples. For the assay, 150 µL of each sample or standard was transferred into a 96-well plate, followed by the addition of 130 µL of Milli-Q water and 20 µL of Griess reagent. The Griess reagent consisted of a 1:1 mixture of 1% sulfanilamide and 0.1% N-(1-naphthyl)ethylenediamine (NED) in 2.5% phosphoric acid.

The plate was incubated at room temperature for 30 minutes, protected from light, to allow the formation of the coloured azo dye resulting from the reaction between nitrite and the Griess reagents. Absorbance was subsequently measured at 548 nm using a microplate reader. Nitrite concentrations in the culture supernatants were determined by comparison with the sodium nitrite standard curve.

The measured nitrite concentrations were used to compare inflammatory responses between treatment groups. In particular, differences between LPS- or IFN-γ-stimulated controls and kawakawa-treated groups provided an indication of whether the extract could modulate stimulus-induced NO production. These results were interpreted together with MTT measurements to account for potential changes in cellular metabolic activity and viability.

2.8 Statistical Analysis

Phase 1, Phase 2 and Phase 3 were conducted as preliminary screening experiments, and statistical analyses were performed to identify effective treatment conditions. Selected conditions were subsequently validated in Phase 4 using three independent biological replicates ($n \geq 3$) for confirmatory analysis. Technical triplicates were included within each biological replicate where applicable. Data were presented as mean ± standard deviation (SD), unless otherwise specified.

2.8.1 Data Preparation and Preliminary Checks

Before performing statistical tests, data normality was assessed using the Shapiro-Wilk test to determine whether parametric methods were appropriate. The homogeneity of variances was evaluated using Levene's test. If the assumptions for analysis of variance (ANOVA) were violated, data transformations such as logarithmic or square root transformations could be applied. In cases where normality could not be achieved through transformation, non-parametric alternatives such as the Kruskal-Wallis test were used.

2.8.2 Phase 1: Co-Treatment Comparisons

To compare multiple treatment groups under co-treatment conditions, a one-way ANOVA was employed. If a significant overall effect was detected, Dunnett's post hoc test was used to compare each treatment group with the control group.

2.8.3 Phase 2 and 3: Time- and Dose-Dependent Effects

A two-way ANOVA was used to examine the effects of both time and dose, as well as their interaction. When a significant interaction was observed, simple effects analysis was carried out to explore specific group differences at individual levels (e.g., comparing doses within a single time point). Post hoc pairwise comparisons were performed using Tukey-adjusted comparisons to control the family-wise Type I error rate.

2.8.4 Phase 4: Multi-Factorial Comparison

To evaluate the effects of multiple experimental factors, such as treatment timing and different stimulus types, a factorial (multi-factor) ANOVA was used. Significant main effects and interactions were further explored using estimated marginal means (EMMs) to compare differences between specific groups. Interaction plots and simple main effect analyses were used to aid interpretation of significant interactions. Additionally, planned contrast tests were applied to assess specific research hypotheses where appropriate.

2.8.5 Software and Significance Thresholds

All statistical analyses were performed using R version 4.5.3 (R Core Team, 2026), with packages including dplyr, tidyr, ggplot2, car, emmeans, multcomp, multcompView, and readxl. The rstatix package was optionally used to streamline hypothesis testing. In addition, GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for complementary statistical analyses and graphical visualisation (GraphPad Software, 2014). Statistical significance was defined as $p < 0.05$, unless otherwise specified. Where applicable, exact p-values and effect sizes were reported to provide context regarding the strength and reliability of the observed effects.

3 Context-Dependent Effects of Hydroethanolic Kawakawa (*Piper excelsum*) Leaf Extract on Nitrite Accumulation in LPS- and IFN-γ-Activated RAW 264.7 Macrophages

3.1 Preface

The literature reviewed in Chapter 1 highlighted the increasing interest in natural products as potential modulators of immune function, particularly their ability to regulate excessive inflammatory responses while maintaining physiological immune activity. Kawakawa (*Piper excelsum*), a traditional New Zealand medicinal plant, has been used for generations in a range of health-related applications, and emerging scientific evidence has suggested that its bioactive constituents possess anti-inflammatory and immunomodulatory properties. However, despite growing interest in kawakawa, the mechanisms involved in its effects on immune cell function remain incompletely understood.

Macrophages are key regulators of innate immunity and play a critical role in coordinating inflammatory responses. While macrophage activation is essential for host defence, inappropriate or sustained activation may disrupt inflammatory homeostasis and contribute to chronic inflammation and tissue damage. Therefore, identifying natural compounds that can modulate macrophage activation without causing cytotoxic effects represents an important area of investigation. Previous studies have demonstrated that kawakawa extracts can influence inflammatory signalling pathways, including NF-κB-associated responses and cytokine regulation; however, most investigations have focused on individual inflammatory pathways or limited cellular models. Whether kawakawa can regulate macrophage responses under different inflammatory activation states remains unclear.

A major consideration when evaluating immunomodulatory activity is that inflammatory responses are highly context-dependent. Different inflammatory stimuli activate distinct but interconnected signalling networks, and the biological effect of a natural extract may depend on both the activation state of the immune cell and the timing of exposure. Therefore, this study was designed to investigate the effects of kawakawa extract using RAW 264.7 macrophages stimulated with different inflammatory conditions, including lipopolysaccharide (LPS), interferon-gamma (IFN-γ), and combined LPS and IFN-γ stimulation. It was hypothesised that kawakawa would suppress excessive macrophage inflammatory activation while maintaining cellular viability, and that its effects would vary according to inflammatory context and treatment strategy.

The study presented in the following chapter demonstrates that kawakawa extract reduced inflammatory nitric oxide (NO) production across multiple macrophage activation models without compromising cell viability. A key observation was that kawakawa activity was strongly influenced by treatment timing, with pre-treatment and co-treatment strategies producing greater inhibitory effects than post-treatment. These results suggest that kawakawa may function primarily as a regulator of macrophage activation by limiting excessive inflammatory responses rather than reversing fully established inflammation.

Although this study provides further evidence supporting the immunomodulatory potential of kawakawa, addi-

tional research is required to identify the active phytochemical constituents and define the molecular pathways involved. Future investigations integrating chemical characterisation, mechanistic studies, and physiological validation will be essential for determining how kawakawa may contribute to approaches aimed at maintaining immune balance.

The study presented in this chapter is formatted as a manuscript prepared for submission to a peer-reviewed journal.

3.2 Abstract

Kawakawa (*Piper excelsum*) is a culturally significant plant of Aotearoa New Zealand used in rongoā Māori. This study investigated the concentration-, time-, stimulus-, and treatment-timing-dependent effects of a hydroethanolic kawakawa leaf extract in RAW 264.7 murine macrophages stimulated with lipopolysaccharide (LPS), interferon-gamma (IFN-γ), or LPS plus IFN-γ. Initial trypan blue exclusion assays detected no statistically significant reduction in the percentage of viable cells following exposure to 0.01-100 μg/mL extract under the screening conditions. Subsequent experiments assessed MTT-reducing metabolic activity and nitrite accumulation in culture supernatants, with nitrite used as an indirect index of nitric oxide production. Kawakawa pre-treatment reduced LPS-induced nitrite accumulation at 24, 48, and 72 h, with the greatest reductions generally observed at 100 μg/mL. However, the inhibitory effect was no longer evident at 120 h. Under IFN-γ stimulation, significant reductions in nitrite accumulation were detected at 48 h but not at 24 h. In experiments comparing treatment schedules, 100 μg/mL kawakawa reduced nitrite accumulation predominantly when administered before or concurrently with inflammatory stimulation, whereas post-treatment produced more limited effects. However, the magnitude and statistical significance of these responses varied according to stimulus, incubation time, and analytical endpoint. MTT-reducing activity was not consistently decreased, although significant reductions occurred under selected conditions at 100 μg/mL. Overall, hydroethanolic kawakawa leaf extract exhibited context-dependent effects on nitrite accumulation in activated RAW 264.7 macrophages, with the most consistent reductions observed at 100 μg/mL and when the extract was administered before or concurrently with inflammatory stimulation. These findings suggest that the observed effects were condition-specific rather than broadly applicable. Further investigation is warranted to determine the underlying mechanisms and bioactive constituents responsible for these effects.

Keywords: *Piper excelsum*, nitrite, macrophage activation, lipopolysaccharide, interferon-gamma

3.3 Introduction

Kawakawa (*Piper excelsum*) is a culturally significant plant endemic to Aotearoa New Zealand that has been used in rongoā Māori for generations (Brooker & Cooper, 1961). Preparations derived from different parts of the plant, including the leaves and stems, have traditionally been used for conditions such as digestive complaints, toothache, and inflammatory skin disorders (Brooker & Cooper, 1961; Obst et al., 2017; Rashidinejad,

2024; Riley, 1994). Phytochemical investigations have identified at least 64 compounds in kawakawa leaves, including phenylpropanoids, lignans, flavonoids, alkaloids, and amides such as pellitorine and piperine (Butts et al., 2019; Jayaprakash et al., 2022). The chemical diversity of kawakawa has prompted interest in its potential biological activities, with reported effects on inflammation, microbial growth, and cellular protection (Butts et al., 2019; Calder et al., 1986; Lei et al., 2015; Ramzan et al., 2022; Tautuiaki et al., 2024; H. Xu, 2018).

Emerging evidence suggests that kawakawa may influence inflammation-related biological processes. Pathway-enrichment analyses following kawakawa consumption have identified changes in mRNA-microRNA networks associated with mitogen-activated protein kinase (MAPK) and mechanistic target of rapamycin (mTOR) signalling (Gojer, 2023; Tautuiaki, 2023; Tautuiaki et al., 2024). However, these findings are associative and do not establish direct regulation of these pathways or demonstrate their involvement in macrophages. Furthermore, existing functional studies predominantly assess bioactivity under fixed, single-parameter conditions. Although NF-κB-related signalling is implicated in the regulation of inflammatory mediators such as IL-6 and NFKB1 (H. Xu, 2018), the cellular actions of kawakawa remain poorly characterised. Previous *in vitro* studies by Newland (2016) suggest that kawakawa leaf extracts can modulate cell viability, with effects varying according to extract concentration and experimental conditions. However, the mechanisms underlying these effects, and whether they depend on the inflammatory stimulus or duration of exposure, remain unclear.

Macrophages are central regulators of inflammatory responses and exhibit substantial functional plasticity in response to signals within their microenvironment (Arora et al., 2018; Locati et al., 2020). Despite that macrophage activation has traditionally been described using the M1/M2 framework, macrophage phenotypes are context-dependent and are better characterised by the stimuli administered and functional or molecular markers measured (Murray et al., 2014). Lipopolysaccharide (LPS) and interferon-gamma (IFN-γ), applied individually or in combination, are commonly employed as defined stimuli to induce pro-inflammatory responses in macrophage models. These stimuli can increase inducible nitric oxide synthase (iNOS) expression and the production of nitric oxide (NO), TNF-α, IL-6, and other inflammatory mediators (Lendeckel et al., 2022). RAW 264.7 macrophages therefore provide a well-established *in vitro* model for exploring how kawakawa extract affects stimulus-dependent inflammatory responses (Noda & Amano, 1997).

In this study, we investigated the effects of a hydroethanolic kawakawa leaf extract on nitrite accumulation and MTT-reducing metabolic activity in RAW 264.7 murine macrophages. The novelty of this work lies in systematically evaluating four critical experimental dimensions within a single study: extract concentration, exposure duration, inflammatory stimulus (LPS, IFN-γ, or combination), and treatment schedule (pre-, co-, or post-treatment). By examining how these factors influenced the cellular response to kawakawa, we demonstrated that kawakawa extract reduced nitrite accumulation under selected experimental conditions, with the greatest effects generally observed at 100 μg/mL and when the extract was administered before or concurrently with inflammatory stimulation. However, the magnitude and persistence of these effects depended on the stimulus, incubation period, and treatment schedule, indicating that the effects of kawakawa on activated macrophages are strongly context-dependent. This work establishes an empirical foundation for understanding the context dependence of botanical extract bioactivity in macrophage models.

3.4 Materials and Methods

3.4.1 Preparation of Kawakawa Extract

Dried commercial kawakawa leaves (OKU NZ Native Herbal Products Ltd) were ground under liquid nitrogen and extracted with 50% ethanol at a plant material-to-solvent ratio of 1:10 (w/v). Briefly, 2 g of powdered leaves was mixed with 20 mL of 50% ethanol and subjected to ultrasound-assisted extraction (UAE) for 30 min using an ultrasonic processor (Hielscher UP100H, Germany) at 50% amplitude with a pulse cycle of 0.5 in a water-ice bath. The extract was centrifuged at 10,000 $\times$ g for 15 min at 4°C, and the clarified supernatant was collected and passed through a 0.45 μm membrane filter. The filtrate was concentrated under a gentle stream of nitrogen for 1 h and subsequently dried at 30°C using a vacuum concentrator (Eppendorf Concentrator Plus, Germany) for 7 h. The dried extract was reconstituted in 100% dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL and sterilised using a 0.22 μm syringe filter before use in cell-based assays. The extraction yielded 0.315 g of dried extract from 2 g of starting material (15.75%, w/w).

Sterility of the final extract was confirmed by culturing aliquots on Plate Count Agar (PCA; 37°C for 48 h) and Sabouraud Dextrose Agar (SDA; 25.5°C for 7 days). No bacterial or fungal colonies were detected, confirming that the extract was suitable for subsequent cell culture experiments.

3.4.2 Cell Culture and Treatment

RAW 264.7 murine macrophages (ATCC TIB-71) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37°C in a humidified atmosphere containing 5% CO_2. Inflammatory responses were induced by treating the cells with lipopolysaccharide (LPS; 500 ng/mL), interferon-gamma (IFN-γ; 20 ng/mL), or a combination of LPS and IFN-γ. Kawakawa extract was diluted in culture medium to the required treatment concentrations, with the final DMSO concentration maintained below 0.1% (v/v) in all treatment conditions. A vehicle control containing 0.1% (v/v) DMSO in culture medium and an extract-only control containing kawakawa extract without inflammatory stimuli were included in all experiments to account for potential effects of the solvent and extract in the absence of inflammatory stimulation, respectively.

3.4.3 Cell Viability (MTT) and Nitric Oxide Production (Griess) Assays

The cytotoxicity of kawakawa extract at the tested concentrations was initially assessed using the trypan blue exclusion assay. In subsequent experiments, cellular metabolic activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with MTT reagent (Invitrogen, M6494), and absorbance was measured at 540 nm. Nitrite accumulation in culture supernatants was quantified as an indirect measure of nitric oxide production using the Griess Reagent Kit for nitrite quantitation (Thermo Scientific, G7921), with absorbance measured at 548 nm.

3.4.4 Statistical Analysis

Statistical analyses were performed to examine treatment effects and to inform the selection of experimental conditions for confirmatory analysis. Selected conditions were subsequently evaluated in two or more independent biological experiments ($n \geq 2$). Where applicable, each biological experiment included three technical replicates, which were averaged to obtain a single value for statistical analysis. Data are presented as mean $\pm$ standard deviation (SD), unless otherwise stated. Statistical significance was assessed using one-way analysis of variance (ANOVA) for single-factor experiments or factorial (multi-factor) ANOVA for experiments involving multiple independent factors. For multiple comparisons, Tukey's honestly significant difference (HSD) test was used for all pairwise comparisons, whereas Dunnett's post hoc test was used for comparisons of multiple treatment groups against a single control group. Both post hoc tests incorporated adjustments for multiple comparisons. Assumptions of normality and homogeneity of variance were verified using Shapiro-Wilk and Levene's tests. All analyses were performed using R version 4.5.3 (R Core Team, 2026). GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for complementary statistical analyses and graphical visualisation (GraphPad Software, 2014). Statistical significance was defined as $p < 0.05$, unless otherwise specified.

3.5 Results

3.5.1 Kawakawa Extract Shows No Detectable Cytotoxicity in RAW 264.7 Cells

To establish a suitable concentration range for subsequent inflammatory-response experiments, the potential cytotoxicity of kawakawa extract was evaluated in RAW 264.7 macrophages. As illustrated in the experimental timeline (Figure 2A), cells were seeded before treatment and assessed after 24 h of exposure to kawakawa extract. Cells were exposed to 0.01-100 μg/mL kawakawa extract for 24 h, after which live and dead cells were quantified using the trypan blue exclusion assay (Figure 2A).

Live cell counts varied among treatment groups but showed no consistent concentration-dependent decline across the tested range (Figure 2B). Although the mean live cell count appeared lower at 100 μg/mL, it was not significantly different from the vehicle control. In contrast, few recoverable live cells were detected following treatment with the positive cytotoxicity control.

Dead cell counts remained low across the untreated, DMSO vehicle control and kawakawa-treated groups, with no concentration-dependent increase after extract treatment (Figure 2C). No significant differences in dead cell count were detected among the treatment groups. The low number of intact dead cells recovered from the positive-control group likely reflected extensive cell detachment or disruption rather than an absence of cytotoxicity.

Total cell counts were broadly comparable among the untreated, vehicle control and kawakawa-treated groups (Figure 2D). Although some variation was observed between replicates and the mean total cell count appeared lower at 100 μg/mL, no significant overall treatment effect was detected. By comparison, few recoverable cells

were present in the positive-control group, consistent with extensive cell loss.

Cell viability remained high across all kawakawa extract concentrations, with no apparent concentration-dependent reduction (Figure 2E). None of the kawakawa-treated groups differed significantly from the untreated control. Collectively, the absence of a concentration-dependent reduction in live cell count, increase in dead cell count or loss of percentage viability indicates that kawakawa extract showed no detectable cytotoxicity in RAW 264.7 macrophages at concentrations between 0.01 and 100 μg/mL under the conditions tested. This concentration range was therefore used in subsequent inflammatory-response experiments.

3.5.2 Co-treatment with Kawakawa Extract Reduces LPS-Induced Nitrite Accumulation at 100 μg/mL

To determine whether kawakawa extract modulates the inflammatory response during macrophage activation, RAW 264.7 cells were co-treated with kawakawa extract (0.01-100 μg/mL) and LPS (500 ng/mL) for 24 h. Cellular metabolic activity and nitrite accumulation were subsequently assessed using the MTT and Griess assays, respectively (Figure 3A).

MTT-reducing activity did not decrease following co-treatment with kawakawa extract at any concentration tested (Figure 3B). MTT-reducing activity, an indicator of cellular metabolic activity and viability, was largely comparable to that of the LPS-stimulated vehicle control. Although the 100 μg/mL co-treatment group showed the highest mean MTT signal, this difference was not statistically significant. Thus, kawakawa co-treatment produced no detectable change in cellular MTT-reducing activity.

Nitrite concentrations were near baseline in the unstimulated medium and 0.1% DMSO vehicle controls but increased markedly in response to LPS stimulation (Figure 3C), confirming macrophage activation. Co-treatment with 0.01-10 μg/mL kawakawa extract did not significantly alter nitrite accumulation relative to the LPS-stimulated vehicle control. In contrast, co-treatment with 100 μg/mL kawakawa extract reduced nitrite concentration from approximately 17 μM in the LPS-stimulated vehicle control to approximately 7 μM, corresponding to an approximately 57% reduction. Nitrite levels nevertheless remained above the unstimulated baseline, indicating partial rather than complete suppression of the LPS response. Thus, co-treatment with 100 μg/mL kawakawa extract partially attenuated LPS-induced nitrite accumulation, whereas lower concentrations had no significant effect. This reduction was not accompanied by decreased MTT-reducing activity, suggesting that the reduction in nitrite accumulation was unlikely to be explained solely by reduced cellular metabolic activity.

3.5.3 Pre-treatment with Kawakawa Extract Reduces LPS-Induced Nitrite Accumulation at 100 μg/mL

To determine whether kawakawa extract could modify the subsequent inflammatory response to LPS, RAW 264.7 macrophages were pre-treated with kawakawa extract (0.01-100 μg/mL) for 2 h before stimulation with LPS (500 ng/mL) (Figure 4A).

At 24 h, MTT-reducing activity was broadly comparable among the LPS-stimulated vehicle control and cells pre-treated with 0.01-100 μg/mL kawakawa extract (Figure 4B). None of the kawakawa concentrations produced a significant reduction in corrected absorbance compared with the LPS-stimulated vehicle control. Similarly, no significant reduction in MTT-reducing activity was observed at 48 h (Figure 4C).

The extended time course showed that MTT-reducing activity varied substantially with incubation time and LPS stimulation (Figure 4D). The unstimulated vehicle and kawakawa-only groups exhibited a pronounced increase in MTT signal at 72 h, whereas the corresponding LPS-stimulated groups remained lower. Within the LPS-stimulated condition, however, pre-treatment with 100 μg/mL kawakawa did not significantly reduce MTT-reducing activity relative to the vehicle control at any time point. By 120 h, the MTT signals of the two LPS-stimulated groups were similarly low. These findings provide no evidence that kawakawa pre-treatment caused a detectable loss of cellular metabolic activity.

Nitrite accumulation was low in unstimulated cells but increased following LPS stimulation. At 24 h, the LPS-stimulated vehicle control accumulated approximately 24 μM nitrite (Figure 4E). Pre-treatment with 100 μg/mL kawakawa reduced this by approximately 57%, resulting in a concentration of approximately 10 μM. In contrast, concentrations of 0.01-10 μg/mL did not significantly reduce nitrite accumulation. The inhibitory effect of 100 μg/mL kawakawa was more pronounced at 48 h (Figure 4F). Nitrite concentration reached approximately 50 μM in the LPS-stimulated vehicle control but remained at approximately 19 μM after kawakawa pre-treatment with an approximately 60% reduction. The lower kawakawa concentrations again did not significantly reduce nitrite accumulation relative to the LPS-stimulated vehicle control.

The time-course analysis showed that nitrite remained near baseline during the first 6 h after LPS stimulation and subsequently increased at 24 h, reaching a maximum of approximately 62 μM at 72 h before declining by 120 h (Figure 4G). Pre-treatment with 100 μg/mL kawakawa significantly attenuated this response at 24, 48 and 72 h. Nitrite nevertheless remained above the unstimulated baseline, indicating partial rather than complete suppression of the LPS response. At 120 h, nitrite concentrations in the kawakawa-pre-treated and LPS-stimulated vehicle groups had converged and were no longer significantly different. These findings indicate that pre-treatment with 100 μg/mL kawakawa transiently reduced LPS-induced nitrite accumulation between 24 and 72 h without a statistically detectable reduction in MTT-reducing activity.

3.5.4 Higher Concentrations of Kawakawa Attenuate the Delayed Nitrite Response to IFN-γ

To determine whether kawakawa extract modulates IFN-γ-induced macrophage activation, RAW 264.7 cells were pre-treated with kawakawa extract (0.01-100 μg/mL) for 2 h before stimulation with IFN-γ (20 ng/mL). MTT-reducing activity and nitrite accumulation were assessed 24 and 48 h after stimulation (Figure 5A).

At 24 h, MTT-reducing activity was generally comparable between the IFN-γ-stimulated vehicle control and cells pre-treated with 0.01-10 μg/mL kawakawa extract (Figure 5B). However, pre-treatment with 100 μg/mL kawakawa significantly reduced MTT-reducing activity. This indicates a reduction in cellular MTT reduction at the highest kawakawa concentration but does not, by itself, establish a loss of viable cells. At 48 h, none of the kawakawa concentrations significantly reduced MTT-reducing activity relative to the IFN-γ-stimulated vehicle control (Figure 5C), indicating that the reduction observed at 24 h with 100 μg/mL was not sustained.

Nitrite concentrations remained low at 24 h, suggesting that a measurable response to IFN-γ had not yet developed (Figure 5D). Kawakawa pre-treatment did not significantly alter nitrite accumulation at this time point. By 48 h, IFN-γ stimulation markedly increased nitrite accumulation compared with the unstimulated controls (Figure 5E). Pre-treatment with 0.01 μg/mL kawakawa did not significantly affect this response. In contrast, pre-treatment with 0.1, 1, 10 and 100 μg/mL kawakawa significantly reduced nitrite accumulation relative to

the IFN-γ-stimulated vehicle control. However, although statistically significant, the reductions were small in magnitude and did not show a clear concentration-dependent relationship across the kawakawa concentrations tested. Collectively, these findings demonstrate that kawakawa pre-treatment moderately attenuated IFN-γ-induced nitrite accumulation at 48 h at concentrations of 1-100 μg/mL. This effect was not accompanied by a concurrent reduction in MTT-reducing activity.

3.5.5 Timing of Kawakawa Administration Determines Its Effect on Stimulus-Induced Nitrite Accumulation

To determine whether treatment timing influenced the response to kawakawa, RAW 264.7 macrophages received kawakawa extract (100 μg/mL) or the corresponding vehicle control (0 μg/mL kawakawa) 2 h before stimulation (pre-treatment), concurrently with stimulation (co-treatment), or 4 h after stimulation (post-treatment). Cells were stimulated with LPS (500 ng/mL), IFN-γ (20 ng/mL), or LPS + IFN-γ, while MTT-reducing activity and nitrite accumulation were assessed at 24 and 48 h (Figure 6).

The effects of kawakawa on MTT-reducing activity were inconsistent in direction and depended on the stimulus and treatment strategy (Figure 6A-C). Under LPS stimulation, kawakawa pre-treatment increased MTT-reducing activity at both 24 and 48 h, whereas no significant differences were detected after co- or post-treatment (Figure 6A). Under IFN-γ stimulation, the only significant difference was a reduction in MTT-reducing activity upon co-treatment at 48 h (Figure 6B). With combined LPS + IFN-γ stimulation, kawakawa reduced MTT-reducing activity following co-treatment and pre-treatment at 24 h but increased it after post-treatment at 48 h (Figure 6C). Thus, kawakawa did not produce a consistent overall reduction in cellular MTT-reducing activity across the experimental conditions. Although several comparisons reached statistical significance, most differences were small in magnitude and may therefore be of limited biological relevance. The notable exception was the marked increase in MTT-reducing activity in response to kawakawa pre-treatment under LPS stimulation at 24 h.

A clearer treatment-timing pattern was observed for nitrite accumulation. Under LPS stimulation, both co-treatment and pre-treatment with kawakawa significantly reduced nitrite concentrations at 24 and 48 h (Figure 6D). In contrast, post-treatment did not significantly alter nitrite accumulation at either time point. Nitrite concentrations remained low during the first 24 h of IFN-γ stimulation, and no significant effects of kawakawa were detected at this time point (Figure 6E). At 48 h, however, nitrite accumulation was significantly reduced by both co-treatment and pre-treatment. Post-treatment again produced no significant reduction. Following combined LPS + IFN-γ stimulation, co-treatment reduced nitrite accumulation at both 24 and 48 h (Figure 6F). Pre-treatment was also effective at 24 and 48 h, whereas post-treatment did not significantly affect nitrite concentrations at either time point. These comparisons demonstrate that kawakawa reduced stimulus-induced nitrite accumulation most consistently when administered before or concurrently with inflammatory stimulation.

3.6 Discussion

During inflammatory responses, activated macrophages produce nitric oxide, which contributes to antimicrobial defence but can also promote tissue injury when generated excessively or for prolonged periods. In this study, a hydroethanolic kawakawa leaf extract was shown to reduce extracellular nitrite accumulation in stimulated RAW 264.7 macrophages. This effect was, however, strongly dependent on the experimental conditions.

Upon LPS stimulation, significant nitrite reduction was observed exclusively at 100 μg/mL. Specifically, pre-treatment at this concentration demonstrated a distinct time-dependent window of efficacy, sustaining nitrite suppression from 24 to 72 h before the inhibitory effect diminished at 120 h. This temporal pattern suggests that kawakawa may establish an anti-inflammatory state before inflammatory activation. Similar preventive effects have been reported for other botanical extracts, where pre-treatment enables bioactive compounds to regulate early signalling pathways, antioxidant responses, and inflammatory gene transcription prior to maximal inflammatory activation (Tong et al., 2019). The reduced effect during prolonged culture may reflect decreased bioavailability of active compounds due to degradation or cellular metabolism over time. Alternatively, long-term culture conditions, including changes in cell proliferation, accumulation of metabolic by-products, and nutrient availability, may explain the decline of the observed inhibitory effect. Stability and medium-replacement controls would help clarify their potential contributions in future work.

Under IFN-γ stimulation, statistically significant reductions were detected at 48 h across several concentrations, although the changes were small and did not exhibit a clear concentration-dependent relationship. This specific effect is consistent with previous reports indicating increased extracellular nitrite production in RAW 264.7 macrophages after 48 h of IFN-γ exposure (Roshick et al., 2006). The treatment-timing experiment further showed that kawakawa was most consistently effective when administered before or concurrently with LPS, IFN-γ, or LPS + IFN-γ. In contrast, administration 4 h after stimulation had no detectable effect on nitrite accumulation. Importantly, reductions in nitrite accumulation generally occurred without corresponding reductions in MTT-reducing activity. This suggests that the lower nitrite concentrations were not simply caused by a loss of metabolically active cells and is consistent with a more selective effect of kawakawa on the NO-related inflammatory response.

The differential efficacy of kawakawa extract under LPS versus IFN-γ stimulation provides valuable insight into its cellular mode of action. LPS activates macrophages primarily through Toll-like receptor 4 (TLR4), triggering MyD88-dependent signalling, IκB-α degradation, NF-κB activation, and subsequent transcription of inflammatory genes including inducible nitric oxide synthase (iNOS) (Han et al., 2023; Kawai & Akira, 2010; Tong et al., 2019; Ying et al., 2013). In contrast, IFN-γ acts via the IFNGR1/2 complex to activate the JAK/STAT1 signalling cascade, promoting a distinct gene expression profile (Hu et al., 2021; Martinez & Gordon, 2014; Murray, 2017; Schroder et al., 2004). In our experiments, kawakawa extract produced a greater and more consistent reduction in nitrite accumulation following LPS stimulation compared with IFN-γ stimulation. This divergence may support the possibility that kawakawa constituents influence pathways associated with TLR4-dependent activation. However, direct effects on NF-κB or STAT1 signalling remain to be established. When combined, LPS and IFN-γ induce synergistic signalling, leading to enhanced *NOS2* transcription and increased production of NO and pro-inflammatory cytokines (Cho et al., 2008; Murray, 2017). Under these intense activation conditions, pre- and co-treatment with kawakawa retained significant effectiveness, further pointing toward an inhibitory action on early signalling events shared during hyper-inflammatory activation.

The concentration profile and temporal sensitivity of the response further clarify the operational window of kawakawa bioactivity. Significant attenuation was largely confined to 100 μg/mL, with lower concentrations (0.01-10 μg/mL) exhibiting minimal or negligible effects. The upper concentration of 100 μg/mL was selected to maximise the opportunity to detect potential biological activity of the crude extract under *in vitro* conditions, particularly given that individual bioactive phytochemicals within crude botanical extracts are present at relatively low concentrations (Jayaprakash et al., 2022; Lee & Park, 2011; Ying et al., 2013). Therefore, a relatively high total extract concentration may be required to reach the threshold for intracellular target modulation. However, the translational relevance of 100 μg/mL remains uncertain, as pharmacokinetic data are lacking to establish whether this concentration can be achieved systemically or at the tissue level after exposure to kawakawa constituents. Thus, the effective concentrations observed should not be interpreted as directly achievable tissue concentrations following traditional use. Future studies incorporating phytochemical characterisation and pharmacokinetic or tissue-distribution analyses will be important for determining whether the bioactive concentrations identified *in vitro* are physiologically relevant. Furthermore, the clear superiority of pre- and co-treatment over post-treatment indicates that kawakawa acts predominantly during the initiation and transcriptional induction phase of the macrophage response. Once downstream inflammatory signalling and NO-producing pathways were established (as in the 4 h post-treatment condition), the extract showed limited ability to reduce subsequent nitrite accumulation. This temporal pattern raises the possibility that kawakawa influences processes involved in the initiation of the inflammatory response, although the specific molecular targets and pathways responsible for this effect remain to be established. Hence, the extract may be more effective at preventing or limiting macrophage activation than at modifying an already established inflammatory response.

The report extends the limited previous evidence that kawakawa may modulate inflammation-associated responses. Earlier cell-based work reported reductions in IL-6 and NF-κB-related gene expression after treatment with kawakawa extracts (Jayaprakash et al., 2022; H. Xu, 2018), while a recent human study found that acute kawakawa tea consumption altered selected inflammation-associated mRNAs and circulating microRNAs (Tautuiaki et al., 2024). Based on these studies, we hypothesised that kawakawa extract would attenuate the response of activated macrophages. Our data now show that kawakawa can reduce nitrite accumulation, but that this activity is not uniformly observed across concentrations, stimuli, time points, and treatment strategies. Accordingly, the current findings support a context-dependent effect on a specific inflammatory readout rather than broad suppression of macrophage activation.

Compared with previous studies, this study provides a more comprehensive evaluation of the context dependence of kawakawa bioactivity by integrating multiple experimental variables within a single macrophage model. This approach revealed that the anti-inflammatory effects of the extract depended on concentration, inflammatory stimulus, treatment schedule, and exposure duration. The concentration-escalation experiments showed that the most substantial LPS-associated reduction occurred at 100 μg/mL, whereas 0.01-10 μg/mL had little or no detectable effect. This pattern is more consistent with a threshold or concentration-specific response than with progressive concentration-dependent inhibition. Furthermore, the absence of a detectable effect at 120 h and the absence of activity after post-treatment indicate that the effect is limited by both time and treatment schedule. Nevertheless, the results consistently show that kawakawa can attenuate nitrite accumulation when present before or during macrophage activation.

Several important limitations of this study should be explicitly acknowledged. A primary limitation is the absence of phytochemical characterisation of the specific extract batch used in this study. The hydroethanolic

extract was evaluated as a whole crude mixture without comprehensive chemical characterisation or quantification of specific bioactive compounds. Kawakawa leaves contain phenylpropanoids, lignans, flavonoids, alkaloids, and amides, and their abundance varies among plant sources and extraction conditions (Jayaprakash et al., 2022). As a consequence, the observed activity may result from one active constituent, the combined action of several constituents, or interactions between compounds with opposing effects. The lack of compositional data, such as total phenolic content or a high performance liquid chromatography (HPLC) fingerprint, also limits assessment of batch-to-batch reproducibility and prevents direct correlation of specific compound classes with the observed bioactivity. In addition, the 50% ethanol extract used in this study is not chemically equivalent to an aqueous infusion used in customary preparations. Human studies have shown that kawakawa constituents can be absorbed and metabolised following consumption, but they have not established tissue exposures equivalent to 100 μg/mL crude extract or demonstrated clinical anti-inflammatory efficacy (Butts et al., 2019; Jayaprakash et al., 2024; Tautuiaki et al., 2024). Future work should therefore focus on chemical characterisation and batch standardisation of the extract, followed by bioactivity-guided fractionation to identify the constituent or combination of constituents responsible for the observed effect.

In addition, interpretation of the MTT and Griess findings requires caution. Trypan blue exclusion showed no detectable cytotoxicity after 24 h exposure to 0.01-100 μg/mL kawakawa, and the reductions in nitrite were not consistently accompanied by lower MTT-reducing activity. This argues against a general loss of metabolically active cells as the sole explanation for the findings. However, the reductions in MTT-reducing activity observed at 100 μg/mL under selected conditions may reflect altered cellular metabolism or metabolic perturbation rather than cell death. Such metabolic changes could potentially contribute to reduced NO production independently of a direct anti-inflammatory effect of the extract. Moreover, the MTT assay measures the reduction of a tetrazolium substrate rather than viable cell number directly, and plant extracts may alter the assay signal through chemical reduction or absorbance interference (Bruggisser et al., 2002; van Tonder et al., 2015). Similarly, the Griess assay measures accumulated nitrite as an indirect indicator of NO production. Lower nitrite concentrations could reflect reduced cellular NO synthesis, but may also result from chemical scavenging, altered conversion of NO to nitrite, or interference with the Griess reaction (Dirsch et al., 1998; Green et al., 1982). Although the absence of detectable cell death by trypan blue supports a lack of overt cytotoxicity, these findings cannot completely exclude metabolic effects that may contribute to the observed reduction in nitrite. Cell-free extract controls, nitrite spike-recovery experiments, and complementary viability or metabolic assays, such as LDH release and ATP quantification, with appropriate normalisation strategies, would help distinguish direct anti-inflammatory effects from altered cellular metabolism and assay interference in future studies.

Moreover, this study was limited to cellular phenotypic readouts and did not directly examine the underlying molecular mechanisms, such as iNOS protein expression, *NOS2* transcript abundance, or NF-κB and STAT1 phosphorylation. Future studies should investigate these pathways alongside downstream cytokine production and additional markers of macrophage activation.

In conclusion, hydroethanolic kawakawa leaf extract reduced extracellular nitrite accumulation in stimulated RAW 264.7 macrophages under selected experimental conditions. The most substantial and consistent effects occurred at 100 μg/mL during LPS stimulation and when kawakawa was administered before or concurrently with inflammatory stimulation. Effects under IFN-γ stimulation were smaller and not concentration dependent, while post-treatment was ineffective under the conditions tested. These findings support further investigation of kawakawa as a source of biologically active constituents, but do not yet establish the cellular mechanism, absence of assay interference, or therapeutic efficacy.

Figures and figure legends

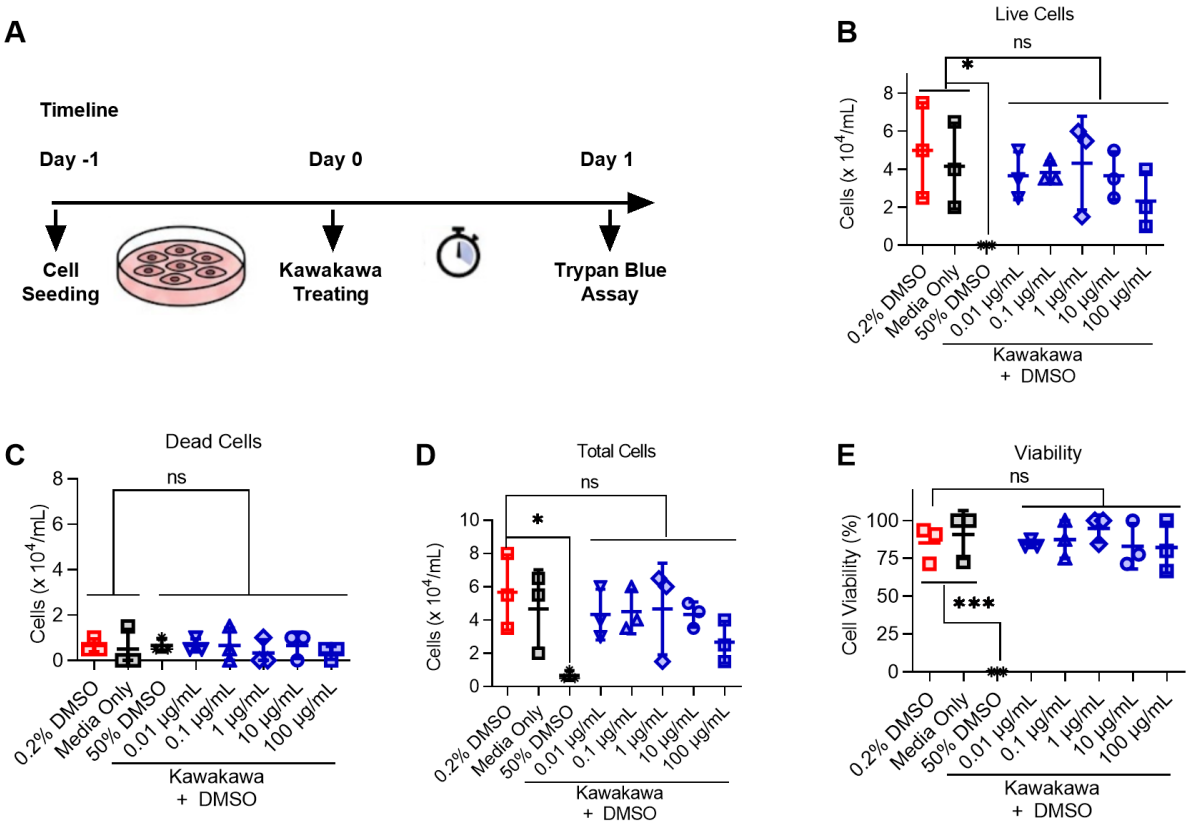


Figure 2: RAW 264.7 macrophage viability is maintained following kawakawa extract treatment. (A) Experimental timeline for assessing the effects of 24 h exposure to kawakawa extract. (B-D) Live, dead, and total cell counts, respectively, determined using the trypan blue exclusion assay. (E) Cell viability, calculated as the percentage of live cells relative to the total number of cells counted. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple-comparisons test. Statistical significance: ns, not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

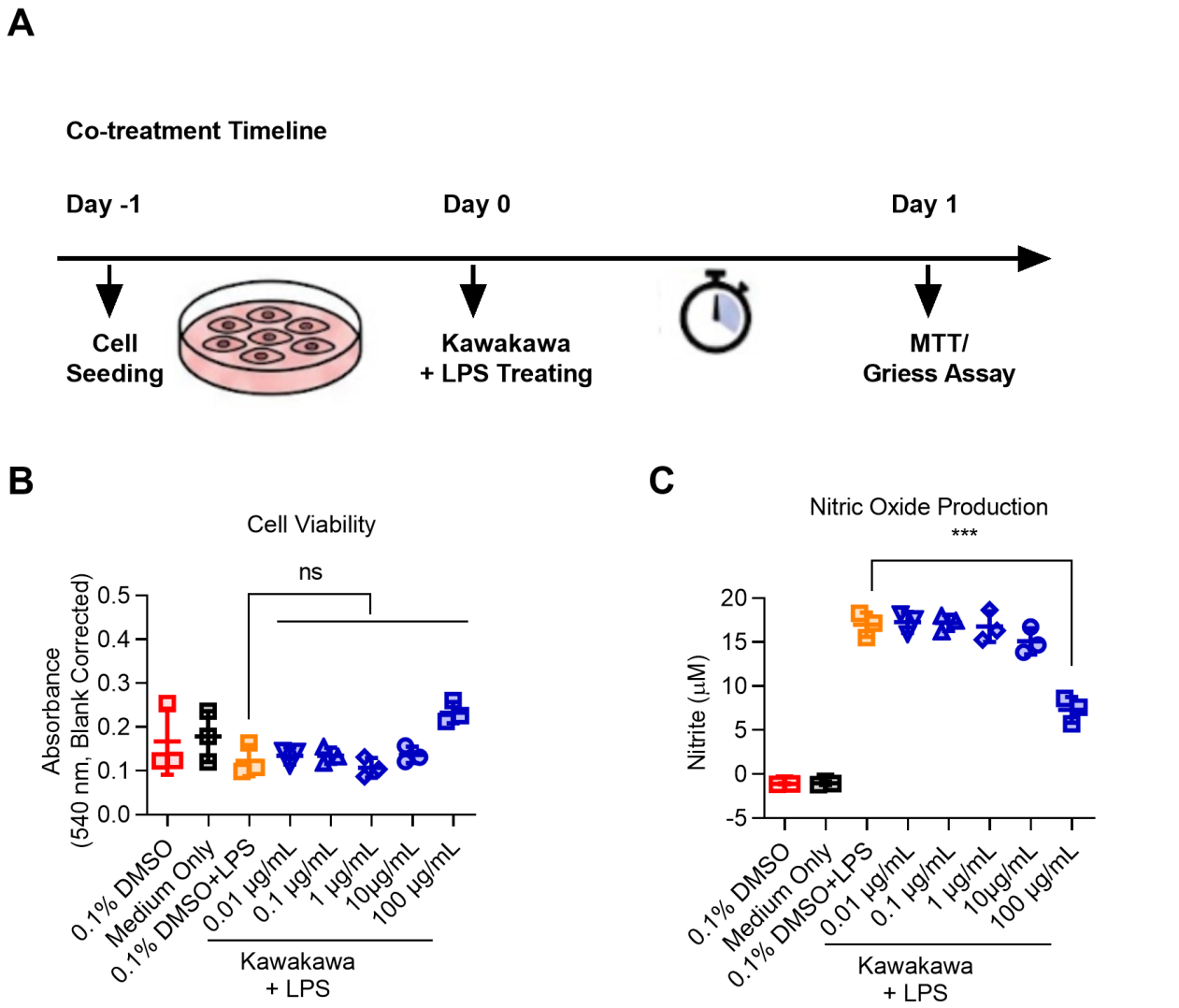


Figure 3: Co-treatment with 100 μg/mL kawakawa extract reduces LPS-induced nitrite accumulation without decreasing MTT-reducing activity in RAW 264.7 macrophages. (A) Experimental timeline. Cells were co-treated with kawakawa extract (0.01-100 μg/mL) and LPS (500 ng/mL) for 24 h. (B) Cellular MTT-reducing activity, expressed as absorbance at 540 nm after subtraction of the assay blank. (C) Nitrite accumulation in culture supernatants, measured using the Griess assay as an indirect indicator of nitric oxide production. Data are presented as mean $\pm$ SD. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple-comparisons test against the relevant control indicated in each panel. Statistical significance: ns, not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

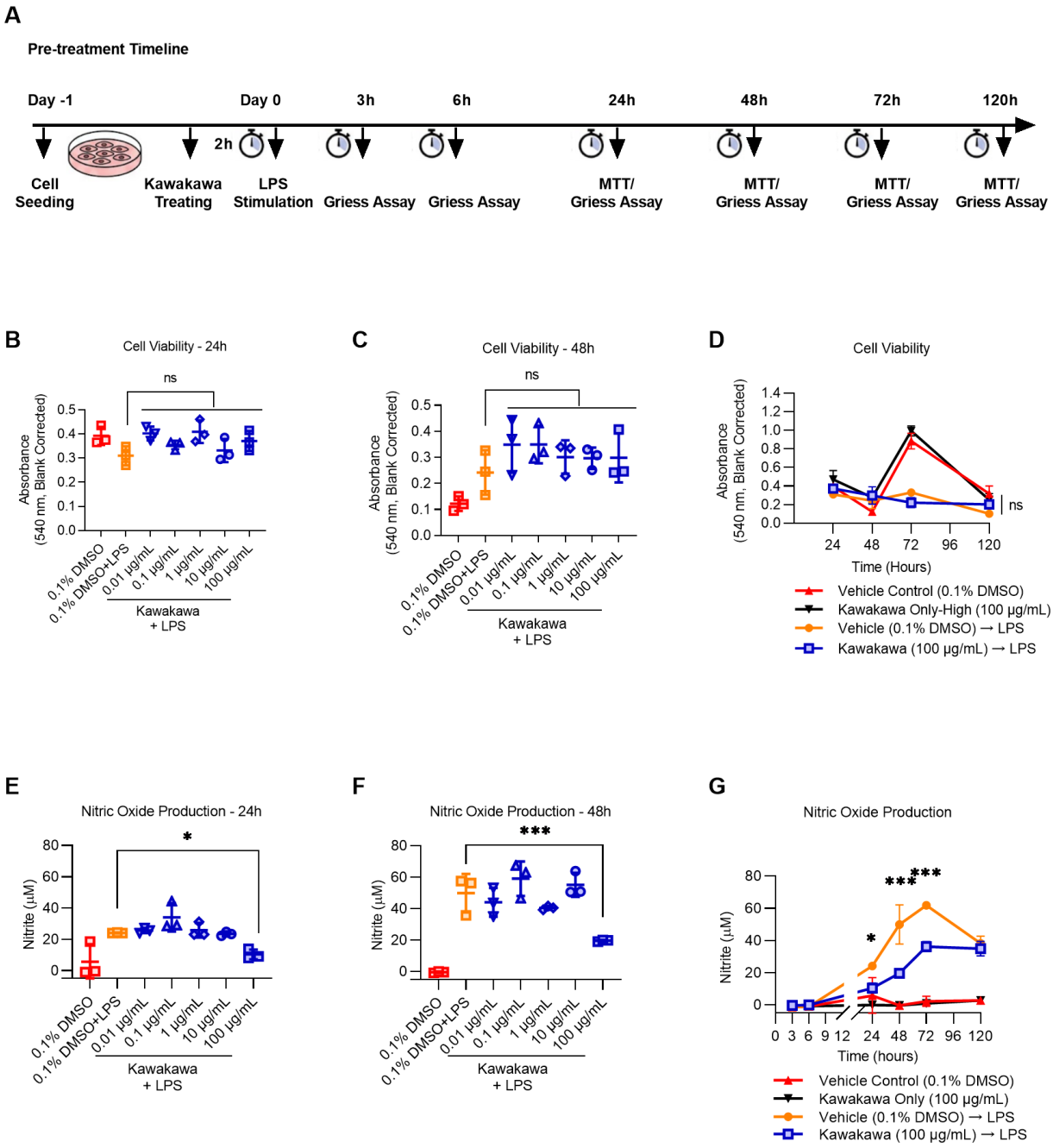


Figure 4: Kawakawa pre-treatment transiently attenuates LPS-induced nitrite accumulation without reducing MTT-reducing activity in RAW 264.7 macrophages. (A) Experimental timeline. Cells were pre-treated with kawakawa extract (0.01-100 μg/mL) for 2 h before stimulation with LPS (500 ng/mL). Following LPS stimulation, cells were maintained under the same experimental conditions without medium replacement throughout the experimental period. Nitrite accumulation was measured at 3, 6, 24, 48, 72 and 120 h after LPS stimulation, whereas MTT-reducing activity was assessed at 24, 48, 72 and 120 h. (B, C) MTT-reducing activity at 24 and 48 h, respectively, reported as absorbance at 540 nm after subtraction of the assay blank. (D) Time course of MTT-reducing activity following pre-treatment with 100 μg/mL kawakawa extract. (E, F) Nitrite accumulation at 24 and 48 h, respectively, measured using the Griess assay as an indirect indicator of nitric oxide production. (G) Time course of nitrite accumulation following pre-treatment with 100 μg/mL kawakawa extract. Data are presented as mean $\pm$ SD. Statistical comparisons are relative to the corresponding LPS-stimulated vehicle control. Statistical significance: ns, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

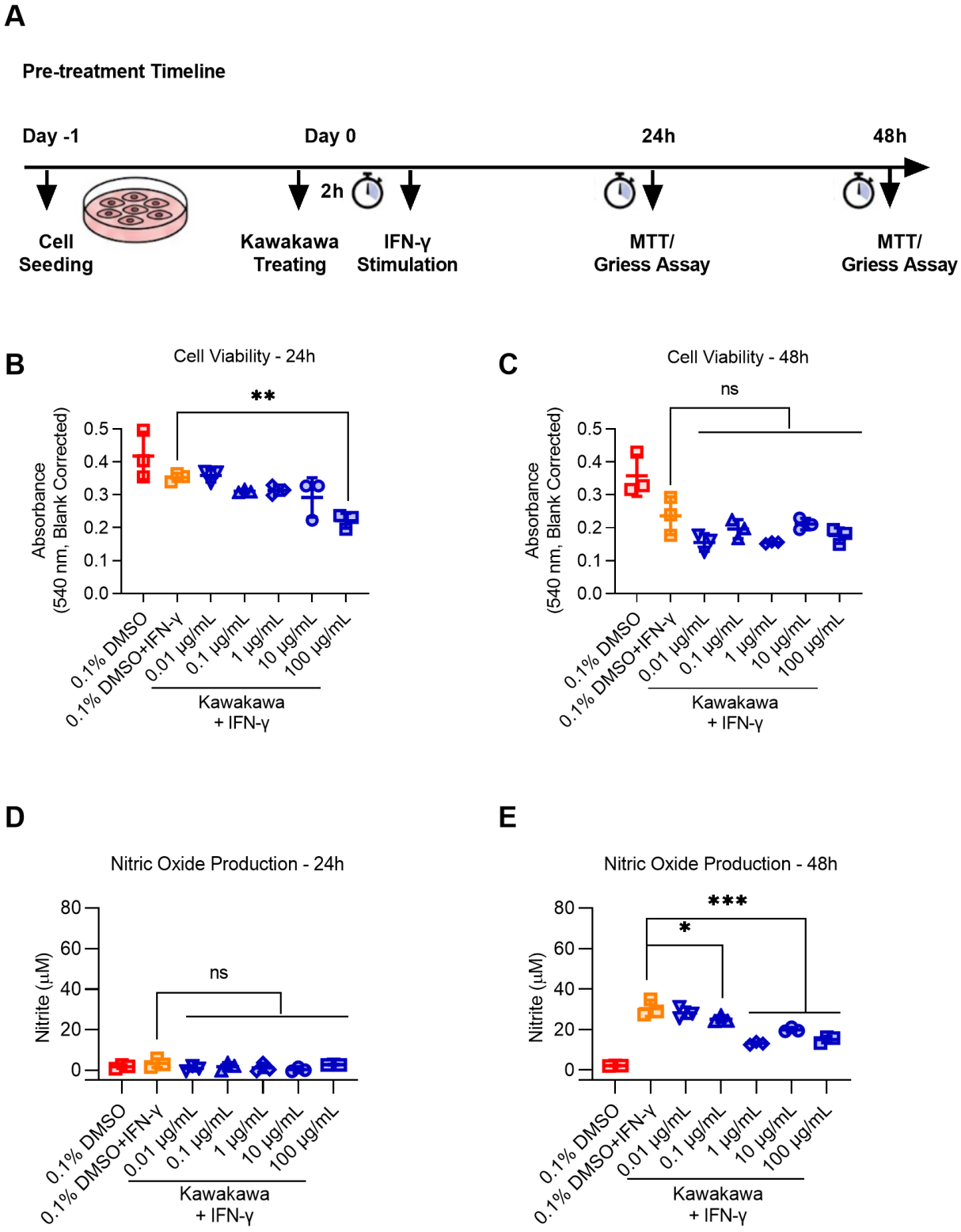


Figure 5: Kawakawa pre-treatment attenuates IFN-γ-induced nitrite accumulation at 48 h in RAW 264.7 macrophages. (A) Cells were pre-treated with kawakawa extract (0.01-100 μg/mL) for 2 h before stimulation with IFN-γ (20 ng/mL). MTT-reducing activity and nitrite accumulation were assessed 24 and 48 h after stimulation. (B, C) MTT-reducing activity at 24 and 48 h, respectively, reported as absorbance at 540 nm after subtraction of the assay blank. (D, E) Nitrite accumulation in culture supernatants at 24 and 48 h, respectively, measured using the Griess assay as an indirect indicator of nitric oxide production. Data are presented as mean $\pm$ SD. Statistical comparisons were made against the corresponding IFN-γ-stimulated vehicle control. Statistical significance: ns, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

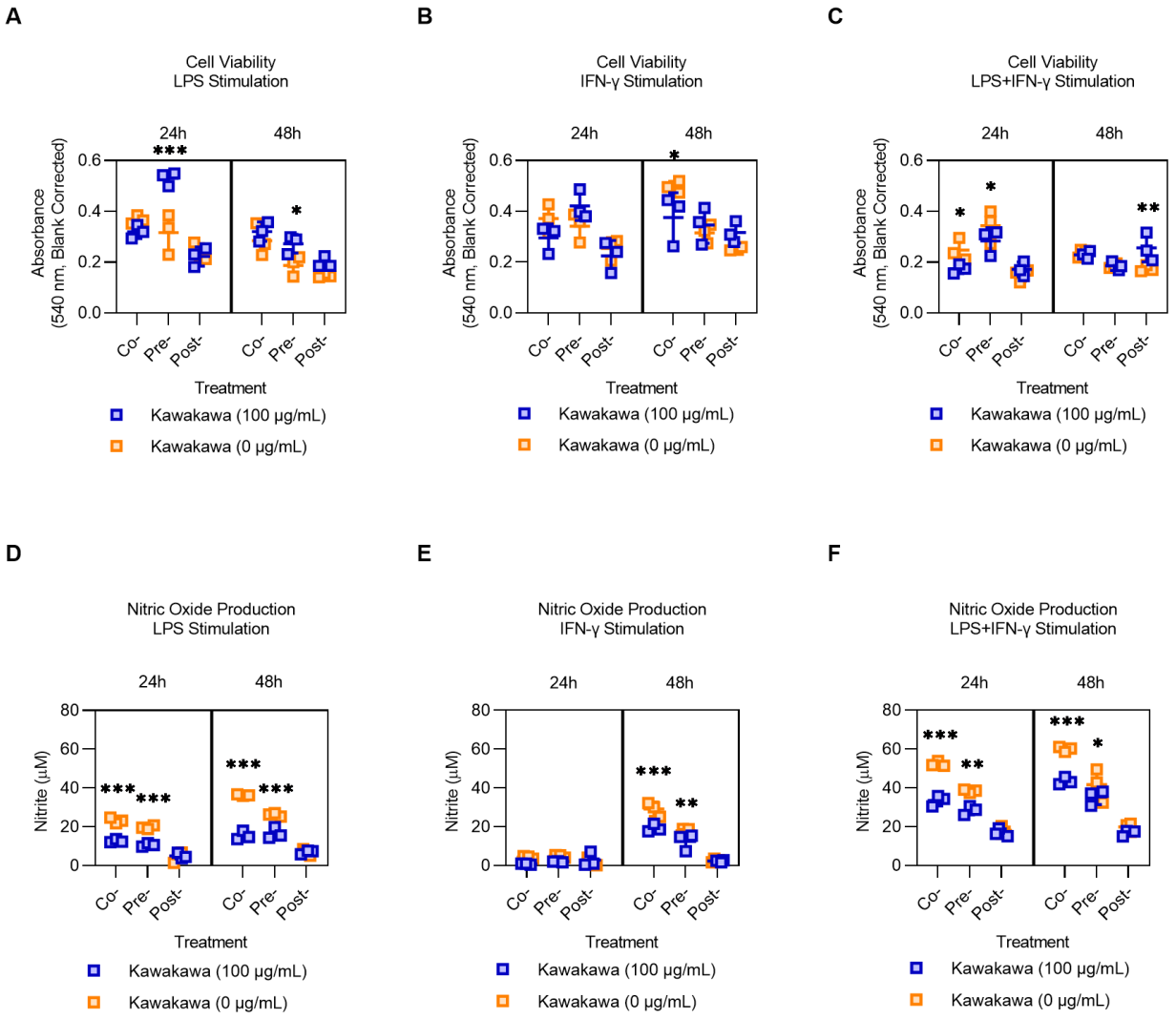


Figure 6: Co- and pre-treatment with kawakawa, but not post-treatment, attenuate stimulus-induced nitrite accumulation in RAW 264.7 macrophages. Cells received kawakawa extract (100 μg/mL) or the corresponding vehicle control (0 μg/mL kawakawa) 2 h before stimulation (pre-treatment), concurrently with stimulation (co-treatment), or 4 h after stimulation (post-treatment). Cells were stimulated with LPS, IFN-γ, or LPS + IFN-γ, and responses were assessed at 24 and 48 h after stimulation. (A-C) MTT-reducing activity following stimulation with LPS, IFN-γ, and LPS + IFN-γ, respectively, reported as absorbance at 540 nm after subtraction of the assay blank. (D-F) Nitrite accumulation following stimulation with LPS, IFN-γ, and LPS + IFN-γ, respectively, measured using the Griess assay as an indirect indicator of nitric oxide production. Data are presented as mean $\pm$ SD. Statistical comparisons were made between kawakawa-treated cells and the corresponding vehicle-treated control (0 μg/mL kawakawa). Statistical significance: ns, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. The data shown are representative of two independent experiments.

4 General Discussion and Conclusion

Building on the findings presented in Chapter 3, this chapter discusses the effects of kawakawa (*Piper excelsum*) leaf extract on nitric oxide-related inflammatory responses in RAW 264.7 macrophages. The findings are considered in relation to existing literature, with particular attention to macrophage activation, cellular metabolic activity, and the potential immunomodulatory effects of the extract. The limitations of the study are also addressed to identify areas for future research.

4.1 Overall Discussion

This study investigated the immunomodulatory potential of kawakawa (*Piper excelsum*) leaf extract through an *in vitro* macrophage model. While previous research has principally described kawakawa as a traditional medicinal plant with anti-inflammatory properties, the present study extends current knowledge by examining its effects on nitric oxide-related inflammatory responses across multiple macrophage activation states and distinct inflammatory stimuli.

A sterile hydroethanolic kawakawa extract was successfully prepared and demonstrated good compatibility with macrophages across the tested concentration range. Throughout this study, reductions in extracellular nitrite accumulation generally occurred without substantial decreases in cellular metabolic activity, which suggests that the observed anti-inflammatory effects are independent of cellular toxicity. Instead, kawakawa appears to modulate macrophage inflammatory responses without substantial loss of cellular metabolic activity. This distinction is critical because effective immune modulation should preserve cellular function while limiting excessive activation. Therefore, the activity observed in this study supports the concept that kawakawa acts as an immunomodulatory agent capable of maintaining inflammatory balance.

A major contribution of this study is the demonstration that kawakawa activity is context-dependent. The biological response varied according to inflammatory environment, exposure timing, and extract concentration. Such observations emphasise the complexity of botanical immunomodulation. Unlike single-target pharmaceutical compounds, plant extracts contain diverse phytochemicals that may interact with multiple cellular pathways. Consequently, the observed biological effects likely reflect the combined actions of multiple constituents and their interactions with interconnected inflammatory signalling pathways.

Another strength of this research is the use of multiple macrophage activation models. Macrophage activation is not a uniform process but involves integration of signals from different receptors and cytokine environments. Investigation of responses induced by distinct inflammatory stimuli revealed that kawakawa activity extends beyond a single inflammatory pathway. The consistent modulation observed across different activation contexts suggests that kawakawa may influence shared regulatory processes involved in macrophage activation and nitric oxide production. However, because the present study was designed primarily to evaluate biological responses rather than molecular mechanisms, definitive conclusions regarding the underlying cellular targets

cannot yet be drawn. More detailed mechanistic investigations were beyond the scope of the present study, particularly given the resource and technical constraints associated with conducting extensive molecular and protein-level analyses. Therefore, the present findings should be best interpreted as evidence of functional immunomodulatory activity, rather than as conclusive evidence for a specific molecular mechanism.

Importantly, these data indicate that kawakawa serves primarily to prevent or regulate excessive inflammatory activation, with a more limited role in directly reversing established inflammation. This interpretation is supported by the stronger effects observed when macrophages were exposed to kawakawa before or during inflammatory stimulation. Such activity may be relevant for approaches aimed at maintaining immune homeostasis or reducing susceptibility to excessive inflammatory responses.

Overall, these findings enrich our comprehension of kawakawa as a natural immune-regulating resource. Through the integration of traditional medicinal knowledge with systematic scientific evaluation, this study reinforces the therapeutic potential of botanical extracts in modern biomedical research.

Limitations of the Study

Several limitations should be considered when interpreting the findings of this study.

First, while the RAW 264.7 macrophage cell line provides a convenient and reproducible model for investigating inflammatory activation, immortalised macrophage lines do not fully represent the complexity of primary macrophages or *in vivo* immune responses. RAW 264.7 cells may exhibit altered signalling sensitivity, proliferation characteristics, and inflammatory responses compared with primary macrophages derived from bone marrow or peripheral blood (Chamberlain et al., 2009). Therefore, validation using primary macrophages or animal models is needed to confirm the biological relevance of kawakawa-mediated immune regulation.

Second, the inflammatory response was primarily evaluated through nitrite accumulation as an indirect indicator of NO production. Although NO is a widely used marker of macrophage activation and iNOS activity, inflammatory responses involve multiple interconnected pathways (Abdulkhaleq et al., 2018). Additional measurements, including iNOS protein expression, NF-κB, MAPK, and STAT1 activation, reactive oxygen species generation, macrophage polarisation markers, and cytokine production (such as TNF-α, IL-6, and IL-1β), would provide a more comprehensive understanding of the mechanisms involved in kawakawa-mediated immunomodulation.

Third, the chemical composition of the kawakawa extract was not characterised in detail. As botanical extracts contain complex mixtures of bioactive compounds, the observed biological activity may result from the combined actions of multiple constituents, with individual compounds potentially contributing to the overall response. Variations in extraction methods, plant origin, and processing may also influence phytochemical composition and bioactivity. Comprehensive chemical profiling using liquid chromatography-tandem mass spectrometry (LC-MS/MS) or HPLC, combined with bioactivity-guided fractionation, will therefore be necessary to identify the compounds responsible for the observed anti-inflammatory effects and to determine whether synergistic or antagonistic interactions contribute to the overall biological activity.

Finally, despite the consistent suppression of inflammatory nitrite production by kawakawa across multiple in-

flammatory models and treatment strategies, the precise molecular mechanisms await further clarification. In particular, it is unclear whether the observed effects arise from modulation of common downstream inflammatory pathways, such as NF-κB and JAK/STAT signalling, or from coordinated regulation of interconnected signalling networks, including MAPK- and microRNA-associated pathways. Further mechanistic studies integrating transcriptomic, proteomic, and functional analyses, as well as validation in primary macrophages and *in vivo* models, will be required to confirm the molecular targets and determine the broader therapeutic relevance of kawakawa in inflammation-associated conditions.

Considerations for Assessing Cell Viability and Metabolic Activity in RAW 264.7 Macrophages

The MTT assay was used to assess cellular metabolic activity and viability in this study. However, interpretation of MTT results in RAW 264.7 macrophages requires caution because this cell type exhibits strong adherence properties and characteristic morphological changes during activation. Inflammatory stimulation can induce changes in macrophage morphology, including increased cell spreading and cytoskeletal remodelling, as well as alterations in cellular metabolism and mitochondrial function. These changes do not necessarily indicate a loss of cell viability (Bussi et al., 2022; Venter et al., 2014).

The MTT assay measures the reduction of MTT to insoluble formazan crystals through cellular reductive activity rather than directly measuring viable cell numbers (Van Meerloo et al., 2011). Therefore, changes in metabolic activity may occur independently of actual cell death. Activated macrophages can undergo metabolic reprogramming, including alterations in mitochondrial activity and glycolytic metabolism, which may influence MTT signal intensity (Ghasemi et al., 2021; Reichner et al., 1995). Furthermore, the strong adherence of RAW 264.7 cells may introduce technical variability during washing, medium replacement, or crystal solubilisation, potentially affecting assay reproducibility.

Similarly, although trypan blue exclusion is widely used to assess membrane integrity, its application to RAW 264.7 cells is also associated with limitations. Since these macrophages adhere tightly to culture surfaces, cell detachment by scraping or enzymatic dissociation may cause incomplete cell recovery or mechanical damage, and lead to variability in cell counts and viability estimates.

In this investigation, MTT results were interpreted with trypan blue exclusion assays performed during preliminary cytotoxicity assessment. However, both methods have inherent limitations when applied to adherent RAW 264.7 macrophages, and the findings should be interpreted cautiously. Nevertheless, neither assay indicated substantial cytotoxicity following kawakawa treatment under the tested conditions. Future studies may benefit from incorporating additional viability approaches, such as resazurin-based assays (Alamar Blue), ATP-based luminescence assays, live/dead fluorescence staining, or automated imaging-based cell counting, which may provide more direct measurements of cell survival in adherent macrophage models (Bouadi & Tay, 2024; Chamberlain et al., 2009; Kamiloglu et al., 2020; Lavogina et al., 2022).

Future Directions

Future studies should focus on identifying the molecular mechanisms and active compounds responsible for kawakawa-mediated immunomodulation. Comprehensive phytochemical profiling combined with fractiona-

tion approaches could identify specific constituents that contribute to the reduction of inflammatory activation. Subsequent mechanistic studies should investigate whether kawakawa regulates key inflammatory pathways, including TLR4/NF-κB, MAPK, JAK/STAT, or Nrf2, and whether it directly influences NOS2 transcription, iNOS protein expression, or enzyme activity. Simultaneous measurement of cytokines, chemokines, oxidative stress markers, and other inflammatory mediators would provide a more complete understanding of its immunomodulatory effects.

Further investigation should also expand inflammatory analysis beyond NO production by measuring cytokine profiles, macrophage polarisation markers, and transcriptomic changes. RNA sequencing or targeted gene expression analysis may provide insight into how kawakawa alters macrophage inflammatory programming.

In addition, validation in more physiologically relevant systems is required. Experiments using primary macrophages, human immune cells, co-culture models, or animal models of inflammation would help determine whether the observed effects translate beyond the RAW 264.7 cell model.

Finally, optimisation of experimental methodologies, particularly viability assessment strategies, will improve future investigations of adherent immune cell models. Combining metabolic assays with direct cell-counting and imaging approaches will provide a more accurate evaluation of treatment effects and reduce the risk of misinterpreting metabolic changes as alterations in cell survival.

4.2 Overall Conclusion

This study provides evidence that kawakawa leaf extract possesses immunomodulatory activity in macrophage models by regulating inflammatory activation without consistently affecting cellular metabolic activity. Under inflammatory conditions, the extract reduced extracellular nitrite accumulation in selected experimental settings, with the greatest and most consistent effects observed under LPS stimulation and when the extract was administered before or concurrently with inflammatory stimulation. In contrast, post-treatment showed little evidence of activity, and responses to IFN-γ stimulation were comparatively modest.

The findings demonstrate that kawakawa does not simply produce a non-specific suppression of macrophage activity, but instead modulates the magnitude of inflammatory responses depending on cellular context. The wider significance of this research lies in supporting the potential of kawakawa as a natural source of bioactive compounds capable of influencing immune balance. Instead of functioning exclusively as an anti-inflammatory agent, kawakawa may represent a multi-component botanical intervention that helps regulate excessive immune activation.

Despite relying on nitric oxide production as the primary indicator of inflammatory activation, the consistent inhibition observed across different stimulation conditions supports the potential of kawakawa as a natural immunomodulatory agent. These findings provide experimental evidence supporting the traditional use of kawakawa, although additional mechanistic and translational studies are required before conclusions regarding therapeutic efficacy can be made.

References

Abdulkhaleq, L. A., Assi, M. A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y. H., & Hezmee, M. N. M. (2018). The crucial roles of inflammatory mediators in inflammation: A review. *Veterinary World*, *11*(5), 627–635. https://doi.org/10.14202/vetworld.2018.627-635

Abughazala, N. (2024). *Bioavailability-enhancing effects of piperinoids upon curcumin uptake in an experimental functional beverage containing kawakawa and turmeric* [Doctoral dissertation, ResearchSpace@Auckland].

Abu-Zurayk, R., Alnairat, N., Khalaf, A., Ibrahim, A. A., & Halaweh, G. (2023). Cellulose acetate membranes: Fouling types and antifouling strategies—a brief review. *Processes*, *11*(2), 489. https://doi.org/10.3390/pr11020489

Akira, S. (2009). Pathogen recognition by innate immunity and its signaling. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, *85*(4), 143–156. https://doi.org/10.2183/pjab.85.143

Alfaro, S., Acuña, V., Ceriani, R., Cavieres, M. F., Weinstein-Oppenheimer, C. R., & Campos-Estrada, C. (2022). Involvement of inflammation and its resolution in disease and therapeutics. *International Journal of Molecular Sciences*, *23*(18), 10719. https://doi.org/10.3390/ijms231810719

Al-Qahtani, W. H., Dinakarkumar, Y., Arokiyaraj, S., Saravanakumar, V., Rajabathar, J. R., Arjun, K., Gayathri, P., & Nelson Appaturi, J. (2022). Phyto-chemical and biological activity of myristica fragrans, an ayurvedic medicinal plant in southern India and its ingredient analysis. *Saudi Journal of Biological Sciences*, *29*(5), 3815–3821. https://doi.org/10.1016/j.sjbs.2022.02.043

Ambarus, C., Krausz, S., Van Eijk, M., Hamann, J., Radstake, T., Reedquist, K., Tak, P., & Baeten, D. (2012). Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. *Journal of Immunological Methods*, *375*(1–2), 196–206. https://doi.org/10.1016/j.jim.2011.10.013

Anderson, N. L., & Anderson, N. G. (2002). The human plasma proteome. *Molecular & Cellular Proteomics*, *1*(11), 845–867. https://doi.org/10.1074/mcp.R200007-MCP200

Anilkumar, S., & Wright-Jin, E. (2024). NF-κB as an Inducible Regulator of Inflammation in the Central Nervous System. *Cells*, *13*(6), 485. https://doi.org/10.3390/cells13060485

Antonelli, M., & Kushner, I. (2017). It's time to redefine inflammation. *FASEB Journal*, *31*(5), 1787–1791. https://doi.org/10.1096/fj.201601326R

Aporosa, S., & Forde, J. M. (2019). Māori and kava: New drug fashion or re-engagement with'kawa'?

Arora, S., Dev, K., Agarwal, B., Das, P., & Syed, M. A. (2018). Macrophages: Their role, activation and polarization in pulmonary diseases. *Immunobiology*, *223*(4–5), 383–396. https://doi.org/10.1016/j.imbio.2017.11.001

Badr, G., Elsawy, H., Amalki, M. A., Alfwuaires, M., El-Gerbed, M. S. A., & Abdel-Moneim, A. M. (2020). Protective effects of myristicin against ulcerative colitis induced by acetic acid in male mice. *Food and Agricultural Immunology*, *31*(1), 435–446. https://doi.org/10.1080/09540105.2020.1739626

Bashir, S., Sharma, Y., Elahi, A., & Khan, F. (2016). Macrophage polarization: The link between inflammation and related diseases. *Inflammation Research*, *65*(1), 1–11. https://doi.org/10.1007/s00011-015-0874-1

Bass, J. J., Wilkinson, D. J., Rankin, D., Phillips, B. E., Szewczyk, N. J., Smith, K., & Atherton, P. J. (2017). An overview of technical considerations for western blotting applications to physiological research. *Scandinavian Journal of Medicine & Science in Sports*, *27*(1), 4–25. https://doi.org/10.1111/sms.12702

Bellavia, L., Kim-Shapiro, D. B., & King, S. B. (2015). Detecting and monitoring NO, SNO and nitrite *in vivo*. *Future Science OA*, *1*(1), FSO36. https://doi.org/10.4155/fso.15.36

Biswas, S. K., & Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. *Nature Immunology*, *11*(10), 889–896. https://doi.org/10.1038/ni.1937

Bouadi, O., & Tay, T. L. (2024). Automated cell counting of macrophages In situ. In E. Mass (Ed.), *Tissue-resident macrophages* (pp. 505–518, Vol. 2713). Springer US. https://doi.org/10.1007/978-1-0716-3437-0_33

Brayton, C. F. (1986). Dimethyl sulfoxide (DMSO): A review. *The Cornell Veterinarian*, *76*(1), 61–90.

Briggs, L. H. (1941). The essential oil of macropiper excelsum (kawakawa). *Journal of the Society of Chemical Industry*, *60*(7), 210–212. https://doi.org/10.1002/jctb.5000600709

Bright-Ohaeri, F. C., Ihebuzo, N. G., Ejeta, K. O., Odimegwu, N., & Ifenze, O. M. (2024). Cell Culture and Laboratory Contaminants, Prevention and Recovery Strategy- A Review. *European Journal of Biology and Medical Science Research*, *12*(2), 32–51. https://doi.org/10.37745/ejbmsr.2013/vol12n23251

Brooker, S. G., & Cooper, R. C. (1961). New zealand medicinal plants. *Economic Botany*, *15*(1), 1–10. https://doi.org/10.1007/BF02906757

Bruggisser, R., von Daeniken, K., Jundt, G., Schaffner, W., & Tullberg-Reinert, H. (2002). Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. *Planta Medica*, *68*(5), 445–448. https://doi.org/10.1055/s-2002-32073

Buccitelli, C., & Selbach, M. (2020). mRNAs, proteins and the emerging principles of gene expression control. *Nature Reviews Genetics*, *21*(10), 630–644. https://doi.org/10.1038/s41576-020-0258-4

Bussi, C., Heunis, T., Pellegrino, E., Bernard, E. M., Bah, N., Dos Santos, M. S., Santucci, P., Aylan, B., Rodgers, A., Fearns, A., Mitschke, J., Moore, C., MacRae, J. I., Greco, M., Reinheckel, T., Trost, M., & Gutierrez, M. G. (2022). Lysosomal damage drives mitochondrial proteome remodelling and reprograms macrophage immunometabolism. *Nature Communications*, *13*(1), 7338. https://doi.org/10.1038/s41467-022-34632-8

Butts, C. A., Van Klink, J. W., Joyce, N. I., Paturi, G., Hedderley, D. I., Martell, S., & Harvey, D. (2019). Composition and safety evaluation of tea from New Zealand kawakawa (Piper excelsum). *Journal of Ethnopharmacology*, *232*, 110–118. https://doi.org/10.1016/j.jep.2018.12.029

Calder, V. L., Cole, A. L. J., & Walker, J. R. L. (1986). Antibiotic compounds from New Zealand plants. III: A survey of some New Zealand plants for antibiotic substances. *Journal of the Royal Society of New Zealand*, *16*(2), 169–181. https://doi.org/10.1080/03036758.1986.10418176

Calucci, L., Pinzino, C., Zandomeneghi, M., Capocchi, A., Ghiringhelli, S., Saviozzi, F., Tozzi, S., & Galleschi, L. (2003). Effects of γ-irradiation on the free radical and antioxidant contents in nine aromatic herbs and spices. *Journal of Agricultural and Food Chemistry*, *51*(4), 927–934. https://doi.org/10.1021/jf020739n

Cambie, R. (1976). A New Zealand Phytochemical Register — Part III. *Journal of the Royal Society of New Zealand*, *6*(3), 307–379. https://doi.org/10.1080/03036758.1976.10421479

Capes-Davis, A., Theodosopoulos, G., Atkin, I., Drexler, H. G., Kohara, A., MacLeod, R. A., Masters, J. R., Nakamura, Y., Reid, Y. A., Reddel, R. R., & Freshney, R. I. (2010). Check your cultures! A list of cross-contaminated or misidentified cell lines. *International Journal of Cancer*, *127*(1), 1–8. https://doi.org/10.1002/ijc.25242

Carlini, V., Noonan, D. M., Abdalalem, E., Goletti, D., Sansone, C., Calabrone, L., & Albini, A. (2023). The multifaceted nature of IL-10: Regulation, role in immunological homeostasis and its relevance to cancer, COVID-19 and post-COVID conditions. *Frontiers in Immunology*, *14*, 1161067. https://doi.org/10.3389/fimmu.2023.1161067

Cavaillon, J.-M. (2021). Once upon a time, inflammation. *Journal of Venomous Animals and Toxins including Tropical Diseases*. https://doi.org/10.1590/1678-9199-jvatitd-2020-0147

Chamberlain, L. M., Godek, M. L., Gonzalez-Juarrero, M., & Grainger, D. W. (2009). Phenotypic non-equivalence of murine (monocyte-) macrophage cells in biomaterial and inflammatory models. *Journal of Biomedical Materials Research Part A*, *88A*(4), 858–871. https://doi.org/10.1002/jbm.a.31930

Chanput, W., Peters, V., & Wichers, H. (2015). THP-1 and U937 cells. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health* (pp. 147–159). Springer International Publishing. https://doi.org/10.1007/978-3-319-16104-4_14

Chen, Y., Wang, B., Yuan, X., Lu, Y., Hu, J., Gao, J., Lin, J., Liang, J., Hou, S., & Chen, S. (2021). Vitexin prevents colitis-associated carcinogenesis in mice through regulating macrophage polarization. *Phytomedicine*, *83*, 153489. https://doi.org/10.1016/j.phymed.2021.153489

Chin, J., & Goldstein, D. (1977). Effects of Low Concentrations of Ethanol on the Fluidity of Spin-Labeled Erythrocyte and Brain Membranes. *Molecular Pharmacology*, *13*(3), 435–441. https://doi.org/10.1016/S0026-895X(25)11220-0

Cho, Y.-C., Kim, H. J., Kim, Y.-J., Lee, K. Y., Choi, H. J., Lee, I.-S., & Kang, B. Y. (2008). Differential anti-inflammatory pathway by xanthohumol in IFN-gamma and LPS-activated macrophages. *International Immunopharmacology*, *8*(4), 567–573. https://doi.org/10.1016/j.intimp.2007.12.017

Cseresnyes, Z., Hassan, M. I. A., Dahse, H.-M., Voigt, K., & Figge, M. T. (2020). Quantitative impact of cell membrane fluorescence labeling on phagocytosis measurements in confrontation assays. *Frontiers in Microbiology*, *11*, 1193. https://doi.org/10.3389/fmicb.2020.01193

Da Cunha, L. R., Muniz-Junqueira, M. I., & Dos Santos Borges, T. K. (2019). Impact of polyphenols in phagocyte functions. *Journal of Inflammation Research*, *Volume 12*, 205–217. https://doi.org/10.2147/JIR.S193749

De León, E. J., Olmedo, D. A., Solís, P. N., Gupta, M. P., & Terencio, M. C. (2002). Diayangambin Exerts Immunosuppressive and Anti-Inflammatory Effects*in vitro*and*in vivo*. *Planta Medica*, *68*(12), 1128–1131. https://doi.org/10.1055/s-2002-36355

De Ménorval, M.-A., Mir, L. M., Fernández, M. L., & Reigada, R. (2012). Effects of Dimethyl Sulfoxide in Cholesterol-Containing Lipid Membranes: A Comparative Study of Experiments In Silico and with Cells (G. Colombo, Ed.). *PLoS ONE*, *7*(7), e41733. https://doi.org/10.1371/journal.pone.0041733

Dervisevic, E., Dervisevic, M., Ang, B., Carthew, J., Tuck, K. L., Voelcker, N. H., & Cadarso, V. J. (2022). Integrated microfluidic device to monitor unseen escherichia coli contamination in mammalian cell culture. *Sensors and Actuators, B: Chemical*, *359*, 131522. https://doi.org/10.1016/j.snb.2022.131522

Dirsch, V. M., Stuppner, H., & Vollmar, A. M. (1998). The Griess assay: Suitable for a bio-guided fractionation of anti-inflammatory plant extracts? *Planta Medica*, *64*(5), 423–426. https://doi.org/10.1055/s-2006-957473

Djaldetti, M. (2021). Piperine – an immunomodulator and inflammation mitigator (Auctores Publishing LLC, Ed.). *Journal of Clinical and Laboratory Research*, *2*(5), 1–4. https://doi.org/10.31579/2768-0487/027

Donohue, T. M., Clemens, D. L., Galli, A., Crabb, D., Nieto, N., Kato, J., & Barve, S. S. (2001). Use of cultured cells in assessing ethanol toxicity and ethanol-related metabolism. *Alcoholism: Clinical and Experimental Research*, *25*(s1). https://doi.org/10.1111/j.1530-0277.2001.tb02380.x

Doyle, A. G., Herbein, G., Montaner, L. J., Minty, A. J., Caput, D., Ferrara, P., & Gordon, S. (1994). Interleukin-13 alters the activation state of murine macrophages *in vitro* : Comparison with interleukin-4 and interferon-γ. *European Journal of Immunology*, *24*(6), 1441–1445. https://doi.org/10.1002/eji.1830240630

Du, Q., Dickinson, A., Nakuleswaran, P., Maghami, S., Alagoda, S., Hook, A. L., & Ghaemmaghami, A. M. (2024). Targeting macrophage polarization for reinstating homeostasis following tissue damage. *International Journal of Molecular Sciences*, *25*(13), 7278. https://doi.org/10.3390/ijms25137278

Earl, E. A., Altaf, M., Murikoli, R. V., Swift, S., & O'Toole, R. (2010). Native New Zealand plants with inhibitory activity towards Mycobacterium tuberculosis. *BMC Complementary and Alternative Medicine*, *10*(1), 25. https://doi.org/10.1186/1472-6882-10-25

Earl, E. A. (2010). *Antibacterial effects of New Zealand plant extracts against mycobacteria* [Doctoral dissertation, Open Access Te Herenga Waka-Victoria University of Wellington].

Elufioye, T. O., Habtemariam, S., & Adejare, A. (2020). Chemistry and Pharmacology of Alkylamides from Natural Origin. *Revista Brasileira de Farmacognosia*, *30*(5), 622–640. https://doi.org/10.1007/s43450-020-00095-5

Evans, S. S., Repasky, E. A., & Fisher, D. T. (2015). Fever and the thermal regulation of immunity: The immune system feels the heat. *Nature Reviews. Immunology*, *15*(6), 335–349. https://doi.org/10.1038/nri3843

Freshney, R. I. (2010, September 20). *Culture of animal cells: A manual of basic technique and specialized applications* (1st ed.). Wiley. https://doi.org/10.1002/9780470649367

Fu, P. P., Xia, Q., Guo, L., Yu, H., & Chan, P.-C. (2008). Toxicity of kava kava. *Journal of Environmental Science and Health, Part C*, *26*(1), 89–112. https://doi.org/10.1080/10590500801907407

Gabay, C., & Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation (F. H. Epstein, Ed.). *New England Journal of Medicine*, *340*(6), 448–454. https://doi.org/10.1056/NEJM199902113400607

Gao, S., Zhou, J., Liu, N., Wang, L., Gao, Q., Wu, Y., Zhao, Q., Liu, P., Wang, S., Liu, Y., Guo, N., Shen, Y., Wu, Y., & Yuan, Z. (2015). Curcumin induces M2 macrophage polarization by secretion IL-4 and/or

IL-13. *Journal of Molecular and Cellular Cardiology*, *85*, 131–139. https://doi.org/10.1016/j.yjmcc.2015.04.025

Ghasemi, M., Turnbull, T., Sebastian, S., & Kempson, I. (2021). The MTT assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. *International journal of molecular sciences*, *22*(23), 12827. https://doi.org/10.3390/ijms222312827

Ginhoux, F., & Guilliams, M. (2016). Tissue-resident macrophage ontogeny and homeostasis. *Immunity*, *44*(3), 439–449. https://doi.org/10.1016/j.immuni.2016.02.024

Gojer, J. S. (2023). *Impact of kawakawa (piper excelsum) on the postprandial levels of circulatory microRNAs related to insulin sensitivity* [Doctoral dissertation, ResearchSpace@ Auckland].

Gordon, S. (2016). Phagocytosis: The legacy of metchnikoff. *Cell*, *166*(5), 1065–1068. https://doi.org/10.1016/j.cell.2016.08.017

Gordon, S., & Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nature Reviews Immunology*, *5*(12), 953–964. https://doi.org/10.1038/nri1733

GraphPad Software. (2014). GraphPad prism version 6.0. *GraphPad Software*. https://www.graphpad.com

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry*, *126*(1), 131–138. https://doi.org/10.1016/0003-2697(82)90118-x

Grievink, H. W., Luisman, T., Kluft, C., Moerland, M., & Malone, K. E. (2016). Comparison of Three Isolation Techniques for Human Peripheral Blood Mononuclear Cells: Cell Recovery and Viability, Population Composition, and Cell Functionality. *Biopreservation and Biobanking*, *14*(5), 410–415. https://doi.org/10.1089/bio.2015.0104

Guo, C., Islam, R., Zhang, S., & Fang, J. (2021). Metabolic reprogramming of macrophages and its involvement in inflammatory diseases. *EXCLI Journal*, *20*, 628–641. https://doi.org/10.17179/excli2020-3053

Gupta, A. (2022). *Discovery of novel plant based compounds to address the drug resistance problem in nematode infested ruminants: A thesis presented in partial fulfilment of the requirements for the degree of doctor of philosophy in chemistry at massey university, manawatū, new zealand* [Doctoral dissertation, Massey University].

Han, H., Kang, J.-K., Ahn, K. J., & Hyun, C.-G. (2023). DMSO Alleviates LPS-Induced Inflammatory Responses in RAW264.7 Macrophages by Inhibiting NF-κB and MAPK Activation. *BioChem*, *3*(2), 91–101. https://doi.org/10.3390/biochem3020007

Hao, J., Hu, Y., Li, Y., Zhou, Q., & Lv, X. (2017). Involvement of JNK signaling in IL4-induced M2 macrophage polarization. *Experimental Cell Research*, *357*(2), 155–162. https://doi.org/10.1016/j.yexcr.2017.05.010

Hao, N.-B., Lü, M.-H., Fan, Y.-H., Cao, Y.-L., Zhang, Z.-R., & Yang, S.-M. (2012). Macrophages in tumor microenvironments and the progression of tumors. *Clinical and Developmental Immunology*, *2012*, 948098. https://doi.org/10.1155/2012/948098

Harrison, D. A. (2012). The JAK/STAT pathway. *Cold Spring Harbor Perspectives in Biology*, *4*(3), a011205–a011205. https://doi.org/10.1101/cshperspect.a011205

Hartley, J. W., Evans, L. H., Green, K. Y., Naghashfar, Z., Macias, A. R., Zerfas, P. M., & Ward, J. M. (2008). Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology*, *5*(1), 1. https://doi.org/10.1186/1742-4690-5-1

Harvey, S. J.-M. (2021). *Investigation of in vitro diabetic inflammation and treatment with piper excelsum tea extract* [Doctoral dissertation, The University of Waikato].

Hasanain, F., Guenther, K., Mullett, W. M., & Craven, E. (2014). Gamma Sterilization of Pharmaceuticals–A Review of the Irradiation of Excipients, Active Pharmaceutical Ingredients, and Final Drug Product Formulations. *PDA Journal of Pharmaceutical Science and Technology*, *68*(2), 113–137. https://doi.org/10.5731/pdajpst.2014.00955

Heidland, A., Klassen, A., Rutkowski, P., & Bahner, U. (2006). The contribution of rudolf virchow to the concept of inflammation: What is still of importance? *Journal of Nephrology*, *19 Suppl 10*, S102–109.

Henslee, E. A., Torcal Serrano, R. M., Labeed, F. H., Jabr, R. I., Fry, C. H., Hughes, M. P., & Hoettges, K. F. (2016). Accurate quantification of apoptosis progression and toxicity using a dielectrophoretic approach. *The Analyst*, *141*(23), 6408–6415. https://doi.org/10.1039/C6AN01596D

Herb, M., Schatz, V., Hadrian, K., Hos, D., Holoborodko, B., Jantsch, J., & Brigo, N. (2024). Macrophage variants in laboratory research: Most are well done, but some are RAW. *Frontiers in Cellular and Infection Microbiology*, *14*, 1457323. https://doi.org/10.3389/fcimb.2024.1457323

Hodge, G., Hodge, S., & Han, P. (2000). INCREASED LEVELS OF APOPTOSIS OF LEUKOCYTE SUBSETS IN CULTURED PBMCs COMPARED TO WHOLE BLOOD AS SHOWN BY ANNEXIN V BINDING: RELEVANCE TO CYTOKINE PRODUCTION. *Cytokine*, *12*(12), 1763–1768. https://doi.org/10.1006/cyto.2000.0790

Hodge, S., Keesing, V., Wratten, S., Lövei, G., Palmer, J., & Cilgi, T. (1998). Herbivore damage and leaf loss in the New Zealand pepper tree ('kawakawa'; macropiper excelsum; piperaceae). *New Zealand Journal of Ecology*, 173–180.

Hoogstraten, C. A., Smeitink, J. A. M., Russel, F. G. M., & Schirris, T. J. J. (2022). Dissecting Drug-Induced Cytotoxicity and Metabolic Dysfunction in Conditionally Immortalized Human Proximal Tubule Cells. *Frontiers in Toxicology*, *4*, 842396. https://doi.org/10.3389/ftox.2022.842396

Hristodorov, D., Mladenov, R., von Felbert, V., Huhn, M., Fischer, R., Barth, S., & Thepen, T. (2015). Targeting CD64 mediates elimination of M1 but not M2 macrophages in vitro and in cutaneous inflammation in mice and patient biopsies. *Mabs*, *7*(5), 853–862. https://doi.org/10.1080/19420862.2015.1066950

Hu, X., Li, J., Fu, M., Zhao, X., & Wang, W. (2021). The JAK/STAT signaling pathway: From bench to clinic. *Signal Transduction and Targeted Therapy*, *6*(1), 402. https://doi.org/10.1038/s41392-021-00791-1

Hudson, M., Milne, M., Reynolds, P., Russell, K., & Smith, B. (2010). Te ara tika: Guidelines for māori research ethics: A framework for researchers and ethics committee members. *Health Research Council of New Zealand*, *9*, 1–29.

Hussain, T., Tan, B., Yin, Y., Blachier, F., Tossou, M. C. B., & Rahu, N. (2016). Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? (V. Rupasinghe, Ed.). *Oxidative Medicine and Cellular Longevity*, *2016*(1), 7432797. https://doi.org/10.1155/2016/7432797

Isailovic, N., Daigo, K., Mantovani, A., & Selmi, C. (2015). Interleukin-17 and innate immunity in infections and chronic inflammation. *Journal of Autoimmunity*, *60*, 1–11. https://doi.org/10.1016/j.jaut.2015.04.006

Iwanaga, S. (2007). Biochemical principle of Limulus test for detecting bacterial endotoxins. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, *83*(4), 110–119. https://doi.org/10.2183/pjab.83.110

Jayaprakash, R. (2023). *Kawakawa (piper excelsum): Human phytochemical metabolism and functional activity* [Doctoral dissertation, University of Auckland].

Jayaprakash, R., Pook, C., Ramzan, F., Miles-Chan, J. L., Mithen, R. F., & Foster, M. (2024). Human Metabolism and Excretion of Kawakawa (*Piper excelsum*) Leaf Chemicals. *Molecular Nutrition & Food Research*, *68*(6). https://doi.org/10.1002/mnfr.202300583

Jayaprakash, R., Ramzan, F., Miles-Chan, J. L., Foster, M., Mithen, R. F., & Pook, C. (2022). Exploring the Chemical Space of Kawakawa Leaf (Piper excelsum). *Nutrients*, *14*(23), 5168. https://doi.org/10.3390/nu14235168

Johnson, P. D., & Besselsen, D. G. (2002). Practical Aspects of Experimental Design in Animal Research. *ILAR Journal*, *43*(4), 202–206. https://doi.org/10.1093/ilar.43.4.202

Joseph, K. (2022). A journey of healing, discovery, and transformation: Hohou te rongo. *Pacific Arts: the Journal of the Pacific Arts Association*, *22*(1).

Jozefczuk, J., & Adjaye, J. (2011). Quantitative real-time PCR-based analysis of gene expression. In *Methods in Enzymology* (pp. 99–109, Vol. 500). Elsevier. https://doi.org/10.1016/B978-0-12-385118-5.00006-2

Kamiloglu, S., Sari, G., Ozdal, T., & Capanoglu, E. (2020). Guidelines for cell viability assays. *Food Frontiers*, *1*(3), 332–349. https://doi.org/10.1002/fft2.44

Katt, M. E., Placone, A. L., Wong, A. D., Xu, Z. S., & Searson, P. C. (2016). In vitro tumor models: Advantages, disadvantages, variables, and selecting the right platform. *Frontiers in Bioengineering and Biotechnology*, *4*. https://doi.org/10.3389/fbioe.2016.00012

Kaur, G., & Dufour, J. M. (2012). Cell lines: Valuable tools or useless artifacts. *Spermatogenesis*, *2*(1), 1–5. https://doi.org/10.4161/spmg.19885

Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. *Nature Immunology*, *11*(5), 373–384. https://doi.org/10.1038/ni.1863

Khan, M. W., Keshavarzian, A., Gounaris, E., Melson, J. E., Cheon, E. C., Blatner, N. R., Chen, Z. E., Tsai, F.-N., Lee, G., Ryu, H., Barrett, T. A., Bentrem, D. J., Beckhove, P., & Khazaie, K. (2013). PI3K/AKT Signaling Is Essential for Communication between Tissue-Infiltrating Mast Cells, Macrophages, and Epithelial Cells in Colitis-Induced Cancer. *Clinical Cancer Research*, *19*(9), 2342–2354. https://doi.org/10.1158/1078-0432.CCR-12-2623

Kim, H. G., Han, E. H., Jang, W.-S., Choi, J. H., Khanal, T., Park, B. H., Tran, T. P., Chung, Y. C., & Jeong, H. G. (2012). Piperine inhibits PMA-induced cyclooxygenase-2 expression through downregulating NF-κB, C/EBP and AP-1 signaling pathways in murine macrophages. *Food and Chemical Toxicology*, *50*(7), 2342–2348. https://doi.org/10.1016/j.fct.2012.04.024

Kleiveland, C. R. (2015). Peripheral blood mononuclear cells. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health* (pp. 161–167). Springer International Publishing. https://doi.org/10.1007/978-3-319-16104-4_15

Koia, J. H., & Shepherd, P. (2020). The Potential of Anti-Diabetic Rākau Rongoā (Māori Herbal Medicine) to Treat Type 2 Diabetes Mellitus (T2DM) Mate Huka: A Review. *Frontiers in Pharmacology*, *11*. https://doi.org/10.3389/fphar.2020.00935

Kołaczkowska, E. (2007). Acute inflammation as a beneficial process—history and recent developments. *Kosmos*, *56*, 27–38.

Kolaczkowska, E., & Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, *13*(3), 159–175. https://doi.org/10.1038/nri3399

Korniluk, A., Koper, O., Kemona, H., & Dymicka-Piekarska, V. (2017). From inflammation to cancer. *Irish Journal of Medical Science*, *186*(1), 57–62. https://doi.org/10.1007/s11845-016-1464-0

Koyasu, S. (2003). The role of PI3K in immune cells. *Nature Immunology*, *4*(4), 313–319. https://doi.org/10.1038/ni0403-313

Lampiasi, N. (2023). Macrophage polarization: Learning to manage it 2.0. *International journal of molecular sciences*, *24*(24), 17409. https://doi.org/10.3390/ijms242417409

Laplante, M., & Sabatini, D. M. (2009). mTOR signaling at a glance. *Journal of Cell Science*, *122*(20), 3589–3594. https://doi.org/10.1242/jcs.051011

Laveti, D., Kumar, M., Hemalatha, R., Sistla, R., Naidu, V., Talla, V., Verma, V., Kaur, N., & Nagpal, R. (2013). Anti-Inflammatory Treatments for Chronic Diseases: A Review. *Inflammation & Allergy-Drug Targets*, *12*(5), 349–361. https://doi.org/10.2174/18715281113129990053

Lavogina, D., Lust, H., Tahk, M.-J., Laasfeld, T., Vellama, H., Nasirova, N., Vardja, M., Eskla, K.-L., Salumets, A., Rinken, A., & Jaal, J. (2022). Revisiting the resazurin-based sensing of cellular viability: Widening the application horizon. *Biosensors*, *12*(4), 196. https://doi.org/10.3390/bios12040196

Lawrence, T. (2009). The Nuclear Factor NF-κB Pathway in Inflammation. *Cold Spring Harbor Perspectives in Biology*, *1*(6), a001651–a001651. https://doi.org/10.1101/cshperspect.a001651

Lee, J. Y., & Park, W. (2011). Anti-inflammatory effect of myristicin on RAW 264.7 macrophages stimulated with polyinosinic-polycytidylic acid. *Molecules*, *16*(8), 7132–7142. https://doi.org/10.3390/molecules16087132

Lei, J., Burgess, E., Richardson, A., Hawkins, B., Baird, S., Smallfield, B., Van Klink, J., & Perry, N. (2015). Cytotoxic Amides from Fruits of Kawakawa, Macropiper excelsum. *Planta Medica*, *81*(12/13), 1163–1168. https://doi.org/10.1055/s-0035-1546106

Lendeckel, U., Venz, S., & Wolke, C. (2022). Macrophages: Shapes and functions. *Chemtexts*, *8*(2), 12. https://doi.org/10.1007/s40828-022-00163-4

Li, M., Wang, M., Wen, Y., Zhang, H., Zhao, G.-N., & Gao, Q. (2023). Signaling pathways in macrophages: Molecular mechanisms and therapeutic targets. *MedComm*, *4*(5), e349. https://doi.org/10.1002/mco2.349

Lianhua, H. E., Huijie, L., Qingxia, Q., Juan, H. E., Jian, C., Yiping, H. U., Yueming, C., Desheng, S., Yu, S., & Qingwen, W. (2022). Shikonin alleviates collagen-induced arthritis mice by inhibiting M1 macrophage polarization. *Journal of Traditional Chinese Medicine = Chung I Tsa Chih Ying Wen Pan*, *42*(6), 932–939. https://doi.org/10.19852/j.cnki.jtcm.20220815.004

Lim, J. J., Grinstein, S., & Roth, Z. (2017). Diversity and versatility of phagocytosis: Roles in innate immunity, tissue remodeling, and homeostasis. *Frontiers in Cellular and Infection Microbiology*, *7*, 191. https://doi.org/10.3389/fcimb.2017.00191

Lindner, B., Burkard, T., & Schuler, M. (2020). Phagocytosis Assays with Different pH-Sensitive Fluorescent Particles and Various Readouts. *BioTechniques*, *68*(5), 245–250. https://doi.org/10.2144/btn-2020-0003

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, *2*, 17023–. https://doi.org/10.1038/sigtrans.2017.23

Liu, Z. (2008). Preparation of botanical samples for biomedical research. *Endocrine, Metabolic & Immune Disorders-Drug Targets*, *8*(2), 112–121. https://doi.org/10.2174/187153008784534358

Locati, M., Curtale, G., & Mantovani, A. (2020). Diversity, Mechanisms, and Significance of Macrophage Plasticity. *Annual Review of Pathology*, *15*, 123–147. https://doi.org/10.1146/annurev-pathmechdis-012418-012718

Lu, J., Xie, L., Liu, C., Zhang, Q., & Sun, S. (2017). PTEN/PI3k/AKT Regulates Macrophage Polarization in Emphysematous mice. *Scandinavian Journal of Immunology*, *85*(6), 395–405. https://doi.org/10.1111/sji.12545

Luo, L., Liang, H., & Liu, L. (2022). Myristicin regulates proliferation and apoptosis in oxidized low-density lipoprotein-stimulated human vascular smooth muscle cells and human umbilical vein endothelial cells by regulating the PI3K/akt/NF-κB signalling pathway. *Pharmaceutical Biology*, *60*(1), 56–64. https://doi.org/10.1080/13880209.2021.2010775

Lux, R., & Shi, W. (2004). Chemotaxis-guided Movements in Bacteria. *Critical Reviews in Oral Biology & Medicine*, *15*(4), 207–220. https://doi.org/10.1177/154411130401500404

Lv, H., Yu, Z., Zheng, Y., Wang, L., Qin, X., Cheng, G., & Ci, X. (2016). Isovitexin exerts anti-inflammatory and anti-oxidant activities on lipopolysaccharide-induced acute lung injury by inhibiting MAPK and NF-κB and activating HO-1/Nrf2 pathways. *International Journal of Biological Sciences*, *12*(1), 72–86. https://doi.org/10.7150/ijbs.13188

Mackaness, G. B. (1962). CELLULAR RESISTANCE TO INFECTION. *The Journal of Experimental Medicine*, *116*(3), 381–406. https://doi.org/10.1084/jem.116.3.381

Majekodunmi, S. O., & Olorunsola, E. O. (2014). Review of some recent advances on filtration in pharmaceutical industry. *Journal of Pharmacy and Biological Sciences*, *9*(4), 30–38. https://doi.org/10.9790/3008-09443038

Mamilos, A., Winter, L., Schmitt, V. H., Barsch, F., Grevenstein, D., Wagner, W., Babel, M., Keller, K., Schmitt, C., Gürtler, F., Schreml, S., Niedermair, T., Rupp, M., Alt, V., & Brochhausen, C. (2023). Macrophages: From simple phagocyte to an integrative regulatory cell for inflammation and tissue regeneration—a review of the literature. *Cells*, *12*(2), 276. https://doi.org/10.3390/cells12020276

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology*, *25*(12), 677–686. https://doi.org/10.1016/j.it.2004.09.015

Mantsounga, C. S., Lee, C., Neverson, J., Sharma, S., Healy, A., Berus, J. M., Parry, C., Ceneri, N. M., López-Giráldez, F., Chun, H. J., Lu, Q., Sellke, F., Choudhary, G., & Morrison, A. R. (2022). Macrophage IL-1β promotes arteriogenesis by autocrine STAT3- and NF-κB-mediated transcription of pro-angiogenic VEGF-A. *Cell Reports*, *38*(5), 110309. https://doi.org/10.1016/j.celrep.2022.110309

Mark, G., Boulton, A., Allport, T., Kerridge, D., & Potaka-Osborne, G. (2022). "Ko Au te whenua, ko te whenua ko Au: I Am the land, and the land is me": Healer/patient views on the role of rongoā māori (traditional māori healing) in healing the land. *International Journal of Environmental Research and Public Health*, *19*(14), 8547. https://doi.org/10.3390/ijerph19148547

Marques, B., Freeman, C., & Carter, L. (2021). Adapting traditional healing values and beliefs into therapeutic cultural environments for health and well-being. *International Journal of Environmental Research and Public Health*, *19*(1), 426. https://doi.org/10.3390/ijerph19010426

Marsit, N. M., Saadawi, S., & Alennabi, K. (2025). Challenges of growth-based microbiological methods in sterility assurance of pharmaceutical product manufacturing. *Discover Pharmaceutical Sciences*, *1*(1), 13. https://doi.org/10.1007/s44395-025-00020-6

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Reports*, *6*. https://doi.org/10.12703/P6-13

Mathew, C., Tesfaye, W., Rasmussen, P., Peterson, G. M., Bartholomaeus, A., Sharma, M., & Thomas, J. (2020). Mānuka oil—a review of antimicrobial and other medicinal properties. *Pharmaceuticals*, *13*(11), 343. https://doi.org/10.3390/ph13110343

Maulitz, R. C. (1978). Rudolf virchow, julius cohnheim and the program of pathology. *Bulletin of the History of Medicine*, *52*(2), 162–182.

McBurnie, L., & Bardo, B. (2004). Validation of sterile filtration. *Pharmaceutical Technology*, *24*, S13.

McKinnon, K. M. (2018). Flow cytometry: An overview. *Current Protocols in Immunology*, *120*(1). https://doi.org/10.1002/cpim.40

Merecz-Sadowska, A., Sitarek, P., Śliwiński, T., & Zajdel, R. (2020). Anti-inflammatory activity of extracts and pure compounds derived from plants via modulation of signaling pathways, especially PI3K/AKT in macrophages. *International Journal of Molecular Sciences*, *21*(24), 9605. https://doi.org/10.3390/ijms21249605

Michael, T., Saaby, L., Moesby, L., & Hansen, E. W. (2013). Considerations regarding use of solvents in in vitro cell based assays. *Cytotechnology*, *65*(5), 887–894. https://doi.org/10.1007/s10616-012-9530-6

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., & Hill, A. M. (2000). M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *The Journal of Immunology*, *164*(12), 6166–6173. https://doi.org/10.4049/jimmunol.164.12.6166

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Murray, P. J. (2017). Macrophage Polarization. *Annual Review of Physiology*, *79*(1), 541–566. https://doi.org/10.1146/annurev-physiol-022516-034339

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., Gordon, S., Hamilton, J. A., Ivashkiv, L. B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F. O., Mege, J.-L., Mosser, D. M., Natoli, G., Saeij, J. P., Schultze, J. L., Shirey, K. A., … Wynn, T. A. (2014). Macrophage activation and polarization: Nomenclature and experimental guidelines. *Immunity*, *41*(1), 14–20. https://doi.org/10.1016/j.immuni.2014.06.008

Mussbacher, M., Derler, M., Basílio, J., & Schmid, J. A. (2023). NF-κB in monocytes and macrophages – an inflammatory master regulator in multitalented immune cells. *Frontiers in Immunology*, *14*, 1134661. https://doi.org/10.3389/fimmu.2023.1134661

Newland, J. (2016). *In vitro cell culture to study microglial inflammation* [Doctoral dissertation, University of Waikato].

Nguyen, S. T., Nguyen, H. T.-L., & Truong, K. D. (2020). Comparative cytotoxic effects of methanol, ethanol and DMSO on human cancer cell lines. *Biomedical Research and Therapy*, *7*(7), 3855–3859. https://doi.org/10.15419/bmrat.v7i7.614

Nims, R. W., & Price, P. J. (2017). Best practices for detecting and mitigating the risk of cell culture contaminants. *In Vitro Cellular & Developmental Biology: Animal*, *53*(10), 872–879. https://doi.org/10.1007/s11626-017-0203-9

Noda, T., & Amano, F. (1997). Differences in nitric oxide synthase activity in a macrophage-like cell line, RAW264.7 cells, treated with lipopolysaccharide (LPS) in the presence or absence of interferon-gamma (IFN-gamma): Possible heterogeneity of iNOS activity. *Journal of Biochemistry*, *121*(1), 38–46. https://doi.org/10.1093/oxfordjournals.jbchem.a021566

Núñez, I., & Matthews, A. A. (2025). Detection bias and the role of negative control outcomes. *BMJ Medicine*, *4*(1), e001336. https://doi.org/10.1136/bmjmed-2025-001336

Obst, K., Lieder, B., Reichelt, K. V., Backes, M., Paetz, S., Geißler, K., Krammer, G., Somoza, V., Ley, J. P., & Engel, K.-H. (2017). Sensory active piperine analogues from Macropiper excelsum and their effects on intestinal nutrient uptake in Caco-2 cells. *Phytochemistry*, *135*, 181–190. https://doi.org/10.1016/j.phytochem.2016.12.016

Oeckinghaus, A., & Ghosh, S. (2009). The NF-κB Family of Transcription Factors and Its Regulation. *Cold Spring Harbor Perspectives in Biology*, *1*(4), a000034–a000034. https://doi.org/10.1101/cshperspect.a000034

Oeckinghaus, A., Hayden, M. S., & Ghosh, S. (2011). Crosstalk in NF-κB signaling pathways. *Nature Immunology*, *12*(8), 695–708. https://doi.org/10.1038/ni.2065

Ono, S., Egawa, G., & Kabashima, K. (2017). Regulation of blood vascular permeability in the skin. *Inflammation and Regeneration*, *37*(1), 11. https://doi.org/10.1186/s41232-017-0042-9

Oronsky, B., Caroen, S., & Reid, T. (2022). What exactly is inflammation (and what is it not?) *International Journal of Molecular Sciences*, *23*(23), 14905. https://doi.org/10.3390/ijms232314905

Pal, R., Mamidi, M. K., Das, A. K., & Bhonde, R. (2012). Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. *Archives of Toxicology*, *86*(4), 651–661. https://doi.org/10.1007/s00204-011-0782-2

Parisi, L., Gini, E., Baci, D., Tremolati, M., Fanuli, M., Bassani, B., Farronato, G., Bruno, A., & Mortara, L. (2018). Macrophage Polarization in Chronic Inflammatory Diseases: Killers or Builders? *Journal of Immunology Research*, *2018*, 8917804. https://doi.org/10.1155/2018/8917804

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.-e., Karandikar, M., Berman, K., & Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions*. *Endocrine Reviews*, *22*(2), 153–183. https://doi.org/10.1210/edrv.22.2.0428

Pelegrin, P., Barroso-Gutierrez, C., & Surprenant, A. (2008). P2X7 receptor differentially couples to distinct release pathways for IL-1β in mouse macrophage. *The Journal of Immunology*, *180*(11), 7147–7157. https://doi.org/10.4049/jimmunol.180.11.7147

Philips, R. L., Wang, Y., Cheon, H., Kanno, Y., Gadina, M., Sartorelli, V., Horvath, C. M., Darnell, J. E., Stark, G. R., & O'Shea, J. J. (2022). The JAK-STAT pathway at 30: Much learned, much more to do. *Cell*, *185*(21), 3857–3876. https://doi.org/10.1016/j.cell.2022.09.023

Piccinini, F., Tesei, A., Arienti, C., & Bevilacqua, A. (2017). Cell counting and viability assessment of 2D and 3D cell cultures: Expected reliability of the trypan blue assay. *Biological Procedures Online*, *19*, 8. https://doi.org/10.1186/s12575-017-0056-3

Pollard, J. W. (2009). Trophic macrophages in development and disease. *Nature Reviews. Immunology*, *9*(4), 259–270. https://doi.org/10.1038/nri2528

PubChem. (2025). PubChem Compound Database of Piperine, Myristicin, Vitexin, Excelsin, and Dopamine. *National Library of Medicine (US)*. Retrieved November 12, 2025, from https://pubchem.ncbi.nlm.nih.gov/

Punchard, N. A., Whelan, C. J., & Adcock, I. (2004). The Journal of Inflammation. *Journal of Inflammation*, *1*(1), 1. https://doi.org/10.1186/1476-9255-1-1

Putriani, N., Perdana, J., Meiliana, & Nugrahedi, P. Y. (2022). Effect of thermal processing on key phytochemical compounds in green leafy vegetables: A review. *Food Reviews International*, *38*(4), 783–811. https://doi.org/10.1080/87559129.2020.1745826

R Core Team. (2026). *R: A language and environment for statistical computing*. Vienna, Austria. https://www.R-project.org

Ramchundar, N., & Nlooto, M. (2020). A comparative qualitative study of the types of traditional treatment of fractures by traditional health practitioners in Kwazulu-Natal, South Africa and the North Island of New Zealand: A survey-based study. *PONTE International Scientific Researches Journal*, *76*(12). https://doi.org/10.21506/j.ponte.2020.12.12

Ramos, G. C. (2012). Inflammation as an animal development phenomenon. *Clinical and Developmental Immunology*, *2012*, 1–8. https://doi.org/10.1155/2012/983203

Ramzan, F., Jayaprakash, R., Pook, C., Foster, M., Miles-Chan, J. L., & Mithen, R. (2022). Acute Effects of Kawakawa (Piper excelsum) Intake on Postprandial Glycemic and Insulinaemic Response in a Healthy Population. *Nutrients*, *14*(8), Article 8, 1638. https://doi.org/10.3390/nu14081638

Rao, K. M. K. (2001). MAP kinase activation in macrophages. *Journal of Leukocyte Biology*, *69*(1), 3–10. https://doi.org/10.1189/jlb.69.1.3

Raschke, W. C., Baird, S., Ralph, P., & Nakoinz, I. (1978). Functional macrophage cell lines transformed by abelson leukemia virus. *Cell*, *15*(1), 261–267. https://doi.org/10.1016/0092-8674(78)90101-0

Rashidinejad, A. (2024). Bioactive compounds from New Zealand's native edible plants: Their role in future functional foods. *Future Postharvest and Food*, *1*(4), 435–440. https://doi.org/10.1002/fpf2.12038

Rather, L. J. (1971). Disturbance of function (functio laesa): The legendary fifth cardinal sign of inflammation, added by galen to the four cardinal signs of celsus. *Bulletin of the New York Academy of Medicine*, *47*(3), 303–322.

Reichner, J. S., Mulligan, J. A., & Bodenheimer, H. C. (1995). Electron Transport Chain Activity in Normal and Activated Rat Macrophages. *Journal of Surgical Research*, *59*(6), 636–643. https://doi.org/10.1006/jsre.1995.1217

Renart, J., Reiser, J., & Stark, G. R. (1979). Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: A method for studying antibody specificity and antigen structure. *Proceedings of the National Academy of Sciences*, *76*(7), 3116–3120. https://doi.org/10.1073/pnas.76.7.3116

Riley, M. (1994). *Māori healing and herbal: New zealand ethnobotanical sourcebook*. Viking Sevenseas NZ.

Rosales, C., & Uribe-Querol, E. (2017). Phagocytosis: A Fundamental Process in Immunity. *BioMed Research International*, *2017*, 1–18. https://doi.org/10.1155/2017/9042851

Roshick, C., Wood, H., Caldwell, H. D., & McClarty, G. (2006). Comparison of Gamma Interferon-Mediated Antichlamydial Defense Mechanisms in Human and Mouse Cells. *Infection and Immunity*, *74*(1), 225–238. https://doi.org/10.1128/IAI.74.1.225-238.2006

Russell, G. B., & Fenemore, P. G. (1973). New lignans from leaves of Macropiper excelsum. *Phytochemistry*, *12*(7), 1799–1803. https://doi.org/10.1016/0031-9422(73)80407-8

Salehi, B., Zakaria, Z. A., Gyawali, R., Ibrahim, S. A., Rajkovic, J., Shinwari, Z. K., Khan, T., Sharifi-Rad, J., Ozleyen, A., Turkdonmez, E., Valussi, M., Tumer, T. B., Monzote Fidalgo, L., Martorell, M., & Setzer, W. N. (2019). Piper Species: A Comprehensive Review on Their Phytochemistry, Biological Activities and Applications. *Molecules*, *24*(7), 1364. https://doi.org/10.3390/molecules24071364

Sanders, E. R. (2012). Aseptic Laboratory Techniques: Plating Methods. *Journal of Visualized Experiments*, (63), 3064. https://doi.org/10.3791/3064

Sandle, T., Skinner, K., & Yeandle, E. (2013). Optimal conditions for the recovery of bioburden from pharmaceutical processes: A case study. *European Journal of Parenteral & Pharmaceutical Sciences*, *18*(3), 84.

Sansbury, B. E., & Spite, M. (2016). Resolution of acute inflammation and the role of resolvins in immunity, thrombosis, and vascular biology. *Circulation Research*, *119*(1), 113–130. https://doi.org/10.1161/CIRCRESAHA.116.307308

Saxton, R. A., Tsutsumi, N., Su, L. L., Abhiraman, G. C., Mohan, K., Henneberg, L. T., Aduri, N. G., Gati, C., & Garcia, K. C. (2021). Structure-based decoupling of the pro- and anti-inflammatory functions of interleukin-10. *Science (New York, N.Y.)*, *371*(6535), eabc8433. https://doi.org/10.1126/science.abc8433

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-gamma: An overview of signals, mechanisms and functions. *Journal of Leukocyte Biology*, *75*(2), 163–189. https://doi.org/10.1189/jlb.0603252

Shah, K., & Maghsoudlou, P. (2016). Enzyme-linked immunosorbent assay (ELISA): The basics. *British Journal of Hospital Medicine*, *77*(7), C98–C101. https://doi.org/10.12968/hmed.2016.77.7.C98

Sharma, L., Wu, W., Dholakiya, S. L., Gorasiya, S., Wu, J., Sitapara, R., Patel, V., Wang, M., Zur, M., Reddy, S., Siegelaub, N., Bamba, K., Barile, F. A., & Mantell, L. L. (2014). Assessment of phagocytic activity of cultured macrophages using fluorescence microscopy and flow cytometry. In I. Vancurova (Ed.), *Cytokine Bioassays* (pp. 137–145, Vol. 1172). Springer New York. https://doi.org/10.1007/978-1-4939-0928-5_12

Sica, A., & Mantovani, A. (2012). Macrophage plasticity and polarization: In vivo veritas. *Journal of Clinical Investigation*, *122*(3), 787–795. https://doi.org/10.1172/JCI59643

Siriwardena, J. (2018). *Potential benefits of traditional maori plant extracts for prostate cancer control, modelled in prostate cancer cell lines* [Doctoral dissertation, University of Auckland].

Soares-Silva, M., Diniz, F. F., Gomes, G. N., & Bahia, D. (2016). The mitogen-activated protein kinase (MAPK) pathway: Role in immune evasion by trypanosomatids. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00183

Starkie, R. L., Rolland, J., Angus, D. J., Anderson, M. J., & Febbraio, M. A. (2001). Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-α levels after prolonged running. *American*

Journal of Physiology Cell Physiology, *280*(4), C769–C774. https://doi.org/10.1152/ajpcell.2001.280.4.C769

Stein, M., Keshav, S., Harris, N., & Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: A marker of alternative immunologic macrophage activation. *The Journal of experimental medicine*, *176*(1), 287–292. https://doi.org/10.1084/jem.176.1.287

Strober, W. (2015). Trypan Blue Exclusion Test of Cell Viability. *Current Protocols in Immunology*, *111*(1), A3.B.1–A3.B.3. https://doi.org/10.1002/0471142735.ima03bs111

Sule, R., Rivera, G., & Gomes, A. V. (2023). Western Blotting (immunoblotting): History, Theory, Uses, Protocol and Problems. *BioTechniques*, *75*(3), 99–114. https://doi.org/10.2144/btn-2022-0034

Sun, J., Zhang, X., Broderick, M., & Fein, H. (2003). Measurement of nitric oxide production in biological systems by using griess reaction assay. *Sensors*, *3*(8), 276–284. https://doi.org/10.3390/s30800276

Tapani, E., Taavitsainen, M., Lindros, K., Vehmas, T., & Lehtonen, E. (1996). Toxicity of Ethanol in Low Concentrations: Experimental Evaluation in Cell Culture. *Acta Radiologica*, *37*, 923–926. https://doi.org/10.1177/02841851960373P296

Tarique, A. A., Tuladhar, N., Kelk, D., Begum, N., Lucas, R. M., Luo, L., Stow, J. L., Wainwright, C. E., Bell, S. C., Sly, P. D., & Fantino, E. (2024). Azithromycin Augments Bacterial Uptake and Anti-Inflammatory Macrophage Polarization in Cystic Fibrosis. *Cells*, *13*(2), 166. https://doi.org/10.3390/cells13020166

Tauber, A. I. (2003). Metchnikoff and the phagocytosis theory. *Nature Reviews Molecular Cell Biology*, *4*(11), 897–901. https://doi.org/10.1038/nrm1244

Tautuiaki, S. (2023). *Effects of acute kawakawa tea (piper excelsum) consumption on markers of inflammation* [Doctoral dissertation, ResearchSpace@ Auckland].

Tautuiaki, S., Gojer, J., Jayaprakash, R., Sharma, P., Pook, C., Foster, M., Miles-Chan, J., Mithen, R., & Ramzan, F. (2024). Anti-inflammatory effects of kawakawa (*Piper excelsum*): An integrative mRNA–miRNA approach. *Food Science & Nutrition*, *12*(11), 8858–8869. https://doi.org/10.1002/fsn3.4450

Tian, Y., Li, Z., Shen, B., Wu, L., Han, L., Zhang, Q., & Feng, H. (2017). The protective effects of Shikonin on lipopolysaccharide/ D -galactosamine-induced acute liver injury via inhibiting MAPK and NF-κB and activating Nrf2/HO-1 signaling pathways. *RSC Advances*, *7*(55), 34846–34856. https://doi.org/10.1039/C7RA03291A

Tilg, H., Trehu, E., Atkins, M., Dinarello, C., & Mier, J. (1994). Interleukin-6 (IL-6) as an anti-inflammatory cytokine: Induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood*, *83*(1), 113–118. https://doi.org/10.1182/blood.V83.1.113.113

Tong, W., Chen, X., Song, X., Chen, Y., Jia, R., Zou, Y., Li, L., Yin, L., He, C., Liang, X., Ye, G., Lv, C., Lin, J., & Yin, Z. (2019). Resveratrol inhibits LPS-induced inflammation through suppressing the signaling cascades of TLR4-NF-κB/MAPKs/IRF3. *Experimental and Therapeutic Medicine*. https://doi.org/10.3892/etm.2019.8396

Tsikas, D. (2007). Analysis of nitrite and nitrate in biological fluids by assays based on the griess reaction: Appraisal of the griess reaction in the l-arginine/nitric oxide area of research. *Journal of Chromatography B*, *851*(1–2), 51–70. https://doi.org/10.1016/j.jchromb.2006.07.054

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., & Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International Journal of Cancer*, *26*(2), 171–176. https://doi.org/10.1002/ijc.2910260208

Turk, J. L. (1994). Inflammation: John hunter's "a treatise on the blood, inflammation and gun-shot wounds". *International Journal of Experimental Pathology*, *75*(6), 385–395.

Unanue, E. R. (1984). Antigen-presenting function of the macrophage. *Annual Review of Immunology*, *2*(1), 395–428. https://doi.org/10.1146/annurev.iy.02.040184.002143

Uribe-Querol, E., & Rosales, C. (2020). Phagocytosis: Our current understanding of a universal biological process. *Frontiers in Immunology*, *11*, 1066. https://doi.org/10.3389/fimmu.2020.01066

Valledor, A. F., Xaus, J., Marquès, L., & Celada, A. (1999). Macrophage colony-stimulating factor induces the expression of mitogen-activated protein kinase phosphatase-1 through a protein kinase C-dependent pathway. *Journal of Immunology (baltimore, Md.: 1950)*, *163*(5), 2452–2462.

Van Meerloo, J., Kaspers, G. J. L., & Cloos, J. (2011). Cell sensitivity assays: The MTT assay. In I. A. Cree (Ed.), *Cancer Cell Culture* (pp. 237–245, Vol. 731). Humana Press. https://doi.org/10.1007/978-1-61779-080-5_20

Vandooren, B., Noordenbos, T., Ambarus, C., Krausz, S., Cantaert, T., Yeremenko, N., Boumans, M., Lutter, R., Tak, P. P., & Baeten, D. (2009). Absence of a classically activated macrophage cytokine signature in peripheral spondylarthritis, including psoriatic arthritis. *Arthritis and Rheumatism*, *60*(4), 966–975. https://doi.org/10.1002/art.24406

van Tonder, A., Joubert, A. M., & Cromarty, A. D. (2015). Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC research notes*, *8*, 47. https://doi.org/10.1186/s13104-015-1000-8

Vara, J. Á. F., Casado, E., De Castro, J., Cejas, P., Belda-Iniesta, C., & González-Barón, M. (2004). PI3K/Akt signalling pathway and cancer. *Cancer Treatment Reviews*, *30*(2), 193–204. https://doi.org/10.1016/j.ctrv.2003.07.007

Venter, G., Oerlemans, F. T. J. J., Wijers, M., Willemse, M., Fransen, J. A. M., & Wieringa, B. (2014). Glucose controls morphodynamics of LPS-stimulated macrophages (P. K. Singh, Ed.). *PLoS ONE*, *9*(5), e96786. https://doi.org/10.1371/journal.pone.0096786

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics*, *13*(4), 227–232. https://doi.org/10.1038/nrg3185

Volokhov, D. V., Graham, L. J., Brorson, K. A., & Chizhikov, V. E. (2011). Mycoplasma testing of cell substrates and biologics: Review of alternative non-microbiological techniques. *Molecular and Cellular Probes*, *25*(2–3), 69–77. https://doi.org/10.1016/j.mcp.2011.01.002

Walsh, S. E., & Denyer, S. P. (2012). Filtration sterilization. In A. P. Fraise, J.-Y. Maillard, & S. A. Sattar (Eds.), *Russell, Hugo & Ayliffe's* (pp. 343–370). Wiley-Blackwell. https://doi.org/10.1002/9781118425831.ch15e

Wang, D., Li, K., & Teo, W. (1999). Preparation and characterization of polyvinylidene fluoride (PVDF) hollow fiber membranes. *Journal of Membrane Science*, *163*(2), 211–220. https://doi.org/10.1016/S0376-7388(99)00181-7

Wang, L.-x., Zhang, S.-x., Wu, H.-j., Rong, X.-l., & Guo, J. (2019). M2b macrophage polarization and its roles in diseases. *Journal of Leukocyte Biology*, *106*(2), 345–358. https://doi.org/10.1002/JLB.3RU1018-378RR

Wasyłeczko, M., Wojciechowski, C., & Chwojnowski, A. (2024). Polyethersulfone polymer for biomedical applications and biotechnology. *International journal of molecular sciences*, *25*(8), 4233. https://doi.org/10.3390/ijms25084233

Weiskirchen, S., Schröder, S. K., Buhl, E. M., & Weiskirchen, R. (2023). A Beginner's Guide to Cell Culture: Practical Advice for Preventing Needless Problems. *Cells*, *12*(5), 682. https://doi.org/10.3390/cells12050682

Wright, T. M. (1997). Cytokines in acute and chronic inflammation. *Frontiers in Bioscience*, *2*(4), d12–26. https://doi.org/10.2741/A171

Wu, X. (2025). Guidelines for anti-inflammatory assays in RAW264.7 cells. *Food Safety and Health*, *3*(1), 128–136. https://doi.org/10.1002/fsh3.12073

Wynn, T. A., Chawla, A., & Pollard, J. W. (2013). Macrophage biology in development, homeostasis and disease. *Nature*, *496*(7446), 445–455. https://doi.org/10.1038/nature12034

Xu, G., Ye, X., Chen, J., & Liu, D. (2007). Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract. *Journal of Agricultural and Food Chemistry*, *55*(2), 330–335. https://doi.org/10.1021/jf0625171

Xu, H. (2018). *Anti-inflammatory and anti-cancer effects of kawakawa (macropiper excelsum) leaves* [Doctoral dissertation, ResearchSpace@ Auckland].

Yamasaki, K., & Eeden, S. F. V. (2018). Lung macrophage phenotypes and functional responses: Role in the pathogenesis of COPD. *International journal of molecular sciences*, *19*(2), 582. https://doi.org/10.3390/ijms19020582

Yang, Y., Kim, S. C., Yu, T., Yi, Y.-S., Rhee, M. H., Sung, G.-H., Yoo, B. C., & Cho, J. Y. (2014). Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses. *Mediators of Inflammation*, *2014*, 1–13. https://doi.org/10.1155/2014/352371

Ying, X., Yu, K., Chen, X., Chen, H., Hong, J., Cheng, S., & Peng, L. (2013). Piperine inhibits LPS induced expression of inflammatory mediators in RAW 264.7 cells. *Cellular Immunology*, *285*(1–2), 49–54. https://doi.org/10.1016/j.cellimm.2013.09.001

Yu, Y., Yue, Z., Xu, M., Zhang, M., Shen, X., Ma, Z., Li, J., & Xie, X. (2022). Macrophages play a key role in tissue repair and regeneration. *PeerJ*, *10*, e14053. https://doi.org/10.7717/peerj.14053

Yunna, C., Mengru, H., Lei, W., & Weidong, C. (2020). Macrophage M1/M2 polarization. *European Journal of Pharmacology*, *877*, 173090. https://doi.org/10.1016/j.ejphar.2020.173090

Zandieh, M., Hosseini, S. N., Vossoughi, M., Khatami, M., Abbasian, S., & Moshaii, A. (2018). Label-free and simple detection of endotoxins using a sensitive LSPR biosensor based on silver nanocolumns. *Analytical Biochemistry*, *548*, 96–101. https://doi.org/10.1016/j.ab.2018.02.023

Zhang, H., Niesel, D. W., Peterson, J. W., & Klimpel, G. R. (1998). Lipoprotein release by bacteria: Potential factor in bacterial pathogenesis. *Infection and Immunity*, *66*(11), 5196–5201. https://doi.org/10.1128/IAI.66.11.5196-5201.1998

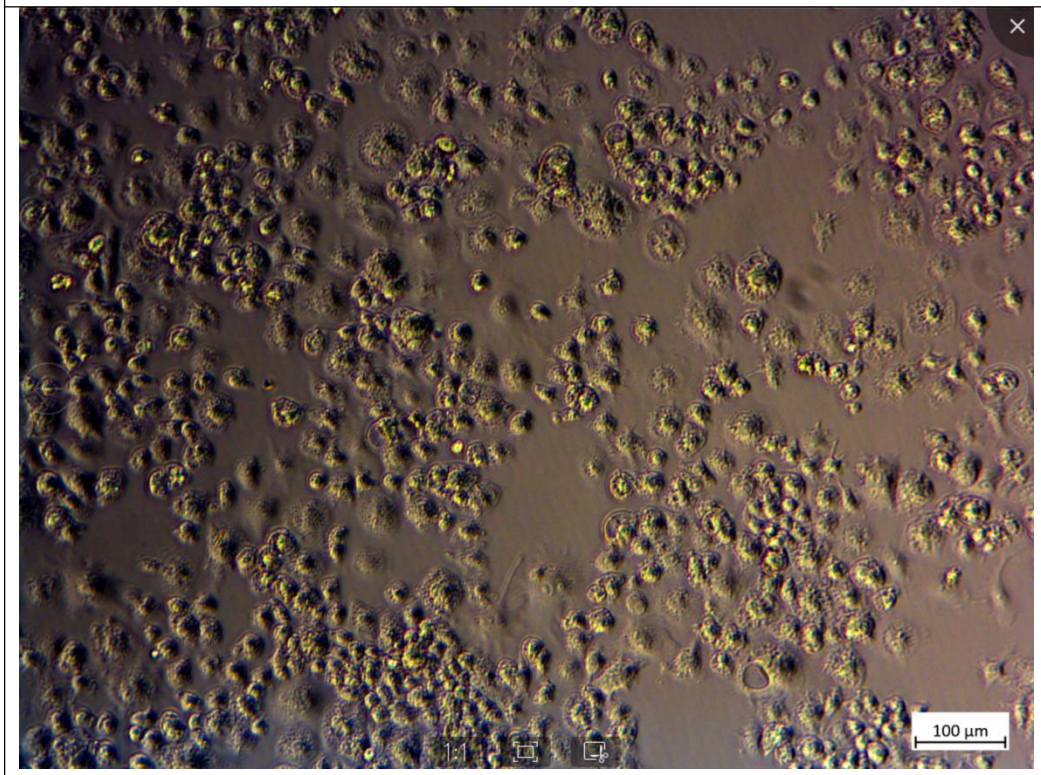
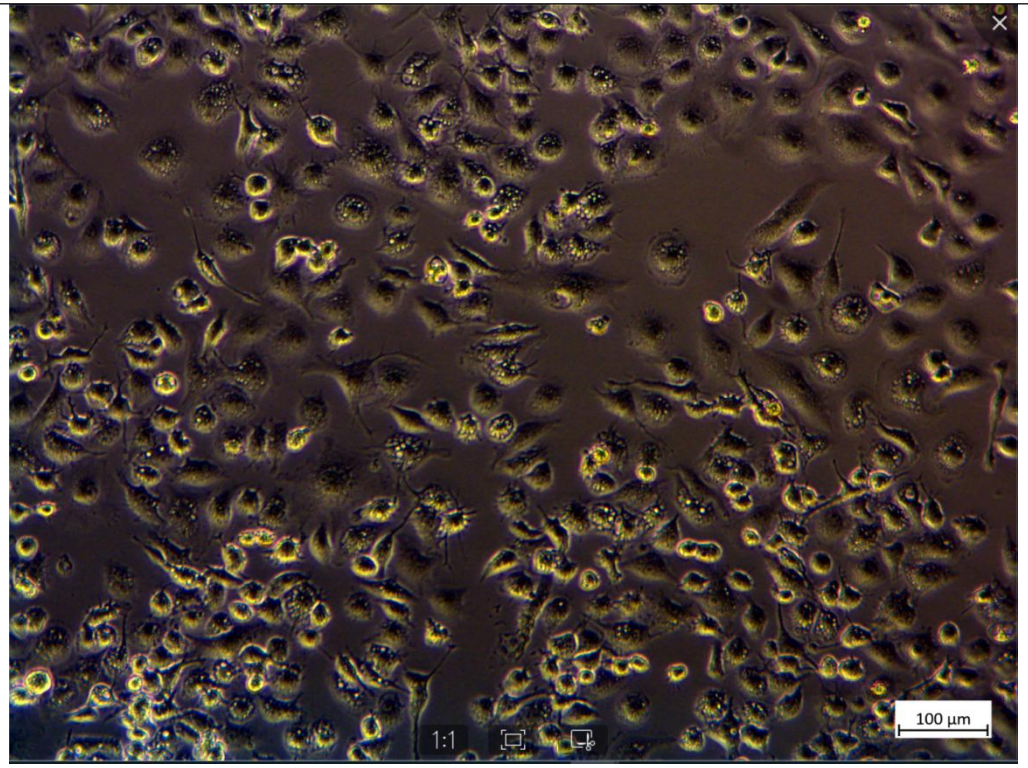
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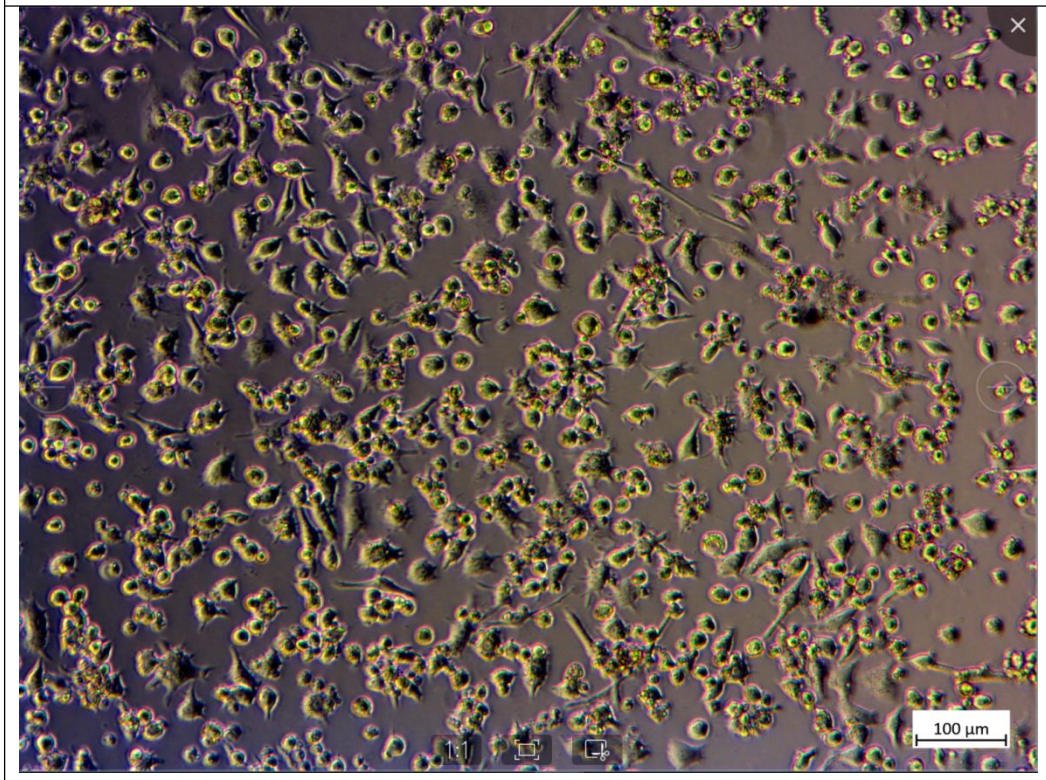
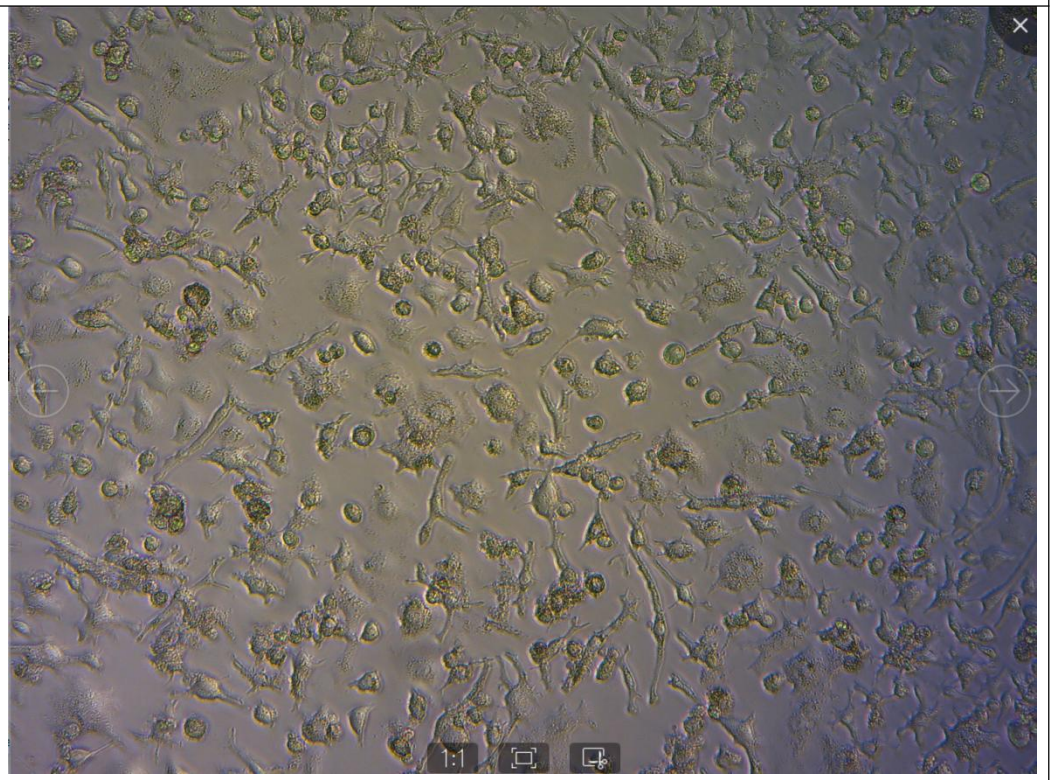
Appendices

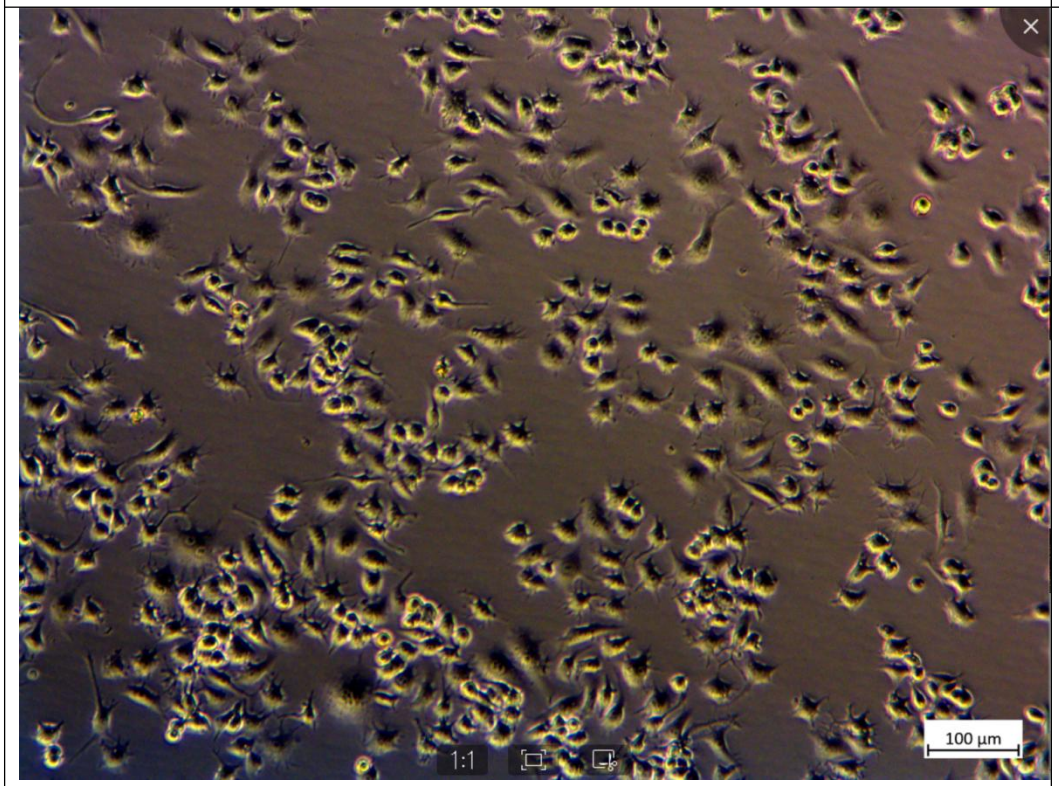
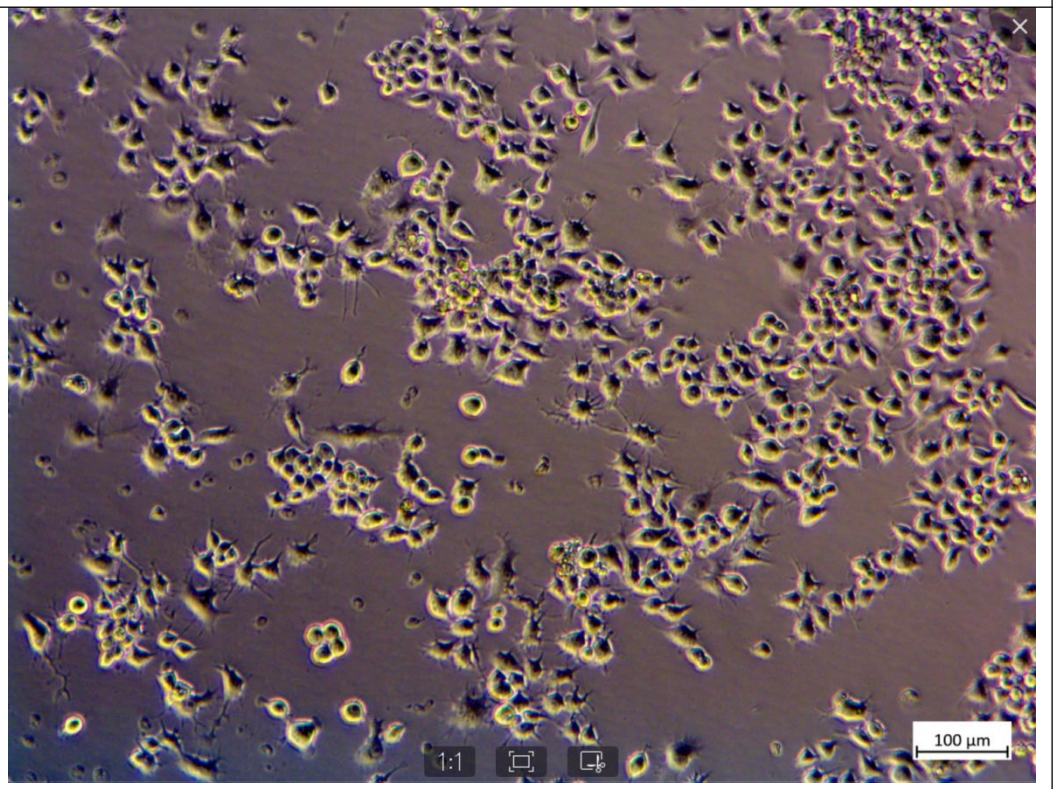
Appendix A. Representative Cell Morphology Images

Representative microscopic images of RAW264.7 macrophages under different treatment conditions are shown below. Cell morphology was examined following exposure to kawakawa extract in combination with either LPS or IFN-γ, as well as under the corresponding control conditions. Representative images were captured at 24, 48, 72, and 120 hours for the 100 μg/mL kawakawa + LPS treatment group, illustrating the temporal changes in macrophage morphology throughout the experiment. Additional representative images were obtained at 24 and 48 hours for the 100 μg/mL kawakawa + IFN-γ treatment group. Images of the vehicle control (0.1% DMSO) and the kawakawa-only control (100 μg/mL) are also included for comparison.

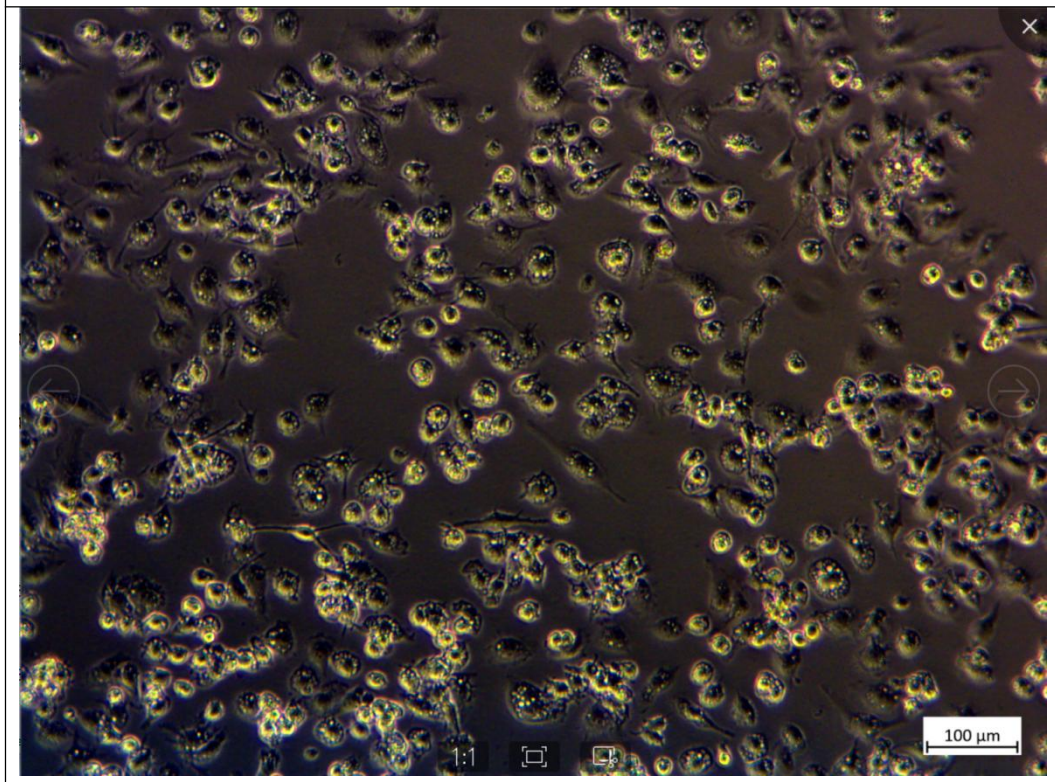
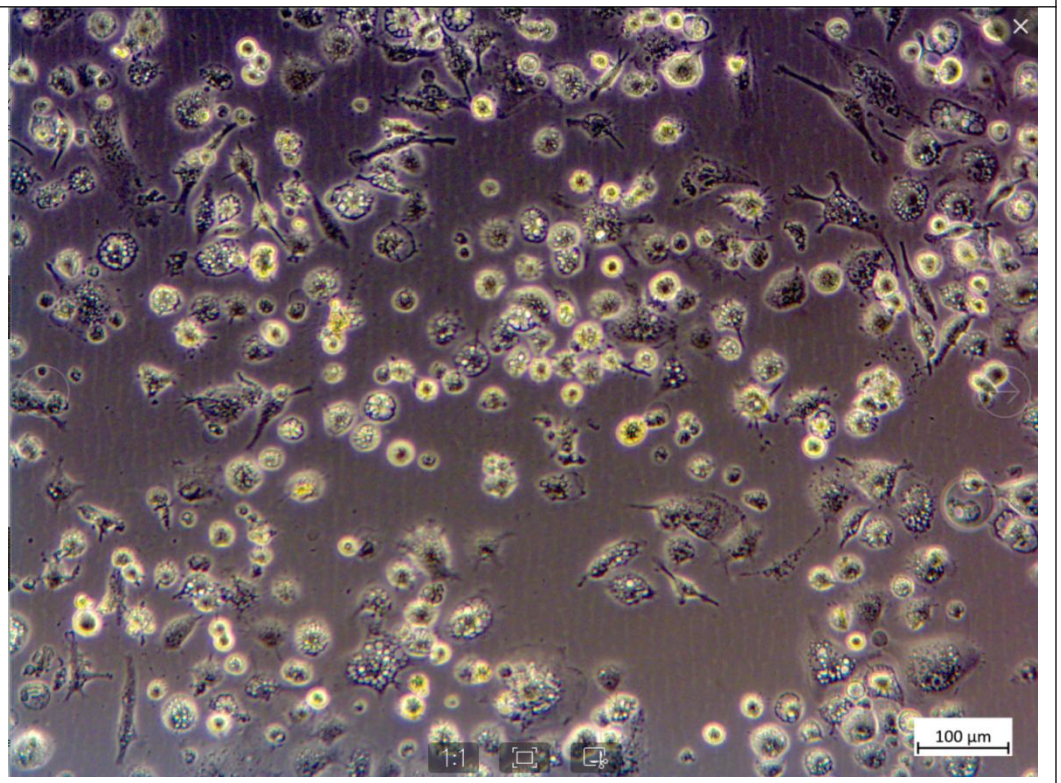
Kawakawa Treatment Groups

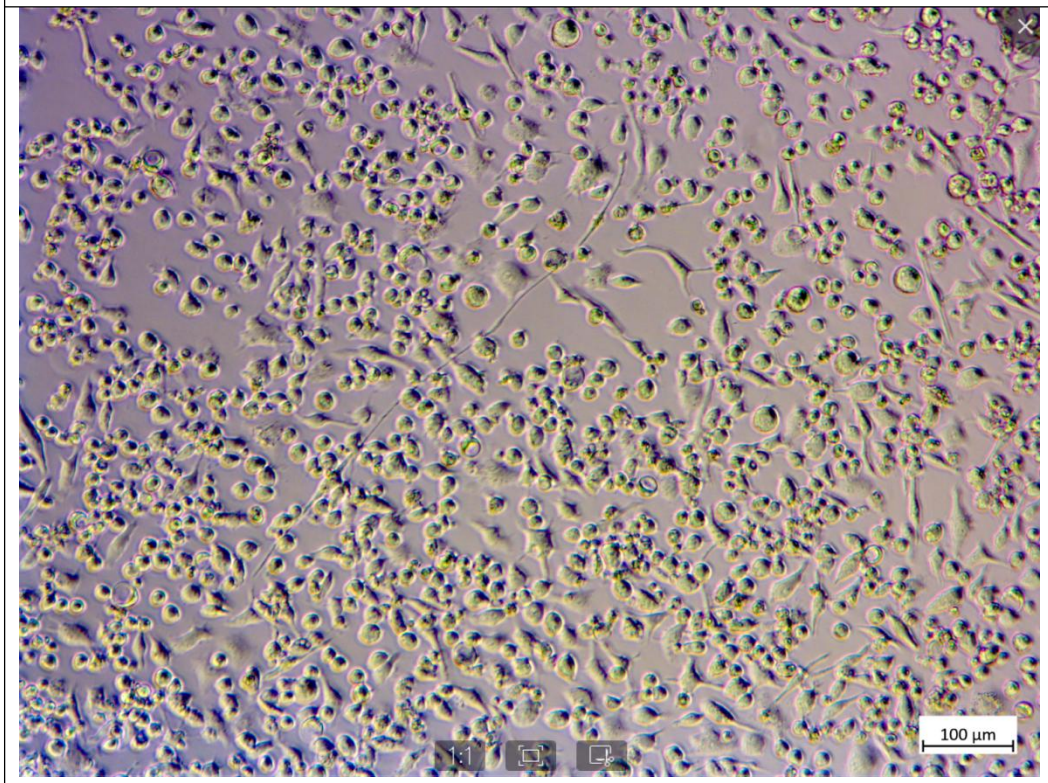
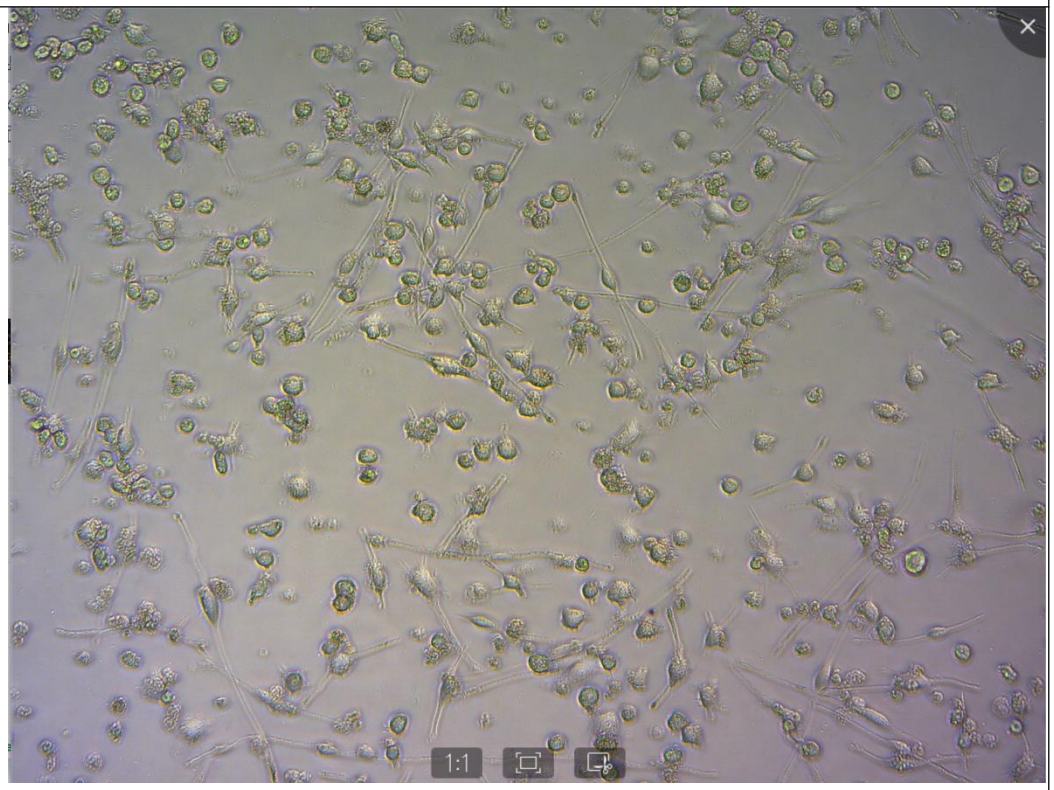
24h, 100 µg/mL kawakawa+LPS	48h, 100 µg/mL kawakawa+LPS
	

72h, 100 µg/mL kawakawa+LPS	120h, 100 µg/mL kawakawa+LPS
	

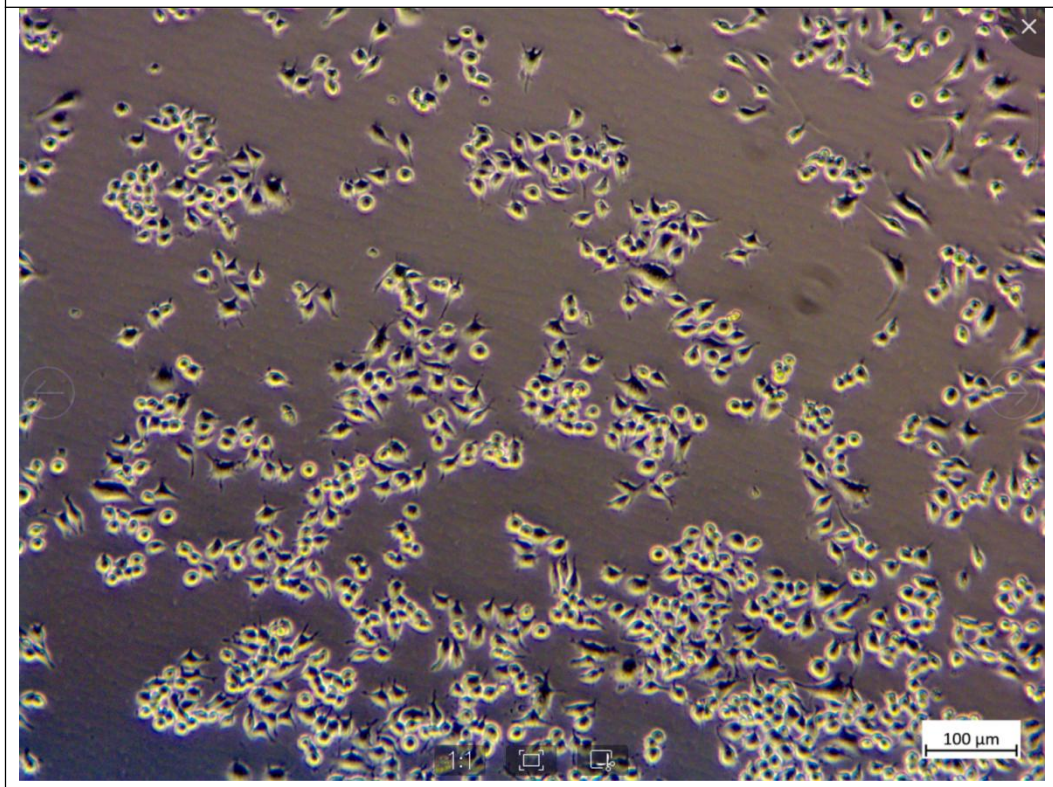
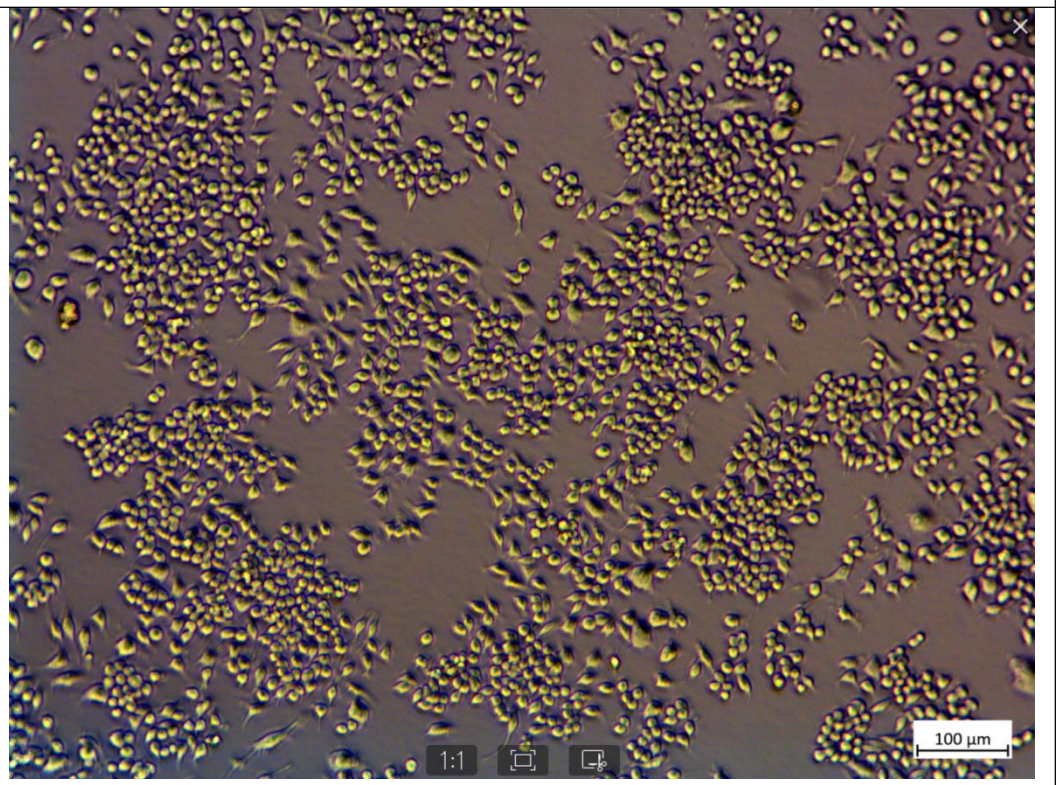
24h, 100 µg/mL kawakawa+IFN-γ	48h, 100 µg/mL kawakawa+IFN-γ
	

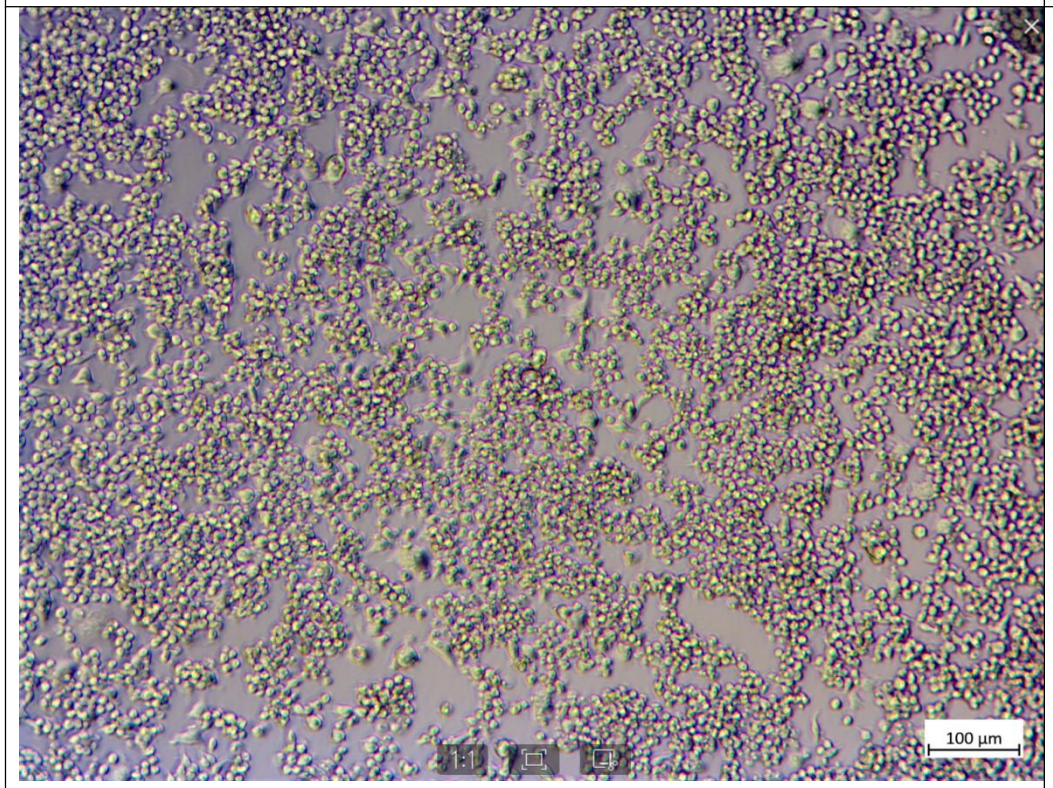
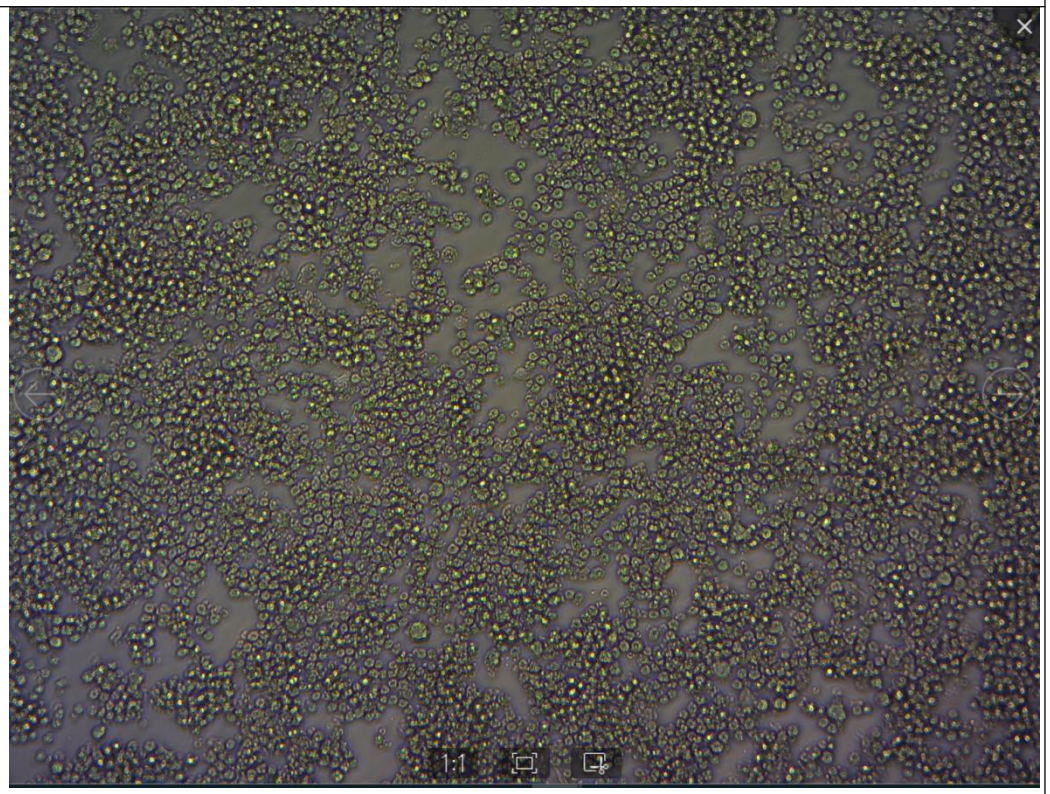
Positive Control Groups

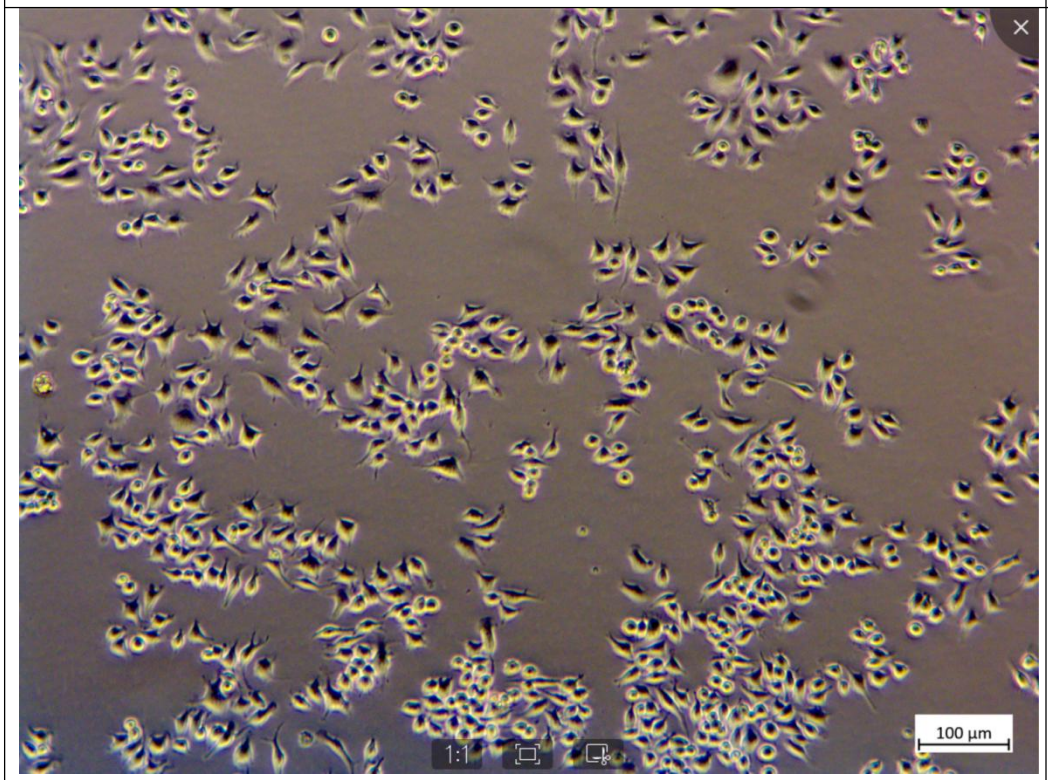
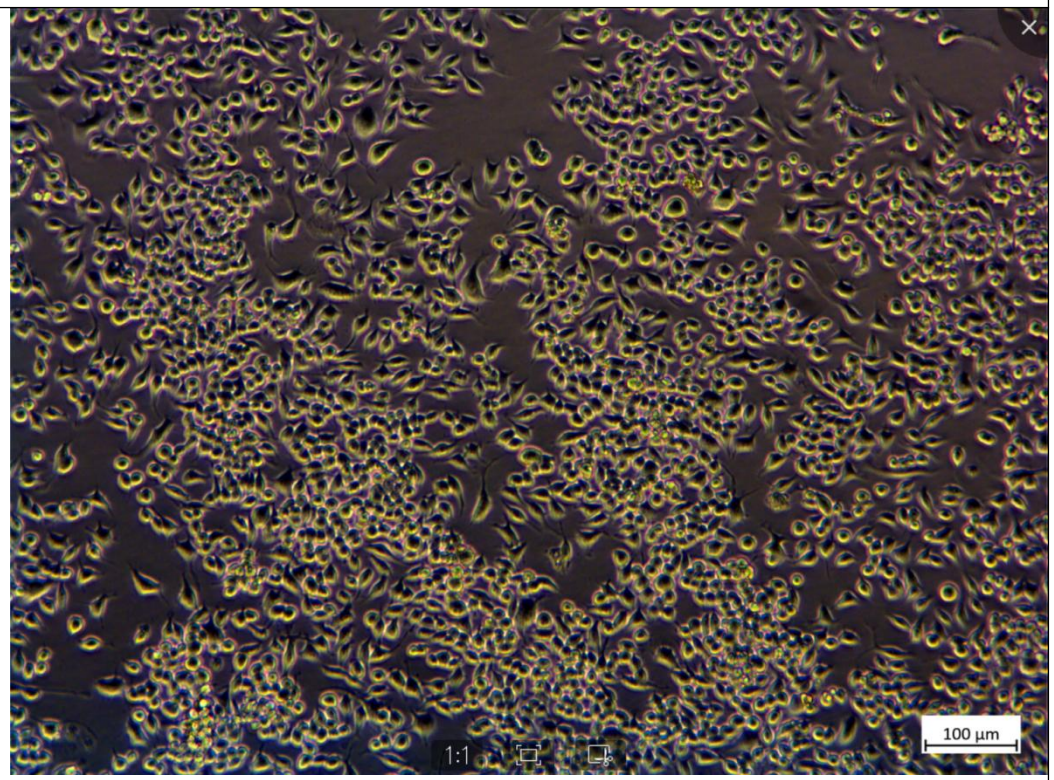
24h, 0.1% DMSO+LPS+IFN-γ	48h, 0.1% DMSO+LPS+IFN-γ
	

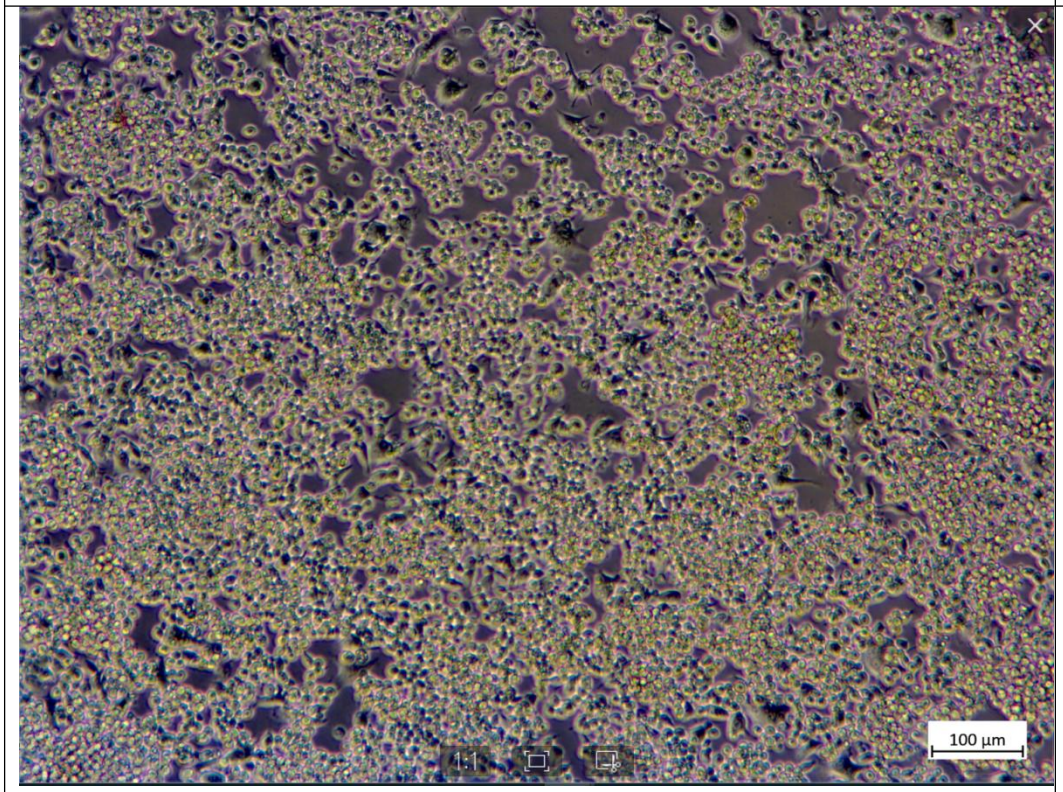
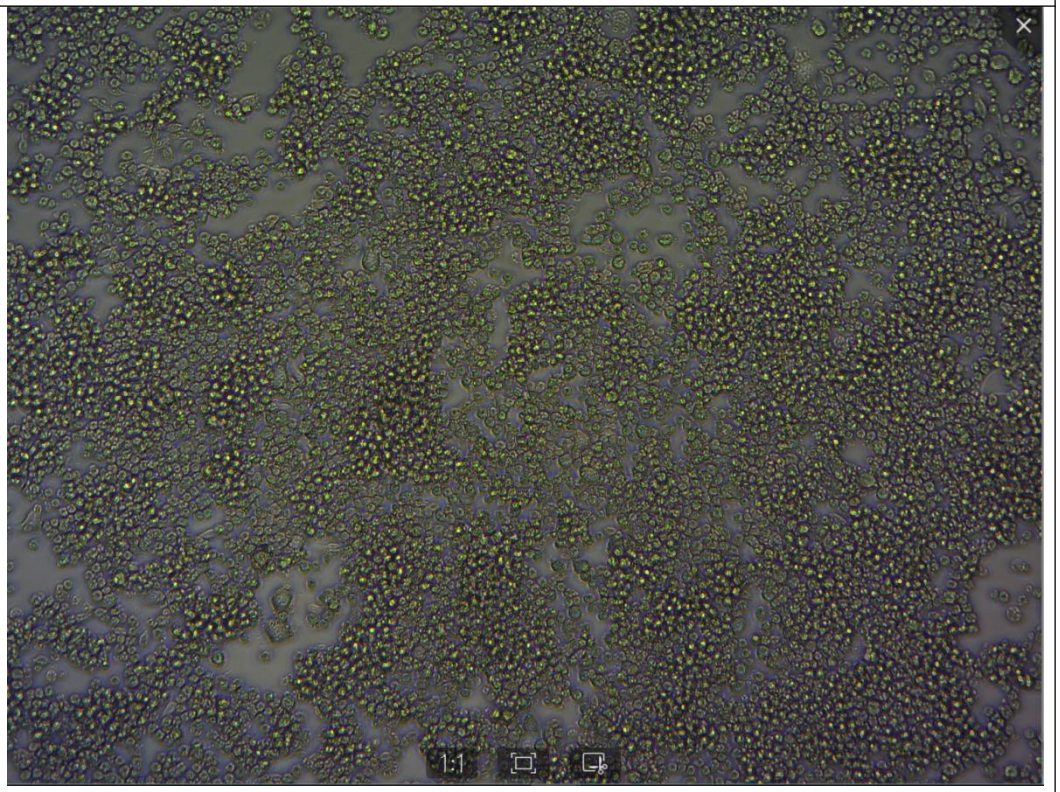
72h, 0.1% DMSO+LPS	120h, 0.1% DMSO+LPS
	

Negative Control Groups

24h, vehicle control (0.1% DMSO)	48h, vehicle control (0.1% DMSO)
	

72h, vehicle control (0.1% DMSO)	120h, vehicle control (0.1% DMSO)
	

24h, kawakawa only control (100 µg/mL)	48h, kawakawa only control (100 µg/mL)
	

72h, kawakawa only control (100 µg/mL)	120h, kawakawa only control (100 µg/mL)
	

Appendix B. Statistical Analysis Details

This appendix contains the detailed statistical outputs generated for all experimental analyses. These supplementary results include assumption tests for model validity, ANOVA summary tables, interaction analyses between experimental factors, and post hoc multiple comparison results. Where applicable, estimated marginal means, effect estimates, test statistics, degrees of freedom, confidence intervals, adjusted P-values, and significance levels are provided to complement the findings reported in Chapter 3 and to support the interpretation and reproducibility of the statistical analyses.

Phase 0 - viability

Table P0.1. Assumption tests for one-way ANOVA (P0)

Variable	Shapiro–Wilk W	P value	Normality	Levene's F	P value	Homogeneity
Viability	0.966	0.573	Yes	0.524	0.804	Yes
Total cells (tc)	0.964	0.520	Yes	0.473	0.834	Yes
Dead cells (dc)	0.935	0.127	Yes	0.262	0.960	Yes
Live cells (lc)	0.959	0.417	Yes	0.752	0.634	Yes

Table P0.2. One-way ANOVA results (P0)

Variable	df (Between, Within)	F value	P value	Significant
Total cells (tc)	7,16	2.370	0.0725	ns
Dead cells (dc)	7,16	0.240	0.968	ns
Live cells (lc)	7,16	2.513	0.0602	ns
Viability	7,16	20.21	7.95×10^{-7}	***

Note: Significance levels: ns, not significant ($P \geq 0.05$); *$P < 0.05$; **$P < 0.01$; **$P < 0.001$.

Table P0.3. Dunnett's multiple comparisons for viability (vs N - negative control)

Comparison	Estimate	t	Adjusted P	Significance
D1 vs N	−0.0869	−0.890	0.905	ns
D2 vs N	−0.0784	−0.803	0.938	ns
D3 vs N	0.0396	0.406	0.998	ns
D4 vs N	−0.0341	−0.349	0.999	ns
D5 vs N	−0.0619	−0.634	0.980	ns
Positive control vs N	−0.9091	−9.315	<0.0001	***
Vehicle vs N	−0.0555	−0.568	0.989	ns

Note: The sign of t indicates the direction of change relative to the control, while the absolute value indicates the strength of the difference. A positive t value represents an increase compared with the control, while a negative t value represents a decrease.

Table P0.4. Dunnett's multiple comparisons for viability (vs V - vehicle control)

Comparison	Estimate	t	Adjusted P	Significance
D1 vs V	−0.0314	−0.322	1.000	ns
D2 vs V	−0.0229	−0.235	1.000	ns
D3 vs V	0.0951	0.974	0.865	ns
D4 vs V	0.0214	0.219	1.000	ns
D5 vs V	−0.0064	−0.066	1.000	ns
Negative control vs V	0.0555	0.568	0.989	ns
Positive control vs V	−0.8536	−8.746	<0.0001	***

Phase 1 - Co-treatment with LPS

Table P1.1. Assumption tests for one-way ANOVA (P1-MTT)

Test	Statistic	P value	Result
Shapiro–Wilk	W = 0.963	0.266	Normal
Levene's test	F = 0.721	0.708	Equal variance

Table P1.2. One-way ANOVA (P1-MTT)

Source	df	F	P value	Significant
Group	11,24	10.38	1.17×10^{-6}	***

Table P1.3. Dunnett's test for MTT (vs untreated control, P1-MTT)

Comparison	Estimate	t	Adjusted P	Significance
K1L	0.0546	1.309	0.766	ns
K2L	−0.0419	−1.005	0.929	ns
K3L	−0.0712	−1.709	0.490	ns
K4L	−0.0437	−1.049	0.911	ns
K5L	−0.0441	−1.058	0.907	ns
KH	0.2354	5.648	<0.001	***
KL	0.1665	3.996	0.0047	**
LPS	0.0104	0.250	1.000	ns
Positive Control	−0.0362	−0.869	0.970	ns
Vehicle	−0.0116	−0.279	1.000	ns
Vehicle + LPS	−0.0552	−1.324	0.756	ns

Table P1.4. Assumption tests for one-way ANOVA (P1-NO)

Test	Statistic	P value	Result
Shapiro–Wilk	W = 0.951	0.114	Normal
Levene's test	F = 0.899	0.555	Equal variance

Table P1.5. One-way ANOVA (P1-NO)

Source	df	F	P value	Significant
Group	11,24	324.5	$<2\times10^{-16}$	***

Table P1.6. Dunnett's test for NO production (vs LPS+vehicle control - VDL, P1-NO)

Comparison	Estimate	t	Adjusted P	Significance
U	−17.988	−18.559	<0.001	***
K1L	−9.667	−9.974	<0.001	***
K2L	−1.883	−1.943	0.349	ns
K3L	−0.222	−0.229	1.000	ns
K4L	0.216	0.223	1.000	ns
K5L	0.278	0.287	1.000	ns
KH	−17.907	−18.476	<0.001	***
KL	−18.222	−18.801	<0.001	***
LPS	1.803	1.860	0.396	ns
Positive control	23.556	24.304	<0.001	***
Vehicle	−18.062	−18.636	<0.001	***

Phase 2 -Pre-treatment with LPS

Table P2.1. Assumption tests for three-way ANOVA (P2-MTT)

Test	Statistic	P value	Conclusion
Shapiro–Wilk normality	W = 0.991	0.858	Residuals normally distributed
Levene's test	F = 0.446	0.985	Homogeneity of variance satisfied

Table P2.2. Three-way ANOVA for cell viability (P2-MTT)

Source of variation	df	F value	P value	Significance
Stimulus	1	125.18	1.87×10^{-15}	***
Dose	5	6.49	9.37×10^{-5}	***
Time	3	88.60	$<2 \times 10^{-16}$	***
Stimulus × Dose	2	2.12	0.130	ns
Stimulus × Time	3	70.58	$<2 \times 10^{-16}$	***
Dose × Time	7	3.88	0.0018	**
Stimulus × Dose × Time	4	7.80	5.29×10^{-5}	***
Residual	52	—	—	—

Table P2.3. Significant pairwise comparisons (Three-way ANOVA, P2-MTT)

Comparison	Significant findings (adjusted P values)
Dose	At 72 h (LPS), Dose 0 vs Dose 100 (p = 0.0412). Under unstimulated conditions, significant differences were observed at 48 h between Dose 0 vs 0.01 (p < 0.0001), Dose 0 vs 100 (p = 0.0136),

Comparison	Significant findings (adjusted P values)
	Dose 0.01 vs 100 ($p < 0.0001$), and at 72 h between Dose 0 vs 100 ($p = 0.0392$).
Time	Under LPS stimulation, significant differences were observed for Dose 0 (24 vs 120 h, $p = 0.0012$; 48 vs 120 h, $p = 0.0455$; 72 vs 120 h, $p = 0.0003$), Dose 1 (24 vs 48 h, $p = 0.0449$), and Dose 100 (24 vs 72 h, $p = 0.0289$; 24 vs 120 h, $p = 0.0108$). Under unstimulated conditions, significant differences were found for Dose 0 (24 vs 48 h, $p < 0.0001$; 24 vs 72 h, $p < 0.0001$; 48 vs 72 h, $p < 0.0001$; 48 vs 120 h, $p = 0.0029$; 72 vs 120 h, $p < 0.0001$), Dose 0.01 (24 vs 48 h, $p = 0.0038$), and Dose 100 (24 vs 48 h, $p = 0.0031$; 24 vs 72 h, $p < 0.0001$; 24 vs 120 h, $p = 0.0007$; 48 vs 72 h, $p < 0.0001$; 72 vs 120 h, $p < 0.0001$).
Stimulus	Significant differences between LPS-treated and unstimulated cells were observed at Dose 0, 48 h ($p = 0.0267$); Dose 0.01, 48 h ($p < 0.0001$); Dose 0, 72 h ($p < 0.0001$); Dose 100, 72 h ($p < 0.0001$); and Dose 0, 120 h ($p = 0.0001$).

Table P2.4. Two-way ANOVA at each time point (P2-MTT)

Time	Effect	df	F value	P value	Significance
24 h	Stimulus	1	7.48	0.0136	*
	Dose	5	1.537	0.228	ns
	Stimulus × Dose	2	0.606	0.556	ns
48 h	Stimulus	1	0.578	0.457	ns
	Dose	5	9.551	<0.001	***
	Stimulus × Dose	2	9.564	0.0015	**
72 h	Stimulus	1	364.15	<0.001	***
	Dose	1	0	0.987	ns
	Stimulus × Dose	1	10.16	0.0129	*
120 h	Stimulus	1	16.863	0.0034	**
	Dose	1	0.28	0.611	ns
	Stimulus × Dose	1	6.598	0.0332	*

Table P2.5. Significant Dunnett comparisons vs. Vehicle + LPS control (P2-MTT)

Time	Reference	Significant comparison	Estimate	Adjusted P
24 h	VD-L-24	KH-24	0.1599	0.0256
48 h	VD-L-48	KL-48	0.3456	<0.001
72 h	VD-L-72	KH-72	0.6637	<0.001
		VD-72	0.5523	<0.001
120 h	VD-L-120	KH-120	0.1499	0.0275
		VD-120	0.2159	0.0042

Table P2.6. Summary of statistical analysis for NO production (P2-NO)

Analysis	Statistical results	Main findings
Three-way	Stimulus: $F = 577.32$, $p < 0.0001$; Dose: $F = 10.27$, $p = 8.96 \times$	All three main effects and all

Analysis	Statistical results	Main findings
ANOVA	10^{-8}; Time: F = 193.81, p < 0.0001; Stimulus × Dose: F = 13.59, p = 7.19 × 10^{-6}; Stimulus × Time: F = 103.36, p < 0.0001; Dose × Time: F = 5.79, p = 1.33 × 10^{-8}; Stimulus × Dose × Time: F = 4.56, p = 0.000115	interaction terms were significant.
Model assumptions	Shapiro–Wilk: W = 0.791, p = 1.96 × 10^{-12}; Levene's test: F = 1.001, p = 0.486	Residuals deviated from normality, whereas homogeneity of variance was satisfied.
Dose comparisons (Tukey)	24 h (LPS): Dose0 vs100 (p=0.0072), Dose0.01 vs100 (p=0.0019), Dose0.1 vs100 (p<0.0001), Dose1 vs100 (p=0.0016), Dose10 vs100 (p=0.0120). 48 h (LPS): Dose0 vs100 (p<0.0001), Dose0.01 vs0.1 (p=0.0025), Dose0.01 vs100 (p<0.0001), Dose0.1 vs1 (p<0.0001), Dose0.1 vs100 (p<0.0001), Dose1 vs10 (p=0.0034), Dose1 vs100 (p<0.0001), Dose10 vs100 (p<0.0001). 72 h (LPS): Dose0 vs100 (p<0.0001).	Dose-dependent reductions in NO production were observed exclusively under LPS stimulation, most prominently at 48 h.
Time comparisons (Tukey)	Significant temporal differences were detected for all LPS-treated dose groups. For every dose, 24 h vs 48 h was significant (Dose0: p<0.0001; Dose0.01: p<0.0001; Dose0.1: p<0.0001; Dose1: p=0.0015; Dose10: p<0.0001). Additional significant differences involving 72 h and 120 h were also observed, particularly at Dose0 and Dose100 (all p≤0.0365).	NO production increased significantly after 24 h, generally peaking at 48–72 h before declining slightly at 120 h.
Stimulus comparisons (Tukey)	24 h: Dose0 (p<0.0001), Dose0.01 (p<0.0001), Dose100 (p=0.0074). 48 h: Dose0 (p<0.0001), Dose0.01 (p<0.0001), Dose100 (p<0.0001). 72 h: Dose0 (p<0.0001), Dose100 (p<0.0001). 120 h: Dose0 (p<0.0001), Dose100 (p<0.0001).	LPS stimulation significantly increased NO production compared with unstimulated controls, particularly at vehicle (Dose0) and the highest dose (100 μg/mL).
Time-specific two-way ANOVA	3 h: no significant effects. 6 h: no significant effects. 24 h: Stimulus (p=3.39×10^{-9}), Dose (p=0.00253), Interaction (p=0.0433). 48 h: Stimulus (p=3.29×10^{-12}), Dose (p=0.000146), Interaction (p=0.00268). 72 h: Stimulus (p=5.43×10^{-10}), Dose (p=1.00×10^{-5}), Interaction (p=2.08×10^{-5}). 120 h: Stimulus only (p=1.40×10^{-7}).	Dose effects emerged after 24 h and were strongest at 48–72 h, whereas only stimulus remained significant at 120 h.

Table P2.7. Two-way ANOVA Model assumptions at each time point (P2-NO)

Time	Stimulus	Dose	Stimulus × Dose	Assumption
3 h	F = 0.915, p = 0.351 (ns)	F = 2.033, p = 0.122 (ns)	F = 0.002, p = 0.998 (ns)	Non-normal residuals; equal variance
6 h	F = 0.014, p = 0.908 (ns)	F = 0.715, p = 0.620 (ns)	F = 1.242, p = 0.312 (ns)	Non-normal residuals; equal variance
24 h	F = 113.31, p = 3.39 × 10^{-9} (***)	F = 5.70, p = 0.00253 (**)	F = 3.76, p = 0.04335 (*)	Non-normal residuals; equal variance
48 h	F = 264.68, p = 3.29 × 10^{-12} (***)	F = 9.48, p = 1.46 × 10^{-4} (***)	F = 8.38, p = 0.00268 (**)	Non-normal residuals; equal variance
72 h	F = 1190.97, p = 5.43 × 10^{-10} (***)	F = 95.60, p = 1.00 × 10^{-5} (***)	F = 78.54, p = 2.08 × 10^{-5} (***)	Normal residuals; equal variance
120 h	F = 291.75, p = 1.40 × 10^{-7} (***)	F = 0.710, p = 0.424 (ns)	F = 0.715, p = 0.422 (ns)	Normal residuals; equal variance

Table P2.8. Significant Dunnett comparisons (P2-NO)

Time	Significant comparisons (vs VD-L control)	t value	Adjusted *p* value
24 h	K1-L-24 vs VD-L-24	-3.26	0.02596
	KH-24 vs VD-L-24	-5.755	<0.001
	KL-24 vs VD-L-24	-6.008	<0.001
	VD-24 vs VD-L-24	-4.352	0.00238
48 h	K1-L-48 vs VD-L-48	-5.447	<0.001
	KH-48 vs VD-L-48	-9.055	<0.001
	KL-48 vs VD-L-48	-9.088	<0.001
	VD-48 vs VD-L-48	-9.049	<0.001
72 h	K1-L-72 vs VD-L-72	-13.18	<0.0000001
	KH-72 vs VD-L-72	-31.32	<0.0000001
	VD-72 vs VD-L-72	-30.67	<0.0000001
120 h	KH-5d vs VD-L-5d	-1.193	<0.0001
	VD-5d vs VD-L-5d	-12.674	<0.0001
	K1-L-5d vs VD-L-5d	-12.676	0.525 (ns)

Phase 3 -Pre-treatment with IFN

Table P3.1. Three-way ANOVA Model Assumption tests (P3)

Assay	Shapiro–Wilk (W)	P value	Levene's test (F)	P value	Conclusion
MTT	0.979	0.451	0.605	0.865	Normality and homogeneity satisfied
NO	0.966	0.118	0.732	0.751	Normality and homogeneity satisfied

Table P3.2. Three-way ANOVA summary (P3)

MTT	Factor	F value	P value	Significant
	Stimulus	23.86	2.13×10^{-5}	***
	Dose	18.50	4.52×10^{-9}	***
	Time	57.54	5.68×10^{-9}	***
	Stimulus × Dose	6.57	0.0037	**
	Stimulus × Time	26.80	8.74×10^{-6}	***
	Dose × Time	1.04	0.408	ns
	Stimulus × Dose × Time	11.61	1.29×10^{-4}	***

NO	Factor	F value	P value	Significant
	Stimulus	408.84	$<2\times10^{-16}$	***
	Dose	20.24	1.10×10^{-9}	***
	Time	770.41	$<2\times10^{-16}$	***
	Stimulus × Dose	18.01	3.45×10^{-6}	***
	Stimulus × Time	401.08	$<2\times10^{-16}$	***
	Dose × Time	15.66	2.84×10^{-8}	***
	Stimulus × Dose × Time	18.44	2.77×10^{-6}	***

Table P3.3. Significant pairwise comparisons (P3)

MTT

Comparison	Significant findings (adjusted P values)
Time	Under IFN-γ stimulation, cell viability was significantly higher at 24 h than at 48 h for Dose 0 ($p = 8.08\times10^{-4}$), 0.01 ($p = 2.43\times10^{-7}$), 0.1 ($p = 9.93\times10^{-4}$), 1 ($p = 1.66\times10^{-5}$) and 10 μg/mL ($p = 0.0160$). Under unstimulated conditions, only 0.01 μg/mL differed significantly ($p = 0.0070$).
Stimulus	IFN-γ significantly affected viability only at 0.01 μg/mL, 24 h ($p = 0.0433$), and at 48 h for 0 μg/mL ($p = 5.32\times10^{-4}$) and 0.01 μg/mL ($p = 2.37\times10^{-8}$).
Dose	Significant dose effects were observed mainly at 24 h. Under IFN-γ stimulation, Dose 0 vs 100 μg/mL ($p = 0.0027$) and Dose 0.01 vs 100 μg/mL ($p = 0.0017$) were significant. Under unstimulated conditions, Dose 0 vs 0.01 ($p = 9.86\times10^{-4}$), Dose 0 vs 100 ($p = 2.94\times10^{-7}$), Dose 0.01 vs 100 ($p = 0.0288$) at 24 h, and Dose 0 vs 100 ($p = 2.05\times10^{-6}$) and Dose 0.01 vs 100 ($p = 1.85\times10^{-7}$) at 48 h were significant.

NO

Comparison	Significant findings (adjusted P values)
Time	Under IFN-γ stimulation, NO production was significantly higher at 48 h than at 24 h for Dose 0 ($p = 2.98\times10^{-20}$), 0.01 ($p = 1.08\times10^{-20}$), 0.1 ($p = 2.56\times10^{-18}$), 1 ($p = 6.61\times10^{-10}$), 10 ($p = 5.77\times10^{-16}$) and 100 μg/mL ($p = 5.54\times10^{-10}$).
Stimulus	IFN-γ significantly increased NO production only at 48 h, including Dose 0 ($p = 3.21\times10^{-21}$), 0.01 ($p = 1.51\times10^{-20}$) and 100 μg/mL ($p = 1.30\times10^{-10}$).
Dose	Significant dose-dependent differences occurred exclusively under IFN-γ stimulation at 48 h. Compared with Dose 0, NO production differed at Dose 0.1 ($p = 0.0088$), 1 ($p = 9.63\times10^{-14}$), 10 ($p = 1.33\times10^{-7}$) and 100 μg/mL ($p = 9.39\times10^{-12}$). Additional significant differences included Dose 0.01 vs 1 ($p = 1.52\times10^{-11}$), 0.01 vs 10 ($p = 1.26\times10^{-5}$), 0.01 vs 100 ($p = 5.47\times10^{-10}$), 0.1 vs 1 ($p = 7.23\times10^{-9}$), 0.1 vs 10 ($p = 0.0088$), 0.1 vs 100 ($p = 3.58\times10^{-7}$), 1 vs 10 ($p = 5.33\times10^{-4}$) and 10 vs 100 μg/mL ($p = 0.0208$).

Table P3.4. Two-way ANOVA at each time point (P3)

Assay	Time	Effect	df	F	P	Sig.
MTT	24 h	Stimulus	1	0.049	0.827	ns
		Dose	5	13.898	<0.001	***
		Stimulus × Dose	2	4.988	0.0189	*
	48 h	Stimulus	1	44.613	<0.001	***
		Dose	5	6.623	0.0012	**
		Stimulus × Dose	2	12.214	<0.001	***
NO	24 h	Stimulus	1	0.023	0.88	ns
		Dose	5	1.859	0.149	ns
		Stimulus × Dose	2	0.8	0.464	ns
	48 h	Stimulus	1	688.77	<0.001	***
		Dose	5	30.32	<0.001	***
		Stimulus × Dose	2	32.38	<0.001	***

Table P3.5. Significant Dunnett comparisons (reference: Vehicle + IFN-γ) (P3)

	Time	Comparison	Estimate	t ratio	Adjusted P	Significance
MTT	24 h	K1	–0.1319	–4.426	0.0021	**
		K2	–0.0610	–2.048	0.256	ns
		K3	–0.0391	–1.313	0.687	ns
		K4	–0.0424	–1.424	0.612	ns
		K5	0.0054	0.181	1	ns
		KH	–0.1477	–4.955	<0.001	***
		KL	–0.0617	–2.071	0.246	ns
		Vehicle	0.0649	2.178	0.206	ns
MTT	48 h	K1	–0.0601	–1.760	0.398	ns
		K2	–0.0248	–0.727	0.973	ns
		K3	–0.0811	–2.376	0.146	ns
		K4	–0.0401	–1.175	0.778	ns
		K5	–0.0806	–2.362	0.15	ns
		KH	–0.0704	–2.062	0.25	ns
		KL	0.1471	4.311	0.0028	**
		Vehicle	0.1219	3.571	0.0134	*
NO	48 h	K1	−0.0538	−10.08	<0.001	***
		K2	−0.0369	−6.92	<0.001	***
		K3	−0.0603	−11.30	<0.001	***
		K4	−0.0185	−3.46	0.0168	*
		K5	−0.0074	−1.39	0.633	ns
		KH	−0.0979	−18.35	<0.001	***
		KL	−0.1018	−19.08	<0.001	***
		Vehicle	−0.0988	−18.52	<0.001	***

MTT (24 h)

Reference: VD-I-24

Comparison	Estimate	Adjusted P	Significant
K1	−0.132	0.0021	**
KH	−0.148	<0.001	***

All remaining groups: ns.

MTT (48 h)

Reference: VD-I-48

Comparison	Estimate	Adjusted P	Significant
KL	+0.147	0.0028	**
Vehicle (VD)	+0.122	0.013	*

All remaining groups: ns.

NO (24 h)

Two-way ANOVA: ns.

All groups: ns.

NO (48 h)

Reference: VD-I-48

Comparison	Estimate	Adjusted P	Significant
K1	−0.0538	<0.001	***
K2	−0.0369	<0.001	***
K3	−0.0603	<0.001	***
K4	−0.0185	0.0168	*
K5	ns	0.633	ns
KH	−0.0979	<0.001	***
KL	−0.1018	<0.001	***
Vehicle	−0.0988	<0.001	***

Phase 4 -Mixed Model

Table P4.1. Assumption Tests and Four-way ANOVA (P4-MTT)

Analysis Category	Description	df	Statistic (W/F)	P value	Significance	Interpretation
Four-way ANOVA	Stimulus	3	45.349	<0.0001	***	Highly significant
	Dose	1	6.133	0.015	*	Significant interaction
	Time	1	0.191	0.6631	ns	Not significant
	Treatment	2	21.313	<0.0001	***	Highly significant
	Stimulus × Dose	3	13.337	<0.0001	***	Highly significant
	Stimulus × Time	3	10.402	<0.0001	***	Highly significant
	Dose × Time	1	2.137	0.1471	ns	Not significant
	Stimulus × Treatment	6	4.797	0.0003	***	Highly significant
	Dose × Treatment	2	0.662	0.518	ns	Not significant

	Time × Treatment	2	23.625	<0.0001	***	Highly significant
	Stimulus × Dose × Time	3	0.619	0.6043	ns	Not significant
	Stimulus × Dose × Treatment	6	3.797	0.0019	**	Significant interaction
	Stimulus × Time × Treatment	6	1.698	0.1297	ns	Not significant
	Dose × Time × Treatment	2	0.058	0.9434	ns	Not significant
	Stimulus × Dose × Time × Treatment	6	1.567	0.1652	ns	Not significant
Assumption Testing	Shapiro–Wilk test (raw residuals)		W = 0.7965	7.239×10^{-13}	***	Significant deviation from normality
	Levene's test (overall interaction groups)		F = 1.0828	0.3650	ns	Homogeneity of variance satisfied
	Levene's test (individual groups)		F = 1.0925	0.3541	ns	Homogeneity of variance satisfied

Table P4.2.Three-way ANOVA Results Stratified by Stimulus Group (P4-MTT)

Stimulus	Source of variation	F value	P value	Significance
LPS	Dose	16.243	0.00049	***
	Time	52.651	<0.0001	***
	Treatment	37.813	<0.0001	***
	Dose × Time	0.069	0.795	ns
	Dose × Treatment	12.987	0.00015	***
	Time × Treatment	12.953	0.00015	***
	Dose × Time × Treatment	6.418	0.00585	**
IFN-γ	Dose	0.2	0.659	ns
	Time	3.013	0.095	ns
	Treatment	16.131	<0.0001	***
	Dose × Time	0	0.994	ns

	Dose × Treatment	6.279	0.0064	**
	Time × Treatment	5.798	0.0088	**
	Dose × Time × Treatment	1.61	0.221	ns
LPS + IFN-γ	Dose	0.248	0.623	ns
	Time	2.144	0.156	ns
	Treatment	9.26	0.0011	**
	Dose × Time	7.009	0.0141	*
	Dose × Treatment	6.342	0.0062	**
	Time × Treatment	23.495	<0.0001	***
	Dose × Time × Treatment	0.005	0.995	ns

Stimulus Group	Outcome Measure	Factor / Interaction	P-value
LPS	Corrected Absorbance	Dose	0.000488
		Time	1.70×10^{-7}
		Treatment	3.82×10^{-8}
		Dose × Treatment	0.000150
		Time × Treatment	0.000153
	Viability	Dose	0.000151
		Treatment	0.000526
		Dose × Treatment	0.000527
IFN-γ	Corrected Absorbance	Treatment	3.63×10^{-5}
		Dose × Treatment	0.00641
		Time × Treatment	0.00883
	Viability	Treatment	0.0199
		Dose × Treatment	0.0199
LI (LPS + IFN-γ)	Corrected Absorbance	Treatment	0.00105
		Dose × Time	0.01410
		Dose × Treatment	0.00615
		Time × Treatment	2.23×10^{-6}
	Viability	Time	0.02145
		Treatment	0.00188
		Dose × Time	0.02132
		Dose × Treatment	0.00188

Table P4.3.Assumption Tests After Stratification by Stimulus Group (P4-MTT)

Stimulus Group	Outcome Measure	Test	Statistic	P-value	Conclusion
LPS	Corrected Absorbance	Shapiro–Wilk	W = 0.9836	0.8599	Normal distribution
		Levene's test	F = 0.6294	0.7864	Homogeneous variance
	Viability	Shapiro–Wilk	W = 0.9817	0.8007	Normal distribution
		Levene's test	F = 0.6294	0.7864	Homogeneous variance
IFN-γ	Corrected Absorbance	Shapiro–Wilk	W = 0.9614	0.2365	Normal distribution
		Levene's test	F = 0.3666	0.9571	Homogeneous variance
	Viability	Shapiro–Wilk	W = 0.9547	0.1469	Normal distribution
		Levene's test	F = 0.3666	0.9571	Homogeneous variance
LI (LPS + IFN-γ)	Corrected Absorbance	Shapiro–Wilk	W = 0.9798	0.7379	Normal distribution
		Levene's test	F = 0.7067	0.7205	Homogeneous variance
	Viability	Shapiro–Wilk	W = 0.9846	0.8862	Normal distribution
		Levene's test	F = 0.7067	0.7205	Homogeneous variance

Table P4.4.Post-hoc Multiple Comparisons (P4-MTT)

Stimulus	Effect	Comparison	Estimate	t	P	Sig.
LPS (MTT absorbance)	Dose	Pre, 24 h, 0 vs 100	−0.2141	−6.514	<0.0001	***
LPS (MTT absorbance)	Dose	Pre, 48 h, 0 vs 100	−0.0855	−2.602	0.0156	*
LPS (MTT absorbance)	Time	Dose 0, Co, 24 vs 48 h	0.0826	2.514	0.0191	*
LPS (MTT absorbance)	Time	Dose 0, Post, 24 vs 48 h	0.0909	2.764	0.0108	*
LPS (MTT absorbance)	Time	Dose 0, Pre, 24 vs 48 h	0.1292	3.931	0.0006	***
LPS (MTT absorbance)	Time	Dose 100, Pre, 24 vs 48 h	0.2578	7.843	<0.0001	***
LPS (MTT absorbance)	Treatment	Dose 0, 24 h, Co vs Post	0.1295	3.941	0.0017	**
LPS (MTT absorbance)	Treatment	Dose 100, 24 h, Co vs Post	0.0977	2.971	0.0176	*
LPS (MTT absorbance)	Treatment	Dose 100, 24 h, Pre vs Co	0.2107	6.41	<0.0001	***
LPS (MTT absorbance)	Treatment	Dose 100, 24 h, Pre vs Post	0.3084	9.381	<0.0001	***
LPS (MTT absorbance)	Treatment	Dose 0, 48 h, Co vs Post	0.1378	4.191	0.0009	***
LPS (MTT absorbance)	Treatment	Dose 0, 48 h, Co vs Pre	0.0972	2.956	0.0182	*
LPS (MTT absorbance)	Treatment	Dose 100, 48 h, Co vs Post	0.1223	3.72	0.003	**
LPS (Cell viability)	Dose	Post, 48 h, 0 vs 100	−0.3504	−2.721	0.0119	*

LPS (Cell viability)	Dose	Pre, 24 h, 0 vs 100	−0.6766	−5.255	<0.0001	***
LPS (Cell viability)	Dose	Pre, 48 h, 0 vs 100	−0.4567	−3.547	0.0016	**
LPS (Cell viability)	Time	Dose 100, Post, 24 vs 48 h	−0.4149	−3.223	0.0036	**
LPS (Cell viability)	Treatment	Dose 100, 24 h, Pre vs Co	0.8051	6.253	<0.0001	***
LPS (Cell viability)	Treatment	Dose 100, 24 h, Pre vs Post	0.7411	5.756	<0.0001	***
LPS (Cell viability)	Treatment	Dose 100, 48 h, Pre vs Co	0.3306	2.568	0.0431	*
IFN-γ (MTT absorbance)	Dose	Co, 48 h, 0 vs 100	0.1208	2.668	0.0135	*
IFN-γ (MTT absorbance)	Time	Dose 0, Co, 24 vs 48 h	−0.1247	−2.754	0.0111	*
IFN-γ (MTT absorbance)	Treatment	Dose 0, 24 h, Co vs Post	0.1172	2.587	0.0413	*
IFN-γ (MTT absorbance)	Treatment	Dose 100, 24 h, Pre vs Co	0.1261	2.783	0.0269	*
IFN-γ (MTT absorbance)	Treatment	Dose 100, 24 h, Pre vs Post	0.1968	4.345	0.0006	***
IFN-γ (MTT absorbance)	Treatment	Dose 0, 48 h, Co vs Post	0.2444	5.395	<0.0001	***
IFN-γ (MTT absorbance)	Treatment	Dose 0, 48 h, Co vs Pre	0.1807	3.99	0.0015	**
IFN-γ (Cell viability)	Dose	-	-	-	ns	ns
IFN-γ (Cell viability)	Time	Dose 100, Post, 24 vs 48 h	−0.3728	−2.783	0.0103	*
IFN-γ (Cell viability)	Treatment	Dose 100, 24 h, Pre vs Co	0.438	3.27	0.0088	**
IFN-γ (Cell viability)	Treatment	Dose 100, 24 h, Pre vs Post	0.3494	2.608	0.0395	*
IFN-γ (Cell viability)	Treatment	Dose 100, 48 h, Post vs Co	0.5011	3.741	0.0028	**
IFN-γ (Cell viability)	Treatment	Dose 100, 48 h, Pre vs Co	0.3443	2.57	0.0428	*
LPS+IFN-γ (MTT absorbance)	Dose	Co, 24 h, 0 vs 100	0.0734	2.564	0.017	*
LPS+IFN-γ (MTT absorbance)	Dose	Pre, 24 h, 0 vs 100	0.0598	2.089	0.0474	*
LPS+IFN-γ (MTT absorbance)	Dose	Post, 48 h, 0 vs 100	−0.0824	−2.879	0.0083	**

LPS+IFN-γ (MTT absorbance)	Time	Dose 100, Post, 24 vs 48 h	−0.0847	−2.958	0.0069	**
LPS+IFN-γ (MTT absorbance)	Time	Dose 0, Pre, 24 vs 48 h	0.1594	5.565	<0.0001	***
LPS+IFN-γ (MTT absorbance)	Time	Dose 100, Pre, 24 vs 48 h	0.0984	3.437	0.0022	**
LPS+IFN-γ (MTT absorbance)	Treatment	Dose 0, 24 h, Co vs Post	0.098	3.423	0.0061	**
LPS+IFN-γ (MTT absorbance)	Treatment	Dose 0, 24 h, Co vs Pre	−0.0968	−3.38	0.0067	**
LPS+IFN-γ (MTT absorbance)	Treatment	Dose 0, 24 h, Pre vs Post	0.1948	6.804	<0.0001	***
LPS+IFN-γ (MTT absorbance)	Treatment	Dose 100, 24 h, Pre vs Co	0.1104	3.855	0.0021	**
LPS+IFN-γ (MTT absorbance)	Treatment	Dose 100, 24 h, Pre vs Post	0.1121	3.913	0.0018	**
LPS+IFN-γ (Cell viability)	Dose	-	-	-	ns	ns
LPS+IFN-γ (Cell viability)	Time	Dose 100, Co, 24 vs 48 h	−0.2627	−2.080	0.0484	*
LPS+IFN-γ (Cell viability)	Time	Dose 100, Post, 24 vs 48 h	−0.3193	−2.528	0.0185	*
LPS+IFN-γ (Cell viability)	Treatment	Dose 100, 24 h, Post vs Co	0.4511	3.571	0.0042	**
LPS+IFN-γ (Cell viability)	Treatment	Dose 100, 24 h, Post vs Pre	0.3279	2.596	0.0406	*
LPS+IFN-γ (Cell viability)	Treatment	Dose 100, 48 h, Post vs Co	0.5077	4.019	0.0014	**
LPS+IFN-γ (Cell viability)	Treatment	Dose 100, 48 h, Post vs Pre	0.4674	3.701	0.0031	**

Table P4.5. Four-way ANOVA Analysis of NO Production (P4-NO)

Factor / Interaction	Degrees of Freedom (Df)	F Value	P Value	Significance
Stimulus	3	1562.447	$<2\times10^{-16}$	***
Dose	1	215.105	$<2\times10^{-16}$	***
Time	1	241.480	$<2\times10^{-16}$	***
Treatment	2	494.627	$<2\times10^{-16}$	***
Stimulus × Dose	3	36.968	5.71×10^{-16}	***

Factor / Interaction	Degrees of Freedom (Df)	F Value	P Value	Significance
Stimulus × Time	3	36.268	9.28×10^{-16}	***
Dose × Time	1	4.502	0.0364	*
Stimulus × Treatment	6	116.204	$<2\times10^{-16}$	***
Dose × Treatment	2	63.151	$<2\times10^{-16}$	***
Time × Treatment	2	52.487	3.97×10^{-16}	***
Stimulus × Dose × Time	3	3.040	0.0327	*
Stimulus × Dose × Treatment	6	8.844	1.12×10^{-7}	***
Stimulus × Time × Treatment	6	12.692	1.69×10^{-10}	***
Dose × Time × Treatment	2	3.206	0.0449	*
Stimulus × Dose × Time × Treatment	6	1.602	0.1550	ns

Table P4.6. Three-way ANOVA Analysis for NO production under different stimulation conditions (P4-NO)

Response	Stimulus	Source of Variation	df	F value	P value	Significance
NO_2^- production	LPS	Dose	1	224.336	<0.001	***
		Time	1	96.152	<0.001	***
		Treatment	2	308.176	<0.001	***
		Dose × Time	1	11.286	0.003	**
		Dose × Treatment	2	69.449	<0.001	***
		Time × Treatment	2	9.618	<0.001	***
		Dose × Time × Treatment	2	9.075	0.001	**
	IFN-γ	Dose	1	21.937	<0.001	***
		Time	1	229.178	<0.001	***
		Treatment	2	75.247	<0.001	***
		Dose × Time	1	5.616	0.026	*
		Dose × Treatment	2	7.812	0.002	**
		Time × Treatment	2	69.605	<0.001	***
		Dose × Time × Treatment	2	0.918	0.413	ns
	LPS + IFN-γ	Dose	1	74.107	<0.001	***
		Time	1	20.681	<0.001	***
		Treatment	2	251.5	<0.001	***
		Dose × Time	1	0.39	0.538	ns
		Dose × Treatment	2	17.452	<0.001	***
		Time × Treatment	2	4.455	0.023	*
		Dose × Time × Treatment	2	0.587	0.564	ns
Inhibition	LPS	Dose	1	16.487	<0.001	***
		Treatment	2	5.282	0.013	*
		Dose × Treatment	2	5.282	0.013	*
	IFN-γ	No significant effects	–	–	>0.05	ns
	LPS + IFN-γ	Dose	1	46.014	<0.001	***
		Treatment	2	3.631	0.042	*
		Dose × Treatment	2	3.631	0.042	*

Table P4.7. Assumption Tests: Normality and Homogeneity of Variance (P4-NO)

Experimental Group	Parameter	Shapiro–Wilk Normality Test	Levene's Test for Homogeneity of Variance	Conclusion
Overall analysis	NO_2 concentration	W=0.8958; P=1.306×10^{-8}	F=1.1184; P=0.3176	Residuals were non-normal, but variances were homogeneous
LPS group	NO_2 concentration	W=0.99577; P=1	F=0.504; P=0.8818	Normal residual distribution and equal variances
LPS group	Inhibition rate	W=0.84386; P=0.0001379	F=1.1623; P=0.3176	Residuals were non-normal, but variances were homogeneous
IFN-γ group	NO_2 concentration	W=0.96009; P=0.2163	F=0.6646; P=0.7568	Normal residual distribution and equal variances
IFN-γ group	Inhibition rate	W=0.71292; P=4.543×10^{-7}	F=1.0108; P=0.4662	Residuals were non-normal, but variances were homogeneous
LPS + IFN-γ group	NO_2 concentration	W=0.90178; P=0.003818	F=1.1413; P=0.3749	Residuals were non-normal, but variances were homogeneous
LPS + IFN-γ group	Inhibition rate	W=0.97222; P=0.4893	F=1.1413; P=0.3749	Normal residual distribution and equal variances

Table P4.8. Significant post hoc comparisons (P4-NO)

Stimulus	Effect	Comparison	Estimate	t	P	Sig.
LPS	Dose	Co, 24 h, 0 vs 100	10.496	7.757	<0.001	***
LPS	Dose	Co, 48 h, 0 vs 100	20.862	15.418	<0.001	***
LPS	Dose	Pre, 24 h, 0 vs 100	9.049	6.688	<0.001	***
LPS	Dose	Pre, 48 h, 0 vs 100	9.568	7.071	<0.001	***
LPS	Time	Dose0, Co, 24 vs 48 h	-13.089	-9.674	<0.001	***
LPS	Time	Dose0, Pre, 24 vs 48 h	-6.469	-4.781	<0.001	***
LPS	Time	Dose100, Pre, 24 vs 48 h	-5.951	-4.398	0.0002	***
LPS	Treatment	Dose0, 24 h, Co vs Post	18.528	13.693	<0.001	***
LPS	Treatment	Dose0, 24 h, Co vs Pre	3.518	2.6	0.04	*
LPS	Treatment	Dose0, 24 h, Post vs Pre	-15.01	-11.093	<0.001	***
LPS	Treatment	Dose100, 24 h, Co vs Post	7.741	5.721	<0.001	***
LPS	Treatment	Dose100, 24 h, Post vs Pre	-5.669	-4.19	0.001	**
LPS	Treatment	Dose0, 48 h, Co vs Post	29.359	21.698	<0.001	***

LPS	Treatment	Dose0, 48 h, Co vs Pre	10.138	7.493	<0.001	***
LPS	Treatment	Dose0, 48 h, Post vs Pre	-19.221	-14.205	<0.001	***
LPS	Treatment	Dose100, 48 h, Co vs Post	8.456	6.249	<0.001	***
LPS	Treatment	Dose100, 48 h, Post vs Pre	-9.611	-7.103	<0.001	***
LPS (Inhibition)	Dose	Co, 24 h, 0 vs 100	-0.452	-2.521	0.019	*
LPS (Inhibition)	Dose	Co, 48 h, 0 vs 100	-0.575	-3.204	0.004	**
LPS (Inhibition)	Dose	Pre, 24 h, 0 vs 100	-0.46	-2.562	0.017	*
LPS (Inhibition)	Treatment	Dose100, 24 h, Co vs Post	0.515	2.869	0.022	*
LPS (Inhibition)	Treatment	Dose100, 24 h, Post vs Pre	-0.522	-2.91	0.02	*
LPS (Inhibition)	Treatment	Dose100, 48 h, Co vs Post	0.581	3.238	0.01	*
IFN-γ	Dose	Co, 48 h, 0 vs 100	9.829	5.419	<0.001	***
IFN-γ	Dose	Pre, 48 h, 0 vs 100	5.407	2.981	0.007	**
IFN-γ	Time	Dose0, Co, 24 vs 48 h	-24.772	-13.657	<0.001	***
IFN-γ	Time	Dose100, Co, 24 vs 48 h	-18.472	-10.183	<0.001	***
IFN-γ	Time	Dose0, Pre, 24 vs 48 h	-13.049	-7.194	<0.001	***
IFN-γ	Time	Dose100, Pre, 24 vs 48 h	-10.488	-5.782	<0.001	***
IFN-γ	Treatment	Dose0, 48 h, Co vs Post	26.389	14.548	<0.001	***
IFN-γ	Treatment	Dose0, 48 h, Co vs Pre	11.194	6.171	<0.001	***
IFN-γ	Treatment	Dose0, 48 h, Post vs Pre	-15.195	-8.377	<0.001	***
IFN-γ	Treatment	Dose100, 48 h, Co vs Post	16.993	9.368	<0.001	***
IFN-γ	Treatment	Dose100, 48 h, Co vs Pre	6.772	3.733	0.003	**
IFN-γ	Treatment	Dose100, 48 h, Post vs Pre	-10.221	-5.635	<0.001	***
IFN-γ (Inhibition)	Dose,Treatment, Time	-	-	-	ns	ns
LPS+IFN-γ	Dose	Co, 24 h, 0 vs	18.854	7.111	<0.001	***

		100				
LPS+IFN-γ	Dose	Co, 48 h, 0 vs 100	16.52	6.231	<0.001	***
LPS+IFN-γ	Dose	Pre, 24 h, 0 vs 100	9.901	3.735	0.001	***
LPS+IFN-γ	Dose	Pre, 48 h, 0 vs 100	6.296	2.375	0.026	*
LPS+IFN-γ	Time	Dose0, Co, 24 vs 48 h	-7.634	-2.879	0.008	**
LPS+IFN-γ	Time	Dose100, Co, 24 vs 48 h	-9.967	-3.76	0.001	**
LPS+IFN-γ	Time	Dose100, Pre, 24 vs 48 h	-6.877	-2.594	0.016	*
LPS+IFN-γ	Treatment	Dose0, 24 h, Co vs Post	34.34	12.953	<0.001	***
LPS+IFN-γ	Treatment	Dose0, 24 h, Co vs Pre	14.024	5.29	<0.001	***
LPS+IFN-γ	Treatment	Dose0, 24 h, Post vs Pre	-20.316	-7.663	<0.001	***
LPS+IFN-γ	Treatment	Dose100, 24 h, Co vs Post	16.712	6.303	<0.001	***
LPS+IFN-γ	Treatment	Dose100, 24 h, Post vs Pre	-11.64	-4.39	0.001	**
LPS+IFN-γ	Treatment	Dose0, 48 h, Co vs Post	40.141	15.141	<0.001	***
LPS+IFN-γ	Treatment	Dose0, 48 h, Co vs Pre	18.387	6.935	<0.001	***
LPS+IFN-γ	Treatment	Dose0, 48 h, Post vs Pre	-21.755	-8.206	<0.001	***
LPS+IFN-γ	Treatment	Dose100, 48 h, Co vs Post	26.729	10.082	<0.001	***
LPS+IFN-γ	Treatment	Dose100, 48 h, Co vs Pre	8.163	3.079	0.014	*
LPS+IFN-γ	Treatment	Dose100, 48 h, Post vs Pre	-18.567	-7.003	<0.001	***
LPS+IFN-γ (Inhibition)	Dose	Co, 24 h, 0 vs 100	-0.36	-4.713	<0.001	***
LPS+IFN-γ (Inhibition)	Dose	Co, 48 h, 0 vs 100	-0.275	-3.605	0.001	**
LPS+IFN-γ (Inhibition)	Dose	Pre, 24 h, 0 vs 100	-0.258	-3.38	0.003	**
LPS+IFN-γ (Inhibition)	Treatment	Dose100, 24 h, Co vs Post	0.292	3.824	0.002	**
LPS+IFN-γ (Inhibition)	Dose (ignoring Time)	Co, 0 vs 100	-0.318	-6.031	<0.001	***
LPS+IFN-γ (Inhibition)	Dose (ignoring Time)	Post, 0 vs 100	-0.112	-2.13	0.042	*

LPS+IFN-γ (Inhibition)	Dose (ignoring Time)	Pre, 0 vs 100	-0.205	-3.887	<0.001	***
LPS+IFN-γ (Inhibition)	Treatment (ignoring Time)	Dose100, Co vs Post	0.205	3.901	0.001	***

Table P4.9. Significant post hoc comparisons for NO inhibition (P4-NO)

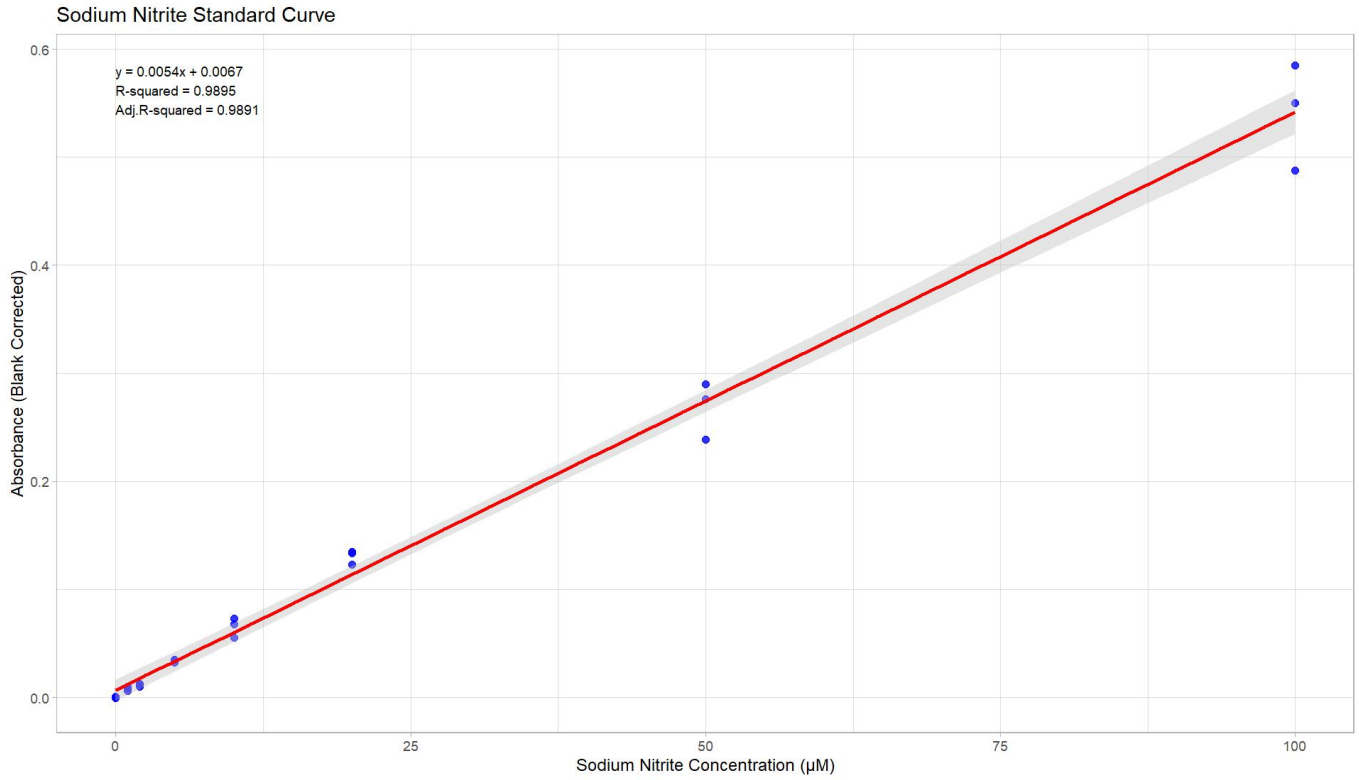
Stimulus	Effect	Comparison	Estimate	t	P
LPS	Dose	Co, 24 h, 0 vs 100	-0.452	-2.521	0.019
LPS	Dose	Co, 48 h, 0 vs 100	-0.575	-3.204	0.004
LPS	Dose	Pre, 24 h, 0 vs 100	-0.460	-2.562	0.017
LPS	Treatment	Dose100, 24 h, Co vs Post	0.515	2.869	0.022
LPS	Treatment	Dose100, 24 h, Post vs Pre	-0.522	-2.910	0.020
LPS	Treatment	Dose100, 48 h, Co vs Post	0.581	3.238	0.010
LPS	Time	-	-	-	ns
IFN-γ	Dose	-	-	-	ns
IFN-γ	Treatment	-	-	-	ns
IFN-γ	Time	-	-	-	ns
LPS+IFN-γ	Dose	Co, 24 h, 0 vs 100	-0.360	-4.713	<0.001
LPS+IFN-γ	Dose	Co, 48 h, 0 vs 100	-0.275	-3.605	0.001
LPS+IFN-γ	Dose	Pre, 24 h, 0 vs 100	-0.258	-3.380	0.003
LPS+IFN-γ	Treatment	Dose100, 24 h, Co vs Post	0.292	3.824	0.002
LPS+IFN-γ	Time	-	-	-	ns

Stimulus	Effect	Significant comparisons for NO inhibition
LPS	Dose	Co 24 h, Co 48 h, Pre 24 h
LPS	Treatment	100 μg/mL: Co > Post (24 h, 48 h); Pre > Post (24 h)
IFN-γ	All	No significant post hoc comparisons
LPS+IFN-γ	Dose	Co 24 h, Co 48 h, Pre 24 h
LPS+IFN-γ	Treatment	100 μg/mL: Co > Post (24 h)

Appendix C. Assay Calibration and Standard Curve Analysis

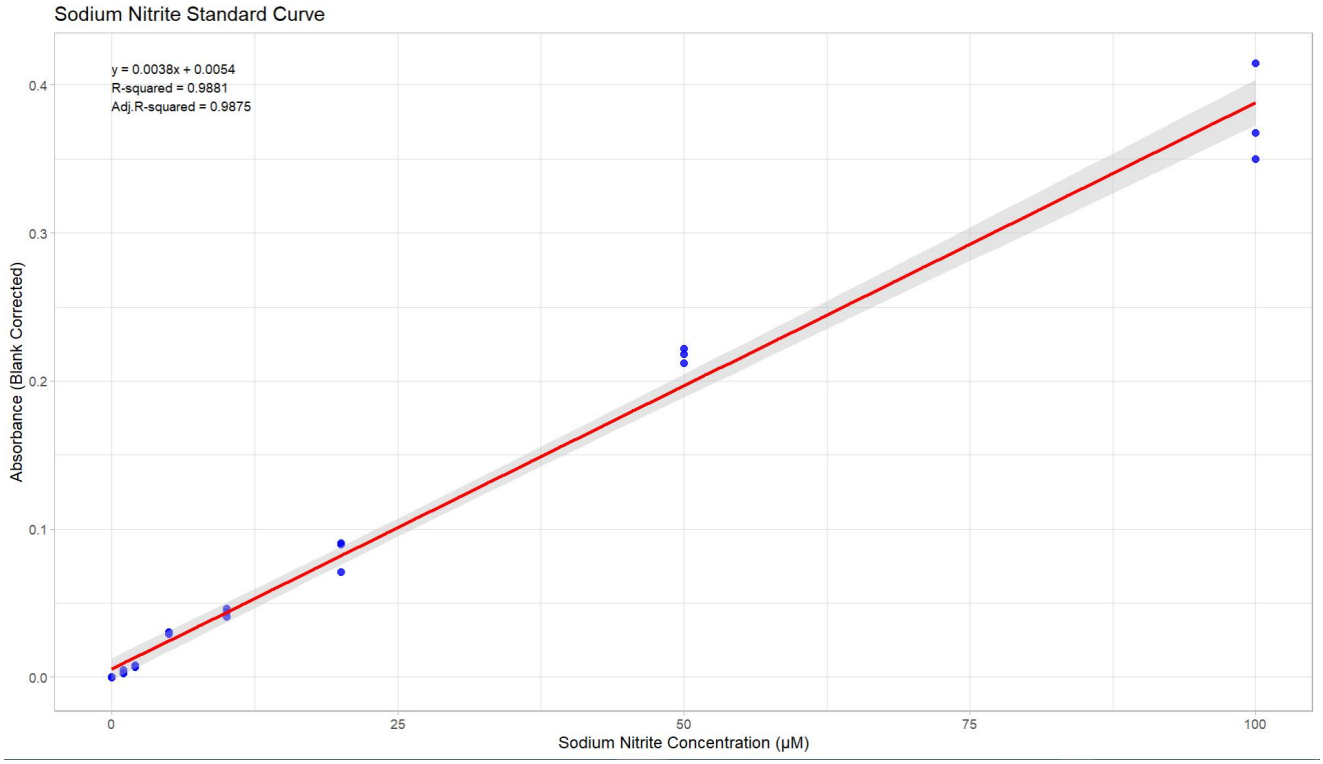
This appendix provides the sodium nitrite standard curves and regression analyses used to support the quantitative measurements obtained from the Griess assays. The included calibration curves demonstrate the relationship between known reference concentrations and measured assay responses, allowing accurate interpolation and quantification of experimental samples. Regression models, including the corresponding equations, goodness-of-fit parameters (such as R^2 values), and statistical outputs, are presented to evaluate the reliability and linearity of the analytical measurements. These supplementary analyses provide validation of the quantitative methods and support the accuracy and reproducibility of the reported experimental results.

Phase 1 Standard Curve



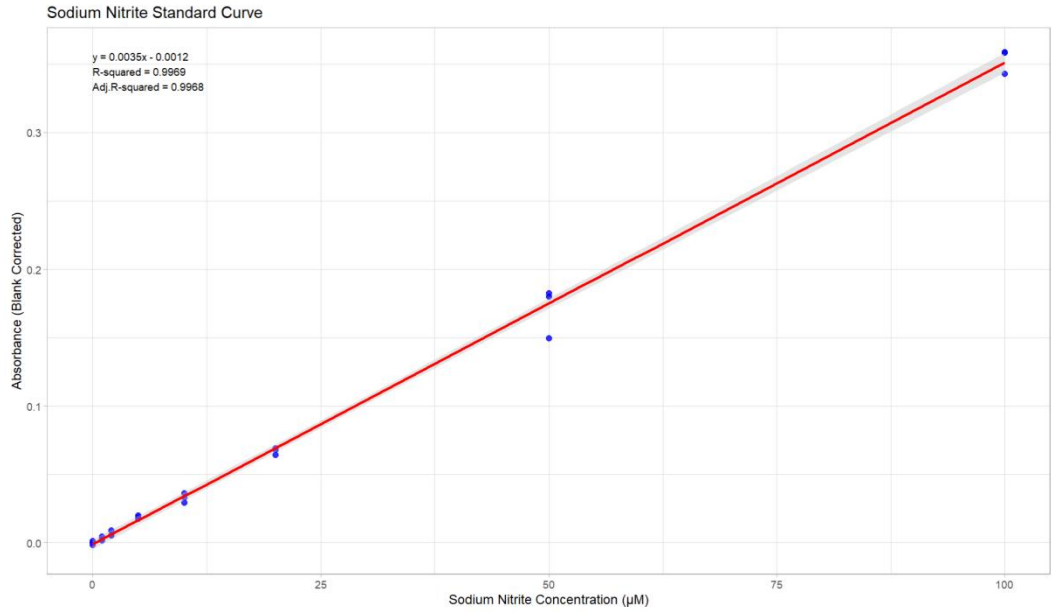
The standard curve was generated using linear regression of corrected assay response against concentration. The fitted regression equation was y = 0.0054x + 0.0067, with a strong coefficient of determination (R^2 = 0.9895; adjusted R^2 = 0.9891). The regression model was highly significant ($F(1,22) = 2082$, $p < 2.2 \times 10^{-16}$), and the concentration term showed a significant positive relationship with assay response ($t = 45.627$, $p < 2 \times 10^{-16}$). Residual analysis identified two potential outliers (observations 23 and 24); however, their influence on model performance was minimal, and the regression demonstrated excellent linearity and suitability for quantitative analysis.

Phase 2 Standard Curve



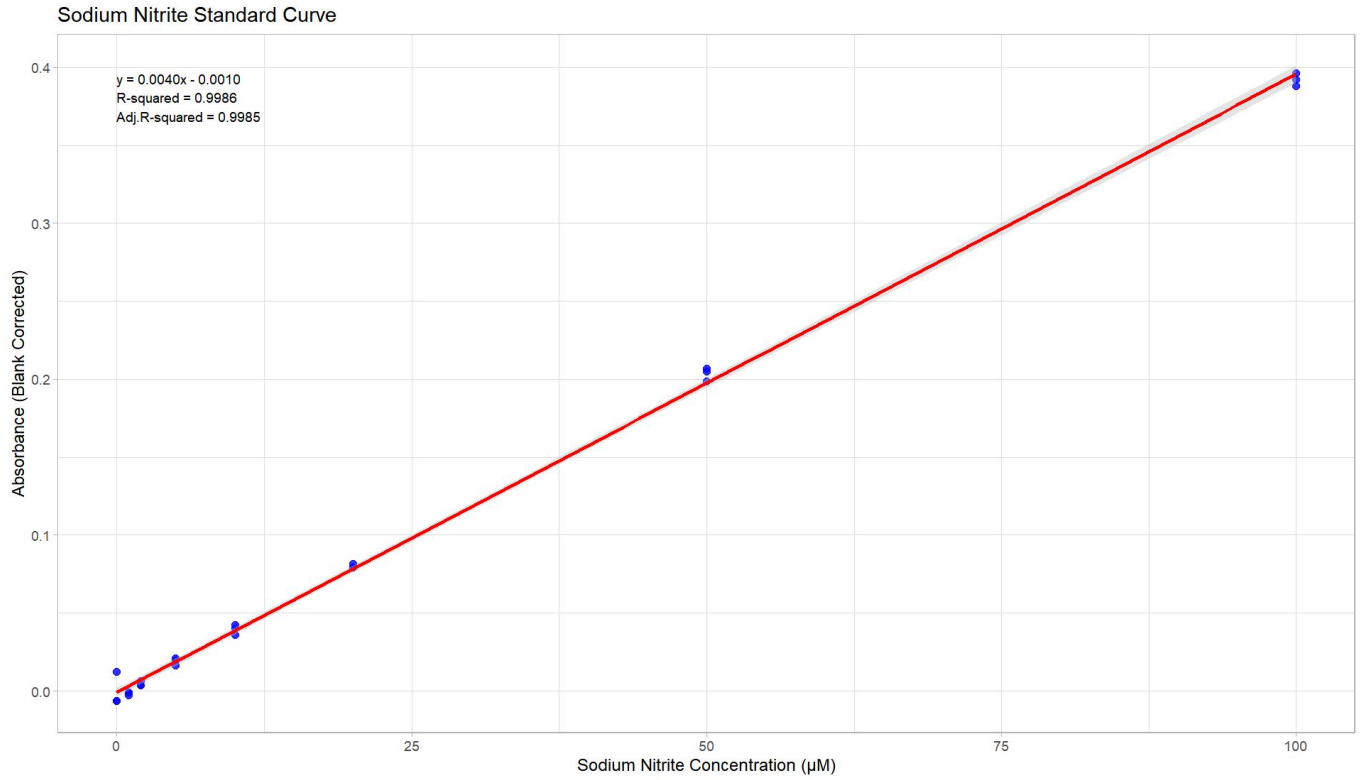
The standard curve was generated using linear regression of corrected assay response against concentration. The fitted equation was y = 0.0038x + 0.0054, with excellent linearity (R^2 = 0.9881; adjusted R^2 = 0.9875). The regression model was highly significant ($F(1,22) = 1822$, $p < 2.2 \times 10^{-16}$), and the concentration term showed a significant positive relationship with assay response ($t = 42.688$, $p < 2 \times 10^{-16}$). The low residual standard error (0.01441) further supports the accuracy and reliability of the calibration curve for quantitative measurements.

Phase 3 Standard Curve



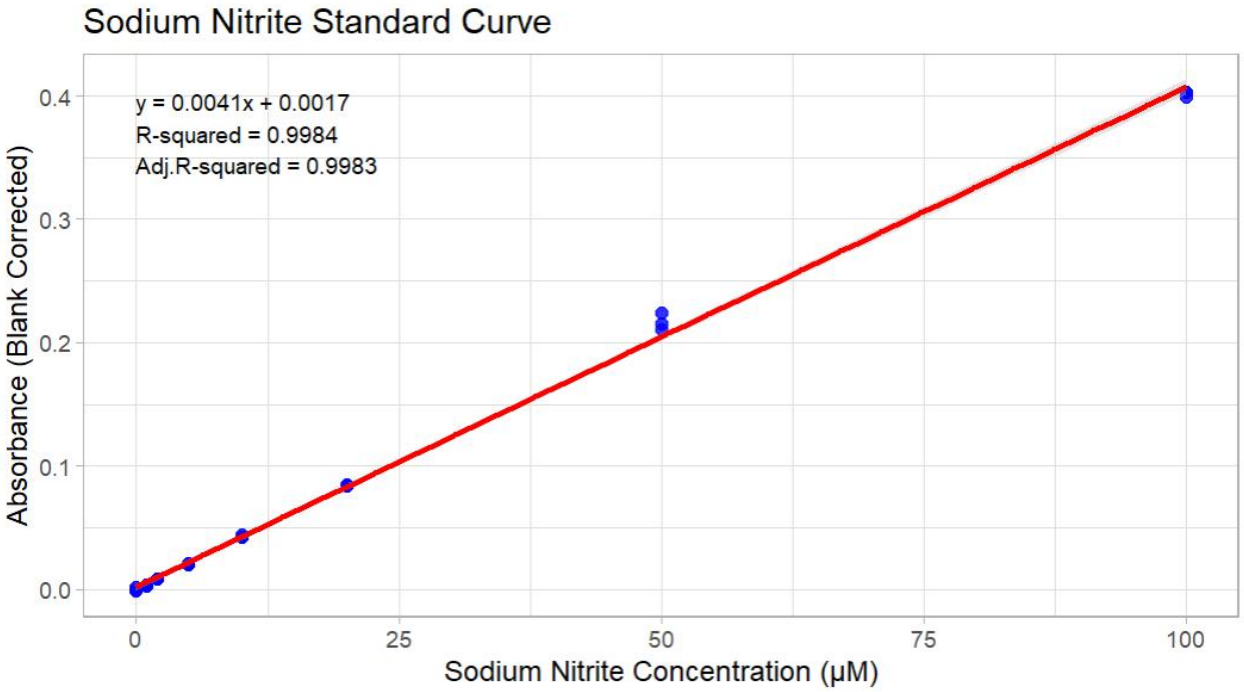
The standard curve was generated using linear regression of corrected assay response against concentration. The fitted regression equation was $y = 0.0035x - 0.0012$, demonstrating excellent linearity ($R^2 = 0.9969$; adjusted $R^2 = 0.9968$). The concentration term showed a highly significant positive relationship with assay response ($t = 84.205$, $p < 2 \times 10^{-16}$), whereas the intercept was not significant ($p = 0.499$). The regression model was highly significant ($F(1,22) = 7090$, $p < 2.2 \times 10^{-16}$), with low residual error (0.006722), supporting the reliability and accuracy of the calibration curve for quantitative measurements.

Phase 4 Standard Curve 1



The standard curve was generated using linear regression of corrected assay response against concentration. The fitted regression equation was $y = 0.0040x - 0.0010$, showing excellent linearity ($R^2 = 0.9986$; adjusted $R^2 = 0.9985$). The concentration term was highly significant ($t = 124.072$, $p < 2 \times 10^{-16}$), while the intercept was not significantly different from zero ($p = 0.426$). The high coefficient of determination and low residual variation demonstrate the accuracy and reliability of the calibration model for quantitative determination.

Phase 4 Standard Curve 2



The standard curve was generated using linear regression of corrected assay response against concentration. The fitted regression equation was $y = 0.0041x + 0.0017$, with excellent linearity ($R^2 = 0.9984$; adjusted $R^2 = 0.9983$). The concentration term was highly significant ($t = 115.706$, $p < 2 \times 10^{-16}$), while the intercept was not significantly different from zero ($p = 0.250$). The regression model showed strong statistical significance ($F(1,22) = 1.339 \times 10^4$, $p < 2.2 \times 10^{-16}$) and low residual variation (residual standard error = 0.005639), supporting the accuracy and reliability of the calibration curve for quantitative measurements.