In vitro cell culture to study microglial inflammation

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Abstract

Inflammation of the central nervous system (CNS) characterised by release of cytokines such as tumour necrosis factor-alpha (TNF-a) and interleukin-1 (IL-1) is an event that is often detrimental to neuronal cells and is thought to contribute to the neuronal loss observed during neurodegeneration.

One objective of this study was to investigate the potential anti-inflammatory properties of kawakawa leaf extracts using *in vitro* model cell systems. Kawakawa leaf extracts anti-inflammatory properties have anecdotally been documented in traditional Maori medicinal practices (Rangoa). However, before this aspect could be carried out, experiments were undertaken to investigate the effects the kawakawa leaf extracts had on the growth rates of two mammalian cell lines (HeLa and THP-1 cells).

Interestingly, the growth assays showed that the extracts to have both a stimulatory and inhibitory effect on cell growth. Toxicity of cells can be categorised as either necrotic or apoptotic cell death. In this study, HeLa cells and THP-1 cells were incubated under standard growth conditions with various concentrations (10 to 100ug/ml) of Kawakawa leave extracts prepared at room temperature or $60^{O}C$.

Growth data as seen in *figure*s 2 and 3 typically have a long lag phase before they start to reach exponential growth between days 4 and 5. The extract having the largest stimulatory effect on HeLa cells as shown by *figure* 6 was 25 ug/ml at $60^{O}C$ and the largest inhibitory effect seen on HeLa cells was seen at 50 ug/ml RT shown by *figure* 6. The extract which has the largest stimulatory effect on THP-1 cells is 25 ug/ml $60^{O}C$ as shown by *figure* 11 whereas the extract producing the largest inhibitory effect is 25 ug/ml RT.

Apoptotic and necrotic cell death mechanisms were examined by using *in vitro* biochemistry techniques. Assessments were largely made based on biochemical changes of HeLa cells and THP-1 cells post treatment of Kawakawa leave extracts.

Measurement of lactate dehydrogenase (LDH assays) in the growth media of cells that were shown to be growth inhibited during exposure to Kawakawa extracts indicated that the growth inhibition resulting from exposure to the extracts was not due to necrotic cell death processes. (ie that cells maintained their membrane integrity) When compared to the control, a lower amount of cytosolic LDH was seen in THP-1 cell lines. However, HeLa cell lines produced a high level of cytosolic LDH which may be due to mycoplasma contamination. On average, Kawakawa concentrations at 12.5ug/ml and 25ug/ml yielded less lysed cells percentage across both cell lines.

The demonstrated cell growth inhibition was unlikely to be due to necrosis and because of this it was decided to see if cell death was caused by mitochondrial processes that would further support apoptotic cell death. The biochemical assay MTT indirectly measures mitochondrial dehydrogenase activity in the presence of kawakawa leave extracts.

The MTT data illustrated mitochondrial impairment where HeLa cells exposed to Kawakawa 60^{O}C concentrations 6.25 ug/ml and 12.5 ug/ml had the lowest average mitochondrial activity. THP-1 cells exposed to Kawakawa 60^{O}C concentrations 12.5 ug/ml and 50 ug/ml had the lowest average mitochondrial activity.

The main results of this study include, the treatment of HeLa and THP-1 cells with kawakawa extracts demonstrated that cytotoxicity was not due to necrosis. The most interesting finding of this study is when kawakawa extracts inhibit cell growth there is

no increase in LDH. This is suggestive that the cellular membrane remains intact when exposed to the extract.

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List of Abbreviations

˚C, degree Celsius, AD, Alzheimer's disease, ATP, Adenosine triphosphate, BBB, Blood Brain Barrier, CAMs, Cell adhesion molecules, CSF, Cerebral spinal fluid, CNS, Central nervous system, CSPGs, Chondroitin sulfate proteoglycans, ECM, extracellular matrix, GFAP, Glial fibrillary acidic protein, IgSF, Immunoglobulin superfamily, IL, Interleukin, LDH, Lactate dehydrogenase, LPS, Lipopolysaccharide, LVD, left ventricular dysfunction, MIP, macrophage inflammatory protein, MMP, Matrix metalloproteinase, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MCP, monocyte chemotactic protein , MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MS, Multiple sclerosis, NTFs, Neurotropic factors, NO, Nitric oxide, NSAIDs, Non-steroidal anti-inflammatory drugs, PAMPs, Pathogen associated molecular patterns, PD, Parkinson's disease, RT, Room temperature, ROs, Reactive oxygen species, SCI, Spinal cord injury, TLRs, Toll-like receptors, TGF, Transforming growth factor, TNF, Tumour necrosis factor, WD, Wallerian degeneration.

1. Literature Review and Research aims

This chapter presents an overview of what central nervous system (CNS) inflammation is and how this could be modulated by specific therapies such as anti-inflammatories. The impact of CNS inflammation on pathology including possible links to neurodegenerative diseases will be examined. Following this, the role primary glial cells play in CNS Inflammation is addressed. Studies having both *in vitro* and *in vivo* elements will be examined to further address primary microglial cells and inflammation. The chapter will then conclude with the intended objectives and aims for this project.

1.1.1 Introduction

Inflammation is a process that involves changes in blood flow through blood vessels, increased capillary permeability and lastly leukocyte exudation. Changes in blood flow and the calibre of blood vessels occur soon after injury and depending on the extent of the injury progress at varying rates. Arterioles are dilated and new capillaries and venular beds are open in the affected area. This opening causes accelerated blood flow that accounts for the classic signals of heat and redness during inflammation. Leakage of protein rich fluid out of small blood vessels and into extravascular fluid is permitted by an increase in the permeability of microcirculation. This leads to the classic inflammation symptom of oedema (Markiewski, 2007).

Leukocytes move to the endothelial lining of small blood vessels in a tightly packed manner. These leukocytes move through endothelial spaces and into extravascular

space. Leukocytes can then move and by a process of chemotaxis are subsequently pulled to the site of the injury (Cathcart, 2009). Neutrophils and macrophages in the area of the injury accumulate and act to neutralize foreign bodies via phagocytosis (Lee, 2003).

In the past, it was believed that the central nervous system (CNS) including the brain was an immune privileged organ. Being an immune privileged organ means that the CNS was not thought to be susceptible to inflammation or immune activation. The CNS was thought to be unaffected by systemic inflammatory and immune responses however, over the span of a decade, this paradigm has been significantly revised (Lucas, 2006). The responses by the brain and CNS to infection and inflammation exhibit different levels of responses when compared to other tissues. The largest difference is seen in the recruitment of leukocyte cells. This is rapid in systemic organs but in the brain, recruitment of leukocytes is slow. In contrast to systemic organs, the brain activates and recruits' microglia cells and releases proinflammatory mediators such as TNFa and IL1-b. The process for inflammation activation typically occurs within hours after CNS trauma (Lucas, 2006).

Inflammation is caused by trauma to the CNS as well as spinal cord injury and is notably one of the primary symptoms. It is important to acknowledge that inflammation of the CNS plays a dual role. Inflammatory reactions are typically associated with causing damage to neurons. The CNS can communicate with the immune system and utilise shared mechanisms and mediators. Immune cells like macrophages on one hand can secrete neuro-destructive molecules such as pro inflammatory cytokines TNFa and IL-1β but these cells can also produce factors which can facilitate neuro-axonal growth, survival and plasticity. This may be a reason for inflammation resulting in injury but also the protection of neurons (Finn, 2013).

The CNS, more specifically the brain demands high levels of energy, it plays an important role for maintaining environmental homeostasis enabling the CNS to handle various metabolic demands. For typical cerebral functions, 20% of the oxygen used by the human body and 25% of the glucose consumed by the human body is required. Though the brain demands so much oxygen and glucose, it makes up a small 2% percent of the total body mass (Belanger, 2011).

The main processes the brain carries out that requires such demanding energy needs include; maintenance and restoration of ion gradients that may have degraded due to signalling processes like postsynaptic and action potentials. Another process that can accounts for high energy demands on the brain is the recycling of neurotransmitters (Belanger, 2011).

The symptoms of inflammation can be subdued by non-steroidal anti-inflammatory drugs (NSAIDs) such as asprin or corticosteroids like cortisone, hydrocortisone and prednisone. However, if left untreated, inflammation can potentially lead to pathology changes resulting in damage to the CNS. The CNS tightly regulates the immune reactions that occur inside the CNS. It does this to protect the brain from events that might be detrimental such as neurotoxicity and axonal damage (Fakhoury, 2015). In recent times, due to the side effects of prolonged treated to NSAIDs, natural remedies such as plant extracts are being looked to for their anti-inflammatory properties (Maroon, 2010).

1.1.2. CNS inflammation

The CNS is characterised by the blood brain barrier (BBB) which is a physical barrier between the CNS and the bloodstream and is composed of endothelial cells. The BBB prevents the majority of molecules from passing through into the CNS. The BBB in the CNS helps to maintain the balance of chemicals in the CNS and it does this by forming a near impermeable layer. The BBB is known to participate in the inflammatory reaction due to the expression of molecules. One class of molecules expressed by the BBB are cytokines. The increase of cytokines such as TNF-α and interleukin-1 beta (IL-1β) during an inflammatory reaction leads to the increased permeability of the BBB (Huber, 2001).

As a result of this permeability, bacteria and viruses can access brain tissue (Fakhoury, 2015). The vulnerable BBB can lead to alterations of the CNS environment which have effects on the ionic and nutritional balance. The more permeable or the larger the opening of the BBB is can lead to the increasing levels of serum protein uptake and edema (Huber, 2001). The permeability of the BBB can be altered by pathological states such as Human immunodeficiency virus-1 encephalitis, multiple sclerosis and hypoxia or glycaemia.

Other molecules that are expressed by the BBB are adhesion molecules, these can be defined as sticky cell surface molecules that have a crucial facilitative role in the communication and intercellular binding of cells. A further molecule that is expressed are serine proteases, these molecules help to regulate local inflammation (Fakhoury, 2015). The BBB limits the exchange of inflammatory cells as well as inflammatory mediators under pathological and physiological conditions (Takeshita, 2012).

CNS inflammation was not thought to associate within the context of SCI; it was thought that inflammation did not occur in the CNS. CNS inflammation is mainly caused by mechanical pressure including; blunt trauma, the introduction of foreign bodies, vibrations and chronic pressure of low intensity (Purves, 2001). Increasing evidence over the past decade has changed this paradigm largely due to the discovery of alternative anti-inflammatory monocytes. Chronic inflammation leads to a change in cell population such as an increase of monocytes.

The integrity of tissue as well as the environmental cues exposed to the cells are supported by the extracellular matrix (ECM). The ECM is a 3 dimensional network composed of secreted molecules such as. The upregulation of the alternative mononuclear (IL-10 producing) phagocytes, instead of the typical pro inflammatory features is seen when tissue is damaged. Chondroitin sulfate proteoglycans (CSPGs) are the predominant extracellular matrix component of the glial scar (Liu et al., 2015).

The glial scar is formed by a process termed reactive astrogliosis. Astrocytes are a CNS cell that respond to trauma to the CNS including infection, physical trauma, ischemia and neurodegenerative diseases. They react by a process called reactive astrogliosis and this involves the changing of molecular expression and morphology and in severe cases, form a scar. The glial scar is made up of multiple cellular components that include: reactive astrocytes, microglial, endothelial cells and fibroblasts as well as a basal membrane this is an extracellular matrix that forms at the centre of the injury (Lucas, 2006).

The glial scar acts as a barrier for re-innervation and isolates the damaged area and therefore it might be an area for therapy to target. It is interesting to note the prevention of glial scar formation increases neuronal loss from the damaged area and can inhibit

the repair of the blood brain barrier (BBB). Axonal re-myelination might also be effected by the prevention of glial scar formation (Lucas, 2006).

Monocyte derived cells acquire matrix degrading enzymes such as MMP13 and the activation of mononuclear phagocytes which produce IL-10 which is dependent on the expression of MMP-13. MMP-13 is a gene on chromosome 11 that codes for the enzyme collagenase 3. It is a member of the matrix metalloproteinase (MMP) family and is involved in the breakdown of extracellular matrices' and tissue remodelling. MMP-13 promotes an environment where axonal regeneration is permitted by degrading the glial scar matrix component, regulating chondroitin sulfate proteoglycan CSPG and promoting glial scar resolution (Page-McCaw, 2009).

These proteoglycans activate microglial cells and macrophages via the CD44 receptor directly. CD44 is a protein that is present on T cells, pre-B cells, monocytes, neutrophils, CNS white matter, fibroblasts, skeletal muscle, and medullary thymocytes (Helmut, 2003).

CD44 is found on chromosome 11q13 where it encodes a cell surface glycoprotein that is involved in cell–cell interactions, cell adhesion and cell migration (Gao, 1997). CD44 is a receptor for hyaluronic acid. This facilitates the secretion of the neurotrophic factors (NTFs) by cells during the first two days of injury. NTFs are a family of biomolecules consisting of mainly proteins or peptides that support the growth, survival and differentiation of both mature and developing neurons (Razavi, 2015).

The interaction of the glial scar formation and the neuro-inflammatory response after SCI is illustrated by current studies and elude to the possibility of controlling neuro-inflammation by manipulating glial scarring (Liu et al., 2015).

Mononuclear cells like monocytes were thought to have the primary role to sense their environment and to replenish tissue macrophages and dendritic cells. Typically, inflammatory monocytes are equipped with a set of Toll-like receptors (TLRs) as well as scavenger receptors. The TLRs recognize pathogen associated molecular patterns (PAMPS) and can remove microorganisms, lipids and dying cells via phagocytosis (Mogensen, 2009).

Inflammatory monocytes produce effector molecules such as cytokines, myeloperoxidase and superoxide which in turn initiates inflammation. When the immune system of the host is healthy and is not susceptible to infection or inflammatory stimuli known as a 'steady state' (Jiyeon Yang, 2014).

Pro-inflammatory mediators like cytokines, reactive oxygen species, and eicosanoids have been shown to increase the levels of cell adhesion molecules (CAMs) including platelet endothelial cell adhesion molecules. CAMs can be divided into four main protein families Ig (immunoglobulin) superfamily (IgSF CAMs), the integrins, the cadherins, and the selectins. In turn, this increase in cell adhesion molecules, increases adhesion between leukocytes and endothelium. Therefore, the CAM-inducing activity of proinflammatory cytokines like TNFa and IL-1 beta play an important role in inflammation. An over activation of CAMs is seen in a number of chronic and acute inflammatory conditions and has opened up another avenue for treatment of inflammation (Figarella-Branger, 2003).

The inflammatory response is similar to the expression of the cytoskeletal protein Glial fibrillary acidic protein (GFAP). Inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) are released by both microglia and

macrophages. Once secreted, these inflammatory cytokines can activate astrocytes which in turn release more inflammatory cytokines (Brahmachari, 2006).

Appropriate levels of inflammatory responses can aid in protecting the body, however, it is important to control the inflammatory response to mitigate the severity of the consequences of CNS and neuro- inflammation. The transforming growth factor (TGF)-β family help to regulate various biological responses including cell growth and differentiation (Nagaraj, 2010).

An important function of TGF-β is the deposition of extracellular matrix (ECM) components. Astrocytes can be affected by TGF-β which can lead to the secretion of the inhibitory ECM chondroitin sulfate proteoglycans (CSPG). In this case, the axons will stop extending downward once they reach the CSPG rich regions of the CNS. To aid in suppressing the consequences of CNS and neuro inflammation the controlling of the ECM secretion is seen as an important factor (Raposo, 2014).

Another important aspect in the process of inflammation includes activation of T lymphocytes. Activated T lymphocytes are seen in processes such as, inflammation, allergic reactions and autoimmune diseases. Interleukin – 2 is the main lymphokine involved in this process. Interleukin-2 lymphokines are involved in multiple intracellular signalling pathways that allow proliferation of activated T lymphocytes (Alberts, 2002). Macrophages are also a key player in the host defence, immunity and inflammatory responses. Macrophages are responsible for producing mediators including; cytokines, oxygen and nitrogen species and high levels of prostaglandin E2 upon induction of cyclo-oxygenase -2 (COX-2) (Bianchini, 2007; Duque, 2014).

Inflammation has been investigated in laboratory settings and this has led to the close examination of cascading events involved in the inflammatory process. This

highlighted not only the pathological process resulting from factors applied but also it should be noted that there is a natural anti-inflammatory process that is active after inflammation has reached a peak (Purves, 2001).

A major consequence of excessive amounts of inflammatory response is a contributor to secondary spinal cord injury (SCI). This includes the process of gliosis involving major cell types like microglia, oligodendrocyte precursors, meningeal cells, astrocytes and stem cells. These types of cells involved in gliosis are known to be activated by damage to the central nervous system (CNS). This causes a glial reaction resulting in the formation of a glial scar (Fawcett, 1999).

When the glial scar forms, it brings about an environment that does not allow axons to regenerate and could also make cellular re-myelination unsuccessful. When damage to the CNS occurs, it contains oligodendrocytes and myelin debris and cells involved in gliosis have been shown to inhibit axon regeneration (Fawcett, 1999).

There are other consequences of CNS inflammation including chronic psychiatric disorders and various neurodegenerative disorders (Lucas, 2006).

An interesting aspect of CNS inflammation that relates to neuronal regeneration is the area of mitochondrial energy. Neuronal regeneration is a process seen to require a large amount of energy. It has been recently eluded to that when axons mature mitochondrial transport declines. In mature neurons, 20-30% of mitochondrial in the neurons are motile. This enables the mitochondrion to temporally supply ATP to presynaptic terminals which alters synaptic energy levels and has influences over ATP-dependent synaptic activities (Lin, 2015).

A correlation between energy requirement and neuronal regeneration has recently been examined to investigate if enhanced metabolic requirements during regeneration

is needed. Recently, a study reveals that as neurons mature, more energy goes into controlling regrowth in injured neurons as opposed to free moving mitochondria (Zhou, 2016).

Axotomy can be defined as cutting or severing an axon this denervation is applied to the physiology of neurons and to examine neuronal death and survival. It can also be used to induce acute mitochondrial depolarisation as well as ATP depletion. As an effect of this, mature neuron-association increases in the protein syntaphilin (SNPD). This is an axonal specific mitochondria-anchoring protein that anchors mitochondria to microtubules found in the cytoskeleton. Mitochondria contain two membranes, an inner membrane and the outer membrane which form the cristae. The presence of mitochondria is dependent on the oxidative phosphorylation of the tissue. Neurons as well as cardiac and skeletal muscle have an abundance of mitochondria which leaves them vulnerable to energy dependant defects that result from mitochondrial abnormalities (Zhou, 2016).

The enhancement of mitochondrial transport via genetic manipulation is seen to increase the regenerative capacity. This in turn replaces unhealthy mitochondria or mitochondria that might have mutations that impair mitochondrial respiratory chain with healthy mitochondria or mitochondria that do not have mutations impairing mitochondrial respiratory chain resulting in increased cellular energy. Mitochondria *In vivo* studies involving sciatic nerve crushes and knock out mice have been demonstrated to have accelerated axon regeneration (Zhou, 2016) .

1.1.2.1 Inflammation of the central nervous system (CNS) and its impact on pathology

There appears to be two outcomes of CNS inflammation it can either be resolved or remain unresolved. The resolution of inflammation involves the removal or reduction of leukocytes and debris from local inflamed areas. The resolution process is initiated after acute challenges by cellular pathways that biosynthesis dual acting anti-inflammatory and pro resolution molecules such as lipoxins, resolvins and protectins. Inflammation must be resolved in order to mitigate the spreading of inflammation resulting in chronic inflammation or inflammation that causes disease (Serhan, 2008).

This section will elude to CNS pathology changes that are caused or catalysed by the unresolved inflammation of the CNS. Neuro diseases can be divided into two sub-categories, acute CNS diseases and chronic CNS diseases.

The role inflammation of the CNS after trauma plays is still under large debate. It has been reported that inflammation is detrimental to CNS neurons while on the other hand, the same inflammation has been reported to play a positive role that promotes CNS regeneration (Mietto, 2015). Cytokines such as TNFa and IL-1 released by CNS inflammation are largely involved in neuronal loss during acute and chronic neurodegenerative disease. However, these cytokines may have a dual role as they are also seen to promote repair and recovery.

Within a few hours following trauma to the CNS a secondary cascade of events takes place. This is defined by normal blood flow interruption causing poor oxygenation leading to haemorrhage, secondary spinal cord injury complications also include edema (swelling) and inflammation. The release of extracellular matrix molecules

including both pro and anti-regenerative factors are also harmful to the spinal cord, another identifier of secondary complications to spinal cord injuries is the activation of programmed cell death known as apoptosis (Mietto, 2015).

Microglial cells are the first in response to various neuro-pathologies such as; Ischemia, chronic pain, Alzheimers disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, Huntingtons disease and brain tumours. Because of such involvement in various neuro-pathologies, it has become an area of interest to study microglial mechanisms (Fakhoury, 2015).

Affected neurons in neurodegenerative diseases are found to share pathological signs that are normally found to be in axons that have had traumatic injury. The pathophysiology of spinal cord injuries (SCI) includes an initial evet that causes neuronal cell death and interrupts axonal connections. The neuron is broken down into two areas, the distal and proximal stumps. The distal stump of the long axon undergoes a degenerative process wallerian degeneration (WD) and the proximal stump is known to retract causing the surviving cell bodies to be more susceptible to subsequent secondary events (Mietto, 2015).

WD is defined as a multi-complex phenomenon that is found to occur within the distal portion of injured nerves. Distal nerves change in morphology after being cut. WD was a term that originally describes axotomized peripheral nerves. WD is known to occur in both central and peripheral axons that have become separated from their parental cell body. WD can be triggered by traumatic injury but it also occurs in various neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (Mietto, 2015).

There is increasing evidence that neurodegenerative diseases like multiple sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD) are found to have high associations with immune system activation and neuro-inflammation. A variety of immunological cells are expressed during neurodegeneration (Fakhoury, 2015).

Cytokines such as TNFa and IL-1B are secreted by macrophages and have the crucial role of repairing processes in the CNS by getting rid of pathogens and they help to reduce the severity of tissue damage. Macrophages eliminate pathogens by the process phagocytosis, the engulfing of bacteria or other material by phagocytes (Lee, 2003).

Microglia and astrocytes also participate in the active immune defence. The cells play a crucial part in regulating tissue homeostasis and also help to preserve the structures and functions characteristic of the brain (Fakhoury, 2015). Chronic activation of microglia (this will be addressed further on) has been seen to cause neuronal damage due to its direct participation in releasing cytotoxic molecules such as reactive oxygen species (ROS), nitric oxide (NO), and a variety of proinflammatory cytokines such as TNFa and IL-1B. Activated microglial produce several chemokines such as macrophage inflammatory protein-1 (MIP-1), monocyte chemotactic protein-1 (MCP-1), these chemokines actively migrate microglia to the injury site and are known to actively participate in the amplification of brain inflammation (Fakhoury, 2015).

Trauma and disease to the CNS can either be acute or chronic by nature:

Acute neuro disease includes stroke and cerebral ischaemia, brain trauma , and can range through to epilepsy (Fakhoury, 2015).

Stroke and cerebral ischaemia:

In the western world, stroke is seen as being in the top three leading causes of death as well as one of the leading causes of permanent disability (Fakhoury, 2015).

Chronic CNS diseases range from multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD). These chronic CNS diseases are more complex in nature when compared to acute neuro diseases (Fakhoury, 2015).

Multiple sclerosis (MS)

MS typically appears in adults in their late 20s or early 30s and causes depression in more than 50% of the cases. This disease affects more than 2.5 million people in North America and Europe (Fakhoury, 2015).

MS is described as a chronic disorder where inflammation plays a key role. The CNS becomes invaded with T cells and macrophages which leads to the death of myelin sheaths that surround axons. This leads to loss of neuronal function and death. MS effects many areas of the CNS and because of this, the clinical symptoms are heterogeneous in nature and can vary widely. Clinical symptoms of MS include fatigue, muscle weakness, areas of numbness and paralysis. The disease is noted to be progressive by cycles of relapse that can be associated with systemic infection and inflammation as well as remission (Lucas, 2006).

It has been suggested by several studies that MS is a disease mediated by inflammation in which the immune system plays a central role. Multiple pharmacotherapies such as glatiramer acetate, natalizumab and mitoxantrone are proven therapies that help to reduce the symptoms of MS. Most of these medications however could cause unwanted side effects including skin irritation, liver damage and hypertension. Because of this, new treatment and strategies must be developed to

better treat MS. More models that include genetic, vial and immune related components need to be developed in order to further understand the pathology of the disease as well as identify possible targets for treatment (Fakhoury, 2015).

Alzheimer's disease (AD)

AD affects individuals over the age of 65 and it is the most common neurodegenerative disease that develops more commonly in woman than men. AD can be characterised by progressive inability the patient has to form new memories as well as the increasing inability to access existing memories. This is largely due to neuronal death in the hippocampus and frontal cortex. Once activated, microglia surround amyloid plagues, amyloid plagues are one of the hallmarks that characterises AD (Fakhoury, 2015; Lucas, 2006).

Having been activated by the amyloid peptide, microglia cells then initiate a cascade of events. They synthesise and release proinflammatory cytokines (IL- 1, IL-6 and TNF-α) and chemokines. This typically leads to the migration of specific cells like monocytes across the blood brain barrier. Higher levels of these released cytokines have been found in the cerebral spinal fluid (CSF) of AD patients. Proinflammatory cytokines TNF-α and IL –I can increase the expression of the amyloid precursor protein and the ab peptide. This is suggestive that the inflammation in the CNS plays a role in amplifying the disease state of AD (Fakhoury, 2015; Lucas, 2006).

Currently, there is no cure for AD and pharmaceuticals and psychological interventions are relied on by patients to reduce some of the symptoms of AD. There is strong evidence for the association AD has with inflammation as well as increased activation of the immune system. The production of beta-amyloid protein is boosted by the

activation of microglial and astrocytes triggered by an inflammatory response (Fakhoury, 2015; Lucas, 2006).

There have been various animal models that are being used to examine pathogenic as well as neurogenic mechanisms of AD. AD can be induced via *in vivo* models by injecting chemical compounds or pharmaceutical agents directly into the brain. A study has demonstrated that intracerebroventricular infusion of beta-amyloid peptides into the rat could result in progressive brain dysfunction as well as neurological damage that is typically seen in AD patients (Fakhoury, 2015; Lucas, 2006).

Parkinson's disease (PD)

PD is the most frequent neurodegenerative disease between the age of 50 and 60 and there is strong evidence suggesting PD is associated with neurodegeneration of neurons caused by CNS inflammation. Immunological activation as well as inflammatory processes are demonstrated to play roles in influencing the severity including pathogenesis and progression of PD. In PD, microglial cells are activated by peripheral inflammation and these cells then mediate the neurodegeneration of neurons in the substantia nigra. PD patients have significantly higher levels of specific proinflammatory cytokines for example, IL-1B, TNF-α, and interleukin – 2 (IL-2) as well as higher amounts of CD4, CD8 and T lymphocytes. The higher levels of these cytokines indicate that inflammation is increased and an activated immune response is seen (Fakhoury, 2015).

Various animal models currently are used to study the pathophysiology of PD, they have been useful models for providing understanding of the molecular mechanisms that are involved in PD. One widely used neurotoxin animal models is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced mouse model. This model is

designed to mimic the biochemical changes occurring throughout PD. MPTP targets involved neurons in PD and causes cellular apoptosis to dopaminergic cells located in the substancia nigra. MPTP also interferes with mitochondrial respiration that can result in cytotoxic consequences. Other *in vivo* models include the 6-hydroxydopamine (6-OHDA) rat model that typically targets dopaminergic pathways located in the substancia nigra (Fakhoury, 2015; Lucas, 2006).

PD is typically characterised by loss of dopaminergic neurones in the substantia nigra, and motor symptoms of tremors, muscle rigidity and bradykinesia. PD is found to have an association with genetic mutations found to encode a-synuclein and parkin. PD is otherwise sporadic, where various environmental factors ranging from pesticides to infections may also contribute to the disease. In patients with PD, inflammation is seen to play a role where activated microglia are found in close proximity to degenerating substantia nigral neurons (Lucas, 2006).

Chronic CNS diseases are typically multi-factorial with environmental factors and genetic backgrounds both contributing to the severity of the disease. These factors can also contribute to inflammation of the CNS and can be further catalysed by ageing. Inflammatory mediators like activation of microglia which in turn produces pro inflammatory cytokines, superoxide radicals NO and components of the complement system. These inflammatory modulators are seen to increase in chronic neuro-degenerative diseases (Lucas, 2006).

1.1.3 The role of microglial cells in CNS Inflammation

To further understand the inflammatory mechanisms and pathophysiology of the central nervous system, it is necessary to study the role microglia have in propagating or at times attenuating pro-inflammatory cascades in the brain (Brawek, 2013; Mietto, 2015). The inflammatory response has many key players involved in the reaction but the microglia are known as the resident immune cells in the CNS. Microglial play a critical role in the inflammatory response occurring in the brain. Microglial cells respond to stimuli from various sources resulting in producing a variety of inflammatory cytokines like TNF-α and IL-1β (Serhan, 2008).

Primary glial cells can be defined as the supporting cells of the central nervous system and they do not conduct electrical impulses unlike neurons. The glial cells surround neurons and provide support for insulation between them. Glial cells are the most abundant cell types in the central nervous system (Purves, 2001).

The total cellular population of the mammalian brain comprises of approximately 12% microglia cells (Tamashiro, 2012). Despite neurons and astrocytes being the major cell types of the nervous system, microglia cells play a significant role in the normal brain physiology by monitoring tissue for debris and pathogens and maintaining homeostasis in the parenchyma via phagocytic activity. The activation of microglia cells occur by various injury and disease conditions this includes neurodegenerative disease, traumatic brain injury and infection of the nervous system (Tamashiro, 2012).

Microglia are cells that go under various names such as mononuclear phagocytes and these cells are specialised phagocytic cells that survey and populate the central nervous system during the embryonic development. Microglia are known for their

ability they have to scan the environment with elaborate mobile processes. Microglia is a type of cell that is known for a rapid responsive reaction to inflammation and injury (Ravikumar, 2014).

Microglia reside in tissue as well as the infiltration of blood-borne macrophages and are activated by the neuro-inflammatory response. This activation further contributes to the secondary damage of the spinal cord, as well as the glial scar and progressive cavitation. The specialised blood spinal cord barrier is typically a monolayer however in the acute stages of a SCI it splits into two layers. An inner endothelial basement membrane and an outer parenchymal basement membrane. Mononuclear phagocytes gather within the space created by the membranes and they infiltrate into the surrounding parenchyma. The infiltrating monocytes exert an inflammatory cascade which occurs during the subacute period following the SCI. This coincides with the formation of the glial scar and glial limitans which could suggest that glial scar aids in regulating the inflammatory reaction (Ravikumar, 2014).

Microglia under various activating conditions these cells increase phagocytic activity, go through morphological and proliferative changes. Microglia can also secrete reactive oxygen and nitrogen species, pro inflammatory cytokines, this can quite often activate the paracrine or the autocrine loop. Microglial activation and response from these cells can contribute to disease pathogenesis in neurological conditions, making research into the mechanisms and environment of microglia cells of large interest (Tamashiro, 2012).

Microglial derive from early embryonic myeloid-monocytic cells that colonise the brain. These cells constantly monitor their micro-environment and can be associated with neuro-plasticity, host defence, homeostasis, wound healing, debris scavenging,

peripheral immune cells recruitment and immune response regulation. As a response to a range of stresses upon these cells, they have migratory capabilities and have the potential to undergo morphological and functional changes (Ginhoux, 2013).

Due to the fact that microglial cells are major immune-competent cells in the brain microglial cells are highly involved in neuroprotection as well as neuro-degeneration. Microglia support the survival of neurons from the secretion of growth factors and anti-inflammatory cytokines such as transforming growth factor beta, interleukin (IL)-1 receptor antagonist. While microglial are beneficial, they can become harmful if exposed to inappropriate pro inflammatory activity. Through secretion, microglial can release neurotoxins like cytokines and chemokines that are pro inflammatory and pro apoptotic in nature. Such neurotoxins include, IL-1B, IL-6, IL12 and tumour necrosis factor alpha (TNF-α). Microglial cells can also be harmful through the production of excitatory amino acids, eicosanoids, reactive oxygen species and nitric oxide (NO) (Rubio-Perez, 2012).

1.1.3.1 Other cell types involved with CNS and neuro-inflammation

As well as microglial cells, inflammation of the CNS is caused by multiple cell types that react to CNS trauma and as a result cause inflammation of the CNS.

The types of CNS cells that can be observed include, oligodendrocytes, astrocytes, ependymal cells, Schwann cells, microglial and Satellite cells.

1.1.4 The use of anti-inflammatory agents to treat CNS inflammation

There is abundant data suggesting inflammation contributes to many CNS diseases and because of this, inflammation is becoming a target for future therapy. The potential for both beneficial and harmful effects yield complications when developing inflammatory therapies. The detrimental effects seen in the application of therapies may be avoided when applied to acute disease like stroke. This is because the application of the treatment can be applied over a short time period. Conversely, the application of treatment to chronic disorders presents a greater challenge (Lucas, 2006).

1.1.4.1 Anti-inflammatories

This section will demonstrate how the use of anti-inflammatory agents can hope to mitigate the effects of CNS inflammation. This is demonstrated by both *in vitro* and *in vivo* models. The advantages and disadvantages of *in vitro* and *in vivo* models will also be addressed.

One way that could mitigate or slow down inflammatory reactions to CNS trauma and injury is to investigate compounds that have possible anti-inflammatory properties.

The use of a novel inflammatory on inflammation seen in the severed CNS is not a new idea. There have been studies conducted to varying degrees of success that use a range of compounds that illustrate and exhibit anti-inflammatory properties. For example, a recent study investigates the use of curcumin for improving neural function

after spinal cord injury by joining intracellular and extracellular components of the glial scar (Yuan et al., 2015) . This study established a spinal cord injury model by using female Sprague-dawley rats. In this model, the spinal cord was exposed and dissected by blunt dissection. The study sorted the rats into specific groups that included, a sham group, a simple injury group, the curcumin treatment group and the methylprednisolone (MP) treatment groups (Yuan et al., 2015).

The methods of this study (Yuan et al., 2015) included behavioural assessment, Immunohistochemical analysis, western blot assays, enzyme-linked immunosorbent assay, real time polymerase chain reaction. The study detected intracellular and extracellular components of glial scar as well as related cytokines that included tumour necrosis factor (TNF)-a, interleukin (IL)-1b, nuclear factor (NF)-kb, transforming growth factor (TGF)-β1, TGF-B2 and gender determining regions like the Y- box (SOX)-9.

The results from this study found that curcumin did inhibit the expression of cytokines with proinflammatory properties like TNF-α, IL-1b and NF-kb (Yuan et al., 2015).

Curcumin was also found to reduce the expression of intracellular components like glial fibrillary acidic protein via anti-inflammation, it also was found to suppress reactive gliosis. Curcumin inhibited the generation of TGF-B1, TGF-B2 and SOX-9 and decreased the deposition of chondroitin sulfate proteoglycan this was by transforming growth factors and transcription factors. This lead on to the improvement of the microenvironment for nerve growth. The study looked at joint inhibition of the intracellular and extracellular components of glial scar and specifically looked at curcumin which significantly reduced glial scar volume and improved locomotor rating and axon growth (Yuan et al., 2015).

Another study (Prasad, Gupta, Tyagi, & Aggarwal, 2014) also reviews the uses and benefits of curcumin also known as the golden spice. The history of this spice dates back thousands of years but only recently has the chemistry been studied (Prasad et al., 2014). The last two decades has seen more than 6000 articles that have been published which have discussed curcumins molecular basis of the antioxidant and anti-inflammatory properties. As well as multiple studies that lead on from this idea, clinical trials have also been conducted to see the effects of this on multiple chronic conditions. Because this study shows the use of anti-inflammatory properties, this promoted the need to investigate other compounds with anti-inflammatory properties (Prasad et al., 2014).

As well as Curcumin, other studies have also used other compounds possessing anti-inflammatory effects on the CNS. This adds to the argument that the glial scar formed in the CNS under trauma is brought about by inflammatory reactions of the immune system. Carvedilol is a beta blocker that treats congestive heart failure left ventricular dysfunction (LVD) and for treating high blood pressure. It has been examined to show how it promotes neurological function, reduces bone loss and attenuates cell damage after acute spinal cord injury. The results of this study indicate that carvedilol does exhibit anti-inflammatory effects and anti-oxidative effects in SCI rats (Liu et al., 2015).

Table 1: Table showing various Anti-inflammatories ("Drugs to Treat Various Pain Conditions," 2016)

Non-Steroidal Anti-Inflammatory drugs (NSAIDs) ("Drugs to Treat Various Pain Conditions," 2016)		
Ant-inflammatory	**Effect**	**Prescription or over the counter (OTC)?**
Aspirin (Anacin, Ascriptin, Bayer, Bufferin, Ecotrin, Excedrin)	Treats cardiovascular disease (CVD), pain, and inflammation	OTC
Choline and magnesium salicylates (CMT, Tricosal, Trilisate)	Treats pain associated with arthritis as well as fever	Prescription
Choline salicylate (Arthropan)		
Celecoxib (Celebrex)	anti-inflammatory properties, treatment of osteoarthritis (OA)	Prescription
Diclofenac potassium (Cataflam)	Treat muscle pain, back pain and various forms of arthritis	Prescription and OTC (Dose dependant)
Diclofenac sodium (Voltaren, Voltaren XR)		

Diclofenac sodium with misoprostol (Arthrotec)		
Diflunisal (Dolobid)	Treatment of pain, Anti-inflammatory	Prescription
Etodolac (Lodine, Lodine XL)		Prescription and OTC (Dose dependant)
Fenoprofen calcium (Nalfon)	Treats pains and fever, Anti-inflammatory	Prescription
Flurbiprofen (Ansaid)	Treatment of joint stiffness, pain and swelling	Prescription
Ibuprofen (Advil, Motrin, Motrin IB, Nuprin)	Treatment of pain, Anti-inflammatory	Prescription and OTC (Dose dependant)
Indomethacin (Indocin, Indocin SR)		
Ketoprofen (Actron, Orudis, Orudis KT, Oruvail)		
Magnesium salicylate (Arthritab, Bayer Select, Doan's Pills, Magan, Mobidin, Mobogesic)	Treatment of joint stiffness, pain and swelling	Prescription

Meclofenamate sodium (Meclomen)	Reduces hormones that increase inflammation	Prescription
Mefenamic acid (Ponstel)	Anti-inflammatory	Prescription
Meloxicam (Mobic)	Treats osteoarthritus pain	Prescription
Nabumetone (Relafen)	Reduces hormones that increase inflammation	Prescription
Naproxen (Naprosyn, Naprelan)	Treatment of pain, Anti-inflammatory	Prescription and OTC (Dose dependant)
Naproxen sodium (Aleve, Anaprox)		
Oxaprozin (Daypro)	Treats arthritis, reduces pain, swelling and stiffness of joints.	Prescription
Piroxicam (Feldene)	Treatment of pain, Anti-inflammatory	Prescription and OTC (Dose dependant)
Salsalate (Amigesic, Anaflex 750, Disalcid, Marthritic, Mono-Gesic, Salflex, Salsitab)	Treats arthritis, reduces pain, swelling and stiffness of joints.	Prescription

Sodium salicylate	Analgesic, antipyretic, induces apoptosis in cancer cells	Prescription
Sulindac (Clinoril)	Treatment of pain, Anti-inflammatory	Prescription and OTC (Dose dependant)
Tolmetin sodium (Tolectin)	Reduces hormones that increase inflammation. Anti-inflammatory, treats arthritic pain.	Prescription

Table 2: Showing natural anti-inflammatory remedies

Natural anti-inflammatory remedies (Maroon, 2010)	
Ant-inflammatory	**Effect**
Omega-3 EFAs (fish oil)	Anti-inflammatory
White willow bark	Analgesic and antipyretic
Curcumin (turmeric)	antioxidant, anti-inflammatory, and antineoplastic
Green tea	Anti-inflammatory and delays progressive joint space narrowing seen in arthritis (chondroprotective)

1.1.4.2 *In vitro* models that demonstrate anti-inflammatory treatment of CNS inflammation

In vitro can be translated to in glass and is related to studies that are in test tubes or petri dishes. An advantage of this type of model is that is yields specific results and has a species – species relationship. The methods used for *in vitro* studies are often simpler, more convenient and are more detailed than studies done by *in vivo* models. One disadvantage of *in vitro* models is that results can be difficult to extrapolate into *in vivo* models that is, how the whole organism might be affected. *In vitro* models are starting to replace studies that use whole animals.

The central nervous system has a large cell population that is heterogeneous in nature and because of the cell population being so varied; it has inhibited the studying and describing biochemical characteristics of well-defined cell populations. The developing of a strategy or protocol to enable the isolating of cell populations into neurons, oligodendrocytes or astrocytes. This isolation of cells into a homogenous population has a variety of benefits.

When looking at the adult brain, it is to be noted that CNS tissues contain a significant amount of myelin which can then make it difficult to isolate these microglial cells in a cell suspension. Myelin is a membrane that insulates axons in the peripheral and central nervous systems and the formation of myelin is continuous through life. Mice who are older will have higher levels of myelin, it is then imperative to remove myelin from the tissues cell suspension prior to cell sorting or staining to ensure clean cell fractions.

One method of isolating and analysing of mouse microglial cells is described a study (Garcia, Cardona, & Cardona, 2014). The study defines microglial cells as phagocytes that have one nuclease and they make up about 10% of the central nervous system. These cells survey and monitor neuronal tissue and are derived from myeloid progenitor cells, they play an important part in homeostasis, inflammatory and immune responses. This study describes multiple microglial cell isolation protocols (Garcia et al., 2014).

Another study looks at *in vitro* models of glial scar inhibition and primarily investigates how astrocytes respond when the central nervous system is injured (Wanner et al., 2008). The study looks at the role of reactive astrogliosis in relation to the formation of the glial scar (Wanner et al., 2008). The proposed model in the study (Wanner et al., 2008) combined two key aspects of CNS injury, mechanical trauma as well as co culture with meningeal cells.

This provided a useful *in vitro* model of the scar from cultures that included highly differentiated astrocytes. To do the analysis, the morphology of cells was looked at for signs of astrocyte reactivity. The increase of markers like glial fibrillary acid protein (GFAP) and noting the expression levels of scar associated markers like phosphacan, neurocan and tenascins were also seen to be increased (Wanner et al., 2008). The model in this study provides a useful tool for investigating the underlying mechanisms of the glial scar formation and it is a useful model as it provides quantifiable inhibition of axonal regeneration (Wanner et al., 2008).

A further protocol for the analysis of primary glial cells created a positive cell culture model which is ideal for the investigation into factors that form the glial scar (Polikov, Su, Ball, Hong, & Reichert, 2009). After the isolation of glial cells for analysis, they

can be subjected to forms of stress, including oxidative stress however other stresses can be used that includes, metabolic stress as well as physical stress (Polikov et al., 2009).

1.1.4.3 *In vivo* models that demonstrate anti-inflammatory treatment of CNS inflammation

In vivo models are models that are done within living animals. Advantages of *in vivo* models include observing the effects of study on the living subject. Clinical trials and animal testing are major *in vivo* elements. *In vivo* models are starting to replace human trials in more recent times. *In vivo* methods more specifically the use of transgenic and knockout animal models have been used in order to study inflammation, infection and autoimmune CNS diseases as well as the relationship between all three (Sohet, 2013)..

A study that used TNF-α transgenic mice showed that overexpression of TNF-α is sufficient enough for the mice to develop neurological disorders that have chronic CNS inflammation as well as white matter degeneration. One useful way to study microglia is by using rodent tissue for *in vitro* culturing. Though there are drawbacks such as yielding low numbers of cells and the methods used are often time consuming for using murine primary microglial culture. These drawbacks limit the types of studies that can be conducted on these cell types (Li, 2003).

1.1.4.4 Specific examination of anti-inflammatory compounds

1.1.4.5 Kawakawa leave extract

The Kawakawa plant or Macropiper excelsum (Forst. F.) Mig is a shrub that is found to be growing in shady areas near the coast throughout New Zealand. Kawakawa can be identified by its shiny green leaves and small yellow fruit and have been exploited by Maori for the shrubs significant healing properties. This shrub belongs to the family Piperaceae and is reported to have medicinal properties (Richardson, 2015) .

This is a native plant found in coastal areas throughout New Zealand. Cuts, wounds, stomach pains and toothache have been reported to be cured by the KawaKawas leaf and bark. The smoke from the slow burning of Kawakawa leaves has on the other hand has been used as an insecticide (Richardson, 2015). Chromatography of extracts of the leaves gave an active fraction from which four neutral lignans have been isolated, one of which is diayangambin (Russell, 1973).

Lignans are natural products that have a range of various biological activity. In the literature, many lignans are found in immunomodulatory, anti-allergic, and anti-inflammatory activities by inhibiting T cell proliferation. Lignans can also inhibit multiple pro inflammatory mediators such as cytokines, eicosanoids and the platelet activating factor. In recent times, the furofuran lignin, diayangambin as well as other lignin derivatives have been isolated from leaves of *Piper fimbriulatum C.D.C.* These plants grow in Panama and belong to a large genus containing ca. 700 species. South American folk medicine use these plants for several purposes including inflammatory processes (Russell, 1973).

The data present in a study shows that diayangambin possesses *in vitro* modulatory activity on lymphocyte proliferation and PGE2 generation of macrophages. The way diayangambin modulates the responses of lymphocytes and macrophages might be a useful way to treat immune and inflammatory responses (Russell, 1973).

Though little research has gone into the composition and biological activity of kawakawa there are two known bioactive molecules, myristicin and diayangambin which are found in the leaves of the plant (Richardson, 2015).

Myristicin is the more common of the two bioactive molecules and it has been reported in other plant sources including nutmeg, parsley and dill. Myristicin has been well studied in terms of its biological activity which helps make evident the medicinal properties of kawakawa. As well as being an anti-inflammatory and an anti-microbial agent Myristicin has also been found to be hepatoprotective (Richardson, 2015).

Myristicin is also a known psychoactive drug and precursor to the psychedelic drug MMDA. This transformation has been suggested by some to play a part during metabolism in the body. As well the significant bioactivity held by myristicin, it has also been found to be an effective insecticide (Richardson, 2015).

The other bioactive molecule in question is diayangambin. Diayangambin in comparison to myristicin is a rare product that also holds significant bioactivity. Diayangambin exhibits anti-inflammatory activity and is also an immunosuppressive agent. Drugs utilising diayangambin as an immunosuppressive agent have been used to treat several conditions where the body’s immune system requires down regulation (Richardson, 2015).

1.1.5 Objectives and aims of our research

The research topic in question for this project is titled "*In vitro* cell culture to study microglial inflammation" the aims for which include investigating novel compounds that could have anti-inflammatory effects on primary glial cell cultures. We aim to investigate inflammation that is expressed by primary glial cells under trauma to the central nervous system (CNS). This project was inspired by an overseas conference where the glial scar and its components was discussed. The context of this project is put into focus with an understanding of how far research has advanced in relation to knowing more about the mechanisms involved in the inflammation of the CNS. Inflammation of primary glial cells cause's problems for future CNS healing and treatments for how this could be treated or mitigated on some level is being examined. Our project uses three cell lines THP1, HeLa and an isolated primary glial cell cultures. These cell lines are used to investigate and demonstrate the effect Kawakawa leaf extracts have on these cell lines. An investigation into what effects Kawakawa leaf extract could have on primary glial cell inflammation.

Given the above research into the literature, it is evident that there is a link between CNS trauma and an inflammatory reaction. The idea of applying novel or new compounds with anti-inflammatory properties is not new and has been done to varying degrees of success. Our research identifies the setting up a primary cell culture of glial cells using post mortem brain tissue from mice/rats. The approach we will take in addressing this issue, is by using novel compounds that have not been applied to primary glial cell cultures which includes the New Zealand Kawakawa plant. The novel compound we will be using in our study is extracts of Kawakawa leaves which are

extracted under two different temperature conditions. A further avenue for study could include the investigation of mitochondria in primary glial cell cultures.

The overall *in vitro* primary glial cell model in mind for our study uses primary glial cell cultures from brains of mice or rats post mortem. The methodology that is used in our project is similar to that of other studies where we set up a primary glial cell culture and will confirm that this is a primary glial cell culture glial cell culture using immunocytochemistry. This method proves effective for isolation of primary glial cell cultures as seen in other studies.

Our way of addressing this issue of CNS inflammation is by investigating modulators of primary glial cell inflammation. Primary microglial cell cultures will be taken from supplied mice/rat brains post mortem. The effects seen by novel or new anti-inflammatory compounds will be monitored. Our research will use specific primers that will be used for detecting TNF-α levels and Inter leukin levels. The monitoring of the maturation of the glial cell culture can be done by using confocal microscopy and fluorescence microscopy. Microscopy will aid in observing proliferation of glial cells and the effect the applied anti-inflammatory compounds could have on CNS inflammation.

2 Materials and Methods

2.1.1 Growth assay

HeLa and THP-1 cells were seeded at a density of x/ml into a 96 well tissue culture plate in a duplicate or triplicate fashion. The plate was incubated at 37^{O}C in a humidity incubator (under standard incubating conditions). HeLa cells were harvested through days 1-5 by trypsinizing the cells and THP-1 cells were harvested through days 1-5 by centrifugation. Viable cells were counted using a haemocytometer.

2.1.2 Cell cultures and dose response curves

2.1.2.1 Cell Cultures

2.1.2.2 HeLa and THP-1 cell culture:

The human cervical cancer HeLa and THP-1 cell lines were borrowed from the University of Waikato's stock of frozen cell samples. In order to grow a culture of HeLa cells, a frozen sample of HeLa cells were thawed in a water bath at 37^{O}C. An aliquot of 1 ml of cells was grown in a T25 flask containing 4mls of complete growth media that was more specifically DMEM media (Gibco) that contained 10% foetal bovine serum (FBS). The cells were then incubated at 37^{O}C in a 5% CO_2 humidified incubator. After 1-2 days of incubation, the flask was checked under the microscope to observe the amount of confluency. If confluent, the cells were harvested to preform various experiments. Cells were harvested by decanting the flask of the complete growth media and washing the cells in 1ml of PBS. A 2 ml aliquot of trypsin/EDTA was added to lift the cells off the flask surface and into solution. To neutralise the trypsin EDTA, complete DMEM media should be added to the solution. The cell solution was then centrifuged (Megafuge 1.0) at 1100 rpm for 5 minutes. The supernatant was then discarded and the cell pellet was re-suspended in 2 mls of pre-warmed DMEM

complete growth media. It should be noted that the experiments carried out use exponentially growing cell cultures or near confluent cell vessels.

The THP-1 cell line was borrowed from the University of Waikato's stock of frozen cell samples. In order to grow a culture of THP-1 cells, a frozen sample of THP-1 cells were thawed in a water bath at $37^{O}C$. An aliquot of 1 ml of cells was grown in a T25 flask containing 4mls of growth media that was more specifically DMEM media (Gibco) that was titled glutaMax. This was supplemented with 10% foetal bovine serum (FBS). The cells were then incubated at $37^{O}C$ and 5% CO_2 in a humidified incubator. After 1-2 days of incubation, the flask was checked under the microscope to observe the amount of confluency. If confluent, the cells were harvested to preform various experiments. Cells were harvested by decanting the flask into a 15mL falcon tube. The cell solution was then centrifuged (Megafuge 1.0) at 1100 rpm for 5 minutes. The supernatant was then discarded and the cell pellet was suspended in 2 mls of glutaMax complete growth media. It should be noted that the experiments carried out use exponentially growing cell cultures or near confluent cell vessels.

An alternative media to use for THP-1 cells consists of 0.5ml 100 mM Sodium pyruvate, 0.5ml 100x penicillin/ streptomycin, 5 mls of FBS and bring up to 50 mls with RPMI 1640 media.

2.1.3 Dose response curves

Does response curves were generated over a 7 day period for HeLa and THP-1 cells. The HeLa cells were grown in complete DMEM media in 96 well plates. THP-1 cells were grown in full RPMI- Glutamax media or THP-1 media and were grown in 96 well plates.

Does response treatments included KawaKawa leaf extracted from two conditions at 60^{o}C and room temperature see section **2.3 Kawakawa extract preparation**.

The cells were exposed to 100ug/ml, 50/ug/ml, 25/ug/ml, 12.5 ug/ml and 6.25 ug/ml concentration of both extracts. Each day two wells per extraction concentration was counted with a haemocytometer using trypan blue exclusion method. The two counts were averaged and plotted to generate a growth curve.

2.2 Cytotoxicity assays

2.2.1 Detection of Apoptosis in HeLa cells and THP-1 cells

2.2.1.2 Introduction

Cellular toxicity can be caused by either necrotic or apoptoic cell death. To study the cytotoxic effects of Kawakawa leave extracts on HeLa cells and THP-1 cells, typical characteristics of apoptosis and necrosis were examined using various biochemical techniques. One of the main characteristics of apoptosis includes the ability of the cell to maintain plasma membrane integrity after death. Biochemical assays like [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and lactate dehydrogenase (LDH) were conducted as well as trypan blue exclusion experiments. Visual characteristics such as morphological changes such as membrane formation and bubbling are seen as cells undergo apoptosis was also examined under the microscope.

2.2.2 LDH Assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released into growth media as a consequence of disruption to the plasma membrane which is used as a marker of necrotic cell death. The Promega CytoTox96 NonRadioactive Cytotoxicity Assay kit was used to determine the levels of LDH present in a solution. The experiment was carried out following the instructions based on the manufacturer's protocol.

Two 96 well plates' were used and in one row each well contained 100ul of blank media (media containing no cells, such as PBS). Another row of wells contained 100ul

of cells in suspension and the last group of wells were inoculated with 100ul of disrupted cells on a separate plate. Plate one will have the blank media and intact cells and the second plate will have disrupted cells. It should be noted that all wells should be repeated in a duplicate or triplicate fashion.

Plate one was incubated overnight in the $37^{O}C$ CO_2 incubator and plate two was placed in the $-20^{O}C$ freezer overnight. The next day, the plates were centrifuged at 250g for 4 minutes. After which, 50ul of supernatant was transferred to new wells and 50 ul of substrate solution was added to the supernatant.

The plates were then incubated in standard incubating conditions for 30 mins. In order to stop this assay 50 ul of stop solution was added to each well and the plates were read by a Model 680 Microplate Reader (Bio-Rad) with an absorbance of 490nm.

The LDH assay is used to obtain the percentage of LDH content in solution that is relative to the control. The data was averaged and then normalised by subtracting the averaged blank media reading. The maximum control was multiplied by 1.1 if need be to account for adding the lysis solution. The final data presented is a percentage which can be interpreted as the percentage of dead cells that are present in the original cell culture if we assume that all cells released equal amounts of LDH.

$$\text{Cytotoxicity (\%)} = 100x \frac{\text{(Experimental LDH release OD490)}}{\text{(Disrupted cells OD490)}}$$

Equation 1: Calculating the percentage of relative cytotoxicity.

2.2.3 MTT Assay

MTT [3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyl tetrazolium bromide] is a tetrazolium dye. When MTT is reduced by the cellular dehydrogenase, blue crystals are formed intracellularly. These crystals can then be dissolved and can also be quantified using spectrometry. The dissolving of the crystals can be an indication for mitochondrial dehydrogenase activity.

The MTT Assay was carried out as per the manufacturer's protocol. Control cells and test compounds with cells were prepared and were loaded onto a 96 well microplate having a 100ul/well final volume. A volume of 10 ul of MTT at 4.5mg/ml concentration solution was added per well, achieving a final concentration of 0.45mg/ml. The microplate was incubated for 30 -45 minutes at 37^{O}C. The incubation period was stopped when purple crystals were seen under the microscope.

After the incubation period 100 ul of stop solution was added to the wells the plate was then placed on a minishaker for 10 minutes. Absorbance was then read at 570nm on a microplate reader. The data was averaged out where the average blank was subtracted from the other data conditions.

Absorbance (corrected) = (Sample OD 570nm) – (Blank OD 570nm)

Equation 2: Calculating the corrected MTT absorbance.

The obtained experimental data was normalised to the control and subsequently expressed as a percentage of mitochondrial dehydrogenase activity.

$$\text{Dehydrogenase activity (\%)} = 100\% \times \frac{\text{Experimental sample absorbance (corrected)}}{\text{Control sample absorbance (corrected)}}$$

Equation 3: Calculating mitochondrial dehydrogenase activity.

2.3 Kawakawa extract preparation

The preparation of the Kawakawa leave extract was done by using three varying methods. The three methods were prepared at room temperature, at 60^{O}C and prepared using a distillation kit.

2.3.1 Method 1: Room temperature preparation of Kawakawa leaf extract

Leaves from a Kawakawa tree were gathered and cut into small fragments where 10g of leaves was weighed out. The fragments were then added into a beaker of 60mls of distilled water. The solution was then stirred at room temperature using magnetic stirrers for 3 hours with a foil cover over the top of the beaker.

2.3.2 Method 2: 60^{o}C temperature preparation of Kawakawa leaf extract

Like method 1, Leaves from a Kawakawa tree were gathered and cut into small fragments where 10g of leaves were weighed out. The fragments were then added

into a beaker of 60mls of distilled water. The solution was then stirred on a heat plate at 60C using magnetic stirrers for 3 hours with a foil cover over the top of the beaker.

2.3.4 Freeze drying samples

After the preparation of the kawakawa extracts the samples were then centrifuged for 5 minutes at 1100rpm to settle the organic material in the sample. After this, 15mls of the liquid was then aliquoted into 50ml falcon tubes and labelled according to each experimental condition. These tubes were then frozen in liquid nitrogen which involved placing the tubes in liquid nitrogen at a relatively flat angle to ensure a thin surface area of frozen solution. Sample tubes were then rolled in the liquid nitrogen and once this was done to all tubes, they were then placed into large cylindrical flasks and put onto the freeze drier machine. This dried the samples over 1-2 days and produced a powder that could be reconstituted and used for experiments. An empty 50 ml falcon tube was weighed and this weight was zeroed after which all other tubes were weighed. The weights of each product was recorded and samples were then placed in a -20 freezer until they are needed to be reconstituted for use.

2.4 Growth experiment exposing HeLa Cells to KawaKawa extracts

2.4.1 Serial dilution for concentrations:

The concentration of Kawakawa extracted wanted for the growth experiment was calculated using

$$C1 \times V1 = C2 \times V2$$

Equation 4: Showing the equation for working out relative concentrations

Serial dilutions of Kawakawa leaf extracts were prepared and were used in growth experiments. The conditions room temperature and 60^{o}C were used as these yielded the most extract once freeze dried. Approximately 0.02g of samples of each condition were reconstituted with 10mls of complete DMEM media which was then put through a syringe filter to decant the solution away from excess organic material.

The starting concentration was again to carry out a serial dilution is calculated by using **Equation 4.**

Once this serial dilution has provided the needed concentration of the extract a growth experiment that uses a 96 well tissue culture plate was set up. The Rows A-D represented the day in this case, Row A is day 1, Row B is day 2, Row C is day 3 and Row D is day 4 and Row E is day 5

The well numbers 1-6 represented the different concentrations with the exception of well 6 which was used as a control that has only cells with complete DMEM media.

One 96 well tissue culture plate was set up for the room temperature kawakawa extracts and another 96 tissue culture plate was set up for the 60^{o}C temperature kawakawa extracts.

The dose experiments were conducted over a five day period and with two cell lines HeLa cells and THP1 cell lines. These experiments were repeated in duplicates and were documented by pictures from wells under each day and condition and was further quantified by conducting MTT and LDH assays.

3. Primary Glial Cell culture

Primary glial cells were used in order to show the effect of the kawakawa leave extract on neuronal cell inflammation. To enhance the growth and proliferation of primary glial cells, the surface of the vessel that was used to grow the culture was coated with polylysine

3.1 Polylysine coating:

The procedure for coating a vessel with polylysine includes diluting 1 part stock solution with 19 parts of water to prepare a 50 g/ml working solution (105 ul stock and 2mls distilled water into a flask). Once this solution is prepared the culture vessel surface was coated with 1.0ml of polylysine and rocked gently to ensure even coating of the surface. After 5 minutes the solution was removed and the vessel was rinsed with 2 mls of distilled water or PBS three times. The liquid was removed from the vessel and this was allowed to surface dry for at least 1 to 2 hours before inoculation with cell suspension.

3.1.2 Glial cell isolation:

Before starting the isolation procedure percoll solutions need to be made up

3.1.3 To create a percoll gradient with a cell interphase:

The molecular weight of NaCl is 58.46 which means 1M of NaCl solution is 58.46 g/L and to make 1.5M of NaCl solution 87.69 g/L. To make a 1.5M NaCl of 10 mls 0.8769 g/10mls is added to 10mls of distilled water. To dilute this solution to 0.15M of NaCl 1 ml of the 1.5M NaCl was added to 9mls of distilled water.

A 1.5M solution of NaCl was made up which is added to percoll at a ratio of 1:9 that is 1ml of 1.5M NaCl and 9mls of percoll. This solution is known as stock isotonic percoll (SIP) as the NaCl makes the percoll isotonic. In order to make further dilutions of this isotonic solution the 0.15M NaCl was used. To make 10mls of 100% percoll, or SIP mix 9mls of percoll and 1ml of 1.5M NaCl. To make 10mls of 70% percoll, mix 7mls of SIP and 3mls of 0.15M NaCl.

In order to culture primary glial cells, fresh brain tissue was needed from mice or rats and the procedure for the removal of the brain tissue as well the procedure for isolating primary glial cells can be found below.

3.2 Brain removal

The head was removed from the body and a midline incision was made in the skin and the skin was then flipped over the eyes to free the skull. A small incision was made going from the top of the skull right down towards the eyes. Taking note that a firm cut through the most anterior part of the skull needs to be made. The parietal bone on both sides as well as the frontal bone was broken off. The brain was then free from the meninges and to do this slide the forceps under the anterior part of the brain and slip the brain upward. The forceps were slipped under to further break the optic nerves and cranial nerves and the brain was gently lifted out of the skull. The brain was then transferred to a petri dish that was on ice to cool the brain down and any excess material was washed off in sterile PBS.

3.3 Primary glial cell isolation procedures

3.3.1 Primary glial cell isolation procedure using percoll

The brain was carefully removed from the skull as mentioned in section 3.2 brain removal. The brain was then transferred to a petri dish and was washed with sterile PBS (the tissue was kept in a petri dish on ice). The tissue from the brain was then homogenized. The cell mix was then transferred to a 15ml falcon tube and made up to 7 mls of complete DMEM growth media. Then 3ml of 100% percoll solution was added to the cell suspension and was mixed by inversion. The solution was then transferred to a 15ml tube containing 2ml of 70% percoll solution (The glial cells will lay atop the 70% layer of percoll).

This tube was then centrifuged for 30 minutes at 550x g at 18^{o}C after which the debris was removed from the top by pipetting. The interphase that contains the mononuclear cells should be located just above the 2ml graduation of the 15ml tube. The interphase (2ml) was then transferred to a 15ml tube containing 8ml of PBS to wash the cells of the percoll and this was mixed by inverting. The tube was centrifuged for 7 minutes at 900 G at 4^{o}C.

The supernatant was discarded and the cell pellet was suspended in 2ml complete growth media. A syringe filter was used to filter the solution and a flask was inoculated with 1 ml of cell suspension and 4 mls of complete growth media. Incubate at 37^{o}C in CO_2 conditions (standard incubation conditions)

3.3.2 Primary glial cell isolation procedure using histopague

An alternative method for glial cell isolation using histopaque and not polylysine was also carried. This procedure involved euthanizing a rat where the head was disinfected in 70% ethanol. The rats brain was removed from the skull and placed in a petri dish where it was washed in sterile PBS. The brain tissue was homogenized and was transferred to a tube containing 4 mls of complete DMEM media. This solution was added as a layer on top of 3mls of histapaque in another centrifuge tube and was then centrifuged at 400g for 30-40 minutes at 18^{o}C.

The top layer after centrifugation was removed which exposed the mononuclear cell layer which is a cloudy white layer. This layer was then transferred to a sterile tube and washed with 8 mls of PBS. The cells were suspended into solution by gently pipetting the solution up and down. The tube was then centrifuged at 400-500g for 10-15 mins at 18^{o}C and the supernatent was removed after centrifugation. The cell pellet was then suspended in 8mls of PBS and centrifuged at 400-500g for 10 mins. After this final cell wash the supernatant was removed and the cell pellet was suspended in DMEM complete media with strep b antibiotic.

The growth of a primary glial cell culture was documented by pictures and once a culture was deemed to be sufficiently mature, immunocytochemistry was carried out.

4.5 Other methods

4.5.1 To split and harvest cells:

Cells should be harvested to perform experiments on or cell splitting is needed when the initial flask is confluent to prevent cell apoptosis. An initial cell count when splitting and harvesting cells is essential to ensure the cell number is known. If a suspension cell line such as THP-1, cells were harvested from the inoculated vessel by centrifuging the cells for 5 mins at 1100 rpm. The cell pellet was then suspended in 2 mls of complete growth media. A cell count was performed to work out how many cells are needed to inoculate the new vessel. These new vessels were then inoculated with 1ml of cell solution at a desired number and 4 mls of complete growth media.

Please note, if using an adherent cell line such as HeLa cells, preliminary steps of washing the cells with PBS and adding TE to lift the cells of the vessel is required.

4.5.2 To freeze cells down:

To freeze cells down each cryogenic tube has a 1ml capacity and a cell count is done when freeing down cells to ensure the cell number is known. Cells were harvested as in section to split and harvest cells 4.5.1 and 950ul of cell solution and 50ul of 100% DMSO was added to cryogenic tube. DMSO is an agent that prevents cells dying from being frozen. This tube was then placed into a -80C freezer.

4.6 Reagents and media recipes:

Table 3: Showing recipes of media and reagents used.

Name	**Composition**
Phosphate-buffered saline (PBS)	NaCl 8g/L (137mM) KCL 0.2 g/L (2.7 mM) Na_2HPO_4 1.42 g/L (10 mM) KH_2PO_4 0.25 g/L (1.8 mM) 800 mls of distilled water to dissolve salts HCL to adjust pH to 7.4 Top up to 1 litre with distilled water
Complete Growth Media (with glucose)	5mls of FBS 45mls of DMEM (with glucose)
1.5 M Tris- HCL, pH 8.8	Dissolve 181.65g Tris base in 800 mL of ddH_2O Adjust pH to 8.8 with concentrated HCL Bring volume to 1 litre with ddH_2O
0.5 M Tris- HCL, pH 6.8	Dissolve 181.65g Tris base in 700 mL of ddH_2O Adjust pH to 6.8 with concentrated HCL Bring volume to 1 litre with ddH_2O
10% Sodium Dodecyl Sulfate (SDS)	5g SDS

	Make up to 50mL with ddH2O water
Acrylamide-bis stock	
Ammonium persulphate	0.05 grams of ammonium persulphate 0.5 mls of distilled water
TEMED	
Trypsin EDTA	For 100 mls 10x Trpsin - EDTA: 0.5g of trypsin power 80 ml of distilled water 10 ml of 10X PBS 0.5 ml of EDTA solution Adjust to 100ml with distilled water 1X Solution Take 1 ml of solution and mix with 9ml of PBS
Tryptophan Blue	
Fetal Bovine Soltion (FBS)	
MTT solution	0.005g of MTT Dissolve in 1 ml of PBS
BCA stock solution	20 mg of BSA 10 ml of PBS
BCA working reagent (WR)	BCA Reagent A 50 parts BCA Reagent B 1 part : Ratio of 50:1
5x Electrode buffer	15g Tris (124mM) 72g glycine (959mM)

	50mL of 10% SDS (0.5%)
Ponceau S.	1g of Ponceau S 50ml acetic acid Make to 1 litre with distilled water
Mammalian Protein Extraction Reagent (m-PER reagent)	
Dimethyl sulfoxide (DMSO)	0.25M EDTA pH 7.5 20%DMSO
Comassie blue stain	200mls methane 50mls acetic acid 250 mls distilled water 0.1g per 100mls of brilliant blue (0.5g)
Destainer	400 mL methanol (40%) 100mL Acetic acid (10%) 500mL of ddH2O
NaCl	NaCl MW = 58.46 1000mls of NaCl 1.5M = 87.69 10mls of NaCl 1.5M = 0.8769g/10mls 10mls of NaCl 0.15M = 1ml of NaCl 1.5M 9mls of distilled water
Percoll	To make 100% (SIP) solution of percoll mix 9mls of percoll and 1 ml of 1.5 NaCl to a tube

	To make a 70% solution of percoll mix 7mls of SIP and 3mls of 0.15 NaCl to a tube.
TBS-Tween(TBST)	100mL 10x TBS 0.5mL Tween20 (0.05%) Make up to 1 L with ddH2O

4.7 Statistical Analysis

The statistics used for this study was carried out in Microsoft Excel. The data was averaged if appropriate and the standard error of the mean (S.E.M) was calculated by finding the standard deviation of the samples, finding the square root of the sample number and subsequently dividing both numbers

$$\text{S.E.M} = \frac{\text{STDEV (samples)}}{\text{SQRT (sample number)}}$$

Equation 5: Showing the calculation for the S.EM.

5. Results

5.1 Cell Culture and Dose Response Curves

HeLa and THP-1 cells were seeded at a density of 7,000 cells/ml onto a 96 well plate containing complete DMEM and THP-1 media respectively in duplicates. The two plates were then placed under standard incubation conditions and the cell number estimated using the tryphan blue dye exclusion assay every 24 hours.

To determine the growth rate of HeLa and THP-1 cells under standard incubation conditions, a standard growth curve was plotted (*figure 1*). The HeLa growth curve showed that the cells remained in a lag-phase during days 1-4, entering the exponential growth phase at day five. The experiment had an N=1 and the error bars represent (±SEM) of cell counts from two wells.

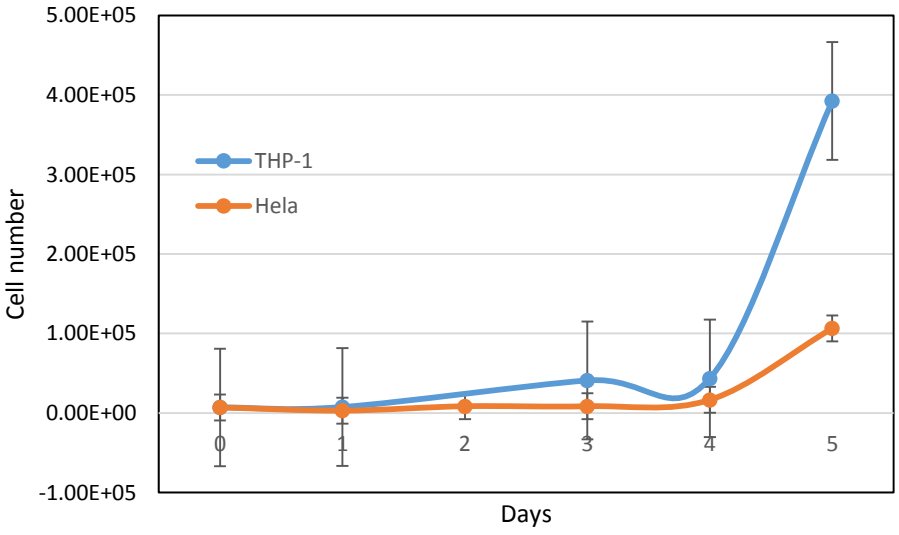

Figure 1: Showing growth curve for THP-1 cells and HeLa cells without extract.

5.2 Cell Culture and Dose Response Curves

Kawakawa extracts were prepared at 60^{O}C and room temperature described in section **2.3 Kawakawa extract preparation.**

HeLa and THP-1 cells were seeded at a density of 7,000 cells/ml onto a 96 well plate containing complete DMEM media and THP-1 media, respectively. The cells were then exposed to Kawakawa extracts at various concentration in duplicates. The two plates were placed in standard incubation conditions and the cell number was estimated using the tryphan blue exclusion method every 24 hours.

5.3 Dose Response of HeLa cells when exposed to Kawakawa extracts

HeLa cells were used as they are a robust cell laboratory cell line, cell culture techniques and methods can be easily carried out on this cell line. HeLa Cells were exposed to varying concentrations of Kawakawa extracts prepared at room temperature or 60^{o}C.

The growth rate of HeLa cells exposed to room temperature Kawakawa extracts at varying concentrations over a 5 day period is shown in *Figure* 2. HeLa cells treated with kawakawa extracts and the untreated control cells exhibited a lag-phase during days 1-4 (similar to that described earlier). Interestingly, the kawakawa extract concentrations (6.25, 12.5, 25 and 50 µg/ml) led to a marked inhibition of cell growth during day 4-5 compared to the untreated control cells, which had entered the exponential growth phase during this period. Cells exposed to the highest Kawakawa extract concentration of 100µg/ml had however, entered an exponential growth phase, at a much reduced growth rate when compared to the control cells. This

experiment had an N=1 and the error bars represent (±SEM) of cell counts from two wells.

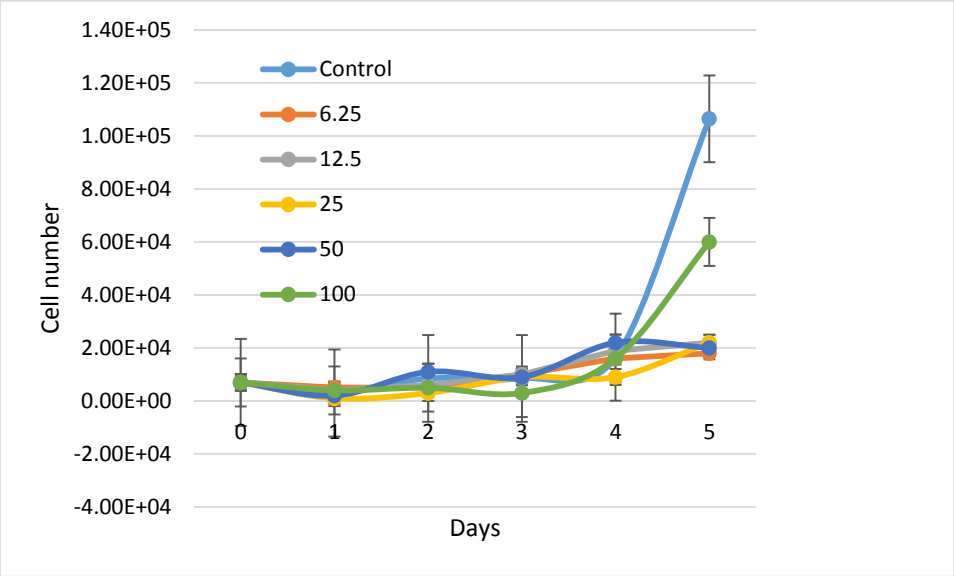

Figure 2: Showing room temperature dose curves of HeLa cells. Error bars show the S.E.M.

The growth rate of HeLa cells exposed to 60^{O}C Kawakawa extracts at varying concentrations over a 5 day period is shown in *Figure* 3. HeLa cells treated with kawakawa extracts and the untreated control cells exhibited a lag-phase during days 1-4 (similar to that described earlier). Interestingly, the kawakawa extract concentrations (6.25 and 25 µg/ml) led to a marked stimulation of cell growth during day 4-5 compared to the untreated control cells. The Kawakawa extract concentration (12.5, 50 and 100µg/ml) led to a marked inhibition of cell growth during day 4-5 compared to the untreated control cells.

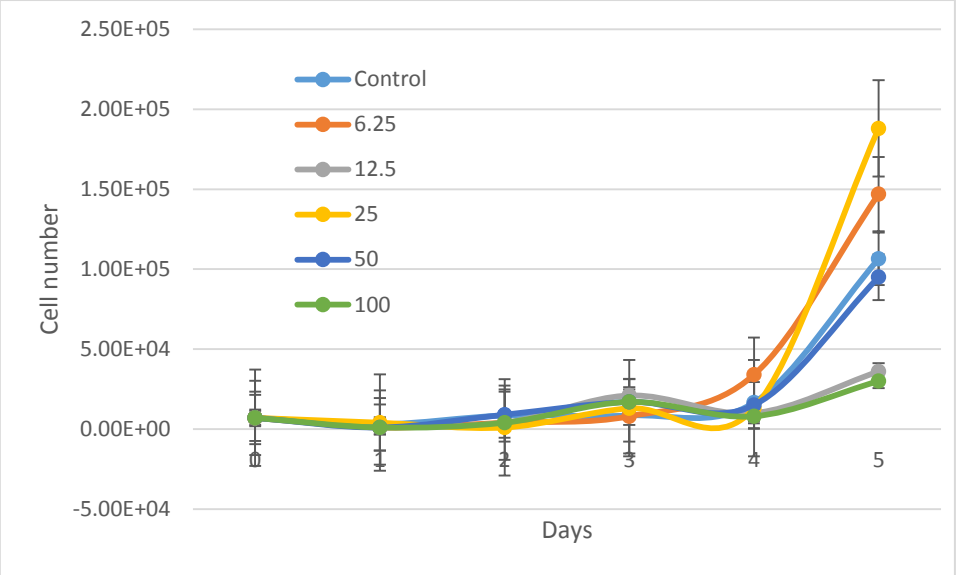

Figure 3: HeLa cell growth exposed to various concentrations of 60oC Kawakawa extract. Error bars showing S.E.M.

To compare cell growth across treatments the rate of cell growth was calculated during the exponential growth phase between days 4 and 5. The slopes for HeLa cells exposed to 60^{O}C Kawakawa extract concentrations were taken from the graph over this time period and shown in *figure 4*. The slopes for HeLa cells exposed to room temperature Kawakawa extract concentrations were taken from the graph over the same time period and is shown in *figure 5*. The gradient of the lines of best fit for HeLa cells at 60^{O}C are expressed in *table 4*. The gradient of the lines of best fit for HeLa cells at room temperature are expressed in *table 5*.

The degree of growth rate inhibition was then determined as shown in *figure 6*. Strong growth inhibition was exhibited by HeLa cells treated with room temperature Kawakawa leave extracts at concentrations of 6.25, 12.5, 25 and 50 ug/ml (97.8%,

96.7%, 85.5%,102%). Slight growth inhibition was exhibited by HeLa cells treated with 100 ug/ml concentration (51%) as shown in *figure* 6.

Strong growth inhibition was exhibited by HeLa cells treated with 60^{O}C Kawakawa leave extracts at concentrations 12.5 and 100 ug/ml (71.1% and 75%). Weak growth inhibition was exhibited by HeLa cells treated with 100 ug/ml concentration (75.5%). Interestingly, strong growth stimulation was exhibited by HeLa cells treated with 60^{O}C kawakawa leave extract 25 ug/ml (91.1%). Slight growth stimulation was exhibited by HeLa cells treated with 6.25 ug/ml (25.5%) as shown in *figure* 6.

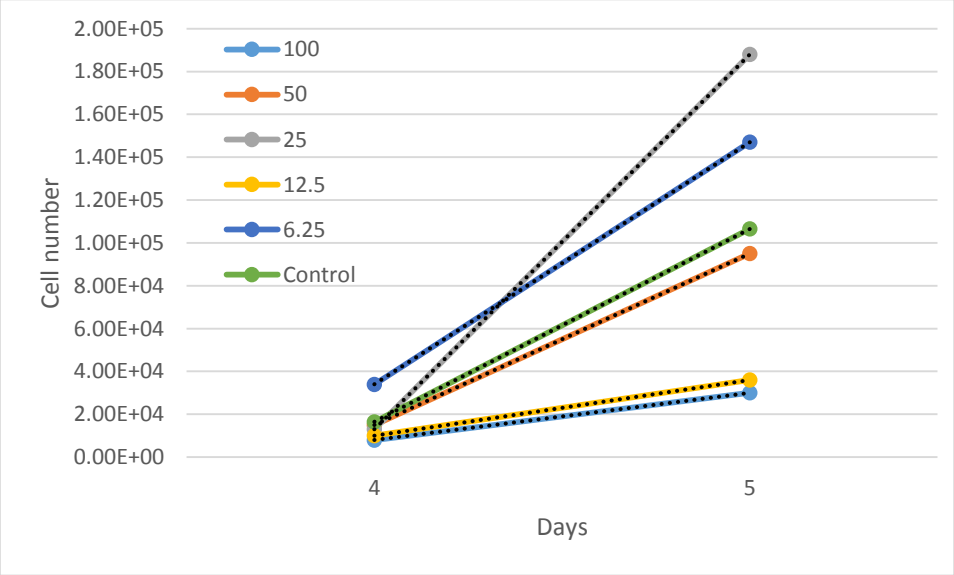

Figure 4: Cell growth of HeLa cells exposed to 60^{O}C Kawakawa extract with lines of best fit.

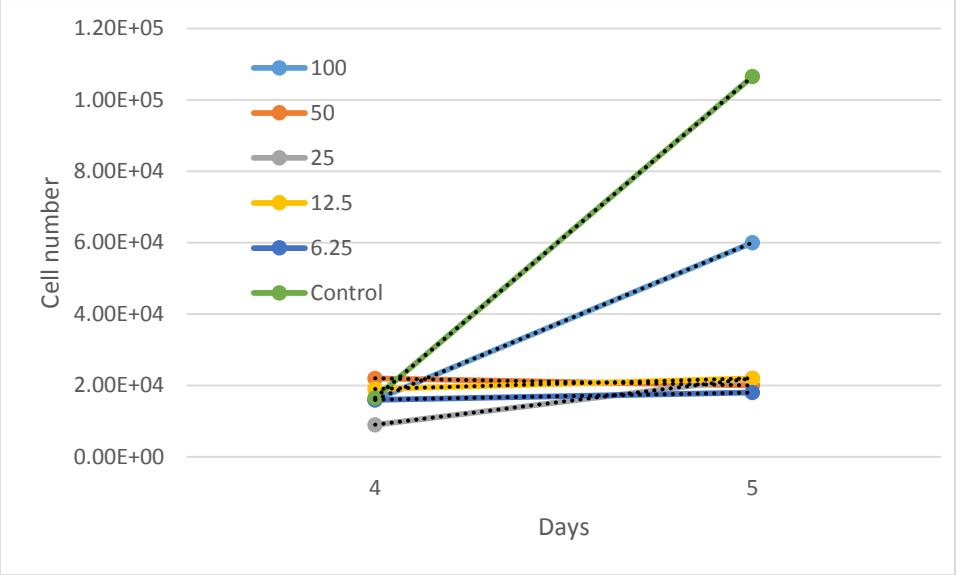

Figure 5: Cell growth of HeLa cells exposed to room temperature Kawakawa extract with lines of best fit.

Table 4: Gradients of lines of best fit from Figure 13 relative to control.

ug/ml	HeLa 60
Control	1.00
6.25	1.25
12.5	0.29
25	1.90
50	0.89
100	0.24

Table 5: Gradients of lines of best fit from Figure 14 relative to control.

ug/ml	HeLa RT
Control	1.00
6.25	0.02
12.5	0.03
25	0.14
50	-0.02
100	0.49

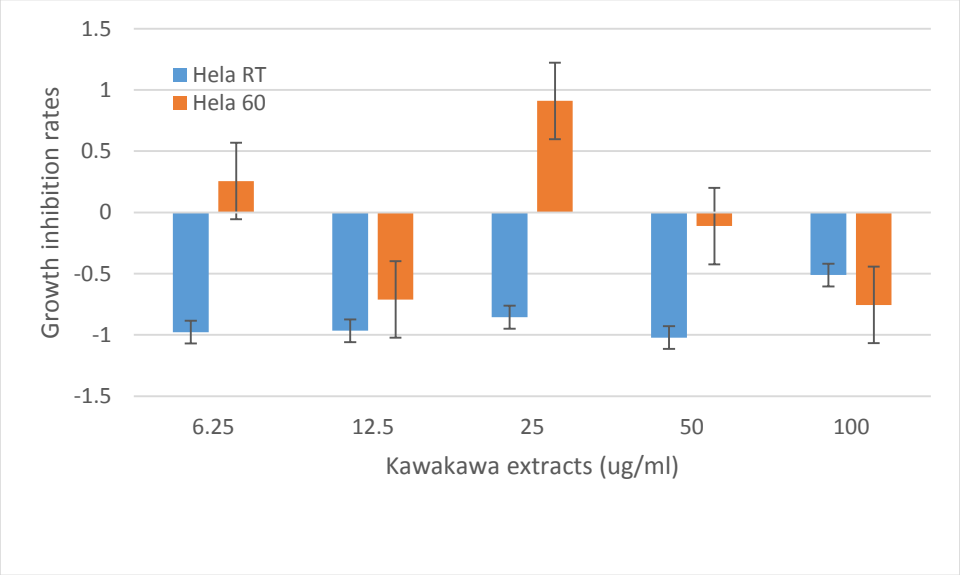

Figure 6: Rates of growth inhibition relative to control in Kawakawa extracts.

5.4 Dose Response of THP-1 cells when exposed to Kawakawa extracts

THP-1 cells were used as they are a cell line that can elicit an inflammatory reaction when exposed to LPS. THP-1 cells were exposed to varying concentrations of Kawakawa extracts prepared at room temperature or 60ºC.

The growth rate of THP-1 cells exposed to room temperature Kawakawa extracts at varying concentrations over a 5 day period is shown in *Figure* 7. THP-1 cells treated with kawakawa extracts and the untreated control cells exhibited a lag-phase during days 1-4. The kawakawa extract concentration (12.5, 25, 50 and 100 µg/ml) led to a marked inhibition of cell growth during day 4-5 compared to the untreated control cells, which had entered the exponential growth phase during this period. The kawakawa extract concentration (6.25 µg/ml) led to a marked stimulation of cell growth during day 4-5 compared to the untreated control cells. This experiment had an N=1 and the error bars represent (±SEM) of cell counts from two wells.

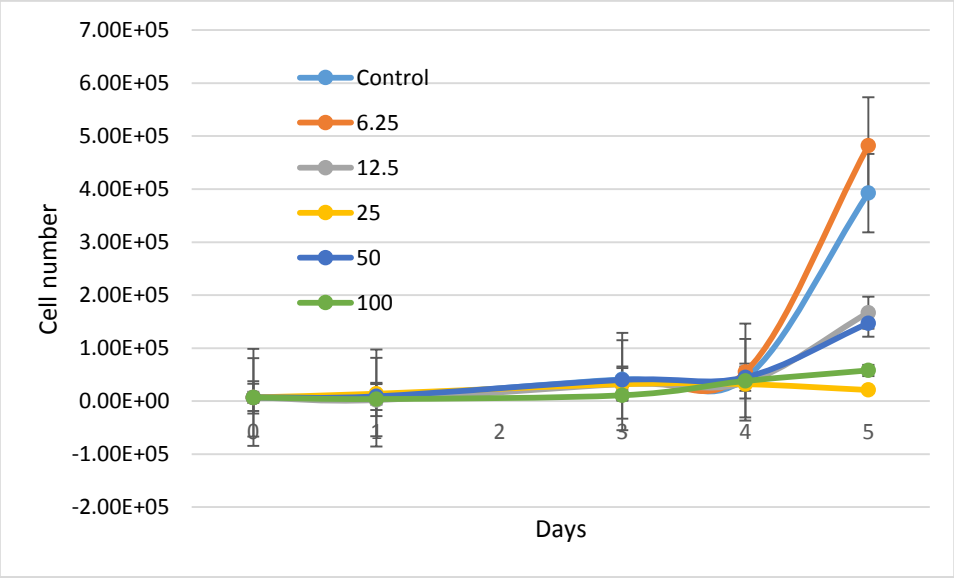

Figure 7: THP-1 cell growth exposed to various concentrations of room temperature Kawakawa extract. Error bars showing S.E.M.

The growth rate of THP-1 cells exposed to 60^{O}C Kawakawa extract at varying concentrations over a 5 day period is shown in *Figure* 8. THP-1 cells treated with kawakawa extracts and the untreated control cells exhibited a lag-phase during days 1-4. The kawakawa extract concentration (6.25 12.5 and 100 µg/ml) led to a marked inhibition of cell growth during day 4-5 compared to the untreated control cells, which had entered the exponential growth phase during this period. The kawakawa extract concentration (25 µg/ml) led to a marked stimulation of cell growth during day 4-5 compared to the untreated control cells.

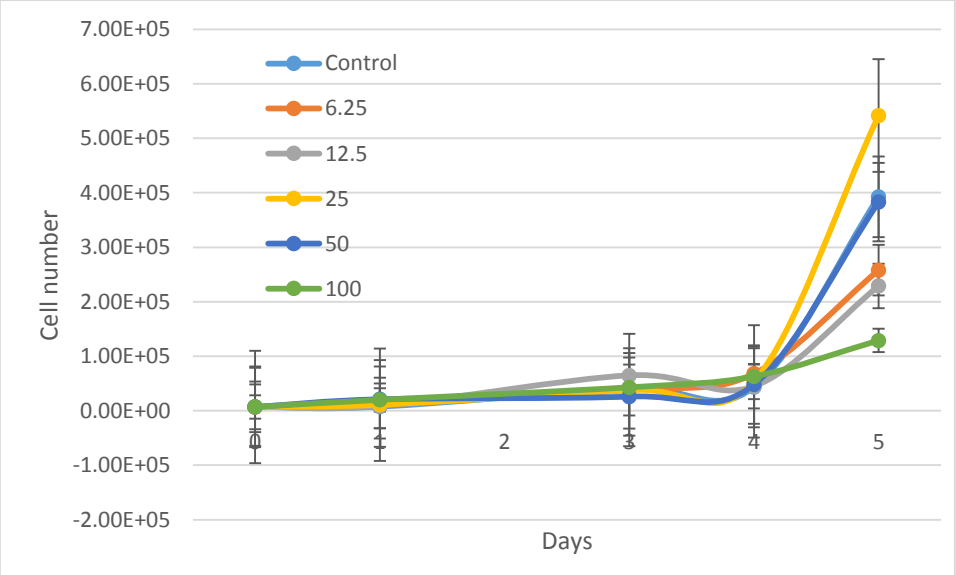

Figure 8: THP-1 cell growth exposed to various concentrations of 60^{O}C Kawakawa extract. Error bars showing S.E.M.

To compare cell growth across treatments the rate of cell growth was calculated during the exponential growth phase between days 4 and 5. The slopes for THP-1 cells exposed to 60^{O}C Kawakawa extract concentrations were taken from the graph over this time period as shown in *figure 9*. The slopes for THP-1 cells exposed to

room temperature Kawakawa extract concentrations were taken from the graph over the same time period as shown in *figure 10*. The gradients of the lines of best fit for THP-1 cells at 60^{O}C are expressed in *table 6*. The gradients of the lines of best fit for THP-1 cells at room temperature are expressed in *table 7*.

The degree of growth rate inhibition was then determined as shown in *figure 11*. Strong growth inhibition was seen on THP-1 cells treated with 60^{O}C Kawakawa leave extracts at concentrations of 6.25, 12.5 and 100 ug/ml (45.5%, 47.3% and 81.1%). Weak growth inhibition was seen on THP-1 cells treated with 50 ug/ml (4.0%) as seen in *figure* 11.

Interestingly, strong growth stimulation was seen on THP-1 cells treated with 60^{O}C kawakawa leave extracts 25 ug/ml (39.8%). Slight growth stimulation was seen on THP-1 cells treated with room temperature Kawakawa leave extracts at the concentration 6.25 ug/ml (22.3%) as seen in *figure* 11.

Strong growth inhibition was seen on THP-1 cells treated with room temperature Kawakawa leave extracts at concentrations 12.5, 25, 50 and 100 ug/ml (62.2%, 103.2%, 70.8%, 94.3%) as seen in *figure* 11.

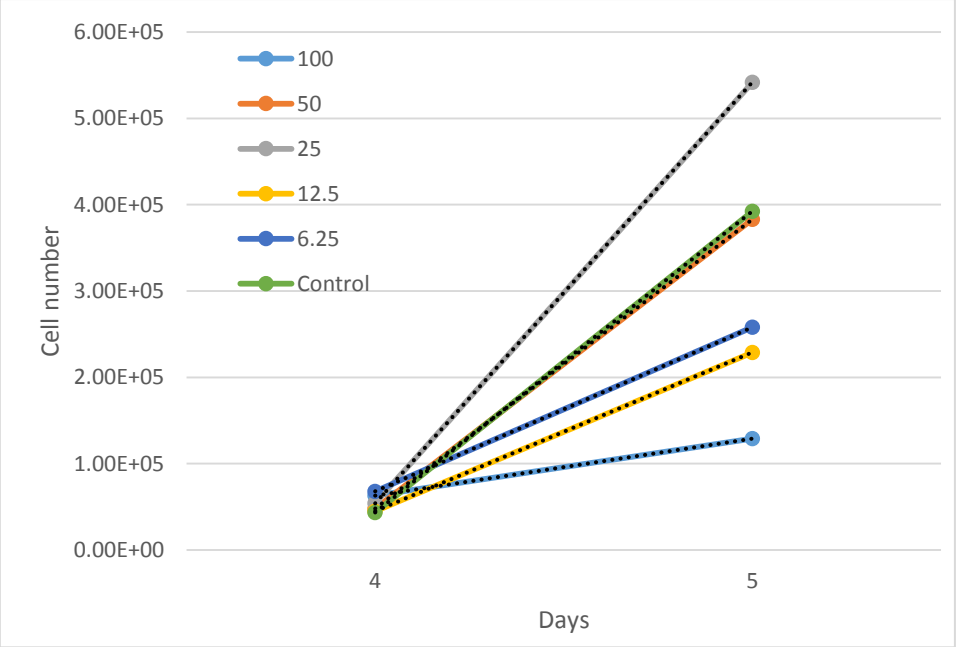

Figure 9: Cell growth of THP-1 cells exposed to 60^{O}C Kawakawa extract with lines of best fit.

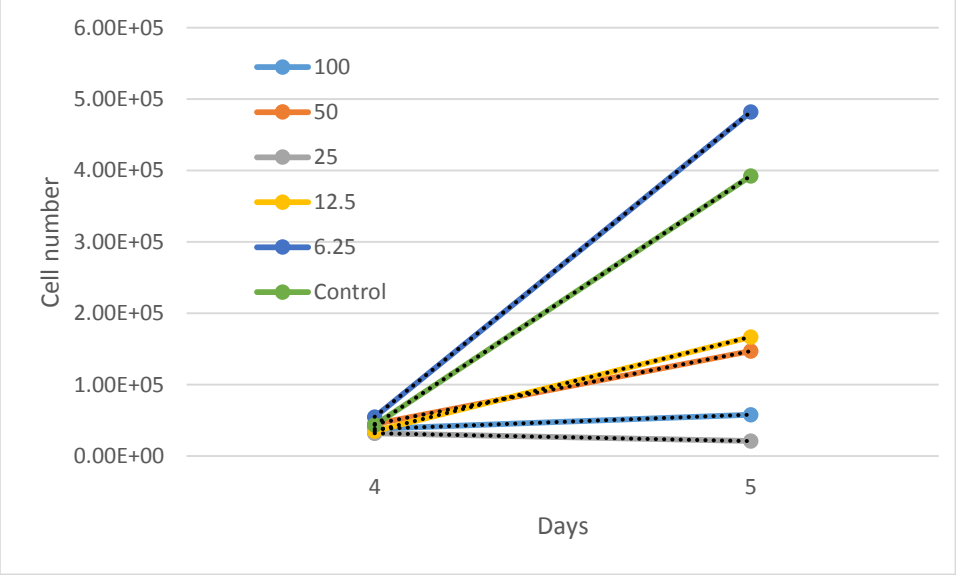

Figure 10: Cell growth of THP-1 cells exposed to room temperature Kawakawa extract with lines of best fit.

Table 6: Gradients of lines of best fit from Figure 18 relative to control.

ug/ml	THP-1 60
Control	1.00
6.25	0.54
12.5	0.52
25	1.40
50	0.96
100	0.19

Table 7: Gradients of lines of best fit from Figure 19 relative to control.

ug/ml	THP-1 RT
Control	1.00
6.25	1.22
12.5	0.38
25	-0.03
50	0.29
100	0.06

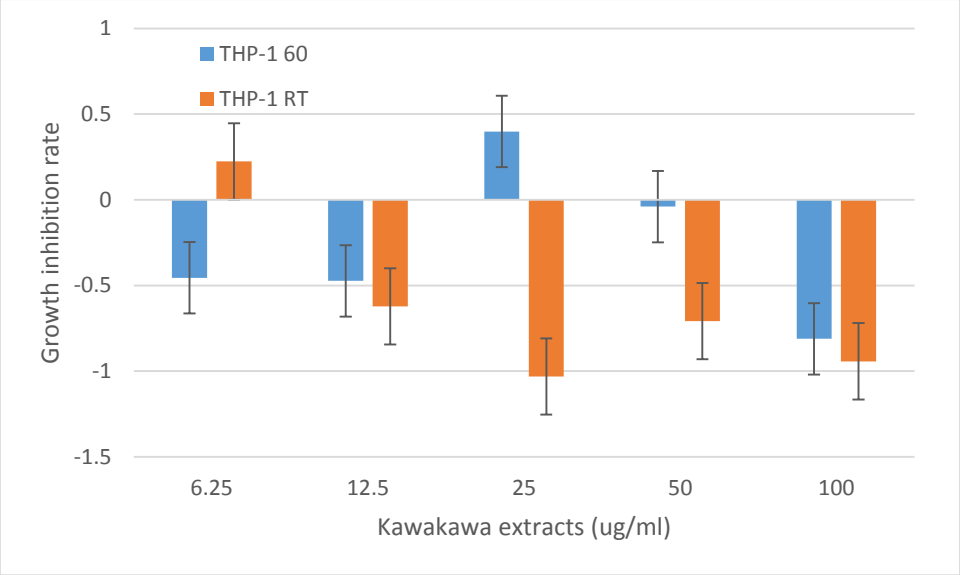

Figure 11: Rates of growth inhibition relative to control in Kawakawa extracts.

5.5. Discussion

The objective of this section was to assess the effect of Kawakawa leave extracts had on the growth of HeLa and THP-1 cells. HeLa and THP-1 cells were incubated with a range of Kawakawa leave extract concentrations, producing both inhibitory and stimulatory effects on cell growth as seen in *figure 6* and *figure 11*.

The main findings this study found was that kawakawa extracts exhibited both stimulatory and inhibitory effects on cell cultures.

Various bioactive compounds such as diayangambin, excelsin, epiexcelsin, Myristicin and demethoxyexcelsin have been isolated from kawakawa leaves. *Figure* 12 shows the chemical structures of Myristicin and Diayangambin. (Richardson, 2015; Russell, 1973).

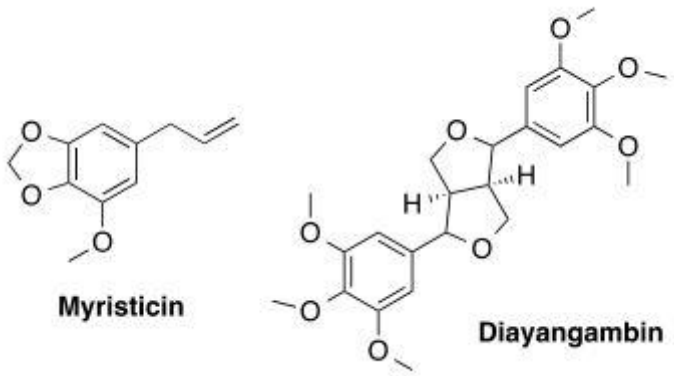

Figure 12: Chemical structures of Myristicin and Diayangambin

In the literature myristicin has been shown to be hepatoprotective. Alanine aminotransferase (ALT) and asparate aminotransferase (AST) are typical markers of liver damage. The levels of the activities for these markers were suppressed by

myristicin at 50, 100 or 200 mg/kg in mice having liver injury induced by LPS (Morita, 2003). As myristicin is a biomolecule found in kawakawa leave extract, this might be the reason for the increase in cell growth seen in *figure 6* and *figure 11*.

In the literature, myristicin has also been found to display apoptotic activity in human leukaemia K562 cells. Apoptotic activity can been characterised by changes in mitochondrial membrane potential, chytochrome c release, caspase- 3 activation, PARP- cleavage and DNA fragmentation (Martins, 2014).

One of the early features of the intrinsic apoptotic pathway is the dissipation of the mitochondrial transmembrane potential which then leads to the release of cytochrome-c. Cytochrome c is thought to be a prominent pro-apoptotic signalling protein that is released from the mitochondria (Martins, 2014).

The bioactive compounds noted above were not isolated and identified in this study. Further characterisation of the compounds present in kawakawa extracts at room temperature and 60^{O}C need to be undertaken.

The results presented here are n=1 and because of this, dose response experiments need to be repeated to determine the validity of trends and reproducibility.

In order to determine if the growth inhibition of the kawakawa extracts was due to either necrotic or apoptotic processes, LDH and MTT assays were undertaken as described in section **5.6 Effects of kawakawa extracts on HeLa and THP-1 cell function**.

5.6 Effects of Kawakawa extracts on HeLa and THP-1 cell function

Since some of the Kawakawa extracts were shown to be inhibitory to cell growth it was decided to see if this inhibition was due to necrotic cell death. To help illustrate the functioning of cells exposed to treatment biochemical assays are used.

Lactate dehydrogenase (LDH) is a strict intracellular enzyme that is only released into the media when the cellular membrane ruptures. The LDH assay was used to determine if the cytotoxicity of kawakawa leave extracts on HeLa and THP-1 cells was due necrotic cell death.

5.7 Results

5.7.1 LDH Assays

The percentage of lysed cells was worked out by dividing the experimental samples with the maximum release of cells (this was obtained by freezing the cells overnight at -20^{O}C) shown by equation 1 in section **2.2.2 LDH assay**. The THP-1 cells without extracts (control sample) had 37.2% lysed cells and was not significantly different from the 6.25 and 100 ug/ml room temperature kawakawa extract groups which had 35.61 and 36.93% of lysed cells. However, treatment with 12.5, 25 and 50 ug/ml room temperature kawakawa extract resulted in decreased percentage of lysed cells compared to the control (28.4, 32.7 and 30.7 %) shown in *figure* 13.

The THP-1 cells without extracts (control sample) had 37.2% lysed cells and was not significantly different from the 50 and 100 ug/ml 60^{O}C kawakawa extract groups which had 33.74 and 34.02% of lysed cells. Treatment with 6.25, 12.5 and 25 ug/ml 60^{O}C

kawakawa extract resulted in a slightly decreased percentage of lysed cells compared to the control (32.8, 32.4 and 30.74 %) shown in *figure* 13. This experiment had an N=1 and the error bars represent (±SEM) of cell counts from two wells.

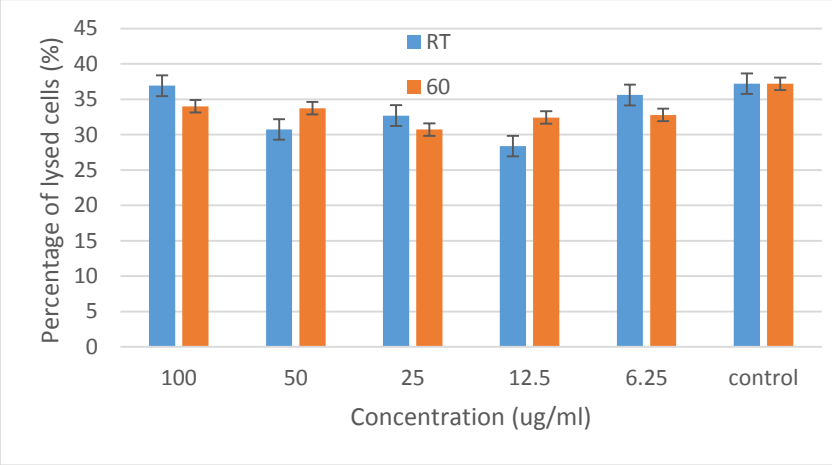

Figure 13: Showing the percentage of lysed THP-1 cells after 48 hours. Error bars show S.E.M.

The HeLa cells without extracts (control sample) had 75.86% lysed cells, because of the high level of dead cells in the control the results are difficult to interpret. The treatment with 6.25, 12.5, 25 and 100 ug/ml room temperature kawakawa extract resulted in decreased percentage of lysed cells compared to the control (74.04, 72.18, 74.53 and 74.93 %) shown in *figure* 14. Treatment with 50 ug/ml room temperature kawakawa extract resulted in increased percentage of lysed cells compared to the control (78.73 %) shown in *figure* 14. This experiment had an N=1 and the error bars represent (±SEM) of cell counts from two wells.

The control sample had 75.86% lysed cells and was not significantly different from the 6.25 and 12.5 ug/ml 60^{O}C kawakawa extract groups which had 76.3 and 76.7%

of lysed cells. However, treatment with 50 ug/ml 60^{O}C kawakawa extract resulted in decreased percentage of lysed cells compared to the control (71.3%) shown in *figure* 14. Interestingly, treatment with 25 and 100 ug/ml 60^{O}C kawakawa extract resulted in a slight increase in percentage of lysed cells when compared to the control (77.19 and 78.48%).

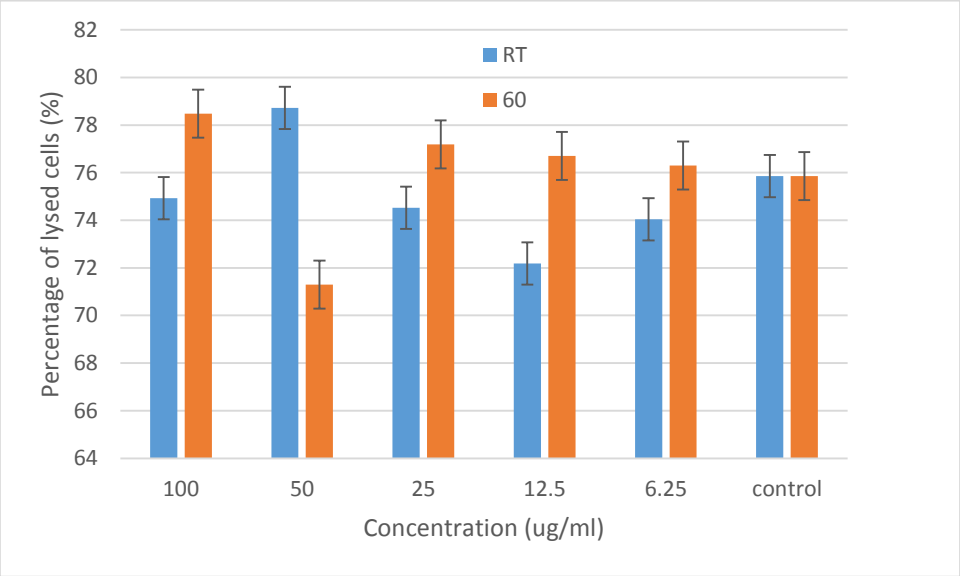

Figure 14: Showing the percentage of lysed HeLa cells (%) after 48 hours. Error bars show S.E.M.

The LDH assay detects how intact the cellular membrane is by measuring the release of the cytosolic enzyme LDH into extracellular space detected when the cell membrane is no longer intact and is ruptured. The LDH assay involves the enzymatic conversion of a tetrazolium salt, INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride], into a water soluble red formazan product. As this is a colourmetric assay, the amount of colour seen relates to the amount of cell lysis.

The LDH assay showed that, THP-1 cells treated with 60^{O}C and room temperature Kawakawa leave extracts compared to the control sample did not have increases of cell lysis after 48 hours. This data suggests that cellular death is not caused by necrosis mechanisms. However different growth rates are seen on HeLa cells and THP-1 cells exposed to various kawakawa extracts, an exponential growth phase is observed between days 4 and 5 see *figure* 2, *figure* 3, *figure* 7 and *figure* 8. LDH needs to be repeated at this time period.

5.7.2 MTT Assays

Given that cell growth inhibition was unlikely to be due to necrosis (see LDH data) it was decided to see if cell death was caused by mitochondrial processes that would further support apoptotic cell death. The biochemical assay MTT was used to indirectly measure mitochondrial dehydrogenase activity in the presence of kawakawa leave extracts.

For each day of the five-day experiment, HeLa cells and THP-1 cells were incubated with MTT for 45 minutes to 1 hr. After this time, the formazan crystals were solubilised, the absorbance was read and the results were presented as a percentage of mitochondrial dehydrogenase activity that was then compared to the control samples. Particular interest was in the MTT results seen at day 5 as this was when the cells entered the exponential phase.

THP-1 cells that are not exposed to kawakawa extract (the control) are assumed to demonstrate maximum mitochondrial activity (100%). Treatment with 50 and 100 ug/ml room temperature kawakawa extract resulted in decreased percentage of

mitochondrial activity compared to the control (90.23 and 85.0 %) shown in *figure* 15. Treatment with 12.5 and 25 ug/ml room temperature kawakawa extract resulted in significantly increased percentage of mitochondrial activity compared to the control (140.8% and 104.02 %) shown in *figure* 15. This experiment had an N=1 and the error bars represent (±SEM) of cell counts from two wells.

The control THP-1 cells was not largely different from the 100 ug/ml 60^{O}C kawakawa extract which had 92% of mitochondrial activity. However, treatment with 6.25, 12.5, 25 and 50 ug/ml 60^{O}C kawakawa extract resulted in decreased percentage of mitochondrial activity compared to the control (78.3, 73.0, 72.12 and 79.65%) shown in *figure* 15.

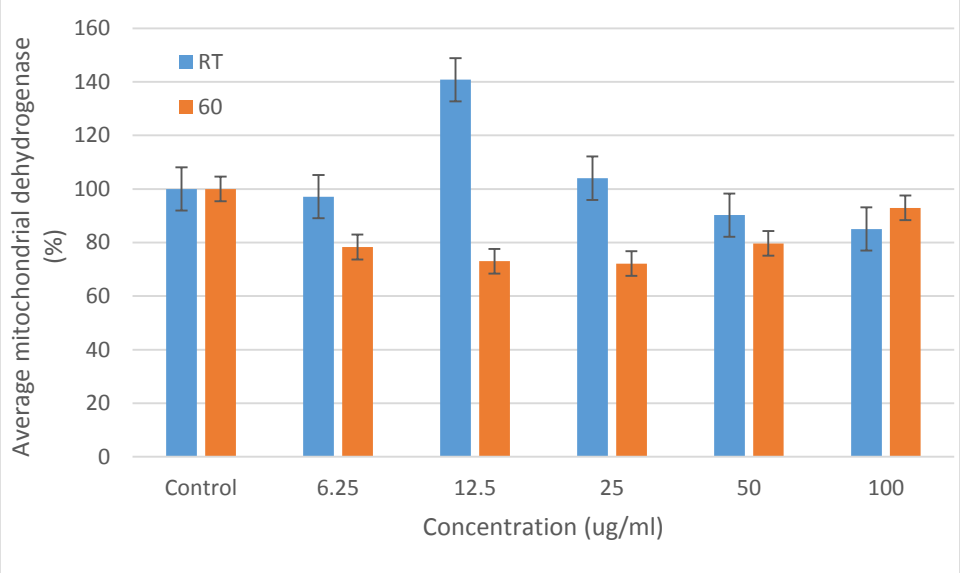

Figure 15: Shows the average percentage of mitochondrial dehydrogenase (%) produced by THP-1 cells for day 5. Error bars show S.E.M.

HeLa cells that have not been exposed to kawakawa extracts (the control) demonstrate mitochondrial activity of 100%, when compared to treatment with 12.5, 25, 50 and 100 ug/ml room temperature kawakawa extract resulted in slight decreased percentage of mitochondrial activity compared to the control (97.5, 98.7, 98.7 and 96.97%) shown in *figure* 16. Treatment with 6.25 ug/ml room temperature kawakawa extract resulted in a percentage of mitochondrial activity comparable to the control (100.53 %) shown in *figure* 16.

Treatment of HeLa cells with 6.25, 12.5, 25 and 50 ug/ml 60^{O}C kawakawa extract resulted in decreased percentage of mitochondrial activity compared to the control (96.4, 97.8, 98.7 and 99.5%) shown in *figure* 25. Treatment with 100 ug/ml 60^{O}C kawakawa extract resulted in an increased percentage of mitochondrial activity compared to the control (109.17 %) shown in *figure* 16.

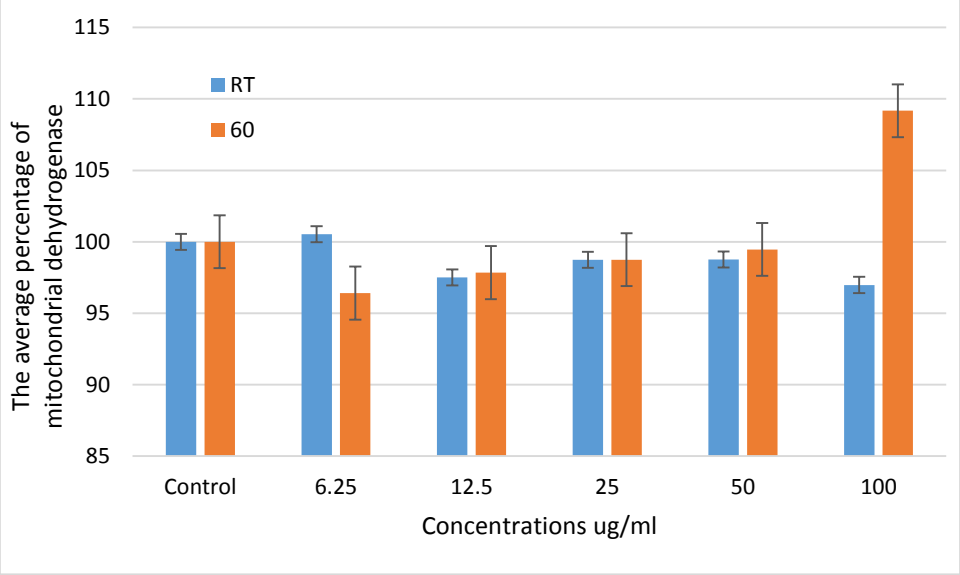

Figure 16: Shows the average percentage of mitochondrial dehydrogenase (%) produced by HeLa cells for day 5. Error bars show S.E.M.

The control THP-1 sample when compared to treatment with 6.25 ug/ml room temperature kawakawa extract resulted in decreased percentage of mitochondrial activity compared to the control (95.86%) shown in *figure* 17. Treatment with 12.5, 25, 50 and 100 ug/ml room temperature kawakawa extract resulted in an increased percentage of mitochondrial activity compared to the control (107.76, 103.11, 102.6 and 102%) shown in *figure* 26.

Treatment with 6.25, 12.5, 25 and 50 ug/ml 60^{O}C kawakawa extract resulted in decreased percentage of mitochondrial activity compared to the control (90.42, 88.89, 90.89 and 93.85%) shown in *figure* 17. Treatment with 100 ug/ml 60^{O}C kawakawa extract resulted in a percentage of mitochondrial activity comparable to the control (99.4%) shown in *figure* 17. This experiment had an N=1 and the error bars represent (±SEM) of cell counts from two wells.

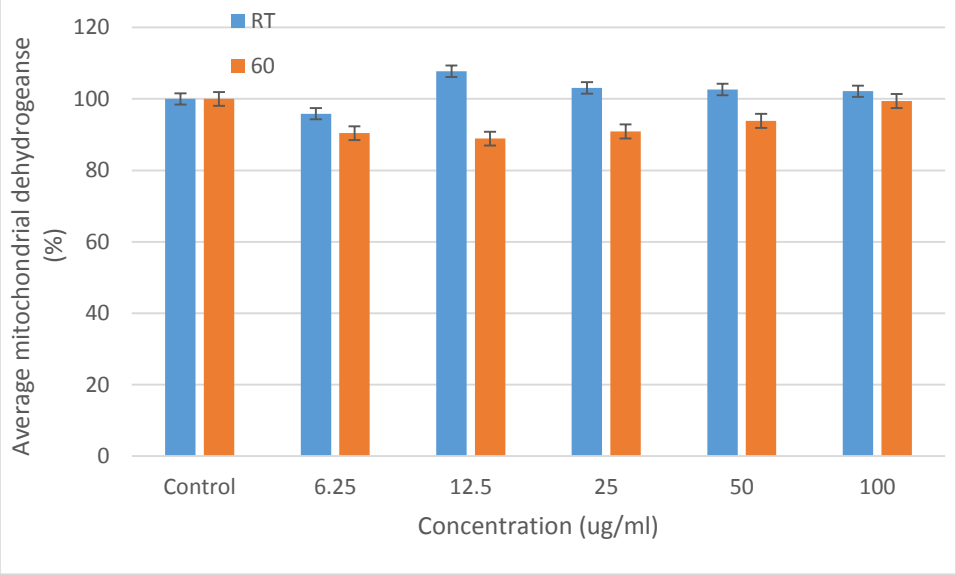

Figure 17: Showing the average mitochondrial dehydrogenase activity produced over 5 days by THP-1 cells when exposed to varying Kawakawa extract concentrations. Error bars showing S.E.M.

The control sample for HeLa cells when compared to treatment with 6.25, 12.5, 25, 50 and 100 ug/ml room temperature kawakawa extract resulted in an increased percentage of mitochondrial activity (106.5, 106, 105.4 105.4 and 102.5%) shown in *figure* 18.

Treatment with 6.25 and 12.5ug/ml 60^{O}C kawakawa extract resulted in decreased percentage of mitochondrial activity compared to the control (98.1% and 98%) shown in *figure* 18. Treatment with 50 ug/ml 60^{O}C kawakawa extract resulted in a percentage of mitochondrial activity comparable to the control (100%) shown in *figure* 18. Treatment with 25 and 100 ug/ml 60^{O}C kawakawa extract resulted in an increased percentage of mitochondrial activity when compared to the control (101 and 102%) shown in *figure* 18.

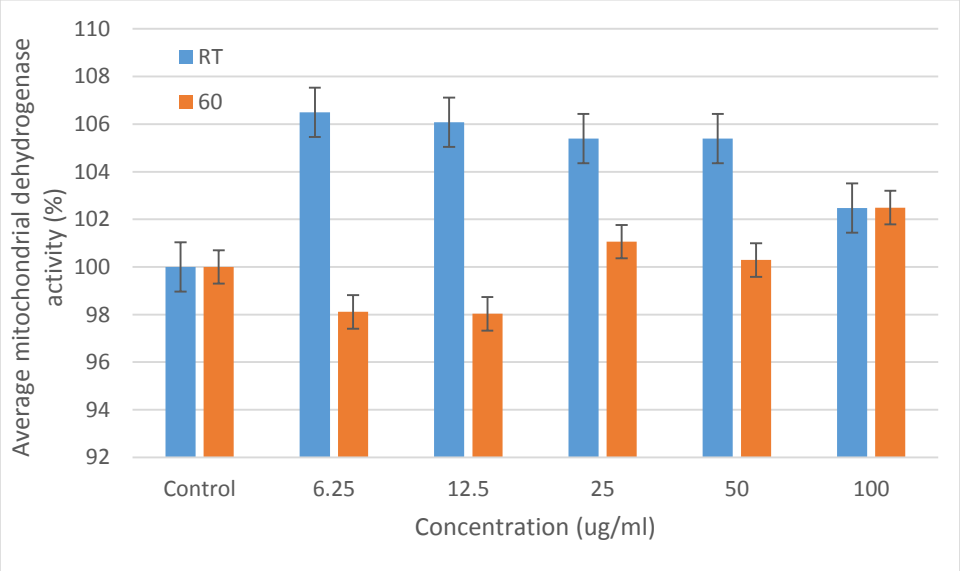

Figure 18: Showing the average mitochondrial dehydrogenase activity produced over 5 days by HeLa cells when exposed to varying Kawakawa extract concentrations. Error bars showing S.E.M.

To determine if the Kawakawa leave extracts had any effects on mitochondrial function, the MTT assay was used. This cell viability assay measures the reduction of MTT (a water-soluble tetrazolium dye) to formazan crystals that are insoluble. This reduction reaction relies on activity from living cells and their activity of functioning mitochondrial dehydrogenase. Therefore the MTT assay is an indicator of cell viability as well as mitochondrial activity.

The MTT assay showed that THP-1 cells treated with 60^{O}C and room temperature Kawakawa leaf extracts for five days led to increases and decreases in the average mitochondrial dehydrogenase activity. Treatment of THP-1 cells with 12.5 ug/ml room temperature kawakawa extract produced an increase in mitochondrial dehydrogenase activity when compared to the control and other treatments. Treatment of THP-1 cells with 100 ug/ml room temperature kawakawa extract produced a decrease in mitochondrial dehydrogenase activity when compared to the control and other treatments. Treatment of THP-1 cells with 60^{O}C kawakawa extract produced decreases in mitochondrial dehydrogenase activity when compared to the control. Treatment of THP-1 cells with 25 ug/ml 60^{O}C kawakawa extract produced the largest decrease in mitochondrial dehydrogenase activity when compared to the control and other treatments. Treatment of THP-1 cells with 100 ug/ml 60^{O}C kawakawa extract produced mitochondrial dehydrogenase activity comparable to or similar to the control.

MTT data for HeLa cells exposed to 60^{O}C and room temperature Kawakawa leave extracts for five days led to increases and decreases in the average mitochondrial dehydrogenase activity. Treatment of HeLa cells with 6.25 ug/ml room temperature kawakawa extract produced mitochondrial dehydrogenase activity similar to and comparable to the control. Treatment of HeLa cells with 12.5 ug/ml, 25 ug/ml 50

ug/ml and100 ug/ml room temperature kawakawa extract produced a decrease in mitochondrial dehydrogenase activity when compared to the control. Treatment of HeLa cells with 60^{O}C kawakawa extract produced decreases in mitochondrial dehydrogenase activity when compared to the control. Treatment of HeLa cells with 6.25 ug/ml 60^{O}C kawakawa extract produced the largest decrease in mitochondrial dehydrogenase activity when compared to the control and other treatments. Treatment of HeLa cells with 100 ug/ml 60^{O}C kawakawa extract increased mitochondrial dehydrogenase activity compared to the control and other treatments.

6 General discussion and future perspectives

One of the objectives in this study was to investigate the effects kawakawa leaf extracts had on cellular inflammation. However, before this could be achieved, preliminary experiments were conducted to find out if the extract was cytotoxic to mammalian cells.

Two main cell lines were used in this study, HeLa and THP-1 cell lines. HeLa cell lines are a standard laboratory cell line that has a quick culture time, this cell line is robust and many laboratory experiments can easily be carried out on the cells. THP-1 cells were used due to their inflammatory reaction with lipopolysaccharide (LPS). LPs is an endotoxin that is from gram negative bacteria and it is known to elicit a strong immune response. An inflammatory reaction via the TLR receptors in THP-1 cells can be induced by LPS allowing experiments to be run to monitor potential anti-inflammatory effects of compounds. LPS induces pro-inflammatory markers such as cytokines IL-6 and TNFa (Huber, 2001). LPS and kawakawa extracts at various concentrations were exposed to cell cultures were they would be incubated for at standard incubating conditions.

The objective of the section **5.6 Effects of Kawakawa extracts on HeLa and THP-1 cell function** was to assess the cytotoxicity that was induced by the Kawakawa leaf extract against HeLa and THP-1 cell lines. Cell growth data with both THP-1 and HeLa cells indicated that kawakawa leaf extracts were cytotoxic at different concentrations.

To assess the mechanism of cell death cytotoxicity assays like LDH and MTT were carried out to determine whether HeLa cells and THP-1 cells undergo apoptosis or

necrosis when exposed to kawakawa leaf extracts. The LDH assay is a colourmetric assay which detects membrane integrity by measuring the amount of cytosolic enzyme LDH released by the cells upon cell lysis. The LDH assay involves enzymatic conversion a tetrazolium salt, INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride], into a water-soluble red formazan product. The amount colour seen is proportional to the amount of cell lysis.

The most interesting finding of this study is when kawakawa extracts inhibit cell growth there is no increase in LDH. This is suggestive that the cellular membrane remains intact when exposed to the extract. The treatment of HeLa cells produced a more variable LDH dataset when compared to the dataset of THP-1 cells.

When comparing the LDH dataset with day two of the dose response curve of THP-1 cells there appears to be a slight correlation. As cell numbers increase, the LDH data decreases and as the cell numbers decrease, the LDH data increases. This correlation is better seen on THP-1 cells exposed to kawakawa extracts prepared at room temperature, see *figure* 16 and *figure* 23.

However as the HeLa cells without treatment expressed a high level of LDH the results are difficult to interpret, see *Figure* 24. One reason for this high level of LDH in the control might be attributed by mycoplasma contamination. Mycoplasma are small bacteria that are parasitic belonging to the class molicute; there are more than 180 species and they infect a large range of hosts. Mycoplasma contamination is one of the biggest issues in cellular research which does not often result in cellular death but it can be responsible for multiple effects seen in cultured cells. Such effects are seen to alter the cells' metabolism including slowed proliferation. This

might also be a reason that an abnormally long lag phase was observed in HeLa dose response curves. See *figure*s 11 and 12.

Mycoplasma contamination is difficult to detect and could compromise the accuracy of the data produced by infected cell lines. It can be detected via PCR that uses primers specific to mycoplasma which can then be amplified and loaded on to a gel (Dobrovolny, 2011; Olarerin-George, 2015).

The dose response curves and the LDH data did not strictly correlate which suggests that the membrane of the cells was intact and cell death was not due to necrosis. As cell death was unlikely to be due to necrosis, it was important to find out if the inhibition of cell growth was due to impaired mitochondrial activity. There was because of this, MTT assays were conducted to assess the mitochondrial activity of the cells. When cell number does not correlate with the increase of the MTT dataset this may suggest that the extract might increase mitochondrial activity and cellular metabolism without directly effecting cell number.

The MTT assay was used to measure the reduction of MTT, a water soluble tetrazolium dye, to insoluble formazan crystals. This reduction is dependent on the function of cellular dehydrogenases in living cells hence this assay is also an indicator of cell viability and mitochondria activity.

When comparing the MTT datasets and the dose response curves for THP-1 cell line there does not seem to be any correlation as cell numbers increase the mitochondrial activity decreases and because of this a correlation cannot be made. See *figure*s 16, 17, 24 and 26.

The MTT datasets for HeLa cell lines exposed to $60^{O}C$ kawakawa extract are similar to the THP-1 cell line and a correlation cannot be made. See *figure*s 11, 12, and 27.

Though the dataset for HeLa cells exposed to room temperature kawakawa extract shows some decreases in MTT activity when cell numbers decrease. See *figure* 11, 12, 25 and 27.

The presented MTT data shows the effects the kawakawa leaf extract had on mitochondrial activity. In order to fully demonstrate cell death by apoptosis, hallmark characteristics need to be measured. These include cytochrome C release, DNA laddering and caspase activation.

The dataset in this study is preliminary, and suggests that the kawakawa leaf extract can be both cytotoxic and provide growth stimulation. This is the first study to document such results.

However, the limitations of the dataset in this study is that the results are based on n=1 and in order to validate this studies results, repetition of the experiments is needed.

In order to investigate the anti-inflammatory properties of the kawakawa leaf extract, it is needed to demonstrate that agents like LPS are able to elicit and inflammatory response from THP-1 cells. THP-1 cells are a transformed cell line, and because of this, the inflammation reaction seen in THP-1 cells may not mirror inflammatory responses seen in primary microglial cells.

Because of this, it was decided that primary microglial cells would be isolated from rodent brain tissue. An attempt to isolate primary microglial cells was made so that experiments concerning the anti-inflammatory properties of kawakawa leaf extracts can be conducted.

In the future, experiments conducted in this study such as dose response curves, MTT and LDH need to be repeated in order to validate the results. Another future

objective is to isolate the bioactive molecules within the kawakawa leaf and address the anti-inflammatory properties of the extract by conducting RNA isolation and subsequent rt-PCR experiments.

6.1 Glial cell isolation

An objective of this study was to isolate and induce inflammation using LPS on primary microglial cells and to have an *in vitro* model set up to see how the kawakawa extracts modulate the inflammatory process.

A challenge to overcome when culturing primary microglial cells is avoiding bacterial contamination. Brain tissue was obtained from rats post mortem and it was important to avoid contamination. To overcome this issue, all equipment was autoclaved, the media and reagents used in this study was sterilised by using syringe filters and gloves were worn at all times.

Another challenge to overcome when culturing primary microglial cells is growing an adherent and viable cell culture. To overcome this issue, the vessels used to culture these cells were coated with polylysine. As primary microglial cells are an adherent cell line, they attach better when grown in vessels that are coated. Polylysine is used to coat cell culturing vessels and it acts as an attachment factor that improves cell adherence. It does this because of the interaction of the positively charged polymer and negatively charged cells.

Primary microglial cells were used as these are central nervous system cells that are involved in numerous neurodegenerative diseases and inflammation pathways.

After overcoming the challenges a stable culture from rat brain tissue was established. *Figure 19* shows cells that are dendritic, elongated in shape and adherent to the culture vessel; these cells were isolated *in vitro*. The method for isolating primary glial cells is seen in *chapter 3*.

After carrying out this procedure two things were altered, instead of making the initial suspension up to 7mls of completed growth media, this volume was brought down to 5mls. The other thing that was altered was increasing the amount of 70% percoll that was used was increased from 2mls to 4mls. These alterations were because of low yields of tissuel that was used in this study.

It is also important to identify the neuronal phenotype of the cells using antibodies such as CD11b/c. CD11b/c is found on chromosome 16 and is able to bind to all granulocytes and to dendritic cells, including cells with microglial morphology in the brain

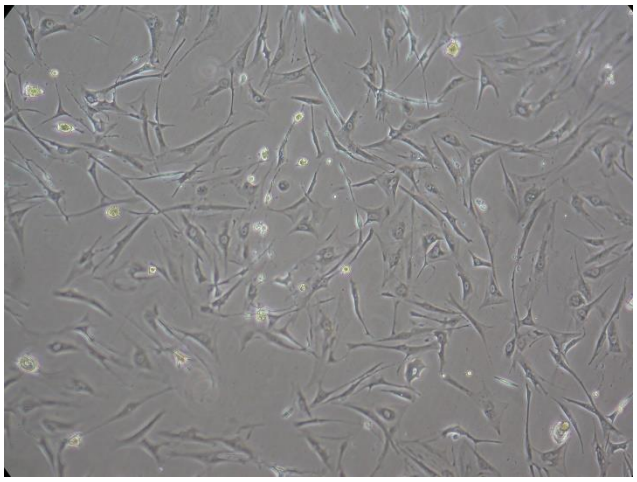

Figure 19: Showing cells isolated from a rat brain

In order to determine if the isolated cell culture in *figure* 28 contained primary microglial cells identification of the cells needs to be carried out via Immunocytochemistry (ICC). ICC involves fixing the cells to the slide, permeabilize these cells and incubate the slide and cells with the primary antibody followed by the secondary antibody. Stain the slide with DAPI and view the slide under florescent microscopy.

To address the anti-inflammatory properties of kawakawa leaf extracts on primary microglial cells, these cells would need to be exposed to both the extract and LPS. After 24 hours of exposing cells to LPS, RNA is needed to be extracted from the cultured cells. If good quality RNA is extracted (proven by running on an electrophoresis gel and nano drop results), the RNA would then be converted to cDNA via rt-PCR. A rt-PCR reaction with TNFa and IL-6 primers would be conducted and the product would then be run on an electrophoresis gel to compare band sizes. Experiments like western blotting can also be conducted in order to examine the kawakawa extract effect on inflammation by assessing protein levels.

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