

Reflective biotechnological report on the anti-inflammatory properties of Kawakawa leaf extracts

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Table of Abbreviations

TNF-α	Tumor Necrosis Factor-alpha	RPMI	Roswell Park Memorial Institute medium
IL-6	Interleukin-6	LPS	Lipopolysaccharide
NO	Nitric Oxide	MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

CMW extract	Methanol/water fraction of the chloroform-methanol-water extract	PBS	Phosphate Buffered Saline
Aqueous extract	Aqueous infusion extract	DMEM	Dulbecco Modified Eagle's Minimal medium
FBS	Fetal Bovine Serum		

1. Context & Issue

The issue for this project came to me on a family holiday in the South Island at Christmas 2011. We were on a bus tour on Farewell Spit and we drove past a grove of Kawakawa, and the bus driver pointed out that Māori thought Kawakawa was a very significant medicinal plant, yet the bus driver stated that there was no scientific evidence to support this.

This made me curious, because I was certain that someone would have conducted research into the medicinal properties of Kawakawa before.

It was at this point that I chose to pursue this issue further, do initial research and develop the initial brief. I chose to do this because this issue was of interest to me as members of my family were Māori, and I identified the opportunity to be able to fuse traditional Māori knowledge with biotechnological science to perhaps provide evidence to support the use of Kawakawa in rongoā. The intended biotechnological outcome would give credence to the medicinal properties of Kawakawa,  therefore support the traditional Māori uses of Kawakawa in rongoā.

2. Initial Research

When we returned to Auckland having searched the internet and having found nothing I searched the University of Auckland Library and found a single study that had tested Kawakawa. When seeking help, I found another study. Neither study showed the level of anti-bacterial or anti-viral activity expected given the significance of Kawakawa in rongoā.

2.1 Previous studies on Kawakawa

There are only 2 known studies of Kawakawa, and neither studied Kawakawa in great depth or identified significant anti-bacterial or anti-viral activity. As a result there is very little scientific evidence to support the claims that are made in rongoā.

The earliest study that tested the medicinal properties of Kawakawa was Antibiotic compounds from New Zealand plants. III: A survey of some New Zealand plants for antibiotic substances (Calder, Cole, & Walker, 1986). This study screened methanol based extracts of the Kawakawa leaf against a variety of bacteria. Kawakawa leaves were freeze dried and were stored at 4°C. 25 g portions of the dried Kawakawa were extracted with methanol using a Soxhlet continuous extractor. The extract was subsequently concentrated in-vacuo to 10 ml before use in an assay. This extract showed minor activity against *Staphylococcus aureus* and *Trichophyton mentagrophytes*, however this activity was significantly less than activity that was seen in other native plants (such as New Zealand passion fruit (*Tetrapathaea tetrandra*)). Anti-bacterial activity is one mechanism by which Kawakawa could act in rongoā.

A similar study, A survey of extracts of New Zealand indigenous plants for biological activity, was conducted (Bloor, 1995), but included viruses and cancers as well as bacteria. In this study any activity shown by Kawakawa was attributed to background tannins in the extracts rather than genuine bioactive compounds. In this study the Kawakawa was dried for 24 hours at temperatures between 30°C and 40°C. The dried Kawakawa, 50g-100g, was extracted twice for 24 hours each time with ethanol and water (in a 4:1 ratio) at room temperature. The extract was then dried in a vacuum at less than 40°C. Anti-bacterial and anti-viral activity are two mechanisms by which Kawakawa could act in rongoā.

There are limitations associated with these studies. Neither study used a method of extraction that reflected the traditional methods of extraction, nor does either specify the concentration at which Kawakawa was applied to cells, something that was done in this study and shown to be of significance. Both studies used organic solvents which may not be the best solvent to extract any bioactive compounds in Kawakawa. The study by Calder, Cole, & Walker (1986) used a method of extraction that didn't necessarily best preserve compounds in the Kawakawa as it used Soxhlet extraction. These limitations may have destroyed thermolabile compounds in the extracts. These compounds destroyed or damaged in this extraction process could be responsible for the activity of Kawakawa in rongoā. Additionally, extremely polar compounds which also may be responsible for the activity of Kawakawa in rongoā, may not be extracted by organic solvents.



These limitations mean that Kawakawa is still a plant of interest, given the mass of historical evidence available on the medicinal uses of Kawakawa.

2.2 Seeking Support

Due to the nature of the initial brief, it was going to be necessary to seek support. Approximately 20, scientific organisations were contacted seeking support.

Out of the organisations and groups contacted, only 2 responded. Jacque Bay at the University of Auckland had contacted others at the University of Auckland. Dr John Taylor at the School of Biological Sciences responded. Dr Taylor was chosen as a supervisor because it was going to be possible for me to conduct much of the testing myself in cell culture laboratories at the University of Auckland.

Ngā Pae o te Māramatanga was identified as a group who might be able to fund this work. The communication with Ngā Pae o te Māramatanga at this stage of the project, identified them as a likely client. The consultation with Ngā Pae o te Māramatanga helped to refine the brief.

It was important to get funding support due to the cost of the work proposed (the cost of the direct consumables and the costs for items necessary for the research such as time on equipment would be \$2520).

It was also important to get Māori support. This is because the project was premised in the traditional uses of Kawakawa in rongoā. It was therefore important to understand rongoā and the traditional uses of Kawakawa in rongoā. In order to contact Kaumātua and other credible Māori sources it was beneficial to have the support of an organisation such as Ngā Pae o te Māramatanga which is an organisation that Māori respect. Later on in the project the support of Ngā Pae o te Māramatanga, as a credible, important Māori research organisation, enabled me to talk to Professor Mike Walker, Dr Rebekah Fuller, Dr Joe TeRito and Dr Marilyn McPherson.

	Paul Davis	1/05/2012
	Chris Ryan	10/04/2012
	Chris Ryan	19/03/2012
	Paul Davis	8/03/2012
	Jacque Bay	3/03/2012
	Jacque Bay	7/03/2012
	David Talbot	7/03/2012
	John Shaw	6/03/2012
	Paul Davis	5/03/2012
	Call2	5/03/2012
	Paula Jameson	4/03/2012
	Karl Crawford	2/03/2012
	Paul Davis	1/05/2012
	Arvind Varsani	1/03/2012
	Hall, Simon	28/02/2012
	Laura Bennet	27/02/2012
	Jacque Bay	26/02/2012
	Gillian Lewis	25/02/2012
	Michelle McConnell	21/02/2012

Figure 1: A list of some of the emails sent to scientific organisations seeking support.

2.3 Initial Brief

Brief statement: To test the anti-viral and anti-bacterial properties of Kawakawa to explain the uses of Kawakawa in rongoā. The outcome will be a Kawakawa extract(s) that demonstrates the anti-viral and anti-bacterial activity, as well as a system for testing the anti-viral and anti-bacterial properties of the Kawakawa extract(s) (or any other plant extract).

Questions to answer: The project would look to address the following questions:

- a) Is there merit to the historical references of Kawakawa curing a variety of ailments?
 - To cures colds
 - To reduce or stop the growth of cancer tumours
 - For urinary complaints
 - For cuts & wounds
 - For gonorrhoea
 - For toothaches
 - For rheumatic pains
 - As a dressing for “bad” wounds
 - As a blood purifier
- b) Do leaves from the Kawakawa bush have antibacterial or antiviral activity?
 - If there is activity & how great is that activity in comparison to penicillin?
 - Is there still activity even when the leaves are exposed to strains that are resistant to penicillin?
- c) Do specifics mentioned in the historical evidence have weight? For example; is there enhanced activity in the leaves that have been chewed by the caterpillars that live on the Kawakawa bush or if the leaves grew in day long sunlight? Why is this?
- d) Can the active ingredient(s) in the Kawakawa leaves be identified and can these ingredients be isolated?

Specifications:

- To justify through biochemical testing and research the belief in rongoā that Kawakawa has medicinal activity.
- A Kawakawa extract that has anti-viral and anti-bacterial activity.
- Kawakawa extracts are not cytotoxic when tested in the MTT cell viability assay.
- Kawakawa extracts demonstrate reductions in the production of bacteria and viruses.

2.4 Challenges with this brief

I was unaware of what each of the assays entailed. This means that the work proposed was very ambitious and scientifically, very complex.

This initial brief was very broad in its scope. As the project progressed it became clear that focussing the research on one area and keeping the other aspects of the project (the extractions and the cell viability tests) as simple as possible would lead to a technological outcome of a higher quality.

Justification for the use of standard methodologies

from the start it was clear that standard methodologies would be needed to conduct the biological assays regardless of whether these were the cell viability testing, anti-viral testing, anti-bacterial testing or the anti-inflammatory testing).

The use of standard methodologies is considered to be good scientific practice. This is because if new, novel methods are developed for testing a well understood parameter (such as NO production, TNF- α production and IL-6 production) there is a significant amount of work (for example a PhD student would spend 3 years of their doctorate developing a new method for testing NO production) involved in the validation of this methodology.

A standard methodology that has been previously validated was used to ensure that the results of this study were reliable and there was no question as to whether the results were dependant on the validity of the method.

Furthermore, it would not have been possible to develop a new, novel methodology within the timeframe of the project and with my level of skill.



3. First Brief Refinement

3.1 Brief statement

To explore the anti-viral properties of water and ethyl acetate extracts of the Kawakawa leaf. To explore the anti-inflammatory properties of water and ethyl acetate extracts of the Kawakawa leaf.

3.2 Specifications

1. To create viable extracts from the Kawakawa using ethyl acetate and water as solvents.
2. To conduct the anti-viral assay.
3. To conduct the anti-inflammatory assay.

3.3 Key Stages

Preparation for the use of Kawakawa in the biological assays

- a. Establishing the solvents and methods to be used in the extraction of the Kawakawa and the biological assays
- b. Gain an understanding of the methodology that will be used.

Preparation with regards to rongoā

- a. Gain an understanding of what rongoā is and protocols of rongoā surrounding the collection, preparation and use of plant material.

Laboratory Work

- a. Collect and prepare the Kawakawa for extraction
- b. Extract the Kawakawa
- c. Conduct the cell viability assay (the MTT assay)
- d. Conduct the anti-inflammatory assay
- e. Conduct the anti-viral assay

Consultation was undertaken with key advisors:

- Professor Mike Walker, Dr Rebekah Fuller, Dr Joe TeRito and Dr Marilyn McPherson were consulted on rongoā and the uses of Kawakawa in rongoā.

- Dr Brent Copp and Associate Professor David Greenwood were consulted on the extraction of Kawakawa
- Dr John Taylor, Carol Wang (Dr Taylors lab assistant)and Jennifer Kuehne (PhD student in Dr Taylors lab) provided assistance on the biochemical assays.

3.4 Who will be involved with each stage of the project

The extraction of the Kawakawa, the cell viability assay, the anti-inflammatory assay, and the anti-viral assay will be undertaken at laboratories in the School of Biological Sciences at the University of Auckland by the author with support from other members of the School of Biological Sciences supervised by Dr. John Taylor a senior lecturer in virology at the School of Biological Sciences at the University of Auckland.

The data analysis will be undertaken by the author but supervised by Dr. John Taylor.

3.5 Justification of the brief

- Anti-bacterial testing was covered in both of the previous scientific studies and so there was less of an opportunity to investigate in this area.
- Anti-inflammatory testing had never been conducted and so there was a very significant opportunity to come to new understandings about Kawakawa.
- The original brief was very ambitious, and given my level of skill and the amount of time available, it was going to be too much of a challenge to complete the original work. This is because discussions with Dr. John Taylor made me aware of how much work was involved with cell culture testing.

4. First Timeline

4.1 Initial Plan of Action

Description of action	Timeframe	Key anticipated issues	Key anticipated outcomes	Reflection
Have a discussion with Ngā Pae o te Māramatanga about how they can support me	August 2012	Ngā Pae o te Māramatanga cannot find anyone with suitable expertise to help me.	A better understanding of rongoā and the uses of Kawakawa in rongoā.	Professor Mike Walker, Dr Rebekah Fuller, Dr Joe TeRito and Dr Marilyn McPherson were successfully consulted on rongoā and the uses of Kawakawa in rongoā. This knowledge was very useful in the development of my technological outcome.
Collect and prepare the Kawakawa for extraction	27 th November 2012	If enough leaves that face east and have been chewed by caterpillars can be found.	Leaves that are suitable for extraction.	This stage was achieved, however later than intended and later brief refinements did not use the leaves on the east of the tree or chewed by caterpillars.

Extract the Kawakawa	28 th November 2012 – 9 th December 2012	The extract takes longer to prepare than anticipated.	A viable Kawakawa extract.	This took significantly longer than expected as the extractions took until early January
Conduct the Cell Viability Assay	3 rd December 2012 – 16 th December 2012	The Kawakawa extract kills the cells and therefore is not viable for use.	A Kawakawa extract that is shown not to compromise the functioning of normal cells.	Due to the delays in the extraction procedures, these took longer. Initially, just the MTT assay was proposed for use, however it became necessary to use the Flow Cytometry and Trypan Blue assays to test cell viability.
Conduct the anti-inflammatory assay	10 th December 2012– 23 rd December 2012 and 14 th January 2013 – 20 th January 2013	My level of skills are not developed enough to carry out these assays. No anti-inflammatory action is observed.	Insight into whether the observed actions of Kawakawa come from its ability to reduce symptoms through reducing the inflammation caused by the body's response to an infection.	Significant amounts of time were spent developing my skills. There were some changes to the anti-inflammatory assays conducted, and this assay was not conducted on schedule.
Conduct the anti-viral assay	21 st January 2013 – 3 rd February 2013	My level of skills are not developed enough to carry out these assays. No anti-viral activity is observed.	Insight into whether Kawakawa affects the ability of a virus to reproduce or whether it helps cells to fight a virus. This should provide insight into if Kawakawa has any anti-viral activity.	These assays were not conducted (see later justifications).
Progress Report for Gold CREST/ Ngā Pae o te Māramatanga	February/ March 2013	None.	Ngā Pae o te Māramatanga (the client) is up to date on the progress of the project.	Ngā Pae o te Māramatanga was aware of progress (despite the project running behind schedule).
Data Analysis	February/ March 2013	None.	An understanding of the results produced. This should enable understanding into the anti-inflammatory and anti-viral properties of Kawakawa.	This was conducted as the assays were conducted. I needed to develop the skills necessary to conduct the “T-test” which was used for statistical analysis and the methods needed to calculate the standard error of the mean of the samples.
Feedback to Ngā Pae o te Māramatanga about the outcomes of this study.	July 2013	None.	Ngā Pae o te Māramatanga has an understanding of what was done, the results and why these results are significant.	Ran behind schedule. Occurred successfully and Ngā Pae o te Māramatanga were pleased with the technological outcome.

4.2 Project Plan

School	Dates	Gold CREST	Expected Specific Breakdown of Time	Notes
School Holidays	September 3, 2012	September 9, 2012	Background Research	This time is obviously significant for preparation for and completion of NCEA Level 2 exams. Therefore no or minimal work will be done through this time.
	September 10, 2012	September 16, 2012		
	September 17, 2012	September 23, 2012		
	September 24, 2012	September 30, 2012		
	Holiday	October 1, 2012	October 7, 2012	
	October 8, 2012	October 14, 2012		
	October 15, 2012	October 21, 2012		
	October 22, 2012	October 28, 2012	Project Proposal (Due 26th October 2012)	
	October 29, 2012	November 4, 2012		
	November 5, 2012	November 11, 2012		
NCEA Level 2 Exams	November 12, 2012	November 18, 2012		Space and support are unavailable during this time, hence no work will realistically occur.
	November 19, 2012	November 25, 2012		
	November 26, 2012	December 2, 2012	Proposal Meeting (Due for completion by the 14th of December)	
	December 3, 2012	December 9, 2012		
	December 10, 2012	December 16, 2012		
	December 17, 2012	December 23, 2012		
	December 24, 2012	December 30, 2012		
	December 31, 2012	January 6, 2013	Christmas and New Years	
	Holiday	January 7, 2013	January 13, 2013	
	January 14, 2013	January 20, 2013		
School Starts	January 21, 2013	January 27, 2013	In the lab - Finish off the anti-inflammatory assay and begin the anti-viral assay	In the lab - Anti-viral assay In the lab - Anti-viral assay If necessary part time in the lab finishing off & begin the Data Analysis Data Analysis
	January 28, 2013	February 3, 2013	In the lab - Anti-viral assay	
	February 4, 2013	February 10, 2013	If necessary part time in the lab finishing off & begin the Data Analysis	
	February 11, 2013	February 17, 2013		
	February 18, 2013	February 24, 2013		
	February 25, 2013	March 3, 2013		
	March 4, 2013	March 10, 2013		

Scholarship Technology Report

5. Further Background Research

I needed to research a number of areas because I needed to understand:

- About rongoā.
- The status of Kawakawa in rongoā.
- How to extract bioactive compounds from plants.
- Previous tests of the medicinal properties of Kawakawa.
- How the inflammatory process worked.
- Experimental methods of measuring inflammation.
- Experimental methods of conducting cell viability assays.

This research was going to guide the development of the technological outcome.

5.1 Kawakawa

Kawakawa (*Macropiper excelsum*) is a New Zealand native plant. It is found in coastal regions of New Zealand (Metcalf, 1972). Kawakawa can be found as far South as Banks Peninsula as well as on Chatham Island (Poole & Adams, 1963). It forms a bush or small tree and grows up to 6 meters in height. The leaves are heart shaped and are often riddled with holes, which are caused by the larva of the native moth, *Cleora scriptaria*. Most other insects find the leaves poisonous and so avoid them (Lucas & Dawson, 2012), although, this is not unique among plants.

5.2 Previous studies on Kawakawa

There are only 2 known studies of Kawakawa, and neither studied Kawakawa in great depth or identified significant anti-bacterial or anti-viral activity. As a result there is very little scientific evidence to support the claims that are made in rongoā.

The earliest study that tested the medicinal properties of Kawakawa was Antibiotic compounds from New Zealand plants. III: A survey of some New Zealand plants for antibiotic substances (Calder, Cole, & Walker, 1986). This study screened methanol based extracts of the Kawakawa leaf against a variety of bacteria. Kawakawa leaves were freeze dried and were stored at 4°C. 25 g portions of the dried Kawakawa were extracted with methanol using a Soxhlet continuous extractor. The extract was subsequently concentrated in-vacuo to 10 ml before use in an assay. This extract showed minor activity against *Staphylococcus aureus* and *Trichophyton mentagrophytes*, however this activity was significantly less than activity that was seen in other native plants (such as New Zealand passion fruit (*Tetrapathaea tetrandra*)). Anti-bacterial activity is one mechanism by which Kawakawa could act in rongoā.

A similar study, A survey of extracts of New Zealand indigenous plants for biological activity, was conducted (Bloor, 1995), but included viruses and cancers as well as bacteria. In this study any activity shown by Kawakawa was attributed to background tannins in the extracts rather than genuine bioactive compounds. In this study the Kawakawa was dried for 24 hours at temperatures between 30°C and 40°C. The dried Kawakawa, 50g-100g, was extracted twice for 24 hours each time with ethanol and water (in a 4:1 ratio) at room temperature. The extract

was then dried in a vacuum at less than 40°C. Anti-bacterial and anti-viral activity are two mechanisms by which Kawakawa could act in ronoga.

There are limitations associated with these studies. Neither study used a method of extraction that reflected the traditional methods of extraction, nor does either specify the concentration at which Kawakawa was applied to cells, something that was done in this study and shown to be of significance. Both studies used organic solvents which may not be the best solvent to extract any bioactive compounds in Kawakawa. The study by Calder, Cole, & Walker (1986) used a method of extraction that didn't necessarily best preserve compounds in the Kawakawa as it used Soxhlet extraction. These limitations may have destroyed thermolabile compounds in the extracts. These compounds destroyed or damaged in this extraction process could be responsible for the activity of Kawakawa in rongoā. Additionally, extremely polar compounds which also may be responsible for the activity of Kawakawa in rongoā, may not be extracted by organic solvents.

These limitations mean that Kawakawa is still a plant of interest, given the mass of historical evidence available on the medicinal uses of Kawakawa. However, these limitations had an impact on my technological practice:

- This previous research which used only methanol extracts of Kawakawa were unlikely to extract the biological compounds from Kawakawa responsible for the medicinal activity of Kawakawa in rongoā. This suggested that methanol based extracts of Kawakawa were unlikely to extract the bioactive compounds responsible for the actions of Kawakawa in rongoā.
- Anti-bacterial testing was not an area where there was an opportunity to be innovative or creative as it had been reasonably well researched in the previous studies.
- It was important to consider the concentration at which the Kawakawa was applied to the cell. It was also important to consider the extraction methodologies.
- It may be important to use methods that do not involve heating.



5.3 Relatives of Kawakawa

Kawakawa is part of the Piperaceae family. A number of the other members of the Piperaceae family have reported medicinal activity. However, there is no scientific evidence to support this activity.

Fijian ‘Kava’ is made from, “the rhizome and roots of *Piper methysticum*” (Brooker, Cambie, & Cooper, 1987). Kava is claimed to have sedative and anesthetic properties (Wikipedia, 2013). In New Guinea *Piper methysticum* is used as a tonic and to treat, “inflammation of the uterus” (Brooker, Cambie, & Cooper, 1987). *Piper methysticum* is also used extensively in Tahiti, Hawaii and in Southeast Asia for its medicinal properties. *Piper bantamense* is described by Brooker *et al* as a rubefacient that was used traditionally as a counter-irritant in a poultice and to treat headaches and other pains (Brooker, Cambie, & Cooper, 1987). *Piper Puberulum*, *Piper graeffei*, *Piper latifolium*, *Piper tristachyon*, *Macropiper timothianum*, and *Macropiper vitiense* are used to heal wounds, treat skin sores, and treat wounds in a number of Pacific nations (Brooker, Cambie, & Cooper, 1987).

This suggests that the compounds responsible for any of the activity that may be observed in Kawakawa may be present in all members of the *Piper* family.

5.4 Traditional & Modern Uses of Kawakawa

In the 18th century the scientist Joseph Banks who travelled with Captain Cook to New Zealand, observed that Māori were in good health and appeared to suffer from few diseases, perhaps due to the effectiveness of rongoā (Riley, 1994).

Rongoā is the traditional Māori way of treating illness and disease. It includes herbal medicine made from plants, physical techniques (like massages), and spiritual healing techniques (Ngāwhare-Pounamu, 2005).

Rongoā involved combating an illness through tapu (natural law), mind, body and spirit. Like most medicine among indigenous groups it was closely associated with the gods. Māori believed that some illnesses were

caused by evil spirits. If a person did something considered bad or wrong, they would get sick. Other illnesses were believed to have physical causes. A tohunga would heal a patient who had any sicknesses. Knowledge of rongoā was passed down through the generations and rongoā is still practiced today (Tourism New Zealand, N.D.).

An important part of rongoā is the use of plants and herbs to combat and prevent both physical and spiritual sickness (Jones, 2012). Early accounts of rongoā indicate that Kawakawa was useful for treating a wide range of ailments as it was, and still is used extensively in traditional Māori medicine (TeRito & McPherson, 2012). Various sources claim that Kawakawa can be used to treat:

- Stomach pains (Brooker, Cambie, & Cooper, 1987)
- Cuts and wounds (Brooker, Cambie, & Cooper, 1987; Riley, 1994; Ngāwhare-Pounamu, 2005)
- Toothache (Brooker, Cambie, & Cooper, 1987)
- Eczema (Brooker, Cambie, & Cooper, 1987)
- Venereal disease (Brooker, Cambie, & Cooper, 1987; Riley, 1994)
- Colds (Brooker, Cambie, & Cooper, 1987; Riley, 1994; Ngāwhare-Pounamu, 2005)
- Bladder complaints (Brooker, Cambie, & Cooper, 1987; Riley, 1994)
- Counter irritant (Brooker, Cambie, & Cooper, 1987)
- Rheumatic pains (Brooker, Cambie, & Cooper, 1987)
- Serious bruises (Brooker, Cambie, & Cooper, 1987; Riley, 1994)
- Chest complaints, such as bronchitis (Brooker, Cambie, & Cooper, 1987; Ngāwhare-Pounamu, 2005; Riley, 1994; New Zealand Association of Medical Herbalists),
- Infected wounds (Brooker, Cambie, & Cooper, 1987; Ngāwhare-Pounamu, 2005; Riley, 1994)

Many of these ailments can be linked to inflammation.

In rongoā, Māori prepared infusions of Kawakawa by boiling the leaves in water or made a hot poultice using the leaves of Kawakawa that were applied to the skin (TeRito & McPherson, 2012).

Kawakawa is still used today in much the same way and is still marketed as an alternative treatment for many of the same ailments that Māori used it for. For example Go Native New Zealand markets Kawakawa extract claiming, "Being anti-inflammatory and antiseptic, Kawakawa is useful as a baby wipe spray, wash for cuts and scrapes, as a soothing facial toner or following waxing and hair removal" (Go Native New Zealand, N.D.).

An understanding of rongoā informed my technological practices:

- One of the methodologies used in the final methodology was a scientific interpretation of traditional Māori practice.
- Kawakawa was traditionally used as an anti-inflammatory agent, suggesting that scientifically investigating the anti-inflammatory properties of Kawakawa would provide evidence to support the traditional uses of Kawakawa in rongoā.



5.5 Inflammation

Inflammation is one of the body's first responses to injury or infection. There are two types of inflammation, acute and chronic inflammation. Acute inflammation is a short term response to a harmful stimulus. Chronic inflammation is a long term inflammation that is characterised by the stimulus destruction and replacement of tissue by the inflammatory process. Inflammation is considered to be part of the bodies innate immune system as it is not specific to the stimuli.

Acute inflammation can be caused by: infections (such as bacterial, viral or parasitic infections), trauma, physical and chemical agents (such as burns or frostbite), and foreign bodies (such as splinters, dirt, sutures) (Zhejiang University, N.D.).

Acute inflammation involves several key processes. The first is the dilation of the arterioles (small branches of arteries that lead to capillaries) resulting in increased blood flow (Nordqvist, 2012). Secondly the capillaries become more permeable meaning that fluids and blood move into the spaces between tissues (Nordqvist, 2012). Thirdly neutrophils migrate out of the capillaries and venules (small veins that go from a capillary to a vein) and move into the spaces between cells (Nordqvist, 2012). Neutrophils produce reactive oxygen species (Butterfield, Best, & Merrick, 2006) which causes the symptoms that characterise inflammation. Following this rapid response by neutrophils, macrophage cells move into the area of inflammation and destroy the remaining microbes that escape the neutrophils and remove all the tissue debris which achieves healing (Institute for Research in Biomedicine Barcelona, 2008).

Macrophages play a critical role in the inflammatory process. They are activated by cytokines (such as TNF- α (Tumor Necrosis Factor-alpha) and IL-6 (Interleukin-6)), bacterial compounds such as lipopolysaccharide, and other chemical mediators (Fujiwara & Kobayashi, 2005; Butterfield, Best, & Merrick, 2006). Macrophages produce a range of biologically active molecules such as nitric oxide (NO) and monokines as they participate in the inflammatory process (Fujiwara & Kobayashi, 2005). NO can function as a pro-inflammatory molecule through several different mechanisms. NO can activate cyclooxygenases (enzymes which are responsible for formation of important biological mediators called prostanoids) and thereby increase prostaglandin production (Pizza, Hernandez, & Tidball, 1998). NO may also function as a pro-inflammatory molecule, "by causing the release of cytosolic molecules (molecules in the intracellular fluid) that can attract or activate inflammatory cells" (Pizza, Hernandez, & Tidball, 1998).

The anti-inflammatory properties of Kawakawa have not been covered by any other study, yet anti-inflammatory activity is one mechanism that could explain the medicinal properties of Kawakawa observed by Māori in rongoā. Inflammation is a result of both viral and bacterial infections, both of which there is circumstantial evidence to suggest that Kawakawa acted against (Brooker, Cambie, & Cooper, 1987).

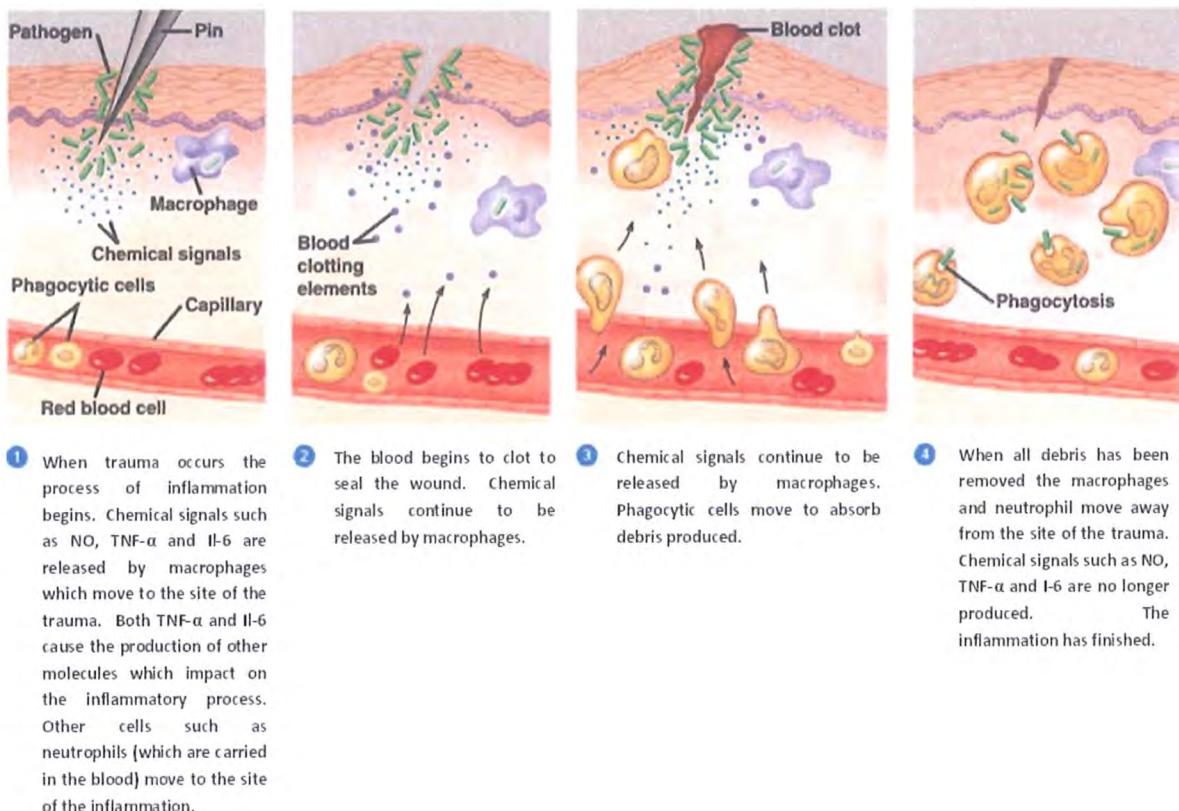


Figure 2: The inflammatory process and the steps associated with acute inflammation.

Adapted from: http://classconnection.s3.amazonaws.com/38/flashcards/1293038/png/inflammatory_response1336076941784.png

Furthermore, inflammation is the main cause of pain or discomfort (Nordqvist, 2012) resulting from an anti-bacterial or anti-viral infection and so an anti-inflammatory agent may reduce the symptoms of the problem. This may account for the evidence that Kawakawa was a potent medicinal plant (TeRito & McPherson, 2012). Some of the ailments that Kawakawa is claimed to treat also point towards Kawakawa acting directly as an anti-inflammatory agent. These include, toothache (Brooker, Cambie, & Cooper, 1987), irritation (Brooker, Cambie, & Cooper, 1987), rheumatic pains (Brooker, Cambie, & Cooper, 1987), serious bruises (Brooker, Cambie, & Cooper, 1987; Riley, 1994) and infected wounds (Brooker, Cambie, & Cooper, 1987; Ngāwhare-Pounamu, 2005; Riley, 1994).

This means that Kawakawa could be having a direct anti-inflammatory action. The idea of plant having anti-inflammatory properties is not new. Traditionally in both India and China *Centella asiatica* is considered to be a potent medicinal herb. It has been shown that aqueous extracts of *Centella asiatica* possess anti-inflammatory activities which justified the traditional use of this plant in the treatment of inflammatory conditions or rheumatism (Gohil, Patel, & Gajjar, 2010). This has been proven by several studies (Somboonwong, Kankaisre, Tantisira, & Tantisira, 2012; Guo, Cheng, & Wing, 2004) which indicate that this anti-inflammatory activity can be both observed and measured via a reduction in nitric oxide concentration.



This informed my technological practice because it suggested that anti-inflammatory testing could be an area of value as anti-inflammatory activity could be a mechanism of medicinal activity.

5.6 Experimental methods for testing in-vitro inflammation

There are a number of methods to test the in-vitro effects of a compound or extract on inflammation. However, 2 were used in this study.

The first is the measurement of NO. NO is produced by macrophages and, the method used to measure the production of NO is the Griess Assay. "One means to investigate NO formation is to measure nitrite (NO_2^-), which is one of two primary, stable and nonvolatile breakdown products of NO" (Promega, 2009). The production of NO_2^- can be measured and related to the production of NO by cells which have been treated with lipopolysaccharide (LPS) as LPS triggers the production of NO. The Griess Reagent System is "based on the chemical reaction which uses sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride (NED)" (Promega, 2009). A decrease in NO production compared to cells not treated with extract would indicate that the extract has anti-inflammatory activity. The Griess Assay was used by Liu, Lin, Deng, Liao, Peng, & Huang (2012) in their study [Antioxidant, Anti-inflammatory, and Antiproliferative Activities of *Taxillus sutchuenensis*](#).

Another method used to measure inflammatory activity is the measurement of cytokines such as TNF- α and IL-6 that are produced during the inflammatory processes. An anti-inflammatory agent would suppress the production of these cytokines. Cytokines such as TNF- α and IL-6 regulate the inflammatory process. When cells become inflamed the macrophages release inflammatory mediators, including TNF- α and IL-6. Their primary role is to regulate the immune cells and induce the symptoms of inflammation. Cytokines control the amount of NO released, they control the production of neutrophils and they control the cells during the inflammatory process. The amount of cytokine produced is indicative of the extent of the inflammation that is occurring. An anti-inflammatory agent would reduce the production of one or both of these inflammatory cytokines.

These methods were chosen because they were likely to provide a comprehensive analysis of the anti-inflammatory properties of Kawakawa. These methods were also likely to be achievable within the timeframe allocated for this work to be completed.

5.7 Cell Viability

Cell viability is the ability of a cell to withstand specific concentrations of a substance. It can be measured by examining the effect of a substance on a cell's metabolic activity or whether the substance is cytotoxic and therefore whether it causes the cell membrane to burst.

Cell viability is important because it is not possible to be confident in any of the conclusions in the later assays without first determining if cells still function in the extract. This is because dead cells or cells with reduced metabolic activity may result in a false result.

There are three main forms of measuring Cell Viability before the use of an extract or compound in in-vitro assays. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay, the Trypan Blue Assay, and flow cytometry.

The MTT Assay is able to determine whether a cell has lost cell function, even if it is still alive (www.dojindo.com, N.D.). The yellow MTT solution is absorbed into the mitochondria of a cell and is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore can be directly related to the number of viable cells. However an apparent reduction in viability may be due to metabolic stress rather than toxicity as the conversion of MTT to purple formazan may be less efficient if the cells are under metabolic stress (Wallert and Provost Lab, 2007). The MTT Assay was used by Liu, Lin, Deng, Liao, Peng, & Huang (2012) in their study [Antioxidant, Anti-inflammatory, and Antiproliferative Activities of *Taxillus sutchuenensis*](#) and by Rehman, Ashfaq, Riaz, Javed, & Riazuddin (2011) in their study [Antiviral activity of *Acacia nilotica* against Hepatitis C Virus in liver infected cells](#), to determine the effect of plant extracts on the cells.

In the Trypan Blue Assay the dye is absorbed into dead cells. This is useful, but its usefulness is limited as the staining does not differentiate between healthy cells and cells that are losing cell function (www.dojindo.com, N.D.).

Another method used to measure the viability of a cell is the use of flow cytometry. Flow cytometry is similar to the Trypan Blue assay because the Propidium Iodide dye can only be absorbed into dead cells. In flow cytometry a laser light of a single wavelength is directed onto a focused stream of liquid. A number of detectors are placed at the point where the stream passes through the light beam. With regards to cell viability flow cytometry provides a method for counting the number of cells that have absorbed the Propidium Iodide dye, which therefore enables the percentage of viable cells to be calculated. It is widely used to determine cell viability. The flow cytometry was used in addition to the previous assays because it was important to be able to be confident that the concentrations of extract used did not negatively affect cell viability.

This informed my technological practices because it showed there were a range of different methods of testing cell viability. It was important that I was aware of all of these methods, because although I originally planned to use only the MTT assay, the results of this assay meant that it was clear to use other methods (the Trypan Blue assay and flow cytometry)

5.8 Bioactive Compounds

Plants are a potent source of bioactive compounds with medicinal activity. It has been stated that 25% of drugs prescribed worldwide have been developed from plants and that over 121 active compounds developed from plants are used in drugs (Rates, 2001). Of the 252 drugs considered to be basic and essential by the World Health Organisation, 11% are exclusively of plant origin and a significant number more are synthetic drugs developed from natural compounds (Rates, 2001). Rates (2001), estimates that 60% of anti-tumour and anti-bacterial drugs already on the market or undergoing clinical trial have a natural origin. Significant examples of drugs developed from plants include morphine and codeine from *Papaver somniferum*, and salicylic acid (the active ingredient in

aspirin) from *Salix* (Willow Tree). In New Zealand it has been shown that Manuka Honey possesses anti-bacterial and anti-inflammatory activity (Advancis Medical, N.D.).

Bernoft (2008) stated that bioactive compounds in plants are often what are known as secondary metabolites. These are produced by the plant and are not required for the daily functioning of the plant and are often thought of as biochemical 'side tracks'. For example, flavonoids can protect against free radicals generated during photosynthesis (Bernoft, 2008). Terpenoids may attract pollinators or seed dispersers, or inhibit competing plants (Bernoft, 2008). Alkaloids may ward off herbivores or insects (Bernoft, 2008).

The following compounds were identified as significant bioactive compounds:

- Phenols (including Anthocyanins, Flavanioids and Proanthocyanidins) (Denny & Buttriss, N.D.)
- Tannins (Visht & Chaturvedi, 2012)
- Resins (Bernoft, 2008)
- Lignans (Bernoft, 2008)
- Mono- and sequi-terpenoids and phylpropanoids (Bernoft, 2008)
- Diterpenoids (Bernoft, 2008)
- Alkaloids (Bernoft, 2008; Visht & Chaturvedi, 2012)
- Proteins and peptides (Bernoft, 2008)
- Furocoumarines & Naphthodianthrones (Bernoft, 2008)
- Carotenoids (Denny & Buttriss, N.D.)
- Plant sterols (Visht & Chaturvedi, 2012)
- Glucosinolates (Denny & Buttriss, N.D.; Visht & Chaturvedi, 2012)

These compounds could be responsible for the anti-inflammatory properties of Kawakawa that were observed in rongoā. Furthermore, if Kawakawa does have anti-inflammatory activity these compounds may be responsible for this activity.

It has also been identified (Rehman, Ashfaq, Riaz, Javed, & Riazuddin, 2011; Bloor, 1995) that most of these bioactive compounds are polar. This links with the practices of preparing Kawakawa in rongoā as Māori prepared infusions of Kawakawa by boiling the leaves in water or made a hot poultice using the leaves of Kawakawa that was applied to the skin (TeRito & McPherson, 2012). The use of water to extract Kawakawa in rongoā would suggest that if there were any bioactive compounds extracted by Māori in rongoā are polar.

A challenge associated with this study is that it is not known what bioactive compounds are in Kawakawa, if any. Consequently the methodology used to extract the Kawakawa in this study must be broad and designed to extract a number of different bioactive compounds.

This informed my technological practice because these bioactive compounds could be responsible for the activity in rongoā, and so in the following section research was conducted into the methods that could extract any or a range of these compounds.

5.9 Extraction of Bioactives

Naturally there are many different ways of extracting these bioactive compounds. Two aspects of research were conducted. The first was general research into possible extraction methods. The second was research into extraction methods used before anti-viral or anti-inflammatory testing.

Most extraction methods use similar principles to extract bioactive compounds from plants. In most cases the method uses the solvent to create a concentration gradient between the plant cells and the solvent which allows the solvent to remove bioactives from the plant as the system seeks to create an equilibrium. Other methodologies seek to break down the cell wall of the plant which would allow any bioactive compounds in the plant to move into the solvent. Heat, agitation, ultrasound, pressure and enzymes are used by various studies to achieve this or to improve or alter this process.

Table 1: Extraction methods

Method		Sources Discussing
Soxhlet Extraction	The repeated washing of the plant material under reflux.	(International Centre for Science and High Technology; Wang & Weller, 2006)
Solid-Liquid Solvent Extraction	The plant material is soaked in a solvent.	(International Centre for Science and High Technology; Vinatoru, 2001)
Enzyme Assisted Extraction	Enzymes (such as cellulase, hemicellulase and pectinase) are added to the plant material before it is extracted.	(Laroze, Soto, & Zuniga; Puri, Sharma, & Barrow, 2012)
Distillation	Steam is passed through the plant material and then condensed. The water and essential oils separate and the essential oils are collected.	(Vinatoru, 2001)
Infusion	The plant material is soaked in water that has just come off the boil.	(International Centre for Science and High Technology)
Percolation	The plant material is soaked in solvent. A tap in the bottom of the container is opened and the solvent is allowed to slowly pass through. New solvent is added continuously to keep the level of solvent in the container constant.	(International Centre for Science and High Technology; TeRito & McPherson, 2012)
Supercritical Fluid Extraction	The plant material is extracted with a solvent (such as CO ₂) that is heated beyond its critical state. It is performed under pressure.	(Wang & Weller, 2006; Visht & Chaturvedi, 2012)
Vorticle/Turbo Extraction	The plant material is placed in the solvent and stirred with a high-speed mixer.	(Visht & Chaturvedi, 2012; Vinatoru, 2001)
Ultrasonic Assisted Extraction	The plant material being extracted is sonicated with ultrasound.	(Vinatoru, 2001; International Centre for Science and High Technology; Wang & Weller, 2006)
Accelerated Solvent Extraction	The plant material is soaked in solvent under high temperature and pressure.	(Wang & Weller, 2006)
Decoction	The plant material is placed in water and boiled.	(International Centre for Science and High Technology)
Expression	The fresh plant material is squeezed and the juices collected.	(International Centre for Science and High Technology)

There are consequently a number of extraction methods that could be employed in this study.

Some of these methods were more practical than others, and some had constraints which limited their suitability for the extraction of bioactive compounds. Certain factors such as heat, time in solution, light, oxygen, and others are widely believed to be enemies of the preservation of biological activity (McCloud, 2010). Consequently consideration should be given to grinding methods, temperature exposure, solvents used for extraction and time in contact with solvent, solvent removal, and how materials would be stored when dried (McCloud, 2010). These were taken into account when designing the extraction methods that were used in this study. The International Centre for Science and High Technology suggests that if constituents are thermolabile, extraction methods such as cold maceration, percolation and counter-current extraction are preferred, and that for thermostable

constituents, the Soxhlet extraction method (if non-aqueous solvents are used) and the decoction method (if water is the solvent) are both useful. However, the source also noted that if extraction methods use heat above room temperature the lowest possible temperature should still be used. This is because some compounds, such as glycosides, are likely to break upon continuous exposure to high temperatures (International Centre for Science and High Technology). The idea of keeping the temperature as low as possible needs to be considered even during the process of drying the leaves before extraction and drying the liquid extract after extraction. Vinatoru (2001) suggested that when drying the plant material it is done in the shade at room temperature or in a hot air oven at no more than 30°C. This source comments that the ultraviolet radiation in sunlight may cause degradation of the bioactive compounds, so direct contact of sunlight should be avoided when drying leaves. Dr Paul Woodgate (2012) commented it would be best to freeze-dry an extract instead of concentrating with rotary evaporator as freeze drying uses lower temperatures which would better preserve any bioactive compounds. These were considered when designing the extraction methods that were used in this study. The extraction methods used in this study combined these factors, from a number of different studies as well as the information provided from consultation with Māori (TeRito & McPherson, 2012), to create the extraction methods used in this study.

A number of sources that extracted plant material, tended to use variations on the solid-liquid solvent extraction method. Tang, Ling, Koh, Chye, & Voon (2012), blended the plant material and consecutively extracted it with methanol in a ratio of 1:5 (g of plant material:mL of solvent) then left in the dark for 3-4 days. The resulting solution was filtered and evaporated at 50°C at reduced pressure. Chen, *et al.* (2011) extracted *Houttuynia cordata* with distilled water in a ratio of 0.1:1 (g of plant material:mL of solvent) for 4 hours at 45°C. The extract was then filtered through a 0.2µm filter then lyophilised. It was then stored at -80°C and resolubilised in sterile water before use. He & Gao (2011), dissolved Dandelion in distilled water at a ratio of 0.1:1 (g of plant material:mL of solvent) at room temperature for 2 hours. This liquid was then extracted twice in water at 100°C for 1 hour. Finally it was filtered through a 0.45µm filter then lyophilised. Rehman, Ashfaq, Riaz, Javed, & Riazuddin (2011) extracted 13 different plants. All were blended and then left to sit in methanol for 24 hours. The solvent was then filtered off and the residue was soaked in methanol for another 24 hours. This was repeated 3-4 times. The extract was partitioned into chloroform, acetone and n-hexane fractions. These methods were used in other studies to extract bioactive compounds from plants and so could be modified for use in this study.

Associate Professor David Greenwood of Auckland University was consulted specifically about extraction methods designed to extract a broad range of bioactive compounds from Kawakawa. Associate Professor Greenwood suggested soaking fresh leaves in a chloroform-methanol-water mixture (in a 1:2:1 ratio) for two days, then removing the chloroform fraction. It was noted that the bioactivity seen by Māori could be synergistic, hence why it is useful to test the bioactivity of a broad extract like the chloroform-methanol-water mixture, as this will extract most bioactive compounds from leaves. Furthermore Associate Professor Greenwood advised it was not always appropriate to macerate the leaf, as cutting the leaf/breaking cell walls means that enzymes in the cell wall could be released, which, could oxidise compounds in the leaf. This was corroborated by McCloud. 

In summary the extraction of bioactive compounds from plants follows this method:

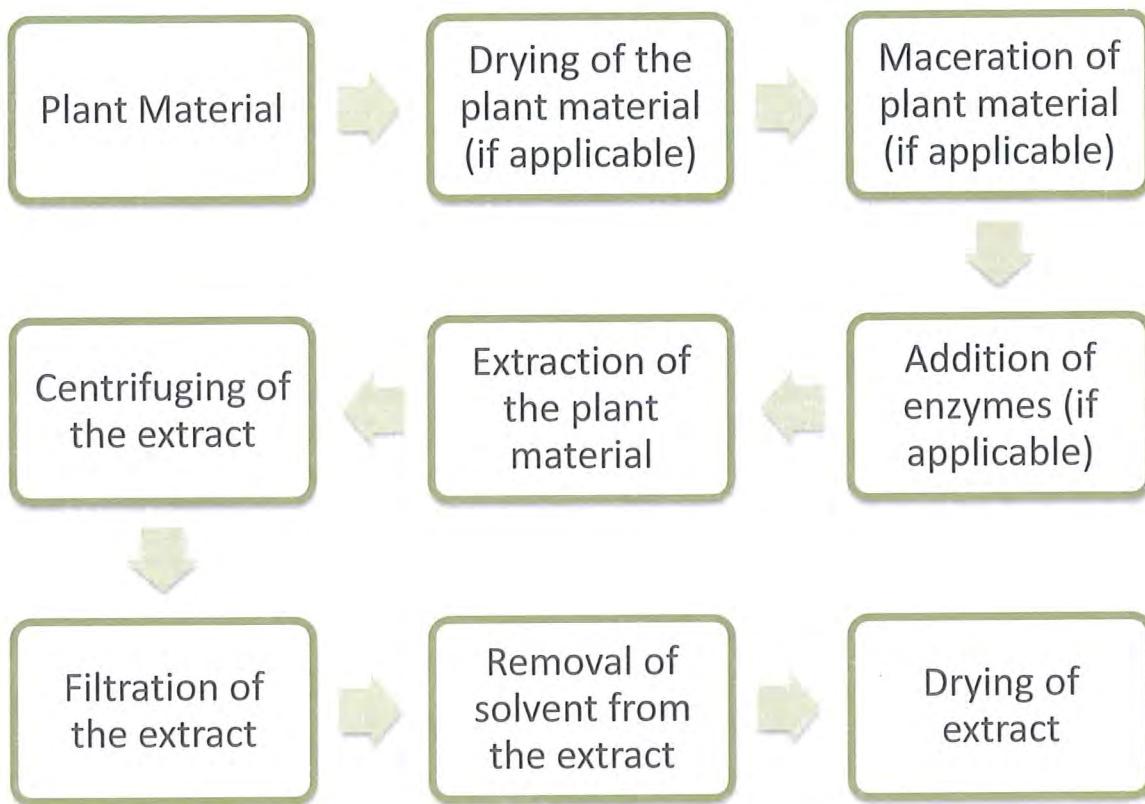


Figure 3: Summary of the process of extracting bioactive compounds from plants.

The extraction is affected by a multitude of factors including:

- Temperature at which the material is extracted at (McCloud, 2010; International Centre for Science and High Technology; Visht & Chaturvedi, 2012)
- Pressure at which material is extracted at (International Centre for Science and High Technology)
- Time length of extraction (McCloud, 2010; Visht & Chaturvedi, 2012)
- The size of the material (Visht & Chaturvedi, 2012)
- Agitation of the plant material (International Centre for Science and High Technology; Visht & Chaturvedi, 2012)
- Polarity of the solvent used (Visht & Chaturvedi, 2012)
- Number of times the material is extracted (International Centre for Science and High Technology)
- Ratio of solvent to plant matter (International Centre for Science and High Technology)
- Alterations to the plant material (such as maceration which could release oxidative enzymes) (McCloud, 2010)
- Solubility of the bioactive in the solvent (Visht & Chaturvedi, 2012)
- pH of the solvent (Visht & Chaturvedi, 2012)
- Ability of the bioactive to move through the cell membrane
- Treatment of the plant material before extraction (such as exposure to light and the time before the plant material is dried and then extracted) (McCloud, 2010)

This knowledge was applied into the development of the extraction methodologies used in this study. Consequently, the research on bioactive compounds and the extraction of these compounds was very significant:

- In the aqueous infusion extract the knowledge of Māori was combined with the knowledge in scientific literature to create a unique and original methodology.
- Extraction methodologies were chosen that:
 - Used a broad solvent that would extract a range of materials from the Kawakawa leaves.
 - The CMW extraction did not involve heat to preserve bioactive compounds.
 - The aqueous extraction method involved heat to replicate traditional Māori methods. Furthermore, this may have enhanced the extraction of bioactive from the Kawakawa leaves.
 - None of the extractions were conducted under pressure.
 - All the material was cut into small bits. This was to increase the surface area of the leaf material and extract more material.
 - All of the plant material was agitated.
 - The ratio of solvent to plant matter was kept as low as possible to have a concentrated solvent solution.
 - Although enzymes were used in some of the methods to assist the extractions, it was kept to a minimum. In the 2 methods that were used in the cell viability and anti-inflammatory assays enzymes had not been added, as enzymes may have damaged bioactive compounds, but also extracted compounds (such as chlorophyll) that were unlikely to be responsible for the bioactivity observed in rongoā.
 - Exposure to light before and during extraction was minimised.

6. Cultural & ethical considerations

Cultural safety was a critical feature of this project. Māori were one of the most significant stakeholders in this project. One of the reasons why it was important to conduct research into rongoā and the uses of Kawakawa in rongoā was because it was important to have an understanding of the Māori perspective towards medicine, Kawakawa and the treatment of ailments. My research suggested that rongoā was a holistic system of living, and so even if Kawakawa had not shown any anti-inflammatory activity, rongoā would still have been an important part of the Māori culture.

One reason why my study was important to Māori was because to some extent rongoā has been persecuted historically. The Tohunga Suppression Act 1907 was an Act of the Parliament of New Zealand aimed to replace tohunga as traditional Māori healers with European medicine and doctors (Wikipedia, 2013). Tohunga were the holders of cultural Māori knowledge including, rongoā. Tohunga were generally experts in the use of medicinal plants and herbs. The perceived loss of power of the tohunga due to the Tohunga Suppression Act 1907 was to become yet another destabilising factor to the traditional Māori lifestyle (Wikipedia, 2013). Consequently, the Act has also been viewed as a breach of the Treaty of Waitangi, as it challenged traditional Māori wisdom – considered to be one of the taonga that Māori were promised under the second article of the Treaty of Waitangi (Rongoā – medicinal use of plants - The impact of colonisation, 2012). Detrimentally, many tohunga declined to pass on their knowledge of rongoā and other cultural traditions. This meant that swaths of māramatanga (knowledge) were lost as Māori was an oral culture (Wikipedia, 2013).



I deemed it to be essential to get the support of Māori and this was achieved through the support provided by Ngā Pae o te Māramatanga. Ngā Pae o te Māramatanga funded this work and they gave the project additional credibility.

One significant aspect of learning was the protocols surrounding communication with Māori and Ngā Pae o te Māramatanga.

RE: Chris Ryan's project on Kawakawa
Daniel Patrick

To: Chris Ryan; Jacqueline Bay; John Taylor;
cc: Daniel Hikuroa;

Kia ora Chris, koutou katoa
Believe we are all good for Wednesday – I am – and I will have Dan Hikuroa, Research Director and Scientists, along with me to discuss some aspects.
We're happy to meet at SBS but have our Board/staff Room here free at Ngā Pae; level 2, room 214, Māori Studies (Building 253), 16 Wynyard St, Auckland.
John/Jacqueline can you please confirm re location?
Thanks
Daniel

Figure 4: An email extract.

Email between myself and Daniel Patrick (the Executive Director of Ngā Pae o te Māramatanga) from 15/10/2012. This illustrates my cultural consultation, my engagement with Māori and with Ngā Pae o te Māramatanga in a respectful way that followed tikanga (protocol).

RE: Kawakawa Project
Daniel Patrick

To: 'Chris Ryan'; Daniel Hikuroa;
Cc: John Taylor;

Kia ora Chris
Sorry for the slow reply – busy and out of town on business.
Great re progressing with discussions of kawakawa preparation and use – two users that we proposed to be on the Advisory group are Dr Joe Te Rito (Senior Fellow at Ngā pae) and Betty McPherson (kaumatua; Māori elder) engaged in research regarding older people.
In regard to Papakura Marae – I will check with our Community Coordinator, Donna, who was following up. Donna outlined a couple of people with rongoā expertise and had raised it with a contact there for the hauora/health clinic.
Shall I make an introduction to Joe and Betty so you can contact and discuss with them? They might know of others to contact too.
Ngā mihi
Daniel Patrick | Executive Director
Ngā Pae o te Māramatanga | New Zealand's Indigenous Centre of Research Excellence
DDI +64 9 923 9729 | Mob +64 21 899 456 | Fax +64 9 373 7928
d.patrick@auckland.ac.nz | www.maramatanga.ac.nz

 NEW ZEALAND'S INDIGENOUS CENTRE OF RESEARCH EXCELLENCE
INDIGENOUS TRANSFORMATION THROUGH RESEARCH EXCELLENCE

Figure 5: An email extract.

An email between myself and Daniel Patrick (the Executive Director of Ngā Pae o te Māramatanga) and Dr Daniel Hikuroa (Research Director of Ngā Pae o te Māramatanga) from 3/12/2012. This illustrates my cultural consultation, my engagement with Māori and with Ngā Pae o te Māramatanga in a respectful way that followed tikanga (protocol).

As it was decided that as the project was to be premised on the traditional uses of Kawakawa it was essential to engage with rongoā in a way that was respectful to Māori culture. Discussions were conducted with Professor Mike Walker, Dr Rebekah Fuller, Dr Joe TeRito and Dr Marilyn McPherson, as to how they personally, and Māori in general, use Kawakawa and its position in rongoā. These groups provided insight into how to fuse māramatanga (knowledge) and science in the extraction methodology.

As part of the funding agreement with Ngā Pae o te Māramatanga there has been communication and reporting back about the progress of the project and the results. The agreement with Ngā Pae o te Māramatanga was that the knowledge of this study would not be patented or commercialised because it is important that this knowledge was accessible and available to all Māori.

Ngā Pae o te Māramatanga also encouraged me to explore my own cultural heritage and I learnt about my own Māori ancestry and came to a greater appreciation of Māori culture.

Overall there was considerable engagement with Ngā Pae o te Māramatanga and their affiliation added credibility to this project. Furthermore, for Ngā Pae o te Māramatanga scientific evidence to support the traditional uses of plants such as Kawakawa and treatments in rongoā fits within Ngā Pae o te Māramatanga's goals.



7. Second Brief Refinement

7.1 Revised Brief

Are the traditional uses of Kawakawa by Māori in rongoā medicine supported by any evidence of anti-inflammatory activity?

7.2 Specifications

- To prove that Kawakawa extracts are not cytotoxic when tested in the MTT cell viability assay.
- To show that Kawakawa extracts demonstrate reductions in the production of inflammatory markers (the measurement of human neutrophils, TNF- α , NO and IL-6)
- To create different Kawakawa extracts to test in the anti-inflammatory assays. 7 proposed methods include:
 - Aqueous infusion using fresh leaves
 - Aqueous infusion using dried leaves
 - Chloroform-methanol-water using fresh leaves
 - Chloroform-methanol-water using dried leaves
 - Enzyme assisted aqueous infusion using dried leaves
 - Enzyme assisted chloroform-methanol-water using dried leaves
 - Aqueous enzyme assisted using dried leaves

7.3 Constraints

7.3.1 Time:

The time available for the work to be conducted in is limited. Time is the main factor that constrains the extent of the work. Time limited the number of anti-inflammatory assays that could be conducted and it limited the number of Kawakawa extracts that could be tested in these assays.

7.3.2 Extracts

The number of solvents used to extract the Kawakawa has to be limited. This is because the assays need to be run for each solvent. Two solvents (water and chloroform-methanol-water) have been chosen. The aqueous solvent was chosen because this method was similar to the methods used by Māori to prepare Kawakawa for use in rongoā. The chloroform-methanol-water solvent was chosen because it would extract a broad range of compounds that are possibly bioactive.

7.3.3 Biological Assay's:

The number biological assay's has been limited. Each new assay creates a significant amount of new work through the creation of new methods which require even more of the limited time available to be carried out. Two assays, the NO assay and the cytokine assay measuring IL-6 and TNF- α production, have been selected for this project. Neither has been conducted to date and so provide an opportunity to gain new insight into Kawakawa.

7.3.4 Skill:

I had no previous experience in conducting the complex biochemical assays. The skills needed to conduct assays of this nature needed to be acquired. This limited the number and the nature of the assays that could be conducted.

7.3.5 Budget:

The cell culture work that was conducted had a cost associated with it. Ngā Pae o te Māramatanga funded this research.

7.3.6 Ethics:

Ethics approval from the University of Auckland was required to conduct the neutrophil testing as it requires the extraction of human neutrophils from human blood.

7.4 Justification for changes

The original plan included the testing of human neutrophils. These were a possible inflammatory marker. However, it was not possible to do the neutrophil assay which was originally proposed. Consultation with Dr Taylor suggested that this assay was not going to be possible to complete as early attempts were unsuccessful, and due to time constraints the time to refine this assay was not available. Furthermore, consultation Dr Taylor also suggested the skills needed to conduct this assay were very complex. Significantly, the use of this assay was absolutely necessary to gain an understanding of the anti-inflammatory properties of Kawakawa.



These 7 extraction methods chosen were selected as the literature review suggested that these extracts would extract the maximum number and maximum variety of bioactive compounds from Kawakawa. These assays were considered to have the best chance of demonstrating anti-inflammatory activity in the later assays. The literature survey suggested that these methods were going to be more appropriate than the Ethyl Acetate solvent originally proposed.

Discussions with the client, Ngā Pae o te Māramatanga, suggested that they would like it if methodologies that replicated traditional Māori preparation methods were included. This meant that the aqueous infusion method using fresh leaves was designed and included.

It was decided not to use the leaves on the east of the tree that had been chewed by the larva of the native moth, *Cleora scriptaria*. This was because it was unlikely that enough of these leaves would be found, but also it would have limited the application of the eventual product to a very specific set of preparation conditions. This would have reduced the relevance of the findings. Leaves were randomly selected from a number of different Kawakawa plants.

8. Third Brief Refinement

8.1 Brief Statement

Are the traditional uses of Kawakawa by Māori in rongoā medicine supported by any evidence of anti-inflammatory activity?

8.2 Specifications

- To prove that Kawakawa extracts are not cytotoxic when tested in cell viability assays
 - The MTT cell viability assay.
 - The Trypan Blue Assay.
 - Flow cytometry to test cell viability.
- To show that Kawakawa extracts demonstrate reductions in the production of inflammatory markers (the measurement of human neutrophils, TNF- α , NO and IL-6)

- To create different Kawakawa extracts to test in the anti-inflammatory assays. These were the same as proposed in the previous brief.

8.3 Constraints

8.3.1 Time:

The time available for the work to be conducted in is limited. Time is the main factor that constrains the extent of the work. Time limited the number of anti-inflammatory assays that could be conducted and it limited the number of Kawakawa extracts that could be tested in these assays.

8.3.2 Extracts

The number of solvents used to extract the Kawakawa has to be limited. This is because the assays need to be run for each solvent. Two solvents (water and chloroform-methanol-water) have been chosen. The aqueous solvent was chosen because this method was similar to the methods used by Māori to prepare Kawakawa for use in rongoā. The chloroform-methanol-water solvent was chosen because it would extract a broad range of compounds that are possibly bioactive.

8.3.3 Biological Assay's:

The number biological assay's has been limited. Each new assay creates a significant amount of new work through the creation of new methods which require even more of the limited time available to be carried out. Two assays, the NO assay and the cytokine assay measuring IL-6 and TNF- α production, have been selected for this project. Neither has been conducted to date and so provide an opportunity to gain new insight into Kawakawa.

8.3.4 Skill:

I had no previous experience in conducting the complex biochemical assays. The skills needed to conduct assays of this nature needed to be acquired. This limited the number and the nature of the assays that could be conducted.

8.3.5 Budget:

The cell culture work that was conducted has a cost associated with it. Ngā Pae o te Māramatanga funded this research.

8.4 Justification for changes

The original plan included anti-viral testing. However, because anti-viral testing is far more technically challenging, and given I had no experience in cell culture testing, I was going to be able to do more of the testing, and the project would be of a higher quality if it focussed on anti-inflammatory testing.

The original plan was intentionally broad in scope to give the project opportunities should the anti-inflammatory testing show no or limited promise. The learning of the complex bio-chemical methods involved with the testing to date has proven to be a substantial challenge, requiring more repetition of tests, which has resulted in additional time requirements. Furthermore, the anti-inflammatory testing is showing promising results. In order to explore this promise and fully substantiate it, testing beyond what was originally planned for is also required (such as the measurement of inflammatory cytokines). If the anti-viral testing was to be conducted the opportunity to explore the anti-inflammatory aspects of Kawakawa would be reduced.

It became necessary to use Flow Cytometry and Trypan Blue cell viability methods, instead of just the MTT cell viability assay due to some of the issues anticipated with this assay as it was conducted.

9. Second Timeline Refinement

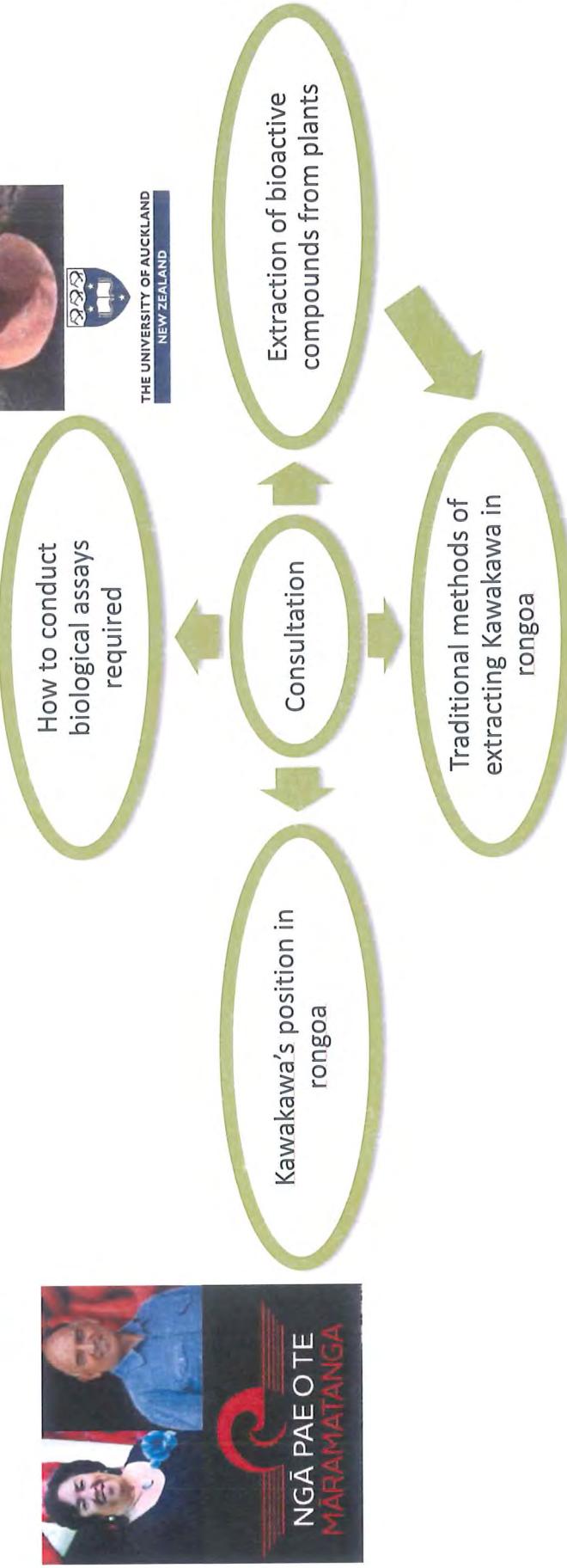
9.1 Project Plan

School	Dates	Gold CREST	Expected Specific Breakdown of Time	Notes
	February 11, 2013	February 17, 2013	Completion of Nitric Oxide experimental work	Work on the introduction, aim, questions, hypothesis and methods of completed work completed and ready for inclusion in the draft report
	February 18, 2013	February 24, 2013	Data Analysis of work to date completed and ready for inclusion in the draft report	
	February 25, 2013	March 3, 2013	Conclusions drawn for work done to date completed and ready for inclusion in the draft report	
March 4, 2013	March 10, 2013	Progress Report Due (15th March 2013)	Discussion of results to date completed and ready for inclusion in draft report	If it fits the new NCEA standard one opportunity would be to submit this work for NCEA Technology 3.1. This involves the creation of a product. This is still under consideration. If it was decided to do this work would need to begin at this point on this
March 11, 2013	March 17, 2013	March 24, 2013	Completion of experimental work - cytokine assay	
March 18, 2013	March 31, 2013	April 7, 2013	Conclusions drawn for work done to date completed and ready for inclusion in the draft report	
March 25, 2013	April 14, 2013	April 14, 2013	Discussion of results to date completed and ready for inclusion in the draft report	
April 1, 2013	April 21, 2013	April 28, 2013	Organisation of report & submission of draft report (target date = 3/6)	
April 8, 2013	May 5, 2013	May 6, 2013	Conclusions drawn for work done to date completed and ready for inclusion in the draft report	
April 15, 2013	May 12, 2013	May 19, 2013	Discussion of results to date completed and ready for inclusion in the draft report	
April 22, 2013	May 26, 2013	May 26, 2013	Organisation of report & submission of draft report (target date = 3/6)	
April 29, 2013	June 2, 2013	June 3, 2013	Gold CREST Draft Report Due on the 19th of July 2013	
School Holidays	June 9, 2013	June 10, 2013	Make changes to draft report	
	June 16, 2013	June 17, 2013		Preparation for the MIT Manukau Science & Technology Fair needs to
	June 23, 2013	June 24, 2013		
	June 30, 2013	July 1, 2013		
	July 7, 2013	July 8, 2013		
	July 14, 2013	July 15, 2013		
	July 21, 2013	July 22, 2013		
	July 28, 2013	July 29, 2013		
	August 4, 2013	August 5, 2013	Gold CREST Report Due on the 30th of August 2013	
	August 11, 2013			

	August 12, 2013	August 18, 2013	Due to mock exams at school, the report will have to be finished earlier than would otherwise be required. Therefore the draft report will have to be good and lots of effort will have to be put into making any changes necessary as quickly as possible. This poses a challenge as the report will be returned by the 2nd of August leaving effectively a week and a half to complete it. This will be made more challenging as preparation for the MIT Manukau Science & Technology has to occur before mock exams as well. This will have to be done in the break between sending the draft report in and getting the draft report back.
Howick College Mock Exams			
	August 19, 2013	August 25, 2013	
	August 26, 2013	September 1, 2013	
	September 2, 2013	September 8, 2013	Final CREST Assessment Meeting (Due for completion by the 27th of September)
	September 9, 2013	September 15, 2013	Feedback to Ngā Pae o te Māramatanga about the outcomes of this study
	September 16, 2013	September 22, 2013	
	September 23, 2013	September 29, 2013	
	September 30, 2013	October 6, 2013	
	October 7, 2013	October 13, 2013	
	October 14, 2013	October 20, 2013	
	October 21, 2013	October 27, 2013	
	October 28, 2013	November 3, 2013	
	November 4, 2013	November 10, 2013	
	November 11, 2013	November 17, 2013	
	November 18, 2013	November 24, 2013	
	November 25, 2013	December 1, 2013	
	December 2, 2013	December 8, 2013	
	December 9, 2013	December 15, 2013	
	December 16, 2013	December 22, 2013	
	December 23, 2013	December 29, 2013	

10. Consultation

Scholarship Technology Re-



As part of initiating this project I emailed scientific organisations seeking support, including Jacquie Bay at the University of Auckland. She identified Dr John Taylor at the School of Biological Sciences as a possible mentor and Ngā Pae o te Māramatanga as a group who might be able to fund this work. Dr John Taylor then supervised me while I conducted the biological assays.

As part of gaining an understanding of rongoā, I spoke with Professor Mike Walker, Dr Rebekah Fuller, Dr Joe TeRito and Dr Marilyn McPherson, as to how they use Kawakawa and its position in rongoā. Rongoā is the traditional Māori way of treating illness and disease including the use of herbal medicine. In rongoā, Māori prepared infusions of Kawakawa by boiling the leaves in water or making a hot poultice using the leaves of Kawakawa that were applied to the skin.

I also spoke to Associate Professor Brent Copp and Associate Professor David Greenwood about possible methods of extracting Kawakawa leaves.

11. Stakeholders

11.1 Māori

Māori were the main stakeholders in this work. The knowledge that was being investigated was traditional Māori knowledge. The outcome of this technological practice could have significant implications for Māori communities. This is because the provision of scientific evidence to support the use of Kawakawa in rongoā strengthens traditional Māori practice.

Māori were also persecuted for 55 years with the Tohunga Suppression Act 1907. Having scientific evidence to support the use of Kawakawa in rongoā would be important in adding back to the knowledge base that was damaged historically.

11.2 Scientific Groups & Organisations

This research was conducted at the School of Biological Sciences at the University of Auckland. The support of Dr John Taylor and the members of the Taylor lab (Jennifer Kuehne and Carol Wang) provided support in obtaining the skills needed to conduct cell culture testing of this nature.

11.3 Ngā Pae o te Māramatanga

Ngā Pae o te Māramatanga funded this research. Ngā Pae o te Māramatanga was also the client.

Professor Mike Walker, Dr Rebekah Fuller, Dr Joe TeRito and Dr Marilyn McPherson, were consulted as to how they use Kawakawa and its position in rongoā. Furthermore, these groups provided some insight into how to fuse māramatanga and science in the extraction methodology.

Furthermore, as part of the funding agreement with Ngā Pae o te Māramatanga there has been communication and reporting back about the progress of the project and the results. This engagement with Māori was important to the success of the project.

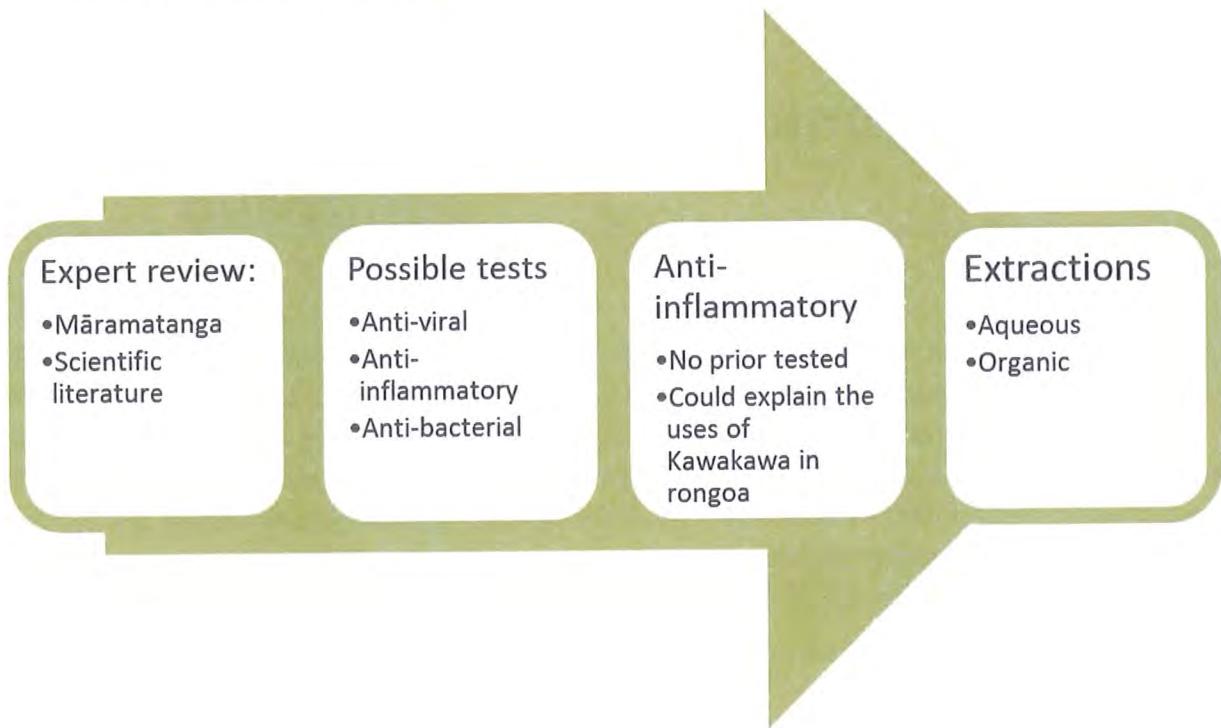
11.4 The wider community

The implications of this research would have impacts on the wider community. The aqueous Kawakawa extract could be included in a naturopathic treatment. Furthermore, the existence of scientific support for the use of Kawakawa in rongoā could lead to wider acceptance of rongoā use of Kawakawa as to the general public the existence of scientific evidence could legitimise the traditional use of Kawakawa. This could lead to the use of Kawakawa in teas or the inclusion of Kawakawa in tea products.

Unlike Chinese traditional medicines, there are very few scientific studies of the medicinal uses of plants traditionally used in rongoā. Where bioactivity is shown it is less than that seen in plants used in other cultures. This means that in the wider community there is uncertainty about the efficacy of rongoā as a medicinal tool. This means that rongoā among the general public has limited credibility as a scientifically proven medicinal tool. Studies like mine which provide evidence to support some of the claims made in rongoā help to change the public's perceptions of rongoā. Studies like mine create the opportunity for scientifically validated therapeutic treatments based on Kawakawa. As is seen with the use of Manuka Honey (Advancis Medical, N.D.) and Horipito in nutraceuticals, the traditional uses of Kawakawa by Māori, and in turn rongoā in general, would gain credibility with the public who, for the most part, have a very limited understanding of rongoā and what it entails.



15 . Project Development Summary



The niche of this project was identified following a review of the scientific literature, which showed there were only 2 previous screening studies on Kawakawa. An expert review (Professor Mike Walker, Dr Rebekah Fuller, Dr Joe TeRito, Dr Marilyn McPherson, Associate Professor Brent Copp and Associate Professor David Greenwood) was conducted to gain an understanding of rongoā but also possible extraction methods.

The plan developed from anti-viral testing to anti-inflammatory testing because anti-viral testing is far more technically challenging, and given I had no experience in cell culture testing, I was going to be able to do more of the testing, and the project would be of a higher quality if it focussed on anti-inflammatory testing.

Anti-bacterial testing was not conducted because this had been well explored in the literature.

The extraction methodologies were then developed. These were original methods that combined Māori knowledge and learning's from another specific literature review on the extraction of bioactive compounds from plants.

15.1 Modification and Improvement

Improvements were made on the work that was done previously. An extraction method that better mirrored the traditional preparation of Kawakawa was used. Extraction methods used in other studies to extract bioactive compounds from plants were modified for use in this study.

Furthermore, within the biological assays there was modification and improvement. The plan was improved based on the outcomes of the previous assays, it was also modified to allow for the repetition of assay's to ensure the reliability of the data. For example the use of flow cytometry to determine cell viability was conducted following the results of the MTT assay and the Trypan Blue Assay. This was done to provide confidence that the concentrations of extract used in the NO and cytokine assay were not impacting on the cell viability. Also, the

16.2 Initial Extractions:

An initial attempt at conducting the extractions, considering these methodologies were developed from scratch, was conducted. Initially the 7 extraction methods proposed in the 3rd brief refinement were used. These methods were: chloroform-methanol-water with fresh leaves, chloroform-methanol-water with dried leaves, enzyme assisted then extracted with chloroform-methanol-water, aqueous infusion with fresh leaves, aqueous infusion with dried leaves, enzyme assisted then aqueous infusion, enzyme assisted aqueous.

However, there were some challenges associated with this initial attempt:

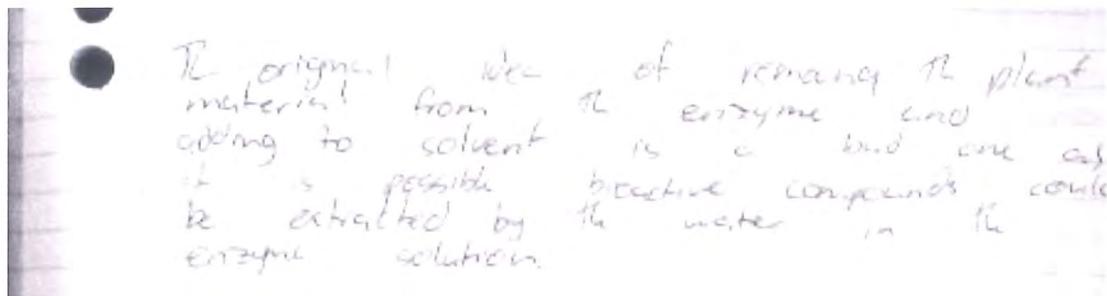


Figure 7: Extract from the lab-book on the 20/12/12.

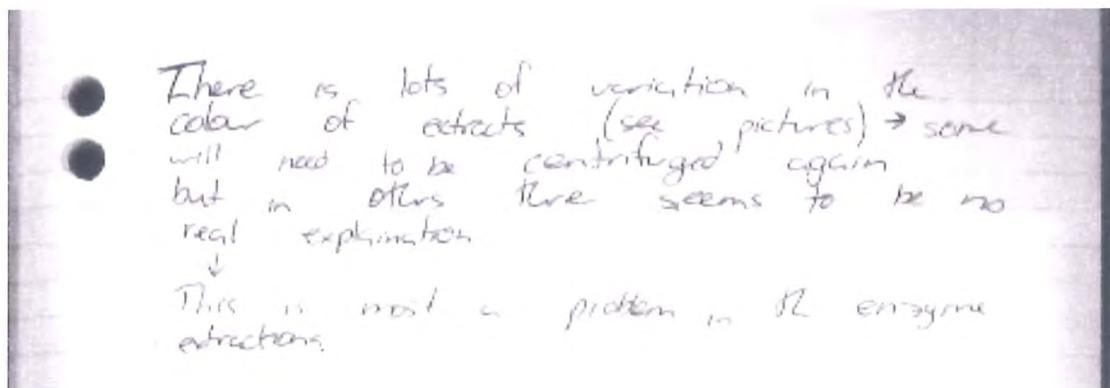


Figure 8: Extract from the lab-book on the 21/12/12

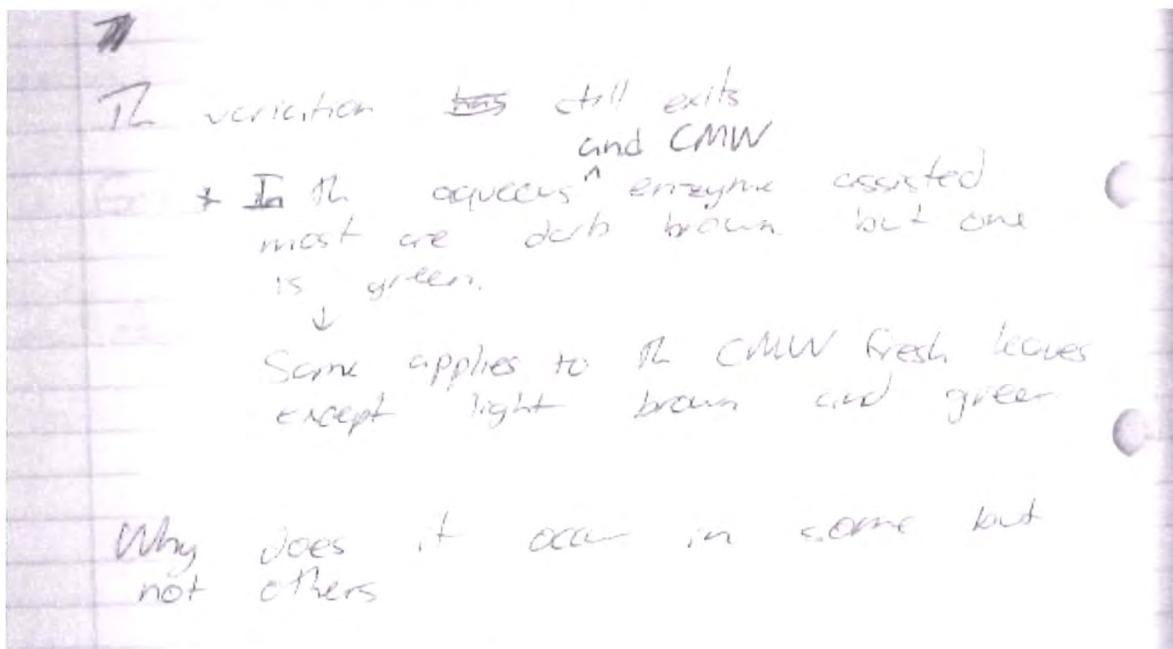


Figure 9: Extract from the lab-book on the 22/12/12

Note: The “variation” referred to is differences in what should have been homologous extracts.

This was an opportunity to refine the methodologies used. For example, repeating them to ensure they were homologous.

As a result of these initial trials, these extractions were repeated and refined. The changes were:

- Constant agitation to prevent the separation of solvents/separation of compounds.
- The aqueous extract was not heated constantly for the entire 30 minutes (as it was in this first trial). This made it more representative of traditional Māori preparation methods.
- In the enzyme assisted trials, the plant material was not removed from the enzyme solution.
- All of the material was cut into small bits. This was to increase the surface area of the leaf material and extract more material.

16.3 Final Extractions:

Purpose: All aspects of the experimental research required the extraction of bioactive compounds from Kawakawa leaves into a solvent system compatible with exposure to living cells. The purpose of these experiments was to extract the Kawakawa leaves into a liquid form.

Summary: A variety of extraction methods were developed for use in this study. Seven different extraction methods were undertaken: chloroform-methanol-water with fresh leaves, chloroform-methanol-water with dried leaves, enzyme assisted then extracted with chloroform-methanol-water, aqueous infusion with fresh leaves, aqueous infusion with dried leaves, enzyme assisted then aqueous infusion, enzyme assisted aqueous. Details of each can be found in the log book.

Enzyme assistance involved soaking the leaves in enzyme solution for an hour before extraction. Typically enzyme assisted extraction methods are used to increase the yield of bioactive material extracted. The yield of material extracted was not an issue in this study as all methods generated sufficient volumes of freeze dried material.

From this collection of extracts, two (the aqueous infusion method and the chloroform-methanol-water extraction method) were chosen and developed further for use in the cell viability and anti-inflammatory assays.

These extraction methods were selected for biological testing on the basis of ease of preparation, similarity to traditional preparation methods, yield of material produced after lyophilisation and the likelihood of being able to extract bioactive compounds. Neither of these methods was based solely upon a method used in a previous study and both combined factors from a number of previous studies. Furthermore, the aqueous infusion extraction method fused traditional Māori knowledge and modern scientific knowledge to create a unique methodology. Finally, no previous scientific study had used an aqueous extraction method when testing the medicinal properties of Kawakawa.

The aqueous extract was prepared from fresh leaves by infusing the leaves in hot water. This method was analogous to making tea.

For the CMW extract fresh leaves were soaked in a mixture of chloroform:methanol:water ([CMW] 1:2:1 vol/vol) to extract. The CMW extract naturally partitioned into two phases; water/methanol and chloroform. The chloroform phase was discarded due to the insolubility of the material after rotary evaporation and because literature suggested that bioactivity tended to lie in the polar fractions (Bloor, 1995; Rehman, Ashfaq, Riaz, Javed, & Riazuddin, 2011). The water/methanol phase was dried by rotary evaporation.



Both extracts were lyophilised and the solid material resuspended in pure water. Stock solutions of both were prepared at 5mg/mL and were filter sterilised before addition to cell cultures.

16.4 MTT Assay:

Purpose: The MTT Cell Viability Assay is designed to show how cell viability varies as Kawakawa extract concentration varies.

Description: It shows the viability of cells treated with extract compared to a control (which is just RPMI cell medium). A reduction in absorbance compared to the control shows that the cells are less viable (the cells in cell medium are assumed to be 100% viable) in the presence of the extract. This is achieved because the yellow MTT solution is absorbed into the mitochondria of a cell and is reduced to purple coloured formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore can be directly related to the number of viable cells. However, an apparent reduction in viability may be due to metabolic stress rather than toxicity as the conversion of MTT to purple formazan may be less efficient if the cells are under metabolic stress.

Method Summary: The MTT assay is conducted in sterile conditions a 96 well plate is seeded with 100,000 RAW247.6 cells per well and incubated for 24 hours at 37.5°C in a UV incubator. After 24 hours 12 different concentrations of extract are added in triplicate. After a further 24 hours of incubation at 37.5°C/5% CO₂ the MTT solution is added to each of the wells. After another 3.5 hours of incubation at 37.5°C/5% CO₂ the extract is removed from each of the wells and the MTT solvent is added and the plate is orbitally agitated for 15 minutes. The absorbance of each well is measured at 590nm and 620nm on a photometer.

16.5 Trypan Blue Assay:

Purpose: The Trypan Blue Cell Viability Assay is designed to show whether a particular concentration of extract is cytotoxic.

Description: Unlike the MTT Cell Viability Assay it only shows whether a cell is dead or not, it does not show if the cells metabolic processes have been compromised. This assay was designed to confirm if the slight reduction in cell viability at the highest concentrations in the MTT assay was due to cytotoxicity or due to the extract placing the cells under metabolic stress.

Method Summary: In sterile conditions a 24 well plate is seeded with RAW247.6 cells and incubated for 24 hours at 37.5°C/5% CO₂ in a UV incubator. After 24 hours, 12 different concentrations of Kawakawa extract are added. After a further 24 hours of hours of incubation at 37.5°C/5% CO₂ the extract is removed and the plate washed out with PBS. A 50% Trypan Blue solution is then added to each of the wells and the plate is incubated at 37.5°C/5% CO₂ for 5 minutes. Each well is then washed out with PBS. The plate is then examined under a microscope. Cells that stain blue are dead.

16.6 Flow Cytometry Assay:

Purpose: The purpose of this assay is to provide another measure of cell viability. It was conducted as it was important to be confident that the extract didn't compromise cell viability, and to confirm the previous results. Additionally, prior to statistical analysis the slight decrease in the absorbance values of the higher concentrations of extract in the MTT assay was a potential cause for concern.

Method Summary: A 24 well plate is seeded with RAW247.6 cells and incubated for 24 hours at 37.5°C/5% CO₂ in a UV incubator. After 24 hours 12 different concentrations of Kawakawa extract are added. After another 24 hours of incubation at 37.5°C/5% CO₂ the cells are removed and stained. They are then run through the flow cytometer.

16.7 Nitric Oxide Assay:

Purpose: To measure the production of Nitric Oxide (NO) by cells exposed to different concentrations of the Kawakawa extracts.

Description: NO is an inflammatory marker. A reduction in the production of NO by cells exposed to a Kawakawa extract would suggest that the extract has *in-vitro* anti-inflammatory activity. The NO assay measures the production of NO through the measurement of nitrite (NO₂⁻). NO is a reactive oxygen species which is a product of inflammation in cells. NO breaks down, and one of the products it forms is nitrite. The Greiss Reagent System Protocol was used (Promega - Technical Bulletin: Griess Reagent System, 2009). This protocol uses a nitrite standard curve to allow the production of nitrite in experimental samples to be related to the production of NO. Cells in the experimental samples are treated with Lipopolysaccharide (LPS) that stimulates the macrophages in cells to produce NO. A reduction in the amount of NO produced would indicate a reduction in the level of inflammation.

Method Summary: In this assay which is conducted in sterile conditions, a 96 well plate is seeded with 500,000 RAW247.6 cells per well and incubated at 37.5°C/5% CO₂ for 24 hours. After 24 hours triplicates of 12 different concentrations of extract mixed in DMEM medium which has been enriched with 10µg/mL LPS are added to the cells. After 24 hours of incubation at 37.5°C/5% CO₂ in an incubator the extract is transferred onto a new 96 well plate. Separately the nitrite standard curve is prepared. The nitrate standard curve is 6 serial two fold dilutions of 0.1M Nitrite Standard prepared 1:1,000 in DMEM cell medium. The Greiss Reagents (Sulfanilamide Solution (1% Sulfanilic acid in 5% phosphoric acid) and NED Solution (N-1-naphthylethylenediamine dihydrochloride)) are added to both the experimental samples and the nitrite standards. The absorbance of each well measured at 550nm on a photometer. The assay determines the nitrite production of cells treated with extract compared to a control (LPS enriched cell medium). Nitrite production is assumed to be representative of NO production (as nitrite is a breakdown product of NO).

16.8 TNF-α/Il-6 Cytokine Assay:

Purpose: The purpose of this assay is to measure the production of the inflammatory cytokines TNF-α and Il-6 by cells exposed to different concentrations of the Kawakawa extracts.

Description: TNF-α and Il-6 are two inflammatory cytokines that indicate inflammation. A reduction in these two cytokines would suggest that the extract has anti-inflammatory activity.

Method Summary: In this assay, which is conducted under sterile conditions, a 96 well plate is seeded with 100,000 RAW247.6 cells per well and incubated at 37.5°C/5% CO₂ for 24 hours. After 24 hours 10 different concentrations of Kawakawa extract in DMEM medium enriched with 20µg/mL LPS are added to the cells. There are 2 controls, the first control contains just DMEM media enriched with 20µg/mL LPS, the second

control is just DMEM cell medium. After 24 hours of incubation at 37.5°C/5% CO₂ in an incubator the supernatant is transferred onto a new 96 well plate which is stored at 4°C in the dark. The "BD CBA Mouse/Rat Soluble Protein Master Buffer Kit" using the mouse treatment, was used to conduct the second part of this assay. Each sample is measured using flow cytometry.

16.9 Statistical Analysis:

Purpose: This test meant it was possible to understand which of the increases and reductions in cell viability or inflammatory markers were significant.

Method Summary: Statistical analysis was undertaken using, "T-test: Two sample assuming unequal variance" to determine the P value. The "P one tail" was calculated.

A hypothesised expected difference of "0" was assumed.

This was done using Microsoft Office Excel 2010. This compared each of the triplicates to the control.

16.10 Challenges faced during carrying out this methodology:

A number of challenges were faced in trying to carry out the methods required to carry out this study. These were mainly derived from my lack of experience at cell culture testing.

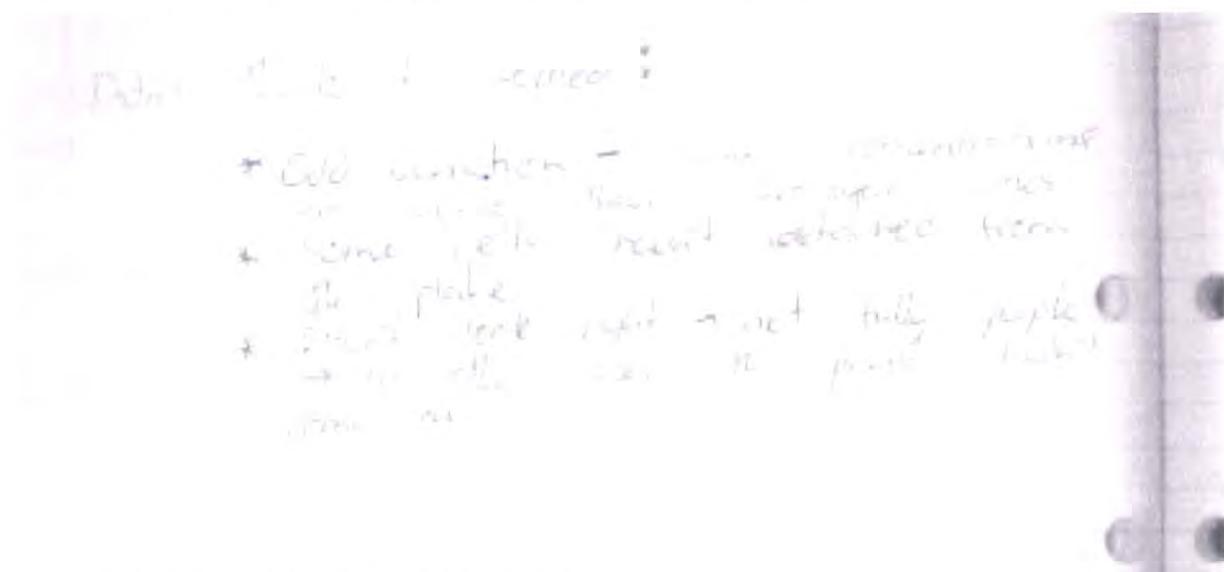


Figure 10: Logbook extract from the 23/1/2012

The cell culture testing skills needed for this study needed to be developed. This required some practicing and as the extracts from the lab-book (figures 10 & 11) show some of the early attempts at the MTT and NO assays were not successful.

When the project began I conducted:

- The initial research
- The background research on Kawakawa and rongoā
- The research on extraction methods
- The extractions
- The MTT Assay
- The Trypan Blue Assay
- The NO Assay
- The data analysis of the data from the MTT assays and the NO assays
- The statistical analysis of all the data from all of the assay's

Practice at repeating the biochemical assays used in this study was necessary to gain the skills that would allow these assays to be repeated successfully.

The skills required to organise and conduct research science were all skills that I needed to acquire. These included the tenacity required to seek support to initiate this project, an understanding of how to gain funding, how to conduct background research and an understanding of the academic environment. It was also necessary to require some of the skills required to approach Māori regarding the uses of Kawakawa in rongoā.

The basic skills required for biochemical cell culture experiments of this nature were all new skills that I needed to acquire. They included:

- Pipetting
 - Counting cells
 - Calculating concentrations
 - Centrifuging
 - Calculating P values
 - Changing cell medium
 - Splitting cells
- Measuring absorbance on a photometer

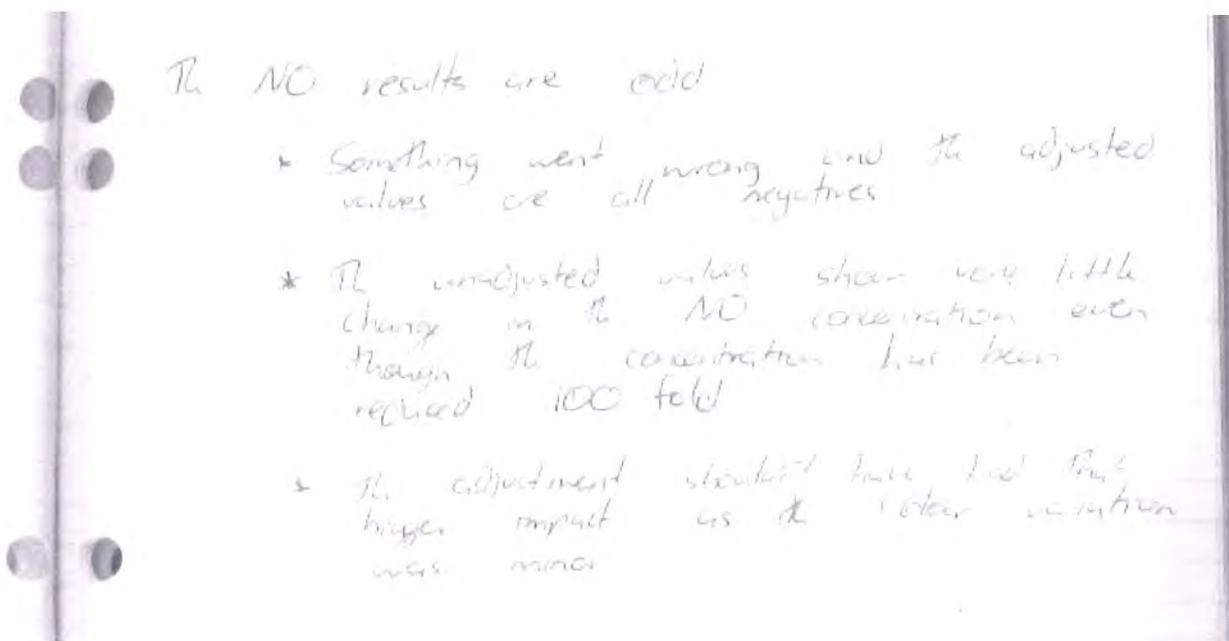


Figure 11: Logbook extract from the 6/2/2013

Note: At this point NO readings were being adjusted for the absorbance of the Kawakawa extract.

These skills were acquired with support from all of those in Dr Taylor's lab.

It was not possible for me to conduct the flow cytometry assay for evaluating the effect of the extract on cell viability or the cytokine assay due to the complexity of these assays (the complexity is such that they are of the level of a PhD student) and the constraints of time which meant that the time available to attain these skills was not available. However, these assays were organised by me and I understand how to conduct these assays, their purpose and the results.

The skills required following the practical work were also new skills. These included the statistical analysis and data processing. The skills required to write the final report were also acquired.

17. Final Brief

17.1 Brief statement

To explore the anti-inflammatory properties of selected extracts of the Kawakawa leaf.

17.2 Specifications

This will be done through:

- Extracting the Kawakawa leaves using CMW and an aqueous infusion methodology.
- Measuring the production of NO in cells exposed to different concentrations of the Kawakawa extracts.
- Measuring the production of inflammatory cytokines (TNF- α and IL-6) in cells exposed to different concentrations of the Kawakawa extracts.

17.3 Constraints

17.3.1 Time:

The time available for the work to be conducted in is limited. Time is the main factor that constrains the extent of the work. Time limited the number of anti-inflammatory assays that could be conducted and it limited the number of Kawakawa extracts that could be tested in these assays.

17.3.2 Extracts

The number of solvents used to extract the Kawakawa has to be limited. This is because the assays need to be run for each solvent. Two solvents (water and chloroform-methanol-water) have been chosen. The aqueous solvent was chosen because this method was similar to the methods used by Māori to prepare Kawakawa for use in rongoā. The chloroform-methanol-water solvent was chosen because it would extract a broad range of compounds that are possibly bioactive.

17.3.3 Biological Assay's:

The number biological assay's has been limited. Each new assay creates a significant amount of new work through the creation of new methods which require even more of the limited time available to be carried out. Two assays, the NO assay and the cytokine assay measuring IL-6 and TNF- α production, have been selected for this project. Neither has been conducted to date and so provide an opportunity to gain new insight into Kawakawa.

17.3.4 Skill:

I had no previous experience in conducting the complex biochemical assays. The skills needed to conduct assays of this nature needed to be acquired. This limited the number and the nature of the assays that could be conducted.

17.3.5 Budget:

The cell culture work that was conducted has a cost associated with it. Ngā Pae o te Māramatanga funded this research.

17.4 Justification for changes

Following discussions with Dr Taylor, fewer extracts of Kawakawa were chosen for use in the biological assays. This was because each the biological assays need to be run for each extraction methodology. In turn this

increases the time pressure. Using more extracts would have reduced the ability to be thorough in the research, which would have made the results less useful.

17.5 Summary Statement

Although modern scientific studies suggest there is no evidence to indicate that Kawakawa has any significant medicinal properties, historical reference, the lack of modern studies, and the variety of ailments it is claimed to treat suggest otherwise and suggest that Kawakawa has some bioactivity making Kawakawa worthy of investigation.

This study seeks to provide scientific evidence that supports the Māori observations of Kawakawa in rongoā. This study will do this by investigating the effect of Kawakawa extracts on inflammation. This will be achieved by measuring the production of three inflammatory markers, NO, TNF- α and IL-6 in the presence of Kawakawa extract. A reduction in the production of these 3 markers would indicate that Kawakawa has in vitro anti-inflammatory activity.

The inflammatory markers used in this study were chosen for measurement because within the constraints that limited the extent of this project they would provide comprehensive insight into the anti-inflammatory properties of Kawakawa.

The two methods of extraction used in this study (the aqueous extraction and the chloroform-methanol-water extraction) were developed because they were likely to extract bioactive compounds from Kawakawa. Neither of these methods was based solely upon a method used in a previous study and both combined factors from a number of previous studies. Furthermore, the aqueous infusion extraction method fused traditional Māori knowledge and modern scientific knowledge to create a unique methodology. Finally, no previous scientific study had used an aqueous extraction method when testing the medicinal properties of Kawakawa before.

18 . Scientific Results

18.1 Extractions

Extraction method	Weight of leaves used	Volume of solvent used	Ratio of leaves: solvent	Yield after freeze drying	Efficiency of extraction	Notes
Aqueous infusion using fresh leaves	15.85g	254mL	1:16	0.7404g	4.67%	Used in the biological assays.
Aqueous infusion using dried leaves	2.73g	43.7mL	1:16	0.3509g	12.85%	
Chloroform-methanol-water using fresh leaves	15.67g	157mL	1:10	0.4683g	2.99%	The yield after freeze drying refers to the methanol/water fraction. Used in the biological assays.
Enzyme assisted aqueous infusion using dried leaves	2.73g	27mL enzyme solution/16.7mL distilled water	1:16	2.9148g	106.77%	
Aqueous enzyme assisted using dried leaves	2.72g	27mL enzyme solution/84mL distilled water	1:40	3.0904g	113.62%	
Chloroform-methanol-water using dried leaves	2.70g	54mL	1:20	These extracts were not rotary evaporated or freeze dried.		
Chloroform-methanol-water enzyme assisted using dried leaves	2.76g	28mL enzyme solution/84mL Chloroform-methanol-water solution	1:40			

Figure 12: Summary of the extraction process.

It shows the effectiveness of the different extraction methods that were trialled. Methods using enzyme methods increased the yield of material compared to other methods.

Extraction method	Efficiency of extraction (weight of fresh leaves used vs. yield after freeze drying)	Amount of fresh leaf used to produce 1mL of 1000µg/mL Kawakawa extract solution	Notes
Aqueous infusion using fresh leaves	4.67%	21.4µg/mL	
Chloroform-methanol-water using fresh leaves	2.99%	33.4µg/mL	Refers to the methanol/water fraction.

Table 2: The amount of fresh leaf in the maximum concentration of extract used.

18.2 MTT Assay

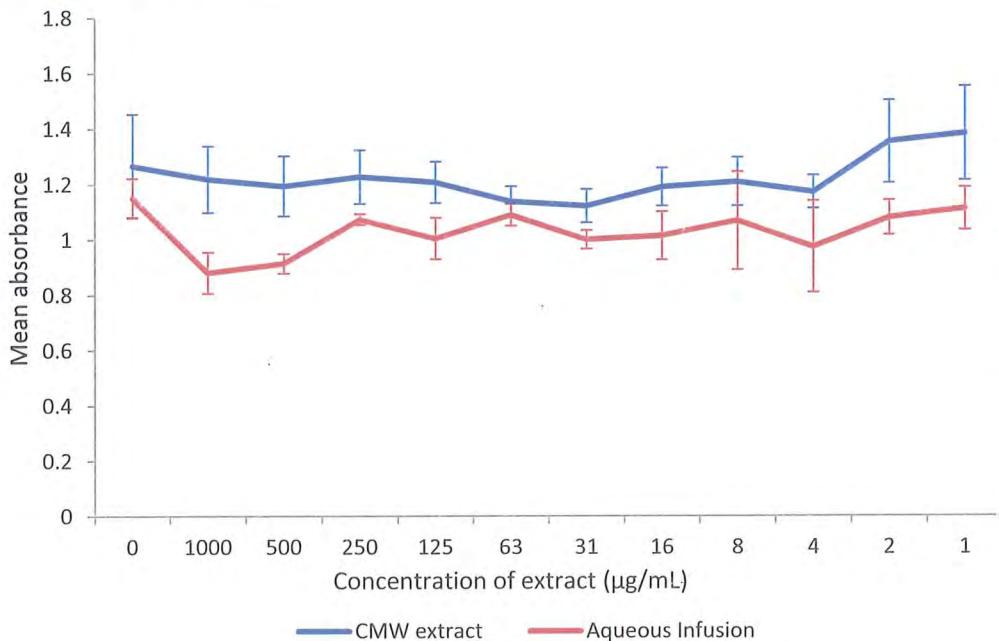


Figure 13: The MTT assay conducted. The 0 $\mu\text{g}/\text{mL}$ sample contains no Kawakawa extract, just cell medium. It is the control. A reduction in absorbance compared to the control is indicative of a reduction in cell viability. None of the data, for either extract, was statistically significant ($P>0.05$). None of the extracts affected the viability of the cells.

18.3 Flow Cytometry Assay

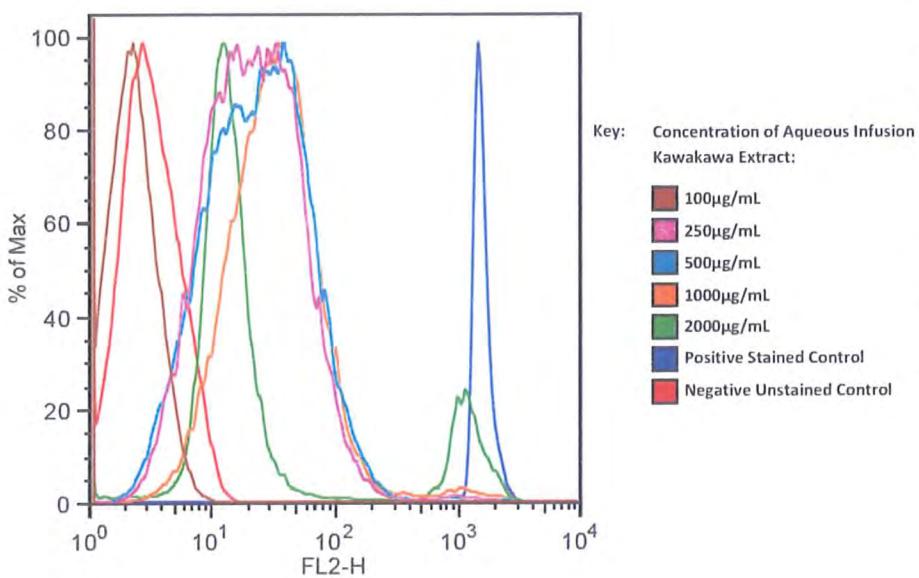


Figure 14: Graph from the flow cytometry assay.

Flow cytometry was measured for the aqueous extract to determine cell viability. Flow cytometry evaluation was used in this instance to confirm viability due to variation in the data (Fig 3).

Blue (positive control) = no cell viability
Red (negative control) = full cell viability.
The extract at a concentration of 2000 $\mu\text{g}/\text{mL}$ was not completely viable, shown by the low peak at 10^3 . Cell viability from 1000- 250 $\mu\text{g}/\text{mL}$ is constant at >99% (Fig 5). Cell viability at 100 $\mu\text{g}/\text{mL}$ is 100% (Fig 5)

Concentration of Aqueous Infusion Kawakawa extract ($\mu\text{g}/\text{mL}$)	Cells stained (%)	Cell Viability (%)
0 (Negative control - Unstained)	99.6	0.4
0 (Positive control - Stained)	0	100
2000	10.4	89.6
1000	0.9	99.1
500	0.28	99.72
250	0.29	99.71
100	0	100

Figure 15: The cell viability from the flow cytometry assay.

This shows the number of dead (stained) cells compared to the control, enabling the cell viability to be calculated. This data was derived from the data in figure 4. The data show that at the concentrations of extract used in the cell viability was greater than 99% except for the highest concentration of extract (2000 $\mu\text{g}/\text{mL}$).

18.4 Nitric Oxide Assay

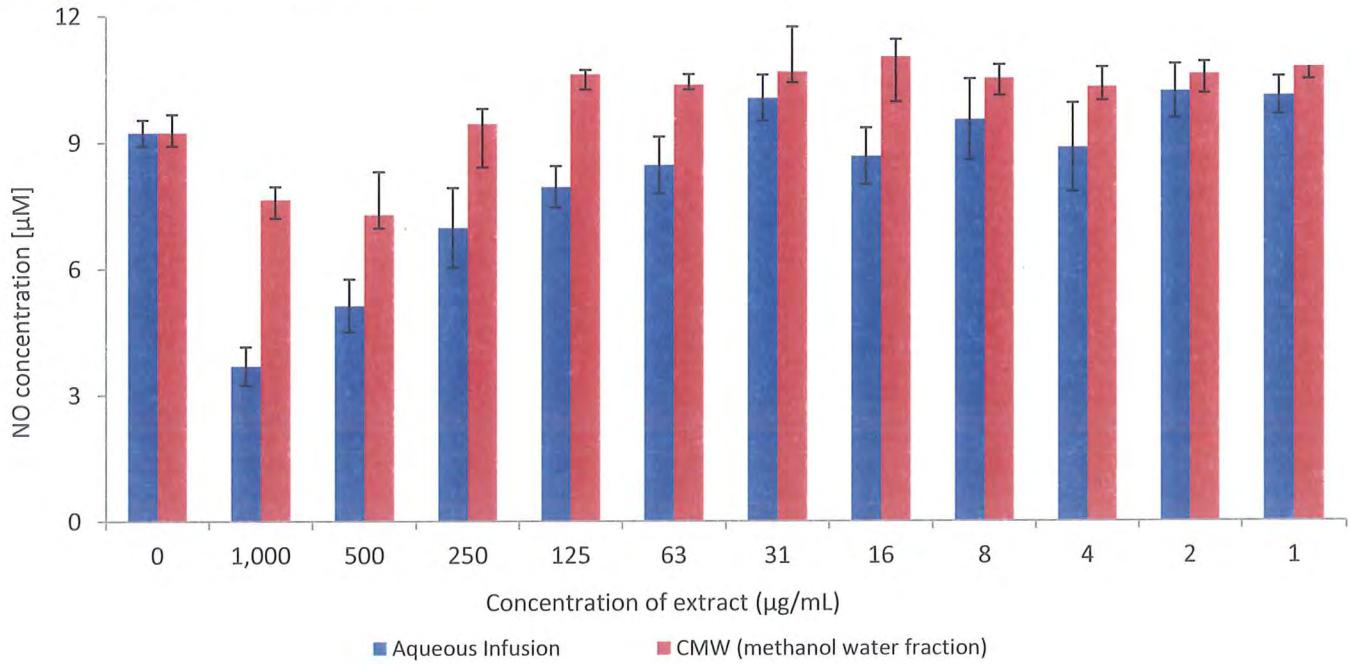


Figure 16: The aqueous extract caused a reduction in NO production.

P<0.05 at 1000μg/mL (60.0% decrease in NO production) and 500μg/mL (44.4% decrease in NO production). The control is the 0μg/mL sample, containing no Kawakawa extract.

18.5 Cytokine Assay

18.5.1 Cytokine production in the presence of the chloroform-methanol-water, methanol/water fraction extract

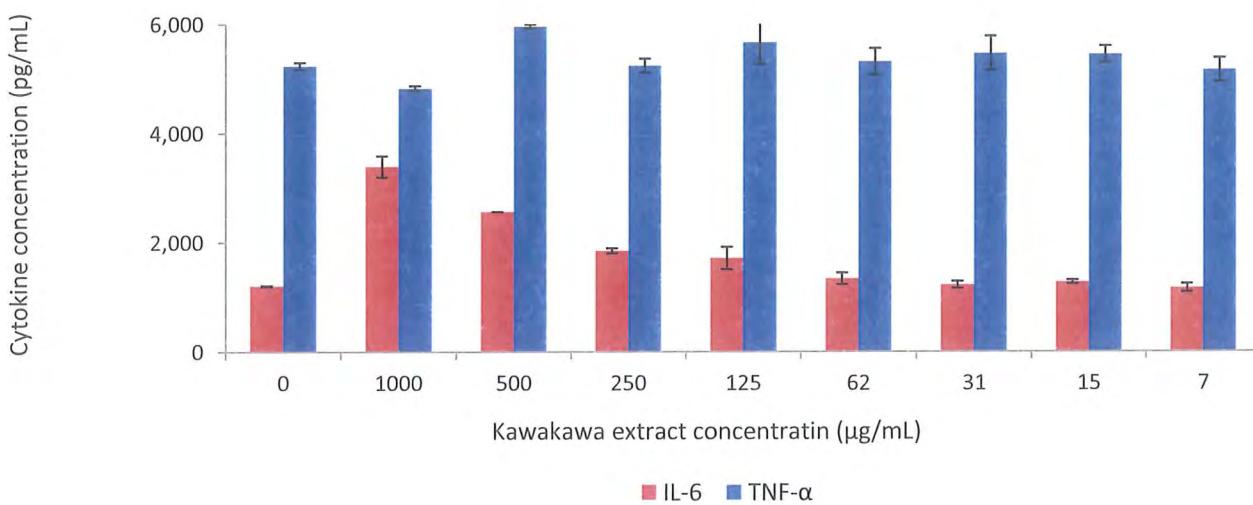


Figure 17: The CMW extract showed pro-inflammatory activity

At 1000μg/mL there was an 8% decrease in TNF-α production, and at 500μg/mL there was a 12% increase in TNF-α production, it is thought this is a result of an abnormally small standard error of the mean. Despite these two results it was concluded the CMW extract had no effect on TNF-α production. However, further research is required to formally exclude an effect of this extract on cytokine production.

At higher concentrations (>250μg/ml), the CMW extract appeared to act as a pro-inflammatory agent as there was a statistically significant increase in the production of IL-6 compared to the control. At 500μg/mL there was a 60% increase in IL-6 production and there was a 68% increase in IL-6 production at a concentration of 1000μg/mL.

The 0μg/mL sample contains no Kawakawa extract, just cell medium and LPS.

18.5.2 TNF- α production in cells exposed to the aqueous infusion extract

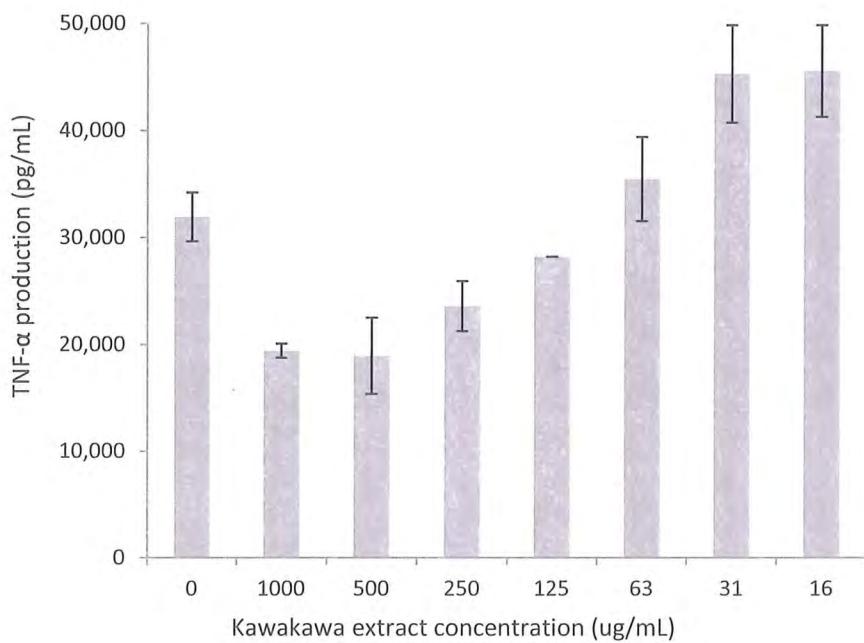


Figure 18: Cells exposed to the aqueous extract showed both pro and anti-inflammatory activity.

$P<0.05$ at $1000\mu\text{g/mL}$ (39.2% reduction), and at $500\mu\text{g/mL}$ (40.7% reduction). At these concentrations the production of TNF- α was inhibited by the addition of aqueous extract to cells.

At a concentration of $15.6\mu\text{g/mL}$ there was a statistically significant ($P<0.05$) increase (42%) in TNF- α production. At $31\mu\text{g/mL}$ there was a 41% increase, however this was not statistically significant.

The $0\mu\text{g/mL}$ sample is the control containing no Kawakawa extract, just cell medium and LPS.

18.5.3 IL-6 production in cells exposed to the aqueous infusion extract

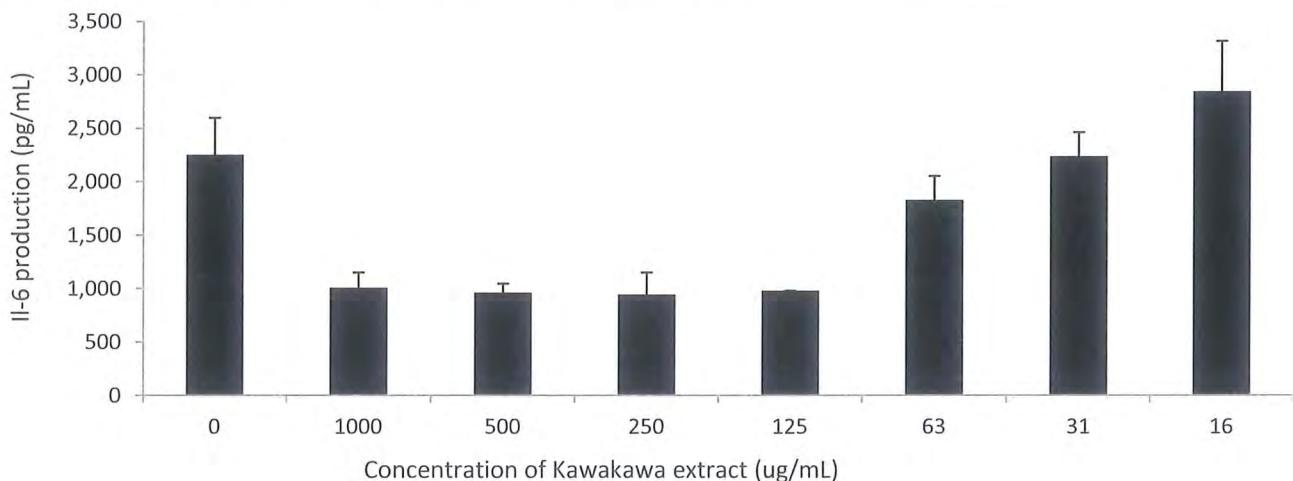


Figure 19: The production of IL-6 was inhibited by the addition of aqueous extract to cells.

The aqueous extract acted as an anti-inflammatory agent. $P<0.05$ at a concentration of $250\mu\text{g/mL}$ (58.2% reduction). There was also a 43% decrease in IL-6 production at a concentration of $125\mu\text{g/mL}$ although there was not enough replicates to calculate statistical significance.

The control is the $0\mu\text{g/mL}$ sample containing no Kawakawa extract.

19 . Scientific Discussion

Three key markers of inflammation were measured in this study to provide insight into whether Kawakawa has anti-inflammatory properties. These three markers were NO, and the inflammatory cytokines TNF- α and IL-6. A reduction in any of these markers would indicate that the extract had anti-inflammatory properties. However, before these tests could be conducted the material in the Kawakawa leaf needed to be extracted from the leaf into a liquid form and then, this extract needed to be tested to ensure it was not cytotoxic.

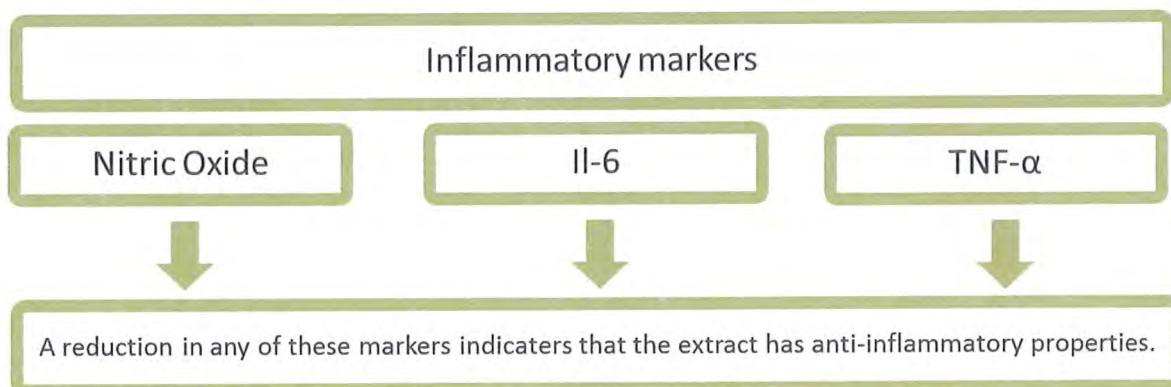


Figure 20: Inflammatory markers that were measured in this study.

19.1 Extractions

Although 7 extraction methods were developed, only 2 were used in the assays that followed: the aqueous infusion extract and the methanol/water fraction of the chloroform-methanol-water (CMW) extract. The specifics of each of these extraction methods are covered in the methodology. However, all of the extraction methodologies in this study were not based solely on any previous methodology.

The knowledge from many previous studies was combined and applied into the development of the extraction methodologies used in this study. As a result of this research the organic solvent extraction method did not involve heat (Vinotoru, 2001; International Centre for Science and High Technology), the leaves were not extracted under pressure, the leaves were agitated, the aqueous solvent was extremely polar and the chloroform-methanol-water solution contained solvents of a range of polarities. Additionally, the exposure of the leaves to light before extraction and before freeze-drying was minimal (McCloud, 2010). This knowledge was applied and a number of different possible extraction methods were considered. Initially 7 extraction methods were developed. These were the:

- Aqueous infusion using fresh leaves
- Aqueous infusion using dried leaves
- Chloroform-methanol-water using fresh leaves
- Chloroform-methanol-water using dried leaves
- Enzyme assisted aqueous infusion using dried leaves
- Enzyme assisted chloroform-methanol-water using dried leaves
- Aqueous enzyme assisted using dried leaves

The two extraction methods used in this study were chosen from these 7. The two methods used were chosen because they were deemed to be the most likely to be successful. The literature survey indicated that the aqueous infusion method best represented the extraction methodology used by Māori in rongoā and so this method fused the knowledge described by Māori in rongoā (TeRito & McPherson, 2012) with the knowledge from previous scientific studies on the extraction of bioactive compounds from plants. For example the leaves were protected from light prior to extraction as Vinatoru (2001) suggested and as Dr Paul Woodgate (2012) suggested the extract was concentrated using freeze drying rather than rotary evaporation.

Furthermore, the CMW extraction was identified as an extraction method that would extract a broad range of bioactive compounds.

The CMW extract naturally partitioned into a methanol/water phase and a chloroform phase by virtue of the relative densities of the solvents used. Only the methanol/water fraction was used because it has been identified that bioactivity tended to lie in the more polar fractions of an extract (Rehman, Ashfaq, Riaz, Javed, & Riazuddin, 2011; Bloor, 1995). The less polar chloroform fraction was likely to have extracted material containing plant compounds such as chlorophyll. These compounds are not likely to be a source of bioactivity (Rehman, Ashfaq, Riaz, Javed, & Riazuddin, 2011; Bloor, 1995; Greenwood, 2013) and they posed a challenge to solubilising the extract in pure water, which carried with it many advantages when adding the extract to cell culture as it meant that solvents such as methanol didn't need to be used. These solvents could have also impacted in cell viability.

Furthermore, as the yield of material produced was acceptable (0.74g (aqueous extract) and 0.47g (CMW extract) of freeze dried material per 15g of fresh leaf), alternative extraction methods, such as the enzyme assisted methods, were not required. The literature survey also suggested that these alternative methods carried with them a risk of damaging bioactive compounds as there was an increased chance of oxidation occurring compared to the aqueous extraction methodology (Greenwood, 2013). Destruction of the cell walls in leaves exposed to the enzyme solution (following placement in the enzyme solution the leaves had been broken down significantly) could have led to any bioactive compounds present in the leaf being exposed to other compounds in the leaf which may oxidise or react with any bioactive compounds present in the leaf. The oxidation of the bioactive compounds in the Kawakawa may reduce their bioactivity. Furthermore, enzymes also have the ability to break down bioactive compounds. Both Pranee & Kunika (2011) and Nattaporn & Pranee (2011) reported that enzymes such as pectinase (which was used in this study) could reduce the antioxidant activities of plant material. Antioxidants are a well described group of bioactive compounds that may have been responsible for the activity observed in this study. However, this was not explored and could be the focus of future studies. Consequently even if yields were not acceptable (which was not the case), other extraction methods were considered to be preferable for use in this study. There was also a chance that the greater extraction yield due to the extraction of cell wall components and lignins from the leaf could effectively dilute any bioactive compounds that were extracted (Greenwood, 2013).

One challenge with these extracts is that they were crude, unrefined extracts that contained a broad range of potentially bioactive compounds. This was demonstrated in the TNF- α assay using the aqueous extract where, at a concentration of 15.6 μ g/mL, there was a statistically significant ($P<0.05$) increase in TNF- α production. This suggested that the extracts contained both pro and anti-inflammatory compounds. The refinement of these extracts would be useful as it is likely that the results of this study are a result of the interactions between pro-inflammatory agents and anti-inflammatory agents present in the extract.

Calder *et al* (1986) screened methanol based extracts of the Kawakawa leaf against a variety of bacteria. This extract showed minor activity against *Staphylococcus aureus* and *Trichophyton mentagrophytes*, however this activity was significantly less than activity that was seen in other native plants (such as New Zealand passion fruit (*Tetrapathaea tetrandra*)) which are not as significant as Kawakawa in rongoā. Bloor (1995) conducted a similar series of tests to Calder *et al* (1986) however, included viruses and cancers as well as bacteria in the screening. In

this study any activity shown by Kawakawa was attributed to background tannins present in the extracts rather than genuine bioactive compounds. Consequently it was concluded that Kawakawa had no anti-bacterial or anti-viral activity. There are limitations associated with these studies. Neither study used a method of extraction that reflected the traditional methods of extraction, nor does either specify the concentration at which Kawakawa was applied to cells, something that was done in this study and shown to be of significance (as the activity observed in this study was shown to be dose dependant).

It is of note that in the NO, TNF- α and IL-6 assays it was only the aqueous extract that demonstrated anti-inflammatory activity. This would suggest that compounds present in the aqueous infusion extract are not present in the CMW extract. This provides some insight into why Bloor and Calder *et al* may have observed no anti-bacterial or anti-viral activity. Both of those studies used organic solvent based extracts, and parts of this research would imply that the bioactive compounds extracted in the aqueous infusion methodology are more conducive to reducing the production of NO, TNF- α and IL-6. This could suggest that the aqueous extract contains a particularly significant bioactive compound or a particular range of bioactive compounds that may mean this extract is more active in a range of different assays. This would also explain why Māori observed medicinal activity when they used a method similar to the aqueous extraction method in rongoā. The aqueous extraction method best represented traditional Māori methods for preparing Kawakawa. This would suggest the bioactive compounds responsible for the activity in this study were thermostable, polar and could only be extracted by the aqueous extraction method. In turn this could suggest that heat may be needed to extract the compounds from Kawakawa. Regardless, there appears to be connection between the extraction method used to extract Kawakawa and whether the extract has bioactivity.

There would be an opportunity for future studies to refine the extracts, fractionate the aqueous extract to isolate the bioactive components and to examine the effectiveness of the other extraction methodologies. This was not done in this study due to the constraints of time and a desire to keep the study focussed.

19.2 MTT Assay

The MTT assay shows the effect of the extract on a cell's metabolism. The yellow MTT solution is absorbed into a cell and is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore can be directly related to the number of viable cells. However an apparent reduction in viability may be due to metabolic stress rather than cell death or a loss of viable cells as the conversion of MTT to purple formazan may be less efficient if the cells are under metabolic stress (Wallert and Provost Lab, 2007). This assay is important because if the extract places metabolic stresses on the cell, this could cause an apparent reduction in the production of the inflammatory markers.

None of the data obtained from this assay showed a statistically significant ($P>0.05$) impact on cell viability. However, with the aqueous extract the apparent reduction in cell viability at the highest concentration was a concern as it suggested that the extract may be having an effect on the metabolism of the cell. To be certain the cells were not negatively affected by the extract, the Trypan Blue Assay and Flow Cytometry cell viability assay were used to determine if the extract was causing cell death.

19.3 Trypan Blue Assay

The Trypan Blue Assay measures cell death. The Trypan Blue dye is only absorbed into dead cells, meaning it provides a measurement of the number of viable cells. No cell death was observed in either of the Kawakawa extracts and there was no increase in the amount of cell death as the concentration of extract increased. This shows that the slight reduction in cell viability observed in the MTT assay for the aqueous extract is not due to cell death.

19.4 Flow Cytometry Assay

Flow cytometry as a measure of cell viability was employed because there was a slight reduction (although not statistically significant) in the absorbance measured in the MTT assay in cells exposed to the aqueous extract. This reduction was consistent across multiple repeats of the assay. This assay was conducted to support the results of the Trypan Blue Assay; the extract did not negatively affect cell viability. Flow cytometry provides a highly accurate measure of cytotoxicity. Flow cytometry is a method of measuring the number of stained cells. The level of cell death in cells exposed to the aqueous extract was constant and very low across the concentrations used in the later assays. This assay showed that the slight drop in apparent cell viability with the aqueous extract is not significant and therefore not relevant.

This assay showed a reduction in cell viability at a concentration of 2000 μ g/mL. This confirms that the aqueous extract does contain cytotoxic compounds meaning it would have not been possible to use this concentration of extract. However, the highest concentration of extract used in the later assays was 1000 μ g/mL where no cytotoxicity was measured. Fractionation of the aqueous extract would identify whether it is the compounds responsible for the anti-inflammatory affects that are cytotoxic or whether the cytotoxicity is a result of additional compounds extracted during the extraction process.

This assay along with the Trypan Blue Assay showed that the concentrations of extract (less than 1000 μ g/mL) used in the NO assay and the cytokine assay were not cytotoxic. Therefore, any reduction or increase in NO, TNF- α or IL-6 was a result of compounds that affect these inflammatory markers rather than cell death.

19.5 Nitric Oxide Assay

Acute inflammation is associated with the production of reactive oxygen species such as NO. Consequently NO is considered to be a key inflammatory marker. If the Kawakawa extract has anti-inflammatory activity, then cells exposed to the extract would show a reduction in the production of NO.

NO is produced by macrophages triggered by the presence of compounds such as lipopolysaccharide (LPS) or by inflammatory cytokines. For a compound to reduce the production of NO it must either:

- Block the site where the LPS or cytokine joins to the receptor that triggers NO production.
- Break down the NO when it is produced to other compounds.
- Interfere with the nitric oxide synthases (an enzyme group).

There were consistent reductions in the NO production for at least the two highest concentrations of the aqueous extract (1000 μ g/mL and 500 μ g/mL) and at times the reduction occurred at lower concentrations. It is interesting to note that in the NO assay the CMW extract showed no activity.

It is also clear that the concentration of extract had an impact on NO, IL-6 and TNF- α production. The greatest suppression of NO and TNF- α production, and therefore the greatest anti-inflammatory activity, occurred at the two highest concentrations of extract (1000 μ g/mL and 500 μ g/mL). Whatever bioactive compound was responsible for inhibiting the production of NO was present at a biologically active concentration in the greatest two concentrations of aqueous extract. The reduced or nil activity at lower concentrations may indicate that there is a critical concentration that is needed to give a biological response, or that at lower concentrations of the Kawakawa extract the anti-inflammatory effect is offset by other compounds present in the extract. Bloor and Calder *et al* may not have used an appropriate extract concentration in their study as the concentration of Kawakawa extracts used are not described.

The presence of an anti-inflammatory affect in the aqueous extract would suggest that the bioactive compounds responsible for this activity is thermostable and highly polar as it was extracted by water but not extracted by less polar organic solvents such as methanol and chloroform.

Kawakawa is likely to contain a variety of compounds, a number of which may be bioactive, responsible for the anti-inflammatory activity and the anti-bacterial actions of Kawakawa observed in rongoā. Consequently, due to the likely presence of a wide range of bioactive compounds in the extract, and the possibility of multiple interactions between these compounds and the cells in this assay it would be wrong to assert that the same compound that inhibited NO production was also responsible for the inhibition/incitement of TNF- α and IL-6 production. However, it is possible that some of the bioactive compounds extracted by the aqueous extract in this study, could also be responsible for some of the other actions of Kawakawa in rongoā, such as anti-bacterial activity.

It is possible that the reduction in NO production could be a result of tannins (Bloor, 1995) as tannins, "could affect the inflammatory response via their radical scavenging activities" (Jeffers, 2006). However tannins are still a legitimate bioactive compound and their actions could explain the reductions in inflammation both in this study and could explain the anti-inflammatory activity observed by Māori in rongoā.

An area for future research would be to use mass spectrometry to investigate the compounds present in the extract to perhaps identify the compound responsible for the anti-inflammatory activity.

The key outcome of this work is to have produced evidence in the NO assay that shows that Kawakawa has anti-inflammatory activity which supports the traditional uses of Kawakawa in rongoā.

19.6 Cytokine Assay

Inflammatory cytokines are another group of key inflammatory markers. Cytokines such as IL-6 and TNF- α are proteins secreted by cells that participate in inflammation in the body and they serve to regulate the control aspects of the inflammatory process and so their measurement provides insight into the extent of an extracts activity. The inflammatory cytokines TNF- α and IL-6, are produced by macrophages in response to trauma or microbial components. A reduction in the amount of IL-6 and TNF- α produced in response to a recognized-inflammatory mediator would indicate anti-inflammatory activity. The results of the cytokine assay are intriguing and add another aspect to the study. As each of the cytokines behaved independently of each other in their response to the Kawakawa extract, each will be dealt with separately.

19.6.1 TNF- α

The results of this assay closely replicated the results of the NO assay.

Although there appeared to be some activity at the highest concentrations of CMW extract in the form of a 8% decrease in TNF- α production at 1000 μ g/mL, and a 12% increase in TNF- α production at 500 μ g/mL, it is thought this is a result of an abnormally small standard error of the mean and a limited number of duplicates. Furthermore, it is odd that there would be anti-inflammatory activity and pro-inflammatory activity in successive concentrations of extract. Therefore, despite these two results it was concluded that the CMW extract had no effect on TNF- α production. Further research is needed to confirm this.

The aqueous infusion extract caused a reduction in TNF- α production at concentrations of 500 μ g/mL and 1000 μ g/mL. At the lowest concentrations of aqueous Kawakawa extract used 15.6 μ g/mL there was a statistically significant ($P<0.05$) increase in TNF- α production. Between 250 μ g/mL and 31.25 μ g/mL there was no statistically significant activity. This would suggest that the activity is dose dependant.

The data suggests that because there was both anti-inflammatory activity (at concentrations of 1000 μ g/mL, and 500 μ g/mL) but also pro-inflammatory activity (at a concentration of 15.6 μ g/mL) that there are compounds in the aqueous Kawakawa extract that can both incite and suppress the production of TNF- α . Therefore, it is likely that at all concentrations the final TNF- α production is the net result of the interactions between pro and anti-inflammatory compounds.

The evidence of both pro-inflammatory and anti-inflammatory activity in the aqueous Kawakawa extract could suggest that these pro-inflammatory and anti-inflammatory compounds extracted affect each of the intracellular mechanisms responsible for the production of TNF- α differently.

A pro-inflammatory compound would not be responsible for the activity observed in rongoā.

Previous studies (Evans & Taylor, 1983) have researched the pro-inflammatory and tumour-promoting diterpenes of the plant families Euphorbiaceae and Thymelaeaceae and shown that pro-inflammatory activity exists. Consequently, the compounds affect what was observed in this study are not unique to Kawakawa.

19.6.2 IL-6

In contrast to the behaviour of the aqueous extract in the NO assay and in the TNF- α assay, the aqueous extract caused a statistically significant reduction in IL-6 production at concentrations of 250 μ g/mL and 125 μ g/mL (although it was not possible to calculate the statistical significance of the data at a concentration of 125 μ g/mL due to having only a single data point). So, at these concentrations Kawakawa extract had anti-inflammatory activity. This reinforces that Kawakawa has significant anti-inflammatory activity. This was different to the NO and TNF- α assay where the anti-inflammatory activity was observed at the highest concentrations of extract. Although there are reductions in IL-6 production at 1000 μ g/mL and 500 μ g/mL that appear to be of a similar magnitude to the reductions at 250 μ g/mL and 125 μ g/mL, these were not statistically significant ($P \approx 0.09$). There would be an opportunity for further research into why this occurred.

In cells exposed to the CMW extract the production of IL-6 increased as the concentration of extract increased. At concentrations of 1000 μ g/mL, 500 μ g/mL and 250 μ g/mL there were statistically significant increases in IL-6 production. Therefore, the CMW extract shows pro-inflammatory activity, suggesting the extract contains pro-inflammatory compounds. Pro-inflammatory activity is unlikely to be responsible for the medicinal uses of Kawakawa in rongoā.

The differences between the CMW extract and the aqueous extract are likely the result of the different extraction methods extracting different types of compounds from the Kawakawa leaf. However, the pro-inflammatory compounds extracted in the CMW extract that affected IL-6 production, did not cause pro-inflammatory activity in the TNF- α or NO assays.

19.6.3 Cytokine Summary

It would not be possible to assume that the same compound is responsible for the activity seen in the NO assay, the TNF- α assay and the IL-6 assay. The difference between the activity in the aqueous extract and the CMW extract could be because the compounds extracted by these different methodologies could affect the intracellular mechanisms responsible for the production of each of the cytokines differently.

Although the effects of Kawakawa appear to differ with respect to TNF- α and IL-6 production, this result is not contradictory. The effects of IL-6 and TNF- α in the body are not the same. Marcinkiewicz *et al* (1994) suggested that, "TNF- α and NO may be stimulated via different receptors to IL-6". Furthermore, Marcinkiewicz *et al* (1994) stated that, "IL-6 and TNF- α play opposite roles during inflammation". This is because TNF- α is a pro-inflammatory cytokine meaning that it creates inflammation in cells when produced. In contrast IL-6 stimulates synthesis of acute-phase proteins, which are produced by the liver in response to inflammation. These acute phase proteins are molecules that are important in limiting inflammation (Marcinkiewicz, Czajkowska, Grabowska, Kaspruwicz, & Kociszewska, 1994). Consequently, IL-6 and TNF- α can show an altered response to same extract. It also means that the production of TNF- α can be limited by a Kawakawa extract even when the production of IL-6 is incited by a Kawakawa extract. This explains how the differences in the results for each of the cytokines can occur even when cells are exposed to the same extract.

This study showed that both the aqueous and CMW Kawakawa extracts each had a different effect on the production of each of the cytokines. The different effects of the extracts on each of the cytokines and the difference between the results in the IL-6 assay and the NO assay are due to complex intracellular mechanisms that govern the production of inflammatory cytokines. However, in both cytokine assays the aqueous Kawakawa extract provide evidence that supports the traditional Māori use of Kawakawa in rongoā.

19.7 Summary

The data produced by this study through the measurement of NO, TNF- α and IL-6 provides a scientific backing to support the actions of Kawakawa observed by Māori in rongoā. This fills a niche in the literature as there has been no previous research into the anti-inflammatory properties of Kawakawa, and nor has any other research demonstrated a scientific basis from which the actions of Kawakawa in rongoā can be explained. Many of the traditional uses of Kawakawa in rongoā could be linked directly to inflammation (such as toothache, irritation, serious bruises, and infected wounds (Brooker, Cambie, & Cooper, 1987)). Furthermore, the anti-inflammatory actions of Kawakawa could mask the symptoms of other medical conditions as inflammation is associated with other ailments such as viral infections.

This research is extremely significant for Māori communities as it supports their cultural practice in rongoā with scientific research for the first time.

20 . Scientific Conclusions

The brief of this investigation was to investigate the effects of Kawakawa extracts on the production of inflammatory mediators produced by immune cells. The results show that:

- The aqueous extract causes a:
 - Significant decrease in nitric oxide production at concentrations of 1000 $\mu\text{g}/\text{mL}$ and 500 $\mu\text{g}/\text{mL}$.
 - Dose-dependent decrease in TNF- α production by macrophages exposed to LPS. This inhibition was maximal at extract concentrations of 1000 $\mu\text{g}/\text{ml}$ and 500 $\mu\text{g}/\text{mL}$.
 - Dose-dependent decrease in IL-6 production by macrophages exposed to LPS. This inhibition was maximal at concentrations of 250 $\mu\text{g}/\text{mL}$ and 125 $\mu\text{g}/\text{mL}$. Above this concentration IL-6 production increased, indicating the presence of pro-inflammatory compounds in the extract.
- The CMW extract:
 - Has no impact on NO or TNF- α production.
 - At concentrations above 250 $\mu\text{g}/\text{ml}$ increased IL-6 production.

These results achieved this brief and proved the hypothesis as the in-vitro production of NO, IL-6 and TNF- α was suppressed in the presence of aqueous Kawakawa extract.

The uses of Kawakawa identified by Māori in rongoā are supported by the anti-inflammatory activity observed in this study through a reduction in NO production, IL-6 production and TNF- α production.

It is significant to note that the extract preparation method has an effect on the results. Both of the extracts had the ability to act in different ways. Furthermore, the different extraction methods have extracted a broad range of both pro and anti-inflammatory compounds and that the compounds within each extract affect the different processes involved with the production of inflammatory markers in different ways.

However, it is important to note that there is some difference between the in vitro work conducted in this study and the in vivo scenarios in which Kawakawa is used as part of rongoā.

Areas of investigation for future studies could involve:

- Mass spectrometry to identify compounds in the extract that are potentially bioactive compounds and what could be responsible for the observed activity and whether this explains the effectiveness of the aqueous infusion extract compared to the CMW extract.
- Refinement of the extracts into more pure substances and isolation of the bioactive compounds.
- Investigating some of the alternative extraction methods to see if they influence the anti-inflammatory properties.
- The measurement of other inflammatory cytokines.
- Using a aqueous extract to test the anti-bacterial and anti-viral properties of Kawakawa.

This study provides, for the first time, scientific evidence that supports the traditional uses of Kawakawa in rongoā.

21. Technological Outcomes

21.1 Nature of the outcome

The technological outcome of this study was an aqueous Kawakawa extract that showed in-vitro anti-inflammatory activity. A secondary outcome was a mechanism to test the ant-inflammatory properties of Kawakawa extracts, which could be applied to any plant extract. Consequently, the brief of this study was achieved.

The system to test the anti-inflammatory properties of Kawakawa (or any other plant extract) has the following attributes:

- Extraction of the plant material
- Testing of the cell viability
 - This establishes the concentrations at which there is no cytotoxicity (cytotoxicity could lead to an artificial reduction in NO, TNF- α and IL-6 production)
 - Use the MTT assay, the Trypan Blue Assay and flow cytometry
 - Based on standard methodology
- Anti-inflammatory testing
 - Use the concentrations that are not cytotoxic (<1000 μ g/mL)
 - Test the anti-inflammatory properties of the extract using the NO, TNF- α and IL-6 assays
 - Based on standard methodology

The primary technological outcome was the aqueous Kawakawa extract:

- Caused no cytotoxicity at concentrations <1000 μ g/mL
- Had anti-inflammatory activity
 - A statistically significant decrease in nitric oxide production at concentrations of 1000 μ g/mL and 500 μ g/mL.
 - Dose-dependent decrease in TNF- α production by macrophages exposed to LPS. This inhibition was maximal at extract concentrations of 1000 μ g/mL and 500 μ g/mL.
 - Dose-dependent decrease in IL-6 production by macrophages exposed to LPS. This inhibition was maximal at concentrations of 250 μ g/mL and 125 μ g/mL. Above this concentration IL-6 production increased, indicating the presence of pro-inflammatory compounds in the extract.
- Nature of the extract

- Ratio of leaves:solvent (water) – 1:16
- Yield after freeze drying – 0.7404g
- Efficiency – 4.67%
- 21.4ug/mL of fresh Kawakawa leaf per 1mL of 1000ug/mL extract solution
- A liquid that can be freeze dried

21.2 Fitness for purpose

The scientific testing conducted provides evidence for the fitness for purpose of the technological outcome.

The technological outcome of this study was an aqueous Kawakawa extract that showed *in-vitro* anti-inflammatory activity. A secondary outcome was a mechanism to test the anti-inflammatory properties of Kawakawa extracts, although this system could be applied to any plant extract.

For the technological outcome to be fit for purpose it needed to demonstrate anti-inflammatory activity.

The CMW extract was not fit for purpose because it did not demonstrate *in-vitro* anti-inflammatory activity against a number of different inflammatory markers. In contrast the aqueous extract was fit for purpose. It demonstrated a:

- Statistically significant decrease in nitric oxide production at concentrations of 1000 μ g/mL and 500 μ g/mL.
- Dose-dependent decrease in TNF- α production by macrophages exposed to LPS. This inhibition was maximal at extract concentrations of 1000 μ g/mL and 500 μ g/mL.
- Dose-dependent decrease in IL-6 production by macrophages exposed to LPS. This inhibition was maximal at concentrations of 250 μ g/ml and 125 μ g/mL.

This anti-inflammatory activity in the aqueous extract fulfils the final brief statement.

Although the aqueous extract demonstrates *in-vitro* anti-inflammatory activity, this technological outcome could be developed into other areas:

- A balm with an aqueous extract concentration of 1000 μ g/mL, 500 μ g/mL or 250 μ g/mL
- A drink with an aqueous extract concentration of 1000 μ g/mL, 500 μ g/mL or 250 μ g/mL
- A tea/beverage with an aqueous extract concentration of 1000 μ g/mL, 500 μ g/mL or 250 μ g/mL

Although, these would not be proven themselves to have activity, this study would support these products having anti-inflammatory activity, although ideally there would be clinical trials into the efficacy of these products.

Alternatively, a rongoā user or a rongoā practitioner who uses Kawakawa, now has scientific support for the use of Kawakawa in rongoā. As discussed previously (in the Stakeholders section, page 30), the general public has a limited understanding of rongoā and rongoā lacks credibility as a medicinal tool among these groups. The primary technological outcome (an aqueous Kawakawa extract that demonstrates anti-inflammatory activity) has the ability to increase the credibility of rongoā. This technological outcome leads to the broader acceptance of Kawakawa's medicinal properties and therefore rongoā. Science is an important tool for acceptance of a product among the general public.

The problem is that there was no scientific evidence to support the use of Kawakawa in rongoā. This technological outcome is extremely significant as there are no existing solutions to the problem which my technological outcome addresses.

Calculating the statistical significance was an important tool for determining the fitness for purpose of these extracts. The CMW extract was not fit for purpose because it did not demonstrate a statistically significant reduction in NO, TNF- α and IL-6 production. The aqueous extract was statistically significant because at

concentrations of 1000 μ g/mL and 500 μ g/mL NO and TNF- α assay it demonstrated anti-inflammatory activity and statistically significant anti-inflammatory activity at concentrations of 250 μ g/mL and 250 μ g/mL in the IL-6 assay.

The scientific evidence and discussion provided in the previous sections demonstrates the aqueous extracts fitness for purpose as this extract demonstrates anti-inflammatory activity.

The secondary outcome of this study, a system for testing the anti-inflammatory properties of Kawakawa extracts, and any plant extract in general, is also fit for purpose.

The methods used in this study (the MTT assay, the Trypan Blue assay, the flow cytometry assay, the NO assay, and the TNF- α and IL-6 assay) were fit for purpose. All of the assays were repeatable, all were standard, previously validated methods and all producing meaningful, realistic results.

The use of previously validated methods meant that the results were consistent and accurate. The repeatability of the results, and that they made sense in the context of the results expected suggests again that the results were accurate.

The cell viability assays and the anti-inflammatory activity could be used with any plant extract (although the concentration of plant extract used in the anti-inflammatory activity would be dependent on the results of the cell viability assays).

21.3 Client Feedback

Early in the project Ngā Pae o te Māramatanga was identified as the client. Following the completion of this project there was communication back to Ngā Pae o te Māramatanga about the outcomes of this project.

Ngā Pae o te Māramatanga sets out to conduct, fund and support excellent research. Within this they have a number of objectives, including:

1. Seek an understanding of the contribution of Māori peoples to new frontiers of knowledge, economic development, environmental sustainability, health and social wellbeing and educational achievement.
2. Build relevant research capacity and capability – create and maintain pathways to research excellence.
3. Give meaningful expression to indigenous knowledge/mātauranga Māori to address issues, needs and opportunities.

After the project was completed Ngā Pae o te Māramatanga was approached for feedback. The feedback from Ngā Pae o te Māramatanga was that they were thrilled and very excited with the biotechnological outcomes of this study. This is because it fulfilled some of their research goals:

1. The knowledge of Māori peoples has contributed to new frontiers of knowledge, and health. This is because the biotechnological outcomes of this project show that the traditional uses of Kawakawa in rongoā are supported by scientific evidence. This technological outcome supports traditional Māori cultural practice. Furthermore, the extract that demonstrated the anti-inflammatory activity (the aqueous extract) was premised on Māori knowledge. This biotechnological outcome adds new knowledge to the body of literature and so creates a new ‘frontier’ of knowledge.
2. The secondary technological outcome of this study (a system for testing the anti-inflammatory properties of a plant extract) is a pathway for research excellence in the future as it allows for the testing of other plants used by Māori in rongoā.
3. The biotechnological outcome of this study is a meaningful expression of indigenous knowledge/mātauranga Māori to which addresses an issue (scientific evidence to support the uses of Kawakawa in rongoā).



Therefore, as it fulfilled these goals, the technological outcome achieved what Ngā Pae o te Māramatanga had desired.

Ngā Pae o te Māramatanga has also indicated that they are interested in supporting and funding the development of the biotechnological outcome developed in this project. They have indicated to Dr Taylor that they would be willing to fund further research. This would include funding the fractionate of the aqueous extract and then testing the anti-inflammatory properties of each fraction with the goal of identifying the compounds responsible for the activity observed, but also trying to increase the magnitude of the anti-inflammatory activity by isolating the anti-inflammatory components.

This technological outcome has created a number of opportunities for Ngā Pae o te Māramatanga as well as the technological outcomes having an inherent value themselves. Consequently, the technological outcome of my study is exciting for them.

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		Dr Ora Emslie	

24. Photo's



Figure 23: The cell culture room in the Taylor lab where the cell culture testing took place.



Figure 22: My desk space in the Taylor lab where the extractions were conducted.

Figure 21: The leaves prior to extraction



Figure 24: Me in the cell culture lab.

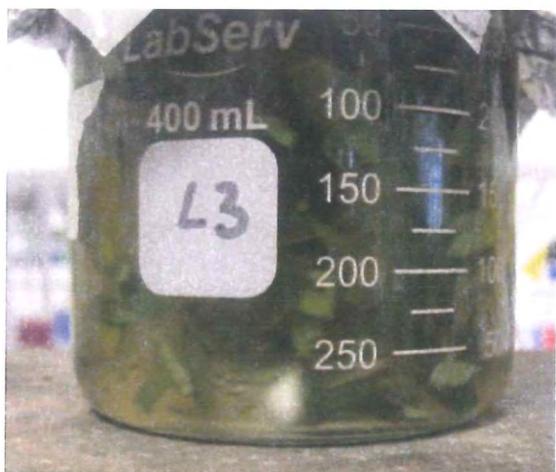
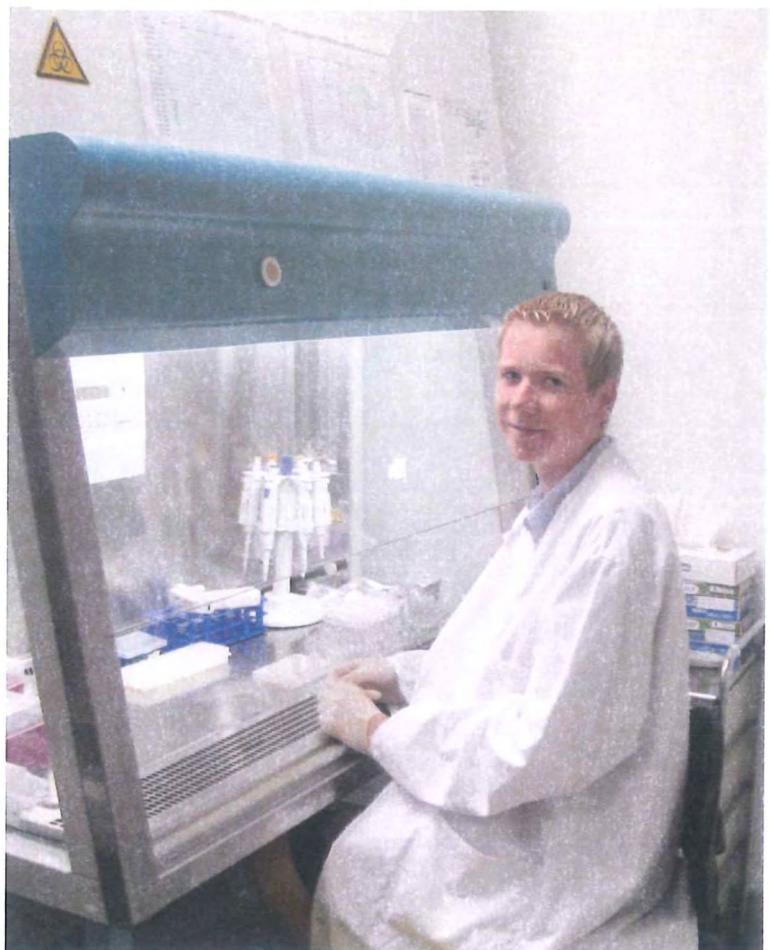


Figure 25: The leaves during the aqueous infusion extraction.



Figure 30: The freeze dried aqueous infusion extract.

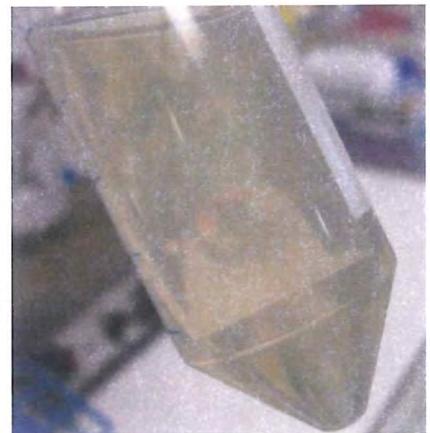


Figure 29: The centrifuged aqueous infusion extract prior to being freeze dried.



Figure 31: The CMW extract after being centrifuged but before being rotary evaporated or freeze dried.



Figure 28: Shows the CMW extract prior to the two fractions being separated, rotary evaporated or freeze dried.



Figure 27: Shows the methanol/water fraction of the CMW extract prior, rotary evaporation or freeze drying.

The dark green layer is the chloroform fraction and the light green layer is the methanol/water fraction. This image is not representative of the volume of extract produced in each of the fractions.



Figure 26: The methanol/water fraction of the CMW extract after rotary evaporation and freeze drying.

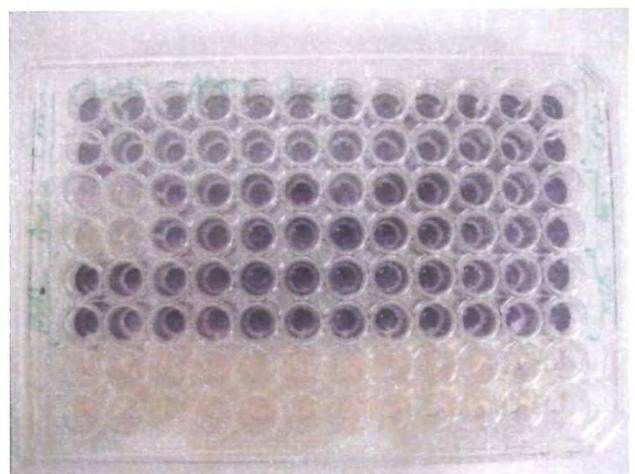


Figure 32: A picture of a 96 well plate used in the MTT assay after treatment with the MTT solvent.

The greater the intensity of the purple colour, the greater the cell viability.