

# **Antibacterial effects of New Zealand plant extracts against mycobacteria**

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## ABSTRACT

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB) has infected approximately 1/3 of the world population, with 9.4 million new TB cases in 2008. In addition to increased cases of drug-resistant strains it is vital that novel antitubercular compounds are discovered in order to treat infections and reduce the time of current TB therapy courses. Natural resources such as plants are now being considered as the focus for discovering new compounds. Plants have long been investigated as a source of antibiotics for the treatment of human disease. New Zealand (NZ) contains a unique and diverse flora; however, to current knowledge no native plants have been examined for antimycobacterial activity. Using ethnobotany as a basis for selection, a total of 58 native plant samples were collected and tested for direct antimycobacterial activity. Samples were extracted with sterile distilled water (SDW), ethanol (EtOH) or methanol (MeOH) and screened for inhibition against the surrogate species, *Mycobacterium smegmatis*. Active plant samples were then validated for bacteriostatic activity towards *M. bovis* BCG and *M. tuberculosis* H37Ra as well as other clinically-important species. Nine extracts from the species *Laurelia novae-zelandiae*, *Lophomyrtus bullata*, *Metrosideros excelsa*, *Myoporum laetum*, *Pittosporum tenuifolium*, *Pseudopanax crassifolius* and *Pseudowintera colorata* were found to be active against *M. smegmatis*. Two active extracts were the bark and cambium extracts of *Laurelia novae-zelandiae* (Pukatea), which were reportedly used by indigenous Māori for the treatment of tubercular lesions. Upon further investigation these extracts also demonstrated

bactericidal activity towards *M. smegmatis* as well as bacteriostatic activity towards the slow-growing species *M. bovis* BCG and *M. tuberculosis*. Purification techniques were then performed to improve the efficiency of activity and initial exploration of delivery systems was also examined. The bioactive extracts determined in this research offer a starting point for identifying their chemical basis of antimycobacterial activity with the objective of potentially discovering new anti-tubercular drugs.

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**ABBREVIATIONS**

|                         |   |
|-------------------------|---|
| <b>AQ</b>               | Aqueous                                 |
| <b>CAP</b>              | Capreomycin                             |
| <b>CHCl<sub>3</sub></b> | Chloroform                              |
| <b>CYS</b>              | Cycloserine                             |
| <b>DNA</b>              | Deoxyribonucleic acid                   |
| <b>EpoA</b>             | Epothilone A                            |
| <b>ETH</b>              | Ethambutol                              |
| <b>EtOAc</b>            | Ethyl Acetate                           |
| <b>EtOH</b>             | Ethanol                                 |
| <b>FCS</b>              | Fetal Calf Serum                        |
| <b>FDA</b>              | Food and Drug Administration            |
| <b>FITC</b>             | Fluorescein Isothiocyanate              |
| <b>GFP</b>              | Green Fluorescent Protein               |
| <b>HIV</b>              | Human Immunodeficiency Virus            |
| <b>HPLC</b>             | High-performance-liquid-chromatography  |
| <b>IC<sub>50</sub></b>  | 50 % inhibitory concentration           |
| <b>INH</b>              | Isoniazid                               |
| <b>IPTG</b>             | Isopropyl β-D-1-thiogalactopyranoside   |
| <b>KAN</b>              | Kanamycin                               |
| <b>KatG</b>             | Catalase-Peroxidase                     |
| <b>LA</b>               | Luria Agar                              |
| <b>LB</b>               | Luria Broth                             |
| <b>OADC</b>             | Oleic Acid, Albumin, Dextrose, Catalase |
| <b>OD</b>               | Optical Density                         |

|               |  |
|---------------|--|
| <b>MDR-TB</b> | Multi-drug resistant TB                            |
| <b>MeOH</b>   | Methanol   |
| <b>MIC</b>    | Minimum Inhibitory Concentration                   |
| <b>MOA</b>    | Mechanism of Action                                |
| <b>MS</b>     | Mass Spectrometry                                  |
| <b>MRSA</b>   | Methicillin-resistant <i>Staphylococcus aureus</i> |
| <b>NMR</b>    | Nuclear magnetic resonance                         |
| <b>NZ</b>     | New Zealand  |
| <b>PZA</b>    | Pyrazinamide                                       |
| <b>RIF</b>    | Rifampin   |
| <b>SARs</b>   | Structural Activity Relationships                  |
| <b>SDW</b>    | Sterile Distilled Water                            |
| <b>SPS</b>    | Solvent Phase Separation                           |
| <b>STR</b>    | Streptomycin                                       |
| <b>TB</b>     | Tuberculosis                                       |
| <b>TET</b>    | Tetrandrine  |
| <b>TMP</b>    | Tetramethylpyrazine                                |
| <b>USA</b>    | United States of America                           |
| <b>WHO</b>    | World Health Organization                          |
| <b>XDR-TB</b> | Extensively drug-resistant TB                      |

# CHAPTER ONE: General Introduction

## 1.1 Background

Tuberculosis (TB) is an infectious disease that primarily affects the lungs and is caused by the slow-growing acid-fast bacillus *Mycobacterium tuberculosis* [1]. Tuberculosis and bacilli infection has afflicted humans for thousands of years [2, 3]. Egyptian mummies have been discovered with signs of TB infection [4] and even 9000 year old human remains had indications of a tubercule contagion [5]. Around the 17<sup>th</sup> Century there was a notable rise in the incidence of TB in England, which was thought to be due to the expansion of cities and general population growth [2, 6]. The increase in close-contact, poor living conditions and inferior quality health-care was thought to have exacerbated the spread of TB [2, 6, 7].

It was not until 1882 that physician Robert Koch identified *M. tuberculosis* as the causative agent of tuberculosis, using his postulates to isolate the bacillus [8].

### 1.1.1 Pathogenesis of Tuberculosis

The primary mechanism of TB infection is by aerosol transmission [1, 9] and can only be spread by people that have contracted the active, not latent form of TB [10]. Approximately 90% of *M. tuberculosis* infections will be latent, when the mycobacteria are considered 'dormant' and do not cause disease [10]. When the bacterium has become active, the disease can be fatal if left untreated. When the microorganisms are inhaled into the lungs, the immune system responds by activation of T-lymphocytes [9]. This causes an accumulation of macrophages which leads to the formation of granulomas, or 'tubercles' [4, 9]. This lesion is a

characteristic feature of TB and in most cases will not cause disease, leading to a latent state of TB [10]. However, up to 5% of infected individuals will progress to an active state of TB within a year of infection and up to another 5% will acquire active TB at some point in their life [10]. The infected tissue undergoes necrosis and leads to irreversible lung damage [9]. Typical symptoms indicative of TB include a chronic cough with blood-tinged sputum, fever, night sweats, loss of appetite, fatigue, weight loss and tubercular ulcers [9]. TB primarily infects lung tissue which is classed as pulmonary TB; though the bacilli can affect other tissues such as the lymph nodes, the circulatory system, the central nervous system, skin and the gastrointestinal tract. This is then classed as extrapulmonary TB [9]. Around 10-15% of active cases are extrapulmonary TB; however, this form of disease is increased up to 50% if the patient is HIV positive [11, 12].

### **1.1.2 Epidemiology**

An estimated one third of the world population is infected with TB bacilli [13]. In 2008 the World Health Organization (WHO) reported 9.4 million cases of TB, including 500,000 cases of multi-drug resistant TB (MDR-TB) [14, 15]. In 2008 approximately 1.8 million people died as a result of TB infection [14]. TB has killed more adults than any other single infectious agent, including AIDS and malaria [16]. TB is responsible for more than a quarter of unnecessary deaths in adults [16].

One of the main problems in the persistence and severity of TB is drug resistance. Co-infection of TB with Human Immunodeficiency Virus (HIV)



is also a major issue, especially in less developed countries where TB is most prevalent [17]. An immunocompromised system can affect the severity of TB onset. Those that are HIV-positive have an increased chance of up to 30 times of progressing to an active state of TB [18]. It is approximated that 1.4 million people infected with TB are HIV-positive [17], with an estimated 500,000 deaths due to TB among HIV patients recorded in 2008 [18]. TB is considered to be the main cause of adult deaths with HIV [13, 17].

In 1993, TB was declared a global emergency by the WHO [19]. The tuberculosis incidence has worsened with the rise in drug-resistant strains of *M. tuberculosis*, increased HIV infections, use of different healthcare schemes among countries and the lack of care in regards to TB control programs [17]. In 2006 WHO, in conjunction with the Stop TB Partnership developed a 'Stop Tuberculosis' strategy, with the aim to "dramatically reduce the global burden of tuberculosis by 2015" and thereby eliminating TB as a global health problem by 2050 [1, 17].

#### **1.1.2.1 Epidemiology in New Zealand**

Tuberculosis is not just an overseas issue. In New Zealand (NZ) there were approximately 7 cases per 100,000 of TB [20] and in central Auckland there was an incident rate of 12.3 cases per 100,000 in 2007 [20]. This is higher than the average number of cases in other developed countries such as Australia, United States of America (USA) and Canada, which have around 4-6 cases per 100,000 [17]. However, this is still lower than the incidence rate of 15 cases per 100,000 in the United Kingdom

[17]. A possible factor for the high incidence of TB cases in NZ is due to high migration rates, with 65% of TB cases due to foreign-born individuals [21].

### 1.1.3 Drug development and treatment

With the continued existence of TB disease in humans there have been a variety of treatments investigated. However, it was not until the 20<sup>th</sup> century that significant events occurred in the development of TB treatment.

In 1912 *Bacillus subtilis* and *Bacillus mesentericus* cultures were shown to inhibit *M. tuberculosis* growth [22]. In 1943 streptomycin was isolated from *Streptomyces griseus*. It had such a high efficacy against a broad range of organisms that it was quickly manufactured on a large-scale by 1944 [23]. There was a sharp decrease in the occurrence of TB after the discovery of streptomycin and it was thought that TB could be controlled if not eradicated [24].

In terms of prevention, Albert Calmette and Camille Guérin were the first to successfully develop a vaccine against tuberculosis using an attenuated bovine-strain labelled 'BCG' between 1905 and 1921 [25, 26]. It was then put into production, immunizing thousands of children in the United Kingdom. It was one of the first major vaccine trials in the United Kingdom and a noticeable decrease in the incidence of TB was observed from 1954 to 1965 [27].

The discovery and production of rifampin (RIF), isoniazid (INH) and pyrazinamide (PZA) soon followed, which are current TB drugs used

today. RIF binds to the  $\beta$ -subunit of the DNA-dependent RNA polymerase enzyme, effectively inhibiting transcription [28]. INH is a powerful pro-drug that inhibits fatty acid synthesis once activated by the enzyme catalase-peroxidase (KatG) [29]. PZA still has a poorly understood mode of action, but it is thought that PZA is also a pro-drug that inhibits fatty acid synthesis [30]. The efficacy and structure of RIF, INH and PZA as well as other major first- and second-line drugs used are shown in Table 1.1.3.

Production of these drugs was significant as they enabled the foundation of combined drug therapy in order to alleviate the levels of TB as well as help avoid the formation of resistant strains [31]. There are now two types of drug regimens currently used for the treatment of tuberculosis. There are first-line drugs, such as ethambutol (ETH), INH, PZA, RIF and streptomycin (STR), as shown in Table 1.1.3. These drugs are first used to combat TB. There are also second-line drugs available such as capreomycin (CAP), cycloserine (CYS) and kanamycin (KAN) that are used when first-line drugs are no longer effective. However, second-line drugs tend to be less potent or more toxic than standard first-line drugs [32].

| <b>TB drug</b>     | <b>Line</b> | <b>MIC against MTB (H37Rv)</b> | <b>Mode of action</b>  |
|--------------------|-------------|--------------------------------|--|
| Capreomycin (CAP)  | Second      | 2 µg/ml.                       | Inhibits protein synthesis <sup>[33]</sup>                                   |
| Cycloserine (CYS)  | Second      | 25 µg/ml.                      | Inhibits cell-wall biosynthesis and maintenance <sup>[34]</sup>              |
| Ethambutol (ETH)   | First       | 0.5 µg/ml.                     | Inhibits cell-wall biosynthesis <sup>[35]</sup>                              |
| Isoniazid (INH)    | First       | 0.025 µg/ml.                   | Pro-drug that inhibits fatty acid synthesis <sup>[29]</sup>                  |
| Kanamycin (KAN)    | Second      | 2 µg/ml.                       | Inhibits protein synthesis <sup>[36]</sup>                                   |
| Pyrazinamide (PZA) | First       | 6 - 50 µg/ml at pH 5.5         | Prodrug that leads to the inhibition of fatty acid synthesis <sup>[30]</sup> |
| Rifampin (RIF)     | First       | 0.4 µg/ml                      | Inhibits protein synthesis <sup>[28]</sup>                                   |
| Streptomycin (STR) | First       | 1 µg/ml                        | Inhibits protein synthesis <sup>[37]</sup>                                   |

**Table 1.1.3. List of current TB drugs used.** First- and second-line TB drugs currently employed with activity against *M. tuberculosis* H37Rv and general mode of action.

Tuberculosis is difficult to treat and requires multiple drugs combined with long courses [1]. The course of treatment currently used is a Directly Observed Treatment Short-course (DOTS) program, involving different combinations of first- and second-line drugs. This course has a period of treatment time of usually 6 to 12 months, longer than most antibiotic treatments [1]. The epidemiological approach for DOTS therapy involves 5 major steps, which the WHO states are [38]:

1. Sustained political and financial commitment.
2. Diagnosis by quality ensured sputum-smear microscopy.
3. Standardized short-course anti-TB treatment (SCC) given under direct and supportive observation (DOT).
4. A regular, uninterrupted supply of high quality anti-TB drugs.
5. Standardized recording and reporting.

This program is applied with the intention of eradicating tuberculosis and to avoid the creation of drug-resistant strains.

#### **1.1.4 Drug resistance**

Although the discovery of streptomycin was hailed as a miracle antibiotic it was not long before resistant-strains appeared [22]. Isolates resistant to RIF, INH and PZA also began to surface not long after their production [39]. Nevertheless the production of these drugs combined with improved health-care standards was thought to be sufficient for controlling the spread of TB [40]. Consequently, there was little appeal in continuing

research in discovering new TB drugs. As a result, for 40 years no new TB drugs were approved by the Food and Drug Administration (FDA) for clinical use [40]. A resurgence of TB in the 1980's, including drug-resistant cases and immunocompromised patients lead to renewed interest in developing novel drugs [40].

There are now two types of drug-resistant TB that have emerged, multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). MDR-TB is resistant to first line drugs RIF and INH, whereas XDR-TB is resistant to second-line drugs such as KAN or CAP as well as first-line drugs [15]. These strains of TB can be up to 250 times more expensive to treat than standard TB as they do not respond to the regular treatment courses provided [41]. MDR-TB and XDR-TB can take years to be treated with more toxic and less effective drugs [41].

There are multiple reasons for the development of drug-resistant strains. In terms of the nature of the bacteria, mycobacteria have highly sensitive drug-efflux systems and can produce enzymes that modify anti-tubercular drugs, for example aminoglycoside acetyl transferases [42]. A distinctive property of mycobacteria which is likely to contribute to its drug resistance is its cell wall, rich in lipids (e.g. mycolic acid), glycolipids, and polysaccharides [43]. This highly hydrophobic cell wall acts as a protective barrier [42]. A particular feature of TB however, is its ability to survive in an active or dormant state, which can provoke resistance [44]. It is these well-developed evasion systems that can provide mycobacteria with the ability to develop resistance. Random mutations within the gene sequence can also occur such as single nucleotide polymorphisms, leading to

development of resistance [45]. HIV can also play a factor with the perseverance of resistant TB, as it has been shown that a patient has a much higher chance of developing MDR- or XDR-TB if they are HIV positive [17]. Other factors for the emergence of resistant strains can be due to circumstances such as poor diagnosis, use of broad-range or poor quality drugs and patients not completing their course of drug treatment [46]. TB therapy is particularly problematic with patients not finishing their course of treatment. This is due to the long treatment time as well as toxic TB drugs. As a result, the patient is more likely to have an aversion to completing the course of medication [17].

With the presence of drug-resistant strains of bacteria, WHO has advised that new drugs are required to treat TB [47, 48]. Natural resources are now being considered as the focus for discovering new compounds with novel modes of activity [49, 50].

## **1.2 Phytotherapy**

Phytotherapy is the study of natural product extracts such as plants that have medicinal uses. Plants have been used for the treatment of human diseases well before antibiotics were discovered.

### **1.2.1 History of Phytotherapy**

Indigenous peoples have known for generations the medicinal value of plants. The earliest discovery of plants thought to be used medicinally were found on the remains of a Neanderthal man (designated as Shanidar IV) from 60,000 B.C.E., from the archaeological site Shanidar Cave in Iraq

[51, 52]. The Sumerians, one of the earliest known civilizations had written records of the study of herbal medicines from over 5000 years ago [51]. Around 2700 B.C.E., during the Han Dynasty the Chinese created a herbal book labelled the *Shennong Bencao Jing*, listing over 300 plants and their medicinal application [51]. One of the plants listed was *Ephedra sinica* that was used for the treatment of asthma and colds. *E. sinica* is still used today for traditional treatment and is the source of the alkaloid ephedrine, one of the active components used in common decongestants today [53]. Around 1500 B.C.E., Egyptians were known to use plants like garlic, wormwood, aloe, coriander, saffron, linseed and opium for medicinal purposes [51, 54]. This information was recorded on the Ebers Papyrus, a document containing approximately 700 descriptions of plants and their medicinal purpose [51, 54]. In ancient Greece, Hippocrates recorded the medicinal practices of the Greeks (around 460 B.C.E.) in the *Corpus Hippocraticum*, detailing the use of herbs, as well as other beneficial medicinal practices such as fresh air and rest [55, 56]. Then around 1025 C.E., a pharmacopoeia titled 'The Canon of Medicine' was written by Avicenna, cataloguing the uses of 800 different plants, drugs and minerals and their preparations [57, 58]. Avicenna was also the first physician to develop a quarantine system after identifying TB as a contagious disease and its possible mode of spread [57, 59].

### **1.2.2 Medicinal value of Phytotherapy**

South Africa is home to about 10% of tracheophyte plant species (plants containing a vascular system) on the planet, yet less than 5% of these



species have been scientifically assessed for pharmacological activity [60]. However, around 70% of all South Africans utilize their endemic plants for medicinal purposes [60]. Bamuamba *et al.* (2008) found that extracts from the South African plant *Buddleja sligna* and *Leyssera gnaphaloides* had significant antibacterial activity, similar to that of the first-line drug RIF [61]. Tabuti *et al* (2010) recently determined that more than 80 plant species have been used by traditional medical practitioners in Uganda to treat tuberculosis [62]. Another study characterised leaf, bark and root material from trees used as traditional anti-tubercular medicines in South Africa [63].

The people of Papua New Guinea used bark from the plant *Evodia elleryana* for the treatment of coughs and fevers [64]. An ethyl acetate extract of this plant revealed that it had significant inhibitory activity against *M. tuberculosis* [64]. It was hypothesized that the inhibitory activity was due to the presence of alkaloid quinoline metabolites in the plant species [65], therefore presenting a potential class of compounds for drug development against *M. tuberculosis* [64].

There is much biodiversity in the Amazonian forest, but few plants have been tested for antibacterial activity [66]. Carneiro *et al* (2008) discovered 12 Amazonian plant extracts to have antibacterial activity against different strains of *Mycobacterium smegmatis*, *Escherichia coli*, *Streptococcus sanguis*, *Streptococcus oralis*, *Staphylococcus aureus* and *Candida albicans* [66, 67]. Methanol and chloroform extractions displayed high antimicrobial activity [66, 67].

A number of plants native to Mexico were used in traditional medicine for the treatment of various respiratory diseases and were tested for antimycobacterial activity [68-71]. Jimenez-Arellanes *et al* (2003) screened Mexican plants that were used to treat respiratory diseases to investigate if they had antitubercular activity against drug resistant strains [68]. It was found that five plants (*Artemisia ludoviciana*, *Chamaedora tepejilote*, *Juniperus communis*, *Lantana hispida* and *Malva parviflora*) had antimycobacterial activity against *M. tuberculosis* H37Rv [68]. *A. ludoviciana* was a particularly important resource in traditional Mexican medicine; used to treat coughs, asthma, bronchitis and other ailments [68, 69]. It was found that the plant *A. ludoviciana* produced sesquiterpene lactones, which were thought to give the plant its antibacterial activity [68, 69]. Extracts of *Citrus aurantifolia*, *C. sinensis* and *Olea europaea*, also from Mexico were found to be active against both drug-susceptible and drug-resistant strains of virulent *M. tuberculosis* [71].

It is estimated that less than 10% of the Earth's higher plant species have been analysed for any kind of bioactivity [18]. Thus, there is a need for more research to be conducted on plants that have been traditionally used by indigenous peoples for the identification of new anti-tubercular drugs.

### 1.2.3 Plant-derived drugs

While today much of the focus has been on screening libraries of synthetic compounds for new lead drugs, plants have often provided a source of anti-bacterial compounds. One of the first drugs created was derived from the compound salicin, found in the bark of *Salix alba* [72, 73].

Salicin was converted into salicylic acid which created aspirin, an effective painkiller and antipyretic [49, 74].

The compound aegicerin was isolated from *Clavija procera* and has been shown to have activity against *Mycobacterium tuberculosis* H37Rv and MDR-TB strains [75]. Rhodomyrtone, a compound from the Southeast Asian plant *Rhodomyrtus tomentosa* was found to have potent antibacterial activity against a number of clinically-important gram-positive species including *Enterococcus faecalis*, *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA) [76]. The drugs tetramethylpyrazine (TMP) and tetrandrine (TET) were derived from Chinese herbal plants and are used for the treatment of cardiovascular disease [77].

Nowadays it is estimated that more than 50% of the principal prescription drugs in the United States of America (USA) are derived from natural plant extracts [78]. Some of the more well-known drugs include the anticancer drugs Paclitaxel (Taxol®), derived from *Taxus brevifolia* [79], Vincristine (Oncovin) derived from *Catharanthus roseus* [80] and Camptothecin (Hycamtin®) which is derived from *Camptotheca acuminata* [81]. In terms of new plant-derived TB drugs there are some promising lead compounds, especially from the class of alkaloids. The alkaloid tryptanthrin, derived from *Strobilanthes cusia*, could lead to a potential TB drug with its considerable activity against the drug sensitive strain *M. tuberculosis* H37Rv [82]. The indole alkaloids ibogaine and voacangine, which were isolated from *Tabernaemontana citrifolia*, displayed promising activity against *M. tuberculosis*, both with a minimum inhibitory concentration

(MIC) of 50 µg/ml [83]. Finally the alkaloid cepharanone B, was isolated from *Piper sanctum* and has potential activity against *M. tuberculosis* H37Rv with an MIC of 12 µg/ml [84]. It is possible that native NZ plants will provide anti-tubercular compounds that could lead to new TB drugs.

#### **1.2.4 Secondary Metabolites in plants**

Secondary metabolites are compounds usually present in plants that are not involved in any primary functions of the plant's requirements, for example, growth, development and reproduction [85]. Secondary metabolites tend to be diverse in size, structure and distribution both within plants and between species, yet appear to have no evident metabolic purpose even though they persist in all flora [85]. Secondary metabolites are likely to have a significant function if the plant expends metabolic energy to produce them [85]. It is proposed that plants have developed complex systems involving secondary metabolites in order to survive the ecological complexity surrounding them. This can include the production of chemicals that facilitate protection against predators, whether it be a deterrent or poison [49]. Hence, although secondary metabolites do not support the essential roles of cell development, the energy spent in the production of them is favourable for the plants overall growth and spread [49, 86].

It is proposed that secondary metabolites could be the source of a plant's antibacterial properties [87]. If these compounds are utilized in the plants vascular system to ward off predators (e.g. as an irritant or poison) or used

to ward off infection from fungi and bacteria then it is likely that these secondary metabolites are a source of antibacterial activity [49].

There are a number of different types of secondary metabolites. The classes found to contain the majority of antibacterial compounds are alkaloids, flavonoids, terpenoids and phenols [88]. Extracts from the South African plant *Buddleja sligna* and *Leyssera gnaphaloides* were thought to have antibacterial activity due to the presence of triterpenoids [61]. The flowers of *Chrysanthemum morifolium* were used in Chinese natural medicine in the treatment of inflammation and fever and it was found that different types of triterpenoids from these flowers demonstrated antitubercular activity [89]. The isoquinoline alkaloid berberine was isolated from *Hydrastis canadensis* and was found to demonstrate antitubercular activity [90]. The leaves of *Galenia africana* also exhibited antimycobacterial activity against *M. tuberculosis* of which the activity was isolated to three flavonoids; (2S)-5,7,2'-trihydroxyflavanone, (E)-3,2',4'-trihydroxychalcone and (E)-2',4'-dihydroxychalcone [91].

Therefore, as plants produce thousands of secondary metabolites there is much potential for investigating flora as a source for antitubercular activity.

### **1.3 New Zealand flora**

NZ has a somewhat diverse range of flora and therefore it is possible that a wide range of novel compounds are present and unique to NZ flora.

#### **1.3.1 Development of our flora**

The biodiversity of NZ's flora can be attributed to the remoteness of its land. The land that was to become NZ was separated from the ancient supercontinent Gondwana around 65 million years ago, allowing the native fauna and flora to evolve in isolation [92]. Consequently, NZ has a distinctive range of plants, with approximately 80% of trees and shrubs and 40% of ferns that are endemic to the country [92]. The genus *Podocarpus* (e.g. Tōtara) and *Nothofagus* (e.g. Beeches) show similarities to biota linked back to the landmass Gondwana [92], suggesting NZ plants are similar to that of ancient flora. Thus, NZ has some of the highest rates of endemic species, which implies that diverse and conserved selection of plants are more likely to contain novel compounds due to greater chemodiversity of secondary metabolites [93]. The diversity is also due to the mild temperate climate and general lack of animal predators that has allowed the fauna and flora to evolve and adapt unimpeded [92]. Despite the years of forestry clearance through the expansion of human settlements, there is still plenty of natural vegetation. Around 18% of NZ is protected conservation land [94] like that of Otari-Wilton bush or Karori Wildlife Sanctuary (Zealandia), therefore providing a wide variety of native plants for selection.

### **1.3.2 Rongoa Māori**

Arrival of the Māori population from Polynesia occurred sometime around 1300 A.D. [95]. With the appearance and settlement of a human population in NZ, the Māori soon became familiar with the native flora, finding uses for it such as tools, building, clothing and medicine [96]. They

employed 'Rongoa Māori', the traditional practice of using native flora for medicinal purposes [96]. This would include the use of leaves, roots, bark and flowers. Faith was also considered an important role in Rongoa medicine. A 'Tohunga' was a form of doctor that had the power of 'tapu' (meaning sacredness) who knew the medicinal uses of plants and was the dispenser of treatments [96]. When European explorers made contact, they found the Māori to be vigorous and strong inhabitants with little sickness present in the community [96]. In fact, it was recorded that the European medicine practitioners were known to rely on the Māori remedies when their own stock of drugs was limited [97].

Many plants were considered important in Rongoa. The NZ flax *Phormium tenax* was utilized in Rongoa Māori for many remedies. The leaves were boiled for the treatment of cuts by applying it as a disinfectant [98]. Flax roots were used for treating abscesses and the gum used for burns and ringworm [96]. *Macropiper excelsum* (Kawakawa) was also considered a principal plant in Māori medicine, as the leaves were used for several remedies. They were applied to wounds as well as used in the treatment of rheumatism, arthritis, bruises, bladder problems and chest difficulties [96, 99, 100]. The leaf of *P. colorata* (Horopito) was used to treat toothache, skin irritations as well as a painkiller [96, 101]. Overall though most of the remedies treated common problems such as pain, toothache, wounds, skin irritations and stomach aches [98].

Rongoa Māori is relevant to this research as it the main basis of selection of native plants for analysis. If activity is identified then it reinforces the

implication that plants used in traditional medicine are likely to contain antibacterial compound(s) [49].

### 1.3.3 Antibiotic activity of native New Zealand plants

Some of the native flora of NZ has already been investigated for various bioactivities. The shrub *Lophomyrtus bullata* (Ramarama) has been identified as having activity towards *Bacillus subtilis* [102]. *P. colorata* is known to have significant activity against *Candida albicans* [103]. Its activity has been isolated to the sesquiterpene dialdehyde polygodial [103]. Liverworts have been investigated for bioactivity. The tea trees Manuka (*Leptospermum scoparium*) and Kanuka (*Kunzea ericoides*) have been shown to have antibacterial properties in their honey [104]. The liverwort *Plagiochila stephensoniana* was found to have antifungal activity, of which its active component was identified as bibenzyl 4-hydroxy-3'-methoxybibenzyl [105]. However, to current knowledge NZ plants have not been assayed for activity against mycobacteria. Thus, there is a potential resource for novel antimycobacterial compounds in NZ flora.

## 1.4 Aims of Research

The aim of this research was to investigate the potential antimycobacterial activity of native plant extracts. This required the following objectives:

- *Collect native plant samples with potential bioactivity*



This will be achieved by compiling a list of native plants with medicinal activity and then collecting the samples from local bush reserves in order to build the plant extract library.

- *Identify native plant extracts with antimycobacterial activity*

The plant samples will be extracted with various solvents and initially screened against surrogate species *M. smegmatis* for the detection of antimycobacterial activity.

- *Validate plant extracts with antimycobacterial and antibacterial activity*

Positive hits will be tested for bacteriostatic activity against species *M. bovis* BCG, *M. tuberculosis* H37Ra, *S. aureus* and *E. coli*. Bactericidal activity will also be determined against *M. smegmatis*.

- *Improve efficiency of extraction and deliver the bioactive extracts in a practical application*

Positive hits will be purified and investigated for improved activity by means of size exclusion fractionation and solvent phase separation. Active plant extracts will also be applied to an aqueous cream for the purpose of delivery of the bioactive extract and assessed for inhibitory activity in a disc diffusion assay.

# CHAPTER TWO: Materials and Methods

## 2.1 Bacterial strains and plasmids

The mycobacterial strains used were *M. smegmatis* mc<sup>2</sup>155, *M. bovis* BCG (Pasteur) and *M. tuberculosis* H37Ra. *M. smegmatis* and *M. bovis* BCG were labelled with a Green Fluorescent Protein (GFP) marker through the introduction of the plasmid pSHIGH + hsp60 by electroporation and selection on media containing kanamycin (50 µg/ml) as described by Miller *et al* (2009) [106]. *Staphylococcus aureus* Newman strain was GFP-labelled as previously described [107, 108] and selected on media containing erythromycin (30 µg/ml) (Sigma). See Appendix 1 Section 'Bacterial strains and plasmids' for more detail [109]. *Escherichia coli* DH5α was labelled with GFP using pOT11 and selected on media containing chloramphenicol (25 µg/ml) (Sigma) as previously described [110].

## 2.2 Culture conditions

*M. smegmatis* mc<sup>2</sup>155 was cultured on a Luria agar (LA) plate with kanamycin (50 µg/ml) (Sigma) at 37°C. Colonies selected were inoculated in Luria Broth (LB) media containing kanamycin (50 µg/ml), Tween 80 (0.1% v/v) (Sigma) and D-arabinose (250 µg/ml) (Sigma).

Both *M. bovis* and *M. tuberculosis* cultures were grown in Middlebrook 7H9 broth supplemented with 10% (v/v) OADC (0.06% Oleic acid, 5% BSA, 2% Dextrose, 0.85% NaCl) (Becton, Dickinson and Company), glycerol (0.5% v/v) (British Drug Houses) and Tween 80 (0.05% v/v).

GFP-labelled *S. aureus* Newman was cultured on LA containing erythromycin (30 µg/ml). When inoculated into media, *S. aureus* was

grown in LB with the addition of erythromycin (30 µg/ml) and xylose (0.5% w/v) (British Drug Houses).

*E. coli* DH5α/pOT11 was cultured on LA containing chloramphenicol (25 µg/ml). Inoculation into LB media involved the addition of chloramphenicol (25 µg/ml) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (100 µg/ml). IPTG was added to induce GFP expression.

### **2.3 Visualisation of bacteria**

Slides of *Mycobacteria smegmatis* were prepared by removing 5 µl from overnight cultures. The bacteria were viewed under a 1000 x (oil immersion) Olympus Provis AX 70TRF Fluorescent Microscope (Olympus America Inc) with a Fluorescein Isothiocyanate (FITC) filter used to visualise the fluorescent cells.

### **2.4 Sample collection**

Plant samples were primarily collected from the Karori Wildlife Sanctuary (Zealandia) and Otari-Wilton Bush Reserve. Other samples were also obtained from Wellington; Kaitoke Regional Park, Upper Hutt and the central Wellington area with permission. Samples were also collected from the Canterbury and Nelson areas with permission. Leaf, flower, seed or bark tissues were collected fresh and stored in plastic bags. Leaves were chosen from the same apical node from each branch with similar size and colouring. Location, date of collection and details of the species were noted.

## **2.5 Solvent extraction**

Approximately 5-10 g of each plant sample was macerated using a blender (Breville) and then dried in roughly 20 g of the granular desiccant calcium sulphate (British Drug Houses). The dried sample was placed in a 50 ml conical tube and sterile distilled water (SDW), ethanol (EtOH) or methanol (MeOH) were added to give a final concentration of 100 mg/ml. Samples were then incubated in a water bath at 55°C for 1 hour before being stored at -80°C for 24 hours. The incubation was performed in order to imitate the preparations used in traditional Māori medicine. The resulting extract was then centrifuged (Alphatech) at 5000 x g for 15 minutes before being sterilized through a 0.22 µm filter (Millipore). The final extract was stored at -80°C.

### **2.5.1 Evaporation of extracts**

Plant samples to be evaporated had 500 µl of extract aliquoted into 1.5 ml eppendorf tubes and were centrifuged (Labconco centrivap concentrator) under vacuum at -100 kPa with 30 bar Hg, at 35°C for 2 hours. The resulting plant material was then resuspended in the desired solvent and sterilized through a 0.22 µm filter.

## **2.6 Purification of extracts**

### **2.6.1 Size exclusion filtration of extracts**

Approximately 2 ml of plant extract in 50% EtOH or MeOH (or 100% SDW if aqueous (AQ) extract) was aliquoted into a 30 kDa filter (Millipore) and

centrifuged at 5000 x g for 20 minutes. The filtrate was then aliquoted into a 3 kDa filter and centrifuged again at 5000 x g for 20 minutes. About 1 ml of SDW was added to each of the filters and sonicated for 10 minutes to remove any extract present (retentate). The resulting filtrate and retentate were then pipetted into fresh eppendorf tubes and evaporated under speed-vacuum, weighed and resuspended in SDW to the desired concentration. The final extract was then sterilized through a 0.22  $\mu\text{m}$  filter and stored at  $-80^{\circ}\text{C}$  until required.

### **2.6.2 Solvent Phase Separation of extracts**

#### *Ethyl Acetate*

Extracts were evaporated and resuspended in SDW to a concentration of 100 mg/ml. Approximately 2 ml of extract was added to a glass test tube and 2 ml of 99% ethyl acetate (EtOAc) was added. The test tubes were then sealed with cotton and parafilm and refrigerated at  $-80^{\circ}\text{C}$  for 24 hours. The AQ (extract) layer was removed and aliquoted into fresh eppendorf tubes, which were then evaporated and resuspended in SDW again to a final concentration of 10 mg/ml. The extracts were then sterilized through a 0.22  $\mu\text{m}$  filter prior to analysis.

#### *Chloroform*

The extracts were evaporated and resuspended in SDW to a concentration of 100 mg/ml. Approximately 2 ml of extract was added to a glass test tube and 2 ml of 99% chloroform ( $\text{CHCl}_3$ ) was added. The test tubes were then sealed with cotton and parafilm and refrigerated at  $-80^{\circ}\text{C}$  for 24 hours. The AQ (extract) layer was removed and aliquoted into fresh

ependorf tubes, which were then evaporated and resuspended in SDW again to a final concentration of 25 mg/ml. The  $\text{CHCl}_3$  layer was also removed and placed into a fresh test tube and evaporated at room temperature. Approximately 2 ml of 99% DMSO (Sigma) was added and sonicated to dissolve the extract. The extracts were then transferred to fresh ependorf tubes, evaporated and resuspended again in DMSO to a final concentration of 10 mg/ml. Extracts were then sterilized through a 0.22  $\mu\text{m}$  filter prior to analysis.

### **2.6.3 Size exclusion filtration and SPS**

Approximately 2 ml of plant extract in 50% EtOH or MeOH (or 100% SDW if AQ extract) was aliquoted into a 30 kDa filter (Millipore) and centrifuged at 5000 x g for 20 minutes. The filtrate was then aliquoted into fresh ependorf tubes (500  $\mu\text{l}$ ), evaporated under speed-vacuum and resuspended in SDW to a concentration of 100 mg/ml. Approximately 1 ml of the filtrate was then aliquoted into a fresh glass test tube. Approximately 1 ml of 99% EtOAc was then added to the filtrate. The test tubes were then sealed with cotton and parafilm and frozen at  $-80^\circ\text{C}$  for 24 hours. The AQ (extract) layer was then removed and aliquoted into fresh ependorf tubes, which were then evaporated and resuspended in SDW to a final concentration of 10 mg/ml. The final extracts were sterilized through a 0.22  $\mu\text{m}$  filter prior to analysis.

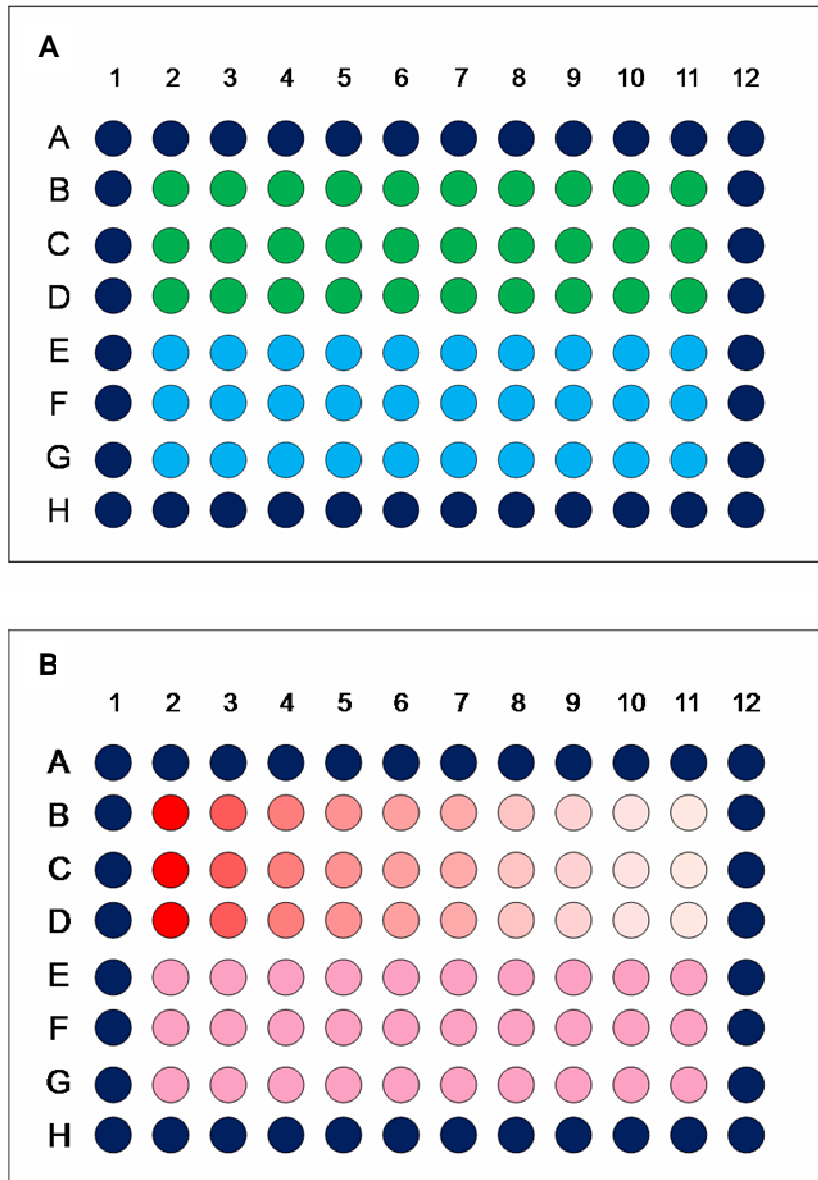
## 2.7 Assay Conditions

### 2.7.1 Bacteriostatic assay

Solvent-extracted and fractionated plant samples were examined in a 96-well microtitre plate (96wp) bacteriostatic assay against *M. smegmatis*, *M. bovis*, *M. tuberculosis*, *E. coli* and *S. aureus*. Figure 2.7.1 illustrates the layout of the bacteriostatic assay. SDW (150  $\mu$ l) was added to the outer lanes of the 96wps to reduce evaporation. The remaining wells were filled with 50  $\mu$ l of liquid media. Plant extracts (10  $\mu$ l) were pipetted into columns 2 and 3 of the microtitre plates with a starting concentration of 2 mg/ml. A two-fold serial dilution was then performed on each extract starting at column 3, aliquoting 30  $\mu$ l from column 3 into column 4 and so on until column 11. Bacterial cells (40  $\mu$ l) were added to rows B-D at a final OD at 600 nm ( $OD_{600}$ ) of 0.2. Rows E-G contained media and extract alone, to measure any background optical density or fluorescence associated with the extract and media. A number of controls were added to ensure assay reliability. Bacterial cells were grown in media alone to allow for comparison of bacteriostatic activity against normal growth for each extract tested. Standard anti-tubercular drugs RIF and STR were used as positive controls, with starting concentrations of 0.1 mg/ml. Solvent controls were also used, as some inhibition of bacterial growth could be exhibited by some solvents. Inhibition of growth due to the ethanol solvent was observed at concentrations of greater than 5% (v/v) for all species tested. *M. smegmatis*, *E. coli* and *S. aureus* also had inhibition due to the methanol solvent at concentrations greater than 5% (v/v), whereas *M.*



*bovis* and *M. tuberculosis* only exhibited inhibition of growth at concentrations greater than 10% (v/v). Thus, extracts tested in ethanol or methanol had a starting solvent concentration of 2% (v/v) corresponding to the final concentration of plant extract. All extracts and controls were tested in triplicate. Plates were sealed with nitrocellulose film (Excel Scientific) and incubated at 37°C with 200 rpm shaking. After 24 hours plates containing *E. coli* or *S. aureus* cultures were read for OD and GFP fluorescence. For the Mycobacterium species, *M. smegmatis* cultures were read after 96 hours, *M. bovis* and *M. tuberculosis* cultures were read after 14 days. Only OD was measured for *M. tuberculosis* as this organism did not contain a plasmid with a GFP insert.



**Figure 2.7.1. Outline of 96wp set-up for bacteriostatic assay.** The outer wells are filled with SDW indicated by the dark blue wells (●). **A)** The green wells (●) indicate cells + media in triplicate and the blue wells (●) indicate media alone in triplicate. **B)** The red wells (●) indicate the 2:1 serial dilution of extract + cells + media in triplicate. The remaining pink wells (●) indicate extract alone in media in triplicate to account for any background absorbance of the extract tested. Drug controls were tested in the same format.

### 2.7.2 Resazurin reduction assay

An aliquot of 30  $\mu$ l of resazurin dye (0.02% w/v) (Sigma) was added to each well at the end-point of the bacteriostatic assay and incubated at 37°C for 2 hours. The plates were then read using the Wallac EnVision® Manager 1.12 software program with a General top mirror and wavelengths of 530 nm excitation and 590 nm emission.

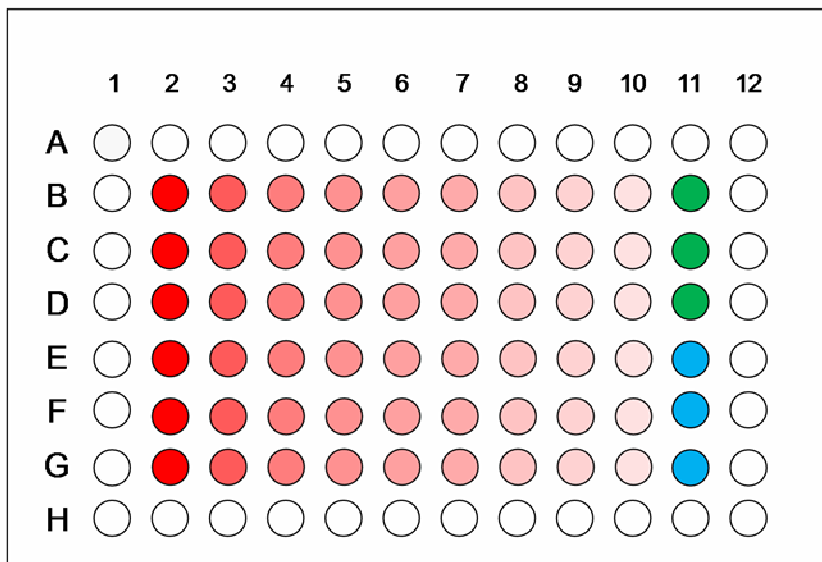
### 2.7.3 Bactericidal assay

*M. smegmatis* was cultured for 8 days in LB containing kanamycin (50  $\mu$ g/ml) until stationary growth phase was reached. The culture was then diluted to an OD<sub>600</sub> of 1.0 with PBS (1x v/v). A 96-wp assay was then set-up as previously described in the bacteriostatic assay protocol, with the exception of LB media being replaced with PBS 1 x (v/v). After 96 hours drug exposure, 30  $\mu$ l of resazurin dye (0.02% w/v) was added and the plates read for any bactericidal activity with wavelengths of 530 nm excitation and 590 nm emission.

### 2.7.4 Cytotoxicity assay

Murine macrophage cell-line J774 cells (ATCC) and human blood cell-line HL-60 (ATCC) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) (Invitrogen) and PenStrep (Pencillin 50 units/ml and Streptomycin 50  $\mu$ g/ml) (Invitrogen). Cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 48 hours. Extracts to be tested were serially diluted twofold in a 50  $\mu$ l volume in a 96 well tissue culture plates (BD biosciences). Cells were then seeded (50  $\mu$ l)

in the 96 wps to produce a final cell concentration of  $10^4$  cells/well. This was counted with a hemocytometer for both cell-lines. For J774 cells an additional step of trypan blue stain (0.4% v/v) (Sigma) was added before counting. A cytotoxic compound, Epothilone A (EpoA) (Merck), was included as a positive control with a starting concentration of 25  $\mu\text{g/ml}$ . A TB drug control RIF was also included with a starting concentration of 0.4 mg/ml. All extracts and controls were tested in triplicate. The plates were incubated for 48 hours followed by the addition of 10  $\mu\text{l}$  of Alamar Blue dye (10% w/v) (Invitrogen). Absorbance readings were then taken after 4 hours at 570 nm using a VersaMax™ microplate reader (Molecular Devices, Inc) and SOFTmax Pro version 3.0 software (Informer Technologies, Inc.). 50% inhibitory concentration ( $\text{IC}_{50}$ ) values were calculated using SigmaPlot® (version 10.0) (Systat Software, Inc).



**Figure 2.7.4. Outline of 96wp set-up for cytotoxicity assay.** The outer wells are blank as indicated by the white wells (○). The green wells (●) indicate cells + media in triplicate and the blue wells (●) indicate media alone in triplicate. The red wells (●) indicate the 2:1 serial dilution of extract + cells + media in triplicate. In this layout rows B-D indicate one extract tested in triplicate and rows E-G indicate a second extract tested in triplicate. Drug controls were tested in the same format.

## 2.8 Data collection and analysis

An EnVision<sup>®</sup> 2102 multilabel plate reader (Perkin Elmer, Inc) and the Wallace EnVision<sup>®</sup> Manager 1.12 software program (Perkin Elmer, Inc) were used to measure the fluorescent and absorbent GFP and OD signals of the microtitre plate cultures. GFP fluorescence was detected using a FITC bottom mirror, with excitation and emission wavelengths of 485 nm and 510 nm, respectively. OD was measured with an excitation light of 590 nm. 12-point scans were performed on each well to minimise intra-well variation, such as ‘clumping’ that can occur with mycobacteria. The intrinsic absorbance and fluorescence readings of extracts in media alone

were also measured to account for any background signals that were later subtracted from the readings for the samples tested. Data was normalised by expressing the absorbance and fluorescence values as a percentage of a no-drug negative control. Sigmaplot<sup>®</sup> 10.0 software (version 10.0) was used to calculate non-linear dose-response inhibitory curves. A simple scatter plot was created with the means of the triplicate data and error bars representing standard deviation. A log (common) scale was applied. A four parameter logistic curve was calculated to fit the plotted data and as a result MIC values and IC<sub>50</sub> values with standard errors were generated.

## 2.9 Disc Diffusion

For *M. smegmatis*, 100 µl of culture at an OD<sub>600</sub> of 0.2 was spread on LA agar containing kanamycin (50 µg/ml) and allowed to dry. For *S. aureus* 100 µl of culture at an OD<sub>600</sub> of 0.2 was spread on LA agar containing erythromycin (30 µg/ml) and allowed to dry. The extract to be tested was mixed with an aqueous cream solution (MedCo), to give a final extract concentration of 0.5% (w/v). Sterile paper discs (Becton, Dickinson and Company) 6 mm in diameter were then dipped in the solution and gently placed onto the agar surface. A negative control of just aqueous cream alone and positive controls extract alone (i.e. no aqueous cream added) and RIF (1 mg/ml) were also tested. The plates were then sealed with parafilm and incubated at 37°C for 24 hours for *S. aureus* and 96 hours for *M. smegmatis*. Any zones of inhibition surrounding the discs were then circled and measured.

# CHAPTER THREE: Development of the Plant Extract Library

### 3.1 Introduction

This research involved collecting native NZ plants and screening them for antimycobacterial activity. Hence, a suitable protocol was developed for the selection and processing of native plants in preparation for screening against *M. smegmatis*.

The selection of plant samples was based on native species and ethnomedicine [49]. Ethnobotany is the study of how a particular culture use and understand plants in terms of food, medicine and religion [111]. Ethnomedicine is the study of an indigenous culture and its use of native plants for medicinal practices [111]. This is a method of targeted collection, selecting plants based on their use in Rongoa Māori (traditional medicine). It would be difficult with the timeframe and financial circumstances to randomly test native NZ plants for antimycobacterial activity. Therefore ethnomedicine was chosen as the focus for selection of plants in this study.

Many bioactive compounds derived from plants are generally flavonoids, alkaloids, terpenoids or phenols [88]. Because of this, the polar solvents ethanol and methanol would be used for solvent extraction. An aqueous solvent was also chosen as many plants were heated in water for various treatments [97].

Plant extracts were initially screened against the surrogate species, *M. smegmatis*. *M. smegmatis* is an acid-fast, aerobic, saprophytic bacterium [112]. It has a genome 1.7 times larger than *M. tuberculosis* and is non-pathogenic, but *M. smegmatis* does share homology with some virulence genes of *M. tuberculosis* [113]. *M. smegmatis* also has a fast doubling



time of 3-4 hours, therefore it can be a rapid and safe model for testing anti-TB compounds in physical containment level-2 laboratories [113].

*M. smegmatis* mc<sup>2</sup>155 strain was used with a pSHIGH plasmid containing a GFP (green fluorescent protein) gene. This protein was isolated from *Aequorea victoria* [114], causing the bacteria to emit a green fluorescence which allows for easy detection of bacterial growth under inspection [114].

## 3.2 Results

### 3.2.1 Medicinal information on native New Zealand plants collected for antimycobacterial screening

A library was compiled of native plants selected for sampling. Table 3.2.1 is a list of the NZ plants collected and any medicinal uses noted.

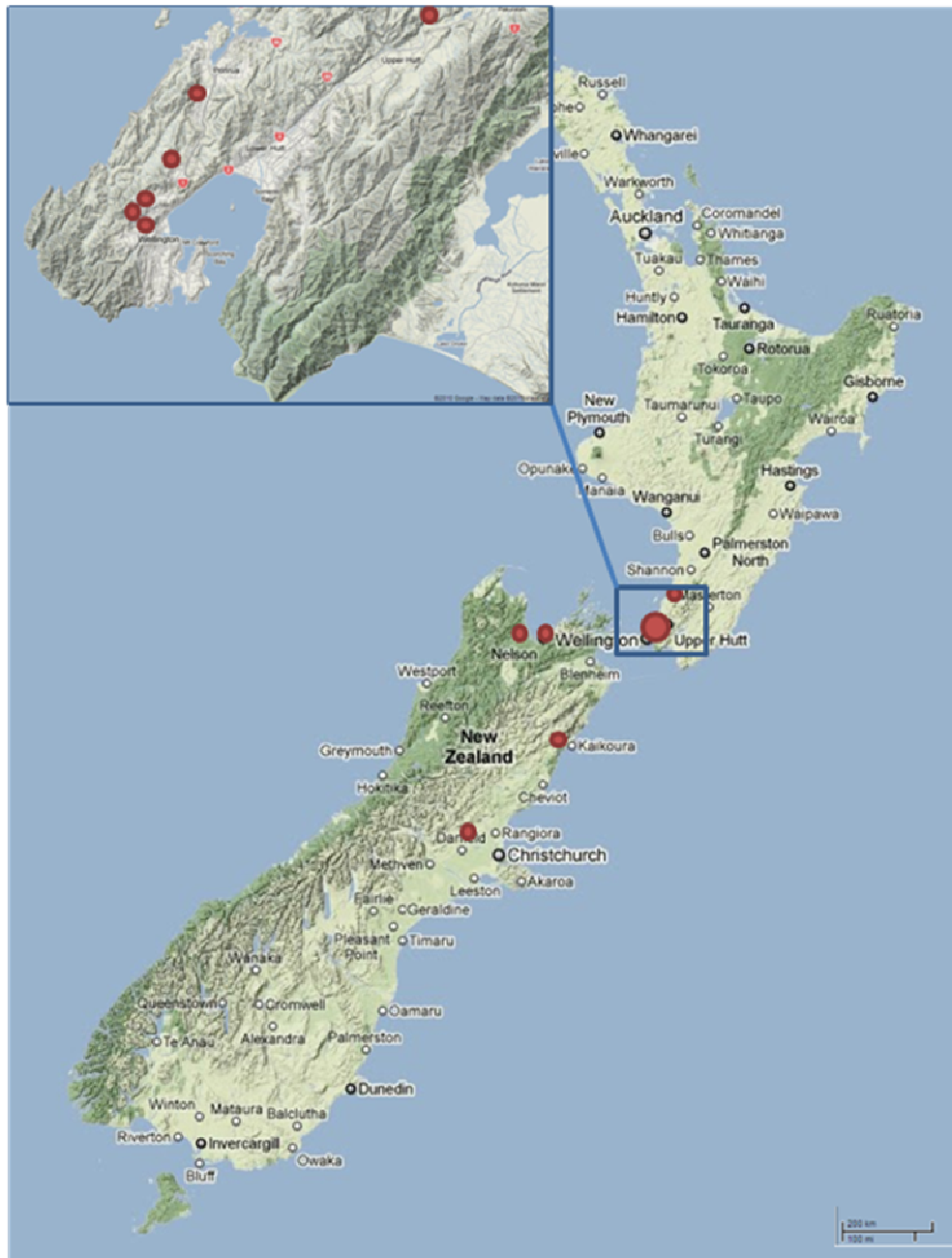
| Plant name (common)                                  | Part           | Medicinal Use  |
|--|----------------|--|
| <i>Adiantum raddianum</i> (Delta maidenhair fern)    | Leaf           | Genus <i>Adiantum</i> used medicinally <sup>[115]</sup>  |
| <i>Agathis australis</i> (Kauri)                     | Leaf           | Presence of antiviral agathisflavone <sup>[116]</sup>  |
| <i>Arthropodium cirratum</i> (New Zealand rock lily) | Leaf           | Used in Rongoa Māori to treat ulcers <sup>[117]</sup>  |
| <i>Asplenium bulbiferum</i> (Hen and chicken fern)   | Leaf           | Genus <i>Asplenium</i> known to have antibacterial activity <sup>[118]</sup>                       |
| <i>Asplenium flaccidum</i> (Drooping Spleenwort)     | Leaf           | Genus <i>Asplenium</i> known to have antibacterial activity <sup>[118]</sup>                       |
| <i>Beilschmiedia tawa</i> (Tawa)                     | Leaf           | Used in Rongoa Māori to treat sore throat, colds, cough <sup>[98]</sup>                            |
| <i>Blechnum fluviatile</i> (Kiwikiwi)                | Leaf           | Used in Rongoa Māori to treat sore tongue and mouth <sup>[98]</sup>                                |
| <i>Brachyglottis repanda</i> (Rangiora)              | Leaf           | Used in Rongoa Māori to treat wounds, ulcers and boils <sup>[98]</sup>                             |
| <i>Carpodetus serratus</i> (Marble leaf)             | Leaf<br>seed   | <i>ni</i>  |
| <i>Clianthus puniceus</i> (Kakabeak)                 | Leaf<br>Seed   | Family <i>Fabaceae</i> has recorded antibacterial activity <sup>[119, 120]</sup>                   |
| <i>Cordyline australis</i> (Cabbage tree)            | Leaf           | Used in Rongoa Māori to treat dysentery, diarrhoea and sores <sup>[121]</sup>                      |
| <i>Cortaderia toetoe</i> (Toetoe)                    | Shoot          | Used in Rongoa Māori to treat diarrhoea, relief of kidney, bladder complaints <sup>[97, 122]</sup> |
| <i>Cyathea dealbata</i> (Ponga)                      | Fronds         | Used in Rongoa Māori to treat boils <sup>[99]</sup>  |
| <i>Dacrydium cupressinum</i> (Rimu)                  | Leaf           | Used in Rongoa Māori to treat sores <sup>[121]</sup>   |
| <i>Dracophyllum longifolium</i> (Inanga)             | Leaf           | Genus <i>Dracophyllum</i> known to have antibacterial activity <sup>[123]</sup>                    |
| <i>Dodonea viscosa</i> (Ake ake)                     | Leaf<br>Seed   | Used in Rongoa Māori to treat toothache, sore throats and skin irritations <sup>[124, 125]</sup>   |
| <i>Dysoxylum spectabile</i> (Kohekohe)               | Leaf           | Used in Rongoa Māori to relieve coughing, colds and fevers <sup>[96]</sup>                         |
| <i>Exocarpus bidwillii</i>                           | Leaf<br>Seed   | Root parasite of other species <sup>[126]</sup>  |
| <i>Hebe stricta</i> (Koromiko)                       | Leaf<br>Flower | Leaf was used in Rongoa Māori to treat skin problems and ulcers <sup>[96, 121]</sup>               |
| <i>Hymenophyton flabellatum</i> (Liverwort)          | Leaf           | Liverworts commonly used medicinally <sup>[127]</sup>  |
| <i>Knightia excelsa</i> (Rewarewa)                   | Leaf           | Used in Rongoa Māori to relieve coughing <sup>[121]</sup>  |

|  |         |   |
|--|---------|---|
| <i>Kunzea ericoides</i> (Kanuka)               | Leaf    | Used in Rongoa Māori to relieve pain, headaches and coughs  |
| <i>Laurelia novae-zelandiae</i> (Pukatea)      | Cambium | Used in Rongoa Māori to treat sores, ulcers and tuberculosis <sup>[97, 99, 121]</sup>               |
|  | Bark    | Used to treat skin conditions, venereal disease and used as a painkiller <sup>[117]</sup>           |
|  | Leaf    | Treated toothache <sup>[121]</sup>  |
| <i>Leptospermum scoparium</i> (Manuka)         | Leaf    | Leaf was used in Rongoa Māori to treat  |
|  | Flower  | colds, pains, urinary troubles <sup>[121]</sup>   |
| <i>Lophomyrtus bullata</i> (Ramarama)          | Leaf    | Used in Rongoa Māori to treat bruises <sup>[121]</sup>  |
| <i>Macropiper excelsum</i> (Kawakawa)          | Leaf    | Used in Rongoa Māori to treat arthritis, rheumatism and chest difficulties <sup>[99, 128]</sup>     |
| <i>Meliclytus ramiflorus</i> (Mahoe)           | Leaf    | Used in Rongoa Māori to treat rheumatism and stomach wounds <sup>[121]</sup>                        |
| <i>Meryta sinclairii</i> (Puka)                | Leaf    | The family <i>Araliaceae</i> has plants used medicinally <sup>[129]</sup>                           |
| <i>Metrosideros excelsa</i> (Pohutukawa)       | Leaf    | Flower was used in Rongoa Māori to treat  |
|  | Flower  | sore throats <sup>[121]</sup>   |
| <i>Metrosideros umbellata</i> (Southern Rata)  | Leaf    | Genus <i>Metrosideros</i> was used in traditional medicine <sup>[121]</sup>                         |
| <i>Microsorium pustulatum</i> (Hound's tongue) | Leaf    | Genus <i>Microsorium</i> was used in traditional medicine <sup>[130]</sup>                          |
| <i>Myoporum laetum</i> (Ngaio)                 | Leaf    | Used in Rongoa Māori to treat bruises and infected wounds <sup>[121]</sup>                          |
| <i>Nothofagus fusca</i> (Red beech)            | Leaf    | Presence of polyphenolic antioxidant nothofagin <sup>[131]</sup>                                    |
| <i>Paesia scaberula</i> (Lace fern)            | Leaf    | Family <i>Dennstaedtiaceae</i> has recorded antibacterial activity <sup>[132]</sup>                 |
| <i>Phormium tenax</i> (New Zealand flax)       | Leaf    | Used in Rongoa Māori to treat cuts and constipation <sup>[98]</sup>                                 |
| <i>Pittosporum tenuifolium</i> (Kohuhu)        | Leaf    | Used in Rongoa Māori to treat eczema, other skin diseases and fever <sup>[121]</sup>                |
| <i>Plagiochila stephensoniana</i> (Liverwort)  | Leaf    | Genus <i>Plagiochila</i> known to have antibacterial activity <sup>[127]</sup>                      |
| <i>Pseudopanax arboreus</i> (Five finger)      | Leaf    | The family <i>Araliaceae</i> contains plants used medicinally <sup>[129]</sup>                      |
| <i>Pseudopanax crassifolius</i> (Lancewood)    | Leaf    | The family <i>Araliaceae</i> contains plants used medicinally <sup>[129]</sup>                      |
| <i>Pseudowintera colorata</i> (Horopito)       | Leaf    | Used in Rongoa Māori to treat toothache, skin irritations, and used as a painkiller <sup>[96]</sup> |
| <i>Pteridium esculentum</i> (Bracken)          | Leaf    | Used as an antiseptic as well as treats sores <sup>[125]</sup>                                      |
| <i>Rubus cissoides</i> (Bush lawyer)           | Leaf    | Used in Rongoa Māori to treat chest congestion, coughs and sore throat <sup>[121]</sup>             |
| <i>Schefflera digitata</i> (Seven finger)      | Leaf    | Contains anti-dermatophyte compound Falcarindiol <sup>[133]</sup>                                   |
| <i>Sophora microphylla</i> (Kowhai)            | Leaf    | Used in Rongoa Māori to treat wounds, back, abdominal and internal pains <sup>[99]</sup>            |
| <i>Urtica ferox</i> (Stinging nettle)          | Leaf    | Genus <i>Urtica</i> has known anti-fungal and anti-viral activity <sup>[134]</sup>                  |
| <i>Vitex lucens</i> (Puriri)                   | Leaf    | Used in Rongoa Māori to treat backache,   |
|  | Fruit   | ulcers, and sore throats <sup>[121]</sup>   |
|  | Seed    |   |
| <i>Weinmannia racemosa</i> (Kamahi)            | Leaf    | Parts of plant used medicinally to treat stomach aches and cuts <sup>[121]</sup>                    |

**Table 3.2.1. Medicinal information on native New Zealand plants selected for antimycobacterial screening.** Information on the native NZ plants was either related to Māori medicinal use or any other known anti-infective properties. *ni* = no information available.

### 3.2.2 Location of collection sites

The majority of samples were collected in the Wellington region, in Wellington central, Karori, Otari-Wilton, Ngaio and Kaitoke as shown below in Figure 3.2.2. From the Wellington region most of the plants collected were from Otari-Wilton bush, a reserve with a wide range of native plants with easy access to them for collection. Other reserves included Karori Wildlife Sanctuary (Zealandia) and Kaitoke Regional Park. Leaves were chosen from the same apical node from each branch with similar size and colouring to reduce the amount of variability of compounds present in each individual sample.



**Figure 3.2.2. Plant sample collection sites in New Zealand.** Collection sites are indicated by the red dots. Inset is an enlarged view of the Wellington region. Map reproduced and adapted from Google Maps, ©2010 Google – and © 2010 MapData Sciences Pty Ltd, PSMA.

### 3.2.3 Conditional information of the samples collected

The location, season and local conditions were recorded for each sample and documented in Table 3.2.3. This information may provide some insight into the plants with activity, such as if there is a correlation between location, season or condition and the levels of inhibitory activity.

| Plant                             | Area         | Season | Conditions                 |
|-----------------------------------|--------------|--------|----------------------------|
| <i>Adiantum raddianum</i>         | Ngaio        | Spring | Filtered light, moist, low |
| <i>Agathis australis</i>          | Otari-Wilton | Summer | Sunny, dry, slightly damp  |
| <i>Arthropodium cirrhatum</i>     | Ngaio        | Spring | Filtered light, moist      |
| <i>Asplenium bulbiferum</i>       | Upper Hutt   | Autumn | Filtered light, damp, low  |
| <i>Asplenium flaccidum</i>        | Upper Hutt   | Summer | <i>ni</i>                  |
| <i>Beilschmiedia tawa</i>         | Upper Hutt   | Autumn | <i>ni</i>                  |
| <i>Blechnum fluviatile</i>        | Otari-Wilton | Summer | Shaded, damp               |
| <i>Brachyglottis repanda</i>      | Karori       | Summer | Filtered light, moist, low |
| <i>Carpodetus serratus</i>        | S. Island    | Spring | Shaded, damp               |
| <i>Clianthus puniceus</i>         | Tawa         | Winter | Sunny, highland            |
| <i>Cordyline australis</i>        | Ngaio        | Winter | Shaded, damp               |
| <i>Cortaderia toetoe</i>          | Karori       | Summer | Sunny, dry                 |
| <i>Cyathea dealbata</i>           | Otari-Wilton | Spring | Shaded, damp               |
| <i>Dacrydium cupressinum</i>      | Upper Hutt   | Autumn | <i>ni</i>                  |
| <i>Dracophyllum longifolium</i>   | Kaikoura     | Winter | Sunny, dry, highland       |
| <i>Dodonaea viscosa</i>           | Otari-Wilton | Autumn | Sunny, damp                |
| <i>Dysoxylum spectabile</i>       | Karori       | Summer | Filtered light, damp       |
| <i>Exocarpus bidwillii</i>        | Canterbury   | Winter | Sunny, dry                 |
| <i>Hebe speciosa</i>              | Otari-Wilton | Summer | Sunny, dry                 |
| <i>Hymenophyton flabellatum</i>   | Otari-Wilton | Summer | Shaded, damp, lowland      |
| <i>Knightia excelsa</i>           | Otari-Wilton | Summer | Filtered light, moist      |
| <i>Kunzea ericoides</i>           | Upper Hutt   | Autumn | <i>ni</i>                  |
| <i>Laurelia novae-zelandiae</i>   | Otari-Wilton | Summer | Shaded, wet, lowland       |
| <i>Leptospermum scoparium</i>     | Kelburn      | Winter | Sunny, dry, highland       |
| <i>Lophomyrtus bullata</i>        | Otari-Wilton | Summer | Filtered light, moist      |
| <i>Macropiper excelsum</i>        | Otari-Wilton | Autumn | Filtered light, damp       |
| <i>Melicytus ramiflorus</i>       | Karori       | Summer | Filtered light, moist      |
| <i>Meryta sinclairii</i>          | Kelburn      | Summer | Shaded, damp               |
| <i>Metrosideros excelsa</i>       | Ngaio        | Summer | Sunny, damp                |
| <i>Metrosideros umbellata</i>     | Kaiteriteri  | Spring | <i>ni</i>                  |
| <i>Microsorium pustulatum</i>     | Karori       | Summer | <i>ni</i>                  |
| <i>Myoporum laetum</i>            | Ngaio        | Winter | Sunny, damp                |
| <i>Nothofagus fusca</i>           | Otari-Wilton | Summer | Sunny, moist               |
| <i>Paesia scaberula</i>           | Otari-Wilton | Summer | Filtered light             |
| <i>Pittosporum tenuifolium</i>    | Ngaio        | Winter | Sunny, dry                 |
| <i>Plagiochila stephensoniana</i> | Otari-Wilton | Summer | Shaded, damp               |
| <i>Pseudopanax arboreus</i>       | Ngaio        | Winter | Shaded, damp               |
| <i>Pseudopanax crassifolius</i>   | Otari-Wilton | Spring | Filtered light, moist      |
| <i>Pseudowintera colorata</i>     | Nelson       | Spring | <i>ni</i>                  |
| <i>Pteridium esculentum</i>       | Karori       | Summer | Filtered light, moist      |
| <i>Rubus cissoides</i>            | Moutere Hill | Spring | <i>ni</i>                  |
| <i>Schefflera digitata</i>        | Ngaio        | Winter | Shaded, damp               |
| <i>Sophora microphylla</i>        | Ngaio        | Winter | Shaded, damp               |
| <i>Urtica ferox</i>               | Otari-Wilton | Summer | Shaded, moist              |
| <i>Vitex lucens</i>               | Kaitoke      | Spring | Filtered light, damp       |
| <i>Weinmannia racemosa</i>        | Upper Hutt   | Autumn | <i>ni</i>                  |

**Table 3.2.3. Information of the samples collected.** The location, season and local conditions were recorded for each sample. *ni* = no information recorded.

### 3.2.4 Development of Solvent Extraction

The leaf of *M. excelsa* was tested in various conditions to validate the protocol that was in development. It was questioned that, under solvent extraction, if dried plant material would have improved antimycobacterial activity compared to fresh. It also was hypothesized that heating the sample during the extraction process would have better activity than if tested fresh. In traditional medicine a lot of plants were boiled before treatment [97]. Thus, it was queried if a period of incubation would help increase the yield of bioactive compounds extracted and thus increase the potential activity of the sample. In the first condition the leaf extract was tested fresh as well as dried with a desiccator. There was no incubation involved at this stage. As shown in Table 3.2.4 it appears that there was a higher yield and lower MIC with the dried extract than when tested fresh. In the second condition a dried sample of leaf was added to MeOH and either incubated at 55°C or had no incubation involved. The solvent of the resulting extracts was then evaporated and the yield of the remaining extract was determined. The extracts were then resuspended in MeOH and analysed for antimycobacterial activity against *M. smegmatis* to give an MIC. The two conditions gave similar yields, but the extract that was incubated had an MIC of 0.75 mg/ml, whereas the MIC was more than 2 mg/ml for the extract with no incubation performed.

| Condition     | Yield (g) | MIC (mg/ml) |
|---------------|-----------|-------------|
| Fresh         | 0.0118    | >2          |
| Dried         | 0.0184    | 0.9         |
| No Incubation | 0.01      | >2          |
| Incubation    | 0.0168    | 0.75        |

**Table 3.2.4. Yield and MIC of *M. excelsa* methanol leaf extract in various extraction conditions.** The leaf MeOH extract of *M. excelsa* was tested in various conditions to validate the protocol that was in development. In the first condition, the leaf extract was tested 'fresh' as well as 'dried' with a desiccator. In the second condition a dried sample of leaf was either incubated at 55°C or had no incubation performed. The resulting yield was weighed and then resuspended in MeOH before screening against *M. smegmatis* to determine the MIC.

### 3.2.5 Initial Screening of Native Plants against *M. smegmatis*

The library of extracts was screened against *M. smegmatis* for initial antimycobacterial activity. The EtOH and MeOH solvents were tested at a starting solvent concentration of 2% which did not affect normal growth of the bacteria. A minimum of 90% inhibition was considered to be an active extract. As shown below in Table 3.2.5 the nine extracts from seven species (*L. novae-zelandiae*, *L. bullata*, *M. excelsa*, *M. laetum*, *P. tenuifolium*, *P. crassifolius*, *P. colorata*) were found to cause  $\geq 90\%$  inhibition.



| Plant                             | Part    | % inhibition |      |      |
|-----------------------------------|---------|--------------|------|------|
|                                   |         | AQ           | EtOH | MeOH |
| <i>Adiantum raddianum</i>         | Leaf    | 14           | 53   | 63   |
| <i>Agathis australis</i>          | Leaf    | 35           | 18   | 21   |
| <i>Arthropodium cirrhatum</i>     | Leaf    | 40           | 16   | 24   |
| <i>Asplenium bulbiferum</i>       | Leaf    | 4            | 12   | 6    |
| <i>Asplenium flaccidum</i>        | Leaf    | 25           | 5    | 0    |
| <i>Beilschmiedia tawa</i>         | Leaf    | 19           | 6    | 11   |
| <i>Blechnum fluviatile</i>        | Leaf    | 35           | 60   | 2    |
| <i>Brachyglottis repanda</i>      | Leaf    | 41           | 27   | 32   |
| <i>Carpodetus serratus</i>        | Leaf    | 3            | 11   | 44   |
|                                   | Seed    | 0            | 21   | 38   |
| <i>Clianthus puniceus</i>         | Leaf    | 0            | 8    | 34   |
|                                   | Seed    | 19           | 56   | 25   |
| <i>Cordyline australis</i>        | Leaf    | 24           | 100  | 79   |
| <i>Cortaderia toetoe</i>          | Leaf    | 15           | 93   | 20   |
| <i>Cyathea dealbata</i>           | Leaf    | 49           | 36   | 41   |
| <i>Dacrydium cupressinum</i>      | Leaf    | 58           | 72   | 79   |
| <i>Dracophyllum longifolium</i>   | Leaf    | 23           | 83   | 56   |
| <i>Dodonaea viscosa</i>           | Leaf    | 54           | 6    | 46   |
|                                   | Seed    | 7            | 22   | 26   |
| <i>Dysoxylum spectabile</i>       | Leaf    | 68           | 57   | 68   |
| <i>Exocarpus bidwillii</i>        | Leaf    | 31           | 30   | 4    |
|                                   | Seed    | 1            | 43   | 33   |
| <i>Hebe speciosa</i>              | Leaf    | 50           | 12   | 94   |
|                                   | Flower  | 89           | 0    | 10   |
| <i>Hymenophyton flabellatum</i>   | Leaf    | 48           | 44   | 7    |
| <i>Knightia excelsa</i>           | Leaf    | 73           | 80   | 37   |
| <i>Kunzea ericoides</i>           | Leaf    | 79           | 39   | 3    |
| <i>Laurelia novae-zelandiae</i>   | Leaf    | 87           | 19   | 68   |
|                                   | Cambium | 70           | 33   | 100  |
|                                   | Bark    | 100          | 90   | 69   |
|                                   | Stem    | 67           | 42   | 0    |
| <i>Leptospermum scoparium</i>     | Leaf    | 56           | 69   | 84   |
|                                   | Flower  | 55           | 62   | 50   |
| <i>Lophomyrtus bullata</i>        | Leaf    | 98           | 97   | 20   |
| <i>Macropiper excelsum</i>        | Leaf    | 41           | 43   | 7    |
| <i>Melicytus ramiflorus</i>       | Leaf    | 57           | 87   | 21   |
| <i>Meryta sinclairii</i>          | Leaf    | 86           | 7    | 4    |
| <i>Metrosideros excelsa</i>       | Leaf    | 90           | 69   | 100  |
|                                   | Flower  | 91           | 100  | 70   |
| <i>Metrosideros umbellata</i>     | Leaf    | 23           | 86   | 80   |
| <i>Microsorium pustulatum</i>     | Leaf    | 0            | 0    | 28   |
| <i>Myoporum laetum</i>            | Leaf    | 100          | 37   | 16   |
| <i>Nothofagus fusca</i>           | Leaf    | 22           | 0    | 11   |
| <i>Paesia scaberula</i>           | Leaf    | 50           | 36   | 33   |
| <i>Pittosporum tenuifolium</i>    | Leaf    | 82           | 100  | 23   |
| <i>Plagiochila stephensoniana</i> | Leaf    | 0            | 37   | 32   |
| <i>Pseudopanax arboreus</i>       | Leaf    | 76           | 40   | 37   |
| <i>Pseudopanax crassifolius</i>   | Leaf    | 7            | 100  | 82   |
| <i>Pseudowintera colorata</i>     | Leaf    | 88           | 94   | 100  |
| <i>Pteridium esculentum</i>       | Leaf    | 65           | 3    | 13   |
| <i>Rubus cissoides</i>            | Leaf    | 50           | 90   | 75   |
| <i>Schefflera digitata</i>        | Leaf    | 64           | 87   | 75   |
| <i>Sophora microphylla</i>        | Leaf    | 33           | 1    | 64   |
| <i>Urtica ferox</i>               | Leaf    | 43           | 51   | 46   |
| <i>Vitex lucens</i>               | Leaf    | 15           | 32   | 28   |
|                                   | Fruit   | 27           | 69   | 7    |
|                                   | Seed    | 48           | 34   | 5    |
| <i>Weinmannia racemosa</i>        | Leaf    | 14           | 34   | 51   |

**Table 3.2.5. Inhibition levels of native New Zealand plants with respect to *M. smegmatis*.** Plants were extracted in an AQ, EtOH or MeOH solvent and screened against *M. smegmatis*. Levels of inhibition were calculated by expressing the GFP fluorescence values as a percentage compared to a no-drug negative control. 100% represents complete inhibition of *M. smegmatis* growth. All extracts were tested at a starting concentration of 2 mg/ml.

### 3.2.6 Summary of Native Plant Extracts active against *M. smegmatis*

Of the 46 native NZ plants initially tested, 7 plant species displayed antimycobacterial activity against *M. smegmatis*. *Laurelia novae-zelandiae*, *Lophomyrtus bullata*, *Metrosideros excelsa*, *Myoporum laetum*, *Pittosporum tenuifolium*, *Pseudopanax crassifolius* and *Pseudowintera colorata* had extracts that showed inhibition towards *M. smegmatis*, with activity specific to one solvent; water, EtOH or MeOH.

| Plant species                   | Common Name | Part    | Location     | AQ | EtOH | MeOH |
|---------------------------------|-------------|---------|--------------|----|------|------|
| <i>Laurelia novae-zelandiae</i> | Pukatea     | Bark    | Otari-Wilton | +  | —    | —    |
|                                 |             | Cambium | Otari-Wilton | —  | —    | +    |
| <i>Lophomyrtus bullata</i>      | Ramarama    | Leaf    | Otari-Wilton | —  | —    | +    |
| <i>Metrosideros excelsa</i>     | Pohutukawa  | Leaf    | Ngaio        | —  | —    | +    |
|                                 |             | Flower  | Ngaio        | —  | +    | —    |
| <i>Myoporum laetum</i>          | Ngaio       | Leaf    | Ngaio        | +  | —    | —    |
| <i>Pittosporum tenuifolium</i>  | Kohuhu      | Leaf    | Ngaio        | —  | +    | —    |
| <i>Pseudopanax crassifolius</i> | Lancewood   | Leaf    | Otari-Wilton | —  | +    | —    |
| <i>Pseudowintera colorata</i>   | Horopito    | Leaf    | Nelson       | —  | —    | +    |

**Table 3.2.6. Antimycobacterial activity of plant extracts against *M. smegmatis*.** ‘+’ indicates  $\geq 90\%$  inhibition of *M. smegmatis* growth at a concentration of 2 mg/ml or lower.

### 3.2.7 Comparison of GFP and OD results of active extracts against *M. smegmatis*

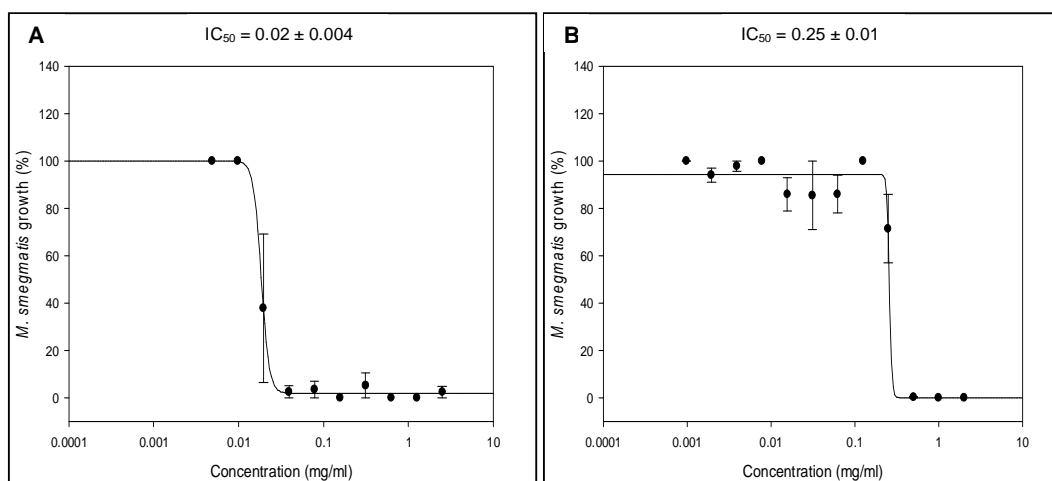
The most active extract was derived from *L. novae-zelandiae* (Pukatea). The bark of *L. novae-zelandiae* generated an IC<sub>50</sub> value of 0.02 mg/ml and the cambium had an IC<sub>50</sub> of 0.25 mg/ml (Table 3.2.7). Significant activity was also observed with respect to the leaf, IC<sub>50</sub> of 0.11 mg/ml, and flower, IC<sub>50</sub> of 0.41 mg/ml, of *M. excelsa*. *P. tenuifolium* and *P. colorata* leaves were less active with IC<sub>50</sub> values of 0.78 mg/ml and 0.74 mg/ml, respectively (Table 3.2.7). The MIC and IC<sub>50</sub> values determined by OD were higher than those determined by GFP.

| Plant                     | Part    | GFP   |                  | OD    |                  |
|---------------------------|---------|-------|------------------|-------|------------------|
|                           |         | MIC   | IC <sub>50</sub> | MIC   | IC <sub>50</sub> |
| <i>L. novae-zelandiae</i> | Bark    | 0.04  | 0.02 ± 0.004     | 0.32  | 0.29 ± 0.13      |
|                           | Cambium | 0.50  | 0.25 ± 0.01      | 0.50  | 0.25 ± 0.02      |
| <i>L. bullata</i>         | Leaf    | 1.00  | 0.31 ± 0.15      | 2.00  | 0.024 ± 0.04     |
| <i>M. excelsa</i>         | Leaf    | 0.63  | 0.11 ± 0.06      | 1.25  | 0.078 ± 0.02     |
|                           | Flower  | 0.63  | 0.41 ± 0.06      | 2.0   | 1.36 ± 1.53      |
| <i>M. laetum</i>          | Leaf    | 0.63  | 0.34 ± 0.02      | 2.0   | 0.46 ± 0.11      |
| <i>P. tenuifolium</i>     | Leaf    | 2.00  | 0.78 ± 0.34      | 2.0   | 1.49 ± 0.41      |
| <i>P. crassifolius</i>    | Leaf    | 0.63  | 0.31 ± 0.01      | 1.25  | 0.74 ± 0.72      |
| <i>P. colorata</i>        | Leaf    | 1.25  | 0.74 ± 0.40      | 2.0   | 1.25 ± 0.17      |
| RIF                       | -       | 0.013 | 0.003 ±<br>0.001 | 0.013 | 0.004 ±<br>0.001 |

**Table 3.2.7. MIC and IC<sub>50</sub> values for GFP and OD of active crude plant extracts with respect to *M. smegmatis*.** Extract and drug control values are in mg/ml. '±' denotes standard error between triplicate results.

### 3.2.7.1 Bacteriostatic inhibition curves of extracts with activity against *M. smegmatis*

Dose-response experiments were performed on the active extracts that were found to have  $\geq 90\%$  inhibition in the initial screen against *M. smegmatis*. Figure 3.2.7.1 depicts the bacteriostatic curves for the two most active extracts, the bark and cambium of *L. novae-zelandiae*. See Appendix 2 for the bacteriostatic curves of the remaining active extracts.



**Figure 3.2.7.1. Bacteriostatic inhibition curves for bark and cambium extracts of *L. novae-zelandiae* against *M. smegmatis*. A) AQ bark extract. B) MeOH cambium extract. Values are based on GFP results. 100% represents complete growth of *M. smegmatis* cells compared to the untreated control. Error bars denote standard deviation between triplicate values.**

### 3.3 Discussion

#### 3.3.1 Construction of Plant Extract Library

Ethnobotany was chosen as the method for selection of plants in order to increase the chances of finding a positive hit. The reasoning behind this was that if the plants were used medicinally then there was a high chance of the plants having antibacterial activity as well as low toxicity [49, 135]. Rongoa Māori can also provide other information such as which parts of the plant were likely to have activity and which season was best for collection. This can make the selection of plants easier to compile in order to increase the chance of attaining a positive hit, considering the thousands of species of NZ flora. Other plants with no traditional use were also selected, based on their physical properties (e.g. *E. bidwilli* is a parasitic plant) or their homology to other plants that were used medicinally (for example, *M. umbellata* compared to *M. excelsa*). Overall, this method of selection appeared to be advantageous as a 15% hit rate of active extracts was obtained from a small selection of extracts collected and tested.

#### 3.3.2 Method Validation

Leaves were mainly chosen for sampling rather than bark, roots or flowers. This was because it was assumed that if secondary metabolites were the compounds present with antibacterial activity then they would logically be in the highest concentrations in the epidermal tissue in order to deter predators or pathogens [85]. Bark or flowers were generally only chosen if traditional medicine used those particular parts. However, the season at

time of collection could have affected the levels of compound present in the plant tissue. In order to conserve energy in the colder seasons the plant could have decreased the levels of metabolites present in the external tissue. An interesting result though was the leaf sample from *M. laetum*, which was taken in the winter and yet it was picked up in the screen as an active hit.

A desiccate was used as many plants were dried first when traditionally used [97]. It was also used to prevent water retention; otherwise the results would be affected by the presence of water remaining in the tissue.

Other non-polar solvents were also attempted for solvent extraction. Toluene, xylene and chloroform ( $\text{CHCl}_3$ ) were used for solvent extraction for some plant samples collected (results not shown). However, these solvents did not yield any positive results, including for those plants which had activity when extracted with polar solvents. Thus, only polar solvents were used for extraction as they were inexpensive and generated extracts with good antimycobacterial activity. EtOH and MeOH solvents were chosen as they have been considered effective solvents for liquid-extraction of plant samples without degrading the tissue [136, 137]. SDW was also chosen as a solvent for extraction even though it does not extract compounds easily through the plants tough cellulose wall due to its high polarity [138]. Water was used for solvent extraction as many of the traditional preparations used in Rongoa Māori involved boiling the plant in water and then using the resulting liquid for treatment [97]. This was also the rationale for the incubation process, as it imitated the preparations

used in traditional Māori medicine with the intention of increasing the potency of the plants activity.

The maceration procedure was performed in order to help increase solvent extraction by increasing surface area of the plant tissue. The heat was also thought to help increase the concentration of compounds extracted from the tissue. This was also facilitated by refrigeration after the incubation process. The extracts activity remained stable when stored at -80°C, that is, they still retained antimycobacterial activity when retested months later.

There were major differences noted in the activity of extracts between solvents used on the same sample. A possible explanation could be that one solvent was able to 'extract more' of the active component present in the sample, thus increasing the antimycobacterial activity. For example, if the active compound was an alkaloid there would be lower concentrations in an aqueous solution than in an alcohol solution. What was especially noted was that many of the aqueous extracts had better activity than the EtOH or MeOH extracts. This could be due to the possibility that the compounds were more stable in a 'natural' state than when in EtOH or MeOH, even though the aqueous solution is more polar and therefore unlikely to extract compounds through the plants cellulose membrane [138]. Overall, the variability between solvents justified the use of more than one solvent in order to maximise the hit rate of active extracts.



### 3.3.3 Initial screening of extracts against *M. smegmatis*

Extracts from 7 plants species, *Laurelia novae-zelandiae*, *Lophomyrtus bullata*, *Metrosideros excelsa*, *Myoporum laetum*, *Pittosporum tenuifolium*, *Pseudopanax crassifolius* and *Pseudowintera colorata* showed inhibition towards *M. smegmatis*. In Rongoa Māori the flower of *M. excelsa* was used to treat sore throats [97, 121]. *Lophomyrtus bullata*, *Myoporum laetum*, *Pittosporum tenuifolium* and *Pseudowintera colorata* were either used for wounds, bruises or skin conditions [96, 121]. This lends to the idea that they potentially have antibacterial compounds. *Pseudopanax crassifolius* was the only extract with activity that was not traditionally used in Rongoa Māori medicine. The bark of *Laurelia novae-zelandiae* (Pukatea) was used by Māori in a number of medicinal remedies [121]. The bark had the best antimycobacterial activity against *M. smegmatis* with an MIC and IC<sub>50</sub> of 0.04 mg/ml and 0.02 ± 0.004 mg/ml, respectively (Figure 3.2.7.1 A), indicating that the bark contained antibacterial compounds. It is possible that during development *L. novae-zelandiae* produced bioactive agents to act as protective measures against predators as well as for maintenance [85], thus signifying it as a good source for novel antitubercular compounds. This would need to be confirmed by validating the activity of the extract against *M. tuberculosis*, as well as establishing its type of activity.

# CHAPTER FOUR: Validation of Active Plant Extracts

(Results in this Chapter were peer-reviewed and published in BMC  
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## 4.1 Introduction

In the previous chapter native plant samples were screened against *M. smegmatis* and seven plant species had extracts with antimycobacterial activity. The aim of this chapter was to then validate the bacteriostatic activity of these extracts against the slow-growing species *M. bovis* BCG and *M. tuberculosis* H37Ra. The extracts were then investigated for bactericidal activity against *M. smegmatis*. The extracts were also tested against *E. coli* and *S. aureus* for antibacterial activity. The active extracts potential cytotoxicity was also assessed. In addition, the potential compounds present in the active extracts are discussed.

*M. tuberculosis* H37Rv is a commonly used strain for the screening of antitubercular compounds in laboratories. However, it is restricted to physical containment level-3 facilities due to its virulent nature. Therefore other species of the mycobacterium genus have been utilized as a surrogate species for *M. tuberculosis* H37Rv. *M. smegmatis* and *M. bovis* BCG are therefore generally used for screening of compounds with potential antimycobacterial activity [139].

*Mycobacterium bovis* is an aerobic, slow-growing organism that is the causative agent of bovine TB in cattle. *M. bovis* is pathogenic and can cause TB in humans. *M. bovis* has a similar genome to *M. tuberculosis* with more than 99.9% homology in sequence [140] and so it is a suitable model when screening for antitubercular compounds. However, *M. tuberculosis* H37Ra was also used as it has the closest homology to the virulent strain *M. tuberculosis* H37Rv. By examining all three species it is possible to get an indication as to what type of inhibition is occurring, e.g.

which of the three mycobacteria species are more susceptible to the active plant extracts. A blue dye, resazurin, that is oxidized to pink (resorufin) in the presence of live cells, was also used to test for the plant extracts bactericidal effect on cell viability [141, 142].

*Escherichia coli* and *Staphylococcus aureus* are part of our natural bacterial flora; however, these species are also the cause of many widespread diseases. *E. coli* is an opportunistic pathogen, usually the common source of food poisoning, but can also be the causal agent for neonatal meningitis [143]. *S. aureus* is one of the most widespread nosocomial infections and can cause the onset of serious diseases such as endocarditis, toxic shock syndrome (TSS) and even sepsis [144-146]. However, there is now the issue of drug-resistant strains of *S. aureus*, one of the major concerns in nosocomial infections [147]. In NZ it appears there is a progressive increase in the number of methicillin-resistant *Staphylococcus aureus* (MRSA) infections [148] and so there is also a need to search a variety of sources for novel antibacterial compounds.

Although plant extracts have been shown to be active against Mycobacteria they must also be non-toxic to humans. As *M. tuberculosis* infects macrophages in the lung, testing against a macrophage cell line presents a more comprehensible indication as to the extracts toxicity. In this research the murine macrophage cell-line J774 was used. A human cell-line HL-60 was also used for examination of the extracts cytotoxicity. HL-60 cells are also commonly used to study the cytotoxicity of anti-cancer drugs; however, they can also be applied for investigating the cytotoxicity of antimycobacterial extracts.

## 4.2 Results

The seven species of active native plants were tested for antimycobacterial activity against *M. bovis* BCG, *M. tuberculosis* H37Ra and for their antibacterial activity against the clinically-important species *E. coli* and *S. aureus*.

### 4.2.1 Summary of initial screening of active extracts against clinically-relevant species

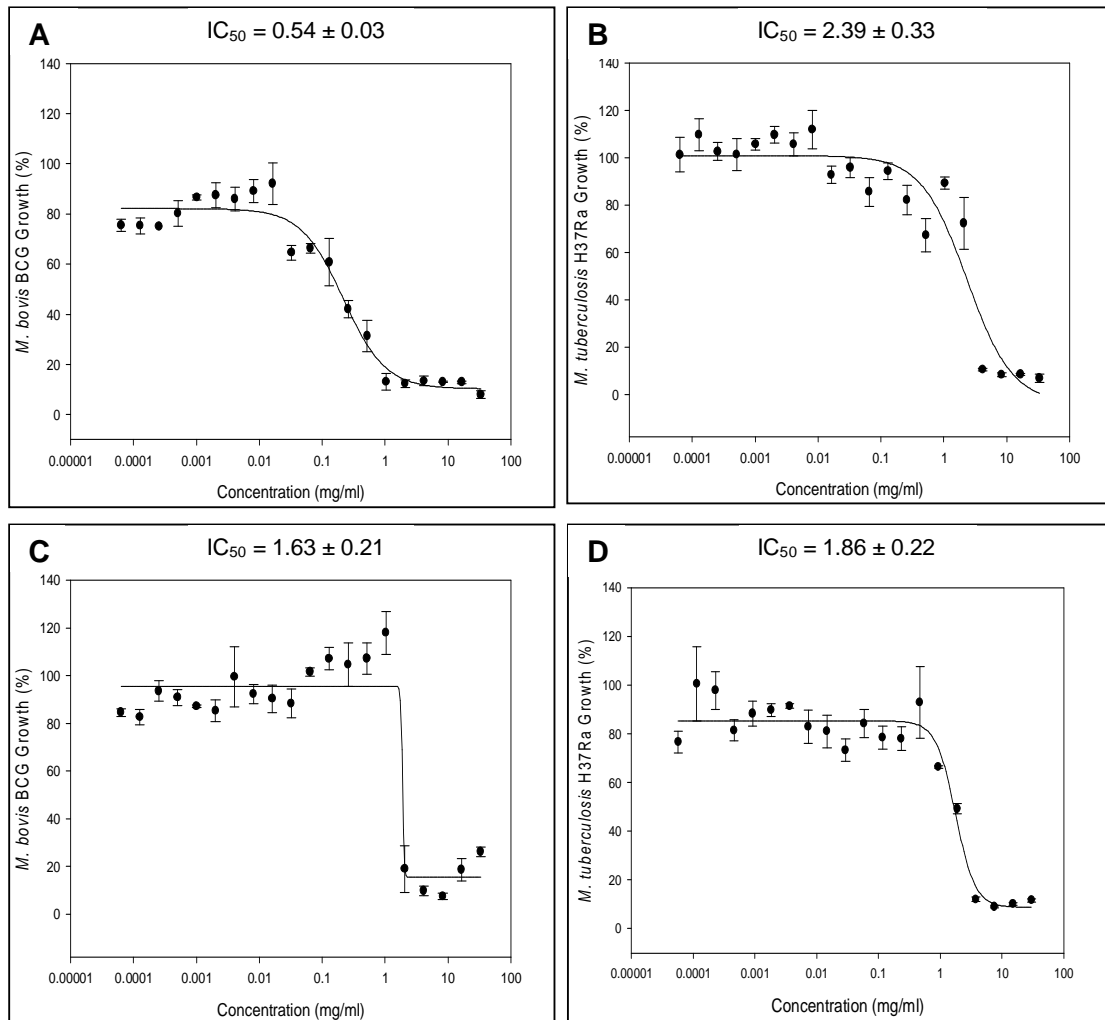
The extracts of *L. novae-zelandiae*, *L. bullata*, *M. excelsa*, *M. laetum*, *P. tenuifolium*, *P. crassifolius* and *P. colorata* were tested against *M. bovis* BCG and *M. tuberculosis* H37Ra. The use of GFP and OD were used to measure growth levels in *M. smegmatis* and *M. bovis* BCG whereas only OD was used for *M. tuberculosis* H37Ra, as it does not contain a plasmid with the GFP gene. However, GFP and OD are considered to have a correlation in measurement of growth levels.

The leaf of *P. tenuifolium* was the most active extract with respect to *M. tuberculosis* with an IC<sub>50</sub> of 0.51 mg/ml (Table 4.2.1). The bark extract of *L. novae-zelandiae* had an IC<sub>50</sub> of 0.54 mg/ml against *M. bovis* and an IC<sub>50</sub> of 2.39 mg/ml against *M. tuberculosis* (Table 4.2.1, Figure 4.2.1). The cambium of *L. novae-zelandiae* was similarly active against both *M. bovis* and *M. tuberculosis* (Table 4.2.1, Figure 4.2.1) with an IC<sub>50</sub> of 1.63 ± 0.21 mg/ml and 1.86 ± 0.22 mg/ml, respectively. The leaf extract from *M. laetum* was not active against *M. bovis* or *M. tuberculosis*. To examine the specificity of the anti-bacterial activity of the plant extracts, the extracts were also tested against *S. aureus* and *E. coli*. The leaf of *M. excelsa*

which was active against *M. smegmatis* and to a lesser extent, *M. tuberculosis*, also displayed antibacterial activity towards *S. aureus*. The leaf of *P. colorata* was the most active of the extracts tested towards *S. aureus* with an  $IC_{50}$  of 0.43 mg/ml. None of the extracts tested exhibited anti-bacterial activity towards *E. coli* up to 10 mg/ml concentration.

| Plant                     | Part    | <i>M. smegmatis</i> |                  | <i>M. bovis</i>         |   | <i>M. tuberculosis</i>  |   | <i>S. aureus</i> |                  | <i>E. coli</i> |                  |
|---------------------------|---------|---------------------|------------------|-------------------------|---|-------------------------|---|------------------|------------------|----------------|------------------|
|                           |         | MIC                 | IC <sub>50</sub> | MIC                     | IC <sub>50</sub>                                  | MIC                     | IC <sub>50</sub>                                  | MIC              | IC <sub>50</sub> | MIC            | IC <sub>50</sub> |
| <i>L. novae-zelandiae</i> | Bark    | 0.04                | 0.02 ± 0.004     | 1.50                    | 0.54 ± 0.03                                       | 4.16                    | 2.39 ± 0.33                                       | >10              | >10              | >10            | >10              |
|                           | Cambium | 0.50                | 0.25 ± 0.01      | 2.50                    | 1.63 ± 0.21                                       | 3.75                    | 1.86 ± 0.22                                       | >10              | >10              | >10            | >10              |
| <i>L. bullata</i>         | Leaf    | 1.00                | 0.31 ± 0.15      | 3.12                    | 2.59 ± 0.43                                       | 6.51                    | 5.59 ± 0.32                                       | >10              | >10              | >10            | >10              |
| <i>M. excelsa</i>         | Leaf    | 0.63                | 0.11 ± 0.06      | 8.35                    | 6.95 ± 3.07                                       | >10                     | 4.83 ± 0.43                                       | 2.20             | 1.17 ± 0.39      | >10            | >10              |
|                           | Flower  | 0.63                | 0.41 ± 0.06      | 3.13                    | 1.12 ± 0.12                                       | 4.18                    | 2.19 ± 0.76                                       | >10              | >10              | >10            | >10              |
| <i>M. laetum</i>          | Leaf    | 0.63                | 0.34 ± 0.02      | >10                     | >10   | >10                     | >10   | >10              | >10              | >10            | >10              |
| <i>P. tenuifolium</i>     | Leaf    | 2.00                | 0.78 ± 0.34      | 10                      | 5.37 ± 1.35                                       | 1.25                    | 0.51 ± 0.11                                       | >10              | >10              | >10            | >10              |
| <i>P. crassifolius</i>    | Leaf    | 0.63                | 0.31 ± 0.01      | 6.25                    | 4.08 ± 1.76                                       | 3.58                    | 1.74 ± 0.18                                       | >10              | >10              | >10            | >10              |
| <i>P. colorata</i>        | Leaf    | 1.25                | 0.74 ± 0.40      | 7.15                    | 5.95 ± 1.28                                       | 7.15                    | 3.66 ± 0.39                                       | 0.75             | 0.43 ± 0.2       | >10            | >10              |
| RIF                       | -       | 0.013               | 0.003 ± 0.001    | 0.12 × 10 <sup>-3</sup> | 4.94 × 10 <sup>-5</sup> ± 5.02 × 10 <sup>-6</sup> | 2.06 × 10 <sup>-4</sup> | 4.84 × 10 <sup>-5</sup> ± 4.03 × 10 <sup>-6</sup> | 0.02             | 0.006 ± 0.001    | 0.04           | 0.03 ± 0.02      |

**Table 4.2.1. Comparison of MIC and IC<sub>50</sub> values of active plant extracts with respect to *M. smegmatis*, *M. bovis*, *M. tuberculosis*, *S. aureus* and *E. coli*.** *M. smegmatis*, *M. bovis*, *S. aureus* and *E. coli* values are based on GFP results and *M. tuberculosis* values are based on OD results. Extract and drug control values are in mg/ml. '±' denotes standard error between triplicate results. MIC, Minimum Inhibitory Concentration. IC<sub>50</sub>, 50% inhibitory concentration.

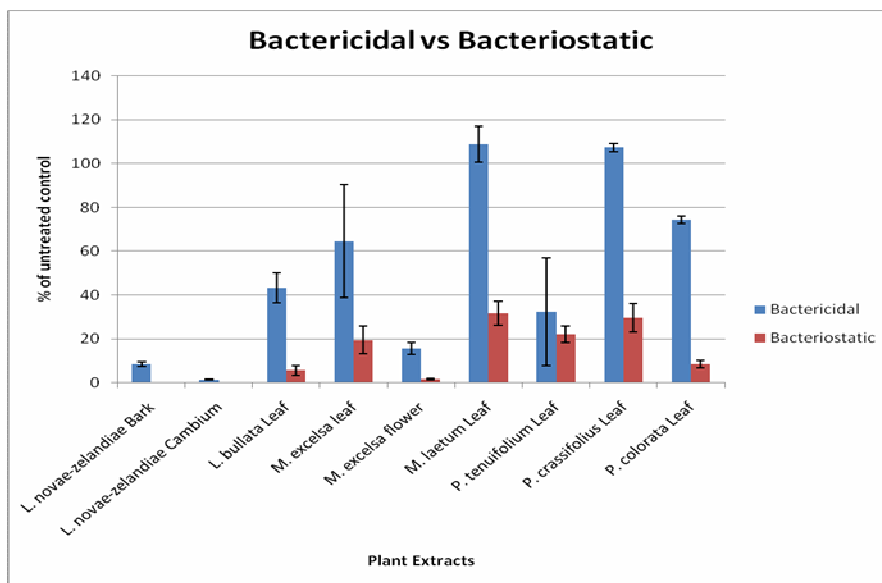


**Figure 4.2.1. Bacteriostatic inhibition curves for bark and cambium extracts of *L. novae-zelandiae* against *M. bovis* and *M. tuberculosis*.** **A)** Inhibitory activity of AQ bark extract against *M. bovis* BCG and **B)** *M. tuberculosis* H37Ra. **C)** Inhibitory activity of MeOH cambium extract against *M. bovis* BCG and **D)** *M. tuberculosis* H37Ra. 100% represents complete growth of *M. smegmatis* cells compared to the untreated control. All values are in mg/ml. Error bars denote standard deviation between triplicate values.



#### 4.2.2 Bactericidal screening of active extracts

To elucidate the type of inhibitory activity, the active extracts were also tested for bactericidal activity. The results shown below in Figure 4.2.2 show the bactericidal activity of each extract compared to its bacteriostatic activity. As bactericidal activity was measured using resazurin reduction the bacteriostatic results were also based on resazurin values. The leaf of *M. laetum*, *P. tenuifolium* and *P. crassifolius* appeared to have lost some bacteriostatic activity with the resazurin reduction, although this was likely due to auto-reduction by the extracts. The bark and cambium extracts of *L. novae-zelandiae* displayed considerable bactericidal activity at concentration of 2 mg/ml. The flower of *M. excelsa* and leaf of *P. tenuifolium* also had some bactericidal activity towards *M. smegmatis*.



**Figure 4.2.2. Bactericidal activity of crude plant extracts with respect to *M. smegmatis*.** All extracts had a starting concentration of 2 mg/ml. Values are based on Resazurin results. 100% represents no inhibition of *M. smegmatis* growth compared to the untreated control. Error bars denote standard deviation between triplicate values.

### 4.2.3 Cytotoxicity of active plant extracts

Active extracts were tested against murine macrophage cell line J774 and human cell line HL-60 to give an indication of cytotoxicity. A cytotoxic compound, EpoA, was included as a positive control. As shown in Table 4.2.3, EpoA had an IC<sub>50</sub> of  $5.32 \times 10^{-6} \pm 8.13 \times 10^{-7}$  mg/ml and  $2.91 \times 10^{-6} \pm 1.11 \times 10^{-6}$  mg/ml against J774 and HL-60 cell lines, respectively. Using this value as the criteria for toxicity, all active extracts could be considered non-toxic. The flower extract of *M. excelsa* had the lowest IC<sub>50</sub> value of  $2.34 \pm \leq 0.001$  mg/ml towards the macrophages, whereas the bark of *L. novae-zelandiae* had the lowest value towards the HL-60 cells with an IC<sub>50</sub> of  $13.64 \pm 0.01$  mg/ml. All of the extracts had lower IC<sub>50</sub> values than the TB drug control RIF, which had an IC<sub>50</sub> of  $1.27 \pm 2.25$  mg/ml against J774 cells and  $0.12 \pm 0.03$  mg/ml against HL-60 cells. The results imply that the extracts were less toxic than the TB drug.

| Plant                     | Part Used | Cytotoxicity (IC <sub>50</sub> )              |   |
|---------------------------|-----------|---|---|
|                           |           | J774  | HL-60   |
| <i>L. novae-zelandiae</i> | Bark      | 0.14 ± 0.02                                   | 13.64 ± 0.01                                  |
|                           | Cambium   | 0.78 ± 0.05                                   | 0.63 ± 0.13                                   |
| <i>L. bullata</i>         | Leaf      | 0.24 ± 0.02                                   | 0.09 ± 0.01                                   |
| <i>M. excelsa</i>         | Leaf      | 0.03 ± 0.05                                   | 0.15 ± 0.001                                  |
|                           | Flower    | 2.34 ± ≤ 0.001                                | 0.08 ± 0.005                                  |
| <i>M. laetum</i>          | Leaf      | 0.42 ± 0.07                                   | 0.94 ± 0.08                                   |
| <i>P. tenuifolium</i>     | Leaf      | 0.02 ± 0.003                                  | 0.04 ± 0.01                                   |
| <i>P. crassifolius</i>    | Leaf      | 0.17 ± 0.008                                  | 0.14 ± 0.007                                  |
| <i>P. colorata</i>        | Leaf      | 0.22 ± 0.02                                   | 0.15 ± 0.03                                   |
| RIF                       | -         | 1.27 ± 2.25                                   | 0.12 ± 0.03                                   |
| EpoA                      | -         | $5.32 \times 10^{-6} \pm 8.13 \times 10^{-7}$ | $2.91 \times 10^{-6} \pm 1.11 \times 10^{-6}$ |

**Table 4.2.3. Cytotoxicity of active extracts against J774 and HL-60 cell-lines.**

The extracts were evaporated and resuspended in SDW to eliminate any bias

effect due to the EtOH or MeOH solvents. All values are in mg/ml. '±' denotes standard error between triplicate results.

### 4.3 Discussion

#### 4.3.1 Screening of active samples against clinically-relevant species

Of the seven plant species found to be active against *M. smegmatis*, only six had antimycobacterial activity against the slow-growing species *M. bovis* and *M. tuberculosis*. The bark and cambium of *L. novae-zelandiae* were particularly active against the mycobacterium species and are discussed further in Sub-section 4.3.1.1. The flower of *M. excelsa* and leaf of *P. tenuifolium* had considerable activity against *M. bovis* and *M. tuberculosis*. In Rongoa Māori, *M. excelsa* was used for the treatment of sore throats and the leaf of *P. tenuifolium* was used to treat skin infections [97, 121]. Therefore, the antimycobacterial activities that were detected for *M. excelsa* and *P. tenuifolium* extracts of *M. excelsa* could relate to their traditional use. The flower of *M. excelsa* and leaf of *P. tenuifolium* also exhibited some substantial bactericidal activity and low cytotoxicity, lending further emphasis that these are extracts to focus on as sources of novel antitubercular compounds. The leaf of *M. excelsa* appeared to have lost activity when tested against *M. bovis* and *M. tuberculosis*, or in the case of the *M. laetum* extract had no activity at all. It could be that the extracts had activity specific to *M. smegmatis*. However, as the extracts did not have activity towards the slow-growing species they may not be considered worthy samples for further analysis for potential lead compounds.

Only the leaf of *P. colorata* and *M. excelsa* displayed activity against *S. aureus*. Interestingly, none of the plant species tested exhibited

antibacterial activity towards *E. coli*. This suggested that the activity of the seven plant species might be specific to mycobacteria. Based on phylogeny with 16S ribosomal RNA, *M. tuberculosis* has similarities with gram-positive bacteria. This would correlate with the antibacterial activity against *S. aureus* that was observed with some of the extracts. However, Fu *et al* (2002) demonstrated that *M. tuberculosis* also has similarities with gram-negative bacteria like *E. coli* [149]. The extracts antimycobacterial activity could therefore be due to other features such as specific virulence factors or pathways.

It should be mentioned that the MIC and IC<sub>50</sub> values of the extracts against the different mycobacteria species (Table 4.2.1) were subject to variation due to differences in growth media used as well as assay incubation time. The activity of the extracts could also vary between mycobacterial species, with higher MIC and IC<sub>50</sub> values towards *M. bovis* and *M. tuberculosis* due to slower metabolism. The compounds in the extract could have degraded over time before being able to fully act upon the mycobacterium's growth. If this was the case then the extracts with higher MICs may not be suitable sources for new anti-TB compounds.

It should also be noted that the MIC and IC<sub>50</sub> of a crude extract is not a reliable indicator of activity. Aliquots of the same extract may have different concentrations of the antimycobacterial compound, or the extract may not have a highly active compound. For example, activity could be due to high concentrations of several moderately active antimycobacterial components rather than an individual component [150]. Further purification

techniques would have to be performed in order to clarify if the activity is due to a single agent or multiple compounds working in synergy.

#### 4.3.1.1 Antimycobacterial activity of *Laurelia novae-zelandiae*

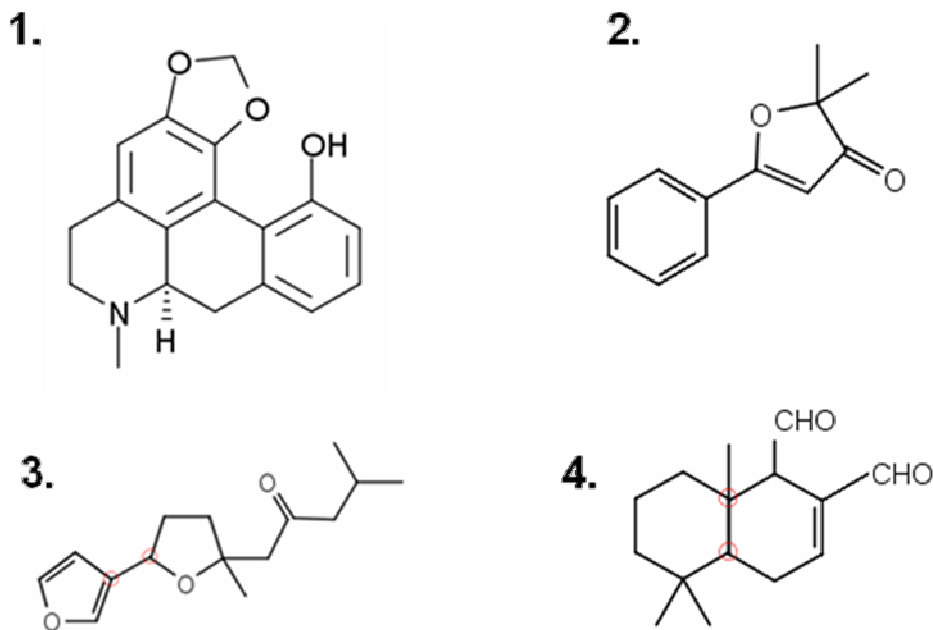
Bark and cambium extracts from *Laurelia novae-zelandiae* (Pukatea) (Figure 4.3.1.1) were the most active against *M. smegmatis* (Table 4.2.1). Furthermore, the bark and cambium extracts also displayed antimycobacterial activity against *M. bovis* and *M. tuberculosis*, exhibiting dose-response inhibition (Figure 4.2.1). The bark of *L. novae-zelandiae* was also the least toxic to HL-60 cells and non-toxic as well towards J774 cells. The activity of Pukatea was of particular interest as the bark was medicinally used in Rongoa Māori to treat tubercular lesions [121]. The pulp of the cambium was boiled in water and the resulting liquid used for treating tubercular ulcers [97]. The antimycobacterial activity of the extract therefore correlated with the use of Pukatea to treat tubercular lesions in Rongoa Māori. The *L. novae-zelandiae* extract also displayed significant bactericidal activity against *M. smegmatis*, reinforcing the possibility that the bark of Pukatea contains previously unknown anti-tubercular compounds.

**Figure 4.3.1.1. *Laurelia novae-zelandiae* (Pukatea) from Otari-Wilton Bush Reserve.** Pukatea is generally found in lowland forests areas [151]. It can grow up to 35 metres and possesses plank buttresses to support the tree's growth in swamp areas [151]. A) Image of Pukatea with two main branches separating from the base of the tree. B) Plank buttress, a typical feature of Pukatea.



### 4.3.2 Potential bioactive compounds present

Although the chemical basis for the anti-tubercular activity of the active extracts needs to be elucidated, earlier chemical studies have identified some compounds, as shown in Figure 4.3.2.



**Figure 4.3.2. Identified compounds in some native plants.** Some compounds have already been identified in four of the plants that have exhibited antimycobacterial activity. 1. = Pukateine, 2. = Bullatenone, 3. = Ngaione, 4. = Polygodial. Pukateine reproduced from Dajas-Bailador F.A. *et al* (1999). Bullatenone, Ngaione and Polygodial adapted and reproduced from Woollard, J.M.R *et al* (2008), Sugimura T. *et al* (1993) and Taniguchi M *et al* (1988). Images created using SktechEl open source software downloadable at [<http://sourceforge.net/projects/sketchel>].

The compounds pukateine, bullatenone and ngaione have so far been isolated from NZ plants. Pukateine is a novel alkaloid isolated from the bark of Pukatea [152]. Pukateine is an analgesic similar to morphine,

which binds to the D<sub>2</sub> dopamine receptor and acts as a dopaminergic agonist [153]. It also acts as an antagonist to the α<sub>1</sub> adrenergic receptor [153, 154]. Bullatenone was isolated from *L. bullata* (Ramarama) [155] and is an antiseptic [98]. Chemical derivatives of bullatenone have been determined to have antibacterial properties against *Bacillus subtilis* [156]. The hepatotoxic sesquiterpene ngaione was found in the leaves of *M. laetum* (Ngaio) [157] and has been used in the treatment of athlete's foot [98]. The leaves of *P. colorata* (Horopito) contain the compound polygodial [158], a drimane sesquiterpene that has been shown to be active against *Candida albicans* [103]. An interesting note is the fern *B. fluviatile* is also known to contain the compound polygodial [127] and yet it did not display any activity. The difference in activity between samples could be due to varying levels of polygodial present in each tissue. Finally, the flower of *M. excelsa* was determined to contain ellagic acid, which is a polyphenol antioxidant [98]. It is possible that compounds such as pukateine, bullatenone, ngaione, polygodial or ellagic acid may contribute to the antibacterial activity of their plant extracts. These compounds offer a starting point for future studies to characterise the chemical basis of the antimycobacterial activity of *L. novae-zelandiae*, *M. excelsa*, *L. bullata* and *P. colorata*.

# CHAPTER FIVE:

## Efficiency and delivery of Plant Extract activity



## 5.1 Introduction

In the previous chapter, eight extracts were validated with antimycobacterial activity against *M. tuberculosis*. In this chapter the efficiency of extraction was investigated to determine if the activity of the extracts could be improved through various purification methods. Possible practical applications of some of the active extracts were also explored.

Size exclusion fractionation is the separation of compounds based on size. This is generally done by chromatography [159, 160] but the protocol has been modified for this research. The fractionation in this research involved centrifuging the extracts through filters of varying sizes. It was hypothesized that the activity of extracts could be improved by removing unwanted compounds. It could also provide information as to the general size of the compound(s) exhibiting antibacterial activity in the extracts.

Solvent Phase Separation (SPS) is a form of liquid-liquid partitioning. This was a procedure used to separate two immiscible liquids in order to extract a substance or compound from one phase to another [161]. Therefore, SPS can be used to 'purify' a sample by removing unwanted compounds [161]. This type of extraction generally involved water and an organic solvent, in this case, Ethyl Acetate (EtOAc). EtOAc was chosen as a solvent for SPS as it is cheap, available and is commonly used in extractions [162]. Chloroform ( $\text{CHCl}_3$ ) was also used as a phase for SPS as it has also been previously used for liquid-liquid extraction with plant samples [163]. Combinations of different fractionation techniques were also applied.

This chapter also involved an initial study involving the utilization of the active extracts in a practical application. The active extracts from *L. novae-zelandiae*, *M. excelsa* and *P. colorata* were added to an aqueous cream and tested against *M. smegmatis* and *S. aureus*. Based on these initial results the active extracts could potentially be used, for example, for the treatment of skin infections in the form of a cream-based solution.

## 5.2 Results

The nine active extracts were subjected to numerous purification techniques. This included techniques such as size exclusion fractionation and solvent phase separation. The leaf of *L. bullata*, *M. laetum*, *P. tenuifolium* and *P. crassifolius* lost antimycobacterial activity upon application of these purification techniques and so were not included in the results. Thus, the following section will focus on the bark and cambium extracts of *L. novae-zelandiae*, the leaf and flower of *M. excelsa* and the leaf of *P. colorata*.

### 5.2.1 Activity of aqueous extracts tested against *M. smegmatis*

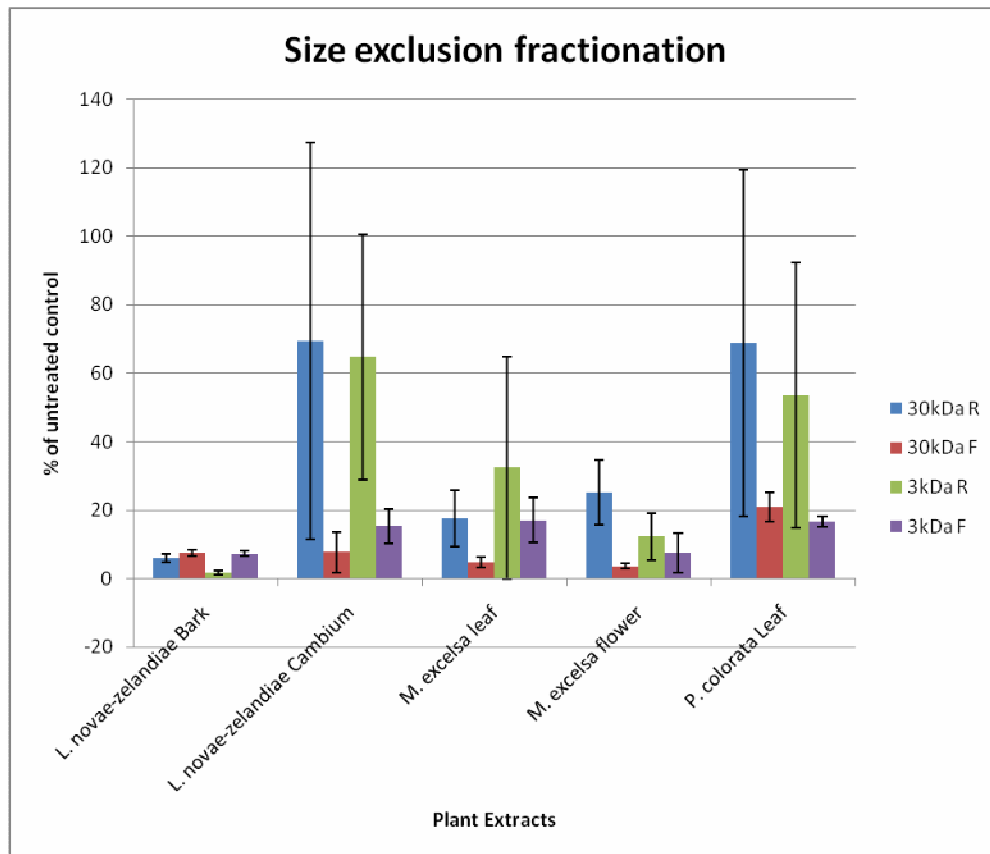
Due to the slight toxicity of some solvents used for extraction, it was investigated if the crude extracts could retain activity when the solvent was evaporated and the sample resuspended in SDW. The rationale was that water extracts were easier to purify, as the filters used would be destroyed by the EtOH or MeOH. Table 5.2.1 gives the activity of the extracts when resuspended in SDW. The bark of *L. novae-zelandiae* was already an AQ extract and so retained antimycobacterial activity. The remaining extracts appeared to have lost activity, with a high MIC and IC<sub>50</sub> when tested against *M. smegmatis*. However, it must be taken into consideration that these values are a measure of potency rather than actual concentration of an active compound, which most likely varies between extracts.

| Plant                     | Part    | Crude |                  | In AQ |                  |
|---------------------------|---------|-------|------------------|-------|------------------|
|                           |         | MIC   | IC <sub>50</sub> | MIC   | IC <sub>50</sub> |
| <i>L. novae-zelandiae</i> | Bark    | 0.04  | 0.02 ± 0.004     | 0.04  | 0.0006 ± 0.004   |
|                           | Cambium | 0.50  | 0.25 ± 0.01      | 2.5   | 1.52 ± 0.18      |
| <i>M. excelsa</i>         | Leaf    | 0.63  | 0.11 ± 0.06      | 10    | 0.33 ± 0.07      |
|                           | Flower  | 0.63  | 0.41 ± 0.06      | 5     | 0.06 ± 0.02      |
| <i>P. colorata</i>        | Leaf    | 1.25  | 0.74 ± 0.40      | 10    | 2.14 ± 1.49      |

**Table 5.2.1. Comparison of MIC and IC<sub>50</sub> values of crude and aqueous plant extracts with respect to *M. smegmatis*.** Extracts were evaporated and resuspended in SDW and examined to determine if activity was retained in an aqueous solution. All values are in mg/ml and based on GFP results. '±' denotes standard error between triplicate results.

### 5.2.2 Size exclusion fractionation of active extracts

Fractionation of active samples was performed via size exclusion. The extracts were evaporated and resuspended in SDW before passed through a 30 kDa filter followed by a 3 kDa filter. The filtrate and retentate for each size were retained. The resulting samples were then evaporated and resuspended in SDW before analysis of antimycobacterial activity against *M. smegmatis* in a bacteriostatic assay. The levels of antimycobacterial activity shown in Figure 5.2.2 indicates that the bark from *L. novae-zelandiae*, flower from *M. excelsa* and leaf from *P. colorata* most likely have bioactive components that are smaller than 3 kDa. The cambium of *L. novae-zelandiae* and leaf of *M. excelsa* appear to have compounds exhibiting antimycobacterial activity that is smaller than 30 kDa but larger than 3 kDa. An interesting note is that the bark of *L. novae-zelandiae* had constant antimycobacterial activity across all fraction sizes. However, size exclusion work is subject to significant variation, especially when working with crude samples where the concentration of the active component(s) is unknown.



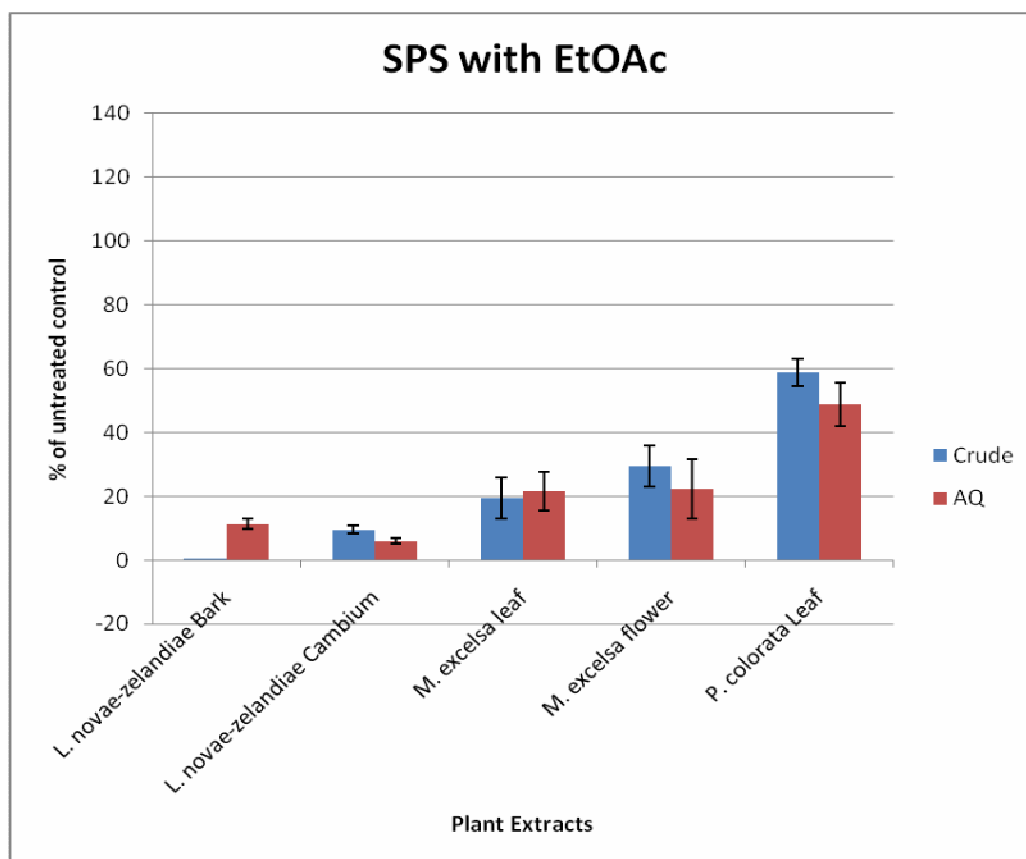
**Figure 5.2.2. Comparison of different size fractions of active plant extracts.** Extracts were resuspended in SDW before fractionation by size exclusion and tested against *M. smegmatis* in a bacteriostatic assay. All extracts were tested at a concentration of 1 mg/ml. These values are based on the GFP results. 100% represents no inhibition of *M. smegmatis* growth compared to the untreated control. Error bars denote standard deviation between triplicate values.

### 5.2.3 Solvent Phase Separation of active extracts

Two different liquids, EtOAc and  $\text{CHCl}_3$ , were used for SPS for the active extracts in order to purify the samples by liquid extraction of unwanted compounds.

#### 5.2.3.1 SPS of active extracts with Ethyl Acetate

Liquid-liquid partitioning with the active samples was performed via SPS with EtOAc. The extracts were evaporated and resuspended in SDW before being purified via liquid partitioning with EtOAc. After solvent phase separation (SPS) the AQ (extract) layer was removed, evaporated and resuspended in SDW again before examination against *M. smegmatis* in a bacteriostatic assay. The crude extracts were also subjected to SPS with EtOAc (i.e. no resuspension in SDW) as a comparison to the original activity of the extracts in its crude form. There was significant bacteriostatic inhibition of mycobacteria growth for the *L. novae-zelandiae* extracts; however, the bark of *L. novae-zelandiae* had better antimycobacterial activity as a crude sample. It should be noted that the EtOAc layer was also tested when solvent phase separated with the AQ and crude layer and was found to have no antimycobacterial activity for either condition (results not shown).

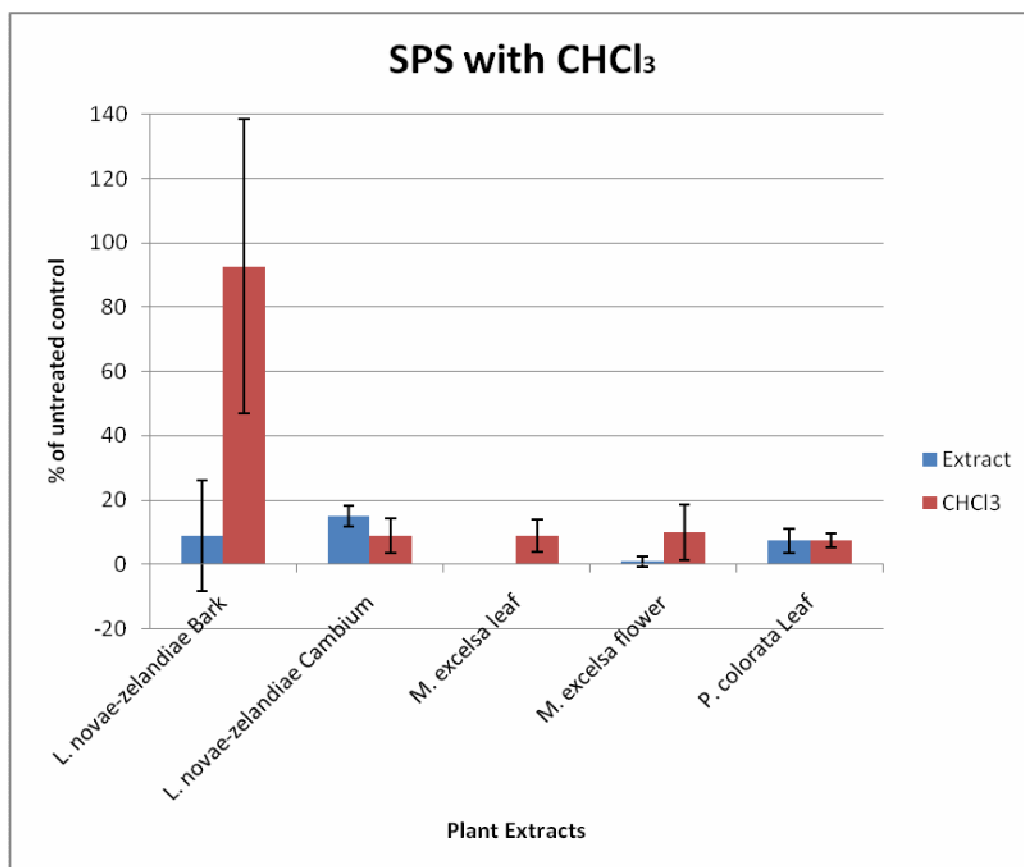


**Figure 5.2.3.1. Comparison of crude and AQ extracts activity when purified with EtOAc through SPS.** Extracts were solvent phase separated from EtOAc and then tested against *M. smegmatis*. All extracts were tested at a concentration of 1 mg/ml. These values are based on the GFP results. 100% represents no inhibition of *M. smegmatis* growth compared to the untreated control. Error bars denote standard deviation between triplicate values.



### 5.2.3.2 SPS of active extracts with Chloroform

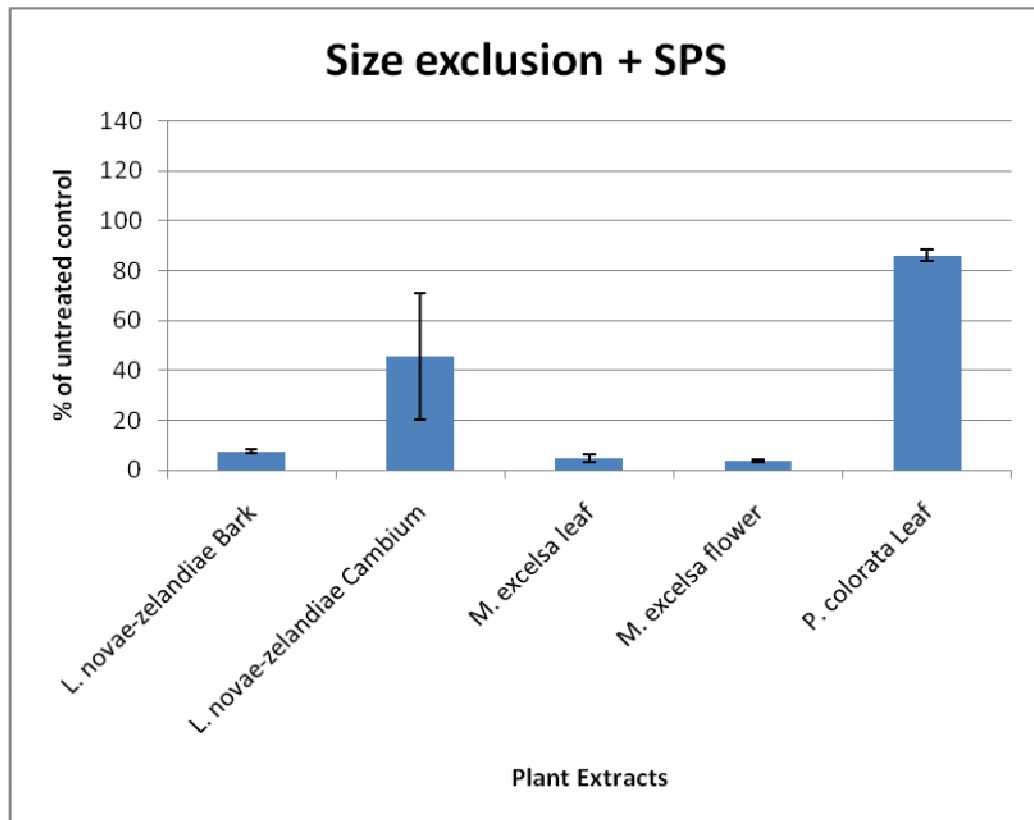
The crude extracts were purified via SPS with  $\text{CHCl}_3$  to determine if activity of the extracts could be improved. After SPS, the extract layer was removed and evaporated before resuspension in SDW. The  $\text{CHCl}_3$  layer was also removed and evaporated before resuspension in DMSO, so as to dissolve any residual plant tissue in the  $\text{CHCl}_3$  layer. The results as displayed in Figure 5.2.3.2 indicate that the extracts from *L. novae-zelandiae*, *M. excelsa* and *P. colorata* had significant inhibitory activity. The  $\text{CHCl}_3$  layer also appeared to have antimycobacterial activity exhibited from some of the extracts. In fact, for the cambium extract from *L. novae-zelandiae* and leaf extract from *P. colorata*, the  $\text{CHCl}_3$  layer exhibited similar or better inhibition levels against *M. smegmatis* than the extract layer.



**Figure 5.2.3.2. Comparison of SPS with Chloroform (CHCl<sub>3</sub>) purified plant extracts.** The extract layer and Chloroform layer were examined against *M. smegmatis* in a bacteriostatic assay. All extracts were tested at a concentration of 1 mg/ml. These values are based on the GFP results. 100% represents no inhibition of *M. smegmatis* growth compared to the untreated control. Error bars denote standard deviation between triplicate values.

### 5.2.3.3 Combination of Purification techniques

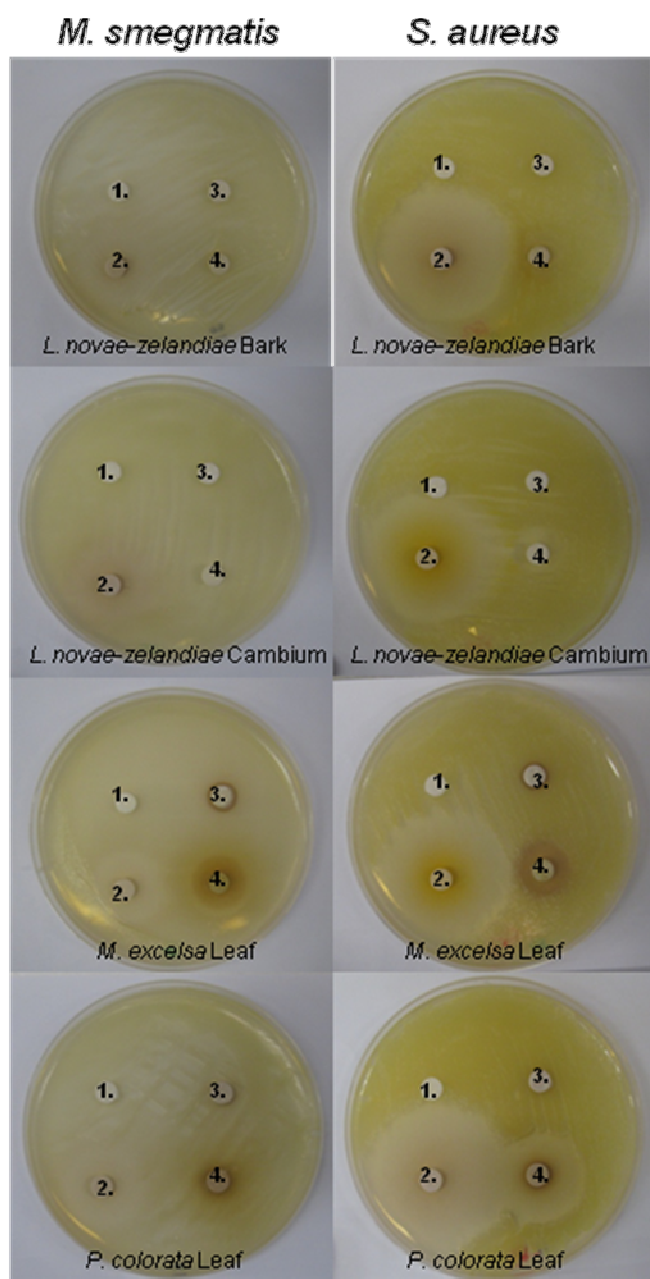
A combination of previous techniques was applied in order to observe an improvement in activity. The extracts were evaporated and resuspended in SDW before purification via size exclusion with a 30 kDa filter. The filtrate was then solvent phase separated with EtOAc. The aqueous layer was removed, evaporated and resuspended again in SDW before being screened against *M. smegmatis* in a bacteriostatic assay. In Figure 5.2.3.3 it can be observed that there was still significant activity exhibited in the bark extract from *L. novae-zelandiae* and the *M. excelsa* extracts. In comparison to the SPS with EtOAc, only the bark extract of *L. novae-zelandiae* and *M. excelsa* extracts had improved activity with the addition of 30 kDa fractionation. It is important to note that this is only an indication of improved efficiency of extraction as there is bound to be variability among results with crude extracts.



**Figure 5.2.3.3. Bacteriostatic activity of purified plant extracts with respect to *M. smegmatis*.** Extracts were subjected to 30 kDa size exclusion fractionation and SPS with EtOAc. All extracts were tested at a concentration of 1 mg/ml. Values are based on GFP results. 100% represents no inhibition of *M. smegmatis* growth compared to the untreated control. Error bars denote standard deviation between triplicate values.

#### 5.2.4 Practical application of *L. novae-zelandiae* and other extracts

Seven plants have been identified with antimycobacterial activity and part of this research has involved improving the efficiency of extraction in order to improve activity. Another aspect to consider is the use of this research. One notion was whether the active hits could be used in a practical application. For example, a moisturizing cream containing natural extracts that could have potential inhibitory effects against skin infections. In this study four of the active extracts were tested in an aqueous cream form against *M. smegmatis* and *S. aureus*. *S. aureus* is known to cause skin infections [144], which is why it was also included in the practical application. The bark and cambium of *L. novae-zelandiae* were tested as they were traditionally used for the treatment of tubercular ulcers on the skin. The leaf of *M. excelsa* and *P. colorata* were also tested as they were the only extracts tested to have activity against *S. aureus*. As shown below in Figure 5.2.4 and Table 5.2.4 it appears that the extracts (with the exception of the leaf extract of *M. excelsa*) lose activity in the aqueous cream form.



**Figure 5.2.4. Disc Diffusion of active extracts in an aqueous cream against *M. smegmatis* and *S. aureus*.** Extracts from *L. novae-zelandiae*, *M. excelsa* and *P. colorata* were tested in an aqueous cream form against *M. smegmatis* (Left column) and *S. aureus* (Right column). Layout is as follows; 1. = Negative control aqueous cream with no extract added, 2. = Positive control Rifampin (1 mg/ml), 3. = Extract added to an aqueous cream (0.5 % w/v), 4. = Extract alone (1 mg/ml).

Table 5.2.4 gives the zones of inhibition that were measured around the discs. The leaf of *M. excelsa* was the only extract to have activity against *M. smegmatis* in the aqueous cream form. None of the extracts had activity against *S. aureus* when added to the aqueous cream solution. All extracts displayed activity against *M. smegmatis* when the extract alone was tested, confirming their activity in liquid and solid conditions. However, all extracts also displayed activity against *S. aureus*, when only the *M. excelsa* and *P. colorata* extracts displayed antibacterial activity against *S. aureus* in the liquid assay.

| Plant Extract             | Condition       | Zone of inhibition (mm) |                  |
|---------------------------|-----------------|-------------------------|------------------|
|                           |                 | <i>M. smegmatis</i>     | <i>S. aureus</i> |
| <i>L. novae-zelandiae</i> | Bark + Cream    | 0                       | 0                |
|                           | Bark            | 1                       | 2                |
|                           | Cambium + Cream | 0                       | 0                |
|                           | Cambium         | 1                       | 4                |
| <i>M. excelsa</i>         | Leaf + Cream    | 2                       | 0                |
|                           | Leaf            | 5                       | 4                |
| <i>P. colorata</i>        | Leaf + Cream    | 0                       | 0                |
|                           | Leaf            | 0                       | 10               |
| Aqueous Cream             | -               | 0                       | 0                |
| Rifampin                  | -               | 10                      | 18               |

**Table 5.2.4. Inhibitory activity of extracts in a cream-based solution.** Active extracts from the plants *L. novae-zelandiae*, *M. excelsa* and *P. colorata* were applied to an aqueous cream and tested against *M. smegmatis* and *S. aureus* for any inhibitory activity in a cream-based solution. Listed are the zones of inhibition (mm) surrounding the discs.

## 5.3 Discussion

### 5.3.1 Purification techniques

The proposition was that the purification techniques used would improve the activity of the bioactive extracts. Due to the harsh properties of the polar solvents used for extraction, the crude extracts had to be resuspended in SDW before the purification techniques were performed. Size exclusion fractionation was employed so that the large compounds with no activity in an extract were removed, thus purifying and increasing the potency of the active compounds. This could be due to an increase in relative concentration of the active compound, or perhaps the reduction of unwanted molecules, allowing 'easier' access to the active compounds target. However, the *L. novae-zelandiae* bark extract displayed similar activity across all fractions. A possible explanation was that the active constituent in the sample was a small compound (less than 3 kDa) that got 'trapped' with the larger, bulkier compounds in the filters. There was also the possibility of synergistic activity occurring, when more than one compound was working concurrently in the extract. If said compounds were of different size range then levels of activity would decrease. Overall, the use of size exclusion fractionation did not show a marked increase in antimycobacterial activity, but it did give an indication as to the general size of the bioactive compounds.

The purification techniques did improve the activity of the crude extracts when they were resuspended in SDW. The bark of *L. novae-zelandiae* was the only extract to display bactericidal activity with any of the purification techniques, notably size exclusion filtration combined with SPS of EtOAc.



However, there was no significant improvement in activity compared to the crude extract (See Table 4.2.1).

Speculation suggests that the loss in antimycobacterial activity was due to the resuspension in SDW. Thus, the chemical environment was changed and therefore disrupted the nature of the bioactive components present in the extract, similar to that of Le Chatelier's Principle [164]. It proposes that if something is altered in the environment, there will be a reaction to make up for that change. *P. colorata* (Horopito) is already marketed for antibacterial purposes as a crude extract, demonstrating that perhaps in order to retain activity the plant extracts are best kept as close to the original state as possible.

The  $\text{CHCl}_3$  layer displayed antimycobacterial activity for seven of the active extracts. The  $\text{CHCl}_3$  layer could consequently provide some novel compounds that could be isolated for further chemical analysis, provided the compounds remain stable in a purified state.

Overall, it appears the purification techniques did not improve the extracts activity in its original solvent. The bark of *L. novae-zelandiae* and leaf of *M. laetum* were AQ extracts that did not have improved activity, instead they had decreased activity upon purification. This suggests that the purification techniques were not suitable for AQ extracts, possibly due to their inorganic nature. Future purification work would involve chemical analysis with more organic solvents.

### 5.3.2 Practical Application

It was investigated as to whether the extracts from *L. novae-zelandiae*, *M. excelsa*, and *P. colorata* could be delivered in a feasible way. The advantage of this application was that the extracts could be used in a crude state, rather than having to work with an individual compound, as many skin cream products use natural extracts. It appeared that when the extracts were added to the aqueous cream, the extracts lost activity against both species tested, with the exception of the leaf of *M. excelsa*. This could possibly be due to the cream interfering with the plant extracts activity. However, it could also have been due to the low concentrations of extract in the aqueous cream, due to limitation of sample quantity available. What was interesting, however, was the inhibition exhibited by both *L. novae-zelandiae* against *S. aureus*, when none was previously exhibited in the liquid bacteriostatic assay. It could be speculated that the *L. novae-zelandiae* extracts had activity against *S. aureus* when the bacteria were grown on solid media than when in culture, suggesting a possible different mode of inhibition occurring of the extracts activity against *S. aureus*.

*M. excelsa* leaf extract displayed activity against *M. smegmatis* in an aqueous cream based solution as well as when tested with extract alone. This was interesting as the extract was resuspended in SDW and has been previously shown to have a drastic reduction in activity when in the extract is in an AQ solution. It could be speculated that the activity of the *M. excelsa* leaf was better in a more solid-based condition. In addition, the bark of *L. novae-zelandiae* was considered a more likely candidate for activity against *M. smegmatis* in the cream form, yet it did not display antimycobacterial

activity when added to the aqueous cream. However, its activity may be dependent on a liquid-based solution. Overall, other factors have to be included in order to create a cream-based product with marketable activity. There is the possibility of combining the active extracts, (*L. novae-zelandiae*, *M. excelsa* and *P. colorata*) together in an aqueous cream. It could be feasible to apply these active extracts in a practical delivery system for antibacterial treatment.

# CHAPTER SIX: Summary and Future Directions

## 6.1 Summary of Research

There is now more emphasis on analysing natural products as sources of novel drugs. The objective of this research was to test native NZ plants for antimycobacterial activity. This involved building a library of native NZ plants, developing a protocol for extracting the potential antitubercular compounds and then testing them against mycobacterial species. A library of 58 native NZ samples from 46 plant species was compiled from various locations and solvent extraction performed to give a total of 174 extracts. Drawing on knowledge from Rongoa Māori, nine extracts from seven native plant species demonstrated activity against *M. smegmatis* in the initial screen. Further validation led to the identification of eight extracts from *L. novae-zelandiae*, *L. bullata*, *M. excelsa*, *P. tenuifolium*, *P. crassifolius* and *P. colorata* that had activity against the clinically relevant slow-growing species *M. bovis* and *M. tuberculosis* H37Ra, suggesting the presence of potential antitubercular compounds. Extracts were also tested against *S. aureus* and *E. coli* for potential antibacterial activity and it was found that the activity suggested specificity to the mycobacteria. In addition, the extracts were non-toxic to murine macrophages and human cell-lines compared to the current TB drug RIF, thus indicating these extracts are worthy candidates for further research.

This study also involved improving the efficiency of extraction as well as investigating a potential delivery system of the active extracts. The efficiency of compound extraction from the plant's tissue was somewhat improved with the various purification techniques used. It was intended that the bioactive compounds have improved antimycobacterial activity

upon purification, but it was found that this was generally not the case. In terms of a practical application such as a skin cream, this was a feasible proposal, as many natural remedies include natural products that are effective in their delivery and efficacy. The results showed that the extract lost activity instead when applied in an aqueous cream; however, other delivery systems of the bioactive extracts could still be explored. For example, the extracts could be taken as an herbal supplement in pill form.

There is an increased interest in identifying the compounds responsible for the anti-mycobacterial activity of plants and developing them as potential new TB drugs. With a high endemic rate of diverse NZ flora this research provides a starting point for discovering unique bioactive extracts that could lead to novel antitubercular drugs, using traditional Māori medicine as a basis for selection.

The flower of *M. excelsa* and leaf of *P. tenuifolium* exhibited substantial bacteriostatic and bactericidal activity against mycobacteria, which could relate to their traditional use in Rongoa Māori. The bark of *L. novae-zelandiae* (Pukatea) was used in traditional medicine for the treatment of TB, correlating with the extracts antimycobacterial activity. For that reason the use of ethnomedicine (Rongoa Māori) was an effective method of sample selection. Furthermore, both the bark and cambium extracts of Pukatea had significant bactericidal activity against mycobacteria, signifying that Pukatea contains potential novel antitubercular compounds. Compounds such as pukateine from *L. novae-zelandiae*, ellagic acid from *M. excelsa* and bullatenone from *L. bullata* could provide insight into the type of activity in their plant extracts. Therefore, the bark of *L. novae-*

*zelandiae*, leaf and flower of *M. excelsa* and leaf of *L. bullata* are excellent candidates for future chemical analysis for the development of novel drugs to help combat TB.

## 6.2 Critique of the Research

Although many drugs today are derived from plants there are limitations to researching natural extracts as a source of lead compounds. It is expensive in time and funding to extract, screen and separate compounds with potent antimycobacterial activity that merit further analysis. Often there is low yield of the desired compound from the extract, or it is not stable in a purified state. The fractionation process was based on the notion that the active compound in the purified extract was simple and stable, retained activity and did not have synergistic activity with other compounds in the extract [49, 150]. There was also the possibility that the compound was not as active *in vivo* compared to activity *in vitro*. With these potential setbacks the potential lead compounds are then less likely to make it to clinical drug trials. For this reason there are still many plants with potential activity that have yet to be properly assessed as sources of lead compounds.

However, it can be speculated that plants that have been used in traditional medicine are more likely to contain potential lead compounds as they have medicinal properties and are likely to be non-toxic to the patient. Furthermore, in traditional medicine crude extracts are used and therefore the bioactive compound must be reasonably effective if the patient receives a low dosage.

Another issue is that even if the bioactive compound is simple and retains activity in an isolated form, it may not be novel and could already have been marketed [49]. Although some compounds have already been identified to be present in other plants (e.g. polygodial in Horopito), it is thought that NZ plants are still likely to contain novel compounds due to the diverse flora and its isolation from other countries. Some compounds already identified have been found to be unique to NZ plants, e.g. Pukateine in Pukatea, Bullatenone in Ramarama and Ngaione in Ngaio. So there is a significant possibility that compounds pertaining to the NZ bioactive extracts activity are not novel.

### **6.3 Future Directions**

There is a growing interest in identifying the compounds responsible for the antimycobacterial activity of extracts used in traditional medicines and developing them as potential new TB drugs. Future studies should therefore focus on isolating the bioactive compounds in extracts with validated antimycobacterial activity, such as Pukatea. This can be done by means of Mass Spectrometry (MS), High-Pressure-Liquid-Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR). HPLC is a rapid and concise technique, providing accurate results with high specificity and good resolution [165, 166]. Bioassay-guided fractionation could be applied in order to ascertain the active constituents in a crude extract [150]. Mass spectrometry would be applied for broad identification of chemical composition of compounds present, whereas NMR would be employed in order to elucidate the structure of novel compounds with



activity. NMR is an effective method as it can quickly identify unique structures with few resources needed for analysis [49]. These active compounds would then be tested against *M. tuberculosis* H37Ra *in vitro* to ensure bacteriostatic and bactericidal activity is retained. The compounds would also be analysed *in vivo* against the virulent strain such as *M. tuberculosis* H37Rv to confirm antimycobacterial activity in an infected host.

Once the compounds of interest have been determined future studies would involve synthesizing derivatives of the compound with the purpose of improving efficacy or decreasing cytotoxicity. It would be desirable to isolate an active compound that has a simple structure, thus allowing basic derivatives to be manufactured. It would also be beneficial to have a novel mechanism of action (MOA) so as to decrease the possibility of cross-resistance with current TB drugs [167].

As the activity of the extracts in the application of a skin cream was ineffective, other delivery systems could still be investigated. The bioactive extracts could be applied in the form of an aerosol spray, or the extracts could be ingested as an herbal supplement. This is a successful application for Kolorex® (New Zealand), which markets kolorXtract™, herbal capsules and teas containing extracts of *P. colorata* which are used to help maintain intestinal microflora [168]. Nutraceuticals are becoming a promising area of research, introducing herbal supplements to the drug market rather than individual compounds [169]. Therefore, NZ plant extracts could be investigated as a source of adjuvants for TB drugs. A medical dictionary describes an adjuvant as an agent that increases the

efficacy of an already existing drug without increasing its cytotoxicity. The drug Dzherelo™ (Immunoxel) is an herbal adjuvant for TB and is derived from a combination of aqueous-alcohol plant extracts including Aloe, Yarrow, Nettle, Dandelion, Marigold, Wormwood, and Juniper fruit [170, 171]. Dzherelo™ is a phytoconcentrate of plant extracts and has shown to increase the efficacy of TB drugs in recent clinical trials [170]. The trials also indicated that the herbal adjuvant decreased cytotoxic damage caused by current TB drugs as well as decreased treatment time. This clinical drug trial also included individuals infected with MDR-TB as well as individuals co-infected with HIV [170-173]. Thus, the addition of Dzherelo™ produced an overall enhanced TB therapy by reducing treatment time and treating drug-resistant cases. Therefore, the bioactive extracts could be tested in combination with current TB drugs and establish if there is improved efficacy and/or decreased toxicity.

Future experiments could also involve bacteriostatic assays against other clinically important organisms such as *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. As Rongoa Māori was an effective form of plant selection it could be used as a starting point for studying the bioactivity of native plants against other bacteria. With many unique plants that were used in Rongoa Māori the potential medicinal value of NZ flora warrants further investigation.

In conclusion, with the use of Rongoa Māori this study identified seven native plant species with antimycobacterial activity, two of which contained antibacterial properties. In particular, the antimycobacterial activity of the bark extract of Pukatea justified its traditional use in Māori medicine. Overall, the native bioactive extracts in this research could be of an interest in drug discovery and offer an excellent source of potential novel anti-tubercular compounds.

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## APPENDICES

### **Appendix 1: Research article in BMC Complementary and Alternative Medicine**

Results from the initial screen of the plant extracts against *M. smegmatis* (Section 3.2.6) and the screening against clinically-relevant species (Section 4.2.1) were peer-reviewed and published in the journal BMC Complementary and Alternative Medicine (2010), 10(1):25. Below is the research article from the journal.

## RESEARCH ARTICLE

## Open Access

# Native New Zealand plants with inhibitory activity towards *Mycobacterium tuberculosis*

Emma A Earl<sup>1</sup>, Mudassar Altaf<sup>1</sup>, Rekha V Murikoli<sup>1</sup>, Simon Swift<sup>2</sup> and Ronan O'Toole\*<sup>1</sup>**Abstract**

**Background:** Plants have long been investigated as a source of antibiotics and other bioactives for the treatment of human disease. New Zealand contains a diverse and unique flora, however, few of its endemic plants have been used to treat tuberculosis. One plant, *Laurelia novae-zelandiae*, was reportedly used by indigenous Maori for the treatment of tubercular lesions.

**Methods:** *Laurelia novae-zelandiae* and 44 other native plants were tested for direct anti-bacterial activity. Plants were extracted with different solvents and extracts screened for inhibition of the surrogate species, *Mycobacterium smegmatis*. Active plant samples were then tested for bacteriostatic activity towards *M. tuberculosis* and other clinically-important species.

**Results:** Extracts of six native plants were active against *M. smegmatis*. Many of these were also inhibitory towards *M. tuberculosis* including *Laurelia novae-zelandiae* (Pukatea). *M. excelsa* (Pohutukawa) was the only plant extract tested that was active against *Staphylococcus aureus*.

**Conclusions:** Our data provide support for the traditional use of Pukatea in treating tuberculosis. In addition, our analyses indicate that other native plant species possess antibiotic activity.

**Background**

Tuberculosis (TB) is the leading cause of death due to a single infectious organism [1]. In 2007, 1.78 million people died from the disease and an estimated 9.27 million new cases were recorded worldwide [2]. TB requires a lengthy treatment period of six months with the first-line drugs rifampicin, isoniazid, ethambutol and pyrazinamide [3]. The availability of new drugs that shortened the course of chemotherapy would improve patient adherence and affordability thus, enabling more favourable treatment outcomes. In addition, alternative drugs are needed to counteract the spread of drug resistant TB which threatens global control programmes [4]. MDR-TB, resistant to rifampicin and isoniazid, now exceeds 0.5 million cases per year [2] and in some states accounts for up to 22% of TB cases [2,5]. Extensively-drug resistant (XDR) strains of *M. tuberculosis*, resistant to both first- and second-line drugs, were first reported in the United

State, Latvia and South Korea in 2006 [6] but are now present in 57 countries [7].

The World Health Organisation has advised that new TB drugs are required to treat TB [8,9]. While much of the focus has been on screening libraries of synthetic compounds for mycobacterial inhibitors, plants have often provided a source of anti-bacterial compounds. For example, Aegicerin, isolated from *Clavija procera*, has been shown to have activity against *Mycobacterium tuberculosis* H37Rv and MDR-TB strains [10]. Rhodomyrtone, a compound from the Southeast Asian plant *Rhodomyrtus tomentosa* was found to have potent anti-bacterial activity against a number of clinically-important Gram-positive species including *Enterococcus faecalis*, *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA) [11].

In this work, solvent extracts were prepared from 45 native New Zealand plants and screened for activity using *Mycobacterium smegmatis*. Active samples were then tested against *Mycobacterium bovis* BCG, *M. tuberculosis* H37Ra, *Escherichia coli* and *Staphylococcus aureus*. A number of plants were identified as containing anti-*Mycobacterium tuberculosis* activity and their potential

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for generating novel compounds for the treatment of tuberculosis is discussed.

## Methods

### Bacterial strains and plasmids

The mycobacterial strains used were *Mycobacterium smegmatis* mc<sup>2</sup>155, *M. bovis* BCG (Pasteur) and *M. tuberculosis* H37Ra. *M. smegmatis* and *M. bovis* BCG were labelled with GFP through the introduction of the plasmid pSHIGH+hsp60 by electroporation and selection on media containing 50 µg/ml kanamycin as previously described [12]. GFP-labelled *Staphylococcus aureus* Newman was constructed as follows: the *fdh* (formate dehydrogenase) promoter was amplified from *S. aureus* Newman using Phusion™ Flash High-Fidelity PCR Master Mix (Finnzymes, Finland) according to the manufacturers' instructions with primers GGGGACAAC TTTGTATAGAAAAGTTGgaagggttaagtgtgataagc and GGGGACTGCTTTTTTGTACAAACTTgtctttaaataag aagttcattaattgttc, where uppercase represents *attB* sites for Gateway cloning (Invitrogen) and lower case represents *S. aureus* DNA. The 275 bp amplified product was cloned into pDONR221 using BP Clonase™ II (Invitrogen) and used to transform chemically-competent *Escherichia coli* TOP 10 (Invitrogen). The insert was sequenced to confirm an error-free amplification. Gateway cloning with LR Clonase™ II (Invitrogen) combined the *fdh* promoter with *gfp*, or *gfpluxABCDE*, and the *rrnBT12* terminator in the destination vector pDEST-pUNK1 as described previously [13]. The correct plasmid constructs from bioluminescent/fluorescent LR-reaction transformants of *E. coli* TOP 10 were confirmed by restriction mapping. The plasmids generated were electroporated into *S. aureus* RN4220 as previously described [14] and then transferred to *S. aureus* Newman by bacteriophage transduction [15]. *S. aureus* strains containing pDEST-pUNK1 derivatives were selected on media containing 30 µg/ml erythromycin. *Escherichia coli* DH5α was labelled with GFP using pOT11 [16].

### Plant Collection and solvent extraction

Plant samples were collected from the Karori Wildlife Sanctuary (Zealandia) and the Otari-Wilton Bush Reserve, Wellington; Kaitoke Regional Park, Upper Hutt; and the Nelson region with permission. The native New Zealand plants tested for anti-mycobacterial activity are listed in Table 1. Plant samples were collected fresh, macerated and dried in a desiccator. For each sample, approximately 1 g of dried plant tissue was placed in a 50 ml conical tube. Sterile distilled water, ethanol or methanol were added to the samples to give a final concentration of 100 mg/ml. The samples were incubated in a water bath at 55°C for 1 hour and then stored at -80°C. The incubation at 55°C was used as Māori heated plant extracts dur-

ing the preparation of some traditional medicines. The resulting crude extracts were sterilized using a 0.22 µm filter prior to anti-microbial analysis.

### Screening plant extracts for bacteriostatic activity towards *M. smegmatis*

Plant extracts were tested for bacteriostatic activity in a 96 well-plate format assay using *M. smegmatis* mc<sup>2</sup>155/pSHIGH+hsp60 as previously described [12]. Optical Density (OD) and GFP fluorescence were used to detect growth inhibition. *M. smegmatis* was cultured for 24 hours in Luria Broth (LB) supplemented with D-arabinose (1 mg/ml), Tween 80 (0.1% v/v) and kanamycin (50 µg/ml). Sterile distilled water (150 µL) was added to the outer lanes of the 96-well microtiter plates to minimise evaporation. Liquid media (50 µL) was added to the remaining wells. The effect of the solvents used on the growth of *M. smegmatis* was tested. Inhibition of *M. smegmatis* growth was observed at concentrations of ethanol and methanol greater than 5% (v/v). Plant extracts were added to columns 2 and 3 of the microtitre plates with a starting concentration of 2 mg/ml corresponding to a solvent concentration of 2% (v/v). A two-fold serial dilution was then performed on each extract starting at column 3. *M. smegmatis* cells were added to rows B-D at a final OD at 600 nm (OD<sub>600</sub>) of 0.2. Rows E-G contained media and extract alone, to measure any background optical density or fluorescence associated with the extract. A number of controls were added to ensure assay reliability, including the use of standard anti-tubercular drugs, rifampicin and streptomycin, as positive controls, each starting at a concentration of 100 µM. All extracts and controls were tested in triplicate. Plates were sealed and incubated at 37°C with 200 rpm shaking for 96 hours, after which the plates were read for OD and GFP fluorescence. For active extracts, dose response experiments were performed to validate activity versus *M. smegmatis*.

### Bacteriostatic assays for *M. bovis*, *M. tuberculosis*, *S. aureus* and *E. coli*

*M. bovis* BCG and *M. tuberculosis* H37Ra cultures were grown in Middlebrook 7H9 broth supplemented with 10% (v/v) OADC (0.06% oleic acid, 5% BSA, 2% Dextrose, 0.85% NaCl), glycerol (0.5% v/v) and Tween 80 (0.05% v/v). A bacteriostatic assay was set up as previously described [12], with cells added to give a final OD<sub>600</sub> of 0.05. All extracts and controls were tested in triplicate. Plates were sealed and incubated at 37°C with 200 rpm shaking for 14 days before the GFP fluorescence and/or OD of the cultures were measured.

GFP-labelled *S. aureus* Newman was cultured in LB containing erythromycin (30 µg/ml) and xylose (0.5% w/v). GFP-labelled *E. coli* DH5α/pOT11 was cultured in LB containing chloramphenicol (25 µg/ml). IPTG (100 µg/



**Table 1: Native New Zealand plants screened for activity against *M. smegmatis*.**

| Plant name (common)                                  | Part       | Documented Traditional Medicinal Use  |
|--|------------|---|
| <i>Adiantum raddianum</i> (Delta maidenhair fern)    | Leaf       | <i>ni</i>   |
| <i>Agathis australis</i> (Kauri)                     | Leaf       | <i>ni</i>   |
| <i>Arthropodium cirratum</i> (New Zealand rock lily) | Leaf       | Ulcers [29]   |
| <i>Asplenium bulbiferum</i> (Hen and chicken fern)   | Leaf       | <i>ni</i>   |
| <i>Beilschmiedia tawa</i> (Tawa)                     | Leaf       | Sore throat, colds, cough [22]  |
| <i>Blechnum fluviatile</i> (Kiwikiwi)                | Leaf       | Sore tongue and mouth [30]  |
| <i>Brachyglottis repanda</i> (Rangiora)              | Leaf       | Wounds, ulcers and boils [30]   |
| <i>Carpodetus serratus</i> (Marble leaf)             | Leaf       | <i>ni</i>   |
| <i>Clianthus puniceus</i> (Kakabeak)                 | Leaf       | <i>ni</i>   |
| <i>Cordylina australis</i> (Cabbage tree)            | Leaf       | Dysentery, diarrhoea and sores [30]   |
| <i>Cortaderia toetoe</i> (Toetoe)                    | Shoot      | Diarrhoea, bladder complaints [23,31]   |
| <i>Cyathea dealbata</i> (Ponga)                      | Fronds     | Treat boils [32]  |
| <i>Dacrydium cupressinum</i> (Rimu)                  | Leaf       | Treat sores [22]  |
| <i>Dracophyllum longifolium</i> (Inanga)             | Leaf       | <i>ni</i>   |
| <i>Dodonaea viscosa</i> (Ake ake)                    | Leaf       | Treat sore throats and skin irritations [33]                                    |
| <i>Dysoxylum spectabile</i> (Kohekohe)               | Leaf       | Relieve coughing, colds and fevers [33]   |
| <i>Exocarpus bidwillii</i>                           | Leaf       | <i>ni</i>   |
|  | Seed       | <i>ni</i>   |
| <i>Hebe stricta</i> (Koromiko)                       | Leaf       | Skin problems, ulcers, diarrhoea, dysentery, kidney and bladder complaints [33] |
|  | Flower     | <i>ni</i>   |
| <i>Hymenophyton flabellatum</i> (Liverwort)          | Leaf       | <i>ni</i>   |
| <i>Knightia excelsa</i> (Rewarewa)                   | Leaf       | Relieve coughing [22]   |
| <i>Kunzea ericoides</i> (Kanuka)                     | Leaf       | Relieve pain, headaches and coughs [22]   |
| <i>Laurelia novae-zelandiae</i> (Pukatea)            | Inner bark | Sores, ulcers, toothache and tuberculosis [23,30,32]                            |
|  | Bark       | Treat sores, skin conditions, venereal disease [29]                             |
|  | Leaf       | Toothache [22]  |
| <i>Leptospermum scoparium</i> (Manuka)               | Leaf       | Colds, pains, urinary troubles [30]   |
|  | Flower     | <i>ni</i>   |
| <i>Lophomyrtus bullata</i> (Ramarama)                | Leaf       | Treat bruises [22]  |
| <i>Macropiper excelsum</i> (Kawakawa)                | Leaf       | Rheumatism, arthritis, diuretic, bruises and chest difficulties [32,34]         |
| <i>Meliccytus ramiflorus</i> (Mahoe)                 | Leaf       | Treat rheumatism and stomach wounds [30]  |
| <i>Meryta sinclairii</i> (Puka)                      | Leaf       | <i>ni</i>   |
| <i>Metrosideros excelsa</i> (Pohutukawa)             | Leaf       | <i>ni</i>   |
|  | Flower     | Sore throats [23,30]  |
| <i>Metrosideros robusta</i> (Northern Rata)          | Leaf       | <i>ni</i>   |
| <i>Metrosideros umbellata</i> (Southern Rata)        | Leaf       | <i>ni</i>   |
| <i>Myoporum laetum</i> (Ngaio)                       | Leaf       | Bruises and infected wounds [30]  |
|  | Leaf       | Bruises and wounds  |
| <i>Nothofagus fusca</i> (Red beech)                  | Leaf       | <i>ni</i>   |
| <i>Paesia scaberula</i> (Lace fern)                  | Leaf       | <i>ni</i>   |



**Table 1: Native New Zealand plants screened for activity against *M. smegmatis*. (Continued)**

|   |      |   |
|---|------|---|
| <i>Phormium tenax</i> (New Zealand flax)      | Leaf | Treat cuts and constipation [28]                      |
| <i>Pittosporum tenuifolium</i> (Kohuhu)       | Leaf | Eczema and other skin diseases, fever and chills [22] |
| <i>Plagiochila stephensoniana</i> (Liverwort) | Leaf | <i>ni</i>   |
| <i>Pseudopanax arboreus</i> (Five finger)     | Leaf | <i>ni</i>   |
| <i>Pseudopanax crassifolius</i> (Lancewood)   | Leaf | <i>ni</i>   |
| <i>Pteridium esculentum</i> (Bracken)         | Leaf | Antiseptic, treats sores [33]                         |
| <i>Rubus cissoides</i> (Bush lawyer)          | Leaf | Chest congestion, coughs and sore throats [30]        |
| <i>Schefflera digitata</i> (Seven finger)     | Leaf | <i>ni</i>   |
| <i>Sophora microphylla</i> (Kowhai)           | Leaf | Wounds, back, abdominal and internal pains [32]       |
| <i>Urtica ferox</i> (Stinging nettle)         | Leaf | <i>ni</i>   |
| <i>Vitex lucens</i> (Puriri)                  | Leaf | Backache, ulcers, and sore throats [30]               |
| <i>Weinmannia racemosa</i> (Kamahi)           | Leaf | <i>ni</i>   |

*ni* = no information available.

ml) was added to induce GFP expression. The bacteriostatic assay for *S. aureus* and *E. coli* were performed as per the *M. smegmatis* bacteriostatic assay method except cultures were incubated for 24 hours at 37°C with 200 rpm shaking post addition of extracts.

#### Determination of inhibitory concentrations of plant extracts

A Perkin Elmer Envision 2102 multilabel plate reader and the Wallace Envision Manager 1.12 software program were used to measure the OD and GFP signals of the microtitre plate cultures. OD was measured at 600 nm. GFP fluorescence was detected using excitation and emission wavelengths of 485 nm and 510 nm, respectively. 12-point scans were performed on each well to minimise intra-well variation. The intrinsic absorbance and fluorescence readings of extracts alone were measured to account for background signal and subtracted from the readings for the test samples. Data were normalised by expressing the absorbance and fluorescence values as a percentage of a no-drug negative control. Dose-response curves were plotted using SigmaPlot (version 10.0) and minimum inhibitory concentration (MIC) and 50% inhibitory concentration (IC<sub>50</sub>) values were calculated.

## Results

### Activity of plant extracts towards *M. smegmatis*

45 plants native to New Zealand (Table 1) were extracted with water, ethanol and methanol and the extracts were tested for their ability to inhibit the growth of the fast-growing species, *M. smegmatis*. Extracts from 6 plants species, *Laurelia novae-zelandiae*, *Lophomyrtus bullata*, *Metrosideros excelsa*, *Myoporum laetum*, *Pittosporum tenuifolium* and *Pseudopanax crassifolius* showed inhibi-

tion towards *M. smegmatis* (Table 2). Dose-response experiments were performed on the active extracts and their MIC and IC<sub>50</sub> values were determined. The most active extract was derived from *L. novae-zelandiae* (Pukatea). The bark of *L. novae-zelandiae* generated an IC<sub>50</sub> value of 0.02 mg/ml, respectively while the cambium had an IC<sub>50</sub> of 0.25 mg/ml (Table 3). Significant activity was also observed with respect to the leaf, IC<sub>50</sub> of 0.11 mg/ml, and flower, IC<sub>50</sub> of 0.41 mg/ml, of *M. excelsa*. The leaf of *P. tenuifolium* was less active with an IC<sub>50</sub> value of 0.78 mg/ml (Table 3).

### Antibacterial activity of plant extracts towards clinically-relevant species

The extracts of *L. novae-zelandiae*, *L. bullata*, *M. excelsa*, *M. laetum*, *P. tenuifolium* and *P. crassifolius* were tested against *M. bovis* BCG and *M. tuberculosis* H37Ra. The leaf of *P. tenuifolium* was the most active extract with respect to *M. tuberculosis* with an IC<sub>50</sub> of 0.51 mg/ml (Table 3). The bark taken from *L. novae-zelandiae* had an IC<sub>50</sub> of 0.54 mg/ml against *M. bovis* and an IC<sub>50</sub> of 2.39 mg/ml against *M. tuberculosis* (Table 3). The cambium of *L. novae-zelandiae* was similarly active against both *M. bovis* and *M. tuberculosis*. The leaf extract from *M. laetum* was not active against *M. tuberculosis*. To examine the specificity of the anti-bacterial activity of the plant extracts, the extracts were also tested against *S. aureus* and *E. coli*. The leaf of *M. excelsa* which was active against *M. smegmatis* and to a lesser extent, *M. tuberculosis*, also displayed antibacterial activity towards *S. aureus* with an IC<sub>50</sub> of 1.17 mg/ml. None of the extracts tested exhibited anti-bacterial activity towards *E. coli* up to the 10 mg/ml concentration tested.

**Table 2: Anti-bacterial activity of plant extracts towards *M. smegmatis* mc<sup>2</sup>155.**

| Plant species                   | Common Name | Part    | Sample location | Extract |         |          |
|---------------------------------|-------------|---------|-----------------|---------|---------|----------|
|                                 |             |         |                 | Aqueous | Ethanol | Methanol |
| <i>Laurelia novae-zelandiae</i> | Pukatea     | Bark    | Otari-Wilton    | +       | -       | -        |
|                                 |             | Cambium | Otari-Wilton    | -       | -       | +        |
| <i>Lophomyrtus bullata</i>      | Ramarama    | Leaf    | Otari-Wilton    | -       | -       | +        |
| <i>Metrosideros excelsa</i>     | Pohutukawa  | Leaf    | Ngaio           | -       | -       | +        |
|                                 |             | Flower  | Ngaio           | -       | +       | -        |
| <i>Myoporum laetum</i>          | Ngaio       | Leaf    | Ngaio           | +       | -       | -        |
| <i>Pittosporum tenuifolium</i>  | Kohuhu      | Leaf    | Ngaio           | -       | +       | -        |
| <i>Pseudopanax crassifolius</i> | Lancewood   | Leaf    | Otari-Wilton    | -       | -       | +        |

'+' indicates  $\geq 90\%$  inhibition of *M. smegmatis* growth at a concentration of 2 mg/ml or lower.

### Discussion

Plants and their extracts have been used by indigenous peoples for the treatment of infectious diseases such as tuberculosis since long before the discovery of antibiotics. A recent study determined that more than 80 plant species have been used by traditional medical practitioners in Uganda to treat tuberculosis [17]. Another study characterised leaf, bark and root material from trees used as traditional anti-tubercular medicines in South Africa. Direct bacteriostatic activity towards *Mycobacterium aurum* was identified for *Acacia nilotica* and *Combretum kraussii* at concentrations ranging from 1.56 to 0.195 mg/ml [18]. A survey of the ethnobotanical literature was used to select plants used by traditional healers in Mexico to test for activity towards *M. tuberculosis*. Extracts of *Citrus aurantiifolia*, *C. sinensis* and *Olea europaea* were

found to be active against both drug-susceptible and drug-resistant strains of virulent *M. tuberculosis* with minimum inhibitory concentrations of between 0.1 and 0.025 mg/ml [19]. Although, the New Zealand shrub *Lophomyrtus bullata* (Ramarama) has been identified as having activity towards *Bacillus subtilis* [20], to current knowledge, Pukatea or other New Zealand plants have not been assayed for activity against mycobacteria [21].

In New Zealand, the bark of *Laurelia novae-zelandiae* (Pukatea) (Figure 1A) was used by Māori in a number of medicinal remedies but it was especially noted for its use against tubercular lesions [22,23]. The pulp of the cambium was boiled in water and the resulting liquid used for treating tubercular ulcers. The timber of Pukatea was also used by Māori to create figureheads for canoes [24]. Pukatea is generally found in lowland forests in the North

**Table 3: MIC and IC<sub>50</sub> values of active plant extracts with respect to *M. smegmatis* and clinically-relevant bacterial species.**

| Plant                     | Part    | <i>M. smegmatis</i> |                  | <i>M. bovis</i> |                  | <i>M. tuberculosis</i> |                  | <i>S. aureus</i> |                  |
|---------------------------|---------|---------------------|------------------|-----------------|------------------|------------------------|------------------|------------------|------------------|
|                           |         | mg/ml               |                  | mg/ml           |                  | mg/ml                  |                  | mg/ml            |                  |
|                           |         | MIC                 | IC <sub>50</sub> | MIC             | IC <sub>50</sub> | MIC                    | IC <sub>50</sub> | MIC              | IC <sub>50</sub> |
| <i>L. novae-zelandiae</i> | Bark    | 0.04                | 0.02 ± 0.01      | 1.50            | 0.54 ± 0.03      | 4.16                   | 2.39 ± 0.33      | > 10             | > 10             |
|                           | Cambium | 0.50                | 0.25 ± 0.01      | 2.50            | 1.63 ± 0.21      | 3.75                   | 1.86 ± 0.22      | > 10             | > 10             |
| <i>L. bullata</i>         | Leaf    | 1.00                | 0.31 ± 0.15      | 3.12            | 2.59 ± 0.43      | 6.51                   | 5.59 ± 0.32      | > 10             | > 10             |
| <i>M. excelsa</i>         | Leaf    | 0.63                | 0.11 ± 0.06      | 8.35            | 6.95 ± 3.07      | > 10                   | 4.83 ± 0.43      | 2.20             | 1.17 ± 0.39      |
|                           | Flower  | 0.63                | 0.41 ± 0.06      | 3.13            | 1.12 ± 0.12      | 4.18                   | 2.19 ± 0.76      | > 10             | > 10             |
| <i>M. laetum</i>          | Leaf    | 0.63                | 0.34 ± 0.02      | > 10            | > 10             | > 10                   | > 10             | > 10             | > 10             |
| <i>P. tenuifolium</i>     | Leaf    | 2.00                | 0.78 ± 0.34      | > 10            | 5.37 ± 1.35      | 1.25                   | 0.51 ± 0.11      | > 10             | > 10             |
| <i>P. crassifolius</i>    | Leaf    | 0.63                | 0.31 ± 0.01      | 6.25            | 4.08 ± 1.76      | 3.58                   | 1.74 ± 0.18      | > 10             | > 10             |

MIC, minimum inhibitory concentration. IC<sub>50</sub>, 50% inhibitory concentration.

Island and the northern areas of the South Island [25]. It can grow up to 35 metres and produces plank buttresses (Figure 1B) to support the tree's growth in swamp or shallow-soil areas [25]. Pukatea has so-called 'toothed' leaves (Figure 1C) and produces small flowers [25].

In this work, we characterised Pukatea and 44 other native plants for activity towards *M. smegmatis*. Extracts from six New Zealand plants were found to have activity against *M. smegmatis*. Bark and cambium samples from Pukatea were the most active against *M. smegmatis* (Table 3). Furthermore, the bark and cambium extracts were also active against *M. tuberculosis*. The direct anti-mycobacterial activity of the Pukatea extract therefore correlates with the use of this plant by Māori to treat tubercular lesions. This strengthens the possibility that the bark of Pukatea contains previously-unknown anti-tubercular compound(s). In Māori medicine, *M. excelsa* was used in the treatment of sore throats. Although the traditional use of *M. excelsa* does not include the treatment of tuberculosis, extracts from *M. excelsa* were nevertheless active against *M. smegmatis* and *M. tuberculosis*. Similarly, the leaf of *P. tenuifolium*, used by Māori to treat skin infections, was active against *M. smegmatis* and *M. tuberculosis*. The anti-mycobacterial activities that were detected for *M. excelsa* and *P. tenuifolium* extracts could relate to their traditional use in anti-infec-

tive therapy. Of the extracts shown to have anti-mycobacterial activity, only the *M. excelsa* leaf extract displayed activity against *S. aureus*. Interestingly, none of the plant species tested exhibited antibacterial activity towards *E. coli*. It should be noted that the MIC and IC<sub>50</sub> values of the extracts in the different mycobacteria (Table 3) will be subject to variation due to species differences, the type of growth medium used, and the assay incubation time.

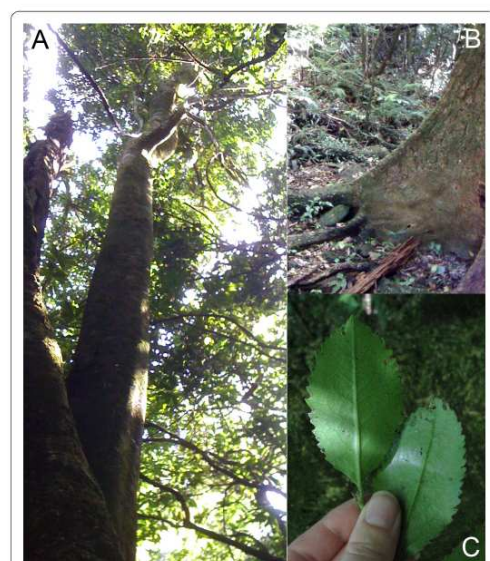
There is growing interest in identifying the compounds responsible for the anti-mycobacterial activity of traditional medicines and developing them as potential new tuberculosis drugs. Although the chemical basis for the anti-tubercular activity of Pukatea needs to be elucidated, earlier chemical studies identified a novel alkaloid, pukateine, from the bark of Pukatea. Pukateine is an analgesic which acts as a D<sub>2</sub> dopamine receptor agonist and α1 adrenergic receptor antagonist [26,27]. A number of the other plant extracts found to have activity against mycobacteria have also been analysed chemically. The flower of *M. excelsa* contains ellagic acid which is a polyphenol antioxidant [28]. Bullatenone, discovered in *L. bullata* (Ramarama), is an antiseptic [28]. Chemical derivatives of bullatenone have been found to have antimicrobial properties against *Bacillus subtilis* [20]. Ngaione, a hepatotoxic sesquiterpene, found in the leaves of *M. laetum* (Ngaio) has been used in the treatment of athlete's foot [28]. It is possible that compounds such as pukateine, ellagic acid, bullatenone or ngaione may contribute to the anti-bacterial activity of their plant extracts. These compounds offer a starting point for future studies to characterise the chemical basis of the anti-bacterial activity of *L. novae-zelandiae*, *M. excelsa*, *L. bullata* and *M. laetum*. They may also assist in identifying ways to improve the efficiency of the extraction of anti-mycobacterial activity from the plant samples and hence, provide extracts with lower MIC values towards *M. tuberculosis*. Such extracts will be integral to any bioassay-guided purification of active compounds.

### Conclusions

It is estimated that less than 10% of the Earth's higher plant species have been analysed for any kind of bioactivity [18]. There is a need for more research to be conducted on plants that have been traditionally used by indigenous peoples to treat tuberculosis, for the identification of new anti-tubercular drugs. New Zealand has a diverse flora which potentially offers many unique bioactive compounds. Drawing on knowledge from traditional medicine, we have identified a number of native plants which contain activity against *M. tuberculosis*. Determining the chemical species responsible for this activity will be the subject of further studies.

### Competing interests

The authors declare that they have no competing interests.



**Figure 1** Images of *Laurelia novae-zelandiae* (Pukatea) specimen located at Otari-Wilton Bush, Wellington. A, branchless trunk and canopy of Pukatea. B, plank buttress of Pukatea. C, examples of "toothed" leaves of Pukatea.



**Authors' contributions**

EAE collected the plant samples, performed the solvent extractions and antibacterial assays with *M. smegmatis*, *E. coli* and *S. aureus*. MA performed the antibacterial assays in the slow-growing mycobacteria, *M. bovis* BCG and *M. tuberculosis* H37Ra. RVM assisted in testing the potency of the plant extracts. SS constructed the GFP-labelled *S. aureus*. ROT conceived and funded the study, and planned and supervised the experimental work. All authors have read and approved the final manuscript.

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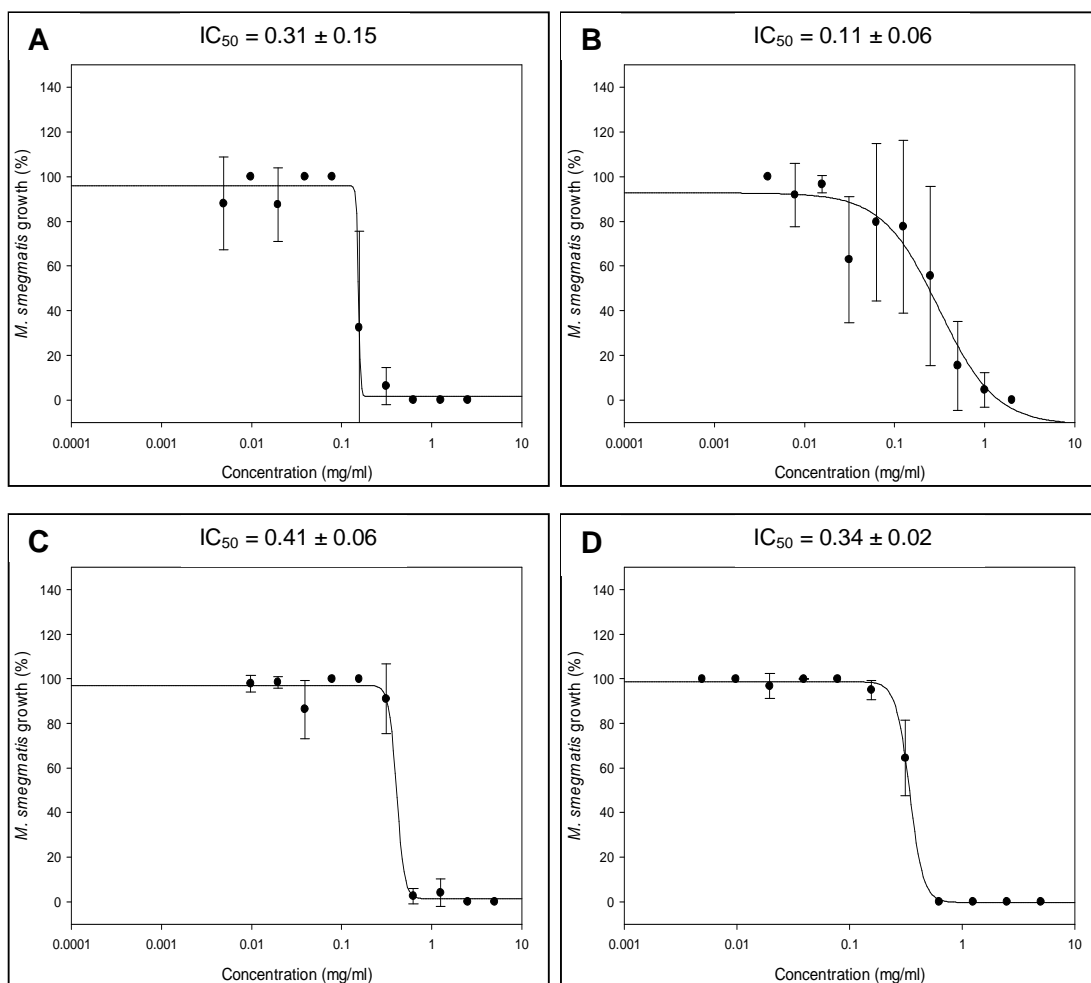
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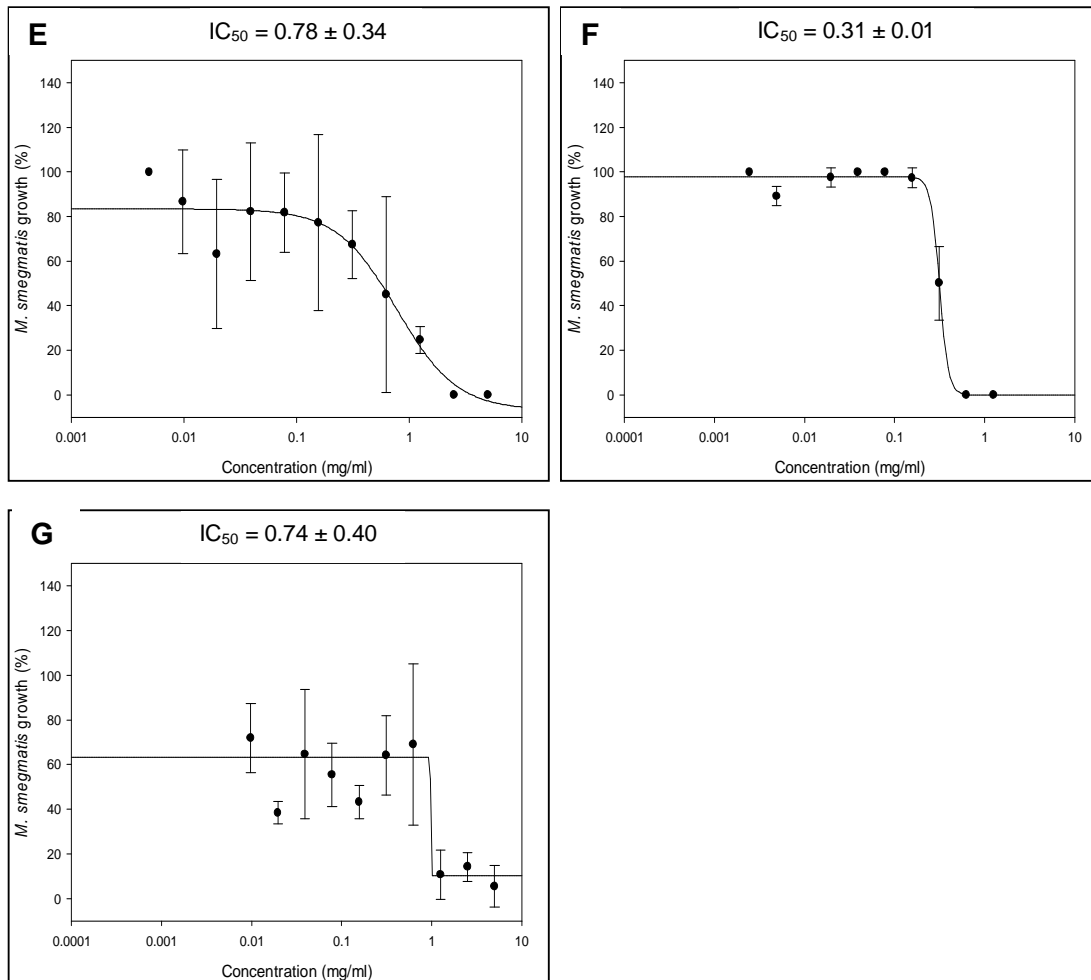
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**Appendix 2: Bacteriostatic inhibition curves of extracts with activity against *M. smegmatis***

Dose-response experiments were performed on the active extracts that were found to have  $\geq 90\%$  inhibition in the initial screen against *M. smegmatis*. Figure A.2 depicts the bacteriostatic curves for the active extracts from *L. bullata*, *M. excelsa*, *M. laetum*, *P. tenuifolium*, *P. crassifolius* and *P. colorata* species.





**Figure A.2. Bacteriostatic inhibition curves of active extracts against *M. smegmatis*.** **A)** MeOH leaf extract *L. bullata*, **B)** MeOH leaf extract of *M. excelsa*, **C)** EtOH flower extract of *M. excelsa*, **D)** AQ leaf extract of *M. laetum*, **E)** EtOH leaf extract of *P. tenuifolium*, **F)** EtOH leaf extract of *P. crassifolius*, **G)** MeOH leaf extract of *P. colorata*. Values are based on GFP results. 100% represents complete growth of *M. smegmatis* cells compared to the untreated control. Error bars denote standard deviation between triplicate values.