Stimulation of macrophage uptake and killing of mycobacteria by compounds derived from the roots of *Pelargonium* spp.

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This thesis describes research conducted in the School of Pharmacy, University of London between September 2003 and September 2006 under the supervision of Professor Peter Taylor. I certify that the research described as original and that any parts of the work that have been conducted in collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date 24.01.08
Acknowledgments

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Many thanks to my friends for being there all the time, I am so grateful to you … you know who you are.

Lastly I would like to say thank-you to my parents for their endless encouragement, patience and love through all the easy and hard times. I’m proud that you are my parents.
Abstract

Background: The development of new treatments for tuberculosis is a public health priority. We have shown that a South African native remedy for respiratory ailments, based on extracts of Pelargonium reniforme and P. sidoides, contain fatty acids with antimycobacterial activity. In order to further assess their potential as a supplement to conventional therapy, we examined root extracts of these plants for the capacity to stimulate the uptake and intracellular killing of mycobacteria by macrophages. Methods: Murine peritoneal macrophages were obtained by lavage and incubated with Mycobacterium fortuitum. Extract-mediated stimulation of bacterial uptake was determined by enumeration of stained intracellular bacteria; the effect of extracts on intracellular killing was evaluated by spread plate counting of bacteria within macrophages. Bioactive extracts, obtained by sequential extraction with solvents of increasing polarity, were subjected to bioassay-guided fractionation using size exclusion and ion exchange chromatography. HPLC, NMR and mass spectroscopy were used to identify compounds of interest. Active compounds were examined for their capacity to stimulate macrophage uptake of M. tuberculosis. Results: Root powders were sequentially extracted with hexane, ethyl acetate, ethanol, 50% ethanol/water and water; aqueous fractions had a greater capacity to stimulate uptake and killing of M. fortuitum compared to other fractions. P. reniforme extracts possessed significantly greater stimulating activity than P. sidoides extracts. Fractions obtained by bioassay-guided fractionation of P. reniforme aqueous extracts contained a small number of chemically distinct structures, the most prominent being gallic acid, methyl gallate, myricetin and quercetin 3-D-glucoside. Highly pure preparations of these compounds (2-25 μg/ml) stimulated macrophage killing of M. fortuitum and M. tuberculosis. Conclusion: Representatives of the plant genus Pelargonium elaborate metabolites that increase the capacity of mice peritoneal macrophages to internalize and kill mycobacteria, including M. tuberculosis.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guérin vaccine</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DOTS</td>
<td>Directly observed treatment strategy</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
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<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IEX</td>
<td>Ion exchange chromatography</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPT</td>
<td>Isoniazid preventative therapy</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multi-drug resistant tuberculosis</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>µl</td>
<td>Microlitre</td>
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<td>mg</td>
<td>Milligram</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>MS/MS</td>
<td>Mass spectrometry/mass spectrometry (tandem mass spectrometry)</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaPO₄</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>P. reniforme</td>
<td><em>Pelargonium reniforme</em></td>
</tr>
<tr>
<td>P. sidoides</td>
<td><em>Pelargonium sidoides</em></td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediates</td>
</tr>
<tr>
<td>ROI</td>
<td>Reactive oxygen intermediates</td>
</tr>
<tr>
<td>RR</td>
<td>Rhubarb root</td>
</tr>
<tr>
<td>PRW</td>
<td>Water Soxhlet extract of <em>P. reniforme</em></td>
</tr>
<tr>
<td>SA</td>
<td>Strong anionic chromatographic extract</td>
</tr>
<tr>
<td>SC</td>
<td>Strong cationic chromatographic extract</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid-phase extraction</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TB/HIV</td>
<td>Tuberculosis-human immunodeficiency virus co-infection</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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INTRODUCTION
1.1 Tuberculosis

Tuberculosis (TB) is an ancient disease; evidence for TB has been found in human bones determined to be over 3000 years old and the disease continues to have an impact on the human population. One-third of the world’s population is currently infected with the TB bacillus (WHO 2002). Annually, there are 2 million deaths and 8 million individuals are newly infected, with 2 million cases occurring in sub-Saharan Africa alone.

In the event of pulmonary tuberculosis, there can be massive damage to the lungs due to the action of the host’s immune response, whereby the high levels of cytokines and phagocytic cells that target the invading organism inadvertently destroy the lung tissue (Dannenberg, Jr. & Collins 2001). Alternatively, the destruction of the pleura surrounding the lungs by tubercular lesions allows the dissemination of the bacteria into the pleural cavity, again triggering the production of cytotoxic components involved in the immune system (Valdes et al. 2003). The accumulation of blood and fluid in the lungs can cause death or other opportunistic bacteria can cause life-threatening infections, such as bronchopneumonia (Alkuja & Miller 2001).

The Health Protection Agency determined the presentation of over 8000 new cases of TB in the United Kingdom in 2005, an increase of 11% over the previous year, with 72% occurring in non-United-Kingdom-born residents (Health Protection Agency 2006). Although the reporting of TB is a statutory requirement, the provision of epidemiological information can be incomplete (Pillaye & Clarke 2003), strongly suggesting that the rate of emergence of new cases identified by the World Health Organisation (WHO) is an underestimate. The high TB
incidence in regions such as sub-Saharan Africa adversely affects control programmes for both TB and other diseases, such as HIV (Corbett et al. 2006). Until recently, the drugs and regimens used to treat TB remained to a large extent unchanged over a period of around 40 years, but recently the invigoration of research activity aimed at discovering and developing new drugs and efforts to extend the use of existing antibiotics for the treatment of TB has resulted in improvements to TB chemotherapy (Check 2007). However, problems presented by the intracellular survival mechanism of TB, the long latency period of the disease and a limited number of cellular targets exploited by current drugs are factors that limit effective chemotherapy. The low level of establishment of clinical trials in countries with high TB infection rates and the difficulty in the translocation of animal model data to clinical human trials also have a negative impact on the development of new treatments (Ginsberg & Spigelman 2007).

These concerns notwithstanding, some effective strategies are in place to maintain control over the disease. The WHO has an objective to gain universal successful diagnosis and treatment of the disease and to reduce the burden of TB on human suffering and resources (WHO 2006). In the United Kingdom, the Chief Medical Officer has released an action plan to reduce the burden of TB in Britain by setting goals, such as increasing TB detection rates and providing information as to how these can be reached (Department of Health 2004).

Although infection with Mycobacterium tuberculosis (M. tuberculosi§), the causative agent of TB, is treatable, the infection carries a high mortality rate (Brewer & Heymann 2005) and therefore is used as a model for other infectious diseases in highlighting diagnosis, treatment and control (Aziz & Wright 2005). In the 1980s in the United Kingdom, it was thought that the disease was close to
eradication as a result of improvements in early detection, successful treatment, segregation of patients, vaccination and improvements in living conditions (NHS 2006). However, the incidence of TB is now rising, prompting the National Institute of Health and Clinical Excellence (NICE) to release documents and guidelines for the control of clinical TB. Developing on the guidelines proposed by the Department of Health report (2004), there is now a standardised protocol for health professionals guiding the diagnosis and treatment of patients (NICE 2006).

Although there are polices in place to control TB in most parts of the world, they are not or cannot be enforced, particularly in sub-Saharan Africa where poor resources limit the monitoring of patient treatment and adherence to the treatment regimen, leading to inadequate control and a failure to contain the infection (Harper et al. 2003).

1.2 Pathogenesis of Pulmonary TB

The cell-mediated immune response is a key line of defence for the elimination of the infectious agent from the human host (Teitelbaum et al. 1999). *M. tuberculosis* is a virulent organism, but it is not highly communicable. However, some carriers are more effective at spreading the disease than others and while factors determining TB carrier rates are not yet completely understood, some individuals may be more genetically predisposed to contracting TB (Secko 2005).

Only 10% of those infected with the bacillus develop the active form of the disease (Frieden et al. 2003). The immune system appears functional and may contain the infection, but the pathogen is not eliminated. The TB bacillus has the capacity to infect most regions of the body including major organs, but in the
majority of cases, TB bacilli are associated with the lungs (Grosset 2003), largely due to the route of infection adopted by the pathogen.

1.2.1 Identification

Although TB has existed for thousands of years, it was only in the mid-19th century that the causative organism was isolated and identified. In 1865, the scientist Villemin inoculated rabbits with material obtained from tubercular lesions and the sputum of TB patients to discover that a similar disease developed in the rabbits. Robert Koch then discovered the bacterium that caused TB in 1882 and the causal organism, which he named *M. tuberculosis* (*Bacterial Infections of Humans, Epidemiology and Control*, 3rd ed., 1998).

*M. tuberculosis* is a Gram-positive, rod-shaped bacterium that has a slow growth rate, a generation time of around 24 h and requires a 3–8-week incubation period for the formation of visible colonies. There is a period of acid-fastness during the growth cycle, due to the presence of mycolic acids at the cell surface, so an acid-fast stain can be used to identify the bacterial cells. The Ziehl–Neelsen stain was developed to identify mycobacterial cells by detecting the presence of mycolic acid associated with the cell wall.

1.2.2 Route of Infection

TB is usually spread by the airborne route, and the bacillus is rapidly taken up by macrophages in the lungs. Therapeutic problems generally arise due to the dormant state of bacilli within viable macrophages, rendering the pathogen resistant to immune attack. Minute particles (droplet nuclei), ranging in diameter from 1 to 5 µm, contain viable *M. tuberculosis* cells that can be transmitted from
person to person by sneezing, coughing and close contact. The primary stage of the infection involves inhalation of the droplets and the mycobacteria are phagocytosed by alveolar macrophages, inducing an immune response, the Th1 response: Th1 is the cellular arm of the immune response that uses specialised T-cells to recognise and destroy infected host cells. Activated macrophages release cytokines to attract T-lymphocytes and other cells of the immune system. Interactions between these cells promote further production of cytokines, inducing increased phagocytosis by macrophages.

The macrophages may kill some of the bacteria, but there are frequently survivors. The ingested mycobacteria are degraded and bacterial antigens liberated by the process are presented by major histocompatibility complexes (MHC) on antigen-presenting cells, attracting T-cells and fibroblasts. Clusters of these cells form a granuloma in an attempt to contain the infection (Flynn & Chan 2001). Inside the granuloma, *M. tuberculosis* expresses proteins that aid its survival. The bacilli may persist within the infected individual indefinitely and these carriers are invariably unaware of their status. The incubation period of the bacteria within the carrier is variable and this latent phase of infection accounts for many of the difficulties of TB control (Chan & Flynn 2004).

Overt symptoms of the disease can be due to exogenous re-infection (Cosma et al. 2004; Chiang & Riley 2005) or may be due to the activation of latent bacteria (Selassie et al. 2005). The cause of reactivation may be difficult to determine, but a decrease in the immune function is known to have an impact on this process, and co-infection with HIV is a frequently cited factor in the emergence of active TB (Narain & Lo 2004). The secondary stage of the infection proceeds, once the bacterial cells have replicated to an extent that enables them to burst through the
confines of the granuloma. Mycobacteria then disseminate via the lymphatic system and the blood to infect other areas of the body. In most cases, the infection remains in the lungs, causing pulmonary TB. Necrosis of cells containing the infectious agent can lead to lung-tissue damage, giving rise to the recognisable symptom of TB termed haematemesis: coughing up of blood. In addition, clusters of bacilli form pulmonary cavities, which are easily identified by chest radiography (Van Dyck et al. 2003).

1.2.3 Role of Macrophages

Macrophages recognise mycobacteria by virtue of determinants expressed at the bacterial cell surface; following ingestion, the bacterial cells are contained within an endocytic vacuole: the phagosome. The environment surrounding the macrophage can influence mycobacterial uptake by macrophages. An acidic pH or anaerobiosis induces an increase in uptake (Li et al. 2002).

The production of surfactant protein A by the MHC induces an increase in macrophage phagocytosis but does not promote the eradication of internalised pathogens (Lopez et al. 2003). Although macrophages can identify foreign material, it is the role of cytokines and antibodies to recognise the invading organisms, inducing further phagocytosis by the macrophages. A lack of certain cytokines, such as interleukin-12 and TNF-α, can lead to a decrease in bacterial uptake.

Macrophages have powerful toxic agents at their disposal and can kill any phagocytosed infective organisms by apoptosis (Oddo et al. 1998). During TB infections, ingested *M. tuberculosis* cause an influx of calcium ions into the host cell (Gil et al. 2003). These ions are a trigger for apoptosis, which is a possible
mechanism for the initial control of a TB infection. Macrophages secrete glutathione which regulates the presentation of antigens by macrophages to other cells and it also induces macrophage apoptosis, thereby killing phagocytosed organisms (Venketaraman et al. 2003). They also secrete serum amyloid P-component which attaches to bacteria, preventing phagocytosis by macrophages and allowing lysis of the mycobacteria to occur (Singh & Kaur 2006).

Macrophages can also produce reactive nitrogen intermediates (RNI) (Flynn & Chan 2003) or reactive oxygen intermediates (ROI) (Rosenvasser et al. 2006); the two effector systems cooperate to destroy the pathogen (Tomioka et al. 1997). While ROI are often responsible for the elimination of bacteria and facilitation of cell apoptosis (Sousa-Franco et al. 2006), their role in fighting *M. tuberculosis* is limited. However, RNI cooperate with fatty acids and other cytotoxic mediators to produce an additive effect (Akaki et al. 2000). Macrophages also contain enzymes that degrade organisms; phagocytosed bacteria are usually destroyed within 2 h of ingestion.

Cytokines increase the acidification and maturation of phagosomes containing mycobacteria, promoting the intracellular killing of bacteria (Hostetter et al. 2002). Cytokines work synergistically to promote the antibacterial effects of macrophages by inducing the activity of the enzyme nitric oxide synthase 2 (Nos2), which produces nitric oxide and the RNI needed to control infections (Ehlers et al. 2003). Mice deficient in the genes coding for different cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) were found to be highly susceptible to a TB infection (Raupach & Kaufmann 2001).
1.2.4 Survival Mechanisms of Mycobacteria

Macrophages phagocytose and usually eliminate infectious agents. However, *M. tuberculosis* can evade these intracellular killing mechanisms, leading to a bacteriostatic, rather than bactericidal, action (Nibbering et al. 1994). The *in vivo* survival of mycobacteria within the hostile environment of the human host is caused by inhibition of macrophage activity (Warner & Mizrahi 2007). Although the mechanisms responsible for this effect are not yet completely understood, bacterial products may be involved in the process. The macrophages can also inadvertently aid mycobacterial survival by providing nutrients for the mycobacteria (Ehrt & Schnappinger 2007).

1.2.4.1 Virulence factors

Although a range of proteins, including a number of enzymes, produced by *M. tuberculosis* have been implicated in the pathogenesis of tuberculosis, many factors remain undetermined. Mycobacterial enzymes may interfere with host-cell function, disrupt communication, or prevent fusion of host-cell components (Hestvik 2005). For example, bacterial tyrosine phosphatase MptpA reduces the uptake of mycobacteria by host macrophages (Castandet et al. 2005) and host-cell proliferation may be compromised by glycolipids on the outer cell wall of *M. tuberculosis* (Saavedra et al. 2006). Mycobacterial enzymes also prevent degradation: methionine sulfoxide reductase A, an antioxidant repair enzyme, reverses the damage caused to the mycobacteria by the host cells (Douglas et al. 2004).

*M. tuberculosis* appears to express some genes in response to challenges found *in vivo*. Fisher et al. (2002) found that the bacillus expressed an additional 81 genes
under conditions approximating those encountered during phagocytosis by macrophages, when compared with mycobacteria grown under standard laboratory conditions. Glycopeptides present in the cell wall of the mycobacteria prevented rapid uptake by the macrophages and permitted further dissemination of the mycobacteria in the lungs. Additionally, a decrease in MHC protein production inhibited the presentation of mycobacterial antigens to T-cells (Etienne et al. 2002). The killing action of macrophages by RNI can be inhibited by *M. tuberculosis* and several genes, such as *M. tb noxRI* and *noxR3*, encode for the enzymes responsible for this effect (Flynn & Chan 2003).

Ingested mycobacteria facilitate the increased permeability of the macrophage vacuole membrane, allowing nutrients to pass from the cytoplasm of the macrophage to the mycobacteria and cytotoxic agents to exit the vacuoles, resulting in continued survival of the intracellular mycobacteria (Teitelbaum et al. 1999). Blocking the fusion of phagosomes and lysosomes, cellular compartments in the macrophage that contain the ingested material and degradation enzymes, respectively, also promotes bacterial survival. Lysosomes contain enzymes that degrade mycobacterial cellular components that function at a low pH. Phagolysosomal fusion can be prevented by a variety of mechanisms (Nguyen & Pieters 2005) that include the inhibition of phagosomal maturation (Kang et al. 2005), by reducing the recruitment of Rab proteins (Smith 2003) and preventing the fusion of the two compartments through the inhibition of calcium-mediated host-cell signalling (Smith, 2003). *M. tuberculosis* may also prevent phagosome–lysosome fusion by facilitating the assembly of the tryptophan-aspartate-containing coat (TACO): intracellular mycobacteria recruit cholesterol to the
phagosomal membrane and promote the assembly of TACO, preventing fusion of the phagolysosome (Amer & Swanson 2002).

The intracellular survival of M. tuberculosis is enhanced by the production of lipids: lipoarabinomannan arrests the maturation of the phagosome by preventing its association with the host’s own various proteins, and phenolioglycolipid is a potent oxygen radical scavenger that prevents ROI from acting on M. tuberculosis (Kang et al. 2005).

1.3 Diagnosis and Treatment of TB

A general TB control guideline produced by the WHO enables health practitioners to identify patients infected with TB and to treat them using adequate chemotherapy, to promote patient recovery and prevent spread of the disease (Potter et al. 2005). The guidelines can be adapted to take into account local resistance patterns or financial limitations (Sant’Anna et al. 2002).

1.3.1 Diagnosis

Tuberculin skin tests detect immunity against TB (Enarson 2004), and testing packs are commercially available. However, recently introduced diagnostic systems such as the MB/BacT, from Organon Teknika, and FASTPlaqueTB™, produced by Biotec Laboratories Ltd (Albay et al. 2003), may also be used to determine the immune status of patients prior to initiation of a treatment regimen and to monitor therapeutic outcome during the course of treatment.

Symptoms of pulmonary TB include: coughing, chest pains, shortness of breath, loss of appetite, weight loss, fever, chills, fatigue and haematemesis. A full patient history helps to confirm the diagnosis and if tuberculosis is suspected, additional
tests may be undertaken. Imaging of pulmonary lesions using chest X-rays also facilitates detection and diagnosis of TB and may be used to investigate atypical patterns of the disease (Van Dyck et al. 2003). The confirmation of results may also include pathogen culture and tissue biopsy (Sant’Anna et al. 2002).

1.3.2 Treatment of Tuberculosis

Prior to the 1960s, there was no known antibiotic for the successful treatment of TB. However, following the introduction of rifampicin, a treatment regime was developed to treat the disease.

1.3.2.1 Chemotherapy

The factors that determine the treatment regimen are the clinical presentation, the antibiotic susceptibility profile of the isolate, and the medical history of the patient. The treatment regimen recommended by the WHO involves two phases of treatment: the initial and continuous phases. The initial phase combats the infection, while the continuous phase treats any residual latent mycobacteria residing within the host’s macrophages (Nuermberger & Grosset 2004). Omitting the continuous phase frequently results in relapse of the disease and the development of antibiotic resistance.

For a fully antibiotic-susceptible stain, the initial phase of therapy using first-line drugs lasts 2 months and is followed by a continuous phase lasting 4 months (Langfield et al. 2004). Table 1.1 lists the first-line drugs used in combination to treat tuberculosis.

The different modes of action of these agents enhance the potential for treatment success. Although the precise modes of action for all the antibiotics are still to be
fully explained, the current evidence for isoniazid favours inhibition of cell-wall biosynthesis (Herman & Weber 1980). Rifampicin blocks transcription through inhibition of DNA-dependent RNA polymerase (Wehrli & Staehelin 1971). Pyrazinamide, an agent used to reduce the duration of treatment, disrupts the mycobacterial membrane transport function (Zhang et al. 2003).

Table 1.1: Doses of the first-line drugs used to treat TB: initial and continuous phases (BNF No. 53, 2007).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
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<tbody>
<tr>
<td>Isoniazid</td>
<td>Initial: 300 mg daily</td>
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<tr>
<td></td>
<td>Continuous: 15 mg/kg three times a week</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Initial: 450–600 mg daily</td>
</tr>
<tr>
<td></td>
<td>Continuous: 600–900 mg three times a week</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>Initial: 1.5–2 g daily</td>
</tr>
<tr>
<td></td>
<td>Continuous: 2 g three times a week</td>
</tr>
</tbody>
</table>

Owing to the emergence of resistant strains of *M. tuberculosis*, second-line drugs must be administered and when used in conjunction with a concurrent TB control programme, their use is feasible and appropriate (Suarez et al. 2002). The addition of streptomycin to the continuous phase has proved effective in some patients but is rarely used, owing to its high toxicity. Ethambutol is recommended in children, especially those co-infected with HIV, but there is a related risk of dose-dependent toxicity (Graham et al. 1998).
1.4 Factors Affecting TB Control

The control and treatment of TB are difficult in densely populated areas, due to an increased likelihood of transmission from person to person (Rodrigues et al. 2006). However, successful TB control can be accomplished by using TB control programmes (Arnadottir 2001).

1.4.1 TB Susceptibility

Close contact with a TB sufferer is a known risk factor for contraction of TB (Chapman et al. 2007), but contact does not always lead to the development of the disease. Gene polymorphisms within the human population alter susceptibility to the development of the infection. For example, the active form of TB can be exacerbated by gene polymorphisms (Fitness et al. 2004). Differences in genes have been found with regard to the level of gene expression in macrophages recovered from subjects identified as susceptible to TB in comparison with those not considered susceptible to the infection (Orlova et al. 2006). The differences in susceptibility of paediatric and adult populations of TB sufferers may be attributable to the roles of genes involved in human development. For example, a polymorphism in the natural resistance-associated macrophage protein gene 1 (NRAMP 1 gene) affects paediatric TB susceptibility but has less effect on the progression of the infection in the adult population (Malik et al. 2005).

The increased susceptibility of an individual to TB may be attributable to inherently low levels of cytokines and other proteins. Gene polymorphisms leading to a decrease in the levels of cytokines such as IFN-γ (Sullivan et al. 2005) and IL (Shin et al. 2005) and of effector molecules, such as monocyte chemoattractant protein (Flores-Villanueva et al. 2005) (involved in multiprotein
nuclear body complex formation of the IFN response; Tosh et al. 2006) can lead to increased susceptibility to TB. A high level of granulocyte recruitment (Keller et al. 2006) or the possession of toll-like receptors with an increased sensitivity to modulation by cytokines (Bafica et al. 2005) also increases a person’s susceptibility to TB.

1.4.2 BCG Vaccine

Vaccination is a key element in the control of TB, and the efficacy of TB vaccines has been optimised and refined over a substantial period of use (Griffin et al. 1999). Vaccination is effective in the prevention of disease development in children (Rodrigues et al. 2005), but there is evidence that the protection it confers to adults decreases with time (Trunz et al. 2006).

Recent modifications to vaccination programmes have focused on the identification and vaccination of individuals at high risk of contracting TB (Department of Health 2005). The Bacille Calmette-Guérin (BCG) vaccine is generally regarded as an important element in the prevention of the disease, with a reported long-term efficacy of 50–60 years (Aronson et al. 2004). It was developed in the early 20th century by French microbiologists, and controlled trials were undertaken in the 1960s. The vaccine utilises an attenuated strain of Mycobacterium bovis, the causative agent of bovine TB, which stimulates a primary response to the infectious agent, but the strain is unable to cause overt symptoms of infection. By 6–12 weeks, a small pustule may form at the site of administration, and the individual is then considered immune.

The tuberculin test confirms immunity and is supplied by manufacturers in a formulation for administration by injection. The active component is derived from
a protein produced by the TB bacillus. A subcutaneous injection of the protein is administered, and patients that have contracted the TB bacillus, or have been immunised against TB, will exhibit an immune reaction in the area surrounding the injection site that manifests as hardening, swelling, and reddening of the skin (Enarson 2004). This reaction to the tuberculosis antigen constitutes the basis of the Mantoux test.

1.4.3 *DOTS*

Supervision of treatment administration is recommended to prevent resistance and reoccurrence of TB and to ensure adequate drug therapy and compliance (Selassie et al. 2005). Such procedures have been implemented to identify emerging complications, such as early-stage liver damage caused by the potent antibiotics. Directly Observed Treatment Strategy (DOTS) is a programme that uses both community and health-facility-based schemes to ensure supervision of patients during their TB treatment (Wandwalo et al. 2005). Although a recent review (Volmink & Garner 2006) comparing the use of DOTS with self-administration concluded that successful TB treatment was not affected by the absence of supervision, there are many case reports detailing the beneficial effects of DOTS and its application in establishing the successful control of TB (Davies 2003).

The WHO has devised a new programme, DOTS-plus, promoting greater support to enable nation states to implement DOTS and to control the spread of multi-drug resistant tuberculosis (MDR-TB) (Pablos-Mendez et al. 2002).
1.4.4 Co-Infection with HIV

The majority of HIV patients are infected with opportunistic bacteria (Eza et al. 2006) and TB is one of the most common causes of mortality in these patients (Reid et al. 2006). HIV can adversely affect successful TB control (Hecht et al. 2006) and increases the risk of the emergence of active TB in patients harbouring latent mycobacteria by a factor of 20 (Reid et al. 2006). Patients given highly active antiretroviral therapy (HAART) have a decreased risk of contracting opportunistic infections (Hung & Chang 2004). However, TB rates remain very high in HIV patients (De Cock & Marston 2005) and in contrast to other opportunistic infections, TB occurs throughout the progression of HIV disease (Sharma et al. 2005).

The incidence of TB and HIV co-infection (TB/HIV) is rising (Langfield et al. 2004) and HIV has been a major contributory factor to the increase in incidence of TB (Silversides 2006). HIV infected children who do not receive antiretroviral treatment and subsequently contract TB, face a high risk of death that is not reduced by a complete course of antitubercular chemotherapy (Hesseling et al. 2005).

A third of people carrying HIV are also infected with the TB bacillus (Nullis-Kapp 2005). However, with HIV carriers in sub-Saharan Africa, this incidence rises to 70% (Ferrari 2004) and Asia also has an increasing TB/HIV population (Vannarith et al. 2005). In response to this worrying scenario, the WHO has established targets for 2007: they recommend that countries with a high incidence of either TB or HIV should develop and initiate a joint TB/HIV implementation plan to institute a process for HIV surveillance among TB patients and for TB surveillance in HIV patients (WHO 2004).
When a patient presents with either TB or HIV, the progression of infection and its transmission to others may be altered due to the change in the patient's susceptibility to infection. The stage of HIV infection affects the clinical presentation of TB, particularly in late-stage HIV, where presentation and clinical symptoms can be atypical of TB. HIV acts by reducing the CD4+ T-lymphocyte (CD4+) count and thus promotes the growth and replication of the mycobacteria. As HIV infection progresses, there is a continued reduction in CD4+ cell numbers, further reducing the control of *M. tuberculosis* proliferation and resulting in wider dissemination of mycobacteria, increasing the risk of extra-pulmonary TB (Narain & Lo 2004).

Latent *M. tuberculosis* may be detected using the tuberculin skin test or a specific interferon-γ test. However, with TB/HIV, false-negative results can occur more frequently than with patients infected only with *M. tuberculosis* (Liberato et al. 2004). For this reason, the implementation of TB and HIV diagnostic procedures is advisable. The outcome of diagnostic tests may be influenced by the *M. tuberculosis* isolate responsible for the infection: some isolates, more frequently encountered in HIV-infected patients, are likely to engender a false-negative smear test in comparison with isolates from non-HIV-infected individuals (Cobelens et al. 2006). However, there is also a high incidence of false-negative results when using the interferon-γ test for TB (Brock et al. 2006).

The rate of resistance can remain relatively low in uncomplicated TB infection. However, the incidence of resistance can more than double when a patient is co-infected with HIV (Nelson et al. 2005). For a variety of reasons, extensively drug-resistant TB can be a cause of death in TB/HIV patients (Gandhi et al. 2006). For
example, isoniazid preventative therapy (IPT) may increase the risk of
development of mycobacterial resistance in TB/HIV.

IPT is recommended for HIV patients with a high risk of active TB where
reactivation of latent bacteria is likely, and this prophylactic approach is effective
in the prevention of TB disease development. However, due to the anergy seen in
TB/HIV, where a response is an all-or-nothing event, there can be a loss of
tuberculin test reactivity, and the presence of latent *M. tuberculosis* may not,
therefore, be detected until reactivation of the bacteria leads to the active form of
the disease (Cobelens et al. 2006). Adherence to IPT can be monitored using in­
house-prepared urine test strips and this surveillance should form part of the TB
control programme (Szakacs et al. 2006). However, as the possibility of the
development of a drug-resistant *M. tuberculosis* is high, IPT should be coupled to
adequate diagnostic and treatment policies in order to identify and treat patients at
risk of developing infection due to resistant strains (Cohen et al. 2006).

TB can also adversely affect an HIV infection, as active TB induces a higher
replication rate of the virus, leading to higher viral loads, increased genetic
diversity of the virus and increased rates of HIV dissemination.

DOTS was conceived prior to the occurrence of TB/HIV (Reid et al. 2006) and
the emergence of TB/HIV has signalled a need for new treatment regimens and
control protocols (Ruiz-Navarro et al. 2005). Drug interactions occur frequently
and limit the use of certain antituberculosis drugs and HAART. Excluding TB
detection and treatment in HIV control policies and the contrary situation, will
reduce their efficacy and limit the success of these policies.
1.4.5 Immigration

In the United Kingdom, children are regularly vaccinated against TB, but those coming from countries where the vaccine is not readily available may not have protection.

Many of the new TB cases diagnosed in this country in recent years are due to *M. tuberculosis* strains that have been imported from overseas rather than due to the transmission of mycobacteria endemic to this country (Lillebaek et al. 2001). Immigrants account for a large number of new cases, both in the United Kingdom and worldwide (Baussano et al. 2006). While there is no significant transmission of TB bacilli between natives and immigrants, there is, nevertheless, a heavy burden placed on health control. In the United Kingdom, 60% of TB sufferers are from overseas; in addition, there is a high incidence among homeless persons (Storey 2004). There is also an increased risk of reactivation of latent *M. tuberculosis* in individuals from overseas, when there is a high TB incidence in the country of origin (Dahle et al. 2005).

1.4.6 Non-Compliance

In the United Kingdom, just under 80% of those receiving treatment complete the prescribed course (Health Protection Agency 2006). The complexity of typical therapeutic dosing regimens and apparent alleviation of symptoms prior to eradication of the TB pathogen sometimes mitigate against completion of the full course of treatment. Non-compliance, due to social and demographic factors such as age and sex (Balbay et al. 2005) or a lack of funds (Dodor & Afenyadu 2005), promotes antimicrobial resistance.
Adverse drug reactions lead to non-compliance, but due to the limited number of available antituberculosis drugs, it may not be possible to discontinue treatment and administer alternative drugs. Table 1.2 highlights the common side-effects of the major antituberculosis drugs.

Mycobacterial persisters in patients who fail to complete the course of treatment often develop resistance to antimycobacterial chemotherapeutics. Resistance to the first-line drugs isoniazid and rifampicin is a major factor in treatment failure and disease relapse (Quy et al. 2003).
Table 1.2: Side-effects of antituberculosis drugs (BNF, No. 53, 2007; New Guide to Medicines and Drugs, Henry, Ed, 1997).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Common side-effects</th>
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| Isoniazid     | Peripheral neuropathy, if there are pre-existing factors  
Liver damage (periodic blood tests usually performed)  
Increased loss of the vitamin pyridoxine (supplements usually taken)  
Vomiting, fatigue, and rashes |
| Rifampicin    | Transient disturbance of liver function initially; serious toxicity occurs at later stages  
Flu-like symptoms  
Acute renal failure  
Coloured secretions  
Oedema, muscular weakness, and jaundice  
(NB: rifampicin reduces the effectiveness of a wide variety of drugs) |
| Pyrazinamide  | Serious liver toxicity  
Ethambutol      | Visual disturbance, usually in the form of colour blindness and restriction of visual fields  
Renal-function impairment |
1.4.7 Resistance

It is clear that a thorough understanding of the basis of antimycobacterial drug resistance will facilitate control of the infection (Harbarth & Samore 2005). *M. tuberculosis* resistance impedes TB treatment and control. The recent increase in TB rates is partly attributable to this factor. Ineffective drug treatment promotes reoccurrence of TB and is largely caused by resistance to first-line drugs (Quy et al. 2003); furthermore, the emergence of new strains by mutations promotes survival and aids dissemination of difficult-to-treat mycopathogens (Livermore 2000).

The molecular basis of resistance to most of the first-line antimycobacterial agents is incompletely understood, but a number of key mutations in *M. tuberculosis* have been found to account for resistance to rifampicin (Jenkins 2005) and related drugs (Maus et al. 2005). Point mutations have been determined to contribute towards resistance in some cases; for example, mutations in the *katG* gene lead to isoniazid (Rouse & Morris 1995) and ethambutol resistance (Lety et al. 1997). A single-point mutation may lead to pyrazinamide resistance, due to inactivation of an enzyme that converts the inactive pro-drug to the active form (Somoskovi et al. 2001).

The use of IPT in patients with latent TB can promote resistance (Balcells et al. 2006) and this therapy is recommended only for those who have a high risk of re-infection and require constant monitoring. An alternative to isoniazid for the treatment of latent TB infection is rifampicin, which has a good efficacy in this situation, but resistance may occur (Reichman et al. 2004).
1.4.7.1 Multi-drug-resistant tuberculosis (MDR-TB)

The development of MDR-TB has been identified as a potentially significant problem for TB control (Reichman 2005). Although resistance to drugs may have developed as a result of poor compliance, there are also other clinical as well as genetic risk factors for MDR-TB development (Sharma et al. 2003). Patients with large high mycobacterial counts, the severity of illness and the nature of the mycobacterium all influence resistance development. MDR-TB is found in most geographical areas, including Central Asia (Cox et al. 2004), Africa, and Europe (Espinal et al. 2001). Particular strains that are prevalent or problematic, such as the Haarlem strain, have the potential to produce a pandemic (Mardassi et al. 2005).

Since the identification of MDR-TB, procedures for diagnosis and treatment have been developed. Different regimens have been implemented in response to the nature of resistance encountered (Mukherjee et al. 2004). Surgical resection, together with the addition of a fluoroquinolone such as ciprofloxacin (Chan et al. 2004) or ofloxacin (Ziganshina et al. 2005), produced a significant improvement in treatment outcome to TB.

A lack of financial resources as well as limited treatment options leads to problems in the control of MDR-TB, which in turn can promote further development of resistance (Pablos-Mendez et al. 2002). At present, there are no randomly controlled trials to determine the efficacy of the use of drugs in patients who are at risk of MDR-TB (Fraser et al. 2006); therefore, the effectiveness of MDR-TB control measures is currently difficult to evaluate.
1.5 New treatments for TB

The current high incidence of TB and the rapid increase in the rate of emergence of drug-resistant bacteria highlight the urgent need for new drugs and treatment options. Although much of the investment in the search for new therapies has focused on the development of new chemotherapeutic agents, alternative forms of therapy should not be ignored.

1.5.1 New Chemotherapeutic Agents

Established interactions between the mycobacteria and potential antituberculosis drugs can provide data for the potential effectiveness of possible new chemotherapeutic agents (Nuermberger and Grosset, 2004). The TB Alliance is a public–private partnership promoting the development of drugs that reduce the length of TB treatment, are effective against drug-resistant forms of *M. tuberculosis* and improve treatment for latent TB. They are supporting the evaluation of a portfolio of potential drug candidates at both the preclinical and clinical stages of development.

A number of established antibiotics are currently being investigated with regard to their potential utility for the treatment of TB. For example, clarithromycin and ciprofloxacin, in combination with isoniazid and rifampicin, have been used clinically with some success (Bhusal et al. 2005). Ciprofloxacin, a drug that has shown promise as an antituberculosis agent, has been associated with treatment failure due to the rapid development of drug resistance rather than to insufficient potency (Gumbo et al. 2005).

There are a number of unique features in the biosynthetic pathways and the metabolism of *M. tuberculosis* that represent promising targets for novel drug
development (Anishetty et al. 2005); biosynthesis of peptidoglycan, necessary for mycobacterial cell walls, and MurD ligase enzyme activity, which catalyses peptide bonds, are two possible targets for inhibition. Novel agents that reduce the length of treatment would be particularly welcome, and derivatives of diarylquinoline and rifamycin have been studied as potentially useful agents in this regard (Zhang et al. 2006). In addition, nitroimidazopyran PA-824, used either alone or in combination with isoniazid and rifampicin, significantly reduces bacterial counts more rapidly, without adversely affecting disease relapse, than conventional therapies (Nuermberger et al. 2006).

1.5.2 Drug-Carrier Systems

Anti-TB therapy is usually administered in oral preparations. However, alternative formulations or drug-carrier systems may provide benefit. Implants or microparticles can promote a reduction in dosing frequency as an aid to patient compliance (du Toit et al. 2006). Nanoparticles are under investigation as oral, inhalation, and intravenous dosage forms (Gelperina et al. 2005) and microspheres with a payload of anti-TB drugs have shown an increased bioavailability, long half-life, and high stability (Pandey & Khuller 2004). Use of these systems may reduce dosing frequency and improve treatment options.

1.5.3 New Vaccines

BCG vaccine is effective in preventing childhood TB but has reduced efficacy in adults (Sierra 2006). Recent advances in the understanding of the interactions of TB bacilli and the immune system, such as establishing the molecular contacts between T-cells and the pathogen by interleukins, aids in the development of new
vaccines that have the ability to potentiate the T-cell response against invading *M. tuberculosis*. An increased understanding can underpin the development of new vaccines (Baumann et al. 2006). Although no new vaccines have been introduced in the recent past, some candidate formulations currently under development may reach the stage of clinical trials in the foreseeable future (Gupta et al. 2007).

1.6 Natural products

Many medicines, both ancient and modern, are based on natural products, and traditional approaches to the treatment of a range of diseases, including autoimmune diseases and infections, utilise materials of plant origin. Almost all anti-infective agents are derived from natural sources. Several unusual sourced materials have been used in this context; for example, polysaccharides of bacterial origin have been proposed as agents for the stimulation of the immune system (Kim et al. 2005). Aspirin and digoxin are two of the most commonly prescribed medicines in the United Kingdom and are examples of drugs that have been derived from plant sources (Phillipson 2001). Evaluation of traditional medicines using scientific procedures should be encouraged in order to discriminate between effective and ineffective preparation. For example, putative herbal cures from Papua New Guinea have been found to be ineffective against infections (Case et al. 2006). However, there is evidence to support certain claims, such as those made for a Ghanian plant, *Chorophytum inornatum*, which was found to possess antimycobacterial activity (O'Donnell et al. 2006). Furthermore, many Mexican plants, such as *Chamaedora tepejilote* and *Lantana hispida*, have been found to exhibit varying degrees of antimycobacterial activity (Jimenez-Arellanes et al. 2003).
Several traditional medicines from countries such as South Africa and other African nation states have been identified as sources of anti-infective medicines (McGaw et al. 2005). Plants have been investigated for antibacterial (Owais et al. 2005), antiviral (Serkedjieva & Hay 1998) and multiple activity (Okunade 2002). Specific classes of compounds derived from plants with antibacterial properties may provide potential drug leads: these include the aurones (Kayser et al. 1999) and tannins (Kolodziej et al. 2001).

Currently there are plants and extracts derived from plants that are in clinical trials for varying medical conditions. The addition of cranberry juice to the conventional triple therapy for \textit{Helicobacter pylori} treatment of omeprazole, amoxicillin and ciprofloxacin was been found to increase eradication rates of the bacteria in women (Shmuely et al. 2007). Cranberry juice was also found to prevent the recurrent urinary tract infections (Bailey et al. 2007). There has also been a randomised control trial using \textit{Arnica Montana} for its putative analgesic effect, where patients could derive its analgesic property following minor surgery (Robertson et al. 2007).

1.6.1 Augmentation of Immunomodulatory Activity

Although many complementary medicines and herbal remedies have not been shown to possess clinical efficacy, they are still widely used. While the continuing search for compounds with direct antibacterial action is essential, infections may be controlled by other means. For example, vaccines are used to augment specifically the immune response to a number of infectious diseases and other procedures could be implemented to increase the protective potential of the immune system. Modifying the immune response, rather than using antibiotics,
may contribute to the control of infection, by either specific or non-specific immunomodulation; for example, the stimulation of macrophages and natural killer cells may provide a means to relieve the microbial burden by processes that reduce or prevent the development of resistance by the pathogen, such as using peptides of natural, synthetic or recombinant origin to increase levels of T cells and induce heightened cytokine activity (Masihi 2000).

There has been considerable interest recently in plants as sources of immunomodulatory compounds. For example, Argentinean plants, for example *Achyrocline flaccida* and *Eupatorium arnottianum*, have been investigated with regard to the identification of immunomodulatory metabolites with the potential for pharmaceutical use (Fernandez et al. 2002). Metabolites from various plants have been shown to possess the capacity to exert modulating effects on a variety of components of the immune system, such as metabolites isolated from *Pestalotiopsis leucothes* increased both interleukins and TNF-α levels, (Kumar et al. 2005) and other metabolites isolated from *Cedrela tubiflora* augmented of the macrophage respiratory burst (Benencia et al. 1999). Such metabolites may also increase nitric oxide production by macrophages (Ignacio et al. 2001) and modulate cytokine production (Choi et al. 2001).

### 1.6.2 Plant-Based TB ‘Cures’

The use of traditional medicines does not necessarily hamper TB control (Oeser et al. 2005). Bioassay-guided procedures may lead to the identification of compounds with antitubercular properties and these procedures have been undertaken with plants from Peru (Graham et al. 2003) and Mexico (Jimenez-Arellanes et al. 2003). An inhaled phytochemical, *Eucalyptus globulus* oil, has
been used with some success as an adjuvant in TB therapy (Sherry & Warnke 2004) and the methanol extract of *Carpobrotus edulis* has shown *ex vivo* activity against intracellular MDR-TB (Martins et al. 2005).

1.6.2.1 Stevens’ cure

In the late 1890s, a doctor sent an English tuberculosis sufferer to South Africa in the hope that warmer climes would aid his recovery, since, at that time, there was no cure for TB. The patient, Charles Stevens, encountered a fellow sufferer, who advised him to visit a local medicine man, who in turn gave Stevens a native medicine. This consisted of the roots of two plants of the geranium species, *Pelargonium sidoides* and *P. reniforme*, the traditional medicine Umckaloabo in native Xosa language. Umckaloabo was and is, used by the indigenous people as treatment for a variety of respiratory ailments, including TB. Stevens took the powdered root concoction twice a day in boiled milk, which caused him to be violently sick for 2 h every morning. However, he continued with the treatment. After 2 months, he felt sufficiently recovered to return to England, where medical practitioners pronounced him free of the disease. As a volunteer in the Boer War, Stevens returned to South Africa and continued to live there after the War, founding a company to sell the root commercially. To promote the sale of the remedy, he wrote to doctors both in South Africa and in England, but was fined for unauthorised medical practice. Returning to England in 1907, Stevens set up to sell the remedy to the general public. However, in 1909, the British Medical Association (BMA) ran a campaign against medically unfounded remedies and published a book entitled ‘Secret Remedies: What They Cost and What They Contain’. The BMA branded Stevens a ‘quack’. They claimed that the powder he
was selling contained a substance called Rhatany, and it could not be proved that his ‘cure’ was of any use for the treatment of TB. Stevens’ business suffered as a result of the accusations and he sued the BMA for defamation. A verdict could not be achieved at this first jury trial and a second trial was scheduled. Although Stevens was supported by expert witnesses in the first trial, these doctors did not attend the second: their testimonies fomented conflict with their professional bodies, in particular the BMA. At the second trial in 1914, the court found in favour of the BMA, thereby damaging Stevens’ reputation. He was ordered to pay substantial fines and appealed against the verdict. Despite this, Stevens continued to sell the cure in national and international marketplaces, renaming the preparation ‘Stevens’ Consumption Cure’ which he formulated in dosage forms such as capsules and lozenges (Helmstadter 1996; Taylor et al. 2005)

1.6.2.2 A scientific basis for Umckaloabo?

In the 1920s, a Swiss physician, Dr Adrien Sechehaye, investigated Umckaloabo after hearing about Stevens’ Cure from a successfully treated TB patient. Initially, one patient was treated with Umckaloabo powder twice a day for 3 months, and then, over the next 9 years, a further 800 patients were treated (Sechehaye, 1931). Not all patients recovered, but failures were attributed to non-compliance or concomitant illnesses. In a later publication by Sechehaye (1954), many patient case files were evaluated, containing details of the presentation of TB, any treatments previously given and the outcome of treatment with Umckaloabo. In subsequent research, Sechehaye found that Umckaloabo produced an apparent cure for TB in patients who had been treated unsuccessfully with conventional medicines. Investigation of the constituent components of ‘Stevens’ Cure’
revealed that it did not contain Rhatany, an extract with the same colour and
tannin content to the *Pelargonium* roots and was known not to have activity
against TB, as stated by the BMA (Helmstadter 1996). From the 1930s, the
widespread commercial use of Umckaloabo commenced and in Germany, it is
currently sold under that name for the treatment of respiratory symptoms, such as
coughs and colds, although it is not marketed as a treatment for TB.

Many of the constituent ingredients of Umckaloabo have been characterised.
There are over 200 essential oils in the leaves of *P. sidoides* and *P. reniforme*
(Kayser et al. 1998) and the roots and leaves contain many components, such as
flavonoids, coumarins and phenolic acids (Kolodziej 2000). *P. reniforme* has
yielded compounds classed as novel ellagitannins (Latte & Kolodziej 2000) and
compounds of the glycosyl flavone (Latte et al. 2002) and coumarin type (Kayser
& Kolodziej 1995) have been identified.

Compounds purified from the two species of *Pelargonium*, such as coumarins and
catechins, have been evaluated in potency assays against a variety of infectious
organisms, including *Escherichia coli*, *Pseudomonas aeruginosa*, and
*Haemophilus influenzae*. The compounds have exhibited varying degrees of
activity against these organisms (Kayser & Kolodziej 1997). However, such
antibacterial effects cannot account for the reported activity of extracts obtained
from the *Pelargonium* species against intracellular pathogens. If Umckaloabo
does possess clinically relevant antituberculosis efficacy, it is probable that, in
addition to conventional bacteriostatic or bactericidal activity, the concoction
contains components with immunomodulatory properties that help in the
eradication of mycobacterial pathogens by phagocytic cells. It is likely that a
combination of both its antibacterial and immunomodulatory properties

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modulated the intracellular parasitic state (Kolodziej et al. 2003). An aqueous extract of *P. sidoides* stimulated macrophages infected with *Leishmania* sp, determined by measurement of an increase of macrophage-associated TNF-α and ROI levels (Kayser, Kolodziej, & Kiderlen 2001). Aurones isolated from *P. sidoides* also exhibited antibacterial activity against extracellular leishmanial organisms and they augmented macrophage function against intracellular leishmanial infection (Kayser et al. 1999). The antiparasitic effect of *Pelargonium* against *Leishmania* has been further elucidated through the identification of tannins that affect both extracellular promastigote and intracellular amastigote viability (Kolodziej et al. 2001). The activity of tannins against leishmanial infection was attributed primarily to their effects on macrophages, as opposed to direct antileishmanial activity (Kolodziej & Kiderlen 2005).

Modulation of gene expression in cells of the immune system initiates a series of events (Trun et al. 2006) that may lead to the induction of nitric oxide synthase (Kolodziej et al. 2005) and to increased levels of reactive intermediates with the capacity to inactivate bacteria (Rosenvasser et al. 2006). Extracts of *P. sidoides* bolster the immune system through the induction of a higher ciliary beat frequency in the mucociliary system, thereby increasing infection control (Neugebauer et al. 2005).

Extracts of the roots of *P. reniforme* and *P. sidoides* have been used clinically in a number of infectious disease states. Extracts of *P. sidoides* have been found to decrease the severity of symptoms of acute bronchitis (Matthys et al. 2006) and have been used to successfully treat tonsillopharyngitis (Bereznoy et al. 2003). The leaves of the plants have also been used as alternative sources of antibacterial
compounds (Lewu et al. 2006). *P. reniforme* extracts possess antibacterial, antifungal and antitubercular properties (Mativandlela et al. 2006).

Bioactive compounds, such as nonanoic derivatives, have been purified from *Pelargonium* spp. and have been shown to have antimicrobial activity (Sahin et al. 2006). Studies in our laboratory have identified fatty acids with antimycobacterial activity, with oleic and linoleic acid exhibiting the greatest activity against mycobacteria (Seidel & Taylor 2004).

The activity of such root extracts against intracellular mycobacteria has not been investigated and there is a possible cost-benefit ratio in using this natural product. As both *P. sidoides* and *P. reniforme* are indigenous to South Africa, locally prepared concoctions could be used to treat the residents and those living in sub-Saharan Africa, supplementing conventional chemotherapy.

In summary, the apparent efficacy of Umckaloabo, as demonstrated by Sechehaye (1954) in his carefully designed clinical studies, could be related to both antimycobacterial and immunomodulatory properties of the preparation and it would be advantageous to investigate such potential activities in order to find additional bioactive molecules that add some benefit in the treatment of TB.

TB is a complex disease, and many factors influence its spread and development in a community. Investigations into new treatments may identify alternative therapies for the containment of the disease and the investigation of the effects of natural products, such as Umckaloabo, increases our options in this important area of research.
1.8 Aims and Objectives

The aim of the project was to investigate the immunomodulatory effects of extracts of *P. sidoides* and *P. reniforme* on the eradication of mycobacteria by macrophages. Based on evidence showing that *Pelargonium* extracts have antitubercular effects (Sechehaye 1954), I hypothesise that compounds in *Pelargonium* roots enter the blood stream following ingestion, by absorption through the gut. Once in the body, they are spread throughout the body where they exert direct and indirect antimycobacterial effects by actions on the individual mycobacterial cells as well as stimulating cells of the immune system. Recently, antimycobacterial compounds have been identified in *Pelargonium* extracts (Seidel and Taylor 2005) and an investigation was conducted to determine the capacity of extracts to enhance the function of macrophages infected with mycobacteria. A systematic bioassay-guided fractionation of *Pelargonium* was undertaken and an attempt made to determine the extent of uptake and eradication of mycobacteria by macrophages in the presence the *Pelargonium* extracts. The compounds responsible for this activity will be identified by a variety of standard chemical methods. This study will contribute towards the long-term objective of our laboratory: to identify and reconstitute the bioactive compounds of *Pelargonium* as a prelude to clinical evaluation in humans.
Chapter 2

MACROPHAGE ASSAY
2.1 Introduction

The initial encounter between mycobacteria and the host occurs in macrophages, with live mycobacteria able to persist in these cells for an indefinite time period. Inhaled TB bacilli reach the alveolar macrophages in the lung periphery and are phagocytosed. Mycobacteria transported to the lymphatic system by the macrophages forms part of the lymph solution, which drains into veins and either circulates back to the lungs or disseminates to other parts of the body by the systemic venous system. The effect of a possible macrophage stimulant could be assessed by the analysis of intracellular mycobacterial levels in macrophages. Alternative parameters can be measured to determine the capability of the stimulants on enhancing macrophage function.

Human macrophage cell lines and murine peritoneal macrophages show comparable reactions, such as phagocytic activity and cytokine production, when subjected to the same conditions (Schepetkin et al. 2005). In this study, peritoneal macrophages harvested from BALB/c mice have been used in the assays that formed part of the bioassay-guided fractionation of Pelargonium root extracts. Macrophages respond to chemical and physical stimulation in a number of ways. For example, engulfment of exogenous particles may induce changes in the ability of the cells to spread on surfaces and to elaborate pseudopodia (Choi et al. 2001). Cytokines, such as TNF-α, IFN and various interleukins, are modulated in response to macrophage activity (Li et al. 2002). These effects may be used to investigate the response of macrophages to exposure to bioactive molecules, for example those associated with Pelargonium fractions. Additionally, the ability of
macrophages to increase or decrease rates of uptake and intracellular killing of mycobacterial pathogens may also provide indicators of utility: in this context, the respiratory burst response, characterised by an increase in reactive nitrogen (RNI) and reactive oxygen intermediate (ROI) levels, representing an increase in bactericidal effect (Benencia et al. 1995), may provide valuable information with regard to the immunological status of these cells.

An increase in the phagocytic activity of macrophages can be measured by enumerating the uptake of synthetic particles (Benencia et al. 1995). Alternatively, lysis of macrophages may be used to facilitate the enumeration of internalised particles (Benencia et al. 1999). Such methods have potential practical use in this project, as evaluation of the intracellular levels of viable mycobacteria should provide evidence of changes in macrophage-killing proficiency in response to external stimuli.

Bacterial surrogates, strains related to problematic pathogens, such as *M. tuberculosis*, that are difficult to employ in everyday laboratory procedures, are commonly used in research and many studies have been performed on avirulent strains of mycobacteria as a model for *M. tuberculosis* (Mehta et al. 2006). Several surrogates are available and although many are not as virulent as *M. tuberculosis*, they have the potential to cause serious infections. Some are relatively fast-growing, with incubation times from 3 days to achieve confluent growth, as opposed to a minimum of 3–4 weeks for *M. tuberculosis*. Owing to a similar genetic complement, surrogates may be assessed for potential vaccine targets and the evaluation of treatments for TB infections (Spratt et al. 2003).
The potency of various chemotherapeutic regimens in TB treatment has been determined by the use of surrogates (Casal et al. 1987). Potential drugs have been screened against a range of different mycobacteria. *M. aurum* has been used in a rapid high-throughput screen in a commercial setting and is a reliable indicator for *M. tuberculosis* responses (Chung et al. 2007). *M. avium* and *M. tuberculosis* showed similar responses when subjected to intramacrophage passage followed by susceptibility testing using different antibiotics (Tomioka et al. 2002). Therefore, mycobacteria deemed appropriate for use as surrogates for *M. tuberculosis* may be employed to standardise susceptibility screening (Woods 2000).

Mycobacterial surrogates survive well in macrophage cultures (Dheenadhayalan et al. 2006). The complex interactions between macrophages and mycobacteria have been extensively characterised using a variety of surrogates such as *M. aurum* (Silva et al. 1987) and *M. smegmatis* (Cougoule et al. 2002). In addition, the viability of the closely related pathogen *Mycobacterium leprae* within macrophages has been evaluated (Vejare & Mahadevan 1987), while *M. marinum* has provided detailed information, at the molecular level, on the process of macrophage invasion by mycobacteria (Mehta et al. 2006). The use of *M. avium* has allowed a greater understanding of the interactions between macrophages and mycobacteria (Tomioka et al. 1997) through the demonstration that lung surfactants increased the rate of mycobacterial uptake (Lopez et al. 2003) and increased the activity of cytokines (Hostetter et al. 2002) and the *MsrA* enzyme (Douglas et al. 2004).
2.2 Materials and Methods

2.2.1 Culturing Mycobacteria

For this project, a number of surrogate mycobacteria were examined for their potential utility in macrophage uptake assays. Blood agar plates, Columbian agar (Sigma, Dorset, UK) supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, UK), were inoculated with mycobacterial strains; *M. fortuitum*, (ATCC 6841) and *M. abscessus* (ATCC 19977) were obtained from the American Type Culture collection (Teddington, Middlesex, U.K.), *M. aurum* A+ was supplied by the Pasteur Institute (Paris, France) and *M. smegmatis* (ATCC 14468) was supplied by De Montfort University (Leicester, U.K.).

2.2.2 Harvesting Macrophages

Macrophage function against mycobacteria was measured as follows. 4-week old, female BALB/c mice (Harlan, Bicester, UK) were sacrificed under CO₂ anaesthesia and the abdomen was sprayed with 70% ethanol to kill contaminating bacteria.

The macrophages were extracted by peritoneal lavage using Medium 199 (Invitrogen, Paisley, UK) supplemented with 10% FBS (Invitrogen, Paisley, UK) and sterilised using a 0.45 μm Milipore membrane filter (Fisher, Loughborough, UK). 10 ml of Medium 199 was injected into the peritoneum using a 23-gauge orange needle. The abdomens were massaged for several minutes and the medium was withdrawn back into a sterile syringe and transferred into a 50-ml Falcon tube (Corning, Artingdon, UK). Successive injections of Medium 199 were performed until approximately 20 ml of macrophage suspension had been collected. The
suspension was centrifuged at 1500 × g, at 4°C, for 10 min. The supernatant was removed using a sterile Pasteur pipette, and the cell pellet was suspended in 10 ml of Medium 199. The macrophages were quantified using a haemocytometer; 10 µl of the macrophage suspension was added to 10 µl of Trypan Blue solution to count the number of viable macrophages. 10 µl of the macrophage/dye suspension was transferred to the chamber of the haemocytometer and the cells were counted. An average of the number of macrophage cells from the corner and centre grids was calculated. The cell suspension was diluted to give a concentration of 1 × 10⁴ cells/ml. 1 ml of the macrophage suspension was transferred into the wells of a Costar 12-well cell-culture plate (Fisher Scientific, Loughborough, UK). Gentamicin (10 µg/ml) was added to each well to suppress bacterial contamination. The culture plates were incubated for at least 2 h at 37°C in a CO₂ incubator (5% CO₂ and 95% humidified air). Macrophages adhered to the bottom of the wells to form a monolayer. To remove any non-adherent materials, the monolayer was washed twice with phosphate-buffered saline (PBS) and overlaid with fresh Medium 199. Thereafter, to test the effect of root extracts on macrophage function, solutions of the extracts and mycobacteria suspensions were added to the macrophage monolayer.

2.2.3 Macrophage Assay

To measure macrophage activity, the macrophage monolayer was exposed to mycobacteria. The mycobacterial suspension was produced by suspending a small amount of the \textit{M. fortuitum} colonies grown on an agar plate in 10 ml of PBS in sterile universals. In lieu of using the virulent \textit{M. tuberculosis}, a non-virulent, fast-growing mycobacterial strain, \textit{M. fortuitum}, was used as a surrogate.
The cells were homogenised by adding sterile 1.5-mm-diameter glass beads and agitated using a vortex mixer. The cells were washed by centrifuging the cell suspension at 1500 x g, followed by the addition of sterile PBS. A concentration of 1 x 10^5 mycobacterial cells/ml was maintained by using a 0.5 McFarland standard.

The extracts obtained from the fractionation procedure of *Pelargonium* roots were then added to the macrophage monolayer. At hourly intervals, the macrophage monolayer was washed with PBS to remove extracellular bacteria and extract. The macrophages were lysed and mixed using 0.5 ml of sterilised distilled water. After 10 min, 100 μl of the water was plated onto blood agar plates. The plates were incubated for 3 days and the number of colony-forming units (CFUs) was counted. The CFUs was expressed as a percentage of the total number of mycobacteria added to the macrophages.

**2.2.4 Ziehl–Neelsen Staining**

In order to demonstrate the uptake of mycobacteria by the macrophages, the Ziehl–Neelsen stain, an acid-fast stain, was used. Macrophages were harvested by peritoneal lavage and plated onto 12-well cell culture plates that had been lined with a glass coverslip. The extracts and mycobacterial suspension was added to the macrophage monolayer. Every hour, the macrophages were washed and the coverslip removed from the cell-culture plates. The coverslips were then attached to labelled microscope slides and the cells were fixed by heat from a Bunsen burner. The cells was covered with carbol-fuchsin stain (Sigma, Dorset, UK) and heated until steam was produced. The stain was allowed to absorb into the cells for 5 min. After washing with deionised water, malachite green (Sigma, Dorset,
UK), containing an alcohol decolouriser was added to the cells. After 1 min, the cells were washed with water and allowed to air dry. 100 macrophages were randomly selected by using the left side of the slide as the point of origin, the slide was moved along to the right to count the mycobacterial content of 10 macrophages, then the slide was moved down by one turn of the wheel that supported the stage holding the slide. After a further 10 macrophages were counted, the stage was moved down again and the process was repeated until the mycobacterial content of 100 macrophages was tallied, and the bacterial content of each macrophage enumerated; the bacterial content of each macrophage was assigned to the following categories: <5, 5–19, or >19 bacteria per macrophage (Speert et al. 1986).

2.2.5 Trypan Blue Exclusion Method

The Trypan Blue stain can be used to evaluate cell viability in an experiment (De Loecker et al. 1998). Macrophages attached to coverslips have also be stained with Trypan Blue to validate an assay (Hellewell et al. 1997). Following staining, dead cells appeared blue, whereas live cells were colourless. This cell-viability assay was used to confirm that the extracts, solvents and mycobacterial suspensions did not reduce macrophage viability. Following the treatment and washing of the macrophages, Trypan Blue stain was added to the cells. After 5 min, the excess dye was removed. 100 cells were observed and those absorbing the stain were counted to calculate the percentage of viable cells.
2.3 Results

2.3.1 Macrophage Assay Development and Validation

An assay was established to determine the effect of *Pelargonium* extracts on the uptake and maintenance of mycobacteria (Figure 2.1). To limit any variation in biological responsiveness BALB/c mice were used, as they are an inbred strain and have a high degree of genetic homogeneity. The degree of variation inherent in the techniques used was evaluated.

In identifying the appropriate mycobacterial surrogate, different strains were grown on blood agar plates. Of these examined, both *M. smegmatis* and *M. fortuitum* had relatively quick growth rates of 3 days. However, the *M. smegmatis* could not be easily enumerated due to the shape the colonies and therefore, *M. fortuitum* selected as the surrogate of choice, as it grew rapidly and formed well-defined, discrete colonies. The value of *M. fortuitum* as a surrogate (Da Silva et al. 2002) stems from a pattern of macrophage infection similar to that seen with *M. tuberculosis* in both human (Tomioka et al. 2004) and murine cell-line models (Parti et al. 2005). *M. fortuitum* has been used as a model for the evaluation of quinolone antibiotics (Gillespie et al. 2001), as a strain resistant to standard regimens in drug-susceptibility testing (Goldstein et al. 1971) and in standard assays for antimycobacterial activity (Ho et al. 1997). Glycolipids from *M. fortuitum* cell wall have also been used in the serodiagnosis of pulmonary TB (Escamilla et al. 1996).
Intracellular *M. fortuitum* levels, shown as colony forming units (CFUs), in the macrophages increased over time. After 4 h, the maximum uptake capacity of the macrophages had been reached.

![Graph showing CFUs over time](image)

**Figure 2.1**: Number of viable intracellular *M. fortuitum* following phagocytosis by murine peritoneal macrophages. (± SEM, n=20)

Extracellular mycobacterial levels were established during the macrophage assay. Prior to washing and lysing the macrophages, at each hourly interval, the supernatant in the cell-culture well was plated onto blood agar plates and the mycobacteria were grown. The results are shown in Figure 2.2.
Figure 2.2: Extracellular bacterial levels during engulfment of *M. fortuitum* by macrophages. (n=6)

Extracellular mycobacterial levels decrease during the macrophage assay as the mycobacteria are phagocytosed by the macrophages.

To determine that all extracellular material, which included mycobacteria, was removed from the macrophage monolayer after washing the cells twice with PBS, each wash was plated and any bacterial colonies were counted during a standard macrophage assay. The results are shown in Figure 2.3.
There are no viable CFUs remained following 2 washes of the macrophages during the macrophage assay, showing washing the monolayer twice is sufficient to remove any adherent extracellular material.

2.3.2 Multiplicity of Infection

To ensure that there were sufficient mycobacteria in the suspensions exposed to macrophages to ensure uptake by the overwhelming majority of phagocytes, the probability of mycobacterial uptake was determined using the Poisson distribution:

\[ P(n) = \frac{m^n e^{-m}}{n!} \]
where ‘$P(n)$’ is the probability that a cell will be infected with a mycobacterium, ‘$m$’ is the multiplicity of infection (MOI), and ‘$n$’ is the number of mycobacteria infecting the macrophages. An MOI of 10 provided the highest probability of infecting the macrophages; this was confirmed by examination of the MOI in the macrophage assays using different bacterial concentrations, as detailed below.

A range of different MOIs were tested. Initially, MOI was increased by unit increments, from 1 to 10, and thereafter by increments of 10, from 10 to 100. The result is shown in Figure 2.4.

The results show that the addition of $1 \times 10^4$ cells/ml gives the highest percentage of uptake of bacteria in macrophages. However, with a steep trend in mycobacterial uptake, there was a high possibility that the macrophages were not fully saturated with ingested mycobacteria. Using an MOI of 100, there was a high count of intracellular mycobacteria cells. However, when the counts were expressed as a percentage of the total number of mycobacteria added to the macrophages, the percentage values were very low and the macrophage assay using an MOI of 100 in the protocol was difficult to validate. When an MOI of 10 was employed in the macrophage assay, the mycobacterial counts were readily determined, showing a quantifiable uptake of mycobacteria when expressed as either CFU or percentage compared to the other MOIs.
Figure 2.4: Effect of MOI on *M. fortuitum* uptake by macrophages. To $1 \times 10^4$ macrophage cells/ml, different numbers of mycobacteria were added to macrophages: (A) (Mycobacterial counts as CFUs) $1 \times 10^4$ cells/ml (MOI = 1) (○); $1 \times 10^5$ cells/ml (MOI = 10) (■); $1 \times 10^6$ cells/ml (MOI = 100) (▲). (B) (Mycobacterial counts as percentage of total mycobacteria added to macrophages) $1 \times 10^4$ cells/ml (○); $1 \times 10^5$ cells/ml (■); $1 \times 10^6$ cells/ml (▲). (+/- SEM, n=6)
2.3.3 Trypan Blue Exclusion Method

Some solvents may have detrimental effects on macrophages; care must therefore be taken in the selection of solvents for solubisation of extracted material and compounds of interest if they are to be incorporated into macrophage bioassays. The Trypan Blue Exclusion method was used to establish macrophage viability and indicated that macrophages tolerated only low concentrations of solvents such as dimethyl sulfoxide (DMSO) and ethanol (EtOH). These two solvents were used to solubilise the material extracted from *Pelargonium* with apolar solvents. Macrophage viability over a 5 h period under assay conditions is indicated in Figure 2.5.

![Figure 2.5: Macrophage viability determined using Trypan Blue stain. (n=6)](image)

Macrophage viability was maintained when the cells were incubated under assay conditions; the addition of either DMSO or EtOH at concentrations greater than 5% resulted in loss of viability (Figure 2.6 and 2.7).
Figure 2.6: Macrophage viability upon addition of different concentrations of DMSO (n=6).

Figure 2.7: Macrophage viability upon addition of different concentrations EtOH. (n=6)
2.3.4 Macrophage Uptake Assay of *M. fortuitum*

Using the Ziehl–Neelsen stain, it was possible to enumerate the uptake of mycobacteria by macrophage preparations. Figures 2.8 and 2.9 shows a Ziehl-Neelsen stain of *M. fortuitum* inside macrophages.

![Figure 2.8: A Ziehl-Neelsen stain of *M. fortuitum* (red, indicated by arrow) inside macrophages (blue) showing a medium count (5-19) of mycobacteria.](image)

Figure 2.8: A Ziehl-Neelsen stain of *M. fortuitum* (red, indicated by arrow) inside macrophages (blue) showing a medium count (5-19) of mycobacteria.

![Figure 2.9: A Ziehl-Neelsen stain of *M. fortuitum* (red, indicated by arrow) inside macrophages (blue) showing a high count (>19) of mycobacteria.](image)

Figure 2.9: A Ziehl-Neelsen stain of *M. fortuitum* (red, indicated by arrow) inside macrophages (blue) showing a high count (>19) of mycobacteria.

To investigate the effect of MOI on uptake, different concentrations of mycobacteria were added to the macrophages (Figures 2.10 and 2.11).
Figure 2.10: Macrophage uptake of *M. fortuitum* at MOI of 10: The histogram shows the percentage of macrophages containing numbers of mycobacteria in the ranges: <5 bacteria (■); 5–19 bacteria (●); >19 bacteria (■). (n=6)

Figure 2.11: Macrophage uptake of *M. fortuitum* at MOI of 100. Total counts of bacterial cells inside the macrophages were classed into three groups: <5 bacteria (■); 5–19 bacteria (●); >19 bacteria (■). (n=6)
When the MOI was increased, there was a comparable increase in the numbers of mycobacteria engulfed by macrophages. In summary, the proportions of macrophages engulfing low, medium or high levels of mycobacteria were found, using the Ziehl–Neelsen stain, to be correlated with the MOI and to be time-dependent.

2.4 Discussion

To investigate the effect of *Pelargonium* extracts on macrophage function, the development of a reliable assay was necessary in order to evaluate bioassay-guided fractionation based on stimulation of macrophage phagocytic activity. Such procedures would facilitate the identification of components with immunomodulating activity.

Test compounds have been applied to various macrophage assays and any change of macrophage function can be indicative of the immunomodulatory potential of the compound. Although the macrophage forms a single component in the immune system, it is a common marker for immunomodulation (Benencia et al. 1999).

Studies have shown that various types of assays have been performed to monitor the potential activity of possible bioactive compounds. The effect of plant extracts or other compounds isolated from bacterial sources on isolated macrophages has been used to evaluate any immunomodulatory potential. Measuring the release of nitric oxide is a common tool to gauge increased macrophage function. Macrophages encountering lipids derived from *Salmonella typhi* (Balasubramanian et al. 2007) were found to have augmented nitric oxide production, indicating an increased killing potential of the macrophages. The
stimulant action of compounds isolated from plant material, such as flavonoids and alkaloids derived from *Desmodium gangeticum*, on macrophages has been used to describe compounds with immunomodulatory capabilities by also showing increased nitric oxide production (Mishra et al. 2005). The activation of macrophages has also been used to demonstrate immunomodulatory capability of compounds by quantifying the release of cytokines, such as interleukins and TNF-α. The immunomodulatory activity of polysaccharides isolated from plant, such as *Juniperus scopolorum* (Schepetkin et al. 2005) and fungal sources, such as *Pestalotiopsis leucothes* (Kumar et al. 2005), have been identified in this manner. Even activated macrophages with internalised parasites have been shown to have increased activity by plant extracts by measuring cytokine and nitric oxide release (Kolodziej & Kiderlen 2005).

Alternatively, a change in counts of viable phagocytosed bacteria has been used as a marker for enhanced macrophage function. The bacteriostatic nature of compounds derived from *Carpobrotus edulis* on both multi-drug resistant *M. tuberculosis* and methacillin-resistant *Staphylococcus aureus* have been identified using macrophages and enumerating their intracellular bacterial colonies (Martins et al. 2005).

However, whilst chemical, such as cytokine release, and physical changes, such as the increased spreading ability of macrophages (Choi et al. 2001), shows that a compound has exhibited an immunomodulatory effect, it does not necessarily confer that any intracellular pathogens will be eliminated. Therefore intracellular counts of bacterial pathogens are necessary.

A new protocol using murine macrophages and the mycobacterial surrogate, *M. fortuitum*, was established to investigate the immunomodulatory activity of
extracts derived from *Pelargonium* roots. The macrophage assay used provided a robust method to test the capacity of extracts to modulate intracellular levels of mycobacteria, as determined by multiple repeats of the assay and the calculation of the standard error of the mean. The Trypan Blue stain was necessary to validate the macrophage assay protocol as well as to determine the concentration of the solvents to be added to the macrophages. A variation in results was likely to be due the innate variation in the cells, such as different enzyme and cytokine concentrations. The inbred strain of BALB/c mice, with a high degree genetic identity, was used to minimise this risk.

Altering the MOI determined the most appropriate concentration of mycobacteria to be added to the macrophages to ensure quantifiable uptake of the mycobacteria in the absence of plant extracts. After testing different MOIs, it was established that a ratio of 1:10 macrophages to mycobacteria was needed in the macrophage assays.

The Ziehl–Neelsen stain was used to investigate the uptake of bacteria as an alternative method to determining intracellular mycobacterial numbers. The percentage of macrophages with high levels of mycobacterial loads increased over time. Although Ziehl–Neelsen staining of intracellular mycobacteria gave some indication of the capacity of macrophages to engulf the bacterium, direct observation provides little information on the true rate and extent of uptake and killing. Several methods are available that would shed further light on dynamic aspects of the interaction of macrophages and mycobacteria. With confocal microscopy, the movement of bacteria within macrophage can be examined and the degree of killing ascertained. Bacteria can be engineered to express green fluorescent protein (GFP) and this permits the tracking of the intracellular
movement of labelled bacteria, including mycobacteria (Parker & Bermudez 1997). Mycobacteria modified to express GFP have been used for the screening the mycobactericidal activity of germicides (Zafer et al. 2001). GFP has also been used to characterise the activity of mycobacteria residing within macrophages exposed to anti-TB drugs (Srivastava et al. 1998) and to study the activity of mycobacterial promoters (Triccas et al. 2001).

Evidence is emerging that Umckaloabo exerts its range of biological effects through their capacity to directly kill infectious organisms and stimulate the immune system (Kolodziej & Kiderlen 2007). Therefore, the development of a suitable macrophage assay was essential. The fractionation of the plant material was also entirely dependent of the feedback from the macrophage assays. A new macrophage assay protocol was developed to identify bioactive fractions to assess macrophage function by enumerating intracellular mycobacteria and can be applied for use with other micro-organisms and adapted for use with different parameters. The data obtained here enabled a comparison of the uptake of mycobacteria by macrophages in the presence and absence of extracts of Pelargonium to be undertaken and a macrophage assay based on this principle was utilised as a key methodology in this study.
Chapter 3

FRACTIONATION OF PLANT MATERIALS
3.1 Introduction

Plants used in traditional medicines from South Africa have been dried and ground to obtain crude extracts using the Soxhlet apparatus, so that they could be screened as antibacterial agents (Reid et al. 2005). The use of solvents of increasing polarity ensures the extraction of a broad range of compounds. By identifying fractions capable of stimulating macrophages, further fractionation can be used to obtain purified bioactive compounds (Grael et al. 2005).

Solid phase extraction (SPE) is used as a chromatographic method to separate compounds or concentrate samples (Zhong 1998). However, it is commonly used to clean a sample before subjecting it to further fractionation. Both water-soluble and apolar compounds can be applied to SPE (Xie et al. 2003).

3.2 Materials and Methods

3.2.1 Fractionation Procedures

Solvents used in the general fractionation of the Pelargonium roots and to dissolve extracts were purchased from Fisher Scientific (Loughborough, UK). Two fractionation procedures were performed to obtain active material based on the method described by Seidel and Taylor (2004): extraction using a Soxhlet apparatus followed by maceration of the roots. The macrophage assay described in Section 2.2.3 was applied to test the extracts for bioactivity. Dried material from the apolar residue, obtained from hexane and ethyl acetate Soxhlet extractions, were dissolved in the least amount of dimethyl sulfoxide (DMSO) or ethanol (EtOH) compatible with complete solubilisation. These solutions were
then diluted with water. The Trypan Blue Exclusion method (Section 2.2.5) was applied to ensure macrophage integrity during bioassay.

### 3.2.2 Soxhlet Extraction

The Soxhlet apparatus uses reflux to heat and cool solvents, enabling maximal compound extraction whilst using the least amount of solvent. It is particularly useful for plant extractions as active compounds may be present only in small quantities and relatively large quantities of plant material can be sourced.

Once the *P. reniforme* (Simply Indigenous, Skeerpoort, South Africa) and *P. sidoides* (Travena Cross Nurseries and Garden Centre, Surrey, UK) were obtained, their identities were confirmed by comparing the leaves, flowers and roots with reference literature (*Geraniums: The Complete Encyclopedia*, 1st ed., 2003). The roots were then washed, cut and allowed to dry to constant weight. The roots were ground to powder using a hammer mill with a 1-mm sieve.

The powder was subjected to Soxhlet extraction with three solvents: hexane, ethyl acetate, and ethanol. Figure 3.1 illustrates the Soxhlet apparatus.

Figure 3.1: Soxhlet apparatus.
The Soxhlet was used for at least one week for each solvent extraction. A thimble to hold the root material was made with Whatman filter paper. As all extracts were coloured solutions, the endpoint of the extraction procedure could be readily determined. The extract, dissolved in the solvent used during Soxhlet extraction, was transferred from the Soxhlet apparatus to a 5-litre flask.

The liquid extracts were decanted into weighed empty 250-ml round-bottomed flasks and subjected to rotary evaporation. The temperature of the water bath was kept to a minimum. However, with ethanol extraction, the higher temperature of 50°C was deemed necessary in order to achieve solvent evaporation. As the solvent was removed, the collecting flask was refilled with more extract solution until all the solvent had evaporated. This required approximately 1 day for the hexane and ethyl acetate extracts. However, the ethanol evaporation required at least 2 days. The flasks were weighed prior to and following evaporation to constant weight in order to establish the dry weight of extracted material.

3.2.3 Maceration

The residual root material remaining after Soxhlet extraction was subjected to maceration with 50% ethanol. The solvent was boiled under reflux and added to the remaining root matter in a 5-litre round-bottomed flask. This was left for 1 week with periodic stirring. The root extracts were collected by pouring the liquid over gauze tissue to remove the large root particles prior to decanting through a Buchner funnel lined with Whatman filter paper to remove smaller particles. Ethanol was removed by rotary evaporation. The remaining aqueous portion of the extract solvent was transferred to pre-weighed 250-ml flasks, which were then freeze-dried to remove water and weighed.
The final general fractionation procedure applied to the residual root material was the addition of sterilized distilled water. After 1 week, the extract solution was decanted and the water in the solution was removed by freeze-drying and the remaining extract weighed.

3.2.4 Solid Phase Extraction

After ascertaining the *Pelargonium* extract obtained from general fractionation with the highest macrophage stimulatory effect using the macrophage assay, solid phase extraction (SPE) was used to further isolate bioactive compounds in the *Pelargonium* root extracts following Soxhlet and maceration. A C18 column (Phenomenex, Macclesfield, UK) was used to elute solvents using a 0 to 100% methanol/water gradient in 10% increments. After loading the column with *Pelargonium* extract solution, 20 ml of the methanol/water mix was added to the column under pressure and the eluate was collected in round-bottomed flasks. UV absorbance was measured using a spectrophotometer to detect the presence of compounds: the UV absorption of PRW was ascertained prior to SPE, and the absorption wavelength was found to range between 256 and 366 nm. The methanol and water were removed from the extracts obtained after SPE and the macrophage assay was performed to identify the fraction containing bioactive compounds.
3.3 Results

3.3.1 General Fractionation of Pelargonium

The dried and powdered roots of the Pelargonium plants were used in bioassay-guided fractionation. The extracts collected from Soxhlet extraction and maceration were dried and weighed. The dry weights of these fractions are presented in Table 3.1.

Table 3.1: Extracts obtained by Soxhlet extraction and maceration of P. reniforme and P. sidoides roots. The yield of extract is expressed as a percentage of the total dry weight of the roots.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. reniforme</td>
</tr>
<tr>
<td>Hexane</td>
<td>0.62</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.08</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7.03</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>2.64</td>
</tr>
<tr>
<td>Water</td>
<td>0.98</td>
</tr>
<tr>
<td>Undissolved residue</td>
<td>87.65</td>
</tr>
</tbody>
</table>

The yields obtained reflect differences in root composition of the two plant species. There is a high probability that a large number of chemically identical compounds are present in both species (Kolodziej 2000), although their relative concentrations may differ.
Table 3.2 lists the abbreviated names allocated to each extract following Soxhlet extraction and maceration. These identifiers are used hereafter when describing a particular extract.

Table 3.2: Designations of *Pelargonium* extracts.

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th><em>P. reniforme</em> (PR)</th>
<th><em>P. sidoides</em> (PS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>PRH</td>
<td>PSH</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>PRE</td>
<td>PSE</td>
</tr>
<tr>
<td>Ethanol</td>
<td>PRAa</td>
<td>PSAa</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>PRAb</td>
<td>PSAb</td>
</tr>
<tr>
<td>Water</td>
<td>PRW</td>
<td>PSW</td>
</tr>
</tbody>
</table>

The capacity of extracts to modulate macrophage uptake of mycobacteria was determined using the macrophage assay described in Chapter 2. Although water or PBS are the preferred solvents for bioassay of material recovered from *Pelargonium* extracts, material obtained using the apolar solvents, hexane and ethyl acetate could only be dissolved in solvents that are lipophilic in nature, such as DMSO and EtOH. High concentrations of these solvents are toxic for macrophages. The Trypan Blue exclusion method (Section 2.3.3) was used to establish that a concentration of less than 5% of DMSO or EtOH in water mix was suitable for use in the bioassay.
The Trypan Blue exclusion method was also used to determine the toxicity level of the *Pelargonium* derived material. Therapeutic levels of *Pelargonium* root extracts have not been established in either animal or human models. Therefore during this study, the highest possible concentration of each extract was tested to study the putative effects of the plant extracts on intracellular survival of *M. fortuitum* in macrophages.

### 3.3.2 Macrophage Assay of Pelargonium Extracts

*Pelargonium* extracts were examined for their capacity to modulate the uptake and killing of *M. fortuitum* by macrophages as described in Section 2.2.3. A concentration of 0.25mg/ml was used for all extracted material. Figures 3.2 and 3.3 show the difference in activity of different extracts of *P. reniforme* and *P. sidoides* on intracellular mycobacterial levels over a 5 h period using the macrophage assay.

Based on anecdotal hearsay that rhubarb had antitubercular effects, rhubarb root extracts, derived by using the same methods to extract the fractions from the Pelargonium roots, were tested as a control using the macrophage assay. The results are shown in Figure 3.2.
Figure 3.2: Effect of extracts of rhubarb root (RR) extracts (0.25mg/ml) on percentage of viable intracellular M. fortuitum following phagocytosis by murine macrophages: Control (no extract) (◊); Hexane extract RR (■); ethyl acetate extract RR (▲); ethanol extract RR (×); 50% ethanol extract RR (★); water extract RR (□). (+/- SEM, n=6)
The extracts derived from fractionation of the rhubarb roots using hexane, ethyl acetate, ethanol, 50% ethanol and water showed the same increasing level of viable intracellular *M. fortuitum* over time. The mycobacterial levels, both in the absence and presence of rhubarb root extracts, remained unchanged.

The *Pelargonium* extract that exhibited the greatest activity, as determined by the macrophage assay, was PRW (see Table 3.2). The difference in activity between the water extracts of *P. reniforme* and *P. sidoides* is shown in Figures 3.3 and 3.4.

Corresponding fractions from each plant exhibited differences in activity, implying either a difference in root composition or in the concentration of active compounds between plant species as shown in Figure 3.5.
Figure 3.3: Effect of extracts of *P. reniforme* (0.25 mg/ml) on intracellular viability of internalised *M. fortuitum*: (A) Control (no extract) (○); PRH (■); PRE (▲); (B) PRAa (×); PRAb (+); PRW (□). (+/- SEM, n=6)
Figure 3.4: Viable intracellular *M. fortuitum* quantified after exposure of murine macrophages to *P. sidoides* extracts: (A) Control (no extract) (○); PSH (■); PSE (▲); (B) PSAa (×); PSAb (●); PSW (□). (+/- SEM, n=6)
3.3.3 Effect of Concentration

The capacity of extracted material to modulate the survival of mycobacteria within macrophages was found to be concentration dependent. Tenfold dilutions of the bioactive extract PRW in water were made and used in the macrophage bioassay (Figure 3.6).

A correlation between decreasing extract concentrations and increasing viable intracellular mycobacterial levels was established.
Figure 3.6: Effect of decreasing PRW concentration on viable intracellular *M. fortuitum* phagocytosed by macrophages; (A) Control (no extract) (○); 2.5 mg/ml (■); 0.25 mg/ml (▲); 0.025 mg/ml (×); (B) Control (no extract) (○); 2.5 mg/ml (■); 2.5 µg/ml (□). (+/- SEM, n=6)
3.3.4 Extracellular mycobacteria

Figure 3.7 shows the effect of PRW-extracted material on the numbers of extracellular mycobacteria present following incubation with macrophages. There were little or no differences in mycobacterial numbers in assays containing extracted material compared to controls.

![Figure 3.7: Proportion of M. fortuitum present in the extracellular milieu: Control (■); PRW 2.5 mg/ml (■). (n=6)](image)

Thus, the addition of material obtained by PRW extraction of P. reniforme and P. sidoides did not affect extracellular mycobacterial levels compared with the untreated control.

Bioassay-guided fractionation revealed that the water extract of P. reniforme (PRW) exhibited the greatest macrophage stimulation, producing a high degree of reduction of intracellular mycobacteria compared with other extracts of both Pelargonium species. Material from this extract was therefore deemed suitable for
further purification of bioactive compounds with a high probability of obtaining single species of bioactive compounds.

3.3.5 **Macrophage Assay of SPE Extracts**

The high probability that the PRW extracted material contained a large number of individual molecular species necessitated further fractionation, which was undertaken using SPE: a C18 column, eluting with a methanol gradient. A discontinuous gradient comprising of 10% increments of methanol was applied to the column. The solvent was removed from the extracts by rotary evaporation. Dried fractions were dissolved in water to give a final concentration of 0.25mg/ml and bioassay undertaken. Table 3.3 shows the result of the bioassay performed on the extracts obtained from SPE.
Table 3.3: Viable intracellular *M. fortuitum* following phagocytosis by murine macrophages exposed to PRW extracts obtained from SPE fractionation using a C18 column. Increasing concentrations of MeOH were passed through the solid-phase column: (A) Control; 0–40% MeOH; (B) 50–100% MeOH.

(A)

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Figure 3.8 shows a representation of some of the results from Table 3.3.

The macrophage assay showed that the fraction with the highest capacity to stimulate ingestion of mycobacteria by was the extract eluted with 0% methanol. This demonstrated that the most bioactive compounds were contained within this extract, which was subjected to further fractionation.
3.4 Discussion

Previously, compounds derived from *Pelargonium* with activity against intracellular leishmaniasis were characterised (Kayser et al. 2001). The beneficial effect of an aqueous extract from the roots of *P. sidoides* have also been established in patients with bronchitis (Matthys et al. 2006) and *Helicobacter pylori* infections (Beil & Kilian 2006).

The analysis of *P. sidoides* extract combined with a macrophage assay has demonstrated the use of macrophages to investigate the possible bioactivity of the plant extracts. However, a systematic analysis of a range of solvent extracts obtained from the fractionation of the roots of both *P. reniforme* and *P. sidoides*, the components of the traditional herbal remedy Umckaloabo, had not been performed. In addition, no compounds with immunomodulatory activity against intracellular mycobacteria had been reported. Therefore, the aim of this research was to identify solvent extracts from both *Pelargonium* species with activity against intracellular mycobacteria.

A methodical approach to fractionating the Pelargonium roots was undertaken by using solvents of increasing polarity. The Soxhlet apparatus has been commonly used to fractionate both root and aerial parts of plants either to characterise the constituent components or establish the bioactive nature of the plants (Romanik et al. 2007). The use of newer fractionation techniques over the classical Soxhlet extraction technique, such as ultra-sound-assisted extraction or supercritical fluid extraction, can provide higher quantitative yields of extracted material (Punin Crespo et al. 2006), but have a limited field of application (Wang & Weller 2007). Antimicrobial extracts have been isolated from *Rosmarinus officinalis* (Celiktas et al. 2005) and antiviral extracts obtained from endogenous Indian medicinal plants,
such as *Aegle marmelos* and *Allium sativum* (Balasubramanian et al. 2007), have been fractionated using the Soxhlet with various solvents, such as ethers, alcohols and water.

Solvent extraction of the roots of *P. reniforme* and *P. sidoides* by Soxhlet distillation indicated significant differences in the composition of the roots of these two species and provides some insight into why the traditional concoction is comprised of material from both species. Although previous experiments have shown that compounds with the capacity to kill mycobacteria could be qualitatively recovered from both species, the situation with regard to compounds with immunomodulatory activity appears more complex; there were large differences in recovery and bioactivity between the two species. It has been suggested that the anti-tubercular activity of Umckaloabo derives from both its antibacterial and immunomodulatory activity (Kolodziej 2000) and it is therefore important to highlight such differences between the two plants that contribute to perceived efficacy.

It can be seen that the water extract of *P. reniforme* has the greatest activity; although the water extract of *P. sidoides* also has a substantial effect on intracellular mycobacteria, its effect was much reduced in comparison to that obtained from *P. reniforme*. This further highlights the differences between the two plants. The active compounds may be identical in both, but the proportions contained in the root appear to differ and this may account for the different activity levels observed.

SPE has been used to separate water-soluble analytes (Buchberger 2007) and aqueous plant extracts (Le Moullec et al. 2007) and provided the first step in a two-phase extraction of proteins (Gu & Glatz 2007). However, SPE has not been
used in fractionating extracts obtained from *P. reniforme* and in the present study, evidence was provided that SPE provided a useful step in isolating the bioactive components derived from *P. reniforme* into a single fraction.

The macrophage assays demonstrated that the water extract of *P. reniforme* exerted the greatest immunomodulatory effect. When compounds with direct antimycobacterial activity were purified from both plant species, fatty acids such as linoleic acid were found in both (Seidel & Taylor 2004). In this study, it is possible that bioactive compounds with immunomodulatory activity are found in both plants as it is known that they share similar compound compositions. However, the differences in either the constituent compounds or their concentration could account for the difference in intracellular mycobacterial levels.

The systematic fractionation of *P. reniforme* and *P. sidoides* using Soxhlet followed by solid-phase extraction to identify extracts with possible immunomodulatory activity has been elaborated for the first time in this study. Although the aqueous extract of *P. sidoides* has been used repeatedly for study (Conrad et al. 2006; Matthys & Heger 2006), the discovery that the water fraction of *P. reniforme* induced a significant reduction in viable intracellular mycobacteria during this research shows evidence-based reasoning behind the inclusion of both *Pelargonium* species in the remedy Umckaloabo.
Chapter 4

CHROMATOGRAPHY
4.1 Introduction

Further purification of bioactive components was facilitated by bioassay-guided fractionation using a number of chromatographic techniques (Merghem et al. 2004).

Chromatography is frequently used to isolate and purify components from complex mixtures and column chromatography was considered the most appropriate practical application for the current task. Different chromatographic procedures employing various phases for partition of molecules of interest are, in combination, powerful tools for resolution of mixtures of compounds with differing physical and chemical characteristics (Uhlig et al. 2005). A variety of phases are employed: modern counter-current chromatography uses liquid phases for separation (Marston & Hostettmann 2006); liquid–liquid chromatography can be applied as an initial fraction step for crude extracts and then subjected to further column chromatography (van der Watt & Pretorius 2001). Fractionation procedures, such as the use of a series of organic solvents that are immiscible with the medium containing crude extracts, are employed in combination with high-performance liquid chromatography (HPLC). However, the use of the solid–liquid phase still predominates. Regardless of the method of choice, procedures need to be tailored in order to optimise the separation of the desired components (Morineau et al. 1997).

To investigate the potential utility of plant-based medicines, it is desirable to undertake an investigation of the bioactivity of crude extracts; for example, the aqueous extracts of some Ethiopian plants, such as *Syzygium guineense* and *Albizia gummifera*, have yielded promising compounds with antimicrobial
activity detected by evaluation of the antimicrobial potential of fractions obtained by guided bioassay (Geyid et al. 2005).

Procedures for the isolation of known components from plant extracts are generally straightforward, precedents have been established and the conditions for separation and purification can be anticipated. For example, reversed-phase HPLC followed by normal-phase liquid chromatography has been used to isolate the cytotoxic principles of fungal contaminants (Uhlig et al. 2005). HPLC can be used to identify individual compounds (Jayaprakasha et al. 2005), but it is only appropriate if the compounds have been previously purified and their structure and physicochemical characteristics established.

Knowledge of the structural identity and the properties of a target compound guide the choice of methodology for its isolation. However, in the current study, information concerning the nature and number of target bioactive compounds is limited; the solvent fractionation procedure employed and evaluation of bioactivity by macrophage assay inform only that the target activity resides with polar compounds. Bioassay-guided fractionation is used regularly for recovery of compounds from plant extracts. Determination of the activity within bioactive fractions aids the isolation of the compounds. While the compounds are unidentified, the extraction procedure is known and so its properties can be exploited.

Column chromatography encompasses a broad spectrum of separation procedures. For examples, the bioassay-guided fractionation and assay of a vasorelaxant compound from the Kaempferia plant (Othman et al. 2006) showed that column chromatography could be employed for its isolation.
The separation and isolation of compounds from crude sources frequently follow a similar path. These procedures involve a series of fractionation steps for isolation of bioactive compounds by a process that involves progressive refinement of the molecular composition of the mixture. Procedures for purification of compounds from apolar and polar fractions are broadly similar, although the solvents and columns employed may differ. These chromatographic procedures may be applied to the separation of compounds in aqueous solution, although separation of highly polar compounds may be problematical. Molecular species in aqueous extracts can be separated in a number of ways; for example, a silica-gel column combined with thin-layer chromatography (TLC) may be employed (Melo et al. 2005).

Size-exclusion chromatography (SEC) is a simple yet effective technique for the fractionation and purification of bioactive components of complex mixtures. Organic material usually contains a substantial number of molecular species of compounds with differing molecular weights. SEC has been used to separate compounds within a molecular weight range (Li et al. 2005). Different solid-phase materials can be used for separation of molecules of interest; these include silica gel and/or Sephadex LH-20 (Ehrlich & Lillehoj 1984). In this context, SEC has also been used in conjunction with mass spectrometry for fractionation and identification of molecules from plant seeds (Koplik et al. 2002).

The biophysical properties of molecules of interest should form the basis for the development of chromatographic procedures designed to optimise purification: matrices used in such separation technology have been, in combination with the mobile phase employed and the conditions imposed, developed to ensure efficient separation of target molecules. Gel-filtration chromatography uses an aqueous
mobile phase to transport a mixture of molecules through a size-exclusion column. Sephadex, a cross-linked dextran gel, is commonly used for this purpose. Sephadex LH-20 has been frequently used for the separation of molecules of a lipophilic (Pottier et al. 2003) or aqueous nature (Mensah et al. 2001).

Hydrophilic interaction chromatography (HILIC) has been used to separate and isolate highly polar compounds of plant origin (Tolstikov & Fiehn 2002). It has also been used in the analysis of metabolites (Idborg et al. 2005) and other components of human and animal origin, such as plasma constituents (Bengtsson et al. 2005). The use of HILIC for the analysis of biological systems indicates that it can be applied reliably to the isolation of bioactive compounds from *Pelargonium*.

Studies with *Pelargonium* have utilised a combination of these techniques (Kayser & Kolodziej 1995). From an initial crude acetone and water extract, fractionation was performed in this study using chloroform, ethyl acetate and n-butanol. Further separation involved the use of Sephadex LH-20, silica gel and RP-18 to obtain coumarins from root material. HPLC has also been used to isolate flavonoids from *Pelargonium* leaves (Williams et al. 1997).

Ion-exchange chromatography (IEX) has been used to purify peptides (Zhang & Lewis 1997) and to separate constituents from their plant sources (Murray, Nichols, & Sassenrath-Cole 2001). Anionic ion-exchange chromatography in combination with cationic ion-exchange columns has been employed for the separation and purification of bioactive compounds (Yun et al. 2005).

As initial extraction techniques frequently involve the use of organic solvents, reverse-phase chromatography has been frequently utilised for the separation of compounds from plant sources (Moulin et al. 2002). Normal-phase HPLC uses
more polar solvents and stationary phases and is more appropriate for use with aqueous extracts.

Thus, the approach chosen in the present study involved the use of a combination of the methods described above for the isolation and identification of bioactive compounds: SEC was initially employed, followed by IEX, with HILIC as the final fractionation procedure.

4.2 Materials and Methods

4.2.1 Size-exclusion chromatography

Gel filtration was employed as the primary step for compound separation; this is a common procedure for initial fractionation of complex mixtures. Sephadex LH20 (Sigma-Aldrich, Gillingham, UK) has been used previously for such procedures, particularly in SEC.

All buffers and samples were filtered prior to use through membrane filters (0.45 μm diameter pore size). Sephadex LH-20 was suspended in distilled water (4 ml/g dry powder) and allowed to swell in excess water for at least 3 h, avoiding excessive stirring in order to prevent the formation of air bubbles. A slurry, with a ratio of 75% settled gel:25% water, was prepared. The settled gel was suspended by gentle agitation and used to fill a stoppered glass column of 1 × 50 cm by running suspended Sephadex over a glass rod, ensuring smooth filling of the column. A reservoir was then attached to the top of the column and topped off with water; the Sephadex was allowed to settle (an appropriate degree of settling could be determined by observing a static column length). The column outlet was opened and the column was washed several times with water. The column was
then examined to verify the absence of air bubbles. Finally, it was equilibrated with the eluting buffer. The column and samples were maintained at room temperature. The optimal sample load of the Sephadex column is 1–2% of the bed volume, where the bed volume is the amount of liquid contained in one column; therefore, 20 ml of PRW was added and allowed to adsorb into the column. Two hundred millilitres of water was then added prior to gravity-fed elution with water. A low flow rate of 1 ml/min was employed to ensure optimal separation of the components of the mixture. One millilitre fractions were collected and eluant monitored spectrophotometrically in the UV range; the UV-absorbance spectrum of the PRW sample indicated maximum absorbance at 220 nm. Fractions were stored at –20°C until required.

After use, the column was washed thoroughly, with at least 2 bed volumes of water. To repeat the separation, the column was washed with 2–3 bed volumes of the eluent and then re-equilibrated. Upon completion, the Sephadex column was equilibrated with 20% ethanol and stored at 4–8°C. The fractions were then examined for their capacity to modulate uptake and killing of mycobacteria by macrophages.

4.2.2 High Performance Liquid Chromatography

The aqueous extract of *Pelargonium* roots displayed the highest level of bioactivity, indicating that at least some presumptive macrophage modulators were highly hydrophilic in nature; this limited the use of columns for separation and purification. Initially, a C18 column and an ODS column purchased from Phenomenex (Macclesfield, UK) were examined for their capacity to effect
separation of bioactive components of the PRW fraction; however, neither column enabled complete purification of the target molecules. Modification of the solvent gradient had little or no effect on compound separation.

Thus, HILIC was used to assess the capacity of this procedure to effect appropriate fractionation of the compound mixture; the ZIC-HILIC column (SeQuant, Theale, UK), which separates highly polar compounds, was employed. The column was attached to the HPLC instrument and washed thoroughly. HPLC-grade water and acetonitrile (ACN) was purchased from Fisher-Scientific (Gillingham, UK). A cleaning step employing water as eluant was used to remove polar impurities and organic solvent residues and the column was then flushed with 0.5 M sodium chloride (NaCl). Water was passed through the column to remove the salt solution and the column was maintained and stored with 80% ACN. Prior to use, the ZIC-HILIC column was equilibrated with 95% ACN. An ACN–water gradient was used to elute molecular species from the column. After the column had been stabilized, 20 μl of extract solution was loaded and a solvent gradient of 95%–5% ACN was applied over varying time periods to allow for maximal separation of compounds. The time was altered during the test runs to determine the optimal separation of compounds in the solution. A large number of peaks were observed; the mixture could not be fully resolved by increasing the time of elution and further fractionation of the water extract was deemed necessary in order to produce satisfactory resolution.

4.2.3 Ion Exchange Chromatography

IEX was used for further fractionation. As the identities of the compounds were unknown, a number of columns were evaluated in order to establish optimal
separation conditions. Both anionic and cationic exchange columns were employed. A selection kit from HiTrap (Amersham Biosciences, Little Chalfont, UK) contained a variety of exchange columns. Columns with high binding capacities were selected; an anionic column, HiTrap QFF, and a cationic column, HiTrap SPFF.

Separation may be effected with IEX with either a pH or an ionic concentration gradient. As fractions obtained following chromatography of the PRW extract were to be evaluated for their effect on macrophage uptake and killing of mycobacteria, separation was undertaken using a salt gradient, raised incrementally in units of 0.1 M and the pH maintained at 7.

The columns employed required different buffers (purchased from Sigma Aldrich (Gillingham, UK)) to maintain pH 7; the cationic column was used with a sodium phosphate (NaPO₄) buffer and the anionic column with a Tris(hydroxymethyl)aminomethane (Tris) buffer. The NaPO₄ buffer was prepared from powder and adjusted to pH 7. Five millilitres of eluting buffer of increasing concentrations of salt (NaCl), from 0.1 to 0.5 M, were prepared. The buffers and plant extracts were filtered to remove particulate matter greater than 0.45 μm and the sample applied to the column at a rate of 1 ml/min. Any eluate was collected. Thereafter, 5 ml of each eluting buffer was applied to the column at a rate of 1 ml/min and fractions collected and desalted using a desalting column. A combination of bioassay-guided fractionation and HPLC indicated that fraction still contained a number of molecular species so ion-exchange chromatography was repeated using an anionic column and the Tris buffer as eluant. The bioactivity of each fraction was examined in the macrophage assay to identify active sub-fractions. Once identified, they were analysed by HPLC using the ZIC-
HILIC column; sufficient separation was achieved to enable compound isolation and identification. HPLC, in conjunction with the bioassay, was therefore used as the final step for isolation of chromatographically pure compounds. After ascertaining that the immunomodulatory activity was confined to a limited number of fractions, the material was subjected to HPLC in order to isolate individual compounds.

4.3 Results

4.3.1 SEC fractions

The bed volume of the Sephadex LH-20 column was collected and found to be 25 ml. One millilitre fractions of the PRW extract were collected and UV absorbance was measured to monitor elution. Figure 4.1 shows the UV absorbance of the eluate. The bioactivity of the 1ml fractions was determined using the macrophage assay described in Section 2.3.3. All fractions were tested for their capacity to increase uptake of *M. fortuitum* by macrophages (Figure 4.2).

![Figure 4.1: UV absorbance (λ 256nm) of eluate following Sephadex LH-20 fractionation of *P. reniforme* PRW extract.](image)
Figure 4.2: Effect of eluted fractions of PRW extract obtained by Sephadex LH-20 chromatography on the viability of intracellular *M. fortuitum* after 5 h following phagocytosis by murine macrophages.

All fractions were examined using the macrophage assay; the bioactivities associated with fractions 26–63 were broadly similar and representative data for these are shown in Fig. 4.2. Therefore, these bioactive fractions were pooled for further investigation. Combining fractions avoided repeated identification of the same compounds, as these are likely to peak across a series of consecutive fractions. HPLC analysis of the pooled fractions 26-63 from PRW extracts subjected to Sephadex LH-20 chromatography is shown in Figure 4.3.
Extraction and subsequent subfractionation led to only a partial purification of polar compounds, as determined by HPLC (Figure 4.3), even though conditions were adopted to optimise resolution of components with polar characteristics. Therefore, the pooled fraction was subjected to sub-fractionation by IEX. Due to the unknown nature of the bioactive components, both anionic and cationic columns were used. Throughout each procedure, to adhere to bioassay-guided fractionation, UV readings and macrophage assays were used to monitor active fractions.

4.3.2 IEX and HILIC fractions

The cationic column HiTrap SP FF was initially used and the fractions obtained were labelled SC (strong cationic), followed by numerical values corresponding to the NaCl concentration of the eluting buffer. The bioactivity of the fractions
obtained following cationic exchange chromatography was determined and the results shown in Figure 4.4.

![Figure 4.4: Effect of PRW extracts obtained following cationic exchange chromatography on viable intracellular *M. fortuitum*. Mycobacterial counts were determined 5 h after addition of extracts to the macrophages. Fractions tested: Control (no extract); sodium phosphate (NaPO₄) buffer; first elute obtained from the column when sample was applied to the column (SCFE); cationic exchange chromatography of PRW with increasing NaCl concentrations 0–0.5 M (SC0.0–SC0.5); final elution with NaPO₄ buffer solution (SCLE).]

The greatest degree of enhancement of intracellular killing of *M. fortuitum* by macrophages was obtained with extracts SC0.0 and SC0.2. Therefore, these extracts were subjected to HPLC to determine the number of molecular species contained therein. The extracts were collected, desalted and then applied to the HILIC column. Figure 4.5 and 4.6 shows the HPLC chromatographs of extracts SC 0.0 and SC 0.2.
Figure 4.5: HPLC spectrum of extract SC0.0. A solvent gradient of 5–95% ACN was applied to the HILIC column to separate the bioactive polar compounds.

Figure 4.6: HPLC spectrum of extract SC 0.2. A solvent gradient of 5–95% ACN was applied to the HILIC column.
SC0.0 contained more than one component. Further IEX was applied to SC0.0 using an anionic column, HiTrap Q FF. Fractions obtained by anionic-exchange chromatography were labelled SA (strong anionic) and numerals corresponding to the NaCl concentration. Figure 4.7 shows macrophage uptake of *M. fortuitum* after 5 h incubation of the macrophages with mycobacteria and the anionic exchange-derived fractions.

![Graph showing macrophage uptake](image)

**Figure 4.7:** Effect of SC0.0 extracts obtained following anionic exchange chromatography on viable intracellular *M. fortuitum*. Fractions tested: Control (no extract); Tris buffer; first elute obtained from the column when sample was applied to the column (SAFE); extracts obtained following anionic exchange of SC0.0 with increasing NaCl concentrations 0–0.5 M (SA0.0–SA0.5); final elution with buffer solution (SALE).
Following fractionation using anionic exchange, the extracts were applied to the HILIC column for further separation and purification of bioactive compounds. Figures 4.8, 4.9 and 4.10 are HPLC chromatographs of SA 0.0, SA 0.1, and SA0.2, showing fractionation of these extracts using HILIC to yield potential bioactive compounds.

Figure 4.8: HPLC spectrum of *Pelargonium* extract SA0.0, an extract obtained following anionic exchange chromatography.

Figure 4.9: HPLC spectrum of *Pelargonium* extract SA0.1 obtained following anionic exchange chromatography.
Following bioassay-guided fractionation of PRW by the application of sequential chromatographic techniques, SEC, IEX and HPLC, five pure compounds were isolated. They were labelled IEX 1–5 and their structures determined by mass spectrometry and nuclear magnetic resonance as described in the following chapter.

4.4 Discussion

The roots of *Pelargonium* species contain hundreds of compounds that have been previously characterised (Kolodziej 2000). However, the bioactive nature of the compounds against intracellular mycobacteria has yet to be ascertained. Previous studies in the biological activity of *P. sidoides* tended to use crude extracts as the test material in bioassays (Conrad et al. 2006; Koch & Biber 2006). However, as shown in this study, the bioactive nature of Umckaloabo against intracellular survival of mycobacteria lay within an extract obtained from *P. reniforme*. 
Therefore, the process of bioassay-guided fractionation in isolating the bioactive extracts likely to be high in bioactive compounds could be achieved by chromatographic fractionation (Pauli et al. 2005).

SEC is often used as an initial step to separate component mixtures. Extracts from organisms, such as barracuda (Pottier et al. 2003), of human origin, such as urinary cortisol (Morineau et al. 1997), or from plant sources, such as Coleus aromaticus (Kumaran & Karunakaran 2005) were successfully fractionated using Sephadex LH-20. The process of applying SEC followed by IEX has been used on extracts obtained from Antarctic krill (Li et al. 2005). Alternative combinations of chromatographic procedures for the fractionation of extracts are usually based on the nature of the extract. The aqueous extract of Veronica anagallis-aquatica used methanolic extraction and medium pressure liquid chromatography to isolate compounds, which were then identified by nuclear magnetic resonance (NMR) (Kupeli et al. 2005). In the isolation of compounds derived Morus root bark, four extraction steps were performed; ethyl acetate extraction, silica gel extraction, reverse-phase liquid chromatography and HPLC (Cui et al. 2006). The use of thin-layer chromatography has also be found to be beneficial isolating compounds in natural products (Lindel & Hochgurtel 2000; Owais et al. 2005).

Much of the previous work on Umckaloabo has focussed on the aqueous extract sourced from P. sidoides, which is designated EPs 7630 and is currently sold as a herbal remedy in Germany. Initial crude extracts from EPs 7630 were obtained using successive liquid-liquid extractions and subsequent fractionations were achieved by solid-phase extractions performed using Sephadex LH-20 and silica gel as the chromatographic substrates. The final purification for the compounds
was conducted using medium pressure liquid chromatography (Kayser & Kolodziej 1995).

The work described in this report shows that different procedures from those used to fractionate *P. sidoides* extracts were needed to isolate the bioactive compounds. A series of chromatographic techniques had been previously used to isolate a protein from an animal origin, whereby, size-exclusion chromatography was followed by ion-exchange chromatography and HPLC (Zeytinoglu et al. 1980). However, the columns and solvents needed to fractionate PRW (water extract of *P. reniforme*) were different and a novel approach used to isolate and purify the bioactive fractions.

In the current study, bioassay-guided fractionation has been employed to identify bioactive fractions within extracts obtained from two species of this genus. The initial bioassay experiments on the crude extracts of Pelargonium roots suggested that the water fraction of *P. reniforme* warranted further fractionation. The unidentified bioactive compounds that were putatively purified were polar in nature and a variety of chromatographic procedures were used to successfully isolate these bioactive compounds from the complex PRW extract. A unique series of chromatographic techniques was established to isolate and purify the compounds from the extracts of the root of *P. reniforme*. The final isolation was individual compounds could be achieved using SEC, IEX then HPLC.

The application of SEC allowed substantial purification to achieve less complex mixtures. The use of IEX, employing cationic and anionic steps in a sequential fashion, allowed further resolution of the bioactive components responsible for the activity of the crude PRW extract. The final fractionation step involved the use of HPLC. The C18 column was found to provide insufficient resolution and a
zwitterionic column was therefore used. HILIC is often employed for separation of polar compounds and the bioactive molecules of interest in this study were purified to a degree sufficient for their isolation and identification.

The specifically designed fractionation procedure described in this study has allowed for the separation of highly polar compounds to be adequately separated for the isolation of single components. No compounds isolated from *P. reniforme* with activity against mycobacteria phagocytosed by macrophages have so far been reported. Therefore, the systematic fractionation of the water extract of *P. reniforme* (PRW) yielded some extracts containing bioactive compounds. The extensive bioassay-guided fractionation separated compound mixtures into individual components that could be further characterised by mass spectrometry and NMR.
Chapter 5

COMPOUND IDENTIFICATION
5.1 Introduction

The bioassay-guided fractionation of the material collected from *P. reniforme* root extraction yielded five compounds with the potential to decrease the numbers of viable intracellular mycobacteria.

A variety of techniques may be used for the identification of molecules of unknown structure; these include mass spectrometry and nuclear magnetic resonance (NMR). The quantity and phase of the compound, combined with the solvent used for its dissolution, will dictate the most effective technique for identification.

Mass spectrometry involves measuring the mass-to-charge ratio of ions of a compound. Basic scans bombard the sample with either negative or positive ions to provide a mass spectrum. However, more detailed scans can be obtained using further mass spectrometry on the parent ion identified in the spectra. The fragmentation of the compound is shown in a mass spectrum, in which the masses of the different fragment ions are also displayed. The structure of the compound can be determined by this analytical technique, together with the orientation and location of any functional groups. Compounds from natural sources have been identified using mass spectrometry (Mooney et al. 2001). Mass spectrometry coupled with either a gas (Popova et al. 2005) or a liquid chromatographic column (Pan et al. 2006) may be used to identify compounds originating from multi-compound samples. Size-exclusion chromatography (SEC) coupled with mass spectrometry has been used to analyse elements from natural products, such as bean seeds (Koplik et al. 2002) and crude plant extracts (Wolfender et al. 1995).
The use of HILIC followed by mass spectrometry has enabled highly water-soluble compounds from plants to be analysed and identified (Tolstikov & Fiehn 2002).

NMR exploits the magnetic properties of an atom’s nucleus using $^1$H and $^{13}$C ions. Initial analysis of the compounds frequently uses $^1$H ions and more detailed studies may be performed using $^{13}$C. NMR has been extensively used for the analysis of purified compounds from plants (Chen et al. 2006). A proton spectrum provides details on the stereochemical orientation of compounds.

NMR is also used in combination with chromatographic columns, such as liquid chromatography for the rapid separation and identification of unknown compounds (Novak et al. 2006) or compounds derived from natural sources (Ye et al. 2007).

A combination of mass spectrometry and NMR may be utilised to confirm the identification of a compound, including highly polar compounds (Preiss et al. 2005) whose chemical nature restricts the use of solvents and chromatographic columns.

In this study, once the bioassays were completed using *M. fortuitum*, the macrophage assay was performed with *Mycobacterium tuberculosis* to ensure that data obtained using the surrogate was applicable to the target pathogen. Assays incorporating the infectious organism associated with TB could provide evidence to support the use of the bioactive compounds as potential TB cures or adjuvants to conventional therapy.
Using the Category 3 laboratory at the Royal Free hospital, *M. tuberculosis* H37RV was chosen as representative of TB pathogens. This strain is virulent, is often used in studies on tuberculosis (Camus et al. 2002) and has been employed to evaluate the bactericidal potential of natural products (Molina-Salinas et al. 2006). In addition, DNA from H37RV has been used to transform avirulent strains of *M. tuberculosis* to virulent derivatives (Pascopella et al. 1994), demonstrating its pathogenetic potential.

### 5.2 Materials and Methods

#### 5.2.1 Mass Spectrometry

HPLC was used in combination with quadruple time-of-flight mass spectrophotometry (Q-TOF); the instruments were used in tandem. Initially, a nominal resolution measurement using mass spectrometry (MS) was taken to estimate the molecular weight of the compounds. Further mass spectrometry, termed tandem mass spectrometry or mass-spectrometry/mass spectrometry (MS/MS), was then performed on the parent ions to identify fragment ions. I collected and prepared the samples and the mass spectrometry performed by the laboratory of Dr William Griffiths (School of Pharmacy, London, UK).

#### 5.2.2 Nuclear Magnetic Resonance

The isolated compounds were dissolved in a 10% D$_2$O: 90% H$_2$O solution (Fisher Scientific, Loughborough, UK). A $^1$H scan was performed on the samples obtained from the bioassay-guided fractionation of *P. reniforme*. Owing to the small amount of material for analysis obtained by HPLC, NMR was used to
confirm the location of the protons in the compound. I collected and prepared the samples and the NMR was performed by the laboratory of Dr Mire Zloh (School of Pharmacy, London, UK).

5.3 Results

Many compounds found in the roots comprising Umckaloabo (P. reniforme and P. sidoides) have been characterised (Kolodziej 2000). Using this library of compounds from P. reniforme and P. sidoides roots, compounds obtained from the water extract of P. reniforme (PRW) with identical mass were investigated, the ion fragments calculated and the fragmentation patterns analysed for analysis. The fragmentation patterns obtained with the various mass spectrometric techniques yielded different ion fragments. Combined with the relative abundance of each fragment, functional group size could be ascertained and, from this, possible structures of the compounds could be assigned.

The compounds isolated from P. reniforme following bioactive-guided fractionation were designated as IEX1 to IEX5, as they were the purified compounds isolated following ion exchange chromatography.

5.3.1 Mass Spectrometry and Nuclear Magnetic Resonance

MS was used predominantly to establish the basic structure of the compounds and $^1$H NMR to confirm structure and establish the location of protons.

Many of the fragmentation patterns yielded ions with a mass size of 162, indicating the presence of hexose sugars, some of which were conjugated to the compounds isolated from PRW. These hexoses were likely to have originated from the natural sugars found in the Pelargonium roots.
A preliminary mass spectrophotometric scan of positive and negative ions yielded information on the basic structure of the compounds. Some compounds possessed a significant number of functional groups, while others were less complex from a structural perspective. Owing to the unknown nature of the compounds, further mass spectrometry, MS/MS, was applied to parent ions.

5.3.1.1 IEX1

Initial negative and positive MS scans were performed on IEX1. A representative spectrum obtained from a negative MS scan is shown in Figure 5.1.

Figure 5.1: MS data of IEX1. The MS analysis data is shown for a mass window $m/z$ 750-1750 Da. $[M - H]^-$ ions for each significant MS peak with a typical mass pattern are indicated.
The spectrum yielded fragmentation patterns that warranted further detailed MS on the parent ions. MS/MS was performed; a typical spectrum obtained is shown in Figure 5.2.

Figure 5.2: Negative ion spectrum of MS/MS data for the deprotonated molecules ([M – H]) of IEX1 (m/z 636).

The fragment ions of IEX1 obtained from MS and MS/MS were identified, resulting in the determination of functional groups and providing potential structural elucidation of the unknown compound (Figure 5.3).

Figure 5.3: Proposed structure of IEX1.
The compound could not be identified using the structure identification tool FX ChemStruct; thus the structure could not be verified.

5.3.1.2 IEX2

Positive and negative MS scans were performed on IEX2 and the spectrum of the positive scan is shown in Figure 5.4.

![Figure 5.4](image_url)

Figure 5.4: MS data of IEX2. The MS analysis data is shown for a mass window $m/z$ 75-580 Da. $[M + H]^+$ ions for each significant MS peak with a typical mass pattern are indicated.

The compound formed multiples in solution and MS/MS was performed on the large fragment ions to determine the functional groups (Figure 5.5).

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Figure 5.5: Negative ion spectrum of MS/MS data for the deprotonated molecules ([M – H]) of IEX2 (m/z 170).

NMR was then performed to determine the location of the hydrogen (H) atoms.

Figure 5.6: $^1$H NMR spectrum of IEX2.
The singlet peak shows that the H atoms were evenly distributed in the compound, indicating that the compound was symmetrical.

Analysis of the spectra obtained for IEX2 provided evidence for the proposed structure, which is shown in Figure 5.7.

![Proposed structure of IEX2](image)

Figure 5.7: Proposed structure of IEX2.

The structure corresponded to that of gallic acid. Gallic acid was purchased from Sigma-Aldrich (Gillingham, United Kingdom) and MS performed to compare the spectrum of IEX2 to that of gallic acid. The MS of gallic acid is shown in Figure 5.8. The high degree of identity of the spectra of IEX2 and gallic acid show that they were the same compound.
Figure 5.8: Negative ion spectrum of gallic acid determined by MS analysis (60 – 190 m/z). All ions are singly deprotonated species. Characteristic ions are indicated.

5.3.1.3 IEX3

Positive and negative MS scans were performed on IEX3: the spectrum of the negative scan is shown in Figure 5.9.

Figure 5.9: MS data of IEX3. The MS analysis data is shown for a mass window m/z 100-480 Da. [M – H] ions for each significant MS peak are indicated.
MS/MS was applied to the parent ion that had been identified by the basic MS scan (Figure 5.10).

Figure 5.10: Negative ion spectrum of MS/MS data for the deprotonated molecules ([M - H]^−) of IEX3 (m/z 184).

^1H NMR was performed on IEX3 and its spectrum is shown in Figure 5.11.

Figure 5.11: ^1H NMR spectrum of IEX2.
The strong singlet peaks indicate that the H atoms in the ring structure are equally spaced, showing that the compound is highly symmetrical.

The structure of IEX3 was determined and is shown in Figure 5.12.

![Figure 5.12: Proposed structure of IEX3.](image)

The structure corresponded to that of methyl gallate, which was purchased (Sigma-Aldrich, Gillingham, United Kingdom) and the MS of the commercial compound determined (Figure 5.13).

![Figure 5.13: Negative ion spectrum of methyl gallate determined by MS/MS analysis (50 – 1000 m/z). All ions are singly deprotonated species. Characteristic ions are indicated.](image)
The spectra of both IEX3 and methyl gallate were identical.

5.3.1.4 IEX4

Positive and negative MS scans were performed on IEX4 and the spectrum of the positive scan is shown in Figure 5.14.

Figure 5.14: MS data of IEX4. The MS analysis data is shown for a mass window $m/z$ 100 - 1600 Da. [M + H]$^+$ ions for each significant MS peak with a typical mass pattern are indicated.

Further fractionation of the compound was performed on several ions (Figure 5.15).
Figure 5.15: Series of positive ion spectra of MS/MS data for the protonated molecules ([M + H]+) of IEX4 (m/z 318).

From the spectra obtained from MS and MS/MS, a structure for IEX4 was determined, shown in Figure 5.16.

Figure 5.16: Proposed structure of IEX4.

The compound was identified as myricetin and was purchased from Sigma-Aldrich (Gillingham, United Kingdom). A mass spectrum was obtained on the commercially available compound (Figure 5.17).
Figure 5.17: Positive ion spectrum of myricetin detected by MS analysis (100 – 1600 m/z). All ions are singly protonated species. Characteristic ions are indicated.

The mass spectra for IEX4 and myricetin were identical.

5.3.1.5 IEX5

Positive and negative MS scans were performed on IEX5 and the spectrum of the positive scan is shown in Figure 5.18.

Once the parent ion of IEX5 was identified (m/z 464), further MS/MS was performed on this parent ion (Figure 5.19).
Figure 5.18: MS data of IEX5. The MS analysis data is shown for a mass window $m/z$ 100 - 1000 Da. $[M + H]^+$ ions for each significant MS peak with a typical mass pattern are indicated.

Figure 5.19: Negative ion spectrum of MS/MS data for the deprotonated molecules $([M-H])$ of IEX5 ($m/z$ 464).
The structure of IEX5 was determined and shown in Figure 5.20.

![Proposed structure of IEX5.](image)

Figure 5.20: Proposed structure of IEX5.

The structure was identified as quercetin 3-D-glucoside and was purchased from Sigma-Aldrich (Gillingham, United Kingdom). The mass spectrum of commercially available quercetin 3-D-glucoside is shown in Figure 5.21.

![Positive ion spectrum of myricetin determined by MS analysis (50 – 1000 m/z). All ions are singly protonated species. Characteristic ions are indicated.](image)

Figure 5.21: Positive ion spectrum of myricetin determined by MS analysis (50 – 1000 m/z). All ions are singly protonated species. Characteristic ions are indicated.

The spectra for both IEX5 and quercetin 3-D-glucoside were identical.
5.3.2 Macrophage assay of target molecules (M. fortuitum)

The MS and NMR data of four of the five purified compounds derived from the roots of *P. reniforme* facilitated their structural elucidation as follows; gallic acid, methyl gallate, quercetin 3-D-glucoside, and myricetin. These commercially purchased molecules were examined with regard to their capacity to modulate uptake and killing of the mycobacterial surrogate, *M. fortuitum*, by macrophages as described in Section 2.2.3. Each compound was tested individually and in different combinations to ascertain their immunomodulatory potential. The highest concentrations that could be incorporated into macrophage assays were determined using the Trypan Blue exclusion method.

Serial dilutions of the compounds were examined: the effect of gallic acid and methyl gallate on the viability of intracellular mycobacterial levels over a 5 h period are shown in Figures 5.22 and 5.23.

![Graph showing the effect of gallic acid on intracellular viability of M. fortuitum phagocytosed by murine peritoneal macrophages. Control (○); 0.025 mg/ml (■); 0.0025 mg/ml (▲); 0.00025 mg/ml (∗). (+/- SEM; n=6)](image)

Figure 5.22: Effect of gallic acid on intracellular viability of *M. fortuitum* phagocytosed by murine peritoneal macrophages: Control (○); 0.025 mg/ml (■); 0.0025 mg/ml (▲); 0.00025 mg/ml (∗). (+/- SEM; n=6)
Figure 5.23: Effect of methyl gallate on intracellular viability of *M. fortuitum* phagocytosed by murine macrophages: Control (○); 0.025 mg/ml (■); 0.0025 mg/ml (▲); 0.00025 mg/ml (▲). (+/- SEM; n=6)

The capacity of myricetin and quercetin 3-O-glucoside to modulate macrophage activity was less than that of gallic acid. Higher concentrations of both compounds could be used in the macrophage assays, as determined by viability assay, and serial twofold dilutions of the compound solution were used. The capacity of these compounds to modulate the uptake and killing of *M. fortuitum* by macrophages was established (Figures 5.24 and 5.25).
Figure 5.24: Effect of myricetin on intracellular viability of *M. fortuitum* phagocytosed by murine macrophages: Control (○); 0.25 mg/ml (■); 0.125 mg/ml (▲); 0.06255 mg/ml (×). (+- SEM; n=6)

Figure 5.25: Effect of quercetin 3-d-glucoside on intracellular viability of *M. fortuitum* phagocytosed by murine macrophages: Control (○); 0.25 mg/ml (■); 0.125 mg/ml (▲); 0.06255 mg/ml (×). (+- SEM; n=6)
A comparison of the activity of the four compounds at the highest concentrations employed is shown in Figure 5.26.

Figure 5.26: Comparison of the four bioactive compounds isolated from *P. reniforme* root extracts on viability of internalised *M. fortuitum*: Control (○); gallic acid 0.025 mg/ml (■); methyl gallate 0.025 mg/ml (▲); quercetin 3-D-glucoside 0.25 mg/ml (●); * myricetin 0.25 mg/ml. (+/- SEM; n=6)

Figure 5.27 shows the effect of the exposure of the four compounds to phagocytosed mycobacteria at a concentration of 0.025 mg/ml.
Figure 5.27: Comparison of the four bioactive compounds isolated from *P. reniforme* root extraction with regard to their capacity to modulate the on intracellular viability of internalised *M. fortuitum*. All compounds at 0.025 mg/ml. Control (○); gallic acid (■); methyl gallate (▲); quercetin 3-D-glucoside (×); myricetin (★). (+/- SEM; n=6)

The capacity of each compound to reduce the intracellular viability of *M. fortuitum* was determined. At 0.025 mg/ml, gallic acid exerted the greatest effect, followed by methyl gallate, myricetin and quercetin 3-D-glucoside.

The roots of *Pelargonium* contain many compounds. Whilst four individual compounds had been isolated and characterised, there was a high probability that a combination of the compounds would produce a greater effect on the viability of intracellular mycobacteria than the individual compounds alone. As any interactions between the compounds have yet to be defined, different
combinations were tested, such as gallic acid combined with methyl gallate or myricetin with quercetin 3-D-glucoside. The results from the macrophage assay using different combinations of the compounds are shown in Figures 5.28–5.31 to show any possible cocktail effect of using different compound mixtures.

Figure 5.28: Effect of a combination of gallic acid and methyl-gallate on intracellular viability of *M. fortuitum* phagocytosed by macrophages: Gallic acid 0.025mg/ml (○); methyl gallate 0.025mg/ml (■); gallic acid 0.025 mg/ml + methyl gallate 0.025 mg/ml (▲); gallic acid 0.0025 mg/ml + methyl gallate 0.0025 mg/ml (×). (+/- SEM; n=6)
Figure 5.29: Effect of a combination of gallic acid and quercetin 3-0-glucoside on intracellular viability of *M. fortuitum* phagocytosed by macrophages: Gallic acid 0.025 mg/ml (○); quercetin 3-0-glucoside 0.25 mg/ml (■); gallic acid 0.025 mg/ml + quercetin 3-0-glucoside 0.25 mg/ml (▲); gallic acid 0.0025 mg/ml + quercetin 3-0-glucoside 0.025 mg/ml (×). (+/- SEM; n=6)

Figure 5.30: Effect of a combination of quercetin 3-0-glucoside and myricetin on intracellular viability of *M. fortuitum* phagocytosed by macrophages: Myricetin 0.25 mg/ml (○); quercetin 3-0-glucoside 0.25 mg/ml (■); quercetin 3-0-glucoside 0.25 mg/ml + myricetin 0.25 mg/ml (▲); quercetin 3-0-glucoside 0.025 mg/ml + myricetin 0.025 mg/ml (×). (+/- SEM; n=6)
Figure 5.31: Effect of various combinations of the different bioactive compounds isolated from material obtained from *P. reniforme* root extraction on intracellular viability of *M. fortuitum* phagocytosed by macrophages: Control (gallic acid 0.025 mg/ml) (○); gallic acid 0.025 mg/ml + myricetin 0.25 mg/ml (■); methyl gallate 0.025 mg/ml + myricetin 0.25 mg/ml (▲); methyl gallate 0.025 mg/ml + quercetin 3-D-glucoside 0.25 mg/ml (×). (+/- SEM; n=6)

The effect of combining all four compounds was also established (Figure 5.32).
Figure 5.32: Effect of the combination of all four bioactive compounds on intracellular viability of internalised mycobacteria: Control (gallic acid 0.025 mg/ml) (○); gallic acid 0.025 mg/ml + methyl gallate 0.025 mg/ml + quercetin 3-D-glucoside 0.25 mg/ml + myricetin 0.25 mg/ml (■); gallic acid 0.0025 mg/ml + methyl gallate 0.0025 mg/ml + quercetin 3-D-glucoside 0.025 mg/ml + myricetin 0.025 mg/ml (▲). (+/- SEM; n=6)

5.3.3 Uptake of M. fortuitum by macrophages

The uptake of *M. fortuitum* by macrophages was investigated using the Ziehl–Neelsen stain, (Section 2.3.4); a range of MOIs was employed to investigate the influence of various concentrations of the bioactive compounds on uptake, as shown in Figures 5.33–5.36.
Figure 5.33: Uptake of *M. fortuitum* by murine macrophages (MOI 10): Control (no extract was added to the macrophages). Numbers of intracellular mycobacteria were grouped as follows: <5 bacteria (■); 5–19 bacteria (○); >19 bacteria (■). (n=6)

Figure 5.34: Uptake of *M. fortuitum* by murine macrophages exposed to gallic acid (0.025 mg/ml) (MOI 10). Numbers of intracellular mycobacteria were grouped as follows: <5 bacteria (■); 5–19 bacteria (○); >19 bacteria (■). (n=6)
Figure 5.35: Uptake of *M. fortuitum* by murine macrophages (MOI 100): Control (no extract was added to the macrophages). Numbers of intracellular mycobacteria were grouped as follows: <5 bacteria (■); 5–19 bacteria ( ); >19 bacteria (■). (n=6)

Figure 5.36: Uptake of *M. fortuitum* by murine macrophages exposed to gallic acid (0.025 mg/ml) (MOI 100). Numbers of intracellular mycobacteria were grouped as follows: <5 bacteria (■); 5–19 bacteria ( ); >19 bacteria (■). (n=6)
The data for all the compounds were comparable. The bioactive compounds did
not change the proportion in the uptake of mycobacteria by the macrophages.

5.3.4 Macrophage assay of target molecules (M. tuberculosis)

The capacity of the compounds to modulate the uptake and killing of M. tuberculosis H37RV by macrophages was determined and compared with M. fortuitum (Figure 5.37).

![Graph showing the comparison of M. fortuitum and M. tuberculosis uptake by macrophages.](image)

Figure 5.37: Viable intracellular M. fortuitum and M. tuberculosis quantified after phagocytosis by murine peritoneal macrophages: M. fortuitum (○); M. tuberculosis (■). (+/- SEM; n=6)

Uptake of M. fortuitum and M. tuberculosis by macrophages was comparable. Therefore, the uptake by macrophages in the presence of the four bioactive compounds was undertaken using M. tuberculosis as shown in Figures 5.38 and 5.39.
Figure 5.38: Effect of gallic acid and methyl gallate on intracellular viability of \textit{M. tuberculosis} phagocytosed by murine peritoneal macrophages: Control (○); gallic acid 0.025 mg/ml (■); gallic acid 0.0025 mg/ml (▲); methyl gallate 0.025 mg/ml (×); methyl gallate 0.0025 mg/ml (●). (+/- SEM; n=6)
Figure 5.39: Effect of quercetin 3-D-glucoside and myricetin on intracellular viability of *M. tuberculosis* phagocytosed by murine macrophages: Control (○); quercetin 3-D-glucoside 0.25 mg/ml (■); quercetin 3-D-glucoside 0.025 mg/ml (▲); myricetin 0.25 mg/ml (×); myricetin 0.025 mg/ml (＊). (+/- SEM; n=6)
5.4 Discussion

The increased incidence of TB associated in particular with multi-drug resistance and HIV co-infection (Quy et al. 2003) has necessitated further investigation into novel anti-tubercular agents with immunomodulatory capabilities.

This study highlights the identity and bioactive nature of compounds isolated and purified from *P. reniforme*. Their capacity for immunomodulation has been established and creating their therapeutic potential as antituberculosis agents. Although many of the constituents of *P. reniforme* roots have been identified (Kayser et al. 1998; Kolodziej 2006), the study of therapeutically relevant bioactivity has remained limited. Antibacterial (Kolodziej & Kiderlen 2006) and anti-oxidative agents (Latte & Kolodziej 2004) have been isolated. New compounds have also been isolated, such as compounds given the designation pelargoniins (Latte & Kolodziej 2000) as well a unique series of glycosylflavones (Latte et al. 2002). However, there has yet to be any study performed against the causative organism of tuberculosis (TB). This present work has shown that *P. reniforme* roots contain compounds capable of significantly decreasing the intracellular survival of two mycobacterial species.

The quantity of material available for analysis played a major role in determining the strategy for structural elucidation of the bioactive components of the extract. As the amount of material recovered by HPLC was very low, mass spectrometry proved an invaluable tool for identification; this procedure requires only small amounts of sample (as low as 1 ng). The use of NMR for structural elucidation was limited, due to the small quantities of material available. There were sufficient quantities available for NMR with two compounds, IEX2 and IEX3. However, for the identification of IEX2 and IEX3 and the location of the H atoms
of these two compounds could be established. The sharp singlet peaks in the spectra of both samples indicated that the H atoms were distributed evenly around the compound. Consequently, the proposed compound structures of gallic acid and methyl gallate could be verified. The peaks in the NMR spectra for samples IEX1, IEX4 and IEX5 were very small and blended with the background noise, rendering verification impossible.

Once the structure of the molecule had been proposed, chemical structure identification software was used to ascertain its systematic name and thereafter, the molecules were labelled with their commonly used names. In order to authenticate the proposed structures, commercially available standards were used for verification. MS was performed on these compounds in order to confirm that the isolated compounds obtained from *P. reniforme* root fractionation and the purchased compounds were the same. Due to the small amounts of purified compounds available following chromatographic purification, the standards were used in the bioassay. The compounds were identified as gallic acid, methyl-gallate, quercetin 3-D-glucoside, and myricetin. Some of these have been previously isolated from other plant sources and shown to be active in various assays. For example, gallic acid had been tested against intracellular leishmanial infections, where it was used to stimulate macrophage cells to kill phagocytosed leishmania (Kolodziej et al. 2003). Compounds containing gallic acid subunits, such as methyl gallate have been tested for immunomodulatory activity, where they found to activate cells of the immune system, such as T cells or macrophages (Nergard et al. 2005). The inhibitory activity of quercetin on nitric oxide (Comalada et al. 2006) and cytokine production (Kim et al. 1999) in macrophages had also been
established. Although quercetin 3-D-glucoside was found to have antibacterial properties against range of infectious organisms (Suzgec et al. 2005), its immunomodulatory potential has not been previously established. A previous study showed that nitric oxide production by macrophages was reduced by myricetin (Wang & Mazza 2002), however, this study showed that the upon addition of myricetin, macrophages were stimulated to kill phagocytosed mycobacteria.

In the current study, the capacity of all four compounds to alter the macrophage’s ability to kill phagocytosed mycobacteria was established.

Upon testing the four compounds against intracellular *M. fortuitum* and *M. tuberculosis*, it was found that at the same concentration (Figure 5.27 and 5.38 respectively), gallic acid induced the greatest degree of eradication of intracellular mycobacteria with methyl gallate, myricetin and quercetin 3-D-glucoside exhibiting a respectively decreasing effectiveness against viable intracellular mycobacteria. However, the toxicity level of the compounds against both the macrophages and mycobacteria was higher for gallic acid and methyl gallate. Therefore, when the compounds were tested at the higher concentrations, gallic acid, quercetin 3-D-glucoside, and myricetin exhibited similar activity in the macrophage assay. Different combinations of the compounds had varying effects on the macrophages. However, when a combination of all the compounds was tested, the lowest level of viable intracellular mycobacteria was obtained.
Although the use of a surrogate provides some indication of their likely effect on other mycobacteria, in particular *M. tuberculosis*, the use of the infective organism provided more evaluation of the potency of the purified compounds isolated from *P. reniforme* extracts on the disease. *M. tuberculosis* was used in macrophage assays after initial testing of the compounds against the surrogate. The compounds exerted the same effect on both the *M. tuberculosis* and *M. fortuitum*. The compounds could stimulate macrophages to kill intracellular *M. tuberculosis*; this may indicate a potential benefit for treatment of TB. The results also corroborated evidence showing that *M. fortuitum* is a suitable surrogate for *M. tuberculosis*.

The *in vitro* studies of compounds derived from *P. reniforme* extracts showed that these compounds increased the bactericidal effect of macrophages against phagocytosed mycobacteria. Moreover, different compounds exhibited different profiles of activity. The cocktail effect of a combination of the compounds could be observed, but it is unclear whether the effect is synergistic or additive. These results are significant in the further understanding of the putative effect of Umckaloabo in TB. So far, four compounds with bioactivity have been characterised. The identity of the unknown compound, IEX1, and establishing its therapeutically relevant concentration is essential to establish a full catalogue of immunomodulatory compounds produced by the roots of *P. reniforme*. 
Chapter 6

DISCUSSION
6.1 Significance

New drugs developed to treat tuberculosis (TB) need to improve the current treatment by any of the following: decrease treatment length, improve the treatment of multi-drug resistant TB or provide a more effective treatment of latent TB (O'Brien & Nunn 2001). Novel approaches to the development of these drugs include compounds that have been synthetically designed to target components unique to mycobacteria or can enhance the host’s protective immune response (Onyebujoh et al. 2005).

A previous study on Pelargonium identified compounds with direct antimycobacterial activity (Seidel & Taylor 2004). Therefore, the present study focused on discovering potential immunomodulatory components. Although bioactive compounds from plant sources with potential immunomodulatory activity have been previously identified (Nergard et al. 2005; Wang et al. 2005), no compounds capable of stimulating macrophages to kill ingested mycobacteria has been characterised so far. Plant extracts have and are currently analysed to discover a potential source for new drugs. Several commonly used medicines have been derived from natural sources, one such example being penicillin, a naturally occurring compound used as an antibiotic. However, the emergence of drug resistance has increased the need to find new antibiotics to fight infections. This has greatly expanded the numbers of plant sources used in such research. As well as the antibacterial effects, antimycobacterial activity of plant extracts has also been widely studied. For example, the hexane extract of Flourensia cernua DC has been reported to show bactericidal activity against MDR-TB (Molina-Salinas et al. 2006). An alternative approach to fighting infections is increasing the host’s
capability of defending against infections. This reduces the need to find new antibiotics and decreases the incidence of resistant organisms. A number of compounds have been investigated for their immunomodulatory activity, for example compounds such as polysaccharides derived from plants (Schepetkin & Quinn 2006).

Previous studies have examined the *Pelargonium* species that of interest in this current study for a variety investigative purposes. An aqueous extract from *P. sidoides* roots, EPs 7630, has shown its effect against bacterial infections of *Helicobacter pylori* (Beil & Kilian 2006) and *Streptococcus tonsillopharyngitis* (Bereznoy et al. 2003) as well as *M. tuberculosis* (Mativandlela et al. 2006). The older literature suggests that the South African herbal remedy Umckaloabo has therapeutic potential in the fight against tuberculosis; we view the identification of the constituents of Umckaloabo with either direct antimycobacterial activity of indirect immunostimulatory capacity as a rational step towards elucidation of the mechanism of its action and eventual clinical utility, either as therapeutics in their own right or as adjuncts to conventional therapy.

Some of the main bioactive constituents of *P. reniforme* and *P. sidoides*, the plants providing the root material of Umckaloabo, have been identified (Kolodziej 2000). Bioactive compounds derived from the roots have been isolated and their actions demonstrated to range from direct antibacterial activity to immunomodulatory activity against intracellular leishmanial infections. Compounds such as scopoletin and umckalin have low minimum inhibitory concentrations against Gram-positive, for example *Streptococcus pneumoniae*, and Gram-negative bacteria, for example *Escherichia coli* (Kayser & Kolodziej
ternal survival of *Leishmania donovani* (Kayser et al. 2001). Compounds with direct antmycobacterial activity have been isolated (Seidel & Taylor 2004). However, the reported effect of the roots against tuberculosis is likely to be due to a combination of antimicrobial and immunomodulatory effects (Kolodziej 2000). Therefore, presumptive immunomodulatory effects of the root extracts have been investigated in this study described herein.

The roots of *P. reniforme* and *P. sidoides* have not been previously subjected to a systematic bioassay-guided fractionation to determine the presence, or otherwise, of chemical constituents with the capacity to engage in the fight against TB. As discussed earlier, *Pelargonium* extracts have been investigated for immunostimulatory activity using fractionation of ethanolic extracts of root material in combination with bioassay-guided fractionation (Kolodziej & Kiderlen 2006). In the present study, the following fractionation techniques were applied for the isolation of bioactive compounds: Soxhlet extraction and maceration of the ground root, solid-phase extraction, size-exclusion chromatography, ion-exchange chromatography and high-performance liquid chromatography. Figure 6.1 shows a schematic representation of the fractionation procedure used for the isolation and purification of bioactive compounds from *Pelargonium* roots.

This approach indicated that the extract with the greatest capacity to induce killing of internalised mycobacteria by rat peritoneal macrophages was the water extract of *P. reniforme* (PRW). Further fractionation of this extract yielded five compounds which were purified to homogeneity; four were identified and possessed the capacity to elicit the bioactive features of the original complex
mixtures; the fifth compound was unidentified and may possess some bioactivity. Owing to the large number of compounds in the root extracts, mostly highly polar in nature, fractionation techniques with the capacity to resolve water-soluble molecular species, such as IEX and HILIC columns, were used.

Aqueous plant extracts frequently contain bioactive constituents, including molecules with the capacity to modulate the immune system or with anti-infective potential. Such aqueous fractionations frequently employ ethanol as the primary solvent. For example, the Soxhlet extraction of *Cochlospermum tinctorium* was performed to investigate the plant’s immunomodulatory potential (Nergard et al. 2005). Polysaccharides were isolated, with gallotannins and ferulic acids found to exhibit bioactivity. However, the water extract of some plants may either inhibit or stimulate the immune system. For example, the water extract of the leaves of *Trichilia glabra* has been shown to decrease macrophage phagocytic activity (Benencia et al. 1999) whereas the water extract of *Phyllanthus tenellus*, a traditional medicine used worldwide, increased macrophage activity by increasing nitric oxide production within the cell (Ignacio et al. 2001). Although water–ethanol extracts of *Pelargonium* have been used in previous studies to establish immunomodulatory activity, the work described in this report demonstrates for the first time the potency of the pure water fraction to promote a greater degree of intracellular killing of mycobacteria by macrophages than extracts produced with less polar constituents.
Figure 6.1: Fractionation scheme for the isolation of bioactive compounds from *Pelargonium* roots. (HPLC denotes high performance liquid chromatography using a ZIC-HILIC column)

Kayser et al. (2001) demonstrated significant immunomodulatory activity against intracellular *Leishmania donovani* of extracts derived from *P. sidoides*. However, the present research showed that extracts of *P. reniforme* root material had a greater effect on the survival of intracellular mycobacteria than extracts of *P. sidoides*. Although gallic acid and methyl gallate are found in both plant species, the effect of the extracts obtained from the initial fractionation performed on the root material (extract PRW compared to PSW) indicated that variations in the composition of the roots may account for the observed differences in macrophage activity against intracellular mycobacteria.
The four isolated compounds identified as bioactive constituents were gallic acid, methyl gallate, myricetin, and quercetin 3-D-glucoside. They are "known" compounds and some have been tested for bioactivity in a variety of assays, such as gallic acid showing potential antileishmanial activity (Kolodziej & Kiderlen 2006) or quercetin 3-D-glucoside showing antibacterial activity against a range of bacteria (Suzgec et al. 2005), although none had been tested against intracellular mycobacterial infections prior to this study. Gallic acid and methyl gallate have been previously examined for their potential to kill intracellular parasites (Kayser et al. 2001) and gallic acid has been found to exhibit an antioxidant effect (Kim 2007).

Flavonoids are known to increase the inflammatory activity of macrophages (Blonska et al. 2003). Flavonoid glucosides and other related molecules were isolated from Desmodium gangeticum but were found to be inactive against intracellular leishmania (Mishra et al. 2005). Naturally occurring flavonoids have been tested for their antibiotic activity: myricetin possessed no significant bactericidal activity against Klebsiella pneumoniae, the minimum inhibitory concentration (MIC) being very high (Lin et al. 2005). However, it was found to possess anti-inflammatory activity by decreasing cytokine production, such as TNF-α which is needed for an inflammatory response (Ueda et al. 2004). There are as yet no reports in the literature with regard to the activity of myricetin against TB, either as an antibiotic or as an immunomodulator.

Quercetin has been examined for antibacterial activity against M. tuberculosis and was found to be inactive (Lall et al. 2006). However, quercetin 3-D-glucoside has not been examined for direct activity against M. tuberculosis and investigations
into the compound’s activity against any pathogenic micro-organisms are scant. Quercetin, the parent compound, was found to exhibit pro-inflammatory effects in macrophage systems (Xagorari et al. 2001), to possess free-radical-scavenging activity (Edenharder & Grunhage 2003) and to stimulate protein synthesis in (Blonska et al. 2003) and spreading ability of macrophages (Orsolic & Basic 2005). It is likely that the derivative quercetin 3-D-glucoside displays bioactivities different from those of the parent compound. In this study, it was found that although quercetin 3-D-glucoside did not exhibit any bactericidal effects it was able, like myricetin, to stimulate the intracellular killing of mycobacteria by macrophages and it would be interesting to determine whether this in vitro effect translates into a capacity to stimulate the immune system of the whole organism. The four compounds isolated were all able to increase the capacity of macrophages to kill engulfed mycobacteria; this activity was greatest when the four molecules were combined. The results shown in this study supports the hypothesis that extracts from Umckaloabo contains bioactive compounds capable of immunomodulatory stimulation. The indirect effects of the compounds against intracellular mycobacteria can, in part, explain the clinical efficacy of Umckaloabo.

The slow growth and virulence of *M. tuberculosis* constrain its use in assays designed to identify agents with appropriate disease-modifying properties. Therefore, surrogate mycobacteria with more convenient growth characteristics and reduced capacity to cause laboratory-acquired infection, such as the strain of *M. fortuitum* used in this work, are employed in the initial phases of drug discovery programmes and there is convincing evidence that they respond in
bioassays in a similar fashion to the primary pathogen *M. tuberculosis* (Chung et al. 2007). The current study showed that *M. fortuitum* was a suitable surrogate for *M. tuberculosis* as bioassay data was essentially identical when either of the two bacterial species was employed (Figure 5.37). Thus, bioassay-guided fractionation using the surrogate proved to be a valid approach to the isolation of bioactive compounds. Further testing of the compounds against *M. tuberculosis* showed that the potential for immunomodulatory activity derived from distinct molecular species elaborated by *Pelargonium* and contained within the roots. This work may open the way for an evaluation of these bioactive compounds for modulation of the course of TB.

The results from the study have implications on the field of TB. The compounds isolated from *Pelargonium* could be reconstituted to form a viable treatment for TB. This research has shown that the putative effect of Umckaloabo could be confirmed by the scientific evidence produced in the present study. The identification of the immunomodulatory components can further the reasoning behind the efficacy of this herbal remedy. By identifying both the antimycobacterial and immunomodulatory agents, the levels of these compounds can be measured *in vivo*. Therefore, the compounds’ route of entry, their degradation profile and concentration in different compartments in the body can be identified. This will, in turn, advance the knowledge in the mechanism of action of Umckaloabo and therapeutic relevant concentrations can be elucidated. The discovery that gallic acid, methyl-gallate, quercetin 3-D-glucoside and myricetin are the immunomodulatory components of Umckaloabo, this may affect
studies into new therapeutic agents for both infectious diseases as well as medical conditions affected by the activity of the immune system.

Although five compounds were isolated, I was not able to undertake structural elucidation of the fifth compound due to the very small quantity recovered by bioassay-guided fractionation. It is not known if the unidentified compound possesses much bioactivity but it was associated with multi-component fractions of the active primary extract. It is essential that its structure be determined before further investigation of its biological properties. The concentration obtained from the final fractionation was too low to be of use in the macrophage assay. As the final experiments showed, the greatest reduction in viable intracellular mycobacterial levels was exhibited when a combination of the four compounds was tested using the macrophage assay. Mycobacterial levels may be further reduced by incorporation of the unidentified compound into the assay mix.

This study has provided significant evidence for efficacy of compounds derived from *P. reniforme* with regard to uptake and killing of *M. fortuitum* and *M. tuberculosis* by macrophages. Further research should be undertaken to explore the effects of these compounds in animal models of infection. In this study the focus has been on the uptake and killing of newly infected macrophages. However, the target disease, TB, is characterised by an often extensive period of latent infection and chemotherapy is implemented for an additional four months following the two months of initial treatment that is undertaken to treat the active infection. It would, therefore, be worthwhile to examine the capacity of the four bioactive molecules identified in this study to modulate the survival of latent mycobacteria in macrophages.
Further studies of the extracts of *P. reniforme*, such as PRAa and PRAb (see table 3.2), both water-soluble extracts, should be undertaken. Although the PRW water extract exhibited the highest level of activity in the bioassay, the other extracts also displayed immunomodulatory activity. Further fractionation of the extracts obtained from *P. sidoides* should also be performed, in particular PSW, as both *Pelargonium* species are used to formulate Umckaloabo. There is a high probability that the same compounds are present in both plants, but it has been shown that some compounds are present in only one plant (Kolodziej 2000). The traditional Umckaloabo concoction is comprised of extracts of both plants and it may be that both are necessary in order to achieve the most beneficial possible outcome. During a previous investigation into the antimycobacterial properties of Umckaloabo (Seidel & Taylor 2004), the fatty acids responsible for this activity were found in both plants.

Bioassay-guided fractionation has provided evidence that bioactive compounds from the roots of *P. reniforme* are able to reduce the numbers of viable mycobacteria within infected macrophages. However, the number of intracellular mycobacteria was the only determinant measured in the assays. Further investigations into the mechanism of action of the compounds should be undertaken as this may shed light on new opportunities for the discovery and development of new anti-TB agents. *Pelargonium* extracts induce cytokines and reactive intermediates during experimental parasitic infections (Kayser et al. 2001). Although the compounds isolated from *P. reniforme* are likely to induce macrophages in a similar fashion, the specific antimycobacterial mechanisms
should be uncovered as they may differ in subtle ways from other protective processes. Further investigations should be performed using methodologies such as the Greiss assay to measure nitric oxide levels or assays to directly measure changes in cytokine levels.

The objective of the work presented here was to support the hypothesis that once the bioactive compounds contained in the root extracts of Pelargonium were spread throughout the body once ingested. Following passage into the blood stream, the compounds would treat a TB infection by stimulating cells of the immune system to kill any phagocytosed M. tuberculosis pathogens whilst exerting direct antimycobacterial action. The objective has been achieved by the careful design and application of bioassay-guided fractionation of both P. reniforme and P. sidoides to isolate and purify immunomodulatory compounds; four compounds isolated were positively identified and found to exhibit bioactivity.

The results obtained here have implications in the treatment of TB. The reconstitution of the bioactive compounds may confer advantages over using the crude extract of Umckaloabo as a cure for TB, as Umckaloabo can induce vomiting in patients, as found discovered by Charles Stevens when he first was given the treatment when in South Africa (Taylor et al. 2005). Eliminating the impurities, that may be cause of the vomiting, could improve patient compliance. The concentration of therapeutic doses of the compounds can also be controlled.

In summary, this is the first report detailing the components of Umckaloabo that are capable of eliminating an intracellular infection of M. tuberculosis, as well as of it surrogate M. fortuitum. New protocols for both the bioassay and fractionation
of plant extracts were designed and executed to isolate and purify bioactive compounds. Any antitubercular effects associated with Umckaloabo are likely to be due to both antimycobacterial and immunomodulatory components. The compounds responsible for these actions are different, but are found in both plants. However, as shown here, the biological outcomes associated with the extracts are almost certainly due to cooperative effects of a number of key constituents. This study has shown that immunomodulatory components of aqueous extracts of the plant may be useful in the fight against TB.
References


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