ANTITUBERCULOSIS ACTIVITY OF MEDICINAL PLANTS BELONGING TO THE FABACEAE FAMILY USED FOR THE TREATMENT OF TUBERCULOSIS IN LESOTHO

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BLOEMFONTEIN
June 2023

DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, Mercy Mpho Khala, identity number _____ and student number _____, do hereby declare that this research project submitted to the Central University of Technology, Free State, for the Master of Health Science in Biomedical Technology degree, is my own independent work; complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules, and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

_____ 23 June 2023

SIGNATURE OF STUDENT **DATE**

DEDICATION

This dissertation is dedicated to God Almighty Jesus Christ, and my family, Makwa, Ntebaleng, Maletsatsi, Ndlala, and Relebohile Khala, for always believing in me.

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ABSTRACT

Tuberculosis (TB) is one of the top 10 causes of death in Southern Africa and 13th leading cause of death worldwide. It remains one of the top priorities of the World Health Organization. Southern Africa has a rich culture of using medicinal plants to treat diseases; however, the safety and efficacy of the medicinal plants used must be investigated and confirmed. In this study, three indigenous medicinal plants were investigated that are commonly used in traditional medicine against TB activity in Southern Africa. Crude plant extracts of *Elephantorrhiza elephantina* (Burch.) Skeels, *Leobordea lanceolata* (E. Mey.), and *Trifolium burchellianum* Ser. subsp. *burchellianum* roots were collected, prepared, and analysed for phytochemical screening, anti-inflammation activity, toxicity levels, and anti-TB activity. It was found that *E. elephantina* and *T. burchellianum* showed anti-inflammation activity at 200 µg/mL for room temperature water (H_2O) extracts, while *L. lanceolata* did not show any anti-inflammatory activity. The *E. elephantina* extracts of methanol and H_2O extracts showed no toxicity levels at the three different concentrations of 50 µg/mL, 100 µg/mL, and 200 µg/mL, but *L. lanceolata* and *T. burchellianum* showed toxicity in room temperature H_2O extracts at 200 µg/mL. Unfortunately, none of the plant extracts showed any anti-TB activity against strains from the Latin American Mediterranean and East African Indian lineages found in the Free-State province.

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LIST OF ABBREVIATIONS

Acyl-CoA	Acyl coenzyme A
AIDS	Acquired immunodeficiency syndrome
ALOX5−/−	5-lipoxygenase
COX	Cyclooxygenase
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DST	Drug susceptibility testing
EAI	East African Indian
ESAT-6	Early Secreted Antigenic Target 6 kDa
EMB	Ethambutol
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma release assay
IL-6	Interleukin-6
IL-12	Interleukin-12
IL-IB	Interleukin-1 beta
INH	Isoniazid
iNOS	Nitric oxide synthase
LAM	Latin American Mediterranean
LPS	Lipopolysaccharide
LTA4H	Leukotriene A4 hydrolase
LTB4	Leukotriene B4
LXA4	Lipoxin A4
MDR	Multi-drug resistant
MGIT	Mycobacteria Growth Indicator Tube
MMP-9	Matrix metalloproteinase-9
Mtb	*Mycobacterium tuberculosis*
MTT	3-(4,5-dimethylimidazol-2,5-diphenyltetrazolium bromide
OADC	Oleic acid, albumin, dextrose and catalase
PGE2	Prostaglandin E2
PI	Propidium iodide
PZA	Pyrazinamide
RIF	Rifampicin
RIP	Receptor-interacting protein

RPMI	Roswell Park Memorial Institute
TB	Tuberculosis
TDM	Trehalose 6,6'-dimycolate
THP-1	Human leukaemia monocytic cell
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TST	Tuberculin skin test
USA	United States of America
WHO	World Health Organization

LIST OF UNITS

°C	Degree(s) Celsius
µg/mL	Microgram per millilitre
µL	Microlitre(s)
µm	Micrometre(s)
cm	Centimetre(s)
g	Gram(s)
m	Metre(s)
mg/mL	Milligrams per millilitre
mL	Millilitre(s)
mM	Millimolar
ng/mL	Nanogram per millilitre
nm	Nanometre

LIST OF SYMBOLS

Br	Bromine
C	Carbon
$C_4H_8O_2$	Ethyl acetate
CH_6N_4	Aminoguanidine
$CHCl_3$	Chloroform
Cl	Chlorine
CO_2	Carbon dioxide
DH_2O	Distilled water

$FeCl_3$	Ferric chloride
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HNO_3	Nitric acid
I	Iodine
iNOS	Nitric oxide synthase
MeOH	Methanol
N	Nitrogen
$NaNO_3$	Sodium nitrate
NH_3	Ammonia
NO	Nitric oxide
O_2	Oxygen
P	Phosphorus
S	Sulphur

CHAPTER 1:
GENERAL BACKGROUND

1.1 INTRODUCTION

Tuberculosis (TB) is an infectious airborne disease caused by *Mycobacterium tuberculosis* (*Mtb*) (Zumla et al., 2015). TB is one of the top 10 causes of death worldwide (World Health Organization [WHO], 2022b). It was hypothesised that *Mycobacterium* originated from *Mycobacterium ulcerans* more than 150 million years ago (Barberis et al., 2017). TB can be traced back 8 000 to 10 000 years ago, during the Neolithic era (Buzic & Giuffra, 2020). In 1865, Jean-Antoine Villemin was the first person to demonstrate the infectious nature of TB (Cambau & Drancourt, 2014). In 1882, Robert Koch was the first scientist to isolate *Mtb*, which helped to propel efforts to eradicate it (Dorhoi & Kaufmann, 2015). These efforts include research, public health systems, governments, and the WHO (Tiberi et al., 2018).

TB spreads when an individual develops active TB (they start developing symptoms) and expels the *Mycobacterium* into the air by coughing, laughing, or talking and another person inhales the bacteria. TB mainly affects the lungs, which causes pulmonary TB, but it can also affect other body parts and cause extrapulmonary TB. For this study, the focus was on pulmonary TB. Approximately 90% of people who develop TB are adults (WHO, 2021b), and can be attributed to a large part of public healthcare and research being centred around adults rather than children (Heemskerk et al., 2015).

TB, a curable disease, can be treated with a six-month drug regime, including isoniazid (INH), rifampicin (RIF), ethambutol (ETH), and pyrazinamide (PZA). Treatment has an approximately 85% success rate (WHO, 2022a). However, drug-resistant TB often results in prolonged treatment regimens, which makes TB treatment more difficult (Bea et al., 2021). In addition, first-line drugs often have harmful side effects that lead patients to discontinue treatment. Side effects include peripheral neuritis, hypersensitivity, visual toxicity, fever, cutaneous reaction, flu-like syndrome, ototoxicity, psychiatric changes, and hepatotoxicity (Imam et al., 2020). This thus necessitates more investigations for novel drug discovery and the

development of alternative TB treatments. One way is to explore medicinal plants due to their novel phytochemical compounds.

Medicinal plants are affordable, naturally occurring, have fewer side effects, and are known as a reservoir of novel active compounds with pharmacological benefits. There is thus a need to investigate medicinal plants as potential anti-TB agents. Traditional healers or herbalists often identify and use medicinal plants and know how various medicinal plants are to be prepared and administered (Mokgobi, 2013). Many traditional plants are therefore presumed safe, but some studies have shown this might not be the case (Mensah et al., 2019). This shows that scientific studies need to be conducted on various medicinal plants to ensure their efficacy and that their cytotoxicity levels are safe for humans.

Phytochemicals are naturally occurring chemical compounds found in plants that provide health benefits to humans other than those attributed to macronutrients and micronutrients (Kose et al., 2015). Phytochemicals help protect plants from disease, damage, and environmental stresses such as drought, ultraviolet light pollution, and pathogenic attacks. In addition, they offer medical benefits when used by humans (Kose et al., 2015).

Plant metabolites can be classified as primary and secondary phytochemicals. Primary metabolites include glucose starch, polysaccharide, protein, lipids, and nucleic acid (Shakya, 2016). Secondary metabolites include flavonoids, tannins, alkaloids, glycosides, carbohydrates, and amino acids. According to Sheeba et al. (2015), flavonoids, tannins, alkaloids, glycosides, carbohydrates, amino acids, and terpenoids contribute to the anti-TB activity of plants. According to Mpofu et al. (2004), *Elephantorrhiza elephantina* (Burch.) Skeels, *Leobordea lanceolata* (E. Mey.), and *Trifolium burchellianum* Ser. subsp. *burchellianum* contain phytochemicals such as flavonoids, tannins, terpenoids, and alkaloids. These phytochemicals play a role in anti-inflammation, antiviral, antibacterial, antimalarial and anticancer activities. They also possess healing agents for skin burns, piles, and gonorrhoea (Agidew, 2022).

Inflammation is defined as the immune system's response to infection and injury of living tissue. Inflammation helps with the restoration of tissue structure and physiological function. Inflammation can either be acute or chronic (Chen et al.,

2018). Acute inflammation involves a quick influx of blood granulocytes, where neutrophils are usually the first to arrive, followed by monocytes that mature into inflammatory macrophages. This causes redness, heat, swelling, and pain. Acute inflammation often results in tissue repair, but failure to repair tissue can lead to chronic inflammation. Chronic inflammation causes damage to the living tissue (Verma, 2016). *Mtb* is known to cause inflammation and tissue damage. The body's response to *Mtb* includes the production of pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). The inadequacy of the IFN-γ pathway often results in severe cases of TB (Mesquita et al., 2016). According to Kose et al. (2015), *E. elephantina*, *L. lanceolata*, and *T. burchellianum* are used to treat TB, but no significant scientific evidence has been presented; hence the importance of investigating these plants for their anti-TB activities.

1.2 PROBLEM STATEMENT

Approximately 80% of the world's population depend on medicinal plants to treat various diseases. In Southern Africa, most people who are dependent on medicinal plants live in rural areas, where transportation to the nearest clinic is not readily available. Some people experience side effects from the medication currently used to treat TB, which often leads to them to discontinue using the TB medication. This leads to incomplete completion of the TB medication regime, which can cause complications in the future as it can lead to the development of multi-drug-resistant (MDR) TB. Medicinal plants are affordable, naturally occurring, have fewer side effects, and are recognised as a source of innovative active chemicals with pharmacological advantages. Investigating medicinal plants as potential TB inhibitors is thus necessary. The anti-TB properties of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* were investigated for this study. These plants all belong to the Fabaceae family and are mainly used to treat TB in Southern Africa (Kose et al., 2015), but there is currently no scientific evidence to support this claim.

1.3 AIM

The aim of this study was to investigate the anti-TB activity of *E. elephantina*, *L. lanceolata*, and *T. burchellianum*.

1.4 OBJECTIVES

The objectives of this study are as follows:

- To investigate the phytochemistry properties of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* by screening for alkaloids, flavonoids, tannins, and terpenoids using a colorimetric assay.
- To investigate the anti-inflammatory activity of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* through 3-(4,5-dimethylimidazol-2,5-diphenyltetrazolium bromide) (MTT).
- To investigate the anti-TB activity of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* through drug susceptibility testing (DST) on susceptible strains of different lineages.
- To determine the cytotoxicity potential of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* on Vero cells.

1.5 STUDY LAYOUT

The critical steps of the research are as follows:

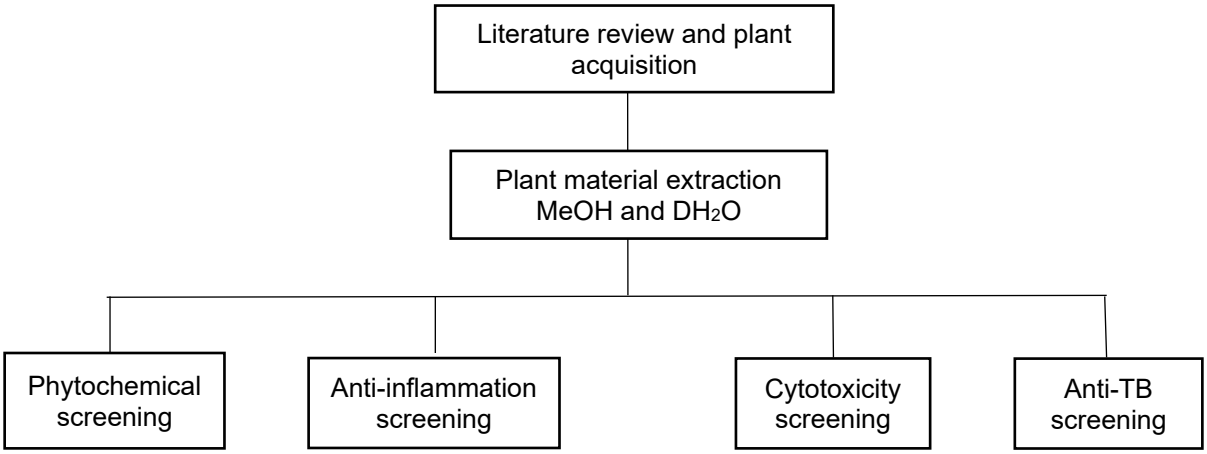


Figure 1.1: A diagrammatic summary of steps in investigating the pharmacological activity of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* plant extracts

1.6 STUDY OVERVIEW

Chapter 1 consists of a brief introduction to TB, the history of TB, and how it spreads. It also outlines the medication used to treat TB and the various side

effects of the disease. It also shows the importance of medicinal plants and phytochemicals and provides a description of the role of anti-inflammation activity in TB. The aim, problem statement, and objectives of the study are also listed in this chapter.

Chapter 2 details the transmission, pathobiology, and inflammatory pathway of TB. It details current medication used for TB and the need to explore medicinal plants. Further details are provided about the three plants chosen for the study, as well as their traditional uses.

Chapter 3 briefly describes the methodology used to investigate the phytochemical, anti-inflammation, anti-TB, and cytotoxicity properties of the selected medicinal plants.

Chapter 4 discusses the study's results.

Chapter 5 provides a detailed description and discussion of the study results and the possible cause(s) for the results.

Chapter 6 presents the conclusion of the study and makes recommendations for future research.

CHAPTER 2:
LITERATURE REVIEW

2.1 OVERVIEW OF TUBERCULOSIS (TB)

TB is the ninth leading cause of death worldwide (Park et al., 2019). In 2021, the WHO estimated that in 2020 TB accounted for 1.3 million deaths among HIV-negative individuals and 214 000 deaths among HIV-positive individuals globally. The alarming increase in TB diagnoses is mainly seen in Asia and Africa, while in Europe and the United States of America (USA), it has decreased gradually (Russel et al., 2013). The leading cause of the increasing number of TB cases globally can be attributed to low socio-economic standards and diseases such as HIV/AIDS (Arya et al., 2014) and diabetes mellitus, to name a few, that compromise an individual's immune system, which renders it weak and highly susceptible to TB infection (Narasimhan et al., 2013).

Densely populated communities, crowding, poor ventilation, lack of sanitation, and a weak immune system encourage TB to thrive. There are many overcrowded areas in Southern Africa that allow TB to thrive. One such place is informal settlements, where most occupants live in shacks (small buildings made from pieces of wood, metal or other materials) that have very little space. Poor sanitation is also primarily associated with informal settlements. Poor ventilation and crowding are also seen in public transportation (Andrews et al., 2013), which easily allow TB transmission. One such case is minibus taxis, which are the primary means of transport for many South Africans. People are mostly seated next to each other, and the windows are not very wide and, in most cases, are not opened. In South Africa, 450 000 people contract TB every year, and 89 000 people die from it, which makes it one of the leading causes of death in South Africa (Vassall, 2022).

2.2 TB TRANSMISSION

Mtb is transmitted through airborne particles, named droplet nuclei, which are 1 to 5 microns in diameter. Infectious droplet nuclei are produced when individuals who have pulmonary TB (TB that affects the lungs) or laryngeal TB (resulting from

pulmonary TB) cough, sneeze, shout, sing, or talk (Fatmawati et al., 2020). These tiny particles may remain suspended in the air for several hours, depending on the environment. The transmission of *Mtb* occurs when a person inhales droplet nuclei containing *Mtb*, and the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the lungs' alveoli, as shown in Figure 2.2 (Centers for Disease Control and Prevention, 2022). There are different infection stages, known as the latent and active TB infection stages.

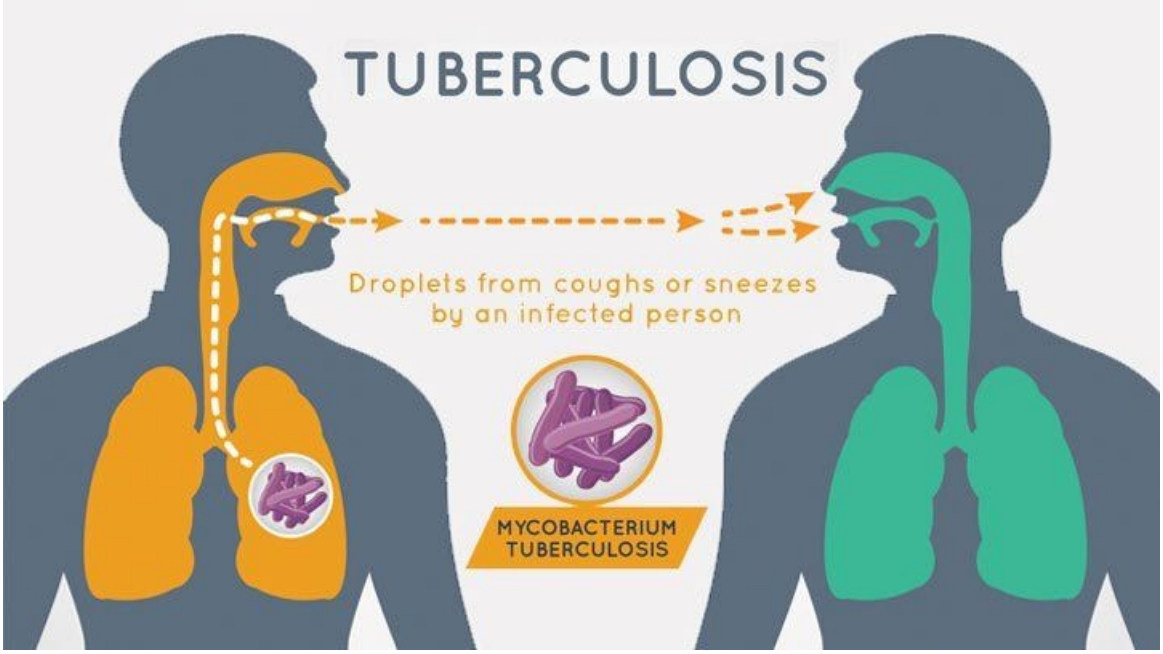


Figure 2.1: Portraying the transmission of TB

Source: Centers for Disease Control and Prevention (2022)

Latent TB infection is when an individual is infected with *Mtb* but it remains dormant, which means that they cannot spread the infection to other individuals. Latent TB infection begins when extracellular bacilli are absorbed by macrophages and presented to other white blood cells. This triggers the immune response, which causes white blood cells to kill or encapsulate most of the bacilli, which leads to the formation of a granuloma. Latent TB infection can be detected using the tuberculin skin test (TST) or an interferon-gamma release assay (IGRA). It can take two to eight weeks after the initial TB infection for the body's immune system to react to tuberculin and for the infection to be detected by the TST or IGRA (Centers for Disease Control and Prevention, 2022). Within weeks of infection, the immune system can usually halt the multiplication of the tubercle bacilli, which prevents

further progression, which means that the individual does not exhibit any TB symptoms. Latent TB can progress to active TB (see Figure 2.2) once an individual's immune system is compromised or due to a change in environmental factors or old age. Active TB is when TB symptoms manifest in the host body. These symptoms include coughing for three or more weeks, coughing up blood or mucous, chest pains (or pain when breathing or coughing), unintentional weight loss, fatigue, fever, night sweats, chills, and loss of appetite (Braian, 2020).

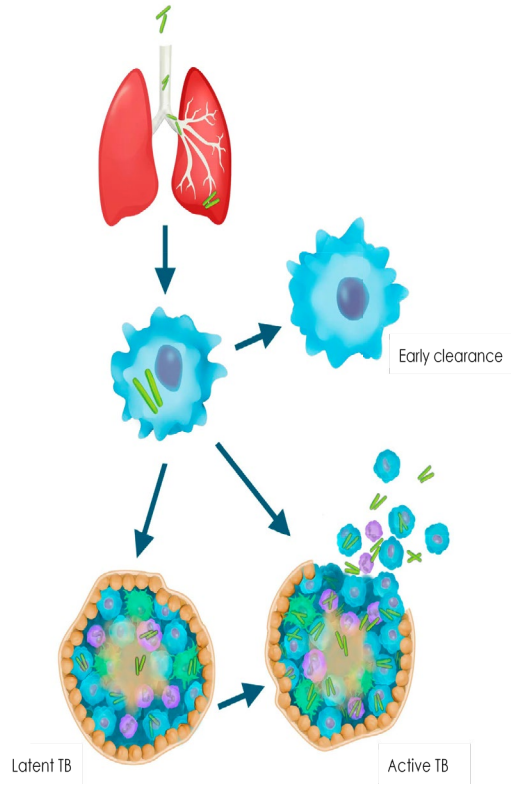


Figure 2.2: Shows that *Mycobacterium tuberculosis* (*Mtb*) spreads infection from the lungs to the alveolar cells, where it develops into active or latent TB

Source: Braian (2020)

The body fights against TB infection by activating its defence systems, which consist of macrophages and dendritic cells. These cells can recognise mycobacterial structures and pathogen-associated molecular patterns using membrane-associated pattern recognition receptors. The most studied pattern recognition receptors are Toll-like receptors (TLRs) such as TLR2, TLR4, and TLR9. When TLRs signal pathways are activated, it predominantly produces pro-inflammatory cytokines such as TNF-α, interleukin-1 beta (IL-IB), interleukin-12 (IL-12), and nitric oxide (NO). Ingested bacteria are destroyed by phagosome-

lysosome fusion and acidification by hydrogen peroxide (H_2O_2) and other reactive oxygen (O_2) intermediates (Heemskerk et al., 2015).

2.3 TB PATHOPHYSIOLOGY IN THE INFLAMMATORY MYELOID CELLS

2.3.1 Formation of granuloma

Mtb is highly associated with granuloma formation in the lungs of infected individuals. The interaction of bacterial and host factors causes granuloma formation. A granuloma is an immunological structure that consists of immune cells, including macrophages, monocytes, dendritic cells, foamy macrophages, epithelioid cells, neutrophils, and multi-nucleated giant cells (see Figure 2.3). When an individual is infected with active *Mtb*, an immune response is triggered that results in granulomatous formation, which can lead to lung damage. Granuloma formation during active TB often results in reduced lung function, even after the completion of TB treatment. The literature indicates that the mycobacterial virulence protein Early Secreted Antigenic Target 6 kDa (ESAT-6) brings about the secretion of host matrix metalloproteinase-9 (MMP-9) by epithelial cells to prompt the introduction of new macrophages into the granuloma (Ndlovu & Marakalala, 2016).

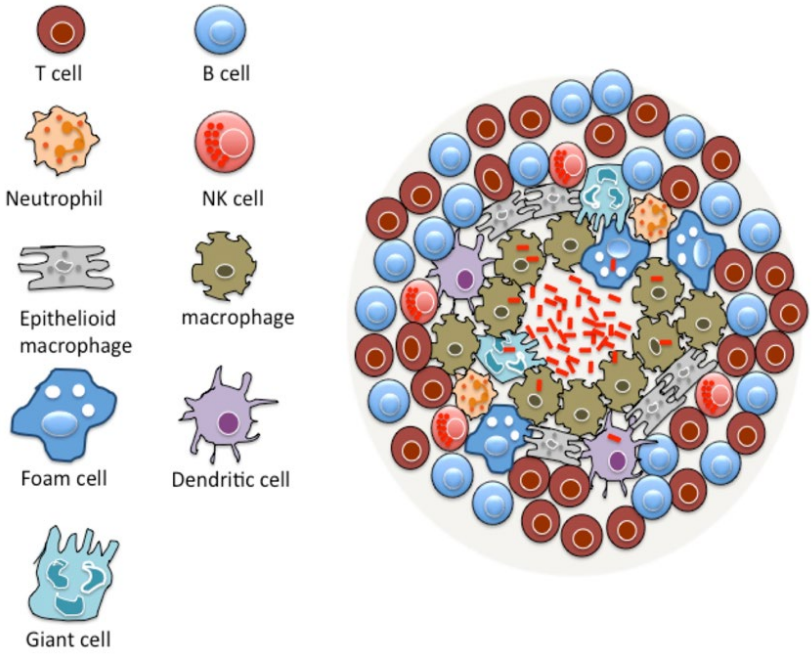


Figure 2.3: Basic structure of granuloma

Source: Ndlovu and Marakalala (2016)

Infected macrophages leave the primary granulomas, which promotes early circulation. Glycolipid and trehalose 6,6'-dimycolate (TDM) of the mycobacterial cell wall can trigger an immune response and granuloma formation. The TDM-led granulomatous response is initiated and maintained by pro-inflammatory cytokines such as TNF-α and interleukin-6 (IL-6). Macrophages within granuloma develop into specialised cell types, including foamy macrophages, epithelial macrophages, and multi-nucleated macrophages (Ndlovu & Marakalala, 2016), which help to contain TB.

Dendritic cells are part of the early-arrival cells at the site of infection, where they engulf the bacteria, bacterial products, and infected dying cells, then leaving the area of infection and travelling through the lymphatic system to the draining lymph nodes. This action could trigger acquired immunity. A study by Harding and Boom (2010) demonstrated that inflammatory dendritic cells infected with Bacilli Calmette-Guérin (BCG) associated with bacteria-specific T cells could form new multi-foci lesions. This highlights that they can play a significant role in granuloma reformation. Furthermore, *Mtb* can modulate dendritic cell function by impairing antigen presentation and moving to the draining lymph nodes; thus delaying the development of the adaptive immune response required to arrest bacterial proliferation (Ndlovu & Marakalala, 2016).

The entrance of the T and B cells characterises granuloma formation. These cells form the lymphocytic cuff at the granuloma periphery, which gives it a solid intact structure (Phuah et al., 2016). The arrival of TB-specific T cells in the lungs coincides with the arrest of bacterial proliferation 14 to 21 days after initiation of infection by producing TNF-α and IFN-γ, which enhance macrophage microbicidal activity (Stewart et al., 2023).

2.3.2 Inflammatory pathway to granuloma caseum

Granuloma formation undergoes changes that lead to the formation of caseum. Caseum is a cheese-like cellular structure containing necrotic material at the centre of the granuloma that is rich in lipids (Sarathy & Dartois, 2020). According to an immunohistological study, cells surrounding the caseum express high levels of proteins involved in lipid metabolism. These include adipophilin, acyl coenzyme A

(acyl-CoA) synthetase long-chain family member 1, and saposin C (Ndlovu & Marakalala, 2016). Damage to the caseum results in liquefaction and granuloma cavitation. This causes the release of infectious *Mtb* into airways, which are aerosolised into cough droplets that can result in *Mtb* infection in new hosts (Marakalala et al., 2016).

Mycobacterial factors can cause granuloma separation, which induces different cell-death modalities in infected cells (Nisa et al., 2022). Infected granuloma macrophages can be destroyed either by apoptosis or necrosis. Apoptotic cell death involves keeping the bacteria encased within dead macrophages that newly arriving uninfected macrophages can readily phagocytose (Roca et al., 2019). Necrotic cell death, known as the loss of cell membrane integrity, results in the leakage of the bacteria into the growth-permissive extracellular environment, where the bacteria thrive by adopting a characteristic cording phenotype, which makes it difficult for new macrophages to engulf the bacteria (Ndlovu & Marakalala, 2016). The inflammatory pathway from necrotic cell death to granuloma caseation is illustrated in Figure 2.4.

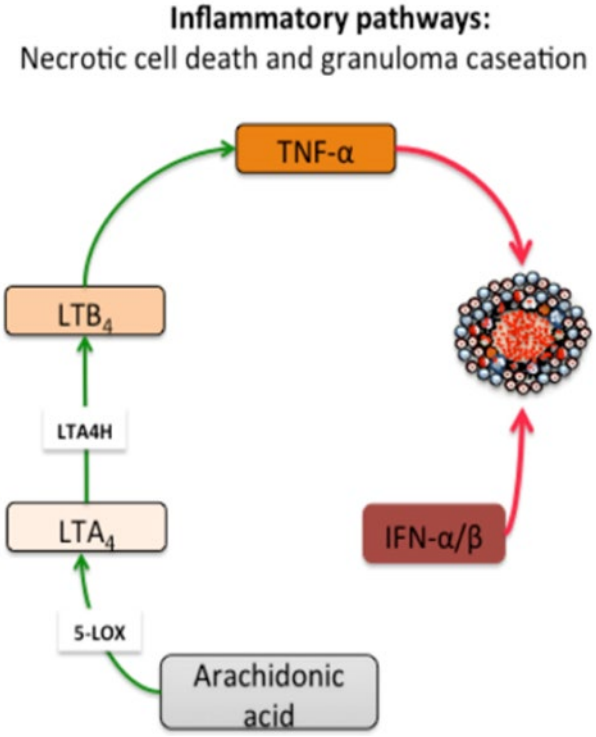


Figure 2.4: Inflammatory pathway from necrotic cell death to granuloma caseation

Source: Ndlovu and Marakalala (2016)

Host-derived TNF-α produces macrophage necrosis during *Mtb* infection (Olsen et al., 2016). A significant amount of TNF-α production increases the necrosis of infected macrophages through a programmed pathway called necroptosis. Necroptosis involves the activation of receptor-interacting protein 1 (RIP1) and RIP3 kinases through the production of reactive O_2 species by the mitochondria (Tobin et al., 2012; Roca & Ramakrishnan, 2013). TNF-α-induced macrophage necrosis is moderated by mitochondrial cyclophilin D (Roca & Ramakrishnan, 2013).

Mycobacterial virulence factors can control macrophage necrosis by producing specific eicosanoids from arachidonic acid (Laval et al., 2021). Lipoxin A4 (LXA4) is created by 5- and 15-lipoxygenases to help encourage necrosis. It is induced by the virulent *Mtb* H37Rv strain. In contrast, H37Ra mutants induce the synthesis of pro-apoptotic prostaglandin E2 (PGE2). It encourages this through cyclooxygenase (COX) 1 and 2 and prostaglandin synthases (Ndlovu & Marakalala, 2016). A previous study reported that lacking 5-lipoxygenase (ALOX5−/−) makes the host more likely to be resistant to infection with the *Mtb* H37Rv strain, which shows that LXA4 is detrimental to the host (Tobin et al., 2010). This is because LXA4 is known to inhibit pro-inflammatory cytokines and cell proliferation (Liu et al., 2017) and PGE2 is known to play a role in the production of inflammation against the disease (Park et al., 2006).

The contribution of eicosanoid metabolites to susceptibility to mycobacterial infection was confirmed by Tobin et al. (2010). Their study identified leukotriene A4 hydrolase (LTA4H), an enzyme involved in the production of leukotriene B4 (LTB4), as a key determinant of host susceptibility to *Mtb* (Tobin et al., 2010). LTA4H mediates host susceptibility through two mechanisms that deregulate TNF-α activity (Laval et al., 2021): excess LTA4H causes overproduction of LTB4, which drives exuberant production of TNF-α, while low or an absence of LTA4H induces the production of anti-inflammatory LXA4, which suppresses TNF-α activity that leads to uncontrolled bacterial proliferation and macrophage necrosis (Tobin et al., 2010; Tobin et al., 2012).

Research has shown protein and lipid signatures associated with granuloma progression, which was accomplished by analysing different granuloma types

(intact, caseous, and cavitary granuloma) from the tissue found in patients who underwent surgery due to severe lung disease (Phuah et al., 2016). Over 3 000 proteins that were differentially expressed between different regions of granuloma types have been identified (Krug et al., 2021). Research has demonstrated more proteomic diversity within instead of between different granuloma types. The proteomic profiles of caseous and cavitary centres were composed largely of pro-inflammatory pathways, which comprise proteins that release pro-inflammatory eicosanoids from arachidonic acid metabolism (Krug et al., 2021). In contrast, cellular regions found at the periphery of granulomas display a more anti-inflammatory signature characterised by the abundance of pathways involved in normal cellular biogenesis and some enzymes involved in the synthesis of prostanoids (Marakalala et al., 2016). Overall, this suggests that the spatial organisation of inflammatory responses within the granuloma may determine the pathologic response to TB.

2.4 TUBERCULOSIS AND CURRENT MEDICATION

The WHO has issued recommendations for the treatment of drug-susceptible TB. The guidelines recommend a six-month treatment regimen comprising the four first-line TB medicines, namely INH, RIF, EMB, and PZA (WHO, 2022a). In the first two months, all four first-line medications are administered, followed by four months of INH and RIF (Suresh et al., 2022). Unfortunately, all anti-TB medications have side effects, as indicated in Table 2.1. Ineffective treatment of susceptible TB may lead to MDR TB in most cases, where the *Mtb* is resistant to INH and RIF. When *Mtb* bacilli are resistant to these drugs, even more toxic drugs need to be used. Treatment of MDR TB includes second-line drugs such as fluoroquinolones, bedaquiline, and linezolid (Ali et al., 2019). This treatment has a longer regimen starting at nine months and up to 20 months (WHO, 2022a), with the success rate of medicine completion being only 54% (Ndjeka et al., 2018).

Table 2.1: Side effects of first-line medication

Drugs	Side effects	References
Rifampin (RIF)	• Fever, hepatitis, thrombocytopenia, rash, renal failure, gastrointestinal upset • Reduces levels of many drugs, including methadone, warfarin, birth control pills, theophylline, dapsone, ketoconazole, and some non-nucleoside reverse transcriptase inhibitors • Discolouration of secretions (sputum, urine, sweat, tears) • Jaundice, acute kidney disease	Imam et al. (2020); Sreejith et al. (2020); Suresh et al. (2022)
Isoniazid (INH)	• Hepatic enzyme elevation, peripheral neuropathy, hepatitis, rash, central nervous system effects, increased phenytoin (dilation) and disulfiram (Antabuse) levels	Imam et al. (2020); Sreejith et al. (2020); Denholm et al. (2014)
Pyrazinamide (PZA)	• Gastrointestinal upset, hepatotoxicity, hyperuricemia, arthralgias, rash, gout	Imam et al. (2020); Sreejith et al. (2020); Kwon et al. (2020)
Ethambutol (EMB)	• Decreased red colour discrimination, decreased red colour acuity (optic neuritis), rash	Imam et al. (2020); Sreejith et al. (2020); Lee and Nguyen (2020)

The wide variety of side effects caused by current TB drugs (Gebreweld et al., 2018) cause people who develop side effects associated with TB medication to discontinue using these medications. The most worrying side effect is hepatitis in children (Sanchez-Codez et al., 2020). It definitely calls for alternative ways to treat TB, such as medicinal plants. Medicinal plants have been used for decades, and are still being used to this day. It is necessary to investigate the scientific evidence as to which plants are the most effective in treating TB and to which extent plants are effective in treating TB in terms of toxicity levels and inflammation activity.

2.5 USAGE OF TRADITIONAL MEDICINAL PLANTS

Since the beginning of time, people have relied on nature for survival in terms of food, shelter, and, most importantly, medicine. Plants have been the backbone of illness remedies, which go hand in hand with traditional African customs. Humans have accumulated knowledge of plant usage through experience, trial and error, and observations. A medicinal plant is a plant that, in one or more of its parts, contains a substance that can be used for therapeutic purposes, or which are a precursor for chemo-pharmaceutical semi-synthesis (Sofowora et al., 2013). The use of medicinal plants can be traced back as early 60 000 years ago and was first

formally documented around 4 000 years ago. Ancient Egypt, China, and India have a rich history of medicinal plant knowledge and usage, which are still being applied today (Karunamoorthi et al., 2013). Moreover, these countries still rely greatly on medicinal plants.

Many developing countries use medicinal plants for their primary healthcare needs. This may be due to living in remote areas where modern medicine is not easily accessible, or due to preference. South Asia accounted for 40% of the world's TB. This can be due to no access to modern healthcare and using traditional medicine (Basnyat et al., 2018). They may belong to communities where their first option is to seek traditional medicine rather than modern medicine; for example, in sub-Saharan Africa, the ratio of traditional healers is 1:500, which is significantly lower than the ratio of medical doctors at 1:40 000. They may believe that traditional medicine is more effective and has fewer side effects. Some may have experienced undesirable side effects with modern medicine and want to try traditional medicine (Karunamoorthi et al., 2013).

The utilisation of medicinal plants is strongly linked with cultural and traditional norms that are actively practised in modern-day South Africa. It is the very reason that there is still a demand for traditional healers, as they have a great deal of knowledge of medicinal plants. Even with the rapid increase in modernisation and urbanisation, educated black people still turn to traditional healers as a first resort and exchangeably utilise modern medicine and traditional medicinal plants (Ndhlala et al., 2011).

2.6 MEDICINAL PLANTS: THE FUTURE OF NEW DRUG DISCOVERIES

It has been estimated that 80% of the world population relies on medicinal plants and that 3.3 billion people in developing countries use medicinal plants regularly (Ahvazi et al., 2012). There has been a renewed interest in research on medicinal plants. This can be due to unmet therapeutic needs, the diversity of the chemical structure and biological activities of secondary naturally occurring metabolites, developmental improvements to detect natural products, and progress made in isolating, purifying, and structurally characterising active constituents (Rout et al., 2009). In the 21st century, naturally occurring products account for more than 50%

of drugs in clinical use, and in the last three decades, 50% of approved herbal medicine came directly or indirectly from plants, microorganisms, fungi, and animals (252 drugs are from plants; therefore 11%) (Shakya, 2016).

2.7 PHYTOCHEMICAL SCREENING

Phytochemicals are secondary metabolites that help plants fight against diseases but it has also been shown that they play a significant role in assisting people in combatting sickness and diseases (Saxena et al., 2013). They have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decreased platelet aggregation, modulation of hormone metabolism, and anticancer properties (De Silva et al., 2017). Phytochemicals may reduce the risk of coronary heart disease by averting the oxidation of low-density lipoprotein cholesterol; thus reducing the synthesis or absorption of cholesterol (Islam et al., 2021). This, in turn, normalises blood pressure and clotting properties, which helps to improve arterial elasticity. In addition, phytochemicals may detoxify substances that cause cancer and TB (Belay & Sisay, 2014).

Secondary constituents such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, and glucosides are usually investigated for their pharmacological activities (Sharma et al., 2019).

2.7.1 Flavonoids

Flavonoids have a large group of polyphenol compounds with a benzoyl-y-pyrone structure. They are bound to sugar(s) as glycosides, are structural derivatives of flavones, and they are phenolic and water (H_2O) soluble (Njeru et al., 2013). There are more than 4 000 flavonoids. Flavonoids are strong antioxidants and therefore play a role in protecting against degenerative diseases (Saxena et al., 2013). They are also known to increase coronary flow, reduce myocardial O_2 consumption, and lower arterial pressure. They are ubiquitous among vascular plants and are consumed by humans and animals. A study has shown that flavonoids have a strong influence against *Mtb* infectivity in human leukaemia monocytic cell

(THP-1) macrophages and in granulomas, including antimycobacterial effects (Cao et al., 2019).

2.7.2 Tannins

Tannins are a heterogeneous group of high-molecular-weight polyphenolic compounds with the capacity to form reversible and irreversible protein complexes. In most cases, tannins form polysaccharides such as cellulose, hemicellulose, protein, alkaloids, nucleic acids, and minerals. Plant extracts that contain tannins are used to treat diarrhoea and duodenal tumours and are used as anti-inflammatory antiseptics and antioxidants (Saxena et al., 2013). Their anti-inflammation activity suggests that they play a role in the pharmacological processes to treat TB (Kahkeshani et al., 2019).

2.7.3 Alkaloids

Alkaloids are one of the largest and main components produced by plants. They contain mostly basic nitrogen (N) atoms. The word “alkaloids” is taken from the word “alkaline”, which is used to describe N-containing bases. Alkaloids not only include carbon (C), hydrogen (H), and N, but can sometimes contain O_2 and sulphur (S) and, in rare cases, other elements such as chlorine (Cl), bromine (Br), and phosphorus (P). Alkaloids are mainly used to treat illnesses that affect the nervous system, act as a local anaesthetic, and possess anti-arrhythmic effects, antimalarial activity, and anticancer activity (Belay & Sisay, 2014).

2.7.4 Terpenoids

Terpenoids are derived from five-carbon isoprene units. The majority of terpenoids contain multicyclic structures that differ from one another by their functional groups and basic carbon skeletons. Terpenoids contain medicinal properties such as anticarcinogenic, antimalarial, anti-ulcer, hepaticidal, antimicrobial, and diuretic activity (Saxena et al., 2013).

2.8 MEDICINAL PLANTS IN THIS STUDY

The Fabaceae family is considered the most diverse plant family in the world, with numerous medicinal benefits to humans (Moteetee et al., 2019). It contains 490 medicinal plants, and is the second largest family of medicinal plants (Dzoyem et al., 2014). The family consists of trees, shrubs, and herbs and is distributed worldwide. *E. elephantina*, *L. lanceolata*, and *T. burchellianum* all belong to the Fabaceae family and their potential as anti-TB agents was investigated in this study. The selected parts of the plants used were aligned with reports by Kose et al., (2015) as traditional healers are reported to use the roots for the treat of TB in Lesotho.

2.8.1 *Elephantorrhiza elephantina* (Burch.) Skeels

2.8.1.1 Description

E. elephantina is considered as underground trees. *Elephantorrhiza* means elephant root. *E. elephantina* has annual stems that grow up to 90 cm tall at ground level, as shown in Figure 2.5. It has a thickened rhizome that is up to 8 m long. It has alternate, bipinnately compound leaves that are almost glabrous with a petiole that grows up to 8 cm long. The leaves comprise two to four pairs of pinnae in the lower leaves and seven to 17 pairs in the upper ones, and the axis is up to 10 cm long. The leaflets are up to 55 pairs per pinna, linear to oblong in shape, 4 to 15 mm long and 0.50 to 2.50 mm wide, with an asymmetric base, apex acute, and usually mucronate (Maroyi, 2017).

E. elephantina has an inflorescence that is an axillary raceme and is often limited to the lower part of the stem, usually solitary or clustered. The flowers are bisexual, with red-brown glands at the base and free petals. The petals are slightly connate at the base. The petals are linear-oblong, 2 to 4 mm long, and approximately 1 mm wide, and are yellow-white. It has 10 stamens that are free, with filaments up to 6.5 mm long. The fruit is a compressed-oblong, straight, or slightly curved pod 5 to 21 cm long and 3 to 6 cm wide, with a red-brown colour, prominently transversely veined, and often swollen over the seeds (Maroyi, 2017).

The *E. elephantina* plant is also known as elands bean, eland's wattle, elephant's root, *baswortel*, *elandsboontjie*, *leerbossie*, *looiersboontjie*, and *olifantswortel*. In Sesotho and Setswana, it is referred to as *mositsane*. In Shona, it is called *mupangara*, and in isiXhosa and isiZulu it is called *intolwane* (Mpofu et al., 2014). The plant is indigenous and is widespread in South Africa, Lesotho, Botswana, Zimbabwe, and Namibia.

Figure 2.5: *E. elephantina,* it is characterised by long green leaves, while the roots grow underground

Source: Author (2020)

2.8.1.2 Traditional uses

In Southern Africa, the roots and rhizomes of *E. elephantina* are boiled in water to treat acne and other skin diseases. The plant can be taken orally to treat blood pressure, clear the air canal, and to treat erectile dysfunction, haemorrhoids, itching, kidney failure, intestinal disorders, menstrual disorders, peptic ulcers, rheumatic conditions, shingles, sores, syphilis, and tonsillitis (Tlotlo & Olivier, 2022). In Botswana, the rhizome or root powder of *E. elephantina* is used to wipe the anus of children with bloody diarrhoea, to clean the womb after abortion, used as a remedy for early menstruating children, and to treat earache, erectile

dysfunction, and sexually transmitted infections (Maroyi, 2017). In Lesotho, a rhizome decoction of *E. elephantina* is used to cleanse the blood and treat breast cancer, herpes, infertility, intestinal disorders, stomach problems, syphilis, and TB (Maroyi, 2017). In Mozambique, leaf, rhizome, and root decoctions of *E. elephantina* are used to treat diarrhoea, anaemia, fever, and dysentery (Maroyi, 2016).

2.8.2 *Leobordea lanceolata* (E. Mey)

2.8.2.1 Description

L. lanceolata is known as *khonathi* in Sesotho. *L. lanceolata* is a perennial plant that produces a cluster of ascending stems that are simple or slightly branched and it can grow 25 to 50 cm tall. This plant species is found in South Africa and Lesotho. In South Africa, it is found in the Free State, KwaZulu-Natal, Limpopo, Mpumalanga, and North West provinces. It is mostly found on grassland (Von Staden, 2011). *L. lanceolata* is found from the subtropical to the warm temperate zones of eastern South Africa. The plant experiences frost in parts of its range (Useful Temperate Plants, 2022). Its synonyms are *Aspalathus lanceolata* and *Lotononis lanceolata.*

This species has a symbiotic relationship with certain soil bacteria, which form nodules on the roots and fix atmospheric nitrogen. Some of this nitrogen is utilised by the growing plant but some can be used by other plants growing nearby (Useful Temperate Plants, 2022).

Figure 2.6: *L. lanceolata* is characterised by yellow flowers and green leaves

Source: Author (2020)

2.8.2.2 Traditional uses

According to indigenous people, *L. lanceolata* is used to treat cancer, TB, asthma, stomach pains, fever, contagious diseases, and diarrhoea (Kose et al., 2015).

2.8.3 *Trifolium burchellianum* Ser. subsp. *burchellianum*

2.8.3.1 Description

T. burchellianum is a rhizomatous, tap-rooted perennial. The stems are glabrate, with the prostrate that sometimes grows up to 60 cm and develops nodal roots. The leaves are more or less globous, and are trifoliolate on long petioles. The leaflets are cuneate-obovate, cuneate-oblong, or cuneate-elliptic shaped, less often oblong, emarginate (sometimes truncate or rounded) at the apex, 2.5-5.0 × 1.9 cm, margin serrulate to denticulate. The inflorescence is many-flowered, the globose capitulum is often 1.5 to 3 cm across; the peduncle is longer than the subtending leaf, there are pilose towards the top and the pedicels are stout, usually 2 to 2.5 mm long, erect in fruit, and the calyx are glabrous except for a few hairs at the margin. The corolla is purple but can be white in rare circumstances, and is 8 to 13 mm long. The pod is approximately 5 mm long and 3 mm wide, with one or

two seeds. The seeds are dark brown, the shape is irregularly oval, and they are approximately 2.1 × 1.8 mm (Tropical Forages, 2022).

T. burchellianum in South Africa is often called African clover, Burchell's clover, cape clover, or wild clover (subsp. *burchellianum*). In Sesotho, it is referred to as *moqophi* or *moroko*, *usithathi* in isiZulu, and *wildeklawer* in Afrikaans (Tropical Forages, 2022). This plant species is mostly found in Angola, Kenya, Tanzania, Uganda, South Africa, and Lesotho (Magama, Lieta & Asita, 2013).

Figure 2.7: *T. burchellianum* is characterised by red flowers

Source: Author (2020)

2.8.3.2 Traditional uses

In Southern Africa, *T. burchellianum* is used to help treat heart problems, sore throat, heartburn, stomach cramps, high blood pressure, and cancer, and to clean the blood (Kose et al., 2015).

CHAPTER 3:
METHODOLOGY

3.1 INTRODUCTION

This chapter describes the methodology applied in this research. The methodology consists of plant material selection, methodology layout, plant extraction method, phytochemical screening, inflammation analysis, nitric acid (HNO_3) testing, and cytotoxicity and anti-TB testing.

3.2 PLANT MATERIAL SELECTION

E. elephantina, L. lanceolata, and *T. burchellianum* are from the Fabaceae family and are used by the indigenous people of Lesotho to treat TB (Moteetee et al., 2019). Unfortunately, these plants have not been scientifically investigated; there is thus a need to investigate these plants to establish the scientific data behind the usage of these plants for TB.

3.3 ETHICAL CLEARANCE

Ethical clearance was sought from the Environment and Biosafety Research Committee (number: UFS-ESD2021/0039) of the University of the Free State (see appendix).

3.4 PLANT MATERIAL COLLECTION

Plant materials were purchased from the nursery due to challenges experienced when applying for the import and export permits (delayed to no response) because of restrictions during COVID-19 pandemic. After collection, plants were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. Plants materials samples were preserved and delivered at the herbarium at CUT with voucher numbers: EE2020CUT for *E. elephantine*, LL2020CUT for *L. lanceolate*, and TB2020CUT for *T. burchellianum*.

3.5 METHODS AND MATERIALS

3.5.1 Plant extraction

The roots of the plants were washed with water to remove the soil and dried at room temperature (22°C). They were then crushed using the POLYMIX® PX-MFC 90 D grinder to make a fine powder. Plant extraction was done using the maceration method. Pulverised plant materials were soaked in methanol (MeOH) and distilled water (DH_2O) (Ace Chemicals, South Africa) for 48 hours, on a Heidolph Unimax 1010 shaker with occasional stirring. The extracts were filtered, and new solvent was added for more extraction until the solvents became clear. The organic extracts were concentrated using Butchi Rotavapor R-300 (Flawil, Switzerland), while aqueous extracts were concentrated using the Julabo F601 rotator evaporator (Seelbach, Germany). The organic extracts were then placed in an incubator for five days at 37°C to allow them to dry.

3.5.2 Phytochemical screening

Qualitative phytochemical screening for the presence of flavonoids, tannins, terpenoids, and alkaloids was carried out using the following methods of Shanmugam et al. (2010).

3.5.2.1 Test for tannins

Powdered plant material of 2.5 g was weighed using SCALTEC balance (Cape Town, South Africa). The plant material was boiled in 20 mL of DH_2O (Ace Chemicals, South Africa). Filtration was accomplished by using a funnel and filter paper. Approximately three drops of 0.1% ferric chloride ($FeCl_3$) (Sigma-Aldrich, USA) solution were added to half the filtrate volume. The appearance of an intense green or brownish green or a blue-black colouration indicated the presence of tannins. It was confirmed by adding a few drops of iodine (I) (Ace Chemicals, South Africa) to the second half of the filtrate to yield a faint bluish colouration that indicates that tannins are indeed present.

3.5.2.2 Test for flavonoids

Powdered plant material of 2.0 g was weighed using SCALTEC balance (Cape Town, South Africa). The powdered plant material was heated with 10 mL of ethyl acetate ($C_4H_8O_2$) (Ace Chemicals, South Africa) over a steam bath for three minutes at 50°C. The mixture was then filtered, 4 mL of the filtrate was shaken, and 1 mL of diluted ammonia (NH_3) solution (Ace Chemicals, South Africa) was added. Yellow colouration indicated a positive test for flavonoids.

3.5.2.3 Test for terpenoids (Salkowski test)

Powdered plant material of 5.0 g was weighed using SCALTEC balance (Cape Town, South Africa). The powdered plant material was mixed with 2 mL of chloroform ($CHCl_3$) (Sigma-Aldrich, USA) and thereafter 3 mL of concentrated sulphuric acid (H_2SO_4) (Ace Chemicals, South Africa) was carefully added to form a layer. Reddish-brown colouration at the interface indicated the presence of terpenoids.

3.5.2.4 Test for alkaloids

Powdered plant material of 0.5 g was weighed using SCALTEC balance (Cape Town, South Africa). The powdered plant material was mixed with 8 mL of 1% hydrochloric acid (HCl) (Ace Chemicals, South Africa), warmed, and filtered. Afterwards, 2 mL of the filtrate was treated with a few drops of freshly prepared Dragendorffs reagent (Merck Pty Ltd, Germany). The presence of precipitate showed a positive result.

3.5.3 Anti-inflammatory screening

The mouse macrophage cell line, RAW 264.7 (Cellonex, South Africa), is a well-characterised and popular model for investigating the anti-inflammatory potential of test samples. Cells were cultured in multi-well plates and activated by exposure to lipopolysaccharide (LPS) (Sigma-Aldrich, USA), which induces the expression of nitric oxide synthase (iNOS) (Ace Chemicals, South Africa) with concomitant NO formation. Changes in NO (Ace Chemicals, South Africa) production were determined by measuring the nitrate levels in the culture medium. Simultaneous

evaluation of cell viability (MTT assay) was used to confirm the absence of cytotoxicity in the test sample.

3.5.3.1 Sample preparation

Three aqueous extracts were solubilised using dimethyl sulfoxide (DMSO) (Ace Chemicals, South Africa) to make a stock of 100 mg/mL. Extracts were stored at 4°C until used. Aminoguanidine (CH_6N_4) (100 µM) was used as a positive control to indicate anti-inflammatory activity.

3.5.3.2 Anti-inflammatory screening protocol

RAW 264.7 (Cellonex, South Africa) cells were seeded in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich, USA) and supplemented with 10% foetal bovine serum (RPMI complete medium) into 96-well plates at a density of 1 x 10^5 cells per well and allowed to attach overnight. The following day, the spent culture medium was removed and the plant extracts (diluted in RPMI complete medium) were added to give final concentrations of 50, 100, and 200 µg/mL. To assess inflammatory activity, 50 µL of LPS (final concentration of 500 ng/mL) containing medium was added to the corresponding wells. Amnioguanidine (CH_6N_4) was used as the positive control at 100 µM. Cells were incubated for a further 24 hours. To quantify the NO (Ace Chemicals, South Africa) production, 50 µL of the spent culture medium was transferred to a new 96-well plate, and 50 µL Griess reagent was added. Absorbance was measured at 540 nm. A standard curve using sodium nitrate ($NaNO_3$) dissolved in an RPMI1640 culture medium (GE Healthcare Life Sciences, USA) was used to determine the NO (Ace Chemicals, South Africa) concentration in each sample.

3.5.4 Cytotoxicity

The cytotoxicity of extracts was performed using the Hoechst 33342/Propidium iodide (PI) dual staining method.

To confirm toxicity, cell viability was assessed using MTT assay. This was done by removing the remaining medium and treatments in each well, replacing it with medium containing 0.5 mg/mL MTT, and further incubating it for 30 minutes at

37°C. The MTT was removed, and 100 µL DMSO was added to each well to solubilise the formazan crystals. Absorbance was measured using a BioTek® PowerWave XS spectrophotometer (Winooski, Vermont, USA) and was read at 540 nm.

3.5.4.1 Plant extract preparation

Dried extracts were reconstituted in DMSO (Ace Chemicals, South Africa) to give a final 100 mg/mL concentration. The samples were sonicated if solubility was a problem and stored at 4°C until needed.

3.5.4.2 Cell line maintenance

The African green monkey kidney cell line, Vero cells (Sigma-Aldrich, USA), was used for cytotoxicity screening. The growth medium consisted of Dulbecco Modified Eagle Media (DMEM) (Marlborough, USA) supplemented with 10% foetal bovine serum (FBS) (Marlborough, USA). Cells were maintained in 10-cm culture dishes in a medium and incubated at 37°C in a Heal Force HF90/HF240 carbon dioxide (CO_2) atmosphere with 5% CO_2 (Shanghai, China).

3.5.4.3 Screening protocol

Cells were seeded into 96-well microtiter plates at a density of 4 000 cells/well using a volume of 100 µL in each well. The microtiter plates were incubated at 37°C, 5% CO_2, and 100% in a Heal Force HF90/HF240 CO_2 (Shanghai, China) using relative humidity for 24 hours before adding test compounds to allow for cell attachment.

3.5.4.4 Treatment

Samples were tested at the concentrations of 50, 100, and 200 µg/mL. A 400-µg/mL dilution of each plant extract was prepared in a complete medium (Marlborough, USA). A three-point dilution series was tested, which resulted in serial dilution. Serial dilution resulted in three concentrations of 100, 200, and 400 µg/mL. Aliquots of 100-µL dilutions were prepared. The aliquots of each dilution were added to 100 µL of attached cells in the 96-well plate. This yielded

final concentrations of 50, 100, and 200 µg/mL. The cells were treated for 48 hours at 37°C, 5% CO_2. Melphalan (100 mM stock) was used as a positive control at 20 µM.

3.5.4.5 Data acquisition and analysis

Quantification of live and dead cells was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) using a 10x Plan Fluor objective and 4′,6-diamidino-2-phenylindole (DAPI) and Texas Red filter cubes. Nine image sites were acquired per well, which are representative of roughly 75% of the surface area of the well. The acquired images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. The acquired data were transferred to a Microsoft Excel spreadsheet, and the data were analysed and processed.

3.5.5 Anti-TB screening

Antimicrobial susceptibility testing is important to determine which drugs a TB strain is resistant to. Drug Susceptibility Testing (DST) was performed using the BACTEC Mycobacteria Growth Indicator Tube (MGIT)™ 960 System (Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA) as described in the MGIT™ 960 System DST (Becton Dickinson, 2004) using *E. elephantina*, *L. lanceolata*, and *T. burchellianum* at these different concentrations of 50 µL, 100 µL, and 200 µL. The BACTEC MGIT™ 960 System is a broth-based, non-radiometric automated method that provides quantitative results within four to 13 days.

3.5.5.1 Sub-culture of the TB strains

The TB strains had to be re-cultured to ensure that they were still viable. For re-culturing, one must first vortex the tube to break up the clumps to allow large clumps to settle. Thereafter, further 1:5 dilutions were made by adding 1 mL plant extract to 4 mL sterile saline. All work was done in an Airstream biological safety cabinet (ESCO, Portland, US) class 2. Aseptically, a fresh MGIT™ tube was prepared by adding a supplement of 0.8 mL BBL MGIT™ OADC™ growth supplement (Becton Dickinson, New Jersey, US) to the labelled MGIT tubes. Thereafter 0.5 mL of the 1:5 dilutions was added. The tube was capped, and mixing

was done by inversion. The TB strains were loaded onto the MGIT™ 960 and monitored daily. The TB strains eventually turned positive, and that day was counted as Day 0.

An H37Rv strain was used as the susceptible control and two well-characterised strains from different lineage families, namely Lineage 2 (East African Indian [EAI]) and Lineage 4 (Latin American Mediterranean [LAM3]) strains, were used to determine if the plant extracts had anti-TB properties. These strains are part of a collection of strains characterised in a previous MMedSc study (Health Sciences Research Ethics Committee ethics number UFS-HSD2018/1034/2509), which are stored in the Medical Microbiology Tuberculosis Research Section at the University of the Free State. These strains included LAM3 (n=1) and EAI (n=1) susceptible isolates.

Plant extracts were weighed and reconstructed in DMSO (Ace Chemicals, South Africa). The TB strains were subjected to grow in the presence of *E. elephantina, L. lanceolata*, and *T. burchellianum* plant extracts. A control was also included without the addition of the plant extracts. A growth control tube, containing the plant extracts at three different concentrations with different strains, and a bar-coded tube carrier holding the set were used. Analysis of the fluorescence, designated as growth value, in a drug-containing tube was compared to the fluorescence of the growth control tube to determine susceptibility results. If the TB strains grow in the control and do not grow in the presence of the drug, they are considered susceptible, but if they grow in both tubes, they are considered resistant to that drug.

3.5.5.2 Treatment protocol

The methanol and water extracts of the plants were reconstituted in 4 mL of DMSO to prepare a stock solution. Finally, four fresh MGITs, one as a growth control (GC) and the other for susceptibility testing of the three concentrations of plant extract, were labelled corresponding to each plant. The growth control (GC) was prepared by adding 100 µL of inoculum to 10 mL of sterile saline to achieve a final dilution of 1:500. A 500 µL volume of the prepared GC was inoculated into the MGIT™ GC without any plant extracts. Then 0.1 mL of each plant stock solution was added into

the MGIT™ tube containing 7 mL growth medium, 0.8 mL of MGIT™ OADC™ and 0.5 mL of the TB strain inoculum to achieve a final concentration of 200 µg/mL, 100 µg/mL and 50 µg/mL. All tubes were incubated at 37°C in the MGIT™ 960 System and monitored for growth until the system detected a growth result. Growth control tubes with a growth unit value of more than 400 caused the BACTEC MGIT™ 960 instruments to indicate that the test was complete; the susceptibility test set was scanned, and a report of the results was printed. The results were reported as susceptible when the tests with growth unit values of 400 or more and a growth unit value of less than 100 for the tubes containing drugs (plant extract), while if the growth unit value of the drug tube was 100 and more, it was reported as resistant. The instrument indicated an X 200 error for tubes with growth unit values of less than 400 within 21 days; this indicated insufficient growth. Growth unit values exceeded 400 in 4 days for growth controls are indicated with an X 400 error by the machine, which indicates contamination. The automatically printed result was then interpreted according to the MGIT™ procedure manual (Siddiqi & Rüsch-Gerdes, 2006).

3.6 Statistical analysis

Statistical analysis was performed using Excel. Each experiment was conducted in quadruplicate. Statistical significance was determined using the Two-tailed Student t-test and $p<0.05$ was deemed significant.

CHAPTER 4:
RESULTS

4.1 PHYTOCHEMICAL SCREENING

The results of the phytochemical screening analysis of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* are reported in Table 4.1. In Table 4.1, a positive and a negative sign were used, where (+) indicates the presence of the phytochemical, (++) showed the intensity of colour change, while (-) indicates the absence of phytochemicals and no colour change.

Table 4.1: Showing which phytochemicals were present and their intensity

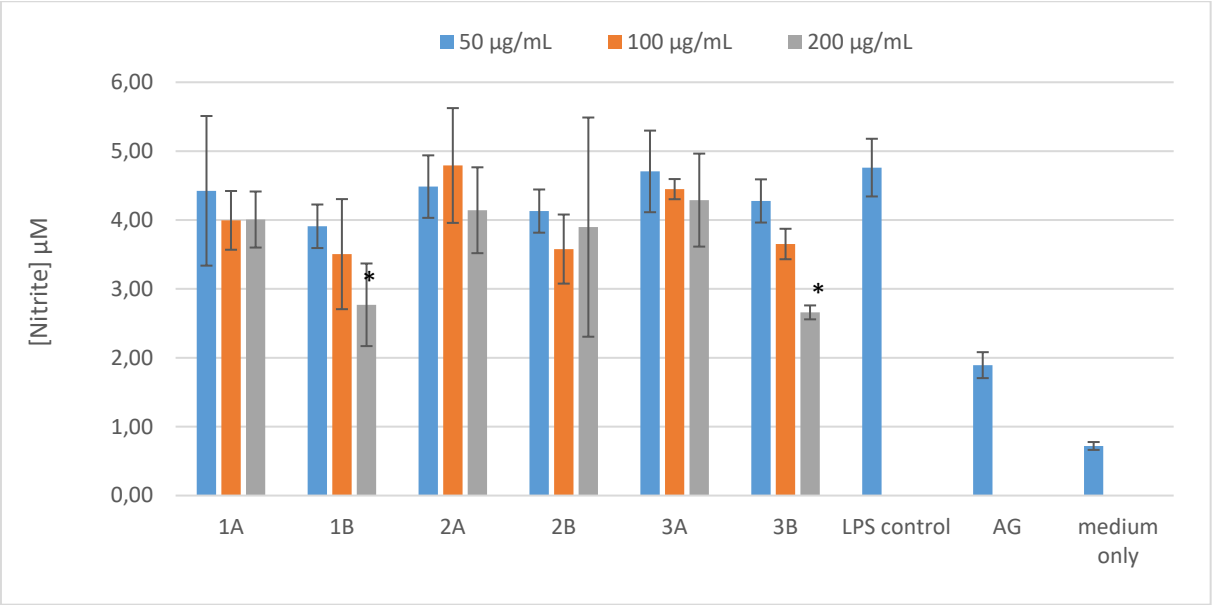
Plant extract	Flavonoids	Tannins	Alkaloids	Terpenoids
E. elephantina	++	++	-	++
L. lanceolata	+	+	-	+
T. burchellianum	+	+	-	+

4.2 ANTI-INFLAMMATORY ACTIVITY

Inflammation is usually associated with a protective or healing response, and many chronic diseases are characterised by persistent inflammation that results in tissue dysfunction. For this reason, the anti- or pro-inflammatory activity of the sample must be considered within the context of the disease in question and the disease progression stage at which intervention will be considered to evaluate the potential therapeutic significance.

The mouse macrophage cell line, RAW 264.7, is a well-characterised and popular model to investigate the anti-inflammatory potential of test samples. Cells were cultured in multi-well plates and activated by exposure to bacterial LPS, which induces the expression of iNOS with concomitant NO formation. Changes in NO production were determined by measuring the nitrite levels in the culture medium. The extracts were screened against RAW 264.7 cells for anti-inflammatory potential. Figure 4.1 illustrates the results of this experiment. To determine the cytotoxic potential against the cells, an MTT assay was performed (see Figure 4.1). Figure 4.1 demonstrates that all three plants promoted NO production at the same level as LPS, which was used as the negative control. However, 1B (*E. elephantina* extract H_2O at 200 µg/mL) and 3B (*T. burchellianum* extract at 200 µg/mL) showed

lower values of the NO production. These plants showed promising anti-inflammatory activity at the highest tested concentration of 200 µg/mL.

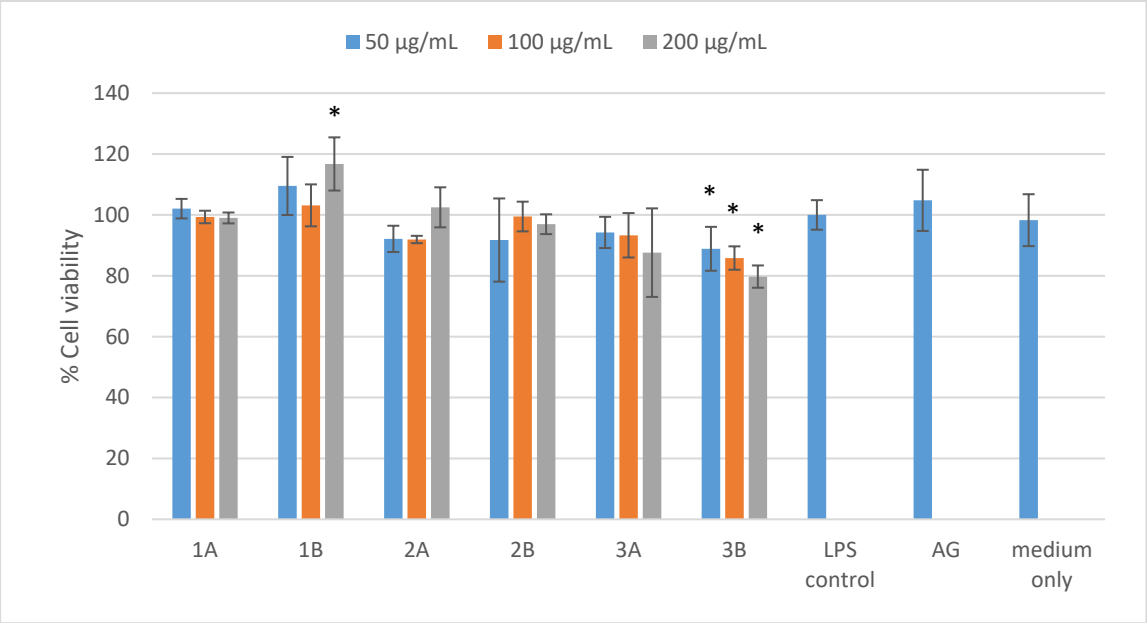


1 represents *E. elephantina*	
2 represents *L. lanceolata*	**A represents** MeOH extracts
3 represents *T. burchellianum*	**B represents** H_2O extracts

Figure 4.1: Nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages treated with different concentrations of extracts. *p-value is <0.05, compared to LPS activated cells.

Figure 4.1 shows that none of the plant extracts inhibited cell proliferation of RAW 264.7 macrophages. The bar graphs in Figure 4.1 represent the quadruplicate values of one experiment and the error bars represent the standard deviation of the mean.

In Figure 4.2, LPS-activated macrophages treated with *E. elephantina* water extracts proliferated more than cells treated with medium only. This indicates that the extract promoted the proliferation of macrophages while producing low levels of NO as shown in Figure 4.1. The bar graphs in Figure 4.2 represent the quadruplicate values of one experiment and the error bars represent the standard deviation of the mean. The results were statistically significant as the p-value was less than 0.05.



1 represents *E. elephantina*
2 represents *L. lanceolata*
3 represents *T. burchellianum*

A represents MeOH extracts
B represents H_2O extracts

Figure 4.2: Cell viability (%) of LPS-activated macrophages after 24 hours of treatment exposure. *p-value is <0.05, compared to LPS activated cells.

Figure 4.3 illustrates a standard curve that shows the relationship between MTT and NO. The regression line demonstrates that the possibility of error is very low, which confirms the accuracy of the results.

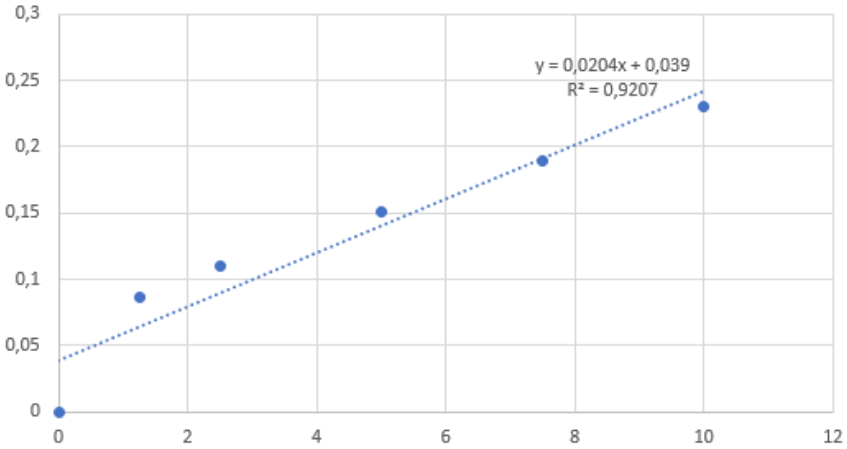
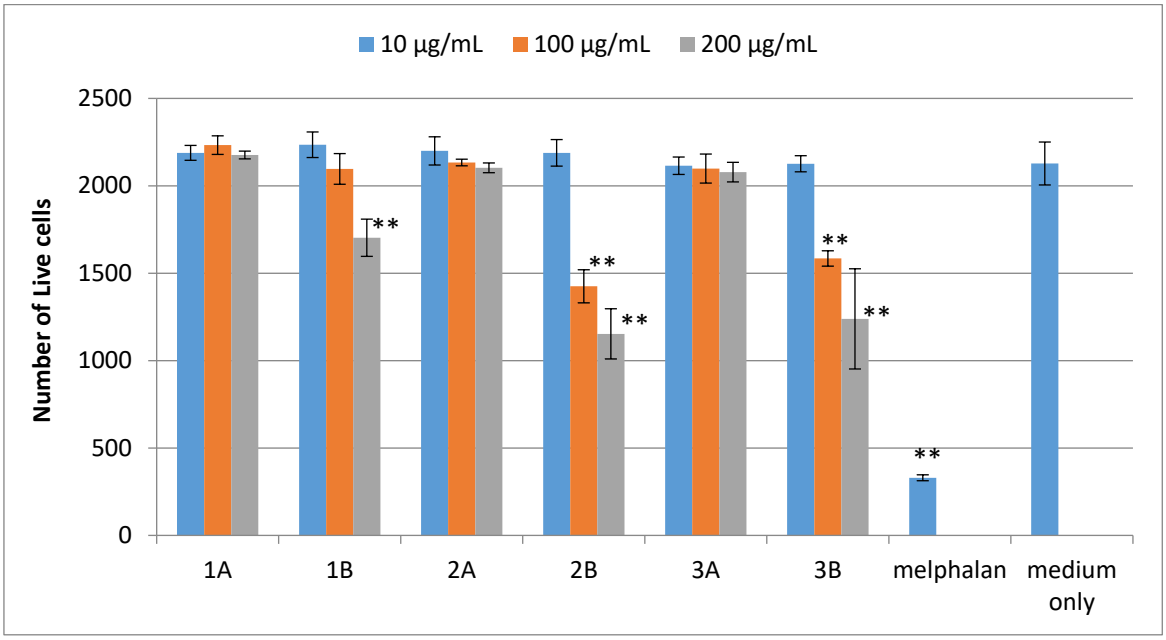


Figure 4.3: Standard curve showing the relationship between 3-(4,5-dimethylimidazol-2,5-diphenyltetrazolium bromide) (MTT) and Nitric oxide (NO)

The cytotoxicity of six samples was assessed using Vero cells (Sigma-Aldrich, USA). Figure 4.4 shows processed live cell data after 48 hours of treatment. The figure shows that the cells were treated against Vero cells, with the plant extracts in 2B (*L. lanceolata* of H_2O extract) and 3B (*T. burchellianum* of H_2O extract) demonstrating toxicity at 200 µg/mL. The cytotoxicity was observed by the decreased growth compared to medium only, but it was not as toxic as melphalan (positive control). The decreased growth confirms the plant extracts to be toxic against Vero cells. Other plant extracts grown indicated no toxicity.



1 represents *E. elephantina*
2 represents *L. lanceolata*
3 represents *T. burchellianum*
A represents MeOH extracts
B represents H_2O extracts

Figure 4.4: The cytotoxicity of six extracts tested against Vero cells. **p-value is <0.001 compared to control cells

The cells were treated for 48 hours. The error bars in Figure 4.4 indicate the standard deviation of quadruplicate values done as a single experiment. The results were statistically highly significant as the p-value was less than 0.001. This supports the validity of the results obtained.

4.3 ANTI-TUBERCULOSIS SCREENING

Anti-TB screening is an effective tool to determine the effectiveness of plants or chemical compounds developed to prove their properties against TB.

DST was performed using the MGIT™ 960 System (Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA) as described in the MGIT™ 960 System DST (Becton Dickinson, 2004). TB susceptibility strains such as LAM and EAI are profound in South Africa and were used and tested against the three plants investigated.

Table 4.2: Results of the susceptibility tests of *E. elephantina* extracts

Plant extract	TB strain	Concentration	Solvents used	Testing tube	Control tube
E. elephantina	LAM	50 µg/mL	MeOH	+	+
			H_2O	+	+
		100 µg/mL	MeOH	+	+
			H_2O	+	+
		200 µg/mL	MeOH	+	+
			H_2O	+	+
	EAI	50 µg/mL	MeOH	+	+
			H_2O	+	+
		100 µg/mL	MeOH	+	+
			H_2O	+	+
		200 µg/mL	MeOH	+	+
			H_2O	+	+

The "+" sign demonstrates positive growth of the TB strain; LAM = lineage 4 LAM3 strain; EAI = Lineage 2 EAI strain.

Table 4.3: Results of the susceptibility tests of *L. lanceolata* extracts

Plant extract	TB strain	Concentration	Solvents used	Testing tube	Control tube
L. lanceolata	LAM	50 µg/mL	MeOH	+	+
			H_2O	+	+
		100 µg/mL	MeOH	+	+
			H_2O	+	+
		200 µg/mL	MeOH	+	+
			H_2O	+	+
	EAI	50 µg/mL	MeOH	+	+
			H_2O	+	+
		100 µg/mL	MeOH	+	+
			H_2O	+	+
		200 µg/mL	MeOH	+	+
			H_2O	+	+

Table 4.4: Results of the susceptibility tests of *T. burchellianum* extracts

Plant extract	TB strain	Concentration	Solvents used	Testing tube	Control tube
T. burchellianum	LAM	50 µg/mL	MeOH	+	+
			H_2O	+	+
		100 µg/mL	MeOH	+	+
			H_2O	+	+
		200 µg/mL	MeOH	+	+
			H_2O	+	+
	EAI	50 µg/mL	MeOH	+	+
			H_2O	+	+
		100 µg/mL	MeOH	+	+
			H_2O	+	+
		200 µg/mL	MeOH	+	+
			H_2O	+	+

These results show that the plants investigated in this study did not have any potential as anti-TB agents for the tested strains, even at the highest tested concentration of 200 µg/mL.

CHAPTER 5:
DISCUSSION OF RESULTS

5.1 DISCUSSION

TB controls are still a challenge globally. Much of it has to do with accessibility and beliefs towards medication (Dheda et al., 2014). Barriers include gender, age, type of disease, social setting, and the ability to pay. Many of these barriers contribute to the difficulty of treating a curable disease, which can lead to it developing resistance and thus making it even harder to treat (Kerr, 2013).

TB has been around since ancient Egyptian and biblical times (Buzic & Giuffra, 2020). Today, TB still affects a large majority of the global population. It has been estimated that one-third of the world's population is infected with TB but the majority of this infection is latent and most people will not develop the disease (WHO, 2022b). A large number of people who do develop TB are mostly situated in the developing world (Bliss, 2014). The current TB medication regimen for first-line drugs such as isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) can be taken for approximately six months (WHO, 2022a). These medications often have severe side effects (Imam et al., 2020), which include hepatitis, cancer, etc. (Sanchez-Codez et al., 2020). This often leads to people abandoning the medication prematurely and developing drug-resistant TB. This prompted the researcher to investigate alternative medication that can be used to treat TB. One such alternative is medicinal plants.

Medicinal plants provide a gateway to break the barriers that TB control faces. In South Africa, medicinal plants have been used for generations and knowledge of how these medicinal plants work has been passed down through the ages, usually through traditional healers and herbalists (Shakya, 2016). The plants were selected for this study based on their reported use for TB treatment (Kose et al., 2015). The study is important because it aimed to address the effectiveness of *E. elephantina*, *L. lanceolata*, and *T. burchellianum* against selected TB strains, their anti-inflammatory activities, and cytotoxicity to non-cancer cells.

Chronic inflammation is very common in TB patients. It is very important for medication to target inflammation and pro-inflammation structures as this will help

to significantly reduce its activity in the host organ, which is the lungs. The potential drug should be able to penetrate the intracellular environment (Muefong & Sutherland, 2020).

Anti-inflammatory activity of selected medicinal plants was determined through the MTT assay. For anti-inflammation studies, both the MeOH and H_2O extracts of plants were tested at the concentrations of 50 µg/mL, 100 µg/mL, and 200 µg/mL, for *E. elephantina* and *T. burchellianum*. It was observed that the *E. elephantina* and *T. burchellianum* H_2O extracts showed good anti-inflammatory activity at 200 µg/mL. The anti-inflammatory activity observed could be due to the presence of flavonoids and anthraquinone as previous studies showed that cell culture composed of anthraquinone exhibited anti-inflammatory activity (Maroyi, 2017). Additionally, *E. elephantina* was found to have anti-inflammatory activity on skin cells (Mabona & Van Vuuren, 2013). Unfortunately, *L. lanceolata* did not show any anti-inflammatory activity. This could be due to low levels of flavonoids, tannins, and terpenoids present in this plant; the colour intensity from the colorimetric assay was not very strong for this plant.

The *E. elephantina* MeOH and H_2O extracts showed no toxicity levels at the three different concentrations of 50 µg/mL, 100 µg/mL, and 200 µg/mL against Vero cells (non-cancer cells). However, the *L. lanceolata* and *T. burchellianum* H_2O extracts showed dose-dependent cytotoxicity against Vero cells at 200 µg/mL. This suggests that *E. elephantina* is non-cytotoxic to normal cells, which corresponds with the literature (Maphosa et al., 2010). Furthermore, all tested plant extracts showed no cytotoxicity at the concentration of 10 µg/mL.

The plant extracts were evaluated for iNOS expression. The iNOS serves as an indication of immune-regulatory agents. The changes in NO production were determined by measuring the nitrate levels. The study showed that *E. elephantina* NO levels were relatively reduced when compared to the LPS control. Both *E. elephantina* extracts (H_2O and MeOH) therefore decreased the LPS-induced production of inflammatory mediators. Furthermore, both these extracts showed no cytotoxic effect on LPS-activated macrophages and Vero cells at the concentrations of 10 µg/mL and 100 µg/mL. A significant decrease in LPS-induced production was by *T. burchellianum* H_2O at the concentration of 200 µg/mL.

However, this concentration was cytotoxic to Vero cells. High concentrations (200 µg/mL) of *T. burchellianum* therefore cannot be considered safe.

Anti-TB studies were conducted using DST to evaluate how effective the tested plant extracts were at inhibiting the TB growth of the two selected TB strains. All the plants studied showed no inhibition of TB growth even though secondary metabolites were identified, such as flavonoids, tannins, and terpenoids. This could be due to the tested plant extracts lacking alkaloids, as alkaloids have been reported to be responsible for anti-TB activity through structural interaction with *Mtb* (Kishore et al., 2009). These results contradict the findings by Mpofu et al. (2014) that reported the presence of alkaloids from *E. elephantina* collected from Eswatini, South Africa, and Zimbabwe.

It is known that geographic location influences the secondary metabolites that a plant produces, which is a result of variations in soil conditions, light, H_2O, CO_2, temperature, and mineral nutrients (Ullrich et al., 2017; Wink, 2003). Plants' ability to adapt to their environment and deal with stress is greatly influenced by secondary metabolites. Harsh conditions such as drought, high salinity, frost, and very high temperatures stimulate plants' production of secondary metabolites. According to Yang et al. (2018), stressed plants produce more secondary metabolites and in larger quantities than unstressed plants. The negative activity observed from this study is because the plants used were purchased from a nursery. Nursery-grown plants are not exposed to very much stress as compared to plants growing freely in their natural habitats (fields and mountains). This could be the reason that the tested plants did not have any alkaloids. Other factors that influenced the anti-TB activity of the extracts are the failure of the extracts to exert destructive effects on the integrity of the mycobacterial cellular structure or testing environmental factors that influenced the interaction of the plant extracts with the LAM and EAI tuberculosis strains.

CHAPTER 6:
CONCLUSION, LIMITATIONS, AND RECOMMENDATIONS

6.1 CONCLUSION

From the results gathered, the *E. elephantina* H_2O extract was found to be one of the most promising plants as a candidate for future anti-inflammation drug discovery and development. The plant roots can be considered safe to use as no toxicity to the Vero cells at three varying concentrations was observed; however, this cannot be concluded without performing an in vivo safety analysis. Inflamed cells are one of the characteristics of patients with TB. *T. burchellianum* showed cytotoxicity at the highest concentration tested on Vero cells but presented anti-inflammation activity at that concentration. Since the *E. elephantina and T. burchellianum* plant extracts showed anti-inflammation activity, they might help to alleviate the symptoms associated with TB in TB patients as their immune system is weakened. *L. lanceolata* showed toxicity to the Vero cells at the highest concentration tested but showed no anti-inflammatory activity. All the plant roots tested showed no anti-TB activity as they all lacked alkaloids, but all presented with phytochemicals such as flavonoids, tannins, and terpenoids. Alkaloids have been reported to have anti-TB activity due to their ability to interact with the TB cell structure.

6.2 LIMITATIONS

It was not possible to test the plant extracts against a larger number of TB strains and cell lines due to the financial implications.

6.3 RECOMMENDATIONS

The study showed no evidence of anti-TB activity using *E. elephantina*, *L. lanceolata*, and *T. burchellianum* root extracts; however, other parts of the plant or any combination of plant extracts might exhibit anti-TB activity. Investigating some parts of the plants and in combination is therefore recommended.

E. elephantina and *T. burchellianum* showed strong anti-inflammatory activity and a reduction of NO production in macrophages. Additional studies should thus be

pursued to investigate the mechanism of action and to investigate these plants for their immunomodulatory activity.

Quantification of secondary metabolites in the tested plants should be undertaken to support the variation in phenolic content between the three tested plants as this led to a lack of anti-inflammatory activity by *L. lanceolata.*

Isolation and characterisation of compounds present in the three tested plants should be performed.

Toxicity tests should be re-conducted in vivo using animal models. Plant-drug interactions should also be conducted.

REFERENCES

Actor, J.K. 2015. Lactoferrin: A modulator for immunity against tuberculosis related granulomatous pathology. *Mediators of Inflammation*, 2015:409596.

Agidew, M.G. 2022. Phytochemical analysis of some selected traditional medicinal plants in Ethiopia. *Bulletin of the National Research Centre*, 46(1):1-22.

Ahvazi, M., Khalighi-Sigaroodi, F., Charkhchiyan, M.M., Mojab, F., Mozaffarian, V.A. & Zakeri, H. 2012. Introduction of medicinal plants species with the most traditional usage in Alamut region. *Iranian Journal of Pharmaceutical Research*, 11(1):185-194.

Ali, M.H., Alrasheedy, A.A., Kibuule, D., Godman, B., Hassali, M.A. & Ali, H.M.H. 2019. Assessment of multidrug-resistant tuberculosis (MDR-TB) treatment outcomes in Sudan: Findings and implications. *Expert Review of Anti-Infective Therapy*, 17(11):927-937.

Andrews, J.R., Morrow, C. & Wood, R. 2013. Modeling the role of public transportation in sustaining tuberculosis transmission in South Africa. *American Journal of Epidemiology*, 177:556-561.

Arya, N., Raut, M.K., Tekale, S.G., Shishoo, C.J. & Jain, K.S. 2014. Tuberculosis: New drug discovery pipelines. *Austin Journal of Analytical and Pharmaceutical Chemistry*, 1014:2381-8913.

Barberis, I., Bragazzi, N.L., Galluzzo, L. & Martini, M. 2017. The history of tuberculosis: From the first historical records to the isolation of Koch's bacillus. *Journal of Preventive Medicine and Hygiene*, 58(1):E9.

Basnyat, B., Caws, M. & Udwadia, Z. 2018. Tuberculosis in South Asia: A tide in the affairs of men. *Multidisciplinary Respiratory Medicine*, 13(1):1-8.

Bea, S., Lee, H., Kim, J.H., Jang, S.H., Son, H., Kwon, J.W. & Shin, J.Y. 2021. Adherence and associated factors of treatment regimen in drug-susceptible tuberculosis patients. *Frontiers in Pharmacology*, 12:625078.

Becton Dickinson. 2004. *Bactec MGIT 960 SIRE Kit for the Antimycobacterial Susceptibility Testing of Mycobacterium Tuberculosis*. Franklin Lakes: Becton Dickinson.

Belay, K. & Sisay, M.J.C.M.R. 2014. Phytochemical constituents and physicochemical properties of medicinal plant (*Moringa oleifera*) around Bule Hora. *Chemistry and Materials Research*, 6(7):61-72.

Bliss, K.E. 2014. *Building New Relationships on TB Control, One BRIC at a Time*. Available at: https://csis-website-prod.s3.amazonaws.com/s3fs-public/legacy_files/files/publication/140604_Bliss_BRICS%26TB_Web.pdf [Accessed: 12 May 2022].

Braian, C. 2020. Innate Immune Responses to *Mycobacterium Tuberculosis* Infection: How Extracellular Traps and Trained Immunity Can Restrict Bacterial Growth (Doctoral dissertation). Sweden: Linköping University.

Buzic, I. & Giuffra, V. 2020. The paleopathological evidence on the origins of human tuberculosis: A review. *Journal of Preventive Medicine and Hygiene*, 61(1 S1):E3-E8.

Cambau, E. & Drancourt, M. 2014. Steps towards the discovery of *Mycobacterium tuberculosis* by Robert Koch, 1882. *Clinical Microbiology and Infection*, 20(3):196-201.

Cao, R., Teskey, G., Islamoglu, H., Gutierrez, M., Salaiz, O., Munjal, S., Fraix, M.P., Sathananthan, A., Nieman, D.C. & Venketaraman, V. 2019. Flavonoid mixture inhibits *Mycobacterium tuberculosis* survival and infectivity. *Molecules*, 24(5):851.

Centers for Disease Control and Prevention. 2022. *Chapter 2: Transmission and Pathogenesis of Tuberculosis*. Available at: https://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf [Accessed: 24 May 2022].

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X. & Zhao, L. 2018. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, 9(6):7204-7218.

Chhetri, A.K., Saha, A., Verma, S.C., Palaian, S., Mishra, P. & Shankar, P.R. 2008. A study of adverse drug reactions caused by first line anti-tubercular drugs used in Directly Observed Treatment, Short course (DOTS) therapy in Western Nepal, Pokhara. *Journal of the Pakistan Medical Association*, 58(10):531-536.

Copenhagen Consensus Center. 2022. *South Africa Perspective: Tuberculosis*. Available at: https://www.copenhagenconsensus.com/publication/south-africa-perspective-tuberculosis [Accessed 17 July 2022].

Denholm, J.T., McBryde, E.S., Eisen, D.P., Penington, J.S., Chen, C. & Street, A.C. 2014. Adverse effects of isoniazid preventative therapy for latent tuberculosis infection: A prospective cohort study. *Drug, Healthcare and Patient Safety*, 6:145-149.

De Silva, G.O., Abeysundara, A.T. & Aponso, M.M.W. 2017. Extraction methods, qualitative and quantitative techniques for screening of phytochemicals from plants. *American Journal of Essential Oils and Natural Products*, 5(2):29-32.

Dheda, K., Gumbo, T., Gandhi, N.R., Murray, M., Theron, G., Udwadia, Z., Migliori, G.B. & Warren, R. 2014. Global control of tuberculosis: From extensively drug-resistant to untreatable tuberculosis. *The Lancet Respiratory Medicine*, 2(4):321-338.

Dorhoi, A. & Kaufmann, S.H. 2015. Versatile myeloid cell subsets contribute to tuberculosis-associated inflammation. *European Journal of Immunology*, 45(8):2191-2202.

Dzoyem, J.P., McGaw, L.J. & Eloff, J.N. 2014. In vitro antibacterial, antioxidant and cytotoxic activity of acetone leaf extracts of nine under-investigated Fabaceae tree species leads to potentially useful extracts in animal health and productivity. *BMC Complementary and Alternative Medicine*, 14:1-7.

Fatima, S., Kumari, A. & Dwivedi, V.P. 2021. Advances in adjunct therapy against tuberculosis: Deciphering the emerging role of phytochemicals. *MedComm*, 2(4):494-513.

Fatmawati, M.A.K., Bonyah, E., Hammouch, Z. & Shaiful, E.M. 2020. A mathematical model of tuberculosis (TB) transmission with children and adults groups: A fractional model. *Aims Math*, 5(4):2813-2842.

Gebreweld, F.H., Kifle, M.M., Gebremicheal, F.E., Simel, L.L., Gezae, M.M., Ghebreyesus, S.S., Mengsteab, Y.T. & Wahd, N.G. 2018. Factors influencing adherence to tuberculosis treatment in Asmara, Eritrea: A qualitative study. *Journal of Health, Population and Nutrition*, 37:1-9.

Harding, C.V. & Boom, W.H. 2010. Regulation of antigen presentation by *Mycobacterium tuberculosis*: A role for Toll-like receptors. *Nature Reviews Microbiology*, 8(4):296-307.

Heemskerk, D., Caws, M., Marais, B. & Farrar, J. 2015. *Tuberculosis in Adults and Children*. London: Springer.

Hoagland, D.T., Liu, J., Lee, R.B. & Lee, R.E. 2016. New agents for the treatment of drug-resistant *Mycobacterium tuberculosis*. *Advanced Drug Delivery Reviews*, 102:55-72.

Imam, F., Sharma, M., Khayyam, K.U., Al-Harbi, N.O., Rashid, M.K., Ali, M.D., Ahmad, A. & Qamar, W. 2020. Adverse drug reaction prevalence and mechanisms of action of first-line anti-tubercular drugs. *Saudi Pharmaceutical Journal*, 28(3):316-324.

Islam, S.U., Ahmed, M.B., Ahsan, H. & Lee, Y.S. 2021. Recent molecular mechanisms and beneficial effects of phytochemicals and plant-based whole foods in reducing LDL-C and preventing cardiovascular disease. *Antioxidants*, 10(5):784.

Kahkeshani, N., Farzaei, F., Fotouhi, M., Alavi, S.S., Bahramsoltani, R., Naseri, R., Momtaz, S., Abbasabadi, Z., Rahimi, R., Farzaei, M.H. & Bishayee, A. 2019. Pharmacological effects of gallic acid in health and diseases: A mechanistic review. *Iranian Journal of Basic Medical Sciences*, 22(3):225-237.

Kaiko, G., Horvat, J., Beagley, K. & Hansbro, P. 2008. Immunological decision-making: How does the immune system decide to mount a helper T-cell response? *Immunology*, 123(3):326-338.

Karunamoorthi, K., Jegajeevanram, K., Vijayalakshmi, J. & Mengistie, E. 2013. Traditional medicinal plants: A source of phytotherapeutic modality in resource-constrained health care settings. *Journal of Evidence-Based Complementary & Alternative Medicine*, 18(1):67-74.

Kerr, P.G. 2013. Plants and tuberculosis: Phytochemicals potentially useful in the treatment of tuberculosis. In M. Rai & K. Kon (Eds.). *Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and Their Components*. Amsterdam: Academic Press.

Kishore, N., Mishra, B.B., Tripathi, V. & Tiwari, V.K. 2009. Alkaloids as potential anti-tubercular agents. *Fitoterapia*, 80:149-163.

Kose, L.S., Moteetee, A. & Van Vuuren, S. 2015. Ethnobotanical survey of medicinal plants used in the Maseru district of Lesotho. *Journal of Ethnopharmacology*, 170:184-200.

Krug, S., Parveen, S. & Bishai, W.R. 2021. Host-directed therapies: Modulating inflammation to treat tuberculosis. *Frontiers in Immunology*, 12:660916.

Kwon, B.S., Kim, Y., Lee, S.H., Lim, S.Y., Lee, Y.J., Park, J.S., Cho, Y.J., Yoon, H.I., Lee, C.T. & Lee, J.H. 2020. The high incidence of severe adverse events due to pyrazinamide in elderly patients with tuberculosis. *PloS One*, 15(7):e0236109.

Laval, T., Pedró-Cos, L., Malaga, W., Guenin-Macé, L., Pawlik, A., Mayau, V., Yahia-Cherbal, H., Delos, O., Frigui, W., Bertrand-Michel, J. & Guilhot, C. 2021. De novo synthesized polyunsaturated fatty acids operate as both host immunomodulators and nutrients for *Mycobacterium tuberculosis*. *Elife*, 10:e71946.

Lee, N. & Nguyen, H. 2020. *Ethambutol*. Florida: StatPearls Publishing.

Liu, X., Wang, X., Duan, X., Poorun, D., Xu, J., Zhang, S., Gan, L., He, M., Zhu, K., Ming, Z., Hu, F. & Chen, H. 2017. Lipoxin A4 and its analog suppress inflammation by modulating HMGB1 translocation and expression in psoriasis. *Scientific Reports*, 7(1):7100.

Mabona, U. & Van Vuuren, S.F. 2013. Southern African medicinal plants used to treat skin diseases. *South African Journal of Botany*, 87:175-193.

Madisha, J.K. 2017. Antimycobacterial Activities of Selected Plants Used in the Management of Tuberculosis in Sekhukhune (Limpopo Province), South Africa (Doctoral dissertation). Bloemfontein: University of the Free State.

Magama, S., Lieta, M.I. & Asita, A.O. 2013. Antioxidant and free radical scavenging properties of four plant species used in traditional medicine in Lesotho. *International Journal of Medicinal Plant Research*, 2(3):170-178.

Maphosa, V., Masika, P.J. & Moyo, B. 2010. Toxicity evaluation of the aqueous extract of the rhizome of *Elephantorrhiza elephantina* (Burch.) Skeels. (Fabaceae), in rats. *Food and Chemical Toxicology*, 48(1):196-201.

Marakalala, M., Raju, R., Sharma, K., Zhang, Y., Eugenin, E., Prideaux, B., Daudelin, I., Chen, P., Booty, M., Kim, J., Eum, S., Via, L., Behar, S., Barry, C., Mann, M., Dartois, V. & Rubin, E. 2016. Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nature Medicine*, 22(5):531-538.

Maroyi, A. 2016. Treatment of diarrhoea using traditional medicines: Contemporary research in South Africa and Zimbabwe. *African Journal of Traditional, Complementary and Alternative Medicines*, 13(6):5-10.

Maroyi, A. 2017. *Elephantorrhiza elephantina*: Traditional uses, phytochemistry, and pharmacology of an important medicinal plant species in Southern Africa. *Evidence-Based Complementary and Alternative Medicine*, 2017:6403905.

Mensah, M.L., Komlaga, G., Forkuo, A.D., Firempong, C., Anning, A.K. & Dickson, R.A. 2019. Toxicity and safety implications of herbal medicines used in Africa. In P.F. Builders (Ed.). *Herbal Medicine*. London: InTech Open.

Mesquita, E.D., Gil-Santana, L., Ramalho, D., Tonomura, E., Silva, E.C., Oliveira, M.M., Andrade, B.B. & Kritski, A. 2016. Associations between systemic inflammation, mycobacterial loads in sputum and radiological improvement after treatment initiation in pulmonary TB patients from Brazil: A prospective cohort study. *BMC Infectious Diseases*, 16(1):1-12.

Mokgobi, M.G. 2013. Towards integration of traditional healing and western healing: Is this a remote possibility? *African Journal for Physical Health Education, Recreation, and Dance*, 2013(S1):47-57.

Moteetee, A., Moffett, R.O. & Seleteng-Kose, L. 2019. A review of the ethnobotany of the Basotho of Lesotho and the Free State Province of South Africa (South Sotho). *South African Journal of Botany*, 122:21-56.

Mpofu, S., Tantoh Ndinteh, D., Van Vuuren, S., Olivier, D. & Krause, R. 2014. Interactive efficacies of *Elephantorrhiza elephantina* and *Pentanisia prunelloides* extracts and isolated compounds against gastrointestinal bacteria. *South African Journal of Botany*, 94:224-230.

Mpofu, S.J., Msagati, T.A. & Krause, R.W. 2014. Cytotoxicity, phytochemical analysis and antioxidant activity of crude extracts from rhizomes of *Elephantorrhiza elephantina* and *Pentanisia prunelloides*. *African Journal of Traditional Complementary and Alternative Medicines*, 11(1):34-52.

Muefong, C.N. & Sutherland, J.S. 2020. Neutrophils in tuberculosis-associated inflammation and lung pathology. *Frontiers in Immunology*, 11:962.

Narasimhan, P., Wood, J., MacIntyre, C.R. & Mathai, D. 2013. Risk factors for tuberculosis. *Pulmonary Medicine*, 2013:828939.

Ndhlala, A.R., Stafford, G.I., Finnie, J.F. & Van Staden, J. 2011. Commercial herbal preparations in KwaZulu-Natal, South Africa: The urban face of traditional medicine. *South African Journal of Botany*, 77(4):830-843.

Ndjeka, N., Schnippel, K., Master, I., Meintjes, G., Maartens, G., Romero, R., Padanilam, X., Enwerem, M., Chotoo, S., Singh, N. & Hughes, J. 2018. High treatment success rate for multidrug-resistant and extensively drug-resistant tuberculosis using a bedaquiline-containing treatment regimen. *European Respiratory Journal*, 52(6):1801528.

Ndlovu, H. & Marakalala, M. 2016. Granulomas and inflammation: Host-directed therapies for tuberculosis. *Frontiers in Immunology*, 7:434.

Nelson, L.J. & Wells, C.D. 2004. Global epidemiology of childhood tuberculosis. *The International Journal of Tuberculosis and Lung Disease*, 8(5):636-647.

Nisa, A., Kipper, F.C., Panigrahy, D., Tiwari, S., Kupz, A. & Subbian, S. 2022. Different modalities of host cell death and their impact on *Mycobacterium tuberculosis* infection. *American Journal of Physiology-Cell Physiology*, 323(5):C1444-C1474.

Njeru, S.N., Matasyoh, J., Mwaniki, C.G., Mwendia, C.M. & Kobia, K.G. 2013. A review of some phytochemicals commonly found in medicinal plants. *International Journal of Medicinal Plants Research*, 105:135-140.

Olsen, A., Chen, Y., Ji, Q., Zhu, G., De Silva, A.D., Vilchèze, C., Weisbrod, T., Li, W., Xu, J., Larsen, M. & Zhang, J. 2016. Targeting *Mycobacterium tuberculosis* tumor necrosis factor alpha-downregulating genes for the development of antituberculous vaccines. *MBio*, 7(3):e01023-15.

Park, J., Pillinger, M. & Abramson, S. 2006. Prostaglandin E2 synthesis and secretion: The role of PGE2 synthases. *Clinical Immunology*, 119(3):229-240.

Park, M., Satta, G. & Kon, O.M. 2019. An update on multidrug-resistant tuberculosis. *Clinical Medicine*, 19(2):135-139.

Phuah, J., Wong, E.A., Gideon, H.P., Maiello, P., Coleman, M.T., Hendricks, M.R., Ruden, R., Cirrincione, L.R., Chan, J., Lin, P.L. & Flynn, J.L. 2016. Effects of B cell depletion on early *Mycobacterium tuberculosis* infection in cynomolgus macaques. *Infection and Immunity*, 84(5):1301-1311.

Roca, F.J. & Ramakrishnan, L. 2013. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. *Cell*, 153(3):521-534.

Roca, F.J., Whitworth, L.J., Redmond, S., Jones, A.A. & Ramakrishnan, L. 2019. TNF induces pathogenic programmed macrophage necrosis in tuberculosis through a mitochondrial-lysosomal-endoplasmic reticulum circuit. *Cell*, 178(6):1344-1361.

Rout, S.P., Choudary, K.A., Kar, D.M., Lopamudra, D. & Jain, A. 2009. Plants in traditional medicinal system – Future source of new drugs. *International Journal of Pharmacy and Pharmaceutical Sciences*, 1(1):1-23.

Russell, G., Merle, C., Cooke, G., Casas, E., Silveira da Fonseca, M. & Du Cros, P. 2013. Towards the WHO target of zero childhood tuberculosis deaths: An analysis of mortality in 13 locations in Africa and Asia. *The International Journal of Tuberculosis and Lung Disease*, 17(12):1518-1523.

Sagbo, I. & Mbeng, W. 2018. Plants used for cosmetics in the Eastern Cape province of South Africa: A case study of skin care. *Pharmacognosy Reviews*, 12(24):139-156.

Sanchez-Codez, M., Hunt, W.G., Watson, J. & Mejias, A. 2020. Hepatitis in children with tuberculosis: A case report and review of the literature. *BMC Pulmonary Medicine*, 20(1):1-4.

Sarathy, J.P. & Dartois, V. 2020. Caseum: A niche for *Mycobacterium tuberculosis* drug-tolerant persisters. *Clinical Microbiology Reviews*, 33(3):e00159-19.

Saxena, M., Saxena, J., Nema, R., Singh, D. & Gupta, A. 2013. Phytochemistry of medicinal plants. *Journal of Pharmacognosy and Phytochemistry*, 1(6):168-182.

Shakya, A.K. 2016. Medicinal plants: Future source of new drugs. *International Journal of Herbal Medicine*, 4(4):59-64.

Shanmugam, S., Sathish, K.T. & Panneer, S.K. 2010. *Laboratory Handbook on Biochemistry*. New Delhi: PHI Learning Private Limited.

Sharma, D.K., Shah, K.R. & Dave, R.S. 2019. Phytochemicals: Promising potential uses in pharmacology. *Recent Trends in Pharmaceutical Sciences*, 6:84-105.

Sheeba, D.G., Gomathi, K.S. & Citarasu, D. 2015. Anti-mycobacterial and phytochemical investigation of methanol extracts of few medicinal plants. *Journal of Chemical and Pharmaceutical Sciences*, 8(3):480-486.

Siddiqi, S.H. & Rüsch-Gerdes, S. 2006. *MGIT™ Procedure Manual For BACTEC™ MGIT 960™ TB System (Also Applicable for Manual MGIT): Mycobacteria Growth Indicator Tube (MGIT) Culture and Drug Susceptibility Demonstration Projects.*

Available at: https://www.finddx.org/wp-content/uploads/2023/02/20061101_rep_mgit_manual_FV_EN.pdf [Accessed 17 July 2022].

Sofowora, A., Ogunbodede, E. & Onayade, A. 2013. The role and place of medicinal plants in the strategies for disease prevention. *African Journal of Traditional, Complementary and Alternative Medicines*, 10(5):210-229.

Sreejith, K., Thasneem, K.M., Maniyan, N., Faris, P.M. & Neena, C.C. 2020. A review on pediatric adverse effects of first line anti-tubercular drugs. *Journal of Drug Delivery and Therapeutics*, 10(6):216-218.

Stewart, P., Patel, S., Comer, A., Muneer, S., Nawaz, U., Quann, V., Bansal, M. & Venketaraman, V. 2023. Role of B cells in *Mycobacterium tuberculosis* infection. *Vaccines*, 11(5):955.

Suresh, A.B., Rosani, A. & Wadhwa, R. 2022. *Rifampin*. Florida: StatPearls Publishing.

Tiberi, S., Petersen, E., Maeurer, M., Ntoumi, F., Yeboa-Manu, D., Mwaba, P., Vilaplana, C., Dar, O., Bates, M., Corrah, T. & Rao, M. 2018. Taking forward the stop TB partnership and world health organization joint theme for World TB Day March 24th 2018 – "Wanted: leaders for a TB-free world. You can make history. End TB". *International Journal of Infectious Diseases*, 68:122-124.

Tlotlo, R. & Olivier, D. 2022. Review on literature of the plant *Elephantorrhiza elephantine* on its healing properties and recent acquired knowledge of its medicinal activities (2000-2020). *Pharmacognosy Journal*, 14(3):715-721.

Tobin, D.M., Roca, F.J., Oh, S.F., McFarland, R., Vickery, T.W., Ray, J.P., Ko, D.C., Zou, Y., Bang, N.D., Chau, T.T. & Vary, J.C. 2012. Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell*, 148(3):434-446.

Tobin, D.M., Vary Jr, J.C., Ray, J.P., Walsh, G.S., Dunstan, S.J., Bang, N.D., Hagge, D.A., Khadge, S., King, M.C., Hawn, T.R. & Moens, C.B. 2010. The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. *Cell*, 140(5):717-730.

Tropical Forages. 2022. *Trifolium spp. African Perennial*. Available at: https://www.tropicalforages.info/text/entities/trifolium_spp._african_perennial.htm [Accessed 4 April 2023].

Ullrich, S.F., Rothauer, A., Hagels, H. & Kayser, O. 2017. Influence of light, temperature, and macronutrients on growth and scopolamine biosynthesis in Duboisia species. *Planta Medica*, 83:937-945.

Useful Temperate Plants. 2022. *Leobordea Lanceolata*. Available at: http://temperate.theferns.info/plant/Leobordea+lanceolata [Accessed 4 April 2023].

Vassall, A. 2022. *South Africa Perspective: Tuberculosis.* Available at: https://www.copenhagenconsensus.com/publication/south-africa-perspective-tuberculosis [Accessed: 17 July 2022].

Verma, S. 2016. Medicinal plants with anti-inflammatory activity. *Journal of Phytopharmacology*, 5(4):157-159.

Von Staden, L. 2011. *Leobordea lanceolata* (E. Mey.) B.-E. van Wyk & Boatwr. *National Assessment: Red List of South African Plants version 2020.1*. Available at: http://redlist.sanbi.org/species.php?species=273-45 [Accessed 12 July 2022].

Wink, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry*, 64:3-19.

World Health Organization (WHO). 2021a. *Treatment of Drug-Susceptible Tuberculosis: Rapid Communication*. Available at: https://www.who.int/publications/i/item/9789240028678 [Accessed 12 July 2022].

World Health Organization (WHO). 2021b. *Global Tuberculosis Report 2021*. Available at: https://www.who.int/publications/i/item/9789240037021 [Accessed 12 July 2022].

World Health Organization (WHO). 2022a. *Consolidated Guidelines on Tuberculosis*. Available at: https://reliefweb.int/report/world/who-consolidated-guidelines-tuberculosis-module-3-diagnosis-tests-tuberculosis-infection?gclid=

Cj0KCQjw7aqkBhDPARIsAKGa0oJPKt3DunQeHwQmTFkBJqjT-vZBVRt_nIs8Hp8sNisvcRHKGm2cXnYaArJoEALw_wcB [Accessed 12 July 2022].

World Health Organization (WHO). 2022b. *Global Tuberculosis Report 2022*. Available at: https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2022 [Accessed 12 July 2022].

Yang, L., Wen, K.S., Ruan, X., Zhao, Y.X., Wei, F. & Wang, Q. 2018. Response of plant secondary metabolites to environmental factors. *Molecules*, 23(4):762.

Yee, D., Valiquette, C., Pelletier, M., Parisien, I., Rocher, I. & Menzies, D. 2003. Incidence of serious side effects from first-line antituberculosis drugs among patients treated for active tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, 167(11):1472-1477.

Zumla, A., Rao, M., Parida, S.K., Keshavjee, S., Cassell, G., Wallis, R., Axelsson-Robertsson, R., Doherty, M., Andersson, J. & Maeurer, M. 2015. Inflammation and tuberculosis: Host-directed therapies. *Journal of Internal Medicine*, 277(4):373-387.

APPENDIX

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Environment & Biosafety Research Ethics Committee

16-Aug-2021

Dear **Miss Mercy Mpho Khala**

Project Title: **Anti-Tuberculosis activity of medicinal plants belonging to the Fabaceae family used for the treatment of TB**

Department: **Biomedical Technology - CUT**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Environment & Biosafety Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2021/0039**

Please note the following:

1. **This ethical clearance is valid for two years from the issuance of this letter.**
2. **If the research takes longer than two years to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

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