Investigating the anti-inflammatory effects of plant-derived compounds in tuberculosis using the THP-1 macrophage model.

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LIST OF ABBREVIATIONS

ADP - Adenosine diphosphate

AFB - Acid fast bacilli

ATP - Adenosine triphosphate

BCG - Bacillus Calmette-Guerin

bEEC - Bovine endometrial epithelial cells

BMDM - Bone marrow derived macrophages

C1 – Concentration 1

COVID-19 – Coronavirus disease 2019

CFU – Colony forming units

DC – Dendritic cells

ddH_2O – Double distilled H_2O

DMSO – Dimethyl sulfoxide

ESAT – Early secreted antigenic target

FBS – Fetal bovine serum

HCL – Hydrochloric acid

HDT – Host-directed therapy

IL – Interleukin

KO – Knock out

LDH – Lactate dehydrogenase

LPS – Lipopolysaccharide

MAPK – Mitogen activated protein kinase

MCAO/R – Middle cerebral artery occlusion/reperfusion

MDR-TB – Multidrug resistant tuberculosis

MMP – Matrix metalloproteinase

MOI – Multiplicity of infection

Mtb – *Mycobacterium tuberculosis*

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

PBA – Plant based antimicrobials

PBS – Phosphate buffered saline

RD1 – Region of difference 1

PKnG – Protein kinase G

PMA - Phorbol 12-myristate 13-acetate

ROS – Reactive oxygen species

RPM – Rotations per minute

RPMI – Roswell Parks Memorial Institute

RT – Room temperature

SLS – Sodium lauryl sulphate

TB – Tuberculosis

TNF – Tumour necrosis factor

VD_3 – Vitamin D3

VEGF – Vascular endothelial growth factor

WA – Withaferin A

WHO – World Health organization

WT – Wild type

XDR-TB – Extensively drug resistant tuberculosis

ABSTRACT

Background

Tuberculosis (TB) is a highly communicable disease that is caused by the bacillus, *Mycobacterium tuberculosis*. TB is responsible for devastating morbidity and mortality worldwide, especially in the developing world. TB can be effectively treated with the currently available drugs; however, poor adherence to therapy and the emergence of drug resistant bacteria has significantly impaired efforts to eradicate TB. Therefore, new therapies and therapeutic strategies are required to combat TB. One of the proposed new strategies is to develop host-directed therapies (HDTs) that are aimed at boosting the host's innate ability to fight the infection and ameliorate the deleterious tissue pathology associated with advanced TB. Repurposed drugs and plant-derived compounds with anti-inflammatory effects have been explored for potential use as HDT's.

Aim

To evaluate the anti-inflammatory effects of plant-derived compounds in macrophages infected with live Mtb strain H37Rv.

Objectives

1. To determine the IC_{50} values of the plant derived compounds on differentiated macrophages.
2. To analyze cytokine levels after macrophages are infected with *Mycobacterium tuberculosis.*
3. To establish mechanisms that may mediate the anti-inflammatory effects in the infected macrophages.

Results

It was found that Ruscogenin failed to modulate cytokine secretion in macrophages that were either infected with live Mtb strain H37Rv or macrophages that were pre-treated with the compound prior to infection with Mtb. Ruscogenin did not lower levels of IL-1β, TNF and IL-6 except for lowering IL-6 secretion in infected macrophages pre-treated with 1.35µM of Ruscogenin. Leonurine significantly lowered the levels of IL-1β, TNF and IL-6 in macrophages that were pre-treated with the compound for 24 hours prior to infection with live Mtb. It was shown that Withaferin A decreased IL-1β and IL-6 secretion in macrophages infected with Mtb prior to treatment with the compound. Finally, pre-treating macrophages with Withaferin A prior to infection with Mtb decreased the secretion of TNF and IL-6 compared to the untreated and infected macrophages.

Conclusion

Leonurine and Withaferin A appear to possess anti-inflammatory effects in macrophages infected with live Mtb. Their potential use as HDTs needs to be explored further in animal models.

CHAPTER 1

LITERATURE REVIEW

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb). It is an age-old disease that has been around for centuries yet remains a global burden. Worldwide approximately 10 million new cases of TB are reported each year, a number that has remained steady for the past couple of years. It is estimated that about a quarter of the world's population is latently infected with Mtb (World Health Organisation, 2021). The aforementioned statistics are a great cause of concern when it comes to the fight against TB. South Africa (SA) is in the top 30 countries that have the highest burden of TB (World Health Organisation, 2021). Moreover, factors such as the HIV/AIDS epidemic and the coronavirus disease 2019 (COVID-19) pandemic have added to the challenge of combating TB. A priority, especially in countries burdened with TB, is to reduce the rate of transmission in addition to maintaining existing treatment strategies and ensuring that treatment is accessible to all (Narasimhan *et al.*, 2013).

1.2 Tuberculosis disease progression and pathogenesis

1.2.1 Transmission and initiation of disease

Mtb is an airborne pathogen that is transmitted via aerosolized droplets that are expelled by an infected person. Transmission occurs when an infected individual coughs, sneezes or sometimes talks in a manner in which saliva droplets are aerosolized and end up being inhaled by another person that is near the infected individual (Ban˜uls, et al., 2015). After aerosolized droplets are inhaled, the microbes travel to the alveoli in the lungs. Cells that are infected first are type 2 pneumocytes, polymorphonuclear neutrophils and alveolar macrophages (Silva Miranda *et al.*, 2012). As is depicted in Figure 1 below, the bacilli are then phagocytosed by alveolar macrophages which does not lead to active TB in most cases. (Fogel, 2015).

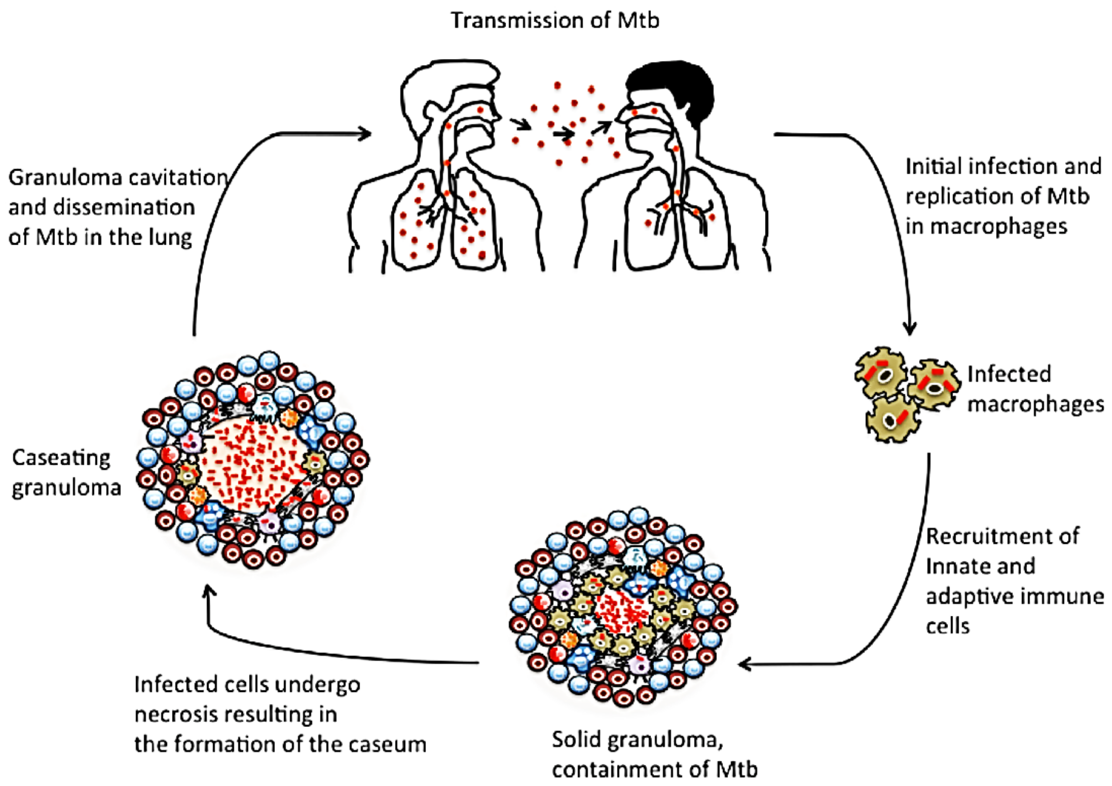


Figure 1: How Mtb is transmitted within the host.
Once an individual is exposed to Mtb, the microbe travels to and resides in the lung. Alveolar macrophages engulf and phagocytose the bacilli which causes an immune response that is responsible for the recruitment of innate immune cells. Different cellular stimuli trigger cells to differentiate into key granuloma constituents. The granuloma serves to contain Mtb while limiting replication but necrosis of infected macrophages result in the formation of caseum and eventually the granuloma erupts and bacilli are transmitted within and between hosts (Ndlovu and Marakalala, 2016).

In individuals who are immunocompromised or have other diseases that may predispose them to TB, the bacilli are not always killed in the initial contact with the host's macrophages (Fogel, 2015). It has been reported that many species of mycobacteria are able to modulate host cellular functioning to create replication niches. Pathogenic mycobacteria can evade lysosomal degradation, restriction and killing (Ernst, 2012). These alveolar macrophages secrete pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukin 8 (IL-8) and IL-6. These cytokines are responsible for the migration of macrophages and other immune cells to the site of infection (Shaler *et al.*, 2013). Dendritic cells (DC) are key players in Mtb infection as they present the antigens to T-cells, which then allows for the onset of the adaptive immune response (Silva Miranda *et al.*, 2012).

1.2.2 The granuloma and how it is formed

It is important to note that the formation or the events that occur in the granuloma are still under debate and not fully understood. Many models such as zebrafish, guinea pigs, mice and rabbits have been used to help scientists understand this process. It is hard to obtain and study human lung biopsies and therefore scientists have used these models to explain what occurs in the human body (Silva Miranda *et al.*, 2012).

The influx of cells and inflammatory cytokines is what ultimately leads to granuloma formation. Granulomas are commonly referred to as the pathological hallmark of tuberculosis and previously, its role was thought to be purely host protective (Ramakrishnan, 2009). However, many studies have reported on the ability of mycobacteria to use this structure to its advantage, specifically as a replicative niche to facilitate dissemination (Taylor *et al.*, 2006; Shaler *et al.*, 2013; Chai, Zhang and Liu, 2018). The interior of the granuloma is composed mainly of innate immune cells such as neutrophils, dendritic cells, macrophages, giant cells and granulocytes (Ndlovu and Marakalala, 2016; Chai, Zhang and Liu, 2018), and the outer regions consists mainly of T and B cells, which form the lymphocytic cuff that is thought to give the granuloma the intact, solid structure. Previous studies have shown that the granuloma is a complex crossing point between the host response and pathogen (Davis and Ramakrishnan, 2009; Volkman *et al.*, 2010; Yang *et al.*, 2012). Figure 2 below shows that the aggregation of immune cells creates the granuloma structure that ''walls off'' the bacteria and the mycobacteria often remain dormant in this structure. The bacteria can also utilise this environment as a protective mechanism against the host's inflammatory responses (Sakamoto, 2012). Mycobacteria use virulence factors such as the type VII secretory system (ESX-1) encoded by the region of difference 1 locus (RD1) to draw uninfected macrophages to the site of infection. The newly recruited macrophages phagocytose the mycobacteria, ultimately leading to an increased infection rate as observed in a study using zebrafish that were infected with *Mycobacterium marinum* (Davis and Ramakrishnan, 2009; Ramakrishnan, 2012).

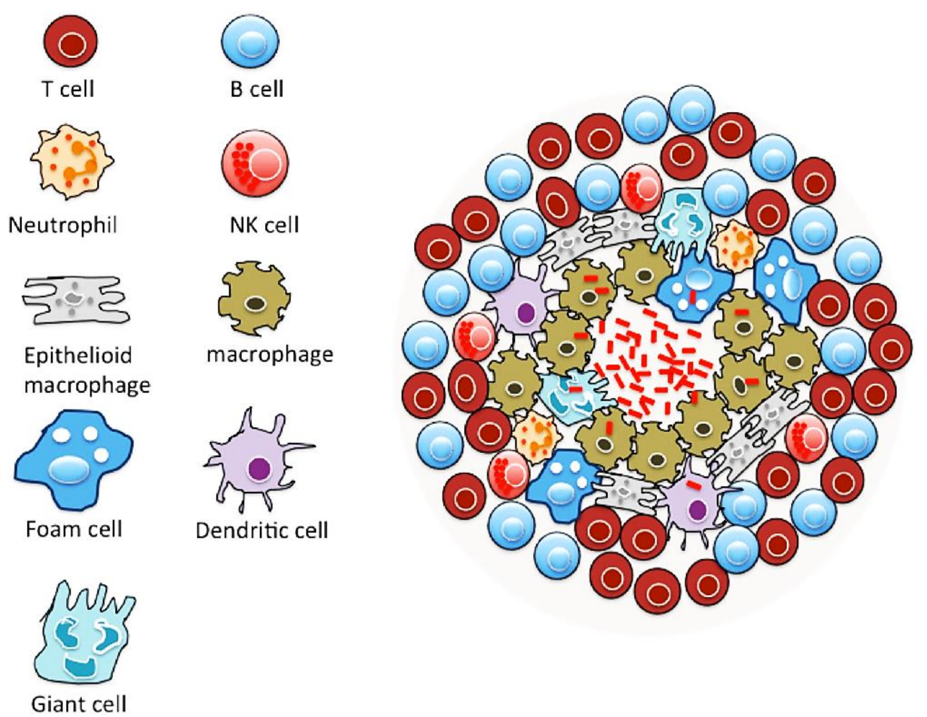


Figure 2: Classical granuloma structure after infection with *Mycobacterium tuberculosis.*

Depiction of the cellular composition of the granuloma and its structure which aims to control infection by walling off the bacteria. The outer cuff is comprised of T and B-cells, natural killer cells and giant cells. Whereas macrophages, dendritic cells, neutrophils, and epithelioid macrophages make up the inner cuff of the granuloma. Bacilli are confined to the centre of this structure (Ndlovu and Marakalala, 2016).

1.2.3 Immunology of the granuloma

A crucial factor in maintaining the protective granuloma is the balance of cytokines, more specifically TNF. Mtb have been found to replicate uncontrollably if the levels of TNF-α are too low, which ultimately leads to macrophages undergoing necrotic cell death (Tobin *et al.*, 2010). Interestingly, when TNF levels are too high, the bacterial burden is low but macrophages still succumb to necrosis via mitochondrial reactive oxygen species (ROS) (Tobin *et al.*, 2010; Roca and Ramakrishnan, 2013). The role of TNF is complex and it is important for the development of host protective immunity, but excessive amounts of TNF can contribute to tissue pathology (Idriss and Naismith, 2000). Flynn *et al.* (1995) revealed that neutralizing TNF-α in test mice resulted in significant mortality compared to control mice. Additionally, uncontrolled replication of Mtb was observed in the lungs and spleen of the mice that were treated with anti-TNF-α neutralizing antibodies compared to control mice (Flynn *et al.*, 1995). These studies highlight how crucial TNF-α is in controlling Mtb infection and not only that, this cytokine has also been found to be involved in regulating the inflammatory response by stimulating IL-1 and IL-6 production (Zuiga *et al.*, 2012).

Interleukin 1β (IL-1β) is another key pro-inflammatory cytokine that if not produced in a controlled amount, can contribute to a worse fate in disease outcome (De Mooij *et al.*, 2017). Not only does IL-1β improve the outcome of Mtb infection in mice, it also plays a role in tissue repair and homeostasis (Yamada *et al.*, 2000; De Mooij *et al.*, 2017). It is present in the beginning stages of infection and is mainly produced by macrophages and monocytes (Duque and Descoteaux, 2014). Blocking IL-1β after *M.marinum* infection improved the lung pathology that was a result of excessive inflammation (Chao *et al.*, 2017).

IL-6 is both pro- and anti-inflammatory and similar to IL-1β, it is also involved in the early stages of immune response to infection (Zuiga *et al.*, 2012). This cytokine is produced by different subtypes of cells, more specifically T cells, B cells, endothelial cells, fibroblasts and even mononuclear phagocytes (Ladel *et al.*, 1997). Although not necessary for protection during *M. bovis* Bacillus Calmette–Guérin (BCG) infection, IL-6 is necessary for prolonged survival in H37Rv infection (Ladel *et al.*, 1997). Bacterial burden also decreases when IL-6 is present (Ladel *et al.*, 1997).

1.2.4 Granuloma dissociation

Mycobacteria can disseminate in the body after granuloma dissociation or break-down. Phagocytes often undergo apoptosis or necrosis during infection, and both types of cell deaths have been implicated in mycobacterial dissemination (Ramakrishnan, 2012). Firstly, if we consider apoptosis; it is commonly viewed as a controlled cell death pathway that usually benefits the host. Macrophages can successfully eliminate intracellular Mtb by undergoing apoptosis (Keane *et al.*, 1997; Fairbairn, 2004). A study that looked at various conditions in which granulomas are known to form, found that the tuberculosis granuloma had a high rate of apoptosis when comparing to leprosy and Chron's disease (Cree *et al.*, 1987). As previously mentioned, mycobacteria make use of the ESX-1 virulence factors to disseminate throughout the body. Uninfected macrophages are drawn to and engulf the apoptotic macrophages that are trying to eliminate the bacteria, which leads to continued bacterial replication in the macrophages and dissemination within the host (Davis and Ramakrishnan, 2009; Ramakrishnan, 2012).

Matrix metalloproteinases (MMPs) have also been implicated in the destruction of the granuloma, more specifically MMP-9. Their functions include being able to degrade collagen and aid in tissue destruction (Martin, Carey and Fortune, 2016). Early secreted antigenic target-6 (ESAT-6), a mycobacterial protein, has been implicated in stimulating the production of MMP-9 by surrounding epithelial cells (Volkman *et al.*, 2010). A study using THP-1 macrophages looked at the effect of ESAT-6 and discovered that it can induce apoptosis by activating certain caspases (Derrick and Morris, 2007). These examples show how Mtb can manipulate and use host machinery to its advantage.

Bacterial factors are not solely responsible for the dissociation of the granuloma, host factors also have a role to play in the destruction of this structure. Cytokine imbalances can also lead to granuloma break-down. As previously mentioned, if the levels of TNF are not optimal, macrophages undergo necrosis as a result of low levels or high levels of TNF (Roca and Ramakrishnan, 2013). Similarly, the level of IL-1β can also contribute to granuloma caseation more specifically, when IL-1β levels are too high(Chao *et al.*, 2017). The necrotizing granuloma or caseating granuloma is the most extreme and often the final fate of the granuloma and can contribute to the lung pathology often seen in the worst cases of tuberculosis (Ravimohan *et al.*, 2018). Thus, it is very important for us to identify ways to prevent granuloma break-down as this will prevent microbial dissemination within and between hosts.

1.2.5 Disease progression

Disease outcome and progression after an individual is infected with Mtb is largely dependent on the state of the individual's immune system. Co-infection with diseases can significantly alter the capability of the hosts immune system to successfully eliminate the mycobacterium pathogen. Figure 3 below depicts the progression of disease, in most cases, an individual clears infection with no consequences after mounting robust innate and adaptive immunity. Latent infection occurs when an individual's immune system can control the infection, but the bacteria still remain viable. The granuloma is formed and at this stage serves to control bacterial replication and contains the infection (Drain *et al.*, 2018). Subclinical TB is similar to latent infection in that it does not result in any clinical presentation of TB. However, there is radiobiological evidence of disease (Drain *et al.*, 2018). Lastly, an individual can advance to active TB which is linked to the dissociation of the granuloma. Mycobacteria actively replicate in the lungs of infected individuals and the host's immune system is incapable of controlling the spread of bacteria throughout the body (Pai *et al.*, 2016).

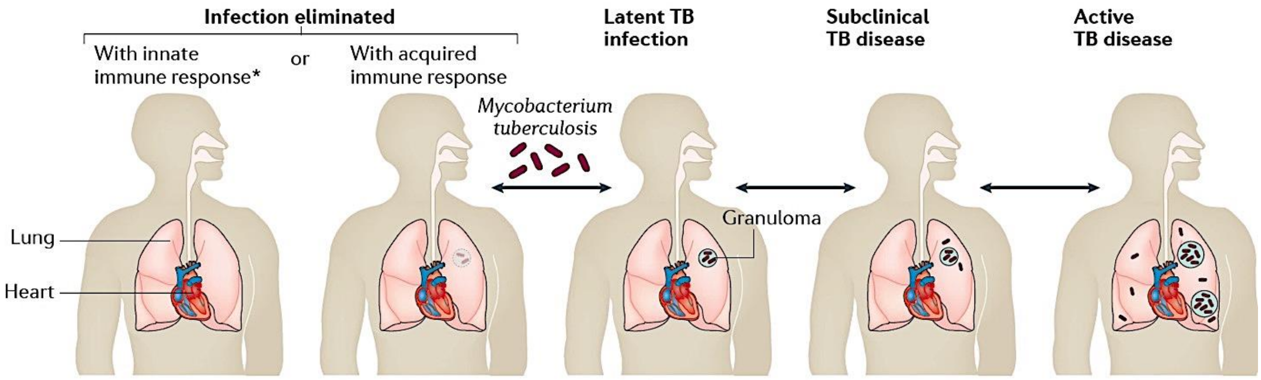


Figure 3: Tuberculosis disease progression.
After and individual is exposed to Mtb, infection is either effectively cleared by the immune system and is what occurs in most cases, or infection can progress into active TB disease. Latent TB disease can be defined as the persistence of mycobacteria within the granuloma but replication and spread is controlled. At the stage of subclinical disease, infection persists, and no clinical presentation of TB is noted, but Mtb infection can be detected using radiobiological techniques. Active TB disease is what we know to be the classical presentation of Mtb infection. There is both clinical and radiobiological evidence of tuberculosis.

1.3 Tuberculosis treatment

1.3.1 Treatment for drug-susceptible TB

Currently, patients with drug-susceptible TB are treated with first-line drugs consisting of rifampicin, isoniazid, ethambutol and pyrazinamide for approximately 6 months (WHO, 2020). In the first two months of treatment, patients are given a combination of the four drugs; thereafter, only rifampicin and isoniazid are administered (Sotgiu *et al.*, 2015). In the first two weeks of this 6-month treatment regimen, infected individuals become non-infectious (Ormerod, 2005). The two major determinants of the effectiveness of TB treatment are adherence and treatment completion. Patients find it hard to adhere to treatment as the anti-TB drugs often cause hepatotoxicity (Mangwani, Singh and Kumar, 2019). In previous treatment strategies, the onus was on the patient to ensure successful adherence or completion of the treatment regimen, but an important and necessary shift has occurred where the responsibility now lies on healthcare workers to follow up with and ensure that patients adhere to treatment programmes (Nahid, Pai and Hopewell, 2006).

1.3.2 Treatment of MDR/XDR tuberculosis

Multi-drug resistance is when mycobacteria are resistant to two of the first-line drugs, more specifically isoniazid and rifampicin (WHO, 2018). Treating patients who have multidrug resistant tuberculosis (MDR-TB) is very challenging. The WHO has put forth that a regimen of pyrazinamide, a second line injectable drug (e.g., Amikacin), a fluroquinolone and cycloserine be administered in the intensive/first phase of MDR-TB treatment, which can last for 8 months. Thereafter, the next phase of treatment should last for a minimum of 20 months, and the combination of drugs administered is largely dependent on the outcome of a culture conversion test after the first phase of treatment is completed (Sotgiu *et al.*, 2015). Factors such as costs, treatment duration and toxic side effects all add to the complexities in trying to obtain the best patient outcomes (Pontali *et al.*, 2019). Patients that suffer from extensively drug resistant TB (XDR-TB) are resistant to isoniazid, rifampicin, fluroquinolones as well as resistance to any one of the three second line injectable aminoglycosides (Pai *et al.*, 2016; Quenard *et al.*, 2017). Advances in technology such as Xpert MTB/RIF assay, have allowed clinicians to screen patients in order to customize their treatment regimens (Sotgiu *et al.*, 2015). In resource rich countries, drug susceptibility testing is recommended so that an effective and efficacious treatment regimen can be prescribed to patients (Ormerod, 2005; Sotgiu *et al.*, 2015).

1.4 Host-directed therapies

Most of the anti-TB drugs currently used to treat patients specifically target the microbe, and with drug resistance being very prominent, it is crucial to look for novel therapeutic strategies. One such strategy is to develop host-directed therapies (HDTs) that are aimed at improving the hosts ability to fight the bacteria by boosting the immune response and reducing the tissue pathology associated with TB (Figure 4). HDTs can be used as adjunctive therapy to complement the current regimens that are used to treat TB. This could possibly help to improve the efficacy of the drugs; thus, assist in shortening the duration of treatment, maintaining lung function, and may reduce the number of drugs a patient has to take if they develop drug resistant TB (Hawn *et al.*, 2013).

Many drugs that are currently on the market are being repurposed as HDTs. Vitamin D is an example of a repurposed therapeutic agent that has been shown to have cathelicidin-dependent antimycobacterial properties (Liu *et al.*, 2007). Another example is metformin, an anti-diabetic drug, which, is capable of reducing intracellular mycobacterial growth in addition to aiding phagosome-lysosome fusion (Singhal *et al.*, 2014). This approach has been proven to be beneficial, especially when using drugs that can target the pathways that are involved in the dissociation of the granuloma as seen in Figure 4 below (Ndlovu and Marakalala, 2016). Animal models have been used to study the effects of these therapeutics and it is evident that by blocking certain players in inflammatory pathway, it is possible to lower inflammation and the related lung tissue pathology (Ndlovu and Marakalala, 2016).

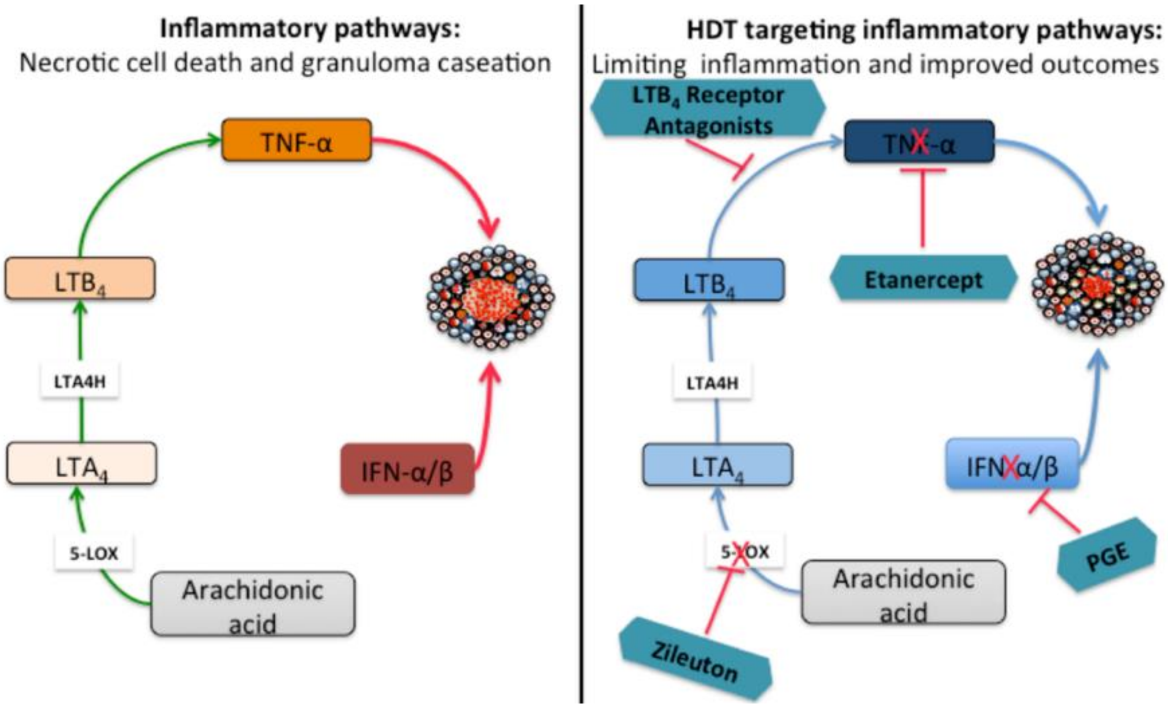


Figure 4: How targeting inflammatory proteins as HDT can improve tissue inflammation.
5-Lipoxygenase (5-LOX) metabolized arachidonic acid (AA) and produces leukotriene 4 (LTA_4). Leukotriene A4 hydrolase (LTA4H) catalyses a substrate of LTA_4 to produce leukotriene B4 (LTB_4), which is highly inflammable. If produced in high levels may induce excessive levels of TNF by macrophage. By using certain receptor antagonists and repurposing drugs, the inflammatory response can be dampened which results in improved patient outcome. (Ndlovu and Marakalala, 2016)**.**

Every treatment strategy has its advantages and disadvantages. HDT priming of the immune system can be beneficial in fighting off any pathogen not just Mtb. However, if certain pathways are targeted or proteins involved in the immune response, there is also a risk of causing a "ripple effect" of events that may impact cell survival or functioning (Dara et al., 2019).

1.5 Plant-derived compounds

Taking the timeline of the development of new antimicrobials into account, it is clear that there has been a decline in antimicrobial discovery and that is largely due to the issue of drug resistance (Bello *et al.*, 2018). Taking drugs can also impact the microbiome directly and as a result; while one disease is being treated, susceptibility to another disease increases (Bello *et al.*, 2018). Plants have been used by traditional and spiritual healers for their medicinal properties since ancient times and now more researchers have started to look at using plants as a means of treatment. In countries like China and India, it is still widely used and accepted, partly due to religious beliefs and cultural practices (Jamshidi-Kia, Lorigooini and Amini-Khoei, 2018). This is an attractive area of research because of the many benefits associated with using medicinal plants. Some of the benefits include the fact that they induce limited side effects, they are inexpensive to produce, they are readily available in addition to being a great source of biologically active compounds. Moreover, there has been no reports of microbes developing resistance to these plant-based antimicrobials (PBAs) (Bello *et al.*, 2018). The compounds that will be investigated for their potential anti-inflammatory effects in this study are discussed below.

1.5.1 Withaferin A

Withaferin A (WA) is a steroidal lactone that is isolated from the *Withania somnifera* plant, more commonly referred to as "Indian ginseng" and even better known as Ashwagandha. This compound is very promising as it has been widely studied for its anti-inflammatory and anti-cancer properties. A common method of extraction includes grinding up the roots or leaves of the *Withania somnifera* plant. Thereafter, solid-phase extraction techniques are applied by diluting the solid-phase of the compound with an alcohol. High performance liquid chromatography (HPLC) proton nuclear magnetic resonance are used to characterize compounds after extraction (Patel, Rao and Hingorani, 2016). Withaferin A has not been used to lower inflammation associated with Mtb infection in THP-1 macrophages but it has been reported that Withaferin A has anti-mycobacterial properties (Adaikkappan, Kannapiran and Anthonisamy, 2012). Moreover, virulent Mtb was treated with different concentrations of WA and plated at various timepoints, colony forming units (CFU) were obtained, and results were converted to percentages. When compared to the control, the highest dose of WA (1mg/ml) showed a 64.47% inhibition (Adaikkappan, Kannapiran and Anthonisamy, 2012). Another reason that this compound was screened in this study was that WA can significantly reduce the growth of Mtb in 7H10 medium (Zhang *et al.*, 2017).. Previous work has demonstrated that Withaferin A, which was isolated from the *Withania somnifera* plant originating from Palestine, exerts its effects at a concentration of 63µg/mL in four different cell-lines, none of which were THP-1 macrophages (Kaileh *et al.*, 2007).

Displaying both anti-mycobacterial and anti-inflammatory properties is very encouraging that this compound can potentially target the mycobacteria and intervene as an HDT in the inflammatory pathway/s involved in causing the excessive inflammation, which could be detrimental to the host. Interestingly, a study looking at the possible inhibitors of protein kinase G (PknG) found that certain withanolides can bind to active sites of PknG and thus possibly inhibit the activity of this kinase (Santhi and Aishwarya, 2011). Among other functions, PknG contributes to the virulence of Mtb in that it can prevent mycobacteria degradation intracellularly (Santhi and Aishwarya, 2011). This is just another example demonstrating that exploring the therapeutic potential of WA is important and could be extremely valuable for improved clinical outcomes in patients with TB.

1.5.2 Ruscogenin

The Chinese herb, *Radix Ophiopogon japonicus,* is made up of many chemical compounds. One of the main biologically active components of this plant are the steroidal saponins, a category in which Ruscogenin is classified in (Chen *et al.*, 2016). Ruscogenin has been used to treat cardiovascular diseases in the past and has been shown to exhibit anti-inflammatory effects (Huang, Kou, Ma, *et al.*, 2008). The effects of Ruscogenin on LPS-induced acute lung injury was demonstrated by pre-treating male ICR mice with Ruscogenin which showed two key results; it reduced lung oedema and by using histology they found a significant decrease in lung damage (Sun *et al.*, 2012). Furthermore, Ruscogenin was also able to lower levels of tissue factor and inducible nitrous oxide both of which are known to adversely contribute to acute liver injury (Sun *et al.*, 2012).

Intriguingly, this compound can inhibit cerebral ischemic injury in mice by decreasing reactive oxygen species (ROS) and inhibiting the mitogen-activated protein kinase (MAPK) signalling pathway in bEnd.3 cells (Cao *et al.*, 2016). Ruscogenin has effects of improving lipid accumulation in HEPG2 hepatocytes and halted hepatic fibrosis in an induced model of non-alcoholic fatty liver disease by acting on the NF-κB pathway (Lu, Tzeng, Liou, Chang, *et al.*, 2014). Lastly, Ruscogenin also possess antithrombotic properties as demonstrated in 2006, rats were administered adenosine diphosphate (ADP) to cause platelet aggregation and treated with Ruscogenin, which showed platelet inhibition. Ruscogenin also displayed a positive effect by preventing venous thrombosis in mice that were treated with this compound in a dose-dependent manner. (Kou *et al.*, 2006). Ruscogenin is a compound that clearly exhibits many therapeutic properties, investigating its effects in the context of TB could prove to be worthwhile.

1.5.3 Leonurine

Isolated from the *Leonuri cardiacae herba* plant and commonly referred to as “motherwort”, Leonurine is an alkaloid that has been shown to possess anti-inflammatory properties (Jin *et al.*, 2019). In the Institute of Cancer Research (ICR) mice, Leonurine has been shown to have many protective effects after ischemic stroke was induced in these mice (Xie *et al.*, 2019). Some of these effects included reducing brain infarct volumes and neurological deficit scores. Leonurine displayed antioxidant activity by lowering the levels of ROS and improved levels of VEGF (vascular endothelial growth factor) in the brain of mice. (Xie *et al.*, 2019). Taken together all these results show that Leonurine has a neuroprotective role.

Moreover, Leonurine has also displayed anti-inflammatory properties in mice and bovine endometrial epithelial cells that had endometriosis stimulated using LPS (Wu *et al.*, 2018). More importantly and in context with that this study aims to investigate, the production of IL-1β and TNF-α was significantly reduced in the uterine tissues of the mice (Wu *et al.*, 2018). These pro-inflammatory cytokines play an important role in the progression of tuberculosis (as discussed in earlier paragraphs). If Leonurine can ameliorate the effects of excessive inflammation as shown by Wu *et al.,*2018, it is then worth examining the effect of this compound in TB and possible use as an HDT.

1.6 Rationale

Mycobacterium tuberculosis has proved to challenge people in many ways. Drug resistant mycobacteria have made it especially more difficult to eradicate TB. Existing treatment regimens are not enough and there is a dire need for the scientific community to explore new therapeutic strategies, and one such method is the use of HDTs.

This study focused on exploring the use of plant-derived compounds to lower inflammation that is associated with TB pathology. Plant-derived compounds have not been used in this context before. This study therefore hopes to provide new insights into the use of these compounds to treat TB..

1.6.1 Hypothesis

We hypothesize that by treating macrophages with Withaferin A, Ruscogenin and Leonurine either before or after infection with Mtb strain, H37Rv will reduce the secretion of pro-inflammatory cytokines and cell death.

1.6.2 Aim and objectives

This study aimed to screen plant-derived compounds for their potential use as HDTs to lower excessive inflammation in THP-1 macrophages after Mtb infection.

Study objectives:

1) To determine the IC_{50} values of the plant-derived compounds on differentiated macrophages.
2) To analyse the levels of cytokine production after macrophages are infected with Mtb and treated with the plant-derived compounds.
3) To investigate the effect of the plant-derived compounds on cell death after infection with Mtb.

CHAPTER 2

METHODS AND MATERIALS

2. 1 Tissue culture

THP-1 monocytes were grown and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium. Media was supplemented with 10% Fetal Bovine Serum (FBS), 1IU/mL penicillin, and 1μg/mL streptomycin. Cells were incubated at 37°C with 5% CO_2 in a water jacket incubator.

2.1.1 Differentiation of THP-1 monocytes

Prior to starting any experiment, the THP-1 monocytes were differentiated into macrophages. Monocytes were differentiated with 100nM Phorbol 12-myristate 13-acetate (PMA) for 72 hours in a 37°C water jacket incubator. Thereafter, cells were washed twice with complete media before fresh media was added which was followed by a period of rest for 24 hours to allow the cells to recover from any mitogenic stress that may have occurred. After this, the next steps of experimentation were carried out.

2.2 Mycoplasma staining

A 6cm Petri dish was used to plate the cells. Before plating, a coverslip was placed in 70% ethanol and left to air dry briefly. Cells were then plated at a density of 1.5×10^6 cells in a final volume of 2mL of RPMI without any antibiotics. The differentiation procedure was followed for the cells to attach to the coverslip. Next, 1mL of fixing solution (methanol: acetic acid in a ratio of 3:1) was added for 10 seconds (s) to the culture dish with the media, and all liquid was pipetted off. Another 1mL of fixing solution was added to the dish for an additional 10s and was pipetted off. Using double distilled water (ddH_2O) in a squirt bottle, the Petri dish was rinsed three times, and water was discarded after each rinse. The dish was left to dry on the benchtop before adding 15μL of mounting fluid onto a slide. The coverslip was then stained with 500μL Hoechst nuclear stain for 10s, followed by three more rinses with ddH_2O. The coverslip was transferred cell side-down to the slide avoiding bubble formation. Cells were viewed using a Zeiss LSM 880 Confocal Microscope and images acquired with Fast Airyscan Technology (Zeiss, Germany).

2.3 Preparation of drugs

The plant-derived compounds were bought from MedChemExpress® (USA). All the compounds were reconstituted in dimethyl sulfoxide (DMSO) which was made up to a final concentration of 10mM and stored at -80°C. Before using the compounds in experiments, working stock solutions were made in complete RPMI.

2.4 MTT Assay

2.4.1 MTT and SLS preparation

MTT was purchased from Sigma-Aldrich® (Merck, USA) and was made up to a final concentration of 5mg/mL in RPMI. SLS powder was also purchased from Sigma-Aldrich® (Merck, USA). 10% (m/v) SLS was prepared by dissolving the powder in ddH$_2$O and 0.01M hydrochloric acid (HCL).

2.4.2 Culturing of the plate

In a 96-well cell culture plate, cells were plated in the presence of PMA at a density of 1 × 10^{5}cells/well in a final volume of 100µL and left to differentiate in the incubator. After a day of rest, cells were treated with different concentrations of a compound for 24 hours. Next, 20µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution and was added and the plates were incubated for 3.5hours before adding 150µL of 10% (m/v) sodium lauryl sulphate (SLS) to the cells. The plate was left in the incubator at 37°C overnight and was read at 595nm using a Biotek microplate spectrophotometer (Winooski, VT, USA).

2.5 *Mycobacterium tuberculosis* infections

Cells were plated at a density of 2.5 × 10^{5}cells/well at a final volume of 500µL in a 48-well cell culture plate and the differentiation protocol was followed to obtain THP-1 macrophages.

2.5.1 Infecting cells

Mycobacterium tuberculosis (Mtb) H37Rv strain stocks were prepared by the Brombacher laboratory at the University of Cape Town. The Mtb stocks had a concentration of 1.2 × 10^{7}cfu/mL. Prior to infecting the cells, the Mtb stock was spun down using a centrifuge for a duration of 5min at 10 000 revolutions per minute (rpm) at 20°C. The pellet was resuspended in 1mL of 1X phosphate-buffered saline (PBS) and spun down using the same centrifuge settings again. Thereafter, the pellet was resuspended in 1mL of antibiotic free RPMI media and used to infect the cells at a multiplicity of infection (MOI) of 5. Once the cells were infected, they were left in the incubator for 4 hours. Subsequently, the cells were washed twice with 1X PBS and new antibiotic free media was added to the cells.

All infections were carried out in a biosafety level 3 laboratory.

2.5.2 Treating cells

The cells were treated before and after infection with the IC_{50} and the concentration at which 100% cell viability was observed (herein referred to as concentration 1 and abbreviated to C1). Each compound was added to the cells and left to incubate at 37°C for 24 hours.

2.5.3 Pre-treatment experiments

The cells were differentiated into macrophages and after a day of rest, they were subjected to pre-treatment with the C1 and IC_{50} concentration of the plant-derived compounds. After a 24 hour incubation period, the cells were then infected using the same infection protocol mentioned above (2.5.1). The cells were left in the incubator for 24 hours, thereafter supernatants were collected using the method explained in the next paragraph.

2.5.4 Collecting supernatants

Following the incubation period, supernatants were collected by means of double filtration using 0.22µm 13mm PTFE hydrophilic filters. Supernatants were removed from the BSL3 laboratory and stored at -80°C for subsequent experiments.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Nunc™-clear, flat bottom immuno-nonsterile 96-well plates were used for this assay. Cytokines (human IL-1β, TNF and IL-6) were detected using the BD OptEIA™ kits (BD, USA) by following the manufacturer's instructions. Briefly, the plates were coated with coating antibody diluted in coating buffer (7.13g $NaHCO_3$, 1.59g Na_2CO_3 in 1L ddH_2O) and incubated for 1 hour at room temperature (RT). The plates were washed (3X) with wash buffer (1XPBS with 0.05% (v/v) Tween 20). Next, the plates were blocked using the assay diluent (1XPBS with 10% (v/v) FBS) and incubated at RT for 2 hours. The plates were washed then the samples and standards (two-fold serially diluted) were added, and the plates were incubated overnight at 4°C. The plates were washed 5X with wash buffer and working detector consisting of a mixture of detection antibody and enzyme (SAv-HRP) was added and incubated for 1 hour at RT. The plates were washed 7X and TMB substrate (SeraCare, USA) was prepared then added to the plates which were incubated for 30min at room temperature (RT) to allow for the colour to develop. Stop solution (25µL, 1M sulphuric acid) was added to each well, and the absorbance was read at 450nm using the Glomax® Discover microplate reader (Promega, USA).

The concentration of samples was determined by means of a standard curve for each cytokine:

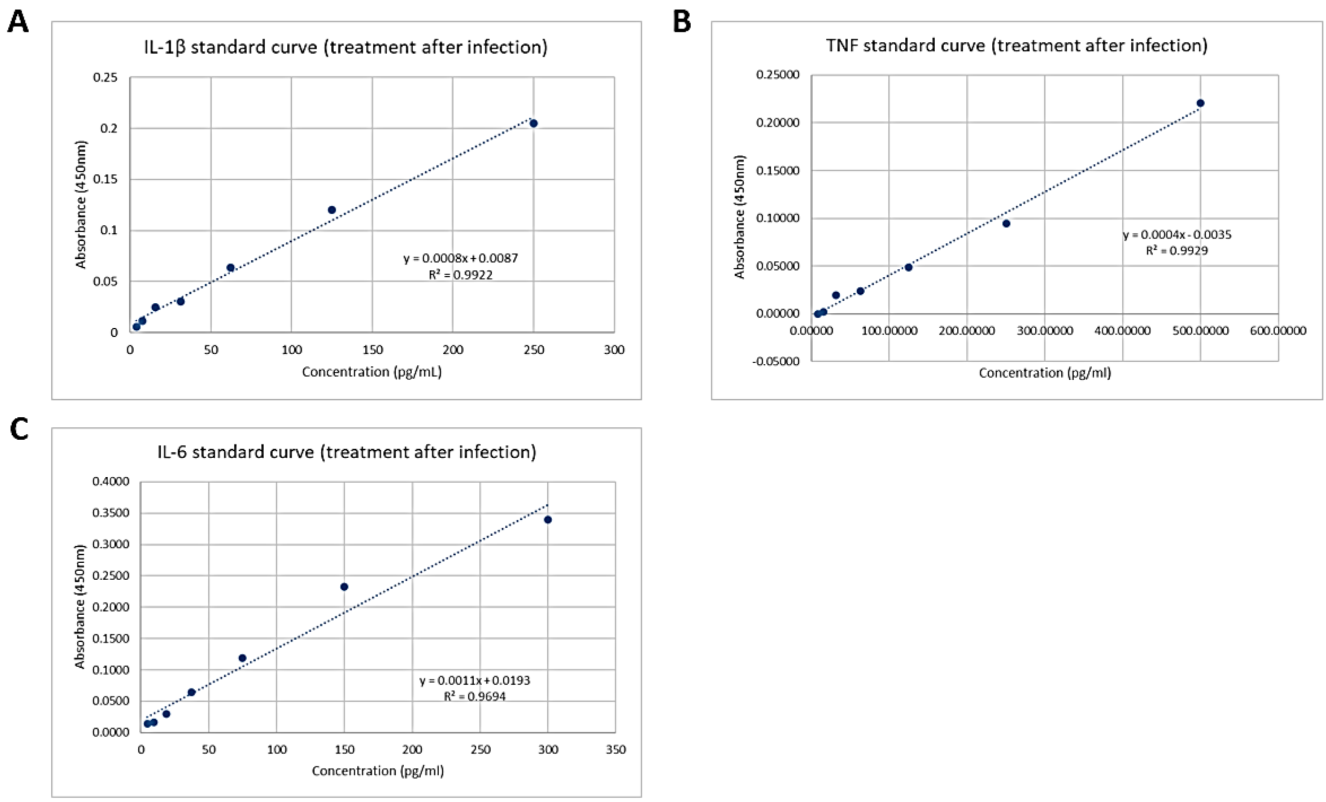


Figure 5: ELISA standard curves for cytokines when macrophages were treated with plant-derived compounds after infection.
Data for the standard curve was obtained by performing serial dilutions of the stock standard of IL-1β, TNF and IL-6. Absorbance was read at 450nm using a Glomax® Discover microplate reader (Promega). Cytokine concentrations were determined using a liner equation.

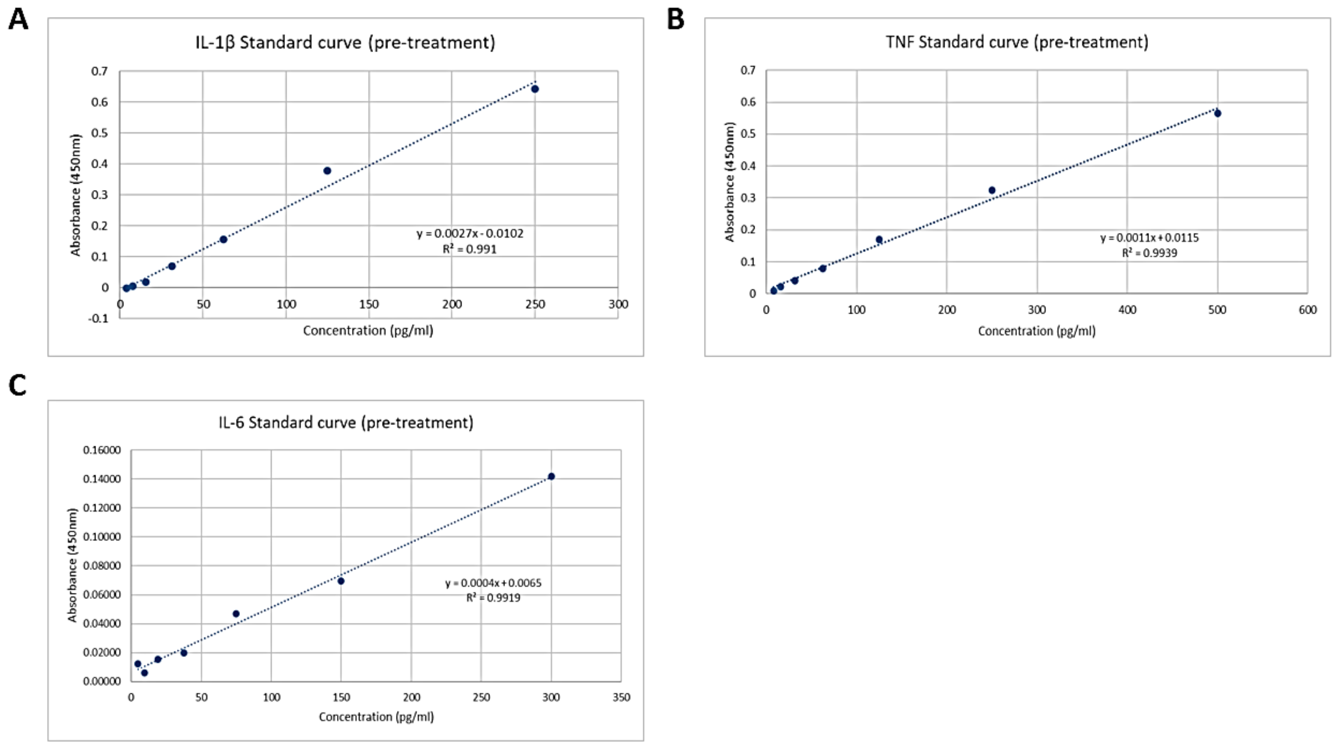


Figure 6: ELISA standard curves for cytokines detected when macrophages were pre-treated with the plant-derived compounds.

Standard curves were determined by performing serial dilutions of the stock standards of each cytokine (IL-1β, TNF and IL-6). Absorbance was read at 450nm and cytokine concentrations determined using linear equations.

2.7 Lactate dehydrogenase (LDH) assay

Macrophages were infected with live Mtb and either treated before or after infection with the plant-derived compounds. Supernatants were collected and filtered as mentioned in section 2.5.3. An LDH assay kit purchased from ThermoFisher Scientific© (USA) was used to perform the assay by following the manufacturer's instructions. The assay controls were plated before performing each assay, thereafter stored supernatants were thawed and 50µL transferred in triplicates to a 96-well plate thereafter 50µL of the LDH reaction mixture (0.6mL assay buffer and 11.4mL substrate mix) was added. The plate was then incubated at room temperature for 30minutes before 50µL of stop solution was added to each well. Absorbance was read at 490nm and 680nm using the Glomax® Discover microplate reader (Promega, USA). LDH activity was determined by subtracting the 680nm absorbance values from the 490nm values.

2.8 Statistics

Data was analyzed using Microsoft Excel and Prism (GraphPad) software. All data are expressed as mean ± SEM. The Student's *t*-test was used to determine statistically significant differences between the untreated and treated samples. Statistical significance was regarded as $p<0.05$.

CHAPTER 3

RESULTS

3.1 Mycoplasma staining

A standard practice in laboratories is to routinely test for Mycoplasma infection in cells during culture. The presence of these organisms in culture can drastically affect the results obtained in the experiments (Young, Sung and Masters, 2010). For this reason, it was critical to rule out the presence of these microbes in the cells. The direct staining protocol mentioned in Chapter 2 was followed and Mycoplasma was detected using confocal microscopy. These microorganisms can be identified by a distinct bead-like network in the cytoplasm surrounding the nucleus (Chen, 1977). It was found that the cells were not infected with Mycoplasma as indicated by the absence of a signal surrounding the nuclei in the cytoplasm of cells (Figure 7).

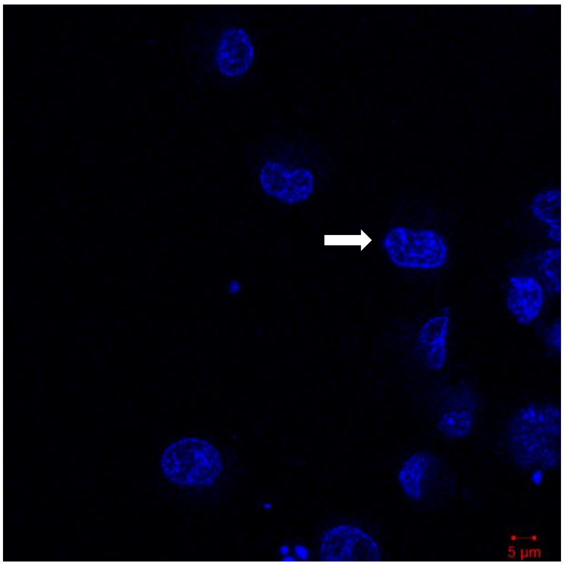


Figure 7: Mycoplasma staining using Hoechst nuclear stain.
Cells were plated at a density of 1.5×10^6 cells/mL to allow for adequate visualisation of any possible Mycoplasmas. The cells were fixed to a coverslip and were stained with Hoechst stain. The nuclei of the THP-1 macrophages stain bright blue (indicated by the white arrow). The images were acquired using the Carl Zeiss LSM 880 confocal microscope with Fast Airyscan Technology.

3.2 Determining the optimal concentration of plant-derived compound to use on the differentiated macrophages

It was critical to establish an appropriate concentration of the plant-derived compounds to use to treat cells. An MTT assay was used, which enabled the determination of concentrations at which these compounds were cytotoxic. The half inhibitory concentration (IC_{50}), where 50% of the cells were dead was also determined from this assay.

3.2.1 Withaferin A

To investigate whether Withaferin A was cytotoxic to macrophages, an MTT assay was conducted to determine the IC_{50} concentration. It was found that the average IC_{50} for Withaferin A was 6.8µM (Figure 8). A logarithmic decrease in cell viability was observed as the dose of Withaferin A increased (Figure 8).

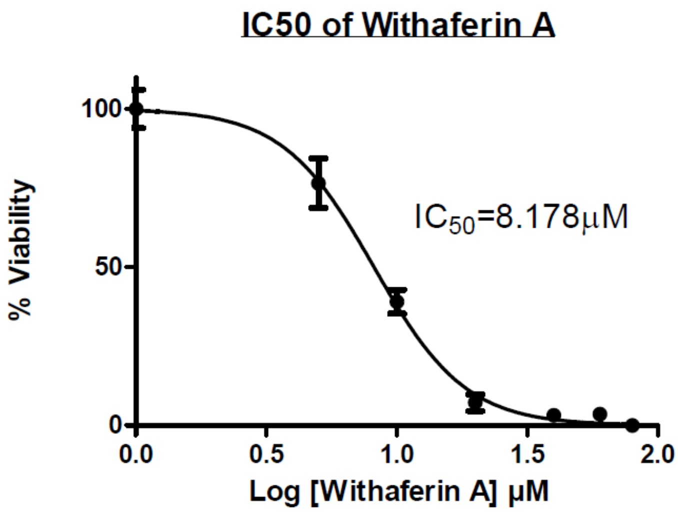


Figure 8: Drug sensitivity curve of Withaferin A.
1 × 10^5 cells were plated in a 96-well plate and allowed to differentiate after the addition of 100nM phorbol 12-myristate 13-acetate (PMA). Subsequently, the cells were treated with several concentrations of Withaferin A for 24hr. After adding the MTT reagent to the culture, the cells were incubated for 3.5hr and then 10% (m/v) SLS was added to dissolve the formazan crystals. The plates were incubated overnight at 37°C and absorbance was read using the Biotek plate reader. Percentage cell viability was calculated on GraphPad Prism, with untreated cells displaying 100% viability. The average IC_{50} was found to be 6.8µM. Data represents two independent experiments.

3.2.2 Ruscogenin

To investigate whether Ruscogenin was cytotoxic to macrophages, an MTT assay was performed with a concentration range from 1 - 80µM. It was found that the average IC_{50} of Ruscogenin was 2.7µM (Figure 9). The results of this experiment lack the classic sigmoidalshaped curve expected to be seen in a dose-dependant cytotoxicity experiment. The concentration range used to treat the cells resulted in cell viabilty being under 50%, with the exception of treatment at 1µM, which had 100% viability (Figure 9).

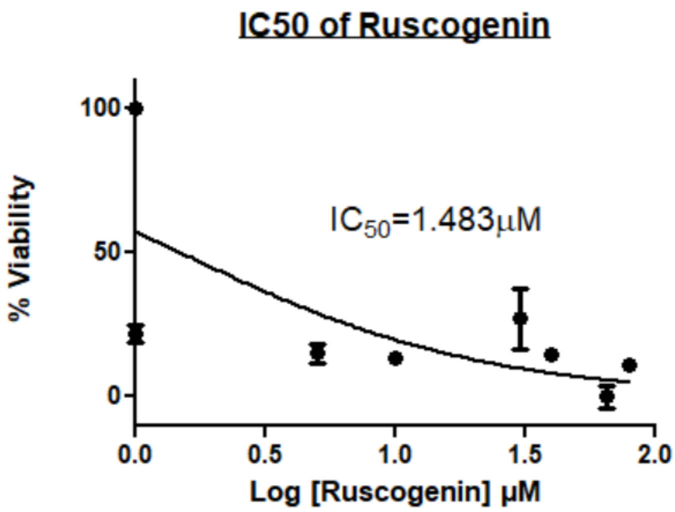


Figure 9: Drug sensitivity curve of Ruscogenin.
Cultured cells were plated at a density 1 × 10^5 cells/well in a 96-well plate and treated with compound for 24hr after differentiation of macrophages. MTT was then added to the cells and absorbance was read using the Biotek plate reader to determine viability. The MTT results yielded an average IC_{50} of 2.7µM. Data represents two independent experiments.

3.2.3 Leonurine

To explore whether Leonurine was cytotoxic to the cells, the MTT assay was initially performed using a concentration range of 5 - 35µM and then 10 - 100µM, and the results were not reproducible and thus the IC_{50} could not be determined. However, reproducible results were obtained after using a much lower concentration range (1 - 10µM). The concentration used to treat the cells was 1.2µM, which was the average IC_{50} of two independent experiments (Figure 10). Similar to Ruscogenin, there was no exponential decrease in cell viability as the concentration of compound increased. Interestingly, treatment with 10µM yielded higher cell viability than the previous three concentrations, which were all lower than 10µM.

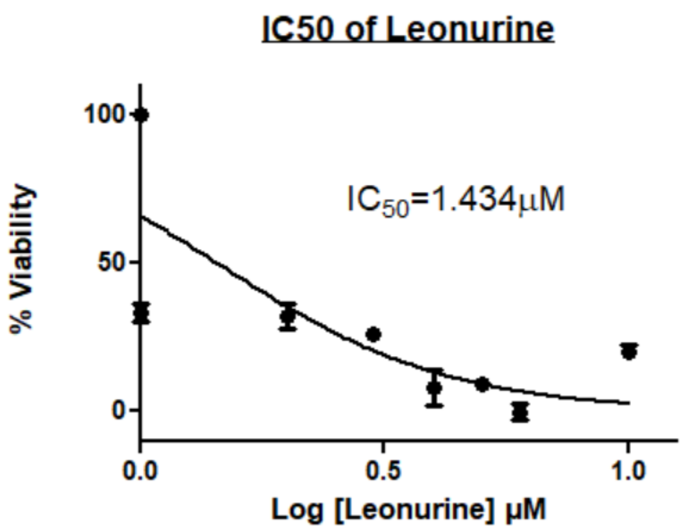


Figure 10: Drug sensitivity curve of Leonurine.
Cells were plated at a density of 1 × 10^5 cells/well and differentiated into macrophages for 72hrs. The macrophages were treated with Leonurine and incubated for 24hrs. MTT reagent was added to the cells and following incubation, absorbance was read at 595nm using the Biotek plate reader. The average IC_{50} was found to be 1.2μM. Data represents two independent experiments.

In diseases like cancer, drugs are designed with the intent of killing the cancerous cells. However, in tuberculosis, all the immune cells that have a role to play in host immunity, are required to be alive to be able to fight the mycobacteria and protect the host. For this reason, treating the cells with the concentration where 100% cell viability was observed on the cell viability curves was explored. This value was determined by averaging the lowest concentrations where 100% cell viability was observed for each compound. This concentration will be referred to as the '"C1" concentration throughout this thesis.

3.3 Mtb infection of cells before and after treatment

Prior to the infection, monocytes were differentiated into macrophages by adding 100nM PMA to the complete media. Monocytes are noticeably smaller in size compared to macrophages (Figure 11). Macrophages are adherent cells and lose proliferative ability as differentiation takes place (Panawala, 2017). Macrophages are also more granular in appearance and their morphology is not as uniform compared to monocytes. The macrophages have some oblong shaped cells, and every cell shape looks different in contrast to the monocytes (Figure 11).

Day 1 monocytes Day 3 macrophages

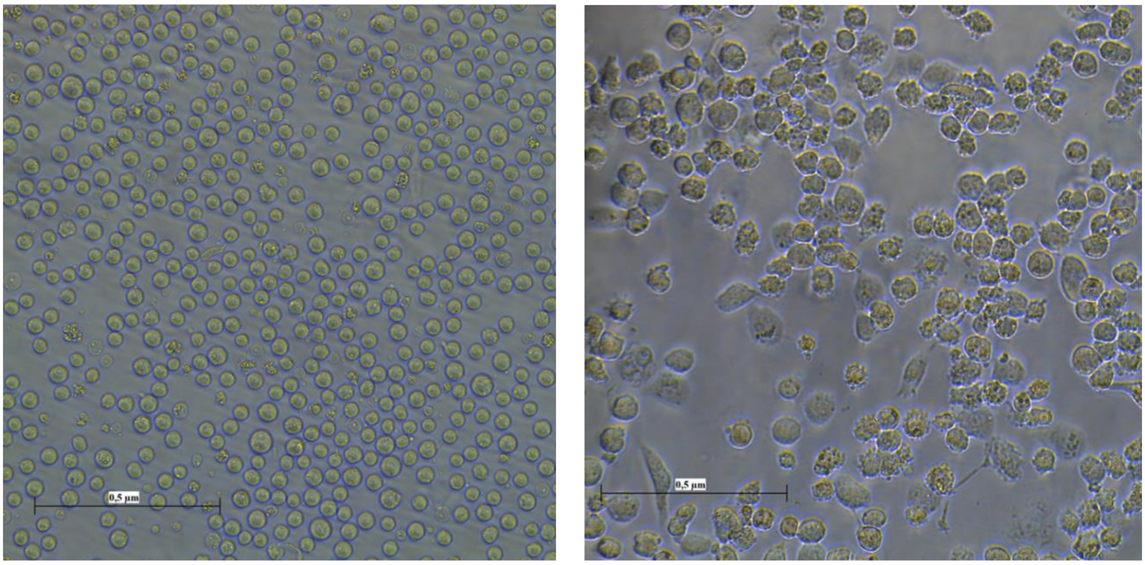
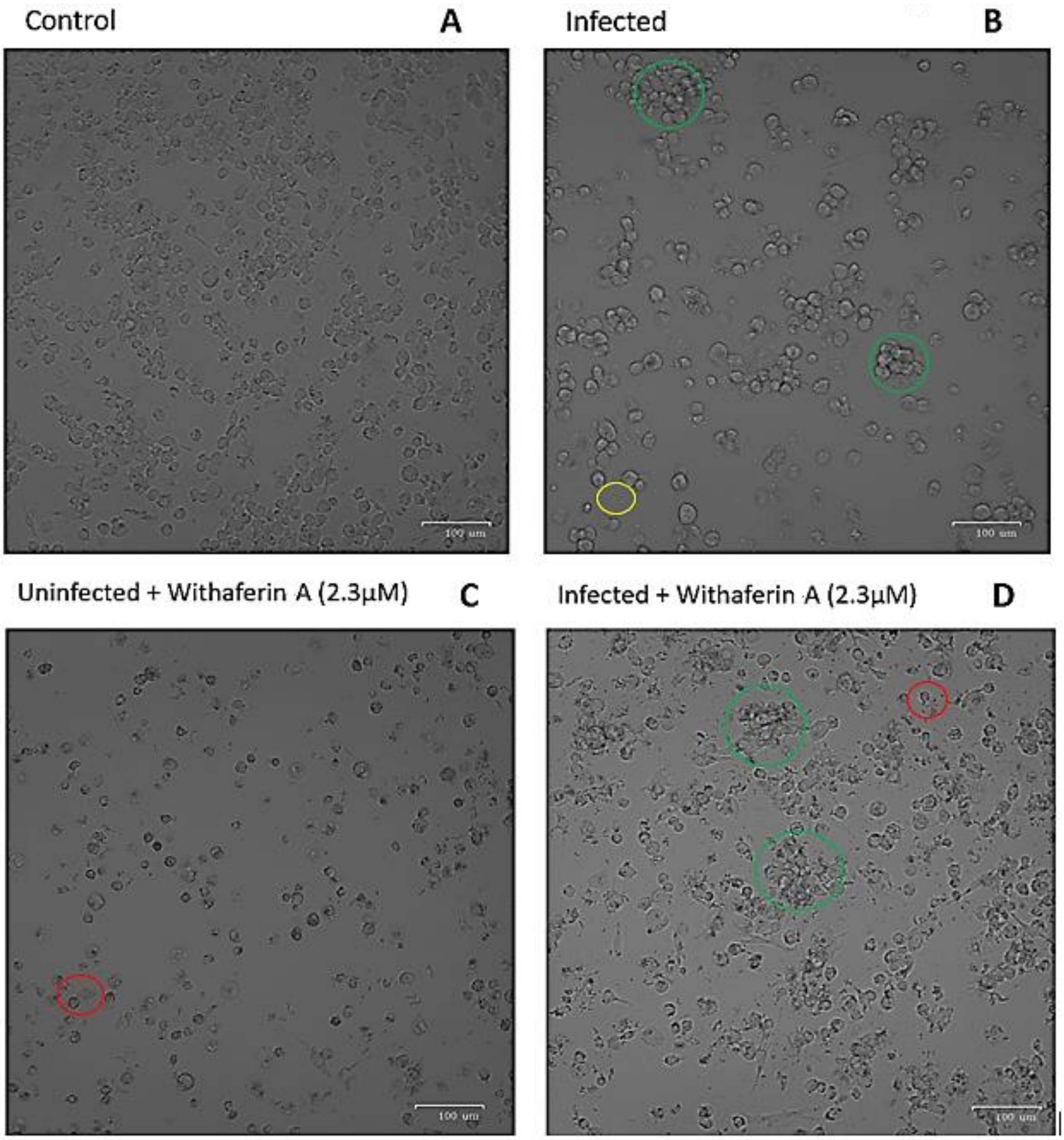


Figure 11: Morphological differences between monocytes (day 1) and macrophages (day 3) the differentiation period.
THP-1 monocytes (left panel) were differentiated into macrophages (right panel) using 100 nM PMA for 72hr. Images were viewed using a Leica DM IL LED inverted microscope at 32× magnification.

After a day of rest in the differentiation protocol mentioned in Chapter 2, the cells were infected with live Mtb strain H37Rv (the laboratory strain) following treatment with the plant-derived compounds for 24 hours. Visually, there were no differences between the control and infected cells as the morphology was similar Figure 12A and B). However, some extracellular bacilli were observed (yellow circle) and clustering of cells in infected cells (green circle, Figure 12A and 12B). The uninfected and Withaferin A treated cells show erupted membranes (red circle) and the distinctive macrophage morphology is not preserved (Figure 12C), suggesting that Withaferin A induces some morphological changes in the cells. However, infected and Withaferin A (2.3μM) treated cells had much more cellular debris and cells can be seen clustered on top of each other (Figure 12D). The uninfected and Ruscogenin (1.35μM) treated cells (Figure 12E) were comparable to the uninfected and Withaferin A treated cells, the blue circle is indicative of an area of cells that are almost unrecognizable possibly due to cell death. In the infected and Ruscogenin treated cells, mycobacteria are visible in the extracellular environment (yellow circle, Figure 12F). Lastly, the Leonurine treated cells do not exhibit great differences between the uninfected and infected cells, besides the presence of bacilli in the infected group (Figure 12G and H). The uninfected cells do not display consistent morphology representative of macrophages. Similarly, infected cells did not retain their typical macrophage-like appearance in addition to being sparse and indicating noticeable membrane damage (indicated with the red circle, Figure 12C, E and G).



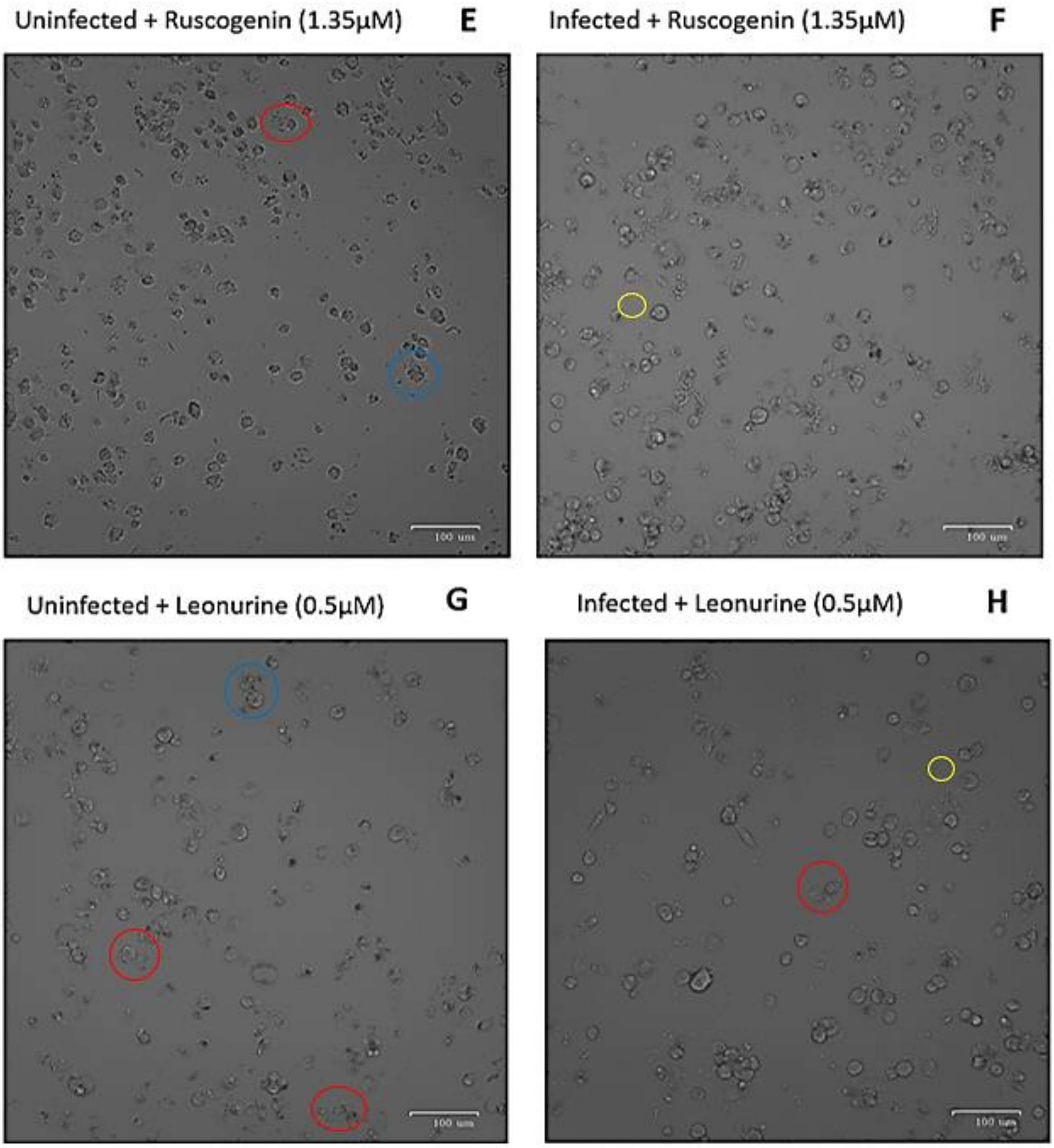
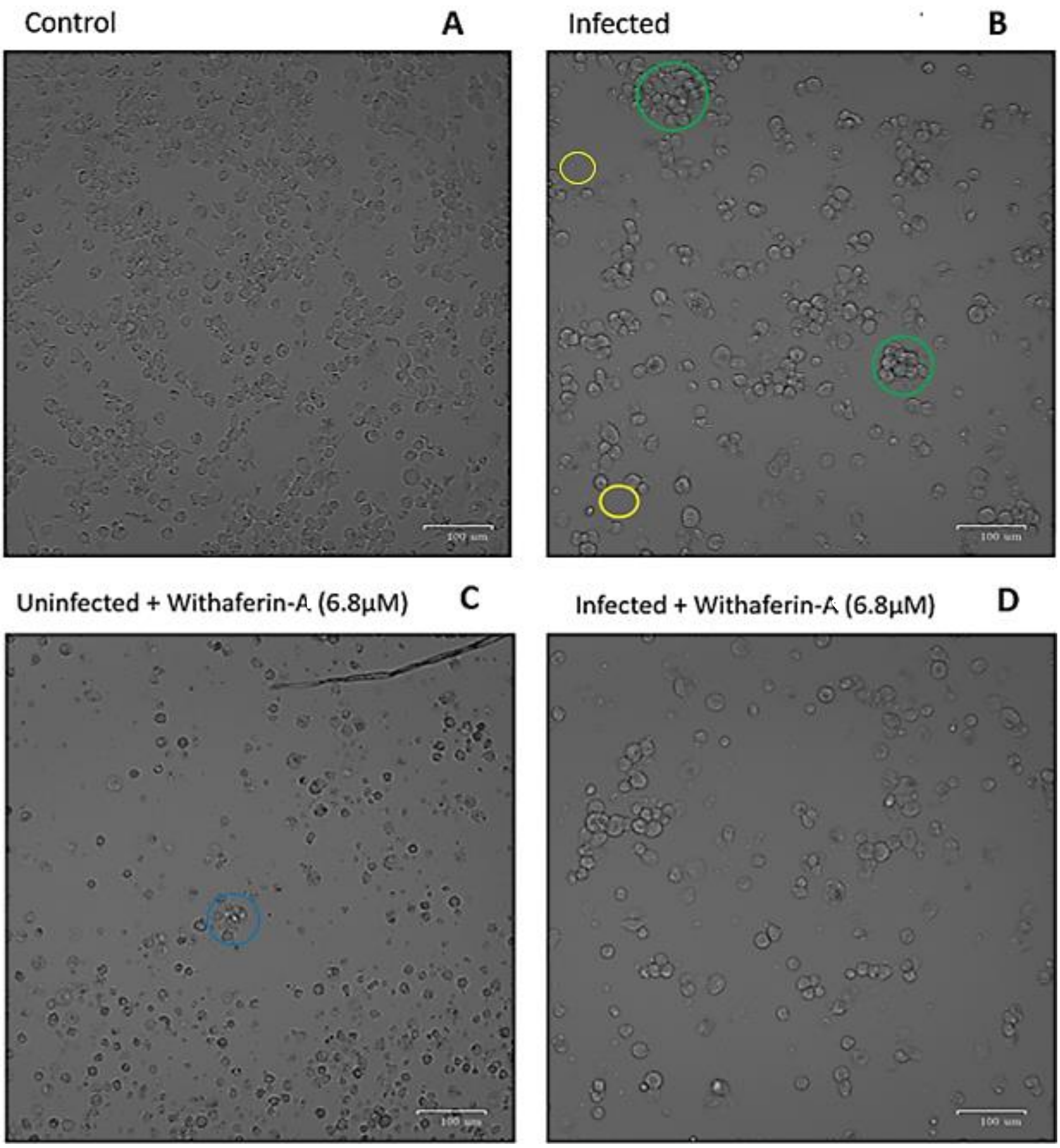


Figure 12: Images of infected THP-1 macrophages taken 24hr after pre-treatment with the C1 concentration of the compounds.

Macrophages were treated for 24hr with the C1 concentration prior to infection with H37Rv Mtb. Images were taken using a Zoe Fluorescent cell imager at 20× magnification. Cell membrane damage is indicated with the red circle. In the infected cells, the presence of bacilli is indicated with the yellow circle. Green circles indicate areas where cells are clustered and blue circles are indicative of expelled cellular content.

The same controls (untreated and infected) were used for both Figure 12 and Figure 13.. All treatment groups were plated in one plate, thus the untreated and infected are the same for both sets of concentrations (C1 & IC_{50}). Uninfected and Withaferin A treated cells showed uncharacteristic macrophage morphology with the cells not being as large as the untreated and uninfected control cells (Figure 13A) and there are many dead/damaged cells (Figure 13C). Compared to the untreated and uninfected control cells, the infected and Withaferin A treated cells exhibited (Figure 13D) morphology that was comparable to that of the infected and untreated cells (Figure 13B). Matching observations can be made for the Ruscogenin treated cells (Figure 13E & F). However, it is worth noting that the infected and Leonurine.



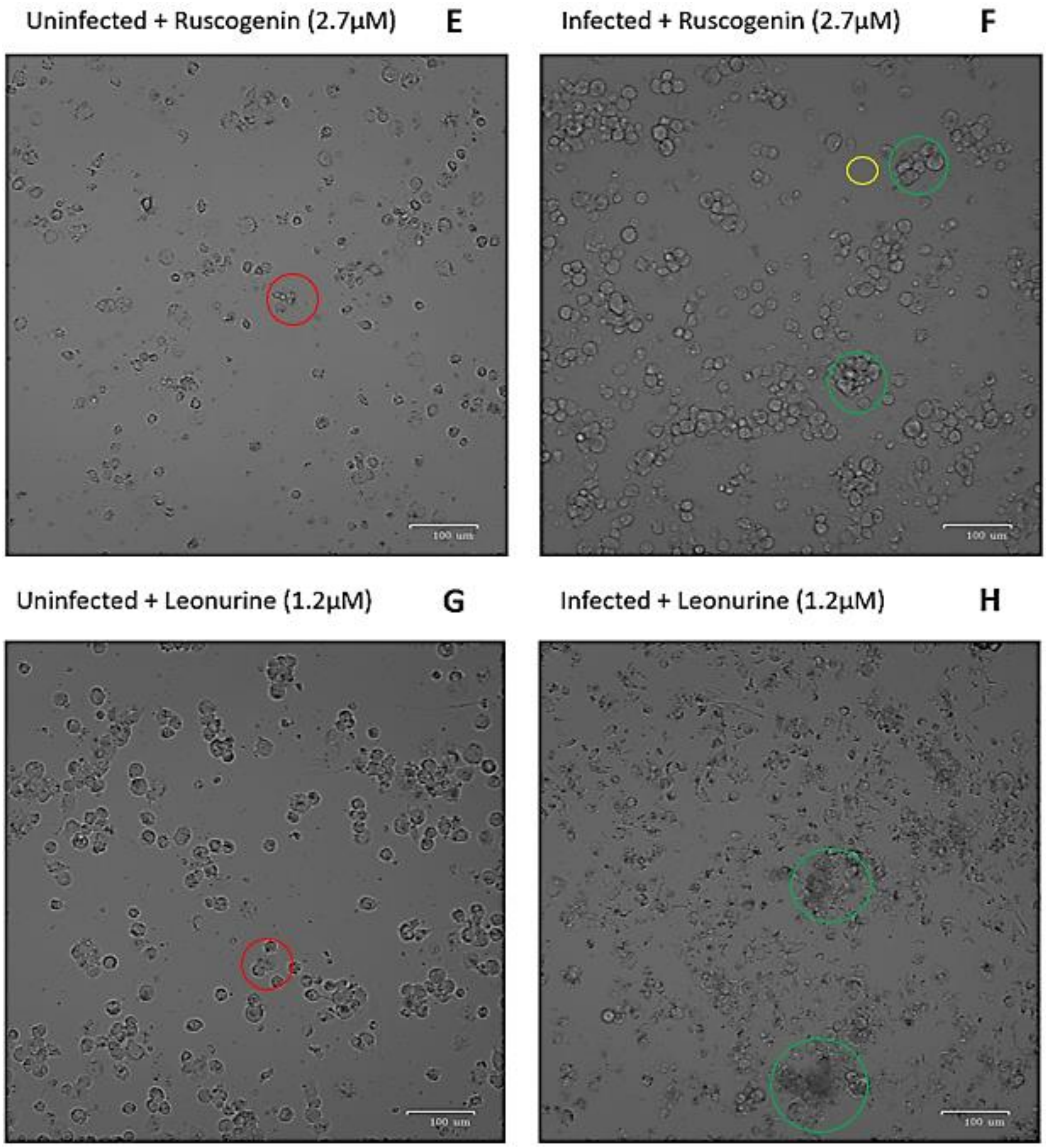


Figure 13: Microscopy images of cells treated 24hr with the IC_{50} of plant-derived compounds before infection with live Mtb H37Rv strain.

Using a Zoe Fluorescent cell imager, pictures were taken at a 20× magnification. These cells were treated with the IC_{50} concentrations of the plant-derived compounds for 24hr before they were infected with Mtb. Red circles are indicative of cell membrane damage and yellow circles show the presence of bacilli, green circles indicate areas where cells are clustered and blue circles are indicative of expelled cellular content.

3.4 Inflammatory profile of H37Rv-infected macrophages in response to treatment with the plant-derived compounds

Excessive cytokine production during tuberculosis can be very unfavourable for the host. Macrophages produce some of these cytokines that can cause harm in uncontrollable amounts (Tobin *et al.*, 2010). Thus, this study examined whether the plant-derived compounds could reduce Mtb-induced cytokine production in macrophages using ELISA. Cells were infected with live Mtb strain H37Rv and cells were treated before or after infection to determine if the plant-derived compounds had anti-inflammatory effects.

3.4.1 Cytokine profile after infected cells were treated with Withaferin A

To investigate whether Withaferin A reduced the secretion of cytokines, macrophages were infected with live Mtb strain H37Rv using a multiplicity of infection (MOI) of 5, supernatants were collected and cytokines detected using ELISA. Significant increases were observed in all cytokines in infected cells compared to the uninfected and untreated control cells (Figure 14A, B and C). A significant reduction was observed in IL-1β secretion by infected macrophages treated with 6.8µM Withaferin A compared with untreated and infected cells (Figure 14A). Lastly, a concentration-dependent decrease in IL-6 production was observed in infected and treated cells compared to infected and the untreated control cells (Figure 14C). Moreover, Withaferin A significantly reduced the secretion of IL-6 even in the uninfected cells compared to uninfected and untreated control cells (Figure 14C

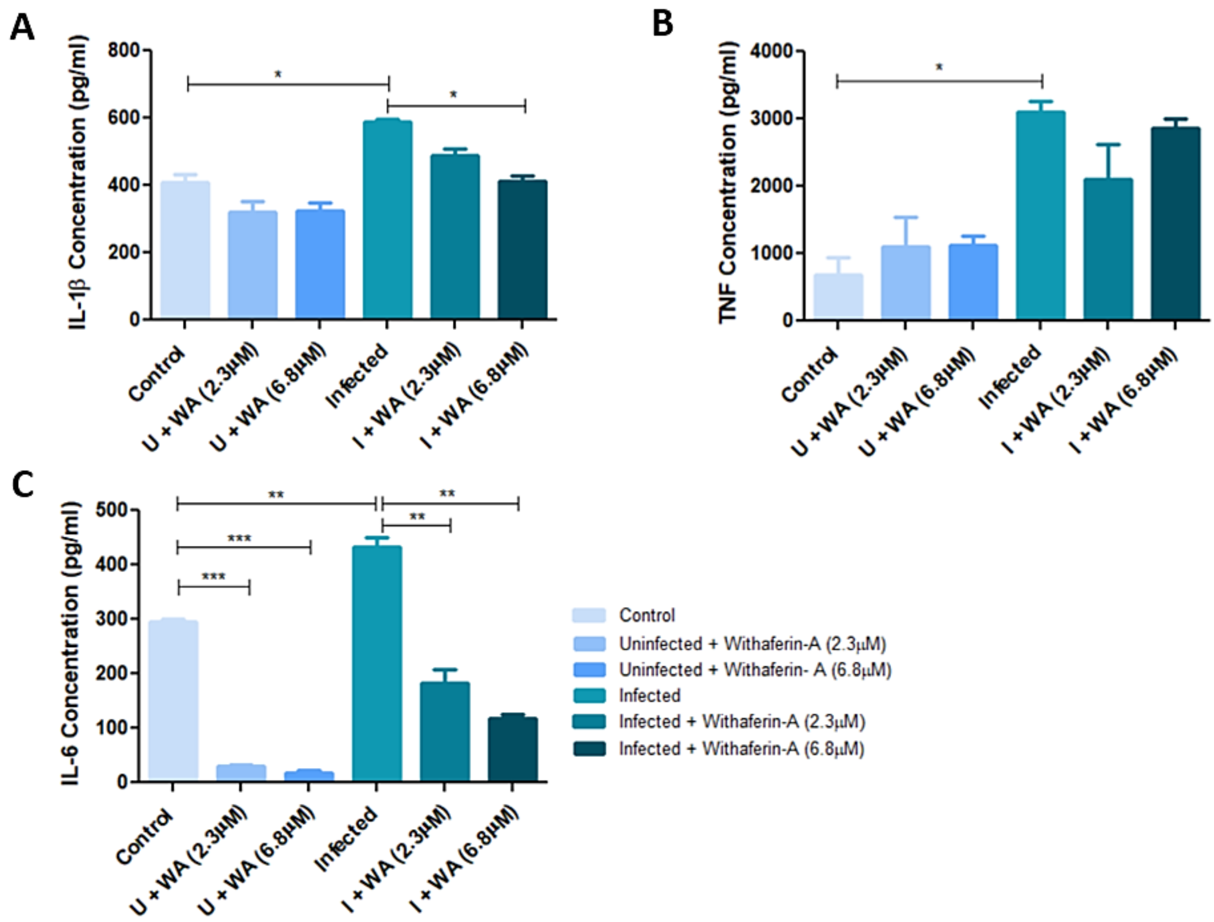


Figure 14: Cytokine profile of infected THP-1 macrophages treated with Withaferin A.
Plated cells were differentiated first before infecting them with Mtb (MOI=5) thereafter, cells were treated with Withaferin A for 24hr before proceeding to collect supernatants. Supernatant was collected to run an ELISA for IL-1β, TNF and IL-6. Absorbance was read at 450nm. Data represents 2 independent experiments. **p*<0.05, ***p*<0.01 using Students *t*-test.

3.4.2 Cytokine profile after infected cells were treated with Ruscogenin

To analyse the anti-inflammatory effects of Ruscogenin, macrophages were infected with live Mtb strain H37Rv and treated the cells with either IC_{50} or C1 concentration of Ruscogenin. The secretion of IL-1β was significantly increased in infected and untreated cells compared to the uninfected and untreated control cells, demonstrating that the infection was successful. However, we observed no significant decrease in cytokine expression in infected and treated cells compared to the infected and untreated cells, suggesting that Ruscogenin had no anti-inflammatory effects (Figure 15A, B and C). It is also important to note that we observed no differences in TNF secretion between the infected and untreated cells and uninfected and untreated control cells, suggesting that infection failed to induce significant increase in TNF secretion (Figure 15B). This challenge is confined only to TNF as no significant increase in IL-6 secretion by infected and untreated cells compared to uninfected and untreated control cells was observed (Figure 15C).

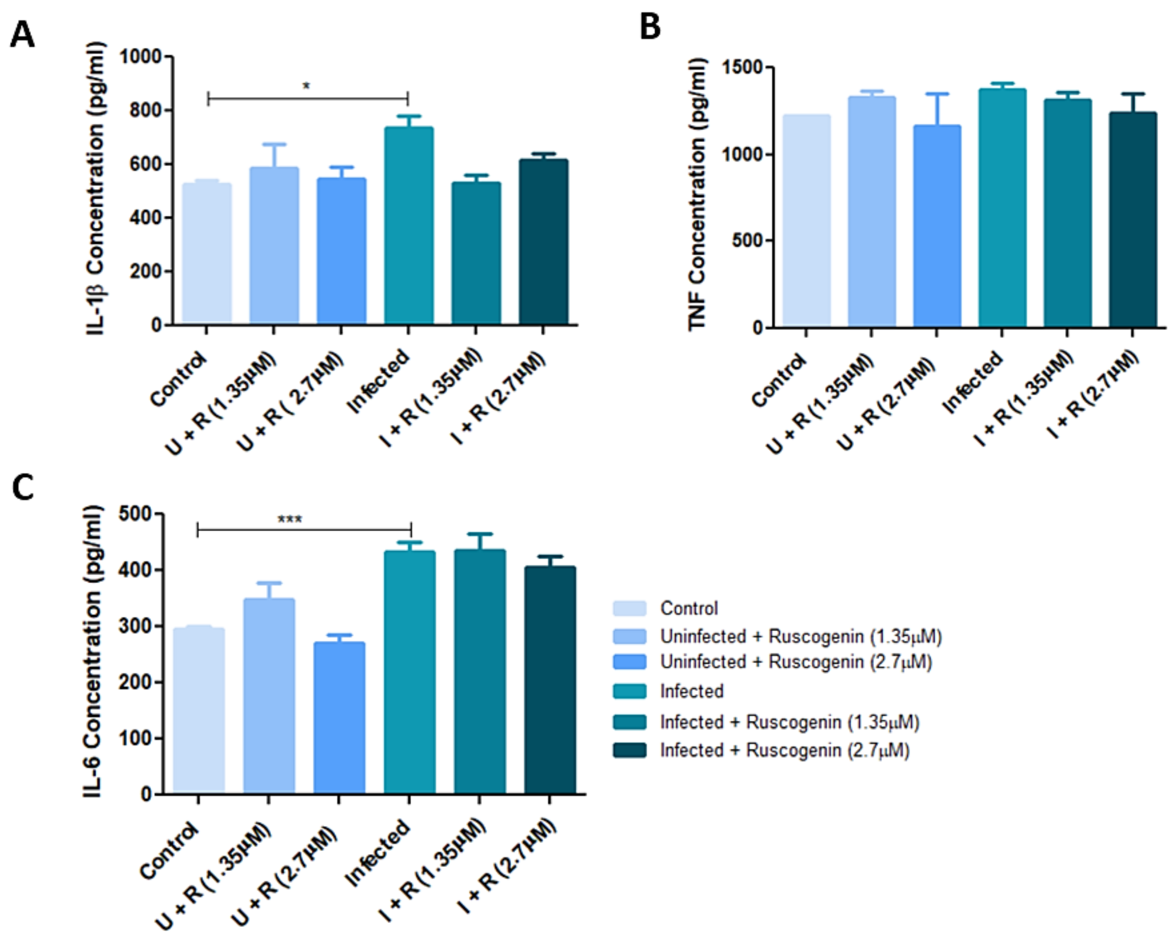


Figure 15: Cytokine profile of infected THP-1 macrophages treated with Ruscogenin.
Plated cells were infected with H37Rv Mtb using an MOI of 5, after which we exposed macrophages to Ruscogenin treatment for 24hr. Cell supernatant was collected and filtered after infection to run ELISAs for IL-1β, TNF and IL-6. Data is representative of 2 independent experiments (n=2). **p*<0.05, ***p*<0.01, ****p*<0.001 using Students *t*-test.

3.4.3 Cytokine profile after infected cells were treated with Leonurine

Lastly, the anti-inflammatory effects of Leonurine using ELISA after collecting supernatants from macrophages infected with live Mtb strain H37Rv were examined. The results demonstrated that Leonurine had no significant effects on any of the cytokines tested as the concentration of the cytokines were similar to those observed in the infected and untreated control (Figure 160A, B and C). However, it was noted that the experiment was successful as the cytokine expression was significantly increased in the infected and untreated cells compared to the uninfected and untreated control cells (Figure 16A, B and C).

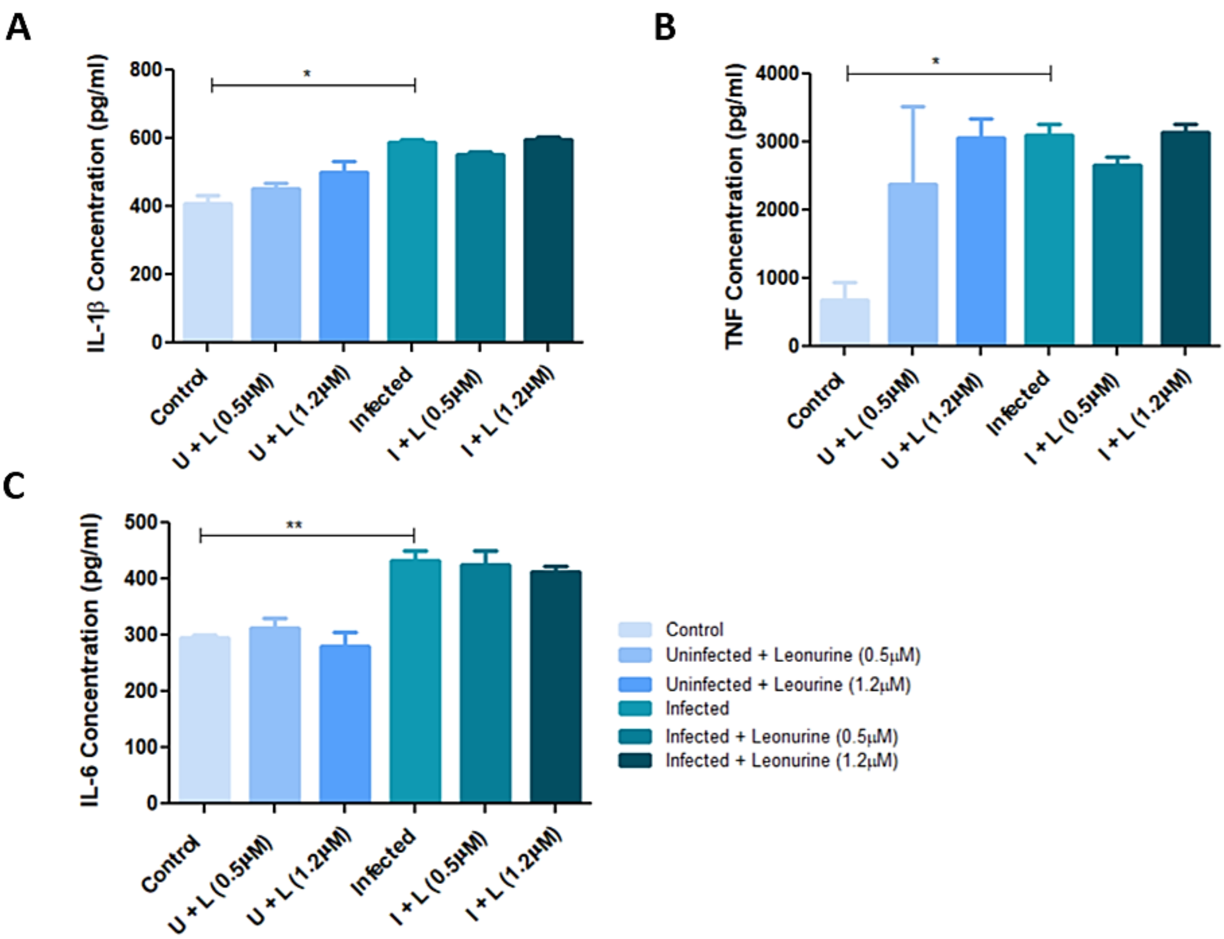


Figure 16: Cytokine profile of infected THP-1 macrophages treated with Leonurine.
Macrophages were infected with Mtb (MOI=5) before they were subjected to Leonurine treatment. Cells were then left for another 24hr until supernatant was collected for the ELISAs. IL-1β, TNF and IL-6 levels were measured. Data represents two independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 using the Student's *t*-test.

3.4.4 Cytokine profile after cells were pre-treated with Withaferin A and infected with live Mtb

After determining the effects of the plant-derived compounds on cytokine secretion after infecting macrophages with Mtb, this study also investigated whether treating cells with the compounds prior to infection will enhance the observed effects. Therefore, macrophages were treated with the IC_{50} or C1 concentration of the compound for 24 hours and subsequently infected the treated cells with live Mtb strain H37Rv at MOI of 5. Supernatants were collected and cytokine secretion determined using ELISA. It is noted that infection alone significantly increased the secretion of all the cytokines compared to the uninfected and untreated cells (figure 17A, B and C). Pre-treatment with Withaferin A had no effect on the secretion of IL-1β at either concentration compared to untreated and infected cells (Figure 17A). Interestingly, a concentration dependent decrease in the secretion of TNF in Withaferin A pre-treated and infected cells compared to the untreated and infected cells was observed (Figure 17B). Moreover, the IC_{50} concentration of Withaferin A significantly reduced the secretion of TNF in pre-treated and uninfected cells compared to the untreated and uninfected control cells (Figure 17B). Finally, pre-treatment with IC_{50} concentration of Withaferin A significantly reduced secretion of IL-6 in infected macrophages compared to the untreated and infected cells (Figure 17C). Moreover, both concentrations of Withaferin A reduced the secretion of IL-6 in pre-treated and uninfected cells compared to the untreated and uninfected control cells (Figure 17C). Therefore, it can be concluded that Withaferin A reduces the secretion of TNF and IL-6 in pre-treated macrophages infected with the laboratory strain of Mtb.

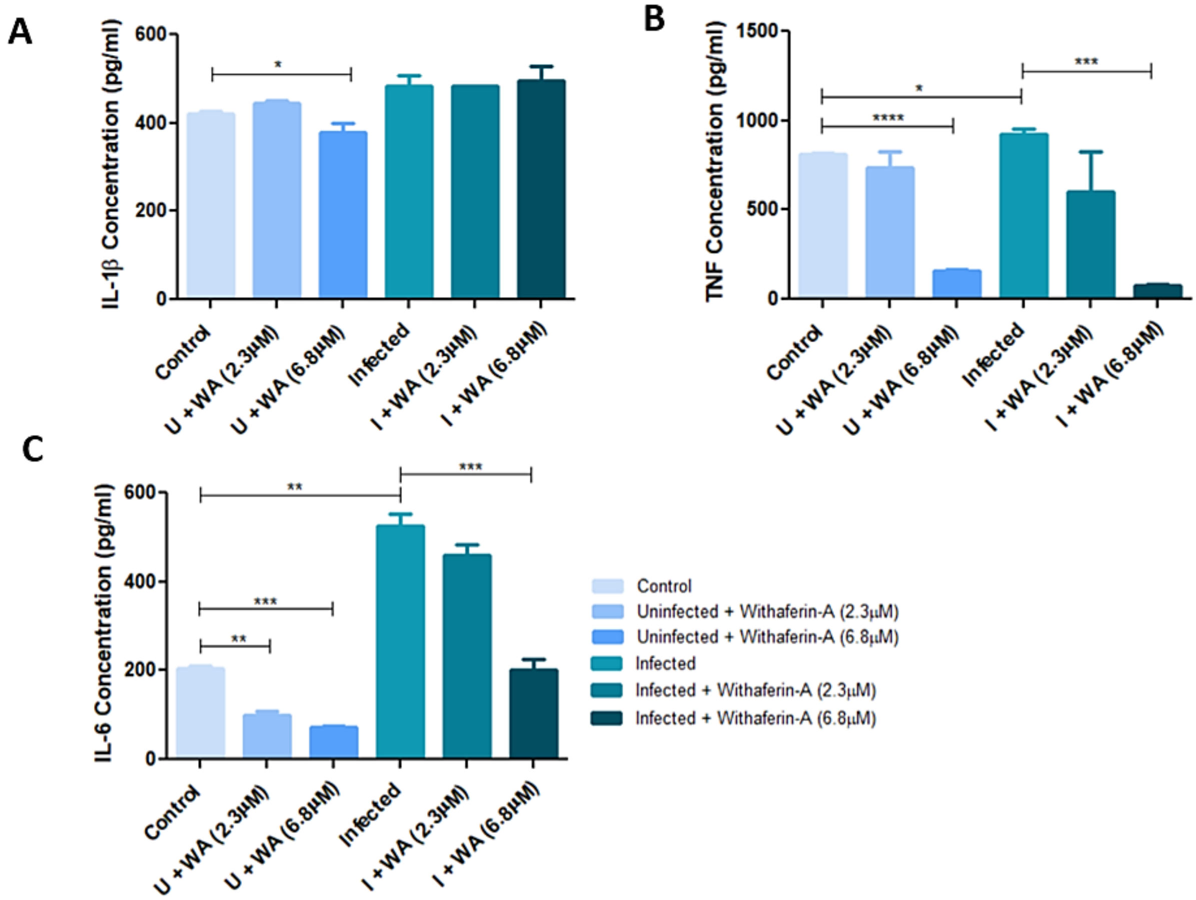


Figure 17: Cytokine profile of THP-1 macrophages pre-treated with Withaferin A and infected with live Mtb strain H37RV.
Cells were treated with Withaferin A for 24hr prior to us infecting them with Mtb (MOI=5). A 24hr incubation time was allowed before supernatant was collected to perform ELISA's. Data represents independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 using the Student's *t*-test.

3.4.5 Cytokine profile after cells that were pre-treated Ruscogenin and infected with live Mtb

This study also investigated whether pre-treatment of macrophages with Ruscogenin prior to infection with Mtb reduced cytokine secretion. It was observed that the infected cells had significantly increased cytokine secretion compared to the untreated and uninfected control cells (Figure 18A, B and C). However, pre-treatment with either concentration of Ruscogenin (IC_{50} and C1) failed to exert any anti-inflammatory effects on pre-treated and infected cells compared to untreated and infected cells, with the exception of IL-6 (Figure 18A, B and C). IL-6 production was significantly reduced when macrophages were pre-treated with 1.35μM Ruscogenin compared to the untreated and infected cells (Figure 18C)

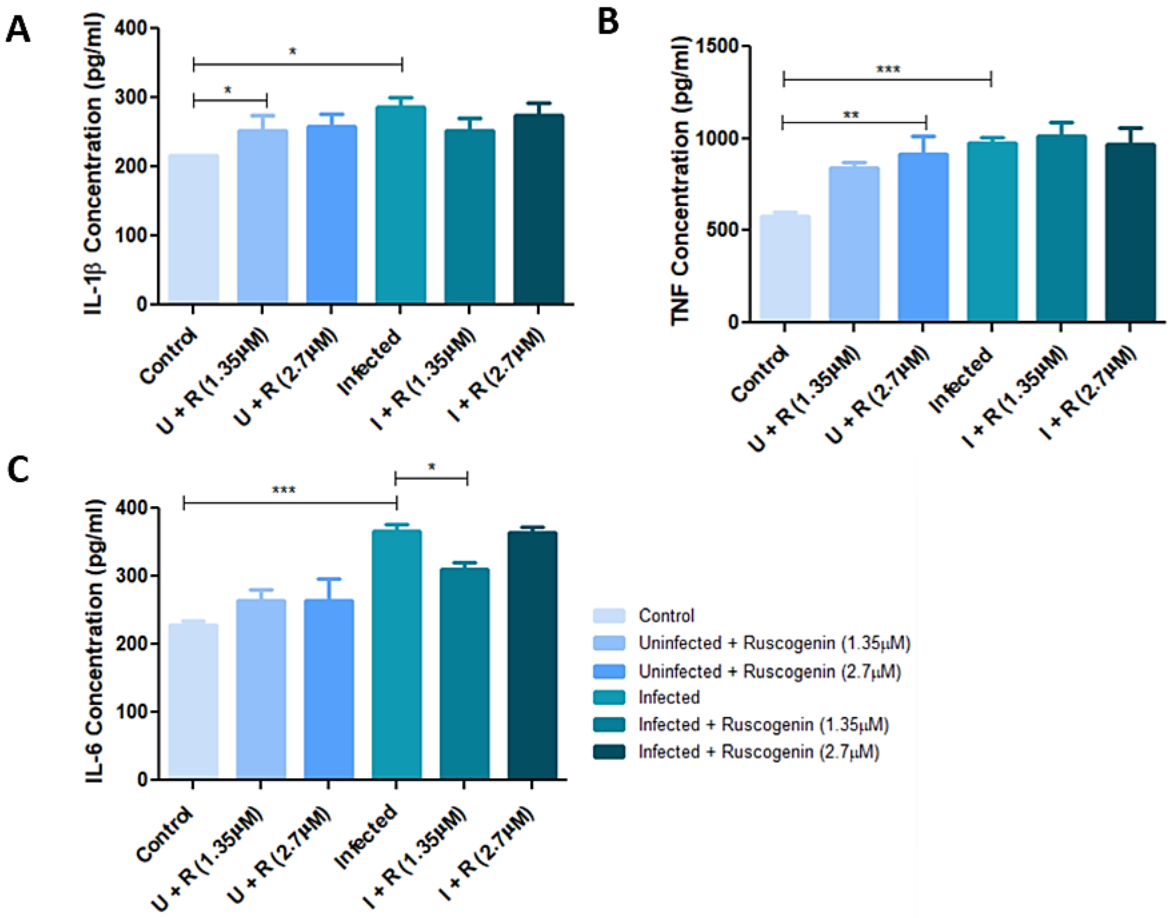


Figure 18: Cytokine profile of THP-1 macrophages pre-treated with Ruscogenin and infected with live Mtb strain H37Rv.
Cells were subjected to treatment with Ruscogenin 24hr before we carried out the Mtb infection (MOI=5). After infection, the cells were left for 24hr. Supernatant was collected to perform the ELISAs for IL1β, TNF and IL-6. Data represents two independent experiments. *p<0.05, **p<0.01, ***p<0.001 using the Student's *t*-test.

3.4.6 Cytokine profile for the cells that were pre-treated with Leonurine and infected with live Mtb

To investigate whether pre-treatment with Leonurine had an anti-inflammatory effect on Mtb-infected macrophages, macrophages were pre-treated with Leonurine for 24 hours and infected cells with the laboratory strain of Mtb 24 hours before collecting supernatants and analysing cytokine secretion using an ELISA. Importantly, these experiments were successful since a significant increase in cytokine secretion was observed in the untreated and infected cells compared to the untreated and uninfected control cells (Figure 19A, B and C). Another interesting observation was that pre-treatment of infected cells with Leonurine at the IC_{50} concentration significantly reduced TNF and IL-6 secretion compared to the untreated and infected control cells (Figure 19B and C). Moreover, it was also observed that pre-treatment with Leonurine at either concentration (IC_{50} and C1) had a significant effect on IL-6 production even in uninfected cells compared to untreated and uninfected control cells (Figure 19C).

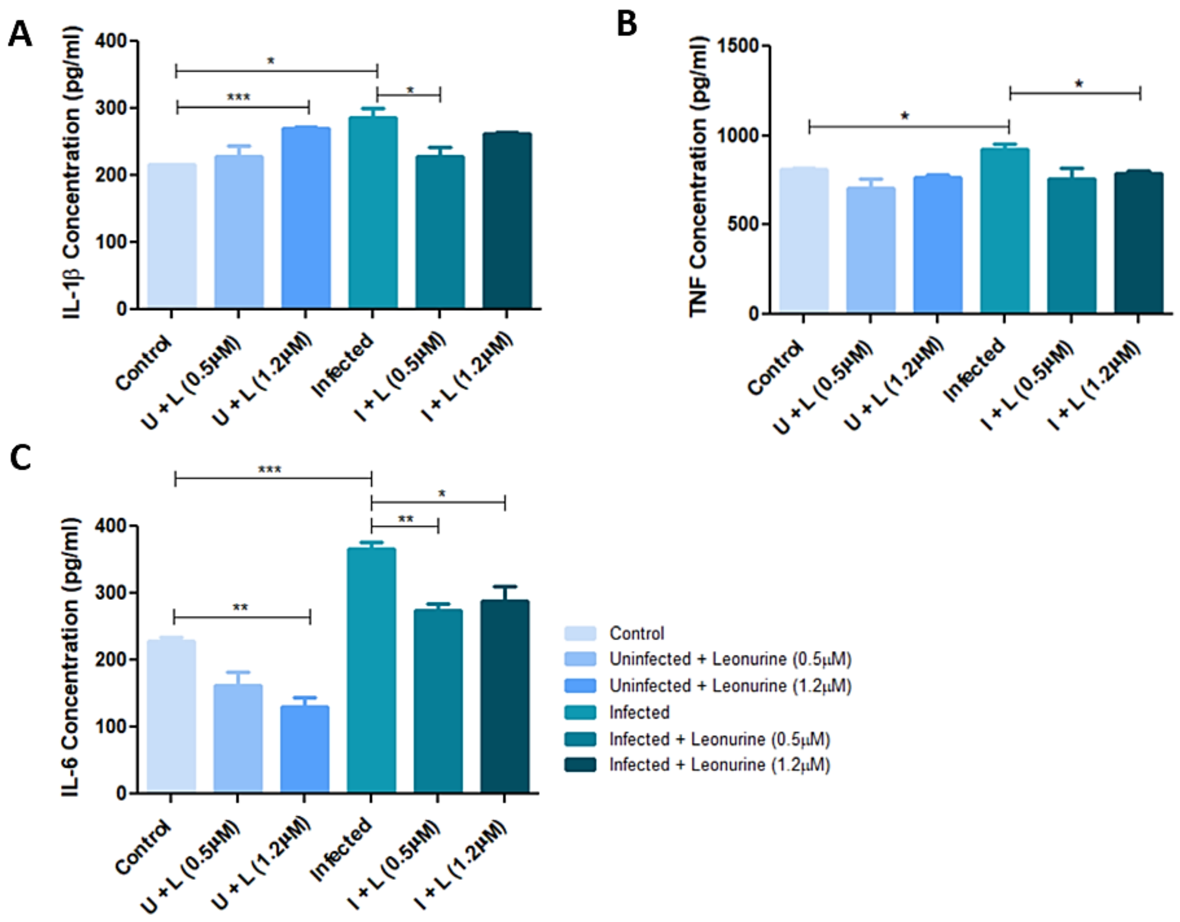


Figure 19: Cytokine profile of THP-1 macrophages pre-treated with Leonurine and infected with live Mtb strain H37Rv.
Treatment with Leonurine was done 24hrs before macrophages were infected with H37Rv Mtb using an MOI:5 and left in the incubator for 24hr before supernatants were collected to run ELISAs. Data represents two independent experiments. *p<0.05, **p<0.01, ***p<0.001 using the Student's *t*-test.

3.5 The effect of compounds on cell death measured with lactate dehydrogenase (LDH) assay

It is known that excessive cytokine production can be undesirable to cells, and may contribute to tissue pathology and disease progression in tuberculosis (Marakalala *et al.*, 2016). Thus, this study had shown that some of the compounds could lower the secretion of pro-inflammatory cytokines by infected macrophages, the next step was to determine if these compounds could in turn prevent cell death. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is released into the extracellular milieu after plasma membrane damage (Chan, Ka-Ming Francis., Moriwaki, Kenta and De Rosa, 2013). To do this, the assay controls were plated and the supernatant obtained from infected cells to measure the amount of LDH released into the supernatant after 24 hours was used. It is worth noting that these experiments were only performed once due to time constraints.

3.5.1 LDH activity in cells either pre-treated or treated with Withaferin A after infection with H37Rv Mtb

Following the collection of supernatants, the LDH assay was carried out for both treatment conditions. It was found that infection with live Mtb strain H37Rv significantly induced cell death compared to the uninfected and untreated control cells (Figure 20). Interestingly, a significant reduction in cell death was observed in infected macrophages treated with 6.8µM Withaferin A compared to infected and untreated cells (Figure 20), suggesting that this compound reduced cell death.

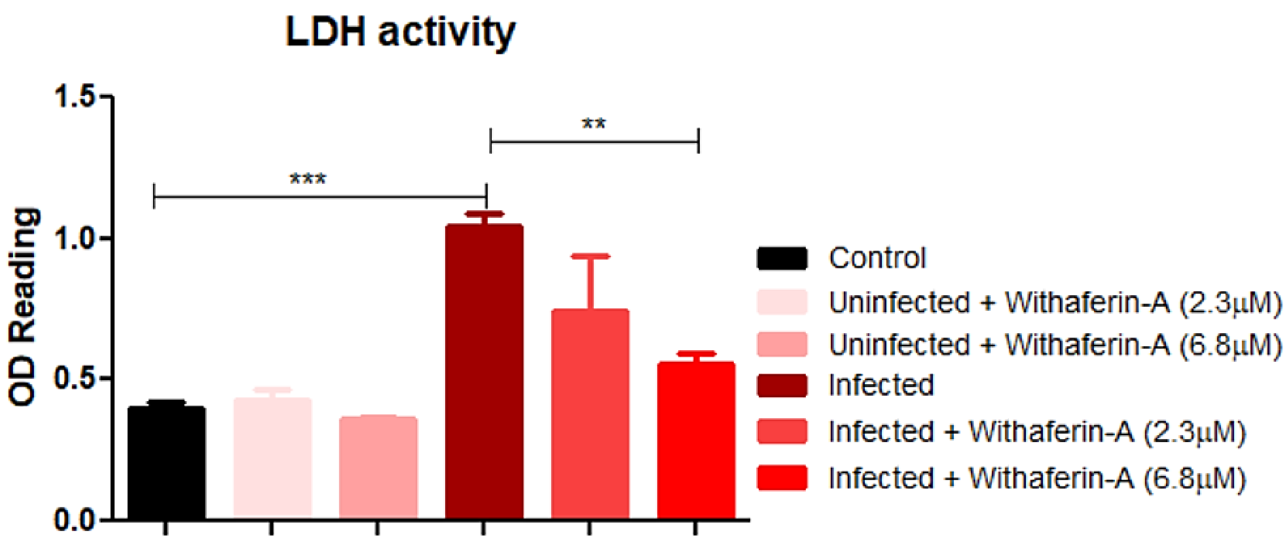


Figure 20: LDH activity in macrophages treated with Withaferin A after Mtb infection.
After macrophages were infected with Mtb, they were treated with both concentrations of Withaferin A (C1 and IC_{50}) for 24hr. Thereafter, the supernatant was collected and the LDH assay was carried out. Data represents one experiment. *p<0.05, **p<0.01, ***p<0.001 using the Student's t-test.

This study sought to investigate whether pre-treating macrophages with Withaferin A would prove beneficial in rescuing them from Mtb-induced cell death. The same procedure was followed to collect the supernatants and these results showed that infection with live Mtb resulted in a significant increase in cell death (Figure 21). Pre-treating the cells with Withaferin A did not have any effect on cell death (Figure 21), in contrast to the ELISA data where pre-treatment significantly lowered TNF and IL-6 (Figure 17).

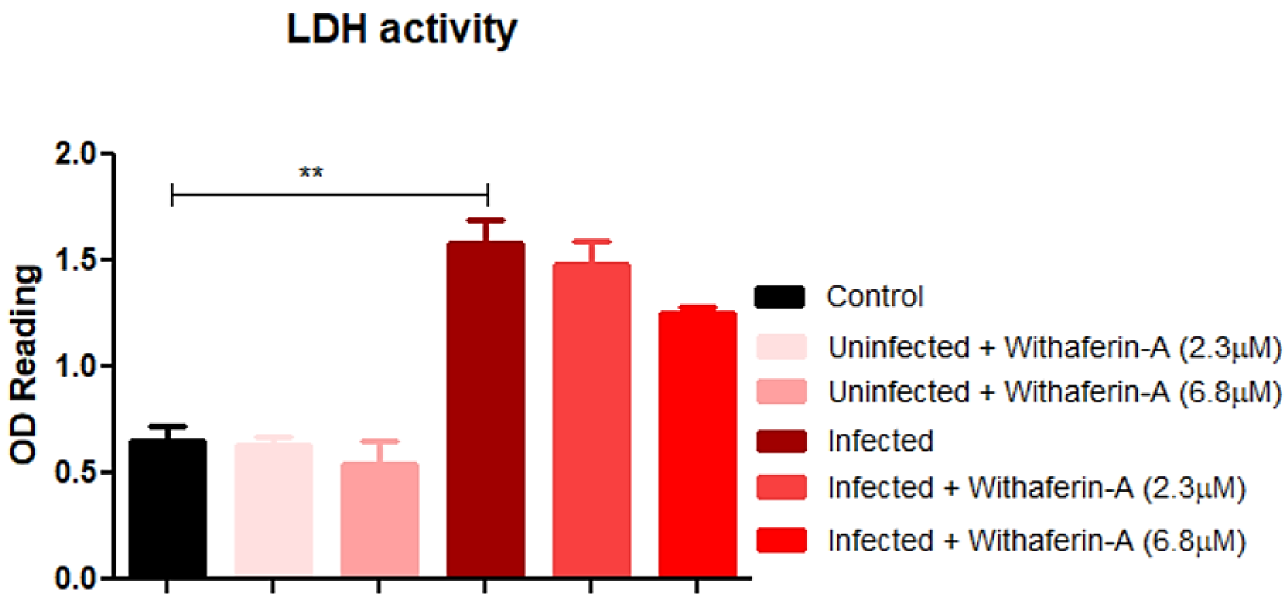


Figure 21: LDH activity in macrophages pre-treated with Withaferin A after Mtb infection.
Prior to infecting macrophages, they were treated with Withaferin A for 24hr. Following infection, the cells were left overnight before supernatants were collected and stored until used for LDH assay. Data represents 1 experiment. *$p<0.05$, **$p<0.1$ using the Student's *t*-test.

3.5.2 LDH activity in cells either pre-treated or treated with Ruscogenin after infection with H37Rv Mtb

Macrophages were infected with live Mtb and then treated with Ruscogenin and left to incubate for 24 hours. Thereafter, supernatant was collected and stored it at -80°C until it was ready to be used for the LDH assays. Cell death was significantly increased following infection (Figure 22) and treatment with the IC_{50} concentration of Ruscogenin significantly rescued macrophages from cell death (Figure 22). At this stage, the results do not seem to mimic what was observed in Figure 15, where Ruscogenin has no effect on inflammatory cytokines.

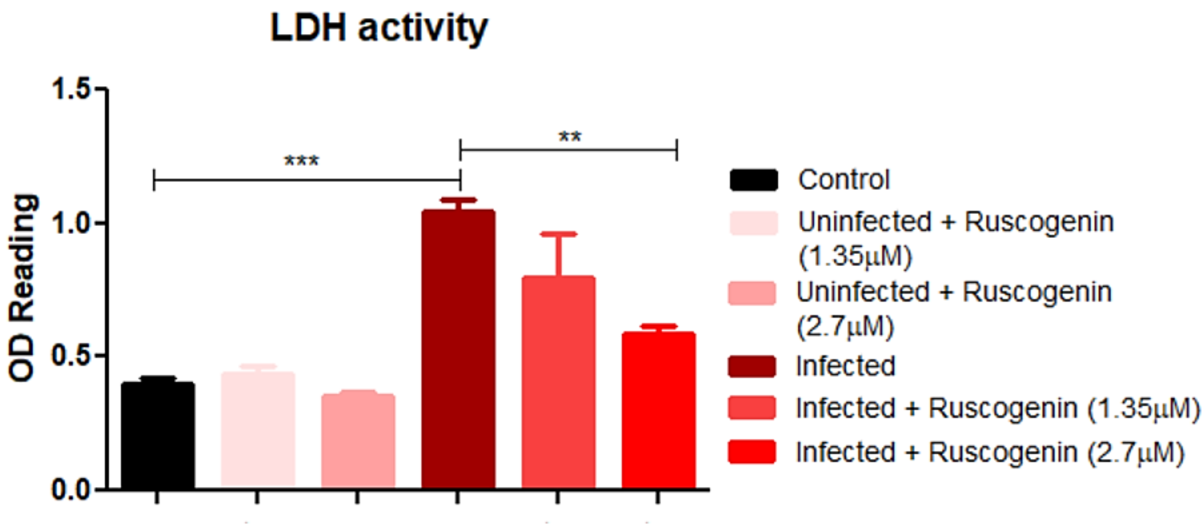


Figure 22: LDH release in macrophages treated with Ruscogenin after infection with Mtb. Cells were infected with the laboratory strain of Mtb and then treated with Ruscogenin**.** After 24hr, the supernatants were collected and the LDH assay was performed. Data represents 1 experiment. *p<0.5, **p<0.01, ***p<0.001 using the Student's *t*-test.

To investigate if pre-treating macrophages with Ruscogenin prevented cell death, macrophages were first treated with Ruscogenin and then infected with live Mtb. Cells were incubated for 24 hours before supernatant was collected in preparation for conducting the LDH assay. The results showed that pre-treatment with Ruscogenin had no effect on reducing cell death in infected macrophages compared to infected and untreated cells (Figure 23). Interestingly, it was observed that treatment with 2.7µM Ruscogenin significantly reduced cell death in uninfected macrophages compared to untreated and uninfected control cells (Figure 23). Taken together with the ELISA data in Figure 18, the data do not correlate at this stage, and it is necessary to conduct more repeats to see if a parallel can be drawn between these two data sets.

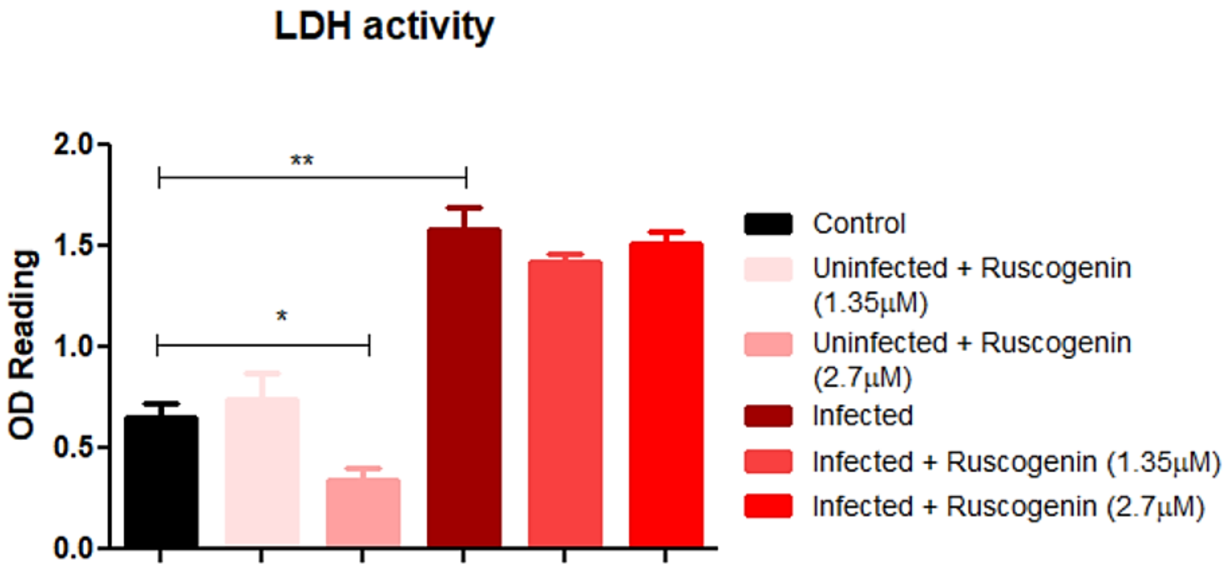


Figure 23: LDH activity in macrophages pre-treated with Ruscogenin and infected with Mtb. Macrophages were infected with the clinical strain of Mtb after they had been exposed to Ruscogenin treatment for 24hrs. Following a 24hr incubation, supernatants were collected and stored until we carried out the LDH assay. Data represents one experiment. *$p<0.05$, **$p<0.01$ using the Student's *t*-test.

3.5.3 LDH activity in cells either pre-treated or treated with Leonurine after infection with H37Rv Mtb

After determining the effects of Leonurine treatment on the pro-inflammatory profile of Mtb-infected macrophages, this study sought to determine if this compound could rescue or prevent cell death in infected macrophages. The results reflect that treatment with Leonurine had no effect on cell death and the only significant observation is that infection with Mtb markedly increased cell death compared to the uninfected and untreated control group (Figure 24). Moreover, these results coincide with what is observed in the ELISA data (Figure 16). It is still too early for to make any conclusions on the effect of Leonurine on cell death.

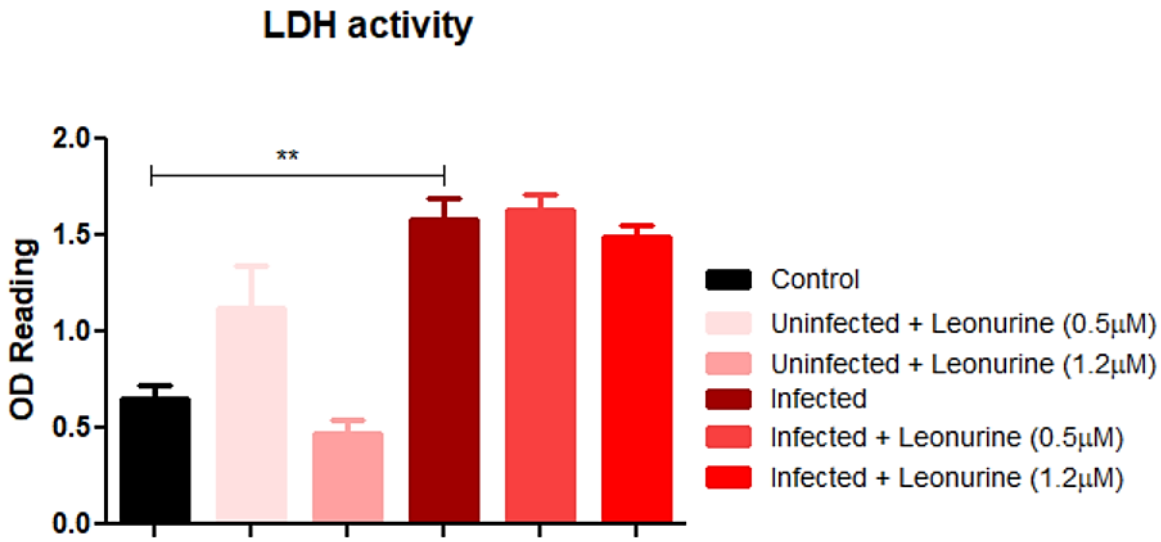


Figure 24: LDH activity in macrophages treated with Leonurine after infection with Mtb.
We infected cells with Mtb and then carried out Leonurine treatment and allowed the cells to incubate for 24hr before collecting supernatants. The LDH assay was carried out according to the manufacturer's instructions. Data represents one experiment. *p<0.05, **p<0.01 using the Students *t*-test.

At the same time, this study investigated if pre-treatment with Leonurine could improve the fate of macrophages after Mtb infection. Following infection, supernatants were collected and the LDH assay was performed at a later stage. The results showed that both concentrations of Leonurine resulted in significant decrease in cell death in infected macrophages compared to the infected and untreated cells (Figure 25). What's more interesting is that even the C1 concentration of Leonurine also significantly reduced cell death in the uninfected group. Thus far, these results reflect what is observed in the ELISA data (Figure 19). Because the LDH experiments were only done once, more repeats need to be done before any valid conclusions can be made.

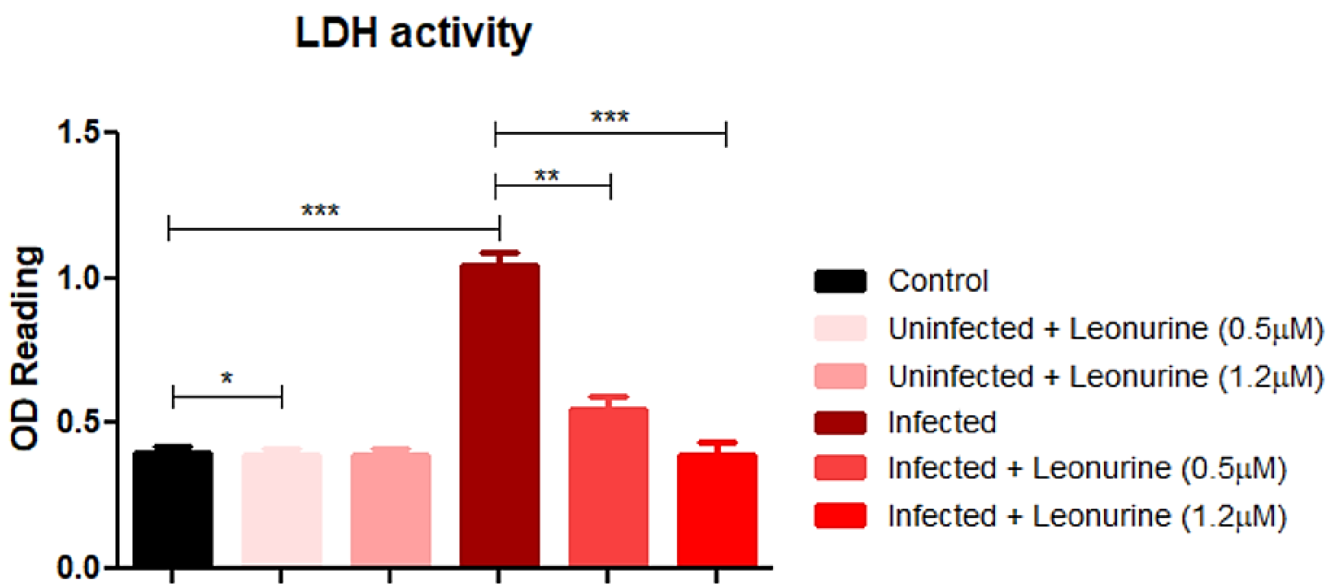


Figure 25: LDH activity in macrophages pre-treated with Leonurine after infection with Mtb.
Prior to infecting the cells, they were subjected to Leonurine treatment for 24hr. After a 24hr incubation period following infection, supernatant was collected. The LDH assay was carried out according to the manufacturer's instructions. Data represents 1 experiment. *$p<0.05$, **$p<0.01$, ***$p<0.001$ using the Student's t-test.

CHAPTER 4

DISCUSSION

Despite TB being a preventable and curable disease, it has remained the main cause of death by a single infectious organism until the coronavirus pandemic (World Health Organisation, 2021). The COVID-19 pandemic has resulted in major setbacks for achieving the global TB targets outlined by the WHO, an increase in TB deaths has been documented in addition to declines in the number of people provided with adequate preventative and treatment for MDR-TB (World Health Organisation, 2021). It is evident that the coronavirus pandemic has added pressure for countries to try and meet TB eradication targets and has created a greater sense of urgency for the exploration of new treatment regimens. At present, the treatment for drug susceptible tuberculosis consists of isoniazid, pyrazinamide, rifampicin and ethambutol for the first 2 months of treatment and the remaining 4 months comprises of rifampicin and isoniazid (Sotgiu *et al.*, 2015). The main challenge in the fight against TB has been the development and persistence of drug resistant strains of mycobacteria. The treatment of MDR-TB comes with its own complexities that make eradicating TB that much more complicated. Some of those challenges include the toxic side effects of the prescribed drugs, long duration and cost of treatment (Caminero, 2006; Sotgiu *et al.*, 2015). Once again, the need for novel approaches to treatment is echoed.

One way to circumvent the rise of antimicrobial resistance is to intervene by targeting the host (Guler *et al.*, 2021). The fate of the pathogen is largely dependent on the hosts immune system and Mtb is known to manipulate host defense mechanisms in order to survive within the host (Young, Walzl and Du Plessis, 2019). Therefore, re-directing the therapeutic approach to target pathways involved in immunity against Mtb can prove to be more promising (Young, Walzl and Du Plessis, 2019). Not only are plants an abundant source of biologically active compounds, they are inexpensive to produce commercially and have less reported side effects (Siddiqui, 2021). Thus, in this present study, the therapeutic potential of plant-derived compounds as HDT for lowering inflammation in Mtb-infected macrophages was investigated.

4.1 Differentiation of monocytes into macrophages

Differentiation of monocytes to macrophages using 100nM PMA was achieved over a period of 72 hours. A study looking at various methods to differentiate monocytic cell lines found that PMA stimulation coupled with a day of rest, produced macrophages that closely resembled human monocyte-derived macrophages (MDMs) as opposed to monocytes that have been differentiated with vitamin D_3 (VD_3) with no rest period (Daigneault *et al.*, 2010). Successful differentiation of monocytes was confirmed by the change in morphology and adherence to the plate. The macrophages are visibly more granular and larger in size (Panawala, 2017). A key feature in differentiation is also the nucleocytoplasmic ratio and in macrophages that ratio is decreased as is seen by the increase in cytoplasmic volume, which is an indicator of cell maturity (Daigneault *et al.*, 2010).

4.2 Determining the optimal concentration of compound to use on the cells

Another important part of this study was to determine an optimal concentration of the compounds to use to treat the cells and we used the MTT assay. Metabolically active living cells convert tetrazolium salts into a formazan by-product, which is soluble and absorbance can be read using an ELISA plate reader (Präbst *et al.*, 2017). The IC_{50}, which is half of the maximal inhibitory concentration, was determined for each compound. These plant-derived compounds possess inhibitory/suppressive properties, the IC_{50} value would represent the concentration required to inhibit certain biological or biochemical functions by half (Caldwell *et al.*, 2012). The secretion of pro-inflammatory cytokines early on in infection is critical for containment of bacterial growth and for the formation of a granuloma. However, it is known that excessive production of pro-inflammatory cytokines can have adverse immunopathological effects (Sasindran and Torrelles, 2011; Torrado, Robinson and Cooper, 2011). For this reason, the IC_{50} concentration is an ideal option as the aim is not to obliterate the inflammatory response, but rather to dampen it.

4.2.1 Withaferin IC_{50}

Adverse side effects and drug toxicity are two very important factors in determining whether a compound/chemical can be considered as a probable therapeutic. A study using female Wistar rats noted that WA had no toxic effects after 14 days of daily administration at 2000mg/kg (Patel, Rao and Hingorani, 2016). A different study reported that Withaferin A inhibits the translocation of Nf-κβ in THP-1 macrophages at concentrations of 5µM, 10µM and 20µM (Dubey *et al.*, 2018). This study reported an average IC_{50} of 6.8µM after conducting two MTT experiments. Although the previously mentioned studies have not used an IC_{50} concentration, it has been reported that ethanol leaf extracts of *Withania somnifera* displayed cytotoxic effects against breast cancer cells (MCF-7) and human liver carcinoma cells (HEPG-2) at IC_{50} concentrations of 9.4µg/mL and 8.5µg/mL (Sharifi-Rad *et al.*, 2021).

4.2.2 Ruscogenin IC_{50}

The average IC_{50} for Ruscogenin was determined to be 2.7µM in THP-1 macrophages but a different study found that Ruscogenin had an IC_{50} of 7µM in two different human pancreatic cancer cell lines (BxPC-3 and SW1990 cells) (Song *et al.*, 2020). That same study demonstrated that Ruscogenin has anticancer activity by inducing ferroptosis, a form of cell death that involves the accumulation of iron and lipid peroxidation (Song *et al.*, 2020). Interestingly, a study exploring the effects of Ruscogenin and its succinylated derivative (RUS-2HS) found that Ruscogenin exerted its effects at different IC_{50} values, depending on what was being studied Ruscogenin reduced TNF-α induced adhesion of HL-60 cells and ECV304 cells at IC_{50} of 6.90nM and an IC_{50} of 3.06nM inhibited the TNF-α dependent upregulation of ICAM-1 mRNA in ECV304 cells (Huang, Kou, Liu, *et al.*, 2008). Despite other studies not making use of the THP-1 cell line in the context of anti-inflammation and TB, we encountered challenges with determining the IC_{50} for Ruscogenin.

4.2.3 Leonurine IC_{50}

Similar to Ruscogenin, the IC_{50} for Leonurine was not easily determined. This experiment was repeated four times and only two out of the four yielded reproducible data. The average IC_{50} concentration for Leonurine was determined to be 1.2µM. A study looking at the anticancer potential of the herb as a whole, found that the herbal extract displayed anticancer properties in several cancer cell lines at IC_{50} concentrations of 25mg/mL and 50mg/mL, respectively when cells were exposed for 48 hours (Chinwala *et al.*, 2003). Currently, there is a lack of literature on this compound and its anti-inflammatory effects on TB, especially in THP-1 macrophages.

Macrophage death, especially during granuloma break-down as a result of necrotizing cells within the granuloma remains a key issue in TB pathology (Ramakrishnan, 2012). Mycobacterial virulence factors are responsible for the death of immune cells at the site of infection, but host factors can also contribute to immune cell death; thus, it was important to explore the potential of using a concentration of plant-derived compound where cell viability was 100% on the IC50 curve. If any of the plant-derived compounds caused significant cell death, it would not be beneficial to explore them as a HDTs as macrophages are key mediators of the immune response to infection (Flynn, Chan and Lin, 2011).

4.3 Morphology of cells when exposed to each compound before or after Mtb infection

Only images of one pre-treatment infection experiment were taken so that the effect of the plant-derived compounds on the cells could be seen before infecting them with live Mtb. After infecting macrophages with Mtb, a 4 hour incubation period was allowed so that the macrophages could phagocytose and thus internalize the bacilli. Thereafter, any extracellular bacilli were washed off. The infected macrophages are clustered together, and some extracellular bacilli were observed, internalized bacilli were hard to see in a picture but could be seen microscopically. Withaferin A pre-treatment (C1 and IC_{50}) resulted in macrophages exhibiting uncharacteristic morphology in addition to some cells having erupted membranes, which could be an indication of necrotic cell death (Zhang *et al.*, 2018). Cells treated with the C1 concentration of Withaferin A had areas where cells were clustered together, and some cells had ruptured membranes with noticeable debris which is expected as treatment plus infection causes the cells to undergo stress. Treatment with the IC_{50} of Withaferin A did not lead to the same morphological observations for the C1 concentration and a huge contrast can be seen when compared to the uninfected and Withaferin A group. Importantly, cells treated with Withaferin A alone at either concentration (C1 or IC50) did not display significant upregulation of cytokine secretion compared to the uninfected and untreated control cells, suggesting that the compound alone did not induce cytokine secretion (Figure 14A, B and C).

Treatment with Ruscogenin (C1) led to cells looking very damaged and cells do not resemble macrophages at both treatment concentrations, they are small and their membranes are not marked or intact in many cells. Based on the morphology of Ruscogenin treatment in the uninfected groups, this compound seems to have adverse effects on the cells. The presence of some mycobacteria were seen both the C1 and IC_{50} treatments in the infected group, despite washing the cells twice to remove any extracellular bacilli. The infected and treated cells looked visibly damaged and there was clumping

The effect of Leonurine on cell morphology was interesting. The C1 treatment resulted in cells looking dead and in some macrophages, cellular content was spilt into the extracellular environment. After infection, the cells looked sparse and bacilli can be seen in the extracellular milieu. While the IC_{50} treatment resulted in cells retaining macrophage morphology with some cells having erupted membranes. However, when cells were infected they looked dead with lots of clumping occurring and noticeable debris. Apart from what has been mentioned in the results section, it is difficult to infer or predict what these compounds are doing to the cells based on morphology only. Generally, treatment with the compounds did not preserve macrophage morphology before infection or after infection. The cells appeared to have suffered a worse fate for both the C1 and IC_{50} concentrations. Therefore, in conclusion by only taking these microscopy images into account, it can be seen that treatment with these compounds may affect macrophages in a detrimental manner as macrophage morphology is not preserved.

4.4 The effect of the Ruscogenin and Leonurine on cytokine expression

4.4.1 TNF

TNF is involved in the formation and maintenance of the granuloma which is essential for the restriction of mycobacterial growth (Clay, Volkman and Ramakrishnan, 2008; Mayer-Barber and Sher, 2015). This study reports that Ruscogenin and Leonurine do not have any effect on TNF secretion after macrophages were infected with Mtb at an MOI of 5. Next, was to investigate if these compounds conferred any protective effects and found that once again Ruscogenin had no effect on TNF secretion when cells were subjected to pre-treatment. The results found in this study contradict the anti-inflammatory effects of Ruscogenin pre-incubation as reported by Hung-Jen Lu and colleagues in 2014. They showed that pre-incubation of HEPG2 cells with Ruscogenin alleviated the palmitic acid induced inflammation by decreasing the levels of TNF, IL-1β and IL-6 (Lu, Tzeng, Liou, Chang, *et al.*, 2014). Leonurine lowered the levels of TNF secretion when cells were pre-treated with the IC_{50} concentration

(1.2µM) before infection with Mtb and no effect was observed on TNF secretion for the C1 concentration. According to a study conducted by Wu et al., in 2018 using Bovine endometrial epithelial cells (bEEC) that were pre-treated with varying concentrations of Leonurine and after stimulation with LPS, had results that showed cells pre-treated with Leonurine had significantly decreased levels of TNF-α mRNA and TNF-α protein levels measured by ELISA (Wu *et al.*, 2018). The results obtained in this study showed that it may be beneficial to further explore the anti-inflammatory properties of Leonurine when macrophages are pre-treated with the compound as opposed to administering Leonurine after infection.

4.4.2 IL-1β

The protective role of IL-1β in mycobacterial infection as demonstrated by Yamada and colleagues, showed that IL-1 knock out (KO) mice developed significantly larger granulomas when compared to wild type (WT) mice in addition to the lungs of IL-1β KO mice having a greater bacterial burden, which showed that IL-1β may be essential to curb bacterial growth and replication (Yamada *et al.*, 2000). In this study, it was demonstrated that treating macrophages with Ruscogenin (C1 and IC_{50} concentrations), both before and after Mtb infection had no effect on IL-1β production. These results diverge from what has been observed in mice that suffered induced middle cerebral artery occlusion/reperfusion (MCAO/R), where pre-treatment with Ruscogenin significantly decreased pro-IL-1β and IL-1β as compared to the sham control (Cao *et al.*, 2016). The same study also observed that Ruscogenin pre-treatment mitigated histopathological damage induced by MCAO/R (Cao *et al.*, 2016). However, this study notes that pre-treatment with Leonurine before infection, using the C1 concentration, lowered the amount of IL-1β secreted by infected macrophages. This study reports no effect on IL-1β secretion when cells were treated with Leonurine after Mtb infection. In contrast to these findings, a previous study demonstrated that Leonurine ameliorated IL-1β induced inflammation in human osteoarthritic cartilage chondrocytes by inhibiting the PI3K/Akt/NF-κβ pathway (Hu *et al.*, 2019).

4.4.3 IL-6

IL-6 is produced by monocytes early on during TB and is known to be both anti-inflammatory and pro-inflammatory. However, in TB, its effects have mainly been reported to be adverse (Zhang, Broser and Rom, 1994; Tsao *et al.*, 1999). This study demonstrates that treating macrophages with Ruscogenin and Leonurine after Mtb infection had no effect on the secretion of IL-6 by Mtb-infected cells. Nonetheless, this study also demonstrated that pre-treating the cells with these two compounds may be beneficial in reducing IL-6 production. Ruscogenin and Leonurine treatment with the C1 concentration significantly reduced IL-6 secretion in infected macrophages, while treatment with the IC_{50} concentration of Leonurine may have also conferred a protective role by significantly reducing IL-6 secretion. The results in the pre-treatment experiments echo what has been previously reported by Lu et al. 2014, where they showed that Ruscogenin treatment in diabetic mice resulted in a decrease in renal secretions of IL-6 along with other cytokines (Lu, Tzeng, Liou, Da Lin, *et al.*, 2014). A recent report has shown that Ruscogenin improves particulate matter induced lung pathology in male ICR mice and decreased IL-6 levels detected in broncho-alveolar lavage fluid (Wang *et al.*, 2021).

4.5 The effect of Withaferin A on cytokine expression

The anti-inflammatory effects of Withaferin A that were observed in this study are worth noting separately. Withaferin A is known to have anti-inflammatory properties and in human islets that were exposed to a combination of various cytokines, treatment with Withaferin A resulted in a decrease in IL-6 levels (Sorelle *et al.*, 2013). Indeed, this study also noted that treating macrophages with Withaferin A (C1 and IC_{50} concentration) after infection resulted in a decrease in IL-6 secretion. Similarly, pre-treating the cells with Withaferin A at IC_{50} concentration before infection with Mtb decreased the amount of IL-6 produced, in addition to suppressing IL-6 in the uninfected cells at both concentrations. It was observed that WA treatment at 6.8μM after Mtb infection decreased IL-1β secretion in macrophages; however, pre-treatment of macrophages with Withaferin A had no effects on IL-1β secretion. Therefore, we can conclude that Withaferin A reduced the secretion of IL-1β and IL-6 in infected macrophages. These results suggest that treating cells after infection with Withaferin A could have the potential to decrease the levels of IL-1β and IL-6 and may even have some inhibitory role in the IL-6 pathway. In THP-1 cells that were primed with adenosine triphosphate (ATP) to activate the NLRP3 inflammasome, an upregulation of IL-1β was seen but in cells that were treated with Withaferin A and ATP, a significant downregulation of IL-1β was observed (Dubey *et al.*, 2018). Other studies have shown that treating bone marrow derived macrophages (BMDMs) infected with *Fusobacterium nucleatum* or *Aggregatibacter actinomycetemcomitnas* reduced IL-6 and TNF production (Noh *et al.*, 2016). Likewise, this study also reports that Withaferin A pre-treatment (both concentrations) of macrophages greatly reduced the secretion of TNF induced by Mtb infection. These results demonstrated that Withaferin A exerts anti-inflammatory effects in Mtb-infected macrophages.

4.6 The effect of the Withaferin A, Ruscogenin and Leonurine when macrophages were infected with a higher dose of Mtb

Next, this study sought to investigate the anti-inflammatory effects of these compounds when macrophages were infected with a higher dose of Mtb using an MOI of 10. Due to the COVID-19 pandemic, which resulted in extremely limited access to the laboratory, these experiments were only performed once. Macrophages were pre-treated with the same C1 and IC_{50} concentrations of the compounds for 24 hours and infected with live Mtb (MOI = 10). Pre-treatment with Withaferin A drastically inhibited the secretion of IL-1β, TNF and IL-6 (Appendix, Figure 26A, B and C). However, pre-treatment with Ruscogenin and Leonurine did not exert any anti-inflammatory effects (Appendix, Figure 27 and 28), contradicting some of the results obtained when we infected the cells with an MOI of 5.

The fate of the granuloma can determine how detrimental Mtb infection would be to the host; caseating granulomas are able to slow down mycobacterial growth but also cause tissue pathology (Fayyazi *et al.*, 2000). Lerner and colleagues demonstrated that after Mtb infection, a large population of macrophages became necrotic. Mtb uses these cells in order to replicate and to facilitate dissemination (Lerner *et al.*, 2017). However, tissue sections from patients that have suffered from TB were studied and revealed that caseous granulomas contain a higher number of macrophages undergoing apoptosis when compared to solid granulomas (Fayyazi *et al.*, 2000). Based on this background information, this study investigated whether Withaferin A, Ruscogenin and Leonurine could protect macrophages from cell death as a result of inflammation induced by Mtb infection.

Withaferin A treatment (6.8µM) after Mtb infection (MOI = 5) rescued macrophages from death and reflected what we observed in the corresponding ELISA data. Despite that, pre-treatment with Withaferin A had no effect on macrophage death and was not in-line with what was observed in the ELISA data. Interestingly, Withaferin A has been reported to induce apoptotic cell death in human cutaneous melanoma cell lines in a Bcl-2-dependent manner (Mayola *et al.*, 2011). Importantly, no conclusive remarks on the effect of Withaferin A on cell death in our study can be made as these experiments were only performed once due to time constraints. More experiments would need to be done so that it would be possible to determine if a parallel can be drawn between what was observed in the ELISA and cell death data.

The effect of Ruscogenin treatment on cell death after Mtb infection indicated that the IC_{50} concentration (2.7µM) rescued macrophages. Intriguingly, the pre-treatment experiment also showed that the IC_{50} concentration prevented cell death in the uninfected macrophages. Ruscogenin has been implicated in inducing ferroptosis in pancreatic cancer cell lines (Song *et al.*, 2020).

Lastly, Leonurine treatment did not prevent macrophage death when treatment was administered after Mtb infection. However, pre-treatment with Leonurine resulted in decreased cell death for both the C1 and IC_{50} concentrations at the same time, the C1 concentration improved cell death in the uninfected group. Although in this study it appears that Leonurine may inhibit or prevent cell death but a previous study demonstrated that Leonurine hydrochloride induced apoptotic cell death in H292 cells (Mao *et al.*, 2015). Once again, it is emphasized that these experiments need to be repeated so that clear confirmation can be made for what is currently being observed in the LDH data for all of the compounds.

Based on the observed effects of these compounds on the pro-inflammatory profile of infected macrophages, it would be beneficial to study the effects of these compounds on the intracellular growth of Mtb. Withaferin A has been reported to have anti-mycobacterial effects as demonstrated by Adaikkappan and colleagues who conducted minimum inhibitory concentration (MIC) experiments (Adaikkappan, Kannapiran and Anthonisamy, 2012). They showed that the aqueous extract of *Withania Somnifera* (0.01 - 1.0mg/mL) significantly inhibited mycobacterial growth in the culture media. Therefore, based on their findings, we postulate that Withaferin A could inhibit mycobacterial growth *in vitro*.

Before even considering the use of these compounds in a clinical setting, important preclinical studies need to be done on these compounds. These findings point to Withaferin A as a therapeutic candidate that needs further exploration in animal models. A study looking at sub-acute and acute toxicity of Withaferin A using Wistar rats reported that standardized

Withania somnifera extract (WSE) had no adverse effects on several clinical pathology parameters (Patel, Rao and Hingorani, 2016).

CONCLUSION

We hypothesized that Withaferin A, Ruscogenin and Leonurine would exhibit anti-inflammatory effects in Mtb-infected macrophages. These plant-derived compounds have not been used in the context of this study. However, many studies have reported on their anti-inflammatory effects in other diseases and conditions. Thus, in this project, these compounds were screened for anti-inflammatory effects in Mtb strain H37Rv infected macrophages.

Withaferin A showed the most potential for use as a HDT by limiting excessive inflammation driven by Mtb infection by decreasing the secretion of IL-1β and IL-6. Withaferin A showed a possible protective role when pre-treating macrophages resulted in a drastic decrease in TNF and IL-6 secretion after Mtb infection. Ruscogenin treatment, both before and after Mtb infection, did not yield any anti-inflammatory effects. Pre-treating macrophages with Leonurine reduced the secretion of IL-1β, TNF and IL-6 secretion after infection, suggesting that it has the potential for use as an HDT in TB treatment.

The main basis of reasoning of this study was to obtain a dynamic balance of pro-inflammatory cytokine secretion so that the harmful effects of excessive cytokine production can limit lung pathology in TB and to offer immunocompromised hosts a better fate in dealing with TB disease.

FUTURE WORK

- To repeat infections where an MOI of 10 is used and to treat macrophages with Withaferin A (C1 and IC_{50} concentrations) either before or after Mtb infection.
- The LDH assay experiments need to be repeated for all compounds so that a conclusion can be made on the effects of Withaferin A, Ruscogenin and Leonurine on cell death in macrophages.
- Future work also involves more infection experiments where supernatant collection for cytokine analysis happens at various time-points and not just at 24 hours.
- To determine if these compounds, particularly Withaferin A have any anti-mycobacterial properties, the bacterial burden needs to be determined by counting the colony forming units after subjecting macrophages to the same treatment conditions as the ELISA experiments.
- Furthermore, flow cytometry experiments can also be performed so that a better understanding of the effect of these compounds on cell death after Mtb infection can be determined and in addition to accurately describe the type of cell death induced by the infection models in this study.
- It would be worthwhile to study how these compounds exert their effects by using mass spectrometry to do a proteomic analysis of proteins that are either up/downregulated after treatment with the compounds.
- Thereafter, the study could seek to explore the effects of Withaferin A in an *in vivo* model of infection.

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APPENDIX

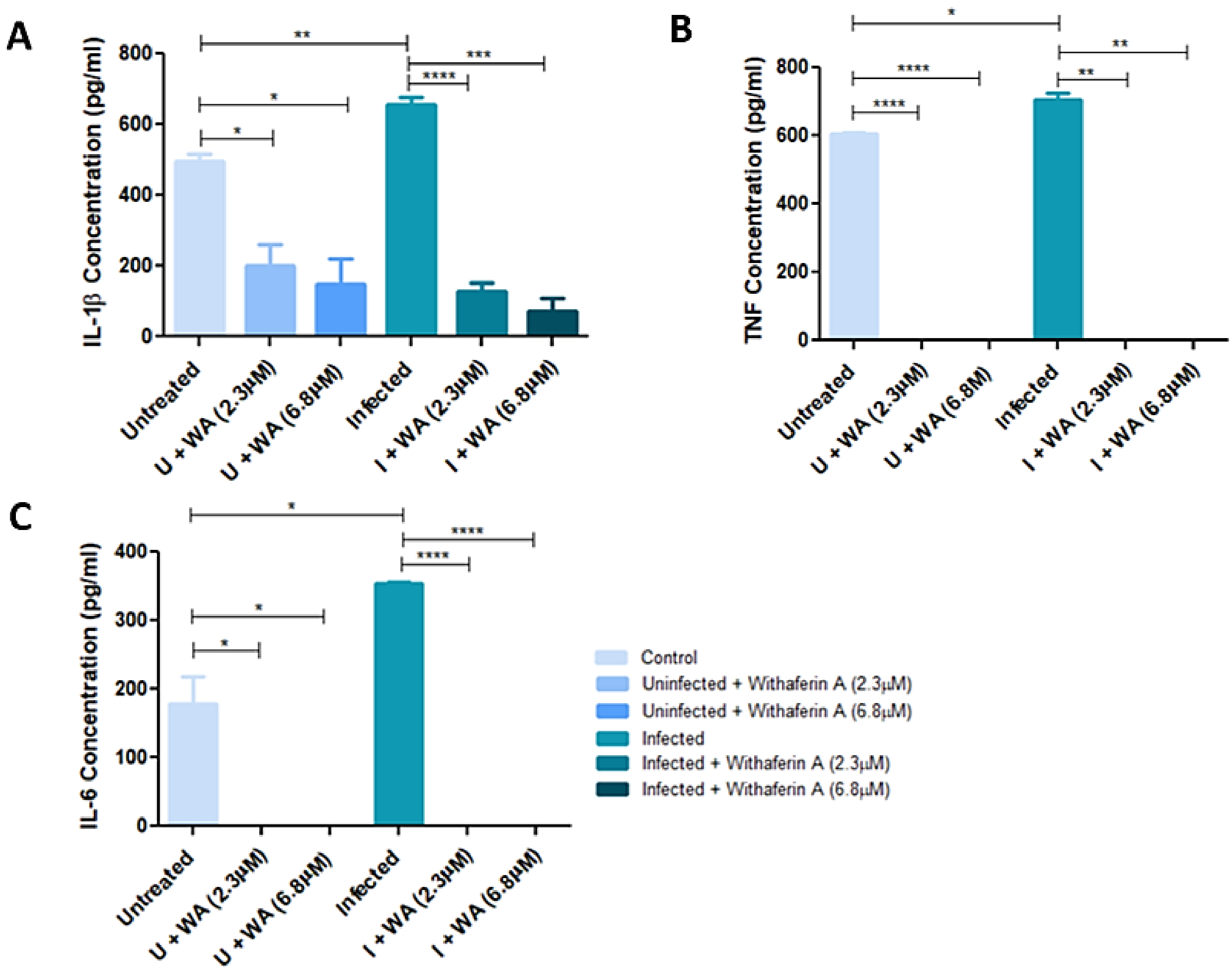

Figure 26: Cytokine profile after macrophages were pre-treated with Withaferin A and then infected with Mtb.

Cells were pre-treated with both the C1 and IC_{50} concentrations of Withaferin A for 24hr. Thereafter, infection with H37Rv Mtb was carried out, using an MO I= 10. Extracellular bacilli were washed off after 4hr into the infection and the cells were left to incubate overnight. Supernatants was collected and stored at -80° until the ELISAs were ran. Data represents one experiment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 using Students t-test.

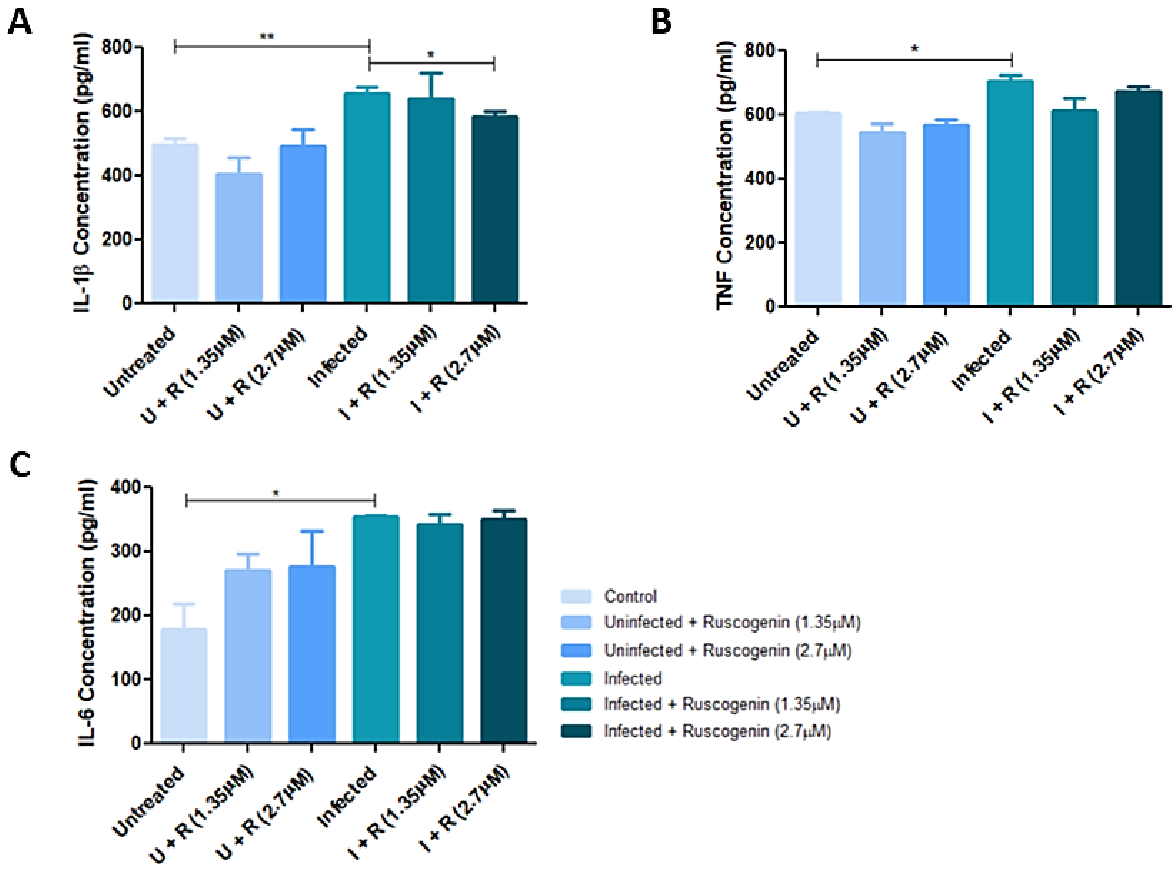

Figure 27: Cytokine profile of macrophages pre-treated with Ruscogenin and then infected with Mtb.

Cells were subjected to treatment with Ruscogenin 24hr before we carried out the Mtb infection (MOI = 10). After infection, the cells were left for 24hr. Supernatant was collected to perform the ELISAs for IL1β. TNF and IL-6. Data represents one experiment. *p<0.05, **p<0.01 using the Student's t-test

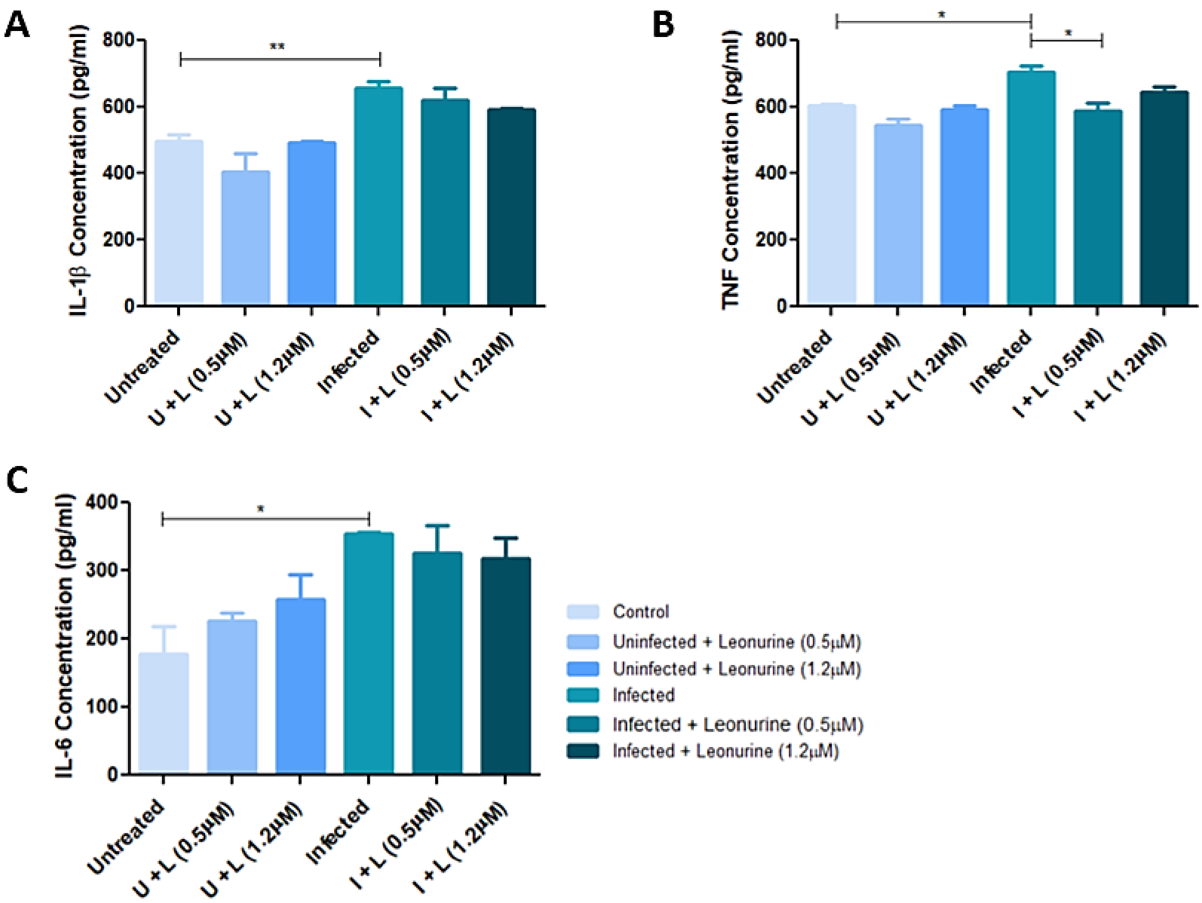

Figure 28: Cytokine profile of THP-1 macrophages treated with Leonurine 24hrs prior to Mtb infection.

Treatment with Leonurine was done 24hrs before macrophages were infected with H37Rv Mtb using an MOI:5 and left in the incubator 24hr before supernatants were collected to run ELISAs. Data represents two independent experiments. **p*<0.05, ***p*<0.01 using the Student's *t*-test.