Addis Ababa University

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In Vitro Evaluation of the Anti-mycobacterial Activity of Selected Medicinal Plants in Ethiopia on *Mycobacterium tuberculosis* and *Mycobacterium bovis* Strains

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This is to certify that the thesis prepared by Liya W/tensay, entitled: *In Vitro* Evaluation of the Anti-mycobacterial Activity of Selected Medicinal Plants in Ethiopia on *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains and submitted in partial fulfillment of the requirements for the Master of Science Degree in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Background: Tuberculosis (TB) is a major global health problem causing ill-health in millions of the world's population each year. The emergence of drug resistant strains is a major challenge to the TB control program. Hence, there is an urgent need for the development of new drugs and in this regard, herbs could be potential sources of anti-TB drugs due to their special attribute as a large source of therapeutic phytochemicals that may lead to novel drugs development.

Objective: The objective of this study was to investigate the *in vitro* anti-mycobacterial activity of the 80% methanol extracts of *Euphorbia candelabrum* (*E. candelabrum*) latex, *Rumex abyssinicus* (*R. abyssinicus*) root, *Otostegia integrifolia* (*O. integrifolia*) leaf and *Erythrina brucei* (*E. brucei*) stem bark, the chloroform extract of *Vernonia amygdalina* (*V. amygdalina*) leaf and the oil of *O. integrifolia* leaf on *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains. Additionally, to test the anti-oxidant activity of *E. candelabrum* extract and to carry out a preliminary phytochemical screening for the plant extracts.

Methods: The 80% methanol extracts were obtained through cold maceration technique, the chloroform extract of *V. amygdalina* was obtained through successive soxhlet extraction and the oil was obtained by employing hydrodistillation extraction technique. Macro-dilution technique was used for determining the minimum inhibitory concentration (MIC). The free radical scavenging ability of *E. candelabrum* was tested by using 2, 2-diphenyl-1-picrylhydrazyl assay. Statistical Package for Social Sciences software was used for data analysis. One way analysis of variance followed by Tukey's post hoc test was employed to test significance.

Results: The MIC of the chloroform extract of *V. amygdalina* leaf was 10mg/ml against the mycobacterial strains. The 80% methanol extracts of *E. brucei*, *O. integrifolia* and *R. abyssinicus* were effective against only *M. bovis* with MIC ranging from 12.5 - 25mg/ml. The oil from *O. integrifolia* leaf was inactive. Although *E. candelabrum* did not show anti-mycobacterial activity, it showed an anti-oxidant activity with a half maximal inhibitory concentration (IC_{50}) of 281µg/ml.

Conclusion: There may be a possibility to develop new compounds from *V. amygdalina* against mycobacterial strains. *E. brucei*, *O. integrifolia* and *R. abyssinicus* showed mycobacterial growth inhibition against *M. bovis* strain. Hence, these plants should be further evaluated for the

development of anti-tuberculosis drugs. *E. candelabrum* may aid in TB treatment through other host directed therapeutic mechanisms rather than direct mycobacterial growth inhibition.

Keywords: Tuberculosis, Mycobacteria, *Vernonia amygdalina, Euphorbia candelabrum, Otostegia integrifolia, Rumex abyssinicus, Erythrina brucei,* anti-mycobacterial activity, resazurin, anti-oxidant activity.

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List of abbreviations

ALIPB – Aklilu Lemma Institute of Pathobiology

AM – Alveolar macrophage

ANOVA – Analysis of variance

BCG - Bacillus of Calmette and Guerin

DMSO – Dimethyl sulfoxide

DPPH - 2, 2-diphenyl-1- picrylhydrazyl

DR-TB – Drug resistant tuberculosis

EPTB – Extra-pulmonary tuberculosis

HDT – Host directed therapy

HIV – Human immunodeficiency virus

IC_{50} – Half maximal inhibitory concentration

LAM - Lipoarabinomannan

LJM – Lowenstein Jensen medium

LTBI - Latent tuberculosis infection

MDR TB - Multidrug-resistant tuberculosis

MIC – Minimum inhibitory concentration

MTC- Mycobacterium tuberculosis complex

OADC - Oleic acid-albumin-dextrose-catalase

ROS – Reactive oxygen species

RR-TB – Rifampicin resistant tuberculosis

TB – Tuberculosis

TDR TB – Totally drug resistant tuberculosis

Th cells – T helper cells

WHO – World Health Organization

XDR TB - Extensively drug-resistant tuberculosis

1. Introduction

1.1 Overview of tuberculosis

1.1.1 Definition and etiology

Tuberculosis (TB) is a disease which is caused by members of the *Mycobacterium tuberculosis complex* (MTC), which includes the tubercle bacillus *Mycobacterium tuberculosis* (*M. tuberculosis), Mycobacterium bovis (M. bovis), Mycobacterium africanum, Mycobacterium microti, Mycobacterium canetti, Mycobacterium caprae and Mycobacterium pinnipedi* (Wani, 2013). Human TB may rarely be caused by the less virulent atypical Mycobacteria (non-tuberculous Mycobacteria) which are resistant to the usual anti-tubercular drugs (Mohan, 2005). The non-tuberculous species of mycobacterium are found in a large diversity of water environments. Some of the atypical mycobacteria are *Mycobacterium avium, Mycobacterium chelonae, Mycobacterium kansasii, Mycobacterium intracellulare, Mycobacterium fortuitum, Mycobacterium marinum, Mycobacterium smegmatis and Mycobacterium abscessus* (Loret and Dumoutier, 2019). *M. tuberculosis* is the most important pathogen of human beings while *M. bovis* usually infects animals and rarely infects humans. They are characterized by being non-motile, rod shaped, acid fast, strict aerobic, non-spore forming bacteria with a waxy coat (Eleanor *et al*., 2016; Knechel, 2009). The picture of *M. tuberculosis* stained using acid-fast Ziehl-Neelsen stain is shown in Figure 1.

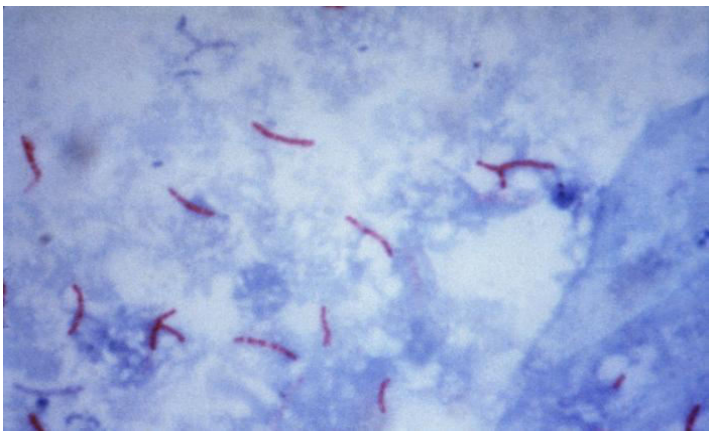

Figure 1 –The picture of *M. tuberculosis* stained using acid-fast Ziehl-Neelsen stain; magnified 1000 x (Singh, 2016).

Mycobacterial cell wall consists of an inner and an outer layer that surround the plasma membrane. The inner compartment is composed of peptidoglycan, arabinogalactan and mycolic acids which are covalently linked together forming an insoluble mycolic acid-arabinogalactan-peptidoglycan complex (essential core of the cell wall) (Hett and Rubin, 2008). One of the fundamental aspects of mycobacteria that confers resistance to antimicrobial agents and promotes its virulence is the thick layer of mycolic acids (Elad *et al*., 2018). The mycolic acid structure also confers the ability to resist destaining by acid alcohol after being stained by certain aniline dyes, leading to the term acid fast bacillus (Wani, 2013).

The outer compartment is composed of free lipids, some with longer fatty acids complementing the shorter α-chains, and some with shorter fatty acids complementing the longer chains. Interspersed are the cell-wall proteins, the phosphatidylinositol mannosides, the phthiocerol containing lipids, lipomannan, and lipoarabinomannan (LAM) (Brennan, 2003). The outer proteins and lipids are soluble components and are referred as the signaling and effector molecules of mycobacteria because of their roles in interacting with the immune system (Hett and Rubin, 2008). The composition and quantity of the cell wall components not only affects the bacteria's virulence but also the growth rate which is extremely slow (doubling time of ~18 h in culture) (Knechel, 2009; Chinta *et al*., 2016).

1.1.2 History of tuberculosis

There is a suggestion in history that TB appeared about 70,000 years ago and that it remained sporadic up to the 18th century. It's believed that it became epidemic during the industrial revolution, owing to the increased population density and the unfavorable living conditions (Banuls *et al*., 2015). Regarding the ancientness of TB, it is stated elsewhere that it has affected mankind for more than 4,000 years (Zaman, 2010). Since hard tissues like bone can be preserved for thousands of years, it has allowed to identify individuals with bone TB who died before 4,000 years ago (Smith, 2003).

TB has also been recorded in history since the Greco-Roman and Egyptian civilizations, with evidence of spinal TB being recorded as long ago as 3,400 B.C (Kahaliw, 2016). Assyrian clay tablets (7th century B.C.) describe patients coughing blood and Hippocrates (5th century B.C.) also wrote of patients with phthisis, i.e., wasting away associated with chest pain and coughing,

often with blood in the sputum. So, the frequency of patients presenting with TB related symptoms at these times indicate that the disease was already well established (Smith, 2003).

The domestication of cattle has been proposed as one possible cause which might have allowed the passage of the pathogen from domesticated livestock to humans, and in this adaptation to a new host, the bacterium would have evolved to the closely related *M. tuberculosis* (Smith, 2003). MTC includes several Mycobacterium species with nearly identical nucleotide sequences and totally identical 16S rRNA (ribosomal ribonucleic acid) sequences. This extreme similarity is a proof that they all have a common ancestor (Banuls *et al*., 2015). *M. canettii* appears to be the ancestor of *M. tuberculosis* (Eleanor *et al*., 2016). And this ancestral form, *M. canettii*, has been isolated only in persons living in Africa. MTC DNA (deoxyribonucleic acid) has been found to be present in 5000-year-old samples from Egypt and pre-Columbian mummies from Ecuador (Mostowy and Behr, 2005). Recent works have demonstrated that *M. tuberculosis*, like *M. bovis* and *M. canettii*, can survive in soil for long periods of time while maintaining its infectious potential (Banuls *et al*., 2015). The German physician Robert Koch discovered the actual infectious agent, the tubercle bacilli, in 1882 (Singh, 2016).

1.1.3 Epidemiology of tuberculosis

TB is a major global health problem (Chatterjee and Pramanik, 2015). It causes ill-health among millions of people each year and ranks alongside the human immune deficiency virus (HIV) as a leading cause of death worldwide (WHO, 2015). Despite international efforts, TB remains a major public health concern, associated with high morbidity and mortality (Liu *et al*., 2017). Developing countries are heavily affected. The highest rates per capita are in Africa, while the largest numbers are in Asia, with nearly 60% of the global burden (Raviglione, 2010). Approximately 2 billion people of the world's population are latently infected with *M. tuberculosis* and are at risk of reactivation to active disease (Nguta *et al*., 2015 (b)).

In 2015, there were an estimated 10.4 million new TB cases worldwide of which people living with HIV accounted for 1.2 million (11%) of all new TB cases. In that same year, there were an estimated 1.4 million TB deaths, 480,000 new cases of multidrug resistant TB (MDR-TB) and an additional 100,000 people with rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment (WHO, 2016). Globally in 2017, there were an estimated 10 million new cases of TB. New cases of RR-TB were estimated to be 558,000, of which almost half were in

three countries: India (24%), China (13%) and the Russian Federation (10%). The absolute number of deaths globally from TB among HIV-negative and HIV-positive people has been estimated to have fallen by 5% and 20% respectively since 2015 (WHO, 2018).

As drug resistant strains of TB are rapidly emerging worldwide, the world health organization (WHO) reports alarming rise of not only MDR-TB (resistance to isoniazid and rifampicin) but also of extensively drug-resistant TB (XDR-TB) globally (WHO, 2009). XDR- TB is caused by strains of mycobacteria which are resistant to isoniazid, rifampicin, fluroquinolones and at least one of the three injectable second line anti-tubercular drugs (amikacin, kanamycin, and/or capreomycin) (Delogu and Fadda, 2009). Furthermore, cases of totally drug-resistant TB (TDR-TB) have been noted in China, India, Africa, and Eastern Europe (Hoagland *et al*., 2016). TDR-TB Centers for Diseases Control and Preventions reported the first cases of TDR-TB in South Africa and they stated that the disease is "virtually untreatable". It is noted that a new strain has been identified in patients in the Eastern Cape Province which is resistant to all current drugs. This strain showed *in vitro* resistance to all first and second line drugs tested (Chatterjee and Pramanik, 2015).

1.1.4 Pathophysiology of tuberculosis

As an obligate pathogen, the persistence of *M. tuberculosis* within the human population depends on the ability to drive successive cycles of infection, disease and transmission (Warner *et al*., 2015). Transmission could be through inhalation, ingestion or inoculation of the mycobacterium. Transplacental route can also result in development of congenital TB in foetus from infected mother but it's a rare mode of transmission (Mohan, 2005). The infection success and the development of pulmonary TB, lungs being the main target of this bacterium, depend on four successive steps: phagocytosis of the bacilli, their intracellular multiplication, the latent contained phase of infection and finally the active lung infection (Banuls *et al*., 2015).

After inhalation of *M. tuberculosis* into the lung, resident alveolar macrophages (AM) are one of the initial target cells of infection in addition to dendritic cells and neutrophils, which traffic to the site of infection (Hawn *et al*., 2013). Endocytosis by the AM occurs through the binding of LAM on the bacterial cell wall through mannose receptors and binding opsonized mycobacteria through complement receptors (Udoh, 2009). Within the AM, fusion of phagosomes with acidic lysosomes is critical for bactericidal activity. However, *M. tuberculosis* uniquely blocks

phagolysosome fusion and acidification, thereby eliciting the rapid recruitment of neutrophils, inflammatory monocytes, interstitial macrophages and T helper cells (Th-cells) in the lungs (Chinta *et al*., 2016). So it is when the innate immune response is insufficient in preventing infection that a T cell-mediated adaptive response is triggered. The T cell subtype, Th1, secretes interferon gamma (IFN-γ) as well as a variety of other cytokines to recruit/activate macrophages and other immune cells to trigger intracellular antimicrobial mechanisms (Teskey *et al*., 2018). When the immune response is partially successful, activated macrophages and other host cells (T cells, B cells, and fibroblasts) surround the *M. tuberculosis*-infected cells in an organized display, a granuloma, creating hypoxic, acidic, nutrient-poor conditions that are less permissive for *M. tuberculosis* replication (Hawn *et al*., 2013).

As the cellular processes occur, TB may develop differently in each patient, according to the status of the patient's immune system (Shi and Sugawara, 2013). In persons with efficient cell-mediated immunity, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial infection in the lung or if a latently infected person's immune system becomes weakened, the granuloma center can become liquefied and then serve as a rich medium by which the now revived bacteria can replicate in an uncontrolled manner (Smith, 2003).

Progression takes place by caseation, liquefaction, excavation and bronchogenic spread. Liquefaction is the most significant pathologic process in progression. Bacilli are numerous in liquefying regions, and drainage of liquefying areas results in excavation and bronchogenic spread to previously healthy parts of the lungs, where the sequence may be repeated (Long, 1940). At this point, viable *M. tuberculosis* can escape from the granuloma and spread within the lungs (active pulmonary TB) and even to other tissues (miliary or extrapulmonary TB) (Smith, 2003). Extrapulmonary TB (EPTB) results from the hematogenous and lymphatic spread of *M. tuberculosis* (Ramirez-Lapausa *et al*., 2015). Some of the possible locations for EPTB include, the blood stream (disseminated), central nervous system, lymphatic, bones, joints, pleura, and genitourinary system (Shi and Sugawara, 2013).

1.1.5 Signs and symptoms of tuberculosis

As the stages include latency, primary disease, primary progressive disease, and extra-pulmonary disease, each stage has different clinical manifestations. People with latent tuberculosis infection

(LTBI) have no signs or symptoms of the disease, do not feel sick and are not infectious (Shi and Sugawara, 2013). The classic clinical features of pulmonary TB include chronic cough, sputum production, appetite loss, weight loss, fever, night sweats, and hemoptysis (Zumla *et al*., 2013). Untreated pulmonary TB continues to spread throughout the respiratory tract and will eventually lead to hypoxia, respiratory acidosis and, often, death (Case, 2006). The clinical presentation of EPTB varies greatly (Ramirez-Lapausa *et al*., 2015). Listed below are some symptoms associated with EPTB with respect to the site of TB infection;

- Skeletal TB (Pott's disease): Possible symptoms are spinal pain and back stiffness, at times paralysis is possible (Eleanor *et al*., 2016)
- TB arthritis: Chronic swelling, pain, loss of joint function that progresses over weeks to months and deformity of the joint (Cantres-Fonseca *et al*., 2018)
- Miliary TB: many small nodules widespread in organs that resemble millet seeds (hence its name) (Eleanor *et al*., 2016)
- Central nervous system TB: headache, seizures, papilledema, or other signs of increased intracranial pressure (Rock *et al*., 2008)
- Genitourinary TB: dysuria, flank pain, increased frequency, masses or lumps (granulomas) (Eleanor *et al*., 2016)
- Laryngeal TB: development of masses, ulcers or nodules in the larynx and vocal cords. The most common clinical manifestation is dysphonia (Ramirez-Lapausa *et al*., 2015)
- Gastrointestinal TB: difficulty swallowing, non-healing ulcers, abdominal pain, malabsorption, diarrhea (may be bloody) (Eleanor *et al*., 2016)
- Pleural TB: empyema and pleural effusions (Eleanor *et al*., 2016).

Since EPTB can affect any organ in the body, it has varied clinical manifestations, and therefore requires a high index of clinical suspicion (Zumla *et al*., 2013). Just as in pulmonary TB, untreated EPTB will spread and often result in death (Case, 2006).

1.1.6 The role of oxidative stress and anti-oxidants in tuberculosis

Studies have linked oxidative stress to various lung disorders, including asthma, chronic obstructive pulmonary disease, acute pulmonary distress syndrome and TB (Shastri *et al*., 2018). Mycobacteria can induce reactive oxygen species (ROS) production by activating phagocytes.

Although this is an important part of the host defense against mycobacteria, enhanced ROS generation may also promote tissue injury and inflammation which may further contribute to immune-suppression particularly in those with impaired antioxidant capacity, such as in HIV infected patients (Pawar *et al*., 2011; Adebimpe *et al*., 2015). Based on a study on guinea pigs, oxidative stress was evident within 2 weeks of infection as measured by a decrease in the serum total antioxidant capacity and blood glutathione levels accompanied by an increase in malondialdehyde within lesions. It was suggested that therapeutic strategies that reduce oxidant-mediated tissue damage may be beneficial as an adjunct therapy in the treatment and prevention of TB in humans (Palanisamy *et al.,* 2011).

Antioxidant supplementation is expected to benefit TB patients by minimizing oxidative stress induced immunosuppression (Bhattacharyya and Banerjee, 2013). An added benefit of antioxidant treatment of patients with TB is the reduction of the toxic side effects of anti-tuberculosis drug therapy, mediated in part by the generation of oxygen free radical in the liver (Zaro *et al*., 2016). It is also suggested in another study that improved nutrition and supplementation with antioxidant therapy in the treatment of pulmonary TB may prevent the oxidative stress and further complications (Moses *et al*., 2008). Another study also suggests that management of oxidative stress will aid healing in TB patients (Wiid *et al*., 2004).

1.1.7 Treatment of tuberculosis

The current TB therapy consists of treatment with a combination of drugs (kahaliw, 2016). The preferred regimen in patients without suspected resistance consists of a two-month intensive phase with daily isoniazid, rifampicin, ethambutol and pyrazinamide, followed by a 4-month continuation phase of isoniazid and rifampicin (Tiberi *et al*., 2018 (b)). Treatment for MDR-TB, defined as resistance to isoniazid and rifampicin (the two most powerful anti-TB drugs), used to be commonly administered for 2 years or longer involving daily injections for six months (Chinta *et al*., 2016, Chatterjee and Pramanik, 2015).

Nowadays, MDR/RR-TB regimens can be offered as either i) a standardized shorter regimen of 9–12 months, where patients with more than one month treatment with second-line drugs or resistance to fluoroquinolones and second-line injectables are excluded; or ii) longer regimens which can last up to 20 months (8 months for the intensive phase) (Mabhula and Singh, 2019). The shorter regimen consists of an intensive phase with gatifloxacin or moxifloxacin,

kanamycin, prothionamide, clofazimine, high-dose isoniazid, ethambutol and pyrazinamide for 4–6 months, followed by a continuation phase of 5 months with gatifloxacin (or moxifloxacin), clofazimine, ethambutol, and pyrazinamide (Tiberi *et al*., 2017).

The first line anti-tubercular drugs being isoniazid, rifampicin, ethambutol and pyrazinamide, WHO categorizes the second-line anti-tubercular drugs as listed in Table 1;

Table 1 – WHO categorization of second-line anti-tubercular drugs recommended for the treatment of RR-TB and MDR-TB (Tiberi *et al*., 2018 (b)).

Categorization of second line anti-tubercular drugs
• Group A: fluoroquinolones ✓ Levofloxacin ✓ Moxifloxacin ✓ Gatifloxacin
• Group B: second-line injectable agents ✓ Amikacin ✓ Capreomycin ✓ Kanamycin ✓ (Streptomycin)
• Group C: other core second-line agents ✓ Ethionamide/prothionamide ✓ Cycloserine/terizidone ✓ Linezolid ✓ Clofazimine
• Group D: add-on agents (not part of the core MDR-TB regimen) • D1 ✓ Pyrazinamide ✓ Ethambutol ✓ High-dose isoniazid

• D2 ✓ Bedaquiline ✓ Delamanid
• D3 ✓ Para-aminosalicylic acid ✓ Imipenem plus cilastatin (requires clavulanate) ✓ Meropenem (requires clavulanate) ✓ Amoxicillin plus clavulanate ✓ Thioacetazone
*HIV negative status required before administering thioacetazone

1.1.7.1 Conventional drugs and vaccines for tuberculosis

Indeed, 70 years ago, there was no drug to treat TB (Banuls *et al*., 2015). Effective drug treatments were first developed in the 1940s. The most effective first-line anti-TB drug, rifampicin, became available in the 1960s (WHO, 2015). The past few years have seen the first approvals of new anti-tubercular drugs in more than 50 years, in large part due to coordinated efforts of government programs, nongovernmental organizations, and support from pharmaceutical companies (Hoagland *et al*., 2016). Bedaquiline was approved by the FDA (Food and Drug Administration) in December 2012 as part of the combination therapy for the treatment of adult patients affected by MDR-TB (Nguta *et al*., 2015(b)). Only one more new drug, delamanid, has been introduced into the market since then, and both drugs were approved for the treatment of DR-TB (Mabhula and Singh, 2019). Clofazimine, linezolid and carbapenems in combination with clavulanate are among the repurposed drugs for the treatment of MDR-TB (Gualano *et al*., 2016; Tiberi *et al*., 2018 (b)). The currently used first and second line drugs are listed in Table 2 along with their mechanism of action and the genes associated with resistance.

Table 2– List of the first and second line anti-tubercular drugs

Class	Drug	Mechanism of action	Genes associated with resistance	References
First line anti-TB drugs to treat drug susceptible TB				
Arylhydrazine	Isoniazid	Inhibits the formation of mycolic acids	*katG, inhA, ndh, ahpC*, *kasA*	Sarkar *et al*., 2016; Patel *et al*., 2012; Peñuelas-Urquides *et al*., 2017
Rifamycins	Rifampicin	Inhibition of RNA synthesis	*rpoB*	Hameed *et al*., 2018; Cole, 1994
Ethylene diamine derivative	Ethambutol	Interfers with the biosynthesis of arabinogalactan	*embCAB operon, DPA, ubiA*	Patel *et al*., 2012; Palomino and Martin, 2014; Mabhula and Singh, 2019; Miotto *et al*., 2018
Nicotinamide analogue	Pyrazinamide	Inhibition of trans-translation and pantothenate and CoA synthesis; Depletion of membrane energy	*pncA, rpsA, panD, clpC1*	Dookie *et al*., 2018; Hameed *et al*., 2018; Damtie *et al*., 2014; Mabhula and Singh, 2019
Second-line anti-TB drugs recommended for treatment of RR-TB and MDR-TB				
Fluoroquinolones	Levofloxacin Moxifloxacin Gatifloxacin	Inhibit the activity of DNA gyrase	-Quinolone resistance determining region of *gyrA* and *gyrB -pstB*	Peñuelas-Urquides *et al*., 2017; Mabhula and Singh, 2019; Miotto *et al*., 2018
Aminoglycosides	Streptomycin	Inhibition of protein synthesis	*rpsL, rrs, gidB*	Damtie *et al*., 2014; Peñuelas-Urquides *et al*., 2017; Dookie *et al*., 2018
	Kanamycin		*rrs, eis*	Mabhula and Singh, 2019; Miotto *et al*., 2018
	Amikacin		*rrs*	Mabhula and Singh, 2019
Macrocyclic polypeptide	Capreomycin	Inhibition of protein synthesis	*rrs, tlyA*	Kolyva and Karakousis, 2012; Palomino and Martin, 2014; Patel *et al*.,

				2012; Dookie *et al*., 2018
Thioamide	Ethionamide/prothionamide	Inhibits mycolic acid synthesis	*ethA*, *ethR, inhA*	Dookie *et al*., 2018; Palomino and Martin, 2014
Isoxazolidinone	Cycloserine/terizidone	Inhibition of formation of D-alanine and its incorporation for peptidoglycan synthesis	*alr, ddlA*, *cycA, ald*	Mabhula and Singh, 2019; Kolyva and Karakousis, 2012; Patel *et al*., 2012; Hameed *et al*., 2018
Oxazolidinone	Linezolid	Inhibits bacterial protein synthesis	rplC and rrl	Hameed *et al*., 2018; Mabhula and Singh, 2019
Riminophenazine	Clofazimine	Inhibits energy production, disruption of membrane and liberating bactericidal levels of ROS	-Rv0678 (up-regulates MmpL5) - *pepQ*	Kana *et al*., 2014; Palomino and Martin, 2014; Hameed *et al*., 2018; Al-Saeedi and Al-Hajoj, 2017
Diarylquinolines	Bedaquiline (TMC-207)	Inhibits ATP synthase	- Rv0678 (up-regulates MmpL5) - *atpE* - *pepQ*	Palomino and Martin, 2014; Miotto *et al*., 2018; Mabhula and Singh, 2019; Hameed *et al*., 2018
Nitro-dihydro-imidazooxazole	Delamanid (OPC-67683)	Inhibits the biosynthesis of methoxy-keto-mycolic acid but not α-mycolic acid	*ddn*, *fgd1*, *fbiA*, *fbiB* and *fbiC*	Kolyva and Karakousis, 2012; Miotto *et al*., 2018
Salicylic acid	Para amino salicylic acid	Inhibits folic acid synthesis	*thyA, folC, ribD, dfrA*	Patel *et al*., 2012; Dookie *et al*., 2018; Miotto *et al*., 2018
Carbapenems	Meropenem and Imipenem plus cilastatin (require clavulanate)	Inhibition of cell wall synthesis as well as *B-lactamase*	*blaC*	Schaaf *et al*., 2011; Mabhula and Singh, 2019; England *et al*., 2012
Penicillin	Amoxicillin (plus clavulanate)	Inhibition of cell wall synthesis	*blaC*	Schaaf *et al*., 2011; Dooley *et al*., 2012
Thiosemicarbazone	Thioacetazone	Inhibits mycolic acid synthesis	*hadABC* and *ethA*	Dooley *et al*., 2012; Palomino and Martin,

	2014; Mabhula and Singh, 2019
katG (catalase-peroxidase KatG), inhA (enoyl-acyl-carrier protein-reductase InhA), ndh (NADH dehydrogenase Ndh), ahpC (alkyl hydroperoxidase C AhpC), kasA (β-ketoacyl ACP synthase KasA), rpoB (RNA polymerase β-subunit RpoB), embCAB operon (arabinosyl transferases EmbABC), DPA (decaprenylphosphoryl -β -D arabinose), ubiA (5-Phospho-α-d-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyltransferase ubiA), pncA (Pyrazinamidase/nicotinamidase PZase PncA), rpsA (30S ribosomal protein S1 RpsA), panD (Aspartate alpha-decarboxylase panD), clpC1 (Caseinolytic protease complex component, clpC1), gyrA, gyrB (DNA topoisomerase II /DNA gyrases GyrA, GyrB), pstB (Phosphate import ATP-binding protein pstB), rpsL (30S ribosomal protein S12), rrs (Ribosomal RNA 16S), gidB (Putative 16S rRNA methyltransferase gidB), eis (Aminoglycoside acetyltransferase eis), tlyA (2'-O-methyltransferase TlyA), ethR (Transcriptional regulatory repressor protein EthR), alr (Alanine racemase alr), ddlA (D-alanyl-D-alanine ligase DdlA), cycA (D-serine / alanine / glycine transporter protein CycA), ald (L-alanine dehydrogenase ald), rplC (50S ribosomal protein L3 RplC), rrl (Ribosomal RNA 23S rrl), Rv0678 (Conserved protein), MmpL5 (Transmembrane transport protien(mycobacterial membrane protein large) mmpl5), atpE (ATP synthase C chain AtpE), pepQ (Cytoplasmic peptidase PepQ), ddn (Deazaflavin-dependent nitroreductase Ddn), fgd1 (Glucose-6-phosphate dehydrogenase fgd1), fbiA (Protein FbiA for flavin cofactor F_{420} biosynthesis), fbiB (Protein FbiB for flavin cofactor F_{420} biosynthesis), fbiC (Protein FbiC for flavin cofactor F_{420} biosynthesis), thyA (Thymidylate synthase thyA), folC (folate synthase FolC), ribD (Bifunctional enzyme riboflavin biosynthesis protein RibD), dfrA (Dihydrofolate reductase dfrA), blaC (β-lactamase gene blac), hadABC ((3R)- hydroxyacyl-acp dehydratases) and ethA (Monooxygenase EthA).	

Bacillus of Calmette and Guerin (BCG) remains the only licensed vaccine available against TB until today (Kaufmann *et al*., 2017). It was first used in humans 90 years ago and has been administered to an estimated 3.5 billion people; unfortunately, it is ineffective for the prevention of pulmonary TB in adults (Philips and Ernst, 2012; Gong *et al*., 2018). It's normally not administered to HIV-infected newborns because it can cause fatal disseminated infection in immunosuppressed patients (Eleanor *et al*., 2016). Therefore, it is very important to develop novel vaccines for TB prevention and control since an effective vaccine could change the face of the current TB epidemic (Philips and Ernst, 2012).

1.1.7.2 Newer anti-tubercular drugs and vaccines under development

Very little TB drug development has occurred over the last years. Pharmaceutical companies lack profit motive and the cost of development and clinical trials is prohibitive (kahaliw, 2016). The cost of developing a new drug is estimated at $115 to $240 million. So, in response to the reluctance of pharmaceutical industries, governments and nongovernmental organizations have started to invest in TB drug research and development (Vanden Boogaard *et al*., 2009). Priorities for TB research and development include a vaccine to lower the risk of infection and a vaccine or new drug treatment to cut the risk of TB disease in the 1.7 billion people already latently infected (WHO, 2018). Strains resistant to several of the novel drugs have already been described even prior to their routine clinical use (Kolyva and Karakousis, 2012). Some of the

drugs under development are listed in Table 3 along with their mechanism of action and the genes associated with resistance.

Table 3 – List of some anti-tubercular drugs under development

Class	Drug	Mechanism of action	Genes involved in resistance	Reference
Nitroimidazopyran	Pretomanid (PA-824) (In phase III clinical trials)	Inhibits the synthesis of proteins and cell wall lipids	*ddn, fgd1, fbiA, fbiB* and *fbiC*	Kolyva and Karakousis, 2012; van den Boogaard *et al*., 2009; Mabhula and Singh, 2019; Tiberi *et al*., 2018 (b)
	Delpazolid (In phase II clinical trials)			Tiberi *et al.,* 2018 (a);
Oxazolidinones	Sutezolide (PNU-100480) (In phase II clinical trials)	Inhibit protein synthesis	rplC and rrl	Tiberi *et al*., 2018 (b); Mabhula and Singh, 2019; Hameed *et al*., 2018
	Contezolid (In phase I clinical trials)			
Benzothiazinone	BTZ043 and PBTZ169 (In phase I clinical trials)	Inhibits arabinogalactan biosynthesis	Over expression of *NfnB* (In *M. smegmatis*), *dprE1(laboratory mutants)*	Palomino and Martin, 2014; Kolyva and Karakousis, 2012; Mabhula and Singh, 2019
Ethylene diamine	SQ-109 (In phase II clinical trials)	Interferes with the assembly of mycolic acids into the bacterial cell wall core	Genes encoding the membrane transporter *mmpl3*	Hameed *et al*., 2018; van den Boogaard *et al*., 2009; Palomino and Martin, 2014; Mabhula and Singh, 2019

Ddn (Deazaflavin-dependent nitroreductase Ddn), fgd1 (Glucose-6-phosphate dehydrogenase fgd1), fbiA (Protein FbiA for flavin cofactor F_{420} biosynthesis), fbiB (Protein FbiB for flavin cofactor F_{420} biosynthesis), fbiC (Protein FbiC for flavin cofactor F_{420} biosynthesis), rplC (50S ribosomal protein L3 RplC), rrl (Ribosomal RNA 23S rrl), NfnB (nitroreductase NfnB), DprE1 (decaprenylphosphoryl-β-D-ribose 2'-epimerase DprE1), mmpl3 (transmembrane transport protein (mycobacterial membrane protein large) MmpL3)

Just like that of the anti-tubercular drugs under development, there are also several TB vaccines which are in various trial stages. More than 200 vaccine candidates have been proposed as the result of work over recent years in experimental laboratory models (Martin, 2005). More than 20 vaccines are currently in clinical trials (Gong *et al*., 2018). The most notable of these new

vaccines is MVA85A, which is the first modern day *M. tuberculosis* vaccine to reach an efficacy trial. In animal models, it was shown to produce a strong Th1 response and provide better protection against *M. tuberculosis* infection than BCG (MacDonald and Izzo, 2015). MVA85A, a viral vector, is a modified recombinant strain of *Vacicinia virus Ankara* expressing the *M. tuberculosis* antigen 85A (Ahsan, 2015). It is in phase IIb trials and since it does not replicate in humans, it has been shown to be very safe (Ahsan, 2015; Orme, 2013). Heat killed whole *Mycobacterium vaccae* (Vaccae), heat killed *Mycobacterium indicus praniis* (MIP) and heat killed *Mycobacterium phlei* (Utilins) are among the vaccines which are in phase III clinical trial (Kaufmann *et al*., 2017; Gong *et al*., 2018).

Recently, attention has also turned to potential host-directed therapy (HDT) in the hope that novel treatment strategies might overcome many of the obstacles faced by antibiotic therapies for TB (Hawn *et al*., 2013). HDT is a new and emerging concept in the treatment of TB, where host response is modulated by treatment with small molecules, with or without adjunct antibiotics, to achieve better control of TB. Since HDT drugs act by directly modulating host cell functions, development of drug resistance by the infecting *M. tuberculosis* is avoided (Kolloli and Subbian, 2017). Particularly in case of MDR or XDR TB, HDTs provide a largely untapped approach as adjunctive anti-TB therapies, either to directly increase the ability of the host immune system to effectively eliminate mycobacteria or to limit collateral tissue damage associated with infection that can result in morbidity and mortality (Tobin, 2015).

The Host-Directed Therapies Consortium Network was launched in April, 2015, with 64 global partners to take forward trials of TB HDTs. It concluded that several drugs approved for other diseases are ready for clinical assessment in phase 2 trials, including imatinib, metformin, doxycycline and CC-11050 (Wallis *et al*., 2016). The adjunctive use of metformin was shown to complement the anti-tubercular properties of rifampicin and reduce the pulmonary load of a resistant mycobacterial strain while potentiating *M. tuberculosis*-directed CD8+ T-cell responses in mice (Rao *et al*., 2019). Doxycycline and CC-11050 have been proposed for further investigation as TB therapeutics due to their potential to inhibit lung damage during the course of the disease (Teskey *et al*., 2018). In another study, the use of HDT with phenylbutyrate and vitamin D_3 as adjunct therapy to standard TB treatment promoted favorable immunomodulation

to improve treatment outcomes (Rekha *et al*., 2018). Aside from the ones mentioned here, the potential of many other immunomodulatory agents are being investigated as adjunct HDTs for MDR-TB treatment (Zumla *et al*., 2016).

1.1.7.3 Herbal therapies

Approximately 60% of the world's population still relies on medicinal plants for their primary healthcare. Plant species still serve as a rich source of many novel biologically active compounds, yet very few plant species have been thoroughly investigated for their medicinal properties, and thus, there is a renewed interest in phytomedicine research (Nguta *et al*., 2015 (a)). The plant kingdom is a virtual goldmine of biologically active compounds and it is estimated that only 10–15% of existing species of higher plants have been surveyed (Bisht *et al*., 2006). Natural products or their semi synthetic derivatives have indeed provided novel drug leads for TB therapy. Examples of such compounds include Streptomycin and Kanamycin from *Streptomyces griseus* and capreomycin isolated from *Streptomyces capreolus*. Rifampicin is a semi-synthetic drug that has been derived from rifamycin, a product of *Amycolatopsis mediterranei* (Bunalema, 2010).

The WHO estimates that about 80% of the populations living in developing countries rely almost exclusively on traditional medicine for their primary healthcare needs (Gupta *et al.,* 2013). Some plants which have traditionally been used for the treatment of TB in different parts of Ethiopia include; *Balanites aegyptiacus* (L.) Delile [Xygophyllaceae], *Calpurnia aurea* (Aiton) Benth. [Fabaceae], *Citrus limon* (L.) Burm.f. [Rutaceae], *Cucumis dipsaceus* Ehrenb. [Cucurbitaceae], *Plumbago zeylanica* L. [Plumbaginaceae], *Zingiber officinale Roscoe* [Zingiberaceae], *Croton macrostachyus* Del. [Euphorbiaceae] (Zenebe *et al*., 2012; Giday *et al*., 2007; Wubetu *et al*., 2017).

The *in vitro* anti-mycobacterial activity of the crude 80% methanolic extracts of the root of *Calpurnia aurea*, seeds of *Ocimum basilicum*, leaves of *Artemisia abyssinica*, *Croton macrostachyus* and *Eucalyptus camaldulensis* were evaluated against *M. tuberculosis* and *M. bovis* strains. The minimum inhibitory concentration (MIC) was reported to be within the range of 6.25 to100 µg/ml (Gemechu *et al*., 2013). On another study, among several plants tested, the methanolic extracts from the aerial parts of *Ambrosia confertiflora* and *Ambrosia ambrosioides*

and the flower of *Guaiacum coulteri* were found to be active with MIC values of 200, 790 and 1000 μg/ml, respectively (Robles-Zepeda, 2013).

Extracts of *Aloe vera* (leaf), *Allium cepa* (bulb and leaves), *Allium sativum* (clove), *Solanum torvum* (unripe fruits and leaves), *Otostegia integrifolia* (root), *Pterolobium stellatum* (root), *Persea americana* (leaves), *Erythrina abyssinica* (stem bark), *Cryptolepis sanguinolenta* (root bark), *Acalypha indica* (leaves), *Adhatoda vasica* (leaves), *Allium ursinum* (bulb), *Dodonaea anguistifolia* (leaf), *Pavetta corymbosa* (leaves and twist), *Canarium schweinfurthii Engl* (leaves), *Piliostigma thonningii Schum* (seed pods), *Syzygium guineense* (root bark), *Vitex dononia* (stem bark), *Erythrina senegalensis* (leaves), *Cassia mimosides* (whole plant) are some other plants with reported activity against mycobacteria (kahaliw, 2016; Nguta *et al*., 2015(a); Bunalema, 2010; Gupta *et al*., 2010; Balcha *et al*., 2014; Nvau *et al*., 2011).

1.1.8 Drug resistance

In the context of *M. tuberculosis*, drug resistance is defined as the ability of >1% proportion of a bacilli to grow in the presence of critical concentration of a drug (Patel *et al*., 2012). Most *M. tuberculosis* resistance observed can be attributed to independent, spontaneous mutations that interfere with the drug binding to the target protein, reduce prodrug-activating enzymes or over express an essential target (Hoagland *et al*., 2016). Intrinsic resistance refers to the innate ability of a bacterium to resist the activity of a drug through its inherent structural (hydrophobic barrier) or functional characteristics (β-lactamase enzymes and efflux mechanisms) (Kolyva and Karakousis, 2012). Studies show that efflux and other intrinsic mechanisms (low cell wall permeability and modifying enzymes) assist in sequential acquisition of low-level drug resistance, followed by high-level resistance through stable mutations in drug target genes (Sriraman *et al*., 2018).

Drug-resistant strains of *M. tuberculosis* arise from spontaneous chromosomal mutations, in particular, single nucleotide polymorphisms at a predictable low frequency (Zumla *et al*., 2013; Hameed *et al*., 2018). The prevalence of RR isolates is 1 in every 10^7 to 10^8 bacilli and ~1 in 10^6 bacilli in case of isoniazid (Gumbo, 2010). Hence, rifampin resistance in the absence of isoniazid resistance is uncommon (Zumla *et al*., 2013). Resistance due to exposure to a single drug suppresses the growth of bacilli susceptible to that drug but permits the multiplication of pre-existing drug-resistant mutants (acquired resistance - resistance among previously treated cases).

Subsequent transmission of such bacilli to other persons may lead to disease that is drug-resistant from the outset, an occurrence known as primary resistance (resistance among new cases) (Damtie *et al*., 2014). Details of genes involved in resistance mechanisms of anti-tubercular drugs are indicated in Table 2 and Table 3.

1.2 Overview of the experimental plants

1.2.1 *Euphorbia candelabrum* kotschy

The Euphorbiaceae is one of the largest families of dicotyledons generally distinguished by the milky sap (Bijekar and Gayatri, 2014; Gupta, *et al*., 2013). *Euphorbia candelabrum (E. candelabrum)* is a succulent species of plant in the Euphorbiaceae family characterized by long, segmented, erect branches, looking like a candelabrum, with spines running along the ridges (Zorloni, 2008). It is found throughout eastern Africa. It grows in Central Africa, East Africa and possibly, Zambia (Holland and Hove, 1975).

Diterpenoids, triterpenoids, steroids, flavonoids, coumarins and phenolic compounds have all been reported as potentially active compounds for different Euphorbia species (Dharani *et al*., 2014). Like most of the euphorbia species, *E. candelabrum* is also known to possess poisonous compounds (Bijekar and Gayatri, 2014; Mwine and Van Damme, 2011). Like other Euphorbiaceae, the latex of *E. candelabrum* is a strong irritant to the eyes and skin, producing acute pain and temporary blindness and this irritant action is due to the presence of several unsaturated diterpene esters, like ingenol and 4-deoxyphorbol (Zorloni, 2008).

It is locally known as K'ulk'wal (Amharic), Carricho (siddama) and Adaamii (Oromo) and different parts of *E. candelabrum* including its latex have been used in traditional Ethiopian medicine (Dharani *et al*., 2014; Regassa, 2013; 'Euphorbia candelabrum', 2016). Some of its uses being; for curing syphilis, to treat the symptoms of leprosy with other medicinal plants as salve, stomach pain, to treat infertility, snake bites, eye tumors (externally), as a drastic laxative, clear out afterbirth and to treat ringworm (Dharani *et al*., 2014; 'Euphorbia candelabrum', 2016; Hedberg *et al*., 1983; Bekele and Reddy, 2015). It is also reported to be used for treating rheumatism, sharp pain and diarrhea in Dale district, Sidama zone (Kewessa *et al*., 2015). In different parts of Africa, it's used to treat malaria, sexually transmitted infections, upper respiratory tract infections, gastrointestinal tract complications, treatment of swellings, external

tumors and for dressing wounds (Mogwasi, 2016; Okello *et al*., 2010; Nanyingi *et al*., 2008; Moshi *et al*., 2003). Despite the many traditional medicinal uses, not much is known concerning the chemistry and pharmacology of *E. candelabrum* (Schmelzer, 2008). The picture of *E. candelabrum* is shown in Figure 2.

Figure 2 - A photograph of the plant *E. candelabrum* (Nasaka, 2014)

1.2.2 *Rumex abyssinicus* jacq

Rumex abyssinicus jacq (*R. abyssinicus*) (family-polygonaceae) is a perennial herb which can grow up to 3 m tall, with a thick and somewhat fleshy rhizome. The Stem is erect, stout, grooved, pale green or reddish (Edwards *et al*., 2000). It's locally known by the name 'Mekmako' (Amharic) and 'Dhangago' (Oromo) (Girma *et al*., 2015; Ayele *et al*., 2015). It is widely spread in the highlands of tropical Africa. It is widespread throughout Ethiopia at altitudes between 1200 and 3300 m. It is a common and tolerated weed in fields and plantations. It also occurs along paths and water, grassland and margins of rain forest (Edwards *et al*., 2000).

R. abyssinicus is a plant used in Ethiopia as a traditional medicine for the treatment of different human ailments. It is used for the treatment of diabetes and lung TB (Fufa *et al*., 2016), gonorrhea (Ayele *et al*., 2015), fever (Zinaye, 2008), pneumonia and cough (Odongo, 2016), bone TB (Meressa, 2018), teeth infections (Zenebe *et al*., 2012), hypertension (Getaneh and Girma, 2014), leprosy (Gulumian *et al*., 2018), itching skin (Giday *et al*., 2007) and vomiting (Mesfin *et al*., 2013).

The genus Rumex contains a large number of chemically complex bioactive compounds such as flavonoids, terpenes, coumarins and steroids (Fufa *et al*., 2016). It is reported that 80 % methanol extract of the rhizomes of *R. abyssinicus* possesses secondary metabolites such as tannins, saponins, flavonoids, steroids and anthraquinones (Mekonnen *et al*., 2010). In particular, different studies reported *R. abyssinicus* to contain a number of anthraquinones (Girma *et al*., 2015). Antraquinones have been reported to elicit varied bacteriostatic and fungicidal activity (Muriuki *et al*., 2013). Betulone, oleic acid, chrysophanol, emodin, rhein, physcion, damnacanthal and catenarin are some of the compounds isolated from the root of *R. abyssinicus* (Fufa *et al*., 2016; Zinaye, 2008; Girma *et al*., 2015).

Different activities have been reported for the plant *R. abyssinicus* like anti-tumor (Worku *et al*., 2013), wound healing and anti-inflammatory (Mulisa *et al*., 2015), anti-bacterial (Fufa *et al*., 2016; de Dieu Tamokou *et al*., 2013; Mohammed *et al*., 2017), anti-oxidant (de Dieu Tamokou *et al*., 2013), chemopreventive effect (Girma *et al*., 2015) as well as diuretic and analgesic activity (Mekonnen *et al*., 2010). The picture of the plant *R. abyssinicus* is shown in Figure 3.

Figure 3 - A photograph of the plant *R. abyssinicus* (Busse and Tefera, 2013)

1.2.3 *Vernonia amygdalina* del.

Vernonia is a genus of about 1,000 species of forbs and shrubs of which *V. amygdalina* is the most prominent species and one of the pan tropical tribes of the family Asteraceae (Tijjani *et al*., 2017). The genus is said to be named after William Vernon, an English botanist who lived in

the 17th and 18th centuries, while the Latin term *amygdalina* means "almond-like" (Zorloni, 2008). It is a small evergreen shrub widespread in Africa and South-East Asia (Ajila and Oloyede, 2012; Iwo *et al*., 2017). They are found naturally in forest margins, woodland, along rivers, lakes, and grassland, up to 2000 m altitude. This plant is commonly known as bitter leaf due to its bitter taste (Okoduwa *et al.,* 2018). *V. amygdalina* is known with different names in Ethiopia which include Girawa (Amharic) and Hecho (Sidamic) (Tadesse *et al*., 1993; Kewessa *et al*., 2015).

It is documented that this plant is commonly used in traditional medicine and the leaves are used for the treatment of malaria, fever, headache, scabies, diarrhea, dysentery, hepatitis, stomach-ache, ascariasis, tonsilitis, cough, bladder distension, as a laxative and as a fertility inducer (Okoduwa *et al.,* 2018; Getaneh and Girma, 2014; Giday *et al*., 2007; Teklehaymanot and Giday, 2007; Wubetu *et al*., 2017). The roots and the leaves are used to treat hiccups and kidney problems. It contains complex active components that are pharmacologically useful (Udochukwu *et al*., 2015). The reported bioactive compounds of *V. amygdalina* are saponins, alkaloids, terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, edotides, tannins, cardiac glycosides and sesquiterpene lactones (Tijjani *et al*., 2017; Okoduwa *et al*., 2016; Lelago *et al*., 2016; Alara *et al*., 2018).

All parts of the plant have been found to be pharmacologically useful (Adesanoye and Farombi, 2014). Anti- bacterial, anti- malarial, anti-parasites, anti-cancer and antioxidant activities of some of the plant parts have been reported in literature. The leaf is also used in the treatment of scurvy, rheumatism, pile, indigestion, blood sugar control, colic pain, loss of appetite, common cold, TB, mouth ulcers and sores amongst others (Ajila and Oloyede, 2012; Udochukwu *et al*., 2015; Enyi-Idoh *et al*., 2012; Adesanoye and Farombi, 2014; Alara *et al*., 2018; Okoduwa *et al*., 2016; Clement *et al*., 2014). The plant has anti-helminthic, anti-tumorigenic, hypo-glycemic, hypo-lipidaemic, anti-inflammatory and anti-nociceptive activities (Alkali and Abdullahi, 2017; Adedapo *et al*., 2014). The leaf extract also has analgesic and antipyretic activities when tested on laboratory animals (Tijjani *et al*., 2017). A freeze dried sample of the extract of *V. amygdalina* is one of the few herbal products that have been formulated into tablets for the control of diabetes mellitus in Nigeria. Another tablet formulation of *V. amygdalina* is in use to

boast the immunity of HIV patients (Toyang and Verpoorte, 2013). A photograph of the plant *V. amygdalina* is shown in Figure 4.

Figure 4 - A photograph of the leaves of *V. amygdalina* (Udochukwu *et al*., 2015)

1.2.4 *Otostegia integrifolia* benth.

The genus *Otostegia* belongs to the family *Lamiaceae*, which consists of about 180 genera and over 3500 species (Tofighi *et al*., 2009; Karunamoorthi, 2014). The *Otostegia* comprises about twenty species, among them fifteen are endemic to the northern part of tropical Africa and Southwestern and Central Asia, while the remaining five species have been reported in the flora of Ethiopia (Karunamoorthi, 2014). *Otostegia integrifolia (O. integrifolia),* commonly known as Abyssinian rose, is endemic to Ethiopia, in the dry evergreen woodlands of Tigray, Gondar, Wollo, Gojjam, North Shewa, Kafa and Hararghe regions, as well as in the dry and moist agro climatic zones of the district known as Dega. It is also endemic to Eritrea and Yemen (Chekol and Desta, 2018). It's locally known as "Tunjit". The plant is easily recognized and stands out due to the grayish color of the leaves and the yellow flowers which are conspicuous when in full bloom (Tadesse *et al*., 2011).

Ethnobotanical surveys in Ethiopia and Eretria found that the local residents have been using *O. integrifolia* as a potential traditional medicine to alleviate various illnesses and disorders (Karunamoorthi, 2014). Reports indicate that the leaves are used for treating lung diseases,

stomachache, malaria and hypertension (Endale *et al*., 2013). There are scientific reports on its anti-oxidant, anti-bacterial, anti-diabetic and anti-malarial activities (Endale, 2012; Chekol and Desta, 2018; Tadesse *et al*., 2011; Shewamene *et al*., 2015).

Methanol extract was found to have a wide range of bioactive compounds including flavonoids, phenols, terpenoids, saponins, steroids and glycosides (Chekol and Desta, 2018). It was reported that a total of 40 constituents (monoterpenes, sesquiterpenes, diterpenes and their derivatives) were identified from the essential oil and the chloroform extract *of O. integrifolia* (Tesso and Konig, 2004). It was reported that the essential oil of *O. integrifolia* is mainly composed of α-pinene (31.33%), 1- octen-3-ol (11.78%) and trans-caryophyllene (11.35%). The oil possesses an excellent broad-spectrum activity against bacteria and fungi and also a significant free radical scavenging activity (Tadesse *et al*., 2011). The picture of *O. integrifolia* is shown in Figure 5.

Figure 5 - Morphological view of the plant *O. integrifolia* (Endale, 2012)

1.2.5 *Erythrina brucei* schweinf

Erythrina is a plant genus belonging to the family Fabaceae (Leguminosae), which comprises of nearly 110 species that are distributed in the tropical and subtropical regions of the world (Gurmessa *et al*., 2018). The origin of the name *Erythrina* comes from the Greek word "erythros" which means red, alluding to the bright red flowers of the trees of the genus (de Araújo-Júnior *et al.,* 2012). *Erythrina brucei* (*E. brucei)* is one of the *Erythrina* species that is endemic to Ethiopia (Gurmessa *et al.,* 2018). It is a tree growing to 15-20m with colorful flowers

which is widely distributed only on the highlands of Ethiopia (Dagne and Steglich, 1984). It is known as 'Korch' in Amharic and 'Woleko' in Sidamic (Wubetu *et al*., 2017; Kewessa *et al*., 2015)

In Ethiopia, the bark of this plant is used to treat ear infection (Chepkirui, 2012). The leaf is used for treating wound with the leaves of other plants (Wubetu *et al*., 2017). The stem bark of *E. brucei* is traditionally used for treating bovine TB and cancer (livestock), in Sidama, Dale district (Kewessa *et al*., 2015). It also improves soil fertility (Aserse *et al*., 2017). Different compounds isolated from the stem bark of *E. brucei* have been reported to possess moderate anti-bacterial activity and moderate to high anti-oxidant activity (Gurmessa *et al.*, 2018).

The genus *Erythrina* produces an enormous array of alkaloids, phenolics and chalcones (Bunalema, 2010). Phytochemical analysis has also demonstrated the presence of terpenes in plants from the *Erythrina* genus (de Araújo-Júnior *et al.,* 2012). From the stem bark of *E. brucei* several alkaloid compounds have been isolated, identified as, crystamidine, 8-oxo-erythraline, erythraline and 10-oxo-erythraline (Chepkirui, 2012). It was also reported on another study regarding the bark of this plant that in addition to erythraline, it contains erysodine, erythrinine and another alkaloid, $C_{18}H_{19}NO_5$ (Dagne and Steglich, 1984). Alkaloids have been reported to be plant constituents having activity against mycobacteria (Nguta *et al*., 2015(a)). Two new isoflavanones, erybrucein A and erybrucein B, have also been isolated from the stem bark and root bark of *E. brucei* along with other compounds (Gurmessa *et al*., 2018). The picture of the leaf and the flower of *E. brucei* are shown in Figure 6.

Figure 6 – A picture of the leaf and the flower of *E.brucei* (Busse and Tefera, 2013; 'Erythrina brucei Schweinf', no date)

1.3 Rationale of the study

Due to the increasing prevalence of MDR and XDR strains, there is an urgent need to search for and develop novel agents against TB (Nguta *et al*., 2015 (b)). Many researches are focused on the discovery and development of new anti-tuberculosis drugs from natural products which can be done by screening novel compound from plant species as they are considered as potential anti-TB agents (Wahyuningrum *et al*., 2017). One of the points incorporated in the Stop TB strategy to reach the global targets developed by WHO is to enable and promote research (Raviglione, 2010). There are five reasons usually given for the need of new anti-tuberculosis drugs as stated by Sanchez and Kouznetsov, 2010;

- To shorten the total duration of treatment,
- To improve the treatment of MDR-TB and XDR-TB strains,
- To provide for more effective treatment of LTBI in programs which are able to implement this practice,
- Side effects, especially hepatotoxicity, are an issue, which in some cases forces an untimely treatment termination, and
- Since the 1960s, there have been few developments in available therapies for the treatment of TB (Sanchez and Kouznetsov, 2010).

Shorter treatment regimens and more effective MDR-TB care regimens are priorities for drug development. An ideal candidate would shorten the treatment duration; have a novel mechanism of action; not interact with anti-retrovirals; be taken orally once daily or intermittently; and be low in cost (Raviglione, 2010).

The use of herbal remedies as an alternative or complementary to industrial medications gains a considerable importance due to their special attributes as a large source of therapeutic phytochemicals that may lead to the development of novel drugs (Ehsanifar *et al*., 2017; Azwanida, 2015). Among the reasons traditional knowledge is considered reliable for the exploitation of herbal remedies is that indigenous communities through a period of long experimentation with herbal medicines are likely to have retained those that are effective and tolerably safe while discarding preparations with low efficacy or acute toxicity (Nguta *et al*., 2015 (a)).

In Ethiopia, there are many plant species reported to be used traditionally to treat TB. One of those being *E. candelabrum*, it is one of the plants which was investigated in this study. The latex (in liquid form) of this plant is claimed to be used by indigenous people of Gemad district, Northern Ethiopia, for the treatment of TB (Mesfin *et al*., 2013). In Central Africa, several drops of latex diluted in water are taken to treat coughs and TB (Schmelzer, 2008). It has also been used for the treatment of bovine TB in animals in Uganda (Nasaka, 2014). The second plant investigated in this study was *R. abyssinicus* which is reported to be traditionally used for the treatment of lung TB and bone TB in different parts of Ethiopia (Fufa *et al*., 2016; Zenebe *et al*., 2012; Moravec *et al*., 2014).

The third plant investigated was the leaf of *V. amygdalina* which has been reported to be used to treat TB (Clement *et al*., 2014). The chloroform extract (by the use of successive soxhlet extraction) of the leaf of *V. amygdalina* was reported to have a significant activity against *M. bovis* strain so this extraction technique was employed here to test its activity against the *M. tuberculosis* strains (Tadesse *et al*., 1993). Although the root extract was found to be inactive against *M. tuberculosis*, the root bark was reported to be active against *M. tuberculosis, M. smegmatis, M. kansasii and M. fortuitum* (Aseffa *et al*., 2017, Mariita *et al*., 2010). *V. amygdalina* has also been reported as one of the locally available herbs used for the treatment of bovine TB in infected animals in Uganda (Nasaka, 2014). The leaf has also been reported to be used in livestock for cough around Dale district Sidama zone, Southern Ethiopia (Kewessa *et al*., 2015).

The last two plants investigated here were the leaves of *O. integrifolia* (80% methanolic extract as well as the essential oil) and the stem bark of *E. brucei*. The root and leaves of *O. integrifolia* have been reported to be used traditionally for the treatment of lung diseases (Karunamoorthi, 2014; Endale, 2012; Tesso and König, 2004; Endale *et al*., 2013). Its root extract was found to have a significant activity against *M. tuberculosis* (Aseffa *et al*., 2017). The essential oil was also investigated for its anti-mycobacterial activity because it's reported that the oil of *O. integrifolia* contains α-pinene and trans-caryophyllene and oils from different plants containing caryophyllene and α-pinene have been reported to have anti-mycobacterial activity (Tadesse *et al*., 2011; Runde *et al*., 2015; Bueno *et al*., 2011; Aşkun *et al*., 2010). The stem bark of *E. brucei*

has been reported to be used in livestock for bovine TB around Dale district Sidama zone, Southern Ethiopia (Kewessa *et al*., 2015).

Aside from the *in vitro* anti-mycobacterial assay, anti-oxidant activity was also done for one of these plant extracts (*E. candelabrum*) since literatures suggest that there is a link between oxidative stress and TB infection and supplementation of anti-oxidants may represent a novel approach for fast recovery (Reddy *et al*., 2004). The anti-oxidant activity was done only for the plant *E. candelabrum* because all the other plants have been tested for their free radical scavenging ability and their anti-oxidant activity have already been reported (Abate and Yayinie, 2018; Gurmessa *et al*., 2018; de Dieu Tamokou *et al*., 2013; Adesanoye and Farombi, 2014).

2. Objectives

2.1 General objective

- To evaluate the *in vitro* anti-mycobacterial activity of the extracts of the selected plants on *M. tuberculosis* and *M. bovis* strains.

2.2 Specific objectives

- To evaluate the *in vitro* anti-mycobacterial activity of the 80% methanolic extract of *E. candelabrum* latex on *M. tuberculosis* and *M. bovis* strains.
- To evaluate the *in vitro* anti-mycobacterial activity of the 80% methanolic extract of *R. abyssinicus* root on *M. tuberculosis* and *M. bovis* strains.
- To evaluate the *in vitro* anti-mycobacterial activity of the 80% methanolic extract of *O. integrifolia* leaf on *M. tuberculosis* and *M. bovis* strains.
- To evaluate the *in vitro* anti-mycobacterial activity of the oil extracted from *O. integrifolia* leaf on *M. tuberculosis* and *M. bovis* strains.
- To evaluate the *in vitro* anti-mycobacterial activity of the chloroform extract of *V. amygdalina* leaf on *M. tuberculosis* and *M. bovis* strains.
- To evaluate the *in vitro* anti-mycobacterial activity of the 80% methanolic extract of *E. brucei* stem bark on *M. tuberculosis* and *M. bovis* strains.
- To test the anti-oxidant activity of the *E. candelabrum* latex extract.
- To carry out a preliminary phytochemical screening for these plant extracts.

3. Materials and methods

3.1 Materials

3.1.1 Chemicals and media

The main chemicals that were used include: distilled water, ethanol, methanol, hexane, anhydrous sodium sulphate, chloroform, glycerol, middlebrook 7h9 broth base, tween 80, dimethyl sulfoxide (DMSO), acetic anhydride, sulphuric acid (H_2SO_4), ferric chloride ($FeCl_2$), hydrochloric acid (HCl), Mayer's reagent, Wagner's reagent, Dragendroff's reagent, lead acetate, ammonia solution, benzene, 2,2-diphenyl-1-picrylhydrazyl (DPPH), vitamin C, oleic acid-albumin-dextrose-catalase (OADC) enrichment, resazurin dye and the standard drug rifampicin. The media used were Lowenstein Jensen medium (LJM) and middlebrook 7H9 broth.

3.1.2 Plant materials

The latex of *E. candelabrum* was collected from Debrezeit located southeast of Addis Ababa. The root of *R. abyssinicus*, the leaf of *V. amygdalina* and the stem bark of *E. brucei* were collected from Addis Ababa and the leaf of *O. Integrifolia* was collected from Ambo located west of Addis Ababa. Identification and authentication of the plant specimens were done by Mr. Melaku Wondafrash, a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where a voucher specimen was deposited for future reference. The name and parts of the plants used in this study along with their site of collection is summarized in Table 4.

Table 4 – The experimental plants used for testing anti-mycobacterial activity

Plant species	**Family**	**Local name (Amh)**	**Parts used**	**Voucher specimen**	**Place of collection**
E. candelabrum	Euphorbiaceae	Qulqual	Latex	L.W - 002	Debrezeit
R. abyssinicus	Polygonaceae	Meqmeqo	Root	L.W - 003	Addis Ababa
V. amygdalina	Asteraceae	Girawa	Leaf	L.W - 004	Addis Ababa
O. integrifolia	Lamiaceae	Tunjit	Leaf	L.W - 005	Ambo
E. brucei	Fabaceae	Korch	Stem bark	L.W - 007	Addis Ababa

3.1.3 Test microorganisms

A culture of rifampicin sensitive *M. tuberculosis* (H37Rv, 44 (h), 03 and SO 0659) and *M. bovis* (btb33) strains on LJM were obtained from Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa, for the *in vitro* anti-mycobacterial assay of the plant extracts.

3.2 Methods

3.2.1 Plant collection

The latex of *E. candelabrum* was collected through longitudinal incisions with a knife in the stem. The collecting glass container was covered with aluminum foil to protect it from direct sunlight. The root of *R. abyssinicus* was collected and the dirt was washed and dried after chopping it into smaller pieces. The stem bark of *E. brucei* was scrapped off from the stem and was washed and dried. The leaves of *V. amygdalina and O. integrifolia* were also cleaned and shade dried after collection.

3.2.2 Plant extraction

The collected latex sample of *E. candelabrum* was spread on a bowl and was shade dried for three weeks. The dried latex sample was then coarsely powdered making it ready for extraction. The coarsely powdered sample was weighed to be 80gm and was suspended in 80% methanol (1:10 w/v ratio of the ground plant sample to solvent) at room temperature as described elsewhere (Tiwari *et al*., 2011). Cold maceration technique was employed. The suspension was allowed to stand for 3 days by placing it on an orbital shaker. Then it was filtered and the marc was re-macerated for a second time by adding fresh solvent. The filtrates from the two batches were combined, concentrated by the use of a rotary evaporator under reduced pressure, frozen and lyophilized.

The dried sample of the root of *R. abyssinicus* was ground and was weighed to be 180gm. The ground *O. integrifolia* leaf sample weighed 80gm and the dried and ground stem bark of *E. brucei* weighed 50gm. The plant samples mentioned above were also extracted by the use of a cold maceration technique as previously described. In case of the plant *O. integrifolia,* in addition to the 80% methanolic extract, the oil was also extracted from fresh leaves by hydrodistillation as described elsewhere (Francezon and Stevanovic, 2017). 400gm of *O.*

integrifolia leaf was used for the extraction of oil. The hydrosol was submitted to extraction with n-hexane. The hydrosol extracts were dried over anhydrous sodium sulphate.

The collected leaves of *V. amygdalina* were cleaned and dried for ten days. The dried leaves were powdered and extracted by the use of the soxhlet apparatus. The solvents employed for the successive extraction were hexane and chloroform. The powdered leaf of *V. amygdalina* (25gm) was placed in a thimble. The thimble was placed in the extraction chamber and the successive extraction was conducted by adding the solvents successively. The extracting solvents were heated until clear liquid contents of the chamber siphoned into the solvent flask. Both fractions were concentrated using rotary evaporator and kept in an oven overnight. All extracts were refrigerated until use. The calculated percentage yield for each plant extract is listed in Table 5.

Table 5 - Percentage yield of each plant extracts

S. No	**Plants**	**Solvent**	**Extraction method**	**Raw material (gm)**	**Yield (%)**
1	*E. candelabrum* latex	80% Methanol	Maceration	80gm	3.5gm (4.4%)
2	*R. abyssinicus* root	80% Methanol	Maceration	180gm	21gm (11.6%)
3	*V. amygdalina* leaf	Hexane	Successive Soxhlet	25gm	2.2gm (8.8%)
		Chloroform			2gm (8%)
4	*O. integrifolia* leaf	Water	Hydrodistillation	400gm	0.6gm (0.15%)
		80% Methanol	Maceration	80gm	33gm (41%)
5	*E. brucei* stem bark	80% Methanol	Maceration	50gm	1.5gm (3%)

3.2.3 Preparation of Inoculum

The *in vitro* anti-mycobacterial assay was done at ALIPB TB laboratory. Before conducting the experiments, the biosafety cabinet's surface was cleaned with alcohol. A splash-proof waste

receptacle with a tuberculocidal disinfectant was placed inside the biosafety cabinet for the disposal of liquids as well as used micropipettes.

First, the middle brook 7H9 broth was prepared by adding 2.35gm of middle brook 7H9 broth base in 450ml of distilled water. Two ml of glycerol was added and the bottle was autoclaved at a pressure of 15lbs (103Kpa) and a temperature of 121^0C for 10 minutes in order for it to be sterilized. Then, it was allowed to cool and 1 vial (100ml) of middle brook OADC growth supplement was aseptically added. This media was allowed to stand overnight in an incubator at 37^0C before sub culturing on it to check for any sign of contamination.

Inoculum preparation was carried out as described by Gemechu *et al*. (2013). The isolates grown on LJM were obtained from ALIPB. It was then sub-cultured in middle brook 7H9 broth supplemented with OADC and was incubated at 37°C for 14 – 21 days. The bacterial suspension was then homogenized by vortex shakeup and the turbidity was adjusted in agreement with tube which is the scales of McFarland no.1 by adding fresh middle brook 7H9 broth medium to the bacterial suspension. The inoculum was then prepared by diluting the adjusted bacterial suspension in the proportion of 1:20 in middle brook 7H9 broth medium (Martins *et al*., 2013). The diluted suspension was used to inoculate each test tube for the detection of the anti-mycobacterial activity of the plant extracts.

3.2.4 Determination of the MIC of the extracts

Resazurin-based MIC determination is widely used as the standard method in TB drug discovery and requires one week to derive the results (Sharma *et al.,* 2014). It was reported that there were no statistically significant differences among MICs determined by fluorometric and visual microplate alamar blue assay as the two colors (blue and pink) can be easily differentiated with the naked eye and visual readout is the most frequently used in evaluation of anti-tuberculosis compounds (Wahyuningrum *et al*., 2017). So this method was used to detect the anti-mycobacterial activity of the extracts in this study.

Resazurin, a phenoxazin-3-one dye with nitrone functional group, reduces in two steps, irreversibly to resorufin and then reversibly to dihydroresorufin (Khazalpour and Nematollahi, 2014; Tratnyek *et al*., 2001). Active bacterial cells reduce the non-fluorescent resazurin (blue) to the fluorescent resorufin (pink) which can be further reduced to hydroresorufin (colorless)

(Elshikh *et al.,* 2016). Reduction of resazurin is believed to be accomplished by reductase or diaphorase-type enzymes from mitochondria and the cytosol (McGaw *et al*., 2014). The structures of resazurin, resorufin and dihydroresorufin are illustrated in Figure 7.

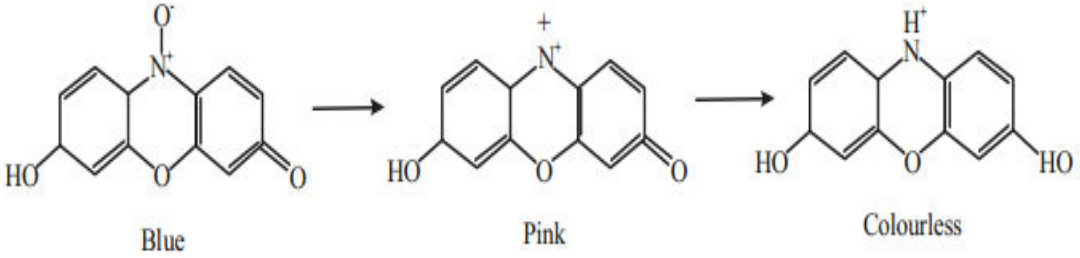

Figure 7 – The structure of resazurin and the resulting color changes (Hassan and Ullah, 2017)

According to literatures, a color change from blue to purple indicates that there is a 25%-75% bacterial growth, a color change from blue to pink indicates that there is 100% growth or 0% bacterial growth inhibition and no color change of the blue dye indicates that there is no growth of bacteria indicating a 100% bacterial growth inhibition (Hassan and Ullah, 2017). So, blue color in the test tube was interpreted as there was no mycobacterial growth (100% growth inhibition) and pink/colorless was scored as 100% growth or 0% inhibition. So, those plants which exhibited 100% inhibition were considered as having a positive activity and those which did not exhibit 100% inhibition were considered as having a negative activity. It is stated elsewhere that, for an extract to be considered as active against mycobacteria, it should have 99% inhibitory effect (Mariita *et al*., 2010).

The chloroform extract of *V. amygdalina* was not soluble in DMSO and tween 80 and according to Tadesse *et al*., 1993, the extract was reconstituted in ethanol to test its anti-mycobacterial activity against *M. bovis* but the concentration of ethanol used wasn't specified. So, in order to detect the activity of ethanol against mycobacteria, a test was implemented by using different concentration of ethanol in distilled water. One strain from *M. tuberculosis* (SO 0659) and one strain from *M. bovis* (btb 33) were used for assessing its anti-mycobacterial activity. A macro-dilution technique was employed as described elsewhere with minor modifications (Rens *et al*., 2016). First, 1ml of 50%, 25%, 15%, 10%, 5% and 1% of ethanol in distilled water was prepared in six different test tubes (for one strain) respectively to assess the anti-mycobacterial activity of the vehicle. Then, into each test tube containing different concentration of ethanol, 1ml of

inoculum was added reducing the final concentrations of ethanol in each test tube by half and it was incubated for a week. On the 7th day, 0.02% w/v of resazurin was added to each test tube and mixed. After incubating it overnight, the result was observed. The activity of ethanol at different concentrations against *M. tuberculosis* and *M. bovis* strains is shown in Figure 8.

Figure 8 – The color change of resazurin in the presence of different concentrations of ethanol against btb 33 and SO 0659 respectively.

As elucidated on figure 8, ethanol turned out to have a mycobacterial growth inhibitory effect at a concentration of 50%, 25% and 15% as there was no color change (blue). At a concentration of 10%, 5% and 1% ethanol, the resulting color change was pink which shows 100% mycobacterial growth. So, 10% ethanol in distilled water was used to solubilize the chloroform extract of *V. amygdalina* at a concentration of 10mg/ml. In case of the 80% methanol crude extracts of *O. integrifolia*, *E. candelabrum*, *R. abyssinicus* and *E. brucei,* the extracts were not totally soluble in distilled water so just like that of ethanol, the activity of methanol was also tested against the mycobacterial strains. Just like that of 10% ethanol, 10% methanol turned out to be inactive against the mycobacterial strains. As the extracting solvent used for these plants was 80% methanol, the extracts were totally soluble while using 10% methanol as a vehicle. So, these extracts (80% methanolic extracts of *O. integrifolia*, *E. candelabrum*, *R. abyssinicus* and *E. brucei*) were dissolved in 10% methanol at a concentration of 25mg/ml. In case of the oil of *O. integrifolia*, it was suspended in 0.02% tween 80 (Hood *et al*., 2003). According to Hood *et al*., 2003, an optimized broth dilution using 0.02% tween 80 to emulsify the oils was developed and was shown to be an accurate method for testing the antimicrobial activity of essential oils providing reliable and correct results which was why 0.02% tween 80 was used for emulsifying

the oil of *O. integrifolia* here. It is also reported elsewhere that the addition of tween 80 to the culture medium did not alter the MIC values obtained (Bueno-Sánchez *et al*., 2009).

Rifampicin served as the positive control. The broth alone served as a sterility control and the inoculum alone served as a growth control. The vehicles 0.02% tween 80, 10% ethanol and 10% methanol served as the negative controls. The tests were done in triplicates. First the anti-mycobacterial activity of all plants were tested against one *M. bovis* strain (btb 33) and one *M. tuberculosis* strain (H37Rv- the laboratory standard rifampicin sensitive strain). After seeing the result, only one plant which showed activity against both *M. bovis* and *M. tuberculosis* strains was further investigated for its activity against other strains of *M. tuberculosis* clinical isolates (44 (h), 03 and SO 0659).

The experiments were carried out by the use of macro-dilution technique in test tubes as described elsewhere (Rens *et al*., 2016). In case of the *V. amygdalina* extract, six test tubes were used for performing the anti-mycobacterial activity assay against one strain (a total of 18 test tubes against one mycobacterial strain, since the test was done in triplicates). To the first test tubes, 600µl from the working solution of the extract was added. Starting from the second test tube up to the sixth test tube, 600µl of broth was added to all for the purpose of serial dilution. Then, to the second test tube, 600µl of the extract was added and mixed by the use of a vortex. After mixing it, 600µl was transferred to the third test tube and this was carried out up to the sixth test tube. Then, 600µl was taken from the sixth test tube and was discarded. Finally, 600µl of inoculum was added to each test tube and mixed by the use of a vortex shake up. The concentration in each test tube was 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml and 0.312mg/ml respectively. Further serial dilutions were not carried out because it was first tested with lower concentrations and failed to show activity against mycobacteria. The starting concentration was 10mg/ml as stated elsewhere (Rajiv *et al*., 2001). The samples were incubated for a week and 0.02% w/v resazurin in distilled water was added on the seventh day and incubated overnight. The result was observed on the next day.

In case of the *E. candelabrum, R. abyssinicus, O. integrifolia* and *E. brucei* extracts, four test tubes were used for performing the anti-mycobacterial activity assay for one plant against one strain (a total of 12 test tubes for one extract against one mycobacterial strain). First, to the first test tubes, 600µl of the dissolved extracts were added by the use of a micropipette. Starting from

the second test tubes to the fourth test tubes, 600µl of broth was added. Then, to the second test tubes, 600µl of extracts was added and mixed with the vortex mixer. After that, 600µl of the mixture was transferred to the third test tubes and mixed again. Then, 600µl from the third test tubes was taken and transferred to the fourth test tubes and mixed by the use of a vortex mixer. Finally, 600µl was taken from the fourth test tubes and was discarded. Then, 600µl of inoculum was added to all test tubes and mixed by the use of a vortex. The final volume in each test tube was 1.2ml and the concentrations in each test tube were 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml respectively. The starting concentration (25mg/ml) was used as described elsewhere (Eldeen and Van Staden, 2008). Further serial dilutions to a lower concentration were not done because it was first experimented with lower concentrations (starting from 2.5mg/ml to 1.56µg/ml) and showed no activity. The samples were incubated for a week and 0.02% w/v resazurin in distilled water was added on the seventh day and incubated overnight. The result was observed on the next day.

In the case of essential oils, it is stated elsewhere that it is important to take into account that the evaporation and the absorption of components by plastic plates could affect the antimicrobial activity. So, it is recommended that broth dilution assays should be carried out under sealed conditions to prevent loss by evaporation, and glass material should be used to prevent loss by absorption (Sánchez and Kouznetsov, 2010). So the *in vitro* anti-mycobacterial assay of the oil of *O. integrifolia* was also assessed by employing the macro-dilution technique by the use of glass test tubes under properly sealed conditions. The same procedure described above was employed to test the anti-mycobacterial activity of the oil of *O. integrifolia* with a starting concentration of 12.5mg/ml.

Rifampicin was prepared at a concentration of 32µg/ml in 1% DMSO (Gemechu *et al*., 2013). Based on a study, the tolerance of mycobacteria for 1% DMSO was tested and this concentration of solvent did not affect growth of the bacteria, so 1% DMSO was used to solubilize the positive control rifampicin (Low *et al*., 2017). The same procedure of anti-mycobacterial assay was performed by the use of the macro-dilution technique. The concentration of rifampicin used ranged from 16µg/ml to 0.5µg/ml (6 serial dilutions). These test tubes were also incubated for a week and resazurin was added on the 7^{th} day and incubated overnight. Test tubes containing the broth alone, the inoculum alone and the vehicles with the inoculum were incubated for seven

days along with the other samples for sterility control, growth control and negative (vehicle) control respectively.

The *V. amygdalina* extract was the only one which showed activity against these two strains so it was further investigated for its activity against the clinical isolate *M. tuberculosis* strains (44 (h), 03 and SO 0659). Further investigation against the *M. tuberculosis* clinical isolate strains was not carried out for the other plant extracts because they did not show activity on H37Rv. The commonly used strain, *M. tuberculosis* H37Rv, has a drug susceptibility profile which is fairly representative of the majority of drug susceptible clinical isolates (Muriuki *et al*., 2013).

3.2.5 Anti-oxidant activity

The anti-oxidant activity was done only for the plant *E. candelabrum* because all the other plants have been tested for their free radical scavenging ability and their anti-oxidant activity have already been reported (Abate and Yayinie, 2018; Gurmessa *et al*., 2018; de Dieu Tamokou *et al*., 2013; Adesanoye and Farombi, 2014). Different concentrations of the 80% methanol extract of *E. candelabrum* were prepared in methanol by two fold serial dilutions with a starting concentration of 50 mg/ml (Enujiugha, 2010). The concentration of extracts prepared ranged from 50mg/ml to 3.12 mg/ml.

The anti-oxidant activity of the extract was determined by using the DPPH free radical scavenging assay as described by Jothy *et al*, (2012) with some modifications. Initially, the DPPH solution was prepared in methanol at a concentration of 0.04% (4mg/100ml). 50 µl was taken from different concentration of the extract solutions prepared and were added to different test tubes containing 5 ml of the 0.04% DPPH in methanol solution to make 500µg/ml, 250µg/ml, 124µg/ml, 62µg/ml and 31µg/ml solutions. After 30 minutes of incubation in the dark, the absorbance was read against a blank at 517 nm. Ascorbic acid (vitamin C) was used for comparison at a starting concentration of 0.5mg/ml (Sellal *et al*., 2019). The concentration of ascorbic acid prepared ranged from 0.5mg/ml to 0.031mg/ml by employing two fold serial dilutions. 50 µl was taken from different concentration of the ascorbic acid solutions prepared and were added to different test tubes containing 5 ml of the 0.04% DPPH in methanol solution to make 5µg/ml, 2.5µg/ml, 1.2µg/ml, 0.6µg/ml and 0.3µg/ml solutions. The assay was carried out in triplicates and the percentage of inhibition was calculated using the following formula (Mahdi-Pour *et al*., 2012);

$$\% \text{ of inhibition} = \frac{(\text{Absorbance of blank} - \text{Absorbance in presence of extract})}{\text{Absorbance of blank}} \times 100$$

IC_{50} (half maximal inhibitory concentration) value was determined from the plotted graph of the scavenging activity of the extract against the different concentrations of extracts used, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50% (Kumar *et al*., 2014).

3.2.6 Preliminary phytochemical screening

A preliminary phytochemical analysis was carried out for the plant extracts; *E. brucei*, *E. candelabrum*, *O. integrifolia* and *R. abyssinicus* for the presence and absence of the phyto-constituents mentioned below. Preliminary phytochemical screening was performed using the standard procedures to identify the constituents as described elsewhere. As for the chloroform extract of *V. amygdalina*, it's stated that terpenoids and flavonoids are the active components that can be found in extracts extracted by the use of chloroform, so the preliminary phytochemical analysis was done to test for the presence or absence of terpenoids and flavonoids (Pandey and Tripathi, 2014).

Test for saponins

The detection for saponins was carried out as described by Banu and Catharine, 2015. The extracts (50 mg) were diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. A two cm layer of foam was indicative of the presence of saponins.

Test for steroids

The extracts were dissolved in 2 ml acetic anhydride followed by careful addition of sulfuric acid. The color change from violet to blue or green in the samples indicated the presence of steroids (Kumar *et al*., 2013).

Test for tannins

About 250mg of the extracts were stirred with 5ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrates. Occurrence of a blue-black, green or blue-green precipitate indicated the presence of tannins (Selvi and Basker, 2012).

Test for alkaloids

The extracts (50mg) were dissolved individually in dilute hydrochloric acid (1% HCl) and filtered. Then, they were treated with Mayer's reagent (Potassium Mercuric Iodide), Wagner's reagent (Iodine in Potassium Iodide) and Dragendroff's reagent (solution of Potassium Bismuth Iodide) individually and were checked for the formation of a yellow/white colored precipitate, a brown/reddish precipitate and a red precipitate respectively for the indication of the presence of alkaloids (Pandey and Tripathi, 2014; Kumar *et al*., 2013; Selvi and Basker, 2012).

In case of the *E. brucei* extract, since it tested negative with the method described above and since it has been previously reported to contain alkaloids from a sample collected from the same site, it was tested again by employing a different method for confirmation. The extract was dissolved in 5% sulfuric acid and filtered. The obtained filtrate was treated with chloroform to remove undesirable matters. The acidic aqueous layer was adjusted to alkaline pH with ammonia solution and the liberated alkaloid bases were extracted with chloroform. After adding chloroform, it was shaken gently to allow the layer to separate. The lower chloroform layer was then taken as alkaloidal fraction and Wagner's reagent was added drop by drop to the filtrate and the formation of a reddish-brown precipitate indicated the presence of alkaloids (Francis *et al.,* 2015; Ewais *et al*., 2016).

Test for phenolic compounds

The plant extracts (50mg) were dissolved in 5ml distilled water. Then, few drops of 5% ferric chloride solution were added to the samples. The presence of a dark green color indicated the presence of phenolic compounds (Selvi and Basker, 2012; Swargiary and Brahma, 2017).

Test for flavonoids

The presence of flavonoids was tested by employing the lead acetate test as described elsewhere. To a solution of extract in distilled water, about 1 ml of 10% lead acetate solution was added. Production of yellow precipitate was considered as positive for flavonoids (Kujur *et al*., 2010).

Test for terpenoids

5mg of the extracts were mixed with 2ml of chloroform and 3ml of concentrated sulphuric acid (H_2SO_4) by carefully adding it to form a layer. The appearance of a reddish brown color in the interface indicated the presence of terpenoids (Vasait Rajendrabhai, 2017).

Test for anthraquinones

100mg of the plant extracts were boiled with 10ml of 1% HCl and filtered. The filtrates were shaken with 3ml of benzene and 2ml of 10% ammonia solution and the mixtures were filtered. The presence of anthraquinones was checked by the presence of pink, violet or red color in the ammonical phase of the solutions (Swargiary and Brahma, 2017).

Test for glycosides

50 mg of the extracts were hydrolysed with concentrated HCl for 2 hours on a water bath and were filtered. The filtered hydrolysates were subjected to Borntrager's test. To 2 ml of the filtered hydrolysates, 3 ml of chloroform was added and shaken. The chloroform layer was then separated and 10% ammomia solution was added to it. Pink color indicates the presence of glycosides (Banu and Catharine, 2015).

3.2.7 Data analysis

Statistical package for social science (SPSS) software version 20 was used for data entry and analysis purpose. The MIC results were presented as mean value. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple comparisons of results among groups (the extracts, the positive control and the negative control). The significance for the difference between the means of the MIC of the active plant extracts and rifampicin against *M. tuberculosis* and *M. bovis* strains were compared. The means of the percentage of growth inhibition of the mycobacteria (100% if resazurin is blue and 0% if resazurin is pink or colorless) obtained by the extracts were compared to the positive (rifampicin) and negative (vehicle and growth) controls. All the data were analyzed at 95% confidence interval and considered as statistically significant if the p value was ≤ 0.05. For data entry of the percent inhibition of the free radicals achieved by the *E. candelabrum* extract and the ascorbic acid along with the respective concentration, Microsoft Excel 2010 was used. The equation to calculate the IC_{50} was derived from the graphs.

4. Results

4.1 Anti-mycobacterial activity of the plant extracts

The 80% methanolic extract of *E. candelabrum* latex failed to show inhibition on the growth of mycobacteria against H37Rv and btb 33 strains at the highest concentration tested (25mg/ml). The 80% methanolic extracts of *O. integrifolia*, *R. abyssinicus* and *E. brucei* failed to show complete inhibition against H37Rv and were found to be active against btb 33 showing complete inhibition at the concentrations tested. However, the oil of *O. integrifolia* did not show any activity against both strains. On the contrary, the chloroform extract of *V. amygdalina* showed activity against both *M. tuberculosis* and *M. bovis* strains at the highest concentration tested (10mg/ml). Table 6 summarizes the activity of the plant extracts against H37Rv and btb 33 strains.

Table 6 - Activity of all plant extracts on H37Rv and btb 33 strains

S. No	**Plant species**	**Highest concentration tested (mg/ml)**	**Activity *on M. bovis***	**Activity on *M. tuberculosis***
1	*E. candelabrum* (latex)	25	Negative	Negative
2	*R. abyssinicus* (root)	25	Positive	Negative
3	*V. amygdalina* (leaf)	10	Positive	Positive
4	*O. integrifolia* (leaf) 80% methanol	25	Positive	Negative
	Oil	12.5	Negative	Negative
5	*E. brucei* (stem bark)	25	Positive	Negative

4.2 Minimum inhibitory concentration of the plant extracts

The visual MIC was defined as the lowest drug/extract concentration that prevented the color change of resazurin reagent from blue to purple/pink/colorless (Hassan and Ullah, 2017). The MICs of the 80% methanolic extracts of *O. integrifolia* leaf and *E.brucei* stem bark were found to be 12.5mg/ml and 25mg/ml and showed some inhibition at a concentration of 6.25mg/ml and 12.5mg/ml respectively against btb 33. The MIC of *R. abyssinicus* root was found to be 25mg/ml and showed 25-75% at 6.25mg/ml against btb33 while it showed some inhibition at 25mg/ml

against H37Rv strain. Since these extracts did not show complete inhibition against *M. tuberculosis* strain, further investigation was not done on other clinical isolates of *M. tuberculosis* strains. In case of the *E. candelabrum* extract and the oil of *O. integrifolia*, 100% growth was observed against both strains. The summary of the MICs of the plant extracts and rifampicin against *M. tuberculosis* and *M. bovis* strains is summarized in Table 7.

Table 7 – MIC of the plant extracts and rifampicin

S. No	**Plant extracts and rifampicin**		**MIC**	
			***M. bovis* (btb 33)**	***M. tuberculosis* (H37Rv)**
1	*E. candelabrum*		N. A	N. A
2	*O. integrifolia*	80 % methanol	12.5mg/ml	N. A
		Oil	N. A	N. A
3	*E. brucei*		25mg/ml	N. A
4	*V. amygdaluna*		10mg/ml	10mg/ml
5	*R. abyssinicus*		25mg/ml	N.A
6	Rifampicin		1 µg/ml	2 µg/ml

N.A – Not active

The color change of resazurin at different concentrations of the plant extracts showing varying degree of mycobacterial growth inhibition is summarized in Table 8 and also shown on the following diagrams for the active ones.

Table 8 – The color change of resazurin in the test tubes containing the inoculum and the extracts

Concentration mg/ml	***E. candelabrum***		***R. abyssinicus***		***O. integrifolia***		***E. brucei***	
	H37Rv	Btb 33	H37Rv	Btb 33	H37Rv	Btb 33	H37Rv	Btb 33
25	Pink	Pink	Purple	Blue	Pink	Blue	Pink	Blue
12.5	Pink	Pink	Pink	Purple	Pink	Blue	Pink	Purple
6.25	Pink	Pink	Pink	Purple	Pink	Purple	Pink	Pink
3.125	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink

Figure 9- The color change of resazurin in the presence of *E. brucei* extract against btb 33 strain

As shown on Figure 9, the test tubes containing different concentrations of *E. brucei* extract showed difference in the resazurin color change. Meaning, the test tube containing the highest concentration (25mg/ml) resulted in no color change (blue), showing 100% mycobacterial growth inhibition. The second test tube containing 12.5mg/ml changed into purple color showing 25-75% growth inhibition. The third and fourth test tubes containing 6.25 and 3.125mg/ml respectively changed into pink color showing 0% inhibition/100% growth.

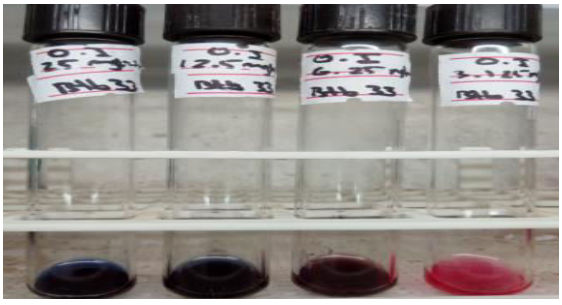

Figure 10 – The color change of resazurin in the presence of *O. integrifolia* extract against btb33 strain

As shown on Figure 10, the test tubes containing different concentrations of *O. integrifolia* extract also showed difference in the resazurin color change. The test tubes containing the concentrations of 25mg/ml and 12.5mg/ml resulted in no color change (blue), showing 100% mycobacterial growth inhibition. The third test tube containing 6.25mg/ml changed into purple color showing 25-75% growth inhibition. The fourth test tube containing 3.125mg/ml changed into pink color showing 0% inhibition/100% growth.

Figure 11- The color change of resazurin in the presence of *R. abyssinicus* extract against btb33 strain

In the test tube containing 25mg/ml of *R. abyssinicus,* as shown on Figure 11, resulted in no color change (blue), showing 100% mycobacterial growth inhibition. The second and third test tubes containing 12.5 and 6.25mg/ml respectively changed into purple color showing 25-75% growth inhibition. The fourth test tube containing 3.125mg/ml changed into pink color showing 0% inhibition/100% growth.

Regarding the chloroform extract of *V. amygdalina*, since it was the only extract which showed activity against both H37Rv and btb 33 strains, further investigation on its activity against other clinical isolates (03, 44 (h) and SO 0659) was conducted. The MIC was found to be 10mg/ml against all strains. But the concentration differed regarding the presence of some inhibition. The color change of resazurin in the presence of *V. amygdalina* is shown on the pictures (H37Rv and Btb 33) below and clearly summarized in Table 9.

Table 9 - Summary of the color changes of resazurin in the presence of *V. amygdalina* extract against the five strains

Concentration	***V. amygdalina* leaf (Chloroform extract)**				
mg/ml	**H37Rv**	**03**	**44 (h)**	**SO 0659**	**btb 33**
10	Blue	Blue	Blue	Blue	Blue
5	Purple	Pink	Purple	Pink	Purple
2.5	Purple	Pink	Purple	Pink	Pink
1.25	Purple	Pink	Purple	Pink	Pink
0.625	Purple	Pink	Purple	Pink	Pink
0.312	Pink	Pink	Pink	Pink	Pink

Figure 12 –The color change of resazurin in the presence of *V. amygdalina* extract against H37Rv strain

Figure 12 illustrates that the test tube containing 10mg/ml showed no color change (blue), hence, 100% growth inhibition. Starting from the second to the fifth test tube containing a concentration of 5mg/ml to 0.625mg/ml, the resulting color change was purple indicating 25-75% inhibition. The last test tube containing 0.312mg/ml was pink showing 0% growth inhibition.

Figure 13 – The color change of resazurin in the presence of *V. amygdalina* extract against btb 33 strain

Figure 13 elucidates that the test tube containing 10mg/ml of *V. amygdalina* showed no color change (blue), meaning 100% growth inhibition. The second test tube containing 5mg/ml exhibited 25-75% inhibition as the resulting color change was purple. Starting from the third to the last test tube containing a concentration of 2.5mg/ml to 0.312mg/ml, the resulting color change was pink showing 0% growth inhibition.

Regarding the activity of the positive control rifampicin on the five strains, since all the mycobacterial strains used were rifampicin sensitive, there was inhibition of mycobacterial growth at low concentrations. The color change of the resazurin containing the inoculum and different concentration of rifampicin as well as the negative (vehicle) control and growth control of the different strains is summarized in Table 10.

Table 10 - Summary of the color changes of resazurin in the presence of rifampicin and the vehicles against the five strains

Controls	**Concentration**	**Color change of resazurin in the test tubes**				
	(µg/ml or %)	**H37Rv**	**03**	**44 (h)**	**SO 0659**	**Btb 33**
Rifampicin (positive control)	16 µg/ml	Blue	Blue	Blue	Blue	Blue
	8 µg/ml	Blue	Blue	Blue	Blue	Blue
	4 µg/ml	Blue	Blue	Blue	Blue	Blue
	2 µg/ml	Blue	Blue	Blue	Blue	Blue
	1 µg/ml	Blue	Pink	Pink	Pink	Blue
	0.5 µg/ml	Pink	Pink	Pink	Pink	Pink
Negative controls (vehicles)	10% ethanol	Pink	Pink	Pink	Pink	Pink
	10% methanol	Pink-colorless	_	_	_	Pink
Growth control	_	pink	Pink - colorless	colorless	colorless	pink

Figure 14 – The color of resazurin in the test tubes containing MIC of rifampicin against the mycobacterial strains.

Shown on the above picture (Figure 14), are the test tubes which contain the MICs of the positive control rifampicin against H37Rv, btb 33, SO 0659, 03 and 44(h) strains. As the mycobacterial strains used were rifampicin sensitive, there was complete inhibition of mycobacterial growth with a low MIC ranging from 1-2µg/ml.

Figure 15 – The color change of resazurin in the presence of 10% methanol and 10% ethanol against the mycobacterial strains (Vehicle control).

In Figure 15, the color change of resazurin in the test tubes containing the vehicles 10% methanol and 10% ethanol shows their activity on the different mycobacterial strains. As shown above, the resulting color changes are pink and pink-colorless in all test tubes indicating that the vehicles are inactive against mycobacteria as it shows 0% growth inhibition.

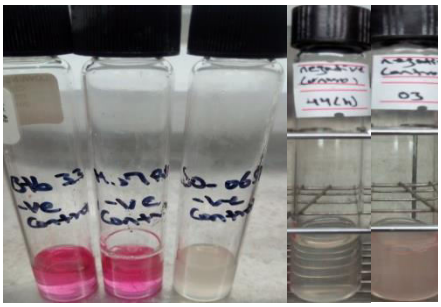

Figure 16 – The color change of resazurin in the test tubes containing only the inoculum (Growth control for the mycobacterial strains)

The color change of resazurin in the test tubes containing the inoculum alone (growth control), as shown in Figure 16, resulted in pink and colorless color changes. This also shows 0% inhibition/100% mycobacterial growth in the absence of the active plant extracts or the positive control rifampicin.

Table 11 – The significance of mean difference among groups in the percentage of mycobacterial growth inhibition

Negative control vs active extracts	Mean difference	Significance	Positive control vs active extracts	Mean difference	Significance
NC vs Ebb	-100.00000*	.000	PC vs Ebb	.00000	1.000
NC vs Rab	-100.00000*	.000	PC vs Rab	.00000	1.000
NC vs Oib	-100.00000*	.000	PC vs Oib	.00000	1.000
NC vs Vab	-100.00000*	.000	PC vs Vab	.00000	1.000
NC vs Va44	-100.00000*	.000	PC vs Va44	.00000	1.000
NC vs Vah37	-100.00000*	.000	PC vs Vah37	.00000	1.000
NC vs Vaso	-100.00000*	.000	PC vs Vaso	.00000	1.000
NC vs Va03	-100.00000*	.000	PC vs Va03	.00000	1.000

NC - Negative control, PC - Positive control, Ebb – *E. brucei* against btb33 strain, Rab – *R. abyssinicus* against btb33 strain, Oib – *O. integrifolia* against btb33 strain, Vab – *V. amygdalina* against btb33 strain, Va44 – *V. amygdalina* against 44(h) strain, Vah37 – *V amygdalina* against H37Rv strain, Vaso – *V. amygdalina* against SO 0659 strain, Va03 - *V. amygdalina* against 03 strain, * The mean difference is significant at $p \leq 0.001$.

As shown in Table 11, the mean difference in the percentage of mycobacterial growth inhibition between the negative controls and the chloroform extract of *V. amygdalina* was statistically significant at $p \leq 0.001$. The mean difference in the ability of the 80% methanolic extract of the plants *E. brucei*, *R. abyssinicus* and *O. integrifolia* to inhibit growth of *M. bovis* were also statistically significant at $p \leq 0.001$ while being compared to the negative controls. There wasn't a significant mean difference in the ability to inhibit growth of mycobacteria (100% inhibition of mycobacterial growth) between the active plant extracts (*V. amygdalina* against all strains and *E. brucei*, *R. abyssinicus* and *O. integrifolia* against *M. bovis*) and the positive control rifampicin showing a comparable activity. However, there was a significant mean difference between the MICs of the extracts and the MICs of rifampicin. While comparing the mean difference of the extracts' MIC with each other, there was a significant mean difference between the MIC of the *V. amygdalina* extract and the MICs of the other extracts at $p \leq 0.001$.

4.3 Anti-oxidant activity and IC_{50} of *E. candelabrum*

The equation to calculate the IC_{50} was derived from the graphs. The following graphs (Figure 17 and Figure 18) show the percentage inhibition of free radicals in the presence of ascorbic acid and *E. candelabrum* with their respective concentration.

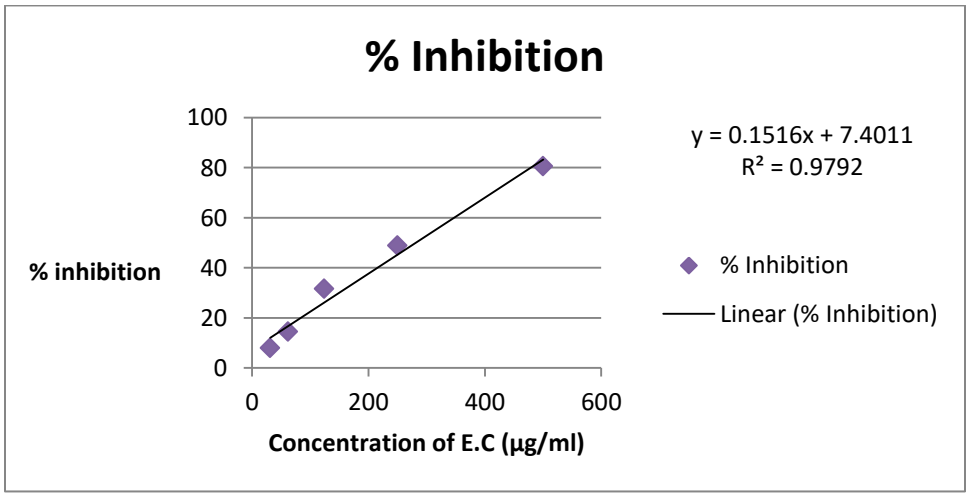

Figure 17 – A graph of the percentage inhibition of free radicals at different concentrations of *E. candelabrum* extract

As shown on Figure 17, the free radical scavenging activity of the crude extract of *E. candelabrum* is concentration dependent. The maximum percentage of inhibition of free radicals

was achieved at the highest concentration (500µg/ml) of *E. candelabrum* used. The percentage of inhibition was 80.6 % at the highest concentration used and 8% at the lowest concentration (31µg/ml) used. The IC_{50} of *E. candelabrum* calculated from the equation derived from the graph was found to be 281µg/ml.

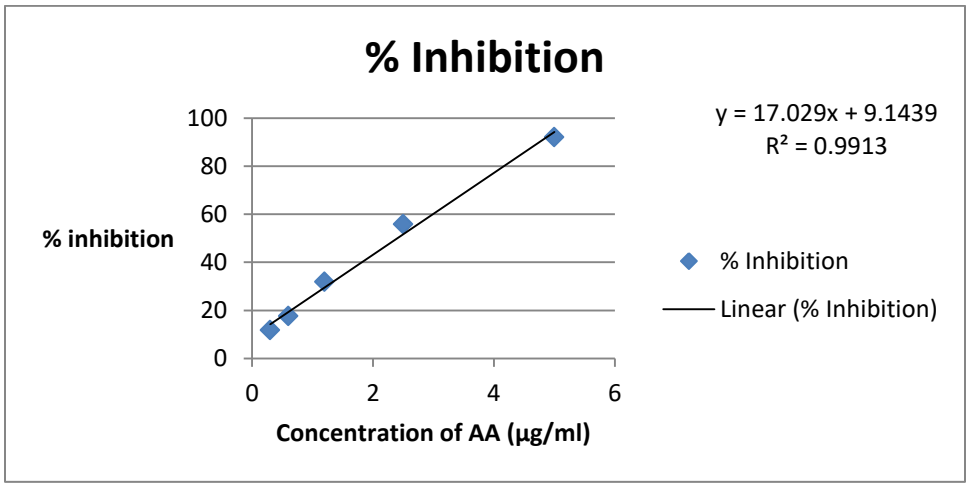

Figure 18 – A graph of the percentage inhibition of free radicals at different concentrations of ascorbic acid

Just like that of the *E. candelabrum* extract, the ascorbic acid also exhibited a concentration dependent inhibition of free radicals showing a linear relationship between the concentrations used and the percentage inhibition of free radicals achieved as illustrated in Figure 18. The maximum percentage inhibition of free radicals obtained was 92% at a concentration of 5µg/ml while the minimum percentage inhibition obtained was 11.8% at a concentration of 0.3µg/ml. The IC_{50} of ascorbic acid was calculated from the equation derived from the graph and was found to be 2.4µg/ml.

4.4 Preliminary phytochemical screening

The preliminary phytochemical analysis of the plant extracts tested revealed that *E. candelabrum* and *E. brucei* contain phenols, saponins, steroids, flavonoids and terpenoids. Tannins, glycosides and anthraquinones were found to be absent in these extracts according to this experiment. Alkaloids were found to be present in *E. brucei* while being absent in the *E. candelabrum* extract. Saponins, tannins, flavonoids, anthraquinones, terpenoids and phenols were found to be present in the *R. abyssinicus* extract while steroids, glycosides and alkaloids were absent.

Regarding the *O. integrifolia* extract, saponins, steroids, tannins, flavonoids, terpenoids and phenols were present while it tested negative for the presence of alkaloids, glycosides and anthraquinones. The chloroform extract of *V. amygdalina* tested positive for the presence of terpenoids while it tested negative for the presence of flavonoids. The preliminary phytochemical screening results are summarized in Table 12 for *E. brucei*, *E. candelabrum, R. abyssinicus* and *O. integrifolia* extracts.

Table 12 – Preliminary phytochemical screening results

Secondary metabolites	***E. brucei***	***E. candelabrum***	***R. abyssinicus***	***O. integrifolia***
Saponins	+++	+++	+++	++
Steroids	+++	++	_	+++
Tannins	_	_	++	+++
Glycosides	_	_	_	_
Flavonoids	+++	+	+	++
Alkaloids	+	_	_	_
Anthraquinones	_	_	+++	_
Terpenoids	+	++	+	+++
Phenoles	+++	++	++	+++

+++: highly present, ++: moderately present, +: Low, -: absent

5. Discussion

The choice of solvents for extraction of these plants (*E. candelabrum, O. integrifolia, E. brucei and R. abyssinicus*) was based on what is reported on different studies for testing anti-mycobacterial activity. As stated elsewhere, even though the most common solvent used by traditional healers is water; its ability to extract non-polar compounds is limited so the use of solvents which are able to extract all compounds covering all range of polarity like methanol is recommended. Methanol extract may contain a wide range of chemical compounds with biological activity such as terpenoids, phenols, flavonoids, saponins, steroids, and others (Singh, 2016; Robles-Zepeda *et al*., 2013). Methanol, either absolute or 80%, has been employed for extraction of plants for *in vitro* anti-mycobacterial activity testing on different researches (Gemechu *et al*., 2013; Mariita *et al*., 2010; Balcha *et al*., 2014; Ehsanifar *et al.*, 2017). Studies have reported that methanol extracts are more active than aqueous extracts in their antibacterial activity (Robles-Zepeda *et al*., 2013).

The extraction method as well as the solvent choice for the plant *V. amygdalina* was different from the others because the base for investigating this plant's activity was not only based on the traditional reports. Based on another study, the chloroform fraction of *V. amygdalina* obtained through successive soxhlet extraction was reported to be active against *M. bovis* strain and was not tested against *M. tuberculosis* strain so this extraction method was simulated (Tadesse *et al*., 1993). Regarding the plant *O. integrifolia*, the oil was also tested in addition to the 80% methanol extract for its *in vitro* anti-mycobacterial activity. This was done because essential oils from different plants containing caryophyllene and α-pinene have been reported for their anti-mycobacterial activity (Runde *et al*., 2015; Bueno *et al*., 2011; Aşkun *et al*., 2010). It was reported that α-pinene and trans-caryophyllene are among the main components present in the essential oil of *O. integrifolia* (Tadesse *et al*., 2011).

As investigated in this study, out of the five plant extracts, the 80% methanol extract of *E. candelabrum* was found to be inactive against both *M. tuberculosis* and *M. bovis* strains. On the contrary, the chloroform extract of *V. amygdalina* was found to have an activity against all the strains tested. The 80% methanol extracts of *E. brucei* and *O. integrifolia* have shown activity against *M. bovis* strain but not against H37Rv. The oil of *O. integrifolia* did not exhibit any activity against both strains. Regarding the crude extract of *R. abyssinicus*, even though it did not

show complete inhibition against the H37Rv strain, there wasn't complete growth either while being compared to the growth control and the negative control. However, it was found to be active against the *M. bovis* strain showing 100% mycobacterial growth inhibition.

It was reported that *E. candelabrum* is one of the locally available plant in Uganda used in the treatment of bovine TB. However, it was also mentioned that it was observed from the respondents' response in that area that they didn't differentiate cough due to TB and cough due to other bacterial infections there by offering the same local remedies in both cases (Nasaka, 2014). According to the current finding, the *E. candelabrum* extract was found to be inactive against these strains at a maximum concentration of 25mg/ml. However, this result cannot be conclusive of its activity *in vivo* in treating TB for the reasons partly mentioned in the following paragraphs.

As stated elsewhere, *in vitro* assays utilizing pathogens such as *M. tuberculosis* (H37R*v*) for anti-TB evaluation represent the most feasible method to determine the underlying biological activity of traditional medicines. However, due to the complexity of the human body, it cannot be assumed that *in vitro* bioassay results can directly translate to human systems making it difficult for conclusion (Case, 2006). A possible explanation of why *in vitro* bioassay results may not translate to human systems could in part be that the anti-mycobacterial effect of a plant may be mediated through immunomodulation rather than direct inhibition of mycobacterial growth. The potential active compounds may also need to be metabolically activated *in vivo* by specific enzymes or may have a pH dependent biological activity (Singh, 2016).

Recent studies have also indicated that new treatment regimens incorporating therapeutics targeting both *M. tuberculosis* and host factors are needed for improving the outcomes of MDR-TB (Zumla *et al*., 2016). They are being developed to improve survival, accelerate eradication of infection and to prevent permanent lung injury and long-term functional disability (Wallis *et al*., 2016; Zumla *et al*., 2016). Consequently, as described by Case (2006), the plant species may be beneficial in the treatment of TB for many other reasons apart from direct anti-mycobacterial activity like providing symptomatic relief from cough or have immune modulating effects).

As stated by O'Connor *et al*. (2016), antioxidants have garnered interest for the treatment of TB for protective properties. Although not traditionally considered as anti-bacterial or

immunomodulatory agents, they have protective properties that may render them useful as a HDT strategy for TB. As investigated here, this plant extract (*E. candelabrum*) was found to have an anti-oxidant activity. This can also serve as one explanation for using it traditionally since many literatures recommend patients with TB to take anti-oxidant supplements because it can prevent oxidative stress there by improving the recovery of the TB patients (Reddy *et al*., 2004). Therefore, the negative results obtained cannot rule out the potential of *E. candelabrum* latex to treat TB traditionally. These explanations may also apply to the other plant extracts experimented here as these are also used *in vivo* traditionally.

Regarding the phytoconstituents of *E. candelabrum* extract, tannins, alkaloids, glycosides and anthraquinones were found to be absent. It is stated that alkaloids are not common in Euphorbiaceae family, which may be why it was found to be absent in this plant extract (Salatino *et al*., 2007). Diterpenoids, triterpenoids, steroids, flavonoids and phenolic compounds have all been reported as potentially active compounds for different Euphorbia species (Dharani *et al*., 2014). The preliminary phytochemical screening showed the presence of terpenoids, steroids, flavonoids and polyphenols. The anti-oxidant activity seen with this plant extract may be associated with the presence of phenolic compounds and flavonoids. Plant polyphenols act as reducing agents and antioxidants by the hydrogen donating property of their hydroxyl groups (Mahdi-Pour *et al*., 2012). However, the presence of flavonoids was low while phenols were moderately present which may be why the plant extract showed weak anti-oxidant activity with an IC_{50} of 281µg/ml. According to Jadid *et al.* (2017), extracts possessing IC_{50} values ranging from 50 - 100µg/ml are considered to exhibit intermediate antioxidant activity, IC_{50} value ranging between 10 - 50µg/ml is considered to possess strong antioxidant activity and those with IC_{50} >100µg/ml are considered to possess weak anti-oxidant activity.

In case of the anti-mycobacterial activity of *R. abyssinicus*, as the resazurin color change observed after incubating it overnight was purple, it can't be considered as 100% inhibition so MIC was not determined for this plant against the *M. tuberculosis* strain but it showed complete inhibition against *M. bovis* strain with a MIC of 25mg/ml. The preliminary phytochemical screening in this research showed the presence of saponins, phenols, tannins, flavonoids, anthraquinones and terpenoids. All these phyto-constituents have been reported to possess anti-mycobacterial activity so the inhibition seen here may be due to the synergistic activity of these

phyto-constituents (Nguta *et al*., 2015(a)). According to literatures, a number of anthraquinone derivatives have also been tested against *M. tuberculosis* and some were found to be active (Muriuki *et al*., 2013). The observed inhibition may also be related to the presence of anthraquinones since different studies have found *R. abyssinicus* to contain a number of anthraquinones and it was also found to be highly present in the preliminary phytochemical screening conducted in this research (Girma *et al*., 2015).

Regarding the extract of the plant *E. brucei*, it was found to be active against the *M. bovis* strain. *Erythrina abyssinica* and *Erythrina Indica,* species from the same genus*,* were also found to show an anti-mycobacterial activity against different mycobacterial strains (Bunalema *et al*., 2011; Waffo *et al*., 2000). Indicanine B, an isoflavonoid isolated from *Erythrina Indica*, and phenolic fractions were reported to be responsible for the anti-mycobacterial activity seen against *M. smegmatis* (Waffo *et al*., 2000). 3-hydroxyisoflavanone compounds, which were isolated from the stem bark of *Dalbergia melanoxylon* (family-Fabaceae), have been tested for their anti-mycobacterial activity and were found to show an activity against *mycobacteria* (Mutai *et al*., 2013). The preliminary phytochemical screening in this research revealed the presence of flavonoids, steroids, saponins, polyphenols, alkaloids and terpenoids. The combination of these different phyto-constituents/compounds may be responsible for the anti-mycobacterial activity seen against the *M. bovis* strain. However, further studies are needed to isolate and identify the compounds which are responsible for the observed anti-mycobacterial activity in the plant *E. brucei.*

Regarding *V. amygdalina*, the ethanolic extract has been reported to contain flavonoids, cardiac glycosides, reducing sugar, terpenoids, saponins, alkaloids and steroids (Evbuomwan *et al.*, 2018). It's stated elsewhere that terpenoids are one of the active components that can be found in chloroform extract (Pandey and Tripathi, 2014). It is also reported that secondary metabolites of terpenoids origin lead the number of natural products with reported anti-mycobacterial activity due to their lipophilic nature and therefore ability to penetrate the mycobacterial cell wall (Bunalema, 2010). Flavonoids, found in *V. amygdalina,* are also one of the active components aside from terpenoids found in chloroform extract (Okoduwa *et al*., 2016; Pandey and Tripathi, 2014). This was why the preliminary phytochemical screening for the chloroform extract of *V. amygdalina* was conducted only for the presence of these two phytoconstituents. The preliminary

phytochemical screening of the extract revealed the presence of terpenoids while it was negative for the presence of flavonoids. So the anti-mycobacterial activity of this plant seen here is possibly related to the presence of terpenoids rather than flavonoids.

It is stated elsewhere that terpenoid lactones have been obtained by successive extractions of plant samples with hexane, chloroform and methanol with activity concentrating in chloroform fraction (Tiwari *et al*., 2011). Sesquiterpenes are C-15 terpenoids that occur in nature as hydrocarbons or oxygenated forms such as alcohols, ketones, aldehydes, acids or lactones (Luna-Herrera *et al*., 2007). Sesquiterpene lactones are a large group of natural compounds which are primarily found in plants of *Asteraceae* family, with over 5000 structures reported to date (Ivanescu *et al*., 2015).

As mentioned earlier, the family Asteraceae contains species with a high concentration of sesqueterpene lactones identified for their wide variety of biological activities, including their anti-mycobacterial effect. Species of the genus Ambrosia (Family-Asteraceae) have been previously reported for their anti-tuberculosis activity, associated to sesquiterpene lactones (Robles-Zepeda *et al*., 2013). Two sesquiterpene lactones which were isolated from *Ambrosia confertiflora,* Reynosin and santamarine, were found to display a mycobactericidal effect against some clinical isolates of *M. tuberculosis* and the reference *M. tuberculosis* H37Rv strain (Coronado-Aceves *et al*., 2016). Costunolide and dehydrocostus lactone, sesqueterpene lactones from Madeira Laurus oil, presented an important inhibitory action against both drug-susceptible and drug-resistant *M. tuberculosis* strains at MICs as low as 6.25 mg/L (range 50–6.25 mg/L) (Luna-Herrera *et al.,* 2007).

Just like that of the other plants in the Asteraceae family, *V. amygdalina* is known to contain different types of sesquiterpene lactones. The active components of the plant have been shown to be mainly sesquiterpene lactones and steroid glycosides (Ojiako and Nwanjo, 2006). Some of the isolated sesqueterpene lactones from *V. amygdalina* include; vernolide, vernodalol, vernolepin, vernodalin, vernomygdin, hydroxyvernolide, vernodalinol, vernomenin, vernolic, 11, 13-dihydrovernodalin, 11, 13-dihydrovernorodeline, 4, 15- dihydrovernodalin, 1, 2, 3, 15, 11, 13, 2', 3'-octahydrovernodalin and epivemodalol (Clement *et al*., 2014; Luo *et al*., 2011; Erasto *et al.,* 2006). So the anti-mycobacterial activity of the chloroform extract of *V. amygdalina* leaf may be

related to the presence of sesqueterpene lactones as they are known compounds to be present in this plant.

As stated previously, one of the biggest concerns with the currently used conventional anti-tuberculosis drugs other than development of resistance is the risk of toxicity. In a previous study, the ethanolic extracts of *V. amygdalina* leaves were shown to protect liver intoxication caused by a combination of the conventional anti-tuberculosis drugs (isoniazid and rifampicin). It was concluded that *V. amygdalina* ethanol extract had a hepatoprotective effect and can be used to protect the liver in combination with these drugs as anti-tuberculosis treatment (Iwo *et al*., 2017). It was also found on different studies that *V. amygdalina* leaf extract had hepatoprotective properties (Sitorus and Nerdy, 2018; Ojiako and Nwanjo, 2006). The hepatocurative potential of sesquiterpene lactones from *Taraxacum officinale* roots (Family - Asteraceae) was evaluated and they were found to have a hepatoprotective effect against acute hepatotoxicity (Mahesh *et al.,* 2010). So, different sesqueterpene lactones from the Asteraceae family were not only reported for their anti-mycobacterial activity but also for their hepatoprotective potential. However, further investigations are required to see the anti-mycobacterial and hepatoprotective activity of sesqueterpene lactones isolated from *V. amygdalina*.

The inflammation process during TB infection generates powerful ROS, making antioxidant supplementation important (Kulkarni and Deshpande, 2016). As the findings of Mohod *et al.* (2011) suggest, suitable antioxidant supplementations are required to protect TB patients from free radical attack. On the other hand, the anti-oxidant activity of *V. amygdalina* extract had been investigated and it was reported that it exhibited a significant free radical scavenging activity (Adesanoye and Farombi, 2014; Alara *et al*., 2018). Sesquiterpene lactones (vernolide and vernodalol) isolated from *V. amygdalina* have also been found to exhibit appreciable antioxidant activities as manifested through their free radical (DPPH) scavenging properties (Erasto *et al*., 2007). Aside from *V. amygdalina*, the other plant extracts (*R. abyssinicus, O. integrifolia and E. brucei*) have also been reported for their anti-oxidant activity (Mohammed *et al*., 2017; Chekol and Desta, 2018; gurmessa *et al*., 2018). The *E. candelabrum* extract was also found to have an anti-oxidant activity as shown here. So, other than having an anti-mycobacterial activity, the

plant extracts may also be able to protect patients with TB from free radical attack by serving as an anti-oxidant supplement.

Out of all the plant extracts investigated here, it was shown that the chloroform extract of *V. amygdalina* leaf has the lowest MIC (10mg/ml) compared to the others. According to Bunalema *et al*. (2011), the chloroform extract of *Erythrina abyssinica* was reported to contain only terpenoids, which could explain why the extract was most active on *M. avium* and a rifampicin resistant strain. It is stated in literatures that the more anti-mycobacterial activity of extracts depend on their lipophilic nature, since the mycobacterial cell wall is lipid-rich and hydrophobic, hydrophobic compounds tend to permeate better and have more efficacy (Gemechu *et al*., 2013; Assefa *et al*., 2017; Primm and Franzblau, 2007). Again on another study, it was found that highly polar molecules exhibit a reduced transport through the outer lipid layer of mycobacteria and, in consequence, lower anti-mycobacterial activity, whereas less polar molecules exhibit higher permeability. So in that study, the results evidenced a higher activity with the chloroform extract compared to dichloromethane, ethyl acetate and methanol extracts (Robles-Zepeda *et al*., 2013). The chloroform extract was found to contain terpenoids in this research. So this may be one reason for the chloroform extract of *V. amygdalina* to show a lower MIC compared to the other plant extracts investigated here.

On the contrary, it is also stated that the problem with many of the studies on medicinal plants is the common misinterpretation of the researcher that a single active compound is responsible for biological activity which ignores the concept of synergy (Case, 2006). This may be one possible explanation of why the essential oil of *O. integrifolia* was found to be inactive and the crude methanol extract of *O. integrifolia* was found to be active at MIC of 12.5 mg/ml. Different compounds of flavonoids, terpenoids, phenols, steroids, saponins and tannins from different plants have been reported to possess anti-mycobacterial activity (Case, 2006, Nguta *et al*., 2015(a)). According to the preliminary phytochemical screening done here, flavonoids, terpenoids, phenols, tannins, steroids and saponins have all been found to be present. So, the activity of the crude methanol extract of *O. integrifolia* leaf may be attributed to the synergistic anti-mycobacterial activity of these different phytoconstituents. However, further investigation is required to test the anti-mycobacterial activity of solvent fractions in order to conclude that the activity seen here is due to synergism.

The MIC of the chloroform extract of *V. amygdalina* leaf against *M. tuberculosis* and *M. bovis* strains investigated here (10mg/ml) is much higher than the MIC stated elsewhere against *M. bovis* (Tadesse *et al.,* 1993). This difference may be accounted to the different *in vitro* experimental methods employed. One difference in the experimental method is that the later was done by employing a radiorespirometric assay technique on a different *M. bovis* strain and not on *M. tuberculosis* strains. The other reason for the difference in their MIC may be attributed to the difference in the concentration of ethanol used to solubilize the extract. In case of the later, it was only stated that the extract was solubilized using ethanol but there was no other information given regarding the concentration of ethanol used and in case of this experiment, a concentration of 10% ethanol in distilled water was used after checking the activity of different concentrations of ethanol.

According to different studies on anti-mycobacterial activity of different plant extracts, there have been different criteria regarding the concentrations to be reported as active. In previous studies, different plant extracts were considered active against *M. tuberculosis* while MIC was ≤ 100µg/mL (Gemechu *et al*., 2013), ≤ 200µg/mL (Robles-Zepeda *et al*., 2013), 3.1-12.5mg/ml (Askun *et al*., 2012), 3.1mg/ml (Eldeen and Van Staden, 2008). A study by Balcha *et al.* (2014) on several medicinal plants in Mekelle, Ethiopia (the starting dose being 1000mg/ml) also reported for some of the extracts to have an anti-mycobacterial activity at MIC of 250mg/ml and 12.5mg/ml (the active ones being *Allium ursinum, Pterolobium Stellatum* and *Dodonaea anguistifolia*). So, compared to that study, the MIC in the present study was found to be very much lower.

According to Ehsanifar *et al*. (2017), the methanol extract of *Capparisspinpsa* fruit was reported to be effective with MIC of 25 mg/ml on several clinical samples including the standard strain H37RV and on another sample, MIC of 10 mg/ml was reported to be effective. This coincides with the MIC of *E. brucei* and *R. abyssinicus* against *M. bovis* strain and *V. amygdalina* against all strains tested, respectively in this study. Results on another research showed that from *Tinospora crispa* (Brotowali) extract and fractions tested, the ethanolic extract exhibited anti-tuberculosis activity against the tested strains with MIC of 12.5mg/ml by which this MIC coincides with the MIC of *O. integrifolia* against *M. bovis* (Wahyuningrum *et al.,* 2017).

According to the study by Wahyuningrum *et al*. (2017), the antimicrobial activity of plant extracts was classified as significant (MIC ≤100µg/mL), moderate (100 µg/ml < MIC < 625µg/mL), or weak (MIC > 625 µg/mL). But as explained elsewhere, even though the bioassay screening of several plant extracts tested showed activity at ≤ 2000µg/ml, it was also pointed out that those with higher MICs cannot equally be ruled out as these may be sources of prodrugs which may be able to produce better activity *in vivo* or if screened by other technique may equally give better results (Nvau *et al*., 2011). So, based on the criteria of Wahyuningrum *et al.* (2017), even if it falls under weakly active because the MICs in this research are much greater than 625µg/ml, the finding in this study cannot be ruled out because of the reasons partly stated above.

As shown on this research, the 80% methanol extracts of *E. brucei*, *O. integrifolia* and *R. abyssinicus* were found to show an activity against *M. bovis* strain and not against *M. tuberculosis* at the maximum concentration tested. This may be linked to difference in susceptibility of these strains. It was previously reported that *M. bovis* was the most susceptible while being compared to that of the *M. tuberculosis* and *M. kansasii* strains which showed lesser susceptibility towards some drugs (Wong *et al*., 1988). So, the *M. tuberculosis* strains may be susceptible to these plant extracts if the concentration were to be further escalated.

Regarding the phytochemical screening of the plant extracts, some results coincide with previous phytochemical screening reports while some don't. For instance, regarding the phytoconstituents of 80% methanolic extract of *O. integrifolia* leaves*,* previous reports from different areas have reported tannins and steroids to be absent while glycosides were reported to be present by which these results do not coincide with the results seen here (Chekol and Desta, 2018; Shewamene *et al*., 2015). Regarding the 80 % methanol extract of the rhizomes of *R. abyssinicus,* it was previously reported that it possesses secondary metabolites such as tannins, saponins, flavonoids, steroids and anthraquinones (Mekonnen *et al*., 2010). The results mostly coincide with what the preliminary phytochemical screening of *R. abyssinicus* revealed here except for the presence of steroids which was absent here. The difference in the results of the phytochemical screening with previous reports may be attributed to different factors as explained below.

Chemical constituents and biological activities of plants have been shown to be affected by geographical location, soil types, growing conditions, nutrient availability, fertilizer applications,

plant's age, season and climate (Khattak and Rahman, 2015; Muraina *et al*., 2008; Verma and Shukla, 2015). Even though plants require light, temperature, soil and minerals for their growth and survival; more or less amount of these abiotic components can cause stress which may lead to variation in the production or accumulation of secondary metabolites (Verma and Shukla, 2015). Different agro-climatic conditions have been shown to have an impact on the phytochemical diversity and antioxidant potential of the plant Aloe vera (Kumar *et al*., 2017). Another study showed that there is a difference in antimicrobial activity of a plant *Cochlospermum regium* which showed the impact of different seasons, plant age and cultivation substrate on biological activity (Inácio *et al*., 2016). Difference in altitude has also been reported to have an impact on terpenoid biosynthesis and oxygenated monoterpenes resulting in different composition of an essential oil constituent of a plant species *Kundmannia anatolica* (Şanli and Karadoğan, 2017).

It was previously stated that studies had mostly reported activity in the plant families of Asteraceae, Lamiaceae, Fabaceae and Apiaceae among others (Bunalema, 2010; Assefa *et al*., 2017). According to this experiment, out of these five plant families (namely; Asteraceae, Lamiaceae, Fabaceae, polygonaceae and Euphorbiaceae) investigated, Asteraceae (*V. amygdalina*), Lamiaceae (*O. integrifolia*), Fabaceae (*E. brucei*) and polygonaceae (*R. abyssinicus*) have shown activity against mycobacteria.

6. Limitations

Even though the MIC was determined, determination of the minimum bactericidal concentration for the active plant extracts was not possible due to the limitation of resources. The other limitation of this research associated with limited resource was that the plant extracts which were active against *M. bovis* strain were not tried with a further escalated dose for their activity against *M. tuberculosis*. As the anti-mycobacterial assay carried out was an *in vitro* assay, it is somewhat difficult to be conclusive of the results to directly correlate with the traditional use as the plants are used *in vivo*.

7. Conclusion

Based on the results of this study, it has been shown that there may be a possibility to develop new compounds against mycobacterial strains from extracts of *V. amygdalina*. Regarding the extracts of *R. abyssinicus*, *O. integrifolia* and *E. brucei,* they have shown anti-mycobacterial activity against *M. bovis* strain. Hence, these plants should be further evaluated for the development of anti-tuberculosis drugs. However, the 80 % methanolic extract of *E. candelabrum* latex was not found to have an anti-mycobacterial activity. But it can't be concluded as having a negative correlation with the traditional knowledge because it may aid in the treatment of TB through other mechanisms rather than direct inhibition of mycobacterial growth. It may be beneficial by acting through other host directed therapeutic mechanisms as it was found to have an anti-oxidant activity.

8. Recommendation

- Further *in vitro* and *in vivo* studies are recommended to see the anti-mycobacterial effect of the chloroform extract of *V. amygdalina* and compounds isolated from it.
- Additional experiments are needed to evaluate the effects of *R. abyssinicus*, *O. integrifolia* and *E. brucei* extracts on mycobacterial strains.
- *In vivo* investigation of the plant *E. candelabrum*, since an *in vitro* test may not be conclusive of its activity *in vivo*.

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