Activity Testing, Toxicity Assay and Characterization of Chemical Constituents of Medicinal Plants Used to Treat Tuberculosis in Ethiopian Traditional Medicine.

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This is to certify that the thesis prepared by Wubayehu Kahaliw, entitled: *Activity Testing, Toxicity Assay and Characterization of Chemical Constituents of Medicinal Plants Used to Treat Tuberculosis in Ethiopian Traditional Medicine* and submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy (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 the leading killer disease worldwide. In 1993, WHO declared TB as a 'global emergency,' which requires emergency action and launched several programs to curb the disease, including the search for newer remedies and/or anti-TB agents to complement currently used agents. Hence, herbal remedies have become the readily available alternatives in the search for new antimycobacterial compounds.

Objective: To investigate antimycobacterial activity and toxicity of selected Ethiopian medicinal plants (*Otostegia integrifolia*, *Vernonia amygdalina*, *Persea americana, Pterolobium stellatum* and *Carissa edulis*) as well as to isolate the main active principles through a bioassay guided process.

Methods: Antimycobacterial activity test was conducted using the broth microdilution and microtitre resazurin assay methods in 96 well microtitre plates and MIC was determined by colony counting and resazurin color change observation for all test materials. Cytotoxicity test was performed based on the CellTiter 96® AQueous One Solution Cell Proliferation Assay on $HepG_2$ cells. Genotoxic effects of extracts were evaluated using SCGE method on $HepG_2$ cells.

Results: Chloroform and 80% methanol extracts of *P. stellatum* and *O. integrifolia* as well as 80% methanol and acetone extracts of *P. americana* had significant antimycobacterial activity ($p < 0.001$) against *M. tuberculosis* H37Rv, while chloroform extract of *V. amygdalina* and *C. edulis* didn't show significant activity compared to negative controls. The MIC of positive control was 0.125 µg/ml against the standard strain. However, MDR-TB clinical isolates were isoniazid resistant. Fractionation and activity testing of the chloroform extract of *P. stellatum* revealed that ethyl acetate

fraction to be the most active fraction against *M. tuberculosis* H37Rv with MIC of 0.195 µg/ml. The MICs of compound 1, 2 and 3 were 1.25, 2.5 and 0.625 µg/ml, respectively. In the cytotoxicity test, *V. amygdalina* chloroform extract showed the highest IC_{50} value (3.202±0.3375), which suggests its safety. *O. integrifolia* and *P. stellatum* chloroform extracts were the most toxic in dose dependent manner as one can see the steepness of the dose-response curve. DNA damage in the form of comet tail has been observed for 1 and 0.5 mg/ml *P. stellatum* chloroform and 80% methanol extracts on $HepG_2$ cells, respectively. The rest of test extracts seemed to be without genotoxic effect up to a concentration of 0.5 mg/ml. Cytotoxicity test was not in the objectives of my study.

Conclusion: *P. stellatum, O. integrifolia* and *P. americana* have potential to be developed into new anti-TB drugs or standardized herbal medicines. *P. stellatum* chloroform extract was the most active extract and hence, three compounds were isolated from ethyl acetate fraction and they were active against *M. tuberculosis* H37Rv. The results have also validated indigenous medical knowledge from the local people regarding the use of these species to treat TB. The IC_{50} value of *P. stellatum* chloroform extract was relatively higher compared to other extracts, suggesting its safety. In addition, its selectivity index was 13.5, which demonstrated > 10 selectivity index, considered as being of interest to the pharmaceutical companies. The genotoxicity assay findings revealed that the chloroform and 80% methanol extracts of *P. stellatum* caused DNA damage at 1 mg/ml and 0.5 mg/ml concentrations. Thus, necessary precautions should be taken during utilization of this plant.

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TABLE OF CONTENTS

ACRONYMS/ABBREVIATIONS IX

LIST OF TABLES XII

LIST OF FIGURES XIII

1. INTRODUCTION 1

1.1 Historical perspectives of tuberculosis 1

1.2 Epidemiology of tuberculosis 1

1.3 Etiology and bacteriology of tuberculosis 4

1.4 Transmission and immunology of tuberculosis 6

1.4.1 Transmission 6

1.2.1 Immune response against *Mycobacterium tuberculosis* 6

1.5 Management of tuberculosis 8

1.5.1 Conventional treatment of tuberculosis 8

1.5.2 Drug resistance and toxicity of antituberculosis drugs 10

1.5.3 New antituberculosis drugs in the pipeline 11

1.5.4 Herbal medicines 15

1.5.4.1 The experimental plants 17

1.5.4.1.1 ***Otostegia integrifolia*** 17

1.5.4.1.2 ***Vernonia amygdalina*** 18

1.5.4.1.3 ***Carissa edulis*** 19

1.5.4.1.4 *Persea americana* 20

1.5.4.1.5 *Pterolobium stellatum* 22

1.6 *In-vitro* assays for evaluation of anti tubercular activity 22

1.6.1 Target organisms 22

1.6.2 *In vitro* assays 23

1.7 Rationale for the study 27

2. OBJECTIVES **29**

2.1 General objective 29

2.1.1 Specific objectives 29

3. MATERIAL AND METHODS **30**

3.1 Materials 30

3.1.1 Chemicals and reagents 30

3.1.2 Plant selection and collection 30

3.1.3 Cells 31

3.1.4 Experimental animals 31

3.2 Methods 32

3.2.1 Plant extraction and isolation 32

3.2.2 Activity testing using colony forming method 36

3.2.3 Activity testing using Resazurin indicator method 39

3.2.4 Acute oral toxicity test 39

3.2.5 Cytotoxicity studies 40

3.2.6 Genotoxicity assay 41

3.2.7 Statistical analysis 45

3.2.8 Ethical considerations 45

4. RESULTS **46**

4.1 Antimycobacterial activity of crude extracts using CFU count method 46

4.2 Antimycobacterial activity against clinical isolates of MDR-TB using Resazurin indicator method 49

4.3 Activity of *Pterolobium stellatum* solvent fractions on *Mycobacterium tuberculosis* H37Rv using Resazurin indicator method 51

4.4 Activity of compounds isolated from *Pterolobium stellatum* against *Mycobacterium tuberculosis* H37Rv using Resazurin indicator method 51

4.5 Mass spectrometric analysis of some isolated compounds 53

4.6 Acute oral toxicity test 55

4.7 Cytotoxic effect of plant extracts against $HepG_2$ cells 55

4.8 DNA damage assay of selected plant extracts against $HepG_2$ cells 59

5. DISCUSSION **65**

6. LIMITATIONS **75**

7. CONCLUSIONS **76**

8. RECOMMENDATIONS **77**

9. REFER.ENCES **78**

10. APPENDIXES **96**

10.1 Appendix I: Examples of plant extracts exhibiting antimycobacterial activity 96

10.2 Appendix II: Antimycobacterial activity of compounds isolated from plants 99

10.3 Appendix III: Cytotoxicity test layout on a 96-well microtiter plate 104

10.4 Appndix IV: Mass spectra of compound 1 105

10.5 Appendix V: Mass spectra of compound 2 105

10.6 Appendix VI: Mass spectra of compound 3 106

ACRONYMS/ABBREVIATIONS

ARVs	Antiretroviral Therapies
AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine Triphosphate
ADC	Albumin Dextrose Complex
AHRI	Armauer Hanson Research Institute
BCG	Bacillus Calmette Gůerin
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CNS	Central Nervous System
CR	Complement Receptor
CRFK	Crandell-Rees Feline Kidney
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DOTS	Directly Observed Therapy Short-Course
EDTA	Ethylenediamine tetraacetic acid
EMB	Etambutol
ESI	Electron Spin Ionization
FCS	Fetal Calf Serum
FDA	Fluorescein diacetate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Chromatography
IC_{50}	Inhibitory Concentration of 50% of cells

IFN-γ	Interferon-γ
IL-1	Interleukin 1
IL-12	Interleukin-12
INH	Isoniazid
iNOS	Inducible Nitric Oxide Synthase
LD_{50}	Lethal $Dose_{50}$
LAM	Lipoarabinomanann
LMA	Low Melting Agarose
MABA	Microplate Alamar Blue Assay
MCF	Michigan Cancer Foundation
MDR	Multi-Drug Resistant
MDR-TB	Multi-Drug Resistant Tuberculosis
MGIT	Mycobacteria growth indicator tube system
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
MTB	Mycobacterium Tuberculosis
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium Salt
MTT	Dimethylthiazoldiphenyltetrazolium
OADC	oleic acid-albumin- catalase

OECD	Organization for Economic Cooperation and Development
PBS	Phosphate Buffer Saline
PMS	Phenazine Methosulfate
PZA	Pyrazinamide
REMA	Resazurin Microplate Assay
RIF	Rifampicin
RPM	Revolutions Per Minute
SCGE	Single Cell Gel Electrophoresis
SRB	Sulforhodamine B
STM	Streptomycin
TB	Tuberculosis
TLC	Thin Layer Chromatography
TLR-2	Toll-like Receptor 2
TLR-4	Toll-like Receptor 4
TNF-α	Tumor Necrosis Factor alpha
TLRs	Toll-like Receptors
TOF	Time-of-Flight
UDS	Unscheduled DNA Synthesis
V-ATPase	Vesicular Proton Pump Adenosine Triphosphatase
WHO	World Health Organization
XDR-TB	Extremely Drug Resistant Tuberculosis

LIST OF TABLES

Table 1: Outcome of extraction in the preparation of crude extracts……………….......33

Table 2: Antimycobacterial activity of experimental plants using colony count method.48

Table 3: Antimycobacterial activity test of *Pterolobium stellatum* chloroform extract against MDR-TB clinical isolates using Resazurin indicator method …………50

Table 4: Test suspension colors obtained from fractions tested against *Mycobacterium tuberculosis* H37Rv in the Resazurin indicator method………………………...52

Table 5: Minimum inhibitory concentrations of solvent fractions against *Mycobacterium tuberculosis* H37Rv………………………………………………………..53

Table 6: Test suspension colors obtained from isolated compounds against *Mycobacterium tuberculosis* H37Rv in the Resazurin indicator method ………54

Table 7: IC_{50} Values (mean ± SED) for extracts against $HepG_2$ cells obtained using the MTS assay for cytotoxicity…………………………………………….............56

Table 8: Mean percentage DNA in tail (tail intensity) of different concentrations of extract treated and control $HepG_2$ cells ……………………………………,,,,,60

LIST OF FIGURES

Figure 1: *Mycobacterium tuberculosis* microscopic morphology (a) Ziehl-Neelsen staining (b) and electron microscopy (c)……………………………………..5

Figure 2: Global tuberculosis drug development pipeline………………………………14

Figure 3: Solvent fractionation of *Pterolobium stellatum* chloroform extract 34

Figure 4: Thin layer chromatographic separation of ethyl acetate fraction of *Pterolobium stellatum* chloroform extract…………………………...............................35

Figure 5: Cytotoxic effect of the chloroform extracts of *Pterolobium stellatum* on $HepG_2$ cells...57

Figure 6: Cytotoxic effect of 80% methanol extract of *Ptrolobium stellatum* on $HepG_2$ Cells……………………………………………………………………………57

Figure 7: Cytotoxic effect of experimental plants……………………………………….58

Figure 8: White spots [(a) comets without tails (b) comets with tails] of the comets that were detected in genotoxicity test……………………………………………59

Figure 9: Genotoxic effect of *Pterolobium stellatum* chloroform extract on $HepG_2$ cells…..…………………………………………………………………...61

Figure 10: Genotoxic effect of *Pterolobium stellatum* 80% methanol extract on $HepG_2$ cells...62

Figure 11: Genotoxic effect of *Otostegia integrifolia* chloroform extract on $HepG_2$ cells……………………………………………………………………..63

Figure 12: Genotoxic effect of *Vernonia amygdalina* chloroform extract on $HepG_2$ cells……………………………………………………………………..64

1. INTRODUCTION

1.1 Historical perspectives of tuberculosis

TB has been recorded in history since the Greco-Roman and Egyptian civilizations, with evidence of spinal TB being recorded as long ago as 3400 BC. It is also mentioned in an ancient Indian scripture (Sanskrit) written sometime between 1500 and 700 BC. It has been postulated that *M. tuberculosis* existed as an unimportant pathogen to man until the industrial revolution. With resulting urbanization and propinquity of living, a new epidemic, described as 'a great white plague', evolved. In the newly industrialized countries, the incidence of TB probably increased sharply from the mid 1700s with subsequent pandemic spread throughout Western Europe over the next century and a peak incidence around 1800. Migration probably resulted in spread to the United States, Central Africa and to South and South-east Asia (Debacker, 2006).

1.2 Epidemiology of tuberculosis

Epidemiological evidence suggests that TB is the most lethal infection worldwide, due to a single agent, *M. tuberculosis* that even surpasses malaria. One-third of the world's population is infected with *M. tuberculosis* (Ojiezeh *et al*., 2015). In 1993, during the world TB Day, WHO declared TB as a 'global emergency,' which requires emergency action and launched several programs to combat the disease, including the search for newer remedies and/or anti-TB agents to complement currently used agents (WHO, 2002). In 2014, TB killed 1.5 million people (1.1 million HIV negative and 0.4 million HIV positive). The toll comprised 890, 000 men, 480, 000 women and 140, 000 children (WHO, 2015).

Globally, there were an estimated 9.27 million incident cases (139 incident cases per 100, 000 population) of TB in 2007. This is an increase from 6.6 million cases in 1990, 8.3 million cases in 2000 and 9.24 million cases in 2006 (WHO, 2009). In 2009, there were an estimated 9.4 million incident cases of TB globally (equivalent to 137 cases per 100 000 population) (WHO, 2010). This figure was 8.8 million in 2010, 8.7 million in 2011, 8.6 million in 2012, 9 million in 2013 and 9.6 million in 2014. There were increasing incident cases from 1990 to 2009 and then decreasing trend from 2010 to 2012. However, this figure was increasing from 2013 to 2014. Death associated with TB was between 1.3 and 1.5 million (WHO, 2010, 2011, 2012, 2013, 2014 and 2015). Globally, 12% of the 9.6 million new TB cases in 2014 were HIV-positive (WHO, 2015). In addition, an estimated 3.3% of new TB cases and 20% of previously treated cases had MDR-TB, a level that has changed little in recent years. In 2014, an estimated 190 000 people died of MDR-TB. XDR-TB had been reported by 105 countries by 2015. An estimated 9.7% of people with MDR-TB have XDR-TB (WHO, 2015).

Of the 9.6 million new TB cases in 2014, 58% were in the South-East Asia and Western Pacific regions. The African Region had 28% of the world's cases in 2014, but the most severe burden relative to population: 281 cases for every 100, 000 people, more than double the global average of 133. India, Indonesia and China had the largest number of cases: 23%, 10% and 10% of the global total, respectively. Globally, TB prevalence in 2015 was 42% lower than in 1990 due to effective control of the disease. The target of halving the rate compared with 1990 was achieved in three WHO regions – the Americas, the South-East Asia Region and the Western Pacific Region – and in nine

high-burden countries (Brazil, Cambodia, China, Ethiopia, India, Myanmar, the Philippines, Uganda and Viet Nam) (WHO, 2015).

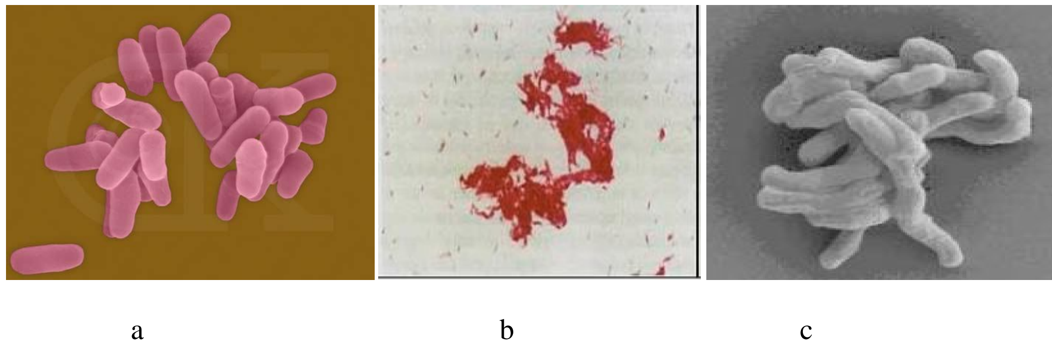
Ethiopia is one of the 22 high burden countries in the world (WHO, 2015). Based on 2011 national population survey, prevalence of smear positive TB among people aged 15 and above was found to be 108/100, 000, whereas prevalence of bacteriologically confirmed TB within the same age group was 277/100, 000. With extrapolation to the total population, including children (using data from routine reporting of case notifications), prevalence of smear positive TB was estimated to be 63/100, 000 population (FMOH, 2011). However, according to the WHO's Global TB Report 2015, TB incidence rate was 207 cases per 100, 000 populations and the prevalence rate was 200/100, 000 including HIV co-infections. Ethiopia is one of the high MDR-TB burden countries. According to WHO Report 2015, new TB cases with MDR-TB were 1.6 % and retreatment cases were 12 % (WHO, 2015).

More than 90% of global TB cases and deaths occur in the developing world, where 75% of cases are in the most economically productive age group (15-54 years). An adult with TB loses on average three to four months of work time. This results in the loss of 20-30% of annual household income and, if the patient dies of TB, an average of 15 years of lost income. In addition to the devastating economic costs, TB imposes indirect negative consequences - children leave school because of their parents' TB, and women are abandoned by their families as a result of their disease (WHO, 2003).

1.3 Etiology and bacteriology of tuberculosis

Three types of Mycobacteria can infect man: the human type, the bovine type and the atypical or opportunistic mycobacteria. There are also two most common groups of Mycobacteria: *M. tuberculosis* complex *(M. tuberculosis, M. bovis, M. africanum and M. microti)* and *Mycobacterium avium* complex (*M. avium, M. intracellularae, and M. kansasii*), which can cause closely related mycobacterium diseases (in AIDS patients) and as a group difficult to distinguish clinically (Brudey, 2006).

*M. tuberculosis*is is an obligate aerobe, with lipid rich cell wall. Its virulence factors are cord factor and sulfatides, making the organism resistant to disinfectants, detergents, common antibacterials and traditional stains. The microscopic appearance does not allow the differentiation of the pathogenic agents of TB, mainly *M. tuberculosis*, from other mycobacteria, although some characteristics may be indicative. In smears stained with carbol fuchsin or auramine and examined under light microscope, the tubercle bacilli typically appear as straight or slightly curved rods (Figure 1). According to growth conditions and age of the culture, bacilli may vary in size and shape from short cocco bacilli to long rods (Barrera, 2007).



a b c

Figure 1: *Mycobacterium tuberculosis* microscopic morphology (a) Ziehl-Neelsen staining (b) and electron microscopy (c) (Barrera, 2007)

M. tuberculosis causes most cases of TB and the reservoir of infection is humans with active disease. Most cases of TB are pulmonary and acquired by person-to-person transmission of air-borne droplets of organisms. Oropharyngeal and intestinal TB contracted by drinking dairy milk contaminated with *M. Bovis* is rarely seen nowadays and usually seen in countries with tuberculous dairy cows and unpasturised milk. Infection with *M. tuberculosis* may not have signs and symptoms of TB disease. In other words, infection, which is the presence of the organism, may or may not cause clinically significant disease. Viable organisms may remain dormant for decades until immunity is suppressed and infection may then be reactivated to produce disease (Udoh, 2009).

The most common clinical manifestation of TB is pulmonary disease; nevertheless, extrapulmonary TB can also occur, but minimally or not contagious. A patient with active pulmonary TB presents with the symptoms of chronic or persistent cough and sputum production. If the disease is at an advanced stage, the sputum will contain blood, and the patient will be diagnosed with lack of appetite, weight loss, fever, night sweats, and thoracic pains. Tools for the diagnosis of active disease include clinical suspicion,

response to treatment, chest radiograph, staining for acid-fast bacilli, culture for mycobacteria, and, more recently, nucleic acid amplification assays (Tibayrenc, 2007).

1.4 Transmission and immunology of tuberculosis

1.4.1 Transmission

M. tuberculosis enters into host cells as live bacteria. When a person with infectious TB disease (TB that can spread) coughs or sneezes, tiny particles (called droplet nuclei) containing *M. tuberculosis* may be expelled into the air. A one-droplet nucleus contains no more than three bacilli, which are so small that they can remain air-borne for extended period. During inhalation of air that contains these droplet nuclei, transmission may occur (Asiimwe 2008). Introduction of *M. tuberculosis* into the lungs leads to infection of the respiratory system; however, the organisms can spread to other organs, such as the lymphatics, pleura, bones/joints, or meaninges and cause extraplumonary TB (Gautam, 2012).

1.2.1 Immune response against *Mycobacterium tuberculosis*

The immune response against TB plays a fundamental role in the outcome of *M. tuberculosis* infection. It is clear that the immune system reacts efficiently in the vast majority of infections. This is particularly evident in the case of TB, where most people infected by the tubercle bacillus (~ 90 %) do not develop the disease throughout their lifetime. Nevertheless, the risk of developing the disease increases considerably when TB infection co-exists with an alteration in the immune system, such as co-infection with HIV (Barrera, 2007).

Even though macrophages are considered the main targets for infection by *M tuberculosis*, it has been recently proposed that other cell populations can also be infected by mycobacteria and therefore may be important in the development of the disease. Neutrophils and mast cells are found within this group of cells (Barrera, 2007).

The initial interactions of the bacilli with the macrophage take place through cellular receptors, such as receptors for Fc, complement, mannose, surfactant protein, CD14, and CD43 (Glickma and Jacobs, 2001). Results of *in vitro* experiments suggest that the macrophage response depends on the type of receptor with which the bacteria interact. Their interaction with Fc receptors increases the production of reactive oxygen intermediates and allows the fusion of the bacteria-containing phagosomes with lysosomes. Bacterial interaction with the CR3 prevents the respiratory burst and blocks maturation of phagosomes harboring the bacteria, thus preventing fusion with lysosomes. TLR-2 and TLR-4 are activated by several *M. tuberculosis* components. Among others, the 19 kilo Dalton lipoprotein and LAM activate macrophages through TLR-2, promoting the production of IL-12 and iNOS. Once the bacteria enter the macrophage, they generally locate themselves in the mycobacterial phagosome. This structure derives from the plasma membrane and presents some cell surface receptors. In contrast to normal phagocytosis, during which the phagosomal content is degraded upon fusion with lysosomes, the mycobacteria block this process (Barrera, 2007).

Another characteristic of the mycobacterial phagosome is its limited acidification. Normally, material transported through an endosomal route finds an acidic medium due

to the action of the V-ATPase in the late endosome. It is suggested that such reduced acidification is the result of a low or zero concentration of V-ATPase in the mycobacterial phagosome. A more recently described property is that this mycobacterial phagosome cannot physically associate with iNOS. The inability of the mycobacterial phagosome to mature has been attributed to the active retention of a protein present in phagosomes, known as tryptophan aspartate coat protein (Barrera, 2007, Meena and Rajni, 2010). The inhibition of phagosome maturation by mycobacteria may be reverted by cytokines, such as IFN-γ and TNF-α which stimulate microbicidal mechanisms, including the production of reactive oxygen and nitrogen intermediates. However, the tubercle bacillus presents molecules, such as LAM and phenolic glycolipid I, which work as oxygen radical scavenger molecules (Barrera, 2007, Meena and Rajni, 2010).

1.5 Management of tuberculosis

1.5.1 Conventional treatment of tuberculosis

The mycobacteria are slow growing organisms that require the administration of a combination of drugs for extended periods to achieve effective therapy and to prevent the emergence of resistance. The risk of adverse reactions therefore must be a major consideration in drug selection. The three basic concepts in TB treatment are as follows: i) Regimens must contain multiple drugs to which the organism is susceptible; ii) Drugs must be taken regularly; and iii) Drug therapy must continue for sufficient time (Somaraju, 2004).

Traditionally, antituberculosis drugs that are classified as first-line drugs are superior in efficacy and possess an acceptable degree of toxicity. These agents include INH, RIF, PZA, EMB and STM. Most patients with TB can be treated successfully with these drugs. Second-line drugs are more toxic and less effective, and they are indicated only when the *M. tuberculosis* organisms are resistant to the first-line drugs. The second-line agents include cycloserine, ethionamide, aminosalicylic acid, rifabutin, ciprofloxacin, moxifloxacin, ofloxacin, trovafloxacin, enofloxacin, Ciprofloxacin**,** moxifloxacin and sparfloxacin capreomycin, viomycin, kanamycin, amikacin and thioacetazone, (Somaraju, 2004 and Kamal *et al*., 2008).

The WHO DOTS strategy has been implemented worldwide and the purpose of this internationally recommended TB control strategy is to provide standardized regimens and proper case management to ensure completion of treatment and cure. Treatment regimens for new patients consist of an initial phase where a fixed-dose combination of INH, RIF, PZA and EMB is given for two months. Patients are expected to become non-infectious within two weeks. This is followed by a continuation phase of sterilizing drugs such as INH and RIF, given for four to six months. Re-treatment patients are given five drugs in the initial and three drugs in the continuation phase (WHO, 2003). Patients are monitored for the entire duration of treatment and take their medication under supervision of a healthcare worker. Such a lengthy treatment period is necessary as a proportion of the infecting organisms are not effectively eliminated by the current TB drugs. The major factor determining outcome of treatment is adherence to the treatment regimen (Lawn *et al*., 1997).

1.5.2 Drug resistance and toxicity of antituberculosis drugs

In recent years, much deadlier MDR-TB and XDR-TB strains have expanded to different regions across the globe. The resistance to two or more of the primary drugs used to treat TB such as INH or RIF characterizes MDR-TB. XDR-TB is resistant to both the first and second-line TB drugs, severely limiting treatment options (Raghvendra *et al.*, 2010). Drug-resistant TB is especially perilous for people with weakened or depleted immune systems, such as those who are HIV positive. In places where MDR-TB and XDR-TB emerge, those that are HIV positive are at a greater risk for infection (Friends of the global fight against AIDS, 2008).

While many surveys report drug-resistant TB around the globe, accurate and comprehensive data are few. Drug-resistant TB is a significant threat to TB control because only a few effective drugs are available against *M. tuberculosis*. In particular, the spread of strains resistant to the two most important drugs, INH and RIF could have serious repercussions on the epidemiology and control of TB. Patients infected with strains resistant to multiple drugs are less likely to be cured. In one study in the USA, treatment failed in 35% of 171 HIV-negative patients with MDR-TB despite the use of multidrug regimens individually tailored by an experienced team (WHO, 1997). The main components of WHO TB initiative end TB strategy are early diagnosis and treatment of all types of TB, patient support, collaborative TB/HIV activities, preventive treatment of persons at high risk and vaccination against TB (Global TB program, 2015).

1.5.3 New antituberculosis drugs in the pipeline

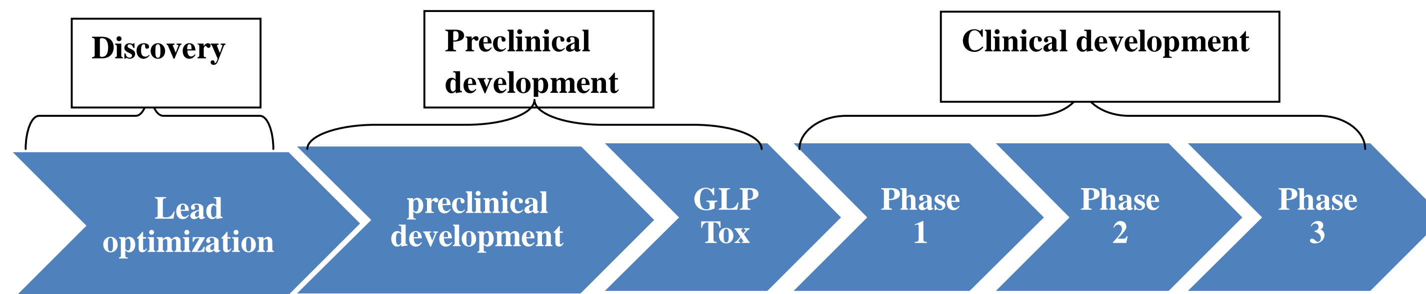
Very little TB drug development has occurred over the last years. Pharmaceutical companies lack profit motive and the cost of development and clinical trials is prohibitive. The Global Alliance for TB Drug Development (GATB, www.tballiance.org) has been established to encourage collaborative research between academic, commercial and non-profit organizations and to facilitate the progression of promising drugs through the development pipeline. Its top priority is the development of a new agent that will shorten the duration of chemotherapy from the current 6–8 months to two months or less, although new drugs with activity against MDR-TB and latent TB are also needed (Duncan and Barry, 2004).

Varying approaches can be used for the discovery of new medicines. These include biodiversity screening, based on either random screening or on ethnopharmacology, to find lead compounds with a pre-defined set of biological activities. Thus, lead compounds can be synthesized or modified to elicit the desired activities (Harvey, 2008). The use of natural products for drug discovery provides a large, structurally diverse pool of potential precursors of new drugs. A survey with the aim of assessing the usefulness of ethnobotany in drug discovery found 122 compounds, 80% of which were used for the same or similar ethnomedical purposes, which were derived from only 94 species of plants (Fabricant and Farnsworth, 2001). These approaches should always be observant of the preservation and diversity of the environment (Rates, 2001).

Drug target identification and throughput screening of antimycobacterial compounds is a new approach. Beside the choice of drug targets, there are many different approaches one should consider and use in anti-TB drug development. One is the way drug screens are designed. Current TB drugs were mostly discovered based on their activity against growing bacilli *in vitro*, with the exception of PZA. However, activity against non-growing persister bacilli is correlated with good sterilizing activity that is responsible for shortening therapy *in vivo*, as shown by PZA and RIF. Thus, novel drug screens that mimic *in vivo* conditions in lesions (i.e. acidic pH and hypoxia) and act against old stationary-phase non-growing bacilli could be important for identifying drugs that kill persisters and thereby shortening TB treatment. In addition, drug combination screens could be performed to identify drugs that have synergistic effects. Along this line of combination screen is the recent interest in the use of system biology approach for drug discovery. Another approach is to make use of the growing knowledge of the unique physiological characteristics of the tubercle bacillus for drug design or screening. Microarray technology can have a role in identifying potential drug targets such as those relevant to persistence of mycobacteria (Zhang *et al*., 2006).

Desirable characteristics of new anti-tubercular drugs include most or all of the following traits. Oral activity, long acting, bactericidal, have novel mechanism of action, potent against both drug sensitive and drug resistant *M. tuberculosis* and non-replicating bacteria, absence of cross-resistance or antagonistic activities with current anti-TB drugs, presence of synergistic or additive effects with current anti-TB drugs that could potentiate efficacy, large therapeutic window and inexpensive to produce. In addition, a new

therapeutic agent has no significant drug-drug interactions with ARVs used in HIV therapy (Protopopova *et al*., 2007). Encouraging work has been performed on fluoroquinolone compounds, oxazolidinone compounds, clofazimine, phenazine, phenothiazines, azoles, peptides, nitroimidazopyrans and long-acting rifamycins (rifapentine, rifabutin, rifalazil). Some of the new drugs under clinical development are illustrated in figure 2 (Hudson *et al*., 2003; Duncan and Barry, 2004).



Nitroimidazoles	CPZEN 45	BTZ043	AZD5847	TMC-207	Gatifloxacin
Mycobacterium	SQ641			PA-824	Moxifloxacin
Gyrase inhibitors	SQ609				Rifapentine
Riminophenazines	DC-159-C			Linezolid	Delamanid
Diarylquinoline	Q201			SQ-109	
Translocase-1 inhibitors				Rifapentine	
Mygrx1 inhibitors				PNU-100480	
InhA inhibitor					
Gyrb inhibitor					
LeuRS inhibitor					
Pyrazinamide analogues					

Figure 2: Global tuberculosis drug development pipeline (Lienhardt *et al*., 2012)

1.5.4 Herbal medicines

Ethnobotanical studies carried out throughout Africa confirm that indigenous plants are the main constituents of African traditional medicines. In Africa 60-90% of populations are reliant on traditional medicine for primary health care (60% in Uganda and 90% in Ethiopia) (ICASA, 2015) and the importance of the role of medicinal plants in the health care delivery is enormous particularly for the respiratory diseases due to the chronic nature of these diseases. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Scientific interest in medicinal plants has burgeoned in recent times because natural products are evolutionary shaped drugs or drug-like molecules. Nature's biosynthetic machinery produces innumerate natural products with distinct biological properties that make them valuable as inhibitors or promoters of biological action. Increased efficiency of new plant derived drugs and rising concerns about the side effects of modern medicine served as impetus for targeting the plant kingdom as sources of new molecular structures that can be used as lead compounds (Mariita *et al*., 2010, Hamisi *et al*., 2009).

Accumulating evidence shows that phytomedicines have great promise in the treatment of intractable infectious diseases, including TB. The aqueous and ethanol extracts of selected medicinal plants used as spices (*Allium sativum, Allium cepa, Syzygium aromaticum, Cinnamomum verum*) had been reported to have anti-TB activity against *M. tuberculosis* H37Ra (Sivakumar and Jayaraman, 2011). Moreover, the ethanol extract of garlic is shown to be active against both non-MDR and MDR *M. tuberculosis* isolates,

with MIC ranging from 1 to 3 mg/ml (Hannan *et al*., 2011). *In vitro* activity of 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 of the crude 80% methanolic extracts ranged from 6.25 to100 µg/ml (Gemechu *et al*., 2013).

Various reviews have been published on antimycobacterial extracts and compounds derived from natural products, examples of which are shown in Appendices I and II. The compound with the greatest activity is a peptide with an MIC of 0.1µg/ml, whereas a norditerpenoid has an MIC of 0.46µg/ml. One could compare these values with the MIC values of the front- line TB drugs, namely INH, RIF, PYR and EMB; have MIC's of 0.05, 0.25, 100 and 3.8µg/ml, respectively. It has been observed that more lipophilic components were associated with increased activities as opposed to more polar substituent since lipophilic compounds easily penetrate lipid rich cell wall of the bacteria. One such hydrophobic compound with antimycobacterial activity is *(E)*-phytol, with an MIC of 2 µg/ml (Cantrell *et al*., 2001). Another example of the activity of lipophilic compounds against mycobacteria was illustrated by compounds isolated from members of the Asteraceae family, specifically members of the tribe Astereae. Three of these compounds exhibited activity against *M. tuberculosis* (Lu *et al*., 1998). In addition, pseudopteroxazole and seco-pseudopteroxazole, active diterpinoid alkaloids containing the uncommon benzoxazole moiety isolated from *Pseudopterogotgia elisabethae*, possess anti-TB activity (Yadvendra *et al*., 2007).

1.5.4.1 The experimental plants

1.5.4.1.1 *Otostegia integrifolia*

Otostegia integrifolia Benth (Lamiaceae (Labiatae)) is herbaceous plant that grows in the wild but is also cultivated in gardens. It is called *Tinjute* (Amharic) and is one of the plants used in Ethiopian traditional medicine. The plant has insecticidal properties and is often used as fumigant for pots and houses (Kidane *et al*., 2013). Traditionally, it is used to treat stomachache (Teklehaymanot *et al*., 2007), tonsillitis, uvulitis and hypertension (Andemariam, 2010), malaria (Giday *et al*., 2007) and antiascariasis (Parvaz and Yadav, 2010) in Ethiopia. The roots and leaves are used for treating lung diseases ((Tesso and König, 2004, Endale *et al*., 2013, Karunamoorthi, 2014). Research has shown that one of the chemical constituents of the plant called stigmasterol may be useful in prevention of certain cancers; including ovarian, prostate, breast, and colon cancers; and inhibits several pro-inflammatory and matrix degradation mediators typically involved in osteoarthritis-induced cartilage degradation. It also possesses potent antioxidant, hypoglycemic and thyroid inhibiting properties (Karunamoorthi, 2014). As reported by Karunamoorthi (2014), stigmasterol is the precursor of oldenone undecylenate, which is used in veterinary medicine to induce growth and is one of the anabolic steroids abused in sports. Regarding *in vivo* toxicity profile of the plant, the methanolic leaf extract possesses no acute toxicity in mice up to a maximum dose of 5000 mg/kg, which indicated that the plant is relatively safe when administered orally (Endale *et al*., 2013).

From the aerial parts of the plant, isolation of eight prefuranic and furanic labdane diterpenes together with iridoid glucoside was reported. These were otostegin A,

otostegin B, 15-*epi*-otostegin B, preleoheterin, leoheterin, and related compounds, including leopersin C, 15-*epi*-leopersin C, ballonigrin, vulgarol, and 8-O-acetylharpagide. In addition, the essential oil and chloroform extract of air-dried leaves of *O. integrifolia* were investigated and a total of 40 constituents including monoterpenes, sesquiterpenes, diterpenes and their derivatives were identified (Tesso and König, 2004).

1.5.4.1.2 *Vernonia amygdalina*

Vernonia amygdalina Del. (Asteraceae) also called grawa (Amharic); vernonia tree, bitter leaf (English), grawa (Tigrigna) is a bushy shrub or well-formed tree up to 7 m in height. Bark is light grey or brown, rather rough and longitudinally flaking, and the branches are brittle (Orwa *et al.*, 2009). It is used to treat tonsillitis and epidemic diseases in northwestern Ethiopia (Teklehaymanot *et al.*, 2007), cough and bleeding (Uzodimma, 2013). In Kenyan folk medicine, the root is used to treat gastrointestinal problems, TB and asthma. *V. amygdalina* is the most potent extract against *M. smegmatis* and *M. fortuitum*, completely inhibiting their growth (Zero GUs) at 0.5, 1.0 and 2.0 mg/ml concentrations. It was also reported to exhibit activity at 2.0 mg/ml against *M. tuberculosis* (Mariita *et al.*, 2010).

Medicinal applications of the plant include the use of the leaf extract as a laxative, anti-oxidant (Anyasor *et al.*, 2010), anthelmintic (Alawa *et al.*, 2010) and antimalarial (Abdrazak *et al.*, 2014), digestive tonic, appetizer, febrifuge and for the topical treatment of wounds (Ijeh and Ejike, 2011). The effects of the leaf extract has been reported to exhibit antithrombotic and hypoglycemic (Ebong *et al.*, 2008), hypolipidaemic (Oboh

and Enobhayisobo, 2009, Akah *et al*., 2009, Ebong et al., 2008), antimicrobial (Mboto *et al*., 2009, Akortha and Nwachukwu, 2009, Ibrahim *et al*., 2009) and anti-breast cancer properties (Yedjou *et al*., 2013). Phytochemical screening of the plant has revealed the presence of saponins, glycosides and tannins, which are known to be bioactive purgative principles. Flavonoids are also present in bitter leaf and have identified three flavones – luteolin, luteolin 7-O-beta-glucuronoside and luteolin 7-O-beta-glucoside. These flavones possess antioxidant activity and may play a beneficial role in cancer prevention and offer some protection against diabetes and atherosclerosis. The high content of the antioxidant vitamin C present in *V. amygdalina* leaves also account for the antioxidant activity (Oboh and Enobhayisobo, 2009). The methanol leaf extract was relatively safe with median lethal dose, $LD_{50} \geq 5000$ mg/kg/ when single dose was administered orally to mice (Yusuf *et al*., 2012).

1.5.4.1.3 *Carissa edulis*

Carissa edulis Vahl (Apocynaceae) is also called agam (Amharic); emir (Arabic); Arabian numnum (English); and agam (Tigrigna). It grows in the tropical African region and Arabia. In Guinea, the boiled leaves are applied as poultice to relieve toothache (Jamilu *et al*., 2007). Root bark is mixed with spices and used as an enema for lumbago and other pains in Ghana; root scrapings are used for glandular inflammation; ground-up roots are used as a remedy for venereal diseases, to restore virility, to treat gastric ulcers, cause abortion, and as an expectorant (Orwa *et al*., 2009). An infusion of roots along with other medicinal plants is used for treating chest pains, and a root decoction is also used for treating malaria and cough (Orwa *et al*., 2009, Nvau, 2011), oedema, epilepsy, ulcer,

worm infestation and it is also used as a source of dye (Jamilu *et al*., 2007). In folk medicine, the root of the plant is used to treat TB, Malaria, Gonorrhea (Kembamo *et al*., 2015), Polio and asthma (Jamilu *et al*., 2007).

Methanol root extracts inhibited mycobacterial growth at 2.0 mg/ml (Mariita *et al*., 2010) and 3.65 mg/ml (Nvau, 2011) against *M. tuberculosis*. In addition, it was potent against *M. smegmatis* and *M. fortuitum*at 0.5, 1.0 and 2.0 mg/ml concentrations (Mariita *et al*., 2010). In the oral acute toxicity test of the crude ethanol extract of *C. edulis* root bark in rats and mice, the LD_{50} was found to be 3, 807.9 mg/kg (Ngulde *et al*., 2013).

The chemical compositions of the plant have extensively been reported. The extract yields benzenoids, penylpropanoid, lignans, sesquiterpenes and coumarins steroids, terpenes, tannins, flavonoids and cardiac glycosides (Ibrahim *et al*., 2007). Roots contain an active ingredient, carissin that may prove useful in the treatment of cancer. The twigs contain quebrachytol and cardioglycosides that are useful as an anthelmintic against tapeworm (Orwa *et al*., 2009) and the roots contain lupeol (has antiviral activity), oleuropein, carissol and β-amyrin (Tolo *et al*., 2010).

1.5.4.1.4 *Persea americana*

Persea Americana Mill (Lauraceae) is a tropical evergreen tree or shrub that can grow 20-30 m tall. Its common name is *Avocado*. The leaves are traditionally used as infusion form for diabetes, diarrhea, inducing abortion, intestinal worms, menstrual cramps, parasites and vaginal infections, and the seed decoction is taken for contraception (Yukes

and Balick, 2010). Balangcod (2011) reported that the leaf decoction is used by the Philippines during cough.

The fruit has been shown to lower total cholesterol levels and is recommended for dyslipidemia and hypercholesterolemia. An avocado-enriched diet has shown glycemic control and plasma lipid triglycerol-lowering effects in patients with non-insulin dependent diabetes mellitus. Avocado and soy unsaponifiables have been shown to alleviate pain, decrease use of painkillers and reduce joint space loss. A cream containing the oil was well-tolerated and showed long-term beneficial effects in the treatment of plaque psoriasis. In preclinical studies, the following effects of this plant have been shown: analgesic, anti-inflammatory, antihemorrhagic, anticancer, hepatoprotective, macrophage-stimulating, uterine muscle stimulant, trypanocidal and vasorelaxant (Yukes and Balick, 2010).

Major chemical constituents include the following: the leaf contains volatile oil, flavonoids and coumarins; the fruit contains sesquiterpenes and carbohydrates; the seed contains fixed oil consisting of vitamins A, D_3, alpha tocopherol and cholesterol. The fruit is a significant source of protein, monounsaturated fatty acids, vitamin A, thiamin, riboflavin, niacin, vitamin B6, vitamin C, vitamin E, folate, vitamin K, pantothenic acid, magnesium, manganese, phosphorus and the amino acids tryptophan, valine, tyrosine, threonine, phenylalanine and methionine (Yukes and Balick, 2010). *Persea Americana* activity against different strains of mycobacterium tuberculosis with MIC of 32.1 µg/ml (hexane extract), against H37Ra strain and H37Rv strains and MIC 125 µg/ml (methanol

extract) against H37Ra strain is demonstrated (Gomez-Flores *et al*., 2008). The LD_{50} in mice of the dry plant material (fruit and leaf) administered orally in single dose is 12.5 g/kg (Yukes and Balick, 2010).

1.5.4.1.5 *Pterolobium stellatum*

Pterolobium stellatum (Forsk.) Brenan. (Fabaceae) is also called *kenteffa* (Amharic). Fresh leaves and roots are chewed for medicinal purposes for tuberculosis and related respiratory diseases (Andualem *et al*., 2014, Balcha *et al*., 2014; Getahun, 1976). The root extract is also used to treat diarrhorea (Flatie *et al*., 2009). It has been reported that the whole plant juice is given orally for one month to treat epilepsy and neuralgia in north-west Ethiopia (Ragunathan and Abay, 2009).

1.6 *In-vitro* assays for evaluation of anti tubercular activity

1.6.1 Target organisms

A well characterized virulent strain, *M. tuberculosis* H37Rv (ATCC 27294) has a drug susceptibility profile which is representative of majority of drug susceptible clinical isolates. Several natural product researchers have also chosen to work with a rapidly growing, avirulent (in rare cases virulent), saprophytic mycobacteria. One such species is *M. smegmatis* (ATCC 607), which has been used extensively. These organisms, however, only possess a limited degree of similarity to *M. tuberculosis* with regard to drug susceptibility. Another alternative is to use the slow growing, avirulent strain known as *M. tuberculosis* H37Ra (ATCC 25177) or the commonly used vaccine strain, *M. bovis* BCG (ATCC 35743). These organisms are more closely related to *M. tuberculosis*

H37Rv than the rapid-growing mycobacteria with respect to both drug susceptibility profile and genetic composition (Gautam *et al*., 2007).

1.6.2 *In vitro* assays

In vitro models of macrophage infection by *Mycobacterium* spp have been used to assay virulence and the intracellular activity of antimycobacterials (Sánchez *et al*., 2010). There are also various methods employed for extracellular antimycobacterial activity testing. These methods are briefly summarized below:

i. Broth/Agar dilution methods

These tests can be performed on broth (Macro/micro broth dilution MIC tests) or agar (agar dilution methods). In these tests, the minimum amount of extract/compound that inhibits the visible growth of an isolate or MIC is determined. Bacterial isolate is subjected to various dilutions of extracts/compounds and the highest dilution of extracts/compounds that has inhibited the growth of bacteria is considered as MIC. In macrobroth dilution tests, serial two-fold dilutions are made in test tubes from zero to maximum concentration that will be achieved *in vivo* without toxic effect. However, in microbroth dilution tests, a polystyrene tray containing 80 wells is filled with small volumes of serial two-fold dilutions of different extract/compound (Sridhar, 2006).

ii) Disk methods

Disk methods (Kirby-Bauer test) comprise the placing of filter paper disks or paper disks containing test compounds on agar plate surfaces previously inoculated with the test

organism. The test molecules or plant extracts then diffuse into the agar and inhibit growth of the test microorganisms. Then, inhibition zones are measured in millimeters and are compared with a standardized chart to determine if the bacteria are resistant, intermediate, or sensitive to the extract or compound. The major disadvantage of using diffusion assays is that the mycobacteria, having a very lipid-rich, hydrophobic cell wall, are often more susceptible to less-polar compounds. Diffusion assays are recommended more for polar rather than non-polar molecules or mixture of compounds such as essential oils (Gautam *et al*., 2007, Sánchez *et al*., 2010).

iii) Radiometric method

BACTEC 460® instrument is a semi-automated radiometric drug susceptibility testing method that measures the $^{14}CO_2$ produced by metabolic breakdown of (1-^{14}C) palmitic acid in a liquid Middlebrook 7H12 medium. With this method, multiple concentrations can be tested and an MIC calculated. However, although results are generally available in 5 days, the tests performed on the BACTEC system are costly and are not suited to the evaluation of large number of compounds. The major disadvantages of these assays are the cost and the isotope disposal in some countries (Sánchez *et al*., 2010).

iv) Fluorescent based methods

FDA staining and flow cytometry are employed for susceptibility testing of *M. tuberculosis*. The method is based on the ability of viable *M. tuberculosis* organisms to hydrolyze FDA and the detection of fluorescent mycobacteria by flow cytometric

analysis. *M. tuberculosis* organisms exposed to antimycobacterial agents hydrolyze significantly less FDA (Norden *et al*., 1995, Sánchez *et al*., 2010).

Another fluorescent-based assay is MGIT. This system relies on an oxygen-sensitive fluorescent compound to detect mycobacterial growth. Essentially, the fluorescent compound is contained in a silicone plug at the bottom of a tube, which contains mycobacterial growth medium. The dissolved oxygen in the medium quenches any fluorescence from the compound. However, inoculation of the medium with mycobacteria and their subsequent growth removes the oxygen and allows the compound to fluoresce. Fluorescence is detected using a long wave ultraviolet lamp or ultraviolet lamp transilluminator (365nm). The system is therefore described as a rapid, non-invasive, non-radioactive method for detection and susceptibility testing of *M. tuberculosis* growth (Gordon *et al*., 1996).

v) Reporter gene assays

Two enzymes capable of producing light are bacterial and firefly luciferase. The light production is dependent on luciferase reaction which is in turn dependent on the reducing power (bacterial) or ATP levels (firefly) of the host cell. Hence, the reaction can be used as a direct reporter of cell viability. Introduction of the luciferase genes into mycobacteria would therefore allow a rapid measurement of drug susceptibility; exposure of luminous mycobacteria to antimycobacterials would block light production in sensitive bacilli but have no effect on resistant bacilli (Gordon *et al*., 1996).

vi) Colorimetric methods

Colorimetric assay for drug susceptibility testing of *M. tuberculosis* is based on the ability of viable bacilli to reduce MTT to formazan that can be measured spectrophotometrically (Gordon *et al*., 1996).

A similar method that relies on the redox dye Alamar blue as the indicator of mycobacterial growth has been developed. The redox indicator is added to cultures of the bacilli containing drugs after 7 to 14 days of growth; a color change from blue to pink indicates growth (Gordon *et al*., 1996). Another colorimetric method relies on the redox dye resazurin, which is the active compound in alamar blue. Thus, REMA and MABA are based on the same chemistry. REMA is the standard and more sensitive method in high throughput screening of compounds against mycobacteria and is the most widely cited one (Primm and Franzblau, 2007, Bueno, 2011).

Correlation studies between REMA and CFU indicated that REMA assay was noted to be superior to the CFU assay in that it distinguished between metabolically active dormant bacteria and non-viable organisms, unlike the CFU assay that could not differentiate between these two populations. In addition, the REMA assay performed with good concordance in both fluorimetric and visual formats to distinguish between bactericidal and bacteriostatic effects of a drug. The REMA assays will be useful for anti-tubercular anti-dormancy compound screening and drug susceptibility testing in a safe, reliable, easy and cost-effective manner particularly in low resource countries (Taneja and Tyagi, 2007).

1.7 Rationale for the study

TB is the leading killer of youths, women, and AIDS patients worldwide. It is a major global health problem which is complicated due to drug resistance (Jim´enez-Arellanes *et al*., 2007, Gupta *et al*., 2010) and HIV epidemic that has dramatically increased risk for developing active TB (Casenghi, 2006). Search for new anti-tuberculosis drugs become obvious due to the above reasons and others such as development of MDR-TB, shortage and expensive nature of TB drugs. Herbal remedies have become the readily available alternative in the search for new antimycobacterial agents.

In contrast with the vast majority of bacterial infections, *M. tuberculosis* is currently treated with combination therapy and for many months. The choice of regimen and its duration depends on the characteristics of the disease (e.g. localized or disseminated to certain sites), the resistance profile of the organism, the potential for drug interactions (a particular potential difficulty in those co-infected with HIV) and the ability of patients to tolerate certain agents. Complex regimens and/or high pill burdens are also a concern for patient compliance. Clearly, simpler and shorter regimens, ideally with less potential for drug interactions and better tolerability are needed for the management of TB.

In addition, treatment of MDR-TB with second-line and newer drugs is both difficult and less cost-effective. Compared to the first-line drugs, they are inferior in efficacy, more toxic and have to be given for at least three times as long and at a hundred times the cost of basic short-course chemotherapy regimens. Since the impact of TB is greatest among the poor, their compliance to treatment remains highly questionable. Although MDR-TB,

in most cases, was assumed to be a sign of poor program performance, one must also be aware of the highly virulent strains spreading rapidly. Moreover, XDR-TB is resistant to first- and second-line drugs and so the treatment options are seriously limited. Thus, there is now a need for new regimens with activity against MDR-TB and XDR-TB *M. tuberculosis* infections.

As part of an effort to come up with a candidate compound for lead optimization and/or new drug development from traditionally used plants having proven efficacy, the present study attempted to undertake activity testing of crude extracts and/or identification and isolation of active principles. The outcome of the research may provide us with a new antimycobacterium tuberculosis drug, which could be used as purified and single chemical entity or as a crude drug form. As a result, limiting issues such as drug resistance, lethal hepato-toxic effects and obscurity of reservoir tubercle bacilli in the brain by the blood brain barrier such as in CNS/cerebral tuberculosis may be effectively averted.

2. OBJECTIVES

2.1 General objective

To investigate antimycobacterial activity and toxicity of selected Ethiopian medicinal plants (*Otostegia integrifolia*, *Vernonia amygdalina*, *Persea americana, Pterolobium stellatum* and *Carissa edulis*) as well as to isolate the main active principles through a bioassay guided process.

2.1.1 Specific objectives

- To investigate *in vitro* activities of crude extracts of the medicinal plants against TB on culture media
- To investigate *in vitro* activities of the fractions from the most active extract
- To evaluate antimycobacterial activity of isolated compounds using a structured approach
- To determine cytotoxic effect of crude extracts
- To determine the genotoxic effect of crude extracts

3. MATERIAL AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Methanol (99.9%), Chloroform, acetone Silica gel, Catechol (1,2-dihydroxybenzene) (Sigma- Aldrich, USA), DMSO (FisherScientific, Spain), deionized water, penicillin (Invitrogen, Sweden), streptomycin (Invitrogen, Sweden), DMEM with glutamax (Invitrogen, Sweden), FCS, trypsin, Trypan blue, GelRed Nucleic Acid Stian ((Sigma-Aldrich, USA), NaCl, EDTA, Tris, Triton X-100, NaOH, LMA, Trizma base and PBS were used as received. In addition, Malachite green (Sigma-Aldrich, USA), Polymyxin B (product of Denmark; Lot No. BCBG 6644v), Fungizone (Sigma-Aldrich, USA), Amoxicillin/Trimethoprim Lactate (Sigma-Aldrich, USA), Middlebrook OADC (BBL^{TM}), (Becton Dickinson and Company Spark, USA) and Middlebrook 7H9 broth base (Difco - Becton Diskinson) have been used. All other chemicals/reagents were purchased from Sigma- Aldrich, USA unless otherwise indicated.

3.1.2 Plant selection and collection

Plants were selected based on their traditional use in Ethiopia to treat symptoms associated with respiratory ailments that could be indicative of TB. Moreover, some of the plants were selected based on evidence in the literature for their *in vitro* activity in preliminary works. The primary databases were mined using search terms, including 'respiratory', 'cough', 'chest', 'asthma', 'catarrh','haemoptysis' and 'antitubercular activity'. Accordingly, five plants were selected. The roots of *O. integrifolia, P. stellatum,* and *C. edulis* were collected from an area near *Angereb* River, Gondar town,

North West Ethiopia, about 730 km away from the capital, Addis Ababa. The root of *V. amygdalina* and the leaves of *P. americana* were collected from Bure town, North West Ethiopia, about 400 km far from Addis Ababa. Part of the root of *V. amygdalina* was cut without cutting the whole tree. The plants were authenticated by a taxonomist. The plants were cleaned from dirt and soil and dried under shade for two weeks. The plants were spread out and regularly turned over to avoid fermenting and rotting. The dried root parts of plants were grinded using 0.75 mm sieve size hammer type mill, while the dried leaves were pulverized using a wooden mortar and pestle. The powdered material was weighed using an analytical balance and stored at room temperature.

3.1.3 Cells

Bacterial cells were *M. tuberculosis* H37Rv (ATCC no 27294, Manassas, VA) and three MDR-TB clinical isolate strains. The three MDR-TB clinical isolates were AOA8W-4, AOZ8W-4 and SO38SW-4. The cell lines were $HepG_2$ cells.

3.1.4 Experimental animals

Swiss albino nulliparous and non-pregnant female mice weighing 31–36 g, and age 8–12 weeks were obtained from the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Addis Ababa University. All animals were housed in an air-conditioned room and allowed to acclimatize for one week before commencement of the study. All the experiments were conducted in accordance with internationally accepted laboratory animal use, care and guideline (ILAR, 2001). Before and during the experiment, mice were allowed free access to standard pellets and water ad libitum.

3.2 Methods

3.2.1 Plant extraction and isolation

i) Crude extract

Information pertaining the preparation of the crude extracts is depicted in Table 1. The air-dried; powdered roots of *P. stellatum*, *O. integrifolia*, *C.edulis* and *V. amygdalina* were exhaustively extracted with chloroform using maceration technique while *P. americana* powdered leaves were extracted with acetone. Maceration was carried out using one liter of the respective solvent for 72 h, with regular shaking. The mixture was filtered with whatman No. 42 filter paper (Whatman No.42, England) and the filtrate was kept at + 4°c. The marc was macerated again in the same solvent two times and filtered. The filtrates were combined evaporated under reduced pressure on a rotary evaporator (Buchi Rota Vapor R-200) and dried in oven at 40°c (Gallenkamp, England).

In parallel, the air-dried and powdered roots of *P. stellatum*, *O. integrifolia* and powdered leaves of *P. americana* were soxhlet extracted with 80% methanol (4:1, methanol: water). The obtained extracts were filtered and evaporated under reduced pressure on a rotary evaporator and lyophilized**.** The extracts were kept refrigerated and away from light. Stock solutions of all extracts were prepared in DMSO at a concentration of 50 mg/ml and stored at -20°C until use.

Table 1: Outcome of extraction in the preparation of crude extracts

Plants	Part (s) used	Solvent	Extraction method	Raw material quantity (g)	Yield in g (%)	Physical appearance
Otostegia integrifolia	roots	chloroform	maceration	300	2 (0.67)	whitish powder
	roots	80% methanol	soxhlet	200	1(0.5)	deep red gumy
Pterolobium stellatum	roots	chloroform	maceration	500	10 (2)	brownish resin
	roots	80% methanol	soxhlet	200	1.5 (0.75)	deep red gumy
Persea americana	Leaves	80% methanol	soxhlet	500	20 (4)	Oily black
		Acetone	maceration	300	3 (1)	gumy black
Carissa edulis	roots	chloroform	maceration	300	2 (0.67)	light green
Vernonia amygdalina	roots	chloroform	maceration	300	1.5 (0.5)	black powder

ii) Fractionation

Antimycobacterial activity evaluation of the crude extract revealed *P. stellatum* chloroform root extract to have a better activity and further fractionation was done using this plant. The dried extract (8 g) was suspended in distilled water (200 ml) and then successively partitioned with n-hexane and ethyl acetate in a separatory funnel (Figure 3). The n-hexane and ethyl acetate fractions were concentrated under reduced pressure using rotary evaporator (Buchi Rota Vapor R-200) and dried in an oven under 40°C, while the water fraction was dried using freeze drier.

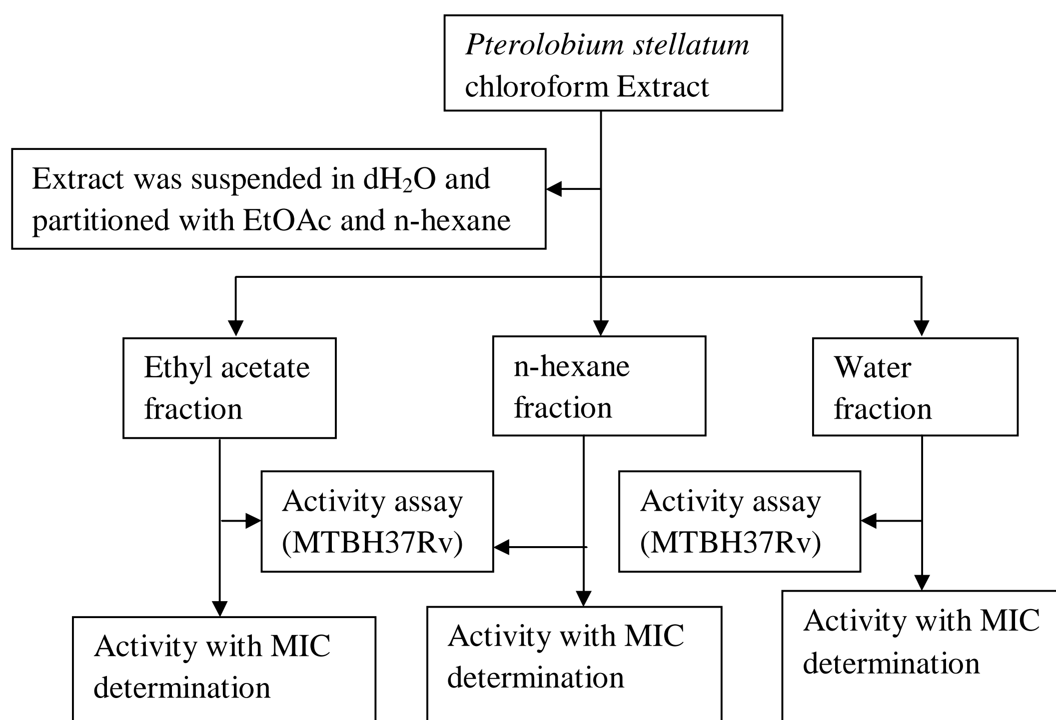


Figure 3: *Pterolobium stellatum* chloroform extract solvent fractionation: EtOAc = ethyl acetate, dH_2O = distilled water, MTB H37Rv = *Mycobacterium tuberculosis* H37Rv.

iii.Isolation

The most active fraction (ethyl acetate fraction) was further subjected to preparative TLC over silica gel. Visible bands were marked under day light and ultraviolet light (254 and 360 nm). The plates were carefully heated at 100°C for optimal color development. Three bands were identified as putative pure compounds because only one band was observed for each on TLC plates (compound **1**, **2** and **3**). The purity of the compounds was monitored using pre-coated analytical TLC (Figure 4).

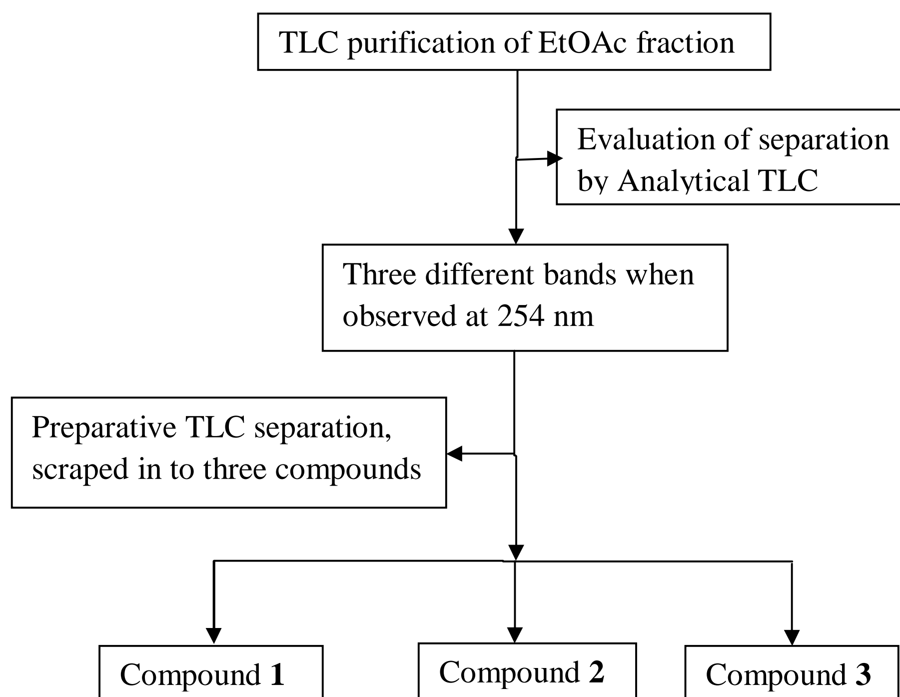


Figure 4: Thin layer chromatographic separation of ethyl acetate fraction of *Pterolobium stellatum* chloroform extract:

iv. Mass spectrometric analysis of some isolated compounds

Semi-preparative HPLC (Shimadzu, USA) and analytical HPLC (shimadzu, USA) were conducted on C18 columns [Jupiter, 250x10 mm (5 µm) and 250x4.6 mm (5 µm] using UV-900 detection at 215 nm. High resolution MS was done in the positive ion mode using ESI on a JEOL JMS SX/SX102A instruments (Waters, USA). Water and acetonitrile was used as HPLC mobile phase with increased acetonitrile gradient.

Preparation of solutions for HPLC and MS analysis: Stock solutions of compound **1**, compound **2** and compound **3** were prepared by dissolving 25 mg of each compound in 5 ml 99.5% ethanol. The solutions were then, filtered and diluted with double distilled water (1:10). Ninety five micro-liters of the diluted solution was injected into an analytical HPLC (Shimadzu, USA) and 50 ml was loaded on a semi-preparative HPLC column. One micro-liter was injected to the MS. In MS analysis, an attempt was made to characterize some of the isolated compounds using the TOF-MS techniques, in which fragmented product ions are used to interpret precursor ion structures.

3.2.2 Activity testing using colony forming method

These tests were performed in a level three bio-safety laboratory at AHRI, Addis Ababa, Ethiopia**.** The antimycobacterial activity of the extracts was evaluated against *M. tuberculosis* H37Rv (ATCC 27294) using CFU method.

i. Culture media preparation

In the preparation of Mycobacteria 7H11 medium (1000 ml), antibiotic solutions were prepared by mixing stock solutions with antibiotics using the method described by

Dubos and Middlebrook, 1947, Middlebrook and Cohn, 1958. Mycobacteria 7H11 Agar (Difco™) (Difco Laboratories, Becton Dickinson and Company Spark, USA; Lot No. 9133794) (18.9 g) and 0.6% malachite green were taken in a beaker and diluted with 780 ml de-ionized water. The mixture was autoclaved at 121ºC for 15 min and then cooled below 50ºC. The antibiotic solution was added to the beaker containing the agar base and mixed slowly with a magnetic mixer for 5 min. Twenty milliliter of the medium was dispensed per sterile petridish using a pipette and an electric aspirator and then closed immediately. It was allowed to solidify for 1 h and sealed with parafilm (Dubos and Middlebrook, 1947, Middlebrook and Cohn, 1958).

To prepare 7H9 broth, Difco Middlebrook 7H9 powder (4.7 g) was suspended in 900 ml of purified water containing 2 ml glycerol. It was then mixed thoroughly and autoclaved at 121°C for 20 min, 100 ml of Middlebrook ADC enrichment was added aseptically to the medium when cooled to 47°C (Middlebrook and Cohn, 1958).

ii. Inoculum preparation

M. tuberculosis was cultured and grown on Mycobacteria 7H11 medium by following a procedure described by Mann *et al*., 2008 and Assefa, 2010. Then, inoculum was prepared by diluting cultures at 1/1000 by adding 25 µl cell culture to 25 ml medium, 7H9 broth (4.7 g of Middlebrook 7H9 broth base [Difco - Becton Diskinson], 2 ml of glycerol in 900 ml water) enriched with ADC when cooled to 47°C (Mann *et al*., 2008).

iii. Antimycobacterial assay

The antimycobacterial effect of chloroform and 80% methanol extract of *P. stellatum* and *O. integrifolia,* chloroform extract of *V. amygdalina* and *C. edulis*, 80% methanol and acetone extracts of *P. americana* were evaluated at concentrations ranging from 0.00244 mg/ml to 2.5 mg/ml. The test concentrations of the extracts were selected based on cytotoxicity and acute toxicity test results in this study and previous studies (Gomez-Flores *et al*., 2008, Mariita *et al*., 2010, Nvau, 2011 and Endale *et al*., 2013). Serial two-fold dilution of extracts was made in microtitre wells. CFUs were counted from triplicate dilutions and duplicate plates were used for each concentration of test extract and controls. The average CFUs were determined from three independent experiments. The percentage inhibition of growth was determined by dividing the CFUs of the test concentration by the CFUs of the negative control (solvent).

Each extract reconstituted with DMSO (50 mg/ml) was further diluted (5 mg/ml) with Middlebrook 7H9 broth supplemented with ADC. Then, activity was measured and MIC determined for extract as well as positive and negative controls following a method described by Mann *et al*., 2008 and Xuan *et al*., 2011. No inhibitory effects were observed in the presence of DMSO at the concentrations used. The MIC was the concentration of extract at which 100% inhibition of mycobacterial growth was observed when compared with the growth control.

3.2.3 Activity testing using Resazurin indicator method

M. tuberculosis H37Rv and clinical isolates of MDR-TB were cultured in Middlebrook 7H9 broth (prepared following the procedure described as above) at 37 °C for two weeks in order to reach logarithmic phase growth. Test inoculums were prepared using a procedure described by Patricia *et al*., 2012.

After incubation at 37^0C for 7 days, 15 µl of 0.01% resazurin (Sigma, St. Louis MO, USA) solution in sterile water was added to the first growth control wells and incubated for 24 h. Once the first sets of growth controls turned pink, the dye solution was added to the second set of growth controls and the test wells, incubated for 24 h at 37^0C. Blue color in the wells containing the test compounds would indicate inhibition of growth and pink would indicate lack of inhibition of growth of *M. tuberculosis* (Deepthi *et al*., 2012). MIC value was expressed as the lowest concentration of compound that caused 100% inhibition of mycobacterium growth (Patricia *et al*., 2012). All assays were run in triplicate and INH was used as positive and DMSO as negative control.

3.2.4 Acute oral toxicity test

As no toxicity data was available for any part of *P. stellatum* chloroform extract, oral toxicity study was conducted using OECD guidelines 423 (OECD, 2001). Briefly, nine mice were randomly divided into three groups of three mice per cage. Before administration of single dose of the extract, the mice were fasted for 3 h and weighed. After administration of a single dose of extract, mice were fasted for 1 h. The first group was given solvent (5% DMSO in distilled water), while the second group were given 2

g/kg (dissolved in 5% DMSO) of the chloroform root extract of *P. stellatum* orally. The mice in the third group were provided with *P. stellatum* root extract 5 g/kg dissolved in 5% DMSO after following the first two groups for 14 days. The mice were observed continuously for 1 h after administration of the extract; intermittently for 4 h, over a period of 24 h, and then frequently for 24 hours for 14 days. Gross behavioral changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality and other signs of toxicity manifestation were observed.

3.2.5 Cytotoxicity studies

Cytotoxicity of crude extracts were carried out by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay method described by Promega Corporation, 2012 on human hematoma ($HepG_2$) cells. The cells were cultured in DMEM containing 10% FCS and 1% pencillin-streptomycin under 5% CO_2 at 37^oc in one T_{75} ($1x10^6$ cells) and two T_{25} ($3x10^5$ cells in each flask) culture flasks. After three days, the cell cultures were washed with PBS warmed at 37^oc, and 2 ml and 1 ml of trypsin was added to T_{25} and T_{75} culture flasks, respectively then incubated under 5% CO_2 for 5 min. Fresh medium was added to the flasks, pooled in to falcon tube and centrifuged at 1100 rpm for 5 min. The supernatant was then discarded and the pellet re-suspended with 3 ml media. Cell count was determined using trypan blue exclusion method, the cells were $1.56x10^6$ cells/ml.

Before the main experiments, pilot studies of solubility were performed to find the appropriate exposure concentrations for the evaluation of the potential cytotoxicity (data not shown). Stock solutions were prepared from which 25 mg/ml, 5 mg/ml and 1 mg/ml

test concentrations were prepared. The final concentrations of extracts were 0.5 mg/ml, 0.25 mg/ml, 0.05 mg/ml and 0.01 mg/ml.

Hundred-microlitre cell suspension (1.2×10^5 cells) was seeded to 96-well microlitre plate in triplicate and incubated under 5% CO_2 at 37^oc for two days. After two days, the culture media was aspirated and test concentrations of extracts and vehicle added. The mixture was incubated under 5% CO_2 at 37^oc for 3 h and 20 µl CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent was added to each well and incubated for 1 h. Reference wells (for background absorbance correction) were prepared that contained 100 µl media and extract. The absorbance of the color complex was recorded at 490 nm with a 96-well plate reader. Three independent experiments were done for each extract and the experimental design is shown in Appendix III. Reference (background) absorbance values were subtracted from sample absorbance values at 490 nm wavelength. Cell viabilities (cell toxicities) were assessed by determining the ratio of the sample values to the control values as described below:

$$\% \text{ Viability} = \frac{\text{Sample Value} \times 100}{\text{Control Value}}$$

3.2.6 Genotoxicity assay

i) Selection of exposure concentrations

Before the main experiments, pilot studies of solubility and viability were done to find the appropriate exposure concentrations for the evaluation of the potential genotoxicity

(data not shown). The solubility of the extracts was tested in different solvents using 0.5 mg/ml as the maximum concentration during exposure. *P. stellatum* chloroform and 80% methanol extracts were easily soluble in 99.5% ethanol where as *O. integrifolia* and *V. amygdalina* chloroform, extracts were soluble in DMSO at 50 mg/ml. The final concentration of vehicle (solvent) was non-toxic and did not exceed 1%. The viability of the cells after 3 h exposure was checked using the Trypan blue exclusion method. According to Tice *et al*. (2000), cell viability below 70% is considered to indicate cytotoxicity and lower cell viability than that should be avoided when evaluating potential genotoxicity in the comet assay. Thus, 0.01, 0.05, 0.25 and 0.5 mg/ml concentrations were used for all test extracts.

ii) Cell culture

$HepG_2$ cells were also used to investigate the genotoxicity of *P. stellatum*, *O. integrifolia* and *V. amygdalina*. The cells were cultured as described earlier under cytotoxicity. Thus, $4x10^5$ cells were suspended in DMEM containing 10% FCS and 1% pencillin-streptomycin then seeded in each well of 6-well plate and incubated under humidified 5% CO_2, 37^oc incubator for two days.

After two days, the medium of each well was thrown away, washed twice with PBS and the cells were treated with the extract, control solutions then incubated for 3 h under 5% CO_2 at 37^oc. The upper medium of each well was thrown away and after trypsinization, they were neutralized by the medium and were poured in five different falcons and

centrifuged. Then, 1 ml of the medium was added to each falcon to use for next stages of the comet assay.

iii) Comet assay

Immediately after exposure and washing, the cells were put on ice until the slides for the comet assay were ready and the cell viability was determined using the Trypan blue technique. Only cell suspensions with viabilities of more than 90% were used for determination of DNA damage.

Each experimental set up was based on three independent electrophoresis runs, and from each electrophoresis run (cell culture); three slides per treatment were prepared for the assay. The DNA damage was evaluated using the alkaline version of the comet assay following a slightly modified protocol of Singh *et al*. (1988) and Tice *et al*. (2000). Immediately after cell viability determination, the cells were centrifuged twice with DMEM, 5% FCS and 1% pencillin-streptomycin. The cells were then re-suspended and mixed with low melting point agarose at 37°c and applied to microscope slides (Menzel-Gläser Diagnostika, Germany), which had been pre-coated with normal melting point agarose. Triplicate slides were applied for each extract and control. The slides were covered with cover slip and placed in cold plate for 15 min at 4°c. The slides were then uncovered and incubated in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10mM Trizma base, 1% Triton X-100, 10% DMSO, pH 10 adjusted with NaOH) at 4°c for 1 h protected from light.

The slides were transferred to an electrophoresis chamber at 4°c where they were treated in the dark with an alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 40 min before subjected to electrophoresis (25 V and 300 mA) in darkness at 4°c for 10 min . After electrophoresis, the slides were washed for 15 min in a buffer (0.4 % Tris, pH 7.5), dried and stored in closed containers until the day of image analysis.

GelRed was added (10 µl) to 50 ml distilled water in a cuvette and the slides were incubated in the cuvette for 30 min in dark. The slides were held in neutralization solution (pH 7.5) for 30 sec in dark. The slides were then kept in a humidity chamber until detection. After staining of the slides, detection was performed with an Olympus BX60 fluorescence microscope with an AVT FireWire camera (Stingray; Allied Technologies, Germany), a CoolLED pE-100 excitation light (536 nm) and the software Comet Assay IV (Perspective instruments, UK). All slides were coded independently and scored blindly. Apparently dead cells (comets without distinct heads: “clouds”) and super imposed comets were not captured during the image analysis. Fifty cells per slide were examined. All tests and controls were used in triplicate and three repeated experiments were performed, which gave 450 examined cells per treatment. The mean tail intensity (showing the percentage of DNA that had moved from the nucleus towards the anode during electrophoresis) was used as an indicator for the level of DNA damage. Relative fluorescence intensity of head and tail, normally expressed as a percentage of DNA in the tail was considered as the parameter for measuring DNA damage in this case. This parameter is linearly related to the break frequency and covers the widest range of damage (Paul *et al*., 2015).

3.2.7 Statistical analysis

The statistical analysis of all results was done using the Statistica 10 and GraphPad Prism 5 softwares and the level of statistical significance was set at $P < 0.05$. Regarding comet data, the average mean tail intensities (TI) from each individual experiment and treatment (usually at least 3 cultures/treatment) were pooled and the average mean TI was used as an indicator of DNA damage and the standard error of mean (SEM) as the measure of variance. For cytotoxicity assay, the results were mean of three independent experiments (mean±SD) and are expressed as percentage cell survival (% control).

Differences in average values between vehicle-treated control cells and extract-exposed bacterial/$HepG_2$ cells were evaluated using a two-tailed unpaired t-test for independent samples, assuming equal variance between the different average mean values and/or one-way analysis of variance (ANOVA). One-way ANOVA followed by Dunnett's multiple comparison test was used to compare the extract and vehicle treated CFUs.

3.2.8 Ethical considerations

Ethical approval was obtained from the Institutional Research Board of the College of Health Sciences, Addis Ababa University and the Armauer Hansen Research Institute (AHRI), Ethiopia.

4. Results

4.1 Antimycobacterial activity of crude extracts using CFU count method

Good growth of *M tuberculosis* H37Rv was evident in the microtitre plate wells containing only liquid Middlebrook 7H9 medium and in the universal Petri dishes containing solid Mycobacteria 7H11 medium, within three to four weeks. DMSO control at 2.5% to 0.0024%, equivalent to solvent concentrations in test extracts, exhibited no inhibitory effect on mycobacterial growth as evidenced by higher CFU/ml (Table 2). Among the different concentrations of isoniazid used, total inhibition of growth of mycobacteria was observed at a concentration of 0.125 µg/ml.

Extracts showed differential effects on the proliferation of mycobacterium. The chloroform extract of *P. stellatum* appeared to be the most active as growth was totally inhibited at concentrations as low as 0.039 mg/ml. Concentrations more than 0.3 mg/ml were required to bring about no growth with the 80% methanol extract of *P. stellatum* and *O. integrifolia* as well as with the chloroform extract of *O. integrifolia*. For the rest of the extracts, increasing concentrations (>1 mg/ml) were associated with growth arrest (Table 2).

The chloroform and 80 % methanol extracts of *P. stellatum* and *O. integrifolia* as well as 80% methanol and acetone extracts of *P. americana* had significant antimycobacterial activity ($p < 0.001$) against *M. tuberculosis* H37Rv compared to vehicle treated group. By contrast, *V. amygdalina* and *C. edulis* chloroform extracts did not have any detectable effect. It is interesting to note that although there was complete inhibition of growth of *M.*

tuberculosis H37Rv at concentrations greater than 1.25 mg/ml for *V. amygdalina* and *C. edulis* chloroform extracts, the difference failed to reach statistical significance ($P>0.05$) (Table 2).

Table 2: Antimycobacterial activity of experimental plants using colony count method

Concentration of extract (mg/ml)	CFU/ ml									
	DMSO ł	***P. stellatum*** **$CHCl_3$ extract**	***P. stellatum*** **MeOH extract**	***O. integrifolia*** **$CHCl_3$ extract**	***O. integrifolia*** **MeOH extract**	**DMSO ǂ**	***V. amygdalina*** **$CHCl_3$ extract**	***C. edulis*** **$CHCl_3$ extract**	***P. americana*** **MeOH extract**	***P. americana*** **acetone extract**
2.50	583	0	0	0	0	354	0	0	0	0
1.25	1194	0	0	0	0	476	0	0	72	0
0.625	2944	0	0	0	0	488	62	43	197	84
0.312	4000	0	0	0	150	742	166	168	64	282
0.156	5944	0	100	334	270	800	260	208	103	94
0.078	8444	0	277	566	650	817	242	222	66	89
0.039	9345	0	302	860	1050	871	496	435	94	344
0.019	1122	100	351	360	886	1275	1033	552	89	1028
0.009	12333	100	421	3334	1334	1517	1733	742	80	28
0.005	19861	100	450	3389	3889	958	2013	813	578	1120
0.0024	16944	100	200	3445	6389	1100	2438	1206	698	1249
Mean ±SEM	7519.45 ± 1984.82	36.36 *** ± 15.21	191.00 *** ± 53.90	1117.00 *** ± 447.70	1329.00 *** ± 608.30	854.36 ± 351.01	767.55 ± 269.50	399.00 ± 117.99	185.55 *** ± 69.34	392.50 * ± 147.70

ł = Solvent CFU for chloroform and methanol extracts of *P. stellatum* and *O. integrifolia*,

ǂ = Solvent CFU for chloroform extract of *V. amygdalina* and *C. edulis* and 80% methanol and acetone extract of *P. Americana*, MeOH = methanol.

* = $p<0.05$, *** = $p< 0.001$

4.2 Antimycobacterial activity against clinical isolates of MDR-TB using Resazurin indicator method

As the chloroform extract of *P. stellatum* demonstrated the highest activity (MIC = 0.039 mg/ml) against the standard strain in the CFU method, screening of the activity of this extract against clinical isolates of MDR-TB was carried out using resazurin indicator method.

The resazurin assay results are depicted in Table 3. Color readings of the growth control wells were pink, demonstrating high levels of mycobacterial growth, while wells with broth alone appeared as blue color, which demonstrated no growth and lack of contamination. The isoniazid containing control wells were pink colors revealing the clinical isolates were isoniazid resistant. Whilst the extract demonstrated the highest activity against AOZ8W-4 strain (MIC 78 µg/ml), it had a moderate activity against both AOA8W-4 and SO38SW-4 strain (MIC 156 µg/ml).

Table 3: Antimycobacterial activity test of *Pterolobium stellatum* chloroform extract against MDR-TB clinical isolates using Resazurin indicator method

		MDR-TB clinical isolates' suspension color		
Plant extracts and controls	**Concentration (mg/ml or %)**	**AOA8W-4**	**AOZ8W-4**	**SO38SW-4**
P. stellatum $CHCl_3$ extract	2.500	blue	blue	blue
	1.250	blue	blue	blue
	0.625	blue	blue	blue
	0.312	blue	blue	blue
	0.156	blue	blue	blue
	0.078	pink	blue	pink
	0.039	pink	pink	pink
	0.019	pink	pink	pink
	0.009	pink	pink	pink
	0.005	pink	pink	pink
	0.0024	pink	pink	pink
DMSO control	2.5%-0.0024%	Pink	Pink	Pink
Isoniazid	1×10^{-3}	Pink	Pink	Pink
	0.5×10^{-3}	Pink	Pink	Pink
	0.25×10^{-3}	Pink	Pink	Pink
	0.125×10^{-3}	Pink	Pink	Pink
	0.06×10^{-3}	Pink	Pink	Pink
	0.03×10^{-3}	Pink	Pink	Pink
Growth control	-	Pink	Pink	Pink
Sterility control	-	blue	blue	blue

- = Not applicable

4.3 Activity of *Pterolobium stellatum* solvent fractions on *Mycobacterium tuberculosis* H37Rv using Resazurin indicator method

As presented in Table 4, n-hexane and aqueous fractions were evaluated in a concentration series of 1000 to 0.977 µg/ml, while ethyl acetate fraction with 200 to 0.195 µg/ml concentrations. The microtitere wells were pink below a concentration of 15.625 µg/ml for the n-hexane fraction, indicating that this fraction was active against the standard strain (MIC = 15.625 µg/ml). By contrast, all wells were pink for the aqueous fraction, suggesting that this fraction was not active against the bacteria within the exposure concentrations used for the experiment. The ethyl acetate fraction was active against *M. tuberculosis* H37Rv (MIC = 0.195 µg/ml), indicating that this fraction is the most active fraction of *P. stellatum* chloroform extract (Table 5). As described above, all wells treated with the vehicle were pink, demonstrating no inhibitory role of the vehicle.

4.4 Activity of compounds isolated from *Pterolobium stellatum* against *Mycobacterium tuberculosis* H37Rv using Resazurin indicator method

As presented in Table 6, compounds **1**, **2** and **3**, isolated from the ethyl acetate fraction of *P. stellatum* were evaluated in a concentration range of 10 to 0.078 µg/ml. Compound **3** was the most active (MIC = 0.625 µg/ml) compound followed by compound **1** (MIC = 1.25 µg/ml) and compound **2** (MIC = 2.5 µg/ml). As described above, all wells treated with the vehicle and the sterility control wells were pink and blue, respectively, demonstrating no inhibitory role of the vehicle and lack of contamination.

Table 4: Test suspension colors obtained from fractions tested against *Mycobacterium tuberculosis* H37Rv in the Resazurin indicator method

n-hexane Fraction		Ethyl Acetate Fraction		Water Fraction		Isoniazid		DMSO	
Concent-ration (µg/ml)	Color	Concent-ration (µg/ml)	Color	Concent-ration (µg/ml)	Color	Concent-ration (µg/ml)	Color	Concent-ration (%)	Color
1000	blue	200	blue	1000	pink	1	blue	2.5	pink
500	blue	100	blue	500	pink	0.5	blue	1.25	pink
250	blue	50	blue	250	pink	0.25	blue	0.625	pink
125	blue	25	blue	125	pink	0.125	blue	0.312	pink
62.5	blue	12.5	blue	62.5	pink	0.06	pink	0.156	pink
31.25	blue	6.25	blue	31.25	pink	0.03	pink	0.078	pink
15.625	blue	3.125	blue	15.625	pink	-	-	0.039	pink
7.813	pink	1.563	blue	7.813	pink	-	-	0.02	pink
3.906	pink	0.781	blue	3.906	pink	-	-	0.01	pink
1.953	pink	0.391	blue	1.953	pink	-	-	0.005	pink
0.977	pink	0.195	blue	0.977	pink	-	-	0.0024	pink

- = Not applicable

Table 5: Minimum inhibitory concentrations of solvent fractions against *Mycobacterium tuberculosis* H37Rv

Solvent fraction	**Minimum Inhibitory Concentration (MIC) (µg/ml)**
n-hexane fraction	15.625
Ethyl acetate fraction	0.195
Water fraction	+++
Isoniazid	0.125
Growth control	+++
DMSO control	+++

+++ = Growth

4.5 Mass spectrometric analysis of some isolated compounds

As depicted in the appendix (Appendices IV-VI), compound **1** gave a base peak (most abundant ion) at mass to charge ratio (*m/z*) of 391.2923. Similarly, the mass to charge ratio (m/z) of the most abundant ions produced from compounds **2** and **3** were 430.16 and 419.04, respectively. Unfortunately, the MS is not enough to do full characterization. The NMR and IR investigation of the compounds is underway to fully characterize and elucidate their structure.

Table 6: Test suspension colors obtained from isolated compounds against *Mycobacterium tuberculosis* H37Rv in the Resazurin indicator method

Compound 1		**Compound 2**		**Compound 3**		**Isoniazid**		**Ethanol**	
Concent-ration (µg/ml)	**Color**	**Concent-ration (µg/ml)**	**Color**	**Concent-ration (µg/ml)**	**Color**	**Concent-ration (µg/ml)**	**Color**	**Concent-ration (%)**	**Color**
5	blue	5	blue	5	blue	1	blue	2.5	pink
2.5	blue	2.5	blue	2.5	blue	0.5	blue	1.25	pink
1.25	blue	1.25	pink	1.25	blue	0.25	blue	0.625	pink
0.625	pink	0.625	pink	0.625	blue	0.125	blue	0.312	pink
0.313	pink	0.313	pink	0.313	pink	0.06	pink	0.156	pink
0.156	pink	0.156	pink	0.156	pink	0.03	pink	0.078	pink
0.078	pink	0.078	pink	0.078	pink	-	-	0.039	pink

- = Not applicable

4.6 Acute oral toxicity test

Acute oral administration of the chloroform extract of *P. stellatum* at different doses showed no overt signs of distress for the 14-day observation period. Moreover, there were no observable symptoms of toxicity or deaths even at a dose of 5000 mg/kg. This indicates that the oral LD_{50} was greater than 5000 mg/kg. All treatment and control group mice gained weight and no significant changes in behavior was noted, suggesting that administration of the crude extract had negligible level of toxicity on the growth of the animals. In addition, gross behavioral changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality and other signs of toxicity manifestations were not observed.

4.7 Cytotoxic effect of plant extracts against $HepG_2$ cells

Cytotoxicity activity of *P. stellatum, O. integrifolia, C. edulis, V. amygdalina and P. americana* were carried out against $HepG_2$ cell line at different concentrations to determine the IC_{50} (50% growth inhibition) by MTS/PMS assay. Results of different concentrations of the extract are presented in Table 7 and Figures 5-7.

Table 7: IC_{50} values (mean ± SED) for extracts against $HepG_2$ cells obtained using the MTS assay for cytotoxicity

Plant extract	**IC_{50} (mg/ml)±SEM**	**Hill slope**	**r^2**
P. stellatum $CHCl_3$ extract	0.5263±14.86	-15.82	0.9711
P. stellatum 80% methanol extract	0.4790 ± 0.07150	-2.174	0.9586
O. integrifolia $CHCl_3$ extract	0.5256 ± 41.77	-18.44	0.5447
O. integrifolia 80% methanol extract	0.4753 ±0.1089	-1.719	0.9355
C. edulis $CHCl_3$ extract	0.2525±0.3305	-0.3476	0.9987
V. amygdalina $CHCl_3$ extract	3.202± 0.3375	-0.5501	0.9799

r^2 = regression coefficient

Chloroform extract of *V. amygdalina* and *C. edulis* exhibited the highest and the lowest IC_{50} value, respectively. In addition, the higher value for the slope of *O. integrifolia* and *P. stellatum* chloroform extracts suggests that the dose-response curves for these extracts are very steep (Table 7).

At the highest concentration used (0.5 mg/ml), the chloroform extract of *P. stellatum* showed a viability of more than 80% (Figure 5). By contrast, at the same concentration, 80% methanol extract of the plant significantly reduced $HepG_2$ cells' viability approximately by 45% (Figure 6). At concentrations below 0.5 mg/ml, neither of the extracts produced discernible effect on $HepG_2$ cell viability, with the chloroform extract providing better viability than the methanol extract.

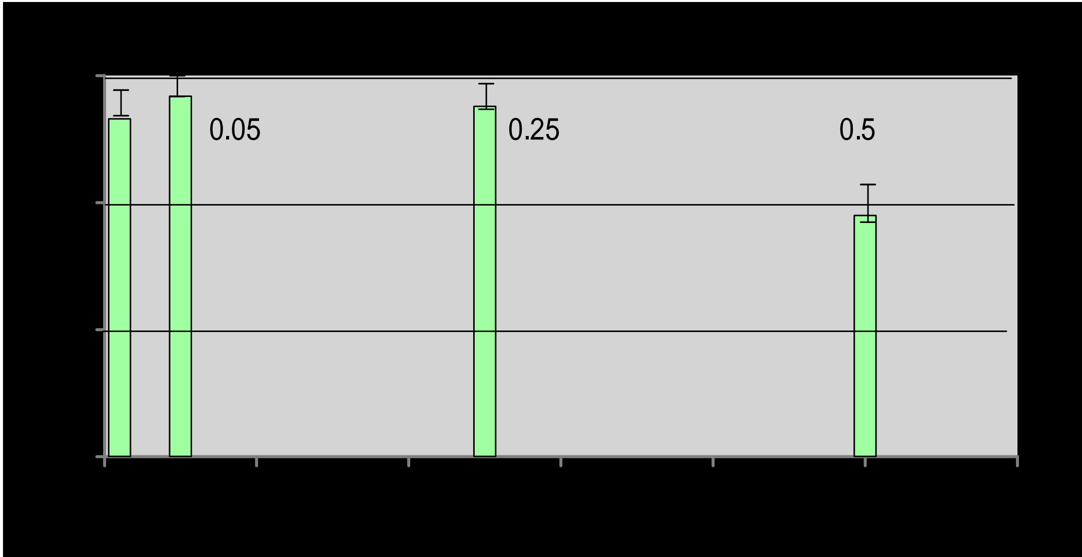


Figure 5: Cytotoxic effect of *Pterolobium stellatum* chloroform extract on $HepG_2$ cells:

pc = *Pterolobium stellatum* chloroform extract

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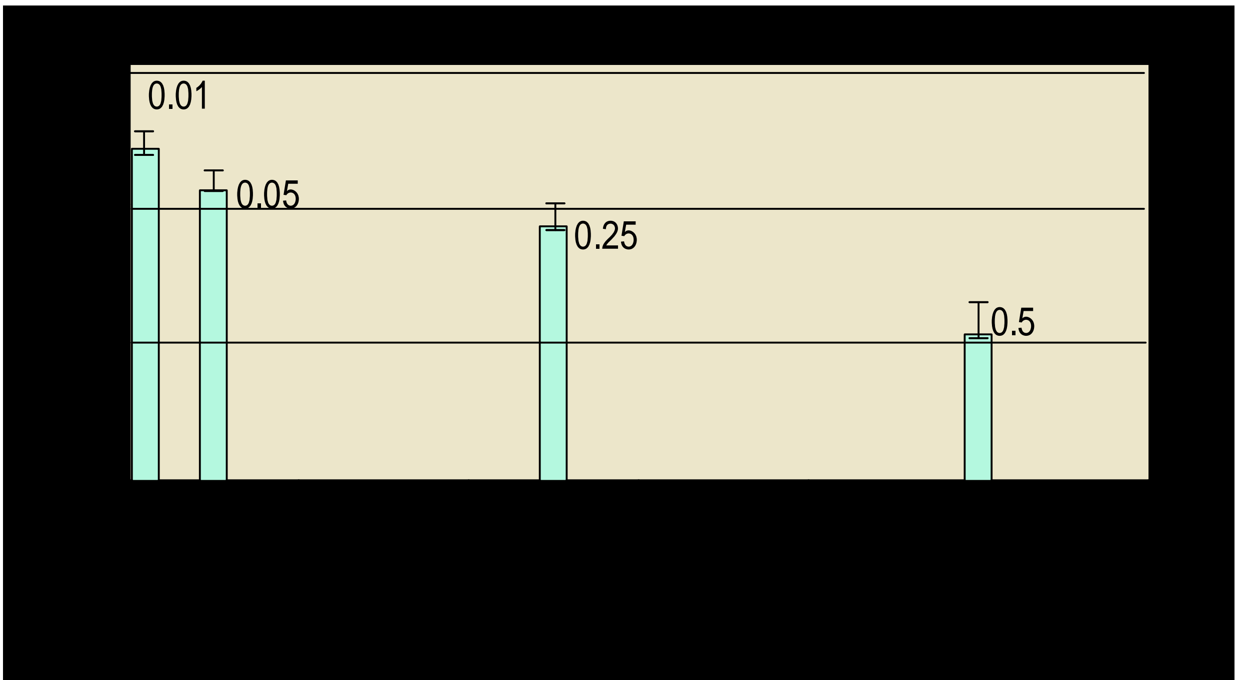

Figure 6: Cytotoxic effect of 80% methanol extract of *Pterolobium stellatum* on $HepG_2$ cells.

At all test concentrations, *O. integrifolia* chloroform extract exhibited toxicity on $HepG_2$ cells, since maximum viability was 72% (Figure 7). However, its methanol extract at the same test concentrations showed sustained cell viability of more than 100% except at 0.5 mg/ml concentration, where viability was < 100%. Sustained cell viability was observed at 0.01 mg/ml, 0.05 mg/ml and 0.25 mg/ml in case of methanol extract of the plant on $HepG_2$ cells. Cell viability reduced as the exposure concentrations of chloroform and methanol extracts increased from 0.05 mg/ml to 0.5 mg/ml (Figure 7).

Chloroform extract of *V. amygdalina* appeared to be non-toxic, as cell viability was > 100% at all concentrations tested. However, toxicity of *C. edulis* chloroform extract seemed to be a function of concentration, since viability was reduced at concentrations > 0.05 mg/ml, and sustained cell viability was achieved at 0.01 mg/ml (Figure 7).

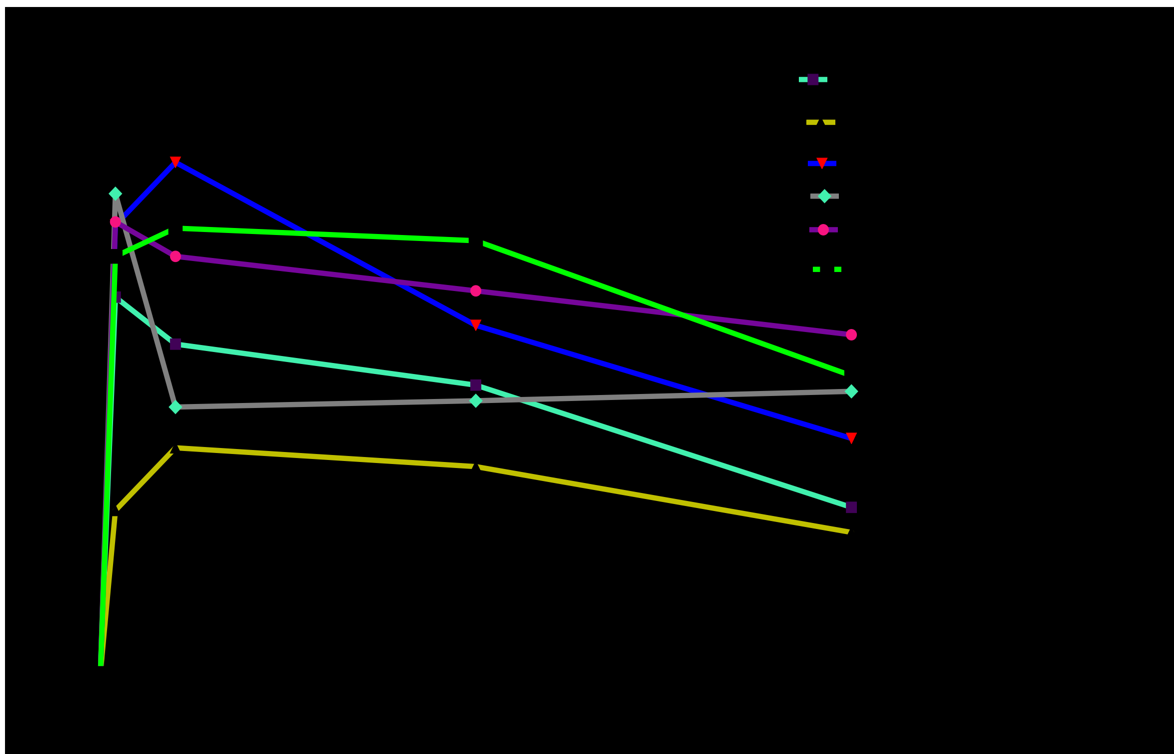

Figure 7: Cytotoxic effect of experimental plants

4.8 DNA damage assay of selected plant extracts against $HepG_2$ cells

White spots were detected in the genotoxicity assay of selected plant extracts as shown in Figure 8. Comets with distinict heads and without tails [Figure 8 (a)] were vehicle treated non- migrated nuclear DNA from individual cells, whereas comets with distinict heads and tails ([Figure 8 (b)] were extract treated and migrated ones.

a **b**

Figure 8: White spots [(a) comets without tails (b) comets with tails] of the comets that were detected in genotoxicity test. The software Comet Assay IV (Perspective instruments, UK) was used to analyse the images.

Percent DNA in tail (tail intensity) in the DNA damage evaluation of the extracts along with the test concentrations are presented in Table 8. DNA damage in the form of comet tail was observed on $HepG_2$ cells exposed to 1 and 0.5 mg/ml of *P. stellatum* chloroform and 80% methanol extracts, respectively. The tail DNA percent ranged from 2.00±0.09 to 4.03±0.57 for *P. stellatum* chloroform extract treated sets at a concentration range of 0.01 to 1 mg/ml. The range was 2.61±0.14 to 2.95±0.02, 2.16±0.26 to 3.02±0.24, and 1.73±0.10 to 2.90±0.18 at test concentrations of 0.01 to 0.5 mg/ml for the methanol and chloroform extract of *O. integrifolia*, and for the chloroform extract of *V. amygdalina*, respectively.

Table 8: Mean percentage DNA in tail (tail intensity) of different concentrations of extract treated and control $HepG_2$ cells

No. Of experiments	***P. stellatum*** **$CHCl_3$ extract**							***P. stellatum*** **80% MeOH extract**						***O. integrifolia*** **$CHCl_3$ extract**						***V. amygdalia*** **$CHCl_3$ extract**					
	Control		Concentration of extract (mg/ml)					Control		Concentration of extract (mg/ml)				Control		Concentration of extract (mg/ml)				Control		Concentration of extract (mg/ml)			
	EtOH 1%	Catechol 3mM	0.01	0.05	0.25	0.5	1	EtOH 1%	Catechol 3mM	0.01	0.05	0.25	0.5	DMSO 1%	Catechol 3mM	0.01	0.05	0.25	0.5	DMSO 1%	Catechol 3mM	0.01	0.05	0.25	0.5
1	2.59	-	2.04	2.04	2.38	2.56	-	2.21	5.03	2.89	2.72	2.90	2.97	2.64	5.60	2.62	2.51	3.25	2.31	2.64	3.76	1.64	1.82	2.07	2.99
2	1.80	5.88	2.13	1.95	2.93	2.00	-	2.05	5.18	2.51	3.07	2.21	2.90	3.08	5.18	2.16	2.43	2.54	3.00	2.63	3.36	1.61	1.57	2.59	2.55
3	1.30	3.20	1.82	2.53	2.31	2.72	2.89	2.37	5.08	2.42	2.38	2.30	2.97	2.57	3.88	1.71	2.97	3.27	3.00	2.60	4.54	1.93	1.22	1.78	3.15
4	1.47	3.16	-	1.54	1.72	2.67	4.51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	3.47	5.82	-	-	3.55	3.94	4.70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean ± SEM	2.12±0.40	4.52±0.77	2.00±0.09	2.02±0.20	2.58±0.31	2.78±0.32	4.03±0.57	2.21±0.09	5.10±0.04	2.61±0.14	2.72±0.20	2.47±0.22	2.95±0.02	2.76±0.16	4.89±0.52	2.16±0.26	2.64±0.17	3.02±0.24	2.77±0.23	2.62±0.01	3.89±0.35	1.73±0.10	1.54±0.17	2.15±0.24	2.90±0.18
p-value	1.00	0.022	0.819	0.827	0.399	0.239	0.030	1.00	0.0001	0.08	0.08	0.33	0.001	1.00	0.02	0.12	0.61	0.42	0.98	1.00	0.02	0.001	0.003	0.11	0.20

The chloroform extract of *P. stellatum* showed increased tail DNA percentage in a concentration dependent manner. It had an increased genotoxic effect after 3 h exposure at a concentration of 1 mg/ml compared to controls. Comet tail length in the extract treated cells (1 mg/ml) was significantly higher by 89% ($p < 0.05$) compared to vehicle treated controls. However, at lower concentrations no apparent genotoxic effect was observed. The extent of DNA damage at 1 mg/ml exposure was comparable to the damage induced by the positive control (3 mM catechol) (Figure 9).

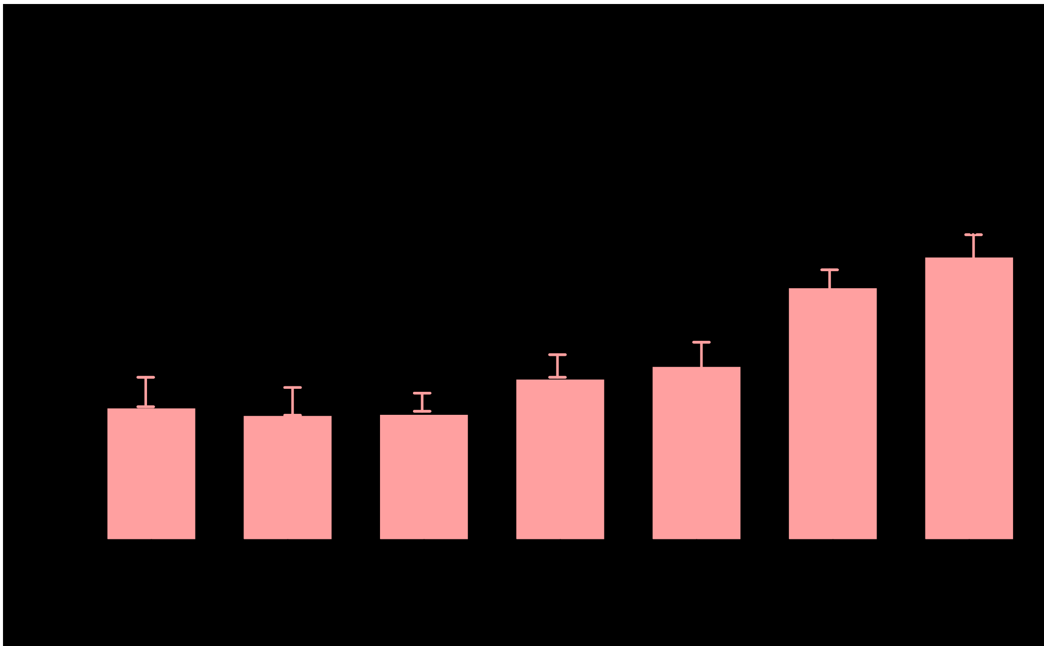

Figure 9: Genotoxic effect of *Ptrolobium stellatum* chloroform extract on $HepG_2$cells: cells were exposed for 3 hours and DNA damage was monitored as an increase in percentage of DNA in the tail (tail intensity) after 10 min of electrophoresis in the comet assay. % vehicle = 1% ethanol (negative control) and 3mM catechol (positive control). The means of percentage of DNA in tail for at list three experiments were compared with the vehicle control using T-test for independent samples, * = $p < 0.05$.

Pterolobium stellatum 80% methanol extract significantly increased DNA damage at a concentration of 0.5 mg/ml ($p < 0.001$) when compared to the vehicle treated cells, although the damage was not concentration dependent (Figure 10). Concentrations lower than 0.5 mg/ml were not significantly associated with DNA damage.

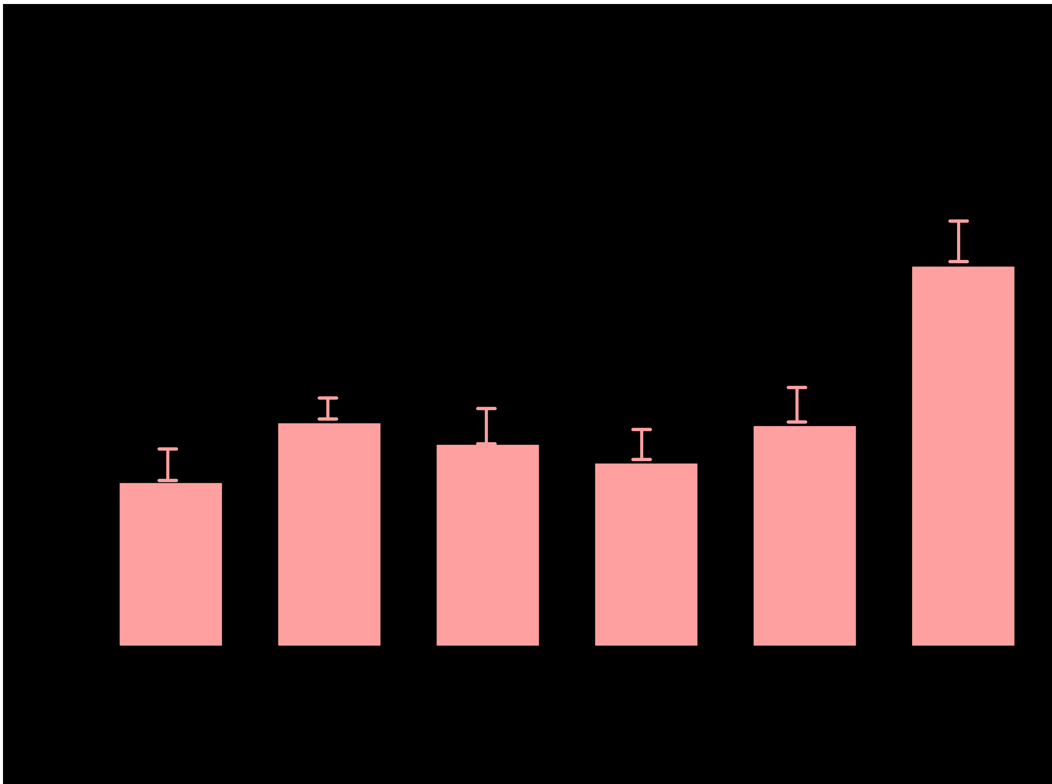

Figure 10: Genotoxic effect of *Pterolobium stellatum* 80% methanol extract on $HepG_2$ cells: cells were exposed for 3 hours and DNA damage was monitored as an increase in percentage of DNA in the tail (tail intensity) after 10 min of electrophoresis in the comet assay. % vehicle = 1% ethanol (negative control) and 3mM catechol (positive control). The means of percentage of DNA in tail for at list three experiments were compared with the vehicle control using T-test for independent samples, * = $p < 0.01$, ** = $p < 0.001$.

Treatment of cells with the choloroform extract of *O. integrifolia* did not show any detectable DNA damage at all exposure concentrations as compared to vehicle treated controls. However, the positive control demonstrated significant damage ($p < 0.05$) (Figure 11).

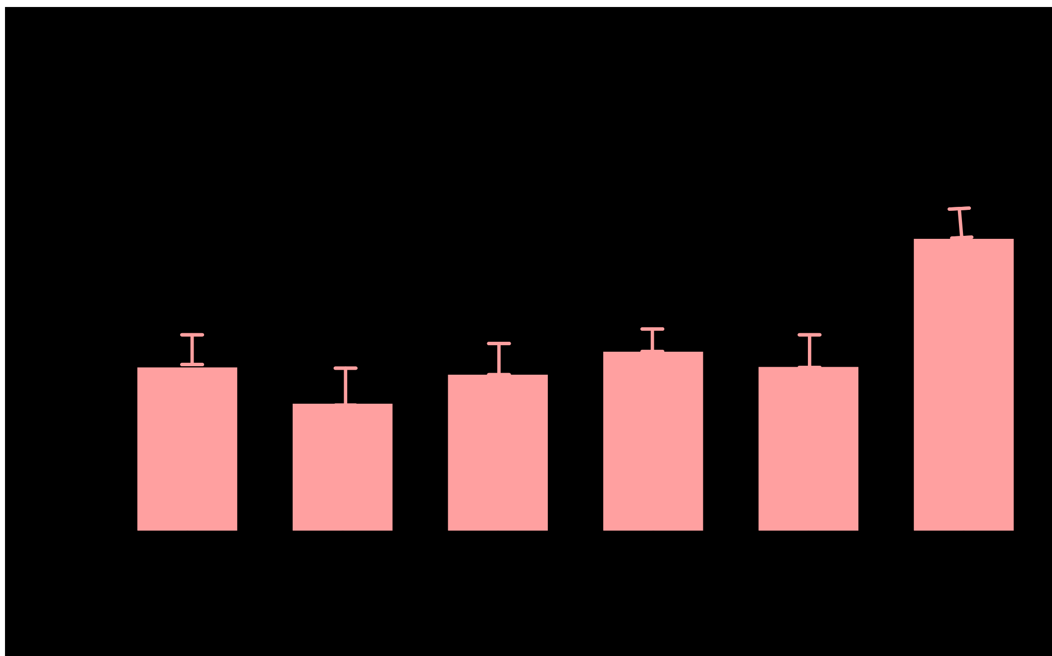

Figure 11: Genotoxic effect of *Otostegia integrifolia* chloroform extract on $HepG_2$ cells: cells were exposed for 3 hours and DNA damage was monitored as an increase in percentage of DNA in the tail (tail intensity) after 10 min of electrophoresis in the comet assay. % vehicle = 1% DMSO (negative control) and 3mM catechol (positive control). The means of percentage of DNA in tail for at list three experiments were compared with the vehicle control using T-test for independent samples, * = $p < 0.05$.

Treatment of $HepG_2$ cells with *V. amygdalina* at 0.01, 0.05 and 0.25 mg/ml concentrations showed tail DNA percentages 1.73±0.10, 1.54±0.17 and 2.15±0.24

respectively. The values were lower than the tail DNA percentage of the negative control (2.62±0.01), while the value (2.90±0.18) at 0.5 mg/ml exposure was greater than the negative control. Treatment of $HepG_2$ cells with the chloroform extract of *V. amygdalina* produced a significant decrease in percentage DNA in tail at 0.01 mg/ml ($p < 0.001$) and 0.05 mg/ml ($p < 0.05$) compared to negative controls. The decrease and increase observed at 0.25 mg/ml and 0.5 mg/ml, respectively, however, was not statistically significant (Figure 12).

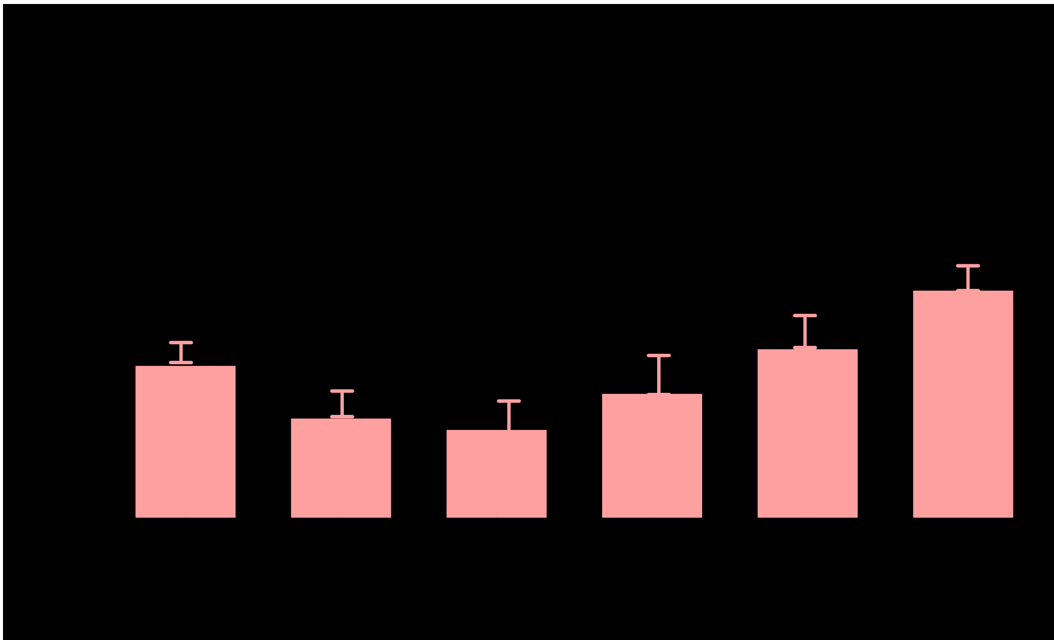

Figure 12: Genotoxic eeffect of *Vernonia amygdalina* chloroform extract on $HepG_2$ cells: cells were exposed for 3 hours and DNA damage was monitored as an increase in percentage of DNA in the tail (tail intensity) after 10 min of electrophoresis in the comet assay. 1% vehcle = 1% DMSO (negative control) and 3mM catechol (positive control). The means of percentage of DNA in tail for at list three experiments were compared with the vehicle control using T-test for independent samples, * = $p < 0.05$, ** = $p < 0.005$.

5. DISCUSSION

Current TB therapy, also known as DOTS, consists of treatment with a combination of drugs. This combination therapy causes hepatotoxicity as the major side effect and development of drug resistance. To avert toxicity and reduce ineffectiveness of current anti-TB drugs, medicinal plants can be considered as potential anti-tubercular agents that can be used in combination with the standard anti-tubercular drugs or alone (Nair *et al*., 2015). Thus, antimycobacterial activity test was conducted on five plant extracts and the result revealed that three of them had significant anti-tubercular activity. The activity was seen with chloroform and methanol extracts of *P. stellatum*and and *O. integrifolia* as well as with methanol and acetone extracts of *P. americana*. In addition, fractions and isolated compounds from the most active plant, *P. stellatum* had demonstrated promising antimycobacterial activity.

MICs of all experimental plant extracts and solvent fractions as well as isolated compounds from the most active plant extract were determined by colony count method and microplate resazurin assay method. As evidenced from Tables 2 and 3, *P. stellatum* chloroform extract was endowed with lower MIC by colony count method than by microplate resazurin assay method, although test organisms were different. It has been reported that the MIC exhibited by a compound/extract depends on the technique used for determination. MICs obtained in a liquid medium are lower than that obtained in solid medium, as the drug has to diffuse through the matrix in the solid medium in order to exert activity (Nair *et al*., 2015).

Thus, for method comparison, activity of *P. stellatum* chloroform extract was evaluated using colony count method and Resazurin indicator method as well as MIC was determined. The finding in the later method revealed that the MICs were 0.078, 0.156 and 0.156 mg/ml against AOZ8W-4, AOA8W-4 and SO38SW-4 MDR-TB clinical isolates, respectively, whereas in the colony count method; the same plant extract had lower MIC (0.039 mg/ml) against the standard strain. The difference in MICs might be ascribed to the difference in susceptibility of the standard strain and MDR-TB clinical isolates towards the extract. Furthermore, this apparent difference might be attributed to more favorable growth conditions provided by broth culture medium to extract-treated bacilli in resazurin indicator method or the drug susceptible standard strain and the drug resistant strain might have different growth conditions.

Another attributable cause for the different MICs of *P. stellatum* chloroform extract could be due to slight difference in the sensitivities of colony count method and resazurin indicator method as reported by Taneja and Tyagi (2007). According to this study , Resazurin indicator assay was noted to be superior to the colony count assay in that it distinguished between metabolically active dormant bacteria and non-viable organisms, unlike the colony count assay that could not differentiate between these two populations.

In this study, *P. stellatum* chloroform extract showed promising activity against *M. tuberculosis* H37Rv and AOZ8W-4 with complete inhibition of *M. tuberculosis* at 0.039 mg/ml and 0.078 mg/ml, respectively, which were within the range stated by the Clinical and Laboratory Standards Institute. Moreover, this extract exhibited promising activity at 0.039, 0.078 and 0.156 mg/ml against both drug susceptible and drug resistant strain with

100% growth inhibitions according to Tosun *et al*. (2004). It was also more active against *M. tuberculosis* H37Rv than the clinical isolates of MDR-TB but less active than the standard drug (isoniazid).

According to either Tosun *et al*. (2004) or Sánchez *et al*. (2010), *P. stellatum* 80% methanol extract (MIC = 0.312 mg/ml) and *P. americana* methanol (MIC = 2.5 mg/ml) and acetone (MIC = 1.25 mg/ml) extracts did not exhibit promising activity against *M. tuberculosis* H37Rv. Potencies of chloroform extract of *O. integrifolia*, methanol extracts of *P. stellatum*, *O. integrifolia* as well as *P. americana* methanol and acetone extracts against *M. tuberculosis* H37Rv strain was low.

Previous studies reported several plants with promising anti-tubercular activity (Tosun *et al*., 2004, Newton *et al*., 2002, Higuchi *et al*., 2008, Billo *et al*., 2005, Jim´enez Arellanes *et al*., 2007, Mann *et al*., 2008). Studies had mostly reported activity in the plant families of Asteraceae, Lamiaceae, Fabaceae and Apiaceae, among others (Gautam *et al.,* 2007). It is noticeable from the present study that plants exhibiting activity belong to Fabaceae, Lamiaceae and Lauraceae families, which is in agreement with the plant families reported by Gautam *et al*. (2007).

Another study indicated that ethanolic extract of garlic prepared by maceration method was active against both non-MDR and MDR *M. tuberculosis* isolates, with MIC ranging from 1 to 3 mg/ml (Hannan *et al*., 2011). The activity obtained in this study from *P. americana* was comparable to the above-mentioned studies, while that of *P. stellatum* and

O. integrifolia was higher. Taking the criteria for plants with promising antimycobacterial activity, the plant extract reported by Hannan *et al*. (2011) could not be considered as promising, while *P. stellatum* chloroform extract could be taken as the plant worth investigating. In addition, the MDR-TB clinical isolates were resistant to isoniazid, while this plant was active against the clinical isolates of MDR-TB, possibly suggesting that the plant could have an obvious advantage over the standard drug.

There is sparse data in the literature about the antimycobacterial activity of *P. stellatum*. Hence, this makes comparison challenging. One study reported that 80% ethanol leaf extract of the plant had shown activity, with an MIC value of 250 mg/ml (Endale *et al.,* 2014). This value is much higher than the one reported in this study for the chloroform extract, indicating that non-polar constituents might be more important for the observed antitubercular activity.

Efforts made to compare activity of the chloroform extract of *P. stellatum* with other plants showed either better or comparable activity. Activity was better compared to stem bark extract of *Anogeissus leiocarpus* (Mann et al., 2008) and comparable to that of the chloroform leaf extract of *Byrsonima fagifolia* (Higuchi *et al*., 2008). Some of the extracts, namely, those from chloroform extract of *P. stellatum* and *O. integrifolia* showed strong antimycobacterial activity. This might be due to non-polar constituents contained in the chloroform extract that could penetrate lipid rich mycobacterial cell wall. It has been demonstrated that the chloroform and 80% methanol extracts of *O. integrifolia* were active against *M. tuberculosis* H37Rv with MICs 312 µg/ml and 625

μg/ml, respectively. The MICs in this finding are lower than a study done on *Pelargonium sidoides* methanol extract (MIC = 5000 μg/ml), *Capparis brassi*, *Entada africana* and *Combretum* species methanol extracts (MIC = 1250 μg/ml each) against *M. tuberculosis* H37Rv by Mann *et al*. (2008). However, the MICs of both extracts were higher than a study done on *Byrsonima fagifolia* chloroform extract (MIC = 62.5 μg/ml) by Higuchi *et al*. (2008) against the same test organism. This difference might be imputed to the fact that different plant species may contain different active constituents at varying amount and/or different *in vitro* methods were used.

In the present study, *P. americana* 80% methanol leaf extract had a lower antimycobacterial activity than the activity reported by Gomez-Flores *et al.* (2008). It has been reported that individual plants within a species may vary depending on a number of factors, including location, temperature, rainfall, season of collection, soil type, length of day, altitude and storage conditions. The extracts might also contain little of the active ingredient. The difference in *in vitro* activity by the plant could be because of the aforementioned factors (Gomez-Flores *et al.,* 2008).

Antimycobacterial activity evaluation revealed that *P. stellatum* chloroform root extract to have a better activity and further fractionation was pursued. It was fractionated in to ethyl acetate, n-hexane and water fractions as well as antimycobacterial activity was evaluated. Ethyl acetate fraction was found to be the most active fraction, which indicated that antimycobacterial constituents were contained in this fraction. As shown in Table 4, the MIC value for this fraction was 0.195 μg/ml, which was more potent fraction

than the n-hexane and water fractions. As to our knowledge, there is no published data concerning the isolation and characterization of antimycobacterial constituents from *P. stellatum* and hence in this study, three compounds were isolated from the most active fraction and activity was evaluated for each compound. Compound **3** was found to be the most potent compound (MIC = 0.625µg/ml), compound **1** (MIC = 1.25 µg/ml) and compound **2** (MIC = 2.5 µg/ml) were also active against the test organism. Research is underway to characterize these compounds. (Activity test of compounds on MDR-TB clinical isolates was not in the study plan)

A study carried out by Green *et al*. (2011) indicated that the n-hexane sub-fraction of ethyl acetate fractions from acetone extracts of *Bridelia micrantha* barks was evaluated using the resazurin microplate assay against two MTB isolates. The result showed 20% inhibition of MTB H37Ra and almost 35% inhibition of an MTB isolate resistant to all first-line drugs at 10 µg/ml. The present finding is comparable with our finding from n-hexane fraction (MIC = 15.625 µg/ml) in terms of active concentration of extracts. This might be an indication of the presence of small amount of antimycobacterial constituents in the n-hexane fraction. On the other hand, the finding from n-hexane fraction is dissonant with the study reported by Mann *et al*., 2008, in which n-hexane fraction of *Anogeissus leiocarpus* and *Terminalia avicennioides* extracts showed antimycobacterial activity with MICs 312 and 200 µg/ml, respectively**.**

MICs for isolated compounds in the present study were lower than the MICs of the compounds isolated from *Byrsonima fagifolia* such as α-amyrin acetate (MIC = 62.5),

dotriacontane (MIC = 62.5 µg/ml) and a mixture of lupeol, α- and β-amyrin (MIC = 31.5 µg/ml) (Higuchi *et al*., 2008). However, compound 2 was found to be comparable in activity with basic acid (MIC = 2.5 µg/ml). This higher activity of compounds in the present study may be attributable to the presence of more antimycobacterial active constituents in *P. stllatum* than *Byrsonima fagifolia* chloroform extract.

In the cytotoxicity study, *O. integrifolia* 80% methanol and chloroform extracts and *P. stellatum* 80% methanol extract decreased cell viability in a dose-dependent manner. Moreover, the IC_{50} value of *C. edulis* chloroform extract (0.2525 mg/ml±0.3305) was relatively lower than the other extracts that raise concern for further investigation and warn potential users. The IC_{50} value of *P. stellatum* chloroform extract, the most active extract against *Mycobacterium tuberculosis*, was relatively safer compared to other extracts. *O. integrifolia* chloroform extract and *P. stellatum* 80% methanol extract might be more toxic when compared to others as shown by the steepness of the slope of the curves (Table 7) and (Figures 6 and 7).

According to Tosun *et al*. (2004), compounds with an MIC of less than 10 µg/ml, and ideally less than 2 µg/ml with a selectivity index (SI = IC_{50}Vero cells/MIC) of >10 is considered as being of interest to pharmaceutical industry (Juan, 2012). *P. stellatum* chloroform and 80% methanol extracts had MICs of 39 and 312 µg/ml and IC_{50} of 526.3 and 479 µg/ml, respectively. Thus, the selectivity indices for the chloroform and methanol extracts were 13.5 and 1.5, respectively. As a result, chloroform extract

demonstrated > 10 selectivity index, which is expected from pure compounds and could be of interest to the pharmaceutical industries for further drug development.

In the present study, *V. amygdalina* chloroform root extract showed antimycobacterial activity (though it was not significant compared to vehcle control) against *M. tuberculosis* H37Rv and cytotoxic effect on $HepG_2$ cells by MTS assay method with MIC 1250 µg/ml (Table 2) and IC_{50} 3, 202 µg/ml (Table 7), respectively. The selectivity index was 2.56, which is comparable with the value (3.07) reported by Omoregie *et al*. (2011). According to Juan (2012), the selectivity indices of the hydroalcoholic extract reported by Omoregie *et al*. (2011) and this study were less than 10 through the criteria focused on pure compounds. Therefore, *V. amygdalina* hydroalcoholic leaf extract and chloroform root extract might be potentially toxic to humans especially at higher doses.

The finding in the present study also revealed that *O. integrifolia* chloroform root extract showed antimycobacterial activity against *M. tuberculosis* H37Rv and cytotoxic effect on $HepG_2$ cells by MTS assay method with MIC of 312 µg/ml (Table 2) and IC_{50} of 525.6 µg/ml (Table 7), respectively; its selectivity index was 1.68. This value is less than 10; hence, the activity observed might be due to toxicity of the extract on the mycobacteria and further investigation on other cell lines is required. The 80% methanol extract of the same plant showed a selectivity index of 0.76 that is much less than 10 and toxicity is higher than the toxicity shown by the chloroform extract. This might be due to the presence of more active toxic constituents in the methanol extract than the chloroform extract.

Carisa edulis chloroform root extract showed antimycobacterial activity (though it was not significant compared to vehicle control) against *M. tuberculosis* H37Rv [MIC of 1250 µg/ml (Table 2)] and cytotoxicity effect on $HepG_2$ cells by MTS assay method [IC_{50} of 252.5 µg/ml (Table 7)], its selectivity index was 0.202. The selectivity index is much less than 10, which is of less interest for pharmaceutical companies. Moreover, *C. edulis* chloroform root extract might be the most toxic plant extract than the other extracts evaluated in the present study.

The genotoxicity assay revealed that the *P. stellatum* chloroform and 80 % methanol extracts increased tail length (percentage DNA in the tail) significantly at 1 mg/ml and 0.5 mg/ml respectively, indicating the potential for DNA damage. However, at the antimycobacterial concentration, these extracts were without genotoxic effect. In addition, *O. integrifolia* and *V. amygdalina* chloroform extracts were without significant genotoxic effect at the test concentrations. At lower test concentrations of *V. amygdalina*, there was decreased tail intensity significantly as compared to the vehicle treated $HepG_2$ cells. This effect might be due to the protective effect of the extract against the background DNA damage.

In previous studies reported by Etebari *et al*. (2012), the aqueous and hydroalcoholic extracts of *Echium amoenum* and *Nardostachys jatamansi* on DNA of $HepG_2$ cells using the comet assay showed that aqueous and hydroalcoholic extracts of *E. amoenum* increased percentage DNA in tail significantly and concentration dependently indicating the genotoxic potential of these plants and their capability of DNA damage. The aqueous

extract of *N. jatamansi* at a concentration of 5 mg/ml increased percentage DNA in tail with a significant difference compared with the control group and hence, this extract is genotoxic at 5 mg/ml. Similarly, the finding in the present study revealed that *P. stellatum* chloroform extract showed significant DNA damage as compared to the negative control at the maximum test concentration (1 mg/ml). However, *P. stellatum* chloroform extract was found to be without genotoxic effect at its antitubercular concentration (MIC = 0.039 mg/ml).

Methanol extract of *P. stellatum* showed significant increase in percentage DNA in tail at 0.5 mg/ml. However, it did not show concentration dependent percentage DNA in tail increase as well as genotoxic concentration was less than that shown by chloroform extract. In addition, methanol extract was found to be more cytotoxic on $HepG_2$ cells than the chloroform extract. This apparent difference between toxic effects of chloroform and methanol extracts of the same plant might be attributed to the presence of more toxic constituents in the methanol extract. Moreover, upon exposure of $HepG_2$ cells to *V. amygdalina* chloroform extract, significant increase in percentage DNA in tail was not induced and hence the extract didn't show genotoxic effect at all test concentrations. The percent DNA in tail decrease at and below 0.05 mg/ml may be attributed to the extract's action to protect the cells from background damage at low concentrations.

6. LIMITATIONS

- Long process of purchasing chemicals, reagents and lab consumables through government foreign procurement system. Hence, many red tapes had been encountered.
- There was limited fund allocated to the project.
- There is sparse data in the literature about the antimycobacterial activity and the constituents of *P. stellatum*. Hence, this makes comparison a little bit challenging.
- Unavailability of fast growing mycobacterial species that could reduce the long time taken to grow slow growing ones.
- Unavailability of *in vivo* model to investigate antimycobacterial activity of extracts due to infectious nature of mycobacterium tuberculosis.

7. CONCLUSIONS

The results of this study have shown that there is potential to develop new compounds against both drug susceptible and MDR-TB from crude extracts of *P. Stellatum, O. integrifolia* and *P. americana*. The present study verifies their use in the treatment of tuberculosis by traditional practitioners and further emphasizes that there is a positive correlation between the antimycobacterial activity results and indigenous medical knowledge on plants used for TB and TB-related diseases in ethnomedicine. However, *Vernonia amygdalina* and *Carisa edulis* chloroform extracts had not significant antimycobacterial activity compared to the vehicle control and hence no correlation between the results and the traditional knowledge on these plants.

The IC_{50} value of *P. stellatum* chloroform extract, the most active extract against mycobacterium tuberculosis, was relatively higher compared to other extracts, suggesting its safety. From the genotoxicity findings, chloroform and 80% methanol extracts of *P. stellatum* caused DNA damage at higher exposure concentrations. Thus, necessary precautions should be taken during utilization of this plant. The selectivity index for *P. stellatum* chloroform extract was 13.5, which demonstrated > 10 selectivity index, considered as being of interest to be pursued as a source of drugs to treat tuberculosis.

8. RECOMMENDATIONS

- Full characterization of isolated active constituents from the most potent plant extract (*P. stellatum* chloroform extract), optimize potential leads and evaluate possible synergism of isolated compounds with current antituberculosis drugs should also be explored.
- Further antimycobacterial evaluation of these plant extracts, especially *P. stellatum* chloroform extract should be performed against different drug susceptible and drug resistant mycobacterium tuberculosis clinical isolates.
- *In vivo* antimycobacterial activity should be evaluated on a mouse model for these plant extracts and isolated compounds.
- In addition, intracellular antimycobacterial activity of the most active plant extracts and active constituents should be done on macrophage cell model.
- Immune boosting properties of extracts and isolated compounds shall be investigated
- Evaluate oral activity, delayed action, bactericidal action and mechanism of action of extracts and isolated compounds
- Evaluation of the activity of extracts and compounds against non-replicating bacteria
- Absence of cross-resistance or antagonistic activities with current anti-TB drugs shall be investigated
- Determination of therapeutic window and evaluation of drug-drug interactions with ARVs as well as determination of LD_{50} of compounds on a rodent model

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10. APPENDIXES

10.1. Appendix I: Examples of plant extracts exhibiting antimycobacterial activity

Plant	Traditional use	Solvent	Organism	MIC	Remarks	References
Anogeissus leiocarpus	Asthma, Cough, Tuberculosis	methanol	MTB	78 µg/ml	Root bark, and Stem bark, Nigeria	Mann *et al*., 2008
Capparis brassii	Tuberculosis	methanol	MTB	1250 µg/ml	Root bark, Nigeria	Mann *et al*., 2008
Combretum spp	Bronchitis, Cough, Tuberculosis	methanol	MTB	1250 µg/ml	Root bark, and Stem bark, Nigeria	Mann *et al*., 2008
Entada africana Guill. & Perr.	Bronchitis, Cough, Whooping cough	methanol	MTB	1250 µg/ml	Root bark, and Stem bark, Nigeria	Mann *et al*., 2008
Euphorbia scarlatica	Stomach ache, common cold, TB	methanol	*MTB*, *M. kansasii, M. fortuitum, M. smegmatis*	500 µg/ml	Stem, Kenya	Mariita *et al*., 2010
Lantana hispida		1:1 mix of hexane and chloroform	*MTB* H37Rv strain, H37Rv STR-resistant, H37Rvv INH-resistant,	12.5 µg/ml for *MTB* H37Rv and 50 µg/ml for the rest	aerial parts, Mexico	Jim´enez Arellanes *et al*., 2007

Appendix I: Continued

Clematis hirsuta		Volataile oil	*Mycobacterium segmentis*	200 µg/ml	aerial parts, Saudi Arabia	Al-Taweel, 2007)
Clematis simensis		Volataile oil	*Mycobacterium segmentis*	400 µg/ml	aerial parts, Saudi Arabia	Al-Taweel, 2007)
Clematis wightiana		Volataile oil	*Mycobacterium segmentis*	150 µg/ml	aerial parts, Saudi Arabia	Al-Taweel, 2007)
Byrsonima fagifolia		chloroform	*M. tuberculosis* H37Rv ATCC 27294	62.5 µg/ml	Leaves, USA	Higuchi *et al*., 2008
Amborella trichopoda	to treat TB-like symptoms	methanol	*M. bovis* BCG	1-2.5µg/ml	-	Billo *et al*., 2005
Chelidonium majus	TB	ethanol	MTB	50µg/ml	aerial parts, Turkey	Tosun *et al*., 2004
Chenopodium ambrosoides	pulmonary diseases	acetone	MDR-MTB	100µg/ml	aerial parts, South Africa	Lall and Meyer, 1999
Commiphora mukul	TB and leprosy	methanol	*M. aurum*	62.5µg/ml	resin	Newton *et al*., 2002
Croton pseudopulchellus	pulmonary diseases	acetone	MTB	100µg/ml	aerial parts, South Africa	Lall and Meyer, 1999

Appendix I: Continued

Ekebergia capensis	pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	bark, South Africa	Lall and Meyer, 1999
Euclea natalensis	pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	roots, South Africa	Lall and Meyer, 1999
Helichrysu m melanacme	pulmonary diseases	acetone	MDR-MTB	100µg/ml	whole plant, South Africa	Lall and Meyer, 1999
Myristica fatua	to treat TB-like symptoms	methanol dichloro methane	*M. bovis* BCG	100µg/ml	100µg/ml lowest concentration s	Billo *et al*., 2005
Nidorella anomala	pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	whole plant, South Africa	Lall and Meyer, 1999
Polygala myrtifolia	pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	aerial parts, South Africa	Lall and Meyer, 1999
Psoralea corylifolia	TB and leprosy	methanol	*M. aurum*	62.5µg/ml	l eaves	Newton *et al*., 2002
Salvia aethiopis	Treatment of TB	ethanol	MTB	50µg/ml	aerial, Turkey	Tosun *et al*., 2004
S. canadensis	TB, leprosy	methanol	*M. aurum*	62.5µg/ml	root	Newton 2002
Stachys sylvatica	TB	ethanol	MTB	50µg/ml	aerial, Turkey	Tosun *et al*., 2004
Ulmus glabra	Treatment of TB	ethanol	MTB	50µg/ml	leaves, Turkey	Tosun *et al*., 2004
Urtica dioica	Treatment of TB	ethanol	MTB	50µg/ml	erial, Turkey	Tosun *et al*., 2004

10.2 Appendix II: Antimycobacterial activity of compounds isolated from plants

Plant	Compound	Organism	MIC	References
Lantana hispida	3-Acetoxy-22-(2_-methyl-2-butenoyloxy)-12-oleanen-28-oic acid	*MTB* H37Rv strain, H37Rv STR-resistant, H37Rv INH-resistant, H37Rv RIF-resistant, H37Rv EMB-resistant	50 µg/ml, 25 µg/ml, 25 µg/ml, 50 µg/ml, 50 µg/ml respectively	Jim´enez-Arellanes *et al*., 2007
Lantana hispida	3-Hydroxy-22 β-(2_-methyl-2-butenoyloxy)-12-oleanen-28-oic acid [reduced lantadeneA]	*MTB* H37Rv strain, H37Rv STR-resistant, H37Rvv INH-resistant, H37Rv RIF, H37Rv EMB-resistant	50 µg/ml to all strains	
Lantana hispida	sitosterol	*MTB* H37Rv strain, H37Rv STR-resistant, H37Rvv INH-resistant, H37Rv RIF-resistant, H37Rv EMB-resistant	25 µg/ml for *MTB* H37Rv strain and 50 µg/ml to the rest	Jim´enez-Arellanes *et al*., 2007
Byrsonima fagifolia	α-Amyrin acetate	*M. tuberculosis* H37Rv ATCC 27294	62.5 µg/ml	Higuchi *et al*., 2008
Byrsonima fagifolia	Dotriacontane	*M. tuberculosis* H37Rv ATCC 27294	62.5 µg/ml	Higuchi *et al*., 2008
Byrsonima fagifolia	Bassic acid	*M. tuberculosis* H37Rv ATCC 27294	2.5 µg/ml	Higuchi *et al*., 2008

Appendix II: Continued

Byrsonima fagifolia	Mixture of lupeol, α- and β-amyrin	*M. tuberculosis* H37Rv ATCC 27294	31.25 µg/ml	Higuchi *et al.*, 2008
Abrus precatorius	abruquinone B	MTB	12.5µg/ml	Limmatvapirat *et al.*, 2004
Ajuga remota	ergosterol-5,8-endoperoxide	MTB	1µg/ml	Cantrell *et al.*, 2001
Caesalpinia pulcherrima	6β-cinnamoyl-7β-hydroxyvouacapen-5α-ol	MTB	6.25µg/ml	Promsawan *et al.*, 2003
Cetraria islandica	protolichesterinic acid	*M. aurum*	=125µg/ml	Ingólfsdóttir *et al.*, 1998
Chamaecyparis nootkatensis	totarol	MTB	16µg/ml	Constantine *et al.*, 2001
Chromolaena odorata	isosakuranetin	MTB	174.8µM	Suksamram *et al.*, 2000
Cladonia arbuscula	ursnic acid	*M. aurum*	32µg/ml	Ingólfsdóttir *et al.*, 1998

Appendix II: Continued

E.roxburgh-iana	engelhardione	clinical MTB MTB	3.125µg/ml 0.2µg/ml	Lin *et al*., 2005
Engelhardia	*roxburghiana* (-)-5-hydroxy-4-methoxy-1-tetralone	clinical MTB	3.125µg/ml 0.2µg/ml	Lin *et al*., 2005
Engelhardia roxburghiana	3-methoxycarbonyl-1,5-dihydroxyanthraquinone	Clinical MTB MTB	6.25µg/ml 4µg/ml	Lin *et al*., 2005
Evodia rutaecarpa	evocarpine	*M. fortuitum, M. smegmatis, M. phlei*	2µg/ml to all	Adams *et al*., 2005
Ferula communis	ferulenol	*M. fortuitum, M. phlei, M. aurum M. smegmatis*	2µg/ml to all 0.5µg/ml	Appendino *et al*., 2004
Haplopappus sonorensis	ermanin	MTB	Inhibited 98% growth at100µg/ml	Murillo *et al*., 2003
Lucas volkensii	(E)-phytol	MTB	2µg/ml	Rajab *et al*., 1998
M. hirsutum	micromolide	MTB H37 R*v* EC90 of 5.6µg/ml	1.5µg/ml	Mata *et al*., 2005
Parmelia saxatilis	salazinic acid	*M. aurum*	=125µg/ml	Ingólfsdóttir *et al*., 1998

Appendix II: Continued

Piper sanctum	2-oxo-14-(3`,4`-methylenedioxyphenyl) dodecane	MTB	6.25µg/ml	Mata *et al*., 2004
Piper sanctum	2-oxo-16-(3`,4`-methylenedioxyphenyl)hexadecane	MTB	6.25µg/ml	Mata *et al*., 2004
Piper sanctum	5,6-dehydro-7,8, dihydromethysticin	MTB	4µg/ml	Mata *et al*., 2004
Piper sanctum	piperolactam A	MTB	8µg/ml	Mata *et al*., 2004
Psoralea corylifolia	bakauchiol	*M. aurum, M. smegmatis, M. bovis BCG*	IC50 = 15.8, = >500, = 21.4µg/ml	Newton *et al*., 2002
Salvia multicaulis	multiorthoquinone	MTB	2µg/ml	Cantrell *et al*., 2001
Sanguinaria canadensis	chelerythrine sanguinarine	*M. aurum, M. smegmatis, M. bovis BCG, M. aurum, M. smegmatis, M. bovis* BCG	IC50 = 7.3µg/ml IC50 = 29, 14.3, 9.61, 41.2, 24.5µg/ml	Newton *et al*., 2002
Sanguinaria canadensis	chelerythrine	*M. aurum , M. smegmatis*	7.3µg/ml 29µg/ml	Newton *et al*., 2002

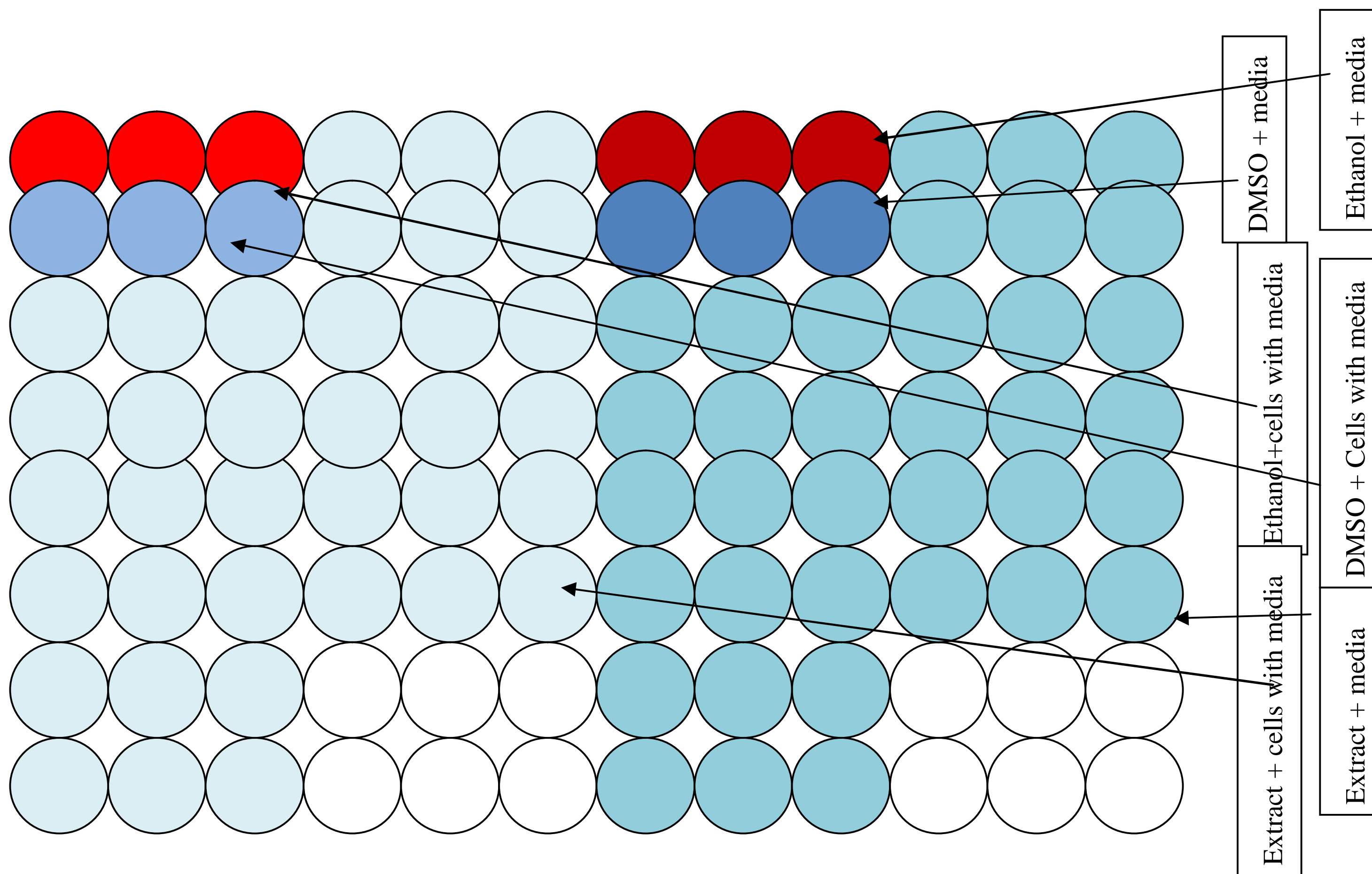
Appendix II: Continued

Saussurea lappa	dehydrocostuslactone	MTB	2µg/ml	Cantrell *et al.*, 2001
Senna obliqua	Quinquangulin, rubrofusarin	MTB	12.0µg/ml	Graham *et al.*, 2004
Stereocaulon alpinum	Atranorin, lobaric acid	*M. aurum*	125µg/ml	Ingólfsdóttir *et al.*, 1998
Tetradenia riparia	8(14),15-sandaracopimaradiene-7α,18-diol	*M. smegmatis*	12.5µg/ml	Van *et al.*, 1986
Warburgia ugandensis	6α, 9α-dihydroxy-4(13	*M. aurum*, *M. fortuitum*, *M. phlei* and *M. smegmatis*	Ranged from 4µg/ml to 128µg/ml	Abebe *et al.*, 2005

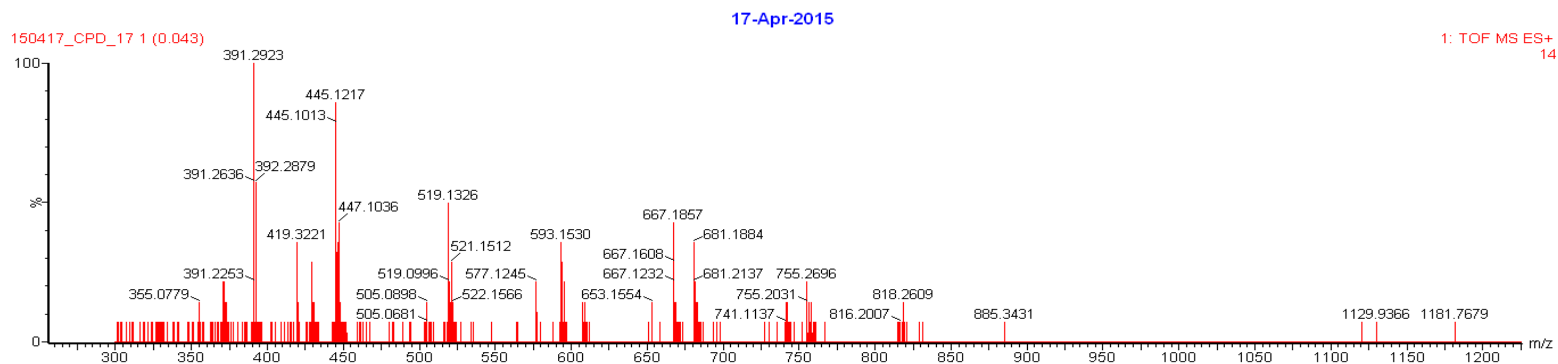
MTB = *M. tuberculosis*

Appendices 1 and 2 show that plants are untapped source of chemically diverse compounds, of which some have promising antimycobacterial properties. These compounds with *in vitro* activities may be lead compounds for further investigation to develop antituberculosisdrugs.

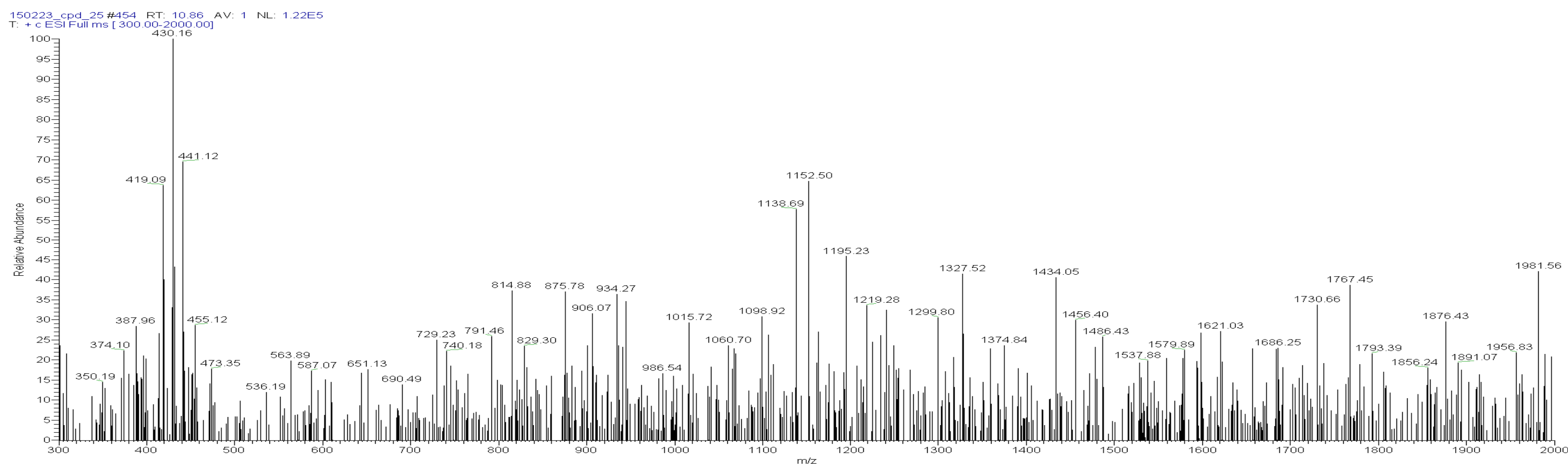
10.3 Appendix III: Cytotoxicity test layout on a 96-well microtiter plate



10.4 Appndix IV: Mass spectra of compound 1



10.5 Appendix V: Mass spectra of compound 2



10.6 Appendix VI: Mass spectra of compound 3