Bioactive compounds from South African plants against *Mycobacterium tuberculosis*

Submitted in fulfillment for the Degree of Doctor of Philosophy (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

Alveera Singh

AUGUST 2016

PROMOTER : **Prof B. Odhav**

CO-PROMOTER : **Prof Y. Coovadia**

REFERENCE DECLARATION

I, Alveera Singh – student number, 20201901 and Prof Bharti Odhav do hereby declare that in respect of the following dissertation:

Title: **Bioactive compounds from South African plants against *Mycobacterium tuberculosis***

1. As far as we ascertain:

a) no other similar dissertation exists;

b) the only similar dissertation(s) that exist(s) is/are referenced in my dissertation as follows:

_____

_____

_____

2. All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

Signature of student	**Date**
Signature of promoter	**Date**
Signature of co-promoter	**Date**

AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Bharti Odhav** and **Prof Yacoob Coovadia**.

_____

Student's signature

DEDICATION

This work is dedicated to my uncle and grandparents

Mr B. Singh

Mr D.G. Singh and Mrs J. Singh

Mr D. Prawalal and Mrs S. Prawalal

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **I**

LIST OF ABBREVIATIONS ..... **IV**

LIST OF FIGURES ..... **VII**

LIST OF TABLES ..... **X**

LIST OF PUBLICATIONS ..... **XI**

LIST OF CONFERENCES ..... **XII**

ABSTRACT ..... **XIII**

CHAPTER 1: INTRODUCTION **1**

1.1 Tuberculosis and Herbal Medicine 1

1.2 Motivation for the study 2

1.3 Objectives of the study 4

1.4 Structure of Thesis 5

CHAPTER 2: LITERATURE REVIEW **6**

2.1 History of Tuberculosis 6

2.2 Mycobacterium species 7

2.2.1 *Mycobacterium tuberculosis* 7

2.2.2 *Mycobacterium smegmatis* 11

2.3 Epidemiology of Tuberculosis 12

2.4 Transmission of Tuberculosis 14

2.5 Pathogenesis of Tuberculosis 16

2.6 Immunology of Tuberculosis 16

2.7 Symptoms of Tuberculosis 17

2.8 TB and HIV Co-infection 18

2.8.1 Impact of HIV on the incidence of TB across the globe 18

2.8.2 Impact of HIV on the incidence of TB in South Africa 19

2.9 Treatment of Tuberculosis 19

2.9.1 Why is there a need for new TB drugs? 22

2.9.2 Why Are Mycobacteria Resistant to Antibiotics? 23
2.9.3 New TB drugs in the Current Market 24
2.10 Medicinal Plants with Antimycobacterial Activity 24
2.10.1 Natural Antimycobacterial Metabolites 31
2.11 Plants Used in this Research for Antimycobacterial Activity 36
2.11.1 *Buddleja saligna* 36
2.11.2 *Capparis tomentosa* 37
2.11.3 *Carpobrotus dimidiatus* 39
2.11.4 *Dichrostachys cinerea* 40
2.11.5 *Ekerbergia capensis* 42
2.11.6 *Ficus sur* 44
2.11.7 *Gunnera perpensa* 45
2.11.8 *Leonotis leonurus* 47
2.11.9 *Tetradenia riparia* 49

CHAPTER 3: METHODOLOGY 51

3.1 Plant Collection and Extraction 51
3.1.1 Plant Collection 51
3.1.2 Plant Extraction 52
3.1.2.1 Aqueous Extraction 52
3.1.2.2 Methanolic Extraction 52
3.2 Antimycobacterial Activity of the Plant Extracts 53
3.2.1 Mycobacterial strains and isolates 53
3.2.2 Preliminary Sensitivity Test 53
3.2.3 Broth Microdilution Assay 54
3.3 Safety Analysis of the Active Plant Extracts 55
3.3.1 Brine Shrimp Lethality Test 55
3.3.1.1 Hatching the Shrimp 55
3.3.1.2 Bioassay 55
3.3.2 Cytotoxicity Screening of the Active Plant Extracts 56
3.3.2.1 Peripheral Blood Mononuclear Cells (PBMCs) 56
3.3.2.2 Mouse BALB/C Monocyte- Macrophage (Line J774.2) 57

3.3.2.2.1 Cell Line 57
3.3.2.2.2 Storage of Cells 57
3.3.2.2.3 Cell Maintenance 57
3.3.2.3 Trypan Blue Exclusion Assay 58
3.3.2.4 MTT Assay 58
3.4 Intracellular Effect of the Active Plant Extracts 59
3.4.1 Cell Culture Conditions 59
3.4.1.1 Cell Maintenance 59
3.4.1.2 Trypan Blue Exclusion Assay 59
3.4.2 Mycobacterial Conditions 59
3.4.3 Infection of the Macrophage with Mycobacteria 60
3.4.4 Harvest of the Infected Macrophage for CFU Plating 60
3.5 Phytochemical Screening of the Plants that have Antimycobacterial Activity 61
3.5.1 Test for Tannins 61
3.5.2 Test for Phlobatannins 61
3.5.3 Test for Saponins 61
3.5.4 Test for Flavonoids 62
3.5.5 Test for Steroids 62
3.5.6 Test for Terpenoids (Salkowski test) 62
3.5.7 Test for Alkaloids 63
3.5.8 Test for Phenols 63
3.6 Thin Layer Chromatography of the Active Plants Extracts 63
3.6.1 Chemicals 63
3.6.2 Thin Layer Chromatography 63
3.7 Bioautography of the Active Plant Extracts against *M. smegmatis* 64
3.7.1 Mycobacterial strains 64
3.7.2 Qualitative Antimycobacterial Activity (Bioautography) 64
3.8 Column Chromatography of the Methanolic Extract from *B. saligna* 65
3.8.1 Extraction 65
3.8.2 Isolation 65
3.8.3 Acetylation Reaction 66
3.9 Characterization of the Isolated Compounds from *B. saligna* 67

3.9.1 Characterization of T1 and T2 compounds by ^{1}H NMR and ^{13}C NMR. 67
3.10 Antimycobacterial Activity of Triterpenes Isolated from *B. saligna* 68
3.11 Molecular Docking of Triterpenes Isolated from *Buddleja saligna* 69

CHAPTER 4: RESULTS 70

4.1 Antimycobacterial Screening of the Nine Plants 70
4.2 Safety Analysis of the Active Plant Extracts 74
4.2.1 Toxicity 74
4.2.2 Cytotoxicity 75
4.3 Intracellular Effect of the Active Plant Extracts against *M. smegmatis* 77
4.4 Phytochemical Screening of the Active Plants 82
4.5 Thin Layer Chromatography of the Active Plant Extracts 83
4.6 Bioautography of the Active Plant Extracts 84
4.7 Isolation and Characterization of Compounds from *B. saligna* 85
4.8 Antimycobacterial Activity of Triterpenes Isolated from *B. saligna* 91
4.9 Molecular Docking of Triterpenes Isolated from *B. saligna* 92

CHAPTER 5: DISCUSSION 95

5.1 Plant Extraction 95
5.2 Antimycobacterial Activity of the Plant Extracts 96
5.3 Safety Analysis of the Active Plant Extracts 98
5.4 Intracellular Activity of the Active Plant Extracts 99
5.5 Phytochemical Analysis of the Active Plant Extracts 100
5.6 Bioautography of the Active Plant Extracts 101
5.7 Identification and Characterization of the Active Compounds 101
5.8 Antimycobacterial Activity of Oleanolic Acid and Ursolic Acid 105
5.9 Molecular Docking Study of Oleanolic Acid and Ursolic Acid 106

CHAPTER 6: CONCLUSION 109

REFERENCES 111

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation and heartfelt gratitude to:

Professor Bharti Odhav, my supervisor and mentor, Department of Biotechnology and Food Technology, Durban University of Technology, for her remarkable insight, constructive criticism, expert guidance and support, time and energy, patience, invaluable assistance during the course of my degree, and for her love and motivation;

Professor Francis Shode, Research Associate, Department of Biotechnology and Food Technology, Durban University of Technology, for his invaluable time and effort, criticism, expert guidance, support and patience;

Professor Yacoob Coovadia, Department of Microbiology, NHLS Laboratory, Inkosi Albert Luthuli Hospital, for all his expert advice, guidance and use of the NHLS laboratory facilities;

Professor H. Baijnath, School of Botany and Zoology, University of Kwa-Zulu Natal, for his assistance, invaluable time and effort, kindness and for obtaining plant material during the course of this project;

Lifelab, Technology Innovation Agency (TIA) and Durban University of Technology (DUT) for funding this project;

Mr Veenash Singh and Mrs Andhira Singh, my parents, for their love, encouragement, financial support, tolerance and guidance throughout this period in my life;

Mr Melendhran Pillay, Department of Microbiology, NHLS Laboratory, Inkosi Albert Luthuli Hospital, for all his assistance with the mycobacterial screening;

Mr Kenneth Uzoma Nwaeze, Department of Pharmaceutical Chemistry, University of Lagos, Nigeria, for all his assistance with the compound analysis;

Dr Venugopala Narayanaswamy, Department of Biotechnology and Food Technology, Durban University of Technology for all his assistance with molecular docking;

Dr John Mellem and Dr Viresh Mohanlall, Department of Biotechnology and Food Technology, Durban University of Technology for their invaluable input, tolerance and assistance;

Mrs Vivienne Clarence, KwaZulu Natal Research Institute for Tuberculosis and HIV, for her invaluable input, proof reading and expert guidance;

Mr Aveen Singh, Miss Arisha Singh and Miss Daneshri Naidoo for their love, support and motivation;

Dr Vashka Hurinanthan, for her love, support, assistance and friendship throughout this project;

The Barmanandh's for their encouragement, support and guidance;

Miss Naazlene Patel and Mr Kabange Kasumbwe for their love and support;

Miss Nkule Mzindle, Miss Berushka Padaychee, Ms Depika Dwarka, Mrs Predeshni Naicker, Mrs Leeantha Govender, Miss Tatum Myles, Mrs Kameshnee Mellem, Mrs Reshma Ramprotal, Mrs Mandy Gopal and, Ms Renuka Ganesh for their love and friendship;

Mrs Priscilla Phillips and Ms Sohana Ranglal for their support, assistance and friendship;

My dogs Roxy, Bonzai, the late Baloo and Bobby for their love and playfulness, taking my mind of the stresses off the day.

“All Glory to God”

LIST OF ABBREVIATIONS

^{1}H	Proton NMR
^{13}C	Carbon NMR
AG	Aarachidonoylglycerol
AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral therapy
ARV	Antiretrovirals
ATCC	American Type Culture Collection
BSM	*Buddleja saligna* (Methanolic Extract)
BST	Brine Shrimp Lethality Test
$CD4^{+}$	Cluster of Differentiation 4
$CD8^{+}$	Cluster of Differentiation 8
CCM	Complete Culture Medium
CDC	Centre for Disease Control and Prevention
CFU	Colony Forming Units
DEET	N, N-diethyl-*meta*-toluamide
DEPT	Distortionless Enhancement by Polarization Transfer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DOT	Directly Observed Therapy
DR-TB	Drug-Resistant Tuberculosis
ETH	Ethambutol
FCS	Fetal Calf Serum
GPA	*Gunnera perpensa* (Aqueous Extract)
HBSS	Hanks' Balanced Salts Solution
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HPTAs	Hydroxyl Pentacyclic Triterpenoic Acids

IFN-γ	Interferon-γ
INH	Isoniazid
INT	Indophenyl Nitrophenyl Tetrazolium
KAN	Kanamycin
KZN	Kwa-Zulu Natal
LAM	Lipoarabinomannin
MABA	Microplate Alamar Blue Assay
MDGs	Millennium Development Goals
MDR-TB	Multi-Drug Resistant Tuberculosis
MIC	Minimum Inhibitory Concentration
MTB	*Mycobacterium tuberculosis*
MTB $H_{37}Rv$	*Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 25177)
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NHLS	National Health Laboratory Service,
NMR	Nuclear Magnetic Resonance
OA	Oleanolic Acid
OAA	Oleanolic Acid Acetate
OADC	Oleic Albumin Dextrose Catalase
OD	Optical Density
OFLX	Ofloxacin
PBS	Phosphate Buffered Saline
PIMs	Phosphatidylmyoinositol mannosides
PMN	Polymorphonuclear
PZA	Pyrazinamide
RIF	Rifampicin
RPMI	Roswell Park Memorial Institute
TB	Tuberculosis
TDR	Total Drug Resistance
TLC	Thin Layer Chromatography

TMS	Tetramethylsilane
TNF-α	Tumour Necrosis Factor alpha
UA	Ursolic Acid
UAA	Ursolic Acid Acetate
UNAIDS	The Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization
XDR-TB	Extensive Drug Resistant Tuberculosis
ZN	Ziehl-Neelsen

LIST OF FIGURES

Figure 1. Colonies of *M. tuberculosis* on Lowenstein-Jensen medium. 8
Figure 2. *M. tuberculosis* cultivated on Middlebrook 7H11 agar plates. 8
Figure 3. *M. tuberculosis* bacteria using acid-fast Ziehl-Neelsen stain; magnified 1000 x .9
Figure 4. Diagram of the mycobacterial cell wall. 10
Figure 5. The acid fast stain of *M. smegmatis* (100 x magnifications). 12
Figure 6. Estimated TB incidence rates (WHO, 2015). 13
Figure 7. A diagram depiction of TB transmission where the dots in the air contain droplet nuclei containing *M. tuberculosis.* 15
Figure 8. Pseudopteroxazole isolated from *Pseudopterogorgia elisabethae* (Rodrıguez and Rodrıguez, 2003). 31
Figure 9. Licochalcone A isolated from *Glycyrrhiza inflata* (Friis-Moller et al., 2002). 32
Figure 10. Secokauranes isolated from *Croton kongensis* (Thongtan et al., 2003). 33
Figure 11: Epidioxysterol isolated from *Morinda citrifolia* (Saludes et al., 2002). 34
Figure 12. Hydroxybenzene analog isolated from *Ardisia japonica* (Huang et al., 1980). 35
Figure 13: Hirsutellide isolated from *Hirsutella kobayasii* (Vongvanich et al., 2002). 35
Figure 14. *Buddleja saligna* at the Steenbok Nature Reserve. 36
Figure 15. *Capparis tomentosa* growing as a shrub in Amanzimtoti, Kwa-Zulu Natal, South Africa. 38
Figure 16. *Carpobrotus dimidiatus* at the Kwa-Zulu Natal Botanical Gardens. 39
Figure 17. *Dichrostachys cinerea* cultivated in Constantia Kloof, Roodepoort. 41
Figure 18. *Ekerbergia capensis* at the Kumbula Indigenous Nursery, in the Eastern Cape.43
Figure 19. *Ficus sur* at Kirstenbosch National Botanical Gardens.....44
Figure 20. *Gunnera perpensa* in Pledge Nature Reserve in Knysna, South Africa. 46
Figure 21**.** *Leonotis leonurus* growing in North Riding, Randburg in South Africa. 48
Figure 22. *Tetradenia riparia* at the Kumbula Indigenous Nursery, in the Eastern Cape. 49
Figure 23. Picture of the column chromatography of methanolic extract from leaves of *B. saligna.*.....67

Figure 24. Microtitre plate of dilutions 250 µg/ml, 125 µg/ml, 62.50 µg/ml, 36.25 µg/ml, 18.02 µg/ml, 9.01 µg/ml, 4.50 µg/ml and 2.25 µg/ml showing the visual effect of OAA and UAA on XDR-TB. Blue depicts the mycobacteria have been killed whilst the pink shows the mycobacteria are alive. 68
Figure 25. Brine shrimp larvicidal activity of the active plant extracts, *B. saligna, C. tomentosa, C. dimidiatus* (methanolic)*, G. perpensa* and *T. riparia* (aqueous) at different concentrations. 75
Figure 26. Cell viability of the active plant extracts *B.saligna, C. tomentosa, C. dimidiatus* (methanolic), *G. perpensa* and *T. riparia* (aqueous) on the BALB/C monocyte macrophage cell line (J774.2).....76
Figure 27. Cell viability of the active plant extracts *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* on the peripheral blood mononucleated cell line (PBMC). Mean ± SD (n=3).....77
Figure 28. Light micrograph of untreated J774.2 macrophage cell line (100 x magnification). 78
Figure 29. Light micrograph of *M. smegmatis* in DMEM supplemented with Fetal Calf Serum (100 x magnification).....78
Figure 30. Intracellular effect of the active plant extracts against *M. smegmatis* within the macrophage (J774.2) and the effect of the active plant extracts against *M. smegmatis* after 24 hours.....80
Figure 31. Petri plates depicting the difference in infection 24 hours after the cells were lysed (A) *M.smegmatis* infected J774.2 Macrophages (B) Effect of Isoniazid on *M. smegmatis* infected J774.2 cells (C) Effect of Rifampicin on *M. smegmatis* infected J774.2 cells (D) Effect of 100 µg/ml of BSM on *M. smegmatis* infected J774.2 cells (E) Effect of 100 µg/ml of GPA on *M. smegmatis* infected J774.2 cells.....81
Figure 32. Petri plates depicting the effect of the plant extracts on *M. smegmatis* after 24 hours (A) *M.smegmatis* (B) Effect of Isoniazid on *M. smegmatis* (C) Effect of Rifampicin on *M. smegmatis* (D) Effect of 100 µg/ml of BSM on *M. smegmatis* (E) Effect of 100 µg/ml of GPA on *M. smegmatis.* 81

Figure 33. TLC of the methanolic extracts of (A) *B. saligna,* (B) *C. dimidiatus,* (C) *C. tomentosa;* aqueous extracts of (D) *G. perpensa,* and (E) *T. riparia* leaves using Hexane : Ethyl Acetate (7:3) as a mobile phase and developed with vanilin spray reagent for visualisation. 83

Figure 34. Bioautograph of the methanolic extracts of (A) *B. saligna,* (B) *C. dimidiatus,* (C) *C. tomentosa;* aqueous extracts of (D) *G. perpensa,* and (E) *T. riparia* leaves using Hexane: Ethyl Acetate (7: 3) as a mobile phase. Plates were sprayed with *M. smegmatis* and clear zones denote a compound of interest. 84

Figure 35. Profile of compounds isolated from the leaves of *B. saligna* using column chromatography combined into 4 subfractions (A, B, C and D) using Hexane: Ethyl Acetate (7: 3) as a mobile phase and developed with conc H_2SO_4 : methanol (9: 1) spray reagent for visualisation 85

Figure 36. Bioautograph profile of compounds isolated from the leaves of *B. saligna* using column chromatography combined into 4 subfractions (A, B, C and D) using Hexane: Ethyl Acetate (7: 3) as a mobile phase. Plates were sprayed with *M. smegmatis* and clear zones denote a compound of interest. 86

Figure 37. The ^{1}H NMR of T1.....87

Figure 38. The ^{13}C NMR of T1.....88

Figure 39. The ^{1}H NMR of T2.....89

Figure 40. The ^{13}C NMR of T2.....90

Figure 41: Binding mode of (2A) ursolic acid; (2B) ursolic acid acetate; and (2C) reference steroid fadA5 inhibitor. 93

Figure 42: Binding mode of oleanolic acid. 94

Figure 43. Chemical structures of (T1) OAA and (T2) UAA.....105

Figure 44 **:** 3D description of the best binding modes of A) Ursolic acid, B) Ursolic acid acetate, C) Oleanolic acid acetate and D) Oleanolic acid. 108

LIST OF TABLES

Table 1. Plants used in the treatment of TB-related ailments 4

Table 2. Summary of first-line drugs used for the treatment of TB 20

Table 3. Traditional plants used in South Africa for treating TB-related diseases adapted from (McGaw et al., 2008).....26

Table 4. Biodata of plants used in this study.....51

Table 5. Antimycobacterial activity of the aqueous and methanolic plant extracts at a concentration of 1 mg/ml.....71

Table 6. Minimum inhibitory concentration of the active plant extracts against *M. smegmatis*, MTB H_{37}Rv (25177), clinical isolate MDR-TB and clinical isolate XDR-TB.....73

Table 7. Phytochemical analysis of the active plants.....82

Table 8. Minimum inhibitory concentration of compounds isolated from *B. saligna* against *M. smegmatis*, *MTB* H_{37}Rv (25177), clinical isolate MDR-TB and clinical isolate XDR-TB 91

Table 9. Docking results of UA, UAA, OA and OAA 92

Table 10. ^{1}H NMR and ^{13}C NMR of T1 (OAA) 102

Table 11. ^{1}H NMR and ^{13}C NMR of T2 (UAA) 103

LIST OF PUBLICATIONS

Singh A., Venugopala K. N., Khedr M. A., Pillay M, Nwaeze K.U, Coovadia Y, Shode F. and Odhav B. (2015) Antimycobacterial and docking studies of pentacyclic triterpenes from *Buddleja saligna* leaves. Journal of Biomolecular Structure & Dynamics. ***Published.***

Alveera Singh, Katharigatta N. Venugopala, Melendran Pillay, Himansu Baijnath, Yacoob Coovadia, Francis Shode and Bharti Odhav. (2016) Antimycobacterial and cytotoxicity activity of nine plants against *Mycobacterium tuberculosis.* Tropical Journal of Pharmaceutical Research. ***Submitted and under review.***

LIST OF CONFERENCES

Singh A, Katharigatta N. Venugopala, Mohammed A. Khedr, Pillay M, Nwaeze K.U, Coovadia Y, Shode F and Odhav B. 17-20 January 2016. Antimycobacterial and docking studies of pentacyclic triterpenes from *Buddleja saligna* leaves. South African Society for Microbiology (SASM) Coastlands Conference Centre, Umhlanga. Poster Presentation

Singh, A., Reddy, L., Coovadia, Y. & Odhav, B. 15 November 2012. Taming Tuberculosis. Durban University of Technology, Faculty of Applied Science Research Day. Full Research Presentation

Singh, A., Pillay, M., Reddy, L., Coovadia, Y. & Odhav, B. 6-9 September 2011. Taming TB. South African Society for Microbiology (SASM) Conference Cape Sun, Cape Town. Poster Presentation

Singh, A., Pillay, M., Reddy, L., Coovadia, Y. & Odhav, B. 10-14 January 2011. Taming Tuberculosis. IOCD Symposium. University of Western Cape, Cape Town. Poster Presentation

ABSTRACT

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB) has infected approximately one-third of the world population, with 9.6 million TB cases in 2014. The emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of MTB has further complicated the problem of TB control. It is now imperative that novel antimycobacterial compounds are discovered in order to treat infections and reduce the duration of current TB therapy courses. For centuries, medicinal plants have been used globally worldwide for the treatment and prevention of various ailments. This occurs particularly in developing countries where infectious diseases are endemic and modern health facilities and services are inadequate. In recent years, the use and search for plant drug derivatives have been fast-tracked. Ethnopharmacologists, botanists, microbiologists, and natural product chemists are trying to discover phytochemicals which could be developed for the treatment of infectious diseases, especially TB. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimycobacterial activity.

In the search for new lead compounds, nine medicinal plant species, *Buddleja saligna, Capparis tomentosa, Carpobrotus dimidiatus, Dichrostachys cinerea, Ekerbergia capensis, Ficus Sur, Gunnera perpensa, Leonotis leonurus* and *Tetradenia riparia* were collected in Kwa-Zulu Natal (KZN) following report of their therapeutic use in traditional medicine to treat symptoms and infections related to TB. They were tested *in vitro* for their activity against *Mycobacterium smegmatis*, *Mycobacterium tuberculosis* H_{37}Rv (ATCC 25177) and three well-characterized clinical isolates of MDR-TB and XDR-TB using the agar incorporation method. The minimum inhibitory concentration of the active plant extracts was determined using the broth microdilution method. Our findings show that five of the nine plants screened have antimycobacterial activity with concentrations ranging from 125 µg/ml to 1000 µg/ml.

The aqueous extracts of *G. perpensa* and *T. riparia*; and the methanolic extracts of *B. saligna, C. tomentosa,* and *C. dimidiatus* possessed significant activity against *M. smegmatis, M. tuberculosis* $H_{37}Rv$ (ATCC 25177) and the three well-characterized clinical isolates of MDR-TB and XDR-TB.

The cytotoxic effect of the active plant extracts was evaluated against the mouse BALB/C monocyte-macrophage (J774.2) and peripheral blood mononuclear cells (PBMCs). The toxic effects of the active plant extracts were evaluated using the brine shrimp lethality assay. Except for a high concentration of *G. perpensa* none of the other plants which possessed antimycobacterial activity showed any toxic or cytotoxic activity. The active plant extracts were thereafter assessed to determine if they had any effect on the survival or death of mycobacterial species, *M. smegmatis*, bound within the macrophage (J774.2) cell line at a concentration of 100 µg/ml. *B. saligna* had inactivated most of the phagocytosed bacilli after 24 hours of treatment therefore, it has a bactericidal effect on the mycobacteria located within the mouse macrophage.

A phytochemical investigation of the leaves of *B. saligna* led to the isolation of two isomeric pentacyclic triterpene compounds namely Oleanolic Acid (OA) and Ursolic Acid (UA) using thin layer chromatography followed by silica gel column chromatography. The structures of these compounds were fully characterized by detailed NMR investigations, which included ^{1}H and ^{13}C NMR. Ursolic acid was isolated from this plant for the first time. Two-dimensional (2D) and three-dimensional (3D) quantitative structure-activity relationship (QSAR) studies were carried out to provide insight on the interaction of the compounds with the enzyme. Molecular docking studies predicted the free binding energy of the triterpenes inside the steroid binding pocket of *Mycobacterium tuberculosis* fadA5 thiolase compared to a reported inhibitor. Thus, their ability to inhibit the growth of *Mycobacterium tuberculosis* was predicted and was confirmed to possess significant antimycobacterial activity when tested against *M. smegmatis, M. tuberculosis* $H_{37}Rv$ (ATCC

25177), clinical isolates of MDR-TB and XDR-TB using the Microplate Alamar Blue Plate (MABA) assay.

The present study has scientifically validated the traditional use of medicinal plant *B. saligna*.

CHAPTER 1: INTRODUCTION

1.1 Tuberculosis and Herbal Medicine

Human tuberculosis (TB) is a contagious, infectious disease caused by *Mycobacterium tuberculosis* (MTB). MTB is an aerobic pathogenic bacterium that usually establishes its infection in the lungs (Ducati et al., 2006). TB is the most commonly notified disease. It is the fifth major cause of death. Green et al. in 2010 reported one in ten of TB cases was resistant to treatment in some areas (Green et al., 2010). Today, TB is the most common cause of adult mortality by an infectious disease. The TB pandemic was thought to have been eradicated in the early second half of the last century. However, during the previous two decades, this disease has reoccurred in developing and industrialized countries. In 1999 Grange stated that TB kills more people than AIDS, malaria, and other tropical diseases. The association between TB with Human Immunodeficiency Virus (HIV) has contributed to the increase in drug-resistant (DR-TB), multi-drug resistant TB (MDR-TB) and extensive drug resistant (XDR-TB) strains being formed. Corbett et al. in 2003 reported that between 1990 and 2003, TB incidence rates tripled in African countries with high HIV prevalence.

In South Africa, MDR-TB has been identified (Green et al., 2010) and these emerging MDR-TB strains complicate treatment (Victor et al., 2007). The current TB chemotherapy consists of an initial two months phase of intensive treatment with all four drugs: pyrazinamide, ethambutol, isoniazid and rifampin. This is followed by continuation phase for four months with rifampicin and isoniazid. This current treatment system is very long and, therefore, is an important co-factor in the increasing rate of DR-TB and MDR-TB development. The lengthy therapy period with multi-drug treatment results in a lack of compliance which causes treatment failure (Sarkar and Suresh, 2011).

Novel antimycobacterial compounds are urgently required to help to control TB; preferably those that could be readily and simply produced from a local source (Lall and Meyer, 1999). The use of antimicrobials from natural vegetation has a great impact on human health care in undeveloped countries. Several approaches currently exist for the search for new anti-TB drugs including the screening of plant materials for bioactive compounds (Telenti and Iseman, 2000, Bamuambaa et al., 2008). Herbal medicines have been used for centuries in rural areas by local healers and have been improved in industrialized countries (Lall and Meyer, 1999).

1.2 Motivation for the study

The elimination of TB has been hindered due to the lack of understanding of the survival strategies used by the pathogen. Thus, research towards discovering new effective antimycobacterial drugs is necessary.

TB control programmes currently emphasize the Directly Observed Treatment Short Course (DOTS) strategy, promoted by the World Health Organization (WHO) and the International Union against TB and lung disease. South Africa adopted the WHO's DOTS strategy in all nine provinces (DoH, 2012). Rural patients delay TB treatment, because they cannot afford to travel to DOTS clinics daily to have a health worker watch them take their drugs (Russell, 2004, Needham et al., 1998). With the rapid increase in infection in sub Saharan Africa and due to the relatively high cost and limited access to synthetically derived drugs, communities in Africa have relied on traditional healers to treat infectious diseases (WHO, 2003). Traditional healers use medicinal plants as their primary source of medicine. Local communities consult traditional healers on a regular basis because they are easily available, are familiar with the patient's culture and the environment and the costs associated with treatments are minimal. Despite the increasing acceptance of traditional medicine in treating TB in South Africa, this indigenous knowledge on

traditional remedies is not adequately documented (Green et al., 2010, Buwa and Afolayan, 2009).

The investigation into traditional medicinal plants is justified by Scott et al. (2004). South Africa is home to about 10% of higher plant species on the planet. However, less than 5% of these species have been scientifically assessed for pharmacological activity (Bamuambaa et al., 2008). It is estimated that 70% of all South Africans use traditional medicines derived from indigenous plant species (Scott et al., 2004). South Africans have used medicinal plants for curing TB related symptoms such as bronchitis, cough, diarrhoea, fever, and even TB (Lall and Meyer, 1999, Scott et al., 2004, Bamuambaa et al., 2008, Springfield et al., 2003, Hutchings and Van Staden, 1994, Vogt, 1995).

A large number of medicinal plants that have been reported in the literature have antimicrobial activity. Some of these plants are listed in Table 1. In our quest for natural and potent antimycobacterial drugs, nine South African medicinal plants namely *Buddleja saligna, Capparis tomentosa, Carpobrotus dimidiatus, Dichrostachys* cinerea, *Ekebergia capensis, Ficus sur, Gunnera perpensa, Leonotis leonurus* and *Tetradenia riparia* were selected to validate their antimycobacterial activities and determine the active components against *Mycobacterium smegmatis, Mycobacterium tuberculosis* H_{37}Rv, MDR-TB, and XDR-TB.

Table 1. Plants used in the treatment of TB-related ailments

Plant	Documented Use	Reference
B. saligna	TB symptoms	(Bamuamba et al., 2008)
C. tomentosa	Chronic cough and chest pain	(Hutchings and Van Staden, 1994)
C. dimidiatus	TB infections	(Springfield and Weitz, 2003)
D. cinerea	Leprosy and coughs	(Vogt, 1995)
E. capensis	Chronic cough, respiratory chest complaints and TB	(Lall and Meyer, 1999)
F. sur	TB	(Hutchings, 1996)
G. perpensa	TB symptoms	(Lall and Meyer, 1999)
L. leonurus	Cough and respiratory ailments	(Scott et al., 2004)
T. riparia	TB symptoms, cough and respiratory ailments	(Scott et al., 2004)

1.3 Objectives of the study

The objectives were to:

(1) prepare aqueous and methanolic extracts of *B. saligna, C. tomentosa, C. dimidiatus, D. cinerea, E. capensis, F. sur, G. perpensa, L. leonurus* and *T. riparia*;
(2) investigate the antimycobacterial activity of the crude extracts against *M. smegmatis, M. tuberculosis* H_{37}Rv, MDR-TB and XDR-TB using the agar incorporation plate assay;
(3) evaluate the minimum inhibitory concentration of the active plant extracts that have antimycobacterial activity against *M. smegmatis, M. tuberculosis* H_{37}Rv, MDR-TB and XDR-TB using the broth microdilution assay;
(4) determine the cytotoxic effect of the active plant extracts against peripheral blood mononuclear cells and the J774 mouse macrophage cell line using the MTT assay;
(5) evaluate the toxic effects of the active plant extracts using the brine shrimp lethality assay;

(6) determine if the active plant extracts can prevent the intracellular growth of *M. smegmatis* in macrophages using colony counts and;

(7) isolate and characterise the active compounds that have antimycobacterial activity from the active plant extracts using TLC-Bioautography and other necessary separation methods including ^{1}H and ^{13}C NMR.

(8) determine the molecular mechanism of action of the active compounds using two-dimensional (2D) and three-dimensional (3D) quantitative structure-activity relationship (QSAR) studies.

1.4 Structure of Thesis

The thesis consists of six chapters.

Chapter one introduces the study and gives the layout of the thesis.

Chapter two comprises of the literature review where we discuss tuberculosis as well as the impact that HIV has on TB, current TB drugs, medicinal plants that have antimycobacterial activity and the plants used for this research.

Chapter three gives a full description of all the methodology utilized in this research.

Chapter four provides the results obtained from all the methods performed.

Chapter five discusses the findings of this study the context of past research and future work.

Chapter six gives the conclusion.

CHAPTER 2: LITERATURE REVIEW

Tuberculosis (TB) is more prevalent in the world today than at any other time in human history. *Mycobacterium tuberculosis* (MTB), the pathogen responsible for TB, uses diverse strategies to live in a diversity of host lesions and evade immune surveillance (Koul et al., 2011).

2.1 History of Tuberculosis

TB is an ancient scourge which has plagued humankind throughout known human history. Consumption, phthisis, scrofula, Pott's disease and the White Plague are all terms used to refer to TB throughout history. It has surged in great epidemics and then receded, thus behaving like other infectious diseases. It is believed that the microorganism came from other, more primitive organisms of the same genus Mycobacterium. The view that TB might contagious had its adherents, however, in 1865 Jean-Antoine Villemin, an army doctor in Paris, showed that it could be transmitted from tuberculous animals to healthy animals by inoculation. German physician Robert Koch discovered the actual infectious agent, the tubercle bacilli, in 1882 and won the Nobel Prize for this discovery in 1905 (Tuberculosis, 2014). Hayman in 1984 stated that *M. tuberculosis* might have killed more people than any other microbial pathogen.

Results from a DNA study, in 2014, of a TB genome reconstructed from remains in southern Peru suggested that human TB not be more than 6,000 years old. A theory from researchers is that humans first acquired it 5,000 years ago in Africa (Zimmer, 2014). According to Zimmer the disease spread to other humans along trade routes. In Africa, it spread to goats, cows, seals and sea lions. The seals and sea lions are believed to have been infected by the disease and carried it across the Atlantic to South America. The first humans to contract the disease would have been the hunters. TB arrived in South Africa

with settlers, missionaries, and colonialists, most of whom were infected by the massive epidemic, which had swept through Europe and North America during the 17th century. Most of them came looking for a cure from the fresh air and sunshine. The previously unexposed, non-immune indigenous people of South Africa quickly developed TB.

In South Africa in the later 1800s, when the gold mines started on the Reef, workers were exposed to silica dust, overcrowded hostel living, poor nutritional status and stress, all of which were major contributors to the development of TB. When these workers became sick, they went home to their families in rural areas and thus spread the disease. It was estimated by 1930 that over 60% of the black population of South Africa was infected. Incidence rates measured from notifications since 1919 rose rapidly and peaked during the 1960s. The rates appeared to decline in the early 1970s when certain sections of the country became independent states and were not included in reported figures (Edington, 2014).

2.2 Mycobacterium species

2.2.1 *Mycobacterium tuberculosis*

TB, caused by *M. tuberculosis* (MTB), is a contagious, infectious disease which primarily infects the lungs. Annually, TB is responsible for approximately nine million cases and two million deaths worldwide (WHO, 2016). MTB is the etiologic agent of TB in humans and we are the only reservoir for the bacterium. MTB is a large nonmotile rod-shaped bacterium. The rods are 2-4 µm in length and 0.2-0.5 µm in width. MTB is an obligate aerobe and due to this, it is always found in the well-aerated upper lobes of the lungs. The mycobacterium which has a slow generation time of 15-20 hours is a facultative intracellular parasite, usually of macrophages and this contributes to its virulence.

Two media are used to grow MTB; namely Middlebrook's medium and Lowenstein-Jensen medium. Middlebrook's medium is agar-based and Lowenstein-Jensen medium is egg-based. When grown on either medium MTB colonies are small and buff coloured (Figure 1 and Figure 2). Both of these media contain inhibitors to keep contaminants from out-growing MTB. It takes 4-6 weeks to get visual colonies on either type of media.

Figure 1. Colonies of *M. tuberculosis* on Lowenstein-Jensen medium.

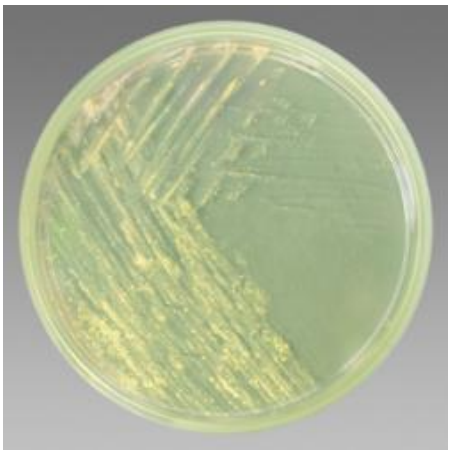


Figure 2. *M. tuberculosis* cultivated on Middlebrook 7H11 agar plates.

Distinctive serpentine cords are formed by chains of cells in smears made from in vitro grown colonies. MTB is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, however, if a Gram stain is

performed on MTB, it stains very weakly Gram-positive. The mycobacteria contain peptidoglycan in their cell wall. The Ziehl-Neelsen stain which is an acid-fast staining method is used for MTB. MTB smear is fixed, stained with carbol-fuchsin, and decolorized with acid-alcohol. The smear is then counterstained with methylene-blue. Acid-fast bacilli appear pink in a contrasting background (Figure 3).

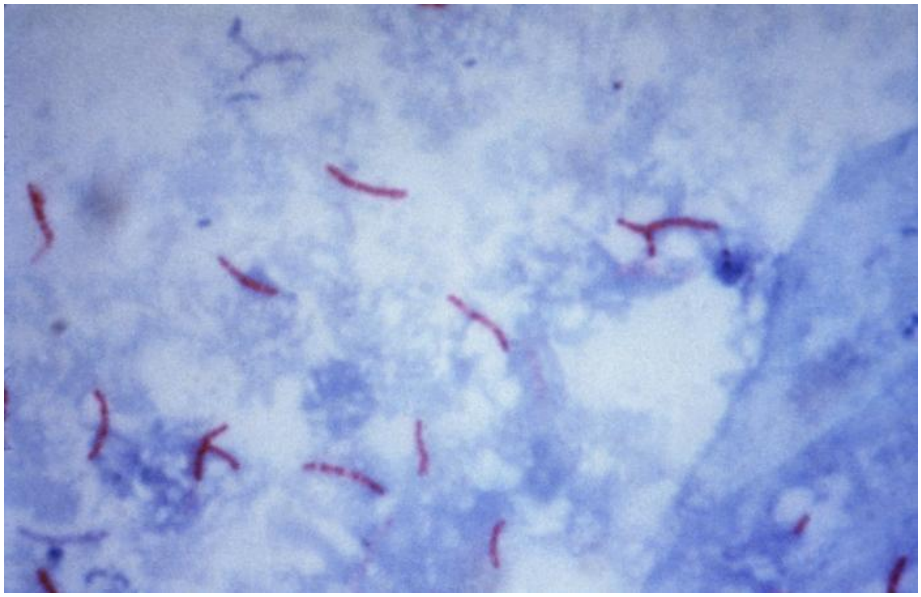


Figure 3. *M. tuberculosis* bacteria using acid-fast Ziehl-Neelsen stain; magnified 1000 x

The cell wall structure of MTB is unique among prokaryotes and it contributes to the virulence of the bacterium. The cell wall complex contains peptidoglycan and complex lipids (Figure 4). The lipid fraction of MTB's cell wall consists of three major components, mycolic acids, cord factor, and wax-D (Todar, 2012). Mycolic acids are unique alpha-branched hydrophobic lipids found in cell walls of *Mycobacterium* and *Corynebacterium* which affect permeability properties at the cell surface. Mycolic acids are thought to prevent an attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. The cord Factor which is abundantly produced in virulent strains of MTB is responsible for the serpentine cording and is toxic to mammalian cells and is

also an inhibitor of PMN migration. The wax-D in the cell envelope is the major component of Freund's complete adjuvant. Due to the high concentration of lipids in the cell wall, MTB is impermeable to stains and dyes, resistant to many antibiotics, acidic and alkaline compounds, and resistant to lethal oxidations and survival inside of macrophages (Todar, 2012).

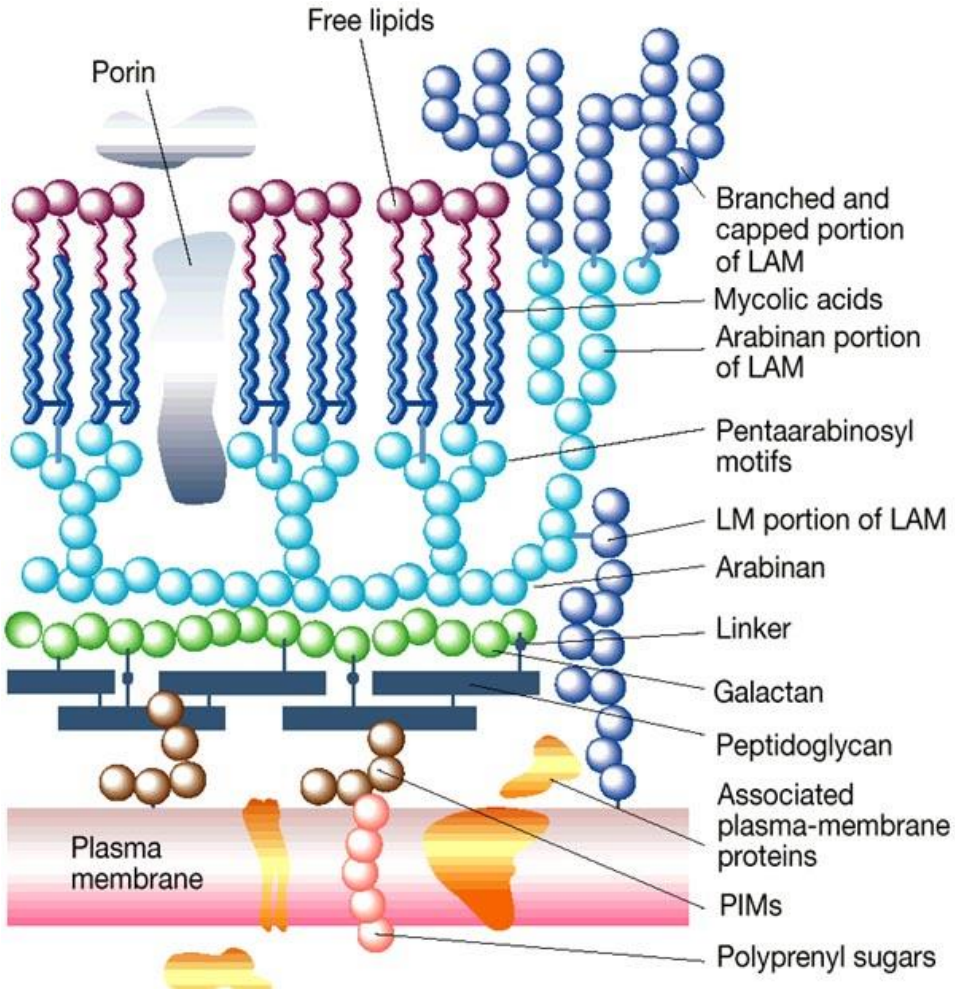


Figure 4. Diagram of the mycobacterial cell wall.

Drugs have been developed for mycobacteria target components of the cell wall. Ethambutol prevents the polymerization step of AG (arachidonoylglycerol) synthesis (Mikusova et al., 1995). Isoniazid is a prodrug activated by a mycobacterial encoded KatG

catalase in the cell wall that, when active, prevents mycolic acid synthesis (Zhang et al., 1992). Ethionamide is a prodrug that, upon activation, prevents fatty acid synthesis required for mycolic acid synthesis (Banerjee et al., 1994).

Researchers often use a close relative, *Mycobacterium smegmatis*, to study TB in the lab due to its virulence. The two types of bacteria share various qualities, which includes the peculiar cell wall, however, *M. smegmatis* is relatively safe because it has low pathogenicity. (Chakradhar, 2012).

2.2.2 *Mycobacterium smegmatis*

M. smegmatis is a rapidly growing mycobacterium. The rapidly growing non-pathogenic microorganism is used as a model in TB experiments compared to severely pathogenic *M. tuberculosis* and *M. avium*. It is also used to study the ability of the pathogenic *M. tuberculosis* to survive inside human macrophages. Although *M. smegmatis* contains the similar structural features of *M. tuberculosis*, its grows much quicker in comparison to the latter (Gordon and Smith, 1953). Recently, it has been reported to cause disease in humans (Kaláb et al., 1995).

M. smegmatis is a nonmotile obligate aerobic organism which requires unique fatty acid biosynthesis to produce the mycolic acids that are present in the cell wall (Megehee and Lundrigan, 2007). They protect themselves with the outer lipid bilayer, which is a thick biological membrane and, therefore, has low permeability making them intrinsically resistant to many antibiotics, acidic and alkaline compounds and the membrane serves as an effective permeability barrier. *M. smegmatis* is a Gram-positive bacteria, characterized by an inner cell membrane and a thick cell wall. Although this mycobacterium is Gram-positive, it has some unique qualities that are divergent from most Gram-positive bacteria. The acids prevent proper gram staining that would normally identify the cell as a gram positive cell because they create a waxy coating so the crystal violet has difficulty

entering the cell (Figure 5), therefore making it seem gram-negative. The cell wall is also abnormal because it is irregularly thick for a gram-positive bacteria and its hydrophobic nature reduces desiccation.

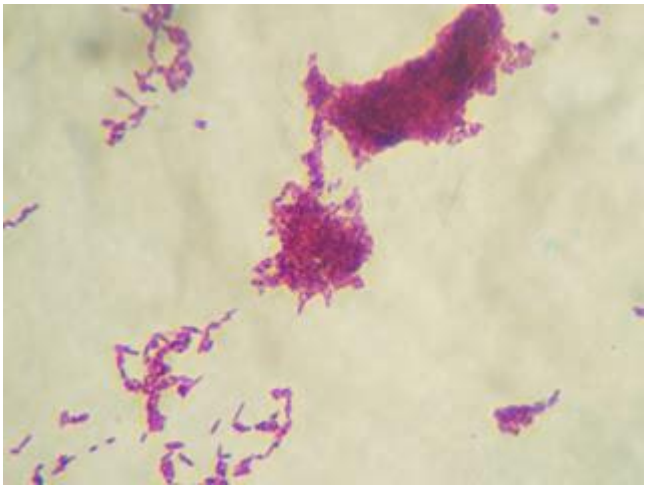


Figure 5. The acid fast stain of *M. smegmatis* (100 x magnifications).

2.3 Epidemiology of Tuberculosis

In 2003, the global incidence of TB peaked and appeared to be declining slowly. However, in 2008 Lönnroth and Raviglione reported that more than two billion people were estimated to be infected with MTB worldwide (Figure 6). in 2012, the World Health Organization (WHO) reported that over eight million individuals became ill with TB and over one million died (WHO, 2013) therefore the disease remains a global scourge. About 75% of these cases were in the African Region. In 2012, an estimated four hundred and fifty thousand people developed MDR-TB and an estimated one hundred and seventy thousand people had died from MDR-TB. The TB incidence rate at country level ranges substantially, with around one thousand or more cases per one hundred thousand people in South Africa and Swaziland, and fewer than ten per one hundred thousand people in

parts of America, several countries in Western Europe, Japan, Australia and New Zealand (WHO, 2013).

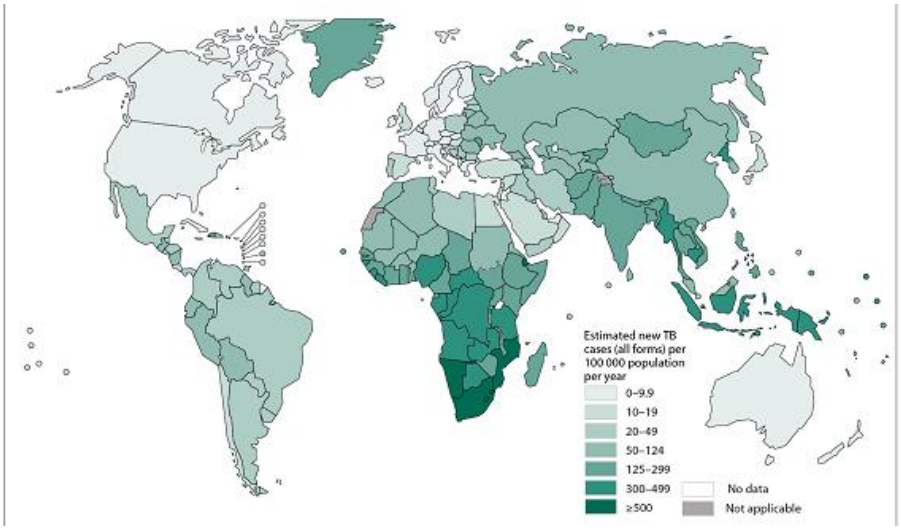


Figure 6. Estimated TB incidence rates (WHO, 2015)**.**

Most TB cases and deaths occur among men, however, globally, TB remains one of the top three killers of women. There were an estimated four hundred and ten thousand TB deaths among women in 2012, including one hundred and sixty thousand among HIV-positive women. In 2012, half of the HIV-positive people who died from TB were women. Of the eight million new cases of TB worldwide in 2012, approximately three million were women. There were an estimated five hundred and thirty thousand TB cases among children and seventy four thousand TB deaths among HIV-negative children in 2012 (Glaziou et al., 2013). In 2014, TB killed one and a half million people (one million HIV-negative and four hundred thousand HIV-positive). The toll comprised eight hundred and ninety thousand men, four hundred and eighty thousand women and one hundred and forty thousand children (WHO, 2015). Worldwide, nine and a half million people are estimated to have contracted TB in 2014. Globally, 12% of the nine and a half million new TB cases in 2014 were HIV-positive. In 2014, six million new cases of TB were reported to WHO, fewer than two-thirds of the nine and a half million people estimated to have fallen

sick with the disease. Of the four hundred and eighty thousand cases of MDR-TB estimated to have occurred in 2014, only one hundred and twenty three thousand were detected and reported (WHO, 2015).

One of the countries with the highest TB burden is South Africa, with the World Health Organization (WHO) providing statistics of an estimated incidence of five hundred thousand of active TB cases in 2011 (WHO, 2013). WHO in 2013 stated that approximately 1% of the population of fifty million develops active TB disease annually. This makes it globally the third highest incidence of any country after India and China, and the incidence has increased by 400% over the past 15 years. It has been estimated that approximately 80% of South Africans are infected with the tubercle bacilli with the vast majority being people living in townships and informal settlements in the 30-39 years old age group. In 2012 statistics released by the South African Department of Health revealed that 73% of TB patients are HIV-positive (DoH, 2012). South Africa has one of the most serious TB epidemics in the world with more than sixty thousand deaths reported in 2010 (Lehohla, 2010). A major TB epidemic in South Africa in the pre-HIV era has been followed by a rising number of TB cases as a result of HIV and TB co-infection. Currently, in South Africa, there is an increase in resistance to some of the antimycobacterial drugs (Padayatchi et al., 2014).

Global targets to reduce the epidemiological burden of TB for 2015 and 2050 have been set within the context of The Millennium Development Goals (MDGs) and the Stop TB Partnership (Glaziou et al., 2013).

2.4 Transmission of Tuberculosis

TB is a communicable infectious disease and the most important source of infection comes from people with pulmonary TB.

Infection is initiated by inhalation of droplet nuclei, which are particles of 1–5 µm in diameter containing *M. tuberculosis*, when people who have pulmonary or laryngeal TB disease cough, sneeze, shout or sing. TB is spread from person to person through the air (Figure 7). The droplet nuclei can remain suspended in the air for several minutes to hours due to their small size. The risk of infection is dependent on four factors; the infectiousness of the source; the closeness of contact; the bacillary load inhaled; and the immune status of the potential host (Frieden et al., 2003).

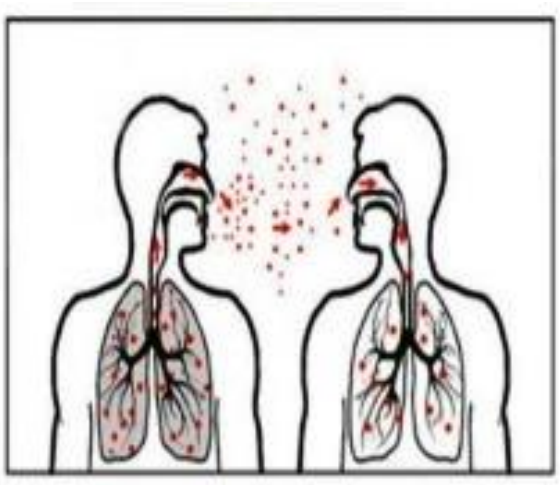


Figure 7. A diagram depiction of TB transmission where the dots in the air contain droplet nuclei containing *M. tuberculosis.*

The primary route of infection involves the lungs. Inhaled droplet nuclei avoid the defences of the bronchi due to their small size, and penetrate into the terminal alveoli where they are engulfed by macrophages and dendritic cells (Mehta et al., 2006). According to García-Pérez et al. (2003) *M. tuberculosis* can also infect non-phagocytic cells in the alveolar space including alveolar endothelial, type 1 and type 2 epithelial cells (pneumocytes) and M cells.

2.5 Pathogenesis of Tuberculosis

Infection occurs when a person inhales droplet nuclei containing tubercle bacilli which reaches the alveoli of the lungs. Most of these bacilli are inhibited or destroyed, however, some may multiply intracellularly and will be released when the macrophages die (CDC, 2005). The live mycobacteria are phagocytosed by alveolar macrophages in the lung and induce a localized proinflammatory response which leads to recruitment of mononuclear cells from neighbouring blood vessels. These cells are the building blocks for the granuloma or tubercle that defines the disease. The granuloma consists of a kernel of infected macrophages, surrounded by macrophages with a mantle of lymphocytes delineating the periphery of the structure (Russell, 2001). This tissue response then enters the 'containment' phase of the infection, during which there are no signs of disease and the host does not transmit the infection to others. Containment fails after a change in the immune status of the host. This is usually a consequence of old age, malnutrition, or HIV-coinfection. Under such circumstances, the center of the granuloma undergoes caseation and spills viable, infectious bacilli into the airways. This leads to the development of a productive cough which facilitates the aerosol spread of infectious bacilli (Russell, 2001).

2.6 Immunology of Tuberculosis

The alveolar macrophages, after entry of *M. tuberculosis*, produce inflammatory cytokines and chemokines which serve as a signal for infection. The monocytes, neutrophils, and lymphocytes migrate to the focal site of infection. However, they are unable to kill the bacteria. According to Chen et al. (2006), the bacilli resist the bactericidal mechanisms of the macrophage by preventing phagosome-lysosome fusion. They multiply in the phagosome and cause macrophage necrosis.

The released bacilli multiply extracellularly and are phagocytosed by another macrophage which is unable to control the growth of *M. tuberculosis*. Dendritic cells with engulfed

bacilli mature, migrate to the regional lymph node, and prime $CD4^+$ and $CD8^+$ against mycobacterial antigens (Bodnar et al., 2001). The specific immune response produces primed T cells which travel back to the focus of infection, guided by the chemokines produced by the infected cells. The accumulation of macrophages, T cells, dendritic cells, fibroblasts, endothelial cells, and stromal cells leads to the formation of a granuloma at the site of infection (Gonzalez-Juarrero et al., 2001).

The granuloma formation walls off tubercle bacilli from the rest of the lung tissue. This limits bacterial spread and provides micro-environment for interactions among macrophages and other cells of the immune system and the cytokines produced by these cells. The $CD4^+$ cells producing interferon-γ (IFN-γ) recognize infected macrophages presenting antigens from *M. tuberculosis* and kill them (Wolf et al., 2008). Even though the infection progression is stopped, some resistant bacilli capable of surviving under the stressful conditions generated by the host escape death. These bacilli enter a state of dormancy and persist by avoiding elimination by the immune system (Hingley-Wilson et al., 2003).

2.7 Symptoms of Tuberculosis

TB symptoms depend on the location of where the mycobacteria are growing in the body. When the disease is in the lungs symptoms such as a bad cough that last three weeks or longer, pain in the chest and coughing up blood or sputum arise. When TB occurs outside your lungs, symptoms vary according to the organs involved. TB of the spine contributes to back pain. TB in the kidneys causes blood in the urine. Other symptoms of the disease include weakness, fatigue, weight loss, lack of appetite, chills, fever and night sweats. People suffering from latent TB infection do not feel sick, do not have any symptoms, and cannot spread TB to others (CDC, 2014).

2.8 TB and HIV Co-infection

Swaminathan et al. in 2000 stated that there was a higher incidence of TB among HIV-infected individuals. People with HIV infection are at increased risk of rapid progression of a recently acquired infection, as well as of re-activation of latent infection. Globally, TB is an opportunistic infection occurring among HIV-positive people (Raja, 2004). TB and HIV infections are both intracellular and known to have a profound influence on the progression of each other. HIV infection brings about the reduction in $CD4^+$ T cells, which play a major role in the immunity to TB. Apart from the reduction in number, HIV also causes functional abnormality of $CD4^+$ and $CD8^+$ cells. Likewise, TB infection also accelerates the progression of HIV disease from an asymptomatic infection to AIDS to death. Matsuyama et al. (1991) showed that a potent activator of HIV replication within T cells is TNF-α, which is produced by activated macrophages within granulomas as a response to tubercle infection. Due to the clinical features of HIV-infected patients with TB being nonspecific, diagnosis can be difficult. The method most widely used, detection of acid-fast bacilli by microscopic examination of sputum smears, is of little use. This is because 50% of HIV-TB cases are negative by acid-fast staining and chest radiographs are normal in up to 10-20% of patients with AIDS (Raja, 2004). The frequent co-infection of TB in HIV patients complicates the selection of an appropriate treatment regimen. The increased pill burden diminishes compliance; drug–drug interactions lead to sub-therapeutic concentrations of antiretrovirals (ARVs); and overlapping toxic side effects increase safety concerns (Koul et al., 2011). The main interaction between HIV and TB anti-infectives is rifampicin induced increased expression of the hepatic cytochrome (CYP) P450 oxidase system (Niemi et al., 2003).

2.8.1 Impact of HIV on the incidence of TB across the globe

In 2010, there were over eight million new cases of TB, with over one million people dying, mostly in developing countries. Three hundred and fifty thousand of these deaths

occurred to those co-infected with HIV (WHO, 2011). In 2012, more than one million people with TB were living with HIV and over one million people died from TB of which three hundred and twenty thousand were HIV-associated TB deaths. In 2013, nine million people around the world became sick with TB disease. There were around one and a half million TB-related deaths worldwide. TB is a leading cause of death among people who are HIV infected. In 2013, over nine thousand cases of TB were reported in the United States of America (CDC, 2014).

2.8.2 Impact of HIV on the incidence of TB in South Africa

South Africa remains the country with the greatest burden of HIV-positive individuals and the second highest estimated TB incidence per capita worldwide (WHO, 2009, UNAIDS, 2008). Barnighausen et al. (2008) stated that approximately one million people in KwaZulu-Natal (KZN), South Africa are HIV-positive individuals. KZN also sustains a high incidence of new adult HIV infections. In 2006, TB notification rates were elevated with one thousand cases per one hundred thousand people. Due to the emerging epidemic of drug-resistant TB, this has received international attention (Gandhi et al., 2006). Drug-resistant TB has been identified as a major menace to the success of antiretroviral therapy (ART) (Andrews et al., 2007). The development of TB resistance remains an important factor driving the current TB epidemic. Worse case scenario will be if TB further encourages HIV resistance and prevents ARTs from impacting on the high mortality rates.

2.9 Treatment of Tuberculosis

Continued drug resistance to current antibiotic therapies is driving the need for new and novel drugs. The main challenge in producing antibiotics against this bacterium is also recognized where the microorganism has an unusually thick, waxy cell wall that is hard to

penetrate. According to Chakradhar in 2012 getting any drugs through this barrier, although possible, is difficult.

Currently, more than twenty drugs are used for the treatment of TB. Most of these drugs were developed years ago. The drugs which are used in differing combinations in different circumstances as some of the drugs are only used for the treatment of new patients who are unlikely to have resistance to any of the current TB drugs. There are other TB drugs that are only used for the treatment of drug-resistant TB (WHO, 2010).

The drugs, most commonly used, RIF and INH, have been losing its effectiveness against the bacterium. Due to this, combinatorial therapy has been in place for almost 50 years however mycobacteria has resisted this as well.

The "first line" TB drugs are INH, RIF, PZA, ETH and Streptomycin (Table 2). These are drugs which are the most effective against TB bacteria because they have the highest activity. Due to this, these drugs are essential to any TB treatment program. These TB drugs are particularly used for someone with active TB disease who has not had TB drug treatment before (WHO, 2010). All the other TB drugs are generally referred to as "second line" or reserve TB drugs.

Table 2. Summary of first-line drugs used for the treatment of TB

Drug	Discovered	Mechanism of action	Active against
Isoniazid (INH)	1912 however anti-TB activity reported on 1951	Inhibiting the cell wall synthesis.	Fast growing population of the bacteria
Pyrazinamide (PZA)	1952	Inhibiting and depleting the membrane energy.	Population of bacteria having low metabolic activity and can survive in acidic pH.
Rifampicin (RIF)	1959	Inhibiting the nucleic acid synthesis.	Population with spurts of metabolism: dormant with occasional short, active period of metabolism

Ethambutol (ETH)	1961	Inhibiting the cell wall synthesis.	Fast growing population and mainly used to minimise INH resistance.

For the two months intensive TB drug treatment phase patients receive INH with RIF, PZA and ETH followed by INH with RIF for the continuation TB drug treatment phase. It is essential to take several TB drugs together. If only one TB drug is taken on its own, then the patient will very quickly become resistant to that drug. It is recommended that patients take the TB drugs daily for six months, although taking them three times a week is possible in some circumstances. It is extremely important that all the recommended TB drugs are taken for the entire time (WHO, 2010). If only one or two of the TB drugs are taken, or the treatment is interrupted or stopped early, then the treatment probably will not work because the mycobacteria that a patient has, develops resistance to the TB drugs. Not only is the patient then still ill, but to be cured they then are required to take drugs for the treatment of drug-resistant TB, and these drugs are more expensive and have more side effects (WHO, 2012).

For the treatment of drug-resistant TB, the current TB drugs are grouped according to their effectiveness, experience of use, and drug class. Group 1 TB drugs are the first-line oral agents which are PZA, ETH, and rifabutin. Group 2 TB drugs are injectable agents which are kanamycin, amikacin, capreomycin, and streptomycin. Group 3 TB drugs are fluoroquinolones which are levofloxacin, moxifloxacin, and ofloxacin. Group 4 TB drugs are oral bacteriostatic second line agents which are para–aminosalicylic acid coming from cycloserine, terizidone, thionamide, and protionamide. Group 5 TB drugs are agents with an unclear role in the treatment of drug-resistant TB (WHO, 2010).

Treatment of drug-susceptible TB involves an initial phase of INH, RIF, PZA and ETH for the first two months followed by a continuation phase of INH and RIF for the last four months. Up to 95% of people with drug-susceptible TB can be cured in six months with this four-drug regimen. MDR-TB is resistant to at least INH and RIF, the two most important first-

line drugs used in the treatment of TB. This may result from either primary infection with drug-resistant bacteria or may develop in the course of a patient's treatment when non-optimal treatment durations or regimens are used. Cure rates for MDR-TB are lower, typically ranging from 50% to 70%. XDR-TB is resistant to INH and RIF as well as any fluoroquinolone and any of the second-line antimycobacterial injectable drugs amikacin, kanamycin or capreomycin). It has very high mortality rates (Koul et al., 2011).

2.9.1 Why is there a need for new TB drugs?

Inadequate, incomplete or improperly supervised treatment regimens, incorrect prescription, and co-infection with HIV are responsible for the emergence of resistant strains of MTB.

Although TB can be treated with the current drugs, treatment is complex and long involving four drugs for two months and two more drugs for at least another four months. Direct Observed Treatment (DOT) as promoted by the WHO to improve compliance with the difficult and long regime can improve cure rates. However, it is demanding for patients and labour intensive for health staff.

Interrupted treatment of TB will result in the transmission of disease. Many patients with TB adhere to treatment until symptoms have resolved and stop. Patients associate the disease will illness and, therefore, the need to continue treatment with illness. The consequent risks of failure of treatment, relapse, death, and drug resistance, threaten patients as well as entire communities (Rouillon, 1972).

When a patient suffers from both HIV and TB it is difficult to take drugs to treat for both diseases simultaneously. Some drugs which are used for the treatment of HIV also interacts with some TB drugs making the treatment more difficult. It is important that the

TB treatment is taken regularly and exactly as the health care provider advised. If the drugs are not taken regularly, the mycobacteria become resistant to the drugs and this can be dangerous and lead to MDR-TB and XDR-TB. It is much more difficult to treat MDR-TB and XDR-TB as well as being more expensive (WHO, 2012).

There is a vital need to develop new and more effective TB drugs. These drugs need to be active against MDR-TB and XDR-TB, target non-replicating bacilli and shorten the length of treatment.

2.9.2 Why Are Mycobacteria Resistant to Antibiotics?

Multi-drug resistance has recently made news headlines. Mycobacteria are inherently resistant to numerous antibiotics because of the cell wall. They have unusually impermeable cell walls that are thought to be an advantage in stressful conditions of osmotic shock or desiccation as well as contributing to their considerable resistance to many drugs (Hett and Rubin, 2008).

Brennan in 1995 found that the permeation ability of a lipophilic molecule is inversely related to the fluidity of the cell wall, which decreases as the length of fatty acids in the mycolic acid layer increases. Lipophilic drugs, such as fluoroquinolones or rifamycins, pass more easily through the lipid-rich cell wall and thus are more active. However, the low permeability is not sufficient to explain the level of innate drug resistance seen in mycobacteria. The surface-to-volume ratio is extremely high for small organisms like bacteria, such that even when low permeability is factored in, mycobacteria must have some other characteristics that make them resistant to antimicrobials (Jarlier and Nikaido, 1994).

Experiments done by Jarlier and Nikaido in 1994 measuring the equilibrium diffusion for mycobacterial drugs show that the drugs reach cytotoxic levels inside the bacteria within a

fraction of the generation time, yet the bacteria are able to survive. Strategies that mycobacteria use in combination with the low rate of diffusion through the cell wall likely include efflux pumps, response regulators, antibiotic-modifying or degrading enzymes such as β-lactamase, target modifying enzymes, and decoys that mimic the drug target (Nguyen and Thompson, 2006).

2.9.3 New TB drugs in the Current Market

Many new TB drugs are currently being developed but the first of these is Sirturo (bedaquiline). This particular drug has only just started to become available outside of clinical trials, and can only be used in very specific circumstances.

A few clinical trials have been carried out of the drugs clofazimine, linezolid, amoxicillin/clavulanate, thioacetazone, imipenem/cilastatin, high dose isoniazid and clarithromycin to see how effective they actually are in the treatment of drug-resistant TB (Dooley et al., 2013). The drug linezolid is an antibiotic usually used to treat severe bacterial infections. The first trial of this drug has just been carried out looking at the use of it in treating XDR-TB. This was a small trial, however, it did show that the drug was effective when added to patients current treatments, although most of the patients experienced side effects (Steenhuysen, 2012).

According to Zhenkun (2010), there are at least, ten compounds in different stages of clinical development for TB. Four of these are existing drugs that are either being redeveloped or repurposed for the treatment of TB, and there are six new chemical compounds that are being specifically developed as TB drugs.

2.10 Medicinal Plants with Antimycobacterial Activity

New and novel drugs are urgently required to combat TB disease and related mycobacterial diseases, globally. Natural products provide leads for the development of novel drugs to treat the rapidly growing numbers of patients with MDR-TB and XDR-TB. Naturally occurring pure compounds as well as plant extracts have showed that inhibitory activity against mycobacteria is widespread in nature.

A summary of the plants adapted from a review article by McGaw et al. in 2008 is presented in Table 3. For the compilation of Table 3 information was supplied by (Watt and Breyer-Brandwijk, 1962), (Hutchings, 1996, McGaw et al., 2008) and Van Wyk et al., 2002 regarding medicinal plants used for treating various TB ailments in Southern Africa.

The potential pharmaceutical value of many compounds derived from medicinal plants remains unknown since data is lacking to show that these compounds are adversely affecting mycobacterial survival mechanisms in humans (Okunade et al., 2004).

Table 3. Traditional plants used in South Africa for treating TB-related diseases adapted from McGaw et al., 2008

Family	Species	Use	Plant part used	Potentially bioactive compounds	Screened for antimycobacterial activity
Aizoaceae	*Carpobrotus* L. spp.	TB, infections (Watt and Breyer-Brandwijk, 1962; Springfield et al., 2003; Springfield and Weitz, 2006)	Leaf juice Tannins, malic acid and citric acid (Watt and Breyer-Brandwijk, 1962)	Flavonoids, hydrolyzable tannins, phytosterols, aromatic acids in *Carpobrotus mellei* (Springfield and Weitz, 2006)	Ethyl acetate extracts of *Carpobrotus mellei* leaves MIC = 15mg/ml, water extract MIC = 30 mg/ml *(M. smegmatis*) (Springfield and Weitz, 2006). *Carpobrotus muirii* and *Carpobrotus quadrifidis* active *(M. smegmatis)* in disc diffusion and bioautography (Springfield et al., 2003)
Aizoaceae	*Galenia africana* L.	Chest pains, TB (Mativandlela et al., 2008)	Leaves	Trihydroxyflavone (Mativandlela et al., 2008)	Ethanol extract of leaves MIC = 0.78 mg/ml (*M.smegmatis*), MIC = 1.2 mg/ml (*M. tuberculosis*); 5,7,2 – trihydroxyflavone MIC = 0.031 and 0.10 mg/ml (*M. smegmatis* and *M. tuberculosis*,)
Asteraceae	*Eriocephalus africanus* L.	Chest complaints, coughs (Salie et al., 1996)	Unspecified parts	Tannins, flavonoids, triterpene steroids (Salie et al., 1996)	Leaf, stem, root extracts inactive (*M. smegmatis*) (Salie et al., 1996)
Asteraceae	Helichrysum spp. (including *Helichrysum nudifolium* (L.) Less., *Helichrysum odoratissimum* (L.) Sweet, *Helichrysum pilosellum* (L.f.) Less., *Helichrysum melanacme* DC., *Helichrysum crispum* (L.) D.Don.)	Coughs, TB (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Salie et al., 1996; Lall and Meyer, 1999; Scott et al., 2004)	Leaves, whole plant	Flavonoids, sesquiterpenoids, acylated phloroglucinols ((Jakupovic et al., 1986, Van Puyvelde et al., 1989); tannins, saponins, cyanogenic glucosides (Salie et al., 1996); caespitate from *Helichrysum caespititium* (Meyer et al., 2002)	*Helichrysum crispum* active (*M. smegmatis*) (Salie et al., 1996). H. melanacme acetone extract MIC = 0.1 mg/ml, water extract MIC = 1 mg/ml (*M. tuberculosis*) (Lall and Meyer, 1999) *Helichrysum odoratissimum* acetone extract MIC = 0.5 mg/ml, water extract inactive (*M. tuberculosis*) (Lall and Meyer, 1999).

Asteraceae	*Leysera gnaphalodes* L.	Respiratory ailments, TB (Scott et al., 2004; Bamuamba et al., 2008)	Whole plant	Oleanolic and ursolic acids (Bamuamba et al., 2008)	No activity of aqueous infusion *(M. smegmatis*) (Scott et al., 2004). Aqueous extract inactive; acetone:water (4:1) extract active in bioautography (*M. aurum*); oleanolic and ursolic acids active (*M. tuberculosis, M. microti, M. avium, M. scrofulaceum*) (Bamuamba et al., 2008)
Buddlejaceae	*Buddleja saligna* L.	TB symptoms (Bamuamba et al., 2008)	Leaves, stems	Oleanolic acid (Bamuamba et al., 2008)	Aqueous extract inactive; acetone:water (4:1) extract active in bioautography (*M. aurum*); oleanolic acid active (*M. tuberculosis, M. microti, M. avium, M. scrofulaceum*) (Bamuamba et al., 2008)
Canellaceae	*Warburgia salutaris* (Bertol. f.) Chiov.	Coughs, fever, chest complaints, respiratoryailments (Watt and Breyer-Brandwijk, 1962; Hutchings and Van Staden, 1994; Hutchings et al., 1996)	Bark or root bark	Drimane sesquiterpenoids including warburganal and polygodial (Watt and Breyer-Brandwijk, 1962; Clarkson et al., 2007; Madikane et al., 2007)	Dichloromethane bark extract, sesquiterpene mixture and 11 - hydroxycinnamosmolide active (*M. tuberculosis* and *M. bovis* BCG) (Clarkson et al., 2007, Madikane et al., 2007)
Chenopodiaceae	*Chenopodium ambrosioides* L.	Cough suppressant, TB (Watt and Breyer-Brandwijk, 1962; Lall and Meyer, 1999)	Aerial parts	Saponins (Watt and Breyer-Brandwijk, 1962) flavonoids, quercetin, oxalic, malic and succinic acids, triterpenoid glycosides, chenopodoside A and B, amino acids, ascaridole (Hutchings et al., 1996)	Acetone extract MIC = 0.1 mg/ml, water extract inactive (*M. tuberculosis*) (Lall and Meyer, 1999)

Clusiaceae	*Garcinia polyantha* Oliv.	Wounds, various uses (Kuete et al., 2007)	Stem bark	1,3,6,7-tetrahydroxyxanthone, 1,3,5-trihydroxyxanthone, bangangxanthone, 1,3,6,7-tetrahydroxyxanthone, 5-hydroxyflavone (Kuete et al., 2007)	Dichloromethane: methanol (1:1) stem bark extract and some isolated compounds active (*M. smegmatis, M. tuberculosis*) (Kuete et al., 2007)
Combretaceae	*Combretum imberbe* Wawra	Combretum species used for chest coughs, fever, infections (Hutchings et al., 1996)	Leaves	Pentacyclic triterpenes triterpene acids and related glycosides (Katerere et al., 2003)	Pentacyclic triterpenes MIC = 1.56–25 g/ml (*M. fortuitum*) (Katerere et al., 2003)
Ebenaceae	*Euclea natalensis A*. DC.	Respiratory chest problems, coughs, TB (Lall and Meyer, 1999)	Roots	Pentacyclic terpenoids, naphthoquinones, triterpenoids (Lall et al., 2005); diospyrin (Lall and Meyer, 2001); octahydroeuclein, 20(29)-lupene-3ß-isoferulate, shinanolone, lupeol, betulin (Weigenand et al., 2004)	Acetone and water extract MIC = 0.1 mg/ml (*M. tuberculosis*) (Lall and Meyer, 1999); isolated compounds active against *M. tuberculosis* and *M bovis* (Lall and Meyer, 2001; Weigenand et al., 2004; Lall et al., 2005)
Fabaceae	*Acacia nilotica* (L.) Willd. ex Del. subsp. kraussiana (Benth.) Brenan	Respiratory ailments, TB, coughs (Hutchings et al., 1996)	Roots, leaf, bark	Hydroxyproline, serine, dimethyl-triptamine, ß-amyrin, betulin and many other compounds in Acacia species (Hutchings et al., 1996)	Leaf, bark and root ethanol and ethyl acetate extracts active, MIC = 0.195–1.56 mg/ml (*M. aurum*) (Eldeen and Van Staden, 2007)
Geraniaceae	Pelargonium spp. (notably *P. reniforme* Curt. and *P. sidoides* DC.	TB, cough (Scott et al., 2004; Seidel and Taylor, 2004)	Tuber	Tannins and other phenolic compounds (Van Wyk et al., 1997), umckalin and related coumarins in P. reniforme tubers, essential oils, flavonoids and phytosterols (Kolodziej, 2000), fatty acids (Seidel and Taylor, 2004)	Aqueous infusions of four species inactive, *P. grossularioides* mildly active (*M. smegmatis*) in disc diffusion assay (Scott et al.,2004); unsaturated fatty acids from *P. reniforme* and *P. sidoides* active (*M. aurum, M. smegmatis, M. fortuitum, M. abscessus, M. phlei*) (Seidel and Taylor, 2004)

Lamiaceae	*Leonotis leonurus* (L.) R. Br.	Coughs, respiratory ailments (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Scott et al., 2004)	Leaves and stems	Volatile oil and diterpenoids (labdane-type lactones) for example marrubin	No activity of aqueous infusion (*M. smegmatis*) (Scott et al., 2004)
Lamiaceae	*Tetradenia riparia* (Hochst.) Codd	Respiratory ailments, coughs, TB symptoms (Hutchings et al., 1996; Lall and Meyer, 1999; Scott et al., 2004)	Leaves, roots	A diterpene diol, ibozol, and related diterpenoids, large amounts of pyrones (Van Puyvelde et al., 1979)	Root acetone and water extracts inactive (*M. tuberculosis*) (Lall and Meyer, 1999); no activity of aqueous infusion (*M. smegmatis*) (Scott et al., 2004)
Meliaceae	*Ekebergia capensis* Sparrm	Chronic coughs, respiratory chest complaints, TB (Watt and Breyer-Brandwijk, 1962; Lall and Meyer, 1999)	Roots, leaves, bark	Unknown (Van Wyk et al., 1997)	Acetone extract of bark MIC = 0.1 mg/ml, water extract inactive (*M. tuberculosis*) (Lall and Meyer, 1999)
Myrtaceae	*Syzygium gerrardii* (Harv. ex Hook. f.)	TB, coughs, chest pains (Watt and Breyer-Brandwijk, 1962; Mativandlela et al., 2008)	Bark	Tannin (Watt and Breyer-Brandwijk, 1962)	Ethanol extract of leaves MIC = 6.25 mg/ml (*M. smegmatis*), not active (*M. tuberculosis*) (Mativandlela et al., 2008)
Rhamnaceae	*Ziziphus mucronata* Willd.	Coughs, chest ailments (Watt and Breyer-Brandwijk, 1962;	Bark	Alkaloids	Ethanol extract of leaves not active (M. smegmatis and *M. tuberculosis*) (Mativandlela et al., 2008)

Rosaceae	*Prunus africana* (Hook. f.) Kalkm.	Chest pain, fever (Mativandlela et al., 2008)	Leaf and bark	B-sitosterol, terpenoids and various other compounds (Van Wyk et al., 1997) ethyl acetate and dichloromethane extracts active, MIC = 0.78–6.25 mg/ml (Mycobacterium aurum) (Eldeen and Van Staden, 2007)	Ethanol extract of leaves not active (*M. smegmatis* and *M. tuberculosis*) (Mativandlela et al., 2008)
Sapindaceae	*Dodonaea angustifolia* L.f.	TB, chest pains, fever (Watt and Breyer-Brandwijk, 1962; Thring et al., 2007; Mativandlela et al., 2008)	Leaves	Dodonic acid, hautriwaic acid and diterpenoids (Sachev and Kulshreshtha, 1984)	Aqueous decoctions and infusions of leaves and stems and ethyl acetate extract MIC = 5 mg/ml; ethanol and methanol extracts MIC = 1.25 mg/ml (*M. smegmatis*) (Thring et al., 2007) Ethanol extract of leaves MIC = 3.13mg/ml (*M. smegmatis*), MIC = 5 mg/ml (*M. tuberculosis*) (Mativandlela et al.,2008).

2.10.1 Natural Antimycobacterial Metabolites

Twenty-three naturally occurring alkaloids and analogs have been found to possess antimycobacterial activities (Newton et al., 2000). The carbazoles obtained from the rhizomes and roots of *Clausena excavata* (Rutaceae) and the other indole alkaloid has modest activity against MTB. The indoloquinoline alkaloid has significant activity against *M. fortuitum* and *M. bovis* (Gillespie et al., 2001). The benzoxazole alkaloids which contain the oxazole moiety found in the synthetic oxazolidinones are strong inhibitors of MTB (Cynamon et al., 1999). The oxazole alkaloid texalin (Figure 8) is significantly active against MTB and two other related species, *M. kansasii* and *M. avium* (Rastogi et al., 1998). The iminium salts demonstrate activity against *M. avium, M. bovis* BCG and *M. smegmatis* and it is believed that the iminium ion improves the lipophilicity and increases the bioavailability of the alkaloids to the organisms. Synthetic analogs of the naturally occurring cleistopholine isolated from *Cleistopholis patens*; and sampangine isolated from *Cananga odorata*, from the Annonaceae family when tested against the AIDS pathogen *M. intracellularae* recorded MICs close to the control drug rifampin (Okunade et al., 2004).

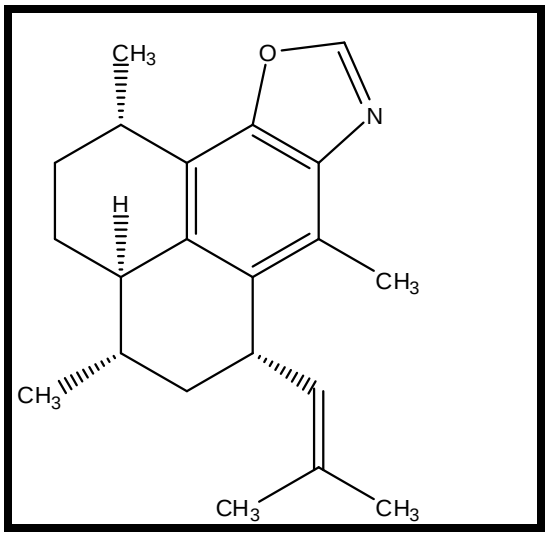


Figure 8. Pseudopteroxazole isolated from *Pseudopterogorgia elisabethae* (Rodrıguez and Rodrıguez, 2003).

Flavonols isolated from *Haplopappus sonoriensis* (Asteraceae) and flavone from *Lysionotus pauciflorus* (Gesneriaceae) are believed to be the active compounds from these plants against *M. tuberculosis* (Murillo et al., 2003). Coumarins are modestly active against *M. tuberculosis* and it is believed that the prenyl at the C-8 position is essential for the antimycobacterial activity. Ostruthin from *Peuceda numostrothium* (Apiaceae) with the prenyl group at C-6 demonstrates significant activity against different strains of the rapidly growing mycobacteria, *M. aurum, M. fortuitum*, *M. phlei* and *M. smegmatis*, with MICs close to those obtained for isoniazid. 7-Hydroxycoumarin is only weakly active against *M. fortuitum* (Schinkovitz et al., 2003). The anthraquinone chromanone exhibits modest activity against mycobacteria in comparison to isoniazid and kanamycin (Kanokmedhakul et al., 2002). *M. avium* and *M. bovis* are strongly inhibited by licochalcone (Figure 9) obtained from *Glycyrrhiza inflata* (Fabaceae).

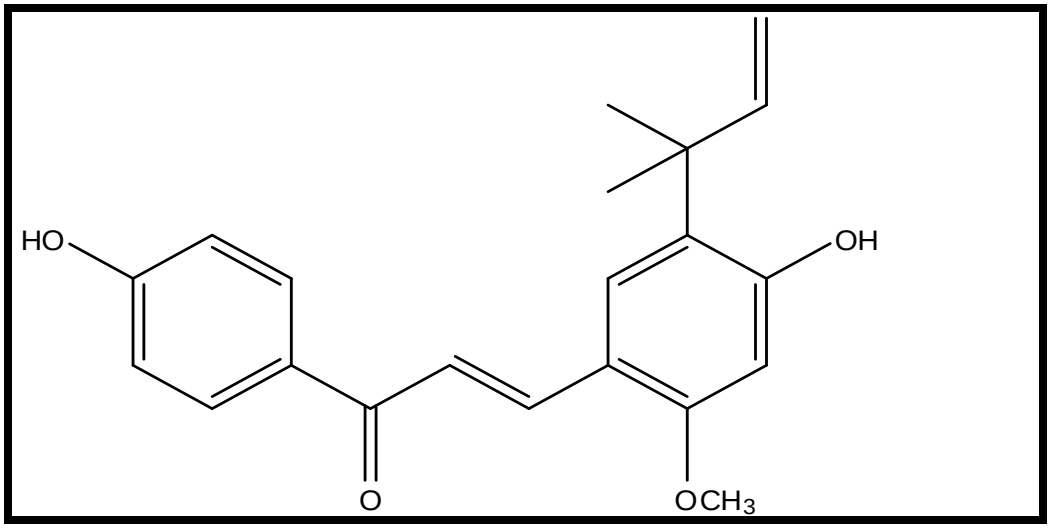


Figure 9. Licochalcone A isolated from *Glycyrrhiza inflata* (Friis-Moller et al., 2002).

One hundred and eighteen synthetic and natural plant terpenoids were reported to have moderate to high antimycobacterial activity against *M. tuberculosis* (Cantrell et al., 2001). The nor-diterpenoid 12-demethylmulticauline has an MIC comparable with

rifampin and even less than that for ethambutol. The serulatane diterpenoids obtained from *Pseudopterogorgia elisabethae*, as well as thiocarbamate, from *Smenospongia aurea*, are significantly active against *M. tuberculosis* (Okunade et al., 2004). The secokauranes (Figure 10) from *Croton kongensis* (Euphorbiaceae) also show significant activity (Thongtan et al., 2003). Totarol from the outer bark of *Xanthocyparis nootkatensis* (Cupressaceae) is reported to have activity against *M. tuberculosis* (Constantine et al., 2001). 8 diterpenes and 3 triterpenes from *Calceolaria pinnifolia* (Scrophulariaceae) were reported to have antimycobacterial activity against *M. tuberculosis* (Woldemichael et al., 2003).

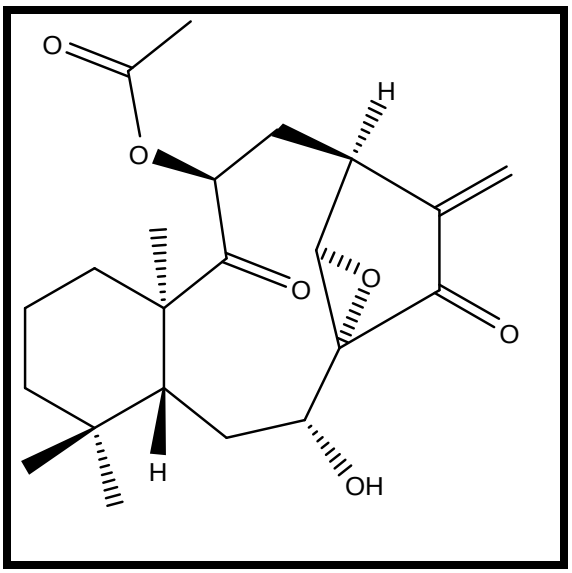


Figure 10. Secokauranes isolated from *Croton kongensis* (Thongtan et al., 2003).

Epidioxysterol (Figure 11) obtained from *Morinda citrifolia* (Rubiaceae) showed significant activity against *M. tuberculosis* (Saludes et al., 2002). Saponins obtained from *Eunicea pinta*, as well as jujubogenin from *Colubrina retusa* (Rhamnaceae) are significantly active while the seco-steroids phasalins from *Physalis angulate* (Solanaceae) are moderately active against *M. tuberculosis* (Okunade et al., 2004).

Sterols from *Ruprechtia triflora* (Polygonaceae) have moderate to very good antimycobacterial activity against *M. tuberculosis* (Woldemichael et al., 2003).

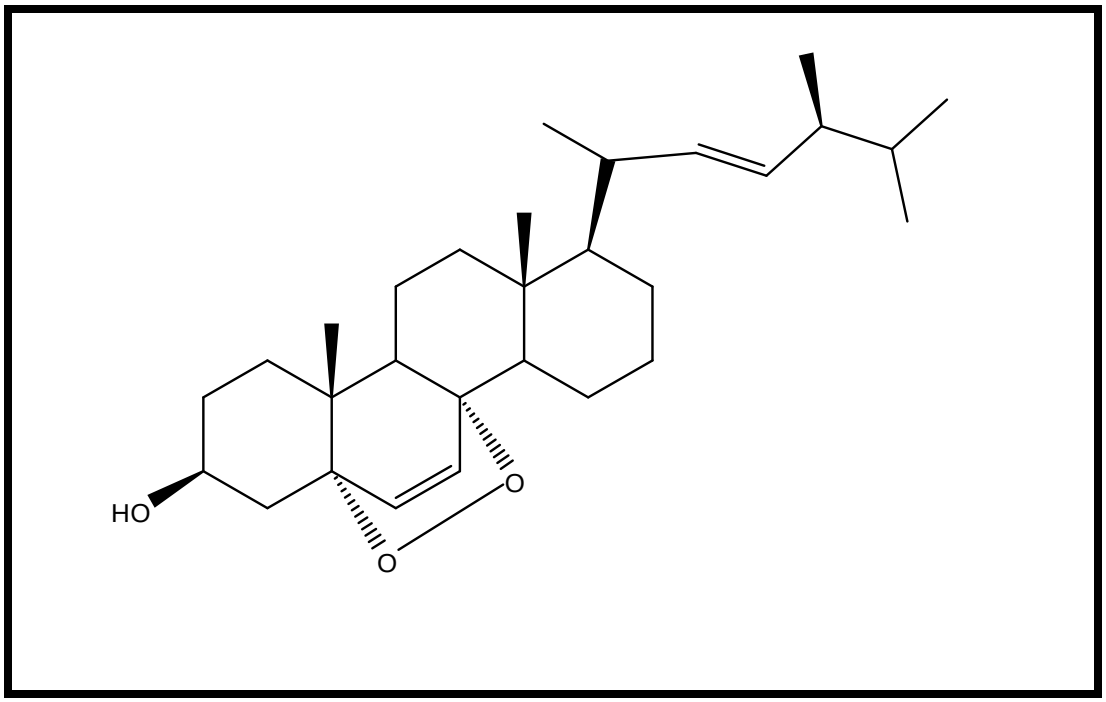


Figure 11: Epidioxysterol isolated from *Morinda citrifolia* (Saludes et al., 2002).

The benzenoid compounds of the type shown in Figure 12 were found to be over 80% effective against mycobacteria (Okunade et al., 2004). Bakuchiol isolated from *Psoralea corylifolia* (Fabaceae) was reported to have significant activity against *M. aurum* and *M. bovis* BCG. The preussomerins have high to moderate antimycobacterial activity against *M. tuberculosis* in comparison to kanamycin (Okunade et al., 2004).

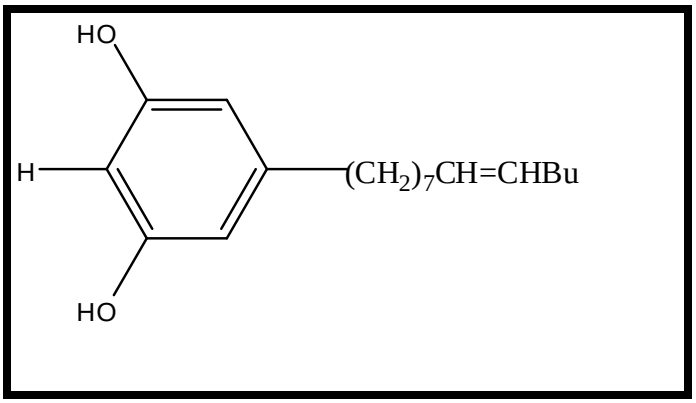


Figure 12. Hydroxybenzene analog isolated from *Ardisia japonica* (Huang et al., 1980).

Cyclodepsipeptides, for example, Hirsutellide, shown in Figure 13 have moderate to high antimycobacterial activity against *M. tuberculosis* (Jackson et al., 2000).

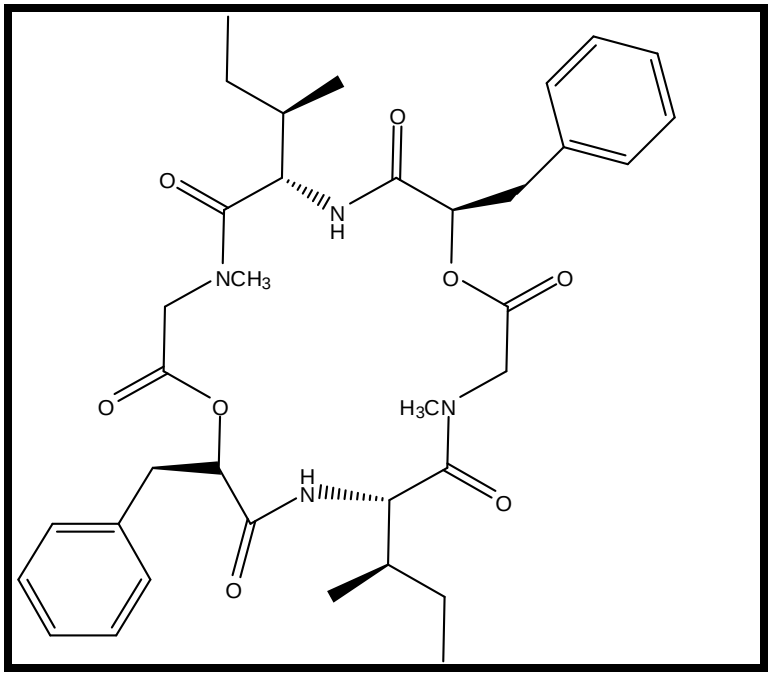


Figure 13: Hirsutellide isolated from *Hirsutella kobayasii* (Vongvanich et al., 2002).

2.11 Plants Used in this Research for Antimycobacterial Activity

2.11.1 *Buddleja saligna*

Description

The young stems of *B. saligna* are four angled and covered with white velvety hairs however the older stems and trunks are creamy or grey to dark brown and fluted with bark peeling in strips. The leaves are long, narrow and textured. The leaf upper surface is hairless and dark green however under the leave is whitish with raised venation. The flowers are tiny, creamy white and have dense sprays at the end of each branch (Van Wyk and Van Wyk, 1997).

Figure 14. *Buddleja saligna* at the Steenbok Nature Reserve.

Nomenclature

Family: BUDDLEJACEAE

Common Name: False-olive, wild elder, bastard olive, olive-sagewood (Zimbabwe) (English); witolienhout , basterolienhout (Afrikaans); motlhwaretshogwana (Tswana); umgqeba (Xhosa); igqeba-elimhlohe, umceba (Zulu); mothlware (Kwena)

Chemical constituents: According to Houghton (1985) there is little in published literature dealing with the chemistry of the plant.

Habitat

B. saligna is endemic to South Africa where it has a wide distribution (Figure 14). It occurs most often in ravines and against outcrops and is distributed from coastal elevations to the central plateau at elevations of < 2000 m (Leeuwenberg, 1979).

Nutritional and Medicinal Uses

The wild olives are used to lower blood pressure in many parts of the world. The bark and leaf decoctions, in southern Africa, are used to treat colic, coughs, colds, sore eyes, urinary problems and as purgatives (Van Wyk and Gericke, 2000). The dichloromethane extract of the leaves of this plant has shown some antimutagenic property (Reid et al., 2006).

2.11.2 *Capparis tomentosa*

Description

C. tomentosa is a robust woody climber which in riverine vegetation may grow to the top of the canopy (Hyde and Wursten, 2010). The stem has sharp, paired, hooked spines and young stems and spines are covered in dense velvety yellow hairs (Pooley, 1993). The leaves are formed between the spines and are alternate, oblong to broadly elliptic, greyish-olive-green and covered in velvet hairs or smooth (Hyde and Wursten, 2010). The petiole is long and velvety (Pooley, 1993). The flowers form in clusters. They are large and scented, with a mass of pinkish-white stamens and may be crimson coloured at the base. The petals are whitish (Hyde and Wursten, 2010). The fruits hang from a long stalk-like branch called gynophores and contain many seeds embedded in a pinkish flesh (Pooley, 1993)

Figure 15. *Capparis tomentosa* growing as a shrub in Amanzimtoti, Kwa-Zulu Natal, South Africa.

Nomenclature

Family: CAPPARACEAE

Common Names: Woolly Caper Bush (English); Wollerige Kapperbos (Afrikaans); Iqwaningi, Umabusane, Inkunzi, Ebomvu and Ukokwane (Zulu); Gwambadzi and Mubadai (Venda); Gamobor (Shona); Mbada paka (Swahilli).

Chemical Constituents: 2.4-ethylcholestan-5-en-3-ol (phytosterol) and N- benzoyl-phenylalanylaninol acetate (dipeptide derivative) (Akoto O. et al., 2008); alkaloid, 1-stachyhydrin (Mishra et al., 2006)

Habitat

C. tomentosa is found in the bushveld and forest (Figure 15) from the Eastern Cape, Kwa-Zulu Natal, Limpopo Province and Mpumalanga in South Africa as well as Mozambique, Botswana, Namibia and Tropical Africa (Pooley, 1993).

Nutritional and Medicinal Uses

Alkaloid, 1-stachyhydrin obtained from seeds, roots bark, flowers, fruits husk and dry fruits of *C. tomentosa* exhibited in vivo antimycobacterial property. The role of this compound has been assigned to increasing blood coagulation, thus shortening bleeding time and blood loss (Mishra et al., 2006). According to Steenkamp et al. (2004), *C. tomentosa* plant extracts showed antimicrobial activity against *Staphylococcus aureus*, *Streptococcus pyogenes, E. coli* and *Pseudomonas aerugenosa.*

2.11.3 *Carpobrotus dimidiatus*

Description

C. dimidiatus is a robust plant with fleshy, green leaves. It may spread over large areas to form a tough ground cover resistant to drought and salt spray. It has large, shiny, mauve flowers which are borne singly on erect flowering branches. The flowers are sharply two-ridged at the base. The fruits have fleshy walls which become dry and tough and never open. The seeds are dispersed by animals which eat the fruits (Pooley, 1998).

Figure 16. *Carpobrotus dimidiatus* at the Kwa-Zulu Natal Botanical Gardens.

Nomenclature

Family: MESEMBRYANTHEMACEAE

Common Names: Natal sour figs (English); ikhambi-lamabulawo, umgongozi (Zulu); gaukum, ghaukum (Khoi)

Chemical Constituents: rutin, neohesperidin, hyperoside, catechin and ferulic acid (Springfield and Weitz, 2006)

Habitat

Natural habitat is the coast of KwaZulu-Natal, South Africa, also stretching south from the Eastern Cape and north into Mozambique (Figure 16).

Nutritional and Medicinal Uses

The plant is regarded as a good source of catechol tannin (Watt and Breyer-Brandwijk, 1962). The leaf juice is astringent and mildly antiseptic and used to treat dysentery and stomach spasms. It is used as a gargle to relieve laryngitis and throat infections (Van Wyk et al., 2002). It is used for skin ailments such as burns, grazes and sunburn, ringworm, eczema, herpes, cold sores and chafing skin (Van Wyk and Gericke, 2003); and soothing blue bottle stings (Watt and Breyer-Brandwijk, 1962)

2.11.4 *Dichrostachys cinerea*

Description

D. cinerea is a tree which grows up to 8 m in height. It has an untidy growth pattern with thorny branches. It forms impenetrable thickets in overgrazed bushveld (Van Wyk and Gericke, 2003). The flowers hang in clusters from branches. There are bright pink fertile flowers in the upper portions of the cluster and sterile, bright yellow flowers are found at the bottom. The curved and coiled seedpods are borne in clusters on long stalks

Figure 17. *Dichrostachys cinerea* cultivated in Constantia Kloof, Roodepoort.

Nomenclature

Family: FABACEAE

Common Name: Sickle bush, Bell mimosa, Marabou-Thorn, Kalahari Christmas tree and Chinese lantern tree (English); Sekelbos (Afrikaans); Ugagane, Umthezane and Umzilazembe (isiZulu); acacia Saint Domingue (French); el marabu (Cuba); Kalahari-Weihnachtsbaum (German)

Chemical Constituents: catechin, quinolizidine alkaloids, including cystisine and sparteine isoflavonoids and coumarin (Raven et al., 1999)

Habitat

D. cinerea is distributed throughout South Africa where it has now intruded upon many overgrown bushvelds regions (Figure 17) (Van Wyk and Gericke, 2003).

Nutritional and Medicinal Uses

The bark is used to treat headaches, toothaches, dysentery, and elephantiasis. Root infusions are taken for leprosy, as an anthelmintic, purgative and strong diuretic (Orwa

et al., 2009). The roots and leaves are used to treat epilepsy, and placed on the sites of snakebites and scorpion stings. The leaves, which are believed to produce a local as a remedy for sore eyes and toothache. Leaves are taken as a diuretic and laxative, and used for gonorrhoea and boils (Orwa et al., 2009). Powder from leaves is used in the massage of fractures (Orwa et al., 2009). The roots are sniffed to stop nose bleeds (Van Wyk and Gericke, 2003). The leaves and roots are smoked to relieve head colds, to treat TB and for the treatment of epilepsy (Van Wyk and Gericke, 2003). As they are rich in nutrients, the plants are often used as fertiliser, particularly in the Sahel region of Africa along riverbanks (Vogt, 1995).

2.11.5 *Ekerbergia capensis*

Description

E. capensis is a tree that grows to a 10-12 m. The bark is grey in colour with a rough texture. It has compound leaves with 7 or 9 oblong leaflets. The flower is pink to white and forms round reddish berries in the summer.

Figure 18. *Ekerbergia capensis* at the Kumbula Indigenous Nursery, in the Eastern Cape.

Nomenclature

Family: MELIACEAE

Common Name: Cape ash (English); umnyamathi (isiZulu)

Chemical Constituents: limonoid ekebergin (Van Wyk et al., 2002)

Habitat

E. capensis range extends from the Eastern Cape of South Africa to Sudan and Ethiopia (Figure 18).

Nutritional and Medicinal Uses

The bark is used in traditional medicine to treat dysentery and heartburn (Watt and Breyer-Brandwijk, 1962). Mulaudzi et al. (2011) stated that the leaves are used to treat venereal diseases, chronic cough, dysentery and skin diseases. Roots are used to treat a chronic cough and scabies (Hutchings, 1996).

2.11.6 *Ficus sur*

Description

F. sur is an evergreen tree, reaching up to 35 m in height. It has large, oval, green leaves borne on a massive, spreading crown. Figs are produced from September to March. They are borne in large clusters mostly low down on the trunk and can even appear at ground level arising from the roots.

Figure 19. *Ficus sur* at Kirstenbosch National Botanical Gardens.

Nomenclature

Family: MORACEAE

Common Name: Broom cluster fig (English); Besem-trosvy (Afrikaans); Mogo-tshetlo (North Sotho); Umkhiwane (Xhosa); Umkhiwane (Zulu)

Chemical Constituents: pentacyclic triterpenoids (Kunle et al., 1999)

Habitat

F. sur is found on riverbanks, riverine forest and woodlands (Figure 19). This species is distributed from North Africa to the Western Cape in South Africa.

Nutritional and Medicinal Uses

The milky latex found in live growth is used to treat lung and throat problems and administered to cows with poor milk production. The tree is used as a magical cure for boils. The root is used to assist when a cow retains part of the placenta after giving birth (Von Breitenbach et al., 2001). According to Kunle et al. (1999), the Zulus consume a preparation of the root and bark for pulmonary TB. In West Africa, diseases of the eye are treated with the leaves of this plant. The latex is used in Zaire for the treatment of burns. In the northern part of Nigeria, the fresh root along with the inner bark is chewed to treat a sore throat.

2.11.7 *Gunnera perpensa*

Description

G. perpensa is a robust, perennial, erect herb up to 1 m tall that always grows near water. The leaves are large, dark bluish green, kidney-shaped and covered with hairs on both surfaces. The margin of the leaves is irregularly toothed. The veins are very noticeable on the lower surface of the leaf. The petioles vary in length from 150 to 750 mm. The flowers are tiny and pinkish reddish brown, borne on a long slender spike, which is taller than the leaves. On each spike there will be female flowers at the base,

male flowers at the top and bisexual flowers in the middle, flowering between September and February (Mendes, 1978).

Figure 20. *Gunnera perpensa* in Pledge Nature Reserve in Knysna, South Africa.

Nomenclature

Family: GUNNERACEAE

Common Names: River pumpkin, Wild rhubarb (English); Rivierpampoen, Wilde ramenas (Afrikaans); Qobo (Sotho); uQobho (Swati); Rambola-Vhadzimu, Shambola-Vhadzimu (Venda); iPhuzi lomlambo, iGhobo (Xhosa); uGobhe, uGobho (Zulu)

Chemical Constituents: Z-venusol, a phenylpropanoid glucoside (Khan et al., 2004)

Habitat

G. perpensa is an obligate wetland plant that grows in shallow water around the edge of pools in marshy areas or along streams (Figure 20). Naturally occurring in central and Southern Africa, Madagascar, New Zealand, Tasmania, Indonesia, the Philippines,

Hawaii, Mexico, Central and South America. It is widespread in tropical Africa from Sudan, Ethiopia, Zaire, Rwanda, Uganda, Kenya, Tanzania, Zimbabwe and Mozambique, extending along the central and eastern areas of Southern Africa down to the Western Cape, including Swaziland and Lesotho (Bergman et al., 1992, Mendes, 1978).

Nutritional and Medicinal Uses

Decoctions of the plant are taken orally to relieve dysuria, rheumatic pains and dyspepsia, as a stomachic, or for colds. Externally it is used as a wound dressing. Infusions may be taken internally or applied externally to treat psoriasis. Root decoctions are used in traditional gynaecological practice as well as traditional veterinary practice to initiate labour, assist delivery or to expel the placenta (Veale et al., 1992, Van Wyk and Gericke, 2000).

2.11.8 *Leonotis leonurus*

Description

L. leonurus is a robust shrub which grows up to 2-3m tall and 1.5m wide. Stems are velvety and woody at the base. The leaves are long, narrow, rough above, velvety below, with serrated edges. The wild dagga flowers profusely in autumn with its characteristic bright orange flowers carried in compact clusters in whorls along the flower stalk. Apricot and creamy white flowered forms are also found.

Figure 21**.** *Leonotis leonurus* growing in North Riding, Randburg in South Africa.

Nomenclature

Family: LAMIACEAE

Common Names: Wild Dagga, Lion's Ear (English); Wildedagga, Duiwelstabak (Afrikaans); Imvovo (Xhosa)

Chemical Constituents: Diterpenoid labdane lactones (Laonigro et al., 1979)

Habitat

L. leonurus is common and widespread throughout South Africa and grows amongst rocks in grassland (Figure 21).

Nutritional and Medicinal Uses

It is widely used in traditional medicine to treat fevers, headaches, coughs and dysentery. It is also used as a remedy for snakebite and as a charm to keep snakes away. The KhoiKhoi used it as tobacco and presented it to settlers as a remedy to help

alleviate chest ailments (Malan and Notten, 2006). The Zulus, Xhosas and Khoikhoi people made a tea with the flowers to sooth a cough and as a cold remedy. It is also used to treat jaundice, cardiac problems, asthma, haemorrhoids, headaches, chest ailments, bronchitis and epilepsy (Van Wyk and Gericke, 2003, Van Wyk et al., 2002). Ojewole in 2005 performed a study that showed that the aqueous leaf extract of *L. leonurus* possessed antinociceptive, anti-inflammatory, and hypoglycemic properties.

2.11.9 *Tetradenia riparia*

Description

T. riparia is a tall, aromatic shrub up to 3 m in height. It is slightly succulent and has an irregular branch pattern. The stems are brown and smooth, except for the younger portions which are covered with glandular hairs and have a ruby tinge. The glandular hairs also cover both surfaces of the leaves and make them slightly sticky to the touch. The leaves spiked and bright green and are slightly heart shaped with the margin irregularly and bluntly toothed.

Figure 22. *Tetradenia riparia* at the Kumbula Indigenous Nursery, in the Eastern Cape.

Nomenclature

Family: LAMIACEAE

Common Name: Misty Plume Bush, Ginger Bush (English); Gemmerbos, Watersalie (Afrikaans); iBoza, iBozane (Zulu)

Chemical Constituents: ibozol (Zelnik et al., 1978), 7 α-hydroxyroyleanone, 8 (14), 15-sandaracopimaradiene-7α,18-diol (Van Puyvelde et al., 1987), umuravumbolide (Van Puyvelde, 1979), tetradenolide (Van Puyvelde and de Kimpe, 1998).

Habitat

The natural habitat of *T. riparia* is along river banks, forest margins, dry wooded valleys and hillsides in areas where there is little frost (Figure 22). The natural distribution ranges from KwaZulu-Natal, Northern Province, Mpumalanga in South Africa to Swaziland, Namibia, Angola and northwards through tropical East Africa into Ethiopia.

Nutritional and Medicinal Uses

The Zulu people have many uses for this plant which include the relief of chest complaints, stomach ache and malaria. Inhaling the scent of the crushed leaves apparently also relieves headaches. Hutchings in 1996 stated that the plant extracts are used to treat respiratory ailments such as coughs, colds, sore throat and mouth ulcers and Watt and Breyer-Brandwijk (1962) reported that the leaves are used to treat stomach ache, diarrhoea, influenza, fever and malaria.

CHAPTER 3: METHODOLOGY

3.1 Plant Collection and Extraction

3.1.1 Plant Collection

The leaves of *B. saligna, C. tomentosa, C. dimidiatus, D. cinerea*, *E. capensis, F. sur, G. perpensa, L. leonurus* and *T. riparia* were authenticated and supplied by Prof H. Baijnath. The plants scientific name, family name, common name, and parts that were used are listed in Table 4. The leaves were separated and washed several times with distilled water until no foreign material remained and air dried for 24 hours. Thereafter, they were dried in an oven (Memmert, South Africa) at 25°C for 7 days. The dried leaves were powdered using an industrial grinder (Retsch Gmbh, West Germany) and stored in Schott bottles in a dark room until required for extraction.

Table 4. Biodata of plants used in this study

Scientific Name	Family Name	Common Name	Location Source	Part Used
B. saligna	Buddlejaceae	False olive	Reservoir hills	Leaf
C. tomentosa	Capparaceae	Woody capper bush	Reservoir hills	Leaf
C. dimidiatus	Mesembryanthemaceae	Natal sour figs	Park Rynie Beach	Leaf
D. cinerea	Fabaceae	Sickle bush	Reservoir hills	Leaf
E. capensis	Meliaceae	Cape ash	Reservoir hills	Leaf
F. sur	Moraceae	Broom cluster figs	Reservoir hills	Leaf
G. perpensa	Gunneraceae	River pumpkin	Reservoir hills	Leaf
L. leonurus	Lamiaceae	Wild dagga	Shongweni Dam	Leaf
T. riparia	Lamiaceae	Ginger bush	Reservoir hills	Leaf

3.1.2 Plant Extraction

The yield per 100 g of plant material was calculated using the following equation:

$$\text{Amount of dry extract per 100 g} = \frac{\text{(Weight of dried crude extract)}}{\text{(Weight of biomass)}} \times 100$$

3.1.2.1 Aqueous Extraction

Leaves (20 g) were powdered and heated in distilled water to boiling point before filtration by gravity using Whatman No. 1 filter paper. The filtrate was then concentrated using a biofreezer (Snijders Scientific, Holland) at -70°C and lyophilized (Virtis Benchtop Freeze Dryer). The lyophilized material was used as a stock and working solutions were prepared for appropriate applications.

3.1.2.2 Methanolic Extraction

The powdered dried leaves were extracted using 80% methanol. The filtrate was concentrated using a Buchi RE Rotoevaporator including a Buchi 461 water bath set at a temperature of 50°C. The concentrate was placed in a biofreezer and lyophilized using a Virtis Benchtop Freeze Dryer. Aliquots were prepared from the dried crude extract and dissolved in solvents depending on the experimental protocol.

3.2 Antimycobacterial Activity of the Plant Extracts

3.2.1 Mycobacterial strains and isolates

The following strains used in this study were obtained from the Department of Microbiology, NHLS laboratory based at the Inkosi Albert Luthuli Hospital in Durban, South Africa: reference drug susceptible strain *M. tuberculosis* (H_{37}Rv ATCC 25177) as a control, well characterized clinical isolates of MDR-TB and XDR-TB; and fast growing *M. smegmatis.* The purity of the mycobacterial strains were checked by Ziehl-Neelsen (ZN) Acid Fast staining before used in any antimycobacterial assays. Susceptibility profile of the clinical isolates against standard antimycobacterial drugs was as follows: MDR-TB resistant to RIF (rifampicin) and INH (isoniazid); XDR-TB resistant to RIF, INH, KAN (kanamycin) and OFLX (Ofloxacin).

3.2.2 Preliminary Sensitivity Test

The plant extracts (100 mg) were dissolved in 1 ml of distilled water and 1 ml of each of the plant extracts was introduced in 100 ml of prepared molten Middlebrook 7H11 agar base. Middlebrook 7H11 (Fluka M0178) agar containing, glycerol (Fluka 49769) and Oleic Albumin Dextrose Catalase (OADC) growth supplement (Fluka M0553) were mixed in a bottle containing distilled water. The agar and plant extracts were mixed and aseptically poured into Petri plates with quadrants and then allowed to solidify at room temperature. The plates were then inoculated with *M. smegmatis* and *M. tuberculosis* H_{37}Rv (ATCC 25177) and incubated at 37°C for 72 hours and 21 days, respectively. Well characterized clinical isolates of MDR-TB and XDR-TB were aseptically inoculated, sealed and incubated at 37°C for 21 days.

3.2.3 Broth Microdilution Assay

The two-fold microdilution method was used with minor modifications to determine the MIC values of the active plant extracts against *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$ (ATCC 25177) in sterile flat-bottomed 96 well microtiter plates (Eloff, 1998). Bacterial inoculums were prepared from five days old cultures in Middlebrook 7H9 broth (Middlebrook 7H9 (Fluka M0178) broth containing glycerol (Fluka 49769) and Middlebrook OADC growth supplement). The turbidity was adjusted to the equivalent of McFarland No 1 ($3x10^8$ CFU/ml). The concentration of the stock solution of all test plant extracts before serial dilutions was 2 mg/ml. The plant extracts were serially diluted two folds with a broth base containing glycerol (Fluka 49769), and Middlebrook OADC and growth supplement (Fluka M0553). The serial dilution was performed by addition of 100 µl of extracts into the first well which had 100 µl of broth base, and thereafter mixed well and transferred 100 µl of the first well sample-broth base mixture to next and subsequent wells of each row. This was followed by the separate inoculation of 100 µl of mycobacterial cultures in each well. Additional wells were used as growth controls (positive and negative controls with no antibiotics). A row with inoculums and rifampicin or isoniazid were used as a positive control. The inoculated microtiter plate containing *M. smegmatis* was incubated at 37°C for 72 hours. To determine the MIC values of the active plant extracts for *M. smegmatis*, 50 µl (0.2 mg/ml) Indophenyl Nitrophenyl Tetrazolium (INT) chloride salt was added to each well and plates incubated at 37°C for 24 hours (Von Breitenbach et al., 2001). Bacterial growth in the wells was indicated by a change in colour, whereas clear wells indicated inhibition by the extracts. The inoculated microtiter plates containing *M. tuberculosis* $H_{37}Rv$ (ATCC 25177) and the well characterized clinical isolates MDR-TB and XDR-TB were incubated at 37°C for 5 days. To determine the MIC values of the active plant extracts for *M. tuberculosis* $H_{37}Rv$ (ATCC 25177), MDR-TB and XDR-TB, 50 µl (1 mg/ml) of freshly prepared reagent mixture 7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (Resazurin) was added to each well and plates re-incubated at 37°C for 24 hours. A colour change from blue to pink

indicated growth and the minimum inhibitory concentration (MIC) was interpreted as the lowest extract concentration which prevented the colour change from blue to pink.

3.3 Safety Analysis of the Active Plant Extracts

3.3.1 Brine Shrimp Lethality Test

The brine shrimp lethality test (BST) was used to predict the presence of toxicity in the plant extracts (Meyer et al., 1982). The experiment was carried out according to Barnighausen et al. (2008).

3.3.1.1 Hatching the Shrimp

Brine shrimp eggs were obtained from Natures Petland in Durban, South Africa. Filtered, artificial seawater was prepared by dissolving 38 g of sea salt in 1 L of distilled water for hatching the shrimp eggs. The seawater was put in a hatching chamber with a partition for dark (covered) and light areas in a small plastic container. Shrimp eggs were added to the dark side of the chamber while the lamp above the other side (light) attracted the hatched shrimp. Two days were allowed for the shrimp to hatch and mature into nauplii (larvae).

3.3.1.2 Bioassay

Solutions of the active plant extracts were made in distilled water, at varying concentrations (50 µg/ml, 100 µg/ml and 1000 µg/ml) and incubated in triplicate vials with the brine shrimp larvae. The volume was then adjusted to 5 ml with artificial sea water. Ten brine shrimp larvae were then placed in each of the triplicate vials. Others were placed in artificial sea water to serve as a negative control. DEET, an organophosphate, was used as a positive control. After 24 hours the nauplii were

examined against a lighted background, with a magnifying glass and the average number of survived larvae was determined. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number and then multiplied by 100%. This served to ensure that the mortality of the nauplii is attributed to the bioactive compounds present in the plant extracts. Data was analyzed by using Microsoft Excel.

3.3.2 Cytotoxicity Screening of the Active Plant Extracts

3.3.2.1 Peripheral Blood Mononuclear Cells (PBMCs)

3.3.2.1.1 Isolation of Monocytes from Whole Blood

The collection, separation and isolation of peripheral blood mononuclear cells (PBMCs) was based on the methods described by Boyum (1968) with modifications.

All procedures were carried out under aseptic conditions. 50 ml of buffy coat sample was obtained from the South African National Blood Services. The buffy coat sample was diluted with an equal volume of prewarmed Hanks balanced salts solution (HBSS) (Bioscience). This mixture was carefully layered onto prewarmed Histopaque 1077 (Sigma) in a ratio of 5:4 in a sterile 15 ml centrifuge tubes (Greiner). The tubes were transferred to a Beckman centrifuge and spun at room temperature at 2000 rpm for 30 minutes. Using a sterile Pasteur pipette, the mononuclear cells were carefully aspirated from the interface between the HBSS and the Histopaque and transferred to a sterile centrifuge tube. The mononuclear cells were washed two times in HBSS and centrifuged each time for 15 minutes at 1200 rpm at room temperature to remove any residual Histopaque. The cell pellets were resuspended in 1 ml RPMI 1640 (Bioscience) supplemented with 2 mM -glutamine and 10% unheated fetal calf serum and 100 IU/ml penicillin and 100 Ig/ml streptomycin until further use.

3.3.2.2 Mouse BALB/C Monocyte- Macrophage (Line J774.2)

3.3.2.2.1 Cell Line

The macrophage cell line was received in an active state from Dr Celia Snyders at the University of Kwa-Zulu Natal in Pietermaritzburg, South Africa and incubated at 37°C in a humidified incubator (Snijders Hepa, United Scientific group, Cape Town, South Africa) with 5% CO_2. When cells were 80% confluent, they were sub-cultured, and stock cultures were stored in a biofreezer (Snijders Scientific, Holland) at -70°C until required.

3.3.2.2.2 Storage of Cells

Cells were stored according to protocols of (Freshney, 1987).

After trypsinization, the cells were pelleted and washed twice with pre-warmed phosphate buffered saline, pH 7.2 (PBS) and resuspended in 0.5 ml fetal calf bovine serum (FCS) and cooled on ice. A 20% DMSO in Dulbecco's Modified Eagle's Medium (DMEM) (v/v 1:4) solution was prepared as the cryoprotecting agent and also placed on ice. Equal aliquots (0.5 ml) of the cell suspension and the cryoprotective agent were added to a cryovial (Corning, South Africa). The vials were transferred to “Mr Frosty” and kept overnight at -20°C. Cells were subsequently transferred to a -70°C bio-freezer and stored until required.

3.3.2.2.3 Cell Maintenance

The maintenance of the cells was performed according to protocols obtained from Freshney (1987). All cell culture procedures were carried out in a laminar flow cabinet containing UV light (Scientific Engineering INC) and were swabbed/sterilized with 70% ethanol (Merck, South Africa) before each use. The cells were grown aseptically in 75 cm^3 tissue culture flasks (Greiner, Germany) using filter sterilized (0.22 mm) 10% Complete Culture Medium (CCM), which comprised DMEM, containing 10% fetal calf

serum and supplemented with antibiotics (penicillin, 10 000 U/ml, streptomycin sulphate, 10 000 U/ml). Cells were incubated in a 5% CO_2 humidified incubator at 37°C and passaged every month.

The culture flasks were examined for colour changes and turbidity of the media every two days, which determined the frequency of media changes. The culture flasks were also examined under an inverted microscope (Nikon) for cell growth. The cells were harvested by trypsinization when the culture was 80% confluent and divided into two separate flasks, more media was then added to each culture flask and incubated at 37°C in a 5% CO_2 humidified incubator until required for experimental use.

3.3.2.3 Trypan Blue Exclusion Assay

Cell viability was determined using the trypan blue dye exclusion method (Patterson, 1979). This test is based on the ability of an intact cell membrane to exclude certain dyes such as safranin, eosin, Congo red, alcian blue nigrosin and trypan blue.

Cell suspensions were prepared by diluting 10 µl of cell suspension and 10 µl of 0.2% trypan blue dye (Biowhittaker, Walkersville (USA)). After 5 minutes, a drop of suspension was placed on a haemocytometer, covered with a coverslip and examined by a light microscope at x400 magnification. Colourless cells were considered to be viable, whilst the blue cells were regarded as non-viable. The cells were harvested if the dye exclusion test showed a viability count of greater than 80%.

3.3.2.4 MTT Assay

The cytotoxic effect of the active plant extracts on mouse BALB/C monocyte-macrophage (J774.2) and peripheral blood mononuclear cells (PBMCs) was measured by microculture tetrazolium (MTT) assay (Mosmann, 1983).

Prior to use in the assay, the cells were grown to 80% confluence and synchronized by incubation in the culture media for 4 hours. The cells (10 000 cells/well) were then incubated with various concentrations (50 µg/ml, 100 µg/ml and 1000 µg/ml) of the active plant extract solutions for 48 hours. After the 48 hour treatment with the plant extracts, aliquots of 20 µl of a 5 mg/ml MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Molecular probes, Life Technologies) solution was added to the wells and microtiter plates were incubated for 4 hours at 37°C in a humidified incubator with 5% CO_2. Thereafter an aliquot of 100 µl of Dimethyl Sulphoxide (DMSO) was then added to the wells to dissolve the formazan crystals that formed in metabolically active cells and incubated for 1 hour. The plates were read at 595 nm on a plate reader (Digital Analogue Systems, Italy) and the data was analyzed by using the Microsoft Excel. The percentage growth inhibition was determined by using the following equation:

$$\text{Percentage growth inhibition} = \frac{\text{(Absorbance of treated cells)}}{\text{(Absorbance of negative control)}} \times 100$$

3.4 Intracellular Effect of the Active Plant Extracts

3.4.1 Cell Culture Conditions

3.4.1.1 Cell Maintenance

The following protocol has been explained above in 3.3.2.2.3.

3.4.1.2 Trypan Blue Exclusion Assay

The following protocol has been explained above in 3.3.2.3.

3.4.2 Mycobacterial Conditions

Inoculated 10 ml culture with 0.2% actively growing *M. smegmatis*. The cultures achieved the expected optical density (OD) in 2 days. Three mls of mycobacterial cultures were

pelleted 3 ml at 2400 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 3 ml fresh 7H9-C media (Middlebrook 7H9 supplemented with glycerol and OADC). The culture was sonicated at power level 2 and pulse level 40 for 5 minutes using the cup sonicator attachment. The OD at 600 nm of the sonicated bacterial suspension was measured and the culture was diluted 1:10. OD readings between 0.050 and 1.000 were within an acceptable range. The multiplicity of infection was calculated using the following conversion:

OD of 1 = 3×10^8 CFU/ml

3.4.3 Infection of the Macrophage with Mycobacteria

The day before the infection 96 well Petri plates were inoculated with 100 µl of 1×10^5 cells/ well (J774.2). 100 µl of infection media which comprised of DMEM 1640 supplemented with 10% Fetal Calf Serum was added to each well. On the day of infection, the 50 µl of the mycobacterial culture was added to each well and the infected macrophages were incubated for 24 hours at 37^{o}C in a 5% CO_2 incubator. After 4 hours to allow for phagocytosis we post treated the cells with 100 µl of the plant extracts and incubated at 37^{o}C in a 5% CO_2 incubator for 24 hours

3.4.4 Harvest of the Infected Macrophage for CFU Plating

After 24 hours a 1:10 dilution of the infection samples were prepared in Middlebrook 7H9-C. 100 µl of the dilution was plated out onto Middlebrook 7H11 agar plates supplemented with glycerol and 10% OADC. The plates were wrapped in aluminium foil and incubated at 37^{o}C for 3 days. The colony forming units were counted as follows:

$$\text{no. of colonies per well} = \frac{\text{no. of colonies}}{\text{dilution factor}}$$

3.5 Phytochemical Screening of the Plants that have Antimycobacterial Activity

The analysis was carried out on the powdered plant samples using standard procedures as described by Harborne (1973).

3.5.1 Test for Tannins

The dried powdered plant samples (0.5 g each) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves were boiled in 20 ml of distilled water respectively. A few drops of 0.1% ferric chloride solution was added to half the volume of filtrate. The appearance of an intense green or brownish green or a blue-black colouration indicated the presence of tannins in the plant sample. This was then confirmed by adding a few drops of iodine in a second portion of the filtrate which yielded a faint bluish coloration.

3.5.2 Test for Phlobatannins

The dried powdered plant samples (1.0 g) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves was mixed in 20 ml of distilled water respectively and filtered. The mixture was thereafter boiled with 1% aqueous hydrochloric acid (HCl). The presence of phlobatannins was observed by the presence of a red precipitate at the base of the test tubes.

3.5.3 Test for Saponins

The dried powdered plant samples (2.0 g) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves was boiled in 20 ml of distilled water in a water bath and filtered. Thereafter 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of

olive oil, shaken vigorously, and observed for the formation of an emulsion. The presence of saponins was indicated by the formation of a heavy emulsion.

3.5.4 Test for Flavonoids

The dried powdered plant samples (2.0 g) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves was heated with 10 ml of ethyl acetate over a steam bath for 3 minutes. The mixture was thereafter filtered and 4 ml of the filtrate was shaken and 1 ml of dilute ammonia solution was added to it. A yellow colouration indicated a positive test for flavonoids.

3.5.5 Test for Steroids

The dried powdered plant samples (0.5 g) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves was mixed with 2 ml of acetic anhydride respectively. This was followed by the addition of 2 ml sulphuric acid. The colour change from violet to blue or green was indicative of the presence of steroids.

3.5.6 Test for Terpenoids (Salkowski test)

The dried powdered plant samples (5 g) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves was mixed in 2 ml of chloroform thereafter 3 ml concentrated sulphuric acid (H_2S0_4) was carefully added to form a layer. A reddish brown coloration at the interface was indicative of the presence of terpenoids. Using this test we could also determine the presence of steroids by visualizing blue-green interfaces between layers.

3.5.7 Test for Alkaloids

The dried powdered plant sample (0.5 g) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves was mixed with 8 ml of 1% HCl, warmed, and filtered. Thereafter, 2 ml of the filtrate was treated with a few drops of freshly prepared Dragendorffs reagent (Sofowora, 1993). The presence of precipitate showed a positive result.

3.5.8 Test for Phenols

The dried powdered plant sample (0.5 g) of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* leaves was mixed with 1 ml of distilled water. A few drops of neutral 5% ferric chloride solution was added to the mixture. A dark green colour indicated the presence of phenols.

3.6 Thin Layer Chromatography of the Active Plants Extracts

3.6.1 Chemicals

All organic solvents used were of HPLC grade. Methanol, hexane and ethyl acetate were purchased from Merck (South Africa). Vanillin was purchased from Sigma-Aldrich (South Africa). Water was distilled and filtered through a Millipore membrane (0.22 µm).

3.6.2 Thin Layer Chromatography

The plant extracts were dissolved in methanol to make up a stock solution of 1 mg/ml. A 5 µl aliquot of each sample was loaded on Merck TLC F254/Silica gel 60 plates that were developed using the following eluent system hexane: ethyl acetate in different ratios to

determine which one gave the best separation. The ratios included 9:1 (v/v), 8:2 (v/v), 7: 3 (v/v), 6:4 (v/v), 5:5 (v/v), 4:6 (v/v), 3:7 (v/v), 2:8 (v/v) and 1:9 (v/v). The separated compounds were visualized using UV light at 254 nm and 366 nm (Camag Universal UV lamp TL-600) or by spraying the plates with vanillin-sulphuric acid spray reagent (15 g vanillin in 1% H_2S0_4 in methanol)(Amarowicz et al., 2005). The TLC plates were then placed in an oven at 100^{o}C until we could visualize the separation of the bands (Carr and Rogers, 1987). Migration rates were calculated using the following equation:

$$R_f = \frac{\text{migration distance of a substance}}{\text{migration distance of a solvent front}}$$

3.7 Bioautography of the Active Plant Extracts against *M. smegmatis*

3.7.1 Mycobacterial strains

The bacterial species was grown and maintained in Middlebrook 7H9 (Sigma) broth containing glycerol (Sigma) and Middlebrook Oleic Albumin Dextrose Catalase (OADC) growth supplement (Sigma). Before analysis, the test culture was grown for three days in a shaking incubator at 37^{o}C. The culture was centrifuged at 2400 rpm for 10 minutes and the supernatant was discarded. Fresh medium was then added and vortexed at a high speed. A McFarland No 1 standard was measured at 600 nm using a UV-Vis spectrophotometer (Biochrom). The purity of the cultures was checked by means of the ZN Acid Fast stain before use in bioautography.

3.7.2 Qualitative Antimycobacterial Activity (Bioautography)

The qualitative antimycobacterial activity was carried out according to a procedure outlined by Begue and Kline (1972).

For bioautographic analysis 5 µl of each extract was loaded on the TLC plates. The plates were developed in mobile phase hexane: ethyl acetate 7:3 (v/v). The chromatograms were dried at room temperature in a dark cupboard for 2 days to remove the solvents used to develop chromatograms. The chromatograms were sprayed with an overnight culture of *M. smegmatis* until completely wet and were incubated at 37^{o}C in a humidified chamber for 24 hours. The plates were sprayed with ρ-iodonitrotetrazolium violet (INT) (Sigma) and incubated for a further 24 hours (Von Breitenbach et al., 2001). The presence of clear bands on the plates against a purple background indicated growth inhibition.

3.8 Column Chromatography of the Methanolic Extract from *B. saligna*

3.8.1 Extraction

The cleaned and oven dried leaf material was blended and extracted using 100% methanol for 5 days. The extract was concentrated in a rotary evaporator and air dried in a glass beaker for 3 days.

3.8.2 Isolation

The air dried methanolic extract (30 g) was mixed with silica gel as a stationary phase and placed in a column (Figure 23). The column was eluted with 6000 ml of a combination of hexane and ethyl acetate with a ratio of 7:3. One hundred and sixteen (116) fractions were collected and TLC analysis was performed on each. Fractions showing a similar profile of compounds were combined. This was then combined into 4 subfractions. Subfractions A, B, C, and D were concentrated in a rotary evaporator and air dried in a glass beaker for 3 days. Subfraction C was then run through a column to further purify the compounds and 30 fractions were collected. Fractions showing a similar profile of compounds were combined into 3 subfractions. Subfractions S1, S2 and

S3 were concentrated in a rotary evaporator and air dried in a glass beaker for 3 days. On further chemical investigations, S1 was predominantly chlorophyll and was not further analysed. However, S2 was the major fraction and it showed chemical characteristics of triterpenes. S3 was very similar to S2 in chemical behavior but much purer. A preliminary NMR investigation confirmed it to be a mixture of isomeric pentacyclic triterpenoic acids which was identified by comparison with authentic oleanolic acid and ursolic acid (Somova et al., 2003) provided by Prof F.O. Shode.

3.8.3 Acetylation Reaction

In order to separate S2 into its components, it was acetylated using pyridine and acetic anhydride (Cheng et al., 2013). Then it was purified using an open column silica gel chromatography and was eluted with hexane and ethyl acetate in a ratio of 9:1 (v/v). Seventy-six fractions were collected and the fractions showing similar TLC profiles were pooled together resulting in two separate subfractions, T1 and T2. These combined fractions were concentrated in a rotary evaporator and air dried in a glass beaker.

Figure 23. Picture of the column chromatography of methanolic extract from leaves of *B. saligna.*

3.9 Characterization of the Isolated Compounds from *B. saligna*

3.9.1 Characterization of T1 and T2 compounds by ^{1}H NMR and ^{13}C NMR.

T1 and T2 were white solids. They were subjected to spectroscopic studies using NMR methods. The NMR measurements were conducted on a Topspin Bruker 400 spectrometer (Germany) operating at 400 MHz for ^{1}H and 600 MHz for ^{13}C using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard.

3.10 Antimycobacterial Activity of Triterpenes Isolated from *B. saligna*

The pharmacological effect of UAA and OAA was evaluated against *M. smegmatis, M. tuberculosis* $H_{37}Rv$, clinical isolate MDR-TB and clinical isolate XDR-TB by a modification of the MABA assay (Eloff, 1998). A stock solution of each compound was prepared in 7H9 broth containing 10% OADC enrichment broth. A volume of 100 µl of the stock solution of UAA and 100 µL of OAA were added to each well, having been thoroughly mixed; thereafter 100 µl of the bacterial suspension adjusted to a McFarland No. 1 tube and diluted in a ratio of 1:10 was added. Controls for each compound were prepared by adding 100 µl of the corresponding stock solution, 100 µl of the culture medium and 100 µl of the same adjusted bacterial suspension. Control for bacterial growth included 100 µl of 7H9 broth and 100 µl of the bacterial suspension. Plates were incubated for 10 days at 37°C; after this period, 30 µl of Alamar blue solution was added to the wells, leaving the plates overnight at 37°C (Figure 24).

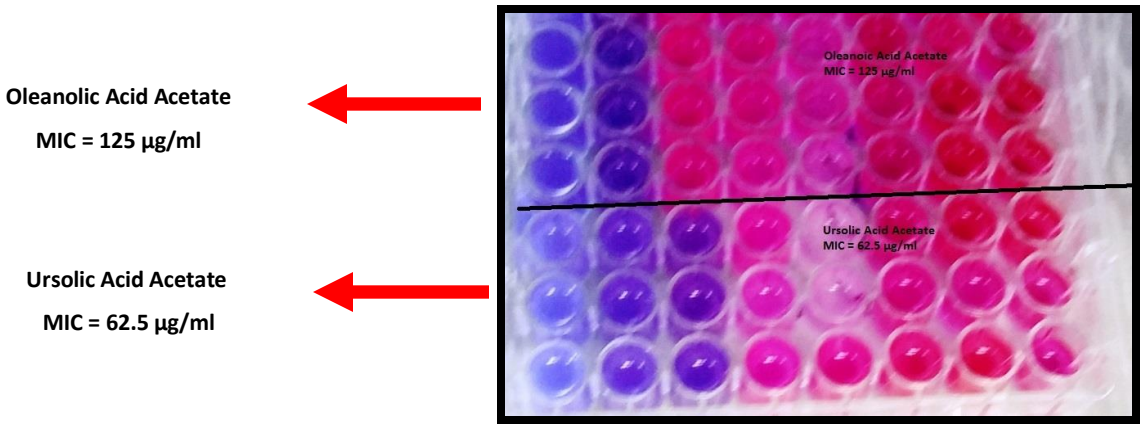


Figure 24. Microtitre plate of dilutions 250 µg/ml, 125 µg/ml, 62.50 µg/ml, 36.25 µg/ml, 18.02 µg/ml, 9.01 µg/ml, 4.50 µg/ml and 2.25 µg/ml showing the visual effect of OAA and UAA on XDR-TB. Blue depicts the mycobacteria have been killed whilst the pink shows the mycobacteria are alive.

3.11 Molecular Docking of Triterpenes Isolated from *Buddleja saligna*

Computational methods are an important tool in designing newer potent molecules (Lokwani et al., 2011) . Two-dimensional (2D) and three-dimensional (3D) quantitative structure-activity relationship (QSAR) studies were carried out. Docking studies provided insight into the interaction of the compounds with the enzyme.

All compounds were built and saved as Mol2. The crystal structure of *M. tuberculosis* fadA5 thiolase enzyme in complex with steroid was downloaded from protein data bank (PDB code entry 4UBT). The protein was loaded into Leadit 2.1.2 (Rarey et al., 1996) and the receptor components were chosen by a selection of chain C as a the main chain which complexed with the steroid. The binding site was defined by choosing steroid as a reference ligand to which all coordinates were computed. Amino acids within radius 6.5 A^o were selected in the binding site. All chemical ambiguities of residues left as default. Ligand binding was driven by enthalpy (classic triangle matching). For scoring, all default settings were restored. Intra-ligand clashes were computed by using clash factor = 0.6. A maximum number of solutions per iteration = 200. Maximum of solution per fragmentation = 200. The base placement method was used as a docking strategy.

CHAPTER 4: RESULTS

4.1 Antimycobacterial Screening of the Nine Plants

From a literature survey carried out on medicinal plants for the treatment of TB in South Africa, nine plants were selected according to their traditional use in treatment of various ailments including chest complaints, pneumonia, cough, bronchitis, and TB in the present study (Bamuambaa et al., 2008, Hutchings, 1996, Springfield and Weitz, 2006, Vogt, 1995, Lall and Meyer, 1999). Eighteen crude extracts were obtained by extraction with distilled water and 80% methanol.

During our screening at a fixed standard concentration of 1 mg/ml five out of the eighteen extracts showed antimycobacterial activity against *M. smegmatis* and *M. tuberculosis* H_{37}Rv (ATCC 25177). These consisted of aqueous and methanolic extracts of the leaf part of the plant. The extracts were aqueous extracts of *G. perpensa* and *T. riparia*; methanolic extracts of *B. saligna*, *C. tomentosa* and *C. dimidiatus*. Table 5 shows the initial screening of the nine plants against *M. smegmatis* and *M. tuberculosis* H_{37}Rv (ATCC 25177).

The initial screening results from this study led us to select the five plant extracts for further antimycobacterial study. The mycobacteria that were tested comprised of three well-characterized clinical isolates of MDR-TB and three well-characterized clinical isolates of XDR-TB. Two of the aqueous plant extracts namely *G. perpensa* and *T. riparia* showed positive antimycobacterial activity against the three different MDR-TB strains (Table 5). Three of the methanolic plant extracts namely *B. saligna*, *C. tomentosa* and *C. dimidiatus* showed positive antimycobacterial activity against the three different MDR-TB and XDR-TB strains (Table 5).

Table 5. Antimycobacterial activity of the aqueous and methanolic plant extracts at a concentration of 1 mg/ml

Plant	Part Used	Extract	*M. smegmatis*	*M. tuberculosis* $H_{37}Rv$	Clinical Isolate MDR-TB	Clinical Isolate XDR-TB
B. saligna	Leaf	aqueous	G	G	G	G
B. saligna	Leaf	methanolic	NG	NG	NG	NG
C. tomentosa	Leaf	aqueous	G	G	G	G
C. tomentosa	Leaf	methanolic	NG	NG	NG	NG
C. dimidiatus	Leaf	aqueous	G	G	G	G
C. dimidiatus	Leaf	methanolic	NG	NG	NG	NG
D. cinerea	Leaf	aqueous	G	G	G	G
D. cinerea	Leaf	methanolic	G	G	G	G
E. capensis	Leaf	aqueous	G	G	G	G
E. capensis	Leaf	methanolic	G	G	G	G
F. sur	Leaf	aqueous	G	G	G	G
F. sur	Leaf	methanolic	G	G	G	G
G. perpensa	Leaf	aqueous	NG	NG	NG	G
G. perpensa	Leaf	methanolic	G	G	G	G
L. leonurus	Leaf	aqueous	G	G	G	G
L. leonurus	Leaf	methanolic	G	G	G	G
T. riparia	Leaf	aqueous	NG	NG	NG	G
T. riparia	Leaf	methanolic	G	G	G	G
Rifampicin			NG	NG	G	G
Isoniazid			NG	NG	G	G
Drug-Free Control			G	G	G	G
Distilled Water			G	G	G	G

(G: Growth, NG: No Growth; n=3)

The minimum inhibitory concentration (MIC) of the active plant extracts was determined for their antimycobacterial activity using INT as an indicator for *M. smegmatis* and resazurin as an indicator for *M. tuberculosis* H_{37}Rv (ATCC 25177) and the well characterized clinical isolates MDR-TB and XDR-TB in 96-well microplates (Table 6).

The MIC of the aqueous extracts provided us with the following: *G. perpensa* (0.250 mg/ml) and *T. riparia* (0.125 mg/ml) for *M. smegmatis*; *G. perpensa* (0.250 mg/ml) and *T. riparia* (0.250 mg/ml) for *M. tuberculosis* H_{37}Rv (ATCC 25177); and *G. perpensa* (0.500 mg/ml) and *T. riparia* (0.250 mg/ml) for MDR-TB. The MIC of the methanolic extracts were as follows: *B. saligna* (0.125 mg/ml), *C. tomentosa* (0.500 mg/ml) and *C. dimidiatus* (0.125 mg/ml) for *M. smegmatis*; *B. saligna* (0.125 mg/ml), *C. tomentosa* (0.500 mg/ml) and *C. dimidiatus* (0.125 mg/ml) for *M. tuberculosis* H_{37}Rv (ATCC 25177); and *B. saligna* (0.125 mg/ml), *C. tomentosa* (1 mg/ml) and *C. dimidiatus* (0.500 mg/ml) for MDR-TB and XDR-TB. The mean MIC results of the aqueous and methanolic crude extracts of each of the active plants showed lower antimycobacterial activities against *M. smegmatis* and *M. tuberculosis* H_{37}Rv (ATCC 25177) in comparison to the positive controls rifampicin and isoniazid (Table 6). Positive controls Rifampicin and Isoniazid were inactive when used for MDR-TB and XDR-TB.

Table 6. Minimum inhibitory concentration of the active plant extracts against *M. smegmatis*, MTB $H_{37}Rv$ (25177), clinical isolate MDR-TB and clinical isolate XDR-TB

Plant	Extract	*M. smegmatis*	MTB H37Rv (25177)	Clinical Isolate MDR-TB	Clinical Isolate XDR-TB
				Activity (mg/ml)	
B. saligna	Methanolic	0.125	0.125	0.125	0.125
C. tomentosa	Methanolic	0.500	0.500	1.000	1.000
C. dimidiatus	Methanolic	0.125	0.125	0.500	0.500
G. perpensa	Aqueous	0.250	0.250	0.500	-
T. riparia	Aqueous	0.125	0.250	0.250	-
Rifampicin		0.001	0.001	-	-
Isoniazid		0.003	0.003	-	-

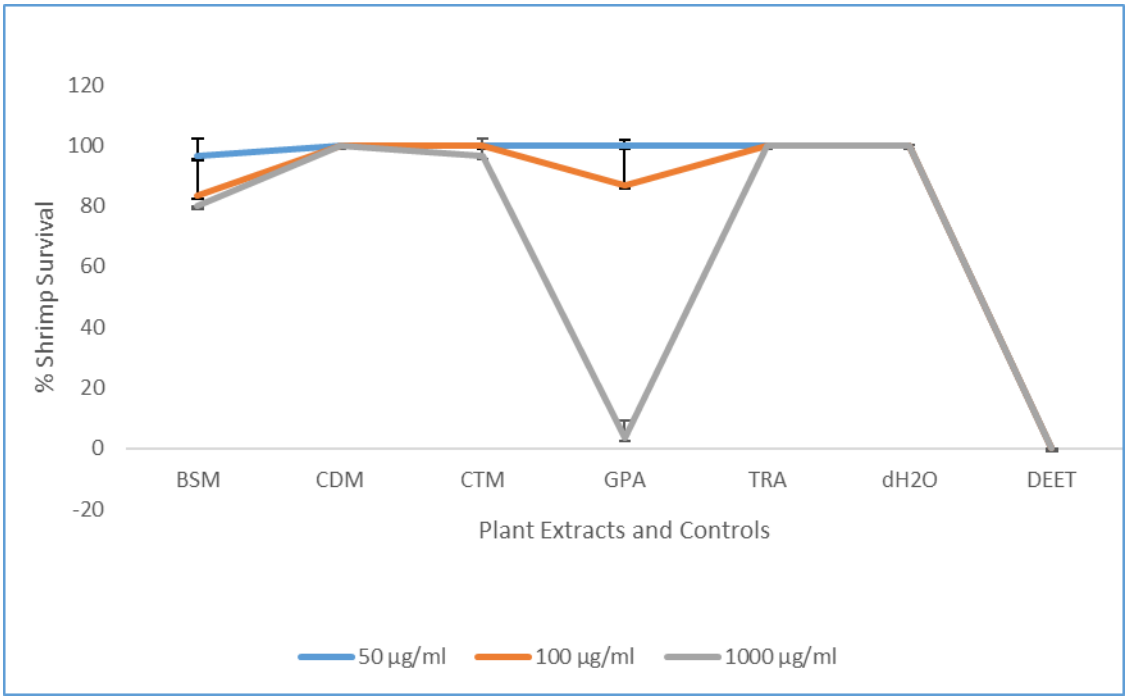
(n=3)

4.2 Safety Analysis of the Active Plant Extracts

4.2.1 Toxicity

The brine shrimp lethality test (BST) was used to predict toxicity properties of the active plant extracts under investigation (Meyer et al., 1982). The procedure determines lethal concentrations of the active plant extracts in brine medium. The activities of a broad range of active plant extracts are manifested as toxicity to the shrimp. In order for a test compound to be considered highly toxic it needs to show shrimp death ≥ 50% and for a compound to be considered slightly toxic it needs to show cell death of between 50 - 70%.

Results revealed that methanolic extracts of *B. saligna, C. tomentosa*, *C. dimidiatus* and the aqueous extract of *T. riparia* were not toxic to brine shrimp larvae at all concentrations tested (50 µg/ml, 100 µg/ml and 1000 µg/ml)(Figure 25). The aqueous extract of *G. perpensa* indicated to be extremely toxic to the shrimp larvae (3.33±5.77%) at a concentration of 1000 µg/ml (Figure 25). All the active plant extracts tested were less toxic to the shrimp larvae in comparison to the positive control, DEET, a well-known organophosphate. Maximum mortality (100%) was observed from the positive control.



Mean ± SD (n=3).

Figure 25. Brine shrimp larvicidal activity of the active plant extracts, *B. saligna, C. tomentosa, C. dimidiatus* (methanolic), *G. perpensa* and *T. riparia* (aqueous) at different concentrations.

4.2.2 Cytotoxicity

Following the promising antimycobacterial activity, the cytotoxic effect of the five active plants extracts namely *B. saligna, C. tomentosa*, *C. dimidiatus*, *G. perpensa* and *T. riparia* against mouse BALB/C monocyte-macrophage (J774.2) and human peripheral blood mononuclear cells (PBMCs) is shown in Figure 26 and Figure 27 respectively. We performed the in vitro cytotoxicity test to measure the damage caused by the antimycobacterial plant extracts on normal living cells. This allowed us to determine whether the plant extracts could be used for therapeutic purposes without excessive damage to host cells.

The main aim of the cytotoxicity assay was to determine which of the plant extracts that had positive antimycobacterial activity were not toxic to J774.2 and PBMCs cell lines. The effect was observed after 48 hours at various concentrations (50 µg/ml, 100 µg/ml and 1000 µg/ml) for the aqueous and methanolic extracts. After 48 hours the methanolic extracts of *B. saligna, C. tomentosa*, *C. dimidiatus* and aqueous extracts of *G. perpensa* and *T. riparia* showed an increase in cell viability for the J774.2 cell line (Figure 26). As the concentration increased so too did cell viability. It can be noted that the active plant extracts stimulated the growth of the cells and was not toxic to the J774.2 cell line at the highest concentrations tested. After 48 hours the methanolic extracts of *B. saligna, C. tomentosa*, *C. dimidiatus* and aqueous extracts of *T. riparia* showed an increase in cell viability for the PBMCs (Figure 27), however, the aqueous extract of *G. perpensa* showed a decrease in cell viability as we increased the concentration of the plant extract. The highest concentration (1000 µg/ml) showed a toxic activity to the PBMCs where there was only 25.26 ± 9.96% cell viability.

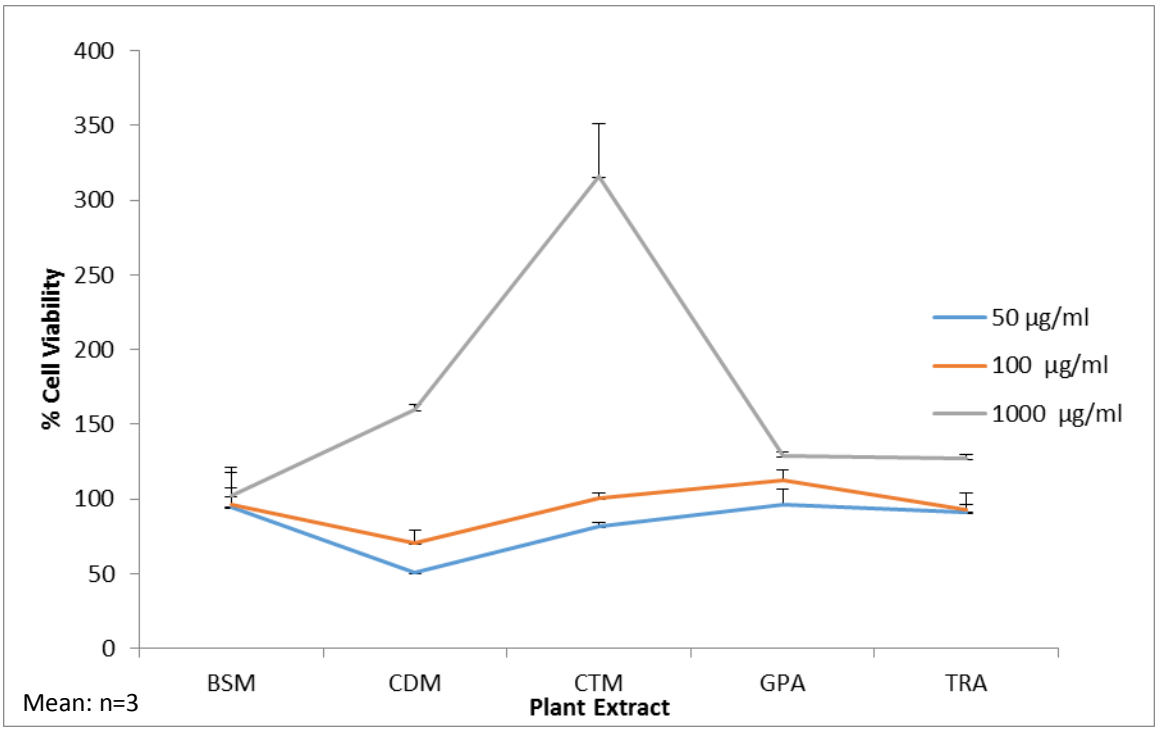
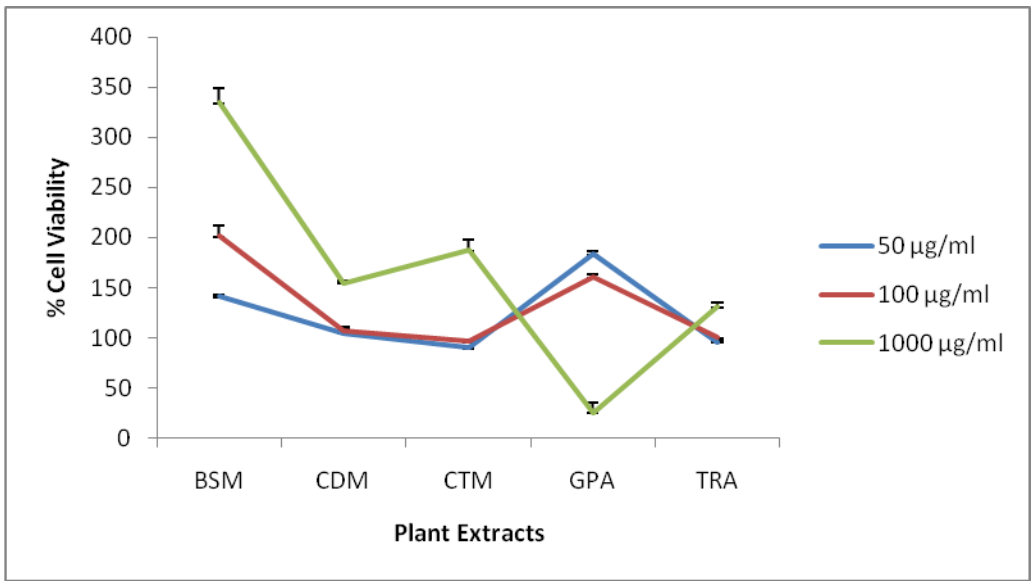


Figure 26. Cell viability of the active plant extracts *B.saligna, C. tomentosa, C. dimidiatus* (methanolic), *G. perpensa* and *T. riparia* (aqueous) on the BALB/C monocyte macrophage cell line (J774.2).



Mean ± SD (n=3)

Figure 27. Cell viability of the active plant extracts *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* on the peripheral blood mononucleated cell line (PBMC). Mean ± SD (n=3).

4.3 Intracellular Effect of the Active Plant Extracts against *M. smegmatis*

Following promising activity from the in vitro antimycobacterial screening the next objective was to assess whether the active plant extracts had any effect on the survival or death of mycobacterial species, *M. smegmatis*, bound within the macrophage (J774.2) cell line at a concentration of 100 µg/ml. Prior to assessing the effect of the plant extracts on the mycobacteria bound macrophage, we performed the trypan blue exclusion assay to verify that the macrophages were > 80% confluent (Figure 28) and checked the purity of *M. smegmatis* (Figure 29) using the Ziel Neelson Acid Fast stain.

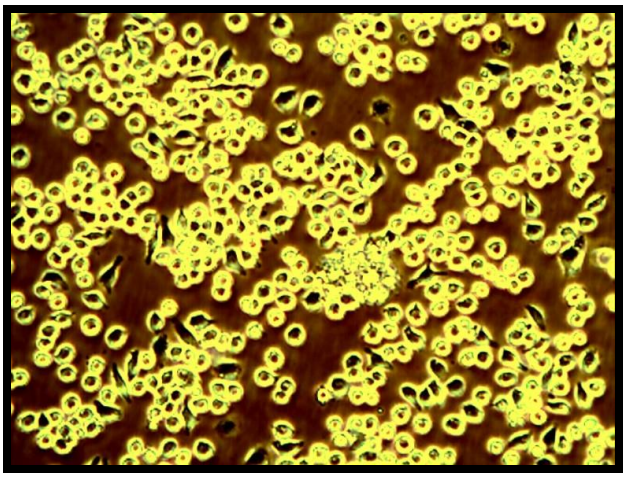


Figure 28. Light micrograph of untreated J774.2 macrophage cell line (100 x magnification).

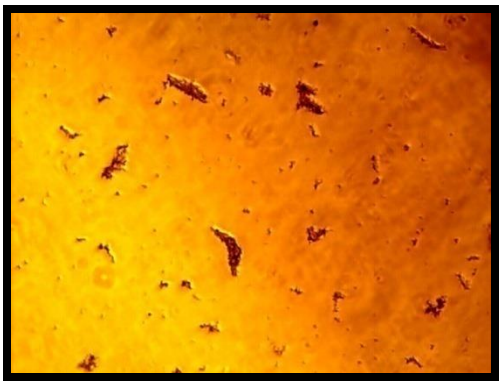
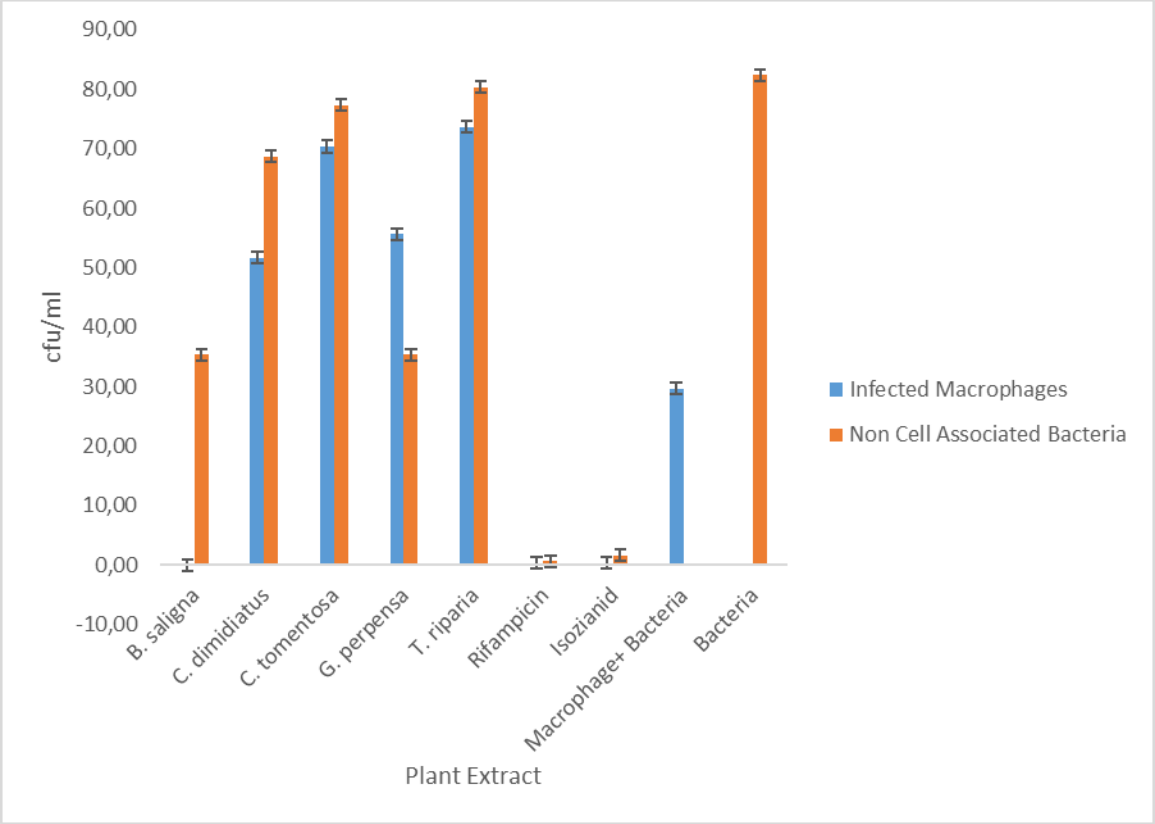


Figure 29. Light micrograph of *M. smegmatis* in DMEM supplemented with Fetal Calf Serum (100 x magnification).

The macrophage cell line J774.2 was infected (MOI 10:1) with *M. smegmatis* and treated with plant extracts from *B. saligna, C. tomentosa, C. dimidiatus* (methanolic), *G. perpensa* and *T. riparia* (aqueous). After 24 hours, cells were lysed and incubated for 72 hours. The intracellular bacilli colony forming units (CFU) were determined using the M7H11 supplemented with OADC agar plates. The colony forming units observed in the experiments with non-cell associated mycobacteria as well as with mycobacteria in macrophages has been plotted in Figure 30. The results obtained from the active plant

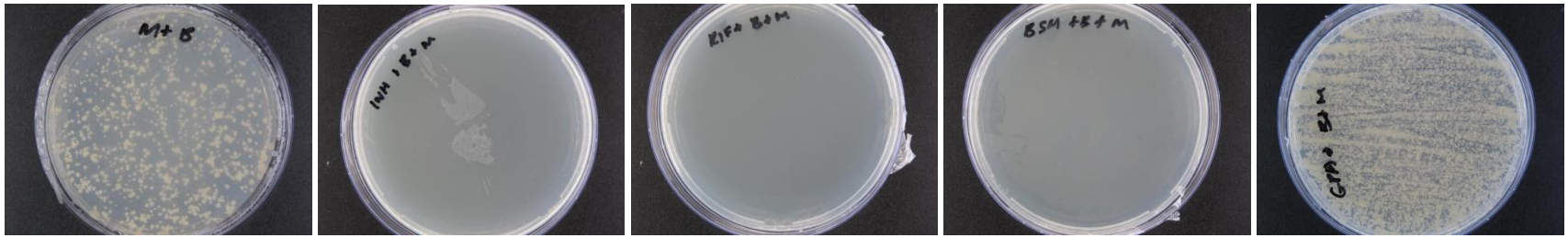
extracts were compared with the control culture (untreated macrophages) and control compounds Rifampicin and Isoniazid.

In order for a plant extract to be considered bactericidal, it has to reduce the viable bacterial counts by 90% or more compared with phagocytosis control. Intracellular mycobacterial growth was observed in the macrophages treated with aqueous extracts of *G. perpensa* (55.66±3.51 CFU/ml), *T. riparia* (73.66±1.52 CFU/ml); and methanolic extracts of *C. tomentosa* (51.66±3.51 CFU/ml) and *C. dimidiatus* (70.33±2.08 CFU/ml). Minimal intracellular mycobacterial growth (3.66±3.05 CFU/ml) was observed in macrophages treated with the methanolic extract of *B. saligna* at a concentration of 100 µg/ml and the control compounds Rifampicin and Isoniazid (Figure 30). The experiments with the non-cell associated bacteria were performed as controls for the experiments in macrophages (Figure 30). Intracellular *M. smegmatis* has the ability to survive and multiply within the untreated and some of the treated macrophages. *B. saligna* had inactivated the phagocytosed bacilli after 24 hours of treatment (Figure 31). From this, we can see that *B. saligna* has a bactericidal effect on the mycobacteria located within the mouse macrophage. None of the other plant extracts tested proved to be bactericidal (Figure 31). Figure 32 shows the colony counts of the non-cell associated mycobacteria treated with the plant extracts.



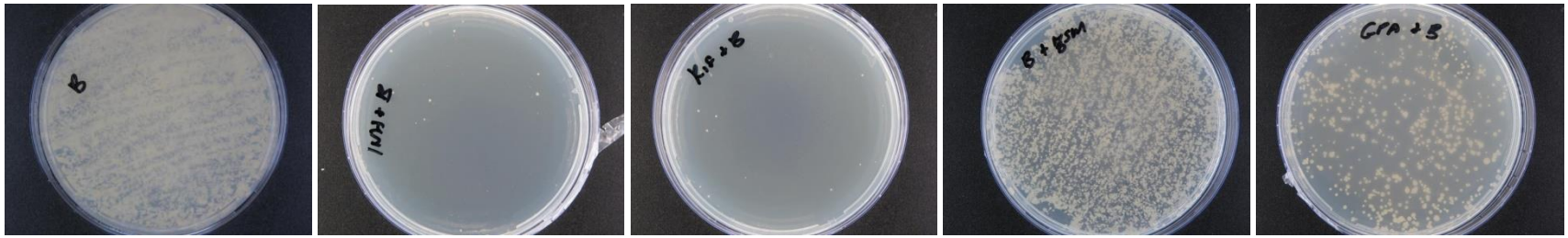
Mean±SD; n=3.

Figure 30. Intracellular effect of the active plant extracts against *M. smegmatis* within the macrophage (J774.2) and the effect of the active plant extracts against *M. smegmatis* after 24 hours.



A B C D E

Figure 31. Petri plates depicting the difference in infection 24 hours after the cells were lysed (A) *M.smegmatis* infected J774.2 Macrophages (B) Effect of Isoniazid on *M. smegmatis* infected J774.2 cells (C) Effect of Rifampicin on *M. smegmatis* infected J774.2 cells (D) Effect of 100 µg/ml of BSM on *M. smegmatis* infected J774.2 cells (E) Effect of 100 µg/ml of GPA on *M. smegmatis* infected J774.2 cells.



(A) (B) (C) (D) (E)

Figure 32. Petri plates depicting the effect of the plant extracts on *M. smegmatis* after 24 hours (A) *M.smegmatis* (B) Effect of Isoniazid on *M. smegmatis* (C) Effect of Rifampicin on *M. smegmatis* (D) Effect of 100 µg/ml of BSM on *M. smegmatis* (E) Effect of 100 µg/ml of GPA on *M. smegmatis.*

4.4 Phytochemical Screening of the Active Plants

The qualitative phytochemical screening of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* is displayed in Table 7 below. All plants analyzed contained alkaloids. *B. saligna* contains a range of phytochemicals such as tannins, phlobtannins, saponins, flavonoids, terpenoids, alkaloids and phenols however there were no steroids present. *C. tomentosa* displayed the presence of saponins, flavonoids, steroids and alkaloids; but did not contain tannins, phlobtannins, terpenoids and phenols. *C. dimidiatus* exhibited the presence of tannins, phlobtannins, saponins, terpenoids and alkaloids; however did not contain any flavonoids, steroids and phenols. *G. perpensa* contains flavonoids, alkaloids and phenols; but do not have tannins, phlobtannins, saponins, steroids and terpenoids. *T. riparia* contained a range of phytochemicals such as tannins, phlobtannins, saponins, flavonoids, steroids, alkaloids and phenols except terpenoids.

Table 7. Phytochemical analysis of the active plants

Plants	Tannins	Phlobtannins	Saponins	Flavonoids	Steroids	Terpenoids	Alkaloids	Phenols
B. saligna	+	+	+	+	-	+	+	+
C. tomentosa	-	-	+	+	+	-	+	-
C. dimidiatus	+	+	+	-	-	+	+	-
G. perpensa	-	-	-	+	-	-	+	+
T. riparia	+	+	+	+	+	-	+	+

[(+) presence of a phytochemical compound (-) absence of a phytochemical compound]

4.5 Thin Layer Chromatography of the Active Plant Extracts

Evaluation of the composition of *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* was done using thin layer chromatography (Figure 33). This process offered effectiveness and a fast outcome that was used to obtain the profile of the plant extracts. Numerous solvent systems were assessed for best separation of the compounds present in the methanolic leaf extracts of *B. saligna, C. tomentosa* and *C. dimidiatus;* and aqueous leaf extracts of *G. perpensa* and *T. riparia*. The mobile phase that gave the best separation was hexane: ethyl actetate (7: 3) sprayed with vanillin sulphuric spray reagent as shown in Figure 33.

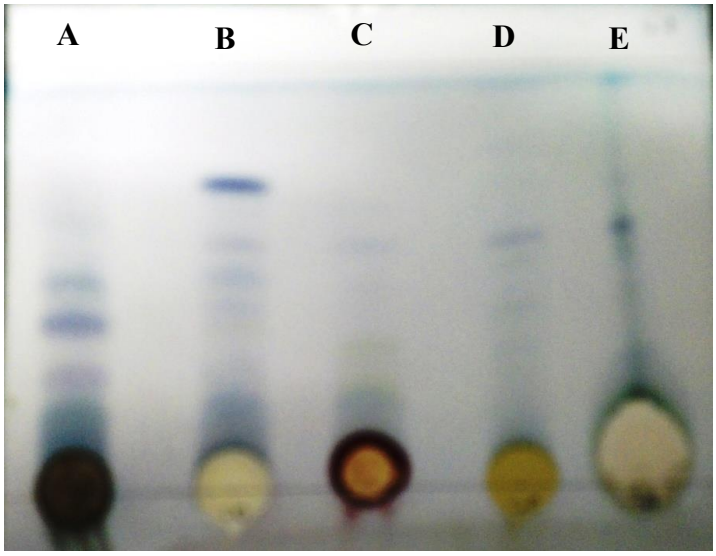


Figure 33. TLC of the methanolic extracts of (A) *B. saligna,* (B) *C. dimidiatus,* (C) *C. tomentosa;* aqueous extracts of (D) *G. perpensa,* and (E) *T. riparia* leaves using Hexane : Ethyl Acetate (7:3) as a mobile phase and developed with vanilin spray reagent for visualisation.

4.6 Bioautography of the Active Plant Extracts

Methanolic extracts of *B. saligna, C. tomentosa* and *C. dimidiatus;* and aqueous extracts of *G. perpensa* and *T. riparia* were analysed by bioautography for qualitative analysis of antimycobacterial compounds using TLC sprayed with *M. smegmatis* (Figure 34). After a period of 24 hours, INT was used as a growth indicator and zones of inhibition was assessed. The bioautographic assays were carried out on three separate occasions for the evaluation of the antimycobacterial properties in order to verify the reproducibility of the results. The methanolic extracts of *B. saligna* and *C. dimidiatus* demonstrated inhibition of growth of *M. smegmatis* on the bioautograph. *B. saligna* displayed four bands that inhibited the growth in the bioautograph. Two bands were observed for *C. dimidiatus* that inhibited the growth of *M. smegmatis*.

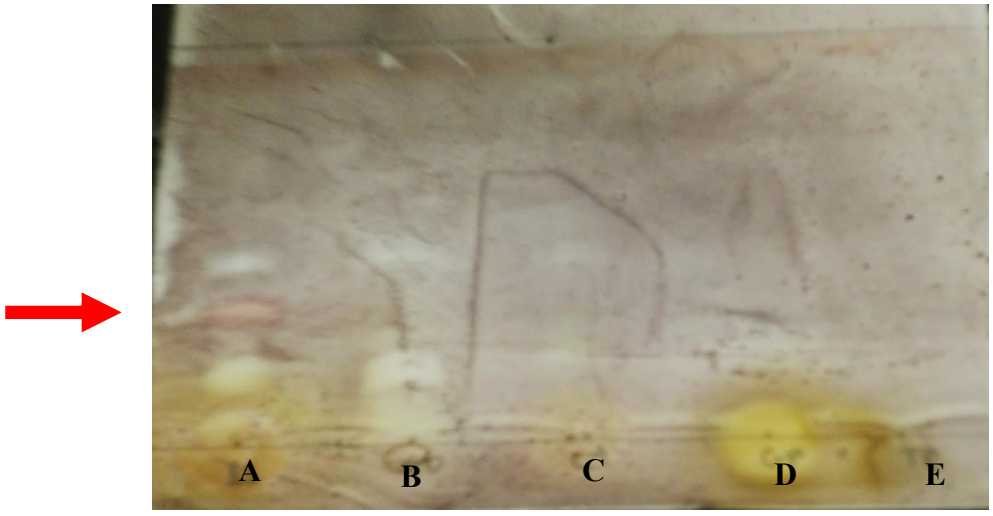


Figure 34. Bioautograph of the methanolic extracts of (A) *B. saligna,* (B) *C. dimidiatus,* (C) *C. tomentosa;* aqueous extracts of (D) *G. perpensa,* and (E) *T. riparia* leaves using Hexane: Ethyl Acetate (7: 3) as a mobile phase. Plates were sprayed with *M. smegmatis* and clear zones denote a compound of interest.

4.7 Isolation and Characterization of Compounds from *B. saligna*

Initial extraction of *B. saligna* proved that the methanolic extract provided the compounds with antimycobacterial activity. Bioautography supported the conclusion and therefore, methanol was used to extract the sample from the isolation process. The column chromatography of the methanolic extract of the *B. saligna* leaves resulted in the separation of plant components using a solvent system of hexane: ethyl acetate (7:3).

A total of one hundred and sixteen fractions of 50 ml each was collected. Fractions showing a similar profile of compounds were combined into 4 subfractions. Subfraction A (0-16), subfraction B (17-29), subfraction C (30-77) and subfraction D (78-116) are shown on a TLC plate in Figure 35 . R was the plant extract *B. saligna* which served as a reference.

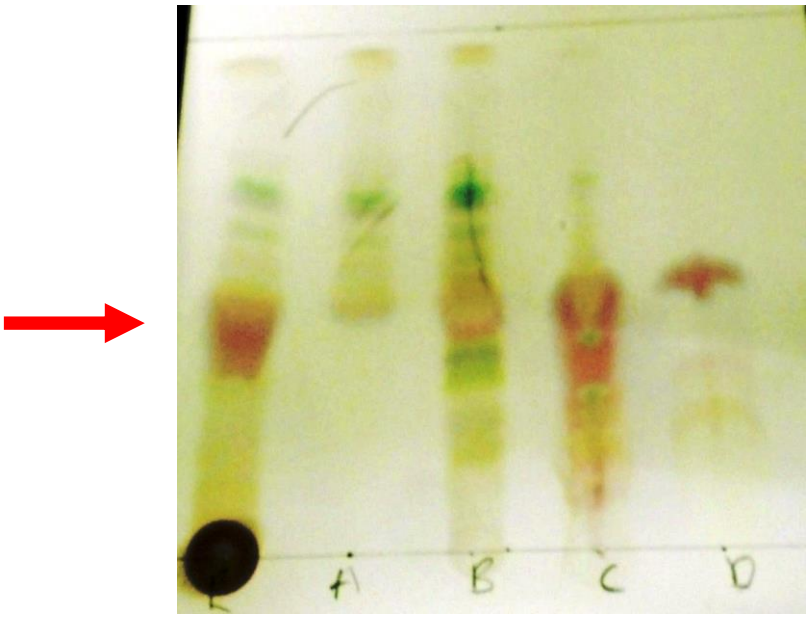


Figure 35. Profile of compounds isolated from the leaves of *B. saligna* using column chromatography combined into 4 subfractions (A, B, C and D) using Hexane: Ethyl Acetate (7: 3) as a mobile phase and developed with conc H_2SO_4 : methanol (9: 1) spray reagent for visualisation

Compounds that solidified after column chromatography were collected and analyzed using bioassay guided fractionation. In bioassay-guided fractionation, the fractions were subjected to bioautography in order to determine any active compounds from the leaves of *B. saligna* (Figure 36).

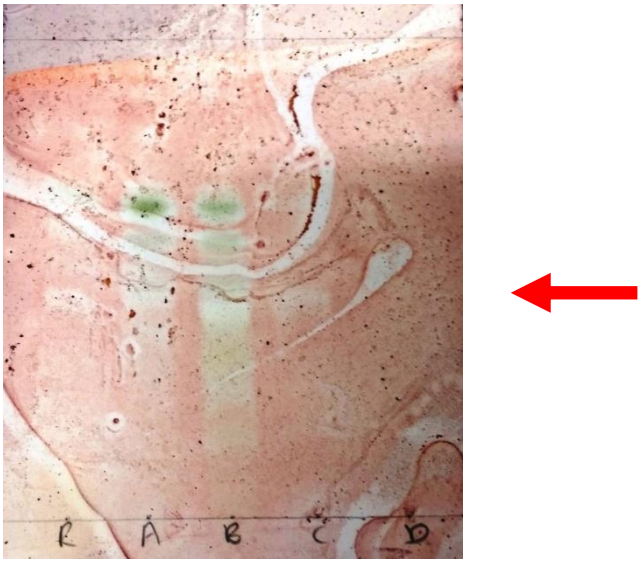


Figure 36. Bioautograph profile of compounds isolated from the leaves of *B. saligna* using column chromatography combined into 4 subfractions (A, B, C and D) using Hexane: Ethyl Acetate (7: 3) as a mobile phase. Plates were sprayed with *M. smegmatis* and clear zones denote a compound of interest.

Of all the active fractions, subfraction C was selected to be further purified using open column silica gel chromatography with hexane and ethyl acetate solvent system (7:3) because of its least complexity and high purity. A total of thirty fractions of 15 ml each was collected and fractions showing a similar profile on TLC were combined into 3 fractions namely, S1, S2 and S3.

The ^{1}H NMR and ^{13}C NMR of T1 are shown in Figures 37 and 38 below.

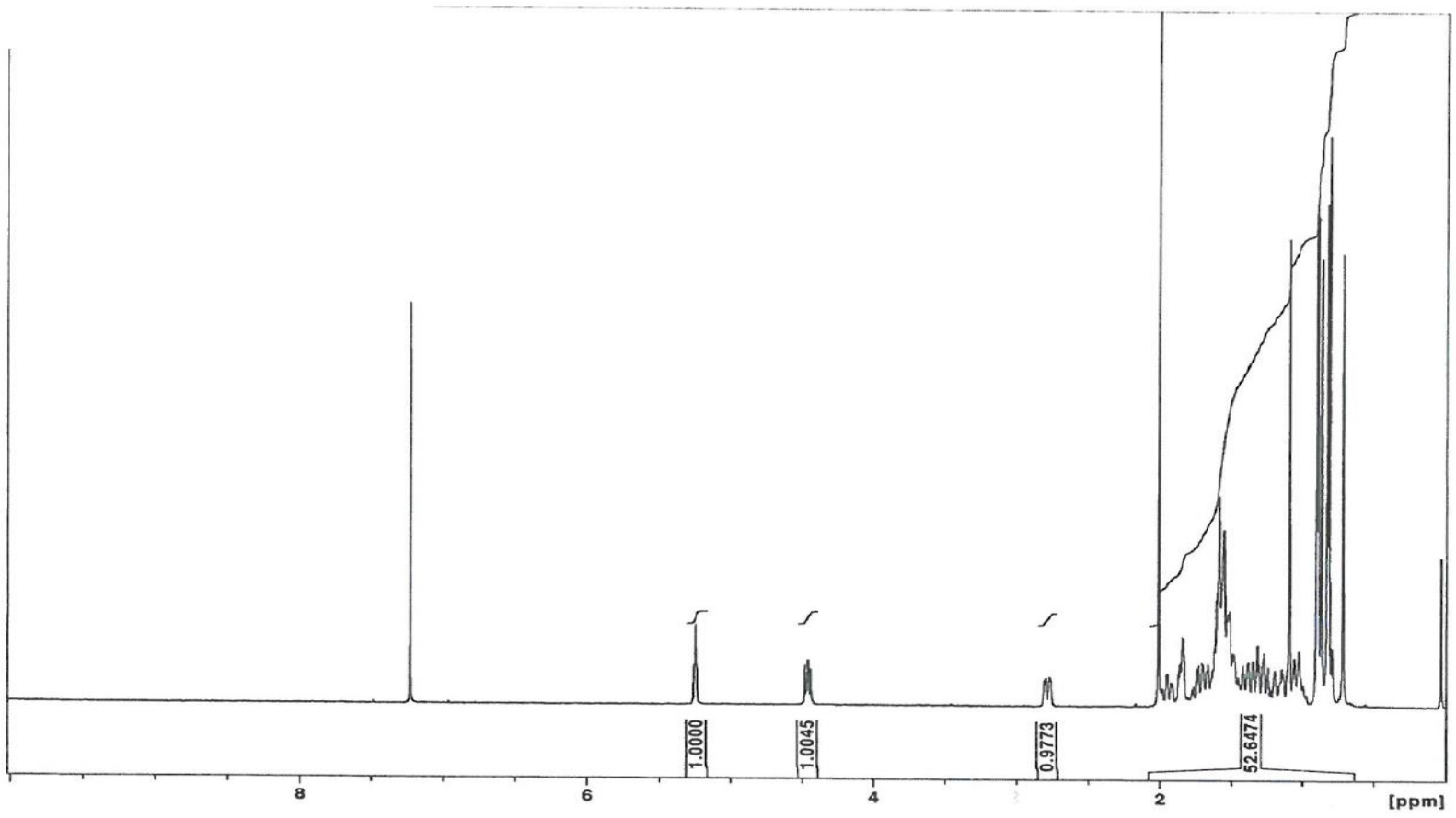


Figure 37. The ^{1}H NMR of T1.

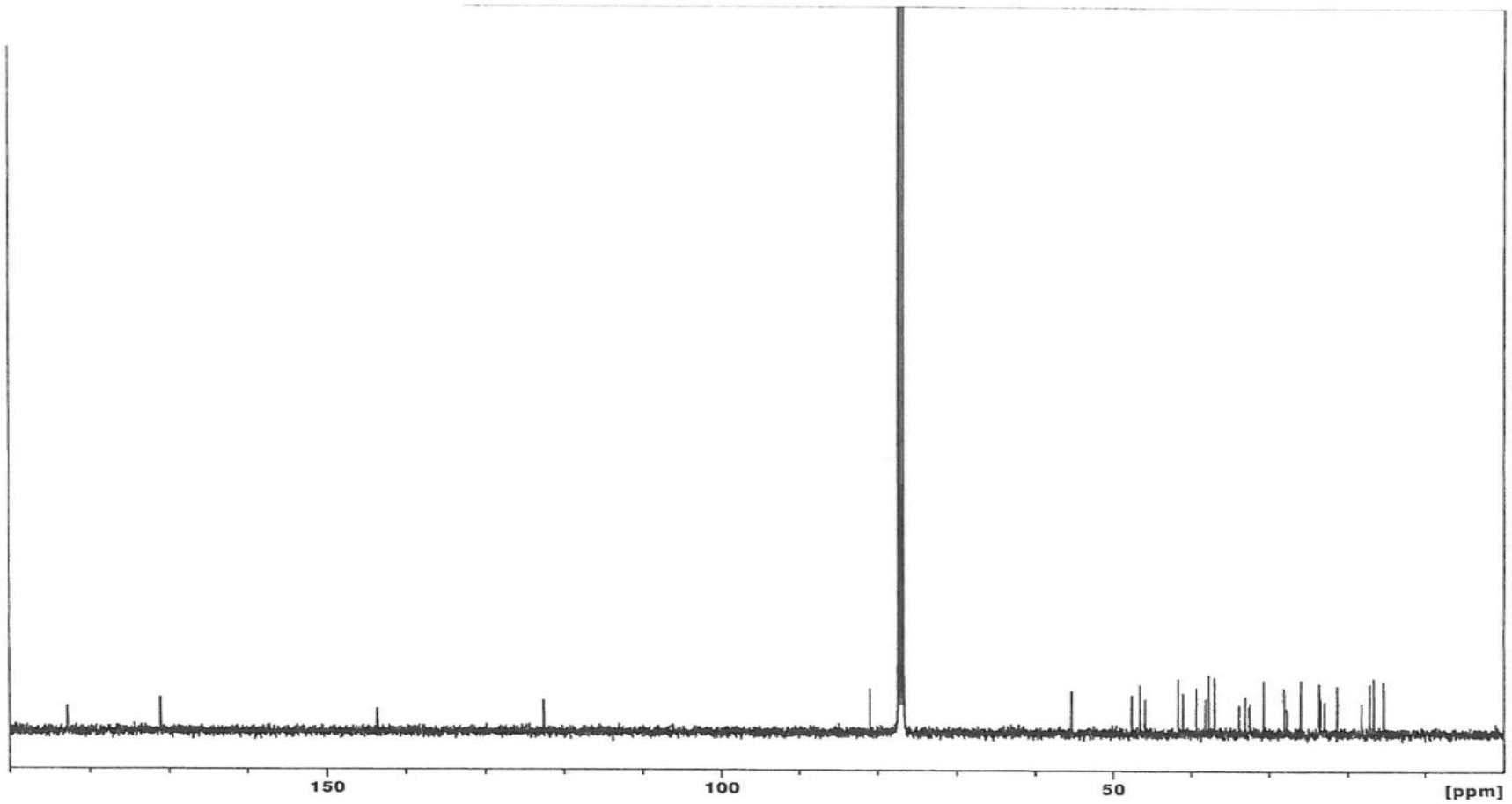


Figure 38. The ^{13}C NMR of T1.

The ^{1}H NMR and ^{13}C NMR of T2 are shown in Figures 39 and 40 below.

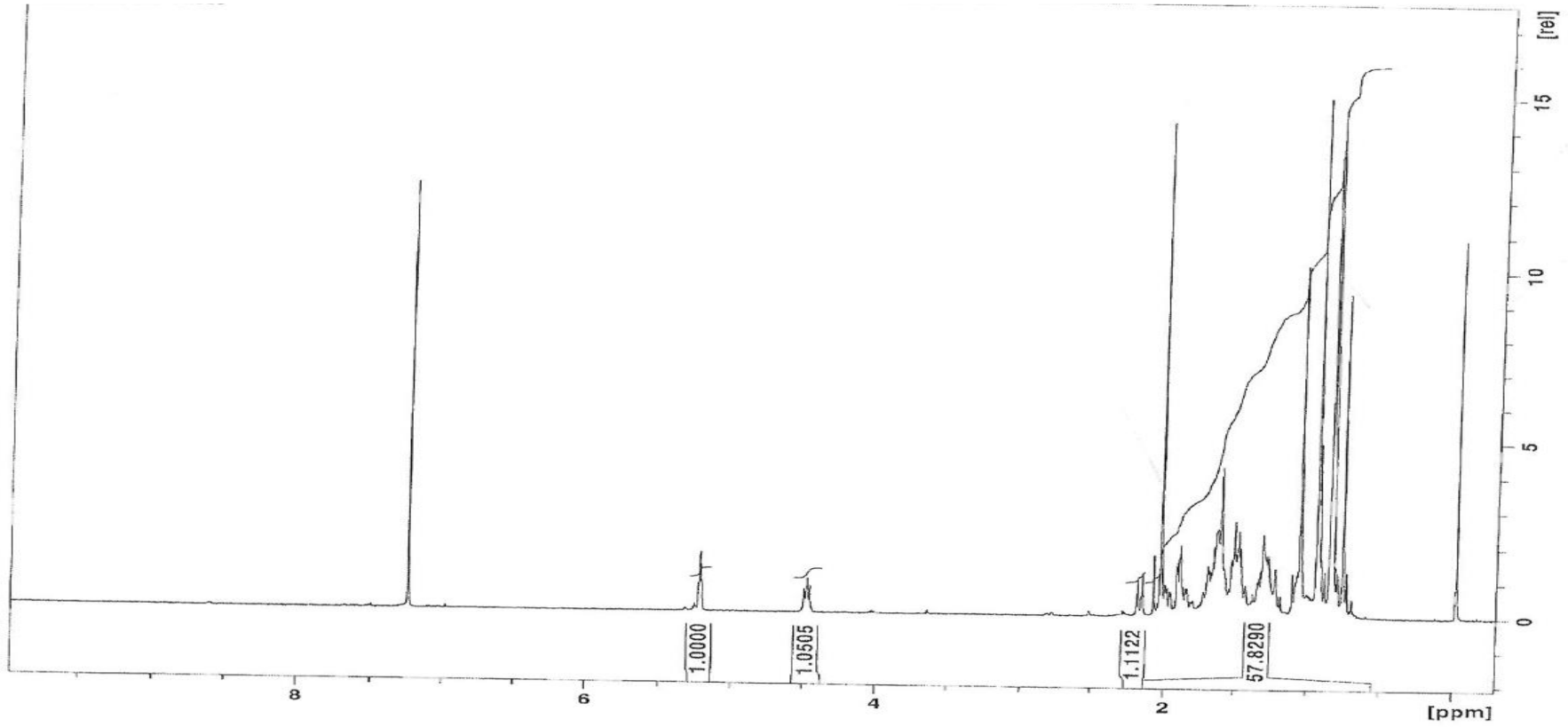


Figure 39. The ^{1}H NMR of T2.

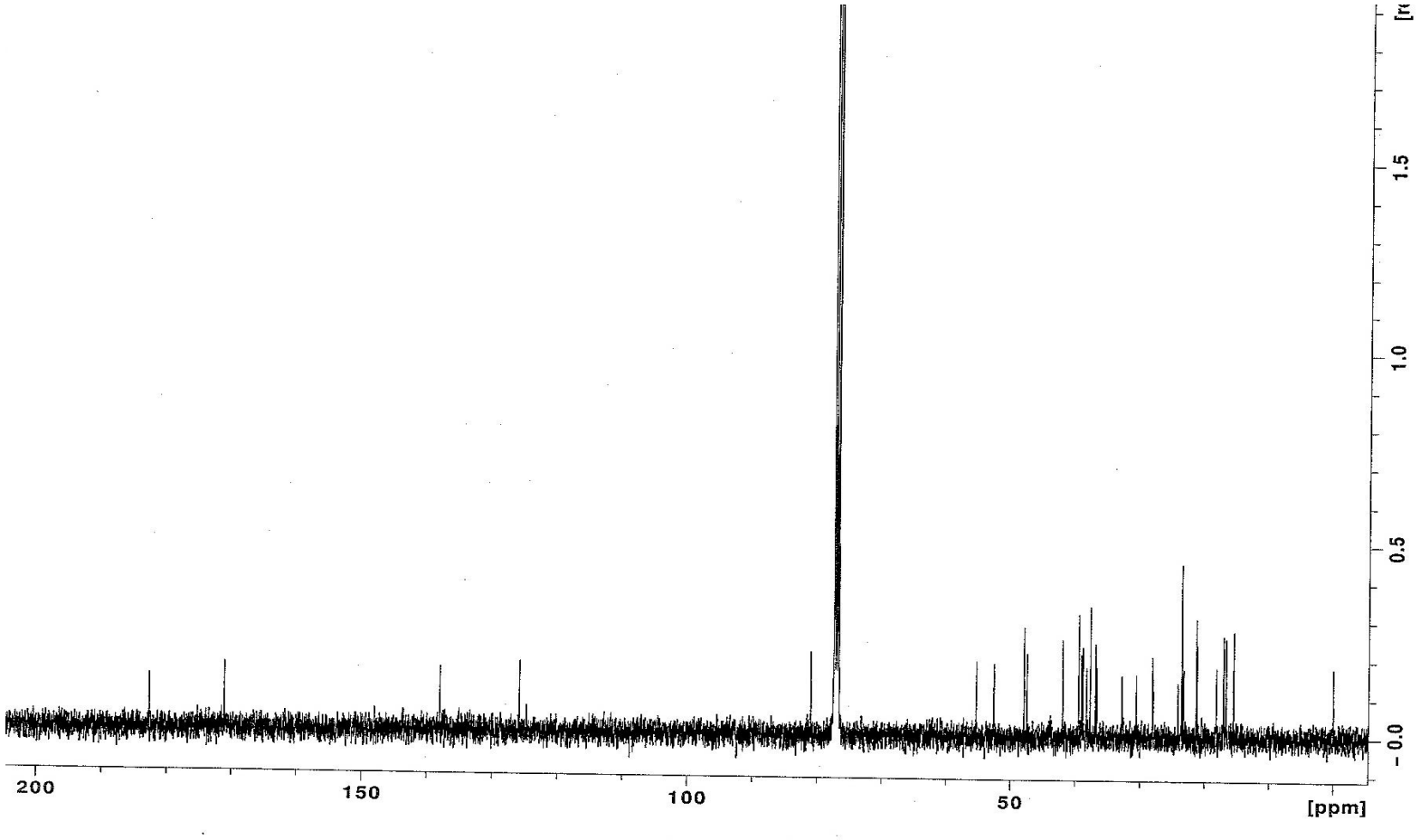


Figure 40. The ^{13}C NMR of T2

4.8 Antimycobacterial Activity of Triterpenes Isolated from *B. saligna*

Table 8 shows the MICs of UAA and OAA determined by MABA. A colour change from blue to pink indicated growth and the MIC was interpreted as the lowest concentration which prevented the colour change from blue to pink (Figure 24). When the reference strain *M. tuberculosis* $H_{37}Rv$ (25177) and *M. smegmatis* were used, UAA (Ursolic Acid Acetate) showed an MIC of 62.5 µg/ml and 125 µg/ml respectively and OAA (Oleanolic Acid Acetate) 125 µg/ml for each of the mycobacterial strains. However, both compounds isolated still had a higher MIC in comparison to the commonly used antimycobacterial drugs Rifampicin and Isoniazid. Interestingly, both compounds were effective against clinical isolates of MDR-TB and XDR-TB which were resistant to RIF and INH where UAA had an MIC of 62.5 µg/ml and OAA had an MIC of 125 µg/ml. UAA and OAA showed synergistic effects against *M. smegmatis*, MTB $H_{37}Rv$ (25177), clinical isolate MDR-TB and clinical isolate XDR-TB. UAA had a positive effect on OAA by decreasing its antimycobacterial activity from 125 µg/ml when alone to 62.5 µg/ml in combination with OAA therefore the synergistic behavior of triterpenic acid ensures smaller doses of each compound is required.

Table 8. Minimum inhibitory concentration of compounds isolated from *B. saligna* against *M. smegmatis*, *MTB* $H_{37}Rv$ (25177), clinical isolate MDR-TB and clinical isolate XDR-TB

Compound	*M. smegmatis*	MTB $H_{37}Rv$ (25177)	Clinical Isolate MDR-TB	Clinical Isolate XDR-TB
	Activity (µg/ml)			
Oleanoic Acid Acetate	125.00	125.00	125.00	125.00
Ursolic Acid Acetate	62.50	62.50	62.50	62.50
UAA/OAA	62.50	62.50	62.50	62.50
Rifampicin	1.00	1.00	na	na
Isoniazid	3.00	3.00	na	na
DMSO	na	na	na	na

(n=3); na: no activity

4.9 Molecular Docking of Triterpenes Isolated from *B. saligna*

Docking studies helped to sort out the compounds with good binding affinity against FadA5. According to the docking results (Table 9), UAA showed the highest free binding energy (-8.11) compared to the four compounds and was close to that of the reference steroid (-8.33). The affinity of both UA and UAA was almost the same (-6.70) and (-6.75), respectively. Clash penalty score for OA and OAA were the highest values, which may be the main cause of decreasing the affinity score. In addition, the Ligand conformation entropy score for UA, UAA and the reference steroid showed low and equal value (2.80) which is favored for the good binding.

Table 9. Docking results of UA, UAA, OA and OAA

	Leadit 2.1.2 Docking					
	Free binding Energy ΔG	**Docking affinity Score kcal/mol**	**Lipo score**	**Clash score**	**Rot score**	**Interacted residues and atoms**
UA	-7.52	-6.70	-11.44	8.52	2.80	His 347, Gly 379
UAA	-8.11	-6.75	10.65	7.32	2.80	His 347, Gln 151, H_2O 804
OA	-6.74	-5.97	-10.82	12.96	4.20	His 347, Ser 93, H_2O 804
OAA	-6.60	-5.87	-10.32	9.77	4.20	Gln 92 , Gly 375
Reference steroid	-8.33	-7.72	-7.65	6.61	2.80	His 347, H_2O 804

The binding modes of UA, UAA and reference steroid fadA5 inhibitor is depicted in Figure 41. The binding mode of UA was through hydrogen bond formation with the conserved His 347 and H_2O 804. While UAA showed an additional hydrogen bond with Gln 151 and H_2O 804. The steroid inhibitor showed a hydrogen bond with His 347 as well in addition to that with H_2O 804.

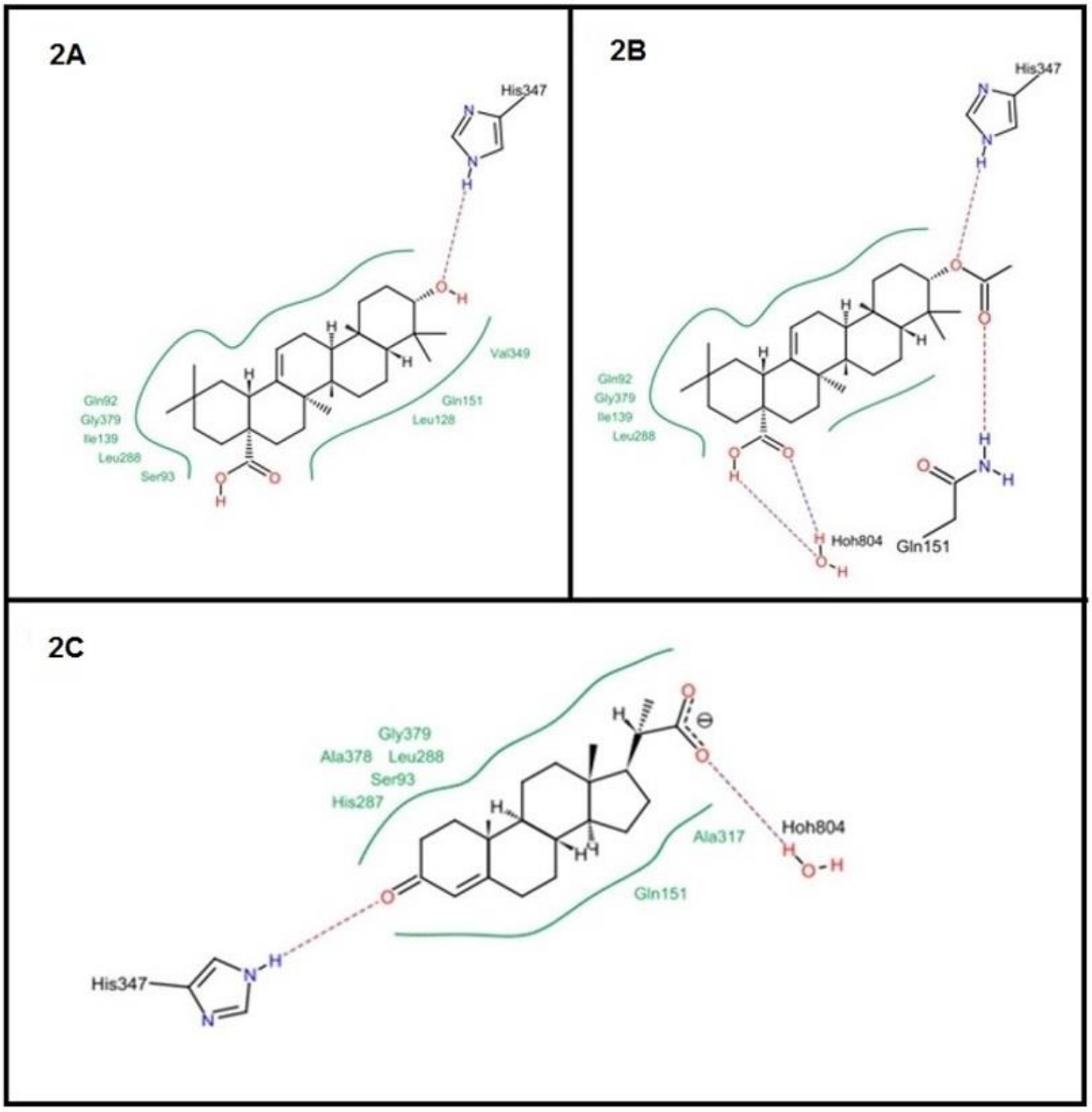


Figure 41: Binding mode of (2A) ursolic acid; (2B) ursolic acid acetate; and (2C) reference steroid fadA5 inhibitor.

OA showed some interesting interactions with His 347, Ser 93, and the water molecule of the oxyanion hole H_2O 804 (Figure 42).

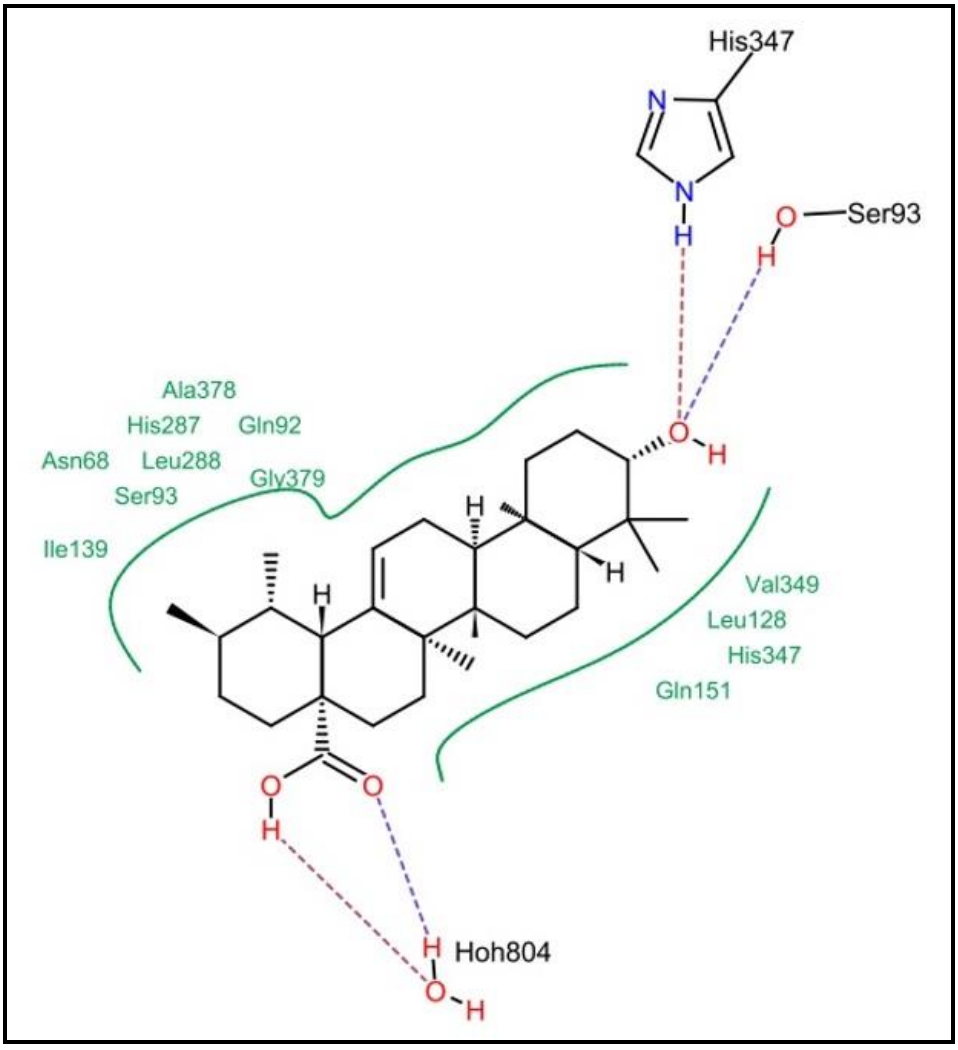


Figure 42: Binding mode of oleanolic acid.

CHAPTER 5: DISCUSSION

The emergence of MDR-TB, XDR-TB and recently reported total drug resistant (TDR) strains of *M. tuberculosis* (Pandya et al., 2012), highlighted an urgent need to find new compounds derived from herbal medicines. Due to this and the side effects of currently available drugs for the treatment of TB, greater efforts have to be put into the search for new and effective anti-TB treatment. Side effects with most MDR-TB and XDR-TB drugs include hepatotoxicity with ethionamide, ototoxicity and nephrotoxicity with aminoglycosides, and dysglycemia with gatifloxacin (Koul et al., 2011). Thus, the current situation demands the immediate identification of new therapeutics that can address emerging resistance. The quest for new effective antimycobacterial drugs motivated the present study to search for natural products from South African medicinal plants for antimycobacterial activity. A total of nine plants namely; *B. saligna, C. tomentosa, C. dimidiatus, D. cinerea, E. capensis, F. sur, G. perpensa, L. leonurus* and *T. riparia* were screened for antimyobcaterial activity.

5.1 Plant Extraction

Two different solvents were used to extract the active compounds from the leaves of *B. saligna, C. tomentosa, C. dimidiatus, D. cinerea, E. capensis, F. sur, G. perpensa, L. leonurus* and *T. riparia*. Evaluating the composition of plant extracts is important in exposing specific compounds that are responsible for their therapeutic property. The solvents used to extract the plant compounds in this study were methanol and water. The most common solvent used by traditional healers is water. However, this is limited due its inability to extract non-polar compounds. Water frequently does not dissolve the intermediate polar to nonpolar components of a dried extract (Masoko and Nxumalo, 2013). The success of determining the biologically active compounds largely depends on the type of solvent used in extraction. Therefore, it is important to use solvents which will extract all compounds, covering all range of polarity. Masoko et al. in 2008

reported methanol extracts had the best extract yield. In this study, it was confirmed that methanol was the best extractant resulting in a greater yield of plant material extracted.

5.2 Antimycobacterial Activity of the Plant Extracts

The increase of resistance to conventional antibiotics by microorganisms has necessitated the search for new, efficient and cost-effective ways to control infectious diseases (Samie et al., 2005, Ndip et al., 2007). Gautam et al. (2007) stated that no new anti-TB drugs have been introduced in the past 30 years. Plant-derived antimycobacterial compounds belong to a wide diversity of classes, including terpenoids, alkaloids, peptides, phenolics and coumarins. Thus medicinal plants serve as an important source to find novel therapeutic agents (Mmushi et al., 2010). The advantages of using these compounds include few side effects, better patient acceptance due to the long history of use, reduced costs and cultivability rendering them renewable in nature (Gur et al., 2006). A number of South African plants have been shown to contain antimycobacterial activities (Mativandlela et al., 2008, Thring et al., 2007, Lall and Meyer, 1999). In this study nine plants were initially screened for antimycobacterial activity and they were selected based on reports according to their traditional use in the treatment of various ailments including a cough, bronchitis, chest complaints, pneumonia and TB. The antimycobacterial activity was determined using the agar incorporation and broth microdilution assays (Eloff, 1998). From the eighteen extracts tested five extracts (Table 5), exhibited varying degrees of antimycobacterial activity against *M. smegmatis, M. tuberculosis* H_{37}Rv (ATCC 25177) and well characterized clinical isolates of MDR-TB and XDR-TB. *M. tuberculosis* H_{37}Rv is a virulent, slow-growing strain. This is an ideal organism for antimycobacterial drug discovery because it has a drug susceptibility profile which is representative of the majority of drug-susceptible clinical isolates. *M. smegmatis* possesses a certain degree of similarity

to *M. tuberculosis* with respect to drug susceptibility (Pauli et al., 2005) and, therefore, it is used as a model for *M. tuberculosis.*

Aqueous extracts of *G. perpensa* and *T. riparia*; methanolic extracts of *B saligna, C. tomentosa* and *C. dimidiatus* showed antimycobacterial activity in this study. Inhibition of growth by these extracts was observed against MDR-TB and XDR-TB which are resistant to first and second line TB drugs, therefore, their antimycobacterial activity appears to be meaningful. Bamuambaa et al. (2008) reported that the acetone/water (4:1) crude extract of *B. saligna***,** exhibited significant activity against *Mycobacterium aurum* A^{+}. Luo et al. (2011) reported an MIC of 125 µg/ml for the 70% ethanol extract *C. tomentosa.* Springfield and Weitz (2006) found that the ethyl acetate extract from the leaves of *Carpobrotus mellei* had an MIC of 15 mg/ml and the water extract had an MIC of 30 mg/ml against *M. smegmatis*. Lall and Meyer (1999) reported that *G. perpensa* and *T. riparia* did not show any positive antimycobacterial activity when screened against *M. tuberculosis* even at a concentration of 5 mg/ml which is contradictory to our study.

The remaining four plant species namely *D. cinerea, E. capensis, F. sur* and *L. leonurus,* in spite of being reported to be used in the treatment of TB and related diseases (Vogt, 1995, Hutchings, 1996, Lall and Meyer, 1999), failed to display any activity against the screened strains in our assays. The possible explanation could be that the antimycobacterial effect of these plants is mediated through immunostimulation or immunomodulation rather than direct inhibition of mycobacterial growth; the type of solvent used in extraction, or that the potential active compounds need to be metabolically activated in vivo by specific enzymes or may have a pH dependant biological activity (Rıos and Recio, 2005, Masoko et al., 2008). The extracts might also contain little of the active ingredient (Apak and Olila, 2006). Cell wall biosynthesis is a key target for antimycobacterial chemotherapy. Due to the complex lipoglycan calyx on the cell surface, which provide a significant physical barrier to intracellular acting

compounds (Ballell et al., 2005), many antibiotics do not work on *M. tuberculosis* (Green et al., 2010). This could explain the lack of activity shown by some of the plant extracts against MTB in this study. Therefore, the negative results obtained could not preclude the potential antimycobacterial effect of those medicinal plants.

Parametric statistics were considered unnecessary since these samples were merely screened to determine antimycobacterial activity. Such results provide evidence that these plants may be potential sources of new antimycobacterial agents even against some resistant strains.

5.3 Safety Analysis of the Active Plant Extracts

Following the promising antimycobacterial activity of the five extracts (Table 5), these were then screened for toxicity and cytotoxicity against the brine shrimp bench-top assay (Meyer et al., 1982) as well as the MTT assay (Mosmann, 1983). Although plants used medicinally are widely assumed to be safe, many are potentially toxic and must be assessed for their safety (Fennel et al., 2004). The brine shrimp assay was used for in vitro cytotoxicity screening tests (Solis et al., 1993). The test is also used routinely for plant extracts (McGaw et al., 2005, Eloff and McGaw, 2006, Fennel et al., 2004) in South Africa. However, some plants known to be toxic to livestock have displayed non-toxicity to brine shrimp (McGaw and Eloff, 2005), thus casting doubt as to whether the brine shrimp assay is capable of detecting toxic effects of plant extracts. Therefore, cell line cytotoxicity (MTT Assay) is applied alongside the Brine Shrimp Assay, as mammalian cells give a better correlation. Except for the aqueous extract of *G. perpensa* at a high concentration of 1000 µg/ml none of the other plant extracts showed any toxicity or cytotoxicity when screened against Artemia nauplii brine shrimp, mouse BALB/C monocyte-macrophage (J774.2) and human peripheral blood mononuclear cells (PBMCs) as depicted in Figures 25, 26 and 27 respectively. All plant extracts except for *G. perpensa* exhibited a stimulatory effect when exposed to the cell lines used.

5.4 Intracellular Activity of the Active Plant Extracts

M. tuberculosis is a facultative intracellular bacterial pathogen which establishes infection after inhalation of bacilli into the alveoli of the lower lungs (Chaparas, 1982). It is suspected that during the initial stages of this process, the bacilli reside principally intracellularly, surviving within the host's phagocytic cells and primarily in the alveolar macrophages (Rook and Bloom, 1994). It has been widely accepted that *M. tuberculosis* bacilli survive, multiply, and are transported through the body via the bloodstream as intracellular parasites of monocytes and macrophages (Dannenberg and Rook, 1994). Since pathogenic mycobacterial strains are intracellular parasites it is important to activate the macrophages and an antimycobacterial agent must be active inside cells (Mangalindan et al., 2000, Skinner et al., 1995).

One of the objectives of this study was to determine if the active plant extracts could prevent the intracellular growth of the model microorganism *M. smegmatis* within the macrophage cell line. The present study clearly demonstrated that the methanolic extract of *B. saligna* exerted an inhibitory effect on the phagocytic capability and intracellular killing of the mycobacteria within the mouse BALB/C monocyte-macrophage (J774.2) cell line. The extract had inactivated most of the phagocytosed bacilli after 24 hours of treatment. This allowed us to conclude that the active plant constituents have a bactericidal effect on the mycobacteria located within the mouse BALB/C monocyte-macrophage. Due to *M. smegmatis* and *M. tuberculosis* having the same sensitivity to antimycobacterial drugs (Mitscher and Baker, 1998), it can be assumed that the extract of this plant would have an inhibiting effect on *M. tuberculosis* infected macrophages. None of the other plant extracts which had an in vitro effect against the mycobacterial strains showed an inhibiting effect on the *M. smegmatis* infected macrophages (Figure 30) One of the aqueous plant extracts namely *G. perpensa,* increased the mycobacterial load within the macrophage in comparison to the in vitro activity (Figure 31 and 32). From the literature studied there are no known

reports on the intracellular effect of the plant extracts analysed therefore this is the first report.

5.5 Phytochemical Analysis of the Active Plant Extracts

The phytochemical analysis of leaves from the plants which showed promising antimycobacterial activity was screened for their protective chemicals and is shown in Table 7. The presence or absence of phytochemicals in different plant samples can be attributed to geographical origin, environmental factors and the storage of the plant material after harvesting (Van Wyk and Gericke, 2000, Van Wyk et al., 2002, Van Wyk and Gericke, 2003).

Regarding the bioactive constituents of plants, we found that most of the leaves predominantly contain tannins, phlobtannins, saponins, flavonoids, steroids, terpenoids, alkaloids and phenols. These phytochemicals are known to demonstrate medicinal activity (Edeoga et al., 2005). There is little in the published literature dealing with the chemistry of *B. saligna*, however, the genus is known to contain iridoids, acetylated iridoids, saponins, sesquiterpenoids and flavonoids (Houghton, 1999). It has previously been reported (Sama and Ajaiyeoba, 2006, Oluwole et al., 2007) that the phytochemicals that are provided by *C. tomentosa* are alkaloids, L-stachydrine, saponin glycosides, tannins, sterol, polyphenols, flavonoids and anthranoids which give it its unique biological activity. Carpobrotus species display a phytochemical profile of flavonoids, tannins, alkaloids, phytosterols and aromatic acids where the flavonoids are rutin, neohesperidin, hyperoside, catechin and ferulic acid (Springfield and Weitz, 2006, Springfield et al., 2003, Van der Watt and Pretorius, 2001). From a study undertaken by Simelane et al. (2010), they found tannins, cardiac glycosides, steroids and flavonoids present in *G. perpensa*. Previous studies for the phytochemical screening of the *T. ripara* revealed the presence of saponins, flavonoids and tannins to be the most prominent secondary metabolites (Khethiwe et al., 2011). It has been proposed that the

antimycobacterial activity of plant extracts may be due to the main bioactive components in the extracts including flavonoids, alkaloids, tannins, saponins, and polyphenols (Arya, 2011, McCarthy and Mahony, 2011). Alkaloids extracted from several plants have been reported to exhibit antimycobacterial activity (Copp, 2003). Plant terpenoids have been reported to exhibit antimycobacterial activity. Sesquiterpene, longifolene, totarol and transcommunic acid, obtained from Juniperus communis showed the highest activity against *M. tuberculosis* $H_{37}Rv$, whereas longifolene and totarol also showed activity against rifampicin-resistant variants (Gordien et al., 2009).

5.6 Bioautography of the Active Plant Extracts

The plant extracts were analysed by bioautography for qualitative analysis of antimycobacterial compounds using thin layer chromatography sprayed with *M. smegmatis.* The methanolic extracts of *B. saligna* and *C. dimidiatus* demonstrated inhibition of growth of *M. smegmatis* on the bioautograph (Figure 31). For the other plant extracts, the acquired ethnopharmacological information provided failed to be scientifically validated by bioautography as the rest of the plants failed to display any activity against *M. smegmatis*. The lack of correlation between the obtained MIC values and bioautography bands can be attributed to the vaporization of volatile active compounds during removal of the TLC eluents or disturbance of synergism between the active constituents caused by TLC separation (Masoko and Eloff, 2006).

5.7 Identification and Characterization of the Active Compounds

Silica gel column chromatography of the methanolic extract of the *B. saligna* leaves resulted in the separation of plant components using a solvent system of hexane and methanol (7:3). Compounds that crystallized after column chromatography were collected analyzed using bioassay guided fractionation. In bioassay-guided fractionation, the fractions were subjected to bioautography in order determine if the compounds

from the leaves of *B. saligna* exhibited the antimycobacterial activity against *M. smegmatis*. The unidentified compound was then acetylated using pyridine and acetic anhydride (Cheng et al., 2013) and further purified using a column which was eluted with hexane and ethyl acetate at a ratio of 9:1 (v/v). Two fractions were separated and analysed using ^{1}H NMR and ^{13}C NMR Spectroscopy. From the resulting peaks (Table 10 and Table 11) these were identified as isomeric pentacyclic triterpenes OAA and UAA as depicted in Figure 43 and the ^{1}H NMR and ^{13}C NMR data are in agreement with the reported data for UAA and OAA (Babalola and Shode, 2013, Mapanga et al., 2009, Kontogiannia et al., 2009).

The ^{13}C NMR spectrum of OAA (Table 10) shows 30 signals, consisting of eight quaternary carbons, four methines, eleven methylenes and seven methyls deduced from the DEPT experiments. The most downfield signal resonated at δ182.75 is attributed to the carboxylic acid (C-28). The appearance of signals at δ122.57 and 143.59 in C-12 and C-13 respectively indicated the presence of a double bond.

Table 10. ^{1}H NMR and ^{13}C NMR of T1 (OAA)

Carbon Position	**δ** ^{13}C (ppm)	Dept	**δ** ^{1}H (ppm)
1	38.07	CH_2	1.05(1H,*m*) 1.58(1H,*m*)
2	27.66	CH_2	1.06(1H.m) 1.70(1H,m)
3	80.93	CH	4.24(1H,*m*)
4	39.28	C	
5	55.29	CH_2	0.79(1H,m)
6	18.18	CH_2	1.30(1H,m) 1.51(1H,m)
7	32.55	CH_2	1.45(1H,m) 1.51(1H,m)
8	36.97	C	
9	47.55	CH	1.58(1H,m)
10	37.69	C	
11	23.52	CH_2	1.94-1.95 (2H,m)
12	122.57	CH	5.15(1H,m)
13	143.59	C	

14	41.59	C	
15	28.04	CH_2	1.09(1H,m) 1.63(1H,m)
16	23.39	CH_2	1.56(1H,m) 1.86(1H,m)
17	46.51	C	
18	41.00	CH	2.79(1H,*m*)
19	45.84	CH_2	1.55(1H,m) 1.69(1H,m)
20	30.67	C	
21	33.79	CH_2	1.30(1H,m) 1.38(1H,m)
22	32.43	CH_2	1.54(1H,m) 1.76(1H,m)
23	22.98	CH_3	0.89(3H,m).
24	16.66	CH_3	0.72(3H,*s*)
25	15.38	CH_3	0.99(3H,*s*)
26	17.13	CH_3	0.84(3H,*s*)
27	25.88	CH_3	1.16(3H,*s*)
28	182.75	C	
29	33.05	CH_3	0.87(3H,s)
30	23.57	CH_3	0.90(3H,s)
1’	171.04	C	
2’	22.93	CH_3	2.06(3H,*s*)

The ^{13}C NMR spectrum of UAA (Table 11) shows 30 signals, consisting of seven quaternary carbons, eight methines, eight methylenes and seven methyls deduced from the DEPT experiments. The most downfield signal resonated at δ182.6 is attributed to the carboxylic acid (C-28). The appearance of signals at δ125.8 and 138.0 in C-12 and C-13 respectively indicated the presence of a double bond.

Table 11. ^{1}H NMR and ^{13}C NMR of T2 (UAA)

Carbon Position	**δ** ^{13}C (ppm)	Dept	**δ** ^{1}H (ppm)
1	38.3	CH_2	
2	24.1	CH_2	
3	80.9	CH	3.43 (1H, brs)
4	37.7	C	

5	55.3	CH	
6	18.2	CH_2	
7	32.9	CH_2	
8	39.5	C	
9	47.9	CH	
10	36.7	C	
11	23.3	CH	
12	125.8	CH	5.50 (1H, brs)
13	138.0	C	
14	41.9	C	
15	30.6	CH_2	
16	23.6	CH_2	
17	47.5	C	
18	52.6	CH	2.52 (1H, d, J = 11.0 Hz)
19	39.0	CH	
20	38.8	CH	
21	30.6	CH_2	
22	36.9	CH_2	
23	23.6	CH_3	1.24 (3H, s)
24	17.1	CH_3	1.02 (3H, s)
25	16.7	CH_3	0.93 (3H, s)
26	17.1	CH_3	1.05 (3H, s)
27	21.3	CH_3	1.22 (3H, s)
28	182.6	C	
29	15.5	CH_3	0.97 (3H, s)
30	21.2	CH_3	0.99 (3H, d, J = 6.1 Hz)
1’	171.0	C	
2’	28.1	CH_3	2.06(3H,s)

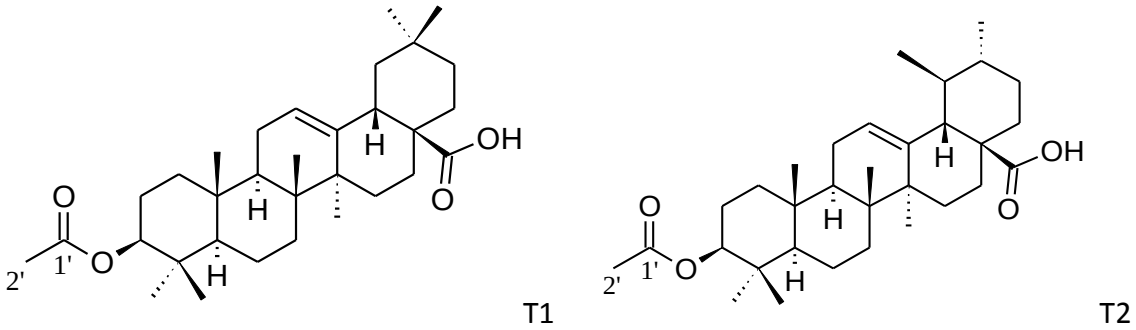


Figure 43. Chemical structures of (T1) OAA and (T2) UAA

5.8 Antimycobacterial Activity of Oleanolic Acid and Ursolic Acid

It is known that the OA (3β-hydroxy-olea-12-en-28-oic acid) and UA (3β-hydroxy-urs-12-en-28-oic-acid) are widely distributed in the plant kingdom (Liu, 2005), however, to the best of our knowledge, the isolation of UA from *B. saligna* has not been reported; Bamuambaa et al. (2008) isolated OA. OA and UA are hydroxyl pentacyclic triterpenoic acids (HPTAs). OA and UA are isomers with similar chemical structures and often exist simultaneously in the same plant as aglycones of saponins and as free acids. These isomeric triterpenic acids mostly occur in medicinal herbs and plants, and naturally in a large variety of vegetarian foods forming an integral part of the human diet. OA and UA have great variation in their distribution between different families, genera and species. OA and UA have been associated with protective effects against periodontal pathogens, antiviral activity against HIV and an antimycobacterial potential against *M. tuberculosis* which has been reportedly associated with OA and UA.

There are numerous important reports of antimycobacterial activity of UA and OA where they are mainly purified from diverse plants (Cantrell et al., 2001, Copp and Pearce, 2007, Bamuambaa et al., 2008). The antimycobacterial activity of UA against *M. tuberculosis* $H_{37}Rv$ and its derivative the fluorescent (GFP) strain $H_{37}Rv$-pFPCA1 was studied using MABA and the green fluorescent protein microplate assay with very high MIC values (65 mg/ml) (Jaki et al., 2008, Changsen C., 2003). Data published by

Bamuambaa et al. (2008) demonstrated antimycobacterial activity of UA and OA with activity fluctuating between 1.25 mg/ml and 2.5 mg/ml. Bacterial species belonging to the genus Mycobacterium are supposedly sensitive to triterpenoids, including OA and UA, because of the high sterol content of their cell envelopes (Daffe and Draper, 1988). Jiménez-Arellanes et al. (2013) reported intracellular activity of OA and UA against *M. tuberculosis* H_{37}Rv and an MDR clinical isolate in a macrophage cell line showing that both compounds, singularly and in combination, were active against intracellular mycobacteria even at low doses. Moreover, when both compounds were used to treat BALB/c mice with TB induced by MTB H_{37}Rv or MDR bacilli, a significant reduction of bacterial loads and pneumonia were observed. Jiménez-Arellanes et al. (2013) reported in vitro antimycobacterial activity of UA against *M. tuberculosis* H_{37}Rv with MICs of 50 µg/ml when evaluated by the radiorespirometric Bactec 460, and 31.0 and 41.9 µg/ml by MABA assay; while MIC values reported for OA were 50 µg/ml when tested by the radiorespirometry method and 30.0, 28.7 and 25 µg/ml by MABA.

The molecular mechanism of the antimycobacterial activity has not yet been determined but it has been proposed by Cantrell et al. in 2001 and Szakiel et al. in 2008 that UA and OA can produce significant disruptions in the bacterial cell wall. Both triterpenes have efficient antilipidic activity on eukaryotic cells (Somova et al., 2003), and perhaps this activity can also affect mycobacteria producing damage on the complex cell envelope, which is rich in lipids.

5.9 Molecular Docking Study of Oleanolic Acid and Ursolic Acid

Of the different targets being explored in mycobacteria for antimycobacterial activity, fatty acid synthesis inhibition is an attractive target for the rational design of new antimycobacterial agents. Mycolic acid is the major component of the *M. tuberculosis* cell wall (Todar, 2012). Enzymes that are responsible for fatty acid biosynthesis are

considered as ideal targets for designing the new antimycobacterial agents (Asgaonkar et al., 2014).

In *M. tuberculosis,* the cholesterol metabolism is an attractive target for the development of new antimycobacterial agents and the cholesterol catabolism is essential for the life of *M. tuberculosis* (Chang et al., 2009). Many genes are involved in the cholesterol catabolism of *M. tuberculosis* (Van der Geize et al., 2007) and fadA5 gene also known as thiolase is highly expressed during the *M. tuberculosis* infection and is involved in the cholesterol catabolism process (Dubnau et al., 2002). The gene fadA5 is responsible for catalyzing the last step of the β-oxidation reaction of the cholesterol side-chain degradation under release of critical metabolites. This is important during the chronic stage of TB infections. It was reported that any mutation in the fadA5 of *M. tuberculosis* strain will result in the reduction of the infective colonies which confirms its importance as a selective target (Nesbitt et al., 2010).

Results indicated that the pentacyclic triterpene compounds UAA and OAA, which are derivatives of UA and OA that were isolated from *B. saligna*, exhibited positive antimycobacterial activity and the compound UAA being the most active at 62.5 µg/mL (Table 8). This was predicted and interpreted by the use of molecular docking approach, which predicts the binding mode of the UA, UAA, OA, and OAA inside the steroid active site of fadA5 of *M. tuberculosis* (Figure 44). The structure of triterpenes obtained from this plant resemble the cholesterol structure. Molecular docking analysis suggested a possible blocking of the steroid active site by binding to His 347 and H_2O 804 conserved molecules required for cholesterol catabolism. Recently, the crystal structure of *M. tuberculosis* fadA5 thiolase was resolved in complex with non-covalently bound steroid (Schaefer et al., 2015). This can give excellent insights toward the inhibition of *M. tuberculosis* growth via binding to fadA5 thiolase at its steroid binding site.

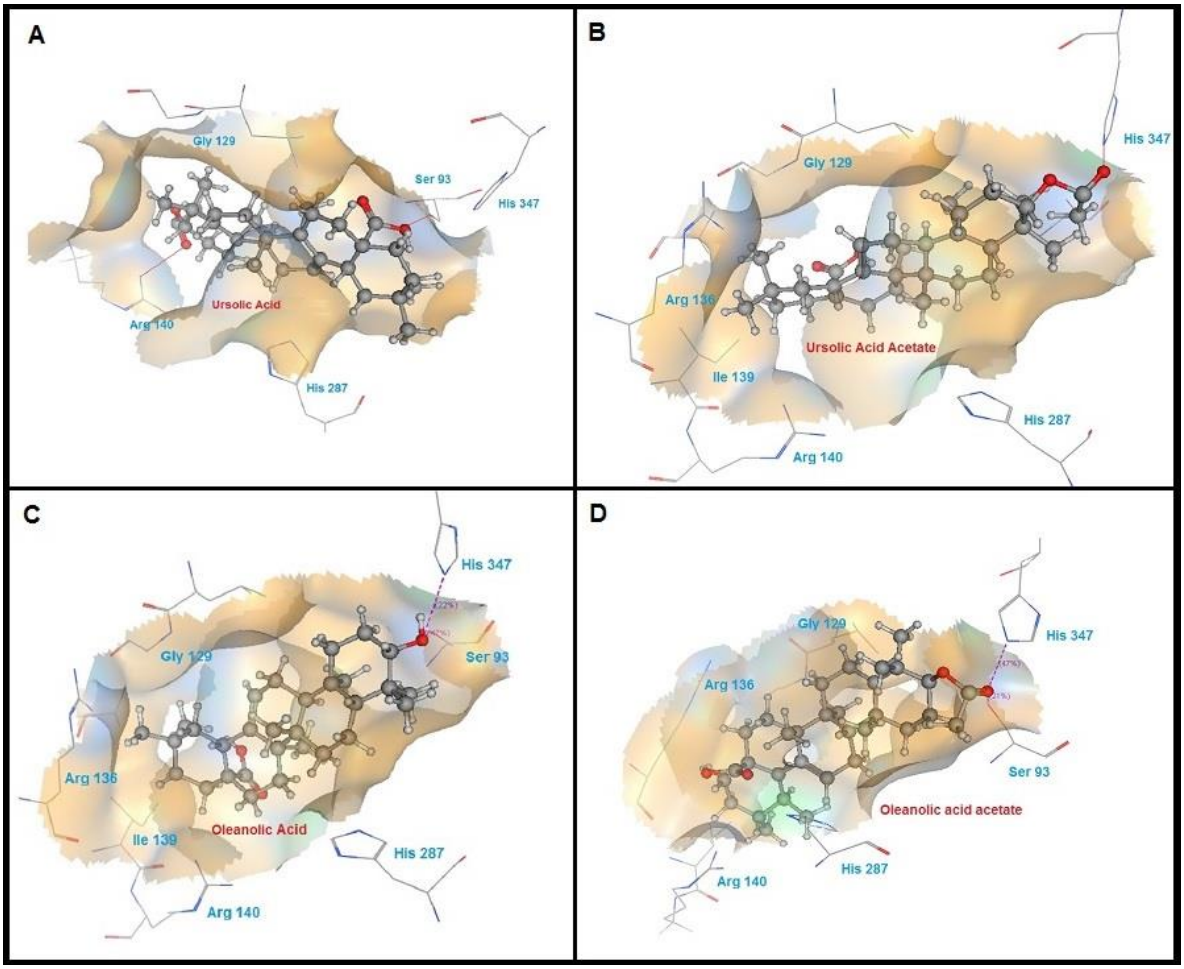


Figure 44 **:** 3D description of the best binding modes of A) Ursolic acid, B) Ursolic acid acetate, C) Oleanolic acid acetate and D) Oleanolic acid.

CHAPTER 6: CONCLUSION

In this study nine plants namely *B. saligna, C. tomentosa, C. dimidiatus, D.* cinerea, *E. capensis, F. sur, G. perpensa, L. leonurus* and *T. riparia* were chosen based on reports from literature (Scott et al., 2004, McGaw et al., 2008, Hutchings, 1996, Hutchings and Van Staden, 1994, Vogt, 1995) to validate their antimycobacterial activities and determine the active components against *M. smegmatis, M. tuberculosis* H_{37}Rv, MDR-TB, and XDR-TB. Only five of the nine plants namely *B. saligna, C. tomentosa, C. dimidiatus, G. perpensa* and *T. riparia* showed in vitro antimycobacterial activity against the strains tested. The safety profile of the five active plants was determined using cytotoxicity and toxicity screening. Except for *G. perpensa,* all the other active plant extracts proved to be non-toxic to the brine shrimp larvae, mouse BALB/C monocyte-macrophage (J774.2) and human peripheral blood mononuclear cells. The phytochemical analysis of the five active plants were determined and these plants showed that the leaves were rich in tannins, phlobtannins, saponins, flavonoids, steroids, terpenoids, alkaloids and phenols. Two plants namely *B. saligna* and *C. dimidiatus* showed zones of inhibition using TLC-Bioautography which denoted positive activity against the mycobacterium tested.

The most potent plant being *B. saligna* showed good antimycobacterial activity against all the strains tested, zones of inhibition when analysed using TLC-Bioautography, as well as intracellular antimycobacterial activity against *M. smegmatis.* Due to this plant having good inhibitory activity as well as showing an intracellular effect, the methanolic extract was subjected to isolation and characterization of compounds. Two compounds identified as isomeric pentacyclic triterpenes were isolated using silica gel column and characterized using NMR Spectroscopy. OAA ($C_{32}H_{50}O_4$) showed 30 signals, consisting of eight quaternary carbons, four methines, eleven methylenes and seven methyls. UAA ($C_{32}H_{50}O_4$) showed 30 signals, consisting of seven quaternary carbons, eight methines, eight methylenes and seven methyls deduced from the DEPT experiments. The binding

mode of the OA, OAA, UA and UAA, inside the steroid active site of fadA5 of *M. tuberculosis,* showed a possible blocking of the steroid active site by binding to conserved molecules required for cholesterol catabolism.

The results from this study provide scientific evidence in support of the use of the extracts of *B. saligna, C. tomentosa, C. dimidiatus* and *T. riparia* as candidates for the herbal formation and indicate that these plants could be possible sources of antimycobacterial drug leads due to their safety and good MIC values. This study also showed that UAA and OAA isolated from the methanolic extract of *B saligna* have a potential for optimization as lead compounds for new antimycobacterial drugs.

REFERENCES

AKOTO O., OPPONG I.V. , ADDAE-MENSAH I. , R., W. & H., A. 2008. Isolation and characterization of dipeptide derivative and phytosterol from Capparis tomentosa Lam. *Scientific Research and Essays,* 3**,** 355-358.

AMAROWICZ, R., MARYNIAK, A. & SHAHIDI, F. 2005. TLC Separation of methylated Epigallocatechin -3-Gallate. *Czech Journal of Food Science,* 23**,** 36-39.

ANDREWS, J. R., SHAH, N. S., GANDHI, N., MOLL, T. & FRIEDLAND, G. 2007. Multidrug-resistant and extensively drug-resistant tuberculosis: implications for the HIV epidemic and antiretroviral therapy rollout in South Africa. *Journal of Infectious Diseases,* 196**,** 482-490.

APAK, L. & OLILA, D. 2006. The in-vitro antibacterial activity of Annona senegalensis, Securidata longepediculata and Steganotaenia araliacea—Ugandan medicinal plants. *African Health Sciences,* 6**,** 31-35.

ARYA, V. 2011. A review on anti-tubercular plants. *International Journal of PharmTech Research,* 3**,** 872-880.

ASGAONKAR, K. D., MOTE, G. D. & CHITE, T. S. 2014. QSAR and Molecular Docking Studies of Oxadiazole-Ligated Pyrrole Derivatives as Enoyl-ACP (CoA) Reductase Inhibitors. *Scientia Pharmaceutica,* 82**,** 71-85.

BABALOLA, I. T. & SHODE, F. O. 2013. Ubiquitous Ursolic Acid: A Potential Pentacyclic Triterpene Natural Product. *Journal of Pharmacognosy and Phytochemistry,* 2**,** 214-222.

BALLELL, L., FIELD, R. A., DUNCAN, K. & YOUNG, R. J. 2005. New small-molecule synthetic antimycobacterials. *Antimicrobial Agents and Chemotherapy,* 49**,** 2153–2163.

BAMUAMBAA, K., GAMMONB, DAVID W., MEYERS , P., DIJOUX-FRANCA , M.-G. & SCOTT, G. 2008. Anti-mycobacterial activity of five plant species used as traditional medicines in the Western Cape Province (South Africa) *Journal of Ethnopharmacology* 117**,** 385–390.

BANERJEE, A., DUBNAU, E., QUEMARD, A., BALASUBRAMANIAN, V., UM, T. K. S., WILSON, D., COLLINS, G. & DE LISLE, W. R. J., JR. 1994. inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. *Science* 263**,** 227–230.

BARNIGHAUSEN, T., TANSER, F., GQWEDE, Z., MBIZANA, C., HERBST, K. & NEWELL, M. L. 2008. High HIV incidence in a community with high HIV prevalence in rural South Africa: findings from a prospective population-based study. *AIDS* 22**,** 139-144.

BEGUE, W. J. & KLINE, R. M. 1972. The use of tetrazolium salts in bioauthographic procedures. *Journal of Chromatography A,* 64**,** 182-184.

BERGMAN, B., JOHANSSON, C. & SÖDERBÄCK, E. 1992. The Nostoc - Gunnera symbiosis *New Phytologist,* 122**,** 379-400.

BODNAR, K. A., SERBINA, N. V. & FLYNN, J. L. 2001. Fate of Mycobacterium tuberculosis within murine dendritic cells. *Infection and Immunity,* 69**,** 800–809.

BOYUM, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest 21 Suppl,* 97**,** 77-89.

BRENNAN, P. J., AND H. NIKAIDO. 1995. The envelope of mycobacteria. *Annual Review of Biochemistry,* 64**,** 29-63.

BUWA, L. V. & AFOLAYAN, A. J. 2009. Antimicrobial activity of some medicinal plants used for the treatment of tuberculosis in the Eastern Cape Province, South Africa. *african Journal of Biotechnology,* 8**,** 6683–6687.

CANTRELL, C. L., FRANZBLAU, S. G. & FISCHER, N. H. 2001. Antimycobacterial plant terpenoids. *Planta Medica,* 67**,** 685–694.

CARR, J. D. & ROGERS, C. B. 1987. Chemosystematic studies of the genus Combretum [Combretaceae], I. A convenient method of identifying species of this genus by a comparison of the polar constituents extracted from leaf material. *South African Journal of Botany,* 53**,** 173-176.

CDC. 2005. Guidelines for preventing the transmission of Mycobacterium tuberculosis in health-care settings. 54.

CDC. 2014. National Tuberculosis Surveillance System Highlights from 2013.

CHAKRADHAR, S. 2012. *Tuberculosis Under the Microscope* [Online]. Harvard College: Harvard Medical School Available: http://hms.harvard.edu/news/tuberculosis-under-microscope-10-24-12 [Accessed 11 September 2014].

CHANG, J. C., MINER, M. D., PANDEY, A. K., GILL, W. P., HARIK, N. S., SASSETTI, C. M. & SHERMAN, D. R. 2009. igr Genes and Mycobacterium tuberculosis cholesterol metabolism. *J Bacteriol,* 191**,** 5232-9.

CHANGSEN C., F. S. G., PALITTAPONGARNPIN P., 2003. Improved green fluorescent protein reporter gene based, microplate screening for antituberculosis compounds by utilizing an acetamidase promoter. *Antimicrobial Agents and Chemotheraphy,* 47**,** 3682-3687.

CHAPARAS, S. D. 1982. The immunity in tuberculosis. *WHO Bulletin,* 4**,** 447–462.

CHEN, M., GAN, H. & REMOLD, H. G. 2006. A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. *The Journal of Immunology,* 176**,** 3707–3716.

CHENG, S.-Y., WANG, C.-M., CHENG, H.-L., CHEN, H.-J., HSU, Y.-M., LIN, Y.-C. & CHOU, C.-H. 2013. Biological Activity of Oleanane Triterpene Derivatives Obtained by Chemical Derivatization. *Molecules,* 18**,** 13003-13019.

CLARKSON, C., MADIKANE, E. V., HANSEN, S. H., SMITH, P. J. & JAROSZEWSKI, J. W. 2007. HPLC SPE-NMR characterization of sesquiterpenes in an antimycobacterial fraction from Warburgia salutaris. *Planta Medica,* 73**,** 578–584.

CONSTANTINE, G. H., KARCHESY, J. J., FRANZBLAU, S. G. & LAFLEUR, L. E. 2001. Totarol from Chamaecyparis nootkatensis and activity against Mycobacterium tuberculosis *Fitoterapia,* 72**,** 572–574.

COPP, B. R. 2003. Antimycobacterial natural products. *Natural Product Reports,* 20**,** 535-557.

COPP, B. R. & PEARCE, A. N. 2007. Natural product growth inhibitors of *Mycobacterium tuberculosis Natural Product Reports,* 24**,** 278-297.

CORBETT, E. L., WATT, C. J., WALKER, N., MAHER, D., WILLIAMS, B. G., RAVIGLIONE, M. C. & DYE, C. 2003. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Archives of Internal Medicine,* 163**,** 1009-1021.

CYNAMON, M. H., KLEMENS, S. P., SHARPE, C. A. & CHASE, S. 1999. Activities of several novel oxazolidinones against Mycobacterium tuberculosis in a murine model. *Antimicrobial Agents and Chemotheraphy,* 43**,** 1189–1191.

DAFFE, M. & DRAPER, P. 1988. The envelope layers of mycobacteria with reference to their pathogenicity. *Advances in Microbiology and Physiology,* 39**,** 131-203.

DANNENBERG, A. M., JR., & ROOK, G. A. W. 1994. Pathogenesis of pulmonary tuberculosis; an interplay of tissue-damaging and macrophage-activating immune responses—dual mechanisms that control bacillary multiplication. *In:* BLOOM, B. R. (ed.) *Tuberculosis: pathogenesis, protection, and control.* Washington DC: ASM Press.

DOH 2012. Annual Performance Plan 2012/13 - 2014/15" *In:* HEALTH, D. O. (ed.). South Africa: Department of Health.

DOOLEY, K. E., OBUKU, E. A., DURAKOVIC, N., BELITSKY, V., MITNICK, C. & NUERMBERGER, E. L. 2013. World Health Organization Group 5 Drugs for the Treatment of Drug-Resistant Tuberculosis: Unclear Efficacy or Untapped Potential? *Journal of Infectious Diseases,* 207**,** 1352–1358.

DUBNAU, E., FONTAN, P., MANGANELLI, R., SOARES-APPEL, S. & SMITH, I. 2002. Mycobacterium tuberculosis genes induced during infection of human macrophages. *Infect Immun,* 70**,** 2787-95.

DUCATI, R. G., RUFFINO-NETTO, A., BASSO, L. A. & SANTOS, D. S. Mem Inst Oswaldo Cruz, 2006 Rio de Janeiro. 697-714.

EDEOGA, H. O., OKWU, D. E. & MBAEBIE, B. O. 2005. Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology* 4**,** 685-688

EDINGTON, M. 2014. Tuberculosis in South Africa. Durban: Health Systems Trust.

ELDEEN, I. M. S. & VAN STADEN, J. 2007. Antimycobacterial activity of some trees used in South African traditional medicine. *South African Journal of Botany* 73**,** 248–251.

ELOFF, J. N. 1998. A Sensitive and quick Microplate Method to determine the Minimum Inhibitory Sensitive and Concentration of Plant Extracts for Bacteria *Planta Medica,* 64**,** 711-713.

ELOFF, J. N. & MCGAW, L. J. 2006. Plant extracts used to manage bacterial, fungal and parasitic infections in southern Africa. *In:* PHYTOMEDICINE, M. (ed.) *Turning medicinal plants in drugs.* Weinheim: Wiley-VCHVerlag GmbH and Co.

FENNEL, C. W., LINDSEY, K. L., MCGAW, L. J., SPARG, S. G., STAFFORD, G. I., ELGORASHI, E. E., GRACE, O. M. & VAN STADEN, J. 2004. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicity. *Journal of Ethnopharmacology,* 94**,** 205-217.

FRESHNEY, R. I. 1987. *Culture of Animal Cells: A Manual of Basic Technique,* New York, Alan R. Liss.

FRIEDEN, T. R., STERLING, T. R., MUNSIFF, S. S., WATT, C. J. & DYE, C. 2003. Tuberculosis. *Lancet,* 23**,** 887-899.

FRIIS-MOLLER, A., CHEN, M., FUURSTED, K., CHRISTENSEN, S. B. & KHARAZMI, A. 2002. In vitro antimycobacterial and antilegionella activity of licochalcone A from Chinese licorice roots. *Planta Medica,* 68**,** 416–419.

GANDHI, N. R., MOLL, A., STURM, A. W., PAWINSKI, R., GOVENDER, T. & LALLOO, U. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 368**,** 1575-1580.

GARCÍA-PÉREZ, B. E., MONDRAGÓN-FLORES, R. & LUNA-HERRERA, J. 2003. Internalization of Mycobacterium tuberculosis by macropinocytosis in non-phagocytic cells. *Microbial Pathogenesis,* 35**,** 49-55.

GAUTAM, R., SAKLANI, A. & JACHAK, S. M. 2007. Indian medicinal plants as a source of antimycobacterial agents. *Journal of Ethnopharmacology* 110**,** 200–234.

GILLESPIE, S. H., MORRISSEYL, E. D. & EVERETT, D. 2001. A comparison of the bactericidal activity of quinolone antibiotics in a Mycobacterium fortuitum model *Journal of Medical Microbiology,* 50**,** 565-570.

GLAZIOU, P., FALZON, D., FLOYD, K. & RAVIGLIONE, M. 2013. *Global Epidemiology of Tuberculosis,* New York, Thieme Medical Publishers.

GONZALEZ-JUARRERO, M., TURNER, O. C., TURNER, J., MARIETTA, P., BROOKS, J. V. & ORME, I. M. 2001. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis. *Infection and Immunity,* 69**,** 1722–1728.

GORDIEN, A. Y., GRAY, A. I., FRANZBLAU, S. G. & SEIDEL, V. 2009. Antimycobacterial terpenoids from Juniperus communis L. (Cuppressaceae). *Journal of Ethnopharmacology,* 126**,** 500-505.

GORDON, R. & SMITH, M. 1953. Rapidly growing, acid fast bacterial species. *Journal of Bacteriology.,* 66**,** 41-48.

GRANGE, J. M. 1999. DOTS with a SMILE. *International Journal of Tuberculosis and Lung Disease,* 3**,** 360.

GREEN, E., SAMIE, A., OBI, C. L., BESSONG, P. O. & NDIP, R. N. 2010. Inhibitory properties of selected South African medicinal plants against *Mycobacterium tuberculosis*. *Journal of Ethnopharmacology,* 130**,** 151–157.

GUR, S., TURGUT-BALIK, D. & GUR, N. 2006. Antimicrobial activities and some fatty acids of turmeric, ginger root and linseed used in the treatment of infectious diseases. *Journal of Agricultural Science,* 2**,** 4.

HARBORNE, J. B. 1973. *Photochemical Methods: A Guide to Modern Techniques of Plant Analysis,* London, Chapman A.& Hall.

HAYMAN, J. 1984. Mycobacterium ulcerans: an infection from Jurassic time? . *Lancet,* 2**,** 1015-1016.

HETT, E. C. & RUBIN, E. J. 2008. Bacterial Growth and Cell Division: a Mycobacterial Perspective. *Microbiology And Molecular Biology Review,* 72**,** 126-156.

HINGLEY-WILSON, S. M., SAMBANDAMURTHY, V. K. & JACOBS JR, W. R. 2003. Survival perspectives from the world's most successful pathogen, Mycobacterium tuberculosis. *Nature Immunology,* 4**,** 949–955.

HOUGHTON, P. J. 1985. Phenylpropanoid glycosides in Buddleja davidii. *Journal of Natural Products,* 48**,** 1005.

HOUGHTON, P. J. 1999. Wound-healing properties of Buddleja globosa (Buddlejaceae) *2000 Years of Natural Products Research: past, present and future.* Amsterdam: Congress.

HUANG, P. H., CHEN, W. S. & HU, Y. 1980. Studies on antituberculosis constituents from Ardisia japonica. *Yaoxue Tongbao* 15**,** 39.

HUTCHINGS, A. 1996. *Zulu medicinal plants,* Pietermaritzburg, Natal University Press.

HUTCHINGS, A. & VAN STADEN, J. 1994. Plants used for stress-related ailments in traditional Zulu, Xhosa and Sotho medicine Part I. Plants used for headaches. *Journal of Ethnopharmacology* 43**,** 89–124.

HYDE, M. A. & WURSTEN, B. 2010. Flora of Zimbabwe: Species information: Capparis tomentosa. Available: http://www.zimbabweflora.co.zw/speciesdata/species.php?species_id=124460 [Accessed 4 October 2014].

JACKSON, C. J., LAMB, D. C., KELLEY, D. E. & STEVEN, S. L. 2000. Bactericidal and inhibitory effects of azole antifungal compounds on Mycobacterium smegmatis. *FEMS Microbiology Letters* 192**,** 159–162.

JAKI, B. U., FRANZBLAU, S. G., CHADWICH, L. R., LAKIN, D. C., ZHANG, F. & WANG, Y. 2008. Purity-activity relationships of natural products: the case of anti-TB active ursolic acid. *Journal of Natural Products,* 71**,** 1742-1748.

JAKUPOVIC, J., KUHNKE, J., SCHUSTER, A., METWALLY, M. A. & BOHLMANN, F. 1986. Phloroglucinol derivatives and other constituents fromSouth African Helichrysum species. *Phytochemistry,* 25**,** 1133–1142.

JARLIER, V. & NIKAIDO, H. 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiology Letters,* 123**,** 11-18.

JIMÉNEZ-ARELLANES, A., LUNA-HERRERA, J., CORNEJO-GARRIDO, J., LÓPEZ-GARCÍA, S., CASTRO-MUSSOT, M. E., MECKES-FISCHER, M., MATA-ESPINOSA, D., MARQUINA, B., TORRES, J. & HERNÁNDEZ-PANDO, R. 2013. Ursolic and oleanolic acids as antimicrobial and immunomodulatory compounds for tuberculosis treatment. *BMC Complementary and Alternative Medicine,* 13**,** 2-11.

KALÁB, M., ALLAN-WOJTAS, P. & SHEA-MILLER, S. 1995. Microscopy and other imaging techniques in food structure analysis. *Trends Food Science and Technology,* 6**,** 117-186.

KANOKMEDHAKUL, S., KANOKMEDHAKUL, K., PHONKERD, N., SOYTONG, K., KONGSAEREE, P. & SUKSAMRARN, A. 2002. Antimycobacterial anthraquinone-chromanone compound and diketopiperazine alkaloid from the fungus Chaetomium globosum KMITL-N080. *Planta Medica,* 68**,** 834-836.

KATERERE, D. R., GRAY, A. I., NASH, R. J. & WAIGH, R. D. 2003. Antimicrobial activity of pentacyclic triterpenes isolated from African Combretaceae. *Phytochemistry,* 63**,** 81–88.

KHAN, F., PETER, X. K., MACKENZIE, R. M., KATSOULIS, L., GEHRING, R., MUNRO, O. Q., VAN HEERDEN, F. R. & DREWES, S. E. 2004. Venusol from Gunnera perpensa: structural and activity studies. *Phytochemistry,* 65**,** 1117-1121.

KHETHIWE, K. J., OYEDEJI, A. O., OPOKU, A. R. & LEWU, F. B. 2011. *Essential Oil Composition and Some Biological Activities of Tetradenia Riparia.* Masters Degree in Chemistry, University of Zululand.

KOLODZIEJ, H. 2000. Traditionally used Pelargonium species: chemistry and biological activity of umckaloabo extracts and their constituents. *Current Topics in Phytochemistry,* 3**,** 77–93.

KONTOGIANNIA, V. G., EXARCHOUB, V., TROGANISC, A. & GEROTHANASSISA, I. P. 2009. Rapid and novel discrimination and quantification of oleanolic and ursolic acids in complex plant extracts using two-dimensional nuclear magnetic resonance spectroscopy—Comparison with HPLC methods. *Analytica Chimica Acta,* 635**,** 188–195.

KOUL, A., ARNOULT, E., LOUNIS, N., GUILLEMONT, J. & ANDRIES, K. 2011 The challenge of new drug discovery for tuberculosis. *Nature Immunology,* 469**,** 483- 490.

KUETE, V., MELI, A. L., KOMGUEM, J., LOUH, G. N., TANGMOUO, J. G., LONTSI, D., MEYER, J. J. M. & LALL, N. 2007. Antimycobacterial, antibacterial and antifungal activities of the methanolic extract and compounds from Garcinia polyantha. *Pharmacology Online,* 3**,** 87–95.

KUNLE, O. O., SHITTUA, U. A., NASIPURIA, R. N., KUNLEA, O. F., WAMBEBEA, C. & AKAHB, P. A. 1999. Gastrointestinal activity of Ficus sur. *Fitoterapia,* 70**,** 542-547.

LALL, N. & MEYER, J. J. M. 1999. In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. *Journal of Ethnopharmacology* 66**,** 347–354.

LALL, N. & MEYER, J. J. M. 2001. Inhibition of drug-sensitive and drug-resistant strains of Mycobacterium tuberculosis by diospyrin, isolated from Euclea natalensis. *Pharmaceutical Biology,* 43**,** 353–357.

LALL, N., MEYER, J. J. M., WANG, Y., BAPELA, N. B., VAN RENSBURG, C. J. E., FOURIE, B. & FRANZBLAU, S. G. 2005. Characterization of intracellular activity of antitubercular constituents from the roots of Euclea natalensis. *Pharmaceutical Biology* 43**,** 353–357.

LAONIGRO, G., LANZETTA, R., PARRILLI, M., ADOLFINI, M. & MANGONI, L. 1979. The configuration of the diterpene spiroethers from Marrubium vulgare and from Leonotis leonurus. *Gazetta Chimica Italia,* 109**,** 145-150.

LEEUWENBERG, A. J. M. 1979. The Loganiaceae of Africa XVIII Buddleja L. *In:* WAGENINGEN, H. V. Z. (ed.) *Revision of the African & Asiatic species.* Nederland.

LEHOHLA, P. J. 2010. Mortality and causes of death in South Africa, 2010: Findings from death notification,. Pretoria: Statistics SA.

LIU, J. 2005. Oleanolic acid and ursolic acid: research perspectives. *Journal of Ethnopharmacology,* 100**,** 92-94.

LOKWANI, D., BHANDARI, S. V., PUJARI, R., SHASTRI, P., SHELKE, G. & PAWAR, V. 2011. Use of Quantitative Structure–Activity Relationship (QSAR) and ADMET prediction studies as screening methods for design of benzyl urea derivatives for

anti-cancer activity. *Journal of Enzyme Inhibition and Medicinal Chemistry,* 26**,** 319–331.

LÖNNROTH, K. & RAVIGLIONE, M. 2008. *Global epidemiology of tuberculosis: prospects for control,* New York, Thieme Medical Publishers.

LUO, X., PIRES, D., AÍNSA, J. A., NA GRACIA, B., MULHOVO, S., DUARTE, A., ANES, E. & FERREIRA, M.-J. U. 2011. Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique. *Journal of Ethnopharmacology,* 137**,** 114– 120.

MADIKANE, V. E., BHAKTA, S., RUSSELL, A. J., CAMPBELL, W. E., CLARIDGE, T. D. W., ELISHA, B. G., DAVIES, S. G., SMITH, P. & SIM, E. 2007. Inhibition of mycobacterial arylamine N-acetyltransferase contributes to anti-mycobacterial activity of Warburgia salutaris. *Bioorganic and Medicinal Chemistry,* 15**,** 3579–3586.

MALAN, C. & NOTTEN, A. 2006. *Leonotis leonurus* [Online]. Available: www.plantzafrica.com [Accessed 10 November 2014].

MANGALINDAN, G. C., TALAUE, M. T., CRUZ, L. J., FRANZBLAU, S. G., ADAMS, L. B., RICHARDSON, A. D., IRELAND, C. M. & CONCEPCION, G. P. 2000. Agelasine F from a Philippine Agelas sp. sponge exhibits in vitro antituberculosis activity. *Planta Medica,* 66**,** 364-365.

MAPANGA, R. F., TUFTS, M. A., SHODE, F. O. & MUSABAYANE, C. T. 2009. Renal Effects of Plant-Derived Oleanolic Acid in Streptozotocin-Induced Diabetic Rats. *Laboratory Studies,* 31**,** 481-491.

MASOKO, P. & ELOFF, J. N. 2006. Bioautography indicates the multiplicity of antifungal compounds from twenty-four southern African Combretum species (Combretaceae). *African Journal of Biotechnology,* 5**,** 1625–1647.

MASOKO, P., MMUSHI, T. J., MOGASHOA, M. M., MOKGOTHO, M. P., MAMPURU, L. J. & HOWARD, R. L. 2008. In vitro evaluation of the antifungal activity of Sclerocarya birrea extracts against pathogenic yeasts. *African Journal of Biotechnology,* 7**,** 3521-3526.

MASOKO, P. & NXUMALO, K. M. 2013. Validation of Antimycobacterial Plants Used by Traditional Healers in Three Districts of the Limpopo Province (South Africa). *Evidence-Based Complementary and Alternative Medicine,* 2013**,** 1-7.

MATIVANDLELA, S. P. N., MEYER, J. J. M., HUSSEIN, A. A., HOUGHTON, P. J., HAMILTON, C. J. & LALL, N. 2008. Activity against Mycobacterium smegmatis and M. tuberculosis by extracts of South African medicinal plants. *Phytotherapy Research* 22**,** 841– 845.

MATSUYAMA, T., KOBAYASHI, N. & YAMAMOTO, N. 1991. Cytokines and HIV infection: is AIDS a tumour necrosis factor disease? *AIDS,* 5**,** 1405-1417.

MCCARTHY, E. & MAHONY, M. O. 2011. What's in a name? Can mullein weed beat TB where modern drugs are failing? *Evidence Based Complementary and Alternate Medicine,* **,** 1-7.

MCGAW, L. J. & ELOFF, J. N. 2005. Screening of 16 poisonous plants for antibacterial, anthhelmintic and cytotoxicity activity in vitro. *South African Journal of Botany,* 71**,** 302-306.

MCGAW, L. J., GEHRING, R., KATSOULIS, L. & ELOFF, J. N. 2005. Is the use of Gunnera perpensa extracts in endometritis related to antibacterial activity? *Onderstepoort J Vet Res,* 72**,** 129-134.

MCGAW, L. J., LALL , N., MEYER , J. J. M. & ELOFF, J. N. 2008. The potential of South African plants against *Mycobacterium* infections. *Journal of Ethnopharmacology 119,* 119**,** 482–500.

MEGEHEE, J. & LUNDRIGAN, M. 2007. Temporal expression of Mycobacterium smegmatis respiratory terminal oxidases. *Canadian Journal of Microbiology,* 53**,** 459-460.

MEHTA, P. K., KARLS, R. K., WHITE, E. H., ADES, E. W. & QUINN, F. D. 2006. Entry and intracellular replication of Mycobacterium tuberculosis in cultured human microvascular endothelial cells. *Microbial Pathogenesis,* 41**,** 119-124.

MENDES, E. J. 1978. Haloragaceae. *Flora Zambesiaca,* 4**,** 79-81.

MEYER, B. N., FERRIGN, R. N., PUTNAM, J. E., JACOBSON, L. B., NICHOLAS, D. E. & MCLAUGHLIN, J. L. 1982. Brine Shrimp: A Convenient General Bioassay for Active Plant Constituents. *Planta Medica,* 45**,** 31-34.

MEYER, J. J. M., LALL, N. & MATHEKGA, A. D. M. 2002. In vitro inhibition of drug-resistant and drug-sensitive strains ofMycobacterium tuberculosis by Helichrysum caespititium. *South African Journal of Botany,* 68**,** 90–93.

MIKUSOVA, K., SLAYDEN, R. A., BESRA, G. S. & BRENNAN, P. J., AND H. NIKAIDO. 1995. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrobial Agents and Chemotheraphy,* 39**,** 2484-2489.

MISHRA, S. N., TOMAR, P. C. & LAKRA.N. 2006. Medicinal and food value of Capparis—a harsh terrain plant. *Indian Journal of Traditional Knowledge,* 6**,** 230-238.

MITSCHER, L. A. & BAKER, W. R. 1998. A search for novel chemotherapy against tuberculosis amongst natural products. *Pure Applied Chemistry,* 70**,** 365-371.

MMUSHI, T., MASOKO, P., MDEE, L., MOKGOTHO, M., MAMPURU, L. & HOWARD, R. 2010. Antimycobacterial evaluation of fifteen medicinal plants in South Africa. *African Journal of Traditional Complimentary and Alternative Medicine,* 7**,** 34–39.

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays *Journal of Immunological Methods,* 65**,** 55-63.

MULAUDZI, R. B., NDHALA, A. R., KULKARNI, M. G., FINNIE, J. F. & VAN STADEN, J. 2011. Antimicrobial properties and phenolic contents of medicinal plants used by the Venda people for conditions related to venereal diseases *Journal of Ethnopharmacology,* 135**,** 330-337.

MURILLO, J. I., ENCARNACION-DIMAYUGA, R., MALMSTROM, J., CHRISTOPHERSEN, C. & FRANZBLAU, S. G. 2003. Antimycobacterial flavones from Haplopappus sonorensis *Fitoterapia,* 74**,** 226–230.

NDIP, R. N., TARKANG, A. E. M., MBULLAH, S. M., LUMA, H. N., MALONGUE, A., NDIP, L. M., NYONGBELA, K., WIRMUM, C. & EFANGE, S. M. N. 2007. In vitro anti-Helicobacter pylori activity of selected medicinal plants from North West Cameroon. *Journal of Ethnopharmacology,* 114**,** 452–457.

NEEDHAM, D. M., GODFREY-FAUSSETT, P. & FOSTER, S. D. 1998. Barriers to tuberculosis control in urban Zambia: the economic impact and burden on patients prior to diagnosis. *The International Journal of Tuberculosis and Lung Disease* 2**,** 811–817.

NESBITT, N. M., YANG, X., FONTAN, P., KOLESNIKOVA, I., SMITH, I., SAMPSON, N. S. & DUBNAU, E. 2010. A thiolase of Mycobacterium tuberculosis is required for virulence and production of androstenedione and androstadienedione from cholesterol. *Infect Immun,* 78**,** 275-82.

NEWTON, S. M., LAU, C. & WRIGHT, C. W. 2000. A review of antimycobacterial natural products *Phytotheraphy Research,* 14**,** 303-322.

NGUYEN, L. & THOMPSON, C. J. 2006. Foundations of antibiotic resistance in bacterial physiology: the mycobacterial paradigm. *Trends Microbiology,* 14**,** 304-312.

NIEMI, M., BACKMAN, J. T., FROMM, M. F., NEUVONEN, P. J. & KIVISTO, K. T. 2003. Pharmacokinetic interactions with rifampicin: clinical relevance. *Clinical Pharmacokinetics,* 42**,** 819–850.

OJEWOLE, J. A. 2005. Antinociceptive, antiinflammatory and antidiabetic effects of Leonotis leonurus (L.) R. BR. [Lamiaceae] leaf aqueous extract in mice and rats. *Methods and Findings in Experimental and Clinical Pharmacology,* 27**,** 257-264.

OKUNADE, A., L.,, ELVIN-LEWIS, M. P. F. & LEWIS, W. H. 2004. Natural antimycobacterial metabolites: current status. *Phytochemistry,* 65**,** 1017–1032.

OLUWOLE, O. G. A., SUKATI, N. A., DLAMINI, P. S. & SIBANDZE, F. G. 2007. Some Swazi plants phytomedicines and their constituents. *African Journal of Biotechnology,* 6**,** 267-272.

ORWA, C., MUTUA, A., KINDT, R., JAMNADASS, R. & ANTHONY, S. 2009. Agroforestree Database:a tree reference and selection guide version 4.0 Available: (http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp).

PADAYATCHI, N., NAIDU, N. & LOVEDAY, M. 2014. Drug Resistant Tuberculosis Control in South Africa – Scientific Advances and Health System Strengthening are Complementary. *Expert Opinion on Pharmacotherapy,* 15**,** 2113–2116.

PANDYA, K., PATEL, P., PATEL, G. & PARIKH, V. 2012. In vitro antimycobacterial study of essential oil of few selected plants. *International Journal of Pharma and Bioscience,* 2**,** 523-529.

PATTERSON, M. K. 1979. Measurement of growth and viability of cells in culture. *Methods Enzymology,* 58.

PAULI, G. F., CASE, R. J., INUI, T., WAND, Y., CHO, S., FISCHER, N. H. & FRANZBLAU, S. G. 2005. New perspectives on natural products in TB drug research. *Life Sciences,* 78**,** 485-494.

POOLEY, E. 1993. *The Complete Field Guide to Trees of Natal, Zululand and Transkei.*

POOLEY, E. 1998. *A field guide to the wild flowers of KwaZulu-Natal and the eastern region,* Durban, Natal Flora Publications Trust.

RAJA, A. 2004. Immunology of Tuberculosis. *Indian Journal of Medical Research,* 120**,** 213-232.

RAREY, M., KRAMER, B., LENGAUER, T. & KLEBE, G. 1996. A Fast Flexible Docking Method using an Incremental Construction Algorithm. *Journal of Molecular Biology,* 261**,** 470-489.

RASTOGI, N., ABAUL, J., GOH, K. S., DEVALLOIS, A., PHILOGENE, E. & BOURGEOIS, P. 1998. Antimycobacterial activity of chemically defined natural substances from the Caribbean flora in Guadeloupe. *FEMS Immunology and Medical Microbiology* 20**,** 267–273.

RAVEN, P. H., EVERT, R. F. & EICHHORN, S. E. 1999. *Families yielding important phytopharmaceuticals,* New York.. W.H. Freeman.

REID, K. A., MAES, J., VAN STADEN, J., DE KIMPE, N., MULHOLLAND, D. A. & VERSCHAEVE, L. 2006. Evaluation of the mutagenic and antimutagenic effects of South African plants. *Journal of Ethnopharmacology,* 106**,** 44-50.

RIOS, J. L. & RECIO, M. C. 2005. Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology,* 100**,** 80-84.

RODRIGUEZ, I. I. & RODRIGUEZ, A. D. 2003. Homopseudopteroxazole, a new antimycobacterial diterpene alkaloid from Pseudopterogorgia elisabethae. *Journal of Natural Products* 66**,** 855–857.

ROOK, G. A. W. & BLOOM, B. R. 1994. Mechanisms of pathogenesis in tuberculosis. *In:* BLOOM, B. R. (ed.) *Tuberculosis: pathogenesis, protection, and control.* Washington DC: ASM Press.

ROUILLON, A. 1972. Problems raised by the organization of an efficient ambulatory treatment for tuberculous patients. *Bulletin of the International Union against Tuberculosis and Lung Disease,* 47**,** 72-87.

RUSSELL, D., G. 2001. Mycobacterium tuberculosis: Here Today, and Here Tomorrow. *Nature Reviews / Molecular Cell Biology,* 2**,** 1-9.

RUSSELL, S. 2004. The economic burden of illness for households in developing countries: a review of studies focusing on malaria, tuberculosis and human immunodeficiency virus/acquired immunodeficiency syndrome. *American Journal of Tropical Medicine and Hygiene,* 71**,** 147–155.

SACHEV, K. & KULSHRESHTHA, D. K. 1984. Dodonic acid, a new diterpenoid from Dodonaea viscosa. *Planta Medica,* 50**,** 448–449.

SALIE, F., EAGLES, P. F. K. & LENG, H. M. J. 1996. Preliminary antimicrobial screening of four South African Asteraceae species. *Journal of Ethnopharmacology* 52**,** 27–33.

SALUDES, J. P., GARSON, M. J., FRANZBLAU, S. G. & AGUINALDO, A. M. 2002. Antitubercular constituents from the hexane fraction of Morinda citrifolia Linn. (Rubiaceae). *Phytotheraphy Research,* 16**,** 683–685.

SAMA, W. & AJAIYEOBA, E. O. 2006. Phytochemical and antimicrobial studies of Capparis thonningii and Capparis tomentosa. *Pharmacognosy Magazine,* 2**,** 119 - 122.

SAMIE, A., OBI, C. L., BESSONG, P. O. & LALL , N. 2005. Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. *African Journal of Biotechnology,* 4**,** 1443– 1451.

SARKAR, S. & SURESH, M. R. 2011. An Overview of Tuberculosis Chemotherapy – A Literature Review. *Jornal of Pharmacy and Pharmaceutical Science,* 14**,** 148 - 161.

SCHAEFER, C. M., LU, R., NESBITT, N. M., SCHIEBEL, J., SAMPSON, N. S. & KISKER, C. 2015. FadA5 a Thiolase from Mycobacterium tuberculosis: A Steroid-Binding

Pocket Reveals the Potential for Drug Development against Tuberculosis. *Structure,* 23**,** 21-33.

SCHINKOVITZ, A., GIBBONS, S., STAVRI, M., COCKSEDGE, M. J. & BUCAR, F. 2003. Ostruthin: An antimycobacterial coumarin from the roots of Peucedanum ostruthium. *Planta Medica,* 69**,** 369-371.

SCOTT, G. A., SPRINGFIELD, E. P. & COLDREY, N. 2004. A pharmacognostical study of 26 South African plant species used as traditional medicines. *Pharmaceutical Biology* 42**,** 186–213.

SEIDEL, V. & TAYLOR, P. W. 2004. In vitro activity of extracts and constituents of Pelargonium against rapidly growing mycobacteria. *International Journal of Antimicrobial Agents,* 23**,** 613–619.

SIMELANE, M. B. C., DJAROVA, T. G. & OPOKU, A. R. 2010. *Lactogenic activity of Gunnera perpensa L. (Gunneraceae) from South Africa* Masters (MSc), University of Zululand.

SKINNER, P. S., FURNEY, S. K., KLEINERT, D. A. & ORME, I. M. 1995. Comparison of activities of fluoroquinolones in murine macrophages infected with Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotheraphy,* 39**,** 750-753.

SOFOWORA, A. 1993. *Medicinal Plants and Traditional Medicines in Africa,* New York, John Wiley & Sons.

SOLIS, P. N., WRIGHT, C., ANDERSON, M. M., GUPTA, M. P. & PHILIPSON, J. D. 1993. A microwell cytotoxicity assay using Artemia salina (brine shrimp). *Planta Medica,* 59**,** 250-252.

SOMOVA, L. O., NADAR, A., RAMMANAN, P. & SHODE, F. O. 2003. Cardiovascular, antihyperlipidemic and anti-oxidant effect of oleanolic and ursolic acids in experimental hypertension. *Phytomedicine,* 10**,** 115- 121.

SPRINGFIELD, E. P., AMABEOKU, G., WEITZ, F., MABUSELA, W. & JHONSON, Q. 2003. An assessement of two Carpobrotus species extracts as potential antimicrobial agents. *Phytomedicine,* 10**,** 434-439.

SPRINGfiELD, E. P. & WEITZ, F. 2006. The scientific merit of Carpobrotus mellei L. based on antimicrobial activity and chemical profiling *African Journal of Biotechnology,* 5**,** 1289–1293.

SPRINGFIELD, E. P. & WEITZ, F. 2006. The scientific merit of Carpobrotus mellei L. based on antimicrobial activity and chemical profiling. *African Journal of Biotechnology,* 5**,** 1289–1293.

STEENHUYSEN, J. 2012. Drug showed promise in clearing drug-resistant tuberculosis. Reuters.

STEENKAMP, V., MATHIVHA, E., GOUWS, M. C. & VAN RENSBURG, C. E. 2004. Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa,. *Journal of Ethnopharmacology* 95**,** 353-357.

SWAMINATHAN, S., RAMACHANDRAN, R., BASKARAN, G., PARAMASIVAN, C. N., RAMANATHAN, U. & VENKATESAN, P. 2000. Risk of development of tuberculosis in HIV-infected patients. *International Journal of Tuberculosis and Lung Disease* 4**,** 839-844.

SZAKIEL, A., RUSZKOWSKI, D., GRUDNIAK, A., KUREK, A., WOLSKA, K. I., DOLIGALSKA, M. & JANISZOWSKA, W. 2008. Antibacterial and antiparasitic activity of oleanolic acid and its glycosides isolated from marigold (Calendula officinalis). *Planta Medica,* 74**,** 1709–1715.

TELENTI, A. & ISEMAN, M. 2000. Drug resistant tuberculosis, what do we do now? *Drugs,* 59**,** 171–179.

THONGTAN, J., KITTAKOOP, P., RUANGRUNGSI, N., SAENBOONRUENG, J. & THEBTARANONTH, Y. 2003. New antimycobacterial and antimalarial 8,9-secokaurane diterpenes from Croton kongensis *Journal of Natural Products,* 66**,** 868-870.

THRING, T. S. A., SPRINGFIELD, E. P. & WEITZ, F. M. 2007. Antimicrobial activities of four plant species from the Southern Overberg region of South Africa *African Journal of Biotechnology,* 6**,** 1779– 1784.

TODAR, K. 2012. Mycobacterium tuberculosis and Tuberculosis. *The Good, the Bad and the Deadly* [Online]. Available: www.textbookofbacteriology.net

TUBERCULOSIS 2014. Tuberculosis. *Encyclopedia Britannica.* Chicago: Encyclopedia Britannica Inc.

UNAIDS 2008. Report on the global AIDS epidemic Geneva.

VAN DER GEIZE, R., YAM, K., HEUSER, T., WILBRINK, M. H., HARA, H., ANDERTON, M. C., SIM, E., DIJKHUIZEN, L., DAVIES, J. E., MOHN, W. W. & ELTIS, L. D. 2007. A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into Mycobacterium tuberculosis survival in macrophages. *Proc Natl Acad Sci U S A,* 104**,** 1947-52.

VAN DER WATT, E. & PRETORIUS, J. C. 2001. Purification and identification of active antibacterial components in Carpobrotus edulis. *Journal of Ethnopharmacology,* 76**,** 87-91.

VAN PUYVELDE, L. 1979. New α-pyrones from Iboza riparia. *Phytochemistry* 18**,** 1215-1218.

VAN PUYVELDE, L. & DE KIMPE, N. 1998. Tetradenolide, an α-pyrone from Tetradenia riparia. *Phytochemistry* 49**,** 1157-1158.

VAN PUYVELDE, L., DE KIMPE, N., COSTA, J. & MUNYJABO, N. 1989. Isolation of flavonoids and a chalcone from Helichrysum odoratissimum and synthesis of helichrysin. *Journal of Natural Products* 52**,** 629–633.

VAN PUYVELDE, L., LEFEBVRE, R., MUGABO, P., DE KIMPE, N. & SCHAMP, N. 1987. Active principles of Tetradenia riparia. Antispasmodic activity of 8 (14), 15-sandaracopimaradiene-7α,18-diol. *Planta Medica,* 52**,** 156-158.

VAN WYK, B. & GERICKE, N. 2000. *Peoples Plants,* Pretoria, Briza Publications.

VAN WYK, B. & GERICKE, N. 2003. *Peoples Plants –A guide to useful plants of South Africa* Pretoria, Briza Publications.

VAN WYK, B., VAN OUDSHOORN, B. & GERICKE, N. 2002. *Medicinal Plants of South Africa,* Pretoria, Briza Publications.

VAN WYK, B. & VAN WYK, P. 1997. *Field Guide to Trees of Southern Africa,* Cape Town, Struik Publishers.

VEALE, D. J. H., OLIVER, D. W., ARANGIES, N. S. & FURMAN, K. I. 1992. South African traditional herbal remedies used during pregnancy and childbirth. *Journal of Ethnopharmacology* 36**,** 185-191.

VICTOR, T. C., STREICHER, E. M. & KEWLEY, C. 2007. Spread of an emerging *Mycobacterium tuberculosis* drug-resistant strain in the Western Cape of South Africa. *International Journal of Tuberculosis and Lung Disease,* 11**,** 195–201.

VOGT, K. 1995. *A field guide to the identification, propagation and uses of common trees and shrubs of dryland Sudan,* United Kingdom, SOS Sahel International.

VON BREITENBACH, J., DE WINTER, B., POYNTON, R., VAN DEN BERG, E., VAN WYK, B. & VAN WYK, E. 2001. *Pocket list of southern African indigenous trees: Including selected shrubs and woody climbers* Pretoria, Briza Publications & Dendrological Foundation.

VONGVANICH, N., KITTAKOOP, P., ISAKA, M., TRAKULNALEAMSAI, S., VIMUTTIPOG, S., TANTICHAROEN, M. & THEBTARANONTH, Y. 2002. Hirsutellide A, a new antimycobacterial cyclohexadepsipeptide from the entomopathogenic fungus Hirsutella kobayasii. *Journal of Natural Products,* 65**,** 1346–1348.

WATT, J. M. & BREYER-BRANDWIJK, M. G. 1962. *Medicinal and poisonous plants of Southern and Eastern Africa,* London, E & S Livingstone Ltd.

WEIGENAND, O., HUSSEIN, A. A., LALL, N. & MEYER, J. J. M. 2004. Antibacterial activity of naphthoquinones and triterpenoids from Euclea natalensis root bark. *Journal of Natural Products* 67**,** 1936–1938.

WHO 2003. Risk Factors for Tuberculosis. Geneva.

WHO 2009. Global Tuberculosis Control Report 2009 SURVEILLANCE planning financing. Geneva: World Health Organization.

WHO 2010. The Global Plan to Stop TB Geneva: World Health Organization.

WHO. 2011. Global Tuberculosis Report 2011.

WHO 2012 Global Tuberculosis Report 2012. Geneva: World Health Organization.

WHO 2013. Global tuberculosis report 2013. Geneva.

WHO 2015. Global Tuberculosis Report 2015. Geneva.

WHO. 2016. *Tuberculosis* [Online]. Geneva: World Health Organization. Available: http://www.who.int/mediacentre/factsheets/fs104/en/ [Accessed 12 January 2016].

WOLDEMICHAEL, G. M., FRANZBLAU, S. G. & ZHANG, F. 2003. Inhibitory Effect of Sterols from Ruprechtia triflora and Diterpenes from Calceolaria pinnifolia on the growth of Mycobacterium tuberculosis. *Planta Medica,* 69**,** 628–631.

WOLF, A. J., DESVIGNES, L. & LINAS, B. 2008. Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *Journal of Experimental Medicine,* 205**,** 105–115.

ZELNIK, R., RABENHORST, E., MATIDA, A., GOTTLIEB, , H.E., L., D., & PANIZZA, S. 1978. Ibozol, a new diterpenoid from Iboza riparia. *Phytochemistry,* 17.

ZHANG, Y., HEYM, B., ALLEN, B., YOUNG, D. & COLE, S. 1992. The catalase peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. *Nature Reviews / Molecular Cell Biology,* 358**,** 591–593.

ZHENKUN, M. 2010. Global tuberculosis drug development pipeline: the need and the reality. *The Lancet* 375**,** 2011-2109.
ZIMMER, C. 2014. Tuberculosis Is Newer Than Thought, Study Says. *New York Times*, 21 August 2014.